ANALGESIC, ANTI-INFLAMMATORY AND TOXICITY STUDIES ON METHANOLIC LEAF EXTRACT OF *ROTHMANNIA LONGIFLORA SALISB* (RUBIACEAE) IN RATS AND MICE

BY

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCES (PHARMACOLOGY)

DEPARTMENT OF PHARMACOLOGY AND THERAPEUTICS, FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA

SEPTEMBER, 2011

DECLARATION

I declare that the work in this thesis entitled; **ANALGESIC, ANTI-INFLAMMATORY AND TOXICITY STUDIES OF METHANOLIC LEAF EXTRACT OF *ROTHMANNIA LONGIFLORA* SALISB (RUBIACEAE) IN RATS AND MICE** has been performed by me in the Department of Pharmacology and Therapeutics, under the joint supervision of Professor J. A. Anuka and Dr. A. U. Zezi. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another degree or diploma at any university.

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CERTIFICATION

This thesis entitled **ANALGESIC, ANTI-INFLAMMATORY AND TOXICITY STUDY ON METHANOLIC LEAF EXTRACT OF *ROTHMANNIA LONGIFLORA SALISB* (RUBIACEAE) IN RATS AND MICE BY MALLAM, DANJUMA** meets the regulations governing the award of the degree of Master of Science (Pharmacology) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my wife (Mrs. Rahab Danjuma Mallam) and our mother Mrs. Mani Mallam

ABSTRACT

The herbalists in several African Countries have used *Rothmannia longiflora* decoctions as febrifugal and analgesic agent. The present study examined the analgesic, anti-inflammatory and toxicological profile of the methanolic extract of the plant in experimental animals.

The preliminary phytochemical screening of the methanolic leaf extract of *R.longiflora* was carried out and revealed the presence of cardiac glycoside, steroids/terpenoids, tannins, saponins, flavonoids and carbohydrates.

The acute toxic effect (median lethal dose values (LD_{50}) of methanolic leaf extract of *Rothmannia longiflora* was carried out using Lork's method in intraperitoneal and oral routes in mice and rats and were found to be above 5000mg/kg body weight.

The anti-nociceptive effects of the extract was studied using acetic acid induced writhing test in mice, hot plate test in mice and formalin induced pain in rats. The anti-inflammatory effect was studied using Carrageenan induced paw oedema in rats.

The extract at doses of 50-1000mg/kg significantly ($P<0.05$) inhibited the number of acetic acid-induced abdominal writhes in mice dose dependently. The highest inhibition of abdominal constriction (64.9%) observed at 1000mg/kg was greater than that of piroxicam (61.5%), the standard non-steroidal analgesic and anti-inflammatory drug used in the study.

The methanolic leaf extract at doses of 250-1000mg/kg *R. longiflora* significantly ($P<0.05$) and dose-dependently protected the mice against thermally induced pain stimulus in mice but there was no significant thermal protection with the dose of 50mg/kg.

The 500 and 1000mg/kg doses of *R.longiflora* extract offered more than 100 and 200% respectively while the standard drug (Morphine sulphate) offered more than 300% protection against thermally induced pain stimulus in mice.

There was no significant inhibition of both the neurogenic pain (early phase) and inflammatory pain (late phase) at 50mg/kg and 250mg/kg doses of the extract.

However, there was significant ($P<0.05$) inhibition of both the neurogenic (early phase) and inflammatory (late phase) pain dose dependently at 500mg/kg and 1000mg/kg with highest inhibition at 1000mg/kg (53.33%). The significant ($P<0.05$) inhibition of the standard drug (Morphine sulphate) at the late phase was 46.67%; this doubled that of the early phase which was 20%.

The methanolic leaf extract of *R.longiflora* significantly ($P<0.05$) inhibited Carrageenan induced paw oedema at doses of 250mg/kg; 500mg/kg and 1000mg/kg dose-dependently. There was no significant inhibition at dose of 50mg/kg.

The percentage anti-inflammatory effects at the peak of the Carrageenan-induced oedema at the third hour were; 39.39%, 51.52%, 63.63% at respective doses of 250mg/kg, 500mg/kg and 1000mg/kg compared to 69.70% for Piroxicam 20mg/kg (the standard anti-inflammatory drug)

Renal function test showed no statistically significant difference ($P>0.05$) of the levels of Sodium (Na^+), Potassium (K^+), Chloride (Cl^-) ions, Urea and Creatinine compared to that of the control at the end of 30 days (sub-chronic) and 90 days (chronic) studies.

In liver function test, there was no statistically significant difference ($P>0.05$) in the levels of AST, ALT, and ALP enzymes in all the treated groups compared to the control group after the 30 days studies. However, there was statistically significant difference with 500mg/kg and 1000mg/kg after the 90 days treatment.

The Histopathological study showed no observable gross lesions during the periods of sub-chronic and chronic studies.

These findings suggest that methanolic leaf extract of *R.longiflora* possesses analgesic and anti-inflammatory potentials/effects that justify the ethnomedical use of the plant in the treatment of painful and inflammatory conditions by the herbal practitioners. In addition, the results of toxicity studies showed that the extract is non-toxic to the kidneys and the heart during the sub-chronic and chronic toxicity studies however; mild fatty change was observed with 500mg/kg in the liver after the chronic studies.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

WHO ----------	World health organization
NSAID----------	Non Steroidal Anti-inflammatory Drugs
GIT----------	Gastro-intestinal tract
e.g ----------	Example
v/v ----------	volume by volume
PG ----------	Prostaglandin
COX-1 ----------	Cyclo-oxygenase-1
COX-2 ----------	Cyclo-oxygenase-2
COX-3----------	Cyclo-oxygenase-3
ESRD ----------	End stage renal disease
LD----------	Lethal Dose
viz:----------	as follows
AST----------	Aspartate aminotransferase
ALT----------	Alanine aminotransferase
ALP----------	Alkaline phosphatase
ICD----------	Isocitrate dehydrogenase
ABD----------	Alpha hydroxybutrate
LHD----------	Lactate dehydrogenase
NARICT----------	National Research Institute for Chemical Technology
Min----------	Minutes
i.p----------	Intraperitoneal
^{0}C----------	Degree centigrade

Tb----------	Control mean
Ta----------	Treated mean
Rmp----------	Revolution per minute
ABUTH----------	Ahmadu Bello University Teaching Hospital
µmol/L----------	Micro mol per litre
IFCC----------	International Federation of Clinical Chemistry
H^+----------	Hydrogen ion
>----------	Greater than
< ----------	Less than
SEM----------	Standard error of mean
Mg/kg----------	Milligram per Kilogram
%----------	Percent
Cm----------	Centimetre
NB----------	Note
Na^+----------	Sodium ion
K^+----------	Potassium ion
Cl^-----------	Chloride
ABU----------	Ahmadu Bello University
H_2O----------	Water
H----------	Hour
B. Pharm----------	Bachelor of Pharmacy

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND INFORMATION

Traditional medicine is defined as "therapeutic practices that have been in existence, for hundreds of years before the development and spread of modern sciences and are still in use (WHO, 2002). Many people in the developing countries rely on traditional healing practices and medicinal plants for their daily health care needs. This is due to the fact that traditional medicine is the most affordable and accessible health care system available. Traditional medicine can therefore be described as the total combination of knowledge and practices, whether explicable or not, used in diagnosing, preventing or eliminating a physical, mental or social disease and which may rely exclusively on past experiences and observations handed down from generations to generations, verbally or written. The practice utilizes vegetables, animals and minerals, and other methods (WHO, 1998). Results obtained from research into substances used in herbal medicine practices led to discovery of relevant plants with useful chemical constituents that can be used in treatment of various diseases (Amos *et al,.* 2001). A medicinal plant as defined by World Health Organization is "any plant in which one or more of its parts contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs" (WHO, 2002).

Quite a number of medicinal plants discovered by herbal practitioners have proved to be important sources of potential therapeutic agents e.g. Quinine (antimalarial agent) from Cinchona bark, digoxin and digitoxin (cardiac activity) from Digitalis purpurea, zingiberone (anti-helmintic) from *Zingiber officinalis*, vincristine and vinblastine alkaloids (anti-leukaemic agents) from *Catharantus roseus*, bourvadine from *Bourvadin ternifolia*, sinocaculine from *Corculus tribolus*, taxol from *Brevifolia* used as anticancer agents etc. (Hardman, 1991)

1.2 JUSTIFICATION

Plants and plant products are extensively used in herbal medicine worldwide, as they are easily available and cheap. Plants have served as valuable starting materials for drug development even in advance countries of the world (Sofowora, A. 1993). Many people in the developing countries rely on traditional healing practices and medicinal plants for their daily health care needs (WHO, 1998). This is due to the fact that traditional medicine is the most affordable and accessible health care system available.

Non-Opioid analgesics (NSAIDS) (examples naproxen, aspirin, ibuprofen) are particularly useful for the treatment of low to moderate pains of muscular skeletal conditions but they exert serious side effects on: GIT (abdominal pain, nausea, gastric ulcers, anaemia, GI perforations and haemorrhage.). Renal/Hepatic (Salt and water retention, oedema, worsening of renal function in renal/cardiac and cirrhotic patients, decrease effectiveness of anti-hypertensives and diuretics medications), while prolonged use of paracetamol at high dose can cause liver damage (Goodman and Gilman, 2006)

Opioid analgesics (examples, pethidine & morphine) are used usually to relieve moderate to severe pain particularly of visceral origin. However, there are serious adverse effects such as: dependence, tolerance, addiction, respiratory depression, hypotension, constipation, drowsiness, etc.

The adverse effects of both opioid and non-opioid analgesics couple with their high cost, limit their uses by the patients in the management of pains hence the need to search for newer analgesics and anti-inflammatory agents which are relatively safe, cheap, available, and potent.

This project therefore, seeks to investigate some of the pharmacological activities of methanolic leaf extract of *Rothmannia longiflora salisb* plant to justify the traditional local uses particularly the claim of febrifugal and analgesic properties, and to assess its toxic effects on the kidney and liver.

1.3 STATEMENT OF RESEARCH PROBLEM

Pain is the most common reason for physician's consultation in various health care delivery systems

in Nigeria and in other countries of the world. Majority of clinical conditions associated with humans are usually accompanied with pains, which can be mild, moderate or severe. Examples of such disease conditions include; cancer, migraine, sickle cell anaemia, gouty arthritis, angina pectoris. Others are; burns (severe or minor) and surgery (post-operative pain). Pain relievers (analgesics) are often used as adjuncts in the therapeutic management of these conditions.

Uncontrolled abuse of analgesics and analgesic combinations may lead to renal damage severe enough to cause end stage renal disease –ESRD- (Linton, 1980) or even the development of urogenital cancer (Mohoney, *et al,* 1977). In United States, the cost of treating ESRD by either dialysis or organ transplantation was $6.6 billion in 1991 (Inglehart, 1993). It has been estimated that patients on therapy for ESRD due to analgesic abuse represent about 3% of cases in Queen land Australia (Buckalow, 1986). In Nigeria such figures are scarcely available for the consumption of the public and regulatory bodies.

Many plants have been identified for their analgesic and anti-inflammatory properties by various researches on medicinal plants but most of these medicinal plants have not been subjected to studies to ascertain their effects on these organs (stomach, kidney, Heart, and liver). Studies of the effects of these plants on these organs will add more value to the efficiencies of these plants as analgesic and anti-inflammatory agents.

1.4 THEORETICAL FRAMEWORK

In recent times, focus on plant research has increased all over the world, and more than 13,000 plants have been studied between 1996 and 2000 (Dahanukar *et al*., 2000).

1.5 MODEL FOR THE STUDIES

The models available for study of pain in experimental animals include:

Acetic acid induced writhing:

This is a sensitive test for detecting substances with analgesic activity. In this test, pain is induced by injection of irritants such as glacial acetic acid (0.6-0.7%) into the peritoneal cavity of a mouse. The animal reacts by characteristic stretching behavour called writhing. Writhes can be described as a wave of constriction and elongation passing caudally along the abdominal wall with twisting of the trunk and extension of the hind limbs in mice. This is due to the nociceptive property of acetic acid (Surender and Mafumdar, 1995). The writhing induced by chemical substances is due to sensitization chemo-sensitive nociceptors by prostaglandins. The test is used to access substances with analgesic properties acting through peripheral mechanism (Sutharson, *et al*., 2007).

Hot Plate method

This method is described by (Lanhers *et al* (1992) and modified by (Mohamed and Ojewole (2004) is employed. A 600ml test beaker is placed on a regulated thermostat hot plate at $55^0C \pm 1^0C$.

Each mouse is placed in the beaker (on the hot plate) and the response to electrical heat-induced nociceptive pain stimulus is obtained. Licking of the paws or jumping out of the beaker is taken as an indicator of the animals' response to heat-induced nociceptive pain stimulus. The time taken for each mouse to lick its paws or jump out of the beaker is noted (reaction time). Each mouse serves as its own control. Before treatment, its reaction time is taken thrice at 1 hour interval and the mean represents reaction time before treatment of the mouse. The mean reaction time of the groups is pooled to obtain the final control mean reaction time (Tb). The test is used to access substances with analgesic properties acting through central mechanism.

Formalin-induced pain:

This test is a chronic pain model which is sensitive to centrally acting analgesic agents (Dubuisson and Dennis, 1977The test also differentiates between inflammatory and non-

inflammatory pains. Huskaar *et al*.,(1985) defines the distinct period of response to include the early response (first phase) and the late response (the second phase). Drugs that act centrally e.g. morphine inhibit both phases equally while peripherally acting drugs inhibit the late phase (Shibata *et al*.,1989; Chan *et al*., 1995). The early phase is probably a result of stimulation of nociceptors in the paw and reflects centrally mediated pain while the late phase is due to the inflammation with release of serotonin, histamine, bradykinin and prostaglandins and at least to some degree, the sensitization of central nociceptive neurons (Tjolsen *et al*., 1992).Activity in this model suggest the activation of the opioids receptors (Gaertner *et al*., 1999).

Anti-inflammatory Activity Studies:

Carrageenan-induced paw oedema:

Carrageenan-induced hind paw oedema is the standard experimental model for acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effect (Chakraborty *et al.,* 2006). Moreover, the model exhibits a high degree of reproducibility (Winter *et al*., 1962). The probable mechanism of action of Carrageenan-induced inflammation is bi-phasic, the first phase is attributed to the release of prostaglandins and lysosome enzymes in 2 to 3 hours (Brooks and Day, 1991).

Toxicology

Toxicology is concerned with the study of the harmful effects that chemical agents produce in living organisms. Chemical substances may evoke one or both of two toxic effects. The first, an acute effect, occurs shortly after contact with a single dose of poison. Chronic effect however occurs when an organism is exposed to repeated small and non-lethal doses of a potentially harmful substance (Hassel, 1982). The acute, sub-acute and chronic toxicity studies were carried out in this work.

1.6 AIM AND OBJECTIVES OF THE STUDY

1.6.1 Aim of the Study

The aim of this study is to scientifically investigate the analgesic and anti-inflammatory activities and toxicity profile of the methanolic leaf extract of *Rothmannia longiflora salisb* plant in experimental rats and mice.

1.6.2 Specific Objectives

(i) To collect and identify the plant *Rothmannia longiflora salisb.*

(ii) To extract the leaves of *Rothmannia longiflora* using pure methanol (100% v/v methanol) as the solvent.

(iii) To conduct preliminary phytochemical screening on the methanolic leaf extracts of *Rothmannia longiflora salisb* so as to identify the various chemical constituents present.

(iv) To carry out Lethal Dose determination (LD_{50}) on the methanolic leaf extract of *Rothmannia longiflora by Lorke's method.*

(v) To screen the leaf extract of *R.longiflora* for analgesic and anti-inflammatory activities.

(vi) To investigate the toxic effects of the extract on the kidney, heart and liver.

1.7 RESEARCH HYPOTHESIS

Ho (Null hypothesis): The methanolic leaf extract of *Rothmannia longiflora salisb* contains active constituents that are responsible for analgesic and anti-inflammatory activity which may cause renal, cardiac and hepatic side effects.

Ha (Alternate hypothesis): The methanolic leaf extract of *Rothmannia longiflora salisb* does not contain active constituents that are responsible for analgesic and anti-inflammatory activity which may not cause renal, cardiac and hepatic side effects.

CHAPTER TWO

LITERATURE REVIEW

2.1 ANALGESICS

Most of the agents which possess anti-inflammatory activity produce analgesia. It is generally accepted that the analgesic action of all NSAIDs is due to inhibition of prostaglandins (PG) formation at peripheral sites. Analgesics are drugs used to relieve pain without producing loss of consciousness or sense of touch. They are broadly classified into two namely, narcotic and non-narcotic analgesics.

Narcotic analgesics are otherwise called opioid analgesics. Opioids are natural or synthetic compounds that produce morphine-like effects. They act by binding to specific opioid receptors in the CNS to produce effects which mimic the action of endogenous peptide neurotransmitters, the opiopentins (e.g. the endorphins, enkephalins and dynorphins). Although, the opioid have a broad range of effects, their primary uses is to relieve intense pain and the anxiety that accompanies it, whether it is from surgery or as a result of injuries or disease, such as cancer (Mycek et al., 2000).

The locus coerulus contains both noradrenergic neurons and high concentration of opioid receptors, and it is postulated to play a critical role in feelings of alarm, fear and anxiety associated with pain (Baumann, 2002). Activity in the locus coerulus is inhibited by both exogenous opioids and endogenous opioid-like peptides (Reisine and Pasternak, 1996).

Non-narcotic analgesics are a group of chemically dissimilar agents that differ in their analgesic, anti-inflammatory and antipyretic activities. These agents are commonly referred to as non steroidal anti-inflammatory drugs (NSAIDs) and are among the most widely used therapeutic agents (Insel, 1996).

NSAIDs are mild analgesics which are particularly effective when inflammation has caused sensitization of pain receptors to normally painless mechanical or chemical stimuli. The inhibition

of prostaglandin Growth hormone (G/H) synthase enzyme (colloquially known as the cyclooxygenase) is the mechanism of action of most currently available NSAIDs. The inhibition of Cyclooxygenase-2 (COX-2) is thought to mediate, in large part, the analgesic, antipyretic and anti-inflammatory action of NSAIDs, while the simultaneous inhibition of COX-l largely but not exclusively accounts for the unwanted adverse effects in the gastrointestinal tract (Burke *et al.,*2006).

Aspirin and NSAIDs do not inhibit the lipoxygenase pathways of arachidonic acid, and hence do not suppress leukotriene formation. Glucocorticoids suppress the induced expression of COX-2, and thus COX-2 mediated PG production. They also inhibit the action of phospholipase A_2, which releases arachidonic acid from the cell membrane (Burke *et al,* 2006).

In spite of its wide use, the mechanism of action of acetaminophen has not been fully elucidated. It is only a weak inhibitor of prostaglandin (PG) synthesis *in vitro* and appears to have very little anti-inflammatory activity, although some reduction of tissue swelling after dental surgery has been reported (Skjelbred *et al*., 1977). Since its potent analgesic and antipyretic actions, it is generally regarded as an NSAID. However, it lacks the other typical actions of NSAIDs, such as antiplatelet activity and gastrotoxicity. Since acetaminophen is

a weak inhibitor *in vitro* of both cyclooxygenase (COX) —1 and COX-2, the possibility exists that it inhibits a so far unidentified form of COX perhaps COX-3.

In animal studies, COX enzymes in homogenates of different tissues vary in sensitivity to the inhibitory action of acetaminophen. This may be evidence that there are greater than 2 isoforms of the enzyme. A variant of COX-2 induced with high concentrations of nonsteroidal anti-inflammatory drugs was shown to be highly sensitive to inhibition by acetaminophen. Therefore, COX-3 may be a product of the same gene that encodes COX2, but have different molecular characteristics (Botting, 2000).

A number of African medicinal plants have been shown to possess analgesic activities in animal

studies. These include *Calliandra pororicensis* (Agunu *et al*., 2005), *Pterocarpus erinaceous* (Aliyu *et al*., 2005) and *Securidaca longenpeduculata* (Wannang *et al.,* 2005). Gossypin, a bioflavanoid from the yellow petals of *Hibiscus vitofolius* has been shown to possess antinociceptive activity similar to morphine and has the advantage of lack of tolerance and dependence liability (Dahanukar *et al*, 2000).

African medicinal plants with anti-inflammatory activities include *Khaya senegalensis* (Lompo *et al*., 2007), Acanthus montanus (Adeyemi *et al,* 2005) and *Desmodium*

gangeticurn (Udeogaranya *et al*., 2005).

Some of the Anti-inflammatory medicines developed from medicinal plants include Ruxiang, or Gummi olibanum, an herbal medicine derived from the gum resin of *Boswellia carterii* of the family Burseraceae, it has been used traditionally in China to alleviate pain and reduce inflammation. Frankincense has been used to reduce pain from arthritis. Compounds in frankincense suppress pro-inflammatory cytokines.

Bisindigotin, a Chinese medicinal herb from an indigo plant called *Isatis indigotica*. The root of indigo plant, popularly known as Ban-Lan-Gen is used in traditional Chinese medicine for seasonal febrile diseases, pestilence, mumps, eruptive diseases, inflammatory diseases with redness of skin, sore throat, etc.

Curcumin Supplement extract from Turmeric plant called *Curcuma longa* - the botanical name. Curcumin prevents interleukins from promoting inflammation; it is used in the therapy of inflammatory bowel disease.

Silymarin capsules, the active extract from *Silybum marianum* (L.) Milk thistle, is a complex of 7 flavonolignans and polyphenols. The most active compound in slymarin is silibinin. It has been extensively used in patients with liver disease (Doreswamy and Darshan, 2004).

2.2 PROSTAGLANDIN

A prostaglandin is any member of a group of lipid compounds that are derived enzymatically from fatty acids and have important functions in the animal body. Every prostaglandin contains 20 carbon atoms, including a 5-carbon ring (Nelson, 2005).

They are mediators and have a variety of strong physiological effects, such as regulating the contraction and relaxation of smooth muscle tissue. Prostaglandins are not hormones, but autocrine or paracrine, which are locally acting messenger molecules. They differ from hormones in that they are not produced at a discrete site but in many places throughout the human body. In addition, their target cells are present in the immediate vicinity of the site of their secretion (of which there are many) (Nelson, 2005). Prostaglandins are produced following the sequential oxidation of AA, DGLA or EPA by cyclooxygenases (COX-1 and COX-2) and terminal prostaglandin synthases. The classic dogma is as follows:

- COX-1 is responsible for the baseline levels of prostaglandins.
- COX-2 produces prostaglandins through stimulation.

However, while COX-1 and COX-2 are both located in the blood vessels, stomach and the kidneys, prostaglandin levels are increased by COX-2 in scenarios of inflammation. A third form of COX, termed COX-3 is thought to exist in the brain and may be associated with relief of Headaches when on NSAID therapy.

Examples of prostaglandin antagonists are:

NSAIDs (inhibit cyclooxygenase), Corticosteroids (inhibit phospholipase A2 production), COX-2 selective inhibitors or coxibs. Cyclopentenone prostaglandins may play a role in inhibiting inflammation (Fabre *et al*, 2001).

2.3 PAIN

First attested in English in 1297, the word pain comes from the Old French peine, in turn from Latin poena, "punishment, penalty" (in L.L. also "torment, hardship, suffering") and that from Greek "ποινή" (poine), generally "price paid", "penalty", "punishment" (Viñuela *et al*., 2007)

Pain is defined as unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Turk and Okifuji, 2000; Baumann, 2002). The body modulates pain through a number of complex processes including the endogenous opiate system, which consist of neurotransmitters (e.g., endokephalins and β-endorphin) and receptors (e.g. Mu, delta, kappa) which are found throughout the central nervous system (Terman and Bonica, 2000).

Pain motivates us to withdraw from potentially damaging situations, protect a damaged body part while it heals, and avoid those situations in the future. Most pain resolves promptly once the painful stimulus is removed and the body has healed, but sometimes pain persists despite removal of the stimulus and apparent healing of the body; and sometimes pain arises in the absence of any detectable stimulus, damage or disease (Raj, 2007)

Pain is the most common reason for physician consultation in the United States and other parts of the world. It is a major symptom in many medical conditions, and can significantly interfere with a person's quality of life and general functioning. Psychological factors such as social support, hypnotic suggestion, excitement in sport or war and distraction can significantly modulate pain's intensity or unpleasantness (Breivik *et al*, 2008).

Pain is one of the cardinals features of inflammation, others being, hyperemia, oedema and heat.

Pain is a sensory modality, which represents the symptom for the diagnosis of several diseases. Morphine is considered to be the king of narcotic analgesics but is also associated with numerous side effects which include; respiratory depression, drowsiness, decreased gastrointestinal motility, nausea and several alterations of endocrine and autonomic nervous system (Almeida *et al*., 2001). Non Steroidal anti-inflammatory drugs (NSAIDS) include different agents of different chemical

classes and are used for reduction of certain types of pain in addition to their anti-inflammatory and antipyretic effects. Other agents with analgesic activities include Tricyclic antidepressants such as imipramine as well as antiepileptic drugs such as carbamazepine, gabapentin and occasionally phenytoin (Rang *et al*., 2003)

Epidemiology of pain

Pain is the main reason for visiting the emergency department in more than 50% of cases and is present in 30% of family practice visits (Hasselström *et al,* 2002). Several epidemiological studies from different countries have reported widely varying prevalence rates for chronic pain, ranging from 12-80% of the population. It becomes more common as people approach death. A study of 4,703 patients found that 26% had pain in the last two years of life, increasing to 46% in the last month (Smith *et al*, 2010).

A survey of 6,636 children (0–18 years of age) found that, of the 5,424 respondents, 54% had experienced pain in the preceding three months. A quarter reported having experienced recurrent or continuous pain for three months or more, and a third of these reported frequent and intense pain. The intensity of chronic pain was higher for girls, and girls' reports of chronic pain increased markedly between ages12 and 14 (Perquin *et al*, 2000).

Nociceptive Pain

Nociceptive pain is caused by stimulation of peripheral nerve fibers that respond only to stimuli approaching or exceeding harmful intensity (nociceptors), and may be classified according to the mode of noxious stimulation; the most common categories being "thermal" (heat or cold), "mechanical" (crushing, tearing, etc.) and "chemical" (iodine in a cut, chili powder in the eyes).

Nociceptive pain may also be divided into "visceral," "deep somatic" and "superficial somatic" pain. Visceral structures are highly sensitive to stretch, ischemia and inflammation, but relatively insensitive to other stimuli that normally evoke pain in other structures, such as burning and cutting. Visceral pain is diffuse, difficult to locate and often referred to a distant, usually superficial, structure. It may be accompanied by nausea and vomiting and may be described as sickening, deep, squeezing, and dull (Urch & Suzuki, 2009).

Deep somatic pain is initiated by stimulation of nociceptors in ligaments, tendons, bones, blood vessels, fasciae and muscles, and is dull, aching, poorly-localized pain. Examples include sprains and broken bones. Superficial pain is initiated by activation of nociceptors in the skin or other superficial tissue, and is sharp, well-defined and clearly located. Examples of injuries that produce superficial somatic pain include minor wounds and minor (first degree) burns (Spanswick and Main, 2000).

Neuropathic Pain

Neuropathic pain is caused by damage or disease affecting any part of the nervous system involved in bodily feelings (the somatosensory system) (Treede *et al,* 2008). Peripheral neuropathic pain is often described as “burning,” “tingling,” “electrical,” “stabbing,” or “pins and needles” Bumping the "funny bone" elicits acute peripheral neuropathic pain (Paice, 2003)

Phantom pain

Phantom pain is pain from a part of the body that has been lost or from which the brain no longer receives signals. It is a type of neuropathic pain. Phantom limb pain is a common experience of amputees (Kooijman *et al,* 2000).

The prevalence of phantom pain in upper limb amputees is nearly 82%, and in lower limb amputees is 54%. One study found that eight days after amputation, 72 percent of patients had phantom limb pain, and six months later, 65 percent reported it. Some amputees experience continuous pain that varies in intensity or quality; others experience several bouts a day, or it may occur only once every week or two. It is often described as shooting, crushing, burning or cramping. If the pain is continuous for a long period, parts of the intact body may become sensitized, so that touching them evokes pain in the phantom limb, or phantom limb pain may accompany urination or defecation (Kooijman *et al,* 2000).

Psychogenic pain

Psychogenic pain, also called psychalgia or somatoform pain is pain caused, increased, or prolonged by mental, emotional, or behavioral factors. Headache, back pain, and stomach pain are sometimes diagnosed as psychogenic. Sufferers are often stigmatized, because both medical professionals and the general public tend to think that pain from a psychological source is not "real". However, specialists consider that it is no less actual or hurtful than pain from any other source (Thienhaus and Cole, 2002).

People with long term pain frequently display psychological disturbance, with elevated scores on the Minnesota Multiphasic Personality Inventory scales of hysteria, depression and hypochondriasis (the "neurotic triad"). Some investigators have argued that it is this neuroticism that causes acute injuries to turn chronic, but clinical evidence points the other way, to chronic pain causing neuroticism. When long term pain is relieved by therapeutic intervention, scores on the neurotic triad and anxiety fall, often to normal levels. Self-esteem often low in chronic pain patients, also shows improvement once pain has resolved (Ramachandran and Roger, 1996).

Pain Pathway

Afferent nociceptive fibers (those that send information to, rather than from the brain) travel back to the spinal cord where they form synapses in its dorsal horn. This nociceptive fiber (located in the periphery) is a first order neuron. The cells in the dorsal horn are divided into physiologically distinct layers called laminae. Different fiber types form synapses in different layers, and use either glutamate or substance P as the neurotransmitter. Aδ fibers form synapses in laminae I and V, C fibers connect with neurons in lamina II, Aβ fibers connect with lamina I, III, & V (Jessell *et al,* 1991).

After reaching the specific lamina within the spinal cord, the first order nociceptive project to second order neurons and cross the midline. The second order neurons then send their information via two pathways to the thalamus: the dorsal column medial-lemniscal system and the anterolateral system. The first is reserved more for regular non-painful sensation, while the lateral is reserved for pain sensation. Upon reaching the thalamus, the information is processed in the ventral posterior nucleus and sent to the cerebral cortex in the brain. As there is an ascending pathway to the brain that initiates the conscious realization of pain, there also is a descending pathway which modulates pain sensory. The brain can request the release of specific hormones or chemicals that can have analgesic effects which can reduce or inhibit pain sensation. The area of the brain that stimulates the release of these hormones is the hypothalamus (Fields *et al,* 1998).

This effect of descending inhibition can be shown by electrically stimulating the periaqueductal grey area of the midbrain. The periaqueductal grey in turn projects to other areas involved in pain regulation, such as the nucleus raphe magnus (which also receives similar afferents from the nucleus reticularis paragigantocellularis (NPG). In turn the nucleus raphe magnus projects to the substantia gelatinosa region of the dorsal horn and mediates the sensation of spinothalamic inputs. The periaqueductal grey also contains opioid receptors which explains one of the mechanisms by

which opioids such as morphine and diacetylmorphine exhibit an analgesic effect (Selbst and Fein, 2006).

Nociceptor neuron sensitivity is modulated by a large variety of mediators in the extracellular space (Hucho and Levine, 2007).

Peripheral sensitization represents a form of functional plasticity of the nociceptor. The nociceptor can change from being simply a noxious stimulus detector to a detector of non-noxious stimuli. The result is that low intensity stimuli from regular activity, initiates a painful sensation. This is commonly known as hyperalgesia. Inflammation is one common cause that results in the sensitization of nociceptors. Normally hyperalgesia ceases when inflammation goes down, however, sometimes genetic defects and/or repeated injury can result in allodynia: a completely non-noxious stimulus like light touch causes extreme pain. Allodynia can also be caused when a nociceptor is damaged in the peripheral nerves. This can result in deafferentation, which means the development of different central processes from the surviving afferent nerve. With this situation, surviving dorsal root axons of the nociceptors can make contact with the spinal cord, thus changing the normal input (Fields *et al,* 1998).

Classification of Pain

In 1994, responding to the need for a more useful system for describing chronic pain, the International Association for the Study of Pain (IASP) classified pain according to specific characteristics: 1. region of the body involved (e.g., abdomen, lower limbs), 2. system whose dysfunction may be causing the pain (e.g., nervous, gastrointestinal). 3. temporal characteristics and pattern of occurrence. 4. intensity and time since onset and 5. etiology (Merskey & Bogduk, 1994).

This system has been criticized by Woolf and others as inadequate for guiding research and

treatment, and an additional category based on neurochemical mechanism has been proposed (Turk and Okifuji, 2001).

Chronic pain

Pain is usually transitory, lasting only until the noxious stimulus is removed or the underlying damage or pathology has healed, but some painful conditions, such as rheumatoid arthritis, peripheral neuropathy, cancer and idiopathic pain, may persist for years. Pain that lasts a long time is called chronic, and pain that resolves quickly is called acute. Traditionally, the distinction between acute and chronic pain has relied upon an arbitrary interval of time from onset; the two most commonly used markers being 3 months and 6 months since the onset of pain (Turk and Okifuji, 2001), though some theorists and researchers have placed the transition from acute to chronic pain at 12 months. Others apply acute to pain that lasts less than 30 days, chronic to pain of more than six months duration, and subacute to pain that lasts from one to six months. A popular alternative definition of chronic pain, involving no arbitrarily fixed durations is "pain that extends beyond the expected period of healing " (Turk and Okifuji, 2001). Chronic pain may be classified as cancer pain or benign (Thienhaus and Cole, 2002).

Pain Thresholds

In pain science, thresholds are measured by gradually increasing the intensity of a stimulus such as electric current or heat applied to the body. The pain perception threshold is the point at which the stimulus begins to hurt, and the pain tolerance threshold is reached when the subject acts to stop the pain (Melzack and Wall, 1996).

Differences in pain perception and tolerance thresholds are associated with, among other factors,

ethnicity, genetics, and sex. People of Mediterranean origin report as painful some radiant heat intensities that northern Europeans describe as non-painful, and Italian women tolerate less intense electric shock than Jewish or Native American women. Some individuals in all cultures have significantly higher than normal pain perception and tolerance thresholds. For instance, patients who experience painless heart attacks have higher pain thresholds for electric shock, muscle cramp and heat (Melzack and Wall, 1996). Women have lower pain perception and tolerance thresholds than men, and this sex difference appears to apply to all ages, including newborn infants (Guinsburg *et al*, 2000).

Pain Assessment

A person's self-report is the most reliable measure of pain, with health care professionals tending to underestimate severity (Prkachin *et al,* 2007). A definition of pain widely employed in nursing, emphasizing its subjective nature and the importance of believing patient reports. "Pain is whatever the experiencing person says it is, existing whenever he says it does". To assess intensity, the patient may be asked to locate their pain on a scale of 0 to 10, with 0 being no pain at all, and 10 the worst pain they have ever felt. Quality can be established by having the patient complete the McGill Pain Questionnaire indicating which words, best describe their pain (Prkachin *et al,* 2007)

Pain- an aid to diagnosis

Pain is a symptom of many medical conditions. Knowing the time of onset, location, intensity, pattern of occurrence (continuous, intermittent, etc.), exacerbating and relieving factors, and quality (burning, sharp, etc.) of the pain will help the examining physician to accurately diagnose the problem. For example, chest pain described as extreme heaviness may indicate myocardial

infarction, while chest pain described as tearing may indicate aortic dissection (Slater *et al,* 1976).

Management of pain

Inadequate treatment of pain is widespread throughout surgical wards, intensive care units, accident and emergency departments, in general practice, in the management of all forms of chronic pain including cancer pain, and in end of life care (Brown *et al,* 2004). This neglect is extended to all ages, from neonates to the frail elderly (Selbst and Fein, 2006). African and Hispanic Americans are more likely than others to suffer needlessly in the hands of a physician; and women's pain is more likely to be undertreated than men's (Deandrea *et al,* 2008).

The International Association for the Study of Pain advocates that the relief of pain should be recognized as a human right, that chronic pain should be considered a disease in its own right, and that pain medicine should have the full status of a specialty. It is a specialty only in China and Australia at this time (Horlocker *et al,* 2008). Elsewhere, pain medicine is a subspecialty under disciplines such as anesthesiology, physiatry, neurology, palliative medicine and psychiatry (Brown *et al,* 2004).

Medication of pain

Acute pain is usually managed with medications such as analgesics and anesthetics. Management of chronic pain, however, is much more difficult and may require the coordinated efforts of a pain management team, which typically includes medical practitioners, clinical psychologists, physiotherapists, occupational therapists, and nurse practitioners (Thienhaus and Cole, 2002).

Sugar taken orally reduces the total crying time but not the duration of the first cry in newborns undergoing a painful procedure (a single lancing of the heel). It does not moderate the effect of pain on heart rate (Stevens *et al* 2010) and a recent single study found that sugar did not significantly affect pain-related electrical activity in the brains of newborns one second after the heel lance

procedure. Sweet oral liquid moderately reduces the incidence and duration of crying caused by immunization injection in children between one and twelve months of age (Harrison *et al* ,2010).

Alternative medicine

Pain is the most common reason for people to use complementary and alternative medicine (Astin, 1998). An analysis of the 13 highest quality studies of pain treatment with acupuncture concluded that there is little difference in the effect of real, sham and no acupuncture (Madsen *et al,* 2009). There is interest in the relationship between vitamin D and pain, but the evidence so far from controlled trials for such a relationship, other than in osteomalacia, is unconvincing (Straube *et al,* 2009).

A 2007 review of 13 studies found evidence for the efficacy of hypnosis in the reduction of pain in some conditions, though the number of patients enrolled in the studies was low, bringing up issues of power to detect group differences, and most lacked credible controls for placebo and/or expectation. The authors concluded that "although the findings provide support for the general applicability of hypnosis in the treatment of chronic pain, considerably more research will be needed to fully determine the effects of hypnosis for different chronic-pain conditions" (Elkins *et al,* 2007).

A 2003 meta-analysis of randomized clinical trials found that spinal manipulation was "more effective than sham therapy but was no more or less effective than general practitioner care, analgesics, physical therapy, exercise, or back school" in the treatment of low back pain (Assendelft *et al,* 2004).

2.4 INFLAMMATION

Inflammation (Latin, inflamatio, to set on fire) is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. Inflammation is not a synonym for infection. Even in cases where inflammation is caused by infection, the two are not synonymous: infection is caused by an exogenous pathogen, while inflammation is the response of the organism to the pathogen (Ferrero-Miliani*., et al*, 2007).

In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, an inflammation that runs unchecked can also lead to many diseases, such as hay fever, and rheumatoid arthritis. It is for this reason that inflammation is normally closely regulated by the body (Dhannanjayan and Thangam, 2003).

Inflammation can be classified as either acute or chronic: Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells, which are present at the site of inflammation, and is characterised by simultaneous destruction and healing of the tissue from the inflammatory process (Doreswamy and Darshan, 2004).

Clinical Signs of Inflammation

Acute inflammation is a short-term process, usually appearing in a few minutes or hours and ceasing once the injurious stimulus has been removed. It is characterized by five cardinal signs rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and functio laesa (loss of function) (Cotran., *et al*, 1998).

2.5 KIDNEY

Kidney is the chief regulator of all body fluids and is primarily responsible for maintaining homeostasis, or equilibrium of fluid and electrolytes in body. The kidney main functions are urine formation, regulation of acid-base balance, excretion of waste products of protein metabolism, protein conservation and hormonal function (First, 1996). Nephrons are lost via toxic, anoxic, or immunological injury that may initially injure the glomerulus, the tubule or both together. Glomerular damage can involve endothelial, epithelial, or mesangial cells and/or the basement membrane (Delaney, *et- a*l, 2006). Newer analgesic combination products are freely found in Nigeria drug market today and their consumption has become a common practice. Systematic review is silent over the renal effects of these newer analgesic combination products. Uncontrolled abuse of analgesics and analgesic combinations may lead to renal damage severe enough to cause end stage renal disease –ESRD- (Linton, 1980) or even the development of urogenital cancer (Mohoney, *et al,* 1977). In United States, the cost of treating ESRD by either dialysis or organ transplantation was $6.6 billion in 1991 (Inglehart, 1993). It has been estimated that patients on therapy for ESRD due to analgesic abuse represent about 3% of cases in Queen land Australia (Buckalow and Schey, 1986). In Nigeria such figures are scarcely available for the consumption of the public and regulatory bodies.

2.6 LIVER

The liver is the major site of metabolism of most substances or compound absorbed through the gastrointestinal tract.

Liver function tests are series of tests which are used to assess the efficiency of various liver functions.

Mathenheimer (1971) identified the function of the liver as follows:

i. Storage of nutrients and their release into the body.

ii. Synthesis of fat from glucose, galactose and other sugars and synthesis of proteins, urea, cholesterol, purines and pyrimidine.

iii. Detoxification of chemical agents by conjugation of toxic substances with glucuronate and sulphate and metabolic conversion of drugs and

iv. Excretion of bile pigments, bile acids and cholesterol.

Liver function tests are based on the measurement of activity changes of certain enzymes in the blood serum. This is, in turn based on the knowledge that all serum enzymes have their origin in the cell, and that the useful ones are normally found in serum in relatively low concentration (Ringler and Dabich, 1980). Increased serum concentration of a particular enzyme thus signifies damage of the cell from which the enzyme was released. Ringler and Dabich, (1980) also observed that serum enzyme responses usually vary with the rate and magnitude of absorption of toxicants hence may serve as an indication of severity of exposure to toxins. A decrease in serum enzyme activity may arise occasionally in the case of a specific enzyme deficiency which could be caused as a result of blockade of de novo enzyme synthesis because of prolonged exposure to some toxicants (Okonkwo, 1991) therefore concluded that a correlation exists between rise in serum enzyme and severity of toxic damage of a particular tissue. Assay of serum enzymes is thus a very important approach to detecting and diagnosing toxicity. It also serves to identify liver disease, to define the nature of the disease process and to locate the cellular and intracellular sites of hepatic pathology (Wilkinson, 1976 and Gowenlock, 1988).

Enzymes are proteins with specific functions accruing naturally as complex compounds that speed up biological reactions that might otherwise progress slowly in their absence. They, thus serve as catalysts (Mathenheimer, 1971). Some enzymes are simple protein while some are conjugated proteins containing metal ions coenzymes or both. Coenzymes serve as carriers of electrons or specific functional group (like hydrogen atoms and amino groups) and aid in their transfer from one substrate to another (Gowenlock, 1988). Wilkinson (1976) classified enzymes into six main groups

according to the type of reaction they catalyse viz:

i. oxidoreductases
ii. transferases (transaminases)
iii. hydrolases
iv. lyases
v. isomerases
vi. ligases

Mathenheimer (1971) observed that the enzymes related to liver function include the transferases (Aspartate aminotransferase (AST), Alanine Aminotransferase (ALT) and alkaline phosphatase (ALP), isocitrate dehydrogenase (ICD), alpha hydroxybutrate dehydrogenase (ABD) and lactate dehydrogenase (LHD). However, in the majority of clinical laboratories transferases and alkaline phosphatase measurements are usually reported in response to a clinical request for liver function tests (Wilkinson, 1976, Ikediobi and Ukoha, 1985). The transferases are the most important group of liver enzymes whose levels in serum are altered in hepatocellular diseases, particularly in acute disease. They are therefore referred to as hepatocellular enzymes. Alkaline phosphatase is referred to as cholestatic enzyme because of its pronounced change in cholestasis (Wilkinson, 1976, Ikediobi and Ukoha, 1985).

2.7 THE PLANT, *ROTHMANNIA LONGIFLORA*

Rothmannia longiflora is found in Gambia, Sudan, Kenya, Tanzania, Angola and is also found in Nigeria, Ghana, Sierra Leone, Democratic Republic of Congo, Ivory Coast, Uganda, Liberia and Cameroon. *Rothmannia longiflora Salisb* is from the family Rubiaceae.

In Nigeria, the common vernacular names of *Rothmannia longiflora* include: **Hausa**, 'Katambiri,' **Igbo:** 'Uli, Alankita uku, mbembe (Ogwashi),' **Yoruba**: 'Edo-pata, Kere buje (Ononchie), Osego ikorun (Millson).'

Rothmannia longiflora is a decorative plant with large, attractive, trumpet-shaped, white or pink

flowers and is worth cultivating as an ornamental.

The fruits of *Rothmannia longiflora* are commonly used to make blue-black markings on the hands, face and body, sometimes to imitate tattooing (Dalziel, 1937). In Central and East Africa fibres of vegetable origin (e.g. of *Raphia*) are coloured by soaking them in swamp mud to which fruits and leaves of *Rothmannia longiflora* have been added. In Nigeria a dye and an ink-like extract ('katambiri') is made from finely crushed seeds. To make a stronger dye and ink, the extract is sometimes mixed with leaves of *Gardenia*, *Indigofera*, Nauclear or *Vitex* and sugar or honey (Dalziel, 1937). A black dye can be prepared from the flowers.

The fruits are edible. In Sierra Leone the stems are used to make shafts of a long- handled chisel ('sundi') used for harvesting fruit bunches of oil palm (Dalziel, 1937).

Rothmannia longiflora is considered to have febrifugal and analgesic properties, and a decoction of the leaves, twigs, bark and roots is applied internally or externally as lotions, washes and baths (Irvine, 1961). In Nigeria the roots are used to treat bowel complaints (Dalziel, 1937). In Democratic Republic of Congo a root infusion is applied as a treatment for throat abscesses, toothache and leprosy. In West Africa like Ghana and Cameroon, leaf pulp is used as an enema against kidney pain and diarrhoea with blood, and drinking the leaf juice is said to help during labour and childbirth. In Sierra Leone leaves are used to treat itching skin diseases and the fruit pulp is said to be emetic (Dalziel, 1937). In Democratic Republic of Congo the seed is used to treat ulcers.



Plate 1: *Rothmannia longiflora* salisb (Rubiaceae) plant in its natural habitat

CHAPTER THREE

MATERIALS AND METHODS

3.1 PLANT MATERIAL

The whole plant, *Rothmannia longiflora salisb* was collected in Gargai, Bagauda Road, Kano, Kano State, Nigeria, in October 2010 by Malam Umar. S. Gallah and was identified by Mallam Musa Mohammed of the Herbarium Section of Biological Science Department of Ahmadu Bello University, Zaria. A voucher specimen number of 2877 was deposited there for future reference. Only the leaves of the plant were used. The leaves were air dried in the shade; it was then crushed into coarse powder with a pestle and mortar. The powdered leaves (500g) were extracted with 2.5Litres of 100% Methanol for 72hours using soxhlet extraction apparatus. The solvent was evaporated to give an average yield of 29.47%w/w. Subsequently, another 500g of the powdered leaves were extracted with another 2.5Litres of 100% Methanol, the same process was used for extraction and evaporation, and another average yield of 29.79% w/w was obtained. The extract was then stored in the freezer at temperature of 0-5^{o}C until needed for the work. Solutions of the extract were prepared freshly for each study.

3.2 ANIMALS

3.2.1 Rats

Strain: Wistar rat

Sex: Both sexes

Body weight: 150-250g

Sources:- Animal House, Department of Pharmacology and Therapeutics, A.B.U-Zaria and National Research Institute for Chemical Technology (NARICT), Basawa, Zaria, Kaduna State.

3.2.2 Mice

Strain: Swiss Albino Mice

Sex: Both sexes

Body weight: 18-30g

Sources:- Animal House, Department of Pharmacology and Therapeutics, A.B.U-Zaria.

The animals were maintained in a well ventilated room in the laboratory animal house under standard laboratory conditions of temperature (25 ± 2^0C) and light (approximately 12/12h light/dark cycle). They were fed on Vital Grower feeds (Vital feeds, Jos-Plateau State) and water *ad libitum*. All experiments performed on laboratory animals in this study followed the ‘’principles of laboratory animal care’’ (NIH Publication, 1985).

3.3 DRUGS AND CHEMICALS

The following drugs were obtained and used for the study:-

Glacial acetic acid solution (Searle Essex, England)

Carrageenan solution (Sigma-Aldrich Laborchemikalein GmBH Germany)

Chloroform (Sigma Chemical Co. USA)

Concentrated hydrochloric acid (BDH Ltd Poole, England)

Distilled water

Dragendorff reagent, chips of magnesium metal (BDH Ltd Poole, England)

Ferric chloride (BDH Ltd Poole, England)

Formaldehyde (Sigma-Aldrich Laborchemikalein GmBH Germany)

Morphine sulphate injection BP 10mg/ml (Martindale Pharmaceuticals, Ramford, Essex RM3 8UG, U.K.PL01883/6138R)

Picric acid regent (BDH Ltd Poole, England)

Sulphuric acid (BDH Ltd Poole, England)

Wagner reagent (BDH Ltd Poole, England)

Cap Piroxicam 20mg (Hovid Pharmaceutical Company Malaysia)

3.4 EQUIPMENT AND OTHER MATERIALS

Animal cages, weighing balance, pestle and mortar, stop clock, syringes (1ml, 2ml, 5ml, 10ml, 20ml) and thermostat hot plate (Gallenkamp thermostat Cat No:Hl054), beakers, plastic bottles, conical flask, retort stand funnel, spatula, water bath, Vernier calliper etc.

3.5 PRELIMINARY PHYTOCHEMICAL SCREENING OF *R. LONGIFLORA*

Phytochemical screening of the methanolic leaf extract of *Rothmannia longiflora* was carried out as follows;

3.5.1 Test for Carbohydrates

Molisch test:

About 0.5g of the extract was dissolved in 10ml distilled water in a test tube. Three drops of 1% σ-naphthol in 80% ethanol were added followed by 5 drops of concentrated sulphuric acid without mixing. Appearance of purple ring at the interface as a result of the reaction between naphthol and furfural and 5- hydroxymethylfurfural aldehydes produced by dehydration of saccharides indicates the presence of carbohydrates (Silva *et al*., 1998).

3.5.2 Test for Flavonoids

Shinoda reduction test (cyanidin test):

About 3ml of the alcoholic solution of the extract was evaporated to dryness. The residue was dissolved in 2ml of 50% methanol with heat. Four (4) drops of concentrated Hydrochloric acid was added followed by some chips of magnesium metal. Immediate appearance of orange color denotes flavones, red — crimson color denotes flavonols and pink — magenta color denotes flavonones (Mahran *et al*., 1980).

Concentrated sulphuric acid:

About 0.2ml of the aqueous methanol extract was dissolved in concentrated sulphuric acid, a deep yellow solution indicates flavones and flavonols, a red or red bluish solution indicates chalcones and aurones and orange to red color indicates flavonones.

3.5.3 Test for Cardiac Glycosides

Keller kiliani test:

About lml of the aqueous methanolic extract was diluted with 20ml of distilled water and lml of strong lead sub acetate solution was added to precipitate pigments, which were filtered off. The filtrate obtained was shaken with an equal volume of chloroform and allowed to separate into two layers in a small separating funnel. The chloroform layer was removed and evaporated to dryness on a water bath. The residue was dissolved in 3m1 of ferric chloride in glacial acetic acid, left for 1min and then transferred into a dry test tube. Six drops of concentrated sulphuric acid was added by the walls of the test tube. On standing, a brown colour at the interface (due to deoxy sugars) and a pale green colour at the upper layer (due to the steroidal nucleus) is a preliminary indication for cardenolides (Brain and Turner, 1975).

3.5.4 Test for Saponins

Froth test:

About 2m1 of the aqueous methanol extract was diluted with twice its volume of distilled water and shaken in a test tube for 15minutes. The occurrence of foam column, of at least 1 cm in height persisted for a minimum of 15minutes indicates the presence of saponins (Silva *et al.,* 1998).

3.5.5 Test for Tannin

Ferric chloride test:

About 1ml of the aqueous methanol extract was diluted with 2m1 of distilled water. Dilute solution of ferric chloride (3 drops) was then added. The occurrence of black-blue or black-green shows the presence of tannins (Evans, 1996).

Lead Sub acetate rest:

About 1ml of the aqueous methanol extract was diluted with 2ml of distilled water. The dilute solution was mixed with lead sub acetate solution. Appearance of a light brown precipitate indicates the presence of tannins (Evans, 1996).

3.5.6 Test for Steroids and Terpenoids

Liebermann-Burchard‘s test:

About 0.5g of the powdered plant material was extracted with 5ml of methanol and then filtered. The filtrate was evaporated to dryness on water bath. The residue was shaken with chloroform and filtered into a clean and dry test tube. 2ml of acetic acid anhydride was added to the filterate and shaken. 1ml of concentrated sulphuric acid was added carefully down the side of the tube to form a lower layer. A brownish-red or violet ring at the zone of contact of the two liquids and the upper layer turning green denotes the presence of sterols and terpenes (Cuilei, 1990).

3.5.7 Test for Alkaloids

About 1ml of the extract of *R.longiflora* was treated with strong ammonia to make alkaline (pH 8) and then extracted twice with 10ml portions of chloroform. The chloroform extracts were combined and concentrated in vacuum to about 5ml. The concentrate was then tested with the following reagents; Dragendorff, Meyer, picric acid and Wagner. The presence of precipitate in most or all of the reagent tests indicates the presence of alkaloids (Evans, 1989), that is;

- Light brown to brownish precipitate - Wagner’s reagent
- White or creamy white precipitate - ‘Mayer’s reagent
- Orange or orange-yellow precipitate -Dragendorff’s reagent

- Yellowish precipitate - picric acid reagent.

3.5.8 Test for cyanogenic glycosides

Gurnard test was conducted; about 05g of the powered drug was mixed with 0.1ml of water in a test tube. A prepared damp sodium picrate paper was suspended at the mouth of the test tube by means of a cork. The test tube was placed in a water bath and allowed for an hour. Absence of brick red colour on the picrate paper indicates the absence of cyanogenic glycoside (Silva l *et al*, 1998).

3.6. Lethal Dose (LD_{50}) Determination in Rats and Mice

LD_{50} determinations were conducted using Lorke's methods (1983) for intraperitoneal and oral routes in mice and rats. This method was carried out in two phases. In the initial phase, 3 groups each containing three mice/rats was treated with methanolic leaf extract of the plant at doses of 10,100 and 1000mg/kg body weight i.p/orally and were observed for signs of toxicity and death for 24 hours. In the second phase, 4 groups each containing one mouse/rat were administered with four more specific doses of the extract 1200mg, 1600mg, 2900mg, and 5000mg/kg body weight based on the result of phase one (initial phase). The LD_{50} value was determined by calculating the geometric mean of the animal survived (0/1 and 1/1).

i.e. the highest non lethal dose (that did not cause death) and the lowest lethal dose for which the rat died.

$LD_{50} = \sqrt{}$ (Highest non lethal dose) × (Lowest lethal dose)

3.7 ANALGESIC STUDIES

3.7.1 Acetic Acid Induced Writhing Test in Mice

This was conducted using the method described by Koster *et al*, (1959). Swiss albino mice were

divided into 6 groups each containing 6 mice. Groups 1 was given 50mg/kg, group 2 was given 250mg/kg, group 3 was given 500mg/kg, and group 4 was given 1000mg/kg of the extract orally; the fifth (5^{th}) group which served as the control was given normal saline equivalent to the vehicle given with the extract orally. The 6^{th} group was given 10mg piroxicam per kg body weight orally as positive control. After 60 mins, mice in all the groups were treated with 0.6% acetic acid (10ml per kg body weight i.p). Five minutes after the acetic acid injection, the mice were placed in individual cages and the numbers of abdominal contractions were counted for each mouse for a period of 10mins after 5 mins latency period using a tally counter. Percentage inhibition of writhing was calculated using the formula:

$$\% \text{ inhibition: } = \frac{\text{Mean number of writhing (control)} - \text{Mean number of writhing (test)}}{\text{Mean number of writhing (control)}} \times 100\%$$

3.7.2 Hot Plate Test Method in Mice

The method described by (Lanhers *et al* (1992) and modified by (Mohamed and Ojewole (2004) was employed. A 600ml test beaker was placed on a regulated thermostat hot plate at $55^0C \pm 1^0C$.

Each mouse was placed in the beaker (on the hot plate) and the response to electrical heat-induced nociceptive pain stimulus obtained. Licking of the paws or jumping out of the beaker was taken as indicator of the animals' response to heat-induced nociceptive pain stimulus. The time taken for each mouse to lick its paws or jump out of the beaker was noted (reaction time). Each mouse served as its own control. Before treatment, its reaction time was taken thrice at 1 hour interval and the mean represents reaction time before treatment of the mouse.

The mean reaction time of the groups was pooled to obtain the final control mean reaction time (Tb).

The mice were divided into 6 groups of 6 mice each, groups 1, 2, 3, and 4 received 50mg/kg, 250mg/kg, 500mg/kg, and 1000mg/kg, of the extract orally respectively while group 5 served as the control that received normal saline and group 6 received the standard drug (5mg/kg morphine

sulphate) subcutaneously.

One hour after treatment; the reaction time of each mouse was again evaluated but only once. The reaction time of the mice in each treatment group and final test mean value (Ta) for each treatment group was calculated using the formula;

$$\% \text{ protection against thermal pain stimulus} = \frac{\text{Treat mean (Ta)- control mean(Tb)}}{\text{Control mean (Tb)}} \times 100$$

3.7.3 Formalin Test in Rats

The method described by Dubuisson and Dennis (1977) and modified by Tjolsen *et al.,* (1992) was used. Adult Wistar rats were divided into 6 groups each containing five rats. Groups 1, 2, 3, and 4 received 50mg/kg, 250mg/kg, 500mg/kg, and 1000mg/kg of the extract orally respectively; the 5th group served as negative control and was given normal saline equivalent to vehicle given with the extract. The 6th group was given 5mg Morphine per kg body weight intraperitoneally (i.p).

One hour (60 minutes) after the treatment above, 50microlitre (0.05ml) of a freshly prepared 2.5% solution of formalin was injected subcutaneously under the planter surface of the left hind paw of each rat. They were placed in an observation chamber and monitored for one hour. Pain responses were scored according to a pain scale. The severity of pain response was recorded for each rat based on the following scale:

0 = rat walked or stood firmly on the injected paw

1 = the injected paw was favoured or partially elevated

2 = the injected paw was clearly lifted off the floor

3 = the rat licked, chewed or shook the injected paw.

Each animal was placed in such a way as to ensure un-obstructed view of the injected paw. Anti-nociceptive effect was determined in two phases;

The early phase (phase1) was recorded in the first 5 minutes while the late phase (phase 2) was recorded during the last 45minutes with a 10 minutes lag period in between both phases.

The early phase indicates the neurogenic pain responses while the late phase indicates the inflammatory pain responses (Huskaar and Hole, 1985).

3.8 ANTI-INFLAMMATORY STUDIES

Carrageenan-induced paw oedema:

The method described by (Winter et al, 1962) was used. Wistar rats of either sex were fasted for 12 hours and deprived of water and were grouped into 6 with each containing 5 rats. Groups 1, 2, 3 and 4 received 50mg/kg, 250mg/kg, 500mgkg, and 1000mg/kg of the extract per kg body weight by oral administration respectively. The 5^{th} group served as control and was given normal saline, while the 6^{th} group was given piroxicam (10mg/kg) body weight orally. One hour later, 0.1ml of freshly prepared Carrageenan suspension (1% w/v in 0.9% normal saline) was injected into the sub planter region of the left hind paw of each rat. The paw diameter was measured with the aid of a vernier calliper at 0, 1, 2, 3, 4, 5 hours, after the injection of Carrageenan.

3.9 TOXICOLOGICAL STUDIES

3.9.1 Sub-Chronic (4 Weeks or 30 Days) Toxicity Studies

Four test doses of the extract within 20% range of the LD_{50} were administered orally to four groups of rats (15 rats per group). Group 1, 2, 3 and 4 received 50mg/kg, 250mg/kg, 500mg/kg and 1000mg/kg respectively, daily for 30days. The control group was administered distilled water which served as vehicle in preparing the extract. On the 31^{st} day 5 rats were removed from each group, and 4.0ml of blood samples were collected into plain containers by cardiac puncture under chloroform anaesthesia from each rat. The blood samples were allowed to clot for two hours, centrifuged at 4000 Revolution per Minutes (rpm) for 10 minutes and serum separated for kidney and liver

function tests. The rest of the rats were disposed or slaughtered by cervical dislocation under the influence of chloroform anaesthesia.

3.9.2 Chronic (12 Weeks or 90 Days) Toxicity Studies

Four test doses of the extract within 20% range of the LD_{50} were administered orally to four groups of rats (10 rats per group from the original 15 rats). Group 1, 2, 3 and 4 received 50mg/kg, 250mg/kg, 500mg/kg and 1000mg/kg respectively, daily for 90 days. The control group was administered distilled water which served as vehicle in preparing the extract. On the 91st day, 5 rats were removed from each group, and 4.0ml of blood samples were collected into plain containers by cardiac puncture under chloroform anesthesia from each rat. The blood samples were allowed to clot for two hours, centrifuged at 4000 Revolution per Minutes (rpm) for 10 minutes and serum separated for both liver and kidney function tests. The same methods and procedures were used for both liver and kidney function tests in Chemical Pathology Department of ABUTH Shika.

3.9.3 Kidney Function Test

Serum sodium and potassium levels were determined by flame photometry method, chloride, urea and creatinine levels were determined according to methods used by Department of Chemical Pathology, Ahmadu Bello University Teaching Hospital, Zaria (Norbert,. 1986), (Edmund,. *et al*, 2006) These methods were as follows:

3.9.3.1 Determination of Sodium and Potassium

0.1 ml of the serum was added into universal bottle and 9.9m1 of distilled water added. 5ml of working standard and 5ml of distilled water added to separate universal bottles respectively. The test bottles were capped with parafilm and mixed by inversion.

To determine sodium the following procedure was followed. Sodium filter was adjusted to 590nm and the galvanometer was switched on. The gas supply was fully turned on and the flame ignited.

The air pressure was regulated to 101b/sq inch. The gas was adjusted smoothly to obtain discrete cones of flame. The galvanometer reading was then set at zero using the working standard and reset at zero. The test was read, checking the standard after 2-3 test readings. The amount of sodium in mmol/L was calculated as galvanometer reading × 2.

In the determinations of potassium, the potassium light filter was adjusted to 770nm. The galvanometer set with potassium working standard to 70 and the procedure continued as for sodium. The amount of potassium mm/L = galvanometer × 0.1.

3.9.3.2 Determination of Chloride

Titration method was used. 0.2rn1 of serum was added to 1 .8m1 of distilled water. 3 drops of diphenylcarbazone indicator was added and mixed. This was titrated with mecuric nitrate to violet-blue coloured end point. The percentage chloride was calculated by comparing the volume of test sample required to volume required of standard sample.

3.9.3.3 Determination of Urea

The test sample was prepared by adding 0.1 ml of serum to 1.0mI of distilled water.2ml of mixed colour reagent (0.02g/ml diacetylmonoxirne and 0.005g/ml thio-semicarbazide and of mixed acid (0.02g/ml ferric chloride in 85% phosphoric acid and 0.43%sulphuric acid) each was added. This is was thoroughly mixed and incubated at 100°C for 20 minutes, cooled and the resulting red mixture was read at 520nm. The urea level in mmol/L was calculated by comparing test sample to standard sample.

3.9.3.4 Determination of Creatinine

The test sample was prepared by adding 1ml of serum to 3ml of distilled water. 1ml of 10% sodium tungstate and 1 ml 2/3N sulphuric acid were added mixed well and centrifuged for 10 minutes. 3m1 of the supernatant was added to 0.75 N sodium hydroxide, 1ml of picric acid was added mixed and

allowed to stand for 1 5minutes (Jaffe's reaction). The resulting red colour was read at 520nm. The creatinine level in μmol/L was calculated by comparing test sample to standard sample.

3.10 Liver Function Test

The liver function test was carried out by the Department of Chemical Pathology in ABUTH Shika-Zaria using an automated machine called; 'Express Plus Clinical Chemistry Analyser, product of Germany'.

The liver enzymes that were analysed include;

Alkaline phosphatase(ALP), alanine aminotransferase(ALT), and aspartate aminotransferase (AST).

Alkaline Phosphatase (ALP):

ALP (IFCC) Reagent: - Bayer Diagnostics Alkaline Phosphatase (IFCC)

Principle: - This application is based on the kinetic procedure reported by Bowers and McComb, and by the International Federation of Clinical Chemistry (IFCC).

P-Nitrophenyl Phosphate + H_2O → p-nitrophenol + H_3PO4

Method: - 300μL of reagent volume was added to 6μL of sample volume and was incubated at 37^0C and readings were taken at every 60 seconds.

Alanine aminotransferase (ALT):

ALT (IFCC) Reagent: - Bayer Diagnostics ALT Reagent (IFCC)

Principle: - This application is → Pyruvate + L-glutamate

Pyruvate + NADH + H^+ → Lactate + NAD^+

Method: - 250μL of reagent volume was added to 25 μL sample volume and incubated at 37^0C and readings were taken at every 100 seconds.

Aspartate aminotransferase (AST):

AST (IFCC) Reagent: - Bayer Diagnostics AST Reagent (IFCC)

Principle: - This application is based on a modification of the methodology proposed by the IFCC.

L-aspartate + 2-oxoglutarae → oxalacetate + L-glutamate

Oxalacetate + NADH + H^+ → L-malate + NAD^+ + H_2O

Method: - 250µL of reagent volume was added to 25µL sample volume and incubated at 37^0C and readings were taken at every 100 seconds.

3.11 HISTOPATHOLOGICAL STUDIES

For the purpose of Histopathological studies, one rat from each group was sacrificed by cervical dislocation and fresh tissues/organs (kidneys, heart and liver) were obtained from both control and treated groups after 30 days of sub-chronic and 90 days of chronic studies and were stored in 10% formalin after careful gross examinations of the organs.

Tissues slices of 3cm thickness were cut and fixed in 10% buffered formalin saline and put in the automatic tissue processor and fixed in 10% buffered formalin-saline solution for 6 hours. They were then dehydrated for 2 hours in each of the ascending grades of alcohol- 70%, 90%, and 100% v/v. The dehydrated tissues were then cleaned with toluene for 2 hours after which the tissues were embedded in paraffin wax and left to cool. The blocks were then trimmed and sectioned on the microtone at 5 microns. The ribbons of sections were then floated in a warm water bath. Suitable section were selected, attached to slide and dried on a hot plate awaiting staining. Haematoxylin and Eosin stain (H and E stain) was used in staining the tissue. The sections were dewaxed in xylene, rehydrated in descending grades of alcohol 100%, 90%, and 70% v/v. They were then stained in haematoxylin for 5 minutes, differentiated in 1% acid alcohol, glued in Scott's tap water and stained with eosin for 3 minutes. They were then rinsed and dehydrated in ascending grades of alcohol: 70%, 90%, and 100% and finally dewaxed in xylene and mounted in a box. The slides were then examined microscopically for pathological lesions, following the method of Arthur and John, 1978.

3.12 ANALYSIS OF DATA

Data were analysed and expressed as means $\pm$SEM (Standard Error of Mean)

Difference between the control and treated groups were analysed using Students T-test. $P<0.05$ were considered to be statistically significant.

CHAPTER FOUR

RESULTS

4.1 PRELIMINARY PHYTOCHEMICAL SCREENING RESULT

4.1 Preliminary phytochemical screening result.

The preliminary phytochemical screening revealed the presence of cardiac glycoside, steroids/terpenoids, tannins, saponins, flavonoids, carbohydrate, and alkaloids but cyanogenic glycoside was absent.

Table 4.1 Phytochemical screening result

Constituents	Remarks
Cardiac glycoside	+
Steroids/terpenoids	+
Tannins	+
Saponins	++
Flavonoids:	
-flavones	+
-flavonols	+
-flavonones	+
Carbohydrate	+
Alkaloids	+
Cyanogenic glycoside	-

+ = Present
++= Highly present
- = Absent

4.2 RESULTS OF LETHAL DOSE (LD_{50})

The intraperitoneal and oral lethal dose values LD_{50} of the methanolic leaf extract of *Rothmannia longiflora* in mice and rats were found to be all above 5000mg/kg body weight.

Table 4.2 Lethal Dose (LD_{50}) values of the methanolic leaf extract of *R.longiflora*.

Route of administration	Animal species	LD50 values mg/kg body weight
Intraperitoneal	Mice	>5000

Oral	Mice	>5000
Intraperitoneal	Rats	>5000
Oral	Rats	>5000

4.3 ANALGESIC STUDIES

4.3.1 The effect of methanolic leaf extract of *R.longiflora* on acetic acid-induced writhing in mice.

The extract significantly (P<0.05) inhibited the number of acetic acid-induced abdominal writhes in mice dose dependently. The highest inhibition of abdominal constriction (64.9%) observed at 1000mg/kg was greater than that of Piroxicam (61.5%), the standard non-steroidal analgesic and anti-inflammatory drug used.

Table 4.3 The effect of the methanolic leaf extract of *R.longiflora* on abdominal constrictions induced by acetic acid in mice.

Treatment	Dose (mg/kg)	Mean abdominal writhes / % inhibition
Normal Saline	1ml (oral)	20.8±4.7
R.longiflora	50 (oral)	16.3±3.2 (21.6)
R.longiflora	250 (oral)	14.7±1.7 (29.3)
R.longiflora	500 (oral)	12.5±2.2 (39.9)
R.longiflora	1000 (oral)	7.3±2.2[a] (64.9)
Cap. Piroxicam	20 (oral)	8.0±3.0[a] (61.5)

Student T-test [a]P<0.05 shows significant statistical difference compared to control (Normal saline); n=6.
Each value is expressed as mean ± SEM.
Figures in parentheses represent percentage inhibition of abdominal constriction.

4.3.2 The effect of methanolic leaf extract of *R.longiflora* on thermally induced pain stimulus in mice.

The methanolic leaf extract of *R.longiflora* significantly (P<0.05) and dose-dependently protected the mice against thermally induced pain stimulus in mice but there was no significant thermal protection at the dose of 50mg/kg.

The 500mg dose of the *R.longiflora* extract offered more than 100% protection; 1000mg offered more than 200% and the standard drug morphine sulphate offered more than 300% protection against thermally induced pain stimulus in mice.

Table 4.4 The effect of methanolic leaf extract of *R.longiflora* on thermally induced pain stimulus in mice (Hot plate method)

Groups	Dose mg/kg (route of admin.)	Mean reaction time (sec)	Protection (%)
Control Group A (Untreated)	-	1.22±0.04	-
Control Group B (Normal Saline)	10ml (oral)	1.33±0.67	9.02
R.longiflora	50 (oral)	1.50±0.22	22.95
R.longiflora	250 (oral)	2.25±0.21[a]	84.43
R.longiflora	500 (oral)	2.83±0.80[a]	131.97
R.longiflora	1000 (oral)	4.33±0.25[a]	254.92
Morphine Sulphate	5 (i.p)	6.00±0.37[a]	391.80

Student T-test [a]P<0.05 shows significant statistical difference compared with the treated control (normal saline); n=6. Each value is expressed as mean ± SEM.

4.3.3 The effect of methanolic leaf extract of *R.longiflora* on formalin test in rats.

There was no significant inhibition of both the neurogenic pain (early phase) and inflammatory pain (late phase) at 50mg/kg and 250mg/kg doses of the extract. Similarly, the 50mg/kg dose and the control group gave the same value which signified zero protection from pain.

But the significant (P<0.05) inhibition of both the neurogenic (early phase) and inflammatory (late phase) pain was dose dependent at 500mg/kg and 1000mg/kg with highest inhibition at 1000mg/kg (53.33%). The significant (P<0.05) inhibition of the standard drug morphine sulphate at late phase doubled 46.67% that of the early phase 20%.

Table 4.5 Effect of methanolic leaf extract of *R.longiflora* on formalin test in rats.

Group	Dose (mg/kg) (Oral route)	Mean pain scores	
		Early phase (0-5min)	Late phase (15-60min)
Control	1ml/kg	3±0	3±0
R.longiflora	50	3±0	3±0
R.longiflora	250	2.8±0.20 (6.67)	2.8±0.20 (6.67)
R.longiflora	500	2.6±0.25[a] (13.33)	2.8±0.20[a] (6.67)
R.longiflora	1000	1.8±0.37[a] (40)	1.4±0.40[a] (53.33)
Morphine sulphate	5 (i.p)	2.4±0.25[a] (20)	1.6±0.51[a] (46.67)

Student T-test [a]P<0.05 significant statistical difference compared with the treated control (normal saline); n=5. Each value is expressed as Mean ± SEM.
Figures in parentheses are percentage protection.

4.4.1 Effect of methanolic leaf extract of *R.longiflora* on Carrageenan-induced paws oedema in rats.

The injection of 1% Carrageenan suspension in the paws of the negative control treated rat with Normal Saline produced a local oedema reaching its maximum at the third (3^{rd}) hour. The methanolic leaf extract of *R.longiflora* significantly (P<0.05) inhibited the paw oedema at doses of; 250mg/kg; 500mg/kg and 1000mg/kg (table 4.6 showed a dose-dependent inhibition). There was no significant (P>0.05) inhibition at dose 50mg/kg.

The percentage anti-inflammatory effects at the peak of Carrageenan-induced oedema at the 3rd hour were: 39.39%; 51.52%; 63.63% at respective doses of: 250mg/kg; 500mg/kg; 1000mg/kg compared to 69.70% for Piroxicam 20mg/kg, the standard anti-inflammatory agent.

4.4 ANTI-INFLAMMATORY STUDIES

Table 4.6 Effect of methanolic leaf extract of *R.longiflora* on Carrageenan-induced paws oedema in rats

Treatment group	Dose (mg/kg)	Oedema diameter (cm) /Time interval				
		1h	2h	3h	4h	5h
Normal Saline	10ml/kg	0.20±0.04	0.26±0.02	0.33±0.03	0.19±0.03	0.17±0.02
R.longiflora	50	0.20±0.01 (0)	0.25±0.02 (3.85)	0.30±0.02 (9.10)	0.14±0.01 (26.32)	0.11±0.01 (35.30)
R.longiflora	250	0.19±0.03 (5.00)	0.16±0.03 (38.46)	0.20±0.03[a] (39.39)	0.09±0.02[a] (52.63)	0.07±0.03[a] (58.82)
R.longiflora	500	0.15±0.02 (25.00)	0.13±0.02[b] (50.00)	0.16±0.02[b] (51.52)	0.05±0.02[c] (73.68)	0.05±0.01[c] (70.59)
R.longiflora	1000	0.12±0.01[b] (40.00)	0.10±0.02[c] (61.54)	0.12±0.01[c] (63.63)	0.04±0.02[c] (78.95)	0.04±0.01[c] (76.47)
Piroxicam	20	0.10±0.02[b] (50.00)	0.08±0.03[c] (69.23)	0.10±0.02[c] (69.70)	0.05±0.01[c] (73.68)	0.03±0.01[c] (82.35)

Student T-test [a]P<0.05; [b]P<0.01; [c]P<0.001 compared to control (Normal saline), n=5. Each value is expressed as mean ± SEM

Figures in parentheses represent percentage anti-inflammatory activity.

4.5 KIDNEY FUNCTION TEST

4.5.1The effect of intake of *R.longiflora* extract on kidney function test during sub-chronic

toxicity studies (4 weeks).

There was no statistically significant difference (P>0.05) of the levels of Sodium (Na^+), Potassium (K^+), Chloride (Cl^-) ions, Urea and Creatinine compared to that of the control at the end of 30 days studies. This showed that *R.longiflora* extract produced no adverse effect on kidney functions during sub-chronic administration for 30 days orally.

Table 4.7 Effect of intake of *R.longiflora* extract on kidney function test during sub- chronic toxicity studies (4 weeks)

Dose (mg/kg)	Na^+ (mmol/l)	K^+ (mmol/l)	Cl^- (mmol/l)	Urea (mmol/l)	Creatinine (µmol/l)
Control	138.40±0.20	3.96±0.16	99.60±1.70	3.66±0.32	71.60±3.70
50	139.00±1.30	3.94±0.14	98.80±0.80	3.56±0.30	63.80±2.40
250	138.40±0.51	4.26±0.11	99.20±0.49	4.16±0.37	76.20±2.52
500	138.00±1.52	3.92±0.20	96.40±1.47	4.50±0.15	73.00±2.78
1000	137.40±0.87	4.00±0.16	98.40±0.75	3.98±0.30	72.40±3.31

Values are Mean ± SEM, n=5
Student's T-test P>0.05

4.5.2 The effect of intake of *R.longiflora* extract on kidney function test during chronic toxicity studies (12 weeks).

There was no statistically significant difference (P>0.05) of the levels of Sodium (Na^+), Potassium (K^+), Chloride (Cl^-) ions, Urea and Creatinine compared to that of the control at the end of 90 days

studies. This showed that *R.longiflora* extract produced no adverse effect on kidney functions during sub-chronic administration for 90 days orally.

Table 4.8 Effect of intake of *R.longiflora* extract on some kidney function test during chronic toxicity studies (12 weeks)

Dose (mg/kg)	Na^+ (mmol/l)	K^+ (mmol/l)	Cl^- (mmol/l)	Urea (mmol/l)	Creatinine (µmol/l)
Control	139.00±0.63	4.10±0.11	99.60±0.75	4.22±0.19	63.60±2.52
50	137.80±1.02	3.98±0.21	98.40±0.75	4.00±0.15	61.20±2.69
250	139.40±1.40	3.94±0.22	99.00±1.41	4.18±0.26	67.20±1.77
500	137.80±1.28	3.88±0.14	98.20±0.80	4.30±0.23	69.60±1.12
1000	137.40±1.33	4.50±0.33	97.02±1.85	5.30±0.50	83.60±6.61

Values are Mean ± SEM, n=5
Student's T-test P>0.05

4.6 LIVER FUNCTION TEST

4.6.1 The effect of intake of *R.longiflora* extract on liver enzymes function test during sub-chronic toxicity studies (30 days)

There was no statistically significant difference (P>0.05) in the levels of AST, ALT, and ALP enzymes in all the treated groups compared to the control group during the 30 days studies.

Table 4.9 Effect of intake of *R.longiflora* extract on liver enzymes function tests during sub-chronic toxicity studies (4 weeks)

Dose (mg/kg)	AST (IU/l)	ALT (IU/l)	ALP (IU/l)

Control	22.40±1.17	45.00±2.17	72.40±1.96
50	19.40±1.67	42.60±2.89	67.80±3.34
250	20.20±1.56	41.00±2.56	71.20±2.48
500	21.20±1.36	42.20±1.28	72.40±2.94
1000	22.40±1.33	41.60±3.78	73.20±3.64

Values are Mean ± SEM, n=5
Student's T-test P>0.05

4.6.2 The effect of intake of *R.longiflora* extract on liver enzymes function test during chronic toxicity studies (90 days)

There was no statistically significant difference (P>0.05) in the levels of AST, ALT, and ALP enzymes treated with 50 and 250mg (groups 1 and 2) compared to the control group during the 90 days studies. However there was statistically significant difference (P<0.05 and 0.001) in groups 3 and 4 treated with 500 and 1000mg respectively compared to the control.

Table 4.10 Effect of intake of *R.longiflora* extract on liver enzymes function test during chronic toxicity studies (12 weeks)

Dose (mg/kg)	AST (IU/l)	ALT (IU/l)	ALP (IU/l)
Control	53.20±5.95	64.60±5.87	64.80±3.14
50	59.60±2.40	74.60±3.30	63.80±1.77

250	61.40±8.13	79.00±4.02	68.40±1.25
500	94.40±2.40[b]	105.20±2.31[a]	77.80±4.65[a]
1000	98.40±1.72[b]	128.60±3.47[b]	88.60±7.66[a]

Values are Mean ± SEM, n=5
Student's T-test [a]P<0.05; [b]P<0.001

4.7 HISTOPATHOLOGICAL STUDIES

4.7.1 Histopathological studies during the sub-chronic studies (30 days) of administration of the crude extract of *R.longiflora.*

There was no statistically significant difference in the liver enzymes in the treated groups compared to the control group hence, the mild steatosis observed in 50, 500, and 1000mg/kg might not be attributed to the toxic effect of the methanolic leaf extract of *R.longiflora.*

Table 4.11 Histopathological results during sub-chronic toxicity studies (30 days) administration of the crude extract of *R.longiflora.*

Groups/Dose mg/kg	Liver	Kidney	Heart
Control	Normal	Normal	Normal
50	Mild steatosis	Normal	Normal
250	Normal	Normal	Normal
500	Mild steatosis	Normal	Normal
1000	Mild steatosis	Normal	Normal

4.7.2 Histopathological studies during the chronic toxicity studies (90 days) of administration of the crude extract of *R.longiflora.*

The liver showed mild steatosis in one of the two sections with 500mg after the period of 90 days (chronic toxicity studies) but one section that showed mild steatosis due to significant rise in liver enzymes alone might not be enough to justify the mild steatosis observed.

Table 4.12 Histopathological result during the chronic toxicity studies (90 days) of administration of the crude extract of *R.longiflora*

Groups/Dose mg/kg	Liver	Kidney	Heart
Control	Normal	Normal	Normal
50 I	Normal	Normal	Normal
II	Normal		
250 I	Normal	Normal	Normal
II	Normal		
500 I	Mild steatosis	Normal	Normal
II	Normal		
1000 I	Normal	Normal	Normal
II	Normal		

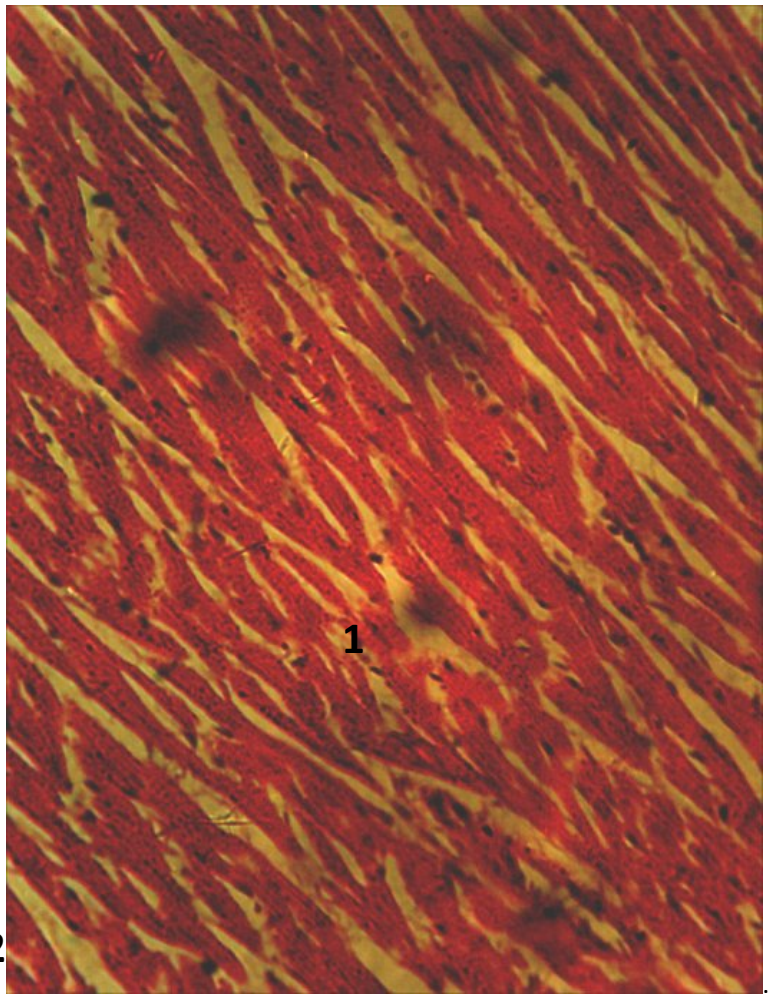


Plate II: Photomicrograph of a section of a normal heart from a rat treated with normal saline for 30 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

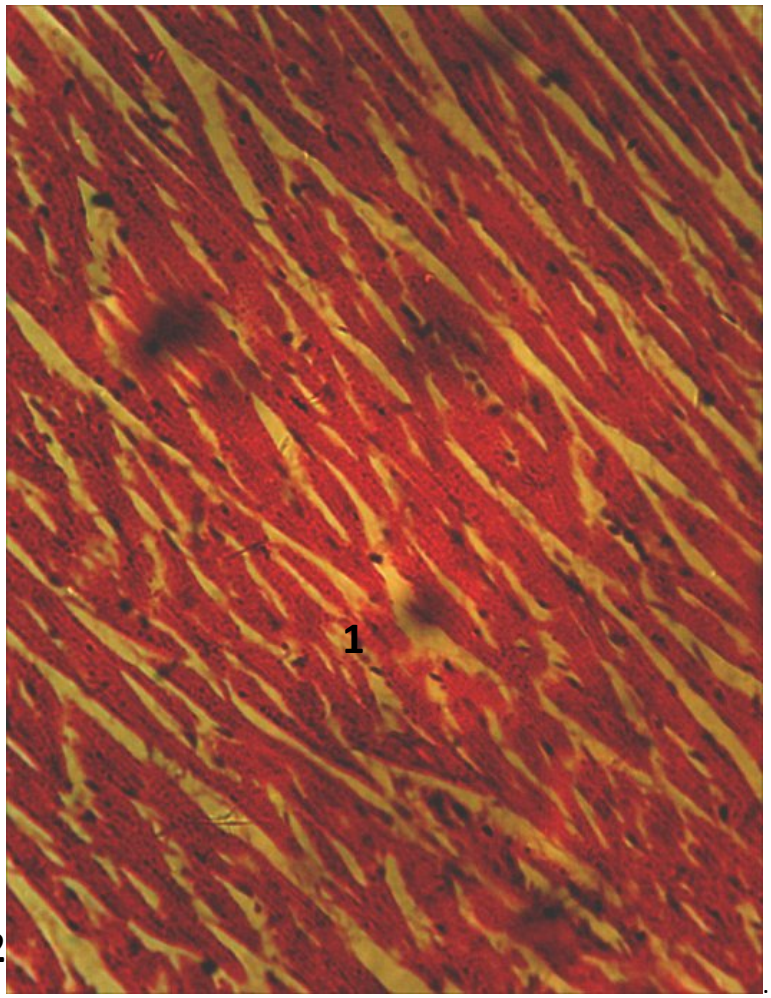


Plate III: Photomicrograph of a section of a normal heart from a rat treated with normal saline for 90 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

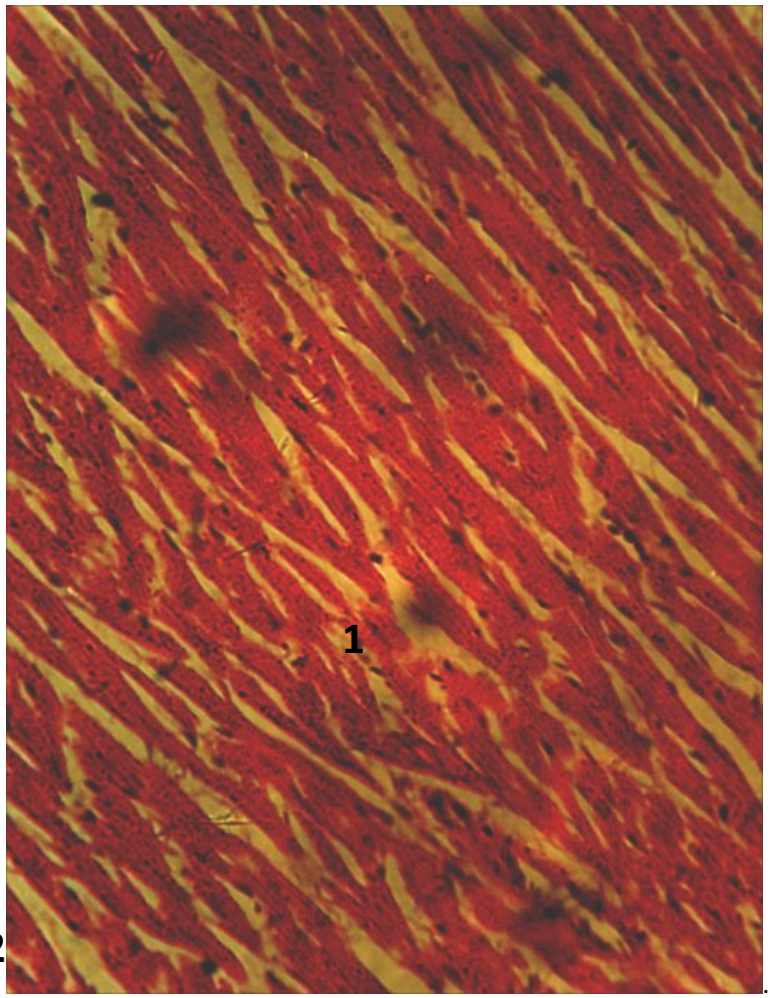


Plate IV: Photomicrograph of a section of a normal heart from a rat treated with 50mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

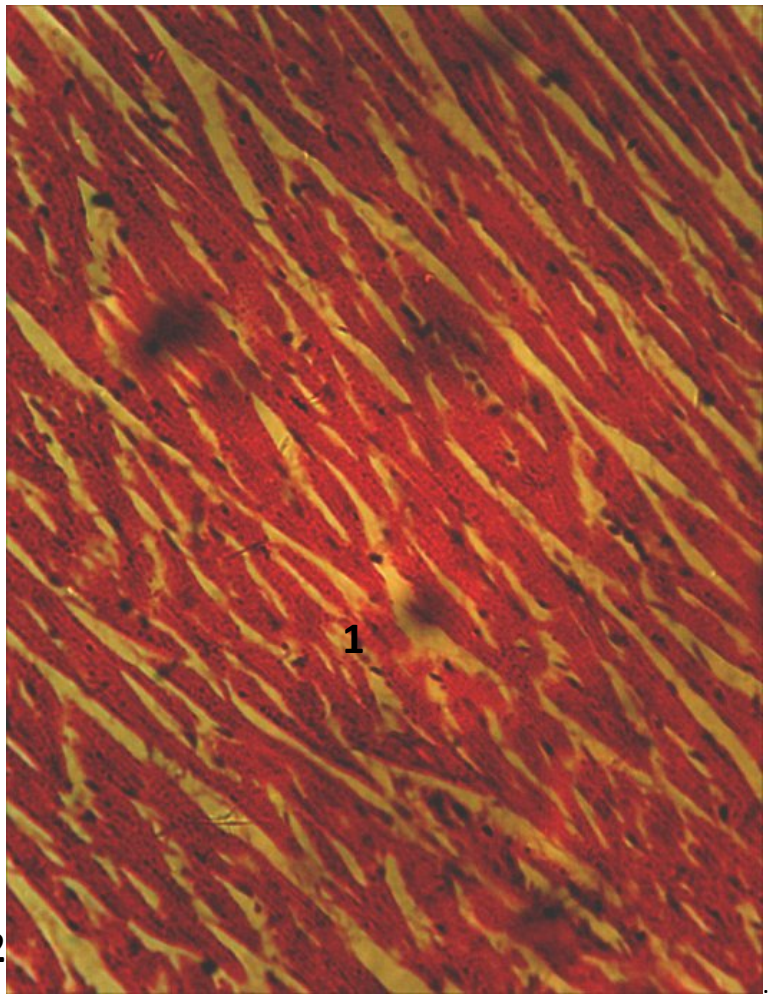


Plate V: Photomicrograph of a section of a normal heart from a rat treated with 50mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

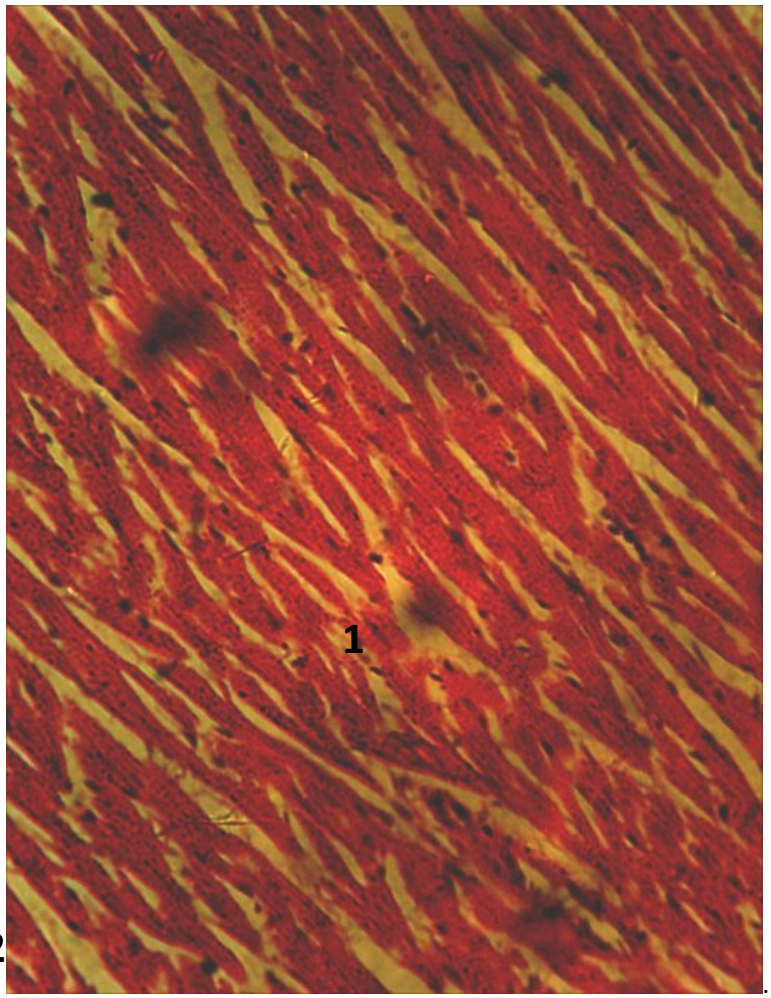


Plate VI: Photomicrograph of a section of a normal heart from a rat treated with 250mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

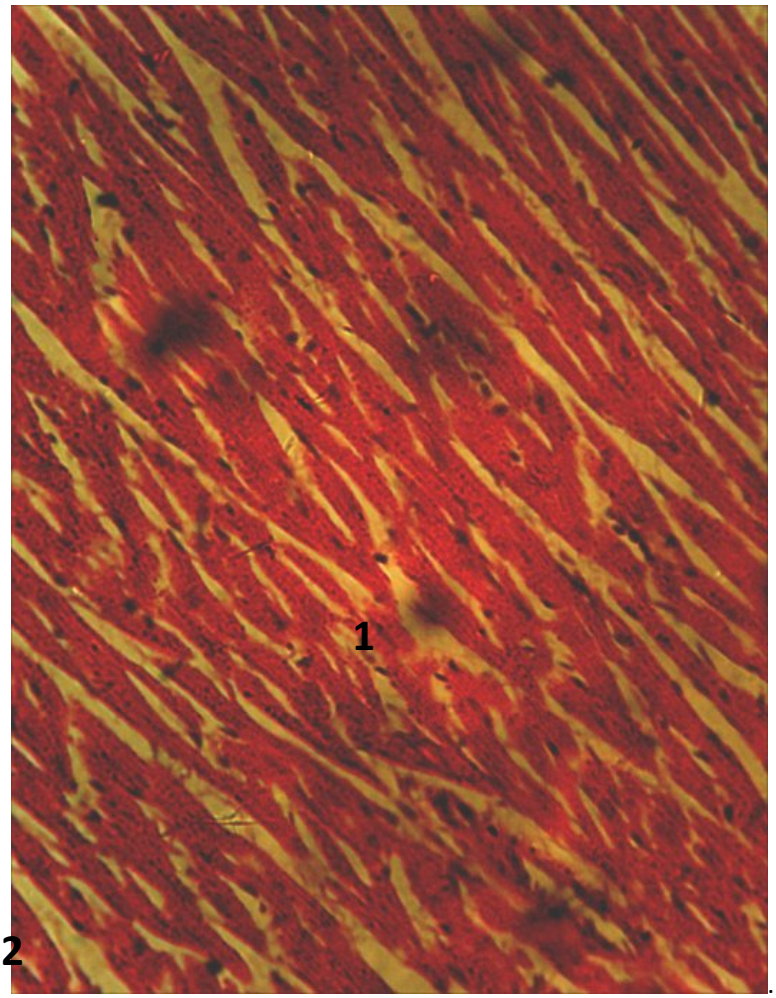


Plate VII: Photomicrograph of a section of a normal heart from a rat treated with 250mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

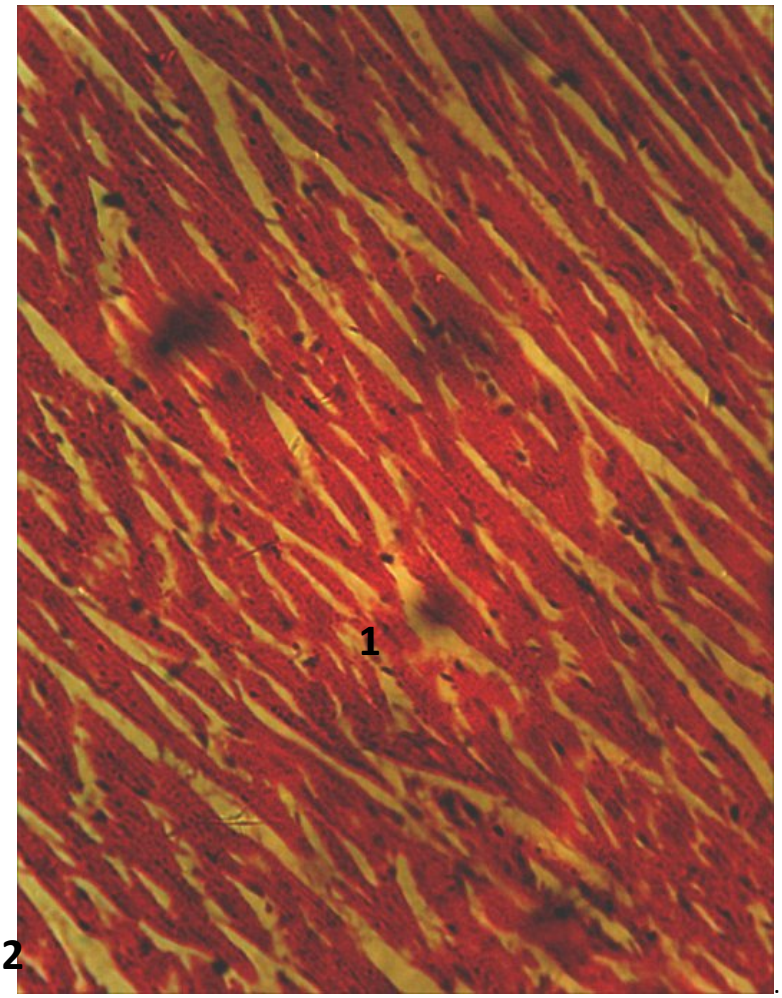


Plate VIII: Photomicrograph of a section of a normal heart from a rat treated with 500mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

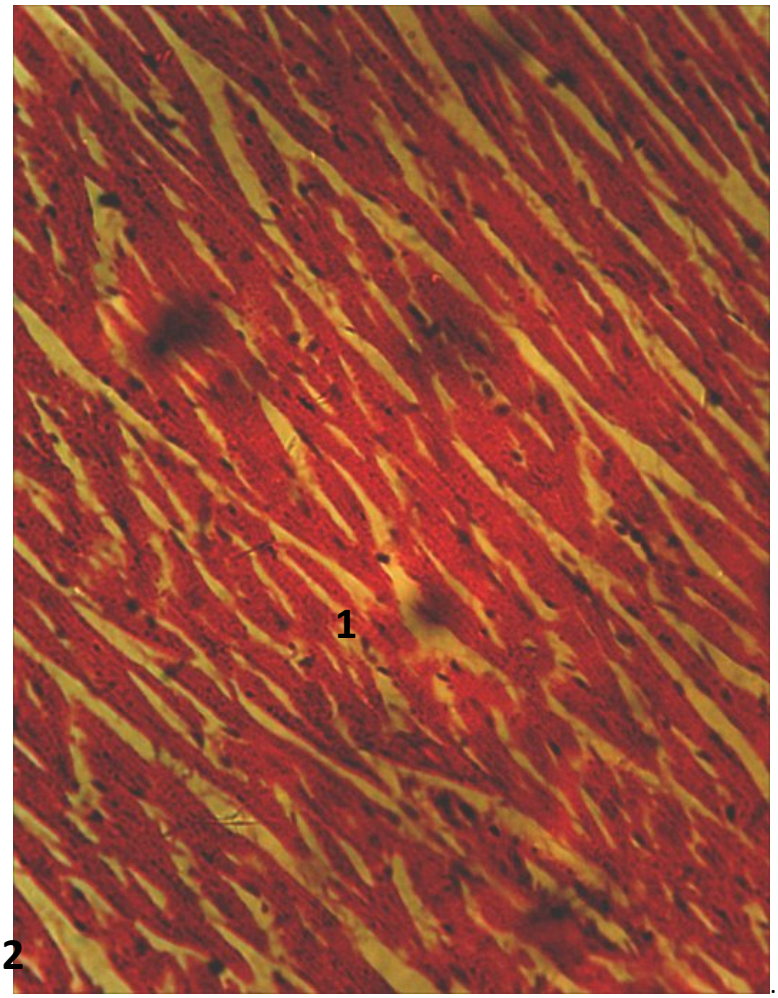


Plate IX: Photomicrograph of a section of a normal heart from a rat treated with 500mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

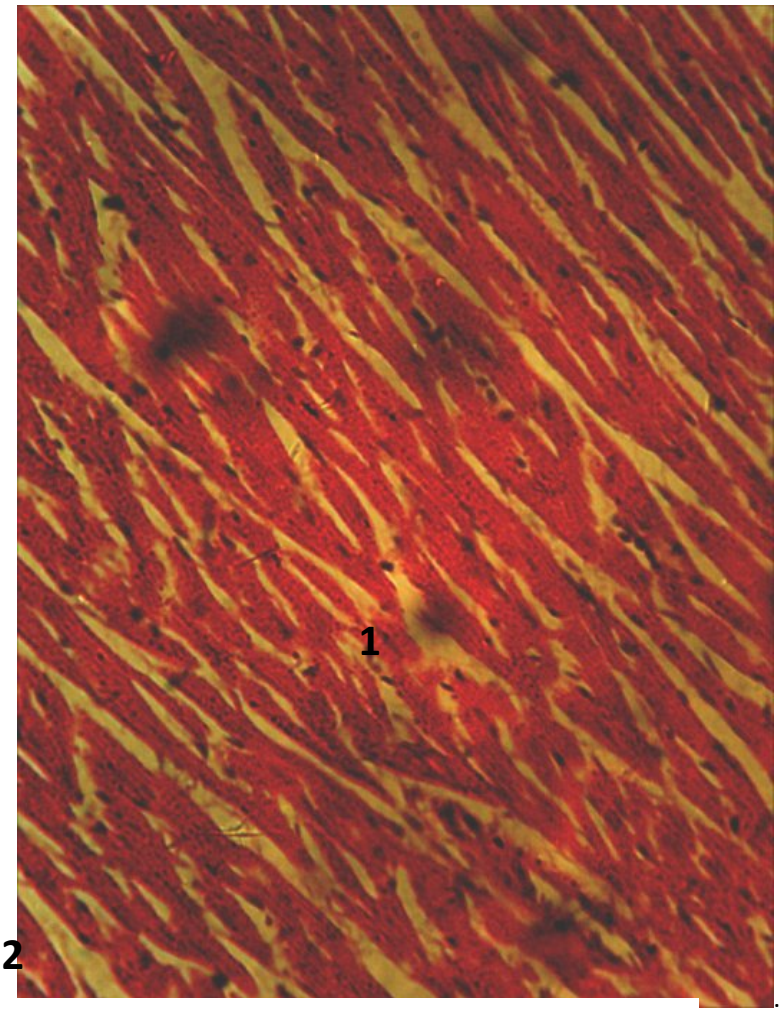


Plate X: Photomicrograph of a section of a normal heart from a rat treated with 1000mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

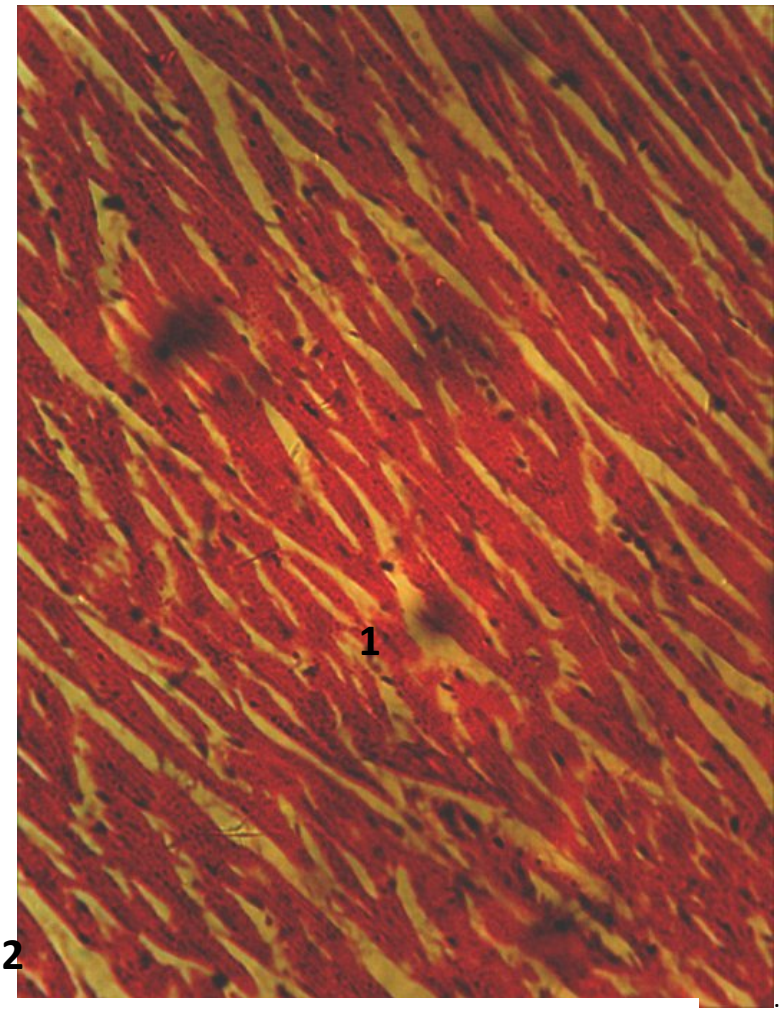


Plate XI: Photomicrograph of a section of a normal heart from a rat treated with 1000mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing the veins (1 & 2) of the cardiac muscles. H & E stain (×400)

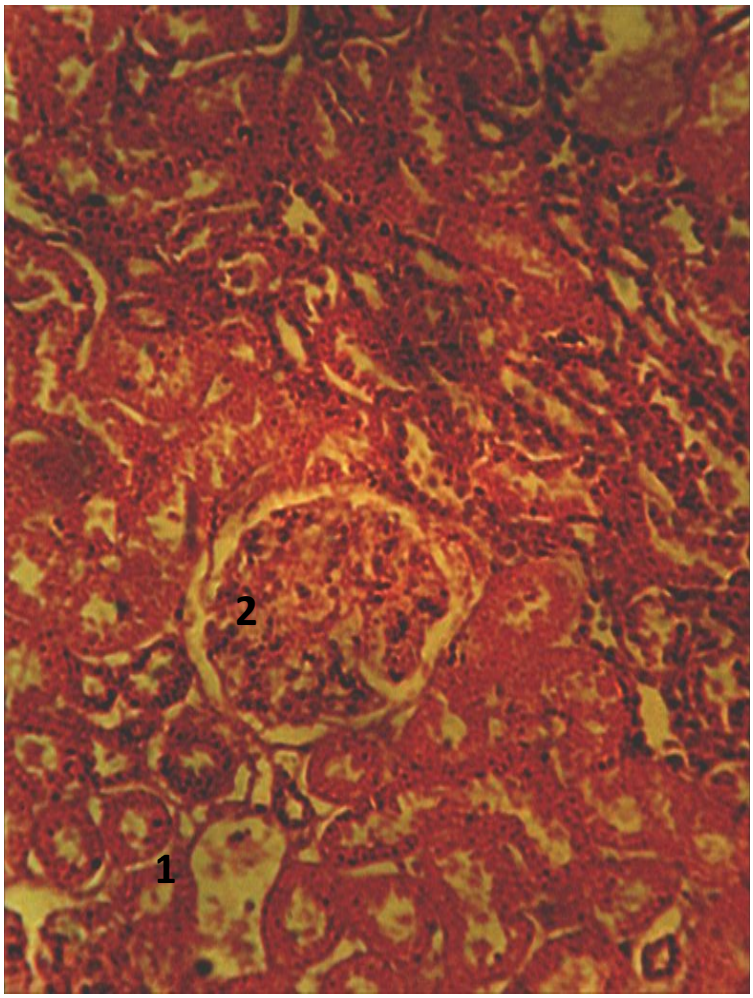


Plate XII: Photomicrograph of a section of a normal kidney from a rat treated with normal saline for 30 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

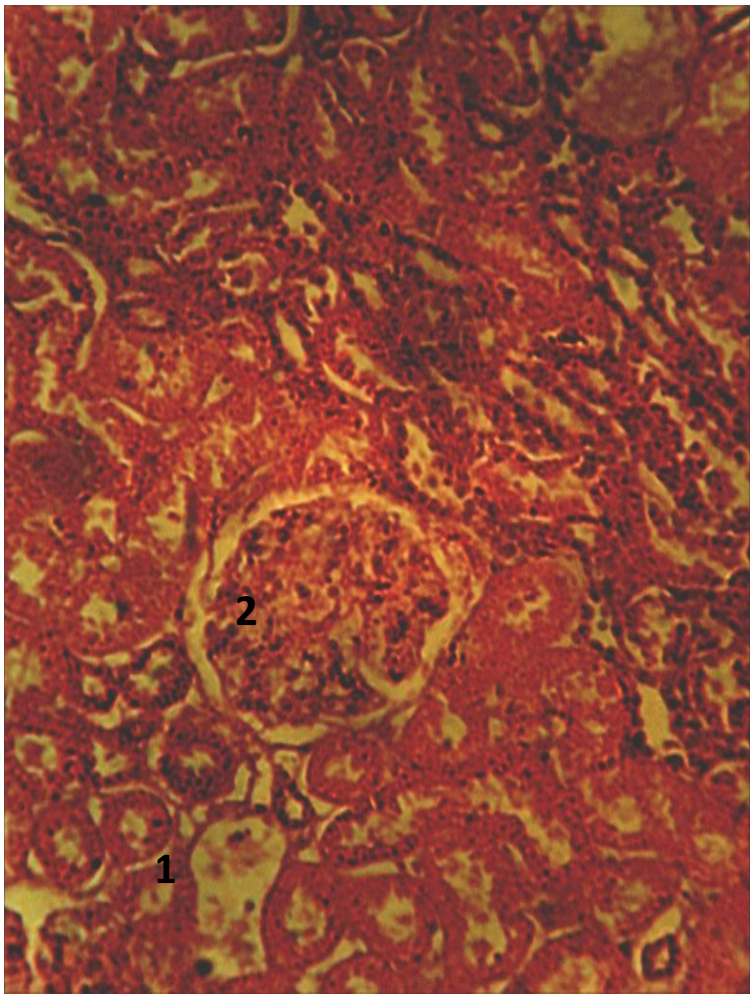


Plate XIII: Photomicrograph of a section of a normal kidney from a rat treated with normal saline for 90 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

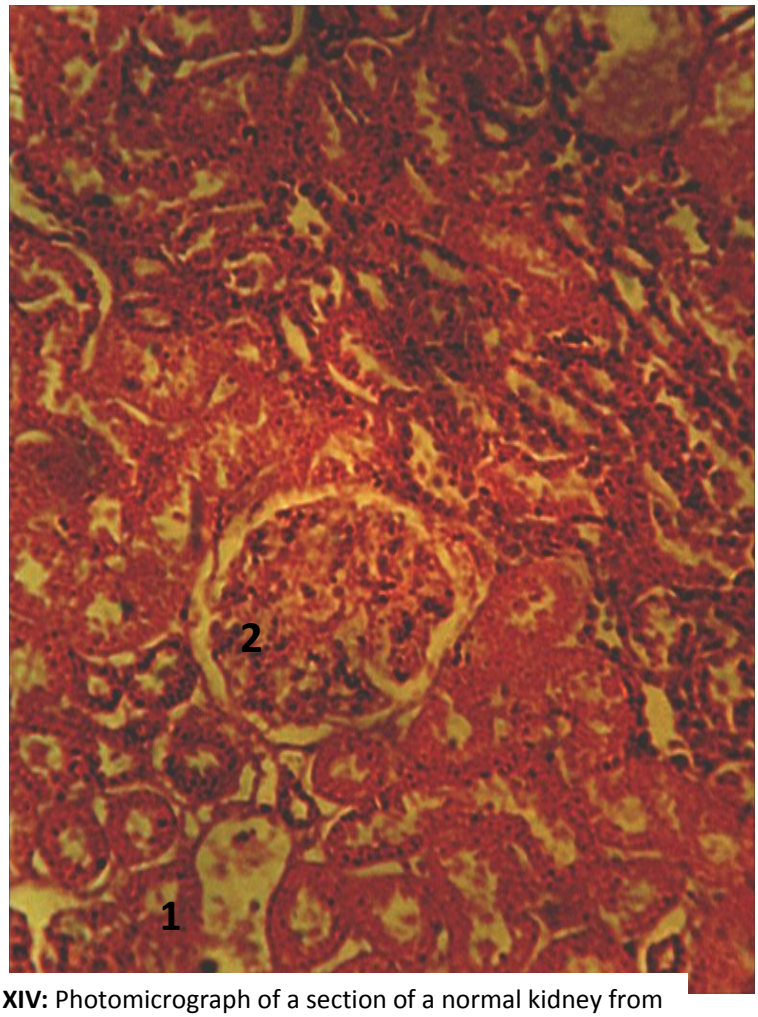


Plate XIV: Photomicrograph of a section of a normal kidney from a rat treated with 50mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

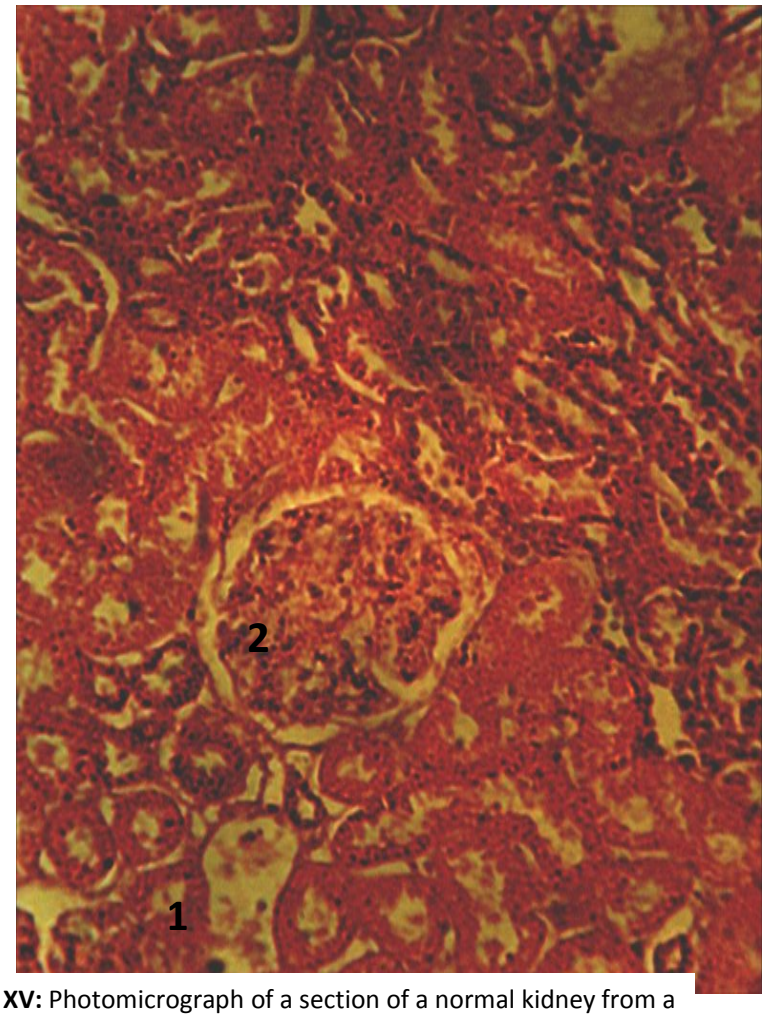


Plate XV: Photomicrograph of a section of a normal kidney from a rat treated with 50mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

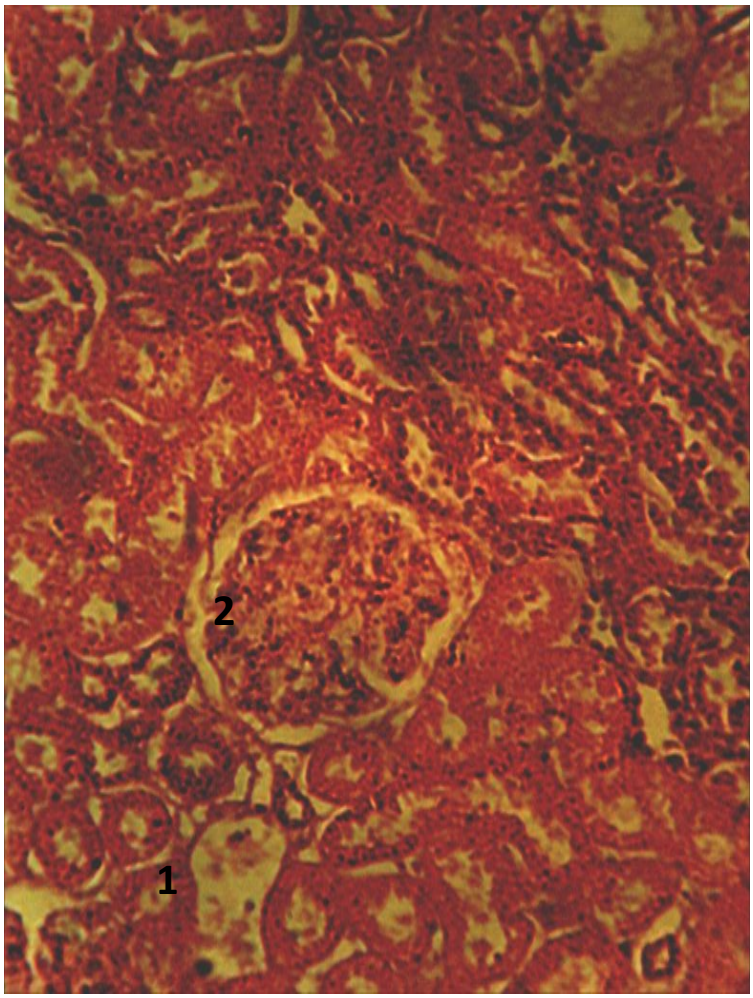


Plate XVI: Photomicrograph of a section of a normal kidney from a rat treated with 250mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

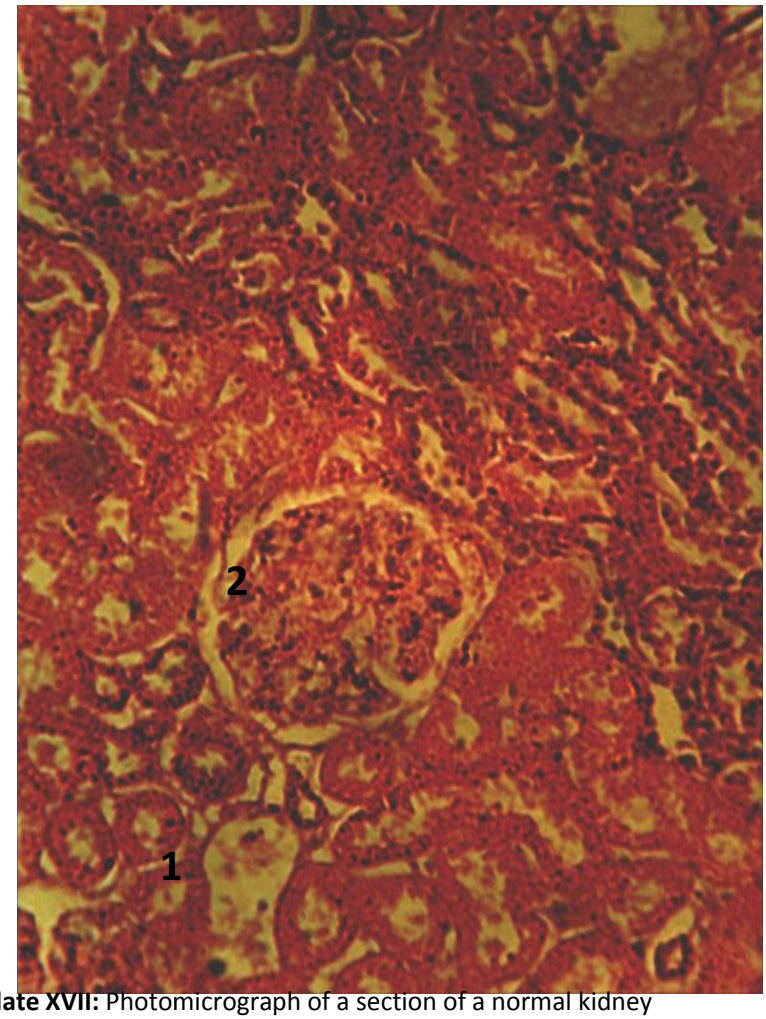


Plate XVII: Photomicrograph of a section of a normal kidney from a rat treated with 250mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

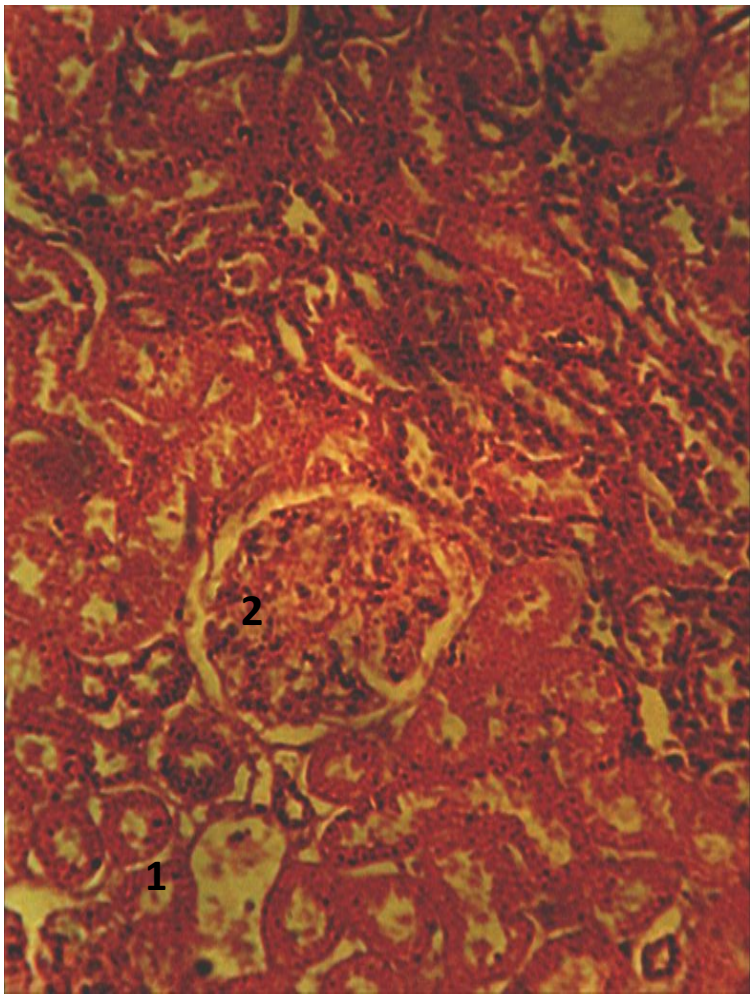


Plate XVIII: Photomicrograph of a section of a normal kidney from a rat treated with 500mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

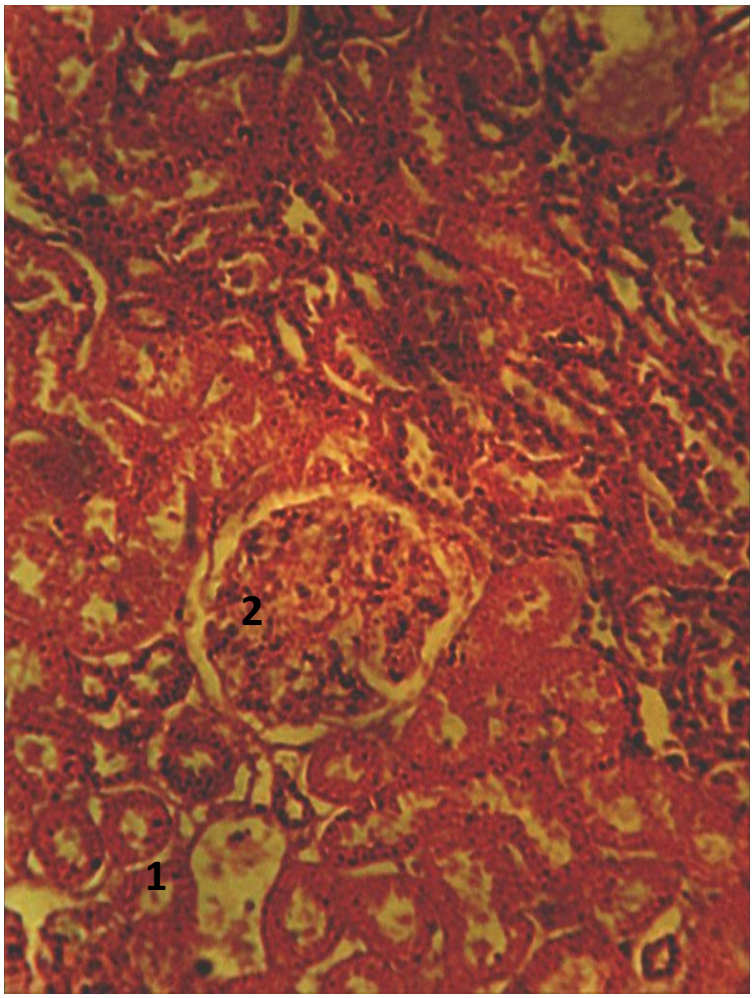


Plate XIX: Photomicrograph of a section of a normal kidney from a rat treated with 500mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

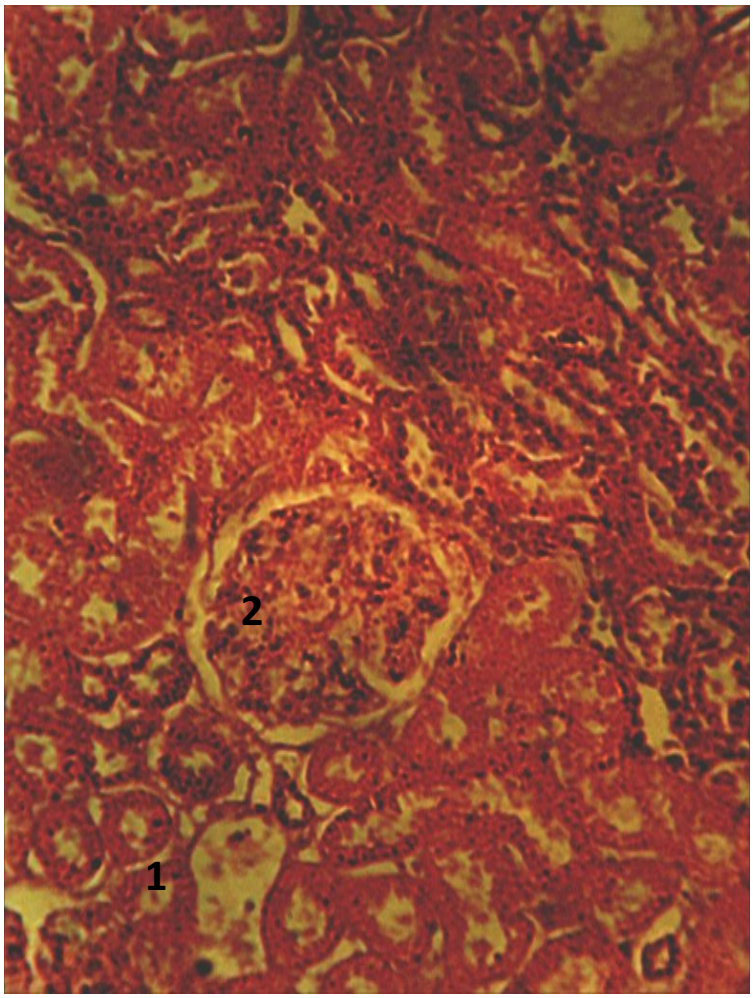


Plate XX: Photomicrograph of a section of a normal kidney from a rat treated with 1000mg/kg of methanolic leaf extract of *R. longiflora* for 30 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

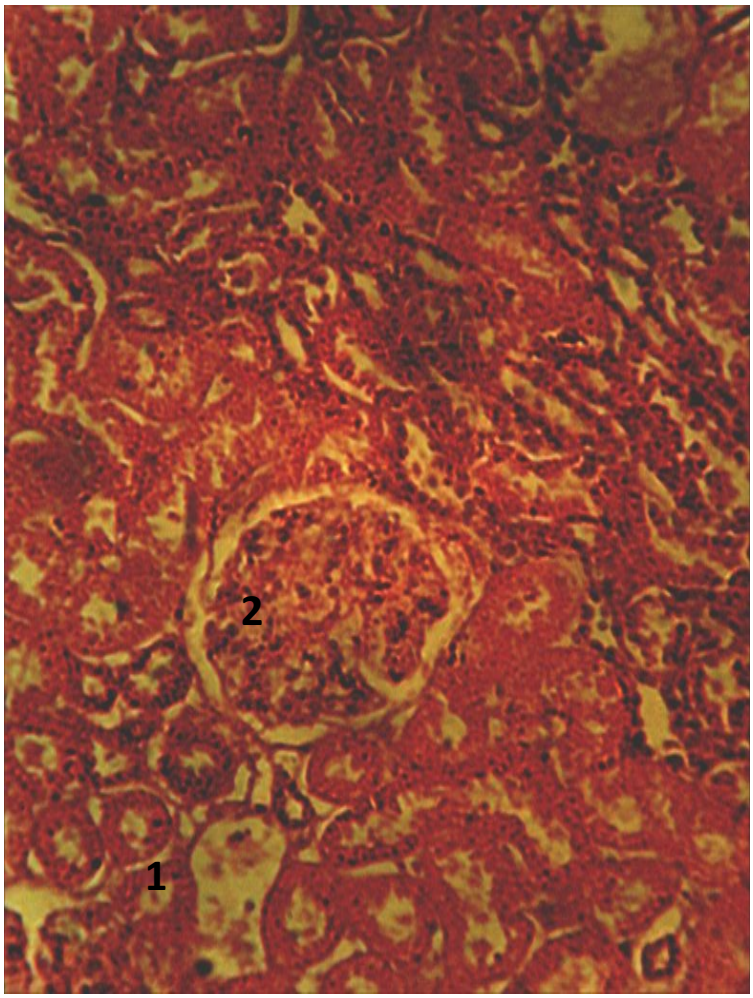


Plate XXI: Photomicrograph of a section of a normal kidney from a rat treated with 1000mg/kg of methanolic leaf extract of *R. longiflora* for 90 days showing (1 =tubule & 2 =glumerulus). H & E stain (×400)

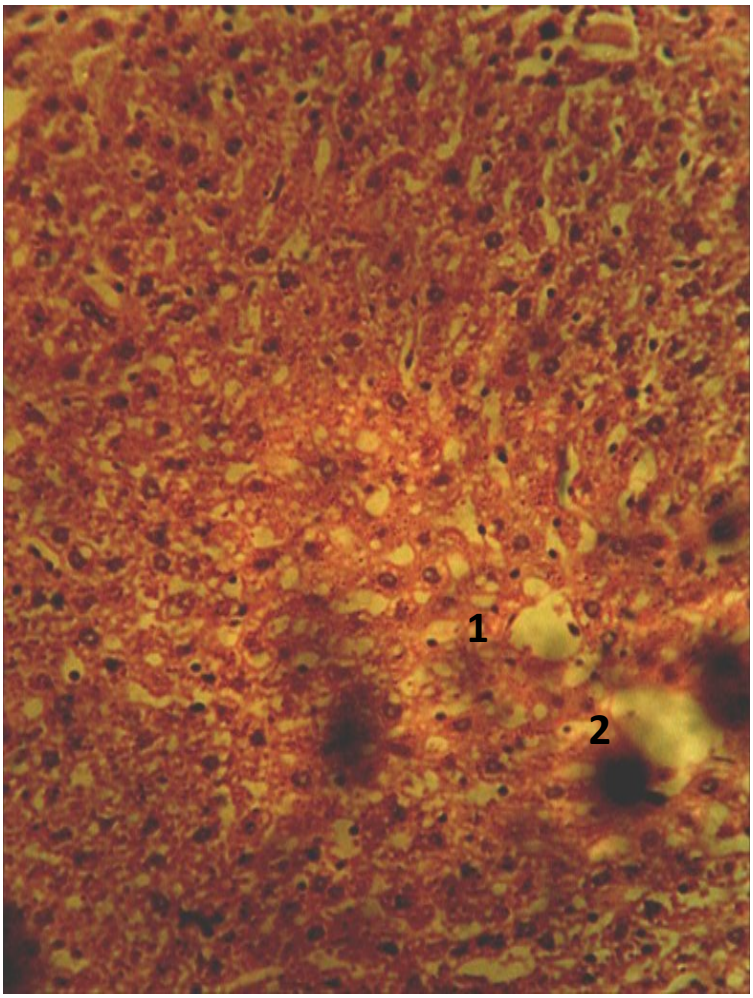


Plate XXII: Photomicrograph of a section of a normal liver from a rat treated with normal saline for 30 days. H & E stain (×400) 1 =Portal tract 2 = Central vein

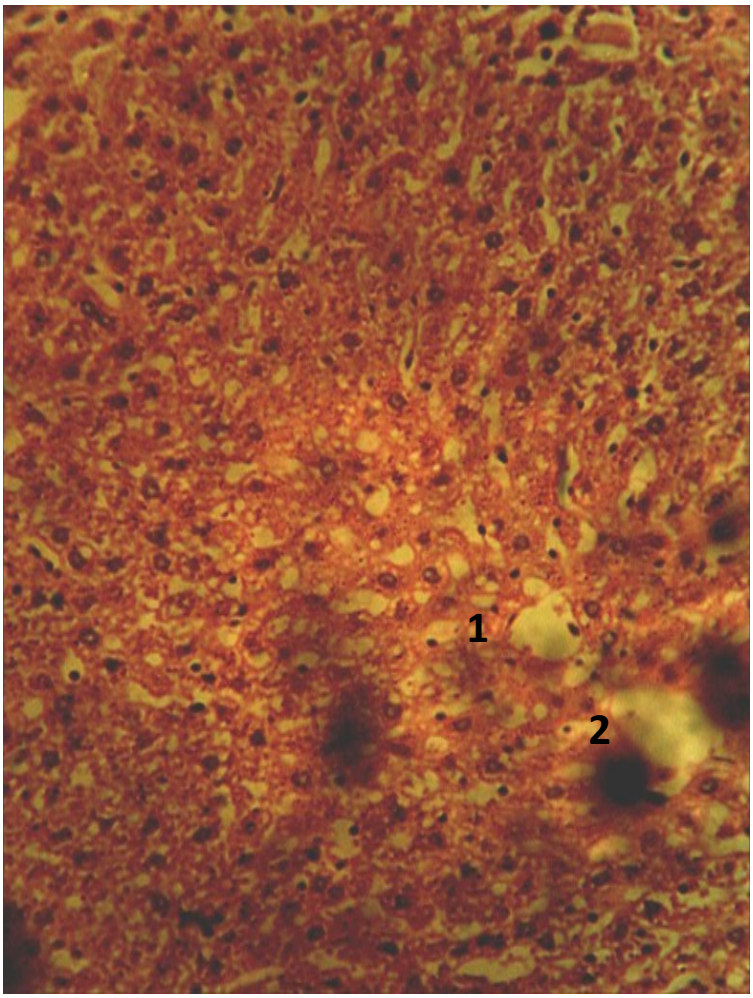


Plate XXIII: Photomicrograph of a section of a normal liver from a rat treated with normal saline for 90 days. H & E stain (×400) 1 =Portal tract 2 = Central vein

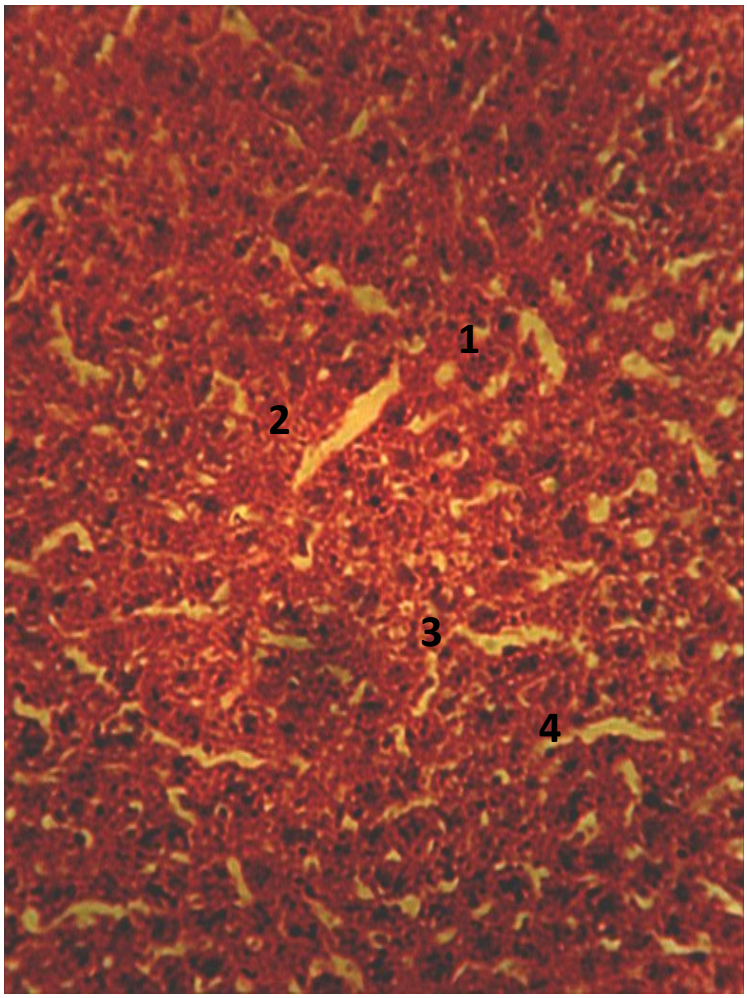


Plate XXIV: Photomicrograph of a section of a liver with mild steatosis from a rat treated with 50mg/kg of methanolic leaf extract of *R. longiflora* for 30 days. H & E stain (×400) 1, 2, 3 and 4 = Mild steatosis (mild fatty change)

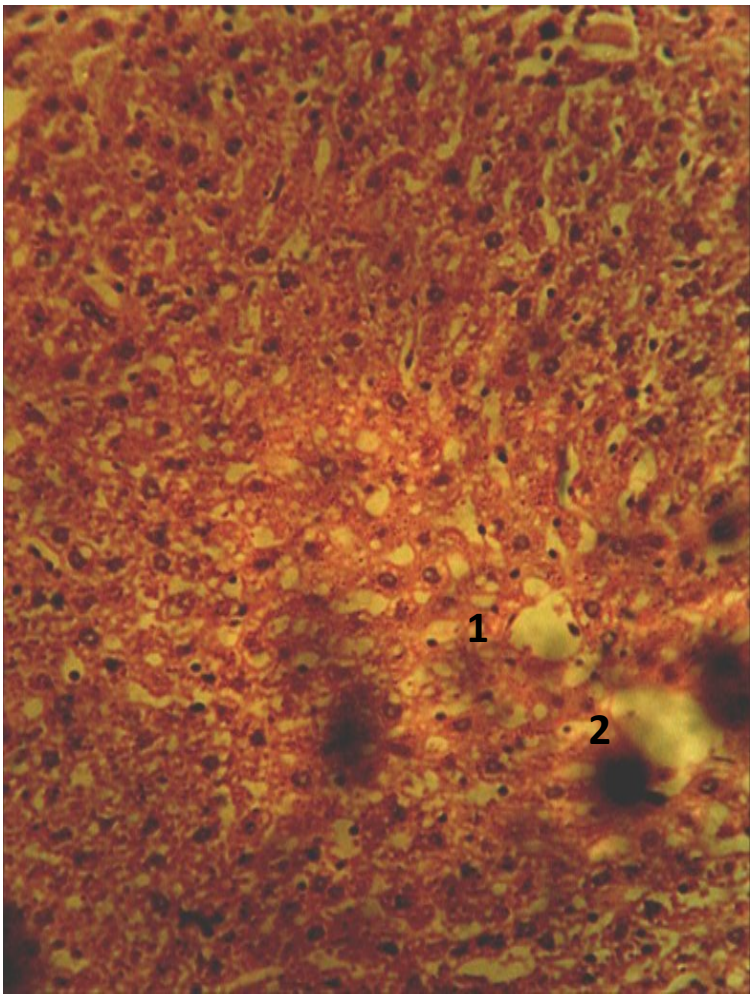


Plate XXV: Photomicrograph of a section of a normal liver from a rat treated with 50mg/kg of methanolic leaf extract of *R. longiflora* for 90 days. H & E stain (×400) 1 =Portal tract 2 = Central vein

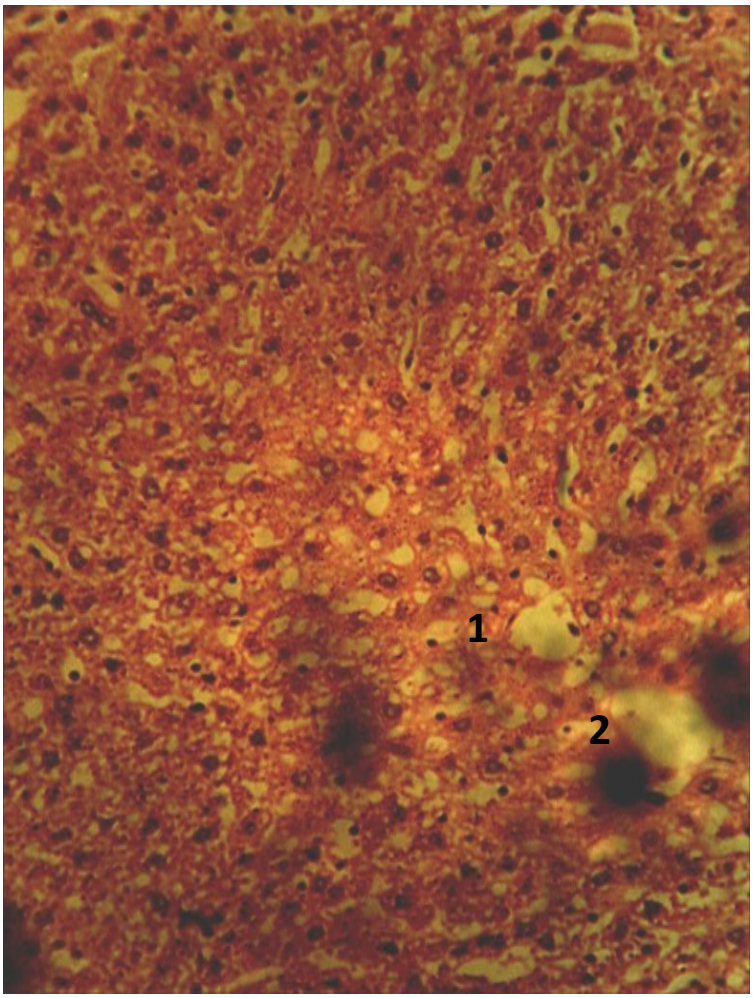


Plate XXVI: Photomicrograph of a section of a normal liver from a rat treated with 250mg/kg of methanolic leaf extract of *R. longiflora* for 30 days. H & E stain (×400) 1 =Portal tract 2 = Central vein

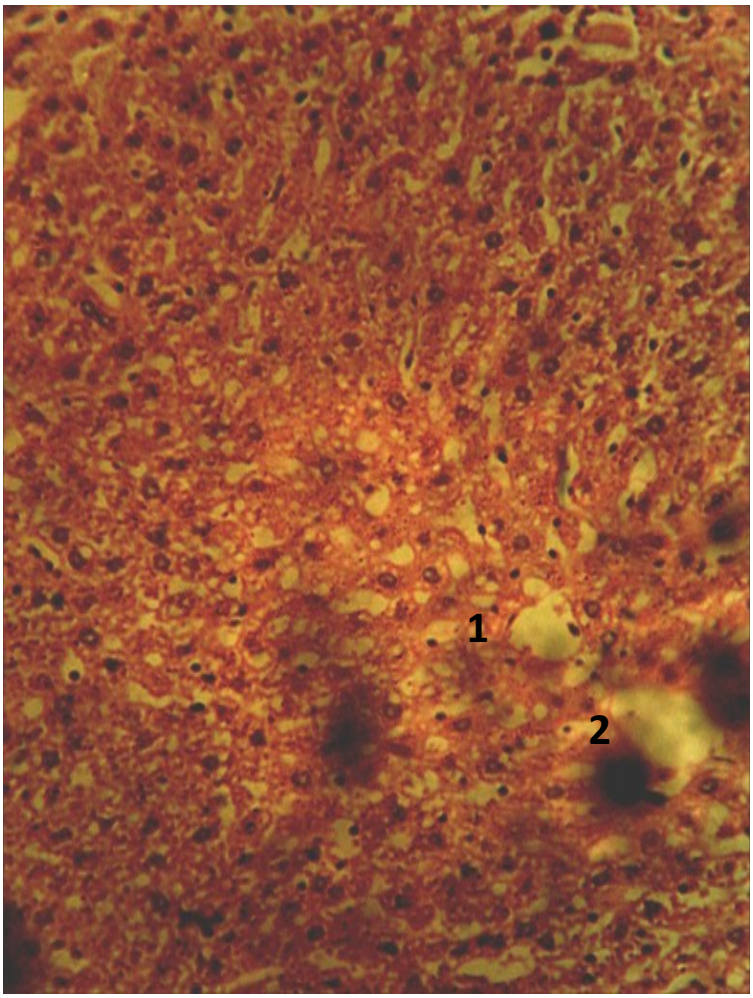


Plate XXVII: Photomicrograph of a section of a normal liver from a rat treated with 250mg/kg of methanolic leaf extract of *R. longiflora* for 90 days. H & E stain (×400) 1 =Portal tract 2 = Central vein

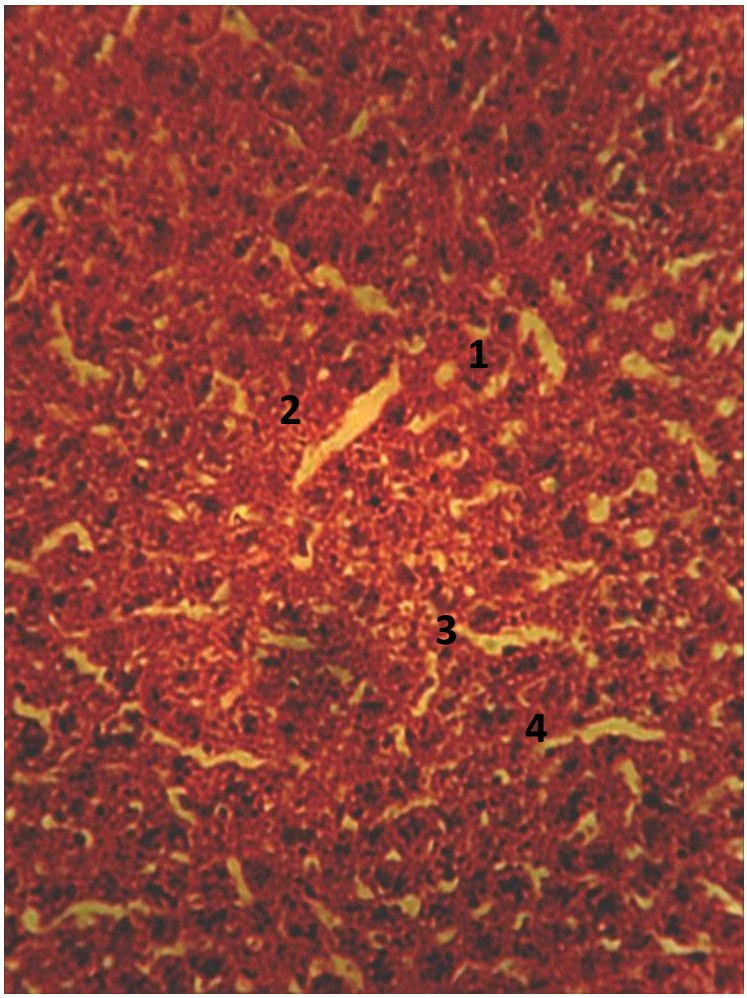


Plate XXVIII: Photomicrograph of a section of a liver with mild steatosis from a rat treated with 500mg/kg of methanolic leaf extract of *R. longiflora* for 30 days. H & E stain (×400) 1, 2, 3 and 4 = Mild steatosis (mild fatty change)

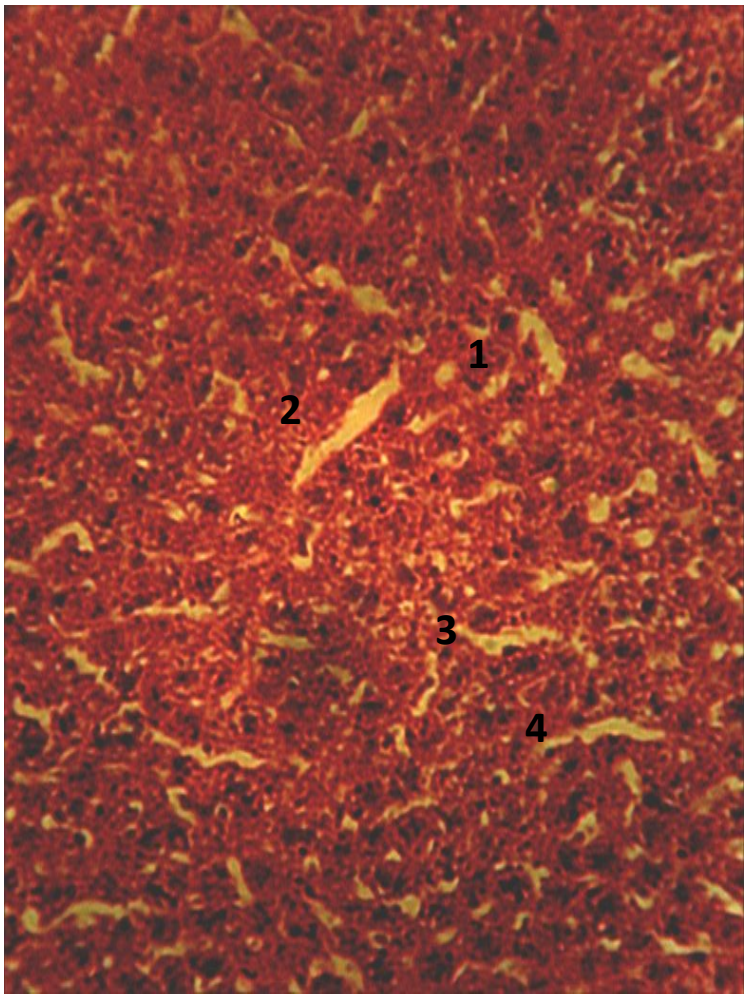


Plate XXIX: Photomicrograph of a section of a liver with mild steatosis from a rat treated with 500mg/kg of methanolic leaf extract of *R. longiflora* for 90 days. H & E stain (×400) 1, 2, 3 and 4 = Mild steatosis (mild fatty change)

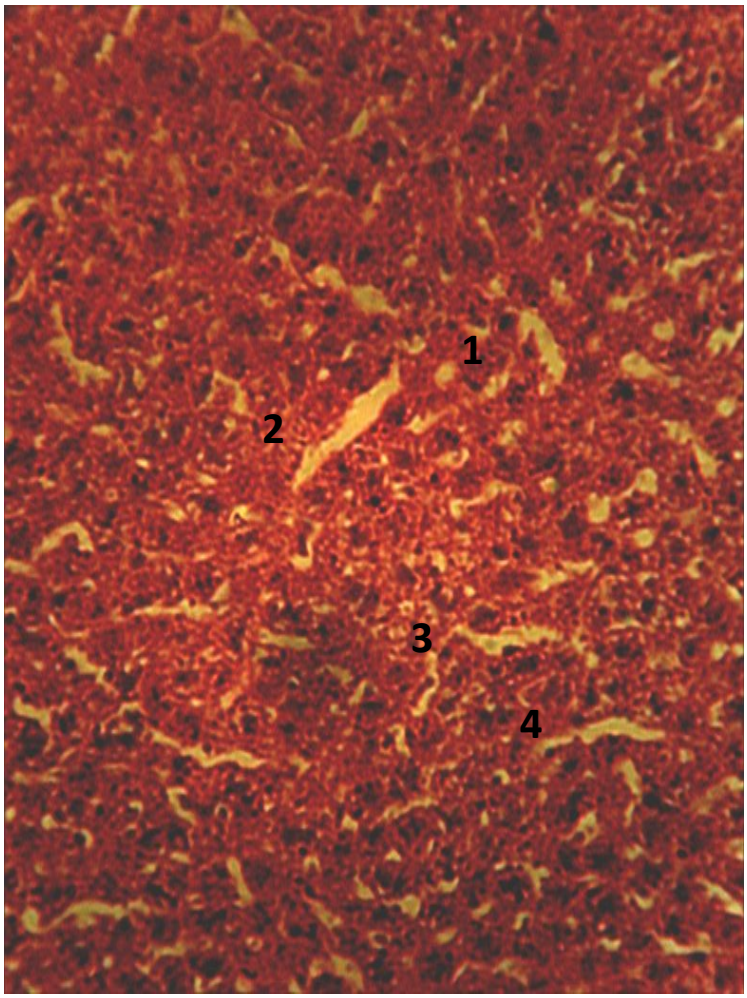


Plate XXX: Photomicrograph of a section of a liver with mild steatosis from a rat treated with 1000mg/kg of methanolic leaf extract of *R. longiflora* for 30 days. H & E stain (×400) 1, 2, 3 and 4 = Mild steatosis (mild fatty change)

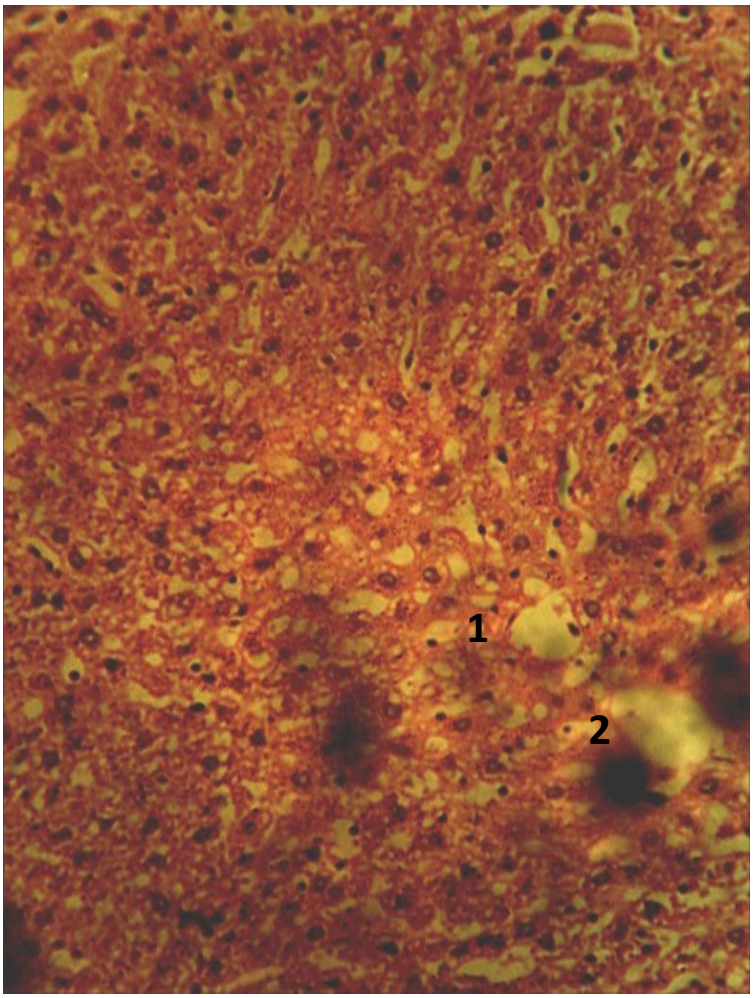


Plate XXXI: Photomicrograph of a section of a normal liver from a rat treated with 1000mg/kg of methanolic leaf extract of *R. longiflora* for 90 days. H & E stain (×400) 1 =Portal tract 2 = Central vein

CHAPTER FIVE

DISCUSSION

The phytochemical screening of *R.longiflora* leaf extract revealed the presence of saponins, flavonoids tannins, cardiac glycosides, alkaloids, and steroids which might be responsible for the observed analgesic and anti-inflammatory activities of the extract.

Analgesic and ant-inflammatory effects have been observed with flavonoids as well as tannins (Ahmadiani *et al.,* 1998; Ahmadiani *et al.,* 2000). There are also reports on the analgesic effects of alkaloids (Reanmongkol *et al.,* 2005) essential oil (De Araujo *et al.,* 2005) and saponins (Hikino *et al.,* 1984; Choi *et al.,* 2005). Flavonoids such as Quecetin are known to be effective in acute inflammation (Rajnarayana *et al.,* 2001; Singh and Pandey, 1997). Certain flavonoids possess potent inhibitory activity against a wide array of enzymes such as protein kinase C, protein tyrosine kinases, phospholipase A_2, phosphodiesterases and others (Middleton, 1998).

Manthey *et al*., (2001) also reported that certain flavonoids inhibit inflammatory processes by inhibiting key enzymes involved in the synthesis of prostaglandins processes. The significant analgesic and anti-inflammatory effect of the extract may be due to the flavonoids saponins and tannins. The alkaloids and saponins present might have also contributed to the analgesic activity of the extract.

The oral and intraperitoneal values of LD_{50} were greater than 5000mg/kg body weight in both rats and mice, suggest that the extract is non-toxic orally and intraperitoneally according to Lorke's 1983 in lethal dose determination.

In lethal dose (LD_{50}) determination by Lorke's 1983, toxic substances are graded as follows:

Substances with lethal dose (LD_{50}) $\geq$ 1mg/kg are considered to be highly toxic, $\geq$ 5mg/kg are considered to be toxic, $\geq$ 100mg/kg are considered to be less or moderately toxic, $\geq$ 1000mg/kg are considered to be slightly toxic and $\geq$ 5000mg/kg are considered to be non-toxic.

The extract significantly ($P<0.05$) inhibited the number of acetic acid-induced abdominal writhes in

mice dose dependently. The highest inhibition of abdominal constriction (64.9%) observed at 1000mg/kg was greater than that of Piroxicam (61.5%), the standard non-steroidal analgesic and anti-inflammatory drug used.

The acetic acid-induced writhing test is very sensitive and able to detect anti-nociceptive effects of compounds at dose level that may appear inactive in other methods like tail flick test (Collier *et al,.* 1968; Bentley *et al.,* 1981). However, the test (tail flick test) is not specific as it does not indicate whether the activity was central and or peripheral (Chan, *et al.,* 1995). The intraperitoneal injection of acetic acid produces an abdominal writhing response due to sensitization of chemo-sensitive nociceptors by prostaglandins (Sutharson *et al.,* 2007). Increase level of prostanoids, particularly PGE_2 and PGF_{2a} (Derardt *et al.,* 1980) as well as lipoxigenase product (Levini *et al.,* 1984; Dhara et al., 2000) have been found in the peritoneal fluid after intraperitoneal injection of acetic acid. The analgesic effects of the extract may therefore be due to its action on visceral receptors sensitive to acetic acid, to the inhibition of the production of algogenic substances or the inhibition at the central level of transmission of painful messages. However, this model may not be able to indicate the mechanism of analgesic effect of the extract because other agents such as antihistamines (Naik *et al.,* 2000) and myorelaxant (Koyama *et al.,* 1997) are able to reduce the pain induced by acetic acid.

The methanolic leaf extract of *R.longiflora* significantly ($P<0.05$) and dose-dependently protected the mice against thermally induced pain stimulus in mice but there was no significant thermal protection at the dose of 50mg/kg.

The 500mg/kg dose of the *R.longiflora* extract offered more than 100% protection; 1000mg/kg offered more than 200% and the standard drug morphine sulphate offered more than 300% protection against thermally induced pain stimulus in mice.

Pain induced by thermal stimulus of the hot plate is specific for centrally mediated nociception (Parkhouse and Pleuvry, 1979). The ability of the extract to prolong the reaction latency to pain

thermally induced in mice by the hot plate further supports its central analgesic activity.

There was no significant inhibition of both the neurogenic pain (early phase) and inflammatory pain (late phase) with 50mg/kg and 250mg/kg doses of the extract. Similarly, the 50mg/kg dose and the control group gave the same value, which signified zero protection from pain.

However, the significant ($P<0.05$) inhibition of both the neurogenic (early phase) and inflammatory (late phase) pain was dose dependent at 500mg/kg and 1000mg/kg with highest inhibition at 1000mg/kg (53.33%). The significant ($P<0.05$) inhibition of the standard drug (morphine sulphate) at late phase doubled 46.67% that of the early phase 20%.

The formalin test showed a distinctive biophasic responses termed early and late phases. Drugs that act primarily on the CNS inhibit both phases equally while peripherally acting drugs inhibit the late phase. (Shibata *et al.,* 1989; Chan *et al.,* 1995) The first phase is probably a direct result of stimulation of nociceptors in the paw. Stimulation of the opioid receptors has also been suggested as a possible mechanism of action against neurogenic pain (Gaertner *et al.,* 1999). This phase therefore reflects centrally mediated pain while the late phase is due to inflammation with release of serotonin, histamine, bradykinin and prostaglandins and at least to some degree, the sensitisation of central nociceptive neurons (Tjolsen *et al.,* 1992). The suppression of both phases as observed in the highest dose tested (1000mg/kg) in this study suggests the presence of both central and peripheral effects. However, the extract has greater inhibitory effect on the peripheral mechanism (inflammatory pain) which showed 53.33% protection at the late phase against the neurogenic pain (central mechanism) 40.0% at the early phase.

The injection of 1% Carrageenan suspension in the paws of the negative control treated rat with Normal Saline produced a local oedema reaching its maximum (0.33 ± 0.03) at the third (3^{rd}) hour. The methanolic leaf extract of *R.longiflora* significantly ($P<0.05$) inhibited the paw oedema at doses of 250mg/kg; 500mg/kg and 1000mg/kg (showed a dose-dependent inhibition). There was no significant ($P>0.05$) inhibition at dose 50mg/kg.

The percentage anti-inflammatory effects at the peak of Carrageenan-induced oedema at the 3rd hour were 39.39%; 51.52%; 63.63% at respective doses of 250mg/kg; 500mg/kg; 1000mg/kg compared to 69.70% for Piroxicam 20mg/kg, the standard anti-inflammatory agent.

At the 4^{th} and 5^{th} hours, there was a general decrease in the volume of the local oedema and correspondent increase in the percentage anti-inflammatory effect, similar effect was observed in the positive control treated with oral piroxicam this indicates that the methanolic leaf extract possesses anti-inflammatory effect that is closely related to that of oral piroxicam (standard drug used in this study). For example, at the 4^{th} hour with 500mg/kg dose, the percentage of anti-inflammatory effect is 73.68%, which is the same with that of the positive control (piroxicam). This further proves that the methanolic leaf extract of *R. longiflora* has similar pattern of anti-inflammatory effect.

Carrageenan-induced hind paw oedema is the standard experimental model for acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effect (Chakraborty *et al.,* 2006). Moreover, the model exhibits a high degree of reproducibility (Winter *et al.,* 1962). The probable mechanism of action of carragenan-induced inflammation is bi-phasic. The first phase is attributed to the release of histamine, serotonin, and kinins in the first hour; while the second phase is attributed to the release of prostaglandins and lysosome enzymes in 2 to 3 hours (Brooks and Day 1991). The methanolic leaf extract of the plant significantly inhibited the Carrageenan-induced inflammation in a dose dependent manner and this effect is comparable to that of piroxicam.

There was no statistically significant difference ($P>0.05$) of the levels of Sodium (Na^+), Potassium (K^+), Chloride (Cl^-) ions, Urea and Creatinine compared to that of the control at the end of 30 days studies. This showed that *R.longiflora* extract produced no adverse effect on kidney functions during sub-chronic administration for 30 days orally.

There was no statistically significant difference ($P>0.05$) of the levels of Sodium (Na^+), Potassium (K^+), Chloride (Cl^-) ions, Urea and Creatinine compared to that of the control at the end of 90 days

studies. This showed that *R.longiflora* extract produced no adverse effect on kidney functions during the chronic administration for 90 days orally.

There was no statistically significant difference ($P>0.05$) in the levels of AST, ALT, and ALP enzymes in all the treated groups compared to the control group during the 30 days studies. This indicates that the extract has no adverse effect on the liver of the rats.

There was no statistically significant difference ($P>0.05$) in the levels of AST, ALT, and ALP enzymes treated with 50 and 250mg (groups 1 and 2) compared to the control group during the 90 days studies. However, there was statistically significant difference ($P<0.05$ and 0.001) in groups three and four treated with 500mg/kg and 1000mg/kg respectively compared to the control. This indicates that that the extract has some adverse effects at higher doses of 500mg/kg and 1000mg/kg and should be used with caution for a long time especially in patients with chronic liver disease.

The histopathological results showed that various tissues/organs of both the treated and the control groups were carefully observed and showed no observable gross lesions during the periods of sub-chronic administration of the extract which lasted for 30 days and chronic studies which lasted for 90 days respectively.

At the end of sub-chronic studies, there were no observable microscopic lesions in the kidneys and hearts of the rats in all the treated groups (Groups 1, 2, 3, and 4) including the control group (group5). They were all normal i.e. there was no pathological feature observed.

However, mild steatosis (mild fatty change) of the liver were observed in the liver of the rats in groups 1, 3, and 4 which were treated with the methanolic leaf extract of *R.longiflora* with 50mg/kg, 500mg/kg, and 1000mg/kg body weight respectively. The mild fatty change observed might not be attributed to the effect of the extract because the levels of the enzymes (AST, ALT and ALP) were within normal range with the control group that was treated with normal saline. Therefore, the mild fatty change could be due to biological variation.

There was no pathological feature observed in the liver of the rat in-group 2, which was treated with

250mg/kg body weight.

Similarly, at the end of the chronic studies, which lasted for 90 days, there were no observable microscopic lesions of the kidneys and hearts of the rats in all the treated groups including the control group, there was no pathological feature observed. However, there was mild steatosis (mild fatty change) in the liver of the rat in the third group, which was treated with 500mg/kg body weight. This could be due to the effect of the extract because there was statistical significant difference in the levels of the liver enzymes (AST, ALT and ALP).

ALT is a cytoplasmic enzyme which is mainly found in the liver (Wilkinson, 1976). Kachmar and Moss (1976) reported that although serum levels of AST and ALT become elevated when the liver cell integrity is affected, ALT is liver specific. Thus, the measurement of ALT activity in serum is of confirming or excluding liver injury (Wilkinson, 1976).

Serum ALP is widely distributed in most of the tissues in the body. It is in high concentration in liver, bone, intestine, kidney, placenta and white blood cell (First, 1996).

Clinically, serum ALP estimation is used in the diagnosis of diseases associated with bones and liver (Kachmar and Moss, 1976).

However, the significant rise of these enzymes (AST, ALP and ALT) did not show any observable mild steatosis with 1000mg/kg. This indicates that the mild fatty change to the liver was not dose-dependent. If the mild injury of the liver was dose-dependent, more of such damage would have been seen with 1000mg/kg dose of the extract but that was not the case, only one of the two sections that indicated mild fatty change with 500mg/kg dose of the extract.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

Based on the data obtained in this study, it may therefore, be concluded that the use of *Rothmannia longiflora* in traditional medicine in Nigeria and other African countries in the management of pain and inflammation is justifiable scientifically. The methanolic leaf extract of *Rothmannia longiflora* has been shown to possess significant analgesic and anti-inflammatory activities, which may be responsible for its use in painful and inflammatory conditions.

Since analgesic and anti-inflammatory drugs usually exact their side effects majorly on the kidneys and other vital organs like the heart and the liver, the result of chemical pathology and histopathology showed that the methanolic leaf extract of *R. longiflora* has no toxic effects on the kidneys and the heart after the sub-chronic (30 days) and chronic (90 days) studies. Therefore, the leaf extract of *R. longiflora* can be used for the treatment of pains and inflammation in Zaria and its environments in Kaduna state (as has been used traditionally) without the fear of injury on the kidneys and hearts as usually noticed with the orthodox analgesic and anti-inflammatory agents.

However, the liver showed mild steatosis in one of the two sections with 500mg/kg after the period of 90 days (chronic toxicity studies) so; the crude extract should not be used for a long period especially in people with chronic liver disease.

It is therefore, recommended that other parameters (total bilirubin, conjugated protein, and albumin) be investigated for liver function test in chronic studies to further confirm the mild fatty change observed in the liver in addition to the rise in liver enzymes to justify the mild steatosis observed.

Further research will involve bioassay-guided fractionation in order to isolate the bioactive components responsible for the observed analgesic and anti-inflammatory activities.

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APPENDICES

Appendix 1 Calculation of Percentage yield of the methanolic leaf extract of *R.longiflora*

The crude drug of 500g was extracted two times using 2.5 litres of pure methanol.

First extraction:

Weight of crude drug that was extracted = 500g

Weight of the crude extract obtained = 147.37g

$$\text{Percentage yield} = \frac{\text{Weight of the crude extract}}{\text{Weight of the crude drug}} \times 100$$

$$= \frac{147.37}{500} \times 100$$

$$= 29.47\%$$

Second extraction:

Weight of the crude drug that was extracted = 500g

Weight of the crude extract obtained = 148.93g

$$\text{Percentage yield} = \frac{\text{Weight of the crude extract}}{\text{Weight of the crude drug}} \times 100$$

$$= \frac{148.93}{500} \times 100$$

$$= 29.79\%$$

Appendix 2 Lethal dose (LD_{50}) determination on methanolic leaf extract of *R.longiflora*

		First phase (n=3)		Second phase (n=1)		
Species	Route of admin.	Doses used (mg/kg)	Quantal mortality	Doses used (mg/kg)	Quantal mortality	LD_{50} (mg/kg)
Mice	Oral	10	0/3	1,600	0/1	>5,000
		100	0/3	2,900	0/1	
		1000	0/3	5,000	0/1	
	Intraperitoneal	10	0/3	1,600	0/1	>5,000
		100	0/3	2,900	0/1	
		1000	0/3	5,000	0/1	
Rats	Oral	10	0/3	1,600	0/1	>5,000
		100	0/3	2,900	0/1	
		1000	0/3	5,000	0/1	
	Intraperitoneal	10	0/3	1,600	0/1	>5,000
		100	0/3	2,900	0/1	
		1000	0/3	5,000	0/1	

NB: $LD_{50} = \sqrt{}$ (Highest non lethal dose) × (Lowest lethal dose)