PHARMACOLOGICAL,CHEMICAL,CHROMATOGRAPHIC AND SPECTROSCOPIC PROFILES OF EXTRACTS AND BIOACTIVE COMPOUNDS FROM SELECTED KENYAN *RUELLIA* SPECIES

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DECLARATION

This thesis is my original work and has not been presented for a Degree in any other university.

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DEDICATION

This thesis is dedicated to the following people: My husband, Engineer Jared Waudo Wangia, and our children; Petronilla Wangia, Engineer Peter Wangia, Dr. Elizabeth Okola, and Elisha Waudo Wangia for their moral support and encouragement as a family. They were there for me when the going got tough and encouraged me to be resilient.

"--With God, **ALL** things are possible"

(Matt. 19:26)

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ACRONYMS AND ABBREVIATIONS

ACPA	Anti-Citrullinated Protein Antibody
ACUC	Animal Care and Use Committee
AIA	Adjuvant-Induced Arthritis
AMU	Atomic Mass Unit
BSA	Bovine Serum Albumin
BUN	Blood Urea and Nitrogen
BW	Body Weight
CAM	Complementary and Alternative Medicines
CFA	Complete Freund's Adjuvant
ECAM-1	Endothelial Cell Adhesion Molecules-1
ED_{50}	Median Effective Dose
DMARDs	Disease-Modifying Anti-rheumatic Drugs
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Erythrocyte Sedimentation Rate
FTIR	Fourier Transform Infra-Red
GC-MS	Gas Chromatography-Mass Spectrometry
GHS	Globally Harmonized System
G.I.T.	Gastrointestinal Tract
GM-CSF-II	Granulocyte/Macrophage-Colony Stimulating Factor
GFR	Glomerular Filtration Rate
5-HT	5-Hydroxy Tryptamine
IC_{50}	Half Maximal Inhibitory Concentration

ICAM-1	Intercellular Adhesion Molecules-1
IL	Interleukin
I.M	Intramuscular
IND	Investigational New Drug
IR	Infra-Red
I.V	Intravenous
JAK	Janus Kinase
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
λmax	Lambda maximum
LC-MS	Liquid Chromatography-Mass spectrometry
LD_{50}	Median Lethal Dose
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Cell Volume
MHC	Major Histocompatibility Complex
MKU	Mount Kenya University
MS	Mass Spectrometry
NADAC	National Average Drug Acquisition Cost
NIST	National Institute of Standards and Technology
Nm	Nanometers
NMR	Nuclear Magnetic Resonance
NSAIDs	Non-Steroidal Anti-inflammatory drugs
OA	Osteoarthritis
OECD	Organization for Economic Cooperation and Development

PBS	Phosphate Buffered Saline
PCV	Packed Cell Volume
PDW	Platelet Counts and Platelet Distribution
RA	Rheumatoid Arthritis
RANK	Receptor Activator of Nuclear Factor-*Kappa* B
RANKL	Receptor Activator of Nuclear Factor-*Kappa* B Ligand
RBK	*Ruellia bignoniiflora*
RBS	Red Blood Cells
RDW	Red Blood Cell Distribution Width
R_f	Retention Factor
RLB	*Ruellia lineari-bracteolata*
ROS	Reactive Oxygen Species
RPM	*Ruellia prostrata*
S.C	Subcutaneous
SEM	Standard Error of Mean
SERU	Scientific and Ethics Review Unit
STAT	Signal Transducer and Activator of Transcription
UoN	University of Nairobi
T.L.C	Thin-Layer Chromatography
TMP	Traditional Medicine Practitioner
UK	United Kingdom
UV	Ultraviolet
UV/VS	Ultraviolet/Visible

ABSTRACT

The growing challenge of non-communicable diseases (NCDs) especially in developing world necessitates search for novel drugs that are not only efficacious but also affordable. Plants have traditionally provided a rich source of medicine, and therefore, there is a need to explore the untapped reservoir of medicinal plants for the management of NCDs. *Ruellia* (Acanthaceae*)* species are wild perennial creepers which have been reported to possess anti-inflammatory, analgesic, antinociceptive, and antipyretic activities. Therefore, the main objective of this study was to investigate the pharmacological, chemical, chromatographic, and spectroscopic profiles of the bioactive compounds present in three Kenyan *Ruellia* species. The selected species *viz. R. prostrata* (RPM), *R. bignoniiflora* (RBK) and *R. linear*i*-bracteolata* (RLB), were studied with special emphasis to antioxidant, analgesic, anti-inflammatory and anti-arthritic activities. Whole plant parts were collected from their natural habitats and both aqueous extracts (hot maceration) and organic extracts (cold maceration) were obtained. The acute toxicity study categorized the aqueous extract of *R. prostrata* to be in Category 5 (>2000-5000mg/kg) as per Globally Harmonised System. The sub-chronic toxicity studies revealed that the oral aqueous extract of *R. prostrata* (1000mg/kg *per os*) had no adverse effects on the internal organs, haematological parameters, and blood biochemistry in Wistar albino rats. The methanolic extract of RLB showed the highest antioxidant activity [1,1-diphenyl-2-picrylhydrazyl radical (DPPH)], scavenging activity, of IC_{50}, 2.9±3.21 µg/mL, comparable to ascorbic acid standard (2.1±0.10 µg/mL). The aqueous extract of RPM showed the highest analgesic activity (Tail flick method) of ED_{50}, 358.90±20.90 mg/kg. The aqueous extract of *R. prostrata* showed the highest anti- antinociceptive activity (Formalin test) in phase-1 (ED_{50} 22.22±0.52 mg/kg), and phase-2 (ED_{50} 7.07±4.10 mg/kg). The aqueous extract of RPM also showed the highest anti-inflammatory activity (Carrageenan test) of ED_{50}, 168.05±3.25 mg/kg. I*n vivo* anti-arthritic studies (Complete Freund's Adjuvant-induced arthritis) revealed no differences ($p>0.05$) compared to the untreated control in hind paw swelling (mL), hind joint thickness (cm), arthritis score on a scale of 0-16, and inhibition of loss of grip strength (secs) as observed on a rotating rotarod. Aqueous extract of RPM did not suppress the bone marrow unlike methotrexate standard. Chemical profiling revealed the presence of terpenoids, saponins, flavonoids, phenolics, and glycosides. No alkaloids were detected in all the three species. Fourier transform infra-red profiling revealed the presence of -OH_{str}, C-H_{str}, C=C_{str} and C-O-C linkages. Some of the compounds in Gas Chromatography-Mass Spectrometry profiling were β-sitosterol, stigmasterol, lupeol, and triterpenes. 13-Docosenamide, (Z)-, l-(+)-Ascorbic acid 2,6-dihexadecanoate, α-D-Glucopyranose, 4-O-. β -D-galactopyranosyl-, squalene, 9-Hexadecenoic acid, methyl ester, (Z), 9-Octadecenamide, (Z)-, 9-Octadecenoic acid (Z)-, methyl ester. These compounds have been documented to possess antioxidant, anti-inflammatory and analgesic activities. The present study concluded that extracts from *Ruellia* species studied possess antioxidant, analgesic, antinociceptive, anti-inflammatory and anti-arthritic activities, with the aqueous extract of RPM showing the highest activities, followed by the aqueous extract of RBK. This is the first scientific report regarding the efficacy of RPM in a rheumatoid arthritis animal model. The antioxidant, analgesic, antinociceptive and anti-inflammatory activities of *R. bignoniiflora* and *R. linear-bracteolata* were reported for the first time. Some compounds identified from the

selected Kenyan *Ruellia* species, such as flavonoids, saponins, phenolics, glycosides, terpenes, β-sitosterol, stigmasterol, and lupeol have been reported in other *Ruellia* species. The present study recommends that the bioactive compounds present in *R. prostrata* and *R. bignoniiflora* should undergo further pre- clinical studies as analgesic and anti-arthritic drugs in a non-human primate before use in man.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Chronic inflammation is due to persistent acute inflammation caused by non-degradable pathogens, persistent foreign bodies or autoimmune reactions and reactive oxygen species. Arthritis (Greek arthro-, joint, and itis, inflammation) is a group of chronic inflammatory conditions involving damage to one or more joints of the body. Arthritis and rheumatism are a general term that represents many types of arthritis namely, osteoarthritis (OA)/degenerative arthritis, rheumatoid arthritis (RA), gout, ankylosing spondylitis, psoriatic arthritis, autoimmune diseases which include lupus, scleroderma and Sjogren's syndrome (Wong *et al.*, 2010).

Rheumatoid arthritis (RA) is a result of chronic inflammation of the joints and is characterized by pain, swelling, and redness of the underlying skin and diminished range of joint motion (Gaffo *et al.*, 2006; Majithia and Geraci, 2007; Walker *et al.*, 2014). Hands, feet, and cervical spine are also affected (Turesson, 2013). Non-joint effects of RA include rheumatoid nodules which affect 30% of patients (Turesson, 2013); amyloidosis, (DeGroot, 2007); liver toxicity which may be due to RA disease or some drugs used in its treatment (Selmi *et al*., 2011); and synovitis, which results into tethering of tissues leading to loss of movement and erosion of joints (Majitha, 2007).

Currently, there is no cure for RA. Pharmacologic approaches for the management of this disease include the use of anti-inflammatory agents called non-steroidal anti-inflammatory drugs (NSAIDs) as well as disease-modifying anti-rheumatic drugs (DMARDs). NSAIDs give symptomatic relief of pain, swelling and joint stiffness in arthritis (Vane and Ferreira, 1978). It was recently discovered that when treatment begins early with DMARDs, which are strong medications, remission of symptoms is more likely. These medicines may slow the progression of the condition when administered early enough before severe joint damage, but have side effects such as

liver damage, severe lung infections, and bone marrow suppression (Katzung, 2017). Due to the many adverse effects of conventional NSAIDs and DMARDs, there is an urgent need to search for drugs which are efficacious and safe. The search for effective and safe anti-rheumatic drugs continues, and plants may provide a rich source for new medicines or provide templates for new medicinal products for the treatment/management of arthritis conditions.

Plants have been used as medicines since the beginning of civilization (Nagavani and Rao, 2010). The reported healing powers of plants indicate that they have medicinal values where they have played a significant role as alternative therapeutic tools for prophylaxis and treatment of both communicable and non-communicable diseases. It has been reported that 80% of the world population has used complementary medication (WHO, 2002; 2003). The discovery of drugs from medicinal plants started from the era when the isolation of drugs such as digitoxin, quinine, cocaine, morphine, and codeine began. These drugs came from an enormous reservoir of biologically active molecules (secondary metabolites) found in medicinal plants, although only a small fraction of these products with medicinal value has been assayed (Harborne, 1998)

Although there are about 300 species of *Ruellia*, only a few have been investigated for their biologic or pharmacologic activities. *Ruellia* is widespread all over the world, and many of its species have been used in folkloric medicine. Significant biological and pharmacological activities have been reported (Afzal *et al.*, 2015). The Kenyan traditional medical practitioners from the plants' natural habitats have not used these species in their practice. There are no local names for these species, which are only known as food for the goats. The medicinal uses of the Kenyan *Ruellia* species *viz. R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* by the traditional medical practitioners have not been documented (Kokwaro, 2009). These species need to be investigated for their biological and pharmacological activities which may reveal lead compounds in treatment of many diseases, including rheumatoid arthritis. The reported use of *Ruellia* species in the treatment of chronic rheumatism suggests the probable utility of these plants against chronic inflammation (Rajan *et al*., 2012). The evaluation of the antioxidant, analgesic, antinociceptive, anti-inflammatory, anti-arthritic

activities, safety of these species, chemical, chromatographic, and spectroscopic profiling of their bioactive compounds forms the basis of this study.

1.2 Statement of the Problem

Rheumatoid arthritis (RA) is a chronic disease that mostly affects joints, such as the wrists and hands symmetrically, followed by the feet, spine, knees, and jaw. In joints, the disease causes inflammation that leads to pain, swelling, stiffness and loss of function. Rheumatoid arthritis is an autoimmune disorder in which the immune system attacks the healthy tissues of the joints. Pain and stiffness often worsen following rest or on waking up in the morning. Non-articular parts of the body are also affected leading to inflammation around the lungs and heart and anaemia (NIH, 2009). Other symptoms come gradually over weeks to months (Majithia and Geraci, 2007). Comorbid conditions with RA include hypertension, diabetes, osteoporosis, and dyslipidaemia (Al-Bishri, *et al.,* 2013). This disease can lead to crippling and deformity of joints such than the patient can not undertake normal chores, and hence finally becomes economically non-productive.

Data on the prevalence of RA around the world indicate the high burden of this disease and the potential concerns for health care needs and health service requirements for people with this disease (Wong *et al*., 2010). About 24.5 million people are affected by RA as of the year 2015 (Vos *et al.,* 2016). This is a prevalence of between 0.5% and 1% of adults in the developed world with 5 and 50 per 100,000 people newly developing the condition each year (Smolen *et al.,* 2016). In 2013, RA resulted in 38,000 deaths up from 28,000 deaths in 1990 (Abubakar *et al.,* 2015).

With no cure currently available, pharmacological management of RA is largely based on the use of NSAIDs and DMARDs. The goals of treatment are symptomatic to reduce pain, inflammation, and improve the overall functioning a person (Allen *et al.,* 2018). NSAIDs alleviate the RA-associated pain and inflammation but have a lot of adverse effects on the gastrointestinal tract (GIT), kidney, liver, and cardiovascular systems. Once RA is confirmed, use of DMARDs is the next option, since these drugs improve RA symptoms, decrease the damage of joints, and improve overall functional abilities (Singh *et al.,* 2016). Steroids are included in RA regimen for quick relief of

the symptoms before the effects of DMARDs are felt. These drugs are used in the shortest term and at the lowest dose possible for flare-ups while waiting for slow-onset drugs to take effect (Criswell *et al.,* 1998). Corticosteroids cause varied adverse effects such as thinning of the bones and weight gain. The disadvantage of DMARDs is that they are slow in onset, taking several weeks before the onset of action. These drugs also have a lot of adverse effects such as GIT, liver damage, bone marrow suppression, severe lung infections and risk of infection (Joseph, 2008; Arya *et al.,* 2011; DiPiro *et al.,* 2014; Katzung, 2017). Should DMARDs not produce relief, biologic modifiers (biologics) are the newer drugs, used alone or in combination with DMARDs (Osthoff *et al.,* 2018). Most of these biologics are given parenterally and patients experience pain or swelling at the injection site, in many cases showing flu-like symptoms. The biologics have a greater risk of adverse effects such as lung infections, liver damage, nausea, and reduced ability to make new blood cells (Ruderman, 2012). The cost of biologics is exorbitant and many average income patients, especially those in developing countries can not afford them. For example, a month supply of adalimumab (Humira) in the United Kingdom as of 2018 was equivalent of Ksh 84,513 (British national formulary: *BNF 76* (76 ed.), whereas in the US, as per 2018, the wholesale cost of adalimumab was equivalent of Ksh 519,223 per month [(National Average Drug Acquisition Cost (NADAC) as of 2019-02-27, Centers for Medicare and Medicaid Services].

Furthermore, inadequacy or unresponsiveness to pharmacologic intervention can lead to debilitating complications such as complete loss of joint mobility, necessitating total hip or joint replacement with the risk of excessive bleeding (Wong *et al*., 2010). Surgery to repair, replace or fuse joints may be useful in certain situations (NIH, 2009). The goals of treatment are, therefore, to reduce pain and inflammation, and improve a person's overall functioning (Allen *et al.,* 2018). Management of RA is associated with adverse effects from any class of drugs selected, with newer drugs costing exorbitantly.

1.3 Justification

Ruellia prostrata has been used in Indian traditional medicine to treat joint disorders of varied aetiology. In addition, extracts of Indian *R. prostrata* exhibited anti-

inflammatory activity *in vivo* (Kaulukusi, 1983; Wangia, 1985). Based on these preliminary findings from the Indian *Ruellia* species, three Kenyan *Ruellia* species viz. *Ruellia prostrata, Ruellia bignoniiflora* and *Ruellia lineari-bracteolata* have been selected for this study with an aim of investigating the pharmacological, chemical, chromatographic, and spectroscopic profiles of the bioactive compounds present in them. To the best of our knowledge, two of the Kenyan species, *R. bignoniiflora* and *R. lineari-bracteolata* have not been studied before. The reported use of *Ruellia* species in traditional medicine and the promising laboratory investigations of *Ruellia* species from other continents provided further justification to study the Kenyan *Ruellia* species.

1.4 Research Questions

i. What are the chemical, chromatographic, and spectroscopic profiles of the organic and aqueous extracts of selected Kenyan *Ruellia* species?
ii. Do the extracts of selected Kenyan *Ruellia* species (*viz. Ruellia prostrata, Ruellia bignoniiflora* and *Ruellia lineari-bracteolata)* possess antioxidant, analgesic, and anti-inflammatory activities?
iii. Is the aqueous extract of Kenyan *Ruellia prostrata* safe on oral administration?
iv. Does the aqueous extract of Kenyan *Ruellia prostrata* possess anti-arthritic activity?

1.5 Objectives

1.5.1 General Objective

To investigate the pharmacological and phytochemical profiles of bioactive compounds from Kenyan *Ruellia* species *viz. Ruellia prostrata, Ruellia bignoniiflora* and *Ruellia lineari-bracteolata*.

1.5.2 Specific objectives

i. To determine the chemical, chromatographic, and spectroscopic profiles of the organic and aqueous extracts of selected Kenyan *Ruellia* species.
ii. To determine the antioxidant, analgesic, and anti-inflammatory activities of

extracts of selected Kenyan *Ruellia* species.

iii. To determine the *in vivo* toxicity of the aqueous extract from Kenyan *Ruellia prostrata.*

iv. To evaluate the *in vitro* and *in vivo* anti-arthritic activities of the aqueous extract from Kenyan *Ruellia prostrata*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of Arthritis

There are several types of arthritis with OA being the most common, followed by RA. Osteoarthritis involves wearing away of the cartilage that caps the bones in the joints, affecting joints in the fingers, knees, and hips leading to cartilage thinning and bony overgrowths (osteophytes), resulting in pain, swelling, and stiffness. On the other hand, RA is a chronic autoimmune disease whose aetiology is unknown and is characterized by joint synovial inflammation and progressive cartilage and bone destruction resulting in gradual immobility (McInnes and Schett, 2017). In RA, the synovium is attacked by the immune system, resulting in inflammation which thickens the synovium (pannus), and eventually destroys the cartilage and bone within the joint. The tendons and ligaments that hold the joint together weaken and stretch, gradually leading to loss of shape and alignment of the joint. RA patients also experience tenderness and warmth in the swollen joints. Joint stiffness is usually worse in the mornings and after inactivity. Fatigue, fever, and weight loss may also occur. The smaller joints of the fingers and toes are the early symptoms of RA, which progress to the wrists, ankles, elbows, hips, and shoulders. The symptoms are symmetric affecting the same joints on both sides of the body (Walker *et al.,* 2014). A radiograph showing a hand with advanced rheumatoid arthritis is illustrated in **Figure 2.1.**

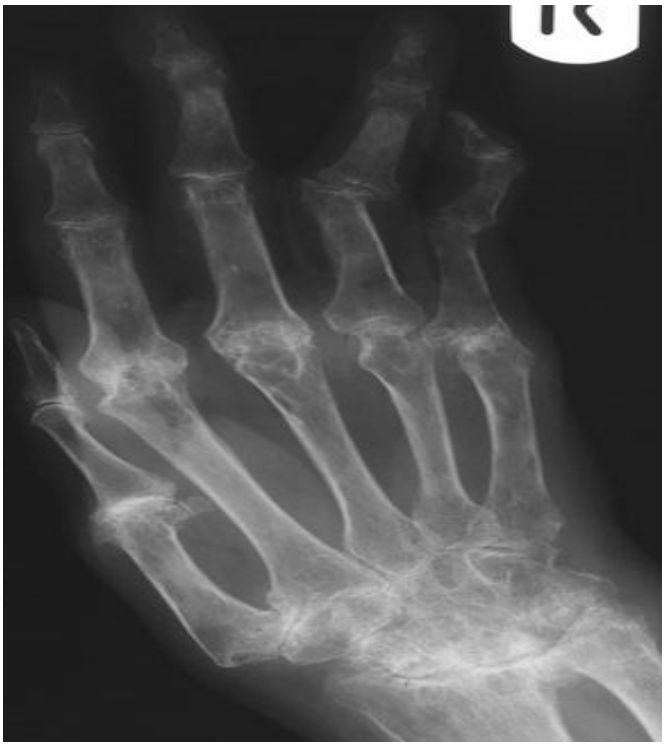


Figure 2.1: A radiograph showing a hand with advanced rheumatoid arthritis.

Radiograph of the hand shows severe destruction and mutilation of the radiocarpal, intercarpal, carpometacarpal, and metacarpophalangeal joints. Intercarpal ankylosis is noted. There is also subluxation and deviation of the fourth and fifth fingers (adapted from Sommer *et al.*, 2005).

A schematic drawing of a normal synovial joint is illustrated in **Fig 2.2.**

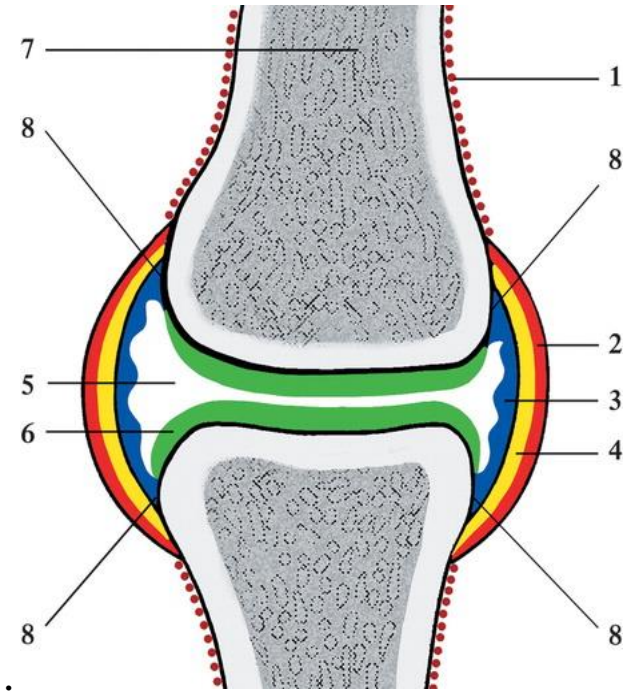


Figure 2.2: Schematic drawing of a synovial joint.

1= periosteum, 2= outer fibrous layer of the capsule, 3= internal synovial layer of the capsule, 4= fat and loose soft tissue, 5= articular space, 6= cartilage, 7= bone, 8= bare area (adapted from Sommer *et al.*, 2005).

There are many complications of RA which are not joint-related such as osteoporosis, rheumatoid nodules around pressure points (Turesson, 2013), dry eyes, infections, heart problems (Murray and Rauz, 2016; Alenghat, 2016), lung problems (Balbir-Gurman, 2006) and risk of lymphoma (Baecklund *et al.*, 2006; Franklin *et al.*, 2006). The likelihood of getting cardiovascular abnormalities such as atherosclerosis, myocardial infarction and stroke is also high (Murray and Rauz, 2016; Alenghat, 2016).

2.2 Epidemiology of Arthritis

About 1% of adults in the developed world are affected with RA, with between 5 and 50 per 100,000 people newly developing the condition each year (Smolen *et al.*, 2016). In 2013, RA resulted in 38,000 deaths up from 28,000 deaths in 1990 (Abubakar *et al.*, 2015). RA onset is uncommon under the age of 15, and from then on, the incidence

rises with age until the age of 80. The prevalence in women affected is three to five times that of men (Turesson, 2013). The age at which the disease most commonly starts in women is between 40 and 50 years, and for men somewhat later (Alamanos and Drosos, 2005). The prevalence of arthritis in some countries reported was as follows; Canada (~10.0%), USA (8.0-16.4 %,), UK (12.5%), Australia, New Zealand, Belgium, and Netherlands (8.0-13%). Physician-confirmed OA in third world countries is small with prevalence ranging between 2.3-11.3%. This low prevalence may be due to a lack of documentation in these countries (Wong *et al.*, 2010).

Very few studies have been done on arthritis in Kenya and Africa (Sikwe-Kunnap, 2012). One such study documented the characteristics of patients with rheumatic diseases in Kenya. The earliest study documented report on the prevalence of rheumatic diseases in Kenya was by Oyoo, (2004). The study revealed that out of 767 patients, who had rheumatologic complaints, 67.1% were female, peak age was 51-60 years; 40% had OA, and 23% had RA. The rest of the patients had low backaches (10%), soft tissues rheumatism (14%), while 4.43% had spondyloarthropathy. Research on rheumatic diseases in Kenya is still a novelty due to shortage of rheumatologists in the whole country, and partly because rheumatic disorders are associated with ageing, and older adults do not go to the hospital to be checked for joint pains (Oyoo, 2004).

2.3 Pathophysiology of Inflammation and Rheumatoid Arthritis

Rheumatoid arthritis is a result of chronic inflammation and inflammation (Latin-*inflamatio*, to set on fire) is part of a complex biological response of vascular tissues to harmful stimuli.

2.3.1 Pathophysiology of Inflammation

The inflammatory process is a protective attempt by the organism which aims to remove the injurious stimuli and to initiate the healing process. This process is a cascade of biochemical events which propagates and matures the inflammatory response and involves the local system, the immune system, and various cells within the injured tissue. Also, the progressive destruction of the tissue would compromise

the survival of the organism. There are various causes of inflammation; burns, chemical irritation, frostbite, toxins, infection by pathogens, physical injury, immune reactions due to hypersensitivity, splinters, dirt, and debris. Without inflammation, wounds and infections would not heal (Stankov, 2012).

Inflammation can either be acute or chronic, depending on the onset, duration and mediators involved. The initial response of the body to harmful stimuli such as pathogens and injured tissues is the acute inflammation. It is achieved by the increased movement of plasma and leukocytes (especially the granulocytes) from the blood into the wounded tissues. The onset is immediate, and duration takes place within a few minutes or hours to a few days and ceases upon the removal of the injurious stimuli. The primary cells are neutrophils and mononuclear cells (monocytes and macrophages), whereas the major mediators are vasoactive amines such as histamine, 5-hydroxy tryptamine (5-HT), and eicosanoids such as prostaglandins (PGs) and leukotrienes (LTs). The outcome is a normal resolution, abscess formation or chronic inflammation. Chronic inflammation is due to persistent acute inflammation due to non-degradable pathogens, persistent foreign bodies or auto-immune reactions and reactive oxygen species (Quiñonez-Flores *et al.,* 2016).

Inflammation is a fundamental defensive reaction of the body to an invasion of pathogens or injury, and it involves a complex array of enzyme reactions; mediators release, extravasations, cell migration, tissue breakdown and repair (Herrero *et al.,* 1997). The cardinal signs of inflammation are pain, redness, heat, swelling and loss of function, and are produced by inflammatory agents such as nitric oxide, prostaglandins, bradykinin, 5-HT, LTs and histamine (Ahmadiani *et al.,* 2000). When inflammation is not controlled and persists, it contributes to the progression of many chronic disease such as multiple sclerosis, rheumatoid arthritis, atherosclerosis, psoriasis, and inflammatory bowel disease (Vittalrao *et al.,* 2011). Although inflammation is a defense mechanism, there are complex events and mediators which are involved in inflammatory reactions and can induce or aggravate many reactions (Dalgleish and O'Byrne, 2002). Pain sensation and inflammation are some of the main reasons why people seek medical attention from medical institution, and they normally do so to get symptomatic relief (Cuartero *et al.,* 2006).

As described by Ashok *et al,* (2010) pain and inflammation are associated with various diseases like arthritis and cancer. The symptoms from these diseases are normally relieved by use of natural products from various traditional medicinal plants. Pain is described as a sensory and emotional experience which is characterized with potential tissue damage. There are many types of pain; neuropathic, inflammatory, nociceptive, and functional (Rajagopal, 2006). Chronic inflammation also results in chronic pain, redness, swelling, stiffness, and damage to normal tissues (Serhan *et al.,* 2005). Pain is a defensive mechanism of the body and is an ill-defined, unpleasant sensation and emotional experience along with acute or chronic tissue damage and is usually induced by an external or internal noxious stimulus.

2.3.2 Pathophysiology of Rheumatoid Arthritis

Although inflammation is a normal, healthy response, inflammatory disorders like RA, which result in the immune system attacking the body's cells or tissues, may cause abnormal inflammation. A state of persistent cellular activation is the start of RA. The disease leads to autoimmunity and immune complexes, which manifest in small joints of the arms and toes and progresses to other organs. The synovial membrane, where swelling and congestion leads to infiltration by immune cells, is the initial site of disease. The progression of RA includes several phases which include the initiation phase due to non-specific inflammation, followed by the amplification phase due to T cell activation, and finally, the chronic inflammatory phase with tissue injury, due to cytokines such as interleukins IL-1, TNF-α and IL-6 (Patel and Bhadoriya, 2011, Shah, 2016).

Altered thresholds in regulation of the adaptive immune response due to genetic mutations are currently understood. These factors allow an abnormal immune response, once initiated, to become permanent and chronic (Smolen *et al.,* 2016). Other environmental factors that appear to modulate the risk of acquiring RA are hormonal factors in the individual which may explain some features of the disease, such as the higher occurrence in women, the infrequent onset after childbirth, and the (slight) modulation of disease risk by hormonal medications. An integral part of RA disease process is the autoantibodies to IgGFc (rheumatoid factors), and antibodies to

citrullinated peptides. These antibodies are abnormally glycosylated and are also present in many other autoimmune diseases (Maverakis *et al.,* 2015). These glycan (oligosaccharide) alterations are believed to promote joint inflammation (Maverakis *et al.,* 2015).

After several years before abnormal immune response becomes established (and before occurrence of symptoms), plasma cells derived from B lymphocytes produce rheumatoid factors and anti-citrullinated protein antibody (ACPA) of the IgG and IgM classes in large quantities, which activate macrophages through Fc receptor and complement binding. This process seems to play an important role in the intense inflammatory response present in RA (Boldt *et al*., 2012). The antibody's N-glycans mediate the binding of an autoreactive antibody to the Fc receptors, which are altered to promote inflammation in people with RA (Maverakis *et al.,* 2015). This in turn, contributes to inflammation of the synovium, reflected as oedema, vasodilation, and infiltration by activated T-cells (mainly $CD4^+$ in nodular aggregates and $CD8^+$ in diffuse infiltrates). An established local immune reaction in the tissue is due to synovial macrophages and dendritic cells which function as antigen-presenting cells, as cells expressing major histocompatibility complex (MHC) class II molecules. The formation of granulation tissue at the edges of the synovial lining (pannus) with extensive angiogenesis and production of enzymes is the ultimate of tissue damage. These mediators are the targets of modern pharmacological treatments of RA. The synovium thickens, with the disintegration of the cartilage and the underlying bone leading to the destruction of the joint after the establishment of the inflammatory reaction.

Apart from TNF-α playing a dominant role in the pathogenesis of RA, other chemical mediators (cytokines) are also likely to be involved in inflammation in RA. Drugs that block interleukins IL-1, IL-6 and IL-15 have shown beneficial effects; and IL-17 may also play an important role (Gaffen, 2017). Immune cells, attracted by cytokines and chemokines, accumulate within the synovial compartment. On the other hand, the activated fibroblasts, accumulated T cells and B cells, monocytes, and macrophages act via signaling through the receptor activator of nuclear factor-*kappa* B ligand (RANKL) and receptor activator of nuclear factor-*kappa* B (RANK) which eventually

trigger the generation of osteoclasts, specialized cells that degrade bone tissue (Smolen *et al*., 2016; Chiu and Ritchlin, 2017).

2.4 Management of Arthritis

There is no cure for RA, but treatments can improve symptoms and slow the progress of the disease. The goals of treatment are to minimize symptoms such as pain and swelling, to prevent bone deformity (for example, bone erosions visible in X-rays), and to maintain day-to-day functioning.

2.4.1 Non-steroidal Anti-Inflammatory Drugs

Use of analgesics such as acetaminophen and NSAIDs may help manage pain (NICE, 2015). They do not change the underlying disease (NICE, 2015; Smolen *et al.*, 2016). The most important mechanism of action of NSAIDs is the inhibition of the biosynthesis of prostaglandins (PGs) through the cyclooxygenase (COX) pathway (Vane, 1971). There are two isoforms of COX enzyme; COX-1 enzyme, the constitutive enzyme, that is involved in gastrointestinal tract (GIT) protection and kidney physiological regulation. The COX-2 enzyme is inducible (either by harmful substances, cytokines, or growth factors) and leads to the synthesis of PGs and LTs that are responsible for pain, inflammation, and fever. NSAIDs can either be non-selective COX inhibitors (traditional NSAIDs such as aspirin or ibuprofen); preferential COX-2 inhibitors (such as meloxicam) or selective COX-2 inhibitors (such as celecoxib). The main disadvantage of selective COX-2 inhibitors is cardiovascular toxicity, hence caution to their use, especially in patients with other cardiovascular diseases.

There are other possible mechanisms of action of NSAIDs other than inhibition of COX pathway which include inhibition of chemotaxis and down-regulation of IL-1 production; inhibition of expression/activity of some molecules and inhibition of generation of free radicals and superoxide. Growth factors like granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-6 and lymphocyte transformation factors may also be inhibited. NSAIDs actions can also lead to stabilization of leukocyte lysosomal membrane and antagonism of kinins as well as

interference of calcium-mediated intracellular events which may also be involved (Tripathi, 2013; Katzung, 2017).

The NSAIDs have shared side effects associated with PG synthesis such as gastric mucosal damage, bleeding due to inhibition of platelet function, delay/prolongation of labour, limitation of renal blood flow; Na^+ and water retention which can lead to kidney failure after chronic use, asthma and anaphylactoid reactions in susceptible individuals (Tripathi, 2013). Other side effects (not associated with PG synthesis), but which depend on the type of the NSAID include nausea and vomiting, diarrhoea, constipation, decreased appetite, rash, dizziness, headache, and drowsiness (Katzung, 2017). Patients with gastrointestinal, cardiovascular or kidney problems should use NSAIDs with caution (McCormack, 2011; Marks *et al.*, 2011; Radner *et al.*, 2012).

Use of topical NSAIDs for relief of pain and swelling is another approach in patients who are above 75 years of age, particularly when few joints are involved (Hoechberg *et al.*, 2012). Topical preparations are believed to penetrate the sub-adjacent tissues attaining high concentrations in affected joints/muscles while maintaining low blood levels (Tripathi, 2013). The rationale for the use of a topical formulation is that when arthritis is localized, a topical preparation would avoid systemic adverse effects which commonly occur with the use of systemic NSAIDs. When topical preparations are used, the gastrointestinal (G.I) systemic adverse effects and first-pass hepatic metabolism are minimized, and patient compliance is increased. For these reasons, many NSAIDs are being marketed in various formulations including gels, creams, and ointments (such as ibuprofen, ketoprofen, and diclofenac gels) for application to painful joints. More findings on the formulation of topical preparations for anti-arthritic conditions are available in the literature (Wang *et al*., 2008; Khuda *et al.*, 2013).

2.4.2 Corticosteroids

Corticosteroids (steroids) are highly effective and potent drugs that can reduce inflammation and help moderate autoimmune activity. These drugs work by mimicking the adrenal hormone, cortisol, which helps in the regulation of inflammation, metabolism, and blood sugar. The glucocorticoids reported for use in

rheumatoid arthritis are prednisone, dexamethasone, cortisone, cortisol, and prednisolone (Mottram, 2003).

Corticosteroids are often prescribed as part of a multi-drug therapy and are delivered either orally or parenterally (i.m, intra-articular or i.v injections). These drugs are only prescribed for short-term use due to the increased risk of side effects, including osteoporosis, weight gain, easy bruising, cataracts, glaucoma, and diabetes. When taken orally, they are prescribed in low doses, in shortest possible time, while waiting for slow-onset DMARDs to take effect due to possibility of flare-ups (Paget, 2002; Singh *et al.,* 2016). These drugs are usually taken in the morning and injections are reserved for acute attacks and used no more than three or four times a year.

2.4.3 Disease-Modifying Antirheumatic Drugs

When treatment starts early and aggressively, best results are obtained when DMARDs are used (Saag *et al.,* 2008). These drugs, grouped by use and convention, are the primary treatment of RA (Singh *et al.,* 2016). DMARDs are a diverse collection of drugs, which improve overall functional abilities by decreasing joint damage (Singh *et al.,* 2016). When DMARDs are started early in the disease, the result is disease remission in approximately half of the people, and improved overall outcomes (Singh *et al.,* 2016). Examples of some DMARDs are methotrexate, hydroxychloroquine, sulfasalazine and leflunomide.

The most used agent is methotrexate, which is the most important and useful DMARD and is usually the first-line treatment. Other agents may be used in combinations (DiPiro *et al.,* 2014; NICE, 2015; Singh *et al.,* 2016). There should be regular monitoring of the adverse effects of methotrexate, especially the effects on the gastrointestinal, blood, lung, liver, and suppression of immunity (DiPiro *et al.,* 2014). Leucovorin can reduce other side effects such as nausea, vomiting or abdominal pain when used together with methotrexate (Shea *et al.,* 2013). When treatment with methotrexate and other conventional drugs is ineffective after a three-month trial, biological modifiers (biologics) are used (Singh *et al.,* 2016).

2.4.4 Biologic Response Modifiers

Compared to other DMARDs, biologic response modifiers (biologics) are associated with a higher rate of serious infections (Singh *et al.,* 2016). These drugs used to treat rheumatoid arthritis include TNF-α blockers, such as infliximab and adalimumab; IL-1 blockers such as anakinra; monoclonal antibodies such as rituximab and tocilizumab; T-cell co-stimulation blockers such as abatacept and etanercept. Biologics are often used in combination with either methotrexate or leflunomide (Singh *et al.,* 2016; Smolen *et al.,* 2016).

Better results are obtained when TNF blockers and methotrexate are used together, although they appear to have similar effectiveness when used alone. Although TNF blockers appear to have equivalent effectiveness, etanercept appears to be the safest (Aaltonen *et al.,* 2012). The biologics have the disadvantages of being costly and associated with infections (Smolen *et al.,* 2016). A monthly supply of adalimumab (Humira) in the United Kingdom as of 2018 was equivalent of Ksh 84,513 (British national formulary: *BNF 76* (76 ed.), whereas in the US, as per 2018, the wholesale cost of adalimumab was equivalent of Ksh 519,223 per month (NADAC as of 2019-02-27, Centers for Medicare and Medicaid Services). This cost is prohibitive, especially in developing countries.

2.4.5 Janus Kinase (JAK) Inhibitors

These are a newer class of non-biologic DMARDs that work by blocking the inflammatory process inside of a cell. Ruxolitinib and tofacitinib are the first JAK inhibitors approved for use in the United States. Both drugs are taken orally. JAK inhibitors are used in people with moderate to severe rheumatoid arthritis who have not responded to methotrexate alone or who have failed on biologics. These drugs interfere with Janus kinases (JAKs) and signal transducer and activator of transcription proteins (STATs) i.e., JAK-STAT signaling pathway, which transmits extracellular information into the nucleus, influencing transcription (Lamb and Rosen 2000; Feist and Burmester 2013).

These drugs are used in combination with methotrexate and taken twice daily. Side effects include headache, fatigue, stomach irritation, flu-like symptoms, diarrhoea, high cholesterol, low white blood cell counts, and an increased risk of infection. Administration of ruxolitinib was reported to result in anaemia and thrombocytopenia, whereas patients treated with tofacitinib were more prone to infections leading to opportunistic pathogens and herpes zoster (Gadina, 2013).

2.4.6 Non-Pharmacological Interventions

Surgery to repair damaged joints is the next alternative when medications fail to prevent or slow joint damage. Surgery can also lead to one's ability to use the affected joint, and correct deformities. The surgical interventions include synovectomy, tendon repair, joint fusion, or total joint replacement, but carry a risk of bleeding, infection, and pain. Synovectomy may be needed to prevent pain or tendon rupture when drug treatment fails, especially for affected fingers, hands, and wrists. Joint replacement, such as knee replacement, may be required when joints are severely affected. After surgery, there is always a need for physiotherapy (Turesson *et al.*, 2003).

Despite the introduction of several highly effective pain-relieving and anti-inflammatory agents, RA and related joint disorders continue to cripple human life. Millions of people all over the world suffering from rheumatoid disorders still require more effective drugs with fewer side effects.

2.5 Medicinal Uses of Natural Products

Eighty per cent of the world population was reported to have used complementary medication (WHO, 2003). Approximately, 50% of drugs used in medicine are of plant origin (Harborne, 1998). Much attention is being paid to use of plant-based drugs in traditional medicine because of their minimal side effects, affordability, easy accessibility, and social acceptability (Lee, 2000; Kumara, 2001; Park *et al.*, 2007; Wagner and Ulrich-Merzenich, 2009). Tradition Medicine/Complementary and Alternative Medicines (TM/CAMs) are commonly used as therapies in low- and middle-income countries because they are generally available and affordable (WHO

2002). Quality control methods for medicinal plant materials have been laid down by Yadav and Prajapati (2011).

Empirical evidence on safety and effectiveness supports the use of many TM/CAM therapies. Sources such as traditional scriptures, clinical experience, pharmacopoeias collected over hundreds of years have led to evidence regarding the use of TM/CAM therapies. There is an increasing number of scientific studies that now support the use of certain TM/CAM therapies. A popular treatment for pain relief, called acupuncture, has been demonstrated both through numerous clinical trials and laboratory experiments (Vickers, 1996; Ernst and Pittler, 1998; White, 2001). Another example is artemisinin, a new antimalarial medicine, which is purified from a traditionally used medicinal plant, *Artemisia annua*. Other examples of TM/CAM therapies with research-derived evidence base are St John's Wort (*Hypericum perforatum*) for mild depression; and Saw Palmetto (*Serenoa repens*) for reducing symptoms of benign prostate hyperplasia (Vickers, 1996; Ernst and Pittler, 1998). It was shown that TM/CAM therapies may cause fewer adverse events than conventional therapies (pharmacotherapy) such as acupuncture (NIH News Release, 1997; Patwardhan, 2005). Patients turn to TM/CAM for complementary care due to the increasing cases of chronic and debilitating diseases for which there is no cure. Scientific studies in human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDs), and cancer patients have revealed that several TM/CAM therapies are effective (Carlsson *et al*, 1985).

Advantages of TM/CAM include its diversity and flexibility, availability and affordability in many parts of the world, widespread acceptance in low- and middle-income countries, comparatively low cost; and the relatively low level of technological input required. Therefore, TM/CAM therapies have the potential to contribute to a better health care system in many countries (WHO, 2002).

An increase in research to improve the evidence-base as regards the efficacy of most TM/CAM therapies is, however needed. Measures are therefore needed to facilitate research efforts which may include the legal recognition of TM/CAM and the

development of appropriate research methods for evaluating some TM/CAM therapies.

2.6 Phytochemistry of Secondary Metabolites

The plant kingdom represents an enormous reservoir of biologically active molecules, and so far, only a small fraction has been assayed for medicinal value (Harborne, 1998). Plants have also been found to produce and accumulate secondary metabolites (organic compounds other than proteins) originally for defence purposes, to fight off herbivores, pathogens, and pests (Theis and Lerdau 2003; Samuni-Blank *et al.*, 2012). These metabolites fall into various groups based on their biosynthetic origin; alkaloids such as morphine, atropine, cocaine; terpenoids such as steroids; saponins such as ginsenosides; phenols such as tannins. Other secondary metabolites that have been discovered belong to the glycosides group such as digitalis; flavonoids such as luteolin. Humans have used plant secondary metabolites as medicines and flavouring agents. The importance of plant compounds in human systems led to phytochemistry or plant chemistry, a branch of chemistry that deals with chemical structures, biosynthesis, turnover and metabolism of plant compounds and their biological functions (Harborne, 1998).

Nearly 50% of drugs used in medicine are of plant origin and higher plants have been found to have ethnobotanical information associated with them. Therefore, there is a need to isolate the phytochemicals in plants for different biological activities such as cytotoxicity activity of Taxol from the bark of *Taxus brevifolia* was discovered via the brine shrimp test, as an assay of anticancer drugs. Alternatively, crude plant extracts can be first assayed for activities and the fractions then analyzed phytochemically. The present study adopted the latter approach.

2.7 The Techniques Used for Profiling of Secondary Metabolites

Use of chromatographic and spectroscopic methods are among the methods used to characterize secondary metabolites of plants and animal origin. Plant extracts contain various types of bioactive compounds which have different polarities.

2.7.1 Chromatographic Profiling

The separation and isolation of these compounds are carried out by use of various chromatographic techniques such as Thin layer chromatography (TLC) and Column chromatography.

2.7.1.1 Thin Layer Chromatography

The principle of thin layer chromatographic profiling depends on the ability of different compounds in the sample mixture to travel at different rates. This is due to the differences in their attraction to the stationary phase and differences in their solubility in the solvent (Sherma and Fried 2003). Separation of compounds is based on the competition of the solute and the mobile phase for binding sites on the stationary phase (Reich and Schibli, 2007).

When normal-phase silica gel is used as the stationary phase, it is considered polar. Therefore, any given two compounds that differ in polarity will have the more polar compounds with a stronger interaction with the silica displace the mobile phase from the available binding sites. As a result, the less polar compounds will move higher up the plate, resulting in a higher retention factor (R_f) value (Sherma and Fried, 2003). When the mobile phase is changed to a more polar solvent or mixture of solvents, it binds better to the polar plate and therefore displace solutes from it. Therefore, all the compounds on the TLC plate will move higher up the plate. Generally, "strong" solvents (eluents) will push the analyzed compounds up the plate, whereas "weak" eluents barely move them. The order of strength/weakness depends on the coating (stationary phase) of the TLC plate. When the stationary phase is polar and the mobile phase is nonpolar, the method is normal-phase as opposed to reverse-phase. This means that if a mixture of ethyl acetate and hexane as the mobile phase is used, adding more ethyl acetate results in higher R_f values for all compounds on the TLC plate.

Detection of compounds is simplest when the compounds of interest are natuarally coloured or fluoresce or absorb ultraviolet (UV) light. Spraying reagents such as vanillin in concentrated sulphuric acid and iodine vapour can produce colour or fluorescence for most compounds. Absorption of UV light is common for most aromatic and conjugated compounds and some unsaturated compounds (Reich and

Schibli, 2007). Such compounds can be inspected at λmax 254 and 365nm on a layer impregnated with a fluorescent detector. In the former λmax, spots appear black on a green background, whereas in the latter, spots are coloured on a blue background.

2.7.1.2 Column Chromatography

Column chromatography is based on the principle that molecules in a mixture applied into the solid stationary phase (stable phase) separate from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid) and affinity or differences among their molecular weights. Due to these differences, some components of the mixture stay longer in the stationary phase, and move slowly in the chromatographic system, while others pass rapidly into the mobile phase, and leave the system faster (Harris, 2012). There are three components that form the basis of the column chromatography technique: stationary phase, which is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support"; mobile phase, which is always composed of "liquid"; and the compounds to be separated.

The basic component effective on separation of molecules from each other is the type of interaction between stationary phase, mobile phase, and substances contained in the mixture. Compounds move through the column at different rates, allowing them to be separated into fractions. The purpose of applying chromatography for separation of compounds, is to achieve a satisfactory separation within a suitable time interval. The separation by column chromatography optimised when TLC is carried out before performing this process. Column chromatography method is used to isolate a single chemical compound from a mixture (Coskun, 2016).

2.7.2 Spectroscopic Techniques

2.7.2.1 Fourier Transform Infra-Red Spectroscopy

The basis of the study is that covalent bonds in molecules are more like stiff springs that can be stretched and bent. These bonds experience a wide variety of vibrational motions, characteristic of their component atoms. As a result, virtually all organic compounds will absorb infrared radiation (IR) that corresponds in energy to these

vibrations. Infrared spectrometers enable absorption spectra of compounds that are a unique reflection of their molecular structure. Some characteristic vibrational modes include symmetric and asymmetric stretching, bending, rocking, and scissoring. Since the energy of molecular vibration is quantized rather than continuous, a molecule can only stretch and bend at certain 'allowed' frequencies. When a molecule is exposed to electromagnetic radiation that matches the frequency of one of its vibrational modes, it will absorb energy from the radiation and jump to a higher vibrational energy state. The amplitude of the vibration will increase, although the vibrational frequency will remain the same. Therefore, the difference in energy between the two vibrational states is equal to the energy associated with the wavelength of radiation that was absorbed. The IR region of the electromagnetic spectrum contains frequencies corresponding to the vibrational frequencies of organic bonds. The technique is very useful as a means of identifying which functional groups, such as carbonyl, alkane, hydroxyl are present in a molecule of interest (Hemmalakshmi *et al.*, 2017).

For a vibrational mode to absorb IR light, it must result in a periodic change in the dipole moment of the molecule. Such vibrations are said to be IR active. Generally, the greater the polarity of the bond, the stronger its IR absorption, such as carbonyl bond, which is very polar and absorbs very strongly. The carbon-carbon triple bond in most alkynes, in contrast, is much less polar, and thus a stretching vibration does not result in a large change in the overall dipole moment of the molecule. Alkyne groups absorb rather weakly compared to carbonyls (Hemmalakshmi *et al.*, 2017).

2.7.2.2 Gas Chromatography-Mass Spectrometry

Gas chromatography–mass spectrometry (GC-MS) is an analytical method which combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample (Sparkman *et al.*, 2011). Applications of GC-MS include identification of unknown samples, detection of drugs and environmental analysis. The GC-MS process allows analysis and detection even of tiny amounts of a substance (Sivakumaran *et al.*, 2019). However, the high temperatures (300°C) used in the GC-MS injection port (and oven) can result in thermal degradation of injected

molecules, thus resulting in the measurement of degradation products instead of the actual molecule (s) of interest (Fang *et al.,* 2015).

The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column whose properties depend on the column's dimensions (length, diameter, film thickness) as well as the phase properties (such as 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then eluted from the column at different times, called called the retention time. This allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-charge ratio (De Hoffmann, 2000; Fabre *et al.,* 2001).

A finer degree of substance identification is achieved when these two components are used together than when either unit used separately. Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Hence, when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis, it typically increases certainty that the analyte of interest is in the sample. Analysis of the GC-MS spectrum essentially compares the given spectrum to a spectrum library to see if its characteristics are present for some sample in the library (De Hoffmann, 2000; Fabre *et al.,* 2001).

2.8 Natural Products in the Management of Arthritis

The search for safer drugs for use in arthritis from natural products continues. White willow (*Salix alba*) is a natural source of salicylic acid. Hippocrates, Galen, and Pliny discovered that the willow bark could ease pain and reduce fever (Norn *et al.,* 2009). Based on preliminary findings, the following are promising herbal medicines which are under investigation for treatments of RA; *boswellic acid* (Sharma *et al.,* 2010;

Abdel-Tawab *et al.,* 2011); *curcumin* (Julie and Jurenka, 2009), and *devil's claw* (Denner, 2007).

Tai Chi has been shown to improve the range of motion of a joint in persons with rheumatoid arthritis (Han *et al.,* 2004). Use of Omega-3 fatty acids and gamma-linolenic acid (as dietary supplements) in RA has been reported (Pirotta, 2010). There are modest benefit and safety in the use of Omega-3 fatty acids and gamma-linolenic acid in reducing pain, tender joint count, and stiffness (Soeken *et al.,* 2003).

Some natural products are used as complementary or alternative medicine in the management of RA and OA. These products are summarized in **Table 2.1.**

Table 2.1: Some herbal preparations used in the treatment of rheumatoid arthritis and osteoarthritis (Wakedar *et al*., 2015)

Name	Source	Description	Compounds
Capsaicin	A herbal medicine extracted from *chilli peppers*.	Available on prescription as cream.	8-methyl-*N*-vanillyl-6-nonenamide
Chondroitin sulphate	A nutritional supplement from the cartilage of cows, pigs, and sharks.	It gives collagen elasticity by helping it retain water and is a vital part of cartilage.	It is a complex sugar – glycosaminoglycan.
Collagen hydrolysate	Extracted from beef, pork or fish bones, and skin.	Stimulates the production of joint cartilage in arthritis joints.	Amino acids
Ginger (*Zingiber officinale)*	A herbal preparation extracted from the rhizome	Anti-inflammatory, antioxidant, antiarthritic	Volatile oils such as ginger oil, monoterpenes
Boswellia serrate **(Indian frankincense)**	An *Ayurvedic* medicine	Anti-inflammatory, anti-arthritic, analgesic.	β-boswellic acid
Aloe barbadensis	Stemless or very short-stemmed plant with thick fleshy green leaves	Anti-inflammatory. Anti-arthritic.	Anthraquinone compounds
Curcuma longa (*turmeric*)	Rhizome	Antioxidant, anti-inflammatory. anti-arthritic.	Curcuminoids such as curcumin

2.9 Evaluation of *In vitro* Antioxidant Activity

This method is based on the generation of reactive oxygen species (ROS), known to take part in inflammatory conditions such as rheumatoid arthritis (Filippin *et al.,* 2008). Regardless of the exact trigger, the ROS have been implicated to be involved in this process (Quinonez-Florez *et al.,* 2016). Scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) is the basis of this test. This compound is a dark-colored crystalline powder composed of stable free radical molecules. Being a well-known radical and a trap ("scavenger") for other radicals, the rate of reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. When test substances are mixed with DPPH and incubated for 30 minutes in a dark room, reduction of DPPH radicals change colour from dark blue colour to yellow coloured solution and absorbances are read at λ517 nm (Ramchoun *et al.,* 2017).

2.10 Evaluation of *In Vitro* Anti-Arthritic Activity

The method is based on the inhibition of denaturation of bovine serum albumin by heat (Mizushima and Kobayashi, 1968; Singh *et al.*, 2011). Bovine serum albumin and test substances are incubated at 57°C and turbidity measured at λ 660nm in a UV–VIS spectrophotometer or ELISA reader. In certain arthritic diseases, production of autoantigens may be due to denaturation of proteins *in vivo* (Umapathy *et al.,* 2010). Therefore, agents that can prevent protein denaturation by heat would be worthwhile for anti-arthritic drug development. The increments in absorbances of test samples with respect to negative control are an indication of stabilization of protein (Jagtap *et al.,* 2011).

2.11 Inhibition of Pain

Pain is an unpleasant feeling usually caused by intense or damaging stimuli (Raja *et al.,* 2020). A common noxious stimulus in models of acute pain is heating. The dependent variable in this model is the latency of the animal's response to the stimulus. The latency responses of treated and negative control are compared. An antinociceptive response, or an analgesic response, is interpreted as a significant

increase in the latency to respond to the heat stimulus after a therapeutic treatment, relative to the negative control. Depending on the stimulus, the latency of response will vary (Bannon and Malmberg, 2007).

2.11.1 Radiant Heat Method

One method is based on the response of rodents to heat by flicking the tail (Tail-flick). This method was initially described by D'Amour and Smith (1941) and later used by Jackson *et al.* (2011), who used warm water as a source of heat. The other method is the hot-plate method which is based on behavioural changes such as biting and licking the paws (Woolfe and MacDonald, 1944; Deuis *et al.,* 2017). Normally a source of heat of 55°C is applied. The nocifensive behaviors in the hot plate method include forepaw or hind paw withdrawal or licking, stamping, leaning posture, and jumping (Espejo and Mir, 1993). The latency of the rodents to respond to a stimulus is considered as a level of analgesic activity. Normally, rodents respond within 3 secs in tail-flick method, and a cut off period of 10 secs is considered to avoid tissue injury (Stein *et al.,*1988**).** The exposure time in the Hot plate method is 45 seconds (Espejo and Mir, 1993).

2.11.2 Formalin Test

In this test, the noxious stimulus is a chemical, and little or no restraining of experimental animals is needed during testing. The nociceptive stimulus and response are persistent rather than transient (Dubuisson and Dennis, 1977). This test, therefore, differs from the hot-plate and tail-flick assays. The formalin test is classified as a model of persistent pain, a good way to determine the potential analgesic activity of test compounds useful in progressive pain.

There are two phases of nociceptive response produced by formalin which can be distinguished pharmacologically. Immediately after the formalin injection is the beginning of phase 1 and lasts ~5-10 minutes. A burst of activity from pain fibers (particularly C fibers) is thought to be responsible for this initial response. Typically, phase 2 begins 15 to 20 minutes after the formalin injection and continues for ≥60 minutes. Peripheral inflammation mediates phase 2 and can be attenuated by NSAIDs.

The second phase behavioural response is also believed to be mediated by central sensitization. This facilitation of central nociceptive processing (such as increased sensitivity of spinal cord neurons) is thought to be a result of the prolonged afferent input to the spinal cord following the formalin injection (Dickenson and Sullivan, 1987). The clinical symptoms, including hyperalgesia, associated with tissue injury are mediated by both the peripheral (inflammation) and central sensitization.

There are several ways of quantifying nociceptive behavior in the formalin test. Counting the number of flinches, bites and licks in the formalin-injected paw may also be used to quantify nociceptive behaviours in rats (Wheeler-Aceto and Cowan, 1991). Flinching behaviour, a characteristic of the formalin test, in rats can be counted easily. Contrary, in mice, the most consistent behavior in the formalin test is licking of the paw (Hunskaar *et al.,* 1985). Time spent licking the paw is, therefore, the most used to quantify formalin-induced nociception in the mouse. The animal must be observed for the desired time interval during both phases 1 and 2, regardless of the behaviuor chosen to score the nociceptive response.

2.12 Screening for Anti-inflammatory Activity

Anti-inflammatory effects are determined by inhibiting oedema caused by carrageenan injection into the hind paw. Carrageenan is the phlogistic agent that has been used for examining anti-inflammatory drugs as it is reported to be antigenic and is devoid of afferent systemic effect. The model exhibits a high degree of reproducibility and is used in determining anti-inflammatory activities of medicinal agents and is well documented for various non-steroidal anti-inflammatory drugs (Winter *at al.,* 1962; Sini *et al.,* 2010).

Mediators such as PGs and LTs are released through the COX pathway (Katzung, 2017). Carrageenan oedema is considered a multi-mediated process that liberates a diversity of mediators. It is believed to be biphasic; with the first phase (1 hr) involving the release of 5-HT and histamine, while the second phase (over 1 hr) is mediated by PGs, the COX products. The transition between the two phases is provided by kinins (Asongalem *et al.,* 2004; Silva *et al.,* 2005; Perianayagam *et al.,* 2006; Brunton *et al.,* 2018). A correlation between the development of oedema induced by carrageenan and

early exudative stage of inflammation has also been reported (Asongalem *et al.,* 2004; Silva *et al.,* 2005). It can, therefore, be argued that suppression of 1st phase may be due to inhibition of release of early mediators, whereas 2nd phase may be explained by inhibition of the COX pathway. There are two main types of COX enzymes: COX-1 and COX-2. The former is constitutive and has gastroprotective activity and homeostatic functions such as renal function, platelet function, macrophage differentiation via cytoprotective prostaglandin, PGE_2. These PGs produced from COX-1 pathway are reported to act by a reduction in acid secretion, stimulating gastric mucus secretion, maintaining mucosal blood flow, and enhancing the resistance of epithelial cells to injury induced by cytotoxins. These PGs can suppress the generation of reactive oxygen metabolites produced by neutrophils thereby reducing inflammation and tissue injury. Further, PGs also contribute to ulcer healing by inducing angiogenesis. There has been accumulating evidence that PGs might contribute to ulcer healing by maintaining the balance between the pro-angiogenic and anti-angiogenic factors (Ananya and Sandip, 2014). The COX-2 enzyme is inducible by stimuli such as cytokines, growth factors and peroxides after injury and is responsible for inflammation, gastric irritation and bleeding, the main adverse effects of non-steroidal anti-inflammatory drugs (Katzung, 2017). The main mechanism of action of NSAIDs is via the inhibition of the COX pathway, hence this explains the common side effects of NSAIDs. Selective inhibitors of the COX-2 enzyme, such as celecoxib, have minimum GIT side effects, although this class of anti-inflammatory agents has adverse effects on the cardiovascular system (Mattia and Coluzzi, 2005).

The complexity of the inflammatory process and the diversity of drugs that have been found useful in modifying the process have resulted in the development of numerous methods of assay capable of detecting anti-inflammatory substances. A few of these methods have achieved popularity because of their simplicity, economic feasibility, and ability to select drugs known to afford some benefits in the clinical management of rheumatoid diseases.

2.12.1 Inhibition of Swelling in the Carrageenan-Induced Inflammation

Screening of anti-inflammatory drugs is based upon the ability of carrageenan (1%w/v) to inhibit oedema produced in rat hind paw after injection. Carrageenan is a mixture of polysaccharides composed of sulphates, galactose units and is derived from Irish Sea Moss (*Chondrus crispus*). Its biological properties have been reviewed by DiRosa (1972) and its use as an oedemogen was introduced by Winter *et al.* (1962) and later used by (Vinegar *et al.,* 1969; Balica *et al.,* 2013).

Use of carrageenan as an oedemogen is a simple and reliable model in the assessment of anti-inflammatory activity of various agents (Perianayagam *et al.,* 2006; Purnima *et al.,* 2010). Prediction of the anti-inflammatory activity of drugs used in human inflammatory disease has been evaluated before by the inhibition of carrageenan-induced inflammation. Doses of NSAIDs in this model correlated well with effective doses in patients (Otterness *et al.,* 1979). This model has allowed the detection of orally active anti-inflammatory agents, particularly in the acute phase of inflammation (DiRosa *et al.,* 1971).

The volume of the paw is measured before and after injection of carrageenan and the measurement of treated animals is compared to the negative control. A substance which shows an ability to reduce paw volume in this model potentially acts as an anti-inflammatory agent by acting through the inhibition of synthesis of inflammatory mediators, such as vasoactive amines like histamine, and 5-HT; PGs and LTs. Usually, the rat is the preferred species (Winter *et al*., 1962).

2.13 Screening for *In Vivo* Anti-Arthritic Activity

Complete Freund's Adjuvant-induced Arthritis (CFA) is an experimental model based on the development of polyarthritis and is based on the model of Carlson *et al.* (1985); Benslay and Bendele (1991); Bendele *et al.* (1999); Snekhalatha *et al,* (2013) and Zhang *et al,* (2017). Complete Freund's Adjuvant is the most effective and widely used adjuvant available for consistently producing high titer antibodies to diverse antigens. It is an irreplaceable and vital for immunology research and antibody production at the present time (Bendele, 2001).

Rat adjuvant arthritis is an experimental model of polyarthritis which has been widely used for preclinical testing of numerous anti-arthritic agents which are either under preclinical or clinical investigation or are currently used as therapeutics in this disease (Pearson, 1956; Carlson *et al.,* 1985; Benslay and Bendele, 1991). The characteristics of this model are reliable onset, and progression of robust, easily measurable, polyarticular inflammation, marked bone resorption and periosteal bone proliferation. Cartilage destruction occurs but is disproportionately mild in comparison to the inflammation and bone destruction that occurs. Although the pathogenesis/reasons for the development of adjuvant disease following injection of arthrogenic preparations are not fully understood, there are numerous studies which have contributed to the understanding of various possibilities; including reactivity to cartilage proteoglycans, heat shock proteins and interactions with intestinal flora (Van Vollenhoven *et al.,*1988; Feige *et al.,* 1994; Van Eden *et al,* 2017; Mantej *et al,* 2019).

2.14 Plants with Reported Anti-Inflammatory and Anti-Arthritic Activities

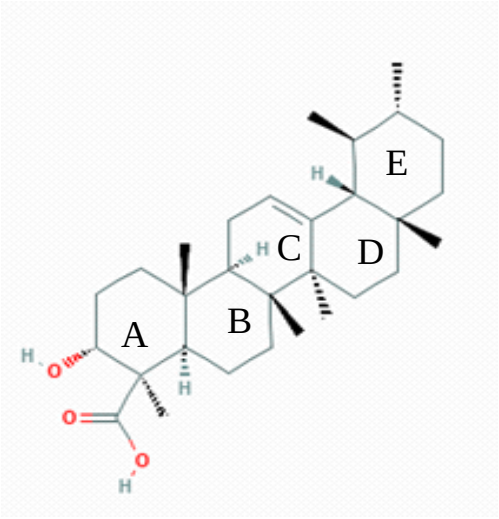
Due to the many adverse effects of conventional NSAIDs, DMARDs, and biologics, there is an urgent need to search for drugs which are efficacious and safe. The search for effective and safe antirheumatic drugs continues, and plants may provide a rich source for new medicines or form templates for new medicinal products for the treatment/management of arthritis conditions.

It has been reported that people suffering from chronic pain, such as RA, are dissatisfied with current treatment and are likely to seek alternative treatments. In fact, an estimated 60–90% of persons with arthritis use complementary and alternative medicine (CAM). There exists a need for an investigation into CAM safety and efficacy with the growing interest in herbal therapies among persons with rheumatoid arthritis (Soeken *et al*., 2003). Worldwide, herbal medicines have recently become popular for the treatment of rheumatoid arthritis (Asif *et al.,* 2011).

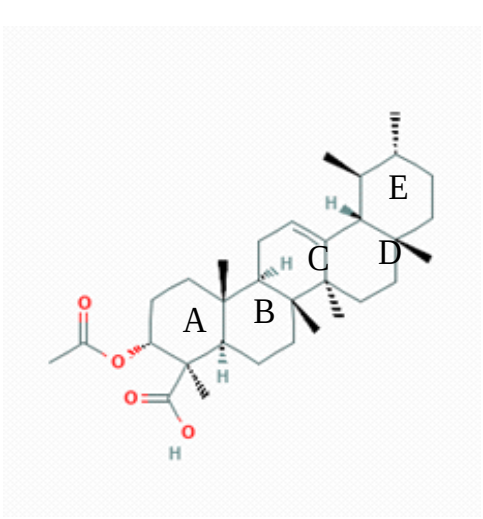
There is a large diversity of phytochemical compounds from medicinal plants which could be used as templates for new drug development (Verpoorte, 1998; Shakya, 2016). In fact, medicinal plants form a source of new compounds which could be developed into new drugs for management of inflammatory conditions among other

diseases. Some of these medicinal plants have been studied, and the findings are summarized in the paragraphs below.

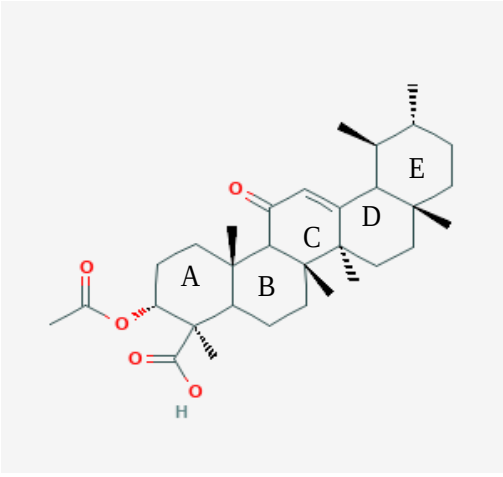
The petroleum ether extract from *Boswellia serrata* Roxb. (Burseraceae) is reported to contain oleogum resin which is pentacyclic triterpenoid in nature and possess boswellic acids (β-boswellic acid, acetyl-β-boswellic acid, keto-β-boswellic acid and acetyl-11-keto-β-boswellic acid). These extracts are used in cancer, inflammation, arthritis, asthma, psoriasis, colitis, and hyperlipidemia (Sharma *et al*., 2010). The chemical structures of these compounds are shown below.



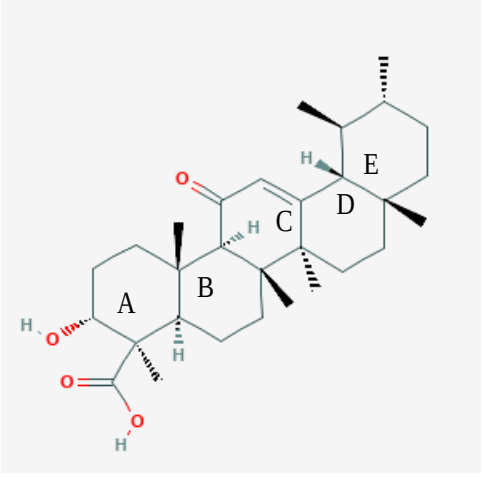
β--Boswellic acid



Acetyl-beta-boswellic acid

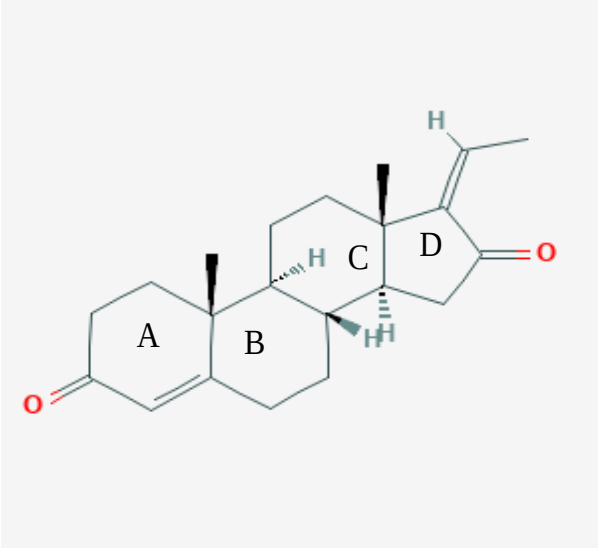


Acetyl-11-keto-β-boswellic acid



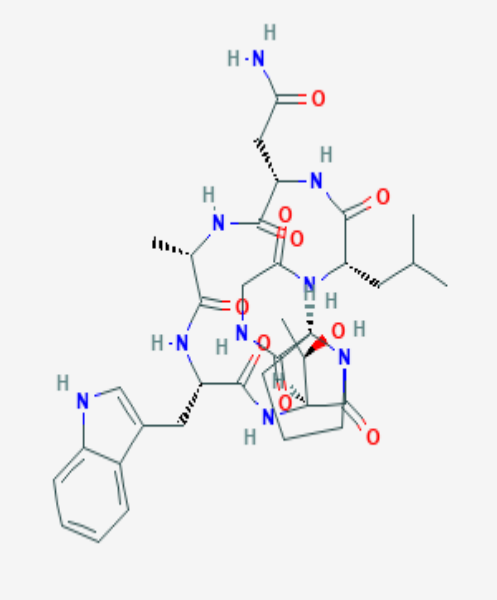
11-keto-β-boswellic acid

The ethyl acetate extracts from the stem of *Commiphora mukul* (Burseraceae) was reported to possess guggulsterones (E- and Z-stereoisomers) and gugu lipid, useful in arthritis, obesity, and other disorders (Mesrob *et al.*, 1998). The chemical structure of guggulsterone is shown below.

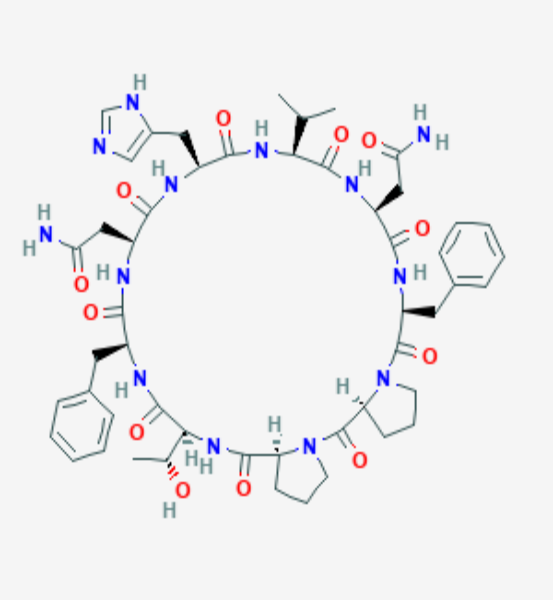


Guggulsterone

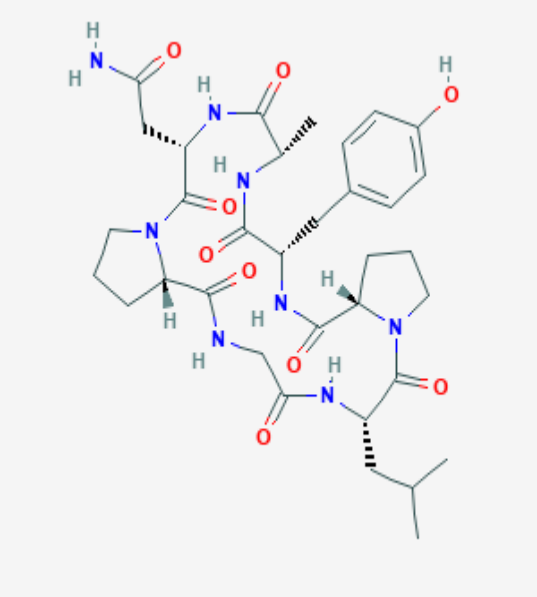
The methanol extract from leaves, fruit, seeds, bark, and roots of *Annona montana* (Annonaceae), have been reported to possess cyclomontanins A-D (1- 4), annomuricatin C (5), and (+)-corytuberine and used as anti-rheumatic, anthelmintic, anticonvulsant, antidepressant, antimicrobial, antineoplastic, antiparasitic, antispasmodic, antiviral, astringent, cardio depressant, cytostatic, cytotoxic, febrifuge, hypotensive, insecticide, nervine, pectoral, sedative, stomachic, vasodilator, vermifuge (Chuang *et al.*, 2011). The chemical structures of Cyclomontanins A, C and D are shown below.



Cyclomontanins A

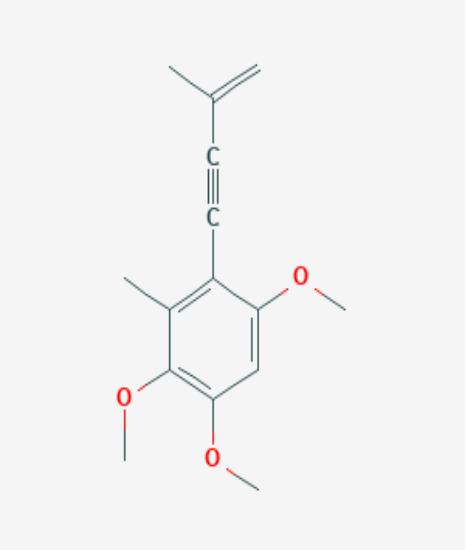


Cyclomontanins C

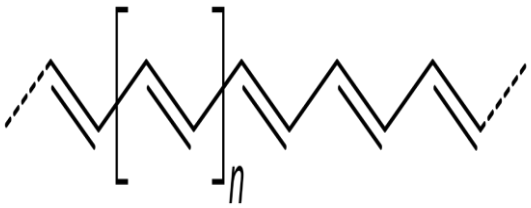


Cyclomontanins D

Methanol extract of fruiting bodies of *Antrodia cinnamomea* (Fomitopsidaceae), a fungus, contains antrocamphin A, ergostane-type triterpenoids and polyacetylenes with anticancer, antioxidant and activity in inflammatory disorders (Kumar *et al.*, 2011). The chemical structures of antrocamphin A and polyacetylene are shown below.

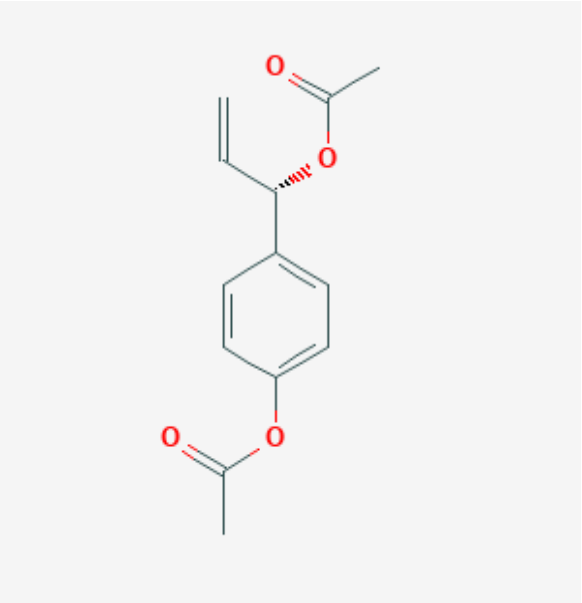


Antrocamphin A



Polyacetylenes

Ethanol extracts of various plants and plant parts have been particularly productive in providing bioactive extracts and phytochemicals. The ethanol extract from rhizomes of *Alpinia conchigera* Griff. (Zingiberaceae) was reported to contain galango flavonoid, 1'acetoxychavicol acetate (galangal acetate), and possess analgesic and anti-inflammatory activities (Lee *et al.,* 2006). The chemical structure of 1'acetoxychavicol acetate is shown below.



1'-Acetoxychavicol acetate

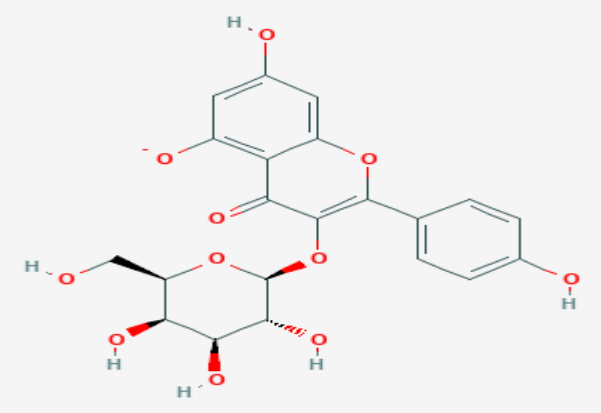
The ethanolic extract (99.9%) from seeds, dried seeds, leaves and roots of *Vernonia anthelmintica* (Asteraceae) showed the presence of alkaloids, flavonoids, steroids, triterpenes, and polyphenols which possess anti-inflammatory and anti-arthritic activities (Otari *et al.,* 2010).

The ethanolic (95%) extract from leaves of *Justicia gendarussa* (Acanthaceae) was reported to possess sterols and flavonoids useful in fever, hemiplegia, rheumatism, arthritis, headache, earache, muscle pain, respiratory disorders, and digestive troubles (Paval *et al.*, 2009).

The ethanolic (90%) extract from wood of *Premna serratifolia* (Verbenaceae) was reported to contain iridoid glycosides, alkaloids, phenolic compounds, and flavonoids useful in cardiovascular diseases, skin diseases, inflammatory diseases, arthritis, gonorrhoea, rheumatism, anorexia, and jaundice (Rajendran and Krishnakumar, 2010).

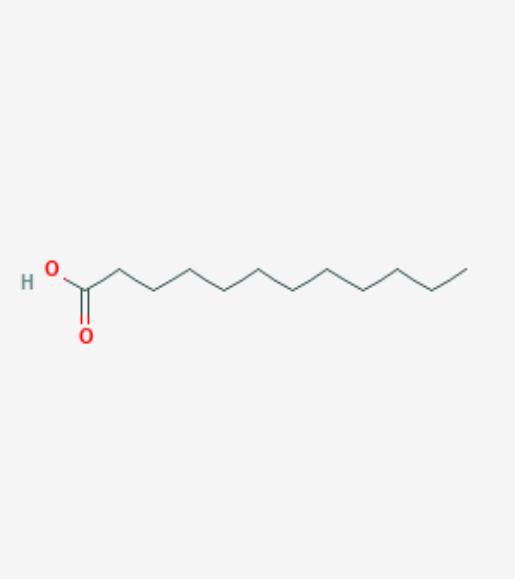
The ethanolic extract from male flowers (inflorescences) of *Borassus flabellifer* L. (Arecaceae) contains alkaloids, terpenoids, spirostane-type steroid saponins and phenolic compounds used as an anti-inflammatory, anti-laprotic, diuretic, antiphlogistic, stomachic, sedative, laxative, aphrodisiac, immunosuppressant (Paschapur *et al.,* 2009).

Ethanol, chloroform, ethyl acetate, n-butanol and water, methanol extracts from aerial parts of *Calluna vulgaris* (Ericaceae) were reported to contain kaempferol-3-O-β-D-galactoside, a common flavonol derivative with anti-inflammatory and antinociceptive, antirheumatic, diuretic, astringent, and treatment of urinary infections (Orhan *et al.,* 2007). The chemical structure of kaempferol-3-O-β-D-galactoside is shown below.

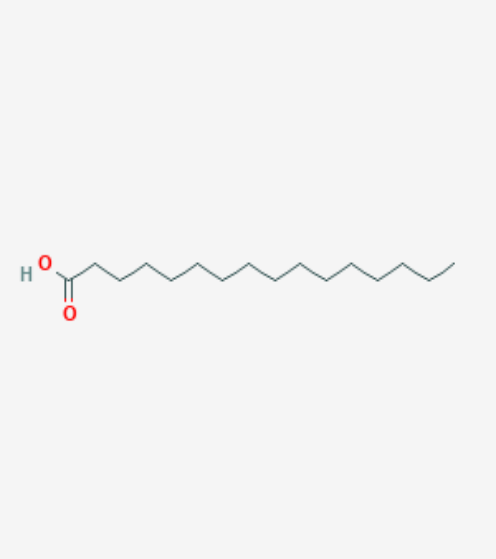


Kaempferol-3-O-β-D-galactoside

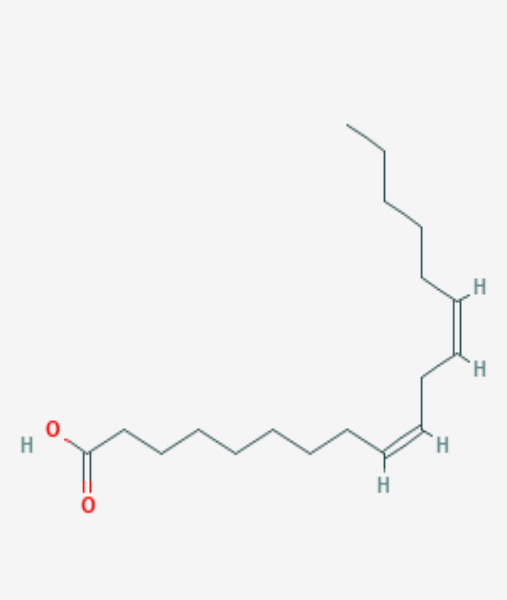
Ethanol, petroleum ether, ethyl acetate from berries (rose hips) of *Rosa multiflora* Thunb (Rosaceae) contain fatty acid, mainly dodecanoic acid (8.72%), hexadecanoic acid (9.24%), pentadecanoic acid (1.58%), linoleic acid (26.04%), oleic acid (22.58%), and octadecanoic acid (6.3%) used in dietary and medicinal purposes like cold, flu, inflammation, osteoarthritis, rheumatoid arthritis, and chronic pain (Guo *et al*., 2011). The chemical structures are shown below.



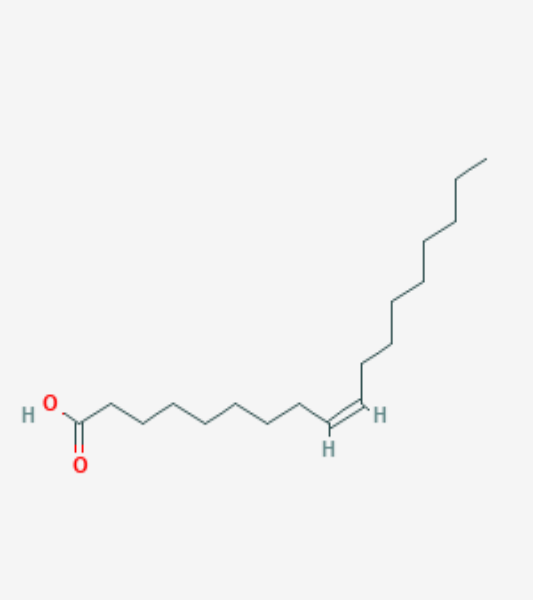
Dodecanoic acid



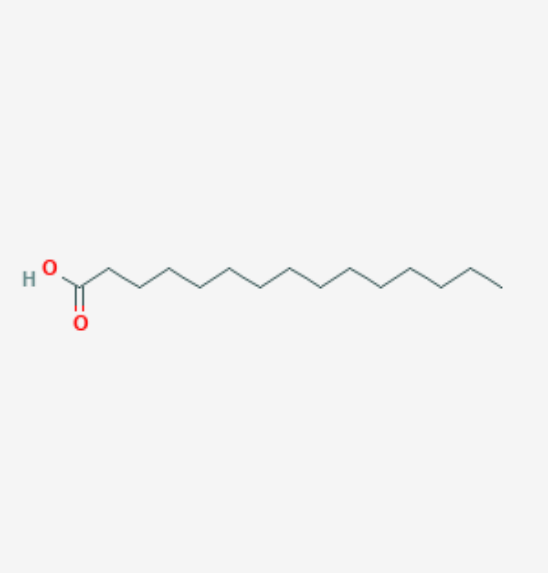
Hexadecanoic acid



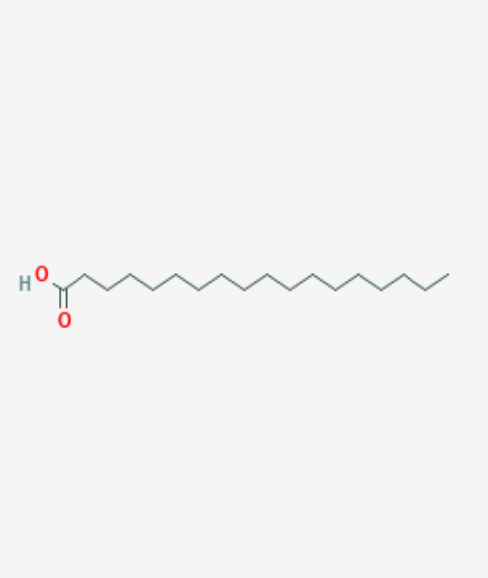
Linoleic acid



Oleic acid

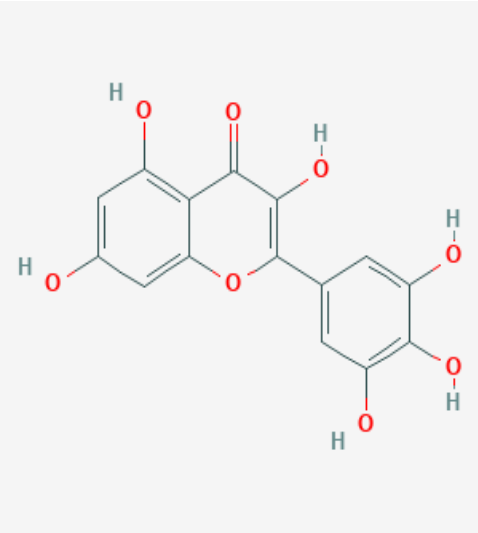


Pentadecanoic acid

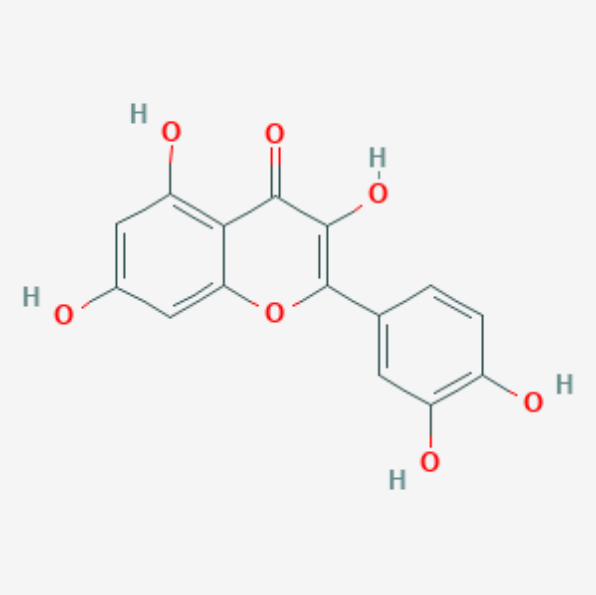


Octadecanoic acid

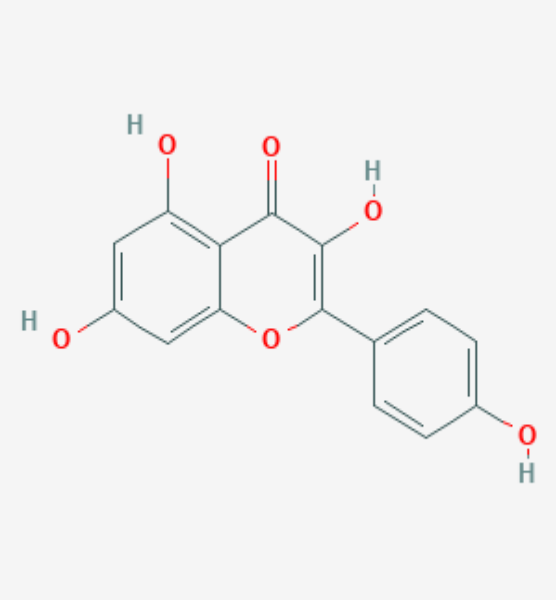
Ethanol extract and its petroleum ether, solvent ether, ethyl acetate, butanol, and butanone fraction from leaves of *Anacardium occidentale* (Anacardiceae) contains myricetin, quercetin, kaempferol, apigenin and glycosides used in diarrhoea, diabetes, swelling, skin diseases, mouth ulcers, inflammation (Patil *et al.*, 2003). The chemical structures of the compounds are shown below.



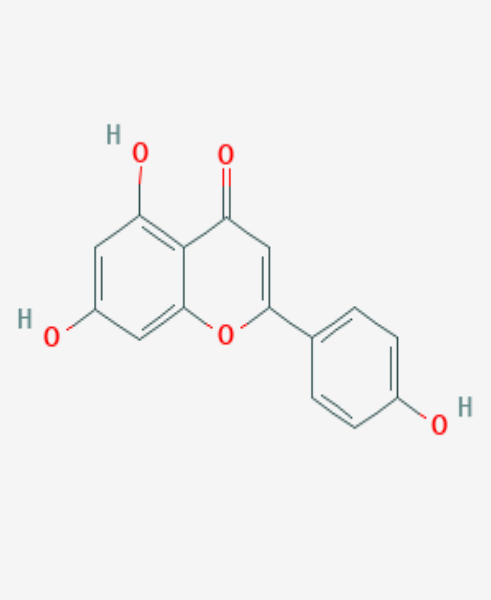
Myricetin



Quercetin

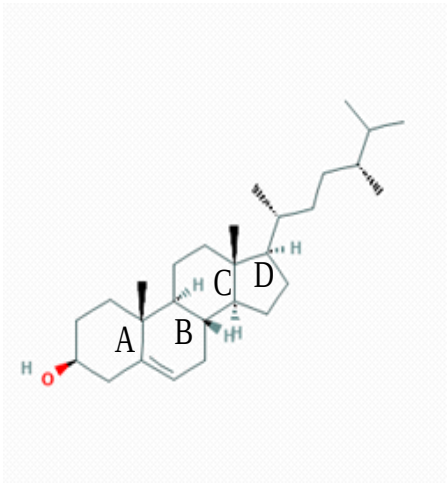


Kaempferol

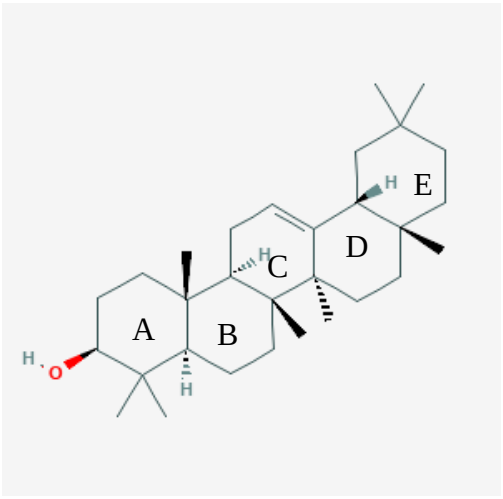


Apigenin

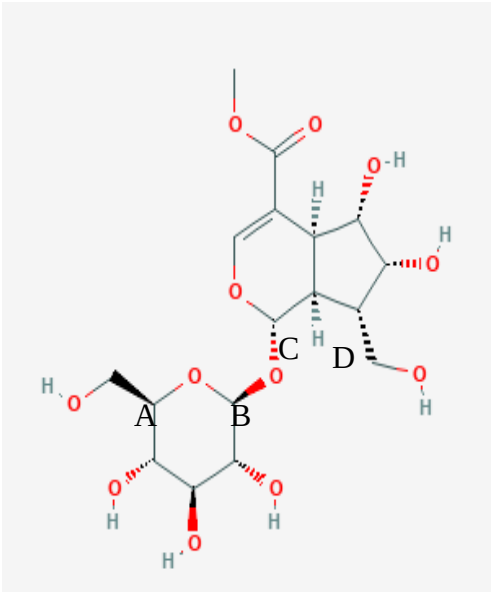
Alcoholic extract from leaves and stems from *Nyctanthes arbortristis* (Oleaceae) was reported to contain nyctanthoside, polysaccharides, iridoid glycosides, ß-sitosterol, ß-amyrin, glycosides useful in sciatica, arthritis, and anti-inflammatory (Saxena *et al.*, 1984). The chemical structures of these compounds are shown below.



ß-sitosterol

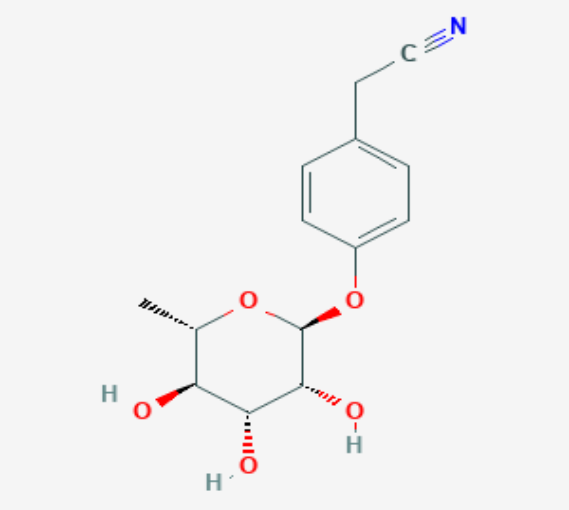


ß-amyrin

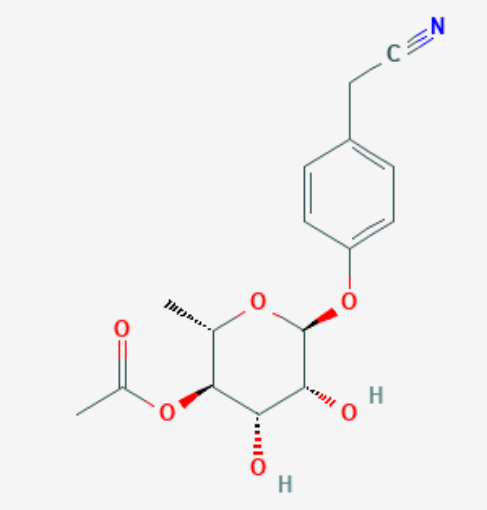


Nyctanthoside

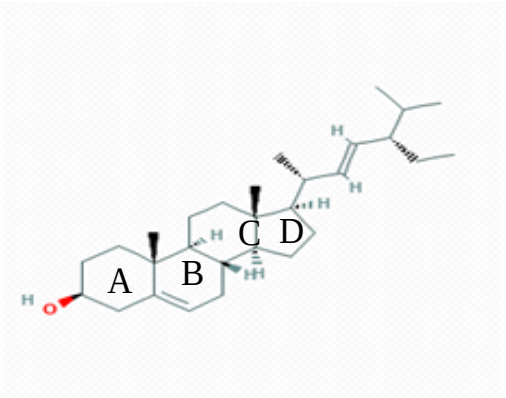
Hydroalcoholic extract from flowers and leaves of *Moringa oleifera* (Moringaceae) was reported to contain nitrile glycosides, niazirin, niazirinin, sterol components–stigmasterol, campesterol which possess anti-arthritic activity (Mahajan and Mehta, 2009). The chemical structures of these compounds are shown below.



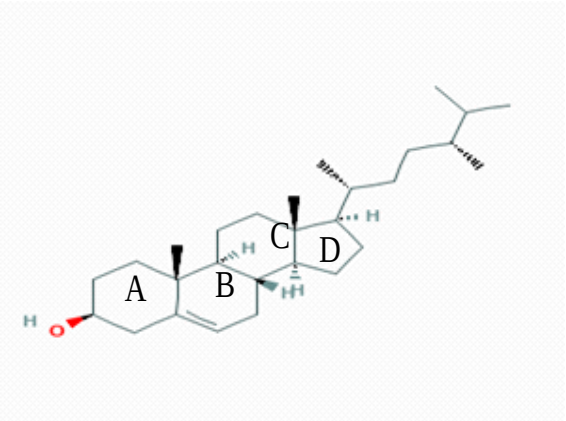
Niazirin



Niazirinin



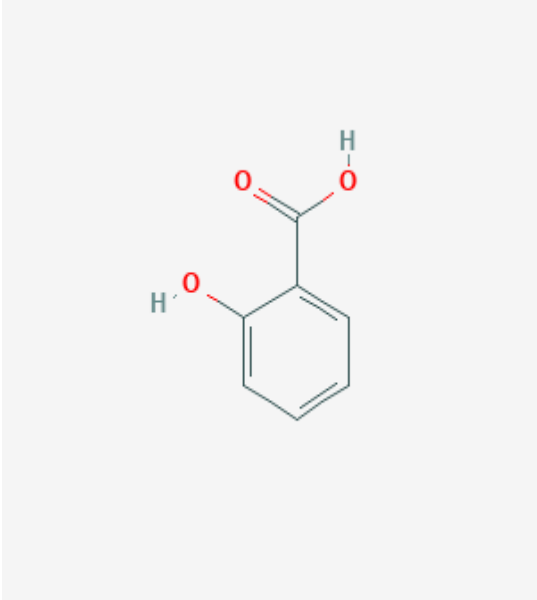
Stigmasterol



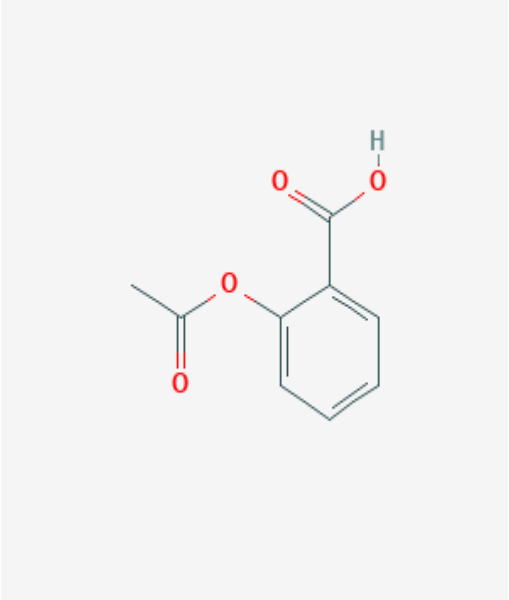
Campesterol

Hydro-alcoholic extract, ethyl acetate and n-butanol fractions of the leaves of *Azadirachta indica* (Meliaceae) contain flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids, and ketones possess anti-inflammatory, antipyretic, antimalarial, antitumour, antiulcer, antidiabetic, antifertility (Mosaddek and Rashid, 2008; Jagadeesh *et al*., 2014).

The bark of White willow (*Salix alba*) was macerated with ethanol to obtain a tincture. Later, the active extract was found to contain salicin, from which salicylic acid and acetylsalicylic acid (aspirin) were derivatized (Mahdi, 2010). The chemical structures of salicin and acetyl salicylic acid are shown below.

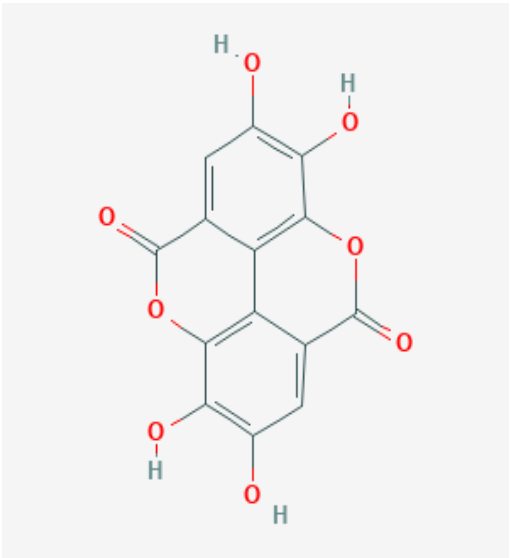


Salicylic acid

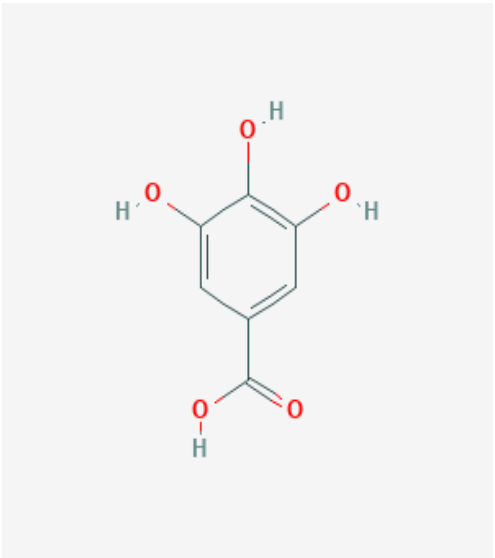


Acetyl salicylic acid

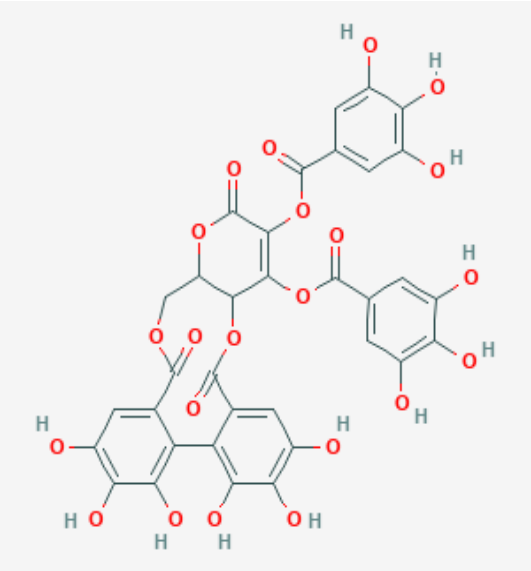
Water extracts from leaves, bark, or fruit of *Phyllanthus emblica* syn. *Emblica officinalis* (Phyllanthaceae) possess flavonoids, kaempferol, ellagic acid and gallic acid; vitamin C and other antioxidants like emblicanin A, emblicanin B, punigluconin, pedunculagin useful in hypercholesterolemic, rheumatoid arthritis and osteoporosis. The chemical structures are shown below.



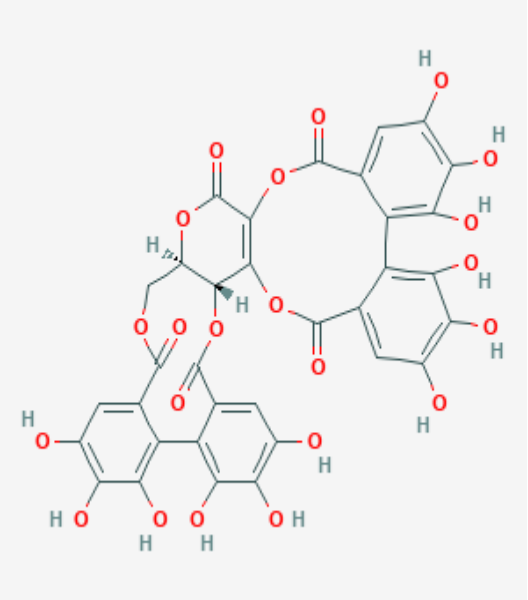
Ellagic acid



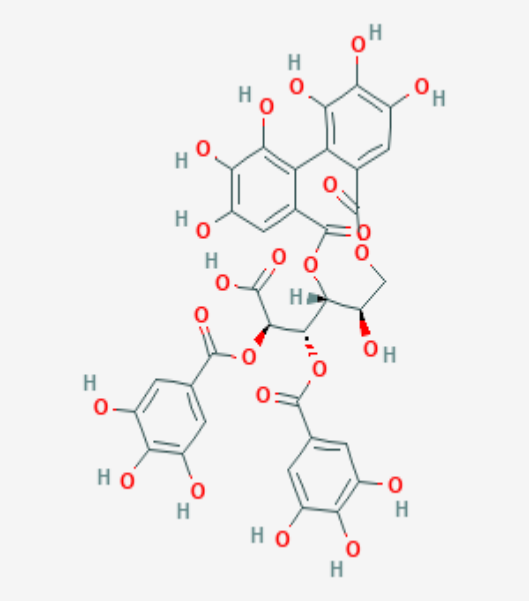
Gallic acid



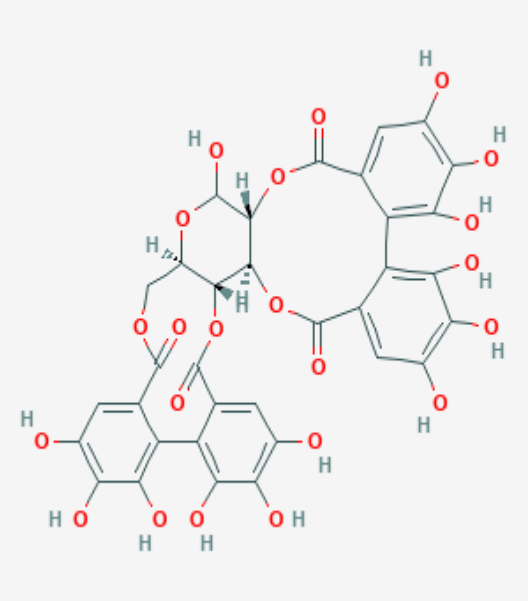
Emblicanin A



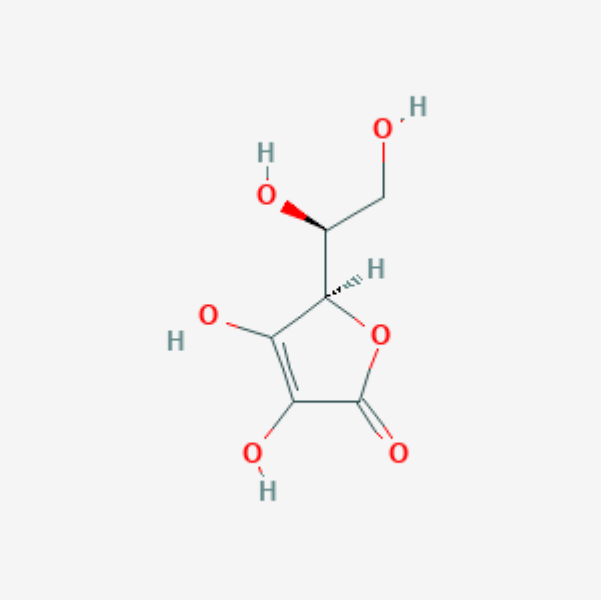
Emblicanin B



Punigluconin



Pedunculagin



Vitamin C

Aqueous extract from roots and leaves of *Butea frondosa* (Fabaceae) contains flavonoids, glucosides, and lectins with anti-inflammatory activity (Mengi and Deshpande, 1995).

A summary of some plant species used in pain and inflammatory disorders are shown in **Table 2.2.**

Table 2.2: Plant species and their therapeutic uses in pain and inflammatory disorders (Wadekar *et al.,* 2015)

Biological source	Part used	Active ingredient (s)	Therapeutic uses	Solvents of Extraction
Annona montana (Annonaceae)	Leaves, fruit, seeds, bark, roots	Cyclomontanins A-D (1- 4), annomuricatin C (5), and (+)-corytuberine	Anti-rheumatic	Methanol
Alpinia conchigera Griff. (Zingiberaceae)	Rhizomes	Galango flavonoid, 1'S-1'-acetoxychavicol acetate, 1'acetoxychavicol acetate (galangal acetate), β-Sitosterol diglucoside (AG-7) and β-sitosteryl Arabinoside	Analgesic and anti-inflammatory	Ethanol
Boswellia serrata (Burseraceae)	Oleogum resin	The resin which is pentacyclic triterpenoid in nature in which boswellic acids (β-boswellic acid, acetyl-βboswellic acid, keto-β-boswellic acid and acetyl-11keto-β-boswellic acid)	Cancer, inflammation, arthritis, asthma, psoriasis, colitis	Petroleum ether
Vernonia anthelmintica (Asteraceae)	Seeds, Leaves and Roots	Alkaloids, flavonoids, steroids, triterpenes, Polyphenols		Ethanol 99.9%
Moringa oleifera (Moringaceae)	Flowers, leaves	Nitrile glycosides, niazirin, niazirinin, sterol components – stigmasterol, campesterol	Anti-arthritic	Hydroalcoholic extract
Calluna vulgaris (Ericaceae)	Aerial parts	Kaempferol-3-O-β-D-galactoside, a common flavonol derivative	Anti-inflammatory antinociceptive, antirheumatic	Water, methanol

2.15 *Ruellia* Species

Ruellia (syn. *Dipteracanthus*) of the Acanthaceae family is a genus of flowering plants commonly known as *Ruellias* or wild petunias. There are about 300 species in this genus, which are distributed in Indonesia, Malaysia, Africa, tropical and temperate regions of both the hemispheres, which include Brazil and Central America, as the leading sources (Afzal *et al*., 2015; Singh *et al.*, 2015).

Ruellia species are also known for their many medicinal and ornamental properties. One of the species, *R. tuberosa*, has been extensively used as a diuretic, antidiabetic, antipyretic, analgesic, antioxidant (Arirudran *et al.*, 2011) antihypertensive, gastroprotective (Roopa *et al.*, 2011), and treatment of gonorrhoea (Suseela and Prema 2007). The use of *R. asperula* in bronchitis, asthma, flu, fever, and uterus inflammation has been reported (Afzal *et al.*, 2015). Use of *R. prostrata* leaf in the treatment of chronic rheumatism, cephalgia, hemiplegia, eczema, and facial paralysis, has been documented. The leaf juice from *R. prostrata* is an efficient remedy in colic of children (Afzal *et al.*, 2015). *R. patula* fresh leaf is pounded and then soaked in water till the solution turns black. Upon decanting, the solution is applied to the ear for ear infections (Tesfaye, 2004).

2.15.1 Description of *Ruellia* Species in this Study

The Acanthaceae family has members with branches angled or often ridged, stem with a distinct transverse ridge at the node. In general, this family has simple, opposite, decussated leaves with entire, toothed, lobed or sometimes spiny margins, rarely whorled or in rosettes and without stipules. Stem and leaves with cystoliths (outgrowths of calcium carbonate is common). Inflorescences variable, but usually of single flowers or cymules or dichasia, spike-like which are often aggregated into axillary or terminal racemoid cymes. Inflorescences with conspicuous bracts; bracteoles often present. Flowers bisexual, zygomorphic (irregular); sepals 4-5, free or fused, rarely reduced; petals 5, fused below and 5 lobed or sometimes 2-lipped above; stamens 2-4, fused to petal tube, with anther sacs normal or solitary, sometimes held at different levels or sometimes nearly actinomorphic (regular). Ovary superior, with 2 locular, or often bearing 1-2 ovules each. Fruit a capsule, usually open explosively

to propel dehiscent seeds, placenta separating from the capsule wall. Seeds, many per locule (1 to over 20 seeds) held on retinaculae from the funicle.

2.15.1.1 *Ruellia prostrata* Poir

Ruellia prostrata Poir belongs to genus *Ruellia* Plum ex L. It is a much-branched herb annual or sometimes perennial, erect, decumbent, or scrambling over other plants 30–160 cm tall. Species stem cylindrical from woody rootstock. Stem internode long with swollen nodes. Young stems puberulous, pubescent or pilose with non-glandular trichomes. Leaves with 1-25 mm petiole long; leave simple, opposite lamina broadly ovate–elliptic, in some leaves narrowly ovate-elliptic, 2-7 x 1.5-4.3 cm with entire margin, ciliate, apex often acute or rarely rounded, base attenuates subglabrous to pubescent, densest along veins. Flowers axillary solitary or in 3–flowered cymes pedicels sensile or 0.1-2 mm long, glabrous cymes. Bracts in cymes, narrowly to broadly ovate, 3-16 mm long with stock pubescent. Calyx 5-20 mm long, glabrous to puberulous, and divided into linear 5 partite. Corolla Pinkish-mauve to purple; tube 15-30 mm long pubescent outside, the basal cylindric part 3-9 mm and the throat 10-20 mm; lobes 5-17 mm across, elliptic with entire to crenate-dentate at the apex. Stamens 4 didynamous included in the throat, anther cells 2, filaments fused at base, free parts 1-4- and 4-9-mm long anthers 1-3.5 mm long. Ovary pubescent; capsule ovate-oblanceolate puberulous, pointed; 13-18 mm long, 5-14 seeded 3mm in diameter, greenish, ovoid to orbicular (Wangia and Muchuku, 2019a). The Voucher specimen for *Ruellia prostrata* is shown in (**Appendix 1).** The natural habitat of *R. prostrata* is shown in **Plates 2.1 and 2.2.**

Plate 2.1: *R. prostrata* in natural habitat

Plate 2.2: *R. prostrata* collected from natural habitat

Locality: Muthetheni Location , Mwala sub-county, Machakos county

Habitat: On a roadside, Open dry bushland of Acacia and combretum species on loam soils.

R. prostrata is a much-branched woody herb to 1metre, leaves stalked, slightly hairly, ovate-elliptic, flowers in stalkless axillary cluster, petals pinkish-mauve to white with bell-shaped tube

The macromorphology features of *Ruellia prostrata* (Wangia and Muchuku, 2019a).are shown in **Figure 2.3.**

Plate 2.3: Macromorphology features of *Ruellia prostrata*

2.15.1.2 *Ruellia bignoniiflora* S. Moore

Multi-stemmed woody perennial herb or subshrub up to 0.5-1.5 m tall. Stems erect or sometimes scrambling on stems of stronger species. Young stems with glandular and non-glandular hairs. Leaves broadly ovate, with sparsely soft hairs leaf petiole 1.5-4.5 cm long. The leaf blade is broadly ovate, largest 8 x 6 cm. Leaf apex is often sub-acute to acute. Leaf base is often sub-cordate or rarely cordate. Leaves are puberulous to pubescent with the densest area being along leaf veins and often with glands. The flower is often singly axillary; pedicel 5-10 mm long. The bracteoles are foliaceous, persistent, and ovate to orbicular 16x18 mm and petiole 3-9 mm long. Calyx 19-21 mm long divided into linear 5 partite (Wangia and Muchuku, 2019b). The Voucher

specimen for *Ruellia bignoniiflora* is shown in (**Appendix 2).** The locality of *R. bignoniiflora is* shown in **Plate 2.4.**

Plate 2.4: Locality of *R. bignoniiflora*- Mombasa road near Simba railway station , Kibwezi district.

Habitat: Dry Acacia-commiphora bushland on loose volcanic soils.

R. bignoniiflora is a tall woody much brached perennial with broady ovate, sparsely soft-hairly stalked leaves,flowers solitary, axillary , stalkless white and night flowering, with a very long petal tube.

The morphological features of *Ruellia bignoniiflora* (Wangia and Muchuku, 2019b) are shown in **Plate 2.5.**

Plate 2.5: Morphological features of *R. bignoniiflora*

2.15.1.3 *Ruellia lineari-bracteolata* Lindau

Ruellia lineari-bracteolata Lindau (Acanthaceae) is a multi-stemmed or multi-branched perennial shrub 50-90 cm tall with bluish-purple flowers. Young stems pale green and pubescent with 0.5-0.8 mm long glandular and eglandular trichomes. Old stem grey. The leaf blade is linear or sometimes lanceolate up to 35-90 x 3-9 mm with prominent veins and with white up to 1.5-1.7 mm. Calyx-lobes with pale mid-vein and broad hyaline margins. Corolla is blue or white at least up to 16-27 mm long and 10-12 mm across, tube funnel-shaped up to 11-13 mm long, lobes with free part 4-6 mm long. Anthers are 1.5-1.6 mm long. Capsule elongate and slender 8-16 seeded up to 13-16 x 2-4 mm, densely pubescent with 0.2 mm long simple trichomes all over as firest described by Agnew (1974). A study by Kilian *et al.* (2004) described *R. lineari-bracteolata* Lindau (Acanthaceae) as a dwarf shrub of dry areas with conspicuous linear leaves and bracts which was reported from South, Central and Northern Somalia. It was reported to occur in the Arabian Peninsula as well, where it was reported as new species (Thulin, 2006).

R. lineari-bracteolata Lindau was also reported as an endangered species in the Southern region of Pakistan (Abbas and Qaiser, 2012). The species is exposed to harsh subjected to multiple threats such as habitat loss, grazing activities and soil erosion, and harsh environmental conditions. Therefore, an urgent conservation strategy is required to avoid its extirpation, which included the study of the species response to different growth hormones, *in vitro*. The study described *Ruellia lineari-bracteolata* is a much-branched perennial herb, which grows up to 80 cm tall with bluish purple flowers. The species is mostly found around the edges of calcareous rocky hills, cliffs, slopes, and canyons. The Kenyan *R. lineari-bracteolata* was collected from a semi - arid area on the outskirts of Isiolo town, where the plant grew up to 48 cm high with perennial base, hairy, stems ridged, leaves narrowly elliptic, opposite, leathery, flowers fine blue, fruit narrowly elongated, pointed. Seeds with circular white rings. The Voucher specimen for *Ruellia lineari-bracteolata* is shown in (**Appendix 3).**

Ruellia lineari-bracteolata was obtained from the outskirts of Isiolo town at Uaso Nyiro river at Action Aid seed germination center.

Habitat: Acacia-Commiphora open bushland rarely on sand flood plain, associated species are Indigofera species, Aerva species, *Acacia mellifera* species and *Barieria acanthoides*.

2.15.2 Medicinal Properties of *Ruellia* Species

Many species of the genus have antinociceptive, analgesic, antioxidant, antiulcer, antidiabetic, antispasmodic, anti-inflammatory properties. The genus has been traditionally claimed to be used for the treatment of joint disorders of varied aetiology, analgesic, high blood pressure, eczema, flu, bronchitis, asthma, fever, antispasmolytic, antiulcer, and diabetes (Afzal *et al.,* 2015; Khan *et al*., 2017). Several *in vitro* and *in vivo* studies have reported on the anti-inflammatory activities of other *Ruellia* species; *R. prostrata* (Samy *et al.,* 2015); *R. tuberosa* (Alam *et al*., 2009) and *R. patula* (Sanjay *et al.,* 2012). These findings reveal a possible trend between *Ruellia* species and anti-inflammatory activity. Currently, there are no reports on the anti-arthritic activities of the three Kenyan species of *Ruellia* in this study (Kokwaro, 2009).

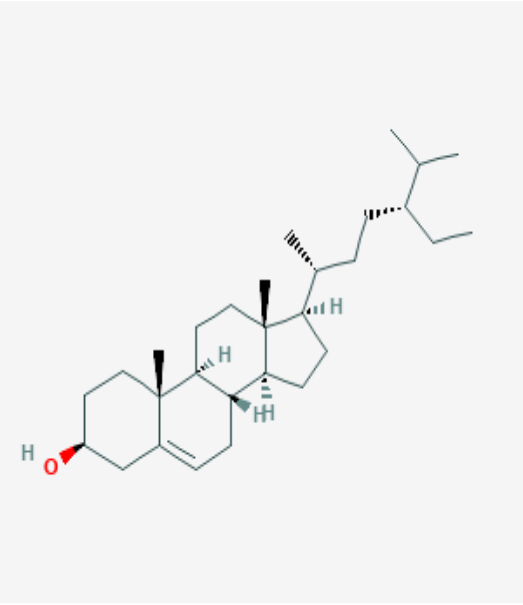
2.16 Phytochemistry of *Ruellia* Species

The phytochemicals isolated from *Ruellia* species include saponins, phenolics, glycosides, alkaloids, flavonoids, and triterpenoids. The chemical constituents of some *Ruellia* species are shown in **Table 2.3**.

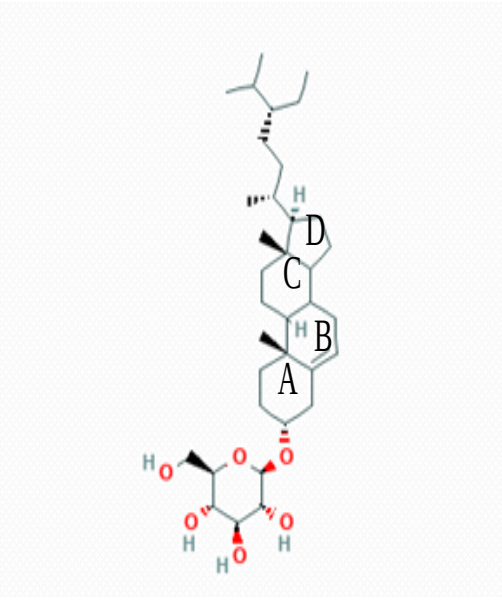
Table 0.3: Chemical constituents from the genus *Ruellia* (Samy *et al.*, 2015)

Class and Name	Species
β-sitosterol	*R. tuberosa, R. prostrata, R. patula*
β-sitosterol glucoside	*R. tuberosa, R. patula*
Stigmasterol	*R. tuberosa, R. prostrata, R. patula*
Campesterol	*R. tuberosa, R. patula*
Luteolin	*R. prostrata*
Luteolin 7-O-glucoside	*R. tuberosa, R. prostrata*
Apigenin 7-O-glucoside	*R. tuberosa, R. prostrata*
Apigenin 7-O-glucuronide	*R. tuberosa, R. prostrata*

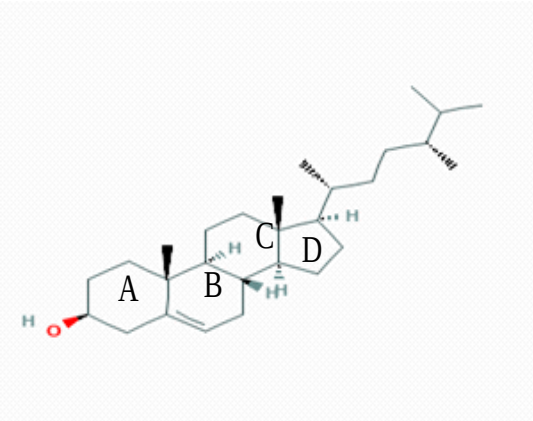
Some chemical structures of compounds commonly found in *Ruellia* species are shown below.



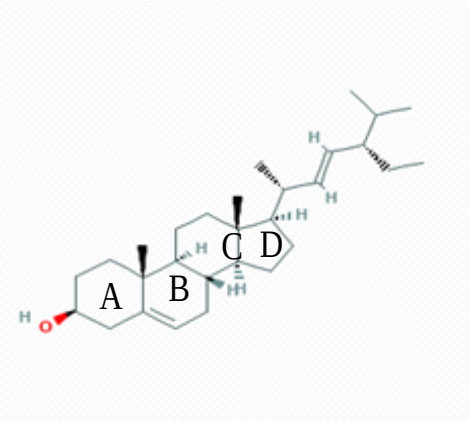
β-Sitosterol



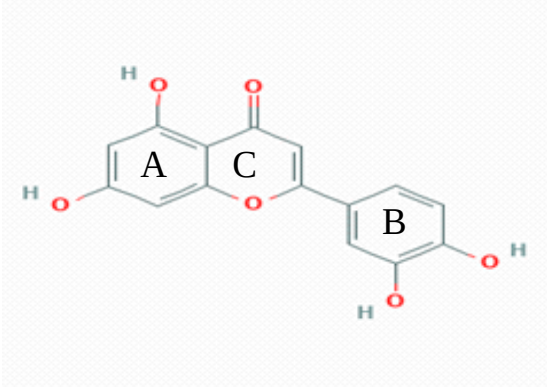
β-Sitosterol glucoside



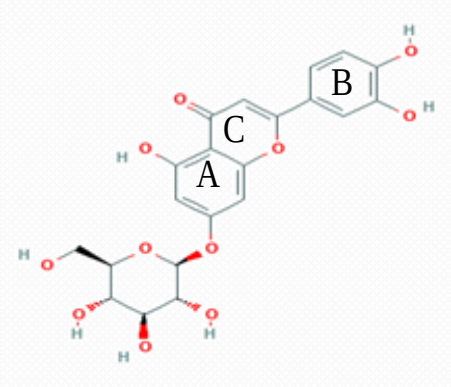
Campesterol



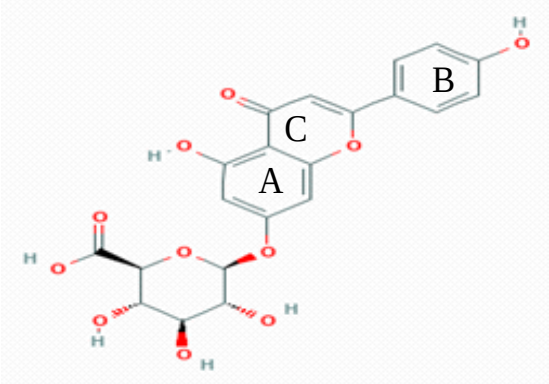
Stigmasterol



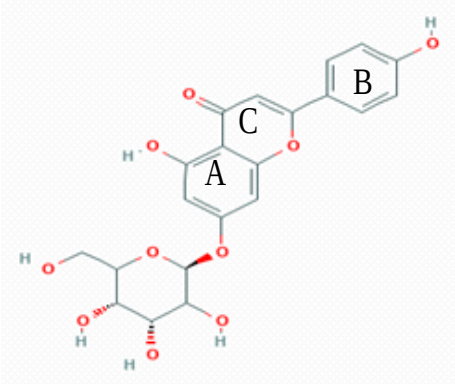
Luteolin



Luteolin 7-O-glucoside



Apigenin 7-O-glucuronide



Apigenin 7-O-glucoside

Structures of selected compounds isolated from *Ruellia* species (Subramanian 1972; Banerjee, 1984).

CHAPTER THREE

MATERIALS AND METHODS

3.1. General Experimental Procedures and Preparation

3.1.1 Collection of Plant Materials

Whole plant parts of the selected Kenyan *Ruellia* species viz. *R. prostrata, R. bignoniiflora*, and *R. lineari-bracteolata* were identified by a plant taxonomist and collected from their natural habitats. *R. prostrata* was collected from Muthetheni and Kavoo (Machakos County), *R. bignoniiflora* from Kibwezi (Makueni County) and *R. lineari-bracteolata* from Isiolo (Isiolo County) in Kenya. The plants were collected in 50-Kg sacks, and a total of 4-5 sacks were collected each time. The plant materials were carried in a personal car to JKUAT green house for drying. The plants were taxonomised and respective Voucher specimens (UoN/ 2010/598 of 16/12/2010 for *R. prostrata* (RPM); UoN/2013/811 of 15/3/2013 for *R. bignoniiflora*, and UoN/2015/003 of 3/7/2015 for *R. lineari-bracteolata*, respectively were deposited at the Department of Botany Herbarium, University of Nairobi (**Appendices 1, 2 and 3**). Whole plant parts were air-dried under shade for 2-3 months until the stems snapped. Once dry, the plant parts were ground into coarse powder by use of an electric grinder and stored in opaque paper bags and sealed to avoid contact with moisture.

3.1.2 Sample Size Determination

Using the resource equation developed by Arifin and Zahiruddin (2017) for sample size determination for animal experiments, the acceptable range of degrees of freedom (DF) for the error term in analysis of variance (ANOVA) was between 10 and 20. The number of species per group was calculated as:

$$n = DF/k + 1$$

Where:

n=number of species per group

k=number of groups

DF-degree of freedom

In the present study, the number of groups were 3. Therefore, the acceptable range of animals was 5-7.

The total sample sizes (N) were calculated as follows:

$$Minimum\ N = Minimum\ n * 3 = 15$$

$$Maximum\ N = Maximum\ n * 3 = 21$$

In the present study, a total of 15 to 21 animals were required to keep the DF within the range of 10 to 20.

3.1.3 Sampling Procedure

A probability simple random sampling method was used to assign animals to test and negative control groups as per Singh and Masuku, (2014). By use of random number tables, individual animals were randomly assigned to the negative control, test, or positive control groups. This sampling method assumed that the population or sampling frame was homogenous. Hence, each animal included in the sample had an equal chance of being included in the sample.

3.1.4 Study Sites

Plant materials were collected from Muthetheni and Kavoo areas (Machakos County), Kibwezi (Makueni County), and Isiolo (Isiolo County). Animal experiments were carried out at JKUAT laboratories. Phytochemical screening, *in vitro* and *in vivo* experiments were conducted at KEMRI and JKUAT laboratories. The map showing study sites where plant materials were collected is shown **in Figure 3.1**.

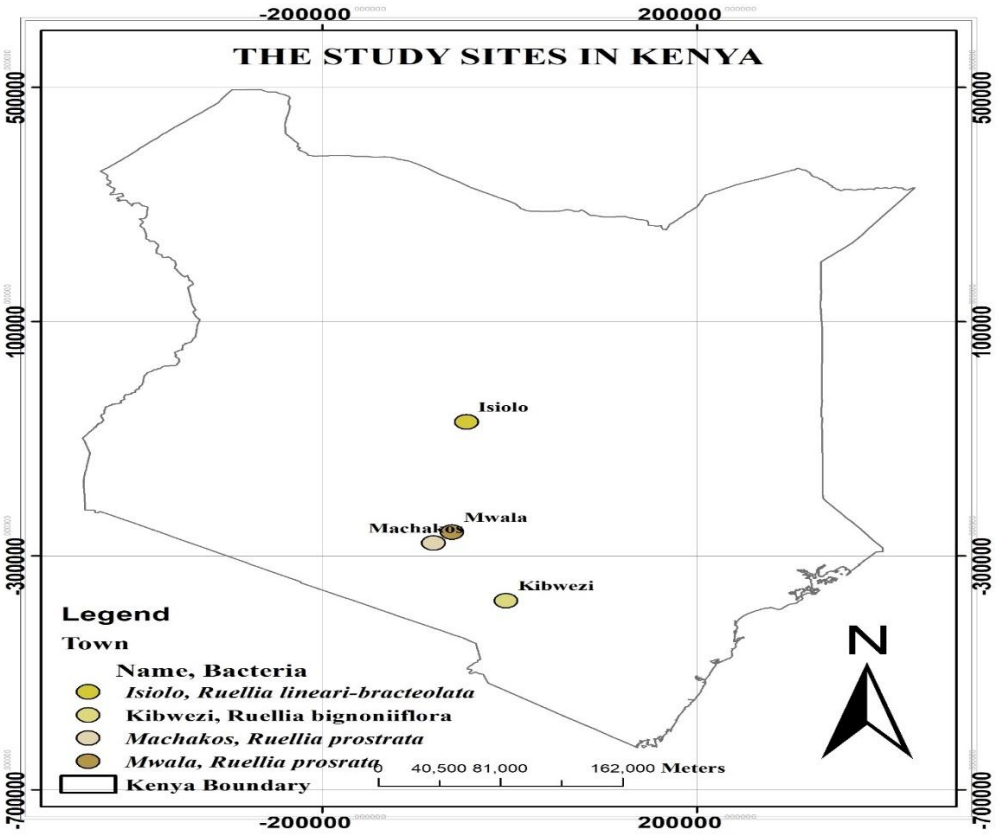


Figure 3.1: Map of Kenya showing study sites where plant materials were collected

3.1.5 Sample Preparation and Extraction

A schematic presentation of sample extraction and analysis is shown in **Figure 3.2.**

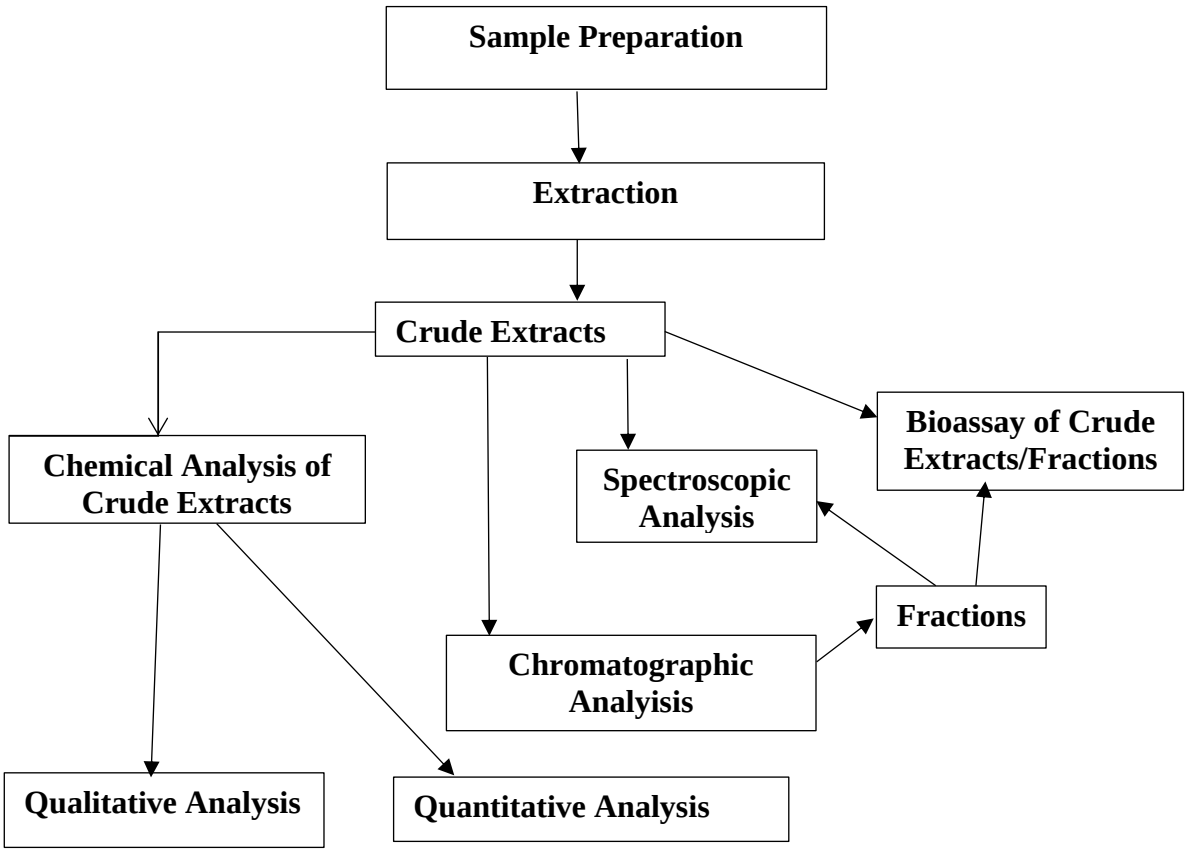


Figure 3.2: A schematic presentation of sample extraction and analysis

3.1.6 Organic Extraction

A quantity of 300 g of plant powder was initially defatted twice using 2000 mL petroleum ether and later extracted twice with 2000 mL of absolute methanol by cold maceration. The methanol contents were shaken using a horizontal mechanical shaker for six hours and allowed to stand overnight. After filtration through Whatman No.1 filter paper using a Buchner funnel, the second lot of 2000 mL was added and shaken for a further six hours. The contents stayed overnight and were filtered. The filtered methanol extracts were pooled together. The solvent was recovered by use of a rotor evaporator (BUCHI R-200, Labortechnik Switzerland) at reduced pressure and extract dried to constant weight in 100 mL beaker in an oven at temperatures between 60-

65°C. The beakers were covered tightly with foil paper and kept in the refrigerator at 4°C until required for *in vitro* and *in vivo* studies.

For column chromatographic analysis, organic extraction was carried out using dichloromethane: methanol (1:1) solvent system. This solvent mixture was able to extract both water-soluble and lipid-soluble compounds. The solvents were recovered by use of a rotor veaporator at reduced at 60-65°C.

3.1.7 Aqueous Extraction

A quantity of 300 g of powdered plant material was heated in 2000 mL distilled water at 70°C for six hours with intermittent shaking. The contents stayed overnight and were filtered through Whatman paper No.1 with the help of a Buchner funnel. The process was repeated once, contents pooled together and freeze-dried (Christ Alpha 1-4 LD). The aqueous extracts were stored at 4°C in a refrigerator.

For both organic and aqueous extractions, the extract % yield was determined using the procedure described by Banso and Adeyemo (2006):

$$\% \text{ Yield} = \frac{\text{Weight of Extract}}{\text{Weight of Plant Powder}} * 100$$

3.2 Chemicals, Solvents and Reference Drugs

The following analytical grade solvents were used: petroleum ether, chloroform, dichloromethane, ethyl acetate, absolute methanol, diethyl ether, n-butanol, absolute ethanol, and ammonia solution. These solvents were sourced from Merck, Germany. Complete Freund's Adjuvant was sourced from Chondrex Inc., USA. Reference drugs: ascorbic acid, acetaminophen, ibuprofen, diclofenac sodium and methotrexate were obtained from Sigma-Aldrich (USA), whereas morphine sulphate was purchased from Martindale Pharma (United Kingdom).

3.3 Laboratory Animals

Swiss albino male mice (6-8 weeks old) of weight 25–30 g and Wistar albino rats of either sex (6-8 weeks old) of weight 150–250 g were used for the study. All animals were purchased from the Small Animal Facility for Research and Innovation (SAFARI) located in JKUAT. These animals were housed in cages of 6 mice/cage and 3 rats/cage. Before any test was done, animals were removed from their home cages and quarantined in the procedure room of SAFARI animal house to acclimatize for five days. Standard laboratory conditions of ambient temperature 22°C±3°C, humidity 50-60% and artificial lighting of a 12- hour light and 12- hour dark cycle was maintained. Conventional laboratory rats and mice pellets were sourced from Unga Limited, Kenya. An unlimited supply of drinking water was provided.

3.4 Chemical Profiling

3.4.1 Qualitative Phytochemical Profiling

Phytochemical qualitative tests were conducted based on standard phytochemical tests (Harborne, 1998; Edeoga *et al.,* 2005; Obianime and Uche, 2008; Boxi *et al.,* 2010; Mojab *et al*., 2010; Deyab *et al.,* 2016; Ismail *et al*., 2016). The aqueous and organic extracts were tested for flavonoids, triterpenoids, steroids, phenolic compounds, saponins, tannins, and alkaloids. The screening tests were based on observation of colour change and/or precipitate formation.

For alkaloid identification, 1 mL of Mayer's reagent (potassium mercuric iodide) was added to 1mL of the test solution. A white precipitate could be a positive indicator for the presence of alkaloids (Mojab *et al*., 2010). To another portion of 1 mL, 3 drops of Dragendorff's reagent (a solution of potassium bismuth prepared from basic bismuth nitrate ($Bi(NO_3)_3$, tartaric acid, and potassium iodide) were added. An orange precipitate could indicate the presence of alkaloids (Boxi *et al.,* 2010).

For tannins identification, 0.5 mL of 5% ferric chloride solution was added to 0.5 mL of the sample solution. Formation of an intense dark green, purple, blue, or black colour would indicate the presence of tannins (Edeoga *et al.,* 2005).

For cardiac glycosides identification (Keller-Killani test), 5 mL of each extracts was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 mL of concentrated sulphuric acid. A brown ring of the interface could indicate a deoxysugar, characteristic of cardenolides (Edeoga *et al.*, 2005).

For steroids and terpenoids (Liebermann-Burchard reaction) identification, 10 drops of acetic anhydride were added to 1mL of methanol and aqueous extracts, followed by 1-2 drops of concentrated sulphuric acid, which was carefully added from the side of the test tube. A pink or violet could indicate presence of terpenoids, whereas a green colour could indicate the presence of steroids (Obianime and Uche 2008; Boxi *et al.*, 2010; Edeoga *et al.* 2015).

For terpenoids (Salkowski test) identification, to 5 mL of methanol and aqueous extracts, 2 mL of chloroform was added. A quantity of 3 mL concentrated sulphuric acid was carefully added, and the layers allowed to separate. A reddish-brown colouration at the interface could indicate the presence of terpenoids (Edeoga *et al.*, 2005).

For flavonoids identification, 5 mL of dilute ammonia solution (10 %v/v) were added to 5 mL of aqueous and methanolic extracts, followed by addition of concentrated sulphuric acid. A yellow colouration observed in each extract could indicate the presence of flavonoids. The yellow colouration could disappear on standing (Edeoga *et al.*, 2005).

For saponins identification, 0.5 g of plant extract was added into 5 mL of distilled water. The mixture was shaken vigorously and observed for the formation of foam that would persist for 15 minutes. Development of a stable emulsion on the addition of three drops of olive oil to the formed foam could confirm the presence of saponins (Edeoga *et al.*, 2005; El Aziz *et al.*, 2019).

The saponins were hydrolyzed by heating an aqueous extract with 1M hydrochloric acid. The sapogenin separated as a residue. On cooling, the supernatant was filtered using Whatman paper No.1 and subjected to both Fehling's and Benedict's tests for

the presence of reducing sugars (Adegoke *et al.*, 2010; Ameh *et al.*, 2010; Herrera *et al.*, 2019).

For reducing sugars identification, Fehling's solution A (a deep blue aqueous solution of copper(II) sulphate) and Fehling's solution B (a colourless solution of aqueous potassium sodium tartrate made strongly alkali with potassium hydroxide) were mixed in equal proportions to form Fehling's solution. These two solutions of Fehling's solution, which are stable separately, were combined because the copper (II) complex formed by their combination was not stable. The active reagent, bis(tartrate) complex of Cu^{2+}, served as an oxidizing agent. The tartrate tetra anions served as bidentate alkoxide ligands. The bis-tartratocuprate (II) complex could oxidize the aldehyde to a carboxylate anion, and in the process the copper (II) ions of the complex could be reduced to copper (I) ions. A quantity of 2 mL Fehling's solution was added to the supernatant (2 mL) and heated on a water bath for 3 minutes. Red copper(I) oxide could then precipitate out of the reaction mixture, which could indicate a positive result. A brick-red precipitate could reveal the presence of reducing sugars (Ameh *et al.*, 2010).

For reducing sugars identification, Benedict's solution (a mixture of sodium carbonate, sodium citrate and copper(II) sulphate pentahydrate) was added to the supernatant and the mixtures heated on a water bath for 3 minutes. Oxidation of the reducing sugar by the cupric (Cu^{2+}) complex of the reagent could produce a cuprous (Cu^{+}) ion, which could precipitate as insoluble red copper (1) oxide (Cu_2O). Colour change from clear blue to brick-red with a precipitate could reveal the presence of reducing sugars (Pataca *et al.*, 2007; Kumar and Gill, 2018).

The foaming index was determined as per Yadav and Prajapati (2011). A quantity of 1g of coarse plant powder (sieve size no. 1250), was weighed and transferred to a 500 mL conical flask containing 100 mL of boiling water. The contents were maintained at moderate boiling for 30 minutes, cooled and filtered into a 100-mL volumetric flask. Enough distilled water was added through the filter to volume. The decoction was poured into 10 stoppered test-tubes (height 16 cm, diameter 16 mm) in successive portions of 1-10 mL, and the volume of the liquid in each tube adjusted 10 mL with water. The tubes were stoppered and shaken in a lengthwise motion for 15 seconds,

two shakes per second, and allowed to stand for 15 minutes. The height of foam in each tube was determined in cm. If the height of the foam in every tube was less than 1cm, the foaming index was less than 100. If the height of the foam was more than 1 cm in every tube, the foaming index was over 1000. In that case, the process could be repeated and determination using a new series of dilutions of the decoction done, to obtain a result. Calculation of the foaming index was done using the following formula:

$$\text{Foaming index} = 100/a$$

Where: a-the volume in mL of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm was observed.

3.4.2 Quantitative Phytochemical Profiling

The concentration of total flavonoids and phenolic compounds in mg/mL and saponin content (%) in Kenyan *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* species were determined.

The total flavonoid concentration was measured by the aluminium chloride colourimetric assay (Marinova *et al.*, 2005). The extract (0.1g) was added to a 20 mL volumetric flask containing 4 mL of double distilled water. To the mixture, 0.3 mL of 5% $NaNO_2$ was added. After 5 minutes, 0.3 mL of 10% $AlCl_3$ was added. After 6 minutes, 2 mL of 1M NaOH was added, and the total volume was made up to 10 mL with double-distilled water. The solution was mixed well, and the absorbance was measured at λ510 nm against a blank. The flavonoid content was determined using catechin as standard.

The determination of total phenols was carried out using the Folin-Ciocalteu method described by Otang *et al.* (2012) and Saxena *et al.* (2013). Dried (finely ground) plant material (200 mg) was put in a 50 mL glass beaker. A quantity of 10 mL of aqueous acetone (70% v/v) was added and extracted while shaking for 20 minutes at room temperature. The contents of the beaker were then transferred to centrifuge tubes and subjected to centrifugation for 10 min (Centurion 6000 Series). The supernatant was

collected and kept on ice and then analysed for total phenolics. Tannic acid was used as the standard to prepare working standards. To the supernatant, 0.3 mL of it was taken in a test tube and 2.2 mL of distilled water added followed by 1.25 mL of Folin-Ciocalteu reagent and then 6.25 mL of sodium carbonate solution. The tube was vortexed, and absorbance recorded at λ 725 nm after 40 minutes in a UV spectrophotometer (UV-1800 Shimadzu). The number of total phenolics was calculated as tannic acid equivalent from the calibration curve and recorded.

Determination of the amount of saponins in the Kenyan *Ruellia* species d was carried out by the procedure of Obadoni and Ochuko (2001). A quantity of 10 gm of the sample was placed in 250 mL conical flask and 200 mL of 20% v/v aqueous ethanol was added and heated over a water bath for 4 hours with continuous stirring at about 55°C. The mixture was then filtered, and the residue re-extracted with another 200 mL 20% v/v ethanol. The combined extracts were reduced to 40 mL over a water bath at about 90°C. The concentrate was transferred into a 250 mL separation funnel and 20 mL of diethyl ether added and shaken vigorously. The aqueous layer was recovered while the diethyl ether layer was discarded. The purification process was repeated once. A quantity of 60 mL of n-butanol was added, and the process repeated once. The combined n-butanol extracts were washed twice with 10 mL of 5% aqueous sodium chloride. The remaining solution was heated on a water bath. After evaporation, the sample was dried in the oven at 110°C to a constant weight and saponin content (%) was then calculated as shown below:

$$\%\ \text{Saponin content} = \frac{\text{Weight of saponin residue}}{\text{Weight of plant material}} * 100$$

3.5 Chromatographic Phytochemical Profiling

3.5.1 Thin Layer Chromatographic Profiling

Thin-layer chromatography (TLC) and column chromatographic profiling methods using silica gel (230:70Å) were used to separate compounds present in Kenyan *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* aqueous and methanolic extracts as per the methods of Harborne (1998); Sherma and Fried (2003); Reich and Schibli

(2007); Debenedetti (2009); Bele and Khale (2011) and Biradar and Rachetti (2013). Commercial TLC plates were made of aluminium foil coated with normal silica gel (230:70Å) in which inorganic fluorescent indicators were incorporated giving rise to fluorescence at λ 254 nm, or λ 365 nm (Reich and Schibli, 2007).

Different solvent systems of different polarities were prepared, and TLC studies were carried out to select the solvent system capable of showing better resolution. Various solvent systems used included n-butanol: glacial acetic acid: water (B:A:W) 6:1:3, lower phase; B:AW (4:1:5), upper phase; methanol: dichloromethane (9.5:0.5); ethanol: 20% ammonia (8:2); n-hexane: ethyl acetate (7:3) and n-hexane: ethyl acetate (6:4). The prepared plant extracts were applied on commercial TLC plates by use of capillary tubes and developed in a TLC chamber using suitable mobile phase. The developed TLC chromatograms were air-dried and observed under UV light at at λ 254 nm, or λ 365 nm (Reich and Schibli, 2007). The chromatograms were later sprayed with different spraying reagents such as vanillin in concentrated sulphuric acid and iodine vapour and heated at 105°C for 5 minutes in hot air oven for the development of colour in separate spots. The retention factor (R_f) values were expressed by determined the movement of the analyte relative to the solvent front. R_f Values were calculated for different samples using the equation below.

$$\mathrm{Rf} = \frac{\text{Distance travelled by compound}}{\text{Diatance travelled by solvent front}}$$

3.5.2 Column Chromatographic Profiling

Column chromatographic studies were carried out as per Harris (2012) and Coskun (2016). Normal silica gel adsorbent was made into a slurry with n-hexane and placed in a cylindrical tube that was plugged at the bottom by a piece of glass wool. A ratio of extract: normal phase silica gel (1:5 to 1:10) was used in packing the column. The organic extracts to be separated were dissolved in n-hexane and introduced at the top of the column and were allowed to pass through the column. As the mixture moved down through the column, the components were adsorbed at different regions depending on their ability for adsorption. The weakly adsorbed components were eluted more rapidly than the others. The different fractions were collected separately.

The component with greater adsorption power was adsorbed at the top and the others were adsorbed at the bottom. The different components could be desorbed and collected separately by adding more solvent at the top. The process of dissolving out of the components from the adsorbent was the elution, whereas the solvent was called the eluent. By the use of a rotary evaporator, the solvents from the different fractions were obtained.

The fractions were collected and pooled together depending on their TLC profiles. The pooled fractions were evaporated at 70-80°C to about 2 mL by use of a rotor evaporator, under reduced pressure. The concentrates were dried under a fan and kept in the refrigerator at 4°C awaiting spectroscopic analyses. Fractions obtained from the column chromatographic separation were subjected to Fourier transform infra-red (FTIR) spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS) for functional groups and compounds identification, respectively.

Since *R. prostrata* showed highest *in vivo* analgesic and anti-inflammatory activities (**Figures 4.4-4.17**), the compounds in this species were further subjected to *in vitro* anti-arthritic activity studies. These fractions were also subjected to FTIR and GC-MS spectroscopy for analysis.

3.6 Spectroscopic Profiling

Fourier Transform Infra-Red and Gas Chromatography-Mass spectrometry techniques were used to determine the nature of the functional groups and compounds present in selected Kenyan *Ruellia* species, respectively.

3.6.1 Fourier Transform Infra-Red Spectroscopic Profiling

Fourier Transform Infra-Red spectroscopic profiling of bioactive compounds was carried out according to Ingle *et al.* (2017); Altemimi *et al.* (2017); Hemmalakshmi *et al.* (2017). and Safitri *et al*. (2019).

Organic extracts were obtained by cold maceration as described in **Section 3.1.6**. Water extracts were obtained by hot maceration as shown in **Section 3.1.7**. In FTIR spectroscopic profiling, solid organic and aqueous extracts of *R. prostrata*, *R.*

bignoniiflora and *R. lineari-bracteolata* were mixed with spectroscopic grade potassium bromide (KBr) in a crucible and made into a pellet. Column fractions were obtained from *R. prostrata* as described in sections **Section 3.5.2**. The infra-red spectra were recorded as frequency (cm^{-1}) using a Shimadzu Fourier Transform Infra-red spectrophotometer of 8000 series in the range of 3500 -500 cm^{-1}).

3.6.2 Gas Chromatography-Mass spectrometry

Spectroscopic profiling of compounds from Kenyan *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* was done using GC-MS and FTIR techniquesas per De Hoffmann, (2000); Fabre *et al.* (2001); Sparkman *et al.* (2011) and Fang *et al.* (2015). A Shimadzu QP 2010-SE GC-MS coupled to an autosampler was used for the analysis. Ultrapure helium was used as the carrier gas at a flow rate of 1mL/minute. A BPX5 nonpolar column, 30m; 0.25 mm ID; 0.25 µm film thickness, was used for separation. The GC was programmed as follows: 60°C (1 minute); 10 °C /min to 290°C (25 minutes). Total run-time was 41.33 minutes. Only 1 µL of the sample was injected. The injection was done at 200°C in split mode, with a split ratio set to 10:1. The interface temperature was set at 290°C. The electron ionization (EI) ion source was set at 200°C. Mass analysis was done in full scan mode, 50-550 atomic mass units (AMU). Raw mass spectra were matched against the NIST 2014 library of mass spectra for possible identification of compounds.

The GC-MS profiling of column organic extracts from *R. prostrata* was diluted in 100 % HPLC grade dichloromethane, filtered through 0.45 µm polytetrafluoroethylene (PTFE) filters and transferred to clean 2 mL auto sampler vials for GC-MS analysis, whereas column organic fractions from *R. prostrata* were constituted in Analar grade ethyl acetate. Column aqueous fractions were reconstituted in 100% methanol. The fractions were filtered through 0.45 µm PTFE filters and transferred to clean 2 mL autosampler vials for GC-MS analysis. A Shimadzu QP 2010-SE GC-MS coupled to an autosampler was used for the analysis as above.

3.7 Pharmacological Assays

3.7.1 Preparation and Administration of Test Preparations and Reference Drugs

For *in vivo* experiments, suspensions of the aqueous and methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* were prepared in commercial phosphate-buffered saline pH 7.4 and vortexed for 10 minutes to obtain homogeneity. The extracts were administered at four different doses of 250-1500 mg/kg, orally using a gastric gavage. The mice were given a volume of extract, of approximately 1 mL per animal, whereas the rats were given a volume of approximately 2 mL per animal as shown in **Table 3.1.**

Table 3.1: Preparation of different doses of extracts for mouse and rat species

Animal species (n=6)	**Average weight (kg)**	**Dose (mg/kg**, orally**)**	**Volume (mL) per animal**	**Quantity prepared (mL)**	**Quantity weighed (mg)**
Mouse	25	1500	1.0	10.0	375
Mouse	25	1000	1.0	10.0	250
Mouse	25	500	1.0	10.0	125
Mouse	25	250	1.0	10.0	62.5
Rat	200	1500	2.0	20.0	3000
Rat	200	1000	2.0	20.0	2000
Rat	200	500	2.0	20.0	1000
Rat	200	250	2.0	20.0	500

Fresh extract solutions were prepared each day in phosphate-buffered saline.

All extracts doses were administered orally by gastric gavage. In acute toxicity studies, analgesic, antinociceptive and anti-inflammatory studies, doses of the extracts were administered as a single dose. In sub-chronic toxicity and anti-arthritic studies, doses were administered daily for 28 days. Both *in vitro* and *in vivo* assays were carried out to determine the biological activities of the phytocompounds present in the Kenyan

Ruellia species in the present study. The methods are discussed in the subsequent sections.

Mice were given a volume of vehicle and standard of approximately 1 mL per animal, whereas the rats were given a volume of approximately 2 mL per animal as shown in **Table 3.2**.

Table 3.2: Preparation of different doses reference standards for mice and rats species

Reference standard	**Animal species (n=6)**	**Weight (g)**	***per os* Dose(mg/kg)**	**Volume per animal (mL/)**	**Quantity prepared (mL)**	**Quantity weighed (mg)**
Acetaminophen	Mouse	25	200	1.0	10.0	50.0
Morphine sulfate injection	Mouse	25	10mg/kg-s.c.	0.02	10mg/mL	10mg/mL
Diclofenac sodium	Rat	200	20	2.0	20.0	40.0
Methotrexate	Rat	200	2	2.0	20.0	4.0
Phosphate buffered saline pH 7.4-vehicle	Mouse	25		1.0		
Phosphate buffered saline pH 7.4-vehicle	Rat	200		2.0		

Fresh standards and extracts were prepared each day in phosphate-buffered saline pH 7.4.

All reference drugs doses were administered orally by gastric gavage, except morphine sulphate which was administered by the s.c. route. In analgesic, antinociceptive and anti-inflammatory studies, doses of the reference drugs were administered as a single dose, whereas in anti-arthritic studies, methotrexate was administered orally twice a week for 28 days.

3.7.2 Evaluation of *In Vitro* Antioxidant Activity

2,2-diphenyl-l-picrylhydrazyl (DPPH) scavenging activity was carried out according to the procedure described by Ramchoun *et al.* (2017). The method was based on the

reduction of DPPH radicals from dark blue colour to the yellow-coloured solution. DPPH solution was prepared by adding 3.94 mg (DPPH) in 100 mL methanol. Concentrations ranging from 3.95-500 µg/mL for both extracts and ascorbic acid standard were prepared. The negative control was methanol. After 30 minutes of incubation in a dark room, absorbance was read at λ 517 nm using an ELISA reader.

The scavenging inhibitory effect of DPPH was calculated according to the following formula:

$$\text{Percentage (\%)Inhibition} = \frac{\text{Absorbance of DPPH} - \text{absorbance of sample}}{\text{Absorbance of DPPH}} * 100$$

Concentration (mg/mL) which caused 50% inhibition (IC_{50}) was calculated using probit regression analysis for aqueous and methanol extracts of *R. prostrata*, *R. bignoniiflora* and *R.lineari-bracteolata*. Graphs showing % inhibition of DPPH radicals *vs* $\log_{10}$ concentrations of aqueous and methanolic extracts were plotted. Ascorbic acid was used as a reference drug.

Although the evaluation of the biological activity of medicinal plants poses many challenges since the animal model chosen for induction of the desired disease syndrome may not be the true replica of the human disorder, the animal models selected for analgesic, antinociceptive, toxicity and arthritic activities are well documented to give reliable results that reflect the disease conditions in humans.

3.7.3. *In Vitro* Anti-Arthritic Activity of Extracts and Column Fractions

Inhibition of protein denaturation was carried out as originally described by Mizushima (1965), and later adopted by Jayaprakasam and Ravi (2012) and Pavithra *et al*. (2015). The reaction mixture (0.5 mL) consisted of 0.45 mL bovine serum albumin, BSA (5% aqueous solution) and 0.05 mL of *Ruellia* extracts or column fractions. The activity was carried out within the concentration range of (1.67-8.33 mg/mL) for all the tests. The pH was adjusted to 6.3 by use of small amounts of 1M HCl. The samples were incubated at 37°C for 20 minutes and then heated at 57°C for 3 minutes. After cooling the samples in ice-cold water, 2.5 mL phosphate-buffered

saline pH 6.3 was added to each tube. Turbidity was measured at λ 660 nm using an ELISA reader (Multiscan GO by Thermo Scientific; Skanit software), relative to negative control. The negative control consisted of 0.5 mL distilled water and 2.5 mL phosphate-buffered saline. The results were compared with diclofenac sodium and methotrexate (1.67-8.33 mg/mL) as standards. The negative control represented 100% protein denaturation, whereas % protection of less than 20% was considered not significant (Mizushima, 1965).

Concentration (mg/mL) which caused 50% inhibition (IC_{50}) was calculated using probit regression analysis for aqueous and organic extracts of *R. prostrata* and *R. lineari-bracteolata* species using diclofenac sodium and methotrexate standards.

3.7.4 Activity-Tail-Flick Method

This method determined the analgesic activity of *Ruellia* extracts in an acute pain model, orignally by D'Amour and Smith (1941) and later used by Jackson *et al.*,(2011). Swiss albino mice were restrained in commercial restrainers. An identification mark was made on the tail of each mouse 5 cm from the tip with water-proof ink. A radiant beam (55°C) from a digital tail-flick analgesiometer (LE 7106, Panlab Harvard Apparatus, Spain) was used as the source of heat. The time (seconds) that the mice took to flick the tail was recorded. Usually, mice flick the tail within 3 seconds. All animals were screened and only those which flicked the tail within 3 seconds were randomly assigned to 7 groups of 6 animals each, as follows: Group 1-positive control (acetaminophen 200 mg/kg *per os*); Group 2-positive control (morphine sulfate 10 mg/kg, s.c); Group 3-negative control (vehicle-treated); Group 4-plant extract (250 mg/kg); Group 5-plant extract (500 mg/kg); Group 6 -plant extract (1000 mg/kg); Group 7- plant extract (1500 mg/kg). All plant extracts were administered orally using gastric gavage.

The reaction time (in seconds) for each mouse to flick the tail was determined at time 0, 5 minutes and 10 minutes after dosing, and then averaged to get the baseline reaction time. A cut-off point of 10 seconds was adopted to avoid tissue injury. Plant extracts (250-1500 mg/kg); acetaminophen (200 mg/kg) and morphine sulfate 10 mg/kg (s.c) were administered 1 hour (extracts) and 30 minutes (standards), respectively, before

the test. The reaction time was determined every 30 minutes for three hours. The percentage increase in reaction time was measured as an indicator of the analgesic activity.

$$\text{Percentage increase in reaction time} = \frac{\text{Ta} - \text{Tb}}{\text{Tb}} \times 100$$

Where:

Ta- the reaction time following the administration of the test drugs

Tb- the baseline reaction time before drug administration

A table was drawn showing the latency in flicking of the tail (sec) in mice in the test groups and the standards with time. Percentage inhibition in the tail-flick was calculated and tabulated.

The highest % inhibition of tail-flick, considered as a maximum activity, was exhibited by morphine sulphate standard. The dose that produced 50% of morphine sulphate maximum dose, also referred to as the ED_{50} in mg/kg (or potency of the extracts) was determined using probit regression analysis for the extracts of the three *Ruellia* species in the present study. Graphs showing % inhibition of reaction time *vs* $\log_{10}$ concentrations of aqueous and methanolic extracts were plotted.

3.7.5 Analgesic Activity-Formalin Test

This assay determined the antinociceptive effects of *Ruellia* species in a progressive pain model. Formalin test is a tonic-pain test in which 0.05 mL of 5% v/v formalin in normal saline was injected subcutaneously into the dorsal surface of the right hind paw in mice, originally by Dubuisson and Dennis (1977) and later by Farouk *et al.* (2008) and Kariuki *et al.* (2012). In this test, the nocifensive behaviour was licking and biting of the formalin-injected paw and flinches, which was measured for 60 minutes in blocks of five minutes. The mean number of flinches, bites, and licks in the first 15 minutes was taken as the pain response in the 1st phase of the formalin test, while the late phase (40 minutes and above) after injection of the formalin was the inflammatory response. The antinociceptive activity of the plant extracts in doses of 250-1500 mg/kg was compared with that of acetaminophen 200 mg/kg and morphine sulphate (10

mg/kg, s.c). Doses of standards and extracts were administered orally 30 minutes and 1hour, respectively before the formalin test.

Mice were randomly assigned into 7 groups of 5 animals as follows: Group 1-positive control (acetaminophen 200 mg/kg); Group 2- positive control (morphine sulphate 10 mg/kg); Group 3- untreated control; Group 4- plant extract (250 mg/kg); Group 5- plant extract (500 mg/kg); Group 6- plant extract (1000 mg/kg); Group 7- plant extract (1500 mg/kg). The percentage inhibition (of flinches, bites, and licks) was calculated using the formula:

$$\text{Percentage inhibition of pain response} = \frac{C - T}{C} * 100$$

Where:

C is the untreated control group value for each phase

T is the extract-treated group value for each phase

The concentration (mg/kg) which was effective in 50% population (ED_{50} mg/kg) was used as a measure of the potency of the *Ruellia* extracts. The (ED_{50} mg/kg) was determined using probit regression analysis during phase 1 and phase 2 in the Formalin-test for aqueous and organic extracts of the three *Ruellia* species. Graphs showing % inhibition of nocifensive activity *vs* $\log_{10}$ concentrations of aqueous and methanolic extracts were plotted.

3.7.6. Evaluation of the Anti-Inflammatory Activity

Evaluation of anti-inflammatory activity of *Ruellia* species was determined by their inhibition in the rat hind paw swelling after injection of carrageenan originally by Winter *at al.* (1962); later by Buadonpri *et al.* (2009); Sini *et al.* (2010) and Balica *et al.*, 2013). A mark using the water-proof pen was made at the tibiotarsal articulation of the rat left hind paw. A quantity of 0.1mL of λ-carrageenan (1% w/v in normal saline) was injected (by use of a disposable needle G26) into the subplantar region of the left rat hind paw. The rat was then held vertically above the digital water plethysmometer (LE 7500, Panlab Harvard Apparatus, Spain) chamber and paw dipped into the chamber up to the mark that was made at the joint. Swelling induced

by carrageenan irritant was determined by volume using a digital water plethysmometer. Measurements were taken every hour for 6 hours (Buadonpri *et al.*, (2009).

Rats were randomly assigned into 7 groups of 6 animals as follows: Group 1-positive control (diclofenac sodium 20 mg/kg); Group 2-positive control (ibuprofen 200 mg/kg); Group 3-untreated control; Group 4- plant extract (250 mg/kg); Group 5- plant extract 500 mg/kg); Group 6- Plant extract (1000 mg/kg); Group 7- plant extract (1500 mg/kg). The extracts were administered by the oral route using gastric gavage 1 hour before carrageenan injection, whereas reference standards were administered 30 minutes before carrageenan injection.

Percentage inhibition of swelling was calculated to determine the anti-inflammatory activity. The difference in the initial 0 hour. and volume at +1hour indicates paw oedema at 1hr. following carrageenan administration. Similarly, the difference from 2 to 6 hours was recorded. The data was recorded in a table showing the doses of the extracts and standards and changes in paw volume caused by the extracts and standards.

$$\text{Percentage inhibition of paw oedema} = \left[1 - \frac{Vt}{Vc}\right] * 100$$

Where:
Vc is the average increase in paw volume of the negative control group of rats at a given time.
Vt is the mean increase in paw volume of the drug-treated rats at the same time.

The concentration (mg/kg) which was effective in 50% population (ED_{50}, mg/kg) was used as a measure of the potency of the extracts. The (ED_{50}, mg/kg) was calculated using probit regression analysis for aqueous and organic extracts of the three *Ruellia* species. Graphs showing % inhibition of swelling *vs* $\log_{10}$ concentrations of aqueous and methanolic extracts were plotted.

3.7.7 *In Vivo* Toxicity Studies

Both acute and sub-chronic toxicity studies were carried out to determine the safety of the aqueous extract of *R. prostrata*, since this species showed highest analgesic, antinociceptive and anti-inflammatory studies. The procedures are doucumented in the subsequent sections.

3.7.7.1 Acute Toxicity Studies

The assessment of the acute toxicity of the aqueous extract of *R. prostrata* was done in the female, nulliparous rats of weight 150-200 gm as per established guidelines (OECD, O, 2001). The female rats were reported to be more sensitive than males (Lipnick *et al.*, 1995). Rats were randomly selected and grouped into 3 rats per holding cage. Food (but not water) was withheld for 1-2 hours before dosing. The rats were weighed before dosing. A starting dose (300 mg/kg body weight) of the plant extracts in PBS pH 7.4 (in a volume of 5.0 mL/kg) was administered as a single dose orally by gavage using a stomach tube. The animals were individually observed after dosing during the first 30 minutes and periodically during the first 24 hours. Particular attention was given during the first 4 hours. A dose of 2000 mg/kg was administered in 3 other animals and observations made like the ones in the 300 mg/kg dose.

Particular attention was paid to, changes in the skin and fur of the animals, eyes and mucous membranes, respiratory, circulatory, and autonomic and central nervous systems, somatoform activity, the pattern of behaviour, tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. Animals were observed for 14 days and all the observations per animal were recorded. Acute toxicity studies were carried out to establish a short-term assessment and evaluation of aqueous extract of *R. prostrata.* The acute toxic class method carried out in this study was a stepwise procedure with the use of 3 animals of single-sex female Wistar albino rat per step. Depending on the mortality and/or the moribund status of the animals, an average 2-4 steps were necessary to allow judgement on the acute toxicity of the test substance. The procedure used very few animals. In the assessment, if 0-1 animal died at the repeat dose of 2000 mg/kg, the test substance would be classified as Category 5(> 2000-5000 mg/kg) by the Globally Harmonised System. This category could translate

to an LD_{50} cut -off point of > 2000 mg/kg. The LD_{50} value in the acute toxicity study determined the range of the doses to be used in more prolonged subsequent *in vivo* studies. The method also provided information needed for the dose selection in prolonged toxicity studies.

3.7.7.2 Sub-Chronic Toxicity studies

The assessment of the sub-chronic toxicity of the aqueous extract of *Ruellia prostrata* (1000 mg/kg) was done in ten albino Wistar rats of either sex of weight 150-200 gm per established guidelines (OECD/OCDE, 2008). The test was a 28-day administration of the aqueous extract of *R. prostrata* at1000 mg/kg. Body weight was determined daily, and any abnormal signs such as changes in the skin and fur of the animals, eyes and mucous membranes, respiratory, circulatory, and autonomic and central nervous systems, somatoform activity, the pattern of behaviour, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma were assessed every morning. Comparisons were made between the untreated control and test group. At the end of the study, blood was drawn by cardiac puncture after carbon dioxide euthanasia. About 5 mL blood was drawn and subjected to both haematological assay and biochemistry using automated analyzers as per the manufacturer's manuals. Gross necropsy was done after excising and weighing the principal vital organs, such as the liver, kidney, lung, heart, intestine, sex organs and spleen. Signs such paleness, tumour, blood congestion in the blood vessels were assessed. The relative organ weight was calculated as a % of the weight of the animal (Wang *et al.*, 2011; Yuet *et al.*, 2013).

The dose of 1000 mg/kg was selected for the sub-chronic toxicity studies because there was no significant difference between the dose of 1000 mg/kg and 1500 mg/kg in the *in vivo* analgesic, antinociceptive and anti-inflammatory activities. In addition, only singe doses were administered in the *in vivo* studies, yet sub-chronic toxicity studies were carried out for 28 days. The lower dose was therefore, adopted to avoid use of the highest dose for 28 days. This dose of 1000 mg/kg was also adopted for future prolonged studies such as *in vivo* anti-arthritis studies.

Biochemical parameters were determined using biochemistry automated analyzer (1 Chroma 2-Korea) as per the manufacturer's manual. The following parameters were assessed; total protein, albumin; total bilirubin, direct bilirubin; alkaline phosphatase, glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and gamma-glutamyl transferase (GGT). Blood chemistry parameters were determined using a Chemistry Analyzer (Dirui-China). The following parameters were assessed, urea and electrolytes, random blood sugar and random blood cholesterol. The purpose of the assessment of blood biochemistry was to determine any significant difference in these parameters between the *R. prostrata*-treated group and the untreated control. Two organs, the liver, and the kidney, are important in disposing off drugs after drug action. The liver deals with drug metabolism, whereas the kidney is the main organ of drug excretion. Therefore, any test substance that interfered with the function of the two organs could led to to drug accumulation in the body leading to toxicity or could interfere with the excretion of the drug leading to drug accumulation and toxicity. These biochemical parameters were indicative of effect of aqueous extract of *R. prostrata* on the liver enzymes, a reflection of hepatotoxicity or impaired creatinine excretion, an indication of renal toxicity.

Haematological parameters were assessed using an automated analyzer (DYMIND-China) as per the manufacturer's manual. The following parameters were assessed; white blood cells (% lymphocytes, % granulocytes, mid-sized %, lymphocyte number, granulocytes number, mid number, mid-sized granulocyte number, red blood cells, haemoglobin, haematocrit, red blood cells indices; mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets, mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT). The purpose of the determination of haematological parameters was to assess the effect of the aqueous extract of *R. prostrata* on the bone marrow, the main organs that produces the blood cells. Suppression of the bone marrow leading to reduced white blood cells, lymphocytes, red blood cells and platelets could lead to propensity of infections, derangement of the immunity, and bleeding conditions. The parameters in the *R. prostrata*-treated and untreated control were compared to determine any significant differences between these parameters in the two groups.

3.7.8 Evaluation of *In Vivo* Anti-Arthritic Activity

Anti-arthritic studies were carried as per Carlson *et al.* (1985); Benslay and Bendele (1991); Bendele *et al.* (1999); Bendele (2001); Snekhalatha *et al,* (2013); Zhang *et al,* (2017); Mahdi *et al.* (2018) and Cui *et al*. (2019). Freund's Complete Adjuvant (CFA) in concentration of 20 mg/mL was vortexed to resuspend the Mycobacterium. The antigens were mixed with an equal volume of normal saline in a test tube and shaken vigorously for an extended period to form a thick emulsion (10 mg/mL). Wistar rats of either sex (165-200 gm) were randomly assigned into 4 groups of 6 animals as follows:

Group 1- Control rats, administered with PBS pH 7.4 orally
Group 2- CFA-induced arthritic rats, treated with *R.prostrata* (1000 mg/kg per day) orally
Group 3- Arthritic rats, induced with CFA treated with methotrexate (2mg/kg weekly) orally
Group 4- Arthritis-induced rats, without treatment

The weights of the rats, hind paw volume in mL (digital water plethysmometer) and ankle thickness in cm (Vernier callipers) were taken before the start of the experiment. A quantity of 0.1 mL (1.0 mg) of Mycobacterium suspension was injected at the base of the tail through the tail vein. Treatments were initiated on day 8 (therapeutic model) and taken daily till Day 28 since the immunological reaction developed in approximately 12 days. Methotrexate in a dose of 2 mg/kg was used as a standard and administered weekly till Day 28. All doses were administered by gastric gavage. In the later stages of the disease (Day 12+), special care was taken to ensure that untreated arthritis-induced rats had access to water and food since the severity of paw swelling could compromise their movement.

3.7.8.1 Assessment of Arthritis Parameters

For assessment of disease progression, both ankle joint circumference and paw volume were taken before the onset of arthritis and twice weekly until the study was terminated on day 28 post-injection of the Adjuvant. The parameters used to describe the course

of CFA-induced arthritis were volume of the paws (water displacement using a digital water plethysmometer) in mL, the weight of the animals, circumference of the ankle joints (determined using Vernier callipers) in cm, grip strength (secs) assessed on a rotarod and arthritis score on a scale of 0-4 (per paw) according to Vijayalaxmi *et al.* (2015); Mahdi *et al.* (2018) and Cui *et al*. (2019) as follows:

0-No reaction (normal paw).
1-Slight swelling and erythema of the limb
2- Mild swelling and erythema of the limb
3- Gross swelling and erythema of the limb
4-Gross deformity and inability of the limb

A score of the 4 limbs was counted and a score of >1 exhibited arthritis, while a maximum score of arthritis was 16

Loss of function in the arthritic rats was assessed by determining the grip strength of animals (Carter *et al*., 2001; Montilla-García *et al.,* 2017; Altarifi *et al.,* 2019) on a rotarod. Each animal was placed on a rotating rod at the speed of 10 revolutions per minute. The rod was raised to 30 cm from the floor. This distance normally discourages the rats from jumping off the rod (Walz *et al* 1971b). The rats were acclimatized to the rotarod for 7 days before the test. Each rat was left on the rotarod and time (secs) recorded when it fell off the rod starting from Day 1 of the arthritis experiment, then twice weekly for 28 days. At the end of the study, the animals were euthanized, blood drawn for haematological parameters analysis, then later humanely killed, and incinerated.

The internal organs such as the liver, kidney, lung, intestine, heart and spleen were weighed and observed to determine any changes such as paleness, tumour, blood congestion in blood vessels between the untreated control and the extract-treated animals.

3.7.8.2 Assessment of Haematological Parameters

Haematological parameters were assessed using an automated analyzer (DYMIND-China) as per the manufacturer's manual as per Mahdi *et al.* (2018) and Cui *et al*. (2019). The following parameters were assessed; white blood cells (% lymphocytes, % granulocytes, mid-sized %, lymphocyte number, granulocytes number, mid number, mid-sized granulocyte number, red blood cells, haemoglobin, haematocrit, red blood cells indices; mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets, mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT).

3.9 Data Analysis

The data that was collected in this study was grouped according to the objectives of the study. The findings for the first objective were qualitative data which included chemical, chromatographic, and spectroscopic profiles of the organic and aqueous extracts of selected Kenyan *Ruellia* species. The data that was collected for antioxidant, analgesic, anti-inflammatory, anti-arthritic, acute and sub-chronic activities of extracts of selected Kenyan *Ruellia* species was quantitative data. For antioxidant assay, DPPH radicals scavenging activity of the extracts was determined by measuring the absorbance at λ517 nm. The analgesic activity of the plant extracts was determined by measuring latency in tail flick in mice at intervals of 30 minutes for 3 hours. Analgesic activity was also determined by measuring the amelioration of formalin-induced pain by counting the number of bites, licks and flinching of the body in mice at intervals of 5 minutes for 60 minutes. The parameter measured for determination of the anti-inflammatory activity of the extracts included swelling of the hind paw in rats at intervals of one hour for six hours.

The acute toxicity and sub-chronic toxicity studies of the extracts were determined by observing mortality and significant signs of toxicity, such as lethargy, diarrhoea, convulsions, coma. At the end of the sub-chronic toxicity study, blood was drawn for blood chemistry and haematology. Gross necropsy of internal organs such as the liver, heart, kidney, spleen, intestines, and sex organs was carried out. Relative weight of the

organs was determined. Daily weight measurements were taken. The anti-arthritic activity of the most potent extract was determined my measuring the following parameters; paw volume, joint circumstance, grip strength, arthritis score, body weight and blood haematology.

The antioxidant experiments were carried out in triplicates whereas analgesic, anti-inflammatory, and anti-arthritic experiments were carried out in five-six animals. Twelve animals were used in sub-chronic toxicity studies. The treatment groups included untreated controls, positive controls, and extract-treated groups. Descriptive statistics for all the quantitative data was generated and expressed as mean ± standard error of the mean (SEM). Statistical comparison of two groups was done using the t-test , whereas more than two groups were compared using ANOVA. Tukey's post-hoc analysis for separation and pair-wise comparison of means followed ANOVA. All the values with p-values ≤ 0.05 were considered statistically significant. The IC_{50} and ED_{50} values for various bioassays were determined by nonli-inear regression analysis. All the analyses were done using MiniTab v18.0 statistical software.

3.10 Ethical Considerations

Ethical approval was granted by the Scientific and Ethical Research Unit (SERU)-KEMRI (Ref: **SSC 2946**) as shown in **Appendix 8.** All animal experiments were performed per internationally accepted principles for the care and use of laboratory animals laid down by the Collaborative Institutional Training Initiative (CITI). The animals were humanely treated during and after the laboratory experiments, as per Euthanasia Guidelines (Underwood *et al.,* 2013).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Extraction Yield with Various Solvents

The chemical profiling involved determination of the presence and quantities of selected secondary metabolites. Sequential extraction using petroleum ether, ethyl acetate, chloroform, methanol, and water gave crude extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* gave different yields as presented in **Table 4.1**.

Table 4.1: % Yields Values of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* Extracts of Different Solvents

Solvent	**Plant solvent extractive value [% Mean (gm/gm) ± SD]**		
	R. bignoniiflora	*R. prostrata*	*R. lineari-bracteolata*
Water	37.60 ±1.94	27.4± 0.69	20.7± 0.69
Methanol	4.38± 0.13	4.4± 0.55	2.3± 0.19
Chloroform	1.43 ± 0.02	1.3± 0.03	0.5± 0.05
Ethyl acetate	1.10 ± 0.17	0.8± 0.01	0.4± 0.04
Petroleum ether	0.52 ± 0.05	0.35± 0.03	0.08± 0.03

The aqueous extraction resulted in the highest yield followed by methanolic extraction, while petroleum ether extraction gave the least yields in all the plants extracted. The aqueous extraction of *R. bignoniiflora* had the highest percentage yield (37.60% ±1.94), compared to *R. prostrata* (27.40% ±0.69) and *R. lineari-bracteolata* (20.7% ±0.69). The percentage yields obtained from the petroleum ether extracts of *R. bignoniiflora* (0.52% ±0.05) followed by *R. prostrata* (0.35% ±0.03) and *R. lineari-bracteolata* (0.08% ±0.03). These findings implied that most of the secondary metabolites present in these species were highly polar and could, therefore, be sufficiently extracted with polar solvents. Based on these findings, the *in vivo* studies were solely carried out using aqueous and methanolic extracts, separately. The

methanolic and aqueous crude extracts obtained from *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata*. were subjected to qualitative phytochemical analysis to determine the nature of the phytochemicals present in them. The findings are as shown in **Table 4.2.**

Table 4.2: Phytochemicals present in *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata*

Phytochemical	Plant species	Type of solvents	
		Methanol	**Distilled water**
Saponins	*R. prostrata*	+	+
	R. lineari-bracteolata	+	+
	R. bignoniiflora	+	+
Tannins	*R. prostrata*	+	+
	R. lineari-bracteolata	+	+
	R. bignoniiflora	+	+
Alkaloids	*R. prostrata*	-	-
	R. lineari-bracteolata	-	-
	R. bignoniiflora	-	-
Glycosides	*R. prostrata*	+	+
	R. lineari-bracteolata	+	+
	R. bignoniiflora	+	+
Flavonoids	*R. prostrata*	+	+
	R. lineari-bracteolata	+	+
	R. bignoniiflora	+	+
Terpenoids	*R. prostrata*	+	+
	R. lineari-bracteolata	+	+
	R. bignoniiflora	+	+

Key: '-' Absent, '+' Present

Phytochemical screening detected the presence of saponins, terpenoids, phenolics, glycosides and flavonoids. However, no alkaloids were detected in all three species using two different qualitatative tests (Mayer's and dragendorff's reaegents). The absence of alkaloids in these Kenyan species concurred with the findings obtained from the Indian *R. prostrata* (Wangia, 1985). Differences in bioactive compounds in

plants are normally determined by several factors including soil composition, geographical locations, seasonal variation, and intra-species variation (Schaffer *et al.*, 2005).

Of the *Ruellia* species reported, the species that were widely studied were *R. tuberosa*, *R. patula* and *R. brittoniana*. In their report, Samy *et al.* (2015) revealed the presence of sterols (β-sitosterol and β-stigmasterol), flavonoids (luteolin, luteoloin-7-O-glucoside, apigenin, and apigenin-7-O-glucoside) as shown in **Table 2.3**. Their study, just like the current one, did not reveal presence of alkaloids. A report by Samy *et al.* (2015) on the chemical constituents and biological activities of the genus *Ruellia*, revealed the presence of sterols, triterpenes, coumarins, flavonoids, lignans, phenolic compounds, megastigmanes, benzoxazinoids. The alkaloids were not detected in the *Ruellia* species studied, a finding that concurred with that in the present study.

A study by Afzal *et al.* (2015) on the importance of the genus *Ruellia* in ethnopharmacology, revealed various phytochemical constituents present in several *Ruellia* species. Similar to the findings in the present study, the presence of glycosides (*R. brittoniana),* flavonoids (*R. tuberosa*), and triterpenoids (*R. brittoniana*) were revealed. But unlike the findings in the present study, alkaloids were detected in *R. tuberosa* and *R. rosea*. A study by Safitri *et al.* (2019) revealed the presence of flavonoids, terpenoids, steroid, phenolics, tannins, saponins and ascorbic acid in hydroethanolic extract of *R. tuberosa*. No alkaloids were reported in the study concurring with the present study.

Among phytochemical compounds present in plant materials; saponins, alkaloids, tannins and flavonoids are important secondary metabolites that are attributed to the medicinal value of plants. As described by Thirumurugan *et al*. (2018), plants have unlimited ability to synthesize secondary metabolites of which over 2,140,000 secondary metabolites are known. These compounds are commonly classified according to their vast diversity in structure, function, and biosynthesis. There are five main classes of secondary metabolites such as terpenoids and steroids, fatty acid-derived substances and polyketides, alkaloids, nonribosomal polypeptides, and enzyme cofactors (Thirumurugan *et al.*, 2018). Most of the isolated compounds have

been used as drugs for many years, such as morphine, atropine, cocaine, steroids, ginsenosides, digitalis and luteolin. Thus, as described by Chew *et al.* (2011), plant phytochemical screening serves as the initial step in predicting the types of potentially active compounds. As reported by Aiyegoro and Okoh (2010), saponins, glycosides, alkaloids and tannins are known to be responsible for anti-inflammatory, antitumor, anti-carcinogenic, antibacterial, and antiviral activities.

Selected secondary metabolites, known for antioxidant, anti-arthritic, anti-inflammatory and antinociceptive activities were quantified. The findings of the quantities of the phytochemicals present in *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* are shown in **Table 4.3**.

Table 4.3: Phytochemical content of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* extracts

Plant Samples	Phytochemical content (mg/mL)		
	Saponins*	**Flavonoids**	**Total phenolics**
R. bignoniiflora	1.25 ± 0.11^{a}	15.10 ± 0.81^{a}	1.20 ± 0.59^{a}
R. prostrata	2.05 ± 0.03^{b}	15.30 ± 0.82^{a}	1.30 ± 0.37^{a}
R. lineari-bracteolata	1.40 ± 0.15^{a}	17.60 ± 0.41^{b}	1.60 ± 0.10^{a}

Key: *Saponins content was in quantified as a % w/w. Superscript Alphabet: Denotes level of significance. Values are expressed as Mean±SEM. Values followed by the same superscript down the column are not significantly different ($p>0.05$).

R. prostrata had the highest content of saponins (2.05 % ±0.03 w/w), followed by *R. lineari-bracteolata* (1.40 ±0.15 mg/mL), while *R. bignoniiflora* had the least saponin content (1.25 ± 0.11 % w/w). However, there was a statistically higher difference ($p\leq0.05$) between the saponins content of *R. prostrata* and that of *R. bignoniiflora* and *R. lineari-bracteolata*. The flavonoid content of *R. bignoniiflora* (15.10 ± 0.81 mg/mL) and *R. prostrata* (15.30 ± 0.82 mg/L) were not significantly different ($p>0.05$). *R. lineari-bracteolata* had the highest flavonoid (17.6 ±0.41 mg/mL) and total phenolic (1.60 ± 0.10 mg/mL) contents. However, the differences in total phenolic contents of all the three plant species were not statistically significant ($p>0.05$).

Even though phytochemical screening had shown the presence of tannins in the *Ruellia* species, their amounts were significantly lower ($p \leq 0.05$) compared to saponins and flavonoids. These little amounts are attributed to the nature of phenolics in form of dried ground powder, which makes them difficult to extract due to their increased fixing into the vacuoles of the plant (Harborne, 1998; Hutzler *et al.,* 1998).

4.2 Chromatographic Profiling of Bioactive Compounds

Thin-layer chromatography (TLC) was used to separate bioactive compounds present in *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* using various solvent systems. The best solvent systems for separation of methanolic extracts were methanol: dichloromethane (9.5:0.5 and 9.0:1.0). The TLC chromatogram for the methanol extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* under visible light is shown in **Plate 4.1,** whreas the same chromatogram viewed under UV light (λ 365nm) is shown in **Plate 4.2**.

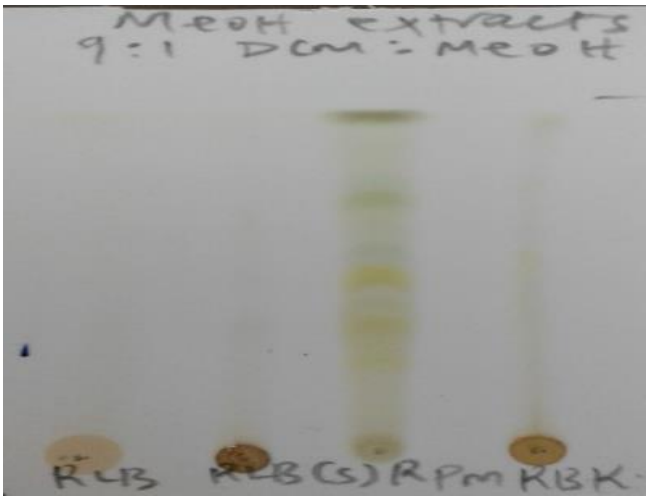


Plate 4.1:TLC chromatogram under visible light

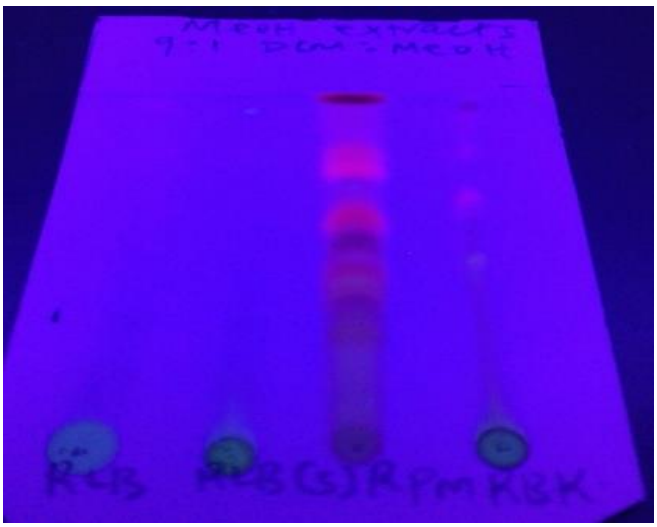


Plate 4.2: TLC chromatogram under UV light

In the visible light, no spots were detected in *R. bignoniiflora* and *R. linearibracteolata* methanolic extracts, whereas *R. prostrata* extract showed seven spots. Flavonoids contribute to many colours found in nature, especially the yellow and orange colours of petals. Even the colourless flavonoids absorb light in the UV spectrum, due to their extreme chromophores **(**Andersen and Markham, 2005). Compounds which possess extensive conjugated bonds, such as phenyl rings, will absorb in the UV-Vis region due to the pi- bonds. These bonds reduce the energy required for the electrons to move from bonding to antibonding π-orbitals. Reduction in photon energy increases the λ max moving from UV shorter λ max (100-400nm)

than that of the visible light of λ max (400-800nm). These compounds could be detected as coloured compounds in the Visible region**.**

The principle of the UV-lamp was to emit light that could either hit the silica gel on the plate or the sample on its surface. The gel emitted a general blue color while the sample absorbed the light and blocked the light from reaching the silica gel. The amount of the sample in the spot determined how much of the silica gel that the UV light was able to reach. If the samples were relatively high, the chemical spots would be dark under UV light. The UV light provided enery that excited the electrons to higher energy levels, which transmitted same energy when they returned to their ground state releasing the absorbed energy as photons. This excitation and emitting of energy gave rise to different colours depending on the wavelength of the energy. The spots that did not show colour under visible light absorbed all the light energy and were not able to transmit the energy. But since UV light has more frequency, hence more energy than visible light, more energy was received which excited the electrons to most likely π-antibonding state and transmitted the light when they came back to the ground state. TLC chromatogram after vanillin and iodine spray is shown in **Plate 4.3** and **Plate 4.4,** respectively.

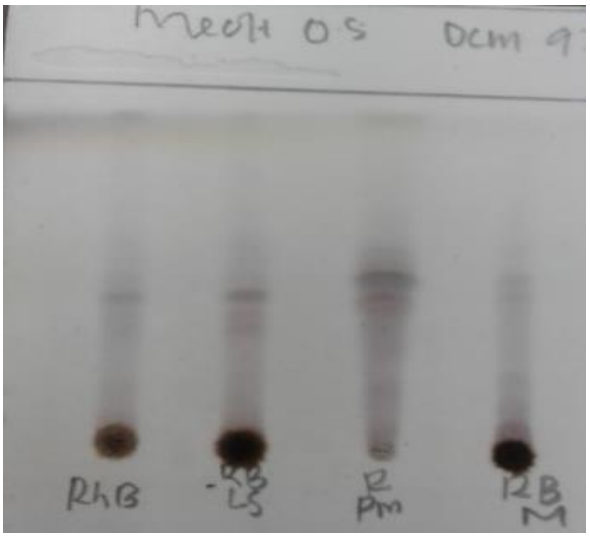


Plate 4.3:TLC chromatogram after vanillin spray

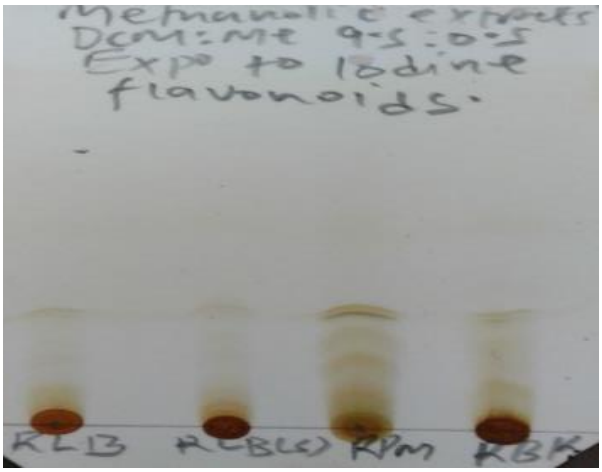


Plate 4.4: TLC chromatogram after iodine spray

Vanillin spay revealed compounds that were invisible in visible light. Presence of terpenoids was confirmed by purple color in all extracts. Vanillin/sulphuric acid (1% v/v) solution is used for the detection of terpenoids and steroids. Maximum colour formation wss obtained when the chromatograms were viewed after heating at 120°C in an oven. Iodine vapor is relatively an unspecific universal reagent for many organic compounds. Spray reagents are mixtures of chemicals, which when applied onto chromatographic plates, react with colourless compounds producing coloured derivatives. After exposure of the TLC chromatogram to iodine vapour, yellow-brown spots indicated presence of flavonoids in all methanol extracts. It was concluded that

although *R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts displayed similar TLC chromatograms, *R. prostrata* displayed similar compounds with several extra compounds that were not in the two species. The R_f values obtained from TLC chromatogram of the methanolic extract of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* are shown in **Table 4.4.**

Table 4.4: R_f Values of methanolic extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata*

Plant species	Solvent system	R_f Values		
		Visible light	UV (λ 365nm)	Vanillin spray
R. prostrata	MeOH: DCM (9.5: 0.5)	0.93, 0.76, 0.69 0.51, 0.39, 0.34 0.24	0.93, 0.79, 0.74 0.58, 0.48, 0.41 0.39, 0.23.	0.62, 0.55, 0.51 0.34, 0.23, 0.11
R. lineari-bracteolata	MeOH: DCM (9.5: 0.5)	No spots visible	0.93, 0.79, 0.74	0.62, 0.55, 0.51
R. bifnonniflora	MeOH: DCM (9.5: 0.5)	No spots visible	0.93, 0.79, 0.74	0.62, 0.55, 0.51

Key: MeOH-methanol; DCM-dichloromethane; R_f-Retention factor

Usually compounds on a TLC chromatogram are not coloured, therefore, a UV lamp is used to visualize the plates. The TLC plates contain a fluorescent indicator in UV light at λ 365 nm, which is an optical brightener, that is stimulated to intense blue fluorescence emission at λmax 365 nm. Observation under UV λ365nm revealed that *R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts compounds had similar R_f values (0.93, 0.79 and 0.74). In addition to the compounds observed in *R. bignoniiflora* and *R. lineari-bracteolata* under UV light λ365nm, *R. prostrata*, methanolic extract displayed extra compounds of different R_f values (0.58, 0.48, 0.41, 0.39 and 0.23). In visible light, *R. prostrata* revealed compounds (R_f values of 0.93, 0.76, 0.69, 0.51, 0.39, 0.34 and 0.24) that were not detected in *R.bignoniiflora* and *R. lineari-bracteolata. R_f* values are characteristic for any given compound, provided that the same stationary and mobile phases are used. *R_f* values can provide corroborative evidence as to the identity of a compound. A solvent system of methanol:

dichloromethane (9.5:0.5) is polar and would push more polar compounds towards the solvent front. Hence, the three *Ruellia* species had several common polar compounds. In addition, *R. prostrata* methanolic extract had some less polar compounds, absent in the *R. bignoniiflora* and *R. lineari-bracteolata* extracts .

After vanillin spray, *R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts showed compounds with similar R_f values (0.62, 0.55 and 0.51). In addition to the three compounds displayed by *R. bignoniiflora* and *R. lineari-bracteolata* after vanillin spray, extra compounds were detected in *R. prostrata* methanolic extract and their R_f values were (0.34, 0.23 and 0.11). Iodine vapour revealed the presence of common compounds in the three *Ruellia* species of R_f values of 0.62, 0.55, 0.51. *R. prostrata* extract revealed extra spots of lower R_f values of 0.34, 0.23, 0.11. Both vanillin and iodine sprays were able to reveal extra compounds in the three *Ruellia* species which were neither detected in visible light nor UV light. These compounds could be different in their chemical composition.

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation. Generally, aromatic compounds or heterocyclic compounds have electron donating or electron withdrawing functional groups and are most fluorescent. Compound fluorescence is induced by higher energy photon (such as UV light). Such inorganic or organic compounds absorb the UV light, and their electrons are “excited” to higher energy state. Since this state is not favorable, the compound tends to lose that energy but instead of going directly to original energy level it first transitions to another energy level that lays between the antibonding and bonding orbitals, releasing that energy as higher wavelength photon which is usually within visible spectrum. The rest of the energy that is needed to get rid of of the energy and to return to starting energy level is dissipated through thermal processes (de Lange *et al.,* 2019).

The results revealed the compounds in *R. prostrata* were coloured in visible light thus the electrons in the phytocompounds were in a more excited state than those in *R. bignoniiflora* and *R. lineari-bracteolata*. This implies that there were more conjugated bonds which were in higher energy levels, leading to visualization in visible light.

Phytocompounds in *R. bignoniiflora* and *R. lineari-bracteolata* needed UV light to excite them to go to higher enery levels, rendering visualization of the spots possible under visible light. The appearance of extra compounds in *R. prostrata* revealed that there were other compounds in this species which needed UV light for their visualization.

Since *R. prostrata* showed highest *in vivo* analgesic and anti-inflammatory activities, the compounds in this species were separated further on column chromatographic techniques on normal silica gel (230:70Å). The separation was achieved using a combination of solvents of increasing polarity that included n-hexane: ethyl and ethyl acetate: methanol. Various fractions collected during column chromatography were pooled together based on their TLC profiles to yield ten combined fractions whose weights are shown in **Table 4.5**.

Table 4.5: Weight of combined fractions from the organic extract of *R. prostrata*

Pooled Column Fractions	Weight (g)
1	1.58
2	3.32
3	2.15
4	0.32
5	3.68
6	2.69
7	1.61
8	4.01
9	19.23
10	17.37

Fractions 1-8 were obtained using n-hexane: ethyl acetate solvent system in 5% gradient from ratios of 100:0 to 0:100, whereas Fractions 9 and 10 were obtained used ethyl acetate: methanol solvent system from ratios of 90:10 to 40:60 and 30:70 to 0:100, respectively, on the same column. The fractions obtained from ethyl acetate: methanol solvent system gave higher yields than those obtained from n-hexane: ethy acetate solvent system. This finding indicated that most of the compounds in the organic extract of *R. prostrata* were polar. The fractions which were more than 1 gm, were sufficient for *in vitro* anti-arthritic activity studies (**Table 4.15).** Pooled Fraction-

9 showed highest *in vitro* anti-arthritic activity in a concentration as low as 3.33mg/mL, with IC_{50} of 0.06 mg/mL. Therefore, this fraction was subjected to GC-MS profiling.

4.3 Spectroscopic Profiling of *R. prostrata*

The results for the spectroscopic profiling of selected Kenyan *Ruellia* species are discussed in subsequent sections.

4.3.1 Fourier Transform Infra-Red Profiles

FTIR spectroscopic profiling was used to identify the functional groups of compounds present in the aqueous, methanolic and 20% v/v ethanolic crude saponin from *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata.* FTIR frequencies of extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* and GC-MS compounds from organic and aqueous extracts of *R. prostrata* are shown in subsequent sections and (**Appendix 4 and 5**). The findings for the FTIR frequency of extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* methanolic, aqueous and saponin are shown in **Tables 4.6.**

Table 4.6: FTIR frequency (cm^{-1}) of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* methanolic, aqueous and crude saponin

		Frequency (cm^{-1})							
Type of solvent`	**Plant species**	**3600-3200 (O-H str)**	**3000-2850 (C-H str)**	**1820-1670 (C=O str)***	**1655-1620 (C=C str)**	**1600-1400 (C=C str) c**	**1389-1377 (C-O str aromatic)**	**1300-1042 (C-O-C str)**	**1000-675 (=C-H bend)**
Methanolic extract	**RPM**	3338.6	2854.5		1627.8			1269.1 1053.1	
	RBK	3382.9	2977.9		1641.3	1550.7 1400.2		1253.6 1118.6	1001.0
	RLB	3631.1 3562.5 3449.5 3258.0	2945.4 2870.2		1628.7	1407.1		1128.7	
Aqueous extract	**RPM**	3400.3	2945.1		1651.0	1564.2 1415.7		1274.9 1082.0 1049.2	893.0
	RBK	3386.8	2939.3		1647.1	1571.9 1413.7		1274.9 1056.9	933.5
	RLB	3425.3, 3392.7	2923.9, 2862.2		1651.0	1560.3 1517.9 1421.4		1255.6 1033.8	918.1, 777.3
20 % v/v Ethanol extract	**Crude Saponin**								
	RPM	3396.4	2977.9		1639.4	1548.7		1255.6 1114.8	989.4, 817.8
	RBK	3406.1 3483.2			1639.3			1269.1 1057.6	
	RLB	3482.4	2978.0		1641.7			1100.0	

Key: RPM-*Ruellia prostrata*, RBK-*Ruellia bignoniiflora*, RLB-*Ruellia lineari-bracteolata* *-C=O functional group was absent.

The aqueous and methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* displayed similar functional groups of -OHstr (3600-3200 cm^{-1}**)**; C-H (3000-2850 cm^{-1}); C=C (1655-1620 cm^{-1}**).** Oligosaccharide linkage C-O-C (1300-1042 cm^{-1}) was evident. Peaks between 1000-675 cm^{-1} indicated the the presence of C-H bending of alkenes were evident in the three *Ruellia* species in the present study (Harika and Radhika, 2019).

The FTIR frequenc findings (cm^{-1}) of two fractions from the organic extract of *R. prostrata* are shown in **Table 4.7**.

Table 4.7: FTIR frequency (cm^{-1}) of the fractions from organic extracts from *R. prostrata*

		Frequency (cm^{-1})							
Type of solvent`	**Plant species**	**3600-3200 (O-H str)**	**3000-2850 (C-H str)**	**1820-1670 (C=O str)**	**1655-1620 (C=C str)**	**1600-1400 (C=C str) c**	**1389-1377 (C-O str aromatic)**	**1300-1042 (C-O-C str)**	**1000-675 (=C-H str)**
n-Hexane: Ethyl acetate 90:10	***R. prostrata***		2916.6 2916.4	1733.6	1461.7	1377.7		1177.0 1169.3	740.2
n-Hexane: Ethyl acetae 80:20	***R. prostrata***		2915.8 2848.9	1733.2	1651.5	1377.8		1169.0	983.7 759.2 729.0

The fractions from the organic extract of *R. prostrata* did not show the OH stretch 3600-3200cm^{-1}, whereas the carbonyl group C=O was detected at 1733 cm^{-1}. The two fractions gave similar spectral characteristics as indicated in **Appendix 6-7**. Overall, the three *Ruellia* species in the present study revealed similar functional groups, despite the

difference in their geographical locations. The similarity in their IR functional groups was revealed in their similar pharmacological profiles (**Section 4.4.4-4.4.5**). The phytocompounds which were detected in *R. prostrata*, *R. bignoniiflora* and *R. linearibracteolata* species in the present study have been reported to have diverse biological activities and could be working synergistically to produce the various biological activities observed (Sim *et al.,* 2004).

The IR region between 1450-3500 cm^{-1}, also called the functional group region, detects groups such as C-H and C=O which have the most common vibrations and can be used to get information about specific functional groups in the same molecule. FTIR frequency peaks in the region of 1500-800 cm^{-1}, also called the fingerprint region by Sim *et al.,* (2004), has the most complex vibrations which are used to distinguish compounds. Apart from enantiomers, no two molecules will give exactly the same IR spectrum. The fingerprint region contains a very complicated series of absorptions mainly due to all manner of bending, vibrations within the molecule.

The FTIR spectral major absorption peaks obtained from the hydroethanolic extracts of *R. tuberosa* (Safitri *et al.,* 2019) revealed functional groups indicative of -OH (3450-3100 cm^{-1}), -C-H- alkane (2950-2850 cm^{-1}), -C=C- and -C=O (1650-1640 cm^{-1}), -C-C- aromatic compounds(1460-1450 cm^{-1}), -C-O- from secondary alcohol (1180-1160 cm^{-1}). These findings agree with the FTIR absorption peaks obtained in the present study.

A study by Sim *et al.* (2004) assessed some herbal medicines based on FTIR spectra and assigned the functional groups as sharp absorption peak (1600–1760 cm^{-1} due to C=O stretching vibration in carbonyl compounds which could be characterized by the presence of high content of terpenoids and flavonoids. The presence of a narrow and sharp peak at approximately 2925 and 2853 cm^{-1} was assigned to C-H and C-H (methoxy compounds) stretching vibration respectively. An absorption of a broad band of hydroxyl (3500–3480 cm^{-1}), ester C-O-C (1270–1150 cm^{-1}) and phenyl -C=C- (1600-1420 cm^{-1}) were evident.

The present study revealed functional groups that concurred with those reported by Sim *et al.* (2004).

A study by Harika and Radhika (2019) on the phytochemical analysis of *R. tuberosa* tuber ethanolic extract using UV-VIS, FTIR and GC-MS techniques revealed different characteristic peak values with various functional compounds in the extract. The peaks were identified at 3329.48, 2922.90, 2852.35, 1708.96, 1624.33, 1519.14, 1406.64, 1342.10, 1103.33, 1035.23, 989.08, and 924.71 cm^{-1}. The band at 3329.48cm^{-1} corresponded to O-H groups of tannins, flavonoids (phenolic compounds) and glucose whereas –NH stretching corresponded to proteins. The band at 2922.90 cm^{-1} corresponded to C-H asymmetric stretching of alkanes. The band at 2852.35 cm^{-1} corresponded to C-H stretching of alkanes. The band at 1708.96 cm^{-1} indicated the C=O stretching of the carbonyl group. The peak at 1624.33 cm^{-1} corresponded to the C=C stretching. The bands at 1519.14 cm^{-1}, 1406.64 cm^{-1}, 1342.10 cm^{-1} corresponds to C-H bending (Mahesha *et al.*, 2015). The bands at 1103.33, 1035.23 cm^{-1} indicated O-H bend. The peaks 924, 873, 825, 769, 713, and 684 cm^{-1} indicated the C-H bending of alkenes. According to Mahesha *et al.* (2015), these groups could majorly be contributed by alkaloids, flavonoids, tannins, phenolic compounds, and carbohydrates present in ethanolic tuber extract. Most of the FTIR functional groups detected in *R. tuberosa* were also detected in *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata*. The findings in the present study revealed that species that belong to the family of Acanthaceae revealed similar compounds, with some differences which could be due to differences in geographical locations, climatic changes, soil composition and intra-species differences (Schaffer *et al.*, 2005).

Maruthamuthu and Ramanathan (2016) identified functional groups based on FTIR spectroscopy as; band at 3432.94 cm^{-1} due to O-H stretching of alcohols and phenols; the band at 2411.00 and 2132.85 cm^{-1} revealed the presence of -C≡C- stretch alkynes. The peak at 1648.74 cm^{-1} was attributed to -C=C – stretching of alkenes, the band 1449.96 cm^{-}

[1] was C-H bend alkanes. The FTIR findings in the present study agreed with their studies. Obtaining an IR spectrum for a compound does not figure out the complete structure of even a simple molecule without a reference spectrum for comparison. But in conjunction with other analytical methods, IR spectroscopy is a very valuable tool, since it provides information about the presence or absence of key functional groups (Safitri *et al.,* 2019).

4.3.2 Gas Chromatography-Mass Spectrometry Profiles

GC-MS analysis was carried out on all crude aqueous and organic extracts and pooled combined fractions obtained from column chromatographic profiling of the organic extract of *R. prostrata*. The findings of GC-MS analysis of compounds from aqueous extract of *R. prostrata* are shown in **Tables 4.8.**

Table 4.8: GC-MS compounds from *R. prostrata* aqueous extract

Retention Time (mins)	Mwt.	Formula	Compound Name
15.50	342	$C_{12}H_{22}O_{11}$	alpha-D-Glucopyranose, 4-O-. beta-D-galactopyranosyl-
15.264	296	$C_{19}H_{36}O_2$	6-Octadecenoic acid, methyl ester, (Z)
16.556	312	$C_{19}H_{36}O_3$	Glycidyl palmitate
17.11	337	$C_{22}H_{43}NO$	13-Docosenamide, (Z)-
17.787	338	$C_{21}H_{38}O_3$	Glycidyl oleate
18.13	330	$C_{19}H_{38}O_4$	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
20.621	440	$C_{25}H_{44}O_6$	9-Octadecenoic acid (Z)-, 2-(acetyloxy)-1-[(acetyloxy) methyl] ethyl ester
23.34	652	$C_{38}H_{68}O_8$	l-(+)-Ascorbic acid 2,6-dihexadecanoate

The compound alpha-D-Glucopyranose, 4-O-.beta-D-galactopyranosyl- obtained from aqueous extract of *R. prostrata* is a disaccharide which could have broken off the main

compound most likely a saponin due to high temperature (250°C) used in the GC-MS process. Being a disaccharide, this compound is most probably attached to one point, C3 of the saponin implying a monodesmosidic saponin (Yoshiki *et al.,* 1998).

The derivative of ascorbic acid, l-(+)-Ascorbic acid 2,6-dihexadecanoate, may have contributed to the antioxidant activity of the *R. prostrata* species. Ascorbic acid (Vitamin C) is reported to have antioxidant activity (Yen *et al.,* 2002). The findings of the GC-MS analysis of compounds from the organic extract of *R. prostrata* are shown in **Table 4.9.**

Table 4.9: GC-MS compounds from the organic extract of *R. prostrata*

Retention time (mins)	Mwt.	Formula	Compound Name
14.02	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
14.31	652	$C_{38}H_{68}O_8$	l-(+)-Ascorbic acid 2,6-dihexadecanoate
14.48	354	$C_{24}H_{50}O$	n-Tetracosanol-1
15.27	296	$C_{19}H_{36}O_2$	9-Octadecenoic acid (Z)-, methyl ester
17.12	281	$C_{18}H_{35}NO$	9-Octadecenamide, (Z)-
20.79	410	$C_{30}H_{50}$	Squalene
21.69	506	$C_{36}H_{74}$	Hexatriacontane
30.41	412	$C_{29}H_{48}O$	Stigmasterol
32.44	414	$C_{29}H_{50}O$	β-Sitosterol
36.51	426	$C_{30}H_{50}O$	Lupeol

In the organic extract of *R. prostrata*, the retention time of the compound l-(+)-Ascorbic acid 2, 6-dihexadecanoate eluted at 14.31mins compared to 23.34 mins in the aqueous extract. This was due to differences in the adsorption on the aqueous column in the GC-MS, with the water extract having the compounds adsorb more tightly than compounds in

the organic extract. GC-MS profiling revealed the compound, lupeol, stigmasterol and β-sitosterol in the organic extract of *R. prostrata*. These compounds were not detected in the aqueous extract of *R. prostrata.*

A study by Saleem (2009) described lupeol as a novel anti-inflammatory and anti-cancer dietary triterpene. Stigmasterol and β-sitosterol are well-known plant phytosterols and have been studied for different biologic activities. The compounds, stigmasterol, β -sitosterol, lupeol are terpenes reported to possess anti-inflammatory activities (Githinji *et al.,* 2012; Pierre and Moses, 2015). Sigmasterol and β-sitosterol are well known plant phytosterols and have been studied for different biologic activities. Isolation and characterization of stigmasterol and β-sitosterol from *Odontonema strictum* (Acanthaceae) by Pierre and Moses (2015) reported these compounds to have anti-inflammatory activities. These triterpenes must have contributed to the anti-inflammatory activities of *Ruellia* species in the present study.

Another study on *R. brittoniana* by Elgindi *et al.* (2015) showed similar compounds in GLC analysis, such as hydrocarbons and sterols like stigmasterol and β -sitosterol, among others. GC-MS analysis of ethanol extract from tubers of *R. tuberosa* by Mohan *et al.* (2014) revealed the presence of lupeol, stigmasterol and α-sitosterol, among others. In the present study, the organic extract of *R. prostrata* revealed the presence of similar compounds such as β -sitosterol and lupeol (*R. tuberosa*), stigmasterol and β -sitosterol (*R. brittoniana*), among others. The findings were not strange because these species belong to the same Acanthaceae Family.

A study by Karthika *et al.* (2016) on the phytochemical analysis of *R. patula* using GC-MS revealed the presence of squalene, α-sitosterol. Studies with *R. patula* have identified c-tocopherol (Karthika *et al.,* 2016) and α-tocopherol (Lakshmi *et al.,* 2017). Another study by Pires *et al*. (2020) on the chemical study, antioxidant activity, and genotoxicity and cytotoxicity evaluation of *R. angustiflora* revealed the presence of campesterol, β-

sitosterol, β-stigmasterol which have been reported in several other species of the genus *Ruellia*. According to Samy *et al.* (2015), *R. patula* presents β--sitosterol, stigmasterol and campesterol. Furthermore, *R. tuberosa* and *R. prostata* have β-sitosterol and stigmasterol in their constitutions. These findings are in congruence with those described in this study for *R. angustiflora.*

Squalene was only detected in the organic extract of *R. prostrata* because it is very lipophilic. Squalene is a precursor of various hormones in animals and sterols in plants (Lozano-Grande *et al.,* 2018). It is a molecule having pharmacological, nutritional, and cosmetic potential. The molecule is reported to prevent the suffering of patients with cardiovascular diseases, and antitumor activity against ovarian, breast, lung, and colon cancer Spanova and Daum (2011). A study by Wangia *et al.* (2019) revealed *in vitro* anticancer activity of *R. prostrata* aqueous extract against breast, prostate, and hepatocellular cancer.

Most of the compounds obtained from GC-MS profiling of *R. prostrata* have been reported to have anti-inflammatory, anticancer, antioxidant activities. A study by Zhou *et al.* (2016) reported that complex formulations of herbal extracts as a whole and/or multiple herbs could offer better efficacies than individual active doses. The same study highlighted the significance of synergistic action in herbal treatments of and/or herbs when used in combination for their synergistic actions. The pooled Fraction-9 which showed highest *in vitro* antiarthritic activity (**Table 4.15**) was also subjected to GC-MS profiling and the compounds obtained are shown in **Table 4.10.**

Table 4.10: GC-MS Compounds from Fraction-9 (organic extract of *R. prostrata*)

RT (mins)	Mwt.	Formulae	Name
11.404	168	$C_{12}H_{24}$	1-Dodecene
15.128	196	$C_{14}H_{28}$	1-Tetradecene
17.329	206	$C_{14}H_{22}O$	2,4-Di-tert-butyl phenol
18.451	310	$C_{16}H_{29}F_3O_2$	Tetradecyl trifluoroacetate
21.421	322	$C_{23}H_{46}$	9-Tricosene, (Z)-
29.54	337	$C_{22}H_{43}NO$	13-Docosenamide, (Z)-
29.64	281	$C_{18}H_{35}NO$	9-Octadecenamide, (Z)-

Key: RT-Retention time

Some compounds obtained from Fraction-9 have been reported to have biological activities. The compound 2,4-Di-tert-butyl phenol is reported to possess antifungal and antioxidant activities (Varsha *et al.,* 2015). Another study by Wangia and Itoobi (2018) revealed the presence of antifungal activity of the aqueous and methanolic extracts of *R. prostrata* against *Candida albicans* using miconazole as a standard. The same study detected the antimicrobial activity of same extracts against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* using ceftriaxone, gentamicin, and chloramphenicol standards. Two amide compounds 13-Docosenamide, (Z)- and 9-Octadecenamide, (Z)- were also obtained in Fraction-9. These two amides were reported to be the major identified compounds from *Achillea filipendulina* which exhibited antioxidant activity (Khan *et al.,* 2019). As per the report of Khan *et al.* (2019), 13-Docosenamide, (Z)- possesses antimicrobial and antinociceptive activities, whereas 9-Octadecenamide, (Z)- possesses antimicrobial activities. These amides could be the compounds responsible for the antimicrobial activity detected by Wangia and Itoobi (2018).

The compound 13-Docosenamide, (Z)- was detected in the GC-MS profile of the aqueous extract of *R. prostrata* (**Table 4.8**), whereas 9-Octadecenamide, (Z)- was detected in the GC-MS profile of the organic extract of *R. prostrata* (**Table 4.9)**, respectively. The compound 9-Octadecenamide, (Z)- was reported to possess anti-inflammatory and antibacterial activities, while stigmasterol was reported to have antidiabetic, antineoplastic, antihypertensive, and anti-retroviral activities (Hadi *et al.*, 2016).

Although the presence of saponins, phenolics, flavonoids, terpenoids was detected in chemical profiling studies, these compounds were not detected in GC-MS profiles. This could be due to a high temperature (250°C) that the extracts were subjected to in GC-MS profiling. These compounds could have been heat-labile, therefore broken into simpler fragments, such as alpha-D-Glucopyranose, 4-O-.beta-D-galactopyranosyl-, a disaccharide that was obtained in the aqueous extract of *R. prostrata*

4.4 Pharmacological assays

4.4.1 *In Vitro* Antioxidant Activity

Antioxidant activity of aqueous and methanolic extracts of Kenyan *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* was determined using *in vitro* DPPH scavenging activity method.

In Vitro Evaluation of Antioxidant Activity of Aqueous Extracts

The findings for percentage inhibition of DPPH radical expressed as (IC_{50} in µg/mL) for the aqueous extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* are presented in **Figure 4.1.**

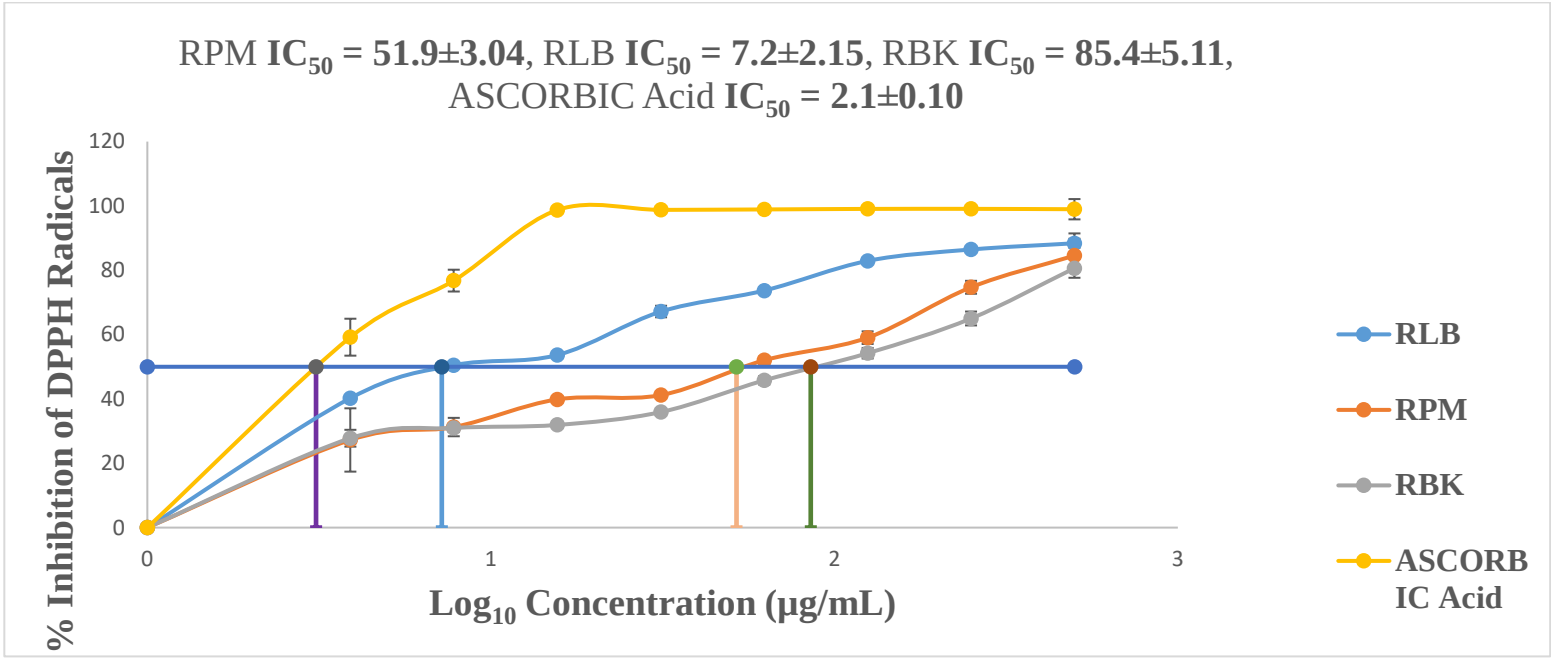


Figure 4.1: Percentage inhibition of DPPH radical expressed as (IC_{50} in µg/mL) for *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* aqueous extracts

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*; **RLB-***R. lineari-bracteolata*

There was a dose-dependent increase in % inhibition of DPPH radical by the aqueous extracts of *R. lineari-bracteolata*, *R. prostrata* and *R. bignoniiflora* and ascorbic acid standard. Ascorbic acid standard attained 98.71% inhibition of DPPH radical at the concentration of 15.6 µg/mL and 98.98% inhibition at 500 µg/mL. The highest inhibition of DPPH radical inhibition was by *R. lineari-bracteolata* (88.39%), followed by *R. prostrata* (84.45%) and *R. bignoniiflora* (80.64%) relative to ascorbic acid (98.97%) at 500 µg/mL. The % inhibition by the *Ruellia* species (at 500 µg/mL) in the present study was similar and about 85% that of ascorbic acid standard.

In Vitro Evaluation of Antioxidant Activity of Methanolic Extracts

The findings for percentage inhibition of DPPH radical expressed as (IC_{50} in µg/mL) for *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts are presented in **Figure 4.**

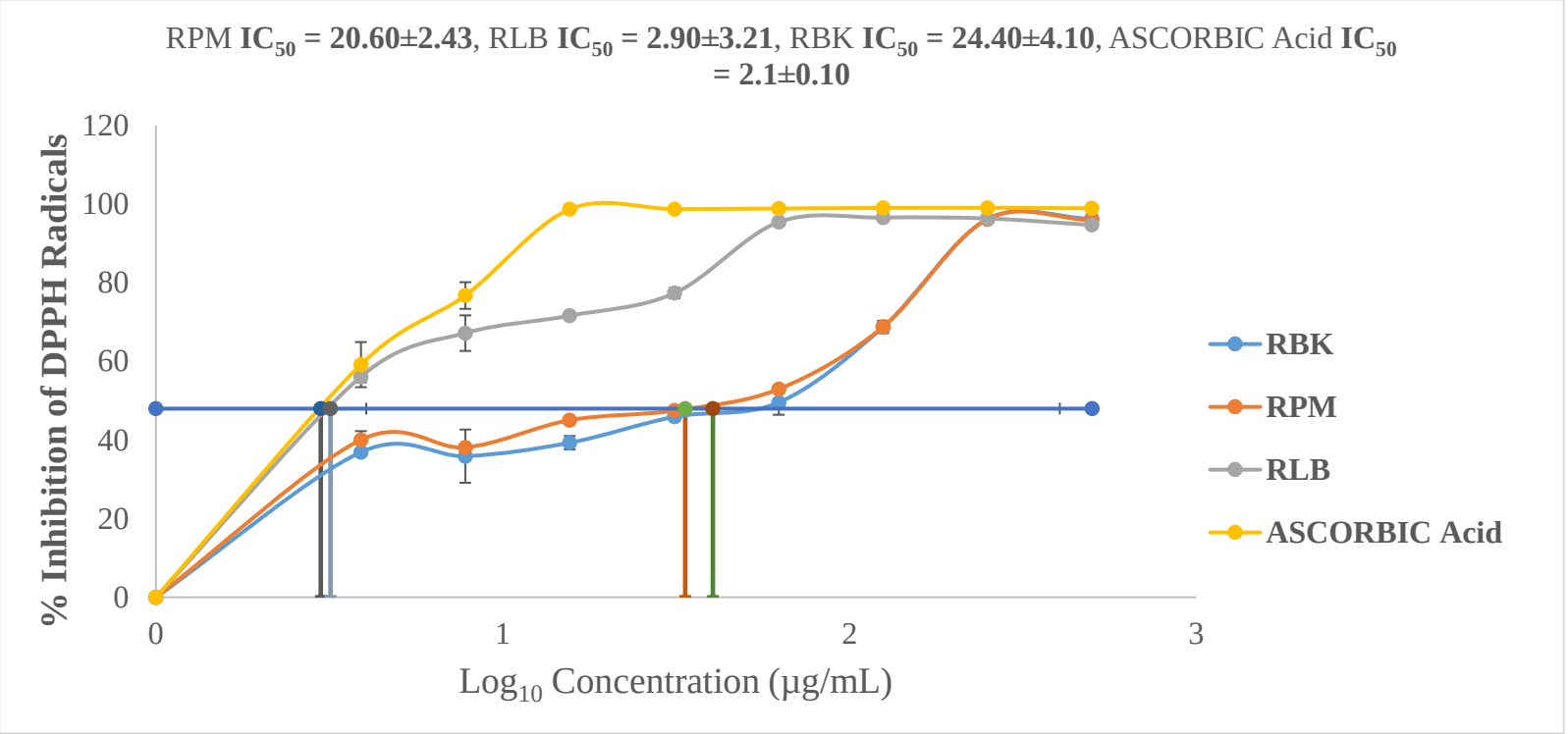


Figure 4.2: Percentage inhibition of DPPH radical expressed as (IC_{50} in µg/mL) for *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*;**RLB-***R. lineari-bracteolata*

The highest inhibition of DPPH radical inhibition by methanolic extracts was *R. linearibracteolata* (94.72%), *R. prostrata* (95.93%) and *R. bignoniiflora* (96.31%) relative to ascorbic acid (98.97%) at 500µg/mL. The % inhibition by the *Ruellia* species (at 500 µg/mL) in the present study was similar and about 95% that of ascorbic acid. Methanolic extracts of the *Ruellia* species in the present study were higher than their respective aqueous extracts. The concentration required to inhibit 50% of DPPH radical, referred to as inhibitory concentration$_{50}$ (IC_{50}), was determined for *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* extracts and the findings are presented in **Table 4.14.**

Ascorbic acid exhibited IC_{50}, 2.1±0.10 µg/mL, whereas the methanolic extract of *R. lineari bracteolata* revealed IC_{50}, 2.9 ±3.21 µg/mL. *R. prostrata* and *R. bignoniiflora* exhibited higher values of (IC_{50} 24.4µg/mL) and (IC_{50}=20.6 µg/mL), respectively, relative to ascorbic acid. Methanolic extracts exhibited lower (IC_{50} µg/mL) values compared to the aqueous extracts. The lower the IC_{50} value, the more potent the antioxidant activity as a lower concentration is required to inhibit 50% of the DPPH radicals.

Analysis of the antioxidant activity of methanolic and aqueous extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* species showed a marked difference in antioxidant activity. This could be attributed to the difference in proportions of the active components that are responsible for the activity. The antioxidant activity findings correlated well with the content of flavonoids and phenolics in the species studied (**Table 4.3**). The difference in antioxidant activity could also be due to the difference in solubility of individual phytochemicals in solvents of extraction. The high antioxidant activity observed at higher concentrations was because increasing the concentration of the plant extracts increased the proportion of the chemical ingredients with pharmaceutical value in the plant extract (Debella, 2002). DPPH has a centred-nitrogen free radical which is stable and can easily and effectively be scavenged by antioxidants. This free radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The antioxidant activity normally occurs when antioxidant molecules react with the radicals resulting in scavenging of the free radical by hydrogen donation (Rackova, 2007). In the

antioxidant activity, flavonoids and phenolics play a significant role since they are highly effective electron donors (Van Acker *et al.,* 1998). These two compounds are also documented to possess anti-carcinogenic and anti-inflammatory properties (Tanaka, 1998; Wong, 2006). Flavonoids are polyphenolic compounds that play a role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen, 1993).

A previous study by Roopa *et al.,* (2011) on the antioxidant potential of fractions from methanol, chloroform, ethyl acetate and n-butanol fractions of aerial parts of *R. prostrata,* confirmed the presence of triterpenoids, glycosides, carbohydrates, tannins, and flavonoids as the bioactive principles responsible for its pharmacological properties. The findings in the study by Roopa *et al.* (2011) revealed the presence of triterpenoids, glycosides, and carbohydrates in the methanolic and n-butanolic extracts of *R. prostrata but* did not report the presence of alkaloids. These findings were in line with the findings in the present study. However, the report by Afzal *et al.* (2015) revealed the presence of alkaloids in *R. tuberosa* and *R. rosea, R. prostrata* was not studied. It was noted that the most studied *Ruellia* species for their biological activities and phytocompounds are *R. tuberosa, R. patula, R. brittoniana* and *R. rosea*. Hence more studies on *Ruellia* species could reveal the nature of the compounds present in this species. Another study by Safitri *et al.* (2019) revealed similar phytocompounds with antioxidant activity from *Ruellia* species similar to those obtained in the present study.

The Indian *R. prostrata* species studied by Wangia (1985) revealed the presence of saponins, glycosides and absence of tannins in the aqueous and ethanolic extracts. No alkaloids were detected in the aqueous and ethanolic extracts using Mayer's, Dragendorff's and Hager's reagents. These reagents are the ones used in qualitative detection of alkaloids from plant materials. Sterols were obtained in the petroleum ether and chloroform extracts. The absence of alkaloids in some *Ruellia* species, such as *R. prostrata, R. bignoniiflora* and *R.lineari-bracteolata* in the present study (**Table 4.2**) could be due to the ability of plants to synthesize secondary metabolites being determined by several factors including soil composition, geographical locations, seasonal variation, and intra-species variation (Schaffer *et al.,* 2005). Plants accumulate their secondary

metabolites mainly for their defence purposes against pathogens, herbivores, or microbes (Theis and Lerdau 2003; Samuni-Blank *et al.,* 2012). The secondary metabolites that are commonly reported in *Ruellia* genera are flavonoids, phenolic compounds, sterols, and triterpenes.

Phenolics and flavonoids are known to be the main groups of compounds that act as primary free antioxidant scavenging radicals (Potterat, 1997; Zheng *et al.,* 2017). *R. prostrata* showed the highest amount of crude saponins compared to *R. bignoniiflora* and *R. lineari-bracteolata,* whereas *R. lineari-bracteolata* showed the highest flavonoid content **(Table 4.3).** Even though phytochemical screening had shown the presence of phenolics in the *Ruellia* species, their amounts were not significant ($p > 0.05$) compared to saponins and flavonoids. These little amounts are attributed to the nature of phenolics in form of dried ground powder, which makes them difficult to extract due to increased fixing of phenolic compounds into the vacuoles of the plants (Harborne, 1998; Hutzler *et al.,* 1998).

4.4.2 *In Vitro* Anti-Arthritic Studies

From the present sub-chronic toxicity studies (**Section 4.4.3**), it was concluded that the aqueous extract of *R. prostrata* was safe and was, therefore, further subjected to anti-arthritic studies. The findings from the *in vitro* anti-arthritic studies are shown in the subsequent section.

4.4.2.1. *In Vitro* Anti-arthritic Activity

In vitro anti-arthritic activity was based on the property of non-steroidal anti-inflammatory drugs to inhibit protein denaturation by heat (Mizushima, 1965; Mizushima and Kobayashi, 1968). The assay was standardized to determine its reliability and the reproducibility of results. The effect of the aqueous and the organic extracts of *R. prostrata* and *R. bignoniiflora* on inhibition of protein denaturation by heat was assessed. Methotrexate, a disease-modifying antirheumatic drug and diclofenac sodium, a non-steroidal anti-inflammatory drug, were used as standards.

To standardize the assay, aliquots from aqueous and organic extracts of *R. prostrata* and *R. bignoniiflora* were analyzed in the *in vitro* anti-arthritic test. The findings on the % inhibition of protein denaturation by heat between aliquots of aqueous extracts of *R. prostrata* are presented in **Table 4.11.**

Table 4.11: Percentage inhibition of protein denaturation of the aqueous extract of *R. prostrata*

Conc. (mg.mL)	Aliquot 1	Aliquot 2	Aliquot 3	Range
		Percentage inhibition		
1.67	48.53±0.00^{b}	51.31±0.64^{a}	52.10±0.06^{a}	**3.57**
2.50	62.56±0.60^{b}	65.97±0.03^{a}	65.05±0.34^{a}	**3.41**
3.33	72.10±0.56^{a}	71.27±1.11^{a}	70.33±1.10^{a}	**1.77**
4.17	75.52±0.17^{a}	71.96±0.64^{b}	75.84±0.16^{a}	**3.88**
5.00	80.38±0.50^{a}	79.43±0.46^{a}	76.92±0.15^{b}	**3.46**
5.83	84.23±0.36^{a}	81.07±0.05^{b}	82.39±0.22^{b}	**3.16**
6.67	88.51±0.09^{a}	84.72±0.07^{b}	86.97±0.40^{c}	**3.79**

Values are expressed as Mean±SEM. Values followed by different superscript row-wise are significantly different ($p \leq 0.05$).

The negative control did not inhibit protein denaturation by heat, and other tests were compared to it. This assay was standardized with a margin of error of less than 20%. The findings of the percentage inhibition of protein denaturation by heat in aliquots of organic extracts of *R. prostrata* are shown in **Table 4.12**.

Table 4.12: Percentage inhibition of protein denaturation in aliquots of the organic extract of *R. prostrata*

Conc. (mg.mL)	Aliquot 1	Aliquot 2	Aliquot 3	Range
		Percentage inhibition		
1.67	93.39±0.13[b]	94.65±0.14[b]	94.43±0.07[b]	**1.26**
2.50	94.51±0.15[a]	95.47±0.18[a]	95.24±0.02[a]	**0.96**
3.33	95.73±0.09[a]	95.72±0.07[a]	95.57±0.14[a]	**0.16**
4.17	96.19±0.10[a]	95.73±0.09[a]	96.48±0.04[a]	**0.75**
5.00	96.26±0.06[a]	96.08±0.00[a]	96.88±0.01[a]	**0.80**
5.83	93.39±0.13[b]	94.65±0.14[b]	94.43±0.07[b]	**1.26**
6.67	94.51±0.15[a]	95.47±0.18[a]	95.24±0.02[a]	**0.96**

Values are expressed as Mean±SEM. Values followed by different superscript row-wise are significantly different ($p \leq 0.05$).

Due to the reproducibility of the results, the method was adopted to determine the *in vitro* anti-arthritic activity of the aqueous and the organic extracts from *R. prostrata* and *R. bignoniiflora*. A comparative analysis of percentage inhibition of extracts from *R. prostrata* and *R. bignoniiflora* was made using diclofenac sodium and methotrexate standards and the findings are shown in **Table 4.13**.

Table 4.13: Comparative % inhibition of protein denaturation by the heat of *R. prostrata* and *R. bignoniiflora* extracts

Type of solvent/chemical	Plant species	Conc. (mg/mL)		
		6.67	**5.00**	**3.33**
		Percentage inhibition		
Organic Extract	*R. prostrata*	96.42±0.22[a]	95.90±0.17[a]	95.35±0.23[a]
	R. bignoniiflora	90.92±0.04[b]	89.10±0.09[b]	86.15±0.23[a]
Aqueous Extract	*R. prostrata*	86.73±0.70[b]	78.91±0.68[b]	71.24±0.54[b]
	R. bignoniiflora	91.25±0.09[a]	88.78±0.20[b]	79.55±0.33[b]
Diclofenac sodium		89.08±0.15[b]	84.33±0.09[b]	68.38±3.10[b]
Methotrexate		97.21±0.01[a]	96.67±0.02[a]	94.82±0.05[a]

Values are expressed as Mean±SEM. Values followed by different superscript column-wise are significantly different ($p \leq 0.05$) and comparisons were made at a concentration of 6.67 mg/mL.

There was a dose-dependent increase in % inhibition of protein denaturation by heat in all tests. Methotrexate showed highest % inhibition of protein denaturation by heat (97.21 ±0.01%) at 6.67 mg/mL, whereas diclofenac sodium standard displayed a lower value of 89.08 ±0.15% at the same concentration. Relative to methotrexate, the organic extract of *R. prostrata* showed inhibition of 96.42 ±0.22%, whereas the organic extract of *R. bignoniiflora* displayed a lower % inhibition of protein denaturation (90.92 ±0.04%) which were not statistically different ($p > 0.05$) at 6.67 mg/mL.

The aqueous extract of *R. bignoniiflora* displayed slightly higher inhibition of protein denaturation by heat (91.25±%) compared to *R. prostrata* (86.73±0.70%). The two extracts displayed a lower % significant ($p \leq 0.05$) inhibition of protein denaturation by heat relative to methotrexate standard (97.21±0.01%) at 6.67mg/mL.

Relative to diclofenac sodium standard, the organic extracts of *R. prostrata* and *R. bignoniiflora* and the aqueous extract of *R. bignoniiflora* displayed higher % inhibition of protein denaturation by heat in all concentrations tested. Aqueous extract of *R. prostrata* displayed lower % inhibition at all concentrations, relative to diclofenac sodium, although the differences were not significant ($p>0.05$). The concentration that caused 50% inhibition of protein denaturation by heat, referred to as (IC_{50}, mg/mL), was determined for *R. prostrata* and *bignoniiflora* aqueous and methanolic extracts and the findings are presented in **Table 4.14**.

Table 4.14: 50% inhibition of protein denaturation by heat expressed as (IC_{50} mg/mL) for *R. prostrata* and *R. bignoniiflora* aqueous and organic extracts

Type of solvent/chemical	Plant species	IC_{50} (mg/mL)
Organic extract	*R. bignoniiflora*	3.90 ± 0.05^{a}
	R. prostrata	0.29 ± 0.01^{e}
Aqueous extract	*R. bignoniiflora*	0.79 ± 0.02^{c}
	R. prostrata	4.13 ± 0.02^{b}
Diclofenac sodium		3.42 ± 0.02^{b}
Methotrexate		$0.17\pm0.02^{e, f}$

Values are expressed as Mean±SEM. Values followed by different superscript column-wise are significantly different ($p\leq0.05$).

Compared to methotrexate standard (IC_{50}, 0.17 mg/mL), *R. prostrata* organic extract displayed (IC_{50}, 0.29 mg/mL) which was 58.62% that of methotrexate. *R. bignoniiflora* aqueous extract (IC_{50}, 0.79 mg/mL), *R. bignoniiflora* organic extract (IC_{50} 3.90 mg/mL) and *R. prostrata* aqueous extract (IC_{50} 4.13 mg/mL) were higher than that of methotrexate standard. Diclofenac sodium showed a higher (IC_{50} 3.42 mg/mL) compared to methotrexate (IC_{50} 0.17 mg/mL). Of the *Ruellia* extracts tested, *R. prostrata* organic extract was the most potent inhibitor of protein denaturation by heat (IC_{50} 0.29 mg/mL). Therefore, *R. prostrata* organic extract was further fractionated on a silica gel column to profile the bioactive compounds using *in vitro* anti-arthritic activities.

A total of ten combined fractions from the organic extract of *R. prostrata* were obtained using column chromatographic method on normal silica gel (230:70Å). Two solvent

systems were used; n-hexane: ethyl acetate (**Fractions 1-7**) followed by ethyl acetate: methanol (**Fractions 8-10**). The % inhibition of protein denaturation by heat by these fractions was determined together with the % that caused 50% inhibition of protein denaturation by heat (IC_{50}, mg/mL). The findings of the % inhibition of protein denaturation by heats are shown in **Table 4.15.**

Table 4.15: Percentage inhibitions of protein denaturation by the heat of *R. prostrata* organic extract column fractions showing (IC_{50} in µg/mL)

Combined Fractions	Concentration (mg/mL)				IC_{50} (mg/mL)
	6.67	**5 .00**	**3.33**	**0**	
	Percentage inhibition				
Fraction-1	39.53±0.47^{d}	36.07±0.00^{f}	30.16±**3**.74$^{c, d}$	0.0	9.63±1.82^{a}
Fraction-2		87.03±2.76$^{d, e}$	53.37±4.69$^{b, c}$	0.00	3.24±0.14$^{b, c}$
Fraction-3	97.35±0.02^{a}	93.20±0.31$^{a, b, c}$	84.05±1.23^{a}	0.00	1.72±0.11$^{b, c}$
Fraction-4			90.82±0.22^{a}	0.00	-
Fraction-5	97.76±0.01^{a}	97.40±0.16^{a}	92.27±0.18^{a}	0.00	0.96±0.08$^{b, c}$
Fraction-6	98.05±0.06^{a}	90.98±0.22$^{c, d}$	32.30±13.50$^{c, d}$	0.00	3.67±0.24$^{b, c}$
Fraction-7		91.53±0.26$^{b, c, d}$		0.00	-
Fraction-8	88.81±0.63^{b}	87.01±0.41$^{d, e}$	12.26±2.96^{d}	0.00	4.11±0.04^{b}
Fraction-9	96.99±0.02^{a}	96.85±0.00$^{a, b}$	96.64±0.13^{a}	0.00	0.06±0.01^{c}
Fraction-10	84.08±0.13^{c}	82.80±0.61^{e}	80.69±0.10$^{a, b}$	0.00	0.31±0.02^{c}

Values are expressed as Mean±SEM. Values followed by different superscript column-wise are significantly different ($p \leq 0.05$).

The % inhibition of protein denaturation by heat as a measure of *in vitro* anti-arthritic activity was dose-dependent. From IC_{50} (mg/mL) values, Fractions 3, 5, 9 and 10 showed high *in vitro* anti-arthritic activity with no significant difference ($p>0.05$) between the fractions**.** When the activities of the pooled fractions were compared at a lower concentration of 3.3 mg/mL, Fraction-9 showed the highest % inhibition of protein denaturation by heat of 96.64 ±0.13% compared to Fractions 3 (84.05±1.23%), 5

(92.27±0.18%) and 10 (80.69±0.10%). However, there was not a significant difference ($p>0.05$) in the *in vitro* anti-arthritiic activity between Fraction 9 and Fractions 3, 5 and 10. Compared to *R. prostrata* organic extract (**Table 4.15**) whose % inhibition of protein denaturation was 96.42 ±0.22% at 6.67mg/mL, the column Fraction-9 showed higher inhibition of protein denaturation by the heat of 96.99 ±0.02% at the same concentration.

The (IC_{50} μg/mL) values of methotrexate and diclofenac standards (**Table 4.14)** were 0.17 mg/mL and 3.42 mg/mL, respectively. Relative to methotrexate, Fraction-9 revealed IC_{50} value of 0.06 mg/mL, whereas Fraction-5 displayed IC_{50} value of 0.96 mg/mL. Fraction-9 displayed the lowest IC_{50} of 0.06 mg/mL, lower than that of methotrexate IC_{50} (0.17 mg/mL and diclofenac sodium IC_{50} 3.42 mg/mL standards. The IC_{50} value value of the organic extract of *R. prostrata* of 0.29 mg/mL was lower than that of diclofenac sodium standard. Overall, Fraction-9 displayed best *in vitro* anti-arthritic activity, better than that of methotrexate and diclofenac sodium standards. The results were automatically obtained using an ELISA reader.

The findings revealed that column fractionation produced pooled fractions that were more potent than their corresponding organic extracts and reference drugs. The findings in the present study revealed that column fractionation could isolate purer and more active compounds compared to their crude extracts. This type of study is called bioassay-guided fractionation, where upon determination of the activity of a plant extract, column fractions are subjected to bioassays to determine which fraction had the expected activity. Such fractionation could lead to isolation and purification of the active principle(s) that could be developed into a pharmaceutical product(s) after structure elucidation (Eloff, 2004).

A study by Umapathy *et al.* (2010) revealed that certain arthritic diseases produce autoantigens which could be due to denaturation of proteins *in vivo.* It was therefore postulated that agents that could prevent protein denaturation by heat could be worthwhile for anti-arthritic drug development. The increments in absorbances of test samples to negative control were reported to be an indication of the stabilization of protein (Jagtap *et al.,* 2011). Therefore, *in vitro* protection of heat denaturation of BSA by known anti-

inflammatory drugs was found to be involved with some interaction with proteins, a property which was utilized as a suitable screen for testing anti-rheumatic drugs. In the present *in vitro* serum turbidity studies, standard anti-rheumatic drug (methotrexate) and a non-steroidal anti-inflammatory drug (diclofenac sodium) were found to be effective, with methotrexate being more potent than diclofenac sodium. This *in vitro* anti-arthritic assay revealed that the method was reliable and reproducible; and could quickly screen potential anti-arthritic drugs before laborious, time-consuming *in vivo* anti-arthritic methods, such as Freund's Complete adjuvant-induced arthritis in rats.

A study by Kamble *et al.* (2017) on the evaluation of *in vitro* anti-arthritic activity of aqueous and ethanolic extract of *Vitex negundo* and *Punica granatum* by BSA and egg albumin denaturation methods concluded that the anti-arthritic effect of the plant extracts was possibly due to the presence of flavonoids, terpenoids and alkaloids. Both flavonoids and terpenoids were present in the plant extracts under the current study and could have been responsible for the anti-athritic activity observed in this study, although alkaloids were absent. Besides, saponins which were present in the *Ruellia* species could have also contributed to the *in vitro* anti-arthritic activity observed in *R. prostrata* and *R. bignoniiflora* extracts. Heat stability of BSA was reported to have been increased by the addition of soy saponin due to electrostatic and hydrophobic interactions (Ikedo *et al.*, 1996). Therefore, it is possible that, even in the current study, the stabilization of BSA in the *in vitro* antia-rthritic study could be due to the presence of saponins. Saponins are naturally occurring surface-active glycosides and are mainly produced by plants and lower marine animals and some bacteria (Yoshiki *et al.*, 1998). These compounds consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methyl pentose, glycosidically linked to an aglycone (sapogenin), which is hydrophobic and maybe triterpenoid or steroid in nature. The aglycone may contain one or more unsaturated C=C bonds. The oligosaccharide chain is normally attached at the C-3 position (monodesmosidic), but many saponins have an additional sugar moiety at position C-26 or C-28, thus are bidesmosidic (Yoshiki *et al.*, 1998).

4.4.3 *In Vivo* Toxicity Studies

Acute and sub-chronic toxicity studies were carried out in Wistar albino rats of either sex to determine the safety of the aqueous extract of *R. prostrata.* The findings are shown in the subsequent sections.

4.4.3.1 *In Vivo* Acute Toxicity Studies

Acute toxicity studies were carried out before the pharmacological studies because the results guided how the pharmacological studies doses were considered. Oral acute toxicity studies were based on OECD, O (2001) Guidelines, whereas oral sub-chronic toxicity studies were based on OECD/OCDE, (2008) Guidelines. The findings from the *in vivo* toxicity studies are discussed in the subsequent sections.

4.4.3.1. *In vivo* Acute Toxicity Studies

Due to lack of resources and unnecessary sacrifice of laboratory animals, one species, *R. prostrata* was studied in the oral toxicity srudies. The findings on the observations of the rats after administratin of 300 mg/kg and 2000 mg/kg of the aqueous extract of *R. prostrata* are presented in **Table 4.16**.

Table 4.16: Observations made after administration of *R. prostrata* aqueous extract (300 mg/kg and 2000 mg/kg)

Observations	*R. prostrata* (300 and 2000 mg/kg)	PBS
Activity	Active	Active
Nature of the fur	Smooth	Smooth
Posture	Normal	Normal
Breathing/Respiration	Normal	Normal
Effects on body weight	None	None
Movement	Normal	Normal
Mortality	None	None
Convulsions	None	None
Salivation	None	None
Diarrhoea	None	None
Lethargy	None	None
Coma	None	None

Key: PBS- Phosphate-buffered saline pH 7.4

The animals in both untreated control and *R. prostrata*-treated rats showed neither observable side effects nor death within 24 hours. After two weeks' observations in oral doses of 300 and 2000 mg/kg body weight, there were no signs of tremor, paralysis, salivation, diarrhoea, convulsions, lethargy, aversive behaviour, coma, or death. Previous studies on Indian *R. prostrata* (Wangia *et al.,* 1985), revealed that oral doses in the range of 1-10g/kg were safe in mice. In that study, the highest oral dose (10g/kg) neither caused death nor any adverse effects in mice.

In the present study OECD, O (2001) protocol was adopted which has got the advantage of using a minimum number of animals. Being a stepwise up-and-down procedure, only three animals were used per dose at a time and increasing or decreasing the dose in another set of 3 animals, depended on whether 2 animals died or showed adverse effects. The safety of the aqueous extract from *R. prostrata* was placed in the highest class of safety as per the Globally Harmonised Classification System (GHS) in mg/kg body weight as Category 5>2000-5000 which was equivalent to LD_{50} value of >2000 mg/kg (OECD, O 2001).

Normally, acute toxicity studies are usually carried out to determine the dose that would cause death or serious toxic manifestations when administered singly or severally at few doses to establish the dose that should be used in subsequent studies (Haschek and Rousseaux, 2013). It is paramount to carry out toxicity testing in the screening of newly developed drugs before they can be used on humans (Arome and Chinedu, 2013). The importance of toxicity testing is to check how safe a test substance is; and to characterize the possible toxic effects it can produce. The guiding principles of toxicity testing is to determine the effect of the test substances on laboratory animals, and the exposure of laboratory animals to high doses could evaluate the new drug's possible hazard on human wthat are exposed to much lower dose. Toxicity testing is important in the development of therapeutic agents.

4.4.3.2. *In Vivo* Sub-ChronicToxicity Studies

Upon determination of the safety of the aqueous extract of *R. prostrata* at 2000 mg/kg, the doses that were adopted in pharmacological studies were in the range of 250-1500 mg/kg. The *in vivo* analgesic and anti-inflammatory studies revealed no statistical difference ($p>0.05$) in activities between the oral dose at 1000 and 1500 mg/kg (**Sections 4.4.3 and 4.4.4**) in the three *Ruellia* species of studied. The aqueous extract of *R. prostrata* showed highest activity in the analgesic and anti-inflammatory studies compared to *R. bignoniiflora* and *R. lineari-bracteolata*. Therefore, this species is the one that was studied in the sub-chronic toxicity studies. Since the doses in the pharmacological studies were single doses, sub-chronic toxicity studies adopted the lower effective dose of 1000 mg/kg as drug administration was for a prolonged period of 28 days. There was no justification to subject the animals to the highest effective dose for prolonged periods when a lower dose of 1000 mg/kg was equally effective.

The animals were observed for any changes as recorded in (**Table 4.16**). There were no toxic effects observed such as urination, lacrimation, fur standing on the body, convulsions, lack of activity or death after 28 days' observation. Blood was drawn on the 28^{th} day by cardiac puncture, and both biochemical and haematological parameters

investigated as useful indices of evaluating the toxicity of the aqueous extract of *R. prostrata* as per Yakubu *et al.* (2008).

Sub-chronic toxicity studies required long term (28 days) administration of aqueous extract of *R. prostrata*, with regular monitoring of physiological, biochemical abnormalities and detailed postmortem examination at the end of the test to detect gross abnormalities. Various biochemical and haematological parameters were investigated. In addition, gross necropsy was carried out on the intenal organs such as the liver, heart, spleen, heart, intestines, and sex organs (testis and uterus). These organs were weighed, and relative organ weight determined.

The use of animal in toxicity testing offered benefits because it involved the examination of a whole functioning organism, a tool that could be used to foresee the future safety of the drug being investigated (Arome and Chinedu, 2013). The effects of the aqueous extract of *R. prostrata* (1000 mg/kg) on the blood biochemistry, haematological parameters, and relative weight of the internal organs after 28 days' administration are discussed in the subsequent sections.

4.4.3.2.1 Effect of the Aqueous Extract of *R. prostrata* on the Liver Enzymes, Total Protein and Bilirubin

The findings on the effects of the aqueous extract of *R. prostrata* (1000 mg/kg) on the liver enzymes, total protein and bilirubin are presented in **Table 4.17**.

Table 4.17: Effect of the aqueous extract of *R. prostrata* (n=12) on the liver enzymes, total protein, and bilirubin

Parameters	Control	*R. prostrata* (1000mg/kg)
TP (g/dL)	8.10±0.60[a]	7.70±0.29[a]
AB (g/dL)	5.35±0.650[a]	4.90±0.178[a]
TB (µmol/L)	17.65±4.25[a]	16.40±1.58[a]
DB (µmol/L)	5.70±1.00[a]	5.18±0.522[a]
AP (U/L)	268.00±23.0[a]	208.75±7.47[a]
SGOT (U/L)	11.00±3.00[a]	8.75±0.85[a]
SGPT (U/L)	4.00±1.00[a]	2.25±0.25[a]
GGT (U/L)	12.50±2.50[a]	11.25±0.63[a]

Key: TP-Total protein; AB-Albumin; TB-Total bilirubin; DB-Direct bilirubin; AP-Alkaline phosphatase; SGOT- Glutamate Oxaloacetate Transaminase; SGPT- Glutamate Pyruvate Transaminase; GGT-Gamma-Glutamyl transferase. Values are expressed as Mean±SEM. Values followed by different superscript across the row are significantly different ($p \leq 0.05$).

There was no significant difference between the untreated control and the *R. prostrata*-treated group for all the biochemical parameters studied; total protein, albumin, total bilirubin, direct bilirubin, alkaline phosphatase (ALP), glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and gamma-glutamyl transferase (GGT). The lack of increase in these blood parameters, especially the activity of liver enzymes; SGOT, SGPT, GGT and AP indicated that the aqueous extract of *R. prostrata* was not harmful to the liver. The enzyme SGPT is a cytoplasmic enzyme found in very high concentration in the liver and an increase of this specific enzyme in the blood indicates hepatocellular damage, while SGOT is less specific than SGPT as an indicator of liver function (Aliyu *et al.*, 2007). The enzyme GGT is usually the first liver enzyme to rise in the blood when any of the bile ducts that carry bile from the liver to the intestines become obstructed. It, therefore, makes the most sensitive enzyme for detecting bile duct disorders. Enzyme GGT is a key enzyme in the gamma-glutamyl cycle which is involved in the synthesis and degradation of glutathione, as well as drug and xenobiotic detoxification (Courtay *et al*., 1992). Enzyme GGT is predominantly used as a diagnostic marker for liver disease, such as chronic viral hepatitis. Elevated serum GGT activity can be found in diseases of the liver, biliary system, and pancreas. The main advantage of GGT over SGOT is in verifying that SGOT elevations are, in fact, due to biliary disease;

although SGOT can also be increased in certain bone diseases, GGT is not (Lum and Gambino, 1972). In the present study, the aqueous extract of *R. prostrata* was found safe on the liver and bile duct.

4.4.3.2.2. Effect of the Aqueous Extract of *R. prostrata* on the Electrolytes

Blood results showing effects of the aqueous extract of *R. prostrata* (n=12) on electrolytes are shown in **Table 4.18**.

Table 4.18: Effect of aqueous extract of *R. prostrata* (n=12) on the electrolytes

Eletrolytes (µml/L)	*R. prostrata* (1000 mg/kg)	Untreated control
Urea	6.69±0.30[a]	6.83±1.74[a]
Potassium	4.71±0.67[a]	4.50±1.56[a]
Sodium	146.75±2.18[a]	137.09±17.0[b]
Chloride	105.25±1.15[a]	98.00±2.52[b]
Creatinine	79.88±2.53[a]	95.67±3.71[b]

Values are expressed as Mean±SEM. Values followed by different superscript across the row are significantly different ($p \leq 0.05$).

There was no significant difference ($p > 0.05$) in the levels of urea and potassium between *R. prostrata*-treated group and the untreated control, whereas a significant difference ($p \leq 0.05$) was observed in creatinine, chloride and sodium levels between the *R. prostrata*-treated group and the untreated control. Creatinine is a breakdown product of muscle creatinine phosphate. Blood measurement of serum creatinine is an important indicator of renal health since it is an easily measured byproduct of muscle metabolism that is excreted by the kidneys unchanged. Creatinine is removed from the blood chiefly by the kidneys, primarily via glomerular filtration and by proximal tubular secretion. When there is little or no tubular reabsorption of creatinine, it means that the filtration in the kidney is deficient, hence, blood creatinine concentrations rise. Therefore, creatinine clearance can be calculated by the determination of the concentration of creatinine in blood and urine. Creatinine levels are used as an indicator of renal failure (Aliyu *et al.,* 2007). There is a correlation between creatinine clearance and glomerular filtration rate (GFR), hence GFR

is a clinically important parameter because it is a measurement of renal function. A low level of blood creatinine indicates nothing more than an efficient and effective pair of kidneys. The low level of creatinine in the blood of the *R. prostrata*-treated rats showed that there was no damage on the kidney leading to the excretion of creatinine, without accumulation in the blood.

A medical test that measures the amount of urea nitrogen found in blood is called the blood urea nitrogen (BUN). The liver produces urea as a waste product of protein digestion in the urea cycle. Blood urea nitrogen is an indication of renal health. The main causes of an increase in BUN are high protein diet, decrease in GFR, suggestive of renal failure, decrease in blood volume, gastrointestinal haemorrhage, and congestive heart failure. In the present study, *R. prostrata* aqueous extract did not show adverse effects on BUN, hence, was safe on the kidneys.

The *R. prostrata*-treated group had higher levels of sodium and chloride compared to the untreated control. A review of the phytochemistry and biological activities of *R. prostrata* by Choudhary and Bassi (2019), revealed that this species, a well-known perennial herb, scattered throughout India, was used traditionally as a powerful diuretic agent. The rats used in the acute toxicity studies in the current study did not show any signs of diuretic effect compared to the untreated control. This action may be related to the effects of aquaretics, novel drugs, that are used to promote aquaresis without electrolyte loss. Aquaretic drugs are used ot treat hyponatraemia (Bolignano *et al.,* 2007). A number of herbal medicines are classified as aquaretics (Awang, 2009; Abdel Baki *et al.,* 2019). Aquaretics were reported to contain volatile oils, flavonoids, saponins or tannins. They function to increase the volume of urine by promoting the flow of blood to the kidney, hence raising the glomerular filtration rate. These agents do not retard the resorption of Na^+ and Cl^- in renal tubules. Therefore, the quantities of these electrolytes are retained in the body and not excreted with water (Awang, 2009). Since the aqueous extract of *R. prostrata* was reported to contain flavonoids, saponins and tannins, these compounds could have induced the significant higher values of Na^+ and Cl^- in the *R. prostrata*-treated re; ative to the untreated control.

Potassium is an essential electrolyte that is critical to the function of nerve and muscle cells**,** including those in the heart. Normally, the kidneys keep a healthy balance of potassium by removing excess potassium out of the body. Therefore, kidney failure is the most common cause of hyperkalemia. When the kidneys fail or do not function properly, they will not be able to remove extra potassium from the body, which can lead to potassium build-up. Since there was no significant difference between the *R. prostrata*-treated rats and untreated control, the aqueous extract of *R. prostrata* did not cause adverse effects on the kidneys, regarding potassium excretion.

Since the liver and kidney play a central place in the metabolism and excretion of drugs, respectively, the aqueous extract of *R. prostrata* in the dose of 1000 mg/kg did not show adverse effects onthese two orgnas. Therefore, *R. prostrata* aqueous extract could be considered a safe herbal alternative.

4.4.3.2.3 Effect of the Aqueous Extract of *R. prostrata* on the Random Blood Sugar

The findings on the effects of the aqueous extract of *R. prostrata* on random blood sugar level are shown in **Table 4.19**.

Table 4.19: Effect of the aqueous extract of *R. prostrata* (n=12) on the random blood sugar level

Dose (mg/kg)	Blood Sugar Levels (mM/L)
Untreated control	6.83 ± 1.12^{a}
R. prostrata 1000	5.87 ± 1.18^{a}

Values are expressed as Mean±SEM. Values followed by different superscript across the column are significantly different ($p \leq 0.05$).

There was no significant difference ($p > 0.05$) in random blood sugar levels between the untreated control and the *R. prostrata*-treated group, suggesting that the aqueous extract of *R. prostrata* did not show any hypoglycemic effect. A similar report which studied streptozotocin-induced diabetes and normal rats reported that in humans, antidiabetic

drugs of the biguanide type such as metformin, decreased blood sugar levels in diabetics, without much effect on normal blood sugar levels (Bailey *et al*., 1985; De Fronzo and Goodman 1995; Stumvoll *et al*., 1995). Another report indicated that the ethanolic extract of *Chrysophyllum albidum* seed cotyledon in alloxan-induced diabetic rats exhibited antihyperglycemic and hypolipidemic effect. There was no hypoglycaemic effect in the non-diabetic rats (Olorunnisola *et al*., 2008). A review of the phytochemistry and biological activities of *R. prostrata* by Choudhary and Bassi (2019), revealed that this species acted as a hypoglycemic agent as used in traditional medicine.

4.4.3.2.4 Effect of the Aqueous Extract of *R. prostrata* on the Fasting Blood Cholesterol

The findings of the effect of the aqueous extract of *R. prostrata* (1000 mg/kg) on fasting blood cholesterol are shown in **Table 4.20**.

Table 4.20: Effect of aqueous extract of *R. prostrata* (n=12) on the fasting blood cholesterol level

Concentration (mg/kg)	Cholesterol (mM/L)
Untreated control	2.74 ± 0.58^{a}
R. prostrata	2.83 ± 0.24^{a}

Values are expressed as Mean±SEM. Values followed by different superscript across the column are significantly different ($p\leq0.05$).

There was no significant difference ($p>0.05$) in fasting blood cholesterol level between the untreated control and the *R. prostrata* -treated group. Cholesterol is the principal sterol biosynthesized by all animal cells and it is an essential structural component of animal cell membranes. Normally, all the cholesterol that the body needs is made by the liver, although some enter the body from food such as animal-based foods like meat, milk, and eggs. Too much cholesterol in the body is a risk factor for atherosclerosis. The present study revealed that aqueous extract of *R. prostrata* did not have a risk to the cardiovascular disease in rats.

4.4.3.2.5 Effect of the Aqueous Extract of *R. prostrata* on the Blood Haematological Parameters

The findings of the aqueous extract of *R. prostrata* (1000 mg/kg) on total blood count are shown in **Table 4.21**.

Table 4.21: Effect of the aqueous extract of *R. prostrata* (n=12) on total blood count

Parameters	Untreated control	*R. prostrata* (1000mg/kg)
WBC (10^{-3}/uL)	10.61±1.37[a]	13.45±1.58[a]
Lym%	92.37±1.10[a]	90.61±1.12[a]
Gran%	2.77±1.03[a]	3.11±0.67[a]
Mid%	4.87±0.35[a]	6.20±0.63[a]
Lym# (10^{-3}/uL)	9.83±1.35[a]	12.20±1.47[a]
Gran# (10^{-3}/uL)	0.26±0.06[a]	0.42±0.11[a]
Mid# (10^{-3}/uL)	0.52±0.09[a]	0.82±0.11[a]
RBC (10^{-6}/uL)	6.91±0.22[a]	8.04±0.20[b]
HGB (g/dL)	15.37±0.93[a]	15.94±0.43[a]
HCT (%)	43.73±3.39[a]	46.45±0.97[a]
MCV (fL)	63.07±2.95[a]	57.89±0.71[b]
MCH (pg)	22.13±0.66[a]	19.80±0.28[a]
MCHC (g/dL)	35.17±0.65[a]	34.21±0.30[a]
RDW-CV (%)	15.10±0.20[a]	15.55±0.22[a]
RDW-SD (fL)	38.03±1.41[a]	35.75±0.74[a]
PLT (10^{-3}/uL)	849.3±56.6[a]	791.6±40.8[a]
MPV (fL)	5.93±0.08[a]	6.06±0.14[a]
PDW	15.20±0.06[a]	15.36±7 0.12[a]
PCT (%)	0.48±0.02[a]	0.50±0.11[a]

Values are expressed as Mean±SEM. Values followed by different superscript across the row are significantly different ($p \leq 0.05$).

Key: WBC-white blood cells; Lym%- % lymphocytes; Gran%-% granulocytes; Mid%- mid-sized %, Lym# -lymphocyte number; Gran#- granulocytes number; Mid#- mid number, mid-sized granulocyte number, RBC -red blood cells; HGB- haemoglobin; HCT-haematocrit.
Red blood cells indices; MCV-mean corpuscular volume; MCH- mean corpuscular haemoglobin; MCHC-mean corpuscular haemoglobin concentration; RDW-red blood cell distribution width; PLT-platelets, MPV- mean platelet volume; PDW- platelet distribution width; PCT- plateletcrit.

There were significant differences in the red blood cells (RBCs) counts between the untreated control ($6.91 \pm 0.22 \times 10^{-6}$/uL) and *R. prostrata* -treated group ($8.04 \pm 0.20 \times 10^{-6}$/uL) in which the *R. prostrata* -treated group showed a higher ($p \leq 0.05$) RBCs count. The increase in the RBCs indicated that *R. prostrata* aqueous extract did not

cause anaemia to the animals, hence was safe to the bone marrow and other blood-forming organs like the liver or spleen.

A significant difference ($p \leq 0.05$) was also observed in the mean corpuscular volume (MCV) between the normal control (63.07 ±2.95 fL) and *R. prostrata*-treated group (57.89 ±0.71 fL), in which the *R. prostrata*-treated group showed a lower ($p \leq 0.05$) MCV. The MCV parameter is a part of a standard total blood count, in which the value is used to categorize patients with anaemia; microcytic anaemia (MCV is below normal), macrocytic anaemia (MCV is above normal) and normocytic anaemia (MCV is within normal range). Normocytic anaemia may occur during acute blood loss or haemolysis, in which bone marrow has not yet responded with a change in volume. Although MCV was significantly lower in *R. prostrata*-treated rats compared to the untreated control in this study, anaemia was ruled out due to the increase in RBCs in this test group compared to the untreated control group. Hemoglobin levels were also comparable between the two groups.

Haematological parameters in CFA- induced arthritis (**Table 4.22)** studies in the present study did not reveal any differences in RBCs counts and MCV value after administration of the aqueous extract of *R. prostrata* (1000 mg/kg) for 28 days. Rheumatoid arthritis is normally associated with different types of anaemia, such as anaemia of chronic inflammation, iron deficiency anaemia, and macrocytic anaemia. Chronic inflammation could lower the production of RBCs in the bone marrow and could also lead to release of certain proteins that could affect how the body uses iron (Nemeth and Ganz, 2014). Inflammation could also affect the way the body uses erythropoietin, a hormone that controls the production of RBCs (Brines and Cerami, 2008). In the present study, the RBCs count was not lowered, ruling out anaemia.

A study by Shatoor (2011) on acute and sub-acute toxicity of *Crataegus aronia* (syn. *Azarolus* (L.) whole plant aqueous extract in Wistar rats revealed a significant increase in the RBC and PCV at a dose of 200 mg/kg compared to the other groups which were treated with different doses. These values were reported to be above the well-established

reference ranges for Wistar rats (Cameron and Watson, 1949). The increase in RBCs could be due to variations in the pharmacokinetics of the antioxidants (flavonoids) present in the extract (Amran *et al*., 2010). The possible mechanism of the increase in RBCs and PCV values could have involved the effect of flavonoids on maintaining the cell membrane through inhibition of peroxidation of polyunsaturated fatty acids (Hasan *et al.,* 2009; Wang *et al.,* 2010). Flavonoids were reported capable of inhibiting the formation of superoxide ions and hydroxyl radicals, which are two strong peroxidation agents that are produced in the body. Under normal conditions, these radicals destroy cells. Therefore, flavonoids may protect both the haematopoietic committed stem cells and the formed blood cells from the attack of the reactive free radicals. The antioxidant activity of flavonoids may maintain the haeme iron in its ferrous state which could enhance erythropoiesis. Since the phytochemical screening in the present study confirmed the presence of flavonoids in the aqueous extract of *R. prostrata*, the increase in the level of RBCs revealed could be due to the role of flavonoids.

The rest of the blood parameters (white blood cells, lymphocytes, granulocytes, and platelets) observed between the untreated control and *R. prostrata*-treated rats did not show any significant differences. These cells are derived from the bone marrow and are useful in defence against infections (WBCs), in immune protective responses (lymphocytes), blood coagulation together with coagulation factors (platelets). Many disease-modifying anti-rheumatic drugs and biologics have adverse effects of suppressing the bone marrow leading to the propensity to infections (Katzung, 2017). Methotrexate-treated rats suppressed the bone marrow (**Table 4.22).** Therefore, the aqueous extract of *R. prostrata* did not suppress the bone marrow and proved a safe herbal alternative as shown in the subsequent section.

4.4.3.2.6 Effect of the Aqueous Extract of *R. prostrata* on Haematological Parameters in Arthritis studies

The findings of the effect of the aqueous extract of *R. prostrata* on haematological parameters in arthritis studiesare shown in **Table 4.22.**

Table 4.22: Effect of the aqueous extract of *R. prostrata* (n=6) on haematological parameters in arthritis studies

PARAMETERS	TREATMENT			
	EX	**NC**	**AI**	**PC**
RBCs (10^6/uL)	7.99±0.23[a]	7.59±0.20[a]	7.78±0.27[a]	7.23±0.44[a]
Hemoglobin (g/dL)	14.10±0.12[a,b]	15.23±0.44[a]	13.38±0.16[a,b]	12.38±0.65[b]
PCV (%)	41.60±0.82[a]	43.27±1.41[a]	38.40±0.62[a]	35.55±2.21[a]
MCV (fL)	53.75±2.53[a]	54.25±2.06[a]	49.75±1.49[a]	49.25±1.03[a]
MCHC (%)	33.88±0.80[a]	35.18±0.30[a]	34.83±0.32[a]	34.88±0.40[a]
MCH (pg)	19.00±0.62[a]	18.48±0.72[a]	17.25±0.58[a]	17.13±0.25[a]
Platelets (10^3/uL)	826.0±23.7[a]	704.5±29.6[b]	725.5±26.7[a,b]	668±16.2[b]
WBC (10^3/μL)	13.55±0.84[a,b]	13.15±0.60[a,b]	15.30±1.81[b,c]	6.80±0.70[c]
Seg. Neutrophil	6.83±1.75[a]	5.56±0.30[a,b]	5.88±0.57[a, b]	2.08±0.39[b]
Lymphocytes (10^3/μL)	11.88±0.89[a]	10.75±1.74[a,b]	8.41±1.22[b,c]	4.26±0.39[c]
Monocytes (10^3/μL)	0.44±0.15[a]	0.40±0.05[a]	0.69±0.34[a]	0.31±0.07[a]
Eosinophils(10^3/μL)	0.16±0.05[b,c]	0.14±0.06[b,c]	0.18±0.03[b,c]	0.08±0.03[c]
Basophils(10^3/μL)	0.08±0.01[b]	0.09±0.02[b]	0.15±0.03[a,b]	0.08±0.01[b]

Key: R_x=Treatment; Ex=*R. prostrata* extract (1000 mg/kg); NC=Untreated control; AI= Untreated arthritis-induced control; PC=Positive control (methotrexate, 2 mg/kg). Values are expressed as Mean±SEM. Values followed by different superscript row-wise are significantly different ($p \leq 0.05$).

Apart from the significantly higher levels ($p \leq 0.05$) in platelets in the *R. prostrata* extract-treated rats ($826.0 \pm 23.7 \times 10^3$/uL) compared to untreated control ($704.5 \pm 29.6 \times 10^3$/uL), there was no significant difference ($p > 0.05$) in other haematological parameters compared to the untreated control. Relative to methotrexate standard, whose platelet level was $668 \pm 16.2 \times 10^3$/uL, there was a lower significant difference ($p \leq 0.05$) in the levels obtained from the *R. prostrata* extract-treated rats.

As reported by Bordoloi *et al*. (2016), drugs that lead to platelet augmentation (thrombocytosis) could have a potential role in improving platelet counts in various thrombocytopenic disorders such as dengue fever. Autoimmune diseases, such as lupus erythematosus, rheumatoid arthritis and pregnancy could also lead to thrombocytopenia (Rodak *et al*., 2013). The aqueous extract of *R. prostrata* could be useful in such medical conditions.

Compared to the untreated control whose haemoglobin level was 15.23 ± 0.44g/dL, methotrexate showed a lower significant ($p \leq 0.05$). level of 12.38 ± 0.65 g/dL. *R. prostrata*-treated rats showed higher significant ($p \leq 0.05$) haemoglobin level of 14.10 ± 0.12g/dL compared to methotrexate. The white blood cells counts of $6.80 \pm 0.70 \times 10^3$/µL, lymphocytes counts of $4.261 \pm 0.39 \times 10^3$/µL, and segmented neutrophils number of 2.08 ± 0.39 in methotrexate-treated rats were significantly lower ($p \leq 0.05$) than those of the untreated control. Relative to methotrexate, *R. prostrata*-treated rats that showed significantly higher ($p \leq 0.05$) white blood cells counts of $13.55 \pm 0.69 \times 10^3$/uL, lymphocytes count of $11.881 \pm 0.89 \times 103/\text{uL}$ and segmented neutrophils number of 6.83 ± 1.75.

In the present study, it was evident that methotrexate caused bone marrow suppression with a reduction in levels of haematological parameters, such as white blood cells, lymphocytes, and segmented neutrophils. The aqueous extract of *R. prostrata* did not show any bone marrow suppression, although the extract induced highest levels in platelets compared to the negative control. Bone marrow suppression by methotrexate is one of the adverse effects of this drug that often leads to patients' non-compliance to medication (Katzung, 2017). Patients whose bone marrow is suppressed are prone to infections (such as lung infections), since white blood cells are involved in defence against pathogens. In the present study, the aqueous extract of *R. prostrata* proved to be a safer alternative and efficacious like methotrexate in the CFA-induced arthritis studies.

4.4.3.2.7 Effect of the Aqueous Extract of *R. prostrata* on the Internal Organs

The findings of the % relative organ weight of animals treated with the aqueous extract of *R. prostrata* are also shown in **Figure 4.3**.

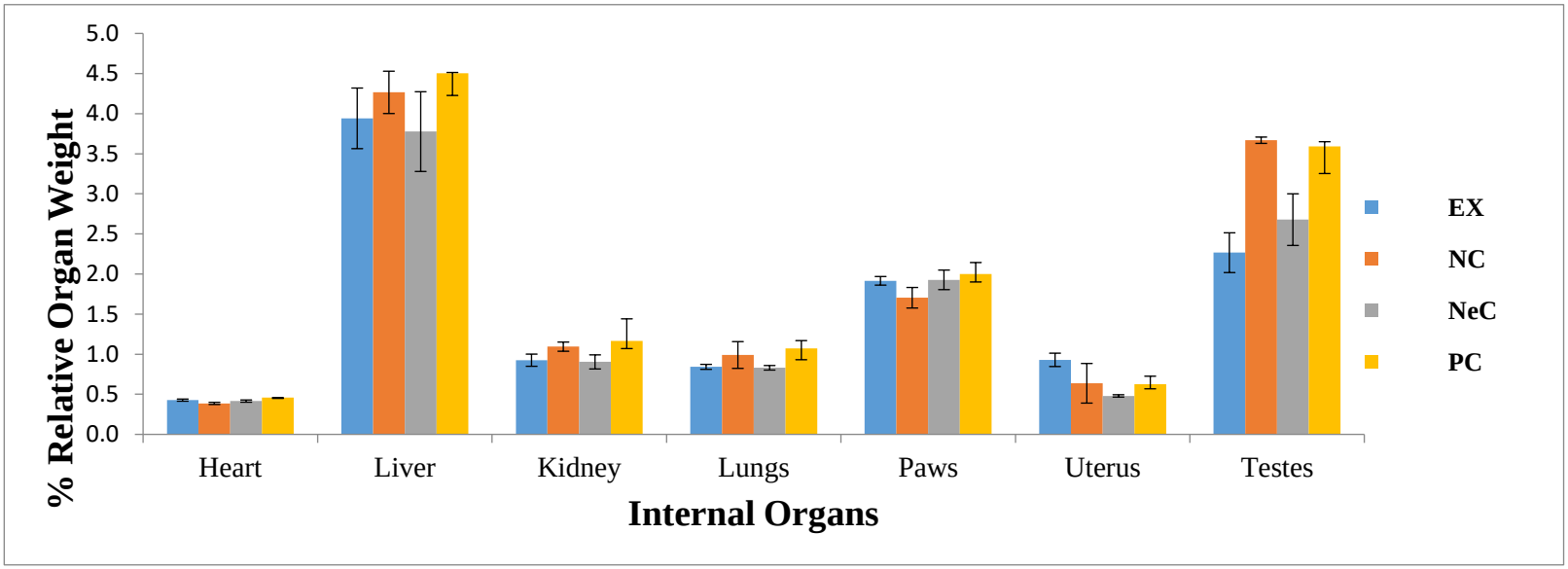


Figure 4.3: Percentage relative organ weight of rats treated with *R. prostrata* aqueous extract.

Key: Ex=RPM Extract, 1000 mg/kg; NC=Untreated control; NeC=Untreated arthritis-induced control; PC=Positive control. (Methotrexate, 2 mg/kg)

The body weight of each rat was assessed using a sensitive balance before commencement of dosing, daily during the dosing period and once on the day of sacrifice (Aniagu *et al.*, 2005). Percentage relative organ weight varied between different treatments. The weight of all organs was not significantly different between the untreated control and *R. prostrata*-treated group ($p>0.05$). The % relative weight of the testes in rats treated with the the aqueous extract of *R. prostrata* (2.66 ±0.25%) was significantly lower ($p\leq0.05$) than the untreated control (3.67 ±0.04%). The positive control had higher significant ($p\leq0.05$) relative organ weight of the heart (0.46 ±0.01%) compared to the untreated control (0.39 ± 0.01).

Organ weight is a very sensitive indicator of an effect of a drug toxicity since significant differences in organ weight between the treated groups and untreated contro animals could occur in the absence of morphological changes **(**Piao *et al.,* 2013).

4.4.4 *In Vivo* Analgesic Activity

Analgesic activity of the aqueous and the methanolic extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* was determined using the Tail-flick tests for acute pain and the Formalin test for progressive pain. To avoid injury to tissue in Tail-flick test, a latency of 10 seconds, was considered 100% inhibition of pain (Stein *et al.,* 1988)**.**

4.4.4.1 Acute Analgesic Activity -Tail-Flick Test

The findings for the % **increase in reaction time** by *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* aqueous extracts are presented in **Figure 4.4.**

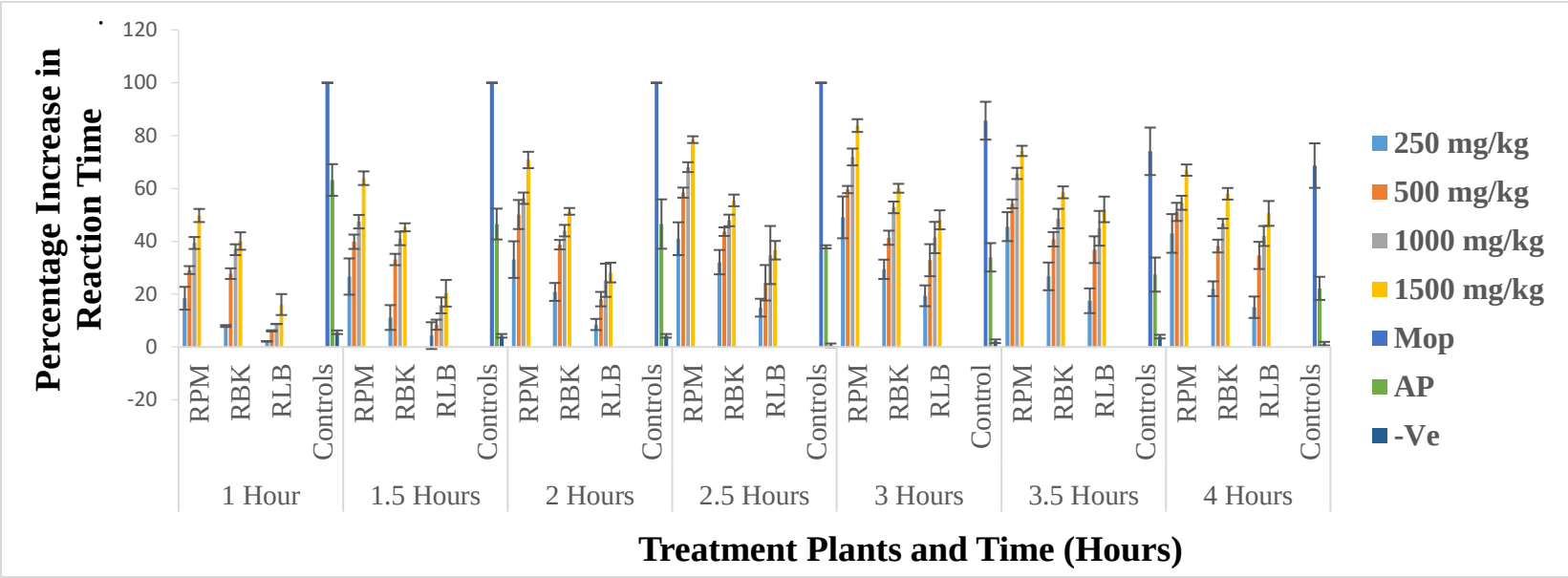


Figure 4.4: Percentage increase in reaction time by the aqueous extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata*

Key: MOP= Morphine sulphate (10 mg/kg); AP=Acetaminophen (200mg/kg); RPM=R. *prostrata;* RBK=*R. bignoniiflora*; RLB=*R. lineari-bracteolata*.

There was a dose-dependent increase in reaction time in tail flick by the aqueous extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* with maximum activity at the 3-hour point. Morphine sulphate standard showed 100.00 ±0.00% increase in reaction time in tail-flick from 1- 2.5 hours, thereafter, declined till the 4th hour. Relative to % increase in reaction time in the tail flick by morphine standard (85.64 ±7.17%) at 3-hour point, the aqueous extracts of *R. prostrata* showed the highest % increase in reaction time (83.80 ±2.42%), followed by *R. bignoniiflora* (60.08 ±1.68%) and *R. lineari-bracteolata* (48.14 ±3.59%) in a dose of 1500 mg/kg. The % increase in reaction time in tail-flick relative to morphine translated to almost 98% for *R. prostrata*, 70% for *R. bignoniiflora* and 56%± for *R. lineari-bracteolata*, respectively at 3-hour point.

Compared to morphine (100.00 ±0.00%), the lowest % increase in reaction time in tail-flick was 18.45 ±4.32% for *R. prostrata*, 7.94 ±2.36% for *R. bignoniiflora* and 2.13% ±7.01 for *R. lineari-bracteolata* in a dose of 250 mg/kg at 1-hour point. Acetaminophen standard (200 mg/kg) displayed lower % increase in reaction time in tail-flick of 63.2 ±6.00 and 33.94±5.37% compared to morphine sulphate standard at 1-hour point and 3-hour point, respectively. Like morphine sulphate, the activity of acetaminophen was maximum in the 1st hour, thereafter, declined till the 4th hour. At 3-hour point, the aqueous extract of *R. prostrata* displayed higher % increase in reaction time in tail-flick at all concentrations tested compared to *R. bignoniiflora* and *R. lineari-bracteolata*. Aqueous extract of *R. bignoniiflora* exhibited a higher % increase in reaction time in tail-flick from 500-1500 mg/kg relative to acetaminophen. Aqueous extract of *R. lineari-bracteolata* exhibited a higher % increase in reaction time in tail-flick at 1000 and 1500 mg/kg relative to acetaminophen at 3-hour point. Overall, aqueous extract of *R. prostrata* was the most potent extract in % increase in reaction time in tail-flick, like morphine sulphate, but better than acetaminophen at the 3-hour point.

The findings for the % **increase in reaction time** by the methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* aqueous extracts are presented in **Figure 4.5.**

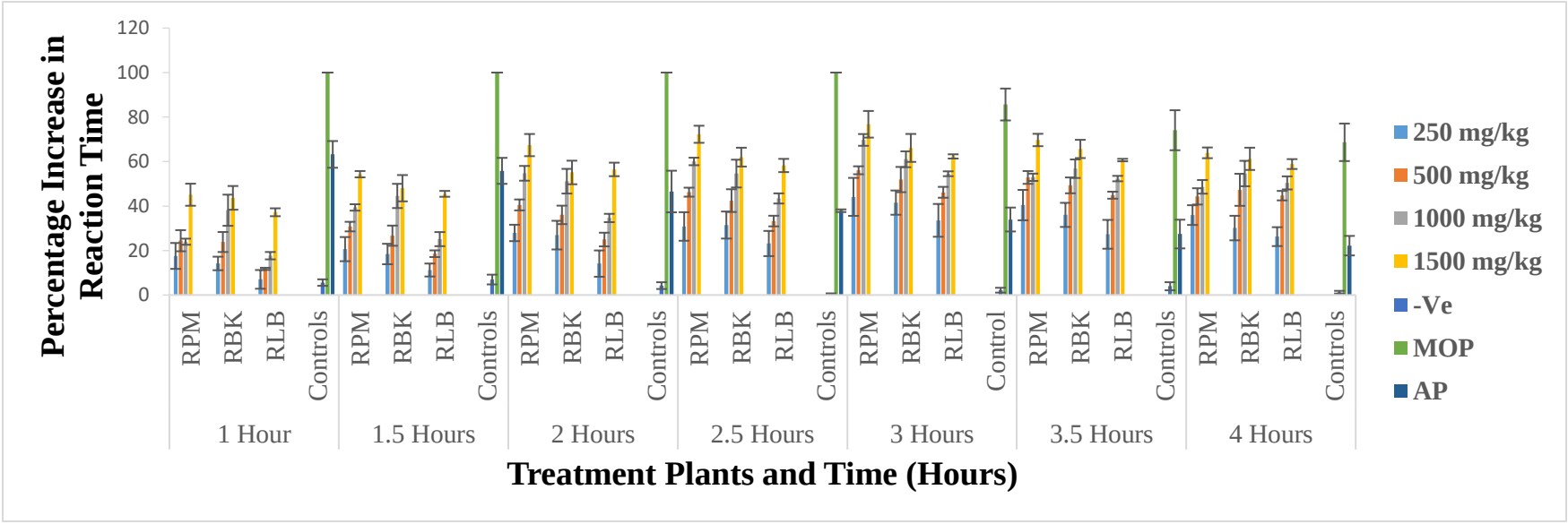


Figure 4.5: Percentage increase in reaction time by the methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata*

Key: MOP= Morphine (10 mg/kg); AP=Acetaminophen (200 mg/kg); RPM=R. *prostrata;* RBK=*R. bignoniiflora*; RLB=*R. lineari-bracteolata*.

The methanolic extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* displayed a dose-dependent % increase in reaction time in tail-flick with maximum activity at the 3-hour point. Relative to % increase in reaction time in tail-flick by morphine standard (85.64 ±7.17%), methanolic extracts of *R. prostrata* showed highest % increase in reaction time (76.75 ±5.98%) followed by *R. bignoniiflora* (66.12 ±6.28%) and *R. lineari-bracteolata* (62.31 ±0.95%) in a dose of 1500 mg/kg at the 3rd hour. The % in increase reaction in tail-flick relative to morphine translated to almost 81% for *R. prostrata*, 77% for *R. bignoniiflora* and 70% for *R. lineari-bracteolata*, respectively at 3-hour point. The lowest % increase in reaction time in tail-flick for *R. prostrata* (17.57 ±5.82%), *R. bignoniiflora* (14.19 ±3.04%) and for *R. lineari-bracteolata* (7.03 ±4.20%) in a dose of 250 mg/kg at 1-hour point respectively, compared to morphine sulphate (100 ±0.00%). It was noted that the methanolic extracts of *R. bignoniiflora* and *R. lineari-bracteolata* displayed higher % increase in reaction time in tail-flick compared to their aqueous extracts, unlike the methanolic extract of *R. prostrata* which displayed lower % compared to the aqueous extract. The methanolic extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* displayed a higher % increase reaction in tail-flick compared to acetaminophen standard at all concentrations tested at 3-hour point.

The highest % increase in reaction time in reaction time in tail flick by morphine standard and was considered as maximum response (100.00%). Therefore, the dose that produced 50% of morphine maximum dose, also referred to as the ED_{50} in mg/kg or potency of the extracts, was determined by plotting $\log_{10}$ concentration of extracts *vs* % increase in reaction time in tail flick. The results of 50 % inhibition of tail flick (ED_{50} in mg/kg) of the aqueous extracts of *R. prostrata, R. bignoniiflora* and *R. linear-bracteolata* at 3-hour are shown in **Figure 4.6.**

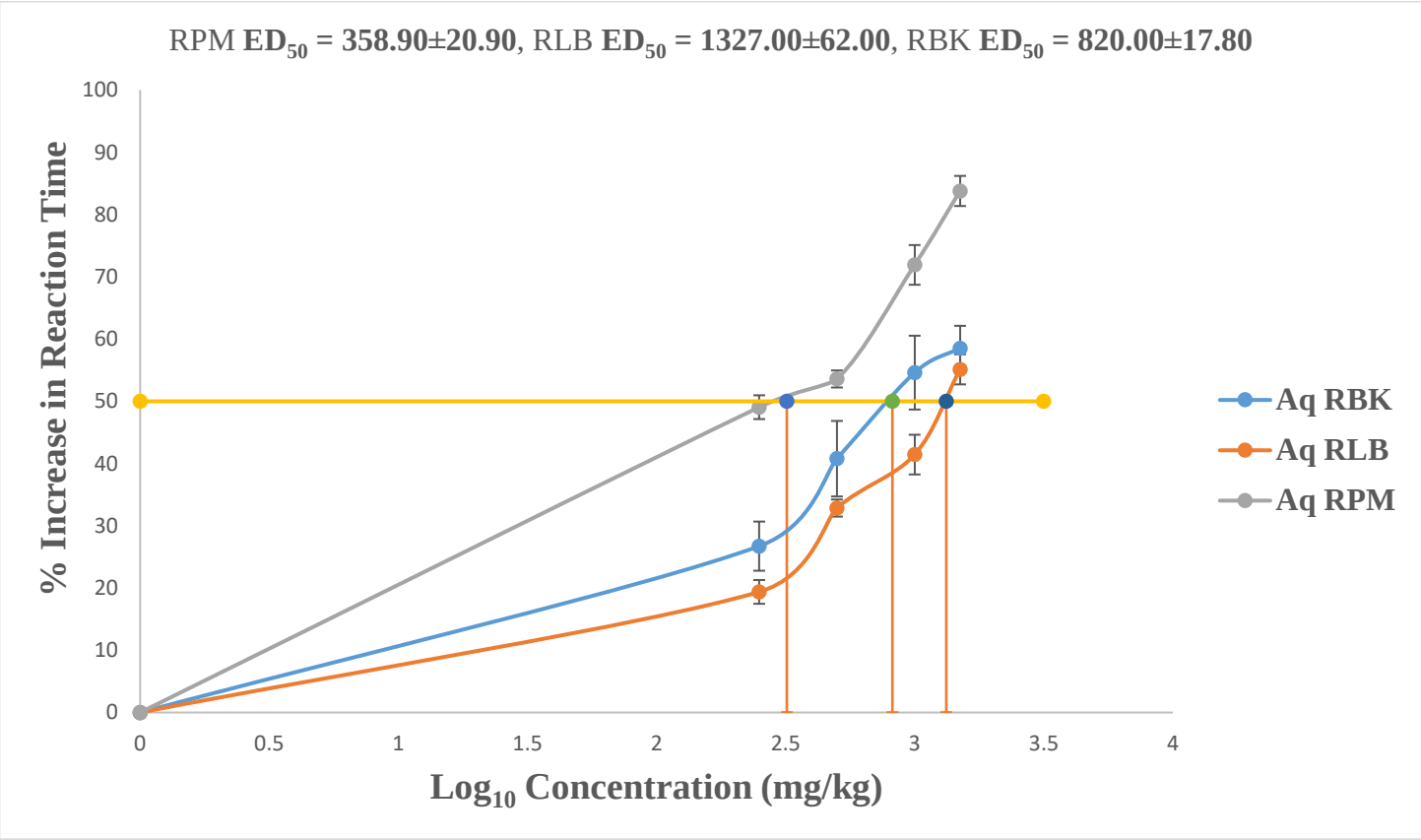


Figure 4.6: 50 % inhibition of tail-flick (ED_{50}, mg/kg) of aqueous extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* at 3-hour

Key: RPM- *R. prostrata;* **RBK**-*R. bignoniiflora*; **RLB**-*R. lineari-bracteolata*

The results of 50 % inhibition of tail flick (ED_{50} in mg/kg) of the methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* at 3-hour are shown in **Figure 4.7.**

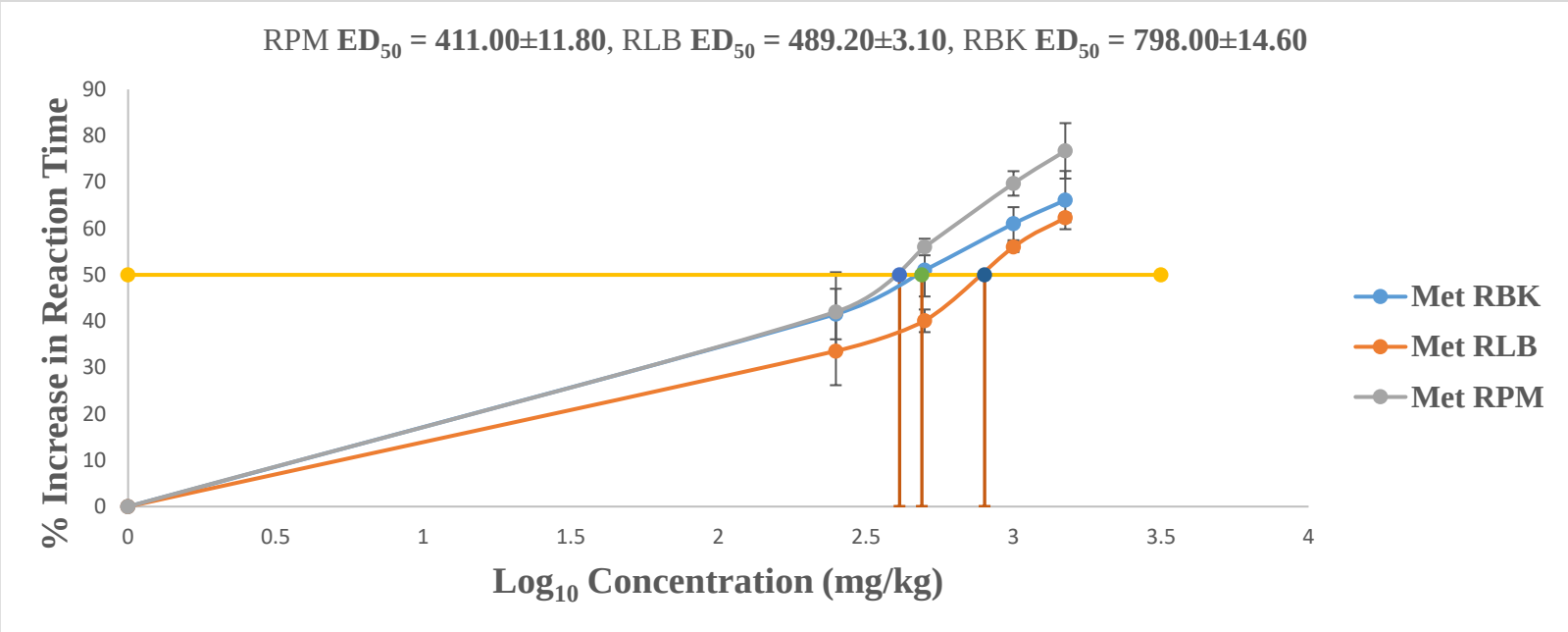


Figure 4.7: 50 % inhibition of tail-flick (ED_{50}, mg/kg) of methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* at 3-hour

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*;**RLB-***R. lineari-bracteolata*

R. prostrata aqueous extract showed lower ED_{50} (358.90 mg/kg) value compared to its methanolic extracts (ED_{50} 411.00 mg/kg). Compared to *R. bignoniiflora* and *R. lineari-bracteolata, R. prostrata* aqueous extract ED_{50} mg/kg was significant ($p \leq 0.05$). The findings imply that *R. prostrata* aqueous extract was more potent ($p \leq 0.05$) than its methanolic extract, R. *bignoniiflora* and *R. lineari-bracteolata* aqueous extracts. The present study also revealed that the aqueous extract of *R. bignoniiflora* was more potent (ED_{50} 820.00 mg/kg) than that of *R. lineari-bracteolata* (ED_{50} 1327.00 mg/kg) and the difference was significant ($p \leq 0.05$). The methanolic extracts of *R. bignoniiflora* and *R. lineari-bracteolata* were more potent than their aqueous extracts ($p \leq 0.05$).

The antioxidant activity of the extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* (IC_{50} values (µg/mL) as shown in **Table 4.6** showed that methanolic extracts had higher activity than their aqueous extracts. The *in vivo* studies revealed that the aqueous extracts of the *Ruellia* species in the present study were more active than the methanolic extracts. This difference could be due to the fact that *in vivo* activity required biotransformation to the active principles, unlike the *in vitro* activity.

Although the aqueous and the methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* showed the highest % increase in reaction time in tail flick at 3-hour point, morphine sulphate and acetaminophen standards displayed the highest activity in the 1st hour, with activity decreasing till the 4th hour. Since the reference drugs were in their active forms, the analgesic effect was observed within the 1st hour. The delay in attaining maximum analgesic response by the *Ruellia* extracts in the present study could be due to *in vivo* biotransformation to active principles by the liver enzymes (Maina *et al.*, 2015). The reduction in % increase in reaction time in tail flick after the maximum activity in the *Ruellia* extracts and reference drugs could be due to liver enzymes metabolism to inactive metabolites. Morphine is usually metabolized to morphine-3-glucuronide and morphine-6-glucuronide via phase II metabolism (Van Dorp *et al.*, 2006).

Acetaminophen (paracetamol) is metabolized primarily in the liver into toxic and non-toxic products in glucuronidation and sulphation (McGill and Jaeschke, 2013) and N-hydroxylation, dehydration then glutathione conjugation (McGill and Jaeschke, 2013). The analgesic activity of both *R. prostrata* and *R. bignoniiflora* was higher than that of acetaminophen (200 mg/kg) standard. At 3-hour point when *R. prostrata* exhibited % increase in reaction time in tail flick of 83.80%, morphine showed a comparable reduction in reaction time of 85.64%. The tail-flick test results revealed that the *Ruellia* species in the present study possess analgesic activity to different degrees in an acute pain model, with aqueous extract of *R. prostrata* (1500 mg/kg) showing highest activity 98%, relative to morphine sulphate standard, at the 3-hour point. It was also noted that there was no significant difference ($p>0.05$) in analgesic activity between the *Ruellia* species in doses of 1000 and 1500 mg/kg. The order of activity coincided with the content of saponins in these species (**Table 4.3**). The saponins in these species were probably metabolized by intestinal esterases to the aglycones, which could be the bioactive moieties.

4.4.4.2. Progressive Analgesic-Formalin Test

Determination of inhibition of progressive pain by the extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* was done using the Formalin test. In this test, inhibition of activities like biting, licking the injected paw and flinching the body was considered % inhibition of progressive pain or antinociceptive activity. The findings of the % inhibition of the nociceptive activity of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* aqueous extracts are presented in **Figure 4.8.**

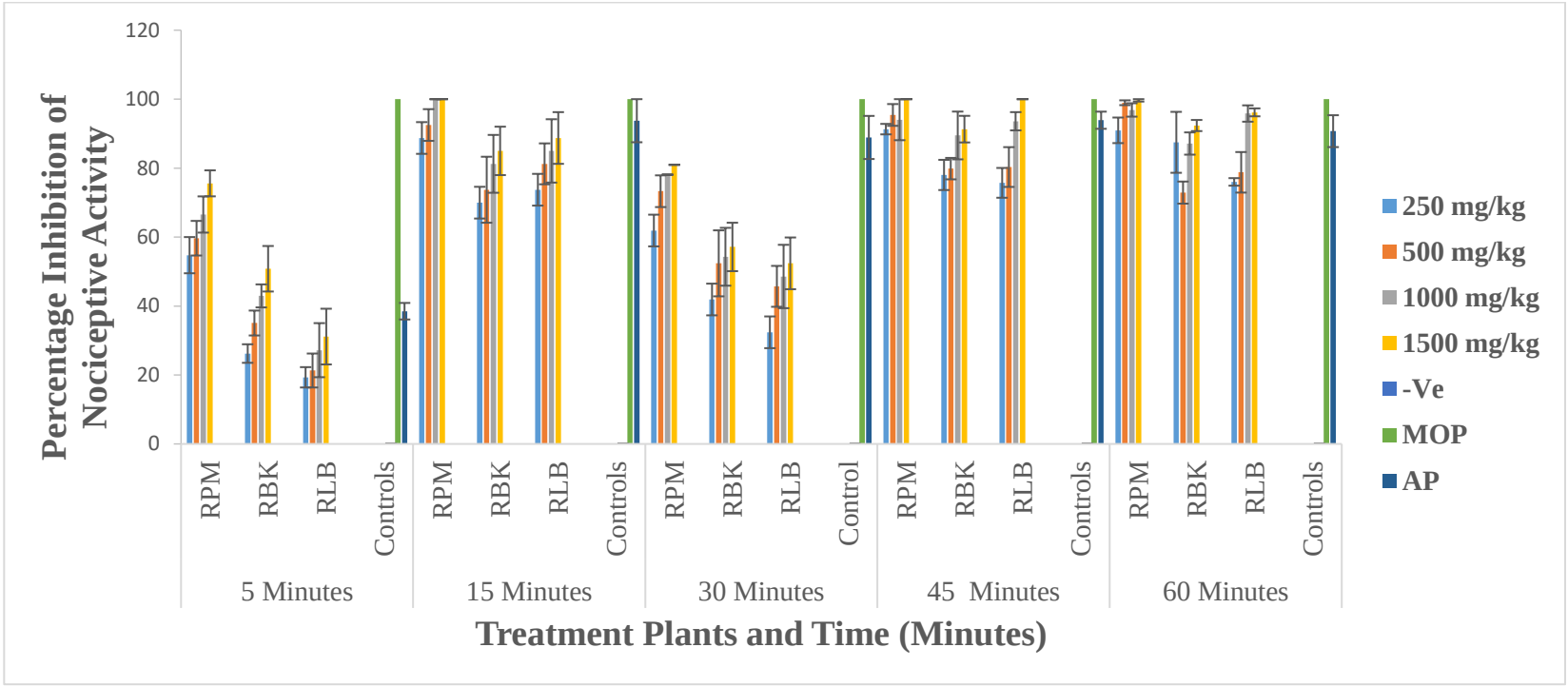


Figure 4.8: Percentage inhibition of formalin-induced pain by *R. prostrata, R. bignoniiflora* and *R. linearibracteolata* aqueous extracts

Key: MOP= Morphine (10 mg/kg); AP=Acetaminophen (200 mg/kg); RPM=R. *prostrata;* RBK=*R. bignoniiflora*; RLB=*R. lineari-bracteolata*.

There was a dose-dependent inhibition of nociceptive activity of formalin by *R. prostrata*, *bignoniiflora* and *R. lineari-bracteolata* aqueous extracts. The inhibition was biphasic, with the highest activity observed at 15 minutes and 45 minutes. Relative to morphine sulphate standard % inhibition of nociceptive pain (100.00 ±0.00%) at 15 minutes, *R. prostrata* aqueous extract showed similar % inhibition (100.00±0.00%), followed by *R. lineari-bracteolata* (88.74 ±7.50%) and *R. bignoniiflora* (84.99 ±7.02%) in a dose of 1500 mg/kg. The % inhibition of pain at a lower dose of 250 mg/kg at 15-minutes by *R. prostrata* (88.74 ±4.60%), *R. lineari-bracteolata* (73.73 ±4.60%) and *R. bignoniiflora* (69.98±4.60%) relative to morphine sulphate (100.00 ±0.00%). There was no significant difference ($p>0.05$) in antinociceptive activity between the *R. prostrata* and *R. lineari-bracteolata* at 250 mg/kg relative to morphine sulphate at 15 minutes.

The % inhibition of nociceptive pain by *R. prostrata* and *R. lineari-bracteolata* extracts in doses of 1500 mg/kg at 45 minutes was like that of morphine sulphate (100.00±0.00%), whereas. *bignoniiflora* showed a lower inhibition (91.30 ±3.86%), which was not significantly different ($p>0.05$). Apart from *R. prostrata* aqueous extract which showed equal inhibition of nociceptive pain at 1500 mg/kg at 15 and 45 minutes, *bignoniiflora* and *R. lineari-bracteolata* extracts showed higher activity at 45 minutes compared to 15 minutes period.

Acetaminophen standard displayed similar biphasic nature in antinociceptive activity, with the highest activity at 15 minutes (93.75 ±6.25%) and 45 minutes (93.89 ±2.49%), respectively. This second standard displayed lower inhibition of nociceptive pain compared with morphine (100.00±0.00%), although the difference was not significant ($p>0.05$). The *Ruellia* species in this study at 1500 mg/kg displayed no difference in activity ($p>0.05$) relative to acetaminophen at 15 and 45 minutes. The *Ruellia* species in the present study showed a higher % inhibition of nociceptive pain at 15 minutes when lower doses of 250 mg/kg were used. The findings of the % inhibition of the nociceptive

activity of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts are presented in **Figure 4.9.**

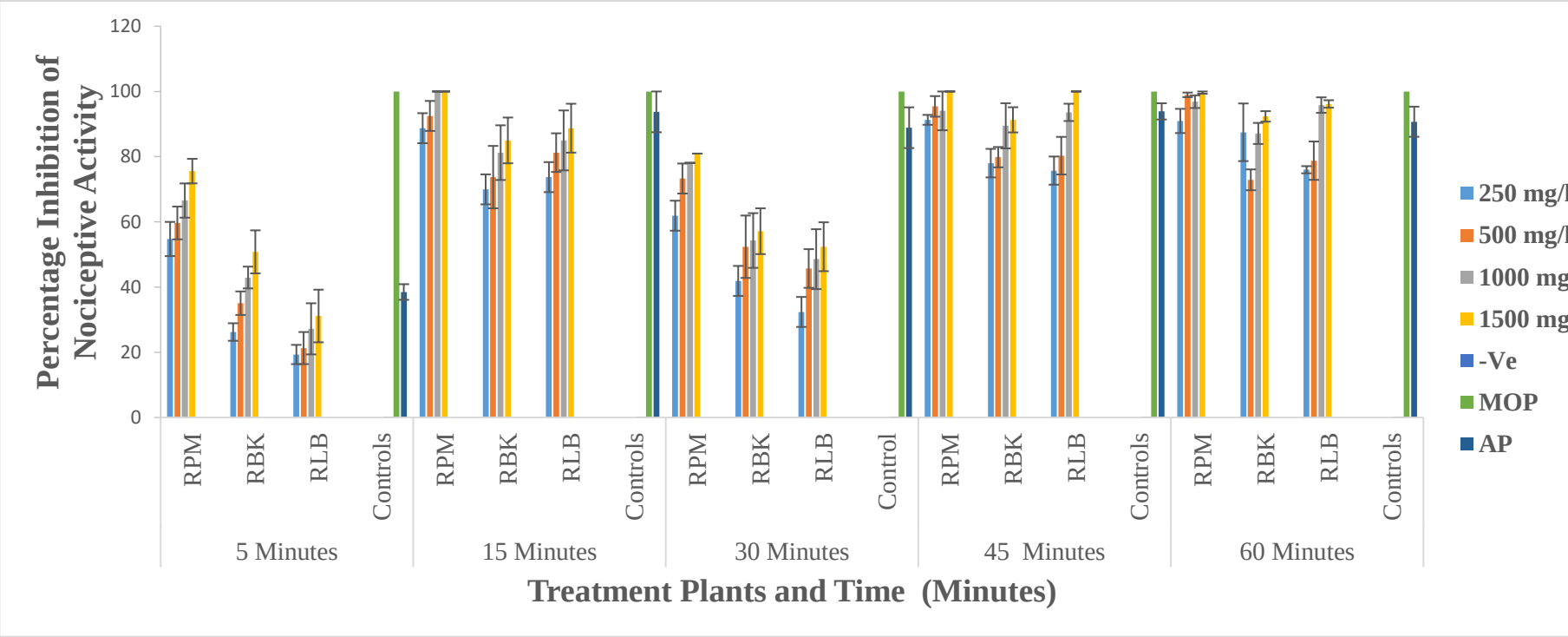


Figure 4.9: Percentage inhibition of the nociceptive activity of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts

Key: MOP= Morphine (10 mg/kg); AP=Acetaminophen (200 mg/kg); RPM=R. *prostrata;* RBK=*R. bignoniiflora*; RLB=*R. lineari-bracteolata*.

There was a dose-dependent inhibition of the nociceptive activity of formalin by *R. prostrata R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts. The inhibition was biphasic, with the highest activity observed at 15 minutes and 45 minutes. Relative to morphine sulphate standard % inhibition of nociceptive pain (100.00 ±0.00%) at 15 minutes, *R. bignoniiflora* methanolic extract in doses of 1500 mg/kg showed similar % inhibition (100.00±0.00%), followed by *R. prostrata* (92.50 ±4.60%) and *R. lineari-bracteolata* (90.62 ±4.20%) The % inhibition of pain at a lower dose of 250 mg/kg at 15-minutes, relative to morphine sulphate was in the order of *R. lineari-bracteolata* (77.49 ±15.0%), followed by *R. prostrata* (77.5 ±15.0%) and *R. bignoniiflora* (73.73 ±7.50%).

The % inhibition of nociceptive pain of *R. prostrata* and *R. bignoniiflora* methanolic extracts in a dose of 1500 mg/kg at 45 minutes was like that of morphine sulphate (10 mg/kg) was 100.00±0.00%, whereas *R. lineari-bracteolata* showed a lower inhibition (86.26±5.32%). Whereas *R. bignoniiflora showed* maximum activity at 15 minutes and 45 minutes, like morphine sulphate, *R. prostrata* methanolic extract showed higher % inhibition of nociceptive pain like morphine at 45 minutes, but lower at 15 minutes in a dose of 1500 mg/kg. Methanolic extract of *R. lineari-bracteolata* showed lower activity at 15 minutes and 45 minutes compared to *R. prostrata* and *R. bignoniiflora.*

Methanolic extracts of *R. bignoniiflora* and *R. lineari-bracteolata* in a lower dose of 250 mg/kg showed lower % significant inhibition ($p \leq 0.05$) of nociceptive pain relative to acetaminophen standard (200 mg/kg). There was no difference in antinociceptive activity between doses at 1000 and 1500mg/kg in the three *Ruellia* species studied. All test groups showed significant differences ($p \leq 0.05$) in activity compared to the negative control.

The highest % inhibition considered as the maximum response was exhibited by morphine (10 mg/kg) reference drug of 100.00±0.00%. The dose that produced 50% of morphine maximum dose, also referred to as the ED_{50} in mg/kg or potency of the aqueous and

methanolic extracts was determined for the extracts of *Ruelli*a species in the present study at 15 minutes and 45 minutes points. The results of 50 % inhibition of pain of the aqueous extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* are aqueous extracts at 15 minutes are shown in **(Figure 4.10)**.

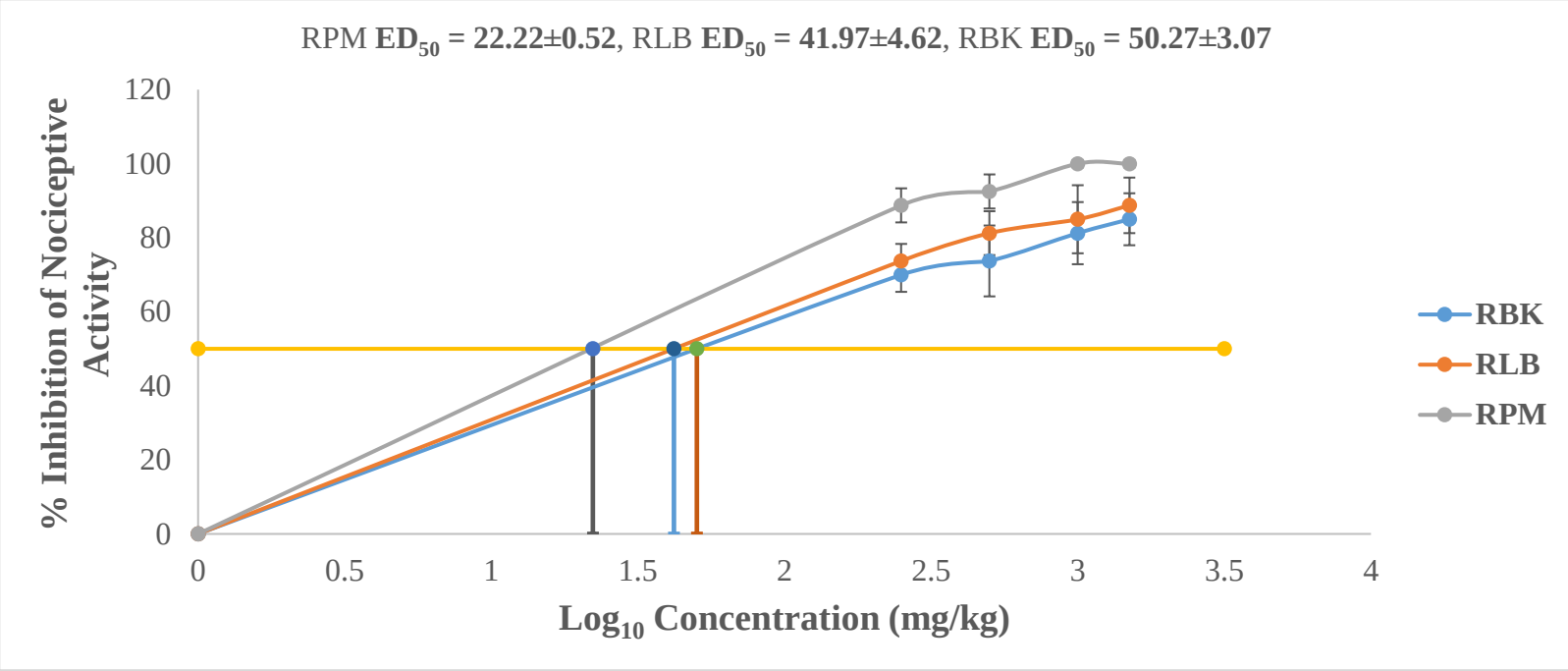


Figure 4.10: 50 % inhibition of pain (ED_{50}, mg/kg) by *R. prostrata, R. bignoniiflora* and *R. linear-bracteolata* aqueous extracts at 15 minutes

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*;**RLB-***R. lineari-bracteolat*

The results of 50 % inhibition of pain of the aqueous extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* are aqueous extracts at 45 minutes are shown in **(Figure 4.11)**.

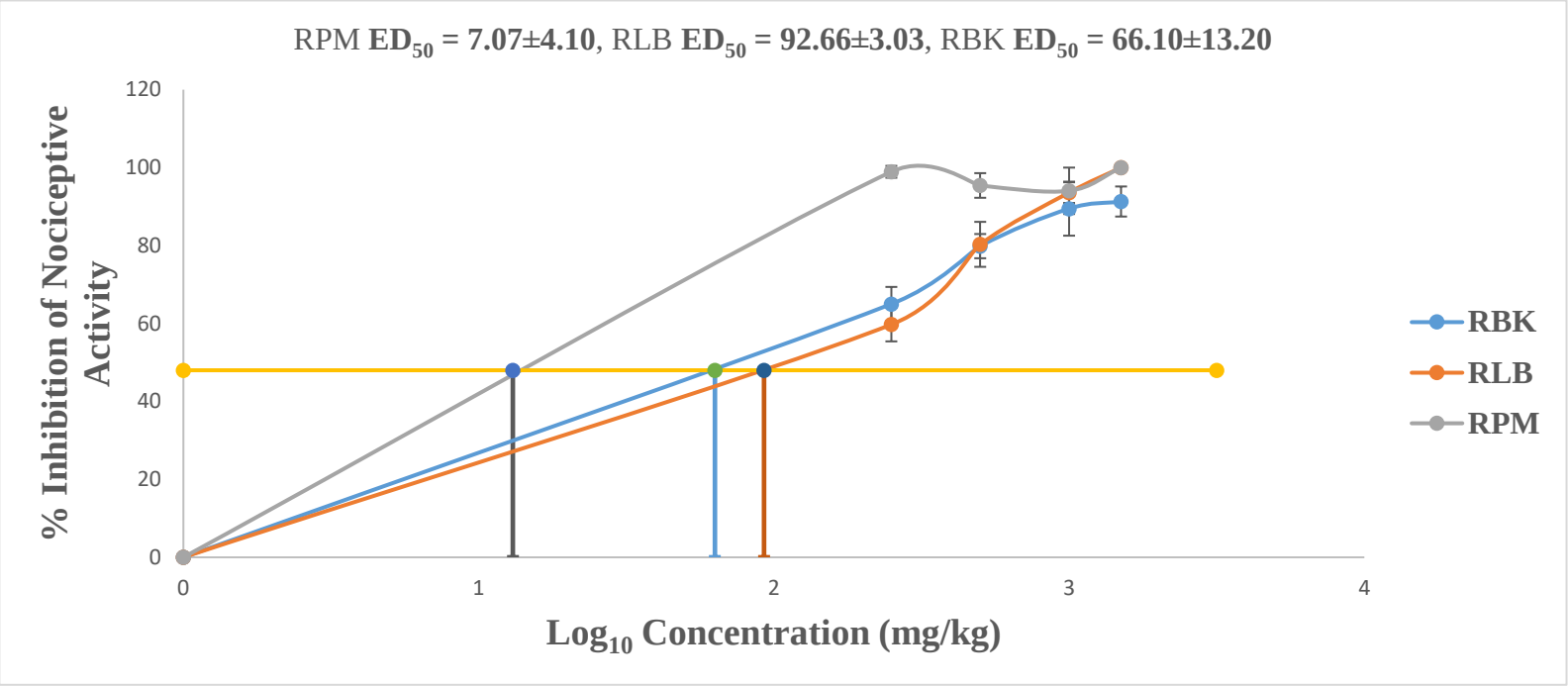


Figure 4.11: 50 % inhibition of pain (ED_{50}, mg/kg) by *R. prostrata, R. bignoniiflora* and *R. linear-bracteolata* aqueous extracts at 45 minutes

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*;**RLB-***R. lineari-bracteolata*

The results of 50 % inhibition of pain of the methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* are methanolic extracts at 15 minutes are shown in **(Figure 4.12.**

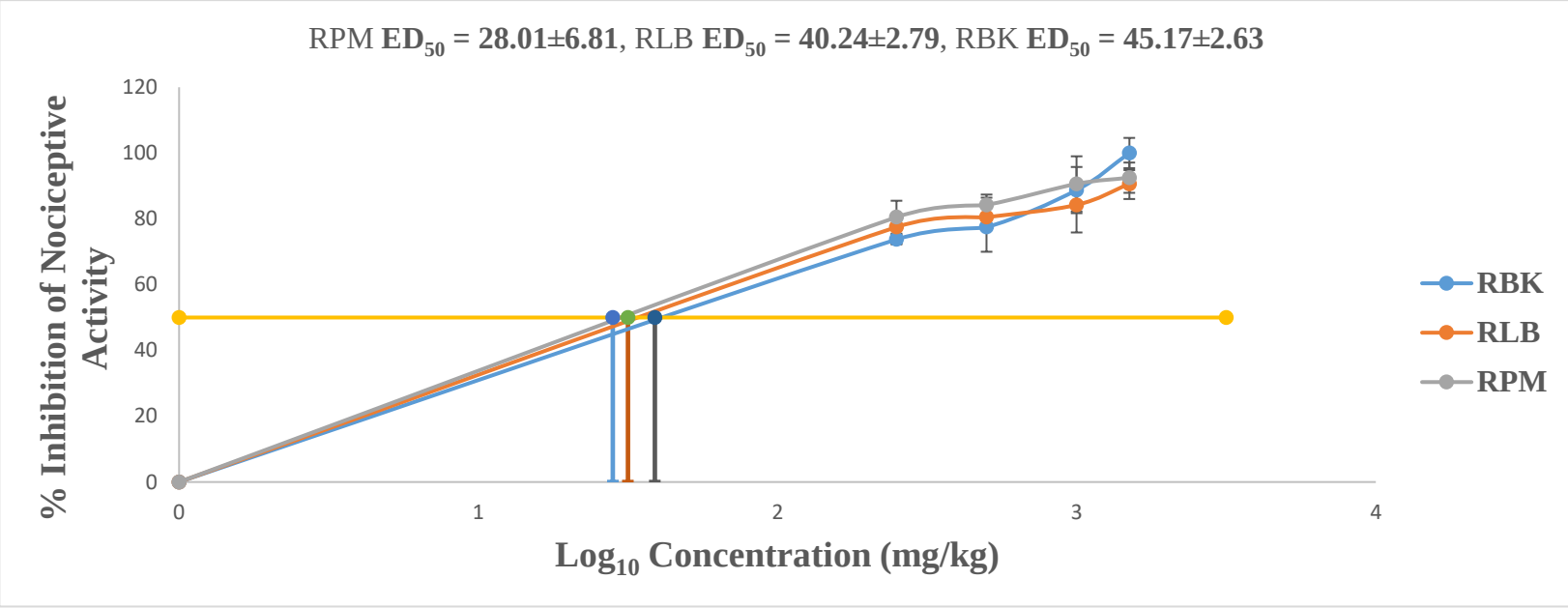


Figure 4.12: 50 % inhibition of pain (ED_{50}, mg/kg) by *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* methanolic extracts at 15 minutes

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*; **RLB-***R. lineari-bracteolata*

The results of 50 % inhibition of pain of the aqueous extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* are methanolic extracts at 45 minutes are shown in **(Figure 4.13)**.

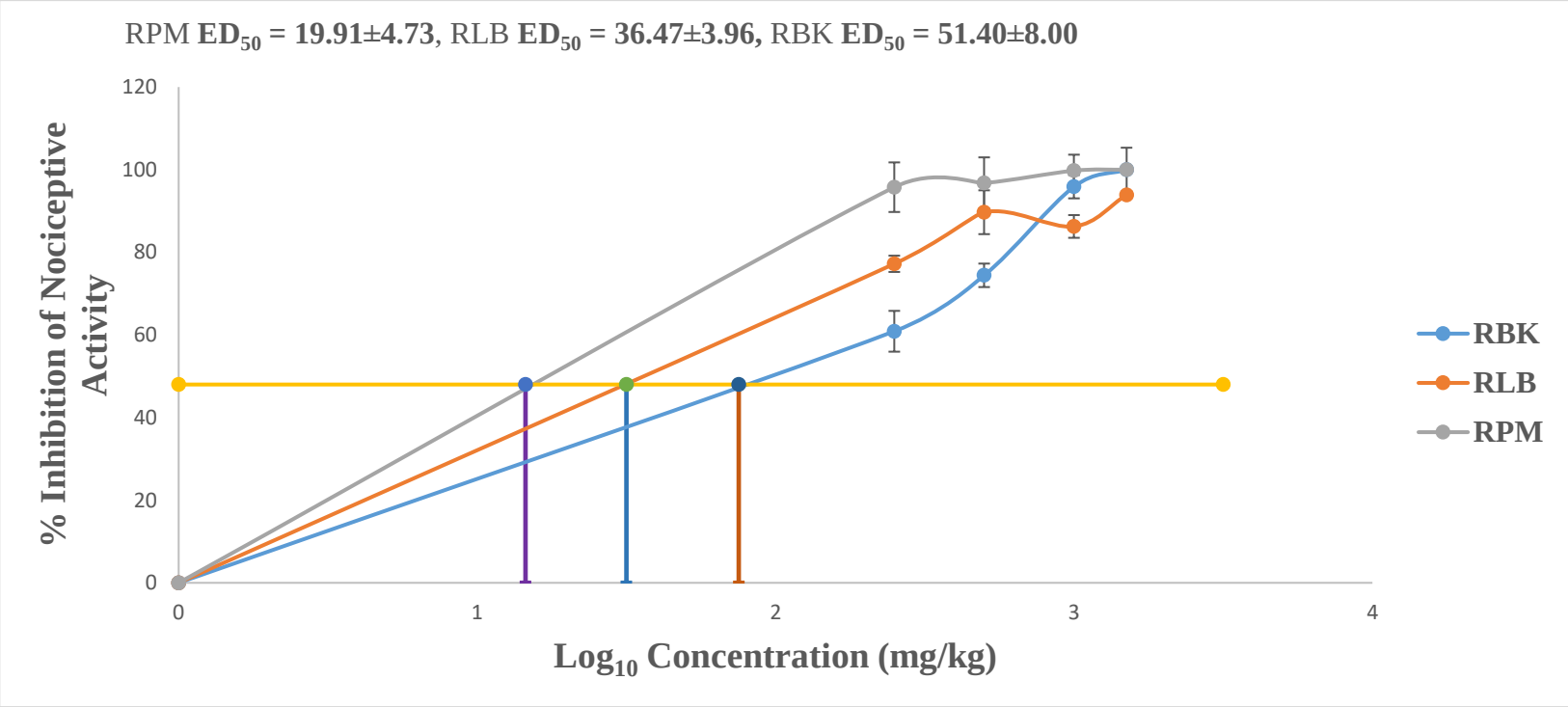


Figure 4.13: 50 % inhibition of pain (ED_{50}, mg/kg) by *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* methanolic extracts at 45 minutes

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*;**RLB-***R. lineari-bracteolata*

Of the three *Ruellia* species studied, *R. prostrata* aqueous extract was the most potent. The activity of the aqueous extract in the 2nd phase (7.07 mg/kg) was higher than that in the 1st phase (22.22 mg/kg). A similar trend, but with lower activity was observed when the methanolic extract of *R. prostrata* was used showing (ED_{50} 19.91mg/kg) at 45 minutes and (28.01 mg/kg) at 15 minutes points, respectively. Aqueous extracts of *R. bignoniiflora* and *R. lineari-bracteolata* showed lower activity which was more potent in the 1st phase than the 2nd phase. The methanolic extracts of *R. bignoniiflora* and *R. lineari-bracteolata* were more potent than their aqueous extracts.

According to Kariuki *et al*. (2012) aqueous extract of *Acacia nilotica*, *Aloe volkensii*, *Cynanchum viminale* and *Urtica dioica* demonstrated a reduction in the formalin-induced paw licking time in both phases. The nociceptive effect in the early phase of formalin test was reported to be due to the direct stimulation of the sensory nerve fibres by formalin, while the nociceptive effect in the late phase was reported to be due to inflammatory mediators, such as histamine, PGs, 5-HT, and bradykinin (Murray *et al.,* 1988, Tjolsen *et al.,* 1992, Dharmasiri *et al*., 2003). The ability of the extracts to attenuate antinociceptive activity in the 2nd phase in formalin test was based on the ability of the extract to inhibit COX enzymes in the peripheral tissues leading to decrease in PGs and LTs synthesis.

Both aqueous and methanol extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* showed a biphasic antinociceptive activity in agreement with the studies of Murray *et al.* (1988); Rosland *et al.* (1990); Tjolsen *et al.* (1992); Dharmasiri *et al*. (2003); Ghannad *et al.* (2005) and Kariuki *et al*. (2012). In their studies, the neurogenic pain could be due to the release of vasoactive amines and Substance P acting on peripheral nerve endings, whereas the inflammatory pain was due to the COX pathway with the release of prostaglandins (such as PGE_2) and leukotrienes (such as LTB_4). The present study findings agree with other reported findings on the biphasic nature of the formalin test. This test is believed to correlate better with clinical pain, hence, a more valid analgesic model (Tjolsen *et al.,* 1992, Ghannad *et al.,* 2005).

The antinociceptive activity of aqueous extract of *R. prostrata* targeted both neurogenic and inflammatory pain, with more activity on the inflammatory pain compared to the neurogenic pain. Compared to morphine sulphate and acetaminophen standards, the aqueous extract of *R. prostrata* exhibited comparable the antinociceptive activity in both 1st and 2nd phases. Morphine sulphate is an opioid analgesic that is useful in severe pain, such as cancer or post-operative pain. It displayed antinociceptive activity of 100.00% in both 1st and 2nd phase in the formalin test. It was observed that all mice that were treated with morphine were hyperactive, pacing around in the cages for the whole test period of 60 minutes. The animals treated with *R. prostrata*, *R. bignoniiflora* and *R. linearibracteolata* (250-1500mg/kg) did not show such hyperactivity. It is most likely that the mechanism of action of the *Ruellia* species in this study may not be via opioid receptors. Acetaminophen (200 mg/kg) showed higher activity in the 1st phase compared to the 2nd phase. Acetaminophen is used for mild and moderate pain, therefore its activity in the fast activity in the 1st phase is relevant to its usefulness as an analgesic for acute pain. Since *R. prostrata* targeted both phases with higher activity in the 2nd phase, this extract could be a good candidate for both acute and progressive pain, such as that observed in chronic conditions like rheumatoid arthritis. A drug that targets both neurogenic and inflammatory pain is an excellent candidate for use in severe painful conditions. Therefore, the Formalin test is an assay that can predict test substances that can be used in conditions with progressive pain such as rheumatoid arthritis.

Several studies have shown the analgesic activity of compounds such as flavonoids and tannins as described by Ahmadiani *et al*. (2000). Flavonoids, on the other hand, are known to be effective in acute inflammation (Rajnarayana *et al*., 2001). Other studies have reported on the analgesic effects of alkaloids, essential oils and saponins (Reanmongkol *et al*., 2005). The analgesic effect of the *Ruellia* extracts in this study may, therefore, be due to the presence of flavonoids, tannins, or saponins (**Table 4.2**). Flavonoids express biological actions which reflect their diverse modes of action in inflammation. Several flavonoids are reported to possess anti-inflammatory activity *in vitro* and *in vivo*. The inhibitory action on mast cells (inflammatory cells), surpasses any other clinically

available compound (Rathee *et al.,* 2009). Flavonoids are reported to modulate the activities of various mediators of inflammation indicating their potential in influencing inflammation. Therefore, there is need to develop anti-inflammatory agents which could provide novel insights into the regulation of the inflammatory process (Rathee *et al.,* 2009). Several cellular action mechanisms are proposed to explain the anti-inflammatory activity of the flavonoids. In addition to antioxidative activity, flavonoids inhibit eicosanoid generating enzymes. Depending on their chemical structures, flavonoids have different action mechanisms, and a single mechanism could not explain all of their *in vivo* activities (Rathee *et al.,* 2009). Another study by (Kim *et al.,* 2004) reported that although flavonoids have multiple cellular mechanisms acting on multiple sites of cellular machinery, the most important contributors to anti-inflammation by flavonoids seem to be the effect on eicosanoid generating enzymes and the effect on the expression of proinflammatory molecules.

4.4.5 *In Vivo* Anti-inflammatory Activity

Anti-inflammatory activity of aqueous and methanolic extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* was established using inhibition of right hind paw swelling in Wistar albino rats after intradermal carrageenan injection into the rat right hind paw.

The findings for % inhibition of swelling in the rat hind paw by the aqueous extracts *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* are presented in **Figure 4.14.**

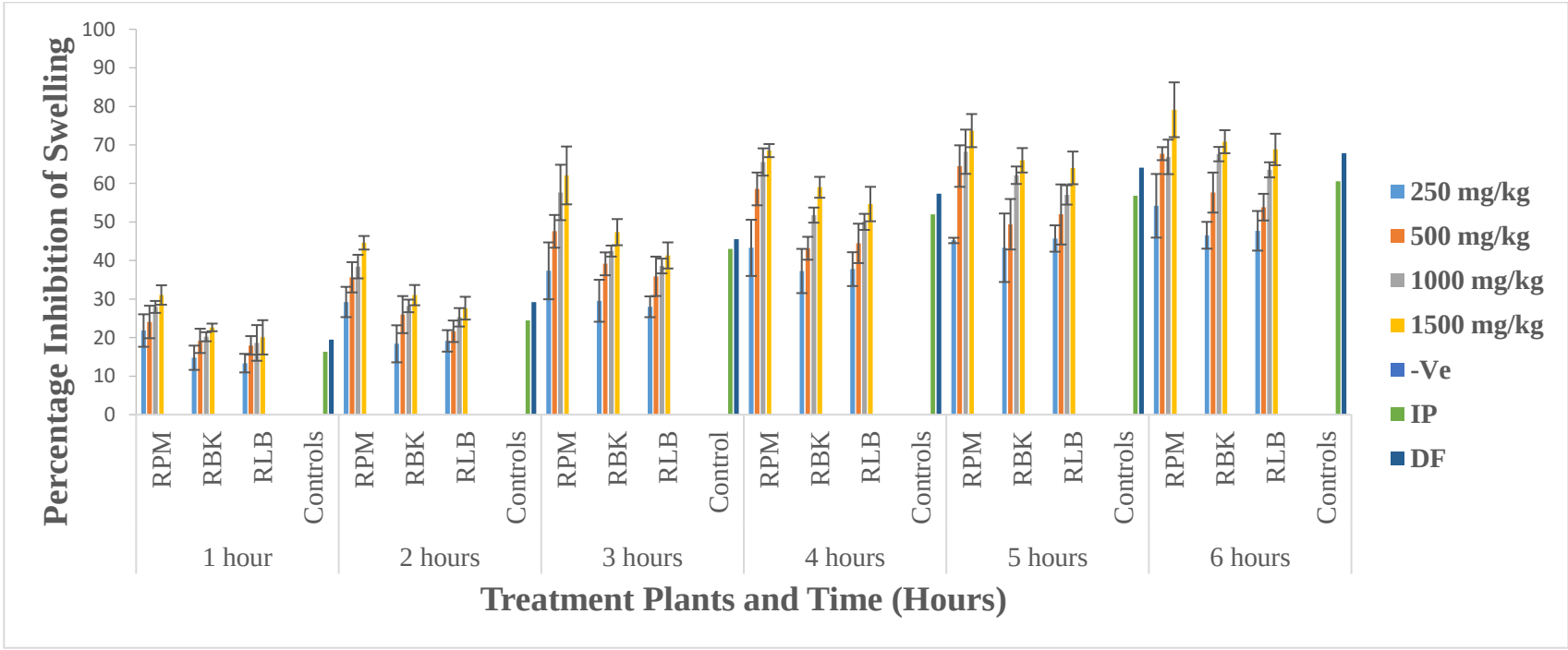


Figure 4.14: Percentage inhibition of swelling in the rat hind paw by the aqueous extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata*

Key: IP-Ibuprofen (200 mg/kg); DF-Diclofenac sodium (20 mg/kg) standard; RPM-*Ruellia prostrata*; RBK-*R. bignoniiflora*; RLB-*R. lineari-bracteolata*.

The % inhibition in rat hind paw swelling by *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* aqueous extracts was dose-dependent, with the highest activity at 6-hour point in doses of 1500 mg/kg. Relative to diclofenac sodium standard whose % inhibition of swelling was 67.87 ±3.27%), the highest % inhibition of swelling was exhibited by aqueous extract of *R. prostrata* (79.12 ±7.11%), followed by *R. bignoniiflora* (70.83 ±2.99%) and *R. lineari-bracteolata* (68.82 ±4.08%). The % inhibition of the *Ruellia* extracts was higher than that of diclofenac sodium (20 mg/kg).

Diclofenac sodium (25 mg/kg) displayed a higher % inhibition in swelling (67.87 ±3.27%) compared to ibuprofen (200 mg/kg) standard (60.53 ±1.84%) at 6-hour point. In lower doses of 250 mg/kg, the aqueous extract of *R. prostrata* showed highest % in swelling (54.20 ±8.26%), followed by *R. lineari-bracteolata (*47.71 ±5.14%) and *R. bignoniiflora* (46.55 ±3.48%), relative to diclofenac sodium exhibited (19.46 ±1.21%) and ibuprofen (16.31 ±1.36%) at 1-hour point. Throughout the study, the aqueous extract of *R. prostrata* displayed a higher % inhibition of swelling in the rat hind paw relative to diclofenac sodium in all doses tested. At the 6-hour point, all aqueous extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* in a dose of 1500 mg/kg displayed a higher % inhibition of swelling in the rat hind paw relative to diclofenac sodium.

There was no significant difference in % inhibition of swelling in the rat hind paw ($p>0.05$) in *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* in doses of 1000 and 1500 mg/kg. All test groups showed higher significant differences ($p\leq0.05$) in activity compared to the negative control.

4.4.5.2 Anti-inflammatory Activity of Methanolic Extracts of Kenyan *Ruellia* species

The findings for the % inhibition of swelling in the rat hind paw by *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts are shown in **Figure 4.15.**

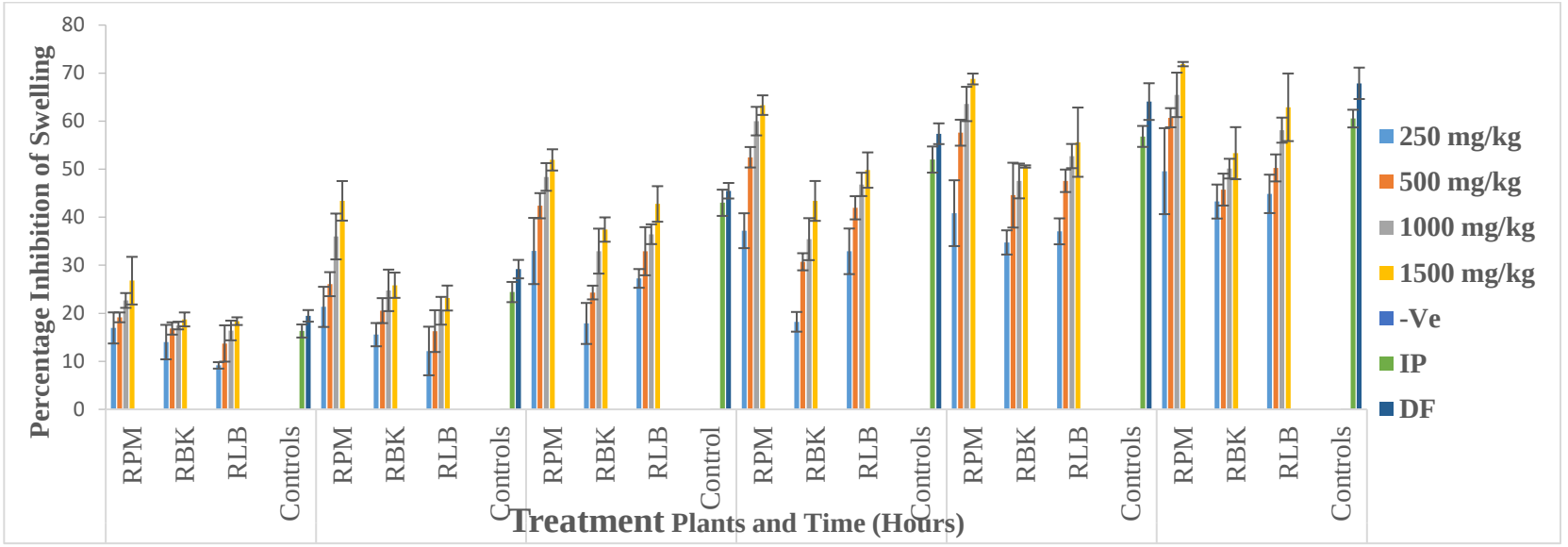


Figure 4.15: Percentage inhibition of swelling in the rat hind paw by the methanolic extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata*

Key: IP-Ibuprofen 200 mg/kg; DF-Diclofenac sodium (20 mg/kg) standard; RPM-*Ruellia prostrata*; RBK-*R. bignoniiflora*; RLB-*R. lineari-bracteolata*.

The % inhibition of swelling in the rat hind paw by *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* methanolic extracts was dose-dependent, with highest activity at 6-hour point in doses of 1500 mg/kg. The methanolic extracts of the three *Ruellia* species in were lower than their aqueous extracts. The % inhibition of swelling exhibited by *R. prostrata* was 71.87 ±0.45%), followed by *R. lineari-bracteolata* (62.88 ±7.04%) and *R. bignoniiflora* (53.33 ±5.42%) in doses of 1500mg/kg were higher than that of diclofenac sodium (20 mg/kg) standard (67.87 ±3.27%) and ibuprofen (200 mg/kg) standards (60.53 ±1.84%). The methanolic extract of *R. prostrata* displayed a higher % inhibition of swelling than diclofenac sodium (20 mg/kg) and ibuprofen (200mg/kg) standard (60.53 ±1.84%) in doses of 1000 mg/kg (65.47 ±4.63%) and 1500 mg/kg (71.87 ±0.45%).

In lower doses of 250mg/kg, methanolic extracts of *R. prostrata* showed % inhibition of swelling (49.59 ±8.95%), followed by *R. lineari-bracteolata (*44.86 ±4.00%) and *R. bignoniiflora* (43.26 ±3.54%) relative to diclofenac sodium (67.87 ±3.27%) %) and ibuprofen (60.53 ±1.84%) %). Of the three *Ruellia* species, *R. prostrata*, methanolic extract in doses of 1500 mg/kg (71.87 ±0.45%) was higher than diclofenac sodium (67.87 ±3.27%) and ibuprofen standards (60.53 ±1.84%) at the 6-hour point.

The highest % inhibition of swelling considered as maximum activity exhibited by diclofenac sodium standard was (67.87%). The dose that produced 50% of diclofenac sodium maximum dose, also referred to as the ED_{50} in mg/kg (or potency of the extracts) was determined for the three *Ruellia* species. The findings of ED_{50} in mg/kg values of the aqueous extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* at 6-hour are presented in **Figure 4.16.**

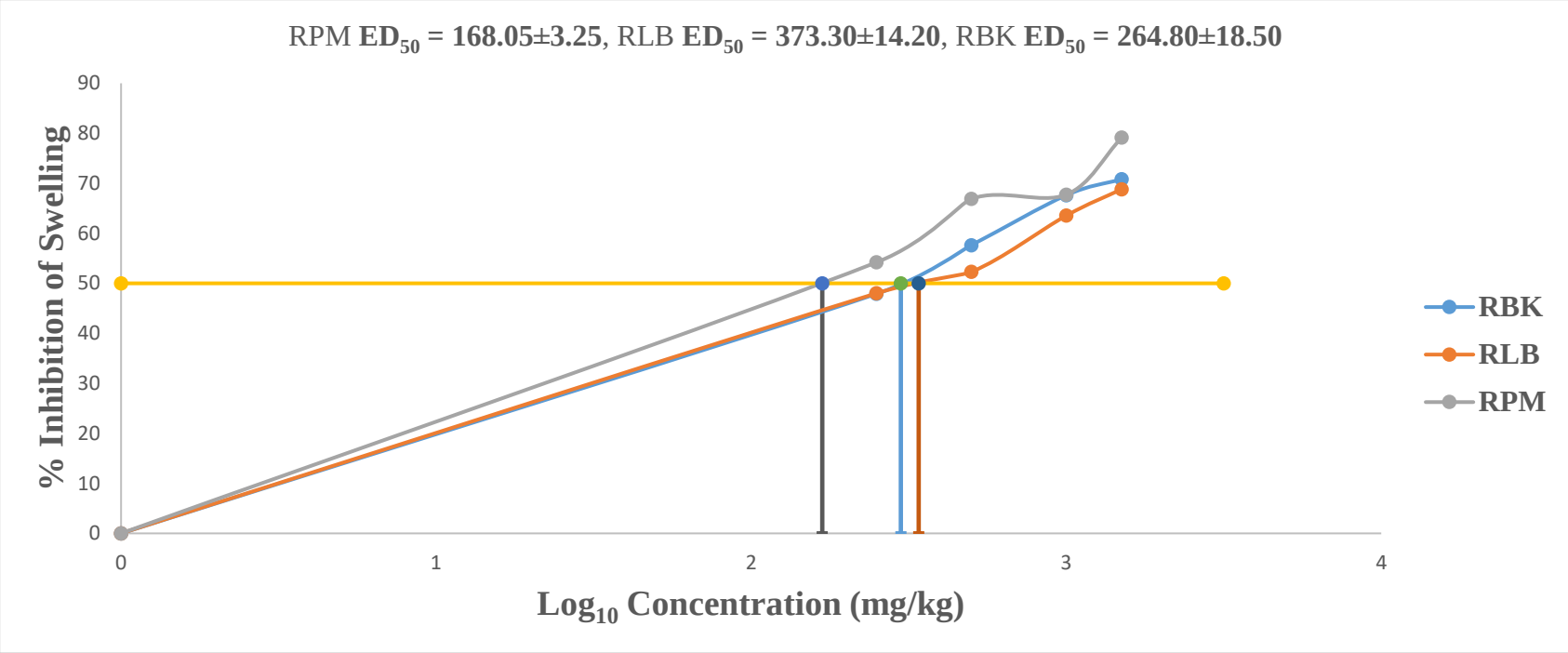


Figure 4.16: 50 % inhibition of swelling in the rat hind paw (ED_{50} in mg/kg) of the aqueous extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* at 6-hour

Key: RPM- *R. prostrata;* **RBK-***R. bignoniiflora*; **RLB-***R. lineari-bracteolata*

The findings of ED_{50} in mg/kg values of the methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. lineari-bracteolata* at 6-hour are presented in **Figure 4.17.**

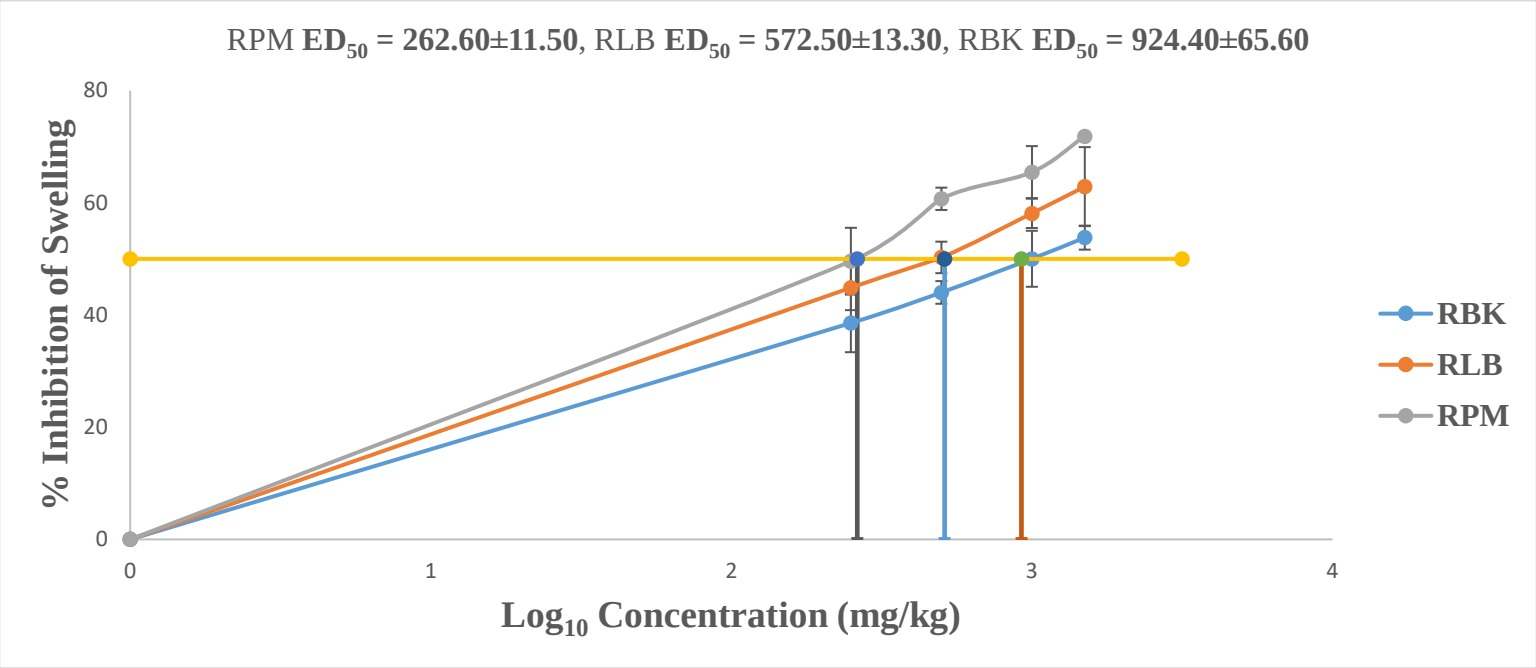


Figure 4.17: 50 % inhibition of swelling in the rat hind paw (ED_{50} in mg/kg) of the methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. linear-bracteolata* at 6-hour

Key: RPM- *R. prostrata;* **RBK**-*R. bignoniiflora*; **RLB**-*R. lineari-bracteolata*

The aqueous extracts of the *Ruellia* species in the present study were more potent than their methanolic extracts. *R. prostrata* aqueous extract was the most potent (ED_{50} 168.05 mg/kg) followed by aqueous extracts of *R. bignoniiflora* (ED_{50}, 264.80 mg/kg) and *R. linear-bracteolata* (ED_{50}, 373.30 mg/kg). However, the methanolic extract of *R. lineari-bracteolata* (ED_{50} 572.50 mg/kg) was more potent than that of *R. bignoniiflora* (ED_{50}, 924.40 mg/kg). Both aqueous and methanolic extracts of *R. prostrata* (ED_{50} 262.60 mg/kg) were more potent than those of *R. bignoniiflora* and *R. lineari-bracteolata*.

Anti-inflammatory effects were determined by inhibiting oedema caused by carrageenan injection into the therat hind paw. Carrageenan is the phlogistic agent that has been used for examining anti-inflammatory drugs as it is reported to be antigenic and is devoid of afferent systemic effect. The model exhibits a high degree of reproducibility and is used in determining the anti-inflammatory activities of medicinal agents and is well documented for various non-steroidal anti-inflammatory drugs (Winter *at al.,* 1962; Sini *et al.,* 2010). It was also reported that the carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation (Sawadogo *et al.,* 2006). The ratios of the potencies of the drugs phenybutazone, aspirin and hydrocortisone were quite close to their respective doses in the treatment rheumatic diseases using the carrageenan-induced oedema protocol (Winter *et al.,* 1962). Another non-steroidal anti-inflammatory drug, indomethacin, was discovered using the carrageenan assay (Winter *et al.,* 1963).

Mediators such as prostaglandins and leukotrienes are released through the cyclooxygenase pathway (Katzung, 2017). Carrageenan-induced oedema is considered a multi-mediated process that liberates a diversity of mediators. It is believed to be biphasic; with the 1st phase (1 hr) involving the release of 5-HT and histamine, while the second phase (>1 hr) is mediated by prostaglandins, the cyclooxygenase products. The transition between the two phases is provided by kinins (Perianayagam *et al.,* 2006, Asongalem *et al.,* 2004, Silva *et al.,* 2005; Brunton *et al.,* 2018). A correlation between the development of oedema induced by carrageenan and early exudative stage of inflammation has also

been reported (Asongalem *et al.*, 2004, Silva *et al.,* 2005). It can, therefore, be argued that suppression of 1st phase may be due to inhibition of release of early mediators, whereas 2nd phase may be explained by inhibition of the cyclooxygenase pathway.

A study by Antonio and Brito (1998) reported that the early phase (1-2 hrs) in carrageenan-induced oedema is mainly mediated by histamine, 5-HT, and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is reported to be sustained by prostaglandins released and mediated by leukotrienes, polymorphonuclear cells, bradykinin and prostaglandins produced by macrophages tissue. It has also been reported that 2nd phase of oedema in carrageenan test is sensitive to most clinically effective anti-inflammatory drugs, which has been frequently used to assess the anti-oedematous effect (Saha *et al.,* 2007) of natural products (Della *et al.*, 1968; Brunton *et al.,* 2018). The reference standards, diclofenac sodium, a phenylacetic acid derivative and ibuprofen (propionic acid derivative) were used in the present study as non -steroidal anti-inflammatory drugs (Katzung, 2017).

The present study showed that the aqueous extract of whole plant parts of *Ruellia* species possessed a significant anti-oedematogenic effect on right hind paw oedema induced by carrageenan, inhibiting the 2nd phase of the inflammatory response. The inhibitory effect of the extracts *Ruellia* species in the present study on carrageenan-induced inflammation over a period of 4 hours is like the effect of most non-steroidal anti-inflammatory drugs. This suggests that the extracts acted in both early and later phase probably by inhibiting the synthesis of arachidonic acid metabolites which produce oedema (Just *et al.,* 1998). The *Ruellia* species and standards in the present study displayed the highest anti-inflammatory activity at 6-hour point, concurring with the report that activity after 3 hours, could be due to inhibition of inflammatory mediators. Although the original study by Winter *et al.* (1962) and later by Ottereness and Moore (1988) reported that subcutaneous injection of a solution of carrageenan in saline in rats induced an acute swelling that gets to a maximum from 3-5 hours, our study showed maximum swelling at the 6-hour point. It was also reported that the carrageenan-induced inflammation model is a significant

predictive test for anti-inflammatory agents acting by the mediators of acute inflammation (Sawadogo *et al.,* 2006).

From the ED_{50} in mg/kg vaues (**Tables 4.9, 4.12 and 4.15**), aqueous extract of *R. prostrata* consistently showed higher analgesic, antinociceptive and anti-inflammatory activities higher than the methanolic axtracts. In analgesic activity and antinocicptive studies, *R. bignoniiflora* and *R. lineari-bracteolata* showed higher activith in their methanolic extracts than their aqueous extracts. The aqueous extracts of all the three species showed higher anti-inflammatory activity than their methanolic axtracts. From the quantitative determination of the phytocompounds (**Table 4.3**), it was noted that the antioxidant activity **(Table 4.6)** of the three *Ruellia* species correlated with their content of flavonoids, whereas highest activity of *R. prostrata* correlated with the highest content of saponins. Flavonoids are polyphenolic secondary metabolites with reported antioxidant activity (**REF**), whereas saponins are glycosides, which might need *in vivo* biotranspormation to active aglycones (**REF**). The difference between *in vitro* antioxidant studies and *in vivo* analgesic, antinociceptive and anti-inflammatory studies observed between *R. prostrata* and *R. lineari-bracteolata* could be due to this difference. The *in vitro* antioxidant studies did not need biotransormation of flavonoids into their active forms. It was also noted that in the anti-inflammatory studies , it took a period of six hours to attain highest activity, a finding that could be in line with *in vivo* biotrnsformation of saponins to their active aglycones.

The three *Ruellia* species in the present study were superior to the standards, diclofenac sodium and ibuprofen. Aqueous extract of *R. prostrata* showed the highest analgesic and anti-inflammatory activities compared to *R. bignoniiflora* and *R. lineari-bracteolata*. A nother study (Wangia and Gowi, 2018) revealed the gastroprotective activity of the aqueous extract of *R. prostrata* in ibuprofen-induced ulcer in rats. In this study, *R. prostrata* aqueous extract (250-1500 mg/kg) showed a dose-dependent protection in the development of ibuprofen-induced ulcers, with the highest activity at 1500 mg/kg. The gastroprotection was like that of ranitidine (Zantac 20 mg/kg i.p). There are reports on the gastroprotective activity of *R. tuberosa* (Roopa *et al.,* 2011). A study by Murugaiyan *et*

al. (2015) reported on anti-ulcer properties of *D. prostratus* (syn. *Ruellia prostrata*). The gastroprotective activity in addition to the analgesic, antinociceptive and anti-inflammatory activities of *R. prostrata,* give this species an advantage over conventional non-steroidal anti-inflammatory agents, which have adverse effects on the gastrointestinal tract.

The anti-inflammatory activity of extracts of *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* species in the present study, may be attributed to the presence of flavonoids, polyphenols and saponins (**Table 4.2**). The results of the present study are an indication that *R. prostrata, R. bignoniiflora* and *R. lineari-bracteolata* species could be effective in acute inflammatory disorders.

Since *R. prostrata* had the largest number of compounds separated on TLC (**Table 4.3**), and highest quantity of saponins (**Table 4.4)** compared to *R. lineari-bracteolata* and *R. bignoniiflora,* the highest anti-inflammatory activity exhibited by this species could be due to the presence several phytocompounds, especially, saponins present in this species. There are two main types of saponins thus steroidal and triterpenoid. Phytochemical studies revealed that the saponins present in *Ruellia* species in this study were likely to be the triterpenoid type (Liebermann-Burchard test). Experiments demonstrating the pharmacological, physiological, immunological properties of saponins have provoked considerable clinical interest in these substances (Francis *et al.,* 2002). Several biological effects have been ascribed to saponins, such as membrane-permeabilising, immunostimulant, hypocholesterolaemic and anticarcinogenic, the effect on growth, feeding intake and reproduction in animals. These structurally diverse compounds have also been observed to kill protozoans and molluscs, are antioxidants, cause hypoglycaemia, and to act as antifungal and antiviral agents (Francis *et al.,* 2002). Glycyrrhizin, a triterpenoid saponin from *Glycyrrhiza glabra* (liquorice), has been reported to show anti-inflammatory activity in experimental models of inflammation and may be beneficial in rheumatoid arthritis and other inflammatory conditions (Evans, 2009). The presence of triterpenoid saponins in *Ruellia* species in the present study could be responsible for the anti-inflammatory activity of these species. *R. prostrata* displayed

the highest % yield of saponins, which correlated with the order of anti-inflammatory activity. Saponins present in *R. prostrata* were likely largely responsible for the high anti-inflammatory activity displayed by this species, whereas flavonoids and phenolics could have been responsible for their antioxidant activities.

A study by Ullah *et al.* (2014) on the antinociceptive, *in-vivo* and *in-vitro* anti-inflammatory activity of ethanolic extract of the rhizome of *Curcuma zedoaria* revealed the presence of tannins, saponins, flavonoids, steroids, and terpenoids in the extract among others. The results obtained from the tests indicate that the plant might have one or more secondary metabolite(s) having central and peripheral analgesic and anti-inflammatory activity. The present study revealed the presence of tannins, saponins, flavonoids, terpenoids which could have contributed to the analgesic, antinociceptive, anti-inflammatory activities.

The aqueous and methanolic extracts of *R. prostrata*, *R. bignoniiflora* and *R. linearibracteolata* were tested for different biological activities including antioxidant, analgesic, antinociceptive, and anti-inflammatory activities using *in vitro* and *in vivo* models. Since the aqueous extract of *R. prostrata* was the most potent in the *in vivo* studies, it was the only extract investigated in the *in vivo* toxicity studies.

4.4.6. *In Vivo* Anti-Arthritic Activity

In vivo anti-arthritic activity was established using Complete Freund's Adjuvant-induced arthritis model. Since the aqueous extract of *R. prostrata* was more potent than *R. bignoniiflora* and *R. linearibracteolata* in the *in vivo* analgesic and anti-inflammatory studies, this species was the only one studied in the *in vivo* anti-arthritic studies. The previous *in vivo* findings (analgesic and anti-inflammatory studies) revealed no significant difference between the doses at 1000 and 1500 mg/kg. The lower dose of 1000 mg/kg aqueous extract of *R. prostrata* was found safe in sub-chronic toxicity studies. Therefore, *in vivo* anti-arthritic studies adopted this oral dose. The findings on the clinical manifestations of arthritis parameters such as hind paw swelling (digital water

plethysmometer in mL), hind paw joint thickness (Vernier callipers in cm), grip strength (on a rotarod in seconds), body weight changes, arthritis score, effects on haematological parameters and non-articular manifestations are represented in subsequent sections.

4.7.1. Clinical Manifestation of Hind Paws

The clinical appearance of the hind paws taken on Day 28 after immunization with Complete Freund's Adjuvant is shown in **Plate 4.2.**

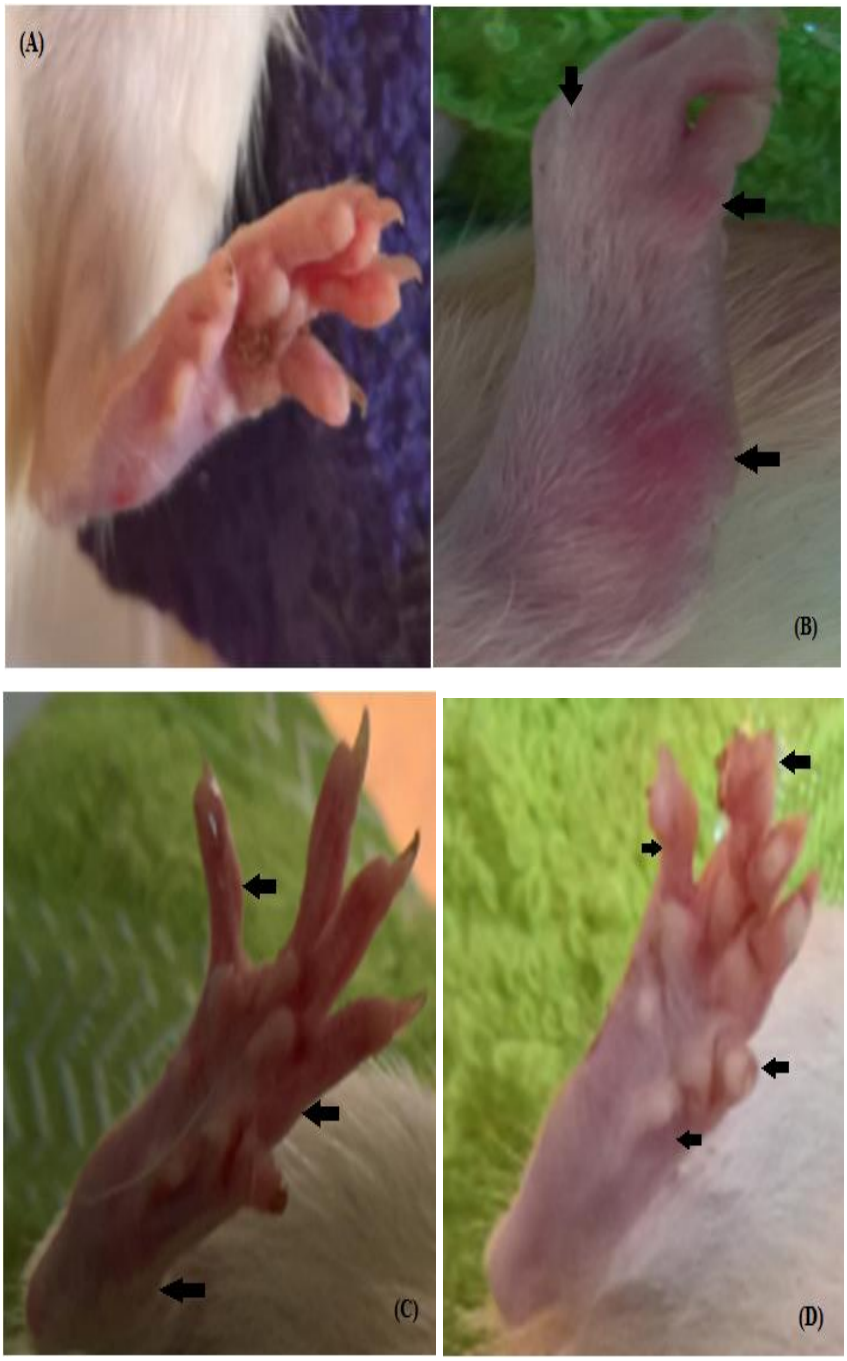


Plate 4.5: Clinical appearance of the hind paws taken on Day 28 after immunization with CFA- Arrows -point to observed swelling

Key: A-Untreated control; B-Untreated arthritis-induced control; C-*R. prostrata* aqueous extract- treated; D-Methotrexate-treated positive control.

Untreated arthritis-induced rats displayed severe soft tissue swelling, erythema and ankylosis in the paws in comparison with the untreated control, methotrexate- and *R. prostrata*-treated rats. There was a reduction in swelling and erythema in *R. prostrata*- and methotrexate-treated rats. Non-ankylosis was observed in rats treated with the aqueous extract of *R. prostrata* and methotrexate.

4.4.6.1. Effect of the Aqueous Extract of *R. prostrata* on Hind Paw Swelling

The findings on the effect of the aqueous extract of *R. prostrata* on % of swelling in the left hind paw are also displayed in **Figure 4.18.**

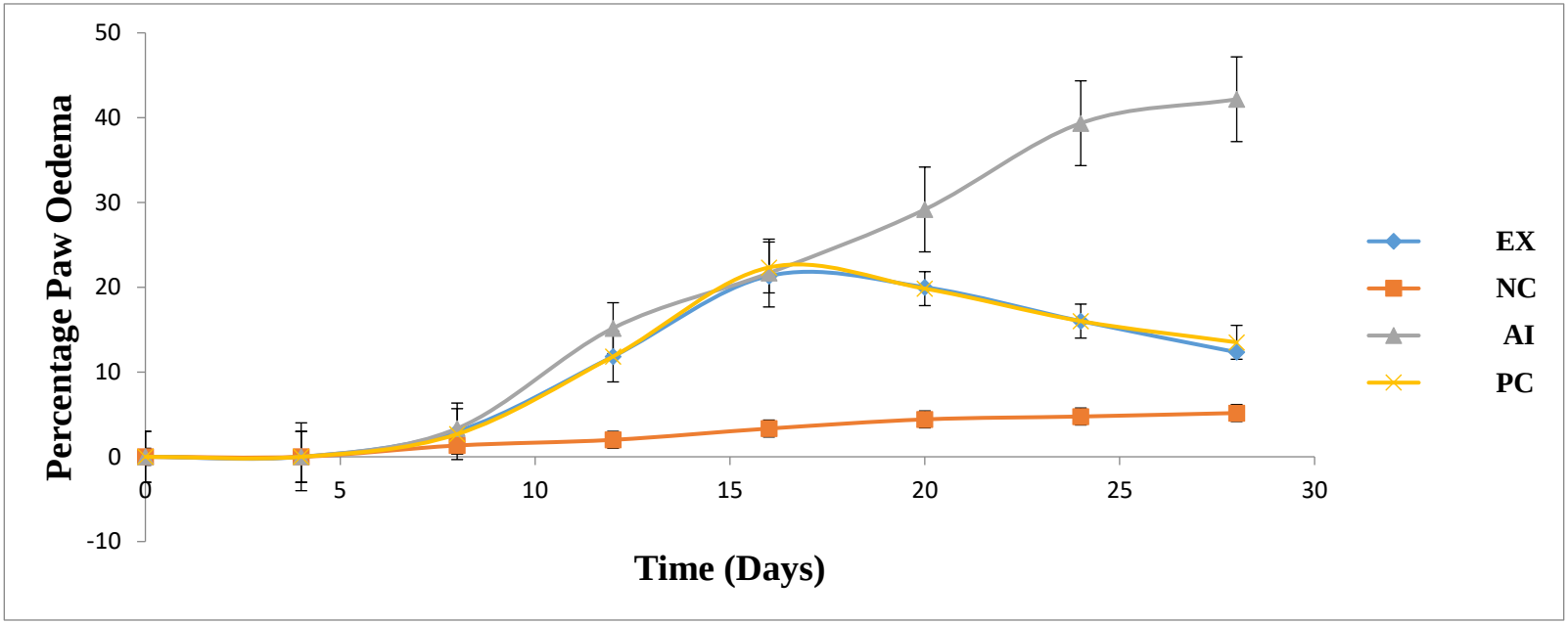


Figure 4.18: Percentage of swelling in left hind paw (n=6) in (mL) in arthritis studies

Key: Ex=*R. prostrata* Extract at 1000 mg/kg; NC=Untreated control; AI= untreated arthritis-induced control; PC=Positive control (methotrexate, 2 mg/kg).

From Day 1 to Day 8 after injection of adjuvant, there was no significant difference ($p>0.05$) in the % of swelling in the left hind paws in all test groups relative to the volume on Day 1. There was no difference in swelling ($p>0.05$) in the untreated control from day 1 to day 28. Arthritis symptoms began from Day 12 to Day 28 when untreated arthritis-induced control showed significant ($p\leq0.05$) increase in % swelling of left hind paw relative to Day 1. The maximum swelling in the left hind paw of untreated arthritis-induced control was on Day 28 when the swelling was the highest (46.2 ±4.11%) relative to Day 1. The *R. prostrata*- and methotrexate standard-treated rats exhibited the highest % of swelling on Day 16 (25.30 ±3.52%) and (25.67 ±3.62%), respectively, which was similar, relative to Day 1, relative to Day 1. The % of swelling in *R. prostrata*-treated rats and methotrexate-treated rats continued to reduce from Day 16 to day 28, the last day of the study (14.46 ±3.57%) and (15.52 ±2.86%), respectively. The *R. prostrata*-treated rats showed a lower % of swelling of left hind paw relative to methotrexate standard on the last day of the study. *R. prostrata* aqueous extract and methotrexate standard protected the rats' left hind paws from developing maximum swelling observed in the untreated-arthritis-induced rats. The untreated arthritis-induced rats continued to show swelling till Day 28, the last day of the study. The findings on the effect of the aqueous extract of *R. prostrata* on % swelling in the right hind paws are also displayed in **Figure 4.19.**

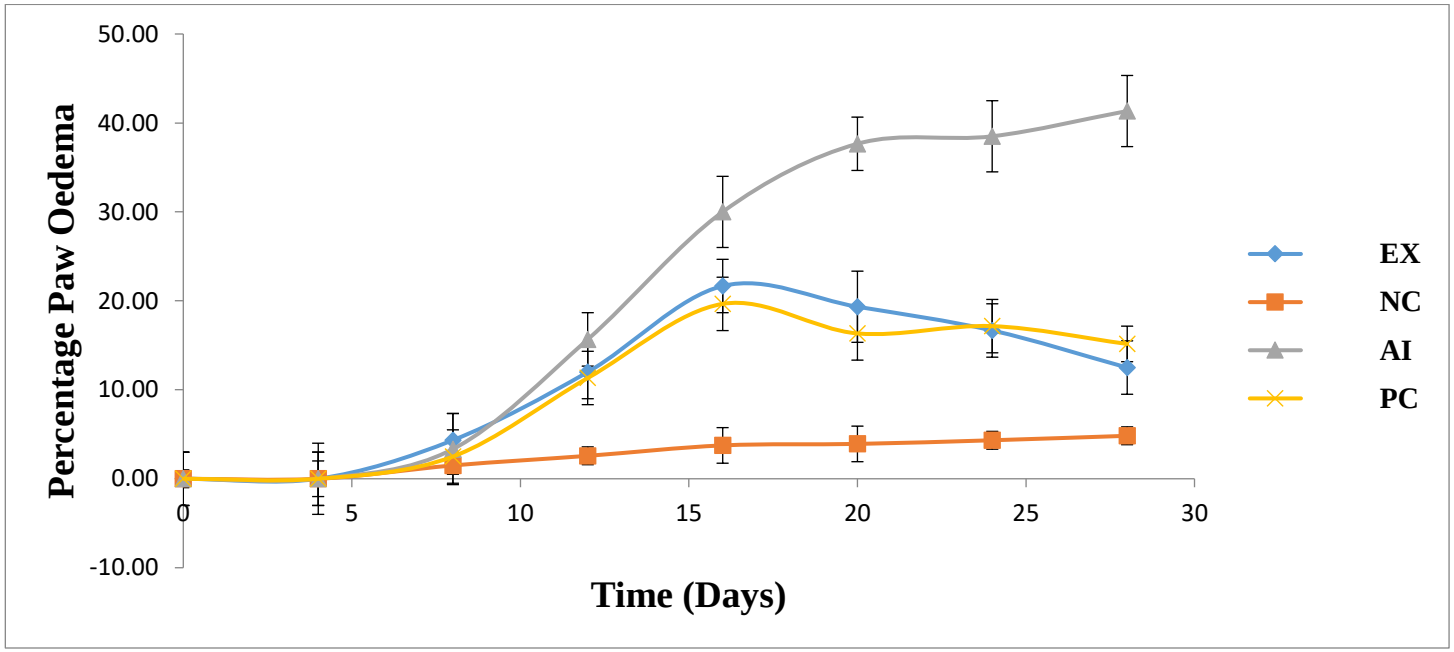


Figure 4.19: Percentage inhibition of swelling (mL) in the right hind paw (n=6)

Key: Ex=*R. prostrata* Extract (1000 mg/kg); NC=Untreated control; AI=Untreated arthritis-induced control; PC=Positive control (methotrexate, 2 mg/kg).

Like the % of swelling in the left hind paw oedema, the right hind paw showed no significant difference ($p>0.05$) in % of swelling in all test groups relative to the volume on Day 1 up to Day 8 of adjuvant injection. Arthritis symptoms began from Day 12 to Day 28 when untreated arthritis-induced control showed significant ($p\leq0.05$) increase in % swelling of right hind paws relative to Day 1. The maximum swelling in the right hind paw of untreated arthritis-induced control was on Day 28 when the % of swelling was highest at 44.75 ±4.92% relative to Day 1. The *R. prostrata*-treated rats exhibited the highest % swelling on Day 16 at 26.22 ±3.30% relative to Day 1, which was higher than that of methotrexate standard-treated rats at 22.22 ±3.70%). The % of swelling in *R. prostrata*- and methotrexate-treated rats continued to reduce from Day 16 to day 28, the last day of the study (15.04 ±3.40%) and (17.05 ±2.58%), respectively, relative to Day 1 of the adjuvant injection. Like in the left hind paw swelling, there was better protection from swelling in the right hind paw on Day 28 by *R. prostrata* relative methotrexate. Overall, *R. prostrata* extract and methotrexate standard protected the rats' right hind paws from developing maximum swelling compared to the untreated-arthritis-induced rats. The untreated arthritis-induced rats continued to show swelling till Day 28, the last day of the study.

The present study was able to determine the reduction in swelling in both left and right hind paws in the CFA-induced arthritis in Wistar rats by the aqueous extract of *R. prostrata* (1000 mg/kg) and methotrexate standard (2 mg/kg). Oral methotrexate (2 mg/kg weekly) was used as a disease-modifying anti-rheumatic drug standard. Use of methotrexate (low dose) has been one of the most successfully used anti-rheumatic agents (Seitz *et al.,* 2003). Compared to *R. prostrata*- and methotrexate-treated groups, the untreated arthritis-induced control showed the highest swelling in the left and right hind paws. These findings determined the significant difference in % inhition of swelling between rats treated with the aqueous extract of *R. prostrata*, methotrexate and the untreated arthritis -induced rats.

4.4.6.2. Effect of the Aqueous Extract of *R. prostrata* on Hind Joint Circumference

The findings on the effect of the aqueous extract of *R. prostrata* on % of thickness in the left hind joint are also displayed in **Figure 4.20.**

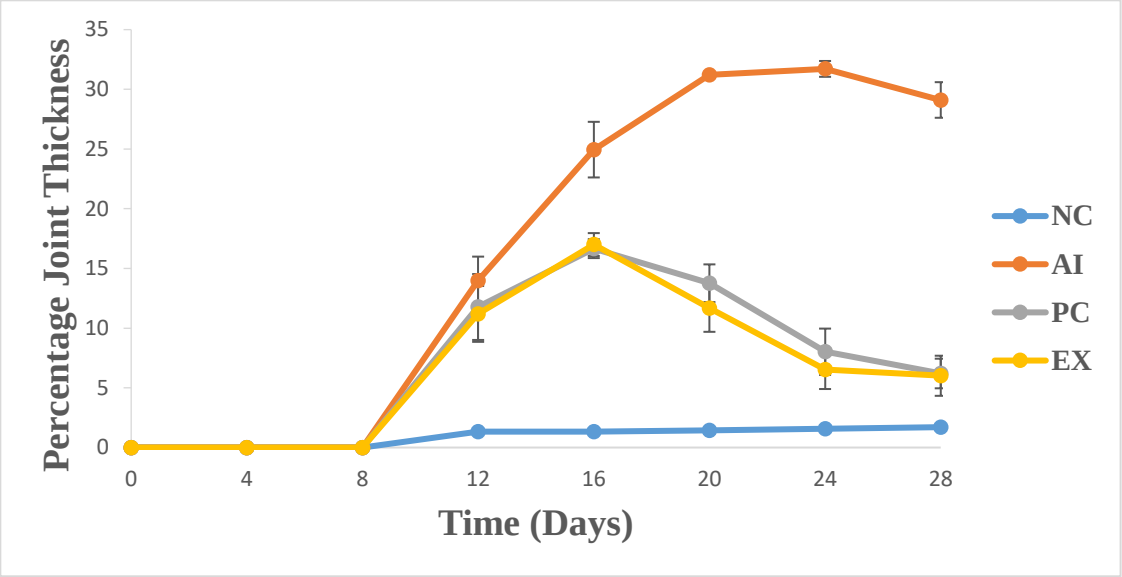


Figure 4.20: Percentage inhibition of thickness in the left hind joint by the aqueous extract of *R. prostrata*

Key: Ex=*R. prostrata* Extract at 1000 mg/kg; NC=Untreated control (normal saline-treated); AI= untreated arthritis-induced control; PC=Positive control (methotrexate, 2 mg/kg).

The was no significant difference ($p>0.05$) in the % inhibition of swelling in the left hind joint circumference from Day 1 to Day 8 after injection of adjuvant, in all treatment groups relative to Day 1. From day 12, arthritis symptoms started to develop and there was a significant difference ($p\leq0.05$) in % swelling of left hind joint circumference to Day 28 in untreated arthritis-induced rats. Maximum % inhibition in swelling in the left hind joint in *R. prostrata*- and methotrexate-treated rats was at 17.0 ±1.96 and 16.66 ±0.79%, respectively, on Day 16 after adjuvant injection. The swelling in the left hind joint continued to decline till Day 28. On Day 28, the last day of the study, there was no significant difference ($p>0.05$) between % swelling in left joint circumference in *R. prostrata* (6.01 ±1.68%) - and methotrexate treated rats (6.20 ±1.24%) relative to Day 1. These findings revealed that these animals were equally protected from left hind joint circumference swelling by *R. prostrata* and methotrexate standard.

The findings on the effect of the aqueous extract of *R. prostrata* on % thickness in the right hind joint are also displayed in **Figure 4.21.**

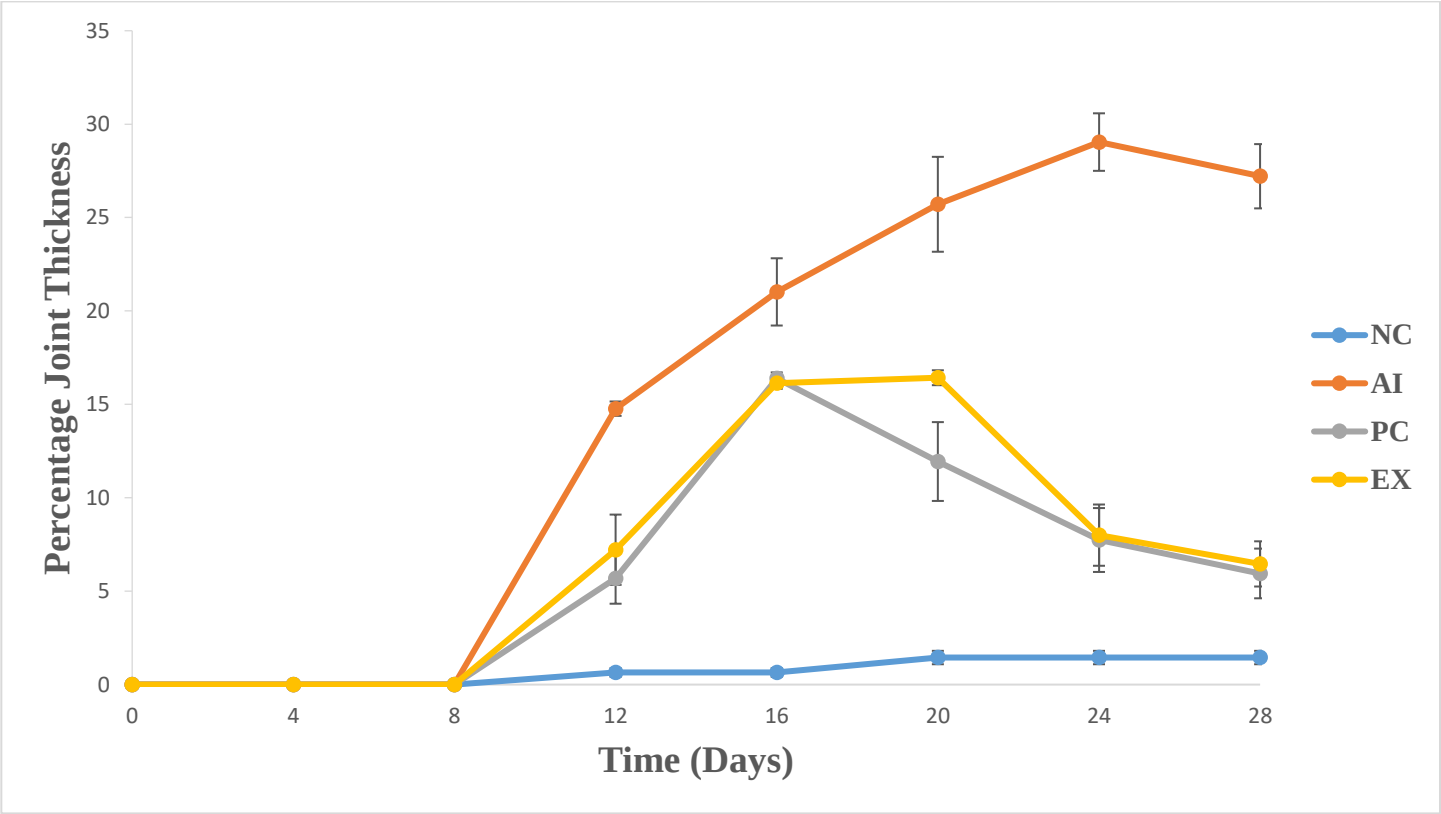


Figure 4.21: Percentage thickness in the right hind joint by the aqueous extract of *R. prostrata*

Key: Ex=RPM Extract (1000 mg/kg); NC=Untreated control; AI=Untreated arthritis-induced control; PC=Positive control (methotrexate at 2 mg/kg).

The was no significant difference ($p>0.05$) in % right hind joint circumference from Day 1 to Day 8 after injection of adjuvant, in all treatment groups relative to Day 1 after adjuvant injection. From day 12, arthritis symptoms started to develop and there was a significant difference ($p\leq0.05$) in right hind joint circumference in untreated arthritis-induced rats till Day 28. Relative to methotrexate-treated right hind joint circumference, *R. prostrata*-treated rats showed a similar trend in % inhibition of swelling of the right joint compared to Day 1 after adjuvant injection till Day 24. Maximum % inhibition of swelling of the right joint in *R. prostrata*-treated rats at 16.43 ±0.40% was on Day 20, whereas methotrexate standard-treated rats displayed a maximum swelling of the right joint earlier on Day 16 (16.39 ±0.32%). The swelling declined with time till Day 28 when *R. prostrata*-treated rats displayed swelling of (6.46 ±1.21%) relative to methotrexate standard at 5.95 ±1.33% compared to Day 1 of adjuvant injection. These findings revealed that the animals in the present study were protected from right hind joint swelling induced by FCA, with *R. prostrata* extract-treated animals displaying maximum swelling later than the methotrexate-treated animals. This finding revealed that the aqueous extract of *R. prostrata* protected the rats from right joint swelling longer than methotrexate standard.

A study by Bendele, (2001) described the use of arthritis induction at either the base tail or in the footpad. In their study, clinical evidence of arthritis occurred between Day 9-10 post-injection of adjuvant. Some studies (Ramadan *et al.*, 2011) injected the adjuvant intradermally in the hind paw. Assessment of the ankle joint width using Calliper measurement of the joint width before onset of arthritis up to termination of the study was reported by Bendele *et al.* (1999). Scoring of arthritis ankles in the study by Bendele *et al.* (1999) was assessed on a scale of 0-5. The present study also used Calliper measurement of joint circumference but instead of using a scale, comparisons of % inhibition of swelling of the hind joints relative to Day 1 when adjuvant was injected were done

4.4.6.3 Effect of the Aqueous Extract of *R. prostrata* on Grip Strength

The time (secs) the rats took to fall off a rotating rotarod was determined as the grip strength. The effect of the aqueous extract of *R. prostrata* on grip strength in rats is also shown in **Figure 4.22.**

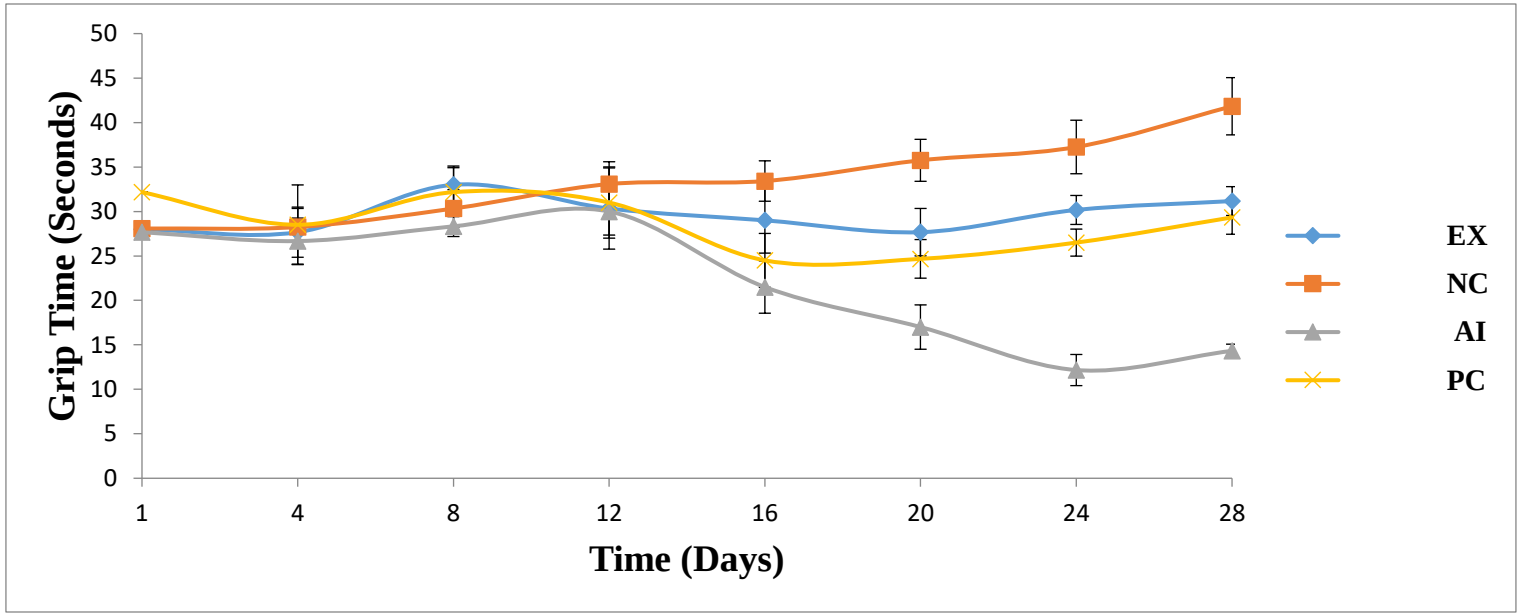


Figure 4.22: Effect of *R. prostrata* aqueous extract (n=6) on grip strength (secs)

Key: Ex=*R. prostrata* extract (1000 mg/kg); NC=Untreated control; NeC=Untreated arthritis-induced control; PC=Positive control (methotrexate at 2 mg/kg).

There was no significant difference ($p>0.05$) in time (secs) the rats in different groups took to fall from a rotating rotarod from Day 1 to Day 12. From Day 16 to 28, there was a significant ($p\leq0.05$) reduction in time of fall in the untreated arthritis-induced control compared to the untreated control. Relative to methotrexate standard-treated rats, there was no significant difference in fall ($p>0.05$) in the *R. prostrata*-treated rats from Day 16 to day 28. The untreated arthritis-induced rats displayed the shortest time of fall (12.17 ±0.75 secs) on Day 24. From Day 16, *R. prostrata*- treated rats showed the shortest time of fall of 27.67 ±1.63 secs on Day 20, whereas methotrexate-treated rats showed time of shortest fall of 24.50 ±2.17 secs earlier on Day16.

Compared to methotrexate standard, *R. prostrata*-treated rats displayed a better grip on the rotarod throughout the study. The untreated control displayed highest grip on the rotarod at 41.83 ± 2.46 secs on Day 28. On the last day of the study, the untreated arthritis-induced rats, *R. prostrata*- and methotrexate-treated rats started to regain their grip strength on the rotarod at 14.33 ±1.23 , 31.17 ±1.70, and 29.33 ±1.33 secs, respectively. These findings implied that the rats used in the present study started to regain their immunity with time from the effect of the adjuvant. Overall, the aqueous extract of *R. prostrata* had better improvement in grip strength in rats compared to methotrexate standard.

The assessment of grip strength on the rotarod was reported to determine motor learning which could be assessed both within and between subjects by comparing the first trial with subsequent trials and is evident as an increased latency to fall over time (Watzman *et al*., 1964; Watzman *et al*., 1967; Pritchett and Mulder, 2003; Rustay *et al*., 2003; Lee *et al.,* 2018). Grip strength determination in arthritis correlates to the impairment of grip function in patients with RA. The crippling effect in RA patients leads to loss of economic activities and may even lead to depression (Margaretten *et al*., 2011; Vriezekolk, 2011). Comorbid depression was reported to be an independent risk factor for mortality in patients with rheumatoid arthritis (Ang *et al.,* 2005). The arthritis model adopted in the

present study was able to reveal the effectiveness of the aqueous extract of *R. prostrata* in improvement of grip strength compared to the untreated arthritis-induced control.

4.4.6.4 Effect of Aqueous Extract of *R. prostrata* on Arthritis Score

Findings for the effect of the aqueous extract of *R. prostrata* on arthritis score are shown in **Table 4.23.**

Table 4.23: Effect of the aqueous extract of *R. prostrata* (n=6) on arthritis score

	Number of days				
	12	**16**	**20**	**24**	**28**
Treatment	**Time of fall (secs)**				
EX	5.00 ± 0.45^{a}	7.83 ± 0.31^{b}	5.67 ± 0.33^{b}	4.00 ± 0.00^{c}	3.50 ± 0.22^{b}
NC	00.00 ± 0.00^{c}	00.00 ± 0.00^{c}	00.00 ± 0.00^{c}	00.00 ± 0.00^{c}	00.00 ± 0.00^{c}
AI	4.67 ± 0.42^{a}	10.83 ± 0.31^{a}	11.50 ± 0.22^{a}	11.67 ± 0.21^{a}	11.50 ± 0.22^{a}
PC	5.33 ± 0.42^{a}	8.50 ± 0.43^{b}	6.33 ± 0.33^{b}	4.67 ± 0.21^{a}	4.17 ± 0.17^{b}

Key: Ex=*R. prostrata* extract (1000 mg/kg); NC=Untreated control; AI=Untreated arthritis-induced control; PC=Positive control (methotrexate 2 mg/kg). Values are expressed as Mean±SEM. Values followed by different superscript column-wise are significantly different ($p\leq0.05$).

Since arthritis symptoms started to develop from Day 12, the determination of arthritis score started from this day. All treatment groups showed a significant difference ($p\leq0.05$) in arthritis score from Day 12 to Day 20 relative to untreated control. The highest arthritis score in the methotrexate-treated (8.50 ±0.43) and *R. prostrata*-treated rats (7.83 ±0.31) was on Day 16. The arthritis score continued to decline till Day 28, when methotrexate-treated rats showed a sore of 4.17±0.17, whereas *R. prostrata*-treated rats showed a lower score of 3.50 ±0.22. On Day 24, there was no significant difference ($p>0.05$) in the arthris score in *R. prostrata*-treated rats (4.00 ±0.00) compared to the untreated control. On this day when *R. prostrata*-treated rats showed significant difference ($p\leq0.05$) in arthritis score compared to methotrexate-treated control (4.67 ±0.21). On Day 24, the untreated arthritis-induced group showed the highest arthritis score (11.67 ±0.21). On the last day of the study (Day 28), there was no significant difference in arthritis score ($p>0.05$) between methotrexate-treated control (4.17 ±0.17) and *R. prostrata*-treated group (3.50 ±0.22).

The aqueous extracts of *R. prostrata* at 1000mg/kg decreased the incidence and severity of the secondary lesions graded in the arthritis score.

A study carried out by Bendele *et al*. (1999), scored the adjuvant arthritis severity based on the arthritic ankles on a scale of 0-5 for inflammation and bone resorption. Their report also indicated that cartilage damage was a minor feature for scoring and was therefore not reliable for evaluation of potential treatment effects. Arthritis score was determined as lesions (thus arthritic signs) on all four paws of each rat and grading was done using a scale of 0-4 per paw according to the extent of both oedema and erythema of the periarticular tissues. A score of 16 was the potential maximum of the combined arthritic scores per animal (Van Eden *et al.,* 1994). Aqueous extract of *R. prostrata* showed a significant improvement in arthritis score (4.00) like that of methotrexate-treated (4.67) control. Therefore, the use of arthritis score was found to correlate with the severity of arthritis symptoms in the rats and related to the arthritis scoring in man (Kay and Upchurch, 2012). Overall, the aqueous extract of *R. prostrata* displayed better arthritis score compared to the methotrexate-treated group, implying a better anti-arthritic drug.

Wistar rats in the present study developed arthritis symptoms from Day 12 after injection of CFA at the base of the tail. These findings concur with the findings by Van Eden *et al.* (1994) who described the induction of arthritis in rats was by injection of adjuvant at the base of the tail as the classical method. Some studies (Ramadan *et al.,* 2011) injected the adjuvant intradermally in the hind paw. Since RA is an immunological disease, injection at the base of the tail gave the best immunologic manifestations, involving all joints, compared to intradermal injection which normally causes local irritation and swelling as an immediate response in the injected paw, before generalized dissemination of antigens from Complete Freund's adjuvant. Two types of treatment approaches were postulated in the study (Van Eden *et al.,* 1994); a prophylactic model, where dosing is initiated on day 0 (day of arthritis induction) or a therapeutic model where dosing starts on day 8. The present study adopted the therapeutic model and arthritis symptoms started to develop from Day 12 after injection of CFA. Aqueous extract of *R. prostrata* (1000 mg/kg) on

28th day showed a decrease in paw swelling and thickness which was the evident significant prevention of paw oedema and joint thickness.

A study by Raman *et al.* (2011) reported on the development of arthritis within 14–18 days after adjuvant injection through cell-mediated autoimmunity in which there was structural mimicry between the mycobacterium capsule and the cartilage proteoglycans (Ramprasath *et al.,* 2005). Their study reported that rat AIA shared several features with human RA including weight loss, oxidative tissue damage and inflammatory infiltration of the synovial membrane in association with joints swelling/destruction (Szekanecz *et al.,* 2000; Gomes *et al.,* 2010). In the present study, RA manifestations started from Day 12 unlike the study of Raman *et al.* (2011) which reported RA manifestations from Day 14-18 after adjuvant injection. The present study confirmed that the adjuvant-induced arthritis in rats was reproducible, reliable, and able to detect anti-arthritic activity of a known disease-modifying anti-rheumatic drug, methotrexate. Therefore, the findings obtained with the aqueous extract of *R. prostrata* in the present study are reliable and predicted the anti-arthritic activity of this extract.

4.4.6.5 Effect of the Aqueous Extract of *R. prostrata* on Body Weight

The results of the effect of the aqueous extract of *R. prostrata* on body weight (gm) in rats are presented in **Table 4.24.**

Table 4.24: Effect of the aqueous extract of *R. prostrata* (n=6) on body weight (g)

	Number of days							
	1	**4**	**8**	**12**	**16**	**20**	**24**	**28**
Treatment	**Body weight (g)**							
EX	176.67±6.5[a]	188.67±7.4[a]	196.50±7.2[a]	206.00±7.3[a]	214.17±7.9[a]	220.17±8.5[a]	226.83±8.1[a,b]	235.33±7.6[a,b]
NC	185.25±3.9[a]	195.33±4.8[a]	206.67±4.7[a]	218.25±4.4[a]	228.25±4.0[a]	236.83±4.0[a]	246.50±3.3[a]	254.00±3.3[a]
AI	195.00±6.2[a]	204.00±5.8[a]	211.00±4.8[a]	219.00±4.6[a]	225.67±4.8[a]	231.17±5.5[a]	236.17±5.8[a,b]	239.00±6.1[a,b]
PC	174.8±10.0[a]	186.2±10.1[a]	196.83±9.2[a]	205.83±9.0[a]	209.17±9.1[a]	215.50±9.3[a]	222.33±9.1[b]	230.17±8.8[b]

Key: Ex= *R. prostrata* extract (1000 mg/kg); NC=Untreated control; AI= Untreated arthritis-induced control; PC=Positive control (methotrexate at 2 mg/kg). Values are expressed as Mean±SEM. Values followed by different superscript column-wise are significantly different ($p \leq 0.05$).

Rats in all treatment groups increased in weight from Day 1 to Day 28. There was no difference ($p>0.05$) in body weight between different groups compared to the untreated control up to Day 20. A significant difference in body weight ($p\leq0.05$) between methotrexate-induced and untreated control was observed from Day 24 to Day 28. Relative to methotrexate-treated rats, *R. prostrata*-treated rats, showed no significant difference in body weight ($p>0.05$) compared to the untreated control throughout the study period. The untreated arthritis-induced control showed a significant difference in body weight ($p\leq0.05$) relative to untreated control on Day 24 and Day 28.

A correlation between changes the impairment in body weight gain and the incidence and severity of RA has been reported (Walz *et al.,* 1971b). There are several features that rat adjuvant-induced arthritis (AIA) shares with human RA. These include weight loss, oxidative tissue damage and inflammatory infiltration of the synovial membrane in association with joints swelling/destruction (Szekanecz *et al*., 2000; Gomes *et al.,* 2010).

The reason why the arthritis rats lose weight is due to immobility because of swollen painful joints. It was, therefore, important in the present study, to determine the changes in body weight of arthritis-induced control rats compared to the untreated control, *R. prostrata*- and methotrexate-treated rats as a measure of assessment of the severity of arthritis. In the present study, care was taken by moving food and water very close to the animals in the untreated arthritis-induced control, because they became immobile after Day 18 (Bendele, 2001). Although food was moved close to the untreated adjuvant-induced control as precaution, this group of animals still had least gain in weight throughout the study period. A study was done by Kim *et al*. (2016), which evaluated the changes in body weight reported a statistically significant difference in reduction in body weight observed in the CFA-control group when compared to the untreated control, a finding that concurred with the findings of the present study.

4.4.6.6 Effect of the Aqueous Extract of *R. prostrata* on Non-articular Symptoms

Non-articular symptoms observed in the rats used in this study were nodules on the ears and tail; vasculitis at the base of the tail and/or in some cases, whole tail. The nodules on the tail and ears were seen mainly in the animals which also had severe secondary lesions. Urethritis was observed in male rats. Towards Day 28 the nodules subsided in some rats except for the thick swelling at the base of the tail. It was also observed that in some arthritis-induced control, the whole tail became thick and hard. Swelling of all digits on either or both front and hind paws was noted in some rats whereas, in others, only the middle digit was swollen. No death was observed in all the rats used in this study, showing the safety of the aqueous extract of *R. prostrata.* Although methotrexate standard caused adverse effects on the bone marrow, it did not produce any death in the rats used in this study. Compared to many disease-modifying anti rheumatic drugs, methotrexate is reported to be first-line treatment and safest. Methotrexate is used alone or in combination with other drugs, such as leflunomide (Strangfeld *et al*., 2009), or hydroxychloroquine (Carmichael *et al.*, 2002).

Rheumatoid arthritis patients have been reported to show non-articular symptoms, such as rheumatoid nodules. The rheumatoid nodule is the most common non-joint feature which occurs in 30% of rheumatoid arthritis patients (Turesson, 2013). Although rheumatoid nodules are most found at points of pressure, subcutaneously, they may also occur in the connective tissue of numerous organs (Ziff, 1990). Since rheumatoid nodules have a special association with RA, it may be assumed that mechanisms involved in the development of rheumatoid synovitis may be like those involved in the formation of the rheumatoid nodule. In the study by Ziff, (1990) it was reported that almost all RA patients who develop rheumatoid nodules are also rheumatoid factor-positive. Therefore, rheumatoid factor complexes may be involved in the pathogenesis of RA. The proposed process of rheumatoid nodule formation involves trauma to small blood vessels with resultant local pooling of rheumatoid factor immune complexes, activation of the immune complex of local macrophages/ monocyte, monocyte chemotactic factors stimulation with resulting mobilization of increased numbers of macrophages (Ziff, 1990).

In the present study, vasculitis was observed in the arthritis rats as ulceration either on the whole tail or t the base of the tail. The rats that were treated with *R. prostrata* did not show non-articular symptoms such as rheumatoid nodules nor vasculitis, conditions that were observed in arthritis-induced rats and some methotrexate-treated rats.

Long-standing and untreated patients with rheumatoid arthritis were reported to possess several forms of vasculitis. The involvement of small- and medium-sized vessels is the most common presentation in established RA with a prevalence of approx.1 to 5%. Rheumatoid vasculitis commonly presents with skin ulceration and vasculitic nerve infarction and has a vast array of clinical manifestations which involve the skin (deep cutaneous ulcers and peripheral gangrene), and mononeuritis multiplex (Genta *et al.,* 2006).

The present study was able to determine the effects of the aqueous extract of *R. prostrata* on the arthritis symptoms developed in Wistar rats after injection of Complete Freund's Adjuvant at the base of the tail. The experimental model for RA revealed all the symptoms that occur in RA patients, both articular and extra-articular. The study revealed that the aqueous extract of *R. prostrata* showed anti-arthritic activity like methotrexate, a conventional DMARD, minus the bone marrow suppression which was observed in methotrexate-treated rats.

Rat adjuvant-induced arthritis model is a model that is widely used for screening anti-inflammatory potential of drugs because of its high degree of similarities that it has with the human disease (Bendele, 2001). This activity is widely used for preclinical testing of many anti-arthritic agents which are either under preclinical or clinical studies. The distinguishing characteristics of this model are reliable onset and progression of robust, easily measureable polyarticular inflammation, marked bone resorption and periosteal bone proliferation (Pearson 1956; Carlson *et al*., 1985; Benslay and Bendele, 1991). Complete-Freund's Adjuvant (CFA)-induced arthritis model in rats was selected in the present study to evaluate the potential anti-arthritic activity of the aqueous extract of *R. prostrata* on the pathogenesis of arthritis induced in rats (Bendele, 2001). The studies by Ramadan *et al.* (2010) also demonstrated the

similarity in several clinical, haematological, radiological, histological, and immuno-inflammatory features which closely resemble the human counterpart RA.

There are many synthetic drugs which have been reported to be used for the treatment of inflammatory disorders and nowadays are of little interest due to their potential side effects and serious adverse effects. These drugs are also found to be highly unsafe for human consumption (Beg *et al*., 2011). Since the last few decades, herbal drugs have regained their popularity in treatment against several human ailments. The problems associated with synthetic preparations have led to immense interest in herbals containing anti-inflammatory activity (Beg *et al*., 2011). Rheumatoid arthritis is a chronic inflammatory and destructive joint disease that affects 1% of the adult population worldwide (Gabriel, 2001). The disease leads to significant disability and a consequent reduction in quality of life, which have a substantial socio-economic impact (Buch and Emery, 2002). Therefore, development of a model that could reliably screen for the efficacy of herbal medicines for treating arthritis was achieved by using the Complete Freund's Adjuvant-induced arthritis in rats (Zhang *et al.,* 2011).

Adjuvant-induced arthritis in rats is a well-established experimental model that has features like human rheumatoid arthritis. This model is a good chronic inflammatory model for the development of potential anti-inflammatory and/or analgesic drugs useful for the treatment of arthritis. Adjuvant-induced arthritis has characteristics of chronic proliferative and inflammatory reactions in synovial membranes, production of pain, disability and eventually destruction of joints. Although the aetiology of rheumatoid arthritis is unknown, it is postulated to be an autoimmune process, in which T lymphocytes are responsible for the generation of adjuvant-induced arthritis. In the present study, the challenge with 0.1mL of CFA (1%) produced poly-articular arthritis, which was evident from the the significant increased paw volume and paw thickness in untreated arthritis-induced control.

The phytochemical compounds in *R. prostrata* which included saponins, flavonoids, tannins, glycosides, terpenoids **(Table 4.2)** could be the bioactive compounds responsible for the biological activities demonstrated by these *Ruellia* species (Samy *et al.,* 2015; Afzal *et al.,* 2015).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The bioactive compounds present in the three *Ruellia* species showed the presence of monodesmosidic triterpenoid saponins, flavonoids, phenolics, such as tannins, glycosides, and terpenoids, such as β-sitosterol, stigmasterol, lupeol and squalene. These compounds have been reported in other *Ruellia* species and implicated in the management of inflammatory non-communicable diseases.

2. The three *Ruellia* species in the present study exhibited antioxidant, analgesic and anti-inflammayory activities, implying that they could be active in a non-human primate. This study was the first report on the antioxidant, analgesic, and anti-inflammatory activities of *R. linear-bracteolata* and *R. bignoniiflora* species.

3. *Ruellia prostrata* showed highest analgesic, and anti-inflammatory activities and was further subjected to acute and sub-chronic toxicity studies. In both these studies, *R. prostrata* was safe, implying that this species could also be safe in a non-human primate.

4. *Ruellia prostrata* exhibited anti-arthritic activity in rats without bone marrow suppression, a setback of many anti-arthritic drugs. Therefore, *R. prostrata* could be further investigated as a new, efficacious, and safe alternative in the management of rheumatoid arthritis, a non-communucable disease.

5.2 Recommendations for Further Work

1. Use of other spectroscopic analyses such as Liquid Chromatography-Mass Spectrometry (LC-MS), High Pressure-Liquid Chromatography (HPLC) on reversed-phase columns, Mass spectrometry (MS), Nuclear Magnetic Resonance (NMR) such as ^{1}H NMR, ^{13}C NMR, or COSY 2D NMR could be used to elucidate the exact structures of the saponins, flavonoids, phenolics and terpenes, compounds which have been reported to be useful in the management of non-communucable inflammatory diseases. Among the compounds obtained in these *Ruellia* species, triterpenoid saponins are complex molecules, which lack chromophores, which limits their detection under UV , hence their structure elucidation will require the use of several spectroscopic techniques.

2. Once the structures (also called lead compounds) are elucidated, use of *in silico* studies will reveal the best pharmacokinetic and pharmacodynamic profiles of these compounds and their derivatization could lead to the prediction of the best drug candidate for investigation. Also, the use of quantitative structure-activity relationships studies will predict the best new drug candidate for further investigation using both *in vivtro* and *in vivo* anti-inflammatory studies.

3. The mechanism of action of the investigational new drug (IND) on several inflammatory mediators, such as enzymes of the cyclooxygenase pathway (COX-2) enzyme inhibition (PGE_2), pro-inflammatory cytokines such as TNF-α, interleukins (IL-1β, IL-2, IL-4, IL-6, IL-11); anti-inflammatory cytokines such as interleukins; IL-5, and IL-10, and IL-17 should be determined using specific ELISA kits, *in vitro*. Effects on other mediators of the immune system such as interferon-gamma (IFN-γ), chemotactic factors; chemokines (IL-8 and leukotriene B_4) *in vitro* and activity on nitric oxide released from macrophages should be determined using respective ELISA kits.

4. Safety of the new drug should be determined using *in vitr*o toxicity studies on cell proliferation of Vero cells and *in vivo* acute and sub-chronic toxicity studies. *In vivo* analgesic and anti-inflammatory studies, using the IND should be done using *in vivo* methods in rats.

5. The IND should go for pre-clinical trials in non-humans primates. Thus, an alternative safe and efficacious drug from *R. prostrata* could be introduced in the market for patients with inflammatory conditions, such as rheumatoid arthritis.

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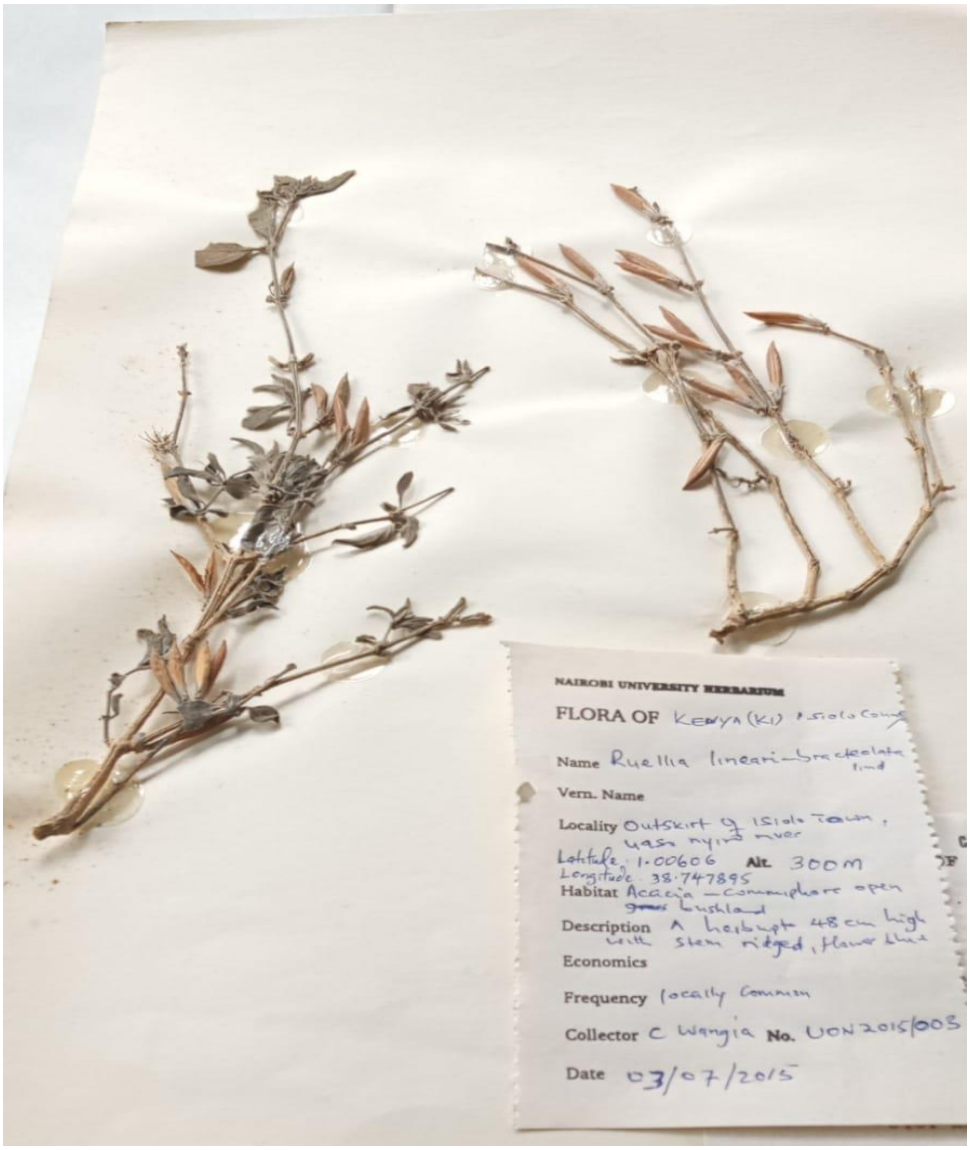
APPENDICES

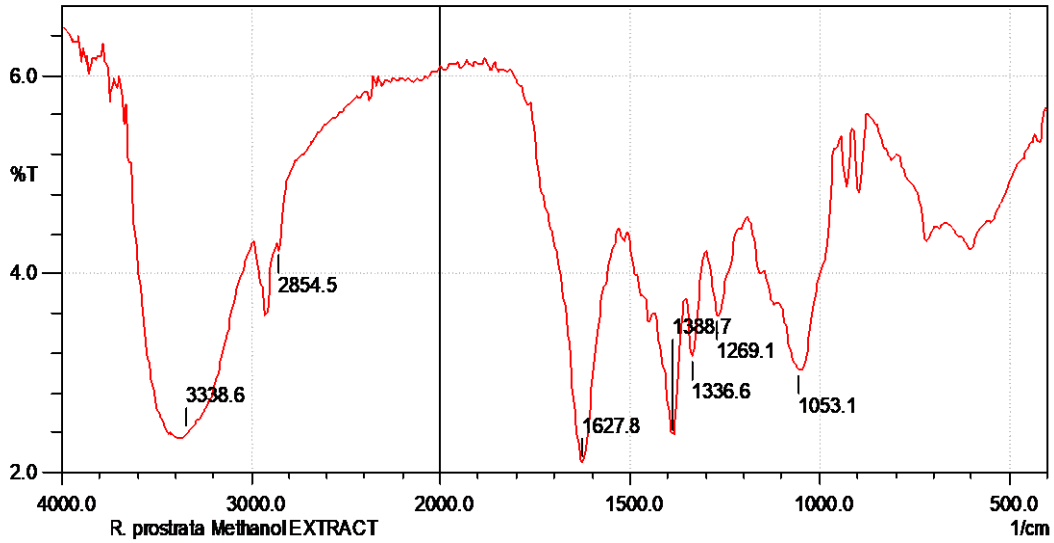
Appendix I: *Ruellia prostrata* Voucher specimen

Appendix II: *Ruellia bignoniiflora* Voucher specimen

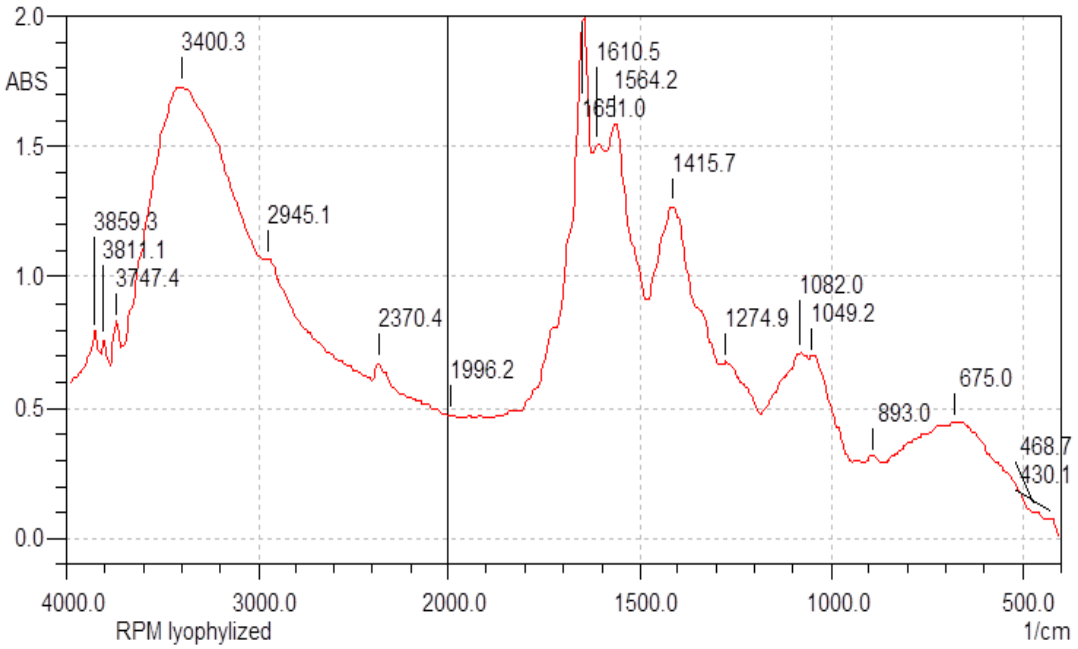
Appendix III: *Ruellia lineari-bracteolata* Voucher specimen



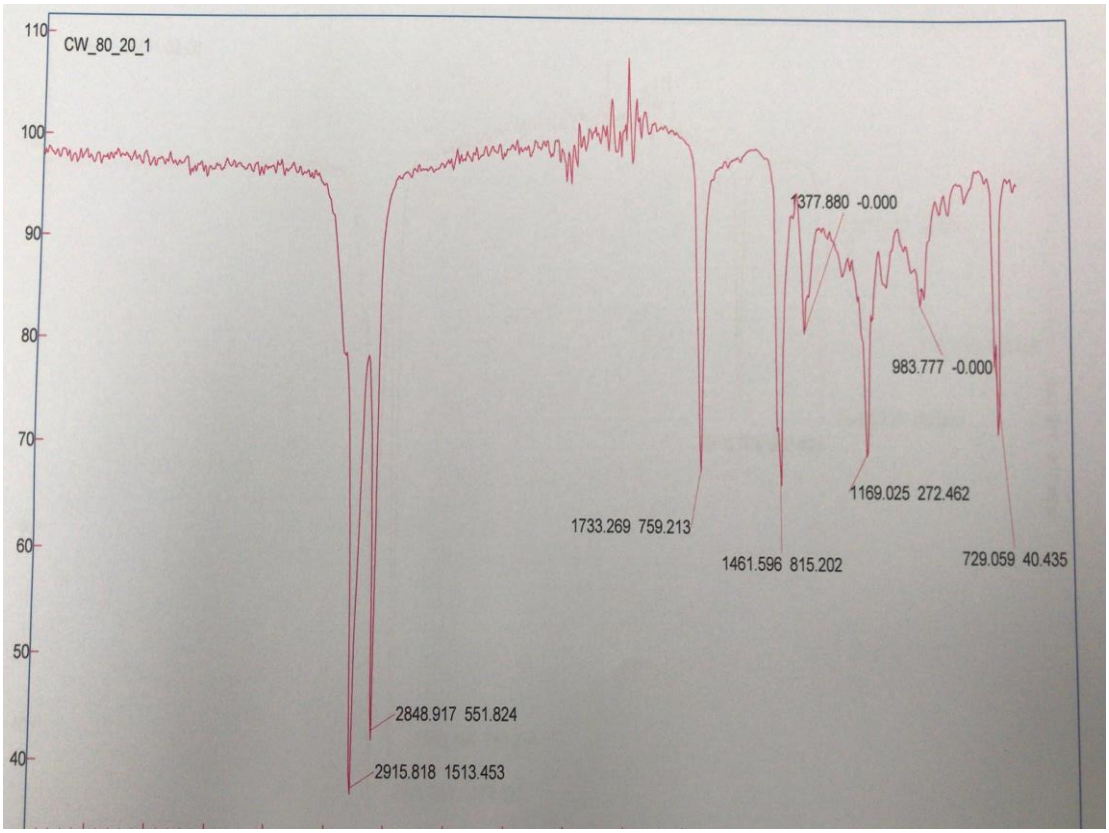
Appendix IV: FTIR spectral frequencies (cm^{-1}) of methanol extract of *R. prostrata*



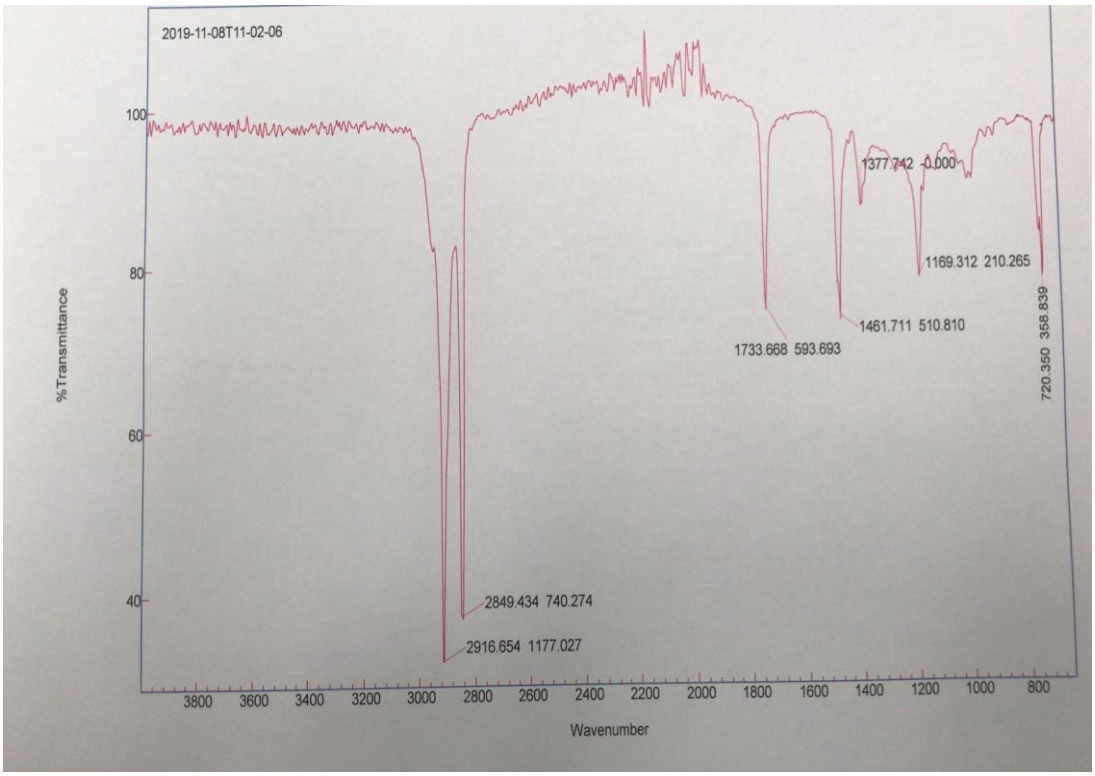
Appendix V: FTIR spectral frequencies (cm^{-1}) of aqueous extract of *R. prostrata*



Appendix VI: FTIR spectral frequencies (cm^{-1}) of n-hexane: ethyl acetate (80:20) pooled fraction from *R. prostrata* organic fraction



Appendix VII: FTIR spectral frequencies (cm^{-1}) of n-hexane: ethyl acetate (90:10) pooled fraction from *R. prostrata* organic fraction



Appendix VIII: Animal Use Approval

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O. Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115, Email: cvr@kemri.org

KEMRI/ACUC/ 01.08.15 **3rd August 2015,**

Dr. Christine Wangia
Center for Traditional Medicine and Drug Research

Dr. Wangia,

RE: Animal use approval for SSC 2946 "Development of a topical analgesic and anti-arthritic formulation from selected Kenyan *Ruellia* species" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately and acknowledges that the use of laboratory animals is justified in achieving the study objectives.

The committee grants you the approval to use laboratory animal species in your study but recommends that you proceed after obtaining all the other necessary approvals that may be required.

Approval is granted for a period of three years starting from when the final ethical approval will be obtained. If you still intend to use laboratory animals after the initial approval, you are required to submit an application for continuing approval to the ACUC 1 month prior to the expiry of this initial approval.

The committee expects you to adhere to all the animal handling procedures as described in the protocol. The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 03 AUG 2015 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....