ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECTS OF STEM BARK EXTRACTS OF *MAERUA ANGOLENSIS* DC (CAPPARACEAE)

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

Maerua angolensis DC of the family Capparaceae is a plant used traditionally for the treatment of inflammatory conditions such as arthritis, pain, gout, boils and wounds. There is however very little scientific data on the usefulness of this plant as an anti-inflammatory agent. This study therefore aims at investigating the anti-oxidant and anti-inflammatory activities of *M. angolensis*. The dried powdered stem bark was extracted using solvents of different polarities to obtain four extracts: 70 % v/v ethanol extract (F1), ethyl acetate extract (F2), Petroleum ether extract (F3) and petroleum ether-ethyl acetate 1:1 (F4).The carrageenan-induced paw oedema, carrageenan-induced pleurisy and acetic acid-induced vascular permeability models were used to assess the possible anti-inflammatory effects of the extracts. Histamine, serotonin, bradykinin and prostaglandin E_2-induced paw oedema was also used to determine the effect of the extracts on early phase mediators in acute inflammation. F1, F2 and F3 (30-300 mg kg^{-1}) dose dependently and significantly suppressed carrageenan induced paw oedema with the highest dose of 300 mg kg^{-1} giving a maximum suppression of 73.45 %, 78.4 % and 68.3 % respectively. Neutrophil infiltration and exudate volumes in carrageenan-induced pleurisy were significantly reduced by F4 (30-300 mg kg^{-1}) with maximum inhibition of 90.9 % and 64.2 % respectively. F4 also preserved normal alveolar architecture in rat lungs in the pleurisy assay with reduced neutrophil infiltration, oedema, hyperaemia and alveolar septal thickening from histopathological assessment. Myeloperoxidase activity, an indicator of neutrophil infiltration was also significantly reduced. Vascular permeability was also attenuated by F4 with marked reduction of Evans blue dye leakage in acetic acid-induced permeability assay. F4 was able to significantly suppress inflammation induced by serotonin, bradykinin and prostaglandin E_2 but not that induced by histamine.

The *in vitro* antioxidant activity of F4 was evaluated with the 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) scavenging test. Total phenolic content and total antioxidant capacity of F4

was also determined. F4 had DPPH radical scavenging activity ($IC_{50;}$ 0.1088 mg $ml^{-}1$), total phenol content (97.24 mg g^{-1} tannic acid equivalent) and total antioxidant capacity (114.2 mg g^{-1} ascorbic acid equivalent). F4 exhibited *in vivo* antioxidant activity in carrageenan induced pleurisy by increasing the levels of glutathione, superoxide dismutase and catalase activity while decreasing lipid peroxidation in lung tissues. The results indicate that *Maerua angolensis* is effective in ameliorating inflammation induced by carrageenan and acetic acid. It also has the potential of increasing the activity of endogenous antioxidant enzymes and also a free radical scavenger. These findings therefore justify the wide use of *Maerua angolensis* in traditional medicine for inflammatory disease conditions.

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DEDICATION

To the memory to my late grandfathers, Jonas Agyei Abankwah and Felix Mensah Asomaning.

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LIST OF ABBREVIATIONS

5HT	5-hydroxytryptamine
5-LOX	5-Lipooxygenase
AA	Arachidonic Acid
BSO	Buthionine sulphoximine
CAM	Cell Adhesion Molecule
COX	Cyclooxygenase
CAT	Catalase
DMARDs	Disease-modifyingAnti-rheumatic Drug
DNA	Deoxyribonucleic Acid
GPCR	G-protein coupled receptors
GPx	Gluthathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
i.p	Intraperitoneal
ICCAM	Intracellular Adehsion Molecule
IL-1	Interleukin 1
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
LT	Leukotrienes
MDA	Malondialdehyde
mPGES-1	Membrane-bound prostaglandin E_2 synthase-1
MPO	Myeloperoxidase
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
O_2^-	Superoxide

OH^-	Hydroxyl Radical
$ONOO^-$	Peroxynitrite Radical
p.o	per os
PAF	Platetlet Activating Factor
PGE_2	Prostaglandin E_2
PLA_2	Phosphopolipase A_2
PMNL	Polymorphonuclear Leukocytes
PUFA	Polyusaturated Fatty Acid
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TNF	Tumour Necrosis Factor
Tx	Thromboxane

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

The use of plants as a source of medicine has been a way of life for humans even before we developed the ability to write (Fabricant *et al.*, 2001). Records from ancient civilizations in Egypt, China, and India have shown well documented use of plants for the treatment of many diseases (Pan *et al.*, 2014). Currently, it is estimated that about 5,000 species of plant have been recognized as medicinal agents in Africa (Iwu, 2014). The World Health Organization (WHO) estimates that 80 % of the world's population especially in Asia and Africa (Tabuti *et al.*, 2012) relies on herbal remedies for majority of ailments. This is partly due to inaccessibility to modern medicine and also to the belief that natural plant medicines are safe and with minor side effects (Shaw *et al.*, 2012). Most of the medicinal plants from folklore have not been assessed for their toxicity, mechanisms of action and interactions with food and other medicines (Iwu, 2014). Hence, it is prudent to conduct further studies into medicinal plants to obtain new data on indications and safety profile.

Maerua angolensis is a plant used traditionally throughout most of the African continent for its medicinal activities. There is not enough scientific data to back many of its folkloric indications. It is therefore necessary to conduct pharmacological assessment of the plant to validate its traditional use and determine its mode of operation.

1.2 MAERUA ANGOLENSIS DC

Botanical Name: *Maerua angolensis* DC **Family**: Capparaceae.

Common names: English – Bead bean

Akan - Osononantini, Koninbere

Nabdam – Pugodugo

Frafra – Pudingo

Hausa - Chichiwaa, Gazare, Zumuwaa, Kiyafa

Fulfulde – Bagushi

Fulani – Leggaelbaal

Arabic - Shegara el zeraf

1.2.1 DESCRIPTION

Maerua angolensis DC is from the family Capparaceae previously Cappariraceae. It is a medium to big tree and grows up to 10 – 20 meters high (Figure 1.1). The leaves are elliptic to lanceolate, up to 7 cm long, glossy green above, paler below with the mid–rib protuberant and whitish, finely textured, hairless; apex round or notched with a hair-like tip; petiole almost as long as the leaf, yellowish with a distension and a curvature below the blade. The stem is white consisting of young branches with conspicuous pale lenticels and straggling branches drooping at the ends and carrying the abundant, conspicuous white flowers. The flowers are axillary, solitary, in terminal spikes or in clusters on small lateral spurs, without petals, stamens plentiful, long and whitish. Flowering time is around December. Fruit is up to 15 cm long, pod–like, usually constrained between the seeds. The wood is hard and heavy, yellowish and fine grained (Burkill, 1995; Mothana *et al.*, 2009; Iwu, 2014).

Figure 1.1 *Maerua angolensis* plant

1.2.2 ECOLOGICAL AND GEOGRAPHICAL DISTRIBUTION

It is commonly found growing in savannah woodland vegetation and around rock boulders. It can also be found in scattered-tree grassland and edges of rain forests. It is widespread in the savannah area of tropical Africa to South Africa and Swaziland. It is a native of tropical Africa found in hot and dry open woodland (Burkill, 1995; Ayo *et al.*, 2013).

1.2.3 TRADITIONAL USES OF MAERUA ANGOLENSIS

The roots, leaves, stem barks and fruits of *Maerua angolensis* are used traditionally in Africa for medicinal purposes.

Fruits: Management of jaundice, hepatitis and liver disease; as a sedative and in insomnia. The raw fruit is used as a laxative (Burkill, 1995).

Leaves: leaf decoctions are useful in anorexia, vomiting and diarrhoea. The leaf sap is dropped into fresh wounds as an antiseptic dressing. A decoction of the leaf is used to prevent abortion. Management of influenza, hydrocele, boils, pimples and skin rashes (Burkill, 1995).

Stem and bark: the stem bark is used in the management of psychosis, epilepsy, paralysis, spasm and convulsions. A decoction of the bark is used in stomach ulcer and dyspepsia. Stem barks are used to relieve headache, toothache, arthritis, gout, swellings and rheumatism (Burkill, 1995).

Roots: The roots are used in decoctions as an aphrodisiac. A decoction of the root is used in nasal infection, influenza, hydrocele, boils ,pimples and skin rashes (Burkill, 1995; Adamu *et al.*, 2007).

1.2.4 PREVIOUS PHARMACOLOGICAL STUDIES ON MAERUA ANGOLENSIS

Saponins, tannins, flavonoids, alkaloids, glycosides, terpenes, carbohydrates and proteins have been detected from phytochemical assessment of stem bark extracts (Ayo *et al.*, 2013). Anti-inflammatory activity has been reported in stem bark extracts in carrageenan–induced oedema in rats (Adamu *et al.*, 2007).The aqueous methanolic stem bark extract possesses anti-diabetic activity in streptozocin-induced diabetes in Wistar rats (Mohammed *et al.*, 2008).Initial gastrointestinal studies on stem bark extracts of *M.angolensis* has been reported to exhibit antidiarrheal property in castor oil–induced diarrhoeal model in mice (Magaji *et al.*, 2008).Hydroalcoholic extracts from the stem bark has been shown to possess central nervous system depressant activities in mice during some neuropharmacological studies (Magaji *et al.*, 2009). Information on the median lethal dose (LD_{50}) of stem bark extracts of *M.angolensis* in mice revealed LD_{50} of 3807.9 mg kg^{-1} orally and > 500 mg kg^{1}intraperitoneally (Magaji *et al.*, 2008). Analgesic activity of *Maerua angolensis* stem bark extract and it's fractions has been reported in morphine dependence in mice (Iliya *et al.*, 2015). Analgesic Property of petroleum-ether-ethyl acetate stem bark extract and fractions of

Maerua angolensis has been reported in Murine Models of Pain (Iliya *et al.*, 2014). Various solvent extracts of *Maerua angolensis* DC stem bark revealed activity in neurogenic and inflammatory pain in mice (Azi *et al.*, 2014).

1.3 INFLAMMATION

Inflammation is a defensive response to injury to curb further damage and to also initiate tissue repair. Celsus described the cardinal signs of inflammation in his works ‘’De Medicina”: *Notae vero inflammationis sunt quattuor* as redness, swelling, heat and pain (Ferrero-Miliani *et al.*, 2007).

Celsus was the first person to record the cardinal signs of inflammation; Virchow further included “*funtio laesa”* (Loss of function) to Celsus’s signs of inflammation (Vogel *et al.*, 2009).

Several years after Celsus, Galen considered inflammation to be useful in injury but his views were contradicted years later by Virchow who subsequently classified inflammation as essentially pathological. Although Celsus's views remains in focus in medical books around the globe, the inflammatory process is much more intricate from recent scientific findings (Karin, 2009).

It is currently understood that inflammation is part of the non-specific immune response that occurs in reaction to any type of cellular injury which may be mechanical (e.g., contusion or abrasion), chemical (e.g., toxins, acid, and alkaline), physical (e.g., extreme heat or cold), microbes (e.g., bacteria, virus and parasites), necrotic tissue, oxidative stress and/or immunological reactions. In the inflammatory response there is increased blood flow, cellular metabolism, vasodilatation, release of soluble mediators, fluid extravasation and cellular influx. Under normal conditions, inflammation is self-limiting but in some disease states there is

persistent injury and further cell damage which then leads to chronic inflammatory disorders (Gilroy *et al.*, 2004; Serhan *et al.*, 2005).

1.3.1 ACUTE INFLAMMATION

Acute inflammation is of comparatively short duration, lasting for minutes, several hours, or days, and it is mainly characterized by oedema formation (fluid and plasma proteins extravasation) and the influx of polymorphonuclear leukocytes, with neutrophils being the most prominent (Hurley *et al.*, 1973; Pulichino *et al.*, 2006).

Acute inflammation can be initiated by a range of factors including infections and bacterial toxins, trauma, physical (radiation, heat etc.) and chemical agents, tissue necrosis, foreign bodies and immunological reactions.(Medzhitov, 2010)

The inflammatory response though complex still follows a predictable sequence of events. When there is damage to blood vessels from an initial trauma (microorganism, toxins etc.), the clotting system is activated through platelet aggregation and leucocyte enrolment in order to form a containment barrier which isolates the injury to allow repairs to take place (Yu *et al.*, 2015).

Acute inflammation has three major components. There are changes in vascular architecture that leads to hyperaemia. This is due to the release of early phase mediators such as histamine which causes vasodilation. Structural changes in the microvasculature allows the influx of plasma proteins and leukocytes from circulation. Fluid exudation into the site of injury attenuates external toxins and toxins generated *de novo* by leucocytes, and allow the inflow of essential serum proteins, comprising components of the complement system and immunoglobulins which stimulate the return to cellular homeostasis. Endothelial cells also play

a significant role in the inflammatory response. In addition to coagulation and permeability factors, they release recruitment factors and the proinflammatory cytokines

(TNFα, IL-1, and IL-6). TNFα and IL-1 causes the endothelial cells to expose adhesion molecules on their lumenal surface to trap blood leucocytes as they pass by the site of injury (Van der Poll *et al.*, 2014). Neutrophils cling on to the endothelium through adhesion molecules (E-, P-, and L-selectins, ICAM-1 VCAM-1, and integrin ligands) and migrate across endothelium (Transmigration) via diapedesis into the site of injury from circulation. They are then activated to counteract the noxious agent. Leucocytes mainly neutrophils traverse from blood vessels to the affected tissue through chemotaxis (IL-8), where they mop up pathogens or injurious agent through phagocytosis and degranulation which resolves the inflammation (Robbins *et al.*, 2010; Telen, 2014).

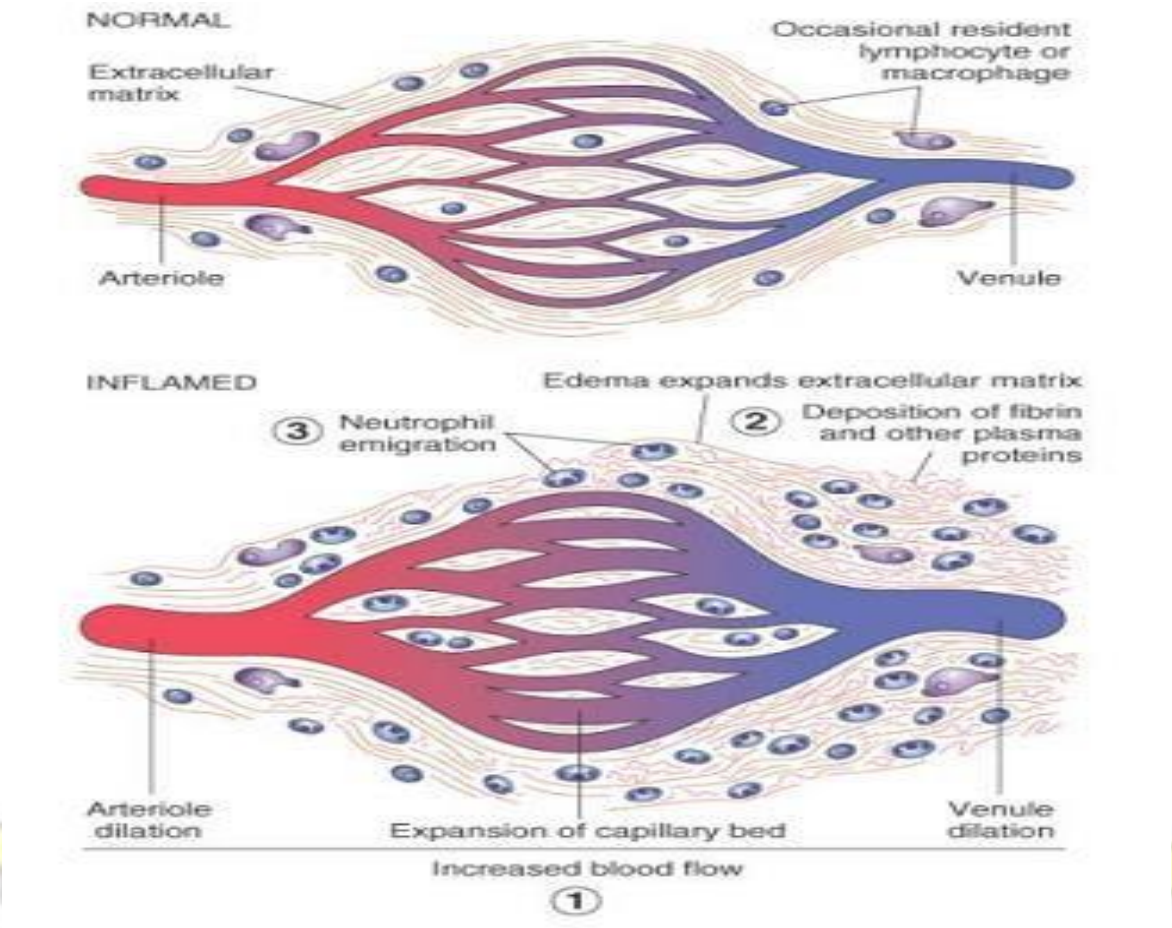

Figure 1.2 The major local manifestations of acute inflammation. Vasodilation followed by increased vascular permeability leads to oedema (adapted from Kumar *et al.* (2014)

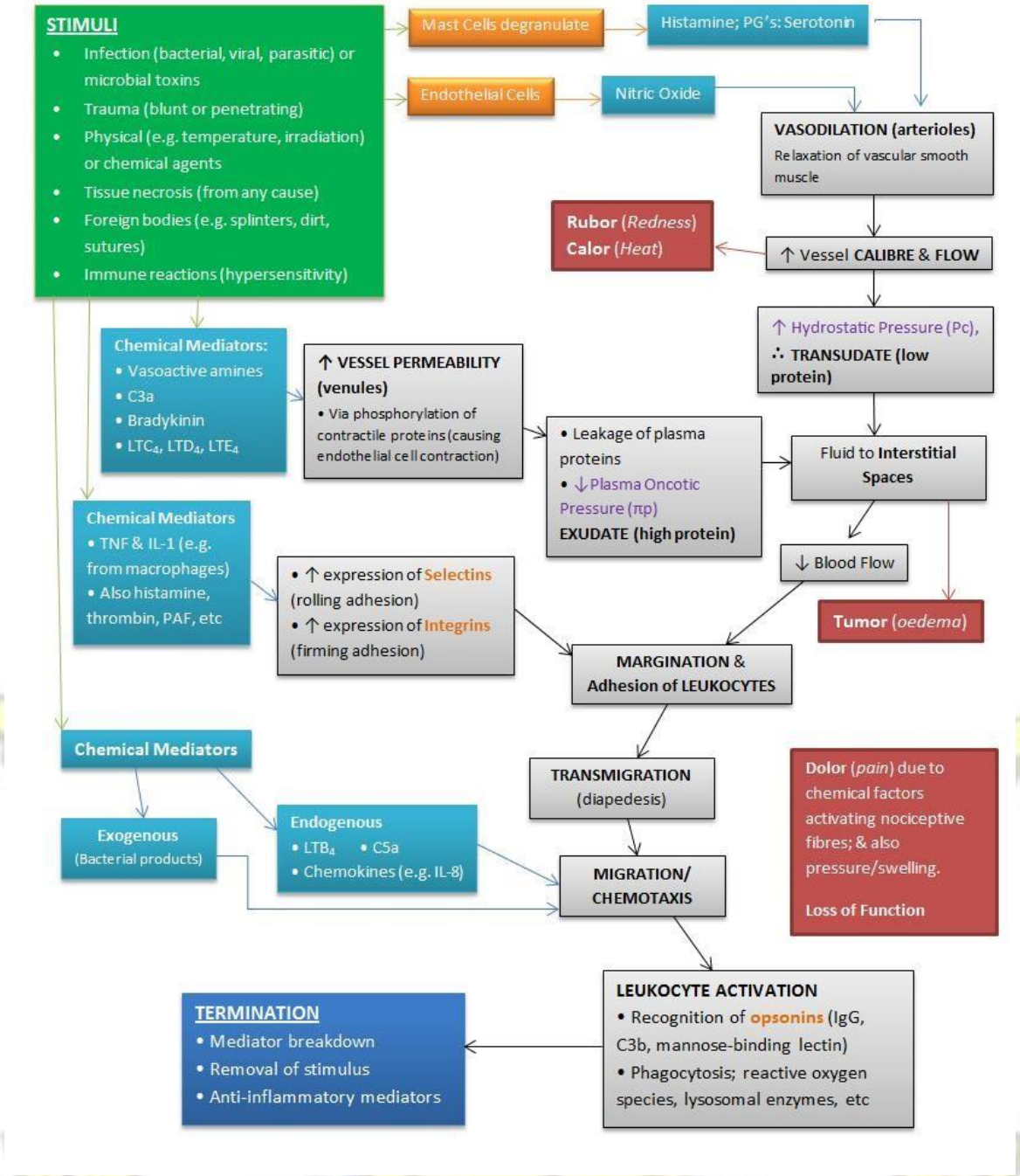

Figure 1.3 Acute inflammation flow chart showing initiation, mediators involved and eventual termination (adapted from Sparshott, 2015)

1.3.2 MEDIATORS OF INFLAMMATION

Many serum and cell-derived compounds perform an essential role in the initiation and magnification of inflammatory responses. A disturbance in cells such as platelets, endothelial

cells, leucocytes and their surrounding connective tissue cells release products which enhances the inflammatory response. For instance, mast cells, which are present in most tissues, are triggered early in inflammation to release histamine and serotonin (rodents). Histamine and serotonin causes the distinctive redness, oedema, warmth and pain, and also leads to the rapid manifestation of endothelial adhesion molecules on the cell surface. Leucocytes generate cytokines, enzymes and other mediators which also enhance the inflammatory process (Lima *et al.*, 2013). Several mediators are generated from arachidonic acid metabolism which further amplifies the inflammatory response. Arachidonic acid is broken down by cyclooxygenases (COX) into prostaglandins and thromboxanes and by lipooxygenases into leukotrienes. These mediators then enhance the inflammation process by increasing vascular permeability, hyperalgesia and pyrexia. Compounds like aspirin are able to slow down this pathway by inhibiting the COX pathway. COX also affects the clotting system by inhibiting platelet aggregation hence long term use of COX inhibitors impairs blood clotting. Inflammatory mediators that affect the clotting system assists leucocyte enrolment (Wakefield *et al.*, 2001).

1.3.2.1 HISTAMINE

Histamine is the first mediator encountered in the genesis of the inflammatory response. It is formed in the Golgi body by histidine decarboxylation via the enzyme L-histidine. It is then stored as granules in mast cells, basophils and platelets. Mast cells can be found in almost all tissues and are responsible for the manifestation of allergic reaction, anaphylaxis and inflammation (MacGlashan, 2003).

Mast cells when activated by physical injury, cytokines, complement proteins and immunoglobulin E degranulate and release histamine. Histamine causes arteriolar dilation, increased vascular permeability and vasoconstriction of large arteries through H_1 receptors (Marone *et al.*, 2002).

1.3.2.2 SEROTONIN

Serotonin is found in most tissues and is synthesized by decarboxylation of tryptophan. It is then stored in enterochromaffin cells. Interestingly, serotonin stores are found in rodent mast cell granules but in humans it is present in platelets. 5-HT is usually stored as a co-transmitter with other compounds such as somatostatin, substance P and Vasoactive intestinal polypeptides (VIP). Serotonin receptors include $5HT_1$, $5HT_2$, $5HT_3$, and $5HT_4$ (Barnes *et al.*, 1998). Platelet aggregation stimulates the release of serotonin. It causes effects similar to histamine such as arteriolar dilation, increase vascular permeability and vasoconstriction of large arteries. 5-HT is also chemotactic for neutrophils (Boehme *et al.*, 2004) and attracts neutrophils to sites of tissue injury by stimulating their adhesion and migration (Kushnir-Sukhov *et al.*, 2006).

1.3.2.3 PLASMA PROTEINS

1.3.2.3.1 THE COMPLEMENT SYSTEM

The complement system is a collection of serum proteins which are triggered by immunological complexes or other substances. They are essential in both innate and adaptive immunity. They are mainly produced in the liver. Components of complement cause increased vascular permeability (C5a and C3b via histamine release), chemotaxis (C5a attracts neutrophils, eosinophils and basophils) and opsonisation (C3b) at the site of injury (Sarma *et al.*, 2011). C5a also trigger the lipoxygenase pathway of arachidonic acid metabolism which enhances the release of more inflammatory mediators (Chen *et al.*, 2010).

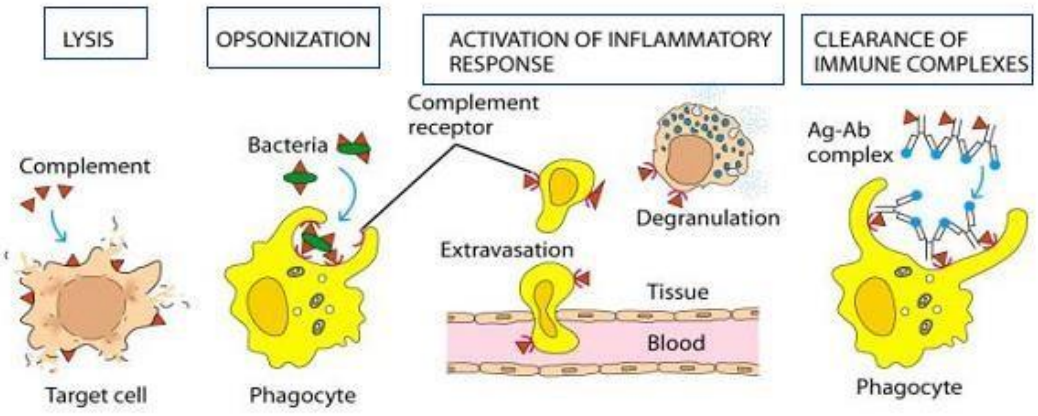

Figure 1.4 Multiple activities of the complement system in enhancing the inflammatory response. (adapted from Kindt *et al.*, 2007)

1.3.2.3.2 THE KININ SYSTEM

The kininogen-kallikrein-kinin (K-k-k) system is of much importance in inflammation, coagulation, nociception and blood pressure regulation. K-k-k mediators cause vasodilation and prostaglandin biosynthesis. The kallikrein-kinin system is multifaceted, with several mediators involved in different section (Schulze-Topphoff *et al.*, 2008).

Kinins activity is mainly due to B_1 and B_2 receptors with B_2 receptors being the most abundant. B_1 receptors are mainly activated by tissue damage. Almost all cells express GPCR-type kinin receptors, which mediate the activities of both bradykinin and kallidin. B Receptor activation causes increased vascular permeability, vascular smooth muscle relaxation, hypotension, contraction of intestinal smooth muscle, and contraction of smooth muscle in airways , nociception, nitric oxide synthesis, release of cytokines by leukocytes and release of eicosanoids from various cell types (Jih-Pyang *et al.*, 1989). The kinins have been implicated in many pathological phenomena including chronic pain, asthma, rheumatoid arthritis, hypertension and many other inflammatory diseases (Saxena *et al.*, 2011).

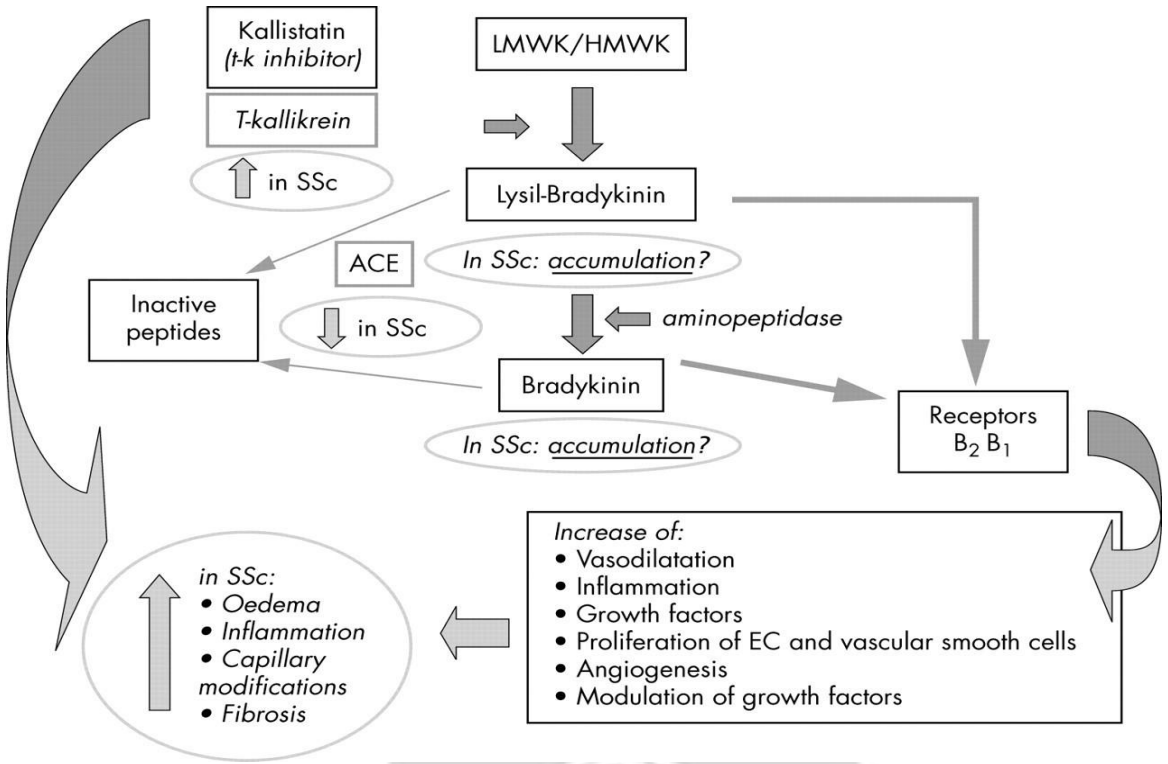

Figure 1.5 The activity of kinins in multiple systems. Activated B receptors causing vasodilation and release of cytokines (adapted from Moreau *et al.*, 2005)

1.3.2.3.3 THE CLOTTING SYSTEM

Inflammation and coagulation play essential roles in host defence. There is a bidirectional relation between inflammation and coagulation in response to tissue injury. Inflammation leads to activation of coagulation pathways while coagulation also significantly affects inflammatory response and vice versa. Coagulation proteases have significant immunomodulatory effects (Boos *et al.*, 2006). When fibrinogen is converted to fibrin threads by thrombin, fibrinopeptides are formed which increases vascular permeability and leucocyte chemotaxis. Thrombin causes leukocytes adhesion and fibroblast proliferation. Plasminogen activator cleaves plasminogen to generate plasmin. Plasmin cleaves fibrin clot to form "fibrin split product". Plasmin also cleaves C3 to produce C3a and C3b and activate Factor XII. C3a causes histamine release from mast cells which leads vasodilation. C3b binds to bacterial cell walls and labels them as targets for phagocytosis (Cheng *et al.*, 2009; Yee *et al.*, 2010).

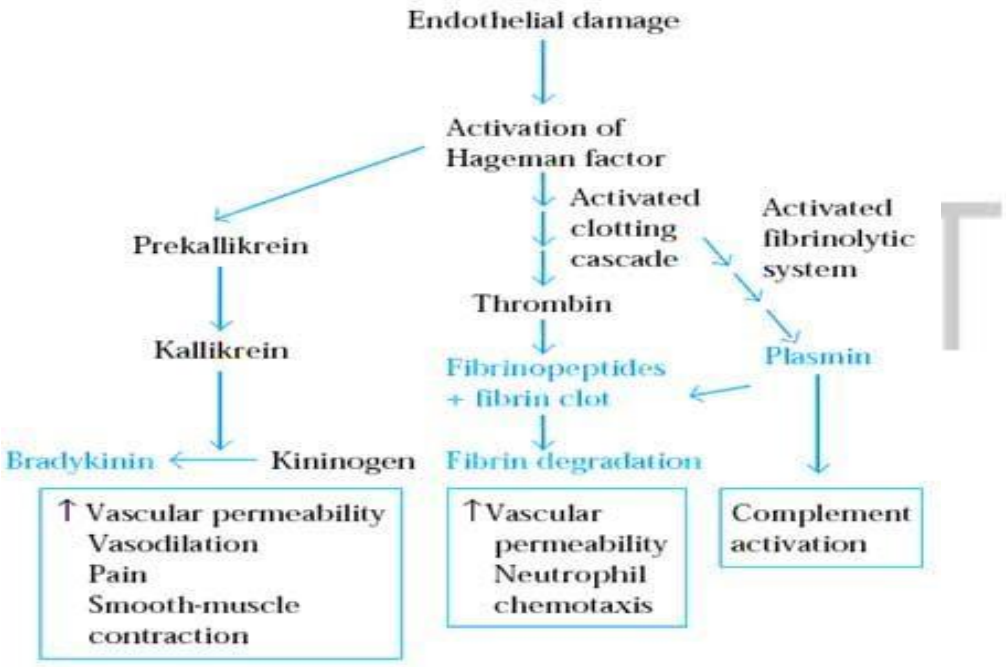

Figure 1.6 Effects of the coagulation cascade on the inflammatory response (adapted from Marinovic, 2008).

1.3.2.4 ARACHIDONIC ACID (EICOSANOIDS)

Cells when injured trigger acyl hydrolases which generate arachidonic acid. Metabolites generated from arachidonic acid metabolism include prostaglandins, prostacyclin, thromboxanes, leukotrienes and lipoxins (Harizi *et al.*, 2008). Eicosanoids unlike histamine are not stored in cells but are produced from phospholipid precursors when needed. Arachidonic acid is the precursor of eicosanoids. It is found in the phospholipids of cell membranes and membranes of subcellular particles. Phospholipase A_2 cleaves and liberates arachidonic acid from cell membrane phospholipids. Phospholipase A_2 is activated when triggered by cellular injury and complement protein C5a (Boyce, 2008).

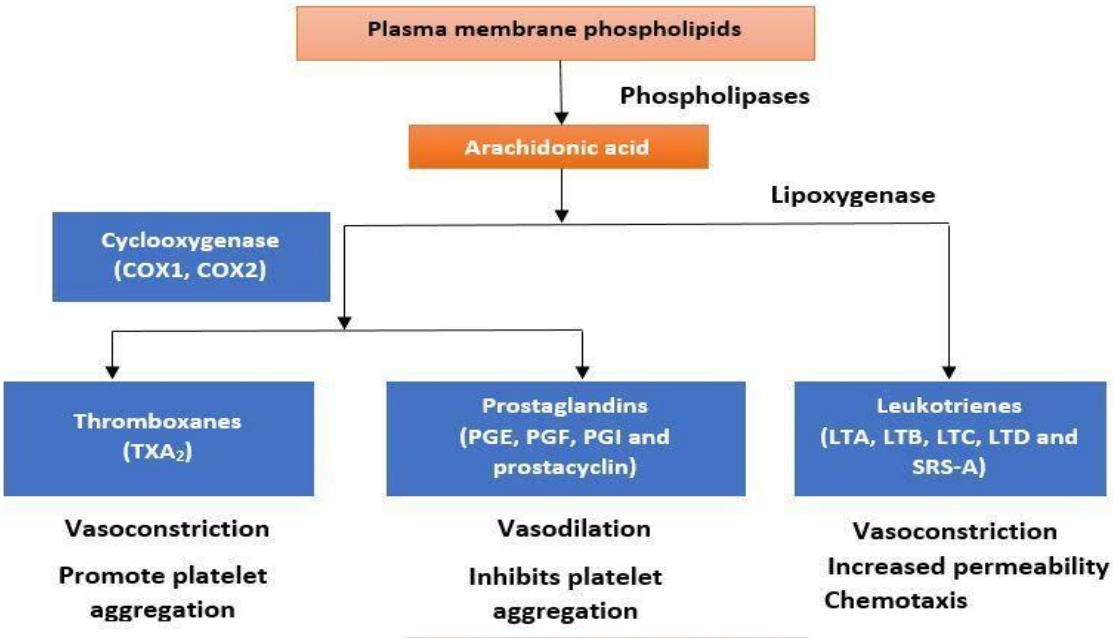

Figure 1.7 The role of arachidonic acid pathway in inflammation. SRS-A, slow-reacting substance of anaphylaxis. (Adapted from Villarreal *et al.*, 2001)

Eicosanoids have been linked to control many physiological processes, and are amongst the most essential mediators and modulators of the inflammatory response (Buckley *et al.*, 2014).

1.3.2.5 CYTOKINES

Cytokine are signalling molecules that facilitate cell to cell communication in immune system during inflammation. Cytokines function locally by autocrine or paracrine mechanisms. They bind and activate specific receptors which are normally upregulated during inflammation (Morimoto *et al.*, 2010). Many cytokines control the expression or release of other cytokines, which in turn enhance or inhibit feedback to regulate the original cytokine. There are many cytokines with TNFα and IL-1 being most significant due to their ability to regulate the induction of other downstream cytokines and mediators (Floege *et al.*, 2012).

1.3.2.6 NITRIC OXIDE

Nitric Oxide (NO) is a free radical derived from endothelial cells, macrophages and brain neurons. It is in form of a soluble gas which allows it to diffuse freely across membranes. It is

produced from L-arginine via the enzyme nitric oxide synthase (NOS). Nitric oxide synthase is activated by influx of calcium ions into cytoplasm of endothelium and neuron with presence of calmodulin. The increase of calcium ions in these cells leads to a rapid synthesis of NO. In macrophages NOS is produced, when cytokines or other agents activate it. NO induces vascular smooth muscle relaxation which increases blood flow and production of ROS. (Laroux *et al.*, 2000). It also exhibits direct cytotoxic effects on certain microbes and tumour cells when released by phagocytes (Hooper, 2004).

1.3.2.7 FREE RADICALS AND REACTIVE OXYGEN SPECIES IN INFLAMMATION

Free radicals are entities which are highly reactive due to the occurrence unpaired number of electrons in their atoms. They exist for short intervals but are able do a lot cellular damage (Watson, 2013). It is worth accentuating that at low or moderate concentrations, free radicals are essential in the development of cellular structures and can act as weapons for the host defence system (Pham-Huy *et al.*, 2008) Free radicals are able to alter the cellular structure of lipids, proteins and DNA and have been recognized as the cause of many diseases. Lipids are highly susceptible damage by free radicals which leads to lipid peroxidation. They cause loss of enzyme activity by damaging proteins. DNA damage leads to mutations and carcinomas.

Reactive oxygen species (ROS) plays a significant part in the variation and regulation of inflammatory reactions. The prime ROS encountered within cells include superoxide anion(O_2^-), hydrogen peroxide(H_2O_2) and hydroxyl radical(OH^-) (Salvemini *et al.*, 1996). Leukocytes are able to release large quantities of superoxide with the intent to eliminate pathogens but it results into endothelial damage ,hyperaemia, oedema which eventually leads to cell death (Tiidus, 1998). Currently, there is evidence that intracellular ROS production plays an important role in variation and regulation of inflammatory mediators. It is has being proposed that ROS may act as second messengers in signal transduction of the inflammatory response.

This is mainly due to the intrinsic expression of non-phagocytic NAD(P)H oxidases in many tissues (Guzik *et al.*, 2003).These enzymes can control adhesion molecule expression on endothelium and inflammatory cells by generating ROS which affect cell enrolment to the sites of inflammation (Fraticelli *et al.*, 1996). They also increase the expression of chemokines and cytokines (Sunil *et al.*, 2012). The effects of ROS are mainly due to their ability to stimulate MAP-kinases activity which results into the activation of a number of transcription factors (Cuzzocrea *et al.*, 1999).ROS activates transcription factors, such as NF-kB which is a rapid-acting transcription factor of genes that code for proinflammatory cytokines (Wang *et al.*, 2007). TNF-α is able increase NAD(P)H oxidase activity and vice versa which further amplifies the inflammatory response (Symons *et al.*, 2003). ROS may also mediate cell damage by separate mechanisms including the initiation of lipid peroxidation, enzyme inactivation and depletion of glutathione. Oxidative stress is a phenomenon that occurs when the production of free radicals and ROS overpowers the body's antioxidant defence systems. This leads to the unopposed destruction of essential biomolecules and cells by radicals which results in cancer and many inflammatory disorders (Denis *et al.*, 2015). Reactive nitrogen species (RNS) are formed when NO is released from mitochondria under hypoxic conditions. RNS leads to the production of other reactive species such malondialdehyde, isoprostanes and 4-hydroxynonenal by causing lipid peroxidation (Reuter *et al.*, 2010).There have been successful attempts develop agents which inhibit intracellular ROS production in order to minimize the inflammatory response. Apocynin, an NAD(P)H oxidase activation inhibitor has been successfully used in controlling inflammation in animal models of rheumatoid arthritis (Kvietys *et al.*, 2012), while decoy peptide, which prevents an association of NAD(P)H oxidase subunits have been shown to be effective in inflammation related to atherosclerosis.

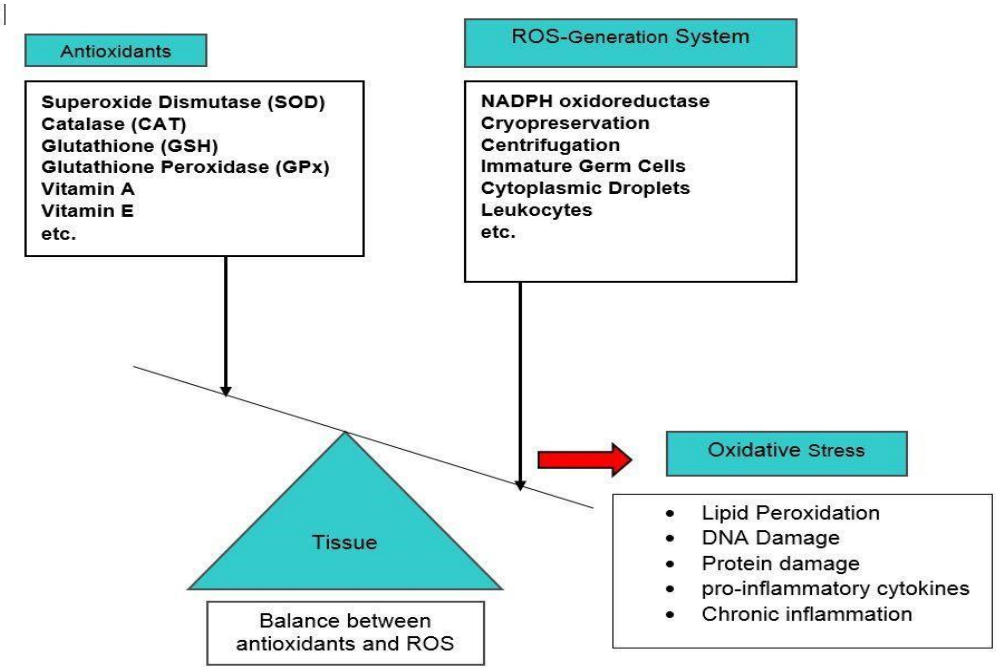

Figure 1.8 Imbalance between antioxidants and ROS generation leads to oxidative stress.

1.3.3 ENDOGENOUS ANTIOXIDANTS

Humans have developed a highly sophisticated and intricate antioxidant protection system in order to protect cells from the damage caused by ROS and RNS. This system involves many compounds which are generated internally in cells or acquired from external sources. The compounds work together to counteract the deleterious effects of free radicals.

These compounds include:

- Food-derived antioxidants such as ascorbates (vitamin C), tocopherols and tocotrienols (vitamin E), Xanthophils and Carotenes, and other low molecular weight compounds such as glutathione and thioctic acid.
- Antioxidant enzymes, e.g., superoxide dismutase ((SOD), Ascorbate reductase glutathione peroxidase, and glutathione reductase, which facilitate free radical scavenging reactions.

- Proteins that bind metal ions, which includes ferritin, lactoferrin, albumin, and ceruloplasmin that trap free iron and copper ions that are capable of catalysing oxidative reactions (Percival, 1997).

1.3.4 MANAGEMENT OF INFLAMMATION

Anti-inflammatory agents have become essential in the management of inflammatory conditions in modern times. Although anti-inflammatory agents do not possess disease modifying property, they are able to reduce pain and swelling and can facilitate exercise and physical therapy (Crowley *et al.*, 2011). There are the classical non-steroidal antiinflammatory drugs (NSAIDS) which non-selectively inhibit COX-1 and COX-2; and the selective COX-2 inhibitors. Selective COX-2 inhibitors have an advantage of being less likely to cause gastrointestestinal ulceration (Hutchins *et al.*, 2001). Disease modifying antirheumatic dugs (DMARDs) therapy when initiated early in chronic inflammation slows down disease progression (Roussy *et al.*, 2013). The common DMARDs in use include methotrexate, sulphasalazine, gold salts, cyclosporine, azathioprine and hydroxychloroquine.

Oral and parenteral corticosteroids are increasingly being used in arthritis conditions (van Vollenhoven, 2009). Corticosteroids have been shown to possess marked efficacy in rapidly decreasing the signs and symptoms of inflammation and reduction of progression of erosion and joint damage when are used early in the disease development (Madureira *et al.*, 2015). Intra-articular corticosteroids are also important adjuncts in inflammatory polyarthritis management. The newest developments in inflammation management are the biological agents targeted against specific cytokines or immunoreactive cells central to the inflammatory process. These agents include etanercept, infliximab and adalimumab which are anti-TNF-α agents (Jurgens *et al.*, 2011). These agents have been shown to slow radiological progression and improve symptoms.

1.4 AIMS AND OBJECTIVES OF STUDY

1.4.1 AIMS

Inflammatory disorders remain one of the main health problems in the world (Sun *et al.*, 2012). Current drug therapies are riddled with many undesirable side effects especially for long term treatments (Vane *et al.*, 1998; Schiro *et al.*, 2015). Hence, there is the need to explore alternative means of drug treatment with multiple modes of action and less side effects (Vane *et al.*, 1995; Gepdiremen *et al.*, 2006). In the past pharmacological activity of plant medicines was mainly attributed to the presence of secondary metabolites like alkaloids, terpenoids and glycosides (Silva *et al.*, 2012). Recent discoveries have elaborated the relevance of metabolites such as flavonoids and other phenolics previously considered unimportant in oxidative stress which is involved in the aetiology of diseases (Iwu, 2014). The role of phenolics in maintaining the antioxidant status in tissues and the prevention of disease progression is better understood now. Antioxidant compounds are of significant importance in attenuating ROS related cell damage. The stem bark extract of *M. angolensis* is used locally in many African countries for the treatment of many inflammatory conditions (Adamu *et al.*, 2007).

The aim of this study is to investigate and establish the anti-inflammatory and antioxidant activities of the stem bark extract of *Maerua angolensis* as informed by its traditional use for inflammatory conditions, pain and wound healing. Despite the folkloric reports of antiinflammatory action, there is little or no pharmacological data on the anti-inflammatory activity of *M. angolensis*. This study therefore seeks to evaluate the anti-oxidant and antiinflammatory properties of various solvent extracts from the stem bark of *M. angolensis* to buttress its traditional indications.

1.4.2 OBJECTIVES

The objective of this study is to carry out pharmacological assessment of the stem bark extracts of *Maerua angolensis* in *in vivo* models of inflammation and *in vitro* and *in vivo* antioxidant assays.

Specific objectives include evaluating the extract for:

- Anti-inflammatory activity.
  i. Carrageenan-induced paw oedema in rats.
  ii. Carrageenan-induced pleurisy in rats.
  iii. Acetic acid-induced vascular permeability in mice iv. Myeloperoxidase assay

- Possible mechanism of action in acute inflammation.
  i. Histamine-induced paw oedema
  ii. Serotonin-induced paw oedema
  iii. Bradykinin-induced paw oedema
  iv. Prostaglandin E_2-induced paw oedema

- *In vitro* and *in vivo* antioxidant activity.
  i. DPPH free radical scavenging assay
  ii. Total phenol assay
  iii. Total antioxidant capacity assay iv. Glutathione assay
  v. Catalase activity assay
  vi. Superoxide dismutase activity assay

CHAPTER TWO

METHODOLOGY

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 PLANT COLLECTION

The stem bark of *Maerua angolensis* was collected from the rocky areas of Kwahu Tafo to Nkyenenkyene road, Eastern Region, Ghana (6.415360 N0.363160 W) and was authenticated by Dr. Kofi Annan of the Department of Herbal Medicine, Faculty of Pharmacy, KNUST, Kumasi. A voucher specimen KNUST/FP/12/051 was deposited at the herbarium of the Faculty of Pharmacy, KNUST.

2.1.2 PLANT EXTRACTION

The stem bark was dried in a room for seven days and pulverized into fine powder. The powder was then packed in a bell jar and extracted serially by cold percolation with 70 % (v/v) ethanol, petroleum ether, ethyl acetate and petroleum ether-ethyl acetate (1:1) to obtain four extracts F1, F2, F3 and F4 respectively. The extracts were them concentrated separately under reduced pressure at 60 °C using a rotary evaporator (Rotavapor R-215, BÜCHI Labortechnik AG, Flawil, Switzerland) into a brown (F1, F2) yellow-brown (F3) and green (F4) viscous mass. Extracts were further dried in a hot air oven at 50 °C for a week and kept in a refrigerator for use.

2.1.3 TLC ANALYSIS OF F1, F2 AND F3

The extracts F1, F2 and F3 (300 mg) were each dissolved in chloroform (2 ml). The extracts were then spotted on TLC plate with capillary tubes at the origin (1 cm above the edge). The spots were dried and placed inside a well-covered chromatank containing the mobile phase

(petroleum ether-ethyl acetate (90:10)). The experiment was allowed to run until the solvent reached a desirable height and the solvent front was marked. The separated compounds were then observed under visible light and ultra violet light (254 nm and 365 nm) followed by spraying with chromogenic agent, anisaldehyde in conc H_2SO_4 (Brain *et al.*, 1975).

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

Sprague-Dawley rats (150 – 220 g) and Imprint Control Region (ICR) mice (20 - 30 g) were procured from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, and housed in the animal facility of the Department of Pharmacology, KNUST, Kumasi, Ghana. The animals were kept in stainless steel cages, with wood shavings as bedding, fed with normal pellet diet and water available, *ad libitum*. Sample size of 5-6 animals per group was utilized throughout the study, following guidelines according to National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85 - 23, revised 1985) All procedures used in this study were approved by the Department of Pharmacology Ethics Committee, College of Health Sciences (CoHS), KNUST, Kumasi, Ghana.

2.2.2 DRUGS AND CHEMICALS

The following drugs and chemicals were used: λ-Carrageenan, histamine, serotonincreatinine sulphate complex (Sigma and Aldrich, St. Louis MO, USA), prostaglandin E_2, bradykinin (Sigma Life Science, USA), diclofenac sodium (KRKR, d.d,Novomesto, Slovenia), dexamethasone and chlorpheniramine (Pharma Nova, Accra), Granisetron (Corepharma LLC, Middlesex, USA),Captopril (Teva ltd, Castleford, UK), Phosphate buffered saline (PBS) (Gibco, Karlsruhe, Germany),Folin-Ciocalteu reagent and 2, 2diphenyl-picrylhydrazyl (DPPH) (Sigma and Aldrich, St. Louis MO, USA). Ascorbic acid, ammonium molybdate, disodium

hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), Tannic acid and acetic acid (BDH, Poole, England) , methanol and ethanol (Fisher Scientific, UK) ; trichloroacetic acid (Amresco®, Solon, USA); liquid paraffin (KAMA Pharmaceutical Industries, Accra, Ghana); paraffin and bluing solution (IHC World LLC, Maryland, USA); Ethyl alcohol (Fisher Scientific, Waltham, 45 UK); haematoxylin and eosin Y (Abbey Color, Philadelphia, USA); xylene, formaldehyde,

thiobarbituric acid, EDTA, mono potassium dihydrogen phosphate, dipotassiummonohydrogen phosphate, monosodium phosphate, disodium phosphate, odianisidine dihydrochloride and Evans blue dye (Sigma-Aldrich, St. Louis MO, USA)

2.2.3. ANTI-INFLAMMATORY ACTIVITY

2.2.3.1 CARRAGEENAN-INDUCED PAW OEDEMA IN RATS

The experiment was conducted as previously described by (Winter *et al.*, 1962) to assess the anti-inflammatory activity of the extracts. Diclofenac and Dexamethasone were used as reference anti-inflammatory drugs. λ-carrageenan (50 µl of a 1 % w/v solution) was injected intraplantar into the left foot paw of rats. Foot volume was measured before and at hourly intervals for 5 hours after carrageenan injection by means of a volume displacement method using a hydro-plethysmometer (IITC Life Science Equipment, Woodland Hills, USA). The oedema component was calculated by measuring the difference in foot volume before and at the various time points after carrageenan injection (Winter *et al.*, 1963; Milanino *et al.*, 1988). The drugs were given preemptively (30 mins for i.p. route and 1 h for oral route) before carrageenan injection. Rats were randomly selected for one of the following groups (n=5): control; diclofenac (10, 30 and 100 mg kg^{-1}, i.p.); dexamethasone (0.3, 1.0 and 3.0 mg kg^{-1}, i.p.) and extract (F1, F2, and F3) (30, 100 and 300 mg kg^{-1}, *p.o.*). Drugs doses were selected

based on previous preliminary work. Extracts were prepared in Tween 80. All drugs were freshly prepared.

2.2.3.2. HISTAMINE-INDUCED PAW OEDEMA

The experiment was conducted as described in the carrageenan-induced paw oedema model with modifications (0.1 mg of histamine as inflammogen) (Mazumder*et al*., 2003). The reference antagonist used was chlorpheniramine. Each group was treated with either F4 (combination of F2 and F3) (30, 100, and 300 mg kg^{-1}, *p.o*.) or Chlorpheniramine (1 – 30 mg kg^{-1}; *p.o*.).The control group received distilled water (1 ml kg^{-1}, *p.o*.). Paw oedema was measured at 30 min intervals for 3 h after treatment and the percentage changes in paw volumes calculated.

2.2.3.3 SEROTONIN-INDUCED PAW OEDEMA

The experimental procedure was similar to that described in the carrageenan-induced paw oedema model but paw oedema (acute inflammation) was induced with 0.1 mg of serotonincreatinine sulphate complex (Mazumder *et al.*, 2003). The reference antagonist was Granisetron. Each group was treated with F4 (30, 100, and 300 mg kg^{-1}, *p.o*.) or Granisetron (10 - 100 µg kg^{-1}*p.o*.). The control group received distilled water (1 ml kg^{-1}, *p.o*.). Paw oedema was measured at 30 min intervals for 3 h post treatment and the percentage changes in paw volumes calculated.

2.2.3.4 BRADYKININ-INDUCED PAW OEDEMA

The experiment was conducted as described by (Campos *et al.*, 1995). Sprague-Dawley rats were pretreated with captopril 1h before bradykinin injection to prevent degradation of the bradykinin. The stock solution for bradykinin was prepared in phosphate buffered saline (PBS) (1-10 mM) in plastic tubes, maintained at 18 °C just before use. Each animal was injected with

0.2 ml of 10 nmol into the right hind paw 30 min after administering the extract and vehicle (distilled water 1 ml kg^{-1}, *p.o.*) as in the carrageenan model. No selective antagonist was given in this model. Paw oedema was measured at 30 min intervals for 3 h post treatment and the percentage changes in paw volumes calculated.

2.2.3.5 PROSTAGLANDIN E_2-INDUCED PAW OEDEMA

Prostaglandin E_2, 0.2 ml (1 nM) was administered into the sub-planter region of the right hind paw of rats, in accordance with the method of (Willis *et al.*, 1973). The paw volume up to the ankle joints were measured plethysmographically before and after 30 min of the prostaglandin E2 injection. Each group was treated with either F4 (30, 100, and 300 mg kg^{-1}, *p.o.*) or diclofenac sodium (10 mg kg^{-1}, i.p.). The control group received distilled water (1 ml kg^{-1}, *p.o.*). Paw oedema was measured at 30 min intervals for 3 h post treatment and the percentage changes in paw volumes calculated and recorded.

2.2.3.6 CARRAGEENAN-INDUCED RAT PLEURISY

Rats (200-220 g) were anesthetised with ether (0.25 ml in a 3 L container) open-drop method and subjected to a skin incision at the left sixth intercostal space. The underlying muscle was cut open, and 1 % w/v λ-carrageenan suspension (0.1 ml) in normal saline was injected into the pleural space (Bus *et al.*, 1978; Vinegar *et al.*, 1982). The skin opening was closed with a stitch, and the rats were allowed to recover. The extract F4 (30, 100 and 300 mg kg^{-1}, *p.o.*), or vehicle (10 ml kg^{-1}, *p.o.*) and diclofenac (10, 30 and 100 mg kg^{-1}, i.p.) were given 1 h and 30 min respectively before the injection of carrageenan. The rats were then sacrificed with excess of ether 6 h after carrageenan injection. The pleural cavity was opened and washed with 2 ml of saline solution containing 0.1 % of EDTA. The pleural fluids were then aspirated and the volumes quantified (ml).The actual exudate volume was determined by subtracting the volume of solution injected (2 ml) from the total volume of fluid aspirated. Pleural fluids tainted with

blood were excluded. The mobilized neutrophils in the exudate were quantified using an automated analyser ABX micros 60-Horiba, Irvine (CA), USA.

2.2.3.6.1 HISTOPATHOLOGY

At the end of pleurisy assay, lung sections were collected and stored in 10 % buffered formalin. The tissue was dehydrated with graded ethanol sections, embedded in paraffin, blocked and sectioned. The sections were stained with haematoxylin-eosin for examination under light microscopy.

2.2.3.6.1.2 HISTOLOGICAL SCORING

Damage to lung tissues of untreated and carrageenan-injected rats was assessed to evaluate microscopic lung damage by light microscopy (Patel *et al.*, 2012). In each treatment group, six random fields of view were analysed by observers unaware of the treatment protocols. The degree of microscopic lung damage induced by carrageenan was assessed. Histological slides were scored according to the following parameters: Hyperaemia, oedema, alveolar septal thickening and neutrophil infiltration. The degree of the disorganization was quantified on a scale of 0–4 (i.e. 0– not present, 1– very mild, 2–mild, 3– moderate, 4– extensive).

2.2.3.7 ACETIC ACID-INDUCED VASCULAR PERMEABILITY

The acetic acid-induced vascular permeability test with slight modifications was conducted as previously described by (Whittle, 1964). Groups of mice (n=6) (25-30 g) were treated with either F4 (30, 100 and 300 mg kg^{-1},*p.o.*), diclofenac (30 mg kg^{-1},i.p) or vehicle. 1 h after treatments, each mouse was injected intravenously with 2 % Evan's blue solution at 0.1 ml/10g body weight through the tail vein. 10 min afterwards, each mouse received 0.6 % acetic acid solution i.p at 0.1 ml/10 g body weight. 30 min after acetic acid injection, the mice were sacrificed and peritoneal cavity washed three times with saline (10 ml). Saline washes were

centrifuged for 5 min at 3500 rpm. The supernatants were collected and their absorbance measured at 590 nm with a plate reader (Synergy H1 Multi-Mode plate reader, Winooski, VT, USA). Evans blue extravasation was enumerated from a standard curve and expressed in µg.

2.2.4 ANTI-OXIDANT ACTIVITY

2.2.4.1 IN VITRO TESTS

2.2.4.1.1 TOTAL PHENOL ASSAY

The total soluble phenols present in the extract were quantitatively determined by colorimetric assay using the Folin-Ciocalteu reagent (Singleton *et al.*, 1999). Tannic acid (0.001953 – 0.125 mg ml^{-1}) was used as the standard drug and methanol was used the blank. 1ml of extract solution (0.007812 - 1 mg ml^{-1}) or 1 ml of the tannic acid solutions and 1ml of distilled water (for the blank) were separately added to 1 ml of Folin-Ciocalteu reagent (1:10 diluted with distilled water) in test tubes.1ml of 2 % w/v sodium bicarbonate solution was added to each mixture. The reaction mixtures were then incubated at room temperature for 2 h. The mixtures were then centrifuged at 600 g for 10 min to obtain a clear supernatant. The absorbance of the supernatants were then determined in triplicates at 760 nm using the UVvisible spectrophotometer (LKB Biochrom, Cambridge, England, Model 4050) against the blank solution. The total phenolic contents were expressed as milligrams per millilitre of tannic acid equivalents (TAEs).

2.2.4.1.2 TOTAL ANTIOXIDANT CAPACITY (PHOSPHOMOLYBDATE METHOD)

3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium carbonate and 4mM ammonium molybdate) was pipetted into test tubes. 1 ml extract (0.007812 - 1 mg ml^{-1}) was then added to the same test tubes, and incubated at 95 ºC for 90 min. After cooling to room temperature the

absorbance of each test tube was measured at 695 nm using the UV-visible spectrophotometer (LKB Biochrom, Cambridge, England, Model 4050) against the blank.

The blank was prepared by adding 3 ml methanol and 0.3 ml reagent and incubating as described above. All measurements were done in triplicates. Ascorbic acid (0.000976 - 0.062500 mg ml^{-1}) was used as the standard and the total antioxidant capacity was expressed as mg per ml of ascorbic acid equivalents (AAE).

2.2.4.1.3 DPPH SCAVENGING ASSAY

The experiment is based on the reduction of DPPH in the presence of an antioxidant observed as a change in colour from purple to yellow. 2, 2'-diphenyl-1-picrylhydrazyl is a stable free radical which gives a maximum absorption at 517 nm. DPPH has an unpaired valence electron which pairs off with another electron when it comes in contact with an antioxidant and is reduced from DPPH (Purple) radical to the $DPPH_2$ form (yellow). The more the intense the yellow colour the more the reducing ability (Sharma *et al.*, 2009). The residual DPPH is then measured through UV spectrophotometry at 517 nm. Absorbance decreases with increasing free radical scavenging activity (Singleton *et al.*, 1999).

The extracts (0.031250 - 1 mg ml^{-1} in methanol) was compared to ascorbic acid (0.0009760.015630 mg ml^{-1} in methanol) as standard free radical scavenger.1ml of either extract was added to 3 ml methanolic solution of DPPH (20 mg ml^{-1}) in a test tube. The reaction was then allowed to proceed for 30 min at 25˚C. The residual DPPH was then quantified from its absorbance at 517 nm in a spectrophotometer. The percentage inhibition was then extrapolated and used to estimate the concentration that results in 50 % reduction in the initial DPPH concentration denoted to as inhibitory concentration (IC_{50}).

2.2.4.2 IN VIVO TESTS

Lung tissues from all the treatment and control groups in carrageenan-induced pleurisy experiment were stored immediately at −80 °C till analysis. Lung tissues were homogenized using a Potter-Elvehjem homogenizer (Ultra-Turrax T25, Janke & Kunkel IKA- Labortechnik, Staufen, Germany) in ice-cold 0.01 M Tris–HCl buffer (pH 7.4) to give a 10 % homogenate. The homogenate was used for myeloperoxidase (MPO), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) assays. The total protein content of the lung tissue homogenate was quantified using the Vitalab Flexor EL 150 (Vital Scientific NV Netherland) chemistry analyser. The protein concentrations were used for the determination of the specific activities of enzymes (SOD, CAT and MPO) and GSH
levels.

2.2.4.2.1 MYELOPEROXIDASE ASSAY (MPO)

Myeloperoxidase activity is an index of neutrophil accumulation. The influence of F4 on MPO activity in lung tissues was measured according to the method described by (Bradley *et al.*, 1982). The assay mixture consisted of 0.3 ml 0.1 M phosphate buffer (pH 6.0), 0.3 ml 0.01 M H_2O_2, 0.5 ml 0.02 M *o*-dianisidine (freshly prepared) in deionized water and 10 µl lung homogenate supernatant in a final volume of 3.0 ml. The supernatant was added last and the change in absorbance at 460 nm was monitored every 1 min for 10 m with a micro plate reader (Synergy H1 Multi-Mode plate reader, Winooski, VT, USA). All measurements were carried out in triplicate. MPO activity was explained as one unit that increases absorbance at a rate of 0.001 min^{-1} and specific activity was expressed as units/mg protein.

2.2.4.2.2 GLUTATHIONE ASSAY (GSH)

Glutathione was assayed as described by (Ellman, 1959). Aliquots 0.1 mL of 10 % tissue homogenate were mixed with 2.4 mL of 0.02 M EDTA solution and kept on ice bath for 10

minutes. Then 2 mL of distilled water and 0.5 mL of trichloroacetic acid (TCA) 50 % (w/v) and centrifuged at 3000 × g for 20 minutes at 4 °C to remove precipitate. The supernatants 1 mL were then mixed with 2.0 mL of Tris buffer (0.4 M, pH 8.9) and 0.05 mL of 5' - dithiobisnitro benzoic acid (DTNB) solution – Ellman's reagent (10 mM) was added and swirled carefully. The absorbance was measured at 412 nm against a reagent blank with no homogenate after addition of DTNB and incubation at room temperature for 5 minutes. Glutathione was then quantified from a glutathione standard curve and expressed as µM/mg of protein.

2.2.4.2.3 CATALASE ACTIVITY (CAT)

Catalase activity was measured as described by (Aebi, 1984). It was determined by measuring the reduction in hydrogen peroxide (20 s interval) concentration at 240 nm for 60 s. Medium consists of 130 µl 50 mM potassium buffer (pH 7.0) and enzyme extract; 65 µl of 10 mM H_2O_2. The blank had 65 µl of the potassium phosphate and 130 µl of sample. The concentration of H_2O_2 was estimated from the absorbance using the following equation:

$$[H_2O_2\ mM] = \frac{Absorbance_{240\ nm}\ x\ 1000}{39.4\ mol^{-1}cm^{-1}}$$

Where 39.4 $mol^{-1}cm^{-1}$ is the molar extinction coefficient for H_2O_2. CAT activity was expressed as U/mg protein.

2.2.4.2.4 SUPEROXIDE DISMUTASE (SOD)

SOD activity was determined as described by (Misra *et al.*, 1972). It is based on the ability of SOD to inhibit autoxidation of adrenaline to adrenochrome. 0.5 ml of tissue homogenate, 0.75 ml of ethanol and 0.15 ml of chloroform (4 °C) were combined in mixture. The mixture was then centrifuged at 2000 rpm for 20 min. Subsequently, 0.5 ml of supernatant, 0.5 ml of 0.6

mM EDTA solution and 1 ml of carbonate bicarbonate buffer (0.1 M, pH 10.2) were added. The reaction was commenced by adding 0.05 ml of 1.3 mM adrenaline and the increase in absorbance at 480 nm due to the adrenochrome formation was measured with a micro plate reader (Synergy H1 Multi-Mode plate reader, Winooski, VT, USA). One unit of SOD activity was defined as the amount of protein causing 50 % inhibition of the autoxidation of adrenaline at 25 °C.

$$\% \ inhibition = \frac{Absorbance_{test} - Absorbance_{reference}}{Absorbance_{test}} x\ 100$$

$$Units\ of\ activity\ per\ mg\ protein = \frac{\% \ inhibition}{50\ x\ weight\ of\ protein}$$

2.2.5 LIPID PEROXIDATION

2.2.5.1 MALONDIALDEHYDE (MDA) ASSAY

Lipid peroxidation is an important marker of oxidative stress in pleuritis. Carrageenan induced pleurisy and lipid peroxidation (as analysed by MDA) have been previously associated (Draper *et al.*, 1993).

The extent of lipid peroxidation in lung tissues was assessed by measuring MDA as described by (Heath *et al.*, 1968). 3 ml of 20 % trichloroacetic acid containing 0.5 % thiobarbituric acid was added to a 1 ml aliquot of lung homogenate supernatant in a test tube. The mixture obtained was heated in a water bath (95 °C) for 30 min and then allowed to cool. The test tube was then centrifuged at 10,000×g for 10 min, and the absorbance of the supernatant at 532 nm measured. The value for the nonspecific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of MDA was calculated using MDA's extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.2.3.8 ANALYSIS OF DATA

Raw scores for right paw volumes were individually normalized as percentage of change from their values at time 0, and then averaged for each treatment group. The time-course curves for paw volume were subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni's post hoc test. Total foot volume for each treatment was calculated in arbitrary units as the area under the curve (AUC) and to determine the percentage inhibition for each treatment, the following equation was used.

$$\%\ inhibition\ of\ edema = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}}\right) x\ 100$$

Differences in AUCs were analysed by ANOVA followed by Dunnett's Multiple Comparison Test. ED_{50} (dose responsible for 50 % of the maximal effect) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$y = \frac{a + (b - a)}{(1 + 10^{(logED_{50} - x)})}$$

Where, X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape. The fitted midpoints (ED_{50}s) of the curves were compared statistically using F test (Miller, 2003). All statistical analysis and ED_{50} calculations were conducted with GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant. Data was presented as the effect of drugs on the time course and the total oedema response for 5 h using GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA,

USA).

CHAPTER THREE

RESULTS

3.1 TLC PROFILE OF F1, F2 AND F3

Thin layer chromatography was performed on F1, F2 and F3 in petroleum ether-ethyl acetate (90:10) solvent. It was observed that F2 and F3 had similar TLC profiles which indicates the elution of constituents with similar Rf values. Ethyl acetate and petroleum ether are non-polar solvents, hence it is possible they eluted similar phytochemicals. From the above observations, extracts, F2 and F3 were combined to form F4. F4 was then used for subsequent experiments after the carrageenan-induced paw oedema experiment.

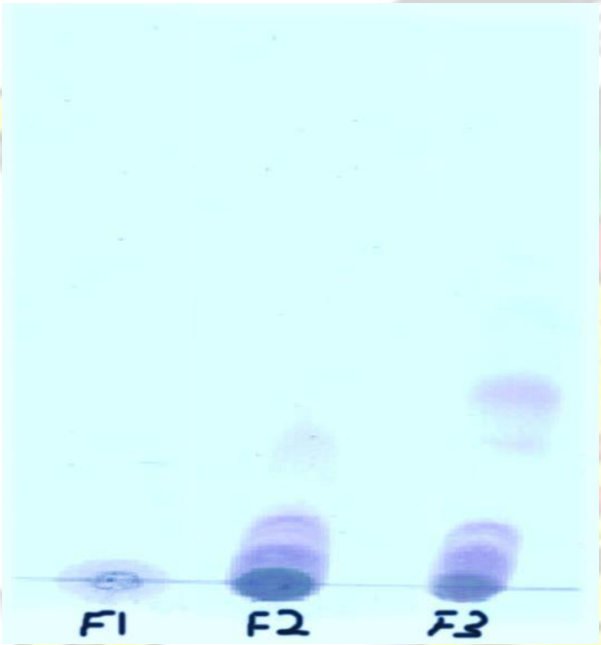

Plate 3.1 TLC profile of F1, F2 and F3 in petroleum ether: chloroform (90:10) solvent.

3.2 CARRAGEENAN–INDUCED PAW OEDEMA

The carrageenan-induced rat paw oedema model has proven to be a reliable animal model for evaluating the anti-inflammatory effect of natural products (Huang *et al.*, 2012).

Sub plantar injection of 50 µl of 1 % (w/v) carrageenan solution into the rat hind paw produced a sustained and measurable oedema which peaked around the 3-4 h and was sustained for 5 h as shown in figure 3.1a,c. and e and figure 3.2 a and c respectively.

Two-way ANOVA (treatment x time) showed a significant effect of drug treatment for F1 ($F_{15, 96}$= 5.71, P=0.0001), F2 ($F_{15, 96}$= 3.16, P=0.0003), F3 ($F_{15, 96}$=6.49, P=0.0001) diclofenac ($F_{15, 96}$= 9.16, P=0.0001) and dexamethasone ($F_{15, 96}$=7.31, P=0.0001) as shown in figure 3.1 a, c and e and Figure 3.2 a and c respectively.

From the total oedema for each treatment expressed in arbitrary units as area under the time course curves (AUC) , F1 and F2 (30-300 mg kg^{-1}) dose dependently and significantly {F1 ($F_{3, 16}$= 12.01, P=0.0002), F2 ($F_{3, 16}$= 10.9, P=0.0004)} reduced the total paw oedema with maximal effect of 73.45 % and 78.4 % respectively (Fig 3.1b and d). F3 also significantly ($F_{3, 16}$= 16.96, P=0.0001) reduced total paw oedema but the effect was not dose dependent with maximal effect of 68.3 % at 300 mg kg^{-1}(Fig 3.1f). The NSAID, diclofenac (10-100 mg kg^{-1}) significantly ($F_{3,16}$=33.95, P=0.0001) and dose dependently reduced the oedema with a maximal effect of 93 % at 100 mg kg^{-1} (Fig 3.2b). Similarly dexamethasone (0.3-3 mg kg^{-1}), a steroidal anti-inflammatory agent, also inhibited the carrageenan-induced oedema significantly ($F_{3,16}$=35.25, P=0.0001) with maximal effect of 83.79 % at 3 mg kg^{-1} (Fig 3.2d).

From the ED_{50} calculated from the dose-response curves (figure 3.3). F1 (ED_{50} = 20.0 ± 12.7) and F2 (ED_{50} = 34.6 ± 6.5) were found to be approximately 2.14× and 3.71× less potent than

diclofenac (ED_{50} = 9.4 ± 5.9) and 745.06× and 1290.28×less potent than dexamethasone (ED_{50} = 0.03 ± 4.7) respectively. F3 (ED_{50} = 6.01 ± 6.7) was found to be approximately 1.56× more potent than diclofenac and 223.74× less potent than dexamethasone.

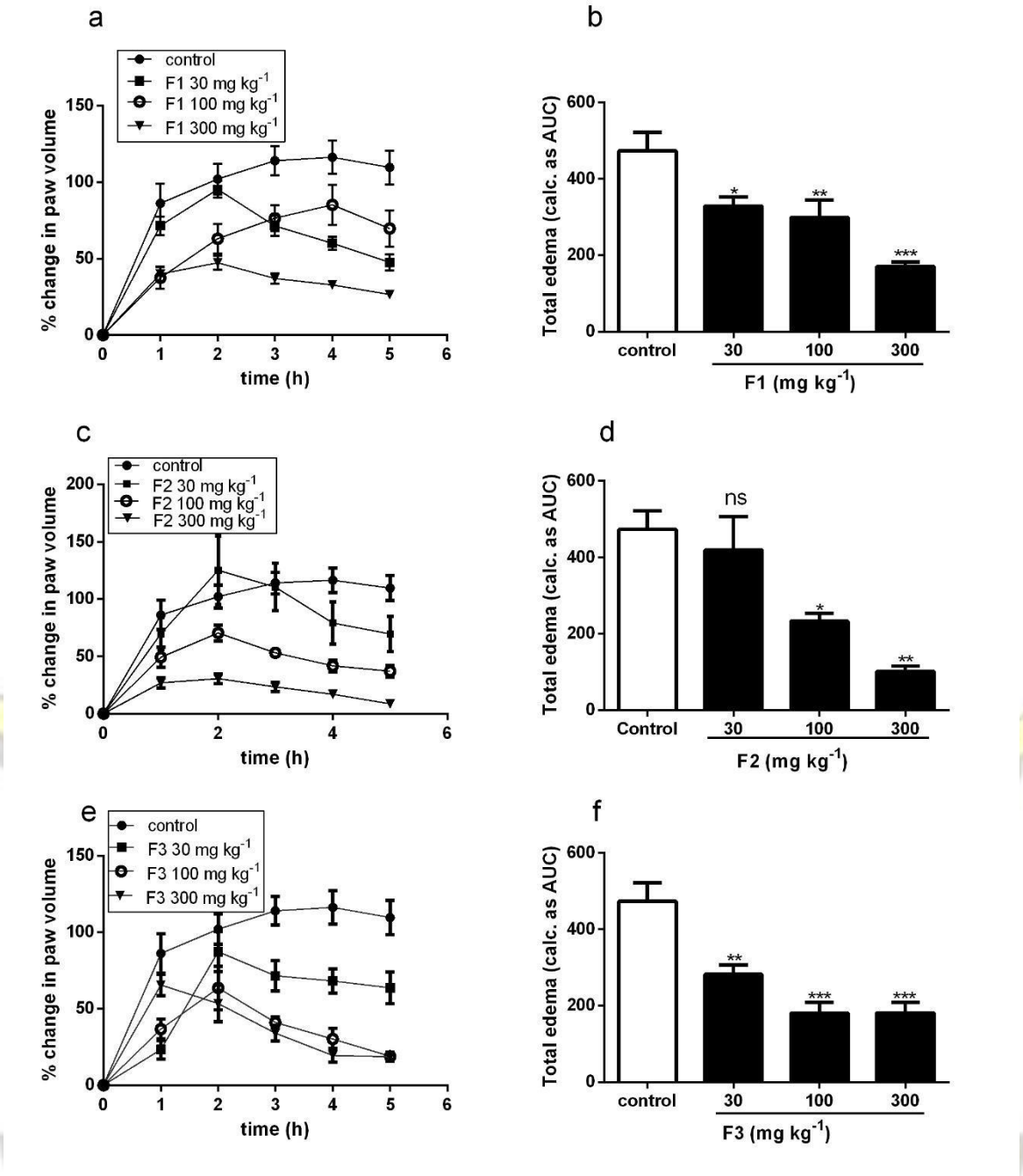

Figure 3.1 Effects of F1, F2 and F3 (10-300 mg kg^{-1}; *p.o.*), on time course curve (a, c and e respectively) and the total oedema response (b, d and f respectively) in carrageenan-induced paw oedema in rats. Values are means ± S.E.M (n=5) ***P<0.0001; ***P<0.0003, ***P<0.0001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). ***P<0.0001;***P<0.0004, ***P<0.0001 compared to vehicle treated group (One-way ANOVA followed Dunnett's multiple comparison test).ns=not significant.

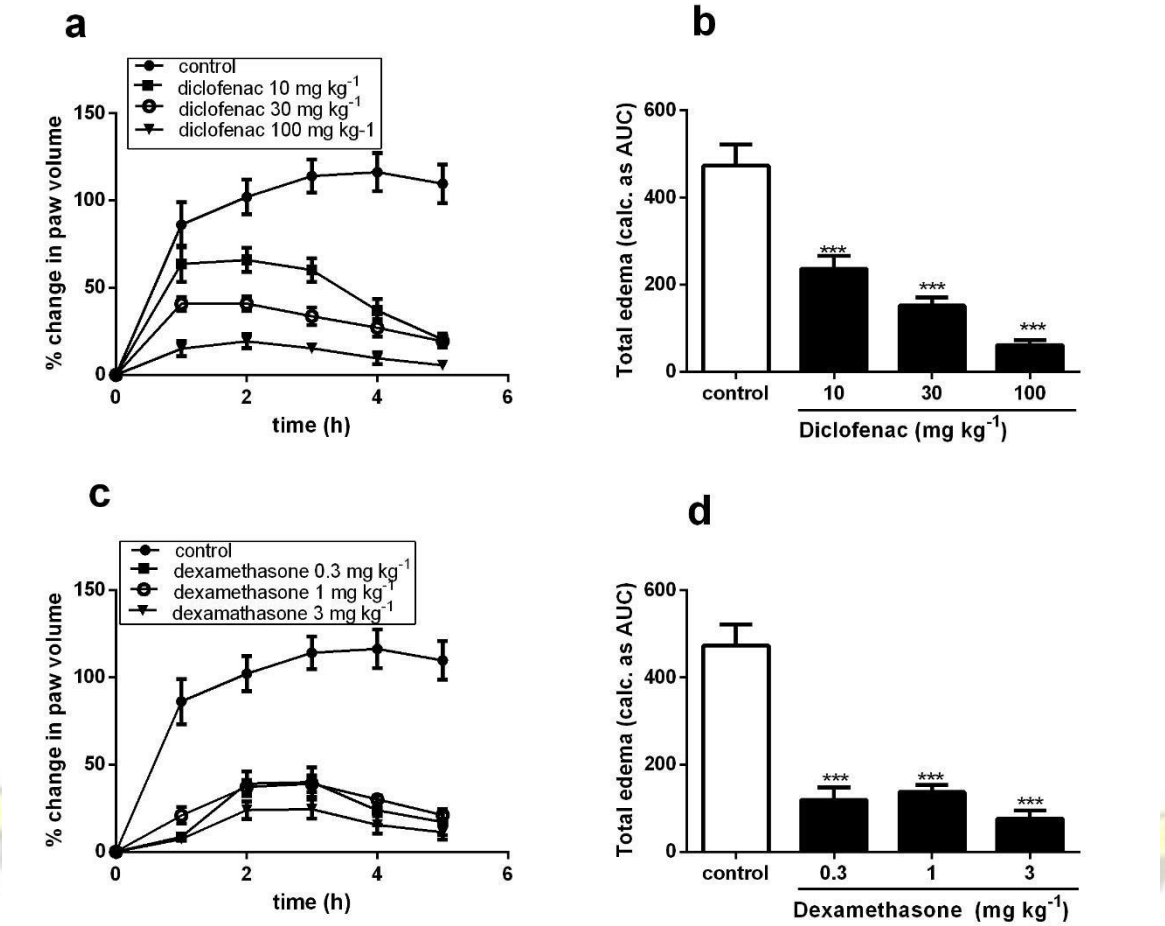

Figure 3.2 Effects of diclofenac (10-100 mg kg^{-1} i.p.) and dexamethasone (0.3-3 mg kg^{-1} i.p.) on time curves (a and c) respectively and total oedema responses (b and d) respectively in carrageenan-induced paw oedema. Values are means ± S.E.M (n=5) ***P<0.0001; ***P<0.0001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). ***P<0.0001;***P<0.0001 compared to vehicle treated group (One-way ANOVA followed Dunnett's multiple comparison test).

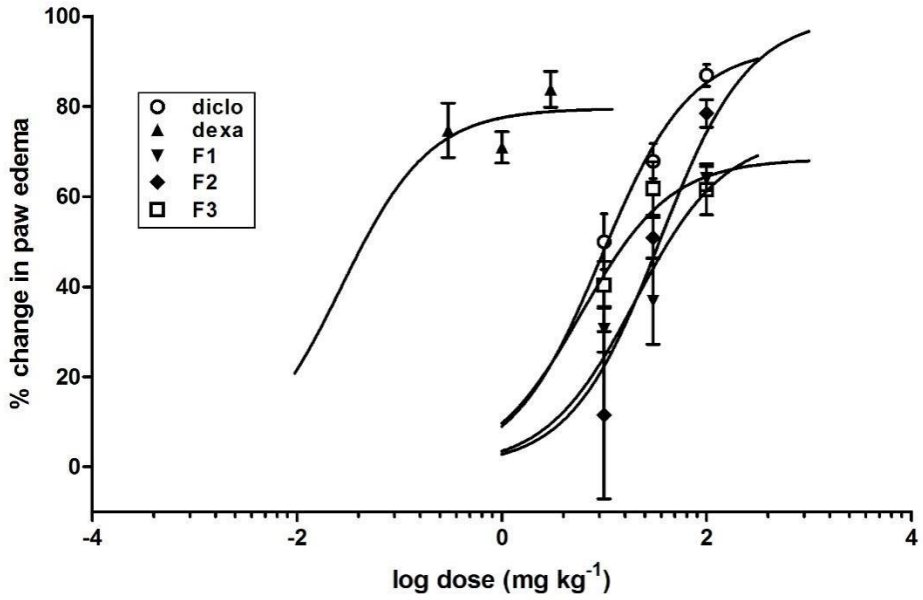

Figure 3.3 Dose response curves for dexamethasone (0.3-3.0 mg kg^{-1} i.p.), diclofenac (10100 mg kg^{-1} i.p.) and F1, F2 and F3 (30-300 mg kg^{-1} *p.o*) on carrageenan-induced paw oedema in rats.

3.3 HISTAMINE-INDUCED PAW OEDEMA

Histamine is known to be involved in the mediation of the first phase of inflammation induced by carrageenan (Di Rosa *et al.*, 1971; Batista *et al.*, 2014).

Injection of histamine resulted in the swelling of the right hind paw of rats within 30 min. Figure 3.4 shows the time course curves and the total oedema response for the effects of F4 and chlorpheniramine in histamine-induced paw oedema. After treatment with the standard drug and the extract, it was observed that F4 (30-300 mg kg^{-1}) treated groups did not exhibit significant reduction (P>0.05) in the histamine-induced paw oedema.(Fig. 3.4).The selective histamine antagonist used chlorpheniramine (10 mg kg^{-1}), caused significant ($F_{3,16}$=11.13, $P\leq$ 0.0007) oedema reduction.

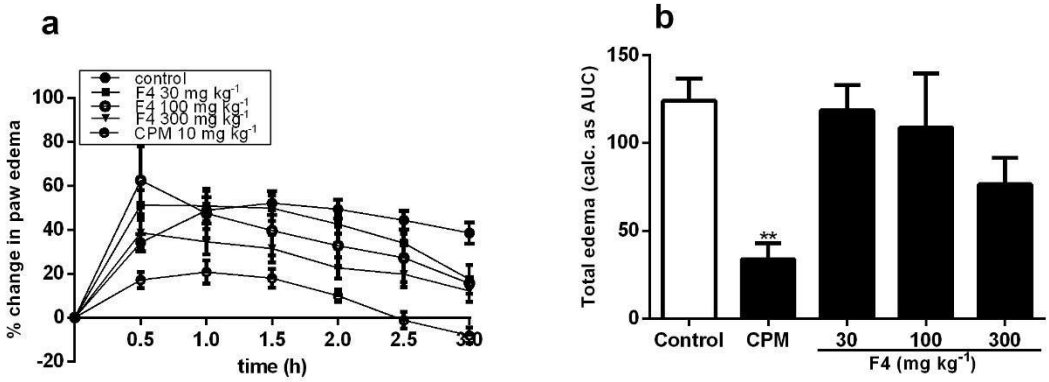

Figure 3.4 Effects of F4 (30-300 mg kg^{-1} *p.o.*) and chlorpheniramine (10 mg kg^{-1} *p.o.*) on time course curve (Fig.3.4a) and the total oedema response (Fig. 3.4b) for 3 h in histamineinduced paw oedema in rats. Values are means ± S.E.M (n=5).*P*> 0.05 ns; ***P*< 0.006 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). *P*>0.05 ns;***P*<0.007 compared to vehicle-treated group (One-way ANOVA followed by Dunnett's multiple comparison test.

3.4 SEROTONIN-INDUCED PAW OEDEMA

Serotonin is involved in the mediation of the first phase of inflammation induced by carrageenan (Holsapple *et al.*, 1980; Albayrak *et al.*, 2013).

After serotonin injection, swelling of the right hind paws of the rats was observed within 30 min and then monitored every 30 min for 3 h. Figure 3.5 shows the time course curves and the total oedema response for the effects of F4, and granisetron in serotonin-induced paw oedema. The oedema peaked in 1.5 h and was then monitored for 3 h. F4 (30 mg kg^{-1}) did not show any significant effect (*P*>0.05). However, F4 (100 and 300 mg kg^{-1}) and granisetron (30 µg kg^{-1}) showed significant activity when compared with vehicle treated group. ($F_{4, 20}$ =5.6, *P*< 0.003), Figure 3.5b, using statistical analysis-one way ANOVA.

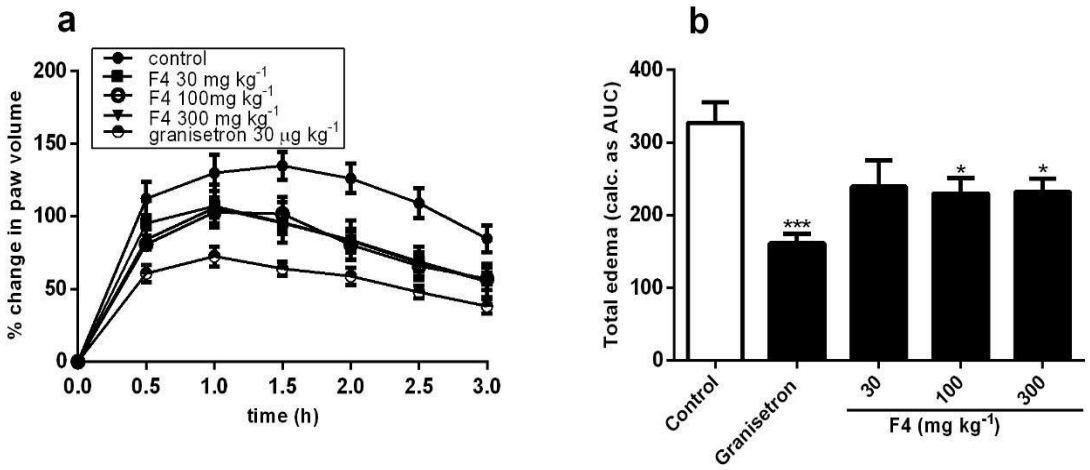

Figure 3.5 Effects of F4 (30-300 mg kg^{-1} *p*.o.) and granisetron (30 µg kg^{-1} *p.o*.) on time course curves (Fig.3.5a) and the total oedema responses (Fig. 3.5b) for 3 h in serotonininduced paw oedema in rats. Values are means ± S.E.M (n=5). **P*<0.04,**P*<.0.02; ****P*< 0.005 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). **P*<0.038, **P*<0.04; ****P*< 0.0005 compared to vehicle-treated group (One-way ANOVA followed by Dunnett's Multiple Comparison test.

3.5 BRADYKININ-INDUCED PAW OEDEMA

Injection of Bradykinin resulted in the swelling of the right hind paws of the rats within the first 30 min. From the time course curves, the oedema peaked within 1 h and dropped gradually every 30 min up to the 3rd h in the controls (Fig.3.6a). Two-way ANOVA showed that F4 (30300 mg kg^{-1}) significantly ($F_{18, 96}$ = 3.722, *P*<0.0001) reduced paw oedema which peaked after 30 m relative to the controls. After treatment with the extract, it was observed that F4 (30-300 mg kg^{-1}) treated groups exhibited significant reduction ($F_{3,16}$=14.34, *P*<0.0001) in total paw oedema relative to the controls (Figure 3.6b).

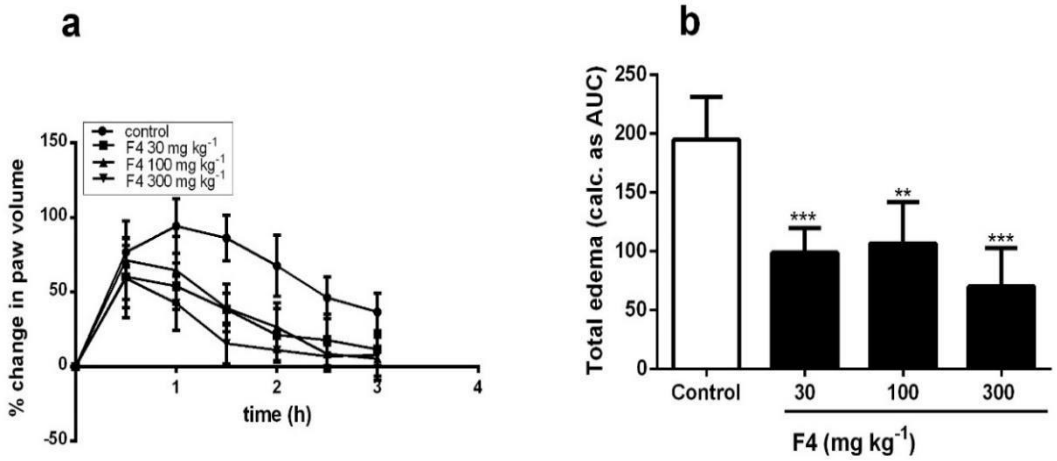

Figure 3.6 Effects of F4 (30-300 mg kg^{-1} *p.o.*) on time course curve (a) and the total oedema response (b) for 3 h in bradykinin-induced paw oedema in rats. Values are means ± S.E.M (n=5). ***P<0.0002, **P<.0.008, ****P< 0.0001 compared to vehicle-treated group (Twoway ANOVA followed by Bonferroni's post hoc test). ***P<0.0006, **P<0.001, ***P< 0.0001 compared to vehicle-treated group (One-way ANOVA followed by Dennett's Multiple Comparison test.

3.6 PROSTAGLANDIN E_2-INDUCED PAW OEDEMA

After prostaglandin challenge, swelling of the right hind paws of the rats was observed within 30 min and then monitored every 30 min for 3 h. The oedema observed for the control rats was sustained and kept increasing for the 3 h duration (Fig 3.7a).Two-way ANOVA showed that F4 (30-300 mg kg^{-1}) and diclofenac significantly ($F_{24, 120}$ = 11.88, P< 0.0001) reduced paw oedema relative to the controls (Fig. 3.7a). F4 (30-300 mg kg^{-1}) and diclofenac (10 mg kg^{-1}) also exhibited significant reduction in total paw oedema relative to control group. ($F_{4, 20}$ =8.59, P< 0.0003), using statistical analysis-one way ANOVA (Fig. 3.7B).

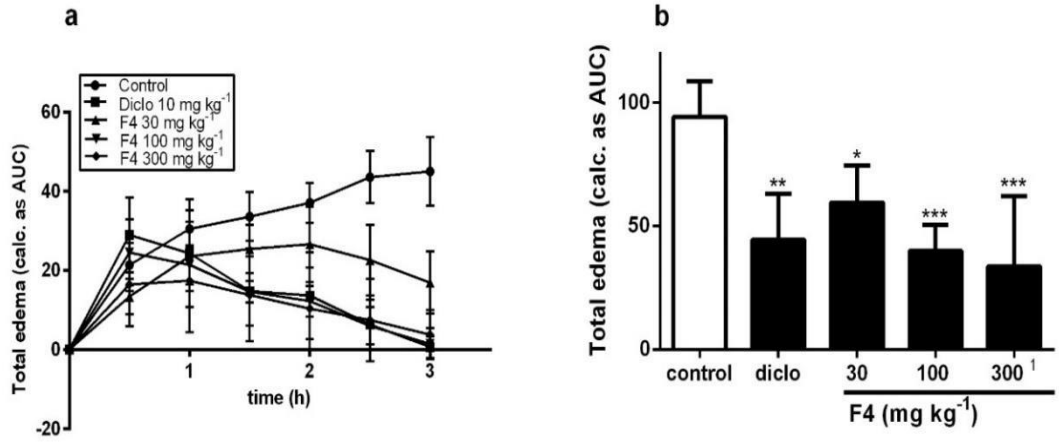

Figure 3.7 Effects of F4 (30-300 mg kg^{-1} *p.o.*) and diclofenac (10 mg kg^{-1} i.p.) on time course curve (a) and the total oedema response (b) for 3 h in prostaglandin-induced paw oedema in rats. Values are means ± S.E.M (n=5), ***P<0.0005, **P<0.004, ****P<0.0001, **** P< 0.0001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). **P<0.001,*P<0.03, ***P<0.0006, ***P<0.0002 compared to vehicle-treated group (One-way ANOVA followed by Dunnett's Multiple Comparison test.

3.7 CARRAGEENAN-INDUCED RAT PLEURISY

The pleurisy model is a widely accepted experimental model employed in the study of acute and subacute inflammation. It allows the assessment of multiple events in the inflammatory process simultaneously (Seegers, 2014).

Injection of carrageenan into the pleural cavity of rats elicited an inflammatory response within 6 h, characterized by the accumulation of fluid that contained large numbers of inflammatory cells (Fig 3.8). F4 at 300 mg kg^{-1} inhibited the inflammatory response as demonstrated by the significant attenuation of exudate formation ($F_{7,20}$=10.84, P<0.0001) and neutrophil infiltration ($F_{7,20}$=8.86, P<0.0001) with maximal effect of 64.2 % and 90.9 %, respectively (Fig 3.8). Diclofenac (100 mg kg^{-1}) as a standard drug also significantly reduced exudate formation ($F_{7,20}$=14.96, P<0.0001) and Neutrophil infiltration ($F_{7,20}$=9.18, P<0.0001) almost completely with maximal effect of 75 % and 96.6 %, respectively (Fig 3.8).

From the ED_{50} calculated from the dose response curves (Fig 3.9a and 3.9b), F4 was found to be approximately 3.8× less potent (ED_{50}=12.26±3.90) than diclofenac (ED_{50}=3.22±4.45) in reducing neutrophil infiltration and 3.5x less potent (ED_{50}=60.57±15.82) than diclofenac (ED_{50}=17.13±17.11) in reducing exudate formation. (Table. 3.1).

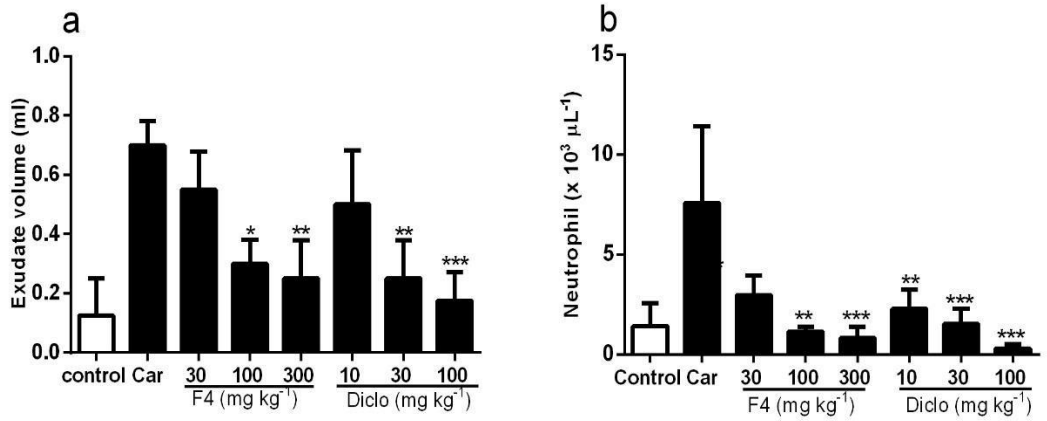

Figure 3.8 Effects F4 (30-300 mg kg^{-1} *p.o.*) and Diclofenac (10-100 mg kg^{-1} i.p.) on exudate volume and neutrophil accumulation in the pleural cavity were assessed 6 h after carrageenan injection. Data are expressed as mean ± SEM. n=5, **** P<0.0001. **** P<0.0001 vs. carrageenan only treated group. (One-way ANOVA followed by Dunnett's Multiple Comparison test.

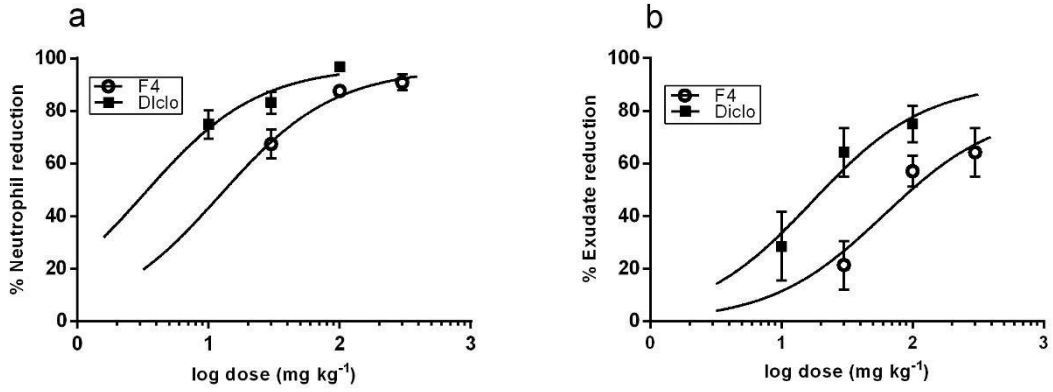

Figure 3.9 Dose-response curves for diclofenac (10-100 mg kg^{-1} i.p) and F4 (30-300 mg kg^{-1} *p.o*) on neutrophil counts and exudate volumes.

Table 3.1 ED_{50} values for carrageenan induced pleurisy in rats.

	Neutrophil ED_{50}(mg kg^{-1})	Exudate ED_{50}(mg kg^{-1})
F4	12.26±3.90	60.57±15.82
Diclofenac	3.22±4.45	17.13±17.11

3.8 HISTOPATHOLOGY

Histological examinations aid in the identification and assessment of microscopic morphological changes in cells and tissues. Serial sections of rat lung tissues were stained with haematoxylin and eosin and evaluated microscopically for neutrophil infiltration, oedema, hyperaemia and alveolar septal thickening.

Vehicle-only treated rats showed normal lung architecture (Plate 3.2A) with little or no signs of neutrophil infiltration (green arrow), oedema (blue arrow), hyperaemia (red arrow) and alveolar septal thickening(white arrow) (Fig 3.10). Vehicle + carrageenan treated rats showed extensive disorganization of alveolar structures (Plate 3.2B) with significant presence of neutrophils, oedema, hyperaemia and alveolar septal thickening (Fig 3.10). When rats were treated with F4 (300 mg kg^{-1}), there was reduced alveolar structural disorganization (Plate 3.2C). This presented with a significantly (P<0.0001) suppressed neutrophil infiltration, oedema, hyperaemia and alveolar septal thickening (Fig 3.10). Rats treated with diclofenac + carrageenan (Plate 3.2D) showed significant (P<0.0001) suppression of neutrophil infiltration with minor or no oedema, hyperaemia and alveolar septal thickening when compared with carrageenan only treated rats (Fig 3.10).

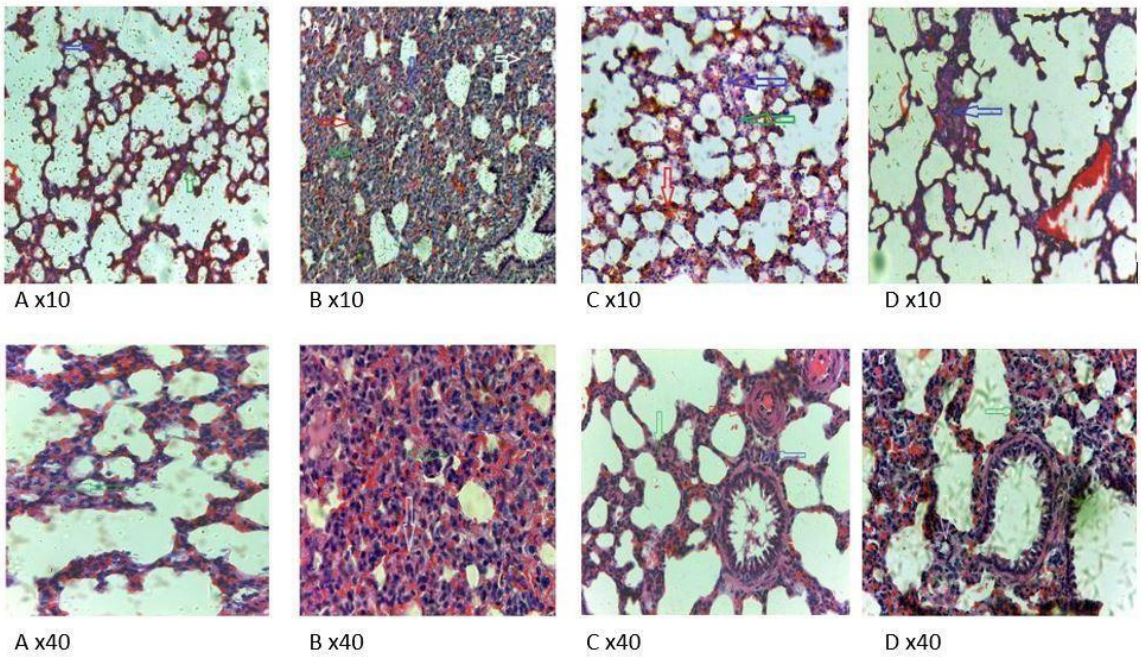

Plate 3.2 Histopathological study in carrageenan induced pleurisy in rats. Histological slides showing vehicle only (A), vehicle + carrageenan (B), F4 (300 mg kg^{-1}) + carrageenan (C), diclofenac (100 mg kg^{-1}) + carrageenan (D).

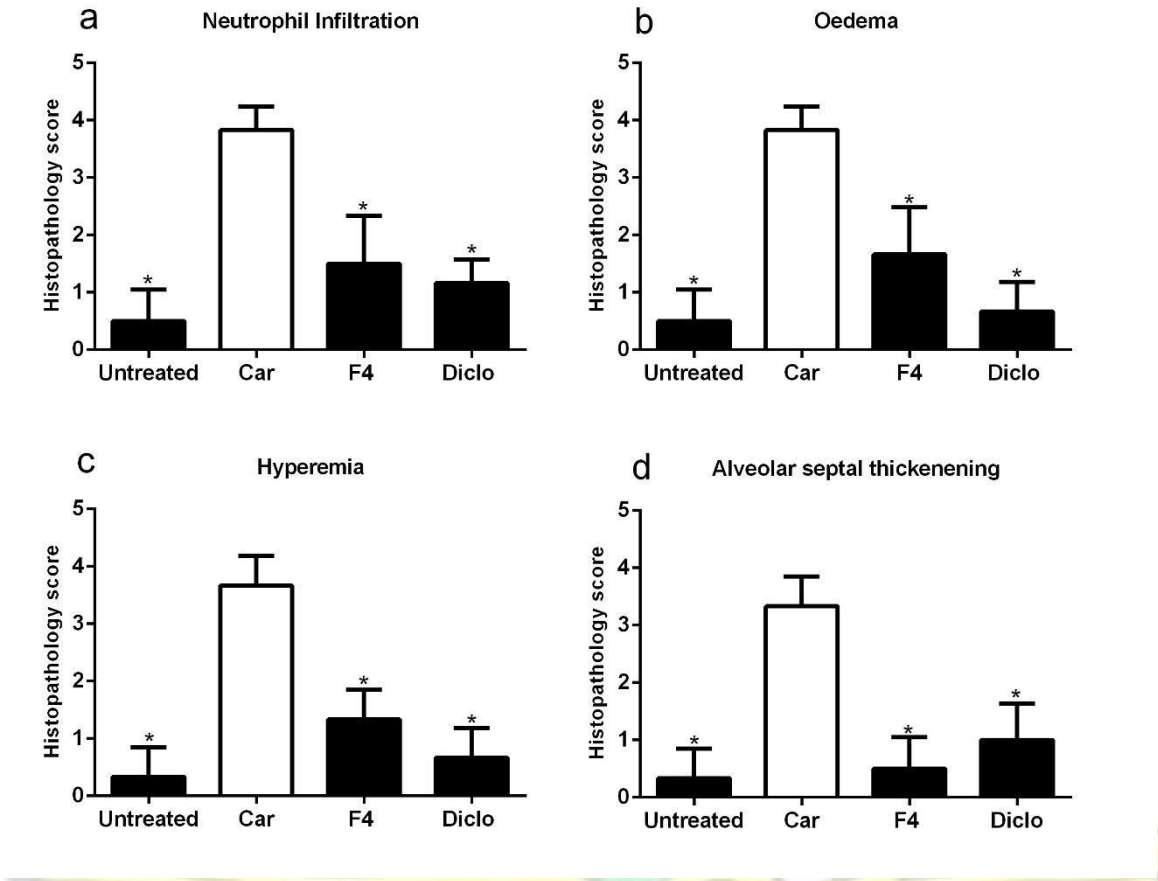

Figure 3.10 Histological scoring of lung injury in carrageenan induced pleurisy**.** F4 and Diclofenac reduced lung injury significantly based on the following scoring indices: neutrophil infiltration (**P*<0.0001), Oedema (**P*<0.0001), Hyperaemia (**P*<0.0001) and alveolar septal thickening (**P*<0.0001) compared to carrageenan only treated groups. The degree of the lung damage was scored on a scale of 0–4. (i.e. 0 – Not present, 1 – Very mild, 2 – Mild, 3 – Moderate, 4 – Extensive).

3.9 ACETIC ACID-INDUCED VASCULAR PERMEABILITY

It has been well established that the intraperitoneal injection of acetic acid greatly enhances vascular permeability and facilitates the vascular leakage of Evans blue dye (Nidavani *et al.*, 2014).

Injection of acetic acid into the peritoneum of mice previously injected intravenously with 2 % Evans blue dye exhibited an inflammatory response after 30 min, characterized by the extravasation of Evans blue dye into the peritoneal cavity of mice. The effect of F4 on dye extravasation in peritoneal fluid after acetic acid challenge was determined with an Evans blue

standard curve as shown in figure 3.11a. There was a significant decrease in dye leakage in mice treated with F4 at 100 mg kg^{-1} (73 % inhibition, P<0.005) and 300 mg kg^{-1} (83 % inhibition, P<0.0025) when compared to controls. Diclofenac at a dose of 30 mg kg^{-1} also caused a significant decrease in dye leakage (78 % inhibition, P<0.003) when compared to controls (Figure 3.11b).

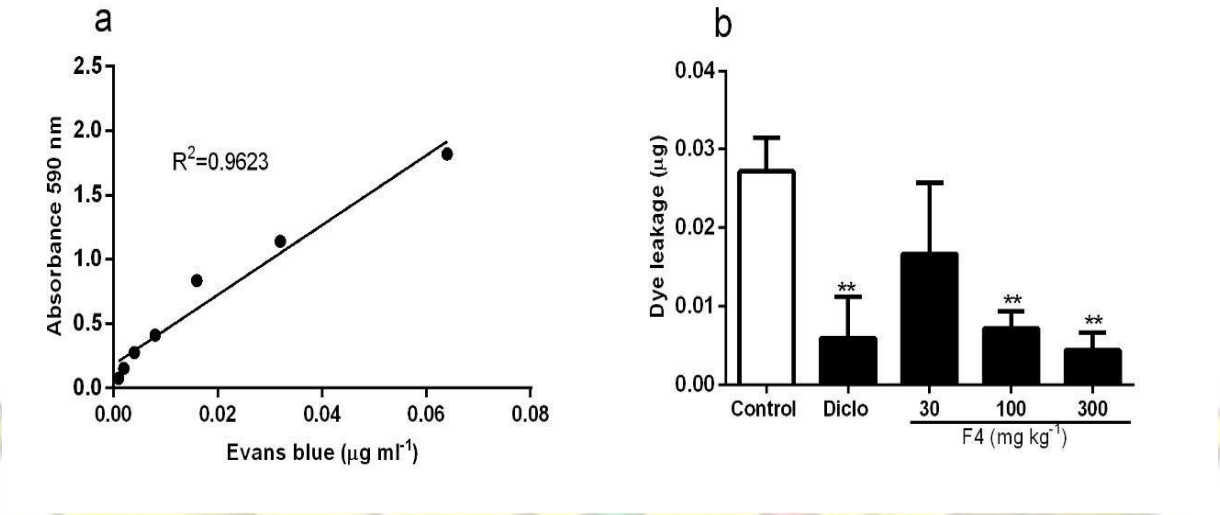

Figure 3.11 Evans blue dye standard curve (a) and Effects of F4 (30-300 mg kg^{-1} *p.o.*) and Diclofenac (30 mg kg^{-1} i.p.) on Evans blue dye extravasation into the peritoneal cavity of mice (b). Data are expressed as mean ± SEM. n=5, ** P<0.0035.vs. vehicle treated group. One-way ANOVA followed by Dunnett's Multiple Comparison test.

3.10 TOTAL PHENOL ASSAY

Polyphenols react with Folin-Ciocalteu reagent to form a blue complex that can be quantified with calorimetric methods. The intensity of the absorption depends on the alkaline solution and the concentration of the phenolic compounds present.

Total phenol content is reported as tannic acid equivalents by reference to the Standard curve, y = 7.112x + 0.08942. The Total phenol content was 88.8 and 97.24 mg g^{-1} of dried extract of F1 and F4 respectively. F4 had a higher phenol content compared F1.

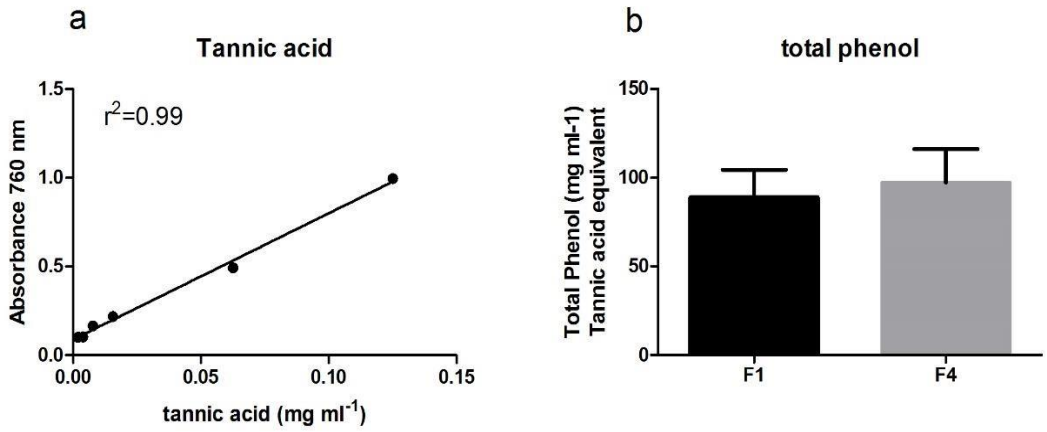

Figure 3.12 Absorbance of tannic acid (0.001953-0.125 mg ml^{-1}) (a) total phenolic content of F1 and F4 expressed as tannic acid equivalents.

3.11 TOTAL ANTIOXIDANT CAPACITY

In the presence of an antioxidant, Molybdenum (VI) is reduced to Molybdenum (V) which is observed as a green complex at acidic pH with a maximum absorption at 695 nm. The higher the antioxidant activity the higher the intensity of the green complex formed (Prieto *et al.*, 2001).

The total antioxidant capacity was calculated as ascorbic acid equivalent by reference to standard curve y = 5.401x + 0.01830 .The total antioxidant capacity of F1 and F4 was 76.01 and 114.2 mg g^{-1} of dried extract respectively

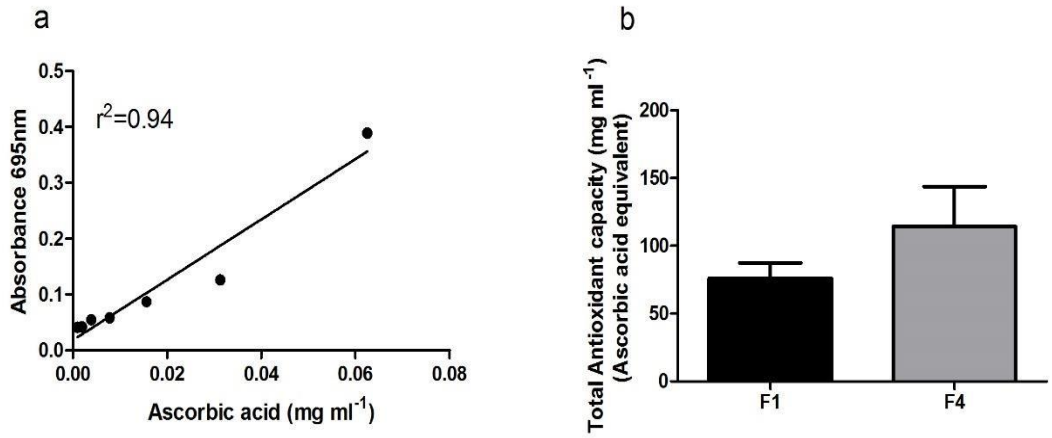

Figure 3.13 Absorbance of ascorbic acid (0.000976-0.015630 mg ml^{-1}) (a) and total antioxidant capacity of F1 and F4 expressed as ascorbic acid equivalents. (b)

3.12 DPPH SCAVENGING ASSAY

F1 and F4 extracts reduced, 2'-diphenyl-2-picrylhydrazyl (DPPH) solution to the yellow coloured product, diphenylpicrylhydrazine, and the absorbance of residual DPPH at 517 nm was then taken. The extracts, F1 and F4 and ascorbic acid exhibited concentration dependent free radical scavenging activity with F4 showing a higher activity as compared to F1 as shown in figure 3.14 and Table 3.3.

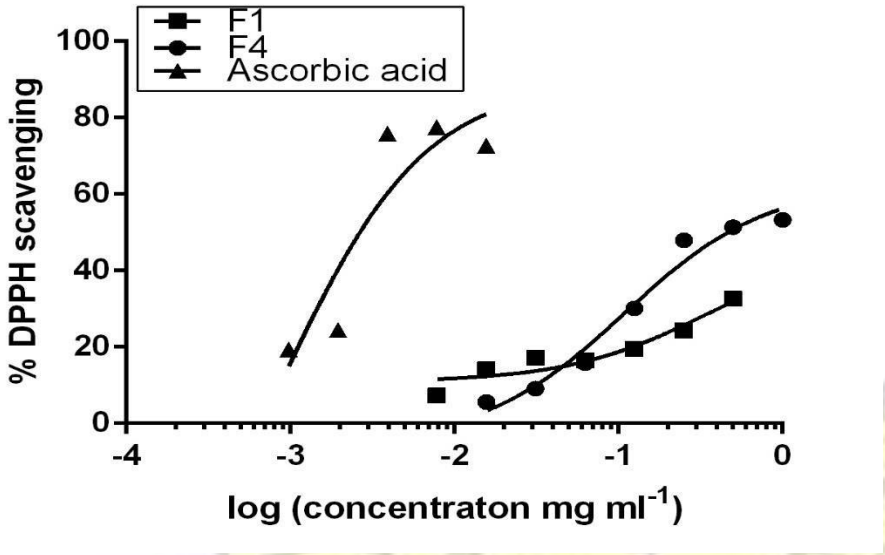

Figure 3.14 Free radical scavenging ability of the extracts, F1 and F4 (0.031250-1 mg ml^{-1}) compared to ascorbic acid (0.000976-0.015630 mg ml^{-1}) in the DPPH scavenging radical assay.

Table

3.2 IC_{50} values for extracts of *M. Angolensis* and ascorbic acid in the DPPH assay.

Drug	DPPH Scavenging IC_{50} (mg ml^{-1})
F1	2.655
F4	0.1088
Ascorbic acid	0.0009089

3.13 MYELOPEROXIDASE ASSAY (MPO)

Myeloperoxidase (MPO) activity, an index of neutrophil accumulation, was determined as previously described. The influence of F4 on myeloperoxidase (MPO) activity in lung tissues was measured according to the method described by (Bradley *et al.*, 1982).

MPO activity in lung tissues significantly increased after carrageenan injection. Compared to carrageenan only treated tissues, there was a significant (P<0.002) reduction in myeloperoxidase activity in F4 (30,100 and 300 mg kg^{-1}) treated lung tissues (Fig. 3.15). The standard drug diclofenac also showed significant (P<0.001) reduction in myeloperoxidase activity at 30 and 100 mg kg^{-1} compared to carrageenan only treated tissues (Fig. 3.15).

From the ED_{50} calculated from the dose response curves (figure 3.16), F4 (ED_{50} =8.49±2.9) was found to be approximately 2.1x less potent than diclofenac (ED_{50} =3.87±1.1) in reducing MPO expression (Table.3.3).

Table ED_{50}

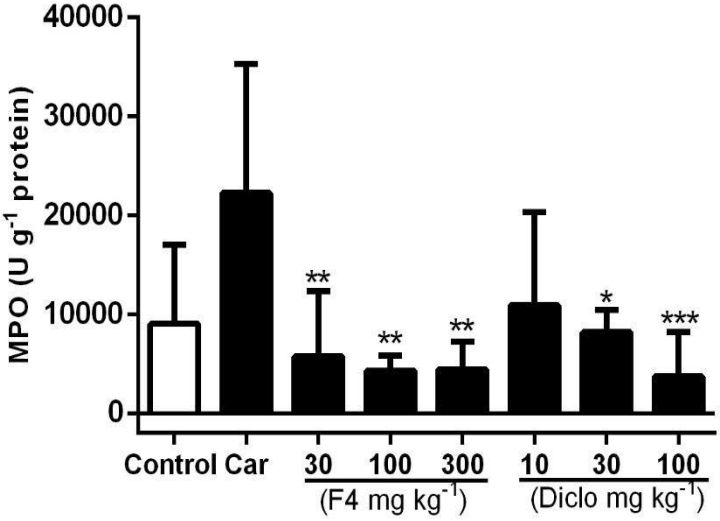

Figure 3.15 Effects F4 (30-300 mg kg^{-1} *p.o.*) and Diclofenac (10-100 mg kg^{-1} i.p.) on MPO activity were assessed from lung tissue 6 h after carrageenan injection. Data are expressed as mean ± SEM. n=5, **P*<0.01, ***P*<0.0012, *** *P*<0.0008 vs. carrageenan only treated group. (One-way ANOVA followed by Dunnett's Multiple Comparison test.

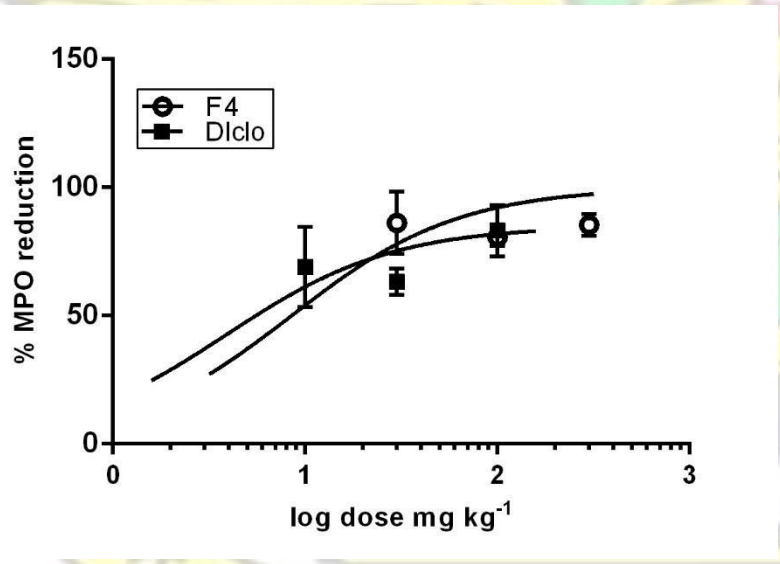

Figure 3.16 Dose-response curves for diclofenac (10-100 mg kg^{-1} i.p) and F4 (30-300 mg kg^{1} *p.o*) on MPO activity.

3.3 values for MPO activity

Test Drug	ED_{50}(mg kg^{-1})
F4	8.49±2.9
Diclofenac	3.872±1.12

3.14 GLUTATHIONE ASSAY (GSH)

Reduced glutathione in lung tissues significantly decreased after carrageenan challenge. Compared to carrageenan only treated lung tissues, there was a significant increase in GSH levels in 30,100 and 300 mg kg^{-1} F4 (P<0.0001) treated lung tissues (Figure 3.17b). The standard drug diclofenac showed significant increase in GSH levels at all doses (10- 100 mg kg^{-1}) compared to carrageenan only treated tissues (P<0.0001) (Figure 3.17b).

From the ED_{50} calculated from the dose response curves (Fig. 3.18), F4 (ED_{50}=7.286±3.87) was found to be approximately 2.4x more potent than diclofenac (ED_{50}= 15.72± 6.65) in increasing GSH expression (Table. 3.4).

Table ED_{50}

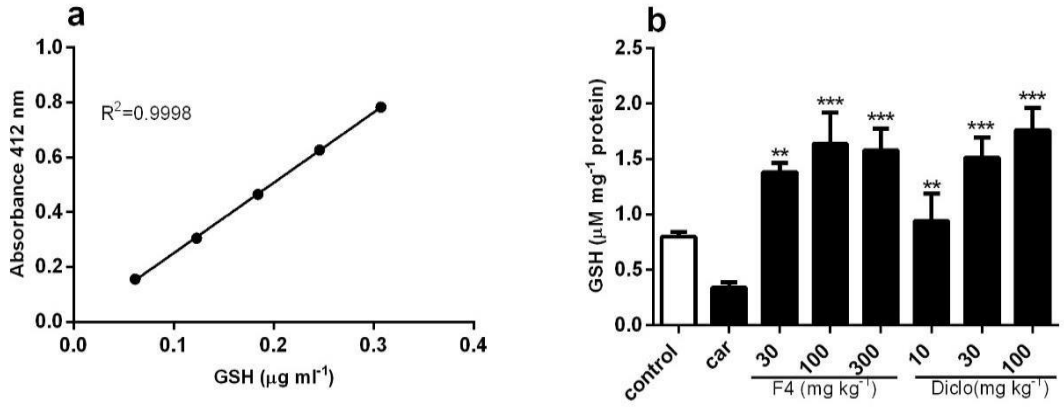

Figure 3.17 GSH Standard curve (0.0002-0.001 µg ml^{-1}) (a) Effects F4 (30-300 mg kg^{-1} *p.o.*) and Diclofenac (10-100 mg kg^{-1} i.p) on GSH levels were assessed from lung tissue 6 h after carrageenan injection. Data are expressed as mean ± SEM. n=5, ***P*<0.001, ****P*<0.0001 vs. carrageenan only treated group. (One-way ANOVA followed by Dunnett's Multiple Comparison test. (b)

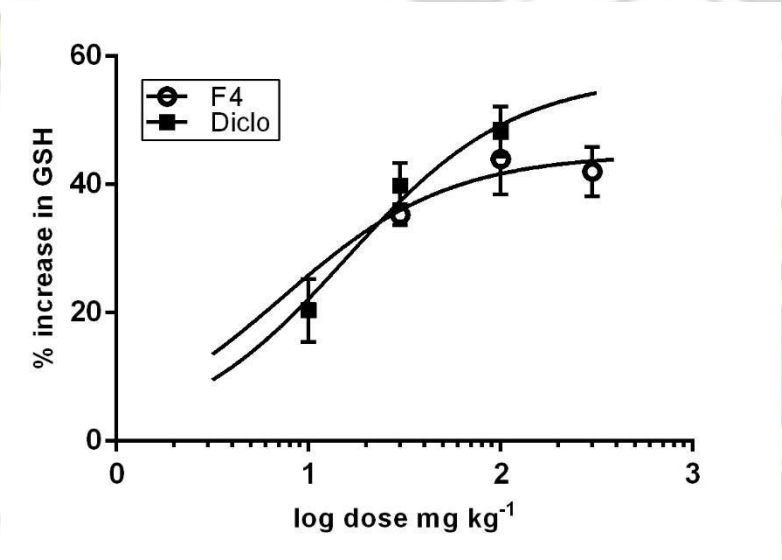

Figure 3.18 Dose-response curves for diclofenac (10-100 mg kg^{-1} i.p) and F4 (30-300 mg kg^{1} *p.o)* on GSH levels.

3.4 values for GSH assay

Test drug ED_{50} (mg kg^{-1})

F4	7.286±3.87
Diclofenac	15.72±6.65

3.15 SUPEROXIDE DISMUTASE (SOD)

SOD in lung tissues significantly decreased after carrageenan challenge. Compared to carrageenan only treated lung tissues, there was a significant increase in SOD activity in 30,100 and 300 mg kg^{-1} F4 (P<0.0001) treated lung tissues (Figure 3.19). The standard drug diclofenac also showed significant increase in GSH levels at 30 and 100 mg kg^{-1} (P<0.0001) compared to carrageenan only treated tissues (Figure 3.19).

From the ED_{50} calculated from the dose response curves (Fig. 3.20), F4 (ED_{50} = 20.96± 9.02) was found to be approximately 2.4x more potent than diclofenac (ED_{50}= 56.46± 31.82) in increasing SOD activity (Table.3.5).

Table ED_{50}

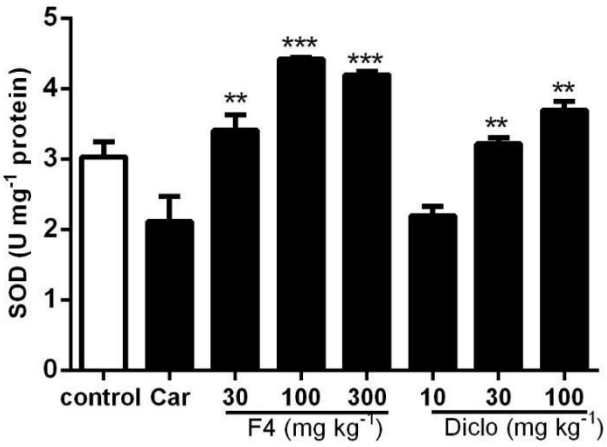

Figure 3.19 Effects F4 (30-300 mg kg^{-1} *p.o.*) and Diclofenac (10-100 mg kg^{-1} i.p) on SOD activity were assessed from lung tissue 6 h after carrageenan injection. Data are expressed as mean ± SEM. n=5, $^{**}P<0.001$, $^{***}P<0.0001$ vs. carrageenan only treated group. (One-way ANOVA followed by Dunnett's Multiple Comparison test.

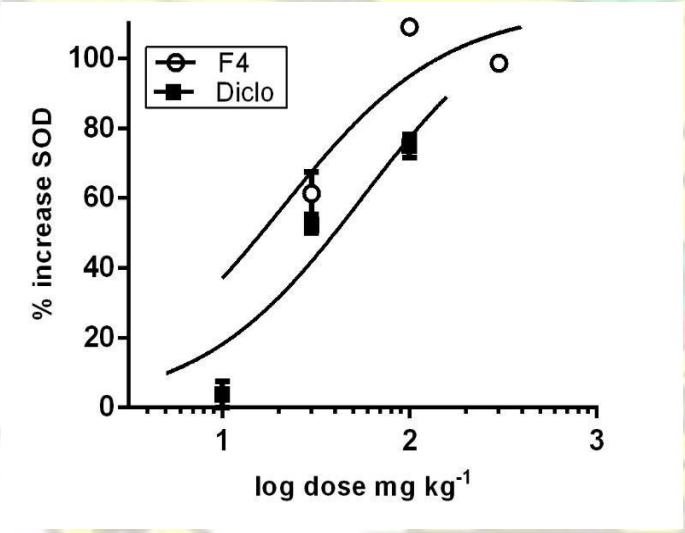

Figure 3.20 Dose-response curves for diclofenac (10-100 mg kg^{-1} i.p) and F4 (30-300 mg kg^{-}

3.5 values for SOD activity

Test drug	ED_{50} (mg kg^{-1})

[1] *p.o*) on SOD activity**.**

F4	20.96± 9.02
Diclofenac	56.46± 31.82

3.16 CATALASE ASSAY (CAT)

Catalase in lung tissues slightly increased after carrageenan challenge. Compared to carrageenan only treated lung tissues, there was a significant dose dependent increase in catalase activity in 30,100 and 300 mg kg^{-1} F4 (P<0.001)treated lung tissues (Figure 3.21). The standard drug diclofenac also showed significant increase in catalase at all doses (10- 100 mg kg^{-1}) (P<0.003) compared to carrageenan only treated tissues (Figure 3.21).

From the ED_{50} calculated from the dose response curves (Fig. 3.22), F4 (ED_{50} = 47.19± 3.18) was found to be approximately 12.8x less potent than diclofenac (ED_{50} = 3.68±2.30) in inducing catalase activity (Table. 3.6).

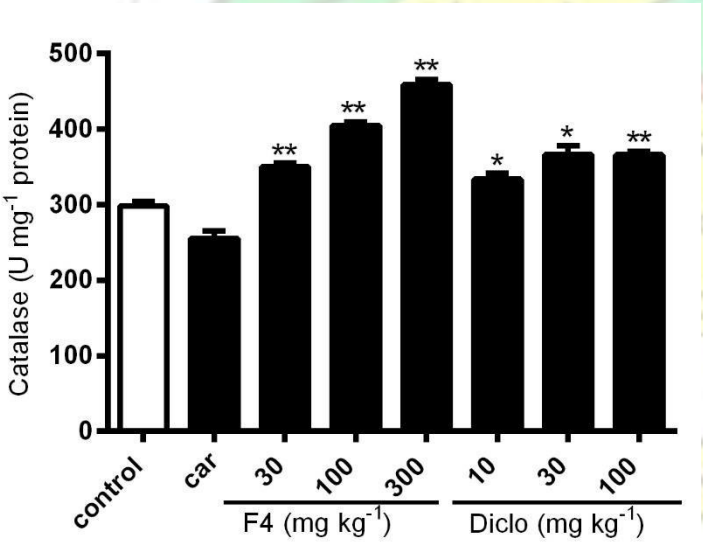

Table ED_{50}

Figure 3.21 Effects F4 (30-300 mg kg^{-1} *p.o.*) and Diclofenac (10-100 mg kg^{-1} i.p.) on catalase activity were assessed from lung tissue 6 h after carrageenan injection. Data are expressed as mean ± SEM. n=5,*P<0.01 ** P<0.001 vs. carrageenan only treated group. (One-way ANOVA followed by Dunnett's Multiple Comparison test.

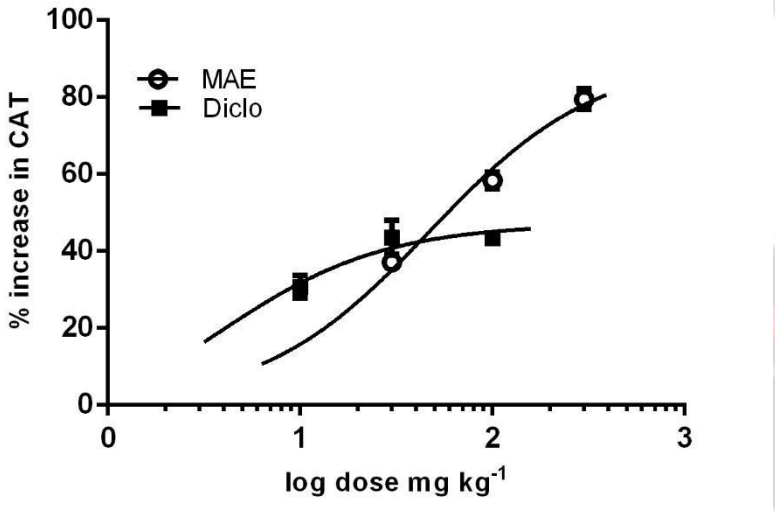

Figure 3.22 Dose-response curves for diclofenac (10-100 mg kg^{-1} i.p.) and F4 (30-300 mg kg^{-1} *p.o.*) on catalase activity**.**

3.6 values for catalase activity

Test drug	ED_{50} (mg kg^{-1})
F4	47.19±3.18
Diclofenac	3.68±2.30

3.17 MALONDIALDEHYDE (MDA) ASSAY

MDA in lung tissues significantly increased after carrageenan injection. Compared to carrageenan only treated tissues, there was a significant reduction in MDA content in 100 and

300 mg kg^{-1} F4 (P<0.0001)treated lung tissues (Figure 3.23). The standard drug diclofenac showed significant reduction in MDA content at 30 and 100 mg kg^{-1} (P<0.0005) compared to carrageenan only treated tissues (Figure 3.23).

From the ED_{50} calculated from the dose response curves (Fig. 3.24), F4 (ED_{50} = 85.02±19.96) was found to be approximately 1.2x less potent than diclofenac (ED_{50} = 65.83±23.35) in reducing MDA expression (Table. 3.7).

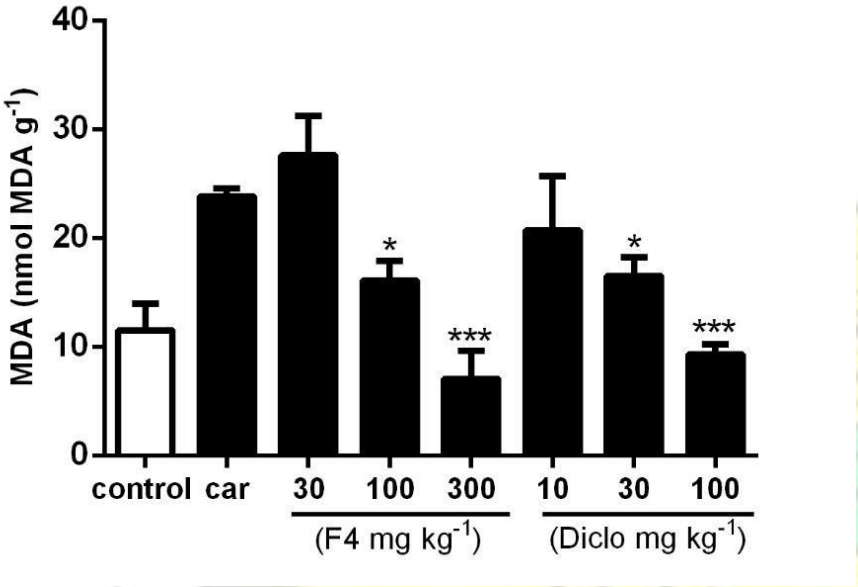

Figure 3.23 Effects F4 (30-300 mg kg^{-1} *p.o.*) and Diclofenac (10-100 mg kg^{-1} i.p.) on MDA levels were assessed from lung tissue 6 h after carrageenan injection. Data are expressed as mean ± SEM. n=5, *P<0.02, *** P<0.0001 vs. carrageenan only treated group. (One-way ANOVA followed by Dunnett's Multiple Comparison test.

Table ED_{50}

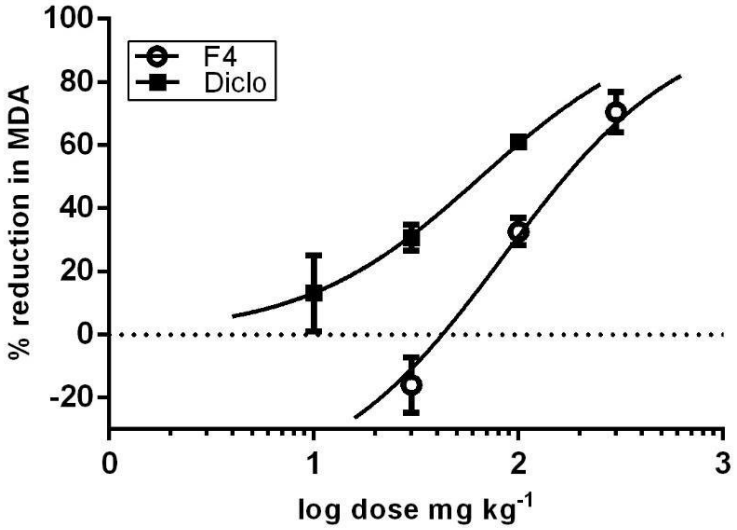

Figure 3.24 Dose-response curves for diclofenac (10-100 mg kg^{-1} i.p.) and F4 (30-300 mg kg^{-1} *p.o.*) on MDA levels.

Table 3.7 ED_{50} values for MDA assay

Test drug	ED_{50} (mg kg^{-1})
F4	85.02±19.96
Diclofenac	65.83±23.35

CHAPTER FOUR

DISCUSSION

The carrageenan-induced paw oedema is a reliable and repeatable experimental protocol for assessing natural products for anti-inflammatory activity (Huang *et al.*, 2012). It has been used over the years in the development of NSAIDS and many potential anti-inflammatory agents (Hernández-Ortega *et al.*, 2012). The initial phase (0-2 h) of carrageenan induced paw oedema has been reported to be mediated by histamine and serotonin followed by kinin and finally through bradykinin, prostaglandins and lysosomes in the compromised tissue's vicinity, while the later phase (2-6 h) is sustained by prostaglandins and mediated by enhanced production of nitric oxide, leukotrienes, polymorphonuclear cells, ROS and cytokines. This has been explained to be due to the rapid induction of COX-2 by carrageenan in the spinal cord and other region of the CNS (Ichitani *et al.*, 1997). COX-2 then catalyses serial reactions, in arachidonic acid metabolism, that leads to the synthesis of prostaglandins.

Oral administration of F1, F2 and F3 significantly and effectively inhibited paw oedema in a dose-dependent manner over the duration of the study which was comparable to that of diclofenac (an NSAID).Diclofenac and dexamethasone, the standard drugs with which the extracts were compared to both showed a dose-dependent inhibition of carrageenan-induced oedema.

F2 and F3 were both extracted with non-polar solvents hence it is possible they contain similar phytochemicals. This was even evident when thin layer chromatography assessment showed the elution of components with similar Rf values. Hence, a combination extract F4 extracted with equal parts of ethyl acetate and petroleum ether was used for subsequent experiments.

In an attempt to identify the specific mediators in the observed anti-inflammatory effects of the extract, inhibitory effect of the extracts on histamine, serotonin, bradykinin and prostaglandin E_2-induced oedema in rats were carried out. F4 did not exhibit any significant inhibition of histamine-induced oedema but was effective in mitigating oedema associated with serotonin, bradykinin and prostaglandin challenge. Serotonin causes vasodilation and vascular permeability through its action on $5HT_3$ receptors in smooth muscles (Motavallian *et al.*, 2013). F4 was able to attenuate oedema from serotonin probably through the inhibition of $5HT_3$ mediated vasodilation and stimulation of sensory neurons that affect the arachidonic acid and kallirein-knin pathways. Bradykinin-induced oedema decreased rapidly in rats that were treated preemptively with F4 extract probably due to interaction of the antiinflammatory constituents in the extract with B_2 receptors which mediates the mechanism by which bradykinin induces rat paw oedema (Campos *et al.*, 1995). PGE_2 exhibits a diverse array of biological effects in multiple tissues through its binding to EP_2 receptors on plasma membrane. COX-2 is induced in the CNS during carrageenan-induced inflammation, this results in the increase of PGs, prostacyclins, and thromboxanes in the initial phase and to a later upsurge in PGE_2 production associated with selective up-regulation of mPGES-1. Hence it can be possibly inferred that F4 inhibited prostaglandin-induced inflammation possibly through inhibition of prostaglandin E receptors and/or down regulation of mPGES-1 activity. These finding show that the extract inhibited carrageenan induced paw oedema by inhibiting serotonin, bradykinin and prostaglandin pathways.

Oedema is just one component of the inflammatory response; increased vascular permeability also plays a significant role. Hence, to further investigate the inhibitory effect of the extracts on the prostaglandin, serotonin and histamine pathways of inflammation, the role of the extract in acetic-acid induced vascular permeability was investigated. In acetic acid-induced vascular

permeability test, acetic acid challenge brings about increases in the level of mediators such as prostaglandins, serotonin, and histamine in peritoneal fluids, which in turn lead to vasodilation and an increase in vascular permeability (Nardi *et al.*, 2007). F4 dosedependently attenuated the capillary permeability induced by acetic acid in mice. These findings suggest that the anti-inflammatory effect of F4 on the acute phase of inflammation might be associated with prevention of vasodilation and inhibition of the release of inflammatory mediators such as histamine and serotonin

Vascular permeability is the mechanism by which plasma and its solutes cross the vascular barrier. It is critical in maintaining the health of normal tissues. Vascular permeability is highly increased in many disease conditions. The inflammatory response comprises of vascular events such as hyperaemia, vascular permeability, and leukocyte influx into compromised tissues (Yamaki *et al.*, 2002). These events are complex but are still to occur quickly and consistently. Evans blue is a basic dye and has a high affinity for nuclei which is acidic. The use of Evans blue dye as a biological marker for vascular permeability, assists the study of the effect of pathological changes that results from inflammatory disorders such as atherosclerosis, rheumatoid arthritis, multiple sclerosis (Nidavani *et al.*, 2014).

Coupled with vascular permeability is the infiltration of pro-inflammatory cells to the site of inflammation. Cellular infiltration is observed when carrageenan is injected into the pleural cavity of rats. There was an inflammatory reaction associated with exudation of fluids into the pleural space accompanied by a high influx of polymorphonuclear leukocytes. This ultimately leads to the increased levels of prostaglandin E_2, TNF-α and IL-1β , ROS and lipid peroxidation (Marzocco *et al.*, 2004). Cell migration occurs as a consequence of several processes including adhesion and cell mobility (Vane *et al.*, 1998). F4-treated groups showed significantly less neutrophils in the pleural exudates than the controls, suggesting the inhibition of neutrophil

infiltration which may be due to inhibition of rolling and adherence of neutrophils, which impaired neutrophil migration from blood vessels. F4 produced a dose dependent inhibition in the influx of polymorphonuclear cells and exudate volume into the pleural cavity similar to diclofenac. PGE_2 is the mediator primarily responsible for the exudation that occurs in carrageenan-induced pleurisy via EP2 and EP3 receptors (Akaogi *et al.*, 2006). Hence, inhibition EP2 and EP3 receptors might contribute to the antiinflammatory activity of F4.

In acute inflammation, there is decreased activity of endogenous antioxidant enzymes (CAT, SOD and GSH), elevated MPO activity and increased lipid peroxidation in tissues as a result of oxidative damage (Posadas *et al.*, 2004). CAT, SOD and GSH also play a crucial role as protective enzymes. (Schreck *et al.*, 1991) demonstrated that antioxidants inhibit, whereas ROS activate nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB), a transcription factor which activates the transcription of several genes involved in inflammation (Baeuerle *et al.*, 1994). MPO is a proinflammatory enzyme found in granulocytes which leads to the generation of cytotoxic compounds such as hypochlorous acid and tyrosil radicals from hydrogen peroxide and tyrosine respectively (Pulli *et al.*, 2013) The protective role of GSH against inflammatory diseases has been proven by depleting endogenous GSH with Buthionine sulphoximine (BSO) (Cuzzocrea *et al.*, 1999) which resulted in aggravating effect on various models of inflammation including carrageenaninduced pleurisy. CAT which is localized in subcellular organelles of peroxisomes, catalyzes the conversion of hydrogen peroxide to water and oxygen (Agarwal *et al.*, 2005); SOD in cells work in conjunction with H_2O_2-removing enzymes such as GPx or CAT to prevent action of H_2O_2, which in turn inhibits the formation of hydroxyl radicals (Limón-Pacheco *et al.*, 2009). Treatment with F4 increased the activities of CAT, SOD and GSH and decreased MPO activity. The above effects resulted in decreased

lipid peroxidation from the low levels of MDA in F4 treated rats thereby significantly reducing the severity of inflammation.

Again, reactive oxygen species have been implicated in the development of Alzheimer's, Parkinson's, cardiovascular and inflammatory diseases and cancer by causing oxidative stress (Laguerre *et al.*, 2007; Conforti *et al.*, 2008). ROS also initiate, sustain or intensify the inflammatory process by up-regulating genes that activate the transcription of proinflammatory cytokines and adhesion molecules. Apart from their ability to enhance the inflammatory response, ROS also stimulate enrolment of more neutrophils and macrophages (Conforti *et al.*, 2008). The above discoveries have elaborated the importance of ROS in the inflammatory response. Hence drugs that are able to mop up or neutralize ROS can lead to the resolution of inflammation.

The antioxidant activity of most plant extracts is mainly due to phenolic compounds such as flavonoids, phenolic acids, etc. Polyphenols are electron-rich compounds which are capable of entering into redox reactions with reactive intermediates (Pham-Huy *et al.*, 2008). The total phenol assay and the total antioxidant capacity assays have demonstrated a high phenol content and ability to resist oxidizing agents by the extract. F4 was able to reduce the absorbance of DPPH radical at 517 nm in a concentration dependent manner which shows it can neutralize free radicals. Phenolics are not the only entities in plants with antioxidant properties, other plant metabolites such alkaloids, carotenoids, lignans, phytosterols and terpene have also been demonstrated to possess antioxidant activity (Amaral *et al.*, 2006).

Histopathology can offer a pronounced structural peculiarity as a pragmatic, univocal and decisively characteristic sign of an inflammatory process (Soren *et al.*, 1987). Henceforth,

histopathological studies on lung sections after carrageenan challenge was carried out which showed the extract was able to preserve normal alveolar architecture with reduced influx of neutrophils and oedema formation. The alveolar walls were markedly less thickened with less hyperaemia. F4 showed ability to attenuate lung injury induced by carrageenan and this is in agreement with Wilson *et al.* (2009) finding which established that materials that act on multiple pro-inflammatory mediators improve lung function in inflammation.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSION

The present study has demonstrated that the stem bark extracts of *Maerua angolensis*

1. has anti-inflammatory activity in acute inflammation by
    I. attenuating carrageenan-induced paw oedema
    II. decreasing acetic acid-induced vascular permeability. III. attenuating carrageenan-induced pleurisy

2. Exhibited both i*n vitro* and *in vivo* antioxidant activity, which may contribute to its anti-inflammatory activity.

5.2 RECOMMENDATIONS

Further experiments to investigate the effect of the *Maerua angolensis* extracts on:

- Nitric oxide production.
- TNF-α, IL-1β, IL-6 and PGE_2 levels.
- iNOs ,COX-2 and 5-LOX levels.
- Animal models of chronic inflammation.

The inflammatory response is a very complicated physiological activity which involves many interconnecting pathways. Data from this study have established that *M. angolensis* exhibits significant anti-inflammatory and anti-oxidant activities in both *in vitro* and *in vivo* models. It is now prudent to determine other signalling pathways, mediators and systemic processes which are involved in the antiinflammatory activity

of the plant. The above experiments will be able to further elaborate other mechanisms of action of *M. angolensis* in inflammation.

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APPENDIX

PREPARATION OF CARRAGEENAN SUSPENSION

A 1 % (w/v) carrageenan suspension was prepared by sprinkling small amounts of the powder (100 mg) evenly over the surface of 10 ml of 0.9 %w/v sodium chloride solution and left to soak in-between additions. It was then left for 2-3 h before use.

PAW VOLUME MEASUREMENT USING PLETHYSMOMETER

The volume transducer is formed by two Perspex tubes interconnected and filled with a conductive solution and a platinum electrode for each chamber. The entire set up is supported by a stand. The water-displacement produced by the immersion of the animal paw in the measuring tube is reflected into the second tube, inducing a change in the conductance between the two platinum electrodes. The Plethysmometer Control Unit (PCU) detects the conductance changes and generates an output signal to the digital display indicating the volume displacement measured (0.01 ml resolution). The value that remains stable on the digital display is recorded. The control unit was zeroed between successive readings.

CALCULATION

% increase in paw volume = $\mathbf{(V_t - V_0)/V_0 \times 100}$ Where, V_t is the paw volume at time t (after injection). V_0 is the paw volume before injection (0 h)

BUFFERS FOR ENZYME ASSAY

0.1 M Sodium Phosphate buffer (pH 6.0)

0.1 M Monosodium phosphate	88.0 ml

0.1 M Disodium phosphate	12.0 ml

50 mM Potassium Phosphate buffer (pH 7.0)

50 mM Potassium Phosphate monobasic	39.0 ml
50 mM Potassium Phosphate dibasic	61.0 ml

0.1 M Carbonate bicarbonate buffer (pH 10.2)

0.1 M Sodium carbonate	70.0 ml
0.1 M Sodium bicarbonate	30.0 ml

DRUG PREPARATION AND ADMINISTRATION

The extract was prepared by emulsifying using Tween-80 and made to volume using normal saline. All the other drugs were prepared by diluting with 0.9 % (w/v) sodium chloride. Extract and reference drugs were prepared in volumes such that not more than 100 µl is administered orally.