ANTI-INFLAMMATORY AND ANTINOCICEPTIVE EFFECTS OF ETHANOL EXTRACT OF *RITCHIEA CAPPAROIDES* ROOT-BARK IN RATS

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TITLE PAGE

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A PROJECT REPORT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR AWARD OF DEGREE OF MASTER OF SCIENCE (M.Sc.) IN PHARMACOLOGICAL BIOCHEMISTRY, UNIVERSITY OF NIGERIA, NSUKKA.

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NOVEMBER, 2018.

CERTIFICATION

This is to certify that Mba, Cornelius Elochukwu, a postgraduate student with registration number, PG/M.Sc./16/81366 in the Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, has satisfactorily completed the requirements for the research work for Master of Science (M.Sc.) degree in Pharmacological Biochemistry. The work embodies in this report is original and has not been submitted in part or full for any diploma or degree of this or any other university.

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DEDICATION

This work is dedicated to the Almighty God who created the world and everything in it.

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ABSTRACT

The effects of ethanol extract of *Ritchiea capparoides* root-back on egg albumin- induced inflammation in rat hind paw, hypotonicity induced haemolysis of human red blood cells, acetic acid–induced nociception in albino mice, phospholipase A_2 activity and calcium chloride-induced platelet aggregation were studied. Twenty five adult albino rats of either sex (120 to 200 g) and 25 adult albino mice of either sex of weights 40-80 g were divided into ten experimental groups of five rats and five mice eac/.h. Five groups of the rats were used for the anti-inflammatory tests, while the other five groups of the mice were used for analgesic test. Inflammation was induced by injecting 0.1 ml of fresh egg albumin into the subplantar surface of the right hind paw of the rats. Ethanol extract of *Ritchiea capparoides* (100, 200 and 400 mg/kg b. w.) and indomethacin (100 mg/kg b. w.) were administered intraperitoneally to separate groups of the rats one hour before inducing inflammation. The control group received equivalent volume of distilled water (vehicle). Nociception was induced in the mice by the administration of acetic acid (50mg/kg b. w.) using standard procedures. *Ritchiea capparoides* extract (100, 200 and 400 mg/kg b. w.) and aspirin (200 mg/kg b. w.) were administered orally to separate groups of the mice thirty minutes before inducing nociception. The control group received equivalent volume of distilled water (vehicle). The effect of the extract on hypotonicity-induced haemolysis, calcium chloride induced platelet aggregation and phospholipase A_2 activity were also evaluated. From the results of the study, rats treated with 100, 200 and 400 mg/kg body weight of the extract showed significant reduction of oedema after five hours of treatment with inhibition of 89.10 %, 85.03 % and 87.00 % respectively and treatment with 200 and 400 mg/kg body weights significantly reduced the number of writhings induced by acetic acid in mice with inhibition of 42.11 % and 99.61 % respectively. The *R. capproides* extract caused a reduction in the haemolysis induced by distilled water, The extract (1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml) significantly ($p < 0.05$) inhibited the lysis of human red blood cells. The absorbance values for the extract were 0.408 ± 0.002, 0.421 ± 0.002, 0.452 ± 0.001, 0.577 ± 0.002 and 0.588 ± 0.003 respectively. The extract reduced acetic acid-induced nociception dose-depently. The extract (100, 200 and 400 mg/kg body weight) significantly ($p < 0.05$) reduced the number of writhings when compared with the control group. The percentage inhibition of the number of writhings for various doses of the extract were (42.11 %) (66.67 %) and 61.4. %) respectively. The extract inhibited phospholipase A_2 activity, the absorbance values for different doses of the extract (1.0), (1.2), (1.4), (1.6) and (1.8 ml) were 0.035 ± 0.002, 0.055 ± 0.001, 0.042 ± 0.002, 0.078 ± 0.001, 0.017 ± 0.000 and 0.056 ± 0.001 respectively. The results showed that the extract at 100, 200 and 400 mg/kg body weights used in this study has potential anti-inflammatory and analgesic effects.

CHAPTER ONE

INTRODUCTION

Medicinal plants continue to play central roles in the healthcare system of large proportion of the world's population. This is particularly true in the developing countries, where herbal medicine has a long and uninterrupted history of use. Recognition and development of medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations (Srinivas *et al,* 2007). gWorld Health Organization (WHO), the specialized agency of the United Nations (UN) that is concerned with international public health, published quality control methods for medicinal plant materials in 1998 in order to support WHO Member States in establishing quality standards and specifications for herbal materials, within the overall context of quality assurance and control of herbal medicines. The anti-inflammatory drugs have their origins in the use of extracts of salicylate-containing plants, especially the bark of the willow tree (*Salix alba*), in the treatment of fever, pain and inflammation. Many plants or plant parts have been reported to have anti-inflammatory effects by many researchers. Such plants include; *Holoptelea integrifolia roxb, Thespesia populnea*, and *Zingiber officiale,* (Sharma *et al.,* 2011; Sen, 1991; Ilavarasan *et al.,* 2012; Sharma *et al.,* 2011 and Shimoda *et al.,* 2011) among others.

Inflammation is a protective strategy evolved in higher organisms in response to detrimental assaults such as microbial infection, tissue injury and other noxious conditions. It is an essential immune response by the host that enables the removal of harmful stimuli as well as the healing of damaged tissue. Acute inflammation has therefore been considered as a part of innate immunity, the first line of host defense against foreign invaders. Mankind has known the classical symptoms of inflammation for hundreds of years, which include redness, pain, swelling and heat (Medzhitov, 2008). Inflammation is the basic process whereby tissues of the body respond to injury (Henson *et al.,* 2005). At present, inflammation is defined by the presence of five macroscopic pathological phenomena, four of them proposed by Celsus as long as 2000 years ago. These are *tumor* - swelling of the tissue, *calor* – elevated tissue temperature, *rubor* – blood color-like redness of vascularized tissue at the inflammation site, *dolor* – intensive sensation of a noxious stimulus, and *functio laesa*, i.e. impaired function of the organ affected. All signs have been regarded as secondary to one

primary pathophysiological event – enhancement of vascular permeability as a direct consequence of tissue injury. Meanwhile, modern natural sciences have comprehensively elucidated biological processes at the biochemical and molecular level that take place in the course of the inflammatory process. In the 1940s, American pathologist Menkin defined the sixth cardinal and at the same time the only essential biochemical sign of inflammation, i.e. proteolysis. However, the significance of his discovery has been largely neglected, leaving considerable confusion in explanations of the cause effect relationships in pathogenesis and in the approaches to treatment of inflammatory diseases (Scott *et al.,* 2004). In particular, it is very often noted that, although inflammation is a defensive, therefore a useful process, its exaggeration or prolonged action may harm the body. However, the reasons for such outcomes are never explicitly stated.

1.1 Profile of *Ritchiea capparoides*

1.1.1 Description and uses of *Ritchiea capparoides*

The plant is an evergreen climber but when alone it is a self-supporting shrub with compound palmate leaves. The leaves can be collected all - year round as the plant can stand dry season. The roots are tuberous and with strong pungent odours when perceived. Like other *Capparidaceae,* this herb is indigenous to the tropics, found mostly in the lowland area of rain forest, especially beside water body and virgin up-lands. As a shrub it grows to a height of few meter(s) and as climber can grow a considerable length of about 5 meters with several branches. The local name springs from the number of leaves (three) present in a leaflet hence it is called Nchi-ato (3-ears) by the Ibo people of Nigeria (Chinedu *et al.,* 2013). *Ritchiea capparoides* (*Capparidaceae*) formerly known as *R. longipedicellata* is an ornamental climbing shrub consisting of two varieties, *Var*. *capparoides* (flower with four petals) and *Var*. *longipedicellata* (flower consists of eight petals or more) (Burkill, 1985). Ethnomedicinally, the roots extract of *R. capparoides* var *longipedicellata* are used as an anthelmintic. In South-Eastern Nigeria, the decoction of its root is used to treat wounds, running stomach, aches and pains. (Chinedu *et al.,* 2013)); in South-Western Nigeria, it is used to treat infectious and parasitic diseases (Taiwo *et al.,* 2012). Most medicinal plants presently employed in traditional medicine are used without scientific evidence. It is therefore necessary to scientifically ascertain the efficacy of *Ritchiea capparoides* root-bark in the treatment and management of inflammatory disorders.

1.1.3 Previous Studies on *Ritchiea capparoides*

Phytochemical screening of *Ritchiea capparoides* root-bark ethanol extract reported the presence of alkaloids, flavonoids, saponins, terpenoid and glycoside (Chinedu *et al*, 2013). Research carried out with 70 % aqueous ethanol extract of dried leaves of *Ritchiea capparoides* reported the antiplasmodial activity of *Plasmodium falciparum* (Taiwo *et al.*, 2013). Also, it was reported that the root-back ethanol extract of *Ritchiea capparoides* has antimicrobial properties (Chinedu *et al.*, 2013). Research demonstrated the presence of stachydrine in the leaves, stem and root-bark of *Ritchea capparoides var longipedicellata* (Taiwo *et al.*, 2013). The compound was found to be active in the antimalarial and antimicrobial assay used (Taiwo *et al.*, 2013). Phytochemical screening of the plant extract showed the presence of tannins, saponins, sesquiterpenes, alkaloids, and phlobatannins (Karanayil *et al.*, 2011). Ethylacetate extract of the leaves of *Ritchiea capparoides* demonstrated activities against *Candida albican* and *Aspergillus niger* (Awoni *et al.*, 2012). Previous chemical investigations have reported cleomin, a mustard oil as glycoside isolated from the root-bark of the plant (Ogwuaka *et al.*, 1981).

1.2 Overview of Inflammation

Inflammation has been known to humankind for at least a few thousand years, in part because it accompanied two major scourges of the past, wounds and infections, and in part because it is rather conspicuous. Although references to inflammation can be found in ancient medical texts, apparently the first to define its clinical symptoms was the Roman doctor Cornelius Celsus in the 1st century AD. These symptoms came to be known as the four cardinal signs of inflammation *rubor et tumor cum calore et dolore* (redness and swelling with heat and pain). Celsus mentions these signs in his treatise *De medicina*, while describing procedures for treating chest pain and in so doing became an often-quoted medical celebrity. Analyzing living tissues under the microscope, Cohnheim observed vasodilation, leakage of plasma, and migration of leukocytes out of blood vessels and into the surrounding tissue (Majno and Joris, 2004). The fifth cardinal sign, *functio laesa* (disturbance of function). Notably, although the four cardinal signs of inflammation only apply to acute inflammation accompanying wounds and infections, *functio laesa* is the only universal sign that accompanies all inflammatory processes. Virchow's main contribution to inflammation research was to establish the cellular basis of pathology, a dramatic departure from the traditional view of disease as an imbalance of the four humors, which had dominated medicine since the time of Hippocrates.

Another major milestone was the discovery of phagocytosis by Elie Metchnikoff and his theory of cellular immunity developed in 1892. Metchnikoff emphasized the beneficial aspects of inflammation and pointed out the key role of macrophages and microphages (neutrophils) both in host defense and in the maintenance of tissue homeostasis.

Allergic inflammation involves a complex interaction of many different inflammatory cells that release a spectrum of chemical mediators ultimately affecting various target tissues. Although the clinical manifestations of the allergic response vary depending on the tissue and antigen involved, it is now understood that the allergic reaction consists of an early-phase response primarily involving mast cell degranulation accompanied by the release of histamine and other mediators including cytokines and a late-phase response that is characterized by the migration of inflammatory cells from the circulation. Eosinophils infiltration is a hallmark feature of allergic rhinitis and distinguishes allergic inflammation from other inflammatory conditions of the upper respiratory tract. The sensitization phase of the allergic response begins when antigen-presenting cells (Langerhans' cells) display allergen fragments to T lymphocytes. In a process that involves the secretion of IL-4 by T lymphocytes, B lymphocytes are activated and mature into plasma cells that produce allergen-specific IgE antibodies. The IgE antibodies then bind to receptors on tissue mast cells and circulating basophils. On subsequent allergen exposure, allergen molecules bind to IgE antibodies, which results in cross-linking of IgE molecules and activates enzyme cascades in the cell membrane involving tyrosine kinase, protein kinase C, phospholipase C, phospholipase A_2 and an influx of calcium ions that results in the release of preformed histamine and newly synthesized chemical mediators (leukotrienes, prostaglandins) in the early phase response. Mast cells also secrete interleukins and other cytokines that regulate the duration and intensity of the immune response by promoting the expression of adhesion molecules and by recruiting inflammatory cells, particularly eosinophils, to the site of the allergic reaction. These inflammatory cells release additional chemical mediators in the late-phase response, leading to chronic inflammation, priming of nasal tissues, and tissue damage (Blanchet *et al*, 2008).

1.2.1 Classification of Inflammation

Inflammation could be classified as either acute or chronic, depending on the type and duration of the antigen challenge and is mediated by some chemical substances such histamine, serotonin, slow

reacting substances of anaphylaxis (SRS-A), prostanglandins and some plasma enzyme systems such as the complement system, the fibrinolytic system, kinin system and clotting system.

1.2.1.1 Acute Inflammation

The initial response of the body to an infection or trauma is called the acute inflammatory response. This response is non-specific and is the first line of defense of the body against injurious substances. It consists of a coordinated local and systemic mobilization of immune, endocrine and neurological mediators. In a healthy response, the inflammatory response becomes activated, clears the pathogen (in the event of infection), begins a repair process and abates. However, inflammation itself can damage otherwise healthy cells which could then further stimulate inflammation. This run away inflammation can lead to organ failure and death. Systemic inflammation accompanied by infection, based on its clinical manifestations, is defined as sepsis (Bone *et al.,* 1992). Sepsis is a common and frequently fatal condition, with 750,000 cases annually in the United States alone in 1995 (Angus *et al.,* 2001). Though much has been learned about the molecular and physiological pathways of the acute inflammatory response, this knowledge has not led to many effective therapies against sepsis. The sole approved drug therapy for severe sepsis is activated Protein-C, which only reduced mortality by 6 % compared with controls in clinical trials (Bernard *et al.,* 2001). One reason for the lack of effective treatments may be that the complex nature of the inflammatory response renders the effect of targeting isolated components of inflammation difficult to predict. Thus, mathematical modeling may provide insights into the global dynamics of the inflammatory process from which therapies may be developed (Klim *et al*, 2008).

1.2.1.2 Chronic inflammation

As inflammation is evolved as a beneficial strategy for the host in response to any potential danger, the inflammatory response is normally terminated once the potential danger is eradicated. Usually the reversion of the inflammatory response to the homeostatic state precedes quite rapidly, a highly regulated process known as the resolution of inflammation. The resolution of inflammation is dominated by many anti-inflammatory mediators such as IL-10, TGF-β and glucocorticoids and it also involves the recruitment of monocytes for the clearance of cell debris (Serhan and Savill, 2005). If the resolution of inflammation fails for any reason, the acute inflammation turns into a chronic stage. Chronic inflammations have been a subject to extensive studies over the decades not only for the growing burdens of the associated pathological conditions in the modern societies but also for

the underlying mechanisms which remain largely unresolved. A chronic inflammation is generally believed to develop if the elimination of the triggering stimulus fails to happen, such as any persistent infection or chronic cellular injury (Kumar *et al.*, 2003; Majno and Joris, 2004). However, the initial trigger for a vast majority of the chronic inflammatory conditions has not been well defined yet, making the understanding of their pathological processes much more complicated due to the lack of any microbial infection or tissue damages. Chronic inflammation is not just a primary cause of these conditions, but it is the major driver of the pathogenesis. In chronic inflammatory conditions, the major damages done to the host are mediated by the host inflammatory response itself, not by the foreign invaders such as pathogens. The importance of chronic inflammatory conditions in this modern era of medical science cannot be overestimated because of their involvement in many diseases such as atherosclerosis, obesity, type 2 diabetes, asthma, inflammatory bowel diseases, neurodegenerative diseases, rheumatoid arthritis and cancer.

1.2.2 Mechanisms of Inflammation

Inflammation consists of a tightly regulated cascade of immunological, physiological, and behavioral processes that are orchestrated by soluble immune signaling molecules called cytokines. The first step of the inflammatory cascade involves recognition of infection or damage. This is typically achieved by the detection of pathogen-associated molecular patterns (PAMPs), which are specifically directed toward general motifs of molecules expressed by pathogens that are essential for pathogen survival. Damage-associated molecular patterns (DAMPs) are endogenous molecules that signal damage or necrosis and are also recognized by the innate immune system. An advantage of detecting these signals is that inadvertent targeting of host cells and tissues is minimized. Unlike adaptive immunity, the innate immune system lacks the ability to distinguish among different strains of pathogens and whether such strains are virulent (harmful to the host). Many damage signals are recognized by germ-line encoded receptors, such as transmembrane Toll-like receptors (TLRs) and intracellular nucleotide binding domain and leucine-rich repeat containing receptors (NOD-like receptors or NLRs. Once recognition of ligands occurs, TLRs activate common signaling pathways that culminate in the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). This transcription factor is found in virtually all cell types and remains in an inactivated state bound to an inhibitor protein, IκB. Upon transduction of the signal, NF-κB is released from IκB and translocates to the nucleus, where transcription is upregulated through binding to target genes.

Importantly, activation of NF-κB does not require new protein synthesis, which permits a rapid response. The NF-κB signaling system is ancient, but there is phylogenetic evidence that regulation of immune function by this pathway in vertebrates evolved independently from invertebrate immune mechanisms. Intracellular NLRs respond to increasing numbers of DAMPs that alert the immune system to cell injury and provide a proximate pathway for sensing exposure to possible toxins or pollutants in the environment. Transcription and translation of genes lead to the third stage of the inflammatory cascade, which is the inducible expression of proinflammatory cytokines, such as interleukin-1-beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α), and others. In conjunction with chemokines (attractants) and various costimulatory molecules, these soluble proteins facilitate the recruitment of effector cells such as monocytes and neutrophils, to the site of disturbance. Neutrophils create a cytotoxic environment by releasing noxious chemicals from cytoplasmic granules (a process called degranulation). Rapid release of these chemicals requires consumption of both glucose and oxygen, known as the respiratory burst. Toxic chemicals released include highly reactive oxygen and nitrogen species (ROS and RNS, respectively) and various proteinases. This is typically achieved by the detection of pathogen-associated molecular patterns (PAMPs), which are specifically directed toward general motifs of molecules expressed by pathogens that are essential for pathogen survival. Damage-associated molecular patterns (DAMPs), are endogenous molecules that signal damage or necrosis and are also recognized by the innate immune system. An advantage of detecting these signals is that inadvertent targeting of host cells and tissues is minimized. Unlike adaptive immunity, the innate immune system lacks the ability to distinguish among different strains of pathogens and whether such strains are virulent (harmful to the host) (Bhadrapura and Serhan, 2016). Many damage signals are recognized by germ-line encoded receptors, such as transmembrane Toll-like receptors (TLRs) and intracellular nucleotide binding domain and leucine-rich-repeat containing receptors (NOD-like receptors or NLRs). Once recognition of ligands occurs, TLRs activate common signaling pathways that culminate in the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). This transcription factor is found in virtually all cell types and remains in an inactivated state bound to an inhibitor protein, IκB. Upon transduction of the signal, NF-κB is released from IκB and translocates to the nucleus, where transcription is upregulated through binding to target genes. Importantly, activation of NF-κB does not require new protein synthesis, which permits a rapid response. The NF-κB signaling system is ancient, but there is phylogenetic evidence that regulation of immune function by this pathway in vertebrates evolved

independently from invertebrate immune mechanisms. Intracellular NLRs respond to increasing numbers of DAMPs that alert the immune system to cell injury and provide a proximate pathway for sensing exposure to possible toxins or pollutants in the environment. Transcription and translation of genes lead to the third stage of the inflammatory cascade, which is the inducible expression of proinflammatory cytokines, such as interleukin-1-beta (IL- 1β), IL-6, tumor necrosis factor-alpha (TNF-α), and others. In conjunction with chemokines (attractants) and various costimulatory molecules, these soluble proteins facilitate the recruitment of effector cells, such as monocytes and neutrophils, to the site of disturbance. Neutrophils create a cytotoxic environment by releasing noxious chemicals from cytoplasmic granules (a process called degranulation). Rapid release of these chemicals requires consumption of both glucose and oxygen, known as the respiratory burst. Toxic chemicals released include highly reactive oxygen and nitrogen species (ROS and RNS, respectively) and various proteinases. These substances are destructive to both pathogens and hosts and essentially induce liquefaction of surrounding tissue to stave off microbial metastasis. These effector mechanisms are thus major contributors to host collateral damage. The net effect of these interactions culminates in the stereotypical cardinal signs of local inflammation: heat, swelling, redness, pain, and loss of function. The effector functions of inflammation are further regulated by the adaptive immune system, which is discussed below. The last phase of inflammation is its resolution, which is critical for limiting collateral damage to the host (Serhan & Savill, 2005). After the first few hours of inflammation, a coordinated program of resolution is set into motion by tissue-resident and recruited macrophages. During acute inflammation, these cells produce proinflammatory prostaglandins and leukotrienes, but rapidly switch to lipoxins, which block further neutrophil recruitment and instead favor enhanced infiltration of monocytes important for wound healing.

1.2.3 Causes of Inflammation

The principal cause of inflammation is a pure mechanical pressure, including blunt trauma, foreign bodies vibrations and chronic pressure of low intensity. The basic mechanism of causing inflammation by pressure is most probably through tissue hypoxia. Namely, tissue oxygen as a liposoluble substance is distributed mainly in lipids and hydrophobic proteinaceous matter. Exposed to pressure, the volume of tissue water and hydrophilic components as virtually incompressible remains unchanged, while hydrophobic matter is compressed, with oxygen being squeezed out.

Upon cessation of pressure, due to its imperfect elasticity the tissue remains more or less shrunk and therefore with a significantly diminished oxygen content for a certain period. Concomitant vascular changes may contribute to hypoxia especially if larger blood vessels are involved. Hypoxia may then generate inflammatory changes through hypoxia-inducible factor (HIF) (Adams *et al.*, 2009).

1.2.4 Mediators of Inflammation and the inflammatory process

A complex interplay of inflammatory cells and chemical mediators is responsible for allergic inflammation. It is now understood that the allergic reaction consists of an early-phase response involving mast cell degranulation with the release of histamine and a late-phase response characterized by the migration of inflammatory cells. This review provides a summary of the early- and late-phase events associated with allergic inflammation and an overview of the principal chemical mediators involved in the inflammatory process.

1.2.4.1 Histamine

Histamine (Imidazolylethylamine) is a vasodilator, a constrictor of smooth muscle, and a potent stimulant of vascular permeability, respiratory mucus, and gastric acid secretion. It exerts its effects on a variety of cell types including smooth muscle cells, neurons, glandular cells (endocrine and exocrine), blood cells, and cells of the immune system (Pearce *et al.*, 1990). In addition to its role in immediate hypersensitivity reactions, histamine can exert H2-receptor- mediated anti-inflammatory activity including inhibition of human neutrophil lysosomal enzyme release, inhibition of IgE-mediated histamine release from peripheral leukocytes and activation of suppressor T. lymphocytes. Histamine is synthesized in the Golgi apparatus of mast cells and basophils by decarboxylation of the amino acid histidine and is then stored in secretory granules in complexes with heparin, protein, or both. In sensitized individuals, histamine release occurs with the binding of specific allergens to IgE antibodies on mast cells and basophils, initiating a Ca+-dependent degranulation reaction. After continuity is established between the secretory granule and the plasma membrane of the mast cell, histamine dissociates from the partially solubilized granular matrix by exchanging with sodium ions in the extracellular environment and is extruded into tissue spaces (Uvnas *et al.*, 1964). Recent studies provide evidence for an alternate secretory process in humans, referred to as piecemeal degranulation, in which histamine is released from mast cells with retention of empty granule containers within the cytoplasm (Dvorak *et al.*, 1997). The release of histamine from mast cells and basophils can occur by immunologic (IgE-mediated) and non immunologic (exercise-induced

asthma) mechanisms, thereby contributing to the symptoms of both allergy and anaphylaxis. Research also suggests that activated T lymphocytes may participate in the process of mast cell degranulation by direct cell-to-cell contact (Bhattacharyya *et al.,* 1998).

1.2.4.2 Leukotrienes

Leukotriene (LT) C4 and its products, LTD4 and LTE4, make up the biologic mixture previously known as the slow-reacting substance of anaphylaxis. Leukotrienes are generated by most cell types that participate in inflammatory reactions including mast cells, basophils, eosinophils, neutrophils, and monocytes (Naclerio, *et al.,* 1991). As chemical mediators of inflammation, they have biologic activity similar to that of histamine. Studies of the effects of H1- receptor antagonists on leukotriene release suggest that the mechanism may involve blocking the activity of receptor-coupled G proteins (Rihoux, *et al.,* 1997). Leukotrienes are derived from arachidonic acid, which is made available from cell membrane phospholipids by the action of phospholipase A_2 or by the sequential action of phospholipase C and diacylglycerol lipase (Kennerly *et al.,* 1979). After being released from cell membranes by the action of phospholipase A_2, arachidonic acid is converted by 5- lipoxygenase to 5*S*-hydroperoxy-6,8-*trans*-11,14-*cis*eicosatetraenoic acid. The same 5-lipoxygenase pathway catalyzes the conversion of 5*S*-hydroperoxy-6,8- *trans*-11,14-*cis*-eicosatetraenoic acid to LT4 (LTA4). LTA4 can then be converted to LTB4 (Borgeat *et al.,* 1979), or conjugated with reduced glutathione to form LTC4 (Corey *et al.,* 1980). In the later phases of the allergic reaction, after transport into the extracellular environment, LTC4 can undergo further metabolism to LTD4 and LTE4 (Naclerio *et al.,* 1991).

1.2.4.3 Prostaglandins

Another group of arachidonic acid-derived molecules that mediate allergic reactions are prostaglandins. The most abundant cyclooxygenase product generated by the immunologic activation of human lung mast cells is PGD_2 (Lewis *et al.,* 1981). PGD_2 is generated by human mast cells after they are activated through the IgE receptor or by calcium ionophore. The biologic effects of prostaglandins generated during mast cell-dependent reactions in tissues include modulation of smooth muscle contractility, vascular permeability, sensations of pruritus and pain, and platelet aggregation and degranulation. Prostaglandins are generated by cyclooxygenase, an enzyme associated with the endoplasmic reticulum of mast cells. Similar to 5-lipoxygenase, cyclooxygenases

catalyzes the formation of relatively unstable intermediates PG_2 and PGH_2 (Hamberg *et al.,* 1974). These intermediates may then be converted non enzymatically or enzymatically by specific isomerase/peroxidase or synthase enzymes to yield the primary prostaglandins PGD_2, PGE_2, and PGF (Schewattz *et al.,* 1984).

1.2.4.4 Kinins

Kinins are potent peptide hormones formed de novo in body fluids and tissues during inflammation. They are derived from 2 globulins (high and low molecular weight kininogens through proteolytic cleavage by a variety of enzymes, the most important of which are plasma and tissue kallikreins (Proud *et al.,* 1988). Three distinct kinins have been identified in humans: bradykinin, kallidin (lysbradykinin), and met-lys-bradykinin. Although some quantitative differences exist, all of the kinins possess the same basic pharmacologic properties including the induction of smooth muscle contractility, vasodilation and increased capillary blood flow. The generation of kinins in the inflammatory response occurs through a plasma pathway, a tissue pathway, and a plasma/tissue-independent pathway. Kinins production by the plasma pathway is initiated by the interaction of activated factor XII (Hageman factor) with prekallikrein and high molecular weight kininogen (Colma *et al.,* 1975). Activated Hageman factor (factor XIIa) initiates the conversion of prekallikrein to kallikrein, which furthers the conversion of factor XII to XIIa. The action of kallikrein to further conversion of factor XII to XIIa is augmented by high molecular weight kininogen. The active kallikrein released by this sequence of events cleaves high molecular weight kininogen to release bradykinin. The tissue pathway for bradykinin synthesis involves tissue kallikreins that are physiochemically and immunologically distinct from plasma kallikrein). Tissue kallikreins release kinins from both high molecular weight kininogens and low molecular weight kininogens, with low molecular weight kininogen making up approximately 70 % of the total human kininogen pool (Proud *et al.,* 1988). The ability of tissue kallikreins to react equally well with high molecular weight kininogen and low molecular weight kininogen provides a larger pool of available substrate than is available to plasma kallikreins. Once activated, tissue kallikreins are less susceptible to inhibition by protease inhibitors than plasma kallikrein, and they are the only known enzymes to generate the bradykinin precursor, kallidin. Kallidin can then be converted to bradykinin by the enzymatic removal of the N-terminal lysine. The independent pathway involves the action of kininogenases other than kallikreins in the cleavage of high and low molecular weight kininogen to form

bradykinin. Although the relevance of kinins as mediators of allergic airway disease is not clearly established, observations that human mast cells and basophils possess IgE-mediated kininogenase activity, that bradykinin receptors are present in nasal mucosa and that elevated levels of kinins are present in nasal lavages of patients with allergic rhinitis suggest a role for these peptides in allergic inflammation (Shin *et al.,* 1966).

1.2.4.5 Platelet activating factor

Platelet activating factor (PAF) is an ether-linked phospholipid, designated as such because of its discovery as a basophil-derived mediator of rabbit platelet activation (Barbaro, *et al.,* 1966). PAF may be produced by several of the cells that participate in the inflammatory response including mast cells, macrophages, neutrophils, and eosinophils (Barnes *et al.,* 1993). The synthesis of PAF occurs as a 2-step pathway in which phospholipase A_2 hydrolyzes a 1-O-alkyl-2-acyl-glyceryl- 3-phosphorylcholine to produce 1-O-alkyl-2-acylglyceryl-3-phosphocholine (lyso-PAF), which is then converted by an acetyltransferase enzyme to PAF. The biologic activities of PAF include platelet activation, activation of neutrophils, and smooth muscle contraction (Schwarttz *et al.,* 1984). Because PAF is rapidly inactivated in vivo, it is likely that it triggers a chain of inflammatory events. PAF stimulates the accumulation of eosinophils to endothelial surfaces, which may be the first step in the recruitment of eosinophils into tissues. Eosinophils also are a source of PAF and can attract additional eosinophils, which can potentiate the inflammatory reaction. PAF stimulates eosinophils to release basic proteins, leading to epithelial cell damage and causes an increased expression of low-affinity IgE receptors on eosinophils and monocytes (Barnes *et al.,* 1993).

1.2.4.6 Cytokines and Chemokines

An increasing body of evidence suggests that cytokines play a critical role in late-phase inflammatory events associated with allergic rhinitis and asthma, and that the mast cell is a source of many multifunctional cytokines (Bradding *et al.,* 1966). Cytokines such as IL-1, -2, -4, -5, -6, TNF-, and GMCSF influence the course of allergic inflammation by modulating the activity of inflammatory cells including macrophages, T cells, B cells, and eosinophils. IL-1 enhances the growth of T-helper cells and the growth and proliferation of B cells, whereas IL-2 causes proliferation of T lymphocytes and activation of B lymphocytes. IL-4 causes differentiation of B lymphocytes into IgE-secreting plasma cells and, along with TNF-□, upregulates both high- and low-affinity IgE receptor expression on antigen presenting cells. IL-5 causes activation of B

lymphocytes and the differentiation and increased longevity of eosinophils, and IL-6 causes increased synthesis and secretion of immunoglobulins by B lymphocytes. In clinical studies in patients with seasonal allergic rhinitis, nasal biopsy specimens showed immunoreactivity for IL-4 and IL-6 localized predominantly to mast cells, whereas IL-5 was found in both mast cells and eosinophils (Brandding *et al.,* 1995). Similar findings of increased levels of cytokines (IL- 4 and IL-10) in nasal fluids were reported in a study of children with seasonal allergic rhinitis (Colma *et al.,* 1975). Chemokines such as IL-8, macrophage inflammatory protein-1, macrophage chemoattractant protein-1, -2, and -3, and RANTES are cytokines that function primarily as chemoattractant molecules for macrophages and circulating leukocytes38 but also induce other inflammatory effects including histamine release from basophils and mast cells and activation of eosinophils. Increased levels of chemokines IL-8, macrophage inflammatory protein-1, and RANTES, which correlated with total symptom scores, together with cytokines TNF-and GM-CSF, have been detected in nasal secretions after allergen challenge in patients with allergic rhinitis (Sim *et al.,* 1995). The inflammatory changes that occur during the allergic response produce priming of the nasal tissues. The resultant nasal hyper-responsiveness leads to escalation of both the symptomatic and inflammatory response to additional allergic or irritant stimulation to the nasal mucosa.

1.2.4.7 Plasma Derived Mediators Of Inflammation

Plasma derived mediators of inflammation are proteins that circulate in the plasma in the form of inactive precursors. The mediator producing systems found in the plasma are complement systems, kinin systems, fibrinolytic systems and clotting systems.

1.2.4.8 Complement System

This is an important part of the innate immune system conferring protection against invading pathogenic agents, such as protozoa, viruses and bacteria. Its role is to generate biologically active products from the pathways of complement activation which include, alternative, classical, lectin, properdin and thombin pathways. Peptides generated by complement activation play a critical role in the elimination of invading pathogens. They include, C3b and C4b which opsonise pathogens for phagocytosis, the anaphylatoxins C3a and C5a which act as chemoattractants for leukocyte and membrane attack complex (MAC or C5b-9) involved in the direct lyses of pathogens. The generation of anaphylatoxins C3a and C5a further induces degranulation of mast cells, basophils,

eosinophils. They also induce the expression of adhesions molecules on endothelial cells and cause smooth muscle contration.

1.2.4.9 Kinin System

Kinis are vasoactive peptides generated through the kinin-generating cascade. Plasma protein and brandykinin are the most important product of this cascade. Prekallikrein is converted to the protease kallikrein by factor XIIa from the clotting cascade. Kallikrein interact with high molecular weight kininogen (HMWK) to bring about the hydrolysis and release of brandykinin. Brandykinin is a powerful vasopermeablity agent. It also causes pain, vasodilation and oedema, all contributing to inflammation.

1.2.4.10 Fibrinolytic System

The coagulation system and fibrolytic systems act in opposite direction to counterbalance clotting system. The fibrolytic system is activated by urinary plasminogen activator (uPA) and tissue plasminogen activator (tPA) which converts plasminogen to plasmin which directly interact with fibrin to bring about the breakdown of fibrin clots as they are formed within the intravascular compartment.

1.2.4.11 Clotting or Coagulation System

The mechanism of coagulation involves adhesion, activation and aggregation of platelets, along with the deposition and maturation of fibrin. The activation of fibrin involves two pathways, the intrinsic and extrinsic cascades. Following vascular injury, the extrinsic clotting cascade is triggered as activated endothelium expresses tissue factor (TF) on their luminal surfaces and expresses increased levels of plasminogen actvator inhibitor-1 which inhibits fibrinolysis. In the presence of cloth activating factor, prothrombin is converted to thrombin by prothrombinase. Thrombin in turn converts fibrinogen to fibrin, which is the major product involved in cloth formation. The intrinsic cascade occurs with the engagement of Hageman factor (fanactor XIII) which interacts with factors Va and VIIIa and becomes converted to its active form (Leslie, 2010).

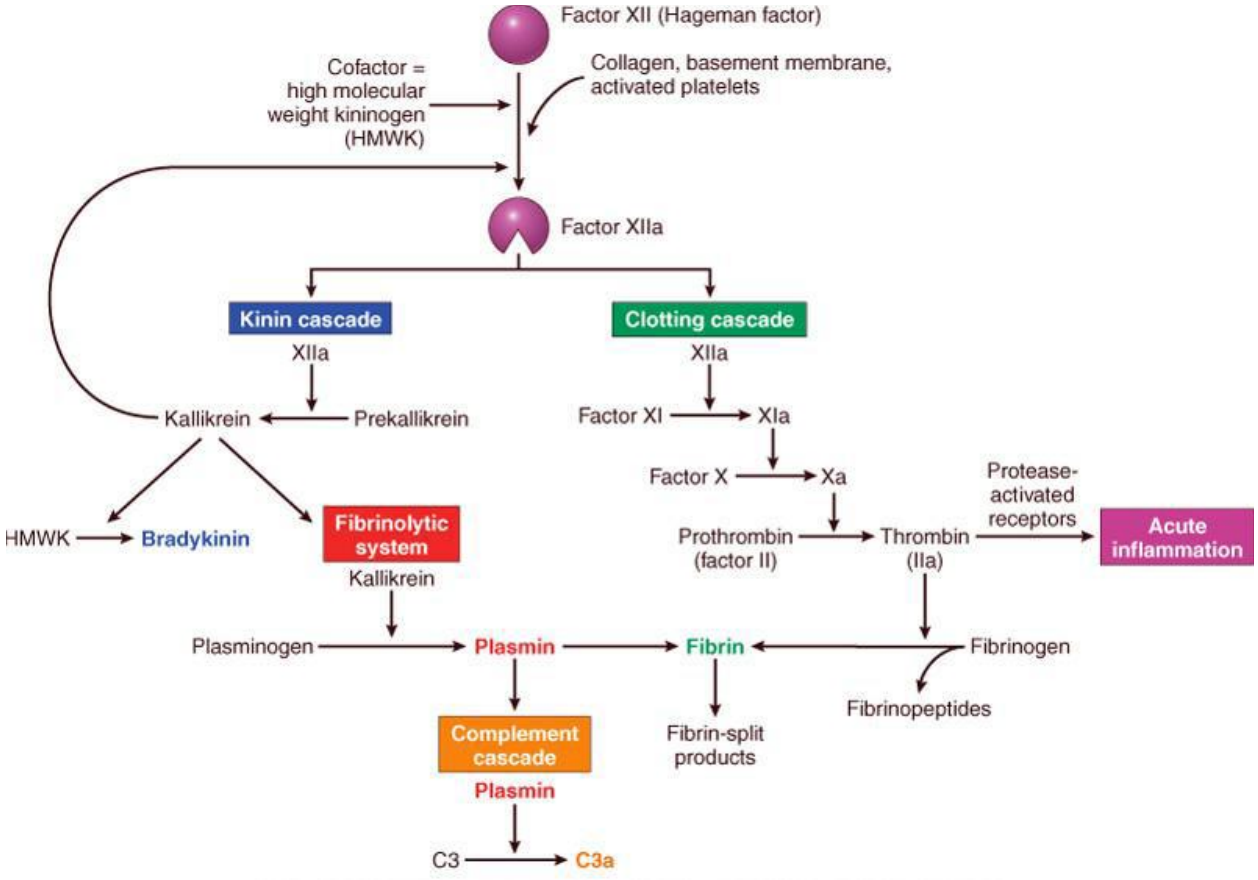

Fig. 2: Clotting system and its role in inflammation

Source: (Pyriyadarshini, *et al.*, 2013)

1.2.5 Cells of Inflammation

Leukocytes are the major cellular components of the inflammatory response and include neutrophils, T and B lymphocytes, monocytes, macrophages, eosinophils, mast cells, and basophils. Specific functions are associated with each of these cell types, but such functions overlap and vary as inflammation progresses. In addition, local tissue cells interact with one another and with inflammatory cells, in a continuous response to injury and infection.

1.2.5.1 Macrophages

Classical or pro-inflammatory activation of macrophages is induced by IFN-γ or LPS while that triggered by IL-4, IL-10 or IL-13 is known as alternative or anti-inflammatory activation. Apart from a series of structural and functional modifications, the main difference between these phenotypes is

the biochemical pathway used for processing the amino acid arginine. IFN-γ or LPS induce NOS2 enzyme, thereby producing nitric oxide (NO), which has great destructive power and in the first phases of inflammation kills microorganisms. In anti-inflammatory macrophages, arginase is induced and produces proline and polyamines, which catalyze the reconstitution of the damaged extracellular matrix, an event that occurs during the final phases of inflammation (Apar *et al*, 2008).

1.2.5.2 Neutrophils

Neutrophils are the most abundant white blood cell, constituting 60-70% of the circulating leukocytes, (Alberts *et al.,* 2002) and including two functionally unequal subpopulations: neutrophil-killers and neutrophil-cagers. They defend against bacterial or fungal infection. They are usually first responders to microbial infection; their activity and death in large numbers form pus. They are commonly referred to as polymorphonuclear (PMN) leukocytes, although, in the technical sense, PMN refers to all granulocytes. They have a multi-lobed nucleus, which consists of three to five lobes connected by slender strands. This gives the neutrophils the appearance of having multiple nuclei, hence the name polymorphonuclear leukocyte. The cytoplasm may look transparent because of fine granules that are pale lilac when stained. Neutrophils are active in phagocytosing bacteria and are present in large amount in the pus of wounds. These cells are not able to renew their lysosomes (used in digesting microbes) and die after having phagocytosed a few pathogens (Pillay *et al.,* 2010). Neutrophils are the most common cell type seen in the early stages of acute inflammation. The life span of a circulating human neutrophil is about 5.4 days (Falcone *et al.*, 2000).

1.2.5.3 Eosinophils

Eosinophils compose about 2- 4 % of the WBC total. This count fluctuates throughout the day, seasonally and during menstruation. It rises in response to allergies, parasitic infections, collagen diseases, and disease of the spleen and central nervous system. They are rare in the blood, but numerous in the mucous membranes of the respiratory, digestive, and lower urinary tracts. They primarily deal with parasitic infections. Eosinophils are also the predominant inflammatory cells in allergic reactions. The most important causes of eosinophilia include allergies such as asthma, hay fever, and hives; and also parasitic infections. They secrete chemicals that destroy these large parasites, such as hook worms and tapeworms that are too big for any one WBC to phagocytize. In

general, their nucleus is bi-lobed. The lobes are connected by a thin strand. The cytoplasm is full of granules that assume a characteristic pink-orange color with eosin staining.

1.2.5.4 Basophils

Basophils are chiefly responsible for allergic and antigen response by releasing the chemical histamine causing the dilation of blood vessels. Because they are the rarest of the white blood cells (less than 0.5 % of the total count) and share physicochemical properties with other blood cells, they are difficult to study (Kumar *et al.,* 2003). They can be recognized by several coarse, dark violet granules, giving them a blue hue. The nucleus is bi- or tri-lobed, but it is hard to see because of the number of coarse granules that hide it. They excrete two chemicals that aid in the body's defenses: histamine and heparin. Histamine is responsible for widening blood vessels and increasing the flow of blood to injured tissue. It also makes blood vessels more permeable so neutrophils and clotting proteins can get into connective tissue more easily. Heparin is an anticoagulant that inhibits blood clotting and promotes the movement of white blood cells into an area. Basophils can also release chemical signals that attract eosinophils and neutrophils to an infection site (Newball *et al,* 1979).

1.2.5.5 Lymphocytes

Lymphocytes are much more common in the lymphatic system than in blood. Lymphocytes are distinguished by having a deeply staining nucleus that may be eccentric in location and a relatively small amount of cytoplasm. Lymphocytes include: B cells make antibodies that can bind to pathogens, block pathogen invasion, activate the complement system, and enhance pathogen destruction.

1.2.5.6 T cells:

CD4+ helper T cells: T cells displaying co-receptor CD4 are known as CD4+ T cells. These cells have T-cell receptors and CD4 molecules that, in combination, bind antigenic peptides presented on major histocompatibility complex (MHC) class II molecules on antigen-presenting cells. Helper T cells make cytokines and perform other functions that help coordinate the immune response. In HIV infection, these T cells are the main index to identify the individual's immune system integrity.

CD8+ cytotoxic T cells: T cells displaying co-receptor CD8 are known as CD8+ T cells. These cells bind antigens presented on MHC I complex of virus-infected or tumour cells and kill them. Nearly all nucleated cells display MHC I. γδ T cells possess an alternative T cell receptor (different from the αβ TCR found on conventional CD4+ and CD8+ T cells). Found in tissue more commonly than in blood, T cells share characteristics of helper T cells, cytotoxic T cells, and natural killer cells.

Natural killer cells are able to kill cells of the body that do not display MHC class I molecules, or display stress markers such as MHC class I polypeptide-related sequence A(MIC-A). Decreased expression of MHC class I and up-regulation of MIC-A can happen when cells are infected by a virus or become cancerous.

1.2.5.7 Monocytes

Monocytes, the largest type of WBCs, share the "vacuum cleaner" (phagocytosis) function of neutrophils, but are much longer lived as they have an extra role: They present pieces of pathogens to T cells so that the pathogens may be recognized again and killed. This causes an antibody response to be mounted (Dahinden, 1994). Monocytes eventually leave the bloodstream and become tissue macrophages, which remove dead cell debris as well as attack microorganisms. Neither dead cell debris nor attacking microorganisms can be dealt with effectively by the neutrophils. Unlike neutrophils, monocytes are able to replace their lysosomal contents and are thought to have a much longer active life. They have the kidney shaped nucleus and are typically a granulated. Some leucocytes migrate into the tissues of the body to take up a permanent residence at that location rather than remaining in the blood. Often these cells have specific names depending upon which tissue they settle in, such as fixed macrophages in the liver, which become known as Kupffer cells. These cells still serve a role in the immune system.

1.2.6 Inflammatory Diseases

Inflammatory diseases are a group of clinical disorders which are characterized by abnormal inflammatory responses (i.e. chronic inflammation) as a major hallmark. Chronic inflammation is so tightly linked to the pathogenesis of inflammatory diseases that it becomes difficult to pinpoint both cause and effect of these disorders. For example, inflammation is caused by obesity, whereas chronic inflammation can lead to obesity-associated diabetes partly because of insulin resistance (Hotamisligil, 2006). Similar feedback mechanisms are also evident for other inflammatory disorders. Thus, the chronic nature of the inflammation associated with these diseases is controlled,

at least in part, by the pathological outcome of these diseases, making them quite different from those induced by persistent infection. Rheumatoid arthritis is another perfect example of a chronic inflammation-associated disorder. In rheumatoid arthritis, the synovium, the lining of the joint, undergoes chronic inflammation by the infiltration of macrophages and lymphocytes and the activation of synoviocytes, synovial cells, which produce synovial fluids. The synovial fluid during rheumatoid arthritis is invaded by billions of neutrophils everyday (Hollingsworth *et al.,* 1967). It has been suggested that neutrophils, which have a half-life of about 4 h, make a significant contribution to the chronic nature of the inflammation in synovial tissue. It is hypothesized that one of the enzymes of neutrophils, cytosolic peptidyl arginine deaminase whose activity depends on the levels of extracellular Ca2+, is released from the dead neutrophils and activated. This enzyme creates citrulline in some proteins by converting the guanidine side chains of Larginine residues to ureido residues. Interestingly, autoantibodies associated with rheumatoid arthritis are found to react with citrullinated proteins. The mechanism of inflammatory response in some inflammatory diseases can be more complex compared to others. For example, asthma and allergic inflammations are caused by dysregulated interactions between mucosal epithelia and innate immune cells. Further studies demonstrated a much wider involvement of the immune systems in asthma pathogenesis than ever thought. Apart from the innate immunity, cells of adaptive immunity such as CD4 T helper 2 (Th2) cells as well as Th2-associated cytokines are found to be strongly linked to asthma (Robinson *et al.,* 1992). The initial cause of asthma seems much more complicated than ever imagined. Even though genetic predisposition to asthma has been reported for years, recent genome-wide studies suggest relatively small hereditary contributions to asthma. Alternatively, environmental factors have been considered as major determinants of asthma risk. For example, numerous reports suggest several viral upper respiratory infections early in life along with genetic predisposition within families influence a significant risk for asthma (Locksley, 2010). The impact of inflammatory disorders our body. To mention a few, inflammation is associated with many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and multiple sclerosis (Glass *et al.,* 2010). Inflammatory bowel disease affects our intestine (Garrett *et al.,* 2010) whereas glomerulonephritis is caused by the inflammation of the glomeruli, small blood vessels, in the kidneys (Fakhouri *et al.,* 2010).

Over the last decade it has become evident that inflammation plays a critical role in promoting cancer, in particular the tumorigenesis, a process of tumor formation. In addition to cancer cells, various types of immune cells are commonly found within tumors. Interestingly, an inflammatory microenvironment is also more frequently found as an essential part of all tumors (Mantovani *et al.*, 2008). It has been demonstrated that the inflammatory response triggered by infection is also associated with increased cancer risk (De Martel and Franceschi, 2009). A recent study has revealed that lung tumorigenesis caused by tobacco smoke is in fact initiated by IKKbeta and JNK1-mediated chronic inflammation, suggesting that the pathways of both tumorigenesis and inflammation are closely linked. It is believed that apart from the tumor-promoting inflammatory response, an active anti-tumor immunity is also present in most tumor microenvironments. It is thus suggested that the progression of tumorigenesis is dictated by the battle between tumor-promoting inflammation and anti-tumor immunity. Obviously, in established tumors, antitumor immunity is profoundly dominated by tumor-promoting inflammation (Smyth *et al.*, 2006). Studies show that inflammatory microenvironments not only promote cancer cell growth but also increase mutation rates, possibly by producing reactive oxygen species and nitrogen intermediates which can cause DNA damage and genomic instability (Grivennikov *et al.,* 2010). The role of inflammation in genomic instability is supported by the observation that activation-induced cytidine deaminase (AID), an enzyme which triggers genomic instability and is usually overexpressed in many cancers, is induced by inflammatory cytokines (Okazaki *et al.,* 2007). Apart from genomic instability, environmental factors such as carcinogens, infectious microbes, tobacco smoke and inhaled pollutants have been considered to play critical roles in inflammation-induced cancers (Aggarwal *et al.*, 2009). Therefore, not all chronic inflammatory diseases are connected to cancer risk. For example, rheumatoid arthritis does not promote cancer whereas inflammatory bowel disease and chronic hepatitis do due to the exposure to dietary and environmental carcinogens. The connection between inflammation and cancer does not operate in one direction only as numerous studies showed that DNA damage can also lead to inflammation. Using the model of hepatocellular carcinoma induced by the carcinogen. An overview of inflammation: Mechanism and consequences diethylnitrosamine (DEN), several reports demonstrated that the DNA damage-induced necrotic cell death lead to inflammation. Cancer-associated oncoproteins such as Ras, Myc and RET can also lead to inflammation by activating signaling pathways involved in the production of proinflammatory cytokines and chemokines (Mantovani *et al.,* 2008). Last of all, tumor-associated inflammation can also be

instigated by the modern cancer therapy itself such as radiotherapy and chemotherapy. These therapies are usually associated with a substantial amount of necrotic death of not only cancer cells but also surrounding normal cells, which in turn activates the inflammatory response (Zong and Thompson, 2006). Therefore, based on the literature, it can be concluded that inflammatory response is an integrated part of cancer biology, right from the beginning of tumorigenesis toward the application of therapeutic interventions.

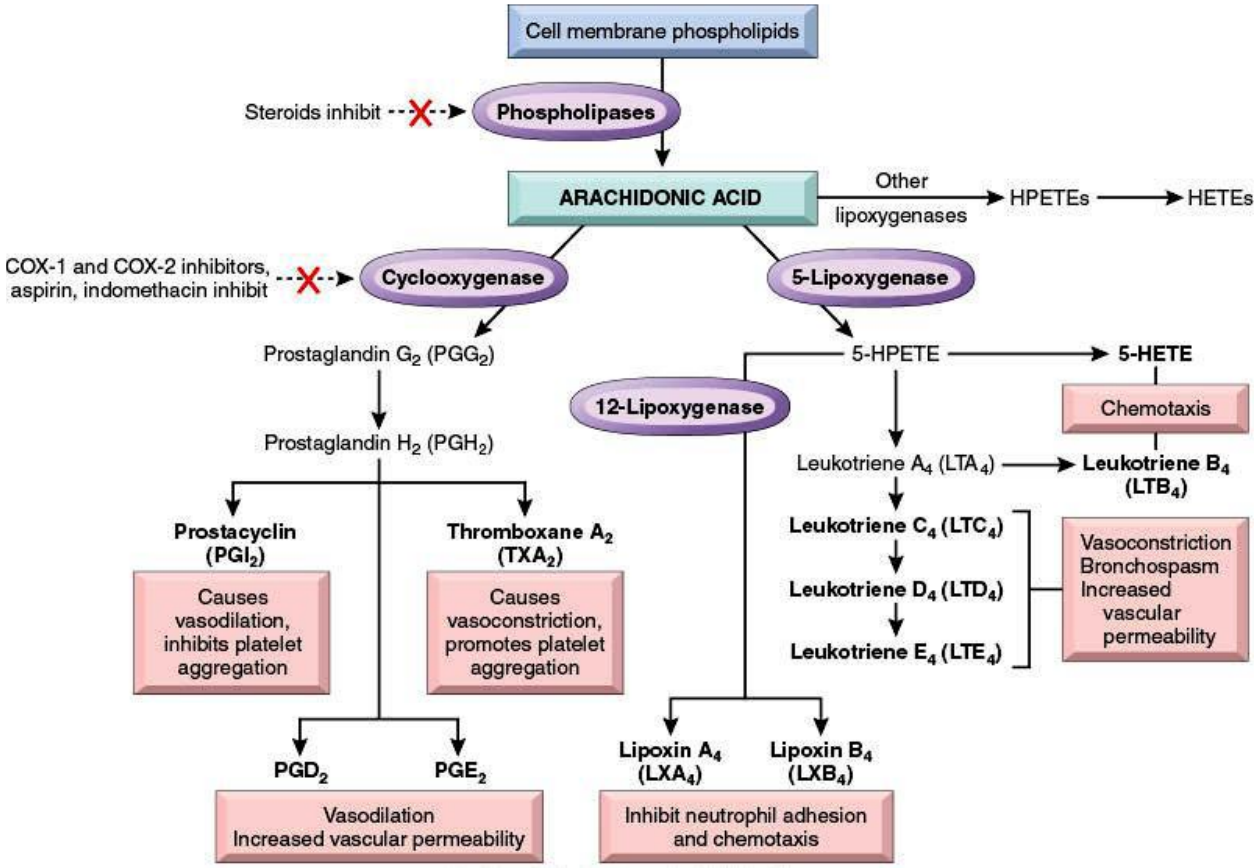

Figure 3: Generation of arachidonic acid metabolites and their roles in inflammation
Source: (Priyadarshini *et al.*, 2013).

1.2.7 Anti-Inflammatory Drugs

The anti-inflammatory analgesic drugs have their origins in the use of extracts of salicylate-containing plants, especially the bark of the willow tree (*Salix alba* and other members of the *Salix* species), in the treatment of fever, pain and inflammatory conditions (Rainsford, 2004). These treatments date from early Chinese, Indian, African and American eras and were initially described in some detail by Roman and Greek medical authorities. During the 17th–19th centuries, the popularity of these plant extracts became evident following the publication by the Reverend Edward Stone in the 17th century of probably what were the first clinical trials of willow bark extract for the treatment of aches or fever. Isolation of the principally active salicylate components followed in the early 19th century and with advances in chemistry in Europe and developments in the German chemical industry in the mid-late 19th century, there followed the synthesis of salicylic and acetylsalicylic acids, the latter being highly successfully commercialized by Bayer AG as Aspirin™ over 100 years ago. The historical aspects of the origins and development of aspirin and other salicylates are told in detail elsewhere. During the period of the exploitation of the by-products of the coal tar industry in Germany in the 19th century came also the development of antipyretic/analgesic agents, antipyrine, aminopyrine, phenacetin and later following recognition of paracetamol (acetaminophen) as the active metabolite of phenacetin, this was eventually commercially developed for use as an analgesic/antipyretic agent in the 1950's. Anti-inflammatory agents are use in the treatment of inflammation. There are two main types of anti-inflammatory agents; namely Glucocorticosteroids and Non- Steroidal Anti-inflammatory Drugs (NSAIDs).

1.2.7.1 Discovery of NSAIDs

The development of the first of the category of what are now known as the nonsteroidal anti-inflammatory drugs (NSAIDs) of which aspirin has now become recognised as the progenitor, was phenylbutazone in 1946 and later indomethacin in the 1960's. Phenylbutazone was initially employed as a combination with antipyrine in the belief it would enhance the actions of the latter. However, it emerged to have greater anti-inflammatory/analgesic activity than antispyrine and was for the best part of 30 years successfully used for the treatment of arthritic and other painful inflammatory conditions until its popularity progressively waned after associations with life-threatening a granulocytosis and bone marrow suppression (still essentially not conclusively proven today), upper gastrointestinal ulcers and bleeding and subsequent popularity of more advanced

NSAIDs. Ibuprofen was developed by Boots (UK) in the 1950–1960's and after establishing its favourable safety profile at dose ranges for analgesic and anti-pyretic efficacy (up to 1200 mg daily) it was the first NSAID (other than aspirin) to be approved for non-prescription (over-the-counter or OTC sale) use in the UK (in 1963), then the USA (in 1964) and later in many other countries worldwide (Rainsford, 1999). Just after ibuprofen was developed, a large number of pharmaceutical companies undertook the discovery and development of NSAIDs with a range of chemical and biological properties (Evans & Wereiamson, 1987). Most of these drugs developed in the 1960's were discovered in the pre-prostaglandin era (i.e. before Vane and his colleagues had discovered the inhibitory actions of aspirin and related drugs on the production of prostaglandins). Their anti-inflammatory, analgesic and antipyretic properties were discovered using animal models with some supportive properties being established in some biochemical systems which were known also to be important in inflammation (e.g. mitochondrial oxidative, intermediary and connective tissue collagen and proteoglycan metabolism; stability of albumin; and later oxyradicals).

1.2.7.2 COX-2 Selective Agents and the Coxibs

Coxibs belong to a class of nonsteroidal anti-inflammatory drugs (NSAIDs) that are used to treat pain and inflammation in a variety of acute and chronic conditions. They have been principally employed for treating rheumatoid and osteo-arthritis, and other arthritic diseases, dental and surgical pain in post-operative settings, dysmenorrhoea, and acute injuries (Kean and Buchanan, 2005). The coxibs have also been explored for the prevention of colorectal and some other cancers as well as Alzheimer's disease (Firuzo and Practico, 2006), although the outcomes of these studies have not been particularly favourable largely through lack of efficacy and/or cardiovascular complications. Indeed, the apparent high risk of myocardial infarctions and the exacerbation of symptoms of hypertension and elevation of blood pressure led to the worldwide dramatic withdrawal of one of the leading members of the coxib class. (Rainsford, 2005). This has been followed by the recommendation of the US Food and Drug Administration in April 2005 that Pfizer Inc, the company manufacturing other leading coxibs (celecoxib and valdecoxib) also withdraw valdecoxib from the US market because of the same adverse events. Questions have now been posed whether a cardio-renal syndrome is associated with the entire class of coxibs – a class effect – that may account for the mortality or non-fatal myocardial infarctions and elevation of blood pressure associated with these drugs, possibly in at risk subjects (as yet undetermined). The US FDA has subsequently

specified a black box warning on the use of celecoxib and all other coxibs (that remain on the market or in clinical trial) and also a general warning of cardiovascular risk with all other NSAIDs. The European Medicines Evaluation Agency (EMEA), now the European Medicines Agency (EMA) has also re-evaluated the cardiovascular risk with the coxibs and has recommended only restricted use of these drugs. Thus, in somewhat over half a decade since their much-heralded introduction as being safer to the gastrointestinal tract and kidneys than traditional NSAIDs and with rofecoxib and celecoxib having achieved worldwide market domination, they have now plummeted from sales to almost obscurity in the therapeutic armamentarium. There are indications, however, that celecoxib may find its way back onto the world markets but the future of rofecoxib is less certain and maybe it were find applications (e.g. in juvenile rheumatoid arthritis in which it was especially effective) but under very strictly restricted conditions. Another Merck drug, etoricoxib, might not be associated with an excess risk of cardio-renal effects and associated myocardial infarction and exacerbation of hypertension. Likewise, although less adequate data are available with lumiracoxib, there are suggestions this drug may not have the same risks as seen with rofecoxib or other coxibs. A key factor that has emerged from the analysis of reasons why rofecoxib, valdecoxib, and celecoxib may have led to development of the cardio-renal syndrome thought to underlie myocardial infarction and hypertension appears to have been that these effects were apparent with high dose levels of these drugs. It is possible that in some of the conditions where they were being used (e.g. a colorectal preventative trials of rofecoxib and celecoxib and post-operative coronary bypass in the case of valdecoxib) may have been conditions where there were appreciable manifestations of disease stress that led to pre-disposition to the development of cardio-renal syndromes and myocardial infarction. A major factor was dosage and the data indicates that the cardio-renal syndrome and cardiovascular risks were only evident with high doses of these drugs. Another factor which has emerged is that the principal mode of action of these drugs, to specifically inhibit the enzyme, cyclooxygenase-2 (COX-2) may have been a major factor causing the development of these site effects – this being an example of what is known as "mechanism-based" toxicology. Coxibs are strictly classed as *functional analogues* since aside from the general chemical features in common with members of this class there are few common specific chemical features that uniformly describe their properties. There are, of course, some features of the biochemical interactions of these with the enzyme, COX-2, which mediates their main pharmacological actions. With the possibly unique exception of lumiracoxib, the other coxibs are tricyclic compounds with high pKa values. These contrast with the

conventional NSAIDs that are weakly acidic compounds with pKa values of about 3–5, derived from either aryl-carboxylic acids or keto-enolic compounds. The coxibs are diaryl-heterocycles that have a *cis*-stilbene moiety substituted in one of the pendant phenyl rings with a 4 methylsulphone (e.g. rofecoxib) or sulfonamide (e.g. celecoxib) substituent. These moieties are critical together with the diaryl heterocyclic structure in determining their actions as highly specific COX-2 inhibitors. The odd drug apparent in these chemical associations within the coxibs is lumiracoxib. This drug is an analogue of the traditional acid NSAID, diclofenac, and does not have the tricyclic character of the other coxibs but is an anilino-phenylacetic acid. The 2 ,6-dichloro-substituents of diclofenac are replaced by 2-chloro, 6-fluoromoieties in lumiracoxib. There are indications that the COX-2 specificity of lumiracoxib. Perhaps this drug should not be classed as a coxib in view of the lack of associations both chemically and possibly pharmacologically with the other coxibs. The term coxib derives logically from ***cox***-inh**ib**it (or) and appears to have been a marketing ploy by the two major companies that developed these drugs to discriminate them from other NSAIDs. Whether such a pharmacological description is justifiable is debatable especially since the claims for markedly improved GI safety with the coxibs are now being increasingly challenged in relation to at least the risk of serious GI adverse reactions observed with low-risk NSAIDs such as diclofenac or ibuprofen (De Leval *et al* 2000).

Rationale for the Discovery of Coxibs

The discovery in 1991 of two COX enzymes that are responsible for the synthesis of inflammatory prostaglandins gave a new basis for understanding how these molecules regulate and mediate inflammatory reactions, pain and fever, as well as a number of diverse physiological reactions such as blood flow, thrombosis, and gastrointestinal, renal and reproductive functions. About two years previously, a unique COX enzyme was discovered that was produced in response to inflammatory stimuli. In a short while, the genes coding for two separate enzyme proteins were isolated and cloned. By convention the enzyme that is responsible for the production of physiologically important prostaglandins and thromboxane A_2 is termed COX-1. The other enzyme, which is responsible for prostaglandins involved in inflammation and pain and is induced upon stimulation with various inflammatory stimuli (lipopolysaccharide, growth factors etc.), is known as COX-2. Actually the term COX refers to the cyclooxygenase enzyme activity and since peroxidase activity is also present, both enzymatic properties exist in one protein which is termed prostaglandin G/H endoperoxide

synthase or PGHS. COX-1 is present in PGHS-1 and COX-2 in PGHS-2. Because the enzymatic activity is the functional response to the gene-regulated and expressed production of prostanoids (prostaglandins and thromboxane A2) it is usual to term the two isoforms COX-1 and COX-2 for short.

Corticosteroids have had considerable popularity since the discovery by Hench at the Mayo Clinic in 1949 that cortisone had a dramatic effect in bed-ridden patients suffering with severe rheumatoid arthritis (Evans & Wereiamson, 1987). Indeed the synthetic developments and large commercial efforts put into the discovery of corticosteroids (or glucocortocoids) made in the 1950s–1980's) (Evans & Wereiamson, 1987) only to yield the relatively few drugs used today is a striking reflection of the complex nature of this class of compounds. Indeed, it was originally considered that corticosteroids acted by immunosuppressive activity, an idea arising due to the effects that occurred at the high doses that were given in former times. Long after their discovery, advances in the molecular biology of inflammatory mediator production have shown that these drugs act principally through their binding to the specific glucocorticoid receptor (GR∗) and consequent inhibition of cellular signalling pathways (especially AP-1 and NF_B) that regulate the production of inflammatory cytokines and chemokines (IL-1, TNF-_, IL-2, IL-5, IFN-_, GM-CSF, MIP-1, MCP-1, Eotaxin, RANTES), cell adhesion molecules (I-CAM, V-CAM, E-selectin), COX-2, cPLA2 and iNOS that control production of eicosanoids and NO, and the production of joint-destructive metalloproteinases. The inhibitory effects of corticosteroids on production of acute phase proteins and erythrocyte sedimentation rate (ESR) are indicators of the effectiveness of these drugs on pathognomic biomarkers of chronic disease. In some respects the corticosteroids are ideal anti-inflammatory drugs for use in chronic disease but their side-effects are proven formidable and often severe or irreversible (e.g. bone damage, immunosuppression and propensity to infection, gastrointestinal ulcers and bleeding, skin thinning) and in part their undoing. While low-dose corticosteroids are now used commonly in rheumatoid arthritis, concerns about their long-term use are still a matter of major concern. In the past two decades, significant advances have been made in the molecular biology of the cells and mediators of chronic inflammatory disease and the biotechnological processes involved in the production of peptides, anti-bodies (including "humanized" monoclonal anti-bodies, or MAbs, from mouse precursors) and the isolation and cloning of genes regulating the production of endogenous inhibitors or soluble receptors that interact with pro-inflammatory cytokines as well as the production of anti-inflammatory cytokines. These

developments have led to a revolution in the therapy of not only rheumatoid arthritis, but also chronic inflammatory diseases affecting a large number of organ systems.

All adrenal corticosteroid are available as drug preparations, as are many synthetic derivatives developed by altering the basic steroid molecule in effort to increase therapeutic effects. When corticosteroids are administered from sources outside the body, they are given mainly for replacement or therapeutic purposes. Replacement involves small doses to correct a deficiency state (physiologic effects). Therapeutic purposes require relatively large doses to exert pharmacologic effect. Drugs effect involves extension of the physiologic effects of endogenous corticosteroids and new effect that do not occur with small, physiologic doses. The most desired pharmacologic effects are ant-inflammatory, immunosuppressive, antiallergic and antistress. These are corticosteroid effects. Mineralocorticoid androgenic effects are usually considered adverse reaction (Abraham *et al.,* 2002). Examples of corticosteroids are hydrocortisone, methylprednisolone, prednisolone, triamcinolone, beclamethasone, betamethasone, cortisone, dexamethasone and fluticasone.

1.2.8 Pain

In recent times, the concept of pain has evolved from one-dimensional to a multi-dimensional entity involving sensory, cognitive, motivational, and affective qualities. Pain is always subjective and every individual use this word through their previous experience related to the injury. Over time, various definitions have been given to describe and understand this pain in medical literature. Task force on taxonomy of the International Association for the Study of Pain (IASP) says that pain is "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. The North American Nursing Diagnosis Association defines that pain is as state in which an individual experiences and reports severe discomfort. (Miller-keane *et al.,* 2003). According to medical dictionary, Pain is defined as an unpleasant feeling that is conveyed to the brain by sensory neurons. The discomfort signals actual or potential injury to the body. However, pain is more than a sensation or the physical awareness of pain; it also includes perception, the subjective interpretation of the discomfort. Perception gives information on the pain's location, intensity, and something about its nature. The various conscious and unconscious responses to both sensation and perception, including the emotional response, add further definition to the overall concept of pain. Pain is an unpleasant sensation localized to a part of the body . It is often described in terms of a penetrating or tissue-destructive process (e.g.: Stabbing, burning,

twisting, tearing, and squeezing) and/or of a bodily or emotional reaction (e.g.: Terrifying, nauseating, and sickening). Pain may be defined as unpleasant emotional experience usually initiated by noxious stimulus and transmitted over a specialized neural network to the central nervous system where it is interpreted. The subject's conscious perception of modulated nociceptive impulses that generate an unpleasant sensory and emotional experiences associated with actual or potential tissue damage or described in terms of such damage. Pain is whatever the experiencing person says it does. The words "pain" and "suffering" have often been used synonymously, but the experience of suffering has been differentiated from pain. Suffering has been defined as indulging the experience of pain but as also including vulnerability, dehumanization, a lost sense of self, blocked coping efforts, lack of control over time and space, and an inability to find meaning or purpose in the painful experience. The peripheral nerve ending (nociceptor) transmits the pain impulse to the dorsal horn of the spinal cord, where it gets modified before onward transmission to the brain. Any pain caused primarily by stimulation of the nociceptor can be said to be nociceptive pain. If pain is not caused by a stimulus applied to the nociceptor, but is caused by impulse generation within the pathway proximal to the nociceptor (this could be in the nerve, the spinal cord or the brain), it is called neuropathic pain. This does not mean that all nociceptive pains are similar in presentation or management. For example pain arising out of smooth muscle spasm can be very different from the pain of skeletal muscle spasm. Nociceptive pain can be subdivided into somatic and visceral pain, depending on site of origin. Neuropathic pain can be of three sub-types; neural injury pain, nerve compression pain and Complex Regional Pain Syndrome (CRPS) According to (Fields and Marthin, 2005).

Neuropathic pain can also be sub-classified into peripheral and central, depending on site of origin of abnormal impulse. The relevance is that central pains often behave differently from peripheral neuropathic pains, particularly in their response to drugs. Central neuropathic pains are commonest in injury to the CNS – for e.g. Spinal cord injuries, stroke etc. It must be remembered that pain originally of peripheral nerve origin, can become centrally established – by somehow altering the CNS. Once this has happened, a peripheral nerve block or neurolysis may not successfully remove the pain.

1.2.9 AIM AND SPECIFIC OBJECTIVE OF THE STUDY

1.2.9.1 AIM OF THE STUDY

The aim of this study was to investigate the anti-inflammatory and analgesic activities of the ethanol extract of root-bark of *Ritchiea capparoides*.

1.2.9.2 SPECIFIC OBJECTIVES OF THE RESEARCH

- To determine the phytochemical composition of the ethanol extract of *Ritchiea capparoides* root-bark.
- To determine the median lethal dose (LD_{50}) of the ethanol extract of *Ritchiea capparoides* root-bark.
- To determine the anti-inflammatory activity of the ethanol extract of *Ritchiea capparoides* root-bark on egg albumin-induced rat paw oedema.
- To determine the effect of the ethanol extract of *Ritchiea capparoides* root-bark on calcium chloride-induced platelet aggregation.
- To determine the effect of the ethanol extract of *Ritchiea capparoides* root-bark on phospholipase A_2 activity.
- To determine the effect of ethanol extract of *Ritchiea capparoides* root-bark on hypotonicity-induced lysis of human red blood cells.
- To determine the analgesic activity of the ethanol extract of *Ritchiea capparoides* root-bark on wistar rats.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant Materials

Fresh *Ritchiea capparoides* root-bark was collected from Umuida, Enugu-Ezike, Igbo Eze North Local Government Area, Enugu State, Nigeria. The plant was authenticated by Mr. Alfred Ozioko of the Bioresources Development and Conservation Program (BDCP) Research Centre, Nsukka, Enugu State.

2.1.2 Animals

Adult Wistar albino rats (30 males) weighing between 130 g to 250 g and mice (48 males) were purchased from the animal house of Veterinary Department, University of Nigeria, Nsukka. The animals were acclimatized under standard laboratory condition in the animal house of the Department of Biochemistry, University of Nigeria, Nsukka, for one week prior to the commencement of the experiment with a 12 hour light and dark cycle and maintained on a regular feed.

The blood samples used for phospholipase A_2 activity, membrane stabilization and platelet aggregation tests were collected from healthy individuals. They were free from drug treatment for at least two weeks before the collection of the sample.

2.1.3 Drugs

Indomethacin (Yangzhou Pharmaceutical Company Limited) was used as the standard drug for platelet aggregation assays and paw oedema.

Prednisolone (Kumid Pharmaceutical Limited) was used as the standard drug for phospholipase A_2 activity assay.

Acetylsalicylic acid (Juhel Pharmaceutical Limited) was used as the standard drug for analgesic activity test.

2.1.4 Chemicals and Reagents

All chemicals used in this study were of analytical grade and products of May and Baker (England) BDH chemicals limited (Poole, England), Sigma Chemical Company (U.S.A). Sterile distilled and deionised water were used in the preparation of the chemicals, reagents and drugs.

2.1.5 Equipment and Instruments

Equipment and instruments used for this study were obtained from the laboratory unit of Department of Biochemistry, University of Nigeria, Nsukka. They include: Refrigerator (thermocool, Nigeria), centrifuge (Vickas Ltd, England), test tube (Pyrex, England), spectrophotometer and weighing balance (Vickas, England).

2.2 Methods

2.2.1 Preparation of Ethanol Extract of *Ritchiea capparoides* Root-Bark.

Fresh *Ritchiea capparoides* root-bark was collected and washed with clean water without using any detergent. The sample was sliced into pieces and air-dried with regular turning to avoid decaying. The dried root-back was pulverized into powdered form using a mechanical grinder. 1000g of the pulverized sample was macerated 95% ethanol, vigorously shaken and was allowed to stand for 72 hours. The mixture was filtered using Whatman no. 1 filter paper and the filtrate was concentrated under reduced pressure using a rotatory evaporator at 45℃ to obtain the crude ethanol extract. The concentrated extract was stored in air-tight container in a refrigerator at 4℃ until required. The percentage yield of the extract was calculated using the formula:

$$\text{Percentage yield} = \frac{\text{Weight of crude extract}}{\text{Weight of dried pulverized sample.}} \times 100$$

2.2.2 Qualitative phytochemical analysis of the root-bark extract of *Ritchiea capparoides.*

The qualitative phytochemical analysis was done using the methods of Harbone (1998) and Trease and Evans (2002).

2.2.2.1 Test for Alkaloids

A quantity of the sample (1 ml) was added into 4 test tubes, to the first test tube, 5 drops of drangendoff reagent were added. Red precipitate indicated the presence of alkaloid.

To the second test tube, 5 drops of Wagner's reagent were added and milkfish precipitate indicates the presence of alkaloid.

To the third test tube, 5 drops of Meryer's reagent were added. Milkfish precipitate indicates the presence of alkaloid.

To the fourth test tube, 5 drops of picrit acid were added. Appearance of milkfish precipitate indicated the presence of alkaloid.

2.2.2.2 Test for Flavonoids

Ethyl acetate (2 ml) was shared into two test tubes containing 1 ml of the sample. Aluminium chloride (1 ml) was put into one test tube, to the second test tube, dilute ammonia (1 ml) was added. Yellow colour of the upper layer indicated the presence of flvonoids.

2.2.2.3 Test for Glycoside

A quantity of the sample (1 ml) was added into a test tube and 5ml of distilled water was also added. The mixture was boiled for 5 minutes and allowed to cool. Fhelins solution A (0.2 ml) and Fhelins solution B (0.2 ml) were added and boiled for 5 minutes. Reddish colour indicated the presence of glycoside.

2.2.2.4 Saponins

A quantity of sample (1 ml) was put into a test tube containing 5ml of distilled water. The mixture was boiled for 5minutes and transferred into two test tubes. In one test tube, two drops of olive oil was added and shake vigorously for 2 minutes and observed for emulsion formation. To the second test tube, 4 ml of distilled water was added and shaken vigorously for 2 minutes.

Observation: To the first test tube, emulsion formation indicates the presence saponins. In the second test tube, emulsion formation also indicated the presence of saponins.

2.2.2.5 Steroids

A quantity of ethanol extract (1 ml) of the sample was shared into 2 test tubes and 3ml of chloroform was added. The mixture was shaken for 1 minute. The upper layers were discarded and 5 drops of Conc. H_2SO_4 were added to one part. The mixture was allowed to stand for 5 minutes. Golden yellow colour indicated the presence of triterpins.

In the second test tube, 5 drops of acetic anhydride were added. The mixture was boiled for

1 minute and allowed to cool. 1 ml of distilled water was added and 1ml of H_2SO_4 was also added down the side of the test tube. Brown ring appeareance at the junction indicated the presence of phytosterol

2.2.2.6 Tannins

A quantity of the methanol extract of the sample (1 ml) was shared into 2 test tubes. In one test tube, 0.5 ml of bromine water and the mixture was observed. Precipitate appearance indicated the presence of tannins.

In the second test tube, 0.5 ml of 2 % ferric chloride was added and the mixture was observed. Precipitate formation indicated the presence of tannins.

2.2.2.7 Phenols

A quantity of methanol extract (1 ml) of the sample was put in a test tube. O.5 % ferric chloride was added. The presences of black colour indicated the presence of phenol.

2.2.3 Quantitative Phytochemical Analysis

2.2.3.1 Estimation of the Concentration of Phenols and polyphenols

The extract (0.5 ml) was put into triplicate tubes (3-test-test), 2 ml of distilled water was added to the test tube. 0.25 ml of foline ciocalteau reagent and 1ml of 20 % sodium carbonate added were added and mixed. It was centrifuged for 10 mins, the absorbance at 650 nm for phenol and 760 nm for polyphenol were taken.

The blank test tube

To the mixture was added 0.5 ml ethanol, 2 ml of distilled water 0.25 ml of foline ciocoutteu reagent, 1ml of 20% sodium carbonate. It was mixed and centrifuged for 10 mins. The absorbance at 750 nm and 650 nm were taken.

2.2.3.2 Estimation of the Concentration of Tannins

The extract (0.5 ml) was put in a test tube, 2 ml of ethanol , 0.15 ml of 0.1 molar ferric chloride in 0.1 molar HCL and 0.1 moles of 0.0008 potassium fericyanide were added and mixed. The absorbance at 720 nm against the blank was taken.

2.2.3.3 Estimation of the Concentration of Flavonoids

The extract (0.5 ml) was put in three test tubes , 1 ml of methanol, 0.1 ml of 1 molar sodium acetate, 2.8 ml of distilled water and 0.1 ml of 10 % aluminum chloride were added, Shaken vigorously for a minute and allowed to stand for 30 minutes. The absorbance at 415 nm was taken.

2.2.3.4 Estimation of the Concentration of Terpeniods

The extract (0.5 ml) was put in three test tubes, 0.5 ml of 5 % aqueous phosphomolybdic acid reagent and 0.5 ml of conc. sulfuric acid gradually were gradually added and mixed. The mixture was allowed to stand for 30 mins. To the mixture, 1 ml of ethanol was added and the absorbance at 700 nm was taken against the blank. The blank contained 1.5 ml of ethanol.

2.2.3.5 Estimation of the Concentration of Saponins

Add 0.5 ml of extract into 3-test tubes. Evaporate to dryness ,allow to cool, add 2 ml of ethylacetate, 1 ml of 0.5 % anisaldehyde in ethylacetate and 1 ml of 50 % sulfuric acid in ethylacetate, incubate at 60 ^{0}C for 20 mins, cool in cold water and take the absorbance at 430 nm against the blank.

2.2.3.6 Estimation of the Concentration of Steroids

The extract (1 ml) was put in a test tube, 2 ml of alkaline potassium hydroxide was added and the mixture was boiled in water bath for 30 mins. It was allowed to cool. To the mixture was added 5 ml of N-hexane and shaken vigorously for 2 mins, transferred 1ml of the upper larger into three test tubes, evaporated to dryness. Allowed to cool, to it was added 2 ml of ethanol and 2 ml of steroid colour reagent, mixed carefully and allowed to stand for 30 mins. The absorbance at 550 nm was taken.

2.2.3.7 Estimation of the Concentration of Carotenoids

The extract (1 ml of ethanol extract) was put in a test tube, 5 ml of N-hexane was added , shaken vigorously, centrifuged for 5 mins and the absorbance of the upper layer at 450 nm against N-hexane at were taken.

2.2.3.8 Estimation of the Concentration of Resins

The extract (0.1 ml) was put into three test tubes, evaporated to dryness, 3 ml of acetonitrite was added and the absorbance at 372 nm against acetonitrite was taken.

2.2.3.9 Estimation of the Concentration of Alkaloids

The extract (0.1 ml) was put three test tubes, 2.5 ml of 60 % sulfuric acid was added and mixed and 2.5 ml of 0.5 % formaldehyde in 60 % sulfuric acid was added, mixed and allowed to stand for 3 hours. The absorbance at 565 nm against was taken against the blank.

2.2.3.10 Estimation of the Concentration of Glycosides

The extract (1 ml of water extract) was put in three test tubes, to each was added 1 ml of alkaline cupper reagent, boiled for 6 minutes in water bath, allowed to cool. To it was added 1 ml of phosphomolybdic acid solution, 7 ml of distilled water and mixed. The absorbance at 420nm against the blank was taken.

2.2.4 Determination of the acute toxicity and lethal dose (LD_{50}) of the root -bark extract of *Ritchiea capparoides.*

Investigation of acute toxicity of the extract with estimation of median lethal dose (LD_{50}) was carried out using the modified method of Lorke (1983). This study was done in two phases and a total of eighteen mice (18) was used. Six (6) groups of (3) mice, each was administered orally, doses of ethanol extract (10, 100, 1000 mg/kg body weight) respectively for the first phase and (1900, 2600 and 5000mg/kg b. w. of the extract) for second phase by oral intobation. The mice was observed for 24 hrs for lethality, neurological and behavioural changes (signs of toxicity). The LD_{50} of the plant was calculated using the formula below.

$$LD_{50} = \sqrt{highest\ \ dose\ that\ produced\ no\ mortality \times lowest\ dose\ that\ produced\ mortality}$$

2.2.5 Determination of the Effect of Ethanol Extract Of *Ritchiea capparoides* Root-Bark on Egg Albumin Induced Rat Paw Oedema.

Principle

Egg albumin releases mediators of acute inflammation responsible for causing oedema. The ability of the ethanol extract to inhibit inflammation mediators is a measure of its anti-inflammatory effect.

Experimental Design for *In vivo* Anti-Inflammatory Study

A total of thirty (30) male albino rat was used for the study. They were divided into five (5) groups of six (6) rats each and treated as follows.

Group 1: received 5ml/kg of 3 % (v/v) tween 80 in distilled water (vehicle).

Group 2: received 10 mg/kg body weight of indomethacin (standard drug).

Group 3: received 100 mg/kg body weight of *Ritchiea capparoides* root-bark ethanol extract.

Group 4: received 200 mg/kg body weight of *Ritchiea capparoides* root-bark ethanol extract.

Group 5: received 400 mg/kg body weight of *Ritchiea capparoides* root-bark ethanol extract

Rats were fasted 18 hours before the experiment to ensure uniform hydration and minimize variability in oedematous response, after which the right paw size of the rats at time zero (before the induction of oedema) was measured using a digital vernier caliper.

One hour after intraperitoneal administration of test substances, acute inflammation was induced by injecting 0.1 ml of freshly prepared egg albumin into the subplantar of the right hind paw of rats.

The increase in the right hind paw size of rats was subsequently measured at 0, 5, 1, 2, 3, 4, 5 hours and 30 minutes after egg albumin injection.

The difference between the paw size of the injected paws at time zero and at different times after egg albumin injection was used to assess the formation of oedema.

These values were used in the calculation of percentage inhibition of oedema for each dose of the extract and for indomethacin at different time intervals using the relation below. Paw oedema= (Vt-Vo), Vo = Paw oedema at time zero. Vt= Paw oedema at time t (0.5, 1,2,2,4 and 5h).

Percentage inhibition of oedema $= \frac{(Vt - V_0)\text{control} - (Vt - V_O)\text{treated groups}}{(Vt - V_0)\text{ control}} \times \frac{100}{1}$

2.2.6 Anti-inflammatory Studies

2.2.6.1 Determination of the Effect of Ethanol Extract of Root-Back of *Ritchiea capparoides* on Phospholipase A_2 Activity

The effect of the extract on phospolipase A_2 activity was determined using modifications of the method of Vane (1971).

Principle

Phospholipase A_2 activity was assayed using its action on erythrocyte membrane. It releases free fatty acids from the membrane phospholipids thereby causing leakage, allowing haemoglobin to flow into the medium in the process. The enzyme activity is thus directly related to the amount of haemoglobin in the medium. This was measured at 418 nm since haemoglobin absorbs maximally at this wavelength.

Enzyme Preparation

Fungal enzymes preparation was obtained from *aspergillus niger* strain culture. The nutrient broth was prepared by dissolving 15 g of sabouraud dextrose agar in 1000 ml of distilled water, homogenized in a water bath for 10 min and dispensed into 250 ml conical flasks. The conical flasks were sealed with cotton wool and foil paper. The broth was then autoclaved at 121 ^{0}C for 15 minutes. The broth was allowed to cool to room temperature and then the organisms in the Petri dishes was aseptically inoculated into the broth and incubated for 72 hours at room temperature. The culture was transferred into test tubes containing 3ml phosphate buffered saline and centrifuged at 3000 rpm for 10 min. The fungal cells were settled at the bottom of the test tube while the supernatant was used as the crude enzyme preparation.

Substrate Preparation

Fresh human blood samples was centrifuged at 3,000 rpm for 10 min and the supernatant (plasma) discarded. The red cells was washed three times with equal volume of normal saline, measured and

reconstituted as a 40 % (v/v) suspension with phosphate buffered saline. This served as the substrate for phospholipase A_2.

Assay Procedure

$CaCl_2$ (2 mM) (0.2 ml), human red blood cell (HRBC) (0.2 ml), 0.2 ml of the crude enzyme preparation and varying concentrations of normal saline, the extract and the reference drug was incubated in test tubes for 1 hr. The control contained the human red blood cell suspension, $CaCl_2$ and free enzyme. The blanks was treated with 0.2 ml of boiled enzyme separately. The incubation reaction mixtures was centrifuged at a speed of 3000 rpm for 10 minutes. Samples of the supernatant (1.5 ml) was diluted with 10ml of normal saline and the absorbance of the solutions read at 418nm. Prednisolone, a known inhibitor of phospholipase A_2, was used as the standard drug..

2.2.6.2 Determination the effect of ethanol extract of *Ritchiea capparoides* root-bark extract on calcium-chloride induced platelet aggregation.

This was achieved following a modification of Born and Cross (1963).

Principle

The aggregation of platelets leads to increased transmittance, therefore less absorbance of light. Calcium chloride-induced platelet aggregation is thus shown by reduced absorbance at 520 nm. Any substance that has anti-aggregatory effect would thus lead to increased absorption by the medium.

Preparation of platelet-rich plasma (PRP)

Blood samples was taken from healthy volunteers. Fresh blood samples (5ml) were drawn intravenously using 5 ml plastic syringe into plastic tubes containing 11 % EDTA as anti-coagulant. The tubes were centrifuged at 3000 rpm for 10 mins and the supernatant was collected, diluted twice with normal saline and used as the platelet-rich plasma (PRP) Nwodo (1981).

Procedure

An aliquot of PRP (0.2 ml) was put into each of the three test tubes containing 1ml each of varying concentrations of extract (0.1, 0.2 and 0.4 mg/ml) in 3 % tween 80 (dissolved in normal saline). Also another set of two test tubes were contain aliquot (0.2 ml) of PRP and 1 ml of each 0.2 and 0.4 mg/ml indomethacin in 3 % in distilled water. The contents of the respective tubes were made up to

2.2 ml with the vehicle. A control tube were contain 2 ml of the vehicle and 0.2 ml of PRP. The tubes were allowed to incubate before the induction of the aggregation by the addition of 0.4 ml of 1.47 % calcium chloride ($CaCl_2$) solution. The test was performed in triplicates. Changes in the absorbance of the solutions were taken at intervals of 2 mins for 18 mins at 520 nm. The blanks were contain indomethacin without PRP.

2.2.6.3 Determination of the effect of *Ritchiea capparoides* root-back extract on hypotonicity induced lysis of human red blood cells.

The effect of extract hypotonicity –induced lysis of human red blood cells was investigated using the method of Shinde et al., (1999). Fresh blood samples (5 ml) were collected from healthy volunteers into plastics tubes containing 0.01 ml of 1 % EDTA to prevent coagulation. These tubes were centrifuged at 3000 rpm for 15 mins. The supernatant was collected and discarded. Twice the volume of normal saline equivalent to the volume of the supernatant was used to dissolve the red cell pellet again. 16 test tubes were used, 8 for the main test and 8 as the blanks for each test tube.

The first test tubes contained 0.1 ml of blood and 2.4 ml of normal saline. The second test tube contained 0.1 ml of blood, 1.9 ml of normal saline and 0.5 ml of distilled water. Test tube three to eight contained the same volume of blood and distilled water but varying volumes of extract and normal saline. The volume of each test tube was 2.5 ml. Subsequently, all the test tube were incubated for 1hr at 37℃ in a water bath. After the incubation, the test-tubes were centrifuged at 3000 rpm for 20 mins to terminate the reaction. The absorbance of the supernatants collected was red at 540 nm. These experiments were done in triplicates and mean absorbance values were taken.

2.2.7 Antinociceptive Activity Test

This was done to determine the antinociceptive acitivity of ethanol extract of *Ritchiea capparoides* root-bark.

Method: It was done according to the method described by Koster *et al.,* (1959)

Method of induction of nociception: Acetic acid-induced nociception.

2.2.8 Experimental Design

A total of thirty (30) male albino rat was used for the study. They were divided into five (5) groups of six (5) rats each and treated as follows.

Group 1: Received distilled water (5 mg/kg)

Group 2: Received aspirin (200 mg/kg b. w.) as standard drug.

Group 3: Received 100 mg/kg body weight of *Ritchiea capparoides* root-bark ethanol extract.

Group 4: Received 200 mg/kg body weight of *Ritchiea capparoides* root-bark ethanol extract.

Group 5: Received 400 mg/kg body weight of *Ritchiea capparoides* root-bark ethanol extract.

Thirty minutes afterwards, 0.6 % acetic acid (10 mg/kg) was intraperitoneally injected into each animal. The number of writhings and stretching was counted over a 20 minutes period.

2.3 Statistical Analysis

The data obtained were analyzed using statistical packages for social sciences (SPSS) version 16.0 and the results were expressed as mean ± standard error of mean. Significant differences of the results were established by one-way and two-way ANOVA and the acceptance level of significance was $p < 0.05$ for all the results.

CHAPTER THREE

RESULTS

3.1 Percentage Yield of Ethanol Extract of *Ritchiea capparoides* Root-Bark

Table 1 represents the percentage yield of ethanol extract of *Ritchiea capparoides* Root-Bark. The extraction of 1000 g (1 kg) of *Ritchiea capparioides* root-bark with 95 % ethanol gave an ethanol extract yield of 27.6 g which represented 0.0276 %.

Table 1: Percentage Yield of Ethanol Extract of Ritchiea Capparoides Root-Bark

Weight of crude sample (g)	weight of extract (g)	percentage yield (%)
1000	27.6	0.0276

3.2 Qualitative Phytochemical Analysis of Ethanol Extract of *Ritchiea capparoides* Root-Bark

Tabe 2 shows the results of the qualitative composition of ethanol extract of *Ritchiea capparoides* root-bark. The result shows that the extract contains high amount of flavonoids, terpenoids and tannins. Glycosides, polyphenols, phenols and alkaloids were moderately present. Steroids, saponins and carotenoids were present in low amount.

Table 2: Qualitative Phytochemical composition of ethanol Extract ethanol extract of *R. capparoides* root-bark

S/N	Phytochemical Constituents	Qualitative Remark
1	Flavonoids	+++
2	Terpenoids	+++
3	Tannis	+++
5	Alkaloids	++
6	Phenols	++
7	Polyphenols	++
8	Glycosides	++
9	Steroids	+
10	Saponins	+
11	Carotenoids	+

Key

+ Present

++ Moderately present

+++ Highly present

3.3 Quantitative Phytochemical Analysis of Ethanol Extract of *Ritchiea capparoides* Root-Bark.

Table 3 shows the result of the quantitative phytochemical analysis of ethanol extract of *Ritchiea capparoides* root-bark. The results obtained correlated with the qualitative results shown above. The ethanol extract of *R. capparoides* constituted the following; flavonoids (1168.2±239.4 mg/100g), terpenoids (1215.9±966.6 mg/100 g), tannins (18.6±29.2 mg/100g), alkaloids (323.3±66.9 mg/100g), phenols (298.1±7.1 mg/100 g), polyphenols (285.1±8.4 mg/100g), glycosides (201.5±25.9 mg/100g), steroids (25.8±1.7 mg/100 g), saponins (13.2±5.7 mg/100 g) and carotenoids (5.4±0.4 mg/100 g).

Table 3: Quantitative Phytochemical Analysis of the *R. capparoides* Extract

S/N	PHYTOCHEMICAL CONSTITUENTS	Concentration (mg/100g)
1	Flavonoids	1168.2±239.4
2	Terpenoids	1215.9±966.6
3	Tannins	18.6±29.2
4	Alkaloids	323.3±66.9
5	Phenols	298.1±7.1
6	Polyphenol	285.1±8.4
7	Glycosides	201.5±25.9
8	Steroids	25.8±1.7
9	Saponins	13.2±5.7
10	Carotenoids	5.4±0.4

Values are mean ± SD (n=3)

3.4 Acute Toxicity Test of Ethanol Extract of *Ritchiea capparoides* Root-Bark.

Table 4 shows that at 10, 100 and 1000 mg/kg body weight of ethanol extract of *R. capparoides*, the was neither death nor any sign of toxicity in the mice used for the first phase. Similarly, at 1900, 2600 and 5000 mg/kg body weight of the extract, there was no record of death or any sign of toxicity.

Table 4: Determination of acute toxicity (LD_{50}) of the *R. capparoides* ethanol extract.

Phases/groups	Dosage of extract (mg/kg body weight)	Mortality rate
PHASE I		
Group 1	10	0/3
Group 2	100	0/3
Group 3	1000	0/3
PHASE II		
Group 1	1600	0/3
Group 2	2900	0/3
Group 3	5000	0/3

3.5 Effect of Ethanol Extract of *R. capparoides* Root-Back on Calcium Chloride-Induced Platelet Aggregation.

Table 4 shows the effect of ethanol extract of *R. capparoides* root-bark on calcium chloride-induced platelet aggregation. The extract significantly ($p < 0.05$) inhibited the response of platelet aggregation. At different doses, the extract inhibited calcium chloride-induced platelet aggregation in concentration-dependent manner. The inhibition of platelet aggregation by the extract was similar to that of the standard drug (indomethacin) used for this study. For example, 0.1 ml showed percentage inhibition of (59.54 %), (55.38 %), (52.81 %), and (51.11 %) at different time interval (2, 4, 6 and 8 minutes). Increase in the concentration of the extract from 0.1, 0.2, 0. 4 and 0.6 mg/ml resulted to an increase the degree of inhibition of human platelet aggregation. At 6 minutes, 0.1, 0.2, 0.4 and 0.6 mg/ml inhibited platelet aggregation with percentage inhibition of (52.81 %), (53.93 %) , (48.31 %), (39.89 %) and (45.51 %) respectively.

Table 5. Effect of ethanol extract *Ritchiea capparoides* Root-Bark on Calcium Chloride-Induced Platelet Aggregation.

Treatments Concentrations (mg/ml)	Δ Absorbance (520nm) 0MINS	2MINS	4MINS	6MINS	8MINS
Control	0.173±0.001^{Ae}	0.173±0.001^{Ae}	0.177±0.001^{Bd}	0.178±0.001^{Bd}	0.180±0.003^{Cd}
Extract					
0.1	0.109±0.001^{Ed} (63.0 %)	0.103±0.001^{Dd} (59.54 %)	0.098±0.001^{Cc} (55.38 %)	0.094±0.001^{Bc} (52.81 %)	0.092±0.001^{Ac} (51.11 %)
0.2	0.096±0.001^{Bc} (55.49 %)	0.101±0.001^{Cc} (58.38 %)	0.096±0.001^{Bc} (54.24 %)	0.096±0.001^{Bc} (53.93 %)	0.090±0.003^{Ac} (50 %)
0.4	0.091±0.001^{Db} (52.60 %)	0.088±0.001^{Cb} (50.87 %)	0.088±0.001^{Cb} (49.72 %)	0.086±0.004^{Bb} (48.31 %)	0.083±0.001^{Ab} (46.11 %)
0.6	0.081±0.002^{Ca} (46.82 %)	0.076±0.001^{Ba} (43.93 %)	0.072±0.002^{Aa} (40.68 %)	0.071±0.001^{Aa} (39.89 %)	0.071±0.001^{Aa} (39.44 %)
Indomethacin					
0.4	0.096±0.002^{Ac} (55.49 %)	0.082±0.001^{Ef} (47.40 %)	0.082±0.001^{De} (46.33 %)	0.081±0.002^{Ce} (45.51 %)	0.081±0.001^{Be} (45,00 %)

n = 3 absorbances
Results expressed as Mean ± SD
Mean values having different lowercase letters as superscripts are considered significant ($p < 0.05$) down the column.
Mean values having different uppercase letters as superscripts are considered significant ($p < 0.05$) across the row.
( ) = % Inhibition of Platelet aggregation.

3.6 Table 5 Shows The Effect of Ethanol Extract of *R. capparoides* Root-Bark On Phosphplipase A_2 Activity.

Table 5 Shows the effect of ethanol extract of *R. capparoides* on phosphplipase A_2 activity. The extract significantly ($p < 0.05$) inhibited the activity of phospholipase A_2. The degree of the inhibition increased with an increase in the concentration of the extract. The activity of the enzyme reduced with an increase in the concentration of the extract. This was shown by the reduction of spectrophotometer reading of the solution as concentration increased. The absorbance values for different doses of the extract (1.0), (1.2), (1.4), (1.6) and (1.8 ml) were 0.035±0.002, 0.055±0.001, 0.042±0.002, 0.078±0.001, 0.017±0.000 and 0.056±0.001 respectively. Prednisolone, a standard ant-inflammatory drug used for this study followed a similar trend.

Table 6: Effect of ethanol extract of *Ritchiea capparoides* root-bark on Phospholipase A_2 activity

Treatment	Extract/Drug volume (ml)	Normal saline (ml)	HRBC (ml)	Crude enzyme (ml)	$CaCl_2$(ml)	Absorbance(418nm)
Control	--	2.0	0.2	0.2	0.2	0.095 ± 0.003^{f}
Extract	1.0	1.0	0.2	0.2	0.2	0.035 ± 0.002^{b}
	1.2	0.8	0.2	0.2	0.2	0.055 ± 0.001^{c}
	1.4	0.6	0.2	0.2	0.2	0.042 ± 0.002^{c}
	1.6	0.4	0.2	0.2	0.2	0.078 ± 0.001^{e}
	1.8	0.2	0.2	0.2	0.2	0.017 ± 0.000^{a}
Drug (Prednisolone)	1.0	1.0	0.2	0.2	0.2	0.056 ± 0.001^{d}

n = 3

Results expressed as Mean ± SD

Mean values having different lowercase letters as superscripts are considered significant ($p < 0.05$) down the column

3.7 Effect of Ethanol Extract *R. Capparoides* On Egg-Albumin-Induced Rat Paw Oedema.

Table 6 shows the effect of *Ritchiea capparoides* root-bark ethanol extract on egg albumin-induced rat paw oedema. The table shows the mean paw oedema and percentage inhibition of egg albumin-induced rat paw oedema over a period of 5 hrs. All the treatment groups showed significant ($p < 0.05$) reduction in the mean paw oedema from 30 minutes to 5 hours when compared to the control. The control groups showed no significant ($p < 0.05$) reduction in the mean paw oedema of the rats at different time intervals. The paw size of the rats treated with increasing doses of the extract and indomethacin significantly decreased with time. The percentage inhibition of oedema for rats treated with 100 mg/kg body weight of the extract at 30 min, 1 h, 2, 3, 4h and 5 h were (18.14 %), (28.34 %), (42’62 %), (65.36 %), (77.30 %) and (89.10 %) respectively. The percentage inhibition of oedema for rats treated with 200 mg/kg body weight of the extract at 30 min, 1 h, 2, 3, 4h and 5 h were (12.58 %), (20.53 %), (46.29 %), (46.81 %), (76.44 %) and (85.03 %) respectively. The percentage of inhibition of oedema for rats treated with 400 mg/kg body weight of the extract at 30 min, 1 h, 2, 3, 4h and 5 h were (18.87 %), (28.52 %), (56.60 %), (69.00 %) (80.19 %) and (87.00 %) respectively. The percentage of inhibition of oedema for rats treated with 10 mg/kg body weight of indomethacin at 30 min, 1 h, 2, 3, 4h and 5 h were (12.63 %), (26.65 %), (46.54 %), (63.30 %), (73.40 %) and (89.10 %) respectively. The inhibitory effects of both the extract and standard drug (indomethacin) on the treatment group was dependent on concentration with 400 mg/kg body weight of the extract showing the highest inhibition of oedema over the period of 5 hours.

Table 7: The Effect of *Ritchiea capparoides* Root-Bark Ethanol Extract On Egg Albumin-Induced Rat Paw Oedema.

GROUPS	Mean Oedema (cm) and Duration					
	30Mins	1Hour	2Hours	3Hours	4Hours	5Hours
Control (distilled water)	4.388 ± 0.22^{bA}	4.608 ± 0.25^{bAB}	4.852 ± 0.51^{bAB}	4.948 ± 0.61^{bAB}	5.118 ± 0.68^{bAB}	5.372 ± 0.73^{bB}
Indomethacin (10mg/kg b. w)	3.834 ± 0.37^{aE} (12.63 %)	3.380 ± 0.87^{aDE} (26.65 %)	2.594 ± 0.81^{aCD} (46.54 %)	1.816 ± 0.67^{aBC} (63.30 %)	1.362 ± 0.37^{aAB} (73.40 %)	0.586 ± 0.62^{aA} (89.10 %)
Extract (100mg/kg b. w)	3.592 ± 0.31^{aE} (18.14 %)	3.302 ± 0.33^{aE} (28.34 %)	2.784 ± 0.39^{aD} (42.62 %)	1.714 ± 0.31^{aC} (65.36 %)	1.162 ± 0.31^{aB} (77.30 %)	0.586 ± 0.62^{aA} (89.10 %)
Extract (200mg/kg b. w)	3.836 ± 0.08^{aD} (12.58 %)	3.662 ± 0.21^{aD} (20.53 %)	2.606 ± 0.53^{aC} (46.29 %)	1.972 ± 0.55^{aB} (46.81 %)	1.206 ± 0.53^{aA} (76.44 %)	0.804 ± 0.17^{aA} (85.03 %)
Extract (400mg/kg b. w)	3.560 ± 0.16^{aD} (18.87 %)	3.294 ± 0.38^{aD} (28.52 %)	2.106 ± 0.30^{aC} (56.60 %)	1.534 ± 0.34^{aB} (69.00 %)	1.014 ± 0.25^{aA} (80.19 %)	0.700 ± 0.17^{aA} (87.00 %)

Results expressed as Mean ± SD n = 5

Mean values having different lowercase letters as superscripts are considered significant ($p < 0.05$) down the column.

Mean values having different uppercase letters as superscripts are considered significant ($p < 0.05$) across the row.

3.8 The Effect Ethanol Extract of *Ritchiea capparoides* Root-Bark On Hypotonicity-Induced Lysis of Human Red Blood Cells.

Table 7 Effect Ethanol Extract of *Ritchiea capparoides* Root-Bark n Hypotonicity-Induced Lysis of Human Red Blood Cells. The extract (1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml) significantly ($p < 0.05$) inhibited the lysis of human red blood cells. The absorbance values for the extract were 0.408 ± 0.002, 0.421 ± 0.002, 0.452 ± 0.001, 0.577 ± 0.002 and 0.588 ± 0.003 respectively. The standard drug used in this study (indomethacin 5.0mg/ml) showed similar effect with absorbance value of 0.649 ± 0.049. The decrease in the absorbance values of both the extract and standard drug when compared with the control showed that the extract and indomethacin inhibited the lysis of human red blood cells.

Table 8 shows effect of the extract of *Ritchiea capparoides* root-bark on hypotonicity-induced lysis of human red blood cells.

Test tube	HRBC (ml)	Normal saline (ml)	Distilled water (ml)	Extract (mg/ml)	Indomethacin (mg/ml)	OD at 540mn
1	0.1	2.4	-	-	-	1.107 ± 0.042^{g}
2	0.1	1.9	0.5	1.0		0.408 ± 0.002^{a}
3	0.1	1.8	0.5	2.0	-	0.421 ± 0.002^{b}
4	0.1	1.7	0.5	3.0	-	0.452 ± 0.001^{c}
5	0.1	1.5	0.5	4.0	-	0.577 ± 0.002^{d}
6	0.1	1.3	0.5	5.0	-	0.588 ± 0.003^{e}
7	0.1	1.7	0.5	6.0		0.918 ± 0.049^{g}
8	0.1	1.5	0.5	-	5.0	0.649 ± 0.049^{f}

n = 3

Result expressed in Mean ± S. Dl

Mean values having different lowercase letters as superscripts are considered significant ($p < 0.05$) down the column

HRB = Human Red Blood Cells

Effect of Ethanol Extract of *R. capparoides* Root-Bark And Aspirin On Acetic Acid-Induced Nociceptive Response in Mice

Table 8 shows the effect of ethanol extract of *R. capparoides* root-bark and aspirin on acetic acid-induced nociceptive response in mice. The extract (100, 200 and 400 mg/kg body weight) significantly ($p < 0.05$) reduced the number of writhings when compared with the control group. The percentage inhibition of the number of writhings for various doses of the extract were (42.11 %) (66.67 %) and 61.4. %) respectively. The standard drug (200 mg/kg body weight of aspirin) showed similar effect with percentage inhibition of (70.18 %). The analgesic effects of both the extract and standard drug (aspirin) were concentration dependent.

Table 9: The effect of *R. capparoides* root-bark extract on nociceptive response induced by acetic acid in mice.

Groups	No of Writhings (Counts/20mins)	% Analgesic activity
Control (distilled water)	11.4 ± 2.7^{b}	
Extract (100 mg/kg b. w)	6.6 ± 4.561^{a}	42.1 %
Extract (200 mg/kg b. w)	3.8 ± 2.4^{a}	66.7%
Extract (400 mg/kg b. w)	4.4 ± 4.8^{a}	61.4 %
Aspirin (200 mg/kg b. w)	3.4 ± 2.4^{a}	70.2 %

n = 4

Results expressed as Mean ± SD

Mean values having different lowercase letters as superscripts are considered significant ($p < 0.05$) down the column.

CHAPTER FOUR

DISCUSSION

4.1 Discussion

The present study investigated the anti-inflammatory and analgesic effects of the ethanol extract of *Ritchiea capparoides* root-bark on egg albumin-induced rat paw oedema and acetic acid induced nociceptive response in experimental animals and in vivo inhibition of phospholipse $A_{2,}$ calcium chloride-induced platelet aggregation and albumin denaturantion.

Phytochemicals are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. It is well-known that plants produce these chemicals to protect themselves, but recent findings showed that many phytochemicals can also protect humans against diseases.

Findings from qualitative and quantitative phytochemical analysis of the extract revealed that *Ritchiea capparoides* is rich in flavonoid, terpenoid, steroids, alkaloids, phenols, saponins, glycosides, soluble proteins, polyphenols, and carotenoids (Narayana, 2001). Without specific knowledge of their cellular actions or mechanisms, phytochemicals have been used as poison and in traditional medicine. For example, salicin, having anti-inflammatory and pain-relieving properties, was originally extracted from the bark of the white willow tree and later synthetically produced to become the common, over-the-counter drug, aspirin.

A lot of plant constituents currently researched are the flavonoids, polyphenol compounds widely spread in nature. To them are assigned various pharmacological activities such as anti-inflammatory, antitumor, anti-allergic, antioxidant, antiviral and antiulcer activities (Sumbul, 2011). The anti-inflammatory function performed by the flavonoids is of considerable importance since these compounds could be a choice option in the treatment of the inflammatory cases and physiological response to a variety of stimuli such as infection and tissue damage (Zhang, 2014). The ability to elicit an inflammatory response is fundamental to survival in view of environmental pathogens and

injury, although in some situations and diseases the inflammatory response may be exaggerated and continued without any apparent benefit (Moldoveanu, 2009).

Acute toxicity studies of oral doses of *R. capparoides* were carried out using experimental animals. The result of the study revealed that the ethanol extract of *Ritchiea capparoides* root-back has a high safety profile, as the extract was tolerated by mice used for the study up to 5000 mg/kg. On administration of the extract, there was no immediate behavioural changes. The animals fed and moved about normally. Piloerection was noticed after 30 minutes and some animals were trying to jump out through the holes in the cages. Acute toxicity studies are designed to determine the dose that will produce either mortality or serious toxicological effects when given once or as multiple administrations. They also serve to determine the doses that should be used in subsequent studies.

In the present study, significant protection against egg albumin-induced rat paw oedema was observed in the animals treated with 200 and 400 mg/kg b. w. of the *R. capparoides* root-kbark ethanol extract. The most widely used primary test to screen new anti-inflammatory agents measure the ability of a compound to reduce local edema induced in the rat paw by injection of an irritant agent (Winter *et al.,* 1962). Egg albumin oedema model has been used in a number of studies. Subcutaneous injection of egg albumin into the rat paw produces oedema resulting from plasma protein-rich fliud exudation along with neutrophils extravasation. The significant inhibitory activity shown by the extract of *Ritchiea capparoides* (200 and 400 mg/kg b. w.) over a period of 4 hours in egg albumin-induced inflammation was quite similar to that exhibited by the group treated with indomethacin. The highest percentage inhibitory activity was found in the dose of 400 mg/kg with the percentage inhibition of 80.19% after 4 hours of extract administration. Previous anti-infammatory studies with some other plants such as *Solanum trilobatum* (Pandurangan *et al.,* 2009), *Plumeria acuminate* and *Thesium chinense* (Parveen *et al.,* 2007) also showed similar effect. The anti-inflammatory effect of the extract observed might be due to the presence of flavonoids in the plant.

The *Rritchiea capparoides* root-bark ethanol extract exhibited high membrane stabilization effect against hypotonicity induced haemolysis of the red cells as is shown by the percent inhibition of haemolysis. The present study showed that the inhibition of haemolysis was dose-dependent, increasing with increased doses of the extract in the medium and was comparable with that of

indomethacin used as standard anti-inflammatory drug. Hypotonicity-induced haemolysis of red blood cells occurs due to water uptake by the cells and this leads to the release of haemoglobin which has its maximum absorption at 418 nm. Hence, the reduced optical density at 418 nm obtained for the various *R. capparoides* test samples is an indication of the stabilization of the red cell membrane caused by the extract. During inflammation, there is lysis of lysosomes which release their component enzymes which produce a variety of disorders (Goodman and Gilman, 2000). Since human red blood cell (RBC) membranes are similar to lysososmal membrane, human RBC stabilization was therefore used as a method to study the mechanism of action of anti-inflammatory agents (Murgesh *et al.,* 1981) . The stabilizing effect of *R. capparoides* extract on lysosomal membranes as seen in this study suggests a possible mechanism of action for its anti-inflammatory effect. Results from this study have shown that R. capparoides has potential as an anti-inflammatory agent.

The *R. capparoides* ethanol extract also showed inhibitory effect on phospholipse A2 activity. Phospholipases A*2* (PLA_2s) are enzymes that cleaves fatty acid in position two of phospholipids, hydrolyzing the bond between the second fatty acid "tail" and the glycerol molecule. This particular phospholipase specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond, releasing arachidonic acid and lysophosphatidic acid. Upon downstream modification by cyclooxygenases, arachidonic acid is modified into active compounds called eicosanoids. Eicosanoids include prostaglandins and leukotrienes, which are categorized as inflammatory mediators. The ethanol extract of R. capparoides root-bark from 1.0 to 1.8ml exhibited a significant ($p< 0.05$) and dose-dependent inhibition phospholipase A_2 activity and this implies that the extract was able to suppress the release of free fatty acids from RBC membrane phospholipids and consequently deprived COX and LOX substrates for inflammatory mediators synthesis, thus preventing inflammation. This effect could be attributed to the presence of flavonoids in the extract as many studies have shown that flavonoids inhibit phospholipase A_2. The anti-inflammatory function performed by the flavonoids is of considerable importance since these compounds could be a choice option in the treatment of the inflammatory cases and physiological response to a variety of stimuli such as infection and tissue damage (Zhang, 2014).

The present study demonstrated that *R. capparoides* ethanol extract inhibited calcium chloride-induced platelet aggregation. This inhibition was concentration and time dependent. This inhibition could be due to the extract's ability to inhibit phospholipase A_2 and COX which are required for the synthesis of TXA_2. The inhibition of TXA_2 is imperative because it is associated with the induction of platelet aggregation by increasing intracellular calcium ion which promotes the fusion of platelet granules with the membrane thereby releasing its contents such as ADP which also promotes the aggregation of platelets. The inhibition of platelet aggregation by the extract could also imply deceased vascular permeability, resulting in oedema, defined as "a swelling tumor", which is one of the first signs of inflammation (Gros *et al.,* 2015). In addition to swelling, the other four cardinal signs of inflammation are rubor (redness), calor (heat),dolor(pain), and functio laesa (loss of function). These signs were first described by Celcus and Gallen. The cardinal signs of inflammation are caused by the release of inflammatory mediators, such as immunomodulatory cytokines, chemokines, and other mediators, from platelets (Jenne *et al.,* 2013). Platelets, also known as thrombocytes, are small anucleate cells that circulate in the bloodstream. They are essential for normal hemostasis and play major roles in inflammation, immunity and wound healing (Leslie, 2010). Platelets play a central role in inflammatory reactions and are involved in responses to a variety of inflammatory diseases. In various inflammatory conditions, platelets contain three types of granules: α-granules, dense bodies and lysosomes. These granules store many important platelet-derived inflammatory and immune mediators that are rapidly released following platelet activation. Notably, platelets can synthesize additional mediators, such as interleukin- 1α (IL-1α) and interleukin-1β (IL-1β). Recently, platelets were shown to contain an assembly of inflammasomes that mediate IL-1β secretion. Platelet activation during inflammation leads to changes in the chemotactic, proteolytic and adhesive properties of endothelial cells. Blood vessel inflammation is caused by interactions between platelets, leukocytes and endo-thelial cells that result in autocrine and paracrine activation. This activation is followed by leukocyte recruitment into the vascular wall and induces an inflammatory reaction via the release of proinflammatory compounds. The anti-platelet aggregatory effect of *R. capparoides* may be due to its phytochemical constituents. A lot of plant constituents currently researched are the flavonoids (polyphenolic compounds) which are widely spread in nature. To them are assigned various pharmacological activities such as anti-inflammatory, antitumor, antiallergic, antioxidant, antiviral and antiulcer activities (Sumbul, 2011).

The analgesic activity of the ethanol extract was studied using acetic acid induced writhing in mice (koster *et al.*, 1959). Acetic acid induced writhing method was adopted for evaluation of analgesic activity. Writhing test is a chemical method used to induce pain of peripheral origin by injection of irritant principles like phenylquinone and acetic acid in mice. Analgesic activity of the test compound is inferred from decrease in the frequency of writhings. The manifestations of abdominal writhings in mice are described as an arching of back, extension of hind limbs and contraction of abdominal musculature. Writhings generated by parenteral administration of acetic acid in mice, are due to profound pain of endogenous nature which recur for a prolonged period of time. Due to irritant nature, these principles are also prone to induce lesions. Writhing is an overt response to the intense pain induced by irritant principles via nociceptors characterized by episodes of retraction of abdomen and stretching of hind limbs. The signals transmitted to central nervous system in response to pain due to irritation, cause release of mediators such as prostaglandins which contributes to the increased sensitivity to nociceptors. The writhing response is considered as a reflexive test and is without clinical counterparts as it cannot be performed in humans and sensations involved are unknown. Immediately after injection of acetic acid, the latency time to the beginning of the first contraction of the abdominal musculature (writhing) was measured and the number of writhins was counted during a 20 minutes observation period. Ethanol extract of *R. capparoides* root-back at 100, 200 and 400mg/kg significantly reduced the number of writhings induced by 0.6% acetic acid in a manner that depended on concentration similar to that of aspirin which was used as a standard drug. This apparently indicated that the extract has analgesic property and its mechanism of action could be by the inhibition of the pathways that lead to prostaglandins generation and local peritoneal inflammation.

4.2 Conclusion

The findings from this study indicate that the plant extract produced significant ($p < 0.05$) anti-inflammatory activity when compared with the untreated control group. The standard anti-inflammatory drug (indomethacin) showed similar effects. The results also showed that the extract has remarkable analgesic activity which is comparable to the standard analgesic drug, aspirin. The results suggest that the mechanisms of the anti-inflammatory effects may be by inhibiting phospholipase A_2 activity, membrane stabilization and platelet aggregation. The findings from this

investigation give credence to the tradomedicinal use of *Ritchiea capparoides* root-back extract in the treatment of inflammation and inflammatory disorders.

SUGGESTIONS FOR FURTHER STUDIES

- Further studies should be carried out to isolate and characterize the active agents present in the plant.
- Further study on the mechanism of anti-inflammatory activity of the plant should be carried out.
- The toxicity profile of the plant should also be determined

REFERENCES

Abraham, E. and Evans, T. (2002). Corticosteroid and septic shopck. *Journal of the Amperican medical Association*, **288**(7), 886-887.

Adams, J. M., Difazio, L. T. and Rolandelli, R. H. (2009). HIF-1: A key mediator in hypoxia. *Acta Physiologica Hungarica*, **96**: 19-28.

Aggarwal, B. B., Vijayalekshmi, R. V. and Sung, B. (2009). Targeting inflammatory pathways for prevention and therapy of cancer: shortterm friend, long-term foe. *Clinical Cancer Reserch*, **15**(2): 425–430.

Alberts, B., Johnson, A., Lewis, M., Raff, k. M., Roberts, K. and Walter, P. (2002). "Leukocyte also known as macrophages functions and percentage breakdown". Molecular Biology of the Cell (4th edition.). New York: Garland Science, **4**:361-369.

Angus, D., Linde-Zwirble, W., Lidicker, J., Clermont, G., Carcillo, J. and Pinsky, M. (2001). Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine*. **29** (**7**), 1303–1310.

Arpa, L, Valledor, A. F., Lloberas, J. and Celada, A. (2008). IL-4 blocks M-CSF-dependent macrophage proliferation by inducing p21Waf1 in a Stat6-dependent way. *European Journal of Immunology*, **25** (5): 600-609.

Ash, A. and Schild H. O. (1966). Receptors mediating some actions of histamine. *British Journal of Pharmacology*, **27**:427-37.

Awoni, C. F, Ikegbunam, M. N., Ezugwu, C. O. and Oche, G. (2012). Antimicrobial activity of ethyl Acetate extract of the leaves of *Ritchiea longipedicellata* FAM. *Cpparidaceae*. *International Journal of Pharmacy*, **2**(4): 700-705.

Barbaro, J. F. and Zvaifler, N. J. (1966). Antigen induced histamine release from platelets of rabbits producing homologous PCA antibody. *Proceedings of the Society for Experimental Biology and Medine*, **122**:1245-7.

Barnes, P. J. Ellis E. F., Adkinson, N. F, Yunginger, J. W. and Busse, W. W. (1993). Pathophysiology of allergic inflammation. Allergy: Principles and practice. 4th ed. St Louis(Mo): Mosby, Pp. 255-6.

Benson, M, Strannegard I. L, Wennergren, G. and Strannegard, O. (1997). Cytokines in nasal fluids from school children with seasonal allergic rhinitis. *Pediatric Allergy and Immunology*, **8**:143-9.

Bernard, G., Vincent, J., Laterre, P., LaRosa, S., Dhainaut, J., Lopez- Rodriguez, A., Steingrub, J., Garber, G., Helterbrand, J., Ely, E. and Fisher, C. J., (2001). Efficacy and safety of

recombinant human activated protein C for severe sepsis. *New England Journal of Medicine*, **344** (10**)**, 699–709.

Bhadrapura, L. D. and Sudharshan, S. N. (2016). The anti-inflammatory activity of standard aqueous stem bark extract of *Mangifera indica* L. as evident in inhibition of Group IA $sPLA_2$. *Anais da Academia Brasileira de Ciências*, **3**: 1-13.

Bhattacharyya, S. P., Drucker, I, Reshef. T., Kirshenbaum, A. S, Metcalfe, D. D. and Mekori, Y. A. (1998). Activated T lymphocytes induce degranulation and cytokine production by human mast cells following cell-to-cell contact. *Journal of Leukocyte Biology*, **63**:337-41.

Blanchet, B., Jullien, V, Vinsonneau, C. and Tod, M. (2008). Influence of burns on pharmacokinetics and pharmacodynamics of drugs used in the care of burn patients. *Clinical Pharmacokinetic*, **47**: 635-54.

Bone, R., Balk, R., Cerra, F., Dellinger, R., Fein, A., Knaus, W., Schein, R. and Sibbald, W. (1992). Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest, **101** (6), 1644–1655.

Borgeat, P, Samuelsson B. (1979). Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. *Journal of Biological Chemistry*, **254**:2643-6.

Born, G. V. R. and Cross, M. J. (1963). Inhibition of aggregation of blood platelets by substances related to adenosine diphosphate. *Journal of Physiology*, **166**: 29-30.

Bradding, P. and Holgate, S. T. (1996). The mast cell as a source of cytokines in asthma. *Annals of New York Academy of Sciences*, **796**:272-81.

Bradding, P., Feather, I. H, Wilson, S., Holgate, S. T. and Howarth, P. H. (1995). Cytokine immunoreactivity in seasonal rhinitis: regulation by a topical corticosteroid. *Americal Journal of Respiratory and Critical Care Medicine*, **151**:1900-6.

Burkhardt, H, Thunnissen M. M. and Holmdahl, R. (2009). Structure and pathogenicity of antibodies specific for citrullinated collagen type II in experimental arthritis. *Journal of Experimental Medicine*, **206**(2): 449–462

Burkill, H. M. (1985). A Revision of Dalziel's Useful Plants of West Tropical Africa. Vol. 1. Kew, UK: Royal Botanical Cardens, pp. 318–388.

Burns, K. A, Vanden Heuvel J.P. (2007). Modulation of PPAR activity via phosphorylation. Biochim Biophys Act, 952-60.

Chinedu, F. A., Utoh–Nedosa, A. U. Onyegbule, A. E. and Chinasa, C. E. (2013). Preliminary phytochemical studies and evaluation of antimicrobial property of the methanol extract of the root-bark of *Ritchiea longipedicellata* Gilg Family, *Capparidacaea, Journal of Pharmacognosy and Phytochemistry*, **1**(6): 6-13.

Colman, R. W, Bagdasarian, A., Talamo, R. C., Scott, C. F., Seavey M. and Guimaraes, J. A. and Williams, T. (1975). Human kininogen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with abnormalities of the Hageman factor-dependent pathways. *Journal of Clinical Investigation*, **56**:1650-62.

Dahinden, C. A., Geiser, T., Brunner, T., Tscharner, V., Caput, D. and Ferrara, P. (1994). Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. *Journal of Experimental Medicine*, **79**:751-6.

De Leval, X., Delarge, J., Somers, F., De -Tullio, P., Henrotin, Y, Pirotte B, and Dogné, J. M. (2000). Recent advances in inducible cyclooxygenase (COX-2) inhibition. *Current Medical Chemistry*, **7**:1041–1062.

De-Martel, C. and Franceschi, S. (2009). Infections and cancer: Established associations and new hypotheses. *Critical Review in Oncology / Hematology*, **70**(3): 183–194

Dvorak, A. M. (1997). New aspects of mast cell biology. *International Archives for Allergy Immunology*, 114:1-9.

Evans, D. and Williamson, W.R. (1987). Chemistry of clinically active anti-inflammatory compounds. In: Anti-Inflammatory Compounds. New York & Basel: Marcel Dekker, 193–302.

Fakhouri, F., Frémeaux-Bacchi, V., Noël, L. H, Cook, H. T. and Pickering, M. C. (2010). C3 glomerulopathy: A new classification. *Nature Review Nephrology*, **6** (8): 494–499

Falcone, F. H., Haas, H. and Gibbs, B. F. (2000). "The human basophil: a new appreciation of its role in immune responses". *Blood*, 96 (13): 4028–38.

Fields HL, Martin JB. (2005). Harrison's Principles of Internal Medicine. 16th edition. New York: McGraw-Hill; Pp. 71-6.

Firuzi, O. and Pratico, D. (2006). Coxibs and Alzheimer's disease: Should they stay or should they go? *Annals of Neuroscience*, **59**:219–228.

Garrett, W. S., Gordon J. I. and Glimcher L. H. (2010). Homeostasis and inflammation in the intestine. *Cell*, **140**(6): 859–870.

Gawaz, M. (2004). Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. *Cardiovascular Research*, **6**: 498-511.

Glass, C. K, Saijo K, Winner, B., Marchetto, M. C. and Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell*, **6**: 279-300.

Goodman, G. and Gilman, G. (2000). The Pharmacological basis of therapeutics. 10th Edn., Hardman JG (edition). The Mcgraw-Hill companies, USA, 686.

Green, K. L. (1978). Quantitative studies on the accumulation of serum albumin and erythrocytes in mouse paw oedema induced by bradykinin or thermal injury. *British Journal of Experimental Pathology*, **59**: 38-47.

Grivennikov, S. I., Greten F. R. and Karin, M. (2010). Immunity, inflammation, and cancer. *Cell*, **140**(6): 883–899.

Gros, A., Ollivier, V. and Ho-Tin-Noé, B. (2015). Platelets in inflammation: Regulation of leukocyte activities and vascular repair. *Frontiers in Immunology*, 5:678.

Hamberg, M., Svensson, J., Wakaboyashi, T. and Samuelsson, B. (1974). Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proceedings of National Academy of Science* USA, **71**:345-9.

Harborne, J. B. (1998). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis 2nd Edition. London: Chapman and Hall Limited. Pp. 282-283.

Henson, P. M. (2005). Dampening inflammation. *Nature Immunology*, **6**: 1179-81.

Hettiaratchy, S. and Dziewulski, P. (2004). ABC of burns: Pathophysiology and types of burns. *British Medical Journal*, **328**: 1427-9.

Hollingsworth, J. W., Siegel, E. R. and Creasey, W. A. (1967). Granulocyte survival in synovial exudate of patients with rheumatoid arthritis and other inflammatory joint diseases. *Yale Journal of Biology and Medicine*, **39**(5): 289–296.

Hotamisligil. G. S. (2006). Inflammation and metabolic disorders. *Nature*, **444**(7121): 860–867.

Ilavasaran R., Mohideen S. and Venkataraman, S. (2012). Analgesic and anti-inflammatory properties of *Thespesia populnea* leaf extracts. *Natural Product Research*, **2**:1616-20.

Jenne, C. N., Urrutia, R. and Kubes, B. (2013). Platelets: Bridging hemostasis, inflammation, and immunity. *International Journal of Laboratory Hematolology*, **35**: 254-261.

Kean, W. F. and Buchanan, W. W. (2005). The use of NSAIDs in rheumatic disorders: A global perspective. *Inflammopharmacology*, **13**:343–370.

Kennerly, D. A., Sullivan, T. J., Sylvester, P. and Parker, C.W. (1979). Diacylglycerol metabolism in mast cells. A potential role in membrane fusion and arachidonic acid release. *Journal of Experimental Medicine*, **150:**1039-44.

Kesersky, S. D., Dewey, W. C. and Harris, L. S. (1973). Anti-pyretic, analgesic and anti inflammatory effect of tetrahydrocarnabinol. *Environmental Journal of Pharmacology*, **24**: 1-7.

Kim, C., San,Y., Todorova., K., Carlson, B., Arpa, L., Celada, A., Lawrence., T., Otsu, K., Brissette, J., Arthur, J. C. and Park, J. M. (2008). The kinase p38α serves cell type-specific inflammatory functions in skin injury and coordinates proinflammatory and anti-inflammatory gene expression. *Nature Immunology*, **9**(9), 1019-27.

Koster, R., Anderson, M. and De-Beer, E. R. (1959). Acetic acid for analgesic screening. *Federation Proceedings*, **18**: 412-416.

Kumar, V., Carey, M. and Robbins, S. L. (2003). Robbins Basic Pathology. 7th edition . New York: Saunders. PP. 243-55.

Leslie, M. (2010). Cell biology. Beyond clotting: The powers of platelets. *Science*, **328**: 562-564.

Lewis, R .A., Holgate, S. T, Roberts, L. J, Oates, J. A. and Austen, K. F. (1981). Preferential generation of prostaglandin D2 by rat and human mast cells. In: Becker EL, Simon AS, Austen, K. F, editors. Biochemistry of the acute allergic reactions. New York: Liss, pp. 239-54.

Locksley, R. M. (2010). Asthma and allergic inflammation. *Cell*, **140**(6): 777–783

Lorke, D. (1983). A new approach to practical acute toxicity testing. *Archives of Toxicology*, **53**:275-280.

Luster AD. (1998). Chemokines: Chemotactic cytokines that mediate inflammation. *New England Journal of Medicine*, **338**:436-45.

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. *Nature*, **454**(7203): 436–444.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature*, **454**(7203): 428–435.

Miller-Keane, M. (2003). Encyclopedia and Dictionary of Medicine, Nursing, and Allied Health. 7th ed. *Philadelphia*, **7**: 39-82.

Moldoveanu, B. (2009). Inflammatory mechanisms in the lun. *Journal of Inflammation Research*, **2**: 1-11.

Murgesh, N., Vembar, S. C. Damadarana, I. Gandludasann, R., Thamasalchew, A. and Baburay, S. (1981). Anti- inflammatory actions of *Lannea coromandelica* by HRBC membrane stabilization. Fitoterapia, **5:** 81-83.

Naclerio, R. M., Baroody, F. M. and Togias, A. G. (1991). The role of leukotrienes in allergic rhinitis: A review. *American Review of Respiratory Disease*, **143** :S91-5.

Narayana, K. R. (2001). Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Indian Journal of Pharmacology*, **33**(1): 2-16.

Newball, H. H., Berninger, R. W., Talamo, R. C. and Lichtenstein, L. M. (1979). Anaphylactic release of a basophil kallikrein-like activity: purification and characterization. *Journal of Clinical Investigation*, **64**:457-65.

Ogwuaka, J. U, Patamia, M., Galeffi, C., Messaria I. and Newletti, M. (1981). Isolation of Cleomin from *Ritchiea longipedicellata. Planta Medica*, **41**(4):410–412.

Okazaki, I. M., Kotani, A. and Honjo, T. (2007). Role of AID in tumorigenesis. *Advances in Immunology*, **94**: 245–273

Pandurangan, A., Khosa R. L. and Hemalatha, S.(2009). Evaluation of anti-inflammatory activity of the leaf extracts of solanum trilobatum Linn. *Journal Pharmaceutical Science and Research*, **1**(1):16-21.

Parveen, Z., Deng., Y, Saeed, M. K, Dai, R., Ahamad, W., Yu, Y. H. (2007). Antiinflammatory and analgesic activities of *Thesium chinense* Turcz extract and its major flavonoids, kaempferol and kaempferol-3-o-glucoside. Yakugaku Zasshi **127**(8):1275-1279.

Pearce F. L. (1990). The mast cell. *Immunology and Allergy Clinics of North America*, **10**:251-62.

Pillay, J., Den-Braber, I., Vrisekoop, N., Kwast, L. M., De- Boer, R. J., Borghans, J. A, Tesselaar, K. and Koenderman, L. (2010). "In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days". *Blood*. **116** (4): 625–7.

Priyadarshini, K., Naidu, K. A. and Raghavendra, R. H. (2013). Screening of anti-inflammatory and ant-platelet aggregation property, studies from *Ipomea staphylina*. *Open Access Scientific Report*, 2(4):1-4.

Proud, D. and Kaplan, A. P. (1988). Kinin formation: Mechanisms and role in inflammatory disorders. *Annal Review of Immunoloy*, **6**:49-83.

Proud, D., Macglashan, D. W., Newball, H. H., Schulman, E. S, and Lichenstein, L. M. (1985). Immunoglobulin E-mediated release of a kininogenase from purified human lung mast cells. *Amerian Review of Respiratory Disea*ses, **132**:405-8.

Pyryadarshini, k., Naidu., K. A. and Raghavendra, R. H. (2013). Screening of anti-inflammatory and anti-platelet aggregation property studies from *pomea staphylina*. *Report*, **9**: 40-53.

Rainsford, K. D. (2005). The discovery, development and novel actions of nimesulide. In: *Nimesulide – Actions and Uses*, K.D. Rainsford, ed. Basel: Birkhäuser Verlag, 1–61.

Rainsford, K. D. (1999). History and development of ibuprofen. In: *Ibuprofen. A Critical Bibliographic Review*, K.D. Rainsford, ed. London: Taylor & Francis. Pp. 1–24.

Rainsford, K. D. (2004). Inhibitors of eicosanoids . In: *The Eicosanoids*, P Curtis-Prior, edition. Chichester: John Wiley & Sons, 189–210.

Rihoux, J. P., Masliah, J., Bereziat, G. and Konig, W. G. (1997). Proteins as biological targets for anti-allergic drugs? *International Archives for Allergy and Immunology*, **113**: 339-41.

Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulo, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R. and Kay, A. B. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *New England Journal of Medicine*, **326**(5): 298–304.

Rouzer, C. A, Matsumoto, T. and Samuelsson, B. (1986). Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. *Proceedings of National Academic Sciences*, USA, **83**:857-61.

Schwartz, L. B, Austen K. F. (1984). Structure and function of the chemical mediators of mast cells. *Progress in Allergy*, **34**:271-321.

Scott, A., Khan, K. M., Cook, J. L. and Duronio, V. (2004). What is "inflammation"? Are we ready to move beyond Celsus? *British Journal of Sports Medicine*, **38**: 248-9.

Sen, T. and Chaudhuri A. K. (1991). Anti-inflammatory evaluation *of Pluchea indica* root extract. *Journal of Ethnopharmacology*, **33**: 135-41.

Serhan, C. N. and Savill, J. (2005). Resolution of inflammation: The beginning programs the end. *Nature Immunology*, **6**(12): 1191–1197.

Sharma S. R., Kapoor, B., Sarka, V. and Rai, S. R. (2011) Phytochemical screening and evaluation of anti-inflammatory activity of leaves extract of *Holoptelea integrifolia* roxb. *International Journal of Research in Pharmacy and Science*, **8**(4) 140-151.

Shimoda, H., Shan, S. J. and Tanaka V. (2010). Anti-inflammatory properties of red ginger (*Zingiber officiale var. Rubra*) extract and suppression of nitric oxide production by its constituents, *Journal of Medicinal Food*, **13**(1) 156-162.

Shin, M. H., Baroody, F., Proud, D., Kagey-Sobotka, A., Lichtenstein, L. M. and Naclerio, R. M. (1966). The effect of azelastine on the early allergic response. *Clinical and Experimental Allergy*, **22**:289-95.

Sim, T. C., Reece, L., M., Hilsmeier, K. A., Grant, J. A. and Alam, R. (1995). Secretion of chemokines and other cytokines in allergen-induced nasal responses: Inhibition by topical steroid treatment. *American Journal of Respiratory and Critical Care Medicine*, **5**(8):62-70.

Srinivas, K., Grierson, D. S. and Afolayan, A. J. (2007). Ethnobotanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province, South Africa. *Current Science,* **92.** Pp. 7.

Sumbul, S., (2011). Role of phenolic compounds in peptic ulcer: An overview. *Journal of Pharmacy and Bioallied Sciences*, **3**(3): 361.

Taiwo, B. J., kinkunmi, E. O. and Omisore, N. O. (2012) Antimicrobial and antiplasmodial activities of a quaternary compound from *Ritchiea Capparoides* Var. *Longipedicellata. Journal Of Traditional, Complementary and Alternative Medicine,* **10**(6): 528–531.

Trease, G. E. and Evans, W. C. (2002). Pharmacognosy. 15th Edition. Saunders Publishers, London. Pp. 42-393.

Uvnas B. (1964) Release process in mast cells and their activation by injury. *Annals of New York Academy of Sciences*, **116**:880-90.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature: New Biology*, **231**: 232-235.

Winter, C. A., Risley, E. A. and Nuss, G. W. (1962). Carrageenin-induced oedema in hind paw of the rats as an assay for anti-inflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine,* **111**: 544-547.

Zhang, X. (2014). Flavonoid apigenin inhibits lipopolysaccharide-induced inflammatory response through multiple mechanisms in macrophages. **9**(9): 107072.

Zong W. X. and Thompson, C. B. (2006). Necrotic death as a cell fate. *Journal of Genes Development*, **20**(1): 1–15.

Lorke, D. (1983). A new approach to practical acute toxicity testing. *Archives of Toxicology*, **53**:275-280.