

TITLE PAGE

**PHYTOCHEMICAL AND ANTI-INFLAMMATORY
PROPERTIES OF METHANOL EXTRACT *OF CRATEVA
ADANSONII* STEM BARK.**

BY

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CERTIFICATION

This to certify that this project research on “PHYTOCHEMICAL AND ANTI-INFLAMMATORY PROPERTIES OF METHANOL EXTRACT OF THE “*CRATEVA ADANSONII*” STEM BARK ON WHITE WISTAR ALBINO RATS. Was carried out by Yahaya Memunat under the supervision of Mr. M. O. Ezenwali in the department of biochemistry, Faculty of natural Sciences Caritas University, Amorji-Nike, Emene Enugu State.

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DEDICATION

This work is dedicated to my “ALL SUFFICIENT GOD” and all aspiring biochemists striving to make a difference.

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My most profound gratitude is to Almighty GOD for the gift of life and intellect which has helped me through my stay in Caritas University.

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ABSTRACT

This research investigated the phytochemical and anti-inflammatory properties of methanol extract of *Crateva adansonii* stem bark. Although several edible and non-edible plants parts are used in inflammatory treatment, many record has been found of the use of *Crateva adansonii* stem bark. For this research, fresh stem bark of *Crateva adansonii* were collected from Asata village in Enugu State. The cuttings were authenticated at the Bioresource development centre. They were then dried at room temperature for one month in an open lab space, grounded into powder and weighed on a beam balance as 460.6g. The powder was soaked for twenty-four to forty-eight hours in methanol to get a methanol extract and then concentrated into paste at a set temperature range of 30-55⁰C in a water bath. A population of twenty adult wistar Albino rats was used for anti-inflammatory test. The rats were divided into five (5) groups of four (4) albino rats each. They were administered 3% tween-80 mixed with dichloromethane extract of *Crateva adansonii* and the control was administered with 0.5ml of 3% tween-80. Acute inflammation was induced an hour after test substances were administered by injecting egg albumin in the subplanter region of the right hind paw and edema assessed by mercury displacement for a period of 0-180 minutes. Anti-inflammatory effect was significant within 30 minutes of induced edema with inhibition occurring in three phases of 0-30, 30-60, 60-90. 90-120 to 180 minutes. Inhibition was highest at the third phase. *Crateva adansonii* barks showed anti-inflammatory effect by inhibiting “prostaglandin” synthesis an inflammatory mediator

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CHAPTER ONE

1.0 INTRODUCTION

Inflammation is a Latin word (*inflammare*) which is translated means to set on fire. It is a complex biological response of vascular tissue to harmful stimuli such as pathogens, damaged cells irritant. Inflammation is the reaction of vascularized tissue to local injury caused by certain stimuli like infections, chemicals and biochemical agents, thermal or other physical trauma, antigen-antibody interaction etc (Carol, 1994). Without inflammatory response, wounds will not heal and minor infections would be over weening. Though inflammation aims at limiting damage and restoring function, some enzymes and toxic products within phagocytic cells are released to the extend of damaging the tissue. The advent of anti-inflammatory agents has made inflammation which as been a threat to human life due to its complex, multivalent, to loose its power. These anti-inflammatory agents or drugs help reduce, pain by inhibiting inflammation as opposed to opioids, which affects the central nervous system. It also prevent repairs, prevent and stop the consequences of inflammation by acting on the body responses without directly antagonizing the causative agent (Stedman, 2000). These anti-inflammatory process involves the process of balancing pro-inflammatory acute-phase reactants (Russell *et al.* 2000), altering biochemical pathway

forming prostaglandins by inhibiting cyclooxygenase enzyme from catalyzing the reaction, as a result, suppress, compensate and correct the mechanical and structural abnormalities by assistive device. (Masumoto *et al.*2009).

The inflammatory reaction is phylogenetically and ontogenetically the oldest defense mechanism. The cells of immune system are widely distributed throughout the body, but if an infection or tissue damage occurs. It is necessary to concentrate them and their products at the site of damage.

Three major events occurring during this response:

1. An increased blood supply to the damaged tissue . It is performed by vasodilation. The inflamed tissue look like containing greater number of vessels.
2. Increased capillary permeability caused by retraction of the endothelial cells. This permits larger molecules than usual to escape from the capillaries, and thus allows the soluble mediators of immunity to reach the site of inflammation.
3. Leukocytes migrates out of the capillaries into the surrounding tissues. In the earliest stages of inflammation, neutrophils are particularly

prevalent, but later monocytes and lymphocytes migrate towards the site of infection (Ashcroft *et al.*1999).

For the possibility of surrounding tissue damage, inflammatory responses must be well ordered and controlled. The body must be able to act quickly in some situations, for example to reduce or stop the loss of blood, whereas tissue repair can begin later. Therefore a wide variety of interconnected cellular and humoral (soluble) mechanisms are activated when tissue damage and infections occur. On the other hand, if injury is negligible, the body must have mechanisms which are able to stop tissue damage when the agent is removed. The development of inflammatory reactions is controlled by cytokines, products of plasma enzymes (complement, the coagulation clotting, kinin and fibrolytic pathways), by lipid mediators (prostaglandin and leukotrienes) released from different cells/ and by vasoactive mediators from the mast cells, basophils and platelets. These anti-inflammatory reactions differ. Fast-acting mediators such as vasoactive amines and the product of the kininsystem, modulate the immediate response. Later, newly synthesized mediators such as leukotrienes are involved in the accumulation and activation of other cells. However, in inflammatory reactions initiated by the immune system, the ultimate control is exerted by the antigen itself in the same ways it controls the immune response itself. For this reason, the

cellular accumulation at the site of chronic infection or auto-immune reactions where antigen cannot ultimately be eradicated, is quite different from the sites where antigenic stimulus can be rapidly cleared.

The nervous system can also participate in the control of inflammation especially axon reflexes, but inflammation may be realized in non-nervous tissues as well.

Inflammation may become chronic in certain settings where the acute process characterized by neutrophil infiltration and swelling gives way to predominance of mononuclear phagocytes and lymphocytes. This probably occurs to some degree with the normal healing process but becomes exaggerated and chronic when there's ineffective elimination of foreign materials as in certain infections (e.g tuberculosis) or following introduction of foreign bodies (example asbestos) or deposition of crystals (example urate crystals). Chronic inflammation is often associated with fusion of mononuclear cells to form multinucleated gigantic cells, which eventually become granuloma. Chronic inflammation is seen under of delayed hypersensitivity (Nathan, 2002).

1.2 CRATEVA ADANSONII AS A PLANT.

The flowering tree *crateva religiosa* (syn *crateva adansonii*) is called the sacred garlic pear and temple plant, and many other names in a variety of dialects, including Balai, lamok, abiyuch, barna, varuna and bidasi. The tree is sometimes called the spider tree because the showy flower bear long, spidery stamens. It is native to Japan, Australia, much of South East Asia and several South east Asia and several South pacific islands. It is grown elsewhere for fruit especially in part of African continent.

The *crateva adansonii* plant is a moderate sized, spreading unarmed, deciduous tree growing to a height of 15 meters. Bark is grey, the wood yellowish-white turning light brown when old. Leaves are clustered at the end of the branchlets with a common petiole 5-10 centimeter long, at the summit of which are three leaflets. Leaflets are ovate-lanceolate or ovate 7.5-12 centimeter long, 4-6 centimeter wide. Pointed at the base rather slender pointer at the tip. Flower occur in terminal corymbs, about 5 centimeters in diameter, greenish yellow, and at length purplish.

Petals are ovate or oblong with the claw haft as long as the limb. Fruit is ovoid or rounded, 3-5 centimeter in diameters, with a hard and rough rind.

Seeds are about 10 centimeter in length, numerous kidney-shaped, and embedded in a yellow pulp.

1.3 DISTRIBUTION

1. In waste places, along streams and in thickets near the sea.
2. Occurs in India, Myanmar, Sri lanka, Malasia, Indonesia and China. (nature serve. 2011).
3. Sometimes planted as ornamentals tree for its beautiful flower.

1.4 SCIENTIFIC CLASSIFICATION

Kingdom:	Plantae
Division:	Angiosperrrrms
Class:	Eudicots
Sub- class:	Rosids
Order:	Brassicales
Family:	Capparaceae

Genues: *Crateva*

Species: *C.religiosa*

Binomial name
<i>Crateva religiosa</i>
Frost .F.

The constituents of the plant have been assessed overtime and it has been found in various parts of the plants to contain:

Bark yield tannin.

Phytochemicals screening of extract of dried leaves yielded allcaloids, carbohydrates, tannin, flavonoids, resins, proteins, oils, steroids and terpenoids.

1.5 PROPERTIES

Generally considered diuretic, anti-inflammatory, laxative, anti-oxidant, hepatoprotective, antilithics, antirheumatic, antiperiodic, contraceptive, anthelmintic.

Bark has a disagreeable smell, the taste slightly bitter, biting and pungent.

Leaves considered stomachic and tonic.

Roots and bark considered laxative, lithotriptic and alternative, promoting appetite and increasing biliary sections.

Leaves are rubifacient, tonic and febrifugal.

1.6 RESEARCH AIM AND OBJECTIVES

This research aim at investigating the anti-inflammatory properties of methanol extract of *crateva adansonii* stem bark. And as objectives, to compare the therapeutic potential, (i.e. anti-inflammatory effect) of the methanol extract of *crateva adansonii* bark against the experimental standard indomethacin.

1.7 BACKGROUND OF STUDY

The plant *crateva adansonii* also known as or also called sacred garlic pear and temple plant. Apart from its medicinal properties which allows the plants to be used as laxative, it is also an edible plant especially the fruits,

berry and young shoot. The plant is used in herbal treatment of tympanites, convulsion, treatment of fever, rheumatism, urinary calculi etc.

Crateva adansonii is capable of suppressing the free radicals, production, it is been suggested to have anti-inflammatory properties which counter the synthesis of inflammatory mediators thus forming the background of this research.

CHAPTER TWO

LITERATURE REVIEW

2.0 DEFINITION OF INFLAMMATION

Inflammation (Latin word “inflammo,” meaning “I set a light, I ignite”. Is the body attempt at self-protection; the aim being to remove harmful stimuli, including damaged cells; irritants (Stedman, 1990). Inflammation is part of the body’s immune response. Initially, it is beneficial when, for example, your knee sustains a blow and tissues need care and protection. However, sometimes inflammation can cause further inflammation; it can become self-perpetuating. More inflammation is created in response to existing inflammation (Guyton, 1990).

2.1 PRINCIPLE OF INFLAMMATION

Inflammation is a complex stereotypical reaction of the body expressing the response to damage of its cells and vascularized tissues, e.g. in normal cornea, the true inflammation does not occur (Smith, 2000).

The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response.

The five basic symptoms of inflammation – redness (rubor), swelling (tumor), heat (calor), pain (dolor) and deranged function (functio laesa) have been known since the ancient Greek and Roman era (Nathan, 2002). These signs are due to extravasation of plasma and infiltration of leukocytes into the site of inflammation. Early investigators considered inflammation a primary host defence system. From this point of view inflammation is the key reaction of the innate immune response but in fact, but inflammation is more than this, since it can lead to death, as an anaphylactic shock or debilitating diseases, as in arthritis and gout (Lentsch & Ward, 2000).

According to different criteria, inflammatory responses can be divided into several categories. The criteria includes:

1. time- hyperacute (peracute), acute, sub acute, and chronic inflammation;
2. the main inflammatory manifestation- alteration, exudation, proliferation;
3. the degree of tissue damage – superficial, profound (bordered, not bordered);
4. characteristics picture- nonspecific, specific;

5. Immunopathological mechanisms

- allergic (reaginic) inflammation,
- inflammation mediated by cytotoxic antibodies,
- inflammation mediated by immune complexes,
- delayed- type hypersensitivity reactions. (Serhan, 2008)

2.2 TYPES OF INFLAMMATION

There are two basic types of inflammation- acute and chronic inflammation.

- Acute inflammation: is of short duration, which could be anything from a few minutes to a few days. Such inflammation is caused by foreign substance entering the body, or by physical damage. A viral infection may also precipitate acute inflammation.
- Chronic inflammation, on the other hand, is long lasting. It may persist for weeks, months or even years. Chronic inflammation may be brought on by acute inflammation or maybe the result of an auto immune disease.

Two other, less common types of inflammation are sub-acute inflammation and granulomatous chronic inflammation.

- Sub- acute inflammation has clinical features of acute and chronic inflammation- it is an intermediary stages between the two.
- Granulomatous chronic inflammation: This is a special type of chronic inflammation that is associated with tuberculosis and some lesser known diseases.

2.3 CATEGORIES OF INFLAMMATION MEDIATED BY THE IMMUNE SYSTEM

The immune processes are probably ongoing and, in producing clinically apparent inflammation indicate that the immune system has encountered either an unusually large amount of antigens, antigen in a n unusual location, or antigen that is difficult to digest. In some diseases, such as rheumatoid arthritis, the initiating agent is unknown or maybe normal host tissue compartments. In others, example systemic lupus erythematosus inherent or acquired immunoregulatory abnormalities may contribute to the sustained nature of the inflammatory process (Litman *et al.* 2005). Coombs and Gell divided inflammatory responses mediated by the immune system into four categories, namely I,II,III, and IV, which represent four distinct immune

mechanisms that result in tissue injury. These same four responses represent in mechanism that result in tissue immune protection from infectious agents.

- I. Immediate hypersensitivity (allergic or reaginic acute inflammation)
- II. Cytotoxic (inflammation mediated by cytotoxic antibodies).
- III. Immune complex (inflammation mediated by immune complexes).
- IV. Delayed hypersensitivity (chronic inflammation mediated by lymphocytes and macrophages).

(A) ALLERGIC (REAGINIC) ACUTE INFLAMMATION

Type 1 hypersensitivity is characterized by an allergic reaction that occur completely following contact with antigen, which is referred to as allergen. The term allergy “changed reactivity” of the host when meeting an “agent” on a second or subsequent occasion. In some individuals certain allergies have a propensity to stimulate production of IgE antibodies. IgE antibodies bind nonspecifically, by their high affinity to the fab receptors, to mast cells and basophils. Subsequent attachment of antigen contents of Cytoplasmic - granules from mast cells and basophils (e.g histamine), as well as in synthesis and secretion of biological active products of arachidonic acid (e.g.

leukotrienes). Mast cell products increase vascular permeability and constrict bronchial smooth muscle. A wheal and flare reaction occurs within seconds to minutes. Neutrophils and eosinophils characteristically predominate and mononuclear cells can also be seen (Strtinova *et al.* 1995).

Reaginic reactions are responsible for such allergic phenomena of acute urticaria seasonal rhinitis (inflammation of mucous membrane asthma and in settings where large amounts of antigens (allergens) enters the host circulation, systemic anaphylaxis. These occur when an IgE response is directed against innocuous environmental antigens, such as pollen, house-dust, mites or animal dander. The resulting release of pharmacological mediators by IgE sensitized mast cell produces an acute inflammatory reaction with symptoms such as asthma or coryzal rhinitis (inflammation of the mucous membrane). The importance of type I reactions in protection from infectious organisms is uncertain, although the increased vascular permeability mediated by these cells to arrive at the infected site. In addition, homocytotropic IgE antibodies and cells containing inflammatory mediators probably participate in the defence against large, non-phagocytatable organisms, most notably the multicellular helminthic parasites (Greenhalgh. 1998).

There is an important question why one individual express a topic diseases and another does not. At least two reasons exist-environmental exposure and genetics. A third a reason-an external exten that atters 1gE regulation may be important in certain clinic situation but may represent a rare cause of a topic diseases (Greenhalgh, 1998).

A topic of diseases, allergic rhinitis, asthma and the topic dermatitis have a genetic component. Some or all of these clinical syndromes can be resented in a single member or in several member of the same family. The natural history of the topic diseases is not known, but it appears that a topic individuals appear to have a relatively high frequency of food allergy than becomes rarer but the patients develop 1gE antibodies to inhalant allergens and manifest allergic rhinitis (Sturtinova *et al.* 1995).

In general, a topic is a hereditary feature manifested by abnormal immediate-type hypersensitivity to a certain allergen or a group of allergens.

B. ACUTE INFLAMMATION MEDIATED BY IMMUNE COMPLEXES

Type III hypersensitivity develops when immune complexes are formed in large quantities, or can not be cleared adequately by the reticulo-endothelial system, leading to serum-sickness type reactions.

Repeated cutaneous injection of antigen was shown by Arthus in 1903 to initiate, within hours, acute local inflammation. This form of inflammation called the “Arthus reaction”, was ultimately shown to require immune complexes. Deposition of immune complexes in local tissues with resultant inflammation is common in rheumatic diseases.

The combination of IgM or IgM or IgG antibodies with antigen activates the complement cascade, generating active peptides such as C5a, which in addition to dilating capillaries and increasing vascular permeability, contracts smooth muscle and mobilizes phagocytic cells. Binding of immune complexes to neutrophils and macrophages also activates the respiratory burst with generation of toxic oxygen products such as hydrogen peroxide, hydroxyl radical, hypochlorous acid, and chloramines. Lysosomal proteolytic enzymes, together with oxygen products, produce a potent system that can damage protein and lead to blood vessel damage with haemorrhagic necrosis and local tissue destruction (Lentsch, 2000).

When large amounts of antigen enter the circulation (as following administration of heterologous serum), a serum sickness reaction may ensue. As antibody is produced, antigen-antibody complex by inflammation and vasculitis. phagocytosis of immune complexes by macrophages can result in release of cytokines, such as IL-1 and TNF- α , which initiate fever,

Desposition of immune complexes in the glomerular basement membrane can lead to glomerulitiis. By similar mechanisms arthritis may result (Plotkin, 2005).

Rheumatoid arthritis has many characteristics of local immune complex reaction, whereas systemic lupus erythematosus has many clinical features of serum sickness.

Diseases resulting from immune complex-formation can be placed broadly into three groups.

1. The combines effects of a low-grade persistent infection (such as occur with U-haemolytic streptococcus Viridans or staphylococcal infective endocarditis, or with a parasite such as plasmodium Vivax, or in Viral hepatitis), together with a weak antibody response, leads to chronic immune complex formation with the eventual deposition of complexes in the tissues.

2. Immune complex disease is a frequent complication of autommune disease where the continued produced of antibody of a self-antigen leads to prolonged immune complex formation. The mononuclear phagocyte, erythrocyte, and complement systems (which are responsible for the removal of complexes) become overloaded and the complexes are deposited in the tissues as occurs in systemic lupus erythematosus (McQuibban *et al.* 2000).

3. Immune complexes may be formed at body surface, notably in the lungs following repeated inhalation of antigen materials from moulds, plants or animals. This is exemplified in farmer's lung and pigeon-fancier's lung, where there are circulating antibodies to the actinomycetes fungi found in mould hay, or to pigeon antigens. Both diseases are forms of extrinsic allergic alveolitis, and they only occur after repeated exposure to the antigen (Cotran *et al.*1998).

(C) CHRONIC INFLAMMATION (DELAYED-TYPE OF HYPER-SENSITIVITY REACTION)

Type IV or delayed type hypersensitivity (DTH), is most seriously manifested when antigen (for examples those of tybercle bacilli) are trapped in a macrophage and cannot be cleared. T cells are than stimulated to elaborate lymphokines which mediates a range of inflammatory responses. Other aspects of DTH reactions are seen in graft rejection and allergic contract dermatitis. DTH I used as a general category to describe all those hypersensitivity reactions which take more than 12 hours to develop, and which involve cell-mediated immune reactions rather than humoral immune

reactions. Whereas allergic reactions occur within seconds and minutes and immune complex reactions occur within several hours to one day, DTH reactions peak at 2 to 3 days (McQuibban *et al.* 2000).

Unlike other forms of hypersensitivity, type IV hypersensitivity cannot be transferred from one animal to another by serum, but can be transferred by T-cells (T_{H1} cells in mice. In humans, transfer from a sensitized to a non-sensitizing individual can be also achieved only by T lymphocytes and, interestingly, by a low molecular weight material extracted from them (transfer factor). Delayed type hypersensitivity is obviously associated with T cell protective immunity but does not necessarily run parallel with it there is no always a complete correlation between delayed hypersensitivity and protective immunity. The T-cells necessary for producing the delayed response are cells which have become specifically sensitized to the particular antigen by a previous encounter, and they act by recruiting other cell types to the site of reaction (Muller and French, 2003).

Three types of delayed hypersensitivity reactions are recognized; contact hypersensitivity and tuberculin-type hypersensitivity both occur within 72 hours of antigen challenge, whereas granulomatous reactions develop over a period of weeks.

The granulomatous are formed by the aggregation and proliferation of macrophages, and may persist for weeks. This reaction is, in terms of its clinical consequences, by far the most serious type of delayed type hypersensitivity response. The position is complicated because these different types of reaction may overlap, or occur sequentially following a single antigenic challenge (Boyton & Openshaw, 2002).

The delayed type hypersensitivity reactions are probably important for host defence against intracellular parasites such as tuberculosis and certain viruses and are prevalent in certain disease such as sarcoidosis, Wegener's granulomatosis and polymyositis. In some diseases, such as chronic granulomatous disease of childhood (Litman *et al.* 2005). granuloma formation can lead to obstruction of vital structures such as the esophagus or ureters. The contact dermatitis is caused by sensitization to certain simple chemicals.

Perhaps the best known example of cell-mediated hypersensitivity is (Cousens & Werbz, 2002) the mantoux reaction obtained by injection of tuberculin into the skin of an individual in who previous infection with the mycobacterium had induced a state of cell-mediated hypersensitivity is (Cousen & Werbz, 2002) the mantoux reaction obtained by injection of tuberculin into the skin of an individual in whom previous infection with the

mycobacterium had induced a stage of cell-mediated immunity. The reaction is characterized by erythema and induration which appears only after several hours and reach a maximum at 24-48 hours, thereafter subsiding. Histologically the earliest phase of the reaction is seen as a perivascular cuffing with mononuclear cells of the monocyte –macrophage series. This contrasts with the essentially “polymorphy” character of the Arthus reaction (Muller & French, 2003).

2.4 MEDIATORS OF INFLAMMATION

Once leukocytes have arrived at a site of infection or inflammation, they release mediators which control the later accumulation and activation of other cells. However, inflammatory reactions initiated by the immune system, the ultimate control is exerted by the antigen itself, in the same way as it controls the immune response itself. For this reason, the cellular accumulation at the site of chronic infection, or in autoimmune reactions (where the antigen cannot ultimately be eradicated), is quite different from that at sites where the antigenic stimulus is rapidly cleared (Serhan, 2008).

There are four major plasma enzyme systems which have an important role in haemostasis and control of inflammation. These are the complement

system, the clotting system, the fibrinolytic (plasmin) system and the kinin system (Alexander *et al.* 2002).

Inflammatory mediators; are soluble, diffusible molecules that act locally at the site of tissue damage and infection, and at more distant sites. They can be divided into exogenous and endogenous mediators (Smith, 1997).

Bacterial products and toxins can act as exogenous mediators of inflammation. Notable among these is endotoxin, or **LPs** of Gram-negative bacteria. The immune system of higher organisms has probably evolved in a veritable sea of endotoxin, so it is perhaps not surprising that this substance evokes powerful responses. For example, endotoxin can trigger complement activation, resulting in the formation of anaphylatoxins, C3a and C5a which cause vasodilatation and increase vascular permeability.

Endotoxin also activates the Hageman factor, leading to activation of both the coagulation and fibrinolytic pathways as well as the kinin system. In addition, endotoxin elicits cell proliferation and has been described as super antigen for T cells (Wiederman U. *et al.* 1996).

Endogenous mediators of inflammation are produced from within the (innate and adaptive) immune system itself, as well as other systems. For example, they can be derived molecules that are normally present in the plasma in an

inactive form, such peptide fragments of some components of complement, coagulation, and kinin systems. Mediators of inflammatory responses are also released at the site of injury by a number of cell types that either contain them as preformed molecules within storage granules, e.g. histamine, or which can rapidly switch on the machinery required to synthesize the mediators when they are required, for example to produce metabolites of arachidonic acid (Wiederman U. *et al.* 1996).

Mononuclear Phagocytes (Monocytes and macrophages) are central to inflammation, as they produce many components which participate in or regulate the different plasma enzyme systems, and hence the mediator of the inflammatory response. They are also actively phagocytic and are involved in microbial killing, as are neutrophils. While the latter can be thought of short-lived kamikaze cells that need to be continually replaced from the bone marrow, mononuclear phagocytes are long-lived basophils are much less phagocytic, but together with platelets, these cells are particularly important for secretion of vasoactive mediators. The function of these cell types is at least partially under the control of cytokines. All inflammatory cells have receptors for Fc Domains of immunoglobulins and for complements, and they possess specialized granules containing an immense variety of products that are released perhaps by common mechanism. Cytotoxic T

lymphocytes and NK cells, In general also posses granules which are involved in the adaptive response to inflammation, and they early events of inflammation are mediated in part by molecules produced by cell of the innate arm of the immune system (Stvrtinova *et al.* 1995).

Early phase mediators are produced by mast cells and platelets. They are especially important in acute inflammation and include mainly histamine, serotonin and other Vasoative substances. **Platets** may contribute to inflammatory responses resulting as a consequence of tissue injury, thorough a variety of mechanisms including:-

1. The release of Vasoactive amines and other permeability factors.
2. The release of lysosomal enzymes,
3. The release of coagulation factors which lead to localized and generalized fibrin deposition and,
4. The formation of platelet aggregates or trombi which result in the blocking of vessels and capillaries.

To the early phase mediators also being chemoattractants (e.g. C5a) and cytokines such as IL-1, IL-6 and TNF-U (May & Machesky, 2001).

Late phase mediators are responsible for the regulation of Vascular events occurring late-from about-6-12 hours after initiation of inflammation. The later Vascular events are mediated, at least in part, by products of arachidonic acid.

The chemical mediators of inflammation are summarized in table below. There is considerable functional redundancy of the mediators by inflammation. This explains why certain patients may have complete absence of a humoral component (e.g. Complement component C3), yet minimal problems with increased susceptibility to infection.

Function	Mediators
Increased Vascular permeability of small blood vessels	Histamine, serotonin, bradykinin C3a,C5a, PGE ₂ , LTC ₄ , LTD ₄ . LTD ₄ prostacyclins, activated Hageman factor, high molecular-weight kininogen fragments, fibrinopeptides.
Vasoconstriction	TXA ₂ , LTB ₄ , LTC ₄ , LTD ₄ , C5a, N- form-Yi peptides
Smooth muscles contraction	C3a, C5a, histamine, LTB ₄ , LTC ₄ , LTD ₄ , TXA ₂ , serotonin, PAF, bradykinin
Increased endothelial cell stickiness	IL-1, TNF-a, MCP, endotoxin LTB ₄
Mast cell degranulation	C5a, C3a
Phagocytes Stem cell proliferation Recruitment from bone. Marrow Adherence/aggregation chemotaxis	IL-3, G-C5F, GM-CSF, M-CSF CSF ₅ , IL-I iC3b, IgG, fibronectin, lectins C5a, LTB ₄ , IL-8 and other chemokines, PAF, histamine for eosinophils, laminin, n-formyl peptides, collagen fragments, lymphocyte-derived chemotactic factor, fibrinmpeptides.
Lysosomal granule release	C5a, IL-8, PAF, most chemoat tractants, phagocytosis C5a, TNF-a, PAF, IL-8, Phagocytic particles; IFN-7 enhances
Production of reactive oxygen intermediates	
Phagocytosis	C3b, iC3b, IgG (Fc portion), fibronectin; IFN-7 increases Fc receptor expression
Granuloma formation	IFN-7, TNF-a, IL-1
Pyrogens	IL-1, TNF-a, PGE ₂ , IL-6
Pain	PGE ₂ , bradykinin

Table 2.3: Mediators of inflammation (Serhan & Savill, 2005).

Edema formation can be separated from phagocytes recruitment vasodilation in response to histamine, bradykinin, PGE² and PG1², and complement fragments C3a and C5a results from a direct action of these substances on endothelial cells and smooth muscle vasulature with reslting leakage of plasma. This is accompanied by release of mediators, such as C5a, LTB⁴, and PAF, that act directly on the phagocytic cells. In addition

N-formyl peptides are released from bacteria and mitochondria of damaged tissues. These mediators are potent chemo attractants that mobilize neutrophils, monocytes and eosinophils, cause release of lysosomal contents, and activate the respiratory burst of the phagocytes with resulting production of toxic oxygen products.

Following intravenous endotoxin, a characteristic change in body temperature and white blood count is observed. The body temperature begins to increase after about one hour and reaches a maximum at about four hours. The leukocyte count shows a characteristic decrease at about 30min, due to neutrophil and monocyte adherence to endothelial cells in the lung and spleen. This is followed by a leukocytosis characterized by the presence of immature neutrophils at about four hours, which can persist throughout 24hr with gradual return to baseline by 48hr. The leukocyte predominantly due to mobilization of immature neutrophils from the bone marrow. The components of the inflammatory response-fever, neutrophil margination in the circular vessels, and then mobilization from the bone marrow-are associated with readily detected changes in circulating levels of certain mediators of inflammation. For example, TNF- α peaks within two hours and is likely the predominant pyrogen associated with the febrile response. Plasma levels of the

chemoattractant IL-8 increase early and peak by four hours. Early increased in IL-8 may relate to the transient decrease in the neutrophil count at 30 min (margination) (Plotkin, 2005). Mediator accumulation at local inflammatory processes in skin blisters is somewhat different from the systemic effects following intravenous endotoxin. Mediators detected in blister fluid within 3 to 5 hours of the inflammatory response included LTB⁴, C5a, IL-8 and IL-6. In contrast IL-1^B, GM-CSF, and TNF- α were not detected until after 8hr in the blister. Thus the endotoxin and skin blister models of inflammation demonstrate that there are clear differences in the mediators that can be detected systemically and locally (Stvrtnova *et al.* 1995).

2.5 HISTAMINE AND SEROTONIN

The most important vasoactive mediators that are stored in mast cell and basophil granules are **histamine** in man, as well as **serotonin** or 5-hydroxytryptamine in rodents. They both are also present in human platelets. Histamine is stored in mast cells and basophils largely complexed to mucopolysaccharide (glycosaminoglycans) such as heparin. Histamine has diverse functions including primary, local dilation of small vessels; widespread arteriolar dilatation; local increased vascular permeability by

contracting endothelial cells; the contraction of nonvascular smooth muscle; chemotaxis for eosinophils; and blocking T lymphocyte function. A number of different cells of the body have receptors for histamine. These can be of three types. H1, H2, and H3. The H1 receptors mediate acute vascular effects together with smooth muscle contraction in the bronchi (histamine act as a “Spasmogen”) and the stimulation of eosinophil chemotaxis. In contrast, the H2 receptors mediate a number of anti-inflammatory effects, including the inhibition of eosinophil chemotaxis, but cause the vasodilation. The H3 receptor is mainly involved in the control of histamine release by different producing cells (May & Machesky, 2001).

Serotonin is also capable of increasing vascular permeability, dilating capillaries and producing contraction of nonvascular smooth muscle. Most serotonin is stored in the gastrointestinal tract and central nervous system but a large amount is also stored in the dense granules of platelets

(Wiederman *et al.* 1996).

2.6 THE COAGULATION MECHANISM

The blood clotting system or coagulation pathway, like the complement system, is a **proteolytic cascade**. Each enzyme of the pathway is present in

the plasma as a zymogen, in other words an inactive factor from the precursor molecule. The coagulation pathway functions as a series of positive and negative feedback loops which control the activation process. The ultimate goal of the pathway is to produce thrombin, which can then convert soluble fibrinogen into fibrin, which forms a clot. The generation of thrombin can be divided into the phases, the intrinsic and extrinsic pathway that provide alternative routes for the generation of factor X, and the final common pathway which results in thrombin formation (figure 1.3):

The intrinsic, extrinsic, and common pathways of the coagulation (clotting) cascade (Strutinova *et al.* 1995).

A pathway is here)

The intrinsic pathway is activated when blood comes into contact with sub-endothelial connective tissues or with negatively charged surface that are exposed as a result of issue damage. Quantitatively it is the most important of the two pathways, but is slower cleave fibrin than the extrinsic pathways. The Hageman factor (factor XII), factor XI, Prekalilkrein, and high molecular kininogen (HMWK) are involved in this pathway of activation. Thus this pathway provides a further of the interrelationship between the various enzyme cascade systems in plasma. The first step is the binding of

Hageman factor to a sub-endothelial surface exposed by an injury. A complex of prekallikrein and HMWK also interacts with the exposed surface in close proximity to the bound factor XII, which becomes activated. During activation, the single chain protein of the native Hageman factor is cleaved into two chains (50 and 28) that remain linked by a disulphide bond. The light chain (28KDa) contains the active site and the molecule is referred to as activated Hageman factor (factor XIIa). There is evidence that the Hageman factor can auto activate, thus the pathway is self-amplifying once triggered (compared with the alternative pathway of complement). Activated Hageman factor in turn activates prekallikrein. The kallikrein produced can then also cleave factor XII, and a further amplification mechanism is triggered. The activated factor XII remains in the close contact with the activating surface, such that it can activate factor XI, the next step in the intrinsic pathway which, to proceed efficiently, required Ca^{2+} . Also involved at this stage is HMWK, which binds to factor XI and facilitates the activation process. Activated factor XI, XIIa and kallikrein are all serine protease, like many of the enzymes of the complement system (Majino *et al.* 1961).

Eventually the intrinsic pathway activates factor X, a process that can also be brought about by the extrinsic pathway. Factor X is the first molecule of the

common pathway and is activated by complex of molecules containing activated factor IX, factor VIII, calcium and phospholipid which is provided by the platelet surface, where this reaction usually takes place. The precise role of factor VIII in this reaction is not clearly understood. Its presence in the complex is obviously essential, as evidenced by the serious consequences of factor VIII deficiency experienced by haemophiliacs (Muller & French 2003). Factor VIII is modified by thrombin, a reaction that can result in greatly enhanced factor VIII activity, promoting the activation of factor X.

The extrinsic pathway is an alternative route for activation of the clotting cascade. It provides a very rapid response to tissue injury, generating activated factor X almost instantaneously, compared to the seconds or even minutes required for intrinsic pathway to activate factor X. The main function of the extrinsic pathway is to augment the activity of the intrinsic pathway (Wiedermal *et al.* 1996).

There are two components unique to extrinsic pathway, tissue factor or factor III, and factor VII. Tissue factor is present in most human cells bound to the cell membrane. The activation process of tissue factor is not entirely clear. Once activated, tissue factor binds rapidly to factor VII which is then

activated to form a complex of tissue, activated factor VII, calcium and a phospholipid, and this complex then rapidly activates factor X.

Clot formation: the end result of the clotting pathway is the production of thrombin for the conversion of fibrinogen to fibrin. Fibrinogen is a dimer soluble in plasma. Exposure of fibrinogen to thrombin results in rapid proteolysis of fibrinogen and the release of fibrinopeptide. A the loss of small peptide A insoluble a process that is required for clot formation, but it tends to form complexes with adjacent fibrin and fibrinogen molecules. A second peptide, fibrinopeptide B, is then cleaved by thrombin, and the fibrin monomers formed by this second proteolytic cleavage polymerise spontaneously to form an insoluble gel. The polymerized fibrin, held together by non covalent and electrostatic forces, is stabilized by the transamidating enzyme factor XIIIa, produced by the action of thrombin on factor XIII. These insoluble fibrin aggregates (clots), together with aggregated platelets (thrombin), block the damaged blood vessel and prevent further bleeding (Cotran *et al.* 1998).

There is an interrelationship between the coagulation pathway and other plasma enzyme systems, contact activation of the coagulation pathway, in addition to promoting blood clotting, results in the generation of plasminogen activator activity, which is involved in fibrinolysis or clot

removal. Activated Hageman factor and its peptides can also initiate the formation of kallikrein from plasma prekallikrein, and this triggers the release of bradykinin from kininogens in the plasma. Kinins are responsible for dilating small blood vessels, inducing a fall in blood pressure, triggering smooth muscle contraction, and increasing the permeability of vessel walls. In addition, activation of the coagulation pathway produces a vascular permeability factor, as well as chemotactic peptides for professional phagocytes (Cotran *et al.* 1998).

2.7 FIBRINOLYSIS: Is a process that prevents blood clots from growing and becoming problematic. This process has two types: primary fibrinolysis and secondary fibrinolysis.

The primary type is a normal body process, whereas secondary fibrinolysis is the breakdown of clots due to a medicine, a medical disorder, or some other cause.

In fibrinolysis, a fibrin clot, the product of coagulation, is broken down. Its main enzyme plasmin cuts the fibrin mesh at various places, leading to the production of circulating fragments that are cleared by other proteases or by the kidney and liver.

2.8 THE KININ-FORMING SYSTEM

The kinins, bradykinin and lysisbradykinin, are important mediators of inflammatory responses. They are liberated from precursor molecules, kininogens, by the action of various proteases, collectively known as **kinnogenases**. Three types of kininogen have been identified: high- and low-molecular weight kininogen (HMWK and LMWK respectively), and T-kininogen. These molecules are synthesized by hepatocytes and are released into the plasma, where in addition to releasing kinins, they function as

- (i) Cofactors in the coagulation pathway.
- (ii) Inhibitors of cysteine protease enzymes; and
- (iii) Part of the acute phase response. The kinins are potent vasoactive basic peptides and their properties are wide ranging, including the ability to increase vascular permeability, cause vasodilation, pain, and the contraction of smooth muscle, and to stimulate arachidonic acid metabolism.

Three different pathways may lead to kinin formation during inflammation: (i) the generation of bradykinin as a result of activation of the Hageman factor and the production of plasma kallikrein;

- ii. The production of Iysylbradykin by tissue kallikreins; and (iii) the action of cellular proteases in kinin formation.

HMWK and prekallikrein circulates in plasma as a 1:1 stoicholmetric complex. This complex, together with the Hageman factor, binds to negatively charged surface or collagen. Once they are exposed by tissue damage, the Hageman factor is activated, prekallikren is converted to kallikrein and HMWk itself is digested to release bradykinin, a nin amino acid peptide.

As bradykininis such a potent vasoactive peptide, its activity and its information must be carefully controlled. Activation of the pathway is controlled internally by the presence of inhibitors for each of the active components.

- (1) Inhibitor controls the activity of the activated Hageman factor, while $\alpha 2$ – macroglobulin and (1 inhibitor act as kallikrein inhibitors. There are a variety of enzymes in plasma that control bradykinin activity, including carboxypeptidase N, which removes the C-terminal arginin residue, thus inactivating the peptide. Kallirein also act directly on the complement pathway with direct cleavage of chemotactically active peptide C5a from the complement component C5. Cleavage of fibrinogen by plasmin results in a

number of products including fibrinopeptide B, which potentiates the action of bradykinin and has also chemotactic activity for phagocytic cells.

Chemotactic factors

The term chemotaxis refers to the movement of leukocytes (or cells in general), induced by a chemotactic stimulus. Besides chemotaxis (stimulated, directed migration), leukocytes also possess two other types of movement: **random migration** (undirected, spontaneous migration) and chemokinesis (stimulated, undirected migration). A chemotactic stimulus is provided by substances that can either attract or repulse the cells. Thus, chemotactic cell movement can be either positive or negative, that is, the cells may move towards the source of chemotactic substances (towards an increasing concentration gradient) or the opposite direction. The positive movement is typical for leukocytes substances possessing chemotactic activity are called chemotactic factor (chemotaxin, chemoattractants). Leukocyte chemotaxis (Leukotaxis) is mainly responsible for their mobilization at the inflammatory site. Both exogenous and endogenous chemoattractant participate in this event.

Exogenous Chemotaxins include bacterial oligopeptides of the FMLP type, lectins, denatured proteins, some lipids and lipopolysaccharides.

Endogenous chemotaxins are produced by the host organism are of humoral complement fragment C5a, C5des Arg and Ba, fibrinopeptides, Kallikrein and plasminogen activator) or cellular type (from different cells-LTB₄, PAF, chemotactic cytokines etc).

Interaction between the chemotactic factor and its corresponding receptor triggers a series of coordinated biochemical events which include changes in the cell transmembrane potential, altered cyclic nucleotide levels and ion flow across the cytoplasmic membrane and increased glucose utilization and oxygen consumption. The composition of membrane phospholipids is altered and arachidonic acid, released by phospholipases, is metabolized into a number of biologically active intermediates and products. Within a few minutes, the leukocyte changes from a round to a triangular shape that is oriented along the direction of chemotactic gradient. Reorganization of cytoskeletal contractile elements, particularly actin microfilaments and microtubular structures, contributes to this shape changes. Activation of the contractile cell system not only results in migration but also in other form of movement such as enhanced adherence, spreading, endocytosis and secretion of lysosomal enzymes.

2.9 INFLAMMAION AND DISEASES

in some diseases, inflammatory process can be triggered even when they are foreign invaders. In auto-immune disease, the body's normally protective immune system damaged its own tissues, as it erroneously recognizes self as foreign and normal as abnormal. Some types of arthritis and other inflammation disorders are as a result of misdirected inflammation (Eming *et al.* 2007).

2.10 WAYS OF TREATING INFLAMMATION

There are various ways of treating inflammatory disorders. Since inflammation as a disease is not new to man, various treating methods includes:

- Treat with ICE
- Foods that have anti-inflammatory effect
- IMSAIDs: Immune Selective anti-inflammatory derivates
- NSAIDs: Non steroidal anti-inflammatory drugs
- Herbs treatment.

ICE TREATMENT

Applying ice or even cold water, to an inflamed tissue has an anti-inflammatory effect and it is often suggested as an injury treatment and pain management technique for athletes. One common approach is rest, ice, comfortable support and elevation (Eming *et al.* 2007).

FOOD

Some advocate the consumption of anti-inflammatory foods as a means of controlling inflammations. A typical anti-inflammatory diet includes a well balanced, varied diet that it is high in vegetables and low in refined carbohydrates and undesirable fats such as saturated fats and trans fat. ([WWW.Foodpyramid. Com](http://WWW.Foodpyramid.Com)).

Anti-inflammatory foods include most colorful. Fruits and vegetables, oil fish (Which contains high level of omega-3 fatty acids), nuts seeds and certain spices such as ginger, extra virgin olive oil contains a chemical, oleocanthal, that acts similarly to Ibuprofen. Those following an anti-inflammatory diet will avoid refined oils and sugar and show a preference for so called anti-inflammatory foods in their meal choices (Porth, 2007).

A diet high in vegetable and low in refined carbohydrate and saturated and trans fat may enhance the creation of prostaglandins. There are three main

types of prostaglandins: PG-E1 and bG-E3 which have anti-inflammatory properties, and PG-E2 which promotes inflammation (Porth, 2007).

2.11 IMMUNE SELECTIVE ANTI-INFLAMMAORY DERIVATIVES (IMSAIDS)

IMSAID_s are a class of peptides which is being developed by biological properties including anti-inflammatory properties by altering the activation and migration of inflammatory cells, which are immune cell responsible for amplifying the inflammatory response (Boyton & Openshaw, 2002).

The IMSDAIDS were discovered by scientist evaluating biological properties of submandibular gland and saliva. The work revealed a pathway which shows an interaction between the immune, nervous and endocrine; the pathway known as cervical sympathetic trunk-submandibular gland (stem) axis which is the regulatory system that plays a role in the systemic control of inflammation. The pathway led to the discovery of three amino acid sequence which are phenylalanine-glutamine-Glycine (FEG) and its D-Isomeric form (FCG) have become the foundation for categorizing IMSAID category (Cortran *et al.* 1980).

2.12 THE USE OF HERBS IN THE TREATMENT OF INFLAMMATION

Reducing inflammation using NSAIDs and other anti-inflammatory drugs often results in the relief of pain for short and significant period analgesic, example, Aspirin have anti-inflammatory effects. Hence they are appropriate for the treatment of both acute and chronic inflammation.
<http://www.natureserve.com>

However, some harmful side effects in the human body have been noted which has led researchers to find novel treatment from natural botanical sources, “folk medicine” which includes seeds, leaves, fruits, roots, stems, of different plants. [www.natureserve .com](http://www.natureserve.com)

Advance improvements have been noted since the advent of medicinal plants have been in use for the past fifty (50) centuries which until the last two and a half centuries was the main source of treatment to man and his domestic animals (Ajitawi & Chi, 2006).

A medicinal plant is one whose parts could be used as a precursor for the synthesis of useful drugs (Sofowara, 1982) and herbal medicine which could be applied in the treatment of various forms of diseases (Ajitiwi & Chi, 2006).

Some herbs which may have anti-inflammatory qualities includes, hyssop, ginger, turmeric, pepprika, garlic, which contains belenalin a sesquiterpene lactone and willow bark, which contains salicylic acid (the active ingredient in aspirin), cannabinoids found in cannabis plants are also known to reduce inflammation. Each treatment has an underlying principle and they could be applied in treatment of anti-inflammation based on the type of inflammation, that is, acute or chronic inflammation. www.natureserve.com.

2.13 ANTI-INFLAMMATORY DRUGS

Anti-inflammatory drugs are mainly known as non-steroidal anti-inflammatory drugs mostly abbreviated as NSAIDs.

NSAIDs are a large class of drug commonly and mostly structurally unrelated, therapeutically useful agents that are widely employed in the treatment of inflammation Disorder (Janeway *et al.* 2005).

They play a major role in the management of inflammation and pains caused by arthritis and those of other etiologies including fever-the reason being:

- Anti-inflammatory properties.
- Anti-pyretic (fever-reducing) properties

- Salicyclates (both acetylated such as aspirin and non acetylated traditional NSAIDS-like herbs and their extracts (Janeway *et al.* 2005).

(A) **HOW NSAIDS WORK**

The mechanism of action of NSAIDS is the inhibition of the enzyme cyclooxygenase which catalyzes arachidonic acid is released from membrane phospholipids as a response to inflammatory stimuli. Phospholipids cause blood vessels as a vital part of the body response to infection or injury or in disease such as arthritis (Janeway *et al.* 2005).

They also cause smooth muscle in the uterus to contract prior to birth and during menstruation, resulting, in painful cramps. NSAID work against prostaglandin production by inhibiting cyclo-oxygenase and essential enzyme involved in the synthesis of prostaglandins thereby leading to a reduction in the amount forced. NSAIDS also inhibit the migration of leukocytes (white blood cells) which influence the inflammatory process. This mechanism may relate to the variation in response among patient. Scientific studies have shown a correlation between concentration of drug and effect but do not explain the difference to individual patient responses. It is tough that the pharmacokinetic (process by which a drug absorbed,

distributed, metabolized and eliminated) differences among the various NSAIDs may account for the variability in response (Ajitawi & Chi, 2006).

(B) THE ENZYME CYCLO-OXYGENASE

The enzyme cyclo-oxygenase also known as COX has revealed that they are two forms as (Cox-1 and COX-2 NSAIDs which affects both forms of cyclo-oxygenase. Cox-1 is involved in maintaining healthy tissue, while COX-2 is involved in inflammatory pathway COX-2 selective inhibitors became the newest subjects of NSAIDs

2.14 PYTOCHEMICALS

Alkaloids

Alkaloids are basic nitrogenous compound with definite physiological and pharmacological activity. Alkaloids solution produces white-yellowish precipitate when a few drops of Mayer's reagents are added (Siddiqui & Ali, 1997).

Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (Evans, 2002).

The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.

2.15 GLYCOSIDE

Glycosides are compounds which upon hydrolysis give to one or more sugars (glycones) and a compound which is not a sugar (a glycone or genine). To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer (Siddiqui & Ali, 1997).

2.16 FLAVONOIDS

This group is formed of pigments without nitrogen. Carotenoids are members of this group, as are the important plant pigments called flavonoids. In leaves, flavonoids selectively admit light wavelengths that are important to photosynthesis, while blocking out ultraviolet light, which is

destructive to cell nuclei and proteins. Flavonoids are also important in flower colour in particular providing red blue pigments. Bright fall colors are produced by the conversion of colorless flavonoids, called flavonols, into coloured forms, called anthocyanins. Quinones provide many yellow, red, and oranges pigments, including several useful dyes derived from insects that feed on plants containing the quinones. Cochineal, for example, is a red pigment obtained from the fat cells of scale insects that feed on cactus plants. Saponins, group of naturally occurring oily glycosides that foam freely when shaken with water. They occur in a wide variety of plants, including acacia, soapwort, soap root, California pigweed and many others. Saponins have been, and sometimes also used as cleaning agents and as foam producers, notably in fire-extinguishing fluids. They have a bitter taste and when ingested orally are practically nonpoisonous to warm-blooded animals. When injected directly into the bloodstream, however, they are dangerous and quickly dissolve red blood cells. Hydrolysis of a saponin, brought about by acids or by enzymes, gives a sugar (often, but not necessarily, glucose) and a sapogenin, the latter being either a triterpene or a steroid. Some of the sugars and saponins are useful as raw materials for synthesis of steroid hormones.

2.17 TANNINS

Tannins, also called tannic acid, common name applied to of group of vegetable products, amorphous and crystalline, obtained from various plants, and important commercially in the tanning of leather. Tannins have variable composition. Some, called condensed tannins, are phenols or moderately complex structure, and others are esters of glucose or some other sugar with one or more trihydroxybenzoic acids. The empirical formula, $C_{14}H_{14}O_{11}$, often given for common tannin, is only an average. Tannins occurs in many trees, and the best sources includes oak galls and the bark of sumac. Extraction with water, or water and alcohol, is the first step in the preparation of tannin. Settling, followed by evaporation at a low temperature, yields the commercial products.

2.18 SAPONINS

Saponin is a group of naturally occurring oily glycosides that form freely when shaken with water. They occur in a wide variety of plants, including acacia, soapwort, soap root, California pigweed, and many others. Saponins have been and are sometimes used as cleaning agents and as foam producers, notably in fire-extinguishing fluids. They have a bitter taste and when

ingested orally are practically non poisonous to warm-blooded animals. When injected directly into the bloodstream, however, they are dangerous and quickly dissolve red blood cells. Hydrolysis of a saponin, brought about by acids or by enzymes, gives a sugar (often, but not necessarily, glucose) and a sapogenin, the latter being a triterpene or a steroid. Some of the sugar and saponins are useful as raw materials for synthesis of steroid hormones.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 EQUIPMENT/APPARATUS USED

The equipment and apparatus used for the research study with the manufacturers name and country include the following:

Equipment	manufacturer
Conical Flask	Pyrex, England
Beakers	West Germany
Electric burner	
Gloves	
Grinder (miiil)	Thomas-Wiley, USA
Reagent bottles	Piove-di-sacro Haly
Resort apparatus	Nigeria
Syringes (Iml, 2ml, oral and 1b inch needle)	
Test-tubes	pyrex, England

Water-bath	Gallenkamp, England
Weighing balance	Wang Taiwan
Bunsen burner	
Laboratory cage	Nigeria (Locally made)
100ML measuring cylinder	Great Britain
10ml measuring Cylinder	Great Britain
500ml measuring cylinder	Great Britain
Chemical balance	
Restort apparatus	Nigeria
Electrical Weighing balance	
Funnel	Nigeria
TLC plate	

3.1.2 Chemicals/Reagent used

Manufacturer/Country

Tween 80

Sigma

Distilled water

Nigeria

Mercury

Nigeria

Methanol

Fluka

Fresh egg (Albumin)

Water

Ferric chloride

Merck

N-hexane

Ethylacetate

Dichloromethane

Olive oil

Mayer's reagent

Magnesium

Concentrated hydrochloric acid

Sulphuri acid

Chloroform

May and Bakor

Ethanol

Bott

BenzeneAcetone

Water

3.1.2 ANIMAL USED

The experimental animals used for the research study were adult albino rats of both sexes. They were about 8-10 months and were all within the average weight range of 50-60g. The rats use for the research study were obtained at Asata village in Enugu State.

The rats were housed in the laboratory cage and maintained on standard pellets (Sonac feeds) and water.

3.2.1 METHODOLOGY

Collection and preparation of plant material

Bark of *crateva adansonii* were obtained from the plant or shrub tree found within the vegetation of Azanta village within the month of June of July 2013. They were authenticated by Mr.Moses Ezenwali. And dried at room temperature in the absence of sunlight for a month after which they were grounded to power using Grinder (mill).

3.2.2 EXTRACTION

After grinding the bark of *crateva adansonii*, a 460.6g of the bark powder was obtained. The weight was accurately weighted using the chemical balance. The bark powder was soaked in methanol solvent for extraction (soaked in methanol) for 24-48hours. The extract was concentrated over a water bath at a temperature range of 30⁰C-55⁰C to obtain a paste-like methanol extract of the bark.

3.2.3 PREPARATION OF REAGENTS FOR PHYTOCHEMICAL ANALYSIS

45% Ethanol:

A quantity, 45ml of absolute ethanol was mixed, with 55ml of distilled water and made up to 100ml.

Dilute Sulphuric acid:

A quantity, 10.4ml, of concentrated sulfuric acid was added to 5ml of distilled and made up to 100ml.

5% ferric Chloride:

A quantity, 2.5g, of ferric chloride was dissolved using 50ml of distilled water.

2% hydrochloric acid:

A quantity, 2ml, of concentrated hydrochloric acid was added to 50ml of distilled water and made up to 100ml.

Mayer's reagent:

A weighed quantity of 1.35g of mercuric chloride was dissolved in 60ml of distilled water. And 5g of potassium iodide was measured and dissolved in 20ml of distilled water. The solutions were mixed and volume made up to 100ml.

3.2.4 PHYTOCHEMICAL ANALYSIS OF THE EXTRACT

3.2.5 Test for alkaloids

A quantity of 0.4g of TLM was stirred with 8ml of 1% HCl (Hydrochloric acid) and the mixture was warmed and filtered. 2ml of filtrate was treated separately with (a) few drops of potassium mercuric iodide (Mayer's reagent) and (b) potassium bismuth (Dragendroff's reagent). Turbidity of precipitation with either of these reagents was taken as evidence for existence of alkaloids.

3.2.6 Test for Saponin

A quantity of 20mg of TLM was boiled in 20ml of distilled water in a water bath for five minute and filtered. 100ml of filtrate was mixed with 5ml of distilled water and shaken vigorously for froth formation. 3 drops of Olive oil were mixed with froth, shaken vigorously and observed for emulsion development.

3.2.7 Test for Terpenoids

Presence of terpenoid in TLM was carried out by taking 5ml (1mg/ml) of TLM and mixed with 2ml of Chloroform, followed by 3ml of concentrated H_2SO_4 . A reddish brown coloration of the interface confirmed the presence of terpenoids.

3.2.8 Test for Anthraquinones

A quantity 200ml of TLM was boiled with 6ml of 1% HCl and filtered. The filtrate was shaken with 5ml of benzene, filtered and 2ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of a pink, violet or red color in the ammoniac phase indicated the presence of free hydroxyl anthraquinones.

3.2.9 Test for Coumarins.

A quantity of 300mg of TLM in a small test tube was covered with filter paper moistened with in NaoH. The test tube was placed for few minutes in a boiling water bath. After removing the filter paper it was examined under UV light, yellow florescence indicated the presence of counmarins.

3.2.10 Test for Phlobatannins

A quantity 80m of TUM was boiled in 1% aqueous hydrocholic acid, the deposition of a red precipitate indicated the presence of phlobatannins.

3.2.11 Test for Flavonoids

A quantity of 50mg of TUM was suspended in 100ml of distilled water to get the filtrate. 5ml of dilute ammonia solution was added to 10ml of filtrate followed by few drops of concentrated H_2SO_4 . Presence of flavonoids was confirmed by yellow coloration.

3.2.12 Test Fortannnins

A quantity, 50mg of TUM was boiled in 20ml of distilled water and filtered. A few drops of 0.1% $FeCl_3$ was added in filtrate and observed for

color change; brownish green or a blue-back coloration was taken as evidence for the presence of tannins.

3.2.13 Test for Saponin

A QUANTITY, 0.5G, of the extract was boiled with 5ml of hot and used for the following test:

- (a) Front Test: 1ml of the filtrate was diluted with 4ml of distilled water. The mixture was shaken vigorously and observed on standing.
- (b) Emulsion Test: Two (2) drops of Olive oil was added to 1ml of the filtrate, shaken and observed for formation of emulsion.

3.2.14 Test for Resins

- (a) Precipitation Test: About 0.2g of the extract was extracted with 15ml of 96% ethanol. The alcoholic extract was poured into 20ml of distilled water in a beaker. A formation of precipitation indicates the presence of resins.
- (b) Colour test: About 0.12g of the extract was extracted using chloroform and the chloroform extract concentrated of dryness. The residue was re-dissolved in 3ml Acetone and 3ml of concentrated HCl was added. The mixture was heated a water bath for 30 minutes.. A pink colouration that changes to magnet red indicates the presence of resins.

3.2.15 TEST FOR STEROIDS AND TERPENOIDS

About 1g of the extract was dissolved with 9ml of ethanol and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5ml on boiling water bath and 5ml of hot water was added. The mixture was allowed to stand for 1 hour and the waxy matter filtered off. The filtrate was extracted with 2.5ml of chloroform using a separate funnel to give the chloroform extract.

To 0.5 ml of the chloroform extraction a test-tube was added 1ml of concentrated sulphuric acid to form a lower layer. A reddish brown interface indicates the presence of steroids.

Another 0.3ml of the chloroform extract was evaporated to dryness on a water bath and heated with a 3ml of concentrated sulphuric acid for 10 minutes on a water bath. A grey colour indicates the presence of terpenoids.

3.2.16 Test for Glycoside

A quantity, 0.5g, of the extract of *crateva adansonii* was mixed with 30ml of distilled water. The mixture was heated on a water bath for 5 minutes and filtered. 0.2ml of fehling's solution A and B were added to 5ml

of the filtrate until it turned alkaline and was heated on a water bath for 2minutes. A brick red precipitated observed indicates the presence of glycosides.

3.2.17 PREPARATION OF REAGENTS FOR ANTI-INFLAMMATORY TEST TWEEN 80

3% of tween 80 was prepared using a careful weighted quantity of tween 80 and was mixed with 100mls of diluted water.

Fresh Egg Albumin

One fresh egg was carefully broken and poured into a beaker avoding the egg yolk to give an undiluted egg albumin solution.

3.2.18 TEST FOR ANTI-INFLAMMATORY ACTIVITY

The rat paw edema method of winer, (Risely & Nuss, 1962) was used for the test. The increase in the right high paw volume induced by the sub-planter rejection of fresh egg albumin (Lokoli & Akah 2004) was the basis of the acute inflammation measured. Adult wistar albino rates the acute inflammation measured. Adult wistar albino rats of both sexes were divided into four (4) groups. This four groups received different does of the extract

and was administered intra-peritoneally. The control group received equivalent volume of 3% tween 80 (1ml).

One hour after administration of the test substances, acute inflammation was induced by injection 1ml of undiluted fresh egg albumin into the sub plantar of the right high paw of the rats. The volume of the paw was measured by mercury displacement immediately after inflammatory induction, 0.30, 60, 90, 120, 150, 180, 210, 240 minutes that is 0-3hours after egg-albumin injection Edema formation was assessed in terms of the difference in zero time saw volume of the inject paw and its volume at the different times after the egg albumin injection. For ach dose of the extract, mathematically relation, inhibition of Edema.

$$\text{Inhibition of Edema (\%)} = 100 \left[1 - \frac{(a-x)}{b-y} \right] \times 100$$

where:

a = mean paw volume of treated rats after egg albumin injection

x = means paw volume of treated rats before egg albumin injection

b = means paw volume of control rats after egg albumin injection

y = means paw volume of control rats before egg albumin injection

p = inflammation

q = control

Inflammation = average paw volume at time (T)

= average paw volume at zero (o) time

% inflammation = average inflammation of treated group at time (T)
Average inflammation of control at same time

3.2.19 THIN LAYER CHROMATOGRAPHY (TLC)

TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC Plate).

A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action.

As the solvent moves past the spot was applied, an equilibrium is established for each component of the mixture between the molecules of the component which are adsorbed on the solid and the molecule which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than other. When the solvent has reached the top of the plate, the

plate is removed from the developed chamber, dried, and the separated components of the mixture are visualized. If the compounds are coloured, visualization is straightforward. Usually the compounds are not coloured, so a UV lamp is used to visualize the plates. (The plate itself contains a fluorescent dye which glows everywhere except where an organic compound is on the plate.)

3.2.20 How to Run a TLC Plate

Step ! prepare the developing container

The developing container for TLC can be a specially designed chamber, a jar with a lid, or a beaker with a watch glass on the top (the latter is used in the undergrad labs at CU). Pour solvent into the chamber to a depth of just less than 0.5cm.

To aid in the saturation of the TLC chamber with solvent vapors, you can line part of the inside of the beaker with filter paper. Cover the beaker with a watch glass, swirl it gently, and allow it to stand while you prepare your TLC plate.

Step 2: Prepare the TLC plate

TLC plates used in the organic chem. Teaching labs are purchased as 5 cm x 20 cm sheets. Each large sheet is cut horizontally into plates which are 5 cm tall by various widths, the more samples you plan to run on a plate, the wider it needs to be. Shown in the photo to the left is a box of TLC plates, a large un-cut TLC sheet, and a small TLC plate which has been cut to a convenient size. Handle the plates carefully so that you do not disturb the coating of adsorbent or get them dirty. Measure 0.5cm from the bottom of the plate. Using a pencil, draw a line across the plate at the 0.5cm mark. This is the origin: the line on which you will spot the plate. Take care not to press so hard with the pencil that you disturb the adsorbent. Under the line, mark lightly the name of the samples you will spot on the plate, or mark numbers for time points. Leave enough space between the samples so that they do not run together; about 4 samples on a 5 cm wide plate is advised.

Step 3 Spot the TLC plate

If the sample is not already in solution, dissolve about 1mg 1ml of a volatile solvent such as hexanes, ethyl acetate, or methylene chloride. As a rule of thumb, a concentration of 1% usually works well for TLC analysis. If the sample is too concentrated, it will run as a smear or streak ; if it is not

concentrated enough, you will see nothing on the plate. Sometimes you will need to use trial and error to get well-sized, easy to read spots.

Obtain a micro capillary. In the organic teaching labs, we used 10uL micro caps-they are easier to handle than the smaller ones used in research labs.

Dip the microcap into the solution and then gently touch the end of it onto the proper location on the TLC plate. Don't allow the spot to become too large-if necessary, you can touch it to the plate, lift it off and blow on the spot. If you repeat these steps, the wet area of the plate will stay small.

This example plate has been spotted with three different quantities of the same solution and is ready to develop. If you are unsure of how much sample to spot, you can always spot multiple quantities and see which looks best.

Step 4: Develop the plate

Place the prepared TLC plate in the developing beaker, cover the beaker with the watch glass, and leave it undisturbed on your bench top. The solvent will rise up the TLC plate by capillary action. Make sure the solvent does not cover the spot.

Allow the plate to develop until the solvent is about half a centimeter below the top of the plate. Remove the plate from the beaker and immediately mark the solvent front with a pencil. Allow the plate to dry.

Step 5: Visualize the spots

If there are any coloured spots, circle them lightly with a pencil. Most samples are not coloured and need to be visualized with a UV lamp. Hold a UV lamp over the plate and circle any spots you see Beware! UV light is damaging both to your eyes and to your skin! Make sure you are wearing your goggles and do not look directly into the lamp. Protect your skin by wearing your goggles and do not look directly into the lamp. Protect your skin by wearing gloves.

If the TLC plate runs sample which are too concentrated, the spots will be streaked and/or run together. If this happens, you will have to start over with a more dilute sample to spot and run on a TLC plate.

The R_f VALUE

The retention factor or R_f is defined as the distance traveled by the compound divided by the distance travelled by the solvent.

$$R_f = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent front}}$$

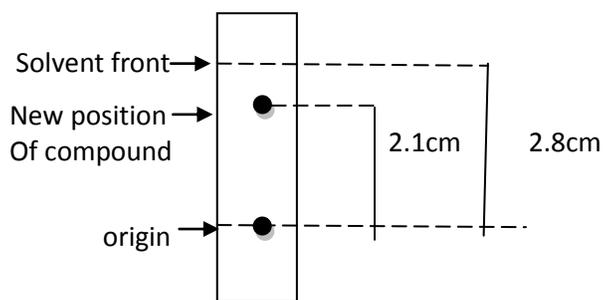
for example, if a compound travels 2.1 cm and the solvent front travels 2.8 cm, the R_f is 0.75:

The R_f Value

The retention factor, or R_f , is defined as the distance travelled by a compound divided by the distance travelled by the solvent.

$$R_f = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent front}}$$

for example, if a compound travels 2.1 cm and the solvent front travel 2.8 cm the R_f is 0.75:



$$R_f = \frac{2.1}{2.8} = 0.75$$

The R_f for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant:

- Solvent system
- Adsorbent
- Thickness of the adsorbent
- Amount of material spotted
- Temperature

Since these factors are difficult to keep constant from experiment to experiment, relative R_f values are generally considered. “Relative R_f ” means that the values are reported relative to a standard, or it means that you compare the R_f values of compounds run on the same plate at the same time.

The larger an R_f of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger R_f is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. Conversely, if you know the structures of the compounds in a mixture, you can predict that a compound of low polarity will have a larger R_f value than a polar compound run on the same plate.

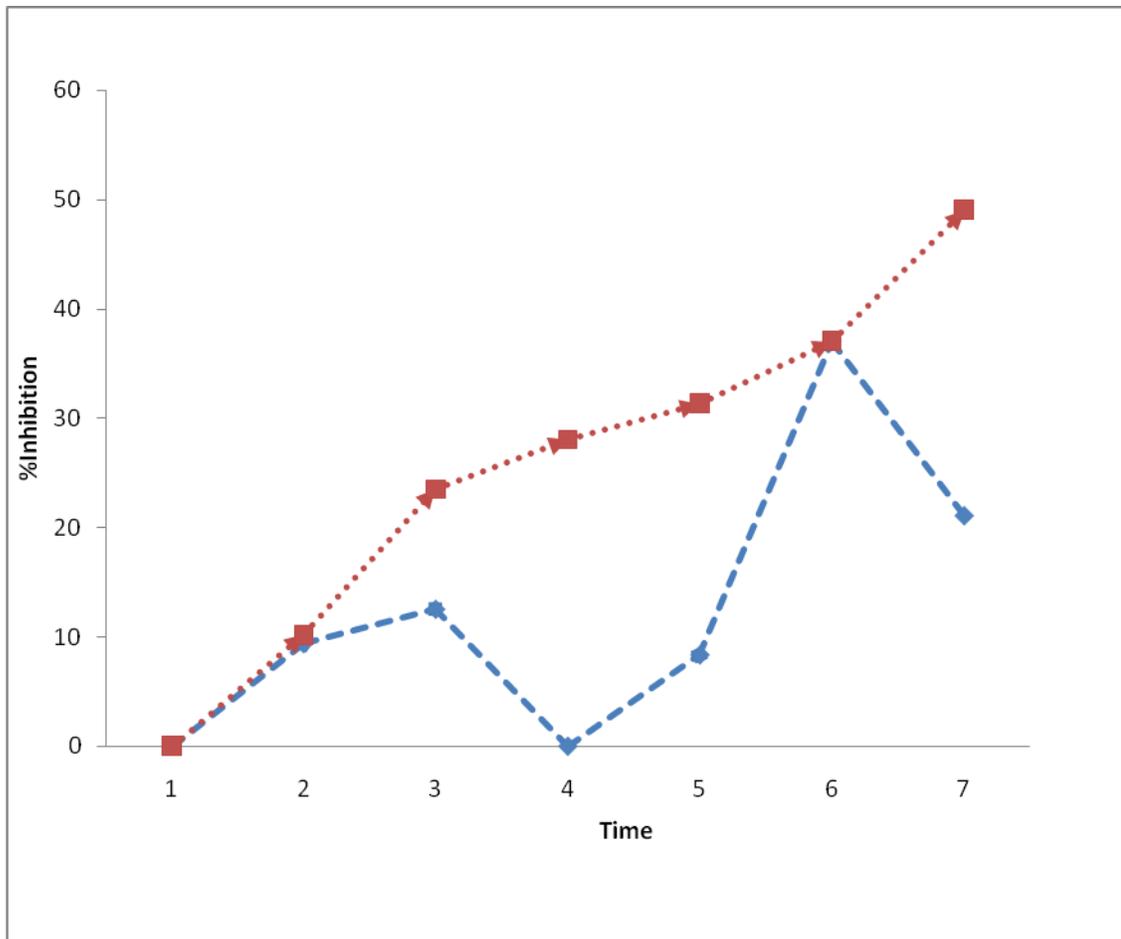
The R_f can provide corroborative evidence as to the identify of a compound. If the identity of a compound is suspected but not yet proven, an authentic sample of the compound, or standard, is spotted and run on a TLC plate side by side (or on top of each other) with the compound in question. if two substances have the same R_f value, they are likely (but not necessarily) the same compound. If they have different R_f values, they are definitely different compounds.

CHAPTER FOUR

RESULTS

4.0 EXTRACTION

Effect of Dichloromethane fraction of *Crateva Adansonii* On Acute Inflammation of Rat Paw.(900Kg .K.b.w of *crateva adansonii*)



Red dotted Line: Ibuprofen (100mg/kg.b.w.)

Rod Blue Line: Dichloromethane fraction (900mg/kg.b.w)

Phytochemical analysis of *crateva adansonii* stem bark extract

phytochemical tests on the methanol extract of *crateva adansonii* bark gave the reaction results shown in table 4.1 below.

Table 4.1: PHYTOCHEMICAL ANALYSIS OF CRATEVA ADANSONII BARK EXTRACT

Constituent/ Test for	Relative presence
Flavonoid	++
<u>Mayer's test</u>	++
<u>Wagner's test</u>	+++
Alkaloid picric acid test	++
Saponin	+
Tannins	–
<u>Resin precipitate test</u>	+++
Colour test	+
Steroid and terpenoids	–
Glycoside	+
Cumarins	–
Phlobatannins	+
Antraquinones	–

CHAPTER FIVE

3.3 DISCUSSION

The anti-inflammatory effect of dichloromethane extract of *crateva adansonii* on egg albumin induced acute inflammation were performed to evaluate the possible inhibition of prostaglandin synthesis. The extract showed a strong protection against the development of acute inflammation of the rat paw. The anti-inflammatory effect was significant within 30 minutes after the inflammation was induced.

The anti-inflammatory effect was low at the second phase as the extracts tend to suppress the inflammatory mediators such as histamine, serotonin and heparin (David, 2001). The inhibition was highest at the third phase when compared with indomethacin.

Several inhibitors of inflammatory diseases modify the inflammatory responses by accelerating the catabolism or antagonizing the action of the inflammatory mediators e.g. histamine.

5.2 CONCLUSION

Following the experimental analysis and result, the methanol extract of *crateva adansonii* stem bark was effective in the treatment of acute inflammation sited in this research. Further researchers are hence encouraged to use other parts of this plant.

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APPENDIX

STEM BARK EXTRACT

S/N	WEIGHT	DOSAGE (mg)	VOLUME (ML)	SEX
1	50.38	45.342	0.51	M
2	47.73	42.957	0.49	M
3	52.14	46.926	0.53	M
4	46.22	41.598	0.49	M

STEM BARK EXTRACT

TABLE 1

Time (mins)	0	30	60	90	120	150	180
	5.20	4.40	4.40	4.60	4.60	4.70	4.80
	5.20	4.70	4.70	4.80	4.90	5.00	5.00
	5.20	4.40	4.40	4.40	4.50	4.70	4.70
	5.30	4.50	4.60	4.70	4.70	4.80	4.90

TABLE II

Time (Mins)	0	30	60	90	120	150	180
	0.8	1.60	1.6	1.40	1.40	1.30	1.20
	0.8	1.30	1.30	1.20	1.10	1.00	1.00
	0.8	1.60	1.60	1.60	1.50	1.30	1.30
	0.7	1.50	1.40	1.30	1.30	1.20	1.10

Table III

0	30	60	90	120	150	180
	0.8	0.8	0.6	0.6	0.5	0.4
	0.5	0.5	0.4	0.3	0.2	0.2
	0.8	0.8	0.8	0.7	0.5	0.5
	0.725	0.7	0.6	0.55	0.425	0.375

Table IV

0	30	60	90	120	150	180
X	0.725	0.7	0.6	0.55	0.425	0.375
Y	0.80	0.80	0.55	0.60	0.675	0.475
%	9.375	12.5	-9.0901	8.33	37.037	21.052

P < 0.05

TABULAR REPRESENTATION OF DICHLOROMETHANE FRACTION OF *CRATEVA ADANSONII* ON ACUTE INFLAMMATION OF RAT PAW. (900KG .B.W OF *CRATEVA ADANSONII*)

Time (minutes)	0	30	60	90	120	150	180	210	240
% Inhibition of <i>crateva adansonii</i> stem bark	-	9.0901	12.5	-9.09	8.33	37.037	21.052	-	-
% Inhibition for Standard	-	0.80	11	-	23	-	28	-	18