Anti-inflammatory, Antipyretic, and Safety Assessment of Aqueous and Ethanolic Leaf Extracts of *Pistia stratiotes* Linn (Araceae)

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. I hereby declare that this thesis is the result of my original research carried out, and humbly wish to affirm that, no part or whole of it has gone in for any such award in any educational setting, and that all sources of information have been duly acknowledged by means of references.

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ABSTRACT

Pistia stratiotes Linn has been reported to have therapeutic effect on arthritis, conjunctivis, and iritis. Thus, this study sought to determine the efficacy of aqueous and ethanolic leaf *Pistia stratiotes* leaf extracts on acute and chronic inflammatory disorders and to establish its safety for use. Carrageenan-induced paw edema, adjuvant and formalin-induced arthritis, endotoxin-induced uveitis and other mediator-induced inflammation using PGE_2, serotonin, bradykinin, and histamine was induced in Sprague-Dawley rat. Antipyretic, acute and delayed and ocular toxicity assessment were also performed. The extracts significantly reduced ($P \leq 0.05$-0.001) paw thickness in all the models of acute inflammation except the 300 mg/kg doses. In adjuvant-induced arthritis, the extracts, except the 300mg/kg ET PSE doses, caused significant ($P \leq 0.05$–0.001) reduction in ipsilateral paw swelling, similar to the effects of methotrexate, dexamethasone, and diclofenac. White blood cell number, erythrocytes sedimentation rate and C- reactive proteins decreased significantly ($P \leq 0.05$–0.01) in arthritic rats treated with the 30 mg/kg dose of aqueous extract and those treated with methotrexate. Histopathological assessment confirmed the efficacy of 30mg/kg dose of the aqueous extract in the management of arthritis in rats. There was significant reduction ($P \leq 0.05$-0.01) in paw thickness of formalin-induced arthritic animals treated with both aqueous and ethanolic leaf extracts with effects comparable to the reference drugs. Lipopolysacharride-induced fever in rats was also significantly reduced ($P \leq 0.05$-0.01) at all doses of the extract treated animals similar to that of acetaminophen. The extracts significantly reduced protein exudation, inflammatory cell infiltration and vasodilation of the iris vessels ($P \leq 0.05$-0.01) associated with uveitis in endotoxin-induced uveitis in rats. Safety assessment showed that the aqueous extract caused reduction in the

number of red blood cells (hemolysis and presence of urobilinogen in urine), proteinuria and microalbuminuria indicating possible acute kidney impairment. But the topical application of the extracts directly into the the conjunctival sac did not show any toxic effect on the ocular tissues.

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Finally my heartfelt gratitude goes to my fiancée Miss Esther Duncan and my colleagues Drs. Stephen Ocansey, Emmanuel Akwesi Abu, Samuel-Bert Boadi Kusi.

DEDICATION

I dedicate this thesis to my mother, Madam Martha Addae.

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LIST OF ABBREVIATIONS

$^1O_2$	Singlet Oxygen
5HT	5-hydroxytryptamine
5-LOX	5-Lipooxygenase
AA	Arachidonic Acid
AIA	Adjuvant-Induced Arthritis
AQ PSE	Aqueous *Pistia stratiotes* Extract
AqH	Aqueous Humour
BN	Brown Norway
BUF	Buffalo
CAM	Cell Adhesion Molecule
CFA	Complete Freud Adjvuvant
CIA	Collagen-Induced Arthritis
CINOD	Cylo-oxygenase Inhibiting Nitric Oxide Donor
COX	Cylo-oxygenase
$cPLA_2$	Cytosolic Phosphplipase A_2
CSIRPM	Centre for Scientific Research into Plant Medicine
DMARDs	Disease-modifying Anti-rheumatic Drug
DNA	Deoxyribonucleic Acid
DSS	Dextran Sodium Sulphate
EDTA	Ethylenediaminetetraacetic Acid
ET PSE	Ethanol *Pistia stratiotes* Extract
FLAP	5-LOX Activating Protein
fMLP	Formylmethionyl leucyl phenylalanine
GAFCO	Ghana Agro Food Comany

GI	Gastroenterology intestinal
GSH	Gluthathione
GSHPx	Gluthathione peroxidase
H_2O_2	Hydrogen peroxide
HCT	Hematocrit
HETE	Hydroxyeicosatetraenoic Acid
HGB	Hemoglobin
HOCl	Hypochlorous Acid
i.p	Intraperitoneal
IBD	Inflammatory Bowel Disease
ICCAM	Intracellular Adehsion Molecule
IF	Interferon
IFA	Incomplete Freud Ajuvant
IL	Interleukin
LH	Lipid hydrogen
LOO	Lipid peroxy radical
LOOH	Lipid hydroperoxide
LPS	Lipopolysaccharide
LT	Leukotrienes
LYM	Lymphocytes
MCH	Mean Corpusular Hemoglobin
MCV	Mean Corpusular Volume
mPGES	Microsomal Prostaglandin E Synthase
MPV	Mean Platelet Volume
mRNA	Messenger Ribonucleic Acid

NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NDGA	Nor-dihydroguaiarectic Acid
NF	Necrosis Factor
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
O_2^-	Superoxide
^-OH	Hydroxyl Radical
OIA	Ovalbumin-Induced Arthritis
ONOO	Peroxynitrate Radical
p.o	Orallly
P_LCR	Platelet Larger Cell Ratio
PAF	Platetlet Activating Factor
PDW	Platetlet Distribution Width
Per os	Orally
PG	Prostaglandin
PIA	Pristane-Induced Arthritis
PLT	Platetlet Count
PMNL	Polymorphonuclear Leukocytes
PUFA	Polyusaturated Fatty Acid
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
RDW-CV	Red Blood Cell Distrbution Width
ROS	Reactive Oxygen Species
SD	Sprague Dawley
SOD	Superoxide Dismutase

TNF	Tumour Necrosis Factor
Tx	Thromboxane
VMPO	Ventromedial Preoptic Area
WBC	White Blood Cell

CHAPTER ONE

INTRODUCTION

This chapter introduces the study and reviews pertinent literature on inflammation and on *Pistia stratiotes.* It also has the justification as well as the aim and objectives for the study.

1.0 GENERAL INTRODUCTION

Inflammation is a usual protective response of living mammalian tissues to injury. It is the body's response to inactivate or raze the invading organism, to eliminate the irritant and to set the stage for repair (Bhitre *et al.*, 2008; Ferrero-Miliani *et al.*, 2007; Highleyman, 2011; Sosa *et al.*, 2002). It is an important pathophysiological parameter in the development, maintenance and aggravation of most infectious and non-infectious disorders of mammals (Sosa *et al.*, 2002; Dutta and Das, 2010). It is involved in disorders in a number of body systems e.g. uveitis (Kalariya *et al*, 2010), atherosclerosis, and asthma (Danese, 2010) and this makes an important concern to clinicians and scientists.

The current treatment of inflammation with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) have serious adverse effects such as immunosuppression (Leung and Bloom, 2003) delayed wound healing, osteoporosis, (Gennari, 1993) cataract formation, and increased intraocular pressure (Friedman and Kaiser, 2007), and gastric ulceration, induction of asthma, bleeding disorders and renal effects,(Cluett, 2009) related to steroids and NSAIDs, reducing their use in some individuals (Juni *et al.*, 2005; Pathak *et al.*, 2005; Singh *et al.*, 2010). Hence, the need to explore alternative means of drug treatment which has moderately less side effects.

Natural product-based anti-inflammatory agents with a transcriptional mode of action, good efficacy, and lower risk of side effects offer promising treatment and prevention option for inflammation-related disorders (Tripathy *et al.*, 2010). Traditionally, *Pistia stratiotes* Linn (Araceae) commonly known as water lettuce, water cabbage, or tropical duckweed has been employed in the management of opthalmia and iritis among some Ghanaians (Abbiw, 1990; Tripathi *et al.*, 2010). This study therefore seeks to evaluate the anti-inflammatory property of the aqueous and ethanolic leaf extracts of *Pistia stratiotes* and to assess its safety for use.

1.1 INFLAMMATION

The term inflammation is derived from the root word "inflammare" which means to burn. Classic inflammatory diseases such as rheumatoid arthritis, uveitis, asthma, colitis hepatitis are major public health concern in the world at large (Cirino *et al.*, 2003, Reiter , Jiang and Christen 2007, Emery, 2009; Poulaki *et al.*, 2007).

Pathophysiologically, inflammation is a series of well synchronized dynamic mechanism consisting of precise vascular, humoral and cellular events that is characterized by the extravasations of fluids, plasma and inflammatory leukocytes to the inflammation site. An array of chemical mediators such as histamine, serotonin, leukotrienes, prostaglandins and oxygen derived free radicals (O_2^-, OH, $ONOO^-$) are produced by inflammatory and phagocytic cells primarily in the sequences which contribute to the onset of inflammation (Tripathy and Grammas, 2009**;** Safayhi and Sailer, 1997). Inflammation response occurs in two phases, these are acute and chronic phases.

1.1.1 Acute inflammation

Acute inflammation lasts from few minutes to hours or one to two days. The cardinal signs of acute inflammation were first described by Celsius which include rubor (redness), calor (heat), tumor (swelling) and dolar (pain) (Jain and Bari, 2010). The process involved in acute inflammation can be explained in two main respects as vascular and cellular processes.

The vascular process takes place in the microvasculature and it becomes evident in 15-30 minutes after an insult or stimulus of inflammation. It is primarily mediated by chemicals such as serotonin and histamine released from mast cells. It is characterized by local vasodilations of venules and capillaries resulting in increased blood flow to the inflamed site hence the redness and heat followed by increased vascular permeability leading to transudation of fluids, plasma and proteins into the site of inflammation causing interstitial oedema (Nathan, 2002).

A number of chemostatic agents including bacterial products possessing amino terminal N-formyl methionyl groups, C5a complement fragment and chemokines e.g. interleukin-8 together with other mediators such as histamine, serotonin (5-HT), leukotriene B_4 (LTB_4) and platelets activating factor (PAF) educe intense polymorphonuclear leukocytes infiltration in a matter of 30-60 minutes (Asako *et al.,* 1992). Neutrophils are the first inflammation cells that are enlisted at the site of inflammation (Phillipson and Kubes, 2011). The process of neutrophil infiltration interact with endothelium in post capillary

venules occurs in multistage it involves the sequential capture rolling along and rigid grip to the endothelium, transmigration through the vessel wall and additional movement in extravascular tissue (Muller, 2003). These cascades of events are engineered by cell adehesion molecules (CAM) which include sclectin, integrin (CD11 and CD18) and intracellular adhesion molecules (ICCAM-1 and-2) (Palmblad and Lerner, 1992**).** The family of CAMs are made up of three members which are expressed on both leukocytes (L-selectin) and endothelial cells during rolling process (Vestweber and Blanks, 1999). Interaction between integrins (CD11 and CD18) and adhesion molecules (CAM-I and CAM-2) mediates high affinity adhesion of leukocytes on endothelial cells. (Ulbrich *et al.*, 2003, Mellado *et al.*, 2008)

1.1.2 Chronic Inflammation

This is marked by the infiltration of mononuclear cells (macrophages and lymphocytes), proliferation of fibroblasts, collagen fibers and formation of connective tissues which at the end of the day lead to the formation of granuloma. In the event of chronic inflammation tissues are degenerated through the mediation activity of reactive oxygen, nitrogen species and protease produced from infiltrated inflammatory cell (Winyard, Blake and Evans 2000). These oxidants are potential mutagen, therefore causes permanent genomic alterations such as point mutations, deletion or rearrangement in case of repeated tissue damage and regeneration (Wiseman and Halliwell, 1996). Available data indicate that p53 mutations occur in rheumatoid arthritis and inflammatory bowel diseases (IBD) at rates comparable to those in tumour (Yamanishi *et al.*, 2002). Other available literature supports

the fact that chronic inflammation eventually advances to carcinoma (Coussens and Werb, 2002; Hussain and Harris 2007).

1.2. MEDIATORS OF INFLAMATION

A number of chemical mediators derived from the blood and inflammatory cells are involved in the process of inflammation. These include vasoactive amines (histamine and serotonin), peptides (bradykinins) and ecosanoids (prostaglandins, leukotrines and thromboxanes).

1.2.1 Vasoactive Amines

Histamine, [2-(4-imidazole) ethylamine] is the first prime mediator implicated in the pathophysiological process of inflammatory diseases. It is initiated by the decarboxylation of histidine catalyzed by the enzyme L-histidine decarboxylase in the Golgi apparatus, from where it is translocated to the granules of mast cells and basophils for storage (Lagunoff, 1983; Akdis and Blaser 2003; MacGlashan, 2003). These mast cells are constituents of almost all organs and tissues and are needed for the development of allergic reaction, anaphylaxis and inflammation by releasing its pro-inflammatory and nociceptive mediators which involves histamine, cytokines, slow reacting substances of anaphylaxis (leukotrienes) and proteolytic enzyme upon activation and eventual degradation of mast cells (Marone *et al*., 2002). Histamine is the most characterized and potent vasoactive substance released by the mast cell. Histamine initiates its biological activity by acting at histamine receptors which are H_1 and H_2 (Hill *et al*., 1997). Well characterized antagonist such as pyrilamine and chlorpheneramine moderates the acute hypersensitivity response

including allergy and rhinitis induced by histamine (Barnes *et al.,* 1998). In the same way mast cell stabilizers example, Sodium cromoglycate prevent the release of histamine from mast cells and is effective in the management of a variety of disorders such as asthma and allergic conjunctives (Day, 1999; Dykewicz *et al.,* 1998".

Serotonin, [5-hydroxytryptamine (5-HT)], which has a similar distribution and activity as histamine is produced by decarboxylation of tryptophan and is stored in secretory granules. Interestingly, serotonin is present in mast cell granules in rodents whiles in humans it is found in platelets. Several serotonin receptors exist and are responsible for its biological activities (Barnes *et al.,* 1998). These include $5HT_1$, $5HT_2$, $5HT_3$, and $5HT_4$.

1.2.2 Peptides

Bradykinin is a nanopeptide derived from plasma Kallikrein-Kininogen system (Bhoola *et al.,* 1992). Bradykinin is produced by a series of proteolytic response stimulated by a variety of factors including tissue damage, allergic reactions, viral infection and other inflammatory proceedings. The two isolated receptors of bradykinins are B_1 and B_2 (Regoli and Barabé, 1980). It is a potent mediator of inflammation as it increases the vascular permeability and effect vasodilation similar to histamine and serotonin. It is also known to enhance the synthesis of prostaglandins and causes localized pain. The strong algesic activity of bradykinins is mediated by the excitation of sensory neurons that incites the release of neuropeptides such as substance P, neurokinin A and calcitonin gene related peptide (Geppetti, 1993). In acute pain, B_2 receptor mediates bradykinin algesia and is considerably reduced by B_2 antagonist but pain of chronic inflammation entails increased

records of B_1 receptors (Regoli and Barabe, 1980; Dray and Perkins, 1993; Regoli *et al.*, 1993).

1.2.3. Eicosanoids

Arachidonic acid, (all-cis-5-8-11-14-eicosatetraenoic acid), is stored in all cells as a component of membrane phospholipids and is a single most essential substrate in the synthesis of biologically active mediators of inflammation dubbed eicosanoids. The ecosanoids comprise of products of 5-lipoxygenase (leukotrienes and 5-hygroxyeicosatetraenoic acid, 12-lipoxygenase (12-hydroxyeicosateteraenoic acid) and cyclooxygenase (prostaglandins and thromboxanes) (Borgeat and Samuelson, 1979).

1.2.3.1 5-Lipoxygenase (5-LOX)

The enzyme 5-lipoxygense was first discovered from glycogen extracted from rabbit polymorphonuclear leukocytes (Borgeat *et al.*,1976). Expression of 5-LOX protein is mainly found in myeloid cells such as polymorphonuclear leukocytes (neutrophils and eosinophils) and mononuclear cells (monocytes, macrophages and lymphocytes) that are involved in inflammation and immune response whereas erythrocytes, platelets endothelial cells and T-cell are 5-LOX negative (Borgeat and Samuelsson, 1979; Claesson and Haeggstrom, 1988; Jakobsson *et al.*, 1992)

A known stimuli that induce 5-LOX activation and leukotrienes synthesis include Ca^{2+} mobilizing agents such as calcium ionophore A2318 (Borgeat and Samuelsson, 1979), formyl-methionyl leucyl phenylalanine (fMLP) (Salari *et al.*, 1985) and soluble

endogenous chemostatic agents such as platelet activating factor (Chilton *et al*., 1982), complement peptide C5a (Clancy *et al.,* 1983), cytokines like IL-8 (Schroder and Christophers, 1989) and phagocytic particles like zymosan (Claësson *et al*., 1983)

1.2.3.2. The Biological Synthesis of Leukotrienes and 5-hydroxyeicosatetraenoic Acid (HETE)

The enzyme 5-LOX is a non-heme iron dioxygenase and is the first important enzyme of the leukotriene pathway. It ensures initial catalytic abstraction of pro-S-hydrogen at C-7 of the arachidonic acid, and subsequent sterospecific insertion of molecular oxygen at C-5 position, resulting in the formation of 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) (Corey and Lansbury, 1983). Ensuing conversion of 5-HPETE to leukotriene A_4 (LTA_4) includes the abstraction of the Pro-R-hydrogen from C-10. On the other hand, 5-HPETE can also be reduced to the corresponding alcohol 5-HETE (Figure 1).

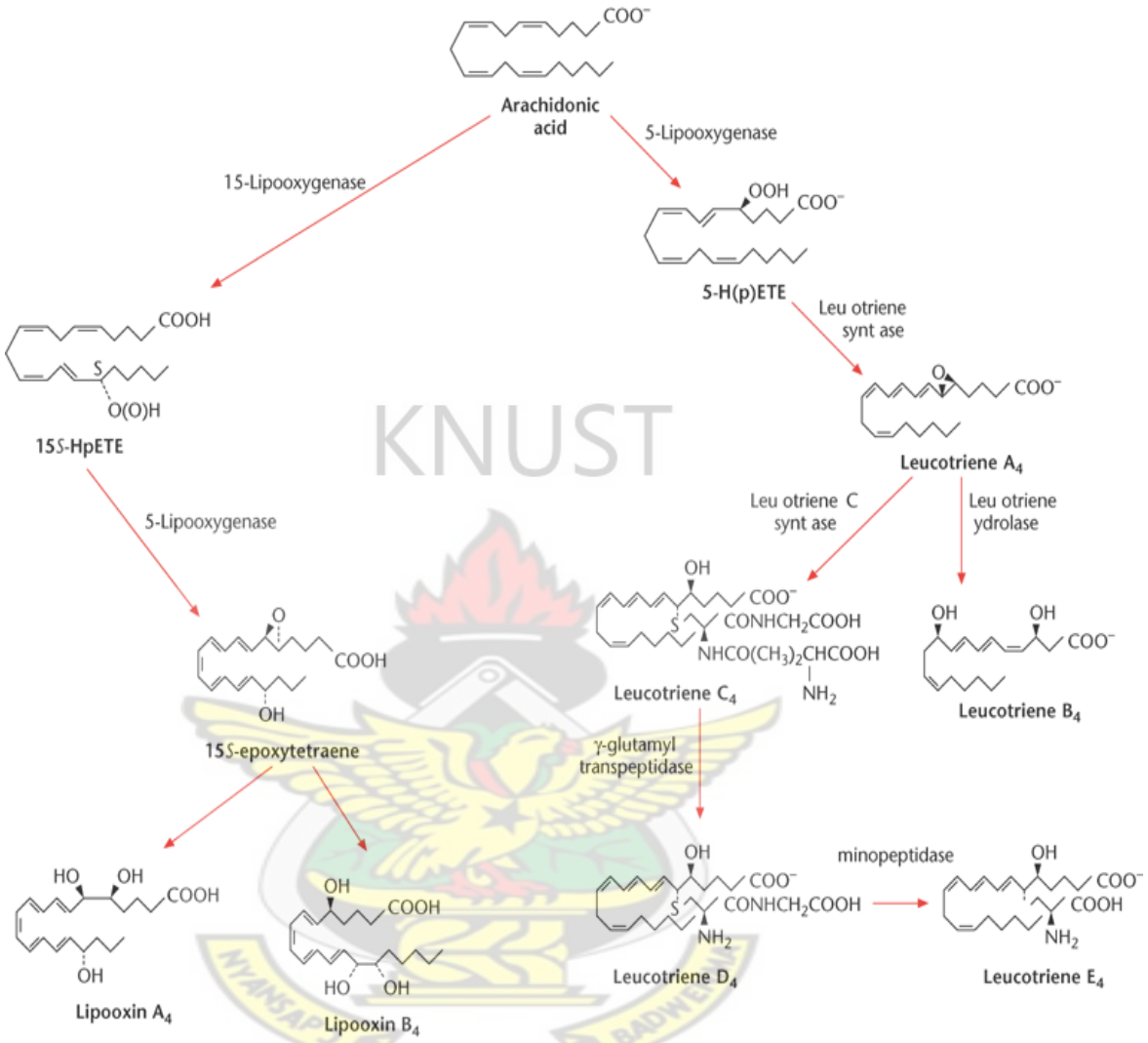


Figure 1: Arachidonic acid lipooxygenation. Lipooxygenase enzymes oxygenate arachidonic acid in different positions, thus they are named the 5-, 8-, 11-, 12-, and 15-lipooxygenases and catalyse reactions that produce messengers. This figure illustrates as an example the synthesis of leucotriene B4 and of sulfidopeptide leucotrienes (LTC_4, D_4 and E_4), the 5-lipooxygenase pathway. It also depicts the synthesis of lipooxins by 15-lipooxygenase.

In the process of the catalyzing the non-heme iron at the active site (harmonized by His-367, His-372, His 550 and the C- terminal) acts as an electron acceptor and donor (Werz, 2002). The action of enzyme 5-LOX ceases after LTA_4 is formed. The downstream reactions of the leukotrine pathway are catalyzed by new enzymes. As a result LTA_4 is

regarded the parent molecule in the leukotriene synthesis that is either converted to LTB_4 by the catalytic action of LTA_4 hydroxylase or conjugated with glutathione to form cysteinyl leukotriene eg. LTC_4 by the enzyme LTC_4 synthase. The catalytic conversion of LTC_4 to cysteinyl leukotriene by the enzyme γ-glutamyltranspeptidase is through the degradation of conjugated tripeptide glutathione to the conjugated dipeptide, cysteinyl-glycine. Finally LTD_4 dipeptidase converts LTD_4 to LTE_4.

Conversely, the enzyme in the succeeding step within the arachidonic acid cascade including LTA_4 hydrolase and LTC_4 synthase are common as they are widely disseminated among different cell types and can synthesize LTB_4 and other cysteinyl leukotrienes using LTA_4 synthezied in the myeloid cells. As a result the liberation of LTA_4 from their site of production (myeloid cell) gives rise to clear increase of leukotrienes at the site of inflammation. A number of available literatures indicate that among different myeloid cells significant disparity exist in type and amount of leukotrine synthesized. Calcium ionosphere stimulation indicated that LTB_4 are found mainly in neutrophils (Byrum *et al.*, 1997), LTC_4 is common in eosinophils (Weller *et al*., 1983) whereas macrophages release both molecules.

Within intact cells the activity and leukotriene production is very much reliant on calcium levels, cytosolic phospholipase A_2 ($cPLA_2$) and 5-LOX activating protein (FLAP). The $cPLA_2$ and 5-LOX are soluble proteins present in cytosol of resting cells but FLAP is sited mainly at the nuclear envelop (Woods *et al.,* 1993). The co-localization of $cPLA_2$, 5-LOX

and FLAP at nuclear envelop are requirement for leukotriene synthesis and calcium is key in ensuring this (Pouliot *et al.,* 1996).

Phosphplipids A_2 consist of a super family of enzyme that is activated by 1-2 micro-molar concentration of Ca^{2+} ions (Uozumi *et al.,* 1997). It causes hydrolysis of the ester bond of membrane phospholipids at the Sn-2 position and provides arachidonic acid as substrate to 5-LOX for leukotriene production in leukocytes (Leslie, 2004). Quite a few studies have shown the participation of $cPLA_2$ in the formation of LTA_4 from neutrophils and macrophages. It has further been established by the use of peritoneal leukocytes from $cPLA_2$ knock out mice upon stimulation of Ca^{2+} ionophore A23187 or LPS to show the marked decrease in the synthesis of eicosanoids (Uozumi *et al.,* 1997).

FLAP is an 18 kDa nuclear membrane bound protein that was identified as a target for MK 886, an inhibitor of FLAP (Miller *et al.,* 1990). FLAP functions as an arachidonic acid binding protein and trigger 5-LOX by transferring its substrate arachidonic acid (AA) to the enzyme for finest synthesis of LTA_4 (Mancini *et al.,* 1993; Ford-Hutchinson *et al.,* 1994). FLAP expression has been detected in all myeloid cells, where 5-LOX mRNA is present and leukotrienes are synthesized (Uhl *et al.,* 2002). The expression of FLAP is greatest in neutrophils, but present in significant amounts in other cell including mast cells, eosinophils and macrophages (Reid *et al.*, 1990; Brock *et al.,* 1995). The necessity of the co-expression of 5-LOX and FLAP for the cellular synthesis of leukotrienes was revealed by the experiment using human osteosarcoma cell line in which 5- LOX and FLAP mRNA were transfected to the cells to increase the production of leukotrienes upon calcium

ionophore stimulation as opposed to the failure of transfected cells with 5-LOX only to produce leukotrienes (Dixon *et al.,* 1990). Additional *in vivo* studies using arachidonic acid –induced ear oedema (Byrum *et al.,* 1997) and collagen-induced arthritis (Griffiths *et al.,* 1997) substantiated the obvious reduction in inflammatory response in FLAP-deficient mice in comparison with wild type mice on the other hand. Inflammation resulting from arachidonic acid induction is chiefly an element of leukotriene production justifying the unconditional requirement of FLAP for cellular leukotrienes production.

Furthermore, the redox state of the cells is also paramount in the activation and regulation of 5-LOX. The 5-LOX is an iron containing enzyme whose activation necessitate the oxidation of Fe^{2+} to Fe^{3+} state which is reliant on threshold levels of hydroperoxide (Rouzer and Samuelson, 1986; Hatzelmann *et al.,* 1989).

1.2.3.3 The Pathophysiological Role of Leukotrienes in the Inflammation Process

Leukotrienes make up a group effective biological mediator in the process of inflammation and anaphylaxis. LTB_4 acts as a chemostatic agent activating the polymorphonuclear neutrophils and kindles their adhesion to vessel wall and elevates cellular permeability (Sha'Afi, 1981; Bray, 1983; Claesson and Dahlén, 1999; Tager *et al*., 2003; Vila, 2004). Studies have shown that LTB_4 trigger the production of pro-inflammatory cytokines such as interferons gamma and interleukins from T- cells and monocytes (Argentieri *et al.,* 1986). A great deal of pathophysiological activities has also been ascribed to cys-LTs (LTC_4, LTD_4 and LTE_4). These include smooth muscles contraction, plasma extravasation, and enrollment of eosinophils, enhanced leakage from post capillary and oedema

formation (Lewis *et al*., 1990). It has been shown to be a thousand fold more potent regarding its contractile effect on airways than histamine. These established properties implicate leukotrienes in the pathophysiological process involved in inflammatory diseases including musculoskeletal disorders eg. arthritis, and respiratory and hypersensitivity disorder such as asthma (Lewis *et al.,* 1990; Byrum *et al.,* 1999).

1.2.4 12-Lipoxygenase

The 12 lipoxygenase enzyme was discovered when platelets were incubated together with arachidonic acid and its metabolites to detect 12-hydrooxyeicosa-5,8,10,14-tetraenoic acid (12-HETE) (Hamberg and Samuelsson, 1974). The enzyme 12-LOX occurs in three isoforms based on tissue distribution such as the type of platelet, type of leukocytes and the type of epithelial tissue (Dailey and Imming, 1999). Stimulation by calcium ionophore A23187 causes cytosolic enzyme translocation to cell membrane to serve as substrate (AA) catalyzing the reaction by integrating molecular oxygen at the C-12 position, as a result producing the metabolites 12 (S)-HPETE, which is transformed to 12-HETE the most abundant metabolite of AA found in platelets (Yamamato *et al*., 1997; Kuhn and Thiele, 1999; Bucar *et al.,*2004).

1.2.4.1 Pathophysiological Function of 12-HETE

Available literature indicates that 12-HETE controls platelet aggregation and neurotransmission (Yoshimato and Takahashi, 2002). It is a potent chemostatic and chemokinetic agent, thus its role in the inception of inflammation (Prieto *et al*., 2003). Increased levels of 12-HETE is also found to be associated with immunoinflammo

disorders such as inflammatory bowel disease and psoriasis (Shannon *et al.*, 1993). On the contrary, HPETE and HETEs have inhibitory consequences on the production of prostaglandins and leukotrienes. This may perhaps modulate its inflammatory response and exercise anti-inflammatory effect as well (Barnes *et al.*, 1998). Also an important pathological role is the involvement of platelet type 12-LOX and 12-HETE in promoting malignant cell growth, proliferation and angiogenesis (Nalarajan and Nadler, 2004; Pidgeon *et al.*, 2002).

1.2.5 Cyclo-Oxygenase (COX)

Cylo-oxygenase (COX), a prostaglandin- endoperoxide synthase is another enzyme responsible for the metabolism of arachidonic acid (AA) and the subsequent production of prostanoids including pro-inflammatory prostaglandins e.g. PDE_2 and PGF2α (Mitchell *et al.*,1994). Studies have shown that in the cells of mammals there exist at least two isoforms of COX i.e COX-1 and COX-2 (Fu *et al.*, 1990; Xie *et al.*, 1991; Langenbach *et al.*, 1995; Quellet *et al.*, 2001). The COX-1which is constitutively expressed in virtually all types of cells such as platelets, stomach, kidney, forebrain, vascular endothelium and uterine epithelium. It is regulated as a house keeping enzyme for various physiological roles. COX-2 on the other hand is inducible and expressed during cell injury or inflammation in response pro-inflammatory cytokines and chemokines such as interleukin-1-beta (IL-1β), tumour necrosis factor-alpha (TNF- α) and interferon gamma (IF-γ) (Akarasereenont *et al.*, 1994; Arias-Negret *et al.*, 1995; Waner *et al.*, 1999; Hood *et al.*, 2003). Despite its implication in the process of inflammation, there is ample evidence that indicates that COX-2 also plays an important functional role in a number of the body's activities and on

the contrary, COX-1 may be induced at inflammation sites as well (Smith *et al.,* 1998). Studies making use of carrageeenan-induced paw oedema model of inflammation amply demonstrated that agents with selectivity for COX-2 were found to ease inflammation at doses which also significantly inhibited COX-1 (Wallace *et al.,* 1998; Seibert *et al*., 1994). These data indicate that COX-1 also contributes to significant fraction of prostaglandins synthesized at the site of inflammation. This assertion is further substantiated by the study involving mice devoid of gene for COX-2 exhibited inflammatory response of similar magnitude to those observed in wild types in carrageenan-induced oedema model of inflammation (Morham *et al.,* 1995; Wallace *et al.,* 1998), in another study employing the same model of inflammation COX-1 deficient mice exhibited diminished inflammatory responses relative to their wild counterpart. (Langenback *et al.*, 1995). Therefore, the unambiguous indication that COX-1 and COX-2 participate in the onset of inflammation.

The human cylo-oxygenase genes have been cloned and consigned to different chromosomes, the COX-1 gene is on chromosome 9 and the COX-2 on chromosome 1 (Kosaka *et al.,* 1994; Tazawa *et al.,* 1994). The two isoforms of cylo-oxygenase are structurally distinct proteins, with their amino acid sequence of their complementary DNA showing about 60% homology (Kurumbail *et al.,* 1996). COX-1 has 576 amino acids and COX-2 have 587 amono acids with molecular mass of ~71 kDa. Although both isozymes have similar active site for their normal substrate arachidonic acids but a minor difference between the isozymes in 3-D structural analysis have been indicated. The smaller valine (an essential amino acid) residue in COX-2 produced a great gap in the enzyme channel,

giving access to site pocket which is thought to be the binding site of many selective COX-2 inhibitory agents (Wong *et al.,* 1997; Marnnett and Kalgutkar, 1999).

1.2.5.1 Biosynthesis of Prostaglandins

The COX isozymes are fundamental membrane proteins and arachidonic acid which is released from membrane is positioned adjacent to the opening of the enzyme channel, is sucked in its hydrophobic area and subsequently converted to prostaglandins and other allied metabolites. The catalytic effect of COX enzyme includes: cyclo-oxygenase activity catalyzing the formation of C-5 ring molecule known as PGG_2 by reacting with two molecules of oxygen, and a peroxidase activity which ensures the reduction of the peroxide group at C-15 location to an alcohol along side the formation of PGH_2 (Funk, 2001; Pulichino *et al.,* 2006) PGH_2 is the antecedent for various biologically active prostaglandins and thromboxanes (Fig. 2). A number of isomerases including, PGD synthase, PGF synthase and PGE synthase catalyzes the conversion of PGH_2 into the different prostaglangins PGD_2, $PGF_{2\alpha}$, PGE_2 in that order. Prostacyclin synthase catalyzes the transformation of PGH_2 into prostacylin, PGI_2 while thromhoxane synthase catalyzes the conversion of PGH_2 into thromboxanes A_2.

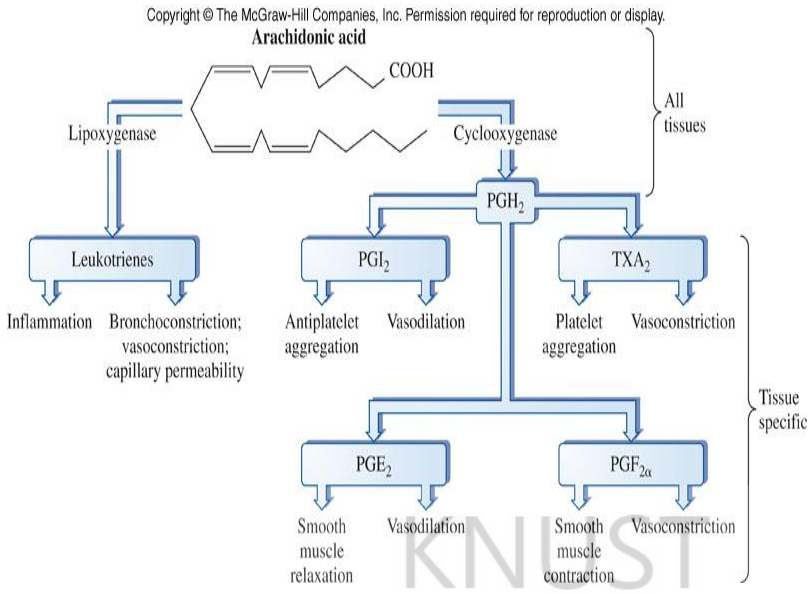


Figure 2: An illustration of the biosynthesis of eicosanoids through the lipoxygenase and cyclooxygenase pathways

1.2.5.2 Pathophysiological Role of Prostaglandins

Protanoids synthesized by COX-1 are essential in many physiological activities including regulating platelet aggregation as thromboxane TXA_2 induces platelet aggregation while PDI_2 exhibits anti-aggregatory properties. It also plays an important role in the gastrointestinal tract (GIT) where PGI_2 and PGE_2 reduces gastric acid secretion, exert vasodilation effect on the gastric mucosa and trigger the synthesis of viscous mucus which forms a protective barrier (Whittle and Vane, 1987; Vane and Botting, 1988). Elsewhere in the kidney prostaglandins including PGI_2, PGD_2 and PGE_2 play a pivotal role in the regulation of renal blood circulation, reducing vascular resistance, dilating the renal vascular beds and enhancing organ perfusion (Whelton, 1999). COX-1 is again seen in all neurons of the brain but more prevalent in the forebrain where PGs are predicted to be involved in complex integrative functions (Yamagata 1993, Breder *et al.*, 1995). It is also

found in the uterine epithelium in early pregnancy and may be essential in ensuring implantation of ovum and angiogenesis needed to establish the placenta (Chakraborty *et al.*, 1996).

However, prostaglandins (PGE_2, PGI_2) are mainly implicated in the maintenance of the inflammatory process by enhancing vascular permeability and magnifying the effect of additional inflammatory mediators such as kin, serotonin and histamine hence, contributing to the cardinal signs described by Celsius. Prostaglandins have implicated in the causation of hyperalgesia through the sensitization of the afferent C-fibres. Besides, PGE_2 are involved in thermoregulatory activity of the hypothalamus, causing pyrexia a systemic manifestation of inflammation. Increased levels of several PGs such as PGE_2 and PGI_2 have been seen in synovial fluids from patients suffering rheumatoid arthritis and osteoarthritis (Pulinchino *et al.*, 2006). In the pathogenesis of many cancerous disorders such as lung, breast and liver cancers, prostaglandin has been shown to play a key role. In these cancerous diseases over expression of COX-2 and excessive production of prostaglandin have been observed (Huang *et al.*, 1998; Achiwa *et al.*, 1999) PGE_2 and PGI_2 mediate pain and inflammation through their action on a variety of receptors. The PGE_2 G-protein coupled receptor subtypes including EPI, EP2, EP3 and EP4 and PGI_2 receptor, IP are some of the receptors identified (McCoy *et al.*, 2002; Lin *et al.*, 2006). The study in which PGI_2 receptor, deficient mice exhibited poor inflammatory response carrageenan-induced paw oedema model of inflammation and acetic acid- induced writhing supports the participation of PGI_2 receptor in the process of inflammation.

1.2.6. Reactive Oxygen Species and Inflammation

The importance of oxygen in aerobic life processes cannot be overemphasized as it is involved in the catabolism of fats, proteins carbohydrates, thereby generating the energy requirement for the body's physical, chemical activities and growth. Nevertheless, an analogous function of oxygen as a toxic agent has been observed in living tissues. An estimated 5% or more of inhaled oxygen is converted to reactive oxygen species (ROS) (Trenam *et al.,* 1992). As a result cells under aerobic state are almost always under threat of insult by ROS. Although, this effect of ROS is sufficiently taken care of by endogenous system of cell without demonstrating an overt effect, any imbalance that occurs between ROS production and endogenous antioxidant defense system exposes the cells to oxidative stress as indicated in certain inflammatory and infectious diseases. Phagocytic cell such as polymorphonuclear leukocytes (neutrophils and eosinophils) mononuclear cells (macrophages and lymphocytes) generate excessive amount of ROS which are essential in host defense approach against microbes. Apart from this notable defensive mechanistic advantage, these excessively produced ROS deregulate cellular functions causing cellular and tissue damage, which turn to worsen situations of inflammation (Trenam *et al.,* 1992; Wu *et al.,* 2006), consequently leading to a range of diseases such as cancer (Wiseman and Haliwell, 1996), atherosclerosis (Witzum, 1994), neurodegenerative disorders (Leboritz *et al.,*1996), Parkinson's disease (Jenner, 2003), rheumatoid arthritis (Halliwell, 1995) and premature aging (Orr and Sohal, 1994).

ROS is adjudged one of the most effective stimuli of inflammation because they also trigger monocytes/macrophages and argument the synthesis of pro-inflammatory cytokines such as TNF-α, IL-8 and IL-1β (Beckman and Koppenol, 1996). All oxygen-derived species including oxygen bearing free radicals and non-radicals are collectively referred to as reactive oxygen species (ROS). Free radicals oxygen derived species include superoxide anion (O_2.), hydroxyl radical (OH.), lipid peroxy radical (LOO.), nitric oxide (NO.), peroxy-nitrite radical (ONOO.) can exist as alone pair electrons with potent reactivity. This reactivity potential is inversely proportional to their stability (Aitken and Fisher, 1994; Squadriato and peyor, 1998). On the other hand, non-radical species comprises of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and lipid hydroperoxides (LOOH) (McCord, 1993; Kerr *et al.*,1996; Gulcin *et al.*, 2003; Bakonyi and Radak, 2004).

Superoxide anion (O^-_2) is synthesized by two enzyme systems indicated as NADPH oxidase and xanthine oxidase, of these two NADPH oxidase (EC 1.6.99.6) is the main superoxide generating system in polymorphonuclear leucocytes, PMNL. It is a multi-component enzymes consist of membrane bound flavocytochrome (cytochrome b_{558}), cytosolic proteins p47 ^{phox} and p67 ^{phox} and a low-molecular weight GTP-binding proteins Rac (Chanock *et al.*, 1994; Choi S-H *et al.*, 2005). The catalytic action of this enzyme is reliant on congregation of all cytosolic apparatus to the membrane. In the cell's state of rest the components are distributed between the membrane and cytosol but upon exposure to suitable inflammatory stimuli or agents such as N-formyl-methionyl-leucyl-phenylalanine, fMLP (a chemostactic peptide) or opsonized zymosan, all the cytosolic

components are translocated to membrane that catalyzes the one-electron reduction of oxygen to O^{-}_{2} at the expense of NADPH.

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2\,O_2^{\cdot} + NADP^{+} + H^{+}$$

Xanthine oxidase has also been suggested to be an important source of O_2^{-} production in tissues. It utilizes molecular oxygen as its acceptor, generating superoxide anion (McCord and Omar, 1993; Prigmore *et al.*, 1995). Additional sources of O_2^{-} synthesis include lipo-oxygenase and cylo-oxygenase catalytic action during the production of eicosanoids (Bagchi and Puri, 1998). Superoxide anions are modest in reacting with biological molecules but it is essential in the synthesis of rather very reactive radical species as in hydroxyl radical (OH) synthesis, which reacts with all bio-molecules with an exceptionally short half life (Cheeseman and Slater, 1993).

Hydroxyl radicals are produced through Haber-Weiss and Fenton reactions. In these reactions hydrogen peroxide (H_2O_2) is the main supply of hydroxyl radials in the presence of either O_2^{-} and Fe_2^{+} (Mello-filho and Meneghini, 1984; Dizdaroglu *et al.*, 1991).

$$H_2O_2 + O_2^{-} \longrightarrow OH + OH^{\cdot} + O_2 \quad \text{(Haber-Weiss reaction)}$$

$$H_2O_2 + Fe_2^{+} \longrightarrow OH + OH^{\cdot} + Fe^{3+} \quad \text{(Fenton reaction)}$$

Therefore, the level of damage at the cellular level caused by H_2O_2 worsens in the presences transition metal ions and O_2^{-} owing to the production of more potent OH ions.

Another oxidizing agent produced by H_2O_2 is hypochlorus acid (Halliwell and Gutteridge, 1992). This hypochlorus acid is synthesized by the catalytic action of myeloperoxidase (EC 1.11.1.17) on chloride ions in the presence of H_2O_2.

$$H_2O_2 + Cl^- \xrightarrow{\text{Myeloperoxidase}} HOCl + OH^-$$

Hypochlorous acid is effective microbicidal agent produced when polymorphonuclear cell are stimulated. Hypochlorous acid is capable of crossing cell membranes. It can also produce hydroxyl radicals in the presences of transitional metal ions by reacting with superoxide anion radicals and ferrous ion (Aruoma, 1994; Pabuçcuoğlu *et al.*, 2003).

$$HOCl + O_2^- \longrightarrow OH + Cl^- + O_2$$

$$HOCl + Fe^{2+} \longrightarrow OH + Cl + Fe^{3+}$$

Peroxy-nitrite radical ($ONOO^-$) is also generated as a result of the release of considerable amount of NO^- and O_2^- from activated neutrophils and macrophages at some point in inflammatory response (Grace, *et al.*, 1998).

$$O_2^- + NO \longrightarrow ONOO^-$$

All the free radicals discussed so far can react with a wide range of bio-molecules including membrane lipids, DNA, proteins and carbohydrates leading to local injury, apoptosis, necrosis and ultimate organ dysfunction eminent upon chronicity of inflammation and cancers (Ivanov and Ivanov 2000).

Biological membranes are known to the extremely susceptible to peroxidation by free radicals because of the presences of poly-unsaturated fatty acids (PUFA) including linolenic acid and arachidonic acid, primarily in the form of esters with phospholipids. An attack by a single free radical on PUFA can initiate the conversion multiple fatty acid side chains into lipid peroxides that ultimately causes cell injury and death (Cheeseman, 1993).

There are three main identifiable stages in free radical mediated lipid peroxidation. These include the initiation process, propagation and termination. The process of initiation begins with the abstraction of an atom of hydrogen from the fatty acid, lipid hydrogen (LH) which is converted into a radical L. Swift oxidation of this radical produces lipid peroxy radical, (LOO) that propagate the reaction by instigating a new chain of oxidation with formation of lipid hydroperoxide (LOOH). This series of reaction if not terminated by an antioxidant's scavenging effect can be detrimental (Atiken and fisher, 1994; Schafer *et al.,* 2000).

Although deoxyribonucleic acid (DNA) is not a sensitive target for free radicals mediated damage, it can damage a specific site of DNA, resulting in disintegration of strands or cause a holdup of the repair before replication occurs which can as well cause mutations, chromosomal aberrations or carcinogenesis (Van-Rensberg *et al.,* 1992; Cheesman and Slater, 1993; Aust and Eveleigh, 1999)

1.3 THERAPEUTIC BASIS OF ANTI-INFLAMMATORY AGENTS

Quiet a number of agents, some on the drug market and others under research are designed to interrupt the process of inflammation by exploiting the physiological landmarks involved in this process to prevent eventual damage to tissue in the case of resolution failure. Some agents are discussed below:

1.3.1 5-LOX Inhibitors

5-LOX inhibitors demonstrate their therapeutic potential in a number of allergic and inflammatory disorders. They are classified as leukotriene biosynthesis inhibitors and leukotrienes receptor antagonists per their mode of action.

1.3.2 Leukotrienes Biosynthesis Inhibitors

These 5-LOX inhibitors are again subdivided into redox active inhibitors, non-redox competitive 5-LOX inhibitors and FLAP inhibitors.

1.3.3 Redox Active Inhibitors

This category is more often than not non- selective antioxidants. They are typically one-electron reducing agent which acts by reduction of active site iron of 5- LOX or interfere with the oxidation of Fe^{2+} to Fe^{3+} therefore, contributing to the uncoupling of catalytic cycle of enzyme (Eleni and Dimitra, 2003). Several compounds containing catecholic groups such as nor-dihydroguaiarectic acid, NDGA isolated from leaves of *Larrea tridentate* (Arteaga *et al.,* 2005), coumarins and flavonoids (quercetin) and caffeic acid have inhibitory effect on 5- LOX (Eleni and Dimitra, 2003).

Another classic example of irreversible iron reducing agent is Phenidone, 1-phenyl-3-pyrozolidone and BW 755C. They are known reference 5-LOX inhibitor in *in vitro* experimentations (Nelson *et al.,* 1991; Eleni and Dimitra, 2003).These compounds have weak selectivity for 5-LOX enzymes. It is known to be effective in *in vivo* inflammatory

disorders associated with oxidative stress and high hydroperoxide levels (Forrest *et al.*, 1998). In spite of this obvious benefit its clinical usage is restricted due to severe side effects e.g. methmoglobin formation by interfering with other biological redox systems and also triggering production of reactive radical species (McMillan and Walker, 1992).

Zileuton, [(N-(1-benzo[b] thien-2lylethyl)-N-hydroxyurea] an iron ligand inhibitor, is a first generation inhibitor of N-hydroxyurea series that selectively inhibit 5-LOX enzyme with weak redox characteristics in *in vivo* and *in vitro* (Rossi *et al.,* 2010). Despite its efficacy in managing specifically provoked asthmatic response by allergens, exercise and cold, its efficacy in the treatment of allergic rhinitis, rheumatoid arthritis and inflammatory bowel disease are yet to be ascertained (Israel *et al.,* 1990; 1993).

1.3.4 Non-Redox Competitive 5-LOX Inhibitors

This category comprises of non-redox type specific 5-LOX inhibitors that competes with AA for binding to 5-LOX. A battery of compounds from dioabicyclooctanyl naphthalene, methoxylkythiazoles and methoxytetrahydropyrans have been found as powerful inhibitors in test tube experiments (McMillan *et al.*,1990; Crawley, 1992; Brooks and Summers, 1996). However, they exhibit only weak potency *in vivo* owning to their poor aqueous solubility and short half life. Another limitation to its clinical uses is its inhibitory effect of leukotrienes synthesis at low peroxide levels as inflammatory response are typified by increased levels of peroxides, thereby the significant lost of efficacy in such disease state (Steinhilber, 1999).

1.3.5 FLAP Inhibitors

They are known to execute their effect by inhibiting the production of leukotirnes in intact cells through antagonizing the FLAP function which involves the binding of AA to FLAP and its subsequent conversion by 5-LOX without actually inhibiting 5-LOX directly. Indole derivatives (MK886) and quinoline derivatives (Bay X 1005) were found to bind to FLAP to inhibit leukotriene biosynthesis in intact cells, not only in *in vivo* experimentations but also *in vitro* studies. Nevertheless, about 1000 fold higher concentration of these indole and quinoline derivatives are needed to inhibit LT synthesis in cell free system as against intact cell (Rouzer *et al.,* 1990; Hatzelmann *et al.,* 1994).

The Bay X 1005 reduces bronchoconstriction in asthma patients (Dahlen *et al.,* 1997). However, they are found to be less effective in the presences of elevated levels of free AA (Fischer *et al.,* 2004). Many agents which therapeutically focus on 5-LOX inhibition were found capable *in vitro* studies but failed to show up on the drug market due to less efficacy and numerous side effects observed during clinical trials. Zileuton is the only 5-LOX inhibitor on the market known for its usefulness in the management of asthma.

1.3.6 Leukotriene Receptor Antagonist

Leukotrienes exercise their physiological effect by binding to and activating specific receptors which include BLT, CysLT1 and CysLT2).LTB_4 mediates chemotaxis by acting on BLT receptor (Yokomizo *et al.,* 2000). Similarlary, most actions of cysteinyl

leukotrienes (LTC_4 and LTD_4) are also mediated by CYS LT1 receptor (Drazen *et al.*, 1999). Drugs able to block these receptors (e.g. montelukast and zafirlukast) are effect against some inflammatory disorders e.g. asthma in the same way, SC-41930, a selective LTB_4 receptor antagonist is effective against colonic mucosal inflammation in animals (Fretland *et al.*,1990).

1.3.7 Isolates of Natural Products That Inhibits 5-LOX

Diverse derivative of plant compounds are emerging as important inhibitors of 5-LOX enzyme. The phytochemical, triterpenoids such as acetyl-11-keto-boswellic acid, an isomer of boswellic acid acts directly on 5-LOX at a sight known to be different from the catalytic AA binding site (Safayhi *et al.*, 1995). The clinical efficacy of the compound acetyl-11-keto-boswellic acid is comparable to zileuton. Similarly, the naturally occurring acylphloroglucinol derivative of hyperforin from the herb *Hypercium perforatum*, St. John's wort, *Hypericum perforatum*, a has been identified as direct 5-LOX inhibitor which acts in a non-competitive fashion (Albert *et al.*, 2002). Alkamides obtained from *Echinacea* and *Achillea* species have demonstrated activity against 5-LOX and relevance in the management of bronchial asthma (Schneider and Bucar, 2005).

1.3.8 Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) in the form of aspirin have been available on the drug market since 1899 for the treatment of treatment of pain and inflammatory disorders (Sneader, 2000).

The landmark progress made in the 1970's regarding the account of the mode of action of NSAIDs relating its inhibitory effect against prostaglandin synthesis through the cyclooxygenase pathway and the identification of two isoforms of COX added in no mean way to the assessment of NSAIDs (Vane and Botting, 1998; Warner *et al.*, 1999; Lin *et al*., 2006). NSAIDs are classified on the basis of their selectivity towards cyclo-oxygenase. These are non-selective COX inhibitors, preferential COX-2 inhibitors and highly selective COX-2 inhibitors.

1.3.9 Non-Selective COX and Preferential COX-2 Inhibitors

Non-selective, NSAIDs are assorted group of chemical substances that inhibit both constitutively expressed COX-1 and inducible COX-2 with about the same potency. Most of these are carboxylic acid containing drugs such as salicylate derivatives e.g. aspirin, carboxylic and heterocyclic acid derivatives e.g. indomethacin, fenamic acid derivatives e.g. mefanamic acid, propionic acid derivatives e.g. ibuprofen, ketoprofen, flurbiprofen and naproxen, phenyl acetic acid e.g. diclofenac, enolic acid containing drugs as such as oxicam derivatives e.g. piroxicam, tenoxicam and meloxicam and pyrazoles e.g. phenylbutazone. These organic acid containing drugs are known to act at the active site of the enzyme and interact with the guanidinium group of Arg-120, thereby preventing the access of AA to the enzyme and bring to a halt the cyclooxygenase pathway (Mancini *et al.*, 1995; Derle *et al.*, 2006)

Although the chemical variety of NSAIDs give rise to a broad range of pharmacokinetic properties, they have general features in common as most of them are well absorbed and

well metabolized by phase I followed by phase II means. Non-steroidal anti-inflammatory drugs, NASIDs are mainly metabolized by CYP2C9 or CYP3A families of P450 enzyme in the liver while renal excretion is the main route for elimination. NSAIDs are available in oral, injectable and topical dosage forms but oral preparations are ideal because of better absorption in relation to other dosage forms. Acetylsalicylic acid, diclofenac sodium and ibuprofen are also available as ointments, widely used to suppress local inflammation and pain. Ophthalmic solution of 0.1% diclofenac, 0.03% flurbiprofen and 0.5% ketorolac are useful in ophthalmic management of inflammatory disorders.

Classical NSAIDs (aspirin like drugs) being the drug of choice in the management of rheumatic disorders and other degenerative inflammatory diseases or as an abuse drug, the United States, U.S alone consumes about 20,000 tones of aspirin (Smith *et al.*,1998; Warner *et al.*, 1999; Steinmeyer, 2000; Pulichino *et al.*, 2006). An estimated 30 million people world wide uses NSAIDs daily with a sales of US$ 5.8 billion (McGettigan and Henry, 2000; Steinmeyer, 2000; Derle *et al.*, 2006). Nevertheless, prolong clinical use elicits numerous side effects , notable amongst them are gastric erosion, ulceration, hemorrhage, bronchospasm, kidney and liver dysfunction (Lin *et al.*, 2006). These unwanted negative effects arise from the obstruction of the physiological effect of prostacyclin (PGI_2), prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2). On the other hand, treatment with NSAIDs shifts the AA pathway towards 5-LOX leading to the production of pro-inflammatory, bronchocontrictive and gastrodamaging leukotrienes (Gilroy *et al.*, 1998; Martel-Pelletier *et al.*,2003).studies have shown that asymptomatic mucosal damage is initially evident in 80% of subjects after NSAIDs therapy (Ehsanullah

et al., 1988) however, upon continuous use of NSAIDs 15-20% of treated patients develop ulcer (Singh *et al*., 1996), and 1-3% received hospital treatment for gastrointestinal, (GI) bleeding or perforation.

1.3.10 COX-2 Selective Inhibitor

A thoughtful consideration of the many unwanted negative effects associated with the use classical NSAIDs has lead to the development of new generation NSAIDs including referocoxib (Vioxx) and celecoxib (Celebrex) as an important anti-arthritic therapy. Referocoxib and celecoxib are 800 and 375 fold more selective for COX-2 than COX-1 respectively. Clinical studies shows that it has a similar efficacy as diclofenac and naproxen but a lower unwanted gastrointestinal effect upon prolong usage (Van Ryn and Pairet, 1999; Bertolini *et al.*, 2002, Alvaro-Gracia. 2004), just when it was thought of have been able to avert the myriad of unwanted side effects accompanying the use of non selective COX inhibitors studies raised alarm about the cardiovascular safety with the use of selective COX-2 inhibitors. Available clinical studies have indicated increased risk of myocardial infarction and stroke among patients who uses selective COX-2 inhibitors over an extended period in managing rheumatoid arthritis (Bombardier *et al.*, 2000; Bresalier *et al.*, 2005). The risk of cardiovascular risk is chiefly attributable to the inhibition of protacyclins (PGI_2) causing severe physiological disproportion between prothrombotic thromboxane A_2 levels (high) and vasodilatory protacyclin levels (low) in the endothelium, supporting platelet aggregation and vasoconstriction (Linton and Fazio, 2004; Lin *et al*., 2006) this has really lead to the ban of Vioxx for general treatment, however available under strict prescription (Krumholz, *et al.*, 2007).

1.3.11 Dual Inhibitors of 5-LOX and COX

It is evident that unwanted negative effects associated with today's analgesic and anti-inflammatory therapy is primarily attributed to the imbalances in metabolites levels of COX-1, 2 and 5-LOX. This eye-opener has prompted the need for the development of multi-target drugs with better efficacy and gastrointestinal tolerability (Celotti and Laufer, 2001; Charlier and Michaux, 2003; Ardoin and Sundy, 2006). This insight in recent past has lead to the discovery of dual 5-LOX and COX inhibitors (Laufer, 2001; Martel-Pelletier *et al.,* 2003). Licofelone; ML-3000 ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl]-acetic acid (Laufer, 2001; Tries *et al.,* 2002). At the moment is under phase –III clinical trials for osteoarthritis and has proved remarkable gastrointestinal safety profile relative to classical NSAIDs (Tries and Laufer 2001; Bias *et al.,* 2004). Besides, it showed no cardiovascular risk associated with the use of selective COX-2 inhibitors as described previously (Alvaro-Gracia, 2004; Rotondo *et al*., 2006). Additionally, licofelone compared to NSAIDs demonstrated an exceptional capability of inhibiting leukocyte rolling and adhesion to endothelium and eventually prevent cellular infiltration during the process of inflammation (Ulbrich *et al.,* 2005).

1.3.12 Cyclooxygenase Inhibiting Nitric Oxide Donors (CINODs)

Nitric oxide (NO) based anti-inflammatory drugs are at center of attention research because they do not exacerbate either gastrointestinal complications or cardiovascular troubles. The design of CINOD drugs is such that nitric oxide donating moiety is conjugated with conventional NSIADs such that after administration the drug is broken metabolically into original NSAIDs and nitric oxide donating moiety (Wallace and Del

Soldato, 2003). Naproxinod, a product of this class of drug is now under phase –III clinical trials (Schnitzer *et al.,* 2005). The worrisome GI toxicity accompanying the use of non-selective NSAIDs are reduced to the barest minimum in the presence of NO. This is because NO stimulate secretion from gastric mucosa that minimizes ulcer formation. Another good thing is that cardiovascular toxicity relating to COX-2 selective NSAIDs are also reduced as NO possesses vascular smooth muscle relaxant properties which curtails vasoconstriction (Naseem, 2005).

A population based study estimates a phenomenal increase in the consumption of non-steroidal anti-rheumatic drug from 380 million to 600 million consumers in the next two decades among the geriatric population alone (Steinmeyer, 2000). This consolidates the need for an urgent search for new safer and efficacious anti- inflammatory agents.

1.3.13 Steroidal Anti-Inflammatory Drugs

Glucocorticoids also known as corticosteroids are widely used among 60-70% of the population for the management of serious inflammatory disorders and rheumatic disorders such as bronchial asthma, systemic lupus erythmatosus, vascilities, Wegener's granulomatosis, giant cell arteritis, psoriasis, rheumatoid arthritis, Grave's disease and multiple sclerosis along with sepsis (Rhen and Cidlowski, 2005). However, the serious associated side effect with chronic corticosteroid therapy limit it use to acute flare-ups of diseases under strict prescription and follow ups.

The steroidal drugs are well absorbed through the intestine and skin, therefore are available in different preparations including oral, injectables and topical forms. Ophthalmic solution

of 0.1% dexamethasone and fluoromethalone are mostly used in the treatment of ocular inflammation. Topical application of glucocorticoid ointment, 1% hydrocortisone is amazingly efficacious in inflammatory dermatoses and eczema. Inhaled corticosteroids such as beclomethasone, betamethasone, budesonide, dipropionate, triacinolone acetonide and flunisolide are currently most effect drugs available for prevention of asthma (Barnes, 1995a, b). Prednisolone and hydrocortisone are widely used orally and intravenously, in numerous inflammatory disorders.

Multiple mechanisms are involved in the suppression of inflammation by glucocorticoids as a result their ability to manipulate all sorts of inflammatory events. Nevertheless, its binding to cytosolic glucocorticoid receptors remain principal mode of action of glucocorticoids. The cortisol-glucocorticoids receptors complex translocate to the nucleus and physically interacts with NF-kB to block its transcriptional activity for multiple inflammatory gene the regulate cytokines, chemokines, cell adhesion molecule complement factor and receptors for these molecules (Barnes, 1999; Mckay and Cidlowski, 1999). NF-kB as well induces the transcription of COX-2, an enzyme key in the production of prostaglandin. Hence glucocorticoids-induced antagonism of NF-kB decreases the expression of COX-2 and subsequent synthesis of its pro-inflammatory metabolites prostaglandins (Tanabe and Tohnai, 2002).

Glucocorticoids also induce anti-inflammatory protein called annexin-1 (lipocortin-1) that physically binds to cell membrane and prevents cytosolic phospholipase A_2 interaction with membrane. This therefore, inhibits $_cPLA_2$ activity, lipocotin-1 blocks the release of

arachidonic acid and it subsequent conversion to inflammatory products i.e. prostaglandins and leukotrienes (Mizuno *et al.,* 1997). Another anti-inflammatory protein MAPK phosphatase 1 also induced by glucocorticoids that dephosphorylates and inactivates the Jun N- terminal kinase. During the process of inflammation and immune response Jun N-terminal kinase is activated and phosphorylates the transcription factor c-Jun.This phosphorylated c-Jun bind to DNA sequences called activator protein 1 and induce the transcription of inflammatory and immune genes (De Bosscher *et al*.,2003) thereby, inhibiting the phosphorylation of c-Jun by glucocorticoids-induced MAPK phosphatase 1 prevents inflammatory and immune response.

Glucocorticoid again causes vasoconstriction when applied topically to the skin, probably suppressing mast cell activation and degranulation (Yoshikawa and Tasaka, 2000). They also decrease capillary permeability through the reduction of histamine released by basophils and mast cells (Cole *et al.,* 2001).

Glucocorticoids in vogue acts non -selectively, therefore their prolonged use and high dose therapy inflict several side effects and impairs numerous anabolic processes. As a measure to curb these adverse effects attention has been shifted towards selectively acting glucocorticoid agents (Rhen and Cidolowski, 2005).

1.3.14 Disease-Modifying Anti-Rheumatic Drug (DMARDs)

The myriad of problems associated with the use NSAIDs in rheumatic diseases has led to the search for alternatives. The understanding that macrophages and T-lymphocytes play a

key role in rheumatoid inflammation through the production of unnecessary amount of cytokines particularly TNF-α and IL-1 involved in joint destruction has led to the quest for DMARDs having immunosuppressant effect (Duff, 1994; Feldmann *et al.,* 1996). This group of compounds belongs to different classes including auranofin (gold compound), chloroquine (ant-malarial), penicillamine (antibiotics) and methotrxate (antimetabolites) entered rheumatology through clinical intuition and have remain for decades though their exact mode of action are still obscure (Bonderson, 1997). However, auronofin and chloroquine have been postulate to act by reducing the formation of IL-1 and TNF-α in monocytes and macropages (Danis *et al.,* 1991; Picot *et al.,* 1991). Penicillamine suppresses the activation of macrophages and production of IL-1 and eicosanoids from these cells. it is also thought to diminish lymphocyte responsiveness, proliferation and mitogen-induced immunoglobulin serection from mononuclear cells (Bonderson, 1997). Methotrexate's mechanism of action in rheumatoid arthritis RA is unlikely to be as a result of direct inhibition of IL-1 and TNF-α synthesis but via increasing the production of anti-inflammatory autocoid adenosine or activating cytokine inhibitor production (Cronstein, 1995).

1.3.15 Antioxidants

The human antioxidant system can be classified into two main classes. These are enzymatic and non-enzymatic. The major intracellular endogenous antioxidant defenses are enzymatic in nature. These include superoxide dismutase (SOD; EC 1.15.1.1) found in the mitochondrial matrix that converts anion to hydrogen peroxide (Ivanova and Ivanov, 2000).

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Catalase (CAT; EC 1.11.1.6) is also located in peroxisomes and metochondria. It is a large tetrametric protein which removes H_2O_2 by catalyzing its conversion to water (Krinsky, 1992)

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Gluthathione peroxidases (GSHPx) are a group of selenium dependent enzymes and require gluthathione (GSH) as a cofactor for detoxification of H_2O_2 (Upasani and Balaraman, 2003).

$$2GSH + H_2O_2 \xrightarrow{GSPHx} GSSG + 2H_2O$$

Non-enzymatic antioxidants can be further classified into two groups: endogenous and exogenous antioxidants. The most important endogenous antioxidants found in human plasma are transition metal binding proteins. These include ceruloplasmin, transferring, hepatoglobin and albumin. They bind with transition metals such as iron and copper and hence control the production of metal catalyzed free radicals (Halliwell and Gutteridge, 1990; Ivanova and Ivanov, 2000).

On the hand, non-enzymatic exogenous antioxidants are generally of dietary source. Notable among them are ascorbic acid, vitamin E and carotenoids. A number of phenolic compounds isolated from plant which include naturally occurring antioxidants such as flavonoids, phenolic acid, polyphenols and quinines. All classes of phenolics possess antioxidant activity, which is dependent on the number of hydroxyl groups present on

benzene rings (Cadenas and Packer, 2002). These compounds exert their antioxidant effect by scavenging free radicals or donating a hydrogen atom. Another group of compounds which include rutin, quercetin, caffeic acid and nordihydroguaiaretic acid chelate redox active metals ions (iron and copper) resulting in termination of the redox cycle of these metals or inhibiting oxidative enzyme such as lipoxygenase, NADPH oxidase and myeloperoxidase. Some antioxidants react with lipid peroxy radicals to yield a relatively stable lipid hydroperoxide (Rice-Evans and Miller, 1996). Therefore protects the membrane from lipid peroxidation induced by damage.

A great deal of studies has been done to investigate the pre-emptive role of antioxidants in diverse diseases. Although the pharmacological half life of superoxide dismutase and catalase are extremely short but have proved to be extremely protective in quite a few ROS mediated inflammation such as carrageenan-induced pleurisy in rats (Salvemini *et al., 2*001; Greenwald, 1990; Lesnefsky, 1992).Many researchers have proved the protective and beneficial role of administered superoide dismutase, SOD mimetics in model inflammation and the decrease in O_2^- ease the inflammatory disorders (Salvemini *et al*., 1996; Salvemini *et al., 2*001).

1.4 FEVER A SYSTEMIC MANIFESTATION OF INFLAMMATION

When infectious microorganisms invade the body through its natural barriers, a range of systemic reactions swiftly develops that alleviates the injurious effects of the invading pathogens and, eventually, restores health. These reactions represent the primary host

defense response to infection; collectively, called the "acute-phase reaction." Fever is the most manifest and recognizable among these early responses; and it closely associated with the defensive role of inflammation. (Blatteis, 1992) The conventional view of the mechanism by which infectious fevers are produced postulates that infectious noxa (e.g., Gram-negative bacteria and/or their products bacterial endotoxic lipopolysaccharides, LPS that plague the body activate mononuclear phagocytes that in turn produce and release pyrogenic cytokines. TNF-α, IL-1β, and IL-6 are considered to be the main pyrogenic cytokines. These, are then transported through the bloodstream to the ventromedial preoptic area, VMPO of the anterior hypothalamus, the ‘‘fever producing center’’, to effect a cascade of events. (Saper, 1998; Roth and De Souza, 2001; Dunn, 2002; Dinarello, 2004). However, these activated cytokines are hydrophilic in nature therefore, direct penetration into the brain is obscured but induces the local generation and release of prostaglandin PGE2, a lipid mediator that is evidently thermogenic when injected centrally (Blatteis, 1997; Ivanov and Romanovsky, 2004). The synthesis of prostaglandin in this scenario has been established to be reliant on the activation of two enzymes, cyclooxygenase (COX)-2 and microsomal PGE synthase (mPGES)-1, which catalyze its conversion from arachidonic acid (AA) present in the membranes of cells (Ivanov *et al.*, 2002). The key manifestation of fever is elevated body temperature; usually by 1-4 ˚C.While the behavioral component include shivering, chills, anorexia, and malaise.

1.5 EXPERIMENTAL MODELS OF ACUTE INFLAMMATION

The common methods used for screening of anti-inflammatory drugs, operates on the basis of the ability of the test agents to inhibit the edema produced in the hind paw of the

experimental animals after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, which include brewer's yeasts (Opas *et al.,* 1987), formaldehyde (Brownlee**,** 1950), dextran (Nasjleti *et al.,* 1984), egg albumin (Nwafor *et al.,* 2007), kaolin (Lewis *et al.,* 1976) sulfated polysaccharides like carrageenan (Winter *et al.,* 1962). In the induction of edema, histamine (Amann *et al,* 1995), xylene (Akindele and Adeyemi, 2007), arachidonic acid (DiMartino *et al.,* 1987), phorbol myristate acetate (Hernández *et al.,* 2009), oxozolone and croton oil (Blazsó *et al.,* 1999) are also used.

Among these methods the most effective and widely used model for inflammation is carrageenan-induced paw edema, Carrageenan is a mixture of polysaccharides composed of sulfated galactose units and is derived from Irish Sea moss, *Chondrous crispus*. Its use as an edemogen was introduced by Winter and Co. Carrageenan initially releases histamine and serotonin followed by release of prostaglandins, protease and lysosomes producing edema.

1.6. EXPERIMENTAL MODELS OF SUB ACUTE INFLAMMATION

Carrageenan induced granuloma pouch model is an outstanding sub acute inflammatory model. The air pouch has the benefit of supplying a suitable space for the induction of inflammatory responses. The injection of irritants, carrageenan into subcutaneous air pouch on the dorsal surface of rats initiates the inflammatory process (Selye, 1953)

Another model is the use of formalin challenge. The nociceptive effect of formalin is biphasic, an early neurogenic component followed by a later tissue-mediated response. In

phase one there is release of histamine, 5-HT and kinin, while phase two is related to the release of prostaglandins (Turner, 1965).

1.7 EXPERIMENTAL MODELS OF CHRONIC INFLAMMATION

The two main models commonly used in chronic inflammatory studies are the rheumatoid arthritic (RA) and inflammatory bowel disease (IBD) models. Some of the common models are;

1.7.1 Collagen-Induced Arthritis (CIA)

This is a standard animal model for evaluation of anti-arthritic activity. The model is about immunization with bovine collagen to develop antibodies against bone and cartilage. The effect which span for average duration of 45 days usually can be studied using mice or rats for both therapeutic and prophylactic reasons. Assessment is based on estimation of arthritic indices, measurement of body weight, paw volume, histopathology, cytokines and collagen titers (Stuart and Dixon, 1983; Wooley *et al*, 1984).

1.7.2 Pristane-Induced Arthritis (PIA)

This a non-immunogenic model that most closely simulates the autoimmune rheumatoid arthritis condition in humans. The study period which take 30 days and recommended for for prophylactic or therapeutic intervention studies. It can be studied in rats (Vingsbo *et al.*, 1996).

1.7.3 Adjuvant-Induced Arthritis (AIA)

This is an economical *in vivo* screening method; one of the longest established animal models for testing arthritis treatments agents. It takes an average study period of 30 days when using this model. Assessment is based on arthritic indices estimation, measurements of body weight, paw volume, histopathological and radiolodical studies. Lewis, Sprague-Dawley (SD), Wister and Brown Norway (BN) strains rats are known as high responders to AIA, while Buffalo (BUF), Fisher 344 (F344) and diabetic resistant subline of diabetic BB (DRBB) rats are medium to low responders to AIA (Pearson, 1956; Kohashi *et al.*, 1977; Cremer, 1990).

1.7.4 Ovalbumin-Induced Arthritis (OIA)

This model offers the advantage of synovial fluid measurement at convenient volumes. Within a 30 to 60 day period the knee width of rodents mostly rabbits are measured. Histopathological studies can also be conducted to ascertain the disease state (Yoshino and Yoshino, 1992).

1.7.5 Dextran Sodium Sulphate (DSS)-Induced Inflammatory Bowel Disease

Human inflammatory bowel disease (IBD) is a chronic, relapsing and remitting inflammatory condition characterized by two overlapping phenotypes — ulcerative colitis (UC) and Crohn's disease (CD). The dextran sulfate sodium (DSS) colitis model is one of the most frequently used rodent IBD models. Mice that are exposed to DSS in drinking water will develop an inflammation of the colon, displaying symptoms such as diarrhea,

rectal bleeding, and weight loss. Acute colitis can be induced by single cycle of DSS exposure to the mice whereas multiple cycles of DSS administration will lead to chronic colitis (Hoffmann *et al.*, 2002).

1.8 EXPERIMENTAL MODELS OF FEVER

Fever is a non –specific clinical manifestation associated with various pathophysiological conditions such as inflammation (Kluger, 1991). The common experimental models used in antipyretic activity studies include subcautaneous injection of brewers yeast (Niemegeers *et al.*, 1975; Smith and Hambourger, 1935) and Lipopolysacchride, LPS (Santos and Rao, 1998.).

1.9 SAFETY ASSESSMENT

Although natural products based pharmaceuticals have been perceived by the public as relatively low risk, there has been more recognition of the potential risks associated with this type of product as the use of these products increases. This has been attributed to the innate toxicity of the plant, as well as from contamination, adulteration, plant misidentification, and interactions with other herbal products or pharmaceuticals The safety of herbal medicines has therefore, become a major concern to both national health authorities and the general public.(Jordan *et al.*, 2010; Dog *et al.*, 2010). In the wake of these concerns animal tests for toxicity are conducted prior to human clinical investigations as part of the non-clinical laboratory tests of pharmaceuticals. These results often represent the only means by which toxicity in humans can be effectively predicted. Standardized assessment procedures have been developed for the following effects:

1.9.1 Acute Toxicity

Acute toxicity tests are generally the initial tests conducted. They provide data on the relative toxicity likely to occur from a single or short exposure. In this protocol three dose levels are usually used and exposures are single doses or fractionated doses up to 24 hours and thereafter observed for signs toxicity daily for 14 day. Observation include evaluation of skin and fur, lethargy, sedation, rhinorrhoea, diarrhoea etc. (Walum, 1998)

1.9.2 Subchronic Toxicity

Subchronic toxicity tests are employed to determine toxicity likely to arise from repeated exposures of several weeks to several months. Detailed clinical observations and pathology examinations are conducted. In this procedure 10 of each sex of rodents are used. The animals are usually treated for a 90 day period (Dirikou *et al.,* 2011).

1.9.3 Chronic Toxicity

Chronic toxicity tests determine toxicity from exposure for a substantial portion of a subject's life. They are similar to the subchronic tests except that they stretch over an extended period of time and involve larger groups of animals, 20 of each sex for rodents over an exposure period of 12 month (OECD, 2009).

1.9.4 Carcinogenicity

Carcinogenicity tests are similar to chronic toxicity tests. However, they extend over a longer period of time and require larger groups of animals in order to assess the potential for cancer. It involves 50 of each sex per dose level. Exposure period last a minimum of 18 months for mice and 24 months for rats (Friedrich and Olejniczak, 2011).

1.9.5 Reproductive Toxicity

Reproductive toxicity testing is intended to determine the effects of substances on gonadal function, conception, birth, and the growth and development of the offspring. The oral route is preferred. It also requires large animals size per group, 20 of each sex per dose levels. The test substance is given to the parental animals (P1) before mating, during pregnancy and through weaning of F1 generation, until the F2 generation is 21 days old (Lamb, 1985).

1.9.6 Developmental Toxicity

Developmental toxicity testing detects the potential for substances to produce embryotoxicity and birth defects. This studies organ development in the fetus for teratogenic effects starting with parents prior to bleeding, and continues through pregnancy for all developmental effects. The offspring are sacrificed and assessed a day to the expected birth for teratogenic effects, growth retardation and abnormal function through infancy (Makris *et al.*, 2011).

1.9.7 Dermal Toxicity

Dermal toxicity tests determine the potential for an agent to cause irritation and inflammation of the skin. This may be the result of direct damage to the skin cells by the substance. It may also be an indirect response due to sensitization from previous exposure. There are two dermal toxicity tests, which are primary dermal irritation which predicts direct toxicity and dermal sensitization which assesses immune hypersensitivity (Vogel *et al.*, 1998).

1.9.8 Ocular Toxicity

Ocular toxicity is determined by applying a test substance for one second to the eyes of 6 test animals, mostly rabbits. The eyes are then carefully examined for 72-hours, using a magnifying instrument to detect minor effects. The ocular reaction may occur on the cornea, conjunctiva, or iris. It may be simple irritation that is reversible and quickly disappears or the irritation may be severe and produce corrosion, an irreversible condition. This referred to as the Draize's test (Green, 1992).

1.9.9 Neurotoxicity

Neurotoxicology is the study of the adverse effects of chemical, biological, and certain physical agents on the nervous system and/or behavior during development and in maturity" (Harry *et al.,* 1998). Many common substances are neurotoxic, including lead, mercury, some pesticides, and ethanol**.**

Neurotoxicity testing is used to identify potential neurotoxic substances. Neurotoxicity is a major toxicity endpoint that must be evaluated for many regulatory applications. Sometimes neurotoxicity testing is considered as a component of target organ toxicity; the central nervous system being one of the major target organ systems. *In utero* exposure to chemicals and drugs can also exert an adverse effect on the development of the nervous system, which is called developmental neurotoxicity.

Like other target organ toxicities, neurotoxicity can result from different types of exposure to a substance; the major routes of exposure are oral, dermal, or inhalation. Neurotoxicity may be observed after a single (acute) dose or after repeated (chronic) dosing. (Crofton *et al.,* 2011).

1.9.10 Genetic Toxicity

Genetic toxicity is determined using a wide range of test species including whole animals and plants (e.g., rodents, insects, and corn), microorganisms, and mammalian cells. A large variety of tests have been developed to measure gene mutations, chromosome changes, and DNA activity (Tice *et al.,* 2000)

1.10 THE USE OF TRADITIONAL MEDICINE

Traditional medicines, including herbal medicines, have been, and continue to be, used in every country around the world in some faculty. In much of the developing world including Asia, Africa, Latin America and the Middle East, 70–95% of the population relies on these traditional medicines for primary care (WHO, 2002). The situation is not different from developed nations of Canada, France, Germany and Italy where available data indicates that some 70% to 90% of their populations use traditional medicines under various nomenclature such as "complementary", "alternative", or "nonconventional" (WHO, 2002 WHO, 2008, Barnes *et al*.,2008). The global market for traditional medicines was estimated at US$ 83 billion annually in 2008, with a suggested rate of growth in recent years amounting to approximately between 5% and 18% per annum (Calixto, 2000; Molly Meri Robinson and Xiaorui Zhang, 2011). This great dependence on traditional medicine is mainly due to accessibility, affordability, acceptance (Patwardhan, 2005) and the motivation that plants are potential source of remedies.

Again, an estimated 25% of all modern medicines are derived, either directly or indirectly, from medicinal plants, largely through the application of modern technology to traditional knowledge. As there are about 500 000 plant species occurring worldwide, of which barely 1% has been phytochemically scrutinized, there is great potential for discovering novel bioactive compounds (Palombo, 2006). In certain cases this projection for medications

derived from medicinal plants is as high as 60% for antitumoral and antimicrobial agents (Sucher and Carles, 2008; Holtz, 2007).

Even though there is growing recognition among policy-makers of the possible use of traditional medicine for the effective treatment or control of certain diagnosed conditions at the international front as one way to reduce the health care burden on the public budget (WHO, 1998), the belief that everything that is natural is safe; the lack of scientific validation and insufficient consumer comprehension remains a major setback (Calixto, 2000; Zhang *et al*, 2008). Research on safety and efficacy is therefore, of prime importance to the continued advancement of traditional medicines in this regard.

1.11 *Pistia stratiotes*

Pistia stratiotes (Family: Araceae), commonly known as Water lettuce, Water cabbage, Tropical duckweed, is one such aquatic flora invading most resourceful water bodies in the tropical and subtropical region of Asia, Africa, and America.(Arber, 2002).

1.11.1 Botanical Description

Pistia stratiotes Linn, (Fig. 3) is a perennial aquatic herb with suckers attached by stolons forming into a free floating rug; rarely anchored by its roots and often blown by wind. It is of symposia structure, but internodes remain short and bear a rosette of large leaves. Leaves are tinged yellowish - green, pubescent, obovate to spathlulate, coatate, up to 15cm long. The flowers are pale green or white; inflorescence is small and bisexual; above it is a

whorl of male flower, each with a synandrium of two stamens. The fruits are small green capsules (Arber, 2002).

Figure 4: *Pistia stratiotes* plants in their natural habitat

1.11.2 Ecology and geographical distribution of *P. stratiotes*

In Ghana, the plant is fast invading most water bodies notable among them is the Fosu lagoon in cape coast in the central region where the plant sample were collected for this study. It is found in Ashanti, Central, Western and Volta regions of Ghana. The plant thrives best in sewage polluted environment and in lime-rich water (Chadha, 1998).

Figure 5: An Adapted Screenshot from Google Earth Showing the Fosu Lagoon in Cape Coast, Central Region, Ghana

1.11.3 Uses of *P. stratiotes*

1.11.3.1 Non-Medicinal Uses

As a source of Food, *P. stratiotes is* fed to ducks, pigs and cattle. It has been used as source of food elsewhere in India by humans during famine (Sculthorpe, 1971). A scientific analysis of leaves and stems revealed the following: moisture 92.9%, protein 1.4%, fat 0.3%, carbohydrate 2.6%, fibers 0.9%, ash 1.9%, calcium 0.2%, phosphorus 0.06%. Leaves are rich in vitamin A and C, and also contain vitamin B (Khare, 2005).

Again, a number of organisms including mollusks and fish, mosses, perihyton and vascular plants has been reported to be used as bioindicators of environmental pollution because of the relatively low cost implication, reliability and simplicity when employed (Zurayk *et al.*, 2001) . Among these vascular plants is *P. stratiotes.* A research report which assessed the effectiveness of three aquatic plants; *P. stratiotes* L. (water lettuce), *Spirodela polyrrhiza* W. Koch (duckweed), and *Eichhornia crassipes* for the removal of five heavy metals (Fe, Zn, Cu, Cr, and Cd) at concentrations (1.0, 2.0, and 5.0 mg 1^{-1}) of metals in laboratory experiment showed *P. stratiotes* as the second to *E. crassipes* in efficiency in removing the selected heavy metals Results from analysis confirmed the accumulation of different metals within the plant and a corresponding decrease of metals in the water without the production of any toxicity or reduction in growth of the plant (Mishra andTripathi, 2008).

1.11.3.2 Medicinal uses

Methanolic extract of the plant *P. stratiotes* possesses antidermatophytic effect against *Tricophyton rubrum, Microsporum gypseum,* and *Epidermophyton floccosum* but inhibited *M. gypseum* at a much lower concentration than *Trichophyton* and *Epidermophyton* spp (Prem kumar and Shyamsundar, 2005).

P. stratiotes leaves possess antifungal properties which explain the use of plant in folk medicines for the treatment of various diseases such as ring worm. (Prem kumar and Shyamsundar, 2005)

Pharmacological activities of *P. stratiotes* were studied and Ca channel blocking activity of methanolic extract of a whole plant was demonstrated using isolated segments of rabbit jejunum and confirmed via inhibition by pretreatment with verapamil. The result showed that plant extract caused a decrease in blood pressure in anesthetized rat. It has also been demonstrated that methanolic extract of *P. stratiotes* showed stronger and broader spectrum of antimicrobial activity as compared to hexane extract. Again extract of *P. stratiotes* has been proven to lower the level of thyroid hormones, therefore the deduction that that plant extract may regulate hyperthyroidism (Achola and Indalo, 1997)

1.12 AIMS AND OBJECTIVES OF THE STUDY

The objective of this study is to evaluate the anti-inflammatory effect and anti-pyretic effect of aqueous and ethanolic leaf extracts of *P. stratiotes* in addition to assessment of its safety for use using animal models.

1.12.1 Specific objectives involve:

1. Evaluation of the anti-inflammatory activity of the extracts using:
    a. Carrageenan – induced paw edema in rats as a model of acute inflammation
    b. Formaldehyde –induced arthritis as a model of subacute inflammation
    c. Adjuvant-induced arthritis in rats as a model of chronic inflammation

2. To predict the possible mechanism of action of its acute anti-inflammatory activity using:
    a. Histamine as a mediator-induced model of inflammation

b. Serotonin as a mediator-induced model of inflammation

c. Prostaglandin as a mediator-induced model of inflammation

d. Bradykinin as a mediator-induced model of inflammation

3. Evaluation of its ocular anti-inflammatory effect using

    a. Endotoxin –induced uveitis model in rats

4. Evaluation of its antipyretic effect using:

    a. Lipopolysaccharide –induced fever model

5. Safety assessment using:

    a. Acute and delayed toxicity studies

    b. Ocular safety assessment model.

1.13 JUSTIFICATION OF THE STUDY

There are only 1,439 health care facilities to serve an estimated population of 24,000,000 people in Ghana (IRIN, 2008). This has been a major problem to access to healthcare and is further compounded by uneven distribution of these facilities across the country, with most rural folks having to travel an average of 16km to consult a doctor even though half of the population mainly urban dwellers live within a 5 km radius (Van den Boom *et al.*, 2004).

Again, despite progress made in the fight against extreme poverty well ahead of the target date of 2015, from 51.7% in 1991/92 to 28.5% in 2005/2006 at the national level the situation is said persist as high as 70% in the three northern region of Ghana (Ghana MDG

Report, 2010). This issue of poverty leaves many with no option than to practice self-medication with traditional medicine without recourse to safety consideration.

Besides, those who have the means to afford orthodox medicine to manage or treat their ailments most of which have inflammatory element as part of their pathophysiology (Serhan, 2004) have had to bear with serious side effects (Lin *et al.*, 2006; Ehsanulla *et al.*, 1988). Traditional health care, which is the oldest medical system in the country, has once again become the initial avenue of accessing care for some 70% of the Ghanaian population (WHO, 2001). The search for alternative treatment for inflammatory disorder has been the preoccupation of scientists for some time now since the conventional treatment involving steroidal and non-steroidal anti-inflammatory drugs are not without issues. *Pistia stratiotes* is reported in Ghana as useful in the treatment of inflammatory disorders (Abbiw, 1990). There is a dearth of scientific proof to validate this claim and its safety for use.

Traditional health care is effective, cost-effective, culturally accepted, and have consistently been argued as an effective means that can aid and complement governments's efforts at ensuring equitable health care. This study is therefore significant as it seek to provide scientific basis for the traditional use of *P. stratiotes* in treatment of inflammatory disorder and to assess its safety for use.

CHAPTER TWO

MATERIALS AND METHODS

This chapter describes the method and materials used in this study which include plant collection of leaf samples, animal husbandry practice, phytochemical screening and the various methods employed in the assessment of the anti-inflammatory property, antipyretic and safety evaluation of the extracts.

2.1 PLANT COLLECTION

Pistia stratiotes was collected from the Fosu lagoon, Cape Coast in the Central Region of Ghana (5°7′ N &1°16′ W) in December 2010. It was identified and authenticated by Mr. G H Sam of the Department of Herbal Medicine, CHS, KNUST, where a voucher specimen bearing the number KNUST/ HM1/11/W002 has been deposited at the herbarium for future reference.

2.2 PREPARATION OF EXTRACTS

The leaves of *P. stratiotes* were washed thoroughly with tap water and sun-dried. The dry leaves were milled into coarse powder using hammer mill (Schutte Buffalo, New York, USA). In preparing the aqueous leaf extract of *P. stratiotes,* 700 g of the leaf powder was mixed with 1litre of water. The mixture was maintained at 80 ^{o}C (in a round bottom flask fitted with a reflux condenser) in a thermostatically controlled water bath for 24 h and then

allowed to cool before filtering. The filtrate was freeze dried with a Hull freeze dryer /lyophilizer 140 sq ft (model 140FS275C, USA) into powder (percentage yield 4.7%) and stored at a temperature of 4 ^{o}C in a refrigerator. This powder was reconstituted in normal saline to a desired concentration and labeled as AQ PSE for dosing in this study. Similarly, 700 g of the leaf powder was soaked with one liter of 70 % ethanol at room temperature (27-29 ^{o}C) for 72 h and filtered. The filtrate obtained was freeze-dried into powder (percentage yield 5.2 %). Quantities of this powder was reconstituted in normal saline at desired concentrations to be referred to and used in this study as the ethanolic leaf extract of *P. stratiotes* or ET PSE.

2.3 ANIMAL AND HUSBANDRY

2.3.1 Rats

Six to eight-week old Sprague Dawley rats of either sex (180-200 g) purchased from the Centre for Scientific Research into Plant Medicine (CSIRPM), Mampong-Akwapim, Ghana, were maintained in the Animal House of Department of Pharmacology, KNUST, Ghana. The animals were housed in polyacrylic cages (34cm × 47cm× 18cm) with soft wood shaving as bedding, under ambient laboratory conditions (temperature 28 ± 2^{o}C, relative humidity 60-70 %, and normal light-dark cycle). Females were non-pregnant. They were fed with normal commercial pellet diet (GAFCO, Tema) water *ad libitum.* All procedures and techniques used in these studies were in accordance with the National Institute of Health for the Care and Use of Laboratory Animals (NIH, Department of

Health and Human Services publication no. 85-23, revised 1985). The protocols for the study were approved by the Departmental Ethics Committee.

2.3.2 Rabbits

New Zealand white rabbits of either sex (2.3-3.0 kg) purchased from the Okyere farms Nakanfoa, Cape Coast, Ghana, was maintained in the Animal House of Department of Pharmacology, KNUST, Ghana. The animals were housed in polyacrylic cages (50cm × 64cm× 30cm) with soft wood shaving as bedding, under ambient laboratory conditions (temperature 28 ± 2^{o}C, relative humidity 60-70 %, and normal light-dark cycle). Females were non-pregnant. They were fed with normal commercial pellet diet (GAFCO, Tema) water *ad libitum.*

2.4 DRUGS AND CHEMICALS

Carrageenan, histamine, serotonin-creatinine sulphate complex (Sigma and Aldrich, St. Louis MO, USA), prostaglandin E_2 and bradykinin (Sigma Life Science, USA). Diclofenac sodium (KRKA, d.d., Novo mesto, Solvenia), a COX inhibitor, chlorpheniramine (Pharmanova, Accra), a H_1 -receptor antagonist, granisetron (Corepharma LLC, Middlesex, USA), a 5-HT_3-receptor antagonist. Formaldehyde (BDH, Poole, England), dexamethasone sodium (Anhui Medihel Co. Ltd), methotrexate sodium (Dabur Pharma, New Delhi, India).

Mycobacterium tuberculosis [strains C, DT and PN (mixed) obtained from the Ministry of Agriculture, Fisheries and Food, UK] , paraffin oil (Ernest Chemist, Accra, Ghana) LPS

from *Escherichia coli* (Axxora, LLC, San Diego, USA) was used to induce uveitis and pyrexia. BCA Protein Assay Reagent kit (Pierce, Rockford, IL. USA) and Prednisolone (Letap Pharmaceuticals Ltd., Accra, Ghana). Ketamine Hydrochloride (Fabriqué par Laborario Sanderson S.A, Chile) and Normal Saline Solution (Intravenous Infusions Ltd., Ghana), dragendorff's reagent (Sigma Aldrick, Canada), mayer's reagent (Sigma Aldrich, USA), chloroform (Daga Global Company Ltd, India), fehling's solution (LP Chemicals, UK).

2.5 PHYTOCHEMICAL SCREENING OF MAJOR METABOLITES

Screening was performed on AQ PSE and ET PSE to ascertain the presence of phytochemicals using standard procedures described by Wagner and Bladt (1996), Glasl (1983), Harborne (1998), Kujur *et al.*, (2010) and Sofowora, 1993.

2.5.1 Test for Alkaloids

To 0.5 g each of AQ PSE and ET PSE, 5 ml of 1 % aqueous hydrochloric acid (HCl) was added and stirred. The solutions were warmed and filtered. A 1 ml sample each of the filtrate was transferred into two test tubes. To one of the test tubes was added a few drops of Mayer's reagent, to the other test tube was added a few drops of Dragendorff's reagent and observed. An orange precipitate indicates the presence of alkaloid.

2.5.2 Test for Tannins

A 0.5 g each of AQ PSE and ET PSE, was boiled with 10 ml of distilled water for 5 minutes, hot-filtered and allowed to cool under room temperature. The filtrate was diluted to 20 ml with distilled water. A 1 ml of the diluted solution was further diluted with distilled water to 5 ml, after which a few drops of 0.1 % ferric chloride solution was added and observations made for intense greenish colour precipitate.

2.5.3 Test for Sterols

A mass of 0.5 g each of AQ PSE and ET PSE was dissolved in 2 ml chloroform in a test tube. Concentrated sulphuric acid was carefully added down the inner sides of test tube which formed a lower layer. The appearance of a reddish – brown colour at the interface was the observed indicator for the presence of steroids.

2.5.4 Test for Saponins

A carefully weighed 0.5g each of AQ PSE and ET PSE was shaken with a 5 ml of water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing.

2.5.5 Test for Glycosides

Five militer of diluted sulphuric acid was added to 0.5 mg of the sample. 2 ml of 20% NaOH was added followed by addition of few drops each of Fehling's solutions A and B.

The mixture was warmed on water bath for two minutes. The formation of a brick-red precipitate was observed as an indicator observed of glycosides.

2.5.6 Test for Terpenoids (Salkowski test)

The detection of terpenoids was made by adding 0.5 mg of each the sample to 2 ml of chloroform in a test tube followed by addition of 1 ml of concentrated sulphuric acid. The reddish-brown coloration was the indicator observed for the presence of terpenoids.

2.5.7 Test for Flavonoids

Five mililiter of ethyl acetate was added to 0.5 mg of the sample in a test tube and warmed. Few drops of dilute ammonia solution were added to the mixture. Appearance of yellowish colour at the bottom of the test tube is indicative of flavonoids.

2.6. CARRAGEENAN- INDUCED PAW EDEMA IN RATS

The experimental animals (8 groups, n=5) were fasted 24 hours prior to induction of edema, water was however available *ad libitum*. Paw edema (acute inflammation) was induced by sub-plantar injection of 0.2 mg of carrageenan in distilled water into the right hind paws of the animals (Winter *et al.*, 1962; Ramesh *et al.*, 2010; Ratnasooriya and Fernando, 2009). They were then put into eight groups of five. Paw edema (expressed as an increase in paw volume) was measured with an electronic von frey plethysmometer (IITC Life Science Inc., California, USA). Each group was treated with one of these; AQ

PSE (30, 100, or 300mg/kg, *per os*), ET PSE (30, 100, or 300mg/kg, *per os*), Diclofenac sodium (0.93 mg/kg, i.p). Paw edema was measured again 1, 2, and 3 hours post-treatment. The control group received distilled water (1 ml/kg, *per os*). Animals were deprived of water during the experimental period to ensure uniform hydration and reduce variability in edematous response.. Percentage changes in paw volume were calculated and recorded using the formula:

$$V = \left(\frac{Vt - Vo}{Vo}\right) \times 100$$

Where V is the percentage change in paw volume, V_t is the paw volume (at different times) after carrageenan challenge and V_0 is the paw volume before carrageenan injection (i.e. time zero)

2.7 HISTAMINE –INDUCED PAW EDEMA

The experimental procedure was similar to that described in the carrageenan-induced paw edema model but paw edema was induced with 0.1 mg of histamine in distilled water (Mazumder *et al.*, 2003). The reference anti-inflammatory agent was chlorpheniramine (0.35mg/kg; *per os*). Each group was treated with either AQ PSE (30, 100, and 300 mg/kg, *per os*) or ET PSE (30, 100, and 300mg/kg, *per os*). The control group received distilled water (1 ml/kg, *per os*). Paw edema was measured 1, 2, and 3 hours post-treatment and the

percentage changes in paw volumes calculated and recorded as in the carrageenan paw edema model of inflammation.

2.8 SEROTONIN-INDUCED PAW EDEMA

The experimental procedure was similar to that described in the carrageenan-induced paw edema model but paw edema (acute inflammation) was induced with 0.1 mg of serotonin-creatinine sulphate complex in distilled water (Mazumder *et al.*, 2003). The reference anti-inflammatory agent was Granisetron (28.5 mcg/kg *per os*). Each group was treated with either AQ PSE (30, 100, and 300mg/kg, *per os*) or ET PSE (30, 100, and 300mg/kg, *per os*). The control group received distilled water (10 ml/kg, *per os*). Paw edema was measured 1, 2, and 3hours post-treatment and the percentage changes in paw volumes calculated and recorded as in the carrageenan paw edema model of inflammation.

2.9 PROSTAGLANDIN -INDUCED PAW EDEMA

Prostaglandin E_2, 0.2 ml (1nM) was administered into the sub-planter region of the right hind paw of rats, in accordance with the method of Willis and Cornelsen (1973). The paw volume up to the ankle joints were measured plethysmographically before and after 30 min of the prostaglandin E_2 stimulation. Each group was treated with either AQ PSE (30, 100, and 300mg/kg, *per os*), ET PSE (30, 100, and 300mg/kg, *per os*), or Diclofenac sodium (0.93 mg/kg, i.p). The control group received distilled water (1 ml/kg, *per os*). Paw edema was measured 30 minutes interval for 2.5 hours post-treatment and the percentage changes

in paw volumes calculated and recorded as in the carrageenan paw edema model of inflammation.

2.10 BRADYKININ -INDUCED PAW EDEMA

Sprague-Dawley rats were pretreated with captopril 1h before bradykinin injection (Marsha-Lyn *et al.,* 2002). The stock solutions for bradykinin were prepared in phosphate-buffered saline, PBS (1-10mM) in siliconized plastic tubes, maintained at 18°C just before use. Each animals was injected with 0.2ml of 10nmol per paw into the right hind paw 30 minutes after administering the extracts i.e AQ PSE (30, 100, and 300mg/kg, *per os*), ET PSE (30, 100, and 300mg/kg, *per os*) and 1 ml/kg, *per os* as vehicle. No selective antagonist was given in this model. Each group was treated with either r distilled water.

2.11 FORMALDEHYDE-INDUCED ARTHRITIS

The test was performed according to the technique developed by Brownlee in 1950. Pedal inflammation was induced by injecting 0.1 ml of 4 % formalin solution below the plantar aponeurosis of the right hind paw of the rats after measuring their paw thickness. The arthritic animals were divided into ten groups of five and treated with either 30, 100, or 300 mg/kg AQ PSE or ET PSE, orally (*per os*, p.o, 30 minutes after intra-plantar injection with formalin on day 1, and then daily), 0.3 mg/kg methotrexate intraperitoneally (i.p every four days), 0.46 mg/kg diclofenac (i.p, daily), 1 mg/kg dexamethasone (i.p, every other day), 1 ml/kg normal saline (p.o, daily), the control, over the experimental period. Percentage changes in paw volume were calculated and recorded using the formula:

$$V = \left(\frac{Vt - Vo}{Vo}\right) \times 100$$

Where V is the percentage change in paw volume, V_t is the paw volume (at different times) after formalin challenge and v_o is the paw volume before formalin injection (i.e. time zero).

2.12 ADJUVANT-INDUCED ARTHRITIS

Arthritis was induced as previously described by Pearson (1956), with slight modification. In this process the initial hind paw volumes (both left and right) of experimental animals were measured by water displacement plethysmography (Fereidoni *et al.*, 2000).The right hind paw of each animal was then injected with 0.1 ml of complete freud adjuvant, CFA. The paw volumes for both the injected paw (ipsilateral) and the non-injected paw (contralateral) were measured on day 1 after injection into the paw, and every other day. A unilateral inflammatory edema of the ipsilateral paw peaking around days 4-6 was indicative of successful induction of adjuvant arthritis.

2.12.1 Experimental Procedure

Nine days after adjuvant-induced arthritis, animals were put into ten groups (A-J) of five and were randomly treated with either 30, 100, or 300 mg/kg of AQ PSE or ET PSE (p.o, daily), 0.3 mg/kg methotrexate (i.p, every 4days), 0.43 mg/kg diclofenac (i.p, daily), 1 mg/kg dexamethasone (i.p, every other day), 1 ml/kg normal saline (p.o, daily) over the

experimental period. A non-arthritic control group (consisting of five animals in which an incomplete arthritis was induced by intra-plantar injection of 0.1ml of sterile paraffin oil (incomplete Freund's adjuvant- IFA) and a normal control group in which there was no induction of arthritis were also studied. The effect of the reference and the tests drugs on the edema component of arthritis was quantified by measuring the difference in paw volume prior to induction of arthritis (day 0) and that at the various time points up to day 27. The arthritic index for ipsilateral and contralateral paw volumes were individually calculated and expressed as percentage change with respect to values at day 0, and then averaged for each treatment group.

Initial body weights of the rats were recorded at day 0 and on day 28. On day 28, the X-ray imaging was performed using a Philips Conventional X-ray machine (Germany) and industrial X-ray film (Fuji Photo Film, Tokyo, Japan) at the radiology unit of the KNUST Hospital. The animals were anesthetized with ketamine hydrochloride. The X-ray apparatus was operated at a 52-kV peak and 10-s exposure with a 45-cm tube-to-film distance for lateral projections.

The rats were sacrificed and blood samples were collected into MediPlus K3 EDTA sample tubes (Sunphoria Co. Ltd., Taiwan) for hematological analysis using the KX-21 N Automated Hematology Analyzer (Sysmex Corporation, Chuo-ku, Kobe, Japan) and Trisodium citrate ESR tubes (Chengdu Rich Science Industry Co., Ltd. China) for estimation of erythrocyte sedimentation rate by the method of Westergren (David and Sykes, 1951). Blood was also collected into glass tubes and centrifuged (temperature:

25°C, speed: 4000 *g*) for 5 minutes using the Mikro 220R [HettichZentrifuge, USA] to obtain the plasma and used to estimate the C-reactive protein levels using the ELISA kit (Fortress Diagnostic Ltd., UK)

After 28 days the right and left hind paws of the animals were amputated and fixed in 4% phosphate-buffered paraformaldehyde, decalcified with 1% EDTA and embedded in paraffin. Sections were stained with hematoxylin –eosin, (Hultqvist *et al.,* 2006; Liu *et al.,* 2009) and fixed on glass slides for microscopic examination at the Pathology Department of the KomfoAnokye Teaching Hospital. Data obtained for the arthritic and radiological indices as well as the histopathological, hematological, and physical profiles were analyzed.

2.12.2 Arthritic Index

Photographs of arthritic rats were taken on day 28 with a camcorder (Everio™ model GZ-MG1300, JVC, Tokyo, Japan). Inflammation in each paw was graded blindly by the same person in all rats on day 28 according to the extent of erythema and edema of the periarticular tissues, using a scale of 0-4 (Table 1).

Table 1: The grading scale for "Arthritic Index"

Score	Description
0	No inflammation
1	Unequivocal inflammation of one joint of the paw
2	Unequivocal inflammation of at least two joints of the paw or moderate inflammation of one joint
3	Severe inflammation of one or more joints
4	Maximum inflammation of one or more joints in the paw

(Kinne *et al.*, 1995; Zhao *et al.*, 2000)

The arthritis score of each rat on day 0 was determined to be 0.The scores for each paw were then added to get the total arthritis score (maximum possible score 16 per animal as the maximum score is 4 per paw) designated "*Arthritic Index*".

2.12.3 Radiological Index

Using the radiographs, the severity of bone and joint destruction was scored blindly by the same person for each hind limb according to the method described by Hoffman *et al*., (1997). Briefly, radiographic scoring was performed by assessing soft tissue swelling,

periosteal new bone formation, joint space narrowing, periarticular osteoporosis and bone destruction on a scale of 0 (normal) to 3 (maximum) per hind limb. The maximum radiographic score was 6 per animal. The radiological score for normal control rats was determined to be 0. The radiological score was termed the radiological index.

2.13 LIPOPOLYSACCHARIDE-INDUCED FEVER

The method of Santos and Rao (1998) was modified and used for the assessment of the anti-pyretic activity of the aqueous and ethanolic extracts of *P. stratiotes*. Animals were fasted overnight prior to induction of fever, but given water *ad libitum*. Rectal temperature was measured using a lubricated ECT-1 digital thermometer (Estar Electronic And Instrument Co., Ltd., ZheJiang, China) inserted 3cm deep into the rectum of the rats. Fever was induced by injecting intramuscularly, 1 mg/kg of LPS into the right thigh of each rat. Rectal temperature was measured again 2 hour latter and animals that showed an increase in temperature of 0.5 ºC and more were selected for the study. The animals with fever were put into eight groups of five and were treated with either 30, 100, or 300 mg/kg AQ PSE or ET PSE, 150 mg/kg acetaminophen, or 1 ml/kg normal saline solution (the control), orally, two hours after LPS-induced fever. Rectal temperature was measured at 1 h intervals for 6h. All experiments were carried out between 08.00 h and 18.00 h in a quiet laboratory with an ambient temperature of 25 ± 2ºC.

2.14 OCULAR ANTI-INFLAMMATORY EFFECT

2.14.1 Pyrexia, Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP) Determination

After baseline body temperatures taken from the rectum of Sprague-Dawley rats were recorded, they were inoculated with 200 µg LPS intraplantarly (100 µg per hind footpad). Two hours after LPS inoculation the body temperatures were again recorded. Six of the rats that showed an increase in body temperature of 0.5 ºC and more were euthanatized and blood samples were collected into trisodium citrate erythrocyte sedimentation rate tubes (Chengdu Rich Science Industry Co, Ltd, Sichuan, China) for estimation of erythrocyte sedimentation rate (ESR) using the Westergren method (David and Sykes, 1951). Blood was also collected from another six rats into glass tubes and centrifuged (temperature 25°C, speed 4000 *g*) for 5 minutes using a Mikro 220R Centrifuge (Hettich Zentrifuge, Tuttlingen, Germany) machine to obtain the plasma, which was used to estimate C-reactive protein (CRP) levels using an enzyme-linked immunosorbent assay kit according to the manufacturer's instruction. (Fortress Diagnostic Ltd, Antrim, Northern Ireland).

2.14.2 Endotoxin-induced Uveitic Rats

Rats which showed an increase in temperature of 0.5 ºC and more after 2 hours of LPS inoculation were selected and grouped into eight (n=6). They were randomized to treatment with 30, 100, or 300 mg/kg of AQ PSE or ET PSE, 30mg/kg Prednisolone, or 10 ml/kg of Normal (uveitic control) saline by oral gavage. A normal control group was kept under experimental condition with no LPS inoculation. After 24h the animals were

anesthetized with Ketamine HCl and the eyes assessed for clinical signs of vasodilation and exudation of proteins and inflammatory cells using an SL500 Shin Nippon Slit Lamp (Ajinomoto Trading Inc., Tokyo, Japan) fitted with a digital microscope camera (Olympus, Tokyo, Japan) to take photographs of the anterior chamber of the rats. The clinical score of inflammation was determined from the photographs using a scale of 0-4 (0, normal; 1, mild; 2, moderate; 3, severe; and 4, very severe (Karim *et al.*, 2009). The animals were euthanatized and the anterior chamber was punctured with a 30 gauge needle, and the aqueous humour was collected from both eyes (15 µl/rat).

2.14.3 Polymorphonuclear Neutrophil Count

A 1:10 dilution (Diluent: Turk solution) of the aqueous humour in the eye of the rats was introduced onto the counting chamber of the Improved Neubauer Haemocytometer (Depth 0.1mm, Area: 1/400 mm^2; Yancheng Cordial Lab Glassware Co. Ltd, Jiangsu, China (Mainland). Polymorphonuclear neutrophils were counted from the four large squares (volume: 0.4 mm^3) of the counting area using Ceti magnum-T/trinocular microscope for fluorescence (Medline Scientific limited, UK) under objective magnification of 40X. The number of PMNs was determined per mm^3 of aqueous humour (taking into consideration the dilution factor).

2.14.4 Estimation of Total Protein Concentration

A BCA protein assay kit (Pierce, Rockford, IL. USA) was used to establish the total protein concentration in the aqueous humour obtained from the enucleated rat eyes. The

protein concentration was determined by pipetting 10 µl of aqueous humour from; LPS inoculated rats treated with 30, 100, and 300 mg/kg of AQ PSE or ET PSE, 30 mg/kg prednisolone, 10 ml/kg normal saline, normal rats without LPS challenge, and standard bovine serum albumin (BSA) into a 96-well microplate. A 200 µl volume of working reagent, constituted according to the manufacturer's instructions, was mixed thoroughly with the content of each well and shaken for 30 seconds. It was then incubated at 37^{o}C for 30 minutes and allowed to cool to room temperature. Absorbances of the mixtures were measured at 562 nm using an ELx800 absorbance microplate reader (BioTek Instruments, Inc., USA). Each determination was in triplicate.

2.14.5 Histopatholgical Assessment

The enucleated eyes of the animals were fixed in 4% phosphate-buffered paraformaldehyde, and embedded in paraffin. Sections were made and stained with hematoxylin and eosin, (Kalariya *et al*., 2010) and fixed on glass slides for microscopic examination.

2.15 ACUTE AND DELAYED TOXICITY TEST

The rats were assigned to six treatment groups with five animals per group. The aqueous extract of *Pistia stratiotes* (AQ PSE) was administered at doses of 10, 30, 100, and 300 mg/kg. The control group (non-treatment group) was given 1 ml/kg distilled water. Observation for clinical and behavioral symptoms of toxicity such as sedation, lethergy, diarrhea, rhinorrhoea etc and mortality were made hourly for 24 hours and then daily

thereafter for 14 days. The time of onset, intensity, and duration of these symptoms, if any, was recorded.

Blood samples from the control and treated rats were collected into EDTA treated sample tubes before treatment, 24 hours after treatment, and then 10 days post-treatment and sent to the KNUST Hospital for hematological assessment using the BC-3000 Plus Auto hematology Analyzer (Mindray, Shenzhen, China). Hematological parameters measured.

A quantitative biochemical test was performed on fresh urine samples obtained from these rats in metabolic cages (Ugo Basile Biological Research Equipment, Comerio,Va., Italy) prior to treatment, 24 hours, and then 10 days post-treatment using H11-MA reagent strips for urinalysis (Durui, The Hague, Netherlands). Parameters measured were urobilinogen, bilirubin, ketone (acetoacetic acid), blood, protein, nitrite, leucocytes, glucose, specific gravity, pH, and microalbumin. The color, appearance and smell of the urine samples were also noted.

2.16 OCULAR SAFETY ASSESSMENT

Ocular safety assessment was performed using Draize test with slight modification (Draize *et al.*, 1944). A 0.5%, 1.0% and 2.0% solution w/v each of the freeze dried sample of the aqueous and ethanolic extracts were prepared using normal saline. Six groups each containing three of New Zealand white rabbits had only their right eye instilled with 0.1 ml of the extracts into the conjunctival sacs. This was after the pH of the solutions were determined to be5.70, 5.71, 5.60 and 5.27, 4.65, 4.75 for 0.5%, 1.0% and 2.0% AQ PSE

and ET PSE respectively. The pH was determined with digital pH meter (Tecpel Co. Ltd, Taiwan). The examined for conjunctival chemosis, redness, discharge, corneal abrasions, iris defects, and lens opacities and papillary reflexes using the head loupe and penlight which were scored using Draize as indicated in Table 2 below. The examination was done 1h, 24h up to the 72nd hour.

Table 2: Draize's Criteria for Determing Ocular Toxicity

LESION	**SCORE**
Cornea	
A. *Opacity – Degree of density (area which is most dense is taken for reading*	
Scattered or diffuse area – details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
B. *Area of cornea involved*	
One quarter (or less) but not zero	1
Greater than one quarter but less than one-half	2
Greater than one-half but less than three quarters	3
Greater than three quarters up to whole area	4
Score equals A x B x 5 Total maximum = 80	
Iris	
A. *Values*	
Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage; gross destruction (any one or all of these)	2
Score equals A x 5 Total possible maximum = 10	

Conjunctiva	
A. *Redness (refers to palpebral conjunctiva only)*	
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. *Chemosis*	
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. *Discharge*	
Any amount different from normal (does not include small amount observed in inner canthus of normal rabbits	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
Score equals (A + B + C) x 2 Total maximum = 20	

[1]From Draize et al. (1944). [2]Scores of 0 are assigned for each parameter if the cornea, iris, or conjunctiva is normal

2.17 STATISTICAL ANALYSIS

Total foot volume for each treatment was calculated as area under the time course curve (AUC). The equation below was used to determine the percentage inhibition of edema.

% inhibition of edema = $\left(\frac{\text{AUCcontrol} - \text{AUCtreatment}}{\text{AUC control}}\right) \times 100$

Differences in AUCs were analyzed by ANOVA followed by Dunnett's *post hoc test* using GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA). All values were expressed as mean ± s.e.m. $P < 0.05$ were considered statistically significant.

CHAPTER THREE

RESULTS

This chapter describes the results obtained after the various experimental procedures were employed.

3.0 QUALITATIVE TEST FOR MAJOR PLANT METABOLITES

Results for the initial phytochemical screening indicated that AQ PSE has tannins, flavonoids, alkaloids, sterols and glycosides. Saponins and triterpenoids were not present in the AQ PSE. ET PSE contains tannins, sterols, glycosides, and flavonoids but has no alkaloids, saponins and triterpenoids (Table 3).

Table 3: Results of phytochemical screening of the aqueous and ethanolic extracts of *P. stratiotes* Linn

	Tannins	Flavonoids	Alkaloids	Sterols	Glycosides	Saponins	Triterpenoids
AQPSE	+	+	+	+	+	-	-
ET PSE	+	+	-	+	+	-	-

"+" implies present, "-" implies absent

3.1 CARRAGEENAN-INDUCED EDEMA IN RATS

Injection of carrageenan resulted in acute inflammation (seen as paw edema) of the right hind paws of the rats within the first hour (Figures 5 and 6). Having treated the rats with the reference drugs and the extracts, it was observed then on that the 30 and 100 mg/kg doses showed significant effects ($P \leq 0.05$-0.01) in the carrageenan-induced inflammation. The 300 mg/kg doses showed no significant reduction ($P > 0.05$) in paw edema (Figure 5 and 6). The reference drug, diclofenac caused very significant reductions ($P \leq 0.001$-0.01). This observation signifies that both extracts have anti-inflammatory properties comparable to the reference drugs.

3.2 HISTAMINE-INDUCED EDEMA IN RATS

Injection of histamine resulted in acute inflammation (seen as paw edema) of the right hind paws of the rats within the first 30 minutes (Figures 7 and 8). After treatment with the reference drugs and the extracts, it was observed that the 30, 100 and 300 mg/kg AQ PSE and ET PSE treated groups had significant reduction ($P \leq 0.01$-0.001) in paw edema in the histamine (Figures 7 and 8). The selective reference antagonist used, chlopheniramine, caused significant reductions ($P \leq 0.001$).

3.3 SEROTONIN-INDUCED EDEMA IN RATS

After serotonin challenge an acute inflammation (seen as paw edema) of the right hind paws of the rats within the first 30 minutes was noted (Figures 9 and 10). After treating the rats with the reference drugs and the extracts, a similar trend as in the case of carrageenan-induced inflammation was made. The 30 and 100 mg/kg doses showed significant effects ($P \leq 0.05$-0.01) and not the 300mg/kg dose of the extracts. The selective reference antagonist, Granisetron caused significant reductions ($P \leq 0.001$).

3.4 PROSTAGLANDIN-INDUCED EDEMA IN RATS

Treatment of the rats with the reference drugs and the extracts, it was observed that the 30, 100 and 300 mg/kg AQ PSE and ET PSE treated groups had significant reduction ($P \leq 0.01$-0.001) in paw edema in the prostaglandin -induced inflammation (Figures 11 and 12). The reference drug, diclofenac caused significant reductions ($P \leq 0.001$).

3.5 BRADYKININ-INDUCED EDEMA IN RATS

The injection of bradykinin resulted in acute inflammation (seen as paw edema) of the right hind paws of the rats (Figures 13 and 14). Having treated the rats with the extracts, it was observed that the 30, 100 and 300 mg/kg AQ PSE and ET PSE pre-treated groups had significant reduction ($P \leq 0.01$-0.001) in paw edema in the bradykinin-induced inflammation (Figures 13 and 14) as compared to the control group.

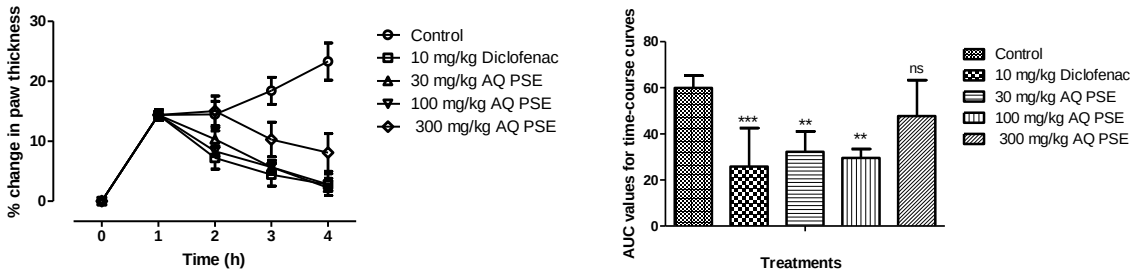


Figure 5: The effects of extracts on carrageenan-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). nsP > 0.05, P< 0.01, ***P< 0.001 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.**

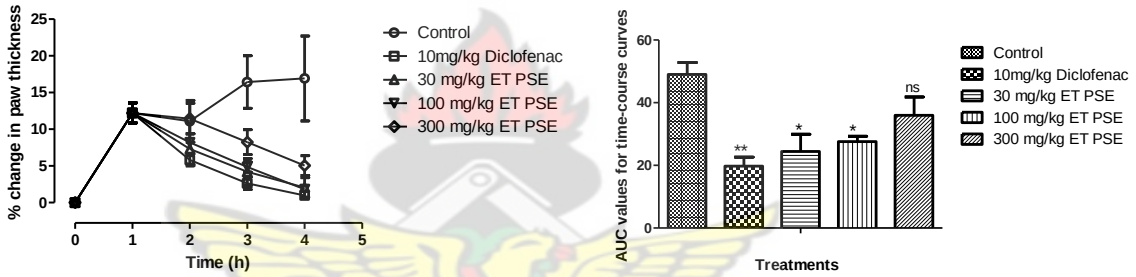


Figure 6: The effects of extracts on carrageenan-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). nsP > 0.05,* P< 0.05, **P< 0.01 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.

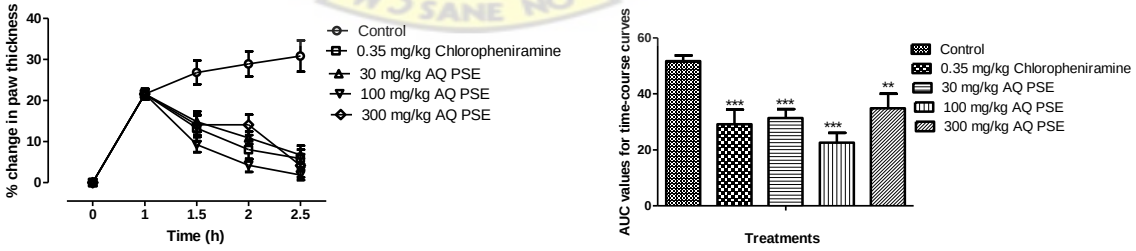


Figure 7. The effects of 30, 100, and 300 mg/kg of AQ PSE on histamine-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). P< 0.01, ***P< 0.001 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.**

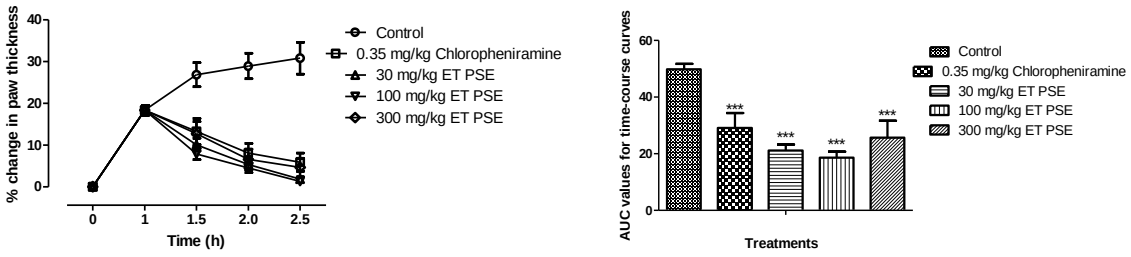


Figure 8: The effects of 30, 100, and 300 mg/kg of ET PSE on histamine-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). ***P < 0.01 is the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.

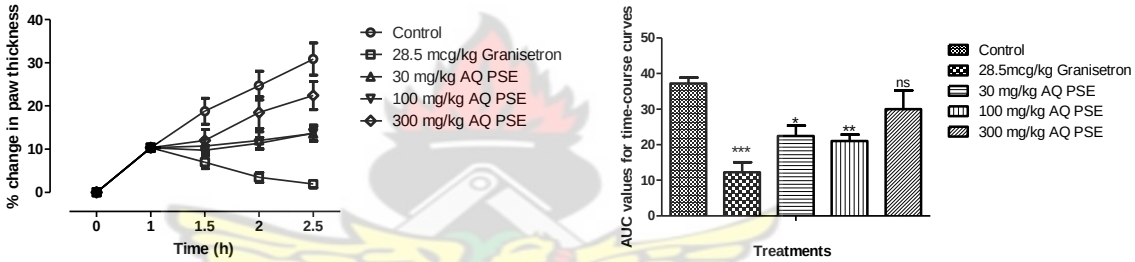


Figure 9: The effects of 30, 100, and 300 mg/kg of AQ PSE on serotonin-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). nsP > 0.05, P< 0.01, ***P< 0.001 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.**

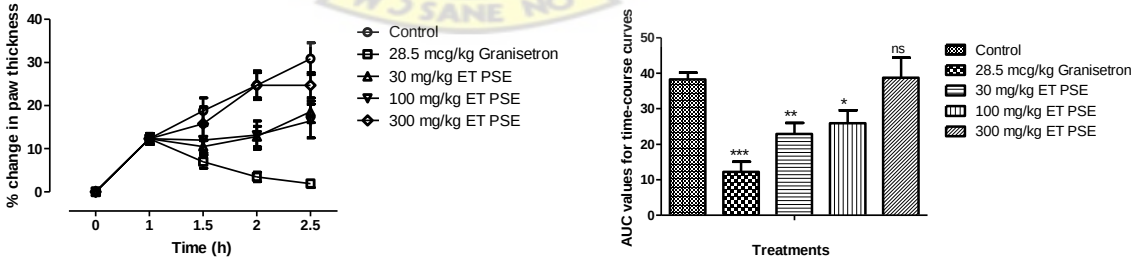


Figure 10: The effects of 30, 100, and 300 mg/kg of ET PSE on serotonin-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). nsP> 0.05, ** P< 0.01, ***P< 0.001 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.

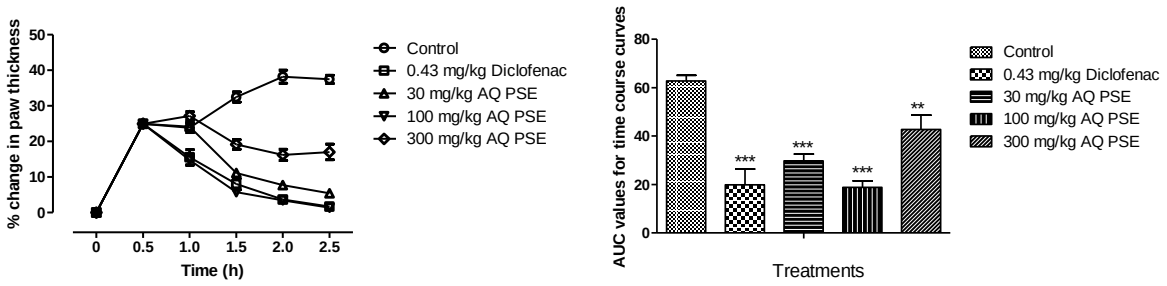


Figure 11: The effects of 30, 100, and 300 mg/kg of AQ PSE on Prostaglandin-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). ** P< 0.01, ***P< 0.001 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.

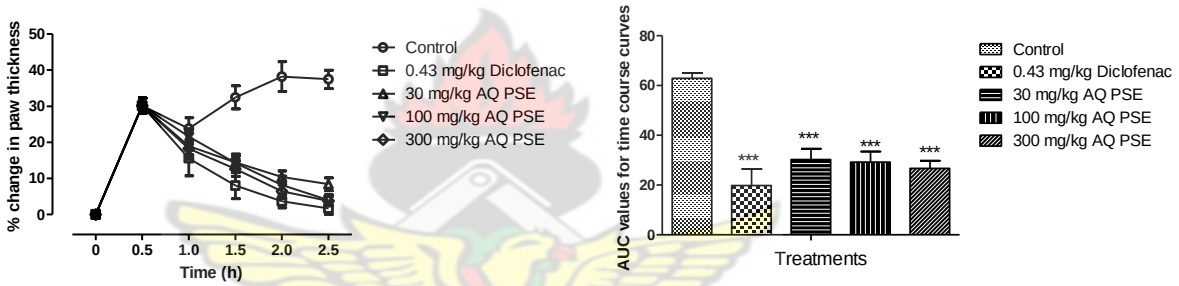


Figure 12: The effects of 30, 100, and 300 mg/kg of ET PSE on Prostaglandin-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). ***P< 0.001 is the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.

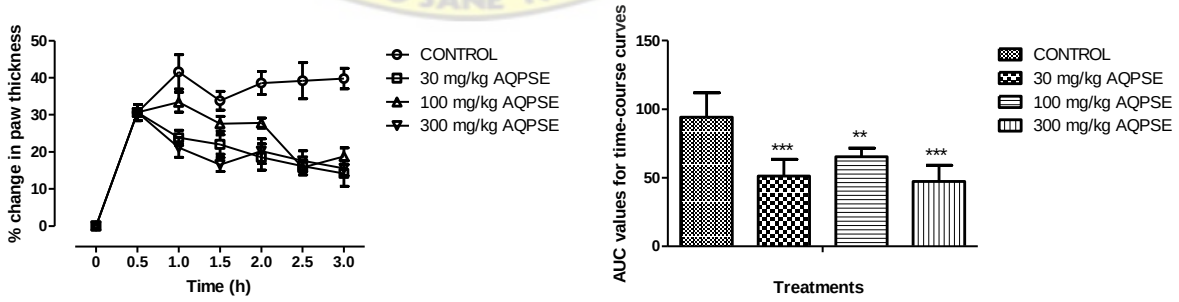


Figure 13: The effects of 30, 100, and 300 mg/kg of AQ PSE on bradykinin-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). ** P< 0.01, ***P< 0.001 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.

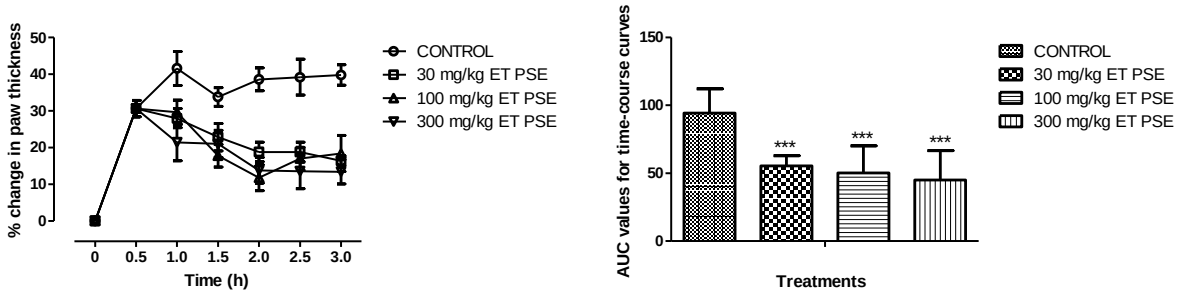


Figure 14: The effects of 30, 100, and 300 mg/kg of ET PSE on bradykinin-induced paw edema in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). ***P< 0.001 is the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test post hoc.

3.6 FORMALDEHYDE-INDUCED ARTHRITIS

There were significant reductions ($P \leq 0.01$-0.001) in paw thickness of formalin-induced arthritic animals treated with both aqueous and ethanolic leaf extracts of *P. stratiotes* compared to the normal saline-treated arthritic animals. Similar significant reductions ($P \leq 0.001$) in paw thicknesses were observed among the methotrexate, diclofenac, and dexamethasone treated arthritic animals (Figure 15 ^{A,B},16^{A,B} and 17 ^{A,B}).

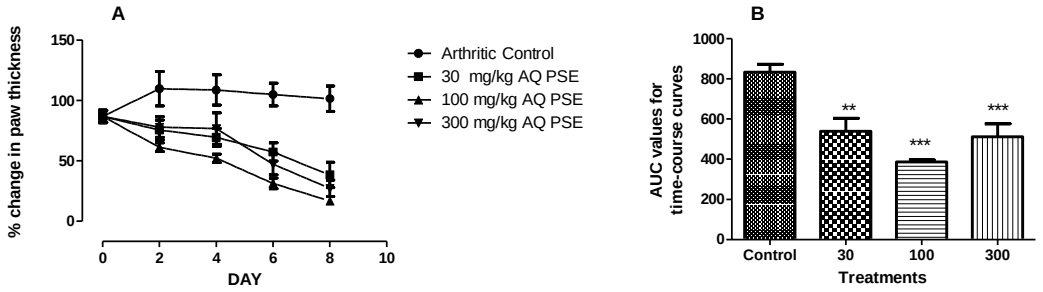


Figure 15: (A) the time-course curves and (B) the area under the time-course curves (AUC) of the effects of 30, 100, and 300 mg/kg of AQ PSE on formalin-induced arthritis in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). ** implies $P \leq 0.01$, ***implies $P \leq 0.001$: the level of significance of paw thickness reduction (compared to the control) analyzed by One-way ANOVA followed by Dunnet's test *post hoc*.

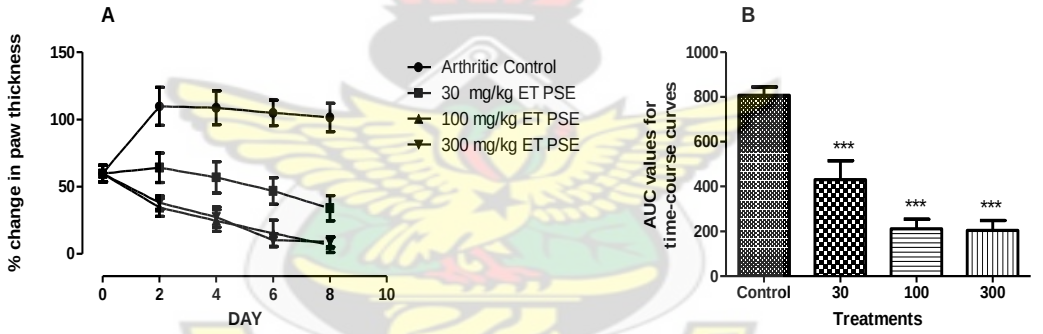


Figure 16: (A) the time-course curves and (B) the area under the time-course curves (AUC) of the effects of 30, 100, and 300 mg/kg of ET PSE on formalin-induced arthritis in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). * implies $P \leq 0.001$; the level of significance of paw thickness reduction (compared to the control) analyzed by One-way ANOVA followed by Dunnet's test *post hoc*.**

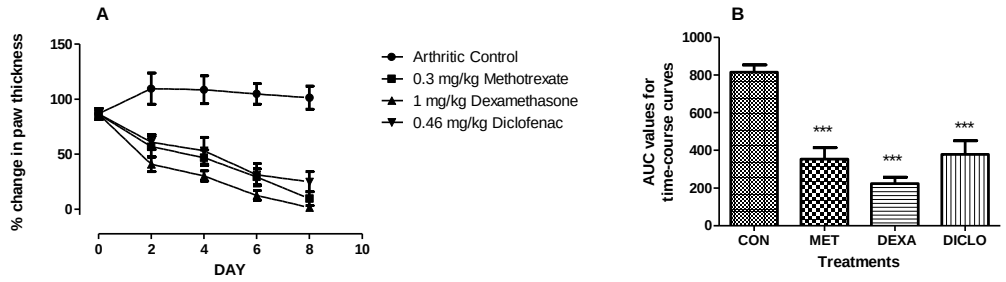


Figure 17: (A) the time-course curves and (B) the area under the time-course curves (AUC) of the effects of 0.3 mg/kg methotrexate, 1 mg/kg dexamethasone, and 0.46 mg/kg diclofenac on formalin-induced arthritis in Sprague-Dawley rats. Data are presented as mean ± SEM (n=5). * implies $P \leq 0.001$; the level of significance of paw thickness reduction (compared to the control) analyzed by One-way ANOVA followed by Dunnet's test *post hoc*.**

3.7 ADJUVANT-INDUCED ARTHRITIS

Intra-plantar injection of CFA induced an inflammatory response characterized by paw swelling in both the ipsilateral and the contralateral paws. The response of the injected paw was biphasic; consisting of an acute and a polyarthritic phases corresponding to days 0-9 and 10-27 post-inoculation, respectively. The acute phase response was characterized by unilateral inflammatory edema of the ipsilateral paw peaking around days 4-6, followed by subsequent polyarthritic phase response which began around day 9 characterized by inflammatory edema of the contralateral paw.

The 30, 100, and 300 mg/kg AQ PSE caused significant reduction ($P \leq 0.01$-0.001) of ipsilateral paw thickness in the CFA-induced arthritic rat however, only the 30 mg/kg AQ PSE caused significant reduction ($P \leq 0.01$) in contralateral paw thickness (Figure 18^{A}, 18^{B}). The ET PSE at doses of 30 and 100 mg/kg effected a reduction ($P \leq 0.05$) in the ipsilateral paw thickness but there was no significant reduction ($P > 0.05$) in contralateral

paw thickness at all dose levels (Figure 19[A], 19[B]). Methotrexate, dexamethasone and diclofenac caused significant reduction ($P \leq 0.05$-0.001) in ipsilateral and contralateral paw thickness of arthritic rats (Figure 20[A], 20[B]). Compared to the arthritic animals, there was no significant increase in ipsilateral and contralateral paw thicknesses of IFA-induced arthritic animals. Normal animals kept under experimental condition did not have any paw swelling (Figure 21[A], 21[B]).

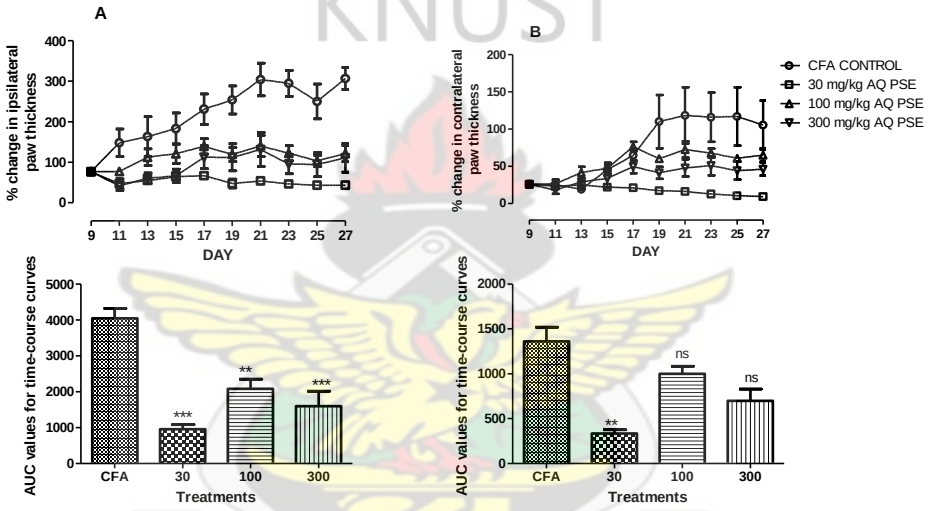


Figure 18: The time-course curves and area under the curves (AUC) for the effects of 30, 100, and 300 mg/kg of AQ PSE on CFA-induced arthritic edema of the ipsilateral paw (A), and contralateral paw (B) in Sprague-Dawley rats. Values plotted are means ± SEM (n=5). Significant reductions in paw edema relative to arthritic rats was analyzed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparisons Test. ^{ns} implies $P > 0.05$, * implies $P \leq 0.05$, implies $P \leq 0.01$, *** implies $P \leq 0.001$.**

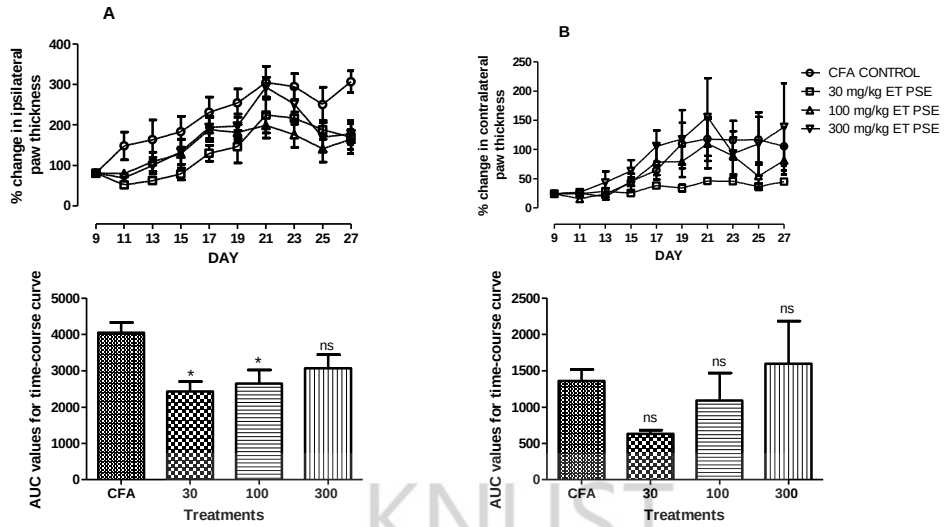


Figure 19: The time-course curves and area under the curves (AUC) for the effects of 30, 100, and 300 mg/kg of ET PSE on CFA-induced arthritic edema of the ipsilateral paw (A), and contralateral paw (B) in Sprague-Dawley rats. Values plotted are means ± SEM (n=5). Significant reductions in paw edema relative to arthritic rats was analyzed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparisons Test. ^{ns} implies $P > 0.05$, * implies $P \leq 0.05$.

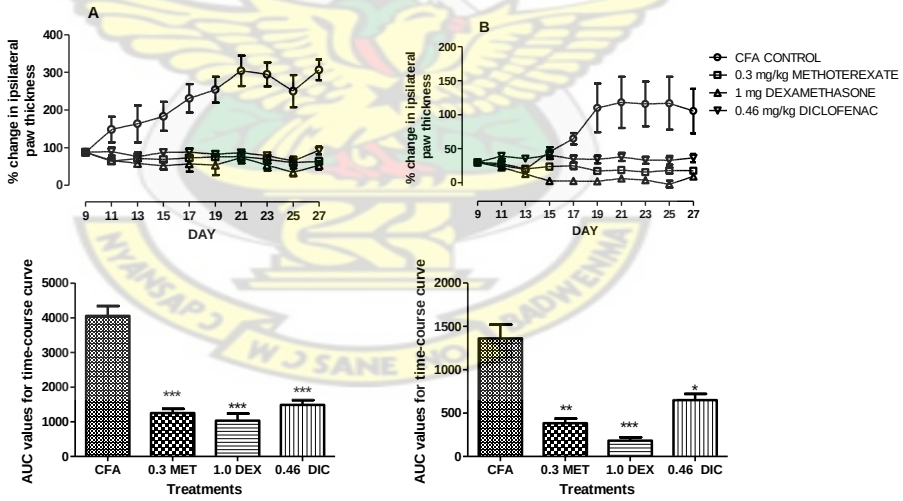


Figure 20: The time-course curves and area under the curves (AUC) for the effects of 0.3 mg/kg methotrexate, 1 mg/kg dexamethasone, and 0.43 mg/kg diclofenac on CFA-induced arthritic edema of the ipsilateral paw (A), and contralateral paw (B) in Sprague-Dawley rats. Values plotted are means ± SEM (n=5). Significant reductions in paw edema relative to arthritic rats was analyzed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparisons Test. ns impliesP > 0.05, ** implies P ≤ 0.01.

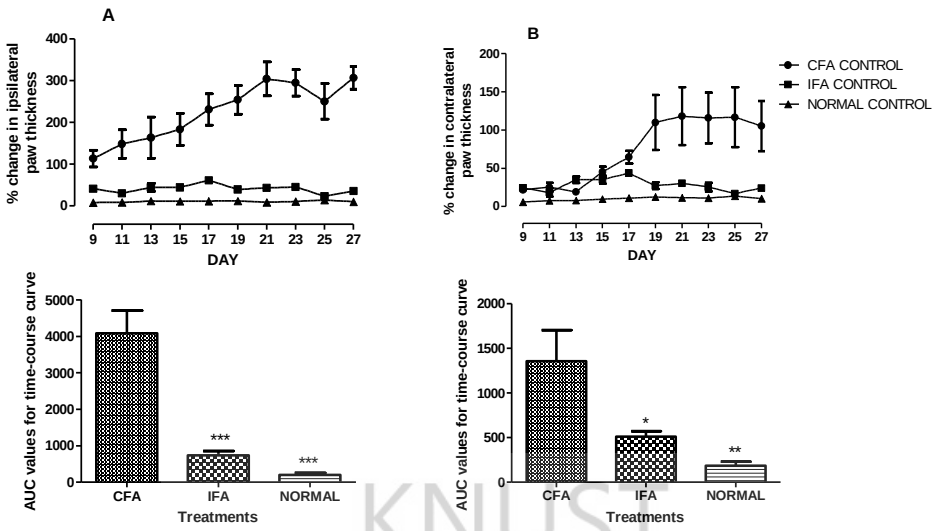


Figure 21: Time-course effects and area under the curves (AUC) of IFA control, and normal control for the ipsilateral (A) and contralateral (B) paws compared to vehicle-treated CFA-induced arthritis in Sprague-Dawley rats. Values plotted are means ± SEM (n=5). Significant reductions in paw edema relative to arthritic rats was analyzed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Comparisons Test. ^{ns} implies$P > 0.05$, ** implies $P \leq 0.01$.

3.7.1 Arthritic and Radiological Index

There were significant reductions in arthritic indices ($P \leq 0.05$-0.001) at all dose levels for extracts and reference drugs compared to the CFA arthritic animals, except the 100 and 300 mg/kg doses of ET PSE (Table 4, 5). This trend of results was also seen with radiological indices recorded (Table 4). Changes in body weight recorded shows significant increments ($P \leq 0.05$-0.01) in only the 30 and 100 mg/kg AQ PSE-treated and the diclofenac-treated arthritic animals (Table 4).

3.7.2 Hematological Assessment

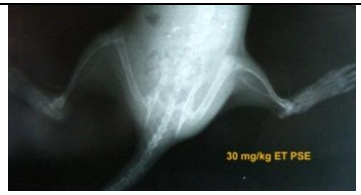
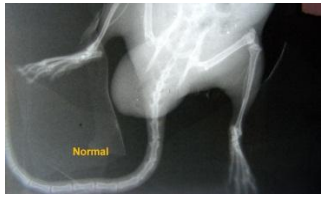
Hematological assessment indicated significantly low levels of white blood cell count ($P \leq 0.05$-0.01) only in 30 mg/kg AQ PSE, methotrexate treated and normal animals kept under experimental conditions (Table 6, 7). The red blood cell count revealed a significant reduction in ($P \leq 0.01$) methotrexate-treated animals, but there was no significant difference in hemoglobin levels at all dose levels of the extract and reference drug-treated groups compared to the CFA-arthritic animals (Table 6, 7). The erythrocyte sedimentation rate, ESR and C-reactive protein, CRP levels were significantly low at all dose level ($P \leq 0.01$-0.001) except 300 mg/kg AQ PSE, 100 and 300 mg/kg ET PSE doses (Table 6, 7).

Table 4: Records of arthritic index, radiological index and change in body weight in CFA and IFA-induced arthritic animals. CFA-induced arthritic animals treated with extracts and reference drugs, and normal animals kept under experimental conditions.

Treatments	Arthritic Index	Radiological Index	Change in weight
CFA	12.0 ± 0.71	5.00±0.58	17.0 ±11.25
IFA	1.4 ±0.50 ***	0.33 ±0.33***	48.0 ±5.83 †
Normal control	0.0 ±0.00 ***	0.00±0.00***	65.0 ±4.18 ††
30 mg/kg AQ PSE	2.8 ±1.24 ***	0.00±0.00***	53.4 ±7.14 †
100 mg/kg AQ PSE	4.4 ±1.12 **	3.00±0.58*	53.4 ±11.83 †
300 mg/kg AQ PSE	3.8 ±1.72 ***	1.00±0.58***	29.0 ±5.10 ns
30 mg/kg ET PSE	6.4 ±1.25 *	3.33±0.67*	37.0 ±6.44 ns
100 mg/kg ET PSE	8.8 ±1.24 ns	3.67±0.33ns	24.0 ±8.86 ns
300 mg/kg ET PSE	9.6 ±1.78 ns	4.33±0.33ns	27.0 ±7.18 ns
0.3 mg/kg Methorexate	2.0 ±0.45 ***	0.00±0.00***	18.0 ±6.04ns
0.43 mg/kg Diclofenac	2.8 ±0.66 ***	0.00±0.00***	44.0 ±1.87 †
1 mg/kg Dexamethasone	2.4 ±0.25 ***	0.33±0.33***	20.0 ±6.52 ns

Values represent the Mean±SEM (n = 5). * implies P ≤ 0.05, ** implies P ≤ 0.01, and * implies P ≤ 0.001, represents significant decrements compared to CFA arthritic condition (One-way ANOVA followed by Dunnet's post hoc test). † implies P ≤ 0.05, and †† implies P ≤ 0.01, represents significant increments.**

Table 5: Radiographs of the ipsilateral and contralateral paws of normal, arthritic but not treated, and methotrexate, diclofenac, dexamethasone, and 30, 100 and 300 mg/kg AQ PSE or ET PSE treated arthritis in Sprague-Dawley rats.

			
CFA control	**0.3 mg/kg Methotrexate**	**30 mg/kg AQ PSE**	**30 mg/kg ET PSE**
			
IFA control	**1 mg/kg Dexamethasone**	**100 mg/kg AQ PSE**	**100 mg/kg ET PSE**
			
Normal	**0.46 mg/kg Diclofenac**	**300 mg/kg AQ PSE**	**300 mg/kg ET PSE**

Radiograph showing the joint architecture of rats upon X-ray imaging.

Table 6: The hematological profile of CFA and IFA-induced arthritic rats, normal rats kept under experimental conditions, and arthritic rats treated with the aqueous and ethanolic leaf extracts of *P. stratiotes*

	Controls			AQ PSE			ET PSE		
	CFA	IFA	Normal	30 mg/kg	100 mg/kg	300 mg/kg	30 mg/kg	100 mg.kg	300 mg/kg
WBC	16.17± 1.89	10.23 ± 0.47	8.13 ± 2.38	7.93 ± 0.12	12.80 ± 2.24	11.90 ± 2.48	10.47 ± 1.97	13.67 ± 1.79	17.70 ± 5.28
($x10^3$/L)		ns	*	*	ns	ns	ns	ns	ns
RBC	8.36 ± 0.18	7.60 ± 0.36	7.64 ± 0.05	7.77 ± 0.41	7.04 ± 0.44	7.73 ± 0.72	7.36 ± 0.36	7.28 ± 0.22	6.99 ± 0.21
($x10^6$/L)		ns	ns	ns	ns	ns	ns	ns	ns
HGB (g/dl)	13.47 ± 0.23	14.13 ± 0.46	14.13 ± 0.18	13.80 ± 0.10	13.90 ± 0.85	13.77 ± 0.98	13.10 ± 0.60	12.30 ± 0.59	11.90 ± 0.52
		ns	ns	ns	ns	ns	ns	ns	ns
MCV (fL)	52.33 ± 1.33	59.83 ± 0.73	59.83 ± 0.73	56.80 ± 1.17	58.13 ± 1.59	58.57 ± 2.01	58.47 ± 1.33	54.43 ± 1.97	56.60 ± 0.57
		**	**	ns	ns	ns	*	ns	ns
ESR (mm)	7.00 ± 1.16	2.00 ± 0.00	1.33 ± 0.33	1.33 ± 0.33	3.67 ± 0.67	5.00 ± 0.58	2.33 ± 0.33	6.67 ± 1.02	6.67 ± 0.88
		***	***	***	**	ns	***	ns	ns
CRP	80.0 ± 16.00	10.0 ± 2.00	6.0 ± 0.00	6.0 ± 0.00	32.0 ± 4.00	56.0 ± 9.12	16.0 ± 4.00	56.0 ± 10.12	64.0 ± 12.32
		***	***	***	**	ns	***	ns	ns

Values recorded are means ± SEM (N=5). * implies P ≤ 0.05, ** implies P≤ 0.01, * implies P ≤ 0.001 are the level of significance of paw edema reduction compared to the CFA control. Analysis was done with One-way Analysis of Variance (ANOVA) followed by Dunnet's multiple comparisons test *post hoc.* White Blood Cell Count (WBC), Hemoglobin (HGB), Red Blood Cell Count (RBC), Mean Corpuscular Volume (MCV)**

Table 7: The hematological profile of CFA and IFA-induced arthritic rats, normal rats kept under experimental conditions, and arthritic rats treated with methotrexate, diclofenac, and dexamethasone.

	Controls			Reference Drugs		
	CFA	IFA	Normal	0.3 mg/kg Methotrexate	0.46 mg/kg Diclofenac	1 mg/kg Dexamethasone
WBC ($x10^3$/L)	16.17± 1.89	10.23 ± 0.47 ns	8.13 ± 2.38*	5.60 ± 0.06 **	15.23 ± 2.00 ns	10.77 ± 0.85 ns
RBC ($x10^6$/L)	8.36 ± 0.18	7.60 ± 0.36 ns	7.64 ± 0.05 ns	6.41 ± 0.01 **	6.96 ± 0.11 ns	8.16 ± 0.25 ns
HGB (g/dl)	13.47 ± 0.23	14.13 ± 0.46 ns	14.13 ± 0.18 ns	12.70 ± 0.1 ns	12.50 ± 0.42 ns	14.27 ± 0.23 ns
MCV (fL)	52.33 ± 1.33	59.83 ± 0.73 **	59.83 ± 0.73 **	61.17 ± 0.12 ***	58.60 ± 1.51 **	56.60 ± 0.56 *
ESR (mm)	7.00 ± 1.16	2.00 ± 0.00 ***	1.33 ± 0.33 ***	1.33 ± 0.33 ***	2.00 ± 0.00 ***	1.33 ± 0.33 ***
CRP	80.0 ± 16.00	10.0 ± 2.00 ***	6.0 ± 0.00 ***	8.00 ± 2.00 ***	22.00 ± 13.00 ***	8.00 ± 2.00**

Values recorded are means ± SEM (N=5). * implies P ≤ 0.05, ** implies P≤ 0.01, * implies P ≤ 0.001 are the level of significance of paw edema reduction compared to the CFA control. Analysis was done with One-way Analysis of Variance (ANOVA) followed by Dunnet's multiple comparisons test *post hoc*.White Blood Cell Count (WBC), Hemoglobin (HGB), Red Blood Cell Count (RBC), Mean Corpuscular Volume (MCV)**

3.7.3 Histopathological Assessment of Paw Tissue

Untreated CFA-induced arthritis showed an intense infiltrate of lymphocytes with foci of necrosis, pus collection and scattered neutrophils in the ipsilateral paws and chronic inflammatory changes dominated by lymphocytes with scattered plasma cells and foci of fibrosis with distortion of the joint architecture of the contralateral paws.

Histological assessment of the ipsilateral paws of rats treated with 30 mg/kg AQ PSE showed no significant inflammatory changes. The contralateral paws however showed only foci of mild chronic inflammatory change characterized by fibrosis with scattered lymphocytes. Tissue in the ipsilateral paws of the 100 and 300 mg/kg AQ PSE-treated animals showed chronic inflammatory changes dominated by lymphocytes with scattered plasma cells. The contralateral paws of the 100 mg/kg AQ PSE-treated animals however showed foci of intense inflammatory changes dominated by neutrophils, plasma cells, lymphocytes and focus of pus collection with necrotic debris while that of the 300 mg/kg AQ PSE-treated animals showed foci of mild chronic inflammatory change characterized by fibrosis with scattered lymphocytes.

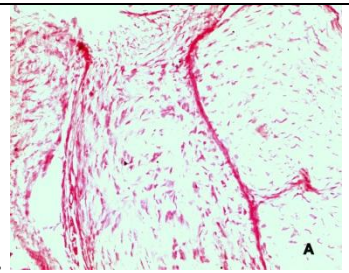
The ipsilateral paws of the 30, 100 mg/kg ET PSE-treated rats showed foci of intense acute and chronic inflammatory change dominated by neutrophils, plasma cells, lymphocytes and focus of pus collection with necrotic debris. That of the 300 mg/kg ET PSE-treated showed an intense infiltrate of lymphocytes within the dermis and in the muscle, and islands of cartilage with synovium. The contralateral paws of all the ET PSE-treated

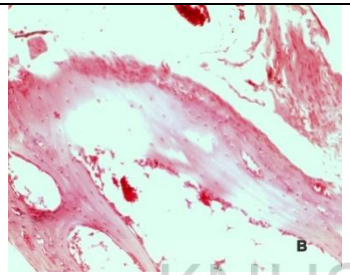
animals manifested chronic inflammatory changes dominated by lymphocytes with scattered plasma cells and foci of fibrosis.

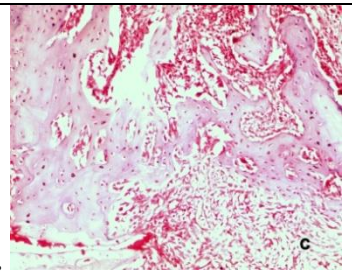
The ipsilateral paws of methotrexate treated rats showed only foci of mild chronic inflammatory change characterized by fibrosis with scattered lymphocytes but the contralateral paw showed no significant inflammatory changes. Diclofenac treatment showed foci of moderate chronic inflammatory change characterized by fibrosis with scattered lymphocytes in the ipsilateral paws, however there was no significant inflammatory changes in the contralateral paws. Dexamethasone showed no significant inflammatory changes in the ipsilateral paws but the contralateral paws showed moderate chronic inflammatory changes dominated by lymphocytes with scattered plasma cells and foci of fibrosis.

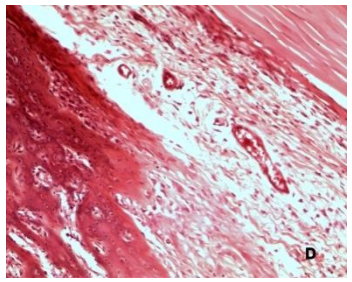
Rats with incomplete arthritis induced by IFA showed mild chronic inflammatory changes dominated by lymphocytes with scattered plasma cells in ipsilateral paw while the contralateral paw showed no significant inflammatory changes. The experimental conditions did not result in any inflammatory condition in animals kept during the study period. Illustrations of these histopathological descriptions are as shown in Table 8

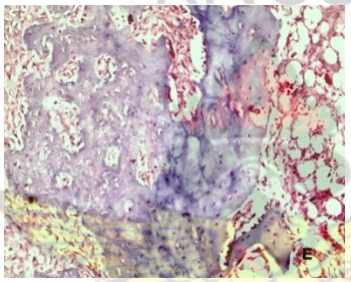
Table 8: Photomicrographs taken from the study showing no inflammatory condition as well as the various intensities of inflammation in the arthritic, and arthritic but treated paws of Sprague-Dawley rats.

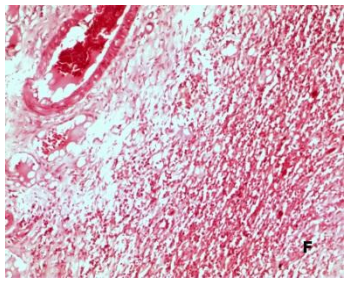
A. 

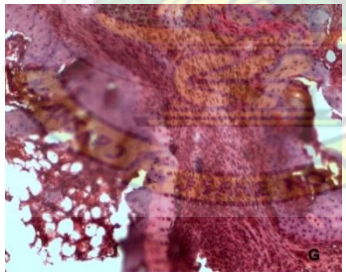
B. 

C. 

D. 

E. 

F. 

G. 

A: No inflammatory condition; B: Mild chronic inflammatory changes dominated by lymphocytes with scattered plasma cells; C: Foci of moderate chronic inflammatory change characterized by fibrosis with scattered lymphocytes; D: Chronic inflammatory changes dominated by lymphocytes with scattered plasma cells and foci of fibrosis; E: Intense infiltrate of lymphocytes with foci of necrosis, pus collection and scattered neutrophils; F: Foci of acute on chronic inflammatory change dominated by neutrophils, plasma cells, lymphocytes and a focus of pus collection with necrotic debris; G: Foci of intense acute on chronic inflammatory change dominated by neutrophils, plasma cells, lymphocytes and focus of pus collection with necrotic debris.

3.8 LIPOPOLYSACHARRIDE –INDUCED FEVER

Lipopolysacharride- induced fever in rats was significantly reduced ($P \leq 0.01$-0.001) at all dose levels of AQ PSE and ET PSE treatment; the effect was similar to that observed for acetaminophen treatment (Figures 22[A, B] and 23[A, B]).

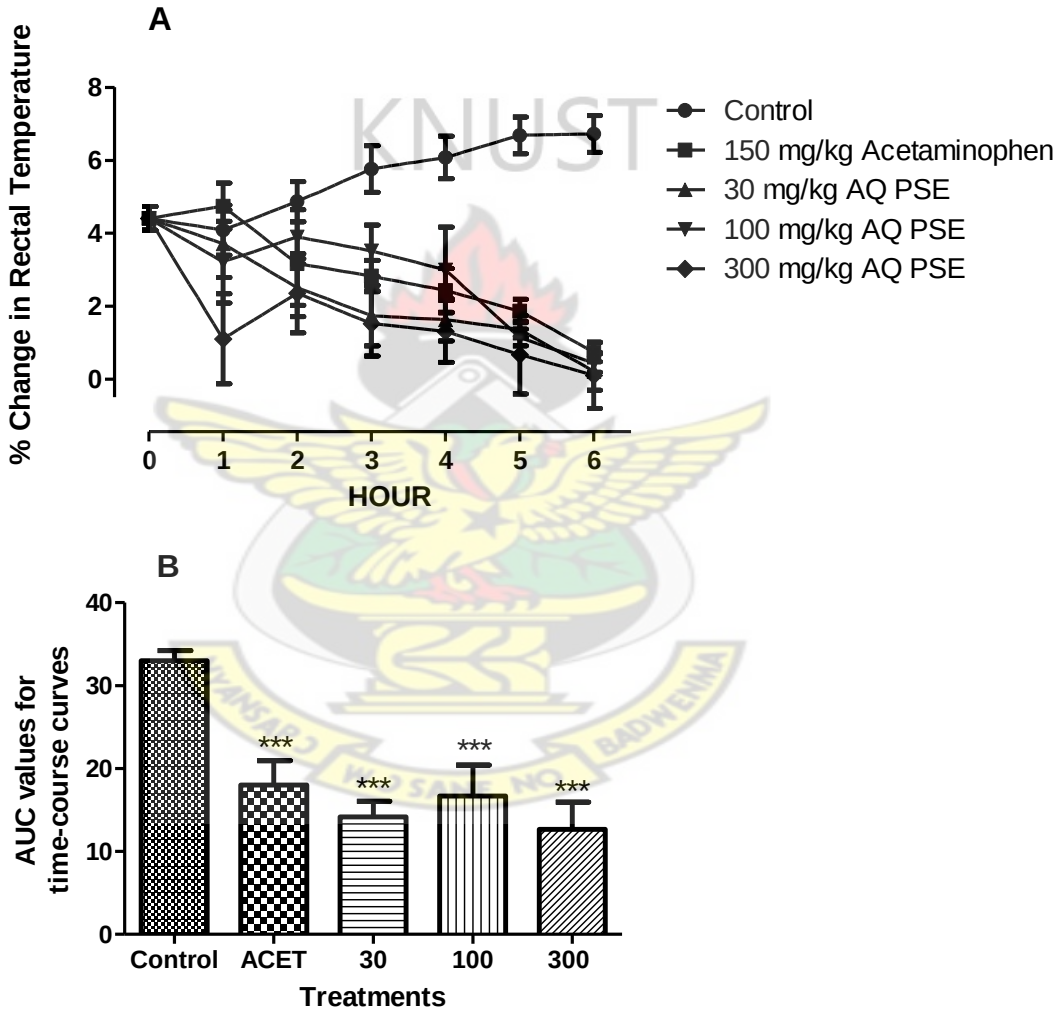


Figure 22: Plots of (A) the time-course curves and (B) the area under the time-course curves (AUC) of the effects of 30, 100, and 300 mg/kg of AQ PSE and 150 mg/kg acetaminophen on LPS-induced fever in Sprague-Dawley rats. Data plotted are means ± SEM (n=5). ***implies $P \leq 0.001$; the level of significance of rectal temperature reduction (compared to the normal saline-treated) analyzed by One-way ANOVA followed by Dunnet's test *post hoc*.

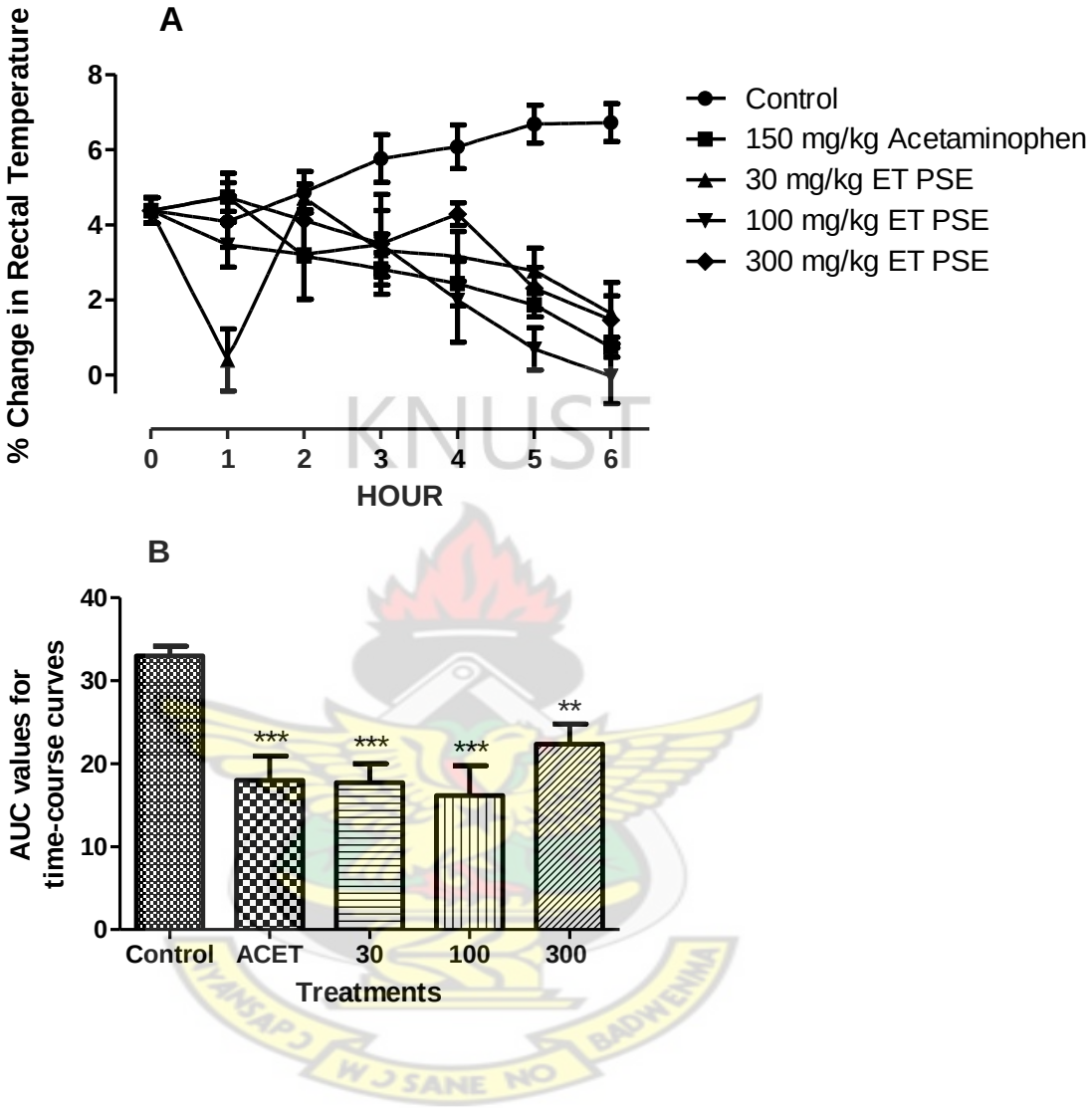


Figure 23: Plots of (A) the time-course curves and (B) the area under the time-course curves (AUC) of the effects of 30, 100, and 300 mg/kg of ET PSE and 150 mg/kg acetaminophen on LPS-induced fever in Sprague-Dawley rats. Data plotted are means ± SEM (n=5). ** implies P ≤ 0.01; ***implies *P* ≤ 0.001: the level of significance of rectal temperature reduction (compared to the normal saline-treated) analyzed by One-way ANOVA followed by Dunnet's test *post hoc*.

3.9 OCULAR ANTI-INFLAMMTORY EFFECT

3.9.1 Pyrexia, ESR and CRP as indicators of inflammation

Intra-plantar injection of LPS was characterized by pyrexia, elevated serum levels CRP and high levels of ESR two hours after injection (Table 9). Elevation of these parameters was considered a satisfactory indication of an inflammatory response hence a basis for selection for endotoxin- induced uveitic studies.

3.9.2 Endotoxin-induced Uveitis

There was significant reduction in vasodilation of the iris vessels and exudation into the anterior chamber relative to the control; seen as significant decrements ($P \leq 0.001$) in clinical scores of inflammation (Table 10) graded from photographs taken upon slit lamp examination (Table 11)

3.9.3 Polymorphonuclear Neutrophil Count

There were significant reductions ($P \leq 0.001$) in the number of polymorphonuclear neutrophil in the reference drug, AQ PSE and ET PSE-treated groups compared to the uveitic control group (Figure $24^{A, B}$). The normal control (without LPS challenge) group showed significantly ($P \leq 0.001$) low numbers of cell infiltration.

3.9.4 Total Protein Concentration

Total protein concentration was significant low ($P \leq 0.001$) in Prednisolone-treated as well as the AQ PSE, ET PSE-treated uveitic rats, and the normal control compared to uveitic rats treated with normal saline (Figure $25^{A,B}$).

3.9.5 Histopathological Assessment of the Anterior Uvea

The histopatological assessment did not show any remarkable signs of in inflammation in anterior uvea in all rats treated with Prednisolone, AQ PSE, ET PSE-treated uveitic rats, and the normal control. However, there were histopathological signs of inflammation characterized by neutrophil infiltration into the uveal tissues (Table. 12).

Table 9: Body temperature, ESR and CRP levels two hours after LPS inoculation in Sprague-Dawley rats

Group	% change in temperature	ESR	CRP
Control	0.001±0.102	0.333±0.211	6.000±0.00
LPS treated rats	0.864±0.110***	3.167±0.477***	28.000±6.693*

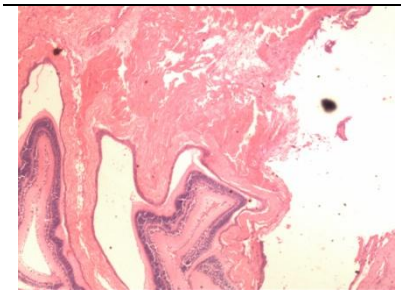
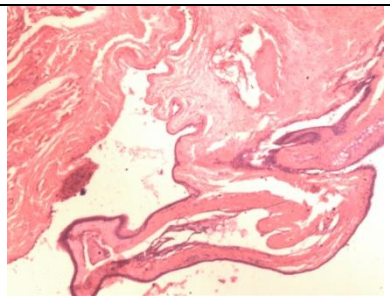
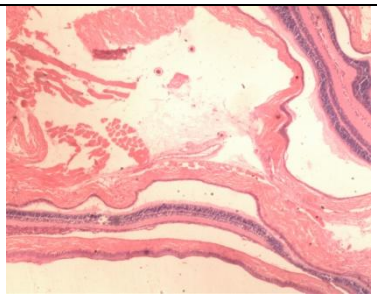
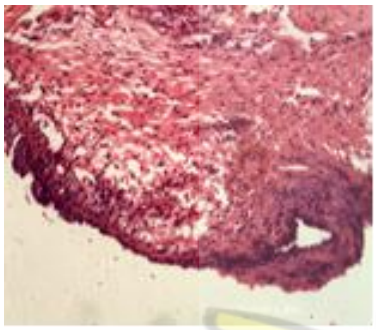
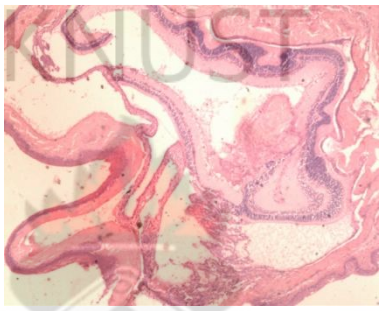
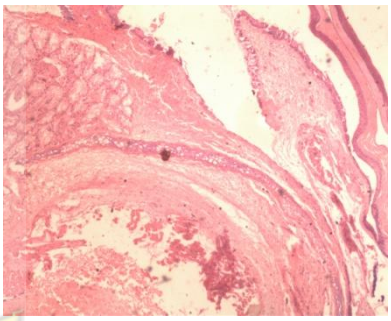
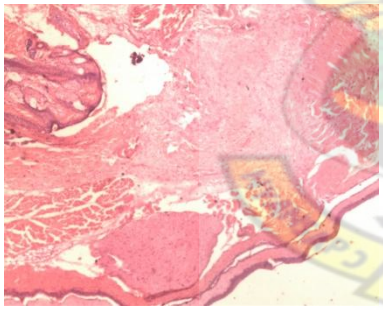
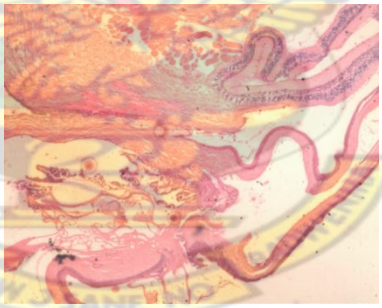
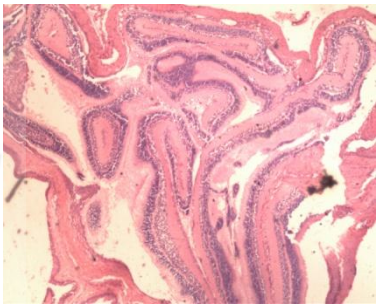
Values are means ± SEM (n = 6). Significant differences between LPS-treated rats and the control were established using one-way analysis of variance followed by Dunnett's post hoc test). * implies $P \leq 0.05$; $P \leq 0.01$; *** $P \leq 0.001$.**

Table 10: Clinical Score of inflammation in Uveitis

Treatments	Clinical Score of Inflammation
Uveitic Control	3.333±0.211
Normal Control	0.167±0.167***
30 mg/kg AQ PSE	0.500±0.224***
100 mg/kg AQ PSE	1.667±0.333***
300 mg/kg AQ PSE	1.667±0.333***
30 mg/kg ET PSE	0.667±0.211***
100 mg/kg ET PSE	1.500±0.342***
300 mg/kg ET PSE	1.167±0.307***
30 mg/kg Prednisolone	0.667±0.333***

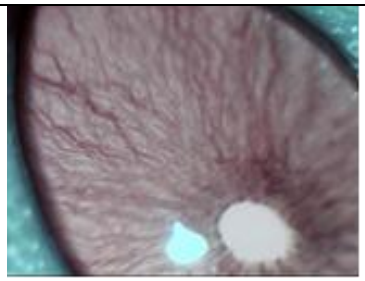
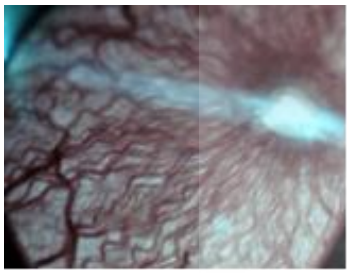
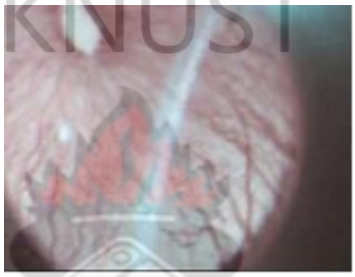
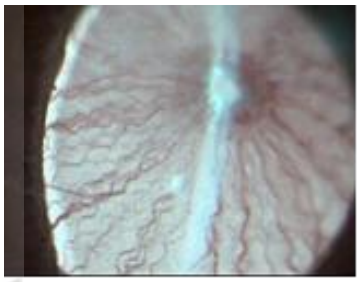
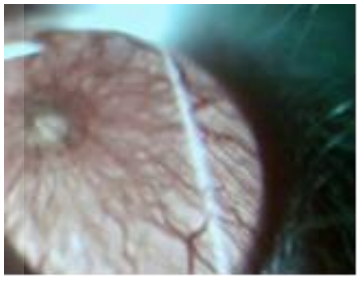
Values are means ± SEM (n = 6). Significant differences between the clinical score of inflammation of the drug-treated and normal control and the uveitic control were established using one-way analysis of variance followed by Dunnett's post-hoc test). * implies $P \leq 0.05$; $P \leq 0.01$; *** $P \leq 0.001$.**

Table 12: Photomicrographs of the anterior uvea of, normal, uveitic, and uveitic rats treated with 30, 100 and 300 AQ PSE, and 30, 100 and 300 ET PSE

Controls	AQ PSE-treated	ET-PSE treated
		
Normal	30 mg/kg	30 mg/kg
		
Uveitic	100 mg/kg	100 mg/kg
		
Prednisolone-treated	300 mg/kg	300 mg/kg

The histological sectioning of the anterior uvea of rats upon staining with hematoxylin and eosin.

Table 11: Photographs of anterior chamber of normal, uveitis rats treated with 30,100 and 300 AQ PSE, and 30,100 and 300 ET PSE

Controls	AQ PSE-treated	ET-PSE-treated
		
Normal	30 mg/kg	30 mg/kg
		
Uveitic	100 mg/kg	100 mg/kg
		
Prednisolone-treated	300 mg/kg	300 mg/kg

Photographs of the anterior chamber showing iris vasodilations upon Slit lamp examination.

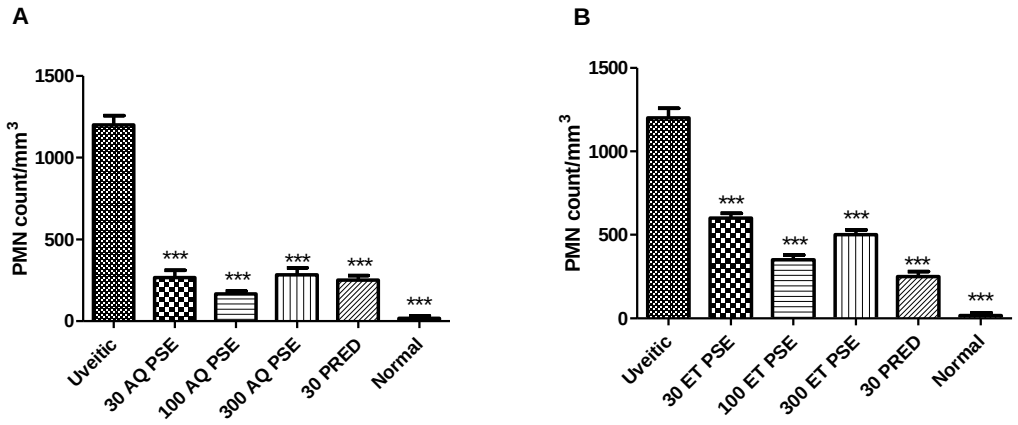


Figure 24: The effect of (A) 30, 100 and 300 mg/kg AQ PSE and 30 mg/kg prednisolone; (B) 30, 100 and 300 mg/kg ET PSE and 30 mg/kg prednisolone on PMN count in the anterior chamber of the eyes of endotoxin-induced uveitic rats. Significant difference between treatment groups, normal controls and controls was established using One-Way Analysis of Variance (ANOVA) followed by Dunnet's post hoc test. *P ≤ 0.05;P ≤ 0.01; ***P ≤ 0.001.**

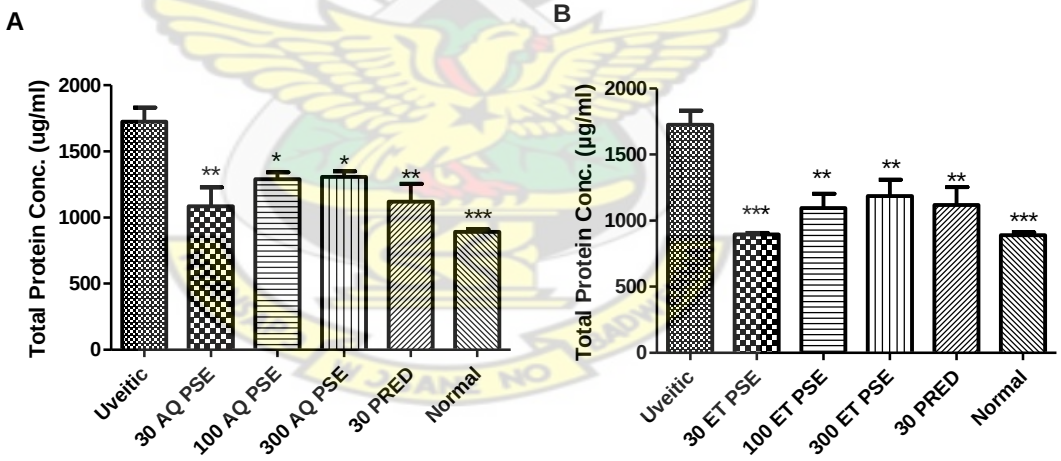


Figure 25: The effect of (A) 30, 100 and 300 mg/kg AQ PSE and 30 mg/kg prednisolone;(B) 30, 100 and 300 mg/kg ET PSE and 30 mg/kg prednisolone on total protein concentration in the aqueous humour of the eyes of endotoxin-induced uveitic rats. Significant difference between treatment groups, normal controls and controls was established using One-Way Analysis of Variance (ANOVA) followed by Dunnet's post hoc test. *P≤0.05;P ≤ 0.01; ***P≤0.001.**

3.10 SAFETY ASSESSMENT

3.10.1 Acute and Delayed Toxicity

Administration of AQ PSE caused no death at all doses over the entire experimental period. There was no labored breathing, constipation, emaciation, skin eruptions, abnormal posture, hemorrhage, sedation, diarrhoea, polyuria, polydipsia, polyphagia, anorexia, rhinorrhoea/nasal congestion, loss of autonomic reflexes, neuromuscular inco-ordination and collapse, hyperesthesia, hypothermia, twitching, spasticity, convulsion, writhing, and respiratory depression. Observations of the gait did not show uncoordinated, staggering, wobbly gait, hind limbs exaggerated, overcompensating, and/or making splayed movements, feet (primarily hind feet) point outward from body, forelimbs dragging and/or showing abnormal positioning, nor walking on toes (the heels of the hind feet are perpendicular to the surface).Hematological assessments revealed significant reductions ($P \leq 0.05$-0.01) in RBC count in all treatment groups over the 10 day period (Table 13). Urine analysis of treated and untreated rat showed no significant changes in measured parameters over the experimental period except urobilinogen and microalbumin which increased significantly in all treatment groups 10 days post-treatment. Urine color changed from straw to amber with in the same period (Table 14).

Table 13: Hematological assessment values obtained before and after treatment of Sprague-Dawley rats with 10, 30, 100, and300 mg/kg of AQ PSE in an acute and delayed toxicity study.

	Control	10 mg/kg		30 mg/kg		100 mg/kg		300mg/kg	
		24 hours	10 days	24 hours	10 days	24 hours	10 days	24 hours	10 days
WBC ($x10^3$/L)	5.63 ± 1.32	10.7 ± 2.43	9.83 ± 2.20	9.53 ± 3.35	7.90 ± 4.25	6.67 ± 2.82	7.33 ± 1.43	9.47 ± 1.02	11.47 ± 2.61 **
HGB (g/dl)	15 ± 0.72	14.43 ± 0.85	13.87 ± 0.40	13.4 ± 1.45	13.67 ± 0.40	13.8 ± 2.02	13.17 ± 0.57*	15.57 ± 0.74	14.8 ± 0.66
RBC ($x10^6$/L)	8.76 ±0.24	7.64 ± 0.29 *	7.50 ± 0.14*	7.20 ± 0.67**	7.65 ± 0.31*	7.46 ± 1.27*	7.50 ± 0.79*	7.65 ± 0.55*	7.28 ± 0.55**
HCT (%)	51.47 ± 0.93	49.37 ± 2.91	46.17 ± 1.13	45.18 ± 5.18	45.90 ± 2.69	45.8 ± 8.41	43.3 ± 3.35 *	52.87 ± 3.23	50.87 ± 2.03
MCV (fL)	58.7 ± 2.43	64.63 ± 1.64	61.53 ± 1.66	62.63 ± 1.77	59.83 ± 2.27	60.4 ± 2.46	61.17 ± 2.80	59.73 ± 0.81	61.53 ± 3.37
MCH (pg)	17.1 ± 0.46	18.43±0.55*	18.47 ± 0.40	18.57±0.61*	17.83 ± 0.51	18.27 ± 0.38	18.67 ± 1.60*	17.6 ± 0.46	17.8 ± 1.28
MCHC (g/dl)	29.17 ± 0.55	29.27 ± 0.23	30 03 ± 0.38	29.67 ± 0.50	29.83 ± 0.95	30.27 ± 1.38	30.47 ± 1.72	29.47 ± 0.55	28.93 ± 0.49
LYM %	74.17 ± 6.48	74.43 ± 7.91	86.7 ± 9.58	75.6 7± 5.52	77.03 ± 7.11	62.17 ± 1.04	63.03 ± 11.85	68.23 ± 8.65	76.60 ± 3.30
LYM#($x10^3$/µL)	4.20 ± 1.25	7.87 ± 1.01	7.90 ± 1.90	7.30 ± 2.86	6.23 ± 3.75	4.10 ± 1.85	4.6 ± 1.14	6.53 ± 1.51	8.83 ± 2.11 *
RDW-CV (%)	12.8 ± 0.72	11.27 ± 0.67	12.5 ± 1.05	11.8 ± 1.65	12.20 ± 0.75	11.8 ± 1.55	12.61 ± 3.38	11.53 ± 0.29	12.33 ± 1.18
RDW-SD (fL)	30.97 ± 0.78	30.77 ± 0.25	30.43 ± 0.94	30.23 ± 2.06	29.77 ± 0.55	29.97 ± 2.82	31.63 ± 5.61	28.83 ± 0.78	30.37 ±0.84
PLT ($x10^5$/L)	7.76 ± 1.3	6.4 ±1.5	9.3 ± 3.1	7.04 ± 0.98	5.92 ± 2.5	4.52 ± 2.5	5. 53 ± 1.9	4.8 ± 2. 6	6.4 ± 1.92
MPV (fL)	6.2 ±0	6.4 ± 0.12	6.27 ± 0.21	6.5 ± 0.36	6.80 ± 0.10	6.70 ± 0.15	6.23 ± 0.40	6.97 ± 0.21	6.63 ± 0.15
PDW	7.1 ± 0 10	7.27 ± 0.15	7.37 ± 0.35	7.57 ± 0.42	8.17 ± 0.23**	8.4 ± 0.75**	7.13 ± 0.60	8.63±0.81***	7.63 ± 0.41
P_LCR (%)	4.07 ± 0.37	5.13 ± 0.76	4.2 ± 0.69	5.13 ± 1.36	6.17 ± 0.91 **	7.07±1.01**	4.13 ± 1.20	8.03±1.22***	6.17 ± 0.25 **

Values recorded are means and standard deviations (N=5). Values obtained for the various parameters before treatment, and 24 h and 10 days post-treatment.* P ≤ 0.05, ** P≤ 0.01 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test *post hoc.* White Blood Cell Count (WBC), Hemoglobin (HGB), Red Blood Cell Count (RBC), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Lymphocytes (LYM), Red Blood Cell Distribution Width (*RDW-CV & RWD-SD*), Platelet Count (PLT), Mean Platelet Volume (MPV), Platelet Distribution Width (PDW), Platelet Larger Cell Ratio (P-LCR).

Table 14: Urinalysis values obtained before and after treatment of Sprague-Dawley rats with 10, 30, 100, and 300 mg/kg of AQ PSE in an acute and delayed toxicity study.

Parameters	Control	10mg/kg		30 mg /kg		100 mg/kg		300 mg/kg	
		24 hours	10 days	24 hours	10 days	24 hours	10 days	24 hours	10 days
UBG (umol/uL)	3.4 0± 0.00	3.40 ± 0.00	17 ± 0.00 ***	7.93 ± 7.85	17.00 ± 0.00 ***	3.4 ± 0.00	3.4 ± 0.00	12.47 ± 7.85 **	17.00± 0.00***
BIL (umol/L)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	17.00 ± 0.00	17.00±0.00n
KET	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
BLD (Ery/uL)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
PROT (g/L)	(-)	2.1 ± 1.56	0.30 ± 1.025	0.20 ± 0.17	0.53 ± 0.40	0.30 ± 1.025	0.53 ± 0.36	0.53 ± 0.40	2.33 ± 1.15
NIT	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
LEU	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
GLU(mmol/L)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
SG	1.03 ± 0.00	1.03 ± 0.00	1.025 ± 0.00	1.02 ± 0.01	1.025 ± 0.00	1.015 ± 0.01	1.02 ± 0.76	1.025 ± 0.01	1.030 ± 0.00
pH	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.17 ± 0.58	6.5 ± 0.00	5.17 ± 0.29	6.83 ± 0.76	6.17 ± 0.29	6.00 ± 0.00
MALB(g/L)	(-)	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00

Values recorded are means and standard deviations (N=5). Values obtained for the various parameters before treatment, and 24 h and 10 days post-treatment. * P ≤ 0.05, ** P≤ 0.01 are the level of significance of paw edema reduction compared to the control, analyzed by One-way ANOVA followed by Dunnet's test *post hoc.* Urobilinogen (UBG);Bilirubin(BIL);Ketones(KET);Blood(BLD);Protein(PROT);Nitrite(NIT);leucocytes(LEU);Glucose (GLU);Specific gravity(SG); Microalbumin(MALB).

3.10.2 Ocular toxicity

Topical administration of 0.1ml of 0.5%, 1.0% and 2.0 % w/v of AQ PSE and ET PSE caused no significant damage to the cornea, iris and the conjunctiva over the entire experimental period (Table 15-18). Statistical analysis was performed to compare the effect of the extract on the right eye to the left eye (control) using paired T test. The extracts showed no significant damage to cornea, iris and conjunctiva tissues at various time points ($t_{(0.05,2)}$ =2.00, p=0.184; $t_{(0.05,2)}$ =1.00, p=0.423; $t_{(0.05,2)}$ =1.02, p=0.417; $t_{(0.05,2)}$ =1.73, p=0.225; $t_{(0.05,2)}$ =1.01, p=0.419; $t_{(0.05,2)}$ =1.51, p=0.270).

Table 15: The ocular toxicological profile of cornea, iris and conjunctival tissues of the eyes of rabbits kept under experimental conditions, with topical administration of aqueous leaf extract of *P. stratiotes* into the right eye .

	Controls			0.5% w/v AQ PSE			1.0% w/v AQ PSE		
Time (h)	Cornea	Iris	Conjunctiva	Cornea	Iris	Conjunctiva	Cornea	Iris	Conjunctiva
1	0.00± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 2.88 ns	3.33 ± 1.67 ns	2.66 ± 1.33 ns	1.67 ± 1.67 ns	1.67 ± 1.67 ns	2.00 ± 1.16 ns
24	0.00 ± 000	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	3.33 ± 1.67 ns	1.33 ± 1.33 ns	0.00 ± 0.00 ns	0.33 ± 0.33 ns	1.33 ± 1.33 ns
48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 ns	1.67 ± 1.67 ns	0.67 ± 0.67 ns	0.01 ± 0.00	0.67 ± 0.33 ns	0.67 ± 0.67 ns
72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 ns	0.33 ± 0.33 ns	0.33 ± 0.33 ns	0.00 ± 0.00 ns	0.33 ± 0.33 ns	0.67 ± 0.33 ns

Values recorded are means ± SEM (n=3). * implies P < 0.05, ^{ns} implies P= 0.05, are the level of significance of ocular tissue damage of the right eye instilled with the test drug compared to the left eye as control.

Table 16: The ocular toxicological profile of cornea, iris and conjunctival tissues of the eyes of rabbits kept under experimental conditions, with topical administration of aqueous leaf extract of *P. stratiotes* into the right eye.

	Controls			2.0% w/v AQ PSE		
Time (h)	Cornea	Iris	Conjunctiva	Cornea	Iris	Conjunctiva
1	0.00± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	1.67 ± 1.67 ns	1.33 ± 0.67 ns
24	0.00 ± 000	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	3.33 ± 1.67 ns	0.67 ± 0.67 ns
48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 1.67 ns	1.67 ± 1.67 ns	0.67 ± 0.67 ns
72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	1.67 ± 1.67 ns	0.67 ± 0.67 ns

Values recorded are means ± SEM (n=3). * implies P < 0.05, ^{ns} implies P= 0.05, are the level of significance of ocular tissue damage of the right eye instilled with the test drug compared to the left eye as control.

Table 17: The ocular toxicological profile of cornea, iris and conjunctival tissues of the eyes of rabbits kept under experimental conditions, with topical administration of ethanolic leaf extract of *P. stratiotes* into the right eye.

	Controls			0.5% w/v ET PSE			1.0% w/v ET PSE		
Time (h)	Cornea	Iris	Conjunctiva	Cornea	Iris	Conjunctiva	Cornea	Iris	Conjunctiva
1	0.00± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 1.67 ns	3.33 ± 1.67 ns	2.67 ± 1.33 ns	12.50 ± 2.50 ns	0.01 ± 0.01 ns	1.33 ± 0.67 ns
24	0.00 ± 000	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	1.67 ± 1.67 ns	2.00 ± 1.16 ns	0.02 ± 0.02 ns	0.01 ± 0.00 ns	1.00± 0.58 ns
48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	1.67 ± 1.67 ns	1.33 ± 0.67 ns	1.68 ± 1.67	0.00 ± 0.00 ns	0.67 ± 0.33 ns
72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	1.67 ± 1.67 ns	0.67 ± 0.67 ns	0.00 ± 0.01 ns	0.00 ± 0.00 ns	0.33± 0.33 ns

Values recorded are means ± SEM (n=3). * implies P < 0.05, ^{ns} implies P= 0.05, are the level of significance of ocular tissue damage of the right eye instilled with the test drug compared to the left eye as control.

Table 18: The ocular toxicological profile of cornea, iris and conjunctival tissues of the eyes of rabbits kept under experimental conditions, with topical administration of ethanolic leaf extract of *P. stratiotes* into the right eye .

	Controls			2.0% w/v ET PSE		
Time (h)	Cornea	Iris	Conjunctiva	Cornea	Iris	Conjunctiva
1	0.00± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.67 ns	1.67± 1.67 ns	0.67 ± 0.67 ns
24	0.00 ± 000	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 ns	1.67 ± 1.67 ns	0.33 ± 0.33 ns
48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00 ns	0.01 ± 0.00 ns	0.67 ± 0.33 ns
72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 ns	0.00 ± 0.00 ns	0.00 ± 0.00 ns

Values recorded are means ± SEM (n=3). * implies P < 0.05, ^{ns} implies P= 0.05, are the level of significance of ocular tissue damage of the right eye instilled with the test drug compared to the left eye as control. Analysis was done with paired T-test

CHAPTER FOUR

DISCUSSION

This Chapter discusses the outcome of the the study after employing standard procedures to evaluate the anti-inflammatory/anti-arthritc, antipyretic and safety of the extracts in rats.

Carrageenan induced paw edema is biphasic in nature with the first phase mediated by histamine and serotonin, the second mediated by prostaglandins particularly the E series and cyclo-oxygenase products which includes prostacyclins and thromboxanes. The continuity between the two phases is ensured through the action of kinins (Silva *et al.*, 2005; Perianayagam *et al.*, 2006). Inhibition of these mediators in effecting their pharmacologic activity is a sure way of curbing the incidence of inflammation at the site of injury. This study has shown that both aqueous and ethanolic leaf extracts of *P. stratiotes* has important anti-edematogenic effect on rat paw edema induced by carrageenan, histamine, serotonin, prostaglandin and bradykinin. Since carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation (Mossai *et al.*, 1995; Sawadogo *et al.*, 2006), the results of this study are an indication that aqueous and ethanolic leaf extracts of *P. stratiotes* can be effective in acute inflammatory disorders, to further ascertain the mechanism by which these extracts may be working histamine, serotonin, prostaglandins and bradykinin were then used as mediators to induce inflammation.

The extracts significantly inhibited histamine, prostaglandin, bradykinin-mediated models of inflammation in rats at all dose levels but its anti-serotonergic activity was observed at relatively lower doses. Histamine is an important inflammation mediator, potent vasodilator substance and also increases the vascular permeability (Vasudevan, 2007). Serotonin (5-hydroxytryptamine) is a vasoactive mediator similar to histamine found in mast cells and platelets in the GI tract and CNS. Serotonin also increases vascular permeability, dilates capillaries, and causes contraction of nonvascular smooth muscle (Borissova *et al.*, 1994). Prostaglandins act as potent pro-inflammatory mediators thereby making it a desirable target for the treatment of cancer, rheumatoid arthritis, intestinal inflammation, Alzheimer's disease and chronic musculoskeletal pain (Yedgar *et al.*, 2007). More importantly, the bradykinin-induced inflammatory response subsided rapidly in rats that were pretreated with the extracts. This inhibitory effect may have resulted from interference of the anti-inflammatory components of the extracts with the B_2 receptor-mediated mechanism by which bradykinin is reported to induce rat paw edema (Campos and Calixto, 1995).

Since the extracts effectively suppressed the edema produced by carrageenan, and subsequent exclusive mediators such as histamine, serotonin, prostaglandin and bradykinin, it showed that the extracts exhibits anti-inflammatory effects by possible inhibition of the synthesis, release or action of these inflammatory mediators.

Preliminary phytochemical screening indicated the presences of flavonoids and sterol, which could possibly be the source of the anti-inflammatory property of *P. stratiotes*. It

has been shown that most flavonoids have anti-inflammatory activity (Pelzer *et al.,* 1998; Funkoshi-Tago *et al.,* 2011).The sterols have structural resemblance to steroids and are known to attenuate inflammation by inhibiting *phospholipase* A_2 which hydrolyzes arachidonic acid from membrane phospholipids, and subsequent formation of prostanoids and leucotrienes via the cyclooxygenase and lipoxygenase pathways and immune dysfunction in experimental models (Mencarelli, 2009).

In attempt to ascertain the efficacy of the extracts on a more sustained model of inflammation, the formalin-induced paw edema in rats was studied. It is established that inhibition of formalin-induced paw oedema in rats is one of the most appropriate modus operandi to screen for anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis (Greenwald, 1991). Injection of formalin subcutaneously into hind paw of rats produces localized inflammation and pain. The nociceptive effect of formalin is biphasic, an early neurogenic component followed by a later tissue mediated response (Wheeler-Aceto and Cowan, 1991). Thus formalin-induced arthritis is a model used for the evaluation of an agent with probable anti-proliferative activity (the ability to prevent the systemic spread of a disorder). This experiment is associated with the proliferative phase of inflammation (Banerjee *et al.,* 2000).

The reference drugs and both extracts of *P. stratiotes* significantly suppressed formalin-induced arthritis.

Furthermore, the adjuvant-induced arthritis in rats, a recommended and a convenient model for preclinical studies of drugs used in the treatment of human arthritis, which has

often been used to study the mechanism of action and preventive effects of a number of disease-modifying antirheumatic drugs was used.(Pearson, 1956;Hoffmann *et al*.,1997;Whitehouse, 2007). The development of adjuvant-induced arthritis in the rat is triphasic, just like human rheumatoid arthritis, starting with the induction phase without evidence of synovitis, followed by early synovitis, and finally late synovitis with progressive joint destruction (Malaviya, 2006; Narendhirakannan *et al.,* 2007; Pine *et al.*,2007). A good antirheumatic agent should be able to suppress one or more of these phases.

This study has demonstrated that curative oral treatment of Sprague-Dawley rats using an aqueous or an ethanolic extract of *P. stratiotes* has potent anti-arthritic properties in adjuvant-induced arthritis. All doses of the aqueous extract and the lower dose of the ethanolic extract suppressed joint inflammation significantly and ultimately reduced destruction of the joints, but the lower dose of the aqueous extract prevented the systemic spread of arthritis. Joint protection and suppression of synovitis are the ultimate goals for the treatment of arthritis. (Hoffmann *et al*., 1997; Atzeni *et al.*, 2007) The extracts used in this study achieved these goals with effects similar to that of the reference drugs.

The reference drugs, ie, methotrexate, dexamethasone, and diclofenac achieved inhibition of adjuvant-induced arthritis and prevented any spread of arthritis, which is consistent with the available literature (Issekutz and Issekutz, 1991; Ozhaion *et al.*, 2006; Swierkot and Szechinski, 2006) Methotrexate, a disease-modifying antirheumatic drug and immunosuppressant, was used for comparison because it is a commonly prescribed front-

line treatment for rheumatoid arthritis and the gold standard against which other systemic medications are compared (Ozhaion *et al.,* 2006). Dexamethasone is known to inhibit the release of proinflammatory cytokines (tissue necrosis factor-α and interleukin-1β), which are known to play a central role in the propagation of the disease process in rheumatoid arthritis. .(Issekutz and Issekutz, 1991). The anti-inflammatory effect of diclofenac is mediated mainly through inhibition of cyclo-oxygenase and prostaglandin production. (Furst and Manning, 2001).

Rheumatoid arthritis is associated with weight loss and loss of lean body mass, known as rheumatoid cachexia. Rheumatoid cachexia is thought to be the end result of cytokine-driven hypermetabolism and is a key comorbidity in rheumatoid arthritis.(Roubenoff *et al.,* 1994; Roubenoff *et al.*, 1997).The loss of lean body mass is associated with decreased physical activity, muscle strength, and endurance in performing activities of daily living.(Rall and Roubenoff, 1996) A loss greater than 40% of existing lean body mass often results in death. Roubenoff and Rall, 1993). Weight gain was observed upon physical assessment of arthritic animals treated with AQ PSE 30 and 100 mg/kg and diclofenac, therefore, is ample evidence of good management of rheumatoid cachexia, minimizing the risk of mortality. The significantly low levels of serum C-reactive protein and erythrocyte sedimentation rate in the AQ PSE 30 and 100 mg/kg, ET PSE 30 mg/kg, and all reference drug-treated arthritic rats indicate remission of inflammation. Serum C-reactive protein is a sensitive but nonspecific marker of inflammation that responds rapidly to changes in underlying inflammatory disease activity, making its measurement an important tool for the detection and monitoring of inflammatory disease.(Pepys and Hirschfield, 2003) The

erythrocyte sedimentation rate is a test that measures inflammation in the body indirectly. It measures the rate of settling or sedimentation of red blood cells in a capillary tube. Proteins produced during inflammation cause erythrocytes to move closer and stack up in a group. When this happens, they become denser and settle faster. The closer and faster the erythrocytes settle, the higher the value of the erythrocyte sedimentation rate.(Van den Hoogen *et al.,*1995) Furthermore, low levels of white blood cells indicate that the 30 mg/kg dose of AQ PSE (with the same effect seen for methotrexate treatment) is a potent antiarthritic treatment, given that elevated white blood cell levels are associated with active inflammation (NIH No. 04-4179).

Radiographs are necessary to verify proper remission of disease and for accurate assessment of disease status. The measurement of paw or joint swelling gives an indication of edematous changes in this region, but the actual damage takes place in the tibiotarsal joint (Escandell *et al.,* 2007). Reduction in bone configuration and increased bone resorption are the causes of bone loss in adjuvant-induced arthritic rats (Aota *et al.,* 1996; Findlay and Haynes 2005; Makinen *et al.*, 2007). The X-rays clearly show that the aqueous extract and a low dose of the ethanolic extract of *P. stratiotes* decreased bone loss, even in cases of insignificant edematous changes in the contralateral paws, and therefore reduced bone degradation in arthritis.

Histopathological studies of the paws strengthen the evidence of complete resolution of arthritis with AQ PSE 30 mg/kg despite evidence of pathology in the arthritic animals treated with the reference drugs. The antiarthritic effect of the aqueous and ethanol leaf

extracts of *P. stratiotes* established in this study could be attributable to the presences of flavonoids, alkaloids, and sterols detected after phytochemical screening of the extracts. This assertion is also supported by reports indicating that the presence of many biologically active phytochemicals, such as triterpenes, flavonoids, alkaloids, steroids, tannins, and glycosides, in various plant extracts may be responsible for their pharmacological properties. (Narendhirakannan *et al.,* 2007; Agarwal *et al.,* 1996; Liu *et al.,* 1996; Mbagwu *et al.,* 2007; Singh B *et al.,* 2002).

Fever is one of the most prominent systemic manifestations of acute inflammation, especially when an inflammation is associated with infection (Romanovsky *et al.,* 2005). These reactions represent the primary host defense response to infection; collectively called the "acute-phase reaction" (Blatteis, 1992). The usual view of the mechanism by which infectious fevers are produced stipulates that infectious noxa e.g. bacterial endotoxic lipopolysaccharides (LPS) that invade the body activate mononuclear phagocytes that then produce and release pyrogenic cytokines including IL-1 β and TNF-α. These are transported via the bloodstream to the ventromedial preoptic area of the anterior hypothalamus, the ''fever producing center'', where they operate (Saper, 1998; Roth and De Souza, 2001; Dunn, 2002; Dinarello, 2004). It is, however, doubtful how cytokines, as hydrophilic peptides, could penetrate the brain. That is to say, it is generally believed that, rather than acting directly, the cytokines induce the local generation and release of prostaglandin E_2, a lipid mediator that is obviously thermogenic when injected centrally (Blatteis, 1997; Ivanov and Romanovsky, 2004). Its production is dependent on the activation of two enzymes, cyclooxygenase (COX)-2 and microsomal PGE synthase-1,

which catalyze its conversion from arachidonic acid present in the membranes of cells (Ivanov *et al.,* 2002).

Acetaminophen is a reputable antipyretic analgesic agent, often administered therapeutically to ease pain and fever (Ayoub *et al.,* 2004). The main mechanism proposed is the inhibition of COX, and recent findings suggest that it is highly selective for COX-2 (Hinz *et al.,* 2008). Paracetamol reduces the oxidized form of the COX enzyme, preventing it from forming pro-inflammatory chemicals (Roberts *et al.,* 2001; Högestätt *et al.,* 2005).This leads to a reduced amount of Prostaglandin E2 in the CNS, thus lowering the hypothalamic set-point in the thermoregulatory centre.

Oral administration of both aqueous and ethanolic leaf extracts of *P. stratiotes* as earlier indicated could possibly be inhibiting COX-2 and subsequent production of prostaglandins thereby exhibiting potent hypothermic effect in LPS-induced fever in Sprague-Dawley rats (Sundeep Kumar *et al.,* 2011).

Uveitis can also occur following focal infections at sites distant from the eye including dysentery caused by *Shigella, Salmonella,* and *Yersinia* and is associated with polyarthritis and the histocompatibility antigen HLA-B27.Certain bacterial products including Gram negative endotoxin and Freund's complete adjuvant when administered systemically in rats will elicit uveitis.The active inflammatory constituent of bacteria cell wall is the lipoplysacharide.

Endotoxin-induced uveitis (EIU), stimulated by injection of lipopolysacharide (LPS) is a useful animal model for acute ocular inflammation. LPS stimulation triggers cellular inflammatory response causing the release of nitric oxide (NO), prostaglandin E2 and tumour necrosis factor (TNF)-α (Chen *et al.,* 2001; Boujedaini *et al.,* 2001; Bello *et al.,* 1996; Murakami *et al.,* 2000; Hoekzema et al., 1992; Tracey and Ceremi, 1994). TNF-α indirectly trigger the production of prostaglandin which mediate the process of inflammation which includes uveitis (Blatteis, 1997; Ivanov and Romanovsky, 2004). LPS could also induce nuclear factor-kappaB (NF-κB) activation and monocyte chemo-attractant protein-1 (MCP-1) expression resulting in inflammation (Hang *et al.,* 2005). LPS footpad challenge marked by pyrexia, elevated serum levels of C-reactive proteins and high ESR was associated with infiltration of inflammatory cells, vasodilation of the iris vessels, protein exudation into the aqueous humour which are classical signs of uveitis.

The curative oral administration of AQ PSE and ET PSE showed anti-uveitic activity indicating ocular anti-inflammatory effect. This conforms to earlier studies in which aqueous and ethanolic extracts of *P stratiotes* have been demonstrated to have anti-inflammatory effect (Sundeep Kumar *et al.,* 2011). It managed well vasodilatation associated with inflammation; seen as significant decrements in clinical scores of inflammation graded from photographs taken upon slit lamp examination. The significant decrease in polymorphonuclear neutrophil infiltration into the aqueous also showed anti-inflammatory activity due to inhibition of vasodilatation. During the acute phase of inflammation, neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation (Cohen and Burns, 2002). They migrate through the blood

vessels, then through interstitial tissue, by chemotatic mechanism initiated by interleukin-8 and other inflammatory mediators (De Larco and Wuertz, 2005).

Neutrophils also release an assortment of proteins (Lactoferrin, Cathelicidin, myeloperoxidase, bactericidal/permeability-increasing protein (BPI), Defensins, and the serine proteases neutrophil elastase and cathepsin G, cathepsin, and gelatinase) by a process called degranulation (Lacy, 2006). This increases the total proteins in an area of inflammation. Treatment with AQ PSE and ET PSE resulted in very significant decrements in total protein concentration compared to the uveitic control. This again indicates that inflammation has been managed.

Histopathological studies also confirmed the anti-inflammatory activity of the extract in a similar manner as prednisolone as seen in the photomicrographs of the histology of uveitic and the uveitic but treated rats. Prednisolone is a corticosteroid drug with predominant glucocorticoid activity, making it useful for the treatment of a wide range of inflammatory condition (Czock *et al.,* 2005). The antiinflammatory action of prednisolone is thought to involve phospholipase A2 inhibitory proteins, collectively called lipocortins. Lipocortins, in turn, control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes by inhibiting the release of the precursor molecule arachidonic acid (Ivanov *et al.,* 2002). The extracts possibly could be exerting their ocular anti-inflammatory effect via a similar mechanism. Earlier studies with the extracts conducted to establish its anti-arthritic effect revealed that AQ PSE and ET PSE had

similar observed effects as dexamethasone (a corticosteroid) and diclofenac (a COX inhibitor).

The presence of biologically active phytochemicals present in both the aqueous and ethanolic extracts of *P. stratiotes* could again have contributed to the ocular anti-inflammatory activity. Tannins (Mota *et al.,* 1985; Owoyele *et al.,* 2010), flavonoids (Borissova *et al.,* 1994; Hämäläinen *et al.,* 2007), sterols (Bouic *et al.,* 1996; Bouic, 1998; Akihisa *et al.,* 2007), alkaloids (Barbosa-Filho *et al.,* 2006) and glycosides (Odontuya *et al.,* 2005; Liu and Wang, 2011) have been documented to have anti-inflammatory effect via several mechanisms

Regarding the safety of the extracts, oral administration to healthy rats showed a comparable glucose concentrations between the extract-treated and control. This implied that the extracts do not induce gluconeogenesis in the liver to cause hyperglycemia. The elevation of urine protein could be a sign of impaired filtration as in glomerulonephritis (Pillitteri *et al.*, 2009), impaired tubular protein reabsorption and degradation capacity of renal tubules (Prakash *et al.*, 2008). The extract possibly could be rather rich in proteins. The elevated levels of microalbumin, however, confirms a possible acute kidney function impairment (Was´En *et al.*, 2004; Luo and Kong, 2005; LTO, 2011) as it is an important prognostic marker for kidney disease. The extract does not induce acidosis or alkalosis as urine pH was not affected. No blood in the urine indicates that the extracts do not cause kidney or bladder calculi, and/or damage to the urinary tract (Raja, 2011). Although quantities of urobilinogen in the urine are quite small, it is an important indicator of liver

function and red blood cell catabolism (Troy, 2005). The elevated urobilinogen levels indicate possible hemolysis of red blood cells caused by the extract confirmed by the significant reduction in RBC count with treatment and the amber colored urine observed.

Nevertheless, pH of the extracts was consistent with that of conventional eye preparation tolerable for topical use and therefore, caused insignificant irritation of the tissues of the eye (Stein *et al.*, 2000). the comparable discharge, hyperemia, chemosis and opacity of the treated and the untreated eye indicates that the extract does not pose treat to the tissues of the eye. This extract was found to be safe for topical use in rats.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSIONS

The results of this study have provided evidence to support the use of *P. stratiotes* leaves as an anti inflammatory/anti-arthritic and antipyretic agent in traditional medicine. These effects might be partially or wholly due to:

- Possible inhibition or interference with the production of some inflammatory mediators, especially prostaglandins, histamine, serotonin and bradykinin.
- No ocular toxicity was observed as the pH of the extracts was found to be consistent with conventional ophthalmic preparations.

5.2 RECOMMENDATIONS

- It is recommended that user stick to lower doses of the extract as it was found to be more efficacious than higher doses
- It is however, recomended that users verify liver and kidney function by performing these tests, as well as a blood count from time to this to ensure maximum safety with the use of this product orally.
- Other physicochemical properties such as melting point, boiling point, specific gravity should also be verified.

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APPENDIX

PREPARATION OF PROSTAGLANDIN E2

Stock solutions of 10 mg/ml was prepared in ethanol and further diluted with 0.1 M phosphate buffer to obtain the desired concentration (the remaining amount of ethanol is usually insignificant).

PREPARATION OF BRADYKININ

An amount of 25mg powdered bradykinin acetate salt was dissolved im 0.1 M acetic acid was futher diluted with 0.1 M phoaphate buffer saline to obatain the requiredconcentration. The preparation was kept at -20 ˚C before and during use.

PREPARATION OF CARRAGEENAN SUSPENSION

A 2% carrageenan suspension was prepared by sprinkling small amounts of the powder (200 mg) evenly over the surface of 10 ml of 0.9% NaCl solution and left to soak between additions. It was then left for 2-3 hours before use.

PREPARATION OF HISTAMINE SOLUTION

A 100 mg powder of histamine was dissolved in 10 ml of 0.9% NaCl solution and was stirred intermittent with a stile glass rod.

PREPARATION OF SEROTONIN SOLUTION

A 100 mg powder of serotonin cratinine sulphate was dissolved in 10 ml of 0.9% NaCl solution and was stirred intermittent with a stile glass rod.

PREPARATION OF COMPLETE FREUND'S ADJUVANT (CFA)

50 mg heat-killed Mycobacterium tuberculosis [strains C, DT and PN (mixed) obtained from the Ministry of Agriculture, Fisheries and Food, U.K] was finely grounded in a mortar using a pestle. Liquid paraffin was added gradually to make 20 ml of 5 mg ml-1 suspension.

PREPARATION OF PHOSPHATE BUFFER

Sodium dihydrogen phosphate monohydrate (8.942 g) and disodium hydrogen phosphate heptahydrate (9.433 g) were dissolved in 500 ml distilled water to make 0.2M sodium phosphate buffer, pH 6.6. Sodium dihydrogen phosphate monohydrate (1.558 g) and disodium hydrogen phosphate heptahydrate (10.374 g) were dissolved in 500 ml distilled water to make 0.1M sodium phosphate buffer, pH 7.4.

FOOT VOLUME MEASUREMENT

A liquid column containing water was placed on a balance. When an object is immersed, the liquid applies a force F to attempt its expulsion. Physically, F is the weight (W) of the volume of liquid displaced by that part of the object inserted into the water. A balance was used to measure this force (F=W). Therefore, the partial or entire volume of any object, for example the inflamed foot of a rats, can be calculated thus, using the specific

gravity of the immersion liquid, at equilibrium mass/specific gravity = volume (V). Since water was used as the immersion liquid in this case, the mass or weight of the foot inserted in the water will be the same as its volume.the extent of oedema at time t (measured as V) will be V1 – V0. The foot being measured was kept away from contacting the wall of the column containing the water whilst the value on the balance was being read.

PREPARATION OF TURK'S STAIN SOLUTION

2ml glacial acetic acid was added to 2% acetic acid in a 100ml volumetric flask. The miture was diluted with distilled water to the 100 ml mark.

Type a title

Table 19: AUCs for carragennan-induced paw edema in rats treated with AQPSE or ET PSE or Diclofenac and the vehicle treated (Control)

	Control	10 mg/kg Diclo	30 mg/kg	100mg/kg	300mg/kg
AQ PSE	59.920±2.383	25.810±7.469	32.240±3.936	29.640±1.736	47.740±6.996
ET PSE	48.960±3.841	19.710±2.940	24.470±5.50	27.560±1.693	35.940±5.851

Table 20: AUCs for Histamine-induced paw edema in rats treated with AQPSE or ET PSE or Chlorophenaraime and the vehicle treated (Control)

	Control	0.35 mg/kg Chlor	30 mg/kg	100mg/kg	300mg/kg
AQ PSE	51.770±1.888	29.140±5.214	31.310±3.254	22.620±3.496	34.860±5.235
ET PSE	49.780±2.004	29.140±5.214	21.060±2.121	18.510±2.188	25.640±5.991

Table 21: AUCs for Serotonin-induced paw edema in rats treated with AQPSE or ET PSE or Granisetron and the vehicle treated (Control)

	Control	10 mg/kg Diclo	30 mg/kg	100mg/kg	300mg/kg
AQ PSE	37.240±1.646	12.290±2.783	22.430±2.939	21.020±1.819	29.980±5.303
ET PSE	38.31±1.857	12.290±2.783	22.910±3.089	25.940±3.637	38.760±5.637

Table 22: AUCs for Prostaglandin-induced paw edema in rats treated with AQPSE or ET PSE or Diclofenac and the vehicle treated (Control)

	Control	10 mg/kg Diclo	30 mg/kg	100mg/kg	300mg/kg
AQ PSE	62.860±2.213	19.860±6.568	29.860±2.722	18.840±2.754	42.790±5.823

ET PSE	62.86±2.213	19.860±6.568	30.320±4.283	29.260±2.273	26.670±3.157

Table 23: AUCs for bradykinin-induced paw edema in rats treated with AQPSE or ET PSE and the vehicle treated (Control)

	Control	30 mg/kg	100 mg/kg	300mg/kg
AQ PSE	94.220±3.988	51.250±5.505	65.350±2.862	47.400±5.281
ET PSE	94.220±3.988	55.340±3.360	50.150±8.940	45.100±9.653

Table 24: AUCs for formalin-induced paw edema in rats treated with AQPSE or ET PSE and the vehicle treated (Control)

	Control	30 mg/kg	100 mg/kg	300mg/kg
AQ PSE	834.100±38.730	538.300±65.311	386.100±9.830	511.400±65.860
ET PSE	807.100±37.270	431.300±82.920	211.000±43.870	204.300±44.810

Table 25: AUCs for formalin-induced paw edema in rats treated with methorexate (MET), dexamethasone (DEX), diclofenac (DICLO) and the vehicle treated (Control)

Control	0.3 mg/kg MET	1 mg/kg DEX	300mg/kg DICLO
815.500±37.600	353.600±61.24	229.900±34.220	379.200±72.120

Table 26: AUCs for adjuvant-induced paw edema in rats treated with AQPSE (ipsilateral and contralateral paw) and the CFA Control

	CFA	30 mg/kg	100 mg/kg	300mg/kg
Ipsilateral	4043.00±279.90	950.40±131.50	2083.00±264.60	1596.00±416.80
Contralateral	1361.00±158.70	331.90±44.10	999.60±85.31	697.90±132.300

Table 27: AUCs for adjuvant-induced paw edema in rats treated with ET PSE (ipsilateral and contralateral paw) and the CFA Control

	CFA	30 mg/kg	100 mg/kg	300mg/kg
Ipsilateral	4048.00±281.00	2433.00±269.40	2647.00±371.60	3066.00±375.00
Contralateral	1360.00±158.70	630.30±51.14	1091.00±376.80	1596.00±589.40

Table 28: AUCs for adjuvant-induced paw edema in rats treated with methotrexate, dexamethasone, doclofenac (ipsilateral and contralateral paw) and the CFA Control

	CFA	0.3 mg/kg MET	1 mg/kg DEX	0.46mg/kgDICL
Ipsilateral	4054.00±280.70	1253.00±120.80	1031.00±205.00	1485.00±135.70
Contralateral	1361.00±159.50	381.70 ±54.41	180.00±39.11	649.70±71.27

Table 29: AUCs for complete freud adjuvant (CFA), incomplete freud adjuvant (IFA) and normal rats kept under experimental condition

	CFA	IFA	NORMAL
Ipsilateral	4080.00±624.50	738.50±112.60	197.00±59.18
Contralateral	1357.00±157347.90	513.00±59.35	186.10±43.46

Table 30: Means and s.m.e of polymorphonuclear neutophil (PMN) count in AqH of uveitic rat, uveitic rat treated with AQ PSE, ET PSE Prednisolone and normal rats kept under experimental conditions

	Uveitic	30 mg/kg	100 mg/kg	300 mg/kg	30 mg/kg Pred	Normal
AQ PSE	1200.00±57.74	266.70±16.76	166.70±16.67	283.30±44.10	250.00±28.87	16.67±16.67
ET PSE	1200.00±57.74	600.00±28.87	350.00±28.87	500.00±28.87	250.00±28.87	250.00±28.87

Table 31: Means and s.m.e of protein count in AqH of uveitic rat, uveitic rat treated with AQ PSE, ET PSE Prednisolone and normal rats kept under experimental conditions

	Uveitic	30 mg/kg	100 mg/kg	300 mg/kg	30 mg/kg Pred	Normal
AQPSE	1726.00±105..50	1085.00±143.60	1289.00±52.83	1308.00±40.43	1118.00±135.50	890.00±21.60
ET PSE	1726.00±105..50	895.10±13.22	1096.00±106.40	1186.00±123.0	1118.00±135.50	890.00±21.60