**KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI, GHANA**

# THE FRUIT EXTRACT OF *XYLOPIA AETHIOPICA* (DUNAL) A. RICH. (ANNONACEAE) AND ITS PRINCIPAL CONSTITUENT, XYLOPIC ACID, MODULATE INFLAMMATION

by

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**DOCTOR OF PHILOSOPHY**

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# DECLARATION

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



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# ABSTRACT

*Xylopia aethiopica* extract is traditionally employed, among other uses, in the treatment of inflammatory conditions. This study aimed at investigating the anti-inflammatory properties of the aqueous ethanol (70% $^{v}/_{v}$) extract of the dried fruit of *Xylopia aethiopica* and its principal constituent, xylopic acid. The anti-inflammatory effect of the extract and xylopic acid were preliminarily established by *in vitro* assay employing the albumen denaturation method. The extract and xylopic acid showed a concentration-dependent inhibition of protein denaturation at the concentration ranges 6.25 - 200 µg $ml^{-1}$ with $IC_{50}$ of 10.12 µg $ml^{-1}$ and 15.55 µg $ml^{-1}$ respectively. The *in vivo* acute anti-inflammatory effect of the extract (30, 100, 300 mg $kg^{-1}$) and xylopic acid (10, 30, 100 mg $kg^{-1}$) were established using the carrageenan-induced acute inflammation model in mice. The inhibition of inflammation obtained were significant, with the highest dose suppressing oedema to 48.13 ± 10.90% and 60.81 ± 3.25% by the extract in both prophylactic and therapeutic models; and 56.58 ± 3.91% and 68.11 ± 3.59% by xylopic acid in the prophylactic and therapeutic models respectively of the inflamed control response. In the allergy studies, passive cutaneous anaphylactic and systemic anaphylactic studies were conducted. Pinnal inflammation reaction model and compound 48/80-induced passive cutaneous anaphylaxis model were employed in the passive cutaneous anaphylaxis studies. The antigen-induced inflammation was significantly suppressed by both the extract and xylopic acid with maximum suppression of 62.83 ± 0.83% and 77.62 ± 2.26% respectively. The extract and xylopic acid inhibited mast cell degranulation and caused a reduction in the extent of mast cell degranulation and skin tissue damage. Compound 48/80-induced systemic anaphylaxis and Lipopolysaccharide (LPS)-induced septic shock models were employed in the systemic anaphylaxis studies. The extract (30, 100, 300, 1000 mg $kg^{-1}$) and xylopic acid (10, 30, 100, 300 mg $kg^{-1}$) offered 40 - 100% and 40 – 90% survival proportion in the compound 48/80-induced systemic anaphylaxis respectively. In the LPS-induced septic shock, the intraperitoneal administration of LPS caused significant mortality in vehicletreated animals. In the extract-treated animals, there was dose-dependent protection against endotoxic shock with maximum protection of 70%, xylopic acid also gave a maximum of 70% protection from endotoxic shock. In the chronic inflammatory study, the role of reactive oxygen species inhibition in the chronic inflammatory process was investigated using the acetic acid-induced colitis model in rats. The extract and xylopic acid showed anti-oxidant activity in acetic acid-induced colitis by decreasing

the activity of *myeloperoxidase* and lowering lipid peroxidation as well as increasing the activities of *catalase*, *superoxide dismutase* and *ascorbate peroxidase in vivo* hence affecting the generation of reactive oxygen species which are involved in the inflammatory process and affects cellular activity and proliferation during the inflammatory process. The anti-arthritic property of the extract and xylopic acid were investigated using adjuvant-induced arthritis model in rats. The extract, at the highest dose, reduced the total limb swelling over 28 days in the ipsilateral limb to 41.07 ± 4.71% prophylactically and 42.90 ± 5.60% therapeutically of the arthritic control rats; with xylopic acid showing a maximal reduction in total limb swelling in the ipsilateral limb to 51.29 ± 8.34% prophylactically and 62.21 ± 11.85% therapeutically of the arthritic control rats with its highest dose administered. The extract and xylopic acid also showed inflammation alleviation in the other indices monitored. With the mechanism elucidation studies carried out, the extract and xylopic acid showed antihistaminic activity with inhibitory effect on histamine-induced inflammation, indirect anti-histaminic activity in the clonidine-induced catalepsy model and possible competitive $H_1$ receptor blockade. The extract and xylopic acid also showed effect on serotonergic, bradykinin and phospholipid/arachidonate pathways of inflammation with inhibitory effect on expression of intercellular adhesion molecule 1 and cellular component recruitment in the inflammatory process. Xylopic acid was identified to inhibit the serum expression of pro-inflammatory cytokines, IL-6 and TNF-alpha, in chronic inflammation. These novel findings show the anti-inflammatory property of the aqueous ethanol extract of the dried fruit of *Xylopia aethiopica* and its principal constituent, xylopic acid, obtained from the bio-fractionation of the petroleum ether extract of the dried fruit of *Xylopia aethiopica*.

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# DEDICATION

To the memory of my beloved friends, Ama Mensimah Abban and Renalyn P. Lanoy.

I pray for the interminable repose of their souls.

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# ABBREVIATIONS

| | |
|---|---|
| AIA | Adjuvant-Induced Arthritis |
| ATCC | American Type Culture Collect |
| BSA | Bovine serum albumen |
| CFA | Complete Freund's adjuvant |
| ELISA | Enzyme-linked immunosorbent assay |
| IFA | Incomplete Freund's adjuvant |
| LPS | lipopolysaccharide |
| SDS-PAGE | Sodiumdodecyl Sulphate-Polyacrylamide Gel Electrophoresis |
| XAE | *Xylopia aethiopica* extract |
| XA | Xylopic acid |

# *Chapter 1*

# INTRODUCTION

## 1.1 *XYLOPIA AETHIOPICA* (DUNAL) A. RICH.

Xylopia has a Greek origin ('xylon pikron') meaning 'bitter wood', while aethiopica makes reference to its Ethiopian origin (Orwa *et al.,* 2009). The dried fruit of *Xylopia aethiopica* belongs to the family Annonacaea and has names which include *"negro pepper"*, *"West African pepper"* (English); "*hab al-zelim*", "*fulful as-Sudan"* (Arabic); *"pimenta-da-áfrica"*, *"pimenta-do-congo"* (Portugese); *"mchofu"* (Swahili). In Ghana, it is known as "*hwentea*" (Twi) (Orwa *et al.,* 2009).

Irvine (1961) reported that *Xylopia aethiopica* is a slim, tall, evergreen, aromatic tree, up to 15–30 m high and about 60–70 cm in diameter with straight stem, many-branched crown and sometimes buttressed. The bark is grey-brown, smooth or finely vertically fissured and peels easily. It has simple leaves which alternates and also oblong, elliptic to ovate, 8-16.5 cm by 2.8-6.5 cm, leathery, bluish-green and with no hairs above, but with fine brownish hairs below, and has entire margin, and glabrous. The petiole is 0.30.6 cm long, thickset and dark in colour. The flowers are bisexual, solitary or in 3-5 flowered fascicles or in strange, sinuous, branched spikes, or cymes, up to 5.5 cm by 0.4 cm and creamy-green. The fruits are small, carpels 7-24, forming dense cluster, twisted bean-like pods, dark brown, cylindrical, 1.5-6 cm long and 4-7 mm thick; the contours of the seeds are visible from outside (Fig 1.1). The seeds are black, 5-8 per pod, kidney-shaped and of approximately 10 mm length with a yellow papery aril. The hull is aromatic, but not the seed itself (Orwa *et al.,* 2009).

**Figure 1.1. The dried fruit of *Xylopia aethiopica*.** The fruits are small, carpels, forming dense cluster, twisted bean-like pods, dark brown, cylindrical, long and thick; the contours of the seeds are visible from outside (Adapted from Gernot Katzer's spice pages).

## 1.2 XYLOPIC ACID

Xylopic acid (Fig 1.2) is a crystalline solid which occurs naturally and can be isolated from the fruit of *Xylopia aethiopica*. It belongs to a class of diterpenes known as kauranes (Ekong and Ogan, 1968). These kauranes have rigid tetracyclic skeleton and form intermediates in the biosynthesis of plant growth hormones such as gibberellins (Bresciani *et al.,* 2004).

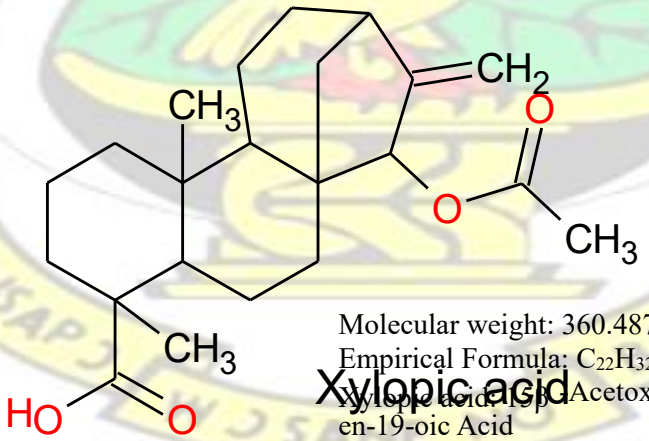


**Figure 1.2. Structure of xylopic acid**

Xylopic acid has a refractive index of 1.547 with a density 1.15 g $cm^{-3}$. Its flash point is 159°C with enthalpy of vaporization of 80.9 kJ $mol^{-1}$. Xylopic acid boils at 475.1°C at

760 mmHg and has a vapour pressure, 2.46 x $10^{-10}$ mmHg at 25°C. When heated to decomposition it emits acrid smoke and irritating vapours (Woode *et al.,* 2012). The melting point of xylopic acid has been established experimentally to be 261 - 262°C and retardation factor (Rf) value of 0.53 using petroleum ether (40 - 60°C): ethyl acetate (9:1) as solvent system (Adosraku and Kyekyeku, 2011) with sparing solubility in petroleum ether, ethanol, methanol and ethyl acetate but soluble in chloroform (Woode *et al.,* 2012).

## 1.3 PREVIOUS STUDIES ON *XYLOPIA AETHIOPICA* AND ITS ISOLATES

The plant contains anonaceine, alkaloids, rutin, volatile aromatic oil and a fixed oil. High amounts of copper, manganese, and zinc have been reported present in *Xylopia aethiopica* (Smith *et al*., 1996). The essential oil has been well characterised with linalool, β-trans-ocimene, α-farnesene, α-pinene, β-pinene, myrtenol, β-phellandrene, and 3-ethylphenol as the major volatile constituents (Tairu *et al.,* 1999). Researchers describe the intense 'pepperish note' of the oil of the fruit to be largely due to linalool and provides that characteristic aroma of the ground, dried, smoked fruits of *Xylopia aethiopica*. The essential oil yield varies from 2.0% to 4.5%. The bark oil consists mainly of α-pinene, trans-pinocarveol, verbenone and myrtenol and differs significantly from that of the leaf oil (spathulenol, cryptone, beta-caryophyllene and limonene). Key constituents are diterpenic and xylopic acids.

The isolates of *Xylopia aethiopica* have been examined for antimicrobial activity against five micro-organisms, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Xylopic acid and two other

diterpene isolates were found to have antimicrobial properties (Boakye-Yiadom *et al*., 1977).

When analysed by gas chromatography/mass spectrometry, essential oils were obtained from the various extracts. Ninety three individual compounds were identified using Kováts indices, mass spectra, and standard compounds. The monoterpene hydrocarbons formed the main portion in all studied samples. β-Pinene was predominant in all cases, while trans-*m*-mentha-1(7),8-diene was the main compound in the essential oils of the leaves and the barks of roots and stems. Their potential antioxidant activity was also investigated and found to be significant in scavenging superoxide anion radical (Karioti *et al*., 2004).

Assays have revealed that kauren-19-oic acid (KA) primarily intercalates with DNA and not with *topoisomerase I*. Fluorescence microscopy using acridine orange/ethidium bromide staining indicated that KA can induce both apoptosis and necrosis in HL-60 cell cultures. However, KA appeared not selective to cancer cells, since it also inhibited the lymphocytes proliferation ($IC_{50}$, 12.6 µg $ml^{-1}$). KA-treated HL60 cells displayed decreased proliferation (5-bromo-2';-deoxyuridine incorporation assay) and *topoisomerase I* activity (DNA relaxation assay) (Cavalcanti *et al*., 2009).

KA has also been documented to have biological activity including analgesia (Block *et al.,* 1998), diuretic, vasorelaxant, anti-inflammatory and anti-pyretic effects in murine models (Somova *et al.,* 2001; Sosa-Sequera *et al.,* 2010). The anti-inflammatory activity of most terpenes has been explained to be as a result of inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity. These terpenes inhibit NF-κB and inhibitory kappa B (IκB) kinase *in vivo*. They, therefore, impair

inflammatory signalling by inhibiting NF-κB inducing kinase, a member of the mitogen activated protein kinase (MAPK) superfamily (Castrillo *et al.,* 2001).

## 1.4 TRADITIONAL USES OF *XYLOPIA AETHIOPICA*

Medicinally, the fruits are used for cough, a calmative, purgative and repulsive to pain. In Liberia, the spice is smoked and inhaled for respiratory ailments. The fruit is often integrated in preparations for enema and external uses, use of its repulsive properties for pains in the ribs, chest and generally for any painful area, neuralgia and in the treatment of boils, skin eruptions and as a liniment for lumbago in Ghana (Irvine, 1961; Abbiw, 1990). The fruit decoction is of use in the treatment of bronchitis and dysenteric conditions, and as a medicine for bulimia. The odiferous roots of the plant are used in tinctures, administered orally as worm expellant and other parasitic animals from the intestines, or in teeth rinsing and mouth wash extracts against toothaches (Burkill, 1985). The seeds of the plant are mixed with other spices, rubbed on the body as cosmetic and scent, and as perfume for clothing (Burkill, 1985). The fruits of *Xylopia aethiopica* when mixed with the roots in a preparation are used in the treatment of rheumatism (Johnkennedy *et al.,* 2011) and other inflammatory conditions in folklore medicine in Nigeria, Ghana and Cameroon (Burkill, 1985).

## 1.5 INFLAMMATION

Inflammation is a complex reaction to harmful agents, such as microbes and damaged necrotic cells and consist of vascular responses, migration and activation of leukocytes, and systemic reactions. Inflammation is a defensive response that begins after cellular injury, which may be caused: mechanically (e.g., by pressure or foreign bodies),

chemically (e.g., by toxins, acidity, and alkalinity), physically (e.g., by temperature), by internal processes (e.g., uremia), by microorganisms (e.g., bacteria, virus, and parasites), necrotic tissue and/or immunological reactions. Inflammation is regulated by mast cells that are in close proximity to autonomic nerves (Wakefield and Kumar, 2001). Inflammation is classified into acute and chronic forms.



### 1.5.1 Acute inflammation

The acute inflammatory reaction is regarded as a series of interconnected and overlying events including coagulation, an increase in blood flow and vascular permeability at the affected site, oedema, localized pain, the migration and accumulation of inflammatory leukocytes from the blood vessels into the tissue, formation of granulation tissue and, finally, tissue repair.

Acute inflammation regularly has a rapid onset, becoming apparent within minutes or at most hours after tissue trauma, and may be characterised by the classical symptoms of redness, heat, oedema and pain (Brenner *et al.*, 2006). The symptoms of acute inflammation symbolise a multipart group of vascular, neurological and cellular responses to the initiating trauma. Inflammation control is mediated to a large extent by soluble proteins (cytokines, growth factors) acting between inflammatory cells and non-haematopoietic cells, such as fibroblasts and vascular endothelial cells, within the traumatised tissue. The cytokines and growth factors normally work in tandem and a harmonised fashion to seize and/or eliminate the injurious agent and then to restore and maintain homeostasis in the tissue. Inability to resolve an acute response results in a chronic event (Villarreal *et al.,* 2001). A component of the acute inflammatory response manifests as allergy.

#### 1.5.1.1 Allergy

Allergy is an altered reactivity of the immune system to foreign body whether it results in immunity or harmful effect (Kay, 2006). Atopic (IgE mediated) allergy arises when an individual produces increasing levels of immunoglobulin E (IgE). Clinical allergy requires efficient cross-linking of high-affinity IgE receptors (FcεRI) on mast cells and basophils (Holt *et al.,* 1999). At least two FcεRI-bound IgE molecules must capture a single allergen (bivalent interaction) to induce mediator release. Acute symptoms of allergy are caused by histamine and there is large release into circulation (e.g. anaphylaxis). Nonatopic (non-IgE mediated) allergy mechanism may involve T Helper 1 (Th1) cells (Lebrec *et al*., 1999). A dramatic form of allergy is anaphylaxis.

#### 1.5.1.2 Anaphylaxis

Anaphylaxis is an acute but mostly severe multisystem allergic reaction with sudden onset upon contact with an allergen. Anaphylaxis may be difficult to diagnose with 20% of cases with no skin manifestation without any signs of vasomotor instability (Sampson *et al*., 2006). After exposure to an allergen, there is stimulated production of IgE antibody. Initial exposure causes decreased concentration of antibodies but IgE binds to mast cells and basophils. If there is further exposure, antigen binds to IgE antibodies causing release of mediators such as histamine, slow reacting substance-A (SRS-A), *tryptase*, prostaglandins and leukotrienes. These mediators then increase vascular permeability, mucous secretion, airway oedema, hypotension, bronchospasm and bronchial smooth muscle tone (Bochner and Lichtenstein, 1991).

### 1.5.2 Chronic inflammation

Chronic inflammation is marked by continuing inflammation and attempted tissue healing by repair which occur simultaneously (Wakefield and Kumar, 2001). At a microscopic level, chronic inflammation may be described as the pattern of cellular response, although this is variable and not altogether reliable. It may either be a resultant effect of acute inflammation or occur without an acute phase. Chronic inflammation may be caused by an irritant that normally induces an acute inflammatory response but fails to be eliminated or is continuously generated locally; or self-antigens that induce an autoimmune response; or an irritant of low intensity but long persistence, which does not induce a significant acute inflammatory reaction.

Important differences between acute and chronic inflammation relate to the relative balance between exudation and cellular recruitment, as well as the types of cells that predominate in the inflammatory response. In chronic inflammation there is typically a less pronounced exudative response and increased inflammatory cellular recruitment, which may be accompanied by local cellular proliferation. The dominant infiltrating cell in all forms of chronic inflammation is the macrophage contrary to neutrophil leukocytes in acute inflammatory response. Depending on the nature of the irritant, different profiles of inflammatory mediators and growth factors are generated locally, giving rise to different morphological patterns of chronic inflammation (Wakefield and Kumar, 2001).

## 1.6 MANAGEMENT OF INFLAMMATION

Anti-inflammatory agents are essential in the management of inflammatory conditions.

Although anti-inflammatory agents do not possess disease modifying property, they are able to reduce pain and swelling and can facilitate exercise and physical therapy. There are the classical non-steroidal anti-inflammatory drugs (NSAIDS); which nonselectively inhibit *cyclooxygenase-1* (COX-1) and *cyclooxygenase-2* (COX-2); and the COX-2 selective inhibitors. COX-2 inhibitors (coxibs) show their main advantage in considerable less potential of causing gastro-duodenal ulceration (Silverstein *et al.,* 2000; Bombardier *et al.,* 2000) when used in acute and chronic inflammatory conditions.

Disease modifying anti-rheumatic dugs (DMARDs) therapy when initiated early in chronic inflammation can retard radiographic progression (Tsakonas *et al.,* 2000; Lard, 2001). The commonest conventional DMARDs in use include methotrexate, sulphasalazine, leflunomide and hydroxychloroquine.

Oral and parenteral corticosteroids are increasingly being used in acute and chronic inflammatory conditions. A number of reviews (Van Everdingen *et al.,* 2002; Landewe *et al.,* 2002) have demonstrated their efficacy in rapidly reducing symptoms and inflammatory signs and reduction of progression of erosion and joint damage when corticosteroids are used early in the disease course. Intra-articular corticosteroids are also important adjuncts in inflammatory polyarthritis management. The newest developments in inflammation management are the biological agents targeted against specific cytokines or immunoreactive cells central to the inflammatory process. These agents include etanercept, infliximab and adalimumab which are anti- tumour necrosis factor α (TNF-α) agents; and vedolizumab which is an alpha 4 integrin inhibitor. These agents have been shown to slow radiological progression and improve symptoms (Lipsky *et al.,* 2000).

## 1.7 JUSTIFICATION, AIMS AND OBJECTIVES OF STUDY

### 1.7.1 JUSTIFICATION

The World Health Organisation estimates that up to 80% of the world's population is reliant on plants for their Primary Health Care (Bandaranayake, 2006). It has become more prudent to undertake more research into plant-based medicine to establish and improve on their efficacy and safety. In most rural communities in Africa and Ghana in particular, primary source of health care continues to play a significant role since it is inaccessible and costly to obtain modern medical services.

Inflammation has always been an integral part of most disease conditions that afflicts man (Serhan, 2004). There are many proven drugs on the market which are very effective therapeutically in the management of inflammation and inflammatory conditions but with varying side effects. Current orthodox treatment of inflammation and allergic conditions accompanied by marked toxic effects, some life threatening (Loewen, 2002; Mirshafiey *et al*., 2005; Wong *et al*., 2005). It is, therefore, prudent that much more viable treatments with milder toxic effects, reliable, efficacious and cost effective herbal-based products made available to the populace due to their continuing wide usage be investigated and possibly used as alternative means of such management.

### 1.7.2 AIM AND OBJECTIVES

The aim of this study is to investigate and establish anti-inflammatory activity of the dried fruit extract of *Xylopia aethiopica* as informed by its traditional use for inflammatory conditions and respiratory ailments. The major constituent of *Xylopia aethipica* is xylopic acid, a kaurene diterpene. Despite the reported anti-inflammatory property of many of the kaurane diterpenes, there is no pharmacological data on the

anti-inflammatory potential of xylopic acid. This study therefore also seek in addition to evaluate the anti-inflammatory property of xylopic acid. *In vitro* and *in vivo* animal models of inflammation will be employed in the study.

Specific objectives of the study include the following;

I. To evaluate, both acute and chronic, the anti-inflammatory activity of the ethanol extract of *Xylopia aethiopica* and xylopic acid.

II. To evaluate the anti-allergic activity of *Xylopia aethiopica* extract and xylopic acid

III. To assess the mechanism(s) of action of the *Xylopia aethiopica* extract and xylopic acid in inflammation.

*Chapter 2*

# PLANT COLLECTION AND EXTRACTION

## 2.1 MATERIAL COLLECTION

The dried fruit of *Xylopia aethiopica* was obtained from the Kwame Nkrumah University of Science and Technology (KNUST) Botanical Gardens (6°41'7" N 1°33'48" W), Kumasi between September and November, 2011. The fruit was authenticated at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Science, KNUST. A voucher specimen (No. FP/09/77) has been kept at the herbarium of the Faculty.

## 2.2 EXTRACTION OF PLANT MATERIAL

Three (3) kg of the dried fruit was pulverised using heavy duty blender (37BL85 (240CB6) WARING Commercial, USA) into fine powder. The powdered material (2.7 kg) was placed in cylindrical jars and macerated with 5 L of 70% ($^{v}/_{v}$) ethanol for 3 days. The filtrate was concentrated using rotary evaporator (Rotavapor R-210, BUCHI, Switzerland) at a temperature of 60°C and further dried in an oven (Gallenkamp OMT Oven, SANYO, Japan). This resulted in a 167 g greenish-solid mass of aqueous ethanol extract of the dried fruit of *Xylopia aethiopica* which was reconstituted as an emulsion using Tween-80 when required and henceforth referred to as *Xylopia aethiopica* extract (XAE).

## 2.3 EXTRACTION AND RECRYSTALLIZATION OF XYLOPIC ACID

The extraction of xylopic acid was carried out based on the method described by Ekong and Ogan (1968). About one (1.37) kg of the dried fruit of *Xylopia aethiopica* was

pulverised using a heavy duty blender (37BL85 (240CB6), WARING Commercial, USA) and placed in cylindrical jars, soaked with 5 L of petroleum ether (40 - 60°C) and allowed to stand for 3 days. The petroleum ether extract was collected and concentrated using a vacuum rotary evaporator (Rotavapor R-210, BUCHI, Switzerland) at a temperature of 50°C. Five (5) ml ethyl acetate was added to the concentrate and allowed to stand for 2 days after which the xylopic acid crystals formed were washed with petroleum ether (40 - 60°C).

The xylopic acid obtained was purified by recrystallization. The recrystallization process involved the dissolution of 32 g impure xylopic acid in 96% ($^{v}/_{v}$) ethanol. The resulting concentrated solution was filtered while hot, and crystals of xylopic acid were deposited after the solution cooled and stood for two days. The yield of the isolated/purified xylopic acid was 1.47% ($^{w}/_{w}$). The purity of the isolated xylopic acid was assessed using high performance liquid chromatography (HPLC) as described by Woode *et al.* (2012). The chromatograph consisted of LC-10AT Shimadzu pump with a programmable absorbance detector (783A Applied Biosystems) and Shimadzu CR501 Chromatopac. The column employed was Phenomenex Hypersil 20 micron C18 200 × 3.20 mm. The mobile phase contained methanol (90%) and water (10%) eluted isocratically at 0.5 ml $min^{-1}$. Twenty (20) µl portions of a suitable concentration of pure xylopic acid were loaded and injected unto the column after dissolving in the mobile phase at 60°C. The eluent was monitored at 206 nm. Similar portions of the *Xylopia aethiopica* extract and the isolated xylopic acid crystals were loaded and injected. The peak(s) were noted as component(s) of the *Xylopia aethiopica* and xylopic acid. The purity of the isolated xylopic acid was 95% ($^{w}/_{w}$). The pure compound when required was constituted as an emulsion using Tween-80 and henceforth referred to as xylopic acid (XA).

# *Chapter 3*

## ACUTE ANTI-INFLAMMATORY ACTIVITY OF THE AQUEOUS ETHANOL EXTRACT OF *XYLOPIA AETHIOPICA* AND XYLOPIC ACID

### INTRODUCTION

Acute inflammatory process is a rapid response to an injurious or phlogenic agent that entails the delivery of mediators of host defense (leukocytes and plasma proteins) to the injury site (Kumar *et al.,* 2014). Acute inflammatory process begins within seconds to minutes following tissue injury. The damage may be restricted to physical damage or may involve the activation of an immune response.

*Xylopia aethiopica*, is reported to be used in the management of inflammation in folklore medicine. The extract and its principal constituent, xylopic acid, in the acute inflammatory process, were therefore evaluated. Use was made of an *in vitro* antiinflammatory assay as described by Mizushima and Kobayashi (1968) and carrageenan-induced paw oedema, an *in vivo* murine model of inflammation as described by Winter *et al.* (1962).

### 3.1 MATERIALS AND METHODS

#### 3.1.1 MATERIALS

##### *3.1.1.1 Animals*

Imprinting Control Region (ICR) mice (20 - 30 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, Ghana.

Mice were maintained in the Animal housing facility of the Department of Pharmacology, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. They were housed in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding, and fed with commercially available pellet diet (GAFCO, Tema, Ghana) and given water *ad libitum*. The animals were considerately handled throughout the experiment in accordance with internationally accepted principles for laboratory animal use and care (EEC Directive of 1986: 86/609 EEC). Additionally all animal experiments were approved by the Department Ethics Committee.

#### *3.1.1.2 Drugs and chemicals*

Lambda-carrageenan and aspirin (Sigma-Aldrich Chemical Co, St Louis, USA); Phosphate buffered saline (PBS) (Gibco, Karlsruhe, Germany) were used together with the test drugs in this study.

### 3.1.2 METHODS

#### *3.1.2.1 Preliminary in vitro anti-inflammatory study: Protein denaturation*

Reaction mixtures (5 ml) consisted of 0.2 ml egg albumen (from fresh hen's egg), 2.8 ml phosphate buffered saline (PBS, pH 6.4) and 2 ml varying concentrations of the test drug XAE or XA to final concentrations of 6.25, 12.5, 25, 50, 100, 200 µg $ml^{-1}$. Five (5) ml of double-distilled water served as control. Mixtures were incubated at 37 ± 2ºC in an incubator (GFL 3032-3033, Jos·Hansen, Hamburg, Germany) for 15 min and heated at 70ºC for 5 min. After cooling, absorbance was measured at 660 nm (ELx808,

Biotek Instrument, USA) by using tween-distilled water emulsion (vehicle) as blank. Aspirin at a concentration of 25, 50, 100, 200, 400, 800 µg $ml^{-1}$ as suspension in doubledistilled water was used as reference drug. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times \left[\frac{(A_t - A_c)}{A_c}\right]$$

Where, $A_t$ = absorbance of test sample, $A_c$ = absorbance of control.

Drug concentrations for 50% inhibition ($IC_{50}$) were determined from the dose response curve by plotting percentage inhibition with respect to control against treatment concentration.

***3.1.2.2 In vivo anti-inflammatory study: Carrageenan-induced paw oedema in mice***

Oedema was induced in the right hind paw of ICR mice (20 – 30 g) by sub-plantar injection of 50 µl of 1% ($^{w}/_{v}$) sterile carrageenan in saline. The thickness of the injected paw was measured before injection and hourly for 6 h using an electronic calipers (Z22855, Milomex Ltd, Bedfordshire, UK).

Inhibition of inflammation was calculated using the relation;

$$\% \text{ change in paw thickness} = 100 \times \left[\frac{(D_t - D_i)}{D_i}\right]$$

Where $D_i$ is paw thickness before carrageenan injection

$D_t$ is paw thickness at time T.

Raw scores for right foot thickness were individually normalised as percentage of change from their values at time 0 and then averaged. Total pedal oedema was calculated in arbitrary units as the area under the curve (AUC) and to determine the percentage inhibition of oedema, the following equation was used:

$$\%\ \text{inhibition of oedema} = \left[\frac{AUC_{(control)} - AUC_{(treatment)}}{AUC_{(control)}}\right] \times 100$$

In the prophylactic protocol, XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$) and aspirin (100 mg $kg^{-1}$ *p.o.*) were given before carrageenan injection. In the therapeutic experimental protocol, drugs were given 1 h after subplantar administration of 50 µl of a solution of 1% ($^{w}/_{v}$) sterile carrageenan in saline. Control animals received normal saline orally

### 3.2 STATISTICAL ANALYSIS

Data was presented as the Mean ± SEM of effect of drugs on the time course curve and the total oedema response for 6 h and the data analysed by two-way ANOVA followed by Bonferonni's *post hoc* tests and one-way ANOVA followed by Dunnett's test. Graphs were plotted using GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA, USA).

## 3.3 RESULTS

### *3.3.1 Preliminary in vitro anti-inflammatory study: Protein denaturation*

Protein denaturation is a documented cause of inflammation. It is established from earlier study by Mizushima and Kobayashi (1968) that a good anti-inflammatory agent does have the ability to inhibit protein denaturation *in vitro*.

This preliminary *in vitro* anti-inflammatory investigation therefore evaluates the potential of XAE and XA to inhibit albumen denaturation. In this test, the aspirin control showed a concentration-dependent reduction in albumen denaturation with an $IC_{50}$ of 203.01 µg $ml^{-1}$ (Fig 3.1 A). XAE showed a concentration-dependent inhibition of albumen denaturation through the concentration range 6.25 - 200 µg $ml^{-1}$. $IC_{50}$ value was obtained to be 10.12 µg $ml^{-1}$ (Fig 3.1 B). Within the same concentration range, XA also showed a concentration-dependent inhibition of albumen denaturation with an $IC_{50}$ of 15.55 µg $ml^{-1}$ (Fig 3.1 C).

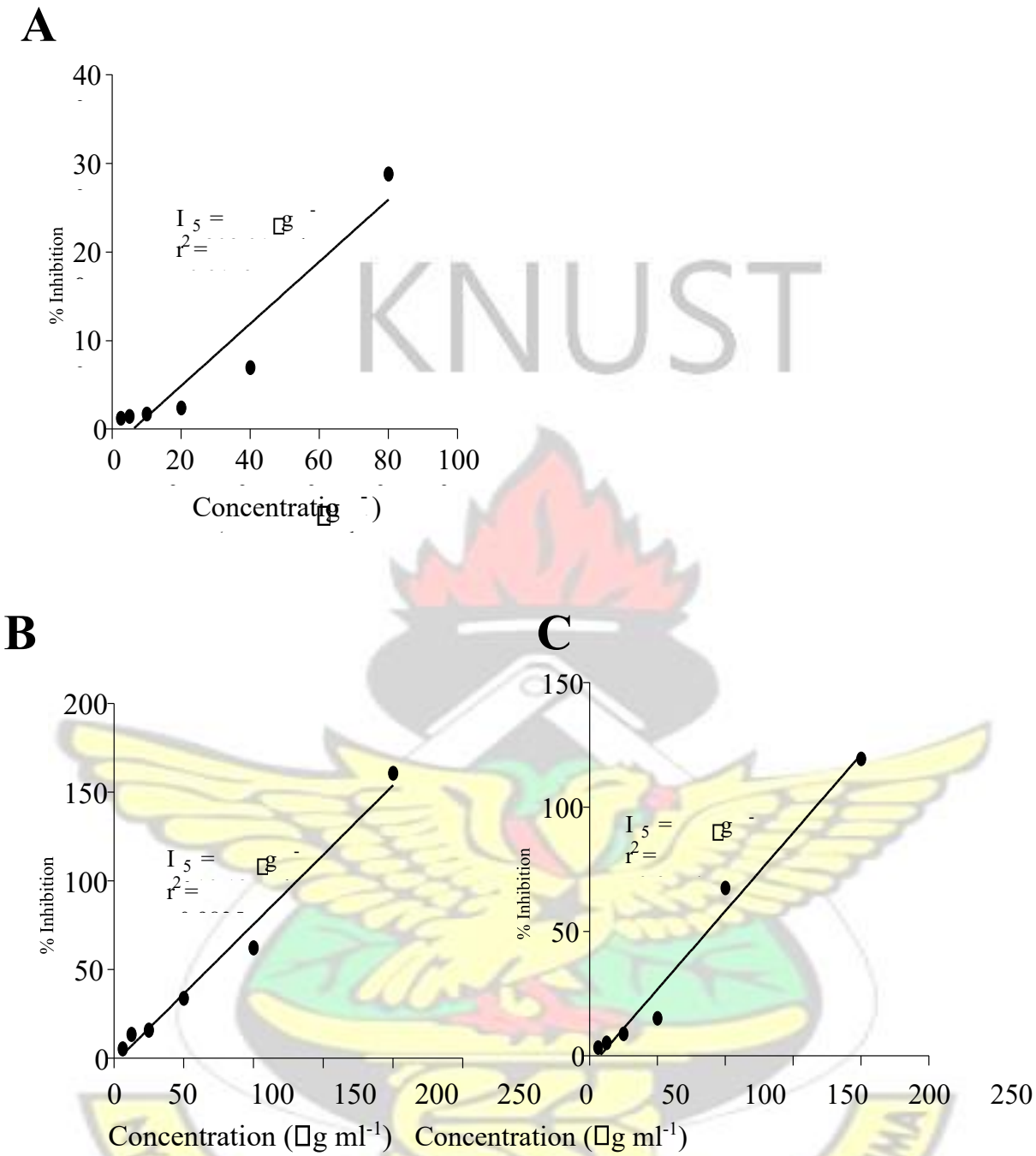


**Figure 3.1. Effect of *X. aethiopica* and xylopic acid on protein denaturation.** 0.2 ml egg albumen (from fresh hen's egg), 2.8 ml phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of the test drug XAE and XA to final concentrations 25, 50, 100, 200, 400, 800 µg ml$^{-1}$ were incubated at 37 ± 2ºC for 15 min and then heated at 70ºC for 5 min (double-distilled water served as control). Absorbance was measured at 660 nm by using tweendistilled water emulsion (vehicle) as blank. Aspirin at the final concentration of 12.5, 25, 50, 100, 200, 400 µg ml$^{-1}$ was used as reference drug. Aspirin (A); XAE (B); XA (C). Triplicates of the results were obtained and data presented as Mean ± SEM.

### *3.3.1 In vivo anti-inflammatory study: Carrageenan-induced paw oedema in mice*

From these results, the preliminary *in vitro* study demonstrated that XAE and XA inhibit protein denaturation, a documented cause of inflammation, and therefore, the study of their effectiveness as acute anti-inflammatory agents were carried out in an *in vivo* model of acute inflammation.

Carrageenan-induced paw oedema, a commonly used primary test to screen new antiinflammatory agents; measures the ability of a compound to reduce local oedema induced in the mice paw by injection of an irritant agent (Winter *et al*., 1962).

In this study, carrageenan was injected into the subplantar tissues of mice as described in section 3.1.2.2. The oedema peaked between 2 - 3 h in control mice (Figs 3.2 A and 3.2 C). XAE (30, 100, 300 mg $kg^{-1}$) when administered before (preventive) the induction of the carrageenan paw oedema caused the mean maximal swelling attained at 2 h to be reduced respectively to 41.02 ± 6.94%, 35.61 ± 4.30% and 29.09 ± 4.90% from the inflamed control response of 55.48 ± 4.05% (Fig 3.2 A). The total paw swellings induced over the 6 h (measured as the area under the time course curve, AUC) were also significantly suppressed to 74.84 ± 14.84%, 63.95 ± 9.37% and 48.13 ± 10.90% of the inflamed control response (Fig 3.2 B). On the other hand, XA (10, 30 and 100 mg $kg^{-1}$) showed a significant reduction of the oedema with the mean maximal swelling attained at 3 h reduced respectively to 44.96 ± 5.12%, 34.63 ± 1.81% and 33.14 ± 1.03% from the inflamed control response of 57.07 ± 3.56% (Fig 3.2 C). The total paw swellings induced over the 6 h were also significantly suppressed to 83.48 ± 12.88%, 62.66 ± 3.38% and 56.58 ± 3.91% respectively of the inflamed control response (Fig 3.2 D).

When administered after the induction of the carrageenan paw oedema (curative), XAE (30, 100, 300 mg $kg^{-1}$) suppressed the mean maximal swelling attained at 2 h respectively to 49.84 ± 3.95%, 43.62 ± 1.01% and 35.97 ± 1.34% of the inflamed control response of 57.65 ± 9.21% (Fig 3.3 A). The total paw swellings induced over the 6 h was significantly suppressed at 100 and 300 mg $kg^{-1}$ to 72.39 ± 4.38% and 60.81

± 3.25% of the inflamed control response respectively (Fig 3.3 B). On the other hand, XA (10, 30, 100 mg $kg^{-1}$) showed a significant inhibition of the oedema with the mean maximal swelling at 2 h reduced respectively to 43.03 ± 3.24%, 41.85 ± 1.92% and 37.42 ± 2.79% of the inflamed control response of 57.65 ± 9.21% (Fig 3.3 C). The total paw swellings induced over the 6 h were also significantly suppressed to 82.30 ± 9.15%, 77.27 ± 7.64% and 68.11 ± 3.59% respectively of the inflamed control response (Fig

3.3 D).

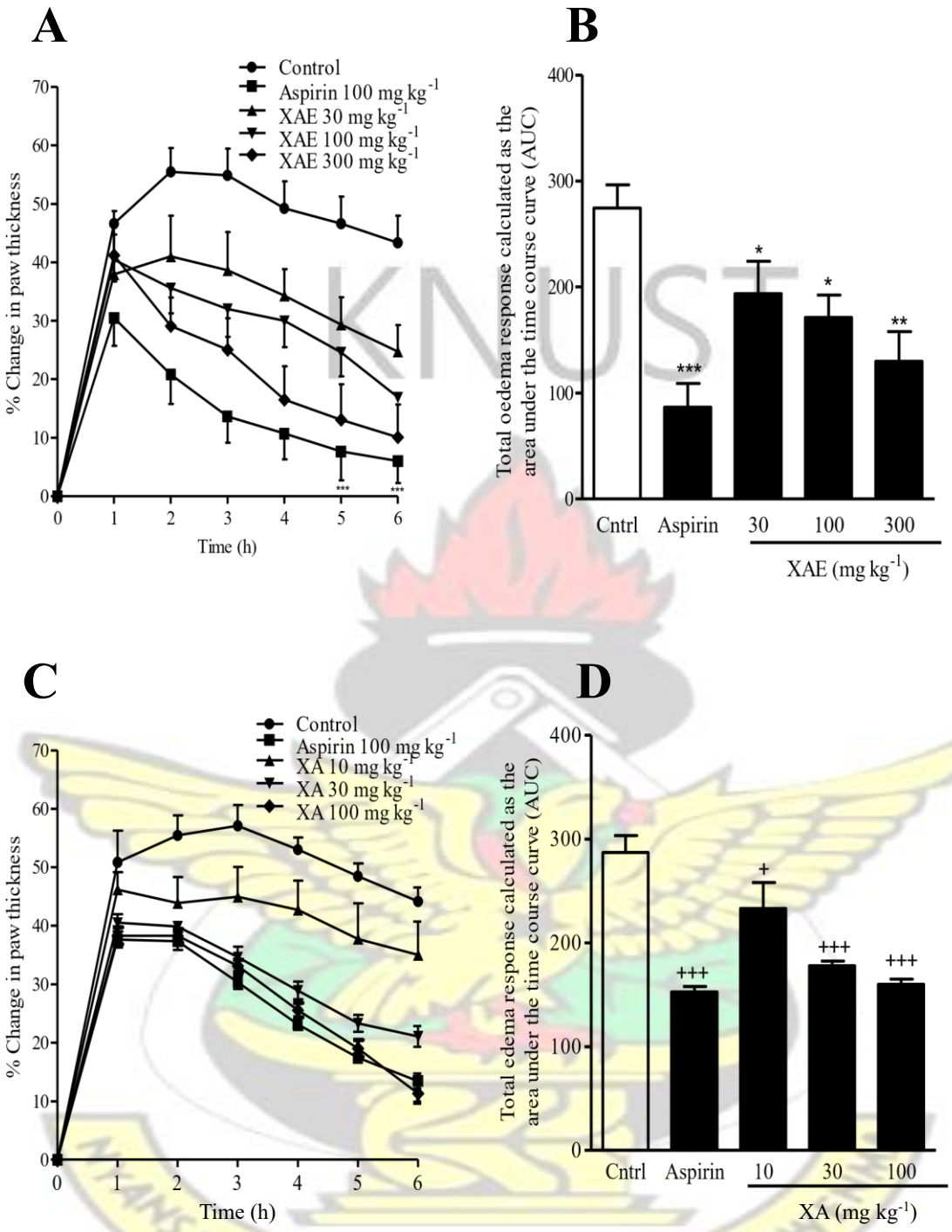


**Figure 3.2. Effect of *Xylopia aethiopica* extract and xylopic acid administered prophylactically on carrageenan-induced oedema in mice.** ICR mice (25 – 30 g) were injected with 50 µl of a 1% carrageenan suspension in normal saline into the sub plantar tissue of the right hind paw. Drug vehicle, XAE (30, 100, 300 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$), and aspirin 100 mg $kg^{-1}$, were given orally 1 h before the induction of the oedema. Oedema was monitored at 1 h intervals over 6 h as percentage increase in paw thickness (A, C). Total oedema induced during the 6 h was calculated as area under the time course curves, AUC (B, D). Drug effects were evaluated by comparing the maximal and total oedema responses attained during 6 h in drug-treated groups with the corresponding values attained in drug vehicle-treated inflamed control groups. Data is presented as Mean ± SEM. (n = 5). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, $^{+}P < 0.01$, $^{+++}P < 0.0001$ when compared with control.

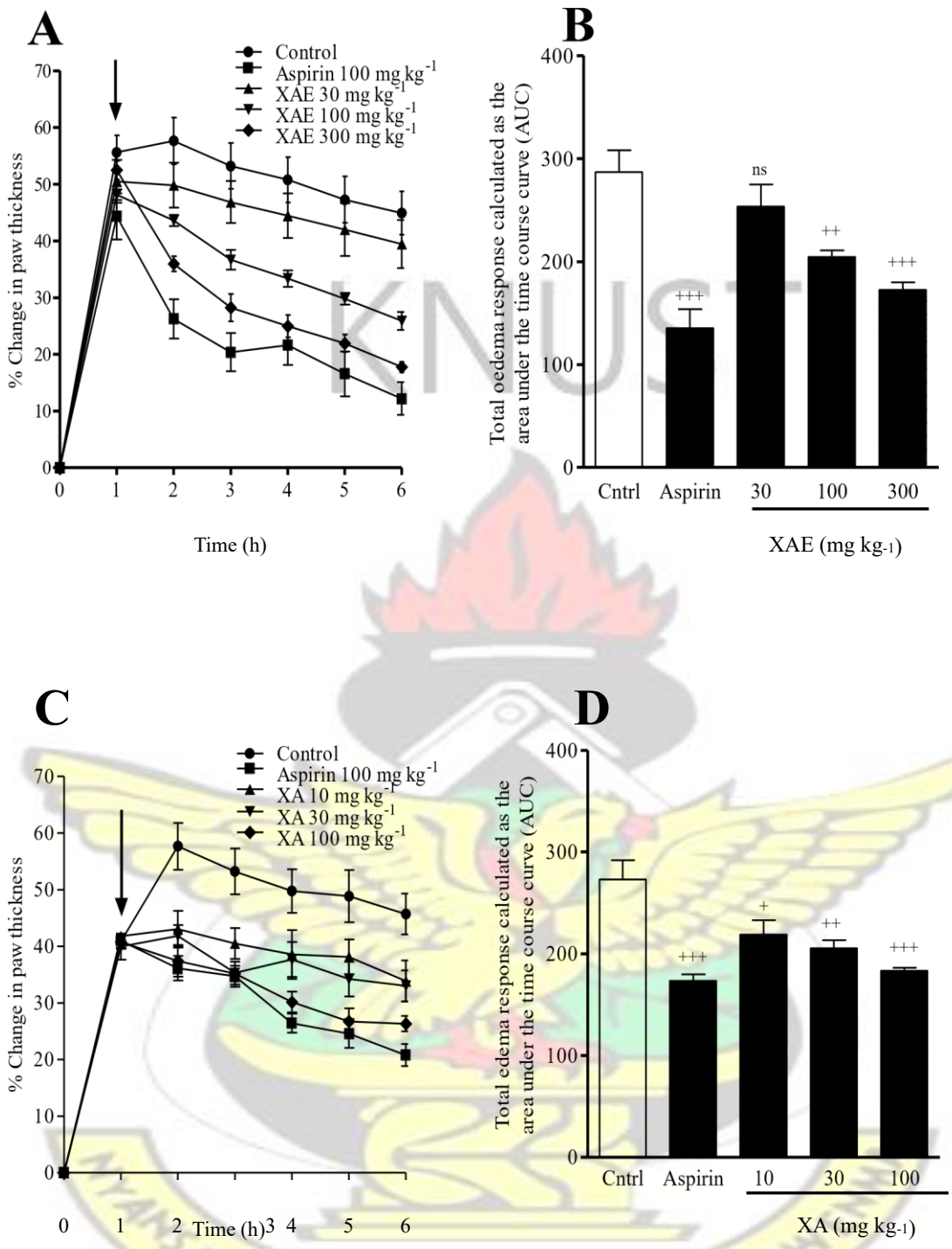


**Figure 3.3. Effect of *Xylopia aethiopica* extract and xylopic acid administered therapeutically on carrageenan-induced oedema in mice.** ICR mice (25 – 30 g) were injected with 50 µl of a 1% carrageenan suspension in normal saline into the sub plantar tissue of the right hind paw. Drug vehicle, XAE (30, 100, 300 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$), and aspirin 100 mg $kg^{-1}$ were done given 1 h post oedema induction. Oedema was monitored at 1 h intervals over 6 h as percentage increase in paw thickness (A, C). Total oedema induced during the 6 h was calculated as area under the time course curves, AUC (B, D). Drug effects were evaluated by comparing the maximal and total oedema responses attained during 6 h in drugtreated groups with the corresponding values attained in drug vehicle-treated inflamed control groups. Data is presented as Mean ± SEM. (n = 5). $^{+}P \leq 0.01$, $^{++}P \leq 0.001$, $^{+++}P \leq 0.0001$

when compared with control. $^{ns}P > 0.05$. Arrow indicates point of Aspirin, XAE or XA administration.

## 3.4 DISCUSSION

Increase in concentration of XAE or XA resulted in increased absorbance and this indicates their ability to stabilise the protein to heat-induced denaturation. This was established in experiment with earlier studies conducted by Mizushima and Kobayashi (1968) which identified phenylbutazone, salicyclic acid and a number of other antiinflammatory agents to show an inhibition of thermally induced protein denaturation.

The carrageenan-induced pedal oedema is the most commonly used model in the evaluation of non-steroidal anti-inflammatory agents (Singh *et al.,* 2000). The development of the carrageenan-induced paw oedema is believed to stem from the release of cytoplasmic enzymes and serotonin from mast cells with an increased release of prostaglandin at the site of inflammation (Vinegar *et al.*, 1987). Thus the role of XAE and XA in inhibiting the release of these mediators of acute inflammation was demonstrated in the carrageenan-induced pedal oedema. Again, Hwang *et al*. (1996) and Lo and Sauf (1987), reported that the initial phase of carrageenan paw oedema is mediated by histamine and serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The later phase is sustained by prostaglandins and mediated by bradykinin, leukotrienes, polymorphonuclear cells (Gupta *et al.,* 2006) and oedema dependent on mobilisation of neutrophils. Recently, acute inflammation has been attributed to the induction of *cyclooxygenase-2* in the tissue (Muniappan and Sundararaj, 2003). The vasoactive substances released in acute inflammation produce an increased vascular permeability and thereby promote accumulation of fluid in tissues that accounts for the oedema (Paschapur *et al.,* 2009). There was a significant inhibition of inflammation by XAE and XA after 2 h, suggesting the ability of the extract and

xylopic acid to act on the latter phase of inflammation. This is not surprising because xylopic acid, a kaurane diterpene, and as earlier reported for these compounds, may exhibit its anti-inflammatory activity through their ability to inhibit prostaglandin $E_2$ synthesis, nitric oxide production, *cyclooxygenase-2*, *inducible nitric oxide synthase* and inhibition of NF-κB. This is informed by earlier findings by Ran *et al.* (2011) that kaurenoic acid, another of the kaurane diterpene, inhibits these mediators of the inflammatory pathway.



## 3.5 CONCLUSION

The aqueous ethanol extract of *Xylopia aethiopica* and xylopic acid demonstrated acute anti-inflammatory action *in vitro* by reducing protein denaturation. *In vivo* carrageenaninduced inflammation study also revealed the inhibitory action of XAE and XA in acute inflammation.

# *Chapter 4* ANTI-ALLERGIC EFFECTS OF THE AQUEOUS ETHANOL EXTRACT OF

## *XYLOPIA AETHIOPICA* AND XYLOPIC ACID

### INTRODUCTION

Allergy is a hypersensitivity reaction of the immune system initiated by immunological mechanisms (WHO, 2002). These are acquired, predictable and rapid response which is one form of hypersensitivity (immediate hypersensitivity). Allergies can range in severity from minor to severe. Anaphylaxis is a serious allergic reaction that is rapid in onset and may cause death (Sampson *et al.,* 2006). It is obvious that anaphylaxis is common and the rate of occurrence is increasing (Decker *et al.,* 2008; Lin *et al.,* 2008; Sheikh *et al.,* 2008). The search for new and more suitable anti-allergic agents continues unabated due to the known side effect and adverse effect profiles of the existing therapies. *X. aethiopica* is traditionally employed in the management of allergic conditions such as asthma.

In this regard, the pinnal inflammation model (Church *et al.,* 1974), compound 48/80induced passive cutaneous anaphylaxis (Choi *et al.,* 2010), systemic anaphylaxis induced with compound 48/80 (Shin *et al*., 1999) and lipopolysaccharide-induced septic shock model as described by Lowry (2005) were employed to evaluate more specific antiallergic actions of both the aqueous ethanol extract of *X. aethiopica* and its principal constituent xylopic acid.

## 4.1 MATERIALS AND METHODS

### 4.1.1 MATERIALS

#### *4.1.1.1 Animals*

Imprinting Control Region (ICR) and C57BL/6 mice (20 – 30 g) and Sprague-Dawley rats (200 – 250 g) provided with the necessary conditions as described in section 3.1.1.1.

#### *4.1.1.2 Bacteria*

*Escherichia coli* (strain: ATCC 25922) was a kind donation from Pharmaceutical Microbiology of the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi.

#### *4.1.1.3 Drugs and chemicals*

Bovine serum albumen (PAA Laboratories, Germany); phosphate buffered saline (Gibco, Karlsruhe, Germany); ether (Fisher Scientific, Waltham, UK); cromoglycate sodium (Ashford lab Ltd, Macau); dexamethasone acetate, Evans blue dye, sodium chloride, aspirin, and compound 48/80 (Sigma-Aldrich Chemical Co, St Louis, USA).

### 4.1.2 METHODS

#### *4.1.2.1 Pinnal inflammation in mice*

ICR mice (25 - 30 g) were immunised with 100 µl solution of bovine serum albumen, BSA (0.05 mg $ml^{-1}$) subcutaneously on day 0. 14 days later immunization was repeated with 100 µl BSA (0.02 mg $ml^{-1}$). The mice were kept for a further period of 7 days, anaesthetised with ether and 200 µl of 1% ($^{w}/_{v}$) Evans blue dye injected into the tail vein. Promptly after this, and while still under anaesthesia, the mice were laid supine

and each pinna was spread out and inoculated with BSA (0.1 mg $ml^{-1}$) using a 21 gauge hypodermic needle. After 30 min the mice were sacrificed and their ears cut off, spread out and the area of the reaction measured by circumscribing the area of extravasation of the blue dye and matching it with the best fit of standard circles. The area of the reaction was taken as the square of the diameter (mm) of the circle of best fit. For treatments, XAE (30, 100, 300 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$), dexamethasone 10 mg $kg^{-1}$ or aspirin 100 mg $kg^{-1}$, were given orally 1 h before second challenge with the antigen (BSA). Vehicle (saline, 100 µl) was injected subcutaneously just before the challenge. The raw score of the diameter of the circle of best fit were normalised as area of extravasation of the Evans blue dye. Percentage inhibition of the inflammatory reaction was expressed as:

$$\% \text{ inhibition of reaction} = 100 \times \left[1 - \frac{A_t}{A_o}\right].$$

Where $A_o$ and $A_t$ are the area of extravasation of the blue dye in the pinna of the saline control and drug or extract-treated mice respectively.

Data was presented as Mean ± SEM of the area of the inflammatory reaction ($mm^2$) and percentage area of extravasation of the Evans blue dye (%).

#### *4.1.2.2 Compound 48/80-induced passive cutaneous anaphylaxis in rats*

Compound 48/80 passive cutaneous anaphylaxis was induced as described by Choi *et al*. (2010). XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) and cromoglycate sodium (50 mg $kg^{-1}$ *i.p.*) were administered to rats 1 h prior to the intradermal injection of compound 48/80 (0.25 µg/50 µl) into their dorsal skins. 30 min after compound 48/80 injection, animals were sacrificed. The lesion portion of the skin

were cut and fixed in 10% buffered formalin. The tissues were stained with 1% ($^{w}/_{v}$) toluidine blue and histopathological analysis blindly done and scored on a scale 0 - 4 according to the extent of tissue damage and degranulation with 4 being the highest score. Scoring was done at ×40 magnification.



#### *4.1.2.3 Compound 48/80-induced systemic anaphylaxis in mice*

C57BL/6 mice (25 - 30 g) were given an intraperitoneal injection of 8 mg $kg^{-1}$ of the mast cell degranulator, compound 48/80. Either vehicle, cromoglycate sodium 50 mg $kg^{-1}$, XAE (30, 100, 300, 1000 mg $kg^{-1}$) or XA (10, 30, 100, 300 mg $kg^{-1}$) was given orally 1 h before compound 48/80 injection. Mortality was monitored for 1 h after induction of anaphylactic reaction. The data was presented as mean survival and survival proportions in percentage.

#### *4.1.2.4 Lipopolysaccharide-induced septic shock in mice*

Male C57BL/6 mice (12 - 14 week old) received intraperitoneal injection of lipopolysaccharide, LPS (*Escherichia coli*, 5 mg $kg^{-1}$ dissolved in PBS). Vehicle, dexamethasone 10 mg $kg^{-1}$, XAE (30, 100, 300 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) was given orally for two consecutive days before initiation of the allergic reaction. Survival rate of the animals was monitored for 7 days after challenge. The data was presented as mean survival and survival proportions in percentage.

### 4.2 STATISTICAL ANALYSIS

All data are presented as the Mean  SEM. In the passive cutaneous anaphylaxis, the difference in the percentage inhibition of the inflammatory reaction was analysed by

one-way ANOVA followed by Dunnett's *post hoc* test. In the Compound 48/80induced anaphylaxis and lipopolysaccharide-induced septic shock, a Kaplan-Maier survival curve was plotted and experimental results analysed using the Log-rank (Mantel Cox) test. Graphs were plotted using GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA, USA).



## 4.3 RESULTS

### *4.3.1 Passive cutaneous anaphylaxis in mice*

The anaphylactic reaction in the pinna of a mouse actively sensitised to bovine serum albumen (BSA) is mediated by immunoglobulin Gi (IgGi) and immunoglobulin E (IgE) antibodies. During the period of 5 - 10 days following sensitization, precipitating antigens appear in the blood. Precipitating antibodies are produced upon sensitization hence there is considerable mast cell damage and histamine release when tissues are brought in contact with antigen (Mota, 1964). Microscopically, there is massive subcutaneous oedema with extensive infiltration of polymorphonuclear leukocytes and haemorrhage in the deep dermis, muscle and subcutaneous connective tissue upon antigen (higher concentration of BSA) challenge after sensitization (Hallstrom *et al.,* 2015).

The capacity of the test drugs to inhibit anaphylaxis was evaluated. It was found that XAE (30 - 300 mg $kg^{-1}$) in a dose-dependent manner inhibited the delayed hypersensitivity reaction with reduced area of the extravasation of Evans blue dye by 23.09 ± 1.52% - 62.83 ± 0.83% (Table 4.1). Similarly, in the XA-treated mice at 10 – 100 mg $kg^{-1}$, the effect was suppressed in a dose-dependent manner which reduced the area of extravasation of the Evans blue dye by 53.02 ± 4.24% - 77.62 ± 2.26% (Table 4.1).

The effect of the aqueous ethanol extract and xylopic acid on mast cell stability after intradermal challenge with a potent mast cell degranulator, compound 48/80, was investigated. This stems from the observation that inflammation is accompanied with the proliferation of mast cells at the site of inflammation which further degranulates to release preformed mediators (Theoharides *et al.,* 2012).

**Table 4.1. Effect of *Xylopia aethiopica* extract and xylopic acid on pinnal inflammation in mice**

| Treatment | Mean reaction area ($mm^2$) | % inhibition of extravasation of Evans blue dye |
|---|---|---|
| Normal Saline (100 µl *p.o.*) | 37.82 ± 0.00 | - |
| Dexamethasone (10 mg $kg^{-1}$ *p.o.*) | 9.74 ± 0.59 | 72.00 ± 0.59* |
| Aspirin (100 mg $kg^{-1}$ *p.o.*) | 10.08 ± 0.28 | 71.02 ± 0.28* |
| XAE (mg $kg^{-1}$ *p.o.*) | | |
| 30 | 26.75 ± 1.52 | 23.09 ± 1.51* |
| 100 | 20.55 ± 1.21 | 40.91 ± 1.21* |
| 300 | 12.93 ± 0.83 | 62.83 ± 0.83* |
| XA (mg $kg^{-1}$ *p.o.*) | | |
| 10 | 15.77 ± 0.82 | 53.02 ± 4.24 * |
| 30 | 10.08 ± 0.43 | 70.37 ± 1.81 * |
| 100 | 7.60 ± 0.65 | 77.62 ± 2.26 * |

ICR mice (20 - 30 g) were sensitised twice as described in methods and treated with aspirin 100 mg $kg^{-1}$, dexamethasone 10 mg $kg^{-1}$, XAE (30 - 300 mg $kg^{-1}$) or XA (10 - 100 mg $kg^{-1}$). 1 h after drug administration, mice were anaesthetised, injected *i.v* with Evans blue dye and immediately challenged with BSA by inoculation into the pinna. Vehicle (saline, 100 µl) was injected subcutaneously just before the challenge. 30 min afterwards mice were euthanised and ears cut off. The reaction area was measured by circumscribing the area of extravasation of the dye and matching it with best fit standard circle. Data represent means of reaction area in 10 ears ± SEM (n = 5). Significance between saline group and drug or extract group denoted by *$P \leq 0.001$.

### *4.3.2 Compound 48/80-induced passive cutaneous anaphylaxis in rats*

Mast cell degranulation can be elicited by a number of secretagogues. The most potent secretagogue (Langunoff *et al*., 1983) includes the synthetic compound known as compound 48/80 and polymers of amino acid (Ennis *et al.,* 1980).

To investigate the effect of XAE and XA in compound 48/80-induced passive cutaneous anaphylaxis, rats were treated and intradermally challenged with compound 48/80 after an hour. Skin samples were fixed in 10% buffered formalin and stained with 1% toluidine blue to determine the extent of tissue damage and mast cell degranulation. From the results, intradermal injection of compound 48/80 causes the degranulation of the skin mast cells with signs of tissue damage (Plate 4.1 B) which is not observed in control animals that were not injected with the compound 48/80 (Plate 4.1 A). Treatment with cromoglycate, the mast cell stabiliser, as expected reduced the extent of mast cell degranulation (Plate 4.1 C) with a significant reduction in tissue damage when compared with compound 48/80 injected rats (Fig 4.1). On treatment with XAE at 30, 100 and 300 mg $kg^{-1}$, there was an inhibition of mast cell degranulation (Plates 4.1 D, 4.1 E and 4.1 F) respectively which when quantified presented a significant and dose-dependent reduction in the extent of tissue damage (Fig 4.1). Also, XA (10, 30, 100 mg $kg^{-1}$) inhibited mast cell degranulation (Plates 4.1 G, 4.1 H and 4.1 I). When counted, it similarly caused a reduction in the extent of mast cell degranulation and tissue damage (Fig 4.1).

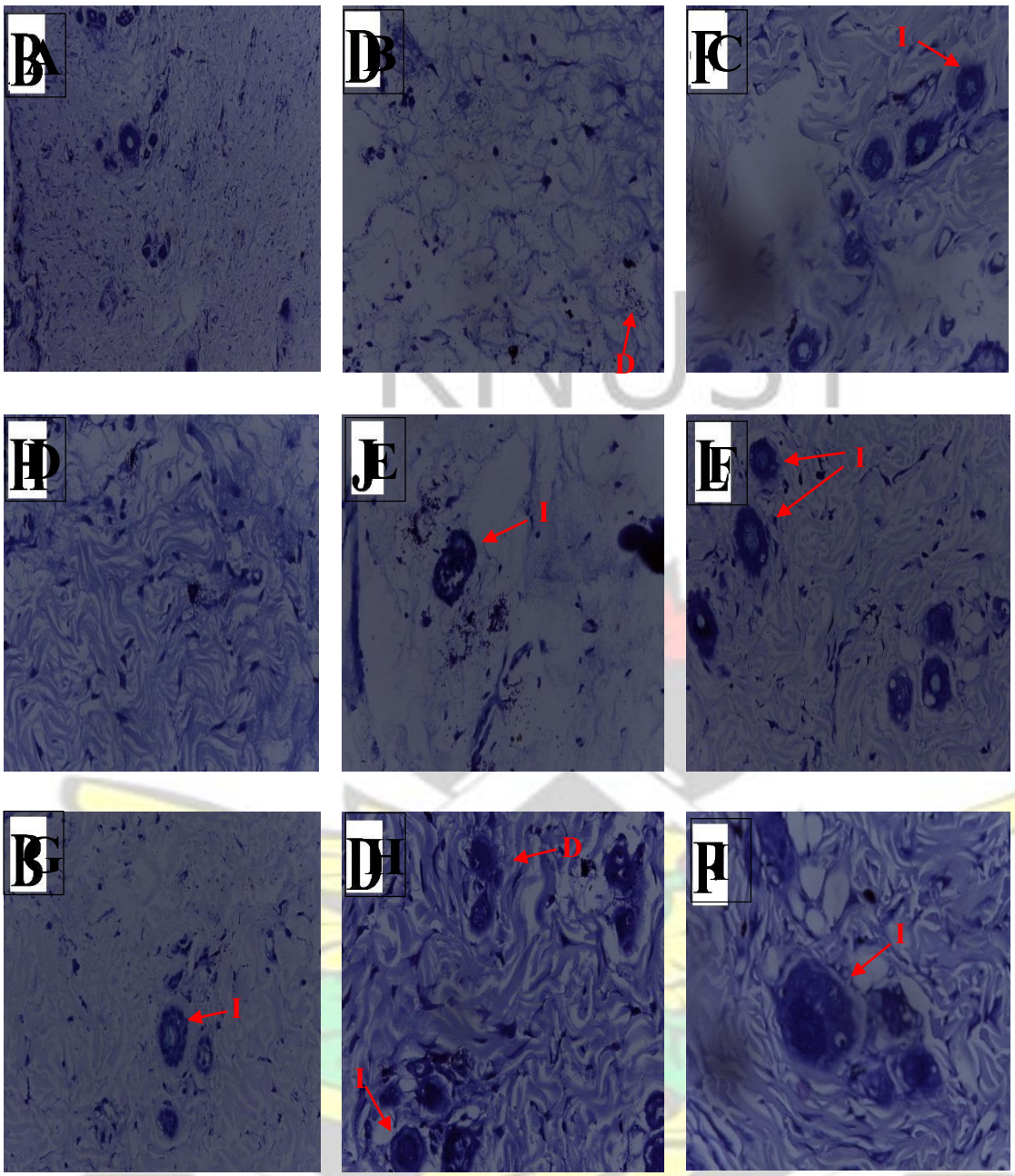


**Plate 4.1. Compound 48/80-induced passive cutaneous anaphylaxis in rats.** Rats received normal saline, cromoglycate sodium (50 mg $kg^{-1}$ *i.p.*), XAE (30 - 300 mg $kg^{-1}$ *p.o.*) or XA (10 - 100 mg $kg^{-1}$) 1 h prior to the intradermal injection of compound 48/80 (0.25 µg/50 µl) into their dorsal skins. 30 min after compound 48/80 injection, animals are sacrificed. The lesion portion of the skin were cut and fixed in 10% buffered formalin. The tissues were stained with 1% toluidine blue and analysed. Normal saline control (no intradermal injection of compound 48/80) (A-×40), normal saline control (intradermal compound 48/80 injection) (B-×40) and cromoglycate sodium 50 mg $kg^{-1}$ (C-×40), XAE 30 mg $kg^{-1}$ (D-×40), XAE 100 mg $kg^{-1}$ (E×40), XAE 300 mg $kg^{-1}$ (F-×40), XA 10 mg $kg^{-1}$ (G-×40), XA 30 mg $kg^{-1}$ (H-×40) and XA 100 mg $kg^{-1}$ (I-×40) (D, degranulated mast cell; I, intact mast cell).

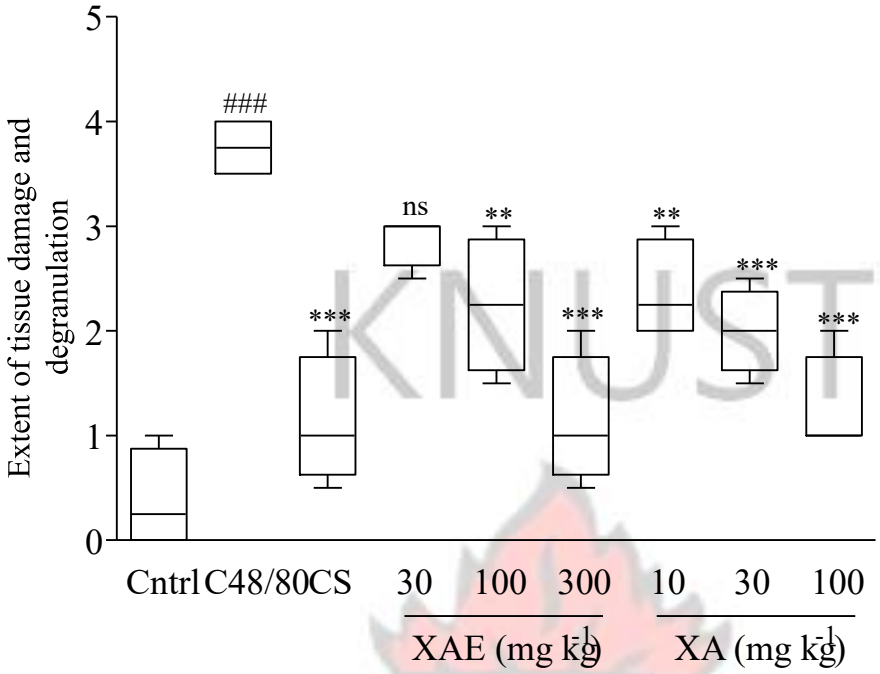


**Figure 4.1. Compound 48/80-induced passive cutaneous anaphylaxis in rats.** Rats received normal saline, cromoglycate sodium (50 mg $kg^{-1}$), XAE (30 – 300 mg $kg^{-1}$ *p.o.*) or XA (10-100 mg $kg^{-1}$) 1 h prior to the intradermal injection of compound 48/80 (0.25 µg/50 µl) into their dorsal skins. 30 min after compound 48/80 injection, animals are sacrificed. The lesion portion of the skin were cut and fixed in 10% buffered formalin. The tissues were stained with 1% toluidine blue and analysed. XAE-treated rats (A); XA-treated rats (B). $^{**}P < 0.001$, $^{***}P < 0.0001$, $^{ns}P > 0.05$ when compared with C48/80-treated control. $^{\#\#\#}P < 0.0001$ when compared with untreated control. Note: C48/80– compound 48/80; CS- cromoglycate sodium.

### *4.3.3 Compound 48/80-induced systemic anaphylaxis in mice*

IgE-dependent and IgE-independent activation of mast cells bring about the process of degranulation with the release of a wide spectrum of mediators which are critical for the development of immediate hypersensitivity reactions and includes anaphylaxis (Petersen *et al.,* 1996) with potential fatality.

In this study Compound 48/80 was used to induce mediator release by aggregation of their cell surface-specific receptor for IgE by a corresponding antigen (Alber *et al*., 1991; Metzger *et al.*, 1986; Chen *et al*., 2002) and mortality monitored for over 1 h.

Intraperitoneal administration of compound 48/80 induced fatal anaphylactic shock in all the control mice within 10 min. When the mice were pre-treated with XAE and XA orally 1 h before compound 48/80 administration there was significant antianaphylactic effects as compared to control with reduced mortality. The extract (30 - 1000 mg $kg^{-1}$) offered 40 – 100% survival proportions in mice. There was a dosedependent increase in the survival proportion of the mice (Table 4.2). Treatment with XA (10 - 300 mg $kg^{-1}$) similarly offered 40 – 90% survival proportions in mice (Table 4.2). Behavioural observation showed a delayed or totally inhibited tremors induced by compound 48/80.

**Table 4.2. Effect of *Xylopia aethiopica* extract and xylopic acid on compound 48/80induced systemic anaphylaxis in mice.**

| Treatment | Deaths/ events | Survival proportion (%) |
|---|---|---|
| Normal Saline (100 µl, *p.o.*) | 10 | 0.00 |
| Cromoglycate (50 mg $kg^{-1}$, *i.p.*) | 0 | 100.00 |
| XAE (mg $kg^{-1}$, *p.o.*) | | |
| 30 | 6 | 40.00 |
| 100 | 6 | 40.00 |
| 300 | 5 | 40.00 |
| 1000 | 0 | 100.00 |
| XA (mg $kg^{-1}$, *p.o.*) | | |
| 10 | 6 | 40.00 |
| 30 | 5 | 50.00 |
| 100 | 4 | 60.00 |
| 300 | 1 | 90.00 |

C57BL/6 mice (25 - 30 g) were pre-treated for 1 h with normal saline, cromoglycate sodium (50 mg $kg^{-1}$, *i.p.*), XAE (30 - 1000 mg $kg^{-1}$) or XA (10 - 300 mg $kg^{-1}$) (n = 10). Compound 48/80 was injected (8 mg $kg^{-1}$ *i.p.*) and mortality monitored for 1 h after induction of anaphylactic shock. Survival curves were significant ($P \leq 0.001$) showing a significant trend ($P \leq 0.0008$).

### *4.3.4 Lipopolysaccharide-induced septic shock in mice*

Lipopolysaccharide, LPS (*Escherichia coli*) is used to study the mechanism of activation of inflammatory pathways during a systemic inflammatory response (Lowry, 2005). In the endotoxaemia model of immuno-mediated anaphylactic shock, mice collapsed to death when challenged with a specific allergen. The male C57BL/6 mice

are known to be very sensitive to LPS-induced septic shock hence served as ideal animals in this model (Zhao *et al.,* 2006, Chi *et al.,* 2006 and Hammer *et al.,* 2006).

LPS was used to induce systemic anaphylactic shock in the male C57BL/6 mice and mortality monitored for 7 days thereafter. From the results, intraperitoneal administration of LPS caused a significant mortality among the vehicle-treated mice starting with 50% mortality after 24 h and increasing through 90% in 72 h to 100% after 96 h. As expected, dexamethasone exhibited maximum protection against endotoxic shock induced with the LPS presenting a survival proportion of 77.78%. The extract, XAE (30, 100, 300 mg $kg^{-1}$) showed a dose-dependent protection against endotoxic shock by 40%, 50% and 70% respectively (Fig 4.2 A). Again, it was noted for XA that it similarly caused a dose-dependent protection against endotoxic shock by 40%, 60% and 70% respectively at 10, 30 and 100 mg $kg^{-1}$ (Fig 4.2 B).

**A** **B**

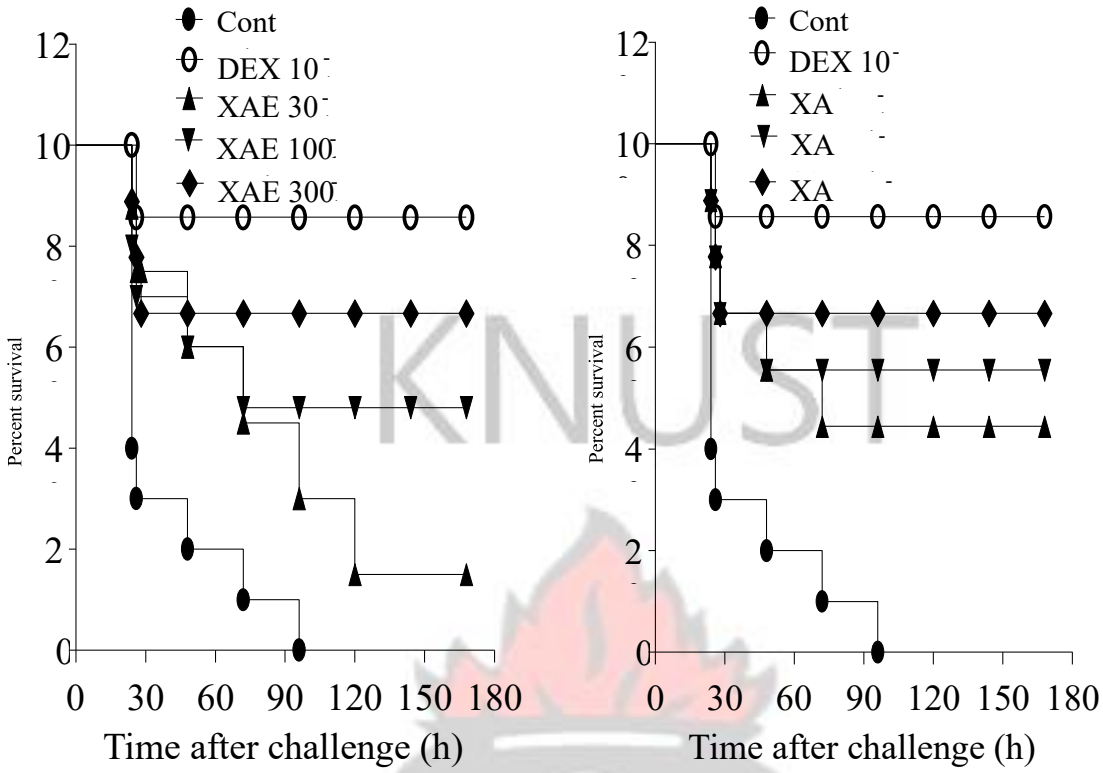


**Figure 4.2. Effect of *Xylopia aethiopica* extract and xylopic acid on lipopolysaccharide (LPS)-induced allergy in mice.** Male C57BL/6 mice (12 - 14 week old) received injection of LPS. Vehicle, dexamethasone 10 mg $kg^{-1}$, XAE 30 - 300 mg $kg^{-1}$ or XA 10 - 100 mg $kg^{-1}$ was given orally for two consecutive days before initiation of the allergic reaction. Survival rate of the mice was monitored for 7 days. Data was analysed using Log-rank (Mantel Cox) test. (n = 10), Survival curves were significant ($P \leq 0.001$) with significant trend ($P \leq 0.0275$).

## 4.4 DISCUSSION

The mouse pinna is an ideal site for study in allergic and inflammatory reaction because it presents a readily accessible area suitable for the study of allergic inflammation reactions (Church and Miller, 1975). BSA at very low concentration was used in sensitization (Church *et al.,* 1974) to raise antiserum to the BSA challenge at higher dose after a latency period of 21 days. This antigenic challenge manifests as increased vascular permeability in the ear. It was observed that in the mouse pinnal anaphylaxis model, XAE and XA suppressed the inflammatory reaction upon antigen challenge. It

is reported in literature that the inflammatory reaction that results is a delayed hypersensitive reactivity to bovine serum albumen (Miller, 1961; Martinez *et al.,* 1962). Thus XAE and XA administration resulted in a decreased ability to develop type III hypersensitivity reaction to bovine serum albumen. This is possibly through inhibition of some stages in the phospholipid/arachidonic acid cascade system and/or as a result of their inhibitory effect on *cyclooxygenase*. This is so informed by the known fact that kaurenoic acid, another kaurane diterpene present in *Xylopia aethiopica*, inhibits prostaglandin $E_2$ synthesis, NF-κB activation and COX-2 (Ran *et al.,* 2011).

The latency of anaphylactic reaction is supposed to be mediated by antibodies found in the serum of sensitised mice (Church and Miller, 1975). Literature reports on experimental studies showing heat-stable IgGi which mediates short latency reaction and a heat-labile reaginic antibody (IgE) which mediates longer latency reaction (Burton and Oettgen, 2011) as the two anaphylactic antibodies produced by the mouse. It is the mouse reaginic antibody, but not mouse IgGi, that binds to mast cell (Konig *et al.,* 1974) and that is possibly inhibited by the treatment with XAE and XA.

Compound 48/80 selectively releases histamine from tissue mast cell by exocytotic degranulation process which requires energy and calcium (Katzung *et al.,* 2012). IgEdependent and IgE-independent activation of mast cells bring about the process of degranulation with the release of wide spectrum of mediators critical for the development of immediate hypersensitivity reactions which includes anaphylaxis which is efficiently averted through the treatment with XAE and XA. There have been many studies confirming that compound 48/80 increases permeability of the lipid bilayer membrane by causing membrane perturbation (Tasaka *et al.,* 1986). XAE and XA possibly inhibits the histamine release from the mast cells through any step along

this cascade of events. XAE and XA may inhibit histamine release by lessening the permeability of mast cell membranes by preventing compound 48/80 binding which could have resulted in membrane perturbation.

Endotoxin is a lipopolysaccharide present in the outer membrane of Gram-negative bacteria such as the *E. coli*. It is considered as the main toxin involved in the pathogenesis of Gram-negative septic shock (Hellman *et al.,* 2000). LPS-induced endotoxic shock is associated with oxidative stress. The lipopolysaccharide interaction with macrophages results in the generation of oxygen-derived free radicals which include peroxide ($H_2O_2$), superoxide anion ($O_2^-$) and hydroxyl radical (OH) which cause oxidative damage to tissues. The anti-oxidant property of *Xylopia aethiopica* has been established by Karioti *et al.* (2004) hence signifying the possible role played by *Xylopia aethiopica* in the inhibition of formation of oxygen-derived free radicals and consistent with this current work.

In an acute LPS-induced endotoxaemic study, it was established that IL-10 plays a very crucial role in the development of endotoxic shock. Indeed IL-10 knock-out mice showed a 20 fold increase in risk of endotoxaemia when compared with the wild type mice (Berg *et al.,* 1995). Therefore, it was postulated that the animals' endogenous IL10 levels determine the amount of endotoxin which constitutes a lethal threshold dose (Berg *et al.,* 1995). It plays a critical role by inhibiting the functions of monocytes/macrophages, including oxidative burst, nitric oxide production and production of pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 12 (IL-12) and inhibitory nuclear factor γ (INF-γ) (Aste-Amezaga *et al.,* 1998). The reduced mortality was attributed to the ability of IL10 to suppress the blood levels of TNF-α and INF-γ. The increased levels of these two

mediators have been observed in mice rendered IL-10 deficient by anti-IL-10 antibody (Marchant *et al.,* 1996); hence, the uncontrolled levels of TNF-α and INF-γ in the absence of IL-10 could be contributing factors for endotoxic susceptibility. The reduced mortality to LPS with XAE and XA treatment could be attributed to their ability to inhibit tissue necrosis and organ failure (both attributed to TNFα) and induction of the enzyme *nitric oxide synthase* which causes production of nitric oxide (Cunha *et al.,* 1992). The uncontrolled production of nitric oxide which results in hypotension and vascular unresponsiveness (Kilbourn *et al*., 1990; Kilbourn, 1990; Nava *et al*., 1992) with subsequent organ failure (Campanile, 1996) which eventually causes death is therefore prevented with the XAE and XA treatment.

## 4.5 CONCLUSION

Taken together, the aqueous ethanol extract of *X. aethiopica* and xylopic acid both showed activity in the passive cutaneous anaphylaxis, compound 48/80-induced anaphylaxis and the LPS-induced septic shock models to affirm their anti-allergic property.

# *Chapter 5* CHRONIC ANTI-INFLAMMATORY ACTIVITY OF THE AQUEOUS

# ETHANOL EXTRACT OF *XYLOPIA AETHIOPICA* AND XYLOPIC ACID

## INTRODUCTION

Chronic inflammation is primarily mediated by monocytes and long-lived macrophages

(Scott *et al.,* 2004). Macrophages and other leukocytes release reactive oxygen species (ROS) and proteases that destroy the source of inflammation. They, however, destroy the body's own tissues with resultant chronic inflammation. In some instances, the body is unable to repair the tissue damage, and the inflammatory cascade continues. Chronic inflammation is deviation from normal bodily response and of no importance to the body and plays a significant role in a number of disease states.

Several human diseases including asthma, Crohn's disease, rheumatoid arthritis, polymyalgia rheumatica, tendonitis, bursitis, laryngitis, gingivitis, gastritis, otitis, celiac disease, diverticulitis, and bowel disease such as ulcerative colitis are inflammatory in nature. Additionally, a number of chronic diseases such as atherosclerosis, obesity, diabetes mellitus, cancer, and perhaps even Alzheimer's disease have inflammatory components (Babio *et al.,* 2009). Hence the search for ideal agents with minimal side and adverse effects for the management of chronic inflammation continues justifiably.

Johnkennedy *et al.* (2011) reported the folkloric use of a mixture of the fruit and root of *Xylopia aethiopica* in the treatment of rheumatism, a chronic inflammatory condition. The chronic inflammation management potential of *Xylopia aethiopica* and xylopic acid, its principal constituent, were thus investigated in rats using the acetic acid-induced ulcerative colitis model as described by Fabia *et al.* (1992) and rat adjuvant-induced arthritis model as described by Pearson (1956).

## 5.1 MATERIALS AND METHODS

### 5.1.1 MATERIALS

#### *5.1.1.1 Animals*

Sprague-Dawley rats (200 – 250 g) were used in this study. Animals were maintained with the necessary conditions as described in section 3.1.1.1.



#### *5.1.1.2 Bacteria*

Heat-killed *Mycobacterium tuberculosis* [strains C, DT and PN (mixed) was obtained from the Ministry of Agriculture, Fisheries and Food, U.K].

#### *5.1.1.3 Drugs and chemicals*

Gelatine (GME, Bruxelles, Belgium); sulphasalazine (Pfizer Inc, New York, USA); hydrogen peroxide (Bell's Healthcare, Cheshire, England); ascorbic acid (Holland and Barrett, Warwickshire, England); trichloroacetic acid (Amresco®, Solon, USA); liquid paraffin (KAMA Pharmaceutical Industries, Accra, Ghana); paraffin and bluing solution (IHC World LLC, Maryland, USA); Ethyl alcohol (Fisher Scientific, Waltham, UK); haematoxylin and eosin Y (Abbey Color, Philadelphia, USA); acetic acid, xylene, picric acid, pyridine hydrochloride, formic acid, formaldehyde, thiobarbituric acid, EDTA, monopotassium dihydrogen phosphate, dipotassium monohydrogen phosphate, monosodium phosphate, disodium phosphate, silver nitrate, *o*-dianisidine dihydrochloride, toluidine blue, tris(hydroxymethyl) aminomethane and pentobarbitone sodium (Sigma-Aldrich Chemical Co, St Louis, USA).

## 5.1.2 METHODS

### 5.1.2.1 *Acetic acid-induced chronic ulcerative colitis in Sprague-Dawley rats*

#### *5.1.2.1.1 Induction of colonic injury and body weight determinations*

Sprague-Dawley rats (200 – 250 g) were randomly divided into nine groups (n = 5). Group I served as control animals which received oral administration of saline (0.9% $^{w}/_{v}$) for 8 days. Group II animals on 4$^{th}$ day received 1 ml 4.0% acetic acid ($^{v}/_{v}$) intrarectally with the aid of a size 6 Ch/Fr paediatric catheter (Flexicare Medical Ltd, Mid Glamorgan, UK). Group III animals were pre-treated with sulphasalazine (500 mg kg$^{-1}$ *p.o.*) for 8 days and 1 ml 4.0% acetic acid administered intrarectally on 4$^{th}$ day. Groups IV-VI and VII-IX were pre-treated with XAE (30 - 300 mg kg$^{-1}$ *p.o.*) and XA (10 - 100 mg kg$^{-1}$ *p.o.*) respectively for 8 days then 1 ml 4% acetic acid was administered as per group III. Treatment in all groups was continued till day 8. Body weight changes were monitored over the 8-day period. Effect of XAE and XA on overall body weight was expressed as area under the curve (AUC).

#### *5.1.2.1.2 Macroscopic and microscopic colonic damage*

Colonic injury with acetic acid was induced in rats as described in section 5.1.2.1.1. At the end of the 8-day period, colons were extirpated and examined for: weight, consistency of the stool found within as well as gross macroscopic appearance and length, measured from 1 cm above the anus to the top of the cecum. Macroscopic damage was assessed as described by Kimball *et al.* (2004) by determining the disease activity index (DAI), calculated as the sum of scores attributed to indices indicated in the table on page 48.

To evaluate microscopic colon damage by light microscopy, samples of the distal colon were fixed immediately in 10% formaldehyde solution, embedded in paraffin, cut into transversal sections and mounted on glass slides. Sections were deparaffinised and stained with haematoxylin and eosin stain (H&E). In each specimen, six random fields of view were analysed by two double-blind observers. The intensity of microscopic colonic damage was assessed.

| | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Stool condition | Normal well-formed fecal pellets | Loosely chaped moist pellets | Amorphous, moist, sticky pellets | Diarrheoa | Presence of blood in stool |
| Colon damage | No inflammation | Reddening, mild inflammation | Moderate or more widely distributed inflammation | Severe and/or extensively distributed inflammation | |
| Colon weight change | 5% | 5 – 14% | 15 – 24% | 25 – 35% | >35% |
| Colon length shortening | 5% | 5 – 14% | 15 – 24% | 26 – 35% | >35% |

#### *5.1.2.1.3 Argyrophylic nucleolar organiser region (AgNOR) staining*

Colonic injury with acetic acid was induced in rats as described in section 5.1.2.1.1. The colons were fixed in formaldehyde and embedded in paraffin as described in section 5.1.2.1.2. Evaluation of silver nitrate stained nucleolar organiser regions in colonic tissues was performed by the method of Rosana *et al.* (2005). Briefly paraffinembedded sections (6.0 µm) were deparaffinised with xylene for 30 min, dehydrated step-wise with ethanol at 10 min time interval. After distilled water washing, slides were immersed in one volume of staining solution (50% silver nitrate, 2.0% gelatine and 1.0% formic acid) in the dark for 60 min and then rinsed in deionised water. The stained sections were dehydrated with ethanol, xylene-cleared and mounted with distrene, plasticiser and xylene (DPX). The appearance of brown or black dots within the nucleus or outside the nucleolus upon silver-staining was examined and counted under Leica ICC50 HD light microscope (Jos.Hansen & Soehne GmbH, Hamburg, Germany). The data were expressed as mean number of AgNORs/nucleus.

#### *5.1.2.1.4 Haematology*

Acetic acid-induced colonic injury in rats was done as described in section 5.1.2.1.1. Rats were euthanised and blood samples collected from the jugular vein. A full blood count was done on the collected blood samples using haematology analyser (BC-2800, Mindray, Shenzhen, China). The Biernacki reaction or erythrocyte sedimentation rate (ESR) was also done using the standard Westergren method. The Fisherbrand™ disposable Westergren pipettes (Thermo Fisher Scientific Inc, Waltham, USA) used met the National Committee for Clinical Laboratory Standards' specification (NCCLS, 1993).

#### *5.1.2.1.5 Mast cell proliferation*

Colonic injury with acetic acid was induced in rats as described in section 5.1.2.1.1. Full thickness segments of colon were fixed in Carnoy's fixative and stained with 1% toluidine blue. Overall morphology was assessed. Mast cells were counted in coded sections at ×40 magnification using a micrometer grid (0.032 $mm^2$). For each rat, 12 contiguous non-overlapping mucosal areas above the muscularis mucosae were evaluated.

#### *5.1.2.1.6 Enzyme assay*

Acetic acid-induced colonic injury in rats was done as described in section 5.1.2.1.1. Colons were stored at −80°C till analysed. Tissue samples were homogenised using a Potter-Elvehjem homogeniser (Ultra-Turrax T25, Janke & Kunkel IKA- Labortechnik, Staufen, Germany) on ice-cold 0.01 M Tris–HCl buffer (pH 7.4) to give a 10% homogenate. The homogenate was used for *superoxide dismutase*, *catalase*, *ascorbate peroxidase*, *myeloperoxidase* and malondialdehyde assays.

##### *5.1.2.1.6.1 Superoxide dismutase (SOD)*

SOD activity was measured by the method of Misra and Fridovich (1972). This test is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. To 0.5 ml tissue homogenate, 0.75 ml ethanol (96% $^{v}/_{v}$) and 0.15 ml chloroform (chilled in ice) were added and centrifuged at 2000 rpm for 20 min. To 0.5 ml supernatant, 0.5 ml 0.6 mM EDTA solution and 1 ml carbonate bicarbonate buffer (0.1 M, pH 10.2) were added. The reaction was initiated by the addition of 0.05 ml 1.3 mM adrenaline and the increase in absorbance at 480 nm due to the adrenochrome formed was

measured with a spectrophotometer. Percentage inhibition of autooxidation of adrenaline was calculated from the formula:

$$\% \text{ inhibition} = \left[\frac{\text{Absorbance}_{\text{test}} - \text{Absorbance}_{\text{reference}}}{\text{Absorbance}_{\text{test}}}\right] \times 100$$



One unit of SOD activity defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 25°C was calculated using the formula:

$$\text{Units of activity per mg protein} = \left[\frac{\% \text{ inhibition}}{50 \text{ x weight of protein}}\right]$$

*5.1.2.1.6.2 Catalase activity (CAT)*

Activity of *catalase* was measured using the method of Aebi (1984). It was determined by measuring decrease, at 20 s interval, in hydrogen peroxide ($H_2O_2$) concentration at 240 nm for 60 s. Medium consists of 130 µl 50 mM potassium phosphate buffer (pH 7.0) and enzyme extract; 65 µl of 10 mM $H_2O_2$. The blank had 65 µl of potassium phosphate and 130 µl of sample. The concentration of $H_2O_2$ was calculated from the absorbance using the following expression:

$$[H_2O_2 \text{ mM}] = \left[\frac{\text{Absorbance}_{240 \text{ nm}} \times 1000}{39.4 \text{ mol}^{-1}\text{cm}^{-1}}\right]$$

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

*5.1.2.1.6.3 Ascorbate peroxidase (APx)*

APx activity was measured by monitoring the decrease in absorbance at 290 nm (extinction coefficient: 2.8 $mM^{-1}cm^{-1}$) for 60 s (measured every 10 s). The assay mixture contained 600 µl 50 mM potassium buffer (pH 7.0), 100 µl 0.1 mM EDTA,

100 µl 1.25 mM $H_2O_2$, 100 µl 0.5 mM ascorbic acid and 100 µl enzyme extract. The blank had all components other than the enzyme extract. The reaction was initiated by adding $H_2O_2$ (Nakano and Asada, 1981).

*5.1.2.1.6.4 Myeloperoxidase (MPO)*

Determined by a modified *o*-dianisidine method. The assay mixture contained 0.3 ml 0.1 M phosphate buffer (pH 6.0), 0.3 ml 0.01 M $H_2O_2$, 0.5 ml 0.02 M *o*-dianisidine (freshly prepared) in deionised water and 10 µl supernatant in a final volume of 3.0 ml. The supernatant was added last and the change in absorbance at 460 nm was followed for 10 min (taken every 1 min). All measurements were carried out in duplicate. One unit of MPO was defined as that giving an increase in absorbance of 0.001 $min^{-1}$ and specific activity was given as U/mg protein.

***5.1.2.1.7 Measurement of malondialdehyde (MDA) levels: lipid peroxidation*** Acetic acid colonic injury in rats was induced as described in section 5.1.2.1.1. Colons were treated as described in section 5.1.2.1.6. Lipid peroxidation levels in tissues were expressed in MDA. Measurement was based on a method by Heath and Parker (1968). Briefly, 3 ml 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added to a 1 ml aliquot of the supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. The tube was centrifuged at 10,000 ×g for 10 min, and absorbance of the supernatant read at 532 nm. The value for the nonspecific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of MDA was calculated using MDA's extinction coefficient of 155 $mM^{-1}cm^{-1}$.

#### *5.1.2.2 Adjuvant-induced arthritis in rats*

Right hind paw of rats (200 – 250 g) were injected intraplantar with 100 µl Complete Freund's adjuvant (CFA). Arthritic control group received only intraplantar injection of CFA, while non-arthritic control group received only intraplantar injection of 100 µl paraffin oil (Incomplete Freund's adjuvant, IFA). Rats were selected for one of the following study groups: aspirin (100 mg $kg^{-1}$ *p.o.*), XAE (100, 300, 600 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*). In the prophylactic treatment group, the test groups were pre-treated (1 h, *p.o.*) with the test drugs prior to CFA inoculation and drugs were administered daily starting from day 0. In the therapeutic protocol, drugs were administered from day 14 with the onset of polyarthritis. Study was carried out in a double-blind manner and for consistency the same trained observer did all measurements throughout the study. Disease progression was monitored from day 0 to day 28 after which rats were sacrificed.

The effect of XAE and XA on the adjuvant-induced arthritis were assessed using six indices;

##### *5.1.2.2.1 Body weight change*

Adjuvant arthritis was induced in rats as described in section 5.1.2.2. Changes in body weight were determined every other day in all treatment groups till day 28.

##### *5.1.2.2.2 Oedema: measured as maximal oedema and total oedema effect*

Rat adjuvant-induced arthritis was done as described in section 5.2.2.2. Foot volume was measured by plethysmometry (Ugo Basile, Comerio, Italy) for both the ipsilateral

(injected hind paw) and the contralateral paw (non-injected hind paw) before intraplantar injection of CFA (day 0) and on every other day up to the 28th day (Binder and Walker, 1998). Raw scores for ipsilateral and contralateral foot volumes were normalised individually as percentage of change from their values at day 0 and then averaged for each treatment group.

Maximal oedema effect was calculated using the formula:

$$\% \text{ change in paw volume} = 100 \times \left[\frac{(V_t - V_i)}{V_i}\right]$$

Where $V_i$ is paw volume before adjuvant injection

$V_t$ is paw volume at time T.

Data was presented as the effect of drugs on the time course curves and the total oedema response calculated in arbitrary units as the area under the curve (AUC) for 28 days. Inhibition of the oedema was calculated from the formula:

$$\% \text{ inhibition of oedema} = \left[\frac{AUC_{(control)} - AUC_{(treatment)}}{AUC_{(control)}}\right] \times 100$$

***5.1.2.2.3 Arthritis score***

***5.1.2.2.3.1 Photography***

Rat adjuvant arthritis was induced as described in section 5.1.2.2. Photographs were taken on day 28 of the affected hind limbs using a digital camera (FE-5050, OLYMPUS, Tokyo, Japan). From the pictures the severity of the arthritis was measured blindly on day 28 on a scale of 0-4 with the hind paw size and erythema as parameters

for scoring (Cai *et al.,* 2007). The arthritis score for IFA -treated group was determined as 0 for all the rats.

#### *5.1.2.2.3.2 Radiography*

Rat adjuvant arthritis was induced as described in section 5.1.2.2. On day 28, rats were anaesthetised by intraperitoneal injection of 50 mg $kg^{-1}$ pentobarbitone sodium. Radiographs were taken with X-ray apparatus (PHILIPS Diagnostic X-ray, Andover, USA) operated at a voltage of 55 kV against 3.2 mA $s^{-1}$ with a tube-to-film distance of 110 cm for lateral projection. The severity of the joint and bone deformation was blindly scored according to the extent of osteoporosis, joint spaces, osteophytes and joint structure (Pohlers *et al.,* 2007). The extent of bone and joint deformation was scored on a scale of 0 - 4, with 0 being score for normal or uninjected control group.

#### *5.1.2.2.4 Haematology*

Rat adjuvant-induced arthritis was done as described in section 5.1.2.2. Animals were euthanised on day 29 and blood samples collected from the jugular vein. A full blood count was done on the collected blood samples using haematology analyser (BC-2800, Mindray, Shenzhen, China). The Biernacki reaction or erythrocyte sedimentation rate (ESR) was also done using the standard Westergren method. The Fisherbrand™ disposable Westergren pipettes (Thermo Fisher Scientific Inc, Waltham, USA) used met the National Committee for Clinical Laboratory Standards' specification (NCCLS, 1993).

#### *5.1.2.2.5 Histopathology*

Adjuvant arthritis was induced in rats as described in section 5.1.2.2 and processed as below.

*5.1.2.2.5.1 Isolation of rat bone tissue*

The Sprague-Dawley rats were sacrificed on day 29 by cervical dislocation. The bones of the hind paws were excised and transferred into sterile Bouin's fluid (1% picric acid (88-89-1), 9.5% formaldehyde (50-00-0), 5% acetic acid (64-19-7) and water).

*5.1.2.2.5.2 Tissue processing and embedding*

Isolated rat tissues were fixed in 4% paraformaldehyde, PFA (for 1 L solution, pH 7.4: 892 ml dd $H_2O$, 108 ml 37% formalin, 11.86 g $Na_2HPO_4$, and 9.07 g $H_2PO_4$) for 16 h at room temperature. The specimens were processed in an automatic tissue processor, hypercenter XP (Thermo Fischer Scientific, Dreich, Germany), programmed to run a series of treatment by immersing the tissues in ethanol, xylene and paraffin in the format:

1. 50% ethanol, 15' immersion, 1h 15' drain
2. 70% ethanol, 30' immersion, 1h 15' drain
3. 95% ethanol, 45' immersion, 15' drain
4. 95% ethanol, 45' immersion, 15' drain
5. 95% ethanol, 45' immersion, 2h drain
6. 100% ethanol, 45' immersion, 15' drain
7. 100% ethanol, 45' immersion, 15' drain
8. 100% ethanol, 45' immersion, 2h drain
9. Xylene, 1 h immersion, 15' drain
10. Xylene, 1 h immersion, 2h drain
11. Paraffin, 1 h immersion, 15' drain
12. Paraffin, 1 h immersion, 2h drain

The principle is to dehydrate the tissues through washing in increasing concentration of ethanol followed by washing steps with xylene to remove the alcohol. Finally tissues were kept in liquid paraffin. After processing, specimens were embedded in paraffin using a Leica EG 1160 embedding machine (Leica Microsystems, Weltzlar, Germany). Paraffin-embedded tissues were kept at 4°C to allow solidification of the paraffin matrix.



*5.1.2.2.5.3 Cutting paraffin-embedded tissue blocks*

Five (5) µm-thick sections were cut from the paraffin-embedded blocks with a Leica RM2155 Microtome (Leica Microsystems, Wetzlar, Germany) at 50°C (10°C below the melting temperature of the paraffin) to remove all the wrinkles and subsequently mounted onto an XTRATM Adhesive Microslide 26 x 76 x 1 mm (Surgipath, Richmond, USA). The slides were placed in a Heraeus oven (Weiss-Gallenkamp, Loughborough, United Kingdom) at 60°C for 1 h to dry.

*5.1.2.2.5.4 Haematoxylin and eosin staining of paraffin-embedded sections*

Five (5) µm-thick sections were deparaffinised and hydrated to distilled water with the following protocol:

| | |
|---|---|
| 1. Xylene, | 5’ immersion |
| 2. Xylene, | 5’ immersion |
| 3. 100% ethanol, | 1’ immersion |
| 4. 95% ethanol, | 1’ immersion |
| 5. 95% ethanol, | 1’ immersion |
| 6. 70% ethanol, | 1’ immersion |

The sections were washed in distilled water and stained with haematoxylin and eosin stain for 2-3 min. Sections were washed in distilled water with 3 changes and quickly dehydrated through dipping in ethanol and finally cleared in xylene as follows:

1. Xylene, 2’ immesion
2. Xylene, 2’ immersion
3. Xylene, 2’ immersion
4. 100% ethanol, 2’ immersion
5. 100% ethanol, 2’ immersion
6. 95% ethanol, 1’ immersion
7. 80% ethanol, 1’ immersion
8. Water, 1’ immersion
9. Haematoxylin, 4’ immersion
10. Water wash, 3’ immersion
11. Differentiate, 1’
12. Water, 3’ immersion
13. Bluing, 1’ immersion
14. Water, 3’ immersion
15. Eosin, 0.30’ immersion
16. 95% ethanol, 0.15’ immersion
17. 100% ethanol, 0.30’ immersion
18. 100% ethanol, 1’ immersion
19. 100% ethanol, 2’ immersion
20. Xylene, 2’ immersion
21. Xylene, 2’ immersion

A glass cover-slip was mounted on top of the slide with the non-aqueous mounting medium Coverquick (Labonord, Monchengladbach, Germany). For microscopy analysis and image acquisition, an Axioscop Zeiss Microscope (Carl Zeiss Microimaging, Heidelberg, Germany) and the software Axiovision (Release 4.5 12/05) were used.



## 5.2 STATISTICAL ANALYSIS

Data was presented as the Mean ± SEM. In the rat adjuvant-induced arthritis, effect of drugs on the time course curve and the total oedema response for 28 days and the data analysed by two-way ANOVA followed by Bonferonni's post hoc tests; one-way ANOVA followed by Dunnett's test was employed in the rat acetic acid-induced colitis and adjuvant-induced arthritis when required. Graphs were plotted using GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA, USA).

## 5.3 RESULTS

### *5.3.1 Acetic acid-induced chronic ulcerative colitis in Sprague-Dawley rats*

#### *5.3.1.1 Induction of colonic injury and body weight determinations*

Production of reactive oxygen species in the inflammatory process was investigated by employing a chronic inflammatory model in which reactive oxygen species (ROS) are implicated. ROS have been reported as the major effector molecules in the colitic process and they destroy the membrane barrier while terminating the process of cell permeable regeneration (Abboud *et al.,* 2008; Liu and Wang, 2011).

In this study, acetic acid-induced colitis was employed in which intrarectal injection of acetic acid induced colitis in rats. Body weight, Disease Activity Index (DAI) and histopathology were assessed. Acetic acid only challenged group presented a steady decrease in the body weight of the animals over the course of the study (Figs 5.1 A and 5.1 C), with a significant total body weight loss when compared with the non-colitic control group (Figs 5.1 B and 5.1 D). Treatment with sulphasalazine resulted in a steady increase in body weight of the rats over the course of the study (Figs 5.1 A and 5.1 C) albeit exhibiting no significant increase in total body weight when compared with the colitic control group (Figs 5.1 B and 5.1 D). XAE-treated animals showed a steady increase in body weight over the duration of the study (Fig 5.1 A) with no significant change in overall body weight when compared with the colitic control at 30 mg $kg^{-1}$ and 100 mg $kg^{-1}$. A significant increase in overall body weight of animals was observed at 300 mg $kg^{-1}$ (Fig 5.1 B). On the other hand, treating rats with XA similarly resulted in an increase in the body weight of the rats over the duration of the study (Fig 5.1 C) but no significant change in total body weight when compared with the colitic control at doses of 10 mg $kg^{-1}$ and 30 mg $kg^{-1}$. At the dose of 100 mg $kg^{-1}$, there was a significant increase in body weight of the rats (Fig 5.1 D).

**A** **B**

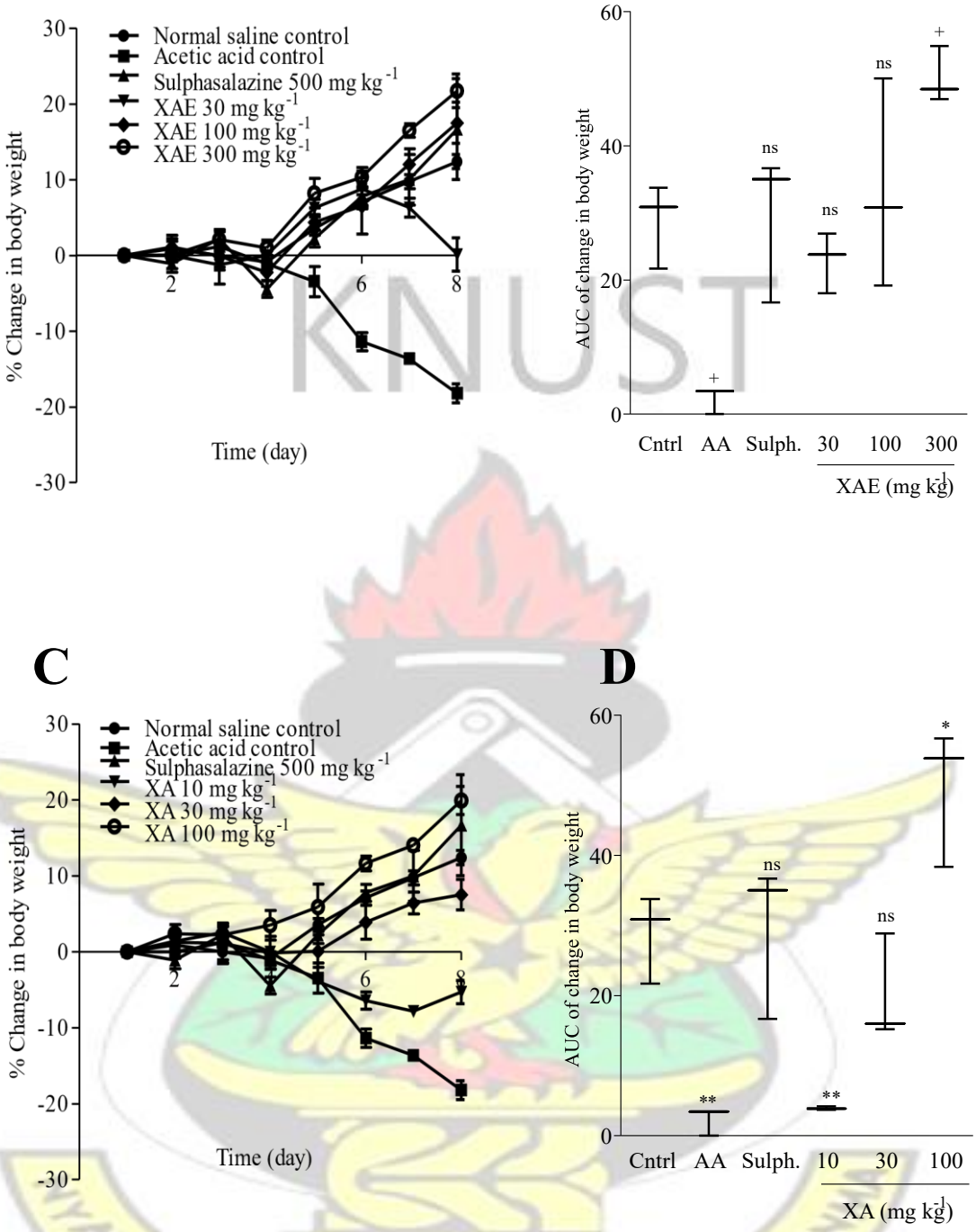


**Figure 5.1. Effect of *Xylopia aethiopica* and xylopic acid on rat body weight.** SprageDawley rats were treated from day 1 with either normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 - 300 mg $kg^{-1}$) or XA (10 - 100 mg $kg^{-1}$) for 8 days. All the groups, except the control animals, on 4$^{th}$ day received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Weight of animals were determined daily. The body weight was monitored as the percentage change in body weight [A, C]. Total body weight measured during the study period was calculated as area under the time course curves, AUC [B, D]. $^{+}P = 0.07$, $*P < 0.01$, $**P < 0.001$, $^{ns}P > 0.05$

### *5.3.1.2 Macroscopic and microscopic colonic damage*

Acetic acid-induced colitis is a model of IBD that bears close resemblance to human IBD in terms of pathogenesis, histopathological features and inflammatory mediator profile (Bitiren *et al.,* 2010; Hartmann *et al.,* 2012). Intrarectal administration of diluted solution of acetic acid causes non-transmural inflammation characterised by increased neutrophil infiltration into the intestinal tissue, massive necrosis of mucosal and submucosal layers, vascular dilation, oedema and submucosal ulceration. These are noteworthy features of human colitis (Nakhai *et al.,* 2007; Daneshmand *et al.,* 2009) which can be assessed together as the disease activity index (DAI).

To evaluate these, acetic acid was used to induce colitis in rats. From the results, the acetic acid control rats exhibited exacerbated colonic injury macroscopically with a significantly high disease activity index when compared with the non-colitic control (Figs 5.2 A and 5.2 B). Treatment with sulphasalazine showed a significant improvement of the macroscopic profile of the disease with decreased DAI when compared with the colitic control (Figs 5.2 A and 5.2 B). XAE-treated animals showed significant improvement of the disease profile macroscopically. The calculated DAI was significantly decreased when compared with the acetic acid-treated group at 30, 100 and 300 mg $kg^{-1}$ (Figs 5.2 A and 5.2 B). In the same manner, the XA-treated animals showed significant improvement of the disease profile macroscopically with decreased DAI when compared with the acetic acid-treated group at doses 10, 30 and 100 mg $kg^{-1}$ (Figs 5.2 A and 5.2 B).

The microscopic observation of the colon in the non-colitic control animals showed no observable signs of inflammation, fibrosis and necrosis with no infiltration of neutrophils (Plate 5.1 A). In the colitic control rats, the histologic ulcers showed

necrosis in the colonic mucosa with submucosal inflammation, with neutrophils and lymphocytes being the predominant infiltrating cells. Moreover, there was an observed crypt abscesses, granulomatous inflammation with fibrosis, and massive thickening of the submucosa. The crypt of the colon in the acetic acid group was elongated with distortion, while exhibiting the loss of epithelial cells, ulceration, lymphocyte infiltration, bowel wall thickening, and goblet cells depletion in the colon (Plate 5.1 B). Treatment with sulphasalazine reduced the mucosal damage with reduced granulomatous inflammation and fibrosis (Plate 5.1 C). Respectively at 30, 100, 300 mg $kg^{-1}$, XAE decreased the gross mucosal injury caused by the acetic acid. There was decreased epithelial cell loss with reduced granulomatous inflammation (Plates 5.1 D, 5.1 E and 5.1 F) respectively. The animals treated with xylopic acid showed a decreased gross mucosal injury caused by the acetic acid at 10, 30 and 100 mg $kg^{-1}$. Reduced granulomatous inflammation and cellular proliferation with treatment with XA (Plates 5.1 G, 5.1 H and 5.1 I) respectively were observed.

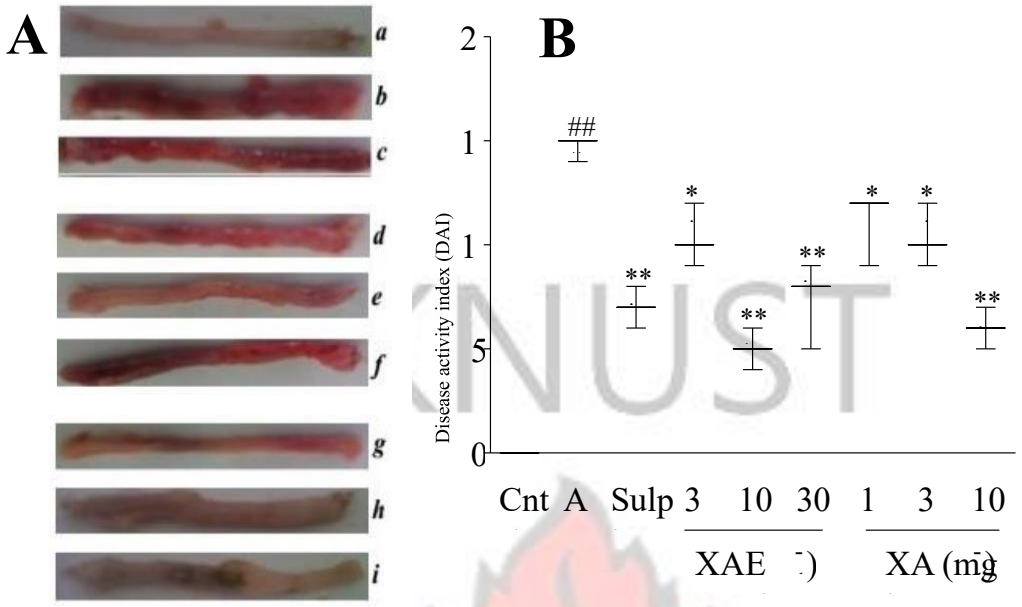


**Figure 5.2. Effect of *Xylopia aethiopica* and xylopic acid on acetic acid-induced colonic damage in rats.** Sprague-Dawley rats were treated with either normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 - 300 mg $kg^{-1}$) or XA (10 – 100 mg $kg^{-1}$) for 8 days. On 4th day received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and examined for: weight, the consistency of the stool found within as well as gross macroscopic appearance and length, **P* < 0.01, ***P* < 0.001 ****P* < 0.0001 when compared with colitic control. ###*P* < 0.0001 when compared with non-colitic control. A; Representative slides of colon (*a*, untreated control; *b*, AA treatment only; *c*, AA + 500 mg $kg^{-1}$ sulphasalazine; *d*, AA + 30 mg $kg^{-1}$ XAE; *e*, AA + 100 mg $kg^{-1}$ XAE; *f*, AA + 300 mg $kg^{-1}$ XAE, g, AA + 10 mg $kg^{-1}$ XA; *h*, AA + 30 mg $kg^{-1}$ XA; *i*, AA + 100 mg $kg^{-1}$ XA). B; Disease activity index of XAE and XA.

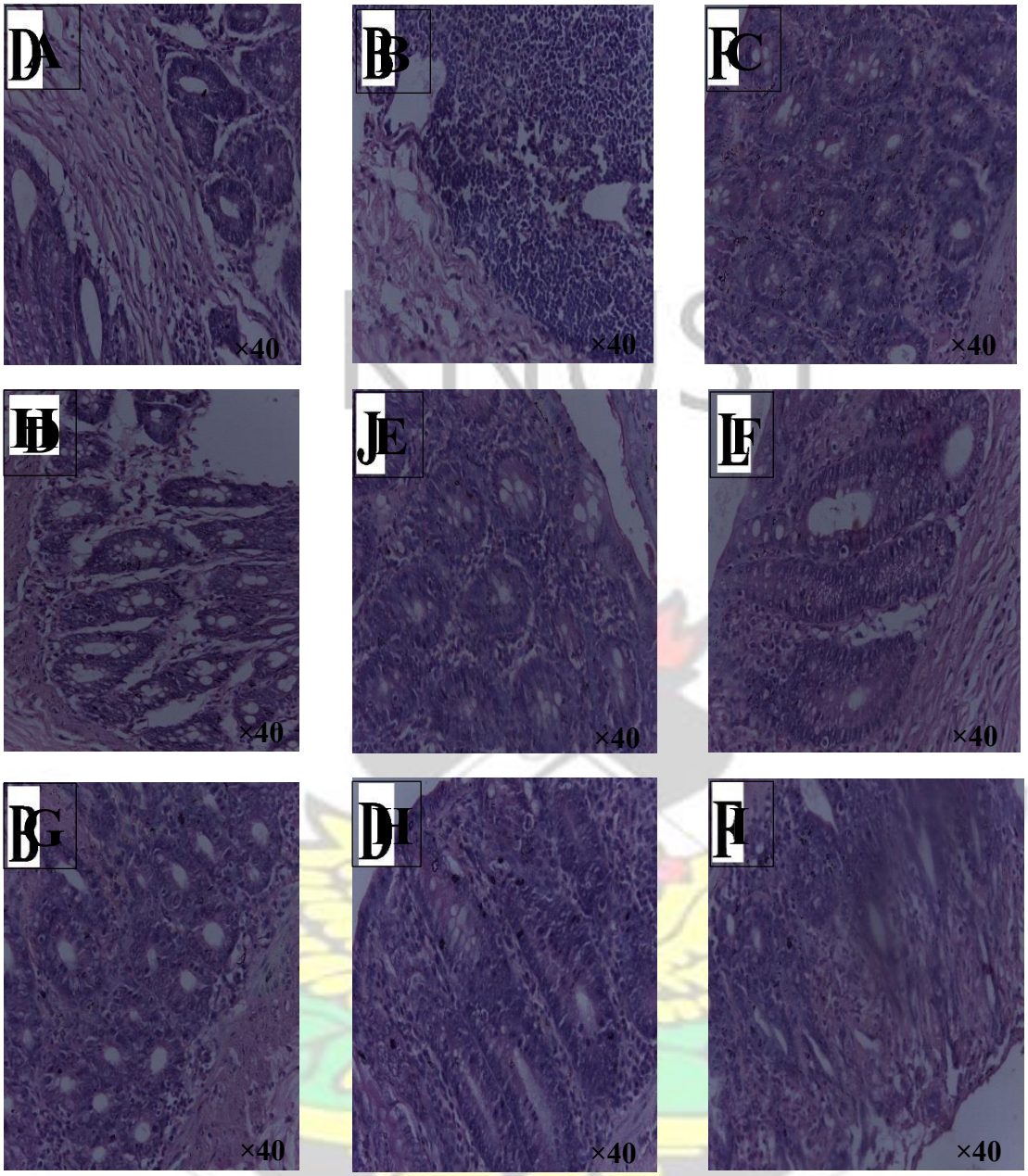


**Plate 5.1. Histopathology of the acetic acid-induced colitis in rats.** Sprague-Dawley rats were treated with either normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 - 300 mg $kg^{-1}$) or XA (10 – 100 mg $kg^{-1}$) for 8 days. Animals, except the non-colitic control animals, on 4$^{th}$ day received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). At the end of the 8-day period, colons were extirpated. Distal colon were fixed immediately in 10% formaldehyde solution, embedded in paraffin, cut into 5mm thick transversal sections, mounted on glass slides, deparaffinised and stained with haematoxylin and eosin stain (H&E). Control (untreated) (A), acetic acid control (B), sulphasalazine 500 mg $kg^{-1}$ (C), XAE 30 mg $kg^{-1}$ (D), XAE 100 mg $kg^{-1}$ (E), XAE 300 mg $kg^{-1}$ (F), XA 10 mg $kg^{-1}$(G), XA 30 mg $kg^{-1}$ (H) and XA 100 mg $kg^{-1}$ (I)

### *5.3.1.3 Argyrophylic nucleolar organiser region (AgNOR) staining*

In the acetic acid-induced ulcerative colitis study, nucleolar organiser region is employed in determining cellular behaviour and proliferation levels (Khan *et al.,* 2009). Studies by Muscara *et al.* (1997) and Yu *et al.* (1992) suggested that the possible thickening of epithelial cells could trigger proliferation rate by liberating excess mucin in colonic region. The colitis results in increased expression of AgNORs in colon tissues because of enhanced transcriptional and cellular activity.

In this study, acetic acid was used to induce colitis in rats and colonic tissues taken. Colonic samples are stained with a staining solution (silver nitrate, gelatine and formic acid) with the silver-staining pattern quantified. As expected, the non-colitic control group presents with a low expression of AgNOR in cells (Plate 5.2 A). The AgNORs/nucleus ratio was accordingly not elevated, relative to the colitic control, when quantified (Fig 5.3). The colitic control showed high proliferation of AgNOR in the cells (Plate 5.2 B) with a corresponding increased expression of AgNORs/nucleus quantitatively (Fig 5.3). Treatment with sulphasalazine significantly reduced the proliferation of AgNOR in the colonic epithelial cells (Plate 5.2 C) with the AgNOR count per nuclei significantly decreased when compared with the colitic control group (Fig 5.3). There were observable reduction in the expression of AgNORs in the XAEtreated groups at 30, 100 and 300 mg $kg^{-1}$ (Plates 5.3 A, 5.3 B and 5.3 C) respectively.

Quantification of AgNORs/nucleus showed a significant decrease ($P < 0.0001$) in the AgNORs/nucleus ratio with XAE treatment (Fig 5.3) to levels comparable with the non-colitic control. Treatment with XA also resulted in decreased colonic epithelial expression of AgNORs at 10, 30 and 100 mg $kg^{-1}$ (Plates 5.3 D, 5.3 E and 5.3 F) respectively with significant decrease ($P < 0.0001$) in the quantitative expression of

AgNORs/nucleus ratio to levels comparable with non-colitic control (Fig 5.3).

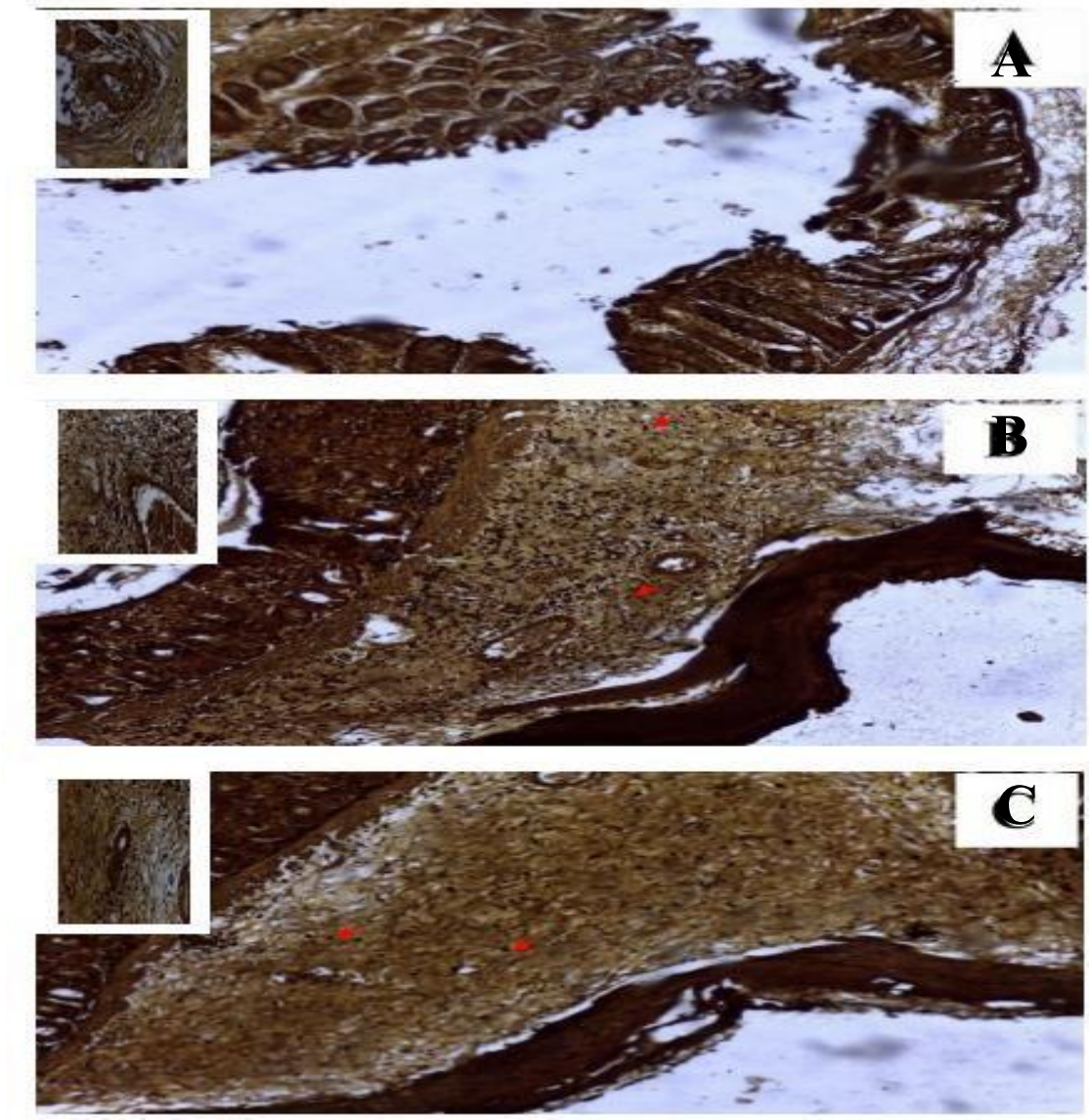


**Plate 5.2. Evaluation of silver nitrate-stained nucleolar organiser region in colonic tissues in rats.** Sprague-Dawley rats were treated with either normal saline or sulpasalazine (500 mg $kg^{-1}$) for 8 days. On 4$^{th}$ day rats received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and fixed immediately in 10% formaldehyde solution and embedded in paraffin. Paraffin-embedded sections were deparaffinised with xylene for 30 min, dehydrated with graded ethanol for 10 min time interval. Slides were immersed with one volume of staining solution in dark for 60 min and then rinsed in deionised water. The stained sections were dehydrated with ethanol, xylene cleared and mounted with DPX. The appearance of brown or black dots within the nucleus or outside the nucleolus upon silver-staining was examined and counted (X10) [insert: X40]. Sample of AgNOR labelled with red arrows. Non-colitic control (A), colitic control (B) sulphasalazine 500 mg $kg^{-1}$ (C)

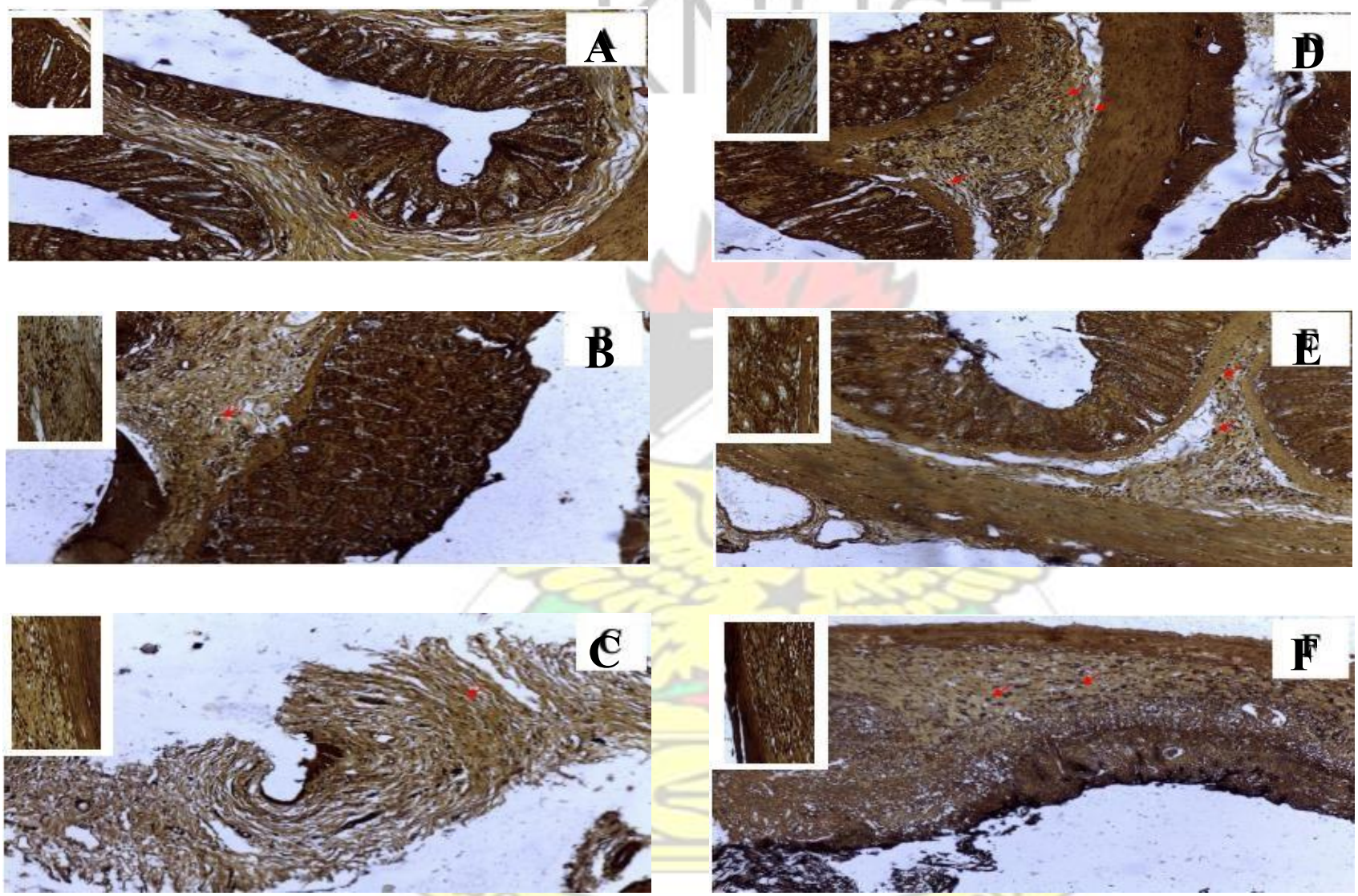


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**Plate 5.3. Evaluation of silver nitrate-stained nucleolar organiser region in colonic tissues in rats.** Sprague-Dawley rats were treated with either XAE (30 - 300 mg $kg^{-1}$) or XA (10 – 100 mg $kg^{-1}$) for 8 days. On 4$^{th}$ day rats received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and fixed immediately in 10% formaldehyde solution and embedded in paraffin. Paraffin-embedded sections were deparaffinised with xylene for 30 min, dehydrated with graded ethanol for 10 min time interval. Slides were immersed with one volume of staining solution in dark for 60 min and then rinsed in deionised water. The stained sections were dehydrated with ethanol, xylene cleared and mounted with DPX. The appearance of brown or black dots within the nucleus or outside the nucleolus upon silver-staining was examined and counted (X10) [insert: X40]. Sample of AgNOR labelled with red arrows. XAE 30 mg $kg^{-1}$ (A), XAE 100 mg $kg^{-1}$ (B), XAE 300 mg $kg^{-1}$ (C), XA 10 mg $kg^{-1}$ (D), XA 30 mg $kg^{-1}$ (E) and XA 100 mg $kg^{-1}$ (F).

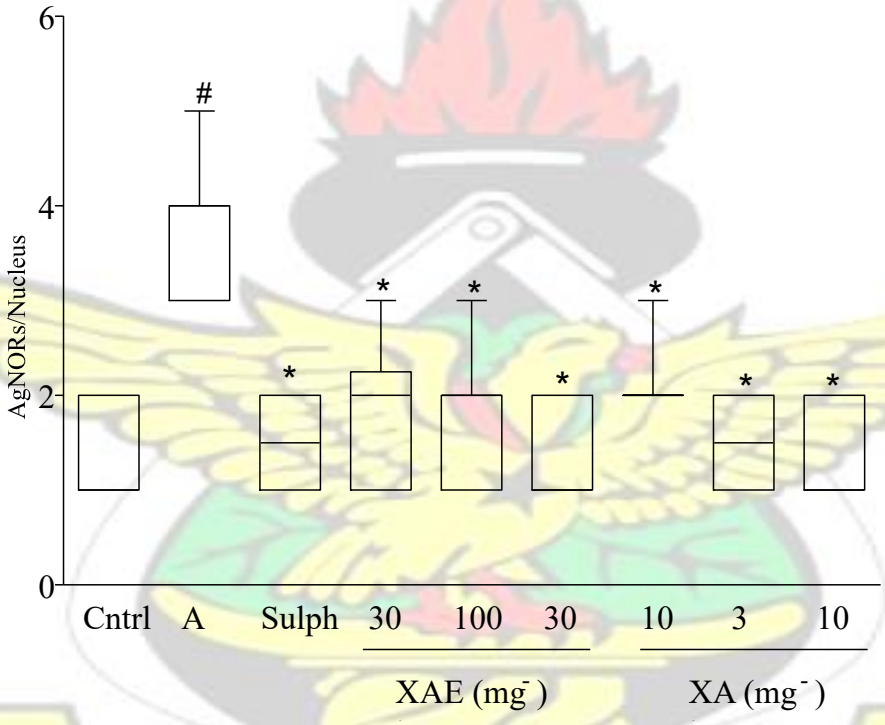



**Figure 5.3. Argyrophilic nucleolar organiser region number per nucleus in *Xylopia aethiopica* extract and xylopic acid-treated rats.** Sprague-Dawley rats were treated with either normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 – 300 mg $kg^{-1}$) or XA (10 - 100 mg $kg^{-1}$) for 8 days. On 4$^{th}$ day rats received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and the distal colon were fixed immediately in 10% formaldehyde solution, embedded in paraffin. Paraffin-embedded sections were deparaffinised with xylene for 30 min, dehydrated with graded ethanol for 10 min time interval. After distilled water washing, slides were immersed with one volume of staining solution in dark for 60 min and then rinsed in deionised water. The stained sections were dehydrated with ethanol, xylene cleared and mounted with DPX. The appearance of brown or black dots within the nucleus or outside the nucleolus upon silver-staining was examined and counted and average count per 10 nuclei determined and presented as Mean ± SEM. *$P < 0.0001$ when compared with colitic control. $^{\#}P < 0.0001$ when compared with non-colitic control.

#### *5.3.1.4 Haematology*

Blood tests have proven important in the initial evaluation of ulcerative colitis. Such parameters include Complete blood count (CBC) (WebMD Medical Reference, 2012).

Effect of extract treatment on improving the haematological profile after colitis induction was investigated by measuring the blood parameters. The non-colitic control showed detectable levels of white blood cells (WBC), lymphocytes (LYM), neutrophils (NEU), haemoglobin (HGB), red blood cells (RBC), haematocrit (HCT) and platelets (PLT). Upon acetic acid treatment, there were significant increases in the levels of WBC, HCT and HGB with the levels of LYM, NEU, RBC and PLT significantly decreased (Table 5.1). Sulphasalazine-treated rats produced a significant increase in LYM, HGB and RBC when compared with the acetic acid-treated rats (Table 5.1). Treatment with XAE resulted in an increase in PLT and a significant decrease in HGB when compared with the acetic acid-treated control rats at doses 30, 100 and 300 mg $kg^{-1}$. There were decreases in WBC count at 30 and 300 mg $kg^{-1}$ and RBC at 30 and 100 mg $kg^{-1}$ of XAE (Table 5.1). Similarly, treating rats with XA resulted in decreased HGB and HCT and an increase in PLT when compared with the acetic acid-treated rats. There were significantly decreased WBC and an increased LYM with 100 and 300 mg $kg^{-1}$ of XA (Table 5.1).

**Table 5.1. Effect of *Xylopia aethiopica* extract and xylopic acid on blood parameters in acetic acid-induced ulcerative colitis in rats**

| Treatment | WBC x$10^3$/µL | LYM% | NEUT% | HGB g $dL^{-1}$ | RBC x$10^6$/µL | HCT % | PLT x$10^3$/µL |
|---|---|---|---|---|---|---|---|
| Control | 10.6 ± 0.10$^{\#}$ | 62.5 ± 7.35 | 10.3 ± 1.37 | 13.7 ± 0.01 | 8.31 ± 0.2$^{\#}$ | 44.0 ± 1.23 | 626 ± 13.72$^{\#}$ |
| Acetic acid | 14.7 ± 0.23* | 44.1 ± 9.25* | 5.7 ± 0.75* | 15.0 ± 0.00* | 5.93 ± 0.01* | 49.0 ± 0.02* | 432 ± 10.92* |
| Sulphasalazine (mg $kg^{-1}$) | | | | | | | |
| 500 | 15.1 ± 0.10* | 62.1 ± 3.48*$^{\#}$ | 4.0 ± 0.43* | 17.1 ± 0.20*# | 9.11 ± 0.11*$^{\#}$ | 47.9 ± 0.23* | 356 ± 3.45* |
| XAE (mg $kg^{-1}$) | | | | | | | |
| 30 | 12.3 ± 0.11*$^{\#}$ | 70.9 ± 3.91*$^{\#}$ | 7.4 ± 1.35*$^{\#}$ | 12.5 ± 0.08* | 7.21 ± 0.05*$^{\#}$ | 37.3 ± 0.12*$^{\#}$ | 1213 ± 20.92*$^{\#}$ |
| 100 | 18.6 ± 0.22*$^{\#}$ | 51.8 ± 2.75* | - | 12.4 ± 0.10* | 6.99 ± 0.15*$^{\#}$ | 38.0 ± 0.07*$^{\#}$ | 1062 ± 15.31*$^{\#}$ |
| 300 | 7.3 ± 0.12*$^{\#}$ | 39.0 ± 1.78* | - | 10.1 ± 0.17*$^{\#}$ | 5.44 ± 0.42* | 29.3 ± 0.75*$^{\#}$ | 1146 ± 21.45*$^{\#}$ |

Table legend on next page

| XA (mg kg$^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | 21.4 ± 3.7* | 32.9 ± 1.52*$^{\#}$ | - | 12.8 ± 0.08*$^{\#}$ | 7.62 ± 0.09*$^{\#}$ | 39.8 ± 0.45*$^{\#}$ | 1683 ± 13.75*$^{\#}$ |
| 30 | 11.6 ± 0.25*$^{\#}$ | 51.3 ± 2.34*$^{\#}$ | 5.1 ± 1.12* | 12.2 ± 0.15*$^{\#}$ | 7.06 ± 0.25*$^{\#}$ | 38.8 ± 0.13*$^{\#}$ | 834 ± 10.92*$^{\#}$ |
| 100 | 4.4 ± 0.32*$^{\#}$ | 52.5 ± 1.98*$^{\#}$ | 5.4 ± 0.57* | 9.8 ± 0.21*$^{\#}$ | 5.08 ± 0.29* | 31.5 ± 0.35*$^{\#}$ | 559 ± 7.35*$^{\#}$ |

Sprague-Dawley rats were treated with either normal saline, sulpasalazine (500 mg kg$^{-1}$), XA (30 – 300 mg kg$^{-1}$) or XA (10 - 100 mg kg$^{-1}$) for 8 days. On 4$^{th}$ day received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). On day 8, animals are sacrificed and blood samples collected for haematological analysis. *$P < 0.0001$ when compared with untreated control; $^{\#}P < 0.0001$ when compared with acetic acid-treated control.

#### *5.4.1.5 Mast cell proliferation*

Mast cells play a central and critical role in the inflammation response. Da Silva (2006) reported that mast cells increase in numbers when there is chronic inflammation.

In this study, acetic acid was used to induce colitis in rats and colonic tissues taken. Colon segments were stained with 1% toluidine blue dye and the proliferation of mast cells due to colitis studied. The results showed that the non-colitic control rats had no increased influx of mast cell to the colonic segment (Plate 5.4 A) and had no elevated levels of mast cells, relative to the colitic control, when quantified (Fig 5.4). There was a very high influx of mast cells in the acetic acid only-treated group (Plate 5.4 B) with high colonic segment count of mast cell significantly higher than as observed in noncolitic control group (Fig 5.4). Treatment with sulphasalazine lowered the proliferation of mast cell in the colonic segment (Plate 5.4 C) with significantly reduced mast cell count when compared with the acetic acid-induced colitic control (Fig 5.4). XAEtreated rats showed a reduced mucosal mast cell infiltration in the range of 30 - 300 mg $kg^{-1}$ respectively (Plates 5.4 D, 5.4 E and 5.4 F) with significant reduction in the mast cell count when compared with the acetic acid-induced colitic control rats (Fig 5.4). Likewise, there was reduced proliferation of mucosal mast cell in the colonic segment of the rats treated with XA (10 – 100 mg $kg^{-1}$) as shown on Plates 5.4 G, 5.4 H and 5.4 I respectively. The count of mast cells showed a significant reduction in the XA-treated groups (Fig 5.4).

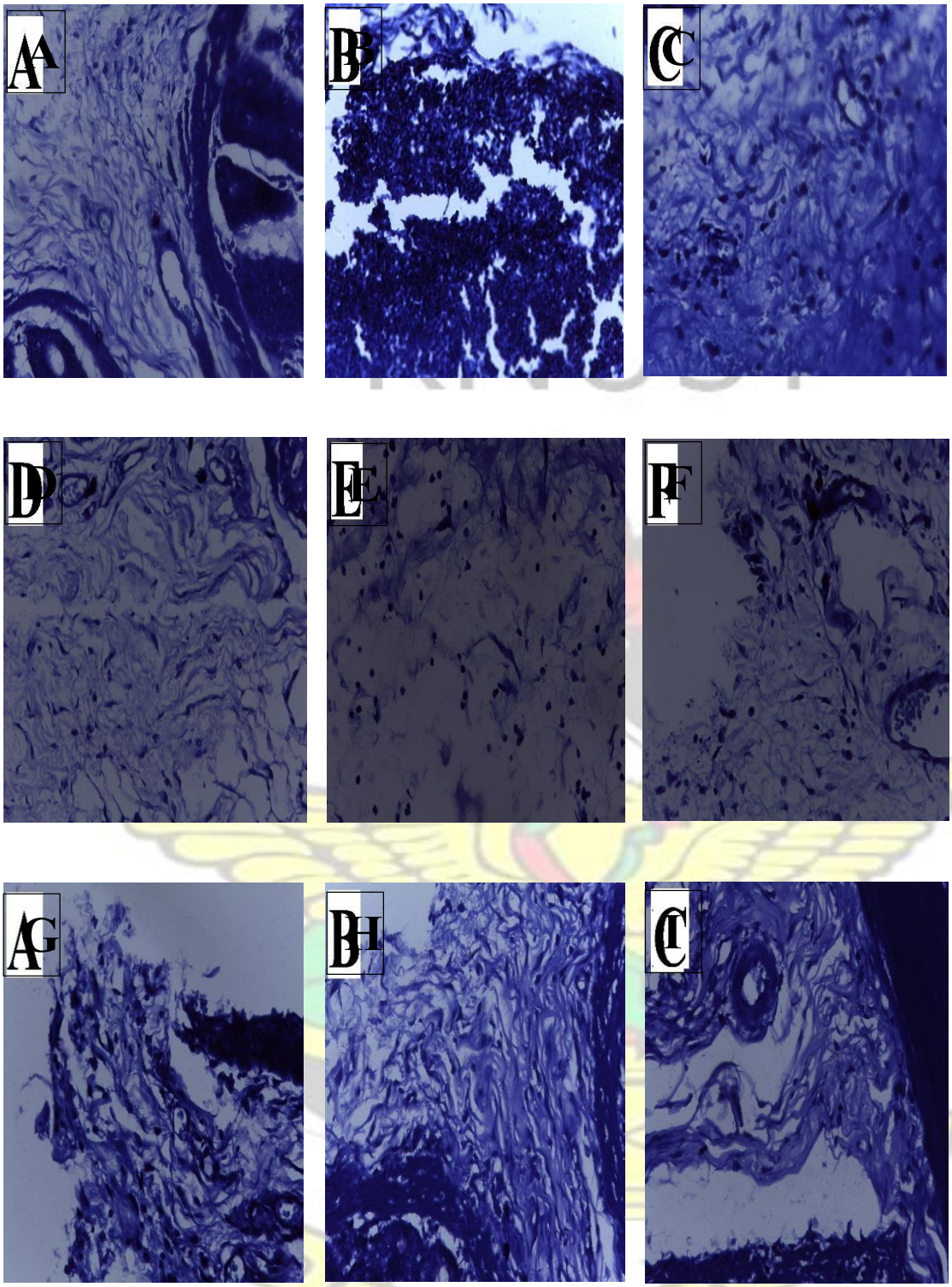



**Plate 5.4. Mast cell proliferation in colon of rats.** Sprague-Dawley rats were treated with either normal saline, sulphasalazine (500 mg $kg^{-1}$), XAE (30 – 300 mg $kg^{-1}$) or XA (10 - 100 mg $kg^{-1}$) for 8 days. On 4$^{th}$ day rats received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and the distal colon were fixed immediately in 10% formaldehyde. Colons were fixed in Carnoy's fixative and stained with 1% toluidine blue. Overall morphology was assessed. Non-colitic control (A), acetic acid-treated control (B), sulphasalazine 500 mg $kg^{-1}$ (C) XAE 30 mg $kg^{-1}$ (D), XAE 100 mg $kg^{-1}$ (E), XAE 300 mg $kg^{-1}$ (F), XA 10 mg $kg^{-1}$ (G), XA 30 mg $kg^{-1}$ (H) and XA 100 mg $kg^{-1}$ (I).

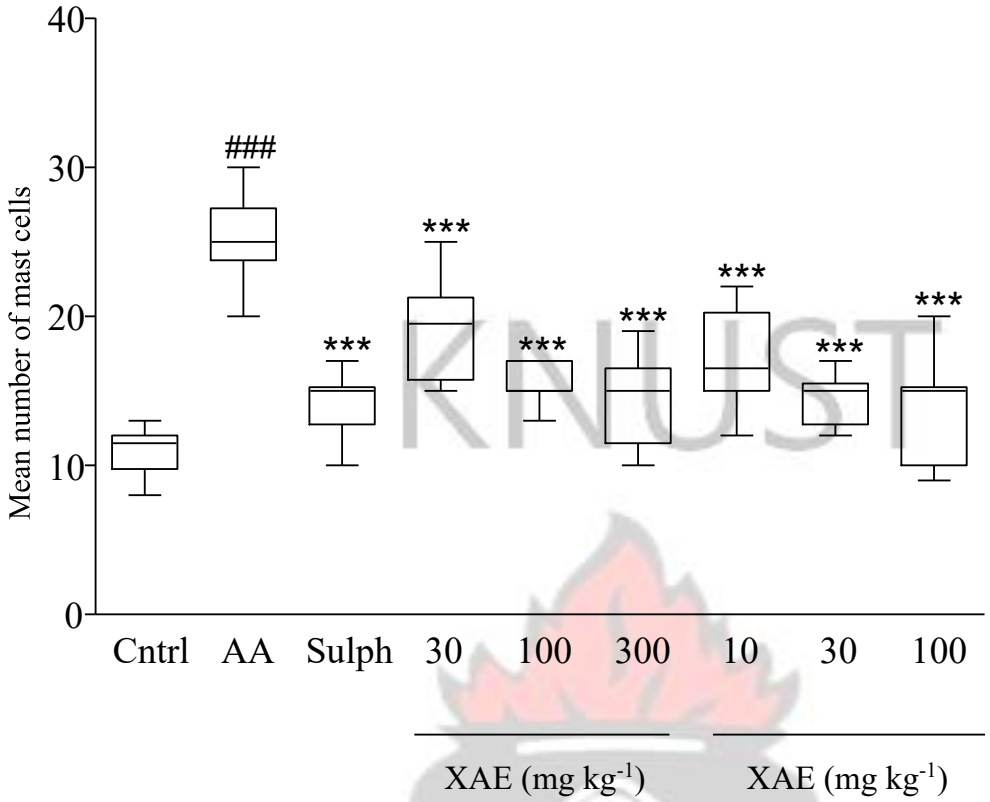



**Figure 5.4. Mast cell count in colon of acetic acid-induced ulcerative colitic rats.** SpragueDawley rats were treated with either normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 – 300 mg $kg^{-1}$) or XA (10 - 100 mg $kg^{-1}$) for 8 days. On 4$^{th}$ day rats received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and the colon fixed immediately in 10% formaldehyde. Full thickness segments of colon were fixed in Carnoy's fixative and stained with 1% toluidine blue. Mast cells were counted in 10 coded sections at X40 magnification. Data presented as Mean ± SEM. ***$P < 0.0001$ when compared with colitic control. $^{###}P <$ 0.0001 when compared with non-colitic control.

### *5.3.1.6 Enzyme assay*

Although the underlining cause of ulcerative colitis remains elusive, it has been reported by Thippeswamy *et al.* (2011) that increased levels of *myeloperoxidase* (MPO) and malondialdehyde (MDA) are prime biomarkers for colon damage coupled with the reduced activity of *superoxide dismutase* (SOD), *ascorbate peroxidase* (APx) and *catalase* (CAT).

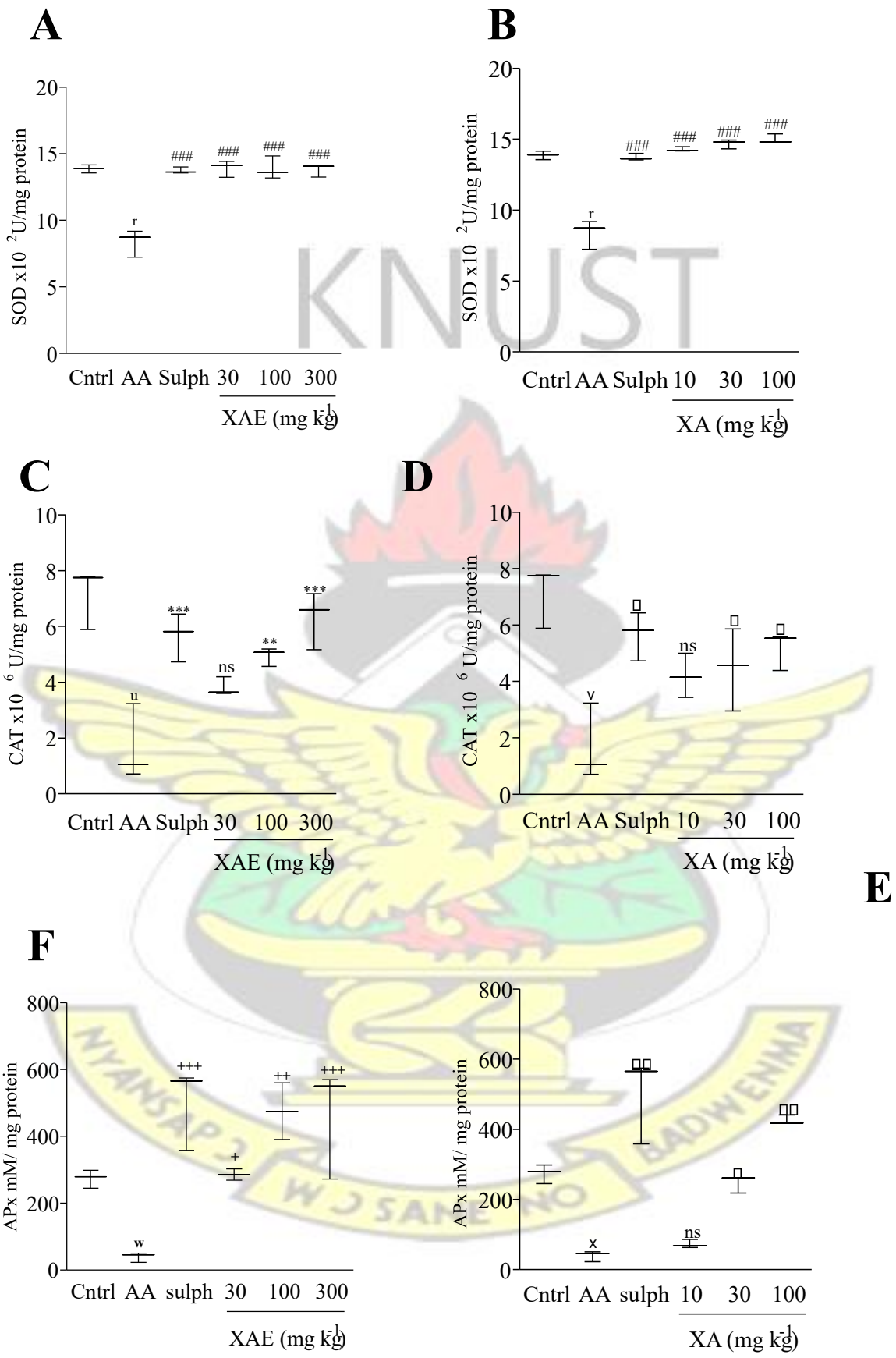
In investigating the effect of XAE or XA on these enzymes, colonic tissue samples were homogenised, centrifuged and from the supernatant the levels of SOD, CAT, APx, and MPO measured. Non-colitic control showed detectable activities of SOD which was significantly reduced upon acetic acid treatment in rats (Figs 4.5 A and 4.5 B). Sulphasalazine treatment resulted in the increased activity of SOD in the colonic tissues (Figs 5.5 A and 5.5 B). Treatment of rats with XAE significantly ($P < 0.0001$) increased the activity of SOD at doses 30 – 300 mg $kg^{-1}$ studied (Fig 5.5 A). Again, treatment with XA also significantly ($P < 0.0001$) increased the activity of SOD at 10 – 100 mg $kg^{-1}$ (Fig 5.5 B).

In the assay of CAT, rats in the non-colitic control group showed significant activity of the enzyme which was decreased when rats were treated with the acetic acid intrarectally (Figs 5.5 C and 5.5 D). Treatment with sulphasalazine increased the activity of CAT significantly (Figs 5.5 C and 5.5 D). In the XAE (30 – 300 mg $kg^{-1}$)treated rats, there was a dose-dependent increased activity of CAT in the rats when compared with rats treated with acetic acid (Fig 5.5 C). Similarly, XA-treated rats showed a dose-dependent increase in the activity of CAT post-colitis at 10 – 100 mg $kg^{-1}$ when compared with rats treated with acetic acid (Fig 5.5 D).

With the assay of the levels of APx, rats in the non-colitic control group showed a significant activity of the enzyme which was decreased with acetic acid treatment. The treatment with sulphasalazine significantly increased the activity of APx (Figs 5.5 E and 5.5 F). On treating rats with XAE (30 – 300 mg $kg^{-1}$), there was increased activity of APx which were significant and dose-dependent (Fig 5.5 E). Likewise, the XAtreated rats showed a significant and dose-dependent increased activity of APx at 10 –

100 mg $kg^{-1}$ when compared with the acetic acid-treated rats (Fig 5.5 F).

The activity of MPO was detectable in the non-colitic control rats and was significantly increased upon acetic acid treatment intrarectally; with sulphasalazine significantly decreasing the activity of MPO relative to the acetic acid-treated rats (Figs 5.5 G and 5.5 H). Treatment of rats with XAE reduced the activity of MPO at 100 and 300 mg $kg^{1}$ (Fig 5.5 G). Similarly, XA-treated rats showed a significant decrease in the activity of MPO at 30 and 100 mg $kg^{-1}$ when compared with the acetic acid-treated rats (Fig 5.5 H).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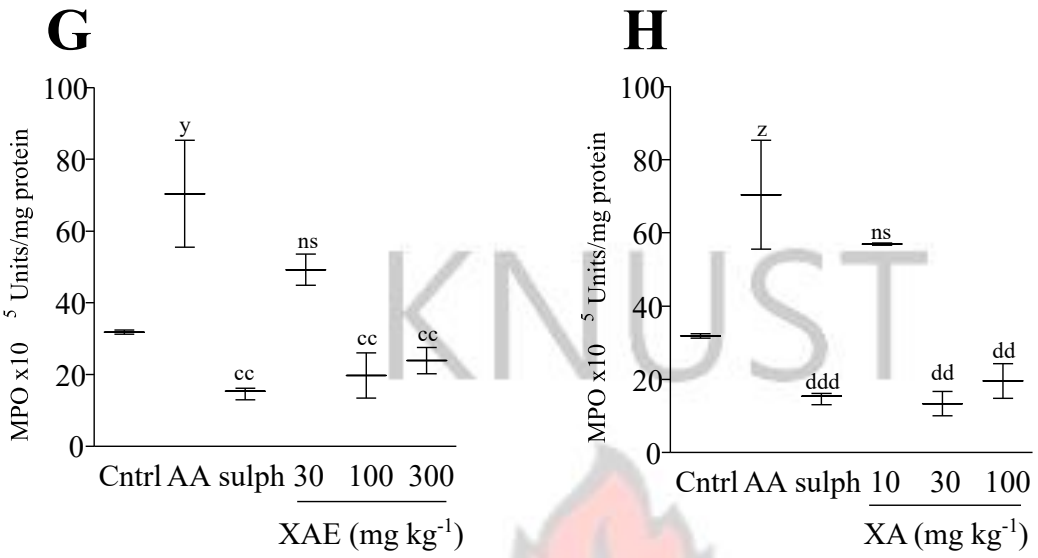


**Figure 5.5. Assay of *superoxide dismutase, catalase, ascorbate peroxidase* and *myeloperoxisade* in acetic acid-induced ulcerative colitic rats.** Sprague-Dawley rats were treated with either normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 – 300 mg $kg^{-1}$) or XA (10 - 100 mg $kg^{-1}$) for 8 days. On 4$^{th}$ day rats received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and homogenised in ice-cold 0.01 M Tris–HCl buffer (pH 7.4). Assay of SOD (A), CAT (C), APx (E) and MPO (G) in XAE-treated rats (left panel); Assay of SOD (B), CAT (D), APx (F) and MPO (H) in XA-treated rats (right panel). $^{###}P < 0.0001$, $^{**}P = 0.001$, $^{***}P = 0.0001$, $^{+}P < 0.05$, $^{++}P = 0.01$, $^{+++}P = 0.001$, $^{\phi}P = 0.012$, $^{\phi\phi}P = 0.0012$, $^{tt}P < 0.001$, $^{ttt}P < 0.0001$, $^{cc}P = 0.0029$, $^{dd}P = 0.01$, $^{ddd}P = 0.001$, $^{aa}P < 0.002$, $^{aaa}P < 0.0002$, $^{b}P = 0.03$, $^{bb}P = 0.003$, $^{ns}P > 0.05$ when compared with colitic control. $^{r}P < 0.0001$, $^{u}P = 0.0001$, $^{v}P = 0.00012$, $^{w}P < 0.05$, $^{x}P < 0.0001$, $^{y}P = 0.029$, $^{z}P < 0.05$ when compared with non-colitic control.

### *5.3.1.7 Malondialdehyde assay: lipid peroxidation*

Paiva *et al.* (2003) reported that an increased *myeloperoxidase* enhances the levels of malondialdehyde, the result of lipid peroxidation. This initiates a cascade of free radical cycle which worsens the inflammatory process and ends in cellular anti-oxidant exhaustion.

Thus in this study, the level of MDA, the byproduct of lipid peroxidation, was determined in the presence of XAE and XA. Briefly, colonic tissue samples were homogenised, centrifuged and the supernatant used to determine the levels of MDA. It

was observed as expected that acetic acid-induced colitis causes a significant increase in lipid peroxidation recorded as increased levels of malondehyde relative to the noncolitic control (Figs 5.6 A and 5.6 B). Treatment with sulphasalazine significantly lowered the malondialdehyde level when compared with acetic acid-induced animals (Figs 5.6 A and 5.6 B). After XAE treatment, there was a significant reduction in the levels of malondehyde at 30, 100 and 300 mg $kg^{-1}$ respectively when compared with the rats that received acetic acid. This implies reduced lipid peroxidation in the extracttreated groups when compared with the acetic acid control (Fig 5.6 A). Again, treatment of animals with XA significantly reduced the expression of MDA at 10, 30 and 100 mg $kg^{-1}$ when compared with rats that received acetic acid (Fig 5.6 B). This signifies reduced lipid peroxidation in xylopic acid-treated groups when compared with the

colitic rats.

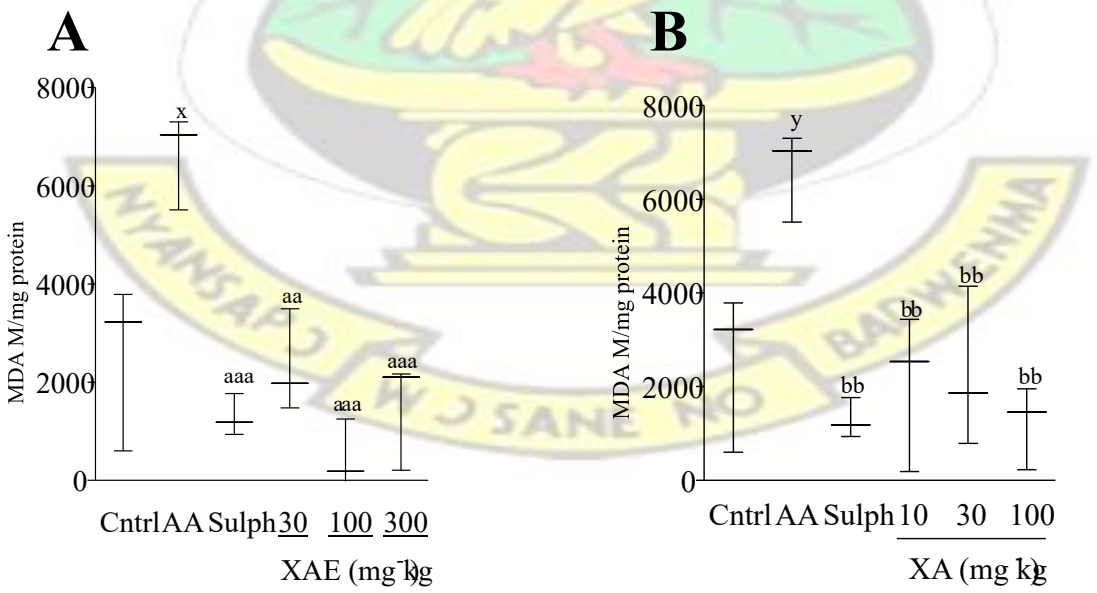


**Figure 5.6. Effect of *Xylopia aethiopica* or xylopic acid-treatment on lipid peroxidation in ulcerative colitis induced by acetic acid in rats.** Sprague-Dawley rats were treated with either

normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 – 300 mg $kg^{-1}$) or XA (10 - 100 mg $kg^{-1}$) for 8 days. On 4th day rats received intrarectally 1 ml 4.0% acetic acid ($^{v}/_{v}$). Colons were extirpated and homogenised in ice-cold 0.01 M Tris–HCl buffer (pH 7.4). Assay of MDA in XAE-treated rats (A) and in XA-treated rats (B). $^{aa}P < 0.002$, $^{aaa}P < 0.0002$, $^{bb}P = 0.003$ when compared with colitic control. $^{x}P < 0.002$, $^{y}P = 0.03$ when compared with non-colitic control.

### *5.3.2 Adjuvant-induced arthritis in rats*

Adjuvant arthritis induced by heat-killed *Mycobacterium tuberculosis* cells is known to simulate the immunological and biochemical features of rheumatoid arthritis (Ramprasath *et al.,* 2006). It is the most frequently studied model of chronic inflammation in rats (Brand, 2005; Escandell *et al.*, 2007; Hughes *et al*., 1989; Wang *et al.*, 2008).

#### *5.3.2.1 Body weight change*

The body weight was monitored over a period of 28 days and percentage changes in body weight determined. From the results obtained, non-arthritic animals showed no significant change in body weight over the period of the study (Figs 5.7 A and 5.7 C). The total body weight of the rats expressed as the AUC also did not significantly change (Figs 5.7 B and 5.7 D). The arthritic control rats showed an initial decrease in body weight with time with a mild increase in body weight from day 8 (Figs 5.7 A and 5.7 C). There was no significant decrease in total body weight of the rats in the CFA group when compared with the non-arthritic rats (Figs 5.7 B and 5.7 D).

Prophylactic treatment of rats with aspirin showed an initial decline in body weights of animals which steadily rose after day 10 (Figs 5.7 A and 5.7 C). The total body weight over the period of study was not significantly higher than was observed in non-arthritic control (Figs 5.7 B and 5.7 D). XAE treatment of rats resulted in an initial decrease in

body weight of the animals with a steady increase in body weight from day 10 to 28 (Fig 5.7 A). There were no significant ($P > 0.05$) changes in total body weights of rats at doses 100, 300 and 600 mg $kg^{-1}$ of XAE over the period of study when compared with the non-arthritic control (Fig 5.7 B). Similarly, treatment with XA yielded an initial decrease in body weight which increased steadily from day 10 (Fig 5.7 C) with no significant increase in total body weight of rats at doses 10 and 30 mg $kg^{-1}$. However, 100 mg $kg^{-1}$ of XA showed a significant increase in body weight when compared with non-arthritic rats (Fig 5.7 D).

In the therapeutic regimen, rats treated with aspirin showed an initial decline in body weights of animals which steadily rose after day 14 (Figs 5.8 A and 5.8 C). The total body weight over the period of study did not increase significantly when compared with the non-arthritic control (Figs 5.8 B and 5.8 D). XAE-treated rats also presented with an initial decrease in body weight of the animals with a steady increase in body weight from day 14 (Fig 5.8 A). There were no significant ($P > 0.05$) changes in total body weights of rats at doses 100, 300 and 600 mg $kg^{-1}$ when compared with the non-arthritic rats (Fig 5.8 B). Also, treatment with XA in the therapeutic protocol resulted in an initial decrease in body weight which increased steadily from day 14 (Fig 5.8 C) with no significant increase in total body weight of rats at doses 10, 30 and 100 mg $kg^{-1}$ when compared with non-arthritic rats (Fig 5.8 D).

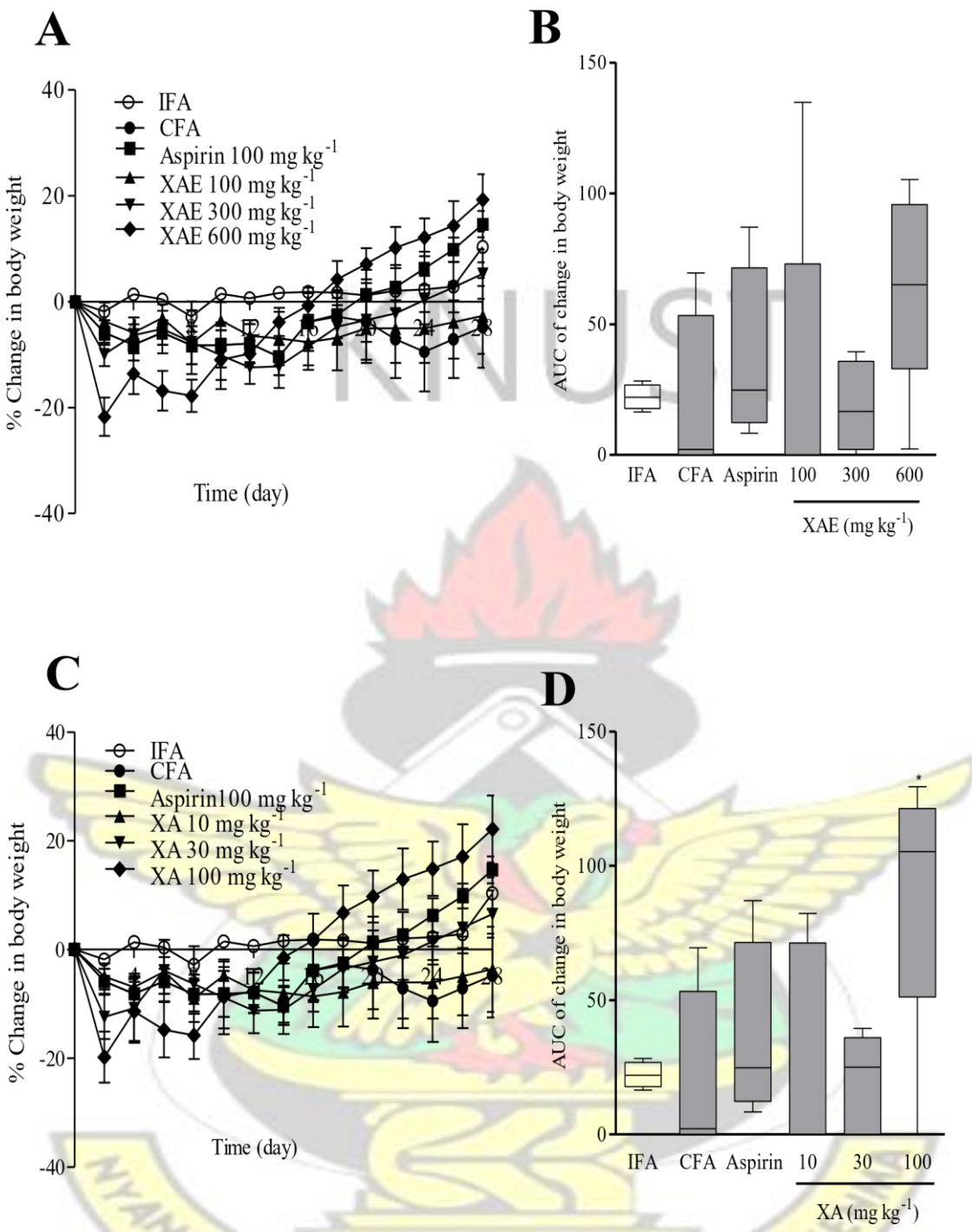


**Figure 5.7. Effect of *Xylopia aethiopica* extract and xylopic acid given prophylactically on body weight in CFA-arthritic rats.** Sprague-Dawley rats (200 – 250 g) (n = 5) were injected intraplantar with 100 µl of Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant into the right hind paw. Their body weights were monitored on every other day as the percentage change in body weight. The drug vehicle and either aspirin 100 mg kg $^{-1}$, XAE (100, 300, 600 mg kg $^{-1}$) or XAE (10, 30, 100 mg kg$^{-1}$) were given orally 1 h before CFA injection. The body weight was monitored as the percentage change in body weight [A, C]. Total body weight measured during the acute and polyarthritis phases was calculated as area under the time course curves, AUC [B, D]. *$P$ = 0.0335, $P$ > 0.05 in all treatment groups.

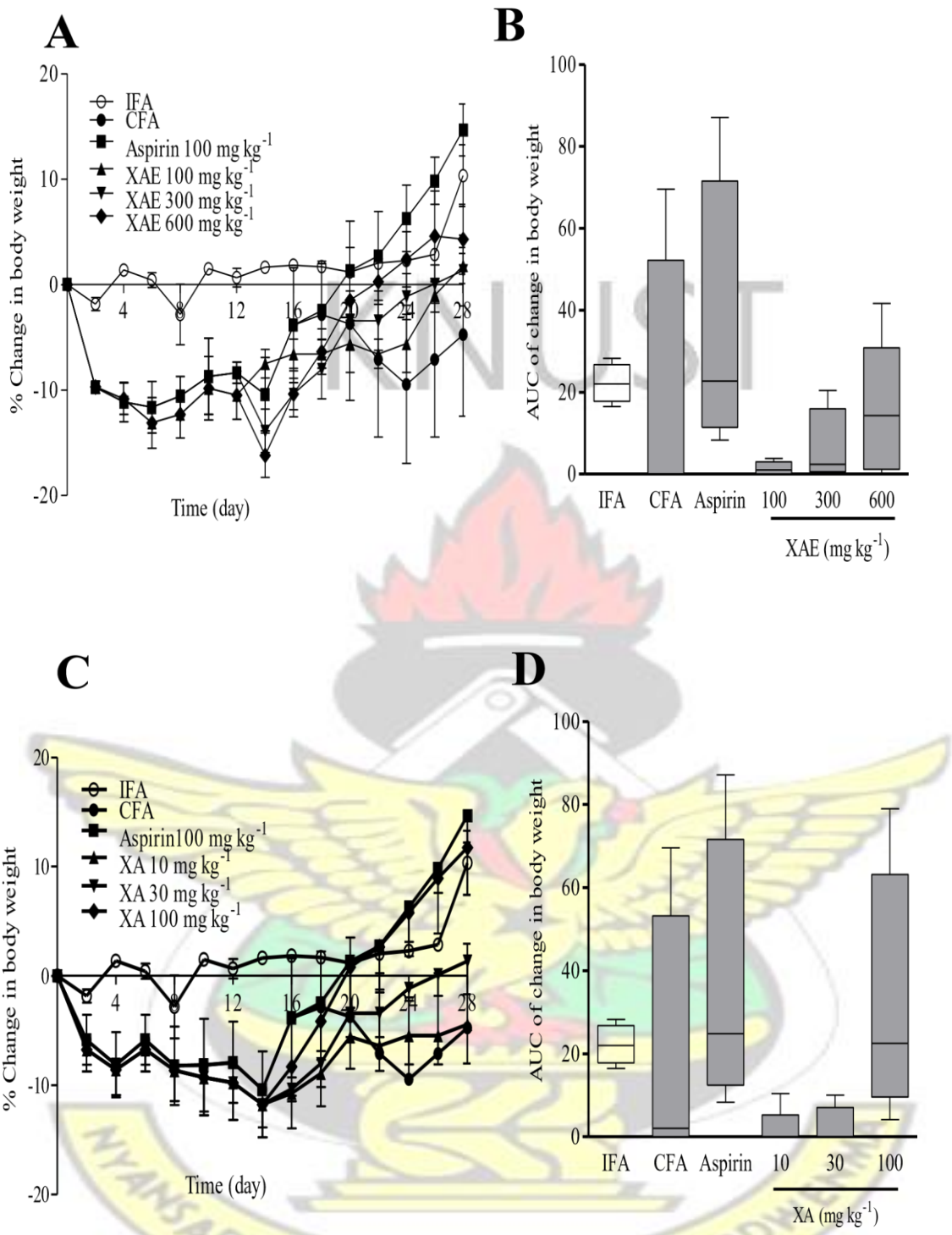


**Figure 5.8. Effect of *Xylopia aethiopica* extract and xylopic acid given therapeutically on body weight in CFA-arthritic rats.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant into the right hind paw. Their body weights were monitored on every other day as the percentage change in body weight. The drug vehicle and either aspirin 100 mg $kg^{-1}$, XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) were given orally starting on the 14$^{th}$ day. The body weight was monitored as the percentage change in body weight [A, C]. Total body weight measured during the acute and polyarthritis phases was calculated as area under the time course curves, AUC [B, D]. $P > 0.05$.

### *5.3.2.2 Oedema assessment*

The validity of the sensitivity of the rat adjuvant-induced arthritis model to antiinflammatory drugs was demonstrated when the daily administration of aspirin suppressed both the maximal oedema response (Fig 5.9 A) as well as the spread of the total oedema response (Fig 5.9 B) in both the ipsilateral and contralateral paws.

In the prophylactic study, the IFA non-arthritic rats showed no signs of oedema during the acute and polyarthritic phase (Fig 5.9 A). The CFA arthritic and drug treated rats showed acute inflammatory oedema at the ipsilateral (injected) paw between days 4 - 6 followed by subsequent chronic polyarthritic phase which was observed around day 12 - 14 and peaked on the 24$^{th}$ day (Figs 5.9 A, 5.9 C and 5.9 E). Total rat knee joint swellings induced over the 28 days were measured as the area under the time course curve, AUC respectively (Figs 5.9 B, 5.9 D and 5.9 F). Respectively, the progress of inflammatory oedema in the contralateral (non-injected) limb was evident from day 13 indicative of a systemic spread of the inflammation (Figs 5.9 A, 5.9 C and 5.9 E, insert). XAE (100 – 600 mg kg$^{-1}$) modified the time course of the oedema significantly ($P$ = 0.01) and showed a significant reduction of oedema induced with the mean maximal swelling attained during the study reducing respectively to 188.34 ± 32.57%, 127.80 ± 39.57% and 130.55 ± 25.64% of the arthritic control rats (Fig 5.9 C). These doses of XAE reduced the total limb swelling over 28 days in the ipsilateral limb respectively to 61.42 ± 14.55%, 46.30 ± 9.78% and 41.07 ± 4.71% respectively (Fig 5.9 D). On the other hand, XA (10 - 100 mg kg$^{-1}$) modified the time course curve significantly ($P$ = 0.0039) and resulted in a significant reduction of oedema with the maximal swelling attained respectively reduced to 143.34 ± 18.45%, 146.28 ± 7.92% and 116.57 ±

24.60% of the arthritic control rats (Fig 5.9 E). The total limb swelling significantly reduced in the ipsilateral limb to 57.93 ± 6.72%, 51.86 ± 11.81% and 51.29 ± 8.34% respectively (Fig 5.9 F).

In the therapeutic protocol, drug treatment commenced on day 14 at the start of the development of the polyarthritic phase of the chronic inflammation. The knee swelling was measured over 28 days. The progress of inflammatory oedema in the contralateral (non-injected) limb was evident from day 13 in arthritic control and drug-treated rats, indicative of a systemic spread of the inflammation (Figs 5.10 B, 5.10 D and 5.10 F). XAE (100 – 600 mg $kg^{-1}$) modified the time course of the oedema significantly ($P$ = 0.0005) and showed a significant reduction of the mean maximal swelling attained reduced to 201.93 ± 36.12%, 143.96 ± 23.41% and 106.96 ± 23.13% respectively (Fig 5.10 C). There was a reduced total limb swelling over 28 days in the ipsilateral limb to 60.93 ± 12.57%, 56.07 ± 9.24% and 42.90 ± 5.60% respectively (Fig 5.10 D). For, XA (10 - 100 mg $kg^{-1}$) there was a significant reduction of oedema with the maximal swelling attained during the study reduced respectively to 172.84 ± 33.28%, 154.01 ± 11.31% and 153.65 ± 10.47% (Fig 5.10 E). The total limb swelling over 28 days was reduced to 79.20 ± 9.20%, 78.66 ± 3.25% and 62.21 ± 11.85% respectively (Fig 5.10 F).

**A** **B**

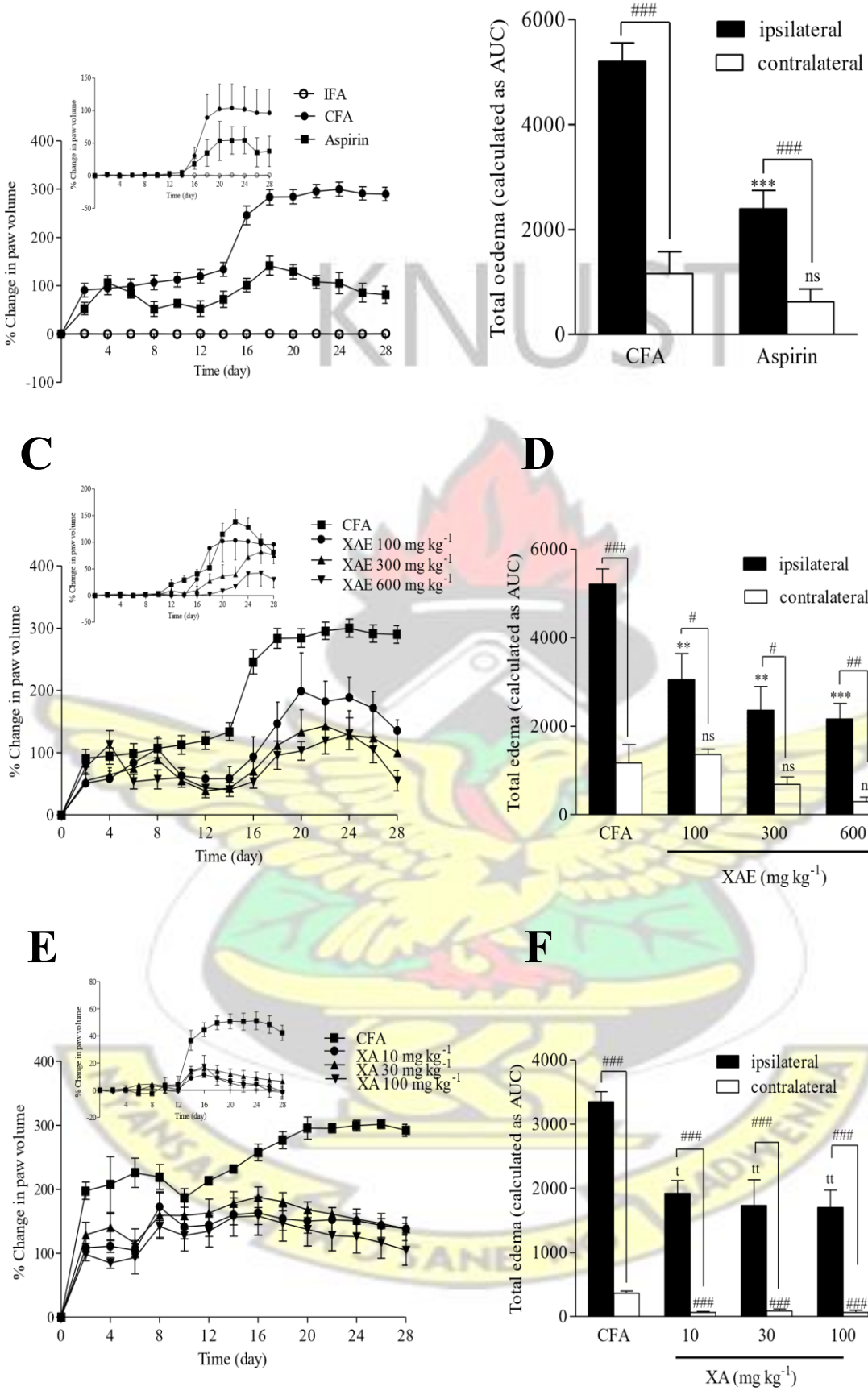


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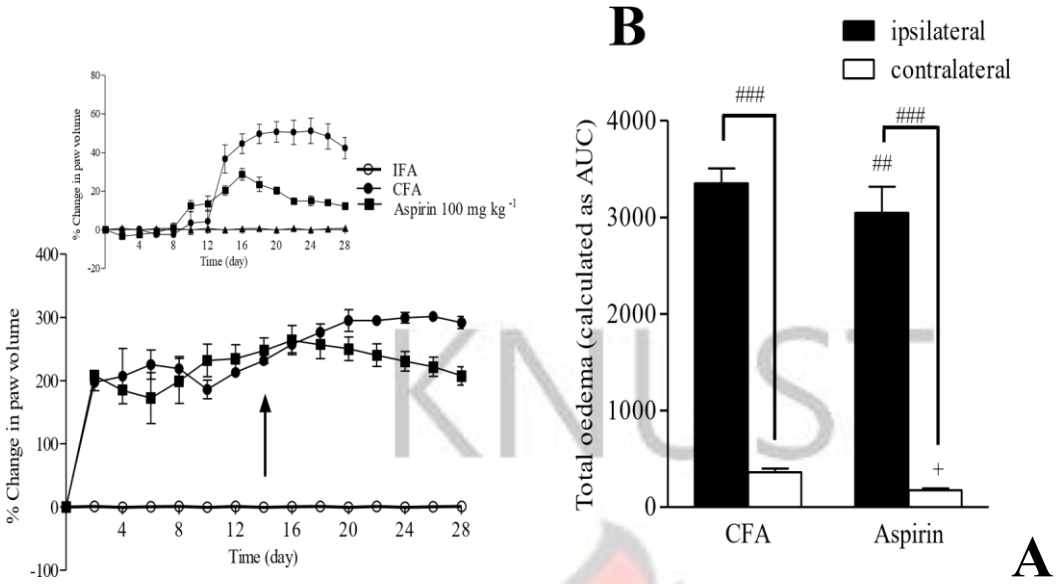
**Figure 5.9. Effect of *Xylopia aethiopica* and xylopic acid administered prophylactically on adjuvant-induced arthritis in rats.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant into the right hind paw. Foot volume was measured by plethysmometry for both the ipsilateral (injected paw) and contralateral paw (non-injected paw) before intraplantar injection of CFA and on alternate days up to the 28$^{th}$ day. The oedema component of inflammation was monitored as the percentage change in paw volume [A, C, E]. Total oedema induced during the acute and polyarthritis phases was calculated as area under the time course curves, AUC [B, D, F]. The drug vehicle, aspirin 100 mg kg$^{-1}$, XAE (100, 300, 600 mg kg$^{-1}$) or XA (10, 30, 100 mg kg$^{-1}$) were given orally 1 h before the induction of the arthritis and daily for 28. Drug effects were evaluated by comparing the maximal and total oedema responses attained during the 28 days in drug-treated groups with the corresponding values attained in drug vehicletreated inflamed control groups. Total oedema is calculated as the area under the time course curve (AUC). Data is presented as Mean ± SEM. (n = 5). Time course curves for paw volume were subjected to Two-way (treatment x time) repeated measure ANOVA followed by Bonferroni's post hoc test). $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.0001$, $^{***}P = 0.0005$, $^{+}P = 0.0338$, $^{t}P = 0.039$, $^{tt}P = 0.0039$, $^{ns}P > 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test).


B
ipsilateral
contralateral
###
###
##
4000
3000
2000
1000
0
Total oedema (calculated as AUC)
+
CFA
Aspirin
A
IFA
CFA
Aspirin 100 mg kg -1
% Change in paw volume
Time (day)
400
300
200
100
0
-100
4
8
12
16
20
24
28

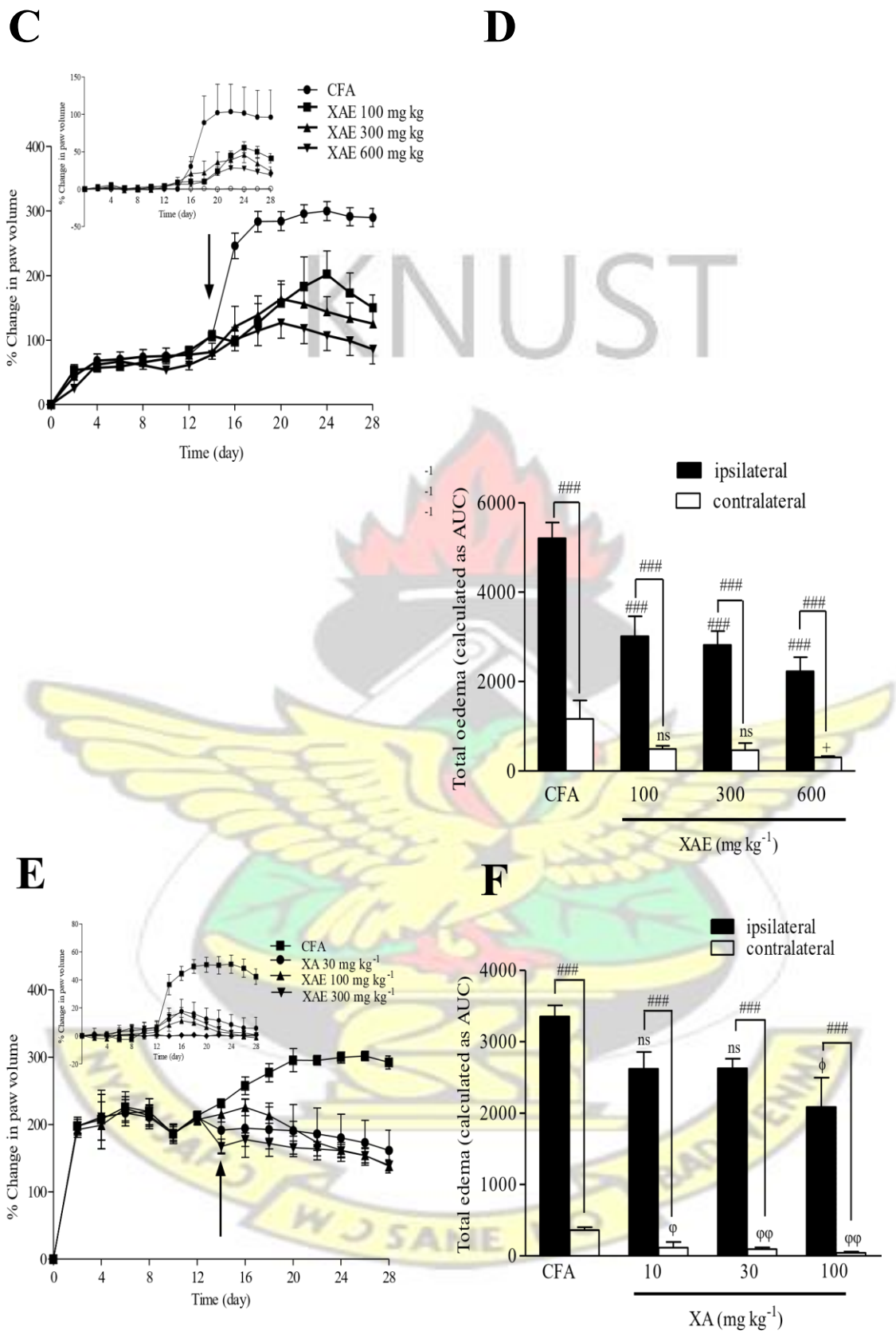



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**Figure 5.10. Effect of *Xylopia aethiopica* and xylopic acid administered therapeutically on adjuvant-induced arthritis in rats.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant into the right hind paw. Foot volume was measured by plethysmometry before intraplantar injection of CFA and on alternate days up to the 28$^{th}$ day. The oedema component of inflammation was monitored as the percentage change in paw volume [A, C, E]. Total oedema induced during the acute and polyarthritis phases was calculated as area under the time course curves, AUC [B, D, F]. The drug vehicle, aspirin 100 mg kg$^{-1}$, XAE (100, 300, 600 mg kg$^{-1}$) or XA (10, 30, 100 mg kg$^{-1}$) and treatment commenced on day 14. Drug effects were evaluated by comparing the maximal and total oedema responses attained during the 28 days in drug-treated groups with the corresponding values attained in drug vehicle-treated inflamed control groups. Total oedema is calculated as the area under the time course curve (AUC). Data is presented as Mean ± SEM (n = 5). Time course curves for paw volume were subjected to Two-way (treatment x time) repeated measure ANOVA followed by Bonferroni's post hoc test). $^{\#\#}P <$ 0.001, $^{\#\#\#}P < 0.0001$, $^{***}P = 0.0005$, $^{+}P = 0.0338$, $^{\varphi}P = 0.0424$, $^{\phi}P = 0.0022$, $^{\phi\phi}P = 0.022$, $^{ns}P >$ 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test). Arrow indicates point of Aspirin, XAE or XA administration.

#### *5.3.2.3 Arthritis score*

##### *5.3.2.3.1 Photography*

From the photographs, the non-arthritic group showed no signs of oedema and erythema (Plate 5.5A). Arthritic control group showed enormous erythema and swelling in both ipsilateral and contralateral hind limbs (Plate 5.5 B). Aspirin administered prophylactically caused a reduced oedema in both ipsilateral and contralateral limbs (Plate 5.5 C). The XAE (100, 300, 600 mg kg$^{-1}$)-treated groups respectively showed reduced erythema and swelling (Plates 5.5 D, 5.5 E and 5.5 F) comparable with that seen in the aspirin-treated group. There were significantly reduced erythema and swelling comparable with that seen in the aspirin-treated group with 10, 30 and 100 mg kg$^{-1}$ of XA respectively (Plates 5.5 G, 5.5 H and 5.5 I).

In the therapeutic protocol, aspirin-treated rats showed reduced erythema and oedema comparable with that in the prophylactically-treated aspirin rats (Plate 5.6 A). XAE treatment resulted in a reduced erythema and swelling respectively at 100, 300 and 600 mg kg$^{-1}$ (Plates 5.6 B, 5.6 C and 5.6 D) which were comparable with the aspirin-treated

group (Plate 5.6 A). Also, XA treatment showed a reduced inflammation and erythema respectively at doses 10, 30 and 100 mg $kg^{-1}$ (Plates 5.6 E, 5.6 F and 5.6 G).

From the physical observation of the photographs, the severity of the arthritis was measured in a blind manner and scored. The non-arthritic (IFA) control rats recorded the lowest score and presented with no swellings (Figs 5.11 A, 5.11 B, 5.11 C and 5.11 D) compared to the arthritic control group which showed enormous erythema and swelling in both ipsilateral and contralateral limbs and thus recorded the highest arthritic score in the prophylactic protocol (Figs 5.11 A, 5.11 B, 5.11 C and 5.11 D). Aspirin-treated rats showed a significantly reduced limb swelling when compared with the arthritic control rats (Figs 5.11 A, 5.11 B, 5.11 C and 5.11 D). Administered prophylactically, XAE significantly *(P* < 0.05) suppressed the swellings thereby reducing the arthritic score in the ipsilateral limb (Fig 5.11 A). This effect of the extract was even more pronounced on the contralateral limbs at 300 and 600 mg $kg^{-1}$ with the arthritic score of the 100 mg $kg^{-1}$ XAE-treated rats not significantly different from the aspirin-treated group (Fig 5.11 A). In XA-treated groups, there was reduction of inflammation in the ipsilateral limb with a significant decrease in contralateral limb inflammation in 100 mg $kg^{-1}$ treated rats (Fig 5.11 B).

In the therapeutic regimen, however, XAE significantly reduced arthritis score at 300 and 600 mg $kg^{-1}$ but not at 100 mg $kg^{-1}$ in the ipsilateral limb (Fig 5.11 C). XAE at 100, 300 and 600 mg $kg^{-1}$ treatment caused a significant ($P < 0.05$) reduction in the arthritic scores in the contralateral limbs (Fig 5.11 C). There was a reduction of paw oedema in the ipsilateral limb in XA-treated rats. However, the results showed that it was only the 100 mg $kg^{-1}$ XA that caused a significant reduction of the arthritis score in the contralateral limb (Fig 5.11 D).

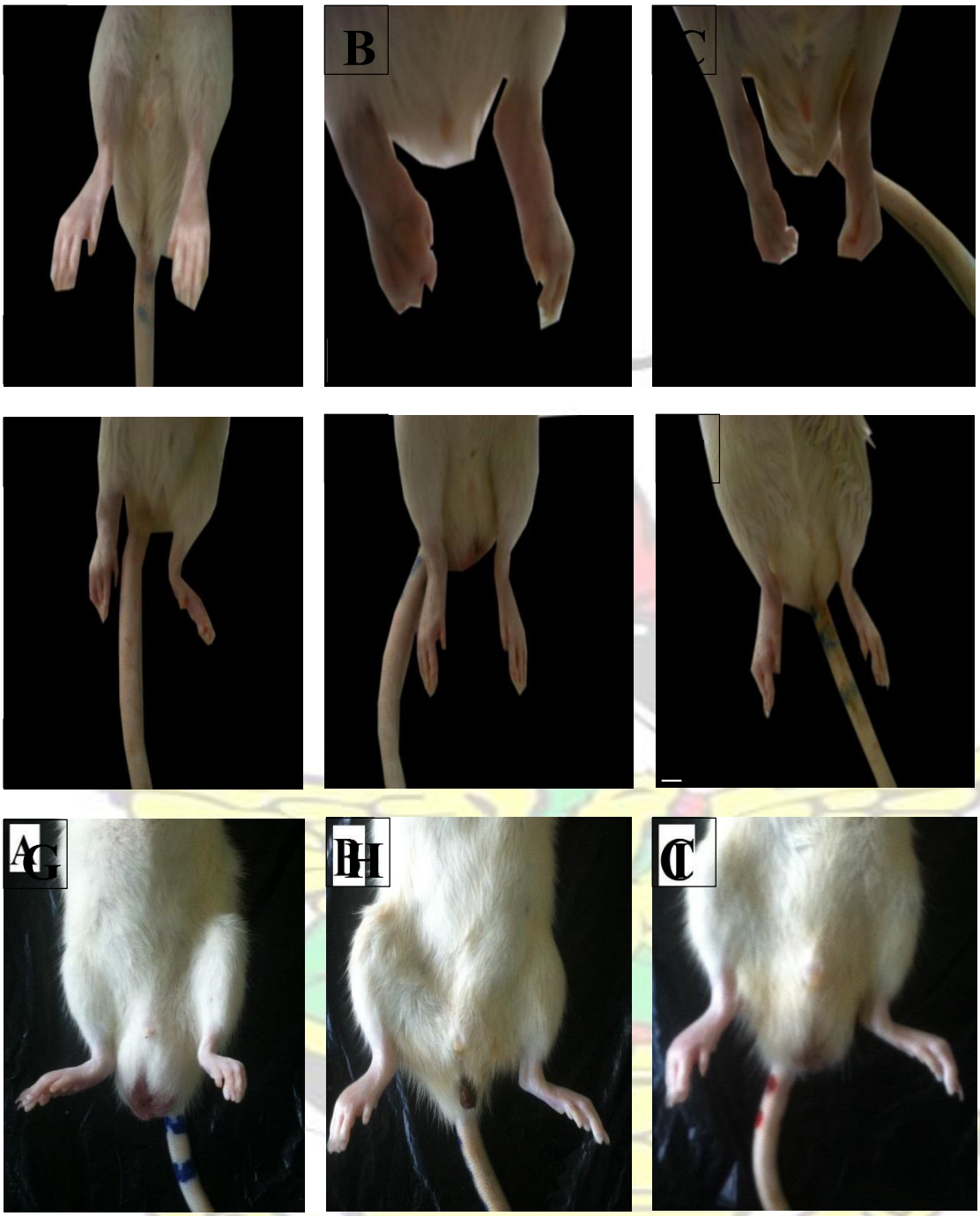



**Plate 5.5. Photographs showing the effect of *X. aethiopica* extract and xylopic acid administered prophylactically in adjuvant-induced arthritis in rats.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw and monitored for 28 days. The drug vehicle, aspirin 100 mg $kg^{-1}$, XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$), were given orally 1 h before the induction of the arthritis and daily for 28 days. Photographs were taken of the hind limbs using a digital camera. The extent of inflammation as observed in the hind paws from the photographs taken scored on a scale of 0 - 4, with 0 being the score of the normal/uninjected paw group. IFA/ non-arthritic control (A), CFA/arthritic control (B), 100 mg $kg^{-1}$ Aspirin (C) 100 mg $kg^{-1}$ XAE (D), 300 mg $kg^{-1}$ XAE (E), 600 mg $kg^{-1}$ XAE (F), 10 mg $kg^{-1}$ XA (G), 30 mg $kg^{-1}$ XA (H) and 100 mg $kg^{-1}$ XA (I).

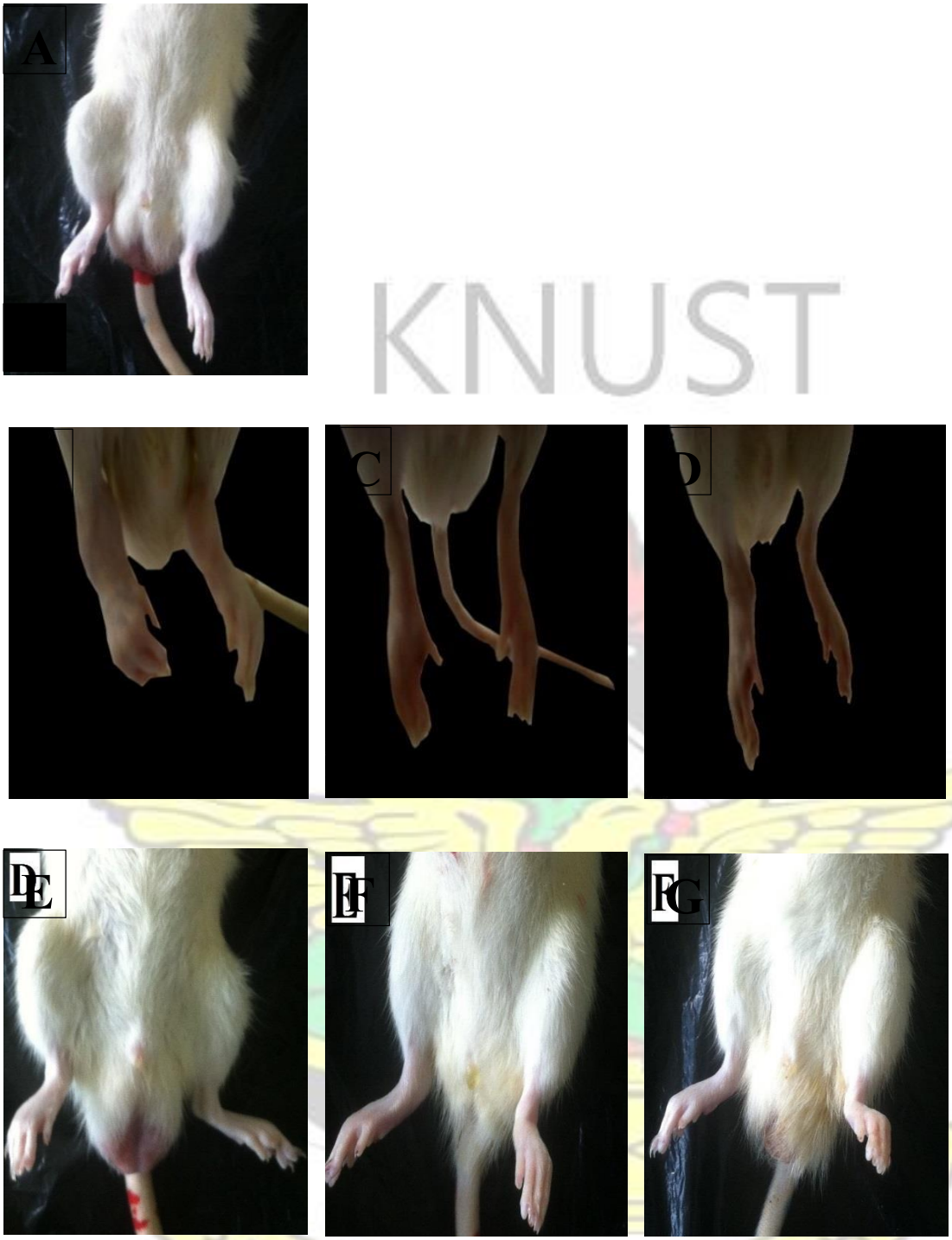





**Plate 5.6. Photographs showing the effect of *X. aethiopica* extract and xylopic acid administered therapeutically in adjuvant-induced arthritis in rats.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw and monitored for 28 days. Aspirin 100 mg $kg^{-1}$; XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30 and 100 mg $kg^{-1}$) were given orally on the 14$^{th}$ day after the induction of the arthritis and daily till 28$^{th}$ day. Photographs were taken of the hind limbs using a digital camera. The extent of inflammation as observed in the hind paws from the photographs taken scored on a scale of 0 - 4, with 0 being the score of the normal/uninjected paw group. 100 mg $kg^{-1}$ Aspirin (A), 100 mg

kg$^{-1}$ XAE (B), 300 mg kg$^{-1}$ XAE (C), 600 mg kg$^{-1}$ XAE (D), 10 mg kg$^{-1}$ XA (E), 30 mg kg$^{-1}$ XA (F) and 100 mg kg$^{-1}$ XA (G).

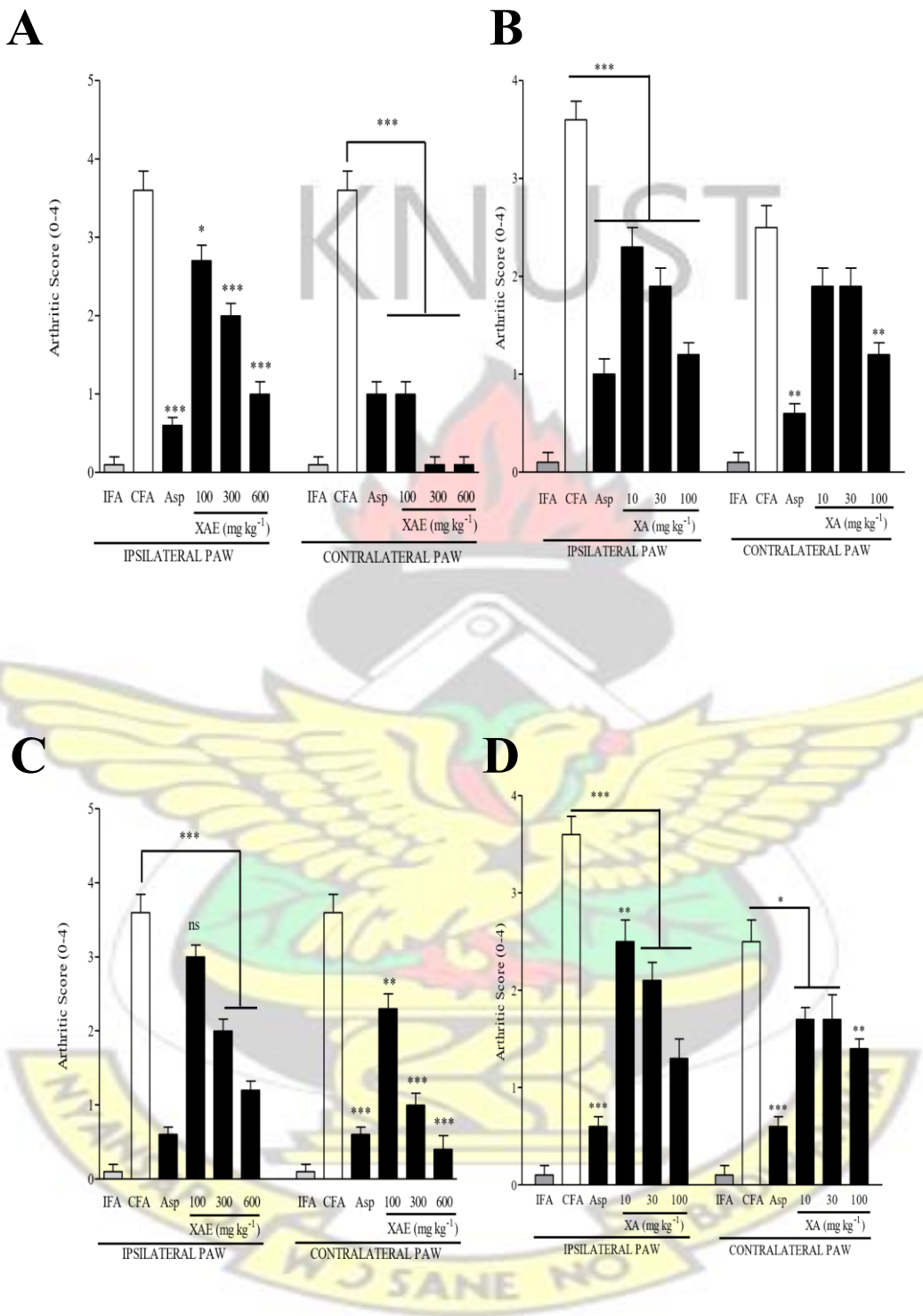



**Figure 5.11. Arthritis scoring of photographs in adjuvant-induced arthritic rats.** SprageDawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw and monitored for 28 days. Aspirin 100 mg kg$^{-1}$, XAE (100, 300, 600 mg kg$^{-1}$) or XA (10, 30, 100 mg kg$^{-1}$) were given orally 1 h before the induction of the arthritis and daily for 28 days in the prophylactic protocol; and treatment commenced on day 14 in the therapeutic protocol. The severity of the arthritis was measured blindly on day 28 on a scale of 0 - 4. The hind paw volume was used as parameter for measurement with severity of arthritic score determined based on extent of erythema and oedema of a given tissue (n = 5). Top panel

[A, B] is prophylactic protocol; Bottom panel [C, D] is therapeutic protocol. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$, $^{++}P = 0.00014$, $^{ns}P > 0.05$.

#### *5.3.2.3.2 Radiography*

Radiography, typically the first imaging study in evaluation for arthritis, offers true remission of disease and accurate evaluation of disease status (Kitamura *et al.,* 2007). It enables differentiation from other joint diseases such as osteoarthritis and is of value when alternative means of assessment of rheumatoid arthritis (RA) is not conclusive (Bohndorf and Schalm, 1996).

Radiographical analyses of rat limbs after XAE and XA treatment will, therefore, offer an accurate evaluation of the arthritis status. Therefore, rat adjuvant arthritis was induced as described earlier. From the radiographs, as was expected, no lesions and joint deformation was observed in the non-arthritic (IFA) control group (Plate 5.7 A). The arthritic control group showed observable signs of inflammation in both the right and left hind limbs including the tissues surrounding the bones of the foot (Plate 5.7 B). There were observable signs of inflammation at the metatarsal-phalangeal joint and the regions in-between the bones of the phalanges and the metatarsals. The rat hind limb shows bone enlargement with active osteophytosis, characterised by very thin trabeculae increasing from bone to connective tissue (1). The osteophytosis is marked on bone metaphysis and linked with lacunae (4). There are no observable joint spaces and some bone islets arise in the connective tissue (2), which is thickened and significantly enlarged (3). Eroding of the phalangeal bone was observed which was absent in the IFA group. Inflammation spread to the fore limbs and slightly affected the carpals (Plate 5.7 B). Prophylactically, aspirin reduced the inflammation and arthritic joint development in rats (Plate 5.7 C). XAE showed prevention of inflammation and subsequent arthritic joint development. Inflammation did not spread to affect tissues

and bones of the fore limbs as was observed in the arthritic control group. In the 100 and 300 mg $kg^{-1}$ XAE-treated groups respectively (Plates 5.7 D and 5.7 E), there was early active osteophytosis in bone metaphysis with decreased bone density, moderate change of joint spaces (5) in a moderately enlarged and dense connective tissue. Regions of minimal stabilised osteophytosis (6) (without newly formed trabecula) were observed in the bone metaphysis and there was persistence of minor joint space alterations in the 600 mg $kg^{-1}$ (Plate 5.7 F) (7). There was reduced signs of bone deformation observed with the increase in doses of the *Xylopia aethiopica* extract. At 100 mg $kg^{-1}$ of XAE (Plate 5.7 D), mild eroding of the phalangeal bone was observed which was comparable with the one observed in the aspirin-treated group (Plate 5.7 C). There was prevention of inflammation and subsequent arthritic joint development in the animals treated with XA (10, 30, 100 mg $kg^{-1}$). Similar to the XAE-treated rats, inflammation did not spread to affected tissues and bones of the fore limbs as was observed in the arthritic control group (Plates 5.7 G, 5.7 H and 5.7 I). In the 10 mg $kg^{1}$ XA rats, there was observable but mild active osteophytosis in bone metaphysis with decreased bone density (Plate 5.7 G), moderate change of joint spaces accompanied with mild bone resorption (1). Respectively, 30 mg $kg^{-1}$ and 100 mg $kg^{-1}$ XA-treatment showed radiological profile comparable with non-arthritic rats (Plates 5.7 H and 5.7 I).

With the therapeutic regimen, aspirin showed an inhibition of inflammation with visible bone metaphysis (Plate 5.8 A). XAE offered prevention of inflammation when compared with arthritic control rats at the doses of 100, 300 and 600 mg $kg^{-1}$ respectively. Inflammation did not spread to affect tissues and bones of the fore limbs as was observed in the arthritic control group. Zones of nominal stabilised osteophytosis

(8) (without newly formed trabecula) were visible in the bone metaphysis (Plates 5.8 B, 5.8 C and 5.8 D). XA administered at doses 10, 30 and 100 mg $kg^{-1}$, on the other hand, resulted in the prevention of inflammation and subsequently reduced the development of arthritic joint. Inflammation did not spread to affected tissues and bones of the fore limbs as was observed in the arthritic control group. XA showed radiological profile comparable with non-arthritic rats at all the dose levels (Plates 5.8 E, 5.8 F and 5.8 G).

In terms of scoring the arthritis from the radiographs, the non-arthritic (IFA) control rats recorded the lowest score and presented with no bone erosion (Figs 5.12 A, 5.12 B, 5.12 C and 5.12 D) compared to the arthritic control model group which showed enormous bone erosion in both ipsilateral and contralateral paws and thus recorded the highest arthritic score (Figs 5.12 A, 5.12 B, 5.12 C and 5.12 D). Prophylactically, aspirin-treated rats showed a significant reduction in bone deformation when compared with the arthritic control rats (Figs 5.12 A, 5.12 B). A significant ($P < 0.0001$) reduction of bone deformation was observed in the ipsilateral paw in XAE (100, 300 and 600 mg $kg^{-1}$) with a significant reduction of bone erosion in the ipsilateral limb (Fig 5.12 A). The contralateral paw showed a reduction of bone deformation (Fig 5.12 A). On the other hand, in XA (10, 30 and 100 mg $kg^{-1}$)-treated groups, there was a significant ($P < 0.0001$) reduction of bone erosion in the ipsilateral paw with a reduction of bone erosion in the contralateral paw (Fig 5.12 B).

Therapeutically, aspirin-treated rats showed a significant reduction in bone deformation when compared with arthritic control rats (Figs 5.12 C and 5.12 D). XAE (100, 300 and 600 mg $kg^{-1}$)-treated animals showed a significant ($P < 0.0001$) reduction in bone loss in the ipsilateral and contralateral limbs (Fig 5.12 C). There was a significant ($P <$

0.0001) reduction of bone erosion in the ipsilateral limb and reduction of bone erosion in the contralateral limbs in animals treated with XA (10, 30 and 100 mg $kg^{-1}$) (Fig 5.12 D).

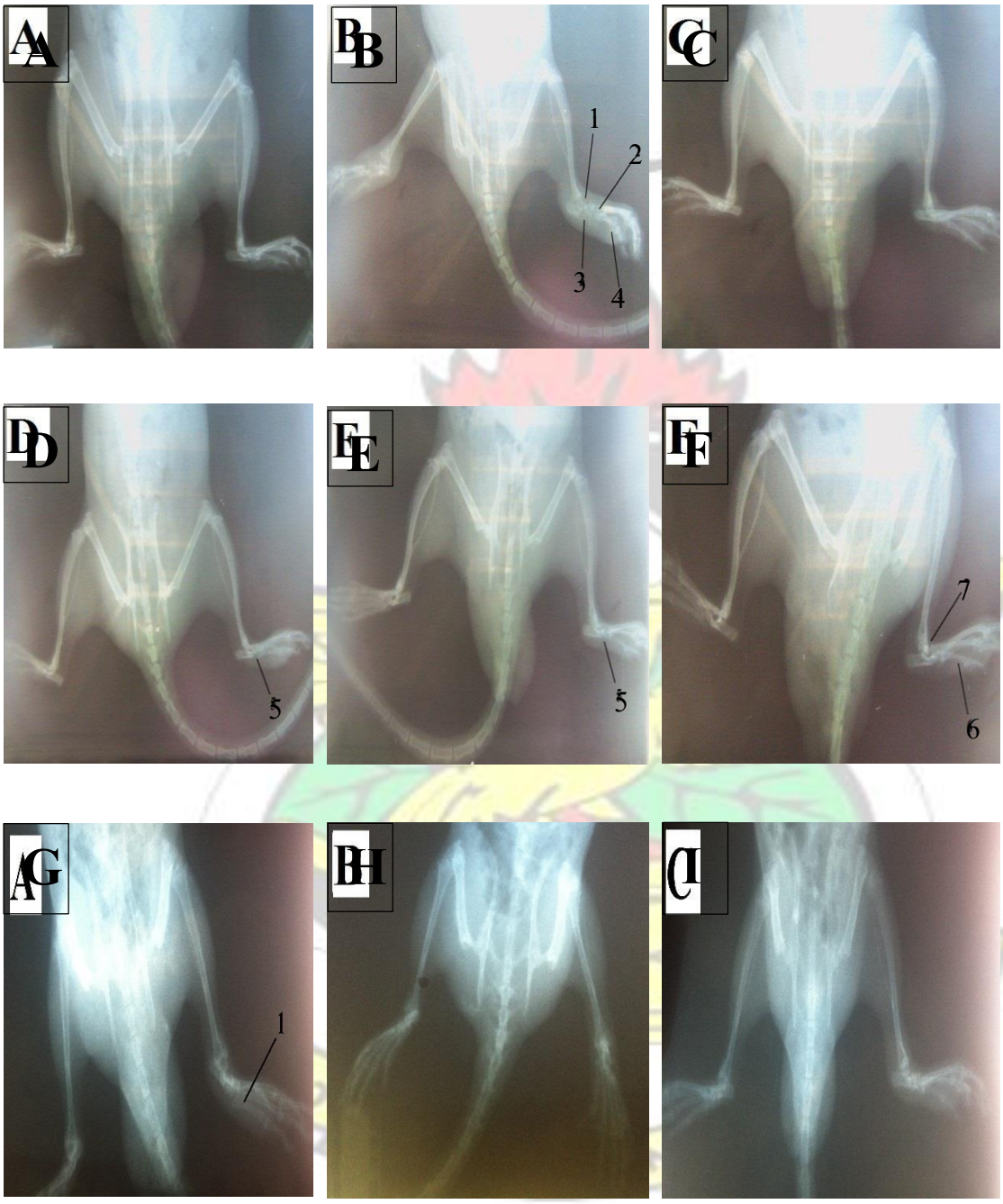



**Plate 5.7. Radiographs showing the effect of *X. aethiopica* extract and xylopic acid administered prophylactically in adjuvant-induced arthritis in rats.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw and monitored for 28 days. The drug vehicle, aspirin 100 mg $kg^{-1}$, XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) were given orally 1 h before the induction of the arthritis and daily for 28 days. Rats were anaesthetised by intra peritoneal injection of 50 mg $kg^{-1}$ pentobarbitone sodium. Radiographs were taken with X-ray apparatus operated at a voltage of 55 kV against

3.2 mA $s^{-1}$ with a tube-to-film distance of 110 cm for lateral projection. IFA/non-arthritic control (A), CFA/arthritic control (B), Aspirin (C), 100 mg $kg^{-1}$ XAE (D), 300 mg $kg^{-1}$ XAE (E), 600 mg $kg^{-1}$ XAE (F), 10 mg $kg^{-1}$ XA (G), 30 mg $kg^{-1}$ XA (H) and 100 mg $kg^{-1}$ XA (I)

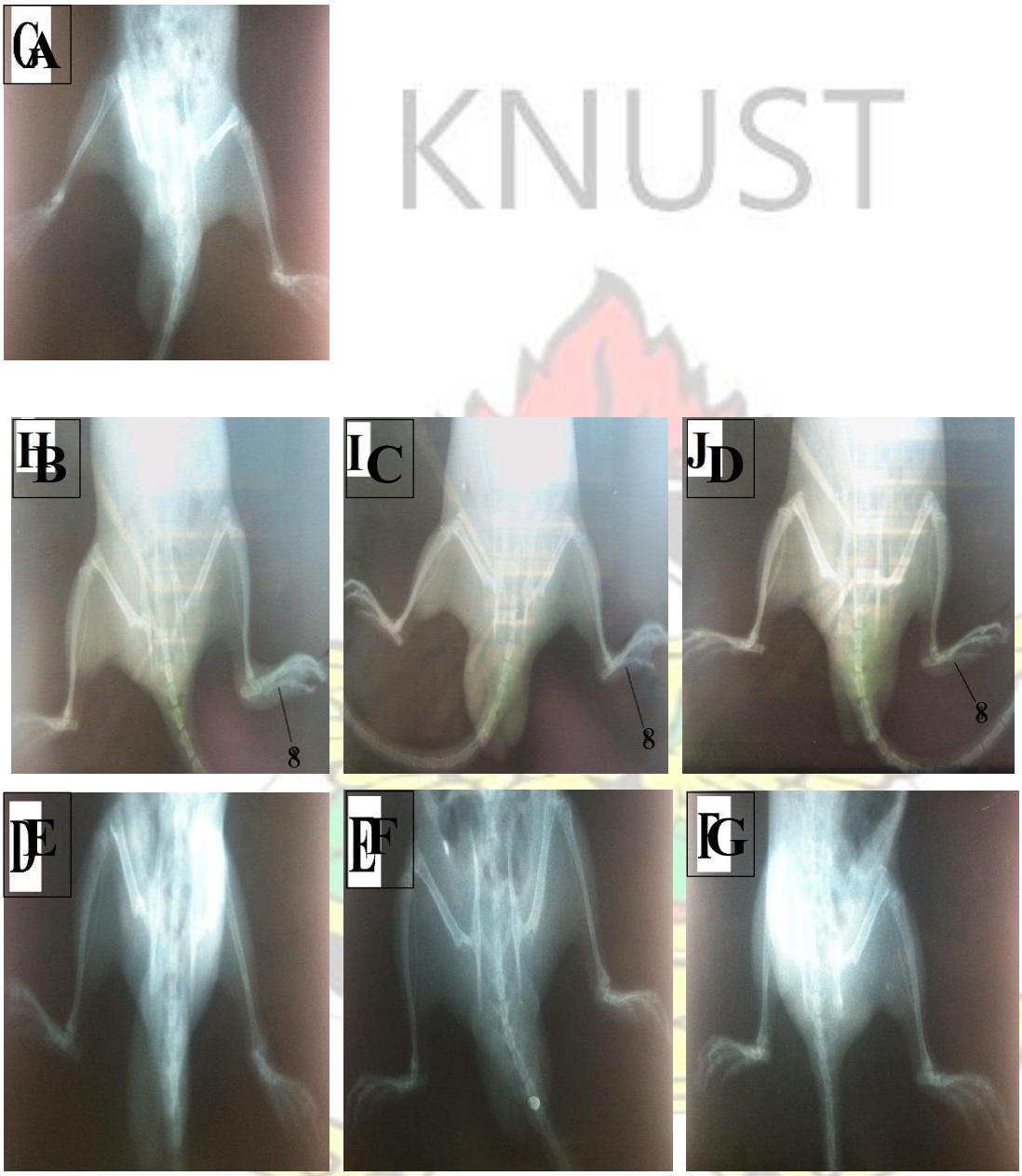





**Plate 5.8. Radiographs showing the effect of *X. aethiopica* extract and xylopic acid administered therapeutically in adjuvant-induced arthritis in rats.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw and monitored for 28 days. Aspirin 100 mg $kg^{-1}$, XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) were given orally on the 14$^{th}$ day after the induction of the arthritis and daily till 28$^{th}$ day. Rats were anaesthetised by intra peritoneal injection of 50 mg $kg^{-1}$ pentobarbitone sodium. Radiographs were taken with X-ray apparatus operated at a voltage of 55 kV against 3.2 mA $s^{1}$ with a tube-to-film distance of 110 cm for lateral projection. 100 mg $kg^{-1}$ Aspirin (A), 100 mg $kg^{-1}$ XAE (B), 300 mg $kg^{-1}$ XAE (C), 600 mg $kg^{-1}$ XAE (D), 10 mg $kg^{-1}$ XA (E), 30 mg $kg^{-1}$ XA (F) and 100 mg $kg^{-1}$ XAE (G)

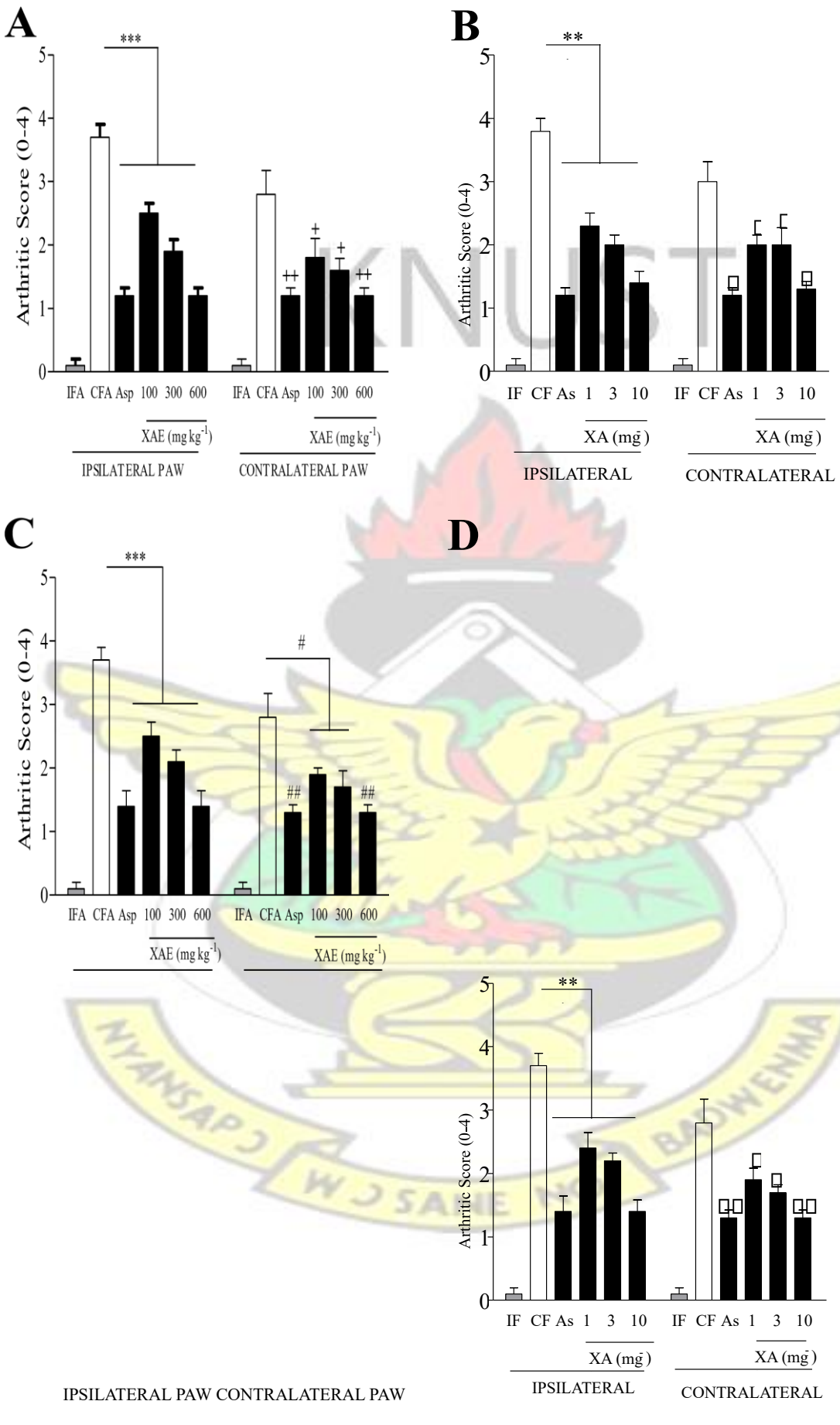

A
B
C
D
Arthritic Score (0-4)
IFA CFA Asp 100 300 600
IF CF As 1 3 10
XAE (mg kg-1)
XA (mg)
IPSILATERAL PAW
CONTRALATERAL PAW
IPSILATERAL
CONTRALATERAL

**Figure 5.12. Arthritis scoring of radiographs in adjuvant-induced arthritic rats.** Sprague Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw and monitored for 28 days. Aspirin 100 mg $kg^{-1}$, XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) were given orally 1 h before the induction of the arthritis and daily and treatment commenced on day 14 in the therapeutic protocol. Rats were anaesthetised by intra peritoneal injection of 50 mg $kg^{-1}$ pentobarbitone sodium on the 28$^{th}$ day. Radiographs were taken with X-ray apparatus operated at a voltage of 55 kV against 3.2 mA $s^{-1}$ with a tube-tofilm distance of 110 cm for lateral projection. The severity of the arthritis was measured blindly on day 28 on a scale of 0 - 4. The radiogaphs of the hind paw was used as parameter for measurement with severity of arthritic score determined based on extent of bone erosion (n = 5). Top panel [A, B] is prophylactic protocol; bottom panel [C, D] is therapeutic protocol. $^{***}P < 0.0001$, $^{+}P = 0.041$, $^{++}P = 0.0041$, $^{\#}P = 0.032$, $^{\#\#}P = 0.0032$, $^{t}P < 0.05$, $^{tt}P = 0.001$, $^{\phi}P = 0.019$, $^{\phi\phi}P = 0.0019$, $^{\phi\phi\phi}P = 0.00019$, $^{ns}P > 0.05$.

#### *5.3.2.4 Haematology*

Blood tests have proven important in the initial evaluation of RA. Such parameters include complete blood count (CBC), erythrocyte sedimentation rate (ESR), Rheumatoid factor (RF), C-reactive protein (CRP) (WebMD Medical Reference, 2014), HLA-B27, Anti-citrullinated peptide (anti-CCP) antibodies (Merck manual, 2008) and Antinuclear antibody (ANA). Among these, CBC and ESR were performed.

Blood samples were taken on the 29$^{th}$ day from the jugular vein of the rats after they were sacrificed by cervical dislocation. The arthritic control group showed an increase in WBC with reduced RBC and haematocrit with a corresponding increase in the ESR (Biernacki reaction) when compared to the IFA controls. Treatment with aspirin resulted in significant decrease in WBC and ESR with an increase in RBC, HCT and HGB when compared with arthritic rats. Similarly, the extract-treated groups showed significant increase in RBC, HCT and HGB as well as a decrease in WBC and ESR when compared with the CFA control group at 100, 300 and 600 mg $kg^{-1}$ (Table 5.2). In the dose range of 10, 30 and 100 mg $kg^{-1}$ respectively, XA showed significant ($P < 0.0001$) decrease in WBC and ESR with an increase in RBC, HCT and HGB when compared with the CFA control group (Table 5.2).

In the therapeutic regimen, aspirin-treated rats showed a significant decrease in ESR and an increase in RBC, HGB and HCT when compared with CFA. The XAE (30, 100, 300 mg $kg^{-1}$)-treated groups also presented with significant ($P < 0.0001$) decrease in WBC and ESR with an increased RBC, HGB and HCT when compared with the arthritic control group (Table 5.3). Likewise, the XA-treated rats at 10, 30 and 100 mg $kg^{-1}$ showed significant ($P < 0.0001$) decrease in WBC and ESR with an increase in RBC, HGB and HCT when compared with the arthritic control group (CFA) (Table 5.3).

**Table 5.2. Blood Analyses of samples taken after chronic inflammation study in prophylactically-treated ratss.**

| Treatment | WBC $x10^3/\mu L$ | HGB g $dL^{-1}$ | RBC $x10^6/\mu L$ | HCT % | ESR mm $h^{-1}$ |
|---|---|---|---|---|---|
| IFA | 10.54 ± 0.08* | 16.58 ± 0.10* | 7.54 ± 0.01* | 52.80 ± 0.12* | 1.10 ± 0.10* |
| CFA | 14.72 ± 0.20 | 10.76 ± 0.27 | 6.32 ± 0.14 | 41.90 ± 0.76 | 2.90 ± 0.33 |
| Aspirin (mg $kg^{-1}$) | | | | | |
| 100 | 10.70 ± 0.10* | 14.50 ± 0.25* | 7.62 ± 0.21* | 55.76 ± 0.30* | 2.30 ± 0.12* |
| XAE (mg $kg^{-1}$) | | | | | |
| 100 | 11.02 ± 0.21* | 12.50 ± 0.11* | 7.27 ± 0.05* | 49.18 ± 0.22* | 2.20 ± 0.12* |
| 300 | 10.64 ± 0.41* | 14.26 ± 0.13* | 7.42 ± 0.11* | 55.58 ± 0.48* | 1.40 ± 0.19* |
| 600 | 9.10 ± 0.23* | 14.90 ± 0.22* | 7.69 ± 0.11* | 54.12 ± 0.84* | 1.40 ± 0.19* |
| XA (mg $kg^{-1}$) | | | | | |
| 10 | 5.7 ± 0.90* | 14.45 ± 0.35* | 8.07 ± 0.12* | 48.7 ± 2.90* | 2.12 ± 0.25* |

| | | | | | |
|---|---|---|---|---|---|
| 30 | 4.4 ± 0.00* | 12.3 ± 0.00* | 6.62 ± 0.00$^{ns}$ | 40.5 ± 0.00 | 1.71 ± 0.13* |
| 100 | 9.55 ± 1.45* | 13.35 ± 0.05* | 7.78 ± 0.17* | 45.8 ± 0.50* | 1.35 ± 0.17* |

Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw. The drug vehicle and either aspirin 100 mg kg$^{-1}$, XAE (100, 300, 600 mg kg$^{-1}$) or XA (10, 30, 100 mg kg$^{-1}$), were given orally 1 h before the induction of the. Rats were euthanised and blood samples collected from the jugular vein on day 29. A full blood count was done on the collected blood samples using blood analyser. The Biernacki reaction or erythrocyte sedimentation rate (ESR) was also done using the standard Westergren method. *$P < 0.0001$, $^{ns}P > 0.05$ when compared to the arthritic control group (CFA).

**Table 5.3. Blood Analyses of samples taken after chronic inflammation study in therapeutically-treated rats.**

| Treatment | WBC x10$^3$/µL | HGB g dL$^{-1}$ | RBC x10$^6$/µL | HCT % | ESR mm h$^{-1}$ |
|---|---|---|---|---|---|
| IFA | 10.54 ± 0.08* | 16.58 ± 0.10* | 7.54 ± 0.01* | 52.80 ± 0.12* | 1.10 ± 0.10* |
| CFA | 14.72 ± 0.20 | 10.76 ± 0.27 | 6.32 ± 0.14 | 41.90 ± 0.76 | 2.90 ± 0.33 |
| Aspirin (mg kg$^{-1}$) | | | | | |
| 100 | 18.1± 0.00$^{ns}$ | 13.0 ± 0.00* | 7.27 ± 0.00* | 46.4 ± 0.00* | 2.41 ± 0.25* |
| XAE (mg kg$^{-1}$) | | | | | |
| 100 | 12.30 ± 0.06* | 11.78 ± 0.10* | 6.84 ± 0.03* | 48.62 ± 0.65* | 2.20 ± 0.12* |
| 300 | 11.52 ± 0.21* | 12.20 ± 0.09* | 7.12 ± 0.06* | 51.98 ± 0.26* | 1.90 ± 0.19* |
| 600 | 10.32 ± 0.08* | 14.92 ± 0.15* | 7.91 ± 0.17* | 58.40 ± 0.88* | 1.50 ± 0.16* |
| XA (mg kg$^{-1}$) | | | | | |
| 10 | 6.35 ± 1.35* | 13.2 ± 0.00* | 7.46 ± 0.11* | 44.5 ± 1.80* | 2.52 ± 0.22* |

| | | | | | |
|---|---|---|---|---|---|
| 30 | 6.6 ± 0.00* | 13.8 ± 0.00* | 8.10 ± 0.00* | 47.6 ± 0.00* | 1.75 ± 0.11* |
| 100 | 5.85 ± 1.35* | 12.45 ± 0.85* | 6.90 ± 0.33* | 42.35 ± 1.75$^{ns}$ | 1.23 ± 0.58* |

Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw. The drug vehicle and either aspirin 100 mg kg$^{-1}$, XAE (100, 300, 600 mg kg$^{-1}$) or XA (10, 30 and 100 mg kg$^{-1}$), were administered from day 14 to day 28 in the therapeutic protocol. Rats were euthanised and blood samples collected from the jugular vein on day 29. A full blood count was done on the collected blood samples using blood analyser. The Biernacki reaction or erythrocyte sedimentation rate (ESR) was also done using the standard Westergren method. *$P < 0.0001$, $^{ns}P > 0.05$ when compared to the arthritic control group (CFA).

### *5.3.2.5 Histopathology*

Histopathology provides a noticeable morphological distinctiveness as a practical and unambiguous pathognomonic sign of RA (Soren, 1980). Histopatholigical analyses of rats treated with XAE and XA will therefore serve as a good assessment of the disease morphology.

To do this, adjuvant arthritis was induced in rats as earlier described. Bones of the right hind limbs were excised after the rats were euthanised and histopathological assessment made. In the non-arthritic group there was intact bone structure with no visible mononuclear cell infiltration nor synovial tissue vasculature (Plate 5.9 A). Mononuclear cell infiltration and vascularity in synovial tissues with characteristic redness, and pannus invasion of the subchondral bone were observed in the arthritic control rats (Plate 5.9 B). In the prophylactic protocol, aspirin-treated rats showed highly vascularised bone tissues with characteristic redness, minimal mononuclear cell infiltration, and brittle bone structure, evident of osteo degeneration (Plate 5.9 C). Treatment with XAE at 100, 300 and 600 mg kg$^{-1}$ showed mononuclear cell infiltration, and fluid infiltration of synovial cavity, and marked cavitation of bone structure, evident of osteoporosis in the XAE 100 mg kg$^{-1}$-treated rats (Plate 5.9 D). The XAE

300 mg $kg^{-1}$-treated group showed mononuclear cell infiltration, and pannus formation and invasion into the subchondral bone (Plate 5.9 E). Remodeling bone structure with intact synovial lining and no visible mononuclear cell infiltration was observable in the XAE 600 mg $kg^{-1}$ treated group (Plate 5.9 F). XA at 10, 30, 100 mg $kg^{-1}$ showed mononuclear cell infiltration, and fluid infiltration of synovial cavity, and marked cavitation of bone structure, evident of osteoporosis at 10 mg $kg^{-1}$ and 30 mg $kg^{-1}$ respectively (Plates 5.9 G and 5.9 H). In addition, signs of bone remodeling was evident at 10, 30 and 100 mg $kg^{-1}$ respectively (Plates 5.9 G, 5.9 H and 5.9 I).

Administered therapeutically, aspirin-treated rats showed mild fluid infiltration of synovial cavity and with signs of osteoclast activity (Plate 5.10 A). XAE treatment caused fluid infiltration of synovial cavity, and marked cavitation of bone structure, evident of osteoclast activity as seen in the 100 mg $kg^{-1}$ treated group (Plate 5.10 B). There was bone reformation structure with large marrow cavity and erythroblasts in the 300 mg $kg^{-1}$ treated rats (Plate 5.10 C). Re-calcified bone structures with osteoblastic excesses were evident in the 600 mg $kg^{-1}$ treated animal (Plate 5.10 D). Treatment with XA showed active signs of bone remodeling at 10, 30 and 100 mg $kg^{-1}$ respectively. There were signs of fluid infiltration at 10 mg $kg^{-1}$ (Plate 5.10 E) and observable signs of bone reformation structure and re-calcified bone structure with osteoblasts excesses respectively at 10, 30 and 100 mg $kg^{-1}$ (Plates 5.10 E, 5.10 F and 5.10 G).

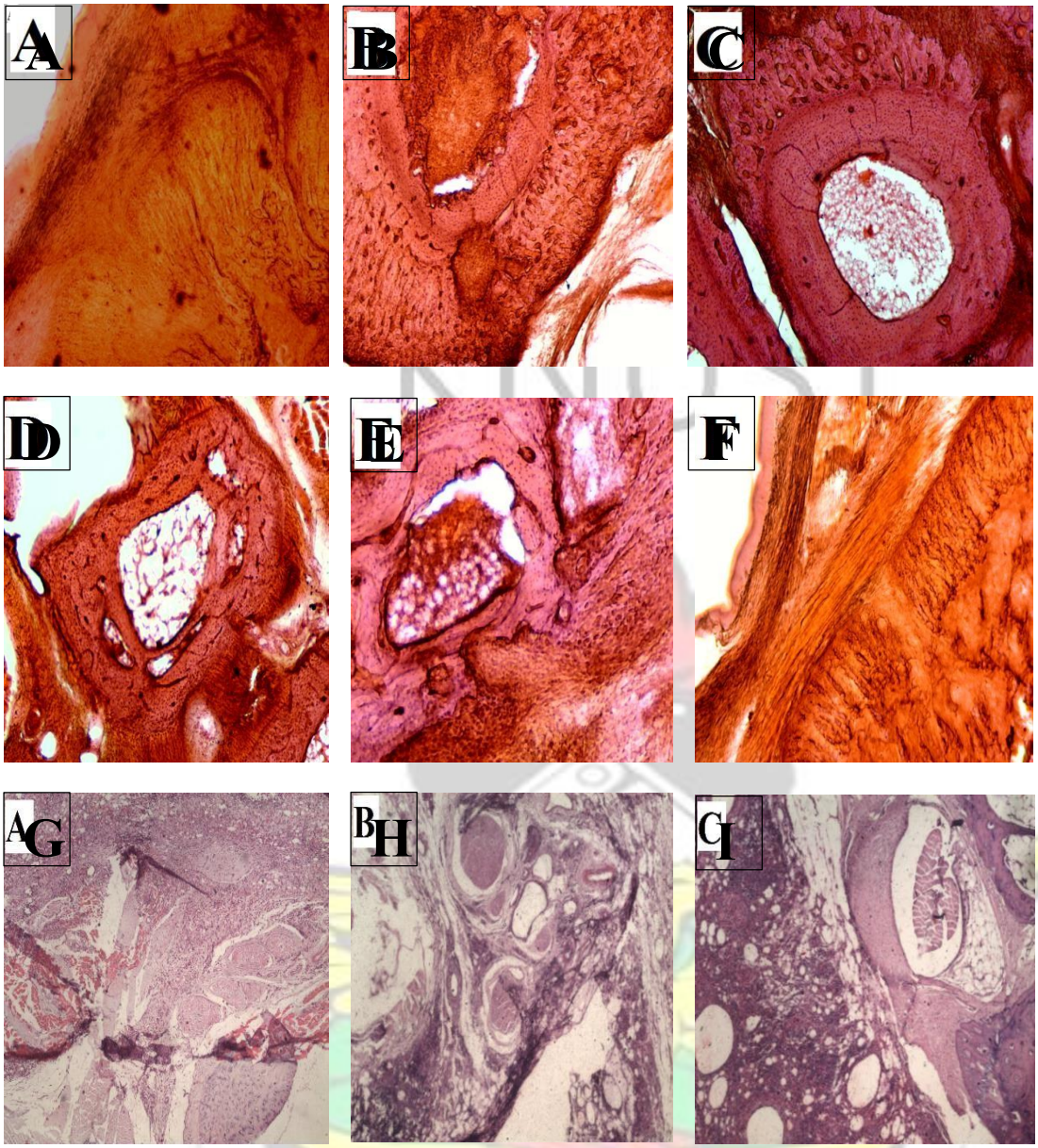



**Plate 5.9. Histopathology of adjuvant-induced arthritis in rats treated prophylactically.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw. Aspirin 100 mg $kg^{-1}$, XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) were given orally 1 h before the induction of the arthritis. Rats were anaesthetised by intra peritoneal injection of 50 mg $kg^{-1}$ pentobarbitone sodium and sections made from the bone of their right hind limb and stained with haematoxylin and eosin and analysed microscopically. IFA (A), CFA (B) treated rats, Aspirin 100 mg $kg^{-1}$ (C), XAE 100 mg $kg^{-1}$ (D), XAE 300 mg $kg^{-1}$ (E), XAE 600 mg $kg^{-1}$ (F), XA 10 mg $kg^{-1}$ (G), XA 30 mg $kg^{-1}$ (H) and XA 100 mg $kg^{-1}$ (I).

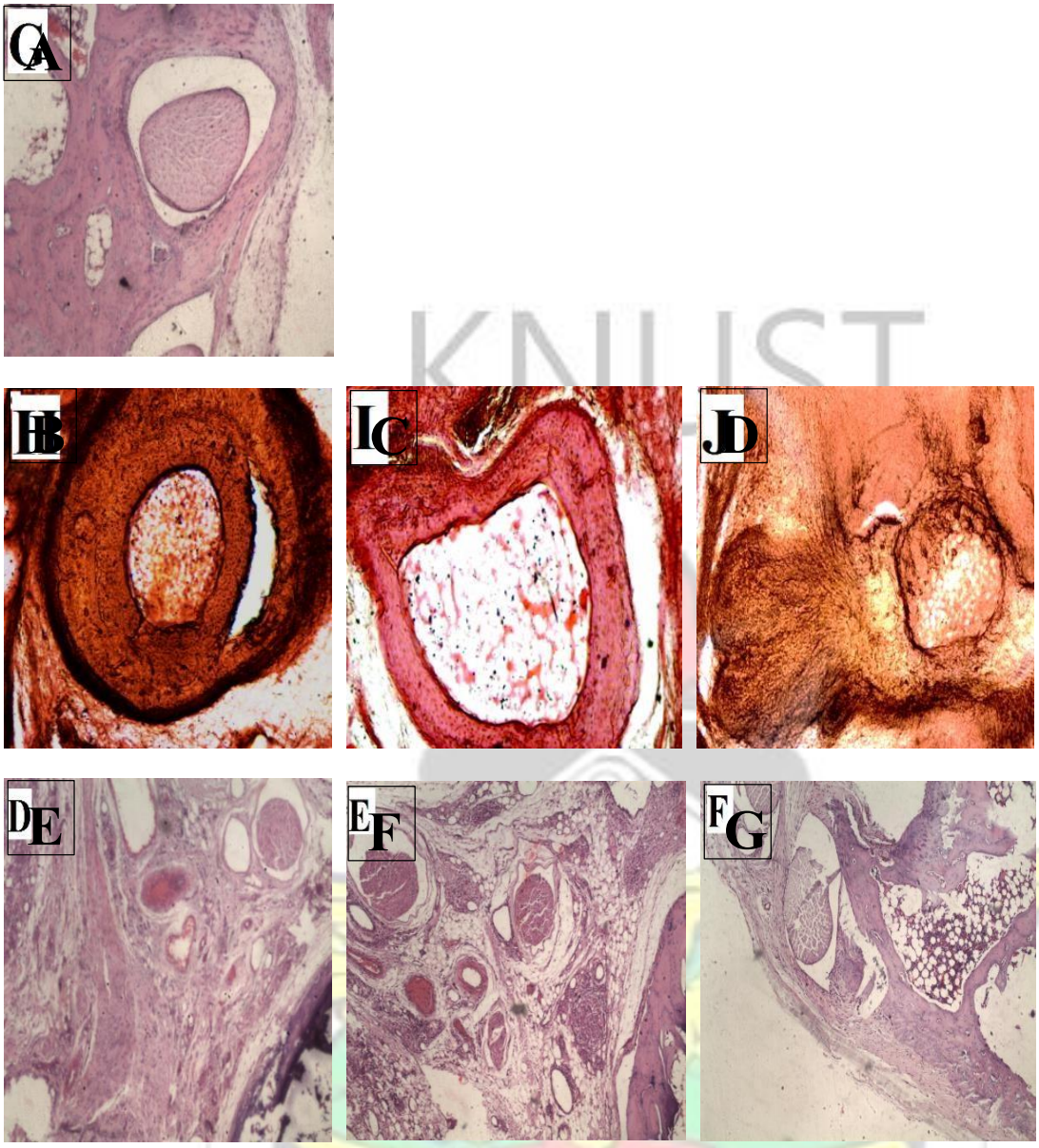



**Plate 5.10. Histopathology of adjuvant-induced arthritis in rats treated therapeutically.** Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of IFA or CFA into the right hind paw. Aspirin 100 mg $kg^{-1}$, XAE (100, 300, 600 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) were given orally on the 14$^{th}$ day after the induction of the arthritis and daily till 28$^{th}$ day. Rats were anaesthetised by intra peritoneal injection of 50 mg $kg^{-1}$ pentobarbitone sodium and sections made from the bone of their right hind limb and stained with haematoxylin and eosin and analysed microscopically. Aspirin 100 mg $kg^{-1}$ (A), XAE 100 mg $kg^{-1}$ (B), XAE 300 mg $kg^{-1}$ (C), XAE 600 mg $kg^{-1}$ (D), XA 10 mg $kg^{-1}$ (E), XA 30 mg $kg^{-1}$ (F) and XA 100 mg $kg^{-1}$ (G).

## 5.4 DISCUSSION

The effect of the aqueous ethanol extract of the fruit of *X. aethiopica* and its principal constituent, xylopic acid, were evaluated on two models of chronic inflammation. In

the acetic acid-induced ulcerative colitis, severe mucosal immune response through ROS-mediated activation of macrophages and neutrophils in the intestinal mucosal tissues (El-Abhara *et al*., 2008; Thippeswamy *et al.,* 2011) are triggered. This results in lipid peroxidation, increased vascular permeability, increase entrance of neutrophils and development of inflammation in the mucosal tissues as reported by Packard *et al.* (2009).



Treatment with the aqueous ethanol extract of *Xylopia aethiopica* and xylopic acid significantly alleviated the colitis thereby decreasing the number of AgNORs count. This is an evidence of reduction in cellular activity and proliferation with extract and xylopic acid treatment. Upon stimulation, mast cells release a heterogeneous group of factors that promote inflammation and influence immune cell proliferation. Mast cells accumulate at sites of injury, further suggesting a critical role in the inflammatory process (Egozi *et al.,* 2003). Treatment with XAE and XA reduced the accumulation of mast cell at the site of injury in agreement with the reduced mast cell proliferation seen in XAE and XA treated groups. Thus contributing to the anti-inflammatory actions of the extract and xylopic acid which is in agreement with earlier findings made by Cavalcanti *et al.* (2009) which identified kaurenoic acid, another kaurane diterpene, to inhibit cell proliferation.

Treatment with XAE and XA reduced the MPO activity and MDA levels. This is possibly due to their anti-inflammatory and anti-oxidant properties. XAE and XA affects the MPO catalysed production of hypochlorous acid which contributes electrons needed for lipid peroxidation. The reduced level of peroxidation caused by XAE and XA treatment resulted in the reduced production of MDA, the byproduct of the lipid peroxidation process.

In ulcerative colitis, there is depleted levels of CAT, SOD and APx in colon tissues as a result of oxidative damage (Kruidenier *et al.,* 2003). CAT, SOD and APx also play a crucial role as protective enzymes. CAT which is localised in subcellular organelles of peroxisomes, catalyses the conversion of hydrogen peroxide to water and oxygen (Limon-Pacheco and Gonsebatt, 2009); SOD is an enzyme shown to exert antiinflammatory effects in inflammatory bowel diseases (Xia *et al.,* 1996; Epperly *et al.,*

2002) by reducing lipid peroxidation, leukocyte rolling and adhesion in colonic tissues (Seguí *et al.,* 2004); APx, an integral component of the glutathione-ascorbate cycle (Noctor and Foyer, 1998), removes peroxides by conjugation using ascorbate as substrate (Raven, 2000). Supplementation via XAE and XA treatment decreased lipid peroxidation thereby significantly increasing activity of anti-oxidant enzymes hence reducing severity of inflammation and thereby exerting anti-inflammatory actions.

It is known that auto-antigens that cross-react with *Mycobacteria* are implicated in the pathogenesis of rat adjuvant-induced arthritis and it is reported by Turull and Queralt (2000) that adjuvant arthritis appears as a consequence of an immune response to the cell wall of the *Mycobacterium*. When compared with normal rats, higher levels of IgG anti-Mycobacterium antibodies were observed in arthritic rats which also presented with delayed skin reactions induced by the soluble fraction of *Mycobacterium*. XAE and XA significantly decreased these humoral immune responses. At the same time, XAE and XA treatment inhibited the delayed-type hypersensitivity seen in arthritic model animals. These significant findings suggest that XAE and XA might exert their effect through their influence on both the cellular and humoral immune response to the *Mycobacterium* in adjuvant-induced arthritic rats. Another characteristics of rat adjuvant arthritis is that there is increased bone resorption and decreased bone

formation which results in bone loss (Findlay and Haynes, 2005; Makinen *et al.,* 2007) and its progression is divided into three phases as in human rheumatoid arthritis. These phases start with the induction phase which has no evidence of synovitis, followed by early synovitis, and finally late synovitis with progressive joint destruction (Hoffmann *et al.,* 1997). A good and classical anti-rheumatic agent should be able to block at least one or more of these phases. This is consistent with my earlier findings reported in this document that XAE and XA suppressed joint inflammation and synovitis. Regions of minimal stabilised osteophytosis were observable in the bone metaphysis and there was persistent minor joint space alterations in the extract-treated groups with reduced inflammation and joint deformation in both preventive and therapeutic protocols.

In RA, there is elevated WBC suggestive of inflammation as a consequence of infection (Braunwald *et al.,* 2001). A lower haematocrit and RBC can be caused by a number of factors or conditions including rheumatoid arthritis (Braunwald *et al.,* 2001). These observations were made in the arthritic control rats which were significantly alleviated in the XAE and XA-treated rats. There is elevated Biernacki reaction in 95% cases of RA (Braunwald *et al.,* 2001). Inflammation causes the body to produce higher proportions of fibrinogen in the blood which makes the red cells clump together, causing them to fall faster than the healthy blood cells. Since inflammation can be caused by conditions other than arthritis, the Biernacki reaction is not a full diagnostic assessment of arthritis however, it does play a significant role in the diagnosis (Braunwald *et al.,* 2001). Nevertheless, XAE and XA decrease ESR in adjuvant arthritis.

The histopathological analysis identified the ability of the bones to re-form upon treatment with XAE and XA. Bone structures re-calcified upon treatment with the extract and xylopic acid. The aqueous ethanol extract of *Xylopia aethiopica* and xylopic

acid exhibited such therapeutic potential from the study results and is therefore consistent with earlier findings that the ability of a drug to suppress inflammation, synovitis and protect a joint is desired in rheumatoid arthritis therapy (Hoffmann *et al.,* 1997; Sharma *et al.,* 2004; Atzeni and Sarzi-Puttini, 2007). This results is supported by the fact that a significant number of the constituent kaurane diterpenes, including kaurenoic acid, are known to inhibit *inducible nitric oxide synthase* (iNOS), COX-2 and TNF-α (Castrillo *et al.,* 2001; Leung *et al.,* 2005; Sosa-Sequera *et al.,* 2010). This is supported by the fact that the principal constituent of XAE is XA, a kaurane diterpene.

Taken together, it is consistent with the findings and not surprising therefore to see significant progressive decreases in thickness of both joints, and suppression of paw oedema in rats treated with XAE and XA compared to vehicle-treated diseased rats. These marked inhibitions in swelling were observed in the time-course of the pathology of adjuvant-induced arthritis in both ipsilateral and the contralateral limbs when XAE and XA was administered orally in both the prophylactic and therapeutic regimens.

## 5.5 CONCLUSION

The aqueous ethanol fruit extract of *Xylopia aethiopica* and xylopic acid are effective in ulcerative colitis and useful anti-arthritic agents experimentally. They increase CAT, SOD and APx activity and decrease MDA levels and MPO activity *in vivo* and suppress joint inflammation and destruction in adjuvant-induced arthritic rats due to their antiinflammatory and anti-oxidant properties. Therefore the possible mechanism of action of XAE and XA as anti-inflammatory agents were evaluated.

# *Chapter 6* MECHANISMS OF ACTION OF *XYLOPIA AETHIOPICA* AQUEOUS

## ETHANOL EXTRACT AND XYLOPIC ACID AS ANTI-INFLAMMATORY AGENTS



### INTRODUCTION

At the molecular level, mast cells express among other surface receptors the high affinity receptor for immunoglobulin E, IgE (FcεRI) which is critical for mast cell development and function (Kinet, 1999). In the absence of an antigen, the FcεRI binds to IgE. Subsequent cross-linking of the IgE-bound FcεRI by a cognate antigen triggers a cascade of signalling pathways resulting in three downstream responses; degranulation, release of arachidonic acid and its subsequent metabolism and finally the release of an array of cytokines (Fig 6.1).

An alternative pathway of activation of the mast cell which is a non-immunologic mechanism is the peptidergic pathway. This is achieved by polycationic compounds such as compound 48/80 referred to as the basic secretagogues of mast cells (Langunoff *et al.,* 1983). The initial phase of inflammation involves the release of preformed mediators histamine and serotonin, with increased synthesis of prostaglandins. This earlier response is then sustained with the eventual synthesis of more prostaglandins, bradykinin, leukotrienes, cytokines and polymorphonuclear cells (Hwang *et al*., 1996; Lo and Sauf, 1987; Gupta *et al*., 2006). Muniappan and Sundararaj (2003) have also established the significant role of the induction of *cyclooxygenase-2* in the development of the acute inflammatory process.

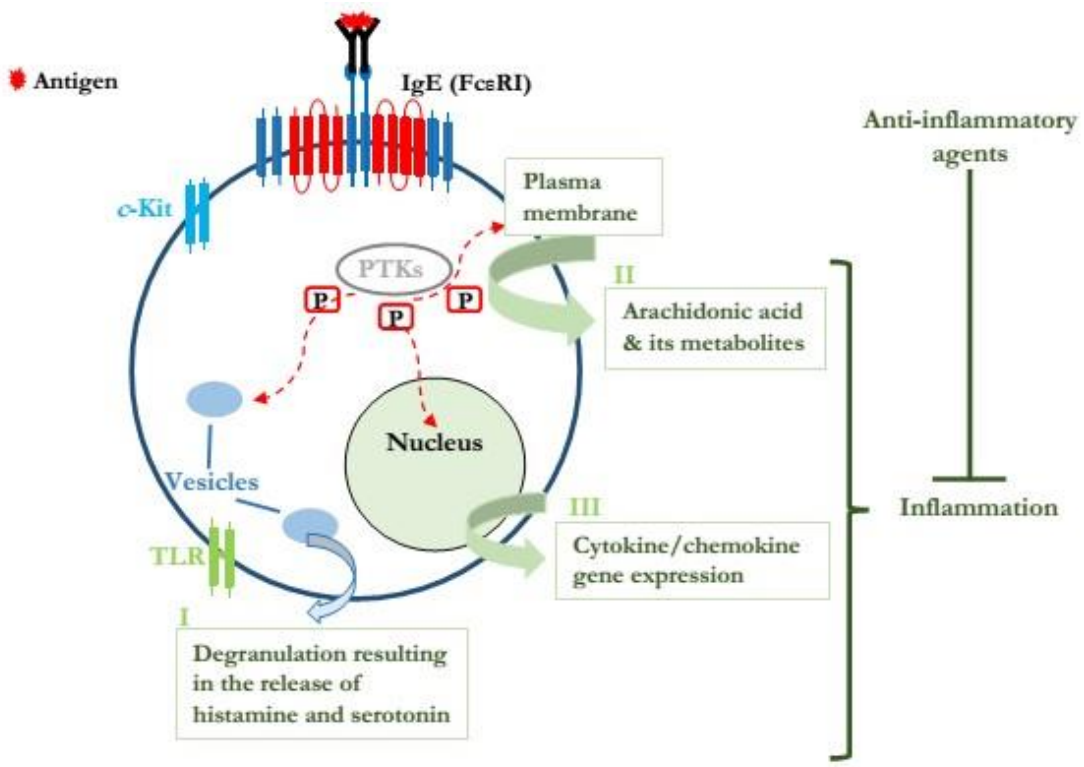



**Figure 6.1. Mast cell activation.** Mast cell activation results in degranulation (release of histamine, 5-hydoxytryptamine, etc.), release of arachidonic acid and its metabolites, and finally the transcription of cytokine and chemokine genes. These proinflammatory compounds mediate the inflammatory response, which is attenuated by anti-inflammatory agents. *c*-Kit, *tyrosine-protein kinase* Kit (CD117); TLR, Toll-like Receptor; IgE, Immunoglobulin E, FcεRI, Fc epsilon RI (high-affinity IgE receptor) are some of the surface receptors on mast cells. (Adapted from Agyare *et al*. (2013)).

Consequently, the effect of the materials on degranulation using mediator-induced paw oedema models, indirect anti-histaminic effect using the clonidine-induced catalepsy model and direct anti-histaminic effect on isolated guinea pig ileum preparation; effect on arachidonic acid pathway using the prostaglandin $E_2$-induced paw oedema model and hydrogen sulphide-induced acute inflammation model; effect on expression of intercellular adhesion molecule 1 in tissue proteome and effect on cytokine gene expression by measuring serum expression of IL-6 and TNF-alpha were investigated, with the aim of establishing their possible mechanism(s) of anti-inflammatory action.

## 6.1 MATERIALS AND METHODS

### 6.1.1 MATERIALS

#### *6.1.1.1 Drugs and chemicals*

PageRuler Plus Prestained Protein Ladder (Thermo Scientific, Waltham, USA); captopril (Teva ltd, Castleford, UK); granisetron hydrochloride (Roche, Basel, Switzerland); diclofenac sodium (Novartis Int AG, Basel, Switzerland); clonidine hydrochloride (Boehringer Ingelheim Inc, Fremont, USA); chlorpheniramine maleate (DWD Pharmaceuticals Ltd, Mumbai, India); Coomassie brilliant blue G-250 (MP Biomedicals, California, USA); methanol (Amresco®, Solon, USA); ammonium persulphate, acrylamide, bis-acrylamide, bromophenol blue, ethylenediaminetetraacetic acid disodium salt, histamine dihydrochloride, sodium hydrogen sulphide, tris(hydroxymethyl) aminomethane base, glycerol, glycine, sodium dodecyl sulphate, bradykinin acetate salt, dithiothreitol, tetramethyl ethylenediamine, serotonin hydrochloride, Tyrode's solution (NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaH_2PO_4$, Glucose), and prostaglandin $E_2$ (Sigma-Aldrich Chemical Co, St Louis, USA).

#### *6.1.1.2 Animals*

Sprague-Dawley rats (200 – 250 g) and Imprinting Control Region (ICR) mice (20 - 30 g) were used. Animals were maintained at the necessary conditions as described in section 3.1.1.1.

## 6.1.2 METHODS

### *6.1.2.1 Degranulation: In vivo mediator-induced oedema*

#### *6.1.2.1.1 Histamine-induced paw oedema*

The experimental procedure is as described in the carrageenan-induced paw oedema model (section 3.1.2.2). Briefly, paw oedema (acute inflammation) was induced with subplantar injection of 0.1 mg of histamine (Mazumder *et al.*, 2003) into ICR mice (20 – 30 g). Mice received normal saline (1 ml $kg^{-1}$ *p.o.*), chlorpheniramine (10 mg $kg^{-1}$ *p.o.*), XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) both prophylactically and therapeutically as earlier described. Paw oedema was measured at 30 min interval for 3 h and the maximal oedema and total oedema response were calculated as described in section 3.1.2.2.

#### *6.1.2.1.2 Serotonin-induced paw oedema*

The experimental procedure is as described in the carrageenan-induced paw oedema model (section 3.1.2.2). Briefly, paw oedema was induced with subplantar injection of 0.1 mg of serotonin (Mazumder *et al.*, 2003) into ICR mice (20 – 30 g). Mice were treated with normal saline (1 ml $kg^{-1}$ *p.o.*), granisetron (100 µg $kg^{-1}$ *p.o.*), XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) both prophylactically and therapeutically as earlier described. Paw oedema was measured at 30 min interval for 3 h and the maximal oedema and total oedema response were calculated as described in section 3.1.2.2.

#### *6.1.2.1.3 Bradykinin-induced paw oedema*

ICR mice (20 – 30 g) were pre-treated with captopril (5 mg $kg^{-1}$ *s.c.*) 1h before bradykinin (10 nmol/paw) injection. The stock solutions for bradykinin were prepared in phosphate-buffered saline PBS (1-10 mM) in siliconised plastic tubes, maintained at 18°C just before use. Mice were injected with 1 µg of the bradykinin subplantar in the right hind paw 30 min after administering normal saline (1 ml $kg^{-1}$), XAE (30, 100, 300 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) both prophylactically and therapeutically as earlier described. Paw oedema was measured at 30 min interval for 3 h and the maximal oedema and total oedema response were calculated as described in section 3.1.2.2. No selective antagonist was given in this model.

#### *6.1.2.1.4 Prostaglandin $E_2$-induced paw oedema*

The experimental procedure is as described in the carrageenan-induced paw oedema model (section 3.1.2.2). Briefly, paw oedema was induced with subplantar injection of 50 µl of 1 nM of prostaglandin $E_2$ (Mazumder *et al.*, 2003) into ICR mice (20 – 30 g). Mice were treated with either normal saline (1 ml $kg^{-1}$), diclofenac (0.93 mg $kg^{-1}$ *p.o.*), XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) both prophylactically and therapeutically as earlier described. Paw oedema was measured at 30 min interval for 2.5 h. Maximal oedema and total oedema responses were calculated as described in section 3.1.2.2.

#### *6.1.2.1.5 Inhibition of arachidonic acid release: Hydrogen sulphide-induced acute inflammation*

##### *6.1.2.1.5.1 Measurement of mouse paw oedema*

This study employed the method described by Bianca *et al.* (2010). ICR mice (20 – 30 g) received an intraplantar injection of NaHS (500 µg per paw) as exogenous source of $H_2S$ or vehicle [30 ml of potassium phosphate buffer (PPS), pH 7.4]. Aspirin (10 mg $kg^{-1}$ *p. o.*), dexamethasone (1 mg $kg^{-1}$ *p.o.*), XAE (30, 100 and 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30 and 100 mg $kg^{-1}$ *p.o.*) were given 90 min before the NaHS (500 µg per paw) (Church and Miller, 1978; Marshall *et al*., 1989). Paw oedema was measured by means of water displacement plethysmometry (Ugo Basile, Comerio, Italy) at 15, 30, 45 and 60 min after the intraplantar injection of NaHS. Percentage changes in paw volume was calculated and the maximal oedema and total oedema response calculated as described in section 3.1.2.2.

##### *6.1.2.1.5.2 Histopathology*

In this study, mice treated with 300 mg $kg^{-1}$ XAE and 100 mg $kg^{-1}$ XA were used. Acute inflammation was induced with hydrogen sulphide as described in section 6.1.2.1.5.1. The mice were sacrificed by cervical dislocation 30 min after the NaHS (500 mg per paw) injection. Mice paws were fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 mm) were stained with haematoxylin-eosin and analysed, by an observer unaware of the treatment protocol. Histological slides were scored according to the following parameters: loss of tissue organization, inflammation, necrosis, cellular degradation and cytoplasmic vacuolation. The degree of the disorganization was quantified on a scale of 0–4 (i.e. 0– not present, 1– very mild, 2– mild, 3– moderate, 4– extensive).

#### *6.1.2.2 Indirect anti-histaminic effect*

##### *6.1.2.2.1 Clonidine-induced catalepsy in mice*

The indirect anti-histaminic activity of the extract was demonstrated in clonidineinduced catalepsy employing the bar test as described by Ferre *et al.* (1990). Clonidine (1 mg $kg^{-1}$ *s.c.*) was administered to ICR mice (25 - 30 g) and their forepaws placed on a horizontal bar (1 cm in diameter, 3 cm above the table). The time required to remove the paws from the bar was noted for each animal. The duration of catalepsy was measured at 30 min intervals up to 3 h after administration of clonidine. In the preventive (prophylactic) protocol, either vehicle (5 ml $kg^{-1}$), chlorpheniramine (10 mg $kg^{-1}$), XAE (30, 100, 300 mg $kg^{-1}$) or XA (10, 30, 100 mg $kg^{-1}$) was given orally for 2 consecutive days ending 30 min before clonidine injection. In the curative (therapeutic) protocol drug treatment commenced 1 h after induction of catalepsy.

#### *6.1.2.3 In vitro studies*

##### *6.1.2.3.1 Studies on isolated guinea pig ileum preparation*

The isolated guinea pig ileum preparation as described by Dhonde *et al*. (2008) was employed. Overnight fasted guinea pigs (200 – 250 g) were sacrificed by a sharp blow to the neck and quickly exsanguinated by carotid artery transaction. The ileum was removed and put into a petri dish containing Tyrode's solution (pH 6.5). Ileum was mounted in an organ bath maintained at 32 ± 0.5°C and containing 20 ml Tyrode's solution under basal tension of 10 mN. The solution was continuously aerated with carbogen (95% $O_2$ + 5% $CO_2$). The tissues were allowed to equilibrate for 30 min, during which, the bathing solution was changed at every 10 min. The contractile

responses of ileum to histamine (0.00001 – 0.4 mg $ml^{-1}$) in the absence and presence of either XAE (4 – 400 µg) or XA (1 – 100 µg) were recorded.

#### *6.1.2.3.2 Gel electrophoresis of tissue proteome*

Colitis was induced in rats (200 – 250 g) as earlier described (section 5.1.2.1.1) and tissues from the colon were stored at -80°C until analysis. Tissue samples were homogenised in Tris-HCl buffer (pH 7.4) and the supernatant used in the proteome analysis. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with tris/glycine buffer system according to Laemmli (1970). 13 µg of total protein of each sample was separated on 10% SDS-PAGE mini gels (10 x 10.5 cm) at 100 V, and constant current of 20 mA/1.5 mm for 1 h and visualised by colloidal Coomassie brilliant blue G-250 staining according to standard protocols. Gels were scanned (16-bit grayscale), and the digitised images were processed with ImageJ (Schneider *et al.,* 2012) software (1D electropherograms). Gels from the same batch were compared for the influence of the temperature and concentration of the disodium EDTA washing solution on gel destaining. Two technical replicates were used for the estimation of sensitivity of different protocols using 1D SDS-PAGE.

### *6.1.2.4 Serology*

#### *6.1.2.3.3.1 Estimation of serum liver function*

Adjuvant arthritis was induced in rats (200 – 250 g) as earlier described (section 5.1.2.2). In these assays, only the pure compound, XA, was used and in the concentration range of 10 - 100 mg $kg^{-1}$. The serum total cholesterol, triglycerides, total bilirubin, *alkaline phosphatase*, *aspartate transaminase*, *alanine transaminase*, high

density lipoprotein, low density lipoprotein and coronary risk as liver function parameters were assayed using Flexor EL150 Chemistry Analyzer System (ELITechGroup, Puteaux, France).

#### *6.1.2.3.3.2 Assay of cytokines (IL-6 and TNF-α) levels*

Adjuvant arthritis was induced as earlier described in section 4.1.2.2 in rats (200 – 250 g). Rats received 10, 30 and 100 mg $kg^{-1}$ of XA. Sera were obtained from blood samples using vacutainer gel and clot activator tubes (Add.Surgifiled Medicals, Meddlessex, England) and stored at -80ºC until analysis were done. The expression of IL-6 and TNFα in the sera of adjuvant-induced arthritic rats were examined using IL-6 (Interleukin-

6) Rat ELISA Kit and TNF alpha Rat ELISA Kit according to the manufacturer's recommendations (Abcam Plc, Cambridge, UK).

## 6.2 STATISTICAL ANALYSIS

Data for the clonidine-induced catalepsy and inflammation studies were analysed by two-way ANOVA followed by Bonferonni's *post hoc* tests and one-way ANOVA followed by Dunnett's test. Graphs were plotted using GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA).

## 6.3 RESULTS

### *6.3.1 Degranulation: In vivo mediator-induced oedema*

#### *6.3.1.1 Histamine –induced paw oedema*

Histamine plays a role in anaphylaxis and its role in the acute inflammatory process was assessed *in vivo*.



In this study, paw oedema (acute inflammation) was induced with subplantar injection of 0.1 mg of histamine. Paw oedema was measured at 30 min interval for 3 h and the change in paw thickness was evaluated in XAE and XA-treated rats as earlier described. It was observed that XAE (30, 100, 300 mg $kg^{-1}$) when administered before the induction of the histamine-induced paw oedema caused the mean maximal swelling attained at 60 min to be reduced to 76.80 ± 11.44%, 79.36 ± 10.95% and 49.57 ± 2.29% respectively relative to the control response of 92.14 ± 6.44% (Fig 6.2 A). The total paw swellings induced over the 3 h (measured as the area under the time course curve, AUC) were also dose-dependently and significantly suppressed by 53.87 ± 4.01%, 32.87 ±10.19% and 57.85 ± 2.22% respectively (Fig 6.2 B). Also, XA (10, 30, 100 mg $kg^{-1}$) caused the mean maximal swelling attained at 60 min to be reduced to 71.68 ± 8.35%, 66.53 ± 7.83% and 55.41 ± 1.54% respectively relative to the inflamed control response (Fig 6.2 C). The total paw swellings induced over the 3 h were also dosedependently and significantly suppressed by 31.13 ± 9.93%, 36.68 ± 10.24% and 49.11 ± 2.84% relative to the inflamed control response respectively (Fig 6.2 D).

In the therapeutic protocol, XAE (30 – 300 mg $kg^{-1}$) suppressed the mean maximal swelling attained at 60 min to 38.99 ± 3.41%, 40.88 ± 4.94% and 28.46 ± 5.56% respectively relative to the inflamed control response of 61.63 ± 4.55% (Fig 6.3 A). The total paw swellings induced over the 3 h were also dose-dependently and significantly

suppressed by 45.71 ± 11.23%, 47.62 ± 5.01% and 51.57 ± 11.88% respectively (Fig 6.3 B). However, XA (10 – 100 mg $kg^{-1}$) suppressed the mean maximal swelling attained at 60 min to 40.96 ± 7.11%, 37.70 ± 6.59% and 33.55 ± 4.80% respectively relative to the inflamed control response (Fig 6.3 C) and the total paw swellings induced over the 3 h by 35.40 ± 14.95%, 44.57 ± 7.50% and 53.71 ± 7.34% relative to the inflamed control response respectively (Fig 6.3 D).

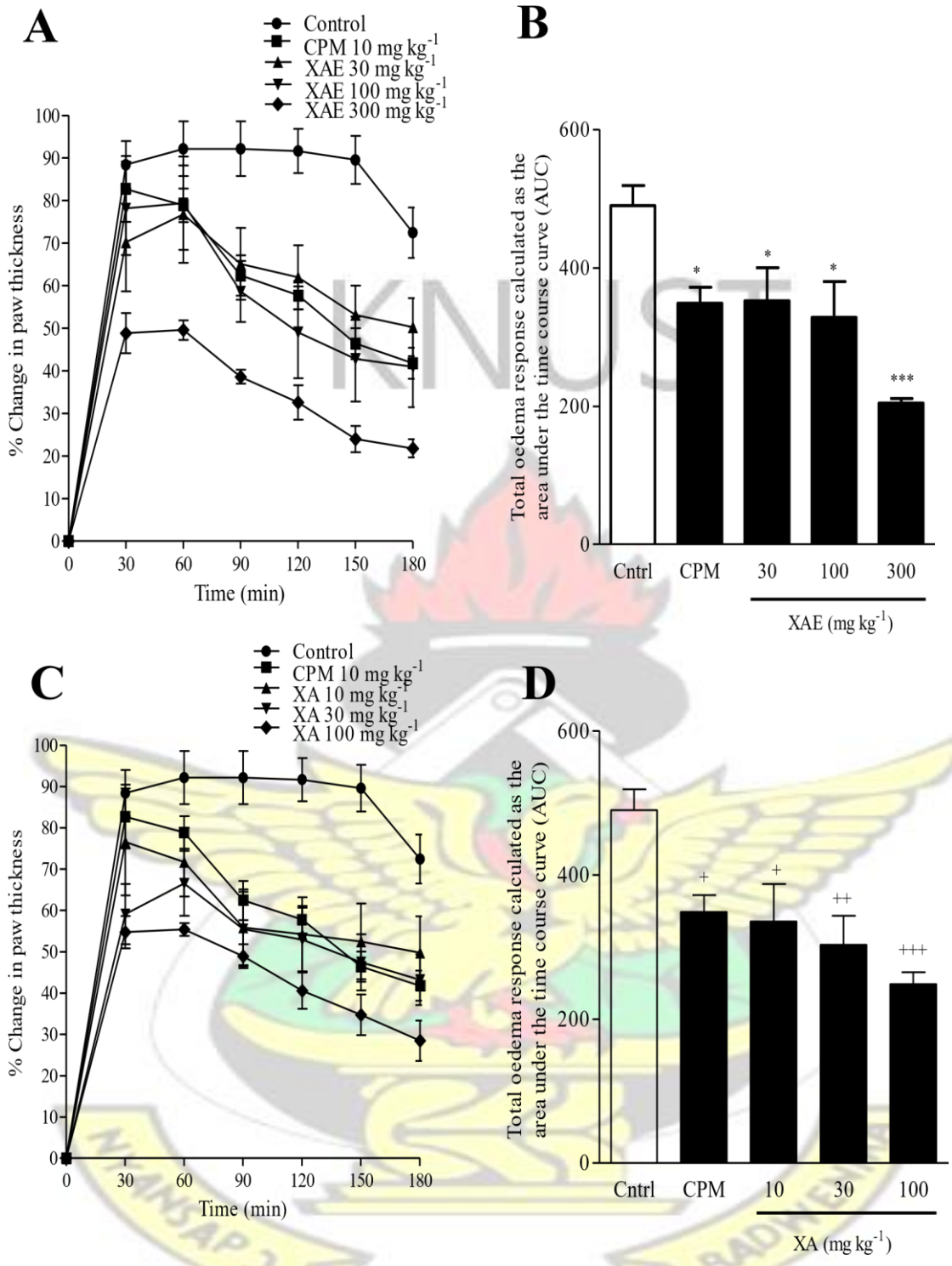



**Figure 6.2. Effect of *Xylopia aethiopica* extract and xylopic acid administered prophylactically on histamine-induced paw oedema in mice.** Chlorpheniramine (10 mg kg[1] *p.o.*), XAE (30, 100, 300 mg kg$^{-1}$ *p.o.*) or XA (10, 30, 100 mg kg$^{-1}$ *p.o.*) was given 1 h before oedema induction. The control group received normal saline (1 ml kg$^{-1}$ *p.o.*). Oedema was induced by injection of 0.1 mg of histamine and monitored at 30 min intervals over 3 h as percentage increase in paw thickness (A, C). Total oedema induced during the 3 h was calculated as area under the time course curves, AUC (B, D) (n = 5). Values are presented as Mean ± SEM.*$P$ = 0.05, ***$P$ = 0.005, $^{+}P$ = 0.012, $^{++}P$ = 0.0012, $^{+++}P$ = 0.00012.

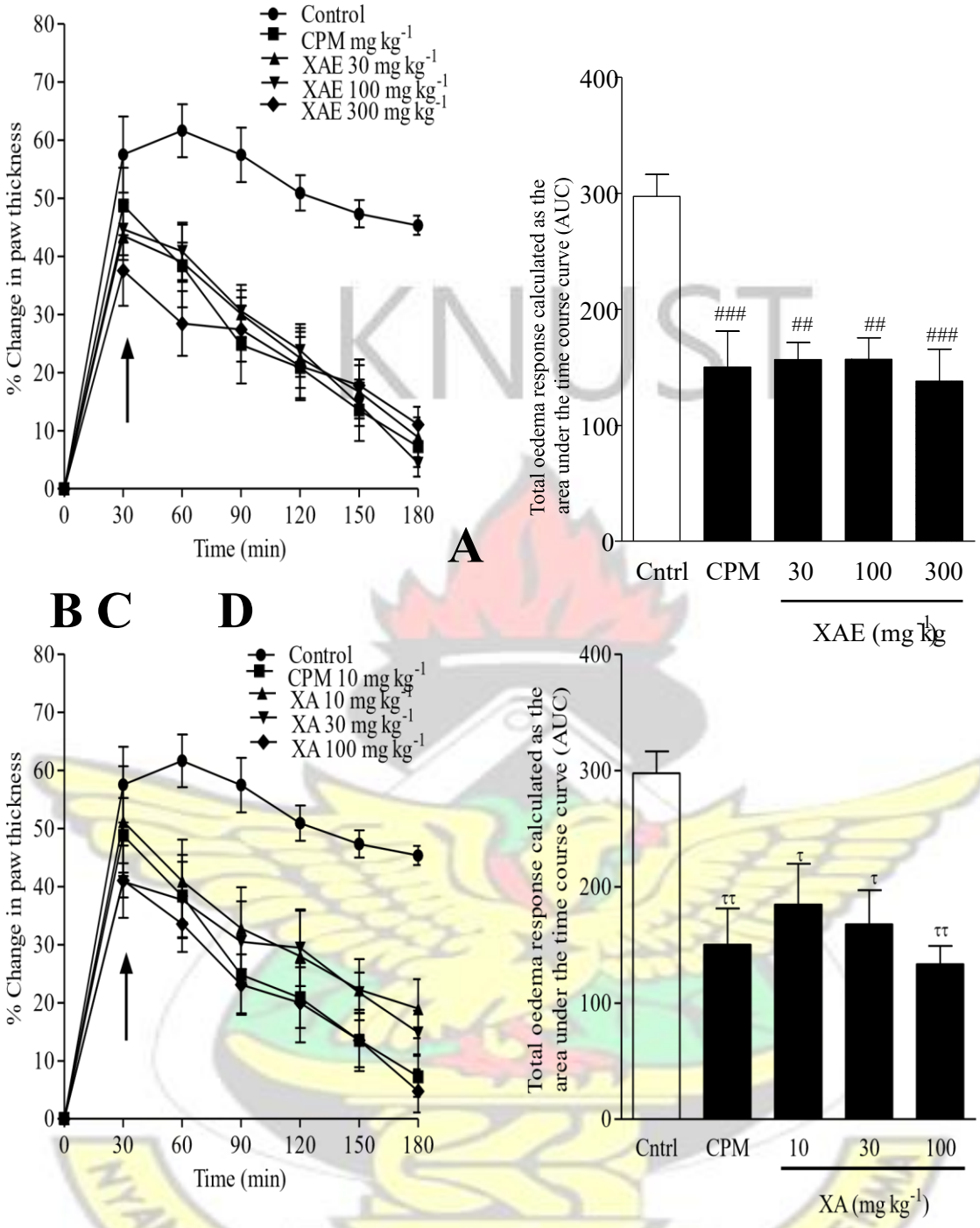



**Figure 6.3. Effect of *Xylopia aethiopica* extract and xylopic acid administered therapeutically on histamine-induced paw oedema in mice.** Chlorpheniramine (10 mg $kg^{-1}$ *p.o.*), XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) was given 30 min after induction. Oedema was induced by injection of 0.1 mg of histamine and monitored at 30 min intervals over 3 h as percentage increase in paw thickness (A, C). Total oedema induced during the 3 h was calculated as area under the time course curves, AUC (B, D) (n = 5). Values are presented as Mean ± SEM. $^{\#\#}P = 0.004$, $^{\#\#\#}P = 0.0004$, $^{\tau}P = 0.03$, $^{\tau\tau}P = 0.003$. Arrow indicates point of Chlorpheniramine, XAE or XA administration.

### *6.3.1.2 Serotonin –induced paw oedema*

Serotonin is a vasoactive mediator of inflammation similar in action to histamine. It increases the vascular permeability, dilates capillaries and plays a role in the oedema response during the inflammatory process (Edwards, 2014). The activity of XAE and XA on the serotonin-induced inflammation was studied.

Accordingly, paw oedema was induced with subplantar injection of 0.1 mg of serotonin. Paw oedema was measured at 30 min interval for 3 h and the percentage changes in paw thickness calculated. XAE (30, 100, 300 mg $kg^{-1}$) when administered before the induction of the serotonin-induced paw oedema caused the mean maximal swelling attained at 90 min to be reduced to 50.74 ± 5.30%, 39.81 ± 3.16% and 34.66 ± 3.35% respectively relative to the inflamed control response of 71.38 ± 5.61% (Fig 6.4 A). The total oedema response induced over the 3 h were also dose-dependently and significantly suppressed by 33.81 ± 9.83% and 45.42 ± 6.69% in the 100 mg $kg^{-1}$ and 300 mg $kg^{-1}$ XAE-treated groups respectively (Fig 6.4 B). When mice were treated with XA (10, 30, 100 mg $kg^{-1}$), the mean maximal swelling attained at 90 min reduced to 49.65 ± 4.58%, 44.45 ± 5.92% and 31.22 ± 4.87% respectively relative to the inflamed control response (Fig 6.4 C). The total paw swellings induced over the 3 h were also dose-dependently and significantly suppressed by 22.57 ± 14.15%, 35.12 ± 11.97% and 58.82 ± 3.56% relative to the inflamed control response respectively (Fig 6.4 D).

XAE (30, 100, 300 mg $kg^{-1}$) when given therapeutically suppressed the mean maximal swelling attained at 90 min to 49.36 ± 6.27%, 44.17 ± 4.51% and 36.39 ± 7.42% respectively relative to the inflamed control response of 71.38 ± 5.61% (Fig 6.5 A). The total paw swellings induced over the 3 h were also dose-dependently and significantly suppressed by 41.01 ± 9.50%, 43.33 ± 8.14% and 54.77 ± 12.26% respectively (Fig 6.5

B). Similarly, treatment with XA (10, 30, 100 mg $kg^{-1}$) suppressed the mean maximal swelling attained at 90 min to 49.53 ± 4.97%, 45.40 ± 5.24% and 35.37 ± 2.55% respectively relative to the inflamed control response (Fig 6.5 C). The total oedema response induced over the 3 h were also dose-dependently and significantly suppressed by 29.91 ± 6.12%, 31.46 ± 10.12% and 47.50 ± 8.51% respectively (Fig 6.5 D).

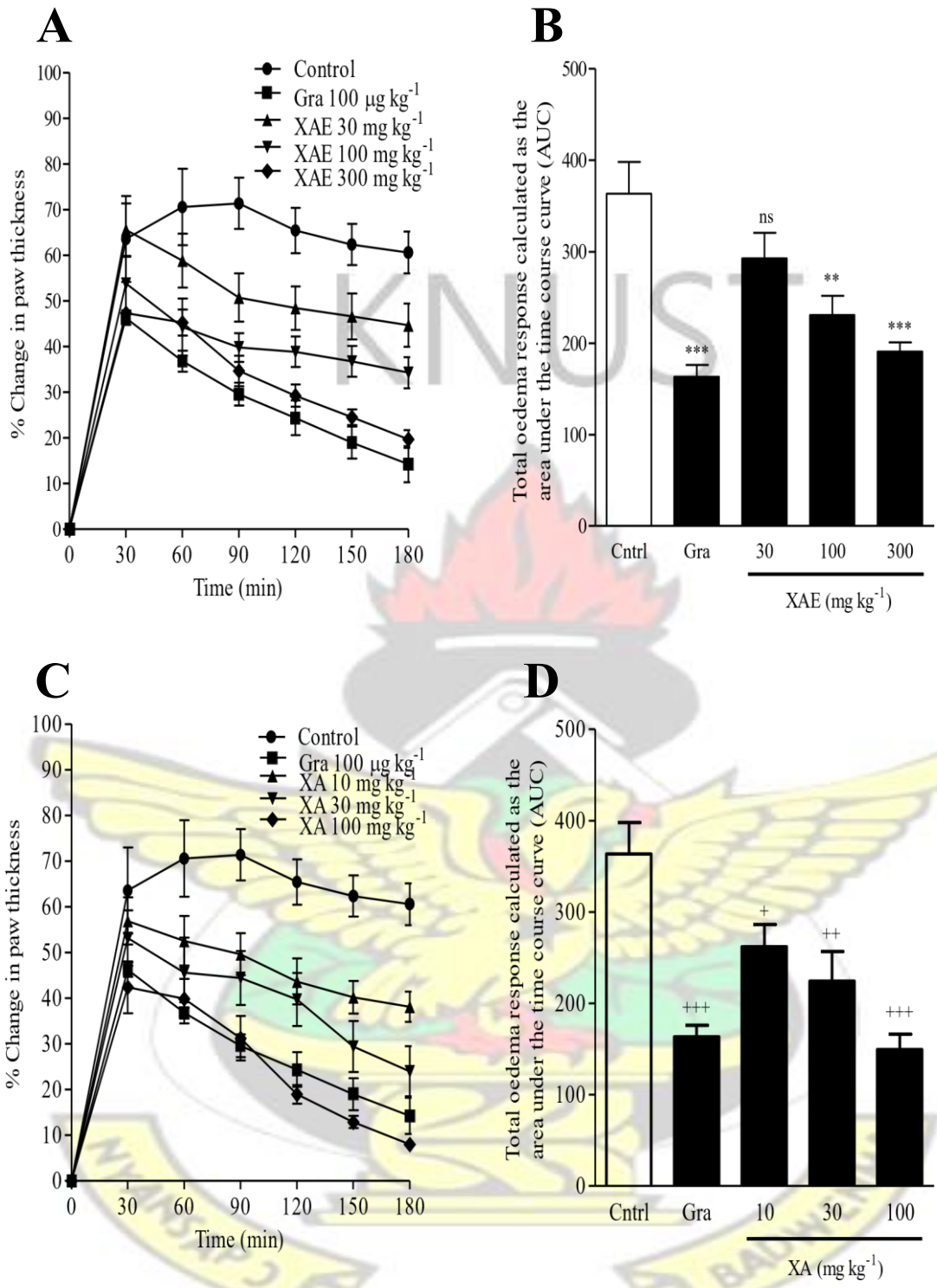



**Figure 6.4. Effect of *Xylopia aethiopica* extract and xylopic acid administered prophylactically on serotonin-induced paw oedema in mice.** Granisetron (100 µg kg$^{-1}$ *p.o.*), XAE (30, 100, 300 mg kg$^{-1}$ *p.o.*) or XA (10, 30, 100 mg kg$^{-1}$ *p.o.*) was given 1 h before oedema induction. Oedema was induced by injection of 0.1 mg of serotonin and monitored at 30 min intervals over 3 h as percentage increase in paw thickness (A, C). Total oedema induced during the 3 h was calculated as area under the time course curves, AUC (B, D) (n = 5). Values are presented as Mean ± SEM. $^{**}P = 0.001$, $^{***}P = 0.0001$, $^{+}P = 0.01$, $^{++}P = 0.001$, $^{+++}P = 0.0001$, $^{ns}P > 0.05$.

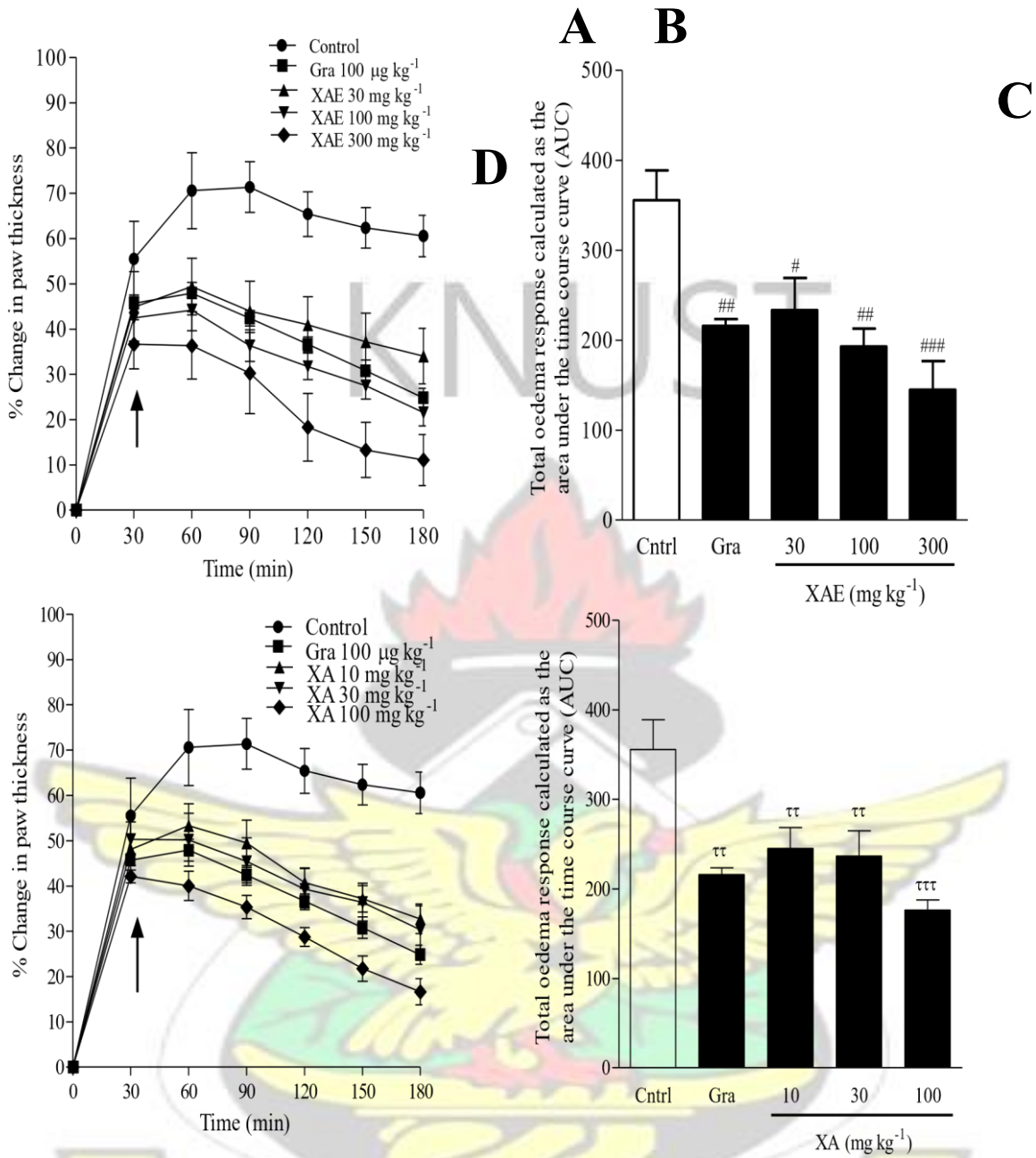



**Figure 6.5. Effect of *Xylopia aethiopica* extract and xylopic acid administered therapeutically on serotonin-induced paw oedema in mice.** Granisetron (100 µg kg$^{-1}$ *p.o.*), XAE (30, 100, 300 mg kg$^{-1}$ *p.o.*) or XA (10, 30, 100 mg kg$^{-1}$ *p.o.*) was given 30 min after oedema induction. Oedema was induced by injection of 0.1 mg of serotonin and monitored at 30 min intervals over 3 h as percentage increase in paw thickness (A, C). Total oedema induced during the 3 h was calculated as area under the time course curves, AUC (B, D) (n = 5). Values are presented as Mean ± SEM. $^{\#}P = 0.05$, $^{\#\#}P = 0.005$, $^{\#\#\#}P = 0.0005$, $^{\tau\tau}P = 0.004$, $^{\tau\tau\tau}P = 0.0004$. Arrow indicates point of granisetron, XAE or XA administration.

### *6.3.1.3 Bradykinin –induced paw oedema*

Bradykinin acts locally by mediating varied actions including pain and inflammation.

It does that by influencing vascular tone and permeability, inducing contractions in

smooth muscles and increasing leukocyte migration to site of tissue damage (Regoli and Barabe, 1980). Bradykinin is a chemical link between the initial phase of inflammation, mediated by histamine and serotonin, and the later stage, mediated by prostaglandins. The effect of XAE and XA on the bradykinin-induced inflammation was therefore studied.

To investigate this, mice were pre-treated with captopril 1 h before bradykinin injection to inhibit *kininase* of the bradykinin. Mice were injected with 1 µg of the bradykinin subplantar in the right hind paw 30 min before and after administering drug treatments and oedema monitored over 2.5 h. Results show that XAE (30, 100, 300 mg $kg^{-1}$) when administered before the induction of the bradykinin-induced paw oedema caused the mean maximal swelling attained at 90 min to be reduced to 18.13 ± 5.84%, 24.26 ± 2.88% and 14.31 ± 5.85% respectively relative to the mean inflamed control response of 45.22 ± 5.38% (Fig 6.6 A). The total oedema induced over the 3 h were also dosedependently and significantly suppressed by 62.06 ± 8.99%, 36.00 ± 18.36% and 62.57 ± 9.08% respectively (Fig 6.6 B). On the other hand, XA (10, 30, 100 mg $kg^{-1}$) caused the mean maximal swelling attained at 90 min to be reduced to 19.84 ± 4.03%, 20.01 ±

1.99% and 23.09 ± 7.45% respectively relative to the inflamed control response (Fig 6.6 C). The total oedema induced over the 2.5 h suppressed by 49.43 ± 13.42%, 41.10 ± 10.79% and 32.52 ± 26.56% respectively relative to the inflamed control response (Fig 6.6 D).

Therapeutically, XAE (30, 100, 300 mg $kg^{-1}$) suppressed the mean maximal swelling attained at 90 min to 47.90 ± 6.19%, 44.23 ± 4.06% and 36.05 ± 8.25% respectively relative to the mean inflamed control response of 70.40 ± 4.74% (Fig 6.7 A). The total oedema induced over the 2.5 h were also dose-dependently and significantly suppressed

by 36.27 ± 9.29%, 36.66 ± 10.59% and 47.54 ± 14.06% respectively (Fig 6.7 B). Also, XA (10, 30, 100 mg $kg^{-1}$) suppressed the mean maximal swelling attained at 90 min to 52.18 ± 4.29%, 50.07 ± 6.29% and 37.02 ± 3.99% respectively relative to the inflamed control response (Fig 6.7 C) while the total oedema induced over the 2.5 h were significantly suppressed by 44.88 ± 8.03% in the mice that received 100 mg $kg^{-1}$ XA relative to the inflamed control response (Fig 6.7 D).

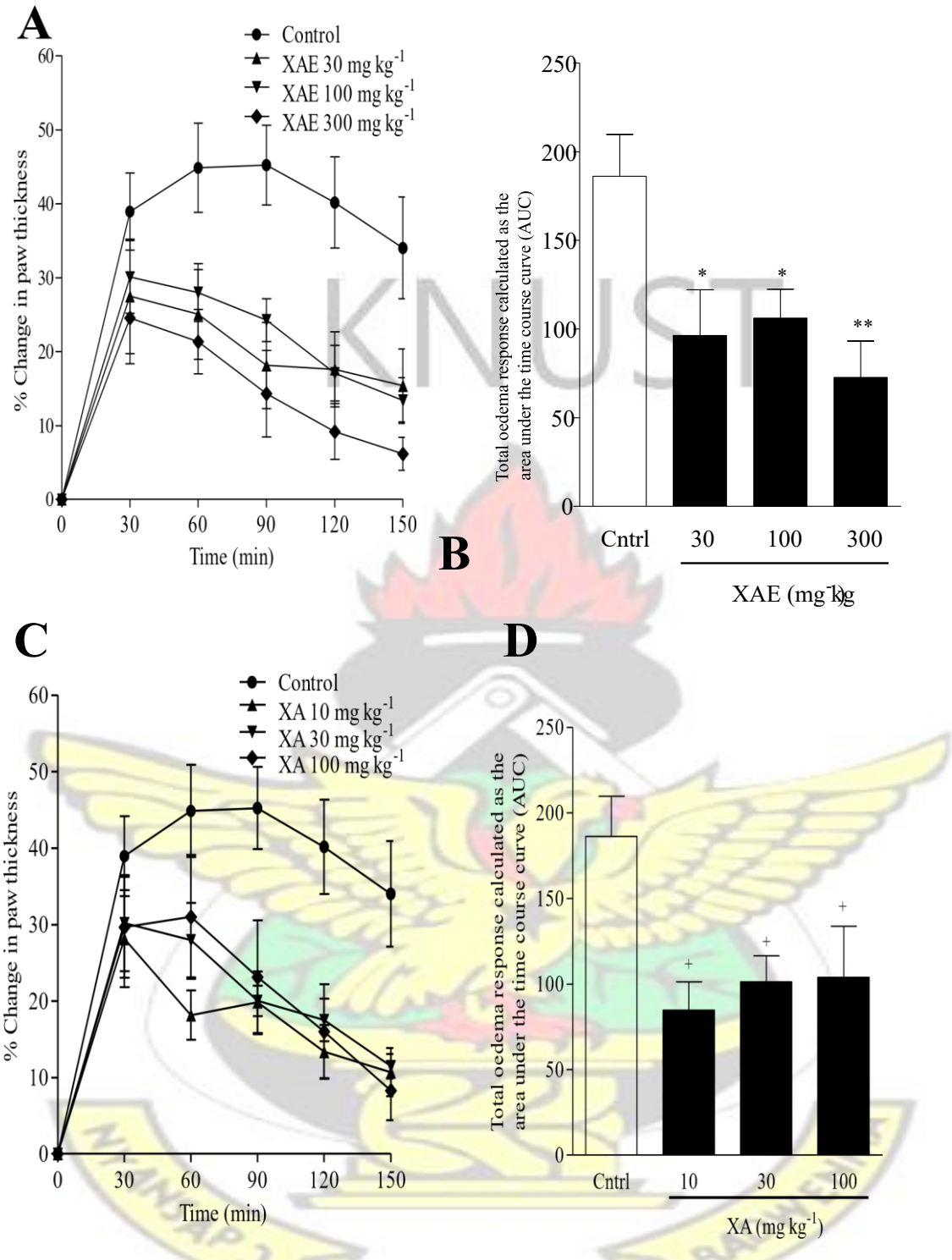



**Figure 6.6. Effect of *Xylopia aethiopica* extract and xylopic acid administered prophylactically on bradykinin-induced paw oedema in mice.** XAE (30, 100, 300 mg kg$^{-1}$ *p.o.*) or XA (10, 30, 100 mg kg$^{-1}$ *p.o.*) was given 1 h before oedema induction. Oedema was induced by injection of 10 nmol of bradykinin and monitored at 30 min intervals over 2.5 h as percentage increase in paw thickness (A, C). Total oedema induced during the 2.5 h was calculated as area under the time course curves, AUC (B, D). No selective antagonist was given in this model (n = 5). Values are presented as Mean ± SEM. **P* = 0.0113, ***P* = 0.00113, $^{+}P$ = 0.0214.

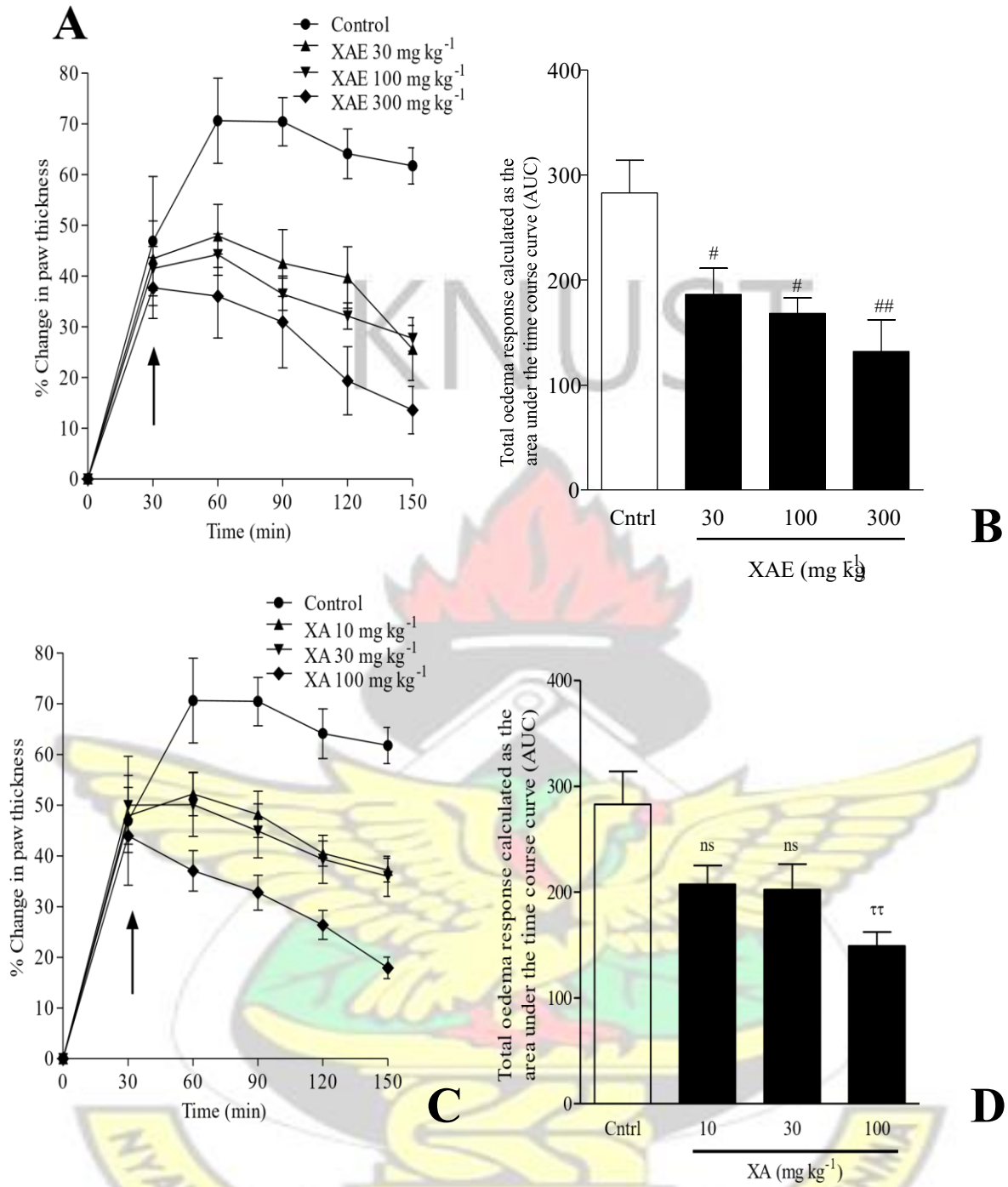



**Figure 6.7. Effect of *Xylopia aethiopica* extract and xylopic acid administered therapeutically on bradykinin-induced paw oedema in mice.** XAE (30, 100, 300 mg kg$^{-1}$ *p.o.*) or XA (10, 30, 100 mg kg$^{-1}$ *p.o.*) was given 30 min after oedema induction. Oedema was induced by injection of 10 nmol of bradykinin and monitored at 30 min intervals over 2.5 h as percentage increase in paw thickness (A, C). Total oedema induced during the 2.5 h was calculated as area under the time course curves, AUC (B, D). No selective antagonist was given in this model (n = 5). Values are presented as Mean ± SEM. $^{\#}P$ = 0.055, $^{\#\#}P$ = 0.0055, $^{\tau\tau}P$ = 0.0064, $^{ns}P$ > 0.05. Arrow indicates point of XAE or XA administration.

### *6.3.1.4 Prostaglandin $E_2$ –induced paw oedema*

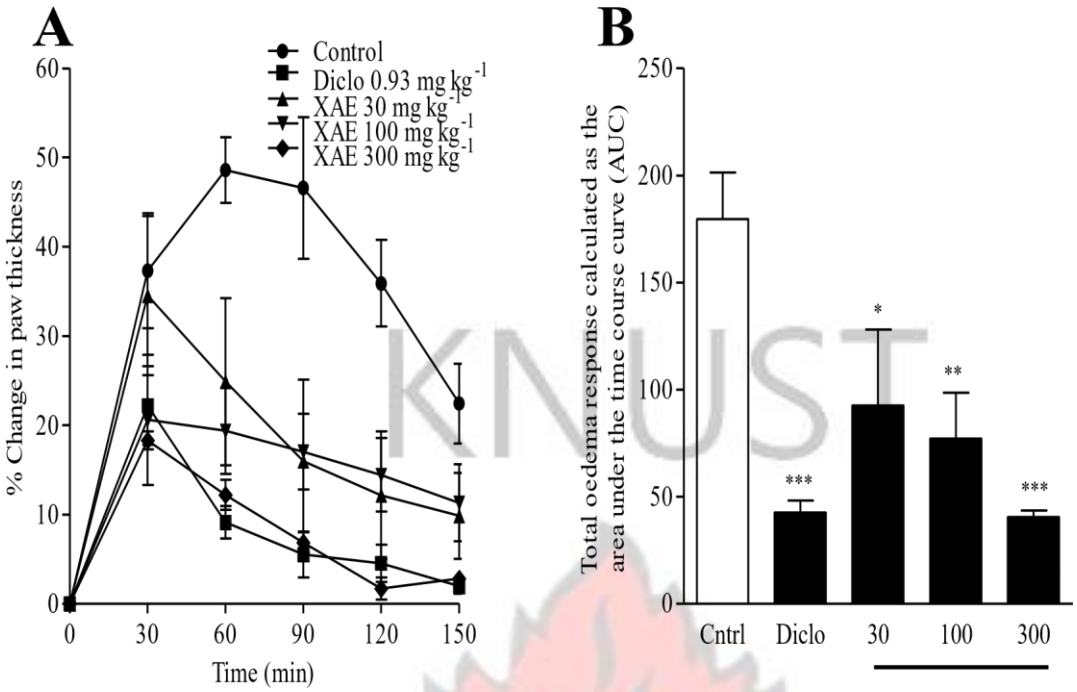
Prostaglandins sustain homeostatic functions and mediate pathogenic mechanisms such as inflammation. Their biosynthesis is increased significantly in inflamed tissues and

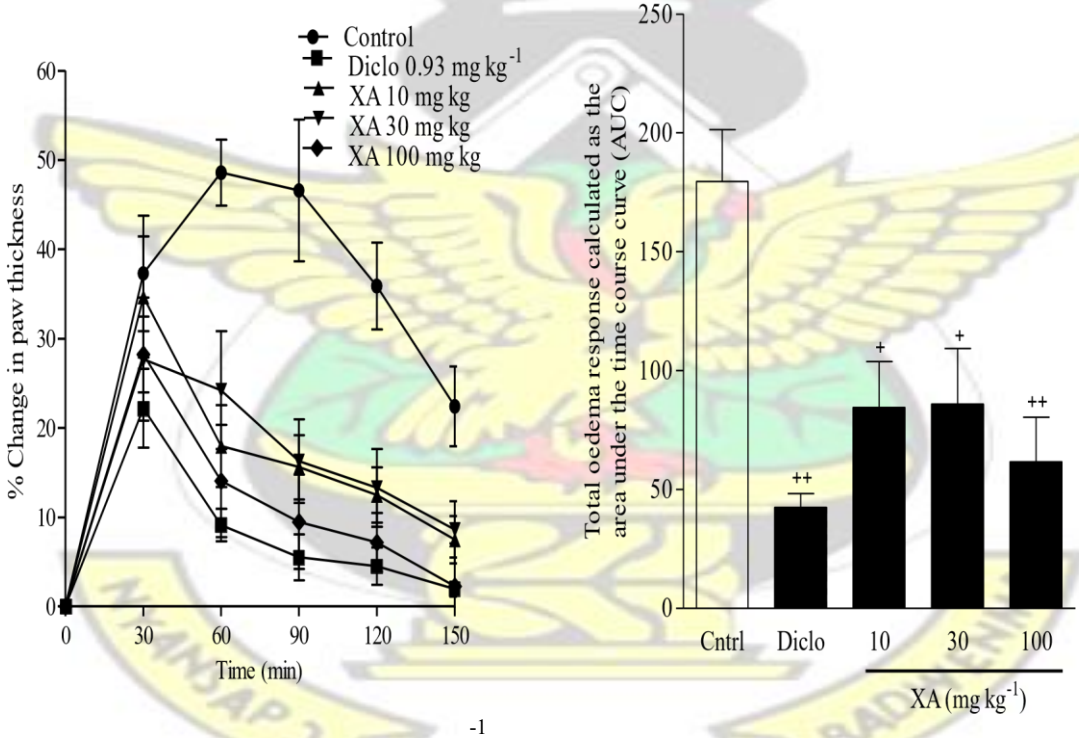
contribute to the development of cardinal signs of acute inflammation (Ricciotti and FitzGerald, 2012). Effect of XA and XAE on role of prostaglandin $E_2$ in acute inflammation was henceforth studied.

To study this, paw oedema was induced with subplantar injection of 50 µl of 1 nM of prostaglandin $E_2$ and oedema monitored as earlier described. Prophylactically, XAE (30, 100, 300 mg $kg^{-1}$) caused the mean maximal swelling attained at 60 min to be reduced to 34.53 ± 8.91%, 20.61 ± 7.29% and 18.32 ± 1.00% respectively compared with inflamed control response of 48.60 ± 3.68% (Fig 6.8 A). The total oedema induced over the 2.5 h were also dose-dependently and significantly suppressed by 62.11 ± 12.82%, 51.99 ± 16.05% and 76.43 ± 2.52% respectively (Fig 6.8 B). Also, XA (10, 30, 100 mg $kg^{-1}$) caused the mean maximal swelling attained at 60 min to be reduced to 34.68 ± 6.79%, 27.70 ± 6.89% and 28.24 ± 4.22% respectively relative to the inflamed control response (Fig 6.8 C). The total oedema induced over the 2.5 h were also dose-dependently and significantly suppressed by 53.97 ± 6.18%, 47.94 ± 19.20% and 63.51 ± 10.46% respectively relative to the inflamed control response respectively (Fig 6.8 D).

In the therapeutic model, XAE (30, 100, 300 mg $kg^{-1}$) treatment suppressed the mean maximal swelling attained at 60 min to 39.66 ± 5.06%, 39.95 ± 3.51% and 27.93 ± 5.18% respectively compared with the inflamed control response of 61.63 ± 4.55% (Fig 6.9 A) with the total oedema induced over the 2.5 h were also dose-dependently and significantly suppressed by 45.10 ± 11.68%, 40.52 ± 5.24% and 48.63 ± 12.52% respectively (Fig 6.9 B). On the other hand, XA (10, 30, 100 mg $kg^{-1}$) treatment suppressed the mean maximal swelling attained at 60 min to 41.29 ± 7.29%, 37.85 ± 6.22% and 33.10 ± 4.44% respectively relative to the inflamed control response (Fig 6.9 C) with the total oedema induced over the 2.5 h also dose-dependently and

significantly suppressed by 41.98 ± 8.60%, 48.55 ± 7.51% in the 30 mg $kg^{-1}$ and 100 mg $kg^{-1}$ respectively relative to the inflamed control response respectively (Fig 6.9 D).

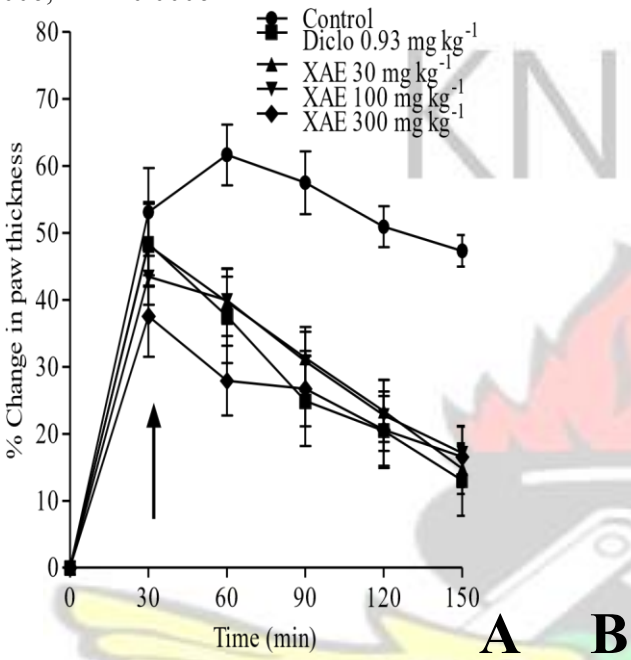



XAE (mg kg-1)

-1
-1
-1

**Figure 6.8. Effect of *Xylopia aethiopica* extract and xylopic acid administered prophylactically on prostaglandin $E_2$-induced paw oedema in mice.** Diclofenac (0.93 mg $kg^{-1}$), XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) was given 1 h before oedema induction. Oedema was induced by injection of 50 µl of 1 nM prostaglandin $E_2$ and monitored at 30 min intervals over 2.5 h as percentage increase in paw thickness (A, C). Total oedema induced during the 2.5 h was calculated as area under the time course curves, AUC (B, D) (n = 5). Values are presented as Mean ± SEM. **P* < 0.05, ***P* = 0.01, ****P* = 0.001, $^{+}P$ = 0.006, $^{++}P$ = 0.0006.



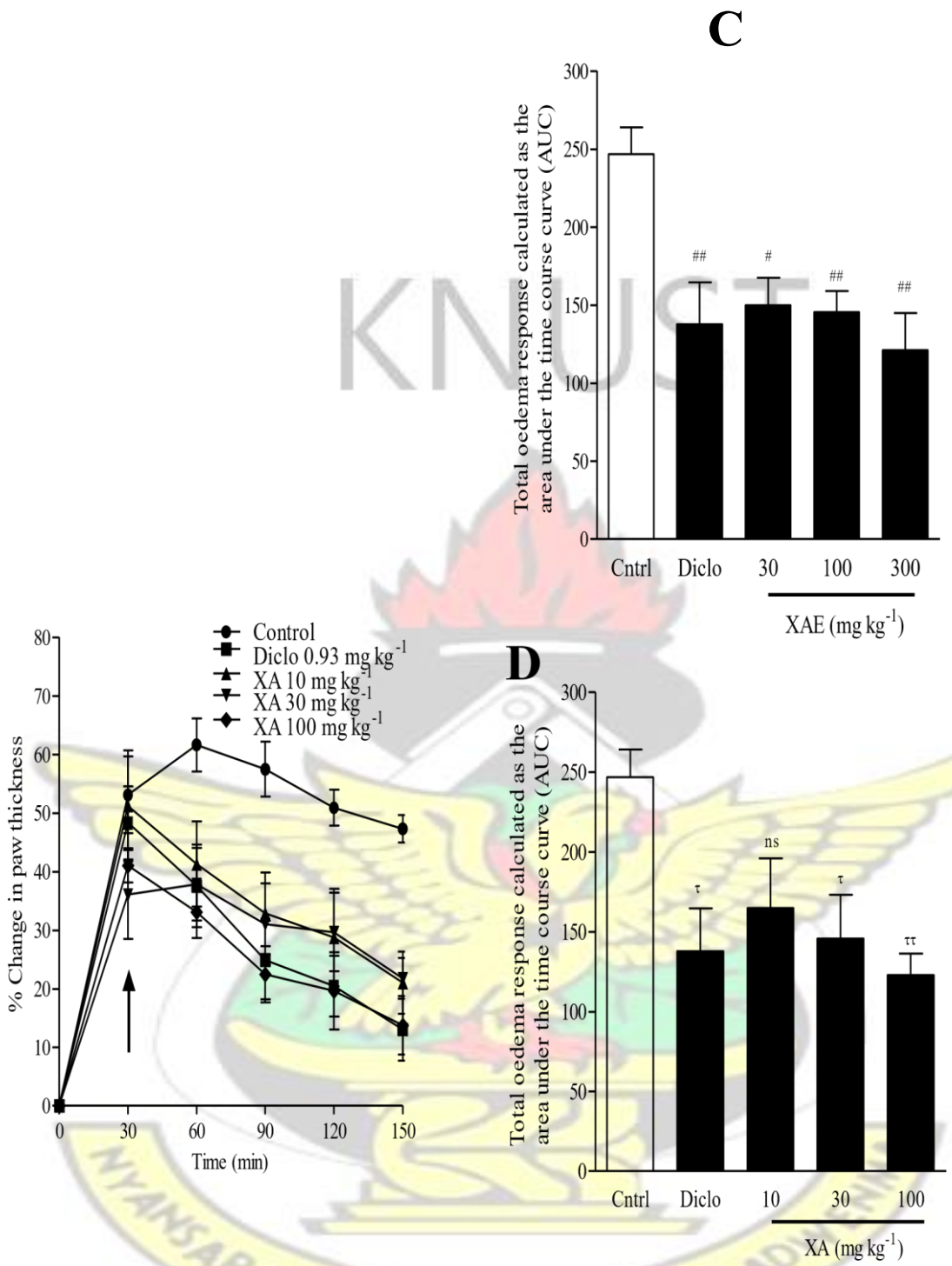



**Figure 6.9. Effect of *Xylopia aethiopica* extract and xylopic acid administered therapeutically on prostaglandin $E_2$-induced paw oedema in mice.** Diclofenac (0.93 mg $kg^1$), XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) was given 30 min after oedema induction. Oedema was induced by injection of 50 µl of 1 nM prostaglandin $E_2$ and monitored at 30 min intervals over 2.5 h as percentage increase in paw thickness (A, C). Total oedema induced during the 2.5 h was calculated as area under the time course curves, AUC (B, D) (n = 5). Values are presented as Mean ± SEM. $^{\#}P = 0.026$, $^{\#\#}P = 0.0026$, $^{t}P = 0.0141$, $^{tt}P = 0.00141$, $^{ns}P > 0.05$. Arrow indicates point of diclofenac, XAE or XA administration.

#### *6.3.1.5 Inhibition of arachidonic acid release: Hydrogen sulphide-induced acute inflammation*

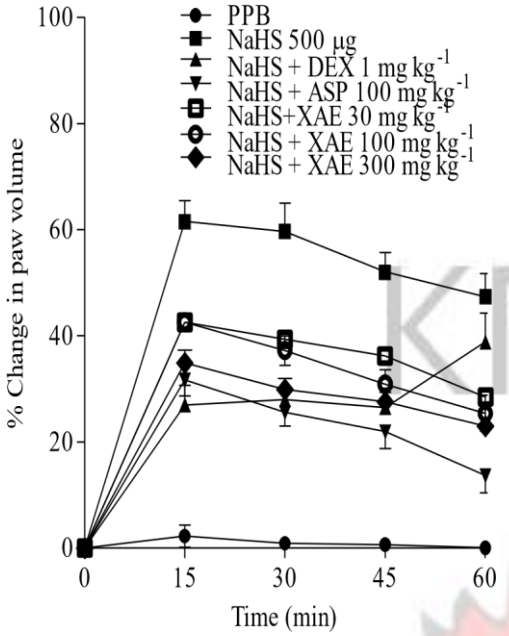
Activation of the mast cells also results in arachidonic acid release. This process starts with the enzymatic action of $PLA_2$ liberating arachidonic acid (AA) from the membrane phospholipid. The so called released AA is metabolised through the COX pathway to produce prostanoids, namely, prostaglandins and thromboxane while the LOX pathway leads to leucotriene systhesis. Western blot analysis has shown an increased activity of *cystathionine beta synthase* (CBS) and *cystathionine gamma-lyase* (CSE) in carrageenan induced acute inflammation, implying the role of the L-cysteins/$H_2S$ pathway (Bianca *et al.,* 2010). Intraplantar injection of $H_2S$ or L-cysteine induces oedema which is of rapid onset (0-60 min) which implies the involvement of the $H_2S$/Lcysteine pathway as reported by Bianca *et al.* (2010).

To investigate this, ICR mice (20 – 30 g) received an intraplantar injection of NaHS (500 µg per paw) as exogenous source of $H_2S$ and paw oedema monitored as earlier described.

##### *6.3.1.5.1 Measurement of paw oedema*

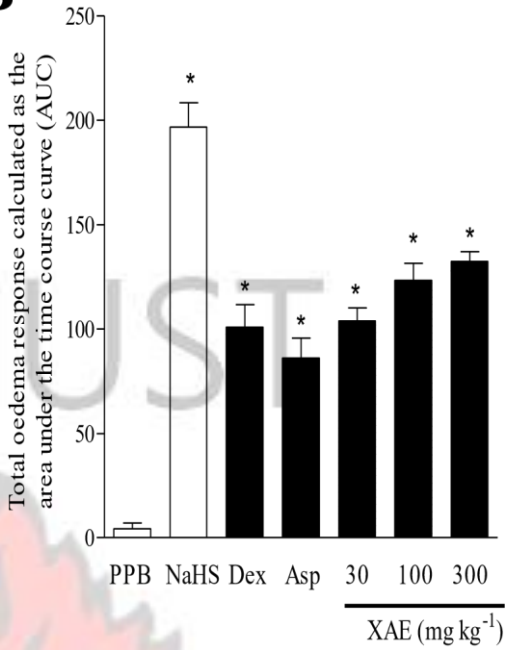
With the intra-plantar injection of NaHS-induced oedema, the peak of the oedematogenic response was evident as early as 15 min after injection and had declined by 60 min (Fig 6.10 A). XAE at doses 30-300 mg $kg^{-1}$ suppressed the mean maximal swelling attained at 15 min to 42.58 ± 1.66%, 42.58 ± 1.66% and 34.91 ± 2.39% respectively compared to the mean inflamed control response of 61.51 ± 3.90% (Fig 6.10 A). The total paw swellings induced over the 1 h were also significantly suppressed by 31.82 ± 5.28%, 36.19 ± 6.01%, and 31.65 ± 5.16% respectively (Fig 6.10 B). On the other hand, XA administered at 10 - 100 mg $kg^{-1}$ suppressed the mean maximal swelling attained at 15 min to 41.75 ± 1.24%, 40.26 ± 1.68% and 38.26 ± 2.52% respectively

relative to the control response (Fig 6.10 C) while the total paw swellings was significantly suppressed by 34.08 ± 4.49%, 38.19 ± 4.50% and 43.40 ± 5.09% respectively relative to the inflamed control response (Fig 6.10 D).


PPB
NaHS 500 µg
NaHS + DEX 1 mg kg-1
NaHS + ASP 100 mg kg-1
NaHS+XAE 30 mg kg-1
NaHS + XAE 100 mg kg-1
NaHS + XAE 300 mg kg-1
% Change in paw volume
Time (min)
0
15
30
45
60
0
20
40
60
80
100

A

B


Total oedema response calculated as the area under the time course curve (AUC)
PPB
NaHS
Dex
Asp
30
100
300
XAE (mg kg-1)
0
50
100
150
200
250

D

C

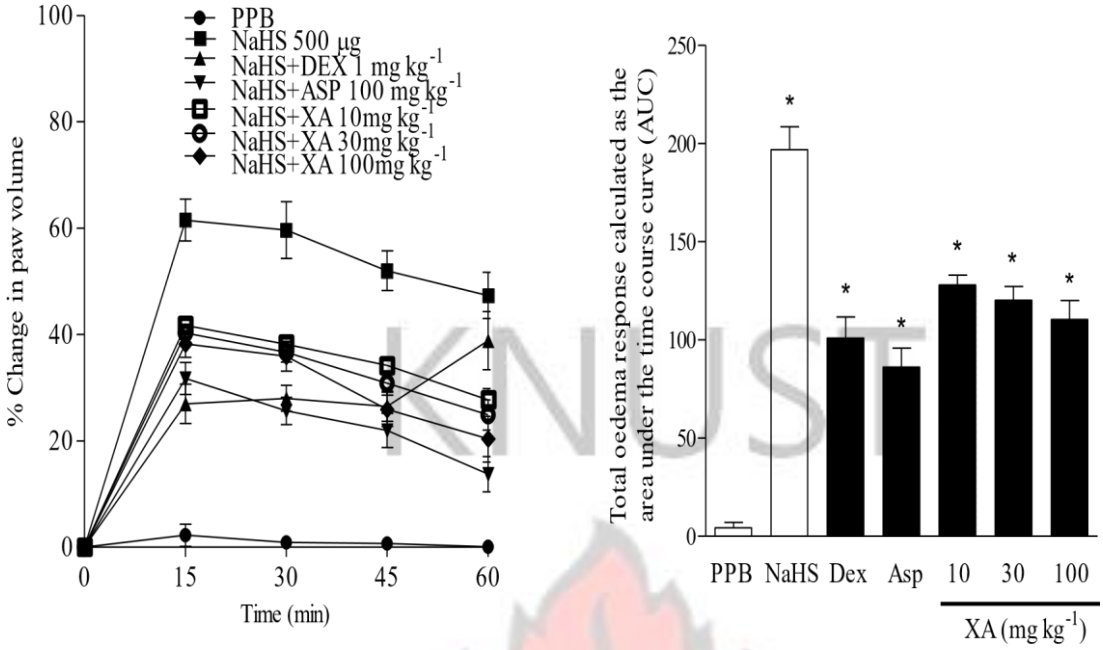



**Figure 6.10. Effect of *Xylopia aethiopica* extract and xylopic acid on $H_2S$-induced paw oedema in mice.** Normal saline (1 ml $kg^{-1}$), dexamethasone (1 mg $kg^{-1}$), aspirin (100 mg $kg^{-1}$), XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) or XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) was given 1 h before oedema induction. Oedema was induced by injection of 500 µg of NaHS and monitored at 15 min intervals over 1 h as percentage increase in paw thickness (A, C). Total oedema induced during the 1 h was calculated as area under the time course curves, AUC (B, D). After the intraplantar injection of NaHS (n = 5). Data presented as Mean ± SEM. *$P < 0.0001$.

***6.3.1.5.2 Histopathology***

Paws of potassium phosphate buffer control mice showed intact cell architecture (Plate 6.1 A) with no signs of cytoplasmic vacuolation, inflammation, cellular degeneration, loss of tissue organisation and necrotic tissues (Fig 6.11). The NaHS-treated mice showed significant loss of tissue organization (Plate 6.1 B) with significant presence of inflamed, necrotic tissue, cytoplasmic vacuolation and cellular degeneration (Fig 6.11). Treating mice with aspirin significantly suppressed the inflammation and cellular degeneration with nearly no necrotic cells present (Plate 6.1 C). Dexamethasone treatment also significantly reduced inflammation and loss of tissue architecture (Plate 6.1 D) with reduced cytoplasmic vacuolation and cellular degenerative activity when compared with the NaHS-treated mice (Fig 6.11). When mice were treated with XAE (300 mg $kg^{-1}$), there was reduced inflammation and cellular degeneration with no loss of tissue organization (Plate 6.1 E). This presented with a significantly reduced

necrosis, cytoplasmic vacuolation and loss of tissue organization (Fig 6.11). Similarly, mice treated with XA (100 mg $kg^{-1}$) showed reduced cellular degeneration and inflammation with absence of necrotic tissues (Plate 6.1 F). Also, there was reduced cytoplasmic vacuolation, necrosis and loss of organization (Fig 6.11).

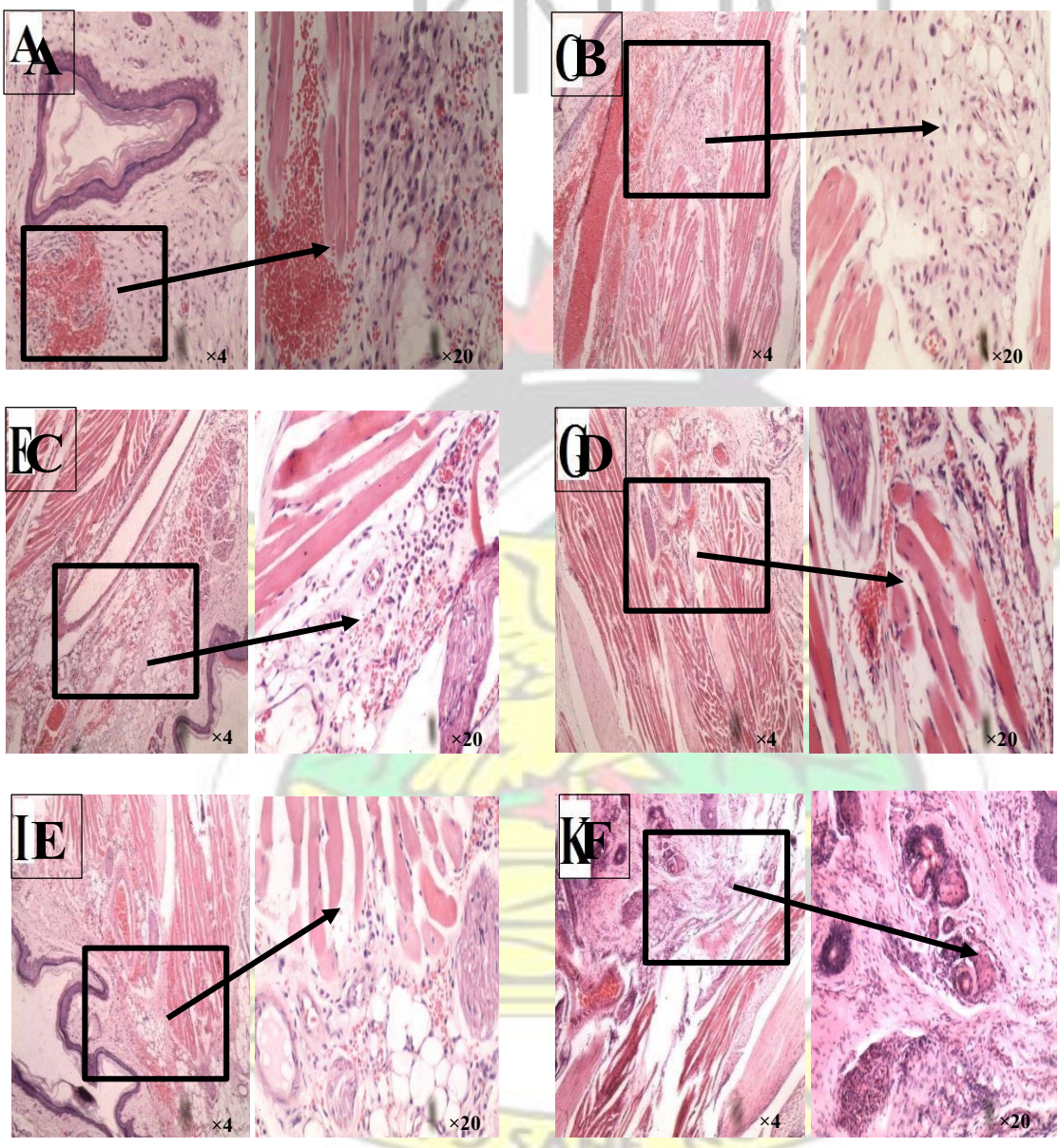



**Plate 6.1. Histopathological study in the $H_2S$-induced paw oedema in mice.** Mice were treated with PPB, aspirin (100 mg $kg^{-1}$), dexamethasone (1 mg $kg^{-1}$), XAE (300 mg $kg^{-1}$) or XA (100 mg $kg^{-1}$) and animals were killed by cervical dislocation 30 min after the NaHS (500 mg per paw), vehicle intra-plantar injection. Mice paws were fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 mm) were stained with haematoxylin-eosin and analysed under light microscopy. (A) PPB; (B) NaHS; (C) NaHS+ASP; (D) NaHS+DEX; (E) NaHS+XAE; (F) NaHS+XA. Scoring was done under ×20 magnification.

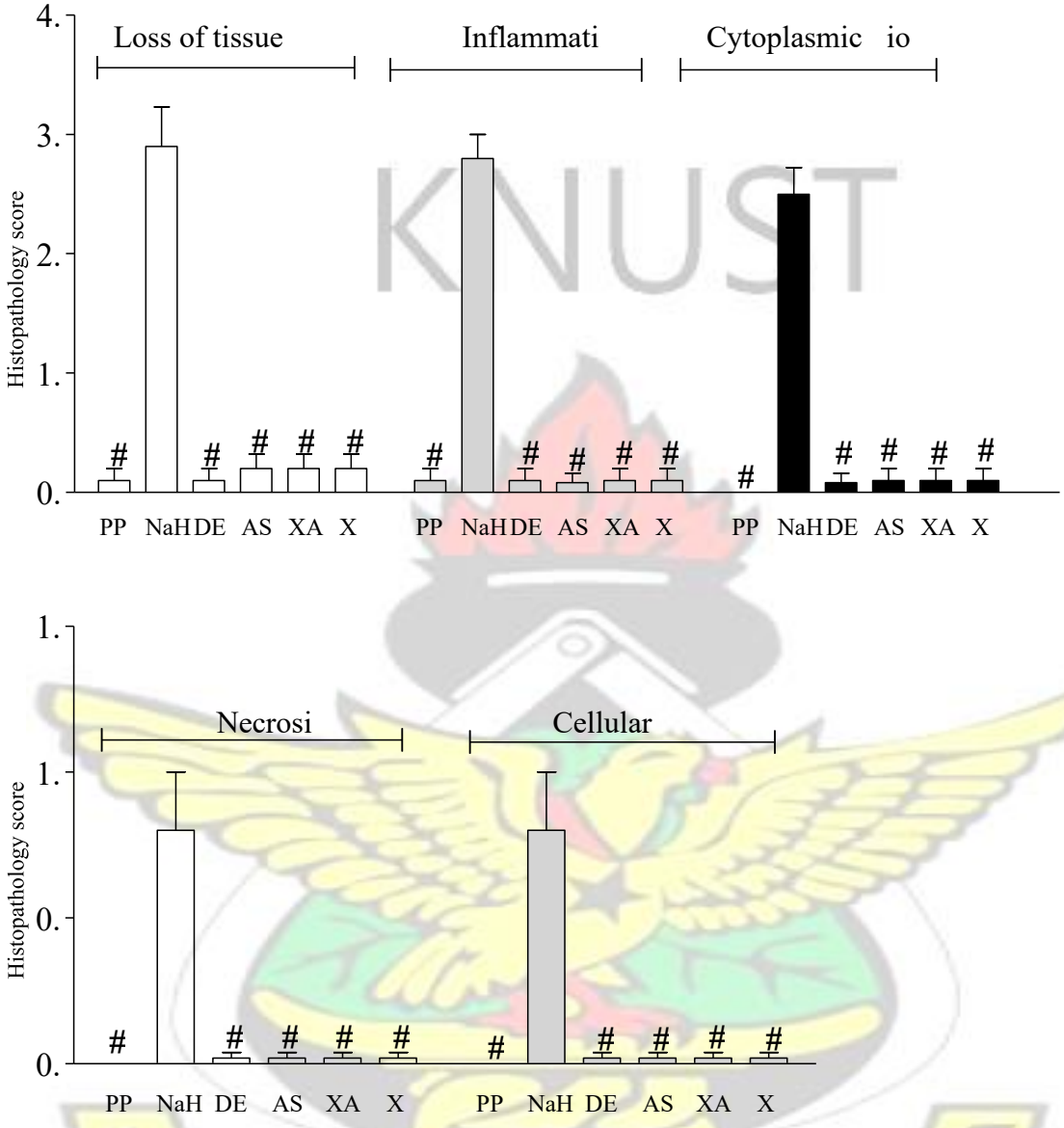



**Figure 6.11. Histopathological score in $H_2S$-induced paw oedema in mice.** Mice were treated with PPB, aspirin (100 mg $kg^{-1}$), dexamethasone (1 mg $kg^{-1}$), XAE (300 mg $kg^{-1}$) or XA (100 mg $kg^{-1}$) and animals were killed by cervical dislocation 30 min after the NaHS (500 mg per paw). Mice paws were fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 mm) were stained with haematoxylin-eosin and analysed under light microscopy, by an observer unaware of the treatment protocol. Histological slides were scored according to these parameters: loss of tissue organization, inflammation, necrosis, cellular degradation and cytoplasmic vacuolation. The degree of the disorganization was scored on a scale of 1–4. (i.e. 0 – Not present, 1 – Very mild, 2 – Mild, 3 – Moderate, 4 – Extensive). $^{\#}P < 0.0001$ (compared with NaHS).

### *6.3.2 Indirect anti-histaminic effect*

#### *6.3.2.1 Clonidine-induced catalepsy in mice*

Clonidine-induced catalepsy is employed to investigate a drugs ability to inhibit the actions of histamine specifically on the $H_1$ receptor. Clonidine causes release of histamine from mast cell (Schwartz *et al.,* 1997). Some of these mast cells have been found to be present in the brain (Gilman and Goodman, 1985). It is known that clonidine causes release of histamine in the brain (Lakadwala *et al.,* 1980) and this plays a definitive role in extrapyramidal motor symptoms of catalepsy. Clonidine is therefore believed to produce the cataleptic effect through its mediated histamine release from brain mast cells (Jadhav *et al.,* 1983).

To investigate the inhibitory effect of the aqueous ethanol extract of *X. aethiopica* and xylopic acid on histamine release from mast cells, clonidine was used to induce catalepsy in the Bar test model as described by Ferre *et al.,* 1990. In the prophylactic protocol, maximum catalepsy was observed in all the groups 90 min after the administration of clonidine (1 mg $kg^{-1}$ *s.c.*). Mice treated with chlorpheniramine (10 mg $kg^{-1}$ *p.o.*) and XAE (30, 100, 300 mg $kg^{-1}$ *p.o.*) showed significant inhibition of clonidine-induced catalepsy (Fig 6.12 A). With XA (10, 30, 100 mg $kg^{-1}$ *p.o.*) there was a significant inhibition of clonidine-induced catalepsy in a dose-dependent manner (Fig 6.12 B).

In a separate experiment, drug treatment was done after the induction of catalepsy in the mice. The maximum catalepsy was observed 90 min post-clonidine administration and inhibition was produced in a linear dose-dependent manner in XAE-treated mice (Fig 6.13 A) and XA-treated mice (Fig 6.13 B).

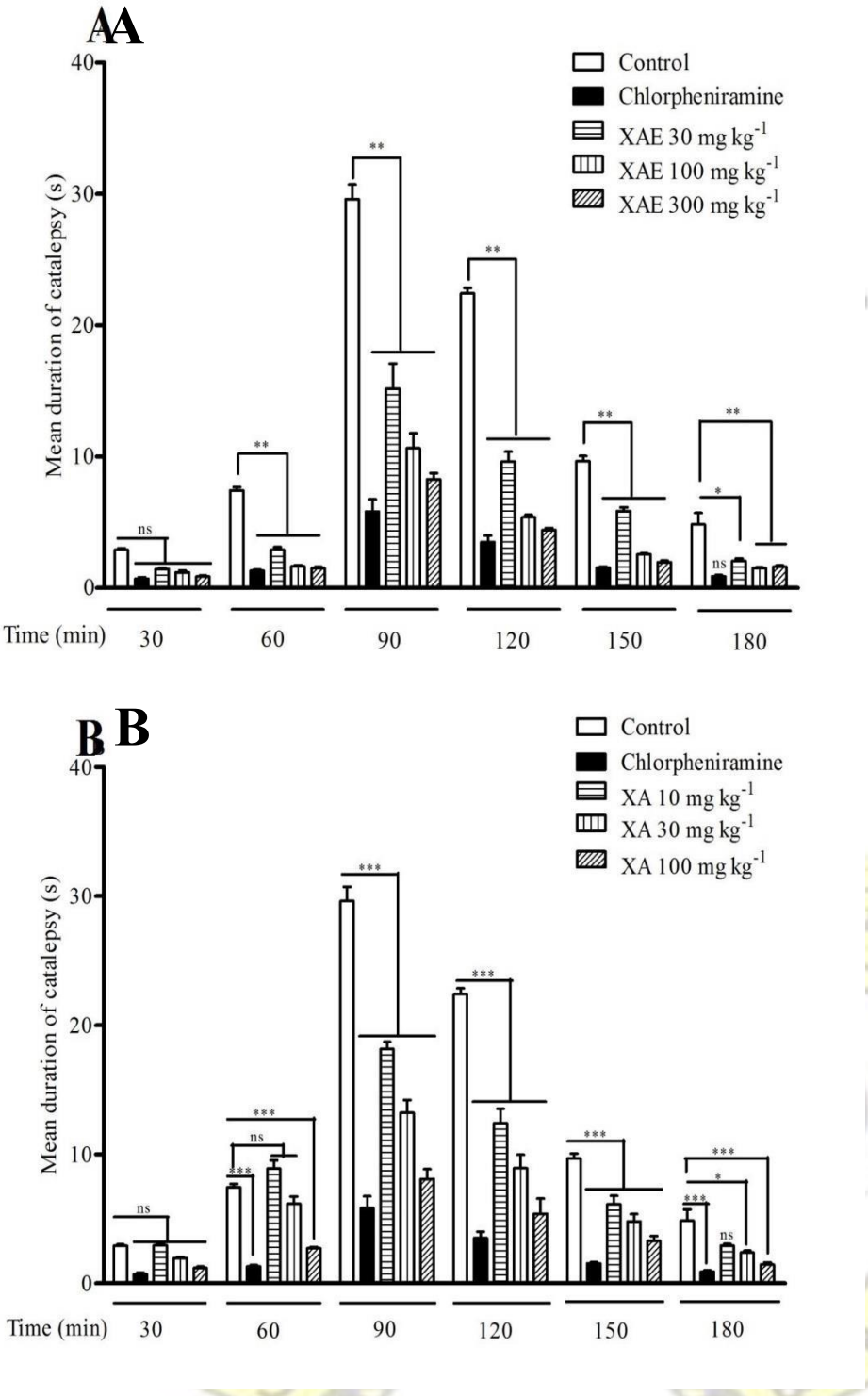



**Figure 6.12. Effect of *X. aethiopica* and xylopic acid administered prophylactically on clonidine-induced catalepsy in mice.** ICR mice (25-30 g) received clonidine 1 mg kg$^{-1}$, *s.c.* and their forepaws placed on a horizontal bar (1 cm in diameter, 3 cm above the table). The vehicle (5 ml kg$^{-1}$), XAE (30, 100, 300 mg kg$^{-1}$), XA (10, 30, 100 mg kg$^{-1}$) or chlorpheniramine (10 mg kg$^{-1}$) was given orally for 2 consecutive days ending 30 min before clonidine injection. The duration of catalepsy was measured at 30 min intervals up to 3 h after administration of clonidine. Values are Mean ± SEM. (n = 5). Significance between vehicle and drug/extracttreated mice denoted by $^{**}$ $P \leq 0.001$ and $^{*}$ $P \leq 0.01$.

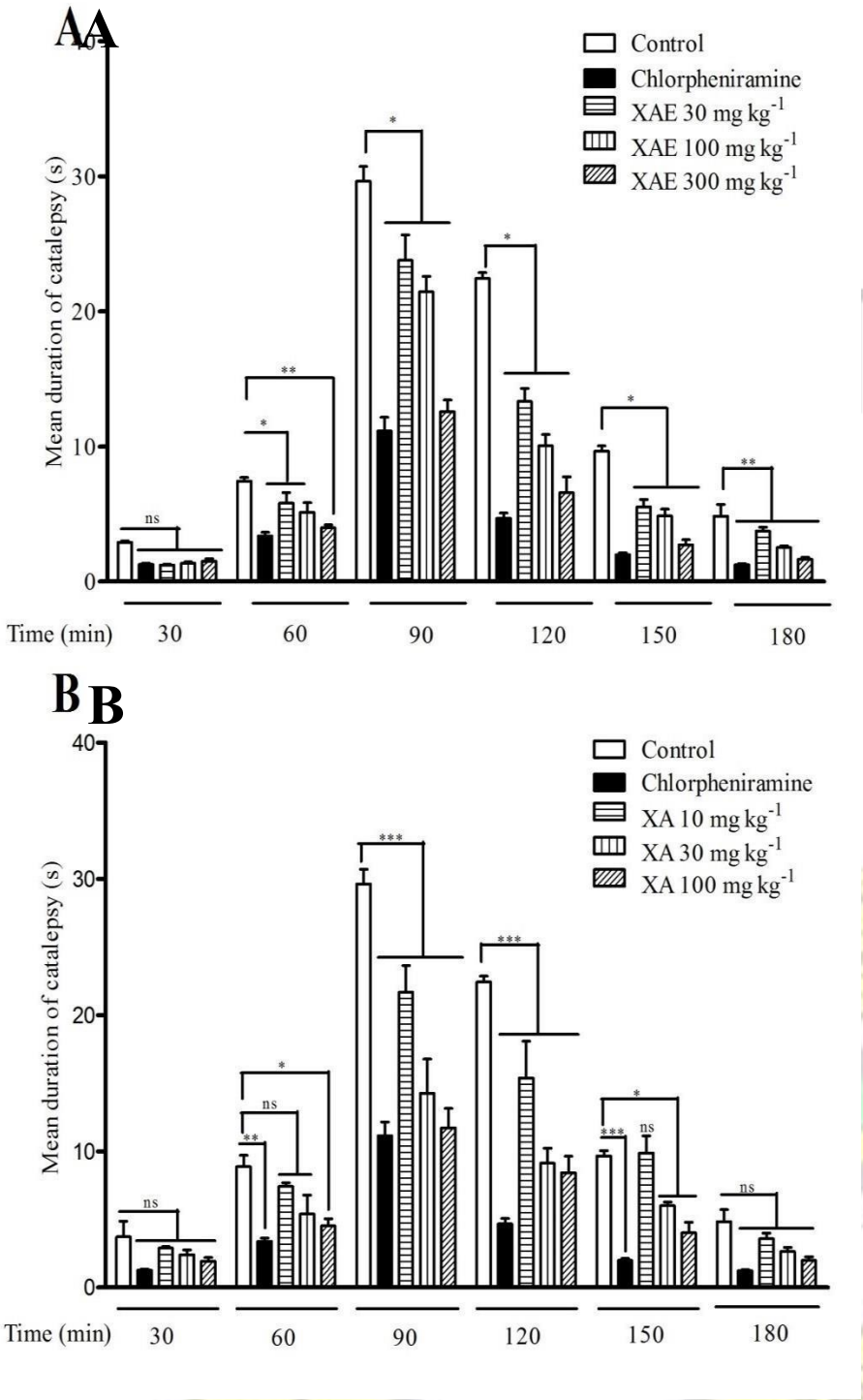



**Figure 6.13. Effect of *X. aethiopica* and xylopic acid administered therapeutically on clonidine-induced catalepsy in mice.** ICR mice (25 - 30 g) received clonidine 1 mg kg$^{-1}$, *s.c.* and their forepaws placed on a horizontal bar (1 cm in diameter, 3 cm above the table). The vehicle (5 ml kg$^{-1}$), XAE (30, 100, 300 mg kg$^{-1}$), XA (10, 30, 100 mg kg$^{-1}$) or chlorpheniramine (10 mg kg$^{-1}$) was given orally 1 h after induction of catalepsy. The duration of catalepsy was measured at 30 min intervals up to 3 h after administration of clonidine. Values are Mean ± SEM. (n = 5). Significance between vehicle and drug/extract-treated mice denoted by *$P \leq$ 0.05, **$P \leq 0.01$ and *** $P \leq 0.01$, $^{ns}P > 0.05$.

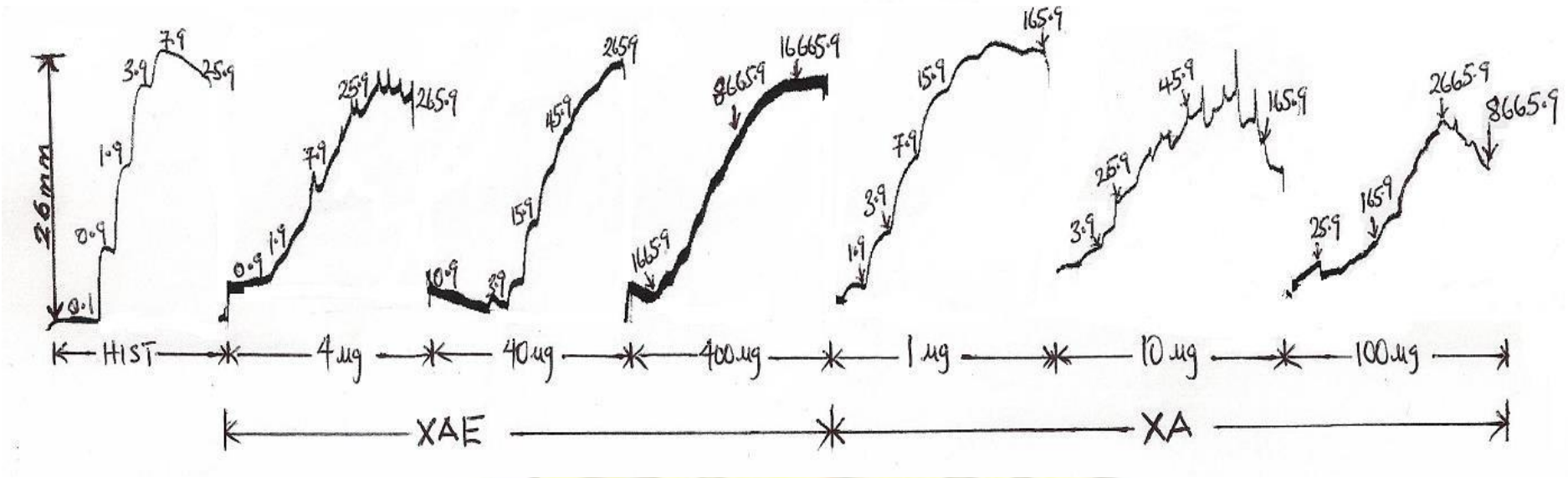
### *6.3.3 In vitro studies*

#### *6.3.3.1 Studies on isolated guinea pig ileum preparation*

Guinea pig ileum is a known sensitive tissue for studying the effect of histamine on $H_1$ receptors. In this study, overnight fasted guinea pigs were sacrificed by cervical dislocation and the ileum was quickly dissected out and mounted in an organ bath maintained at 32 ± 0.5°C and containing 20 ml Tyrode's solution under basal tension of 10 mN.

Cummulative dose-response curves for histamine were obtained (Fig 6.14 A) and repeated in the presence of XAE or XA. From the tracing, XAE exerted antagonistic effect on histamine-induced contraction of smooth muscles. There was a parallel shift of the histamine response in the presence of XAE at 4, 40 and 400 µg, indicating a competitive antagonism (Figs 6.14 B, 6.14 C and 6.14 D) respectively. Similarly, XA exerted antagonistic effect on histamine-induced contraction at 1, 10 and 100 µg with a parallel shift of the histamine response in the presence of XA indicating a competitive antagonism (Figs 6.14 E, 6.14 F and 6.14 G).

From the schild analysis, the slope of approximately 1 was obtained, representing a competitive antagonism by XAE. The $pA_2$ value was calculated to be 4.40 which implies that XAE acts as an antagonist of low potency on the $H_1$ receptor (Fig 6.15 A and 6.15 B). Likewise, XA gave a slope of approximately 1 in the schild analysis to confirm its competitive antagonism. The $pA_2$ was obtained to be 4.16 suggesting a low potency on the histaminic receptor on the guinea pig ileum (Figs 6.15 C and 6.15 D).




**A** **B** **C** **D** **E** **F** **G**

**Figure 6.14. Kymograph of isolated guinea pig ileum contraction in response to histamine.** Isolated guinea pig ileum preparation was set up as described earlier. The contractile responses of ileum to histamine (0.00001 – 0.4 mg $ml^{-1}$) in the absence and presence of XAE (4 – 400 µg) and XA (1 – 100 µg) were recorded. Representative tracing are shown: Histamine (A), histamine + XAE 4 µg (B), histamine + XAE 40 µg (C), histamine + XAE 400 µg (D), histamine + XA 1 µg (E), histamine + XA 10 µg (F) and histamine + XA 100 µg (G). [Doses indicated on tracing are selected concentrations $x10^{-6}$ mg $kg^{-1}$]

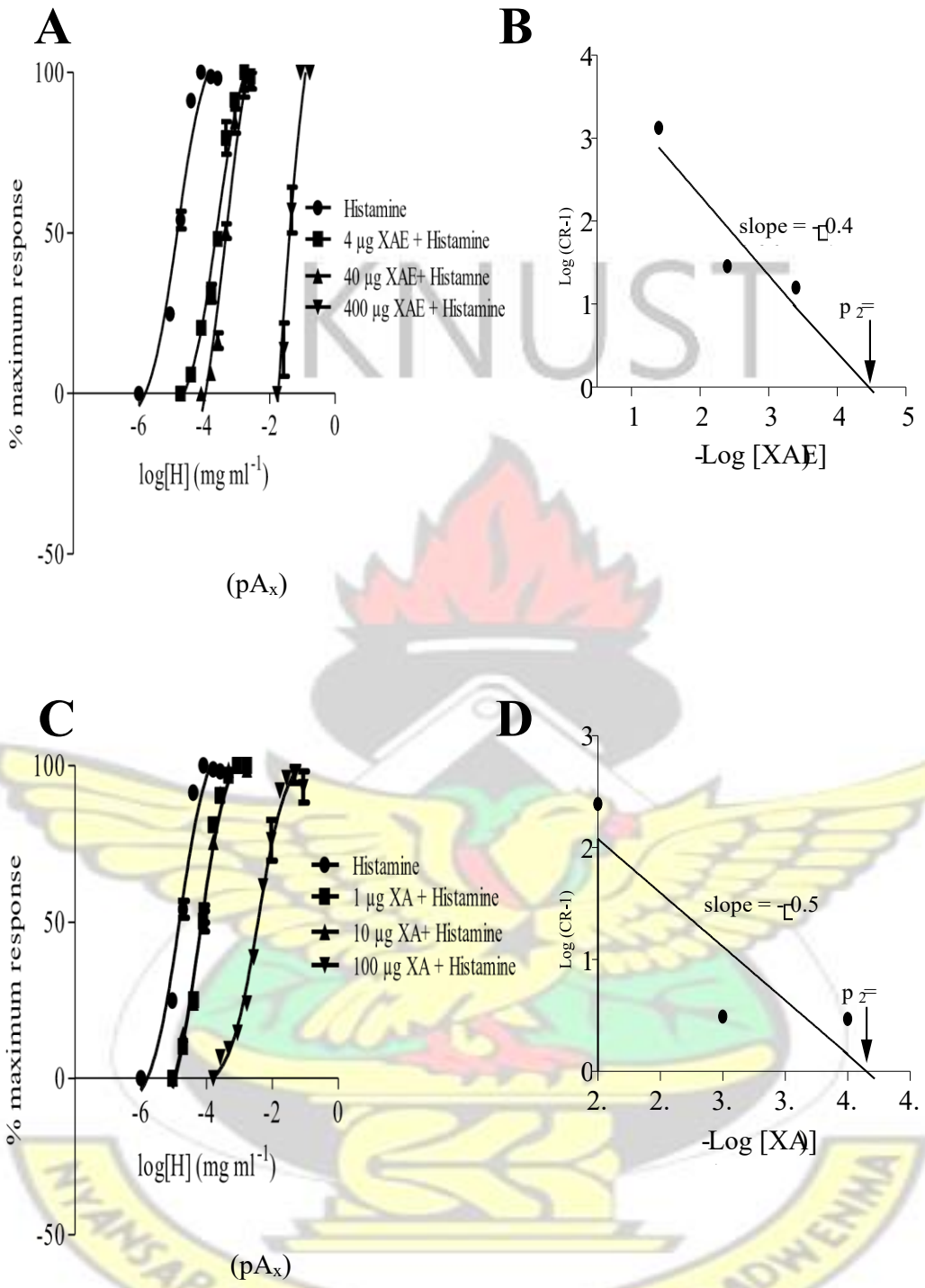



**Figure 6.15. Effect of *Xylopia aethiopica* extract and xylopic acid on *in vitro* histamineinduced contractions on isolated guinea pig ileum preparation.** Isolated guinea pig ileum preparation was set up as described earlier. The contractile responses of ileum to histamine (0.00001 – 0.4 mg $ml^{-1}$) in the absence and presence of either **A.** XAE (4 – 400 µg) were recorded. **B.** Schild plot for the antagonism of histamine-dependent contractions by XAE in the guinea pig isolated ileum, **C.** XA (1 – 100 µg) were recorded. **D.** Schild plot for the antagonism of histamine-dependent contractions by xylopic acid in the guinea pig isolated ileum

#### *6.3.3.2 Gel electrophoresis of serum proteome*

SDS-PAGE in combination with Coomassie brilliant blue G-250 staining is a useful means of determining qualitatively the levels of protein in a given sample. The mobility of a protein is a function of length, conformation and charge on the molecule (Rath *et al.,* 2007). The separated proteins and polypeptides can then be identified with their molecular weights and the band densities quantified using appropriate software.

In this study, colitis was induced with acetic acid and colon subjected to SDS-PAGE. The separated proteins were identified by molecular weight (Fig 6.16 A). The expression of intercellular adhesion molecule 1 (ICAM-1, Mwt~ 60 kDa) were quantitated from the band densities (Fig 6.16 B). Treatment with acetic acid caused an increased expression of ICAM-1 when compared with the non-colitic group (compare lanes 2 and 4, Figs 6.16 A and 6.16 B). The expression of ICAM-1 was significantly decreased when colitic rats were treated with sulphasalazine (compare lanes 3 and 4, Figs 6.16 A and 6.16 B). Upon XAE (30, 100, 300 mg $kg^{-1}$) treatment, there was a significant and dose-dependent reduction of colon expression of ICAM-1 evident of the inhibitory effect on adhesion molecules expression during the chronic inflammatory process when compared with the colitic control (compare lanes 5, 6 and 7 to lane 4, Figs 6.16 A and 6.16 B). Similarly, XA treatment significantly and dose-dependently reduced the colon expression of ICAM-1 at doses 10, 30 and 100 mg $kg^{-1}$ indicative of the inhibitory effect on the expression of ICAM-1 (compare lanes 8, 9 and 10 to lane 4, Figs 6.16 A and 6.16 B).

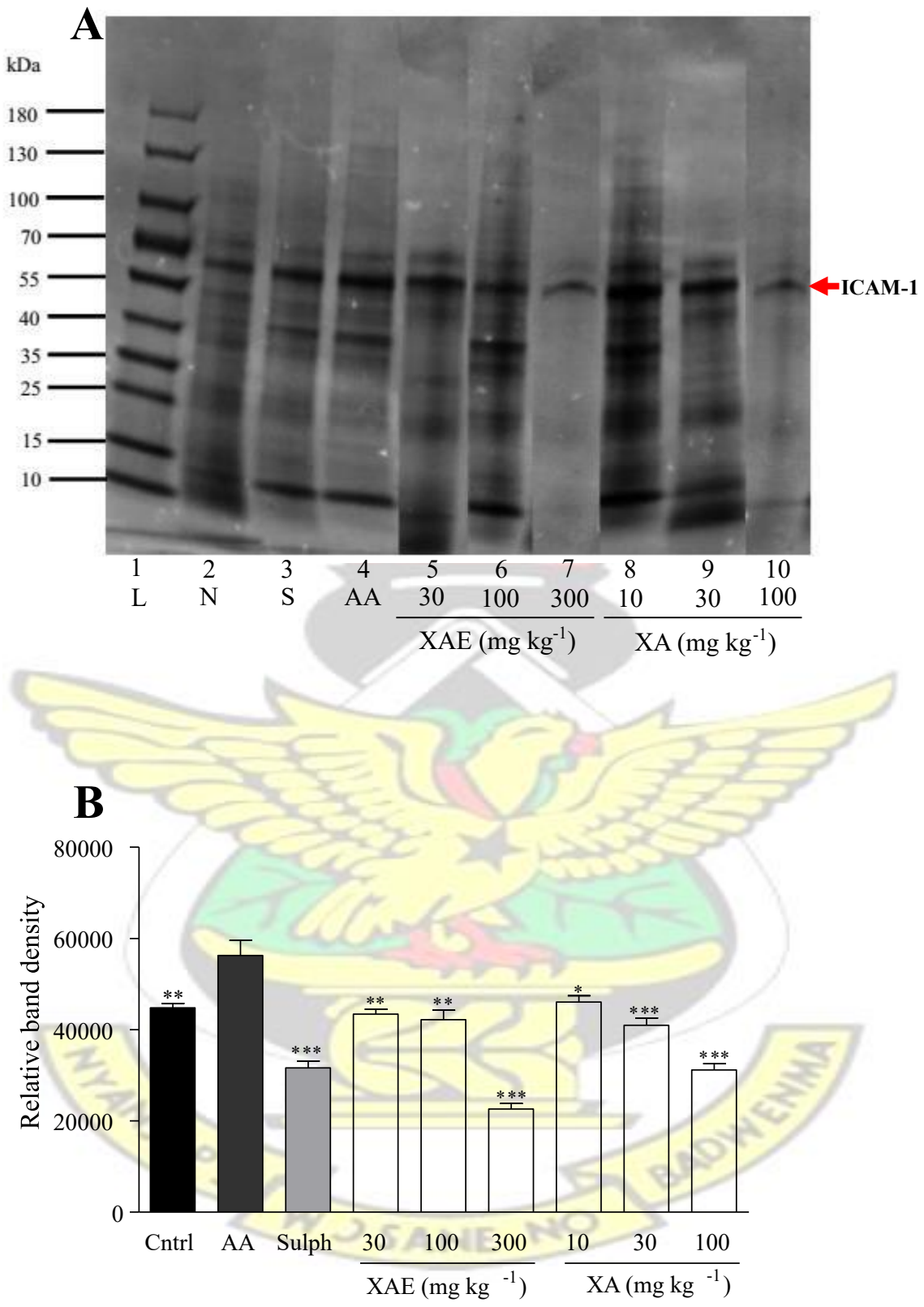



**Figure 6.16. Expression of ICAM-1 in acetic acid-induced colitic rats.** Colitis was induced as described earlier. Normal saline, sulpasalazine (500 mg $kg^{-1}$), XAE (30 - 300 mg $kg^{-1}$) or XA (10 – 100 mg $kg^{-1}$) was given for 8 days. Colons were homogenized and supernatant subjected to SDS-PAGE. The colon expression of ICAM-1 (Mwt~60 kDa) was quantified using image J.

Lane 1: ladder; 2: normal saline-treated control; 3: sulphasalazine-treated control; 4: acetic acid-treated control; 5 - 7: XAE (30, 100 and 300 mg $kg^{-1}$) respectively; 8 – 10: XA (10, 30 and 100 mg $kg^{-1}$) respectively. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$

### *6.3.4 Serology*

#### *6.3.4.1 Serum liver function*

Changes in the liver function profile can be attributed to certain diseased states through higher than normal levels of some of these liver enzymes. Those worth noting include *aspartate aminotransferase* (AST), *alkaline phosphate* (ALP), *alanine aminotransferase* (ALT). Other liver function indicators that signal pathological condition include bilirubin levels, cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and coronary risk.

For this test, the full serum biochemistry were determined using sera obtained at the end of the arthritis study in XA-treated rats. Arthritic control rats showed no change in the activity of ALP and concentrations of LDL and TGL, reduced activity of AST and ALT and concentrations of HDL, total bilirubin and cholesterol with an increased coronary risk when compared with the non-arthritic rats (Table 6.1). Prophylactically, when rats were treated with aspirin, there were significant elevation in activity of AST and ALT and levels of HDL and total bilirubin when compared with arthritic control rats with a reduced coronary risk. There was however no significant change in activity of ALP and serum concentration of LDL, TGL and cholesterol expression. Serum tests showed no significant increase in activity of ALP. There was however increased activity of AST and ALT with increased concentration of total bilirubin when compared with CFA but a significantly reduced activity of ALP and serum concentration of cholesterol at 30 mg $kg^{-1}$; LDL at 100 mg $kg^{-1}$ and coronary risk and TGL at 10 – 100 mg $kg^{-1}$ XA (Table 6.1).

In the therapeutic protocol, aspirin-treated rats showed no significant increase in the activity of ALP and serum concentration of LDL, total bilirubin and TGL. There was a reduced cholesterol level with a reduced coronary risk when compared with the arthritic control rats. There was however a significant elevation in the activity of AST and ALT. Also, treatment with XA resulted in the elevation in the concentration of HDL, TGL, total bilirubin and cholesterol and increased activity of AST and ALT with a reduced coronary risk and LDL. There was however no significant change in the ALP activity (Table 6.1).



**Table 6.1. Effect of xylopic acid treatment on liver function parameters in adjuvant-induced arthritic rats**

| Treatment | ALP | ALT/GPT | AST/GOT | Bil total | Chol | HDL | CR | LDL | TGL |
|---|---|---|---|---|---|---|---|---|---|
| CFA | > 900 | 113.10± 5.01 | 73.40 ± 0.10 | 0.90 ± 0.05 | 1.32 ± 0.27 | 0.38 ± 0.16 | 4.80 ± 1.25 | 0.47 ± 0.18 | 1.04 ± 0.41 |
| IFA | > 900 | 184.75 ± 0.45* | 225.95 ± 0.15* | 2.33 ± 0.08* | 1.71 ± 0.02* | 0.87 ± 0.05* | 2.27 ± 0.00* | 0.47 ± 0.18 | 1.04 ± 0.41 |
| Aspirin (prophylaxis) | > 900 | 141.35 ± 5.91* | 150.31 ± 4* | 1.05 ± 0.39 | 1.52 ± 1.12* | 0.51 ± 0.02* | 4.21 ± 1.08* | 0.55 ± 0.17* | 1.03 ± 0.51 |
| Aspirin (therapeutic) | > 900 | 138.50 ± 4.59* | 147.60 ± 5* | 1.10 ± 0.27 | 1.37 ± 1.35 | 0.43 ± 0.13* | 4.40 ± 1.50* | 0.51 ± 0.11 | 0.96 ± 0.08 |
| Prophylactic XA treatment | | | | | | | | | |
| 10 | > 900 ± 0.00 | 185.25 ± 10.55* | 169.85 ± 11.35* | 2.15 ± 0.05* | 1.72 ± 0.07* | 0.67 ± 0.02* | 3.60 ± 0.10* | 0.65 ± 0.04* | 0.92 ± 0.20* |
| 30 | 825 ± 8.0* | 122.60 ± 5.30* | > 250* | 1.70 ± 0.13* | 1.25 ± 0.08* | 0.58 ± 0.01* | 3.0 ± 0.05* | 0.43 ± 0.00 | 0.51 ± 0.00* |
| 100 | 891 ± 9.00 | 213.70 ± 4.80* | 243.25 ± 6.75* | 2.35 ± 0.65* | 1.39 ± 0.15 | 0.60 ± 0.04* | 3.20 ± 0.10* | 0.32 ± 0.09* | 1.02 ± 0.03 |

Legend on next page





| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Therapeutic XA treatment | | | | | | | | | | |
| 10 | | > 900 | 183.65 ± 0.30* | 0.15* | 0.03* | > 250* | 1.60 ± 0.20* | 1.53 ± 0.19* | 0.59 ± 0.14 | 3.60 ± 0.44 ± 1.11 ± 18.05* |
| 30 | > 900 | 192.30 ± 10.13* | 185.80 ± 13.10* | 1.90 ± 0.13* | | 1.59 ± 0.32* | 0.60 ± 0.01* | 3.60 ± 0.00* | 0.38 ± 0.06* | 1.33 ± 0.23* |
| 100 | > 900 | > 250* | > 250* | 2.10 ± 0.60* | | 1.43 ± 0.27* | 0.57 ± 0.11* | 3.45 ± 0.05* | 0.43 ± 0.10* | 0.95 ± 0.13 |

Sprague-Dawley rats (200 – 250 g) were injected intraplantar with 100 µl of Incomplete Freund's adjuvant or Complete Freund's adjuvant (CFA) into the right hind paw. The drug vehicle XA 10, 30 and 100 mg $kg^{-1}$, were given orally 1 h before the induction of the arthritis and daily for 28 days in the prophylactic protocol; and administered from day 14 to day 28 in the therapeutic protocol. Rats were euthanised and blood samples collected from the jugular vein on day 29. Sera were obtained and serum analysis done.*$P < 0.0001$, $^{ns}P > 0.05$ when compared to the arthritic control group (CFA). Note. ALP, alkaline phosphate; ALT/GPT, *alanine aminotransferase*; AST/GOT, *aspartate aminotransferase*; Gamma GT, *gamma-glutamyl transpeptidase*; Bil, bilirubin; Chol, cholesterol; HDL, high-density lipoprotein cholesterol; CR, coronary risk; LDL, low-density lipoprotein cholesterol; TGL, triglycerides.

### *6.3.4.2 Assay of cytokine (IL-6 and TNF-α) levels*

At the molecular level, the array of cytokines manifested to play key roles in inflammatory development and bone erosion of rheumatoid arthritis include TNF-α which is an autocrine stimulator and a potent paracrine inducer and thus increases the expression of other inflammatory mediators such as IL-1β, $PGE_2$ and NO (Feldmann and Maini, 2008) and interleukin-6 (IL-6) to mediate and/or amplify their effects in peripheral organs (Cawthorn and Sethi, 2008).

In light of this, the serum expression of IL-6 and TNF-alpha were determined by employing a sandwitched enzyme-linked immunosorbent assay at the end of the adjuvant-induced arthritis study in rats. CFA control rats showed an increase in the levels of cytokines IL-6 and TNF-alpha in both treatment protocols when compared to their levels in the IFA rats (Figs 6.17 A – D). Serum IL-6 and TNF-alpha levels in the CFA control rats increased from 172.50 ± 41.59 pg $ml^{-1}$ to 512.60 ± 46.43 pg $ml^{-1}$ and 406.80 ± 60.50 pg $ml^{-1}$ to 7741.00 ± 125.90 pg $ml^{-1}$ upon the induction of arthritis respectively. With prophylactic treatment with XA, there was a significant reduction in the serum expression of IL-6 at 30 and 100 mg $kg^{-1}$ (Fig 6.17 A) and reduction of the TNF-alpha expression at 10, 30 and 100 mg $kg^{-1}$ (Fig 6.17 C).

Similarly, therapeutic administration of XA (10 – 100 mg $kg^{-1}$) resulted in the decreased serum expression of IL-6 (Fig 6.17 B). Also, the expression of TNF-alpha was significantly reduced with XA treatment in rats (Fig 6.17 D).

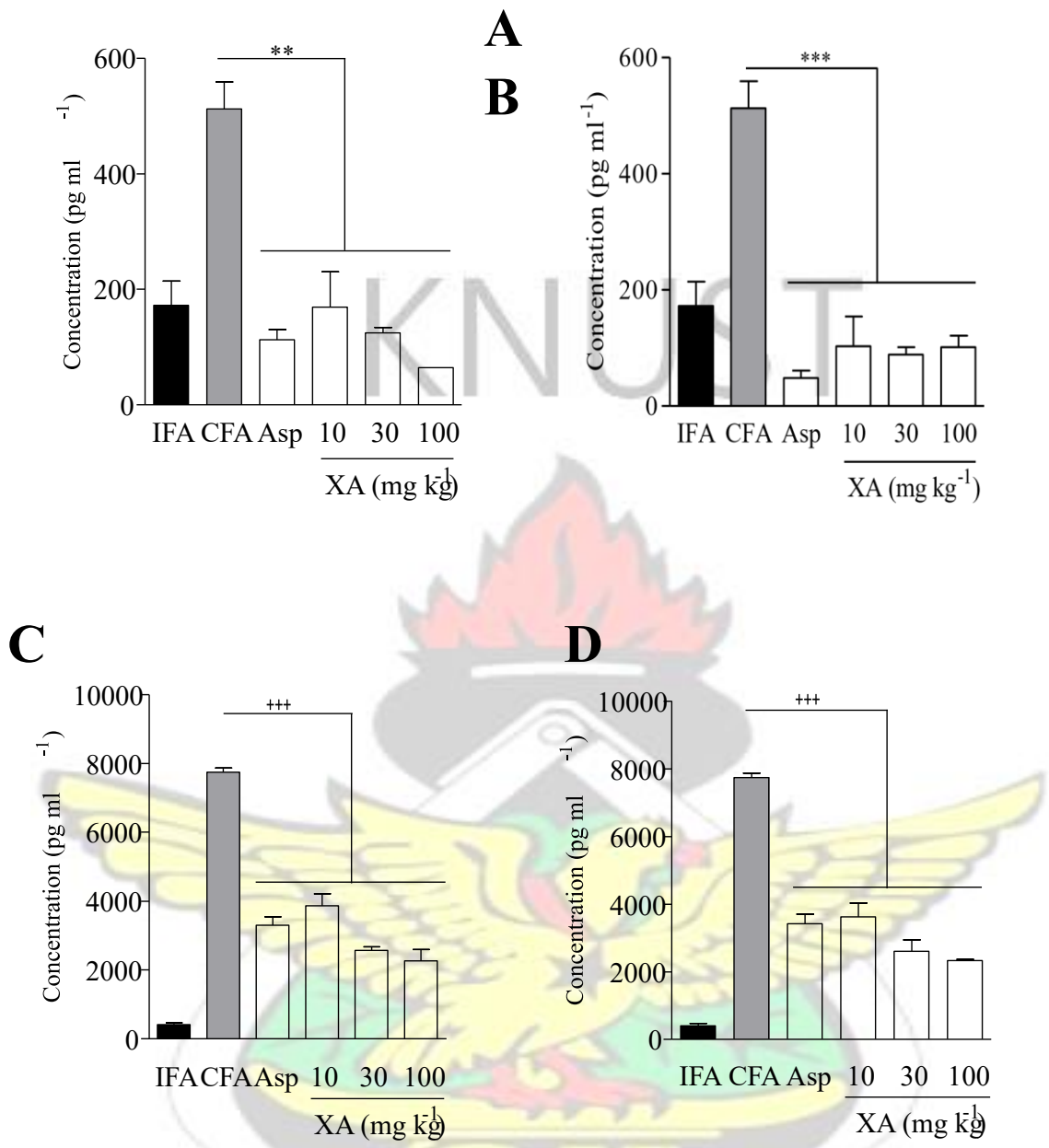



**Figure 6.17. Effect of xylopic acid on the serum expression of IL-6 and TNF-α in adjuvantinduced arthritic rats.** Arthritis was induced as described earlier. The drug vehicle, XA (10, 30, 100 mg $kg^{-1}$) or aspirin 100 mg $kg^{-1}$, was given orally 1 h before the induction in prophylactic protocol [A, C]; and from day 14 in therapeutic protocol [B, D] and daily till day 28. The expression of IL-6 and TNF-α in the sera of adjuvant-induced arthritic rats were examined using IL-6 (Interleukin-6) and TNF alpha Rat ELISA Kit respectively, according to the manufacturer's recommendations. The total amount of IL-6 levels in the sera were expressed as Mean ± SEM. Assay of serum IL-6 [top panel]. Assay of serum TNF alpha [bottom panel]. $^{**}P < 0.0012$, $^{***}P < 0.0006$, $^{+++}P < 0.0001$ [Newman-Keul's multiple comparison test].

## 6.4 DISCUSSION

Involvement of histamine in allergy and inflammation is very significant (Bachert *et al*., 1998). Anti-histaminic drugs that have $H_1$ receptor blocking effect have anti-allergic and anti-inflammatory effects and do inhibit histamine release from mast cells (LeviSchaffer, 2009). Chopra and Dandiya (1975) identified that each stage of catalepsy corresponded with given brain levels of histamine. Lakadwala *et al*., (1980) have shown that clonidine releases histamine from mast cell in a similar manner to a selective mast cell degranulator such as compound 48/80. Jadhav *et al*. (1983) and Lakadwala *et al*. (1980) have also showed that clonidine, a $\alpha_2$-adrenoreceptor agonist induces dosedependent catalepsy in mice which is inhibited by histamine $H_1$ receptor antagonist but not by $H_2$ receptor antagonist. Consistent with the findings in this study about the inhibitory effect of XAE and XA, Dhanalakshimi *et al.* (2004) showed that extracts having anti-histaminic or mast cell stabilizing effect inhibit clonidine-induced catalepsy. Clonidine releases histamine from mast cells which is responsible for different asthmatic conditions. Catalepsy produced by clonidine is mediated by histamine via $H_1$ receptor and hence can be blocked by $H_1$ receptor antagonist such as the extract and xylopic acid.

Different agonists like acetylcholine, histamine, 5_hydroxytryptamine (serotonin) and bradykinin are responsible for contractile responses (Sheth *et al.,* 1972). In isolated guinea pig ileum preparation, there is a right side shift of dose response curve of histamine in the presence of the extract or xylopic acid. Histamine antagonism modulated by the endothelial relaxing factors involved and may be due to the suppression of histamine $H_1$-receptor (Shi *et al.,* 2001). Histamine and serotonin are potent vasodilators which increase vascular permeability and play an important part in the development of inflammation (Vasudevan *et al*., 2007). XAE and XA showed

histaminic receptor blocking activity which was competitively expressed though they exhibited low potency, per the obtained $pA_2$ values (Salmon, 2014), histaminic receptor blockade and also prevented development of serotonin-induced inflammation.

Bradykinin is a nonapeptide which is released in the early stages of tissue damage and evoke the cardinal signs of inflammation. Its production is as a results of a cascade of biochemical reactions from very large molecular weight precursors (kininogens) through the action of kallikreins (Colman, 1986). The varied effect of bradykinin on different cell types are mediated by $B_1$ and $B_2$ receptors (Roberts, 1989; Burch *et al.,* 1990) though its $B_2$ effect appears to be responsible for its most relevant biological actions (i.e. pain and inflammation). Several properties of kinins point to their involvement in the inflammatory process; by inducing vasodilation, enhancing vascular permeability, inducing pain, stimulating the synthesis and release of arachidonic acid metabolites and growth factors and up-regulate the immune responses which can eventually result in tissue damage (Regoli and Barabe, 1980; Proud and Kaplan, 1988; Marceau *et al.,* 1983). Bradykinin has been reported to stimulate the production of prostacyclin and $PGE_2$ from fibroblast and endothelial cells (Bareis *et al.,* 1983; Conklin *et al.,* 1988) with other report indicating their ability to release IL-1 and TNFalpha from macrophages (Tiffany and Burch, 1989; Burch *et al.,* 1989). The present study showed the *X. aethiopica* extract and xylopic acid's ability to reduce inflammation induced with bradykinin signifying their ability to affect the bradykinin involvement in the development of inflammatory response. This is achieved possibly through their ability to affect the $B_2$ receptor activity of the exogenously administered bradykinin.

XAE and XA were able to suppress prostaglandin $E_2$-induced inflammation possibly by interfering with the activity of $PGE_2$ on its cognate receptors, E prostanoid receptors 1-4 (EP1-4) as supported by reports in literature. For example, prostaglandins syntheses usually are low in uninflamed tissues but their expression increase in acute inflammation prior to the recruitment of leukocytes and the infiltration of immune cells (Ricciotti and FitzGerald, 2012). They exert their effects by activating rhodopsin-like seven transmembrane spanning G protein-coupled receptors. $PGE_2$ is one of the most abundant of the prostaglandins and is of particular interest because it is involved in all processes leading to the classic signs of inflammation: redness, swelling and pain. Redness and oedema result from increased blood flow into the inflamed tissue through $PGE_2$-mediated increase of arterial dilatation and increased vascular permeability (Funk, 2001).

XAE and XA inhibition of $H_2S$-induced inflammation therefore suggests the possible effect on secreted phospholipase $A_2$ ($sPLA_2$) activation and eventually may inhibit *cyclooxegenase* enzymatic action on arachidonic acid hence affects the sysnthesis of eicosanoids. Consistent with this, Landucci *et al*. (2000) and Thimmegowda *et al*. (2007) reported that $H_2S$-induced oedema has a similar profile to that of $PLA_2$-induced oedema and its isoform $sPLA_2$ plays a major role in the inflammatory process. The activation of $sPLA_2$ proceeds through the orientation of a water molecule by hydrogen bonding to the active site histidine. Adjacent to this histidine, there is a conserved aspartate residue, which, together with a $Ca^{2+}$-binding loop, acts as a ligand cage for $Ca^{2+}$ (Murakami and Kudo, 2002). Thus, $Ca^{2+}$ and water are two key elements implicated in $PLA_2$ activation. Interestingly, $H_2S$ could provide both elements necessary for $PLA_2$ activation. $H_2S$ has a three-dimensional structure close to that of

$H_2O$, but weaker intermolecular forces, and $H_2S$ also induced entry of extracellular $Ca^{2+}$ (Zhao and Wang, 2002). Thus, it is feasible that $H_2S$ could activate $PLA_2$, either through $Ca^{2+}$ entry and/or substituting for a molecule of $H_2O$. Additionally, oxidative modification of phospholipids can alter the physiological state of the membrane, which in turn affects the susceptibility of oxygenated and non-oxygenated fatty acid residues towards $sPLA_2$ attack in a multifaceted way (Murakami and Kudo, 2002). Therefore, an alternative or additional explanation for $PLA_2$ activation by $H_2S$ could be that $H_2S$, being a reducing agent, may alter the cellular redox status, triggering prostaglandin production. Moreover, because the isoform of $PLA_2$ involved in the inflammatory process depends on the type of inflammation considered, $H_2S$ could interact with both secretory and cytosolic $PLA_2$, without distinguishing between the isoforms. It has henceforth been shown from earlier study that the pro-inflammatory action of $H_2S$ in mouse hind paw involves $PLA_2$ activation with a consequent increase in $PGE_2$ levels. This effect appears to be independent of preformed mediators such as histamine and 5hydroxtryptamine.

AST and ALT are recognised as non-specific indicators of acute hepatocellular injury with their high plasma levels possible indication of hepatobiliary disease (Aida, 1993). The treatment with XA did not alter AST and ALT significantly when compared with levels expressed in non-arthritic animals indicative of their inhibitory effect on development of hepatocellular injury. However, this argument may not be credible for this study since markers of hepatocellular injury, such as ALP and AST, were not significantly increased in arthritic rats.

Mostly, inflammatory responses begin with infiltration of neutrophils and macrophages (Hanauer, 2006) with the activated macrophages producing a combination of broadly

active inflammatory cytokines which includes TNF-alpha and IL-6 as well as cell adhesion molecules such as intracellular adhesion molecule 1, ICAM-1 (Podolsky, 2002). For example, while *in vitro* experiments have shown IL-1β potently induces matrix *metalloproteinases* generation and osteoclasts activation (Barksby *et al.,* 2007) directly resulting in bone erosion, evidences also exist for the potential degradation of cartilage and bone by TNF-α (Zwerina *et al.,* 2006). It is also believed that TNF-alpha stimulates fibroblast to express adhesion molecules, including ICAM-1 (Zhang *et al.,* 2015). Therefore inhibiting the production and function of pro-inflammatory mediators is thought to be an effective method to treat RA. The extract and xylopic acid suppressed the expression of the intracellular adhesion molecules while XA was identified to suppress the expression of IL-6 and TNF-alpha in chronic inflammation.

### 6.5 CONCLUSION

Taken together, XAE and XA have anti-histaminic activity with inhibition of serotonin, bradykinin and prostaglandin-mediated inflammatory process and do have inhibitory effect on the arachidonate pathway. They also have inhibitory effect on the expression of pro-inflammatory cytokines such as IL-6 and TNF-alpha as well as expression of cellular adhesion molecules such as ICAM-1.

## *Chapter 7*

## GENERAL DISCUSSION

In the current study, the aqueous ethanol extract of the dried fruit of *Xylopia aethiopica* and xylopic acid, the principal product from its bio-fractionation, demonstrated antiinflammatory activity in both acute and chronic models of study due to their effect

on the action of pro-inflammatory mediator (histamine, serotonin, bradykinin and prostaglandin), free radical generation and the expression of pro-inflammatory cytokines and cell adhesion molecules. The extract and xylopic acid elicited these effects possibly through similar mechanisms and affected diverse pathways of inflammation.

Catalepsy in animals can be induced by several drugs. Chopra and Dandiya (1975) identified the role played by acetylcholine and histamine in perphenazine-induced catalepsy and postulated the anticholinergic activity of antidepressant might be due to its increase in dopamine levels in the brain or their ability to inhibit the action of acetylcholine (Chopra and Dandiya, 1975). Irrespective of this finding, a defect in the function of acetylcholine (Klemm, 1985), γ-amino butyric acid (Somani *et al.*, 1999; Ghaisas *et al.,* 2008) and serotonin (Silva *et al.,* 1995) have been identified to result in catalepsy. In addition to these dysfunctional causes, many clinical and preclinical studies have also pointed out the involvement of reactive oxygen species in catalepsy (Polydoro *et al.,* 2004) which gives credence to the role of the extract and xylopic acid in catalepsy.

*Xylopia aethiopica* fruit extract and xylopic acid administration resulted in a decreased ability to develop type III hypersensitivity reaction to bovine serum albumen. This indicates a possible inhibitory effect on some stages in the phospholipid/arachidonic acid cascade system and/or as a result of their inhibitory effect on cyclooxygenase. Lipopolysaccharide activates Toll-like receptor 4 (TLR4) which results in downstream expression of transcription factors NF-κB and Activator protein 1 (AP1) (Newton and Dixit, 2015). Henceforth, the inhibitory effect of the extract and xylopic acid on septic shock induced by the administration of *E. coli* (source of LPS), signifies their inhibitory

effect on the cascade of events that lead to the expression of the above mentioned transcription factors and eventual expression of pro-inflammatory mediators. The observation is informed by the known fact that kaurenoic acid and linearol, known kaurane diterpenes, inhibit prostaglandin $E_2$ synthesis, NF-κB activation and COX-2 (Ran *et al.,* 2011).

*Xylopia aethiopica* fruit extract and xylopic acid possibly inhibit the histamine release from the mast cells through any step along the cascade of events leading to mast cell degranulation initiated by compound 48/80. Compound 48/80 has been documented to increase permeability of the lipid bilayer membrane by causing membrane perturbation (Tasaka *et al.,* 1986). XAE and XA may inhibit histamine release by lessening the permeability of mast cell membranes by preventing compound 48/80 binding which could have resulted in membrane perturbation. Again, the mouse reaginic antibody that binds to mast cell (Konig *et al.,* 1974) is possibly inhibited by the treatment with XAE and XA.

The extract and xylopic acid affect the arachidonate pathway of inflammation as was realised from the $H_2S$-induced inflammation study. The anti-allergic and antiinflammatory activities of the extract and xylopic acid were further established with their inhibitory role on histamine-induced oedema and further confirmation by their competitive histaminic ($H_1$) receptor antagonism using isolated guinea pig ileum though they were also identified to have weak binding ability to the $H_1$-receptor. Their inhibitory effect on oedema development in the bradykinin and prostaglandin $E_2$induced inflammation further confirmed their anti-inflammatory activity.

The anti-inflammatory activity of the extract and xylopic acid are also related to their anti-oxidant activity which helps reduce the generation of reactive oxygen species *in*

*vivo*. This was achieved by reducing activity of MPO which contributes to lipid peroxidation; and increasing activities CAT and APx which are involved in the glutathione-ascorbate cycle to remove peroxides (Noctor and Foyer, 1998). These contribute to the extract and xylopic acid inhibition of oxidative damage and eventual reduction in inflammation. Again, the lipopolysaccharide interaction with macrophages results in the generation of oxygen-derived free radicals with resultant oxidative damage to tissues. The anti-oxidant property of *Xylopia aethiopica* has been established by Karioti *et al.* (2004) hence signifying the possible role played by *Xylopia aethiopica* in the inhibition of formation of oxygen-derived free radicals and consistent with this current work.

Not only did the extract and xylopic acid increase the activity of anti-oxidant enzymes, they affected cellular components of the inflammatory process through the inhibition of the rate of cell proliferation during the inflammatory process and inhibited the expression of intracellular adhesion molecule-1 at the site of inflammation. Also, treatment with the aqueous ethanol extract of *Xylopia aethiopica* and xylopic acid decreased the proliferation of nucleolar organizer region. This signifies a reduction in the cellular activity and proliferation with extract and xylopic acid treatment. Upon stimulation, mast cells release a heterogeneous group of factors that promote inflammation and influence immune cell proliferation.

Mast cells accumulate at sites of injury, further suggesting their critical role in the inflammatory process (Egozi *et al.,* 2003). Treatment with XAE and XA reduced the accumulation of mast cell at the site of injury in agreement with the reduced mast cell proliferation seen in XAE and XA treated groups which further supported the extract and xylopic acid's inhibitory effect on cellular activity and proliferation in

inflammation. This contributes to the anti-inflammatory actions of the extract and xylopic acid which is in agreement with earlier findings made by Cavalcanti *et al.* (2009) that identified kaurenoic acid, another kaurane diterpene, to inhibit cell proliferation.

In all the studies, the aqueous ethanol extract of *X. aethiopica* and xylopic acid demonstrated anti-inflammatory action when administered prophylactically and therapeutically. This establishes a genuine mechanism of anti-inflammation. Kaibara *et al.* (1983) could demonstrate that cyclosporine, an immunosuppressive drug, prevented the onset of collagen-induced arthritis in rats; however, when used after the disease had been established, it exacerbated the condition. Again as shown by Larsson *et al.* (1990) limonide, an experimental drug developed against heterologous collagen-induced arthritis, exhibited similar paradoxical effects. These earlier findings strongly suggest that demonstrated anti-inflammatory effect of a drug given prior to the induction of inflammation does not necessarily guarantee the same effect when given after the induction.

Taken together, a proposed mechanism of action is prescribed below:

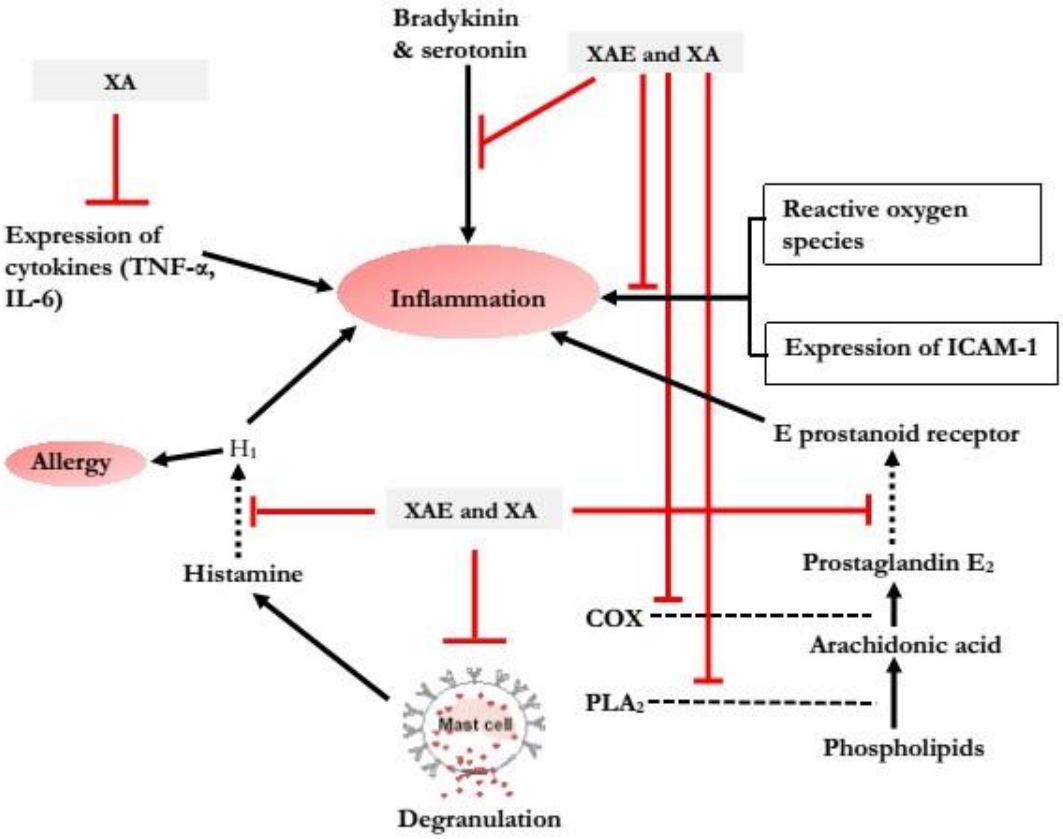



**Figure 7.1. Proposed mechanism of anti-allergic and anti-inflammatory activity of *X. aethiopica* and xylopic acid.** The extract and xylopic acid have inhibitory effect on histaminic, serotonergic, bradykinin and arachidonate pathways of inflammation. They also have inhibitory effect of the generation of reactive oxygen species, *in vivo*, and the expression of intracellular adhesion molecule-1. They exhibit anti-allergic property through the inhibition of mast cell degranulation. Xylopic acid has inhibitory effect on the serum expression of IL-6 and TNF-α.

## CONCLUSION

This study has provided pharmacological evidence to support the folkloric use of *Xylopia aethiopica* in the management of inflammatory conditions. It also provides evidence that the anti-inflammatory activity of the fruit extract is partly due to the presence of the kaurane diterpene, xylopic acid, which is the major constituent obtained by bio-fractionation from the dried fruits.

The established anti-inflammatory activity is identified from this study to be mediated by:

I. The inhibitory effect of the extract and xylopic acid on acute inflammation.

II. The ability of the extract and xylopic acid to stabilise mast cells hence preventing degranulation and release of pre-formed mediators of inflammation.

III. The inhibitory effect on development of hypersensitivity reaction through inhibition of antigen-antibody response and systemic induced sepsis.

IV. The inhibitory effect on histaminic pathway of inflammation generation and competitive antagonism at $H_1$-receptor.

V. The inhibitory effect on arachidonate, bradykinin and serotonergic pathways of inflammatory response

VI. The inhibition of expression of pro-inflammatory cytokines and cellular adhesion molecules during the inflammatory process.

## RECOMMENDATIONS

This study can however not be conclusively extrapolated to human subjects, therefore further works need to be done to improve on the acquired knowledge on the antiinflammatory actions of *Xylopia aethiopica* and xylopic acid.



I. The possible effect on down regulation, or otherwise, of IgE receptor during mast cell activation.

II. The effect on signalling pathways of inflammation such as the MAPK (i.e. extracellular signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinases (JNKs)) pathway could be investigated.

III. Other possible mechanism(s) of the anti-allergic and anti-inflammatory activity should be investigated employing cell line-based approaches using other molecular techniques such as real-time polymerase chain reaction, flow cytometry, western and northern blotting.

## REFERENCES

Abbiw D (1990) Useful Plants of Ghana. Intermediate Technology. Royal Bot. Gard., Kew pp337.

Abboud PA, Hake PW, Burroughs TJ, Odoms K, O'Connor M, Mangeshkar P, Wong HR and Zingarelli B (2008) Therapeutic effect of epigallocatechin-3-gallate in a mouse model of colitis. Eur J Pharmacol 579: 411–417.

Adosraku RK and Kyekyeku JO (2011) Charactarization and HPLC quantification of xylopic acid in the dried fruit of *Xylopia aethiopica*. Int J Pure Appl Chem 6(2): 209-213.

Aebi H (1984) Catalase *in vitro*. Methods Enzymol 105: 121-126.

Agyare C, Obiri DD, Boakye YD and Osafo N (2013) Analgesic Activities of African Medicinal Plants. In: Medicinal plant research in Africa. Elsevier Inc. 726pp.

Aida S (1993). Alkaline phosphatase isoenzyme activities in rheumatoid arthritis: hepatobiliary enzyme dissociation and relation to disease activity. Ann Rheum Dis 52: 511-516.

Alber G, Miller L, Jelsema C, Varin-Blank N and Metzger H (1991) Structure/function relationships in the mast cell high-affinity receptor for IgE (FcεRI): Role of cytoplasmic domains. J Biol Chem 266: 22613–22620.

Aste-Amezaga M, Ma X, Sartori A and Trinchieri G (1998) Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. J Immunol 160: 5936–5944.

Atzeni F and Sarzi-Puttini P (2007) Early rheumatoid arthritis. Reumatismo 59(2): 100117.

Babio N, Bullo M and Salas-Salvado J (2009) Mediterranean diet and metabolic syndrome: the evidence. Public Health Nutr 12(9A): 1607-17.

Bachert C, Virchow CJ and Plenker A (2002) Desloratadine in the treatment of seasonal allergic rhinitis: results of a large observational study. Clin Drug Invest 22 (Suppl. 2): 43–52.

Bandaranayake WM (2006) Quality control, screening, toxicity and regulation of herbal drugs in modern phytomedicine. Turning medicinal plants into drugs eds Ahmad I, Aqil F, Owais M, editors. (Weinheim: Wiley-VCH GmbH & Co. KGaA;) 25-57 10.1002/9783527609987.ch2

Bareis DL, Mangaoiello VC, Hirata F, Vaughan M and Axelrod J (1983) Bradykinin stimulates phospholipid methylation. Calcium influx. Prostaglandin formation and CAMP accumulation in human fibmblasts. Proc Natl Acad Sci 80: 25142518.

Barksby HE, Lea SR, Preshaw PM and Taylor JJ (2007) The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol 149(2): 217–225.

Berg K, Wood-Dauphinee S and Williams JI (1995) The Balance Scale: reliability assessment with elderly residents and patients with an acute stroke. Scand J Rehabil Med 27: 27-36.

Bianca Rd'EdV, Coletta C, Mitidieri E, De Dominicis G, Rossi A, Sautebin L, Cirino G, Bucci M and Sorrentino R (2010) Hydrogen sulphide induces mouse paw oedema through activation of phospholipase $A_2$. Br J Pharmacol 161: 1835–1842.

Binder W and Walker J (1998) Effect of the peripherally selective kappa-opioid asimadoline, on adjuvant arthritis. Br J Pharmacol 124: 647–654.

Bitiren M, Karakilcik AZ, Zerin M, Ozardali I, Selek S, Nazligül Y, Ozgonul A, Musa D and Uzunkoy A (2010) Protective effects of selenium and vitamin E combination on experimental colitis in blood plasma and colon of rats. Biol Trace Elem Res 136: 87-95.

Block LC, Santos AR, de Souza MM, Scheidt C, Yunes RA, Santos MA, Monache FD and Filho VC (1998) Chemical and pharmacological examination of antinociceptive constituents of Wedelia paludosa. J Ethnopharmacol 61: 85–89.

Boakye-Yiadom K, Fiagbe NI and Ayim JSK (1977) Antimicrobial properties of some West African medicinal plants iv. Antimicrobial activity of xylopic acid and other constituents of the fruits of *Xylopia aethiopica* (Annonaceae) Lloydia. 40: 543–5.

Bochner BS and Lichtenstein LM (1991) Anaphylaxis. N. Engl. J. Med 324(25):1785–1790.

Bohndorf K and Schalm J (1996). Diagnostic radiography in rheumatoid arthritis: benefits and limitations. Baillieres Clin Rheumatol 10(3): 399-407.

Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK and Schnitzer TJ (2000) Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. N Eng J Med 343: 1520–1528.

Brand DD (2005) Rodent models of rheumatoid arthritis. Comp Med 55(2): 114-122.

Braunwald E, Fauci AS, Kasper DL, Hauser SL, Longo DL and Jameson JL (2001) Harrisons's Principles of Internal Medicine. 15th ed. McGraw-Hill.

Brenner M, Braun C, Oster M and Gulko PS (2006) Thermal signature analysis as a novel method for evaluating inflammatory arthritis activity. Ann Rheum Dis 65(3): 306-311.

Bresciani LF, Yunes RA, Burger C, De Oliveira LE, Bof KL and Cechinel-Filho V (2004) Seasonal variation of kaurenoic acid, a hypoglycemic diterpene present in *Wedelia paludosa* (Acmela brasiliensis) (Asteraceae). Z Naturforsch C 59: 229–232.

Burch RM, Connor JR and Tiffany CW (1989) The kallikrein- kininogen-kinin system in chronic inflammation. Agents Actions 27: 258-260.

Burch RM, Farmer SG and Steranka LB (1990) Bradykjnin receptor antagonists. Med Res Rev 10: 237-269.

Burkill HM (1985) The Useful Plants of West Tropical Africa. White Friars Press Ltd. Great Britain pp960.

Burton OT and Oettgen HC (2011) Beyond immediate hypersensitivity: evolving roles for IgE antibodies in immune homeostasis and allergic diseases. Immunol Rev 242(1): 128–143.

Cai X, Zhou H, Wong YF, Xie Y, Liu ZQ, Jiang ZH, Bian ZX, Xu HX and Liu L (2007) Suppression of the onset and progression of collagen-induced arthritis in rats by QFGJS, a preparation from an anti-arthritic Chinese herbal formula. J Ethnopharmacol 110 (1): 39–48.

Campanile F, Giampietri A, Grohmann U, Belladonna ML, Fioretti MC and Puccetti P (1996) Evidence for tumor necrosis factor a as a mediator of the toxicity of a cyclooxygenase inhibitor in Gram negative sepsis. Eur J Pharmacol 307: 191199.

Castrillo A, de Las Heras B, Hortelano S, Rodriguez B, Villar A and Bosca L (2001) Inhibition of the nuclear factor kappa B (NF-kappa B) pathway by tetracyclic

kaurene diterpenes in macrophages. Specific effects on NF-kappa B-inducing kinase activity and on the coordinate activation of ERK and p38 MAPK. J Biol Chem 276: 15854–15860.

Cavalcanti BC, Bezerra DP, Magalhães HI, Moraes MO, Lima MA, Silveira ER, Câmara CA, Rao VS, Pessoa C and Costa-Lotufo LV (2009) Kauren-19-oic acid induces DNA damage followed by apoptosis in human leukemia cells. J Appl Toxicol 29(7): 560-568.

Cawthorn WP and Sethi JK (2008) TNF-alpha and adipocyte biology. Feder Eur Biochem Soc Lett 582: 117–131.

Chen P, Li J, Barnes J, Kokkonen GC, Lee JC and Liu Y (2002) Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide stimulated macrophages. J Immunol 169: 6408-6416.

Chi H, Barry SP, Roth RJ, Wu JJ, Jones EA, Bennett AM and Flavell RA (2006) Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase-1 (MKP-1) in innate immune responses. Proc Natl Acad Sci USA 103: 2274–2279.

Choi YH, Chai OH, Han EH, Choi SY, Kim HT and Song CH (2010) Lipoic acid suppresses compound 48/80-induced anaphylaxis-like reaction. Anat Cell Biol 43(4): 317-324.

Chopra YM and Dandiya PC (1975) The relative role of brain acetylcholine and histamine in perphenazine catatonia and influence of antidepressants and diphenhydramine alone and in combination. Neuropharmacol 14: 555-560.

Church MK and Miller P (1975) Simple models of anaphylaxis and of histamine and 5-hydroxytryptamine induced inflammation using the mouse pinna. Brit J Pharmac 55: 315.

Church MK and Miller P (1978) Time courses of the anti-anaphylactic and antiinflammatory effects of dexamethasone in the rat and mouse. Br J Pharmacol 62: 481–486.

Church MK, James GWL and Miller P (1974) The inhibitory effect of insulin on pinnal anaphylaxis in the mouse. Br J Pharmac 52: 454.

Colman RW (1986) ln: Greenbaum LM, Margolius HR, ads. Advances in Experimental Medicine and Biolo. New York: Plenum Vol: l-10.

Conklin BR, Burch RIM, Steranka LR and Alexrod J (1988) Distinct bradykinin receptors mediate stimulation of prostaglandin synthesis by endothelial cells and fibroblasts. Pharm Exp Ther 244: 646.

Cunha FQ, Poole S, Lorenzetti BB and Ferreira SH (1992) The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. Br J Pharmacol 107: 660–664.

Da Silva CA (2006) Stem cell factor expression, mast cells and inflammation in asthma. Fundam Clin Pharmacol 20(1): 21-39.

Daneshmand A, Rahimian R, Mohammadi H, Ejtemaee-Mehr S, Tavangar SM, Babaei Kelishomi R and Dehpour AR (2009) Protective effects of lithium on acetic acid-induced colitis in rats. Dig Dis Sci 54: 1901-1907.

Decker WW, Campbell RL, Manivannan V, Luke A, St Sauver JL, Weaver A, Bellolio MF, Bergstralh EJ, Stead LG and Li JT (2008) The etiology and incidence of anaphylaxis in Rochester, Minnesota: a report from the Rochester Epidemiology Project. J Allergy Clin Immunol 122: 1161-1165.

Dhanalakshmi S, Khaserao SS and Kasture SB (2004) Effect of ethanolic extract of some anti-asthmatic herbs on clonoidine and haloperidol-induced catalepsy in mice. J Orient PharmExperiment Med 4: 1-5.

Dhonde SM, Siraskar BD, Burande MD, Kulkarni AV, Kulkarni AS and Bingl SS (2008) Anti-asthmatic activity of ethanolic extract of stem bark of *Bauhinia variegate* Linn. Ad Pharmacol and Toxicol 9: 131-138.

Edwards SH (2014) Chemical Mediators of Inflammation. Merck Manual.

Egozi EI, Ferreira AM, Burns AL, Gamelli RL and Dipietro LA (2003) Mast cells modulate the inflammatory but not the proliferative response in healing wounds. Wound Repair Regen 11(1): 46-54.

Ekong DE and Ogan AU (1968) Chemistry of the constituents of *Xylopia aethiopica*. The structure of xylopic acid, a new diterpene acid. J Chem Soc C Organic 311–312.

El-Abhara HS, Hammad LNA and Abdel Gawad HS (2008) Modulating effect of ginger extract on rats with ulcerative colitis. J Ethnopharmacol 118: 367–372.

Ennis M, Pearce FL and Weston PM (1980) Some studies on the release of histamine from mast cells stimulated with polylysine. Br J Pharmacol 70: 329–334.

Epperly MW, Sikora CA, DeFilippi SJ, Gretton JE, Bar-Sagi D, Archer H, Carlos T, Guo H and Greenberger JS (2002) Pulmonary irradiation-induced expression of VCAM-I and ICAM-I is decreased by manganese superoxide dismutaseplasmid/liposome (MnSOD-PL) gene therapy. Biol Blood Marrow Transplant 8: 175-187.

Escandell JM, Recio MC, Manez S, Giner RM, Cerda-Nicolas M and Rios JL (2007) Cucurbitacin R reduces the inflammation and bone damage associated with adjuvant arthritis in lewis rats by suppression of tumor necrosis factor-alpha in T lymphocytes and macrophages. J Pharmacol Exp Ther 320(2): 581-590.

Fabia R, Willén R, Ar'Rajab A, Andersson R, Ahrén B and Bengmark S (1992) Acetic acid-induced colitis in the rat: a reproducible experimental model for acute ulcerative colitis. Eur Surg Res 24(4): 211-225.

Feldmann M and Maini SR (2008) Role of cytokines in rheumatoid arthritis: an education in pathophysiology and therapeutics. Immunol Rev 223: 7–19.

Ferre S, Guix T and Prat G (1990) Is experimental catalepsy properly measured? Pharmac Biochem Behav 35: 753-757.

Findlay DM and Haynes DR (2005) Mechanisms of bone loss in rheumatoid arthritis. Mod Rheumatol 15(4): 232-240.

Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871–1875.

Ghaisas MM, Ninave PB, Ganu GP, Zopa VS, Tanwar MB and Deshpande AD (2008) Effect of *Randia dumetorum* Lam on clonidine and haloperidol-induced catalepsy in mice. Pharmacologyonline 2: 42-50.

Gilman AG and Goodman LS (1985) The Pharmacological Basis of Therapeutics, 7th ed. Macmillan Publishing Co. New York 176-210.

Gupta M, Mazumder UK, Gomathi P and Thamilselvan V (2006) Anti-inflammatory evaluation of leaves of *Plumeria acuminate*. BMC Compl Alt Med 6(36):1472-6882.

Hallstrom KN, Srikanth CV, Agbor TA, Dumont CM, Peters KN, Paraoan L, Casanova JE, Boll EJ and McCormick BA (2015) PERP, a host tetraspanning membrane protein, is required for Salmonella-induced inflammation. Cellular Microbiol 1462-5822.

Hammer M, Mages J, Dietrich H, Servatius A, Howells N, Cato AC and Lang R (2006) Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. J Exp Med 203: 15-20.

Hanauer SB (2006) Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. Inflamm Bowel Dis 12 (Suppl 1): S3–S9.

Hartmann RM, Morgan Martins MI, Tieppo J, Fillmann HS and Marroni NP (2012) Effect of *Boswellia serrata* on antioxidant status in an experimental model of colitis rats induced by acetic acid. Dig Dis Sci 57: 2038-2044.

Heath RL and Parker L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stiochiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics 125: 189-198.

Hellman J, Loiselle PM, Tehan MM, Allaire JE, Boyle LA, Kurnick JT, Andrews DM, Sik KK and Warren HS (2000) Outer membrane protein A, peptidoglycanassociated lipoprotein, and murein lipoprotein are released by *Escherichia coli* bacteria into serum. Infect Immun 68: 2566-2572.

Hoffmann JC, Herklotz C, Zeidler H, Bayer B, Rosenthal H and Westermann J (1997) Initiation and perpetuation of rat adjuvant arthritis is inhibited by the anti-CD2 monoclonal antibody (mAb) OX34. Ann Rheum Dis 56(12): 716-722.

Holt PG, Macaubas C, Stumbles PA and Sly PD (1999) The role of allergy in the development of asthma. Nature 402(6760 Suppl): B12–B17.

Hughes P, DeVirgilio J, Humber LG, Chau T, Weichman B and Neuman G (1989) Synthesis and biological evaluation of 4,6-diethyl-1,3,4,5-tetrahydropyrano[4,3-b]indole-4-acetic acid, an isomer of etodolac. J Med Chem 32(9): 2134-2137.

Hwang S, Lam M, Li C and Shen T (1996) Release of platelet activating factor and its involvement in the first phase of carrageenan rat foot oedema. Eur J Pharmacol 120: 33-41.

Irvine FR (1961) Woody Plants of Ghana. Oxford Univ. Press Ltd., London. 863 pp.

Jadhav JH, Balsara JJ and Chandorkar AG (1983) Involvement of histaminergic mechanism in the cataleptogenic effect of clonidine in mice. Pharm Pharmacol 35: 671-673.

Johnkennedy N, Emejulu A, Amaechi A and Chukwunyere NE (2011) Influence of *Xylopia aethiopica* Fruits on Some Hematological and Biochemical Profile. Al Ameen J Med Sci 4(2): 191-196.

Kaibara N, Hotokebuchi T, Takagishi K and Katsuki I (1983) Paradoxical effects of cyclosporin A on collagen arthritis in rats. J Exp Med 158(6): 2007–3015.

Karioti A, Hadjipavolou-Litinna D, Merlin LK, Fleischer TC and Skaltsa H (2004) Composition and Antioxidant Activity of Essential oils of *Xylopia aethiopica* (Dun) A. Rich. (Annonacae) Leaves, Stems, Bark, Root Bark, Fresh and dried Fruits Growing in Ghana. Agric Food Chem 52: 8094–8098.

Katzung BG, Masters SB and Trevor AJ (2012) Histamine, Serotonin, & the Ergot Alkaloids In: Basic and clinical pharmacology 12ed, McGraw Hill USA p275.

Kay (2006) Clinical and Experimental Allergy 36, 2006, “100 years of ‘Allergy’: can von Pirquet’s word be rescued?” pp 555-559.

Khan MR, Wajiha R, Khan GN, Khan RA and Shaheen S (2009) Carbon tetrachlorideinduced nephrotoxicity in rats: protective role of *Digera muricata*. J Ethnopharmacol 122: 91–9.

Kilbourn RC (1990) Reversal of Endotoxin-Mediated Shock by $N^G$-Methyl-LArginine, an inhibitor of Nitric Oxide Synthesis. Biochem Biophysical Res Comm 172(3): 1132-1138.

Kilbourn RC, Gross SS, Jurdan A, Adams J, Griffith OW, Levi R and Lodato RF (1990) $N^G$-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. Proc Natl Acad Sci U.S.A. 87: 3629.

Kimball ES, Wallace NH, Schneider CR, D’Andrea MR and Hornby PJ (2004) Vanilloid receptor 1 antagonists attenuate disease severity in dextran sulphate sodium-induced colitis in mice. Neurogastroenterol Motil 16: 811–818.

Kinet JP (1999) The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. Ann Rev Immunol 17: 931-972.

Kitamura T, Hashimoto J, Murase T, Tomita T, Hattori T, Yoshikawa H and Sugamoto K (2007) Radiographic study of joint destruction patterns in the rheumatoid elbow. Clin Rheumatol 26(4): 515-519.

Klemm WR (1985) Evidence for a cholinergic role in haloperidol-induced catalepsy. Psychopharmacol (Berl) 85: 139-42.

Konig W, Okudaira H and Ishizaka K (1974) Specific binding of mouse IgE with rat mast cells. A Immunol 112: 1652.

Kruidenier L, Kuiper I, Van Duijn W, Marklund SL, van Hogezand RA, Lamers CB and Verspaget HW (2003) Differential mucosal expression of three superoxide dismutase isoforms in inflammatory bowel disease. J Pathol 201: 7-16.

Kumar V, Abbas A and Jon Aster (2014) Acute and chronic inflammation, In: Robbins and Cotran Pathologic Basis of Disease, Professional Edition, Saunders, An Imprint of Elsevier 9th ed.

Laemmli UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227: 680-685.

Lakadwala AD, Dadkar NK and Dohadwala AN (1980) Action of clonidine on mast cells of rats. Pharm Pharmacol 32: 790-791.

Landewe RB, Boers M, Verhoeven AC, Westhovens R, van de Laar MA, Markusse HM, van Denderen JC, Westedt ML, Peeters AJ, Dijkmans BA, Jacobs P, Boonen A, van der Heijde DM and van der Linden S (2002) COBRA combination therapy in patients with early rheumatoid arthritis. Arthritis Rheum 46:347–356.

Landucci EC, Toyama M, Marangoni S, Oliveira B, Cirino G, Antunes E and de Nucci G (2000) Effect of crotapotin and heparin on the rat paw oedema induced by different secretory phospholipases $A_2$. Toxicon 38: 199–208.

Langunoff D, Martin TW and Read G (1983) Agents that release histamine from mast cells. Annu Rev Pharmacol Toxicol 23: 331-51

Lard LR (2001) Early versus delayed treatment in patients with recent onset rheumatoid arthritis: results of two meta-analyses. Am J Med 111:446–451.

Lebrec H, Bachot N, Gaspard I, Kerdine S, Guinnepain MT, Laurent J and Pallardy M (1999) Mechanisms of drug-induced allergic contact dermatitis In. Cell Biol Toxicol pp 57-62.

Leung CH, Grill SP, Lam W, Han QB, Sun HD and Cheng YC (2005) Novel mechanism of inhibition of NF-kappa B DNA-binding activity by diterpenoids isolated from *Isodon rubescens*. Mol Pharmacol 68: 286-297.

Levi-Schaffer F (2009) Mast Cell Stabilizing Properties of Anti-histamines. J Invest Dermatol 129: 2549-2551.

Limon-Pacheco J, Gonsebatt ME (2009) The role of antioxidants and antioxidant related enzymes in protective responses to environmentally induced oxidative stress. Mutat Res 674: 137–147.

Lin RY, Anderson AS, Shah SN and Nurruzzaman F (2008) Increasing anaphylaxis hospitalizations in the first 2 decades of life: New York State, 1990-2006. Ann Allergy Asthma Immunol 101: 387-393.

Lipsky P, Van Der Heide DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, Smolden JS, Weisman M, Emery P and Feldmann M (2000) Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study group. N Eng J Med 343:1594–1602.

Liu X and Wang J (2011) Anti-inflammatory effects of iridoid glycosides fraction of *Folium syringae* leaves on TNBS-induced colitis in rats. J Ethnopharmacol 133: 780–787.

Lo TN and Sauf SS (1987) Carrageenan stimulated release of arachidonic acid and of lactate dehydrogenase from rat pleural cells. Biochem Pharmacol 36: 24052513.

Loewen PS (2002) Review of the selective COX-2 inhibitors celecoxib and rofecoxib: focus on clinical aspects. Cjem 4(4): 268-275.

Lowry SF (2005) Human endotoxemia: a model for mechanistic insight and therapeutic targeting. Shock 24: 94–100.

Makinen H, Kautiainen H, Hannonen P, Mottonen T, Leirisalo-Repo M, Laasonen L, Korpela M, Blafield H, Hakola M and Sokka T (2007) Sustained remission and reduced radiographic progression with combination disease modifying anti-rheumatic drugs in early rheumatoid arthritis. J Rheumatol 34(2): 316321.

Marceau F, Lussier A, Regoli D and Giroud JP (1983) Pharmacology of kinins: their relevance to tissue injury and intlammation. Gen Pharmac 14: 209-229.

Marchant A, Amraoui Z, Gueydan C, Bruyns C, Le Moine O, Vandenabeele P, Fiers W, Buurman WA and Goldman M (1996) Methylprednisolone differentially

regulates IL-10 and tumour necrosis factor (TNF) production during murine endotoxaemia. Clin Exp Immunol 106: 91-96

Marshall LA, Chang JY, Calhoun W, Yu J and Carlson RP (1989) Preliminary studies on phospholipase $A_2$-induced mouse paw oedema as a model to evaluate antiinflammatory agents. J Cell Biochem 40: 147–155.

Martinez C, Kersey J, Papermaster BW and Good RA (1962) Skin homograft survival in thymectomised mice. Proc Soc Exp Biol Med 109: 193–196.

Mazumder UK, Gupta M, Manikandan L, Bhattacharya S, Haldar PK and Roy S (2003) Evaluation of anti-inflammatory activity of *Vernonia cinerea* Less. Extract in rats. Phytomedicine 10:185-188.

Merck manual (2008) Rheumatoid Arthritis (RA). Last full review/revision February by Roy D. Altman, MD.

Metzger H, Alcaraz G, Hohman R, Kinet JP, Pribluda V and Quarto R (1986) The receptor with high affinity for immunoglobulin E. Annu Rev Immunol 4: 419–470.

Miller JFAP (1961) Immunological function of the thymus. Lancet ii, 748–749.

Mirshafiey A, Cuzzocrea S, Rehm BH and Matsuo H (2005) M2000: a revolution in pharmacology. Med Sci Monit 11(8): PI53-PI63.

Misra HP and Fridovich I (1972) The role of superoxide anion in the auto-oxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247: 3170-3175.

Mizushima Y and Kobayashi M (1968) Interaction of anti_inflammatory drugs with serum proteins, especially with some biologically active proteins. J Pharma Pharmacol 20: 169_173.

Mota I (1964) The mechanism of anaphylaxis. I. Production and biological properties of 'mast cell sensitising' antibody. Immunol 7: 681.

Muniappan M and Sundararaj T (2003) Anti-inflammatory and antiulcer activities of *Bambusa arundinacea*. J Ethnopharmacol 88(2-3): 161-167.

Murakami M and Kudo I (2002) Phospholipase $A_2$. Biochem 31: 285–292.

Muscara M, Giuffre G, Barresi G and Tuccari G (1997) The AgNOR count in ulcerative colitiswith and without dysplasia. Pathol 29(2): 136–140.

Nakano Y and Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplast. Plant Cell Physiol 22: 867-880.

Nakhai LA, Mohammadirad A, Yasa N, Minaie B, Nikfar S, Ghazanfari G, Zamani MJ, Dehghan G, Jamshidi H, Boushehri VS, Khorasani R and Abdollahi M (2007) Benefits of Zataria multiflora Boiss in experimental model of mouse inflammatory bowel disease. Evid Based Complement Alternat Med 4: 43-50.

National Committee for Clinical Standards (1993) Reference procedure for erythrocyte sedimentation rate (ESR) test. 3rd ed. H2-A3. Villanova, Pa.: NCCLS.

Nava E, Palmer RMJ and Moncada S (1992) The role of nitric oxide in endotoxic shock: Effects of $N^G$-monomethyl-L-arginine. J Cardiovasc Pharmacol 20: S132–S134.

Newton K and Dixit VM (2012) Signaling in innate immunity and inflammation. Cold Spring HArbour Laboratory Press. doi: 10.1101/cshperspect.a006049

Noctor G and Foyer CH (1998) ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49: 249–279.

Orwa C, Mutua A, Kindt R, Jomnadass R and Simons A (2009) Agroforestry Database: a tree reference and selection guide version 4.0 (http://www.worldagroforestry.org/af/treedb/)

Packard RR, Lichtman AH and Libby P (2009) Innate and adaptive immunity in atherosclerosis. Semin Immunopathol 31: 5–22.

Paiva LAF, Gurgel LA, Silva RM, Tome AR, Gramosa NV, Silveira ER, Santos FA and Rao VS (2003) Anti-inflammatory effect of kaurenoic acid, a diterpene from *Copaifera langsdorffii* on acetic acid-induced colitis in rats. Vascul Pharmacol 39: 303–307.

Paschapur MS, Patil MB, Kumar R and Patil SR (2009) Evaluation of antiinflammatory activity of ethanolic extract of *Borassus flabellifer* L. male flowers (inflorescences) in experimental animals. J Med Plant Res 3(2): 49–54.

Pearson CM (1956) Development of arthritis, periarthritis and periostitis in rats given adjuvants. Proc Soc Exp Biol Med 91(1): 95-101.

Petersen LJ, Mosbech H and Skov P (1996) Allergen-induced histamine release in intact human skin *in vivo* assessed by skin microdialysis technique: Characterization of factors influencing histamine releasability. J Allergy Clin Immunol 97: 672-679.

Podolsky DK (2002) Inflammatory bowel disease. N Engl J Med 347: 417–429.

Pohlers D, Beyer A, Koczan D, Wilhelm T, Thiesen HJ and Kinne RW (2007) Constitutive upregulation of the transforming growth factor-beta pathway in rheumatoid arthritis synovial fibroblasts. Arthritis Res Ther 9(3): R59.

Polydoro M, Schröder N, Lima MNM, Caldana F, Laranja DC and Bromberg E (2004) Haloperidol and clozapine-induced oxidative stress in the rat brain. Pharmacol Biochem Behav 78: 751-6.

Proud D and Kaplan AP (1988) Kinin formation: mechanisms and role in inflammatory disorders. Annu Rev lmmunol 6: 49-83.

Ramprasath VR, Shanthi P and Sachdanandam P (2006) Immunomodulatory and antiinflammatory effects of *Semecarpus anacardium* LINN. Nut milk extract in experimental inflammatory conditions. Biol Pharm Bull 29(4): 693-700.

Ran JC, Eun MS, Hyun AJ, Jae SC and Yeong SK (2011) Inhibitory effects of kaurenoic acid from Aralia continentalis on LPS-induced inflammatory response in RAW264.7 macrophages. Phytomedicine 18(8–9): 677–682

Rang HP, Dale MM and Ritter JM (1999) Anti-inflammatory and immunosuppressant drugs, In: Pharmacology. 5th ed, Churchil Livingston Edinburgh London p248.

Rath A, Glibowicka M, Nadeau VG, Chen G and Deber CM (2007) Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proc Nat Aca Sci 106(6): 1760-1765.

Raven EL (2000) Peroxidase-catalyzed oxidation of ascorbate. Structural, spectroscopic and mechanistic correlations in ascorbate peroxidase. Subcell Biochem 35: 317–49.

Regoli D and Barabe J (1980) Pharmacology of bradykinin and related kinins. Pharm Rev 132: l-46.

Ricciotti E and FitzGerald GA (2012) Prostaglandins and Inflammation. Arterioscler Thromb Vasc Biol 31(5): 986–1000.

Roberts RA (1989) Bradykinin receptors: characterization, distribution and mechanisms of signal transduction. Progress Growth Factor Res 1: 237-252.

Rosana FRC, Maria DA, Soma M, Sotto MN, Jose Jr AS and Neto CF (2005) Nucleolar organiser region staining patterns in paraffin embedded tissue cells from human skin cancer. J Cutan Pathol 32: 323–328.

Salmon DM (2014) Smooth Muscle Preparations, In: Practical Pharmacology for the Pharmaceutical Sciences. John Wiley & Sons, Ltd p 55-92

Sampson HA, Muñoz-Furlong A, Campbell RL, Adkinson NF Jr., Bock SA, Branum A, Brown SG, Camargo CA Jr, Cydulka R, Galli SJ, Gidudu J, Gruchalla RS, Harlor AD Jr, Hepner DL, Lewis LM, Lieberman PL, Metcalfe DD, O'Connor R, Muraro A, Rudman A, Schmitt C, Scherrer D, Simons FE, Thomas S, Wood JP and Decker WW (2006) Second symposium on the definition and management of anaphylaxis: summary report—Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. J Allergy Clin Immunol 117(2): 391-397.

Schneider CA, Rasband WS and Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671–675 doi: 10.1038/nmeth.2089.

Schwartz JC (1997) Annual review of Pharmacology and Toxicology. In: Elliot HW, George R and Okun R editors. Palo Alto. Ann Reviews Inc.p. 325-339.

Scott A, Khan KM, Roberts CR, Cook JL and Duronio V (2004) What do we mean by the term "inflammation"? A contemporary basic science update for sports medicine. Br J Sports Med 38(3): 372-380.

Seguí J, Gironella M, Sans M, Granell S, Gil F, Gimeno M, Coronel P, Piqué JM and Julián Panés (2004) Superoxide dismutase ameliorates TNBS-induced colitis by reducing oxidative stress, adhesion molecule expression, and leukocyte recruitment into the inflamed intestine. J Leukoc Biol 76(3): 537-544.

Serhan CN (2004) A search for endogenous mechanisms of anti-inflammation uncovers novel chemical mediators: missing links to resolution. Histochem Cell Biol 122: 305-321.

Sharma PK, Hota D and Pandhi P (2004) Biologics in rheumatoid arthritis. J Assoc Physicians India 52: 231-236.

Sheikh A, Hippisley-Cox J, Newton J and Fenty J (2008) Trends in national incidence, lifetime prevalence and adrenaline prescribing for anaphylaxis in England. J R Soc Med 101:139-143.

Sheth UK, Dadkar NK and Kamath NG (1972) Selected topics in experimental pharmacology. Vol 5, Kothari Book Depot, Bombay, India p63.

Shi CC, Liao JF and Chen CF (2001) Spasmolytic effects of three harmala alkaloids on guinea-pig isolated trachea. Pharmacol Taxicol 89(5): 259-264.

Shin TY, Park JH and Kim HM (1999) Effect of *Cryptotympana atrata* extract on compound 48/80-induced anaphylactic reactions. J Ethnopharmacol 66: 319–325.

Silva SR, Futuro NHA and Pires JGP (1995) Effect of 5-HT3 receptor antagonists on neuroleptic-induced catalepsy in mice. Europharmacol 34: 97-9.

Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM and Geis GS (2000) Gastrointestinal toxicity with celecoxib vs non-steroidal anti-inflammatory drugs for osteoarthritis and rheumatoid

arthritis: the CLASS study: A randomized controlled trial. Celecoxib Longterm Arthritis Safety Study. JAMA 284(10): 1247–1255.

Simons FER, Sampson HA (2008) Anaphylaxis epidemic: fact or fiction? J Allergy Clin Immunol 122: 1166-1168.

Singh H, Kumar S, Dewan S and Kumar VL (2000) Inflammation induced by latex of Caleotropis procera - a new model to evaluate anti-inflammatory drugs. J Pharmacol Toxicol method 43: 219-224.

Singh S, Taneja M and Majumdar DK (2007) Biological activities of *Ocimum sanctum* L. fixed oil--an overview. Indian J Exp Biol 45(5): 403-412.

Smith G, Clegg M, Keen C and Grivetti L (1996) Mineral values of selected plant foods common to southern Burkina Faso and to Niamey, Niger, West Africa. Int J Food Sci Nutr. 47: 41-53.

Somani RS, Kasture VS and Kasture SB (1999) Haloperidol inhibits (-) bicucullineinduced seizures and bicuculline potentiates haloperidol-induced catalepsy in mice. Indian J Pharmacol 31: 434-436.

Somova LI, Shode FO, Moodley K and Govender Y (2001) Cardiovascular and diuretic activity of kaurene derivatives of *Xylopia aethiopica* and *Alepidea amatymbica*. J Ethnopharmacol 77: 165–174.

Soren A (1980) The diagnostic significance of certain histopathologic changes for rheumatoid arthritis. Z Rheumatologie 39(3-4): 74-78.

Sosa-Sequera MC, Suarez O and Dalo NL (2010) Kaurenic acid: An *in vivo* experimental study of its anti-inflammatory and antipyretic effects. Indian J Pharmacol 42:293–296.

Tairu AO, Hofmann T and Schieberle P (1999) Identification of the key aroma compounds in dried fruit of *Xylopia aethiopica*. In: J. Janick (ed.), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. p. 474– 478.

Tasaka K, Mio M and Okamoto M (1986) Intracellular calcium release induced by histamine releasers and its inhibition by some antiallergic drugs. Ann Allergy Asthma Immunol 56: 464-469.

Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, Asadi S, Vasiadi M, Weng Z, Miniati A and Kalogeromitros D (2012) Mast cells and inflammation. Biochim Biophys 1822(1): 21-33.

Thimmegowda NR, Dharmappa KK, Kumar CS, Sadashiva MP, Sathish AD, Nanda BL, Vishwanath BS and Rangappa KS (2007). Synthesis and evaluation of tricyclic dipyrido diazepinone derivatives as inhibitors of secretory phospholipase A2 with anti-inflammatory activity. Curr Top Med Chem 7: 811–820.

Thippeswamy BS, Mahendran S, Biradar MI, Raj P, Srivastava K, Badami S and Veerapur VP (2011) Protective effect of embelin against acetic acid induced ulcerative colitis in rats. Eur J Pharmacol 654(1): 100–105.

Tiffany CW and Burch RM (1989) Bradykinin stimulates tumor necrosis factor and interleukin-I release from macrophages. FEBS Lett 247: 189-192.

Treré D (2000) AgNOR staining and quantification. Micron 127–131

Tsakonas E, Fitzgerald A, Fitzcharles MA, Cividino A, Thome JC, M'Seffar A, Joseph L, Bombardier C and Esdaile JM (2000) Consequences of delayed therapy with second-line agents in rheumatoid arthritis; a three year followup on the hydroxychloroquine in early rheumatoid arthritis (HERA) Study. J Rheumatol 27:623–629.

Turull A and Queralt J (2000) Selective cyclooxygenase-2 (COX-2) inhibitors reduce anti-Mycobacterium antibodies in adjuvant arthritic rats. Immunopharmacol 46(1): 71–77.

Van Everdingen A, Jacobs JW, van Reesema SD and Bijlsma JW (2002) Low dose prednisone therapy for patients with early active rheumatoid arthritis: clinical efficacy, disease modifying properties, and side effects. Ann Intern Med 136: 1–12.

Vasudevan M, Gunman KK and Parle M (2007) Antinociceptive and antiinflammatory effects of *Thespesia populnea* bark extract. J Ethnopharmacol 109: 264-270.

Villarreal G, Zagorski J and Wahl SM (2001) Inflammation: Acute. Encyclopedia of Life Sciences. [Online] Nature Publishing Group, London, United Kingdom. http://www.els.net/doi:10.1038/npg.els.0000943.

Vinegar R, Truax JF, Selph JL, Johnston PR, Venable AL and McKenzie KK (1987) Pathway to carrageenan-induced inflammation in the hind limb of the rat. Fed Proc 46(1): 118-126.

Wakefield D and Kumar RK (2001) Inflammation: Chronic. Encyclopedia of life Sciences. Nature Publishing Group. p 1-6.

Wakefield D and Lloyd AR (1992) The role of cytokines in the pathogenesis of inflammatory eye disease. Cytokine 4: 1–5.

Wang L, Gu Q, Xu Y, Li S, Gui J, Yang J, Yao Q and Ji Y (2008) Effects of Yunke (technetium-99 conjugated with methylene diphosphonate; (99)Tc-MDP) and/or colloidal chromic phosphate phosphonium-32, alone and in combination, in rats with adjuvant arthritis. Clin Exp Pharmacol Physiol 35(1): 23-28.

WebMD Medical Reference (2014). Reviewed by Varnada Karriem-Norwood, MD on May 16. http://www.webmd.com/rheumatoid-arthritis/tc/rheumatoid-arthritisreferences

Williams RO (2007) Collagen-induced arthritis in mice: a major role for tumor necrosis factor-alpha. Methods Mol Biol 361: 265-284.

Winter CA, Risley EA and Nuss GW (1962) Carrageenan-induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc Soc Exp Biol Med 111: 544-547.

Wong M, Chowienczyk P and Kirkham B (2005) Cardiovascular issues of COX-2 inhibitors and NSAIDs. Aust Fam Physician 34(11): 945-948.

Woode E, Ameyaw OE, Boakye-Gyasi E and Abotsi KMW (2012) Analgesic effects of an ethanol extract of the fruits of *Xylopia aethiopica* (Dunal) A. Rich (Annonaceae) and the major constituent, xylopic acid in murine models. J Pharm BioAll Sci 4(4): 291-301.

World Health Organisation (2002) Prevention of Allergy and Allergic Asthma. WHO publications p3.

Wu LH, Xu ZL, Dong D, He SA and Hong Y (2011) Protective effect of Anthrocyanins extract from blubbery on TNBS-induced IBD of mice. Evid Based Complement Altern Med 52: 54-62.

Xia B, Deng CS, Chen DJ, Zhou Y and Xiao JQ (1996) Role of copper zinc superoxide dismutase in the short-term treatment of acetic acid-induced colitis in rats. Acta Gastroenterol Latinoam 26: 227-230.

Yu J, Du Y, Xa M, Hu D, Wang L and Xing H (1992) Studies of nucleolar organiser regions and mucin histochemistry in ulcerative colitis. Chin J Cancer Res 4(2): 43–7.

Zhang X, Sun J, Xin W, Li Y, Ni L, Ma X, Zhang D, Zhang D, Zhang T and Du G (2015) Anti-inflammation effect of methyl salicylate 2-O-β-D-lactoside on adjuvant induced arthritis rats and lipopolysaccharide (LPS)-treated murine macrophages RAW264.7 cells. Int Immunopharmacol 25(1): 88-95.

Zhao H, Liu S, Huang D, Xu Q, Shuto T and Iwamoto Y (2006) The protective effects of incadronate on inflammation and joint destruction in established rat adjuvant arthritis. Rheumatol Int 26(8): 732-740.

Zhao W and Wang R (2002) $H_2S$-induced vasorelaxation and underlying cellular and molecular mechanisms. Am J Physiol Heart Circ Physiol 283: 474–480.

Zwerina J, Hayer S, Redlich K, Bobacz K, Kollias G, Smolen JS and Schett G (2006) Activation of p38 MAPK is a key step in tumor necrosis factor-mediated inflammatory bone destruction. Arthritis Rheumatol 54(2): 463–472.

# APPENDIX

## PREPARATION OF CARRAGEENAN SUSPENSION

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

**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 LIPOPOLYSACCHARIDE**

5 mg $kg^{-1}$ of *Escherichia coli* was dissolved in phosphate buffer saline to form the LPS solution administered.

**PAW DIAMETER MEASUREMENT USING DIGITAL VERNIER CALIPERS**

Digital Vernier calipers work by having a digital encoder attached to a small gear which turns on a rack gear (long, linear gear) which runs along the length of the caliper.

The digital encoder has a large number of alternating opaque and transparent bands arranged radially around a disk which is turned by the small gear. Two led/phototransistor pairs monitor the passage of the bands. By watching in pattern of light transmission, and which phototransistor sees the change first, a small digital circuit determines how many bands have passed from the zero point.

Because the gear ratios are fixed, and the bands on the disk are fixed, the circuit is programmed to translate one band into a certain distance of the caliper. This distance is converted to the appropriate millimeter value, and displayed on the liquid-crystal display (LCD).

CALCULATION:

$$\% \text{ increase in paw diameter} = \left[\frac{D_t - D_i}{D_i}\right] x\ 100$$

Where $D_i$ is paw diameter before carrageenan injection and $D_t$ is paw diameter at time T.



**PAW VOLUME MEASUREMENT USING PLETHYSMOMETER**

The volume transducer is formed by two Perspex tubes interconnected and filled with a conductive solution and a platinum electrode for each chamber. The entire set up is supported by a stand.

The water-displacement produced by the immersion of the animal paw in the measuring tube is reflected into the second tube, inducing a change in the conductance between the two platinum electrodes. The Plethysmometer Control Unit (PCU) detects the conductance changes and generates an output signal to the digital display indicating the volume displacement measured (0.01 ml resolution). The value that remains stable on the digital display is recorded. The control unit was zeroed between successive readings.

CALCULATION

$$\% \text{ increase in paw volume} = \left[\frac{V_t - V_0}{V_0}\right] x\ 100$$

Where, $V_t$ is the paw volume at time t (after injection). $V_0$ is the paw volume before injection (0 h)

**DRUG PREPARATION AND ADMINISTRATION**

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



**BUFFERS FOR ENZYME ASSAY**

**0.1 M Sodium Phosphate buffer (pH 6.0)**

| | |
|---|---|
| 0.1 M Monosodium phosphate | 88.0 ml |
| 0.1 M Disodium phosphate | 12.0 ml |

**50 mM Potassium Phosphate buffer (pH 7.0)**

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

**0.1 M Carbonate bicarbonate buffer (pH 10.2)**

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

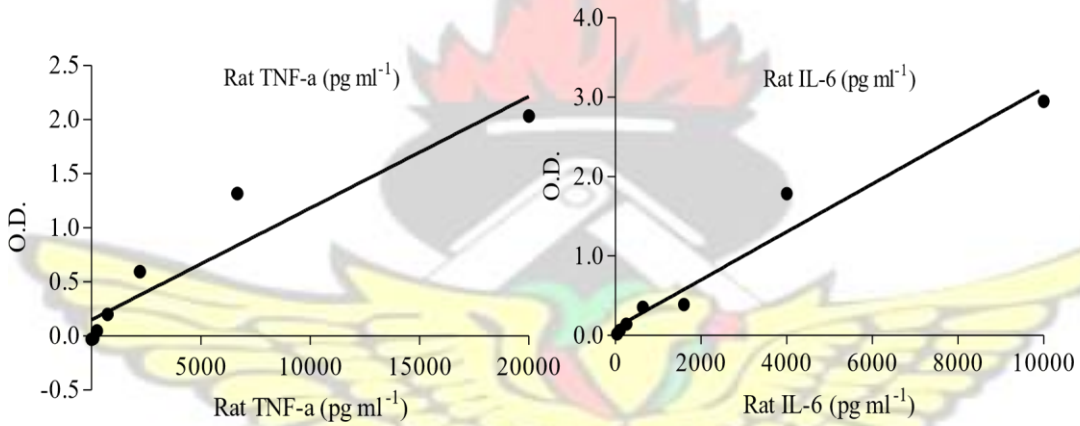
**SANDWICH ELISA ASSAY**

This assay employs an antibody specific for Rat TNF-α or IL-6 coated on a 96-well plate. Standards and samples are pipetted into the wells and TNF-α or IL-6 present in a sample is bound to the wells by the immobilised antibody. The wells are washed and biotinylated anti-Rat TNF-α or anti-Rat IL-6 antibody is added. After washing away unbound biotinylated antibody, *Horseradish peroxidase* (HRP)-conjugated streptavidin is pipetted to the wells. The wells are again washed, a 3, 3', 5, 5'-tetramethylbenzidine (TMB) one-step substrate solution is added to the wells and color develops in

proportion to the amount of TNF-alpha bound. The Stop Solution (0.16M sulphuric acid) changes the color from blue to yellow, and the intensity of the color is measured at 450 nm a plate reader (ELx808, Biotek Instruments, USA).

These ELISA kits show no cross-reactivity with the following cytokines tested as stated by the manufacturers: rat CINC-2, CINC-3, CNTF, fractalkine, IL-1α, IL-1β, IL-4, IL-10, GM-CSF, IFN-γ, leptin, lix, MCP-1, MIP-3α, β-NGF, and TIMP-1.

**Standard curves:**




**SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)**

The gel matrix used for SDS-PAGE is polyacrylamide, which is a good choice because it is chemically inert and, crucially, can easily be made up at a variety concentrations to produce different pore sizes giving a variety of separating conditions that can be changed depending on your needs.

In an applied electrical field, the SDS-treated proteins will move toward the cathode at different rates depending on their molecular weight. These different mobilities will be exaggerated in the high-friction environment of a gel matrix.

**Sample preparation**

Samples for SDS-PAGE by the Laemmli procedure are prepared in diluted gel buffer containing SDS, dithiothreitol, glycerol and bromophenol blue tracking dye. Stock solution of buffer containing everything except dithiothreitol is first prepared and this reagent is added right before use. Enough of the protein of interest should be loaded on the gel for it to be subsequently located. Detection in gels require on the order of 1 µg of protein for easily visibility of bands stained with Coomassie Brilliant Blue G-250.



**SDS-PAGE BUFFERS & PROCEDURE**

**30:0.4 Acrylaminde/bis-acrylaminde Stock**

| | [Final] | Mr/[Stock] | /100ml |
|---|---|---|---|
| 30% (w/v) acrylamide | | 71.08 | 30.0g |
| 0.4% (w/v) bis-acrylaminde | | 154.17 | 0.4 g |

• Dissolve in ~70 ml water by stirring until dissolved.

• DEIONISE with MIXED BED RESIN (Sigma, M-8032) and filter.

• Store protected from light in brown glass bottle.

**4X CONCENTRATED Stacking Gel Buffer, pH 6.8**

| [Final] | Mr/[Stock] | /100ml |
|---|---|---|
| 0.5 M Tris-HCl, pH 8.8 | 121.1 | 6.06g |

(Dissolve 6.06 g of tris base and 0.4 g of 0.4% SDS in 80 ml water, adjust the solution to pH 6.8 with HCl and add water to final volume of 100 ml)

**4X CONCENTRATED Resolving Gel Buffer, pH 8.8**

| [Final] | Mr/[Stock] | /100ml |
|---|---|---|
| 1.5 M Tris-HCl, pH 8.8 | 121.1 | 18.17g |

(Dissolve 18.17 g of tris base and 0.4 g of 0.4% SDS in 80 ml water, adjust the solution to pH 8.8 with HCl and add water to final volume of 100 ml)

**Tris-glycine-SDS buffer**

10X electrode buffer stock

| | |
|---|---|
| Tris base | 30.3 g |
| Glycine | 144.0 g |
| SDS | 10.0 g |

Dissolve in water to a total volume of 1 litre. Do not adjust the pH. Store at room temperature. To prepare 1X electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3), dilute 100 ml of 10X buffer stock with 900 ml of water.



**Sample Buffer**

0.075 M tris-HCl, pH 8.8, 2% SDS, 0.5 M dithiothreitol, 10% glycerol, and 0.01% bromophenol blue.

| | |
|---|---|
| Resolving gel 4X buffer stock | 0.5 ml |
| 10% SDS | 2.0 ml |
| 50% (w/v) Glycerol | 2.0 ml |
| 0.25% Bromophenol blue | 0.2 ml |
| Water | 4.8 ml |

Store at room temperature. Prior to use, add 0.5 M of dithiothreitol to 950 µl of sample buffer stock.

**Coomassie brilliant blue solution (1 L)**

| | |
|---|---|
| Coomassie brilliant blue G-250 | 2 g |
| Methanol | 450 ml |
| Glacial acetic acid | 100 ml |
| Distilled water | 450 ml |

**Destainer (1 L)**

| | |
|---|---|
| Methanol | 50 ml |
| Glacial acetic acid | 70 ml |
| Distilled water | to 1000 ml |

**Resolving gel preparation**

| Components | Volume: 10 ml resolving gel solution | | |
|---|---|---|---|
| | 8% gel | 10% gel | 12% gel |
| Deionised water | 4.73 ml | 4.13 ml | 3.43 ml |
| 30% acrylamide/bis | 2.7 ml | 3.3 ml | 4 ml |
| 1.5 M tris-HCl containing 0.4% SDS, pH 8.8 | 2.5 ml | 2.5 ml | 2.5 ml |
| 10% Ammonium persulphate | 60 µl | 60 µl | 60 µl |
| TEMED | 13 µl | 13 µl | 13 µl |



**Stacking gel preparation**

| Components | Volume: 5 ml stacking gel solution |
|---|---|
| Deionised water | 3 ml |
| 30% acrylamide/bis | 700 µl |
| 1.5 M tris-HCl containing 0.4% SDS, pH 8.8 | 1.25 ml |
| 10% APS | 25 µl |
| TEMED | 20 µl |

**Procedure**

1. The glass plate sandwich is assembled. The resolving gel solution is prepared as described above.

2. APS and TEMED are added last and carefully mixed to avoid formation of bubbles.

3. Important Note. Polymerization begins as soon as APS is added to the mixture, so all subsequent actions are performed promptly.

4. The gel solution is poured between the glass plates with a pipette, about 1/4 of the space is left free for the stacking gel. The top of the resolving gel is carefully covered with 50% isopropanol, 0.1% SDS solution or water, and waited until the resolving gel polymerises (~30 min). A clear line appears between the gel surface and the solution on top when polymerization is complete.

5. The water, isopropanol or SDS solution is discarded and gently washed with doubledistilled water.

6. The stacking gel solution (prepared as described above, add APS and TEMED last) is carefully poured with a pipette to avoid formation of bubbles.

Important Note. Polymerization begins as soon as APS is added to the mixture, so all subsequent actions must be performed promptly.

7. A comb is inserted and the gel allowed to polymerise for at least 60 min.

8. Comb is then carefully removed and the gel placed in the electrophoresis tank, and tank filled (bottom and top reservoirs) with fresh 1X Tris-glycine-SDS Buffer.

Precaution is taken that the gel wells are covered with the buffer.

9. The protein ladder/marker and probes were then loaded.

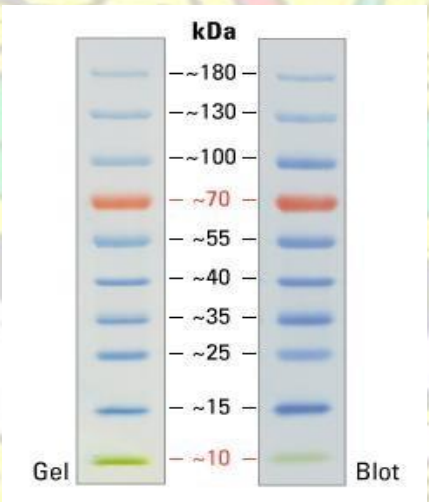
10. An appropriate voltage and current were set depending on how many gels are being run. The power is increased when the dye front reaches the running gel.

11. The electrophoresis run is stoped when the dye front reaches the bottom of the gel.

The gel sandwich is disassembled and the gel stained.



**Protein ladder for protein identification**




**SDS-PAGE band profile of the Thermo Scientific PageRuler Prestained Protein Ladder.** Images are from a 4-20% Tris-glycine gel (SDS-PAGE) and subsequent transfer to membrane.