STUDIES ON THE ANTI-INFLAMMATORY PROPERTIES OF THE AQUEOUS ETHANOL EXTRACT OF THE STEM BARK OF *BOMBAX COSTATUM* P.V

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DECLARATION

I, Meshack Antwi-Adjei, hereby declare that the experimental work described in this thesis was carried by me at the Department of Pharmacology, KNUST. This work has neither partially nor wholly been submitted elsewhere for any other degree.

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ABSTRACT

The stem bark of *Bombax costatum* (family: Bombacaceae) is used traditionally in Ghana for the treatment of many inflammatory diseases. The present study evaluated the effect of aqueous ethanol stem bark extract of *Bombax costatum* on carrageenan-induced paw oedema, cotton-pellet tissue granuloma formation and adjuvant-induced arthritis. The work also established its possible mechanisms of action using clonidine and haloperidol-induced catalepsy, PGE_2-induced paw oedema; and determination of serum cytokines levels after acetic acid-induced colitis in rats. Carrageenan-induced foot oedema in chicks was used to assess the effect of the extract on acute inflammation. Extract of *Bombax costatum* (EBC) showed maximal inhibition of 21.22 ± 1.18 %, 18.39 ± 1.31 % (prophylactic study) at 50 and 100 mg kg^{-1} and 24.97 ± 1.89 % (curative study) at 100 mg kg^{-1} respectively. For cotton-pellet granuloma formation; EBC (10-100 mg kg^{-1}) significantly reduced the total granuloma formation in both wet and dry cotton-pellets by 49.01 % and 54.53 % at 100 mg kg^{-1} respectively. In adjuvant-induced arthritis, EBC significantly inhibited maximal oedema to mean maximal inhibition of 139.49 ± 8.04 %, 136.96 ± 9.71 % and 132.14 ± 12.33 % at 10, 50 and 100 mg kg^{-1} respectively. Cataleptic responses induced in mice using clonidine was inhibited by EBC with maximal percentage inhibition of 17.50 ± 1.94 % and 12.75 ± 0.85 % at doses of 50 and 100 mg kg^{-1}. EBC reduced the total oedema in PGE_2-induced inflammation remarkably by 53.06 % at 100 mg kg^{-1}. EBC reduced total serum levels of IL-6 and TNF-α significantly by 47.32 %, 70.57 % and 37.91 %, 50.32 % at 50 and 100 mg kg^{-1} respectively. Preliminary phytochemical analysis of aqueous ethanol stem bark extract of *Bombax costatum* indicated the presence of terpenoids, saponins, tannins, phytosterols, alkaloids, reducing sugars and flavonoids which could possibly be attributed to its anti-inflammatory effects on acute and chronic inflammation.

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Finally, my heartfelt gratitude goes my family for their prayers and support.

DEDICATION

I dedicate this work to my wife, Roberta Antwi-Adjei, and my sons, Mikel Faith Antwi-Adjei and Manuel Antwi-Adjei. Also to my parents and all siblings for their loyal support and prayers.

Ultimately, I dedicate this work to the Almighty God who has been my help and strength.

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ABBREVIATIONS

AP-1	Activator protein 1
cAMP	Adenosine 3',5'-cyclic monophosphate
EBC	Extract of *Bombax costatum*
COX	Cyclooxygenase
CRP	C-reactive protein
DNA	Deoxyribonucleic acid

DMARD	Disease modifying anti-rheumatoid drug
DHA	Docosahexanoic acid
EPA	Eicosapentanoic acid
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte macrophage-colony stimulating factor
HCT	Hematocrit
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
PLA_2	Phospholipase A_2
ROS	Reactive oxygen species
NAOH	Sodium hydroxide
H_2SO_4	Sulfuric acid
TNFα	Tumor necrosis factor α
WBC	White blood cells
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1. INFLAMMATION

Inflammation is the adaptive response of the body to pernicious stimuli and conditions such as infections and foreign substances like bacteria and viruses (Majno and Joris, 2004; Kumar *et al*., 2003; Medzhitov, 2008). The body's defense against tissue injury or invasion of pathogens is a means of tissue repair, removal or destruction of the perilous substance, prevention of the spread of infection and preparation of healing mechanism. Inflammation is traditionally characterized by four cardinal signs as *rubor* (redness), *tumor* (swelling), *calor* (heat), *dolor* (pain) respectively (Brenner *et at.,* 2006), and *functio laesa* (loss of function) which was later proposed by Virchow (1871). It is generally thought that a controlled inflammatory response is beneficial (for example, in providing protection against infection), but it can become detrimental if deregulated (for example causing diseases such as septic shock, etc.) (Nairn and Helbert, 2007). The inflammation process entails a diverse nature of biological systems that modulate physiological response, eventually leading to familiar clinical symptoms. At the inflammatory site, molecular signals released by inflamed cells cause a number of physiological changes in the affected area: vasodilation; increased vascular permeability; fluid exudation which contains proteins and invasion by leukocytes, a type of white blood cells (Singh *et al.*, 2008). Inflammation process is divided into acute and chronic phases.

1.1.1 Acute inflammation

Acute inflammation is a normal process of the body's defense against injury or invasion of pathogens. During acute inflammation, blood vessels dilate and vascular permeability increases leading to an increased blood flow to the site of injury (Pulichino *et al.,* 2006). At the site of injury,

certain cells involved in inflammation such as mast cells, platelets, endothelial cells, nerve endings, signal molecules and chemo-attractants recruit leukocytes to the affected area. Neutrophils, a granulocyte, most often appear at the site of injury first. They engulf and destroy invading pathogens by releasing non-specific toxins such as hypochlorite, hydroxyl radicals and superoxide radicals that kill microorganisms and other abnormal cells (Pulichino *et al.,* 2006; Schmid-Schönbein, 2006). Neutrophils also release cytokines which in turn induce the synthesis of reactant proteins and systemic inflammatory responses. There are two different responses of acute inflammation: First, vascular response occurs as a result of vasodilation, increased vascular permeability and blood flow produced by pro-inflammatory agents There is vasodilation at the arteriole level, generating to the capillaries which lead to a net increase in the quantity of blood present causing redness and heat. Increased vascular permeability leads to the movement of plasma into tissues resulting in stasis due to the high concentration of cells within blood; a typical condition of large blood vessels packed with cell (Pulichino *et al.,* 2006). The stasis allows leukocytes to migrate along the endothelium (Nourshargh and Alon, 2014). Unlike vascular response, cellular response occurs over a period of hours after excessive tissue damage or infection. The main cellular processes involved in acute inflammation are leukocyte extravasation and phagocytosis (Muller, 2013). The leukocytes are delivered to the site of tissue injury due to the accumulation of white blood cells, and undergo the process of phagocytosis where offending agents, bacteria and other microbes are damaged (Nourshargh and Alon, 2014). The key phagocytes involved are the neutrophils, a type of white blood cells that contain granules of cell-destroying enzymes and proteins. A vast number of neutrophils reach the site of injury first, and hours after secretion, monocytes released which eventually mature into macrophages (Sunderkötter *et al.,* 2004).

1.1.2 Chronic inflammation

The deregulation of acute inflammation results into chronic condition which can persist for days, months, or years. Chronic inflammation is believed to be a prolonged duration of untreated inflammation in which tissue destruction, repair and healing processes occur simultaneously (Weiss, 2008). Many disease conditions such as cardiovascular disorders, chronic lung diseases, tuberculosis, and rheumatoid arthritis occur as a result of chronic inflammation (Agarwal and Brenner, 2006). During chronic inflammation, the body's tissues are persistently exposed to the bacterial infection and/or the harmful agents. The immune system can also under certain conditions initiate an inflammation process against itself, leading to autoimmune diseases. In chronic inflammation, cells such as lymphocytes, macrophages, and other cellular agents play active role which results in the proliferation of blood vessels, tissue necrosis, and fibrosis (Medzhitov, 2008).

1.1.3 Mediators of inflammation

Inflammatory mediators are usually present in an inactive form within the body and mostly activated by inflammatory reactions. These pro-inflammatory mediators generally cause vasodilation, alter vascular permeability and attract leukocytes. Examples are histamine, serotonin, prostaglandins, cytokines and kinins.

1.1.3.1 Histamine

Histamine is largely stored in mast cells, platelets and basophils, and exists in complex forms with mucopolysaccharide (glycosaminoglycan) such as heparin (Marone *et al.*, 2003). Histamine is synthesized through decarboxylation of L-hisdine by the enzyme decarboxylase in the Golgi apparatus. Histamine is a vasoactive substance which causes local vasodilation, local contraction

of the endothelial cells resulting in increased vascular permeability, nonvascular smooth muscle contraction, eosinophil chemotaxis and inhibiting the functions of T-lymphocytes (Haas *et al*., 2008). Histaminic effects are mostly exhibited during the early phase of inflammation. There are three subtypes of histamine receptors: The H_1-histaminic receptors cause acute vascular effects as well as bronchial constriction of the smooth muscles (spasmogenic action) and activation of eosinophil chemotaxis. Unlike H_1, the H_2 receptors regulate some anti-inflammatory processes including the hindrance of eosinophil chemotaxis, but can also induce vasodilatation. The H_3histaminc receptors participate in controlling histamine release from different producing cells; their actions are brief relatively and primarily occur as a result of intermediate-transient response from mild injury (Akdis and Blaser, 2003).

1.1.3.2 Serotonin

Serotonin, [5-hydroxytryptamine (5-HT)] is produced by decarboxylation of tryptophan and is essentially released from mast cells. In rodents, serotonin is stored in mast cell granules whereas in humans, it is present in the central nervous system (CNS) as well as the gastrointestinal tract (GIT) but most are found in dense granules of platelets. Serotonin, like histamine, is capable of increasing vascular permeability, dilating capillaries and producing contraction of the nonvascular smooth muscles. A variety of serotonin receptors exist which oversee its biological functions (Barnes and Neumaier, 2011).

1.1.3.3 Kinins

Kinins are mostly characterized into two bioactive peptides: bradykinin which is synthesized in plasma and tissues by the action of kininogenase on killikrein precursors, and its metabolites, des-Arg^9-bradykinin. Bradykinin binds selectively to kinin B_2 receptors (Leeb-Lundberg *et al*, 2005).

Bradykinin and killikrein are usually activated in the synovial fluid of patients affected by various arthropathies such as rheumatoid arthritis, osteoarthritis, psoriatic arthritis and gout (Nishimura *et al*, 2002; Meini and Maggi, 2008; Rahman *et al*, 1995). Bradykinin is implicated in inflammatory responses causing alteration of the joints either in the synovium, cartilage or bones since the kinin B_2 receptors are activated by synovial cells, chondrocytes and osteoblast (Meini and Maggi, 2008; Brechter and Lerner, 2002). During inflammation, bradykinin causes pain, recruitment of leukocytes, increased vascular permeability and vasodilation resulting in heat and oedema (Pawlak *et al*, 2008; Lo *et al*, 1999; Cambridge and Brain, 1995). Bradykinin is involved in bone resorption, cartilage matrix homeostasis and endothelial cell proliferation (Colman, 2006; Meini and Maggi, 2008; Brechter and Lerner, 2007).

1.1.3.4 Prostaglandins

Prostaglandins exist in the form of unsaturated fatty acids with a 20-carbon chain backbone (Ricciotti and FitzGerald, 2011). Prostaglandins cause a number of changes in the biological systems and contribute to the clinical signs of inflammation such as pain, redness, fever and oedema (Ricciotti and FitzGerald, 2011). They are synthesized rapidly via the COX pathway in several types of cell in reaction to various stimuli such as tissue injury and pathogens (Funk, 2001). Prostaglandins are lipid-soluble hormone-like molecules produced by different types of cells such as the macrophages and monocytes, and exist in four different bioactive isoforms: prostaglandin E_2 (PGE_2), prostacyclin I_2 (PGI_2), prostaglandin D_2 (PGD_2) and prostaglandin F_2 (PGF_2) (Ricciotti and FitzGerald, 2011). PGE_2 is a key COX product which exhibits a broad range of biological actions in diverse tissues via binding to specific receptors on the plasma membrane (Hata and Breyer, 2004).

1.1.3.5 Cytokines

Cytokines are said to be non-immunoglobulin in nature and soluble glycoproteins released by host cells, acting non-enzymatically in molar concentrations via specific receptors to modulate function of the host cell. Different cell types of the immune system generate, store and transport numerous cytokines such as TNF-α, ILs (interleukins), INF (interferons), monokines and lymphokines. Cytokines play a major role in controlling the direction, amplitude and period of immune responses and to manage the remodeling of tissues. Individual cytokines may possess pleiotropic (multiple), overlapping and occasionally contradictory functions based on their concentration, the type of cell they act, and the presence of other mediators and cytokines (Mclnnes and Schett, 2007).

1.2 MANAGEMENT OF INFLAMMATION

Inflammation is self-limiting in healthy humans, where numerous cell types and tissues are involved in the initiation and termination of the acute stage (Schwab and Serhan, 2006).

However, inflammation usually results in tissue injury due to destruction of tissues or the activation of a repetitive process that alters tissue function (Schmid-Schönbein, 2006). Some recommended drugs used for the treatment of inflammation include the non-steroidal antiinflammatory drugs (NSAIDs), the disease modifying anti-rheumatoid drugs (DMARDs), and corticosteroids.

The NSAIDs like aspirin and ibuprofen are structurally diverse group that provide symptomatic relief in active osteoarthritic diseases and other inflammatory joint disorders which in effect reduce swelling, joint stiffness and pain and improve joint motility, but these have little effect on the underlying tissue degenerative process that lead to bone damage and cartilage loss (Ding, 2002). NSAIDs mostly inhibit cyclooxygenase (COX), an enzyme that catalyzes arachidonic acid metabolism into prostaglandins and thromboxanes (Green, 2001). NSAIDs are known to cause

gastrointestinal disorders in some regular individual users. Inhibition of thromboxane by NSAIDs results in decreased blood clotting capability of individual users which could have clinical importance (e.g. high risk of stroke). Moreover, the use of COX-2 specific NSAIDs can cause increased risk of cardiovascular disorders regardless of the low gastrointestinal risk associated with their use (Green, 2001).

The DMARDs have a complex mechanism of action with variations and in many cases the actions are unclear, but they all retard the process of joint destruction in rheumatoid arthritis. The usage of DMARDs can cause skin, liver, kidney and gastrointestinal side-effects ((Day *et al.,* 1987).

Glucocorticoids (GCs) on the other hand, remain at the forefront of anti-inflammatory and immunosuppressive therapies. These are extensively used to treat acute and chronic inflammatory diseases including psoriasis, multiple sclerosis, eczema, rheumatoid arthritis, allergic disorders, inflammatory bowel disease as well as immunological disorders (Quax *et al*., 2013; Clark and Belvisi, 2012). Glucocorticoids (GCs) such as dexamethasone, cortisol, prednisolone etc. inhibit the synthesis of pro-inflammatory proteins as well as to induce *de novo* production of inflammatory mediators. The production of inflammatory cytokines such as interleukins-1, -2, -6, tumor necrosis factor-α, interferon-$_{\gamma}$, chemokines, like IL-8, adhesion molecules is remarkably inhibited by glucocorticoids. GCs inhibit vasodilation and increased vascular permeability following inflammatory event and reduce leukocyte migration into inflamed sites and also modify leukocyte distribution (Perretti and Ahluwalia, 2000). GCs exert their anti-inflammatory effects by binding to glucocorticoid receptors (GRs) which results in the inhibition of pro-inflammatory mediators such as nuclear factor-κB (NF-κB) or activator protein1 (AP-1) (Luecke and Yamamoto, 2005; Busillo and Cidlowski, 2013). However, GCs are known to cause undesirable side effects such as euphoria, psychosis, gastrointestinal disorders, muscle weakness, skin rashes etc. (Schimmer and

Parker, 2006; Guyton and Hall, 2006). These adverse effects exhibited from the use of conventional drugs have currently built the prevalence interest in natural products particularly herbal preparations for the management of inflammatory diseases, and *Bombax costatum* has become one of the plants under scientific investigation to ascertain its therapeutic activity in the management of inflammatory diseases.

1.3 THE *BOMBAX COSTATUM* (Pellegr. et Vuillet)

1.3.1 Botanical Description

The *Bombax costatum* (Figure 1.1) belongs to the family Bombacaceae and is locally called redflowered silk-cotton tree or red kapok tree. It is a deciduous tree growing straight up to about 30 m tall and 100 cm in diameter. The crowns from the young trees are storeyed, developing into irregular and hardy in older trees. The bark is thick, rough, corky, greyish brown in colour, and covered with conical pointed spines on the stem and branches. The leaves are digitally compound, ovate with 5-7 leaflets and the petioles are about 8-15 cm long. At both ends, the leaflets are partly acuminate and partly ovate with lateral nerves of 8-10 pairs. The flowers are solitary, bisexual and 5-7 cm with deep red, orange or yellow, tulip-shaped, glabrous peduncles. The calyx are cup-shaped, 12-17 mm long, truncate, 5- toothed. The plant has 5 oblong-linear petals 4.5 cm x 1.5 cm with a round apex. The fruits are contained in ellipsoidal capsule darkbrown in colour with variable shapes. The fruits are embedded in white floss called kapok and contain several small seeds (Oyen, 2011).

Figure 1.1 Stem bark of *Bombax costatum* (*source: www.worldagroforestry.org*).

1.3.2 Ecological and Geographical distribution

Bombax costatum is a characteristic fire resisting tree of the savannas and dry woodlands from Senegal to central Africa, from Guinea across Ghana and Nigeria to southern chad. It grows on stony and gravelly lateritic soils, and often in cropland and close to settlements. Its thick, corky bark protects it against fire (Oyen, 2011).

1.3.3 Traditional Uses

A macerated powder of the root is used to treat epilepsy (Orwa *et al.*, 2009). Medicinal stem bark preparations promote wound healing (Orwa *et al.*, 2009). The stem bark and roots, in Sierra Leone and Senegal, are said to possess diuretic properties. The stem bark is also utilized to treat trichomoniasis, amoebiasis and dysentery in other forms. A powdered preparation of the stem bark is used to treat headache. The stem bark can also be prepared in a bath to treat insanity (Orwa *et al.*, 2009). The bark is also used for the treatment of skin diseases. In Mali, a decocted preparation

of the bark, leaves and other parts are used to solve menstrual problems. In children, decocted preparations of the leaves may be used to ameliorate fever. The leaves are also used in the treatment of hookworm and the flowers active against taenia. Natural preparations of the leaves and other medicinal plants are used to treat leucorrhoea and diarrhea (Orwa *et al.*, 2009). An extract formulation of the leaves is used to treat convulsions and other problems during childbirth. A decocted formulation of the leaves, roots or stem bark is used to treat severe cases of oedema. Additionally, the leaves and twigs are decocted to treat jaundice and rickets in children. Various plant parts are used for promoting lactation and as a tonic against fatigue. Mixture of the leaves and shea butter is used to attenuate leprosy of the skin (Orwa *et al.*, 2009).

In veterinary medicine, leaves are given to sheep bitten by a snake. In Burkina Faso, a macerated leave preparation is given to animals with retained placentae. In Niger, a cold decocted formulation of leaves and twigs is given to animals with piles (Paré *et al*., 2010).

A study conducted on *Bombax costatum* by Atawodi *et al*., (2012) had shown that the plant possesses anti-trypanosomal activity.

1.4 JUSTIFICATION, AIMS AND OBJECTIVES OF STUDY

1.4.1 Justification

Chronic inflammation has been found recently to contribute to the development of many disorders such as cardiovascular, respiratory, cancer and neurodegenerative disorders (Agarwal and Brenner, 2006). The frequency of life-threatening diseases associated with inflammation has increased worldwide and is becoming an important cause of morbidity and mortality especially in immunocompromised patients often in developing countries such as Ghana (Nkrumah, 2008). There are many pharmacotherapy approaches used conventionally for the management of inflammation and these include the use of glucocorticoids (GCs) and non-steroidal

antiinflammatory (NSAIDs) agents. It is estimated that about 30 million people worldwide resort to the use of NSAIDs for the treatment and management of certain diseases (Derle *et al.,* 2006; McGettigan and Henry, 2000). However, they often produce significant side-effects, which include gastric ulcer, renal damage, bronchospasm and cardiac abnormalities, thus limiting their use (Burke *et al*., 2006).

The use of natural products as complementary and alternative medicine is now on the increase in developing countries such as Ghana in response to the World Health Organization directives in several pre-clinical and clinical studies that have provided the scientific basis for the use of plants in folkloric medicine to treat diseases (Vijaya *et al*., 1997; Dilhuyday, 2003). The research and analysis of plants employed as anti-inflammatory agents in traditional enthnomedicine is one of the productive and logical strategies in the search for natural plant products (Elisabetsky *et al.*, 1995; Vongtau *et al.*, 2004).

Unfortunately, fewer studies have been conducted on this plant and hence, this study is significant as it seeks to give the scientific evidence necessary for the safer utilization of *Bombax costatum* traditionally in the management of inflammation.

1.4.2 Aims and objectives

The present study aimed at assessing the anti-inflammatory effect of the aqueous ethanol extract of the stem bark of *Bombax costatum* (EBC) using chicks and murine models of inflammation.

1.4.2.1 Specific objectives involve:

1. Screening of the phytochemical constituents of the plant extract.
2. Evaluation of anti-inflammatory activity of the extract in acute model using carrageenaninduced paw oedema in chicks.

3. Evaluation of anti-inflammatory activity of the extract in chronic models using:
    a. Cotton-pellet granuloma inflammation in rats
    b. Adjuvant-induced arthritis in rats
4. Establishment of possible mechanisms of action of the aqueous ethanol extract of *B. costatum* using:
    a. Prostaglandin E_2-induced oedema in rats
    b. Serum cytokine (IL-6 and TNF-α) level determination
    c. Clonidine-induced catalepsy in mice
    d. Haloperidol-induced catalepsy in mice

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant collection

The stem bark of *Bombax custatum* was collected from Nkawkaw in the Eastern Region, Ghana between February and April, 2014 and authenticated by Dr. Henry Sam, Department of Herbal Medicine, Kwame Nkrumah University of Science and Technology, with voucher specimen (No. KNUST/ HM1/ 2014/ WP006) kept in the Department of Herbal Medicine herbarium. The plant material was air-dried at room temperature for 2 weeks and milled into a semi-powdered material using a hammer-mill.

2.1.2 Sample extraction

Six hundred grams (600 g) of the powder was extracted by cold maceration using 2.0 L of 70 % (v/v) ethanol for 3 days. The filtrate was concentrated at 60 °C and under low pressure to a dark-

brown liquid using the rotary evaporator. The dark-brown liquid was finally dried in an oven at 60 °C for 24 h to give a semi-solid concentrate with a final yield of 8.76 % ($^{w}/_{w}$). The extract was reconstituted in 2 % tragacanth mucilage (dissolved in normal saline) and referred to as aqueous ethanol *Bombax custatum* extract (EBC in this project) and stored at 4 °C until use.

2.2 Chemicals and drugs

Carrageenan sodium salt (Sigma-Aldrich, St. Louis, MO, USA), Dexamethasone (Pharm-Inter, Brussels, Belgium), Diclofenac (Trogue, Hamburg, Germany), Methotrexate sodium (Dabur-Pharma, Uttar Pradesh, India), Paraffin oil (Ernest Chemist, Accra, Ghana), Acetic acid (SigmaAldrich, St. Louis, MO, USA), Salphasalazine (Shire Pharmaceuticals Inc., MA, USA), Clonidine (Teva Ltd, Wakefield, UK); Haloperidol (Alkem, Numbai, India), Chlorpheniramine maleate (Unimark Remedies Ltd, Numbai, India), Prostaglandin E_2 (PGE_2); IL-6 and TNF-α ELISA kits (Boster, CA, USA).

2.3 Animals

Cockerels (*Gallus gallus*; strain shaver 579); (50-60 g) were obtained one-day post-hatch from Atiwa Farms, Kumasi, Ghana. The chicks were kept for 6 days in the animal house of the Department of Pharmacology, KNUST, Kumasi and fed with food (Chick Mash) purchased from GAFCO, Tema, Ghana and water *ad libitum*. The chicks were kept and maintained at a room temperature of 26 ± 2 °C and incandescent illumination was provided on 12-h light-dark cycle. On the 6th day, the healthy chicks were selected and grouped in cages (34 cm × 57 cm × 40 cm) made of stainless steel containing soft wood shavings as bedding, and each cage contained 5 or 6 chicks per cage.

Sprague Dawley rats (200-250 g) and Imprint Control Region mice (ICR) (25-30 g) of both sexes were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana and maintained in the animal house of the Department of Pharmacology, KNUST. The animals were housed in groups of five (5) or six (6) in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under standard laboratory conditions.

2.4 Drug preparation

All drugs (extract and standard) were prepared by suspension in 2 % tragacanth mucilage dissolved in normal saline.

2.5 METHODS

2.5.1 PHYTOCHEMICAL ANALYSIS

The presence of saponins, phytosterols, tannins, alkaloids, terpenoids, flavonoids and glycosides in EBC were tested using simple qualitative methods as previously described by Trease and Evans (2002).

2.5.1.1 Saponins

The powdered extract (0.4 g) of *Bombax custatum* was added to 10 ml of water in a test tube; the mixture was shaken vigorously for 5 min and observed for the presence of froth which persisted for more than 10 min without breaking.

2.5.1.2 Phytosterols (Lieberman's test)

The powdered stem bark of *Bombax custatum* (0.4 g) was extracted with chloroform in a test tube followed by filtration and 2 ml of acetic anhydride was added to the filtrate. A few drops of conc. H_2SO_4 were added along the sides of the test tubes cautiously and the appearance of blue coloration at the interface indicated the existence of steroids.

2.5.1.3 Tannins

The powdered stem bark of *Bombax custatum* (0.4 g) was boiled with 15 ml of water for about 5 min. The mixture was then allowed to cool and filtered. 5 drops of 1 % lead acetate was added to 1 ml aliquot of the filtrate in 10 ml of water. An intense greenish coloration suggested the presence of tannins.

2.5.1.4 Alkaloids

The powdered stem bark of *Bombax custatum* (0.4 g) was extracted with 20 ml of hydrochloric acid and filtered. The filtrate was evaporated to dryness and rendered alkaline with dilute ammonia solution. Chloroform was added to the alkaline solution in a separating funnel for partitioning and the chloroform layer was separated and evaporated to dryness. The residue was dissolved in 1 % H_2SO_4 and a few drops of Dragendorff's reagent added. Appearance of orange colour indicated the presence of alkaloids.

2.5.1.5 Terpenoids (Salkowski test)

The powdered stem bark of *Bombax custatum* (0.4 g) was extracted with 70 % ethanol and mixed with 2 ml of chloroform in a test tube. This was then warmed for 30 min. 1 ml conc. H_2SO_4 was added to the solution and the appearance of a reddish-brown colouration suggested the presence of terpenoids.

2.5.1.6 Flavonoids

The powdered stem bark of *Bombax custatum* (0.4 g) was extracted with 70 % ethanol and 5 ml of ethyl acetate was added to the extract in a test tube. The mixture was warmed for 2 min and few drops of dilute ammonia solution were then added. A yellowish colour appeared at the bottom of the test tube indicating the presence of flavonoids.

2.5.1.7 General test for Glycosides (Reducing sugars)

About 0.4 g of the *Bombax* extract was added to 5 ml of dilute H_2SO_4 in a test tube. 2 ml of 20 % NaOH was added to the mixture after which few drops of Fehling's solutions A and B were added. The mixture was warmed on water bath for 2 min. Appearance of a brick-red precipitate at the bottom of the test tube indicated the presence of glycosides.

2.5.2 ANTI-INFLAMMATORY EFFECT OF AQUEOUS ETHANOL EXTRACT OF *BOMBAX COSTATUM* ON ACUTE INFLAMMATION

2.5.2.1 Carrageenan-induced foot oedema in chicks

The anti-inflammatory effect of EBC was evaluated by employing carrageenan-induced foot oedema model in chicks as previously reported by Roach and Sufka (2003). Oedema was induced

by injecting 0.1 ml of carrageenan (1 % (w/v) in saline) into the subplantar region of the right footpad of the chicks (50-60 g). The paw thickness of right footpad was quantified before oedema induction and hourly interval for 5 h using digital venier caliper (VC1346i, MP Lab Equip, U.S.A). The increase in paw thickness was expressed as the mean percentage change of paw thickness using the formula:

$$\% \text{ change in paw thickness} = \left[\frac{\text{PWo}-\text{PWt}}{\text{PWo}}\right] \times 100$$

Where PWt is the Paw thickness (at various time points) post carrageenan injection and PWo is the Paw thickness before carrageenan injection (i.e. time zero).

Total oedema was determined as the area under the time course curve (AUC) during the 5 h of carrageenan injection. The percentage inhibition of the total oedema was calculated using the formula:

$$\% \text{ Inhibition of oedema} = \left[\frac{AUC\ control - AUC\ treated}{AUC\ control}\right] \times 100$$

In the prophylactic study, chicks were treated with EBC (10, 50 and 100 mg/ kg, *p.o*), dexamethasone (0.3, 1.0 and 3.0 mg/ kg, i.p) and diclofenac (10, 30 and 100 mg/ kg, i.p) before carrageenan injection. In the curative study, drugs were administered 1 h post-subplantar inoculation of carrageenan (0.1 ml of 1% in saline). Control chicks received normal saline.

2.5.3 ANTI-INFLAMMATORY EFFECT OF AQUEOUS ETHANOL EXTRACT OF *BOMBAX COSTATUM* ON CHRONIC INFLAMMATION

2.5.3.1 Cotton pellet granuloma in rats

In this model, inflammation was induced using cotton pellet implantation method as previously described by Swingle and Shideman (1972). Briefly, rats (200-250 g) under light ether were anesthetized with pentobarbital (20 mg/ kg) and the groin region of the skin shaved and disinfected with 70 % ethanol. An incision was made and by blunt forceps subcutaneous tunnels were formed into which sterilized cotton pellets (40 ± 1 mg) were implanted bilaterally on both sides. The rats were monitored for 7 days and sacrificed on the 8th day by cervical dislocation.

The pellets were removed (along with granular tissue formed around) and freed from extraneous tissue, weighed immediately for wet weight. Then, pellets were dried in an incubator at 60°C for 24 h to obtain a constant dry cotton pellet weight. The granuloma formation is considered to be the mean weight of granuloma tissue formed around each cotton pellet (wet and dry weight). The percentage mean change of granuloma formation was calculated using the formula:

$$\% \text{ change of granuloma formation} = \left[\frac{Wc - Wd}{Wc}\right] \times 100$$

Where Wc is the difference in pellet weight of the control group and Wd is the difference in pellet weight of the treated group.

Total tissue granuloma formed during the 7 days was determined as the area under the time course curve (AUC). The percentage inhibition of the total tissue granuloma was calculated using the formula:

$$\% \text{ Inhibition of tissue granuloma} = \left[\frac{AUC\ control - AUC\ treated}{AUC\ control} \right] \times 100$$

After recovering from anesthesia, rats were treated with either EBC (10, 50 and 100 mg/ kg, *p.o*, daily) or dexamethasone (0.3, 1.0 and 3.0 mg/ kg, i.p, daily). The vehicle control group received normal saline. Drugs were administered for 7 consecutive days (starting from day 0). The effect of EBC on cotton pellet tissue granuloma was assessed on the following clinical indices;

2.5.3.1.1. Haematological analysis

Blood samples were collected before and at the end of the experiment for total blood count.

2.5.3.1.2 Body weight change

Change in body weight in the various treatment groups was noted daily.

2.5.3.1.3 Spleen weight/ 100 g of body weight of rats

Spleen of the individual animals in the various groups were removed and weighed.

2.5.3.2 Complete Freund's Adjuvant-induced arthritis in rats

Adjuvant arthritis was induced as previously described by Pearson (1956). The right hind limbs of the rats (200-250 g) were intraplantarly inoculated with 0.1 ml of Complete Freund's Adjuvant (CFA). Arthritic control group was injected with 0.1 ml of CFA, whilst non-arthritic control group was inoculated with 0.1 ml of Incomplete Freund's Adjuvant [(IFA) (sterile paraffin oil)]. The CFA was prepared by suspending 3 mg/ ml of heated killed Mycobacterium tuberculosis [strains C, DT

and PN (mixed)] in paraffin oil. After 9 days of CFA injection, arthritic rats were divided into different treatment groups and drug administration commenced on the 14th day till the day 28. Animals were treated with EBC (10, 50 and 100 mg/ kg, *p.o,* daily), dexamethasone (3.0 mg/ kg, i.p every other day) and methotrexate (1.0 mg/ kg, i.p every 4 days) according to the respective groups. Disease progression was observed from day 0 till the 28th day. The test drugs were prepared and administered in such a way that not more than 0.5 ml was given for the reference drugs and not more than 1 ml for EBC. All drugs were freshly prepared for each treatment on the day of drug administration. The effect of EBC on adjuvant-induced arthritis was evaluated adopting four (4) indicators;

2.5.3.2.1 Oedema: measured as maximal and total oedema effects

Hind limb volume was measured by water displacement plethysmometry (model 7140, Ugo Basile, Italy) for both injected hind limb and non-injected hind limb before CFA inoculation (day 0) and every other day till day 28 (Binder and Walker, 1998). Maximal oedema effect was expressed as the percentage change in paw volume using the formula:

$$\% \text{ change in paw volume} = \left[\frac{PVt - PVo}{PVo}\right] \times 100$$

Where PVo is paw volume before adjuvant injection and PVt is the paw volume at various time, T.

Data obtained was shown as the effects of drugs on the time course curves and the total oedema responses calculated as the area under the curve (AUC) in arbitrary units for 28 days. The percentage inhibition of the total oedema was calculated using the formula:

$$\% \text{ Inhibition of oedema} = \left[\frac{AUC\ control - AUC\ treated}{AUC\ control} \right] \times 100$$

2.5.3.2.2 Body weight change

Body weight of rats was taken every 4 days till day 28.

2.5.3.2.3 Arthritic index

2.5.3.2.3.1 Photography

On the 29th day, a digital camera was used to take photographs of the paw limbs. Arthritic scoring was blindly made on the photographs by the same person and the severity of inflammation was classified according to the degree of oedema formation of the periarticular tissues and erythema (Kinne *et al*., 1995), using the scale of 0-4 per limb where 0 = no inflammation; 1 = unequivocal inflammation of 1 joint of the paw; 2 = moderate inflammation of 1 joint of the limb; 3 = pronounced inflammation of 1 joint of more and 4 = utmost or excess inflammation of 1 or more joints of the limb with joint rigidity. The arthritic score of each animal on day 0 was estimated to be 0. Arthritic score and the hind paw volume were used as the measurement of inflammation.

2.5.3.2.3.2 Radiography

Radiological assessment of the severity of cartilage and bone destruction was done on the 29th day. Radiographs of the hind paw were taken after rats were anesthetized by intraperitoneal injection with pentobarbitone sodium (20 mg/ kg) and radiographs captured using an X-ray apparatus

(Softex, Tokyo, Japan) and industrial X-ray film (Fuji Photo Film, Tokyo, Japan). The X-ray apparatus functioned at 30-kV peak and 10-s exposure with 45-cm tube-to-film distance for lateral projections. The severity of bone and cartilage damage was scored for each hind paw as stated by the method previously described by Hoffmann *et al*. (1997). The radiographical index was based on extent of bone damage, paw tissue swelling, osteoporosis, and peritoneal formation of new bone on the scale of 0 = normal to 3 = maximum per hind paw.

2.5.4 ESTABLISHMENT OF POSSIBLE MECHANISMS OF ACTION OF AQUEOUS ETHANOL EXTRACT OF *BOMBAX COSTATUM*

2.5.4.1 Indirect anti-histaminic activity of *Bombax costatum* using Clonidine- and Haloperidol-induced catalepsy in mice

Clonidine- and haloperidol-induced catalepsy tests as previously described by Ferre *et al*. (1990) were used to evaluate the indirect anti-histaminic effect of EBC. Either clonidine or haloperidol (1 mg/ kg) was injected subcutaneously into ICR mice (25-30 g, n=5) and their forepaws were placed on a horizontal bar (1 cm in diameter, 3 cm above a table). The time taken for the mice to remove their paws from the bar was recorded and duration of catalepsy was measured at 30, 60, 90, 120, 150 and 180 min. Mice received either EBC (10-100 mg/ kg, *p.o*, 1 h) or chlorpheniramine maleate (5 mg/ kg, i.p, 30 min) after catalepsy induction. The control group was given (1 ml/ kg, *p.o*) of normal saline.

2.5.4.2 Effect of *Bombax costatum* on Prostaglandin E_2-induced paw oedema

Paw oedema was induced by injecting 0.2 ml (1nM) Prostaglandin E_2 into the subplantar tissue of the right hind limb of the rats (200-250 g, n=5) as previously described by Willis and Cornelsen (1973). Measurement of the paw volume was recorded before PGE_2 induction and 30 min interval

over 3 h by water displacement plethysmography. Oedema was estimated from the percentage change in paw volume over the different time points. Rats were treated with either EBC (10, 50 and 100 mg/ kg, *p.o*, 1 h) or diclofenac (30 mg/ kg, i.p, 30 min) after PGE_2 injection. The control group received normal saline (1 ml/ kg, *p.o*). The maximal oedema and total oedema responses were estimated as described in the carrageenan-induced paw oedema model (section 2.5.2.1).

2.5.4.3 Effect of *Bombax costatum* on serum cytokine (IL-6 and TNF-α) levels

The effect of EBC on cytokine (IL-6 and TNF-α) serum levels was assessed on acetic acidinduced colitis model as previously described by Mousavizadeh *et al*. (2009) with slight modification. Colitis was induced in rats (200-250 g, n=5) with 1 ml of 4 % of acetic acid (AA) ($^{v}/_{v}$) solution on the 5th day using a 2.7 mm soft catheter trans-rectally under light ether anesthesia. Rats were held horizontally for 1-2 min to avoid leakage and randomly allocated into groups. Animals were treated with either EBC (10, 50 and 100 mg/ kg, *p.o*) or sulphasalazine (300 mg/ kg, *p.o*) commencing on day 0 for 7 consecutive days (Harputluoglu *et al*., 2006). The non-colitic (NC) control group received only 1 ml of normal saline without AA inoculation. On the 8th day, blood samples were collected into vacuum container gel and activator tubes and allowed to clot at room temperature. The blood samples were then centrifuged (model 5415C, Medizin & Labortechnik GmbH, Schnakenberg, Germany) at × 1000 g for 15 min. Sera formed were aliquoted into eppendorf tubes and stored at -20 ℃ before analysis. Serum levels of the cytokines (IL-6 and TNF-α) were measured in duplicates with appropriate rat ELISA kit in accordance with the recommendations outlined by the manufacturer. Briefly, 0.1ml each of standard (at different concentrations) and test samples were pipetted into wells in micro-titre plates pre-coated with monoclonal antibodies specific for IL-6 and TNF-α respectively, sealed and incubated (Heracell VIO5, 160 CO_2 Incubator, 230V 50-60Hz, Thermo Electron LED GmbH, Langenselbold,

Germany) at 37 ℃ for 90 min with gentle shaking. After incubation, the plate contents were discarded and subsequently blotted onto paper towel. Either IL-6 or TNF-α antibody (0.1 ml of biotinylated anti-rat) working solutions were pipetted into each well and incubated at 37 ℃ for 60 min; plates were washed 3 times with 0.01 M PBS (washing buffer) and at each time, buffer was let to stay in the wells for 1-2 min before discarding contents. After washing, 0.1 ml of Avidin-Biotin-Peroxidase Complex (ABC) was added to each well and incubated at 37 ℃ for 30 min and then the plates were washed 5 times with washing buffer (0.01 M PBS) after the incubation. Contents of each plate were discarded onto paper towel and 90 µl of prepared 3,3,5,5 Tetramethylbenzidine (TMB) colour developing agent was subsequently added to each well and incubated at 37 ℃ in the dark for 15-20 min. TMB stop solution (0.1 ml) was pipetted into each well which resulted in colour change into yellow colouration immediately. The optical density (OD) absorbance of the samples was read at 450 nm using a micro-plate spectrometer reader (Spectramax 190 Micro-plate Spectrometer, 90-250V 50-60Hz, Molecular Devices, CA, USA).

2.6 STATISTICAL ANALYSIS

All graphs were plotted using GraphPad prism for windows version 6.0 (GraphPad software, San Diego, CA, USA). Data obtained was statistically estimated through test of significance using both one-way and two-way analysis of variance (ANOVA) followed by Dunnet *post hoc* test. All data were presented as the effect of drugs on the maximal and total oedema responses over time period and data expressed as mean ± SEM. ED_{50} (dose responsible for 50 % of maximal effect) was determined by using an iterative computer least square method, with the non-linear regression (three-parameter logistic) equation:

$$Y = \frac{a + (b - c)}{(1 + 10^{(LogED_{50} - X)})}$$

Where X is the logarithm of dose and Y is the response. Y begins at a (the bottom) and goes to b (the top) with a sigmoid shape. The fitted midpoints (ED_{50s}) of the curves were compared statistically using Dunnet *post hoc* test.

CHAPTER THREE

RESULTS

3.1 RESULTS

3.1.1 PHYTOCHEMICAL SCREENING OF *BOMBAX COSTATUM*

Table 3.1 Chemical constituents of aqueous ethanol extract of *B. costatum.*

TEST	RESULTS
Saponins	+
Phytosterols (Lieberman's test)	+
Tannins	+
Alkaloids	+
Terpenoids (Salkowski test)	+
Flavonoids	+
Glycosides (General test)	+

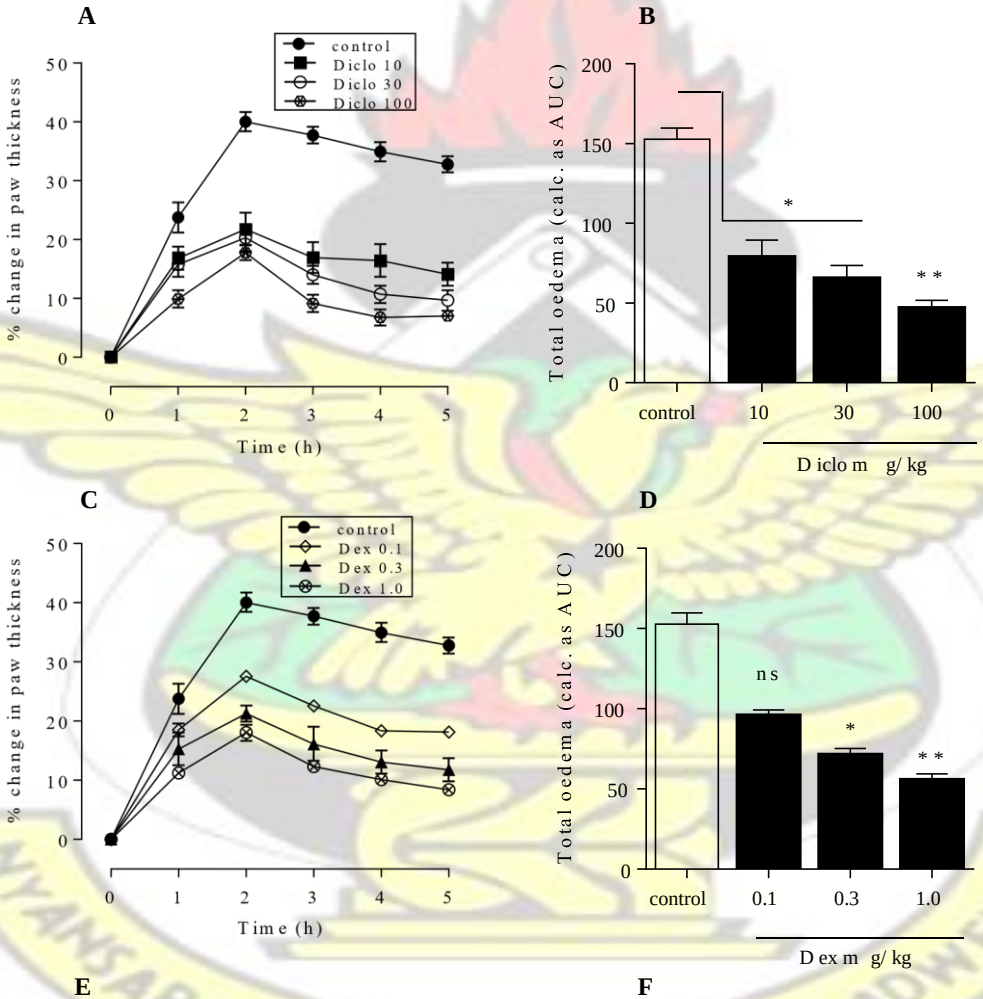
+: Present, —: Absent.

3.1.2 ANTI-INFLAMMATORY EFFECT OF AQUEOUS ETHANOL EXTRACT OF

***BOMBAX COSTATUM* ON ACUTE INFLAMMATION**

3.1.2.1 Carrageenan-induced oedema in chicks

Injection of carrageenan (0.1 ml of 1 % (w/v) in saline) resulted in moderate inflammatory oedema in the footpad of the week old chicks peaking either at 2 h or 3 h. In the prophylactic study, the mean maximal oedema attained at 2 h was reduced significantly to 21.75 ± 2.75 %, 20.31 ± 2.28 %, 17.79 ± 1.32 % for diclofenac at 10, 30 and 100 mg/ kg; and 21.26 ± 0.61 %, 18.05 ± 1.35 % for dexamethasone at 0.3 and 1.0 mg/ kg respectively as compared to the inflamed control response of 40.03 ± 1.65 % (Figure 3.1[A, C and E]). Similarly, EBC dosedependently decreased the mean maximal swelling significantly to 21.22 ± 1.18 % and 18.39 ± 1.31 % at 50 and 100 mg/ kg respectively as compared to inflamed control response of 40.03 ± 1.65 % (Figure 3.1[A, C and E]). The total paw oedema over the 5 h period (illustrated as AUC of the time-course curves in arbitrary units) was significantly reduced by 48.31 %, 57.04 %, 69.16 % for diclofenac at 10, 30 and 100 mg/ kg; 53.17 %, 63.44 % for dexamethasone at 0.3 and 1.0 mg/ kg; and 53.29 %, 66.07 % for EBC at 50 and 100 mg/ kg respectively in a dosedependent manner (Figure 3.1[B, D and F]).


A
control
Diclo 10
Diclo 30
Diclo 100
% change in paw thickness
Time (h)
B
Total oedema (calc. as AUC)
control
10
30
100
Diclo mg/kg
C
control
Dex 0.1
Dex 0.3
Dex 1.0
% change in paw thickness
Time (h)
D
Total oedema (calc. as AUC)
ns
control
0.1
0.3
1.0
Dex mg/kg
E
F

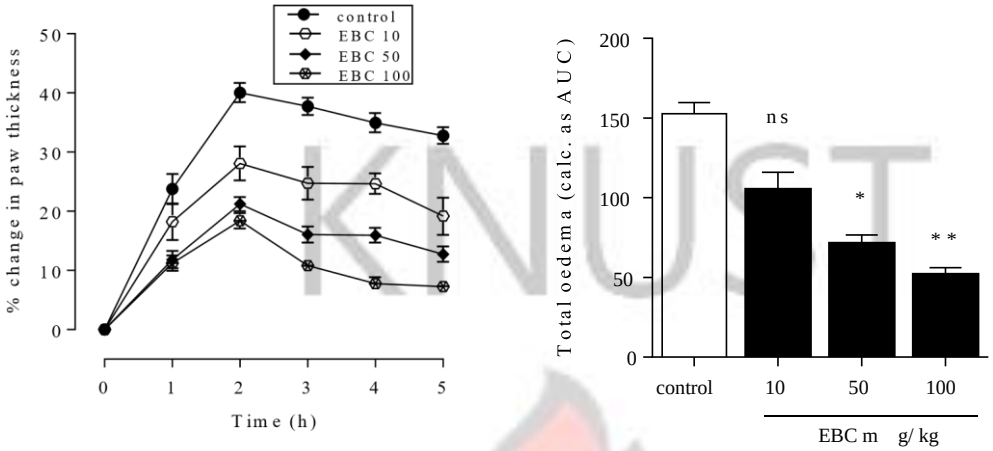
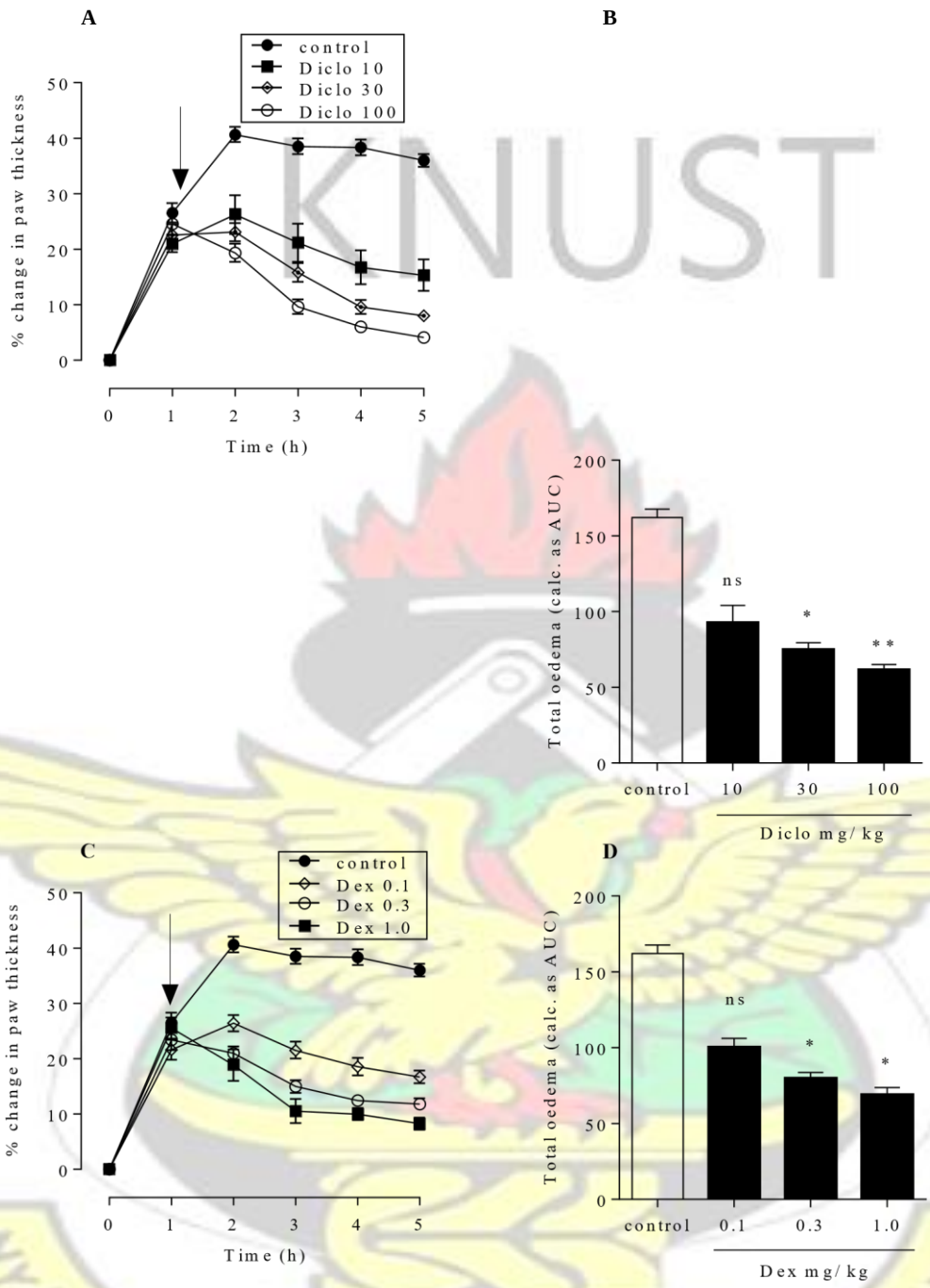


Figure 3.1 Effect of *Bombax costatum* extract on the maximal (A, C and E) and the total oedema responses (B, D and F) respectively in the prophylactic study of carrageenan-induced oedema in chicks. Values are mean ± SEM (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

In the therapeutic study, the mean maximal oedema attained at 2 h was significantly reduced to 23.09 ± 1.62 %, 24.54 ± 1.89 % for diclofenac at 30 and 100 mg/ kg; and 23.42 ± 1.90 %, 25.54 ± 1.89 % for dexamethasone at 0.3 and 1.0 mg/ kg respectively as compared to the control with a mean maximal oedema of 40.64 ± 1.38 % (Figure 3.2[A, C and E]). Similarly, EBC dosedependently reduced the mean maximal paw swelling significantly to 24.97 ± 1.87 % at 100 mg/ kg as compared to the inflamed control response of 40.64 ± 1.38 % respectively (Figure 3.2[A, C and E]). The total paw oedema attained over the 5 h period (illustrated as AUC of the timecourse curves in arbitrary units) was dose-dependently suppressed significantly by 53.71 %, 61.99 % for diclofenac at 30 and 100 mg/ kg; 50.70 %, 57.40 % for dexamethasone at 0.3 and 1.0 mg/ kg; and 56.83 % for EBC at 100 0 mg/ kg respectively (Figure 3.2[B, D and F]).



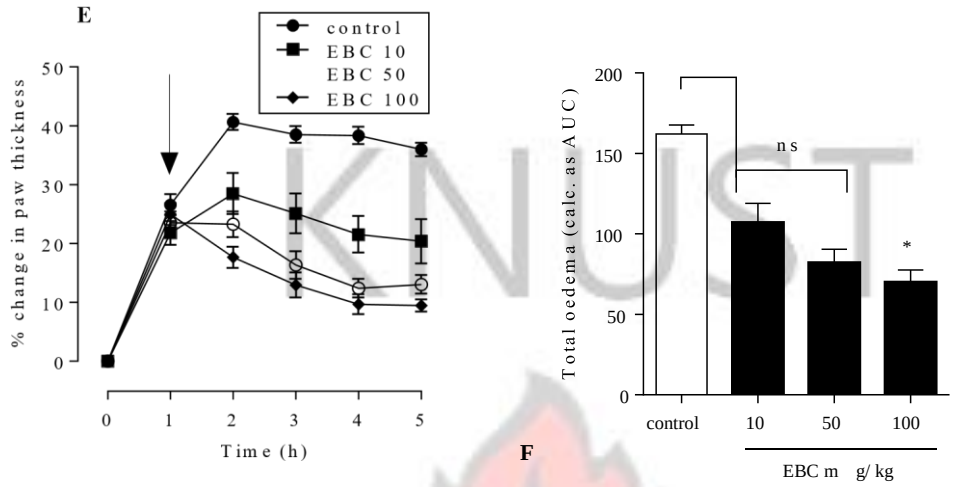


Figure 3.2 Effect of *Bombax costatum* extract on the maximal (A, C and E) and the total oedema responses (B, D and F) respectively in the therapeutic study of carrageenan-induced foot edema in chicks. Values are mean ± SEM (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test). Arrow denotes the time of drug administration.

The ED_{50} (dose responsible for 50 % of maximal effect) for the various drugs administered in both prophylactic and therapeutic studies of carrageenan-induced paw oedema was determined (as shown in Figure 3.3[A and B]). In the prophylactic study, the ED_{50} for diclofenac (10-100 mg/ kg, i.p), dexamethasone (0.1-1.0 mg/ kg, i.p) and EBC (10-100 mg/ kg, *p.o*) were 5.05 ± 1.597, 1.663 ± 0.300 and 14.47 ± 1.460 respectively (Figure 3.3A). In the therapeutic study, the ED_{50} of 6.225 ± 1.546 for diclofenac (10-100 mg/ kg, i.p), 1.327 ± 0.179 for dexamethasone (0.1-1.0 mg/ kg, i.p) and 9.209 ± 1.631 for EBC (10-100 mg/ kg, *p.o*) were recorded (Figure 3.3B). Dexamethasone and diclofenac were more potent than EBC; however, dexamethasone was more potent than diclofenac (Figure 3.3[A and B]).

A Prophylactic **B** T herapeutic

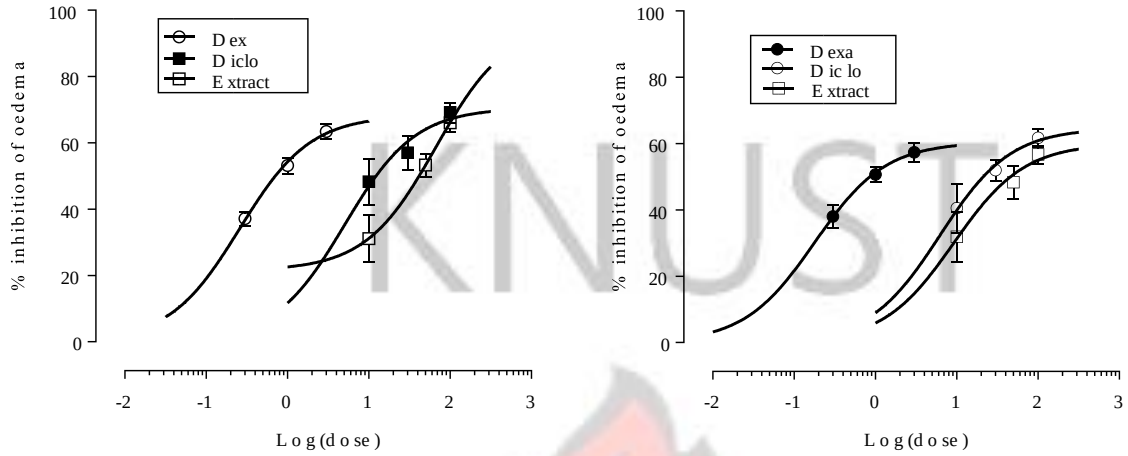


Figure 3.3 Dose-response curves for *Bombax costatum* extract, Diclofenac (10-100 mg/ kg, i.p), and Dexamethasone (0.1-1.0 mg/ kg, i.p) in both prophylactic and therapeutic studies of carrageenan-induced foot oedema in chicks.

3.1.3 ANTI-INFLAMMATORY EFFECT OF AQUEOUS ETHANOL EXTRACT OF *BOMBAX COSTATUM* ON CHRONIC INFLAMMATION

3.1.3.1 Cotton pellet granuloma tissue formation

Tissue granuloma formation was studied in this experiment, where granuloma formation was classified into different stages of inflammation over 8-day experimental period. Total granuloma tissue formation and the percentage inhibition for wet and dry cotton pellet weights of the various treatment groups are shown in Table (3.2).

In the wet weight analysis, the mean maximal tissue granuloma formed was dose-dependently suppressed significantly to 2.75 ± 0.18, 2.30 ± 0.77, 1.99 ± 0.30 for dexamethasone at 0.3, 1.0 and 3.0 mg/ kg; and 2.63 ± 0.18, 2.46 ± 0.11, 2.18 ± 0.19 for EBC at 10, 50 and 100 mg/ kg respectively as compared to the control with a maximal tissue granuloma formation of 4.27 ± 0.15 mg (Table

3.2). The total granuloma tissue attained was dose-dependently reduced significantly by 35.52 %, 46.11 %, 53.49 % for dexamethasone at 0.3, 1.0 and 3.0 mg/ kg; and
38.49 %, 42.41 %, 49.02 % for EBC at 10, 50 and 100 mg/ kg respectively (Table 3.2).

In the dry weight analysis, the mean maximal tissue granuloma formed was dose-dependently suppressed significantly to 1.28 ± 0.15, 1.03 ± 0.31, 0.88 ± 0.10 for dexamethasone at 0.3, 1.0 and 3.0 mg/ kg; and 1.45 ± 0.05, 1.19 ± 0.15, 1.00 ± 0.05 for EBC at 10, 50 and 100 mg/ kg respectively as compared to the control with a maximal tissue granuloma formation of 2.00 ± 0.05 mg (Table 3.2). The total granuloma tissue attained was dose-dependently reduced significantly by 41.67 %, 53.28 % 60.10 % for dexamethasone at 0.3, 1.0 and 3.0 mg/ kg; and
34.09 %, 45.71 % 54.55 % for EBC at 10, 50 and 100 mg/ kg respectively (Table 3.2).

Table 3.2 Effects of *B. costatum* on Cotton pellet granuloma inflammation in rats.

GROUPS	Mean weight of Wet cotton pellet (mg)	% Inhibition of tissue granuloma formation	Mean weight of Dry cotton pellet (mg)	% Inhibition of tissue granuloma formation
Control	4.27 ± 0.15	-	2.00 ± 0.05	-
Dexamethasone				
0.3 mg/ kg	2.75 ± 0.18*	35.52 %	1.28 ± 0.15**	41.67 %
1.0 mg/ kg	2.30 ± 0.77**	46.11 %	1.03 ± 0.31***	53.28 %
3.0 mg/ kg	1.99 ± 0.30**	53.49 %	0.88 ± 0.10***	60.10 %
EBC				
10 mg/ kg	2.63 ± 0.18*	38.35 %	1.45 ± 0.05*	34.09 %

50 mg/ kg	2.46 ± 0.11*	42.41 %	1.19 ± 0.15**	45.71 %
100 mg/ kg	2.18 ± 0.19**	49.02 %	1.00 ± 0.05***	54.55 %

Data presented as mean ± SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

In this study, the ED_{50} was determined for the various treatment protocols (wet and dry) (as shown in Figure 3.4). Dexamethasone (0.3-3.0 mg/ kg, i.p) recorded ED_{50} value of 3.156 ± 0.1784 for wet analysis (Figure 3.4A) and 2.401 ± 0.1543 for dry analysis (Figure 3.4B). EBC (10-100 mg/ kg, *p.o*) recorded ED_{50} value of 2.600 ± 1.987 for wet (Figure 3.4A) and 6.664 ± 1.579 for dry analysis (Figure 3.4B) respectively. Dexamethasone (0.3, 1.0 and 3.0 mg/ kg) was however more potent than EBC (10, 50 and 100 mg/ kg) in both treatment protocols.

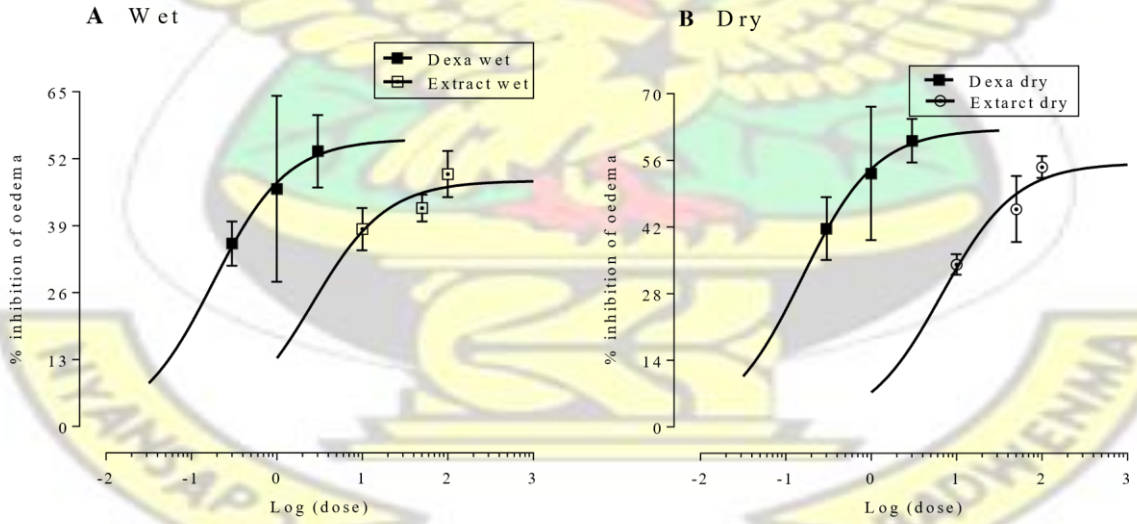


Figure 3.4 Dose-response curves for *Bombax costatum* extract and Dexamethasone (0.3-3.0 mg/ kg, i.p) in cotton pellet tissue granuloma formation study in rats.

3.1.3.1.1 Haematological analysis

Whole blood assessment of each treatment group for days (0 and 8) of the experimental period is shown in table 3.3. Results attained were analyzed and compared to the control-treated group. Analysis of blood sample on day 0 showed no significant change in blood indices compared to the control for dexamethasone (0.3, 1 and 3 mg/ kg, i.p) and EBC (10, 50 and 100 mg/ kg, *p.o*) (Table 3.3). However, on the 8th day, changes occurred in certain indices of the blood analysis compared to the control (Table 3.3). Dexamethasone-treated group significantly decreased the total WBC concentration at 0.3, 1.0 and 3.0 mg/ kg as compared to the control with total WBC of 7.40 ± 1.25 ($\times 10^3$ μL). Similarly, EBC significantly decreased WBC migration to the inflammatory site at 10, 50 and 100 mg/ kg as compared to the control (7.40 ± 1.25 × 10^3 μL) (Table 3.3).

Table 3.3 Haematological analysis of rats in cotton pellet granuloma inflammation.

	DAY 0				DAY 8			
	WBC ($\times 10^3/\mu L$)	HCT %	RBC ($\times 10^{-6}/\mu L$)	HGB ($g\ dL^{-1}$)	WBC ($\times 10^3/\mu L$)	HCT %	RBC ($\times 10^{-6}/\mu L$)	HGB ($g\ dL^{-1}$)
Control	5.83 ± 0.15	53.73 ± 2.52	9.13 ± 0.54	14.97 ± 0.22	7.40 ± 1.25	38.20 ± 7.50	6.20 ± 0.43	11.77 ± 0.73
Dexamethasone 0.3 mg/ kg	5.80 ± 0.32	51.40 ± 1.82	8.14 ± 0.19	14.93 ± 0.23	4.93 ± 0.18*	41.47 ± 0.27	7.44 ± 0.39	13.47 ± 0.32
1.0 mg/ kg	5.52 ± 0.17	53.80 ± 1.67	8.05 ± 0.20	14.70 ± 0.29	3.53 ± 0.32***	43.07 ± 4.19	7.56 ± 0.64	13.63 ± 1.43
3.0 mg/ kg	5.50 ± 0.23	52.47 ± 2.21	8.01 ± 0.21	14.74 ± 0.15	3.01 ± 0.05***	38.90 ± 1.44	7.21 ± 0.14	13.07 ± 0.15
EBC 10 mg/ kg	6.76 ± 0.50	49.70 ± 1.55	8.23 ± 0.36	14.27 ± 0.12	5.29 ± 0.58*	42.10 ± 2.19	7.66 ± 0.34	12.43 ± 0.98
50 mg/ kg	6.40 ± 0.53	52.03 ± 1.74	8.04 ± 0.15	14.73 ± 0.28	4.91 ± 0.12*	38.50 ± 0.50	7.59 ± 0.45	12.07 ± 0.44
100 mg/ kg	5.61 ± 0.32	48.63 ± 1.16	8.31 ± 0.31	14.57 ± 0.23	4.36 ± 0.29**	40.50 ± 2.86	7.54 ± 0.56	12.53 ± 1.05

Cotton pellets (40 ± 1 mg) were implanted subcutaneously (bilateral) in the groin regions of rats. The drug vehicle, dexamethasone (0.3-3.0 mg/ kg, i.p), and *Bombax costatum* extract were administered daily for 7 consecutive days. Blood sample was collected on the 8th day and a whole blood count was carried out. Data presented as mean ± SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to control-treated group. One-way ANOVA followed by Dunnet *post hoc* test was statistically performed.

3.1.3.1.2 Body weight change

Body weights of the rats in the various treated groups were observed over the 8-day experimental period. From the study, there was a general body weight loss in all the study groups over the 8day study period (Figure 3.5[A, B, C and D]). Dexamethasone-treated rats exhibited no significant change in both maximal and total body weight when compared to the control (Figure 3.5[A and B). Similarly, EBC-treated rats showed a loss in body weight over the study period (Figure 3.5C) except a steady increase in total body weight by 33.02 ± 7.46 % at 10 mg kg^{-1} when compared to the control (52.22 ± 10.69 %) (Figure 3.5D).

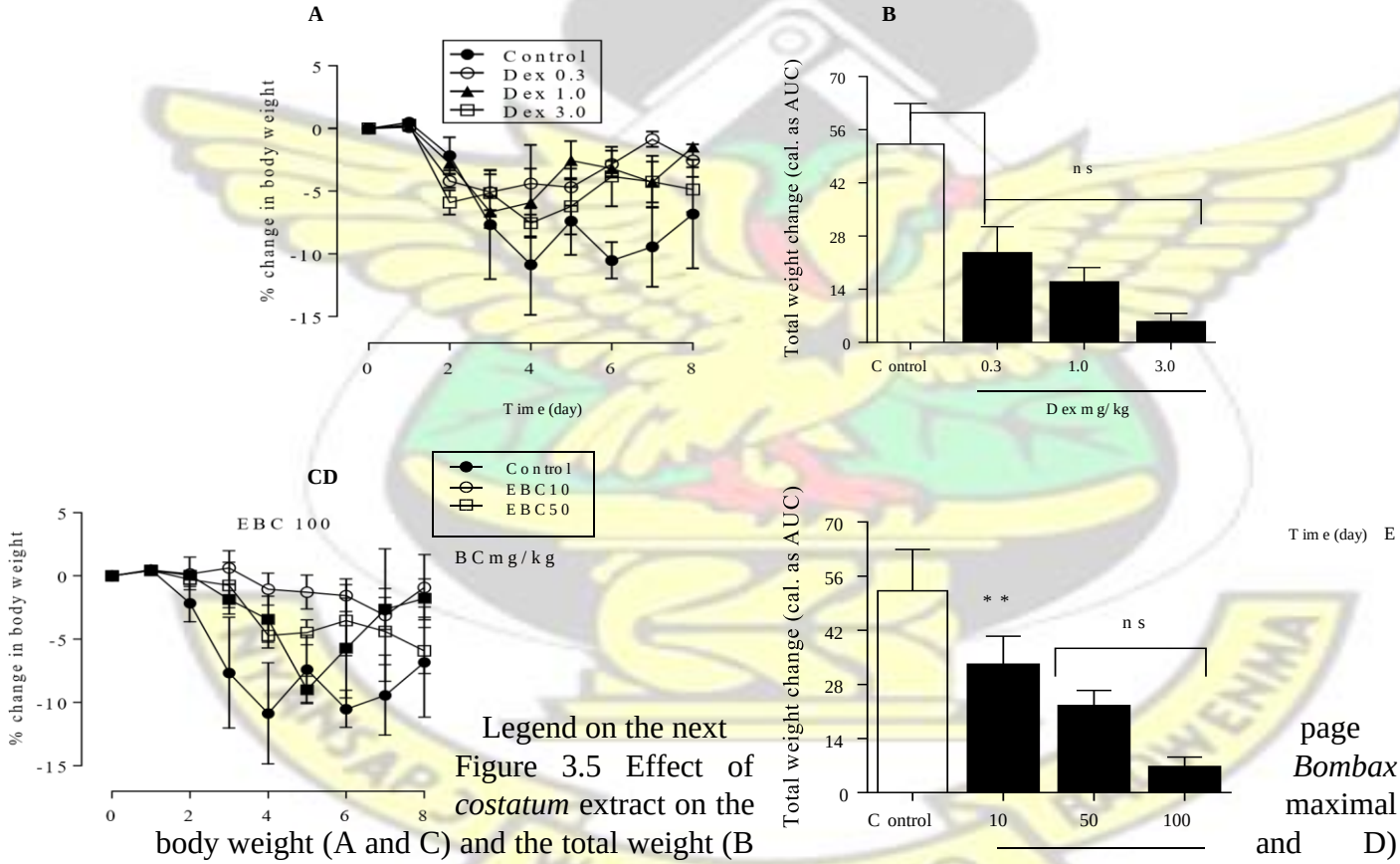


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Figure 3.5 Effect of *Bombax costatum* extract on the maximal body weight (A and C) and the total weight (B and D) respectively in cotton pellet granuloma formation in rats. Data presented as mean ± SEM (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

3.1.3.1.3 Spleen weight/ 100g of body weight of rats

The weight of spleen/ 100g of body weight of the rats were monitored in the various groups (Table 3.4). From the study, there was a general reduction of total spleen weight of the rats in each treatment group when compared to the control. Dexamethasone-treated group reduced the total spleen weight significantly by 18.63 %, 22.10 % and 28.00 % at 0.3, and 1.0 and 3.0 mg/ kg as compared to the control (Table 3.4). Similarly, EBC reduced the total spleen weight significantly by 13.41 % 15.05 % and 21.39 % at 10, 50 and 100 mg/ kg as compared to the control respectively (Table 3.4).

Table 3.4 Spleen weight/ 100g of body weight of rats in cotton pellet granuloma inflammation.

GROUPS	SPLEEN WEIGHT/ BODY WEIGHT
Control	0.93 ± 0.02
Dexamethasone	
0.3 mg/ kg	0.76 ± 0.03 **
1.0 mg/ kg	0.73 ± 0.04 ***
3.0 mg/ kg	0.67 ± 0.03 ***
EBC	
10 mg/ kg	0.81 ± 0.01 **
50 mg/ kg	0.79 ± 0.01 **
100 mg/ kg	0.73 ± 0.02 ***

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Cotton pellets (40 ± 1 mg) were implanted subcutaneously (bilateral) in the groin regions of rats. The drug vehicle, dexamethasone (0.3-3.0 mg/ kg, i.p), and *Bombax costatum* extract were administered daily for 7 consecutive days. The spleens were removed on the 8th day and weights

taken. Data presented as mean ± SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to control group. One-way ANOVA followed by Dunnet *post hoc* test was statistically performed.

3.1.3.2 Complete Freund's adjuvant-induced arthritis in rats

3.1.3.2.1 Oedema assessment: measured as maximal and total oedema effects

Complete Freund's adjuvant inoculation in rats resulted in the formation of oedema of the injected paw (ipsilateral) and spread systemically to the non-injected paw (contralateral) with time (Figure 3.6A, C and inserts). Paw volumes of the contralateral and ipsilateral limbs were measured before inducttion of arthritis (day 0) and every other day till day 28. Drug treatment commenced on day 14 after injection of CFA when the polyarthritic stage had begun. No significant change in paw volume of the IFA (non-arthritic group) was observed (Figure 3.6A). Methotrexate (1.0 mg/ kg, i.p) and dexamethasone (3.0 mg/ kg, i.p), significantly decreased the mean maximal oedema attained to 158.64 ± 11.54 % and 162.54 ± 10.18 % respectively as compared to the CFA (arthritic) control with a mean maximal swelling of 222.93 ± 13.42 % (Figure 3.6A). EBC dose-dependently suppressed the mean maximal oedema effect to 139.49 ± 8.04 %, 136.96 ± 9.71 % and 132.14 ± 12.33 % (Figure 3.6C) at 10, 50 and 100 mg/ kg respectively as compared to the control-arthritic group. The total oedema response was significantly reduced by 28.33 % for methotrexate (1.0 mg/ kg) and 35.29 % for dexamethasone (3.0 mg/ kg) respectively (Figure 3.6B). Similarly, EBC significantly decreased the total oedema attained by 26.14 %, 34.07 % and 40.00 % at 10, 50 and 100 mg/ kg in a dose-dependent manner respectively (Figure 3.6D).

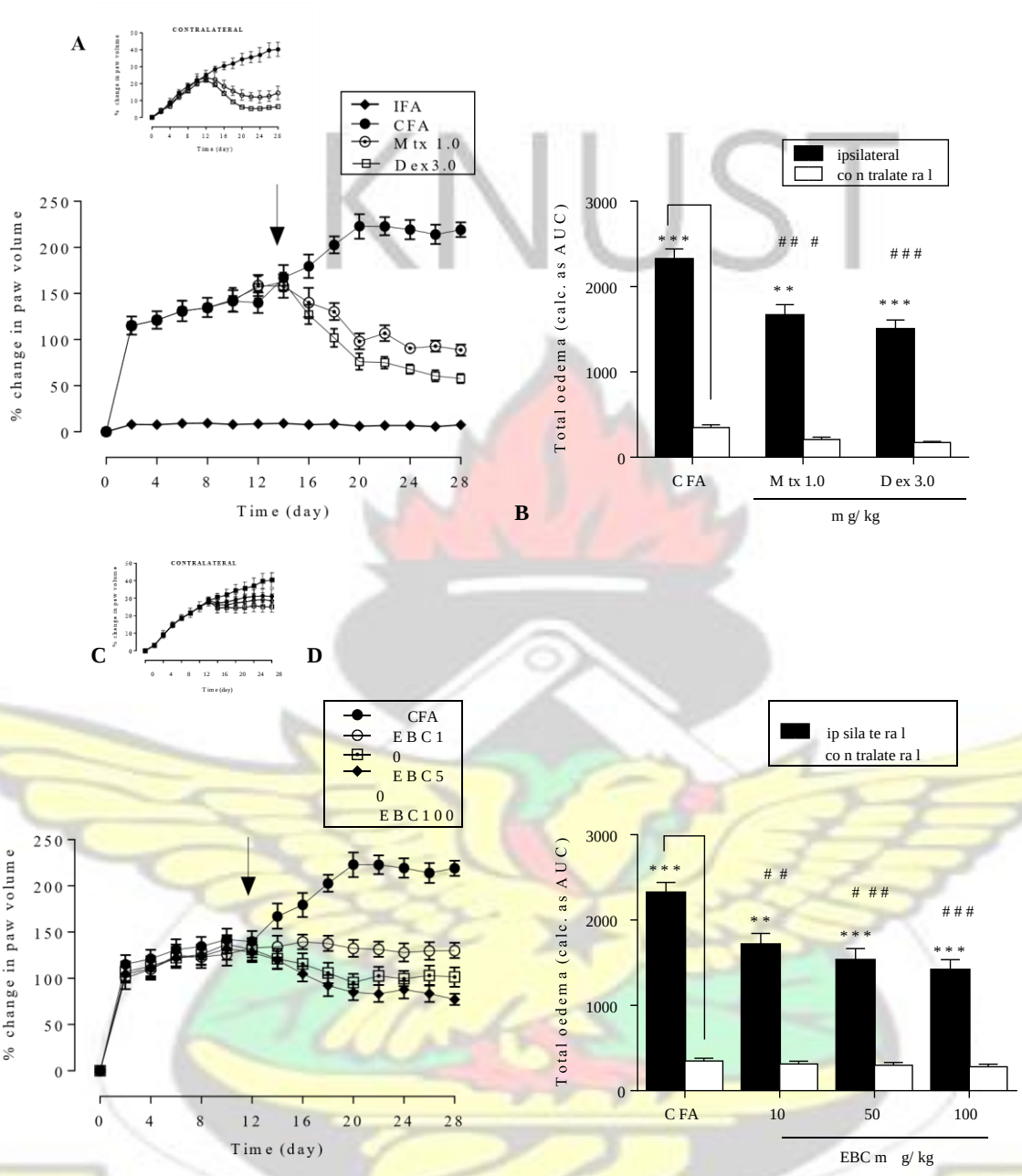
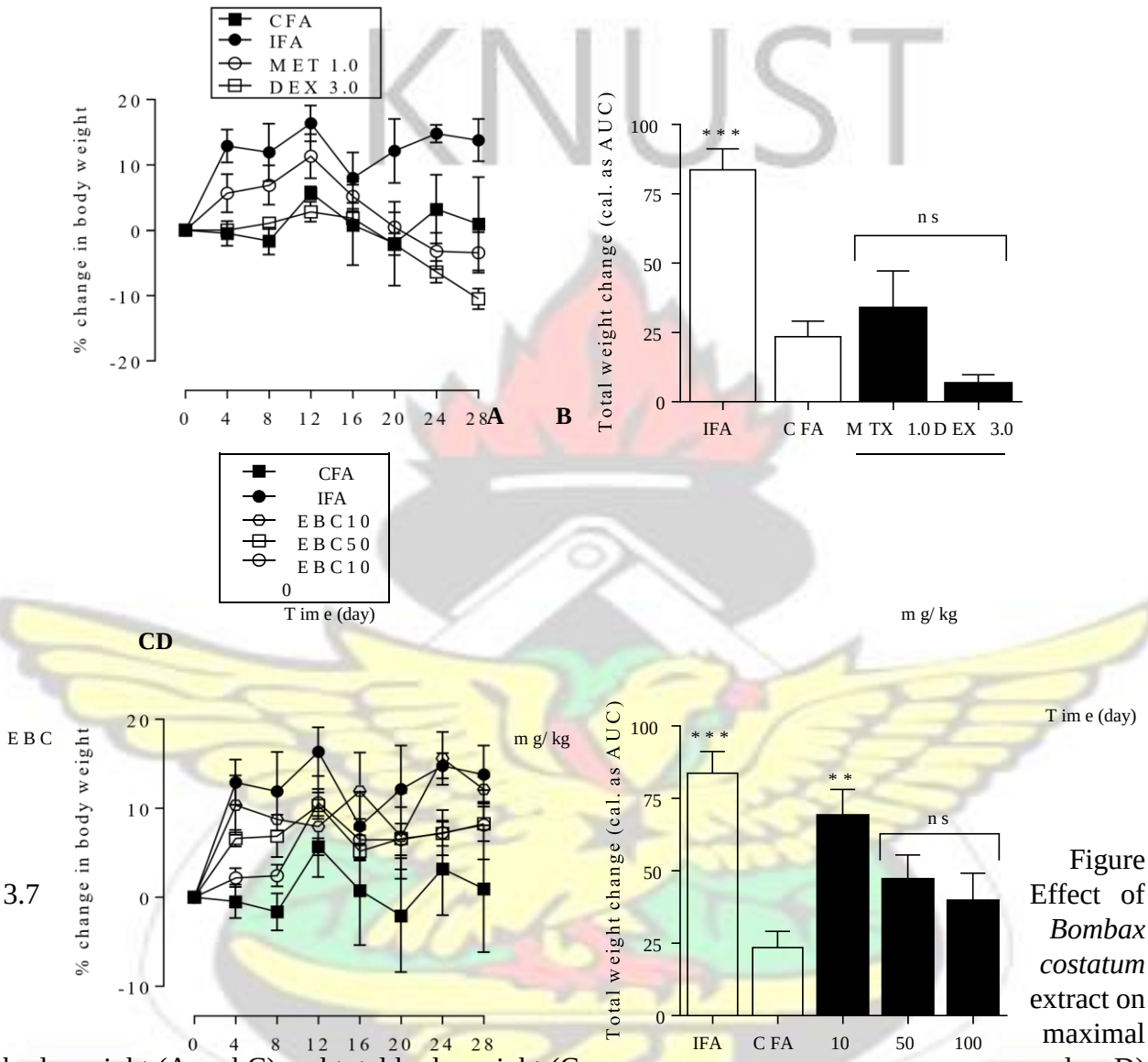


Figure 3.6 Effect of *Bombax costatum* extract on the maximal (A and C) and total oedema responses (B and D) in adjuvant-induced arthritis in rats respectively. The total oedema was calculated as AUC over 15-day treatment period. Data presented as mean ± SEM (n=5). #*P*<0.05; ##*P*<0.01; ###*P*<0.001 compared to control-treated group (Two-way ANOVA followed by Dunnet *post hoc* test). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to control-treated group (One-way ANOVA followed by Dunnet *post hoc* test). Arrow indicates day of drug administration

3.1.3.2.2 Body weight change

Body weights of rats were monitored every 4 days over the 28-day period of adjuvant-induced arthritis. The maximal and total change in body weights of rats were determined respectively (Figure 3.7[A, B, C and D]). CFA (arthritic control) group showed massive decrease in both maximal and total body weights of rats (Figure 3.7[A, B, C and D]). IFA (non-arthritic) group showed an increase in maximal body weight of 16.35 ± 2.75 % when compared to the CFA (5.71 ± 0.96 %) (Figure 3.7A). There was no significant change in body weight for methotrexate (1.0 mg/ kg) and dexamethasone-treated (3.0 mg/ kg) rats (Figure 3.7[A and B]). EBC-treated rats similarly showed an increase in maximal body weight of 15.59 ± 2.99 %, 10.31 ± 1.49 % and 10.61 ± 1.53 % at 10, 50 and 100 mg/ kg in a dose-dependent manner when compared to the CFA (5.71 ± 0.96 %) respectively (Figure 3.7C). IFA (non-arthritic) group increased the total body weight of rats by 83.68 ± 7.54 % when compared to the CFA (23.53 ± 5.57 %) (Figure 3.7B). There was a significant increase in total body weight for EBC by 69.06 ± 9.01 % at 10 mg/ kg when compared to the CFA (23.53 ± 5.57 %) (Figure 3.7D).



3.7 Figure Effect of *Bombax costatum* extract on maximal body weight (A and C) and total body weight (C and D) respectively in adjuvant-induced arthritis in rats. Data presented as mean ± SEM (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

3.1.3.2.3 Arthritic index

3.1.3.2.3.1 Photography

From the study, photographs of the injected paw of arthritic rats in CFA (arthritic control) and drug/ extract-treated groups were taken (Plate 3.1). There was no evidence of erythema and paw swelling in the IFA (non-arthritic) group (Plate 3.1A). CFA (arthritic control) group exhibited severe paw swelling and erythema of both injected (ipsilateral) and non-injected (contralateral) paw limbs (Plate 3.1B). Methotrexate (1.0 mg/ kg, i.p) decreased the paw swelling of both ipsilateral and contralateral paws significantly (Plate 3.1C). Dexamethasone-treated rats however, showed virtually no characteristic of paw swelling (Plate 3.1D). EBC (10-100 mg/ kg, *p.o*) reduced the erythema and paw swelling in the ipsilateral in a dose-dependent manner (Plate 3.1[E, F and G]).

In evaluating the arthritic index from the photographs, statistical analysis of significance was performed on the blind arthritic scored values for the IFA (non-arthritic) group, CFA (arthritic control) group and drug/ extract treatment groups as shown in Table 3.5. Paw swelling and erythema were significantly reduced by methotrexate (1.0 mg/ kg), dexamethasone (3.0 mg/ kg) in both ipsilateral and contralateral paw; and EBC (10-100 mg/ kg) reduced paw swelling and erythema in the ipsilateral limb but no significant score of the contralateral as compared to the CFA (arthritic control) group (Table 3.5) respectively.

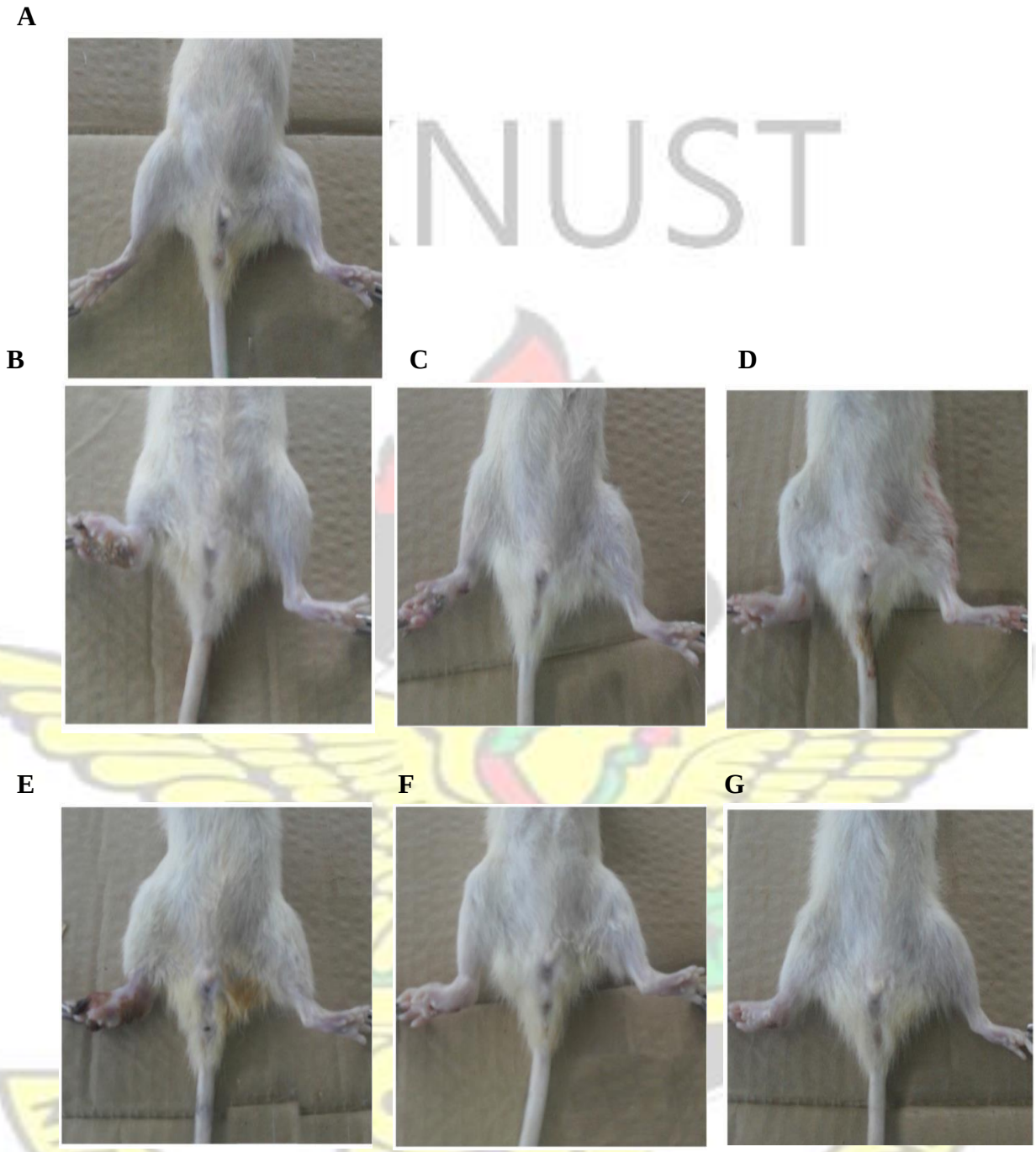


Plate 3.1 Effect of *Bombax costatum* extract on adjuvant-induced arthritis in rats. On day 29, rats were sacrificed by cervical dislocation and photographs of the paw limbs were taken. IFA (nonarthritic) group (A), CFA (arthritic control) group (B), Methotrexate (1.0 mg/ kg, i.p) (C), Dexamethasone (3.0 mg/ kg, i.p) (D) and EBC (10-100 mg/ kg, *p.o*) (E-G) respectively.

Table 3.5 Arthritic index for rats in Adjuvant-induced arthritis evaluated from photographs.

Groups	Arthritic (photograph) Index	
	ipsilateral	contralateral
IFA	0	0
CFA	3.80 ± 0.20	2.80 ± 0.20
Methotrexate		
1.0 mg/ kg	2.20 ± 0.37**	0.60 ± 0.24***
Dexamethasone		
3.0 mg/ kg	1.80 ± 0.37***	0.40 ± 0.24***
EBC		
10 mg/ kg	3.60 ± 0.24	2.40 ± 0.25
50 mg/ kg	2.60 ± 0.25*	2.00 ± 0.32
100 mg/ kg	2.40 ± 0.24**	1.60 ± 0.25

One-way ANOVA followed by Dunnet *post hoc* test. Data presented as mean ± SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to CFA control group.

3.1.3.2.3.2 Radiography

From the study, effect of EBC on X-ray radiography was assessed and shown in Plate 3.2. There were no characteristic signs of joint destruction, cartilage damage and bone loss of both ipsilateral and contralateral limbs in the IFA (non-arthritic control) group (Plate 3.2A). CFA (arthritic control group) showed the presence of severe joint destruction, bone loss and cartilage damage in the ipsilateral limb, which subsequently spread systemically to the contralateral limb over time during the experimental 28-day period (Plate 3.2B). Methotrexate (1.0 mg/ kg, i.p) and dexamethasone (3.0 mg/ kg, i.p) suppressed the systemic spread of arthritis characterized by massive bone loss, cartilage destruction and joint damage (Plate 3.2[C and D]) respectively. EBC showed significant inhibitory effect on bone loss, cartilage damage and joint destruction in the ipsilateral limb at 100

mg/ kg as compared to the CFA but however, there was no significant inhibition observed in the contralateral limb as compared to the CFA (arthritic control group) (Plate 3.2[E, F and G]).

In evaluating the arthritic index from the X-ray radiographs, statistical analysis of significance was performed on the blind arthritic values for the IFA (non-arthritic) group, CFA (arthritic control) group and drug/ extract treatment groups (Table 3.6). Characteristic arthritic conditions of cartilage destruction, bone loss and joint deformation were significantly inhibited systemically by methotrexate (1.0 mg/ kg), dexamethasone (3.0 mg/ kg) in both ipsilateral and contralateral paw limbs. EBC showed significant suppression on bone loss, cartilage damage and joint deformation at 100 mg/ kg when compared to the CFA (arthritic control group) (Table 3.6).

A

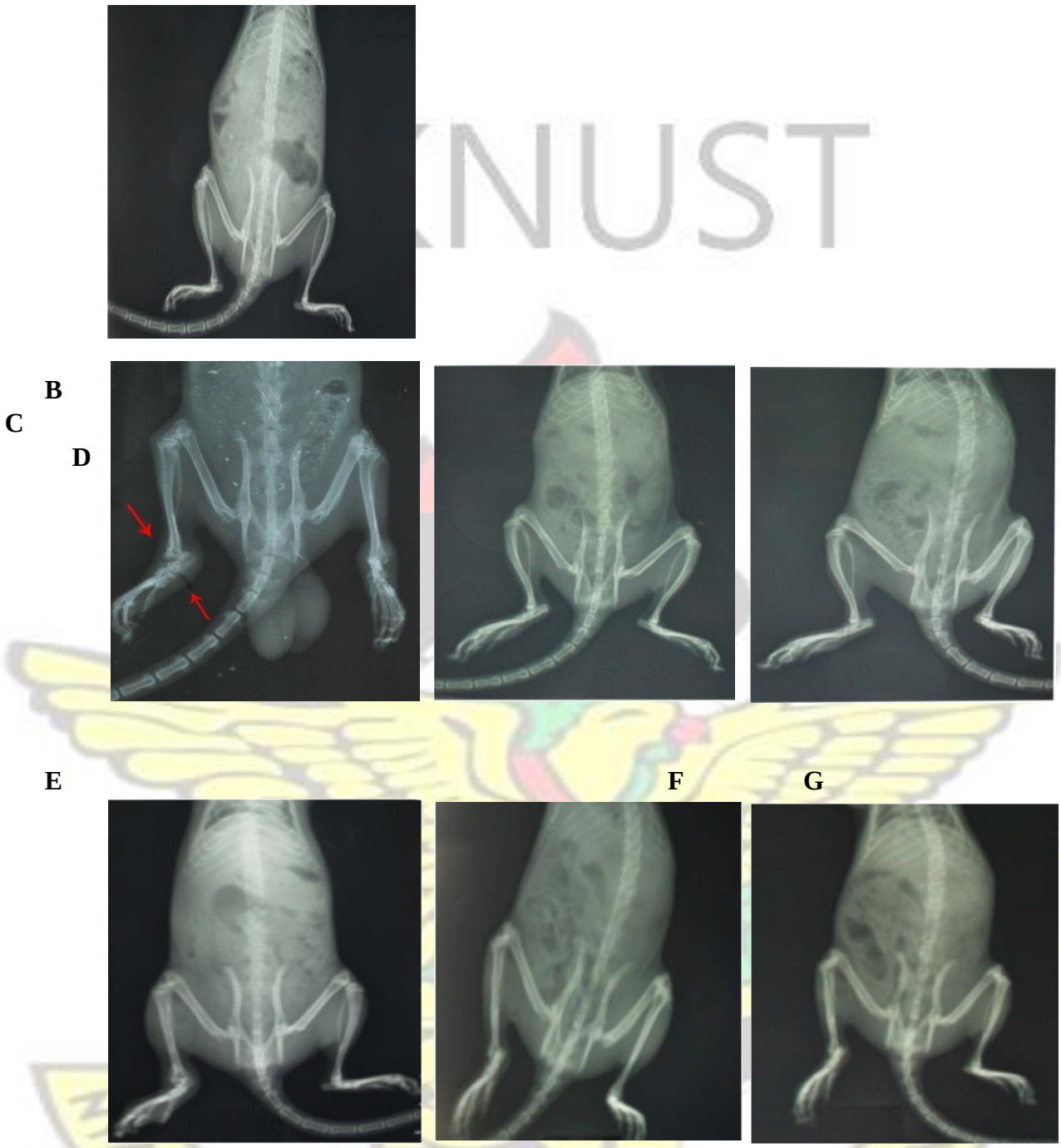


Plate 3.2 Effect of *Bombax costatum* extract on adjuvant-induced arthritis in rats. On day 29, rats were sacrificed by cervical dislocation and X-ray radiographs of the paw limbs were taken. IFA (non-arthritic) group (A), CFA (arthritic control) group (B), Methotrexate (1.0 mg/ kg, i.p) (C), Dexamethasone (3.0 mg/ kg, i.p) (D) and EBC (10-100 mg/ kg, *p.o*) (E - G) respectively.

Table 3.6 Arthritic index for rats in Adjuvant-induced arthritis evaluated from radiographs.

Groups	Radiological Index

	ipsilateral	contralateral
IFA	0	0
CFA	2.60 ± 0.25	2.40 ± 0.25
Methotrexate		
1.0 mg/ kg	0***	0***
Dexamethasone		
3.0 mg/ kg	0***	0***
EBC		
10 mg/ kg	2.40 ± 0.24	2.20 ± 0.20
50 mg/ kg	1.80 ± 0.20	2.00 ± 0.31
100 mg/ kg	1.40 ± 0.24*	1.80 ± 0.20

One-way ANOVA followed by Dunnet *post hoc* test. Data presented as mean ± SEM (n=5). *P<0.05; ** P<0.01; ***P<0.001 compared to CFA control group.

3.1.4 POSSIBLE MECHANISMS OF ACTION OF AQUEOUS ETHANOL EXTRACT OF *BOMBAX COSTATUM*

3.1.4.1 Indirect anti-histaminic activity of *Bombax costatum* in Clonidine- and Haloperidolinduced catalepsy in mice

Injection of clonidine or haloperidol (1 mg/ kg) subcutaneously into ICR mice (25-30 g) resulted in a cataleptic effect as previously described by Ferre *et al*. (1990). From the study, Chlorpheniramine (5 mg/ kg) decreased significantly the maximum duration of cataleptic response (30.00 ± 4.74 s) attained to 9.00 ± 0.82 s (Figure 3.8A). Similarly, EBC (10, 50 and 100 mg/ kg, *p.o*) dose-dependently reduced the maximum duration of cataleptic response (30.00 ± 4.74 s) attained significantly to 17.50 ± 1.94 s and 12.75 ± 0.85 s at 50 and 100 mg kg^{-1} respectively (Figure 3.8A). Chlorpheniramine (5 mg/ kg) suppressed the total cataleptic response produced over the 3 h by 69.80 % and EBC dose-dependently decreased the total cataleptic response

significantly by 48.63 % and 57.84 % at 50 and 100 mg/ kg when compared to the control respectively (Figure 3.8B).

From the study, chlorpheniramine (5 mg/ kg) and EBC- treated groups showed no inhibitory effect on the maximum and total cataleptic responses attained in haloperidol-induced catalepsy (Figure 3.8[C and D]).

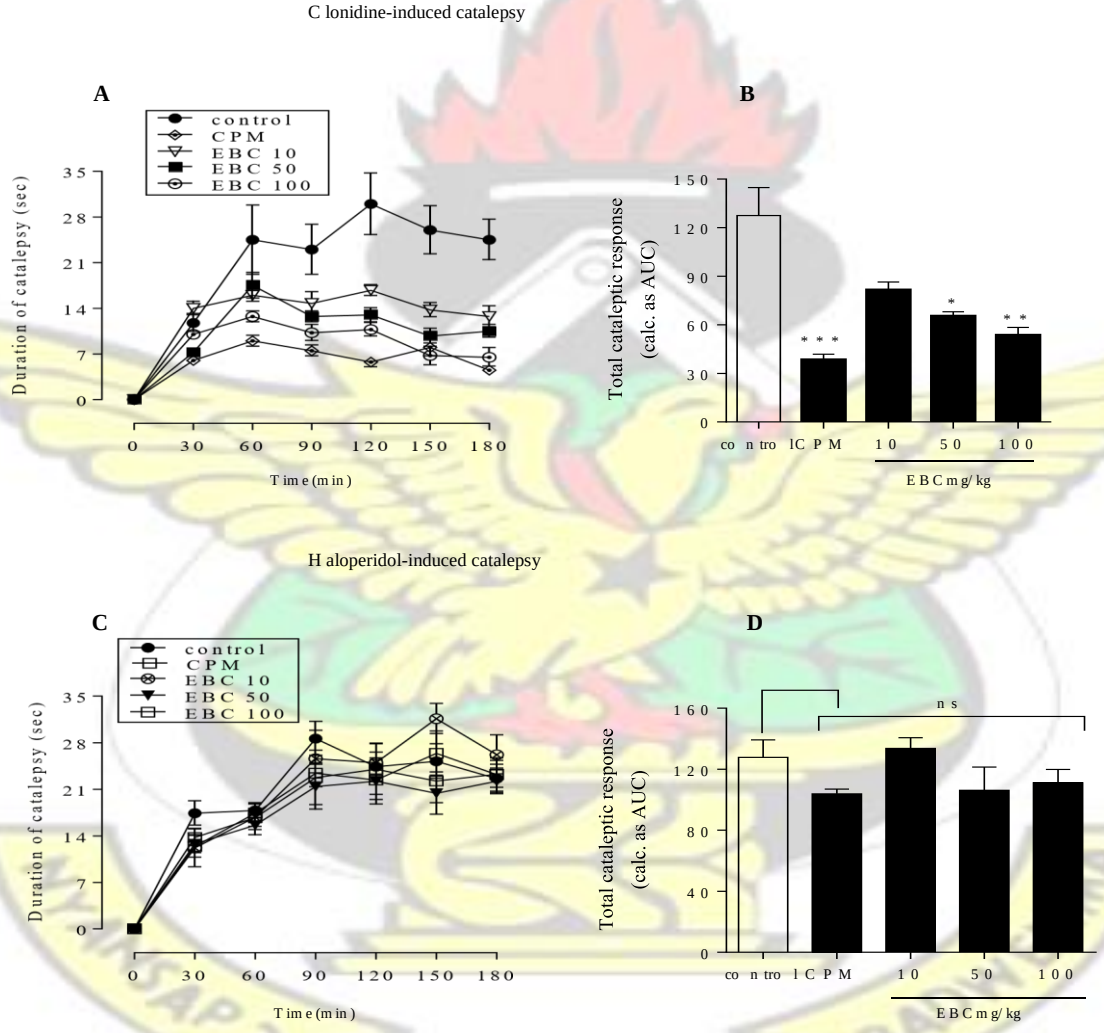


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Figure 3.8 Effect of *Bombax costatum* extract on the maximal (A and C) and the total cataleptic responses (B and C) respectively in the therapeutic study of clonidine- and haloperidol-induced

catalepsy in mice. Data presented as mean ± SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

3.1.4.2 Effect of *Bombax costatum* on PGE_2-induced paw oedema

Injection of 0.2 ml (1nM) prostaglandin E_2 into the subplantar tissue of right hind limb of rats (200-250 g) resulted in oedema formation. The effect of EBC (10-100 mg/ kg, *p.o*) on prostaglandin E_2-induced inflammation was investigated as shown in Figure 3.9. From the study, EBC significantly reduced the mean maximal paw oedema (80.31 ± 8.15 %) attained at 60 min to 40. 91 ± 5.73 % and 36.95 ± 7.14 % at 50 and 100 mg/ kg respectively in a dose-dependent manner (Figure 3.9A). Also, EBC significantly suppressed the total oedema induced over 3 h by 39.08 % and 53.06 % at 50 and 100 mg/ kg respectively in a dose-dependent manner (Figure 3.9B).

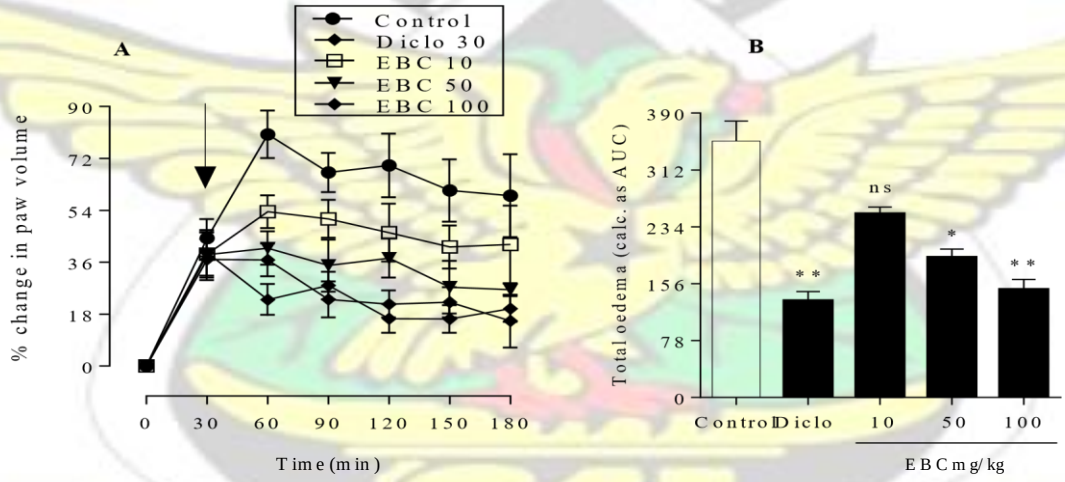

Figure 3.9 Effect of *Bombax costatum* extract on the maximal (A) and total oedema responses (B) respectively in the therapeutic study of prostaglandin E_2-induced paw oedema in rats. Data presented as mean ± SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to the controltreated group (One-way ANOVA followed by Dunnet *post hoc* test). Arrow denotes the time of drug administration.

3.1.4.3 Effect of *Bombax costatum* on serum cytokine (IL-6 and TNF-α) levels

Administration of 1 ml (4 % (v/v)) acetic acid solution trans-rectally into rats (200-250 g) resulted in prolonged activation of the intestinal immune system which plays a major role in the pathological events of chronic inflammation (Sartor, 1997). From this study, there was a significant rise in serum levels of IL-6 and TNF-α in acetic acid-induced colitic (AC) rats when compared to non-colitic (NC) rats. Serum level of IL-6 increased from 3.68 ± 1.07 pg ml^{-1} to 36.55 ± 2.40 pg ml^{-1} upon induction of colitis (Figure 3.10A). Sulphasalazine (300 mg/ kg) reduced significantly IL-6 serum level to 21.38 ± 2.67 pg ml^{-1} (Figure 3.10A). Similarly, EBC dose-dependently reduced IL-6 serum level significantly to 27.57 ± 1.91 pg ml^{-1}, 22.50 ± 1.70 pg ml^{-1} and 18.90 ± 2.11 pg ml^{-1} at 10, 50 and 100 mg/ kg when compared to the control respectively (Figure 3.10A).

TNF-α serum level increased from 16.34 ± 3.79 pg ml^{-1} to 84.23 ± 3.06 pg ml^{-1} upon induction of colitis (Figure 3.10B). Sulphasalazine (300 mg/ kg) significantly reduced TNF-α serum level to 51.70 ± 4.75 pg ml^{-1} and EBC dose-dependently reduced TNF-α serum level significantly to 69.85 ± 2.89 pg ml^{-1}, 60.25 ± 3.51 pg ml^{-1} and 51.39 ± 2.50 pg ml^{-1} at 10, 50 and 100 mg/ kg when compared to the control respectively (Figure 3.10B).

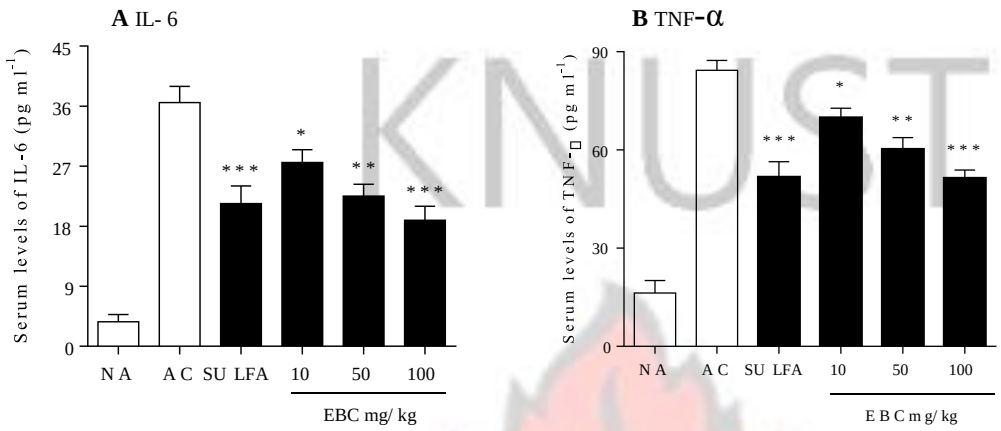

Figure 3.10 Effect of *Bombax costatum* extract on the total serum cytokine IL-6 (A) and TNF-α (B) levels in acetic acid-induced colitis in rats. Data presented as mean ± SEM (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

CHAPTER FOUR

DISCUSSION

Even though the various phytochemical constituents present in EBC have not been isolated and clinically tested therapeutically, yet the anti-inflammatory activity of *B. costatum* can be attributed to the existence of secondary metabolites present which is consistent with the findings observed in this study. Phytochemical analysis of the aqueous ethanol stem bark extract of *B. costatum* showed the presence of steroids, glycosides, triterpenoids, saponins, tannins, alkaloids, and flavonoids. These results agreed with the literature reported on the plant which showed these chemical constituents to be present by Atawodi *et al*., (2012). Phytochemical constituents of plants are known to illustrate anti-inflammatory effects through a series of mechanisms of action such as regulation of inflammatory cells (mast cells, neutrophils, macrophages and lymphocytes), anti-oxidative and radical scavenging activities (Yuva *et al*., 2013). They also regulate pro-inflammatory enzyme activities such as phospholipase A_2 (PLA_2), cyclooxygenase (COX), lipooxygenase (LOX) and nitric oxide synthase (NOS); and regulate the synthesis of other pro-inflammatory agents and gene expression (Yuva *et al*., 2013).

Saponins, for example, are found widely among plant materials and possess a wide range of medicinal activities including membrane-permeabilising, immunostimulant, anti-carcinogenic and anti-inflammatory (Francis *et al*., 2002). Tannins are astringenic agents known to form a protective layer in the skin and mucous membrane which is insoluble and can resist disease. This emphasizes the use of natural products containing tannins in the treatment of cuts and wounds, and inflammatory conditions of the digestive tract (Watal *et al*., 2014). Flavonoids are known to possess numerous medicinal activities including anti-nociceptive, anti-oxidant and antiinflammatory mechanisms by targeting reactive oxygen species (ROS) and arachidonic acid metabolism which are involved in the late phase of acute inflammation (Hesham *et al*., 2007;

Rotelli *et al*., 2003; Guardia *et al*., 2001; Rajnarayana *et al*., 2001). Flavonoids inhibit the phosphodiesterases implicated in cell activation and most of these effects are upon the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to the site of injury. Flavonoids inhibit the synthesis of prostaglandins which are involved in various immunologic responses and are the end products of the COX and LOX pathways (Moroney, *et al*., 1988; Meli *et al*., 1990). Alkaloids, which are the largest single class of phytochemical constituents, possess a range of remarkable therapeutic properties including anti-cancer, analgesic and ant-inflammatory activities (Gomes *et al*., 2009; Zulak *et al*., 2006; Barbosa-Filho *et al*., 2000). Therefore, it is not surprising that EBC exhibited anti-inflammatory effects on acute and chronic inflammation.

The most extensively used preliminary test in search for new, complementary and alternative anti-inflammatory drugs measures the ability of an agent to reduce oedema induced in animals by injection of a phlogistic substance (Chakraborty *et al*., 2004). Carrageenan-induced inflammation in chicks was employed initially to investigate the anti-inflammatory activities of the aqueous ethanol extract of *Bombax costatum* in both prophylactic and curative studies. This is an acute model which was previously described by Winter *et al*., (1962) and exhibit potential degree of reproducibility. Chicks were used in this study instead of the usually used rodents, validated by Roach and Sufka (2003). The use of chicks for this experiment is more economical and easy to handle. Additionally, it has been shown that intraplantar inoculation of carrageenan in 7-day old chicks presents measurable, reliable and relatively short-lived oedema state which is distinctively potentiated by systemic administration of typical inflammatory substance relating closely to the commonly used rodents (mice and rats) in the search for agents with antiinflammatory properties (Roach and Sufka, 2003). The carrageenan-induced inflammation produces a biphasic response in which the action of COX products of arachidonic acid metabolism and ROS (reactive oxygen

species) are well established. The first phase (from 0 to about 2 h) is mediated through the release of pro-inflammatory agents such as histamine, serotonin and bradykinin affecting vascular permeability (Crunkhon *et al*., 1971) whereas the second phase (> 2.0 h) is related to the release of prostaglandins and the production of inducible COX 2 peaking at 3 h (Panthong *et al*., 2004; Marrassini *et al*., 2010) and declined afterwards until the 5 h. EBC showed a significant dose dependent inhibitory effect on carrageenan oedema in both prophylactic and therapeutic studies. EBC was able to moderately reduce or/and inhibit inflammation during the early phase (first) which is mainly mediated by the release of histamine, serotonin and bradykinin. The late phase (second) is mainly mediated by the release of prostaglandins which is a major product of arachidonic acid metabolism via COX pathway (Rouzer and Marnett, 2009; Jawaid *et al*., 2011). According to Legler *et al*., 2010 and Claudino *et al*., 2006, deregulation of prostaglandins (PGs) synthesis causes a variety of inflammatory effects such as redness, swelling and pain as a result of increased vasodilation and microvascular permeability. EBC also induced a more significant inhibition of oedema in the late phase and this is supported by earlier observed findings in this study where EBC exhibited its anti-inflammatory effect either by inhibiting PGE_2 formation or release of PGE_2 in the metabolic arachidonic acid pathway.

The cotton pellet granuloma test is an inflammatory model used to assess anti-proliferative activity of agents capable of managing chronic inflammation (Dhawn *et al*., 2000). The cotton pellet implanted in rats subcutaneously undergo three stages of inflammatory response: a transudate stage that occurs during the first 3 h; an exudative stage that occurs between 3-72 h after inserting the cotton pellet and lastly, the proliferative stage that occurs between 3-6 days after cotton pellet implantation (Swingle *et al*., 1972). The proliferative phase is quantified as the increase in dry weight of tissue granuloma formation whilst the moist wet weight of the pellets correlates to

transudative phase (Kalpana *et al*., 2010). The cotton pellet is known to stimulate the immune system, producing antibodies and cytokines such as interleukins. This stimulation eventually results in the formation of granuloma tissue and proliferation of lymphocytes (Suleyman *et al*., 2003; Hosseinzadeh *et al*., 2000). Kinin, a vasoactive substance, is implicated to be main pro-inflammatory mediator of granuloma (Afsar *et al*., 2013). Hence suppression of the proliferative phase is an indication of a possible decrease in production of fibroblast, infiltration of cells, interleukins, kinins and synthesis of collagen and monopolysaccharides during granuloma tissue formation (Swingle *et al*., 1972; Perez *et al*., 2005). EBC effectively produced a dose dependent inhibitory effect on the granuloma tissue formation at the site of inflammation which is an indication of its positive activity on both the transudative and proliferative stages of inflammation consistent with literature reported by Lau *et al.*, (2009). EBC could possibly reduce the production of antibodies; inhibit interleukins, kinins and suppressed cell infiltration. This is supported by earlier findings in this study where EBC significantly decreased serum level of IL-6. It was noted that the higher doses of EBC showed marginal inhibitory effects on granuloma tissue formation.

During inflammatory reaction, more blood cells migrate to the site of inflammation. EBC showed no significant change in blood parameters at day 0. However, on the final day (8^{th} day), significant changes in blood indices of EBC was observed when compared to control-treated group. During inflammatory response, WBC migration and accumulation at the site of injury is a biological marker (Mishra *et. al*., 2010). Accumulation of leukocytes at the inflammatory sites is said to cause granuloma tissue formation (Owoyele *et al*., 2005). By the 8^{th} day, EBC significantly reduced the migration of WBC such as leukocytes and lymphocytes to the site of inflammation which eventually decreased the infiltration of neutrophils and exudation of fluid, and subsequently reducing oedema which is evidently consonant with literature reported by Perez *et al.*, (2005).

The weight of the spleen was examined at the end of the study. In cotton pellet granuloma inflammation, the spleen is known to enlarge as a result of its phagocytic nature (Mishra *et al*., 2010). EBC showed a significant reduction of the spleen weight compared to the control-treated group which revealed an enlarged spleen with an increase in weight which is consonant with literature reported by Mishra *et al.*, (2010). Consequently, EBC has been shown to possess inhibitory effect on the phagocytic nature of the spleen during inflammatory response.

Adjuvant-induced arthritis (AIA) is an experimental chronic inflammatory model widely used for preclinical screening of anti-inflammatory agents used in treating rheumatoid arthritis (Ferraccioli *et al*., 2010; Kelly and Genovese, 2013). The disease is characterized by infiltration of synovial membrane in association with destruction of joints which resembles rheumatoid arthritis in humans (Astusi *et al*., 2005; Plasqui, 2008). Bansod *et al*, 2011 reported that rheumatoid arthritis develops in three stages; the swelling of the synovial lining and swelling around the joints, the rapid division and growth of cell, or pannus, and finally, the inflamed cells release enzymes digesting the bone and cartilage resulting in shape loses in joints and loss of movement. Inflammatory reaction results in the production of prostaglandins E_2 (PGE_2), cytokines (IL-1ß and TNF-α), interferons (INF) and other reactive oxygen species (Zhang *et al*., 2015). In this study, EBC administered orally reduced the synovitis, swelling and joint inflammation as seen from the arthritic indices (photographs and X-ray) representations. Deductions made from the X-ray image representation revealed that EBC moderately modified systemic spreading of inflammation thereby inhibiting further destruction of the cartilages, joints and bones which is in accordance with literature reported by Astusi *et al*. (2005). Since PGs, cytokines such as interleukin-1, -2, and -6, TNF-α are implicated in the arthritis disease, EBC inhibited these pro-inflammatory proteins mainly produced

by macrophages and usually involved in up-regulation of inflammatory responses during inflammatory disorders such as rheumatoid arthritis (Zhang and An, 2007). This is supported by earlier observed findings of this study where EBC mitigated significantly the serum levels of IL-6 and TNF-α which is consonant with literature reported by Sundaram *et al*., 2015. EBC probably exhibited largely its antinflammatory effect on the arthritic condition without any immunologic effect on disease progression. Dexamethasone, a glucocorticoid, reduced increased bone resorption, oedema swelling, cartilage and synovial membrane destruction. It inhibited TNF-α, IL-1ß, IL-2, IL-6, prostaglandins and also exhibited immunologic effect on the spread of arthritis systemically from ipsilateral to the contralateral (Kirwan *et al*., 2007). DMARD, disease-modifying antirheumatoid drug such as methotrexate, is reported to be widely used as 'front-line' treatment for rheumatoid arthritis diseases (Svensson *et al*., 2003; Osiri *et al*., 2003; Alarcón, 2000; Emery *et al*., 2000). Moreland and O'Dell (2002) reported that methotrexate decreases joint destruction by regulating the functions of macrophages, and also inhibit the synthesis of pro-inflammatory cytokines such as IL-1, IL-2 and IL-6, interferon (INF)-γ and suppresses TNF-α activity (Omoigui *et al*. 2014; Mottram, 2003). From the study, EBC could be suggested to emulate the activity of dexamethasone and methotrexate by its inhibitory effect on pro-inflammatory cytokines IL-6 and TNF-α activities; and additionally, regulating the role of macrophages and modifying the disease progression. This assertion needs to be investigated further to confirm this action of EBC.

As reported by Syed-Uzair *et al*, (2013), change in body weight of the rats is a clinical indication of assessing disease progression and severity. Generally, EBC showed a loss in body weight in cotton pellet granuloma which is in an agreement with literature reported by Schopf *et al*., (2006) but not in adjuvant-induced arthritis. There was a marginal increase in body weight at 10 and 50

mg kg^{-1} as compared to the arthritic-treated (CFA) group. The loss of body weight by EBC at 100 mg kg^{-1} could be attributed to adverse effects such as loss of appetite and low rate of metabolism in the rats and this observation needs to be ascertained to justify the toxicity effects of *Bombax costatum*.

According to Boini and Guillemin, (2001), radiographs are used as an outcome measure to assess the severity and progression of arthritic disease, and also to establish the effects of treatment. This has become an important exercise providing a permanent data with which the disease can be sequentially evaluated (Sharp, 1989; Brower, 1990; Van der Heijde *et al*., 1999). EBC significantly reduced massive bone loss which is as a result of increased bone resorption and decreased bone formation and this is consonant with literature reported by Gravallese *et al*., (2000).

Clonidine, presynaptic α_2-adrenoceptor agonist, usually induces catalepsy in a dose-dependent fashion in mice by releasing histamine from mast cells in the brain. This is blocked by Histamine H_1 receptor antagonist but not antagonist of H_2 receptors (Jadhav *et al*., 1983). Schwartz (1997) further carried out investigations to discover histamine containing mast cells in the brain. Mast cell degranulation results in the release of pro-inflammatory mediators such as histamine, prostaglandins, cytokines as well as leukotrienes (LTs) which are involved in inflammatory reactions (Bradding *et al*., 2006; Dawicki and Marshall, 2007; Abraham and St. John, 2010; Theoharides *et al*., 2012). Uvnas (1969) investigated the correlation between mast cell degranulation and the release of histamine after administration of Compound 48/80, a mast degranulating agent. Chopra and Dandiya (1975) indicated the direct correlation of the different stages of catalepsy with brain histamine content. According to Benly, (2015), histamine is known to cause many inflammatory effects such as increased vascular permeability, smooth muscle contraction, and vasodilation. Numerous drugs are said to induce catalepsy in animals but with

different mechanisms, however, neuroleptic agents are known to induce catalepsy by suppressing expression of dopamine D_2 receptors in the substantia nigra (Sanberg, 1980). As reported by Herbert, (2002), unlike clonidine, haloperidol is a typical neuroleptic agent that induces catalepsy in rodents and extrapyramidal adverse effects in humans. This is a known non-selective Dopamine D_2 antagonist that primarily induces catalepsy via blockade of D_2 receptors in the straiatum. Neuroleptic-induced catalepsy is mainly inhibited by agents that increase dopamine transmission since it is associated to a blockade of postsynaptic striatal D_1 and D_2 receptors (Herbert, 2002; Farde *et al*., 1992). EBC exhibited significant inhibitory effect on the cataleptic responses produced in clonidine-induced catalepsy in the mice and this is in agreement with literature reported by Dhanalakshmi *et al*., (2004) which showed that clonidine-induced catalepsy is inhibited by extracts having anti-histaminic activity. EBC possibly inhibited histamine in clonidine-induced catalepsy which is evident in the earlier findings and in agreement with literature reported by Lakadwala *et al*., (1980). The histaminic inhibitory effect of EBC on catalepsy may be attributed to a mast cell stabilizing property it could possess. Nevertheless, EBC showed no inhibitory effect on haloperidol-induced catalepsy, only suggesting an indirect anti-histaminic effect rather than acting on the dopaminergic transmission.

4.2 CONCLUSION

Preliminary phytochemical analysis of EBC revealed the presence of steroids, tannins, glycosides, saponins, triterpenoids, alkaloids, and flavonoids. EBC exhibited inhibitory effects on both acute and chronic inflammation, and the arachidonate pathway of inflammation. EBC has been shown to possess anti-histaminic activity and decreased serum cytokine (IL-6 and TNFα) levels during inflammatory response.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The outcome of this study has supplied the scientific proof to endorse the use of the stem bark of *B. costatum* as anti-inflammatory agent in the management of inflammatory conditions. The antiinflammatory effects might be partially or wholly due to:

- The inhibitory effects of EBC on carrageenan-induced foot oedema (acute), cotton pellet granuloma formation and adjuvant-induced arthritis (chronic) inflammation.
- The inhibitory effects of EBC on histaminic pathway and competitive antagonistic effect at H_1-receptor.
- The inhibitory effects of EBC on prostaglandin E_2-induced paw oedema and as a result inhibit the arachidonate pathway of inflammation.

- The significant inhibitory effect on pro-inflammatory cytokine (IL-6 and TNF-α) levels during inflammatory response.

5.2 RECOMMENDATIONS

Further research investigations need to be carried out on *B. costatum* to improve on the acquired scientific knowledge. The areas to be considered are:

- Further assessment should carried out on the toxicity profile of *B. costatum* both acute and chronic studies in order to ascertain the safety in its folkloric use.
- Isolation and characterization of the active compounds responsible for the antiinflammatory activity of *B. costaum* should be done.

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