EVALUATION OF THE ANTI-INFLAMMATORY EFFECTS OF THE HYDROETHANOLIC EXTRACT OF *HOLARRHENA FLORIBUNDA* IN MURINE MODELS OF INFLAMMATION

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DECLARATION

I, Stephen Antwi, hereby declare that except for references of other people, which has been duly recognized and acknowledged, this research is the result of my own investigations, and that, this work has neither wholly nor partially been submitted elsewhere for another degree.

……

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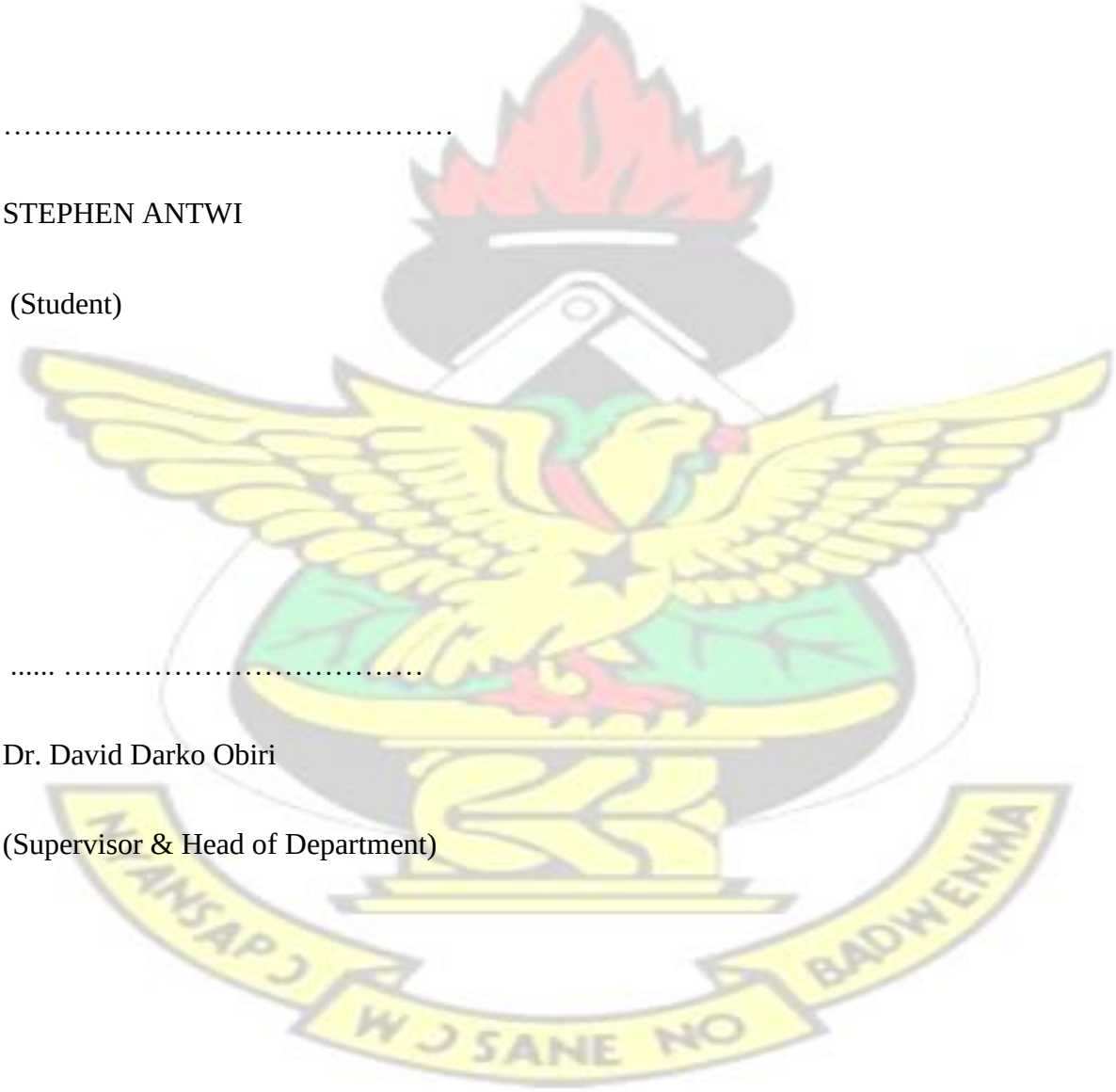

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ABSTRACT

Holarrhena floribunda in Ghanaian folk medicine is claimed to be used to treat inflammatory diseases. To validate its anecdotal use, a 70% ethanolic extract of *Holarrhena floribunda* (HFE) was prepared and evaluated on murine models of inflammation. The effect of HFE on acute inflammation was evaluated using the carrageenan-induced paw oedema model in rats. HFE (50, 200 and 500 mg/kg) dose-dependently and significantly inhibited paw oedema. The total paw oedema induced over 4 h was inhibited by 39.85 ± 11.19, 61.00 ± 7.35 and 65.41 ± 3.85 % respectively prophylactically and by 33.43 ± 6.83, 48.33 ± 6.83 and 56.55 ± 2.05 % respectively when administered therapeutically. Administration of HFE dose- dependently suppressed compound 48/80-induced mouse systemic anaphylactic shock (0, 16 and 50 % respectively for the 50, 200 and 500 mg/kg HFE). HFE dose dependently protected rats from LPS-induced anaphylactic shock on both prophylactic and therapeutic approaches with maximum protection of 20 % and 40 % respectively. Pinnal inflammation reaction model was employed in the passive cutaneous anaphylaxis study. The antigeninduced inflammation was significantly ($p<0.001$) suppressed in a dose dependent (44.77%, 69.89% and 80.69%) manner. The anti-arthritic effect of HFE was evaluated using the adjuvant-induced arthritis model in rats. HFE (50, 200 and 500 mg/kg) dose-dependently and significantly inhibited swelling of the injected paw and prevented the systematic spread of arthritis to the uninjected paw. The maximal inhibition in total paw oedema in rats given prophylactic and therapeutic treatments was observed to be 78.21 % and 67.92 % respectively. HFE also showed inflammation alleviation properties in indices monitored such body weight, radiology, histology, and haematology. HFE dose dependently inhibited histamine-induced paw oedema and exhibited an indirect anti-histaminic activity in clonidine-induced catalepsy test. HFE also showed inhibitory effects on serotonin and prostaglandin E_2-induced paw oedema. HFE inhibited the serum expression of IL-1α, IL-6 and protein kinase C and enhanced the expression of IL-10, Cyclic adenosine monophosphate and protein kinase A levels in chronic inflammation. Phytochemical screening of the ethanolic extract of the stem bark of *Holarrhena floribunda* indicated the presence of saponins, phenolic compounds, alkaloids and reducing sugars which possibly could be responsible for its observed inhibitory effects on acute and chronic inflammation.

DEDICATION

This work is dedicated to the memory of my late father Mr. Dominic Dodoo Antwi, my mum Cecilia Afua Ayorkor, Jarred-Stephen Antwi my Son, Theodosia Akuokor Antwi my wife, Clara Efia Lewis a dear friend and all my siblings for their loyal support throughout my education and life. The ultimate dedication goes to the Almighty God who has been my help and „all that to me.“

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ABBREVIATIONS

·OH	hydroxyl radical
CCL11:	C-C motif chemokine 11
COX:	Cyclooxygenase
CYP P450	Cytochrome P450
CYP2C9:	Cytochrome P450 2C9
CYP3A:	Cytochrome P450 3A
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
ICR:	Imprinting Control Region
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IL	Interleukin
LD_{50}	lethal dose fifty

CHAPTER ONE

INTRODUCTION

1.1 INFLAMMATION

Inflammation is a defensive mechanism in organisms which causes the local build-up of products of catabolism, which leads to the elevation of tissue osmotic pressure and it attendant fluid attraction, with or without the production of heat (Stankov, 2012). It involves the body‟s attempt to rid itself of harmful substances, injury or uncharacteristic stimulus and initiates the process of healing. Inflammation is a precursor in many disease conditions and its outcomes happen to be dreadful if treatment is unsuccessful and is allowed to progress from the initial acute phase to the chronic phase. Inflammatory cells together with mediators have been indicated to be critical in the pathophysiology of numerous diseases, particularly rheumatoid arthritis, asthma, chronic dermatitis, coronary artery disease, rhinitis and multiple sclerosis (Theoharides and Cochrane. 2004; Van-Assche *et al.*, 2011; Mandhane *et al*., 2011; Chung, 2012).

Inflammation occurs in host organism when the organism responds to microorganisms invasion or following damage to tissue (Alessandri *et al.*, 2013). It is a defensive reaction and is usually restricted to the cells/tissue in the area of infection (Iwalewa *et al*., 2007). It is complex and initiated by a countless number of factors of a physical, biological and chemical nature and usually result in cell/tissue damage or death (O‟Byrne and Dalgleish, 2001) if not properly controlled. The process usually involve the action of host cells, blood vessels and proteins working together to eradicate the original cause of the damage, eliminate the damaged cells/tissue and also commence the process of repairing the damage caused to cells /tissue.

The progression of inflammation is very intricate but divided mainly into two distinct parts namely, acute and chronic inflammation.

1.1.1 Acute inflammation

Acute inflammation is the first step of the inflammatory cascade. The cascade begins with primarily a vascular response after infection or damage to sterile tissue. The main processes that occur during this phase of inflammation include a brief localized constriction of arterioles stimulated by the contractions in smooth muscles around these arterioles. Subsequent, dilatation of the arterioles that supply blood to the damaged region causes increased localized blood flow causing hyperemia. Increases in the permeability of outer walls of blood vessels lead to extravasation of plasma proteins and fluid into the affected area. The acute inflammation episode is very rapid and happens within minutes of the infection or damage to cells (Cotran *et al.*, 1999; Lawrence *et al*., 2002). The cells involved in acute inflammation are mainly mast cells, neutrophils, eosinophils, macrophages and monocytes. These leucocytes in the presence of infection or damaged tissue interact with the endothelial cells of capillaries. The interaction is mediated by selectins on the leucocytes (L selectins) and the endothelial cells (E and P selectins) (Petri *et al*., 2008). This interaction leads to further interaction with chemotractant factors such as eotaxin/CCL11 and leukotriene on the endothelium which leads to up regulation of integrins which promote further binding of leucocyte to the endothelial cells. Consequently, leukocytes move on vascular endothelial walls where they find suitable openings and they migrate into the interstitial spaces (Phillipson *et. al.*, 2006). The migrated leucocytes in tissue provide a wide array of inflammatory mediators which range from vasoactive amines such as histamines and serotonin, lipid

mediators (eicosanoids), cytokines, chemokines, and reactive oxygen species which propagate the inflammatory process.

A component of the acute inflammatory response sometimes manifests as allergy. An allergic reaction which is life-threatening is termed anaphylaxis. Most anaphylactic reactions are immunologic in nature and mediated by the action of immunoglobulin E (IgE) on mast cells. The mast cell is the main effector cell among other cells such as the basophil, neutrophils and oesinophils that mediate allergic responses as well as fatal anaphylactic reactions (Amira *et al.*, 2011). The activation of mast cells causes a series of signalling mechanisms resulting in an initial degranulation causing the expulsion of mediators stored in vesicles such as histamine, tryptase heparin and chymase. This is followed by the breakdown of membrane phospholipids to form arachidonic acid which is metabolised by cyclooxygenases and lipoxygenases to form prostaglandins, thromboxanes and leukotrienes and later the gene expression of a range of cytokines and chemokines (Ogawa and Grant, 2007). These locally generated mediators lead to a local increase in vascular permeability which is one of the characteristic features of allergic inflammation (Burke and Miles, 1958). The mediators are paracrine in nature and as such initiate, recruit and activate other cells and mediators which contribute to the advancement of the symptoms observed in anaphylactic reaction (Ogawa and Grant, 2007).

As reported by Hallett *et al*., (2008) acute inflammation predominantly protects the host organism, is self-regulatory and as such advances to the point where the inflammation is resolved. Alessandri *et al.*, (2013) stated that the process of resolving inflammation is an active one encompassing various inflammatory cells/mediators and mechanisms that control the cessation of the reaction by reducing the recruitment of various leukocytes and the reversal of the dilation of blood vessels as well as vascular permeability. Alessandri and his team further

stated that other pathways include the shift from the generation of proinflammatory mediators to anti-inflammatory ones; the termination of signals linked with cytokine generation and leukocyte survival, apoptosis of recruited inflammatory cells and their subsequent clearance. The fate of acute inflammation as documented by Freire and Van Dyke (2013) dwells on the balance between mediators/cells that augment or control the inflammatory reaction. Deregulation or incessant triggering of acute inflammation, tend to be detrimental to the cells/tissue and subsequently leads to progression from an acute to a more chronic situation that is termed inflammatory disease which results in necrosis and fibrosis (Alessandri *et al.*, 2013; Freire and Van Dyke, 2013).

1.1.2 Chronic inflammation

The causes of chronic inflammation include persistent infections, prolonged exposure to potentially toxic agent‟s and autoimmunity. Persistent infection and prolonged exposure to toxic substances lead to delayed hypersensitivity reaction which prevents acute inflammation from resolving leading to granulomatous reactions. Auto-antigens and common environmental substances also incite immune responses to an organisms own cells/tissues leading to chronic inflammatory diseases like rheumatoid arthritis, multiple sclerosis and chronic allergic diseases, such as bronchial asthma. Chronic inflammation is implicated in the development of several degenerative diseases such as atherosclerosis, arthritis, inflammatory bowel disease, aging and other neurodegenerative central nervous system depression (O‟Byrne and Dalgleish, 2001; Dalgleish and O‟Byrne, 2002).

A typical protracted inflammation episode is characterized by infiltration with numerous leukocytes such as monocytes, macrophages, lymphocytes and plasma cells as a result of persistent infections, prolonged exposure to potentially toxic agent‟s and efforts at restoring

homeostasis or normal condition leading to replacement of damaged tissue with necrotic and fibrous tissue. According to Akbar and Salmon, (1997), the resolution phase in chronic inflammation is disordered, and leads to the continuous presence of inflammatory exudate, tissue damage and scarring.

Chronic inflammation is primarily mediated by cells and chemical mediators that perpetuate the pro-inflammatory response. There is an interplay of mast cells, monocytes, macrophages, T and B lymphocytes and chemical mediators, including histamine, thrombin, cysteinyl leukotrienes, oxidants, IL-1, TNF-alpha, and eicosanoids. Monocytes mature into macrophages on entering tissues, phagocytize bacteria, foreign substances, and also discharge various mediators that perpetuate the pro-inflammatory response. Infected cells are destroyed by T lymphocytes whilst antibodies are produced by B lymphocytes against attacking microbes for destruction at the later stages of chronic inflammation. The persistence of a large number of these cells and mediators in the tissue propagate the chronic inflammatory episode.

1.1.3 Mediators of inflammation

The body‟s reaction to tissue damage encompasses a myriad of interactions involving various antibodies, cells, proteins, antimicrobial peptides and connective tissue features that prevent tissue damage and eventually restores normal tissue integrity (Davidson, 1992).

During the initial phase of a local inflammatory response, after tissue injury or infection, there is an initial vasodilation and increase in permeability in vascular endothelium leading to large numbers of leukocytes being recruited from peripheral blood to the inflamed tissue site (Butcher, *et al.*, 1999). Cells involved in the inflammatory process include mast cells, basophils, neutrophils, eosinophils, macrophages and monocytes.

These cells in tissue are essentially the source of a range of inflammatory agents such as histamine, serotonin, bradykinin, cytokines, chemokines, lipid mediators (eicosanoids) and reactive oxygen species (ROS) (Grutzkau *et al*., 1998; Boesiger *et al*., 1998, Galli, *et al.*, 2005). The role of inflammatory cells and numerous mediators in the inflammation process is very critical both for perpetuating the process and also resolving the inflammation.

1.2 MANAGEGENT OF INFLAMMATORY DISEASES

The management of inflammation involves a diverse group of agents with each agent having a specific therapeutic basis for its usage and as such target specific cells and mediators involved in the inflammatory process. There are the leukotriene receptor antagonists which reduce the effect of the leukotrienes such as constriction of airways in the lungs, the excessive secretion of mucus, mucosal edema, enhance airway hyper-reactivity, chemoattractants effects for eosinophils in the airway (Gauvreau *et al*., 2001). The antagonists, therefore, improves the functioning of the lung, reduce excessive responsiveness of the bronchus, and reduce the number of attacks in asthmatic patients (Barnes, 2000).

The non-steroidal anti-inflammatory drugs (NSAIDs) are a group of chemically unrelated compounds with similar biological capabilities used in the treatment of pain and inflammation (Burke *et al.*, 2006). The NSAIDs reduce or eliminate the erythema, swelling, elevated temperature and pain caused by a variety of inflammatory stimuli. Based on their selectivity towards cyclooxygenase, NSAIDs are categorized into three main groups, the non-selective COX inhibitors, the highly selective COX-2 inhibitors and lastly the preferential COX-2 inhibitors. The anti-inflammatory effects of NSAIDs are primarily achieved through the inhibition of COX-2 and subsequently the synthesis of prostaglandins (Vane and Ferreira,

1979). NSAIDs are mainly metabolized by CYP2C9 or CYP3A families of P450 enzyme in the liver while renal excretion is the main route for elimination. Prolong clinical use elicits numerous side effects , notable amongst them are gastric erosion, ulceration, haemorrhage, bronchospasm, kidney and liver dysfunction (Lin *et al.*, 2006). These unwanted negative effects arise from the obstruction of the physiological effect of prostacyclin, prostaglandin E_2 and thromboxane A_2. On the other hand, treatment with NSAIDs shifts the arachidonic acid pathway towards 5-LOX leading to the production of pro-inflammatory, broncho-constrictive and gastro-damaging leukotrienes (Gilroy *et al*., 1998; Martel-Pelletier *et al*., 2003).

The most effective anti-inflammatory and immunosuppressive therapeutic agents available are the natural and synthetic glucocorticoids. Glucocorticoids are extensively used to treat inflammatory diseases such as bronchial asthma, systemic lupus erythematous, viscidities, Wegener„s granulomatosis, giant cell arthritis, psoriasis, rheumatoid arthritis, Grave„s disease and multiple sclerosis along with sepsis (Rhen and Cidlowski, 2005). The Glucocorticoids are plagued with numerous adverse effects ranging from metabolic diseases and osteoporosis to increased risk of cardiovascular disease (Wei *et al.,* 2004; Souverein *et al.,* 2004; de Vries *et al.,* 2007; Vegiopoulos and Herzig, 2007). Despite these many adverse effects the glucocorticoids remain a mainstay for reducing inflammation.

The Disease-modifying anti-rheumatic drugs (DMARDs) are another class of drugs which are used mainly because of their long term beneficial effects in preventing the progress of disease activity in chronic inflammation. They are not used for their immediate analgesic or anti-inflammatory. DMARDs interfere directly with immune cells or their function to reduce inflammation. According to Kemper *et al.,* (2012) the mechanism of action of the DMARDs involves suppression the body's overactive immune systems. The DMARDs are characteristically identified as either biologic or non-biologic (Burfeind, 2008).

Endogenous antioxidants e.g. ceruloplasmin, transferring, hepatoglobin, albumin and enzymes such as superoxide dismutase and catalase, found in human plasma bind with transition metals like iron and copper and control the production of metal catalysed free radicals (Halliwell and Gutteridge, 1990; Ivanova and Ivanov, 2000). Exogenous antioxidants such as ascorbic acid, vitamin E, carotenoids and phenolic compounds isolated from plants such as flavonoids, phenolic acid, polyphenols and quinines exert their antioxidant effect by scavenging free radicals or donating a hydrogen atom. Superoxide dismutase and catalase have proved to be extremely protective in quite a few ROS mediated inflammation such as carrageenan-induced pleurisy in rats (Greenwald, 1990; Lesnefsky, 1992; Salvemini *et al.,* 2001).

Currently there is renewed interest in natural compounds, particularly food supplements and drugs of plant origin for the management of inflammation (Reynolds et al., 1995). The mechanisms of action of these remedies are reported to involve inhibiting cyclooxygenase, nuclear factor-kB (NF-kB), tumour necrosis factor alpha and interleukin-1 inflammatory pathways. Omega-3 essential fatty acids, Curcumin, Green tea, and Boswellia serrata resin are a few documented natural compound used in the management of inflammation.

Holarrhena floribunda is one of such plant which is used in Ghanaian folkloric medicine to manage inflammatory diseases.

1.3. *HOLARRHENA FLORIBUNDA*

1.3.1. Botanical description

Holarrhena floribunda is small tree that that is found mainly in West Africa and grows up to 25 m tall. The tree is laden with copious quantities of white latex in all parts giving it the

common name “false rubber”. Its flowers are white and scented and the fruits are made up of two long and slender follicles, which are light grey to brown in colour (Burkill, 1985).

Fig. 1.1 The stem, whole plant leaves and flowers of *H. floribunda*

Source: www.worldagroforestry.org

1.3.2 Geographical distribution and uses

H. floribunda occurs in relic deciduous, closed woodland forests, sometimes in fringing and riverine forests in West Africa (Hutchinson and Dalziel, 1937).

The leaf extracts has been used to treat convulsion in Nigeria and a combination of the stembark and leaves are used to treat various afflictions such as malaria, fever, dysentery, amoebic diseases, diarrhoea, female sterility, amenorrhea and diabetes (Arbonnier, 2002;

Fotie *et al.*, 2006; Bayala *et al.*, 2006). The stems are used for the building of granaries due to its ability to resist the damaging effect of *Prostephanus truncatus*, a pest of stored grain (Kossou, 1992). In Ghana the white wood is used for making carvings, combs, axe handles (Abbiw, 1990).

1.3.3 Phytochemical constituents

Badmus *et al*., (2010) has documented that *Holarrhena floribunda* contains a myriad of alkaloids ranging from those derived from conanine in the stem and root barks to those derived from pregnane in the leaves and conessine and conamine in the seeds. It also contains active compounds of saponins, tannins, and steroidal glycosides and active compounds of trichothecenes Badmus *et al*., (2010).

1.3.4 Pharmacological properties

H. floribunda is reported to poses several biological and pharmacological properties. Saponins, steroidal glycosides and alkaloids from the plant inhibited the growth of *Escherichia coli*, *Bacillus subtilis* and *Candida albicans* (Goutarel, 1964; Bogne *et al.*, 2007). Conessine isolated from the stem bark is used to treat dysentery resulting from *Entamoeba histolytica* (Bogne *et al.*, 2007) and also has demonstrated activity against drugresistant strains of *Plasmodium falciparum* (Fotie *et al.*, 2006). Antioxidant activity against radicals like $OH^{\bullet}$, DPPH, $NO^{\bullet}_2$, and lipid peroxidation inhibition of the methanolic leaf extract of HF, have also been reported (Badmus *et al*., 2010). *H. floribunda* also contains polyphenolic compounds, which inhibit platelet aggregation (Bogne *et al.*, 2007).

1.4 JUSTIFICATION, AIMS AND OBJECTIVES OF STUDY

1.4.1 JUSTIFICATION

Current therapeutic agents for the management of inflammatory ailments target cells and mediators but are reported to lack specificity and are plagued with untoward side effects (Dhikav *et al.,* 2002). The NSAIDs are plagued with the usual adverse effects on the gastrointestinal tract. Oral disease modifying anti-rheumatic drugs like TNFα blockers and immunosuppressants have proven to be partially efficacious and toxic which has adversely prohibited further development (Genovese, 2009). These agents however have demonstrated clear efficacy in translational animal studies (Kumar *et al.*, 2003). The glucocorticoids are also reported to be ineffective in anaphylactic situations (Choo *et al*., 2010) and become effective when administered in conjunction with an inhibitor of phosphatase (Obiri *et al.,* 2012). Glucocorticoids also lead to the development of symptoms of the Cushing‟s effect. In the light of these adverse effects there is the urgent need for the development of potent alternatives with fewer side effects.

Newman and Gordon, (2007) stated that products of a natural origin are pivotal in the unearthing of leads for the research and development of drugs for the treatment of human diseases and that 63% of small molecules chemical entities released between 1981 and 2006 were natural products, derivatives of natural products or synthetic products based on the pharmacophore of natural products and their derivatives. In recent times, herbal medicines have gained importance in the treatment of many diseases due to their significant effect and lesser side effects as compared to allopathic medicines (Gill *et al.,* 2011). Yuri *et al.,* (2007) posits the rise in usage and popularity of medicines of plant origin stems from the fact that these medicines are perceived to be efficacious. Hills *et al.,* (2006) have also reported on an increase in the demand for traditional medical services, and attributed this increase to the

formalization of these medical services, improved quality and safety standards in the preparation, and the use of plant materials as an alternative therapy. Traditional medicine is used by about 80% of Africans to meet primary healthcare needs (Kumara, 2001). It is also established that the use of medicinal plants and traditional health care is affordable, familiar and is available at the local level (Danquah *et al.*, 2011). There are medicinal plants of interest, which herbal medicine practitioners claim are useful in the management and prevention of several diseases. The setback in the usage of these herbal medicines in Africa is the lack of documentation on the safety and efficacy (Adebayo *et al.*, 1997). One of such plants which is used in Ghanaian folkloric medicine to manage inflammatory diseases is *Holarrhena floribunda*

1.4.2 AIMS AND OBJECTIVES

Though *H. floribunda* has been used in Ghanaian traditional medicine in the treatment of inflammatory conditions, it has not been validated scientifically. This work, therefore, seeks to evaluate *H. floribunda* to determine its effects on various models of acute and chronic inflammation to validate its anecdotal use.

The objectives of the study include the following:

1. To evaluate the effect of the ethanolic extract of *H. floribunda* on both acute and chronic inflammation.
2. To assess the mechanism(s) of action of the *H. floribunda*

CHAPTER TWO

PLANT COLLECTION, EXTRACT PREPARATION, PHYTOCHEMICAL SCREENING AND ACUTE TOXICITY STUDIES

2.1 PLANT COLLECTION AND EXTRACT PREPARATION

2.1.1 Plant collection

The stem bark of *Holarrhena floribunda* was collected from Abetifi in the Eastern region of Ghana in September 2012 with the help of an herbalist. The plant was identified and authenticated by the Plant Development Department of the Centre for Plant Medicine Research (CPMR), Mampong Akuapem. A specimen voucher numbered 05/12 has been kept at the herbarium.

2.1.2 Preparation of ethanolic extract *of Holarrhena floribunda*

The stem bark of *H. floribunda* was washed thoroughly, chopped into pieces, allowed to airdry and milled into a coarse powder. The powdered stem bark (1 kg) was macerated in five litres (5L) of 70 % v/v ethanol with periodic stirring, decanted after 72 hours and filtered. The ethanol was evaporated on a rotary evaporator and the aqueous concentrated extract freeze-dried to obtain a 73.33 g powdered extract representing a yield of 7.33 % (w/w) of raw plant material. The powder was referred to as hydroethanolic extract of *H. floribunda* (HFE) and stored at 4^{o} C until used.

2.2 PHYTOCHEMICAL SCREENING

The chemical constituents of *H. floribunda* were determined according to the methods described by Trease and Evans (1989). The classes of compounds that were analysed were either hydrophilic or lipophilic compounds.

2.2.1.1 Phytochemical screening for hydrophilic compounds

Two (2) g of HFE was reconstituted in 20 ml water, aliquoted and used for the following tests

2.2.1.1.1 Saponins

A 2 ml aliquot was shaken for between 30 seconds to 1 minute in a test tube. The appearance of froth that persisted for about 2 minutes indicated a positive test for saponins.

2.2.1.1.2 Cyanogenic glycoside

To 2 ml aliquot a drop each of chloroform and picric acid were added and placed in a water bath. A change in colour of picric acid from yellow to brownish red indicated the presence of cyanogenic glycosides.

2.2.1.1.3 Phenolic compounds

$FeCl_3$ (2 -3 drops) was added to a 2 ml aliquot. A blue black colouration indicated the presence of phenolic compounds.

2.2.1.1.4 Polyuronides

Two drops of acetone was added to a 2 ml aliquot and observed. A precipitate indicated the presence of polyuronides.

2.2.1.1.5 Reducing sugars

Equal volumes of Fehling‟s A and B solutions were added to a 2 ml aliquot, allowed to stand in a water bath for 10 minutes and observed for precipitate to indicate the presence of reducing sugars.

2.2.1.1.6 Alkaloids

To a 2 ml aliquot, 1 ml ammonia (NH_3) solution was added and shaken in a separating flask. Chloroform in a ratio 1:1 was added and swirled. This was allowed to settle and the organic layer evaporated and reconstituted with 5 ml HCl. The solution was then filtered and 1 ml Mayer‟s reagent added. The presence of a precipitate in a creamy solution indicated alkaloids.

2.2.1.2 Phytochemical screening for lipophilic compounds

The presence of lipophilic compounds was determined after refluxing to rid the extract of lipids. To reflux, 10 ml of 2 N HCl was added to 100 ml of reconstituted HFE and placed in a water bath for 2 hours. To 2 ml of the refluxed HFE in separating flask 20 ml of diethyl ether was added, swirled and separated into an organic and inorganic layer.

2.2.1.2.1 Triterpenes/Phytosterols

Four (4) ml of the organic layer was evaporated to dryness and then reconstituted with equal volumes of acetic anhydride and chloroform (1:1) and then dehydrated with anhydrous Na_2SO_4. Three (3) drops of concentrated H_2SO_4 was then added. A wine colouration indicated

the presence of triterpenes whereas green colour indicated phytosterols and a brownish layer indicated a positive test for both triterpenes and pytosterols.

2.2.1.2.2 Flavonoids

Four (4ml) of the organic layer was evaporated to dryness and reconstituted with 5 ml methanol. A Mg ribbon and 2 drops of concentrated HCl were then added. A reddish or pink colouration indicated flavonoids.

2.3 DETERMINATION OF MEDIAN LETHAL DOSE (LD_{50})

A single dose of 5000 mg/kg *HFE* was administered orally to Sprague-Dawley rats (200 – 220 g, n=6) and Imprint control region (ICR) mice (20 – 30 g, n=6). The rats and mice were observed over a 48 h period for number of deaths and general behaviour. Surviving animals were observed further for a period of 12 days for signs of toxicity such as the arrangement of hairs, condition of eyes, movement and breathing.

2.4 RESULTS

2.4.1 Phytochemical analysis

Table 2.1 Phytochemical constituent of *Holarrhena floribunda*

Groups of Phytochemicals	Results
Saponin	Present

Reducing Sugar	Present
Triterpenes	Absent
Phytosterols	Absent
Flavanoids	Absent
Phenolic compounds	Present
Polyuronides	Absent
Alkaloids	Present
Cyanogenic glycosides	Absent

2.4.2 Determination of median lethal dose (LD_{50})

No deaths were recorded within 48 h after a single oral dose of 5000 mg/kg of HFE was administered to the rats and mice. There were also no physical signs of toxicity as evidenced by normal movement and breathing of the rats and mice. There were also no signs of bulging of eyes or any lachrymatory effects and no piloerection.

2.5 DISCUSSION

The phytochemical screening of the hydroethanolic extract of *Holarrhena floribunda* revealed the presence of saponins, reducing sugars, alkaloids and phenolic compounds in agreement with preliminary phytochemical screening done elsewhere on the plant (Badmus *et al.*, 2010; Gnangoran *et. al.,* 2012). These active metabolites are possibly responsible for its pharmacological effect. The pharmacological and biological properties of various plants are ascribed to the existence of secondary metabolites such as phenolic compound, reducing sugars, flavonoids, tannins, and terpenoids in the plants.

For example saponins, a diverse set of active glycosides found principally in lower marine animals, plants and some bacteria (Francis *et al.,* 2002) have been documented to possess several pharmacological properties. Prominent among these properties is the antiinflammatory property. This is supported by the findings that saponins isolated from particular parts of *Schwenkia americana, Asparagus africanus,* D*ichrostachys cinerea*, *Ficus iteophylla* and *Indigofera pulchra* have inhibitory effects on carrageenan-induced inflammation (Hassan *et al.,* 2012). Similarly phenolic compounds, an important group of diverse secondary metabolites produced by plants and characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups are confirmed to possess biological effects. Prominent among these effects are the anti-inflammatory, anti-allergic, anti-arthritic, antimicrobial, anti-diabetic, and analgesic properties (Miles *et al*., 2005; Cheeke *et al*., 2006; Song-Chwan *et al*., 2008; Sergent *et al*., 2010).

Badmus *et al*., (2010) reported that *Holarrhena floribunda* contains a myriad of alkaloids, mainly conanine from the stem bark and root bark, steroidal alkaloids in the leaves and conessine, norconessine, conamine and conarrhimine in the seeds. Alkaloids are one of the major groups of plant secondary metabolites with a wide range of pharmacological and biological properties. The anti-inflammatory properties of several alkaloids isolated from plants have been documented by several researchers (Souto *et al.,* 2011).

The median lethal dose (LD_{50}) of HFE was determined to be greater than 5000 mg/kg. At this dose of the extract no deaths and physical signs of toxicity were recorded in Sprague Dawey (SD) rats and Imprint control region (ICR) mice. The implication of this observation is that doses less than the 5000 mg/kg might be relatively safe when administered to SD rats and ICR mice.

2.6 CONCLUSION

Preliminary phytochemical screening of the hydroethanolic extract of the stem bark of *Holarrhena floribunda* indicated the presence of saponins, phenolic compounds, reducing sugars and alkaloids. The LD_{50} has been found to be greater than 5000 mg/kg in both rats and mice.

CHAPTER THREE ACUTE ANTI-INFLAMMATORY ACTIVITY OF THE HYDROETHANOLIC

EXTRACT OF *HOLARRHENA FLORIBUNDA*

3.0 INTRODUCTION

Acute inflammation is the first step of the inflammatory cascade and it is primarily a vascular response after infection or damage to sterile tissue. The main processes that occur during this phase of inflammation include dilatation of arterioles and an increase in the permeability of outer walls of blood vessels that supply blood to the damaged tissue. These processes result in the extravasation of plasma proteins and fluid into the affected tissue causing oedema. The acute inflammation episode is very rapid and happens within minutes of the infection or damage to cells (Cotran *et al.*, 1999; Lawrence *et al*., 2002). The cells involved in acute inflammation are mainly mast cells, neutrophils, eosinophils, macrophages and monocytes. The greatest setback in currently available potent orthodox drugs (mainly the NSAIDs, and

glucocorticoids) used in the management of acute inflammation lie in their toxicity. The search for alternatives remains a major priority of research and development, with a great diversity of plants used locally to manage inflammation providing a useful source for the discovery of new compounds.

Holarrhena floribunda is one of such plants used in Ghanaian folkloric medicine for the management of inflammation. A hydroethanolic extract of the plant was therefore evaluated for its effects on acute inflammation by means of the carrageenan-induced paw oedema test, 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

Male Sprague-Dawley rats (SDR, 200 – 220 g) were obtained from the Animal house of the Centre for Plant Medicine Research (CPMR) and fed on feed obtained from the Ghana Agro Food Company in Tema, Ghana. The animals were housed in metallic cages with soft wood shavings as bedding, under ambient laboratory conditions (temperature 28°C ± 2°C, relative humidity 60% – 70%, and a normal light/dark cycle of 12 h each) and allowed access to sterilized drinking water *ad libitum*. The animals were handled gently throughout the experimentation period in accordance with internationally accepted principles of laboratory animal use and care (EEC Directive of 1986: 86/609 EEC). Additionally all animal experiments were approved by Ethics Committee of the CPMR, Mampong Akuapem.

3.1.1.2 Drugs and chemicals

λ-Carrageenan and diclofenac (Sigma-Aldrich, St Louis, USA).

3.1.2 Methods

3.1.2.1 Carrageenan-induced paw oedema

Paw oedema was induced by a method earlier described by Winter *et al*., (1962). SDRs (n=6) were injected with 1 % (w/v) sterile carrageenan in normal saline (0.1 ml, s.c.) into the subplantar tissue of the right hind paw. Oedema was monitored at a 1 h interval for 4 h with a plethysmometer (7140, UGO Basil ltd, Camerio VA, ITALY). Control rats received sterilized distilled water while drug/extract-treated groups received either diclofenac (100 mg/kg) or HFE (50, 200 and 500 mg/kg). In the prophylactic protocol, drug/extract were given orally 1 h prior to oedema induction while in the therapeutic protocol, drug/extract were given 1 h post oedema induction. Oedema was calculated as:

$$\text{Percent change in paw volume} = 100 \times \left[\frac{(V_t - V_0)}{V_0}\right]$$

Where V_0= Volume of paw before carrageenan injection (t=0)

V_t = Volume of paw at time t

Raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0 and then averaged. Total foot oedema during the 4 h period was calculated in arbitrary units as the area under the curve (AUC).

The percentage inhibition of total oedema was calculated using the following equation:

$$\text{Percent inhibition of oedema} = 100 \times \left[\frac{\left(AUC_{(Control)} - AUC_{(Treatment)}\right)}{AUC_{(Control)}}\right]$$

3.2 STATISTICAL ANALYSIS

Data were presented as the Mean ± SEM of the effect of drugs on the time course curve and the total oedema response over the 4 h period. Data obtained were subjected to statistical tests of significance using the one way analysis of variance, two way analysis of variance and independent sample t-test to assess significant variation in groups tested. Probabilities less than 0.05 ($p< 0.05$) were considered statistically significant. All statistical analysis and graphs were performed using the GraphPad prism software for Windows Version 5.01 (GraphPad, San Diego, CA, USA)

3.3 RESULTS

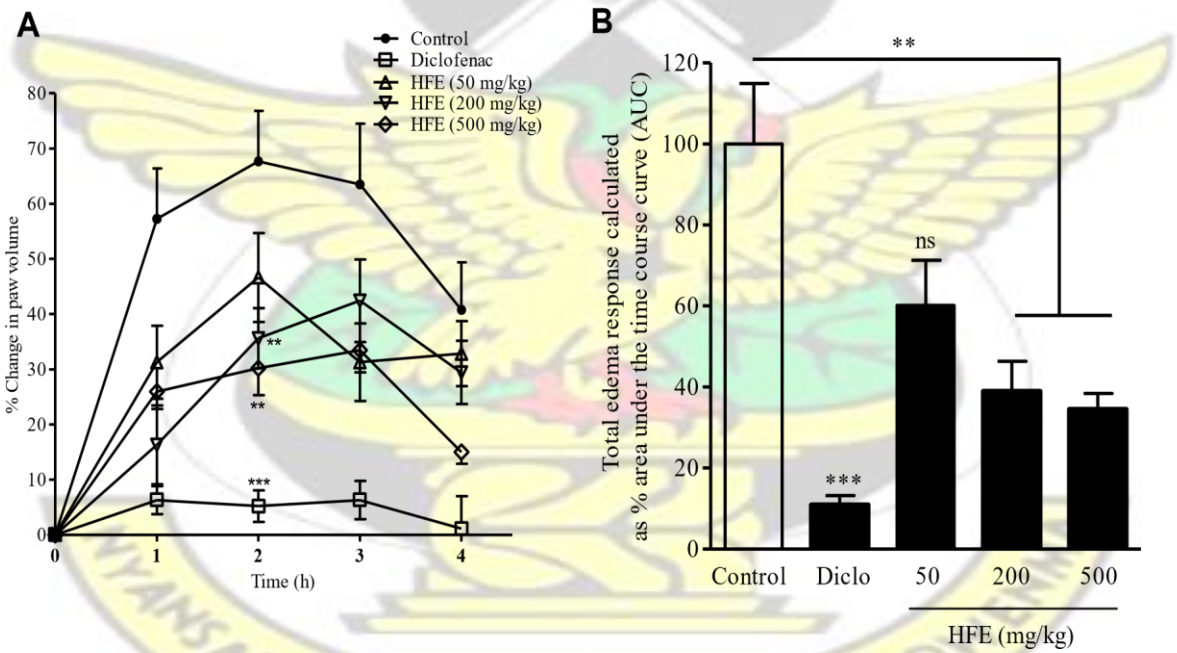
3.3.1 Carrageenan-induced paw oedema

Carrageenan–induced paw oedema is the most commonly used model of acute inflammation. Carrageenan is a sulphated polymeric carbohydrate or polysaccharide obtained from seaweed (Rhodophyceae), which produces oedema and inflammation owing to its ability to trigger the release of histamine, serotonin, bradykinin and prostaglandins (Kulkarni, 2007). This test measures the ability of an agent to inhibit the formation of local oedema that is produced in the rat paw by injection of an irritant, carrageenan (Winter *et al*., 1962).

In this study, subplantar injection of carrageenan into the right hand paw of rats as described in section 3.1.2.1 produced oedema that peaked between 2-3 h in control mice (Figs 3.1 A and 3.1 C). In the prophylactic approach the percentage mean maximal oedema attained for the inflamed control group at 2 h was 67.69 ± 9.13 % (Fig. 3.1 A). HFE at 50, 200 and 500 mg/kg, dose-dependently reduced the percentage mean maximal oedema attained at 2 h to 46.44 ± 8.06 %, 35.62 ± 5.45 % and 30.22 ± 4.93 % respectively (Fig. 3.1 A). In a similar manner 50-500 mg/kg HFE, dose-dependently inhibited the total paw oedema induced over 4 h (measured

as the area under the time course curve, AUC) by 39.85 ± 11.19 %, 61.00 ± 7.35 % and 65.41 ± 3.85 % of the mean control value (Fig 3.1 B). The inhibitions in total oedema for the 200 and 500 mg/kg of HFE were significantly different ($P < 0.01$) from the control.

On therapeutic administration the percentage mean maximal oedema attained for the inflamed control group at 3 h was 59.21 ± 7.60 % (Fig. 3.1 C). For this protocol, HFE dosedependently reduced the percentage mean maximal oedema attained at 3 h respectively to 35.30 ± 3.23 %, 27.36 ± 2.31 % and 14.76 ± 1.99 at 50, 200 and 500 mg/kg (Fig. 3.1 C). The total paw oedema induced over 4 h was also dose-dependently and significantly ($P < 0.001$) suppressed by 33.43 ± 6.89 %, 48.33 ± 6.83 % and 56.55 ± 2.05 % of the mean control value (Fig 3.1 D).



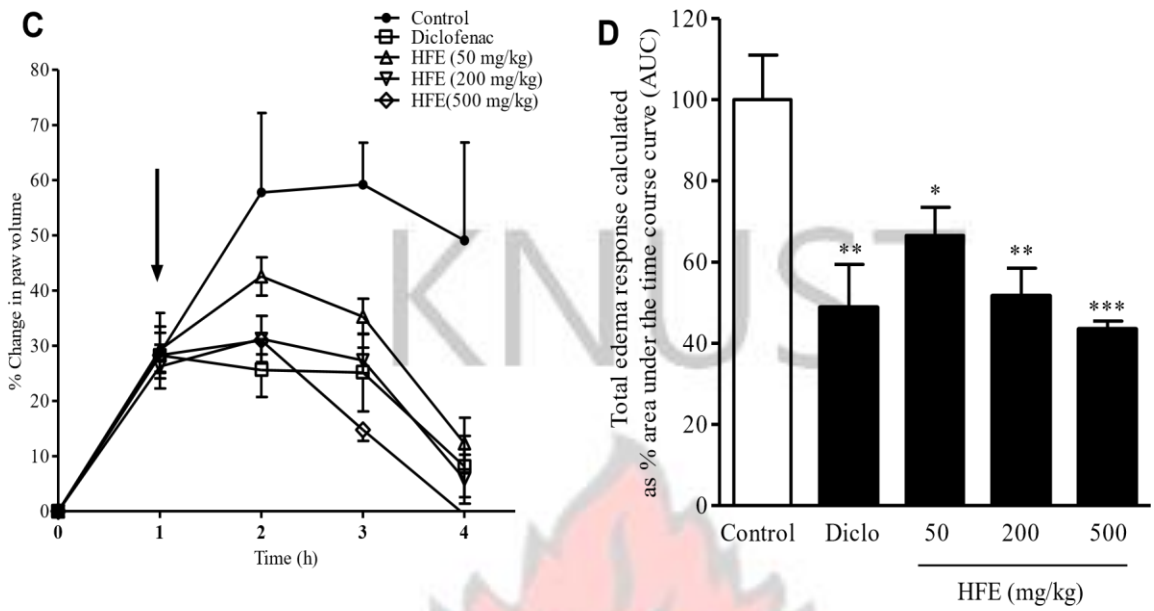


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Fig. 3.1 Effect of *Holarrhena floribunda* extract on carrageenan-induced oedema in rats. Sprague-Dawley rats (200–220 g) were injected with 0.1 ml of a 1 % carrageenan into the subplantar tissue of the right hind paw. Oedema was monitored at 1 h intervals over 4 h as percentage increase in paw thickness (A and C). Total oedema induced during the 4 h was calculated as area under the time course curves, AUC (B and D). In the prophylactic protocol (upper panel), drug vehicle, HFE 50, 200 and 500 mg/kg, and diclofenac, 100 mg/kg, were given orally 1 h before the induction of the oedema while in the therapeutic protocol (lower panel), treatment was done 1 h post oedema induction. Data are presented as Mean ± S.E.M. (n=6). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, when compared with control. ns: not statistically significant P 0.05. Arrow indicates point of extract administration in the therapeutic protocol.

3.4 DISCUSSION

The carrageenan-induced oedema test has characteristics features similar with acute inflammation where there is basically an increase in extravasation of cells and fluid into tissues. The test typically assesses compounds for their acute anti-inflammatory activity. The test is a conventional model of inflammation and an increased sensitivity to pain, which has been largely used over the years to assess the anti-inflammatory properties of NSAIDs and

selective COX-2 inhibitors (Vinegar *et al.,* 1969; Ichitani *et al.,* 1997). The progression of oedema after the administration of carrageenan is a biphasic, age-weight dependent event. Histamine, serotonin and bradykinin are the first mediators to be detected in the early phase (Crunkhorn and Meacock, 1971) and a late phase sustained by prostaglandins is mediated by bradykinin, leukotrienes and polymorphonuclear cells (Gupta *et al.*, 2006). The induction of inducible cyclooxygenase-2 in the hind paw has also been reported to be a contributing factor to oedema formation in the late phase in addition to the elevated levels of prostaglandins reported earlier (Nantel *et al.*, 1999). The significant inhibition of inflammation by HFE before and after 2 h of oedema induction, suggests the ability of the extract to act on both the initial and the late phases of acute inflammation. HFE significantly and dose dependently inhibited carrageenan oedema formation in rats in both prophylactic and therapeutic administration. The inhibition of paw oedema by HFE was more pronounced when administered prophylactically than therapeutically. Administration of HFE before the induction of oedema might have inhibited the release of vasoactive mediators that cause inflammation and as such protected rats to a larger extent than when administered therapeutically. Studies by Kaibara *et al*., (1983) on cyclosporine demonstrated that an established inflammatory property of a medication administered prior to the start of inflammation does not essentially indicate an ability to act when administered after the commencement of inflammation. In their study, cyclosporine administered prophylactically inhibited collagen-induced inflammation but worsened the condition when administered therapeutically. The ability of HFE to have activity in both prophylactic and therapeutic administration is an indication that it might be a genuine anti-inflammatory agent

3.5 CONCLUSION

The hydroethanolic extract of *Holarrhena floribunda* has acute anti-inflammatory property, demonstrated by its inhibitory effects on carrageenan-induced paw oedema in SpragueDawley rats.

CHAPTER FOUR ANTI-ALLERGIC ACTION OF THE HYDROETHANOLIC EXTRACT OF

HOLARRHENA FLORIBUNDA

4.0 INTRODUCTION

Allergy is a hypersensitive reaction of the immune system and a component of the acute inflammatory process. An allergic reaction which is life-threatening is termed anaphylaxis, with reactions being mostly immunologic in nature (Johansson *et al.*, 2004) and mediated by immunoglobulin E (IgE) activation of mast cells. The mast cell is the main effector cell among other cells such as the basophil, neutrophils and eosinophils that mediate allergic responses as well as fatal anaphylactic reactions (Amira *et al.*, 2011). The activation of mast cells generates a series of mechanisms that result in degranulation and release of proinflammatory preformed mediators such as histamine, a wide variety of mediators including neutral proteases, proteoglycans, arachidonic acid and its metabolites and an array of cytokines which elicits symptoms of anaphylactic reactions (Ogawa and Grant, 2007). The rate of occurrence of

anaphylaxis is increasing (Decker *et al.*, 2008) and the search for new and more suitable anti-allergic agents has also increased.

The ability of HFE to inhibit various murine models of anaphylaxis was investigated in this chapter to validate its anecdotal use in folkloric medicine. The effect of *Holarrhena floribunda* on anaphylaxis was ascertained systemically with compound 48/80 and lipopolysaccharide (LPS) and in passive cutaneous anaphylaxis.

4.1 MATERIALS AND METHODS

4.1.1 Materials

4.1.1.1 Animals

Imprint Control Region (ICR) and C57BL/6 mice (20 – 30 g) and Sprague-Dawley rats (200 – 220 g) were provided with the necessary conditions as described in section 3.1.1.1.

4.1.1.2 Microorganism

Escherichia coli (strain: ATCC25922) was obtained from the Microbiology Department of CPMR

4.1.1.3 Drugs and Chemicals

Bovine serum albumin, BSA, (PAA Laboratories, Germany) Phosphate buffered saline, PBS, (Gibco, Karlsruhe, Germany), Compound 48/80, aspirin and dexamethasone (SigmaAldrich, St Louis, USA), sodium cromoglycate (Ashford lab Ltd, Macau).

4.2 Methods

4.2.1 Compound 48/80-induced systemic anaphylaxis

Compound 48/80-induced systemic anaphylaxis was examined as previously described (Kim *et al.*, 2005). Male C57BL/6 mice (n=6) received an intraperitoneal injection of compound 48/80 (8 mg/kg, i.p). The control group received sterilized distilled water while drug/extract treated groups received either sodium cromoglycate (50 mg/kg) or HFE (50, 200 and 500 mg/kg) orally 1 h prior to administration of compound 48/80. Survival rates and anaphylactic symptoms were monitored for 1 h after induction of anaphylactic shock.

4.2.2 Lipopolysaccharide (LPS)-induced anaphylactic shock

LPS-induced systemic anaphylaxis was examined as illustrated previously by Lowry (2005) with few modifications. Male SDRs (n=6) received intraperitoneal injection of 25 mg/kg LPS, (*Escherichia coli* dissolved in PBS). The control group received sterilized distilled water while drug/extract-treated groups received either dexamethasone (0.3 mg/kg) or HFE (50, 200 and 500 mg/kg). In the prophylactic approach drug/extract was orally administered twice; a day and 1 h before LPS challenge. In the therapeutic approach drug/extract was administered 1 h post LPS challenge. The survival rates of the animals were monitored for 168 h after LPS challenge.

4.2.3 Passive cutaneous anaphylaxis (Pinnal inflammation)

The pinnal inflammation model previously described by Church *et al.*, (1974) was adopted. ICR mice (20 – 30 g, n=6) were subcutaneously immunized on day zero with 100 µl of 0.05 mg/ml bovine serum albumin, BSA. On day 14 immunization was repeated with 100 µl of 0.02 mg/ml of BSA. On day 21, mice were treated orally with either 0.3 mg/kg dexamethasone, 100 mg/kg aspirin or 50, 200 and 500 mg/kg HFE. An hour after treatment

the mice were anaesthetized with ether and 200 µl of a 1 % w/v of Evans blue dye injected into the tail vein of all the mice. Immediately after this, and while still under anaesthesia, the pinna of each mouse was spread out and inoculated with 0.1 mg/ml BSA using a 21 gauge hypodermic needle. The mice were euthanized by cervical dislocation after 30 min and their ears cut off, spread out and the area of the reaction was 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.

Percentage inhibition of the inflammatory reaction was calculated using the following equation

$$\text{Percent inhibition of oedema} = 100 \times \left[1 - \frac{A_t}{A_0}\right]$$

Where A_o is the area of extravasation of the blue dye in the pinna of the saline control mice

A_t is the area of extravasation of the blue dye in the pinna of the drug or extracttreated mice

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 (%).

4.2 STATISTICAL ANALYSIS

All graphs and analysis were performed with the GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA). Data are expressed as mean ± standard error of mean. Compound 48/80-induced systemic anaphylaxis and LPS-induced septic shock data was analysed using Log-rank (Mantel Cox) test. Pinnal inflammation was analysed using one way analysis of variance followed by Newman-Keul‟s *post hoc* test. Differences in means were considered statistically significant at $P < 0.05$.

4.3 RESULTS

4.3.1 Compound 48/80 – induced systemic anaphylaxis

Compound 48/80 is the most potent secretagogue (Langunoff *et al.,* 1983) which specifically activates and degranulates mast cells, leading to the discharge of preformed mediators stored in vesicles such as histamine and other chemical mediators, including eicosanoids, cytokines and chemokines which provokes the symptoms associated with anaphylaxis. Nishikawa and Kiana, (2008) reported that challenge with compound 48/80 results in connective tissue-type mast cells and rat peritoneal mast cells (RPMCs) releasing large amount of inflammatory substances. According Kubes and Granger, (1996) administering compound 48/80 evokes responses in various animal models leading to the development of allergic response including anaphylaxis.

To investigate the inhibitory effect of HFE on systemic anaphylaxis, 8 mg/kg of compound 48/80 was injected intraperitoneally to mice pre-treated with drug/extract and monitored. Results show that administration of compound 48/80 successfully induced anaphylactic death within 1 h after intraperitoneal injection. Mortality in mice began six min after administration in the control group and within 25 min all control mice were dead. Pretreatment with HFE dose-dependently protected mice from anaphylactic shock compared with the controls (Fig 4.1). This was evident by the increasing survival proportion (0, 16 and 50 %) respectively at 50, 200 and 500 mg/kg HFE and delayed symptoms of anaphylactic shock such as twitches and isolation of mice and eventual death with increasing doses of

HFE treatment. However mice treated with HFE (50 mg/kg) died before control mice.

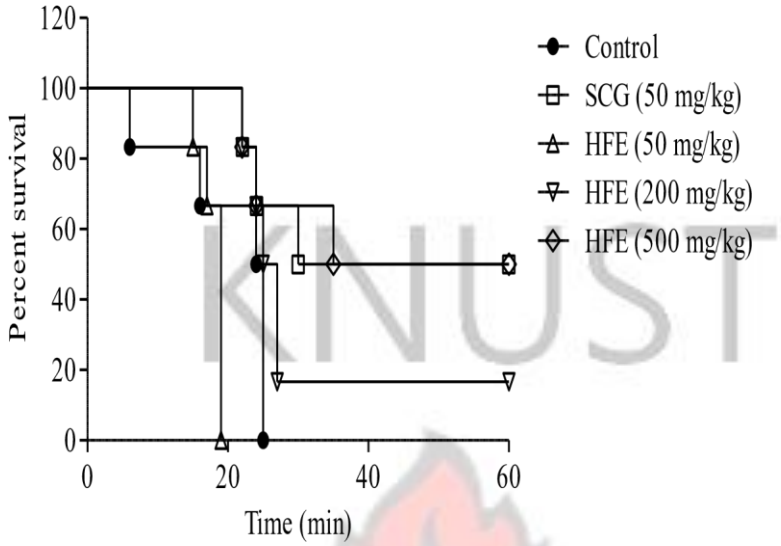

Fig.4.1 Effect of *Holarrhena floribunda* extract on compound 48/80-induced systemic anaphylaxis in mice**.** C57BL/6 mice (20 – 30 g) received vehicle, sodium cromoglycate, (SCG) 50 mg/kg or HFE 50, 200 and 500 mg/kg orally 1 h before injection of compound 48/80 (8 mg/kg, i.p). Survival rate of the mice was monitored for 1 h. Data was analysed using Log-rank (Mantel Cox) test. (n=6), Survival curves were significant ($P \leq 0.001$).

4.3.2 Lipopolysaccharide (LPS)-induced anaphylactic shock

Lipopolysaccharide, an endotoxin found in the outermost layer of gram negative bacteria, causes systemic anaphylaxis and death. Intravenous injection of LPS promotes a quick reduction in the circulating thrombocytes levels, resulting from the aggregations or accumulation of these thrombocytes in pulmonary and hepatic capillaries (Morrison and Ulevitch, 1978) leading to anaphylactic shock and eventual death.

To evaluate the effect of HFE on LPS-induced systemic anaphylactic shock, rats were treated with drug/extract before or after LPS challenge and mortality monitored for 168 h. It was observed that the administration of LPS intraperitoneally to rats caused endotoxic or anaphylactic shock and eventual death in the rats. In the prophylactic model, there was 100 % mortality in vehicle-treated rats 12 h after LPS challenge. Drug/extract-treated rats survived

beyond the 12th h, indicating the protective effect of the drugs/extract against LPS- induced anaphylactic shock. Survival proportions for the 50, 200 and 500 mg/kg HFE extract were respectively 0, 20, and 20 % (Fig 4.2 A).

When treated after LPS challenge (therapeutic) all vehicle-treated rats died (100 % mortality) before 10 h. HFE dose-dependently protected rats against LPS shock with survival proportions of 0, 20, and 40 % respectively for the 50-500 mg/kg HFE (Fig 4.2 B). Survival proportion for dexamethasone against endotoxic shock was the highest (60 %) on therapeutic administration.

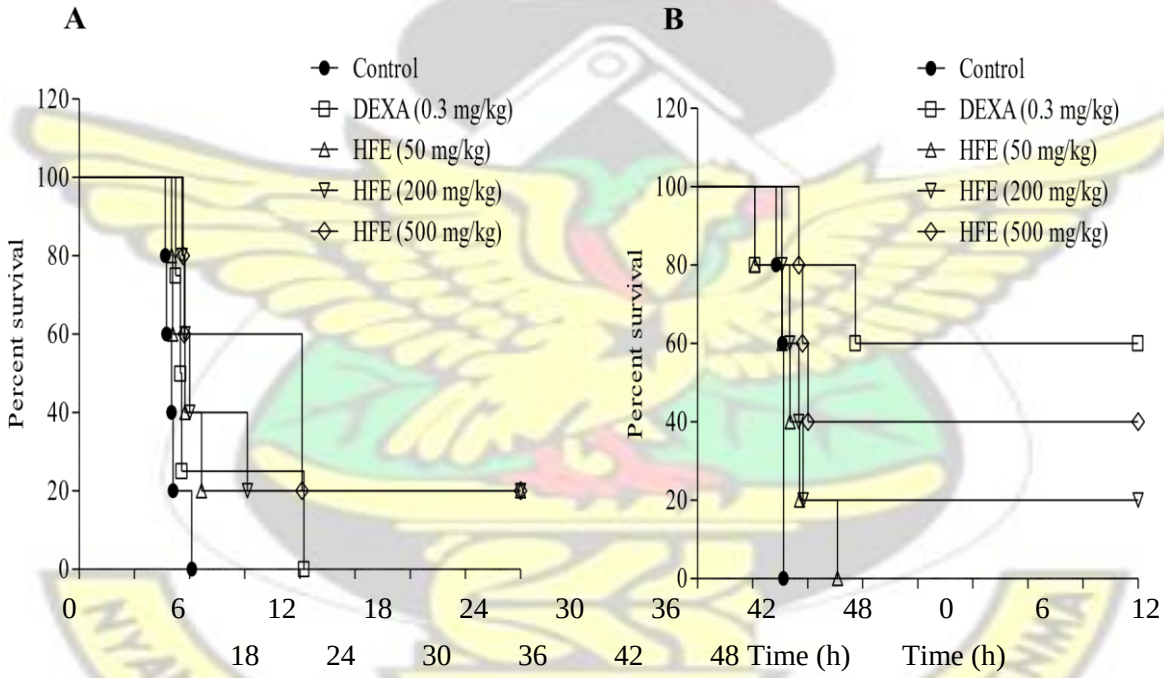

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Fig. 4.2 Effect of *Holarrhena floribunda* extract on LPS-induced anaphylactic shock in rats**.** Sprague-Dawley rats (200 -220 g) received injection of lipopolysaccharide, LPS (*Escherichia coli*, 25 mg/kg dissolved in PBS, i.p). Vehicle, dexamethasone 0.3 mg/kg or HFE 50, 200 and 500 mg/kg were given orally for two consecutive days before LPS challenge in the prophylactic model (A) and an hour after LPS challenge in the therapeutic model (B). Survival

rate of the mice was monitored for 168 h. Data was analysed using Logrank (Mantel Cox) test. (n=6), Survival curves were significant ($P \leq 0.001$) with significant trend ($P \leq 0.02$)

4.3.3 Passive cutaneous anaphylaxis (Pinnal inflammation)

Passive cutaneous anaphylaxis (PCA) is used as a tool to study sensitivity to allergens and determine efficacy of compounds in ameliorating allergic responses. It involves a response of the skin specifically the dermis to a foreign substance and proceeds through an interaction between the foreign substance and IgE leading to an increased permeability of vessels within the skin.

The ability of the HFE to inhibit passive cutaneous anaphylaxis was evaluated. Briefly mice were sensitised twice as described earlier in section 4.2.3 and treated with drug/extract. From this experiment it was observed that the administration of 50, 200 and 500 mg/kg of HFE extract dose-dependently and significantly ($P < 0.001$) inhibited the extravasation of Evans blue dye by 44.69 %, 69.66 % and 83.17 % respectively (Fig. 4.3). Dexamethasone (0.3 mg/kg) and aspirin (100 mg/kg) suppressed the extravasation of the Evans blue dye by 80.50 % and 85.71 % respectively (Fig 4.3)

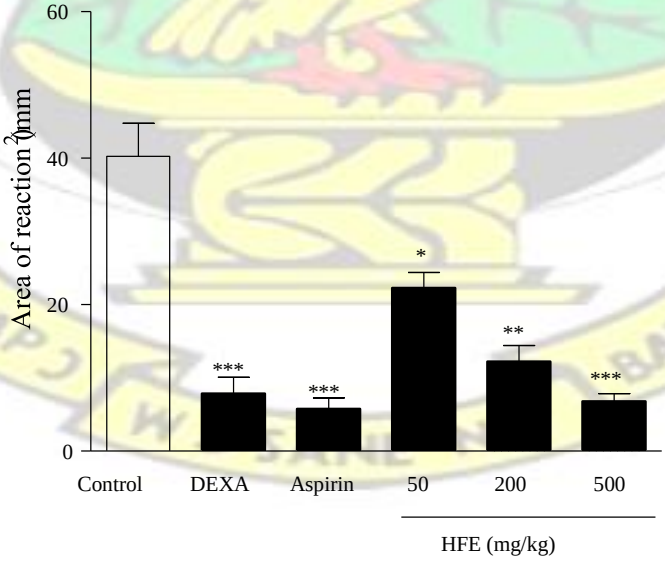

Fig 4.3 Effect of *Holarrhena floribunda* extract on Pinnal inflammation in mice**.** ICR mice (20-30 g) were sensitised twice as described earlier and treated with either vehicle, aspirin 100 mg/kg, dexamethasone 0.3 mg/kg or HFE 50, 200 and 500 mg/kg, p.o. An hour later the mice were anaesthetized, injected i.v with Evans blue dye and immediately challenged with bovine serum albumin by inoculation into the pinna. 30 min afterwards the mice were euthanized 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 5 ears ± S.E.M (n= 5). Significance between saline group and drug or extract group denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.4 DISCUSSION

Anaphylaxis is an allergic reaction which can potentially cause death if not controlled (Johansson *et al.*, 2004). It is mediated by immunoglobulin E (IgE) activation of mast cells. The mast cell is the main effector cell among other cells such as the basophil, neutrophils and eosinophils that mediate allergic responses as well as fatal anaphylactic reactions (Amira *et al.*, 2011). They are mostly found in connective tissue and mucosal surfaces. The effect of *Holarrhena floribunda* on anaphylaxis was ascertained systemically with compound 48/80 and lipopolysaccharide (LPS) and in passive cutaneous anaphylaxis.

Compound 48/80, a mixed polymer of phenethyl-amine and polymers of basic amino acids, such as substance P are potent stimulators (Ennis *et al.*, 1980) of degranulation of mast cells, releasing about 90 % of preformed histamine, arachidonic acid and its metabolites and a variety of cytokines associated with anaphylactic symptoms making it a very potent agent for investigating the mechanism involved in allergic and anaphylactic reactions (Allansmith *et al.*, 1989). The release of histamine and other mediators from tissue mast cell is by an exocytotic degranulation process which requires energy in the form of guanisine triphosphate-gamma S (GTP) γS binding to G-proteins (Palomäki and Laitine, 2006) and triggering the activation of protein kinase C and Ca^{2+} signalling (Katzung *et al.*, 2012). Tasaka *et al.*, (1986) reported that a perturbation of the mast cell membrane by compound 48/80 reduces the

stability of the membrane and makes it porous to preformed mediators which leak out. In the present study pre-treatment with HFE protected mice from death associated with compound 48/80-induced endotoxic shock possibly by inhibiting processes leading to the activation of protein kinase C and Ca^{2+} signalling thereby stabilizing the lipid bilayer of mast cell, preventing degranulation and subsequent release of vasoactive mediators and the expression of various cytokines required for anaphylaxis.

The extract, (HFE) significantly and dose-dependently protected rats from LPS-endotoxic shock in both prophylactic and therapeutic models. The reduced number of deaths observed in animals that received the extract when challenged with LPS in the study indicated the probable ability of the extract to prevent cardiovascular injury, tissue necrosis, organ failure and hypotension which have been reported by different studies to be the main causes of death in LPS anaphylaxis (Tracey *et al.,* 1986, 1987; Cunha *et al.,* 1992; and Vanhoutte, 2001). Lipopolysaccharide is an endotoxin found in the external wall of gram-negative bacteria and as reported by Barton and Jackson, (1993) is shown to cause fatal septic or anaphylactic shock in the absence of an overt infection. According to Morrison and Ulevitch, (1978) the intravenous injection of LPS induces a rapid fall in platelet levels in circulation as a result of the accumulation of these platelets in pulmonary and hepatic capillaries. The accumulation is highest in the lung with a maximum of about 80 % of the platelets lost from the blood accumulating in the lung (Shibazaki *et al.,* 1996; and Endo *et al.,* 1997). Anaphylactic shock occurs as a result of this accumulation in the lung, with the severity of the shock paralleling the quantity of platelets accumulated in the lung (Shibazaki *et al.,* 1996, Endo *et al.,* 1997). The effect of LPS *in vivo* has also been reported to be a result of mediators produced by the host's cells. Tumour necrosis factor alpha (TNFα) is a cytokine produced by macrophages during septic shock and has been implicated in the cardio-vascular injury and

death (Tracey *et al.,* 1986, 1987). TNFα also causes tissue necrosis and organ failure and the induction of the enzyme nitric oxide synthase, NOS (Cunha *et al.,* 1992). The expression of NOS II (iNOS) isoform of nitric oxide synthase is induced by immunological stimuli and is calcium independent (Moncada and Higgs, 1993). The synthesis of iNOS results in the production of nitric oxide (NO), and free radicals which cause myocardial dysfunction with resulting systemic hypotension and tissue injury (Vanhoutte, 2001; Chen *et al.,* 2003). The effect of the extracts on anaphylaxis demonstrated with the inhibition of compound 48/80 and LPS- induced systemic anaphylaxis might be attributed to inhibitory effects on the expression of cytokines such as tumour necrosis factor alpha and the enzyme, nitric oxide synthase. This is in agreement with work done by

Yamazaki and Kawano (2011) where alkaloids of plant origin inhibited levels of tumor necrosis factor alpha and nitric oxide produced in lipopolysaccharide-stimulated raw 264 macrophages.

The anti-anaphylactic effect was further confirmed with the IgE-mediated pinnal passive cutaneous anaphylactic test. This is a unique *in vivo* model of anaphylaxis in a local allergic reaction (Wershil *et al.,* 1987) in which anaphylaxis is mainly induced by vasoactive mediators such as histamine from mast cells. It is reported that the inflammatory reaction that results in passive cutaneous anaphylaxis is a delayed hypersensitive reaction to bovine serum albumen (Martinez *et al.,* 1962). There was a dose-dependent and significant ($p<0.001$) inhibition of the passive cutaneous anaphylactic shock by the extract. The extract might be inhibiting the hypersensitivity reactions either through its stabilizing effect on the mast cell or its ability to inhibit the effects of the products of mast cell degranulation.

4.5 CONCLUSION

The hydroethanolic extract of *H. floribunda* has inhibitory effects on the compound 48/80induced systemic anaphylaxis, LPS-induced anaphylactic shock and passive cutaneous anaphylaxis models. The extract could thus be said to be anti-allergic.

CHAPTER FIVE

EFFECT OF HYDROETHANOLIC EXTRACT OF *HOLARRHENA FLORIBUNDA* ON RAT ADJUVANT-INDUCED ARTHTRITIS

5.0 INTRODUCTION

The deregulation or failure to resolve acute inflammatory reactions lead to chronic inflammation which results in necrosis and fibrosis (Alessandri *et al*. 2013; Freire and Van Dyke, 2013). One model of studying chronic inflammation is the rat adjuvant-induced arthritis. Rheumatoid arthritis (RA) is a chronic inflammatory disorder that is characterized by an inflamed synovial membrane, cartilage destruction, change in joint integrity, polyarthritis with functional impairment and disability. Though the exact cause remains unclear, pro-inflammatory mechanisms involving cytokines particularly tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL- 1β) have been implicated by several studies to be associated with the disease progression and joint destruction (Eastgate *et al.*, 1988; Saxne *et al.*, 1988). The global prevalence and economic burden of chronic inflammatory diseases particularly RA keep worsening with increasing levels of morbidity, mortality and a reduced quality of daily life of affected people. Current therapeutic agents for the management of chronic inflammatory diseases target inflammatory cells and mediators but lack specificity and are plagued with untoward side effects (Dhikav *et al.*, 2002) making it necessary for the

search of alternatives. Consequently the effect of HFE on rat adjuvant induced arthritis was assessed to determine its effect on chronic inflammation.

5.1 MATERIALS AND METHODS

5.1.1 Materials

5.1.1.1 Animals

Sprague-Dawley rats (200 – 220 g) maintained with the necessary conditions as described in section 3.1.1.1 were used.

5.1.1.2 Drugs and Chemicals

Dexamethasone (Sigma-Aldrich, St Louis, USA); Paraffin oil (KAMA Pharmaceutical Industries, Ghana).

5.1.1.3 Microorganism

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

5.1.2 Methods

5.1.2.1 Anti-arthritic effect of *Holarrhena floribunda*

Arthritis was induced as previously described by Pearson (1956), with slight modification. The right hind paw of Sprague-Dawley rats (n=6) maintained as arthritic groups were injected subplantar with 0.1 ml of a 5 mg/ml suspension of heat-killed *Mycobacterium tuberculosis* triturated in paraffin oil, referred to as Complete Freund‟s adjuvant (CFA). The non-arthritic control group received subplantar injection of 0.1 ml of sterile paraffin oil

(Incomplete Freund‟s adjuvant (IFA).
Drug/extract doses administered were either dexamethasone (0.3 mg/kg) or HFE (50, 200, 500 mg /kg). All drug/extract were administered orally by gavage and rats received sterilized distilled water *ad libitum.* Disease progression was monitored from day 0 to day 28 after which rats were sacrificed. In the prophylactic protocol drug/extract was given orally 1 h before the induction of arthritis and daily for 28 days. In the therapeutic protocol rats were injected with 0.1 ml of CFA or IFA subplantar on day zero. On day 14 after induction of arthritic rats were treated with daily doses of drug/extract until day 28.

The effect of HFE on the adjuvant-induced arthritis was assessed using the six indices indicated below;

5.1.2.1.1 Change in body weight

Adjuvant arthritis was induced as earlier described in section 5.1.2.1 Rats were weighed on day zero (baseline) and weekly thereafter till day 28.

5.1.2.1.2 Oedema: measured as maximal oedema and total oedema

Rat adjuvant arthritis was induced as described earlier in section 5.1.2.1 Foot volumes of the ipsilateral and contralateral paws were measured prior to induction of arthritis (day 0) and every other day throughout the experimental period using a plethysmometer (7140, UGO Basil Ltd, Camerio VA, ITALY).

Maximal oedema was calculated using the formula:

$$\text{Percent change in paw volume} = \left[\frac{V_t - V_0}{V_0}\right] \times 100$$

Where V_t = Volume of paw at time t

V_0= Volume of paw at baseline (t=0)

Total oedema induced during the experimental period was measured as an area under the time course curves (AUC) and the percentage inhibition of oedema was calculated using the following equation:

$$\text{Percent inhibition of oedema} = 100 \times \left[\frac{\left(AUC_{(Control)} - AUC_{(Treatment)}\right)}{AUC_{(Control)}}\right]$$

5.1.2.1.3 Arthritic score

The arthritic indexes for the ipsilateral and contralateral paws were determined using photography and radiography.

5.1.2.1.3.1 Photography

Adjuvant arthritis was induced in rats as earlier described in section 5.1.2.1 and monitored for 28 days. Photographs of the limbs were taken on the 28th day with a digital camera and the extent of inflammation blindly quantified on a scale of 0- 4 (0- uninjected paw without swelling, 1- slight swelling and/or erythema, 2-low to moderate oedema, 3- pronounced oedema with limited joint use, 4- excess oedema with joint rigidity).

5.1.2.1.3.2 Radiologic index

Rat adjuvant arthritis was induced as earlier described in section 5.1.2.2 and monitored for 28 days. On the 28th day rats were euthanized by cervical dislocation. X-ray images were taken with a conventional X-ray machine (Philips, Eindhoven, The Netherlands) and industrial X-ray film (Fuji Photo Film, Tokyo, Japan). The X-ray apparatus operated at a 52 kV. The extent of periarticular swelling, oesteolysis and joint damage were blindly quantified by a radiologist

on a scale of 0-3, with 0: being score for IFA non-arthritic control group; 1: mild; 2: moderate; and 3: severe.

5.1.2.1.4 Histology of bones tissue

Rat adjuvant arthritis was induced as earlier described in section 5.1.2.1 and monitored for 28 days. Rats were euthanized by cervical dislocation, and arthritic paws were amputated above the ankle and placed in 4% formalin. The paws were trimmed and placed in decalcifying solution of Ethylenediaminetetraacetic acid for 10 days. Paws were embedded in paraffin, sectioned at 4 µm, stained with haematoxylin and eosin and studied under a light microscopy (Dialux 22; Leitz, Wetzlar, Germany) to examine the histopathological changes. Changes in the joints were quantified blindly by a pathologist on a scale of 0-3 (0-absence of synovial hyperplasia, pannus, bone erosion, and presence of inflammatory cells, 1- mild presence of synovial hyperplasia, pannus, bone erosion, and presence of inflammatory cells, 2- moderate presence of synovial hyperplasia, pannus, bone erosion, and presence of inflammatory cells and 3-severe presence of synovial hyperplasia, pannus, bone erosion, and presence of inflammatory cells).

5.1.2.1.5 Haematology

Adjuvant-induced arthritis was induced in rats as described earlier in section 5.1.2.2 and monitored for 28 days. Blood samples from rats were collected by tail bleeding into either ethylenediamine tetra-acetic acid coated tubes for haematological analysis or trisodium citrate tubes (Quest Scientific, Swaziland) for the estimation of erythrocyte sedimentation rate using the Westergren method.

5.2 STATISTICAL ANALYSIS

All graphs and analysis were performed with the GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA). Data are expressed as mean ± SEM. All data were analysed using one way analysis of variance followed by Newman-Keul‟s *post hoc* test.

Differences in means were considered statistically significant at P< 0.05, 0.01 and 0.001.

5.3 RESULTS

5.3.1 Anti-arthritic effect of *Holarrhena floribunda*

The use of the Complete Freund Adjuvant in the induction of arthritis is by far the most widely used model of arthritis because of the several characteristics in common with human rheumatoid arthritis with outcomes being tissue destruction, and the potential for pain and distress in the host animal (Stills, 2005). The effect of HFE on the indices below was assessed to determine its effect on rat adjuvant arthritis.

5.3.1.1 Change in body weight

Body weights of the rats were monitored weekly over a period of 28 days and percentage changes in body weight determined.

From the study there was a general increase in body weight with time for all groups. The increase in body weight in the non-arthritic group was significantly ($P < 0.01$) higher compared to that of the arthritic groups (Figs. 5.1 A and 5.1 C). The maximum change in body weight attained for the IFA non-arthritic control group was 29.89 ± 3.38 % while that for the CFA arthritic control group was 6.19 ± 2.64 % (Figs. 5.1 A and 5.1 C). Total body weight expressed as AUC over the experimental period was significantly ($P < 0.001$) higher in the

IFA non-arthritic control group (69.66 ± 7.04) compared to that of the CFA arthritic control group of 20.48 ± 7.26 % (Figs. 5.1 B and 5.1 D).

Prophylactic treatment with dexamethasone as expected resulted in a reduction in maximum body weight attained compared to the CFA arthritic control group (Fig. 5.1 A). In a similar manner total body weight measured as AUC was reduced compared to the CFA arthritic control group (Fig. 5.1 B). However, treatment with HFE at 50-500 mg/kg resulted in a dose-dependent increase in maximum change in body weight to 10.52 ± 2.73 %, 13.30 ± 3.45 % and 15.31 ± 5.94 % respectively compared to the CFA arthritic control group (Fig. 5.1 A). Similarly, treatment with HFE at the same doses, dose-dependently increased total change in body weight calculated as AUC to 38.88 ± 6.14 %, 41.30 ± 9.50 % and 59.79 ± 15.36 % respectively (Fig. 5.1 B). These increases were however not significantly ($P > 0.05$) different from the CFA arthritic control group except the 500 mg/kg HFE.

In the therapeutic model treatment with dexamethasone resulted in a reduction in maximum body weight attained compared to the CFA arthritic control group (Fig. 5.1 C). In a similar fashion total body weight measured as AUC was reduced compared to the CFA arthritic control group (Fig. 5.1 D). The maximum change in body weight attained in HFE (50-500 mg/kg) treated groups were 3.46 ± 4.29 %, 7.29 ± 2.04 % and 9.27 ± 2.94 % respectively (Fig. 5.1 C). Likewise, treatment with HFE dose-dependently increased the total change in body weight over 28 days to 20.09 ± 9.10 %, 28.80 ± 4.91 % and 46.74 ± 10.3% respectively compared with the CFA arthritic control group (Fig. 5.1 D). The increases in body weight were however not significant ($P > 0.05$) compared with the CFA arthritic control group.

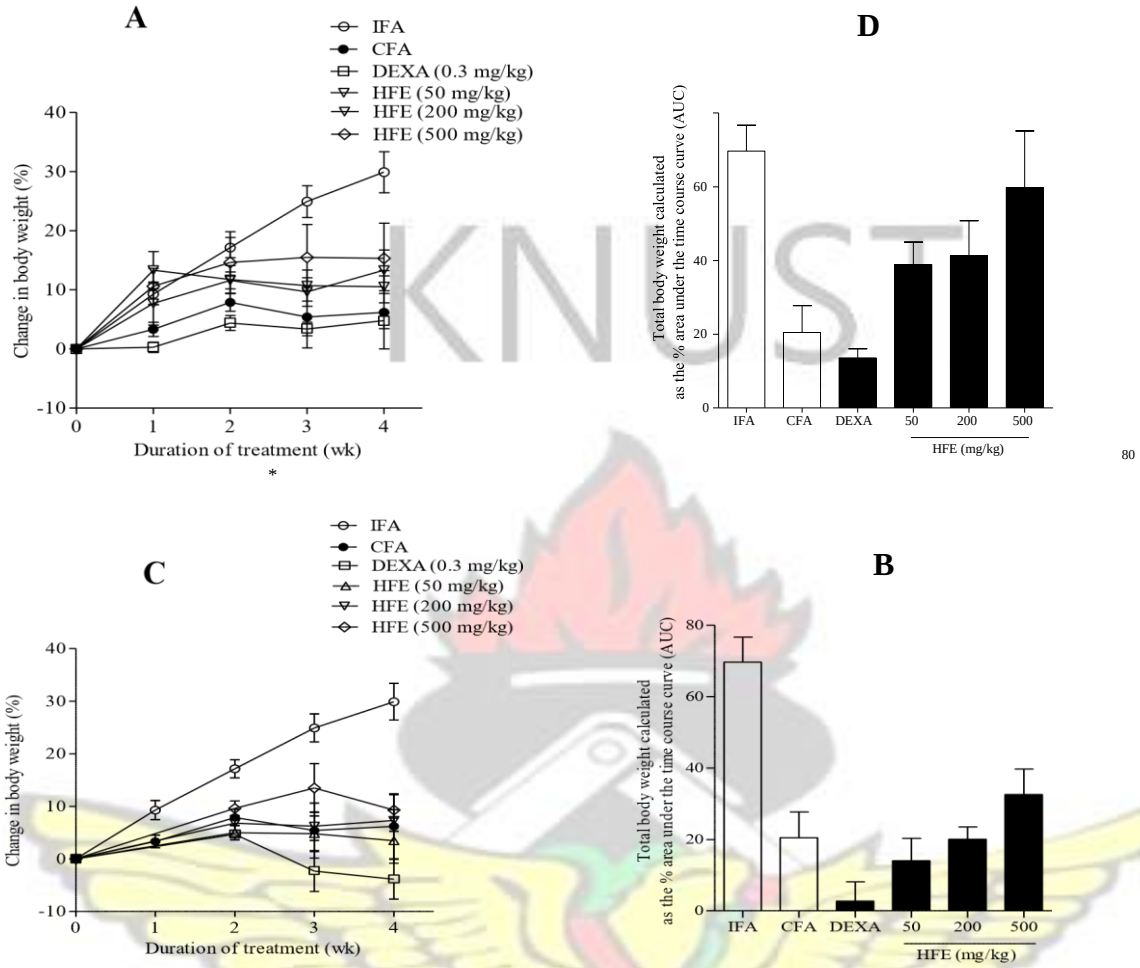

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Fig. 5.1 Effect of *Holarrhena floribunda* on body weight in adjuvant-induced arthritic rats. Sprague-Dawley rats (200 – 220 g) were injected subplantar with 0.1 ml of Complete Freund‟s Adjuvant (CFA) or Incomplete Freund‟s Adjuvant (IFA) into the right hind paw. Their body weights were monitored weekly as the percentage change in body weight (A and C). Total body weight measured during the acute and polyarthritis phases were calculated as area under the time course curves, AUC (B and D). The vehicle and either dexamethasone (0.3 mg/kg) or HFE (50, 200, 500 mg/kg) were given orally 1 h before induction of arthritis in the prophylactic model (upper panel) and starting on the 14th day after arthritis induction in the therapeutic model (lower panel).Values are Mean ± S.E.M (n= 6), * ($P < 0.05$) values significantly different from CFA arthritic control group

5.3.1.2 Oedema assessment: measured as maximal oedema and total oedema

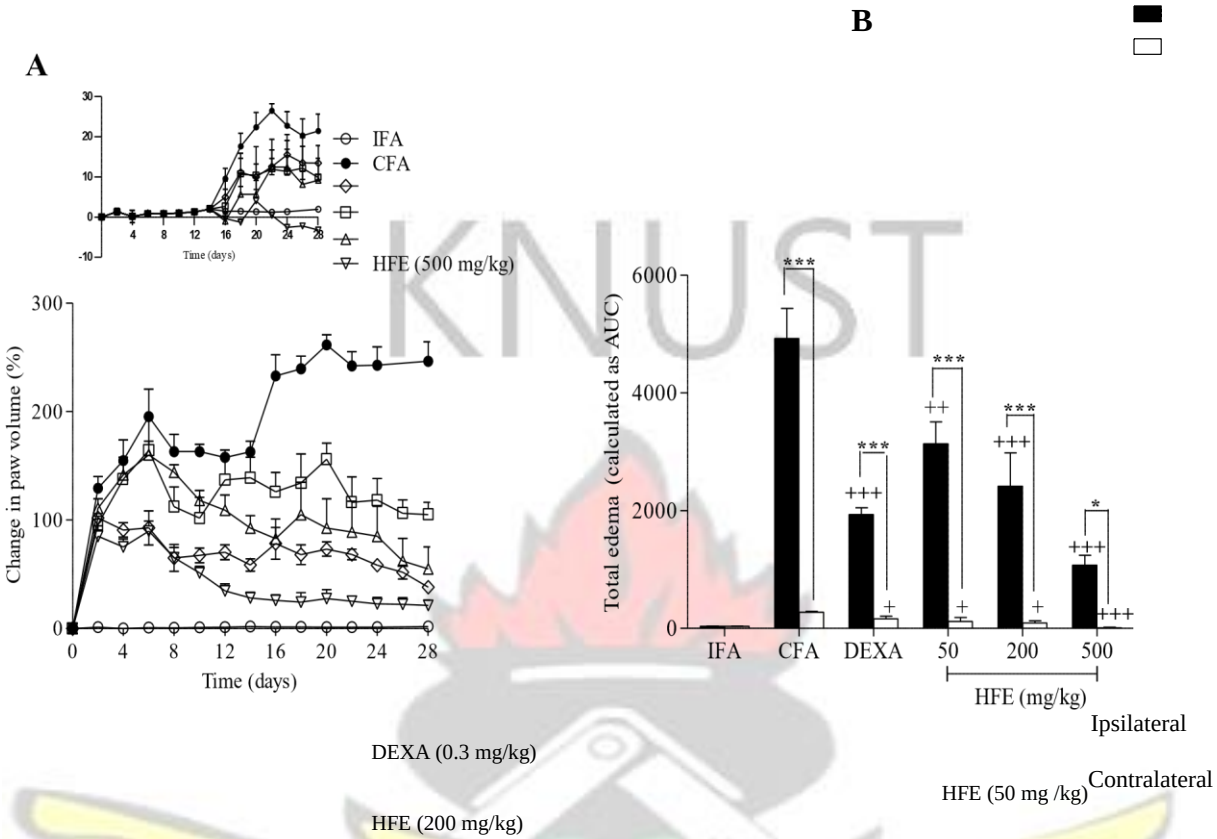
To assess the effect of *Holarrhena floribunda* on paw oedema, rat adjuvant arthritis was induced as described earlier in section 5.1.2.1. Paw volumes of the ipsilateral and contralateral paws were measured prior to induction of arthritis (day 0) and every other day throughout the experiment.

It was observed from the study that the IFA non-arthritic control group recorded no significant changes in paw volume over the experimental period (both acute and polyarthritic phase) (Figs 5.2 A and C). Injection of the Complete Freund Adjuvant resulted in a progressive increase in swelling of the injected paw of rats with time and a systematic spread of inflammation to the uninjected paw starting at day 14 (Figs 5.2 A and C inserts). The CFA arthritic control and drug/extract-treated rats recorded acute inflammatory oedema of the ipsilateral (injected) paw between day 4-6 with a subsequent chronic polyarthritic phase which was observed around day 14 - 16 and peaked on the 20th day (Figs 5.2 A and C). Evidence for the polyarthritic phase was the progress and development of oedema in the contralateral (non-injected) limb in CFA arthritic control and drug-treated rats.

In the prophylactic study the percentage mean maximal oedema attained for the CFA arthritic control group was 261.57 ± 9.30 % (Fig 5.2 A). Treatment with HFE at 50-500 mg/kg dose-dependently and significantly ($P < 0.05$) reduced the maximal oedema attained to 156.11 ± 14.78 %, 95.58 ± 27.10 % and 27.63 ± 8.28 % respectively (Fig 5.2 A). Total oedema measured as the area under the curve for the ipsilateral paw was dose-dependently and significantly ($P < 0.05$-0.001) reduced to 63.68 %, 48.96 % and 21.79 % respectively relative to the controls (Fig. 5.2 B). Similarly in the contralateral paw, total oedema was dose-

dependently and significantly ($P < 0.05$-0.001) reduced to 42.84 %, 33.28 % and 3.50 % mg/kg with the same doses of HFE (Fig. 5.2 B)

In the therapeutic protocol, drug administration was started on day 14 after induction of arthritis during which the polyarthritic phase or a systemic spread of the inflammation had started. The administered doses of HFE (50-500 mg/kg) dose-dependently and significantly ($P < 0.001$) modified the time course of the oedema and resulted in significant reduction of the mean maximal swelling attained by the CFA arthritic control group of 261.89 ± 7.60 % to 145.29 ± 12.57 %, 89.14 ± 11.19 % and 53.80 ± 7.48 % respectively (Fig 5.2 C). There was a dose-dependent and significant ($P < 0.001$) reduction in total limb swelling of the injected paw to 68.18 %, 52.15 % and 38.02 % with the same doses of HFE (Fig. 5.2 D). In a similar fashion total oedema induced over the 28 day period was reduced to 73.39 %,
23.60 % and 10.95 % respectively in the uninjected paw (Fig 5.2 D).



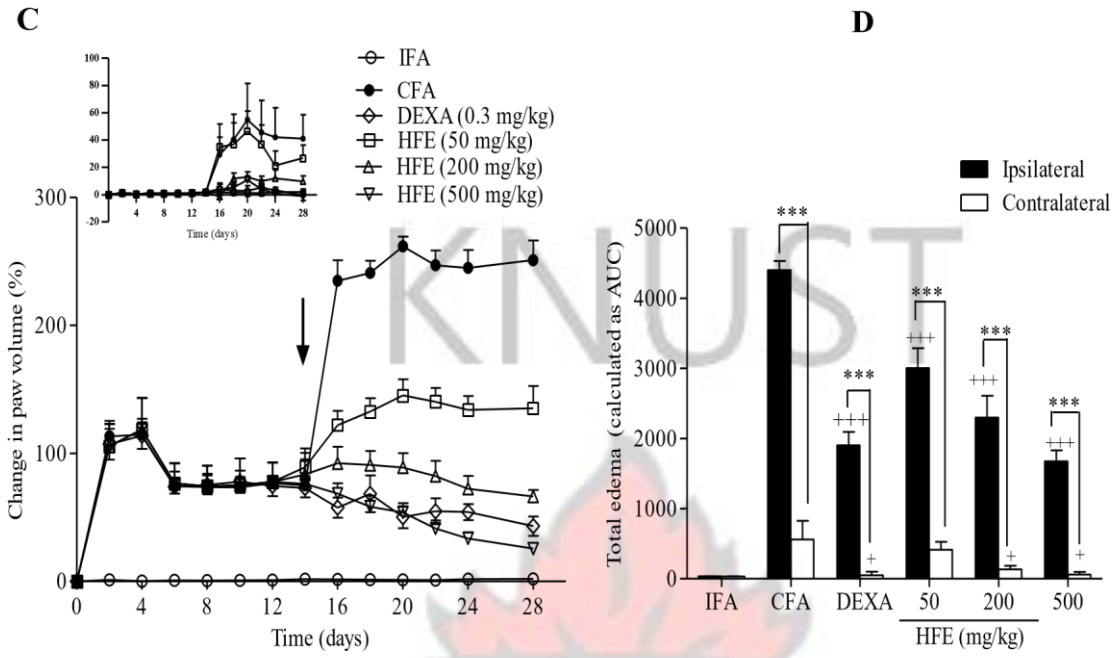


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Fig. 5.2 Effect of *Holarrhena floribunda* on adjuvant-induced arthritis in rats. Sprague Dawley rats (200 – 220 g) were injected subplantar with 0.1 ml of CFA or IFA into the right hind paw. Paw volumes were measured by plethysmometer for both the ipsilateral (injected paw) and contralateral paw (non-injected paw) before subplantar injection of CFA and IFA on alternate days up to the 28th day. The oedema component of inflammation was monitored as the percentage change in paw volume [A, C]. Total oedema was calculated as area under the time course curves, AUC [B, D]. The drug vehicle, dexamethasone (0.3 mg/k) and HFE (50, 200, 500 mg /kg) were given orally 1 h before the induction of arthritis and daily for 28 in the prophylactic model (upper panel) and commenced on the 14th day after the induction of the arthritis and daily for 28 in the therapeutic model (lower panel). Values are mean ± SEM (n=6). Time course curves for paw volume were subjected to Two-way (treatment x time) repeated measure ANOVA followed by Newman-Keul‟s post hoc test) *** ($P< 0.001$) values significantly different from ipsilateral, + ($P<0.05$), ++ ($P<0.01$), +++ ($P<0.001$) values significantly different from CFA arthritic control group (One-way ANOVA followed by Newman-Keul‟s post hoc test). Arrow indicates point of drug/extract administration.

5.3.1.3 Arthritic Score

5.3.1.3.1 Photography

From the photographs of the limbs of rats, there were no signs of oedema and erythema in the IFA non-arthritic control group (Plate 5.1 A). The CFA arthritic control group showed the presence of severe erythema, lesions, and swelling in both injected and uninjected hind limbs (Plate 5.1 B). Prophylactic treatment with dexamethasone resulted in reduced erythema, lesions, and swelling in both injected and uninjected hind limbs compared to the CFA arthritic control group (Plate 5.1 C). HFE at 50 - 500 mg/kg dose-dependently reduced erythema, lesions and swelling in both injected and uninjected hind limbs (Plates 5.1 D, 5.1 E and 5.1 F) when compared with the CFA arthritic control group.

In the therapeutic protocol, HFE at 50-500 mg/kg dose-dependently reduced erythema, lesions and swelling (Plates 5.1 G, 5.1 H and 5.1 I) when compared with that of the CFA arthritic control group.

The extent of arthritic swelling was scored blindly on the 28th day of experiment from the photographs of hind paws. The IFA non-arthritic control group scored the lowest with no erythema, swelling and lesions while the CFA arthritic control group recorded the highest scores with the severe presence of erythema, swelling and lesions in both the ipsilateral and contralateral limbs (Figs 5.3 A and 5.3 B). Rats treated with dexamethasone recorded moderate levels of erythema, soft tissue swelling and lesions in both the ipsilateral and contralateral limbs when compared with the CFA control group (Figs 5.3 A and 5.3 B).

When administered prophylactically, 50 - 500 mg/kg HFE dose-dependently reduced scores for the injected paw swelling (Fig. 5.3 A). The reductions, were, however significant ($P <$ 0.01-0.001) for only the 200 mg/kg and 500 mg/kg doses of HFE when compared with the CFA arthritic control group. Similarly HFE dose-dependently reduced paw oedema and subsequently scores for the non-injected paw (Fig. 5.3 A).

When administered therapeutically HFE at 50 - 500 mg/kg dose-dependently and significantly ($P < 0.001$) reduced scores for the ipsilateral paw when compared with the CFA arthritic control group (Fig. 5.3 B). There was a dose-dependent reduction in arthritic scores for the contralateral paw. However, the reductions were not significantly different when compared with the CFA arthritic control group (Fig. 5.3 B).

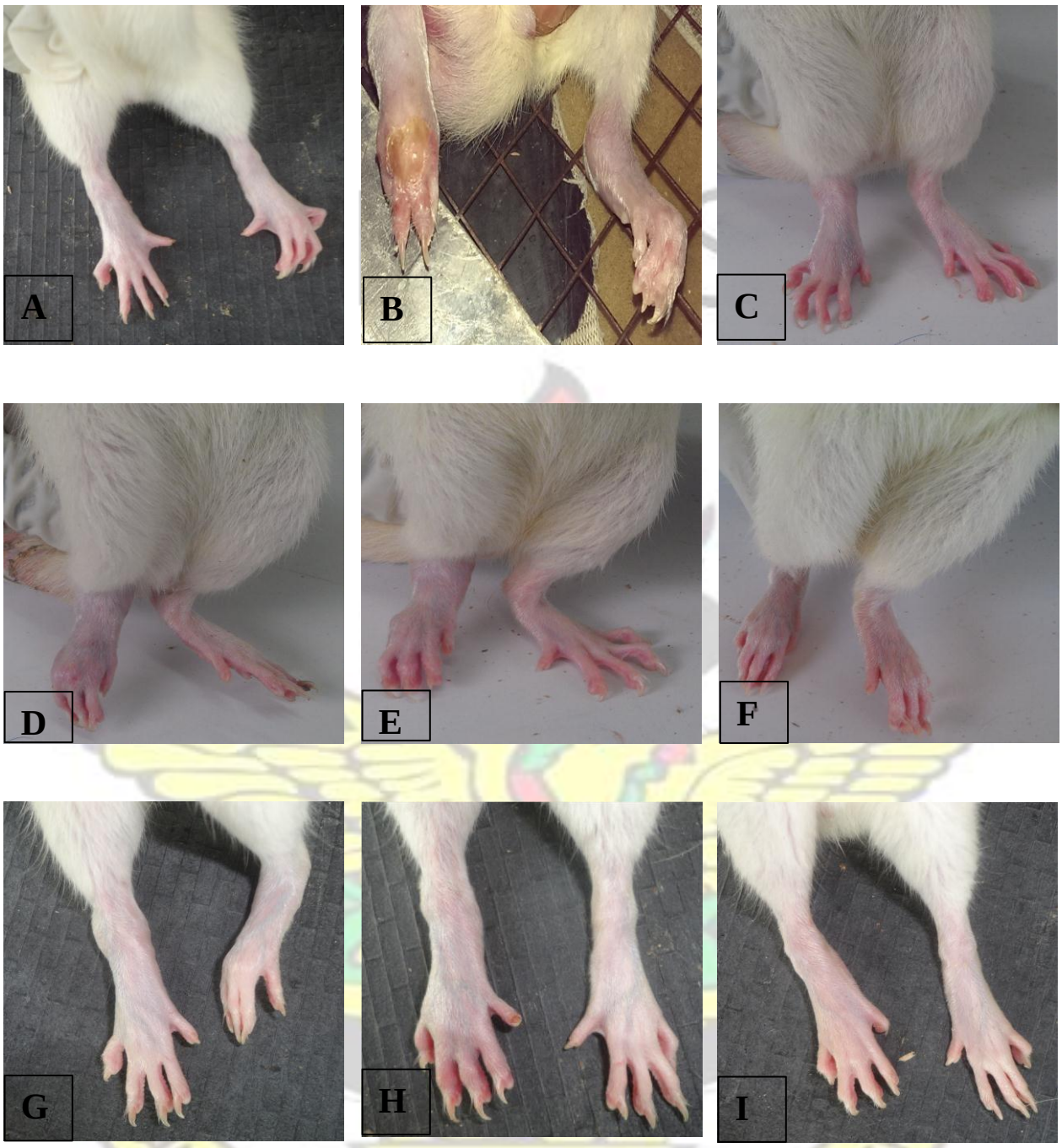


Plate 5.1 Effect of *Holarrhena floribunda* on adjuvant-induced arthritis in rats**.** Sprague Dawley rats (200 - 220 g) were injected subplantar with 0.1 ml of IFA or CFA into the right hind paw. The drug vehicle, drug/extract were administered orally 1 h before the induction of the arthritis and daily for 28 days in the prophylactic model and commenced on the 14th day after the induction of the arthritis and daily till the 28th day in the therapeutic model. Rats were euthanized by cervical dislocation and photographs were taken of the limbs with a digital camera. (A) IFA/non-arthritic control, (B) CFA/arthritic control, (C) Dexamethasone, (D – F) 50 - 500 mg/kg HFE prophylactic, (G – I) 50 - 500 mg/kg HFE therapeutic.

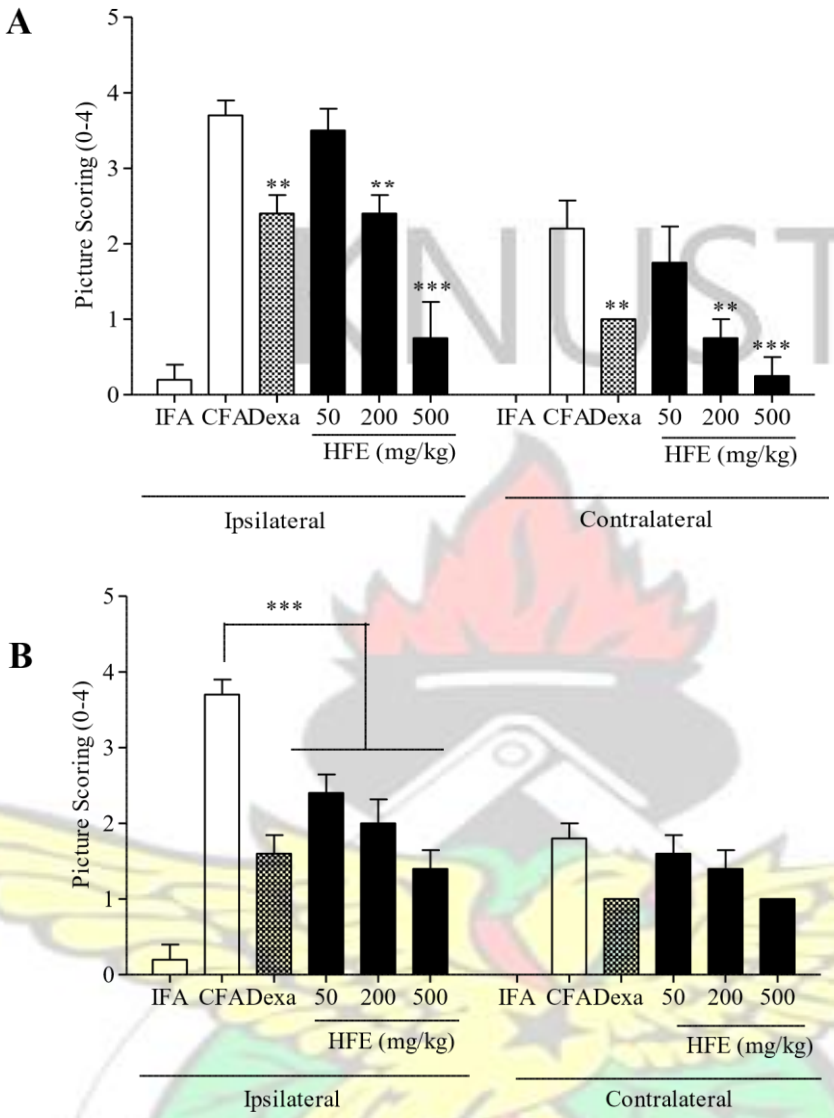


Fig. 5.3 Arthritic score of photographs in adjuvant-induced arthritic rats. Sprague-Dawley rats (200 - 220 g) were injected subplantar with 0.1 ml of IFA or CFA into the right hind paw. The drug vehicle, dexamethasone (0.3 mg/kg) and HFE (50, 200, 500 mg/kg) were administered orally 1 h before arthritis induction and thereafter until the 28th day in the prophylactic model (top panel) and commenced on the 14th day after the induction of arthritis and daily till the 28th day in the therapeutic model (bottom panel). Rats were euthanized by cervical dislocation and photographs were also taken of the limbs with a digital camera and blindly scored 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. Values are Mean ± S.E.M of n= 6. *** ($P < 0.001$), ** ($P < 0.01$), values significantly different from CFA arthritic control group.

5.3.1.3.2 X-ray radiography

The detection and quantification of bone erosion constitutes a major tool for disease diagnosis and for monitoring and evaluation of the efficacy of drug therapy in rheumatoid arthritis.

The effect of HFE on X-ray radiography was determined as described in section 5.1.2.1.3.2. In the IFA non-arthritic control rats, no joint damage or osteolysis of bone was seen in the right and left hind paws (Plate 5.2 A). In the CFA arthritic control group (Plate 5.2 B) there was evidence of severe peri-articular soft tissue swelling in both the injected and noninjected hind limbs. There was also osteolysis of tarsal and metatarsals bone (1) as evidenced by the reduced bone density and demineralization of the bones. There were observable signs of inflammation at the metatarsal-phalangeal joint and the regions inbetween the bones of the phalanges and the metatarsals. Eroding of the phalangeal bone was also observed. Moderate affectation of distal tibia (2) and joint deformation were also seen. Prophylactic treatment with dexamethasone (0.3 mg/kg) resulted in reduced soft tissue swelling, osteolysis of tarsal and metatarsals bone, moderate affectation of distal tibia and reduced joint deformation (Plate 5.2 C). HFE at 50-500 mg/kg dose-dependently reduced peri-articular soft tissue swelling, radiographic joint lesion, bone demineralisation and erosion and joint deformation respectively (Plates 5.2 D, 5.2 E and 5.2 F). HFE prevented the spread of inflammation, joint destruction and osteolysis of bone from the injected limb to the uninjected limb and the fore limbs as was observed in the CFA arthritic control group. In the 50 mg/kg HFE treated rats, there was observable but mild bone demineralization and erosion with decreased joint deformity (Plate 5.2 D). The 200 and 500 mg/kg HFE treatment groups showed radiological profile comparable with IFA non-arthritic control rats (Plate 5.2 F).

Therapeutic treatment with dexamethasone (0.3 mg/kg) resulted in reduced soft tissue swelling, osteolysis of tarsal and metatarsals bone, mild affectation of distal tibia and reduced

joint deformation (Plate 5.2 C). HFE at 50 - 500 mg/kg dose-dependently prevented soft tissue inflammation and subsequent arthritic joint destruction (Plates 5.2 G, 5.2 H and 5.2 I). Treatment with HFE prevented the spread of inflammation of soft tissue and bones deformation from the injected limb to the uninjected limb and fore limbs as was observed in the CFA arthritic control. Rats treated with 500 mg/kg HFE showed radiological profile comparable with IFA non-arthritic rats (Plate 5.2 I)

A blind X-ray score of the radiographs of rats showed the IFA non-arthritic control rats with the lowest score and presented with no peri-articular swelling, osteolysis and joint damage compared to the CFA arthritic control group which showed severe peri-articular swelling, osteolysis and joint damage and thus recorded the highest arthritic score (Table 5.1). Prophylactically, dexamethasone-treated rats showed significant reduction in bone and joint deformation when compared with the CFA arthritic control rats (Table 5.1). HFE (50 - 500 mg/kg) dose-dependently reduced peri-articular swelling, osteolysis and joint damage compared with the CFA arthritic control group (Table 5.1).

When administered therapeutically, dexamethasone-treated rats showed significant reduction in soft tissue swelling, bone and joint deformation when compared with the CFA arthritic control rats (Table 5.1). The extract at the doses administered dose-dependently reduced peri-articular swelling, osteolysis and joint damage compared with the CFA arthritic control group (Table 5.1).

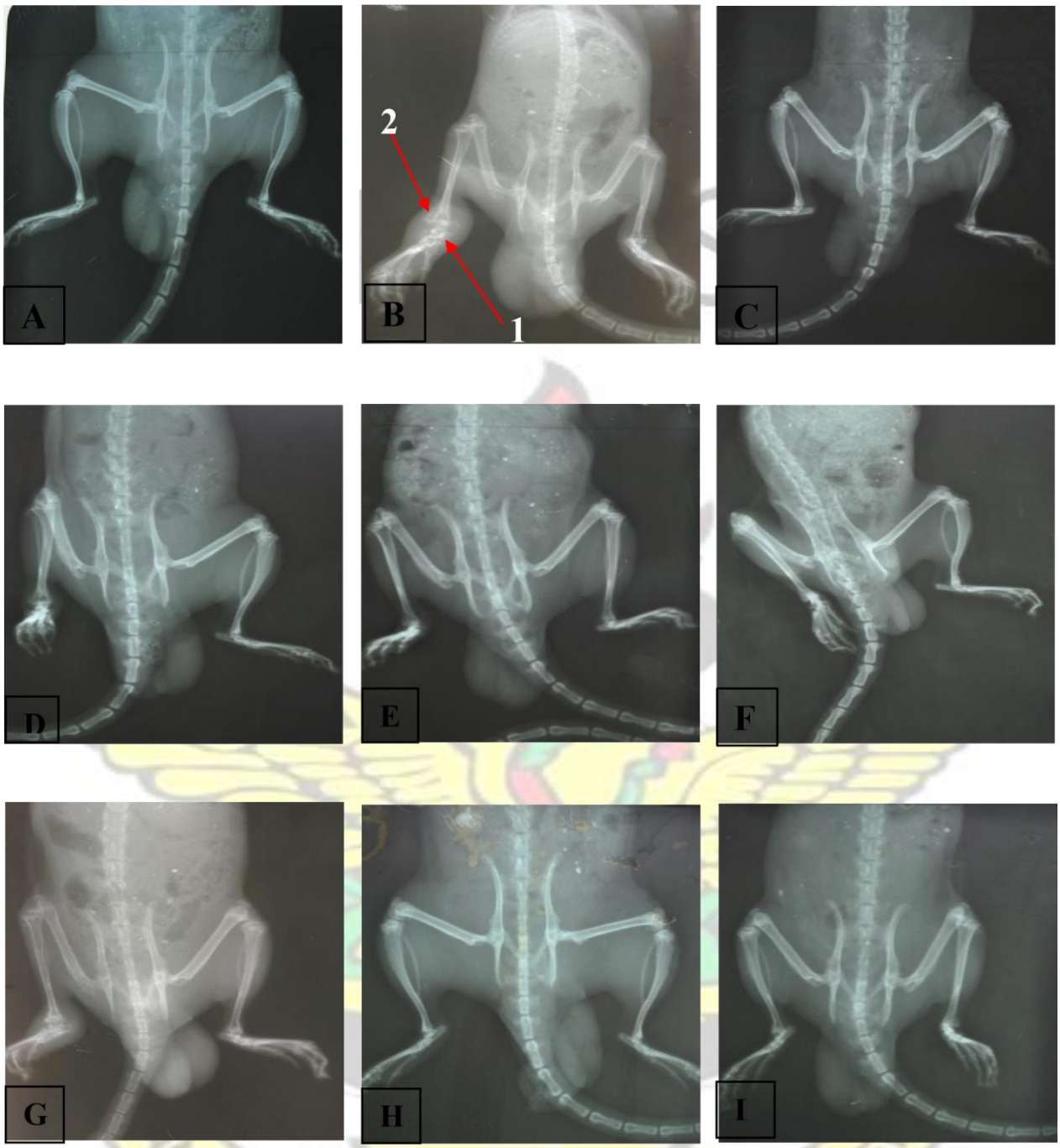


Plate 5.2 Effect of *Holarrhena floribunda* on radiograps of rat paws in adjuvant-induced arthritic rats. Sprague-Dawley rats (200 – 220 g) were injected subplantar with 0.1 ml of IFA or CFA into the right hind paw and monitored for 28 days. The vehicle, drug/extract were administered orally 1 h before the induction of arthritis and daily for 28 days in the prophylactic model (middle panel) and commenced on the 14th day after the induction of the arthritis and daily till the 28th day in the therapeutic model (bottom panel). Rats were euthanized by cervical dislocation and radiographs taken. (A) IFA/non-arthritic control, (B) CFA/arthritic control, (C) Dexamethasone, (D – F) 50-500 mg/kg HFE prophylactic, (G – I) 50-500 mg/kg HFE therapeutic.

Table 5.1 Radiologic score of adjuvant-induced arthritic rats

		Peri-articular swelling	Osteolysis	Joint destruction
	IFA	0	0	0
	CFA	3	3	3
	Dexamethasone (0.3 mg/kg)	1	1	2
Prophylactic treatment	HFE (50 mg/kg)	3	2	2
	HFE (200 mg/kg)	2	2	2
	HFE (500 mg/kg)	1	1	1
Therapeutic treatment	HFE (50 mg/kg)	3	2	2
	HFE (200 mg/kg)	2	2	2
	HFE (500 mg/kg)	1	1	1

Sprague-Dawley rats were injected subplantar with 0.1 ml of IFA or CFA into the right hind paw. The drug vehicle, dexamethasone (0.3 mg/kg) and HFE (50, 200, 500 mg/kg) were administered orally 1 h before arthritis induction and thereafter until the 28th day in the prophylactic approach and started on the 14th day after the induction of arthritis and daily till the 28th day in the therapeutic approach. Rats were euthanized by cervical dislocation and radiographs taken. The extent of peri-articular swelling, oesteolysis and joint damage of the radiographs were blindly scored by a radiologist on a scale of 0-3, where 0: no damage; 1: mild; 2: moderate; and 3: severe.

5.3.1.4 Histology of bones

Histopathological studies of the paws of rats corroborate the evidence of resolution of arthritis and provide a noticeable morphological distinction as a practical and unambiguous pathognomic sign of rheumatoid arthritis (Soren, 1980). To assess the effect of *Holarrhena floribunda* on the histology of paw bone and soft tissues, adjuvant arthritis was induced as earlier described in section 5.1.2.1 and monitored for 28 days. Amputated paws were then processed for histopathological assessment.

Non-arthritic control rats (IFA) had intact bone structure with no visible necrotising granulomatous inflammation and mononuclear cell infiltration (Plate 5.3 A). The CFA arthritic control group had severe presence of necrotising granulomatous inflammation of the synovial membrane, mononuclear cell infiltration with mostly lymphocytes and multinucleated giant cells, vascular proliferation, presence of macrophages and bone erosion (Plate 5.3 B). Treatment with dexamethasone (0.3 mg/kg) both prophylactically and therapeutically resulted in reduced arthritic changes compared to the CFA control group (Plate 5.3 C). When administered prophylactically HFE at 50 - 500 mg/kg dose dependently ameliorated arthritic changes observed in the CFA arthritic control group respectively (Plates 5.3 D, 5.3 E and 5.3 F).

Similarly when administered therapeutically HFE at the same doses, dose-dependently reduced arthritic changes observed in the CFA arthritic control group (Plates 5.3 G, 5.3 H and 5.3 I).

The extent of histopathological damage to bone and joint structure was blindly scored. The IFA non-arthritic control group as expected scored the lowest with the absence of synovial hyperplasia, pannus, bone erosion and inflammatory cells while the CFA arthritic control group recorded the highest score with the severe presence of synovial hyperplasia, pannus, bone erosion, and inflammatory cells (Table 5.2). Prophylactic treatment with 50 - 500 mg/kg HFE resulted in a dose-dependent reduction in histopathological scores with a progressive reduction in synovial hyperplasia, pannus, bone erosion and inflammatory cells (Table 5.2).

When administered therapeutically HFE at the same doses, dose-dependently reduced histopathological scores when compared with the CFA arthritic control group (Table 5.2).

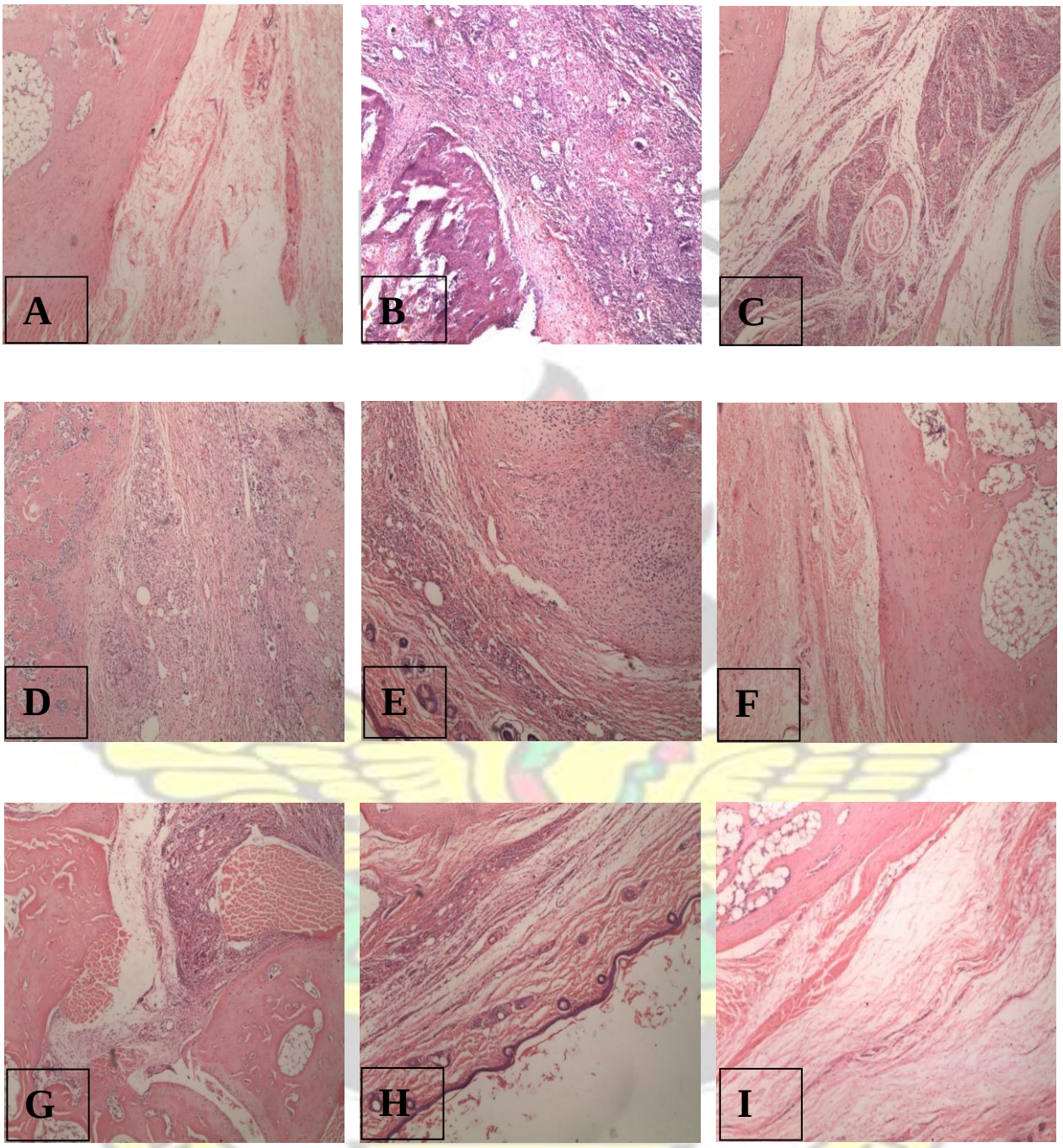


Plate 5.3 Histopathology of paws of adjuvant -induced arthritic rats. Sprague Dawley rats were injected subplantar with 0.1 ml of IFA or CFA into the right hind paw. The vehicle, drug/extract were administered orally 1 h before the induction of the arthritis and daily for 28 days in the prophylactic model and commenced on the 14th day after the induction of the arthritis and daily till the 28 th day in the therapeutic model. Rats were euthanized by cervical dislocation and sections made from th e bone of their right hind limb, stained with haematoxylin and eosin and analysed microscopically. (A) IFA/non -arthritic control, (B) CFA/arthritic control, (C) Dexamethasone, (D _ F) 50 - 500 mg/kg HFE prophylactic, (G _ I) 50 - 500 mg/kg HFE therapeutic

Table 5.2 Histhopathological scores of adjuvant-induced arthritic rats

	Histopathological score	
	Prophylactic approach	Therapeutic approach
IFA	0	0
CFA	3	3
Dexamethasone (0.3 mg/kg)	1	1
HFE (50 mg/kg)	2	2
HFE (200 mg/kg)	2	2
HFE (500 mg/kg)	1	1

Sprague-Dawley rats were injected subplantar with 0.1 ml of IFA or CFA into the right hind paw. The drug vehicle, dexamethasone (0.3 mg/kg) and HFE (50, 200, 500 mg/kg) were administered orally 1 h before arthritis induction and thereafter until the 28th day in the prophylactic approach and started on the 14th day after the induction of arthritis and daily till the 28th day in the therapeutic approach. Rats were euthanized by cervical dislocation, sections made from the bone of their right hind limb, stained with haematoxylin and eosin, analysed microscopically and blindly scored on a scale of 0 - 3. Values are Mean of n= 6

5.3.1.5 Haematology

Changes in peripheral haematological profile of rats were observed on the 28th day of experiment.

Induction of arthritis caused an increase in white blood cell (WBC) and Erythrocyte sedimentation rate (ESR) and a decrease in red blood cell (RBC), haemoglobin (HGB) and haematocrit (HCT) in the CFA control group when compared with the IFA non-arthritic control group. In the prophylactic protocol, treatment of arthritic rats with HFE at 50 - 500 mg/kg dose-dependently and significantly ($P < 0.05$) reduced levels of WBC and ESR and

enhanced levels of RBC, HGB and HCT when compared with the CFA arthritic control group (Table 5.3).

In a similar manner when administered after the induction of arthritis HFE reduced levels of WBC and ESR and enhanced levels of RBC, HGB and HCT when compared with the arthritic control group (Table 5.3).

Table 5.3 Effect of *Holarrhena floribunda* on haematological profile of adjuvant-induced arthritic rats

		WBC ($x10^3/\mu L$)	RBC ($x10^6/\mu L$)	HGB ($g\ dL^{-1}$)	HCT (%)	ESR (mm/h)
	IFA	13.58 ± 1.25	8.18 ± 0.14	15.15 ± 0.22	47.15 ± 0.79	0.40 ± 0.24
	CFA	18.94 ± 0.85	6.83 ± 0.395	12.96 ± 0.25	40.26 ± 1.33	10.00 ± 1.87
	Dexa(0.3mg/kg)	7.00 ± 0.56#	8.31 ± 0.18¥	14.74 ± 0.25#	45.34 ± 0.97¥	2.20 ± 0.94#
PT	HFE(50 mg/kg)	16.65 ± 1.8	7.81 ± 0.14¥	13.85 ± 0.18*	42.38 ± 0.86	6.50 ± 1.19*
	HFE(200mg/kg)	14.68 ± 0.2*	8.08 ± 0.22¥	14.80 ± 0.37#	45.35 ± 0.87¥	3.40 ± 0.93#
	HFE(500mg/kg)	13.97 ± 1.32*	8.29 ± 0.26¥	14.96 ± 0.11#	46.24 ± 0.27¥	1.60 ± 0.68#
TT	HFE(50 mg/kg)	18.78±0.75	7.86±0.39*	14.83±0.44¥	43.93±2.22	6.40 ± 0.81*
	HFE(200mg/kg)	17.55±1.14	7.94±0.10*	14.33±0.24*	44.43±0.44¥	4.00 ± 0.55#
	HFE(500mg/kg)	15.50±0.39	8.19±0.21¥	15.45±0.57#	47.00±0.35¥	2.20 ± 0.37#

Sprague-Dawley rats were injected subplantar with 0.1 ml of IFA or CFA into the right hind paw. The drug vehicle, dexamethasone (0.3 mg/kg) and HFE (50, 200, 500 mg/kg) were administered orally 1 h before arthritis induction and thereafter until the 28th day in the prophylactic model (PT) and commenced on the 14th day after the induction of arthritis and daily till the 28th day in the therapeutic model (TT). Blood was collected from the tail vein on the 28th day and a full blood count was done using a haem analyser. Erythrocyte sedimentation rate (ESR) was also determined using the standard Westergren method. Data are presented as Mean ± S.E.M. (n=6). * ($P < 0.05$), ¥ ($P < 0.01$), # ($P < 0.001$): values significantly different when compared with CFA arthritic control.

5.4 DISCUSSION

The effect of *Holarrhena floribunda* on adjuvant - induced arthritis, a model for the study of chronic inflammation was evaluated. The Complete Freud Adjuvant (CFA)-induced arthritis in rats, reported by Stills (2005) to be the most widely used model of arthritis because of characteristics in common with human rheumatoid arthritis was adopted. The presence of excessive pain, long-lasting inflammatory reaction that appeared at the site of injection, lesions that eventually developed into ulcers, peri-articular erythema, oedema, and a reduction of paw function was observed in the CFA arthritic control groups. This is in agreement with findings made by Ramprasah *et al.*, (2006) that CFA stimulates the immunological and biological features of rheumatoid arthritis and that of Stills (2005) who further stated that the outcome of immune-modulation by CFA is inflammation, tissue destruction, and the potential for pain and distress in the host animal. *Holarrhena floribunda* significantly reduced these biological and immunological reactions. Adjuvant-induced arthritis develops in two phases: an acute peri-articular inflammation phase followed by a chronic inflammation phase of joint and bone involvement (Pearson, 1956; Jacobson *et al* 1999). In both approaches adopted in the study the CFA arthritic control group showed acute inflammation in the injected limb between days 4-6 followed by a chronic polyarthritic inflammation phase which started after day 14. HFE at the doses used dose-dependently and significantly inhibited joint inflammation and eventually prevented joint and bone destruction in rats in agreement with Atzeni and Sarzi-Puttini‟s (2007) claim that antiarthritic medications achieve cardinal objectives of protection of joint integrity and suppression of synovitis.

A characteristic feature of rheumatoid arthritis is weight loss and the loss of body cell mass called rheumatoid cachexia. Prevention of weight loss in HFE treated rats is indicative of the ability of the extract to prevent rheumatoid cachexia thereby preventing the associated

mortality. The loss of body cell mass involves a net catabolic process, with negative energy, protein, and micronutrient balances over a period of time. The presence of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α have been reported to be the mediators behind body wasting that results with chronic inflammation (Clowes *et al* 1983; Baracos *et al*., 1983; Warren *et al* 1987; and Moldawer *et al* 1992; Ershler and Keller 2000). A loss of greater than 40% of existing lean body cell mass results in death (Roubenoff and Rall, 1993) and its been reported that patients with rheumatoid arthritis have body cell mass 13% lower compared with healthy adult suggesting that approximately one-third of mobilisable body cell mass is lost in these patients with clinically well controlled rheumatoid arthritis (Roubenoff *et al.*, 1994). This is in agreement with observation made in the study where arthritic rats had significantly lower body weights compared to non-arthritic rats.

Holarrhena floribunda dose-dependently caused a reduction in soft tissue swelling, articular space narrowing, extent of joint deformity or damage and demineralization or erosion of bones. This is significant because bone erosion and joint deformation are central features of rheumatoid arthritis and are associated with disease severity. A disparity between calcium reabsorption in bone by osteoclast and calcification of bone by osteoblast leads to bone erosion (Makinen *et al.*, 2007). The progression of bone erosion involves an initial phase of induction with no evidence of synovitis, followed by early synovitis and a concluding phase of synovitis with continuous joint destruction (Hoffmann e*t al.* 1997). Rheumatoid arthritis targets the synovial lining of joints and as the disease progresses a continuous destruction of the structural components of joints such as the articular cartilage and the bone at the margins of the joint occurs. The ability of HFE to inhibit joint deformity and subsequently bone erosion makes it a good candidate for the management of rheumatoid arthritis.

Histopathological analysis of the paws of rats provides a noticeable morphological distinction as a practical and unambiguous pathognomic sign of the resolution of rheumatoid arthritis (Soren, 1980). It also demonstrates the ability of bone structures to re-calcify upon treatment with an extract. The histographs for the CFA arthritic control group showed distinctive signs of severe necrotising granulomatous inflammation with massive infiltration of lymphocytes and multinucleated giant cells. There was vascular proliferation and presence of macrophages with severe bone and cartilage destruction, and pannus. Administration of HFE both prophylactically and therapeutically suppressed inflammation, synovitis and also bone erosion, consistent with documented findings that the capability of a drug to suppress inflammation, synovitis and maintain joint integrity is desired in rheumatoid arthritis therapy (Hoffmann *et al.*, 1997; Atzeni and Sarzi-Puttini, 2007).

A characteristic feature of anaemia of chronic disorders is a reduction in iron levels, red blood cells survival, iron-binding capacity and the failure of bone marrow to increase erythrocyte production. This is usually associated with chronic infection, inflammation, and malignancy (Cartwright, 1966). The progression of rheumatoid arthritis is associated with a reduction in levels of red blood cells, haemoglobin, and haematocrits (Braunwald *et al.*, 2001). This extract demonstrated the ability to resolve rheumatoid arthritis and the associated anaemia. This was evidenced by reduced white blood cells count and increased levels of red blood cells, haemoglobin, and haematocrits significantly when compared to the CFA control groups. HFE also significantly inhibited erythrocyte sedimentation rate (ESR), an indirect means of ascertaining inflammation in the body when compared with the CFA arthritic control group. ESR measures the rate at which erythrocytes settles or sediments. Inflammatory

proteins present during an inflammatory episode causes erythrocytes to stack up, become denser and as a result settle faster. These effects observed on blood indices indicate the potential of HFE at inhibiting the progression of rheumatoid arthritis.

5.5 CONCLUSION

The hydroethanolic extract of *Holarrhena floribunda* has exhibited inhibitory effects on chronic inflammation. This was demonstrated with its effect on various indices used in measuring adjuvant-induced arthritis in Sprague-Dawley rats.

CHAPTER SIX POSSIBLE MECHANISMS OF ACTION OF HYDROETHANOLIC EXTRACT OF

HOLARRHENA FLORIBUNDA AS AN ANTI-INFLAMMATORY AGENT

6.0 INTRODUCTION

The role of the mast cell in inflammation at the molecular level is unequivocal. The accumulation and upregulation of high affinity receptor (FcεR1) bound IgE leads to mast cells stimulation and activation (Stone *et al.,* 2010). Subsequent crosslinking of the FceRIbound IgE on mast cells generates a series of mechanisms that result in the degranulation of the mast cells, the release of arachidonic acid from the membrane of the mast cell and its subsequent

metabolism and cytokine expression in the nucleus (Galli and Tsai, 2003, Grutzkau *et al.*, 1998; Kemp and Lockey, 2002).

As reported by Moore and Willoughby (1995) concentrating on a particular group of mediators for the management of inflammation results in other mediators compensating for the deficit thereby reducing the potency and effectiveness of the treatment. A good antiinflammatory drug should therefore have multiple effects on various mediators of inflammation. Consequently mediator-induced paw oedema was used to assess the effect of HFE on degranulation. Prostaglandin E_2-induced oedema was also used to assess the effect of the extract on the arachidonic acid pathway. For cytokine gene expression, serum levels of IL-1α, IL-6 and IL-10 were determined. Since inflammation involves the recruitment of cells to the inflammatory site serum levels of protein kinase C (PKC), protein kinase A (PKA) and cyclic adenosine monophosphate (cAMP) were also determined.

6.1 MATERIALS AND METHODS

6.1.1 Materials

6.1.1.1 Drugs and chemicals

Clonidine (Boehringer Ingelheim Inc, USA); Chlorpheniramine (DWD Pharmaceuticals Ltd, India); Granisetron hydrochloride (Roche, Basel, Switzerland); Haloperidol (Incas Pharmaceuticals Ltd , India); Histamine dihydrochloride, diclofenac, serotonin hydrochloride and prostaglandin E_2 (Sigma-Aldrich Chemical Co, St Louis, USA); IL-1α, IL-6, and IL-10 ELISA kits (Abcam Plc, Cambridge, UK), Cyclic adenosine monophosphate (cAMP), Protein kinase C (PKC) and Protein kinase A (PKA) ELISA kits (Mybioscience, San Diego, California, USA)

6.1.1.2 Animals

Sprague-Dawley rats (200 - 220 g) and Imprint Control Region (ICR) mice (20 - 30 g) were used. The animals were maintained with the necessary conditions as described in section 3.1.1.1.

6.1.2 Methods

6.1.2.1 Effect of *Holarrhena floribunda* on the degranulation process

6.1.2.1.1 Histamine-induced paw oedema

The effect of HFE on histamine-induced oedema was measured with histamine employed as a phlogistic agent (Singh and Pandey, 1996). Paw oedema was induced in Sprague-Dawley rats (200 -220 g, n=6) by subplantar injection of 0.1 ml of freshly prepared histamine (1 % w/v) in the right hand paw and oedema was monitored at a 1 h interval for 4 h with a plethysmometer (7140, UGO Basil ltd, Camerio VA, ITALY). Chlorpheniramine (CPM,

4 mg/kg) and HFE (50, 200 and 500 mg/kg) were administered orally 1 h prior to oedema induction. Control rats received sterilized distilled water. The maximal oedema and total oedema responses were calculated as described in section 3.1.2.1

6.1.2.1.2 Indirect anti-histaminic effect of *Holarrhena floribunda*

6.1.2.1.2.1 Clonidine-induced catalepsy

Clonidine-induced catalepsy test (Ferre *et al*., 1990) was carried out to determine the indirect anti-histaminic activity of the extract. Clonidine (5 mg/kg, s.c.) was administered to ICR mice (20 – 30 g, n=6). The mice were made to grip a horizontal bar (1 cm in diameter, 3 cm above the table) with their fore paws. The time taken for the mice to remove their paws from the bar

was recorded as the duration of catalepsy and was measured at a 30 min interval for 3 h. Drug/extract administered was either 4 mg/kg chlopheniramine (CPM) or 50, 200 and 500 mg/kg HFE. The control group received sterilized distilled water. In the prophylactic model drug/extract was administered 1 h before catalepsy induction whereas in the therapeutic model drug/extract was administered 1 h post catalepsy induction.

6.1.2.1.2.2 Haloperidol-induced catalepsy

Haloperidol-induced catalepsy test (Ferre *et al.*, 1990) was carried out as described for the clonidine-induced catalepsy in section 6.1.2.1.2.1. A dose of 5 mg/kg of haloperidol was used to induce catalepsy instead of clonidine.

6.1.2.1.3 Serotonin-induced paw oedema

Paw oedema was induced by a method earlier described (Singh and Pandey, 1996). SDRs (n=6) were injected with 1 % (w/v) serotonin in normal saline (0.1 ml, s.c.) into the subplantar tissue of the right hind paw. Oedema was monitored at a 1 h interval for 4 h with a plethysmometer (7140, UGO Basil ltd, Camerio VA, ITALY). Control rats received sterilized distilled water while drug/extract-treated groups received either granisetron (100 µg/kg) or HFE (50, 200 and 500 mg/kg) orally 1 h prior to oedema induction. The maximal oedema and total oedema response were calculated as described in section 3.1.2.1

6.1.2.2 Effect of *Holarrhena floribunda* on arachidonic acid pathway

6.1.2.2.1 Prostaglandin E_2 (PGE_2) -induced paw oedema

Paw oedema was induced by a method earlier described (Singh and Pandey, 1996). SDRs (n=6) were injected with 1 % (w/v) Prostaglandin E_2 in normal saline (0.1 ml, s.c.) into the

subplantar tissue of the right hind paw. Oedema was monitored at 1 h interval for 4 h with a plethysmometer (7140, UGO Basil ltd, Camerio VA, ITALY). Control rats received sterilized distilled water while drug/extract-treated groups received either diclofenac (100 mg/kg) or HFE (50, 200 and 500 mg/kg). Drug/extract was given orally 1 h prior to oedema induction. The maximal oedema and total oedema response were calculated as described in section 3.1.2.1

6.1.2.3 Effect of *Holarrhena floribunda* on cytokine gene expression

6.1.2.3.1 Quantitative determination of serum cytokine (IL-1α, IL-6 and IL-10) levels Adjuvant arthritis was induced as previously described in section 5.1.2.1 in Sprague-Dawley rats. Blood from the tail vein was collected into vacutainer gel and clot activator tubes (SG Biotech, Meddlessex, England) on the 28th day, allowed to clot at room temperature and then centrifuged at ×1000 g for 10 min. The sera obtained were aliquoted into eppendorf tubes and stored at -80^{o}C until analyzed. Serum levels of the cytokines IL-1α, IL-6 and IL10 were measured in duplicates with the appropriate rat ELISA kit according to the manufacturer‟s recommendations. Briefly 100 µl each of standard and samples were pipetted into appropriately labelled wells in micro titre plates pre-coated with monoclonal antibodies specific for IL-1α, IL-6 and IL-10 respectively and incubated overnight at 4°C with gentle shaking. After washing away any unbound substances, 100 µl of prepared biotin anti-rat antibody specific for IL-1α, IL-6 and IL-10 respectively were added to each well and incubated for 1 h at room temperature with gentle shaking. Wells were then thoroughly washed and 100 µl of prepared Streptavidin solution was added to each well and incubated for 45 min at room temperature with gentle shaking. Washing was repeated and 100 µl of 3,3,5,5 Tetramethylbenzidine (TMB) One-Step substrate reagent added to each well and incubated

for 30 min at room temperature in the dark to develop colour in proportion to the amount of bound cytokine. The enzyme-substrate reaction was terminated by the addition of 50 µl of Stop solution to each well and optical density read immediately in an Ultra Microplate Reader ELx 808IUI (Bio-Tek Instruments, Inc, USA) at 450 nm.

6.1.2.4 Effect of *Holarrhena floribunda* on enzymes and secondary messengers

6.1.2.4.1 Quantitative determination of serum enzyme (PKA and PKC) levels

Adjuvant arthritis was induced as earlier described in section 6.1.2.3 in Sprague-Dawley rats. Serum levels of Protein kinase A (PKA) and Protein kinase C (PKC) were measured in duplicates with the appropriate rat ELISA kit according to the manufacturer‟s recommendations. Briefly 100 µl each of standard and sample were pipetted into appropriately labelled wells in micro titre plate pre-coated with monoclonal antibodies specific for PKA and PKC respectively and 50 µl of Conjugate added to each well and incubated for 1 h at 37°C. Wells were then washed and 50 µl of Substrate A and 50 µl of Substrate B added subsequently. The wells were then covered and incubated for 10 min at 37°C in the dark. The enzyme-substrate reaction was terminated by the addition of 50 µl of Stop solution to each well, mixed well and optical density read immediately in an Ultra Microplate Reader ELx 808IUI (Bio-Tek Instruments, Inc, USA) at 450 nm.

6.1.2.4.2 Quantitative determination of serum secondary messenger (cAMP) levels Adjuvant arthritis was induced as earlier described in section 6.1.2.3 in Sprague-Dawley rats. Serum levels of cyclic adenosine monophosphate (cAMP) were measured in duplicates with the appropriate rat ELISA kit according to the manufacturer‟s recommendations.

Briefly 100 µl each of standard or sample were pipetted to the appropriate wells in the antibody pre-coated microtiter plate. 100 ul of PBS (pH 7.0-7.2) was added in the blank control well. 50 µl of Conjugate was added to each well (blank control well excluded) and incubated for 1 h at 37°C. Wells were washed and 50 µl of Substrate A and 50 µl of Substrate B added subsequently. The wells were then covered and incubated for 10 min at 20-25°C in the dark. The enzyme-substrate reaction was terminated by the addition of 50 µl of Stop solution to each well, mixed well and optical density read immediately in an Ultra Microplate Reader ELx 808IUI (Bio-Tek Instruments, Inc, USA) at 450 nm.

6.2 STATISTICAL ANALYSIS

Data obtained were subjected to statistical tests of significance using the one way analysis of variance (ANOVA), followed by the Newman-Keuls post-test to assess significant variation in groups tested. Probabilities less than 0.05 ($P < 0.05$) were considered statistically significant. All statistical analysis was performed using the GraphPad prism software version 5.01.

6.3 RESULTS

6.3.1 Effect of *Holarrhena floribunda* on the degranulation process

6.3.1.1 Histamine-induced paw oedema

Histamine is the most characterized mediator involved in the early phase inflammatory reactions (Petersen *et al.*, 1996). Its role was therefore determined in-vivo by assessing the effect of HFE on histamine-induced paw oedema. To do this, oedema was induced with the injection of 100 µl of 1 % histamine into subplantar tissue of the right hind paw of rats. Paw

volumes were measured by plethysmometer at a 1 h interval for 4 h as earlier described in section 6.1.2.1.1.

From this test, the percentage mean maximal oedema attained for the inflamed control group was 70.89 ± 4.47 % (Fig. 6.1 A). HFE given at 50, 200 and 500 mg/kg, dose-dependently reduced the percentage mean maximal oedema attained to 50.27 ± 8.19 %, 43.88 ± 4.26 % and 42.03 ± 2.33 % respectively (Fig. 6.1 A). These same doses of the extract, dosedependently inhibited the total paw oedema induced over 4 h by 22.04, 28.29 and 34.86 % respectively (Fig 6.1 B). The observed inhibitions were significantly ($P < 0.05$-0.01) different from the inflamed control group.

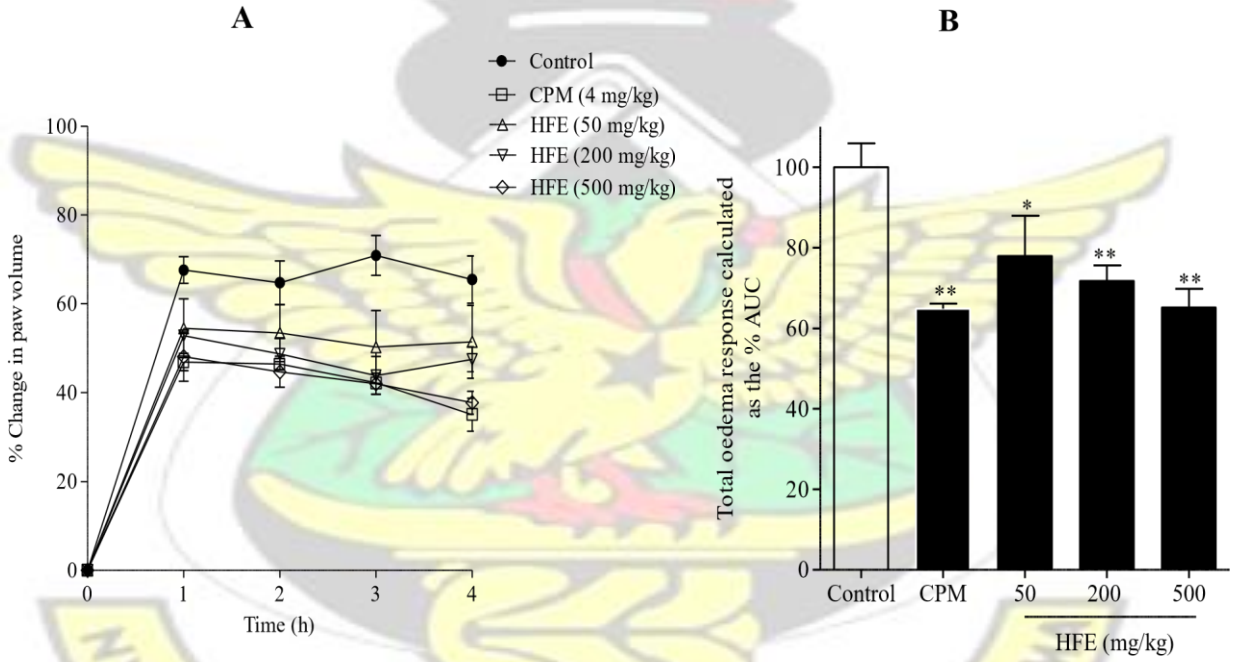


Fig 6.1 Effect of *Holarrhena floribunda* extract on histamine-induced paw oedema in rats. Oedema was induced by injection of 0.1 ml of 1 % (w/v) histamine and monitored at a 1 h interval for 4 h as percentage increase in paw thickness (A). Total oedema induced during the 4 h period was calculated as area under the time course curves, AUC (B). Chlorpheniramine (CPM, 4 mg/kg *p.o.*) or HFE (50, 200, 500 mg/kg *p.o.*) was given 1 h before induction of oedema. Data are presented as Mean ± S.E.M. (n=6). * $P < 0.05$, ** $P < 0.01$ values significant when compared with control.

6.3.1.2 Indirect anti-histaminic effect of *Holarrhena floribunda*

6.3.1.2.1 Clonidine-induced catalepsy

Clonidine, an α_2-adrenergic agonist stimulates the histamine release from mast cells in the brain which causes catalepsy. This catalepsy is inhibited by antagonists of the histamine H_1 receptor but not H_2 receptor antagonist (Lakdawala *et al.,* 1980, Jadhav *et al.,* 1983). The clonidine-induced catalepsy test was used in assessing the inhibitory effect of HFE on histamine release by mast cells.

To do this clonidine was administered to mice and the effect of HFE on catalepsy determined.

Results in the prophylactic protocol show that maximum catalepsy was observed in the control group 2 h after induction of catalepsy. From the study HFE administered at 50 - 500 mg/kg, dose-dependently and significantly ($P<0.05$) inhibited clonidine- induced catalepsy in a time-dependent fashion (Fig. 6.2 A). A maximum duration of catalepsy of 30.80 ± 8.10 s attained in the control group was reduced in a dose-dependent manner (6.8 ± 1.16, 5.8 ± 1.07 and 4.8 ± 0.73 s) respectively with HFE treatment (Fig. 6.2 A). Total catalepsy induced calculated as the AUC over the 3 h period was also dose-dependently and significantly ($P<0.05$) reduced by 75.64, 77.14 and 84.12 % respectively at the same doses of the extract (Fig. 6.2 B).

When drug/extract were administered an hour after catalepsy induction, maximum catalepsy of 19 ± 4.49 s attained in the control group was respectively reduced to 8.00 ± 1.78, 7.00 ± 0.91 and 6.20 ±1.24 s with HFE (50, 200 and 500 mg/kg) (Fig. 6.2 C). Total catalepsy induced over the 3 h period was also dose-dependently and significantly ($P<0.05$-0.001) reduced by 61.47, 64.01 and 70.19 % respectively (Fig. 6.2 D).

A **B**

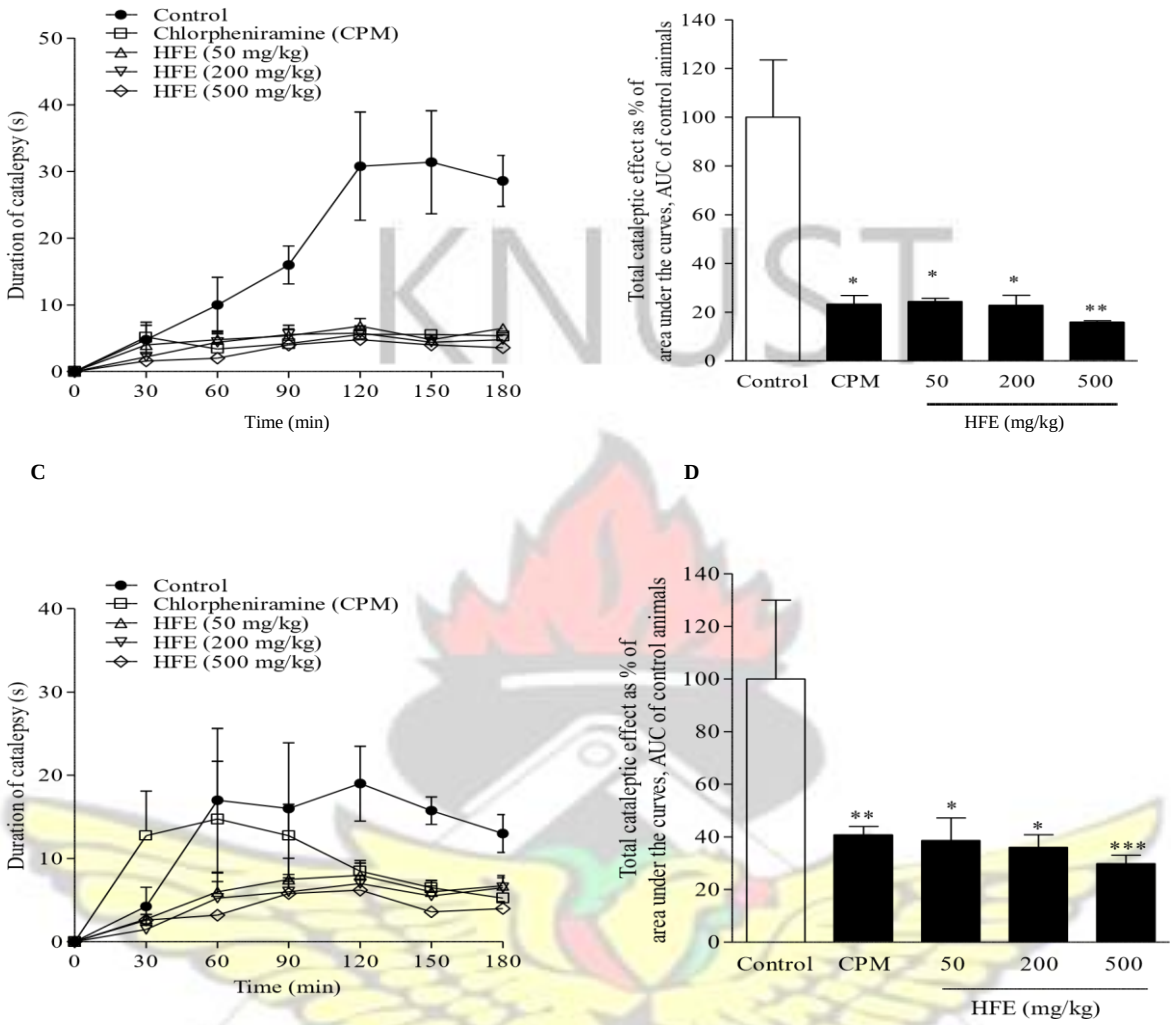


Fig. 6.2 Effect of *Holarrhena floribunda* on clonidine-induced catalepsy in mice. ICR mice (20-30 g) received clonidine 5 mg/kg, s.c. and their fore paws placed on a horizontal bar (1 cm in diameter, 3 cm above the table). The duration of catalepsy was measured before the administration of clonidine and at a 30 min interval up to 3 h and presented as duration of catalepsy (A and C). Total catalepsy induced during the 4 h was calculated as area under the time course curves, AUC (B and D). In the preventive protocol (upper panel), vehicle, chlorpheniramine (4 mg/kg) and HFE (50, 200 and 500 mg/kg), and were given orally for 2 consecutive days ending 30 min before clonidine injection. In the curative protocol (lower panel) drug/extract treatment commenced 1 h after induction of catalepsy. Values are Mean± S.E.M. (n=6). Significance between vehicle and drug/extract treated mice denoted by $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$

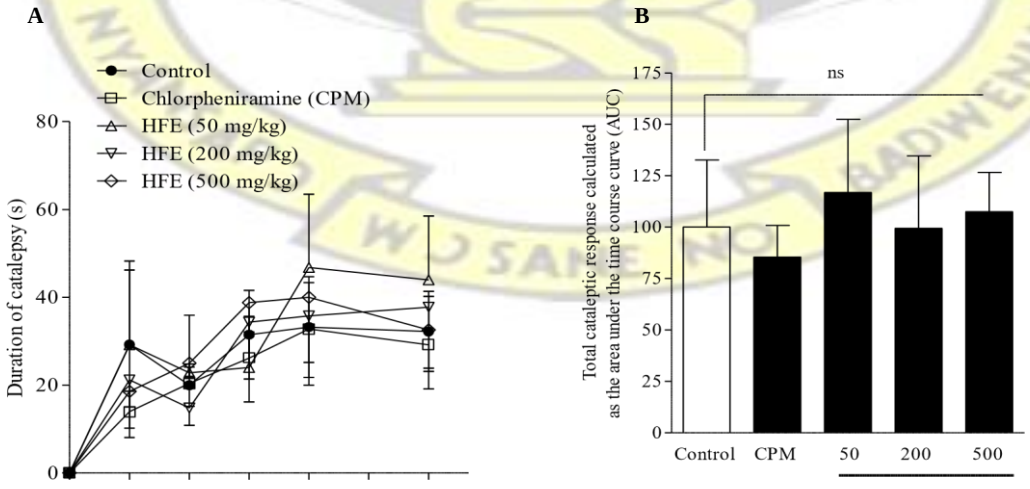
6.3.1.2.2 Haloperidol-induced catalepsy

Haloperidol causes catalepsy mediated through its effects on dopamine receptors localised postsynaptically on striatal neurons (Sanberg, 1980). This cataleptic episode cannot be blocked by histamine H_1 receptor antagonist because it does not involve histamine release from mast cells. This test was run to confirm that the extract has histamine H_1 receptor blocking activity.

To perform this investigation, haloperidol was administered to mice as described earlier and the effect of the extract on catalepsy was determined.

Results from the study indicated that chlopheniramine, a known histamine H_1 receptor antagonist had no inhibitory effect on both maximum catalepsy attained (Figs 6.3 A and 6.3 C) and total catalepsy calculated as AUC (Figs 6.3 B and 6.3 D). HFE administered prophylactically at doses of 50-500 mg/kg did not have any inhibitory effect on the maximum catalepsy attained when compared to the control group (Fig 6.3 A). Total catalepsy induced over 3 h was also not inhibited by HFE (Fig 6.3 B).

Therapeutic administration of HFE at the same doses did not have any inhibitory effect on the maximum catalepsy attained compared to the control group (Fig 6.3 C). Total catalepsy induced over the 3 h period was also not inhibited by HFE (Fig 6.3 D).



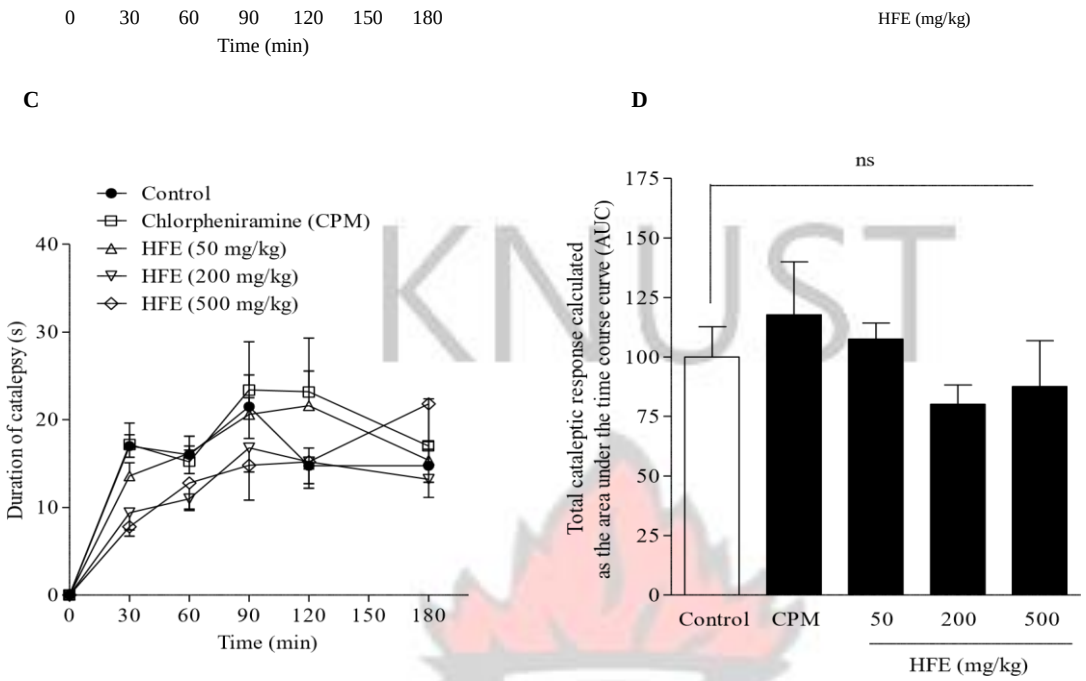


Fig. 6.3 Effect of *Holarrhena floribunda* extract on haloperidol-induced catalepsy in mice. ICR mice (20-30 g) received haloperidol 5 mg/kg, s.c. and their fore paws placed on a horizontal bar (1 cm in diameter, 3 cm above the table). The duration of catalepsy was measured before the administration of haloperidol and at a 30 min interval up to 3 h and presented as duration of catalepsy (A and C). Total catalepsy induced during the 4 h period was calculated as area under the time course curves, AUC (B and D). In the preventive protocol (top panel), vehicle, chlorpheniramine (4 mg/kg) and HFE (50, 200 and 500 mg/kg) were given orally 30 min before haloperidol injection. In the curative protocol (bottom panel) drug/extract treatment commenced 1 h after induction of catalepsy. Values are Mean± S.E.M. (n=6). Significance between vehicle and drug/extract treated mice denoted by $^{**}P \leq 0.001$, and $^{*}P \leq 0.01$

6.3.1.3 Serotonin-induced paw oedema

Serotonin is a monoamine which is very critical in inflammatory and allergic reactions with effects similar to that of histamine (Vasudevan, 2007; Beer, *et al.*, 1984). The inhibitory effect of *Holarrhena floribunda* on serotonin was therefore evaluated with the serotoninduced paw oedema test by Singh and Pandey (1996).

Briefly, oedema was induced with subplantar injection of 0.1 ml of 1 % serotonin in the right hind paw of rats and monitored as earlier described in section 6.1.2.1.3. From the results it was observed that compared to the control group with a percentage mean maximal oedema

response of 59.22 ± 8.08, HFE (50, 200 and 500 mg/kg) dose-dependently and significantly ($P < 0.001$) reduced percentage mean maximal oedema attained to 28.16 ± 4.09 %, 14.98 ± 4.07% and 10.99 ± 2.33 % respectively (Fig. 6.4A). In a similar manner HFE at the same doses inhibited total paw oedema induced over 4 h by 51.07, 68.42 and 80.82 % respectively (Fig 6.4B).

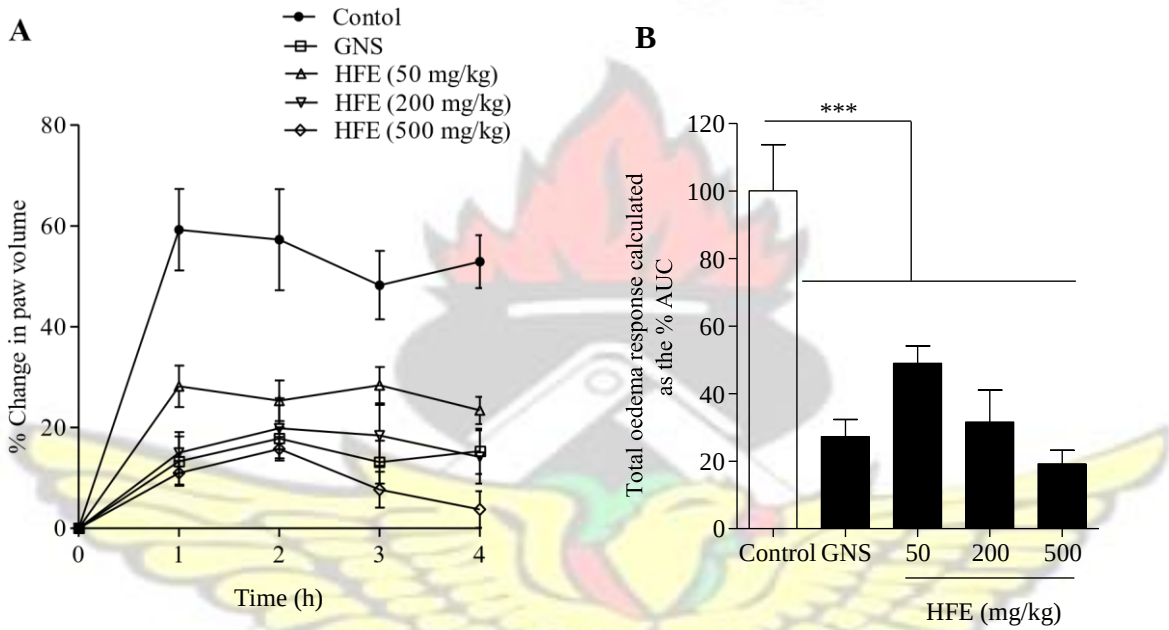


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Fig 6.4 Effect of *Holarrhena floribunda* extract on serotonin-induced paw oedema in rats. Oedema was induced by injection of 0.1 ml of 1 % (w/v) serotonin and monitored at 1 h interval for 4 h. Percentage increase in paw thickness was determined (A) and total oedema induced during the 4 h period was calculated as area under the time course curves, AUC (B). Granisetron, GNS (100 µg/kg p.o) or HFE (50, 200, 500 mg/kg p.o.) was given 1 h before induction of oedema. Data are presented as Mean ± S.E.M. (n=6). *** $P < 0.001$ values significant when compared with control.

6.3.2. Effect of *Holarrhena floribunda* on arachidonic acid pathway

6.3.2.1 Prostaglandin E_2 (PGE_2)-induced paw oedema

The development of the cardinal signs of acute inflammation is increased with the synthesis of prostaglandins (Ricciotti and FitzGerald, 2012). Prostaglandins are reported to mediate a

number of effects including the dilation and constriction of blood vessels (Giles and Leff, 1988), the repression of the aggregation of thrombocytes (Whittle *et al.*, 1985), as well as the relaxation and contraction of smooth muscle (Narumiya and Toda, 1985). The effect of HFE on prostaglandin E_2 was consequently determined using PGE_2-induced paw oedema test.

To do this a subplantar injection of 1 nM of prostaglandin E_2 was performed in the paw of rats and oedema monitored. In this study the control group had a percentage mean maximal oedema response of 75.38 ± 1.54 %. When treated with doses of 50 - 500 mg/kg of HFE, the percentage mean maximal oedema attained was dose-dependently reduced to 32.79 ± 8.96 %, 26.44 ± 3.60 % and 16.49 ± 6.04 % respectively (Fig. 6.5 A). Similarly total paw oedema induced over 4 h was dose-dependently and significantly ($P < 0.001$) inhibited by 54.36 %, 69.02 % and 79.43 % respectively (Fig 6.5 B)

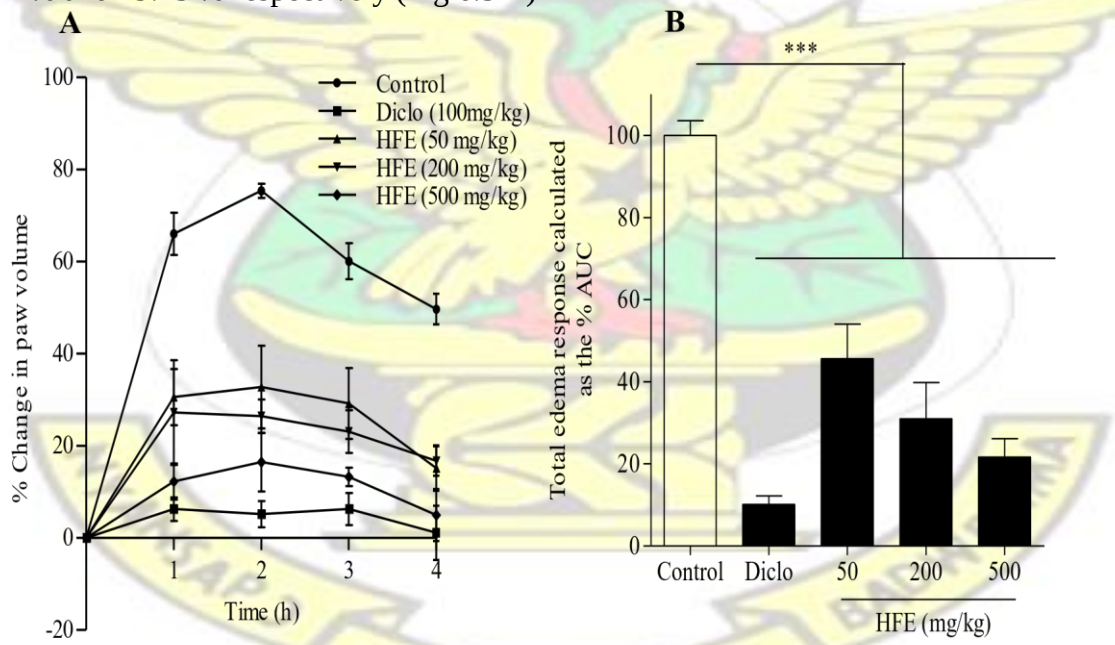


Fig 6.5 Effect of *Holarrhena floribunda* on prostaglandin E_2-induced paw oedema in rats. Oedema was induced by injection of 0.1 ml of 1 nM prostaglandin E_2 and monitored at a 1 h interval for 4 h. Percentage increase in paw thickness was determined (A) and total oedema induced during the 4 h period was calculated as area under the time course curves, AUC (B). Diclofenac (100 mg/kg p.o.) or HFE (50, 200, 500 mg/kg p.o.) was given 1 h before induction

of oedema. Data are presented as Mean ± S.E.M. (n=6). *** P < 0.001: values significantly different when compared with control.

6.3.3 Effect of *Holarrhena floribunda* on cytokine gene expression

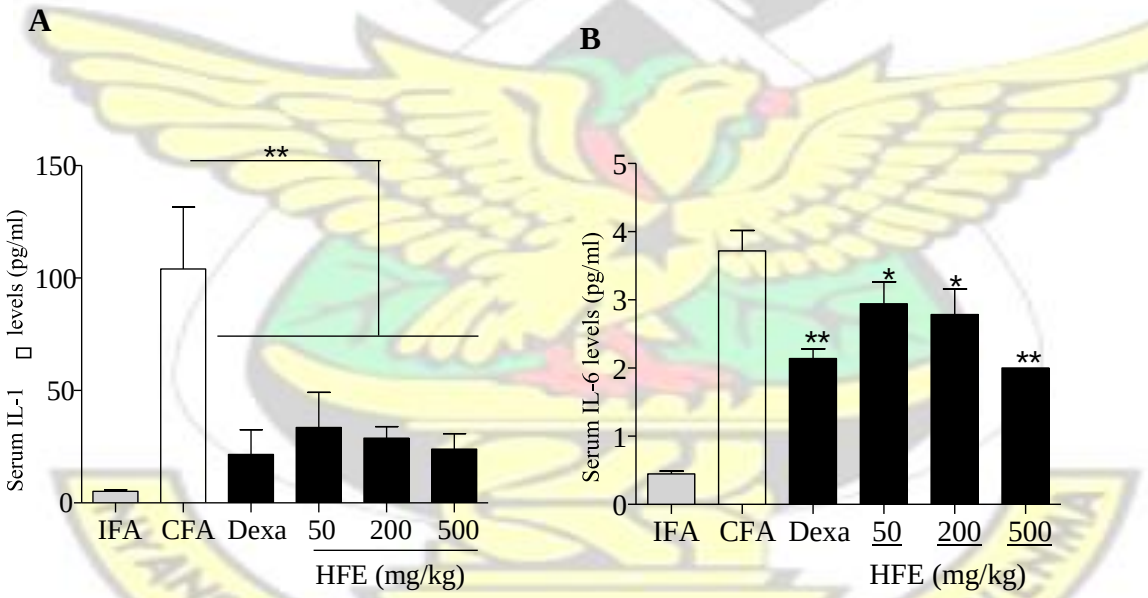
6.3.3.1 Quantitative determination of serum cytokine (IL-1α, IL-6 and IL-10) levels

During chronic inflammation, the presence of various cytokines and their interactions leads to the influx of monocytes to the site of injury which becomes activated into macrophages. Macrophages contribute to inflammation by chronically increasing levels of IL-1 and TNFα which are elicits the signs such as eating disorders, loss of body cell mass, and fever, and also act as potent paracrine inducers of other cytokines (Feldmann and Maini, 2008)

The effect of *Holarrhena floribunda* on cytokines was therefore determined. To do this arthritis was induced in rats as described in section 6.1.2.1 and serum levels of IL-1α, IL-6, and IL-10 determined by particular sandwich enzyme-linked immunosorbent assay kits. From this study the induction of arthritis caused a significant rise in serum levels of IL-1α from 5.23 ± 0.59 pg/ml in the IFA non-arthritic control group to 104.05 ± 27.46 pg/ml in the CFA arthritic control group. Treatment with 50, 200 and 500 mg/kg HFE resulted in dosedependent and significant (P < 0.01) reduction (33.59 ± 15.58, 28.86 ± 5.04 and 23.99 ±6.85 pg/ml) in serum levels of IL-1α respectively (Fig. 6.6 A)

Similarly it was also observed that IL-6 levels in the IFA non-arthritic control group increased from 0.45± 0.04 pg/ml to 3.71± 0.30 pg/ml in the CFA arthritic control group (Fig 6.6 B). There was a dose-dependent and significant (P < 0.05-0.001) reduction (2.94 ± 0.32, 2.79 ± 0.37 and 2.00 ± 0.00 pg/ml) in IL-6 levels with 50, 200 and 500 mg/kg HFE treatment respectively (Fig 6.6 B).

The induction of arthritis led to significant reductions in IL-10 levels of the CFA arthritic control group (25.17 ± 5.17 pg/ml) when compared to the IFA arthritic control group (657.66 ± 21.73 pg/ml). Treatment of arthritic rats with HFE (50-500 mg/kg) resulted in a dose-dependent increase (24.13 ± 4.13, 60.28 ± 0.28 and 221.90 ± 58.32 pg/ml) in serum IL10 levels (Fig 6.6 C) relative to the arthritic control group. The increase in the 500 mg/kg HFE was significantly different from the arthritic control group.



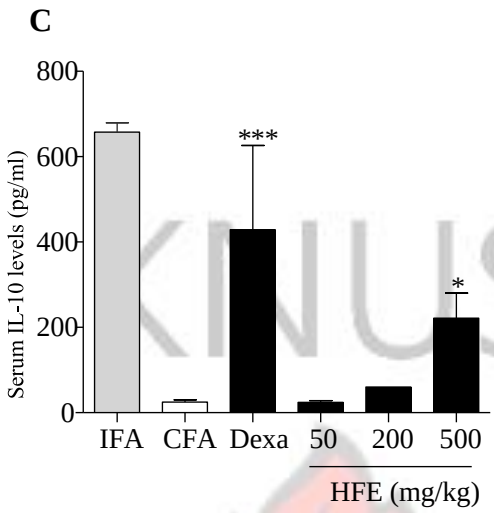


Fig. 6.6 Effect of *Holarrhena floribunda* on serum levels of IL-1α, IL-6 and IL-10 in adjuvant-induced arthritic rats. Sprague-Dawley rats (200 – 220 g) were injected subplantar with 0.1 ml of CFA or IFA into the right hind paw. Dexamethasone 0.3 mg/kg, HFE (50, 200, 500 mg/kg) were given orally 1 h before induction of arthritis and daily till the 28th day. IL-1α (A), IL-6 (B) and IL-10 (C) levels in serum obtained at the end of the study were determined using rat ELISA Kit according to the manufacturer‟s recommendations. Data are presented as Mean ± S.E.M. (n=6). * P < 0.05, ** P < 0.01, *** P < 0.001: values significant when compared with control.

6.3.4 Effect of *Holarrhena floribunda* on enzymes and secondary messengers

6.3.4.1 Quantitative determination of serum enzyme (PKA and PKC) levels

The serum level of PKA in the IFA non-arthritic control group (25.41 ± 2.07 ng/ml) was significantly (P<0.01) reduced in the CFA arthritic control group (7.71 ± 0.64 ng/ml). Administration of 50 - 500 mg/kg HFE dose-dependently and significantly (P < 0.05-0.001) increased the serum levels of PKA to 10.88 ± 0.35, 13.76 ± 1.46 and 17.18 ± 1.32 ng/ml respectively (Fig. 6.7A).

From the study, serum PKC levels in the IFA non-arthritic control group was 0.38 ± 0.04 ng/ml. This was significantly (P<0.05) increased to 1.78 ± 0.37 ng/ml in the CFA arthritic

control group. Arthritic rats treated with 50, 200 and 500 mg/kg HFE had PKC levels decreasing dose-dependently but insignificantly ($P > 0.05$) to 1.77 ± 0.29, 1.32 ± 0.23 and 1.21 ± 0.17 ng/ml (Fig 6.7B)

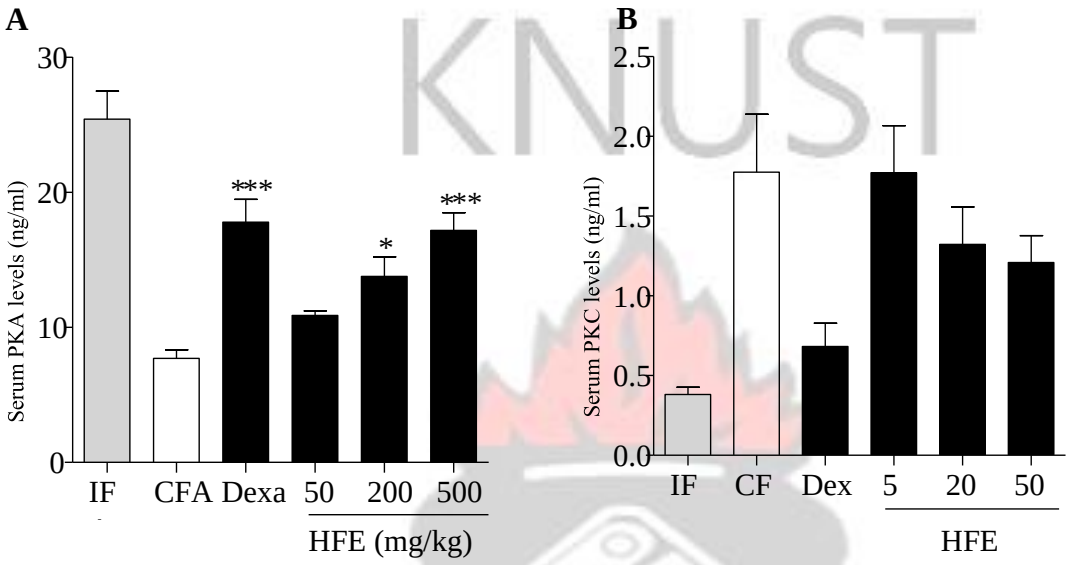


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Fig. 6.7 Effect of *Holarrhena floribunda* on the serum levels of PKA and PKC in adjuvantinduced arthritic rats. Sprague-Dawley rats (200 – 220 g) were injected subplantar with 0.1 ml of CFA or IFA into the right hind paw. Dexamethasone 0.3 mg/kg or HFE (50, 200, 500 mg/kg) was given orally 1 h before induction of arthritis and daily till the 28th day. PKA (A) and PKC (B) levels in serum obtained on the 28th day were determined using rat ELISA Kit according to the manufacturer‟s recommendations. Data are presented as Mean ± S.E.M. (n=6). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$: values significant when compared with control.

6.3.4.1 Quantitative determination of serum secondary messenger (cAMP) levels

The induction of arthritis resulted in a significant reduction in cAMP levels from 16.53 ± 1.72 pg/ml in the IFA non-arthritic control group to 4.95 ± 0.32 pg/ml in the CFA arthritic control group (Fig. 6.8). Treatment with HFE caused a dose-dependent increase in cAMP levels with the 50, 200 and 500 mg/kg respectively to 1.93 ± 0.3, 6.52 ± 0.25 and 8.36 ±

0.63 pg/ml. The increases in the 200 and 500 mg/kg HFE groups were significantly different (P<0.01-0.005) from the CFA arthritic group (Fig. 6.8).

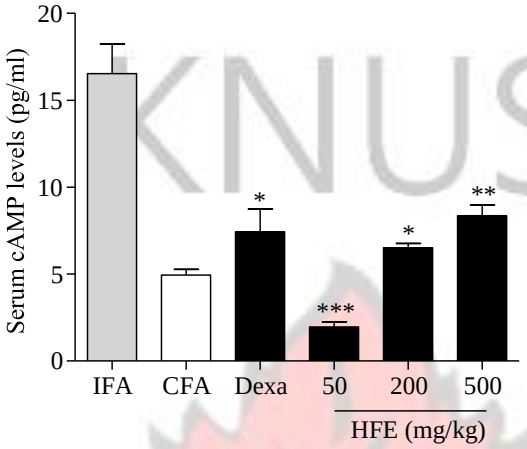

Fig. 6.8 Effect of *Holarrhena floribunda* on the serum levels of cAMP in adjuvant-induced arthritic rats. Sprague-Dawley rats (200 – 220 g) were injected subplantar with 0.1 ml of CFA or IFA into the right hind paw. Dexamethasone 0.3 mg/kg or HFE (50, 200, 500 mg/kg) was given orally 1 h before induction of arthritis and daily till the 28th day. cAMP levels in serum obtained on the 28th day was determined using rat ELISA Kit according to the manufacturer"s recommendations. Data are presented as Mean ± S.E.M. (n=6). * P < 0.05, ** P < 0.01, *** P < 0.001 when compared with control.

6.4 DISCUSSION

Mast cell activation initiates a complex of signaling events that lead to the secretion of numerous vasoactive and pro-inflammatory mediators like histamine, serotonin, tumour necrosis factor alpha (TNFα), kinins, proteases such as tryptase, chymase, lipid-derived mediators such as the eicosanoids like prostaglandins and leukotrienes (Galli. and Tsai, 2003) and finally cytokines including IL-1, 2, 5, 6, 8, 9, 13 (Grutzkau *et al.*, 1998).

Histamine is a monoamine which is very critical in inflammatory and allergic reactions (Beer *et al.*, 1984; Petersen *et al.*, 1996 Vasudevan *et al.*, 2007). The histamine H_1 receptor is found in various human tissues including airway, intestinal and vascular smooth muscle and brain (Hill, 1990) and is involved in contraction of smooth muscle, vasodilation, increasing vascular permeability, bronchoconstriction, increased pruritus and nociception (Raffa, 2001; Togias,

2003 Zhang *et al,* 2007) and on endothelial cells histamine is reported to be involved in leukocyte rolling and vascular protein leakage (Asako *et al* 1994). The inhibitory effect of HFE on histamine-induced paw oedema was established. HFE significantly and dose-dependently inhibited histamine-induced inflammation. Antihistaminic drugs with anti-allergic and anti-inflammatory effects have H_1 receptor blocking effect and inhibit histamine release from mast cells (Levi-Schaffer, 2009). H_1 receptors could have been blocked by HFE and inhibited the release of histamine from mast cells. The effect of HFE on histamine demonstrated with inhibition of histamine-induced paw oedema was further confirmed with the clonidine-induced catalepsy test. Clonidine, an α_2-adrenergic agonist stimulates histamine release, from mast cells in the brain which causes catalepsy. Histamine release, from mast cells by clonidine is comparable to that of compound 48/80 (Lakdawala *et al.,* 1980) a basic secretagogue which causes mast cell degranulation. The various stages of catalepsy have been established to be paralleled with given brain levels of histamine (Chopra and Dandiya, 1975). Haloperidol on the other hand is an agent that also causes catalepsy mediated through its effects on dopamine receptors localised postsynaptically on striatal neurons (Sanberg, 1980). The inhibitory effect of HFE and the antihistamine drug chlorpheniramine on clonidine induced catalepsy have been demonstrated. Whereas HFE significantly and dose-dependently inhibited clonidine-induced catalepsy when administered before and after catalepsy induction, it had no inhibitory effect on the haloperidol-induced catalepsy. This is indicative of the inhibitory effect of HFE on histamine which is released from the mast cell when clonidine is administered and causes catalepsy. Lakdawala *et al.*, (1980) reported that catalepsy as a result of clonidine is suppressed by antagonists of histamine H_1 receptor but not H_2 receptor antagonist therefore HFE possibly has antagonistic effects on the H_1 receptor of histamine.

Serotonin is a monoamine which is very critical in inflammatory and allergic reactions (Beer *et al.,* 1984; Petersen *et al.,* 1996; Vasudevan, 2007). Villalon and Centurion (2007) further reported that serotonin functions in the cardiovascular system where it controls processes like vasoconstriction and heart rate by interacting with seven distinct classes of receptor proteins based on structural and operational characteristics (Hannon and Hoyer, 2008). HFE significantly and dose-dependently inhibited serotonin-induced inflammation possibly by blocking one or more of the seven serotonin receptors.

According to Ricciotti and FitzGerald (2012), levels of prostaglandins increase significantly during inflammation and the development of the cardinal signs of acute inflammation: redness, swelling and pain are attributed to increasing levels of PGE_2 (Funk, 2001). The inhibitory effect of HFE on PGE_2 was established using PGE_2-induced inflammation. HFE significantly and dose-dependently inhibited PGE_2-induced inflammation by possibly interfering with the activity of PGE2 on one or more of its cognate receptors, E prostanoid receptors 1-4 (EP1-EP4)(Trebino *et al,* 2003).

The interactions between histamine, serotonin and PGE_2 and their receptors have been established to promote inflammatory reactions by regulating inflammatory cytokines levels (Grutzkau *et al.*, 1998; Mazzoni *et al.,* 2001; Kubera *et al.,* 2005; Babaev *et al.,* 2008;). Elenkov and Chrousos (2002) stated that the expression and changes in cytokines levels is a primary step in the response of inflammatory cells to inflammatory stimuli. The production of pro-inflammatory cytokines by cells of the immune system is known to play a very critical role in mediating the initial inflammatory response. However, the inability to resolve or halt the initial responses to inflammation leads to deleterious chronic inflammatory diseases with predominant upsurge in the levels of pro-inflammatory cytokines and a reduction in anti-inflammatory cytokines (Howard *et al.,* 1993; Berg *et al.,* 1995). In this study HFE

significantly and dose dependently supressed the levels of IL-1α and IL-6 and PKC and enhanced serum levels of IL-10, cAMP and PKA. The ability of HFE to suppress pro-inflammatory cytokines (IL-1 α and IL-6) and enhance or increase levels of antiinflammatory cytokines (IL-10) points to its ability to resolve acute inflammation. It was observed in the study that treatment with HFE resulted in dose-dependent increases in serum cAMP levels in arthritic rats. cAMP is an intracellular second messenger which has been shown to act on a variety of hormones, inflammatory mediators and cytokines. Several *in vitro* studies have demonstrated the ability of cAMP to reduce the release of histamine and leukotrienes from mast cells (Marone *et al.,* 1987) and release of cytokines (Renz *et al.,*

1988) from macrophages. cAMP is reported to be pivotal in an array of endogenous processes involved in down-regulating and preventing the beneficial effects of acute inflammation from progressing to the deleterious effect on tissue seen in chronic inflammation (Moore and Willoughby, 1995). Elevated levels of cAMP are also associated with the activation of protein kinase A (PKA) which exerts anti-inflammatory activity. HFE dose-dependently reduced levels of PKC in arthritic rats suggesting possible effect in regulating gene expression, differentiation and proliferation of cells involved in the inflammatory process. Protein Kinase C (PKC) is a prominent group of enzymes which phosphorylates serine threonine residue in amino acids and plays a key role in the functioning of cells in an organism such as the release of arachidonic acid through the activation of phospholipase A_2 , eicosanoid production, gene expression, growth regulation, differentiation and proliferation of the cells. The expression of PKC results in pathological conditions such as inflammation, cancer and cardiovascular diseases (Churchill *et al,* 2008; Ali *et al.* 2009). PKC also regulates pro-inflammatory gene expression leading to the production of several cytokines involved in inflammation (Chand *et al.,* 2012).

6.5 CONCLUSION

The hydroethanolic extract of *Holarrhena floribunda* (HFE) has inhibitory effect on histamine, serotonin, and prostaglandin E_2-mediated inflammatory process. HFE also inhibited the expression of IL-1α, IL-6 and protein kinase C. It, however, enhanced the expression of IL-10, cAMP and protein kinase A.

CHAPTER SEVEN

GENERAL DISCUSSION

In this study the anti-inflammatory effect of the hydroethanolic extract of *H. floribunda* (HFE) has been shown in rodents. It has been established that the LD_{50} of the hydroethanolic extract was greater than 5000 mg/kg. Various murine models of inflammation were used to validate the anecdotal use of the plant in Ghanaian folkloric medicine. The extract has demonstrated inhibitory effects on acute and chronic inflammation.

The anti-inflammatory and anti-allergic property of HFE has been established. Carrageenaninduced oedema is a biphasic, event involving histamine, serotonin and bradykinin in the early phase (Crunkhorn and Meacock, 1971) and mainly prostaglandins in the late phase (Gupta *et al.*, 2006). Hypersensitivity reactions also involve preformed mediators like histamine; serotonin and prostaglandins involved in the immediate reaction and leukotrienes, chemokines, and cytokines in the late-phase reaction (Ogawa and Grant, 2007). *Holarrhena floribunda* inhibited the progression of acute inflammation and hypersensitivity reaction possibly through its inhibitory effects on histamine, serotonin and prostaglandin E_2. This was demonstrated by the inhibition of histamine, serotonin and prostaglandin E_2- induced

oedema in rats. The effect on histamine was further confirmed in an indirect manner using the clonidine-induced catalepsy test which is reported to be inhibited by histamine H_1 receptor antagonists (Lakdawala *et al.,* 1980) and as such HFE possibly has antagonistic effect on the histamine H_1 receptor.

Holarrhena floribunda also inhibited chronic inflammation, demonstrated by marked effects on various indices of Freund‟s adjuvant-induced arthritis. The progression from acute to chronic inflammation involves the interplay of several mediators. These include histamine, serotonin, lipid mediators, cytokines, enzymes and secondary messengers. The demonstrated decrease in levels of IL-1α, IL-6 (pro inflammatory cytokines) and PKC as a result of treatment with HFE presupposes that HFE has the ability to resolve acute inflammation. HFE also enhanced the expression of IL-10, cAMP and PKA which are an anti-inflammatory cytokine, a secondary messenger and an enzyme respectively, whose function involves mainly resolving chronic inflammation.

Moore and Willoughby (1995) have stated that concentrating on a particular group of mediator for the management of inflammation results in other mediators compensating for the deficit thereby reducing the potency and effectiveness of the treatment. In this study HFE has shown activity against an array of mediators ranging from the preformed, proinflammatory and anti-inflammatory mediators to serine threonine kinases and secondary messengers.

An initial phytochemical analysis of the hydroethanolic extract of the stem bark of *Holarrhena floribunda* indicated the presence of saponins, phenolic compounds, alkaloids and reducing sugars which possibly could be responsible for its anti-inflammatory activity in agreement with earlier studies (Francis *et al.,* 2002; Miles *et al*., 2005; Cheeke *et al*., 2006; Song-Chwan *et al*., 2008; Badmus *et al*., 2010; Sergent *et al*., 2010; Hassan *et al.*, 2012). The prophylactic and therapeutic approaches were adopted for majority of the experiments performed in the

study. Studies by Kaibara *et al*., (1983) on cyclosporine demonstrated that an established inflammatory property of a medication administered prior to the start of inflammation does not essentially indicate an ability to act when administered after the commencement of inflammation. In their study, cyclosporine administered prophylactically inhibited collagen-induced inflammation but worsened the condition when administered therapeutically. In all studies where both approaches were adopted HFE demonstrated antiinflammatory activity, giving credence to its potential as a genuine anti-inflammatory agent.

CONCLUSION

The study has provided a scientific basis for the anecdotal use of *Holarrhena floribunda* in the management of inflammatory conditions. The presence of saponins, phenolic compounds, alkaloids and reducing sugars possibly could be responsible for its antiinflammatory activity investigated and listed below:

- The inhibitory effect on acute inflammation.
- The ability to inhibit hypersensitivity reactions through inhibition of systemicinduced anaphylaxis.
- The inhibitory effect on prostaglandin E_2, the histaminic and serotonergic pathways of inflammatory response.
- The inhibition of the expression of pro-inflammatory cytokines and the enhancement of the expression of anti-inflammatory cytokines, secondary messengers and enzymes in the chronic inflammatory process.

RECOMMENDATIONS

This study is not conclusive and as such further studies should be carried out to improve on the acquired knowledge. Areas that can be explored include:

- The toxicity profile of *Holarrhena floribunda* should be determined on both acute and chronic administration in animals. This will provide scientific evidence of its safety to add up to its established efficacy.

- Herbal practitioners who have an interest in the commercial production of antiinflammatory medicines can consider *Holarrhena floribunda* as a very good candidate.

- Pharmaceutical companies interested in the development of new therapeutic agents can pursue research to isolate and characterize the active constituents responsible for the anti-inflammatory properties of *Holarrhena floribunda.*

- Possible receptor binding interactions with *Holarrhena floribunda* should be explored.

- Other pharmacological properties aside the anti-inflammatory effect of *Holarrhena floribunda* should be explored since the phytochemical analysis revealed the presence of a number of constituents which could have other biological properties.

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