UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

PHARMACOLOGICAL EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF AQUEOUS EXTRACT OF THE ROOT BARK OF

CAPPARIS ERYTHROCARPOS

BY

BAAH JOSEPH

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF PHILOSOPHY IN PHARMACOLOGY
DECLARATION

I hereby declare that the experimental work described in this thesis is the result of my own work produced from research under supervision of the undersigned lecturers. Except for references to other peoples work and statements or quotations attributed to specified sources, which have been duly acknowledged, no part of this work has been presented for another degree in the University or elsewhere.

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ABSTRACT

Background: *Capparis erythrocarpos* is a medicinal plant that is used extensively in folklore medicine in Ghana to manage various inflammatory conditions. Previous pharmacological study showed that an ethanolic extract of the roots of *Capparis erythrocarpos* may have anti-inflammatory activity. However, no attempt has been made to investigate the basis of the effect(s), if any, of the aqueous extract on the inflammatory processes or ascertain its safety.

Aim: The aim of this study was to verify claims in folklore medicine that the aqueous extract of the root bark of *Capparis erythrocarpos* has anti-inflammatory activity, as well as to ascertain its safety in animal models.

Methodology: The extract was prepared by extraction of the pulverized root of *C. erythrocarpos* with water in a Soxhlet extractor. Reaction time, volume of paw oedema, weight of section of mouse pinna, and white blood cells (WBC) count in inflammatory exudates, were used as measurable indicators of the inflammatory processes, namely, perception of pain, increased capillary permeability, vasodilation and recruitment of white blood cells. In each of the respective inflammation studies, 25 rats (150 - 230 g) or mice (24 - 30 g) of both sexes were randomly selected and assigned to 5 treatment groups of 5 rats each (cohort). One group received normal saline (control) whiles two other treatment groups received specified dose levels of the extract (20 – 80 mg/kg). In all cases, an internal standard (morphine, indomethacin or celecoxib) was employed. The animals were subjected to the appropriate stimulus, the individual responses were measured and the mean responses of the cohorts determined.
Plasma prostaglandin E$_2$ (PGE$_2$) concentration of indomethacin-, celecoxib- and extract-treated rats was also determined using Enzyme-Linked Immunosorbent Assay (ELISA). The mean plasma PGE$_2$ concentration of a treatment group was compared to that of control, and the percent inhibition caused by a treatment determined. The toxicity of the extract was studied in rats given the extract orally for a period of 28 days, followed by histopathological examination of the liver and kidneys under light microscope.

**Results:** The extract (20 – 80 mg/kg), like indomethacin, significantly increased pain perception threshold, reduced paw oedema and inhibited PGE$_2$ biosynthesis (p $<$ 0.05) in the animal models of acute pain and inflammation used in this study, though not in a dose-dependent manner. The aqueous extract was also found to inhibit recruitment of inflammatory cells to the site of injury, which contributes to the validation of its anti-inflammatory activity. The extract (50 – 200 mg/kg) did not cause any significant alteration in the histoarchitecture of the liver and kidneys of extract-treated rats compared to controls when the extract was administered orally over a period of 28 days.

**Conclusion:** The results indicate that the aqueous extract of the root of *C. erythrocarpos* exhibited significant analgesic and anti-inflammatory properties. The extract, at the stated dose range, was neither hepatotoxic nor nephrotoxic in rats over the 28-day observation period. The findings support ethnomedical claims of therapeutic efficacy of the extract in management of pain and inflammatory conditions.
DEDICATION

This work is dedicated jointly to my Supervisors, Mr. Johnson Kwadwo Baah Jnr and his wife, Cecilia Kusi, Mr. Michael Baah, Mr. Umar Said Alhassan, Miss Alice Charwudzi, and especially to my wife, Mrs. Beatrice Frimpomaa Baah.
ACKNOWLEDGEMENTS

I am grateful to the Head of Department of Pharmacology, Prof. K. A. Bugyei, who found me worthy of the training and nominated me for sponsorship by my employers, the University of Ghana Medical School, Korle Bu. Profound gratitude also goes to my thesis supervisors, Dr. A. B. A. Prempeh, Prof. A. C. Sackeyfio and Prof. G. D. Lutterodt for their constructive criticisms, advice and tireless efforts to get the project to its completion. I am particularly indebted to Prof. G. D. Lutterodt for his invaluable pieces of advice, encouragement and support throughout the period of my studies. Thanks also to all Technical staff of the Department of Pharmacology, University of Ghana Medical School, especially to Nana Perseus Asare, Madam Ruth Addy, Mr. Ebenezer Boateng and Ilyas Ahmed Bilal, for their assistance during data collection.

The encouragement and support for my studies by the Head of Department, Dr. I. J. Asiedu-Gyekye, and all other Lecturers of the Department of Pharmacology & Toxicology, University of Ghana School of Pharmacy, is greatly appreciated. Thanks to Prof. Ben A. Gyan of the Department of Immunology, Noguchi Memorial Institute for Medical Research (NMIMR), who facilitated the procurement of ELISA kit for one of the module experiments. I would like to also recognize staff of the Clinical Virology Unit of the Department of Microbiology, University of Ghana Medical School, particularly Prince Pappoe, and Rashid Adams of the Pathology Laboratory, School of Allied Health Sciences, who offered assistance to my laboratory work. I am most grateful for the permission granted me to use their laboratories for part of the work.

To Mr. Thomas Ansah of Kwame Nkrumah University of Science and Technology, Kumasi, who donated λ-carrageenin for the module experiments through my friend, David Nyarko, I say
God richly bless you for your benevolence. The financial support given to me by the Board of Trustees of the College of Health Sciences Postgraduate Endowment Fund and Prudential Bank Limited is also gratefully acknowledged.
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<td>DPX</td>
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<td>MAC</td>
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<td>MBL-associated serine proteases</td>
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<td>MBL</td>
<td>Mannose- binding lectin</td>
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<td>NMR</td>
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<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<td>PAF</td>
<td>Platelet Activating Factor</td>
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<td>PGE₂</td>
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<td>SAID</td>
<td>Steroidal anti-inflammatory drug</td>
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<td>SEM</td>
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<td>sIL-6R</td>
<td>Soluble Interleukin-6 Receptor</td>
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<td>TNF-α</td>
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<td>TXA₂</td>
<td>Thromboxane A₂</td>
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<tr>
<td>UV</td>
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ABSTRACT

**Background:** *Capparis erythrocarpos* is a medicinal plant that is used extensively in folklore medicine in Ghana to manage various inflammatory conditions. Previous pharmacological study showed that an ethanolic extract of the roots of *Capparis erythrocarpos* may have anti-inflammatory activity. However, no attempt has been made to investigate the basis of the effect(s), if any, of the aqueous extract on the inflammatory processes or ascertain its safety.

**Aim:** The aim of this study was to verify claims in folklore medicine that the aqueous extract of the root bark of *Capparis erythrocarpos* has anti-inflammatory activity, as well as to ascertain its safety in animal models.

**Methodology:** The extract was prepared by extraction of the pulverized root of *C. erythrocarpos* with water in a Soxhlet extractor. Reaction time, volume of paw oedema, weight of section of mouse pinna, and white blood cells (WBC) count in inflammatory exudates, were used as measurable indicators of the inflammatory processes, namely, perception of pain, increased capillary permeability, vasodilation and recruitment of white blood cells. In each of the respective inflammation studies, 25 rats (150 - 230 g) or mice (24 - 30 g) of both sexes were randomly selected and assigned to 5 treatment groups of 5 rats each (cohort). One group received normal saline (control) whiles two other treatment groups received specified dose levels of the extract (20 – 80 mg/kg). In all cases, an internal standard (morphine, indomethacin or celecoxib) was employed. The animals were subjected to the appropriate stimulus, the individual responses were measured and the mean responses of the cohorts determined.
Plasma prostaglandin E$_2$ (PGE$_2$) concentration of indomethacin-, celecoxib- and extract-treated rats was also determined using Enzyme-Linked Immunosorbent Assay (ELISA). The mean plasma PGE$_2$ concentration of a treatment group was compared to that of control, and the percent inhibition caused by a treatment determined. The toxicity of the extract was studied in rats given the extract orally for a period of 28 days, followed by histopathological examination of the liver and kidneys under light microscope.

**Results:** The extract (20 – 80 mg/kg), like indomethacin, significantly increased pain perception threshold, reduced paw oedema and inhibited PGE$_2$ biosynthesis (p < 0.05) in the animal models of acute pain and inflammation used in this study, though not in a dose-dependent manner. The aqueous extract was also found to inhibit recruitment of inflammatory cells to the site of injury, which contributes to the validation of its anti-inflammatory activity. The extract (50 – 200 mg/kg) did not cause any significant alteration in the histoarchitecture of the liver and kidneys of extract-treated rats compared to controls when the extract was administered orally over a period of 28 days.

**Conclusion:** The results indicate that the aqueous extract of the root of *C. erythrocarpos* exhibited significant analgesic and anti-inflammatory properties. The extract, at the stated dose range, was neither hepatotoxic nor nephrotoxic in rats over the 28-day observation period. The findings support ethnomedical claims of therapeutic efficacy of the extract in management of pain and inflammatory conditions.
CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Drugs have been the mainstay of treatment of inflammatory conditions since the introduction of aspirin into clinical practice in 1899 (Sneader, 2000). Recent estimates indicate that worldwide annual consumption of aspirin alone is about 40,000 metric tons, which translates to the equivalent of about 120 billion standard aspirin tablets (Warner and Mitchell, 2002). Today, other anti-inflammatory drugs have found pervasive use due to the fact that inflammation underlies a large number of diseases (Rainsford, 2007).

In Ghana, anti-inflammatory drugs, unlike other orthodox medicines, are readily available and widely used in both rural and urban areas. However, the increasingly high cost of drugs has rendered indigenous systems of treatment, including herbal medicine, particularly preferred alternative to orthodox medicine. Consequently, many herbal medicines abound for the treatment of inflammatory conditions. *Capparis erythrocarpos* is a medicinal plant of the genus *Capparis* that is used extensively in folklore medicine to manage various inflammatory conditions, including conjunctivitis, mastitis, otitis and rheumatoid arthritis (Danquah et al., 2011). Mshana et al. (2000) reported that aqueous extract of the whole root is traditionally used to treat rheumatoid arthritis, but use of the powdered root bark extract is rather suggested elsewhere (Irvine, 1961).

Previous pharmacological study showed that ethanolic extract of the root of *Capparis erythrocarpos* exhibited dose-dependent analgesic activity in formalin-induced nociception in
mice. The analgesic effect was not antagonized by naloxone but by theophylline, suggesting an adenosinergic mechanism may underlie the analgesic effect (Woode et al., 2009). The ethanolic extract also significantly reduced carrageenin-induced foot oedema in chicks, and reversed baker’s yeast-induced pyrexia in rats (Danquah et al., 2011). These two published reports (possibly the only ones in the literature) confirm, though not unequivocally so, that the extract has anti-inflammatory activity. Because the extract does not contain a steroid (Danquah et al., 2011), it would seem the active principle is a non-steroidal anti-inflammatory agent.

Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting cyclooxygenase (COX) and, thereby, interrupt the synthesis of prostaglandins. The deficiency of prostaglandins interrupts inflammatory processes responsible for the cardinal signs of inflammation, namely, pain, reddening, warmth and oedema (swelling). Because the active principle of ethanolic extract of the roots of C. erythrocarpos seems to be acting similarly to the NSAIDs, the present study focused on the effect of crude aqueous extract (not ethanolic extract) on inflammatory processes, namely, perception of pain, vascular response to inflammation, migration of inflammatory cells and prostaglandin synthesis.

1.2 STATEMENT OF THE PROBLEM

Despite the extensive use of the aqueous extract of the root bark of C. erythrocarpos in folklore medicine to treat variety of inflammatory conditions, claim of its efficacy and safety remains to be rigorously tested and validated. Aside the study on the analgesic and antipyretic effect of the ethanolic extract, no attempt has been made to elaborate the effect(s), if any, of the extract on the other processes of inflammation, viz, vascular response, migration of inflammatory cells and
synthesis of prostaglandins with the view to elucidating the mechanism(s) of action and, hence, the pharmacological and possible toxic effects. There are also conflicting reports as to whether the root bark or whole root possessed anti-inflammatory activity. The active principle, if concentrated in the root bark, could be diluted by compounds in the woody core when the whole root is used, making it less efficacious. Furthermore, water, but not ethanol, is used as the vehicle for the extract in folklore medicine. It is essential that the method employed to prepare the extract replicates that used in folklore medicine.

1.3 JUSTIFICATION

The conventional anti-inflammatory drugs being used currently are associated with serious undesired effects, whilst the newer relatively safe COX-2 selective inhibitors are very expensive and, therefore, beyond the reach of majority of Ghanaians. Consequently, the search for alternative, efficacious, safe and accessible anti-inflammatory agents, in consonance with the tenets of rational pharmacotherapy, is essential. It was envisaged that this study would elucidate some of the possible mechanism(s) of action underlying the anti-inflammatory activity of the crude aqueous extract of the root bark of C. erythrocarpus and hence the pharmacological effects; provide putative data for its safety and thus confirm the therapeutic potential of Capparis erythrocarpus as an anti-inflammatory agent.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 INFLAMMATION

Inflammatory diseases represent one of the greatest burdens of suffering and economic cost in the world (Shapira et al., 2010). It is now recognised that inflammation contributes to other major health care problems, including diabetes, cardiovascular diseases, Parkinson’s disease and neoplastic transformation (Wellen and Hotamisligil, 2005; Libby, 2006; Hirsch and Hunot, 2009; Colotta et al., 2009). Drugs have been the bedrock of the treatment of inflammatory conditions. Accordingly, a major preoccupation of pharmacologists has been the development of safe and efficacious anti-inflammatory drugs.

Inflammation is a vascularized living tissue response to harmful nonspecific insults caused by chemical, physical, or biological stimuli (Ferrero-Miliani et al., 2007). The response is characterised by four cardinal signs: redness, warmth, pain and swelling. The redness and warmth are due to increased blood flow consequent to vasodilation; pain is caused by algesic substances released during the inflammatory process, and the swelling is the accumulation of fluid in the tissue resulting from fluid and protein exudation into the interstitium following increased capillary and venular permeability.

Following an injury, the inflammatory process begins with vascular response which comprises: 1) transient vasoconstriction followed by arteriolar dilatation, resulting in increased capillary blood flow to the injured or infected site; 2) retraction of endothelial cells of capillary and

4
postcapillary venules, which widens the intercellular gaps and thus exposes the basement membrane. Because the latter lacks selective permeability, capillary permeability is increased (Kumar et al., 2010). The consequence of increased capillary permeability is accumulation of protein-rich fluid exudates and phagocytic cells in the interstitium. The net effect of the vascular response is excess fluid in the tissue (oedema) associated with inflammation.

Tissue damage triggers release of phospholipase $A_2$ that catalyses hydrolysis of membrane phospholipids of affected and surrounding cells to produce inflammatory mediators and oxygen-derived free radicals. It also incites proinflammatory cytokine release, including TNF-α, IL-1, and IL-6 from neutrophils and resident tissue macrophages (Umukoro and Ashorobi, 2006; Ramaiah and Jaeschke, 2007). Under the influence of chemical mediators or chemotaxins, white blood cells are attracted to the site of injury. Neutrophils are the first cells to migrate followed by monocytes and then lymphocytes. It has been observed that monocytes begin to predominate 24 hours after the initial neutrophilic infiltration. The mechanism of transition from neutrophil to monocyte recruitment during acute inflammation has not been well elucidated. However, it has been determined that IL-6, together with its soluble receptor (sIL-6R), decreases neutrophil infiltration and favors monocyte recruitment (Hurst et al., 2001).

Chemical mediators or chemotaxins may be cell-derived or plasma-derived (Kumar et al., 2010). The cell-derived chemical mediators (i.e. secreted from both injured tissues and sensitized phagocytic cells recruited to the injured tissue) are histamine, 5-hydroxytryptamine (5-HT), platelet activating factor (PAF), eicosanoids and cytokines. The plasma-derived chemical mediators are by-products of the kinin and complement systems. In order to appreciate the role
of these chemical mediators in propagating the inflammatory response, it is essential to review, briefly, their pharmacologic actions.

2.2 CHEMICAL MEDIATORS OF INFLAMMATION

2.2.4 Eicosanoids

Among the major substances that play pivotal role in inflammation are oxygenated metabolites of arachidonic acid called eicosanoids. These mediators are thromboxanes, prostaglandins, prostacyclin and leukotrienes (Lawrence et al., 2002; Serhan et al., 2004). Knowledge of the metabolic fate of arachidonic acid and the functions of its many metabolites is relevant to understanding how inflammatory processes can be modulated or interrupted to advantage.

Arachidonic acid, the most abundant and important of the eicosanoid precursors, is a 20-carbon (C20) tetraenoic acid. In resting cells, arachidonic acid is stored esterified with glycerol of phospholipids within the plasma membrane and is released from the membrane phospholipids by activated phospholipase A2 (PLA2) (Smith et al., 2000; Six and Dennis, 2000). Alternatively, membrane phospholipid may be hydrolysed by phospholipase C (PLC), yielding diacylglycerol (DAG), which is subsequently cleaved by diacylglycerol lipase to produce arachidonic acid (Tang et al., 2006).

Following release from membrane phospholipids, arachidonic acid is oxygenated by four separate routes: the cyclooxygenase, lipoxygenase, P450 epoxygenase, and isoprostane pathways (Brash, 2001). The cyclooxygenase and lipoxygenase pathways are the most important with
respect to the propagation of the inflammatory response. The cyclooxygenase and lipoxygenase pathways lead to the formation of prostaglandins and leukotrienes respectively (Fig. 2.1).

Cyclooxygenase (prostaglandin G/H synthase) catalyzes stepwise conversion of arachidonic acid into two short-lived intermediates, prostaglandin G and prostaglandin H respectively. The latter is further metabolized into prostaglandins D$_2$, E$_2$, F$_{2\alpha}$, prostacyclin (PGI$_2$), and thromboxane A$_2$ (TXA$_2$) by the activity of specific enzymes, prostaglandin synthases (DuBois et al., 1998). Both PGE$_2$ and PGI$_2$ are potent vasodilators that mediate vascular response in inflammation. Prostacyclin inhibits platelet aggregation in vivo and acts synergistically with histamine, serotonin and bradykinin to increase vascular permeability. On the other hand, PGE$_2$ induces pain, sensitizes nociceptors in the skin and potentiates the algesic actions of bradykinin (Ulmann et al., 2010). PGE$_2$ is also a pyretic agent which mediates the heat associated with inflammation.

In humans the lipoxygenase pathway comprises three distinct routes, each catalysed by separate lipoxygenases, namely, 5-lipoxygenase (5-LOX), 12-lipoxygenase (12-LOX), and 15-lipoxygenase (15-LOX) (Jillian and Dino, 2007). Each lipoxygenase forms a distinct hydroperoxy-eicosatetraenoic acid (HPETE) which rapidly transforms to hydroxy derivatives (HETEs) and leukotrienes. The most actively investigated leukotrienes are those produced by the 5-lipoxygenase present in inflammatory cells (polymorphonuclear leukocytes, basophils, mast cells, eosinophils, macrophages). These include leukotrienes B$_4$, C$_4$, D$_4$, and E$_4$ (Talahalli et al., 2010; Mandal et al., 2004). Leukotriene B$_4$ (LTB$_4$) mediates leucocyte chemotaxis and adhesion to endothelial cells whereas leukotrienes C$_4$, D$_4$, E$_4$ increase vascular permeability, thus enhancing oedema formation in inflammation. It is also known that 5-HPETE can be
metabolized by 12-lipoxygenase to produce lipoxins A₄ and B₄ (LXA₄, LXB₄), which cause vasodilation and inhibit white blood cell recruitment (Levy and Serhan, 2002) (Fig. 2.1).

2.4 MEDICINAL PLANTS AS POTENTIAL ANTI-INFLAMMATORY AGENTS

Concerns over the safety and expense of anti-inflammatory drugs have renewed interest in natural products as potential alternative to conventional drugs. Results from clinical trials are providing increasing evidence of improved clinical outcomes in the management of inflammatory conditions using medicinal plants (Haghighi et al., 2005; Basnet and Skalko-Basnet, 2011). Some medicinal plants and herbs, such as *Camellia sinensis*, *Moringa oleifera*, *Ruta graveolens*, *Microtrichia perotitii*, and *Eugenia jamolana*, have shown demonstrable anti-inflammatory activity (Ndiaye et al., 2002; Lambert et al., 2007; Ratheesh and Helen, 2007; El-Shenawy, 2009; Nuhu et al., 2010).
CHAPTER THREE

3.0 METHODOLOGY

3.3.1 Phytochemical Screening

Previous phytochemical screening of the ethanolic extract of the roots of *C. erythrocarpos* revealed the presence of alkaloids and flavonoids (Woode *et al.*, 2009). The aqueous extract was subjected to phytochemical tests in order to verify the presence of possible bioactive compounds documented by the previous authors.

3.3.1.1 General methodology

The presence of phytochemical constituents were determined using protocols for simple qualitative tests as described - see Brian and Turner (1975); Sofowora (1993); Trease and Evans (1989).

3.3.1.2 Pre-treatment of aqueous extract

Phytochemical tests were carried out on a concentrate of the aqueous extract to determine the presence of hydrophilic compounds (i.e. saponins, cyanogenic glycosides, reducing sugars, phenolic compounds and polyuronides). The aqueous extract concentrate (40 ml) was hydrolysed by refluxing with 20 ml of 2 N HCl for 1 hour. Aliquots of the hydrolysed aqueous extract were used to screen for lipophilic compounds, such as flavonoids, alkaloids, anthracenosides, phytosterols and triterpenes.
3.3.4 Comparative Anti-inflammatory Activity of the Root Bark and Whole Root of *C. erythrocarpos*

Preliminary screening showed apparent anti-inflammatory activity of the aqueous extract of both the root bark and roots of *C. erythrocarpos* at 20 mg/kg, with the roots being more potent. This observation supports literature report that both the root bark and roots could be useful in the management of inflammation (Irvine, 1961; Mshana *et al.*, 2000). However, the root bark exhibited marked proinflammatory activity at 40 mg/kg (Fig. 3.6) and was, therefore, deemed not suitable for subsequent main studies. Considering that the phytochemical constituents of both the root bark and roots were the same, differences in activity could be due to the relative quantities of the active principles and/or the ratio of anti-inflammatory and proinflammatory compounds present.
3.4 STUDY SITES

The study was conducted in the laboratories of the Department of Pharmacology and the Clinical Virology Unit of the Department of Microbiology, University of Ghana Medical School; and the Centre for Scientific Research into Plant Medicine, Mampong-Akuapem.

3.5 LABORATORY ANIMALS

Healthy young adult Sprague-Dawley rats (150 – 230 g) and BALB/c mice (24 - 30 g) used were obtained from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. The animals were kept, at least 7 days, in the animal house of the University of Ghana Medical School, Korle Bu. During this period the animals were maintained under standard laboratory conditions (day and night cycle, temperature 27 °C ± 4 °C and relative humidity 60 -
70%) and had access to feed and water *ad libitum*. The animals were fasted 12 hours before and during the experiments. The laboratory temperature was maintained at 20°C ± 1°C.

### 3.6 INCLUSION/EXCLUSION CRITERIA OF LABORATORY ANIMALS

Sprague-Dawley rats (150 g – 230 g) and BALB/c mice (24 g - 30 g) of both sexes, aged between 8-10 weeks, were used. Animals were examined carefully prior to the experiments and those found with swelling or reddening of the paws and ears, or showing signs of ill-health were excluded from the study. Male and only nulliparous female rats and mice were used. Mice selected for the hot plate experiments all passed initial sensitivity tests. Animals that were hyperactive, hypersensitive or insensitive to thermal stimulus, as described (see page 26), were excluded. Mice were declared insensitive on failing reaction response within 12 seconds contact with the hot plate.

### 3.7 ETHICAL ISSUES

Pain was inflicted on live animals in the study. All efforts were made to minimize animal suffering in the conduct of the experiments. Ethical clearance for the study was sought and approved by the Ethical and Protocol Review Committee of the University of Ghana Medical School (Protocol Identification number: MS-Et/M.5 - P 4.1/2011-12). In the absence of locally documented guidelines on animal experimentation, the procedures performed on the animals conformed to the ethical guidelines for the care and investigation of laboratory animals (Zimmermann, 1983).
MAIN ANIMAL EXPERIMENTS

3.8 ANALGESIA

3.8.1 Hot Plate Method

Twenty-five mice weighing 25 - 30 g of both sexes that passed initial sensitivity test were randomly selected and assigned to 5 treatment groups of 5 mice each (3 males and 2 females). One group (control) received normal saline orally on weight basis, 10 ml/kg. Two other groups were given two dose levels of morphine, 5 mg/kg and 10 mg/kg, subcutaneously (internal standard); and the remaining two groups given the extract at two dose levels, 20 mg/kg and 40 mg/kg, respectively. Both doses of the extract were given orally. Each mouse was gently placed on a hot plate (55 ± 0.5 °C) and the latent period of response to thermal pain (reaction time) recorded. Licking of paws or jumping, whichever occurred first, was used as the endpoint. The reaction time was determined before treatment (time 0) and at 30 and 60 minutes post-treatment. A cut off period of 30 seconds exposure to thermal stimulus was observed to prevent damage to the paw tissue (Woolfe and MacDonald, 1944; Vaz et al., 1997). The experiment was repeated two times at 3-day intervals, and the average results of the two independent experiments used. The mean reaction time for each treated group was determined and compared with that obtained for each group before treatment and the controls.

3.10.3 Histology of Paw Tissues

The positive controls and the extract-treated rats were euthanized after collection of the paw inflammatory exudate. Sectioning of the soft tissues of the paw were made perpendicularly to the subplantar surface and immediately placed in 10% buffered formalin (pH = 7.2) to fix at room temperature (25 °C) for 48 hours. The sections were dehydrated through graded concentrations of
ethanol, cleared with xylene and embedded in paraffin wax at 56 °C. Serial sections of the paw tissue (4 µm approximately) were carried out and stained using routine hematoxylin-eosin (H & E) techniques. The sections were then mounted on microscope slides using DPX mountant. Sections of the stained tissue obtained from control animals were compared with those from treated animals for histological differences in neutrophilic infiltration and other morphologic features of acute inflammation. Evidence of inflammation of the tissues was assessed by a pathologist who was blinded to the treatments.

3.11 INHIBITION OF THE BIOSYNTHESIS OF PROSTAGLANDIN E₂

3.11.1 Drug administration, blood collection and storage

Forty rats of both sexes (180 – 230 g) were randomly selected and assigned to 8 treatment groups of five rats each (3 males and 2 females). The groups of rats were given four different treatments orally: two groups received normal saline (control), 10 ml/kg; two other groups were given two dose levels of indomethacin (internal standard for non-selective COX inhibitors), 20 mg/kg and 40 mg/kg, respectively; another two groups were given celecoxib (internal standard for selective COX-2 inhibitors) at two dose levels, 20 mg/kg and 40 mg/kg, respectively; and the remaining two groups given two dose levels of the extract, 20 mg/kg and 40 mg/kg respectively.

One percent (1% w/v) suspension of carrageenin in normal saline (0.1 ml) was used to induce inflammation in the right hind paw of one group of control rats and the treated groups, one hour after treatment. The non-carrageenin treated control group of rats served as negative control, whereas the carrageenin-treated control group of rats served as positive controls. The rats were anaesthetised with ether three hours after the injection of carrageenin. The animals were then
dissected to expose the heart. Blood sample (5 ml) was collected from each animal by cardiac puncture and was immediately put into an EDTA-containing tube. The blood was mixed thoroughly with the EDTA by gently inverting the tube eight times. The blood samples were centrifuged at room temperature for 15 minutes at 2,000 rpm within 30 minutes of collection. The plasma was pipetted into Eppendorf tubes for storage at -20 °C, avoiding freeze-thaw cycles during the period of storage.

### 3.11.2 Measurement of Plasma PGE$_2$ concentration

Commercial Rat Prostaglandin E$_2$ (PGE$_2$) ELISA kit (Cusabio$^R$) with lower detection limit of 0.5 pg/ml and high specificity for rat PGE$_2$ was procured for the plasma PGE$_2$ assay. All reagents and samples were brought out and allowed sufficient time to attain room temperature before use. The preparation of the samples and reagents were in accordance with the manufacturer’s instructions.

**Assay Procedure:** The frozen samples were allowed to thaw and centrifuged again before the assay. Next, a 96-well microtitre plate sufficient to run all the samples, including PGE$_2$ standards supplied and blank wells without any solution was mounted. Fifty microlitres of standard or sample was added per well. Then 50 μl of Horseradish peroxidase conjugate was pipetted into each well, except the blank well. This was followed by the addition of 50 μl antibody to each well. The microtitre plate was then incubated for 1 hour at 37 °C. Each well was aspirated and washed thoroughly, ensuring that all the wells were completely filled and emptied at each wash. Briefly, each well was filled with 200 μl wash buffer, using a multi-channel pipette and allowed to stand for 10 seconds. This was followed by complete aspiration of each well content, repeating the process thrice, using a multi-channel pipette. Any residual wash buffer in the wells
was removed by inverting the plate and blotting it dry against clean blue tissue paper. Enzyme Substrate A and Substrate B (50 μl each) were added to each well and agitated briefly on a microplate shaker to mix thoroughly. The plate was securely sealed with an adhesive strip and incubated for 15 minutes at 37 °C. Finally, 50 μl of acidic stop solution was pipetted into each well, and the plate gently tapped to ensure thorough mixing and to terminate the reaction. The optical densities (OD) of the PGE2 standards, together with those of the blank and sample wells, were determined using ELISA plate reader set at 450 nm. All samples and standards were assayed in duplicate.

Standard calibration curve was plotted from the concentrations and respective absorbances of the standard PGE2 supplied in the kit, using an immunoassay software package "professional Curve Expert 1.4" capable of generating four parameter logistic (4-PL) curve-fit. The levels of plasma PGE2 in the samples were interpolated from the standard calibration curve.

The percentage inhibition of PGE2 biosynthesis (I%) was calculated, using the formula:

\[
I\% = \{1 - (\text{conc. } S/ \text{conc. } C)\} \times 100
\]

Where conc. S = concentration of plasma PGE2 in drug- or extract-treated rats

conc. C = concentration of plasma PGE2 in carrageenin-treated control rats.
CHAPTER FOUR

4.0 EXPERIMENTAL RESULTS

4.1 PHYTOCHEMICAL INVESTIGATION

The results of phytochemical screening indicated presence of saponins, reducing sugars, polyuronides and triterpenes in the aqueous extract of the whole root and root bark of *C. erythrocarpos*, whereas flavonoids, alkaloids, tannins and cyanogenic glycosides were absent (Table 4.1). The results of phytochemical tests conducted on aqueous extract of the roots without the bark (woody core) were similar to the root with bark. Significantly, flavonoids were detected in only the ethanolic extract of the whole root. Most of the phytoconstituents were polar compounds.

**Table 4.1: Results of phytochemical screening of various extracts of *C. erythrocarpos***

<table>
<thead>
<tr>
<th>Phytoconstituent</th>
<th>Aqueous extract of root bark</th>
<th>Aqueous extract of whole roots</th>
<th>Ethanolic extract of whole roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyanogenic glycosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polyphenols/tannins</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Polyuronides</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anthracenosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: (+) present; (-) absent
4.2 ANALGESIA

Tukey’s post hoc test (after-test ANOVA) revealed statistically significant differences in the mean reaction time among the groups of mice at 30 minutes post-drug administration (p<0.05), except between those administered 5 mg/kg morphine and 40 mg/kg extract (p = 0.979). Similarly, the Analysis of Variance (ANOVA) showed the mean reaction time differed significantly among the different treatment groups of mice administered different dose levels of the extract and morphine at 60 minutes post-drug administration (p<0.05). However, at 60 minutes post-drug administration, the multiple intra- and inter-group comparisons using the post hoc test (after-test ANOVA) showed no significant difference in mean reaction time between mice treated with 5 mg/kg morphine and 40 mg/kg extract (p = 0.574), and between those treated with 20 mg/kg and 40 mg/kg extract (p = 0.204).

Table 4.2: Effect of the aqueous extract of *C. erythrocarpus* and morphine on reaction time in mice subjected to the hot plate test

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg)</th>
<th>Reaction time (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time (0 min)</td>
</tr>
<tr>
<td>Control</td>
<td>0.9% saline</td>
<td>7.46 ± 0.13</td>
</tr>
<tr>
<td>Morphine</td>
<td>5</td>
<td>7.79 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.58 ± 0.16</td>
</tr>
<tr>
<td>Extract</td>
<td>20</td>
<td>7.65 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>7.52 ± 0.15</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM from 6 animals in each experimental group for two independent experiments. P<0.05 was considered statistically significant.
4.3.1 Carrageenin-induced Inflammation in Rat Paw

In the control group of rats given normal saline, subcutaneous injection of the suspension of carrageenin led to sustained increase in paw volumes over the 6-hour observation period, indicating that the acute inflammatory response was successfully induced. As shown in Fig. 4.1, the increase in hind paw oedema peaked at 3 hours and gently decreased beyond 4 hours. The increase in paw volumes with time was generally less in the extract- and indomethacin-treated groups. Increase in paw volumes was marked by initial gentle slope which peaked at 4 hours after carrageenin injection, followed by a relatively sharp declining slope up to 6 hours. This trend was repeated for the doses of the extract and indomethacin tested, except the 80 mg/kg dose of extract (Figs. 4.1 & 4.2). The 80 mg/kg dose of the extract was relatively ineffective in reducing paw oedema as the paw volume continuously increased throughout the 6 hours observation period. However, the 40 mg/kg dose of the extract exhibited significant reduction in paw volume beyond 1 hour.
In contrast with the trend for extract-treated rats, the different doses of indomethacin showed varying degrees but similar pattern of anti-inflammatory activity. Indomethacin caused significant reduction in rate of increase of paw volumes which peaked at 2 hours for both the 20 mg/kg and 40 mg/kg, and at 3 hours for the 10 mg/kg-treated animals respectively. There was significant reduction in paw volumes of the rats beyond 2-3 hours (Fig. 4.2). Comparing with the extract-treated groups, percentage reduction in paw volumes recorded 4 hours after injection of the inflammatory agent for 10, 20 and 40 mg/kg indomethacin were 31.06%, 25.55% and 18.88% respectively, suggesting a dose-dependent trend (Fig. 4.2).
4.5 HISTOPATHOLOGICAL EXAMINATION OF PAW TISSUES

Plates 4.1 (a-e) show the photomicrographs of histological sections of the paw tissue of control rats, and rats treated orally with the extract or indomethacin (20 - 40 mg/kg).

In control rats whose paws were not injected with carrageenin (Plate 4.1 a), sections show histological features of normal paw tissue. There were no acute inflammatory changes involving the full thickness of the tissue from the superficial dermis to the muscle layer and the intermuscular stroma. The vascular caliber was normal. In contrast, the sections of carrageenin-treated positive control rats showed severe acute inflammatory changes involving the full thickness of the tissue from the superficial dermis to the subcutaneous layer. The inflammatory response is characterized by infiltration of numerous neutrophils within the dermis, subcutaneous layer and spaces between muscle bundles and around nerves. There is also severe oedema with
numerous congested capillary and moderate-sized vessels (Plate 4.1 b). Similarly, histological sections of paw tissue of rats administered the extract (20 mg/kg) showed severe acute inflammatory changes characterized by an intense band of cell infiltrates composed mainly of neutrophils, with severe oedema and vascular congestion (Plate 4.1 c). Histological sections of rats treated with the extract at a dose of 40 mg/kg, however, showed mild acute inflammatory changes characterized by a relatively few infiltrate of neutrophils in the dermis and the intermuscular stroma with moderate oedema and vascular congestion. The paw tissue of rats administered 20 mg/kg indomethacin showed histological features similar to those of rats administered 40 mg/kg extract (Plate 4.1 d & e).
Plate 4.1: Photomicrograph of the sections of the paw tissue in negative controls (a), positive control rats (b), rats treated orally at a dose of 20 mg/kg (c) and 40 mg/kg (d) of the aqueous extract, and rats administered 20 mg/kg indomethacin (e) (H & E, ×200). The dermis of carrageenin-treated rat paws show infiltration of inflammatory cells and oedema.

4.6 INHIBITION OF PROSTAGLANDIN E\(_2\) BIOSYNTHESIS

The plot of concentration of standard PGE\(_2\) against the respective mean absorbances was carried out using the four parameter logistic (4-PL) curve-fitting software "professional Curve Expert 1.4". The plot yielded a curvilinear standard curve (Fig. 4.9).
Fig. 4.9: Standard calibration curve used to determine plasma levels of PGE\(_2\).

4.7 ASSESSMENT OF TOXICITY OF THE EXTRACT

In the acute toxicity study, there were no significant changes noted in behaviour, activity, posture, or external appearance of the mice and rats. Also, all the animals survived throughout the 24 hours study period. Similarly, in the subacute toxicity study, no remarkable signs of extract-related toxicity were observed either immediately or during the post-treatment period, even at the highest dose of 200 mg/kg.

Gross pathological examination of the liver and kidney harvested from rats that were sacrificed at the end of the 28-day treatment period did not reveal any sign of abnormality or damage in the extract-treated groups compared to the controls. The morphology of the capsule of the isolated organs was normal, with no evidence of tear or deterioration. Furthermore, histological assessment of H & E stained sections of these organs did not show any significant differences or abnormality in their histoarchitecture (Plate 4.2 & 4.3).

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Plate 4.2: Photomicrograph of the sections of the liver in control rats (a), and rats treated orally at a dose of 50 mg/kg (b), 100 mg/kg (c) and 200 mg/kg (d) of the aqueous extract of *C. erythrocarpus* for 28 days (H & E, ×200).

Key: H = Hepatocyte; P = Hepatic portal vein; B = Central vein
Plate 4.3: Photomicrograph of the sections of the kidney in control rats (a), and rats treated orally at a dose of 50 mg/kg (b), 100 mg/kg (c) and 200 mg/kg (d) of the aqueous extract of *C. erythrocarpos* for 28 days (H & E, ×200).

Key:
- G = Glomerulus
- T = Renal tubule
- C = Corpuscular space
CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

Medicinal plants are widely patronized in Ghana for management of pain and inflammatory conditions. These plants constitute an untapped source of structurally diverse and novel compounds that may serve as lead molecules for the synthesis of various drugs (Verma and Singh, 2008). It is, therefore, a useful strategy to investigate medicinal plants acclaimed to be useful for management of pain and inflammation for their potential therapeutic value and safety. This is in recognition of the fact that previously discovered prototype analgesics and anti-inflammatory drugs, such as morphine and salicylic acid, were plant-derived (Sneader, 2000; Katzung et al., 2012).
5.1.6 SUMMARY OF FINDINGS

The study sheds light on the following key findings:

1. That both the aqueous extract of the root and root bark exhibit demonstrable anti-inflammatory activity within limited dose range.

2. That the aqueous extract does not contain flavonoids despite its significant anti-inflammatory activity, hence, a different bioactive agent, possibly a steroidal saponin or polyuronide, may be implicated in the anti-inflammatory activity.

3. The study indicated that pro-inflammatory agent of the extract is concentrated in the root bark and that pro-inflammatory activity of low doses of the root bark extract, just as higher doses of the root extract, could be due to bioconversion of a saponin or polyuronide active principle in the extract.

4. This study documents significantly, the first report of inhibition of the recruitment of inflammatory cells and \textit{in vivo} inhibition of prostaglandin E$_2$ biosynthesis by the aqueous extract.

5. That the extract acts by non-selective inhibition of cyclooxygenase, but preferentially selective for COX-2.

6. The extract (50 – 200 mg/kg) has very low or no significant hepatotoxic and nephrotoxic effects in rats when administered once daily over the 28-day study period.
5.2 CONCLUSION

The aqueous extract of the root of *C. erythrocarpos* exhibited significant analgesic and anti-inflammatory properties in all animal models of acute pain and inflammation used in this study. The aqueous extract was also found to possess white blood cell anti-migration properties, which contribute to the validation of its anti-inflammatory activity. The scope of the present investigation further revealed the involvement of the extract in inhibition of prostaglandin synthesis as well as possible interaction with fast-release inflammatory mediators. The findings support ethnomedical claims of therapeutic efficacy of the extract in the management of pain and inflammatory conditions.
5.4 LIMITATIONS OF THE STUDY

Due to the low yield of the extract and financial constraints, the acute toxicity study was conducted in only rats and mice whiles the subacute toxicity study was limited to rats. Consequently, the results may not be justifiably extrapolated to humans. It would be necessary to replicate the study in other laboratory animals including guinea pigs and rabbits. Also, special stains are a routine part of renal biopsy histopathology interpretation and may help to reveal subtle minute and delicate changes, but these were not employed in the study.
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Proinflammatory Gene Induction by Platelet-Activating Factor Mediated Via Its Cognate


xanthoxyloides* as an anti-inflammatory agent. Thesis submitted for the Award of Master of
Philosophy degree, University of Ghana, Legon.


