DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY
SCHOOL OF BIOLOGICAL SCIENCES

COLLEGE OF BASIC AND APPLIED SCIENCES

UNIVERSITY OF GHANA, LEGON

ANTI-INFLAMMATORY ACTIVITY AND THE MECHANISM OF ACTION OF *MORINDA LUCIDA* BENTH.

BY

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THIS THESIS IS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY, COLLEGE OF BASIC AND APPLIED SCIENCES, UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL BIOCHEMISTRY DEGREE

DECEMBER, 2016

DECLARATION

I, Frederick Ayertey, do hereby declare that except for references to other people's work, for which I have acknowledged, this report is the product of my own research carried out at the Department of Biochemistry, Cell and Molecular Biology, Center for Plant Medicine Research and Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, under the supervision of Prof. Laud Kenneth Okine and Dr. (Mrs.) Regina Appiah-Opong.

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DEDICATION

I dedicate this thesis to the Almighty God for his protection and guidance, my parents, siblings and all friends for their loyal support.

ACKNOWLEDGMENTS

I acknowledge my supervisors Prof. Laud Kenneth Okine and Dr. (Mrs.) Regina Appiah-Opong for their key guidance and support throughout the research period. Such a height would not have been reached without the advice and encouragement of Dr. Alfred Ampomah Appiah also.

A special thank goes to Mr. Stephen Antwi, Mr. Bismark Asante (Pharmacology Department), Mr. Henry Brew-Daniels, Mr. Benjamin Asante, Peter Bola (Phytochemistry Department), Mr. Sylvester Kamintah (Microbiology), staff of the Animal house and all other staff of Centre for Plant Medicine (CPMR), Mampong-Akwapim for the immense contributions to this research work.

My sincere appreciation goes to Mr. Ebenezer Ofori-Attah (Clinical Pathology Department), Dr. Mitsuko Ohashi and Mr. Michael Amoa-Bosompem (Parasitology Department), Dr. Kusi Asamoah (Immunology Department) and all other staff of the Clinical Pathology, Parasitology and Immunology Departments of Noguchi Memorial Institute for Medical Research (NMIR), Legon-Accra, for their special assistance.

I am most grateful for the kind contributions of Prof. Takuhiro Uto of Nagasaki International University, Japan, the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology (BCMB) and Japan Agency for Medical Research and Development (AMED).

Finally, I would like to thank the staff and colleagues of the Department of Biochemistry, Cell and Molecular Biology.

To everyone I say ‘Ayekoo’.

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ABBREVIATIONS AND ACRONYMS

AP:	Activator protein
BHT:	Butylated Hydroxytoluene
C/EBP:	CCAAT/Enhancer-Binding Protein
CAM:	Cellular Adhesion Molecules
cGMP:	Cyclic Guanosine Monophosphate
CNS:	Central Nervous System
COX:	Cyclooxygenase
CPM:	Chlorpheniramine
CPMR:	Center for Plant Medicine Research
CRE:	Cyclic AMP Response Element
DMEM:	Dulbecco's Modified Eagle's Medium
DNA:	Deoxyribonucleic Acid
DPPH:	2, 2-Diphenyl-1-picryl hydrazyl
EDRF:	Endothelium-derived Relaxing Factor
ELISA:	Enzyme-linked Immunosorbent Assay
eNOS:	Endothelial Nitric Oxide Synthase
FBS:	Fetal Bovine Serum
GAE:	Galic Acid Equivalence
GIT:	Gastrointestinal Tract
GPCR:	G-protein Coupled Receptor
GRA:	Granisetrone
HEML:	Hydro-ethanolic leaf extract of *Morinda lucida.*

HRP: Horse Radish Peroxidase

IFN-ɣ: Interferon-gamma

IL: Interleukin

iNOS: Inducible Nitric Oxide Synthase

LAM: Lipoarabinomannan

LD_{50}: Lethal Dose at 50%

LFA: Lymphocyte Function Associated Antigen

LOX: Lipooxygenase

LPS: Lipopolysaccharide

MAC: Membrane Attack Complex

MAPK: Mitogen-Activated Protein Kinase

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NF-κB: Nuclear Factor Kappa B

NKC: Natural Killer Cells

NMIMR: Noguchi Memorial Institute for Medical Research

nNOS: Neuronal Nitric Oxide Synthase

NO: Nitric Oxide

NSAIDs: Non-Steroidal Anti-inflammatory Drugs

PAF: Platelet-Activating Factors

PAMPs: Pathogen Associated Molecular Patterns

PGE_2: Prostaglandin E_2

PGI: Prostacyclin

PKC: Protein Kinase C

PRR:	Pathogen Recognition Receptors
PSGL:	P-selectin Glycoprotein Ligand
QE:	Quercetin Equivalence
ROS:	Reactive Oxygen Species
SDR:	Sprague-Dawley Rat
SDS-PAGE:	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SFN:	Sulforaphane
TNF-α:	Tumour Necrotic Factor- alpha
TXA:	Thromboxane A

ABSTRACT

A commonly used medicinal plant in African folk medicine for the treatment of various diseases including inflammation is *Morinda lucida* Benth. However, scientific data supporting its anti-inflammatory activity is scarce. In the current study, the anti-inflammatory activity of the hydroethanolic leaf extract of *Morinda lucida* Benth (*HEML*) was assessed using carrageenan-induced paw edema in female Sprague-Dawley rats (SDRs). The potential mode of action of *HEML* was determined by assessing its effect on the levels of PGE_2, NO, IL-1β, TNF-α and IL-10, and also expressional levels of COX-2 and iNOS in RAW 264.7 cells *in vitro*. Its phytochemical constituents were determined by standard methods, and its antioxidant activity was investigated by DPPH free radical scavenging assay. The ability of *HEML* to significantly reduce rat paw edema caused by carrageenan demonstrated its anti-inflammatory activity. *HEML* also inhibited paw edema induced by histamine or serotonin, and further suppressed LPS-induced fever in the SDRs, which indicates that both early and late phases of acute inflammation were affected by the extract. Persistence of the late phase mediators may plunge the organism into a chronic state of inflammation. Results obtained through the determination of levels of PGE_2, NO, IL-1β, TNF-α and IL-10 in culture supernatant of LPS-activated RAW 264.7 cells showed that *HEML* reduced NO and PGE_2 concentrations by downregulating iNOS expression but not COX-2. It also suppressed the levels of pro-inflammatory cytokines IL-1β and TNF-α possibly by boosting levels of the anti-inflammatory cytokine IL-10. *HEML* contained saponins, reducing sugars, polyphenolics including flavonoids. It possesses antioxidant activity, which may be due to its polyphenolic content, particularly flavonoids. The current findings provide evidence-based scientific data to support the anecdotal use of *M. lucida* as treatment agent for inflammation.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Inflammation is a complex mechanism and defensive process where biological tissues respond to dangerous stimuli produced by pathogens, damaged cells or irritants (Ferrero-Miliani *et al*., 2007). It provides the required physiology to get rid of infected or damaged cells, but the aftermath can be undesirable. This is because, uncontrolled inflammation forms the basis of some pathophysiological conditions such as gastritis, esophagitis, hepatitis, atherosclerosis, as well as cancer (Schottenfeld and Beebe-Dimmer, 2006). Thus, what begins as a harmless process to protect organisms, if not controlled can lead to deleterious consequences.

Evidence shows that prolonged inflammation is critical in the development of some pathophysiological conditions like cancer (Khansari *et al.,* 2009). Mast cells, basophils, neutrophils, eosinophils, macrophages and monocytes are major cells which participate in the process of inflammation. These cells form the important source of a range of chemicals including histamine, serotonin, bradykinin, cytokines, lipid mediators (eicosanoids), vascular endothelial growth factors and reactive oxygen species (ROS) which mediate the inflammatory processes (Boesiger *et al.,* 1998; Grutzkau *et al.,* 1998; Galli *et al.,* 2005).

A major composition of bacterial (gram negative) outer membrane is made up of an endotoxin lipopolysaccharide (LPS). The lipid part of LPS is found to activate many genes that express inflammation including that of cyclooxygenases (*COXs*), inducible nitric oxide synthase (*iNOS*) and cytokines among others (Kubes and McCafferty, 2000; Tanabe and Tohnai, 2002). Prostanoids (prostaglandins) formation from arachidonic acid (obtained after the hydrolysis of

membrane lipid by phospholipases) is catalyzed by COXs. Accumulated data indicate that there are two isoforms of COXs, which are COX-1 and COX-2 expressed by different genes. Constitutively, most tissues express COX-1 which synthesis basal levels of eicosanoids to ensure homeostasis (Funk *et al.,* 1991). Meanwhile, COX-2 expression is inducible and can be triggered by inflammatory stimuli such as LPS (Kong *et al.,* 2002). Increased expression of COX-2 is attributed to most disorders associated with inflammation including lupus erythematosus, multiple sclerosis, arthritis, Alzheimer's disease and cancer (Kapoor *et al.,* 2005; Wang *et al.,* 2007). Clinically, it has been shown that prostaglandins levels and COX-2 expression are higher in transformed tissues than in normal tissues, indicating an important role of COX-2 in tumourigenesis (Taketo, 1998; Buskens *et al.,* 2002). Malignant cell proliferation can be stimulated by enhanced levels of COX-2 (Subbaramaiah *et al.,* 1998) which can also promote angiogenesis (Lee *et al.,* 2003), inhibit immune surveillance (Thomas *et al.,* 2000) and suppress apoptosis (D'Acquisto *et al.,* 1997).

During arginine metabolism by nitric oxide synthases (NOSs), NO is generated endogenously to play a significant role in killing of tumour cells, host intracellular defense against pathogens, neurotransmission and prevention of platelet aggregation (Lorsbach *et al.,* 1993; Kroncke *et al.,* 1998). In a biological system, NO is produced through a chemical reaction catalyzed by three major isoforms of NOSs. These include neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) which are expressed constitutively in neuronal and endothelium cells, respectively. However, the expression of the third isoform, inducible nitric oxide synthase (iNOS) is inflammation dependent and can be generated in stressed cells or cells (macrophages, hepatocytes and endothelial cells) induced with LPS endotoxins, pro-inflammatory cytokines (Nathan and Xie, 1994). The expression of iNOS leads to the production of large amount of NO.

Excess NO production may play a significant role in the pathogenesis of cancer; thus can damage DNA directly or indirectly by several mechanisms, interfere with DNA repairs and cause post-translational modification by nitrosylation to initiate tumour formation and further drive the process of tumourigenesis (Tamir *et al.*, 1996; Wink *et al.*, 1998). Thus NO production by iNOS may reflect the degree of inflammation and cancer.

1.2 PROBLEM STATEMENT AND JUSTIFICATION

A common practice in the therapeutic approach to alleviate the symptoms associated with both acute and chronic inflammatory diseases is the use of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Unfortunately, most NSAIDs have adverse effects on coagulation of blood, gastrointestinal lining and the renal systems due in part to the inhibition of the housekeeping enzyme COX-1 (Rainsford, 1999). Thus, most available therapeutic agents for the treatment of inflammatory diseases lack specificity and are plagued with untoward side effects (Dhikav, 2002).

While current treatment modality targets inflammatory cells and mediators, there is evidence that the extent of success with this approach could have been reached using plants (Dhikav, 2002). Limitations of NSAIDs, including the usual adverse effects on the gastrointestinal tract and renal system have sensitized the need for an alternative approach. Moreover, some newer COX-2 specific inhibitors, claimed to be devoid of these adverse effects, have not lived up to these claims (Celotti and Laufer, 2001), and hence the continuous efforts in search of more potent anti-inflammatory drugs with minimal side-effects.

The increasing optimism in Ghana and other developing countries for the use of herbs and safety-modified natural metabolites in the treatment of various diseases including inflammatory

conditions has come of age. *M. lucida* Benth. (Rubiaceae) is one of the most popular medicinal plants widely distributed in Africa. Although *M. lucida* has been used in Ghanaian folk medicine in the treatment of inflammation, there is little scientific data to validate its use as such. This work, therefore, seeks to evaluate the anti-inflammatory properties of hydroethanolic leaf extract of *M. lucida (HEML)* and determine its possible mechanism. This would provide some scientific evidence to support the use of the plant in the treatment of inflammation.

1.3 HYPOTHESIS

M. lucida has anti-inflammatory activity and this is expressed through the suppression of pro-inflammatory mediators and/or elevation of anti-inflammatory mediators.

1.4 AIM/ SPECIFIC OBJECTIVES

1.4.1 Aim

To evaluate scientifically the anti-inflammatory effect of *HEML* and its possible mechanism of action.

1.4.2 Specific Objectives

- To determine the phytochemical constituents and antioxidant activity of the *HEML*.
- To assess the anti-inflammatory activity of *HEML* using the Carrageenan-induced paw oedema rat model.
- To determine the possible mechanisms of action of *HEML* as an anti-inflammatory agent by examining its effect on selected inflammatory mediators.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MEDICINAL PLANT

It is believed that about 80% of the world's population use medicinal plants for their primary health care needs (Segar, 2012). Medicinal plants have long formed a key composition in traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental, Native American Indian medicine and African folklore. Records indicate that 74% of plant-derived pharmaceutical medicines used in modern medicine directly correlates with their traditional uses as plant medicines by native cultures. This has placed an urgent need on modern pharmaceutical companies to engage in extensive research on the pharmacological values of medicinal plants since these plants are utilized in herbal medicine.

The reason for high patronage of herbal medicines include its perceived lesser side effects, affordability, accessibility and availability. They are also thought to be more effective especially for chronic disease conditions that do not respond well to orthodox medicine (Grunert, 2011). However, the most fascinating issue to discredit the world of herbal medicine is the inadequate data and documentation to explain its anecdotal use. There is also the risk of self-dosing and slow response to ailment as compared to modern medicine, which can treat sudden illnesses and emergency conditions much more effective at a faster rate (Grunert, 2011), but often with deleterious consequences. A typical example of such medicinal plant exploited for its medicinal values and other economic gains in Africa is *M. lucida*.

2.2 BOTANICAL DESCRIPTION OF *M. LUCIDA*.

M. lucida is a medicinal plant which belongs to the Rubiaceae family. It is known commonly as Brimestone tree due to the yellow color of its wood. In Nigeria, especially among the Yoruba natives of south-western Nigeria, it is referred to as 'Oruwo' (Dalziel, 1937), while in Ghana it is popularly known as 'Konkroma' by the Akans and 'Amake' by the Ewes (Addo-Fordjour *et al.*, 2008). The medium sized tree or evergreen shrub is about 18-25 meters high, with the branches often crooked or gnarled, projecting from a stem covered with both smooth and rough-forming irregular grey-brown patches at the bark, often showing purple divisions (Abbiw, 1990). In addition, the plant has slender branchlets and a dense crown. The leaves are broad, ovate and tapering to the end, with sizes ranging normally from 7-15 cm long and 3.5-7.5 cm wide. The display of the leaves around the branches is opposite and simple.

As a flowering plant, *M. lucida* produces white aromatic scented flowers from January to July and September to October and also bears fruits from March to April (Irvine, 1961). The flowers are bisexual and have narrow glabrous corolla tube of about 2.5 cm. The fruits produced are classified as drupe; several are arranged together into an almost globose succulent syncarp 1-2.5 cm in diameter, which is soft and black when matured, pyrene compressed ovoid, up to 6.5 mm x 4 mm, dark red brown and one seeded.

A

B

Fig.2.1: *M. lucida* plant; (A) showing the whole plant and (B) showing the leaves.

2.3 GEOGRAPHICAL DISTRIBUTION OF *M. LUCIDA*

M. lucida thrives on grassland, exposed hillsides, thickets, forests, and often on termite mounds, sometimes in areas which are regularly flooded, from sea-level up to 1300 m altitude. The plant also grows in fringe forests and sometimes it takes over secondary clearings in rain forests.

2.4 TRADITIONAL USES OF *M. LUCIDA*

The indigenous style of treating or managing specific disease conditions from the native's perspective which may reflect the living conditions of people in a given environment is said to be ethnomedicine. The concept of ethnomedicine is proven useful in studying indigenous therapeutic agents particularly during drug discovery or development. This has enabled researchers to understand fairly and suggest possible scientific explanations to the basis of most native treatment modalities (Neuwinger, 2000).

Various parts of *M. lucida* have been used extensively in traditional medicine in West Africa. Nigerians use it as one of the top four medicinal plants in various preparations for the treatment of fever. Decoctions and infusions made from the leaves, roots and bark may serve as remedy against fever during childbirth and infectious diseases such as malaria, yellow fever, trypanosomiasis among others (Adesida and Adesogan, 1972). Other disease conditions such as diabetes, hypertension, cerebral congestion, dysentery, stomach-ache, ulcers, leprosy and gonorrhea are also well managed or treated with *M. lucida* (Adesida and Adesogan, 1972).

As one of the ways of treating ringworm infections and itches in DR Congo, the leaves or stem bark is used in combination with the root bark as dressings. Decoction of the leaves is also used as remedy against jaundice in Côte d'Ivoire (Abbiw, 1990). Adewunmi *et al.* (1984) reported that aqueous extract of the leaves is applied to the breast of women during weaning of their infants not only due to its bitterness, but also to prevent infections. Traditionally, *M. lucida* is reported to be used generally as laxative, analgesic and febrifuge while it is used locally in Ghana and Nigeria for the treatment of irregular menstruation, jaundice, insomnia, wound infections, abscesses and chancre (Makinde and Obih, 1985; Burkill *et al.*, 1997; Raji *et al.*, 2005), however, the specific part was not stated. Some herbalists in Ghana often cold macerate the fresh leaves in palm wine to form a bitter concoction. This is administered orally for few days to patients with diabetes.

Apart from the medicinal values, the yellowish to red dye extracted from the wood of *M. lucida* has significant benefits to the textile industries. For example, the root remains an important source of yellow dye to feed the Kasai Province of Democratic Republic of Congo, where it may be used with or without a mordant. In both Gabon and Nigerian, scarlet red of textiles is made using the root bark as dye. In Ghana, the indigenes of the Ashanti Region during the grief period

over the death of a chief require special clothing locally made and known as ‘Kobene’. ‘Kobene’ is cotton clothing dyed red using the root bark of *M. lucida* and represents the official dress for the indigenes of the Ashanti kingdom for mourning a dead chief. By the process of fermentation and reduction which are some steps in dyeing, the root of *M. lucida* is added to indigo vats to generate a darker blue color in Cote d’ Ivoire. This process may not be complete without adding leafy twigs of *Saba comorensis.* To obtain a pale green color of basket during basket weaving, the young leaves of *M. lucida* is combined with the leaves of Philenoptera species.

In the food industry, the roots are used as chewing stick and flavoring agent in alcoholic beverages. The hardy woody nature of *M. lucida* makes it very useful in energy generation hence is an excellent source of charcoal (Burkill *et al.*, 1997).

2.5 SOME CHEMICAL CONSTITUENTS OF *M. LUCIDA*

It has been documented that the major composition of *M. lucida* extracts are alkaloids, anthraquinones and anthraquinols (Adewunmi *et al.,* 1984). Among the 18 anthraquinones known to have been isolated from the wood and bark of *M. lucida* are red dyes 1-methylether-alizarin, rubiadin and their derivatives, lucidin, soranjidiol, damnacanthal, nordamnacanthal, morindin, munjistin and purpuroxanthin. Other compounds such as Oruwalol and Oruwal have been isolated and characterized from the stem (Adesogan, 1973). Recently in Ghana, a tetracyclic iridoid named molucidin and its derivatives have been isolated and characterized from the leaves *of M. lucida* through a bioassay guided fractionation process in the quest for drug development against parasitic infections such as trypanosomiasis, malaria and leishmeniasis (Suzuki *et al.,* 2015; Kwofie *et al.,* 2016). Kwofie *et al.* (2016) studied extensively the mechanism of action of molucidins on trypanosome parasites. Other groups of phytochemicals such as tannins, flavonoids and saponins have been detected and some isolated (Adewumi and

Adesogan, 1984). Oruwacin has also been reported (Evans *et al.,* 2002) to be isolated from the roots of *M. lucida* and further confirmed the presence of anthraquinones also in the roots. Furthermore, work done on the leaves showed the presence of ursolic and oleanolic acids which are also classified as triterpenes (Evans *et al.,* 2002).

2.6 PHARMACOLOGICAL PROPERTIES OF *M. LUCIDA*

Various *in vitro* models have been used to assess the biological and pharmacological effects of extracts of *M. lucida* and further confirmed its activity by pre-clinical trials using animal models. Most of these activities reflect and explain the traditional applications of extracts from the plant. A typical example is as cited in a study, which demonstrated that extracts of the roots of *M. lucida* possess analgesic and consequently a sedative effect in mice (Younos *et al.,* 1990). Zimudzi and Cardon (2005) also reported the antihypertensive activity of extracts of the leaf and stem bark of *M. lucida.* Although the extracts showed a strong antihypertensive activity, it was short acting, which may possibly be due to rapid metabolism and faster elimination of metabolites from circulation but had no data to ascertain that. It was, therefore, recommended that extracts of *M. lucida* has potentials as treatment agent for hypertension and cerebral complications due to its strongly marked diuretic and tranquilizing ability. Most especially, *M. lucida* extract did not only cause a remarkable reduction in contraction of the smooth muscles of the uterus of both pregnant and non-pregnant women but also suppressed contractions induced by both oxytocin and acetylcholine in the uterine walls (Elias *et al.*, 2007).

The anthraquinone content has molluscidal activity against fasciola and schistosoma sp., which has been attributed to the presence of oruwacin (Adewumi and Adesogan, 1984). The anti-trypanosomal property of the hydroethanolic leaf extract has been demonstrated to be pronounced in mice infected with *Trypanosoma brucei* and treated at a dose of 1000 mg/kg

intraperitoneally (Asuzu and Chineme, 1990). In a bioassay-guided fractionation, Suzuki *et al.* (2015) isolated a tetracyclic iridroid and two other derivatives from the chloroform fraction of 50 % (v/v) hydroethanolic leaf extract of *M. lucida.* These compounds have activity against trypanosomes and were named as Molucidin, ML-2-3 and ML-F52. The side chain of the ML-2-3 at the C-4 has carboxylic acid functional group. The other two derivatives (ML-F52 and Molucidin) have functional groups which are methyl and ethyl esters of the main compound ML-2-3, respectively. They also established possible mechanisms of action of ML-2-3 and ML-F52 on *Trypanosoma brucei* as induction of apoptosis and suppression of the expression of paraflagellar rod protein 2 (PFR-2). Their research suggested that, the compounds isolated were not only active against trypanosomes but other protozoans. They, however, failed to prove that the isolate could be active against other protozoans since they had no data to support it.

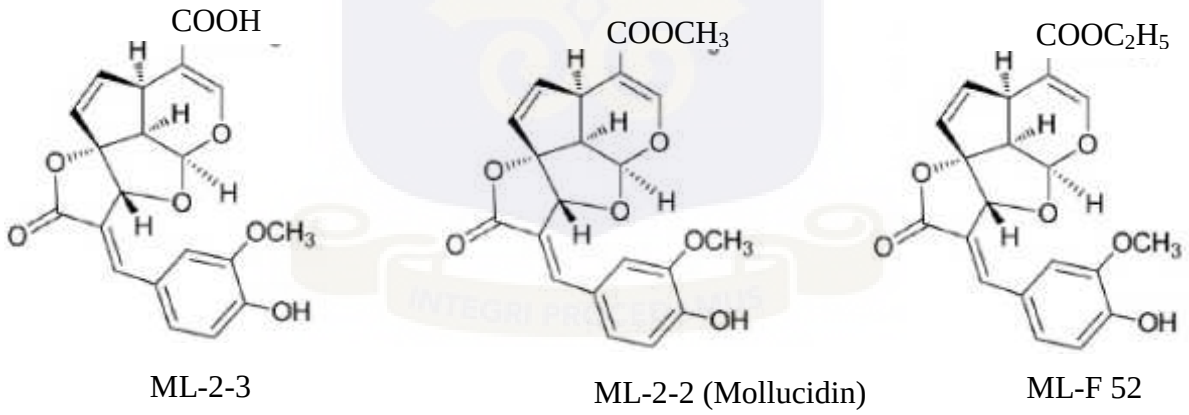


Fig.2.2: Chemical structures of tetracyclic irridroids isolated from *M. lucida.* (Suzuki et al., 2015).

During infection, there is inflammation triggered by the immune system just to notice and get rid of the infection (Ferrero-Miliani *et al*., 2007). One key manifestation of inflammation during infections is fever due to the release of prostaglandins E_2 (PGE_2). Although *M. lucida* has been reported and evaluated for various pharmacological activities, there is limited research to show

its anti-inflammatory, anti-fever and pain relieving effect, even though other species from the Rubaecia family has been evaluated for their anti-inflammatory effect (Awe *et al.,* 1998). The leaf extracts of *M. lucida* has also been reported to have anti-malarial activity against *Plasmodium falciparum in vitro* (Adeneye *et al.,* 2008) and *Plasmodium bergei* in mice where the leaf extract had significant inhibition on the schizontal stage of the parasite (Makinde and Obih, 1985; Obih *et al.,* 1985).

2.7 INFLAMMATION

As part of a complex defensive mechanism in an organism, the local build-up of catabolic products leading to subsequent rise in tissue osmotic pressure and the concomitant attraction of fluids accompanied with or without heat is said to be inflammation (Stankov, 2012). It can be reduced to five macroscopic pathological phenomena, characterized by tumour or swellings of the tissue resulting from fluid accumulation, elevated temperature and tissue redness due to increased blood flow to the affected area, intense sensation due to noxious stimulus and finally loss of function of organ(s) involved resulting from combination of factors. For example, in the process of recovery from a toxic insult in the liver, there may be high levels of collagen disposition into the extracellular space of the hepatocytes. This may harden the liver and impair its function, leading to a disease condition referred to as liver cirrhosis.

The process of inflammation is key in the displacement of offending factors and total restoration of tissue structure for proper functioning of the body. Quite often, the supposed beneficial process has gone out of control plunging the organism involved into dreadful and irreversible pathophysiological conditions. The inflammatory mechanism is, therefore, complex and may lead to cell death or tissue damage. A number of factors including physical, biological and chemical factors may trigger the whole cascade. Host cells, blood vessels and plasma proteins

are some of the major players in the initiation and resolution stages of the mechanism. Progression of inflammation is very intricate and can be divided into acute; where inflammation lasts for only few days and chronic; where inflammation persist for longer duration.

2.7.1 Acute Inflammation

The initial stage of a typical inflammatory process is marked by an acute phase, where the cascade begins with primary response of the immune and vascular system right after infection or damage to sterile tissues. It represents the first line of defense against injury, nonspecific but immediately lethal which is characterized by various changes in microcirculation, thus plasma exudation and emigration of leukocytes from blood vessels to the site of injury (Lawrence *et al.,* 2002). The response is rapid and persists for a short while, normally before the immune response becomes established. This is because, the primary role of acute inflammation is targeted at eliminating the injurious agent as soon as possible. Acute inflammation also acts as a homeostatic mechanism to the benefit of the host in a reparative process where affected tissues are restored to normal (Gabay and Kushner, 1999). A hallmark of acute inflammation is the infiltration of polymorphonuclear leukocytes, thus leukocytes with looped nucleus (Maton *et al.,* 1997). Leukocytes are group of white blood cells (WBCs) which make up the cells of the immune system. They consist of the broadest category of cells including neutrophils, eosinophils, basophils, lymphocytes and monocytes. They are derived from hematopoietic stem cells from the bone marrow and aid in protection against invasion. They are present throughout the body in circulation, thus the blood and lymphatic systems (Maton *et al.,* 1997).

Sentinel cells are basic guard cells which include mast cells, dendritic cells and resident macrophages. Their major role is to protect the body against invasion and this makes them critical players during inflammation. Pathogens possess pathogen associated molecular patterns

(PAMPs) which recognize and bind to pathogen recognition receptors (PRRs) present on the surface of the sentinel cells (Janeway and Medzhitov, 2002; Kawai and Akira, 2010). This cause their activation and consequent release of acute phase mediators such as histamine, bradykinin and serotonin from mast cell especially, interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF- α) by resident macrophages and dendritic cells. Unlike macrophage and dendritic cells, mast cell activation is more rapid because mediators involved are already synthesized and stored in the vesicles. Macrophage and dendritic cell activation is followed by the release of mediators such as IL-1, IL-6, TNF-α, prostaglandins (PGs) and NO which are synthesized in delayed process.

Histamine released from degranulation of mast cells act on endothelial cells lining the walls of terminal arterioles and capillaries and post-capillary venules to release nitric oxide (NO) and prostaglandins (e.g. prostacyclin-2 (PGI_2) and prostaglandin E_2 (PGE_2)). The NO and prostanoids generated cause the relaxation of vascular smooth muscle cells leading to vasodilatation of the blood vessels. This enables more blood to rush through causing redness of the inflamed area. Vasodilation of blood vessels means the pulling apart of actin filaments at adherence junction linking endothelial cells from each other, creating gaps to allow plasma exudates into the interstitial spaces and hence tumour or edema formation. Plasma exudate contains proteins like that of the complement system, kallikrein-kinin system and coagulation factors.

The C3b complement protein found among plasma exudate coats the pathogen membrane surface for easy opsonization and phagocytosis by phagocytes (Martin and Blom, 2016). Other complement proteins such as C3a and C5a are regarded as anaphylatoxin and are essential for the formation of membrane attack complex (MAC) in the membrane of the pathogen to cause

osmotic lysis. C3a and C5a also have their receptors located on mast cells. They bind to them and cause positive feedback loop by activating mast cells to release more histamine. Kallikrein-kinin system also consists of proteins such as bradykinin and kallidin. Bradykinin and kallidin in plasma exudate act on sensory neurons and cause pain and loss of function. They also act on B2 receptors on endothelial cells to cause positive feedback loop activation.

Coagulation factors have no specific role to play in circulation but once they are leaked into the interstitial spaces and come into contact with collagen and tissue factors, the intrinsic or extrinsic coagulation cascade is set-up. This results in the transformation of fibrinogen (factor-1) into fibrin (factor 1A). Fibrin then polymerizes into fibrin strands which are joined together to form a very dense mesh network known as fibrin mesh work (Doolittle, 1973). Fibrin mesh work restricts the spread of pathogens and also prevents external pathogen from entry.

To recruit phagocytes (e.g. neutrophils and macrophages) to the site of injury, endothelial cells release already synthesized and sequestered receptors stored in weibel-palade bodies. These receptors are known as P-selectin. Selectins are cellular adhesion molecules (CAMs) with single-chain transmembrane glycoproteins, which facilitate attachment of cells and are calcium-dependent in binding (Cotran and Mayadas-Norton, 1998). During the process, the weibel-palade bodies fuse with the apical membrane of the endothelial cells and release the P-selectin onto its surface. Neutrophils for example have on their membrane surface P-selectin glycoprotein ligand-1 (PSGL-1) which binds to P-selectin on the surface of the endothelial cells to slow down the rate of movement. The process of rolling of neutrophils begins by breaking and re-bonding with other P-selectin secreted on the surface of endothelial cell, until a weak interaction is formed between lymphocyte function associated antigen-1 (LFA-1) on the neutrophil membrane surface and intracellular adherence molecule-1 (ICAM-1). The weak interaction is due to the non-

activation of LFA-1. Platelet activating factors (PAF) released by the endothelial cells activate LFA-1 by binding to a platelet activating factor receptor (PAFR) present on the neutrophil. This finally halts the rolling neutrophil to enable it to push through the space created in between the endothelial cells and gets into the interstitial space in a process known as diapedesis. C3b receptors on the neutrophil membrane bind to C3b protein coatings on pathogen membrane and invaginate to form a phagosome. Lysozymes stored in vesicles in neutrophils are released to fuse with phagosome and digest the pathogen. Persistence of acute phase mediators due to the injurious agent may set the system into the chronic stage of inflammation.

2.7.2 Chronic Inflammation

Chronic inflammation is a prolonged (takes weeks, months or years) and an active inflammation in which tissue injury and healing proceed simultaneously (Lazzarino *et al.*,2016). It is characterized by infiltration of mononuclear cells such as macrophages, lymphocytes and plasma cells. It is caused by prolonged infection and can be better explained by the pathogenesis of tuberculosis.

Microorganisms can escape clearance by the immune system leading to prolonged infection and activation of several immune cells resulting in chronic inflammation. For instance, during *Mycobacterium tuberculosis* infection in the lungs, resident alveolar macrophages and tissue dendritic cells engulf the mycobacterium to form a phagosome and tend to eliminate it by fusing the lysosome containing hydrolytic enzymes with the phagosome (Steinberg and Grinstein, 2009). Mycobacterium cell wall component; lipoarabinomannan (LAM), prevents the fusion process by suppressing phagosomal maturation by the alveoli macrophages. This affects and interferes with the associated cell signaling cascade to switch cytokines response from pro-

inflammatory to anti-inflammatory (Nigou *et al.,* 2001; Briken *et al.,* 2004; Pathak *et al.,* 2005; Vergne *et al.,* 2005) to enable the microorganism to survive.

The pro-inflammatory cytokines released at the initial phase of the infection leads to the recruitment of more inflammatory cells from the blood stream (Briken *et al.,* 2004). Infected cells become activated and release IL-12 and IL-18 at a local lymph node where they activate T-lymphocytes. The activated T-lymphocyte release interferon gamma (IFN-γ) which induces natural killer (NK) cell activity and enhance the fusion of lysosome with the phagosome, just to eliminate the microorganism. The IFN-γ released also acts on endothelial cells lining blood vessels to attract monocytes from tissues to differentiate into macrophages where they are recruited to the site of infection. The consequent activation of the macrophages is associated with the release of pro-inflammatory cytokines such as TNF-α and IL-1 and results in high inflammation (North and Jung, 2004; Korbel *et al.,* 2008). The activated macrophages crowd and fuse to form giant cells. The site of infection becomes necrotic and surrounded by activated T-lymphocytes causing reactions leading to granuloma formation and granuloma inflammation. Chronically activated macrophages release cytokines which stimulate fibroblast proliferation and collagen production in a healing process. This produces a scar (fibrosis) at the inflammatory site and impair the normal tissue function of the specific organ involved (Welin *et al.,* 2011).

Apart from persistent infections, other factors such as autoimmunity and prolonged exposure to potentially toxic substances are of importance in chronic inflammation. These factors delay hyperactivity and prevent the system from attaining the optimum resolution during acute inflammation (O'Byrne and Dalgleish, 2001; Dalgleish and O'Byrne, 2002). Chronic inflammatory diseases such as rheumatoid arthritis, multiple sclerosis and chronic allergic diseases (e.g. bronchial asthma) might have occurred through the activation of immune cells by

auto-antigens and common environmental agents like smoke, dust and hay. In most autoimmune diseases, there is a 'self-antigen' which continually activates T cells and results in significant tissue damage. Chronic inflammation has been attributed to the development of 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).

2.8 MEDIATORS OF INFLAMMATION

Immune reaction elicited by the body to tissue damage involves a myriad of antibodies, cells, proteins, antimicrobial peptides and connective tissue features which interact to prevent the damage thereby restoring normal tissue integrity (Davidson, 1992). Vasodilation and consequent increase in vascular endothelial permeability at the initial phase of local inflammation, as a result of infection or tissue injury is associated with the recruitment of large number of leukocytes from peripheral blood to the site inflamed (Butcher *et al.,* 1999). The leukocytes involved in the inflammatory process include basophils, neutrophils, eosinophils and monocyte which differentiate into macrophages at the inflammatory site. Other cells which mediate inflammation are mast cells and dendritic cells. Upon activation, these cells release chemical agents which do not only propagate the whole inflammatory process but also act as crucial players for resolving it. Some of these chemical mediators are histamine, serotonin, bradykinin, cytokines, chemokines, lipid mediators (eicosanoids) and reactive oxygen species (ROS) (Boesiger *et al.,* 1998; Grutzkau *et al.,* 1998; Galli *et al.,* 2005).

2.8.1 Histamine

Histamine [2-(4imidazolyl)-ethylamine] is a vasoactive and endogenous short acting biogenic amine which is obtained from the catalysis of histidine by the enzyme histidine decarboxylase. It

is synthesized and stored in granules of mast cells, basophils and platelets and known to regulate Th-1 and Th-2 antigen specific immune responses (Packard and Khan, 2003). It was first described as an anaphylactogen due to its high tendency of mimicking anaphylaxis and since then, research has clearly elucidated its role as far as inflammatory response is concerned. For example, during allergic reaction, histamine acts as a chemical neurotransmitter to cause skin, nose, throat and lung irritation (Zampeli and Tiligada, 2009). The ability of histamine to bind its specific receptors is key to affecting cellular activities and this may represent a basic regulatory mechanism to control the undesirable effects elicited. There are four major G-protein coupled receptors (GPCR) subunits designated as H1, H2, H3 and H4 known to be acted upon by histamine (Dy and Schneider, 2004; Akdis and Simons, 2006). These receptors are widely distributed and differentially expressed in various cell types such as endothelial cells, macrophages, dendritic cells, T- and B-lymphocytes (Zampeli and Tiligada, 2009).

Activation of H1 receptor results in histamine-induced vascular permeability (Zampeli and Tiligada, 2009). Just like H1 receptor, H2 receptor type in addition to mediating vascular permeability also serve as a more important secretagogin to stimulate gastric acid secretion. H3 receptors are known to be localized in the CNS but very little is known about their role in humans (Parsons and Ganellin, 2006). H4 receptors are found on cells with hematopoietic origin and hence are regarded as major players during inflammation (Parsons and Ganellin, 2006). Due to this, H4 and H2 receptor antagonists may serve as promising drug candidates for treating inflammatory conditions and lower the risk of gastrointestinal tract disorders (e.g. peptic ulcer) like those associated with treatment of inflammation by non-steroidal anti-inflammatory drugs (NSAIDs).

2.8.2 Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT) formed from tryptophan. It is a monoamine molecule found in mast cells and platelets distributed in the gastrointestinal tract (GIT) and the central nervous system (CNS). It serves as a neurotransmitter and so modulates behaviors such as cognition, mood, aggression, mating, feeding, and sleep in the brain (Nichols and Nichols, 2008). It also acts as vasoactive amine, which increases vascular permeability and act as vasodilator of capillaries to bring about contraction of nonvascular smooth muscle (Borissova *et al.,* 1994). Thus, during acute inflammation, serotonin plays a major role by increasing vascular permeability through vasodilation of blood vessels. To facilitate and accelerate the rate of leukocytes emigration from microcirculation to the site of inflammation, serotonin increases the levels of intracellular cyclic guanylate monophosphate (cGMP) to induce a chemotactic effect so that polymorphonuclear granulocytes migrate to the inflamed site (Paegelow *et al.,* 1985; Kelm and Schrader, 1990). McCorkle *et al.* (1990) reported that the presence of 5-HT antagonist could suppress leukocyte emigration by blocking serotonin effect.

2.8.3 Bradykinin

Bradykinin is a peptide which is endogenously produced through the kallikrein-kinin system (Raidoo and Bhoola, 1998). Its effect is mediated in the body through B_1 and B_2 receptors (Chao *et al.,* 1987; Hall, 1997). These receptors are transmembrane and members of G-protein coupled receptors (GPCR). The B_2 receptor is expressed constitutively on various cell types such as endothelial cells, nerve fibers, leukocytes, and mast cells (Bhoola *et al.,* 1992; Calixto *et al.,* 2000) while the B_1 receptor is known to be inducible and expressed in most stressed tissues. The expression of the latter may be enhanced by cytokines production during shocks and inflammation (Marceau *et al.,* 1998; McLean *et al.,* 2000).

Bradykinin is an acute phase inflammatory mediator. During inflammation, bradykinin released by endothelial cells binds to the B_2 receptor and activates it by exchanging a guanosine diphosphate (GDP) bound to the receptor for a guanosine triphosphate (GTP). The alpha subunit (αq) of the heterotrimeric protein coupled to the receptor dissociates and binds to activate membrane phospholipase-Cβ (PL-Cβ). Activated PL-Cβ act on and hydrolyze membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce inositol triphosphate (IP_3) and diacylglycerol (DAG) (Hilgemann, 2007). Both IP_3 and DAG serve as secondary messengers and important signaling molecules which control diverse cellular events especially by stimulating the release of intracellular calcium from the endoplasmic reticulum of endothelial cells. Influx of calcium in the cytosol leads to activation of protein kinase C (PKC), which may control signaling activity of the cell. DAG may also serve as a precursor to arachidonic acid generation and hence prostaglandins formation. Thus, there is enough evidence to suggest that bradykinin has a direct effect on cellular levels of arachidonic acid (Burch and Kniss, 1988; Kaeser *et al.*, 1988) and induction of calcium dependent release of glutamate and cytokine expression (Parpura *et al.*, 1994; Schwaninger *et al.*, 1999). Bradykinin also induces the expression of adhesion molecules, vasodilation of arteries, facilitation of leukocyte infiltration and protein extravasation in post capillary venules (Calixto *et al.*, 2000). B_2 receptor activation can lead to the co-expression of B_1 receptors (Phagoo *et al.*, 1999). This, however, suggests that the induced expression and activation of B_1 receptors can take part in the cascade resulting in tissue injury and systemic inflammation.

2.8.4 Cytokines

Cytokines can be defined as small proteins which are specific in their actions and are secreted by cells to mediate cellular communications. Cytokines released by lymphocytes are known as

lymphokines, those released by monocytes are termed as monokines, those released by leukocytes or act on leukocytes are known as interleukins, while chemokines are cytokines with chemotactic activity. As far as inflammation is concerned, cytokines can be classified as pro-inflammatory or anti-inflammatory.

Pro-inflammatory cytokines are those which promote inflammation whereas those cytokines which reduce or prevent inflammation by suppressing the activities of pro-inflammatory cytokines are called anti-inflammatory cytokines (Charles and Dinarello, 2000). This concept is fundamental to cytokine biology and may be based on the fact that, during inflammation, certain encoding genes are upregulated. For example, genes responsible for the synthesis of pro-inflammatory mediators such as phospholipase A_2 (PL-A_2), cyclooxygenases-2 (COX-2) and inducible nitric oxide synthase (iNOS) are increased in expression during inflammation. This increases the expression of enzymes and proteins responsible for the synthesis of pro-inflammatory mediators.

Interleukine-1 (IL-1), intereukine-6 and tumour necrotic factor alpha (TNF-α) or cachectin are some few examples of pro-inflammatory cytokines which may exert their effects through the induction of endothelial adhesion molecules for leukocyte interaction. Cytokine expression can be induced by endotoxins such as LPS, but other cytokines can be formed through stimulation by IL-1α, IL-1β and TNF-α and in some cases, interferon-ɣ (IFN-ɣ) after their expression. During fever, TNF-α, IL-1α and IL-1β enhances the production of PGE_2 through the vascular endothelium of the hypothalamus (Warren, 1990) and stimulate T-cell proliferation. TNF-α also serve as a trimer (Smith and Baglioni, 1987) to regulate apoptotic pathways, nuclear factor-kB (NF-kB) activation of inflammation and active stress-activated protein kinases. It is one of the

products of activated macrophages/monocytes, fibroblasts, mast cells, and some T and natural killer (NK) cells (Aggarwal, 1992).

Anti-inflammatory cytokines are regarded as immunoregulatory peptides which control the response of pro-inflammatory cytokines by acting in concert with specific cytokine inhibitors and soluble cytokine receptors (Zhang and An, 2007). Some anti-inflammatory cytokines include IL-4, IL-10, IL-11, IL-13 and transforming growth factor-beta (TGF-β) which suppress the expression of pro-inflammatory cytokines and vascular adhesion molecules. This implies that, a ratio closer to 1.0 may ensure a proper balance between the pro- and anti-inflammatory cytokines, which is critical in determining the outcome of a disease (Charles and Dinarello, 2000). IL-10 happens to be one of the potent anti-inflammatory cytokines which represses the expression of TNF-α, IL-6 and IL-1 by activated macrophages. It can counter-regulate both the synthesis and function of pro-inflammatory cytokines in multiple states by increasing the expression of endogenous anti-cytokines and down-regulating the receptors for pro-inflammatory cytokines. It has been realized that low levels of IL-10 and other anti-inflammatory cytokines such as IL-4 in the blood could be key to chronic pain (Uceyler *et al.,* 2006) while acute administration of IL-10 protein suppressed the development of pain mediated by the spine in different animal models (Wieseler-Frank *et al.,* 2004).

2.8.5 Lipid Mediators (Eicosanoids)

The lipid mediators are generated through the catabolism of arachidonic acid, where the availability of arachidonic acid is a rate-limiting factor to the pathway. Arachidonic acid is produced in all cells from the hydrolysis of membrane phospholipids. It acts as the single but essential substrate to the production of active mediators of inflammation known as eicosanoids. Eicosanoids are made up of products of cyclooxygenases; prostaglandins (PG) and thromboxane

(TXA), 5-lipoxygenase (5-LOX); leukotrienes and 5- hydroxyeicosatetraenoic acid and 12-lipoxygenases(12-LOX); 12-hydroxyeicosateteraenoic acid (Borgeat and Samuelsson, 1979).

2.8.5.1 Prostaglandins and inflammation

Prostaglandins are twenty-carbon fatty acid derivative formed from the enzymatic metabolism of arachidonic acid through the enzyme cyclooxygenases (COXs). They act generally in both autocrine and paracrine manner and mediate various physiological processes including mucosal defense. Prostaglandins released in the stomach stimulate mucus and bicarbonate secretion, increase mucosal blood flow and resistance of cytotoxin-induced injury to epithelial cells. As a result, the inhibition of prostaglandins especially in the stomach can be very detrimental and a critical event to consider in the development of pathophysiological conditions related to the stomach as those elicited by the NSAIDs administration.

There are two isoforms of the COXs namely COX-1 and COX-2 which are encoded by different genes. COX-1 is constitutively expressed and is known to generate basal levels of prostanoids in order to maintain homeostasis (Funk *et al.*,1991). On the contrary, COX-2 is only found in many cells associated with inflammation such as macrophages, endothelial cells and fibroblast and is expressed upon stimulation (Hla *et al.*, 1993). Tumour and transformed cells also express high level of COX-2 and so have increased levels of prostaglandins (Lupulescu, 1996). COX-2 for this reason has important role to play in inflammation and carcinogenesis, hence its regulation may be relevant and very promising to avoiding diseases associated to inflammation.

Regulation of COX-2

COX-2 can be regulated at the transcriptional level. The *cis*-acting elements present in the promoter region of the COX-2 gene include NF-kB site, CCAAT/enhancer-binding protein

(C/EBP) site and cyclic AMP-response element (CRE) site which are critical in the regulatory process. The NF-kB pathway leads to the activation of transcriptional factor NF-kB upon LPS-stimulation and mediates the activation of many cytokines and other products of inflammation. It has been reported that, the inhibition of NF-kB impairs transcription of COX-2 mRNA (D'Acquisto *et al.*, 1997; Inoue and Tanabe, 1998). The C/EBP is a family of proteins (transcriptional factors) which interacts with the CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine) box motif present in the promoter region of the COX-2 gene (Sorli *et al.*, 1998). CRE is also essential for both basal and induced COX-2 transcriptional levels in most cells by binding to cyclic AMP response element binding proteins (CREB) and activator protein-1 (AP-1) (Wadleigh *et al.*, 2000).

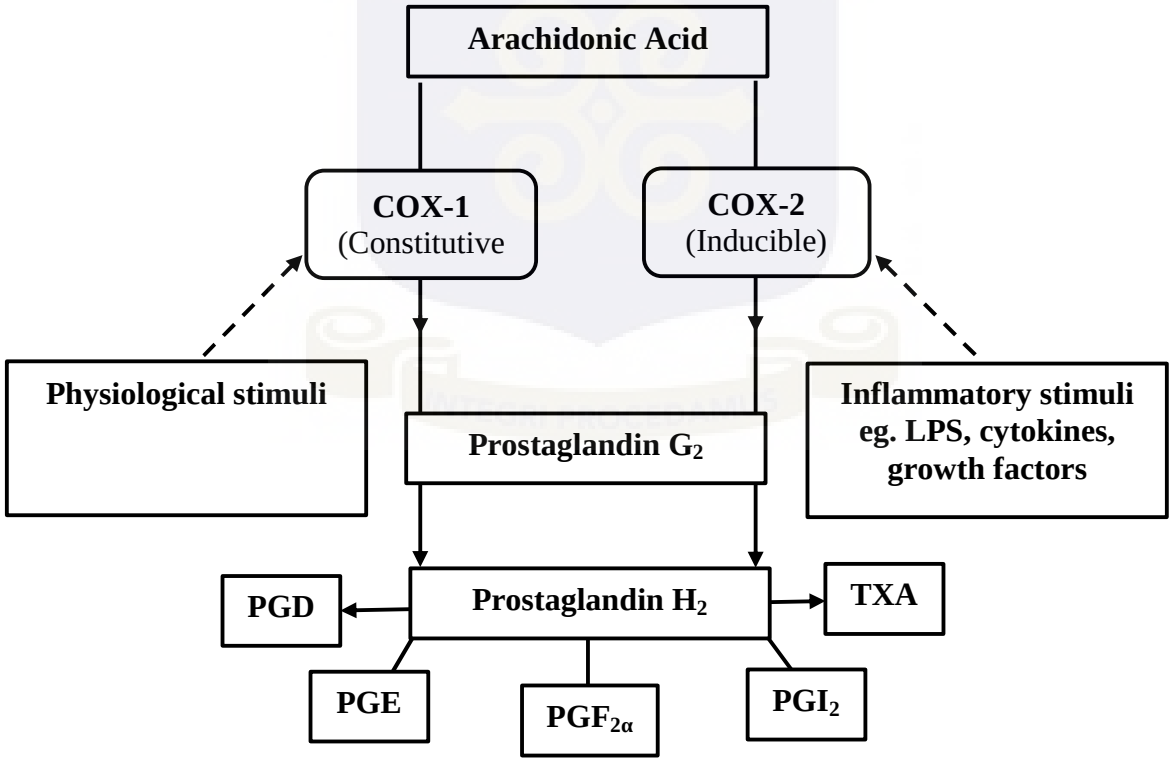


Fig. 2.3: The metabolic pathway of arachidonic acid mediated by COX-1 and COX-2. (Uto *et al*., 2012).

2.8.6 Free Radicals

Molecular species which are independent in their existence because they contain in their atomic orbital unpaired electrons are classified as free radicals. Cellular activity requires oxygen to generate energy in the form of adenosine triphosphate (ATP) by the mitochondrial complexes through the electron transport chain (ETC) system. Consequently, in the process, free radicals are generated as by products which include reactive oxygen species (ROS). Although these species can be both beneficial and detrimental to the organism, a balance between the two effects may be crucial to the life of an organism. Thus, ROS may be beneficial at lower or moderate levels as far as cellular activity is concerned. However, they exert a deleterious effect at increased levels by causing oxidative stress (Halliwell and Gutteridge, 2007).

2.8.6.1 Reactive oxygen species (ROS) and inflammation

It is estimated that at least 5% of the oxygen inhaled into the human system is channeled into ROS generation (Trenam *et al.,* 1992). This means that cells under aerobic condition are likely to experience the toxic insults of ROS. Superoxide anion (O_2.), oxygen singlet ($^1O_2$), hypochlorite (HOCl), hydroxyl radical (OH.), nitric oxide (NO.), peroxy-nitrite radical ($ONOO^-$), hydrogen peroxide (H_2O), among others are some potent examples of ROS. The level of stability of these species determines the degree of reactivity (Aitken and Fisher, 1994).

Even though endogenous systems may be available to cater for ROS without the cell being affected, any imbalance between ROS generated and the available endogenous antioxidant system may put the cell under stress resulting in oxidative damage as observed in most diseases associated with inflammation. Mononuclear cells such as macrophages and lymphocytes as well as polymorphonuclear leukocytes like neutrophils and eosinophils, due to their phagocytic action tend to generate excessive quantity of ROS, which is essential to combat any microbial attack.

The excessive ROS generated in the process affect cellular function and results in cell or tissue damage by elevating the level of inflammation (Trenam *et al.,* 1992) leading to pathological states like cancer (Wiseman and Halliwell, 1996), atherosclerosis (Witztum, 1994), neurodegenerative disorders (Lebovitz *et al.,* 1996), Parkinson disease (Jenner, 2003), rheumatoid arthritis (Halliwell, 1995) and premature aging (Orr and Sohal, 1994). ROS act as activators of immune cells monocytes/macrophages specially to synthesize TNF-α, IL-1β and IL-6 to promote inflammation (Beckman and Koppenol, 1996), which initiate the inflammatory cascade.

Nitric Oxide (NO) and inflammation

It was reported that intact endothelium is required for the relaxation of blood vessels in response to acetylcholine (Furchgott and Zawadzki, 1980) and the endothelium-derived relaxing factor (EDRF) responsible was identified as NO or its derivatives (Palmer *et al.,* 1987). NO is a signaling molecule which plays a significant role in regulation of physiologic processes including vasodilation, neurotransmission and inflammation (Prast and Philippu, 2001; Ignarro, 2002).

Some biological importance of NO such as killing of tumour cells, defense of host system against intracellular pathogens, inhibition of platelet aggregation and acting as a neurotransmitter (Moncada *et al.,* 1991; Alderton *et al.,* 2001) cannot be overemphasized. However, excess production of NO in a biological system has long been identified as a potent mediator of inflammation (Korhonen *et al.,* 2005). Excessive NO levels can also affect the DNA of an organism by direct or indirect damage through several mechanisms (Tamir *et al.,*1996), interfering with their repair (Jaiswal *et al.,* 2000) and also causing nitrosylation of DNA, which is a good post-translational modification to potentially initiate and drive tumourigenesis (Wink *et*

al., 1998), and hence cancer. Under chronic conditions sustained and high levels of NO could confer multiple damages and lead to accumulation of mutated genes such as that of the tumour suppressor gene p53 (Forrester *et al.,* 1996; Chazotte-Aubert *et al.,* 2000) which contributes to malignant transformation. As a result, NO has been described as endogenous mutagen (Wink *et al.,* 1998), inhibitor of apoptosis and oncogene enhancer (Li *et al.,* 1997; Ambs *et al.,* 1998).

2.8.7 Enzymes Involved in NO Synthesis

Endogenous synthesis of NO in living systems is mediated by the different isoforms of nitric oxide synthases (NOSs) through the oxidative deamination of L-arginine (Sharma *et al.,* 2007) to citrulline and then the consequent release of NO. The three isoforms of NOS include endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS). Both eNOS and nNOS are constitutively expressed in endothelial and neuronal tissues, whilst iNOS is inducible mostly in macrophages, hepatocytes (Kupffer cells) and endothelial cells during inflammation (Nathan and Xie, 1994; Alderton *et al.,* 2001). Excess generation of NO has been attributed to the activation of iNOS, which is believed to play a key role in inflammation (Maeda and Akaike, 1998). Unlike eNOS and nNOS, iNOS is calcium-independent and can easily be activated by the release of proinflammatory cytokines such as IL-1,TNF-α, IFN-γ and also other endotoxins such as lipopolysaccharides (LPS) (Nakayama *et al.,* 1992).

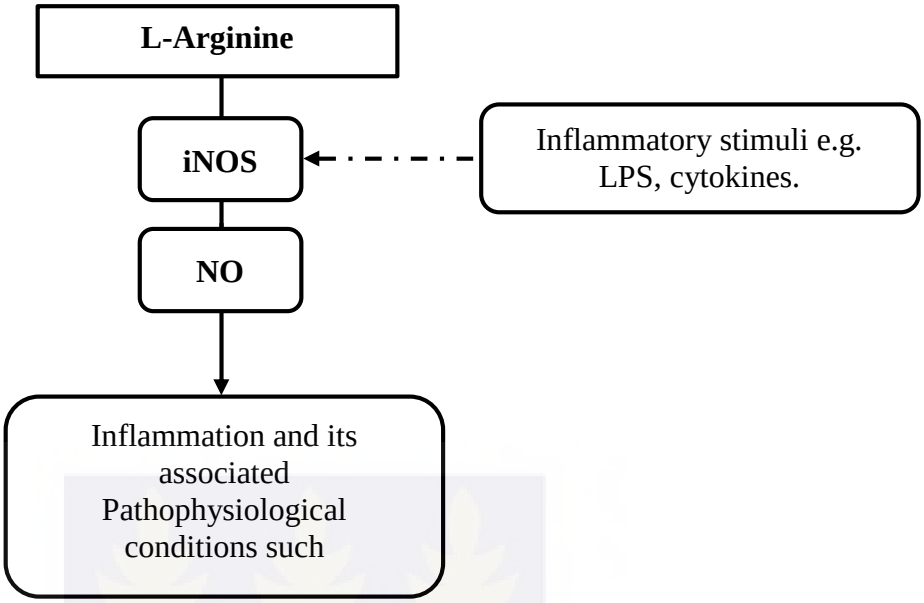
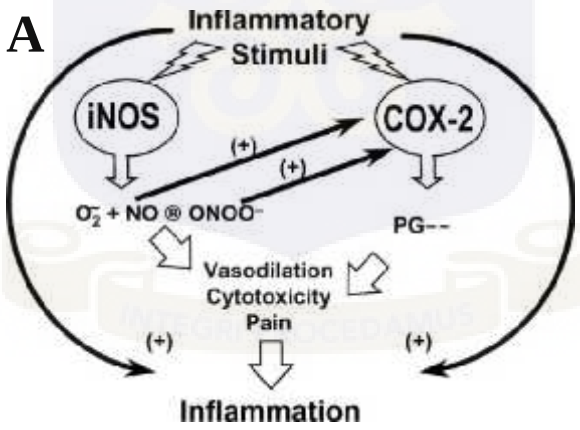


Fig 2.4: Generation of NO catalyzed by iNOS. (Uto *et al*., 2012)

2.8.7.1 Regulation of iNOS synthesis

The active form of iNOS with catalytic activity is a homodimeric protein of approximate size of 260 kD (Mollace *et al.,* 2005). The regulation of NO production by iNOS is mainly at the transcriptional level and its mRNA stability (Xie *et al.,* 1993). This is because the mechanism of iNOS activation involves *de novo* transcription and biosynthesis of new protein. Thus, transcription may occur through binding of transcriptional factors, including activator protein-1 (AP-1) and nuclear factor kappa-B (NF-kB) to the *cis*-acting elements of the promotor region (Marks-Konczalik *et al.,* 1998). AP-1 is a transcriptional factor, minimally activated under normal physiologic conditions but dramatically activated under inflammatory stimuli including that of bacterial LPS (Guha *et al.,* 2001). NF-kB represents another transcription factor to regulate the iNOS gene and is activated by inflammatory responses elicited during viral and bacterial infections (Marks-Konczalik *et al.,* 1998).

There is about 66% homology between murine and human iNOS at the 5' flanking region, which has sequences conserved for binding transcriptional factors like that of NF-kB (Chartrain *et al.*, 1994). In a review by Mollace *et al*. (2005), it was reported that further release of NO during inflammation can activate the NF-kB pathway and lead to the co-activation of COX-2 (Fig 2.4). Similar immunological stimuli which activate iNOS are also found to induce guanosine triphosphate cyclohydrolase. Guanosine triphosphate cyclohydrolase is an enzyme which synthesizes and supply iNOS with the cofactor tetrahydrobiopterin (BH4) (Salvemini and Masferrer, 1996). However, many antioxidants (e.g. pyrrolidine-dithiocarbamate and diethydithiocarbamate), glucocorticoids such as dexamethasone, thrombin, macrophage deactivation factor, TNF-α, platelet-derived growth factor, IL-4, IL-8 and IL-10, all inhibit iNOS induction (Di Rosa *et al.*, 1990; Mollace *et al.*, 2005).



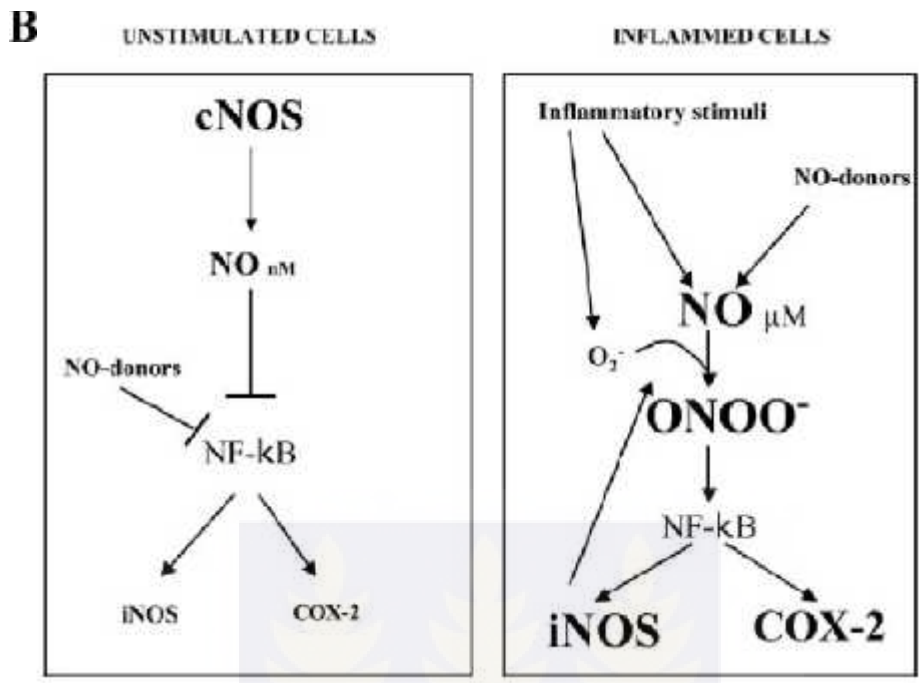


Fig. 2.5 Co-activation of COX-2 by iNOS. Increased NO levels by iNOS during inflammation co-activates COX-2 (**A**) through the NF-κB pathway (**B**). (Mollace et al., 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

Female Sprague-Dawley Rats (SDRs) of weight 180-200 g were acquired from the Animal Experimentation Session of the Centre for Plant Medicine Research (CPMR), Mampong-Akwapem, Ghana. Animals were fed *ad libitum* with feed obtained from Ghana Agro Food Company, Tema, Ghana and housed in metallic cages lined with soft wood shavings as beddings. The animals were kept under standard laboratory conditions (temperature 28 ºC ± 2 ºC, relative humidity 60-70 % and normal 12 h cycle of light and dark) and allowed access to sterilized drinking water *ad libitum.* This animal study was approved by the Ethics Committee of the CPMR, Mampong-Akwapem, Ghana. The animals were handled in accordance with internationally accepted principles of laboratory animal use and care (EEC Directive of 1986: 86/609 EEC).

3.1.2 Plant Collection

Dried leaves of *Morinda lucida* Benth were obtained from the Plant Development Department (PDD) of the CPMR, Mampong-Akwapem, Ghana. It was authenticated by a Senior Botanist in the department and a voucher specimen number CSRPM/8510 was assigned and kept at the herbarium of the PDD.

3.1.3 Chemicals and Reagents

Antibodies against COX-2 and iNOS were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fetal bovine serum (FBS) came from Equitech-Bio (Kerrville, TX). LPS

(*Escherichia coli*) was purchased from Sigma-Aldrich (St. Louis, Island, NY). Carrageenan, diclofenac, histamine and serotonin were obtained from Sigma-Aldrich (St. Louis, USA). Absolute ethanol and methanol were purchased from Hayman Limited (Loughborough, England, UK). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Fluka BioChemika (Buchs, Switzerland). TLC plates (Kieselgel 60 F_{254}) were purchased from Merck, Damstadt, Germany.

3.2. METHODS

3.2.1 Extract Preparation

The air-dried pulverized leaves of *M. lucida* Benth (500 g) were cold-macerated in 5 L of 70 % v/v ethanol-water for 72 h with periodic stirring. It was then filtered and the crude extract obtained was concentrated under low temperature (40 ºC ± 2 ºC) and reduced pressure using the rotary evaporator (EYELER) to remove ethanol. The residue was re-extracted with the 70% ethanol three times to ensure exhaustive extraction. The aqueous-based concentrated crude extract was freeze-dried and the powdered extract was labelled as hydroethanolic extract of *M. lucida* (*HEML*) and kept until used in an air-tight desiccator, with silica gel as desiccant, in a cool dry place. This was to prevent possible absorption of moisture, which may facilitate the growth of molds and compromise the general stability of the extract.

3.2.2. Phytochemical Screening of *HEML*

Some phytochemical constituents present in *HEML* were ascertained with reference to methods described previously by Trease and Evans (1989), in which 10 cluster of compounds screened for were grouped as hydrophilic or lipophilic.

3.2.2.1. Qualitative phytochemical screening for hydrophilic constituents

The *HEML* (2 g) was reconstituted in 20 ml of distilled water, sonicated to dissolve and aliquoted for testing for the presence of hydrophilic phyto-constituents qualitatively.

Test for saponins

An aliquot (2 ml) of the reconstituted extract in water was shaken for 30 seconds to 1 minute. The formation of a froth which persisted for about 10 minutes indicated a positive test for saponins.

Test for reducing sugars

Equal volumes of Fehling's solutions A and B (1 ml each) were mixed freshly and added to 2 ml aliquot of *HEML*. The mixture was heated for 10 minutes in a water bath and the formation of a brick-red precipitate was indicative of a positive test for reducing sugars.

Phenolic compounds

To 2 ml of reconstituted extract was added 2-3 drops of ferric chloride ($FeCl_3$). A blue-black coloration indicated the presence of phenolic compounds.

Test for cyanogenic glycosides

To an aliquot of the reconstituted extract (2 ml) was added 4 ml of chloroform. A strip of filter paper stained in picric acid was placed at the neck of the test tube containing the mixture making sure that it did not touch the contents of tube. The tube was plugged loosely with a clean cotton wool to keep the picric paper firmly in place and to prevent any possible extrusion of mixture during heating. The preparation was heated on a water bath for about an hour. A change in color of picric paper from yellow to brownish-red was indicative of a positive test for cyanogenic glycosides.

Test for alkaloids

An aliquot (5 ml) of the reconstituted extract was basified with 1 ml of 25% ammonia (NH_3) solution in a separating funnel. An equal volume of chloroform was added, shaken and left to settle. The chloroform layer was collected into a test tube and evaporated off on water bath. The residue was reconstituted in 5 ml of 2 M HCl and filtered. Mayer's reagent (1 ml) was added and the appearance of a creamy precipitate was indicative of a positive test for alkaloids.

Test for polyuronides

The presence of violet or blue precipitate upon addition of about 2-3 drops of acetone to 2 ml of reconstituted *HEML* was indicative of a positive test.

3.2.2.2 Qualitative phytochemical screening for lipophilic constituents

Dry extract of *HEML* (10 g) was suspended in 100 ml of distilled water and sonicated for 5 minutes to dissolve. The reconstituted extract was refluxed on water bath for 2 h after adding 10 ml of 2 M HCl to extract lipids. The refluxed solution of *HEML* (5 ml) was shaken with 20 ml of diethyl ether in a separating funnel and left to settle. The inorganic layer was carefully siphoned off, leaving the organic layer (diethyl ether) which was screened for lipophilic phytochemicals.

Test for flavonoids

About 4 ml of the diethyl ether layer were evaporated on a water bath to dryness. The residue was dehydrated using anhydrous Na_2SO_4, reconstituted in 8 ml methanol and divided into two portions of 4 ml each. Magnesium (Mg) ribbon and concentrated HCl were added to one portion. A reddish or pinkish coloration indicated the presence of flavonoids.

Test for phytosterols and triterpenes

About 4 ml of the diethyl ether layer was aliquoted into a test tube and placed on a water bath to evaporate completely the diethyl ether. Anhydrous Na_2SO_4 was added to absorb any moisture present. The residue was dissolved in equal volumes of chloroform and acetic anhydride (1:1). Drops (2-3) of concentrated H_2SO_4 were added to the mixture. A green coloration indicated the presence of phytosterols but a wine coloration indicated the presence of triterpenes. A brownish layer dividing the content of the test tube indicated the presence of both phytosterols and triterpenes.

Test for anthraquinones

To 2 ml aliquot of the diethyl ether layer was added 1-2 drops of 25% NH_3 solution (v/v). A red coloration was a positive test.

3.2.2.3 Thin layer chromatography profiling of *HEML*

The hydroethanolic extract obtained was spotted both on a normal and reverse phase silica gel coated on aluminum sheet and dried with a hand drier (Revlon Handheld drier, RVDR5034, USA). The normal phase (NP) TLC plate was run with chloroform/methanol (7:1 v/v) and the reverse phase (RP) TLC plate was run with methanol/water (2:1 v/v). Both plates were developed with 10% (v/v) H_2SO_4 and heated at 110 ºC for visualization and the retention factor (Rf) was calculated as:

$$Rf = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by solvent (solvent front)}}$$

3.2.3 Determination of Antioxidant Property of *HEML*

3.2.3.1 Quantification of total phenolic content

Total phenolic compounds or polyphenols present in *HEML* was measured using the Folin-Ciocalteau (FC) colorimetric method (Pattanayak *et al.*, 2012). Gallic acid was used to plot a standard curve (Appendix I) from which the total phenolic compounds present in the extract was estimated under the same conditions. In brief, 10 µl of 5 mg/ml of *HEML* dissolved in distilled water was mixed with 0.79 µl of distilled water and 50 µl of FC reagent in a 96 well plate. After 8 minutes of incubation, 150 µl of 0.09 M Na_2CO_3 was added and incubated for further 2 h at room temperature. The absorbance of the mixture was read at 750 nm with a microplate reader (Infinite m200pro TECAN, Grodig, Austria) and in triplicate. Results were expressed as microgram of Gallic acid equivalent (GAE) per milligram of dry sample.

3.2.3.2 Quantification of total flavonoids

The total flavonoid content of the *HEML* was estimated by colorimetry (Pattanayak *et al.*, 2012). Quercetin was used to plot a standard curve (Appendix II) from which the total flavonoid content present of the extract was estimated under the same conditions. About 100 µl *HEML* (5 mg/ml in methanol) were added to 100 µl of 2% $AlCl_3$ (w/v) in methanol and incubated for 20 minutes at room temperature. The absorbance of the mixture was read at 415 nm using a microplate reader and in triplicate. The total flavonoid content was expressed as microgram of quercetin present per milligram of dry sample.

3.2.3.3 DPPH free-radical scavenging activity

The free radical scavenging activity of *HEML* was determined according to the method described previously by Appiah-Opong *et al.* (2015). About 100 µl of 2, 2-diphenyl-1-picryl hydrazyl

(DPPH) in methanol (0.5 mM) was added to 100 µl each of varying concentrations of *HEML* and allowed to stand for 20 minutes at room temperature (25 ºC) in the dark. Absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) (0.97-500 µg/ml) was used, under identical conditions, as a positive control. Percentage antioxidant was determined by the equation:

$$\% \text{ Antioxidant} = \frac{(A - B)}{A} \times 100\%$$

Where A optical density of blank, and B is the optical density of sample.

The concentration of *HEML* or positive control (BHT) at 50% antioxidant activity was determined; effective concentration at 50% value (EC_{50}). The experiment was carried out in triplicate.

3.2.4 Determination of Median Lethal Dose (LD_{50})

The acute toxicity of *HEML* was explored using a standard scale described by Hodge and Sterner, (2005). A single dose of 5000 mg/kg *HEML* was administered orally to female SDRs of body weight 180-200 g (n=6). The rats were observed over 48 h period for number of deaths and general behavior. Surviving animals were observed for additional 12 days for signs of toxicity such as abnormalities in feeding, breathing, locomotion, lachrymation, and pilo-erection.

3.2.5 Effect of *HEML* on Carrageenan-induced Paw Edema

The effect of *HEML* was evaluated using the method described previously by Winter *et al*. (1962) with slight modifications. Female SDRs of body weight 180-200 g were grouped into 5 different metallic cages with each cage containing 6 animals (n=6). The initial paw volume of the right hind limbs of the animals was taken at baseline using the plethysmometer (7140, UGO Basil Ltd, Camerio VA, ITALY). Sterile carrageenan, 0.1 ml 1% (w/v) in normal saline, was injected (s.c) into the sub-planter tissue of the right hind paw of each rat. The paw volume of the

right hind paw of the rats was taken after 30 minutes to ascertain some level of edema after which drug/extract was administered. Paw edema was monitored 1 h post drug/extract administration at 1 h interval for 4 h. Treatment groups received either diclofenac (100 mg/kg) as positive control or *HEML* (100, 300 and 600 mg/kg) while control rats received sterilized water. Percentage change in paw edema was calculated in both controls and treatment groups as follows:

$$\% \text{ Change in paw edema} = \frac{(V_t - V_o)}{V_o} \times 100$$

Where V_t is the paw volume at time *t*, V_0 is the paw volume at baseline.

3.2.6. Anti-histaminic Activity of *HEML*

The anti-histaminic effect of *HEML* was assessed in a histamine-induced paw edema assay using histamine as a phlogistic agent in a method described earlier (Singh and Pandey, 1996). Freshly prepared histamine (1% w/v dry histamine in saline, 0.1 ml) was injected (s.c) into the sub-planter tissue of the right hind paw of the SDRs (180-200 g, n=6) to cause edema formation. Edema was monitored after the first 30 min and then 1 h interval for 4 h using a plethysmometer (7140, UGO Basil ltd, Camerio VA, ITALY) after edema induction. Control animals received distilled water (1 ml) while treated groups received chlorpheniramine (CPM, 4 mg/kg) or *HEML* (100, 300 and 600 mg/kg) orally 1 h prior to induction of edema. Paw edema was calculated as described in section 3.2.5.

3.2.7 Anti-serotogenic Activity of *HEML*

The effect of *HEML* was assessed in serotonin-induced paw edema assay, where serotonin was used as a phlogistic agent in a method described earlier (Singh and Pandey, 1996). Freshly prepared serotonin (1% w/v serotonin in saline, 0.1 ml) was injected (s.c) into the sub-planter tissue of the right hind paw of each SDR (180-200 g, n=6) to induce edema. Paw edema was

monitored post induction at first 30 min and then at 30 min intervals for 4 h using a plethysmometer (7140, UGO Basil Ltd, Camerio VA, ITALY). Control animals received distilled water (1 ml) while treated groups received serotonin antagonist Granisetron (0.1 mg/kg) as positive control or *HEML* (100, 300 and 600 mg/kg) orally 1 h prior to inducing edema. Paw edema was calculated as described in section 3.2.5.

3.2.8 Anti-pyretic Activity of *HEML*

The disruption of *E. coli* cell wall to expose lipopolysaccharide (LPS) was done according to methods described previously (Rezania *et al.*, 2011) with slight modifications. *E. coli* (ATCC25922) suspension containing 1.29 x 10^9 cells was obtained from the Microbiology Department of CPMR. It was centrifuged at 10,000×g for 5 minutes and the pellet was washed twice and re-suspended in 5 ml of 0.5 M PBS (pH=7.2) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The mixture was sonicated on ice for 15 minutes. The anti-pyretic effect of *HEML* was assessed using method described earlier (Santos and Rao, 1998) with slight modifications. Briefly, female SDRs were fasted overnight. The rectal temperature of the animals was taken in a temperature controlled room at 25± 2ºC prior to injection of LPS exposed from *E. coli* cell wall in PBS (15 mg wet weight *E. coli* per ml of PBS; i.p) with a digital thermometer. The rectal temperature of the test animals was taken after 30 min of LPS injection and animals with differences in temperature were randomly selected into five different groups (n=6). The groups were treated orally with or without *HEML* at 100, 300 and 600 mg/kg or acetaminophen at 150 mg/kg suspended in distilled water. The control group received only distilled water. The rectal temperatures of the rats were monitored at 1 h intervals for 4 h after *HEML* or acetaminophen treatment. The percentage change in temperature was calculated as:

$$\% \text{ Change in Temperature} = \frac{(T_1 - T_0)}{} \times 100$$

T_0

Where T_0 is the basal temperature reading and T_1 is the temperature reading at each hour.

3.2.9 Cell Culture

Murine macrophage-like RAW 264 cells were obtained from the Clinical Pathology Department, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon. The cells were cultured in a humidified incubator at 37°C in 5% CO_2 using Dulbecco's modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution. To eliminate LPS and growth factors interferences on the true features of macrophages, all experiments were carried out under serum-free conditions.

3.2.10 Cell Survival Assay

Cell viability was determined by MTT assay (Uto *et al.*, 2012). RAW 264.7 cells (2×10^4 cells/ well) were seeded in 96 well micro-titer plates and incubated for 24 h, after which cells were starved by culturing in serum-free medium for another 2.5 h to eliminate the influence of FBS. They were then treated with or without 50, 16.67, 5.56, 1.85 and 0.62 mg/ml of *HEML* or sulforuphane (SFN) and incubated for 30 min. The cells were stimulated with or without 50 ng/ml LPS and incubated for 12 h. To each well, 20 µl of MTT solution in PBS was added and further incubated for 4 h to generate formazan product. The formazan product was made soluble by adding 150 µl of acidified isopropanol (0.04 N HCL-isopropanol) in 1 % v/v Triton-X and incubated overnight in the dark. The levels of formazan product generated was determined by measuring absorbance at 570 nm using a microplate reader (Infinite m200pro TECAN, Grodig, Austria). The data generated was presented as a ratio of the optical density of treatment group to that of the control group. Percent cell survival was evaluated using the formula:

$$\% \text{ Cell survival} = \frac{(A_1 - A_o)}{A_2} \times 100$$

Where A_1 = the absorbance of the test experiment (cells pretreated with *HEML* or SFN), A_O = the absorbance of the test control (reaction mixture with test sample but without cells) and A_2 = the absorbance of the negative control (untreated cells).

3.2.11 Measurement of Cellular Nitric Oxide (NO) Levels

The concentration of nitric oxide in culture supernatant was determined by measuring the concentration of nitrite produced in an assay using the Griess reaction (Archer, 1993). RAW 264.7 cells (1 x 10^6 cells/well) were seeded in a 48-well plate and left overnight for attachment. The cells were then starved for 2.5 h by aspirating the culture medium and replacing with serum-free medium just to eliminate FBS influence. The cells were treated with or without *HEML* (50, 16.67, 5.56, 1.85 and 0.62 mg/ml) for 30 min and stimulated with 50 ng/ml of LPS and incubated for further 12 h. The cell culture supernatant was transferred into microtubes. Equal volumes of cell culture supernatant (100 µl) were mixed with the Griess reagent (1% w/v sulfanilamide in 5% v/v phosphoric acid and 0.1% w/v naphthylethylenediamine dihydrochloride in water) in a 96 well plate and incubated for 10 min. The absorbance was read at a wavelength of 550 nm using (Infinite m200pro TECAN, Grodig, Austria). Concentration of nitrite generated in cell culture supernatant was estimated by extrapolation from a standard curve (Appendix III) plotted with the optical density of sodium nitrite at concentrations (0, 1.25, 2.5, 5, 10, 20, 40 µM).

3.2.12 Determination of PGE_2 in Cell Culture Supernatant

The levels of PGE_2 in cell culture supernatant was determined using PGE_2 enzyme-linked immunoassay (ELISA) kit (R&D systems, USA) and according to the manual provided by the manufacturer. In brief, RAW 264.7 cells (1 x10^6 cells) were seeded in 6 cm^3 petri dish overnight and incubated for 24 h. Cells were then starved by replacing spent medium (DMEM) with a

serum-free medium and incubated for 2.5 h, just to avoid the interferences FBS. Cell treatment was done with or without *HEML* at 16.67, 5.56, 1.85 µg/ml or SFN at 40 µM (positive control) for 30 min before stimulation with 50 ng/ml LPS for 12 h. Culture supernatant was removed and the quantity of PGE_2 released was determined by reading absorbance at 450 nm with a microplate reader (Infinite m200pro TECAN, Grodig, Austria). The concentration of PGE_2 was extrapolated from a PGE_2 standard curve (Appendix V).

3.2.13 Determination of Cytokine Levels in Culture Medium

The concentrations of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-10) cytokines released into the cell culture medium were determined using sandwich enzyme-linked immunoassay (ELISA) kit (R&D systems, USA) and according to the manual provided by the manufacturer. In brief, RAW 264.7 cells (1 $x10^6$ cells) were seeded in 6 cm^3 petri dish overnight and incubated for 24 h. Cells were then starved by replacing spent medium (DMEM) with a serum-free medium for 2.5 h, just to avoid the influence of FBS. Cells were then treated with or without *HEML* at 16.67, 5.56, 1.85 µg/ml or SFN at 40 µM for 30 min before stimulated with 50 ng/ml LPS for 12 h. Culture supernatant was removed to determine the amounts of IL-1β, TNF-α and IL-10 by reading absorbance at 450 nm with an Ultra Microplate Reader ELx 808IUI (Bio-Tek Instruments, Inc, USA) with their concentrations estimated from their respective standard curves (Appendices VI, VII and VIII).

3.2.14 Western Blot Analysis

Western blot analysis was performed according to methods described previously by Hou *et al.* (2004). Cultured cells (RAW 264.7, 1 $x10^6$ cells) starved in serum-free medium were incubated with or without *HEML* at 16.67, 5.56, 1.85 µg/ml or SFN at 40 µM for 30 min before exposure to 50 ng/ml of LPS for 12 hrs. The cells were removed from medium, washed with phosphate

saline buffer (PBS), harvested and lysed using a RIPA buffer. Cell lysate was centrifuged at 10,000 g for 10 min at 4 ºC and the supernatant was collected. Protein concentration in the lysate (supernatant) was determined from a BSA standard curve (Appendix IV) and according to method described by (Bradford, 1976). Cell lysates (supernatant) were denatured at 70 ºC for 10 minutes in SDS sample buffer and equal protein (40 µg/ml) was loaded and run using Invitrogen NuPAGE 12 % Bis-Trisgel (SDS-PAGE). The proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore, USA) to blot. The membrane after blotting was incubated overnight at 4°C with specific primary antibody; anti-goat iNOS or COX-2 at 1:200 dilutions. It was further incubated for an hour using a secondary antibody anti-goat horseradish peroxidase (HRP) antibodies at 1:2,000 dilution. Substrate solution (Immobilon Western; Millipore, USA) was added to the membrane. A camera system EzCapture II coupled with ATTO cooled charge device (CCD) (ATTO Corporation, Japan) was used for the detection of bound antibodies. The intensities of the bands were quantified using Image J software. The band intensities of the individual samples were normalized to that of the internal control (Actin bands). The relative density of the bands (cells treated *HEML*/SFN and exposed to LPS) were determined with respect to the negative control (cells exposed to only LPS).

3.2.15 Statistical Analysis

Significant variations between all treatment and untreated groups were performed using one-way analysis of variance (ANOVA). In order to identify where significance lies, Newman-Keuls post-test was performed just after the ANOVA. P values of ≤ 0.05 were considered significant. All graphs and analysis were done using GraphPad prism software version 5.01.

CHAPTER FOUR

4.0 RESULTS

4.1. EXTRACT YIELD

The aqueous-based concentrated crude extract prepared from 500 g of air-dried pulverized *M. lucida* leaves yielded 46.04 g of powdered extract. This represents 9.21 % (w/w) yield.

4.2. PHYTOCHEMICAL SCREENING

Phytochemical screening of *HEML* showed the presence of saponins, reducing sugars, polyphenols and flavonoids but the absence of polyuronides, cyanogenic glycosides, alkaloids, antraquinones, triterpenes and phytosterols (Table 4.1).

Table 4.1: Some groups of phytochemicals in *HEML*

GROUP OF PHYTOCHEMICALS	RESULT
Saponins	Present
Polyuronides	Absent
Cyanogenic Glycosides	Absent
Reducing Sugars	Present
Alkaloids	Absent
Polyphenols	Present
Antraquinones	Absent
Flavonoids	Present
Triterpenes	Absent
Phytosterols	Absent

4.3 TLC PROFILING OF *HEML*

The TLC profile of *HEML* was displayed on both normal (Plate A) and reversed (Plate B) phase silica gels coated on aluminum sheet to reveal the possible non-polar and polar constituents of the extract (Fig. 4.1). There were 8 spots with the following Rf: 0.92, 0.87, 0.77, 0.75, 0.74, 0.66, 0.40 and 0.26 when separated on the normal phase and 7 spots with the following Rf: 0.87, 0.78, 0.71, 0.62, 0.20, 0.15 and 0.09 when separated on the reversed phase silica gels.

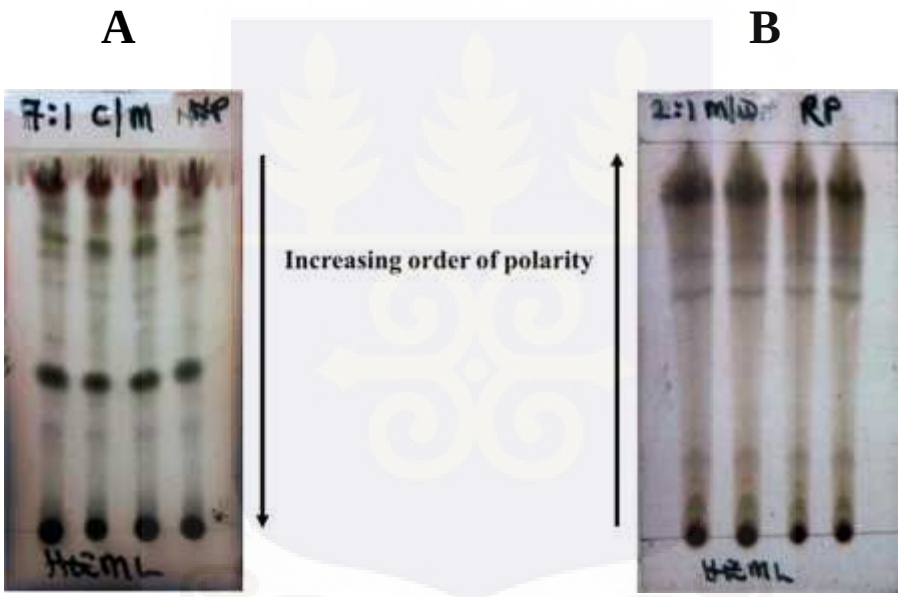


Fig. 4.1: TLC profile of *HEML*: The extract was spotted on both normal phase (NP) plate **A** and reversed phase (RP) plate B. The NP plate was run with chloroform/methanol (7:1) and developed with 10% v/v H_2SO_4 and heated at 110 ºC to display the components (8 spots). The RP plate was run with methanol/water (2:1) and developed with 10% v/v H_2SO_4 and heated at 110 ºC to show the constituents (7 spots).

4.4 TOTAL PHENOLIC AND FLAVONOID CONTENTS/ANTIOXIDANT ACTIVITY OF *HEML*

4.4.1 Total Phenolic Content

The total phenolic present was estimated to be 2.23 mg GAE/100 mg of *HEML* from a standard curve plotted with 0.39- 250 µg/ml of Gallic acid (Appendix I).

4.4.2 Total Flavonoid Content

The total flavonoid content was estimated to be 0.15 mg QE/100 mg of *HEML* from a standard curve plotted with 0.78-50 µg/ml of Quercetin (Appendix II).

4.4.3 DPPH Free Radical Scavenging Activity

The effective concentration at 50% inhibition (EC_{50}) of *HEML* to that of the standard butylated hydroxytoluene (BHT) estimated from Figs. 4.2 A & B were 0.017 ± 0.004 and 0.962 ± 0.024 mg/ml, respectively.

EC_{50}= 0.017± 0.004 mg/ml

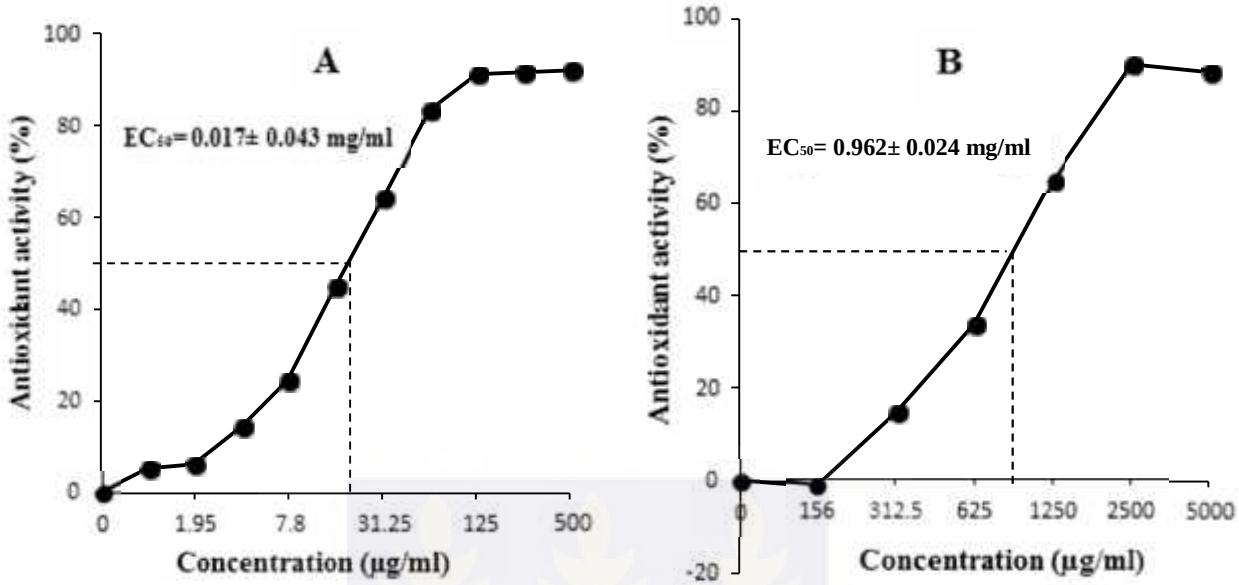


Fig. 4.2: Antioxidant activity expressed as DPPH free radical scavenging activity of (A) *HEML* and (B) BHT. Results are means of triplicate determinations. The EC_{50} value was estimated to be 0.017± 0.004 mg/ml for BHT (**A**) and 0.962± 0.024 mg/ml for *HEML* (**B**).

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

A single oral dose of 5000 mg/kg *HEML* administered to the Sprague- Dawley rats did not cause any mortality after 48 h and 12 days thereafter. There were no observable signs of toxicity since experimental animals showed normal locomotion, breathing, gaiting, lachrymation and no signs of pilo-erection. The median lethal dose of *HEML* was, therefore, estimated to be greater than 5000 mg/kg

4.6 CARRAGEENAN-INDUCED PAW EDEMA IN RATS

There was a 100% increase in paw volume in untreated control animals within 2 hours with a 10% dip by the 4th hour. There was a decrease in paw volume with the different doses of *HEML* in 2 hours (30-50%) with relatively no further change in paw volume in the 300 and 600 mg/kg *HEML* doses by the 4th hour but a steady decline in respect of the 100 mg/kg dose at this hour

(20%). The positive control, diclofenac, showed a trend similar to the *HEML* dose at 100 mg/kg but with slightly higher reductions in paw volume (Fig. 4.3 A). These results are depicted in the areas under the curve (AUC) over the 4-hour period (Fig. 4.3 B), which showed diclofenac with a 50% significant reduction ($p<0.001$) in paw volume followed by *HEML* at 100 mg/kg (40%; $p<0.01$) and 300 mg/kg (35%; $p<0.05$). The reduction in paw volume seen with *HEML* at 600 mg/kg (20%) was not statistically significant ($p>0.05$) from the untreated control.

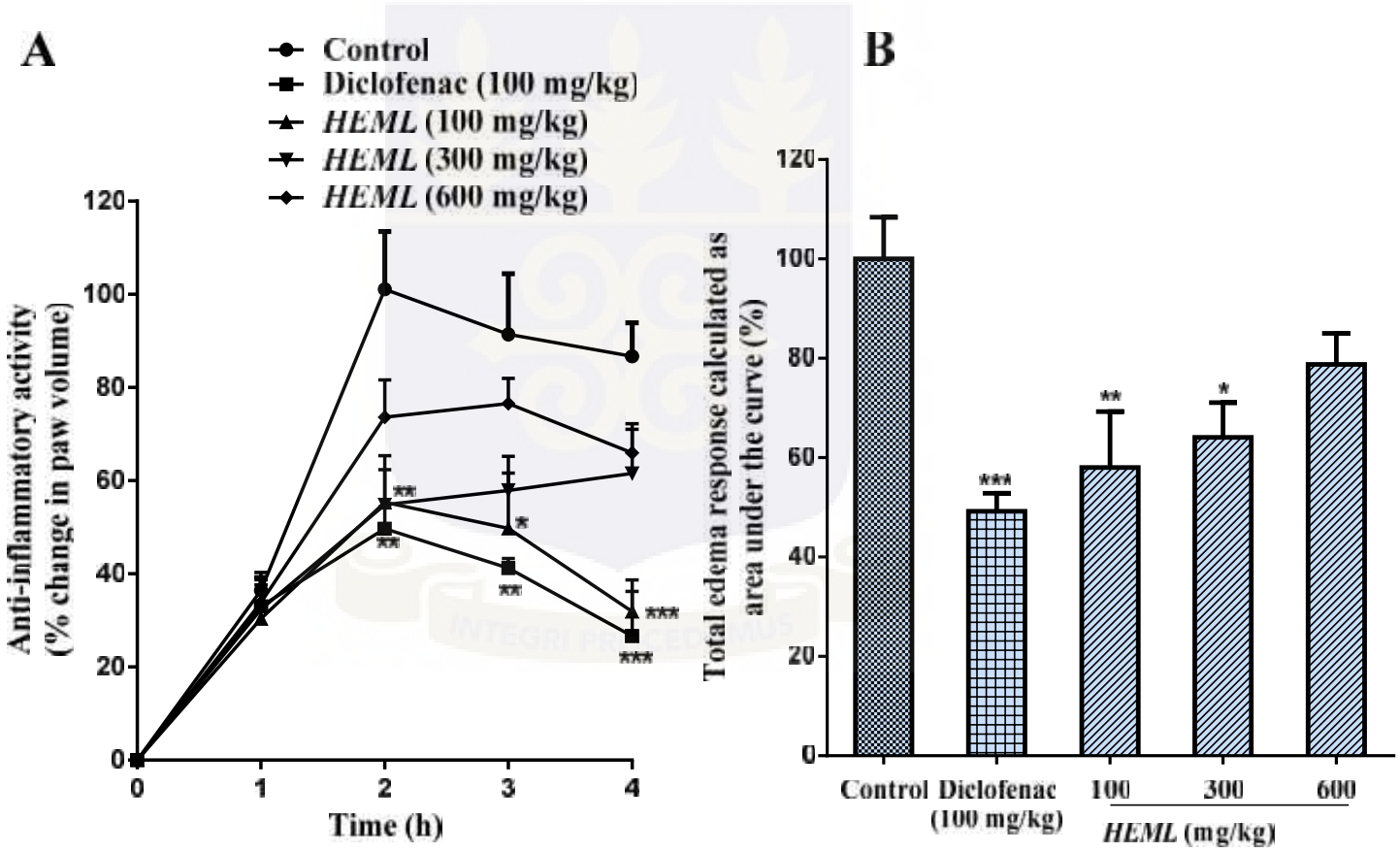


Fig. 4.3: Effect of *HEML* and diclofenac on carrageenan-induced paw edema in rats; (A) time-course curves and (B) areas under the curve (AUC). Data are presented as means ± S.E.M. (n=6). Value statistically significant compared with untreated control; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.7 HISTAMINE-INDUCED PAW EDEMA IN RATS

Pretreatment of animals with or without *HEML* or Chloramphineramine (CPM) as positive control followed by histamine challenge 1 hour later, showed an increase (62%) in paw volume of untreated histamine-challenged control animals relative to normal controls within 30 minutes followed by reduction in paw volume to 50% by 4 hours post-histamine challenge. All the other animal treatment groups showed a similar trend. The changes seen with the *HEML*-treated groups at 300 and 600 mg/kg (60% at 30 minutes to 30-35% at 4 hours) were not significantly different from the untreated control. However, the increase in paw volume seen with *HEML* at 100 mg/kg at 30 minutes after histamine challenge was similar to CPM at 4 mg/kg (42%) which decreased to about 5% by the 4^{th} hour, albeit to a slightly higher degree with CPM within the period (Fig. 4.4A). These changes were reflected in the AUC in which there were significant reductions in paw volume as seen with *HEML* at 100 mg/kg (65%; $p<0.01$) and CPM (75%; $p<0.001$) relative to the untreated control. No significant reductions in paw volume were seen with *HEML* pretreatment at 300 and 600 mg/kg over the period after histamine challenge (Fig. 4.4B).

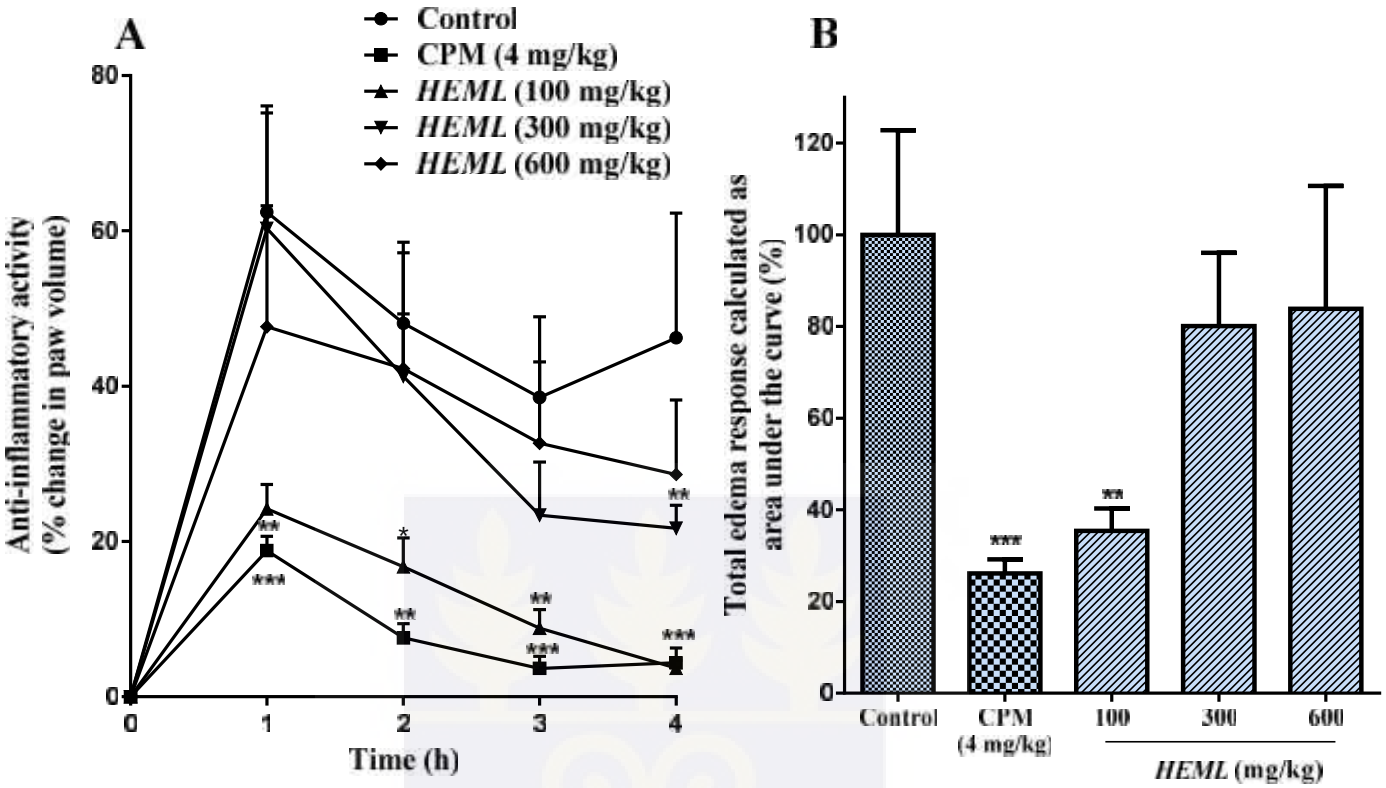


Fig. 4.4: Effects of pretreatment with *HEML*/Chlorpheniramine (CPM) on suppression of histamine-induced paw edema in rats with time post-histamine challenge; (A) time-course curves and (B) areas under the curve (AUC). Data are presented as means ± S.E.M. (n=6). Value statistically significant compared with untreated control; ** $p \leq 0.01$, *** $p \leq 0.001$.

4.8 SEROTONIN-INDUCED PAW EDEMA IN RATS

Pretreatment of animals with or without *HEML* or Granisetron (GRA) as positive control followed by serotonin challenge 1 hour later, showed an increase (25%) in paw volume of untreated serotonin-challenged control animals relative to normal controls within 30 minutes followed by a gradual increase in paw volume to 55% by 4 hours post-serotonin challenge. All the other animal treatment groups showed a first rise in paw volume (22-35%) 30 minutes post-

serotonin challenge, with the *HEML* at 600 mg/kg being 10% higher than the control, followed by a second rise 1 or 1½ hours later. There were gradual declines in these animal treatment groups thereafter to 5-25% change in paw volume (Fig. 4.5A). These results are depicted in the AUC in which there were significant reductions in paw volume seen with *HEML* at 100 and 300 mg/kg (40-42%; $p<0.05$) and GRA (42%; $p<0.05$) relative to the untreated control. No significant reduction ($p>0.05$) in paw volume was observed with *HEML* pretreatment at 600 mg/kg over the period after serotonin challenge (Fig. 4.5B).

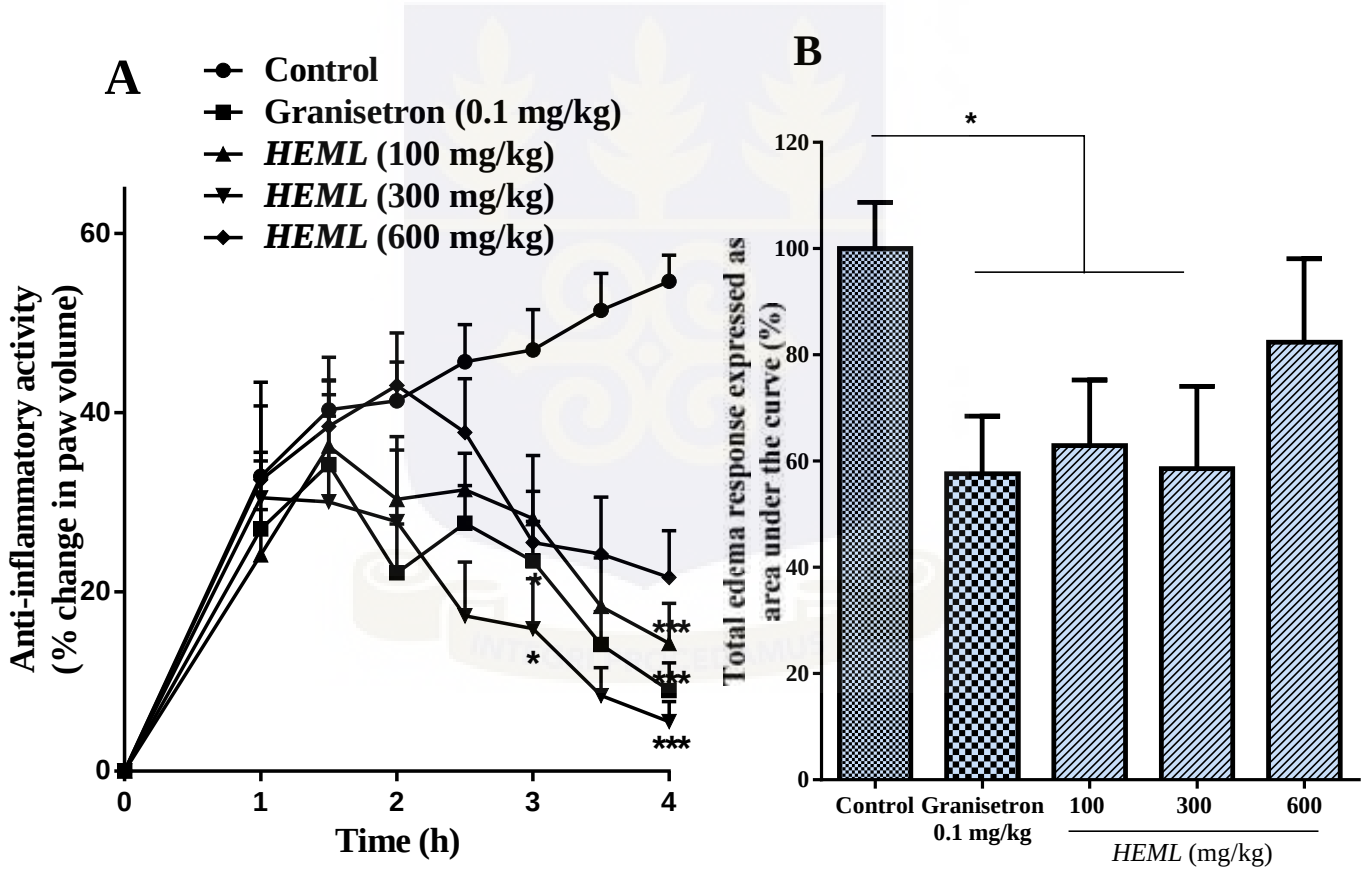


Fig. 4.5: Effects of pretreatment with *HEML* or Granisetron (GRA) on suppression of serotonin-induced paw edema in rats with time post-serotonin challenge; (A) time-course curves and (B) area under the curve (AUC). Data are presented as means ± S.E.M. (n=6). Value statistically significant compared with untreated control; ** $p \leq 0.01$, *** $p \leq 0.001$.

4.9 LPS-INDUCED FEVER IN RATS

Injection of LPS from *E. coli* (i.p) caused a rise in rectal temperature of normal control animals by 30% within 1 hour followed by a decline (18%) by the 4th hour. That of the 300 and 600 mg/kg *HEML* groups followed a similar trend with 20-25% increases in rectal temperature at 1 hour followed by declines to 5.9-8.2% by the 4th hour. The 100 mg/kg *HEML* and acetaminophen groups showed a peak increase of 15% at 30 minutes followed by a decline to 5.9-7.7% by the 4th hour (Fig. 4.6A). These results are depicted in the AUC in which there were significant ($p<0.05$-0.01) reductions in rectal temperatures with respect to acetaminophen and the 100 and 300 mg/kg *HEML* (38-52%) relative to normal control with insignificant reductions in case of the 600 mg/kg *HEML* group (Fig. 4.6B).

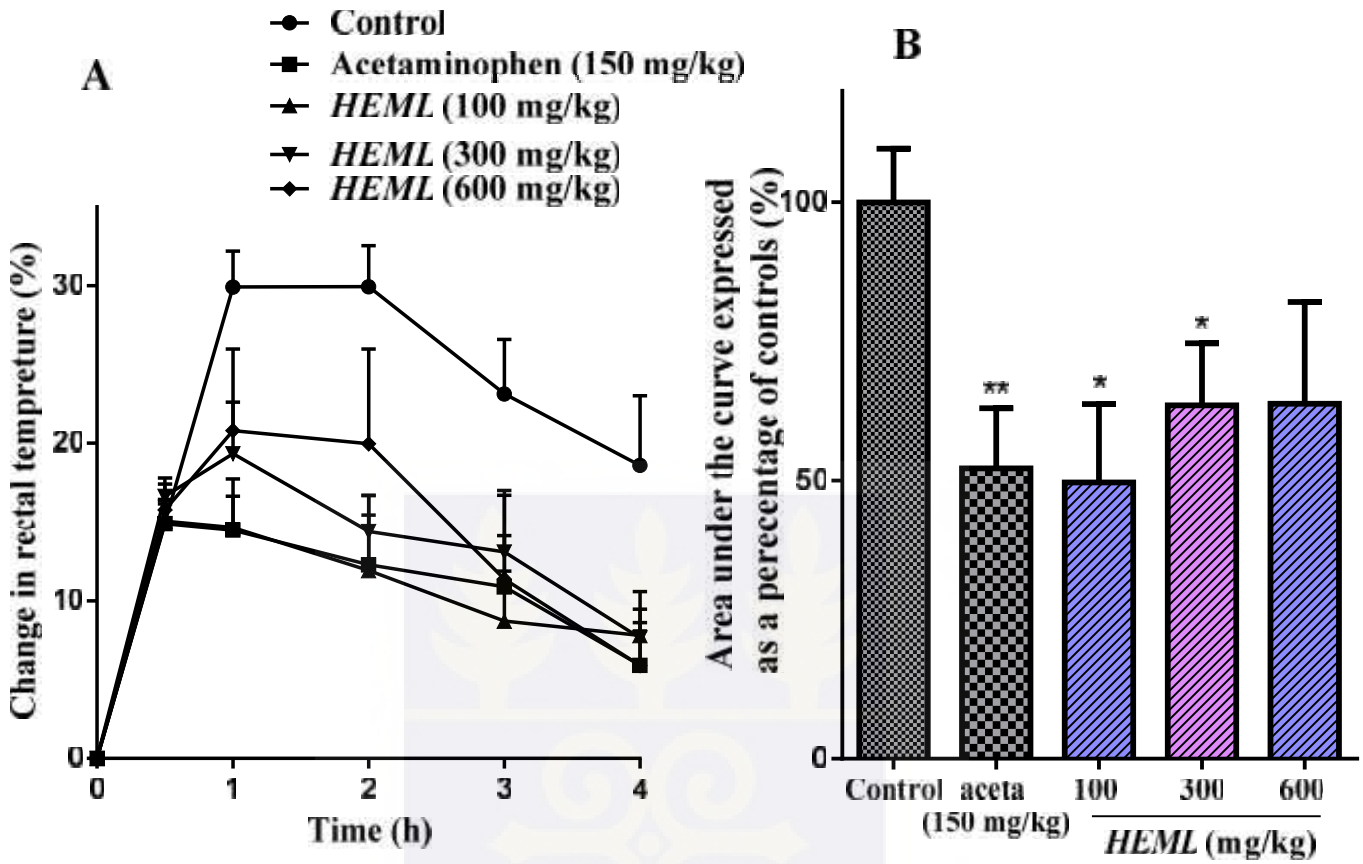


Fig. 4.6 Effect of *HEML* on LPS-induced fever in rats; (A) time-course curves and (B) areas under the curve (AUC) in arbitrary units. Data are presented as means ± S.E.M. (n=6). Value statistically significant compared with untreated control; * $p \leq 0.05$, ** $p \leq 0.01$. Aceta = acetaminophen (150 mg/kg).

4.10 CELL VIABILITY OF *HEML* ON LPS-ACTIVATED RAW 264.7 MACROPHAGES

In order to indicate that the concentration of *HEML* used were not toxic to the cells, a cell viability assay was carried out using RAW 264 cells in the presence of *HEML* in an MTT assay. There was no significant difference ($p > 0.05$) between cells treated with LPS alone and cells treated with *HEML* at concentrations of 0.62, 1.85, 5.56 and 16.67 µg/ml as shown (Fig. 4.7).

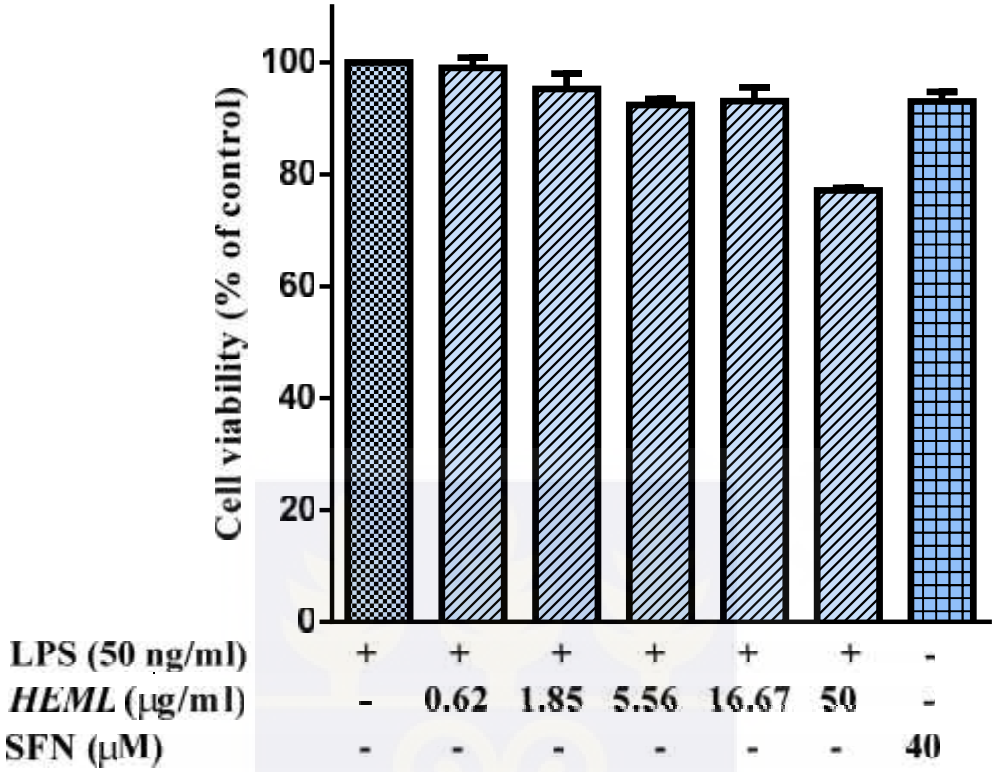


Fig. 4.7: Effect of *HEML* on cell viability of LPS-activated RAW 264 cell. Data was presented as mean ± SEM of n=3. Data generated was presented as a ratio of the optical density of treatment group to that of the control group.

4.11 PGE_2 RELEASE AND COX-2 EXPRESSION

The *HEML*, at 16.65 µg/ml, markedly suppressed (33.4%) LPS-induced release of PGE_2 into the culture supernatant (Fig. 4.8A), but did not inhibit the expression of COX-2 (Fig.4.8B). On the other hand, *HEML* at 1.85 and 5.55 µg/ml neither affected the levels of PGE_2 nor inhibited COX-2 expression. The positive control sulforaphane (SFN), on the other hand, almost completely suppressed PGE_2 release (98%) and strongly inhibited COX-2 expression (84%).

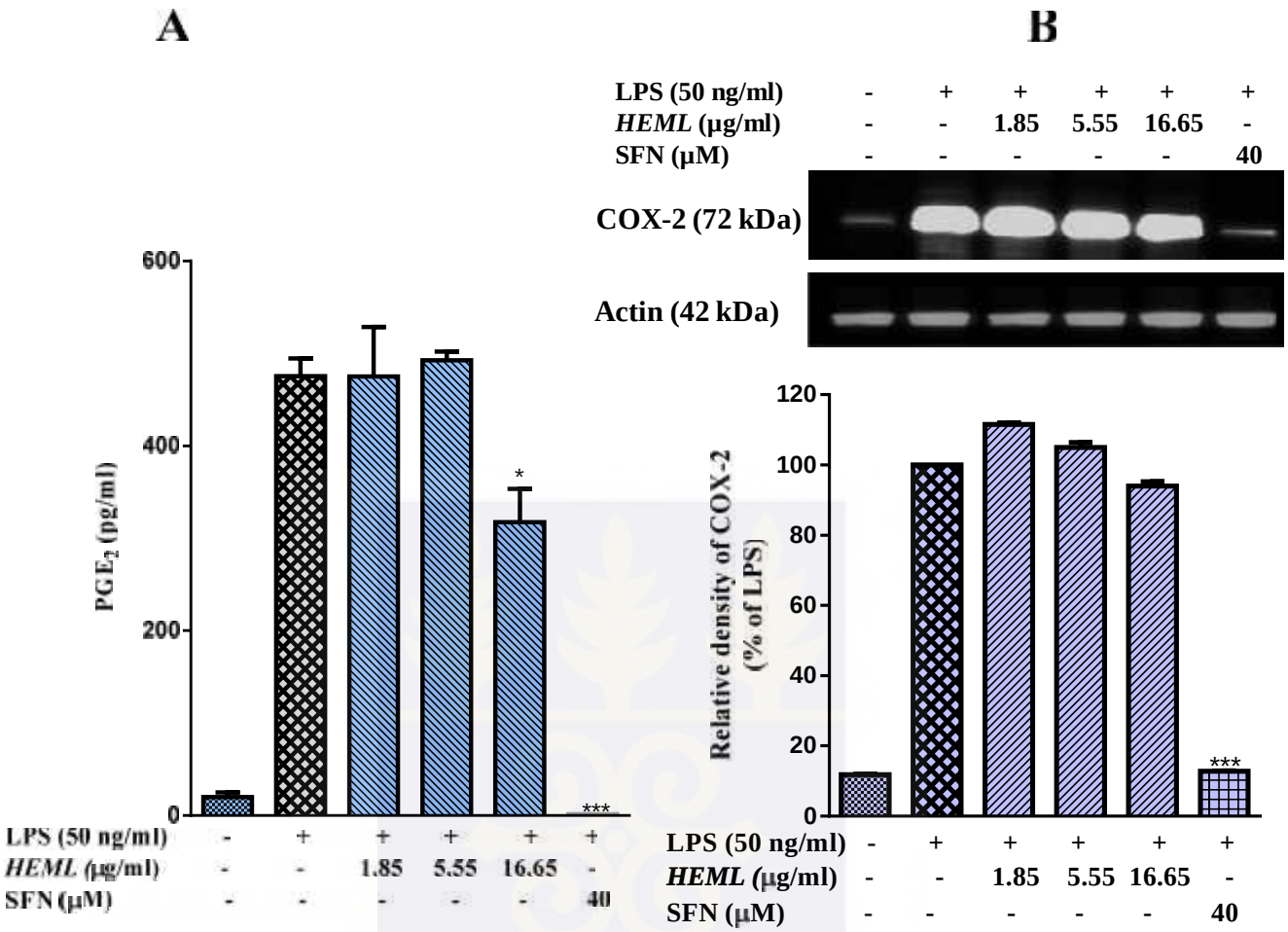


Fig. 4.8: Effect of *HEML* or sulforaphane (SFN) on PGE_2 release (A) and COX-2 expression (B) in LPS-induced RAW 264.7 macrophages. Cells were treated with varying concentrations of *HEML* (1.85-16.65 µg/ml) and fixed concentration of SFN (40 µg/ml) and incubated in the presence of LPS (50 ng/ml). Blank (without LPS and *HEML*/SFN) and control (with LPS but without *HEML*/SFN) were also investigated. The relative density of COX-2 band intensity (B) was quantified using image J software and normalized in the controls. Data is presented as the mean ± S.E.M (n=3). ** $p \leq 0.01$, *** $p \leq 0.001$

4.12 NITRIC OXIDE (NO) PRODUCTION AND iNOS EXPRESSION

HEML caused a non-concentration dependent reduction (16-36%) in NO production in culture medium compared to 84% by SFN (Fig. 4.9A). Expression of iNOS in the RAW 264.7 cells (Fig. 4.9 B) was increased 100% after LPS exposure but was inhibited in cells pretreated with

HEML at 5.55 µg/ml (40%) and 16.65 µg/ml (70%) or 40 µM SFN (100%). *HEML* at 1.85 µg/ml, on the other hand, showed a 20% increase in iNOS expression.

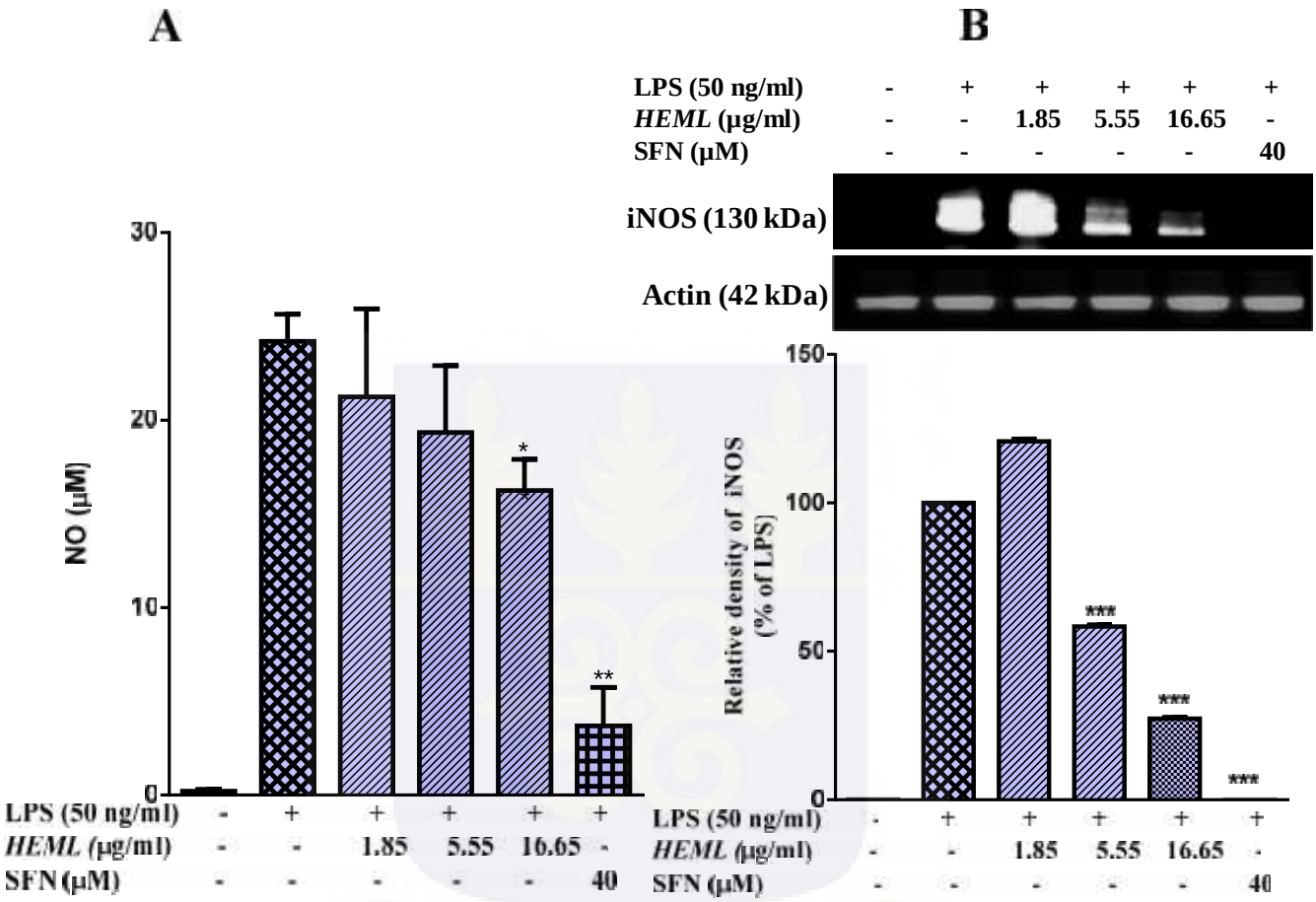


Fig. 4.9: Effect of *HEML* or sulforaphane (SFN) on NO production (A) and iNOS expression (B) in LPS-induced RAW 264.7 macrophages. Cells were pretreated with varying concentrations of *HEML* (1.85-16.65 µg/ml) and fixed concentration of SFN (40 µg/ml) and incubated in the presence of LPS (50 ng/ml). Blank (without LPS and *HEML*/SFN) and control (with LPS but without *HEML*/SFN) were also investigated. The relative density of iNOS band intensity (B) was quantified using image J software and normalized in the controls. Data is presented as the mean ± S.E.M. (n=3). ** $p \leq 0.01$, *** $p \leq 0.001$

4.13 LPS-INDUCED CYTOKINE EXPRESSION

The cells treated with LPS alone without *HEML*/SFN expressed 2 to 4.8-fold increase in IL-β and TNF-α levels but a 60% reduction in IL-10 levels compared to unstimulated cells (cells without LPS). *HEML* at the three concentrations used reduced IL-1β by 19-33% (Fig 4.10 A). TNF-α was markedly reduced by about 87% at *HEML* concentrations of 5.55 and 16.65 µg/ml but was increased by 15% at 1.85 µg/ml of *HEML* (Fig. 4.10 B). The standard drug SFN markedly reduced IL-β and TNF-α by 100% and 95%, respectively. *HEML* caused about 7 to 10-fold increase in IL-10 levels whilst SFN caused a 10-fold increase (Fig. 4.10 C). The changes caused by *HEML* in all the cytokines measured were not concentration-dependent.

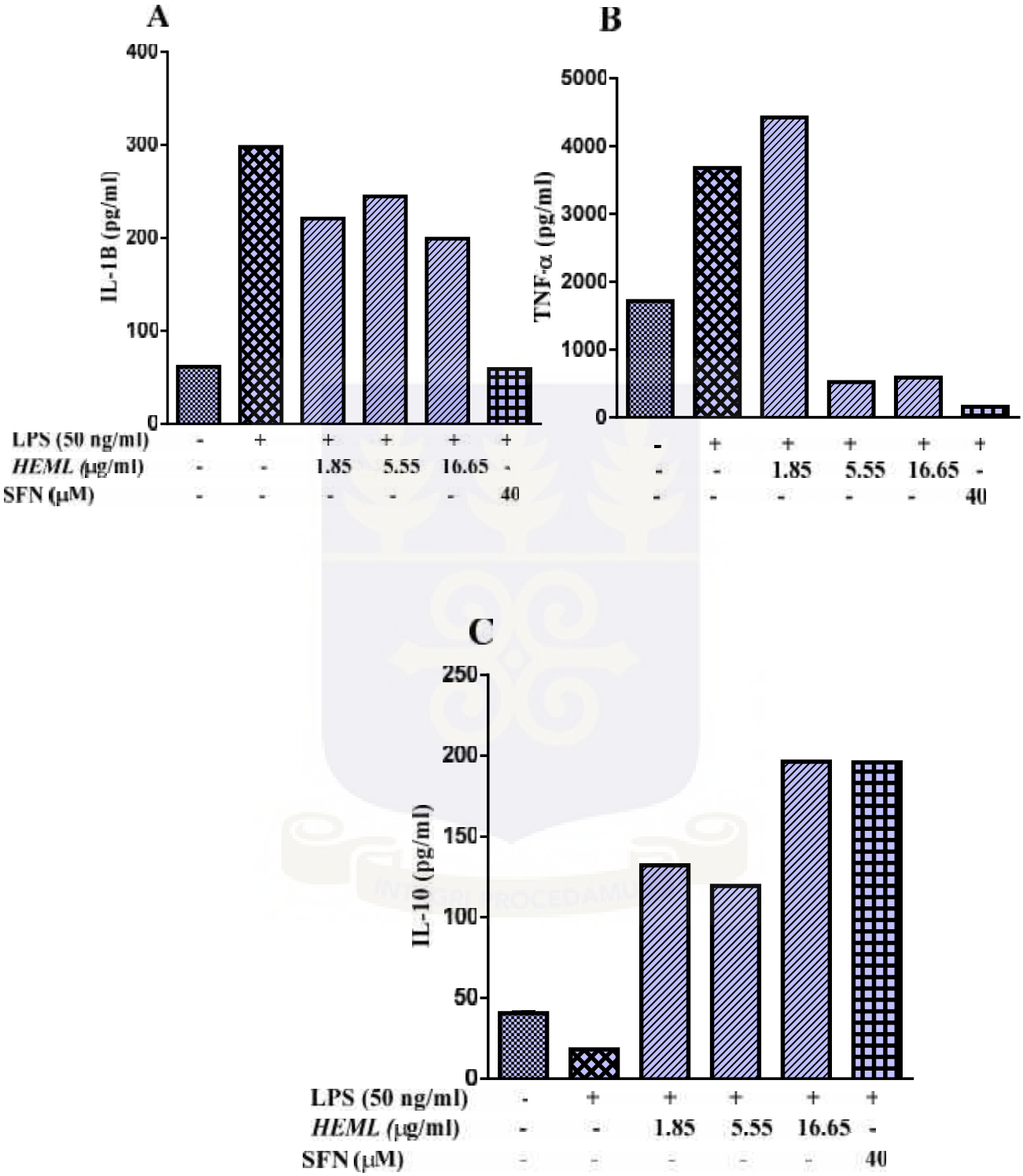


Fig. 4.10: Effect of *HEML* or SFN on cytokine levels of LPS-activated RAW 264 cell; IL-1β (A), TNF-α (B) and IL-10 (C) levels. Data are presented as means duplicate determinations.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The induction of paw edema by carrageenan is reported to show a biphasic pattern of inflammation (Vinegar *et al.,* 1969), such that mediators released at the first phase include histamine and serotonin and then proceeds to a second phase with the release of other mediators such as NO and prostaglandins, especially the E-series and other products of the enzyme COXs. The kallikrein-kinin system is relevant to the cascade and ensures the synthesis and release of mediators (e.g. bradykinin) whose action tends to bridge these two phases (Silva *et al.,* 2005; Perianayagam *et al.,* 2006). Inflammatory responses represent a principal mechanism by which the body defends itself. This mechanism is basically controlled by the mediators NO, PGE_2 and various cytokines like that of IL-1β, IL-6, TNF-α (Dewanjee *et al.,* 2013). For this reason, inflammation could bypass the acute phase into the chronic state when certain levels of mediators of the late phase of acute inflammation are sustained. A promising way to remedy the incidence is obviously the inhibition of these mediators occurring both at the early and to some extent late phases of inflammation either by influencing their pharmacological activities or by interfering with their synthetic or metabolic pathways.

The current study was to assess the anti-inflammatory activity of *M. lucida (HEML)* and propose its possible mechanisms of action. The LD_{50} of *HEML* has been estimated to be greater than 5000 mg/kg since it did not cause any death in the SDRs during the acute toxicity test. Hence the doses of 100, 300 and 600 mg/kg were chosen for the study. Using a classical acute model of inflammation, the anti-edematous effect of *HEML* was demonstrated in female SDRs, and its ability to reduce the edema formation during the injury time confirmed its anti-inflammatory

effect. It was established that *HEML* inhibited paw edema caused by carrageenan in an inverse dose-dependent manner (Fig. 4.3). Thus, as the dose of *HEML* increased from 100 mg/kg to 600 mg/kg, anti-inflammatory activity was found to decrease (Fig. 4.3B). The significant suppression of paw edema before and after the second hour of *HEML* administration in the rats indicates that, *HEML* may act on both the initial and late phases of acute inflammation. *HEML* also reduced significantly paw edema caused by histamine and serotonin (Figs. 4.4 and 4.5), which goes to support its effect on the initial phase of acute inflammation in the rats.

During infection or a challenge with an inflammatory stimulus, especially at the periphery of the body, various responses tend to be generated within the central nervous system (CNS). Other than localized, inflammation may be systemic where the innate immune cells respond by elevating levels of prostaglandins, cytokines, NO and complement factors in circulation, which communicate with the brain through multiple humoral and neural routs to cause neuro-immune activation leading to fever (Blatteis, 2007).

Fever is an adaptive defense mechanism in response to inflammation or infection. The increase in body temperature is thought to prevent propagation of infectious microorganisms and activates the host's immune system (Kluger *et al.*, 1998). In mammals, body temperature is regulated by controlling heat production to adjust to a set temperature by the hypothalamus around 37 °C. The 'resetting' of the set temperature above the normal threshold leads to excessive generation of heat and prevention of heat loss, leading to increased muscle tone, shivering and peripheral vasoconstrictions until the body temperature reaches the new set point (Cabanac, 2006). The process is mediated by the elevation of pyretic cytokines such as TNF-α, IL-1β, and IL-6 in circulation and consequent stimulation of PGE_2 production which is considered as important mediator to the triggering of fever (Netea *et al.*, 2000).

Some prominent late phase mediators produced in the carrageenan-induced paw edema include PGE_2, NO and pro-inflammatory cytokines. If *HEML* affects both phases (Fig 4.3), then there is the possibility that *HEML* suppressed inflammation at the late phase by affecting levels of one or combinations of or all the late phase mediators (PGE_2, NO and pro-inflammatory cytokines). LPS trigger systemic inflammation and may lead to the release of PGE_2, pro-inflammatory cytokines and NO, which results in fever or hyperthermia (Kluger, 1991). The significant suppression of rectal temperature in rats treated with different doses of *HEML* as shown in the LPS-induced fever assay (Fig. 4.6), indicates that *HEML* inhibited the late phase inflammatory mediators.

To examine which specific late phase mediator was affected, *in vitro* assays were conducted using LPS-induced RAW 264.7 cells. At the concentrations of *HEML* used in these studies no cytotoxic effects were observed (Fig. 4.7). *HEML* significantly reduced PGE_2 levels in LPS activated RAW 264.7 cells without affecting COX-2 expression (Fig. 4.8). This suggests that, *HEML* does not affect the *de novo* synthesis of COX-2 but rather interferes with its catalytic activity, which may explain why PGE_2 levels reduced in culture supernatant of cells treated with *HEML*. COX-2 is inducible and its over-expression has been associated with inflammation and cancer (Maier *et al.,* 1990) due to increased PGE_2 production. Inhibiting PGE_2 overexpression may provide a promising remedy to solving such pathophysiological conditions (Masferrer *et al.,* 1994; Oshima *et al.,* 1996). Due to the standard limitations of the conventional NSAIDs, newer COX-2 specific inhibitors are being sought after (Vane, 1994), while available ones (e.g. Celecoxib and Rofecoxib) have still not lived up to the claim that they are devoid of adverse effects (Celotti and Laufer, 2001).

Data gathered from clinical studies indicates that, some COX-2 specific inhibitors accelerate vascular events leading to cardiovascular related problems such as heart attack or non-fatal myocardial infarction, stroke and even death; hence the withdrawal of these drugs from the market (Kearney *et al.*, 2006; Antman *et al.*, 2007). The control of PGE_2 levels in a biological system, without affecting homeostasis provides a key concept in dealing with inflammation, where the available conventional NSAIDs and COX-2 specific inhibitors happen to fail. This may account for some of these adverse side effects associated with their treatment. As a result, the reduction in PGE_2 levels by *HEML* without affecting COX-2 expression may denote an effective approach of curbing the numerous problems associated not only with treatment of inflammation by conventional NSAIDs but also COX-2 specific inhibitors. However, more data is needed to establish this claim.

Nitric oxide is another potent inflammatory mediator and excess generation is due to higher expressions of iNOS, which promote tumour formation by transforming cells with normal growth pattern into malignant tumour (Ohshima and Bartsch, 1994). As a result, the interference of iNOS activity or its complete inhibition may be a reasonable approach to controlling inflammation. In the current study, *HEML* was shown to lower NO levels in culture supernatant by downregulating the expression of iNOS (Fig.4.9) suggesting that it may suppress the *de novo* synthesis of iNOS.

Interestingly, some group of researchers (Mollace *et al.*, 2005) have indicated a cross-relationship between iNOS and COX-2. They explained that, during stimuli, inflammatory cells such as macrophages generate ROS and increase iNOS expression, which consequently increases NO levels. The NO produced combines with superoxide anion (a specific form of ROS) to form

peroxy-nitrite ($ONOO^-$). Although $ONOO^-$ generated has important role in parasitic and bacterial infection, it also has the ability to activate the NF-κB pathway which leads to the activation and transcription of COX-2 and iNOS genes. On the other hand, when the NF-κB pathway was studied at lower levels of endogenous NO concentration in cells, the pathway was kept inactive (Togashi *et al.*, 1997). Thus, *HEML* by its ability to reduce NO levels and as a result of its oxyradical scavenging activity (Fig. 4.2) may significantly reduce ONOO, which in turn may prevent the co-activation of the inducible COX-2 and thus reduce inflammation. The antioxidant activity of *HEML* may be due to its phenolic content, particularly flavonoids (Table 4.1; Fig. 4.2). By means of extrapolations, *HEML* may have the ability to stabilize superoxide anion and other free radicals including NO generated by the immune cells, thereby controlling inflammation to some extent. It is, however, possible that some of the phyto-constituents present in *HEML* antagonize the actions of other components responsible for its anti-inflammatory effect at higher concentrations. This may explain why *HEML* at 100 mg/kg showed significant anti-inflammatory activity but did not show such significant activity at 300 and 600 mg/kg (Fig. 4.3). This observation could be resolved with the isolation of the active component(s) of *HEML* responsible for its anti-inflammatory activity.

As part of the immuno-regulatory process, inflammatory response proceeds with increasing levels of circulating cytokines, where pro-inflammatory cytokines predominate initially, but their levels are later regulated by increasing anti-inflammatory cytokines (Zhang and An, 2007). Based on this, a good anti-inflammatory agent should have the ability of suppressing pro-inflammatory cytokines but proportionally elevate the levels of anti-inflammatory cytokines, just to maintain a balance. *HEML* reduced the levels of IL-1β and TNF-α (pro-inflammatory cytokines) whilst boosting IL-10 (anti-inflammatory cytokine) levels markedly (Fig.4.10). This

is consistent with the inferences from the initial animal studies which showed that *HEML* resolved acute inflammation.

5.2 CONCLUSIONS

The hydroethanolic extract of *M. lucida (HEML)* contains groups of phytochemicals such as saponins, phenolics, flavonoids and reducing sugars. *HEML* has antioxidant activity, which may be due to the presence of phenolic compounds particularly flavonoids. It showed anti-inflammatory activity by affecting both early and late phases of acute inflammation. The potential mechanism of action of *HEML* as an anti-inflammatory agent may be through (1) the down regulation of iNOS expression leading to reduction in NO formation, (2) the reduction in PGE_2 production without directly affecting COX-2 expression, but probably through inhibition of its catalytic activity, (3) elevation of anti-inflammatory cytokine (IL-10) levels and reduction in pro-inflammatory cytokine (IL-1β and TNF-α) levels and (4) mopping up of ROS. These findings provide scientific evidence for the anecdotal use of *M. lucida* in the treatment of inflammation.

5.3 RECOMMENDATIONS

Further data may be needed to explain and improve on the knowledge acquired so far. It is therefore recommended that;

- A dose response assay be carried out to determine the effective dose at 50 (EC_{50}) which will help to establish the therapeutic dose of *HEML*.
- The effect of *HEML* on the promotor region of COX-2 gene and COX-2 enzyme activity should be examined in order to explain why PGE_2 levels reduced without affecting COX-2 protein expression.

- Detailed signaling mechanisms should be conducted to investigate the effect of *HEML* on iNOS transcription and protein expression.
- The toxicity profile of *HEML* be established pre-clinically using both acute and chronic modules.
- The active component responsible for the anti-inflammatory effect should be isolated and its structure elucidated.
- The effect of *HEML* on the inherent COX-1 expression in gastro-intestinal and renal cells especially should be assessed. This will provide scientific evidence to indicate that treatment of inflammation with the extract is not accompanied with side effects like that of conventional NSAIDs.

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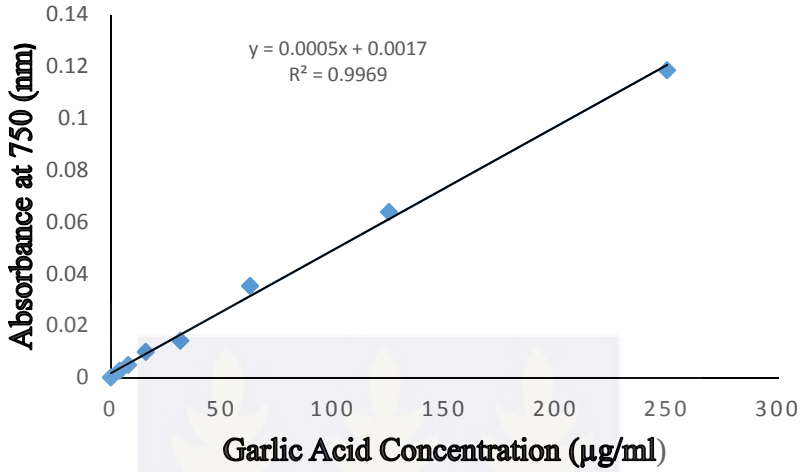
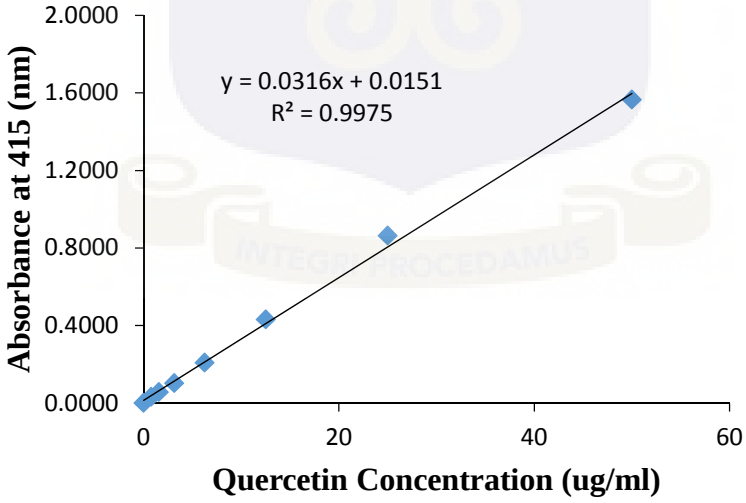
APPENDICES

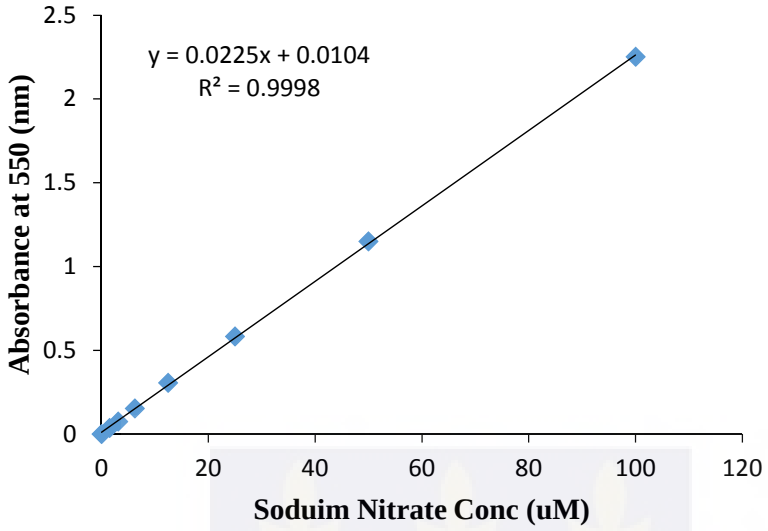
Appendix I: Standard Curve for Gallic Acid



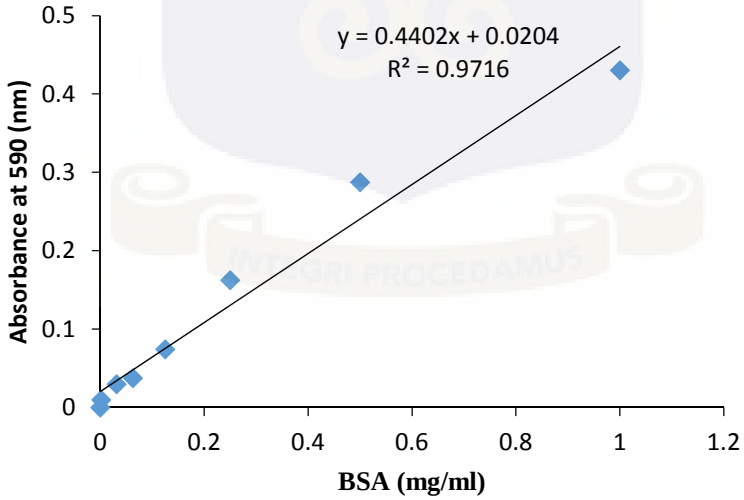
Appendix II: Standard Curve for Quercetin



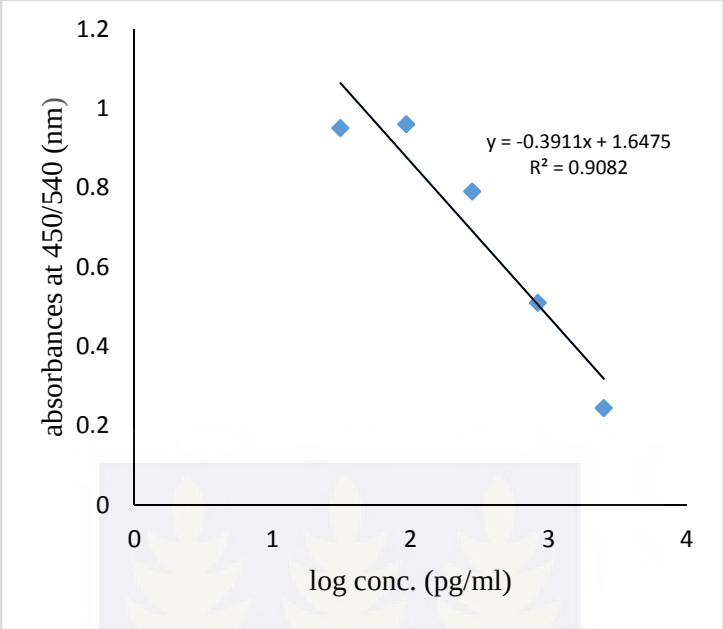
Appendix III: Standard Curve for Sodium Nitrite



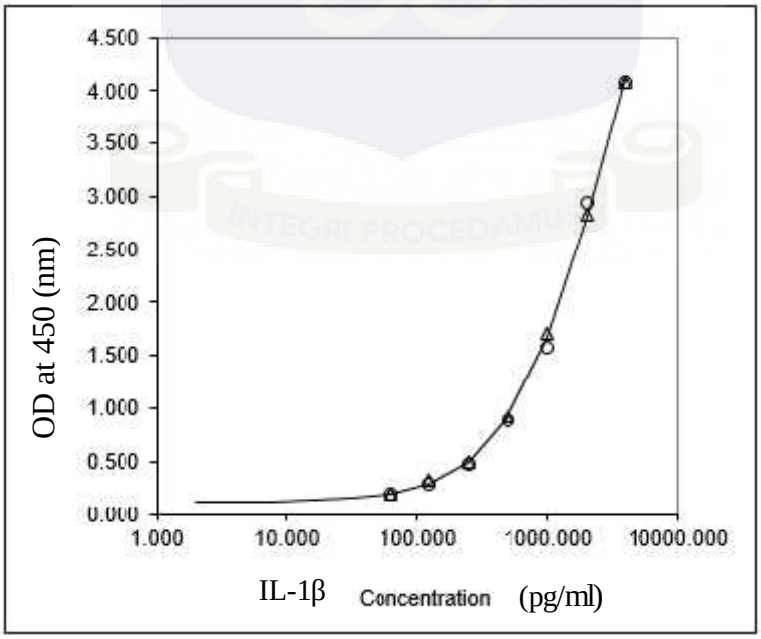
Appendix IV: Standard Curve for Bovine Serum Albumin (BSA)



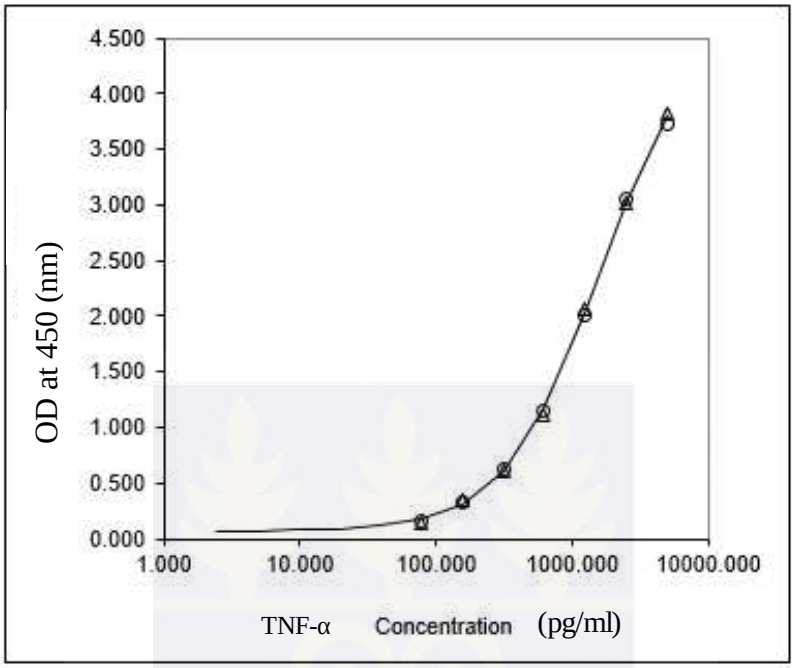
Appendix V: Standard Curve for PGE_2



Appendix VI: Standard Curve for IL-1B



Appendix VII: Standard Curve for TNF-α



Appendix VIII: Standard Curve for IL-10