UNIVERSITY OF CAPE COAST

HYDROETHANOLIC LEAF EXTRACT OF *PERSICARIA LANIGERA* R. BR. SOJÁK (POLYGONACEAE) EXHIBITS ANTI-INFLAMMATORY EFFECTS IN EXPERIMENTAL MODELS OF INFLAMMATION

MESHACK ANTWI-ADJEI

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BY

MESHACK ANTWI-ADJEI

Thesis submitted to the Department of Biomedical Sciences of the School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfillment of the requirements for the award of Doctor of Philosophy degree in Drug Discovery and Toxicology

MAY, 2022

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidates Signature …….. Date……..

Name: Meshack Antwi-Adjei.

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisors' Signature……... Date……..

Name: Prof. Elvis Ofori Ameyaw.

Co-Supervisors' Signature …… Date……..

Name: Dr. Ernest Obese.

ABSTRACT

Persicaria lanigera extract (PLE) is traditionally used to manage inflammatory disorders. This study aimed at evaluating the anti-inflammatory and oral safety of a hydroethanolic (70 % $^{v}/_{v}$) leaf extract of *Persicaria lanigera*. The anti-inflammatory effects of the extract were assessed using both acute and chronic inflammatory models while the toxicity profile was performed using acute and sub-acute toxicity studies. From the acute inflammatory studies, PLE (100-600 mg kg^{-1}, *p.o.*) administered pre-emptively suppressed the mean maximal oedema to 59.10±4.94 %, 56.08±3.65 %, and 48.62±3.27 % at 100, 300 and 600 mg kg^{-1}, and total inflamed paw by 43.72 %, 52.34 % and 61.58 % at the same doses in carrageenan-induced paw oedema. In the chronic inflammatory studies, PLE suppressed the disease activity index (DAI) score in acetic acid-induced colitis from 84.00±5.09 to 58.00±5.39, 50.00±7.07 and 43.00±5.38 at 100, 300 and 600 mg kg^{-1}, and similarly inhibited the granuloma tissue formation by 20.65 %, 22.61 % and 30.14 % at the same doses respectively. PLE significantly suppressed the Complete Freund's Adjuvant (CFA)-induced arthritic ipsilateral paw to 126.58±10.91 %, 113.82±11.46 % and 106.45±34.85 % at 100, 300 and 600 mg kg^{-1} respectively when administered prophylactically. In the therapeutic study, PLE decreased the CFA-induced inflammation in the ipsilateral limb to 568.50±91.18 %, 545.50±71.88 % and 541.83±70.21 % dose-dependently at the same doses respectively. In the toxicity studies, PLE was confirmed to be relatively safe and non-toxic with no clinical signs or treatment related toxic effects including haematological, biochemical, histological changes and death.

KEY WORDS

Anti-inflammatory drugs

Toxicity

Cytokines

Phytoconstituents

Prophylaxis

Therapeutic

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Finally, I want to express my gratitude to my mother, father, siblings, and everyone else who assisted me in completing this project.

DEDICATION

To my lovely children, Mikel Faith Antwi-Adjei, Manuel Antwi-Adjei, Miracle Bless Antwi-Adjei, and wife, Dr. (Mrs.) Roberta Antwi-Adjei.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

WHO	World Health Organization
NSAID	Non-steroidal anti-inflammatory drug
DMARD	Disease modifying anti-rheumatoid drug
CFA	Complete Freund's Adjuvant
DPPH	2,2-diphenyl-1-picryl-hydrazil
IFA	Incomplete Freund's Adjuvant
H_2O_2	Hydrogen peroxide
ELISA	Enzyme-linked immunosorbent assay
TNF-α	Tumor necrosis factor alpha
IL	Interleukin
NF-κB	Nuclear factor kappa B
COX	Cyclooxygenase
IFN	Interferon
ECM	Endothelial cell membrane
TLR	Toll-like receptor
AGE	Glycation end product
ROS	Reactive oxygen species
PLA	Platelet activating factor
GC	Glucocorticoid
PGE	Prostaglandin E
cAMP	Cyclic adenosine monophosphate
PKC	Protein kinase C

5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
NEU	Neutrophils
LYM	Lymphocyte
PGI	Prostacyclin
NGF	Nerve growth factor
SCF	Stem cell factor
THPO	Thrombopoietin
PLT	Platelet
fMLP	N-Formylmethionyl-leucyl-phenylalanine
GPCR	G-protein coupled receptor
PSGL	P-selectin glycoprotein ligand
TxA	Thromboxane
ADP	Adenine dinucleotide phosphate
CXCL	C-X-C Motif Chemokine Ligand
MIP	Macrophage inflammatory protein
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RA	Rheumatoid arthritis
PGHS	Prostaglandin H synthase
AA	Arachidonic acid
RNS	Reactive nitrogen species
DNA	Deoxyribonucleic acid
HIF	Hypoxia-induced transcription factor

AP-1	Activator protein 1
MAPK	Mitogen-activated protein-kinase
NFAT	nuclear factor of activated T cells
CYP	Cytochrome P
NADPH	Nicotinamide adenine dinucleotide phosphate
SOD	Superoxide dismutase
CAT	Catalase
MDA	Malondialdehyde
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GPx	Glutathione peroxidase
GSH	Glutathione
LDL	low-density lipoprotein
TBA	Thiobarbituric acid
FTC	Ferric thiocyanate
LD_{50}	50 % of Lethal dose

CHAPTER ONE

INTRODUCTION

This chapter captures the description of the plant species, *Persicaria lanigera*, its geographical distribution, and the traditional uses of the plant. The problem statement states the existing toxic effects associated with the usage of orthodox medication in the management of inflammation and other related inflammatory conditions. The problem further indicates the need for alternative and complementary sources of treating inflammation accompanying minimal or no adverse effects and highly affordable not forgetting accessibility.

The purpose and justification of the study under this section explain the life-threatening issues accompanying the wide usage of conventional therapy in the management of inflammation which therefore becomes imperative for more research and investigations to be conducted on medicinal plant species such as *Persicaria lanigera* to establish its anti-inflammatory activity and safety profile to treat inflammation. This chapter also captures the aim and specific objectives for the study.

Background to the Study

Herbal medicine has been the dominant traditional form of health care assessed by humans. Today, in about 80 % of the world's population, plant medicine has been used the primary form of medicine and, many people resort to herbal therapy (World Health Organization, 2002). Mostly, certain parts of plants such as the leaves, roots, flowers, fruits, stems and seeds are used either in herbal formulations or as pure active substances prepared into favourable preparations. Several medicines used currently are from herbal sources and nonetheless, numerous prescribed drugs of about 25 % are compose of not less

than one active ingredient obtained from plant material (Vitalini, Tomè, & Fico, 2009). In addition, medicinal plants either in the form of an extract, a derivative or pure compound give remarkable opportunities for the development of new drugs (Yuet-Ping, Darah, Chen, Sreeramanan, & Sasidharan, 2013).

Globally, in health care system, natural products have played a major role since herbal-derived medicines are used extensively in all civilizations including other cultures. In many developing countries, the traditional ways of herbal therapy are part of the culture and considered to be the main methods in disease management. The usage of herbal products, with a remarkable degree of effectiveness, is accepted socially and economically feasible, and most at times becomes the only available source (WHO, 2003). Numerous herbal products used in folkloric medicine have solid scientific evidence in relation to their biological activities (Yuet-Ping *et al*., 2013).

The *Persicaria lanigera* plant

Botanical name: *Persicaria lanigera* R. Br. Soják

Family: Polygonaceae

Common name: Smartweed

Local name: Okwatakyie (Akan)

Figure 1: The *Persicaria lanigera* plant (photo from sample site by Meshack).

Description

The genus *Persicaria* or *Polygonum* is a cosmopolitan group of species that comprises of about 100-150 species. *Persicaria lanigera* (= *Polygonum lanigerum* R. Br. Soják) was discovered by Tabone (2008) from Wied Sara and Wied tal-Grazzja, Gozo in October 1992. It is an erect and villous perennial herb, grows tall to about 200 cm in height. It has a branch stem with moderate to dense woolly pubescent. The leaves are shortly petiolated, lanceolate or ovate-lanceolate about 10-25×2-6 cm in size with apex acute to acuminate, base attenuate, margin ciliate and the upper surfaces are moderately woolly pubescent whiles the lower surfaces are moderate to dense woolly pubescent. The petiole is densely woolly pubescent, sessile or up to 1 cm long. The ochreae is short, villous, cylindrical about 1-2.5 cm long, glabrous or sparsely woolly pubescent, truncate or virtually oblique at the apex, glabrous or ciliate. Inflorescences are terminal or in the axil of upper leaves, they become spiciform racemes, which are 1-5 cm long and usually branch 1-3 times. These form bracts cup-shaped, 2-2.5 cm long, acuminate at the apex with green ridges along the

midvein, densely pubescent, oblique at the apex and have short hairs at the margins. Peduncle is 0.5-5 cm long. The flowers are pink or white in 5-6 flowered fascicles; bract membranous, cylindrical, 1-2 mm long, truncate; pedicles 1.5-2 mm long; perianth 4-parted, pale yellow, 2-3.5 mm long, apex obtuse with densely glandular dots at the center of the tepals. It has 6 stamens; 0.8-1.5 mm long filaments; 0.2 mm long discoid anthers; 1 style, 2 deep clefts of about 1.5-2 mm long and 2 stigmas. The achenes are smooth, brown, 2-2.5 mm long that are slightly biconcave and broadly ovoid in outline [Figure 1] (Chantaranothai & Tubtimtong, 2001).

Ecological and geographical distribution

Habitat: The genus, *Persicaria* or *Polygonum*, is a group of hardy annuals and perennial herbs located mostly in the temperate regions and few others can be found in the tropical and sub-tropical regions at various levels from the sea to different altitudes (Heywood, Brummitt, Culham, & Seberg, 2007).

Location: They are usually distributed in Europe, West Asia as well as Africa (Mifsud, 2011) including Ghana. The plant was obtained from Jukwa, a suburb in the Lower Denkyira District (latitude 1º22′41.78″W, longitude 5º15′5.76″N) of the Central Region for this research work (Figure 2).

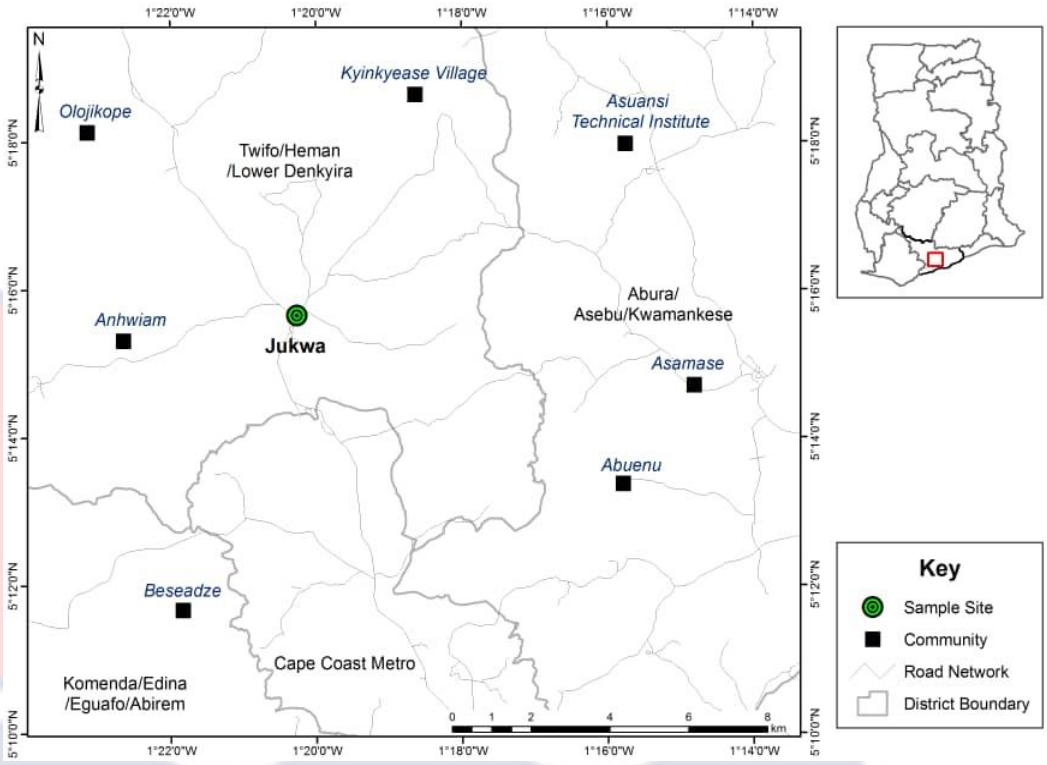


Figure 2: Geographical location of *Persicaria lanigera* R. Br. Soják in the Central Region.

Traditional Uses

Traditionally, *Persicaria lanigera* has been used to treat numerous disease conditions. Certain parts of the plant are used domestically for various purposes.

Medicinal uses

Persicaria (= Polygonum) is known to be a relevant group of medical plant species used for several purposes (Soodabeh, Hoda, Pantea, Fahimeh, & Gholamreza, 2011) and has been reported to contain active secondary metabolites with anti-microbial, anti-leukaemic, anti-inflammatory, anti-cancer, anti-oxidant, analgesic and tyrosinase-inhibitory properties (Salama & Marraiki, 2010). In addition, *Persicaria* has been reported to possess anti-fungal activity (Derita & Zacchino, 2011) and also, it is used in traditional medicine to

manage cough, gonorrhea, hypertension, atherosclerosis, dermatitis (Yi, Leung, Lu, Zhang, & Chan, 2007).

Non-medicinal uses

Persicaria lanigera can be used as a special food during famine because of its easy availability and high nutritional content. Usually, the polygonum species are considered to be an essential source to prevent nutritional deficiency when food becomes scares (Hameed, Dastagi, & Hussain, 2008). It is also used as dye (Coffey, 1993).

Previous studies on *Persicaria lanigera*

Few scientific studies have been conducted on the plant. These include; the biochemical and molecular genetic characterisation of four species of Polygonaceae (Soliman, El-Amier, & Fayed, 2015), a review of Polygonaceae on its ethnopharmacological and phytochemical perspectives (Mohamed, Narasimhulu, & Reddy, 2014), the genus *Persicaria* in Maltese Island (Mifsud, 2011), the tepal surface micromorphology and its taxonomic implications in some species of Polygonaceae (Shiha, 2019), an investigation into a new combination of *Persicaria* and a new record (Chantaranothai & Tubtimtong, 2001), validation of the ethnopharmacological uses of *Persicaria* for its anti-fungal activities (Derita & Zacchino, 2011), taxonomy and traditional practices of Polygonaceae family (Uddin, Mahbubur-Rahman, & Rafiul-Islam, 2014) and, anti-nociceptive activity of hydroethanolic leaf extract of *Persicaria lanigera* via cytokine and glutamatergic pathways *in vivo* (Obese *et al*., 2021).

Statement of the Problem

Inflammation is a major component of numerous medical disorders (Dos Santos *et al*., 2018). The prevalence of inflammatory diseases such as pain, hay fever, periodontitis, ischaemic heart disease, asthma, arthritis, ulcer, atherosclerosis, rheumatoid arthritis, dermatitis, cancer etc. caused by irritants, infections (pathogens) and other unwanted immune reactions is high among people from developing countries. Treatment for inflammatory disorders currently is the use of conventional anti-inflammatory drugs which lead to clinical improvement in the inflammatory conditions but often, are associated with numerous undesirable adverse effects which are life-threatening and consequently affect quality of life. For example, prolong use of non-steroidal anti-inflammatory drugs (NSAIDs) causes heartburns, dizziness, headache, gastrointestinal disoders (GIT) such as stomach ulcers and bleeding (Tabas & Glass, 2013). Although cycloogenase 2 (COX-2) selective inhibitors such as celecoxib has minimal GIT toxicity, however, prolong use may lead to impaired renal flow, decreased pressure infiltration and kidney malfunction (Kumari, Weerakoon, Handunnetti, Samarasinghe, & Suresh, 2014). Similarly, prolong use of glucocorticoids (GCs) is associated with hypertention, osteoposrosis, hyperglycemia, immunosppressive effects as well as growth retardation (Giles *et al*., 2018). Furthermore, the long-term usage of disease modifying anti-rheumatoid drugs (DMARDs), and other biologics leads to hepatotoxicity, kidney disorders and immunosuppression (Kivity *et al*., 2014).

The current available conventional drugs contribute to a major problem of drug-related toxicity and recurrence of symptoms on discontinuation (Nunes *et al*., 2020). It is therefore imperative to search for new and alternative source

of medicinal substances with potential anti-inflammatory properties to manage inflammation and its associated disorders. This scientific search remains a subject of great interest to discover and develop safer anti-inflammatory agents from natural products.

Aim

This study is aimed at evaluating the anti-inflammatory activities of *Persicaria lanigera* in both acute and chronic inflammatory models.

Specific objectives

The specific objectives of the study is to:

1) assess the anti-inflammatory activities of PLE in both acute and chronic inflammation using;
    a) Carrageenan-induced paw oedema in rats.
    b) Zymosan-induced acute knee joint arthritis in rats.
    c) Acetic acid-induced ulcerative colitis in rats.
    d) Cotton pellet-induced granuloma tissue formation in rats.
    e) Complete Freund's Adjuvant (CFA)-induced arthritis in rats.
2) establish the possible mechanisms of action using mediator-induced model and enzyme-linked immunosorbent assay kit assays (ELISA kit assays);
    a) Histamine-induced paw oedema in rats.
    b) Determination of TNF-α, IL-1β, NF-κB, COX-2 levels in serum.
3) assess the *in vitro* anti-oxidant capacities of PLE using;
    a) Total anti-oxidant capacity.
    b) DPPH (2, 2-diphenyl-1-picryl-hydrazil) scavenging activity.
    c) H_2O_2 (Hydrogen peroxide) scavenging activity.

4) identify the phytoconstituents in PLE using qualitative phytochemical tests.
5) assess the toxicity profile of PLE in acute and sub-acute toxicity studies in rats.

Significance of the Study

It has been established that the usage of conventional medication for the management of inflammation is associated with numerous adverse effects such as gastrointestinal tract disorders, obesity, skin rashes and other unwanted effects. An estimated 10-20 % of patients who use orthodox drugs to manage inflammation experience some form of dyspepsia (Green, 2001). For this reason, the practice of complementary and alternative medicine is now on the increase in developing countries in response to World Health Organization directives culminating in several pre-clinical and clinical studies that have provided scientific basis for the efficacy of many plants used in folk medicine to treat various ailments (Dilhuyday, 2003). Therefore, the significance of the study is to provide the scientific basis of data to support the folkloric use of *Persicaria lanigera* in the management of inflammation and its related disorders. In addition, the active components from *Persicaria lanigera* after phytochemical screening can be isolated and characterised, and further formulated into natural medicinal products such as gel, syrup, ointments, capsules and tablets.

Justification

The use of plants in traditional medicine has become the preferred primary choice of health care in several communities, with about 60 % of the world's population and 80 % in developing countries depend mainly on natural

plants for their health needs (Shrestha & Dhillion, 2003). This can be attributed to many reasons including accessibility, affordability and less expensive (Asase *et al*., 2008). Many scientists worldwide have carried out various research works on the anti-inflammatory activities of certain plants, and many of them have proven to be therapeutically effective due their medicinal properties (Adriana, Almodóvar, Pereira, & Mariângela, 2007). Although traditional and scientific knowledge have enormously contribiuted to the discovery and development of numerous drugs presently used in managing inflammatory diseases, however, low availability, higher cost and adverse effects of these drugs have essentially motivated scientists to undertake more research into natural medicinal plants to establish and improve on their efficacy as well as safety. Therefore, it is imperative to search for new, alternative and complementary sources with lesser adverse effects, reliable, low cost and efficacious for the treatment of inflammation.

One plant of interest is the *Persicaria lanigera* which has been extensively used traditionally to manage several inflammatory disorders. In Ghana, decoctions of the leaves of the plant are used to treat joint pain, swelling and other pathogenic infections (Obese *et al*., 2021). Even though the plant has become useful in traditional medicine, there is no available scientific data and safety profile of the plant. Therefore, the study seeks to establish the scientific validation and oral safety of *Persicaria lanigera*, and the data obtained from this study would be relevant in the scientific world as a validation of its anti-inflammatory activities. Furhermore, this study shall provide essential information to traditional medicine practitioners on the folkloric use the plant

as new, complementary or alternative remedy for inflammatory diseases in addition to its safety use.

Hypothesis

The study seeks to test the following hypothesis in attempt to achieving the objectives stated:

1. H_1: The 70 % hydroethanolic leaf extract of *Persicaria lanigera* can be safe and non-harmful for medicinal purposes.

2. H_1: The leaf extract of *Persicaria lanigera* can possess anti-inflammatroy activites in both acute and chronic inflammatory conditions

3. H_1: The *Persicaria lanigera* extract will be able to inhibit pro-inflammatory mediators and downregulate gene expression of pro-inflammatory cytokines.

Delimitation of the Study

In this study, the plant crude extract prepared was administered to animals only and this can not be extraporated in human unless further cilincal trials are carried out.

Limitation of the Study

Isolated active compounds of *Persicaria lanigera* were not used since these phytochemical constituents have not been isolated and characterised from the plant.

Definition of terms

To avoid misinterpretation, certain terms in this thesis are defined as follows:

Inflammation: is an adaptive response of the body that is triggered by perilous stimuli and conditions such as infections and foreign substances like bacteria and viruses.

Cytokines: are small secreted proteins that are generated by practically every cell to control and impact immune response.

Oedema: is a fluid build-up in the body that causes the afflicted tissue to swell.

Histopathology: is the diagnosis and investigations of diseased tissues and/ or cells under a microscope.

Organization of the Study

The thesis is didvied into five chapters. Chapter one consists of the background of the study, introduction and other biological activities conducted on the plant species, *Persicaria lanigera*. The problem statement in this chapter states the key problems of the study. It underscores the major adverse effects associated with the conventional therapy in the management of inflammatory diseases.

Chapter two is the Literature Review. This chapter captutres the signs of inflammation, causes and the management of inflammatory disorders. Furthermore, cells involve in inflammatory response and the effects of pro-inflammatory mediators in inflammation have been explained. Procedures used in toxicological studies of biological samples were as well mentioned. Chapter three of the study consists of collection, extraction and preparation of sample. In addition, detailed scientific inflammatory models used to assess the anti-inflammatory potential of *Persicaria lanigera* were outlined.

Chapter four gives detailed Results and Discussion. Results obtained were explained thoroughly and compared to a standard agent.

Chapter five captures the Summary, Conclusion and Recommendation. Conclusions based on the objectives of this study were made and further

investigations on *Persicaria lanigera* along other signaling pathways were recommended.

Chapter Summary

There is no scientific literature reported on the anti-inflammatory activities of *Persicaria lanigera*. Scientific investigations on its other biological activities have been stated in this chapter. Inflammation disorders are well-known to be associated with numerous medical conditions and the conventional therapy of inflammatory diseases have been outlined in this chapter.

CHAPTER TWO

LITERATURE REVIEW

Introduction

This chapter reviews the relevant literature in relation to the study. Inflammation and its pathological effects, mediators involve and drugs use in the management of inflammatory conditions as well as toxicological studies of agents with potential anti-inflammatory activities.

Inflammation

Inflammation is an adaptive response of the body that is triggered by perilous stimuli and conditions such as infections and foreign substances like bacteria and viruses (Majno & Joris, 2004; Medzhitov, 2008). This is the basic mechanism available for tissue repair following an injury that comprises of cascade of cellular and vascular reactions leading to removal of damage tissues and regenerate new ones. The cascade of events includes increased vascular permeability, adhesion of circulating cells to the vessels at the site of tissue injury, migration of several cell types, cell apoptosis and regeneration of new tissues and blood vessels. Inflammation is traditionally characterised by four classical cardinal signs including acute pain sensation (*dalor*), heat (*calor*), swelling (*tumor*), and redness (*rubor*) which were first listed by a roman, Celsus in the first century (Brenner, Braun, Oster, & Gulko, 2006).

Later, a fifth cardinal sign, loss of function (*function laesa*), was added by Virchow (1871). These cardinal signs together with eventual healing of the damaged tissues during inflammation may appropriately refer inflammation to as cascade of inflammatory reactions. This entails a long chain of reactions as well as cellular events that lead to tissue repair in several circumstances of life,

from a minor skin cut or repair of tissue after birth to healing of the most severe tissue injuries (Figure 3). Inflammatory responses, for example, underlie life-threatening hypersensitivity reactions to insect bites, toxins, drugs as well as other common chronic diseases such as liver cirrhosis, lung fibrosis, atherosclerosis, cancer and rheumatoid arthritis.

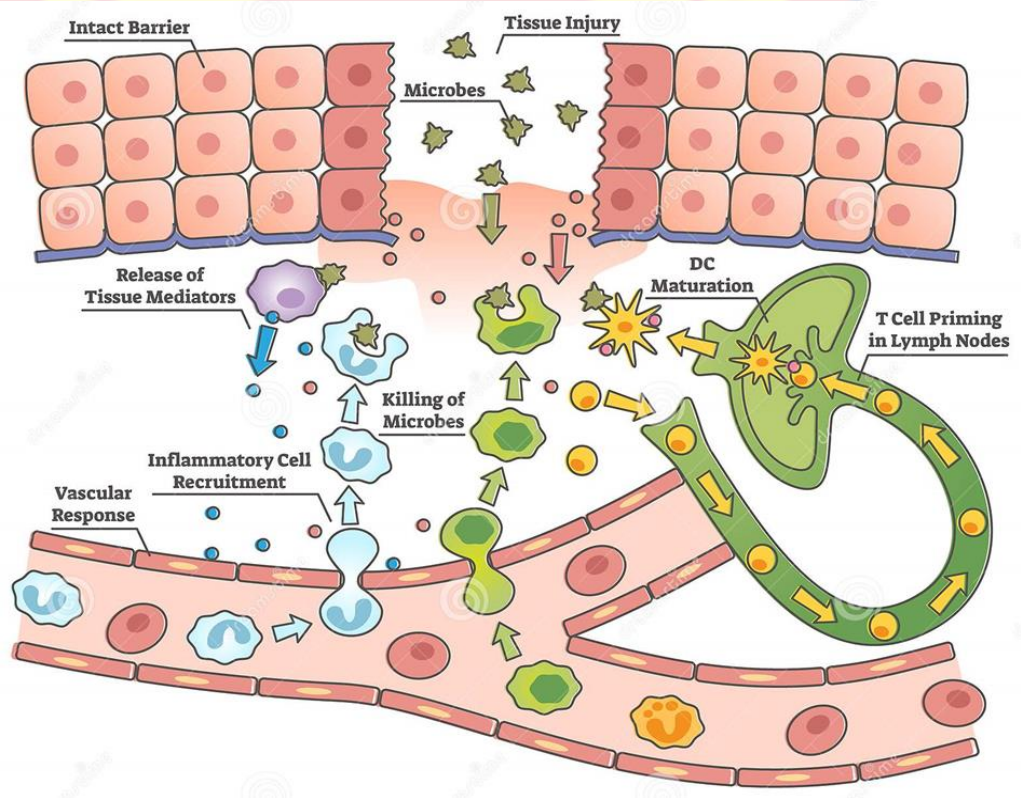


Figure 3: The inflammatory processes (Shaykhiev, 2007).

For this reason, anti-inflammation drugs are produced to ideally enhance inflammatory effects yet control its harmful sequelae. Inflammation process is categorised into acute and chronic stages:

Acute inflammation

Acute inflammation is a process of the body's defense against invasion of pathogens or injury. The process is somewhat short, lasting for minutes, several hours, or in some cases few days which is characterised by fluid exudation, plasma proteins, and the emigration of leukocytes, mainly

neutrophils (Pulichino *et al*., 2006). Neutrophils, a granulocyte, most often appear at the site of injury first. They engulf and destroy invading pathogens by releasing non-specific toxins such as hypochlorite, hydroxyl radicals and superoxide radicals that kill microorganisms and other abnormal cells (Pulichino *et al*., 2006; Schmid-Schönbein, 2006). Neutrophils also release cytokines that in turn induce the synthesis of reactant proteins and systemic inflammatory responses (Pulichino *et al*., 2006). Acute inflammation has three majors parts: (i) alterations in vascular caliber that results in increased blood flow, (ii) structural adjustment in the microvasculature that allows the plasma proteins and leukocytes to leave the circulation, and (iii) emigration of the leukocytes from the microcirculation and their aggregation in the site of injury (Pulichino *et al*., 2006; Schmid-Schönbein, 2006).

There are two different events of acute inflammation: (1) *vascular event*- this occurs as a result of vasodilation, increased vascular permeability and blood flow produced by pro-inflammatory agents. There is vasodilation at the arteriole level, generating to the capillaries that lead to a net increase in the quantity of blood present causing redness and heat. Increased vascular permeability leads to the movement of plasma into tissues resulting in stasis due to the high concentration of cells within blood; a typical condition of large blood vessels packed with cell [Figure 4] (Pulichino *et al*., 2006). The stasis allows leukocytes to migrate along the endothelium (Nourshargh & Alon, 2014). (2) *Cellular events* - usually occurs over a period of hours following severe infection or tissue damage. Leukocyte extravasation and phagocytosis are mainly involved in cellular response (Muller, 2013). Leukocytes are present at the site of injury due to accumulation of white blood cells, and invading agents, bacteria and

other microbes are damaged via phagocytosis (Nourshargh & Alon, 2014). Neutrophils are the major phagocytes that reach the site of injury first, and after hours of secretion, monocytes are released which consequently mature into macrophages [Figure 4] (Sunderkötter *et al*., 2004).

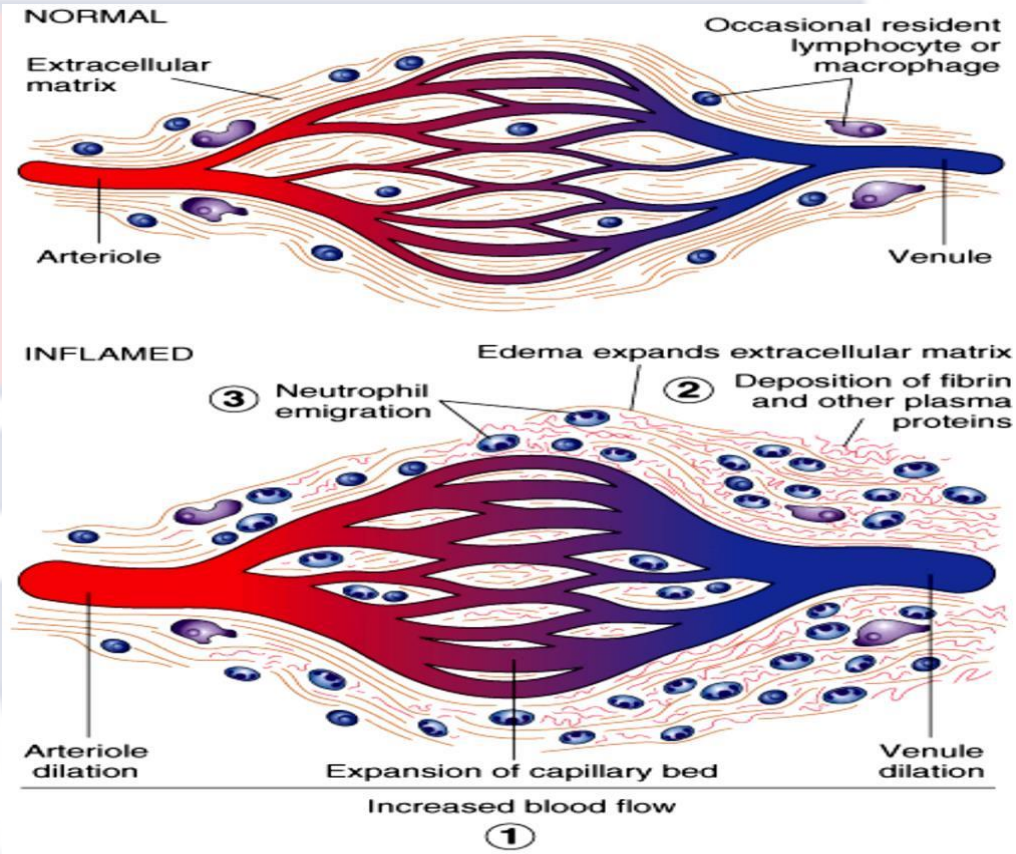


Figure 4: Acute inflammatory response: 1) increased blood flow through capillaries, 2) extravasation and deposition of plasma proteins and fluid (oedema), 3) leukocyte emigration and accumulation (Vinay, Abul, & Nelson, 2005).

Chronic inflammation

Chronic inflammation usually occurs when acute inflammation is deregulated persisting for days, weeks, months, or years. Chronic inflammation is believed to be untreated inflammation of prolonged duration in which tissue destruction, repair and healing are proceeding simultaneously (Weiss, 2008). Numerous diseases including chronic lung diseases, tuberculosis,

atherosclerosis, rheumatoid arthritis and chronic liver diseases occur as a result of chronic inflammation (Agarwal & Brenner, 2006).

Mullazehi, Mathsson, Lampa & Rönnelid (2007) outlined the various ways in which chronic inflammation occurs:

1. Persistent infection by certain microorganisms such as *treponema pallidum*, tubercle bacilli, and other fungi;
2. Continuous exposure to the potentially harmful agent, either endogenous or exogenous like particulate silica. Atherosclerosis is believed to be a chronic inflammatory response of the induced arterial wall, partly by endogenous toxic plasma lipid components.
3. Autoimmunity: immune reactions, under certain conditions, acts against the individual's tissues, resulting in autoimmune diseases. Normally, in autoimmune diseases, auto antigens trigger a self-acting immune reaction that leads to several chronic inflammatory diseases including rheumatoid arthritis and lupus erythematosus.

In chronic inflammation, cells such as lymphocytes, macrophages, and other cellular agents play active role that results in the proliferation of blood vessels, tissue necrosis, and fibrosis (Medzhitov, 2008).

Mediators of inflammation

The production of several inflammatory mediators is activated by inflammatory inducers, which sequentially modify the activities of many organs and tissues (i.e. the downstream effectors of the inflammatory pathway). Several inflammatory mediators have common effects on the recruitment of leukocytes and on the vasculature. There are numerous inflammatory mediators that are either derived from plasma proteins or secreted by cells [Figure 5]

(Medzihtov, 2008). These mediators are released during tissue injury. Plasma- and cell-derived mediators function to stimulate cells by binding to specific receptors, recruiting cells to the site of injury, and causing the release of more soluble mediators. The mediators are relatively short-lived, or can be intrinsically suppressed by certain mechanisms, effectively halting the response and allowing the process to resolve. The plasma-derived mediators are usually produced in the liver into circulation whereas cell-derived mediators are released at the site of inflammation (Jian, Pandey, & Shukla, 2015). Cell-derived mediators consist of several chemical groups such as lipid mediators (prostaglandins and leukotrienes), platelet activating factor (PLA), vasoactive amines (histamine and serotonin), and cytokines (interleukins [ILs], tumor-necrosis factor-α [TNF-α]) [Figure 5] (Jian *et al*., 2015).

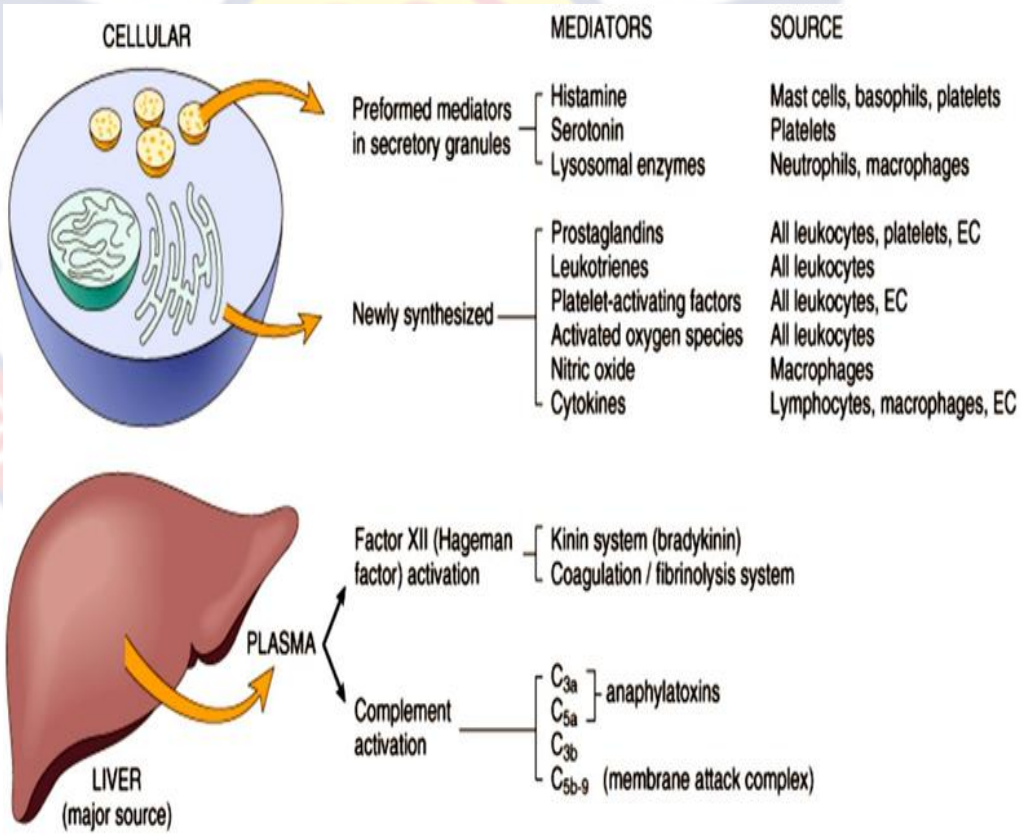


Figure 5: Mediators of inflammation (Vinay *et al*., 2005).

Histamine

Histamine (β-Imidazolylethylamine), a biogenic amine, is synthesised through decarboxylation of histidine (an amino acid) via enzyme L-histidine decarboxylase in the Golgi apparatus. They are mostly stored in platelets, basophils and mast cells, and exist in complex forms with mucopolysaccharide (glycosaminoglycan) as heparins (Marone, Granata, Spadaro, Genovese, & Triggiani, 2003). A rise in cytosolic Ca^{2+} triggers the release of histamine however, certain agents such as β-adrenoceptor agonists that increase cAMP (cyclic adenosine monophosphate), inhibit histamine secretion (Rang & Dale, 2007). Histamine is a vasoactive substance that causes local vasodilation, endothelial gap formation resulting in increased vascular permeability, nonvascular smooth muscle contraction, eosinophil chemotaxis and inhibiting the functions of T-lymphocytes (Haas, Sergeeva, & Selbach, 2008). Histaminic effects are mostly exhibited during the early phase of inflammation and cause bronchospasm, pruritus and severe oedema.

There are three subtypes of histamine receptors, which are G-protein coupled receptors. The H_1 receptors cause acute vascular effects as well as bronchial constriction of the smooth muscles (spasmogenic action) and activation of eosinophil chemotaxis. H_1 receptors activate the stimulation of protein kinase C (PKC) through the hydrolysis of phosphoinositide (IP). H_2 receptors become coupled to adenylyl cyclase in the lungs due to increased levels of cAMP (Jian *et al*., 2015). Unlike H_1, the H_2 receptors regulate some anti-inflammatory processes including the blockage of eosinophil chemotaxis, but can also induce vasodilatation. The H_3 receptors participate in controlling histamine release from different producing cells; their actions are brief

relatively and occur primarily as a result of intermediate-transient response from mild tissue injury (Akdis & Blaser, 2003). In addition, it acts as an inhibitory receptor on the central nervous system (Jian *et al*., 2015).

Serotonin

Serotonin [5-hydroxytryptamine (5-HT)] is produced by decarboxylation of tryptophan and is essentially released from mast cells, and stored in secretory granules. 5-hydroxyindoleacetic acid (5-HIAA), which is formed through degradation by monoamine oxidase, is eliminated in urine and serves as an indicator of 5-HT production in the body (Jian *et al*., 2015). In rodents, serotonin is stored in mast cell granules whereas in humans, is present in the central nervous system (CNS) as well as the gastrointestinal tract (GIT) but most are found in dense granules of platelets (Barnes & Neumaier, 2011). Serotonin is categorised into seven classes (5-HT [1-7]), one of these (5-HT-3) is a ligand-gated ion channel whiles the rest are G-protein coupled receptors (Rang & Dale, 2007). Serotonin, like histamine, is able to increase vascular permeability, dilate capillaries and produce contraction of the nonvascular smooth muscles. A variety of serotonin receptors exist which oversee its biological functions (Barnes & Neumaier, 2011). It stimulates the peripheral nociceptive endings, causes increase in gastrointestinal motility, excitation/ inhibition of the CNS, and platelet aggregation (Jian *et al*., 2015).

Prostanoids

Prostanoids are synthesised from arachidonic acid via COX (Cyclooxygenase), an enzyme associated with the endoplasmic reticulum of mast cells. They trigger the release of inflammatory mediators from inflammatory cells which activate inflammatory mediators' release from

monocytes, eosinophils, neutrophils and mast cells. Prostanoids include thromboxanes (Tx) and prostaglandins (PGs). COX catalyses the formation highly unstable endoperoxides such as prostacyclin-2 (PGI_2) and prostaglandin H-2 (PGH_2). They are non-enzymatically converted by isomerase synthase enzyme to yield the primary prostaglandins including prostaglandin D-2 (PGD_2), prostaglandin E-2 (PGE_2), and prostaglandin F-2 α ($PGF_{2\alpha}$) which are bioactive end-products of this reaction (Jian *et al*., 2015). In injury and many inflammatory diseases, arachidonic acid plays a key role.

PGE_2 is a key COX product which exhibits a broad range of biological actions in diverse tissues via binding to specific receptors on the plasma membrane (Hata & Breyer, 2004). It causes contraction of the gastrointestinal smooth muscles, bronchial, intestinal smooth muscles, dilatory action on the prostanoid receptor EP-2, and inhibition of gastric acid secretion. PGI_2 causes renin release, vasodilation and blockage of platelet aggregation. PGD_2 causes the release of pituitary hormones, relaxation of gastrointestinal and uterine muscles, inhibition of platelet aggregation, and vasodilation. $PGF_{2\alpha}$ is known to cause luteolysis, bronchoconstriction in some animals, and myometrial contractions in humans (Jian *et al*., 2015).

Kinins

Kinins are mostly characterised into two bioactive peptides. They are synthesised from the high and low molecular weight kininogens in plasma and tissues by the action of serine proteases, kallikreins. Plasma kallikrein, apart from hepatocytes, is found in several endocrine and epithelial cells whiles tissues kallikrein is mostly found in neutrophils, epithelial and endocrine cells. These peptides (bradykinins and lys-bradykinin [kallidin]) are degraded by

kininases rapidly in tissues. The numerous cellular actions of kinins are normally mediated by the activation of two distinct receptors, B_1 and B_2 (Bhoola & Fink, 2006). These receptors are G-protein coupled receptors that mediate specific but similar effects (Rang & Dale, 2007). Kinins are known to be associated with secondary production of other mediators including cytokines, nitric oxide, prostanoids, and mast cell-derived products. Bradykinin selectively binds to B_2 receptor (Leeb-Lundberg, Marceau, Müller-Esterl, Pettibone, & Zuraw, 2005).

Usually, bradykinin is activated in the synovial fluids of patients affected by various arthropathies such as osteoarthritis, psoriatic arthritis, rheumatoid arthritis, and gout (Meini & Maggi, 2008). Bradykinin is implicated in most pathological conditions, and usually causes vasodilation and increased vascular permeability leading to heat and oedema. Also, it's a potent pain-producing agent and plays a key role in bone resorption, cartilage matrix homeostasis and endothelial cell proliferation (Brechter & Lerner, 2007; Meini & Maggi, 2008).

Cytokines

Inflammatory cytokines are said to be non-immunoglobulin in nature and soluble glycoproteins released by host cells, acting non-enzymatically in molar concentrations via specific receptors to modulate function of the host cell during inflammation. They are produced by many cell types mainly from macrophages and mast cells. Cytokines comprise of tumor necrosis factor-α (TNF-α), chemokines, interleukins (ILs), interferons (IFNs), colony-stimulating factors, and growth factors (GFs). Cytokines play a major role in networks that involve antagonistic and synergistic interactions, and have both positive and

negative effects on numerous target cells. Individual cytokines may possess pleiotropic (multiple), overlapping and occasionally contradictory functions based on their concentration, the type of cell they act, and the presence of other mediators and cytokines [Figure 6] (Mclnnes & Schett, 2007). Except chemokines that bind to G-protein coupled receptors, all the rest activate kinase-linked receptors to regulate gene expression.

Chemokines are chemoattractant cytokines that regulate leukocyte migration, and function as traffic coordination in inflammatory and immune reactions. The main primary cytokines implicated in acute and chronic inflammatory processes including resolution and tissue repair are TNF-α and IL-1 (Jian *et al.*, 2015).

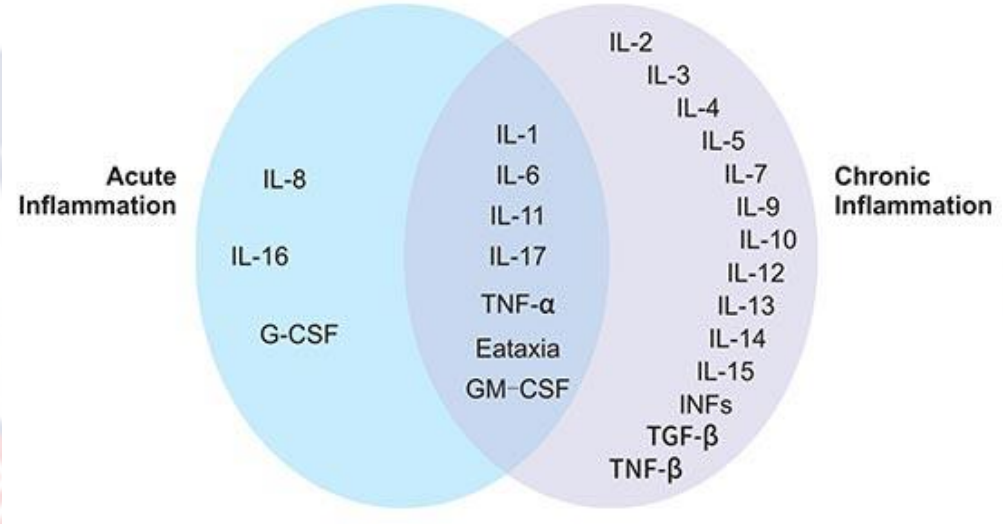


Figure 6: Cytokines involved in inflammatory responses (Feghali & Wright, 1997).

Cells involved in inflammation

Cells that play key roles in inflammation are either naturally present in tissues or mostly carried to injury site through the blood during inflammatory reaction. These include neutrophils, macrophages, mast cells and platelets.

Neutrophils

Neutrophils are major cells critical in inflammatory reactions during inflammatory conditions such as acute, chronic, autoimmune, infectious, and non-infectious disorders (Kolaczkowska & Kubes, 2013). In innate immunity, neutrophils are known to play a key function. However, recent studies have revealed that neutrophils are implicated during adaptive immunity to facilitate the recruitment and activation of antigen-presenting cells or direct acting T-cells. Neutrophils are the huge number of leukocytes in inflamed joints, and vital in the initiation and progression of inflammation (Wright, Moots, & Edwards, 2014). Hence, neutrophils play a relevant function in joint inflammation and the regulation of their effects is imperative as a possible target for pharmacological intervention in arthritis (Rosas, Correa, & das Graças Henriques, 2017).

Macrophages

The first cell type that leaves the marrow and enters the peripheral blood in an incomplete differentiated state is called monocytes. These cells reach the extravascular tissue and undergo transformation into macrophages which are larger phagocytic cells. Apart from its phagocytic activity, macrophages have the ability of being activated that results in increased cell size, enhanced lysosomal enzyme production, high active metabolism, and extensive potential to phagocytose and kill ingested microbes. These cells mature and form macrophages once they reach tissues. After activation by external stimuli such as microorganisms, macrophages may exhibit distinct morphology and functional features. Activated macrophages can combine to form multinucleated giant cells. Furthermore, they release cytokines including

interleukins (IL-6, IL-12, IL-10, IL-18, IL-1), TNF-α, and interferons (IFNs) that are implicated in the regulation of inflammatory reactions. In all organs and connective tissues, macrophages occur including microglial cells in the CNS, Kupffer cells in the liver, alveolar macrophages in the lungs and osteoclast in the bone (Fujiwara & Kobayashi, 2005). Functionally, macrophages exhibit antigen presentation, phagocytosis and immunomodulation (Firestein, 2004; Kasahara & Matsushina, 2001). Additionally, they synthesise biologically active molecules that play major roles in innate and adaptive immune response (Fujiwara & Kobayashi, 2005).

Mast cells

Mast cells are derived from specific precursors in the bone marrow which mature under the control of local tissue micro-environmental conditions through different cytokines such as stem cell factor [SCF] (Kitamura & Ito, 2005). Degranulation and production of cytokines are mostly triggered by mast cells through their cross-links with high affinity surface receptors for immunoglobulin E [IgE] (i.e. FcεRI) which does not induce degranulation by its own (Figure 7). Nerve growth factors (NGF) are also known to promote mast cell maturation that acts through tyrosine kinase receptors distinct from the C-kit activated by stem cell factor (SCF). Numerous vasoactive and pro-inflammatory meditators are secreted by mast cells upon activation including histamine, kinins, serotonins, cytokines (i.e. tumor necrosis factor-α [TNF-α], interleukins [ILs]), prostaglandins, leukotrienes, platelet activated factors and certain proteases stored in secretory granules [Figure 7] (Theoharides *et al*., 2012). Mast cells exert a key action in innate or acquired immunity (Galli *et al*.,

2005), bacterial infection (Galli & Tsai, 2010) and autoimmunity (Rottem & Mekori, 2005).

In addition, mast cells are responsible for Th17 cell maturation which is implicated in autoimmune disorders (O'Connor Jr, Zenewicz, & Flavell, 2010).

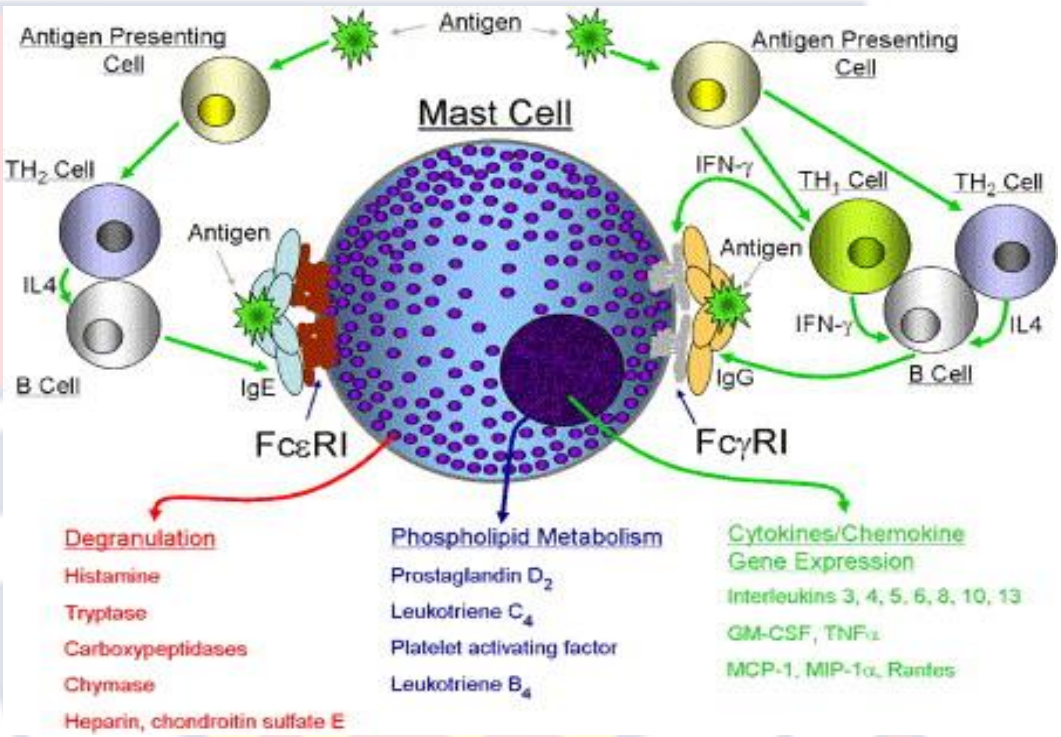


Figure 7: Regulation of mast cell activation and mediator release (Gilfillan & Beaven, 2011).

Platelets

Platelets play a critical role in maintaining vascular integrity and homeostasis. Platelets are discoid-shape fragments formed from bone marrow megakaryotes. Usually, platelets circulate in the blood for 7-10 days approximately after been released under the regulation of thrombopoietin [THPO] (Kaushansky, 2005) and are either eliminated from the spleen (Ab-mediated) and liver (Fc-dependent, desialytation, dependent), or in the lungs, and the brain under certain condition. In addition, they can be phagocytosed by macrophages in a von Willebrand factor (vWF)-dependent manner (Clautier *et*

al., 2018; Geys *et al*., 2018). At rest, prostacyclin (PGI_2) and nitric oxide (NO) are secreted by the endothelium and these inhibit platelet activation and aggregation. On platelet membranes, vWF forms links between the platelet glycoprotein (GP/Ib/V/IX) complex and exposed collagen which induce activation via binding to platelet receptors (GPIa/IIa and GPVI) (Thomas & Storey, 2015). Usually, THPO activates platelets, induces platelet-leukocyte aggregation, and causes the release of neutrophils by enhancing fMLP (N-Formylmethionyl-leucyl-phenylalanine)-mediated reactive oxygen species [Figure 8] (Tibbles, Navara, Hupke, Vassilev, & Uckun, 2002). Platelet activation through G-protein coupled receptor (GPCR) causes the release of soluble agonists including ADP and thromboxanes (TxAs) as well as inflammatory mediators. P-selectins also interact with leukocytes in endothelial cells via its P-selectin glycoprotein ligand 1 (PSGL-1) and are reported to express monocytes, neutrophils, eosinophils and hematopoietic cells [Figure 8] (Jenne *et al*., 2013; Thomas & Storey, 2015).

In addition, P-selectin cross-links between the platelets and leukocytes stimulate the upregulation release of pro-inflammatory cytokines such as ILs (IL-1[β, α]) and TNF-α (Yun, Eun-Hye, Ri-Young, Joo-In, & Jin-Yeong, 2016). Activated platelets also express CD40L, Platelet Factor 4 (PL4), chemokines (C-X-C Motif Chemokine Ligand) including CXCL1, CXCL5, CXCL7, CXCL12, and macrophage inflammatory protein (MIP)-1α as well as Regulated upon Activation, Normal T-cell Expressed and Secreted [RANTES] (Yun *et al*., 2016). Platelets are implicated in rheumatoid arthritis, neuro-inflammatory diseases and other inflammatory disorders [Figure 8] (Jenne & Kubes, 2015).

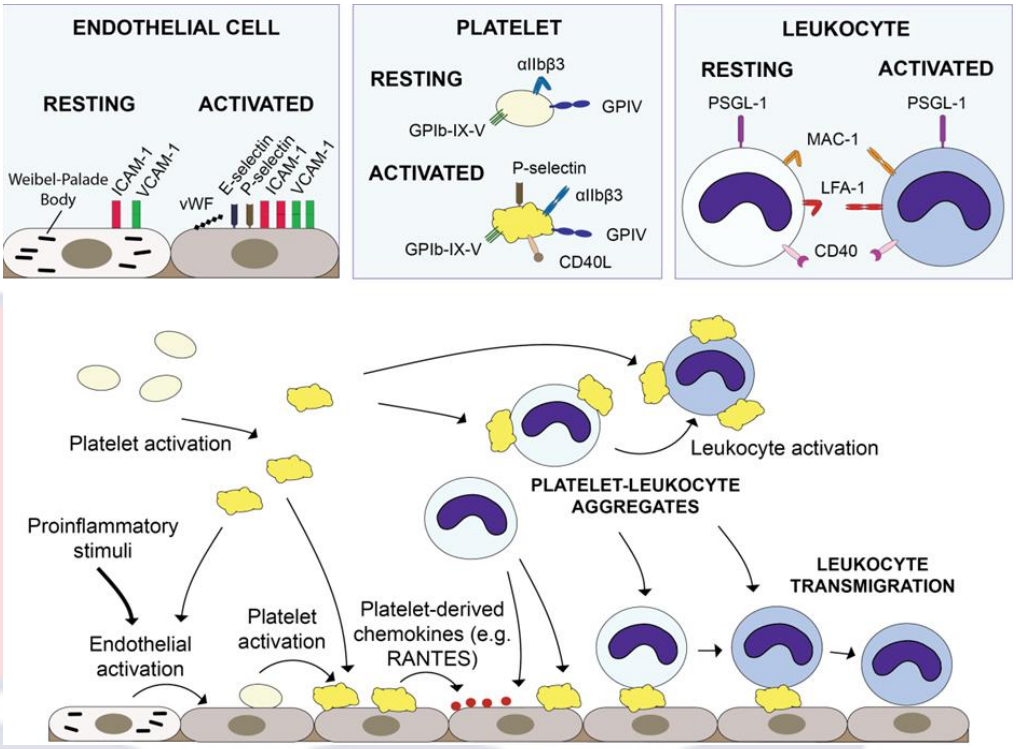


Figure 8: Platelet cross-links between the endothelium and leukocytes (Arman, Payne, Ponomaryov, & Brill, 2015).

Management of inflammation

In healthy state of numerous tissues and cell types involved in the initiation and termination of acute stage, inflammation is self-limiting (Schwab & Serhan, 2006). However, inflammation usually leads to tissue injury as a result of a direct damaging effect or the initiation of repair processes that modify the tissue function (Schmid-Schönbein, 2006). Anti-inflammatory agents are useful in the management of inflammation and some recommended drugs used to treat inflammatory diseases include non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and disease modifying anti-rheumatic drugs (DMARDs).

NSAIDs constitute an essential group of drugs that have been used for several centuries with potential therapeutic activities. They are used to manage various inflammatory conditions including rheumatoid arthritis (RA),

osteoarthritis and inflammatory pain (Rao & Knaus, 2008) but these agents have little effect on the primary tissue degenerative process that leads to bone and cartilage damage (Ding, 2002). NSAIDs act mainly by inhibiting the enzymatic actions of COX (cyclooxygenase) which is involved in the synthesis of prostaglandins resulting in their analgesic, anti-pyretic and anti-inflammatory effects. They inhibit both COX-1 and COX-2 or prostaglandin H synthase (PGHS) but the newer selective agents of COX (selective COX-2 inhibitors) predominantly block COX-2 [Figure 9] (Day & Graham, 2013). Prolong usage of NSAIDs (i.e. aspirin, naproxen, ibuprofen, diclofenac, celecoxib, indomethacin) is associated with gastrointestinal tract toxicities (perforations, ulcers, bleeding) and this has become a major drawback in their long-term therapy (Tamblyn *et al*., 1997).

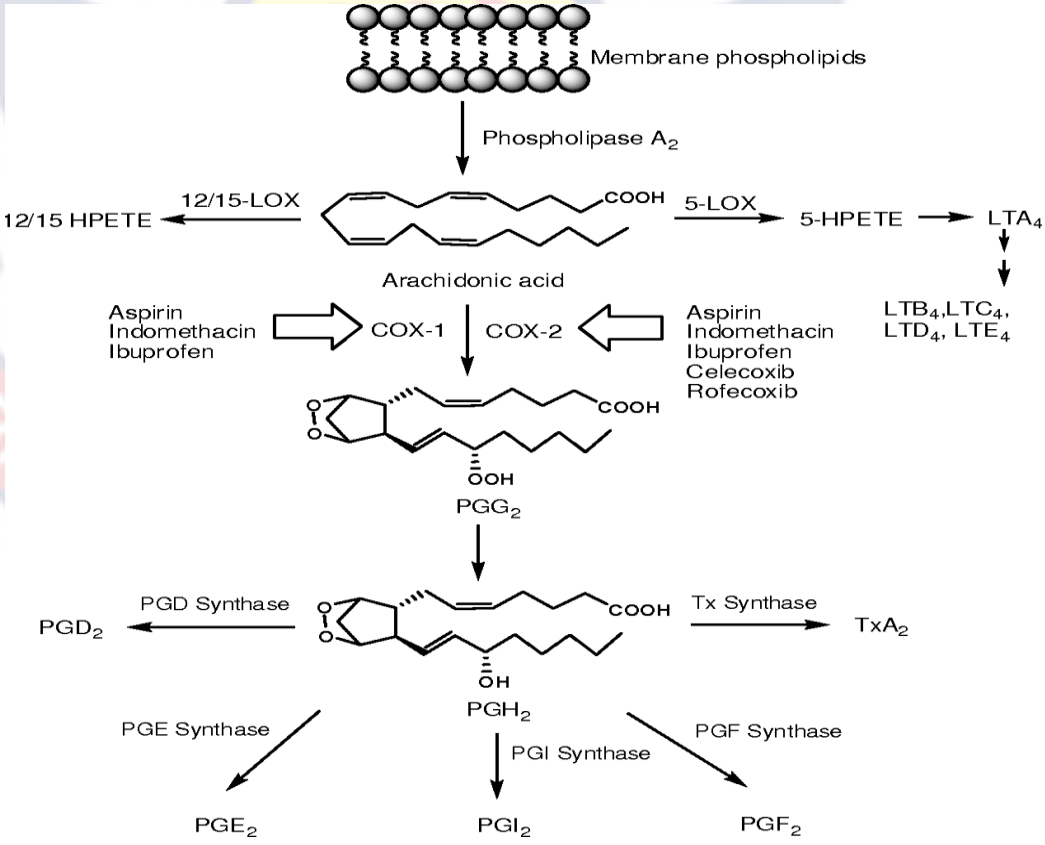


Figure 9: Biosynthetic pathway of prostaglandin (PG) from arachidonic acid (AA) via enzymatic actions COX-1/ COX-2 isoform (Rao & Knaus, 2008).

Glucocorticoids (GCs), a category of cholesterol-derived hormones, are released from the adrenal glands. They are mainly implicated in a wide range of immunosuppressive, anti-inflammatory, metabolic and cognitive signaling activities leading to homeostasis and preservation of normal organ function (Scherholz, Schlesinger, & Androulakis, 2019). GCs are the most relevant agents of systemic inflammation and maintain appropriate balance between pro-inflammatory and anti-inflammatory mediators which can trigger severe infections or diseases (Liu *et al*., 2013). They are widely used to manage both acute and chronic inflammatory disorders including eczema, rheumatoid arthritis, multiple sclerosis, allergic reactions, crohn's diseases, asthma, and immunological conditions (Scherholz *et al*., 2019). GCs including dexamethasone, prednisolone, cortisol etc. block pro-inflammatory cytokine production (i.e. ILs, TNF-α, INF-γ, chemokines) and modulate pro-inflammatory mediators under normal physiological conditions (Cruz-Topette & Cidlowski, 2015; Straub & Cutolo, 2016). Additionally, they regulate blood pressure, energy homeostasis, the cell cycle and bone resorption (Yan *et al*., 2013). GCs inhibit increased vascular permeability, vasodilation and reduce leukocyte migration as well as distribution as a result of inflammatory reactions (Perretti & Ahluwalia, 2000). Exerting their anti-inflammatory effects, GCs bind to glucocorticoid receptors (GC-receptors) which results in the blockade of nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) (Busillo & Cidlowski, 2013). Despite the numerous clinical benefits, GCs are associated with serious adverse effects including depression, obesity, osteoporosis, gastritis, glaucoma, cardiovascular disorders, and drug-induced hyperglycemia (Liu *et al*., 2013; Oster *et al*., 2017).

Disease modifying anti-rheumatic drugs (DMARDs) are a class of drugs used to manage various arthritic disorders such as rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis in order to arrest the disease progression as well as providing pain relief (Joshi & Dhaneshwar, 2014). They can also be used to manage other clinical conditions such as systemic sclerosis, inflammatory bowel diseases, systemic lupus erythromatosus, inflammatory myositis, vasculitis and other types of cancers (Abbasi *et al*., 2019). DMARDs are immunosuppressive and immunomodulatory drugs classified into either biologic or non-biologic disease modifying anti-rheumatic agents (Benjamin, Bansal, Goyal, & Lappin, 2021). Currently, non-biological DMARDs including azathioprine, sulfasalazine, hydroxychloroquine and methotrexate are used to relief pain and inhibit disease progression whiles biological DMARDs such as infliximab, abatacept, toclizumab and adalimumab have proven to be more efficacious with little side effects but less accessible and expensive. Generally, DMARDs act by the inhibition of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α, induction of apoptosis of inflammatory cells, elevation of chemotactic factors, suppression of synovial collagenase gene expression, depletion of B-cells, inhibition of T-cell activation, pyrimidine metabolism and purine synthesis (Joshi & Dhaneshwar, 2014).

Oxidants and anti-oxidants

Oxidants

Oxidants include reactive nitrogen species (RNS) and reactive oxygen species (ROS) that are variety of free and non-free radicals generated both in the body and environment usually as a result of aerobic metabolism (Fang, Yang, & Wu, 2002). Recently, more attention is being given to free radicals in

experimental or clinical medicine and biology (Schaller, 2005). The etiology of several human chronic diseases has been attributed to the actions of these chemical species (Figure 10) including cancer, neurodegenerative diseases, cardiovascular disorders, atherosclerosis and inflammatory lesions.

Oxidants and transcription factors

In addition to the regulatory function of oxidants, they are also involved in the binding of transcription factors. The intermediate production of hydrogen peroxide (H_2O_2) triggers the breakdown of I*k*B kinase (inhibitory component) and either tyrosine phosphorylation or increased ubiquitination. For nuclear factor-kappa B (NF-κB) to bind to deoxyribonucleic acid (DNA), redox-sensitive mechanisms are involved to allow binding via the reduction of a highly preserved cysteine residue (Lys-Cys-Arg). Activation of NF-κB promotes increased expression of series of genes that participate in inflammation, stress and infection. Nonetheless, this has been attributed to cellular protection from apoptotic death which is caused by chemotherapeutic substances, irradiation and TNF-α, as a result of formation of oxidants. Oxidants can also activate activator protein 1 (AP-1) which consist either homodimers or heterodimers of *Fos* and *Jun* proteins. The binding of DNA to either homodimers or heterodimers is usually regulated by the reduction-oxidation of one conserved cysteine residue present in the DNA-binding domain. Degradation of hypoxia-induced transcription factor 1 (HIF-1) is also possible, and DNA binding of HIF-1 is regulated via the redox-sensitive mechanisms in the same manner as those of the NF-κB system. Hence, several transcription factors are activated by oxidants and their capacity to bind to DNA at higher concentrations in the nuclear compartment can be blocked. Since activator protein 1 (AP-1) is

involved cellular proliferation and NF-κB is critical in inflammation, the regulation of these oxidant-induced transcription factors is a useful target for selective pharmacological intervention [Figure 10] (Comhair & Erzurum, 2002).

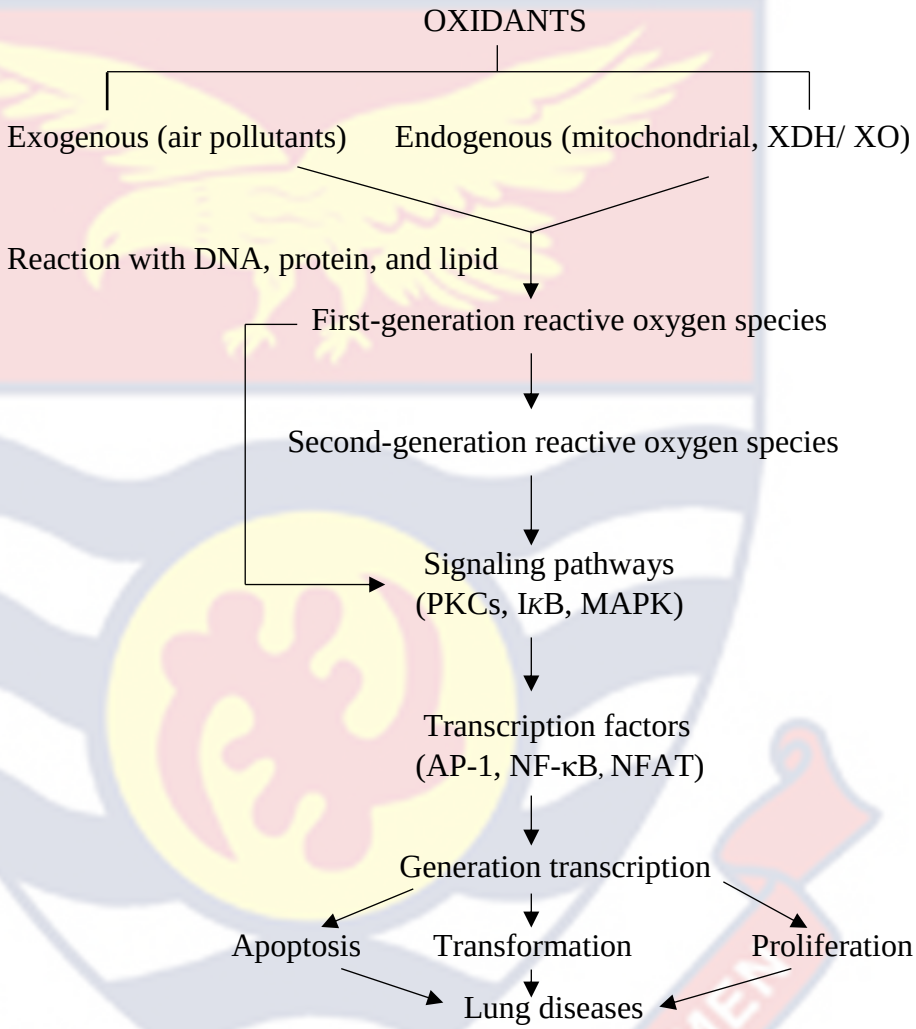


Figure 10: Regulation of cell signalling pathways and transcription factors by oxidants leading to several lung diseases. AP-1 (activator protein 1), NF-κB (nuclear factor kappa B), MAPK (mitogen-activated protein-kinase), PK-C (protein kinase C), NFAT [nuclear factor of activated T-cells] (Comhair & Erzurum, 2002).

Free radicals, Reactive oxygen species (ROS) and oxidative stress

Free radicals are group of molecules, atoms or ions that have an unpaired electron in the outer layer. These agents are unstable and very reactive, and usually when formed quickly interact with their surrounding molecules such as

proteins, nucleic acids and fats, causing structural changes and eventually damage the cell, lipids, proteins and DNA (Hazra, Biswas, & Mandal, 2008). Also, their activities normally lead to impaired organ function (Gemma, Vila, Bachstetter, & Bickford, 2007).

Reactive oxygen species (ROS)

These are derivatives of oxygen including both radical and non-radical species that are involved in a series of radical chain reactions (Genestra, 2007). Many genes, which undergo differenciation, development, cell-cell adhesion and encode transcription factors, are stimulated by ROS. They also stimulate cell signalling, involve in vasoregulation, increase anti-oxidant enzyme expression and cause fibroblast proliferation (Freitas, Gomes, Porto, & Fernandes, 2010). Though cells exhibit different defence mechanisms and repair processes against ROS, inadequate regulation can lead to oxidative stress in which anti-oxidant defences of the organisms are destroyed by ROS (Cui, Luo, Xu, & Ven Murthy, 2004). Superoxide (O_2^-) and hydroxyl (OH) are examples of radical oxygen species.

***Superoxide (O_2^-)*:** This is an anion formed via incomplete reduction of oxygen. Superoxide is the precursor of other reactive oxygen species and normally not harmful. It is very soluble in lipids and cannot cross membranes due its electronegative charge. Superoxide is produced from enzymatic reactions of CYP_{450} (cytochrome P_{450}) which catalyses the hydroxylation of substrates such as fatty acids, steroids and xenobiotics (Dröge, 2002). Superoxide anion can also be formed during respiratory burst in phagocytes specifically by the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase:

$$NADPH + O_2 \rightarrow NADP^+ + H^+ + O_2^-$$

Superoxide anion causes damage through the production of ·OH (hydroxyl radical) in a cascade of ion-catalysed reaction such as the Haber-Weiss reaction:

$$\cdot O_2 + H_2O_2 \rightarrow O_2 + \cdot OH + \cdot OH$$

***Hydroxyl radical (·OH)*:** Hydroxyl radical is produced in the ion-catalysed reaction of the Fenton reaction in which Fe^{2+} is catalysed by the transformation of H_2O_2 into ·OH:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{+3} + OH^- + \cdot OH$$

The formation of hydroxyl radical occurs also after the exposure of γ-ray in cells. After *in vivo* formation of ·OH, it usually reacts close to its site of production (Valko *et al*., 2007). ·OH can easily penetrate cell membrane (Dröge, 2002) with a very short half-life of 10^{-9} *in vivo* approximately (Pastor, Weinstein, Jamison, & Brenowitz, 2000) and is considered to be the most harmful ROS with a high ability to cause damage. Hence, elimination of these radicals is necessary to avoid tissue damage (Dröge, 2002).

Non-radical oxygen species include hydrogen peroxide (H_2O_2), ozone (O_3) and singlet oxygen (O_2) that can lead to free radical reactions quickly in living organisms (Genestra, 2007).

***Ozone (O_3)*:** This is a harmful oxygen species that oxidises proteins, lipids and nucleic acids (Cui *et al*., 2004).

***Single oxygen ($^1O_2$)*:** During biological reactions such as absorption of energy, peroxidation and respiratory burst in phagocytes, single oxygen is formed. It is known to be non-radical but more reactive that can cause damage. Sometimes, excess energy of single oxygen is passed on to other molecules which transforms $^1O_2$ to its 'rest' triplet state (Dröge, 2002). The action of $^1O_2$

on polyunsaturated fatty acids has been reported to induce several carcinogenic, genotoxic and mutagenic effects (Cui *et al*., 2004).

***Hydrogen peroxide (H_2O_2)*:** This is the most stable oxidant and less reactive. However, it is more damaging, moves far from its site of production and rapidly diffuses across membranes (Phaniendra, Jestadi, & Periyasamy, 2015). In the presence of metal ion under favourable conditions, H_2O_2 can be converted to reactive ·OH (Aprioku, 2013). H_2O_2 is produced through non-enzymatic or superoxide dismutase (SOD)-catalysed reaction as follows:

$$O_2{}^{\cdot -} + O_2{}^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Also, H_2O_2 can be produced specifically by other oxidase enzymes such as amino acid oxidase and monoamine oxidase. In peroxisomes, H_2O_2 production is abundant during long chain fatty acid degradation. H_2O_2 can react with chloride ions (Cl^-) to form hypochlorite (ClO^-) and hydroxyl radicals through a reaction as follows:

$$H_2O_2 + Cl^- \rightarrow HClO + \cdot OH$$

ClO- is highly reactive and causes oxidative damage to cellular apparatus (Marnett, Riggins & West, 2003). It is a potent oxidant which goes through series of oxidative reactions (Winterbourn, 2002) and also, inactivates anti-proteinases such as α-2-macroglobulin (Siddiqui, Zia, Ali, Ahsan, & Khan, 2018). In disease conditions such as atherosclerosis, ClO^- modified proteins can be detected (Van der Veen, de Winter, & Heeringa, 2009) and are involved in the pathology of health conditions such as arthritis and Alzheimer's disease (Wyatt *et al*., 2014).

Oxidative stress takes place when there is an imbalance between pro-oxidant and inhibitory anti-oxidant defences. Both acute oxidative stress

together with chronic oxidative stress are involved in several human degenerative disorders (Figure 11) that alter a vast range of physiological activities (Cui *et al*., 2004). Oxidative stress can cause damage to almost all target agents such as lipids, DNA and proteins. The tissue injury cause by oxidative stress occurs by either apoptosis or necrosis mechanism (Guetens, De Boeck, Highley, van Oosterom, & de Bruijn, 2002). This plays a major role in inflammation, affects ageing and promote various degenerative disorders [Figure 11] (Galli *et al*., 2005).

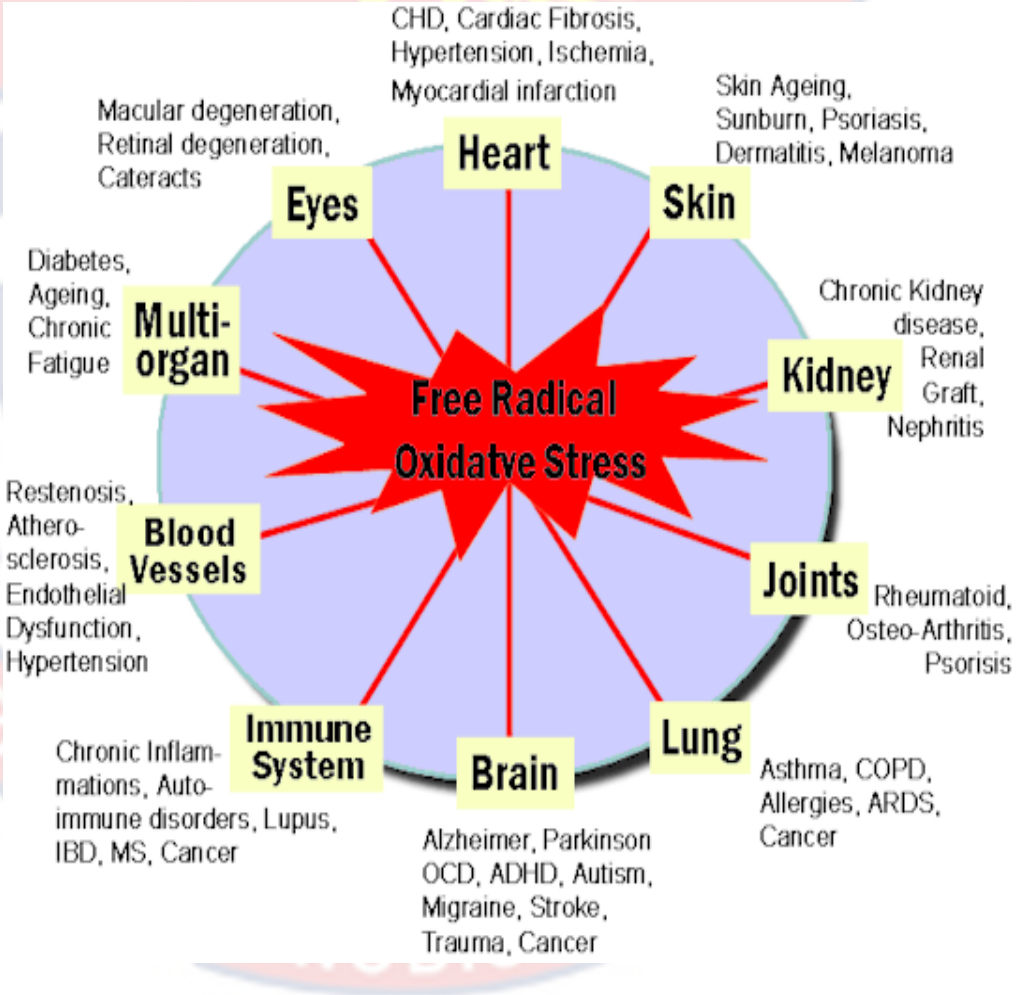


Figure 11: Human diseases caused by the production of free radicals (Galli *et al*., 2005).

Anti-oxidants

Anti-oxidant is an agent or substance that at low concentration is capable to significantly inhibit or delays oxidative stress activity. In the same manner, anti-oxidants prevent oxidative damage to target cells (Halliwell & Gutteridge, 1995). These molecules inhibit, reduce or eliminate completely the action of free radicals and oxidants thereby protecting the body against oxidative damage (Lobo, Patil, Phatak, & Chandra, 2010). Several reports have been made to support the fact that anti-oxidants are useful radioprotectors that act to prevent numerous human diseases including rheumatoid arthritis, cancer, atherosclerosis, neurodegenerative diseases, stroke and diabetes (Fang *et al*., 2002). The defence mechanism exhibited by anti-oxidants is universal within plant and animal tissues or cells at different concentrations depending on the type of anti-oxidant (Ali *et al*., 2020). Anti-oxidants exert their activities either through enzymatic [i.e. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)] and non-enzymatic (glutathione, uric acid, vitamin E, carotenoids, vitamin C etc.) mechanisms [Figure 12] (Gomes, Silva, & Oliveira, 2012).

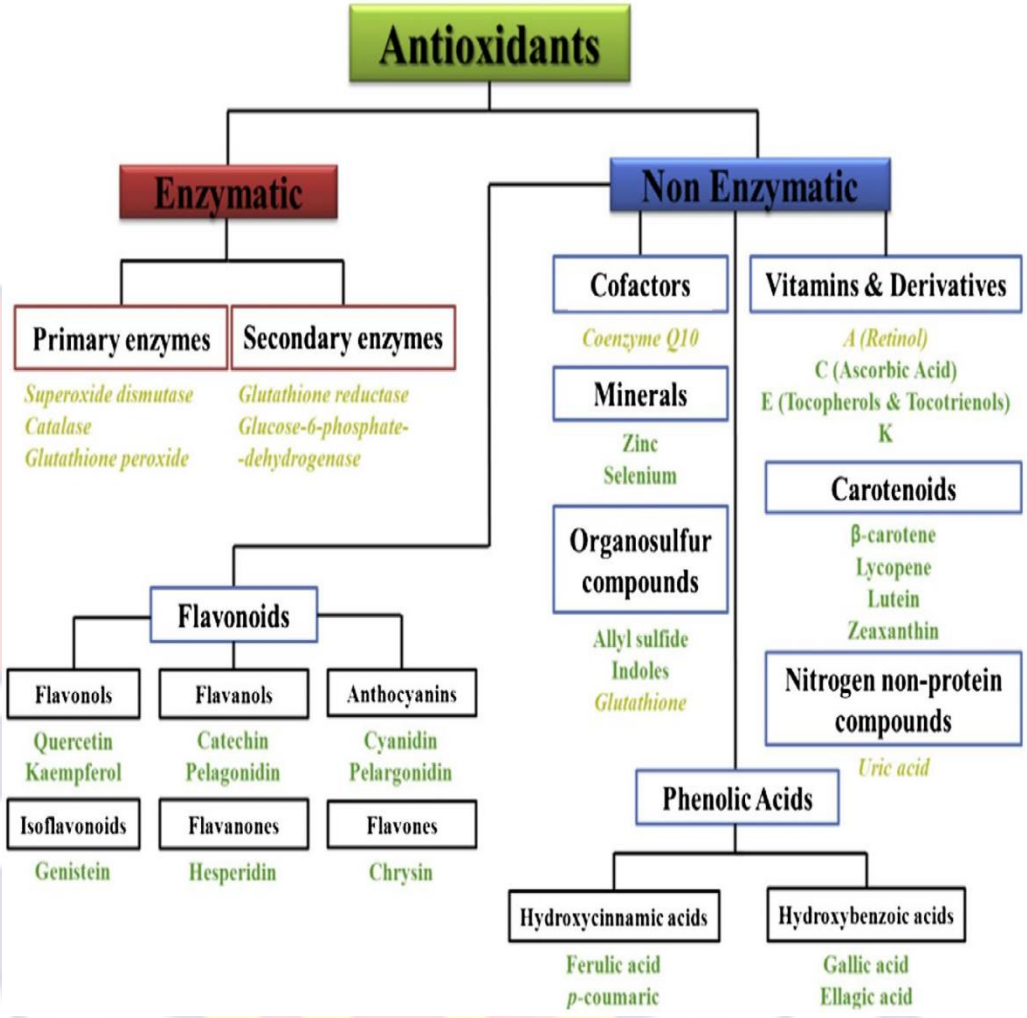


Figure 12: Classification of anti-oxidants (Carocho & Ferreira, 2013).

Enzymatic anti-oxidants

Superoxide dismutase (SOD)

This is an enzyme widely distributed in all tissues (65-85 % in cytosol, 15-35 % in mitochondria) with extensively high activity. SOD catalyses the conversion of superoxide ($\cdot O_2^-$) into oxygen (O_2) and hydrogen peroxide (H_2O_2). The reaction requires two superoxide anions and two protons.

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

It is a metalloenzyme that needs a cofactor for its activity and depending on the type of metal ion (iron, manganese or copper) involve but several forms exist. SOD is categorised in three forms: a) Fe-SOD, primarily present in

prokaryotes and chloroplast of some plants, b) MnSOD is found in mitochondria and cytosol of bacteria, and c) CuSOD is mainly present in cells and crucial for aerobic life (Dringen, Pawlowski, & Hirrlinger, 2005).

Catalase (CAT)

This is a haemoprotein extensively distributed within cells including blood cells, liver, bone marrow and kidney. CAT has peroxisomes (small organelles) carrying oxidases that produce hydrogen peroxide. The main function of CAT is the dismutation H_2O_2 into H_2O and O_2 [Figure 13]:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

However, CAT has a little affinity for H_2O_2 (Powers & Jackson, 2008) and this is a critical cell defence action against oxidative damage by H_2O_2 (Glorieux & Calderon, 2017) since H_2O_2 is implicated in several biological processes including proliferation, apoptosis, changes in morphology, and signaling [i.e. NF-κB] (Sies, 2017). Also, CAT can be found in microsomes, cytosol and mitochondria at low concentrations (Glorieux & Calderon, 2017). CAT has been reported to degrade peroxynitrite (Heinzelmann & Bauer, 2010) and oxidise nitric oxide (NO) to nitrite (NO^{2-}) (Brunelli, Yermilov, & Beckman, 2001).

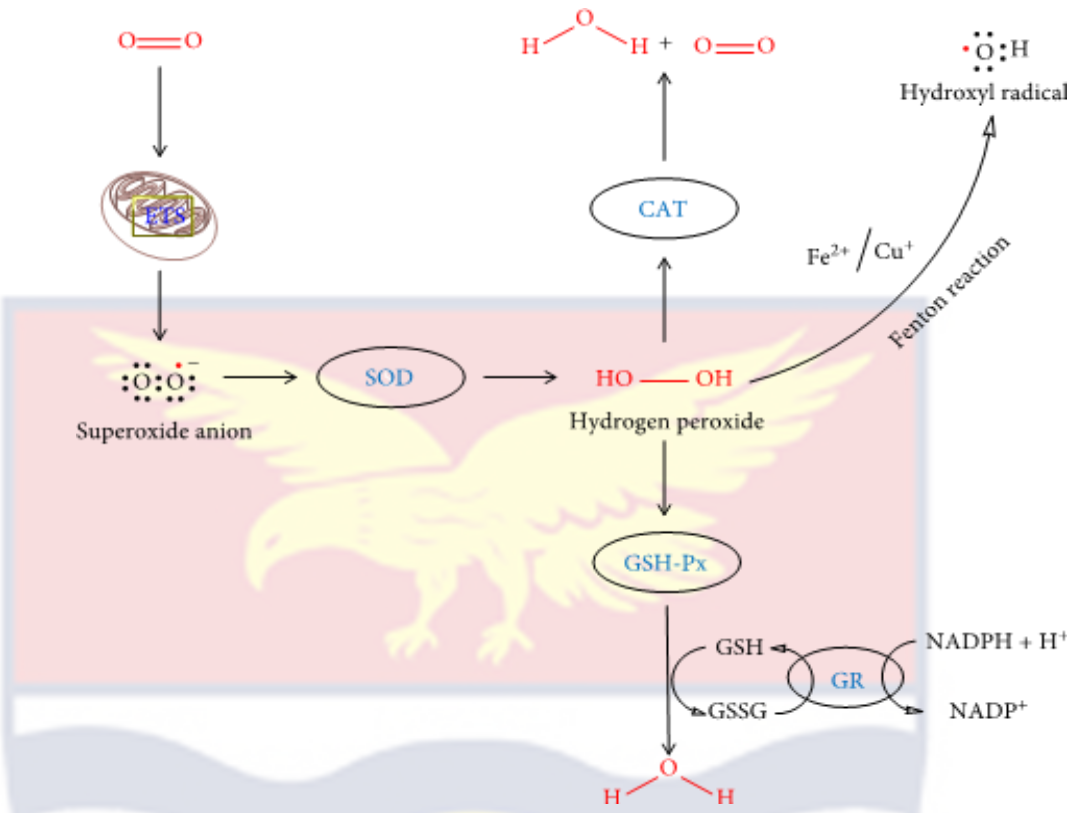


Figure 13: Relationship between catalase and other anti-oxidant agents (Nandi, Yan, Jana, & Das, 2019).

Glutathione peroxidase (GPx)

GPx is an enzyme that provides a second-line of defence against oxidative stress. GPx catalyses the reduction of H_2O_2 (hydrogen peroxide) and ROOH (organic hydroperoxides) to H_2O and alcohol that requires GSH (glutathione) as a substrate (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012):

$$ROOH + 2GSH \rightarrow GSSG + ROH + H_2O$$

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

GPx is present in mitochondria and cytosol. GPx, like CAT, is found in most animal cells (Sharma, Jha, Dubey, & Pessarakli, 2012). There are two classic forms of GPx: selenium dependent and selenium independent. In mammalian tissues, four different kinds of selenium-dependent GPx isozymes

are found. These include: (i) classical GPx [GPx1], (ii) gastrointestinal GPx [GPx2], (iii) plasma GPx [GPx3], and (iv) phospholipid GPx [GPx4]. Organs such as kidney, liver, lungs as well as red blood cells contain GPx1. GPx2 and GPx3 can be found everywhere in the body but are mostly present in the heart, lung, muscle, epididymis, kidney, vas deferens, seminal vesicle and placenta (Margis, Dunand, Teixeira, & Margis-Pinheiro, 2008). GPx is critical in providing a multicomponent anti-oxidant defence system as a protection for cell membrane polyunsaturated fatty acids. It reduces fatty acid hydroperoxides (Gathawala & Aggarwal, 2016). Under high concentrations of ROS, GPx is mostly activated first and also implicated vitally in the detoxification of ROS (Duggett *et al*., 2016; Halliwell & Gutteridge, 2015). GPx alters the oxidation of sulfhydryl groups in proteins as well as other compounds. Furthermore, an NADPH-dependent enzyme (i.e. glutathione reductase) reduces oxidised glutathione (GSSG) and GPx plays an important role to maintain GSH-GSSG ratio. High levels of GSSG in cells often lead to lipid peroxidation, DNA breakage and protein denaturation (Zitka *et al*., 2012).

***In vitro* assay methods of anti-oxidant activity**

To assay anti-oxidants, several *in vitro* methods are used. The methods can be grouped into two: direct and indirect methods (Laguerre, Lecomte, & Villeneuve, 2007).

Direct methods: In direct anti-oxidant assay, an oxidisable substrate is required. When a substrate in a test system subjected to natural or accelerated conditions, the inhibitory activity of a possible anti-oxidant agent can be assessed in these methods on the oxidative degradation of the substrate. Mostly, the oxidisable substrate comprises either single or mixed lipids, chromophores, fluids

containing biological active species, DNA, low-density lipoproteins (LDLs) or plant proteins and biological membranes including fluorophores. One example of the direct anti-oxidant assay methods is the lipid peroxidation (linoleic acid autoxidation method).

The *in vitro* anti-oxidant assay processes are relevant in evaluating mechanism of anti-oxidant effect of test anti-oxidant sample.

Indirect methods: In these methods, an oxidisable substrate is not needed. Generally, the ability of a potential anti-oxidant agent to reduce a transition metal (easily by electron transfer) or a stable artificial free radical (by hydrogen or electron transfer). Examples include total anti-oxidant capacity test, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing anti-oxidant assays.

Inhibition of Linoleic acid autoxidation method (lipid peroxidation)

This is a chain reaction in which oxidative destruction of polyunsaturated fatty acids occurs via addition of oxygen radical or hydrogen abstraction. The fatty acid radical (i.e. product) is stabilised by readjustment into a conjugated diene to maintain high stable products such as alkanes, hydroperoxides, alcohols as well as aldehydes. Malondialdehyde (MDA) is extensively used oxidised derivative among the many different aldehydes that are produced in the lipid peroxidation as an indicator for free radical damage in an assay with thiobarbituric acid (TBA). The degree of lipid peroxidation reduction is as a result of the incorporation of an anti-oxidant in the reaction. At room temperature, linoleic acid (polyunsaturated fatty acid) oxidises slowly. Peroxidation reaction is accelerated during incubation at a higher temperature (normally 40 ℃). At the primary phase, early degradation products that are formed include a variety of cyclic peroxides and hydroperoxides. During the

primary stage of lipid peroxidation, the quantity of peroxides is determined by ferric thiocyanate (FTC) methods whiles thiobarbituric acid (TBA) methods quantify the generation of carbonyl compounds degraded from the peroxides at the secondary stage (Nieto, Ros, & Castillo, 2018). During linoleic acid oxidation, the peroxides produced in the FTC method oxidise blue Fe^{2+} (in $FeCl_2$) to reddish brown Fe^{3+} (in $FeCl_3$). The absorbance of the Fe^{3+} ions formed is measured at 500 nm after quantifying the ions by complexing with SCN^- (from ammonium thiocyanate). The anti-oxidant activity is inversely proportional to the number of peroxides produced from linoleic acid and the Fe^{3+} ions. Thus, lower absorbance signifies a greater state of anti-oxidant activity.

Melondialdehyde (MDA) formation forms the principle for notable TBA method usually employed to assess the degree of lipid peroxidation. MDA binds to TBA at low pH and high temperature (100 °C) to develop a pink complex (TBA-MDA adduct) in which the absorbance is measured at 532 nm (Figure 14). The degree of peroxidation of linoleic acid depends on the intensity of pink colouration produced. Low levels of chromogenic product (TBA-MDA) is formed as a result of anti-oxidant activity.

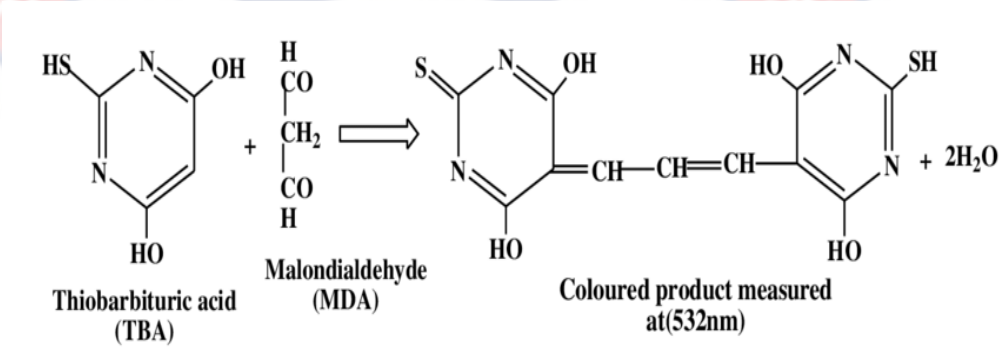


Figure 14: Reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) forming MDA-TBA complex [coloured product] (Alhamadany Mahdy, Hamid, Yousif, & Alkass, 2013).

The percentage inhibition of linoleic acid oxidation can be determined by:

$$\% \text{ Inhibition} = \frac{(FRM-L)-(DRUG/\ EXTRACT\ -DRUD/\ EXTRACT\ ALONE)}{(FRM-L)}$$

Where

FRM implies the extent linoleic acid oxidation without an anti-oxidant.

L indicates the initial peroxidation of linoleic acid prior to the onset of accelerated autoxidation at 40 ℃ incubation.

Extract/ Drug alone indicates the absorbance of extract/ drug samples being examined.

Total anti-oxidant capacity

This test is a spectrophotometric method emplyed to determine anti-oxidant activity quantitatively (Prieto, Pineda, & Anguilar, 1999). The principle is based on Mo (VI) reduction to Mo (V) using the anti-oxidant agent and the resultant generation of a Mo (V) complex (green phosphate) at acidic pH with a maximum absorption level of 695 nm. The greater the anti-oxidant capacity, the greater the absorption of the green complex. Similar to the total phenol assay method, this method is quantitative and is determined as the amount of equivalents of α-tocopherol or ascorbic acid.

Total phenol assay

The principle is based on reducing phosphomolybdate-phosphotungstate salts of Folin-Ciocalteau reagent in alkaline medium by phenolic substances (Slinkard & Singleton, 1977). Using spectrophotometer, the reduced blue colour of Folin-Ciocalteau reagent can be measured at 760 nm. Hence, the greater the levels of phenolic substances, the higher the extent of reduction and the greater the absorbance.

Reducing power test

To assess anti-oxidant activity of a substance, reducing power test is one of the methods used. As described by Oyaizu (1986), the method is based on the capacity of the test sample to reduce Fe^{3+} to Fe^{2+}. In the formation a Prussian blue complex measured at 700 nm spectrophotometrically, the reduced ions (Fe^{2+}) further react with ferricyanide ion.

$$Fe^{3+} \rightarrow Fe^{2+}$$

$$K_3Fe(CN)_6(aq) + Fe^{2+}(aq) \longrightarrow KFe[Fe(CN)_6(s) + 2K^+(aq)$$

The higher the reducing capacity, the higher the concentration of blue complex and the greater the absorbance.

DPPH scavenging assay

As described by Blois (1958), DPPH (2, 2-diphenyl-1-picrylhydrazyl hydrate) assay, is very simple, quick and efficient process to assess the free radical scavenging capacity of anti-oxidant substances in natural extract from plants. Characteristically, DPPH is a violet colouration and a very stable radical. In the presence of a free radical quencher, DPPH is changed to yellow via the appearance of reduced 2, 2-diphenyl-1-picrylhydrazine (Figure 15).

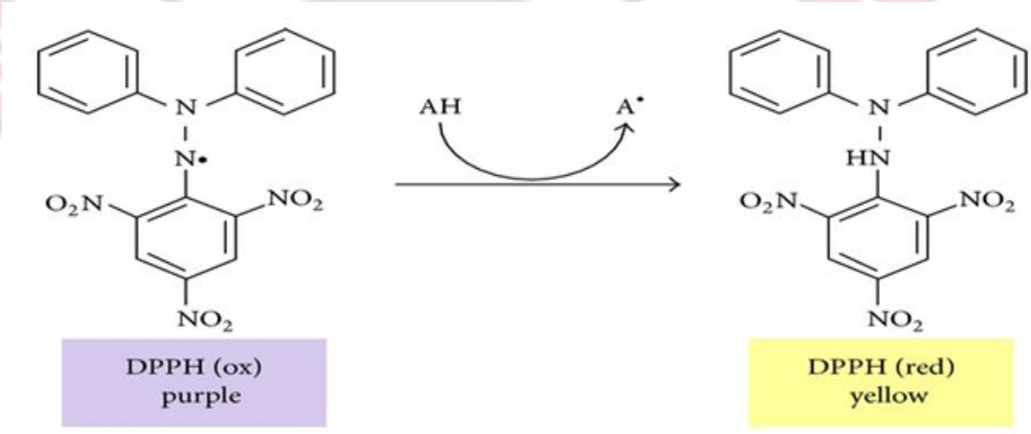


Figure 15: Schematic diagram of DPPH scavenging free radical capacity (Teixeira, Gaspar, Garrido, & Borges, 2013).

An extract with free radical scavenging capacity can degrade free radicals and any excess free radicals, and absorbance is determined spectrophotometrically. The percentage scavenging activity can be expressed as:

$$\% \text{ Scavenging} = \left(\frac{ABSORBANCE_{Control} - ABSORBANCE_{Test}}{ABSORBANCE_{Control}}\right) \times 100$$

Plant as source of anti-oxidant

Plants contain remarkable quantity of anti-oxidants to inhibit oxidative stress via oxygen and photons during photosynthesis (Auddy *et al*., 2003). Anti-oxidants incorporated in natural plant sources consumed in diets daily can be suitable remedy to resolving numerous human health conditions. As a preventive medicine, natural anti-oxidant plant sources can be used since they provide protection against the generation of free radicals and are critical therapeutic compounds that reduce disorders associated with oxidative stress (Ravipati *et al*., 2012). Many researchers have recently reported that dietary consumption of antioxidant-rich food is inversely linked to the prevalence of human diseases (Krishnaiah, Sarbatly, & Nithyanandam, 2011). Plant derived-anti-oxidants include vitamins (Vit A, Vit E, Vit C), polyphenols (flavonoids and non-flavonoids), small molecules (glutathione, ubiquinone) and other trace elements [selenium] (Chabert, Auger, Pincemail, & Schini-Kerth, 2014). Flavonoids and phenolic acids are said to be the major anti-oxidant compounds in vegetables and fruits (Wu & Ng, 2008). Also, other studies have reported that trace of metals including Mn, Zn, Se, Cu and Mg exert significant functions in anti-oxidant system (Ravipati *et al*., 2012).

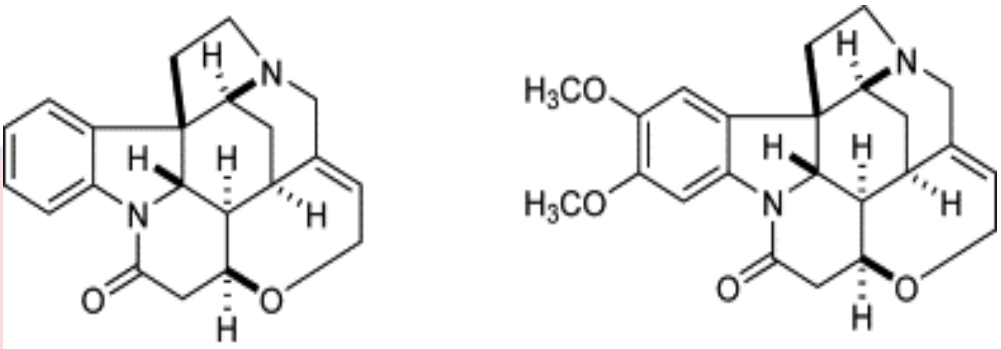
Phytochemical constituents

Numerous new drugs derived from plant phytochemical constituents are used to manage many diseases (Menichini *et al*., 2009). In recent years, plant secondary metabolites have gained much interest and attention due to their numerous health benefits including anti-inflammatory and anti-oxidant activities (Harbourne, Marete, Jacquier, & O'Riordan, 2013). Examples of such secondary metabolites include alkaloids, tannins, saponins, flavonoids, glycosides and terpenoids.

Alkaloids

Alkaloids, a very diverse class of phytochemicals, are nitrogenous compounds (i.e. possess a baisic nitrogen atom) of low molecular weight such as strychnine and brucine [Figure 16] with a wide range of pharmacological activities (Alves de Almeida *et al*., 2017; Fernando, Nah, & Jeon, 2016) including anti-depressant, anti-hypertensive, diuretic, anti-microbial, myorelaxant, analgesic, anti-tumor, anti-viral, anti-ulcer and anti-inflammatory effects (Alves de Almeida *et al*., 2017; Sobarzo-Sánchez, 2014). They have a phenolic hydroxyl end (functional group) [Figure 16] that explains its unique bioactivity (Cushnie, Cushnie, & Lamb, 2014). Alkaloids have high ionisation profile with potential ability to form stable salts making them good therapeutic agents (Souza, Bezerra, & Souto, 2020) of desirable pharmacokinetic properties (Thomas, 2007). Alkaloids are considered to be potent anti-oxidants (Czapski *et al*., 2014; Li *et al*., 2016) and mostly, reported to exhibit their anti-inflammatory activity via downregulation of NF-κB, TNF-α, IFNγ, KC (keratinocyte chemoattractant) and IL-17 levels (Somani *et al*., 2015), and

inhibition of enzymes such as iNOS and COX-2 involved in inflammatory responses (Beg, Swain, Hasan, Barkat, & Hussain, 2011).

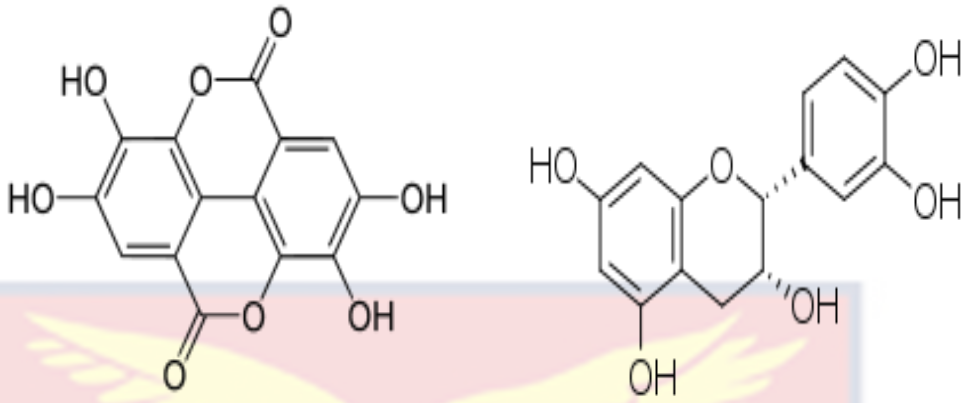


A **B**

Figure 16: Chemical structures of alkaloids. A [Strychnine] and B [Brucine] respectively (O'Connor, 2010).

Tannins

Tannins, a diverse and heterogeneous group of phytochemicals, are reported to possess numerous biological activities including anti-oxidant, anti-inflammatory (Fraga-Corral *et al*., 2021), anti-bacterial (Mainasara, Aliero, Aliero, & Yakubu, 2012), anti-cancer, anti-hepatotoxic, anti-atherosclerosis, and anti-HIV replication activities (Landete, 2011). Generally, they are known to contain phenolic rings with hydroxyl groups [e.g. gallic acid and procyanidin] (Figure 17) having high ability to bind to several molecules, and act as scavengers against radical species (De Hoyos-Martínez, Merle, Labidi, & Charrier-El Bouhtoury, 2019; Voulo, Lima, & Maróstica Junior, 2019). Also, tannins are astringenic compounds known to form protective layer in the skin and mucous membranes which is hydrophobic and can prevent disease. This confirms the use of natural products containing tannins in the management of wounds and cuts as well as intestinal inflammation (Watal, Gupta, Chatterji, & Yadav, 2014).



A **B**

Figure 17: Chemical structures of tannins. A [gallic acid] and B [procyanidin] respectively (Seigler & Seigler, 1998).

Saponins

Saponins, glycosylated and high molecular weight phenolic compounds with both hydrophilic glucidic chain and lipophilic structures (Figure 18), are known to possess numerous pharmacological activities (Liu & Henkel, 2002) including anti-viral, anti-bacterial (Sparg, Light, & van Staden, 2004), anti-tumor, anti-microbial, hepatoprotective, anti-ulcer, anti-allergic and anti-inflammatory effects (Fang, Li, Yang, Fang, & Zhang, 2020; Tian, Zhang, Long, & Zhang, 2017). They are also reported to regulate oxidative stress-related disorders (Biapa, Agbor, Oben, & Ngogang, 2007). Saponins inhibits cAMP phosphodiesterase (Tian *et al*., 2017) and exerts their anti-inflammatory effects via the inhibition of pro-inflammatory cytokines including TNF-α, IL-6, NF-κB, and Janus kinase-2 (JAK2) signaling pathway (Fang *et al*., 2020), synthesis of pro-inflammatory mediators such as prostaglandins (Yassin, Melek, Selim, & Kassem, 2013), and COX (Moghimipour & Handali, 2015).

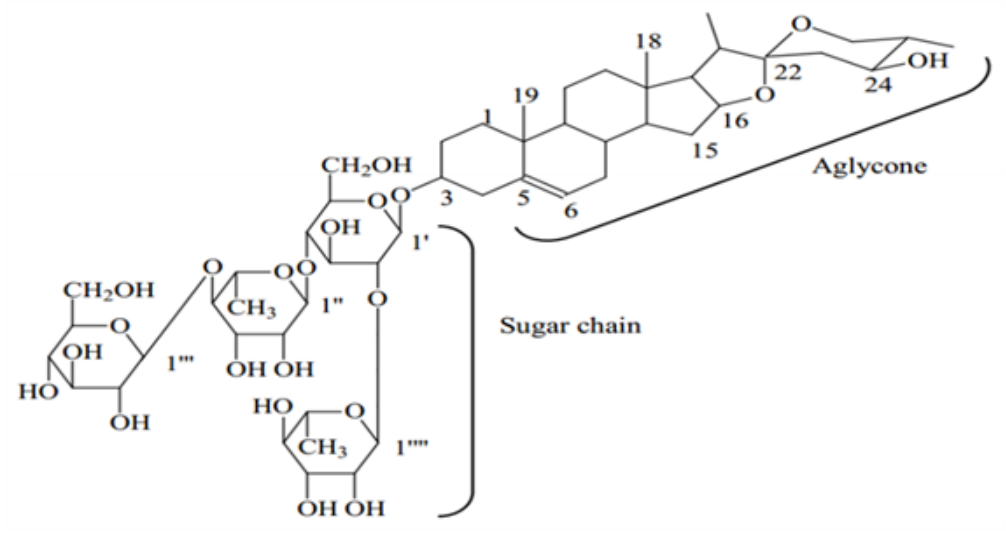


Figure 18: The chemical structure of saponins (Moghimipour & Handali, 2015).

Flavonoids

Flavonoids are hydroxylated and low molecular weight phenolic compounds such as flavones, flavonols and flavanonols (Figure 19) responsible for numerous pharmacological activities (Mahomoodally, Gurib-Fakim, & Subratty, 2005) including anti-inflammatory, anti-oxidant, anti-carcinogenic, anti-bacterial, free radical-scavenging, anti-atherosclerosis, anti-mutagenic and anti-allergic activities (Jucá *et al*., 2020; Karak, 2019; Tiwari & Husain, 2017) as well as providing security against respiratory disorders (Tiwari & Husain, 2017). They are reported to possess health-promoting effects and prevent chronic degenerative diseases (Tiwari & Husain, 2017). Flavonoids are known to exhibit their anti-inflammatory action through the inhibition of platelet aggregation and capillary permeability (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Alvarez, 2008), and several enzymes such as LOX, COX, xanthine oxidase (OX), aldose reductase, Ca^{2+} ATPase, iNOS, tyrosine, serine-threonine protein kinases and phosphoinositide 3-kinase that are involved in the generation of an inflammatory response (Kumar & Pandey,

2013; Viuda-Martos *et al*., 2008). They are also reported to reduce the synthesis of pro-inflammatory mediators such as prostaglandins (Agati, Azzarello, Pollastri, & Tattini, 2012), inhibit pro-inflammatory cytokines such as IL-6, TNF-α, IFNγ and NF-κB (Kumar & Pandey, 2013; Pan, Lai, & Ho, 2010), and upregulate the synthesis of anti-inflammatory cytokine such as IL-8 (Pan *et al*., 2010).

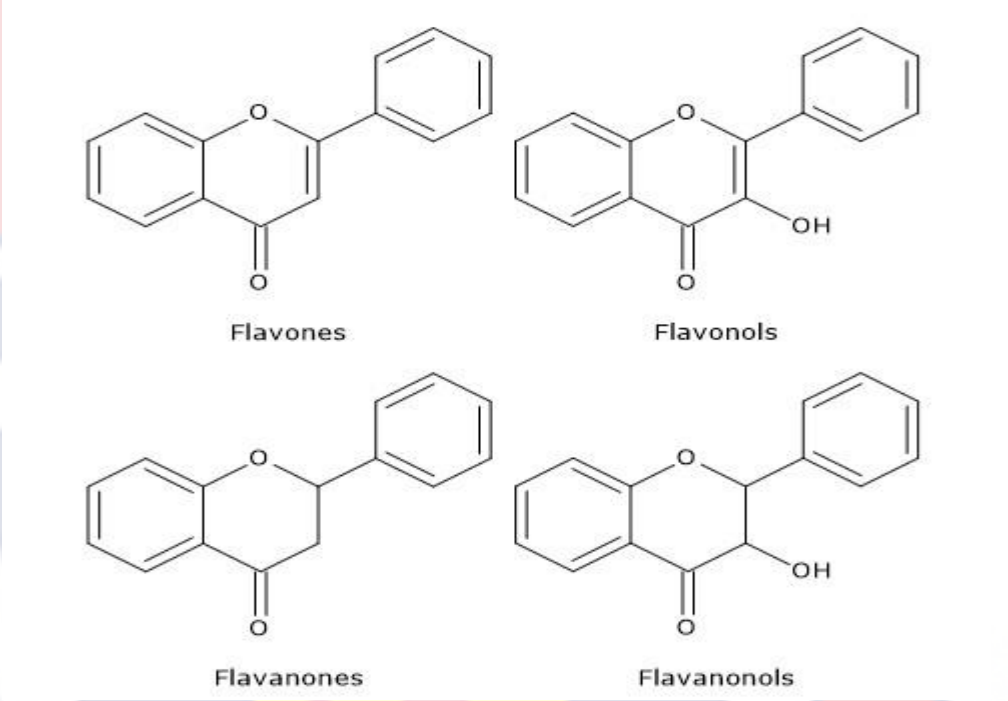


Figure 19: Examples of flavonoids with their chemical structures (Kumar & Pandey, 2013).

Toxicity studies

Toxicity assessment is relevant for the development of new and alternative drugs for the widening of existing molecules of therapeutic potentials. In drug process, toxicity screening of new agents is important and the test of these substances can be observed by (a) investigating the accidental exposures to the compound (b) *in vitro* studies using cells or cell lines (c) *in vivo* exposure on experimental animals. In 21st century, toxic effects of food

substances, chemicals, pharmaceuticals etc. have gained much importance (Parasuraman, 2011). Toxicity studies can be acute, sub-acute and chronic testing.

Acute toxicity studies

This is done to assess the effect of a single dose of a substance on a specific animal species. Generally, acute toxicity study is recommended to be done using two kinds of animal species (one rodent and one non-rodent). In acute toxicity study, the test sample is given at various dose levels and the effect is monitored for 14 days. After the testing, all mortalities related to the test agent are recorded and also, morphological, biochemical as well as histopathological changes are evaluated. In this study, 50 % of the lethal dose (LD_{50}) of the test sample is allowed to be determined. To determine LD_{50}, a large number of animals is used and usually there is high mortality rate (Parasuraman, 2011).

Sub-acute toxicity studies

To assess the sub-acute toxicity of a compound, rodents and non-rodents are used. The investigative sample is orally administered for about 6 weeks, and weekly variations in body weight, biochemical and cardiovascular parameters as well as the behavioral changes, are noted. Observations are made on gross pathology and histopathological analyses are done on all tissues. Between individual animals during acute toxicity testing, there is little variation and a range of ±20 % is allowed for weight variation (Parasuraman, 2011). Sometimes in this study, a satellite group may be added and there are both a control group and a high-dose group in this group (Muralidhara *et al.*, 2001).

Chronic toxicity studies

To conduct chronic toxicity study, at least one rodent and one non-rodent species are used. The test substance to be investigated is orally administered for 90 days or more, and periodically, the animals are observed. Usually, inferences concerning the long-term effect in animals of the investigative sample are provided by chronic toxicity study and this can be infered to human safety of the test sample. Between individual animals during chronic toxicity study, little variation is permitted and a range of ±20 % is allowed for weight variation. In this study, normal physiological activities, behavioral changes and modifications in biochemical parameters are critically monitored. Tissues are taken at the end of the study from all parts of the animals for histological screening (Jaijoy *et al*., 2010).

Chapter Summary

This chapter focused on inflammation and its related disorders. The pro-inflammatory mediators and other cells implicated in inflammatory responses including the actions of pro-inflammatory cytokines during inflammatory reactions. This chapter also focuses on the plant, *Persicaria lanigera*, its distribution, and its traditional uses and described the various toxicological studies to assess the safety of agents for phytomedicinal purposes.

CHAPTER THREE

MATERIALS AND METHODS

Introduction

This chapter describes the materials, equipment, reagents and drugs used for the study, and all the various methods employed. The methods were carried out either by *in vitro* or *in vivo* analysis to screen the hydroethanolic leaf extract of *Persicaria lanigera* for its possible anti-inflammatory activities as well as establishment of its possible mechanisms of action. In addition, the toxicity profile of *Persicaria lanigera* was assessed using both acute and sub-acute toxicity studies.

Plant collection

The fresh leaves of *Persicaria lanigera* were obtained from Jukwa, Lower Denkyira District, Central Region between September and November, 2018. The leaves were authenticated by Mr. Francis Otoo and Mr. Felix Fynn from the herbarium section in the School of Biological Sciences, College of Agricultural and Natural Sciences, University of Cape Coast. A voucher specimen (no. MAA003) has been kept at the herbarium of the School.

Preparation and extraction of plant material

The leaves were air-dried for a week and pulverised with a heavy-duty blender (3628GL72- 430CB2- Waring, USA) into fine powder. The powder (2.8 kg) was extracted by cold maceration with 2.0 L of 70 % ($^{v}/_{v}$) ethanol over a period of 72 h and the resulting filtrate was then concentrated under a reduced pressure at a low temperature of 60 ℃ using rotary evaporator (Model: R-290, BUCHI, Switzerland). It was further dried into a 262 g dark greenish semi-solid mass in a hot air oven (Gallenkamp OMT oven, Sanyo, Japan) at 50 ℃ and later

kept in a refrigerator (LG Haiser 220L Freezer, Ningbo Haiser Electronic Appliance Co. Ltd., Zhejiang, China) until it was ready to be used. This dark greenish semi-solid mass aqueous ethanol extract of the leaves of *Persicaria lanigera* was reconstituted as an emulsion using Tween 80 when required and henceforth referred to as *Persicaria lanigera* extract (PLE). The final yield was 9.3 % ($^{w}/_{w}$).

Chemicals, reagents and drugs

Carrageenan sodium salt from Sigma-Aldrich, St Loius, MO, USA, Zymosan A from Carbosynth Ltd., Compton, UK, Dexamethasone tablets from Pharm-Inter, Brussels, Belgium, Paraffin oil from Asgin Pharmacy, Cape Coast, Ghana, Aspirin tablets from MP-Biomedicals, California, USA, Acetic acid from Ernest Chemist, Accra, Ghana, Sulphasalazine from Shire Pharmaceuticals Inc., MA, USA, Ethanol from Ernest Chemist, Accra, Ghana, Histamine powder from Sigma-Aldrich, St Loius, MO, USA, IL-1β, TNF-α, COX-2, NF-kB from Genesis Biotechnologies, Inc., MD, USA, and Complete Freud's Adjuvant from Santa Cruz Biotechnology Inc., Dallas, Texas, USA.

Animals

Sprague Dawley rats (150-200 g) of both sexes were purchased from Center for Plant Medicine Research, Mampong-Akwapim, Ghana and maintained in the animal house of the Department of Biomedical Sciences, UCC. The animals were housed in groups of five (5) in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (AGRICARE, Tema, Ghana), given water *ad libitum* and maintained under standard laboratory conditions.

Phytochemical analysis of PLE

The presence of tannins, alkaloids, phytosterols, terpenoids, flavonoids, general test for glycosides (reducing sugars), anthracene glycosides, and saponins were tested by simple qualitative and quantitative methods.

Tannins

About 0.5 g of powdered *Persicaria lanigera* leaves was boiled with 25 ml of water for 5 min., cooled and filtered. 1 ml aliquot of the aqueous extract was diluted with 10 ml of water and 10 ml of 10 % NaCl solution added. 5 drops of 1 % gelatin solutin were added. A cloudy (white) precipitate was observed indicating the presence of tannins (Trease & Evans, 2002; Usman, Abdulrahman, & Usman, 2009).

Alkaloids

About 0.5 g of powdered *Persicaria lanigera* leaves was shaken with 10 ml of amoniacal alcohol in a test tube for 5 min. The mixture was filtered into a different test tube and 10 ml of dilute HCl, filtered and the filtrate made distinctly alkaline with dilute ammonia solution. The filtrate was the partitioned with chloroform and the chloroform layer separated. The chloroform extract was evaporated to dryness, reconstituted with 10 ml of dilute HCl, and transferred into a new test tube. To 1 ml of the filtrate, 3 drops of Dragendorff's reagent (potassium bismuth iodide solution) was added, shaken and the appearance of orange-brown precipitate formation indicates the presence of alkaloids (Trease & Evans, 2002; Usman *et al*., 2009).

Phytosterols (Lieberman-Burchard's test)

The powdered leaves of *Persicaria lanigera* (0.5 g) was extracted with 10 ml of chloroform. To the extract, 2 ml of acetic anhydride was added and

few drops of concentrated H_2SO_4 were cautiously added at the side of the test tube. A brown coloured ring appeared at the interface between the chloroform-acetic anhydride layer and the concentrated H_2SO_4 and a blue to green colour in the upper layer indicating the presence of phytosterols (Jana & Shekhawat, 2010; Sofowora, 1993).

Terpenoids (Salkowski test)

The powdered leaves of *Persicaria lanigera* (0.5 g) was extracted with 10 ml of chloroform in a test tube and then 1 ml of concentrated H_2SO_4 was subsequently added. The mixture was mixed properly and a reddish-brown coloration at the interface shows a positive result for the presence of terpenoids (Jana & Shekhawat, 2010; Sofowora, 1993).

Saponins

The powdered leaves of *Persicaria lanigera* (0.5 g) was extracted with water and filtered. The filtrate was shaken intensely in test tube and the presence of froth that persisted for over 5 minutes without breaking upon standing indicated the presence of saponins (Trease & Evans, 2002).

Glycosides (General test)

The powdered leaves of *Persicaria lanigera* (0.5 g) was boiled with dilute H_2SO_4 on a water bath for 5 min. and then cooled. The mixture was filtered and distinctively rendered alkaline with 2 to 5 drops of 20 % NaOH. To the filtrate, 1 ml of Fehling's A and B solutions were added and then heated on a water bath for 2 min. The appearance of a brick-red colouration shows the presence of glycosides (Sofowora, 1993; Trease & Evans, 2002).

Flavonoids (Ammonia test)

The powdered leaves of *Persicaria lanigera* (0.5 g) was extracted with 10 ml of water and filtered. About 5 ml of dilute ammonia solution was added to the aqueous filtrate and observed for the appearance of yellow colouration which disappeared upon the addition of concentrated H_2SO_4 indicating the presence of flavonoids (Ayoola *et al*., 2008).

Acute anti-inflammatory effects of PLE

a) Carrageenan-induced paw oedema in Sprague-Dawley rats

A method previously described by Winter, Risley, & Nuss (1962) with minor modifications was used to evaluate the effect of PLE on carrageenan-induced paw oedema in rats. Briefly, oedema was formed in the right hind limbs of rats (150-200 g, n=5) by sub-plantar injection of 100 µl of 1 % ($^{w}/_{v}$) sterile carrageenan (suspended in 0.9 % saline). Initial basal paw thickness of animals was measured using electronic digital venier caliper (VC1346i, MP Lab Equip, USA) prior to carrageenan injection. Paw thickness of injected limb was then measured at hourly interval for 5 h.

Inhibition of oedema was expressed as:

$$\% \text{ Change in paw thickness} = \left(\frac{(P_t - P_o)}{P_o}\right) \times 100$$

Where *Po* is the paw thickness before carrageenan injection (i.e. time zero).

Pt is the paw thickness (at various time intervals) post carrageenan injection.

Percentage change of paw thickness for each animal was calculated from the raw scores at time 0 and then averaged. Total oedema was expressed as the area

under the time course curve (AUC) and the percentage inhibition of oedema was calculated using the formula:

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

In the prophylactic study, either PLE (100, 300, 600 mg kg^{-1}, *p.o.*) or aspirin (100 mg kg^{-1}, *p.o.*) was administered prior to carrageenan injection. In the curative study, drugs were administered 1 h post sub-plantar injection of carrageenan solution. Control rats orally received Tween 80 in normal saline.

b) Zymosan-induced acute knee arthritis in rats

Induction of acute arthritis

Sprague-Dawley rats (150-200 g, n=5) were randomly selected into six (6) groups (I-VI). Prior to induction of arthritis, hairs around the knee joint were carefully shaved to expose the joint and also, cleaned with 70 % alcohol. A method previously described by Mortada & Hussain (2014) with slight modifications was used. Briefly, initial right knee joint thickness (transverse dimeter, mm) of each rat in all the groups (I-VI) was measured using digital venier caliper (VC1346i, MP Lab Equip, USA). Either PLE (100-600 mg kg^{-1}, *p.o.*) or aspirin (100 mg kg^{-1}, *p.o.*) was administered 1 h prior to arthritis (intra-articular injection of zymosan [ZM]) induction. Acute knee joint arthritis was induced with 500 µg of ZM in 25 µl of normal saline (dissolved in 0.9 NaCl) in each knee joint cavity of the right limb.

Group I (naïve) : received only 1 ml of Tween 80 in normal saline.

Group II (arthritic) : received only 500 µg of zymosan in 25 µl of saline.

Group III : received 100 mg kg^{-1} of aspirin + 500 µg of zymosan

in 25 µl of saline.

Group IV : treated with PLE (100 mg kg^{-1}).

Group V : treated with PLE (300 mg kg^{-1}).

Group VI : treated with PLE (600 mg kg^{-1}).

Knee joint swelling was evaluated by measuring the knee joint thickness of each rat in all treatment groups at hourly interval for 5 h. Inhibition of joint swelling was determined same as the procedure earlier expressed above (in section [a]). The following parameters were assessed:

i) Determination of total leukocyte and neutrophil infiltration

Total leucocyte and neutrophil infiltration levels into knee cavity were evaluated after 5 h of intra-articular injection of ZM. Under light ether anaesthesia, blood samples were collected from each knee joint synovial cavity into ethylenediaminetetraacetic acid (EDTA) tubes. Blood samples were analysed for neutrophils and total leukocytes using automated haematology cell diagnosis analyser (HP-HEMA6500A, Zhengzhou Hepo International Trading Co. Ltd, Henan, China).

ii) Histopathological assessment of knee joint

Procedure below was used to prepare and assess the histopathology of the test samples after induction of arthritis at the end of observation period.

Isolation of bone tissue

The Sprague Dawley rats were euthanised after 5 h by cervical dislocation. Tissues of bones from the knee joint of the right hind limbs were excised and transported into sterile Bouin's solution (1 % picric acid, 9.5 % formaldehyde, 5 % acetic acid and water).

Tissue processing and embedding

Tissues isolated from the rats were fixed in paraformaldehyde, PFA (for 1 L solution, pH 7.4: 892 ml H_20, 108 ml 37 % formalin, 11.86 g Na_2HPO_4, and 9.07 g H_2PO_4) for 16 h at room temperature. An automated tissue processor (Rd-1301 CE, Biobase Biozone Co. Ltd, Shandong, China) was used to process the specimens operated to run several treatments by immersing the tissues in serially concentrated ethanol, xylene and paraffin in the format:

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

The concept is to dehydrate the tissues in increasing concentration levels of ethanol followed by washing with xylene to eliminate the ethanol. The tissues were finally kept in liquid paraffin. After processing, the tissues were embedded using an embedding machine (Leica EG 2210, Leica Microsystems, Weltzlar, Germany) in paraffin. The paraffin-embedded tissues were kept at 4 ℃ to allow solidification of the paraffin matrix.

Cutting paraffin-embedded tissue blocks

At 50 ℃ (10 ℃ below the melting point of the paraffin), the paraffin-embedded tissue blocks were cut into 5 µm-thick sections using a microtone (Leica RL3211, Leica Microsystems, Wetzlar, Germany) to eliminate all wrinkles and further mounted onto an XTRATM adhesive microslide 25.4×76.2×1 nm (Nanjing Amada Instrument Co. Ltd, Jiangsu, China). The slides were placed in an oven (TOB-DHG-9023A Forced Air Dry oven, TOB Machine, Xiamen City, Fujian Province, China) at 50 ℃ for 60 min to dry.

Haematoxylin and eosin (H & E) staining of paraffin-embedded sections

The following procedure was used to deparaffinise and hydrate the 5µm-thick sections obtained using distilled water.

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

For 2-3 min, the sections were stained with H & E stain after being washed in distilled water. The sections were washed in distilled water with 3 changes and immediately dehydrated by dipping in alcohol and finally removed with xylene as follows:

1. Xylene, 2’ immersion
2. Xylene, 2’ immersion
3. Xylene, 2’ immersion

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

A glass cover-slip was mounted on top of the slide with non-aqueous mounting medium Coverquick (VWR Chemicals, Leicestershire, England, UK). Image analysis and acquisition was performed using a microscope (BS-2040fb LED, Movel Scientific Instrument Co. Ltd, Zhejiang, China).

Determination of mast cell proliferation in knee joint

Thick segmented sections of the knee joint tissues were fixed in Carnoy's fixative and stained with 1 % toluidine blue. Using micrometer grid (0.042 mm^2), mast cells were counted in coded sections at ×20 magnification.

Chronic anti-inflammatory effects of PLE

a) Acetic acid-induced ulcerative colitis in rats

Induction of ulcerative colitis

Sprague-Dawley rats (150-200 g) were randomly selected into six (6) groups (n=5). A method previously described by Shalkami, Hassn, & Bakr (2018) with slight modification was used. Briefly, control animals (group 1) received oral administration of Tween 80 in normal saline (0.9 % NaCl) for 8 days and groups II-VI animals received 1 ml of 4.0 % acetic acid ($^{v}/_{v}$) intrarectally using sterilised paediatric catheter (size: 6Ch/Fr) (SAFER-Shanghai SNWI Medical Co. Ltd, Shanghai, China) on the 4th day. Rats were positioned upside down for 60 s to prevent leakage of acetic acid solution. Group III animals were pre-treated with sulfasalazine (500 mg kg^{-1}, *p.o.*) for 8 days prior to intrarectal injection of 1 ml of 4 % acetic acid on day 4. Groups IV-VI were pre-treated with PLE (100-600 mg kg^{-1}, *p.o.*) daily for 8 days before 1 ml of 4.0 % acetic acid was injected as per group (on day 4) respectively. Drug administration was carried out till day 8 and the parameters evaluated include:

Change in body weight

Change in body weight of rats was observed over the 8-day period. The effect of PLE on general body weight was determined as the area under the time course curve (AUC).

Macroscopic ulcerative colonic damage

Colons from the animals induced with colitis were removed, cleaned, washed and assessed for: stool consistency, weight and length (1 cm above the anus to the apex of the caecum) as well as gross macroscopic appearance. Using Kimball, Wallace, Schneider, D'Andrea, & Hornby (2004) model, colonic damage was assessed by calculating the disease activity index (DAI), expressed as the sum of values obtained from the indices indicated in the table (on page 68).

Macroscopic colonic damage scoring criteria

Condition	0	1	2	3	4
Stool consistency	Normal	Loose and moist	Moist and sticky	Diarrheoa	Presence of blood
Colon damage	No Inflammation	Mild inflammation	Moderate or slightly spread inflammation	Severe or widely spread inflammation	Severe and widely spread inflammation
Colon weight change	<5 %	5-14 %	15-24 %	25-35 %	>35 %
Colon length shortening	<5 %	5-14 %	15-24 %	25-35 %	>35 %

Maximum score for ulcerative colonic damage =16.

Haematological analysis

The rats were sacrificed by cervical dislocation and blood samples were collected via cardiac puncture into EDTA tubes. Complete blood count analysis was performed using automated haematology cell diagnosis analyser (HP-HEMA6500A, Zhengzhou Hepo International Trading Co. Ltd, Henan, China).

Histopathological analysis

Distal colons (1 cm) from the animals were removed, cleaned and washed thoroughly. Procedure is thoroughly explained above (refer to pages 61-64).

Mast cell proliferation

Thick segmented sections were fixed in Carnoy's fixative and stained with 1 % toluidine blue. Using micrometer grid (0.042 mm^2) at ×20 magnification, mast cells were counted in coded sections. For each rat, 10 adjacent non-overlapping mucosal portions above muscularis mucosae were assessed.

Enzyme Activity

The colons were cleaned, washed thoroughly and stored at -80 ℃ untill analysis. Using a homogenizer (BSL Pre-PROC homogeniser, BioBase Biozone Co. Ltd, Shandong, China), tissues were homogenised on ice-cold in 0.01 M Tris-HCl buffer (pH 7.4) to give a 10 % homogenate. The homogenate was used for catalase, superoxide dismutase and lipid peroxidation assays.

i) Catalase (CAT) activity

Using a method previously described by Aebi (1984), catalase activity was measured. This was determined by measuring for 60 s reduce levels of H_2O_2 concentration at 240 nm, at 20 s intervals. Medium contained 0.13 ml 50 mM

potassium phosphate buffer (pH 7.0) and enzyme extract; 0.065 ml of 10 mM H_2O_2. The blank had 0.065 ml of potassium phosphate and 0.13 ml of the sample. The concentration of H_2O_2 was calculated from the absorbance using the formula:

$$[H_2O_2 \text{ mM}] = \left(\frac{Absorbance_{240} \times 1000}{39.4\ mol^{-1}cm^{-1}}\right)$$

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

ii) Superoxide dismutase (SOD) activity

A method previously described by Misra & Fridovich (1972) was used to measure SOD activity. The principle of the test is mainly the ability of SOD to prevent autoxidation of adrenaline to adrenochrome. 0.75 ml ethanol (95 % $^{v}/_{v}$) and 0.15 ml chloroform (child on ice) were added to 500 µl of the tissue homogenate, and centrifuged (HERMLE Z366 Series Lab Centrifuge, BenchMark Scientific, Sayreville, NJ, USA) at 3000 × g rpm for 10 min. 0.5 ml of 0.6 mM EDTA solution and 1 ml carbonate bicarbonate buffer (0.1 M, pH 9.8) were added to 0.5 ml of the supernatant. The reaction was triggered by the addition of 0.05 ml 1.3 mM adrenaline and the increase in absorbance at 480 nm as a result of the adrenochrome formed was determined. Percentage inhibition of autoxidation of adrenaline was expressed as:

$$\% \text{ Inhibition} = \left(\frac{Absorbance_{(test)} - Absorbance_{(reference)}}{Absorbance_{(test)}}\right) \times 100$$

One unit of SOD activity expressed as the quantity of protein causing 50 % inhibition of autoxidation of adrenaline at 25 ℃ was determined using the formula:

Units of activity per mg protein = $\left(\frac{\%\ inhibition}{50 \times weight\ of\ protein}\right)$

iii) Lipid peroxidation: measurement of MDA (malondialdehyde)

Lipid peroxidation concentrations were measured in MDA. A method previously described by Heath & Parker (1968) was used for the determination of MDA levels. To 1 ml aliquot of the supernatant, 3 ml of 20 % trichloroacetic acid and 0.5 % thiobarbituric acid were added. Mixture was heated for 30 min at 90 ℃ and then immediately cooled on ice bath. This was centrifuge at 10,000 × g rpm for 10 min, and at 532 nm absorbance was measured. The score for the non-specific absorption at 600 nm was subtracted from the 532 nm reading. The level of MDA was expressed with MDA's extinction coefficient of 155 nM^{-1} cm^{-1}.

b) Cotton pellet granuloma tissue formation in Sprague-Dawley rats

In this model, anti-proliferative effect of PLE was assessed using cotton pellet-induced granuloma tissue formation in rats. Cotton pellet granuloma tissue was formed in the rats using a method previously described by Swingle & Shideman (1972) with slight modifications. Briefly, rats (150-200 g, n=5) were divided into six (6) groups and allowed to fast overnight with only water administered *ad libitum*. Under light ether, rats were anaesthetised with pentobarbitone (20 mg kg^{-1}, i.p) and the abdomen clearly shaved after which the area was disinfected with 70 % ($^{v}/_{v}$) ethanol. At the groin region, an incision was made and using a blunt forceps, sterilised cotton (weighing 40±1 mg) was inserted subcutaneously on each side of the abdomen bilaterally. The incisions were closed with sutures. Group I (naïve group) received Tween 80 in normal saline (1 ml, *p.o*, daily); Group II (negative control) received Tween 80 in

normal saline (1 ml, *p.o.*, daily); Group III treated with dexamethasone (3 mg kg^{-1}, *p.o.*, daily); Group IV-VI received PLE (100-600 mg kg^{-1}, *p.o.*, daily) respectively. Animals were monitored for one week and on day 8, all animals were sacrificed by cervical dislocation. The pellets were carefully removed (together with granular tissue, freed from immaterial tissue) and weighed immediately for wet weights. At 60 ℃, pellets were incubated for 24 h to obtain a constant weight. Weight of exudate amount (mg) was calculated as a difference between the weight of wet pellet and weight of dry pellet. The granuloma tissue formation was determined by subtracting weight of sterilised cotton (40±1 mg) from dry weight of pellet (Subash, Veeraraghavan, Sali, Bhardwaj, & Vasanthi, 2016). The effect of PLE on cotton pellet granuloma tissue formation was assessed on the following clinical parameters;

Change in body weight

Change in body weight of rats was monitored daily for 7 days as previously described (refer to page 66).

Haematological analysis

Blood samples were collected from rats on day 8 after sacrifise via cardiac puncture into EDTA tubes. Complete blood count was performed (refer to procedure on page 69).

Spleen weight/ body weight of rat

The spleen from each animal in all treatment groups was removed and weighed. The relative spleen weight for each animal was determined.

Enzyme activity

Serum for enzyme assay was made from blood samples collected from each animal in all treatment groups. Blood samples were put into vacuum

separator tubes and centrifuged at 3000× g rpm for 15 min at 5 ℃. The sera samples were put in Eppendorf tubes and stored at -80 ℃ until use. The following anti-oxidant activities were determined:

Catalase (CAT) activity

Procedure same as earlier described above (refer to page 69).

Superoxide dismutase (SOD) activity

Procedure same as earlier described above (refer to page 70).

Lipid peroxidation: measurement of MDA (malondialdehyde)

Procedure same as previously described above (refer to page 71).

a) Complete Freund's Adjuvant-induced arthritis in rats

Induction of arthritis

Right hind paw of the rats (150-200 g, n=5) were inoculated intraplantarly with 100 µl of Complete Freund's adjuvant (CFA) using a method previously described by Snekhalatha, Anburajan, Venkatraman, & Menaka (2013). Arthritic control group received 100 µl of CFA only whereas the non-arthritic control group received 100 µl of Incomplete Freund's adjuvant (IFA) [paraffin oil] only. Rats were selected randomly for the various experimental groups:

Group I : Non-arthritic (IFA [paraffin oil]-treated) control

Group II : Arthritic (CFA-treated) control

Group III : Dexamethasone (3 mg kg^{-1}, *p.o.*)

Group IV : Aspirin (100 mg kg^{-1}, *p.o.*)

Group V : PLE (100 mg kg^{-1}, *p.o.*)

Group VI : PLE (300 mg kg^{-1}, *p.o.*)

Group VII : PLE (600 mg kg^{-1}, *p.o.*)

In the pre-emptive study, the rats were pre-treated with test drugs (1 h, *p.o.*) prior to CFA injection and drug administeration commenced (daily) on day 0. In the curative study, the test drugs were administered from the 14^{th} day after the onset of polyarthritis. The experiment was carried out for 28 days during which disease progression was critically monitored and the rats were sacrificed for further analyses. The effects of PLE on adjuvant-induced arthritis were evaluated using the following indices;

Change in body weight

The change in body weight of rats was determined every four days in all treatment groups for 28 days.

Paw oedema measured as maximal and total oedema effects

Paw thickness was measured using digital venier caliper (VC1346i, MP Lab Equip, U.S.A) for both injected hind paw (ipsilateral) and non-injected hind paw (contralateral) prior to the intraplantar injection of either CFA or IFA and every other day till day 28. Raw values for ipsilateral and contralateral paw thickness were normalised separately as percentage change for their scores at day 0 and then averaged for each test group. Maximal oedema effect was calculated using the formula:

$$\% \text{ Change in paw thickness} = \left(\frac{(Pt - Po)}{Po}\right) \times 100$$

Where

P*o:* paw thickness before CFA injection.

P*t*: paw thickness at time T.

Data obtained was expressed as the maximal oedema effect (i.e. on the time course curves) and the total oedema responses calculated as the area under the time course (AUC) in arbitrary units for 28 days. The percentage inhibition of total oedema was calculated using the formula:

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

Arthritic score

Photography

Photographs of the hind limbs were taken on the 28th day using a digital camera (Sony DSC-w830 Cyber-20.1MP Silver, Aya Nagar, Delhi, India). Arthritic scoring was blindly made on the photographs and severity of arthritis was determined on day 28 on the scale of 0-4 with the degree of oedema formation of periarticular tissues and erythema as parameters for scoring (Cai *et al*., 2007) where 0= no inflammation; 1= absolute inflammation of 1 joint of the limb; 2= moderate inflammation of 1 joint of the limb; 3= pronounced inflammation of 1 joint or more and 4= severe or excessive inflammation of 1 joint or more with joint rigidity. The arthritic score for each animal on day 0 was estimated to be zero (0). Similarly, the arthritic scores for IFA-treated group were determined as 0 for each rat.

Radiography

Radiographical assessment of the severity of bone and cartilage damage was carried out on day 29 after anesthetisea (20 mg kg^{-1} of pentobarbital, i.p). Radiographs were taken with X-ray apparatus (50 Hz Digital, 125 KVP, AK Industries, Navi, Mumbai, Thane, India) and industrial x-ray film (Fujifilm DIHL X-ray film, Asian Reprographics Ltd, Chennai, India) functioned at 30-

kV peak and 10 s exposure with 45 cm tube-to-film distance for lateral projections. The severity of joint and bone destruction was scored blindly accordingly to the degree of osteoporosis, osteophytes, joint spaces and joint structure (Pohlers *et al*., 2007). The degree of joint and bone deformity was scored on the scale of 0-4, with 0 being scored as normal.

Haematology and biochemistry

Haematological and biochemical analyses were done after 28-day of observation period. Complete blood count and serum biochemical analysis were performed (refer to procedure on page 69).

Histopathology

Hispathological analysis was performed to assess the histopathology of the test samples using the same procedure earlier described above (refer to pages 62-65).

Eye assessment

Drug administration commenced on day 14 till the 28th day. The eyes of rats in all treatment groups were examined every other day using ophthalmic slit lamp (My-V038c-N, Guangzhou Maya Equipment Co. Ltd, Guangdong, China) from day 0 until the 28th day of the experiment for ocular integrity, especially on a condition called uveitis.

Mast cell proliferation

Thick segmented sections were fixed in Carnoy's fixative and stained with 1 % toluidine blue. Using micrometer grid (0.042 mm^2) at ×20 magnification, mast cells were counted in coded sections.

In vitro anti-oxidant properties of PLE

Total anti-oxidant capacity

The total anti-oxidant capacity of PLE was assessed by phosphomolybdate method using ascorbic acid as a standard (Prieto, Pineda, & Anguilar, 1999). Briefly, 0.5 ml of PLE from serial concentrations (15.625-250 µg g^{-1}) solution was mixed with 5 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in test tubes. The mixture was incubated at 95 ℃ for 1 h 30 min. The samples were cooled and absorbance was measured at 695 nm against a blank (i.e. 5 ml of reagent solution and required volume of solvent). All assays were in triplicate. Total anti-oxidant capacity was determined as the number of µg of equivalent of ascorbic acid (AAE) per g of extract.

DPPH (2, 2-diphenyl-1-picryl-hydrazil) scavenging activity

The free radical scavenging activity of PLE was evaluated using a method earlier described by Jedrejek, Lis, Rolnik, Stochmal, & Olas (2019) with slight modification. In brief, solutions of PLE were prepared at different concentrations (15.625-250 µg ml^{-1}) with 80 % methanol. The standard solution (ascorbic acid) was also prepared at different concentrations (1.5625-6.2500 µg ml^{-1}) using the same solvent. Then, 0.1 ml of PLE (15.625-250 µg ml^{-1}) solutions were mixed with 1.9 ml of 0.2 nm solution of DPPH in methanol. The mixture was incubated for 30 min in a dark room at room temperature and absorbance was measured at 517 nm against a blank solution. Measurement of assays was done in triplicates.

The percentage scavenging activity was determined (Azhari, Xu, Jiang, & Xia, 2014) as:

$$\% \text{ SA} = \left(\frac{ABSORBANCE_{(Control)} - ABSORBANCE_{(sample)}}{ABSORBANCE_{(Control)}} \right) \times 100$$

Where SA is scavenging activity.

H_2O_2 (Hydrogen peroxide) scavenging activity

To determine the H_2O_2 scavenging activity of PLE, a method previously described by Siddeeg, AlKehayez, Abu-Hiamed, Al-Sanea, & Al-Farga (2021) was used with slight modifications. Briefly, 100 ml of PLE solutions at different concentrations (15.625-250 µg g^{-1}) was mixed with 0.002 % of H_2O_2 before 100 mM NaCl and 0.8 ml of 0.1 M phosphate buffer were added. After 10 min of incubation at room temperature, 0.2 mg ml^{-1} of phenol red dye and 0.1 mg ml^{-1} of horseradish peroxide (HRP) were added to the mixture. The mixture was then incubated again for 15 min at 37 ℃ after which 1 ml of 1 M NaOH was added to terminate the reaction. Absorbance was measured at 230 nm.

Toxicity screening of PLE

Toxicological study of new agents is vital for drug development process. Usually, preclinical toxicity assessment on different biological systems discloses species-, organ- and dose-specific harmful effects of the testing compound (Parasuraman, 2011). Toxicity profile of PLE were performed using acute and sub-acute toxicity studies in rats.

Acute toxicity study

Acute toxicity study of PLE was performed in accordance to the procedures as described by OECD guildlines-425 (Lorke, 1983; Organisation for Economic Co-operation and Development [OECD], 2008) with slight modifications. Sprague-Dawley rats (150-200 g, n=5) of both sexes were randomly selected and divided into six (6) groups. Animals were allowed to

acclimatised to the laboratory environment for 1 week and fasted overnight, but with access to water *ad libitum* and weighed before oral administration of PLE in doses of 100 mg kg^{-1} (Group II), 300 mg kg^{-1} (Group III), 1000 mg kg^{-1} (Group IV), 3000 mg kg^{-1} (Group V) and 5000 mg kg^{-1} (Group VI) of body weight. Control group (Group I) received 1 ml of Tween 80 in normal saline. Individual animals in all treatment groups were critically monitored for a period of 24 h for changes in general behavior, physiological function including mortality. Using the method previously described by Irwin (1968), animals were monitored at 0, 15, 30, 60, 120, 180, 240 min, and 24 h for convulsion, tremor, excitement, respiratory changes, unusual locomotion, agitation, ataxia, aggression, sedation, salivation, urination and defecation after PLE administration. Using the Basante-Romo, Gutiérrez-M, & Camargo-Amado (2021) scaling method with minor modifications, observations made for all toxicity signs were scored on the scale of 0-3. The lethal dose (LD_{50}) of PLE was determined using the formula (Osagie-Eweka, Orhue, Omogbai, & Amaechina, 2021):

$$LD_{50} = \sqrt{\frac{D_0 + D_{100}}{2}}$$

Where D*o* is the maximum dose that resulted in 0 % mortality; D_{100} is the minimum dose that resulted in mortality.

Sub-acute toxicity study

The experiment was performed in accordance to the procedures previously described by OECD Guidelines-425 (OECD, 2008) and using previously described method (Kpemissi *et al*., 2020) with slight modifications. Briefly, rats (150-200 g, n=5) of both sexes were randomly selected and divided

into six (6) groups. Animals were allowed to acclimatised to the laboratory environment for 7 days and their initial weights recorded before oral administration (daily) of PLE in doses of 100 (Group II), 300 (Group III and 1000 (Group IV) mg kg^{-1} of body weight for 28 days consecutively. Control group (Group I) received 1 ml of Tween 80 in normal saline. Rats were monitored critically every day during the experimental period for morbidity or mortality, physiological and behavioral changes. Change in body weight of rats was determined as percentage change in body weight of rats using the formula:

$$\% \text{ Change in body weight } = \left(\frac{Final\ weight\ (g) - Initial\ weight\ (g)}{Initial\ weight\ (g)}\right) \times 100$$

Animals were euthanised on 29th day and blood samples as well as some vital organs (liver, heart, spleen, stomach, kidney) were collected for the following analyses:

i. Relative organ weight (ROW)

Rats were euthanised by cervical dislocation on the 15th day and vital organs including heart, liver, spleen, stomach and kidney were removed and weighed. The weight of each organ was determined as a relative percentage of organ weight of the rats (Markman, Bacchi, & Kato, 2004) using the formula:

$$\text{ROW} = \left(\frac{absolute\ organ\ weight\ (g)}{weight\ of\ animal\ on\ sacrifise\ day\ (g)}\right) \times 100$$

ii. Haematological and biochemical analyses

Procedure same as previously described above (refer to page 69).

iii. Histopathological assessment

Vital organs selected (heart, liver, spleen, stomach, kidney) were removed, cleaned and washed thoroughly using the procedure earlier explained

(refer to pages 62-65).The organs were then assessed microscopically for any histological variations.

Establishment of possible mechanisms of action of PLE

To investigate the possible mechanisms of action of PLE, compound-induced acute inflammatory paw oedema models and serum cytokine gene expression of TNF-α, IL-1β, NF-κB, COX-2 levels were assessed.

***In vivo* mediator-induced acute inflammatory paw oedema**

Effect of PLE on histamine-induced paw oedema in rats

Anti-inflammatory (anti-histaminic) effect of PLE was investigated using a method previously described by Sowemimo, Onakoya, Fageyinbo, & Fadoju (2013) with slight modifications. In brief, rats (150-200 g, n=5) of both sexes fasted overnight and grouped into five (5) groups in metallic cages. Initial paw thickness of both limbs of animals was measured using venier caliper (VC1346i, MP Lab Equip, U.S.A) and oedema was induced by injecting 0.1 ml of 1 % histamine (freshly prepared in normal saline) into the subplantar tissues of the right hind paw of rats. Paw thickness of rats was measured at 30, 60, 90, 120, 150 and 180 min. Treatment commenced 60 min before histamine injection (for pre-emptive study) and 60 min post histamine injection (for therapeutic study). Group I (control group) received 1 ml of Tween 80 in normal saline, Group II (positive control) orally received 4 mg kg^{-1} chlorpheniramine (dissolved in normal saline) and Group III-V were treated orally with PLE 100-600 mg kg^{-1} respectively. Paw thickness was calculated using the formula previously stated (in carrageenan-induced paw oedema model [refer to pages 60-61]).

Effect of PLE on serum cytokine levels (TNF-α, IL-1β, NF-κB, COX-2)

The effect of PLE on serum cytokine (TNF-α, IL-1β, NF-κB and COX-2) levels was assessed on CFA-induced arthritis in rats. Arthritis was induced as previously described (in CFA-induced arthritis method) by intraplantar injection of 100 µl of CFA into the right hind limbs of rats. Drug administration commenced on day 14 till the 28th day. Blood samples were collected into EDTA tubes from each experimental group and centrifuged (HERMLE Z366 Series Lab Centrifuge, BenchMark Scientific, Sayreville, N.J, U.S.A) at 3000 × g rpm for 10 min. Sera formed were aliquoted into Eppendorf tubes and stored at -20 ℃ until use. Serum cytokine ELISA assay was performed in duplicates with appropriate rat ELISA kit using the protocol described by the manufacturer. Briefly, 10 wells were set for the standards in a microelisa strip plate. In well 1 and 2, 100 µl standard solution and 50 µl standard dilution buffer were added and mixed thoroughly. In well 3 and 4, 100 µl solution from well 1 and 2 were added after which 50 µl standard dilution buffer were added to the wells respectively and mixed thoroughly. 50 µl solution was discarded from well 3 and 4, after which 50 µl from the same wells (3 and 4) were added to wells 5 and 6. 50 µl standard solution buffer were added to 5 and 6, and mixed thoroughly well. In wells 7 and 8, 50 µl solution from 5 and 6 and 50 µl standard dilution buffer were added respectively and mixed well. In wells 9 and 10, 50 µl solution from 7 and 8 were added in addition to 50 µl of the standard dilution buffer and mixed respectively. 50 µl of the solution was discarded from wells 9 and 10. After dilution, total volume in all the wells was 50 µl. In microelisa strip plate, a well was left as a blank control. In sample wells, 40 µl of sample dilution buffer and 10 µl of test samples (dilution factor of 5) from each treatment groups

(Group I- VII) were loaded into the wells and mixed thoroughly well after shaken gently. Microelisa stripplate was incubated at 37 ℃ for 30 min. and later washed (repeated 5 times). 50 µl of horseradish peroxidase (HRP)-conjugate reagent was added to each well except the blank control well. Incubation and washing were repeated. For colour (blue colouration), 50 µl of chromogen solution A and B were added to each well, mixed thoroughly well and incubated for 15 min. at 37 ℃ (light was avoided). For termination, 50 µl of stop solution was added to each well to terminate the reaction. The colour changed from blue to yellow in all wells. Reading (for absorbance O.D) was done within 15 min using a microtiter plate reader (BIOBASE-EL10A Elisa Microplate Reader, Biobase Group, Jinan, Shandong, China) at 450 nm.

Statistical analysis

All data were expressed as the mean ± SEM (n=5) of the effect of drugs on the time course curves (i.e. maximal response) and total oedema effects over time period. Data were analysed statistically through a test of significance using both one-way and two-way ANOVA (analysis of variance) followed by Dunnet's *post hoc* test. All graphs were plotted using GraphPad Prism version 7.0 (GraphPad, San Diego, USA).

Chapter Summary

This chapter focused on the materials, chemicals and reagents used in the study. The methods used for crude extraction and various experimental models employed to investigate the anti-inflammatory effects of *Persicaria lanigera* leaf extract were explained. Additionally, anti-oxidant and phytochemical analyses including toxicological studies were described appropriately.

CHAPTER FOUR

RESULTS AND DISCUSSION

Introduction

This chapter discusses the results obtained from the various methods used in chapter 3 to evaluate the anti-inflammatory effects of the 70 % hydroethanolic leaf extract of *Persicaria lanigera*. The results were analysed in comparison to well-known standard drugs used in the management of inflammatory diseases and other related disorders.

Phytochemical screening of PLE

Table 1: Phytochemical constituents of 70 % hydroethanolic leaf extract of *Persicaria lanigera*.

CONSTITUENTS	TEST	INFERENCE
Tannins	Ferric chloride test	+
	Lead acetate test	+
Alkaloids	Dragendorff's test	+
Phytosterol (steroids)	Leibermann-Burchard's test	+
Terpenoids	Salkowski test	+
Saponins	Frothing test	+
Glycosides	General (Fehling's) test	+
Flavonoids	Ammonia test	+

-: Not detected, +: Present.

Carrageenan-induced paw oedema in rats

From this study, carrageenan inoculated into the subplantar tissues of rats (as earlier described) caused oedema of the limb peaking between 2-3 h in

the control rats. (Fig. 20). In the prophylactic study, the percentage mean maximal oedema formed in control rats was 99.01±12.59 % (Fig. 20A). Aspirin (100 mg kg^{-1}, *p.o.*) significantly reduced the mean maximal oedema to 41.84±9.25 % (Fig. 20A). PLE (100-600 mg kg^{-1}, *p.o.*) administered pre-emptively similarly decreased the mean maximal swelling to 59.10±4.94 %, 56.08±3.65 % and 48.62±3.27 % at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control group respectively (Fig. 20A). The total paw oedema formed after 5 h of oedema induction was significantly reduced by 43.72 %, 52.34 % and 61.58 % at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control group respectively (Fig. 20B).

In the therapeutic study where either aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}) was administered post carrageenan induction, the mean maximal swelling attained by the control group was 101.86±13.1 % (Fig. 20C). Aspirin (100 mg kg^{-1}, *p.o.*) suppressed the mean maximal oedema formed significantly to 61.29±8.43 % relative to the control group (Fig. 20C). Similarly, PLE (100-600 mg kg^{-1}, *p.o.*) significantly suppressed the mean maximal oedema formed to 61.97±7.75 %, 61.42±7.59 % and 65.24±8.07 % at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control group respectively (Fig. 20C). The total paw oedema attained was significantly reduced by 43.72 %, 52.3 % and 61.58 % at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control group respectively (Fig. 20D).

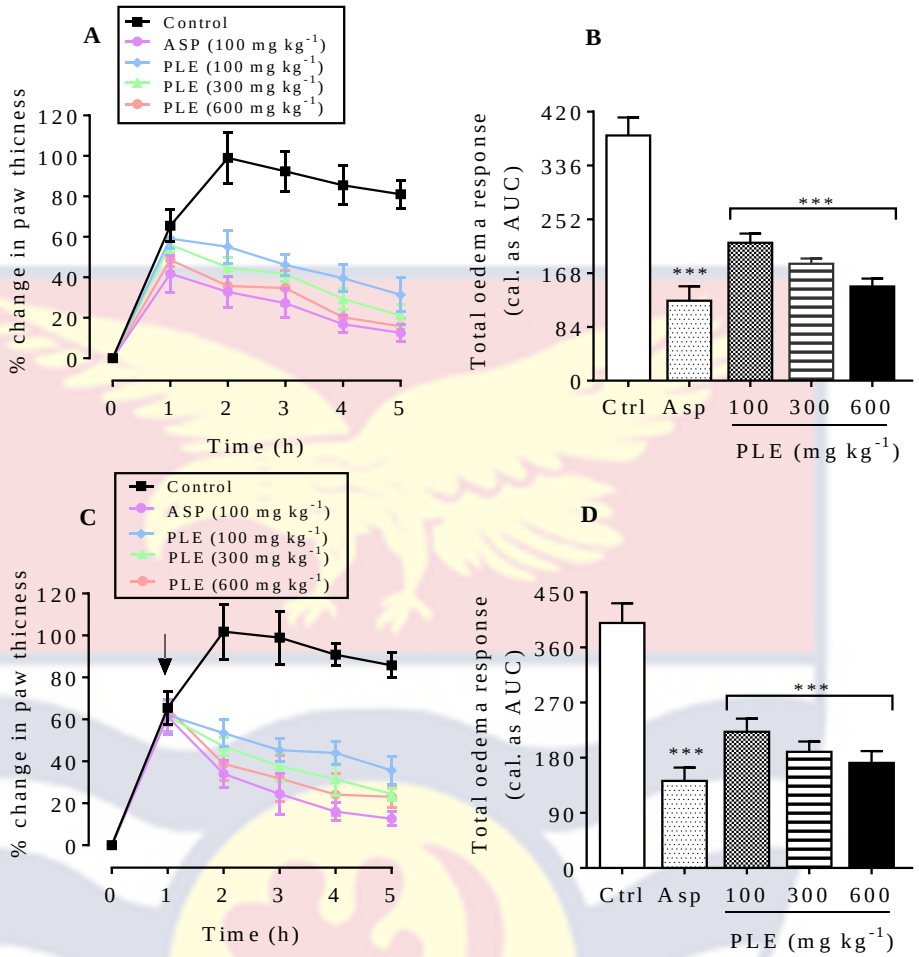


Figure 20: Effect of PLE (100-600 mg kg^{-1}, *p.o.*) on carrageenan-induced paw oedema in rats. Paw oedema formation was observed at 1 h intervals for 5 h as the time course curves (maximal response) of paw thickness (A, C). Total paw oedema was determined as AUC (B, D) and data was presented as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001 compared to inflamed control response (Two-way ANOVA followed by Dunnet's *post hoc* test). Arrow denotes time of drug administration. Ctrl= Control; ASP= Aspirin.

Zymosan-induced acute knee joint arthritis in Sprague-Dawley rats

Knee Joint swelling assessment

From the study, induction of acute knee joint arthritis as a results of intra-articular injection of ZM (as earlier described) caused swelling of the knee joint. In this study, naive group showed no swelling of the knee joint relative to the control (zymosan-treated) group (Fig. 21). However, there was a severe swelling of knee joint of rats in the negative control group that attained a mean

maximal knee joint thickness of 62.43±5.73 % (Fig. 21A). Aspirin (100 mg kg^{-1}, *p.o.*) significantly decreased the mean maximal knee joint swelling attained to 19.28±5.78 % relative to the control group (Fig. 21A). Similarly, PLE (100-600 mg kg^{-1}, *p.o.*) significantly decreased the mean maximal knee joint thickness attained to 32.07±2.98 % and 24.33±8.58 % at 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control response respectively (Fig. 21A).

The total knee joint swelling attained after 5 h of acute knee joint arthritis induction was suppressed significantly by 37.51 %, 47.27 % and 60.46 % at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control group in a dose-dependent manner respectively (Fig. 21B).

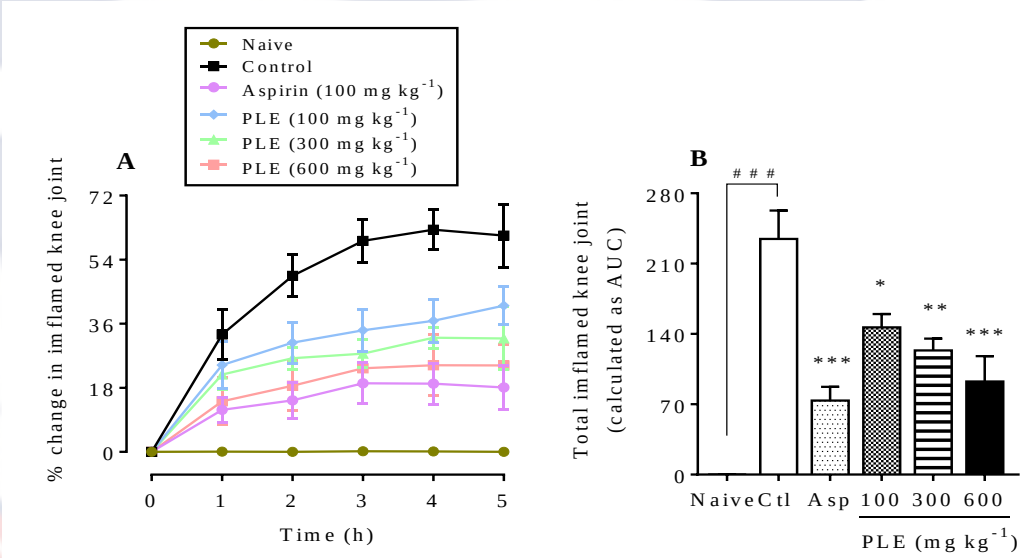


Figure 21: Effect of PLE (100-600 mg kg^{-1}, *p.o.*) on zymosan-induced acute knee joint arthritis in rats. Data was presented as mean ± SEM. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to inflamed control response (Two-way ANOVA followed by Dunnet's *post hoc* test). Ctrl= Control; Asp= Aspirin.

Neutrophil and leukocyte infiltration in knee joint

Intra-articular injection of zymosan is known to induce articular inflammatory response which is characterised by massive neutrophil influx and increased total leukocyte migration into the synovial cavity (Rosas *et al.*, 2015).

From the study, there was elevated infiltration of neutrophil and leukocyte levels in the knee joint cavity of the control group. Influx of neutrophils in the synovial joint of the knee increased to 8.51±0.50 in the control group (Fig. 22A). Aspirin (100 mg kg^{-1}, *p.o.*) significantly reduced neutrophil infiltration in the knee cavity to 3.28±0.73 relative to the control (Fig. 22A). PLE (100-600 mg kg^{-1}, *p.o.*) similarly decreased neutrophil influx in the knee cavity significantly to 4.93±0.71 and 3.72±0.54 at 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control group respectively (Fig. 22A). Total leukocyte infiltration into the knee cavity increased enormously to 10.46±0.39 in the control group (Fig. 22B). Aspirin (100 mg kg^{-1}, *p.o.*) exhibited a decreased of total leukocyte infiltration significantly to 5.29±0.60 relative to the control group (Fig. 22B). Similarly, PLE (100-600 mg kg^{-1}, *p.o.*) showed a significant reduction of the total leukocyte infiltration in the knee joint cavity to 7.42±0.47, 6.50±0.51 and 5.51±0.49 at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} relative to the control group respectively (Fig. 22B).

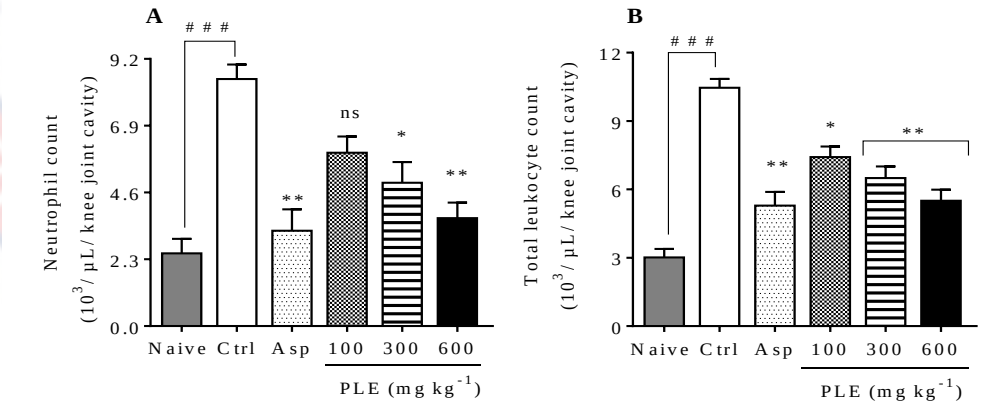


Figure 22: Effect of PLE (100-600 mg kg^{-1}, *p.o.*) on neutrophil (A) and total leukocyte (B) infiltration of knee joint cavity in zymosan-induced acute knee joint arthritis in rats. Data was presented as mean ± SEM. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to control group (Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant; Ctrl= Control; Asp= Aspirin.

Histopathological assessment of knee joint

Microscopic examination of the knee joint after 5 h of intra-articular injection of ZM was performed. From the study, the naïve group showed normal articular cartilage, bone and synovium with no signs of pathological arthritis relative to the control (ZM-treated) group (Fig. 23A). The negative control (ZM-treated) group exhibited severe synovitis of pathological arthritis characterised by marked increased inflammatory cell infiltration, decreased chondrocytes, damaged cartilage and synovium hypertrophy (Fig. 23B). However, aspirin (100 mg kg^{-1}, *p.o.*) attenuated the pathological arthritis of the knee joint by reducing cartilage and bone destruction, inflammatory cell infiltration and synovium hypertrophy relative to the control group (Fig. 23C). In a similar manner, PLE (100-600 mg kg^{-1}, *p.o.*) ameliorated the histological changes associated with pathological arthritis by decreasing synovium hypertrophy, inflammatory cell infiltration and improving knee joint and cartilage architecture at all doses relative to the control group (Fig. 23[D-F]).

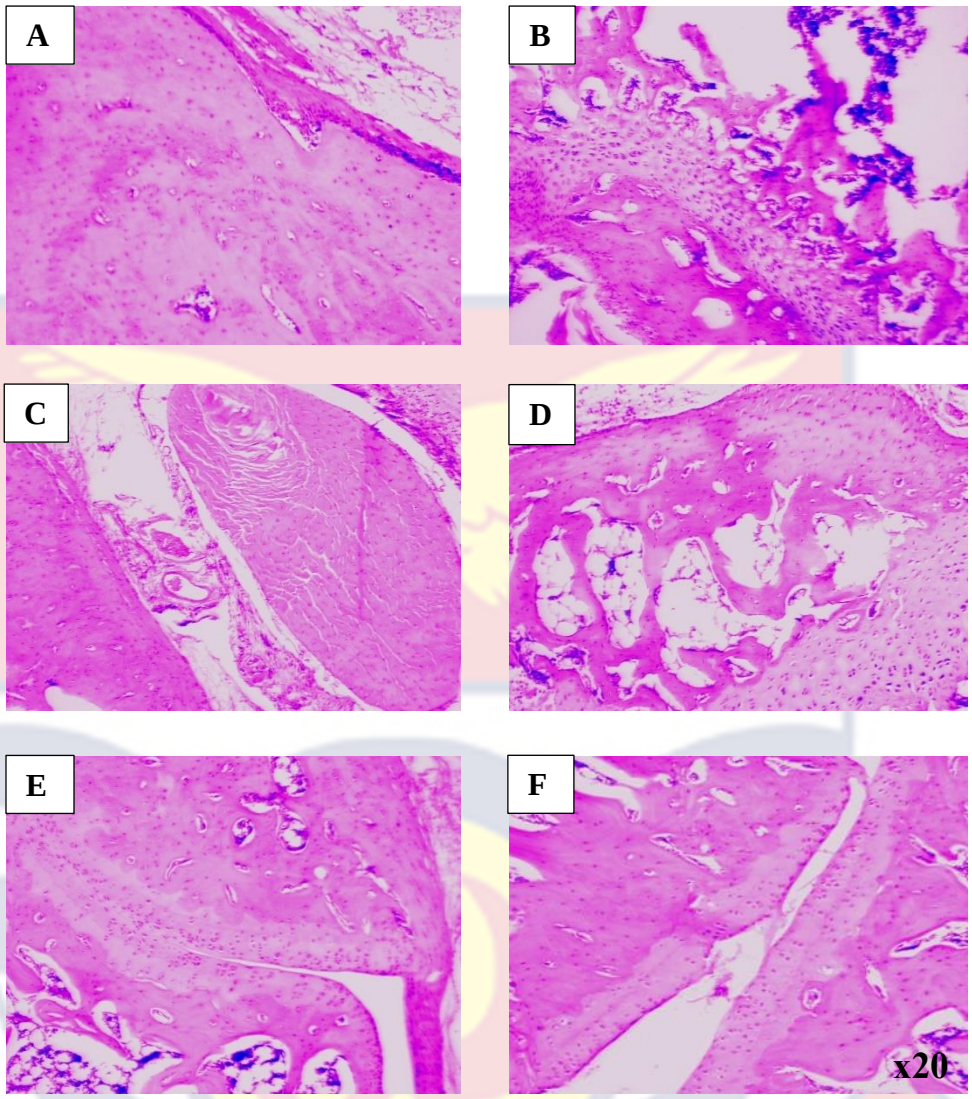


Figure 23: Histopathological evaluation of PLE on zymosan-induced acute knee joint arthritis in Sprague-Dawley rats. Sections made from knee joint of the right hind limbs, and stained using H & E stain. Naïve control (A), ZM-treated (acute knee-joint arthritic) control (B), Aspirin 100 mg kg^{-1} (C), PLE (100-600 mg kg^{-1}) [D-F] respectively.

Mast cell proliferation in knee joint cavity

In this study, after arthritis formation in rats as described earlier, knee joint tissues were taken. Tissue sections were stained with 1 % toluidine blue dye and the proliferation of mast cells due to knee joint arthritis were assessed. From the study, the naïve control group showed no increased influx of mast cells to joint cavity (Fig. 24A) and significantly recorded a baseline mean total number of mast cell of 6.60±1.33 relative to knee joint arthritis (ZM-treated)

control group (Fig. 25). Knee joint arthritis (ZM-treated) control group showed elevated influx levels of mast cells to the knee joint cavity (Fig. 24B) with high mean total number of mast cells count of 30.80±3.06 (Fig. 25). However, aspirin (100 mg kg^{-1})-treated groups decreased the proliferation of mast cells at the knee joint cavity (Fig. 24C) and significantly reduced the mean total number of mast cells count to 15.20±1.66 relative to arthritic (ZM-induced) control group (Fig. 25). In a similar manner, PLE-treated (100-600 mg kg^{-1}) group showed a decreased influx of mast cells to the joint cavity (Fig. 24[D-F]) with a reduction of the mean total number of mast cells count significantly to 21.20±3.34, 18.20±2.85 and 17.00±2.00 at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} relative to ZM-treated (acute knee joint arthritic) control group respectively (Fig. 25).

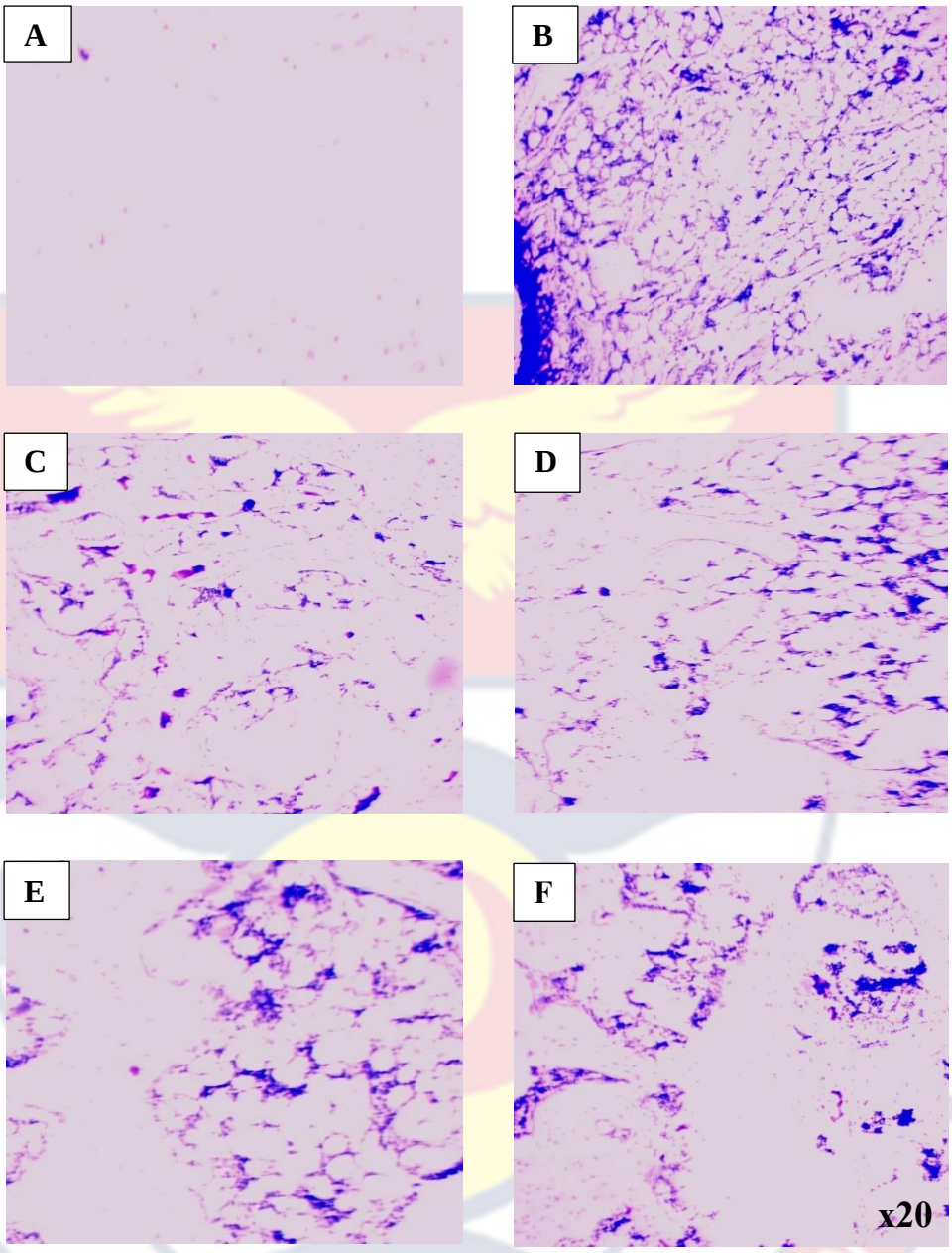


Figure 24: Mast cell proliferation in zymosan (ZM)-induced acute knee joint arthritis in Sprague-Dawley rats. Sections made from knee joint of the right hind limbs, and stained using 1 % toluidine blue dye. Naïve control (A), ZM-treated (acute knee-joint arthritic) control (B), Aspirin 100 mg kg^{-1} (C), PLE (100-600 mg kg^{-1}) [D-F] respectively.

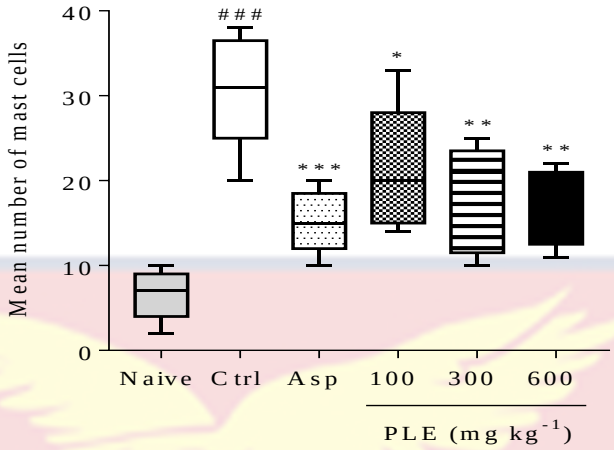


Figure 25: Effect of PLE (100-600 mg kg^{-1}, *p.o.*) on mast cell proliferation in zymosan (ZM)-induced acute knee-joint arthritis in Sprague-Dawley rats (n=5). Data was presented as mean ± SEM. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to control group (Two-way ANOVA followed by Dunnet's *post hoc* test). Ctrl= Control; Asp= Aspirin.

Acetic acid-induced ulcerative colitis in rats

Change in body weight

Colitis was induced in rats by intrarectal injection of 1 ml of 4 % acetic acid. Changes in body weight were recorded daily till day 8. From the study, there was a general loss of body weight in all the treatment groups [Group I-VI] (Fig. 26). Acetic acid challenged group (Group II) exhibited a remarkable reduction of body weight in rats over the time course of the study (Fig. 26A), with corresponding total body weight loss relative to the non-acetic group [Group I] (Fig. 26B). Sulfasalazine (500 mg kg^{-1})-treated group exhibited a decrease in body weight of rats in both the time course of the study (Fig. 26A) and the total body weight (Fig. 26B) relative to the colitis control group respectively. PLE (100-600 mg kg^{-1})-treated group showed a marginal gain in body weight at 600 mg kg^{-1} in both the mean (Fig. 26A) and total body weights of rats (Fig. 26B) relative to the colitis control group.

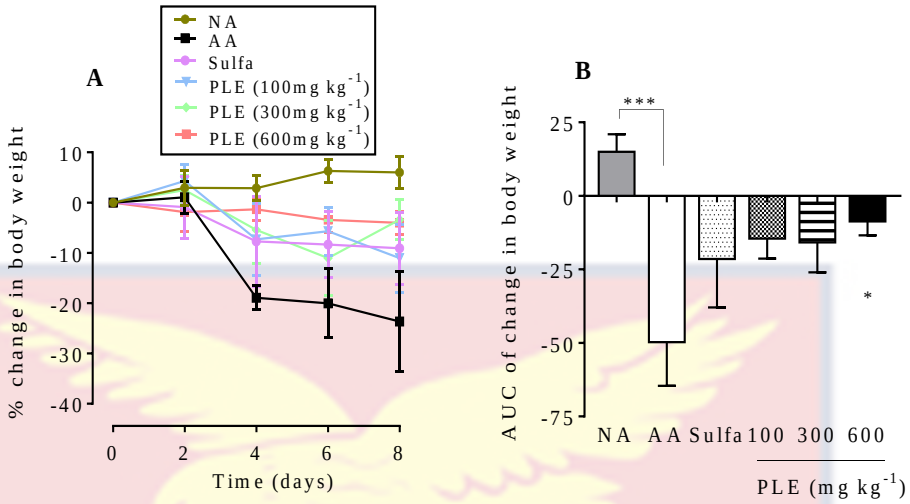


Figure 26: Effect of PLE (100-600 mg kg^{-1}, *p.o.*) on the body weight of rats in acetic acid-induced colitis. Data was prsented as mean ± S.E.M (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to colitis control group (Two-way ANOVA followed by Dunnet's *post hoc* test). NA and AA denote non-colitic and acetic acid-induced colitis groups respectively.

Disease Activity Index (macroscopic analysis)

Intrarectal injection of 1 ml of 4 % acetic acid causes mucosal colonic ulceration characterised by increased infiltration of neutrophils, severe mucosal necrosis, submucosal colitis, oedema and vasodilation. These are the main features of human IBD (Daneshmand *et al*., 2009) evaluated all together as the disease activity index (DAI). To assess these, colonic damage was induced with 4 % of acetic acid in rats. From the study, macroscopic assessment revealed that acetic acid control group showed extremely severe colonic injury (refer to appendix A[b]) with a significantly high disease activity index (84.00±5.09) as compared to the non-acetic control group (Fig. 27A). Non acetic acid-induced group showed no colonic damage (refer to appendix A[a]) and thus attained significantly lower disease activity index of 2.00±1.40 (Fig. 27A).

Sulfasalazine (500 mg kg^{-1})-treated group exhibited a significant improvement of the disease activity index (37.00±10.44) macroscopically (refer

to appendix A[c]) when compared to the acetic acid control group (Fig. 27A). PLE-treated rats (100-600 mg kg^{-1}) exhibited an improvement of the macroscopic status (refer to appendix A[d-f]) of the disease significantly with decreased DAI (58.00±5.39, 50.00±7.07 and 43.00±5.38) at 100, 300 and 600 mg kg^{-1} dose-dependently relative to the acetic acid control group respectively (Fig. 27A). Colon weight-to-length ratio was evaluated and from the study, sulfasalazine-treated (500 mg kg^{-1}) group showed a significant decrease in the colon weight-to-length ratio to 0.28±0.06 relative to the acetic acid-treated control group (0.67±0.05) (Fig. 27B). Similarly, PLE-treated group showed a reduction of colon weight-to-length ratio significantly from 0.67±0.05 to 0.32±0.10 and 0.25±0.11 at 300 mg kg^{-1} and 600 mg kg^{-1} dose levels respectively relative to the acetic acid-induced control group (Fig. 27B).

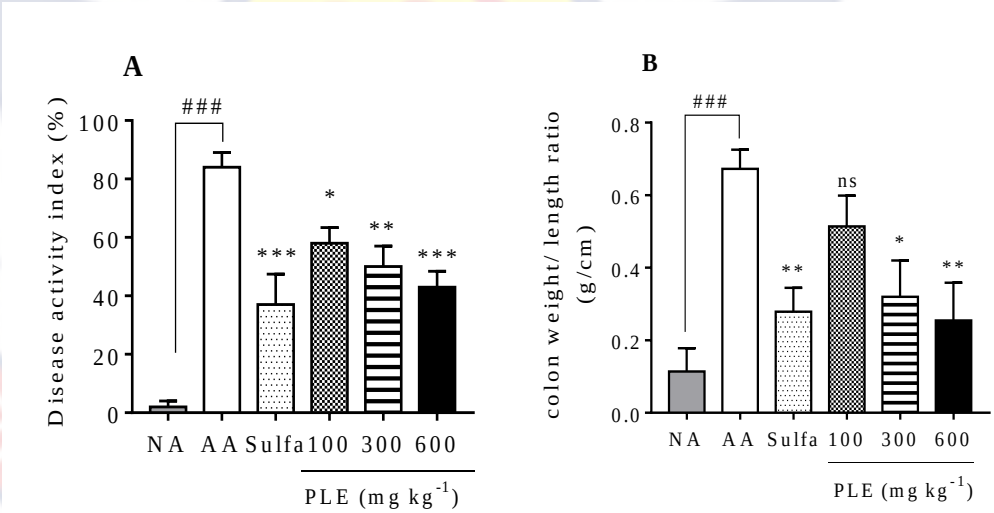


Figure 27: Effect of PLE (100-600 mg kg^{-1}, *p.o.*) on acetic acid-induced ulcerative colitis in rats (n=5). DAI (Fig. 27A) and colon weight-length ratio (Fig. 27B) were determined as the area under the time course curves (AUC). Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to colitis control group (Two-way ANOVA followed by Dunnet's *post hoc* test). NA and AA indicate non colitis and acetic acid-induce colitis groups respectively. ns denotes non-significant.

Haematology

PLE (100-600 mg kg^{-1}) effects on haematological parameters after colitis induction was evaluated by analysing blood samples for Full Blood Count (FBC). Detectable levels of red blood cells (RBC), lymphocytes (LYM), platelets (PLT), white blood cells (WBC), haematocrit (HCT), haemoglobin (HGB) and neutrophils (NEU) were observed in non-acetic acid control group. There were remarkably high levels of WBC, LMP and NEU in acetic acid-induced colitis group relative to the non-acetic acid control group (Table 2). Sulfasalazine-treated (500 mg kg^{-1}) group showed significant reduced levels of WBC, LYM and NEU relative to acetic acid-treated control group (Table 2). Treatment with PLE (100-600 mg kg^{-1}) resulted in a significant decrease in the levels of WBC at all dose levels, LYM at 300 and 600 mg kg^{-1}, and NEU at 600 mg kg^{-1} when compared to the acetic acid-treated control group respectively (Table 2).

Table 2. Effect of PLE on blood indices in acetic acid-induced ulcerative colitis in rats.

Treatment	WBC (10^3/ µL)	HGB (g/ dL)	RBC (10^3/ µL)	PLT (10^3/ µL)	LYM (10^3/ µL)	HCT (%)	NEU (10^3/ µL)
Non-acetic acid	4.19±0.99**	11.34±1.03	6.44±0.55	485.6±88.21	3.44±0.39	43.54±3.89	0.59±0.22**
Acetic Acid	10.8 2±1.32	11.93±0.82	5.39±0.67	572.8±147.7	5.11±0.71	43.94±3.34	2.52±0.88
Sulfa (500 mg kg^{-1})	4.47±0.57**	10.57±1.75	5.74±0.76	381.8±147.0	2.23±0.43*	39.63±2.90	0.73±0.23*
PLE (100 mg kg^{-1})	5.99±1.02*	11.60±0.67	5.88±0.44	388.0±116.3	2.93±1.09	39.48±2.49	1.71±0.37
(300 mg kg^{-1})	5.38±1.43*	12.02±0.92	6.13±0.48	471.2±152.5	2.43±0.52*	41.38±3.23	1.38±0.46
(600 mg kg^{-1})	4.64±1.79**	11.96±0.54	5.83±0.43	421.0±162.2	2.05±0.41*	39.54±2.94	0.67±0.17*

Sprague-Dawley (150-200 g, n=5) rats received either sulphasalazine [Sulfa] (500 mg kg^{-1}, *p.o.*) or PLE (100-600 mg kg^{-1}, *p.o.*) for 8 days. Colitis was induced intrarectally on day 4 using 1 ml of 4 % acetic acid ($^{v}/_{v}$). On day 9, animals were euthanised and blood samples were collected for haematological analysis. *P<0.05; **P<0.01; ***P<0.001 when compared to colitis control group.

Histopathological assessment

Histopathological examination was performed on the colons for colonic integrity in rats of all the treatment groups after colitis had been induced by injecting 1 ml of 4 % acetic acid ($^{v}/_{v}$) through the rectum into the colons.

In this study, the non-acetic control group after microscopic examination showed intact colon with normal mucosa, submucosa and muscularis propria (Fig. 28A). Thus, colonic integrity was maintained when compared to the acetic-acid control group. Acetic-acid control group showed severe colonic damage characterised by increased infiltrated inflammatory cells, mucosal ulceration, necrosis, oedema and epithelial layer disruption (Fig. 28B). Treatment with sulfasalazine (500 mg kg^{-1}, *p.o.*) ameliorated histological damage by significantly decreasing infiltrated inflammatory cells, oedema and necrosis. Also, mucosal and submucosal damage was inhibited relative to the acetic-acid control group (Fig. 28C). PLE (100-600 mg kg^{-1}, *p.o.*) significantly reduced cell necrosis, oedema and increased infiltrated inflammatory cells. Mucosal and submucosal ulceration as well as distortion of the epithelial layer were reduced at 300 and 600 mg kg^{-1} relative to the acetic acid-induced control group (Fig. 28[D-F]).

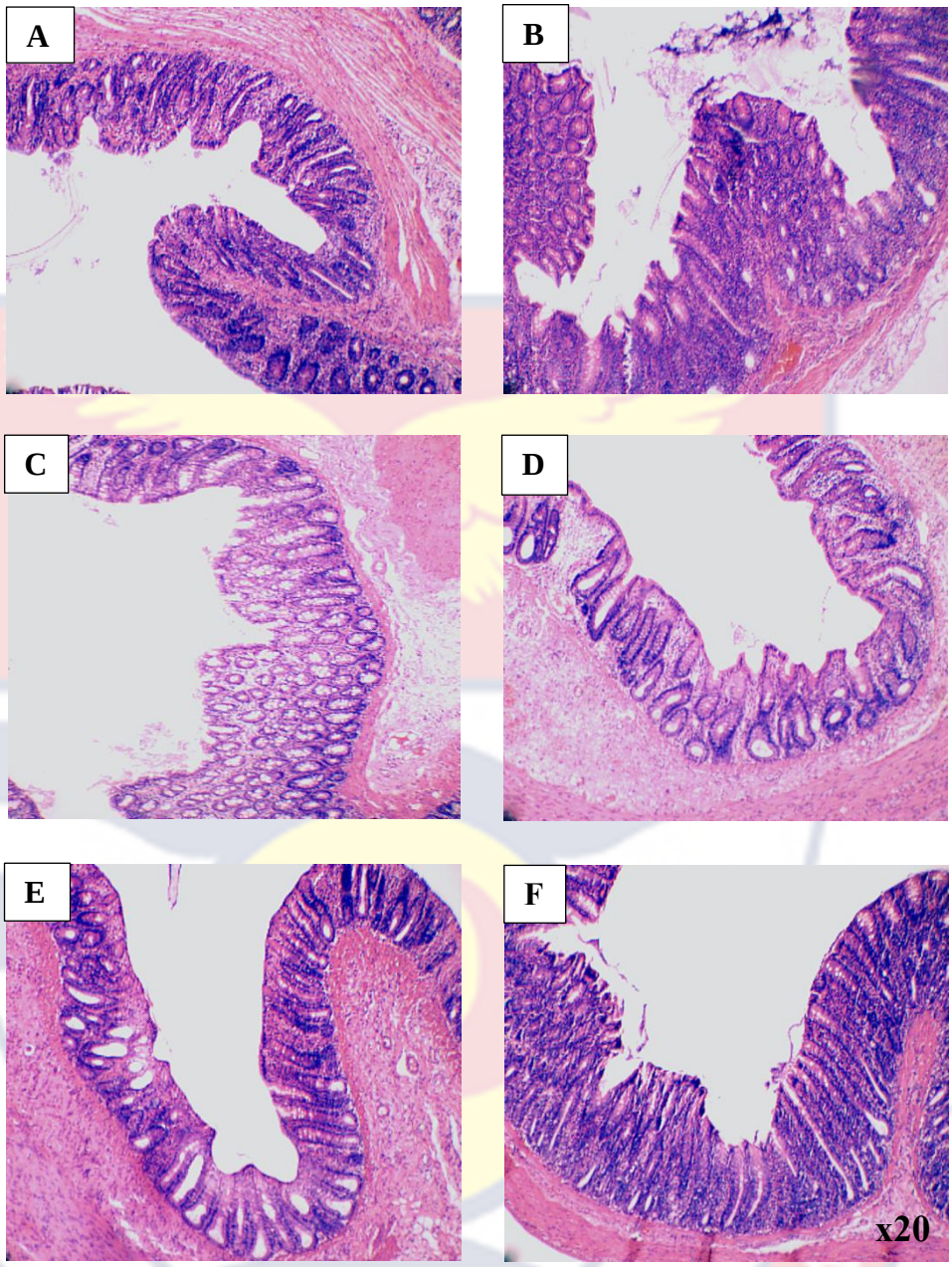


Figure 28: Effect of PLE on histopathological assessment of acetic acid-induced ulcerative colitis in rats (n=5). Either normal saline, sulfasalazine (500 mg kg^{-1}) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered for 8 days. Colitis was induced (except non-acetic acid control animals) on 4th day intrarectally with 1 ml of 4 % acetic acid ($^{v}/_{v}$). At the end of the 8-day period, distal colons were extirpated. Distal colons were fixed immediately in 10 % formaldehyde solution, embedded in paraffin, cut into 5mm thick transversal sections, mounted on glass slides, deparaffinised and stained with H & E stain. Non-acetic acid control (A), colitis control group (B), sulphasalazine 500 mg kg^{-1} (C), PLE (100-600 mg kg^{-1}) [D-F] respectively.

Mast cell proliferation

Mast cells are known to play major function in inflammatory reactions. Theoharis *et al*. (2012) reported that mast cells are implicated in chronic inflammation.

In this study, after colitis formation in rats as described earlier, colonic tissues were taken at the end of observation period. Colon sections were stained with 1 % toluidine blue dye and the proliferation of mast cells due to colonic injury assessed. From the study, the non-acetic acid induced control showed no increased influx of mast cells to the colonic site of injury (Fig. 29) and recorded mean total number of mast cell count of 4.36±0.89 when compared to the acetic acid-treated control group [20.46±2.43] (Fig. 30). In the acetic acid-treated control group, there was elevated influx levels of mast cells to the colonic injured site (Fig. 29B) with significantly higher mean total number of mast cell count [20.46±2.43] (Fig. 30). Sulfasalazine-treated (500 mg kg^{-1}) group decreased the proliferation of mast cells to the site of colonic injury (Fig. 29C) with significantly reduced mean total mast cell count of 7.39±1.54 when compared to the acetic acid-induced control group (Fig. 30). Similarly, PLE-treated (100-600 mg kg^{-1}) groups showed a decreased influx of mast cells to the colonic injured site (Fig. 29[D-F]) with a significant reduction of mean total number of mast cells to 12.07±2.21, 9.88±2.24 and 8.59±1.79 at 100 mg kg^{-1}, 300 mg kg^{-1} and 600 mg kg^{-1} in a dose-dependent manner when compared to the colitis control group (Fig. 30).

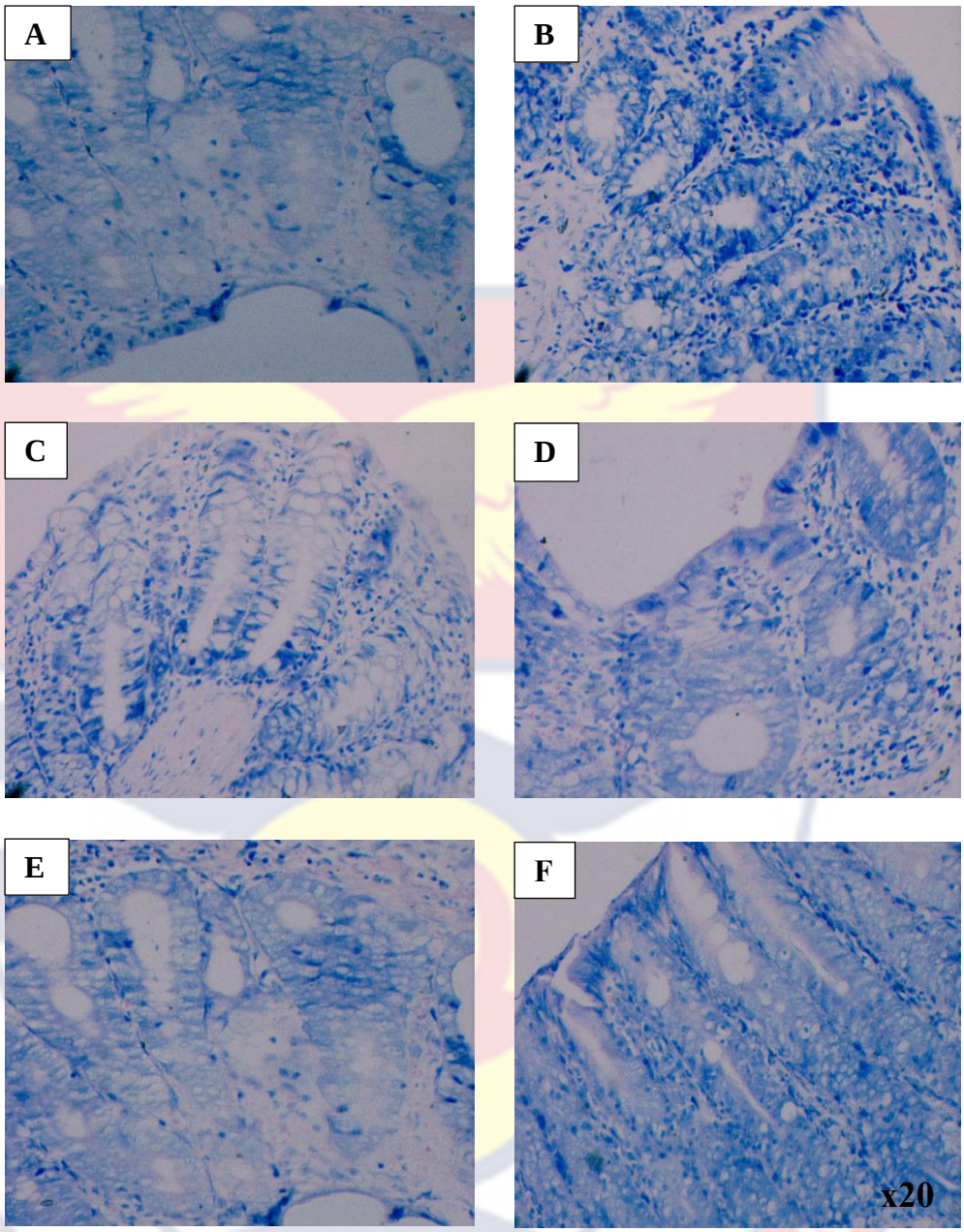


Figure 29: Mast cell proliferation in acetic acid-induced ulcerative colitis in rats (n=5). Either normal saline, sulfasalazine (500 mg kg^{-1}, *p.o.*) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered for 8 days. Colitis was induced (except non-acetic control animals) on 4^{th} day intrarectally with 1 ml of 4.0 % acetic acid ($^{v}/_{v}$). At the end of the observation period, colons were extirpated. Distal colons were fixed immediately in 10 % formaldehyde solution, embedded in paraffin, cut into 5 mm thick transversal sections, mounted on glass slides, deparaffinised and stained with 1 % toluidine blue dye. Non-colitis control (A), Colitis (acetic acid-tretaed) control (B), sulfasalazine 500 mg kg^{-1} (C), PLE (100-600 mg kg^{-1}) [D-F] respectively.

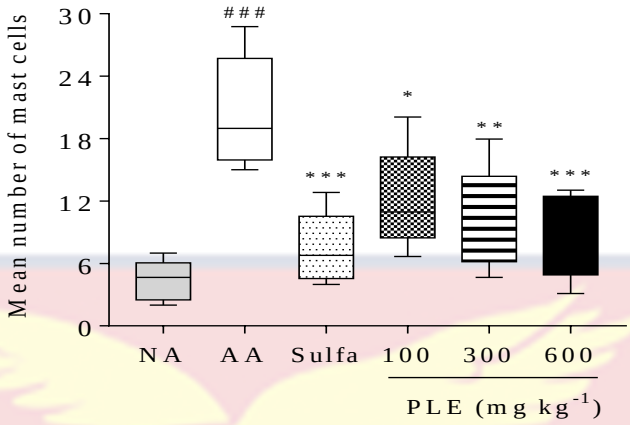


Figure 30: Effect of PLE on mast cell proliferation in acetic acid-induced ulcerative colitis in rats. Distal colons were fixed immediately in 10 % formaldehyde solution, embedded in paraffin, cut into 5 mm thick transversal sections, mounted on glass slides, deparaffinised and stained with 1 % toluidine blue dye. Mast cells were counted using electronic microscope. Data was presented as mean ± SEM. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to colitis group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). NA and AA indicate non-acetic coltis and acetic acid-induced colitis groups respectively.

In vivo anti-oxidant activities of PLE

The anti-oxidant capacity of CAT, SOD enzymes and MDA content in colon samples of rats were tested to assessed the anti-oxidant potential of PLE (100-600 mg kg^{-1}) under different treatment groups.

Catalase (CAT) activity

CAT is an essential anti-oxidant enzyme in the body. From the study, there was a drastic reduction in the expression of CAT enzyme in the colons of rats in the control group (0.36±0.14 units/ mg protein) (Fig. 31A). However, sulfasalazine (500 mg kg^{-1}, *p.o.*) showed a significant increase of CAT enzyme expression to 2.49±0.27 units/ mg protein when compared to the control group (Fig. 31A). Similarly, PLE (100-600 mg kg^{-1}, *p.o.*) significantly increased the expression of CAT enzyme to 1.16±0.10 units/ mg protein, 1.40±0.22 units/ mg

protein and 1.93±0.13 units/ mg protein at 100, 300 and 600 mg kg^{-1} dose-dependently when compared to the control group respectively (Fig. 31A).

Superoxide dismutase (SOD) activity

Like CAT, SOD enzyme is known to be important in the body. In this study, the level of SOD enzyme expression in the acetic acid-induced control group was greatly reduced [0.033 ± 0.006 units/ mg protein] (Fig. 31B). It was observed that sulfasalazine (500 mg kg^{-1}) significantly increased the SOD enzyme expression to 0.192±0.012 units/ mg protein when compared to the control group (Fig. 31B). Similarly, PLE (100-600 mg kg^{-1}, *p.o.*) significantly increased the expression of SOD enzyme to 0.159±0.042 units/ mg protein at 600 mg kg^{-1} when compared to the control group (Fig. 31B).

Lipid peroxidation (MDA) activity

Oxidative stress mostly could be linked to the MDA content in the body. From the study, MDA content in the colon tissues of rats in the control group was drastically increased to 94.34±5.22 nmol/ mg protein (Fig. 31C). However, sulfasalazine (500 mg kg^{-1})-treated group showed a significant decrease of MDA content to 17.95±5.07 nmol/ mg protein when compared to the control group (Fig. 31C). Similarly, PLE (100-600 mg kg^{-1})-treated groups showed a decrease of MDA content significantly to 60.25±6.77 nmol/ mg protein, 53.08±11.82 nmol/ mg protein and 18.19±8.35 nmol/ mg protein at 100, 300 and 600 mg kg^{-1} when compared to the control group respectively (Fig. 31C).

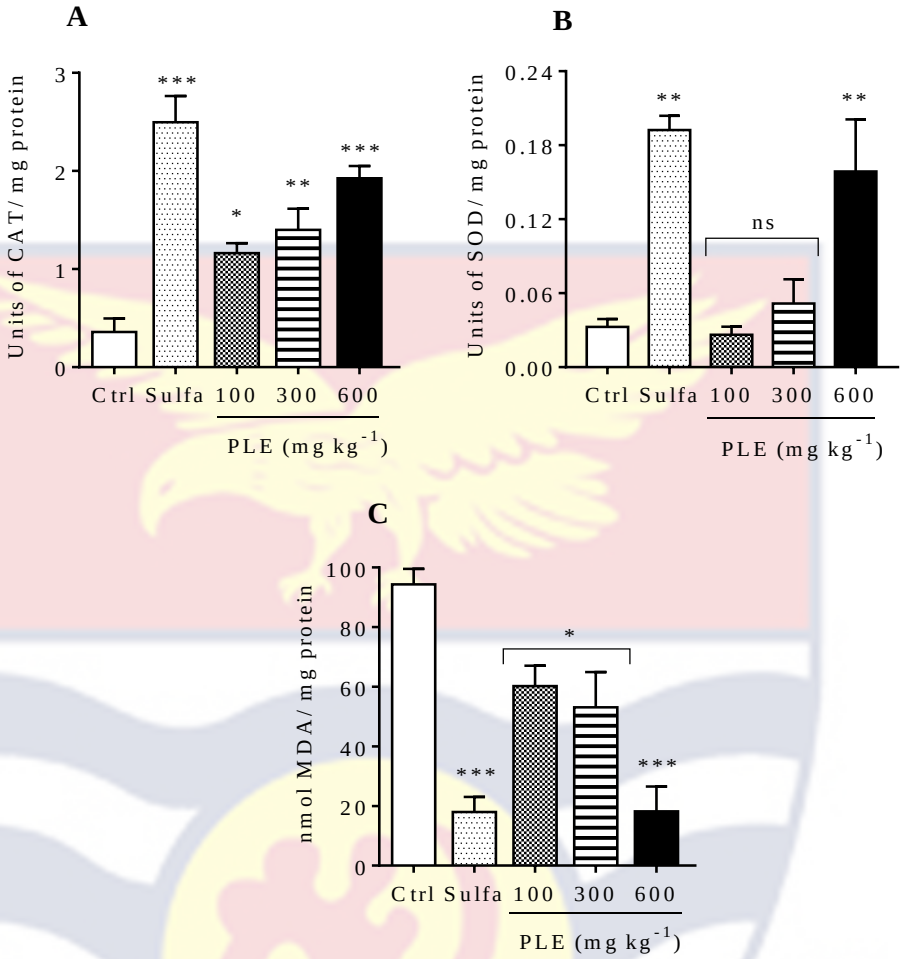


Figure 31: *In vivo* anti-oxidant activity of PLE in acetic acid-induced ulcerative colitis in Sprague-Dawley rats (n=5). Colitis was induced by intrarectally inoculating 1 ml of 4 % acetic acid into rats' colons. Either sulfasalazine [Sulfa] (500 mg kg^{-1}, *p.o.*) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered from day 4 until the 8^{th} day. Rats were euthanised on day 9, blood samples were collected and sera were prepared for anti-oxidant activities. Anti-oxidant analysis was performed for Catalase activity (CAT) [A], Superoxide dismutase (SOD) [B] and Malondialdehyde (MDA) [C] respectively. All data were presented as mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared to colitis group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Ctrl= Control; Sulfa= Sulfasalazine.

Cotton pellet granuloma tissue formation in rats

Granuloma tissue formation is caused by the cellular infiltration and proliferation of fibroblast (Subash *et al*., 2016). The anti-proliferative effect as a measure of anti-inflammatory effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) was then evaluated. From the study, mean exudate amount of the control group after 7 days of observation period was 835.30±35.38 mg (Table 3). Dexamethasone (3 mg kg^{-1}, *p.o.*, daily) decreased the mean exudate amount (Fig. 32) to 441.30±33.51 mg and significantly inhibited the exudate formation by 47.17 % (Table 3). PLE (100-600 mg kg^{-1}) similarly decreased the mean exudate amount (Fig. 28) to 572.70±31.34 mg, 496.30±65.90 mg and 446.00±60.85 mg and, inhibited exudate formation by 31.44 %, 40.58 % and 46.61 % at 100, 300 and 600 mg kg^{-1} relative to the control respectively (Table 3). In the control group, the granuloma tissue formed at the end of observation period (Fig. 32) was 132.70±7.51 mg (Table 3). Dexamethasone (3 mg kg^{-1}) significantly suppressed the mean granuloma tissue formed to 36.67±4.63 mg with percentage granuloma tissue inhibition of 72.34 % relative to the control group (Table 3). Similarly, PLE (100-600 mg kg^{-1}) suppressed the mean granuloma tissue formed significantly to 105.30±4.18 mg, 102.70±7.22 mg and 92.67±4.91 mg with percentage granuloma tissue inhibition of 20.65 %, 22.61 % and 30.14 % at 100, 300 and 600 mg kg^{-1} relative to the control group respectively (Table 3).

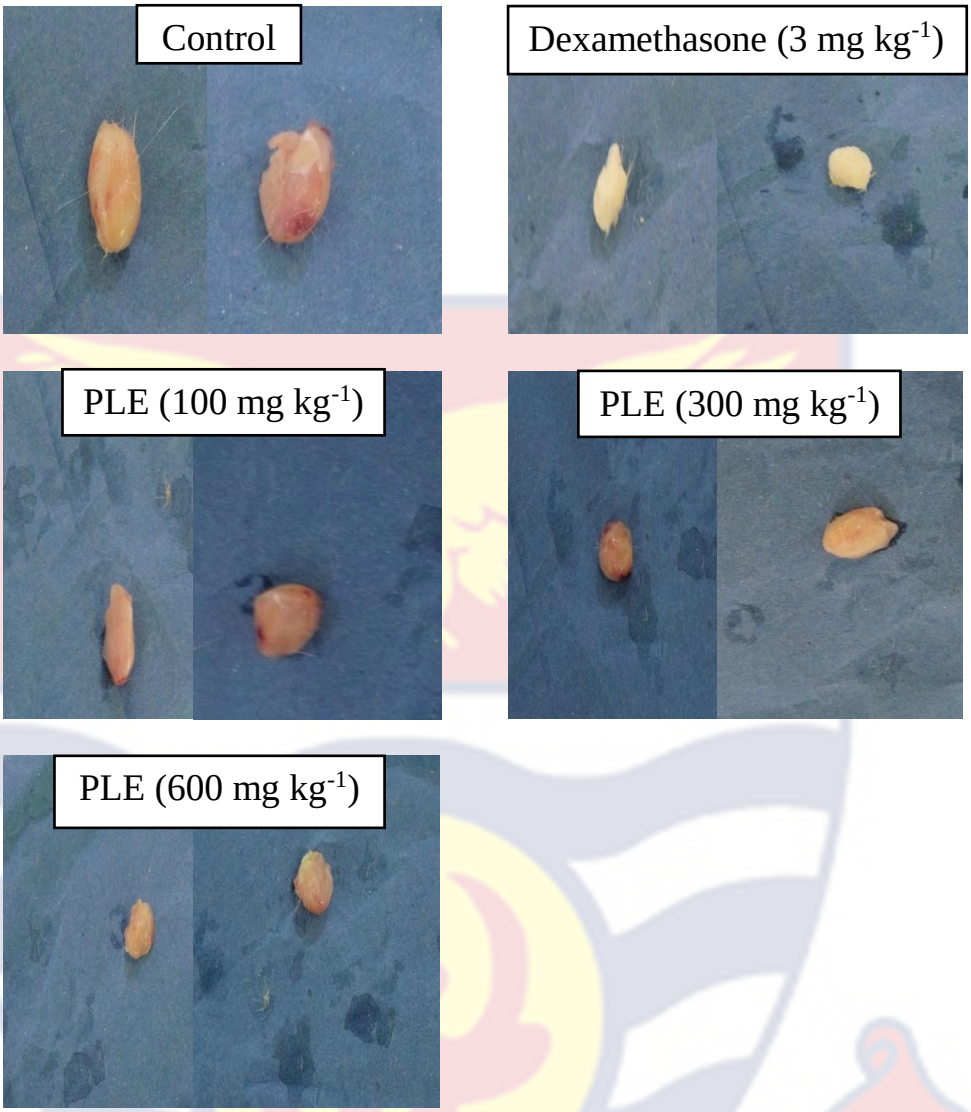


Figure 32: Photographs of cotton pellets removed from rats in all treatment groups in cotton pellet-induced granuloma tissue formation. Sterilised cotton (40±1 mg) was implanted into subcutaneous tissue of the abdomen of Sprague-Dawley rats (150-200 g, n=5). Cotton pellets were removed and weighed immediately for wet weight.

Table 3: Effect of PLE on granuloma tissue formation in rats.

Treatment groups	Exudate (Wet weight)/ mg	% Inhibition	Granuloma tissue (Dry weight)/ mg	% Inhibition
Control	835.30±35.38	-	132.70±7.51	-
Dex (3 mg/ kg)	441.30±33.51***	47.17 %	36.67±4.63***	72.34 %
PLE (100 mg/ kg)	572.70±31.34**	31.44 %	105.30±4.18*	20.65 %
(300 mg/ kg)	496.30±65.90**	40.58 %	102.70±7.22*	22.61 %
(600 mg/ kg)	446.00±60.85***	46.61 %	92.67±4.91**	30.14 %

Sterilised cotton (40±1 mg) was inserted into subcutaneous tissue of the abdomen of rats (150-200 g, n=5). Cotton pellets were weighed for wet weight and incubated for 24 h at 60 ℃ to obtain a constant weight. Dry weight was determined by deducting sterilised cotton (40±1 mg) from the dried pellets. Data was presented as mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared to control group. Dex = Dexamethasone.

Change in body weight

Body weight of rats was recorded (daily) for 7 consecutive days. From the study, the naïve group (animals without cotton implant) increased the mean maximal weight significantly when compared to the control group [acetic acid-induced colitis] (Fig. 33A). There was a general loss of body weight in all treatment groups but the control group remarkably loss weight over the time course of the study (Fig. 33A) as well as the total body weight of rats (Fig. 33B). Dexamethasone (3 mg kg^{-1})-treated group significantly increased the body weight in both the mean maximal (Fig. 33A) and the total body weight of rats in relation to the control group (Fig. 33B). Similarly, PLE (100-600 mg kg^{-1})-treated group showed a significant increase of body weight in both the mean maximal (Fig. 33A) and the total body weight of rats at 300 and 600 mg kg^{-1} when compared to the control group respectively (Fig. 33B).

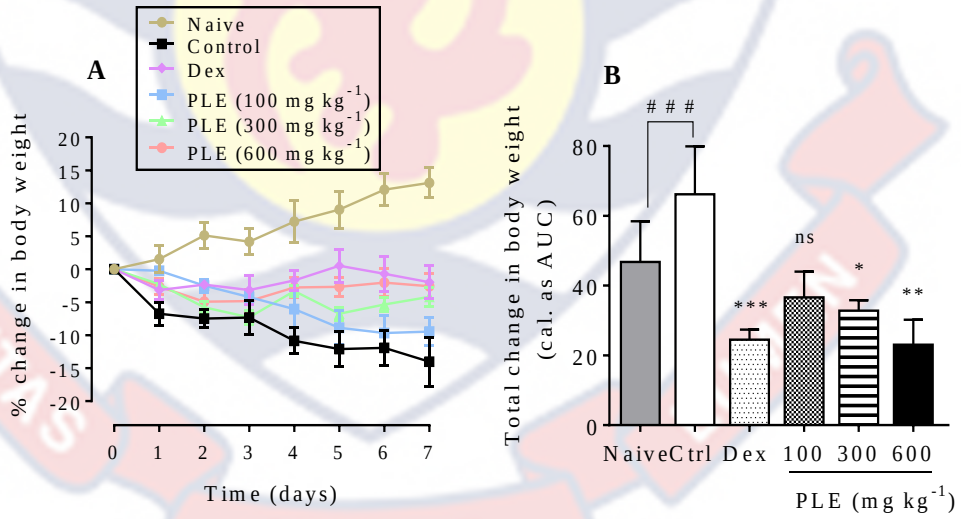


Figure 33: Effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) on the body weight of rats in cotton pellet-induced granuloma tissue formation. Mean maximal change in body weight (A) and total body weight (B) of rats were determined. Data was presented as mean ± SEM. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). Dex= Dexamethasone; Ctrl= Control. ns denotes non-significant.

Haematological examination

PLE (100-600 mg kg^{-1}) effects on haematological parameters after sterilised cotton (40±1 mg) was inserted bilaterally into the subcutaneous tissue of abdomen of rats to induce granuloma tissue formation was assessed by analysing blood samples collected from all treatment groups for Full Blood Count (FBC). There were detectable normal levels of white blood cells (WBC), mean corpuscular volume (MCV), haemoglobin (HGB), lymphocytes (LYM), red blood cells (RBC), neutrophils (NEU) and platelets (PLT) recorded in blood samples from rats of the naïve group (Table 4).

In this study, the control group showed high levels of WBC, PLT and NEU after 7 days of observation period (Table 4). However, dexamethasone (3 mg kg^{-1}, *p.o.*, daily)-treated group significantly decreased the levels of WBC, PLT and NEU when compared to the control group (Table 4). Similarly, PLE (100-600 mg kg^{-1}, *p.o.*, daily)-treated group significantly reduced the levels of WBC at 300 and 600 mg kg^{-1}, PLT at 600 mg kg^{-1}, and NEU at 100, 300 and 600 mg kg^{-1} when compared to the control group respectively (Table 4).

Table 4: Haematological assessment of PLE on cotton pellet granuloma tissue formation in Sprague-Dawley rats.

Groups	WBC (10^3/ µL)	RBC (10^3/ µL)	HGB (g/ dL)	PLT (10^3/ µL)	MCV (fL)	NEU (%)	LYM (%)
Naïve	7.66±0.61#	7.86±0.81	14.21±1.19	557.0±44.0##	58.45±2.05	21.90±1.89##	45.60±2.90
Control	16.73±0.68	5.66±0.36	11.44±1.16	896.0±24.0	47.64±2.44	51.64±5.89	54.51±3.75
Dex (3 mg kg^{-1})	8.69±0.67*	7.39±0.38	14.60±0.90	679.50±31.5*	61.35±2.25	27.77±2.27**	48.87±2.06
PLE (100 mg kg^{-1})	10.13±1.89	6.54±0.35	13.66±0.66	729.0±60.0	56.56±3.26	34.99±1.91*	47.32±2.78
(300 mg kg^{-1})	9.79±2.02*	7.24 0.58	13.35±1.05	702.5±10.5	52.57±2.55	31.09±3.55*	41.69±4.25
(600 mg kg^{-1})	9.09±1.91*	6.94±0.61	13.94±0.87	673.0±51.0*	55.02±4.87	28.22±3.10**	39.78±2.22

Sterilised cotton (40±1 mg) was implanted into subcutaneous tissue of the abdomen of rats (150-200 g, n=5) to induce granuloma tissue formation. Either dexamethasone (3 mg kg^{-1}, *p.o.*, daily) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered for 7 days. On day 8, animals were euthanised and blood samples were collected for haematological analysis. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). Dex= Dexamethasone.

Spleen weight/ body weight of rat

In all treatment groups, the weight of spleen/ body weight of rats was determined and recorded (Table 5). From the study, there was an enlargement of spleen in rats of control group and thus, recorded a high total spleen weight (Table 5). However, the naïve group recorded a normal spleen weight when compared to the control group (Table 5). Dexamethasone (3 mg kg^{-1})-treated group significantly reduced the total spleen weight by 48.53 % when compared to the control group 1(Table 5). Similarly, PLE (100-600 mg kg^{-1})-treated group decreased the total spleen weight significantly by 42.65 % at 600 mg kg^{-1} when compared to the control group (Table 5).

Table 5. Spleen weight/ body weight of rats in cotton pellet granuloma tissue formation.

Groups	Spleen weight/ body weight of rat
Naïve	0.21±0.03***
Control	0.68±0.05
Dex (3 mg kg^{-1})	0.35±0.04**
PLE (100 mg kg^{-1})	0.57±0.03
(300 mg kg^{-1})	0.53±0.06
(600 mg kg^{-1})	0.39±0.07*

Sterilised cotton (40±1 mg) was inserted into subcutaneous tissue of the abdomen of Sprague-Dawley rats (150-200 g, n=5) to induce granuloma tissue formation. Either dexamethasone (3 mg kg^{-1}, *p.o.*, daily) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered for 7 days. On day 8, rats were euthanised, spleens were removed and weighed. *P<0.05; **P<0.01; ***P<0.001 compared to control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). Dex= Dexamethasone.

***In vivo* anti-oxidant Enzyme activity of PLE**

The anti-oxidant enzyme activities of CAT, SOD and inhibition of lipid peroxidation (MDA content) of PLE (100-600 mg kg^{-1}, *p.o.*) was evaluated using sera samples from rats in cotton pellet-induced granuloma tissue formation.

Catalase (CAT) activity

From the study, CAT enzyme expression in the sera of rats of the control group was enormously reduced to 0.68±0.17 units/ mg protein (Fig. 34A). However, dexamethasone (3 mg kg^{-1}, *p.o.*)-treated group increased the CAT enzyme expression significantly to 1.91±0.11 units/ mg protein when compared to the control group (Fig. 34A). PLE (100-600 mg kg^{-1}, *p.o.*)-treated group similarly showed a significant increase in the CAT enzyme expression to 1.27±0.08 units/ mg protein, 1.31±0.14 units/ mg protein and 1.82±0.10 units/ mg protein at 100, 300 and 600 mg kg^{-1} when compared to the control group respectively (Fig. 34A).

Superoxide dismutase (SOD) activity

SOD enzyme activity in this study was decreased in the sera of rats of the control group to 0.074±0.019 units/ mg protein (Fig. 34B). However, dexamethasone (3 mg kg^{-1})-treated group increased the SOD enzyme activity significantly to 0.252±0.178 units/ mg protein when compared to the control group (Fig. 34B). PLE (100-600 mg kg^{-1})-treated group similarly increased the SOD enzyme expression significantly to 0.188±0.031 units/ protein, 0.229±0.010 units/ mg protein and 0.236±0.017 units/ mg protein at 100, 300 and 600 mg kg^{-1} when compared to the control group respectively (Fig. 34B).

Lipid peroxidation (MDA) activity

From the study, it was shown that there was a massive lipid peroxidation activity in the sera of rats of the control group which resulted in a high MDA content of 98.06±8.70 nmols/ mg protein (Fig. 34C). Dexamethasone (3 mg kg^{-1}, *p.o.*) significantly inhibited the lipid peroxidation activity and thus, reduced the MDA content to 22.11±4.05 nmols/ mg protein when compared to the control group (Fig. 34C). Similarly, PLE (100-600 mg kg^{-1}) suppressed lipid peroxidation activity significantly resulting in a reduction of the MDA content to 62.29±6.91 nmols/ mg protein, 45.01±9.53 nmols/ mg protein and 26.44±8.67 nmols/ mg protein at 100, 300 and 600 mg kg^{-1} dose-dependently when compared to the control group respectively (Fig. 34C).

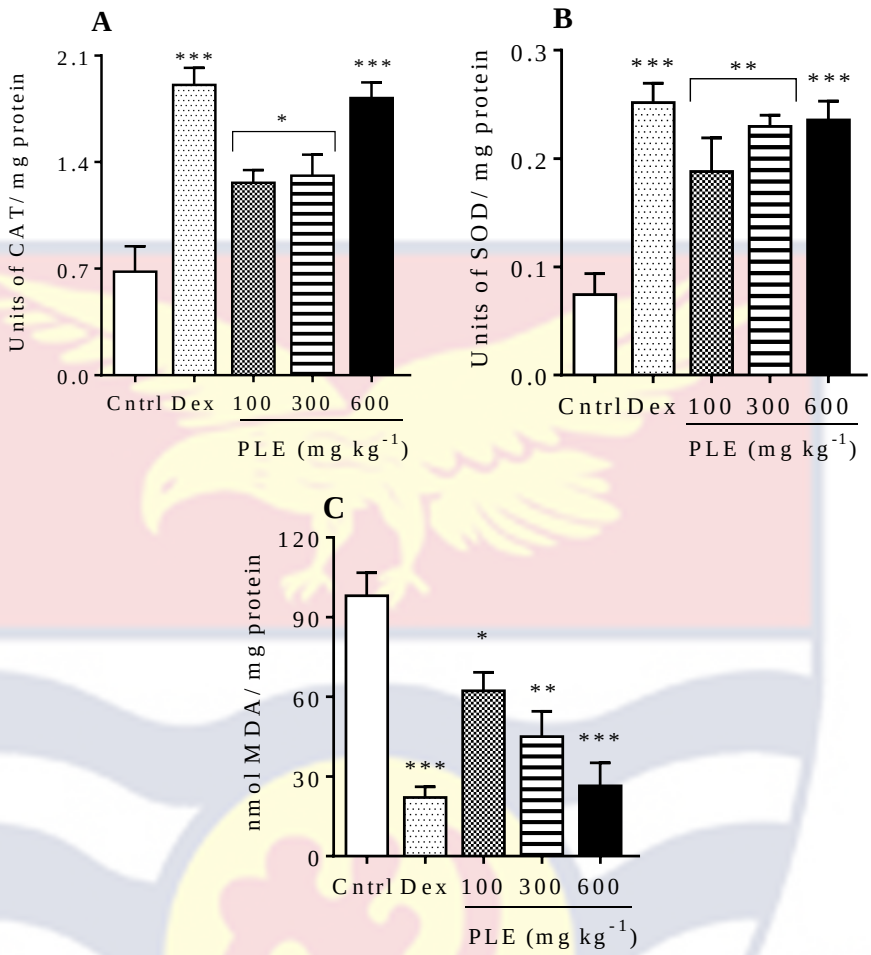


Figure 34: *In vivo* anti-oxidant activity of PLE (100-600 mg kg^{-1}, *p.o.*, daily) on cotton pellet-induced granuloma tissue formation in Sprague-Dawley rats. Anti-oxidant analysis was performed for Catalase activity (CAT) [A], Superoxide dismutase (SOD) [B] and Malondialdehyde (MDA) [C] respectively. Data was presented as mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared to colitis group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). Dex= Dexamethasone.

CFA-induced arthritis in Sprague-Dawley rats

Biochemical and immunological signs of rheumatoid arthritis are stimulated in adjuvant arthritis via induction of heat-killed *Mycobacterium tuberculosis* cell (Ramprasath, Shanthi, & Sachdanandam, 2006). Adjuvant rheumatoid arthritis is the most widely studied model of chronic inflammation in rats (Wang *et al*., 2008).

Change in body weight

Change in body weight of the rats was observed every 4 days in all the experimental groups till day 28. From the study, the non-arthritic (IFA-treated) group exhibited a significant increase in body weight of rats (Fig. 35[A, C]). The total body weight of rats in the IFA group (calc. as AUC) increased significantly when compared to the arthritic (CFA-treated) group (Fig. 35[B, D]).

In the prophylactic study, dexamethasone (3 mg kg^{-1})-treated group exhibited a significant change in body weights in both the mean maximal to 23.35±4.47 % and total body to 65.89±.18.01 % (cal. as AUC) weight levels of rats when compared to the arthritic-induced control group (-17.14±9.56 %) respectively (Fig. 35[A, B]). Unlike dexamethasone, aspirin-treated (100 mg kg^{-1}) rats showed no significant change of body weight in both the mean maximal and total body weights of rats when compared to the CFA-treated control group (Fig. 35[A, B]). PLE (100-600 mg kg^{-1})-treated groups similarly showed a significant steady increase in maximal body weight to 12.31±3.08 % and 14.19±10.04 %, and total body weights (cal. as AUC) of rats to 38.55±13.56 % and 54.81±35.83 % at 300 and 600 mg kg^{-1} when compared to the arthritis control group respectively (Fig. 35[C, D]).

In the curative study, IFA (non-arthritic) group showed a significant change in body weights of rats (Fig. 36[A, C]). The total body weight of rats (cal. as AUC) also increased significantly relative to the CFA-treated (arthritic) control group [-28.19±2.44 %] (Fig. 36[B, D]) respectively. From the study, a general decrease of body weights in rats was observed and this was evidenced in dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups which

exhibited no significant change of body weight in both the mean maximal and total body weights (cal. as AUC) of rats respectively (Fig. 36[A, B]). Similarly, there was no significant change of body weight in both the mean maximal and total body (cal. as AUC) weights in PLE (100-600 mg kg^{-1})-treated rats when compared to the the CFA-treated (arthritic) control group respectively (Fig. 36[C, D]).

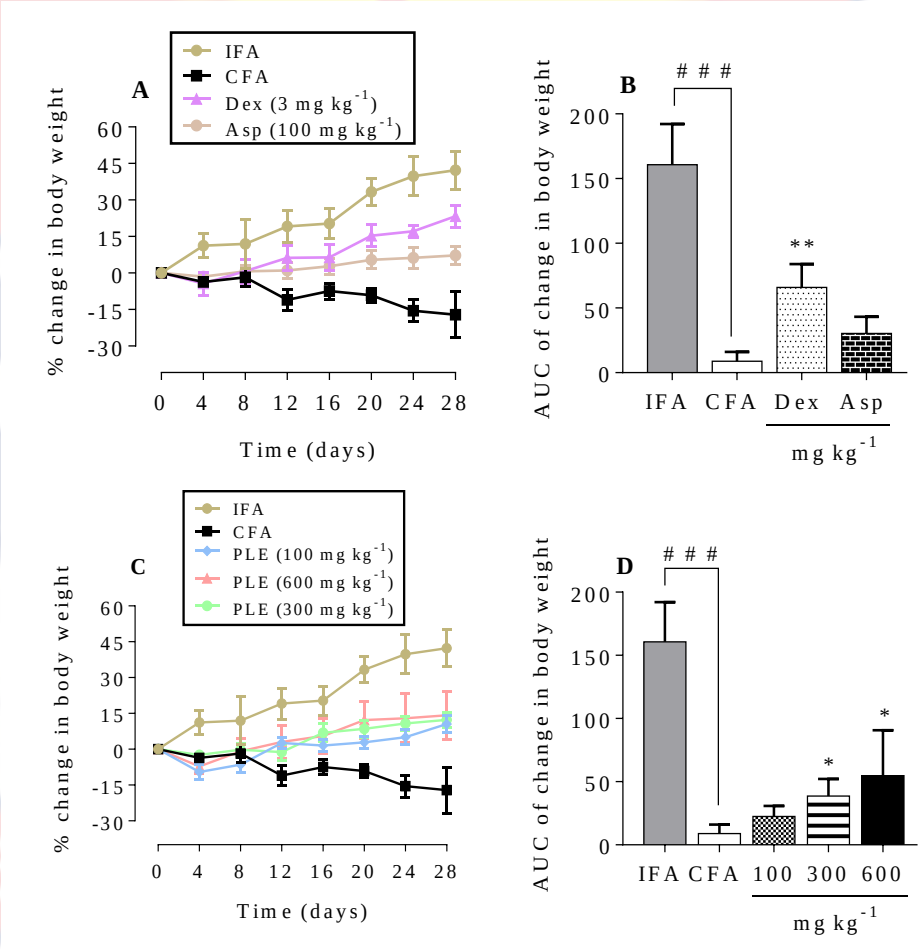


Figure 35: Effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) administered prophylactically on body weight in CFA-induced arthritis in rats. Total body weight was determined as the area under the time course curves, UAC (C, D). All data expressed as mean ± S.E.M (n=5). #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to arthritis control group (Two-way ANOVA followed by Dunnet's *post hoc* test). Dex and Asp denote Dexamethasone and Aspirin respectively.

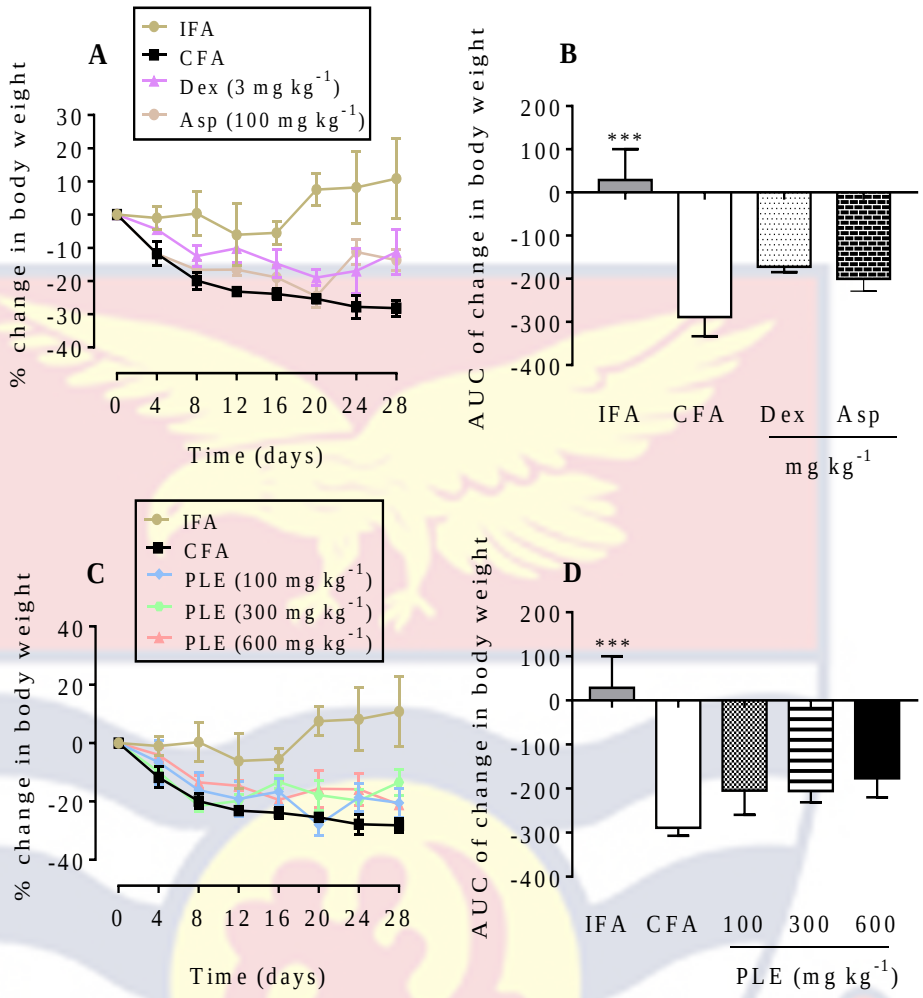


Figure 36: Effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) administered therapeutically on body weight in CFA-induced arthritis in rats. Total body weight was determined as the area under the time course curves, UAC (C, D). All data expressed as mean ± S.E.M (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to CFA-treated control group (Two-way ANOVA followed by Dunnet's post hoc test). Dex and Asp denote Dexamethasone and Aspirin respectively.

Paw oedema evaluation

Paw oedema of rats in the CFA-induced arthritic model was assessed to verify the validity of the sensitivity of the drugs to alter both the maximal (Fig. 37A) and total paw oedema responses (Fig. 37B) in both injected (arthritic) and non-injected (non-arthritic) paws.

In this study, IFA (non-arthritic)-treated group in the prophylactic protocol (i.e. prior to CFA induction) revealed no observable signs of inflammation during the study (Fig. 37). The arthritic control (CFA-treated) group showed severe mean maximal acute oedema (193.05±16.03 %) at the ipsilateral (injected) paw from day 4 to 8 and subsequent polyarthritic stage peaking between day 12 and the 24th day (Fig. 37[A, C]). The spread of oedema in the contralateral paw progressed from day 12 suggestive of systemic spread of inflammation (Fig. 37[A, C]). Dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups reduced the mean maximal oedema significantly to 92.41±20.39 % and 105.49±18.39 % when compared to the arthritic (CFA-treated) group respectively (Fig. 37A). Similarly, PLE (100-600 mg kg^{-1}, *p.o.*)-treated group exhibited a significant reduction of mean maximal inflammatory oedema to 126.58±10.91%, 113.82±11.46 % and 106.45±34.85 % at 100, 300 and 600 mg kg^{-1} when compared to the arthritic (CFA-treated) control group respectively (Fig. 37C). The total joint oedema formed over the 28 days was determined as the AUC (Fig. 37[B, D]) respectively. From the study, dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups suppressed the oedema formed over the 28-day period significantly by 46.32 % and 35.63 % respectively (Fig. 37B). PLE (100-600 mg kg^{-1})-treated group suppressed the inflammatory oedema attained over the 28-day period by 25.33 %, 35.39 % and 43.75 % at 100, 300 and 600 mg kg^{-1} respectively (Fig. 37D).

In therapeutic study, drug administration commenced on the 14th day at the onset of polyarthritis phase. Dexamethasone(3 mg kg^{-1})-treated group exhibited a significant decrease in the mean maximal paw oedema attained (854.50±67.00 %) to 536.17±44.49 % relative to the arthritis control group (Fig.

38A). Like dexamethasone, aspirin (100 mg kg^{-1})-treated group significantly reduced the paw swelling attained (854.50±67.00 %) to 544.67±66.15 % relative to the arthritis control group (Fig. 38A). Similarly, PLE (100-600 mg kg^{-1}) significantly decrease the paw oedema attained to 568.50±91.18 %, 545.50±71.88 % and 541.83±70.21 % dose-dependently at 100, 300 and 600 mg kg^{-1} relative to the arthritis control group respectively (Fig. 38C). The total paw swelling formed over 28 days was decreased by 37.79 % when treated with dexamethasone (3 mg kg^{-1}) and 28.20 % when aspirin (100 mg kg^{-1}) was administered respectively (Fig. 38B). PLE (100-600 mg kg^{-1}) similarly reduced the total paw oedema significantly by 20.98 %, 27.11 % and 33.45% at 100, 300 and 600 mg kg^{-1} respectively (Fig. 38D).

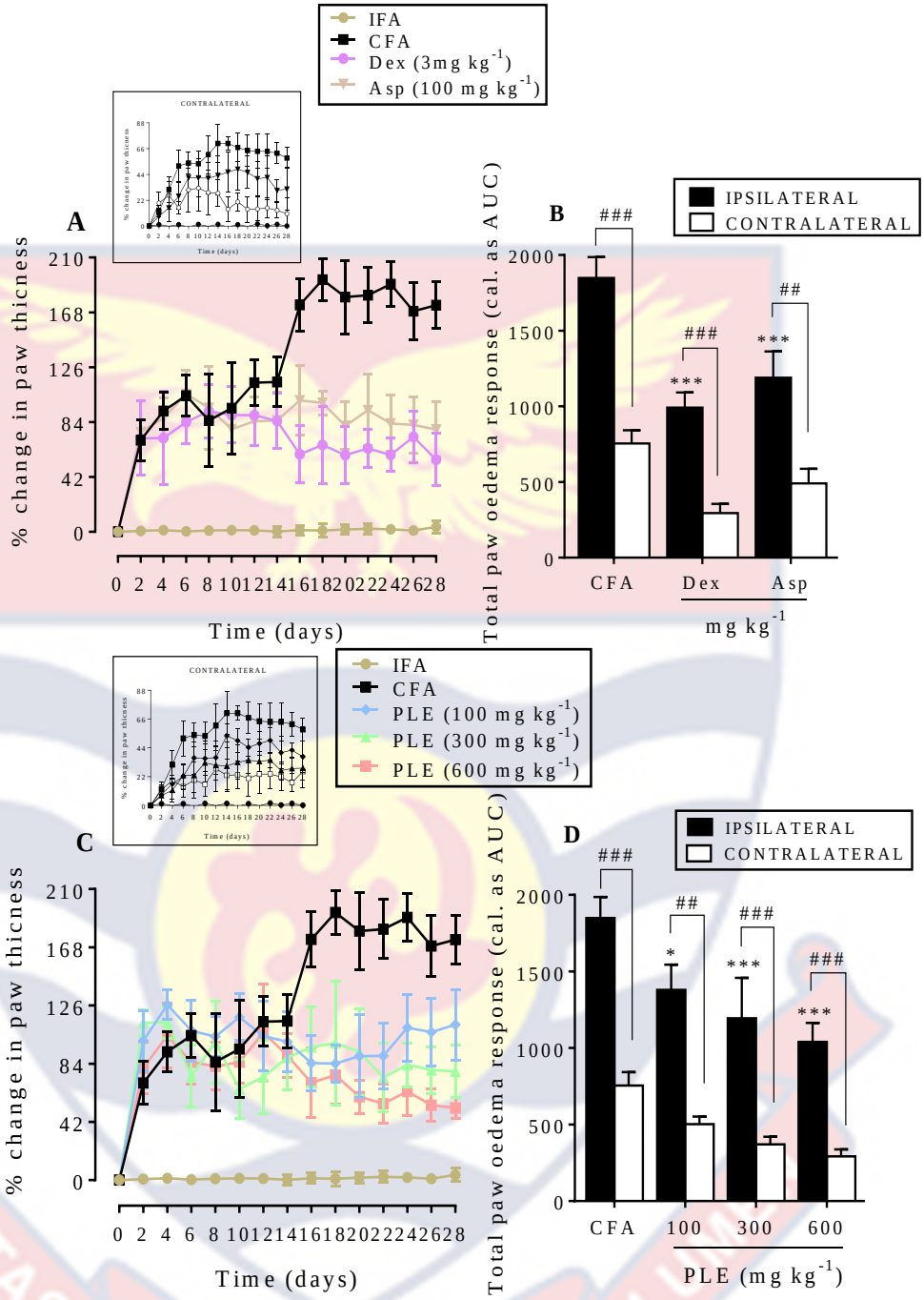


Figure 37: Effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) on oedema formation in prophylactic study of CFA-induced arthritis in rats. Oedema was expressed as the percentage change in paw thickness. Total oedema was calculated as AUC. Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to arthritic control group (Two-way ANOVA followed by Dunnet's *post hoc* test). Dex and Asp indicate Dexamethasone and Aspirin respectively.

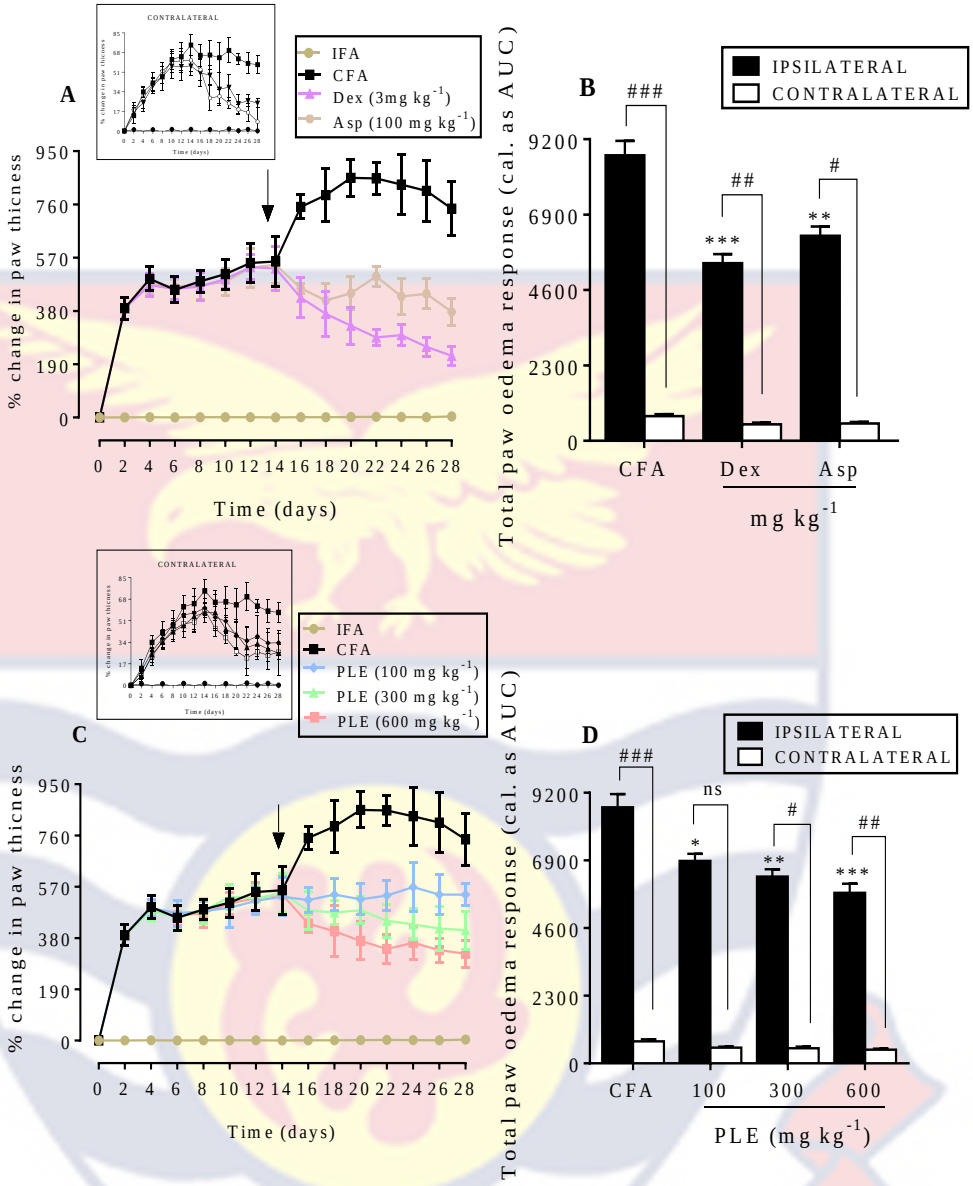


Figure 38: Effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) on oedema formation in therapeutic study of CFA-induced arthritis in rats. Oedema was expressed as the percentage change in paw thickness. Total oedema was calculated as AUC. All data were presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to arthritic control group (Two-way ANOVA followed by Dunnet's *post hoc* test). Dex and Asp indicate Dexamethasone and Aspirin respectively.

Arthritic score

Photography

From the study, photographic evaluation revealed no signs of oedema and erythema in non-IFA-treated group (refer to appendice B[a] and C[a]). CFA-induced control (arthritic control) group exhibited severe erythema and oedema in both ipsilateral and contralateral limbs (refer to appendice B[b] and C[b]). In the prophylactic study, dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups decreased oedema and erythema in both ipsilateral and contralateral paws (refer to appendix B[c, d]) when compared to the arthritis control group respectively. Similarly, PLE (100-600 mg kg^{-1})-treated groups showed a significant decrease of erythema and oedema (refer to appendix B[e, f, g]) at 100, 300 and 600 mg kg^{-1} when compared to the arthritis control group respectively.

In the curative study, dexamethasone- (3 mg kg^{-1}) and aspirin- (100 mg kg^{-1}) treated groups exhibited reduced oedema and swelling in both contralateral and ipsilateral limbs relative to the arthritis control rats respectively (refer to appendix C[c, d]). PLE (100-600 mg kg^{-1}, *p.o*)-treated groups comparably decreased the oedema and erythema in both ipsilateral and contralateral limbs at 100, 300 and 600 mg kg^{-1} relative to the arthritis control group respectively (refer to appendix C[e, f, g]).

Severity of arthritis was evaluated blindly and scored from the physical observation of the photographs. From the study, there was lower score (0.20±0.20) for non-arthritic group and no oedema or erythema observed in both ipsilateral and contralateral limbs (Fig. 39 and 40) when compared to the arthritic-induced control group (Fig. 39 and 40). Arthritic (CFA-treated) control

group recorded the highest arthritic scores (3.60±0.25 and 3.40±0.24) after physical observation in both ipsilateral and contralateral paws with severe swellings and eryhtema respectively (Fig. 39 and 40). In the prophylactic treatment protocol, lower scores (1.80±0.37, 1.40±0.24 and 2.00±0.32, 1.60±0.24) were recorded for both dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups with significant decrease in paw swellings and erythema in both ipsilateral and contralateral paws when compared to the arthritis control group respectively (Fig. 39[A, B]). Similarly, PLE (100-600 mg kg^{-1}) administered prophylactically suppressed the paw oedema and erythema and thus, reduced the arthritic scores (2.40±0.24, 2.20±0.37, 1.80±.20 and 2.20±0.37, 2.00±0.45, 1.60±0.40) significantly in the ipsilateral limb dose-dependently at 100, 300 and 600 mg kg^{-1} as well as the contralateral limb at the same doses when compared with arthritis control group respectively (Fig. 39[C, D]).

In the therapeutic treatment protocol, dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups suppressed paw swelling and erythema and thus, recorded significant reduction of arthritic scores (1.60±0.25, 1.40±0.24 and 2.00±0.32, 1.60±0.24) in both ipsilateral and contralateral limbs when compared to the CFA-treated (arthritic) control group respectively (Fig. 40[A, B]). PLE (100-600 mg kg^{-1}, *p.o*)-treated groups similarly showed a reduced paw oedema and erythema in both ipsilateral and contralateral limbs and thus, recorded significant reduction of arthritic scores (2.00±0.31, 2.20±0.37, 1.80±0.37 and 1.80±0.37, 1.60±0.40) in the ipsilateral limb at all doses, however, at 300 and 600 mg kg^{-1} in the contralateral paw when compared to the CFA-treated (arthritic control) group respectively (Fig. 40[C, D]).

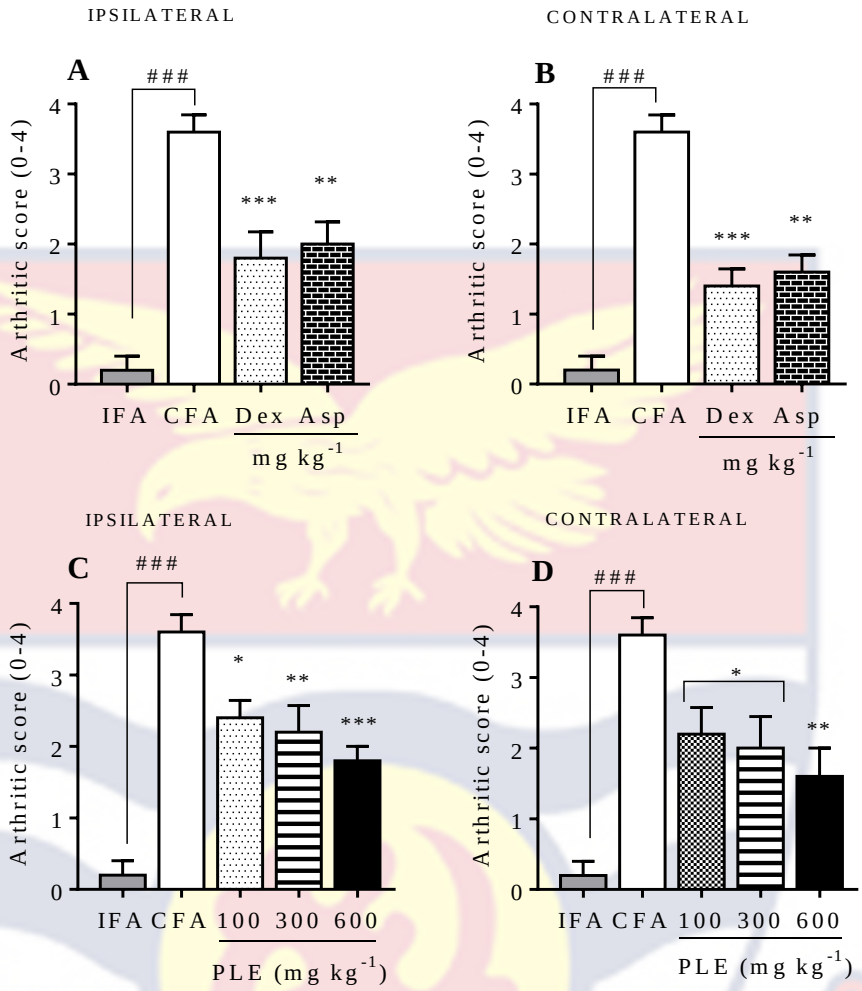


Figure 39: Arthritic scoring of photographs of prophylactic administration of PLE in CFA-induced arthritis in rats. Rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Either saline (1 ml, *p.o.*), dexamethasone (3 mg kg^{-1}, i.p.), aspirin (100 mg kg^{-1}, *p.o.*), or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered prior to arthritis induction until 28th day and the severity of arthritis was blindly measured on a scale of 0-4. Arthritic score was estimated based on the degree of erythema and paw swelling. Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to arthritic control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). Dex and Asp indicate Dexamethasone and Aspirin respectively.

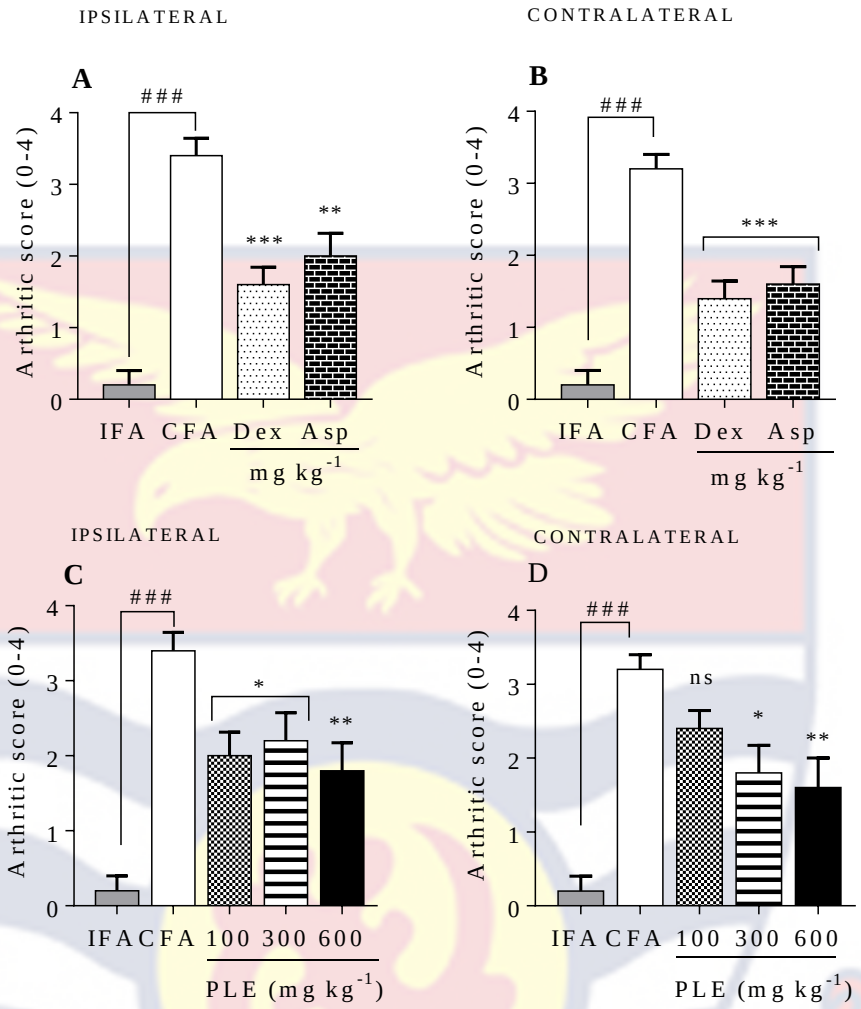


Figure 40: Arthritic scoring of photographs of therapeutic administration of PLE in CFA-induced arthritis in rats. Rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Either saline (1 ml, *p.o.*), dexamethasone (3 mg kg^{-1}, i.p.), aspirin (100 mg kg^{-1}, *p.o.*), or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered post arthritis induction from day 14 till the 28th day and the severity of arthritis was blindly measured on a scale of 0-4. Arthritic score was estimated based on the degree of erythema and paw swelling. Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared to arthritic control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Dex and Asp indicate Dexamethasone and Aspirin respectively.

Radiology

Radiography gives the true picture of disease remission and precise assessment of the disease condition and is primarily the initial imaging study in evaluation of arthritis (Jacobson, Girish, Jiang, & Resnick, 2008). Radiological evaluation of the rat hind paws after PLE treatment will further give concise and better assessment of the arthritis profile.

From the radiographs, there were no joint destruction, synovial articulation or cartilage damage observed in the non-arthritic (IFA) control group (Fig. 41[a] and 42[a]). The arthritic control (CFA-treated) group portrayed remarkable signs of bone erosion with severe bone/ joint space, cartilage and synovial destruction in both ipsilateral and contralateral paws that resulted in joint inflammation when compared to the non-arthritic (IFA) group (Fig. 41[b] and 42[b]). The rat right limb showed periarticular osteopenia, narrow joint space loss, bone erosion, soft-tissue swelling and increased bone resorption. Inflammation at the metatarsal-phalangeal joints was observed which led to bone proliferation. Dexamethasone (3 mg kg^{-1}) and aspirin (100 mg kg^{-1}) administered prophylactically reduced the joint inflammation and arthritic formation in the rats when compared to the arthritic (CFA-treated) control group respectively (Fig. 41[c, d]). PLE (100-600 mg kg^{-1})-treated groups similarly reduced joint inflammation, bone resorption and arthritic formation in the ipsilateral limb significantly at 100, 300 and 600 mg kg^{-1} dose levels, however, at doses of 300 and 600 mg kg^{-1} in the contralateral limb when compared to the arthritic (CFA-treated) control group respectively (Fig. 41[e, f, g]). In addition, PLE (100-600 mg kg^{-1})-treated rats exhibited improved bone remodeling which resulted in renewal of bone tissues.

In the therapeutic study, dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups exhibited significant suppression of joint inflammation, bone erosion, bone loss and arthritic formation in both ipsilateral and contralateral paws of rats when compared to the arthritic control group respectively (Fig. 42[c, d]). Comparably, PLE (100-600 mg kg^{-1})-treated group similarly showed a significant reduction of joint inflammation, bone erosion, arthritic formation and bone resorption which could result in bone loss in both ipsilateral and contralateral limbs of the rats when compared to the CFA-treated (arthritic) control group respectively (Fig. 42[e, f, g]). There was also enhanced bone remodeling observed in rats treated with PLE (100-600 mg kg^{-1}).

In terms of arthritic scoring from the radiographs, the IFA-treated group recorded the least score (0.20±0.20) and showed no bone erosion nor bone loss when compared to the arthritic control group (Fig. 43 and 44). The arthritic (CFA-induced) control group presented severe bone erosion in both ipsilateral and contralateral limbs and hence, recorded higher arthritic scores [3.20±0.20 and 3.40±0.25] (Fig. 43 and 44). In the prophylactic protocol, dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups reduced bone deformation and bone loss significantly in both the ipsilateral and contralateral paws and hence, recorded lower arthritic scores (1.40±0.25, 1.60±0.24 and 2.00±0.31, 2.00±0.32) when compared to the control group respectively (Fig. 43[A, B]). Similarly, PLE (100-600 mg kg^{-1}) showed a significant decrease of bone erosion as well as bone loss and hence, scored lower arthritic values (2.20±0.37, 1.60±0.24, 1.40±0.25) in the ipsilateral limb in a dose-dependent manner at 100, 300 and 600 mg kg^{-1}dose levels, however, low arthritic scores (2.00±0.44, 1.60±0.25) were recorded at 300 and 600 mg kg^{-1} in the contralateral limb when

compared to the CFA-induced (arthritic) control group respectively (Fig. 43[C, D]).

In the therapeutic treatment study, dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups showed a significant reduction of bone erosion as well as bone loss and hence, recorded lower arthritic scores (1.20±0.20, 1.60±0.24 and 1.80±0.37, 2.00±0.32) in both the ipsilateral and contralateral paws relative to the CFA-induced control group respectively (Fig. 44[A, B]). Comparably, PLE (100-600 mg kg^{-1})-treated exhibited a significant suppression of bone erosion as well as bone deformation and thus, recorded lower arthritic scores (2.20±0.37, 2.00±0.31, 1.80±0.20) in the ipsilateral paw at 100, 300 and 600 mg kg^{-1} doses, however, lower arthritic scores (2.00±0.32, 1.60±0.25) were recorded at 300 and 600 mg kg^{-1} in the contralateral limb relative to the arthritic control group respectively (Fig. 44[C, D]).

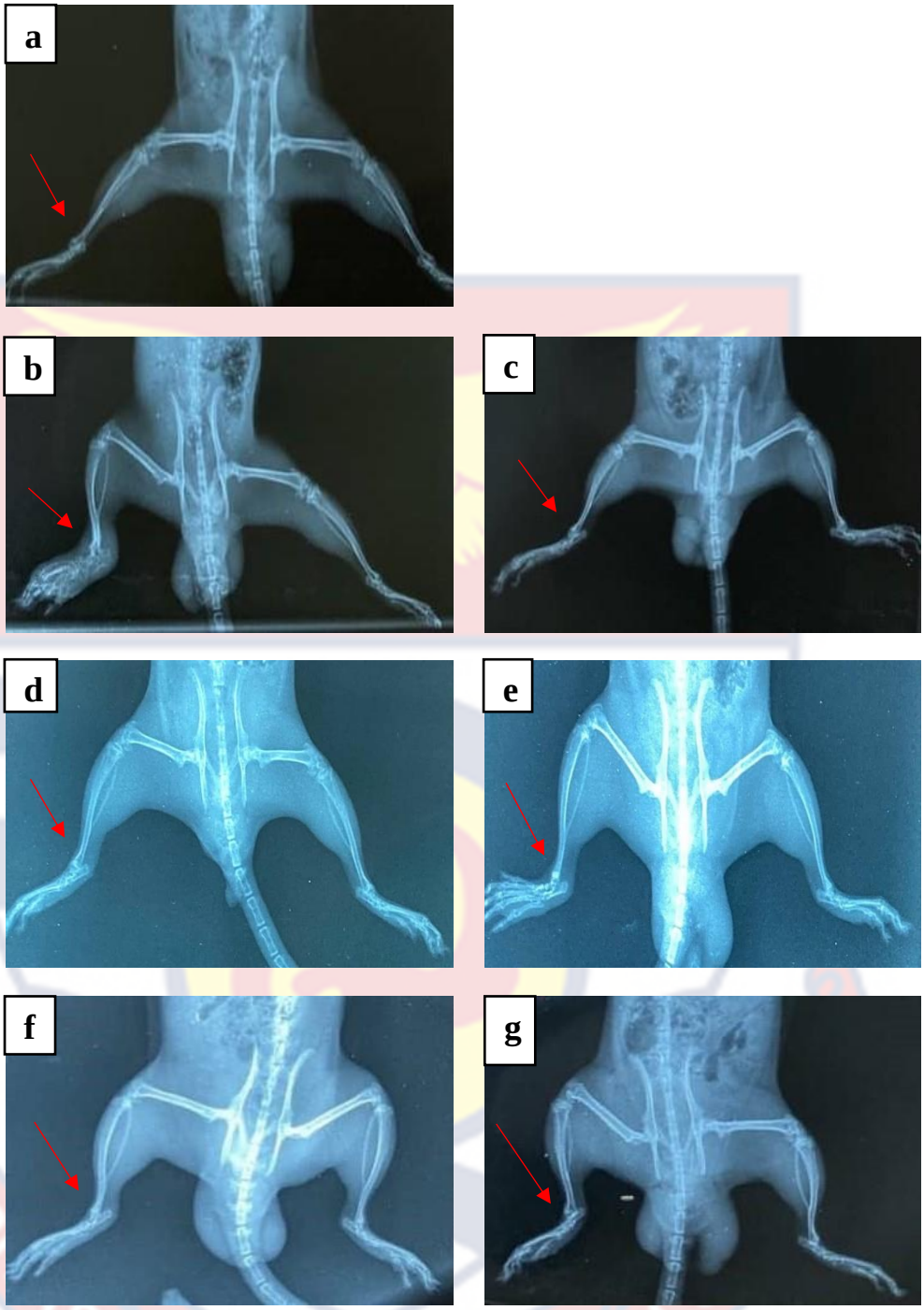


Figure 41: Radiography showing the prophylactic effects of PLE on CFA-induced arthritis in rats. Rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Photographs were taken using digital camera. The intensity of inflammation was scored on the scale of 0-4. **a**= IFA-treated, **b**= CFA-treated, **c**= dexamethasone (3 mg kg^{-1}), **d**= aspirin (100 mg kg-^{1}), **e-f**= PLE (100-600 mg kg^{-1}) respectively. Arrow indicates extent of joint erosion.

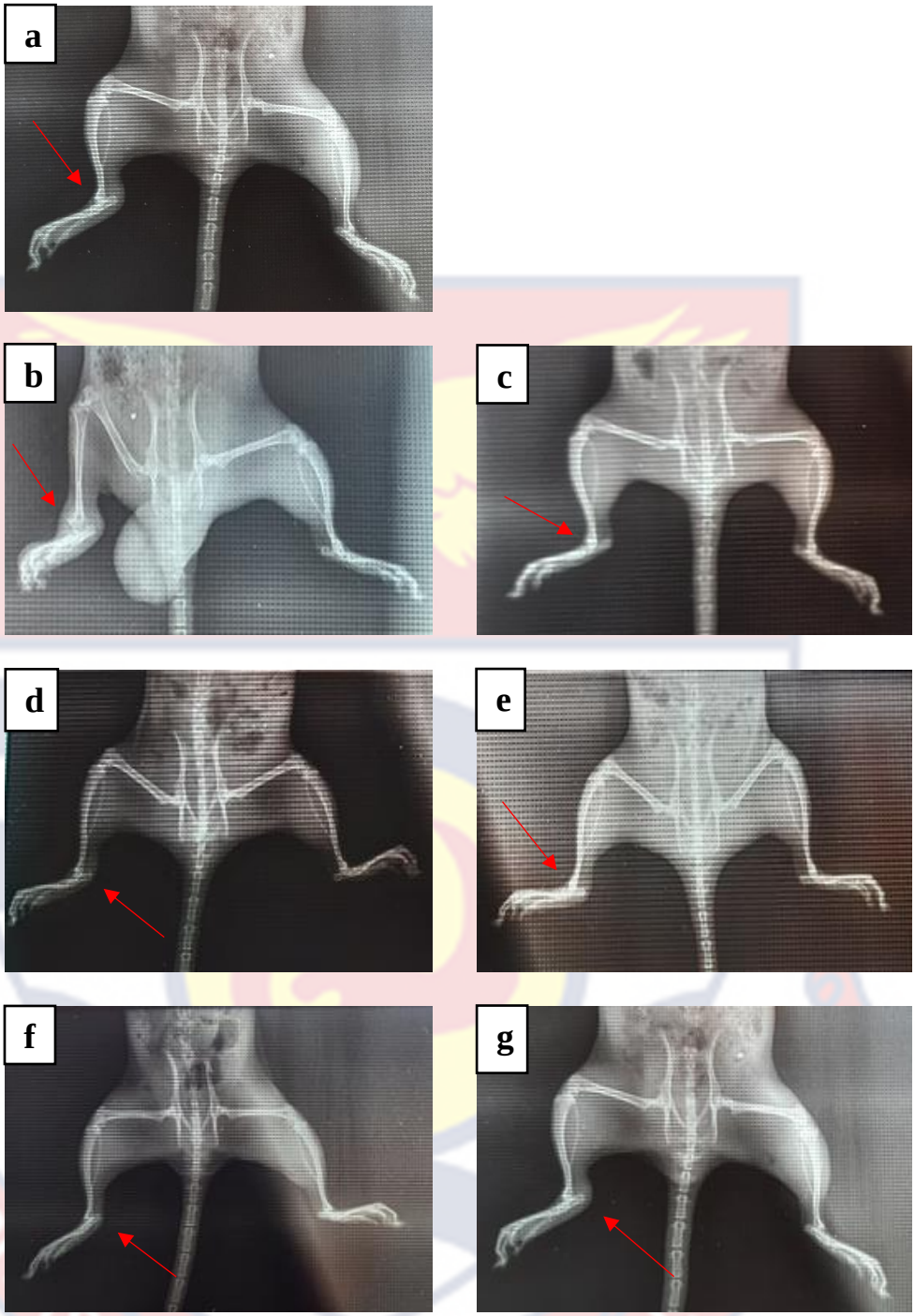


Figure 42: Radiography showing the therapeutic effects of PLE on CFA-induced arthritis in rats. Rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Photographs were taken using digital camera. The intensity of inflammation was scored on the scale of 0-4. **a**= IFA-treated, **b**= CFA-treated, **c**= dexamethasone (3 mg kg^{-1}), **d**= aspirin (100 mg kg^{-1}), **e-f**= PLE (100-600 mg kg^{-1}) respectively. Arrow indicates extent of joint erosion.

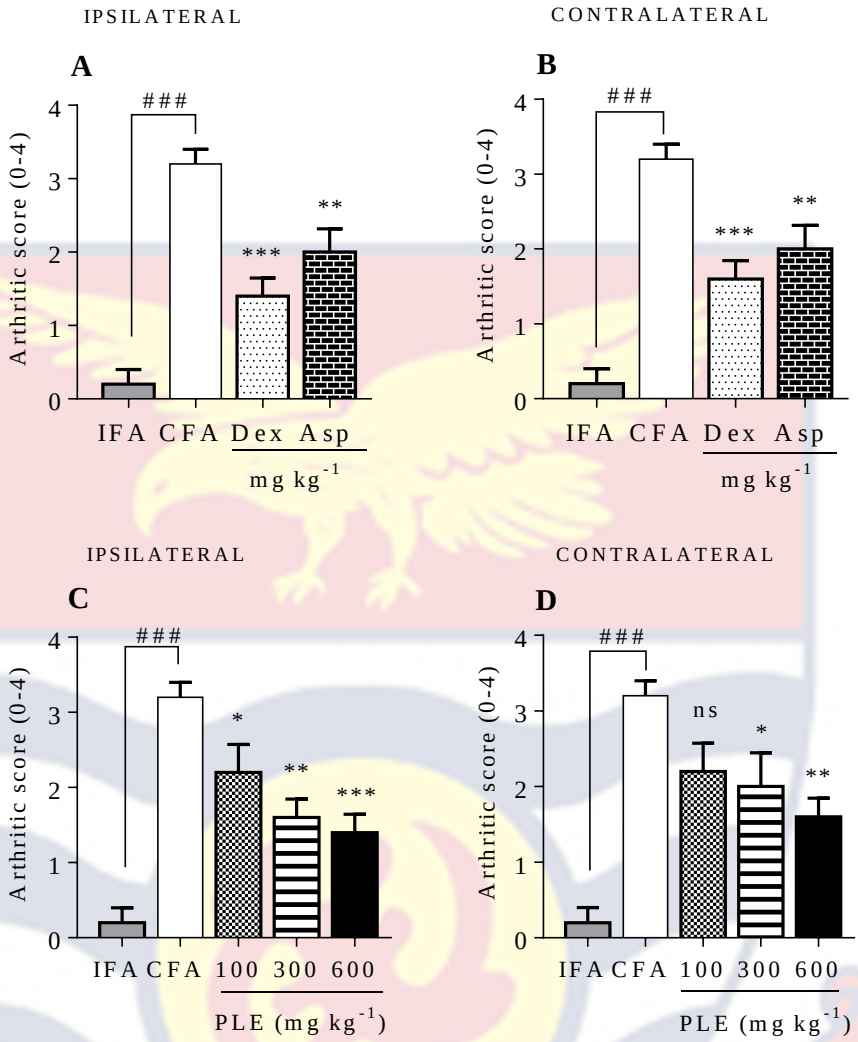


Figure 43: Arthritic scoring of radiographs of prophylactic administration of PLE in CFA-induced arthritis in rats. Rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Either saline (1 ml, *p.o.*), dexamethasone (3 mg kg^{-1}, i.p.), aspirin (100 mg kg^{-1}, *p.o.*), or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered prior to arthritis induction until 28th day and the severity of arthritis was blindly measured on a scale of 0-4. Arthritic score was estimated based on the degree of erythema and paw swelling. Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared with arthritic control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Dex and Asp denote Dexamethasone and Aspirin respectively.

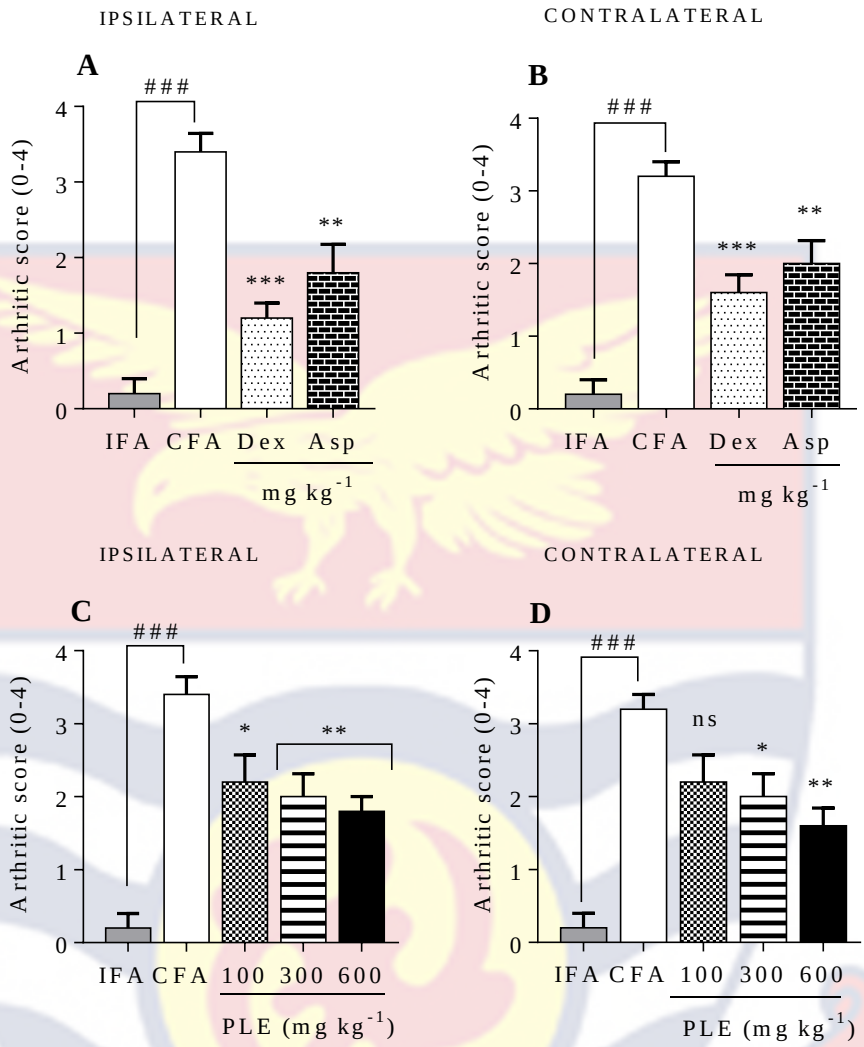


Figure 44: Arthritic scoring of radiographs of therapeutic administration of PLE in CFA-induced arthritis in rats. Rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Either saline (1 ml, *p.o.*), dexamethasone (3 mg kg^{-1}, i.p.), aspirin (100 mg kg^{-1}, *p.o.*), or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered post arthritis induction from day 14 till the 28th day and the severity of arthritis was blindly measured on a scale of 0-4. Arthritic score was estimated based on the degree of erythema and paw swelling. Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared with arthritic control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Dex and Asp denote Dexamethasone and Asp respectively.

Histopathological analysis

Accurate histopathological assessment is critical in evaluating the potency of agents used for reducing the severity of rheumatoid arthritis in animal models (McNulty *et al*., 2012). Histological studies of PLE will therefore serve as a vital assessment of the disease severity. This was done after inducing arthritis with 100 µl of CFA in the right hind limbs of rats intraplantarly as earlier described (in CFA-induced arthritis). Bones of the right hind limbs were excised after animals were euthanised and histological evaluation made. Non-arthritic (IFA-treated) group no visible synovial tissue hyperplasia nor cartilage erosion and no infiltration of mononuclear cells of the joint. Hence, bone structure was intact when compared to the arthritic (CFA-treated) control group (Fig. [45A and 46A]). Arthritic (CFA-treated) control group showed severe mononuclear cell infiltration, cartilage erosion of the joint, synovial hyperplasia, bone erosion and high joint space with pannus formation (Fig. [45B and 46B]). Dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups reduced severity of bone and joint inflammation through the inhibition of synovial hyperplasia, cartilage and bone erosion, vascular pan formation and reduced joint narrow space when compared to the CFA-treated arthritis group (Fig. 45 and 46[C, D]). PLE (100-600 mg kg^{-1}) similarly decreased joint narrow space of the ankle, cartilage and bone erosion. The presence of vascular pan formation and hyperplasia of synoviocytes were remarkably inhibited. In addition, regeneration of the damaged stratum and stratum granulosum was observed, and bone resorption was reduced which showed evidence of bone remodeling at 300 and 600 mg kg^{-1} doses when

compared to the arthritic (CFA-treated) control group respectively (Fig. 45 and 46[F, G]).

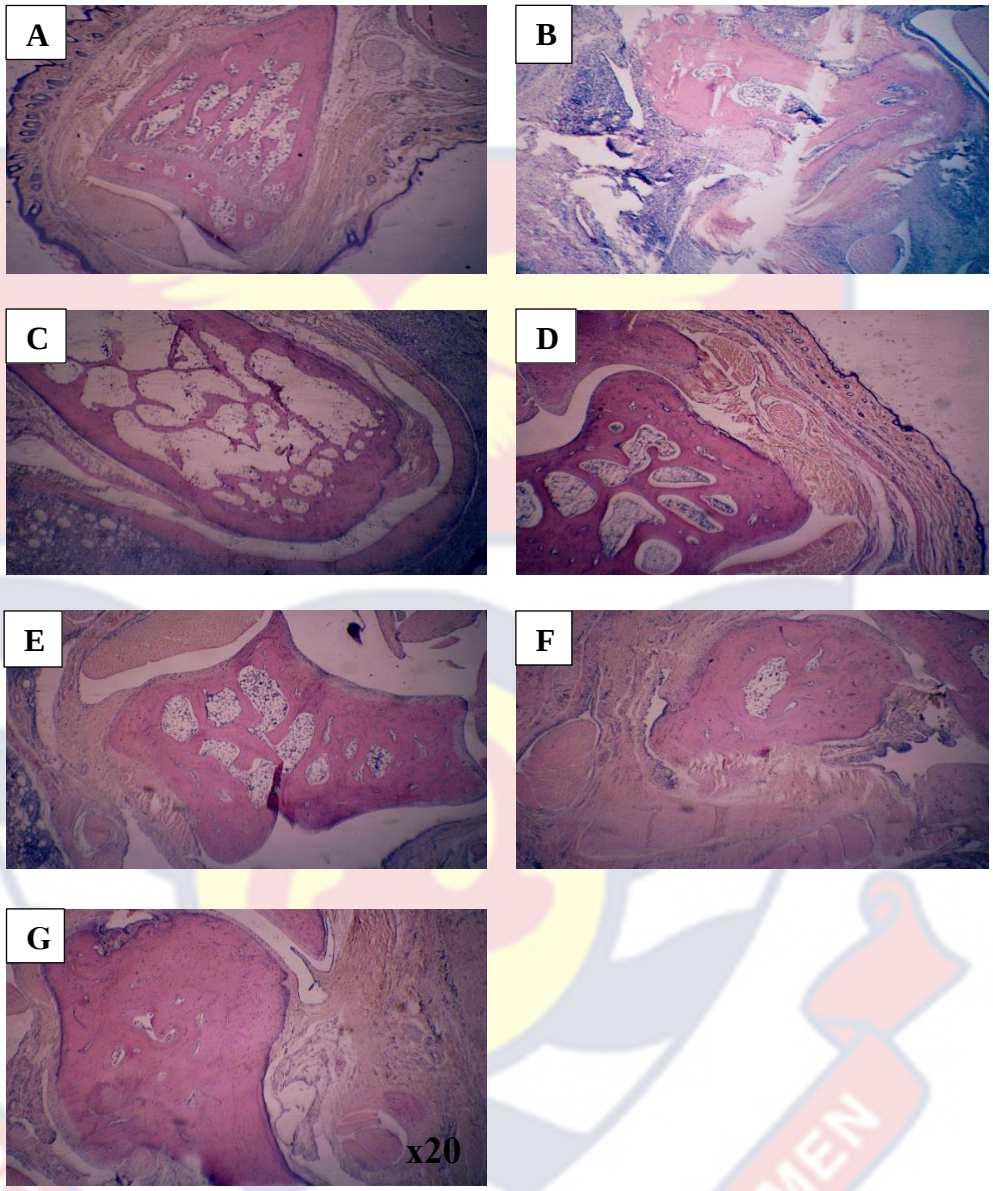


Figure 45: Histopathological assessment of PLE in prophylactic study of CFA-induced arthritis in rats. Arthritis was induced by intraplantarly inoculating 100 µl of CFA or IFA into the right hind limbs of rats. Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}) was administered from day 0 prior to CFA induction until the 28th day. Using pentobarbitone (20 mg kg^{-1}), rats were anaesthetised, sections made from bone tissue of the right hind paw, and stained with H & E stain.

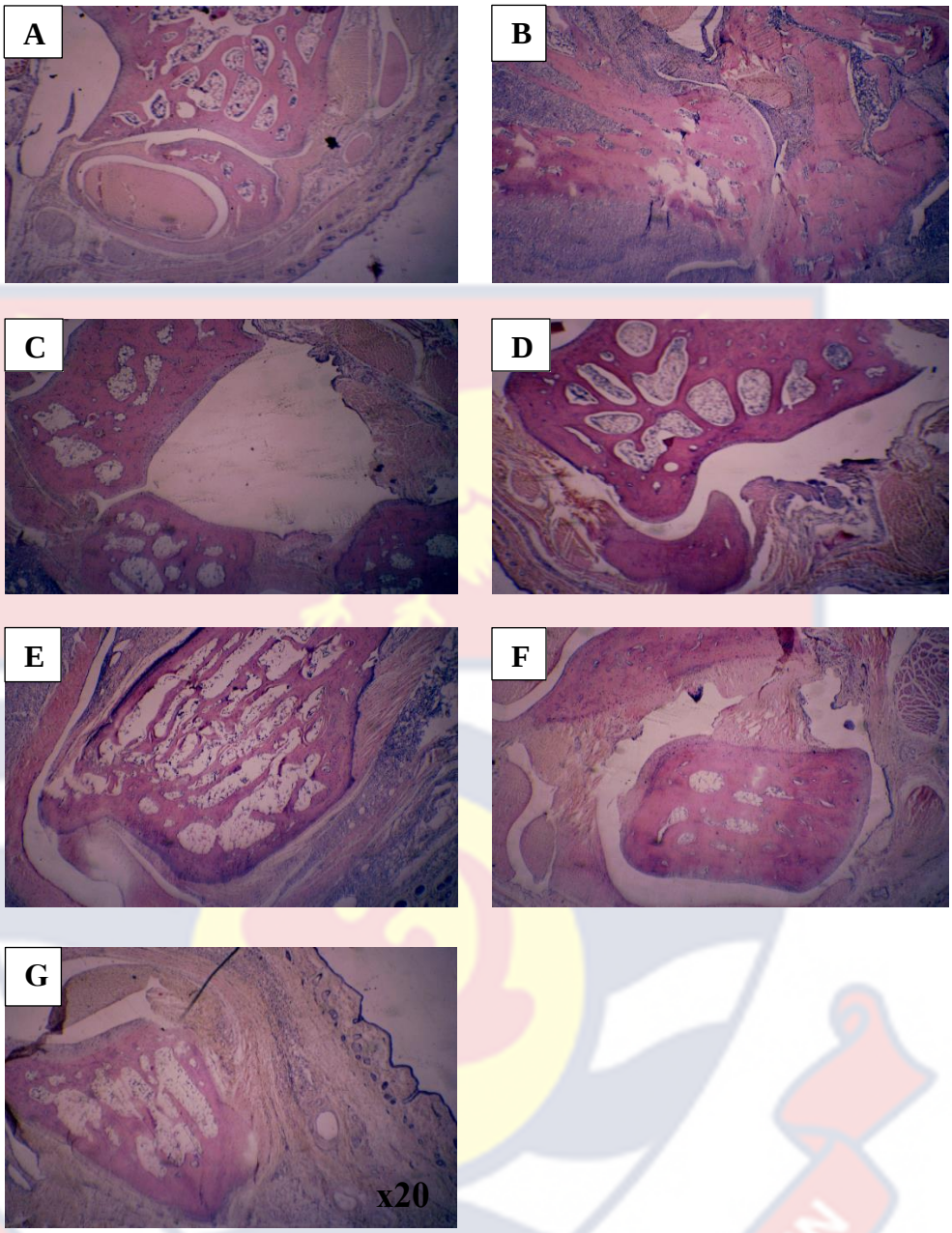


Figure 46: Histopathological assessment of PLE in therapeutic study of CFA-induced arthritis in rats. Arthritis was induced by intraplantarly inoculating 100 µl of CFA or IFA into the right hind limbs of rats. Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}) was administered from day 0 prior to CFA induction until the 28^{th} day. Using pentobarbitone (20 mg kg^{-1}), rats were anaesthetised, sections made from bone tissue of the right hind paw, and stained with H & E stain.

Haematology and biochemistry

Haematological assessment

Recently, haematological markers have been reported to be critical in evaluating treatment response of pharmacological agents (Javad *et al*., 2018). Thus, blood test has proven to be essential in the primary assessment of rheumatoid arthritis. From the study, the arthritic (CFA-treated) control group showed increased levels of white blood cells (WBC), platelets (PLT), lymphocytes (LYM) and neutrophils (NEU), and reduced concentrations of HGB and RBC when compared with non-arthritic (IFA-treated) group (Table 6 and 7) respectively. In the prophylactic study, treatment with dexamethasone (3 mg kg^{-1}) resulted in a significant reduction of WBC, PLT and NEU levels, and treatment with aspirin (100 mg kg^{-1}) equally showed significant reduction of WBC and NUE when compared to the arthritic control rats respectively (Table 6). Similarly, PLE (100-600 mg kg^{-1}) significantly decreased the concentrations of WBC and NEU at 300 and 600 mg kg^{-1} dose levels when compared to the control group respectively (Table 6).

In the therapeutic protocol, dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups showed a significant decrease of WBC, NEU levels and increased the concentrations of RBC and HGB (Table 7) respectively. Comparably, PLE (100-600 mg kg^{-1})-treated groups reduced the levels of WBC and NEU significantly and increased the levels of HGB and RBC at 300 and 600 mg kg^{-1} when compared to the CFA-induced (arthritic) control group respectively (Table 7).

Table 6. Haematological analysis of blood samples taken from CFA-induced arthritis in Sprague-Dawley rats in prophylactic study.

Treatment	WBC (10^3/ µL)	HGB (g/ dL)	RBC (10^3/ µL)	PLT (10^3/ µL)	LYM (10^3/ µL)	HCT (%)	NEU (10^3/ µL)
IFA	5.12±0.74*	13.22±0.54	7.58±0.29	716.6±50.75	4.39±0.81	38.91±1.61	0.66±0.15***
CFA	12.58±1.87	11.58±0.41	7.10±0.47	965.6±50.81	6.08±0.52	42.04±1.51	3.51±0.35
Dex (3 mg kg^{-1})	4.84±1.75**	12.41±0.56	6.24±0.39	708.8±55.54*	3.71±0.77	40.29±2.08	1.50±0.19**
Asp (100 mg kg^{-1})	6.43±1.23*	12.09±0.53	6.92±0.28	786.4±52.79	3.81±0.40	42.59±1.44	1.58±0.30**
PLE (100 mg kg^{-1})	7.59±1.76	12.20±0.48	6.75±0.64	897.8±59.54	5.36±0.60	40.44±1.28	2.28±0.55
(300 mg kg^{-1})	5.74±1.20*	11.95±0.36	7.48±0.28	810.0±59.75	3.79±0.61	42.00±1.18	2.18±0.35*
(600 mg kg^{-1})	5.64±1.71*	12.18±0.45	6.54±0.39	799.8±51.60	3.92±0.53	41.36±1.99	1.18±0.33**

Sprague-Dawley rats (150-200 g, n=5) were inoculated with 100 µl of IFA or CFA intraplantarly into the right hind limb. Animals received either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}), or PLE (100-600 mg kg^{-1}) prior to CFA induction and continued till day 28. Rats were euthanised and blood samples collected via cardiac puncture on day 29. A full blood count was performed using blood analyser. *P<0.05; **P<0.01; ***P<0.001 when compared with CFA-induced control group. Dex and Asp indicate Dexamethasone and Aspirin respectively.

Table 7. Haematological analysis of blood samples taken from CFA-induced arthritis in Sprague-Dawley rats in therapeutic study.

Treatment	WBC (10^3/ µL)	HGB (g/ dL)	RBC (10^3/ µL)	PLT (10^3/ µL)	LYM (10^3/ µL)	HCT (%)	NEU (10^3/ µL)
IFA	6.09±0.95**	12.98±0.32	7.01±0.264	680.8±53.28	4.76±0.74	42.18±1.55	0.64±0.21**
CFA	13.62±2.44	10.48±0.87	5.01±0.40	884.8±79.36	5.66±0.96	41.96±1.94	3.58±0.89
Dex (3 mg kg^{-1})	5.53±1.44***	13.48±0.26**	7.09±0.28**	696.8±50.48	3.11±0.88	40.78±2.19	0.82±0.38**
Asp (100 mg kg^{-1})	6.98±1.34*	12.48±0.59*	6.71±0.50*	649.6±35.63	3.53±0.42	42.06±2.98	0.83±0.14**
PLE (100 mg kg^{-1})	8.04±2.38	12.01±0.17	5.78±0.48	926.4±92.99	5.55±1.68	40.50±2.02	1.82±0.61
(300 mg kg^{-1})	6.45±1.08*	13.34±0.34**	7.12±0.24**	711.8±91.19	2.72±0.66	40.84±2.87	1.42±0.65*
(600 mg kg^{-1})	5.96±1.39*	13.36±0.44**	7.13±0.20**	673.0±100.40	3.50±0.62	42.42±3.09	1.37±0.45*

Sprague-Dawley rats (150-200 g, n=5) were inoculated with 100 µl of IFA or CFA intraplantarly into the right hind limb. Animals received either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}), or PLE (100-600 mg kg^{-1}) prior to CFA induction and continued till day 28. Rats were euthanised and blood samples collected via cardiac puncture on day 29. A full blood count was performed using blood analyser. *P<0.05; **P<0.01; ***P<0.001 when compared with CFA-induced control group. Dex and Asp indicate Dexamethasone and Aspirin respectively.

Biochemical analysis

Biochemical analysis was conducted on sera taken from rats in all treatment groups on day 29. There were no significant changes statistically *(P<0.05)* between the drug-treatment groups (dexamethasone [3 mg kg^{-1}], aspirin [100 mg kg^{-1}], PLE [100-600 mg kg^{-1}]) for the biochemical parameters (albumin [A], total proteins [B] and globulin [C]) measured in both prophylactic and curative protocols when compared to the CFA-treated control group (Fig. 47 and 48[A-C]) respectively.

Ratio of albumin-to-globulin levels was determined for all treatment groups. No significant *(P<0.05)* change recorded statistically in all the drug-treatment groups when compared to the arthritic control group (Fig. 47D and 48D).

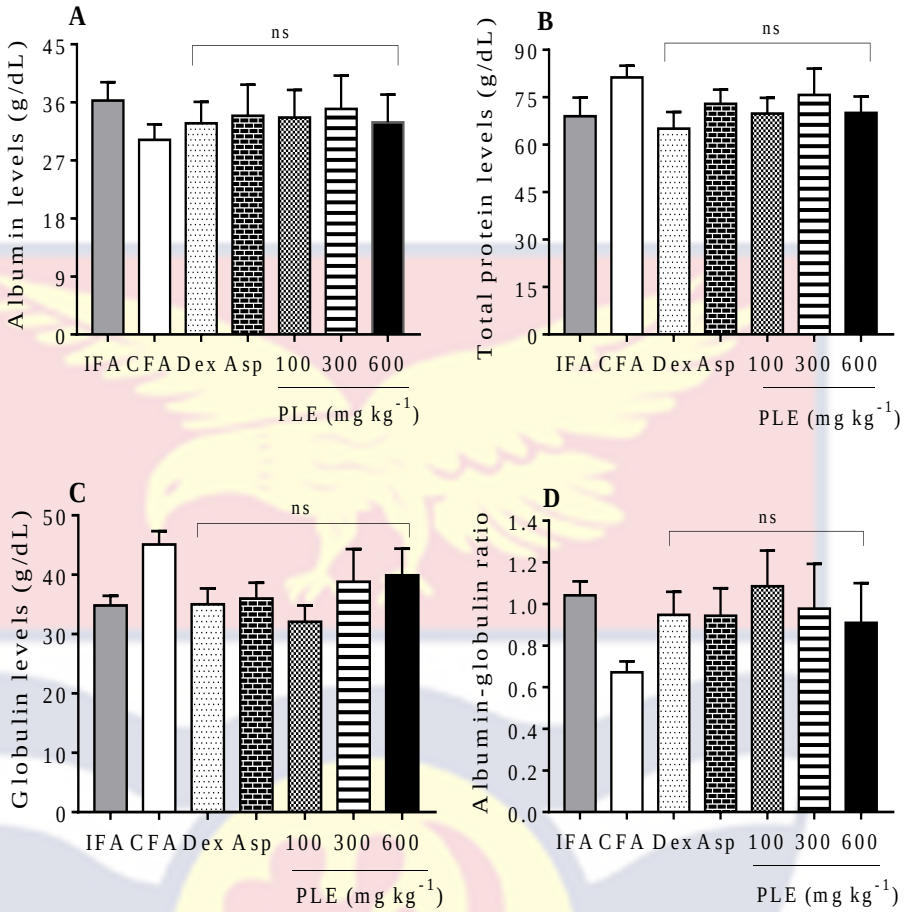


Figure 47: Effect of PLE on serum biochemical analysis of rats (n=5) in CFA-induced arthritis in a prophylactic study. 100 µl of CFA was injected into right hind paw to induce arthritis. Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}, *p.o.*, daily). Serum was collected for albumin (A), total protein (B), and globulin (C) analyses. Data was presented as mean ± S.E.M. Statistically at $P<0.05$ compared with arthritic control group (Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Dex and Asp denote Dexamethasone and Aspirin respectively.

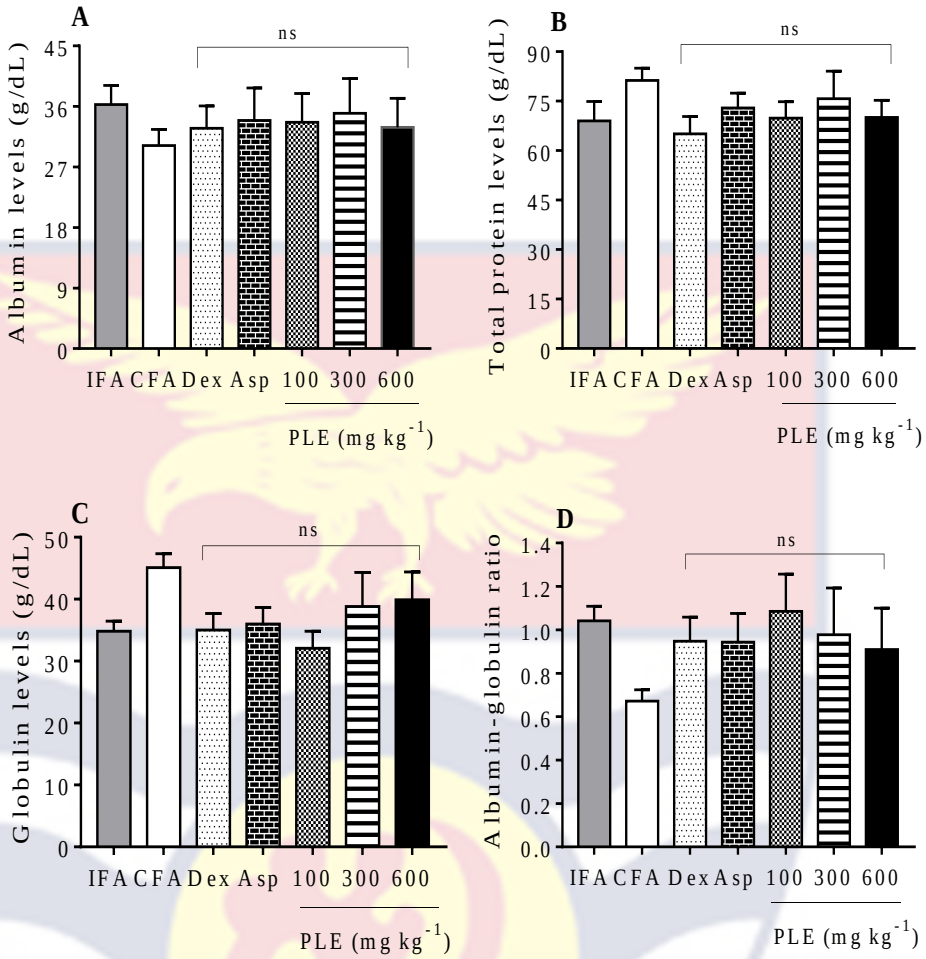


Figure 48: Effect of PLE on serum biochemical analysis of rats in CFA-induced arthritis in a therapeutic study. 100 µl of CFA was injected into right hind paw to induce arthritis (n=5). Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}, *p.o.*, daily). Serum was collected for albumin (A), total protein (B), and globulin (C) analyses. Data was presented as mean ± S.E.M. $P<0.05$ compared with arthritic control group (Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Dex and Asp denote Dexamethasone and Aspirin respectively.

Eye assessment in CFA-induced arthritis

In 2016, Murray & Ruaz reported that rheumatoid arthritis is associated with possible sight-threatening inflammatory eye disorder. This condition can cause dry eye, scleritis or uveitis leading to corneal damage and eventually affect vision when untreated. Arthritis was induced as described earlier. Drug administration commenced on day 14 till the 28th day. Uveitis of the eye of rats

in all the groups was examined based on hyperemia (redness), flare, cells in the anterior chamber (AC), hypopyon (pus in the anterior chamber) and miosis. Ophthalmic assessment was performed using slit lamp biomicroscope (My-V038cN Ophthalmic Slit Lamp, Guangzhou Maya Equipment Co. Ltd, Guangdong, China).

From the study, there were no uveitis observed in the eyes of rats in non-arthritic (IFA-treated) group when compared to the arthritic (CFA-treated) control group (Fig. 49). Arthritic (CFA-treated) control group showed severe inflammation in both eyes (especially, oculus sinister), the left eye than the right eye (oculus dextrus). There were massive hyperemia, increased pus cells and other cells in the anterior chamber, high miosis and flare which are characteritic signs of uveitis (Fig. 49[A-E]). Dexamethasone (3 mg kg^{-1}) significantly suppressed all signs of uveitis in the eyes of rats when compared to the arthritic control group (Fig. 49[A-E]). Aspirin (100 mg kg^{-1}) showed similar effect by decreasing the signs of uveitis significantly when compared to the arthritic control group (Fig. 49[B-E]). However, there was no significant difference in flare of the eye when compared to the arthritic control group (Fig. 49A). PLE (100-600 mg kg^{-1}) significantly suppressed the signs of uveitis in the eyes of rats when compared to the arthritic control group (Fig. 49[A-E]). Flare in the eye (3.6±0.25) was significantly decreased to 2.6±0.24 at 600 mg kg^{-1} (Fig. 45A). Cells in the anterior chamber of the eye (4.0±0.32) was reduced significantly to 2.4±0.51 and 2.2±0.58 at 300 and 600 mg kg^{-1} respectively (Fig. 49B). The level of hypopyon (4.2±0.37) was significantly decreased to 2.8±0.37 and 2.4±0.25 at 300 and 600 mg kg^{-1} respectively (Fig. 49C). Hyperemia (redness) level of the eye (3.8±0.20) was suppressed significantly to 2.4±0.40,

2.2±0.20 and 2.0±0.32 at 100, 300 and 600 mg kg^{-1} respectively (Fig. 49D). PLE at 600 mg kg^{-1} suppressed significantly level of miosis (3.4±0.25) to 2.2±0.37 when compared to the arthritic control group (Fig. 49E).

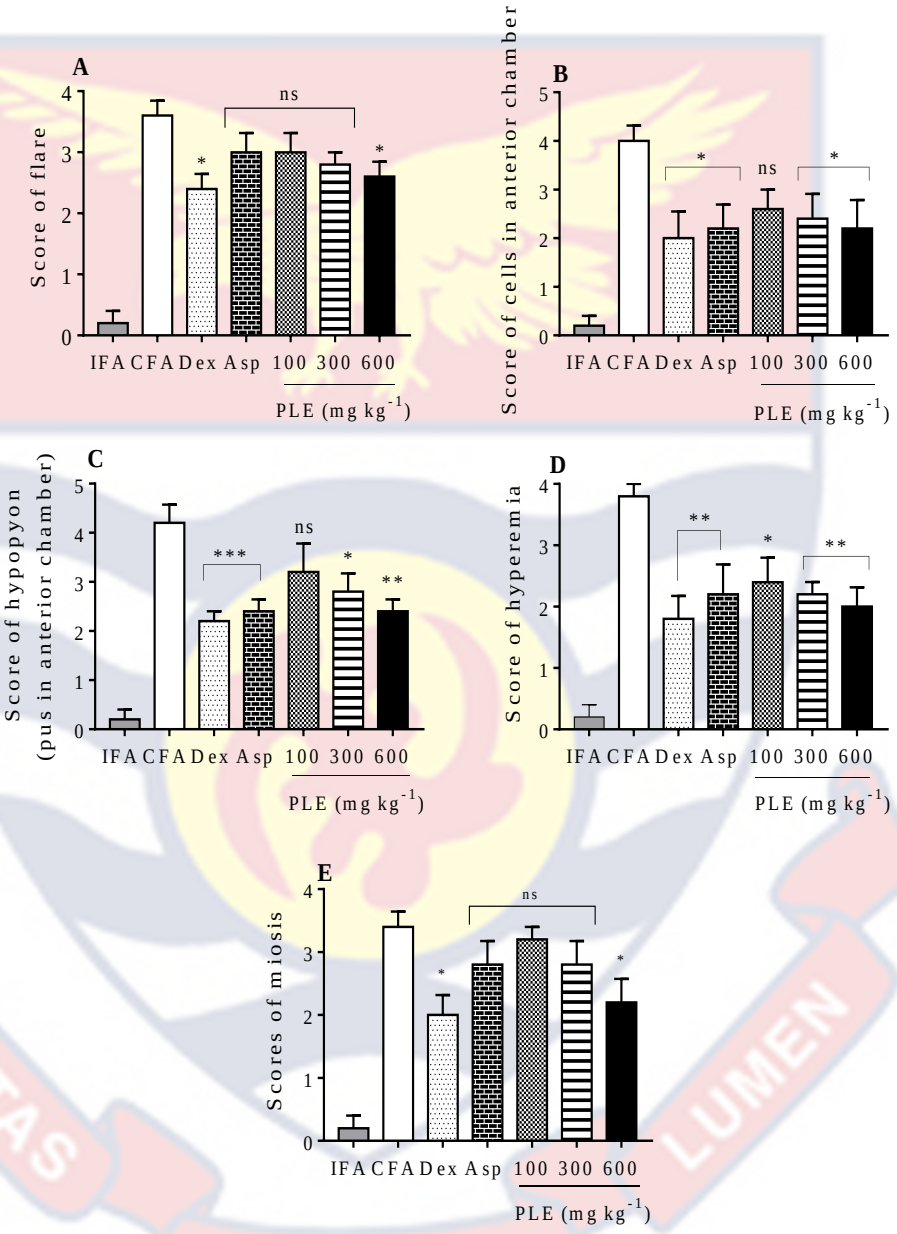


Figure 49: Effect of PLE on the eyes of rats in CFA-induced arthritis. Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered post arthritis induction. Ophthalmic evaluation was carried for Flare (A), Cells in AC (B), Hypopyon (C), Hyperemia (D), Miosis (E). Data was presented as mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared with arthritic control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Dex and Asp indicate Dexamethasone and Aspirin respectively.

Mast cell proliferation assessment

Usually, mast cells are implicated in inflammatory reactions and also, known to play major roles in chronic inflammation, (Theoharis *et al*., 2012). In this study, bone tissues were taken after study period. Bone sections were stained with 1 % toluidine blue dye and the proliferation of mast cells due to joint inflammation was assessed. From the study, the IFA-treated control showed no increased influx of mast cells to arthritic joint (Fig.50[A] and Fig. 51[A]), and significantly attained a mean total number of mast cell count of 7.20±1.72 when compared to the arthritic control group (Fig. 52). In the prophylactic study, CFA-treated (arthritic) control group showed elevated influx levels of mast cells to the arthritic joint (Fig. 50B) with higher mean total number of mast cells count of 38.80±5.16 (Fig. 52A). Dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups decreased mast cell proliferation at site of arthritic joint (Fig. 50[C and D]) with a significant reduction of the mean total number of mast cell count to 13.20±2.82 and 18.40±4.71 when compared to the CFA-induced control group respectively (Fig. 52A). Similarly, PLE (100-600 mg kg^{-1})-treated groups at all doses showed a decreased of mast cell influx to the arthritic joint (Fig. 50[E-G]) with a significant decrease in the mean total number of mast cell count to 19.60±3.27, 18.80±3.26 and 14.60±3.59 at 100, 300 and 600 mg kg^{-1} when compared to the CFA-treated (arthritic) control group respectively (Fig. 52A).

In the therapeutic study, CFA-treated (arthritic) control group showed higher influx of mast cell (Fig. 51B) and the mean total number of mast cells at arthritic joint was 40.00±4.60 (Fig. 52B). From the study, Dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups reduced the proliferation of

mast cells at inflamed joint (Fig. 51[C, D]) with a significant decrease in the mean total number of mast cell count to 11.60±3.12 and 17.80±3.43 when compared to the CFA-induced control group respectively (Fig. 52B). PLE (100-600 mg kg^{-1})-treated groups similarly showed a reduced influx of mast cells to the arthritic joint (Fig. 51[E-G]) with a significant decrease in the mean total number of mast cell count to 23.00±5.24, 19.00±5.43 and 14.20±4.62 at 100, 300 and 600 mg kg^{-1} when compared to the CFA-treated (arthritic) group in a dose-dependent manner respectively (Fig. 52B)

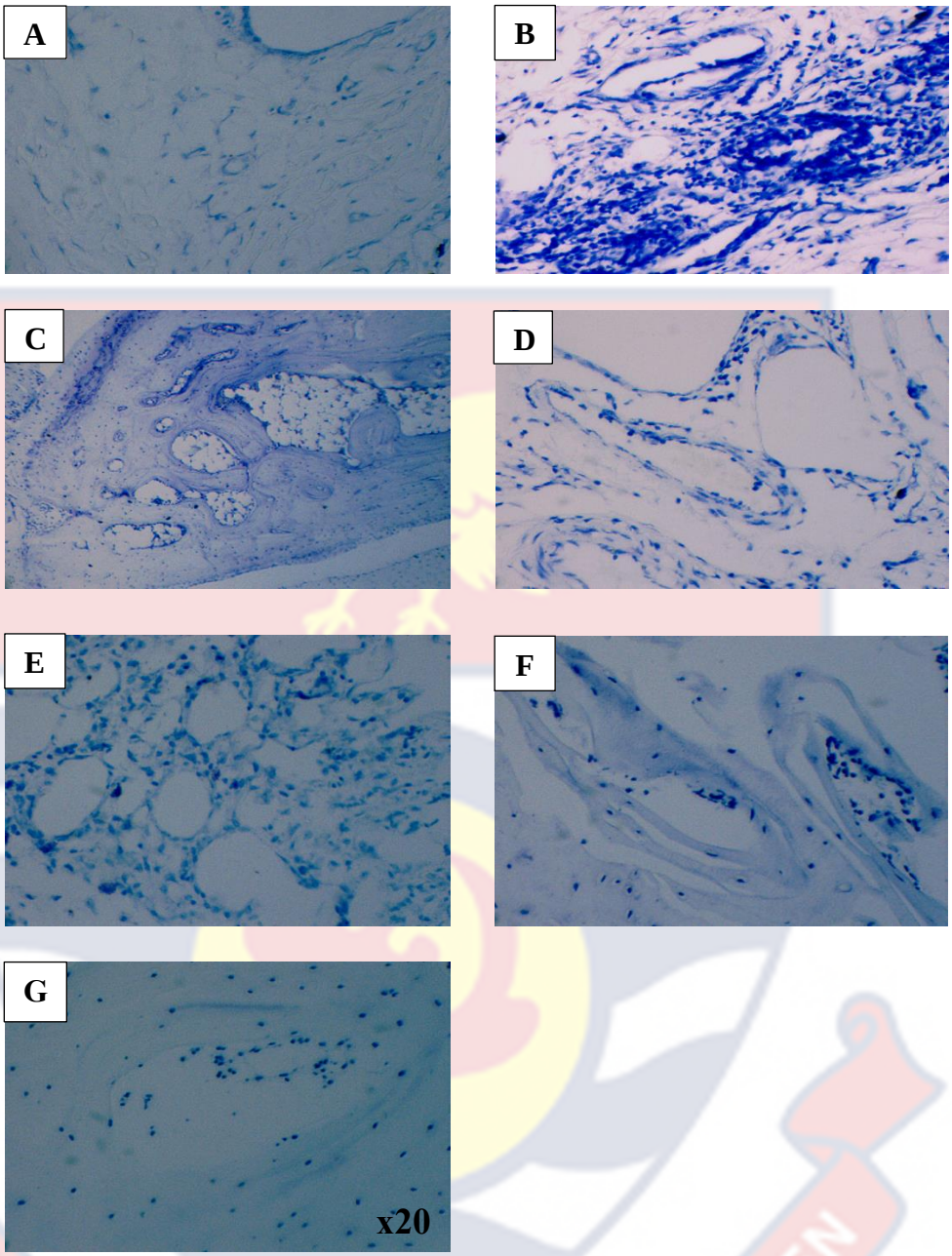


Figure 50: Mast cell proliferation in a prophylactic study of CFA-induced arthritis in rats. Arthritis was induced by intraplantarly inoculating 100 µl of IFA or CFA into the right hind limbs of rats (n=5). Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered from day 0 prior to CFA induction until the 28^{th} day. Using pentobarbitone 40 mg kg^{-1}, rats were anaesthetised and sections made from bone tissue of the right hind paw, fixed and stained with 1% toluidine blue. IFA (non-arthritic) group (A), CFA-treated (arthritic) group (B), Dexamethasone (3 mg kg^{-1}) (C), Aspirin (100 mg kg^{-1}) (D), PLE (100-600 mg kg^{-1}) [E-F] respectively.

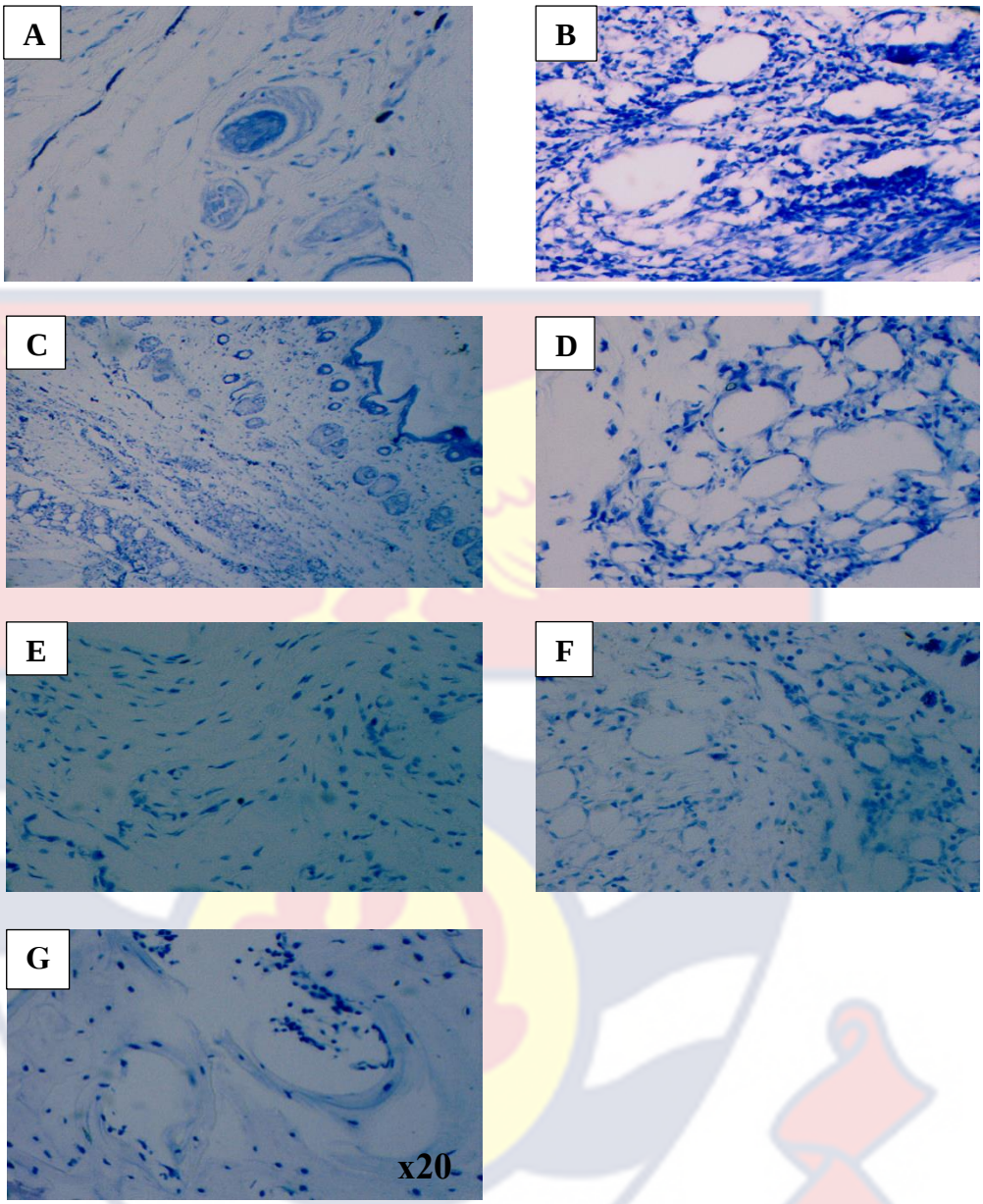


Figure 51: Mast cell proliferation in a therapeutic study of CFA-induced arthritis in rats. Arthritis was induced by intraplantarly inoculating 100 µl of IFA or CFA into the right hind limbs of rats (n=5). Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered from day 0 prior to CFA induction until the 28^{th} day. Using pentobarbitone 40 mg kg^{-1}, rats were anaesthetised and sections made from bone tissue of the right hind paw, fixed and stained with 1% toluidine blue. IFA (non-arthritic) group (A), CFA-treated (arthritic) group (B), Dexamethasone (3 mg kg^{-1}) (C), Aspirin (100 mg kg^{-1}) (D), PLE (100-600 mg kg^{-1}) [E-F] respectively.

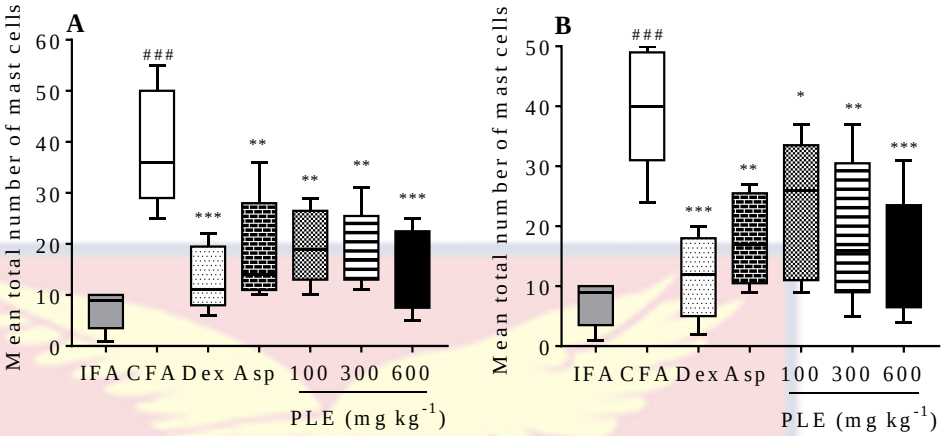


Figure 52: Effect of PLE on mast cell proliferation in prophylactic (A) and therapeutic (B) studies of CFA-induced adjuvant arthritis in rats. Arthritis was induced by intraplantarly inoculating 100 µl of IFA or CFA into the right hind limbs of rats (n=5). Either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) was administered from day 14 until the 28^{th} day. Using pentobarbitone 40 mg kg^{-1}, rats were anaesthetised and sections made from bone of the right hind paw, fixed and stained with 1 % toluidine blue dye. Mast cells were counted using electronic microscope. Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared with arthritic control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test. Dex and Asp denote Dexamethasone and Aspirin respectively.

In vitro anti-oxidant activity of PLE

Total anti-oxidant capacity

PLE (0.015625-0.250 µg ml^{-1}) exhibited an increase in total anti-oxidant activity in a concentrated-dependent manner when measured as ascorbic equivalents (Fig. 53). Thus, the total anti-oxidant capacity of PLE was estimated to be 39.13±6.80 µg ascorbic acid equivalent per g of PLE (Table 8).

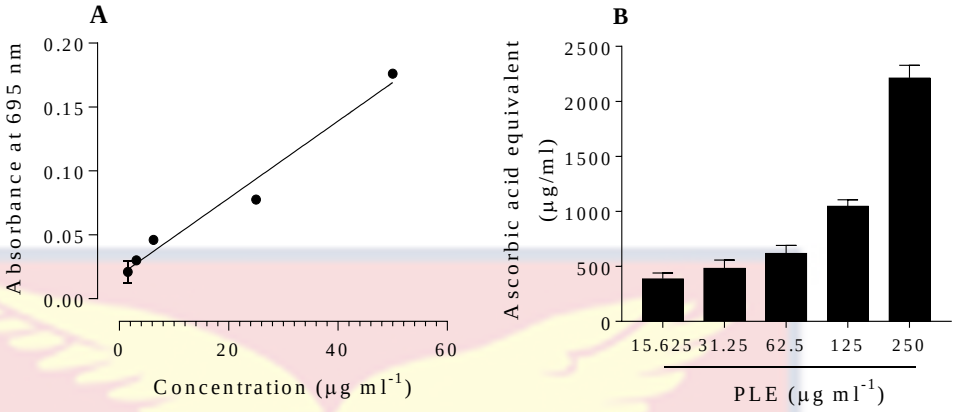


Figure 53: Absorbance of ascorbic acid (1.5625-50 µg ml^{-1}) [A] and PLE (15.625-250 µg ml^{-1}) [B] respectively, expressed as ascorbic acid equivalent [B] and total anti-oxidant capacity at different concentrations of PLE. Data presented as mean ± S.E.M (n=5).

Table 8: Total anti-oxidant capacity.

Measurement (µg ml^{-1})	Anti-oxidant capacity value
Total anti-oxidant capacity	39.13±6.80

Total anti-oxidant capacity was determined as µg ascorbic acid ml^{-1} of extract (PLE). Value was mean ± S.E.M (n=5).

DPPH scavenging activity

The DPPH scavenging assay measures the anti-radical potential of natural products (Jha, Panda, Ramaiah, & Anand, 2014). PLE (15.625-250 µg ml^{-1}) showed a high anti-radical activity similar to that of ascorbic acid (Table 9). The percentage scavenging activity of PLE was 81.87±8.25 % when compared to ascorbic acid, 87.37±6.57 % respectively (Table 9). However, ascorbic acid was more potent than the extract.

H_2O_2 svavenging property

PLE (15.625-250 µg ml^{-1}) exerted a potent scavengigng anti-oxidant effect on H_2O_2 in a similar manner as ascorbic acid (Table 9). The extract scavenged H_2O_2 radical species at an absorbance of 0.0340±0.0093 when

compared to ascorbic acid at 0.0484±0.0192 respectively (Table 9). However, ascorbic acid was more potent than the extract at 230 nm absorbance.

Table 9: EC_{50} values for PLE and ascorbic acid in *in vitro* anti-oxidant experiment.

Test agent (µg ml^{-1})	DPPH scavenging activity (%)	H_2O_2 scavenging active
PLE	81.87±8.25	0.0340±0.0093
Ascorbic acid	87.37±6.57	0.0484±0.0192

Anti-oxidant capacity was determined as µg ascorbic acid ml^{-1} of extract (PLE). Value was mean ± S.E.M (n=5).

Establishment of mechanisms of action of PLE

***In vivo* mediator-induced paw oedema in Sprague-Dawley rats**

Histamine-induced paw oedema in rats

Oedema formation in this study was induced by injecting 100 µl of 1 % histamine (freshly prepared in saline) into the subplantar tissues of the right hind paw of rats. Paw oedema was measured at an interval of 30 min for 180 min and the difference in inflamed paw was determined as previously described. From the study, it was observed that PLE (100-600 mg kg^{-1}, *p.o.*, daily) administered prohpylactically (prior to oedema induction) caused the mean maximal inflamed paw oedema formed at 60 min to be suppressed significantly to 61.53±9.17 %, 54.21±9.38 % and 54.22±9.37 % at 100, 300 and 600 mg kg^{-1} relative to the inflamed control response (97.38±14.87 %) respectively (Fig. 54A). Also, the total inflamed paw oedema attained over 180 min (cal. as AUC) was equally reduced significantly by 42.88 %, 51.44 % and 58.12 % at the same doses relative to the inflamed control paw

respectively (Fig. 54B). In the therapeutic study, PLE (100-600 mg kg^{-1}) decreased the mean maximal oedema formed at 60 min to 80.89±12.60 % and 56.59±6.77 % at 300 and 600 mg kg^{-1} when compared to the inflamed control paw (101.38±21.87 %) respectively (Fig. 54C). Similarly, the total inflamed paw attained over 180 min (cal. as AUC) was also reduced by 46.46 % and 57.62 % at the same doses when comapred to the inflamed control paw respectively (Fig. 54D).

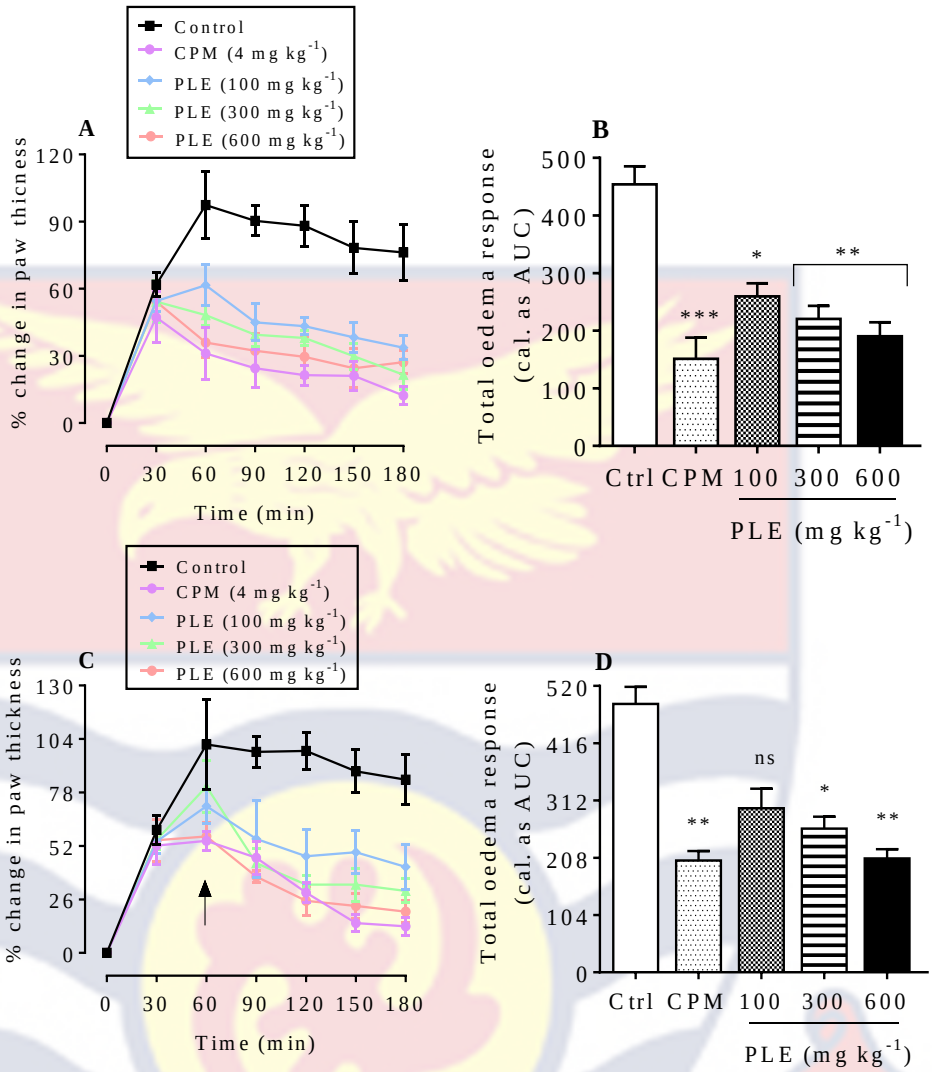


Figure 54: Effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) on histamine-induced paw oedema in rats. Rats (150-200 g, n=5) were inoculated with 100 µl of 1% histamine (suspended in saline) into subplantar tissues of right hind limbs of rats. Either Chlorpheniramine (4 mg kg^{-1}) or PLE was administered (1 h) prophylactically (A, B) prior to oedema induction or therapeutically (C, D) 1 h after histamine injection. Paw oedema formation was observed at an intervals of 30 min for 180 min as mean maximal change in paw thickness (A,C) and total paw oedema, cal. as AUC (B, D). Data was expressed as mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared with inflamed control response (Two-way ANOVA followed by Dunnet's *post hoc* test). Arrow denotes time of drug administration. ns indicates non-significant. Ctrl and CPM denote Control and Chlorpheniramine respectively.

Effect of PLE on serum cytokine (TNF-α, IL-1β, NF-κB, COX-2) levels

It has been reported that inoculation of CFA in rats stimulates the synthesis of pro-inflammatory cytokines such as TNF-α, NF-κB, IL-1β and IL-6 which promote irreversible joint damage, synovitis, arthritic joint, tissue and cartilage destruction as well as bone erosion (Mo, Panichayupakaranant, Kaewnopparat, Nitiruangjaras, & Reanmongkol, 2013). From the study, CFA-induced control group showed elevated gene expression of TNF-α, IL-1β, NF-κB, and COX-2 levels when compared to the IFA-treated (non-arthritic) control (Fig. 55[A-D]). Serum levels of TNF-α, IL-1β, NF-κB and COX-2 increased from 14.11±5.94, 8.55±4.65, 0.58±0.35 and 27.99±12.41 to 118.00±19.96, 130.30±30.81, 5.98±1.03 and 252.60±27.09 respectively (Fig. 55[A-D]). Dexamethasone (3 mg kg^{-1})- and aspirin (100 mg kg^{-1})-treated groups significantly reduced serum levels of TNF-α, IL-1β, NF-κB and COX-2 to 43.29±9.76, 36.91±9.02, 1.92±0.69, 83.12±27.22 and 61.31±7.01, 51.76±24.83, 2.42±0.92, 113.90±20.09 relative to the CFA-treated control group respectively (Fig. 55[A-D]). PLE (100-600 mg kg^{-1}, *p.o.*, daily)-treated groups similarly exhibited a significant decrease in TNF-α serum levels to 65.71±9.01 and 51.06±9.37 (Fig. 51A), IL-1β levels to 28.14±10.87 and 26.44±10.27 (Fig. 55B), NF-κB serum levels to 2.73±0.61 and 2.00±0.43 (Fig. 55C), and COX-2 serum levels to 114.60±40.28 and 103.00±42.77 (Fig. 55D) at 300 and 600 mg kg^{-1} relative to the CFA-treated control group respectively.

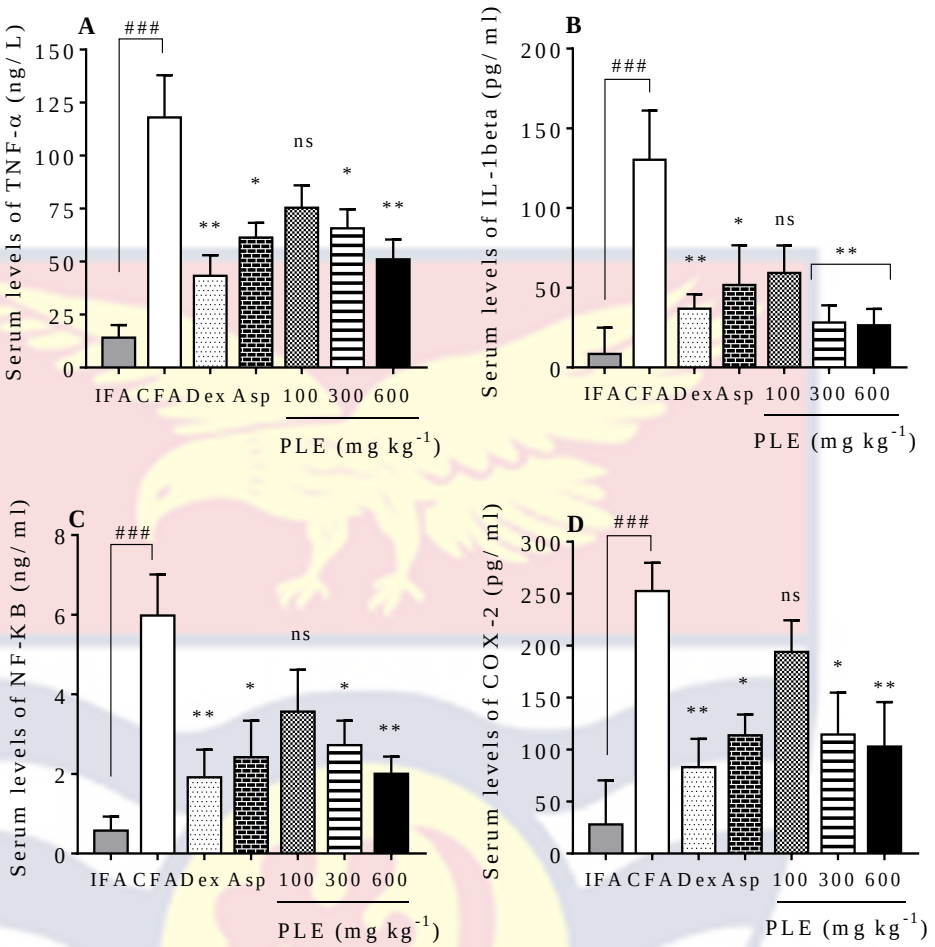


Figure 55: Effect of PLE (100-600 mg kg^{-1}, *p.o.*, daily) on serum levels of TNF-α, IL-1β, NF-κB and COX-2 in CFA-induced arthritic rats. Rats received either dexamethasone (3 mg kg^{-1}), aspirin (100 mg kg^{-1}), or PLE (100-600 mg kg^{-1}, *p.o.*, daily) and non-arthritic (IFA) group received saline only. The levels of TNF-α, IL-1β, NF-κB and COX-2 in sera of CFA-induced arthritic rats were determined using rat ELISA assay kit specific for each cytokine in accordance to the manufacturer's instructions. Data was presented as mean ± S.E.M. #P<0.05; ##P<0.01; ###P<0.001; *P<0.05; **P<0.01; ***P<0.001 compared with CFA-treated control group (One-way or Two-way ANOVA followed by Dunnet's *post hoc* test). ns denotes non-significant. Dex and Asp denote Dexamethasone and Aspirin respectively.

Phlogistic agents (Carrageenan, Zymosan A, Acetic acid, Cotton pellet, CFA)

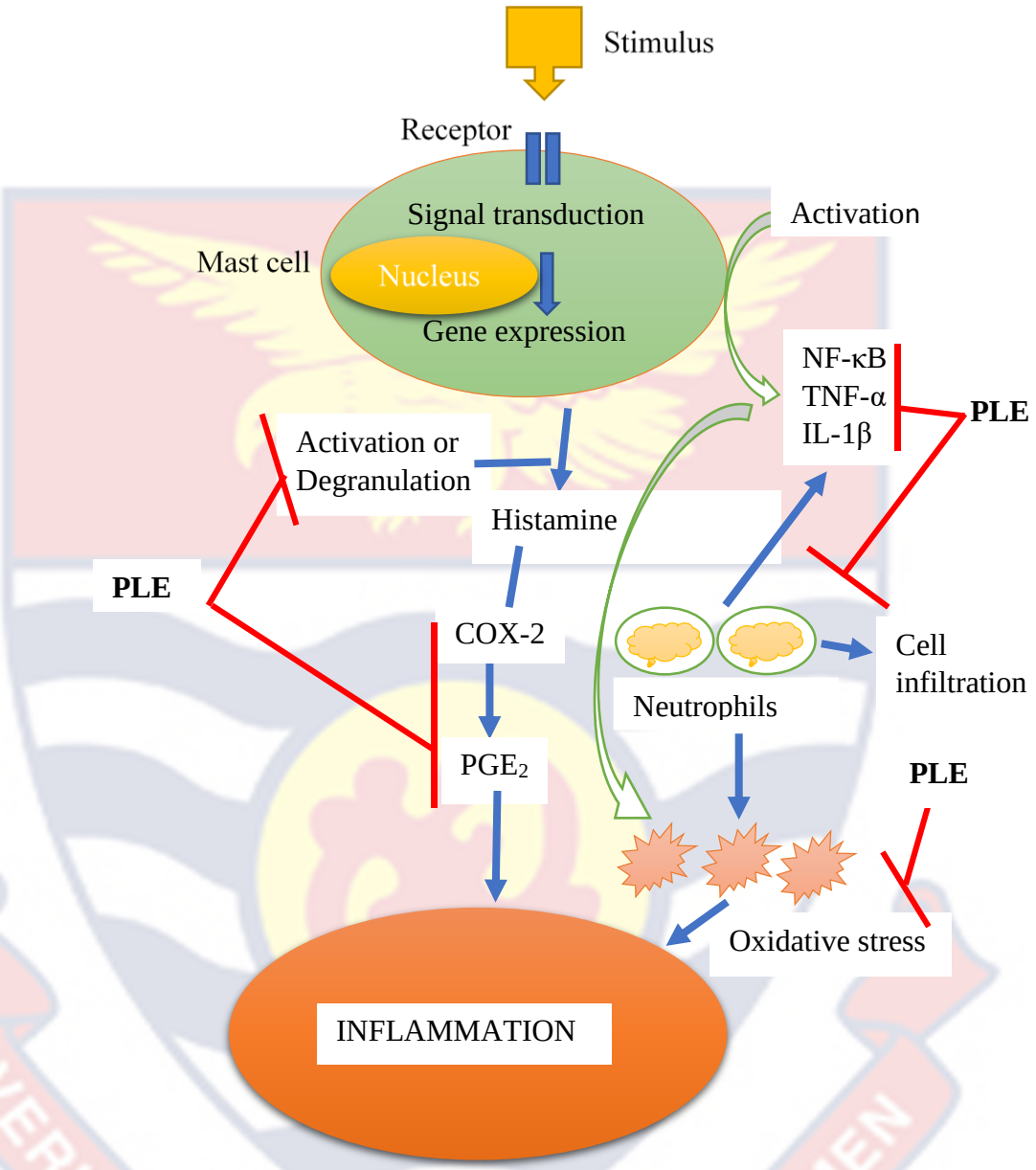


Figure 56: Schematic diagram showing the possible mechanisms of action of PLE following the innoculation of carrageenan, zymosan A, acetic acid, cotton pellet, or CFA. These compounds trigerred the activation of the macrophage via interaction with the receptor (s) to induce signal transduction causing the release of pro-inflammatory mediators such as histamine, serotonin, COX-2 (synthesizes PGE_2 from arachidonic acid) and cytokines including NF-κB, TNF-α and IL-1β. Neutrophils and macrophages generate free radicals that cause oxidative stress. In this study, PLE inhibited the activities of histamine, serotonin and PGE_2. In addition, PLE reduced the serum levels of COX-2, NF-κB, TNF-α, IL-1β and prevented oxidative stress.

Toxicity profile of PLE

Acute toxicity evaluation of PLE

Preliminary acute toxicity assessment of PLE at doses of 100, 300, 1000, 3000 and 5000 mg kg^{-1} showed no critical or clinical effects that could lead to death. This in fact, suggest that the LD_{50} value of PLE could exceed >5000 mg kg^{-1} and thus, practically makes the extract non-toxic. Therefore, PLE (100-5000 mg kg^{-1}, *p.o.*) exhibited no noticeable behavoiural, physiological or clinical signs that could lead to death which is indicative that PLE was acutely safe (Table 10). However, 8 h post-oral administration of PLE, there were only mild signs of urination and defeacataion observed in rats of PLE-treated groups, 3000 and 5000 mg kg^{-1} (Table 10) but these signs wore off gradually until the 24^{th} h. Thus no mortality was recorded at the end of the observation period in all PLE-treated groups (Table 10). The doses used in the pharmacological activity test of PLE were tested based on the LD_{50} value.

Table 10: Observations in the acute toxicity study post oral administration of PLE in rats.

Treatment groups						
Toxicity signs	Control	100	300	1000	3000	5000
Mortality	NØ	NØ	NØ	NØ	NØ	NØ
Latency (h)	-	-	-	-	-	-
Tremor	0	0	0	0	0	0
Excitement	0	0	0	0	0	0
Convulsion	0	0	0	0	0	0
Respiratory abnormality	0	0	0	0	0	0
Aggression	0	0	0	0	0	0
Agitation	0	0	0	0	0	0
Unusual locomotion	0	0	0	0	0	0
Ataxia	0	0	0	0	0	0
Sedation	0	0	0	0	0	0
Salivation	0	0	0	0	0	0
Defeacation	0	0	0	0	1	1
Urination	0	0	0	0	1	1
Diarrhea	0	0	0	0	0	0
Reactivity to touch	0	0	0	0	0	0

0= normal; 1= mildly impaired; 2= moderately impaired; 3= severely impaired. NØ= no death.

Sub-acute toxicity evaluation of PLE

Oral administration of PLE (100-1000 mg kg^{-1}, *p.o.*, daily) for 28 consecutive days revealed no significant changes in the behavioural, physiological or clinical conditions of the PLE-treated rats when compared to the control group. In fact, all rats in PLE-treated groups (100-1000 mg kg^{-1}) survived until the last day of the observation period.

Changes in body weight

Generally, from the study, it was observed that oral administration of PLE (100-1000 mg kg^{-1}) in the rats did not remarkably change the body weights of rats when compared to the control group (Fig. 57A). However, PLE-treated group at dose 300 mg kg^{-1} exhibited a significant marginal increase in both the mean maximal percentage (35.28±1.40 %) and total (154.00±13.50 %) change in body weights of rats when compared to the control group (17.72±2.18; 71.17±13.66) at the end of 28-day study period respectively (Fig. 57[A, B]).

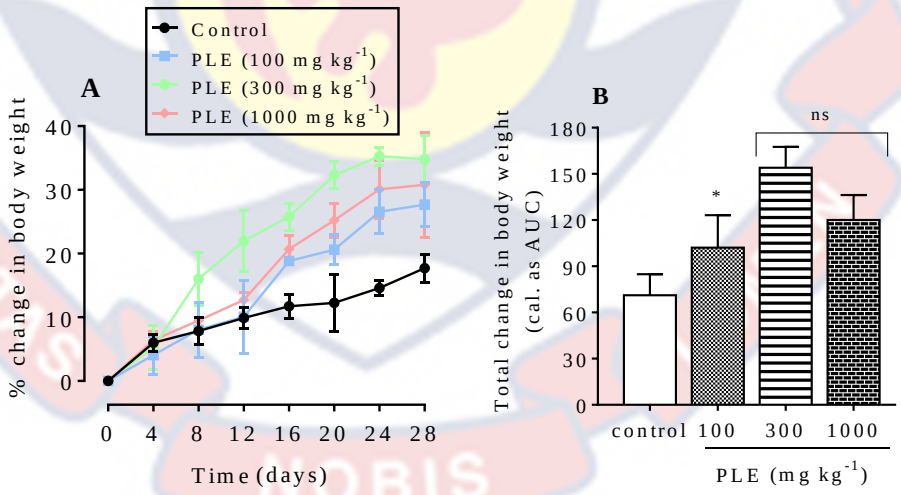


Figure 57: Effect of PLE on the mean maximal percentage (A) and total body (B) weights of rats (n=5) in sub-acute (28-day) toxicity study. Data was presented as mean ± S.E.M. PLE-treated groups (100-1000 mg kg^{-1}, *p.o.*, daily) were compared with control group using a one-way ANOVA followed by Dunnet's *post hoc* test. Statistically ($P<0.05$) significant. ns denotes non-significant.

Relative organ weight (ROW)

Initial macroscopic observations of the organs (liver, spleen, stomach, heart, kidney) of PLE-treated rats exhibited no pathological abnormalities, changes in colour or texture when compared to the control group during necropsy in the rats on day 29 at the end of the study. All organs appeared normal post PLE (100-1000 mg kg^{-1}) oral treatment (Fig. 58). Similarly, after 28 consecutive days of repeated oral PLE (100-1000 mg kg^{-1}) administration, the relative organ weight of each vital organ (i.e. liver, spleen, stomach, heart, kidney) of rats in PLE-treated groups determined at necropsy did not differ significantly when compared to the control group (Fig. 58) at the end of observation period.

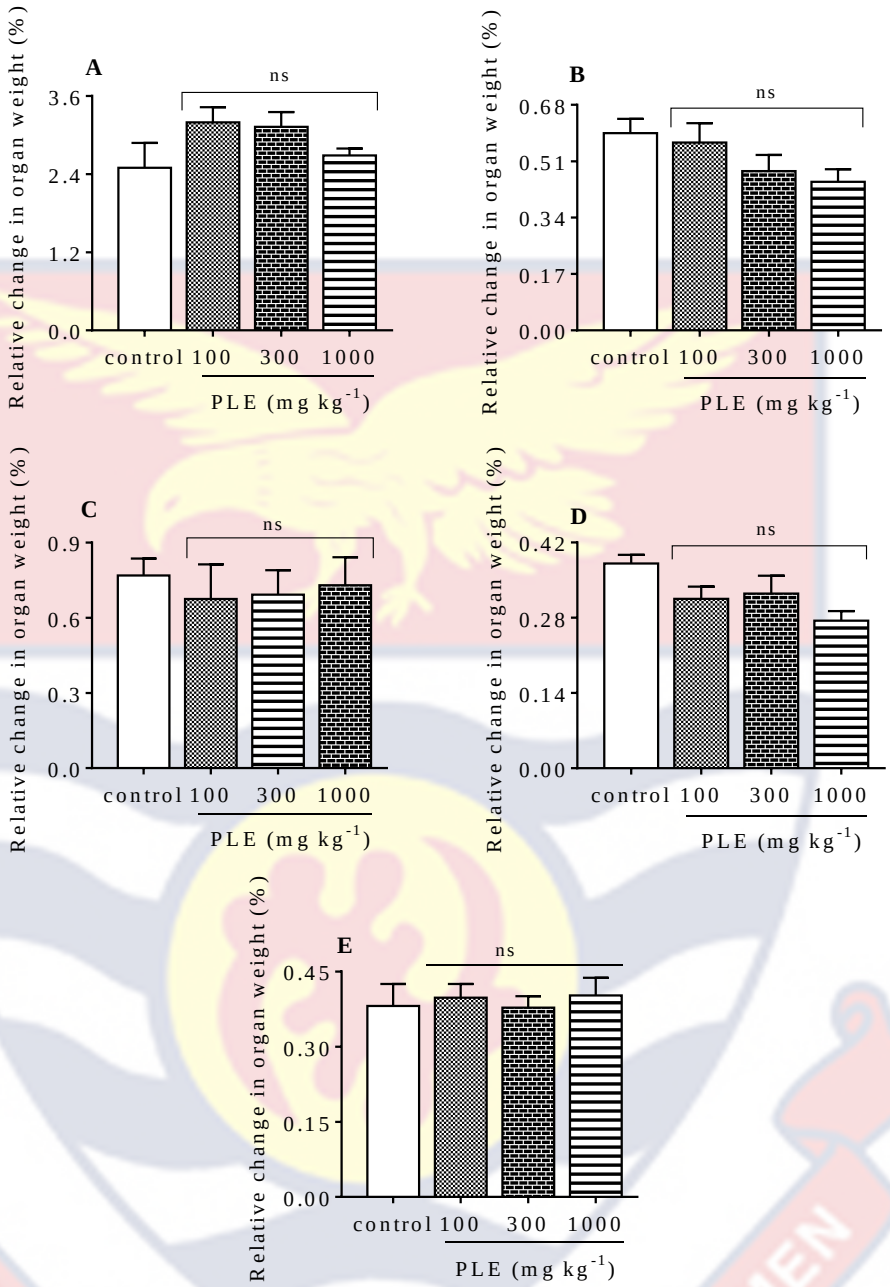


Figure 58: Effect of PLE on the relative organ weight (ROW) in sub-acute toxicity study in Sprague-Dawley rats (n=5). Data was expressed as mean ± S.E.M. PLE-treated groups (100-1000 mg kg^{-1}, *p.o.*, daily) were compared with control using a one-way ANOVA followed by Dunnet's *post hoc* test. Statistically ($P<0.05$) significant. Vital organs: Liver (A), Spleen (B), Stomach (C), Heart (D), Kidney (E) respectively. ns denotes non- significant.

Haematological examination

After 28 days of observation period for sub-acute toxicity study, all the haematological parameters of PLE-treated (100-1000 mg kg^{-1}, *p.o.*, daily) groups did not differ significantly from the control group at the same doses (Table 11).

Table 11. Haematological evaluation of sub-acute toxicity studies of PLE in Sprague-Dawley rats.

Groups	WBC (10^3/ µL)	RBC (10^3/ µL)	HGB (g/ dL)	PLT (10^3/ µL)	MCV (Fl)	MCH (pg)	LYM (%)
Control	12.17±1.29	7.84±1.05	13.55±0.46	858.0±53.0	54.70±3.20	16.35±0.95	68.40±2.50
PLE (100 mg kg^{-1})	12.48±2.56	7.69±0.56	12.79±0.76	796.5±108.5	62.37±2.34	17.75±1.45	65.95±2.95
PLE (300 mg kg^{-1})	12.99±2.01	7.51±0.43	12.55±0.45	698.5±67.5	59.35±2.85	17.60±0.80	65.70±7.40
PLE (1000 mg kg^{-1})	10.30±2.10	7.32±0.76	13.15±0.75	751.5±46.5	55.90±3.80	17.15±0.75	63.40±7.80

Sprague-Dawley rats (150-200 g, n=5) received PLE (100-1000 mg kg^{-1}, *p.o.*, daily) for 28 days. On the 29th day, animals were euthanised and blood samples were collected via cardiac puncture from each treatment group for Full Blood Count (FBC). P>0.05 when compared with control group.

Biochemical evaluation

From the study, the 28-day sub-acute toxicity assessment of PLE-treated (100-1000 mg kg^{-1}, *p.o.*, daily) exhibited no significant variations in all the biochemical parameters analysed at the same doses when compared to the control group (Table 12).

Table 12. Biochemical analysis of sera samples from a 28-day sub-acute toxicity studies of PLE in Sprague-Dawley rats.

Groups / Biochemical parameters	Control	PLE 100 (mg kg^{-1})	PLE 300 (mg kg^{-1})	PLE 1000 (mg kg^{-1})
Alkaline phosphate (U/l)	293.40±25.35	179.50±53.95	172.60±60.20	152.90±29.75
Alanine transaminase (U/l)	59.5±1.90	57.25±2.05	52.95±7.55	56.85±4.55
Aspartate transaminase (U/l)	130.30±2.90	127.30±5.35	140.30±19.75	117.20±4.55
Albumin (g/l)	29.15±1.55	31.55±1.35	30.10±1.80	28.80±1.90
Globulin (g/l)	46.85±2.25	47.12±1.72	44.18±2.28	41.84±2.53
Total protein (g/l)	76.05±3.75	77.55±4.15	76.60±1.80	76.20±3.10
Total bilirubin (µmol/l)	2.67±0.46	2.74±0.27	3.25±0.87	3.93±0.71
Direct bilirubin (µmol/l)	0.52±0.03	0.45±0.04	0.36±0.11	0.33±0.11
Indirect bilirubin (µmol/l)	2.80±1.08	2.53±0.53	2.58±0.45	2.96±0.19
Urea (mmol/l)	8.87±0.34	8.61±0.73	7.70±0.57	7.45±0.46
Creatinine (µmol/l)	80.70±7.40	77.50±3.30	79.75±3.65	76.35±6.75
Blood urea nitrogen	102.20±7.37	114.50±11.21	105.10±6.56	111.20±8.49

Sprague-Dawley rats (150-200 g, n=5) received PLE (100-1000 mg kg^{-1}, *p.o.*, daily) for 28 days. On the 29th day, animals were euthanised and blood samples were collected via cardiac puncture from each treatment group. Serum was prepared by centrifugation at 3000× g rpm for 15 min. and stored (-20 ℃) until use. Biochemical analysis was performed. P>0.05 when compared with control group.

Histopathological analysis

Figures (59-63) show the histopathological evaluation of the isolated organs of rats (liver, spleen, stomach, heart, kidney) of PLE (100-1000 mg kg^{-1})-treated and control groups post 28 days of sub-acute toxicity study. Generally, from the study, all the organs (i.e. liver, spleen, stomach, heart, kidney) isolated from PLE-treated rats exhibited normal histological features without aberrations in the architecture when compared to the control group (Fig. 59-63[B-D])). The control group microscopically exhibited no alterations in the organs isolated and thus, preserved organ integrity (Fig. 59-63[A]).

At the end of 28-day observation period of sub-acute toxicity study, the histological assessment of the liver of PLE-treated rats did not show any histological alterations when compared to the control group (Fig. 59[B-D]). From the study, the central and portal veins appeared normal with hepatocytes lined by endothelial cells. The hepatocytic sinusoids appeared normal with no vascular congestion in the veins, no sinusoidal dilation, mononuclear cell infiltration and focal necrosis (Fig. 59[B-D]) like the control group (Fig. 59A).

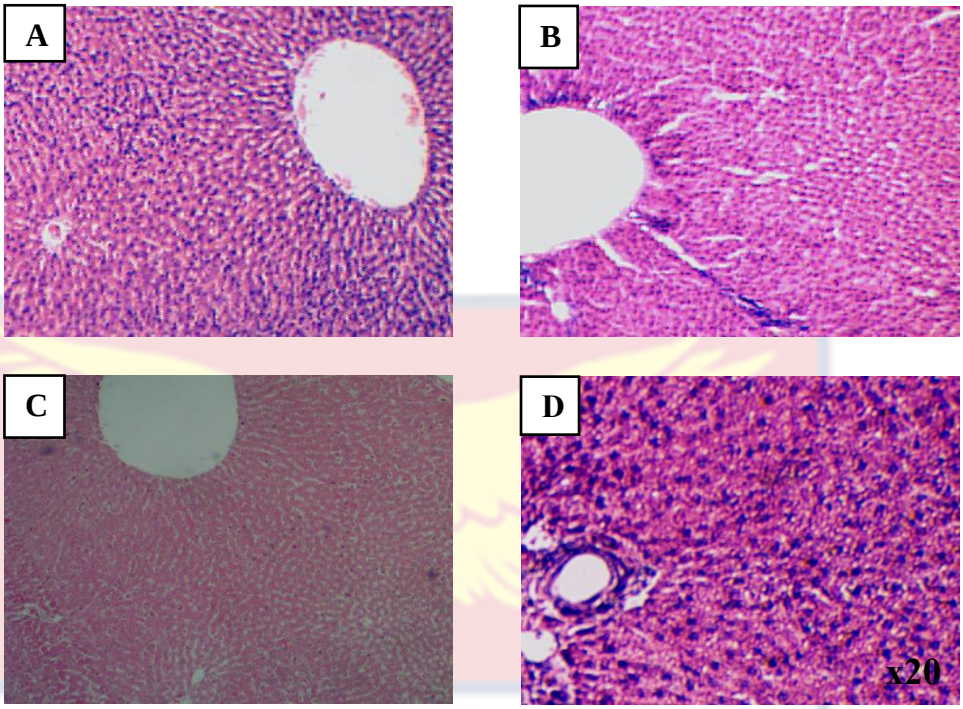


Figure 59: Photomicrograph showing sections of the liver in a 28-day sub-acute toxicity study in Sprague-Dawley rats (n=5). Rats orally received PLE (100-1000 mg kg^{-1}, *p.o.*, daily). (B-D)= PLE-treated groups respectively. Control group (A) received normal saline (1 ml of Tween 80 in normal saline, *p.o.*, daily).

Similarly, the architectural of the spleen after oral PLE (100-1000 mg kg^{-1}, *p.o.*, daily) administration for 28 days revealed normal splenic histoarchitecture. In this study, the spleen isolated from PLE-treated rats showed no haemorrhage or splenic congestion. The red pulp of the spleen consisted of a number erythrocytes and lymphocytes. In addition, marginal zone was normal, and the white pulp contained visible irregular mass of lymphoid tissues with no histological aberrations of the architecture (Fig. 60[B-D]) similar to the control group (Fig. 60A).

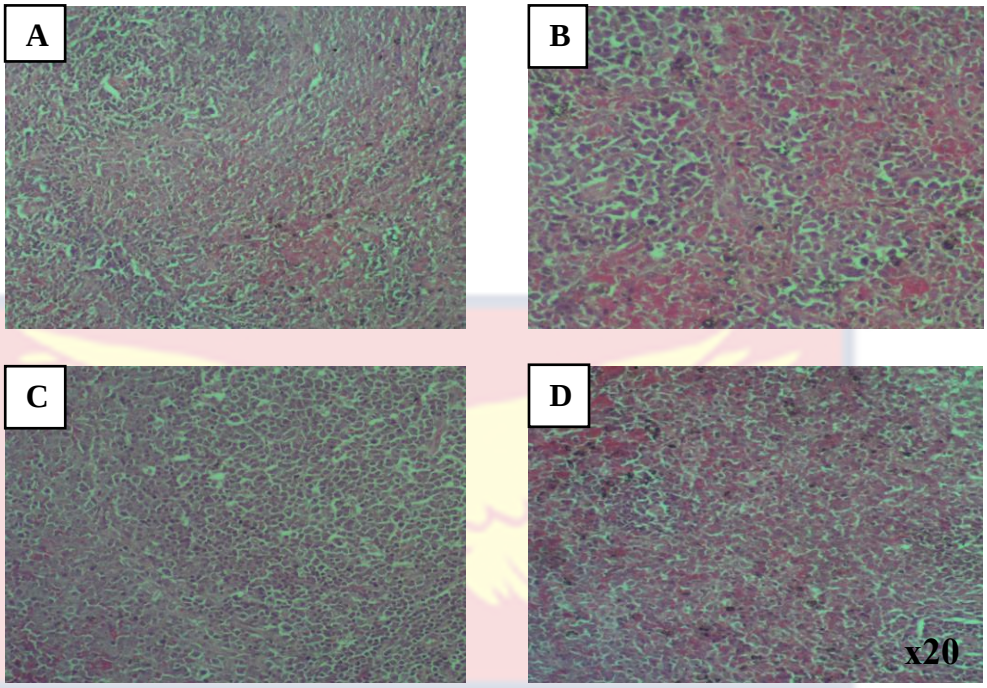


Figure 60: Photomicrograph showing sections of the spleen in a 28-day sub-acute toxicity study in Sprague-Dawley rats (n=5). Rats orally received PLE (100-1000 mg kg^{-1}, *p.o.*, daily). (B-D)= PLE-treated groups respectively. Control group (A) received normal saline (1 ml of Tween 80 in normal saline, *p.o.*, daily).

From the study, cross sections of the stomach of PLE (100-1000 mg kg^{-1}, *p.o.*, daily)-treated and the control group exhibited normal mucosa architecture with intact epithelial structure (Fig. 61). Histopathological evaluation of cross sections of the stomach post oral PLE (100-1000 mg kg^{-1}) administration showed preserved mucosal and submucosal structures with normal simple columnar epithelium of the gastric pit and glands. The laminal and muscularis propria were normal with no epithelial erosion, mucosal necrosis or distortion of the gastric glands (Fig. 61[B-D]) similar to the control group (Fig. 61A).

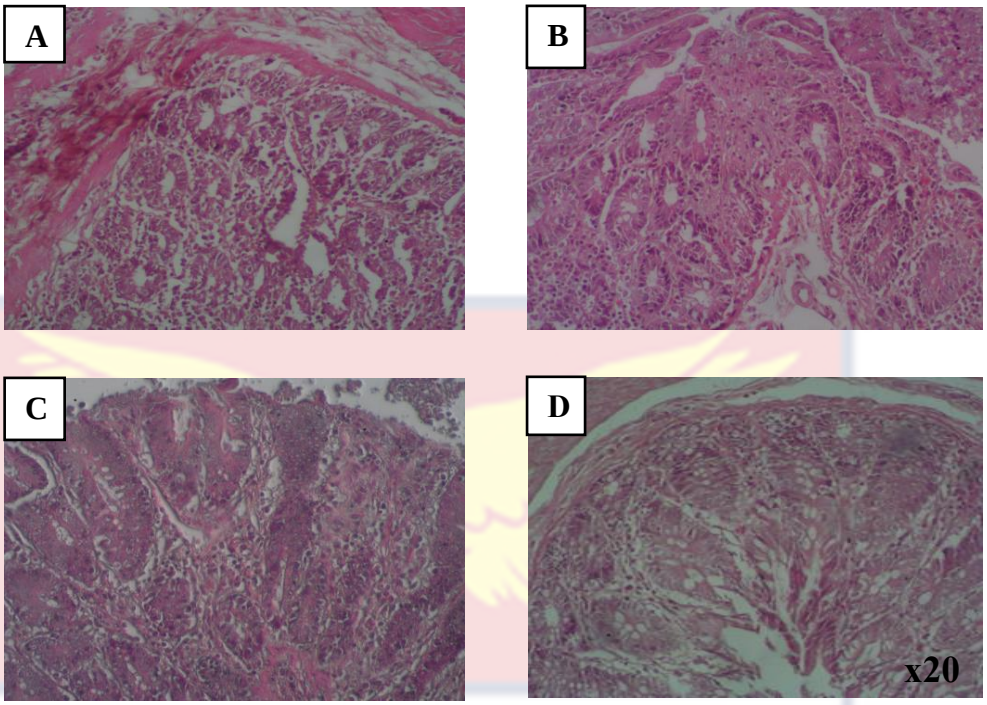


Figure 61: Photomicrograph showing sections of the stomach in a 28-day sub-acute toxicity study in Sprague-Dawley rats (n=5). Rats orally received PLE (100-1000 mg kg^{-1}, *p.o.*, daily). (B-D)= PLE-treated groups respectively. Control group (A) received normal saline (1 ml of Tween 80 in normal saline, *p.o.*, daily).

Photomicrograph of heart sections of the PLE (100-1000 mg kg^{-1}, *p.o.*, daily)-treated and control rats showed no architectural alterations upon examination (Fig. 62). From the study, post 28 days of PLE (100-1000 mg kg^{-1}) administration revealed normal heart histoarchitecture with cardiac fibers containing concise cardiomyocytes and intact myofibers. In addition, muscle fibers were organized into clusters, disks that linked closely to adjacent cardiac cells with numerous layers of muscle cells present (Fig. 62[B-D]) similar to the control group (Fig. 62A).

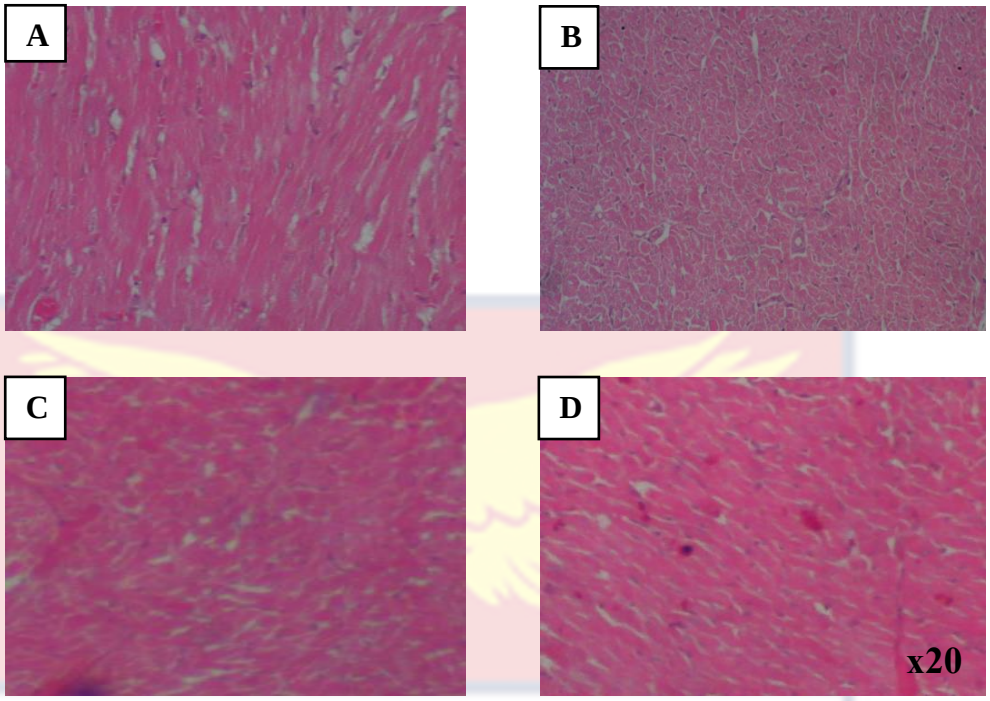


Figure 62: Photomicrograph showing sections of the heart in a 28-day sub-acute toxicity study in Sprague-Dawley rats (n=5). Rats orally received PLE (100-1000 mg kg^{-1}, *p.o.*, daily). (B-D)= PLE-treated groups respectively. Control group (A) received normal saline (1 ml of Tween 80 in normal saline, *p.o.*, daily).

At the end of sub-acute (28-day) toxicity study, microscopic examination of the photomicrograph of kidney sections in PLE-treated (100-5000 mg kg^{-1}, *p.o.*, daily) rats exhibited no remarkable histoarchitectural variations after treatment when compared to the control group (Fig. 63). Oral administration of PLE (100-1000 mg kg^{-1}) repeatedly showed no histological changes of the kidney with no inflammation or congestion of the glomerulus. The glomerulus appeared normal with parietal layered bowman's space, and cuboidal epithelial lining of the convoluted tubules (Fig. 63[B-D]) similar to the control group (Fig. 63A).

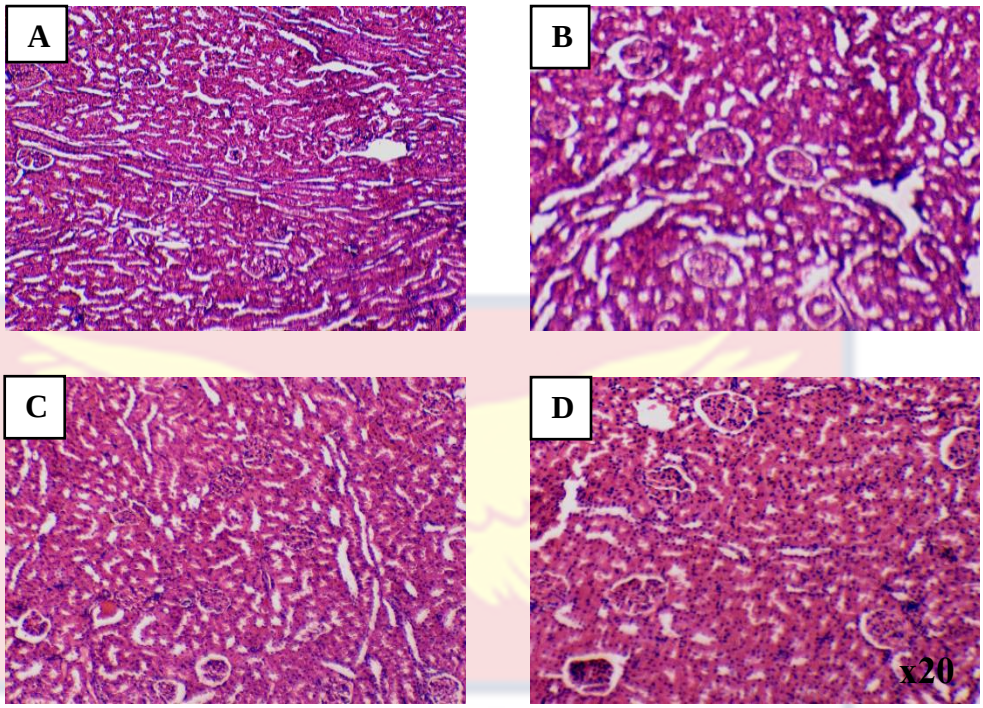


Figure 63: Photomicrograph showing sections of the kidney in a 28-day sub-acute toxicity study in Sprague-Dawley rats (n=5). Rats orally received PLE (100-1000 mg kg^{-1}, *p.o.*, daily). (B-D)= PLE-treated groups respectively. Control group (A) received normal saline (1 ml of Tween 80 in normal saline, *p.o.*, daily).

DISCUSSION

Anti-inflammatory effects of PLE on acute inflammation

Carrageenan-induced paw oedema in rats

In inflammation studies, many experimental models have been used to assess inflammation. Usually, the methods used to determine whether a compound has anti-inflammatory activity are to test it in animal, and biochemical models (Souto *et al*., 2011). The most extensively used preliminary test in search for new, complementary and alternative anti-inflammatory drugs measures the ability of an agent to reduce oedema induced by injection of a phlogistic substance in animals (Chakraborty, Devi, Rita, Sharatchandra, & Singh, 2004). Therefore, to test the anti-inflammatory activities of PLE on acute inflammation, carrageenan-induced paw oedema and zymosan-induced acute knee joint arthritis in rats were used.

Carrageenan-induced paw oedema is a typical acute inflammatory model used in animal studies to evaluate anti-inflammatory activities (Akinnawo, Anyasor, & Osilesi, 2017). It is also a very sensitive and reproducible model that is used to assess new compounds with anti-inflammatory properties (Dzoyem, McGaw, Kuete, & Bakowsky, 2017). Carrageenan-induced paw oedema causes local and acute inflammatory responses that is useful for discovering orally active anti-inflammatory compounds, thereby making it an important model for anti-inflammatory compounds that act through acute inflammatory mediators (Dzoyem *et al*., 2017). It has also been reported that carrageenan-induced paw oedema is sensitive to inhibitors of both COX and LOX enzymes (Calhoun, Chang, & Carlson, 1987). The injection of carrageenan induces oedema biphasically over

time, and the anti-inflammatory effect is normally determined after 6 h due to the depletion of kininogen (an inflammatory cofactor) after this period (Lee *et al*., 2017; Akinnawo *et al*., 2017). In the early phase, which occurs between 0-2 h (after carrageenan injection), pro-inflammatory mediators such as histamine, bradykinin and serotonin (5-HT) are involved. The late phase (>2 h, after carrageenan injection), also known to be a complement-dependent reaction, is mediated by the release of prostaglandins (PGs), nitric oxide (NO), TNF-α, free radical oxygen species, and leukotrienes (Silva *et al*., 2017; Zuntini Viscardi *et al*., 2017). Characteristic effects such as increased vascular permeability and accumulation of fluid in tissues that account for the oedema are as a result of the production of vasoactive substances during acute inflammation (Paschapur, Patil, Kumar, & Patil, 2009). From the present study, administration of PLE showed a significant inhibition of oedema both in the prophylactic (pre-emptive, 2 h before carrageenan injection) and curative studies (2 h after carrageenan injection). PLE was a potent inhibitor of the initial phase suggestive of its anti-inflammatory action which is related to the inhibition of pro-inflammatory mediators including histamine and 5-HT. The final phase is mainly mediated by PGs, and according to Silva *et al*. (2017) and Zuntini Viscardi *et al*. (2017), the production of PGs in the arachidonic pathway is sustained by high levels of COX. It has also been reported that deregulation of PGs causes redness, pain and swelling as a result of increased vasodilation and microvascular permeability (Legler, Bruckner, Uetz-von Allmen, & Krause, 2010). PLE significantly inhibited the synthesis of PGs in the late phase which showed its high inhibitory effects on the action of COX in the metabolic arachidonic acid pathway. The potential effects of PLE on the late phase also

implies that other pro-inflammatory mediators such as NO and leukotrienes were inhibited.

Zymosan-induced acute knee joint arthritis in Sprague-Dawley rats

Zymosan-induced acute knee-joint arthritis is widely used to evaluate the anti-inflammatory activities of various compounds (Gegout, Gillet, Terlain, & Netter, 1995). According to Underhill (2003), during inflammatory responses as a results zymosan-induced inflammation, actions of phagocytosis, cell migration and the synthesis of pro-inflammatory mediators can be studied. Zymosan is known to activate phagocytic cells which leads to increase secretions of lysosomal enzyme levels, promotes the release of pro-inflammatory cytokines such as TNF-α and IL-6, enhances leukotriene production of monocytes (Sato *et al*., 2003), chemokines such as Chemokine-C-X-C motif [CXCL-1] (Conte *et al*., 2008), matrix metalloproteinase-9 (Kolaczkowska, Arnold, & Opdenakker, 2008) and monocyte chemoattractant protein [MCP-1] (Takahashi, Galligan, Tessarollo, & Yoshimura, 2009). Intra-articular injection of zymosan in rodents causes a biphasic arthritis comprising of early stage that is characterised by vascular permeability, oedema formation, leukocyte infiltration, influx of neutrophils and synthesis of pro-inflammatory mediators, and the late stage is mediated by increased lymphocytes and macrophages (Asquith, Miller, McInnes, & Liew, 2009). Previous reports have also shown that pro-inflammatory cytokines such as NF-κB, TNF-α, IL-1β, IL-6 (Rasquel-Oliveira *et al*., 2020; Rosas *et al*., 2015) and ROS (Rasquel-Oliveira *et al*., 2020) are involved in zymosan-induced acute knee joint arthritis in rats. In addition, eicosanoids such as prostaglandins and leukotrienes participate in tissue destruction, infiltration of neutrophils and pathogenesis of arthritis

(Brennan & Mclnnes, 2008). From the study, pre-emptive oral administration of PLE significantly reduced the knee joint thickness in the right limbs of the rats. Thus, oedema formation due to intra-articular injection of zymosan was remarkably inhibited and consequently, pro-inflammatory mediators such as prostaglandins including cytokines and ROS involved in acute knee joint inflammation were inhibited. This primary anti-arthritic effect of PLE could be attributed to the inhibition of several pro-inflammatory mediators, cytokines and other enzymes implicated in arthritic inflammation. It can therefore be established that PLE could possess anti-arthritic activity and this is consistent with earlier literature that established the anti-inflammatory potential of plants against inflammatory disorders including arthritis (Lama & Saikia, 2011). Conte *et al*. (2008) reported that intra-articular injection of zymosan stimulates massive migration of neutrophils into the synovial tissue and fluids of inflamed joints. Neutrophils are reported to exert a key action in the pathogenesis of joint arthritis and promote cartilage damage as well as increased bone resorption in the joints via the production of ROS in association with other proteolytic enzymes (Wright *et al*., 2014). It has also been reported that high levels of leukocyte migration into the inflamed cavity of the knee joint occurs during zymosan-induced inflammatory response (Mortada & Hussain, 2014; Rasquel-Oliveira *et al*., 2020). According to Milanova, Ivanovska, & Dimitrova (2014), neutrophils trigger numerous surface receptor expressions necessary for adhesion molecules, ILs, complements such as C3 and C5 that promotes activation and recruitment. Neutrophils are also reported to induce the release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and chemokines including CXCL-1 that cause bone and cartilage damage (Brennan & Mclnnes,

2008; Rasquel-Oliveira *et al*., 2020). Therefore, antagonism of neutrophil infiltration or activation is a critical alternative to treating arthritis (Rosas *et al*., 2015). From the study, oral treatment with PLE significantly attenuated the influx of neutrophils and migration of leukocytes into the synovial cavity of the knee joint and thus, inhibited the production of pro-inflammatory mediators and other pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 that could cause cartilage and bone damage. The reduction of neutrophil and leukocyte levels by the extract in the knee cavity and fluids of the inflamed joints contributed to the management of the arthritic condition. This is in consonant with literature previously reported that established attenuation of increased neutrophil infiltration is relevant in the management of arthritis (Rosas *et al*., 2015).

According to Babu *et al*. (2021), histopathological changes of an arthritic knee joint are characterised by severe cell infiltration, loss of synovial space, cartilage and bone erosion, and distortion of the synovial membrane lining. Hence, these pathological alterations are known to be clinical features of degenerative joint disorder (Dobson *et al*., 2018). Also, it has been reported that bone and cartilage erosion in arthritic knee joint is mediated by pro-inflammatory mediators and cytokines (Kim *et al*., 2018). From the study, PLE showed a significant improvement of the histological changes in the knee joint of rats by relatively maintaining the bone and cartilage tissues, synovial membrane lining and synovial space. This was evident in its inhibitory effects against cartilage and bone degradation, decreased synovial space, inflammatory cell infiltration and pro-inflammatory cytokines as well as mediators implicated in knee joint arthritis. Hence, bone remodeling was upregulated by the extract

to maintain bone integrity, and therefore, this is in agreement with earlier literature reported that established the ability of plant extracts to maintain histopathological architecture of the bone and cartilage during arthritic inflammation (Babu *et al*., 2021).

Chan *et al*. (2014) reported that mast cells are implicated in the pathogenesis of osteoarthritis and their levels are proliferated in osteoarthritic bone. They are also said to be present in the synovium and synovial fluids of patients with knee osteoarthritis (de Lange-Brokaar *et al*., 2016; Fusco, Skaper, Coaccioli, Varrassi, & Paladini, 2017). Mast cells, upon activation, release a series of pro-inflammatory mediators such as histamine, pro-inflammatory lipids (prostaglandins), chemokines and cytokines [TNF-α, IL-6] (Theoharides *et al*., 2012; Yu, Blokhuis, Garssen, & Redegeld, 2016) which regulate bone metabolism potentiating bone resorption (Chiappetta & Gruber, 2006). Therefore, amelioration of mast cell proliferation or activation is a beneficial effect on the pathogenesis of osteoarthritis (Chan *et al*., 2014). On the basis of this assertion, the anti-proliferative effect of PLE on mast cell proliferation or activation in this study was very significant. PLE remarkably reduced the mast cell levels in the synovium and synovial fluid of the knee joint cavity and consequently, inhibited the pro-inflammatory cytokines and mediators that could be released to modulate bone metabolism leading to increased bone resorption to cause bone and cartilage erosion. This is consistent with literature earlier reported that established the inhibitory effects of medicinal plants to suppress mast cell activation or proliferation in the bone tissue as a key mechanism in arthritic therapy (Chan *et al*., 2014).

Anti-inflammatory effects of PLE on chronic inflammation

Acetic acid-induced ulcerative colitis in rats

The initial response of inflammation may provide a protection for the host and somewhat self-limiting that progress to an utmost tissue repair or resolution (Duffin, Leitch, Fox, Haslett, & Rossi, 2010). Acute inflammation, if deregulated however, can lead to the pathogenesis of a more inflammatory chronic and autoimmune diseases including gastrointestinal tract inflammation, atherosclerosis, respiratory inflammation and rheumatoid arthritis (Nathan & Ding, 2010); eventually this causes tissue or organ damage and fibrosis (Sousa, Alessandri, Pinho, & Teixeira, 2013). Therefore, the effect of PLE on chronic inflammation was evaluated on chronic inflammatory models using acetic acid-induced ulcerative colitis, cotton pellet granuloma tissue formation and CFA-induced arthritis in rats.

Acetic acid-induced ulcerative colitis is a well-known model used to study IBD (Aleisa *et al*., 2014) and this may resemble human IBD in terms of its pathophysiological characteristics but differ etiologically. Pathophysiological features such as colonic damage associated with mucosal ulceration, oedema, weight loss, diarrheoa and hemorrhage following intrarectal injection of 3-5 % acetic acid in rats are related to human IBD (Hartmann *et al*., 2012). Additionally, increased infiltration of neutrophils into the injured colon, mucosal and submucosal colon damage, synthesis of pro-inflammatory mediators including cytokines, prostaglandins, and generation of ROS that lead to oxidative damage can be found in both diseases (Ali, Abd Al-Haleem, Khaleel, & Sallam, 2017). From the study, intrarectal injection of acetic acid caused inflammation in colon tissues, lipid peroxidation and increased

microvascular permeability. Colonic damage is reported to result in bowel wall thickening, reduced colon length, increased colon weight-to-length ratio (Abdel-Daim, Farouk, Madkour, & Azab, 2015), and loss in body weight.

Loss of body weight in colitis occurs either due to loss of appetite, malnutrition, malabsorption, or maldigestion as a result of protein loss or metabolic stress, and excessive loss of body fluid via colorectal bleeding and diarrheoa associated with intestinal inflammation (Owusu *et al*., 2020). Hunschede, Kubant, Akilen, Thomas, & Anderson (2017) reported that pro-inflammatory cytokines such as TNF-α and IL-6 are implicated in body weight loss in IBD due to the release of neuropeptides that affect appetite and precipitate cachexia. Remission maintenance is critical in ulcerative colitis treatment (Kamat *et al*., 2019). From this study, treatment with PLE showed no significant change in body weight but instead overall loss of body weight was recorded. After macroscopic assessment, colonic damage showed a significant increase in colon weight-to-length ratio of rats which could be attributed to increased infiltration of inflammatory cells, necrosis, severe colon-wall oedema and goblet cell hyperplasia (El-Abhar, Hammad, & Gawad, 2008). PLE-treated rats, after intestinal macroscopic content assessment, showed improved colon weight change, colon length shortening, stool consistency and reduction of colonic damage. There was no visible blood or mucus stains, and this could be linked to the protection of the mucus layer and suppression of loss of excess blood being hallmarks of anti-ulcerative compounds (Al-Rejaie Abuohashish, Ahmed, Aleisa, & Alkhamees, 2012) and also, consistent with reports of other researchers (Bastaki, Al Ahmed, Al Zaabi, Amir, & Adeghate, 2016). It is therefore not surprising that PLE significantly reduced the DAI score (El-Abhar

et al., 2008) and preserved mucosal integrity via its promising healing effects in ulcerative colitis, and thus evidently validate its folkloric use in gastrointestinal disorders.

In the initial assessment of ulcerative colitis, blood test such as full blood count has been considered to be critical. In addition, Olamilosoye, Akomolafe, Akinsomisoye, Adefisayo, & Alabi (2018) and Javed & Jabeen (2021) reported that anomalies in haematological variables are characteristic indications of ulcerative colitis. Hence, evaluation of haematological parameters can be used to assess the severity of colitis (Jagtap, Niphadkar, & Phadke, 2011). From the study, acetic acid-induced colitis group showed a significant increase in WBC, LYM and NEU. These variations in the haematological parameters contributed to the extensive mucosal ulcerations and therefore, treatment with PLE significantly restored the WBC, NEU and LYM count due its ability to reduce their elevated levels. Thus, PLE significantly attenuated the haematological aberrations of the ulcerative colitis and this is in a line with the literature reported by Olamilosoye *et al*. (2018).

Regarding histological examination, microscopic lesions in colonic tissues (Goyal, Rana, Ahlawat, Bijjem, & Kumar, 2014) as well as other histological complications are observed after intrarectal injection of acetic acid in animals, and this is consistent with other animal studies of IBD (Dejban, Rahimi, Takzare, & Dehpour, 2020; Rashidian *et al*., 2020). From the study, acetic acid-induced colonic damage was characterised by vital histological features such as increased inflammatory cellular infiltration, thickening of the smooth muscle, damaged mucosal layer, oedema of the submucosal, necrosis, epithelial damage and ulceration (Chamanara *et al*., 2019; Babitha, Bindu,

Nageena, & Veerapur, 2019). Administration of PLE ameliorated the histological signs detected in acetic acid-induced colitis and exhibited a protective effect for the mucosal layer of the colon. The protective ability of PLE could be attributed to its significant suppression of cellular infiltration of inflammatory cells, ulcerations, necrosis, oedema of the submucosal, distortions in the goblet cells, and enhancing regeneration of the crypt structures. Thus, colon integrity was maintained and this was evidenced with the haematological, DAI score (macroscopic) and microscopic outcomes. Consequently, it could be established that PLE possesses anti-ulcerative properties.

In this study, intra-rectal administration of 4 % acetic acid causes high influx of mast cells into the site of colonic injury. Mast cells are known to play key roles in inflammatory responses (Blank, Essig, Scandiuzzi, Benhamou, & Kanamaru, 2007) such as lesions of the gastrointestinal tract and systemic mast cell syndrome (Frenzel & Hermine, 2013) after tissue injury and also, release a variety of preformed mediators including pro-inflammatory cytokines and growth factors upon stimulation (Theoharis *et al*., 2012). Costa de Oliveira *et al*. (2011) reported that inhibition of mast cell secretion and activation provides a good therapeutic strategy to treatment of inflammatory disorders. From the study, acetic acid-induced colitis group showed high influx of mast cell at the site of colonic injury and subsequently, recorded increased mast cell count. Treatment with PLE significantly decreased mast cell accumulation at colonic injury site in line with the reduced mast cell proliferation score recorded, and this is consistent with literature reported on anti-inflammatory effects of other novel agents on mast cell activation and proliferation (Jeong *et al*., 2009).

In the etiology of numerous diseases, oxidative stress which is a state of imbalance between reactive oxygen species (ROS) generation and anti-oxidant levels is likely implicated (Sundaram *et al*., 2019; Tang, Wei, Deng, & Lei, 2017). Reuter, Gupta, Chaturvedi, & Aggarwal (2010) reported that oxidative stress triggers inflammatory pathways, and in the development of drugs, determination of anti-oxidant capacity of biological substances is an intriguing way of assessing the safety of these substances (Siddeeg *et al*., 2021). To compare the anti-oxidant contents and the potency of anti-oxidants for the management of oxidative stress-related chronic diseases, the effective approach is to determine their levels in biological samples such as serum and tissues (Apak *et al*., 2007). Generally, the techniques used to assess anti-oxidant capacity depend on factors such as scavenging the peroxyl radicals (e.g. total reactive anti-oxidant potential [TRAP], oxygen radical absorbance capacity assay [ORAC], 2, 2-diphenyl-1-picrylhydrazyl [DPPH]) (Karadag, Ozcelik, & Saner, 2009) and testing other products following oxidation reactions such as thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) (Karadag *et al*., 2009). It has also been reported that MDA, a metabolic product of lipid peroxidation, increases as a results of oxidative stress (Subash *et al*., 2016) and a chain of anti-oxidant agents including SOD, CAT and GSH act on ROS or free radicals when generated (Essel, Obiri, Osafo, Antwi, & Duduyemi, 2017). Ferreira, Barros, & Abreu (2009) reported that high anti-oxidant capacity reduces the risk of chronic diseases such as cardiovascular disorders, diabetes, cancer, among others and also provide a defensive system against oxidative injuries (Antonisamy, Agastian, Kang, Kim, & Kim, 2019). Nonetheless, inhibition of depletion and/ or augmentation of SOD, GSH and CAT generation

as a result of their increase due to oxidative stress has been established (Birben *et al*., 2012). Thus, amelioration of ROS generation and free radicals is an essential activity in numerous pathological conditions (Subash *et al*., 2016). From the study, the anti-oxidant capacity of PLE on CAT, SOD enzyme activities and MDA levels were assessed. In this study, it was shown that PLE blocked the effect of oxidative stress by upregulating the enzymatic activities of SOD and CAT while reducing MDA content in both serum and colon tissues due to the inhibition of lipid peroxidation reaction. It is therefore not surprising that PLE was shown to possess high anti-radical capacity by scavenging free radicals generated from *in vitro* DPPH and H_2O_2 activities. In addition, the total anti-oxidant capacity (i.e. a measure of the capacity of anti-oxidants in biological samples) of PLE was found to be high and this confirms that, PLE is an anti-oxidant agent with potent anti-lipidperoxidative effect consistent with earlier reports suggesting that plants contain various substances such as derivatives of polyphenolic, saponins and flavonoids which possess anti-inflammatory and anti-oxidative effects (Dogan & Anuk, 2019; Wattanathorn *et al*., 2017). Consequently, anti-oxidant and high anti-radical scavenging capacities of PLE could be credited to its secondary metabolites present.

Cotton pellet-induced tissue granuloma formation in rats

One of the established characteristic feature of chronic inflammatory reaction is cotton pellet-induced granuloma tissue formation (Sengar, Joshi, Prasad, & Hemalatha, 2015). Cotton pellet-induced granuloma tissue formation model is used widely to evaluate the proliferative, transudative, and exudative stages of chronic inflammation (Boonyarikpunchai, Sukrong, & Towiwat, 2014). In response to the subcutaneous implantation of sterilised cotton, three

phases are involved: 1) the first phase, transudative phase, occurs within 3 h and characterised by leakage of fluid containing low proteins from blood vessels as a result of increased vascular permeability; 2) the intermediate phase, exudative stage, continues after 2-3 days, characterised by infiltration of fluid containing proteins from bloodstream due to a repair process towards altered vascular permeability and 3) the final phase, the proliferative stage, which occurs within 3-6 days and characterised by the formation of granulomatous tissue as a result of the release of pro-inflammatory mediators (Swingle & Shideman, 1972; Pingsusaen *et al*., 2015). In the formation of granulomatous tissue, inflammatory mediators such as IL-1α, TNF-α, IL-6 (Fujiwara & Kobayashi, 2005), eicosanoids (Kamei *et al*., 2004) and chemokines (Lukacs *et al*., 1994) are involved and also, release of lysosomal enzymes and ROS occur (Patil & Patil, 2016). The transudative phase measures the change in the wet weight of cotton pellet whiles an increase in dry weight correlates with the proliferative stage [i.e. amount of granuloma tissue formed] (Valderramas *et al*., 2008). From the study, PLE demonstrated a significant reduction of both dry and wet weights of granuloma tissue which reflected its inhibitory effects on both the transudative and proliferative phases of inflammatory response. Consequently, this was an indicative of the anti-prolifeartive effect of PLE on pro-inflammatory cytokines such as IL-1α, TNF- α, IL-6 (Fujiwara & Kobayashi, 2005), the proliferation of fibroblasts, infiltration of monocytes and neutrophils, collagen and mucopolysaccharide synthesis (More, Lande, Jagdale, Adkar, & Ambavade, 2013) which are natural components in the formation of granuloma (i.e. vascularised red mass). During the inflammatory response, the generation of ROS as result of mediator release (Patil & Patil, 2016) was significantly

suppressed by PLE which was evident in its increasing effect on CAT and SOD enzymatic activities and a significant reduction capacity on MDA levels. This is consistent with earlier literature reported by Patil & Patil (2016).

It has been reported that variation in body weight is a clinical parameter for assessing severity and progression of diseases (Syed-Uzair, Huma, Shahid, Shazia, & Shabana, 2013). From the study, a marginal gain of body weight generally in PLE-treated rats could be attributed to increase in appetite and somewhat metabolic rate and disagrees with the reported earlier suggested that adverse effect of a test agent is delineated by a decrease in body weight due to loss of appetite (Teo *et al*., 2002). However, further investigations are needed to ascertain these findings.

Jorum, Piero, & Machocho (2016) reported that assessment of haematological indicators is relevant in explaining blood-related actions of plant extracts. It has also been reported that blood indicator evaluation shows the clinical risk assessment of haematological variations (Oslon *et al*., 2000) and easily reveals alterations in the metabolic activities of the body, providing useful information on the body's response to infection, injury, stress or deprivation (Fazio *et al*., 2015). During inflammatory response, increase migration of WBC at the site of inflammation and elevated levels of WBC count serve as inflammatory biomarkers (Aryaa & Viswanathswamy, 2017). Furthermore, blood parameters such as neutrophils, lymphocytes and platelets play an essential role in inflammation and their peripheral levels are useful in the determination of disease activity (Erre *et al*., 2019). Neutrophils are also known to stimulate the production of cytokines (IL-1β, IL-6, TNFα), proteases and NO (Sampson, 2000). From the study, PLE-treated group significantly

decreased the levels of WBC, NEU and PLT and thus, inhibited the inflammatory activities of these blood cells. Also, all other haematological parameters assessed in the cotton pellet-induced granuloma formation were normalised by the extract when compared to the control group.

In cotton pellet-induced granuloma tissue formation, enlargement of the spleen occurs as a result its phagocytic nature (Mishra *et al*., 2011). PLE in this study significantly reduced the weight of the spleen relative to the enlarged spleen of the control group (with an increase in weight) and this is in consonant with literature earlier reported (Mishra *et al*., 2011). Hence, PLE has shown to exhibit inhibitory effect on the phagocytic nature of the spleen during inflammatory reactions.

CFA-induced arthritis in rats

Several studies have established the relationship between the immune response to mycobacterial infections and autoimmune disorders, particularly rheumatoid arthritis (Shoenfeld & Isenberg, 1988). Also, other studies conducted have revealed the presence of *Mycobacterium* antigens in the joints of RA patients (Erre *et al*., 2014). Additionally, it has been reported that adjuvant arthritis occurs via the interaction between the cell wall of the *Mycobacterium* and immune response (Turull & Queralt, 2000). Adjuvant arthritis caused by heat-killed *Mycobacterium tuberculosis* cell is believed to have similar biological and immunological characteristics of RA (Ramprasath *et al*., 2006). The clinical symptoms of human RA such as joint inflammation, synovitis, pain, tissue and bone erosion, and loss of joint function, are known to resemble that of CFA-induced arthritis model. This, therefore, makes CFA-induced arthritis model useful to study disease pathogenesis and to assess a

potential therapeutic agent beneficial for the treatment of RA (Kumar, Dhaliwal, Dhaliwal, Dharavath, & Chopra, 2020). CFA induction in rats produces remarkable joint inflammation and severe oedema of the hind paw (Das, Bose, & Das, 2020). Arthritis induced by the injection of CFA is a biphasic; the initial phase is acute, occurs between 0-10 days and arises due to the release of 5-HT, kinins, histamine and PGs by immune cells causing vascular exudation phenomenon, and the late phase is chronic, lasting for 11-28 days which results in the secretion of numerous pro-inflammatory mediators including IL-6, IL-1β, TNF-α, INF-γ, PGE_2, granulocyte-macrophage colony-stimulating factor, causing increased infiltration of inflammatory cells, hypertrophy, cartilage and bone erosion, synovitis, joint oedema (Foyet *et al*., 2015). The characteristic feature of RA after inflammation is the oedema of the joint (Duer-Jensen *et al*., 2011), and thereby, paw oedema and arthritis scores are used to assess anti-arthritic properties of a drug (Zhao *et al*., 2016). The key focus in the management of RA is basically to improve symptoms, decrease joint destruction, and improve quality of life (Shinde, Venkatesh, Kumar, & Shivakumar, 2014). It has been reported that an anti-arthritic agent must inhibit joint inflammation and reduce the expression of pro-inflammatory cytokines thereby preventing joint destruction (Cui *et al*., 2019). Several reports have established that medicinal plants and their products offer good help to decreasing the effects exhibited during arthritic condition and are comparable with orthodox agents particularly by mitigating the symptoms of RA (Bihani, Rojatkar, & Bodhankar, 2014; Patel & Pundarikakshudu, 2015). Hence, from the study, CFA-induced arthritis group showed severe joint oedema of the hind paw with high arthritic score. Oral treatment with PLE significantly decreased

the paw thickness and this indicates suppression of humoral response leading to reduced membrane permeability (Peng *et al*., 2020). To estimate the severity of the disease state in RA and confirm disease remission, radiographs are used (Uttra *et al*., 2019). In CFA-induced arthritis, the reduction in bone architecture and increased bone resorption (Xu, Zhou, Zhang, Sun, & Cheng, 2017) which are observed in the radiographic pattern of the hind limbs of the CFA-treated rats exhibiting joint space narrowing with soft tissue oedema is indicative of cartilage damage. From the study, treatment with PLE showed significant reduction in narrowing of the joint space with an improved radiographical pattern. These activities of PLE were mediated via inhibition of pro-inflammatory mediators such as histamine, PGs and other cytokines (Gupta *et al*., 2014) which eventually accompanied with reduction of the arthritic score in the rats.

Patil & Suryavanhsi (2007) reported that the decrease in body weight in induced arthritic rats is due to inflammation and the severity of the disease. This emphasises the direct link between weight loss and the severity of joint inflammation. Thus, change in body weight of CFA-induced arthritic rats is considered to be a key marker for assessing the effect of drug and duration of the disease during inflammation (Naik & Wala, 2014). From the study, there was a severe weight loss observed in CFA-induced arthritic rats and this could be due to intestinal malabsorption, poor protein and lipid metabolism, loss of appetite, allodynia, disease-associated distress, and hyperalgesia (Taksande, Gawande, Chopde, Umekar, & Kotagale, 2017). In this study, oral treatment with PLE significantly protected animals against loss of body weight which consequently improved body weight through increased appetite of the rats and

probably enhanced metabolism. This could be attributed to the key role of PLE in improving intestinal absorption of ^{14}C-glucose and ^{14}C-leucine nutrients, and thereby decreasing the disease severity (Choudhary, Kumar, Gupta, & Singh, 2014). This is consistent with earlier reports that have established that an anti-inflammatory compound can regain the impended absorption possibility of the intestine (Alamgeer-Uttra & Hassan, 2017). Furthermore, it has been reported that regaining body weight after treatment is a usual phenomenon that occurs following herbal medicine therapy (Patil & Suryanashi, 2007).

Haematological and biochemical differences RA can be determined using CFA-induced arthritis model (Uttra *et al*., 2019). Bihani *et al*. (2014) reported that numerous haematological and biochemical alterations considered to be vital factors in an arthritic disease condition showing a change in the immune system are seen in patients with RA. From the study, CFA-induced arthritic rats showed high significant variations in the haematological parameters such as a rise in WBC and NEU levels, and a decrease in RBC and HGB count. Saleem, Saleem, & Akhtar (2020) suggested that one of the key extra-articular indicators of RA is reduction of HGB and RBC. Furthermore, Kumar *et al*. (2016) reported that a rise in WBC is an indication of inflammation as a result of infection, and a decrease in RBC level could occur due to some conditions including RA. The reduction of RBC and HGB levels observed in CFA-induced arthritic rats could represent an anaemic condition as a result of diminished erythropoietin, bone marrow failure, and reduced plasma iron (Uttra *et al*., 2019) since anaemia is reported to be a common haematological disorder in RA patients due to loss of blood (Ali, Jabbar, & Mohammed, 2019). It has also been reported that blood inflammation is associated with patients with RA,

and indicators such as TNF-α and IL-6 levels rises remarkably in synovial tissues causing joint destruction by inducing increased infiltration of neutrophils and osteoclast maturation (Smolen, Aletaha, & McInnes, 2016). Additionally, neutrophils, a haematological parameter, are known to play a significant role in the pathogenesis of RA and their variations provide a surrogate measure of the disease activity. They are said to be involved in the activation of antigen-preventing cells, generation of pro-oxidative mediators and lytic enzymes in the arthritic joint (Erre *et al*., 2019). In this study, PLE significantly reverted all increased haematological indicators such as WBC and NEU to near normal, and elevated RBC and HGB levels thereby preventing the development of anaemia in the rats. The restoration of the haematological parameters in PLE-treated rats could be attributed to the suppression of leukocyte migration into inflamed site and the inhibition of pro-inflammatory mediators implicated in joint inflammation (Rani *et al*., 2006). These effects exhibited by PLE is in line with other reports establishing the fact that herbal medications used to manage arthritis and inflammation exhibit gradual changes in the haematological indicators after treatment (Tatiya, Saluja, Kalaskar, Surana, & Patil, 2017).

Histopathology gives a remarkable morphological difference as practicable and obvious pathognomonic symptom of RA (Soren, 1980). In RA, cartilage and bone damage leads to a decrease in joint space causing bone fusion (Lad & Bhatnagar, 2016). From the study, histopathological examination of CFA-induced rats exhibited high synovial hyperplasia with increased infiltration of inflammatory cells in the synovium, intense pannus formation, and severe tissue destruction leading to cartilage and bone damage. In this study, oral administration of PLE significantly showed decreased synovial hyperplasia

of the articular tissue, and infiltration of inflammatory cells to injury site with no or little pannus formation thereby improving the arthritic condition and eventually providing protection for the epithelial cells of the cartilage. Furthermore, PLE could be said to provide lubrication to reduce cartilage and bone degradation serving as an additional benefit to delay the progression of the disease which would lead to an improve quality of life. The observable anti-arthritic effects of PLE could be linked to its inhibitory actions on pro-inflammatory cytokines in CFA-induced arthritic rats (Singh, Kumar, Jain, & Gupta, 2015) and oxidative stress (Das *et al*., 2020). Hence, the immunomodulatory effects of PLE against CFA-induced arthritis is indicative of its anti-arthritic property and this is in consonant with previous literature that suggested a drug's capacity to reduce synovitis, inflammation, and protect a joint is essential in the management of RA (Atzeni & Sarzi-Puttini, 2007).

It has been established that there is a clear relationship between ocular complications and RA (Murray & Rauz, 2016). Gutowski, Wilson, Van Gelder, & Pepple (2017) reported that *Mycobacterium tuberculosis* has been used to induce uveitis in animal model to assess drug delivery and it is reproducible in part, in managing human disease. Usually, the ocular manifestation seen in patients with RA is dry eye (Murray & Ruaz, 2016) which is a symptom of uveitis (Qin *et al*., 2014). Uveitis is an ocular inflammatory disorder that affects the iris, ciliary body, and/ or the choroid (Castro *et al*., 2020), sometimes as a result of atypical immune responses against certain specific auto-antigens of ocular tissues (Ayyildiz, Durukan, Ozgurtas, & Gunal, 2015). It has also been reported that pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and NF-κB are implicated in ocular inflammation (Castro *et al*., 2020). From the study,

ophthalmological evaluation of the eyes of CFA-induced arthritic rats showed severe uveitis characterised by hyperemia, flare, and cells in the anterior chamber (AC), hypopyon in the AC, and high miosis which could lead to loss of vision (Murray & Rauz, 2016). The principle based on uveitis therapy focuses on regulating intra-ocular inflammation, preventing visual difficulties and attenuating patients' discomfort (Castro *et al*., 2020). Therefore, in this study, treatment with PLE significantly showed improvement of uveitis with accompanied reduction of hyperemia, flare, and cells of the AC, and massive blockade of miosis in the eyes suggestive of its potential therapeutic effect and additionally, providing ocular safety and ensuring patients' visual comfort. This is consistent with others studies conducted to establish the ability of herbal products to ameliorate ocular inflammation (Kim, Han, Han, & Chung, 2018).

Nigrovic & Lee (2005) reported that chronically inflamed tissue such as the synovium of RA patient is characterised by increased proliferation of mast cells. Together with proliferative number of mast cells, high levels of mast cells mediators are also found in the synovial fluid of inflamed joint (Nigrovic & Lee, 2005). As part of systemic autoimmune diseases, RA appears to exhibit considerable action for mast cell. Several cells of the innate immune system are implicated in inflammatory responses including fibroblast-like synovial cells, dendrite cells, and macrophages. Also, T- and B-cells play a vital role in generating the inflammatory response. Hence mast cells could be implicated in this regulatory process (Frenzel & Hermine, 2013). Mast cell, upon activation, releases numerous pro-inflammatory mediators including histamine, serotonin, proteases, PGs, cytokines such as TNF-α, IL-1, IL-6, IL-16, IFN-γ etc. chemokines, PAF, kinins, leukotrienes, reactive oxygen species (Theoharides *et*

al., 2012) and vascular growth factors (Grutzkau *et al*., 1998) which are produced *de novo* and secreted several hours after activation. These mediators are critical for the pathogenesis of inflammatory diseases and subsequently cause tissue damage (Theoharides *et al*., 2012). From the study, CFA-induced arthritic rats showed elevated number of mast cells at site of tissue injury and consequently, attained higher mean number of mast cell count. Nevertheless, treatment with PLE significantly reduced proliferation of mast cells to the site of the inflamed joint and therefore, attained a lower mean number of mast cell count. This implies that PLE exhibited significant inhibitory actions on the secretion of pro-inflammatory mediators implicated in arthritic inflammation of the joint, and this further emphasises its anti-arthritic potential which is in line with similar findings that reported the ability of the plant species, *Cordia verbenacea*, to inhibit mast cell proliferation (Costa de Oliveira *et al*., 2011).

Possible mechanisms of action of PLE

Mast cells have high affinity for IgE receptor, FcεRI, which is found on their surface and can be activated by antigen-regulated aggregation of IgE bound to the receptor. Usually, antigen results in degranulation of mast cells causing the release of a variety of inflammatory mediators including cytokines/ chemokines and biogenic amines which are implicated in inflammatory responses (Choi *et al*., 2020). The activation of inflammatory pathways triggers the release of pro-inflammatory mediators such as COX-2, TNF-α, IL-1, COX-2, NF-κB, NO, PGE_2 as well as ROS (Balkwill & Mantovani, 2001). These mediators can lead to inflammatory responses that can cause cellular damage and promote pathophysiology of numerous inflammatory and neurodegenerative disorders (Javed *et al*., 2019). Thus, mechanisms to elucidate

these abnormal inflammatory responses could help to prevent the onset of these disease conditions. Therefore, to elucidate the possible mechanisms of action of PLE, phlogistic agent-induced paw oedema in rats using histamine, and serum cytokine gene levels of TNF-α, IL-1β, COX-2 and NF-κB were determined.

Histamine is a potent vasoactive agent that regulates a series of physiological activities by playing a major role in the body's inflammatory responses (Lieberman, 2011) and other several pathomechanisms of inflammatory diseases (Patel & Mohiuddin, 2021). Histamine exerts its biological function through the activation of either H_1, H_2, H_3 or H_4 receptors. The activation of H_1 receptor causes local vasodilation of the capillaries and venules leading to increase in blood flow to the inflamed site (Benly, 2015). H_3 receptor is reported to modulate pain via series of mechanisms (Hough & Rice, 2011). The H_4 receptor, located on cells of haemetopoietic origin, causes vasodilation and increases vascular permeability leading to an increase in blood flow. This results in fluid, plasma and protein extravasation which causes interstitial oedema and consequently, leads to redness, heat, and swelling (Branco, Yoshikawa, Pietrobon, & Sato, 2018). From the study, pro-inflammatory mediator like histamine which played diverse roles in acute inflammation as described earlier in carrageenan-induced paw oedema, was significantly inhibited by PLE via downregulation of their production, release as well as effects and this is consistent with earlier study reported on the plant by Obese *et al*. (2021).

Moreover, it has been reported that cytokines are the major agents implicated in all stages of inflammatory response (Hackett, Holloway, Holgate, & Warner, 2008) and mostly, are proliferated during initial stage of

inflammation (Zuo, Xia, Li, & Chen, 2014). In CFA-induced arthritis, T-cell is triggered which in turn stimulates macrophages and monocytes continuously, thus, increases lysosomal enzymes, and synthesis of pro-inflammatory cytokines that cause irreversible joint damage, synovitis, apoptosis in arthritic joint and bone erosion (Mo *et al*., 2013). Also, the expression of IL-1β, PGE_2 and IL-6 genes (Obiri, Osafo, Ayande, & Antwi, 2014), synovial hyperplasia, synthesis of matrix-degrading enzymes, stimulation of collagenase, stromelysin, and osteoclast differentiation that promote the progression of arthritic erosion are increased due to the overproduction of TNF-α (Cheng *et al*., 2015).

According to Barksby, Lea, Preshaw, & Taylor (2007), IL-1β enhances osteoclast stimulation and matrix metalloproteinase production which cause bone damage. Shabbir *et al*. (2014) reported that NF-κB, which is a transcription factor, plays a key role in osteoclast stimulation and differentiation resulting in a defective cell death, Th1 response, loss of bone and synovial proliferation. Considering PGE_2, synthesised through the metabolism of arachidonic acid via COX-2, is critical in rheumatoid arthritis since higher levels cause vasodilatation, oedema, pain, extravasation of fluid and plasma, redness, cartilage, and bone destruction (Cheng *et al*., 2015; Zhao *et al*., 2016). COX-2 is known to be an inducible enzyme which causes inflammation and tissues damage due to its overproduction (Jacob & Manju, 2020). Therefore, any therapeutic agent that specifically inhibits the effects of IL-1β, TNF-α and IL-6 (Shanahan, Moreland, & Carter, 2003), COX-2 (Saleem *et al*., 2020), and NF-κB (Shabbir *et al*., 2014) has high therapeutic capacity and is feasible for anti-arthritic therapy in human diseases including RA. Hence in this study, oral

administration of PLE in arthritic rats significantly downregulated gene expression of IL-1β, TNF-α, NF-κB and further inhibited the enzymatic action of COX-2 for the excess production of PGE_2 which is consistent with previous studies reported on the plant by Obese *et al.* (2021). The inhibitory effects of PLE on pro-inflammatory cytokines and arachidonic acid metabolism strongly demonstrate its anti-arthritic potential and, accordingly indicate that it could be an alternative source for managing rheumatoid arthritis.

Phytochemical constituents of PLE

Plants with medicinal activities contain a high quantity of phytochemicals such as phenolic compounds (Butt, Nazir, Sultan, & Schroën, 2008). Usually, the biological activities of medicinal plants are attributed to their phytochemicals (Issa, Volate, & Wargovich, 2006). Phytochemical metabolites of plants are reported to exhibit anti-inflammatory effects through series of mechanisms of action including regulation of inflammatory cells (neutrophils, mast cells, lymphocytes, macrophages), anti-oxidant, and free radical scavenging effects. Also, these compounds regulate pro-inflammatory enzymes including nitric oxide synthase (NOS), cyclooxygenase (COX), phospholipase A_2 (PLA_2), and lipoxygenase (LOX); and modulate gene expression and other pro-inflammatory compounds (Bellik *et al*., 2012). They mediate inflammation via kinases including MAP-K (mitogen-activated protein kinase) and PK-C (protein kinase C) thereby altering the DNA-binding ability of NF-κB (Zhu, Du, & Xu, 2018). Phytochemical constituents have been reported to modulate the immune response, and different transcription factors, or reduce cytokine secretion (Somani *et al*., 2015). Phytochemical analysis of PLE showed the presence of tannins, saponins, steroids, alkaloids, glycosides,

triterpenoids and flavonoids. Even though individual phytochemical constituents have not been isolated in PLE and tested pharmacologically, it is therefore not surprising that the anti-inflammatory activities exhibited by PLE could be attributed to the presence of one or more secondary metabolites, and this is consistent with literature as reported by Saeed, Muhammad, & Khan (2010).

Toxicity profile of PLE

The toxicity or safety profile of a compound is critical in assessing its risk to humans (Kamsu *et al*., 2021). To increase human safety, more scientific data on medicinal plants' toxicological status and their extracts are relevant to promote their use in the development of pharmaceuticals as well as medicinal agents (Yuet-Ping *et al*., 2013). This is necessary because the use of traditional medicine for primary health care has increased and about three-fourth of herbal drugs used in the world are derived from medicinal plants (Verma & Singh, 2008). Consequently, screening of potential toxicity profile of medicinal plants using animal models is critical for the validation of their use in regular therapy (El-Kabbaoui *et al*., 2017) since the effects exhibited by the animals due to the exposure to chemical agents could be correlated to humans (Kamsu *et al*., 2021). For this reason, toxicity profile of PLE was evaluated to ascertain its safety using both acute and sub-acute toxicity studies in rats.

In the acute toxicity study, all the animals survived after oral administration of PLE at all doses throughout the study period. Rats treated with PLE (100-5000 mg kg^{-1}, *p.o*, daily) showed no behavioural, physiological, clinical changes or any adverse effects including death. However, mild transient signs such as defaecation and urination were observed in rats at doses of 3000

and 5000 mg kg^{-1}, but these were reversed in a period of 24 h post PLE treatment. In this study, the data attained showed that the LD_{50} of PLE was projected to be above 5000 mg kg^{-1}. It can therefore be established that PLE is relatively safe for acute folkloric use which conforms to the scale based on the Hodge and Steiner toxicity scale (OECD, 2000).

Generally, the observation of adverse effects particularly mortality in acute toxicity study with plant products is inadequate since plant products are not pure as compared to the synthetic agents that appear to exhibit toxicity in acute assays (Feres *et al*., 2006). According to Innalegwu, Amuda, Gara, & Muhammad (2020), advanced toxicity evaluation such as sub-acute or sub-chronic studies of plant extracts in animal models is useful to predict the possible adverse effects of these plant products. In addition, conforming to the OECD guidelines for sub-acute oral toxicity studies, toxic signs of a test sample can be assessed by using different parameters such as food consumption, weight and histopathological changes of organs, body weight, biochemical profile, and haematological screenings that give the understanding of the no observed adverse effect level (NOAEL) and low observed adverse effect level [LOAEL] (OECD, 1998a). In line with this, the repeated doses of PLE in sub-acute toxicity study for 28 days was performed and the results showed no noticeable toxic effects, death or clinical changes observed after treatment. Nevertheless, changes in body weight and relative weight of organs are useful clinical tools for the evaluation of primary signs of toxicity of a substance (Berenguer *et al*., 2013). Hence, from the study, there was a significant increase of body weight of rats after 28 days of PLE administration at 300 mg kg^{-1} dose level. This marginal gain in body weight could be attributed to the normal growth rate with

age of rats or improved feeding habits of the rats which disagrees with a literature earlier report suggesting that a reduction in body weight is indicative of toxicity after exposure to a substance (Agatemor & Nwodo, 2021) however, the data obtained showed marginal weight increase of PLE-treated rats which is consistent with literature previously reported by Kamsu *et al*. (2021). Hence, this further confirmed the good health condition of PLE-treated rats (Heywood, 1987). In 1995, Auletta reported that liver, spleen, heart, kidney and lungs are the key organs targeted by any toxic agent metabolically. According to Jeong *et al*. (2019), organ weight may be a useful index of treatment-related variations with or without any correlating histological assessment in continuous toxicity analysis and can present precise evidence for pathological alterations indicative of a drug's effect on organs This can also provide clinical information on organ atrophic or hypertrophic conditions (Miaffo, Wansi, Ntchapda, & Kamanyi, 2020) and a decrease in organ weight indicates necrosis of the target organ (Teo *et al*., 2002). However, macroscopic examinations of the liver, spleen, stomach, heart, and kidney during necropsy isolated from the PLE-treated rats in this study showed no visible changes in size, shape, texture and colour. In addition, the relative weights of these organs in all treatment groups after PLE administration showed no significant variations in absolute or relative organ weights of the rats and also, no lesions or noticeable signs of toxicity was noted in the various isolated organs when compared with control group. Thus, this demonstrates that PLE showed no treatment-related toxic effects on the organs, providing the evidence for its non-toxic nature and relative safety when consumed sub-acutely.

According to Nigatu, Afework, Urga, Ergete, & Makonnen (2017), changes in hematological and biochemical biomarkers are used mostly as parameters of toxicities in acute and chronic toxicological studies. Furthermore, Arika, Nyamai, Musila, Ngugi, & Njagi (2016) reported that haematological assessment is an essential and sensitive indicator considered to be critical in toxicity investigations during extrapolations of experimental data to clinical analysis. It has also been reported that consumption of harmful agent or plants can cause variations in the haematological biomarkers (Zahmati & Shokooh-Saljooghi, 2016) and drugs associated with adverse effects could result in organ dysfunction as well as vital differences in haematological profiles (Arome & Chinedu, 2013). Moreover, assessment of blood indices such as RBC, HCT, and HGB is crucial in the diagnosis of anaemia (Miaffo *et al*., 2020). In this study, oral treatment with PLE in sub-acute toxicity study did not cause any significant alterations in the haematological parameters when compared to the control group. This shows that the extract may possess no toxic substance that can cause blood abnormalities. Thus, the normal haematological profile of PLE justifies its non-toxic nature.

According to Loha, Mulu, Abay, Ergete, & Geleta (2019), biochemical parameters play important roles as indicators in toxicological studies since clinical signs and symptoms are exhibited because of their response to toxicants. To assess the toxic effects of plants or drugs, primary hepatic and renal function analyses are critical (Loha *et al*., 2019) with one being useful in the metabolism of ingestion and the other for excretion of metabolic waste, respectively (Dramane *et al*., 2019; Sureshkumar, Begum, Johannah, Maliakel, & Krishnakumar, 2018) and both are relevant for an organism survival (Al-Afifi,

Alabsi, Bakri, & Ramanathan, 2018). According to Miaffo *et al*. (2020), serum biochemical parameters such as blood urea nitrogen, uric acid, blood creatinine, and renal clearance are major components for diagnosis of renal function; and high elevated levels of these indicators in blood are accompanied by low renal function and increased kidney failure. It has also been reported that creatinine and urea are widely used biomarkers for assessing renal injury function since creatinine, a residual product of creatine, is released into body fluids after being produced endogenously at a constant rate whiles urea (about 90 % eliminated by kidneys) is the main metabolite of protein degradation (Sa′ *et al*., 2015). From the study, oral administration of PLE in repeated doses for 28 days showed no significant differences statistically in the levels of creatinine, serum urea, and blood urea nitrogen when compared to the control group and this reveals that PLE has no toxic effect on the kidney and its function. It is therefore not surprising that this finding was strongly supported by the microscopic examination of the kidney which showed normal architecture with no alterations or lesions of the histological structure. It can also be said that the integrity of the kidney was maintained and thus, PLE is non-nephrotoxic.

Furthermore, the function of the liver can be evaluated by assessing the levels of serum biomarkers such as bilirubin, albumin, and total protein in which a reduction in these parameters is associated with reduced synthetic function, characteristic of liver disease or damage (Ugwah-Oguejiofor *et al*., 2019). According to Adeoye & Oyedapo (2004), cellular integrity can be described with liver enzymes (i.e. AST and ALP) whereas liver function can be explained using total protein and albumin. Serum levels of ALP and AST are known to increase as a result of the destruction of hepatocytes (Yang, Schnackenberg,

Shi, & Salminen, 2014) and however, due to the special abundance nature of ALP levels in the cytoplasm of hepatocytes, ALP is mostly used as a key biomarker to assess cholestasis or biliary interference (El-Hilaly, Israili, & Lyoussi 2004) and suspected liver cell damage (Yang *et al*., 2014). In 2007, Thapa & Walia suggested that bilirubin, a by-product of haemaglobin metabolism, causes clinical disorders such as primary biliary cirrhosis and liver cholestasis when its serum levels are high . Also, abnormal level of total proteins is known to cause liver infections or chronic inflammation (Tatefuji, Yanagihara, Fukushima, & Hashimoto, 2014). In this study, the serum levels of total protein, albumin, AST, ALP, and ALT in PLE-treated rats showed no significant changes or anomalies relative to the control which strongly suggest that administration of PLE in sub-acute toxicity study exhibited no alterations in the liver cells or its metabolic activities. In addition, other serum biomarkers such as total bilirubin, indirect and direct bilirubin, and globulin did not reveal any significant alterations in their levels relative to the control. This was further supported strongly by the findings seen in the histopathological analysis of liver which revealed no histological alterations or abnormalities indicating no liver injury or hepatoxicity and thus, maintained liver integrity.

Histopathological evaluation of vital organs such as stomach, liver, heart, and kidney is considered to be an essential test primarily done to assess the safety profile of an agent (Sindete *et al*., 2021). Furthermore, histopathological assessment following single or repeated doses of plant extract gives true evidence of organ toxicity and also the correlation with observed alterations in biochemical parameters which is much needed can be provided (Husain, Latief, & Ahmad, 2018; Mukherjee & Ahmed, 2018). In this study,

histopathological assessment of the heart, liver, kidney, spleen and stomach were examined after orally administered PLE for 28 day of observation period. According to Babaei, Arshami, Haghparast, & Danesh-Mesgaran (2014), the spleen is among the key sites where most primary immune responses for activation of B lymphocyte and the synthesis of antibodies are initiated. From the study, microscopic examination of the spleen in PLE-treated rats showed no abnormalities or histopathological alterations when compared with control. These results indicate that PLE did not cause any treatment-related toxic effects on the spleen and thus, has no toxic effects on the spleen. In addition, the microscopic evaluation of the heart and stomach equally showed an indistinguishable cellular architecture or histoarchitecture between the PLE-treated rats and control group indicative its non-adverse effect on these vital organs. Consequently, putting all these observations together, PLE can be justified to be non-toxic in nature and this is consistent with a literature previously reported on a plant extract which confirmed its non-adverse effects on vital organ such as the kidney, liver, heart, and spleen following histological analysis (Morales, Paredes, Olivares, & Bravo, 2014).

Chapter Summary

This chapter focused on all the results of the experimental inflammatory models in both acute and chronic inflammation that established the anti-inflammatory activities of *Persicaria lanigera* leaf extract (PLE) as well as its anti-oxidant effects. Moreover, the possible mechanisms of action of *Persicaria lanigera* was established. Phytochemical screening of PLE revealed the presence of alkaloids, tannins, saponins, glycosides, terpenoids, and flavonoids.

The oral safety of the plant was also comfirmed and LD_{50} was estimated to be above 5000 mg kg^{-1}.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

Summary

The anti-inflammatory effects of hydroalcohol (70 % $^{w}/_{v}$) leaf extract of *Persicaria lanigera* (PLE) were established through its inhibitory effect on acute inflammation by a significant suppression of the mean maximal oedema to 59.10±4.94 %, 56.08±3.65 % and 48.62±3.27 % at 100, 300 and 600 mg kg^{-1} when administered prophylactically, and also decreased the inflamed swelling to 61.97±7.75 %, 61.42±7.59 % and 65.24±8.07 % at the same doses relative to the control when administered therapeutically in carrageenan-induced paw oedema respectively. This inhibitory effect of PLE was repeated in zymosan-induced acute knee joint arthritic in which it significantly reduced the knee joint thickness to 32.07±2.98 % and 24.33±8.58 % at 300 and 600 mg kg^{-1} respectively. In the chronic inflammatory studies, PLE showed a significant inhibition of the DAI to 58.00±5.39, 50.00±7.07 and 43.00±5.38 at 100, 300 and 600 mg kg^{-1} respectively. In addition, PLE exhibited a significant reduction of both the transudative and proliferative granuloma formation phases by 20.65 %, 22.61 % and 30.14 % at 100, 300 and 600 mg kg^{-1} respectively in the cotton pellet-induced granuloma tissue formation. Similar inhibitory effect of PLE was again revealed in its anti-arthritic effect on chronic arthritic inflammatory condition in which the joint swelling was reduced significantly to 126.58±10.91 %, 113.82±11.46 % and 106.45±34.85 % at same doses when administered prophylactically. Similarly, when PLE was given therapeutically, the inflamed arthritic paw oedema was suppressed significantly to 568.50±91.18 %,

545.50±71.88 % and 541.83±70.21 % at 100, 300 and 600 mg kg^{-1} dose-dependently in CFA-induced arthritis in rats respectively.

The toxicity profile of PLE revealed that the extract was relatively non-toxic and safe to use in traditional medicine therapy since there was no behavioural, physiological or clinical alterations including death in the rats treated with the extract. Sub-acute toxicity study showed that PLE exhibited no systemic treatment-related adverse effects in the haematological, biochemical parameters and histoarthitectural features of the vital organs after oral repeated treatment with PLE. This further emphasised the safety and non-toxic nature of PLE in the management of inflammatory disorders.

Conclusion

It can be concluded that hydroalcohol (70 % $^{w}/_{v}$) leaf extract of *Persicaria lanigera* (PLE) possesses anti-inflammatory effects and this has provided the pharmacological evidence to support its folkloric use in the management of inflammatory disorders. The establishment of the anti-inflammatory effect of PLE observed from this study to be mediated by:

I. The inhibitory effects of PLE on acute and chronic inflammation.
II. The inhibitory effect of PLE on histaminic pathway through the inhibition of histamine-induced paw oedema.
III. The inhibitory effects of PLE on gene expression of pro-inflammatory cytokines during inflammatory response.
IV. The possession of anti-oxidant activities.
V. The presence of phytochemical constituents in PLE.

Furthermore, the study strongly established that PLE has no significant oral toxicity in rats. This was evident in the results recorded in histopathological,

haematological and serum biochemical analyses performed on PLE-treated rats in sub-acute toxicity study. Thus, PLE is confirmed to be non-toxic and relatively safe for folkloric use.

Recommendation

The study cannot be conclusive since further works need to be carried out before extrapolating to humans in order to improve on the acquired knowledge on the anti-inflammatory activities of *Persicaria lanigera* leaf extract (PLE).

I. Further investigations should be conducted to isolate and identify the active secondary metabolites or compounds responsible for its anti-inflammatory effects.

II. Pharmaceutical and other chemical companies can formulate, prepare and develop new drugs using the isolated active compounds.

III. The possible effect on down and upregulation of certain inflammatory receptors during mast cell activation.

IV. The possible effects on signaling pathways of inflammation such as Janus kinase/ signal transducer and activator of transcription (JAK/ STAT), mitogen-activated protein kinase (MAPK), p38 and P13K signaling pathways could be evaluated.

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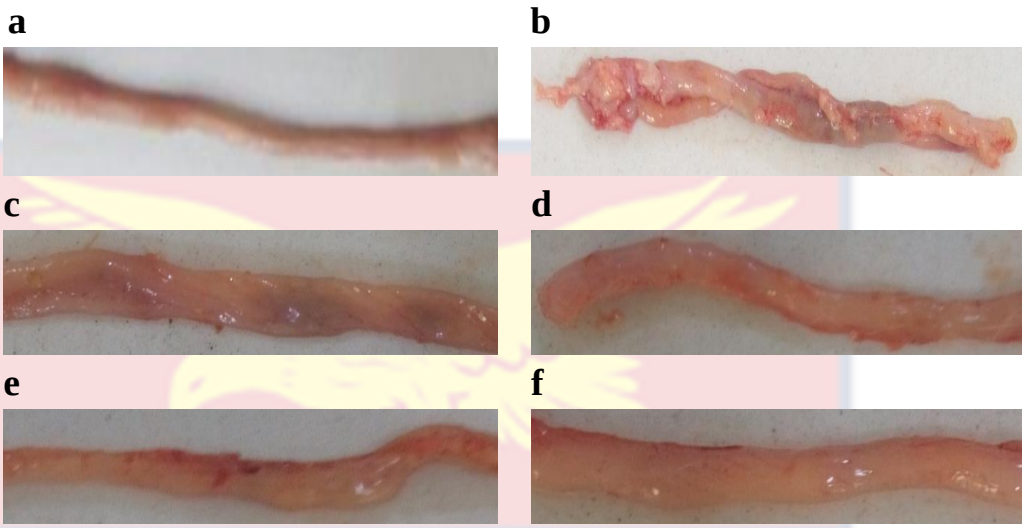
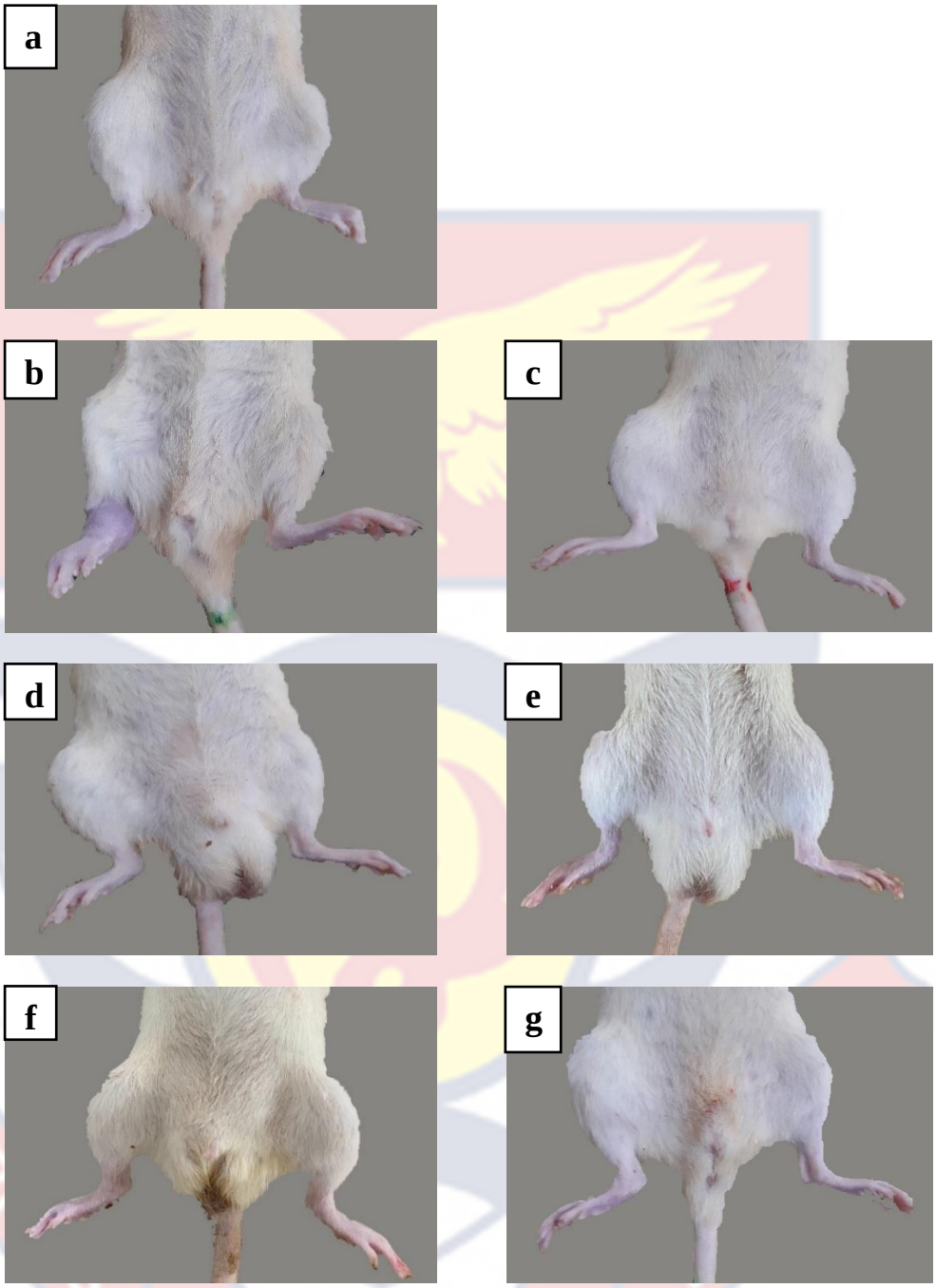
APPENDICES

Appendix A



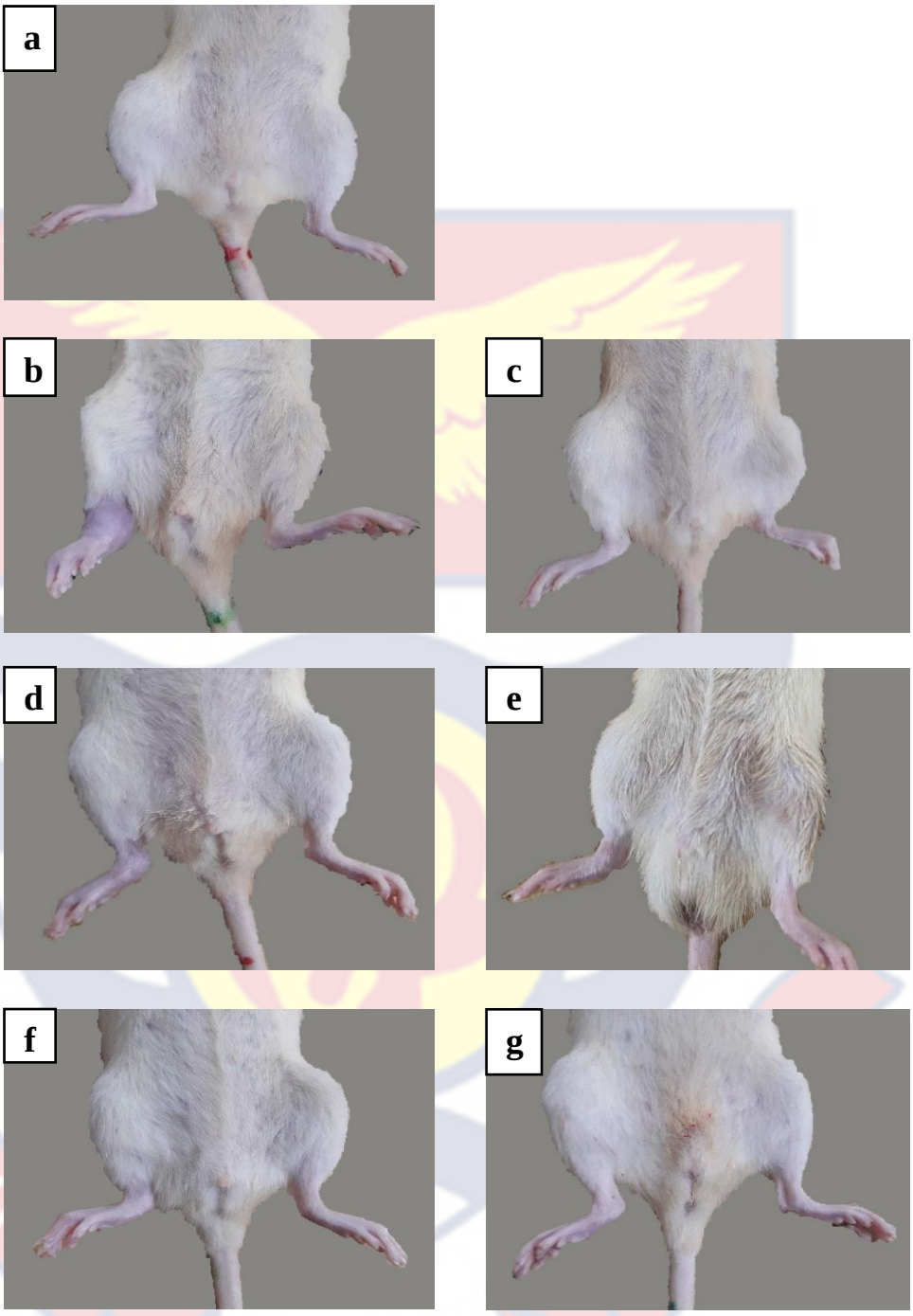
Photographs of colons in acetic acid-induced ulcerative colitis in rats. Colonic damage was induced by intrarectal injection of 1 ml of 4 % acetic acid into rats (n=5) on day 4. Rats received either sulfasalazine (500 mg kg^{-1}, *p.o.*) or PLE (100-600 mg kg^{-1}, *p.o.*, daily) from day 1 till 8^{th} day. Colons were extirpated and assessed macroscopically (for disease activity index). Representative slides of colon **a**= non-acetic acid group; **b**= acetic acid-treated group; **c**= AA + sulfasalazine (500 mg kg^{-1}); **d-f**= AA + PLE (100-600 mg kg^{-1}) respectively.

Appendix B



Photographs showing the prophylactic effects of PLE on CFA-induced arthritis in rats. Sprague-Dawley rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Photographs were taken using digital camera. The intensity of inflammation was scored on the scale of 0-4. **a**= IFA-treated, **b**= CFA-treated, **c**= dexamethasone (3 mg kg^{-1}), **d**= aspirin (100 mg kg^{-1}), **e-f**= PLE (100-600 mg kg^{-1}) respectively.

Appendix C



Photographs showing the therapeutic effects of PLE on CFA-induced arthritis in rats. Sprague-Dawley rats (150-200 g, n=5) were inoculated with 100 µl of either CFA or IFA intraplantarly into the right limb and observed for 28 days. Photographs were taken using digital camera. The intensity of inflammation was scored on the scale of 0-4. **a**= IFA-treated, **b**= CFA-treated, **c**= dexamethasone (3 mg kg^{-1}), **d**= aspirin (100 mg kg^{-1}), **e-f**= PLE (100-600 mg kg^{-1}) respectively.

Appendix D

A: Carrageenan solution preparation

Carrageenan (1 % $^{w}/_{v}$) solution was prepared by suspending 100 mg carrageenan powder in 10 ml of normal saline (0.9 % $^{w}/_{v}$ NaCl solution). The mixture was left for 3 h before use.

B: Preparation of CFA

50 mg of heat-killed *Mycobacterium tuberculosis* [strains C, DT and PN (mixed) obtained from Santa Cruz Biotechnology Inc., Dallas, Texas, USA] was suspended in liquid paraffin to carefully make 20 ml of 5 mg ml^{-1} suspension.

C: Measurement of paw thickness using venier caliper

Digital venier calipers work using a digital encoder linked to a small gear that turns a rack gear running along the length of the caliper. The digital encoder has many alternating opaque and transparent bands radially fixed around a disk which is turned using the small gear. Two phototransistor pairs regulate the passage of the bands. By monitoring the pattern of transmission and which phototransistor sees the first change, a small digital circuit measures the number of bands that have been to point zero. The gear ratios and the bands on the disk are fixed and the circuit is programmed to move one to another distance on the caliper. The distance is converted to a suitable millimeter value which is seen on the liquid-crystal display (LCD).

CALCULATION: % change in paw thickness = $\left(\frac{(P_t - P_o)}{P_o}\right) \times 100$

Where;

Po is the paw thickness before carrageenan injection (i.e. time zero).

Pt is the paw thickness (at various time intervals) post carrageenan injection.

D: Drug preparation and administration

The extract was prepared by reconstituting as an emulsion using Tween-80 and made to the required volume using normal saline. All other drugs were prepared by suspending in saline. Standard drugs and extract were prepared in volumes such that not more than 1 ml was orally administered.

E: Preparation of buffer for enzyme assay

0.1 M Sodium Phosphate buffer (pH 6.0)

0.1 M Monosodium phosphate 88.0 ml

0.1 M Disodium phosphate 12.0 ml

50 mM Potassium Phosphate buffer (pH 7.0)

50 mM Potassium Phosphate monobasic 39.0 ml

50 mM Potassium Phosphate dibasic 61.0 ml

0.1 M Carbonate bicarbonate buffer (pH 10.2)

0.1 M Sodium carbonate 70.0 ml

0.1 M Sodium bicarbonate 30.0 ml