ANTI-INFLAMMATORY AND ANTI-ANAPHYLACTIC EFFECTS OF *TRICHILIA MONADELPHA* (THONN.) J. J. DE WILDE. EX OLIV.*PP* (*MELIACEAE*) EXTRACTS IN RODENTS

A THESIS PRESENTED IN FULFILMENT OF

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By

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MAY, 2013

DECLARATION

I by this declare that I am the sole author of this thesis. The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST and the Department of Pathology, Komfo Anokye Teaching Hospital. This work has not been presented for any other degree. This is a true copy of the thesis, including any needed final revisions, as accepted by my examiners.

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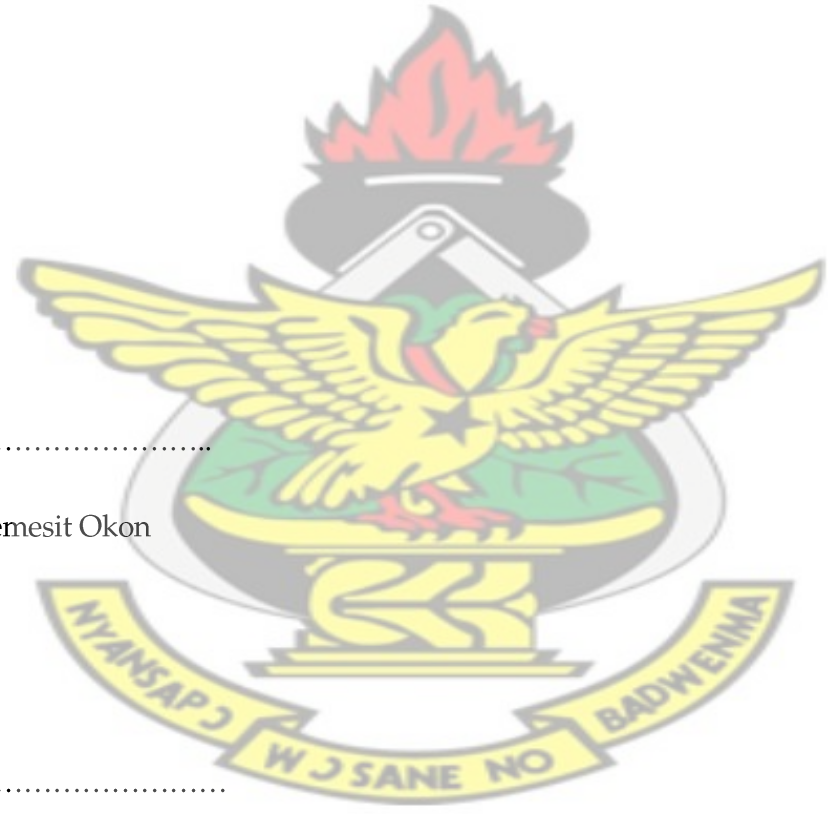

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Prof. Eric Woode

ABSTRACT

Trichilia monadelpha (Meliaceae), in Ghana and other parts of Africa, is used locally to manage various inflammatory and pain conditions. Three extracts (petroleum ether extract, PEE, ethyl acetate extract, EthE, and ethanol extract, EAE) obtained from this plant and evaluated pharmacologically. Sprague Dawley rats and C57BL/6 mice were experimental animals used for throughout the study.

Effects of stem bark extracts of Trichilia monadelpha on acute inflammation was performed using rodent models of acute inflammation, pyrexia and mediators (histamine, serotonin, bradykinin and PGE_2)-induced inflammation. PEE, EthE and EAE (10 – 100 $mgkg^{-1}$) significantly ($F_{3,16}$=12.20 P<0.001; $F_{3,16}$=8.98 P<0.01; $F_{3,16}$=13.34 P<0.001, respectively), reduced paw oedema induced by carrageenan. The extracts also significantly ameliorated inflammation induced by specific mediators (histamine, serotonin, PGE_2 and bradykinin) produced in the 2-phase reaction of carrageenan-induced inflammation. The extracts also reduced pyrexia and CRP levels.

The extracts effects on bone morphology, histology, cytokines release, haematology and oxidative stress biomarkers were also evaluated. It was observed PEE, EthE and EAE significantly (P<0.0001; P<0.01; P<0.01, respectively) and dose-dependently reduced the polyarthritic phase of adjuvant-induced arthritis. PEE and EAE also significantly (P<0.0001; P<0.01, respectively) minimized oedema spread from acute phase (day 0 to 10) to polyarthritic phase (day 10 to 28). EthE improved body weight. PEE, EthE and EAE, significantly (P<0.01; P<0.05; P<0.05, respectively) improved arthritic score, reducing erythema, swelling and joint rigidity. The extracts also significantly (P<0.001; P<0.01; P<0.05, respectively) reduced hyperplasia, pannus formation, exudation of inflammatory cells, observed as accumulation of abundant monomorphonuclear and polymorphonuclear cells into the synovial spaces. PEE, EthE and EAE significantly (P<0.0001; P<0.01; P<0.01, respectively) reduced elevated WBCs, neutrophils, lymphocytes, and reduced RBC and haemoglobin (associated with arthritis) to normal. Treatments significantly and dose-dependently reduced high levels of TNF-α and IL-6, as well as MDA and MPO associated with arthritis. SOD activity increased significantly with treatments.

Anti-anaphylactic effect of Trichilia monadelpha was evaluated, using models of anaphylaxis; Compound 48/80-induced systemic anaphylaxis; Passive cutaneous anaphylaxis, PCA; Evans Blue method of vascular permeability evaluation; Histopathological analysis; and assessment of cytokines levels (TNF-α and IL-6) using ELISA. PEE significantly (P<0.0001, P<0.05) reduced compound

48/80-induced mortality dose-dependently. The extracts (PEE and EAE) delayed or totally inhibited tremors induced by compound 48/80 in systemic anaphylaxis. PEE and EAE also significantly ($F_{3, 8}$= 159.9 P< 0.0001; $F_{3, 8}$= 194.4, P< 0.0001, respectively) inhibited PCA reactions and compound 48/80-induced extravasation. The extracts also significantly ($F_{4, 5}$ = 65.14, P<0.001; $F_{4, 5}$ = 15.70, P<0.0001, respectively) stabilised mast cell, preventing mast cell degranulation and significantly (TNF-α: $F_{3, 8}$ =25.17 P<0.001; $F_{3, 8}$ =13.21 P <0.01; IL-6: $F_{3, 8}$ =26.59 P<0.001; $F_{3, 8}$ =32.74 P <0.0001, respectively) inhibited TNF-α and IL-6 secretion.

Phytochemical screening and evaluation of antioxidant properties in vitro (phenol test, reducing power test and DPPH-scavenging free radical assay) of stem bark extracts of Trichilia monadelpha was carried out. Alkaloids, terpenoids, phytosterols, reducing sugars and coumarins were present in PEE. EAE showed the presence of tannins, alkaloids, terpenoids, phytosterols, reducing sugars, flavonoids, cardiac glycosides, anthraquinones and saponins while EthE contained tannins, alkaloids, reducing sugars, cardiac glycosides, anthraquinones, terpenoids and phytosterols. Total phenol contents were estimated to be 7.51±0.87 mg tannic acid equivalent/g of PEE, 34.14±0.78 mg tannic acid equivalent/g of EthE and 119.30±3.20 mg tannic acid equivalent/g of EAE. The extracts showed a concentration-dependent reduction of Fe^{3+} to Fe^{2+} in the reducing power test as well as concentration-dependent DPPH free radical scavenging. EAE had the most antioxidant activity.

In conclusion, Trichilia monadelpha is a promising therapeutic agent for clinical studies since it showed it had anti-inflammatory and anti-anaphylactic effects and contained important secondary metabolites.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine
ACPAs	anti-citrullinated peptide antibodies
ACTH	Adrenocorticotropic hormone
AGEs	advanced glycation end product
AIA	adjuvant-induced arthritis
AIDS	acquired immune deficiency syndrome
ALP	alkaline phosphatase
ALT	alanine transaminase
AP-1	Activator protein-1
APPs	acute phase proteins
APR	acute phase response
AST	aspartate transaminase
AUC	area under the curve
BHA	butylatedhydroxyanisole
BHT	butylatedhydroxytoluene
Br^-	bromide ion
cAMP	cyclic adenosine monophosphate
CAT	catalase

CFA	Complete Freund's Adjuvant
cGMP	cyclic guanosine monophosphate
CHF	congestive heart failure
Cl^-	chloride ion
CNS	central nervous system
CO	carbon monoxide
COX	cyclooxygenase
CRP	C-reactive protein
DCs	dendritic cells
DMARDs	disease modifying anti-rheumatoid drugs
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EAE	ethanol extract
ECM	endothelial cell membrane
ED_{50}	effective dose for 50 % maximal effect/potency of drug
ELISA	enzyme-linked immunosorbent assay
E_{max}	maximal effectiveness of drug/efficacy of drug
EP_3	prostaglandin E receptor 3
ERM	ezrin, radixin, and moesin proteins

ESAM	endothelial-cell selective adhesion molecule
EthE	ethyl acetate extract
FLS	fibroblast-like synovial
G6PD	glucose-6-phosphate dehydrogenase
GPCRs	G-protein coupled receptors
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase
GTPase	guanosine triphosphate
H_2O_2	hydrogen peroxide
Hb	Haemoglobin
HOCl	hypochlorous acid
I^-	iodide ion
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFA	Incomplete Freund's Adjuvant
IgE	immunoglobulin E

IL	interleukin
JAM	junctional adhesion molecule
LFA1	lymphocyte function-associated antigen-1
LK	leukotrienes
LOX	lipoxygenase
LPS	*Lipopolysaccharide*
LYM	lymphocytes
MAC	macrophagen antigen
MADCAM	mucosal vascular adhesion cell-adhesion molecule
MAPK	mitogen-activated protein kinase
MIP	macrophage inflammatory protein
MMPs	matrix metalloproteinases
MPO	myeloperoxidase
MS	multiple sclerosis
MYD88	myeloid differentiation primary-response protein-88
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NEUT	neutrophils
NF-κB	nuclear factor-κB

NFAT	nuclear factor of activated T cells
NGF	nerve growth factor
NO	nitric oxide
NO^{2-}	nitrite
NO^{3-}	nitrate
NOS	nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
NT	neurotensin
O^{2-}	superoxide anion
OA	osteoarthritis
OHOOH	peroxynitrous acid
OH^{-}	Hydroxyl radical
$ONOO^{-}$	peroxynitrite anion
PACAP	pituitary adenylatecyclase activating polypeptide
PAF	platelet activating factor
PAMPs	pathogen-associated molecular pattern
PAR	protease-activated receptor
PCA	passive cutaneous anaphylactic reaction
PCV	packed cell volume

PDCF	platelet-derived growth factor
PECAM	platelet/endothelial-cell adhesion molecule
PEE	petroleum ether extract
PGs	prostaglandins
PI3Kγ	phosphoinositide-3-kinase-γ
PLA_2	phospholipase A_2
PLT	platelet
PMNL	polymorphonuclear leucocyte
PMNs	polymorphonuclear leukocytes
PSGL1	P-selectin glycoprotein ligand 1
RA	rheumatoid arthritis
RANTES	regulated upon activation, normal T cell expressed and presumably secreted
RBCs	red blood cells
RHO	RAS homologue
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCF	stem cell factor
SCN^-	cynanide ion

SMCD	systemic mast-cell disease
SOD	superoxide dismutase
SP	substance P
TNF-α	tumour necrosis factor-α
Trk	tyrosine kinase receptors
TRL4	Toll-like receptor-4
VCAM	vascular cell-adhesion molecule
VE-cadherin	vascular endothelial cadherin
VLA1	very late antigen 4
VVOs	vesiculo-vacuolar organelles
WBC	white blood cells
XO	xanthine oxidase

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

There has been great interest in research into plant medicine in all continents especially in the Asian continent: China, India and Russia, where over 45 per cent of all drugs come from plants (Busia, 2007; WHO, 2002). Globally, based on mostly ancestral use (Baliga *et al*., 2004), plants identified for medicinal use are more than 85,000 species (Liu *et al*., 2007). Many medicinal plants provide relief of symptoms with little or no side effects compared to allopathic medicines. This is mainly because of belief that herbal medicine may have fewer side effects and can supplement for conventional agents or be an alternative treatment (Desai *et al*., 2003). Although these medicinal plants offer promising effects however scientific documentation of their ethno medical and pharmacological uses is virtually unavailable.

Despite some odds noted from clinical practice, there are metabolites in screened plants and isolated compounds from plants that are potent and efficacious against mediators of inflammatory responses (Molla *et al*., 2007; Rödel *et al*., 2007; Sonis, 2002). This research is, thus carried out on a plant, *Trichilia monadelpha* which have been shown traditionally to have potent medicinal properties.

Trichilia monadelpha (Thonn.) J. J. de Wilde is used traditional for arthritis, rheumatism, cutaneous and subcutaneous parasitic infection, dropsy, oedema and many other disease conditions (Burkill, 1985; Dennis, 2002; Mshana, 2000). The bark decoction or the pulped bark is applied externally to wounds, sores, skin infections including yaws, lumbago and oedema. A bark decoction is drunk to soothe cough, as an analgesic and anthelmintic, and to treat syphilis, whereas small amounts of pulped bark are eaten or applied as an enema to treat gastrointestinal complaints. Bark decoctions serve as an aphrodisiac, ecbolic and abortificients. A leaf decoction is taken to treat heart complaints, and pounded leaves to

treat gonorrhoea and lumbago. The roots constitute the ingredients prepared to treat dysentery (Lemmens, 2008).

The stem bark of the plant which was used for this study is usually brown with aromatic odour and a bitter taste. It has lignified fibres with prismatic crystal sheaths, rosette and prismatic calcium oxalate crystals; starch grains are present as simple or compound grains. It also has oil cells, few cork cells and rich parenchymatous cells (Busia, 2007). Chemical constituents include reducing sugars, tannins (pyrocatechuic acid), limonoids (dregeanin, heudelobin and related limonoids) (Busia, 2007).

There are pharmacological evidences that *Trichilia monadelpha* inhibits carrageenan-induced acute inflammation in seven–day old chicks and inflammation associated with adjuvant-induced arthritis (Ainooson *et al*., 2012). There are also evidences it has antimalarial (Atindehou *et al*., 2004) and antimicrobial effects (Aladesanmi *et al*., 2000). Though from the various interesting findings and speculations on the pharmacological effect of the plant, there is, however, no evidence, to the best of my knowledge, on the possible mechanism of action to which the plant may show its effect especially its anti-inflammatory properties. The present study is therefore to explore the possible mechanism of action of the stem bark extracts of the plant using rodent models such as mediators-induced inflammation, models of acute inflammation and chronic arthritis, anaphylaxis and pyrexia to further support the ethno medical use of the plant in managing inflammatory associated diseases.

1.2 TRICHILIA MONADELPHA

Botanical name: *Trichilia monadelpha* (Thonn) J. J. de Wilde. ex Oliv.*pp*

***Family*:** *Meliaceae*

Local names: *otan-nuru; Otan-aduru* (Ashanti Twi, Ghana),
tenuba (Nzema, Ghana),
ako rere/rere, ajanrere, janrere, akika, olomi (Yoruba, Nigeria)
agbigben (Itsekiri, Nigeria)
ovien urhaen (Sobo, Nigeria)
ekwinsu (Sierra Leone)
jasui, njawa (Mende, Sierra Leone)
nekp'e, njags (Loko, Sierra Leone)
nendo (Kissi, Sierra Leone)
tepel-le (Bulom (Sherbro), Sierra Leone)
anzebuia, ekem (Fang, Equatorial Guinea/ France)
banayi (Ivory Coast/ France)
lebonda (Cameroun/ Gabon) (Mshana, 2000; Odugbemi *et al*., 2008; Wilks *et al*., 2000)

1.2.1 Description

Trichilia monadelpha is a fast-growing hairless perennial tree. It is a small tree that grows up to 20-60 feet high but commonly bushy and shrubby around settlements and secondary forests (Figure 1.1). It reproduces from seeds and vegetative from basal portions of cut stems. It is a woody plant, a flora of West Tropical Africa. The stem is woody, usually low branching, irregularly twisted, smooth and with knobby twigs. The stem bark is grey; slash dark pink with slight exudates of whitish latex and flowers greenish yellow, with its fruits buffed colour. The leaves are compound, pinnate, opposite or occasionally whorled about 50 cm long. The rachis is swollen at the nodes and has two glands on the petiole which is about 8 cm long. There are 3 - 6 pairs of leaflets. Each leaflet is oblanceolate, 10 - 20 cm. The leaf blade is papery, smooth and prominently veined. Each pinnate leaf is borne on a petiole

that is about 8 cm long. The inflorescence is a dense, terminal, racemose-panicle with reddish-pink or purple, tubular flowers about 5-6 cm, often attractive to ants. The fruit is a long, pendulous, dehiscent capsule about 30 cm long, dotted with glands that attract ants (Figure 1.1).

Trichilia comprises about 90 species, most of them in tropical America. In Africa 18 species occur, in Madagascar 6 species have been found (Lemmens, 2008). Of these species, the ones that have been identified in Africa are discussed in the following paragraphs.

Trichilia djalonis (A.Chev.), a shrub or small tree up to 15 m tall found in Guinea and Côte d'Ivoire, closely resembles *Trichilia monadelpha*, but differs in fewer, glabrous leaflets and smaller fruits. A reddish dye can be prepared from the bark of *Trichilia djalonis*, and possibly its wood is used for similar purposes as that of *Trichilia monadelpha* (Lemmens, 2008).

Trichilia ornithothera (J. J.de Wilde), is a small tree up to 15 – 20 m tall, occurring from Sierra Leone to Ghana, and closely resembles *Trichilia monadelpha.* The wood is used for similar purposes and the bark is used as a traditional medicinal substance. This specie is characterised by larger and more hairy leaves in comparison with *Trichilia monadelpha*, and by its usually 2-celled ovary (Lemmens, 2008).

Trichilia tessmannii (synonyms: *Trichilia lanata* (A. Chev.), *Trichilia mildbraedii*) has almost the same distribution area as *Trichilia monadelpha*, but less common in most areas. It is a medium-sized tree up to 30 m tall, with straight, cylindrical bole up to 80 cm in diameter, and is most simply recognized by its twigs having reddish brown, flaking bark. The wood is used in building because it is resistant to termites. The bark is used to treat stomach ache and as a purgative. In DR Congo the fruits from this species are cooked and eaten. In Nigeria the seeds are used for rattles and tambourines (Lemmens, 2008).

In the past *Trichilia welwitschii* (C.DC.) has been confused with *Trichilia monadelpha*, but it differs in its 2-celled ovary and fruit. It is a small to medium-sized tree up to 30 m tall with a cylindrical bole up to 45 – 60 cm in diameter, which occurs from Nigeria to eastern DR

Congo and northern Angola. The wood is used for similar purposes as that of *Trichilia monadelpha*. In DR Congo bark decoction is administered as an enema to treat haemorrhoids and other abdominal disorders, and as an abortificients, whereas pounded young leaves are applied to syphilitic sores (Lemmens, 2008).

Trichilia gilletii (de Wilde) occurs in roughly the same region and closely resembles *Trichilia welwitschii*, from which it differs in fewer and glabrous leaflets. Its wood is used similarly, whereas its bark is used to treat fever and as a purgative, and its seed oil as an emetic (Lemmens, 2008).

Trichilia monadelpha (Thonn.) J. J. de Wilde is a common understorey tree in lowland evergreen and semi-deciduous secondary forest, up to 650 m altitude, also occurring in forest edges, sometimes in deciduous forest, along rivers and in other moist localities (Lemmens, 2008). It is used in traditional medicine in Ghana for treating a spectrum of human diseases. However, much scientific assessment or scrutiny has not been documented. There is therefore the need for a more elaborate scientific assessment and validation of the pharmacological activities of *Trichilia monadelpha*.

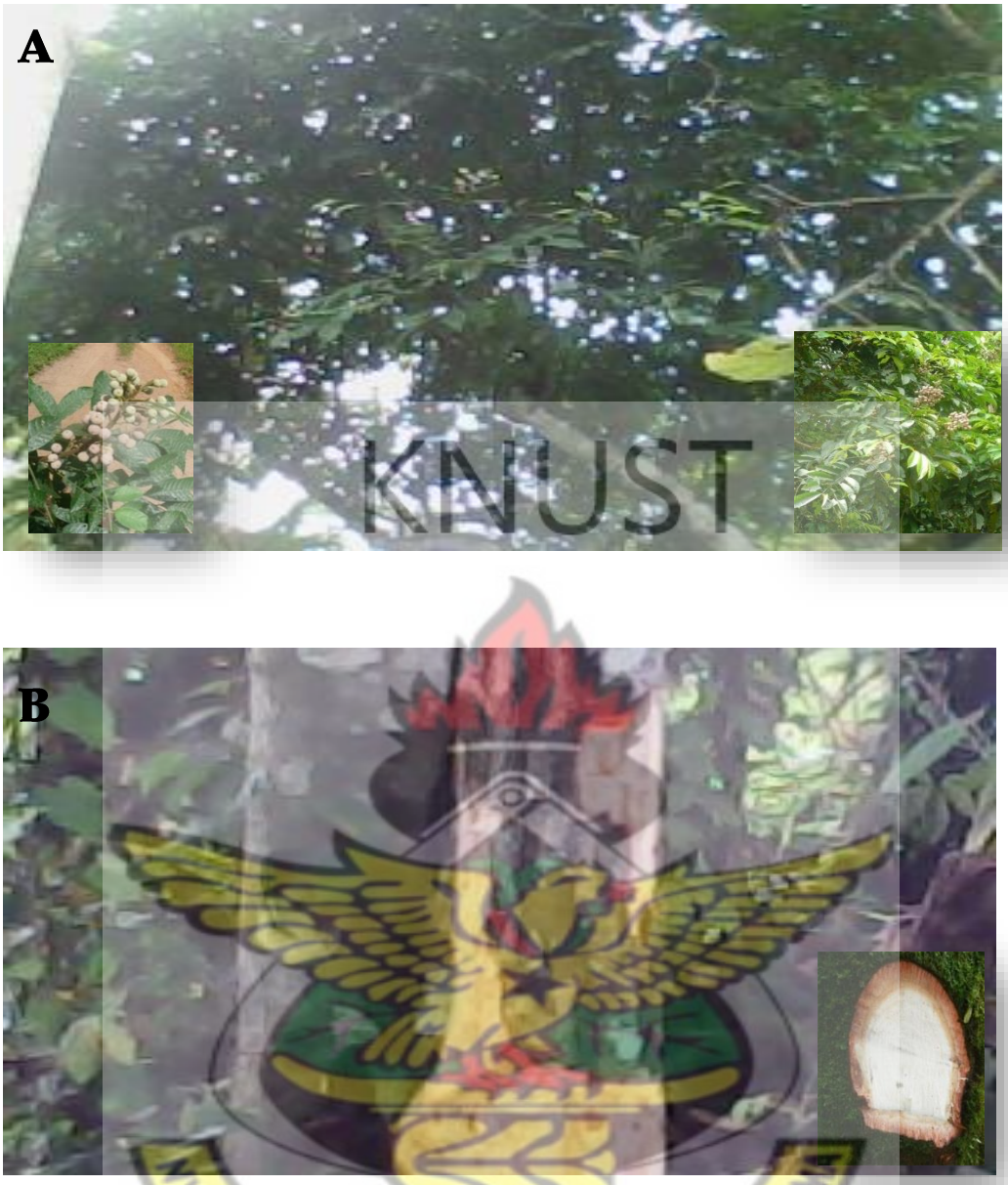


Figure 1.1: (A) *Trichilia monadelpha* tree showing the leaves and flowers (insets). (B) The stem tree and stem bark (inset) removed from the stem.

1.2.2 Ecological and Geographical Distribution

***Habitat*:** common in moist understorey of rainforest, especially in secondary regrowth types.

***Location*:** Throughout Brong-Ahafo and Ashanti Regions. The plant was obtained from Bomaa, Tano North District (latitude $2^{0}10'01.63''$ W, longitude $7^{0}05'06.82''$ N, 787 feet above sea level), of the Brong-Ahafo Region for this research work.

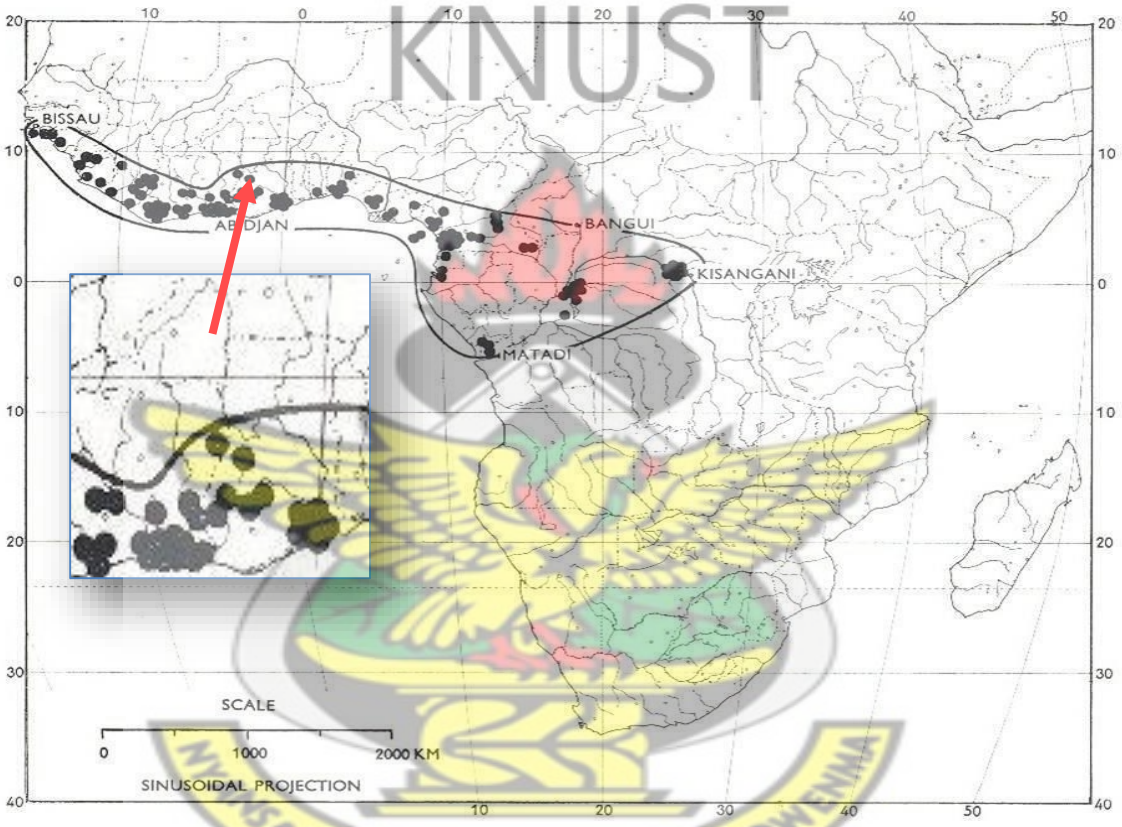


Figure 1.2: Geographical distribution of *Trichilia monadelpha* (Thonn.) J. J. de Wilde.
The plant is mostly found distributed throughout the rainforest region of West Africa. INSET: *Trichilia monadelpha* distribution in Ghana (Fauna *et al.*, 2011).

1.2.3 Traditional Uses

Trichilia monadelpha has been used traditionally for managing different disease conditions. Some parts of the tree have also been used domestically.

1.2.3.1 Non-medicinal Uses

Trichilia monadelpha fruit serves as a feeder for birds (Burkill, 1985). Other useful products from the tree are; dyes, stains, inks and mordants (from the bark), gums, resins from exudation of the stem bark (Burkill, 1985; Lemmens, 2008). The wood or twig also serves as chewing-stick (Burkill, 1985). The seed oil is used in cooking and the tree is useful for soil protection and soil improvement (Lemmens, 2008).

1.2.3.2 Medicinal Uses

Gastrointestinal tract disorders: The root is used to treat dysentery, diarrhoea (Burkill, 1985; Lemmens, 2008), dyspepsia, ulcers and gout (Busia, 2007; Dennis *et al.*, 2004; Mshana *et al.*, 2000). Small amounts of pulped bark are eaten or applied as an enema to treat gastrointestinal complaints (Lemmens, 2008).

Disorders of the respiratory system: In Ghana and Nigeria, the stem is chewed for cough (Burkill, 1985). A bark decoction is also drunk to sooth cough (Lemmens, 2008).

Disorders of the Central nervous system: The stem bark decoction when used is sedative and can cause debility. It has also been used as an alcoholic beverage and stimulant (Burkill, 1985).

Disorders of the genito-urinary system: The dried bark and young twigs, pounded and cooked with light soup has been used as embolic for pregnant women, serving as an abortificients (Burkill, 1985). The bark and the roots have also been used as genital stimulants or depressants and considered as an aphrodisiac (Burkill, 1985; Lemmens, 2008).

Infectious diseases: The stem bark is used to treat yaws, cutaneous and subcutaneous parasitic infection, venereal disease (gonorrhoea and syphilis) (Burkill, 1985; Lemmens, 2008), candidiasis, skin ulcers (Addo-Fordjour *et al*., 2008; Busia, 2007; Dennis, 2002; Mshana, 2000).

Musculoskeletal and joint disorders: Arthritis, rheumatism, dropsy, waist pain, lumbago (Burkill, 1985; Busia, 2007; Dennis *et al*., 2004; Lemmens, 2008; Mshana *et al*., 2000).

Other medicinal uses: The leaves have been used for heart problems (Burkill, 1985), malnutrition, painkiller, wound, analgesic and anthelmintic (Busia, 2007; Lemmens, 2008).

1.2.4 Phytochemical Constituents of *Trichilia Monadelpha*

Phytochemicals are natural and non-nutritive bioactive compounds produced by plants that act as protective agents against external stress and pathogenic attack (Chew *et al*., 2009). Secondary metabolites are important for plant defences (*e.g.* an antioxidant or antimicrobial agent) which has enabled plants to survive. Based on their biosynthetic origin, Phytochemicals are divided into several categories: phenolics, alkaloids, steroids, terpenes, saponins. Phytochemicals could also show other bioactivities such as antimutagenic, anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory properties (Yen *et al*., 1993). These plant-derived phytochemicals with therapeutic properties could be used as single therapeutic agent or as combined formulas in drug development. Some of the main bioactive agents or constituents of medicinal plants are: Alkaloids, Flavonoids, Saponins, Tannins, Phenols, Polyphenols, Anthraquinones and Glycosides (Harborne, 1998; Sofowora, 1993; Srinivas *et al*., 2003; Trease *et al*., 1989; Yen *et al*., 1993).

The chemical constituents identified from *Trichilia monadelpha* include reducing sugars, tannins (protocatechuic acid), limonoids (Busia, 2007). There is little information on scientific investigation on these chemical constituent. However, some investigations on these compounds from plants of the *Meliaceae* family and from the genus *Trichilia* have been reported (Nakamura *et al*., 2000; Roy *et al*., 2006).

The simple phenol, protocatechuic acid, 2, 3-dihydroxybenzoic acid (Figure 1. 3 A) is one of the major benzoic acid derivatives from edible plants and fruits (mostly of the family *Meliaceae*) and shows a strong anti-oxidative effect, 10-fold higher than that of α-tocopherol (Nakamura *et al*., 2000).

The term limonoids stemmed from limonin, the first tetranortriterpenoid obtained from citrus bitter principles (Devakumar *et al*., 1996; Roy *et al*., 2006). Hundreds of limonoids (Figure 1.3 B) have been isolated from various plants. These limonoids are confined to only plant families of the order *Rutales* and more richly in *Meliaceae* and *Rutaceae* plant family and less often in *Cneoraceae* and *Harrisonia* sp. of *Simaroubaceae* (Roy *et al*., 2006).

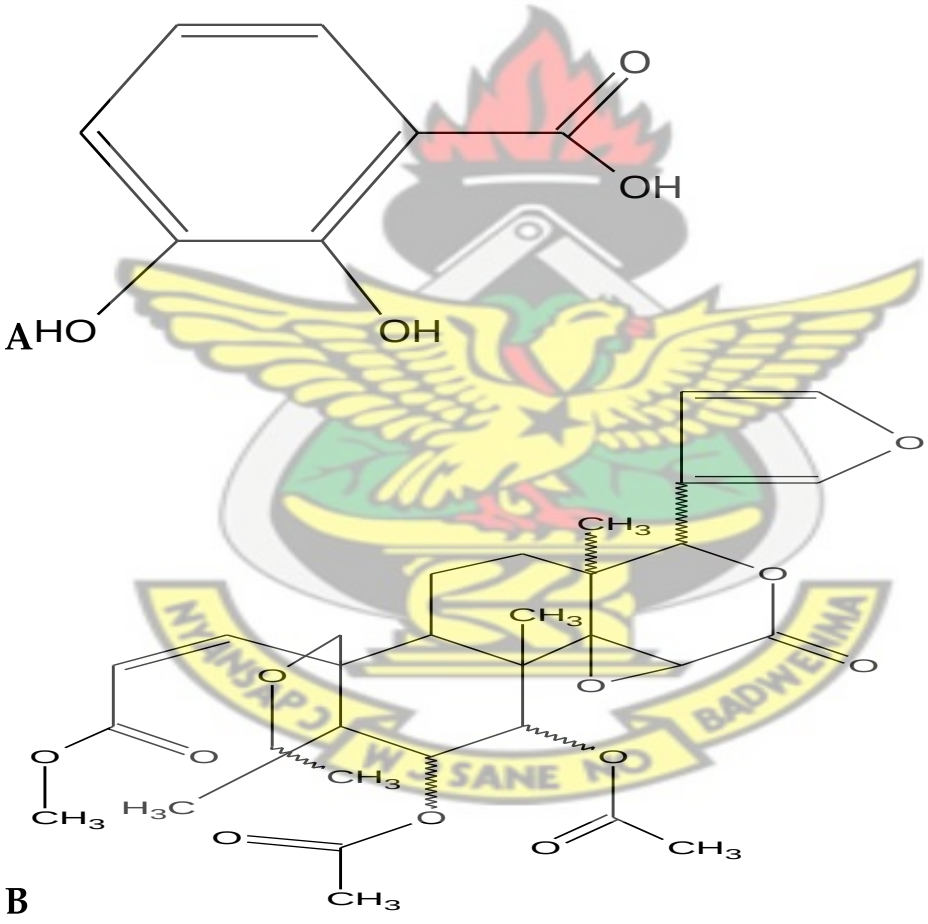


Figure 1.3: Two reported chemical constituents of *Trichilia monadelpha*.
(A) Protocatechuic acid (phenolic compound). (B) Limonoid (tetranortriterpenoid) (Busia, 2007).

Limonoids are important group of metabolically altered triterpene, which are limited in their distribution. The medicinal properties reported include anticancer, antimalarial, antimicrobial, anti-HIV, antiviral and several others (Roy *et al*., 2006). Published reports on effects of limonoids of other genus of *Meliaceae* (e.g. Neem tree, citrus family) are extensive but not on *Trichilia monadelpha*.

1.2.5 Previous Studies on *Trichilia monadelpha*

Trichilia monadelpha has been used locally to manage different disease conditions. There are few scientific reports on the plant. These reports on the plant show its ability to inhibit acute and chronic inflammation and hyper analgesia. The reports also showed the plant improved sperm viability with little or no toxic effect on important organs of the system.

1.2.5.1 Anti-inflammatory Effect

This study focused on the effect of extracts (petroleum ether extract, aqueous extract and hydro alcoholic extract) from the bark of *Trichilia monadelpha* on acute and chronic inflammation (Ainooson *et al*., 2012). Carrageenan-induced acute footpad oedema in 7-day-old chicks was the model used in the study. Carrageenan-induced acute footpad oedema in laboratory animals (Winter *et al*., 1962) is a model of acute inflammation widely used to evaluate non-steroidal anti-inflammatory drugs (Di Rosa *et al*., 1971; Donadieu *et al*., 2005). The rat adjuvant arthritis model was the model for chronic inflammation in the study. It is a model of chronic inflammation for screening NSAIDs, steroids and immunosuppressive drugs (Crofford *et al*., 1992; Cronstein, 1995; Hayward *et al*., 1989; Segawa *et al*., 1997).

The plant (the petroleum ether, aqueous and hydro-alcoholic extracts) inhibited inflammatory oedema in the two models (Ainooson *et al*., 2012). The precise mechanism by which *Trichilia monadelpha* extracts inhibited inflammation was outside the scope of the study (Ainooson *et al*., 2012). However, reports show that plant constituents such as alkaloids, flavonoids, glycosides, terpenoids, steroids and many other secondary plant

metabolites may present anti-inflammatory effects (Calixto *et al*., 2004; Calixto *et al*., 2003; Rios, 2008). Phytochemical studies of the plant bark showed it contains alkaloids, tannins, saponins, steroids, flavonoids, terpenoids and glycosides (Ainooson *et al*., 2012). These plant metabolites may be responsible for the antiarthritc effects of *Trichilia monadelpha* extracts (Ahmed *et al*., 2005; Mythilypriya *et al*., 2008; Puratchikody *et al*., 2011). Whereas it is clear *Trichilia monadelpha* extracts effectively reduced inflammation in adjuvant arthritis, it remained uncertain whether this translated into an improvement in indices of joint integrity such as bone and cartilage degradation (Ainooson *et al*., 2012).

1.2.5.2 Anti-nociceptive Effects

The analgesic or anti-nociceptive activity of the plant (petroleum ether, aqueous, and hro-alcoholic extracts of the stem bark of the plant) was studied (Ainooson *et al*., 2012), using the formalin test model of analgesic (Dubuisson *et al*., 1977). This is a widely used model of analgesic studies and most predictive of acute pain (Le Bars *et al*., 2001) and valid model of clinical pain (Costa-Lotufo *et al*., 2004; Vasconcelos *et al*., 2003). In the study, intradermal injection of 10 µl of 4 % formalin evoked a biphasic response characterised by flinching, licking or biting of the injected paw (Dubuisson *et al*., 1977; Wheeler-Aceto *et al*., 1990). The first phase of the biphasic response observed (0-5 min) depicts acute neurogenic pain and the second phase (15-30 min) shows acute inflammatory pain (Asongalem *et al*., 2004; Mino *et al*., 2004). The first phase is through direct stimulation of nociceptive afferent fibres therefore sensitive to drugs such as those that interact with the opioid system. The second phase is through involvement of inflammatory pain mediators (kinin, histamine and serotonin) that can possibly activate small afferent neurons. These are sensitive to analgesic-anti-inflammatory drugs (Hunskaar *et al*., 1987; Le Bars *et al*., 2001; Malmberg *et al*., 1992) and corticosteroids (Vasconcelos *et al*., 2003). Centrally acting analgesic drugs inhibit both phases but peripherally acting analgesic drugs inhibit only the second phase (Mino *et al*., 2004).

Trichilia monadelpha, in the study (aqueous and petroleum ether extracts) like morphine, significantly reduced the duration of the animals' response to pain in both phases and the hydro-alcoholic extracts only reduced response of the first phase. It is possible the plant has compounds that were inhibiting pain transmission in the central and peripheral nervous systems (Ainooson *et al*., 2012).

The mechanism by which *Trichilia monadelpha* elicits its analgesic effect is possible by its antinociceptive effects which act by inhibiting local synthesis, release and/or action of nociceptive endogenous mediators including prostaglandins and proinflammatory cytokines (Iwalewa *et al*., 2007). Hydro-alcoholic, ethyl acetate and petroleum ether extracts of *Trichilia monadelpha* were used in the study carried out by Woode *et al*. (2012). Here the hydro-alcoholic extract produced analgesic effects through mechanism that involved interaction with the opioid, adenosinergic, muscarinic, cholinergic pathway and ATP-sensitive K^+ channels. The ethyl acetate and petroleum ether extracts, however, produced analgesic effects that involve muscarinic and cholinergic system (Woode *et al*., 2012).

1.2.5.3 Effects on Reproductive indices (Sperm characteristics and testosterone secretion)

Oyelowo *et al.* (2011) studied the effects of aqueous extract of *Trichilia monadelpha* stem bark on male rat reproductive indices such as the sperm characteristics and testosterone secretion. This study was based on the findings that many antimalarial agents have various degrees of antifertility activities (Raji *et al*., 1997) and the bark extracts of *Trichilia monadelpha*, has potent antiplasmodial activity against chloroquine and pyrimethamine-resistant *Plasmodium falciparum* strains (Oyelowo *et al*., 2011). With this, the research showed the plant significantly increased both the sperm motility and viability with no significant difference in the sperm counts (Oyelowo *et al*., 2011).

Oyelowo *et al.* (2011), also showed that *Trichilia monadelpha* showed the presence of alkaloids, tannins and simple sugars. These phytochemicals identified are associated with some properties; alkaloids are associated with abnormal sperm morphology even in human adults (Ejebe *et al*., 2008). The simple sugars is involved in increased sperm motility and

viability through the metabolism of simple sugars like glucose that results in producing pyruvate, a substrate necessary for the activity and survival of sperm cells (Egbunike *et al*., 1986).

According to Oyelowo *et al*. (2011), rats treated with *Trichilia monadelpha* had increased levels of serum testosterone. This supports the tradomedical use of the plant as an aphrodisiac, possibly due to steroidogenic potentiation (Oyelowo *et al*., 2011). Testosterone is essential for male reproductive organs development and maintenance (Mooradan *et al*., 1987). It acts in association with follicle-stimulating hormone, on the seminiferous tubules to cause and preserve spermatogenesis (Christensen, 1975). The study therefore, suggested that *Trichilia monadelpha* could improve sperm performance with no spermatoxic effect (Oyelowo *et al*., 2011).

1.2.5.4 Toxicity Studies

The oral treatment of aqueous extract of *Trichilia monadelpha* had no significant effect on the haematological parameters assessed in the study carried out by Ainooson *et al*. (2012). In the study, liver function was assessed as the liver is the largest organ in the human body with a role of preserving the internal body environment within a limited range (homeostasis). To achieve this, the liver metabolises, synthesises and detoxifies several compounds including proteins, glucose, fats and natural products. Some toxic compounds produced may cause damage to the liver. Levels of aspartate transaminase (AST) and alanine transaminase (ALT) in the serum were determined as these two enzymes are linked to finding out hepatocellular damage (Lyoussi *et al*., 2004).

Alkaline phosphatase (ALP) levels reach spectacular height in biliary cirrhosis, hepatitis and in disease characterised by inflammation, regeneration and obstruction of intrahepatic bile ductules (Panthong *et al*., 2003). In the study there was no significant change in the levels of ALP, ALT and AST showing the plant had no marked adverse effect on the liver. The liver synthesises most of the plasma proteins such as albumin and globulin and low levels show liver damage. Bilirubin, the key pigment formed from the breakdown of haemoglobin in

RBCs is conjugated in the liver and secreted into bile. Decreased secretion of bilirubin or obstruction of the bile ducts is detected as increased bilirubin levels (Panthong *et al*., 2003). This high levels leads to haemolytic jaundice. In the study no significant changes in the levels of the liver enzymes and other parameters assessed was observed. This possibly showed the plant had no adverse effect on the liver (Ainooson *et al*., 2012).

In the 14-day toxicity study, no death or any abnormal behaviour was observed. The gross body weight changes of the treated rats were not significant compared to the control group. Weight changes of the stomach, liver, kidney and spleen were also not significant. Change in body weight is a sign of adverse effect of drugs (Tofovic *et al*., 1999). The study thus claimed that continual oral administration of the extract up to 1000 mg kg^{-1} for two weeks was non-toxic (Ainooson *et al*., 2012). The pentobarbitone-induced sleeping model showed animals treats with plant extracts had prolonged sleeping time measured as sedation (Ainooson *et al*., 2012). It was postulated the plant caused sedation due to activation of the opioid receptors (μ, δ and κ) of the midbrain known to cause sedation in the CNS (Christie *et al*., 2000).

1.3 INFLAMMATION

The human system is prone to tissue injury caused by physical trauma, harmful chemicals, or microbiologic agents. This has made the system, evolutionarily and genetically, to create processes – inflammation - that would seek to preserve and manage this tissue injury. Thus inflammation, by definition, is a normal, protective response to tissue injury (Finkel *et al*., 2009). It is the body's effort to inactivate or destroy invading organisms, remove irritants, and set the stage for tissue repair, referred to as healing. When healing is complete, the inflammatory response usually subsides (Finkel, *et al*., 2009).

This inflammatory response is characterised by four signs or cardinal signs. First listed by Celsius, a Roman writer of the first century, these signs are redness (***rubor***), heat (***calor***), swelling (***tumor***), and pain (***dolor***). A fifth clinical sign, loss of function (***functio laesa***), was added by Virchow (Hurley, 1972). These clinical signs of inflammation are the macroscopic result of molecular and cellular processes, of which over the last 120 years, is reproduced in convenient experimental systems *in vitro* (Spector *et al*., 2009). Besides the cardinal signs that appear at the site of injury, systemic manifestations (*e.g*., fever) may occur as chemical mediators produced at the site of inflammation enter the circulatory system. The constellation of systemic manifestations is known as the *acute-phase response* (Chandrasoma *et al*., 1998; Fantone *et al*., 1999; Mitchell *et al*., 2003). The unique feature of the inflammatory process is reaction of blood vessels, leading to build-up of fluid and leucocytes in extravascular tissues (Figure 1.4) (Vinay *et al*., 2005).

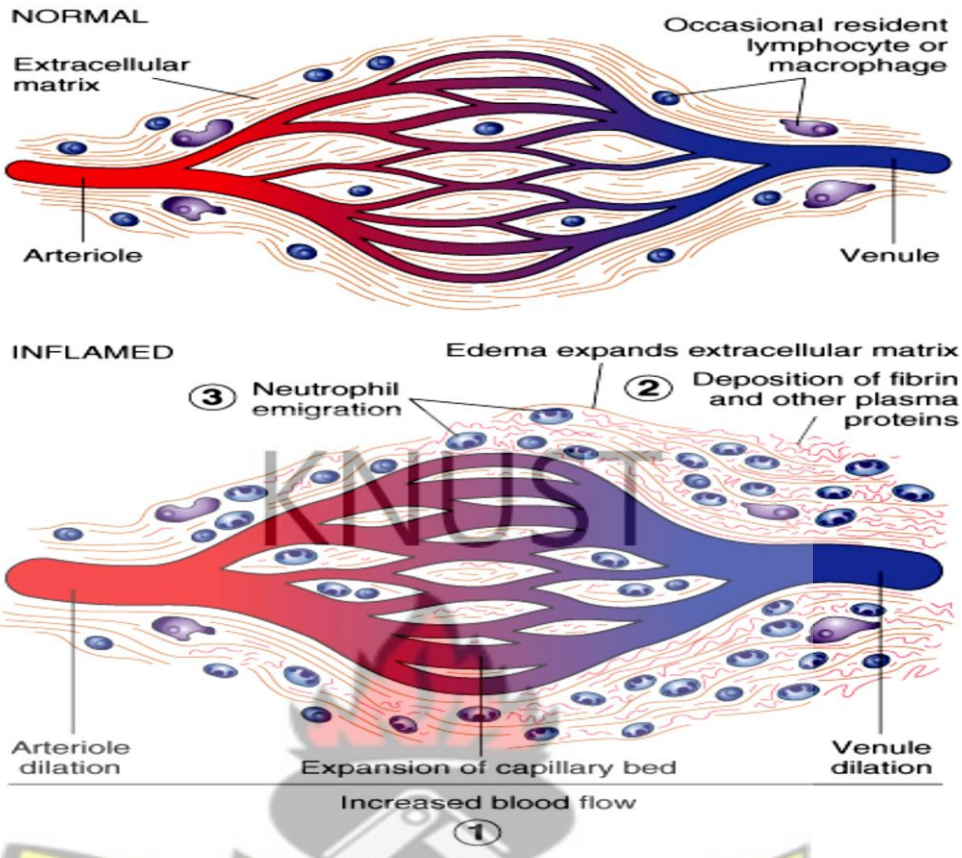


Figure 1.4: The major local manifestations of inflammation compared to normal.
(1) Vascular dilation and increased blood flow (causing erythema and warmth), (2) extravasation and deposition of plasma fluid and proteins (oedema), and (3) leucocyte emigration and accumulation at the site of injury (Vinay *et al.*, 2005).

Inflammatory processes aim at combating tissue injury or pathogen invasion. These often lead to diseases and disorders such as; atherosclerosis, arthritis, allergies, fever, diabetes, acquired immune deficiency syndrome (AIDS) mediated by the Human Immunovirus, asthma, neoplasia, degenerative and cardiovascular diseases. Epidemiological data suggest that lower incidences of these diseases are associated with frequent intake of fruits and vegetables and stem bark of most plants (Ames *et al.*, 1993; Choi *et al.*, 2002; Chu *et al.*, 2002). Biological effects of several naturally occurring substances from foods, herbs and other natural sources have been implicated in acute and chronic diseases through animal studies (Weisburger, 2002). Some of these naturally occurring bioactive substances with antioxidant properties, plant phenols, vitamins, carotenoids, phytoestrogens and terpernoids have anti-inflammatory

activity and may play an important role in disease prevention and immune promotion, especially in chronic inflammatory diseases (Weisburger, 2002).

Phytomedicine constituents from plant extracts that inhibit any of the molecular targets of inflammation such as the chemical mediators, have the potential to inhibit or reduce the inflammatory process. Understanding the mechanism and involvement of chemical mediators in inflammatory process is important to appreciate the action of this process in the system. These mediators, depending on the duration of injury determine the severity of inflammation and are termed pro-inflammatory fundamental factors (Coleman, 2002). These substances bind to specific target receptors on the cells and may increase vascular permeability, promote neutrophil chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain and mediate oxidative damage (Coleman, 2002). Even though the innate cascade process of inflammation is complex, it is mainly divided into two parts i.e. acute and chronic which could either be beneficial or detrimental (Peter, 2010).

Acute inflammatory response, involves a complex orchestration of events. These include leakage of water, salt, and proteins from the vascular compartment; activation of endothelial cells; adhesive interactions between leucocytes and the vascular endothelium. It also includes activation of tissue macrophages; activation of platelets and their aggregation; activation of the complement; clotting and fibrinolytic systems; and release of proteases and oxidants from phagocytic cells. All these may aid in coping with the state of injury (Mitchell *et al*., 2003; Peter, 2010; Rang *et al*., 2007; Vinay *et al*., 2005). Whether due to physical or chemical causes the earliest *in vivo* hallmark of the acute inflammatory response is neutrophils (polymorphonuclear leucocytes, PMNs) adhering to the vascular endothelium (Peter, 2010).

Chronic inflammatory response is defined according to the nature of the inflammatory cells appearing in tissues, not necessarily the duration of the inflammatory response (Fantone *et al*.,

1999; Mitchell *et al*., 2003). This response is observed by the expression histologically, of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis. The persistent chronic inflammation develops into degenerative diseases such as rheumatoid arthritis, cancer, congestive heart failure (CHF), multiple sclerosis (MS), diabetes, infections (bacteria, fungi, and parasites), gout, ageing and other neurodegenerative CNS depression. These are associated with immunopathological features that appear to play a key role in the onset of the condition (Dalgleish *et al*., 2002; O'Byrne *et al*., 2001).

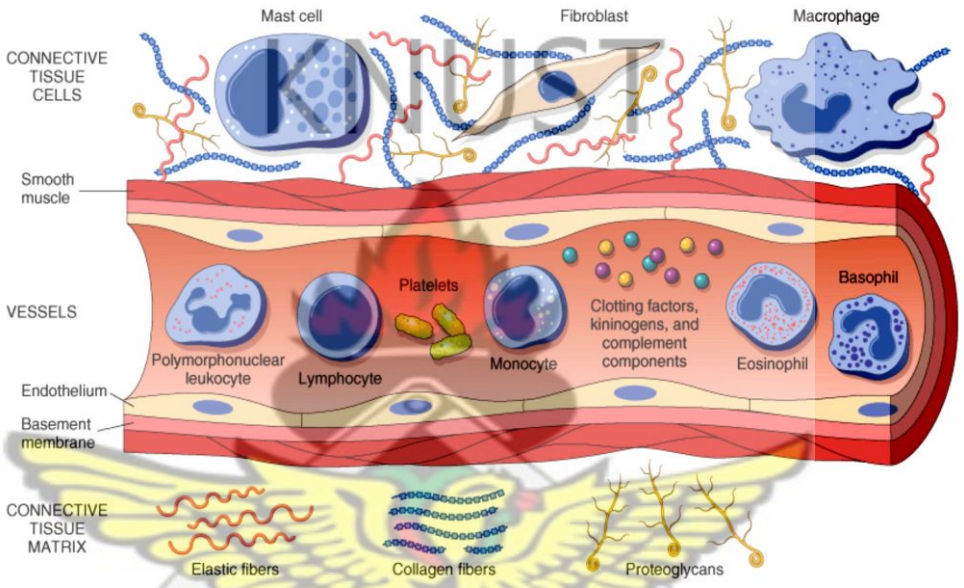


Figure 1.5: Components of acute and chronic inflammatory responses.
Circulating cells and proteins, cells of blood vessels, and cells and proteins of the extracellular matrix are shown (Vinay *et al*., 2005).

1.3.1 Inducers and Mediators of Inflammation

The ability of the body to sustain injury, resist attack by microbial agents and repair damaged tissue is dependent on the inflammatory reaction which is coordinated by a large range of mediators that form complex regulatory networks. To separate these complex systems, it is helpful to place these signals into practical classifications and to distinguish between inducers and mediators of inflammation (Medzhitov, 2008).

1.3.1.1 Inducers of inflammation

Inducers of inflammation can be exogenous or endogenous (Figure 1.6). Exogenous inducers are classified into two groups: microbial and non-microbial. Microbial inducers, e.g. lipolysaccharide of Gram-negative bacteria (e.g. *E. coli*), *Mycobacterium tuberculosis*, mostly have pathogen-associated molecular patterns (PAMPs) recognised by pattern recognition or Toll-like receptors on host tissue macrophages (Medzhitov *et al*., 1997). In contrast to PAMPs, virulence factors are restricted to pathogens; they are not sensed directly by dedicated receptors. The effects of their activity, their adverse effects on host tissues, are responsible for triggering the inflammatory response. Typical activities of virulence factors can be detected by specialised sensors (Medzhitov, 2008). Exogenous inducers of inflammation that are of non-microbial origin include allergens, irritants (carrageenan, dextran, and zymosan), foreign bodies and toxic compounds (Dostert *et al*., 2008; Rizki *et al*., 1992).

Endogenous inducers of inflammation are signals produced by stressed, damaged malfunctioning tissues. They probably belong to various classes according to the nature and the degree of tissue anomalies on which they report (Bianchi, 2007; Rock *et al*., 2008). Another class of endogenous inducer, more relevant to chronic inflammatory conditions includes crystals of monosodium urate and calcium pyrophosphate dehydrate, AGEs (advanced glycation end products) and oxidised lipoproteins (such as high-density lipoproteins and low-density lipoproteins). Formation of such crystals is facilitated in certain connective tissues, which provide an appropriate surface for crystal nucleation (Rock *et al.,* 2008).

Another group of endogenous inducers of inflammation consist of breakdown products of the ECM (endothelial cell membrane) that are produced during tissue malfunction or damage. Tissue injury promotes its breakdown into low molecular weight fragments, which are inflammatory, activating TLR4 and promoting a tissue-repair response (Jiang *et al*., 2005). Several endogenous pathways that start the inflammatory response depend on reactive oxygen species, ROS (Medzhitov, 2008).

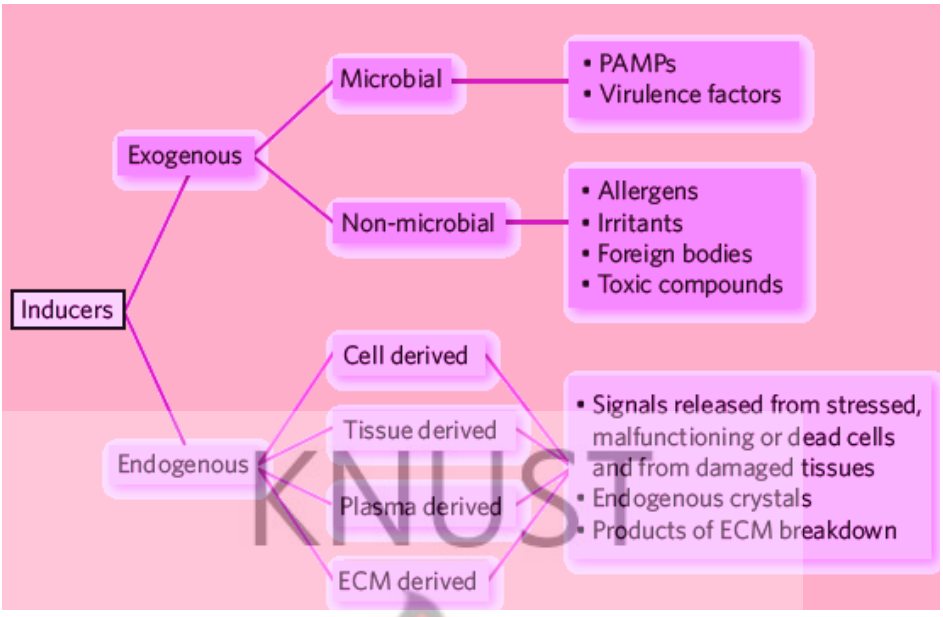


Figure 1.6: Classification of Inducers of inflammation.
ECM, extracellular matrix; PAMP, pathogen-associated molecular pattern. (Medzhitov, 2008).

1.3.1.2 Mediators of inflammation

Mediators originate either from plasma or from cells (Figure 1.7). Plasma-derived mediators (e.g., complement proteins, kinins) are present in plasma in precursor forms that must be activated, usually by a series of proteolytic cleavages, to acquire their biological properties. Cell-derived mediators are normally sequestered in intracellular granules that need to be secreted (e.g., histamine in mast cell granules) or are synthesised *de novo* (e.g., prostaglandins, cytokines) in response to a stimulus. The major cellular sources are platelets, neutrophils, monocytes/ macrophages, and mast cells, but mesenchymal cells (endothelium, smooth muscle, fibroblasts) and most epitheliums can also be induced to elaborate some of the mediators (Majno *et al.*, 2004; Vinay *et al.*, 2005).

Mediators act on few target cell types and have diverse targets and effects on different types of cells. Once activated and released from the cell, most of these mediators are short-lived. They quickly decay (e.g., arachidonic acid metabolites) or are inactivated by enzymes (e.g.,

kininase (specifically angiotensin-1 converting enzyme, ACE or kininase II) inactivates bradykinin), or they are otherwise scavenged (e.g. antioxidants scavenge toxic oxygen metabolites) or inhibited (e.g., complement regulatory proteins break up and degrade activated complement components). There is thus a system of checks and balances in the regulation of mediator actions. Most mediators have the potential to cause harmful effects (Vinay *et al.*, 2005).

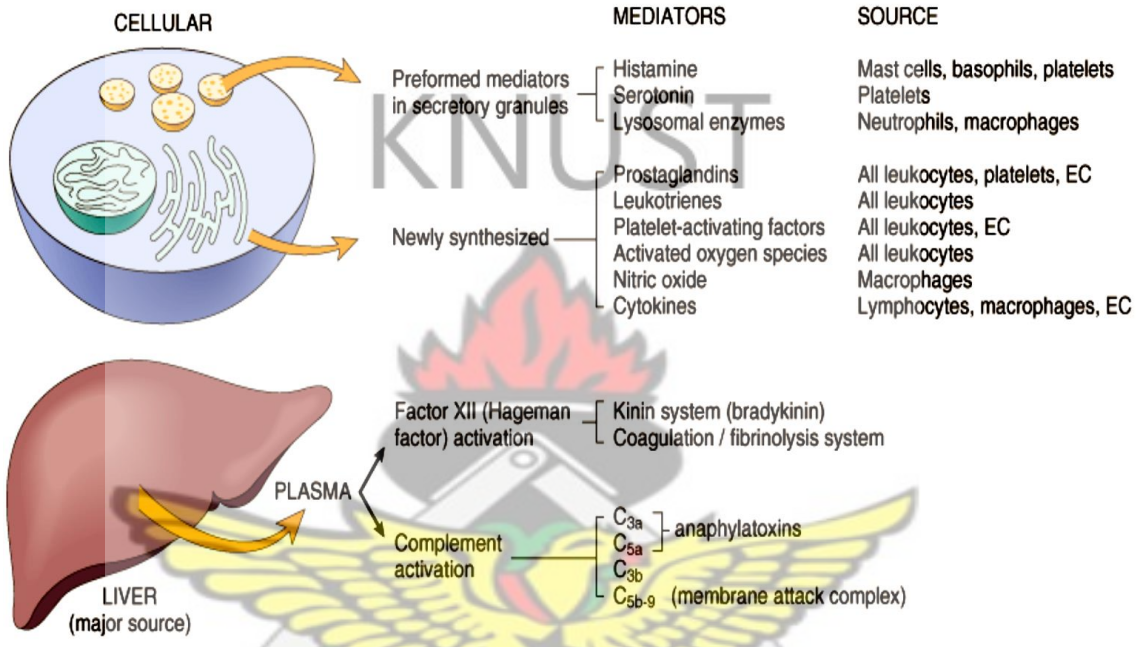


Figure 1.7: Chemical mediators of Inflammation (EC – endothelial cells) (Vinay *et al.*, 2005).

Inflammatory mediators are classified into seven groups according to their biochemical properties as follows: vasoactive amines; vasoactive peptides; fragments of complement units; lipid mediators; cytokines; chemokines and proteolytic enzymes (Majno *et al.*, 2004; Vinay *et al.*, 2005).

Vasoactive Amines: These, such as histamine and serotonin, are secreted in an all-or-none manner when mast cells and platelets degranulate. These secreted amines cause increased

vascular permeability and vasodilation, or vasoconstriction. These vasoactive amines, injected intradermally, cause 'triple response': ***reddening*** (local vasodilatation), ***weal*** (direct action on blood vessels) and ***flare*** (from an 'axon' reflex in sensory nerves releasing a peptide mediator). The effect of their release by mast cells can be damaging in sensitised organisms, causing vascular and respiratory collapse during anaphylactic shock (Rang, *et al*., 2007; Medzhitov, 2008).

Vasoactive Peptides: These are stored in an inactive form in secretory vesicles (for example, substance P) or produced by proteolytic processing of inactive precursors in the extracellular fluid (for example, kinins, fibrinopeptide A, fibrinopeptide B and fibrin degradation products). Substance P is released by sensory neurons and cause mast cell degranulation. Other vasoactive peptides are produced through proteolysis by the Hageman factor, thrombin or plasmin. These cause vasodilation and increased vascular permeability (either directly or inducing the release of histamine from mast cells). The Hageman factor works as both a sensor of vascular damage and an inducer of inflammation. It also activates the kallikrein–kinin cascade, with the main product, bradykinin, affecting the vasculature, as well as having a potent pro-analgesic (pain-stimulating) effect. Pain sensation importance in inflammation is alerting the organism to the abnormal state of the damaged tissue (Damas, 1980; Pulsatelli *et al*., 2006).

Complement Fragments: The complement fragments C3a, C4a and C5a (also known as anaphylatoxins) are produced by several pathways of complement activation. C5a (and to a lesser extent C3a and C4a) stimulate granulocyte and monocyte recruitment and induce mast cell degranulation (Low *et al*., 2005).

Lipid Mediators: Lipid mediators (eicosanoids and platelet-activating factors) are derived from phospholipids, such as phosphatidylcholine, present in the inner leaflet of cellular

membranes. After stimulation by intracellular Ca^{2+} ions, cytosolic phospholipase A_2 produces two precursors, eicosanoids and platelet-activating factor, the two classes of lipid mediator, from phosphatidylcholine. Arachidonic acid is metabolised to form eicosanoids either by cyclooxygenases (COX-1 and COX-2), which produce prostaglandins and thromboxanes, or by lipoxygenases, which produce leukotrienes and lipoxins (Vinay *et al*., 2005). The prostaglandins PGE_2 and PGI_2, cause vasodilation, and PGE_2 is hyper analgesic and a potent inducer of fever (Higgs *et al*., 1984). Lipoxins (and dietary ω-3-fatty-acid-derived resolvins and protectins) inhibit inflammation and stimulate resolution of inflammation, and tissue repair (Serhan, 2007). The second classes of lipid mediator are platelet-activating factors. These are produced by the acetylation of lysophosphatidic acid and activation of several processes that occur during the inflammatory response, including recruitment of leucocytes, vasodilation and vasoconstriction, increased vascular permeability and platelet activation (Oh-ishi, 1997; Teather *et al*., 2002).

Inflammatory Cytokines: These include tumour-necrosis factor-α (TNF-α), interlukins-1 (IL-1), interlukins-6 (IL-6) and many others, which are produced by many cell types, most notably by macrophages and mast cells. Their role in inflammatory response includes activation of the endothelium and leucocytes and induction of the acute-phase response (Akama, 2007; Bao *et al*., 2001).

Chemokines: Chemokines are formed by many cell types in response to inducers of inflammation. They regulate leucocyte extravasation and chemotaxis towards the affected tissues (Chen *et al*., 2004).

Proteolytic Enzymes: Several proteolytic enzymes (including elastin, cathepsins and matrix metalloproteinases) have different roles in inflammation, such as degrading the extracellular

matrix and basement-membrane proteins. These proteases have vital roles in many processes, including host defence, tissue remodelling and leucocyte migration (Fiedorczyk *et al*., 2006).

1.3.2 Site of Inflammation

At the site of inflammation, neutrophils, lymphocytes and monocytes are recruited and recirculated. These units entail adhesion and transmigration through blood vessel walls. Discovery of integrins, selectins and their respective ligands, chemokines and chemokine receptors led to proposal of three steps; selectin-mediated rolling, chemokine-triggered activation and integrin-dependent arrest. These steps, considered as the classical model of leucocyte adhesion, suggested the cascade achieved combinatorial specificity (Butcher, 1991; Springer, 1995). The original three-step leucocyte adhesion cascade has been expanded, to include; slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration (Figure 1.8) through the basement membrane (Ley *et al*., 2007). This is because of new insights (Kinashi, 2005; Laudanna *et al*., 2002), into the post-adhesion events that support leucocyte attachment to the endothelium, and into the molecules that are involved in leucocyte transendothelial migration (Imhof *et al*., 2004; Muller, 2003).

Selectin-ligand interactions with their particular ligands allow leucocytes to adhere to inflamed endothelium. This occurs in blood flow as they bind with specially high on- and off-rates (which regulate the speed with which bonds are formed and broken, respectively) (Alon *et al*., 1995). Present is also the active involvement of endothelial cells in rolling leucocytes. This consist the expression of E-selectin and P-selectin, important rolling molecules, through the expression of phosphoinositide 3-kinase-γ (PI3Kγ) (Puri *et al*., 2005). Integrins such as, α4β7-integrin and α4β1-integrin contribute to rolling and mediation of firm leucocyte adhesion (Berlin *et al*., 1995; Kerfoot *et al*., 2002; Ley *et al*., 2007; Vajkoczy *et al*., 2001).

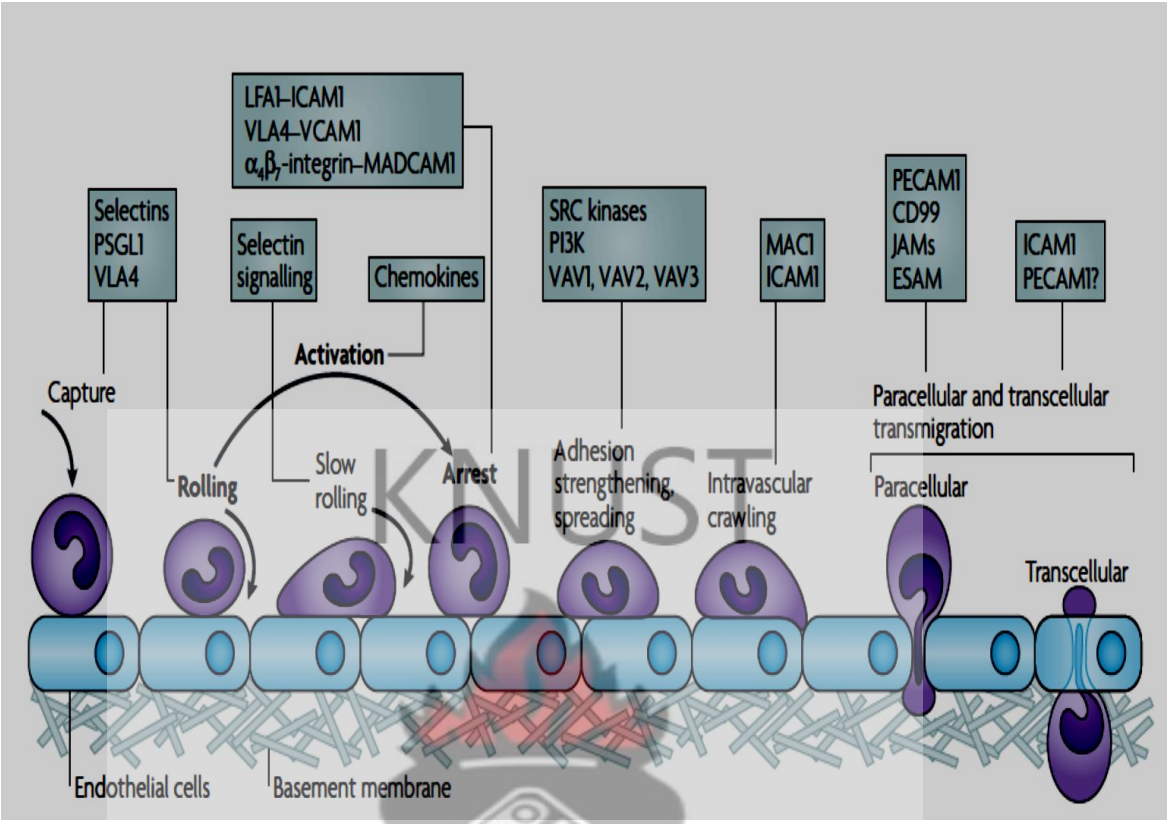


Figure 1.8: The updated leukocyte adhesion cascade.
Bolded words indicate the original three steps. Expanded steps include: Capture (or tethering), Slow rolling, Adhesion strengthening and Spreading, Intravascular crawling, and Paracellular and Transcellular transmigration. Key molecules involved in each step are itemized in boxes (Ley *et al.*, 2007)

During inflammation, endothelial cells are activated; this in turn causes the synthesis and expression of chemokines, lipid chemoattractants and adhesion molecules that exist on the luminal surface. Proteolytic cleavage of activated mast cells and platelet can also result in generation of chemoattractants. These can be transported to endothelial cells through circulating microparticles or exocytosis of intracellular granules on to the inflamed endothelium thus, trigger the arrest of rolling monocytes (Huo *et al.*, 2003; von Hundelshausen *et al.*, 2001).

Leucocytes membrane protrusions extend into the endothelial-cell body and junctions (Barreiro *et al.*, 2002; Carman *et al.*, 2004). These extensions, mediating ligation of ICAM1, is triggered by MAC1 (Phillipson *et al.*, 2006; Schenkel *et al.*, 2004). ICAM ligation is associated

with increased intracellular Ca^{2+} and activation of p38 MAPK and RHO GTPase. This response promotes transmigration through opening of endothelial-cell contacts via activation of myosin light-chain kinase (Huang *et al*., 1993; Muller, 2003). This leads to improved endothelial-cell contraction and therefore, opening of interendothelial contacts (Figure 1.8) (Ley *et al*., 2007). These events may promote leucocyte migration through endothelial junctions also known as paracellular route. Through this route, molecules that do not support leucocyte migration, and might cause difficulty to emigrating cells (such as VE-cadherin), may be spread away from the junctional regions (Shaw *et al*., 2001).

Leucocytes migrate through the endothelial basement membrane and collagen type IV, linked with molecules such as nidogen-2 and the heparin sulphate proteoglycan pelican (Hallmann *et al*., 2005) (Figure 1.8) and, in most venules, the pericyte sheath. Low-expression sites are co-localized with gaps between pericytes and so neutrophil migration occurs specifically at regions of least resistance, that is, gaps between adjacent pericytes and regions of low protein deposition within the extracellular matrix (Wang *et al*., 2008).

1.3.3 Phagocytosis

Phagocytosis is the process by which macrophages, dendritic cells and other myeloid phagocytes internalise diverse particulate targets. This is a central regulator of cell responds to a particle, thus a key mechanism of innate immunity. The role phagocytosis has in cell or tissue injury is gathering information about the particle, determining what threat it poses and stimulating a set of responses that are appropriate to the threat (Underhill *et al*., 2012).

Clearance of pathogenic microorganisms involves phagocytes detecting the microorganisms using surface receptors such as Fc receptors and dectin, internalising them. Also, some phagocytes internalise targets to get antigens for presentation to T cells, and most produce pro-inflammatory cytokines and chemokines that coordinate local and systemic inflammatory responses and direct adaptive immunity to develop (Underhill *et al*., 2012).

Internalisation of particulate targets varies and depends on the target and its location (Figure 1.9). Specific receptors on the cell surface initiate separate types of phagocytosis. For example, 'reaching' phagocytosis (also known as 'zippering' phagocytosis) is initiated by Fc receptors and dectin 1, while 'sinking' phagocytosis is initiated by complement receptors. Another process, macropinocytosis (also known as 'cell drinking') can be triggered by various microbial and self-stimuli. This result in the internalisation of particles bound to the cell surface (Underhill *et al.*, 2012). Intracellular membranes engulf organelles and cytoplasmic debris, by a process called autophagy, and this process can engulf intracellular microorganisms into a phagosome. In each case, internalised particles are degraded as the compartment acidifies and is filled with proteolytic enzymes. Neutrophil internalisation is improved by fusion of the phagosome with pre-packaged storage granules that have densely packed antimicrobial peptides and enzymes. Also units of the phagocyte NADPH oxidase may accumulate on the phagosome and produce antimicrobial reactive oxygen species (ROS) to enable killing (Underhill *et al.*, 2012).

The process of phagocytosis is in four stages; 'tasting', 'feeling', 'and swallowing' and 'digesting' (Figure 1.9). By 'tasting' and 'feeling' a target, phagocytes collect information about its chemical and physical properties. This is observed as contact of target with surface of phagocytic cells. This contact trigger two events: chemical sampling of the target through surface receptor engagement ('tasting') and physical exploration of the target by the plasma membrane ('feeling'). The recruitment and oligomerization of receptors that identify specific chemical constituents of the target leads the target to phagocyte surface. This starts certain intracellular signalling pathways, such as activation of NF-κB (nuclear factor-κB) by TLRs via MYD88 (myeloid differentiation primary-response protein 88). Such signalling may be necessary to induce cytokine production. Extension of pseudopodia and engagement of specific phagocytic receptors, describes the physical exploration of phagocytes. The ability of receptors to engage their ligands and propagate downstream signals (Rosas, 2008) is affected by several processes. These include the size of the particle bound to the cell surface, geometry and topography of target (Doshi *et al.*, 2010), strength, speed and duration of target binding

and internalisation. These also have an important influence on the inflammatory immune response to be activated (Brewer *et al.*, 2004).

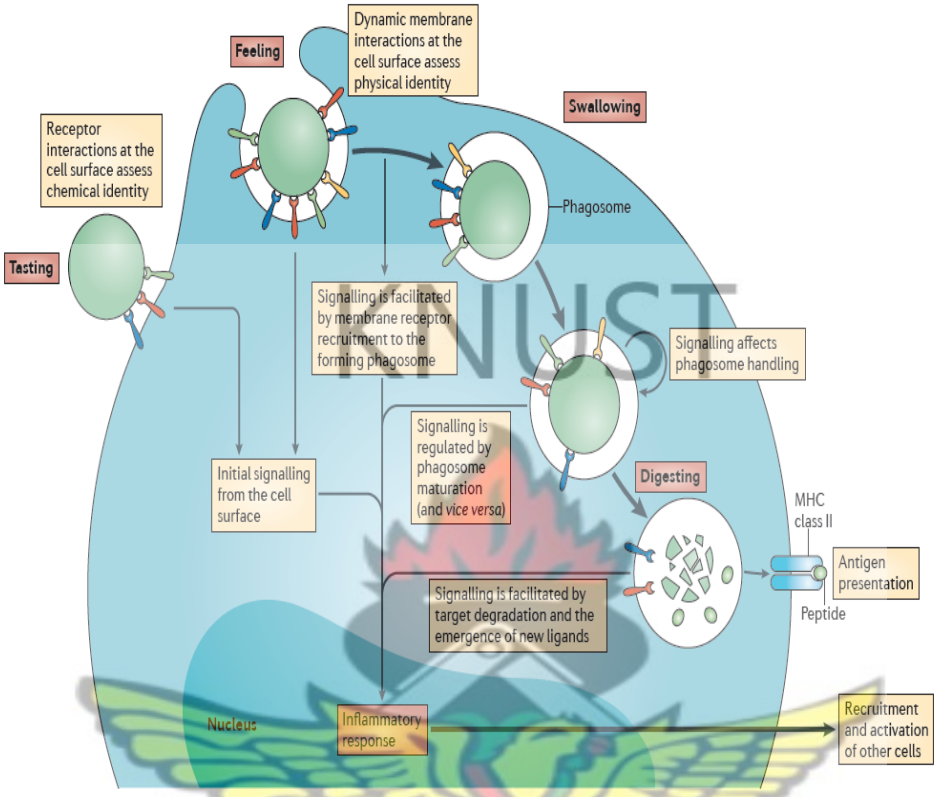


Figure 1.9: Information processing at different stages of phagocytosis. (Underhill *et al.*, 2012).

To enact immune response, receptors sample the chemical constituents of the particle and membrane dynamics facilitate an assessment of its physical properties at myeloid cell surface. Further information is gathered as phagosome nips off from the plasma membrane and as it matures through interactions with other intracellular compartments. Finally, target degradation exposes ligands that were not formerly accessible, releasing ligands into the cytosol for recognition by intracellular receptors (Underhill *et al.*, 2012).

In 'swallowing', when a target is engulfed and internalised, phagocytes further evaluate its form and chemical composition, and this regulates its fate within the cell. Under such circumstances, phagocyte control how the cell died. Cells that die by necrosis caused by injury or infection alert phagocytes to the presence of danger and signal that immune activation is required to deal with the threat (Torchinsky *et al.*, 2010). On the other hand, if cells die by apoptosis and there is no sign of infection or other danger, then they are removed and recycled, without inducing cytokine and chemokine production (Sauter *et al.*, 2000).

Digesting the target releases additional ligands that further inform the cell about the target and control the response of the cell (Underhill *et al.*, 2012). These additional released ligands could have previously been obscured. However, at this point, they are detected inside the cell through lysosomal rupture by either receptors on the phagolysosomal membrane or by sensors in the cytosol (Ip *et al.*, 2008; Underhill, 1999). Two different pieces of information could be delivered to cytosolic sensors by lysosomal rupture. First, components of the digested target that are released could be recognised as 'danger' signals, and second, disruption of the integrity of the intracellular compartment itself could be recognised as 'damage' (Hornung *et al.*, 2008).

1.3.4 Oxygen Radicals in Inflammation

Phagocytes can trigger the production of reactive oxygen species, ROS, most especially through TLR stimulation from phagosomes. The phagosomal ROS stimulation enhances bacteria killing or internalisation (West *et al.*, 2011). Pro-inflammatory cytokines such as interferon-*gamma*, interleukin-1 *beta*, and other cytokines are modulators of the inflammatory reactions and many of them promote induction of oxidative stress through the production of ROS (Nathan, 1992).

ROS include oxygen derived small molecules such as the oxygen radicals: superoxide, hydroxyl, peroxyl, and alkoxyl; or the non-radicals: hypochlorous acid, ozone, singlet oxygen, and hydrogen peroxide (Bedard *et al*., 2007). ROS production can either occur as a by-product of cellular metabolism (e.g., in mitochondria through autoxidation of respiratory chain units) or formed by enzymes with the primary function of ROS generation (Rojkind *et al*., 2002). Enzymes that quickly increase local H_2O_2 levels include the family of NADPH oxidases (Bedard *et al*., 2007) and other oxidases such as xanthine oxidase (Pritsos, 2000) and 5-lipoxygenase (Demiry¨urek *et al*., 1999).

Neutrophils produce two superoxide anions and transport two electrons from one NADPH across the membrane to the extracellular or intraphagosomal space causing an NADPH-oxidase-mediated respiratory burst. Superoxide produced is further converted into hydrogen peroxide either through spontaneous dismutation. This involves the expenditure of two protons, or promoted by the catalytic action of superoxide dismutase via the Fenton reaction in the presence of transition metals, in biological systems (Figure 1.10) (Maeda *et al*., 1998). Hydrogen peroxide alone and in union with the increase of myeloperoxidase (MPO) is responsible for bacterial killing (Figure 1.10) (Klebanoff, 2005; Nauseef, 2001). MPO, richly present in phagocyte granules, catalyses the conversion of halides and pseudo-halides such as Cl–, I–, Br–, and SCN– to form hypohalous acids or pseudo-hypohalous acids. HOCl, however, is the primary MPO product in neutrophils responsible for bacterial killing (Wittmann *et al*., 2012). The chief sources of reactive species in all cells are mitochondria, cytochrome P450 and peroxisome (Kröncke, 2003; Nathan, 2003).

Singlet oxygen ($^1O_2$), mainly involved in photochemical reactions, is reactive, though it does not contain unpaired electrons and therefore not a free radical. *In vivo*, the unstable singlet oxygen is dissociated to produce the superoxide anion radical by enzymatic activation of oxygen. This occurs through arachidonic acid pathway; by the mitochondrial respiratory chain reaction; oxidation of xanthine and hypoxanthine by xanthine oxidase; or by NADPH

oxidase.(Figure 1.10) (Ames *et al.*, 1993; Cui *et al.*, 2004). Reaction of this anion radical with nitric oxide by nitric oxide synthase (NOS) or dismutation by superoxide dismutase (SOD) can generate peroxynitrite or less reactive hydrogen peroxide respectively (Figure 1.10) (Cui *et al.*, 2004).

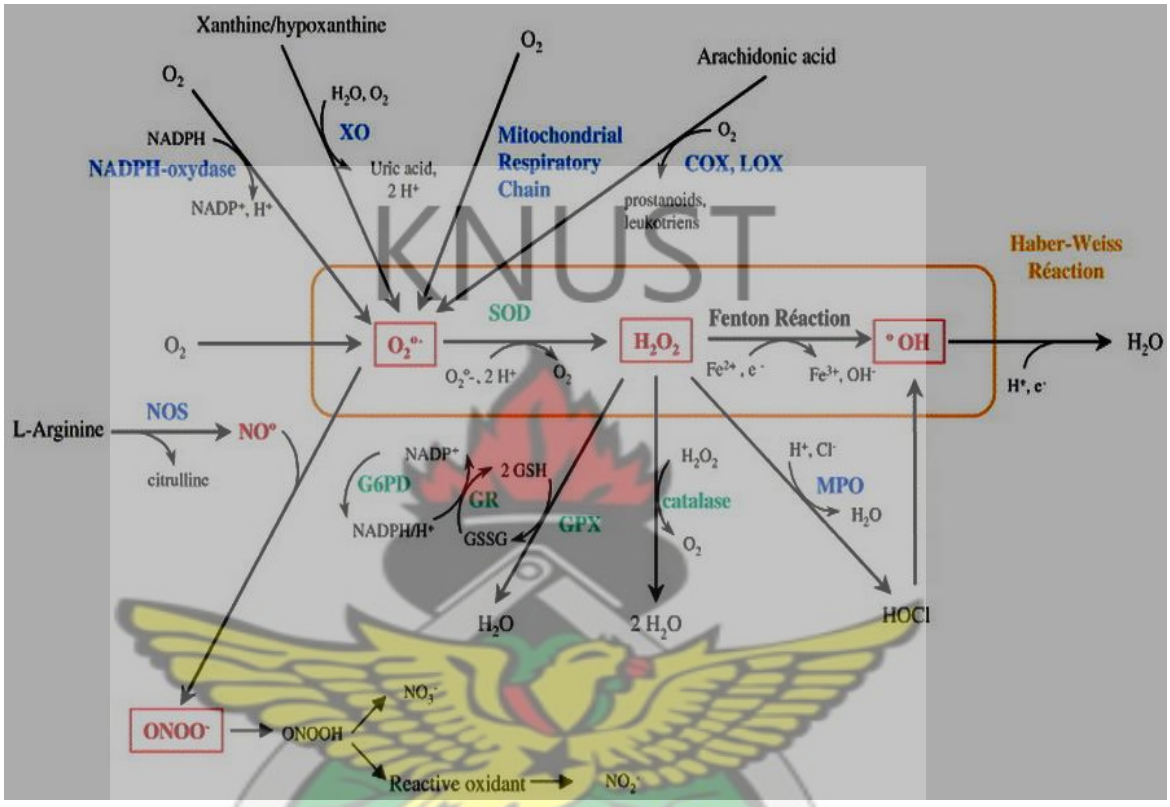


Figure 1.10: *in vivo* enzymatic activation of oxygen (Cui *et al.*, 2004)

Hydrogen peroxide is also an inactive molecule. Unlike superoxide, it can easily cross cell membranes. Hydrogen peroxide and superoxide could undergo further transformations in the presence of transition metals (mostly iron and copper) to produce the reactive hydroxyl radicals, by the Haber–Weiss or Fenton reactions (Figure 1.10) (Cui *et al.*, 2004). The imbalance in redox status and the improved production of intermediate reactive species gradually consumes the antioxidant defences, leading the cells to develop oxidative stress. Cells might react to these insults by improving their antioxidant potential or by activating the caspases that induce programmed cell death (apoptosis). Oxidative stress can also induce cell necrosis

and oncosis through the release of cytochrome c and the depletion of ATP at mitochondrial levels (Herrera *et al.*, 2001). For homeostasis to occur in cell, a series of protective molecules and systems, known as antioxidant defences, are produced. The antioxidant defences are produced as enzymes such as; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST), proteins that sequester transition metals, reduced glutathione (GSH), cysteine, thioredoxin, vitamins, etc. (Valko *et al.*, 2007).

1.3.4.1 Antioxidants

Antioxidants are substances able to counter the damaging effects of oxidation in body tissues (Scheibmeir *et al.*, 2005). Excess ROS is inactivated by endogenous or exogenous antioxidant molecules that have the capacity, even at low concentrations, to inhibit oxidation of a substrate (Cui *et al.*, 2004). This involves removing or lowering the local concentrations of one or more of the substrate in this reaction, such as oxygen, ROS, or metal ions (Fe^{3+} , Cu^{2+}, etc.), which catalyse oxidation. Inactivation also occurs as antioxidants interfere with the chain reaction that spreads oxidation to neighbouring molecules. Antioxidants can also improve endogenous antioxidant defences of the cell (Cui *et al.*, 2004).

Antioxidants are classified into; enzyme antioxidants, preventive antioxidants and scavenging or chain-breaking antioxidants. The enzyme antioxidants are enzyme cells with large number of antioxidants to prevent or repair damage caused by ROS. In mammalian cells the primary antioxidant enzymes found are SOD, CAT and GPx, a substrate-specific peroxidase (Figure 1.10) (McCord *et al.*, 1971). The SODs convert $O_2^{\bullet -}$ into H_2O_2 (Banniste *et al.*, 1987; Zelko *et al.*, 2002), whereas the catalases (CAT) and glutathione peroxidases (GPx) convert H_2O_2 into water (Brigelius-Flohe, 1999; Chelikani *et al.*, 2004). GPx requires some secondary enzymes, such as glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G-6-PD), and cofactors, such as reduced glutathione (GSH), NADPH and glucose 6-phosphate and the presence of its five isoenzymes, to be effective (Weydert *et al.*, 2010). SOD and catalase do not need co-factors to function. In the glutathione system, glutathione

reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) do not act on ROS directly, but they promote the GPx, in a coupling reaction pathway, to function (Liu *et al*., 2004).

Manganese-containing SOD (MnSOD) is localised in the mitochondria; copper- and zinc-containing SOD (Cu-ZnSOD) is located in the cytoplasm and the nucleus; and extracellular SOD (EcSOD) is expressed in some extracellular tissues (Corpas *et al*., 2001; Corpas *et al*., 2006; Wuerges *et al*., 2004). CAT, found in peroxisomes and cytoplasm and GPx, localised in many subcellular compartments like mitochondria and nucleus are also compartmentalised. To reduce oxidative stress in the various parts of the cell, antioxidant proteins with related enzymatic activity may have special effects after modulation because of different localisations within cells (Weydert *et al*., 2010). Another important enzyme is MPO. It is a major constituent of the azurophilic cytoplasmic granules (Bainton *et al*., 1971). It is also a classical heme peroxidase that uses hydrogen peroxide to oxidise variety of aromatic compounds (RH) by a 1-electron mechanism to give substrate radicals (R·) (Dunford, 1987; Heinecke *et al*., 1993; Hurst, 1991; Marquez *et al*., 1996). Most of the hydrogen peroxide produced by neutrophils is consumed by MPO (Kettle *et al*., 1997; Klebanoff, 1992), quickly oxidising chloride ions to the strong non-radical oxidant, HOCl (Harrison, 1976). HOCl is the most bactericidal oxidant formed by the neutrophil (Iyer *et al*., 1961; Klebanoff, 1968). Many species of bacteria are phagocytised by an MPO/hydrogen peroxide/chloride system (Albrich *et al*., 1982).

The preventive antioxidants, another class of antioxidant, act by binding to and sequestering oxidation promoters and transition metal ions, such as iron and copper, which have unpaired electrons and strongly hasten free radical formation. Examples include transferrin and lactoferrin (which bind ferric ions), ceruloplasmin (which binds Cu, catalyses the oxidation of ferrous ions to ferric due to its ferroxidase activity, and increases binding iron to transferrin). Other examples include haptoglobins (which bind haemoglobin), haemopexin (which binds heme), and albumin (which binds copper and heme) (Cui *et al*., 2004). The scavenging or chain-breaking antioxidants, on the other hand, present themselves for oxidation at early

stages in the free radical chain reaction. Thus result in low energy products that may not reproduce the chain further (Scheibmeir *et al.*, 2005). Lipid-soluble and water-soluble scavengers act in cellular sites that are either hydrophobic or hydrophilic, respectively. The major lipid-soluble scavengers are vitamin E (α-tocopherol), β-carotene, and coenzyme Q (CoQ), while ascorbic acid, various thiols, uric acid, and bilirubin are effective in the aqueous milieu (Cui *et al.*, 2004). Melatonin (*N*-acetyl- 5-methoxytryptamine), an endogenous substance formed in the pineal gland, is a powerful antioxidant, believed to be of significance to the nervous system (Reiter *et al.*, 2003; Shen *et al.*, 2002).

1.3.4.2 Plants as Source of Antioxidants

Protection of food against oxidation led to production of synthetic and natural food antioxidants. Prominent examples of synthetic antioxidants include phenolics such as, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). These phenolics are exploited in food, cosmetics and therapeutic industries. High volatility and instability at high temperature of BHT and BHA, led to setting up strict legislation on the use of synthetic food additives. This has led to the growing interest in natural antioxidants (Branen, 1975; Grice, 1986; Imida *et al.*, 1983; Ito *et al.*, 1983; Papas, 1999). The use of natural antioxidants in food, cosmetic, and therapeutic industry now serves as a promising alternative for synthetic antioxidants because of low cost, compatible with dietary intake and no harmful effects inside the human body. Many antioxidant compounds, naturally in plant sources have been identified as free radical or active oxygen scavengers (Brown *et al.*, 1998).

Plants produce large amount of antioxidants to prevent the oxidative stress caused by photons and oxygen during photosynthesis (Auddy *et al.*, 2003). The major group types include; carotenoids (that suppress ROS) ascorbate (that scavenge ROS and inhibit ROS-related damage) and mostly thiols, which are involved in repair. A vast number of other compounds such as the phenolic substances (mainly the flavonoids) have strong antioxidant activities *in vitro* (Jeon *et al.*, 2008). These compounds have recognised antioxidant, anticarcinogenic,

antimutagenic, antitumor, antiartherogenic, antiplatelet aggregation, antibacterial, antiviral and anti-inflammatory properties (Jeon *et al*., 2008; Ozgová *et al*., 2003).

1.4. INFLAMMATION AND ACUTE-PHASE AND OTHER SYSTEMIC RESPONSE

Many changes, distant from the site or sites of inflammation and involving many organ systems, accompany inflammation (Gabay *et al*., 1999). In 1930 interest concentrated on these changes by the discovery of C-reactive protein (so named because it reacted with the pneumococcal C-polysaccharide) in the plasma of patients during the acute phase of pneumococcal pneumonia (Tillett *et al*., 1930). These systemic changes have since been referred to as the acute-phase response (Kushner, 1993), even though they come with both acute and chronic inflammatory disorders. New acute-phase responses are recognised, and the mechanisms mediating them are becoming better understood (Gabay *et al*., 1999).

Conventionally, to protect the body against infection on tissue injury, inflammation brings about recruitment and activation of leucocytes and plasma proteins at the site of infection to remove the infectious agent (Kindt *et al*., 2004). The infectious microorganisms, after gaining bodily access to the site of injury, cause local inflammation (Lundberg *et al*., 2010). The local inflammatory response with major systemic response are known as acute phase response (APR) (Male *et al*., 2006). This response is distinct by inducing fever, anorexia, solmonescence, drowsiness. Other features include increased synthesis of hormones such as adrenocorticotropic hormone (ACTH) and hydrocortisone (Willey *et al*., 2008), increased leucocytosis and altered production of large number of proteins in liver (Zheng *et al*., 1995). Those proteins whose levels change during inflammation are termed acute phase proteins (APP) (Gabay *et al*., 1999).

Several bacterial components and products such as peptidoglycans, lipoteichoic acid, exotoxins, lipoproteins and glycolipids can begin the local inflammatory processes (Paul, 2008). Following bacterial invasion, various cell types living in the mucosa or skin may produce molecules important in controlling infections (Baumann *et al*., 1994). Among the main resident host cells are the mast cells, known for their stores of histamine, serotonin (Moshage, 1997) and for containing preformed TNF-α and various cytokines (Arnett *et al*., 2010). On contact with various bacterial products mast cells release these pro-inflammatory cytokines, which are important in recruiting neutrophils to the site of inflammation (Abbas *et al*., 2007). Key cytokines are IL-1, IL-6, and TNF-α, which have marked behavioural, neuroendocrine and metabolic effects (Rich, 2008). The concentration gradient of various tissue products released activates the vascular system and inflammatory cells. These responses in turn are related to production of more cytokines and other inflammatory mediators which spread to the extracellular fluid compartment and circulate in the blood (Ceciliani *et al*., 2002). Aside the cytokine mediated-rise of clinical symptoms, a chain of changes occur. These are changes in concentration of several APPs, activation of complement cascades, increased value of ACTH and glucocorticoids, and decreased serum levels of calcium, zinc, iron, vitamin A, and α-tocopherol (Moldawer *et al*., 1997). Acute phase proteins mediate opsonisation of microorganisms and activate complement units, while others scavenge cellular remnants and free radicals or neutralise proteolytic enzymes (Nairn *et al*., 2002).

Activation of complement cascade form complement products that act as chemotactic agents to recruit neutrophils (Finckh, 2009). Complement anaphylatoxins (C3a, C5a) induce local mast cells degranulation to release histamine, causing vasodilation and smooth muscle contraction (Gonda *et al*., 2009). Leucocytes, kallikrein and bradykinin move out through blood vessels causing swelling. Bradykinin then binds to close capillary cells and stimulates the synthesis of prostaglandins which then binds to free nerve endings making them to begin pain impulse (Quinton *et al*., 2009). Together, they all contribute to the rise of classical signs of inflammation: redness, increased heat, swelling, pain, and loss of function (Khan *et al*., 2010).

1.4.1 Acute Phase Response induced by Cytokines

Cytokines, intercellular signalling polypeptides produced by activated cells have several sources, many targets, and various functions. The cytokines formed during and taking part in inflammatory processes are chief stimulators of acute-phase proteins production. These inflammation-associated cytokines include IL-6, IL-1β, TNF-α, interferon-γ, transforming growth factor β (Kushner, 1993), and possibly IL-8 (Wigmore *et al*., 1997). They are produced by various cell types, but the most important sources are macrophages and monocytes at inflammatory sites (Gabay *et al*., 1999).

IL-6 is the chief stimulator of most acute-phase proteins production (Gauldie *et al*., 1987), whereas the other associated cytokines influence subgroups of acute-phase proteins. For example, in mice unable to express IL-6 (knockout mice), IL-6 role in stimulating the production of acute-phase proteins depends on the nature or site of the inflammatory stimulus (Fattori *et al*., 1994). This is similar with IL-1β knockout mice, presumably because IL-1β is vital in stimulating the production of IL-6 after administering turpentine (Zheng *et al*., 1995). These studies provide further evidence that patterns of cytokine production and the acute-phase response differ in different inflammatory conditions (Richards *et al*., 1996).

Cytokines work both as cascade and as network in stimulating the production of acute-phase proteins. Many cytokines can regulate the synthesis of other cytokines and cytokine receptors. For example, TNF-α is the main stimulator of IL-1 production in patients with rheumatoid arthritis (Feldmann *et al*., 1996). IL-1β may increase or decrease expression of its own receptors (Dinarello, 1996). The IL-6 response to turpentine injection in mice requires IL-1β; and IL-6 inhibits TNF-α expression (Xing *et al*., 1998).

The effects of cytokines on target cells may be inhibited or improved by other cytokines, hormones, or by cytokine-receptor antagonists and circulating receptors. Combinations of cytokines have additive, inhibitory, or synergistic effects on cellular responses (Mackiewicz *et al*., 1991). Thus CRP and serum amyloid A induction in some models requires both IL-6 and either IL-1 or TNF-α. The induction of fibrinogen by IL-6 is inhibited by IL-1, TNF-α,

and transforming growth factor β (Mackiewicz *et al*., 1991). IL-6 improves the effect of IL-1β in inducing the expression of IL-1–receptor antagonist (Gabay *et al*., 1997), and IL-4 inhibits induction of some acute-phase proteins by other cytokines (Loyer *et al*., 1993). Soluble IL-6 receptor-α molecules improve the effects of the ligand (Mackiewicz *et al*., 1992), whereas other soluble receptors, such as those for TNF-α and IL-1, are inhibitory. Glucocorticoids usually improve the stimulatory effects of cytokines on the production of acute-phase proteins (Baumann *et al*., 1987), whereas insulin decreases cytokine effects on the production of some acute-phase proteins (Campos *et al*., 1994).

1.4.1.1 Febrile Response

Fever suggests the neuroendocrine changes that characterise the acute-phase response. Though several cytokines may induce fever, IL-6 produced in the brainstem is essential for the final steps leading to fever (Dinarello, 1997). Fever depends on humoral signals from the body (Figure 1.11). It is coordinated by the hypothalamus and involves the orchestration of a wide range of endocrine, autonomic, and behavioural responses (Saper *et al*., 1994). Characteristic of fever is rise in body temperature, usually by 1 to 4 °C. The rise in temperature by even a few degrees may improve the efficiency of leucocyte chemotaxis and probably hinder replication of many offending microorganisms (Milton, 1982). Fever is a common symptom linked with various types of cerebral inflammation, including meningitis and encephalitis. Under such inflammatory conditions, one important process leading the fever is activation of the arachidonic acid cascade in the brain (Milton, 1982), in which membrane phospholipid is enzymatically converted to several kinds of bioactive prostaglandins (PGs). Among the PGs, PGE_2 is mainly the mediator of fever because it is pyrogenic when injected into the cerebral ventricle or into the preoptic area, the thermoregulatory centre and a presumed locus of PGE_2 action (Milton, 1982; Stitt, 1986).

Cytokines take part in signalling a fever (Conti *et al*., 2004). Peripheral cytokines signal the brain through four mechanisms to induce fever (Conti *et al*., 2004). They can enter the brain through regions lacking blood-brain barrier (specialised areas along the cerebral ventricular

surface). Cross the blood-brain barrier by specific transport mechanisms. Transmit a signal to the brain via the vagus nerve; and activate brain vasculature stimulating release of mediators such as prostaglandins (PGE), NO, or cytokines (IL-1β), which act on brain parenchymal cells.

Several exogenous substances can evoke fever and other brain-controlled sickness signs or symptoms when injected systemically into experimental animals. Such fever-inducing agents are called exogenous pyrogens. All these substances induce the production and release of immunoregulatory proteins, called cytokines. Circulating cytokines are the endogenous mediators of fever in response to administration of an exogenous pyrogen (Kluger, 1991; Roth *et al.*, 2001). *Lipopolysaccharide*s (LPS) of Gram-negative bacteria (e.g. E.coli) are used as exogenous pyrogen in most experimental studies. LPS is the principal part of outer membrane of Gram-negative bacteria e.g. *E. coli*. Depending on the route and injected dose of LPS, TNF-α is the first cytokine which appears in the circulation (Janský *et al.*, 2008; Kluger, 1991; Roth *et al.*, 2001). Followed by traces of IL- 1ß (Janský, *et al.*, 2008), and high amounts of IL-6 (LeMay *et al.*, 1990 ; Roth *et al.*, 1993) and IL-8 (van Zee *et al.*, 1991).

Bacterial LPS and pro-inflammatory cytokines up-regulate several enzymes in the brain which catalyse formation of small signal molecules such as nitric oxide (NO), carbon monoxide (CO) and PGE_2. The synthesis of PGE_2 depends on the activity of the two isoforms of cyclooxygenase (COX), the constitutively expressed COX-1 and the inducible form COX-2 (Cao *et al.*, 1995; Elmquist *et al.*, 1997). Production of PGE_2 via induction of COX-2 or via constitutive COX-1 is an important step in febrile response to LPS. Central injection of prostaglandins evokes fever (Blatteis *et al.*, 1997; Kluger, 1991; Zeisberger, 1999). The levels of PGE_2 in the cerebrospinal fluid from the third ventricle (Coceani *et al.*, 1988) and in microdialysis samples from the preoptic area/anterior hypothalamus (Blatteis, *et al.*, 1997) rise more or less comparable to the LPS-induced febrile response. Drugs which block prostaglandin synthesis could also effectively inhibit the febrile response (Blatteis *et al.*, 1997; Kluger, 1991; Zeisberger, 1999). Following strong evidence of the role of central PGE_2 in fever

induction, the following chain of events has been suggested: LPS appearing in the blood induces circulating cytokines. These, circulating cytokines and LPS induce another source of cytokines as well as COX-2 within the brain. Centrally produced cytokines are further triggers for COX-2 induction thus prolongs formation of PGE_2 within the preoptic area and the hypothalamus (Roth, *et al*., 2001). Studies show that intraperitoneally injected LPS (50 µg kg^{-1}) induced fever and kinin B_1 receptor mRNA expression in the rat hypothalamus (Souza *et al*., 1988). Sub-diaphragmatic vagotomy inhibits both fever and B_1, but not B_2 kinin receptor or ß-actin mRNA expression, signifying a participation of central hypothalamic kinins in the pyrogenic response to LPS (Souza *et al*., 1988). By acting on its constitutive B_2 and newly expressed B_1 receptors, bradykinin might trigger prostaglandin synthesis, which would act as one of the final mediators of fever (Roth *et al*., 2001).

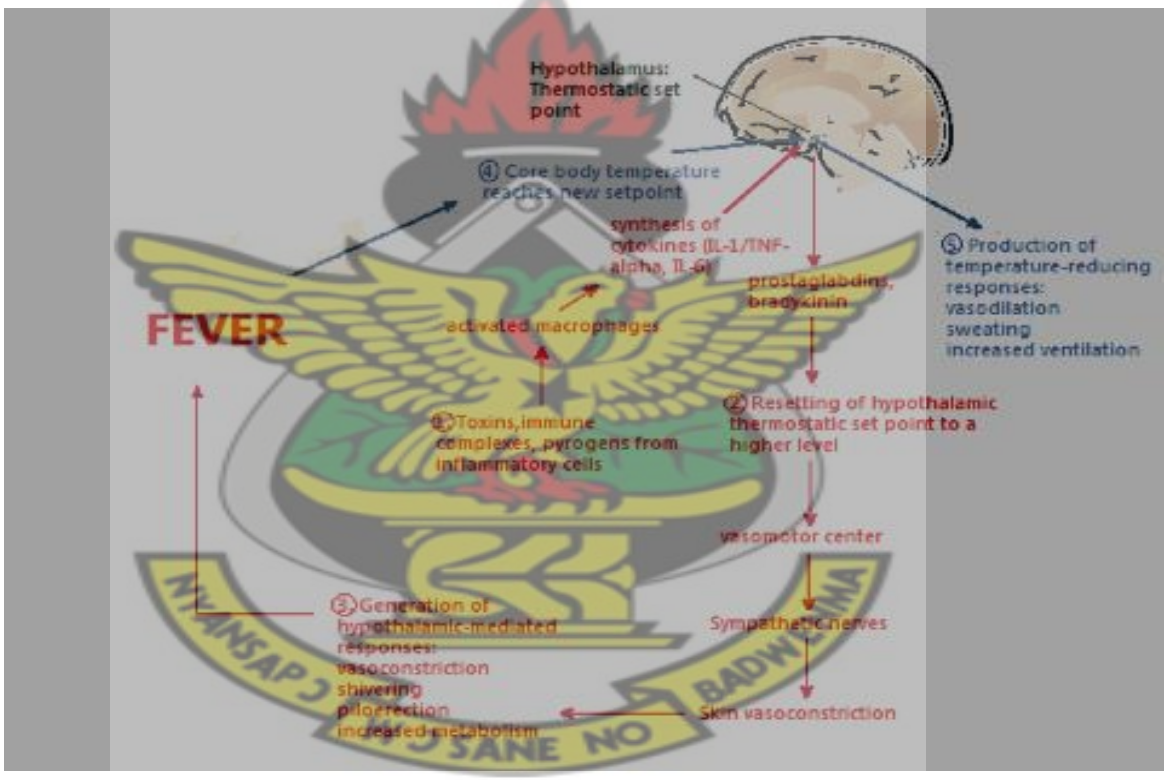


Figure 1.11: The Mechanism of fever

Regulation of body temperature requires a delicate balance between heat production and loss of heat, and the hypothalamus regulates the set point at which body temperature is

maintained. In fever this set point raises and antipyretics like paracetamol are used to reduce it (Gupta *et al*., 2005). Evidence of the participation of prostaglandins in the beginning of fever led to the use of NSAIDs as antipyretics as the action of these drugs depend on their ability to inhibit COX-1 and/or COX-2 activity (Vane *et al*., 1998).

1.5 THE ROLE OF MAST CELLS IN INFLAMMATION AND ANAPHYLAXIS

Most of the mediators synthesized or released in inflammatory responses are observed in anaphylactic responses with mast cell as the primary source of most of these mediators that trigger the release of other mediators. Mast cells are found in the skin and in all mucosal tissues at homeostasis, and numbers are raised in asthmatics lungs (Amin *et al*., 2000) gastrointestinal tract of inflammatory bowel disease (Amin, 2012). First described by Ehrlich in 1878, mast cells were identified and described based on their unique staining characteristics and large granules (noted as most of the body's histamine), with their cytoplasm stuffed with granular material. These are mesenchymal cells of the immune system that stain metachromatically with some blue dyes (Elhrlich, 1878). Their central role in inflammatory and immediate allergic reactions is the ability to release of potent inflammatory mediators. These mediators include; histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid that act on the vasculature, smooth muscle, connective tissue, mucous glands and inflammatory cells (Borish *et al*., 1992). The body's encounter with toxic substance and recognition of tissue injury by mast cells mediate the release of histamine. The histamine released by these means causes nearby blood vessels to dilate allowing more blood to get to the site of the injury or infection (Amin, 2012).

In circulation, mast cells are found as immature cells in tissues (Moon *et al*., 2010). Under the effect of local tissue micro-environmental conditions, mast cells differentiate into granules. These further mature through the activity of various cytokines such as stem cell factor (SCF),

a growth factor that binds to the c-Kit tyrosine-kinase receptor on the mast cell membrane (Chen *et al*., 2005; Kitamura *et al*., 2005). On its own SCF would not induce mast cell degranulation. However it enhances mast cell degranulation and cytokine production through cross-linking of their high affinity surface receptors for IgE (FcεRI) (Blank *et al*., 2004; Hundley *et al*., 2004; Kraft *et al*., 2007; Siraganian, 2003). Molecules such as nerve growth factor, NGF (Langford *et al*., 2003) promote mast cell maturation (Aloe *et al*., 1977), via tyrosine kinase receptors (Trk A, B, C), different from the c-kit activation by SCF (Tam *et al*., 1997). IgE and antigen immunoglobulin free light chains (Blank *et al*., 2004; Redegeld *et al*., 2005; Redegeld *et al*., 2002), anaphylatoxins, hormones are released under stress. Neuropeptides (Theoharides, 1996; Theoharides *et al*., 2004), such as, substance P (SP) (Matsuda *et al*., 1989) are also released under stress. Hemokinin (Zhang *et al*., 2000), neurotensin (NT) (Carraway *et al*., 1982), NGF (Bienenstock *et al*., 1987; Tal *et al*., 1997) are also released under stress (De Simone *et al*., 1990). Pituitary adenylate cyclase activating polypeptide (PACAP), (Odum *et al*., 1998; Seebeck *et al*., 1998) can trigger mast cell secretion (Foreman, 1987; Goetzl *et al*., 1990; Janiszewski *et al*., 1994).

Mast cells are intricately involved in regulating inflammatory process. This occurs as mast cells in innate immune responses, through their toll-like receptors (TLRs) identify bacterial, viral, and parasitic peptides such as pathogen-associated molecular patterns (PAMPs) and prompt the synthesis of pro-inflammatory cytokines (Frenzel *et al*., 2012). Mast cells also influence the humoral response by interacting with B-cells via CD40/CD40L and certain cytokines, inducing B-cell activation and proliferation and, most importantly, isotypic immunoglobulin recombination into IgE. The IgE thus formed activate the mast cells by binding to the FcεI receptor. Equally, mast cells can contribute to the activation, proliferation, and migration of dendritic cells, which are the main antigen-presenting cells, thus regulating the immune response (Frenzel *et al*., 2012).

Among systemic autoimmune diseases, rheumatoid arthritis (RA) engages the greatest role for mast cells (Frenzel *et al*., 2012). The pathophysiology of RA is characterised by chronic

inflammation of the synovial membrane, whose progressive hyperplasia into the typical rheumatoid pannus results in joint destruction. Many cells of the innate immune system contribute to the inflammatory response, including fibroblast-like synovial (FLS) cells, macrophages, and dendritic cells. T cells and B-cells play a crucial role in perpetuating the inflammatory response. Thus, mast cells may be involved in this regulatory process (Frenzel *et al*., 2012). In the collagen-induced arthritis mouse model, mice lacking mast cells do not develop arthritis (Lee *et al*., 2002). By producing TNF-α and IL-6, mast cells may induce macrophage activation, causing the synthesis of more pro-inflammatory cytokines. Also, the release by mast cells of tryptase and tryptase-heparin complexes may activate the fibroblast-like synoviocytes (FLS) cells via protease-activated receptor-2 (PAR-2). This activation may induce the release of several chemotactic factors that attract neutrophils and may contribute to inhibit the Fas/FasL-dependent apoptosis pathway, taking part in the synovial hyperplasia (Palmer *et al*., 2007; Shin *et al*., 2009). Finally, the FcεI receptor on the mast cell surface may display high affinity for the IgE/anti-citrullinated peptide anti-bodies (ACPAs) complex. Thus, among patients with RA, those with ACPAs may have a greater degree of mast cell activation than those without ACPAs (Suurmond *et al*., 2011).

1.6 MODELS OF INFLAMMATION

Developing new drugs to treat inflammatory diseases relies on the existence of suitable animal models to assess these new drug agents (Anthony *et al*., 1986). Several inflammatory models have been developed to study the intricate nature of the inflammatory process with both theoretical and clinical relevance (Peter, 2010). Agents known to induce inflammation include; carrageenan, Freunds adjuvant (subplantar injection of heat-killed bacteria, such as *Mycobacterium tuberculosis*), zymosan, dextran, yeast, turpentine, formalin, monosodium urate crystals (Higgs, 1989; Singh *et al*., 2000). Other agents include; injection of polymorphonuclear leucocyte (PMNL), chemotactic factors (e.g. N-formyl-leucyl-phenylalanine), leucotriene B4, vasoactive agents (e.g. platelet activating factor, serotonin, histamine, bradykinin), arachidonic acid (in acetone) and PGE_2 (Issekutz *et al*., 1989).

1.6.1 Models of Acute Inflammation

Although the most severe and unbearable inflammatory disease state is chronic in nature, study of acute inflammation is important. Models of acute inflammation are important for the following reasons: the discovery and screening of novel therapeutic agents and new drug agents and for studies of mechanisms involved in the transition of acute to chronic inflammation (Anthony, *et al*., 1986). Acute inflammatory responses may be induced by injection of inflammatory agents into various body parts of experimental animals. Examples of these are heat-killed bacteria (*Escherichia coli*), zymosan (Araico *et al*., 2007; Lucas *et al*., 2003), vasoactive agents (e.g. platelet activating factor and histamine, serotonin, prostaglandin, bradykinin) (Vasudevan *et al*., 2007). Arachidonic acid (Kang *et al*., 2008), carrageenan (Duwiejua *et al*., 1994; Jeon *et al*., 2008; Winter *et al*., 1962) are other examples. Yeast (Ushiyama *et al*., 2008; Zakaria *et al*., 2007), dextran (Lima *et al*., 2007; Melgar *et al*., 2007) and latex (Higgs, 1989; Shivkar *et al*., 2003) are other examples. Of these, the carrageenan-induced acute footpad oedema in laboratory animals first introduced by Winter *et al*. (1962) has been widely used to screen new anti-inflammatory drugs. This model remains an acceptable preliminary screening test for anti-rheumatic activity (Singh *et al*., 2000). Many mediators are involved in the inflammatory response elicited by various inflammogens. The early phase of acute

inflammation involves cellular influx associated with the release of mediators like histamine and serotonin. This is followed by production of bradykinin (BK) and prostaglandins (PGEs) (Di Rosa *et al*., 1971). All these mediators produce inflammation when injected subcutaneously in the rat paw (Vogel *et al*., 1997).

Traumatic injury triggers an acute inflammatory response in inflammatory sites. This produces systemic symptoms such as fever, increased numbers of circulating neutrophils (neutrophilia), increased heart rate (tachycardia), and sometimes a feeling of anxiety (Peter, 2010). Several exogenous substances can evoke fever and the other listed brain-controlled sickness signs or symptoms when injected systemically into experimental animals. Such fever-inducing agents are called exogenous pyrogens (Peter, 2010).

Lipopolysaccharide (LPS) has been used as exogenous pyrogen in most experimental studies. Depending on the route and injected dose of LPS, TNF-α is the first cytokine which appears in the circulation (Janský *et al*., 2008; Kluger, 1991; Roth *et al*., 2001). This is followed by traces of IL-1ß (Jansky, *et al*., 2008), and high amounts of IL-6 (LeMay *et al*., 1990 ; Roth *et al*., 1993). Also observed are IL-8 (van Zee *et al*., 1991) as well as other cytokines such as, macrophage inflammatory protein-1 (MIP-1) (Ziegler *et al*., 1991).

1.6.2. Models of Chronic Inflammation

The most studied models of chronic inflammation have been models of arthritis, the polyarthritis induced in rat with *Mycobacterium* (Freund's adjuvant arthritis) (Brand, 2005; Escandell *et al*., 2007; Hughes *et al*., 1989; Wang *et al*., 2008). Other models of arthritis developed over the last decade include polyarthritis induced by type II collagen in rats and mice (Bajtner *et al*., 2005; Griffiths *et al*., 2007; Subramanian *et al*., 2005; Williams, 2007). A condition resembling gout may be produced by injecting urate crystals into the synovial fluids of joints (Kannan *et al*., 2005; Scott *et al*., 2006). Chronic inflammatory reactions can be

produced by implantation of cotton wool pellets subcutaneously. These are afterwards removed and weighed to determine the extent of granulation (Khanna *et al.*, 2001). Injection of turpentine oil (into pleural cavity or subcutaneous pouch) provides a long-standing inflammatory reaction (Singh *et al.*, 2007). Of these models, the adjuvant induced arthritis was employed in this study.

Adjuvant induced arthritis (AIA) in rats, a chronic inflammatory disease characterised by infiltration of the synovial membrane and associated with destruction of the joints, resembles rheumatoid arthritis in humans (Behar *et al.*, 1995; Kumar *et al.*, 2002). AIA is induced by heat-killed cells of *Mycobacterium tuberculosis* and it mimics the immunological and biochemical features of Rheumatoid arthritis (RA) in which self-antigens are recognised as foreign bodies (Aota *et al.*, 1996; Ramprasath *et al.*, 2006). Its popularity stems from: its reproducibility; its relatively short time course (2-4 weeks) and its adaptability to drug screening patterns (Ishikawa *et al.*, 2005). Although the clinical course of AIA is different, it shares important features with RA: Poly synovitis, characteristic histological findings and finally erosive bone destruction (Aota *et al.*, 1996). In addition, the clinical response to anti-inflammatory agents is similar in RA and established AIA (Behar*et al.*, 1995). The only difference between rheumatoid and adjuvant disease are the absence of rheumatoid factor and the lack of genital or skin lesions in RA, common to adjuvant disease (Bersani-Amado *et al.*, 1990; Dick, 1972; Pearson, 1963; Pearson *et al.*, 1963). However, AIA in rats is widely used as a model for therapeutic and pathogenetic studies of chronic forms of arthritis (Pearson, 1963).

1.7 MODELS OF ANAPHYLAXIS

Vasoactive amines (histamine and serotonin) are produced all-or-none, when mast cells and platelets degranulate. They have complex effects on the vasculature, causing increased vascular permeability and vasodilation, or vasoconstriction, depending on the context. The immediate effects of their release by mast cells can be damaging in sensitised organisms, resulting in vascular and respiratory collapse during anaphylactic shock (Medzhitov, 2008;

Ruslan, 2008). Mast cells are important components in various biological processes of allergic diseases and anaphylaxis (Sang-Hyun *et al.*, 2005).

Anaphylaxis is an acute systemic allergic reaction mediated by the release of histamine in response to the antigen cross-linking of immunoglobulin E (IgE) bound to the FcεI receptor on the mast cells. The mast cells start the process of degranulation after activation via the FcεI receptor. This results in the release of a wide variety of other inflammatory mediators such eicosanoids, proteoglycans, proteases and several proinflammatory (Artuc *et al.*, 1999; Royer *et al.*, 2001; Royer *et al.*, 2001; Stassen *et al.*, 2001). Chemotactic cytokines such as TNF-α, IL-1, IL-4, IL-6, IL-8 and IL-13 and transforming growth factor-β (Artuc *et al.*, 1999; Royer *et al.*, 2001; Royer *et al.*, 2001; Stassen *et al.*, 2001) are also released. Recent studies showed that IL-6 up-regulates histamine production rather than increases its storage and is an important inducing factor in expressing IgE FcεI receptor (Conti *et al.*, 2002).

Activation of mast cells leads to phosphorylation of tyrosine kinase and mobilisation of internal Ca^{2+}. This is followed by the activation of protein kinase C, an increase of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB), and release of inflammatory cytokines. In addition, mast cells-mediated histamine release correlates with decreased cAMP levels (Alm, 1984; Botana *et al.*, 1994). The transcriptional factor NF-κB is an important mediator of cellular responses to extracellular signals and plays an important role in regulating pro-inflammatory molecules of cellular responses, especially TNF-α and IL-6 (Azzolina *et al.*, 2003).

Cell co-culture assays have been useful in revealing the molecular basis of mast cell effect. These *in vitro* results in disease are often vague. The most definite proof of mast cell contributions *in vivo* is drawn from research using two strains of mast cell-deficient mice; (WBxC57BL/6) F1-Kit^{W}/Kit^{Wv}(W/W^{v}) and C57BL/6Kit^{Wsh}/Kit^{Wsh}(W-sash). These mice carry mutations in the *c-kit* gene (white spotting locus, *W*) (Galli *et al.*, 1987; Grimbaldeston

et al., 2005; Kitamura *et al.*, 1978; Kneilling *et al.*, 2009; Nakano *et al.*, 1985). Tyrosine kinase-dependent c-kit signalling, suitable for mast cell development and survival are reduced in these mice (Galli *et al.*, 1987; Grimbaldeston *et al.*, 2005; Kitamura *et al.*, 1978; Kneilling *et al.*, 2009; Nakano *et al.*, 1985). Both W/W^v and W-sash mutations also show other phenotypic abnormalities; W/W^v mice are anaemic, neutropenic, have weakened melanogenesis and are sterile (Galli *et al.*, 1987). W-sash mice are fertile and not anaemic. They have neutrophilia, defects in skin pigmentation, show anxiety-like phenotype and show a time dependent loss in mast cells with full deficiency achieved only at 10–12 weeks of age (Grimbaldeston *et al.*, 2005; Nautiyal *et al.*, 2008).

A process known as "knock-in" is employed to prove mast cell contributions to a specific phenotype. In this process mast cell populations can be reconstituted in these mice without prior irradiation by systemic or local injections of either whole bone marrow or bone marrow-derived mast cells. Such "knock-in" strategies can also be exploited to find out which local mast cell subpopulation is significant in disease. However this is difficult because of the widespread nature of mast cells in many tissues (Nakano *et al.*, 1985; Sayed *et al.*, 2010; Tanzola *et al.*, 2003).

This research therefore employed models that are less complex, reproducible and effective in demonstrating the role of mast cells in inflammation, though further study into mast cells contribution to specific phenotype, as is recommended.

1.7.1 Compound 48/80-induced systemic and passive anaphylactic reaction

Mast cell degranulation can be stimulated by the synthetic compound 48/80. This is used as a direct and convenient reagent to study the mechanism of anaphylaxis (active and passive) (Ennis *et al.*, 1980). Compound 48/80 causes anaphylaxis which arises from the activation of

mast cells and basophils involving cross-linking of immunoglobulin (Ig) E and aggregation of FcεI receptor. On activation, mast cells and basophils rapidly release preformed mediators from secretory granules; histamine, tryptase, carboxypeptidase A, and proteoglycans (Peavy *et al*., 2008). Downstream activation of phopholipase A_2 (PLA_2), followed by COX and LOX, produces arachidonic acid metabolites (prostaglandins, leukotrienes, and platelet activating factor) (Kostopanagiotou *et al*., 2009). The inflammatory cytokine, TNF-α is released as a preformed mediator, and as a late-phase mediator with other cytokines and chemokines (Peavy *et al*., 2008). Many of these mediators account for the pathophysiology of anaphylaxis (Peavy *et al*., 2008). Characteristic features are noted during anaphylactic reaction as certain mediators are synthesised and regulated. Histamine stimulates vasodilation, and increases vascular permeability, heart rate, cardiac contraction, and glandular secretion. Prostaglandin D_2 is a bronchoconstrictor, pulmonary and coronary vasoconstrictor, and a peripheral vasodilator. Leukotrienes produce bronchoconstriction, increase vascular permeability, and promote airway remodelling. PAF is also a potent bronchoconstrictor and increases vascular permeability. TNF-α activates neutrophils, recruits other effector cells, and enhances chemokine synthesis (Ogawa *et al*., 2007). These intersecting physiological effects contribute to the general pathophysiology of anaphylaxis that is observed as generalised urticaria and angioedema, bronchospasm, respiratory symptoms, hypotension, syncope, cardiovascular symptoms, nausea, cramping, and other gastrointestinal symptoms (Peavy *et al*., 2008).

In humans, anaphylaxis independently of IgE, show alternative mechanisms such as complement anaphylatoxin activation, neuropeptide release, immune complex generation, cytotoxicity, T-cell activation, or even multiple mechanisms (Simons *et al*., 2007). Mouse models have offered an alternative mechanism for anaphylaxis in which two mechanisms of anaphylaxis have been proved. One pathway involves IgE, the cross-linking of FcεI receptors, mast cell degranulation, and the release of histamine and PAF. Another pathway involves IgG, the IgG receptor, FcγRIII, and the release of PAF, not histamine, as the major mediator (Baeza *et al*., 2007; Finkelman, 2007; Finkelman *et al*., 2005; Nauta *et al*., 2007; Strait *et al*., 2002). Mouse models for studying mast cell activation, degranulation and anaphylaxis offer the

chance to study knockout models to determine the role of specific genes to general signal transduction pathways (Peavy *et al*., 2008).

Another way to test the anaphylactic reaction is to induce passive cutaneous anaphylaxis, PCA (Cypcar *et al*., 1996). PCA is an anti-IgE antibody induced *in vivo* model of anaphylaxis in local allergic reactions in which mast cells play an important role. Mediator secretions in PCA differ from that in compound 48/80-induced systemic anaphylaxis. It results from aggregation of specific IgE receptors (FcεI) on the surface of mast cells by the corresponding antigen (Dai *et al*., 2004). TNF-α and IL-6 released from activated mast cells further contribute to allergic inflammation (Kim *et al*., 2005). TNF-α is an autocrine stimulator as well as inducer of IL-6 (Halayko *et al*., 2003; Van Wagoner *et al*., 1999). TNF-α also contributes to allergic rhinitis in mice (Metcalfe *et al*., 1981). IL-6 up-regulates histamine production, induces the expression of FcεI receptor and enhances IgE-dependent histamine release from mast cells (Kikuchi *et al*., 2002). To understand and examine the mechanism of action of anaphylaxis, vascular permeability of the skin can be examined with Evans blue dye which stains extravasated cells blue (Choi *et al*., 2010; Agnieszka *et al*., 2010). The extent of degranulation, measured as mast cell stability can be measured through histological means with toluidine blue staining (Crookham *et al*., 1991; Luna, 1968; Sheehan *et al*., 1980).

Enzyme-linked immunosorbent assay, ELISA, can also be used to find out the effect of new compound or drug substance on the IgE-mediated cytokine secretion levels. Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilised on a solid support (usually a polystyrene microtiter plate). This is done either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilised, the detection antibody is added, forming a complex with the antigen. The detection antibody covalently links to an enzyme, or is detected by a secondary antibody, linked to an enzyme through bio-conjugation. Between each step, the

plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which suggests the quantity of antigen in the sample (Adler *et al*., 2009; Leng *et al*., 2008; Lequin, 2005).

1.8 MANAGEMENT OF INFLAMMATION

Acute inflammation is self-limiting, with many cell types and tissues involved in initiation and termination of the acute phase (Schwab *et al*., 2006). However, inflammation often results in tissue injury because of a direct destructive action or activation of a reparative process that alters tissue functions (Schmid-Schönbein, 2006). The principal anti-inflammatory drugs are non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs (glucocorticoids). Others are the anti-rheumatoid drugs and drugs used for gout (Fairchild *et al*., 2004; Furst *et al*., 2001). Among drugs used to treat rheumatic diseases, distinction is made among the NSAIDs, the DMARDs, and glucocorticoids. The NSAIDs are structurally diverse group of drugs that provide symptomatic relief in inflammatory joint and active osteoarthritis (OA). These reduce swelling, joint pain and stiffness and improve joint mobility. But have little effect on the underlying tissue degenerative processes that lead to cartilage loss and bone damage (Day *et al*., 2004).

DMARDs, on the other hand, have little or no acute anti-inflammatory or analgesic properties, but act, usually over weeks or months, to slow down or stop progresses to RA (Fries *et al*., 1996). The mechanisms of action of DMARDs are complex and vary, often remaining unclear, but they all result in slow progression of joint destruction and, in some patients, remission. Glucocorticoids also have multiple sites of action in rheumatic diseases. Although they inhibit disease progression (Kirwan, 1995), they are restricted to use in severe cases because of long-term side effects. Side effects are disadvantages of the other groups of drugs as well. NSAID use is often associated with gastrointestinal toxicity and DMARDs can cause skin, liver, kidney and gastrointestinal side effects (Day *et al*., 1987). Therefore, in mild cases of RA and

in OA (which is associated with less intense inflammatory lesions), simple analgesics, such as paracetamol, are often prescribed, mainly in Anglo-Saxon countries (Hochberg *et al*., 1995). Repeated attempts have been made to develop drugs that either protect cartilage or stimulate cartilage repair and have few adverse effects.

In treating RA, various approaches are used. These are used in different combinations and at different times during the disease and are chosen according to the patient's individual situation (Chen *et al*., 2005; Tlustochowicz, 2006). All treatment approach targets the same set of goals. Relieve pain, reduce inflammation, slow down or stop joint damage and improve a sense of well-being and ability to function (Atzeni *et al*., 2007; Smolen *et al*., 2005). Current treatment strategies include pain-relieving drugs and medications that slow joint damage. A balance between rest and exercise allow most people with the disease to lead active and productive lives (Kirwan *et al*., 2007; Lee *et al*., 2006; Wells *et al*., 2008).

Scientists studying rats with condition that resembles rheumatoid arthritis in humans aim at finding the genetic basis of rheumatoid arthritis (Kumar *et al*., 2002). Researchers are searching for new drugs or combinations of drugs that can reduce inflammation and slow or stop the progression of rheumatoid arthritis with few side effects. New therapeutic strategies for chronic forms of arthritis have to aim at both, suppression of inflammation and bone protection. To achieve these goals it is important to understand the different stages of disease progression and to identify relevant targets—that is, most relevant cell type(s) or cytokine(s) or both (Goldblatt *et al*., 2005; Moreland, 2005).

A report from mailonline.com reported by Mashta (2011) under the headline "latest drug treatment for rheumatoid arthritis" named a new drug Remicade, as a breakthrough in treating RA. It is administered either self-injected by patients weekly, or given through an intravenous drip once every two months in hospitals. It describes the drugs ability to ease

pain and prevent disease from progressing. The drug was developed as a TNF-α blocker since TNF-α is noted to be a key trigger for inflammation, especially RA. Remicade acts by switching off TNF-α, neutralising its action. It helps the joint to recover but does not cure RA. This report also stated that 20 % of patients do not respond to the drug, reasons yet unknown. The drug is expensive (cost up to £ 7, 000 per patient, per year), though available as a subcutaneous injection (similar to diabetes injection).

Recently a protein, known as iRHOM2, has been identified as a potential new target for drugs to treat RA patients. The protein is encoded by the gene Rhbdf2, and it regulates the maturation of the TNF- α convertase, TACE, which controls shedding or release of TNF- α and its biological activity *in vivo*. TACE is a possible target to treat TNF- α –dependent diseases, such as RA. Concerns are raised about its potential side effects if targeted, since TACE also protects the skin and intestinal barrier by activating epidermal growth factor receptor (EGFR) signaling (Blaydon *et al*., 2011; Issuree *et al*., 2013). To prevent compromising the protective functions of TACE, models were used to investigate how it can be selectively targeted. It was identified that molecules that regulate TACE are iRHOM1 and iRHOM2. Inactivation of Rhbdf2 caused a down-regulation of iRHOM2 and allowed tissue-specific regulation of TACE to selectively prevent its maturation in immune cells, without affecting its homeostatic functions in other tissues. This inactivation did not affect the expression of iRHOM1, which is widely expressed, except in haematopoietic cells, supporting TACE maturation and shedding of the EGFR ligand TGF- α in Rhbdf2-deficient cells (Issuree *et al*., 2013). Mice lacking Rhbdf2 showed attenuation of inflammatory arthritis in K/BxN mouse RA model, to the same extent as mice lacking TACE in myeloid cells or TNF- α -deficient mice (Issuree *et al*., 2013). The underlying mechanism showed that two main drivers of K/BxN arthritis complement C5a and immune complexes, stimulated iRHOM2/TACE-dependent shedding or release of TNF-α in mouse and human cells. Thus the findings revealed that iRHOM2 and myeloid-expressed TACE play a significant role in inflammatory arthritis suggesting iRHOM2 as a potential therapeutic target for selective inactivation of TACE in myeloid cells. Therefore it was proposed that antibodies or pharmacological compounds that can block the function of

iRHOM2 can functionally help RA patients (Issuree *et al.*, 2013). Such reports support identification and production of new drug agents with same or better efficacy and potency which is accessible and affordable to more people and can target specific cells.

1.9 MAST CELLS AND THERAPEUTIC POSSIBILITIES

By its pivotal position in the inflammatory process, the mast cell may serve as a treatment target in certain chronic inflammatory diseases. Effects on mast cells can be exerted at three different levels (Figure 1.12):

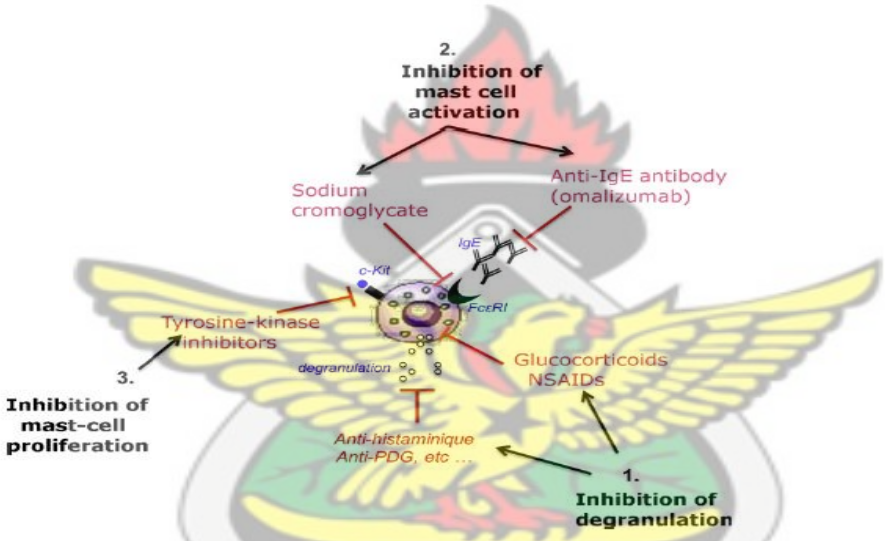


Figure 1.12: The three levels of action of anti-mast cell treatments.
Degranulation inhibition by antihistamines, glucocorticoids, or nonsteroidal anti-inflammatory agents. Inhibition of mast-cell activation by IgE antibodies and sodium cromoglycate. Inhibition of mast cell proliferation by tyrosine-kinase inhibitors targeting the c-Kit receptor, such as imatinib or masitinib (Paul *et al.*, 2010).

•**1.** ***Molecules produced by mast cells***: Some of these molecules can be inhibited directly, for instance by antihistamines or antagonists to PDGF receptors or to chemoattractants (e.g., CysLT), which can interfere in inflammatory cell recruitment by mast cells. Anti-inflammatory agents such as glucocorticoids inhibit the production of mediators by mast cells;

•**2.** ***Mast cell activation, via interaction between the FcεI receptor and IgE:*** This effect is the main mechanism of action on mast cells of the anti-IgE antibody omalizumab, used to treat asthma. Sodium cromoglycate may also act at this level, by controlling mast cell membrane stability via the flow of calcium. Tyrosine-kinase inhibitors of Src or c-Kit may block the production of these mediators;

•**3.** ***Mast cell proliferation:*** Several tyrosine-kinase inhibitors are under evaluation or already available on the market (e.g. imatinib, masitinib, and midaustorin), whose efficacy in SMCD seems well established. These tyrosine-kinase inhibitors act on c-Kit to block mast cell proliferation (Dubreuil *et al*., 2009; Paul *et al*., 2010).

1.10 AIMS AND OBJECTIVES

1.10.1. Justification

Most of the drugs presently used as anti-inflammatory, antipyrectic and antianaphylactic agents include the non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying antirheumatic drugs (DMARDs), glucocorticoids and some drugs that target the immediate-type of hypersensitivity. The several and lethal side effects of most of these agents have created more research into other effective anti-inflammatory and anti-anaphylactic agents with insignificant side effects at therapeutic doses and probable therapeutic benefits.

Beginning from ancient times, plants have been used as medicine by man. Even in the age of modernisation and civilisation, plants still serves as a valuable part of the society especially medically. Developing countries dwell on plant-based medicines through folklore or advice from plant medicine specialists. New drug agents are also developed from plants. African native herbal medicines are extensively used all over the African continent, even though an obvious lack of scientific proof for their quality, safety and efficacy (Johnson *et al*., 2007).

Intense search for plants with medicinal value started decades ago and it still advancing. Exploration in this part of the world, specifically, Ghana revealed many plants with therapeutic features (Duwiejua *et al*., 1994). Of such is *Trichilia monadelpha* (*Meliaceae*). This plant is a potent and efficacious agent in many disease conditions (Ainooson *et al*., 2012; Oyelowo *et al*., 2011). It also has several non-medicinal properties (Burkill, 1985; Dennis *et al*., 2004; Mshana *et al*., 2000).

1.10.2. Aim

The aim of this study is to explore the possible mechanism of action of the stem bark extracts of the plant using rodent models. Such as mediators-induced inflammation, models of acute inflammation and arthritis, anaphylaxis and pyrexia to further support the ethno medical use of the plant in managing inflammatory associated diseases.

1.10.3. Objective

Several discoveries and assumptions on the pharmacological effect of the plant are made. However there is no scientific information on the probable mechanism of action presented by the plant particularly its anti-inflammatory properties. The objective is to explore the possible mechanism of action of the stem bark extracts of *Trichilia monadelpha* using animal models.

Specific objectives include:

- Anti-inflammatory activities of extracts in acute inflammation and its associate mediators released and synthesised during the acute inflammatory response. These mediators associated with carrageenan-induced oedema in the two-phase mechanism involve histamine, serotonin (5-HT), prostaglandin E_2, PGE_2 and bradykinin, and levels of C-reactive protein, CRP, as a marker of acute phase proteins.

- Effects of extracts against systemic inflammatory response observed as pyrexia.

- Anti-inflammatory activities of extracts in chronic inflammation and some important features such as effects of extracts on levels and activity of antioxidant enzymes, pro-inflammatory cytokines, and bone morphology.

- Anti-anaphylactic effects of extracts were also evaluated

- Phytochemical screening and further *in vitro* antioxidant properties were evaluated such as: phenol test (Benklebia, 2005), reducing power test (Oyaizu, 1986) and DPPH-scavenging free radical assay (Govindarajan *et al.*, 2003)

Chapter 2

PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Plant collection

The stem bark of *Trichilia monadelpha* was obtained from Bomaa in the Tano North District (latitude 2°10′01.63″ W, longitude 7°05′06.82″ N, 787 feet above sea level) (State Geographer, 2012) of the Brong-Ahafo Region, Ghana between August and October, 2010. The leaves of the plant were authenticated by Dr. Sam, and verified by Dr. K. Annan of the department of Herbal Medicine, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. A voucher specimen was kept in the Faculty of Pharmacy Herbarium (No. FP/079/10).

2.1.2 Preparation of stem bark extracts

The plant bark (50 kg) was chopped into pieces, sun-dried for fourteen days and pulverised into fine powder (15.3 kg). The powder was soxhlet-extracted serially with petroleum ether, ethyl acetic acid and 70 % (v/v) ethanol, for four days. The extracts got were labelled, as follows; EAE (ethanol extract), PEE (petroleum ether extract) and EthE (ethyl acetic acid extract). The resulting extracts were concentrated under reduced pressure at 40 – 60 ˚C to a dark-brown syrupy mass in a rotary evaporator. The syrupy mass was then dried using water bath and kept in a desiccator. The final yields were 9.6 %, 0.9 %, and 0.7 % respectively.

2.2 PHYTOCHEMICAL ANAYLSIS

Phytochemical analysis of the extracts was as described in literature (Tiwari *et al*., 2011; Trease *et al*., 1989).

2.2.1 Test for Tannins and Phenolic compounds

About 0.5 g of each of the plant extracts (PEE, EthE, and EAE) was boiled with 25 ml of water for 5 minutes. It was then cooled, filtered and the volume adjusted to 25 ml. To 1 ml aliquot of each extracts, 10 ml of water and 5 drops of 1 % lead acetate solution was added. White precipitate indicated the presence of tannins (Kokate, 2005).

a) The boiling, filtering above were repeated. To 1 ml aliquot of each extracts 3-4 drops of neutral 5 % ferric chloride solution was added. Formation of dark-green colour indicated the presence of phenols (Kokate, 2005).

b) To about 1 g of each extracts, 1 % gelatin solution containing sodium chloride was added. Formation of white precipitate indicated the presence of tannins (Tiwari *et al*., 2011).

2.2.2 Test for Alkaloids

Five grams of the each extracts was stirred with 5 ml of 1 % aqueous HCl on water bath and then filtered. Of the filtrates, 1 ml of each extract filtrates were taken into test tubes and tested for the presence of alkaloids (Fulton, 1932).

a. To 1 ml of each extracts, 1 ml of Dragendroff's reagent (potassium bismuth iodide solution) was added. An orange-red precipitate indicated the presence of alkaloids.

b. To 1 ml of each extracts, 2 ml of Wagner's reagent (iodine in potassium iodide) was added. A reddish brown coloured precipitate indicated the presence of alkaloids.

2.2.3 Test for Steroids

a) One gram of extracts was dissolved in 10 ml of chloroform and filtered. The filtrates were treated with few (3-4) drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicated the presence of triterpenoids. There was also a bluish red to cherry colour in the chloroform layer and green fluorescence in the acid layer noted. This observation shows the presence of steroidal components. This test is known as the Salkowski's test (Wall *et al*., 1954).

b) One gram of extracts was dissolved in few drops of chloroform, 3 ml of glacial acetic acid and 3 ml of acetic anhydride were added. This solution was warmed and cooled under running tap water. Few drops of Conc. Sulphuric acid were added along the side of the test tubes. Appearance of bluish-green colour showed the presence of phytosterols. This test is known as the Libermann-Burchard's test (Wall *et al*., 1954).

2.2.4 Test for carbohydrates

One gram of extracts was dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates (Tiwari *et al*., 2011). One gram of extracts was dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates (Tiwari *et al*., 2011).

a) **Benedict's test:** 1 ml of filtrates was added to 5 ml Benedict's reagent and heated gently for 2 minutes and cooled. Orange red precipitate indicated the presence of reducing sugars

b) **Fehling's Test:** 1 ml of filtrates was hydrolysed with dil. HCl, neutralised with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicated the presence of carbohydrates.

2.2.5 Test for Flavonoids

a. **Shinoda's test:** About 1 g of the extracts was further dissolved with 5 ml of ethanol (98 %). To this was added a small piece of magnesium foil metal, this was followed by drop wise addition of concentrated hydrochloric acid. Magenta colour indicated the presence of flavonoids (Brain *et al*., 1975).

b. Few drops of sodium hydroxide solution were added to each extracts in test tubes. Formation of yellow colour precipitate indicated the presence of flavones, yellow to orange indicated the presence of flavonones and blue to violet indicated the presence of anthocyanines (Tiwari *et al*., 2011).

c. In a test tube 1 g of extracts were placed and covered with filter-paper moistened with dil. NaOH, then heated on water bath for a few minutes. The filter-paper was examined under UV light, yellow fluorescence indicated the presence of coumarins (El-Tawil, 1983).

2.2.6 Test for glycosides

Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

a) **Keller-Killiani's's test:** 1 ml of each extracts was mixed with 5 ml of 70 % alcohol for 2 minutes. This was filtered and to the filtrates was added 10 ml of water and 0.5 ml of lead acetate. This was filtered and the filtrate was shaken with 5 ml chloroform. The chloroform layers were separated in a porcelain dish and the solvent removed by evaporation. This was cooled and dissolved in 3 ml glacial acid containing 2 drops of 5 % ferric chloride solution. The solution was carefully transferred to the surface of 2 ml Conc. Sulphuric acid. A reddish brown layer formed at the junction of the two liquids and the upper layer which slowly became bluish green and darkening with standing indicated the presence of cardiac glycosides (Harborne, 1998).

b) **Borntrager's test:** Few drops of dilute sulphuric acid were added to 1 ml of the extracts, boiled and filtered. The filtrate was extracted with chloroform. The chloroform layer was treated with 1 ml of ammonia. The formation of red colour on the ammonical layer showed the presence of anthroquinone glycosides (Harbourne, 1984; Sofowora, 1993).

2.2.7 Test for Saponins

a) **Froth Test:** Extracts (1 g) were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins (Tiwari *et al*., 2011).

b) **Foam Test:** 0.5 g of extracts was shaken with 2 ml of water. Foam produced which persisted for ten minutes indicated the presence of saponins (Trease *et al*., 1983).

2.3 RESULTS

The presence of alkaloids, tannins, saponins, flavonoids, terpenoids, and glycosides in the EAE, and alkaloids, steroids, and terpenoids in PEE, confirms findings by Ainooson *et al*. (2012). Other compounds found to be present in PEE include; coumarins, phenols, phytosterols and reducing sugars. For EAE, other compounds found to be present include; reducing sugars, phytosterols, cardiac glycosides and phenols. EthE showed the presence of steroids, tannins, alkaloids, glycosides and reducing sugars, confirming the study by Woode *et al*.(2012) (Table 2.1).

Table 2.1: Phytochemical constituents of stem bark extract of *Trichilia monadelpha*

TESTS	PEE	EthE	EAE
Tannins and Phenolic compounds			
• Lead acetate test (tannins)	-	+	++
• $FeCl_3$ test (phenols)	-	-	++
• Gelatin test (tannins)	-	+	++
Alkaloids			
• Dragendroff's test (alkaloids)	+	+	+
• Wagner's test (alkaloids)	+	+	+
Steroids			
• Salkowski's test (triterpenoids)	++	++	+
• Libermann-Burchard's test (phytosterols)	++	++	+
Carbohydrates			
• Benedict's test (reducing sugars)	+	+	+
• Fehling's test (carbohydrates)	+	+	+
Flavonoids			
• Shinoda's test (flavonoids)	-	-	++
• Alkaline Reagent test (flavonones, yellow to orange ppt.)	-	-	++
• Fluorescence test (courmarins)	+	-	-
Glycosides			
• Keller-Killiani's test (cardiac glycosides)	-	+	++
• Borntrager's test (anthraquinone glycosides)	-	+	++
Saponins			
• Frost test (saponins)	-	-	++
• Foam test (saponins)	-	-	++

–: Not detected, +: Present in low concentration, ++: Present in moderate concentration.

2.4 DISCUSSION

The phytochemical screening carried out confirmed that carried by Ainooson, *et al.* (2012) and Woode, *et al*. (2012). New compounds were found to be present; reducing sugars, coumarins, cardiac glycosides and anthroquinone in PEE; phenols, reducing sugars, cardiac glycosides

and anthroquinone in EAE. Ethyl acetate, a lightly polar solvent, was used and this possibly extracted compounds which were lightly polar from the stem bark of the plant. EthE contained triterpenoids, reducing sugars, tannins, alkaloids, cardiac glycosides and anthraquinones were indicated to be present. The phytochemical constituents detected in the three extracts of *Trichilia monadelpha* could contribute to its traditional therapeutic use of the whole stem bark.

Flavonoids, a group of polyphenols, are free radical scavengers, antioxidants which have anti-inflammatory activity, prevent oxidative cell damage through their water soluble property and have strong anticancer activity (Okwu, 2001; Okwu, 2001; Salah *et al*., 1995). Coumarins are potential antioxidants, according to studies, with the ability of scavenging free radicals and chelating metal ions (Tseng, 1991). Saponins (mostly responsible for the bitter taste in plants), glycoside of triterpenes or steroids (possess anti-inflammatory effects) including the group of cardiac glycosides (acting on cardiac muscles) and steroidal alkaloids are said to be anti-infecting agents (Mamta *et al*., 2012). These are related to sex hormones such as oxytocin involved in control of birth inducement and subsequent release of breast milk (Santhi *et al*., 2011; Shi *et al*., 2004). Phenolic compounds are one of the largest and most common groups of plant metabolites. There are several biological properties of phenolics such as; anti-apotosis, anti-ageing, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection, inhibition of angiogenesis and cell proliferation as well as improve endothelial function (Han *et al*., 2007). Due to the physiological activity, such as antioxidant, antimicrobial and anti-inflammatory properties, tannins have received great attention in many fields especially in the fields of nutrition, health and medicine. Tannins have astringent properties (speed up inflamed mucous membrane and wound healing) and antidiarrheal properties (Killedar *et al*., 2010; Santos-Beulga *et al*., 2000). Triterpenoids have analgesic and anti-inflammatory properties (Savithramma *et al*., 2012). Anthraquinone glycosides have purgatory effects (Santhi *et al*., 2011; Shi *et al*., 2004). However, more research is needed to determine the specific roles of these phytoconstituents present in *Trichilia monadelpha*.

2.5 CONCLUSION

Preliminary phytochemical screening of PEE, EthE and EAE of *Trichilia monadelpha* show that the stem bark of the plant contains important secondary metabolites that could contribute to its therapeutic effects.

Chapter 3

EFFECTS OF EXTRACTS OF *TRICHILIA MONADELPHA* ON ACUTE AND SYSTEMIC INFLAMMATION

3.1 INTRODUCTION

Pharmacological evidences show that petroleum ether and ethanol or hydro-alcoholic extracts of *Trichilia monadelpha* inhibits carrageenan-induced acute inflammation in seven – day old chicks (Ainooson *et al.*, 2012). This chapter gives insight into the effects of the stem bark plant extracts on mediators of inflammation when carrageenan is induced in rat.

3.2 EXPERIMENTAL PROCEDURE

3.2.1 Acute Anti-inflammatory Effect

3.2.1.1 Animals

Male Sprague-Dawley rats (150 – 200 g) were bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, and kept in the animal house of the Department of Pharmacology, KNUST, Kumasi, Ghana. Animals were housed in aluminium cages, with rat diet (GHAFCO, Tema, Ghana) and water available, *ad libitum*. Sample size of 5 animals per group was utilised throughout the study. Guidelines on animal use was observed as instructed by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised 1985). This was approved by the Departmental Ethics Committee.

3.2.1.2 Drugs

The extracts were triturated with Tween-80 (3 drops) in normal saline (NS), filtered and administered orally to rats at doses ranging from 3 to 100 mg kg^{-1}. The following chemicals were used as inflammogens in the study: λ– carrageenan, histamine, serotonin (5-HT)

creatinine sulphate, PGE_2, bradykinin acetate salt (Sigma-Aldrich, Inc. St. Louis MO USA). Lipopolysaccharide from *Escherichia coli*, serotype 0111:B4 (Sigma-Aldrich, Inc. St. Louis MO USA) was used to induce pyrexia. CRP-latex slide test (Biolabo Reagents., Maizy, France) was used for qualitative and semi-quantitative test for CRP levels. Phosphate buffer saline (PBS) (Sigma-Aldrich, Inc. St. Louis MO USA) were used as a buffer reagent to prepare some of the inflammogens. The reference drugs used were; diclofenac (Bliss GVS Pharma, India), chlorpheniramine, aspirin (Kama Pharmaceuticals, Accra, Ghana), ondansetron and captopril (Dabur Pharma, India), dexamethasone (Wuhan Grand, China). Animals of the extract-treated groups received ≤1 ml of extract solution. Animals of the standard drugs-treated groups received ≤1 ml of drug solution. Control group received only intraplantar injection of inflammogens suspension and were given normal saline. All drugs and extracts were freshly prepared.

3.2.1.3 Carrageenan-induced Inflammation

The procedure (Winter *et al.*, 1962) with modifications (Abotsi *et al.*, 2010; Woode *et al.*, 2009) was to reaffirm the anti-inflammatory properties of the extracts. Briefly, λ - carrageenan (50 µl of a 0.5 % solution in saline) was injected intraplantar into the left foot paw of rats. Foot volume was measured at hourly intervals for 6 hours after carrageenan injection by a volume-displacement method using a hydroplethysmometer (IITC Life Science Equipment, Woodland Hills, USA). The oedema component of inflammation was quantified by measuring the difference in foot volume before and after carrageenan injection and at the various time points (Milanino *et al.*, 1988; Winter *et al.*, 1962). The drugs were given curatively (30 mins for *i.p.* route and 1 h for oral route) after carrageenan challenge.

3.2.1.4 Lipopolysaccharide, LPS-induced pyrexia

Systemic inflammation model (LPS-induced pyrexia), measuring hyperthermia as a marker of systemic inflammation (Chan *et al.*, 1997) was the model used to evaluate effects of extracts on pyrexia. Sterile saline solution (0.5 ml) containing 0.1 mg kg^{-1} LPS was given *i.p.* to all animals. Temperature ($T\ ^0C$) of the rectum was monitored once every hour for 5 hours just before and after injecting the LPS with a rectal thermometer (Panlab model 0331;

Barcelona, Spain). The drugs were given curative (30 min for *i.p.* route and 1 hr for oral route) after LPS challenge. Rats in the respective dosing groups were treated with aspirin (3, 10, 30 and 100 mg kg^{-1}) and extracts (3, 10, 30, and 100 mg kg^{-1}).

3.2.1.5 Vasoactive amines (Histamine and serotonin), PGE_2 and Bradykinin-induced Inflammation

The procedure was similar to carrageenan-induced model. Histamine and serotonin, PGE_2 and Bradykinin (BK) were the inflammogens (Amann *et al.*, 1995; Vogel *et al.*, 1997), with some modifications, used to evaluate the anti-inflammatory properties of the extracts (1 – 30 mg kg^{-1}). Chlopheniramine and ondansetron were reference drugs for histamine-induced model and serotonin -induced model respectively. Histamine (0.1 ml of a 0.1 % solution in saline) and serotonin (0.1 ml of a 0.02 % solution in saline) were injected sub-plantar into the left foot paw of rats. PGE_2 (1 nmol/paw solution in PBS) and BK (10 nmol/paw solution in PBS) were also injected into left paws of rats. To avoid degradation of the BK, animals were pretreated with captopril (5 mg kg^{-1}) before induction of inflammation with BK. Foot volume was measured before injection and thirty (30) minutes intervals for 3 hours after injection by a volume displacement method using a hydroplethysmometer (IITC Life Science Equipment, Woodland Hills, USA). From the results of the carrageenan-induced inflammation, PEE and EAE were more efficacious than EthE, thus PEE and EAE were used for these tests. The oedema component of inflammation was quantified by measuring the difference in foot volume before the inflammogens injection and at the various time points. The drugs were given pre-emptively (30 min for *i.p.* route and 1 h for oral route) before inflammogens challenge and curatively (30 mins for *i.p.* route and 1 hr for oral route) after inflammogens challenge. Control group received only subplantar injection of the inflammogens suspension and treated with 0.9 % normal saline *p.o.*

3.2.1.6 C-reactive protein, CRP, agglutination test

Animals randomly grouped into 5 per group, were pretreated with extracts, dexamethasone (0.1 mg kg^{-1}) and diclofenac (30 mg kg^{-1}). From the results of the carrageenan-induced inflammation, PEE and EAE were more efficacious than EthE, thus PEE and EAE (10 -100 mg kg^{-1}) were used for this test. After 30 minutes, inflammation was induced by injection of carrageenan into the paw of the rats. Blood was drawn from animals through cardiac puncture after 30 minutes and hourly interval for 6 hours. The blood got was stored in tubes and centrifuged at 3421 *g* for 15 min at room temperature and the serum separated into eppendorf tubes. CRP agglutination test was carried out as describe in the manual of the CRP-latex slide test. Briefly, the qualitative and semi-quantitative are described as follows:

***Qualitative method*:** One drop of the negative CRP control was placed on one of the circle of the agglutination slide. A drop of the positive CRP control was placed on the adjacent circle of the slide. One drop of samples was placed on other six circles. One drop of latex reagent was placed beside the control and the serum samples. These were mixed gently with separate stirrers. These slides were transferred to a rocker and rocked for 2 minutes. Each circle was observed for agglutination after two minutes.

***Semi-quantitative method*:** A twofold serial dilution of the serum samples was prepared using saline and the test was performed in the same way as the qualitative method. Table 3.1 below gives a summary of the procedure and the calculations;

Table 3.1: Semi-quantitative determination

Dilutions	1/2	1/4	1/8	1/16
Saline	100 µL	100 µL	100 µL	100 µL
Specimen	100 µL	-	-	-
	→	100 µL →	100 µL →	100 µL →
Transfer onto a circle of a test slide :				
Diluted Specimen	50 µL	50 µL	50 µL	50 µL
Latex Reagent	50 µL	50 µL	50 µL	50 µL
Calculate the result as follows :				
6 x N° of dilution	6 x 2	6 x 4	6 x 8	6 x 16
Results : mg/L	12	24	48	96

The titre is expressed as the reciprocal of the highest dilution showing macroscopic agglutination.

3.2.4 Statistical Analysis

All changes in ipsilateral paw volume of the carrageenan and mediators-induced inflammation are presented as percentage increase from the pre-treatment value obtained on time 0, which was calculated according to the formula;

% Increase in foot volume = $[V_x - V_0)/V_0] \times 100$ %

Where V is the mean volume measurement, x is the time after drug injection and 0 (zero) is the time before the carrageenan/inflammogens injection.

All rectal temperature changes were presented as percentage increase from the pre-treatment value obtained on time 0, which was calculated according to the formula;

% change in rectal core temperature (Tc) = $[T_x - T_0)/T_0] \times 100$ %

Where T is the rectal temperature, x is the time after drug injection and 0 (zero) is the time before the LPS injection.

Data was presented as the effect of drugs on the time course and the total oedema response for 3-6 h of inflammation induced by inflammogens and on the time course and Fever Indices (calculated for each rat, as area under the ΔTc curves, ^0C h) of the rats' response to the treatment followed by LPS or saline injection for 5 h.

The ED_{50} (dose responsible for 50 % of the maximal effect, used as a measure of the drug's potency), E_{max} (maximal effectiveness of the drug which defines the efficacy of the drug) and inhibitory effects of drugs were analyzed by using an iterative computer least squares method, with the following nonlinear regression (four-parameter logistic equation) of a sigmoid dose-response model.

$$y = \min + \frac{\max - min}{1 + (\frac{x}{EC50})^{-hillslope}}$$

Where; Min - bottom of the curve

Max - top of the curve

EC_{50} – the x value for the curve point that is midway between the max and min parameters. It is called the half-maximal effective concentration. Equivalent definitions are ED_{50} (half-maximal effective dose) and for inhibition curves IC_{50} (half-maximal inhibitory concentration).

Hillslope – characterizes the slope of the curve at its midpoint. Large values result in a steep curve whereas small values a shallow curve.

X is the logarithm of concentration and Y is the response and starts at ***min***, and goes to ***max*** with a sigmoid shape.

The fitted midpoints (ED_{50}s) of the curves were compared statistically using F test (Motulsky *et al*., 2003; Motulsky *et al*., 2003). GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used for all statistical analyses, ED_{50} and E_{max} determination. Levels of significance were determined by one-way

analysis of variance (1-way ANOVA) and Holm-Sidak's *post-test* using SigmaPlot version 12.3 (Systat Software Inc. Chicago USA, webmaster@cranessoftware.com). All values were expressed as mean±SEM. $P<0.05$ and higher F values ($F<4.0$) were considered significant in all analysis.

The maximal effects of the treatment given were calculated according to the formula below, using the means caluculated through column statistics:

$$\% \text{ maximal effect} = [Mean_{control} - Mean_{treatment}) / Mean_{control}] \times 100\ \%$$

3.3 RESULTS

3.3.1 Acute Anti-inflammatory Effect

3.3.1.1 Carrageenan–Induced Foot Edema

Injection of carrageenan (10 µl, 0.5 % suspension) induced moderate inflammation resulting in foot oedema rats peaking at 5 h as described by Winter *et al.*, 1962. One-way ANOVA (*control group* × *treatment groups*) revealed a significant effect ($F_{3, 16}$=12.20 $P<0.001$; $F_{3, 16}$=8.98 $P<0.01$; $F_{3, 16}$=13.34 $P<0.00$; $F_{3, 16}$=19.14 $P<0.0001$, respectively,) of drug treatment for the extracts; PEE, EthE, EAE and diclofenac. (Figure 3.1)

Total oedema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves. PEE showed maximal inhibitory effect of 74.7 % at dose 100 mgkg^{-1}. Maximal inhibitory effect of EthE was 55.0 % at dose 100 mgkg^{-1} and EAE of 76.7 % at dose 100 mgkg^{-1}. Also, diclofenac showed maximal inhibitory effect of 90.3 % at dose 100 mgkg^{-1}. (Figure 3.1 a, b, c and d).

Dose-response effects for the inhibition of foot oedema are shown in Figure 3.2. The extracts showed different potency and efficacy (PEE: ED_{50}: 55.66±59.37 mg kg^{-1}, E_{max}: 115.9; EAE: ED_{50}: 24.78±25.52 mg kg^{-1}, E_{max}: 101.4; EthE: ED_{50}: 3.69±7.03 mg kg^{-1}, E_{max}: 55.56). Diclofenac also displayed a high potency (ED_{50}: 4.31±4.38 mg kg^{-1}) and high efficacy (E_{max}: 90.50).

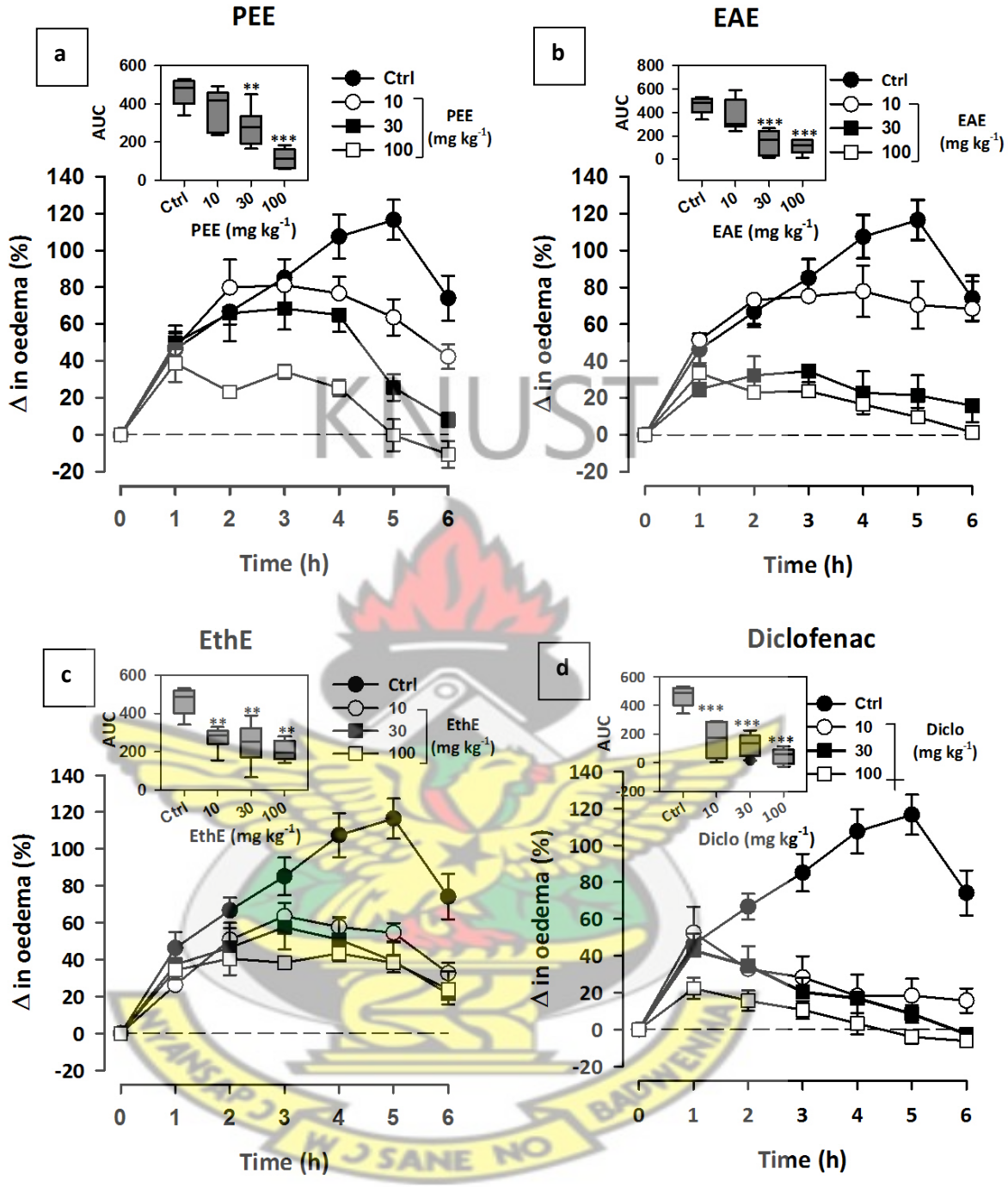


Figure 3.1: Effects of *Trichilia monadelpha* extracts on carrageenan-induced paw oedema
Time course curve effects of (a) PEE, (b) EAE, (c) EthE and (d) diclofenac. a – d insets are the boxplot derived from the time course curves, showing the total oedema response for 6 h [defined as the area under the time course curves (AUC)]. Each point and boxplot (showing the 1st quartile to the 3rd quartile. The vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± s.e.m (n=5). *** $P< 0.001$, ** $P< 0.01$: * $P< 0.05$, determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

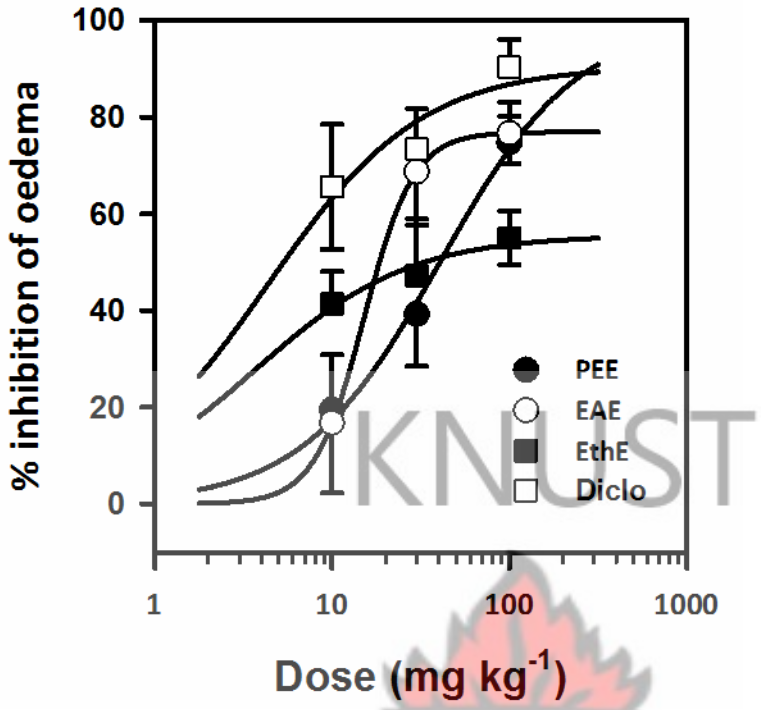


Figure 3.2: Dose-response curve of extracts of *Trichilia monadelpha* and diclofenac on carrageenan-induced paw oedema
Each extract exhibited different potency and efficacy, observed as the high or low steepness of the Hillslope of the curve. The data points reflect mean values of n= 3 repeated trials ± standard error. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

3.3.1.2 Lipopolysaccharide, LPS-induced pyrexia

In control animals (vehicle + LPS), 2 hours after the LPS injection, *T* °C (2 °C increase from basal values) started to increase, and remained raised until the end of the experiment. One-way ANOVA (*control group x treatment group*) showed that PEE, EthE and EAE significantly ($F_{4, 20}$=2.92 P<0.05; $F_{4, 20}$=3.46 P<0.05; $F_{4, 20}$=7.99 P<0.001, respectively) reduced febrile response. Aspirin significantly ($F_{4, 20}$=6.67, P<0.01), also caused a reduction in febrile response. (Figure 3.3 a - d)

Total change in rectal temperature produced by each treatment is expressed in arbitrary units as AUC of the time-course curve (Figure 3.3 a – d insets). PEE showed maximal anti-pyretic

effect of 60.9 % at 30 mg kg^{-1}. EthE maximal effect was 55.4 % at 30 mg kg^{-1}. EAE also showed maximal anti-pyretic effect of 69.2 % at 100 mg kg^{-1}. Aspirin showed maximal anti-pyretic effect of 69.2 % at 30 mg kg^{-1}.

Dose-response effects for the reduction of rectal temperature are shown in Figure 3.4. Different potency and efficacy was observed for the extracts (PEE: ED_{50}: 6.38±9.78 mg kg^{-1}, E_{max}: 64.92; EthE; ED_{50}: 3.11±4.16 mg kg^{-1}, E_{max}: 64.39; EAE: ED_{50}: 13.98±11.23 mg kg^{-1}, E_{max}: 82.28). Aspirin also was potent (ED_{50}: 1.10±1.92 mg kg^{-1}) and efficacious (E_{max}: 72.76) in reduction of rectal temperature.

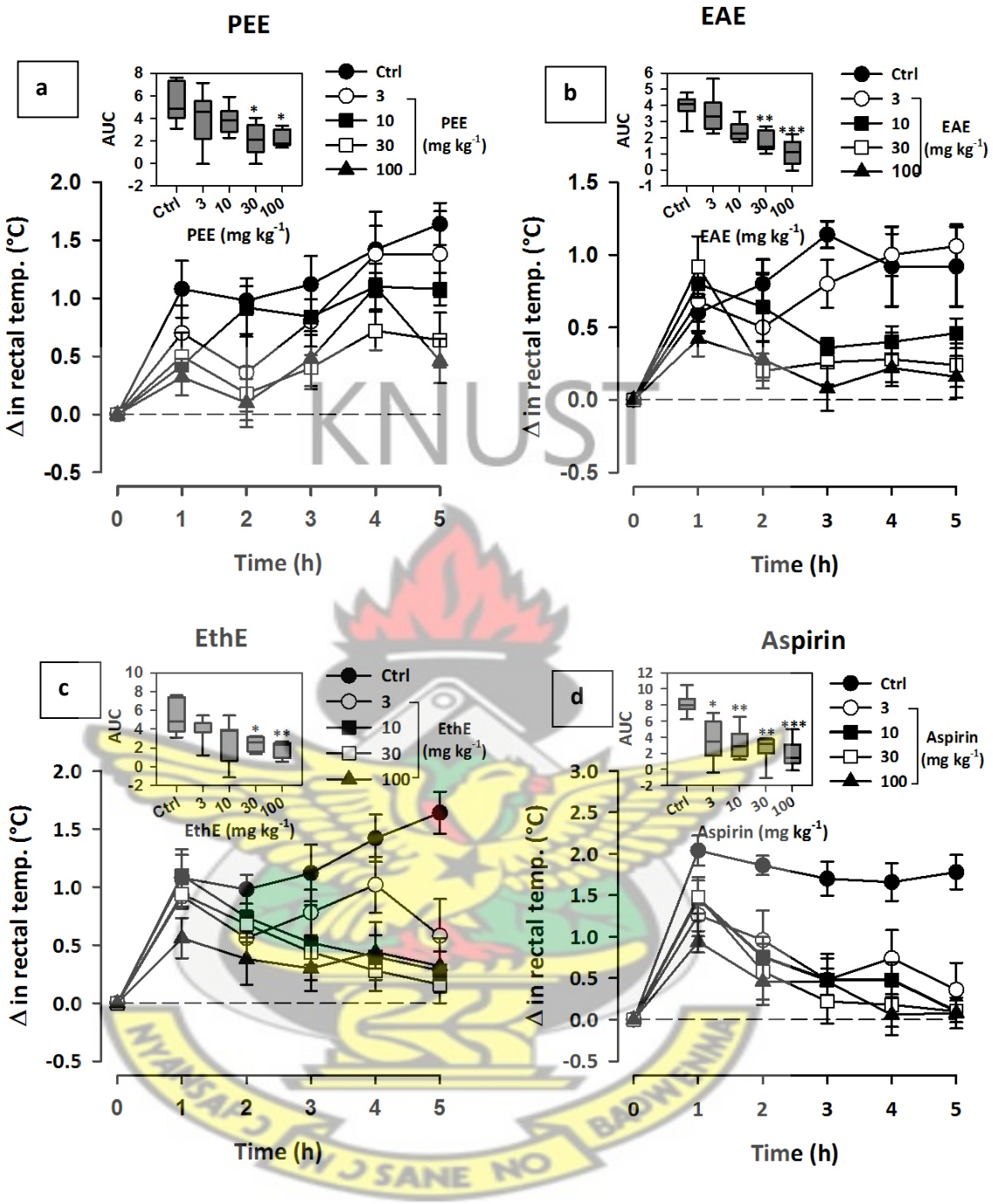


Figure 3.3: Effects of *Trichilia monadelpha* extracts on LPS-induced pyrexia
Time course curve effects of the change in rectal temperature (*T*c) response in animals treated with (a) PEE, (b) EAE, (c) EthE and (d) aspirin. a – d insets are the boxplot derived from the time course curves, showing Fever Indices (calculated as area under the Δ*T*c curves) of the rats' response to the treatment. Each point and boxplot (showing the 1st quartile to the 3rd quartile. The vertical line is drawn at the 2ndquartile which is the median of the data) represents the mean ± s.e.m (n=5). *** *P*< 0.001, ** *P*< 0.01: * *P*< 0.05, determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

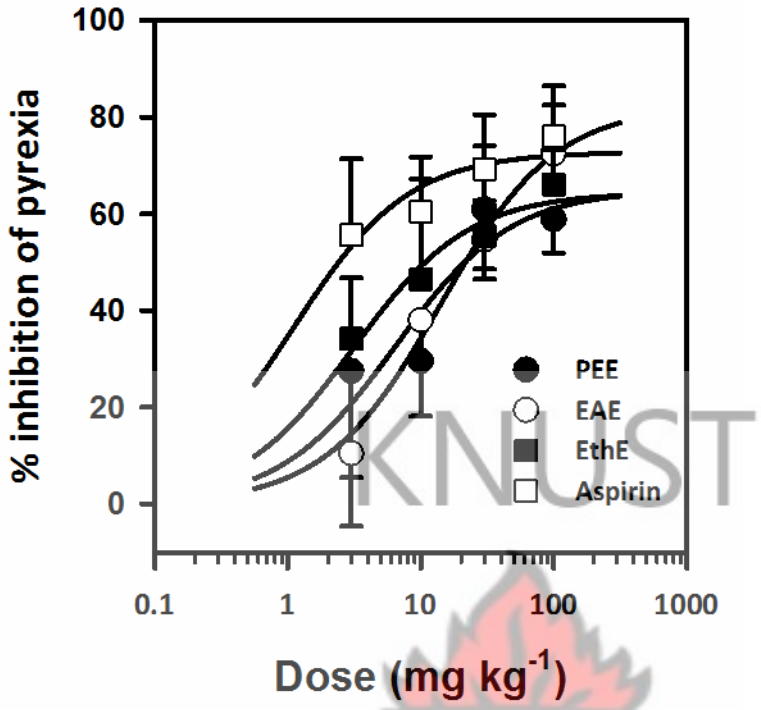


Figure 3.4: Dose-response curve of extracts of *Trichilia monadelpha* and aspirin on LPS-induced pyrexia
Each extract exhibited different potency and efficacy, observed as the high or low steepness of the hillslope of the curve. The data points reflect mean values of n= 4 repeated trials ± standard error. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

3.3.2 Mediators-induced Inflammation and CRP levels

Extracts were given curatively and pre-emptively to rats, to determine if the plant extracts effectively reduced established effects or it prevented the release or effects of released mediators. The effects of inflammogens; histamine and serotonin, PGE_2 and Bradykinin (BK)-induced paw edema in rats are shown in Figures 3.5 – 3.11.

3.3.2.1 Histamine-induced inflammation

Figures 3.5 (a – c) and (d – f) show the time course curves for effects of PEE, EAE and chlorpheniramine on histamine-induced oedema for the curative and prophylactic processes, respectively. One-way ANOVA (*control group* × *treatment groups*) revealed a significant

(curative: $F_{4, 20}$=16.83 P<0.0001; $F_{4, 20}$=7.69 P<0.0001; $F_{4, 20}$=10.27 P<0.0001; prophylactic: $F_{4, 20}$=13.55 P<0.0001; $F_{4, 20}$=12.26 P<0.0001; $F_{4, 20}$=11.53 P<0.0001, respectively) effect of drug treatment for PEE, EAE, and chlorpheniramine.

Total oedema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves (Figure 3.5 (a – f) insets). PEE (3-100 mg kg^{-1}, *p.o.*) significantly reduced foot oedema with maximal effect of 80 % at 100 mg kg^{-1} and 115 % at 100 mg kg^{-1} for curative and prophylactic treatments respectively. EAE (3-100 mg kg^{-1}, *p.o.*) significantly reduced foot oedema with maximal effect of 72 % at 30 mg kg^{-1} and 61 % at 30 mg kg^{-1} for curative and prophylactic treatments respectively. Also, H_1-specific antagonist, chlorpheniramine (1-30 mg kg^{-1}, *i.p.*) dose dependently reduced the oedema with maximal effect of 85 % at 100 mg kg^{-1} and 144 % at 100 mg kg^{-1} for curative and prophylactic treatments respectively.

Dose-response effects for the inhibition of foot oedema are shown in Figure 3.6. Comparing the ED_{50} values of extracts on established oedema, PEE was 3-fold less potent and 2-fold less potent than the reference drug used for the curative and prophylactic process, respectively (curative: ED_{50}: 1.57±0.44 mg kg^{-1}; prophylactic: ED_{50}: 1.29±1.02 mg kg^{-1}), was efficacious (curative E_{max}: 87.68; prophylaxis E_{max}: 98.89) in decreasing oedema. EAE was 10-fold and 11-fold less potent than chlorpheniramine in both treatment process (curative: ED_{50}: 4.59±2.79 mg kg^{-1}; prophylactic: ED_{50}: 6.39±3.44 mg kg^{-1}) and less efficacious (curative E_{max}: 77.83; prophylaxis E_{max}: 74.87). Chlorpheniramine also displayed a high potency (curative: ED_{50}: 0.48±0.34 mg kg^{-1}; prophylactic: ED_{50}: 0.56±0.48 mg kg^{-1}) and high efficacy (curative E_{max}: 89.39; prophylaxis E_{max}: 101.10).

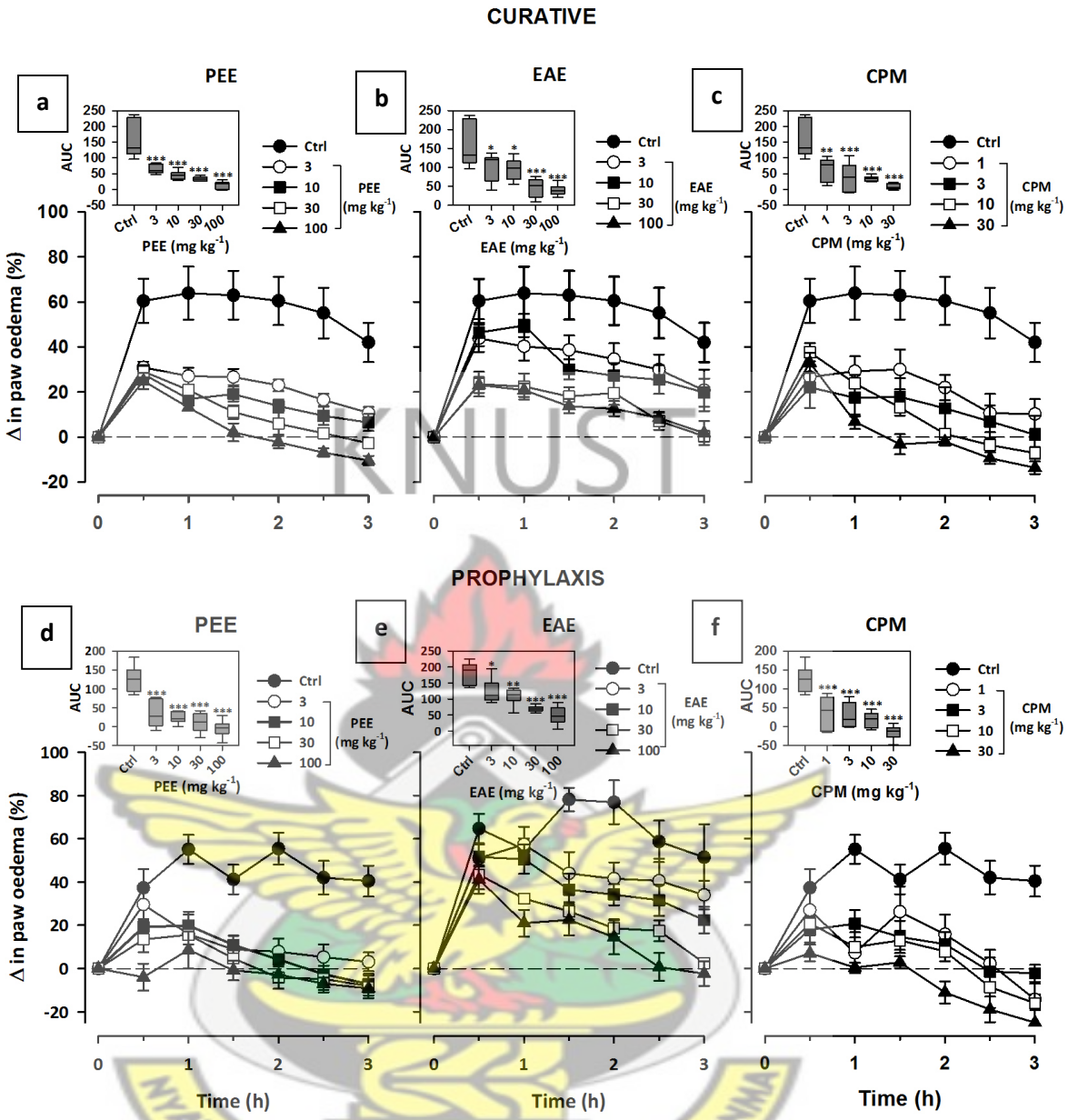


Figure 3.5: Effects of *Trichilia monadelpha* extracts on histamine-induced paw oedema
Time course curve effects of (a, d) PEE, (b, e) EAE, and (c, f) chlopheniramine for the curative and prophylactic processes, respectively. a – f insets are the boxplot derived from the time course curves, showing a dose-dependent total oedema amelioration for 3 h of each treatment [defined as the area under the time course curves (AUC)]. Each point and boxplot (showing the 1st quartile to the 3rd quartile. The vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± s.e.m (n=5). *** $P<0.001$, ** $P< 0.01$: * $P< 0.05$, determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

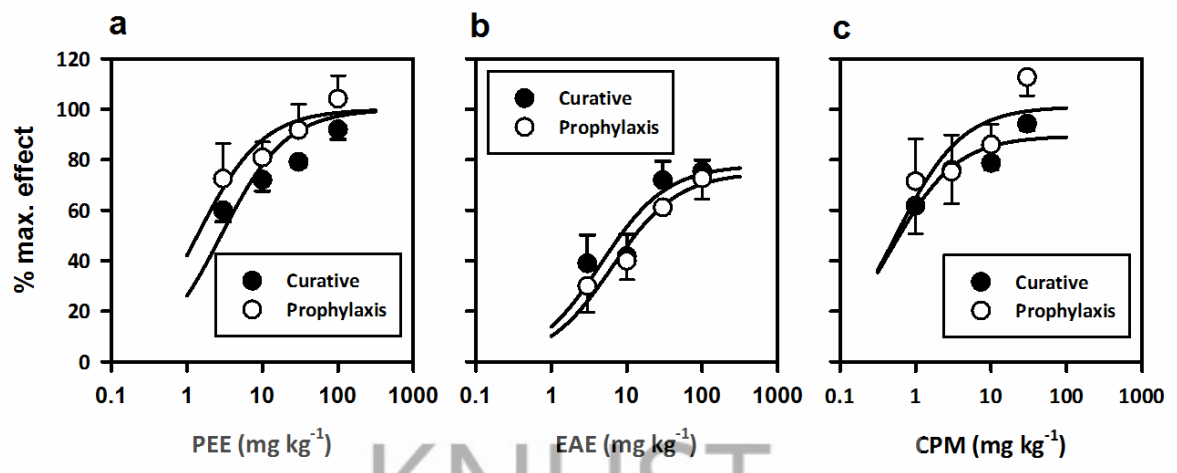


Figure 3.6: Dose-response curve of extracts of *Trichilia monadelpha* and chlopheniramine on histamine-induced paw oedema
Each extract exhibited different potency and efficacy, observed as the high or low steepness of the hillslope of the curve. PEE was effective prophylactically than curative, while EAE was effective curative than prophylactically. However PEE was most efficacious. The data points reflect mean values of n= 4 repeated trials ± standard error. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

3.3.2.2 Serotonin -induced inflammation

Figures 3.7 (a – c) and (d – f) show the time course curves for effects of PEE, EAE and ondansetron on serotonin-induced oedema for the curative and prophylactic processes, respectively. One-way ANOVA (*control group* × *treatment groups*) revealed a significant effect (curative: $F_{4, 20}$=8.48 P<0.001; $F_{4, 20}$=5.35 P<0.01; $F_{4, 20}$=7.84 P<0.001; prophylactic: $F_{4, 20}$=5.99 P<0.01; $F_{4, 20}$=10.04 P<0.001; $F_{4, 20}$=9.60 P<0.001, respectively) of drug treatment for PEE, EAE, and ondansetron.

Total oedema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves (Figure 3.7 (a – f) insets). PEE (3-100 mg kg^{-1}, *p.o.*) significantly reduced foot oedema with maximal effect of 70 % at 100 mg kg^{-1} and 77 % at 30 mg kg^{-1} for curative and prophylactic treatments respectively. EAE (3-100 mg kg^{-1}, *p.o.*) significantly reduced foot oedema with maximal effect of 51 % at 30 mg kg^{-1} and 56 % at 100 mg kg^{-1} for curative and prophylactic treatments respectively. Also, $5HT_3$-specific antagonist, ondansetron (1-30 mg

kg^{-1}, *i.p.*) dose dependently reduced the oedema with maximal effect of 82 % at 30 mg kg^{-1} and 72 % at 30 mg kg^{-1} for curative and prophylactic treatments respectively.

Dose-response effects for the inhibition of foot oedema are shown in Figure 3.8. PEE exhibited approximately the same potency as ondansetron for both treatment processes (curative: ED_{50}: 3.84±2.57 mg kg^{-1}; prophylactic: ED_{50}: 0.53±2.29 mg kg^{-1}). EAE was 1-fold more and 5-fold less potent than ondansetron for both treatment processes (curative: ED_{50}: 1.76±1.86 mg kg^{-1}; prophylactic: ED_{50}: 10.04±6.54 mg kg^{-1}). However, in both curative and prophylactic processes, PEE was more efficacious (curative E_{max}: 78.75; prophylaxis E_{max}: 79.40) than EAE (curative E_{max}: 54.69; prophylaxis E_{max}: 68.05), in decreasing oedema. Ondansetron also displayed a high potency (curative: ED_{50}: 2.59±1.88 mg kg^{-1}; prophylactic: ED_{50}: 1.92±1.17 mg kg^{-1}) and high efficacy (curative E_{max}: 96.31; prophylaxis E_{max}: 81.61).

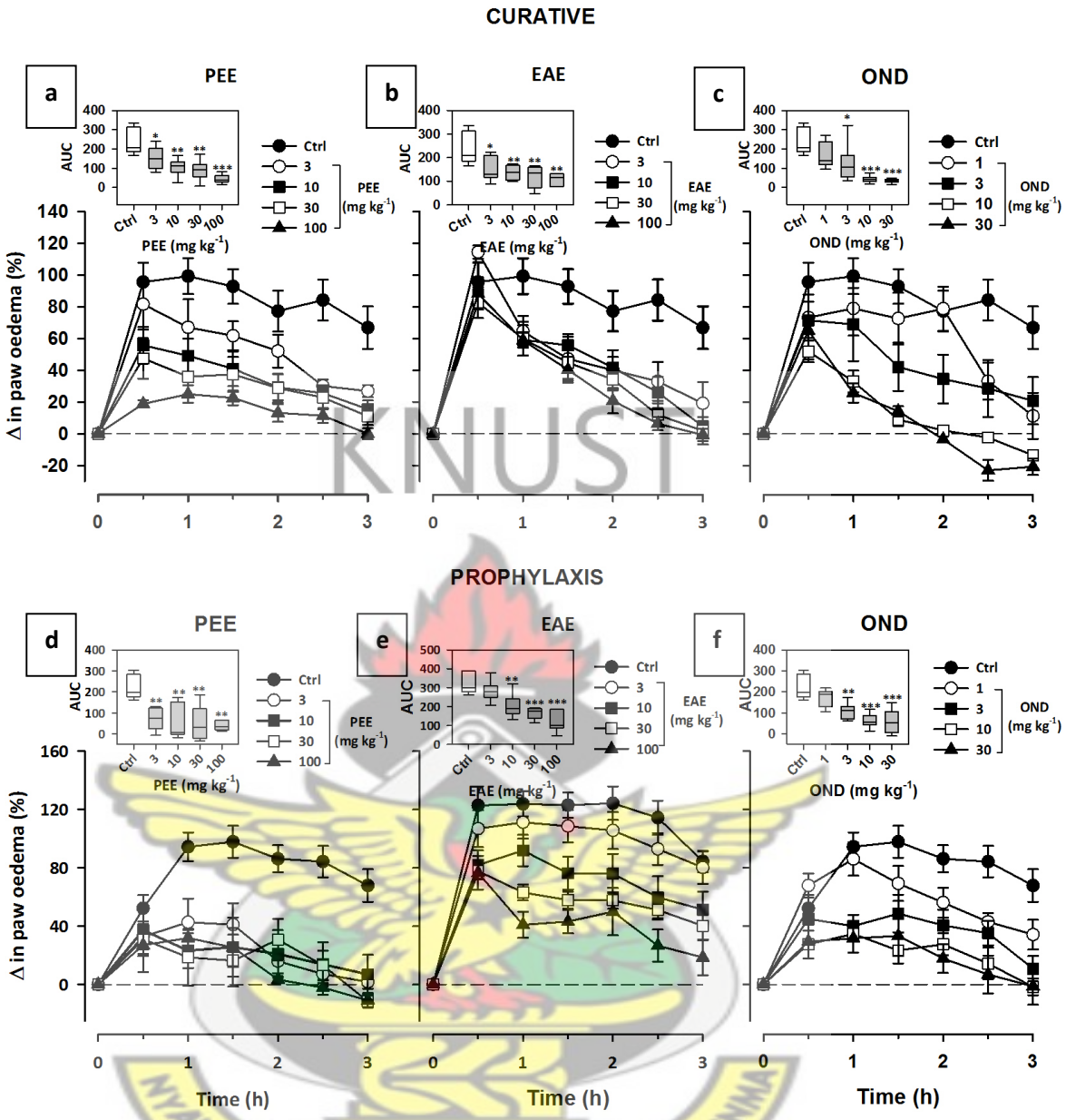


Figure 3.7: Effects of *Trichilia monadelpha* extracts on serotonin-induced paw oedema
Time course curve effects of (a, d) PEE, (b, e) EAE, and (c, f) ondansetron for the curative and prophylactic processes, respectively. a – f insets are the boxplot derived from the time course curves, showing a dose-dependent total oedema amelioration for 3 h of each treatment [defined as the area under the time course curves (AUC)]. Each point and boxplot (showing the 1st quartile to the 3rd quartile. The vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± s.e.m (n=5). *** P<0.001, ** P< 0.01: * P< 0.05, determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

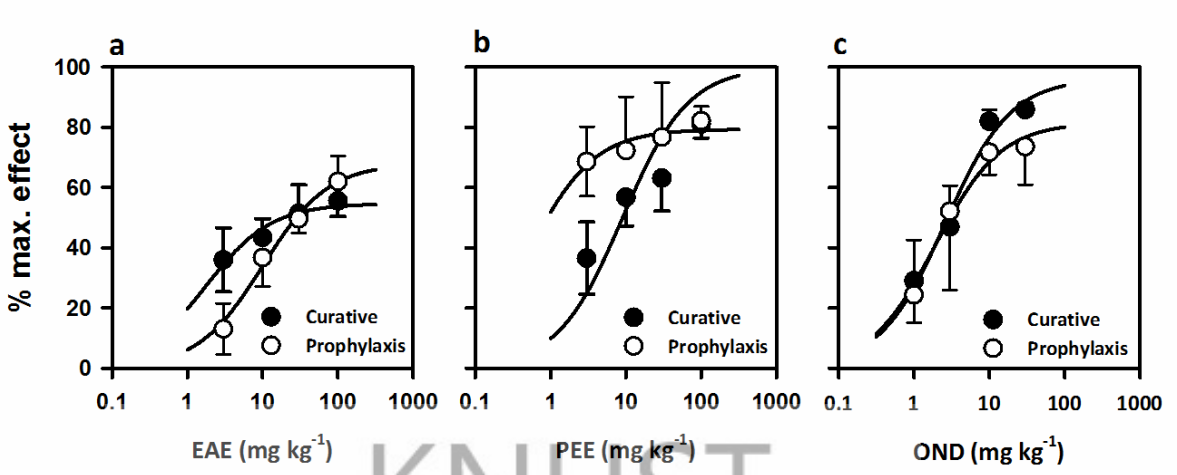


Figure 3.8: Dose-response curve of extracts of *Trichilia monadelpha* and ondansetron on serotonin-induced paw oedema
Each extract exhibited different potency and efficacy, observed as the high or low steepness of the hillslope of the curve. PEE was effective prophylactically than curative, while EAE was effective curative than prophylactically. However PEE was most efficacious. The data points reflect mean values of n= 4 repeated trials ± standard error. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

The results however, show that PEE effectively ameliorated inflammation of mediators that are known to be involved in mediating the first phase of inflammation induced by carrageenan.

3.3.2.3 Prostaglandin E_2 (PGE_2)-induced inflammation

Figures 3.9 (a, b) and (c, d) show the time course curves for effects of PEE and EAE on PGE_2-induced oedema for the curative and prophylactic processes, respectively. One-way ANOVA (*control group* × *treatment groups*) revealed a significant effect (curative: $F_{4, 20}$=3.47 P<0.05; $F_{4, 20}$=6.69 P<0.01; prophylactic: $F_{4, 20}$=15.66 P<0.0001; $F_{4, 20}$=16.09 P<0.0001, respectively) of drug treatment for PEE and EAE.

PEE (3-100 mg kg^{-1}, *p.o.*) significantly reduced foot oedema with maximal effect of 70 % at 30 mg kg^{-1} and 332 % at 100 mg kg^{-1} for curative and prophylactic treatments respectively. EAE (3-100 mg kg^{-1}, *p.o.*) significantly also reduced foot oedema with maximal effect of 60 % at 30 mg kg^{-1} and 86 % at 10 mg kg^{-1} for curative and prophylactic treatments respectively. (Figure 3.9 a - d insets).

Dose-response effects for the inhibition of foot oedema are shown in Figure 3.11 (a, b). The potency (curative: ED_{50}: 3.00±3.92 mg kg^{-1}, 2.18±2.09 mg kg^{-1}; prophylactic: ED_{50}: 2.75±1.85 mg kg^{-1}, 2.14±1.49 mg kg^{-1}, respectively) of PEE and EAE were relatively close, with EAE slightly more potent than PEE in both processes. However, PEE, in both processes was more efficacious (curative E_{max}: 77.04, 54.33; prophylaxis E_{max}: 130.7, 84.61) than EAE.

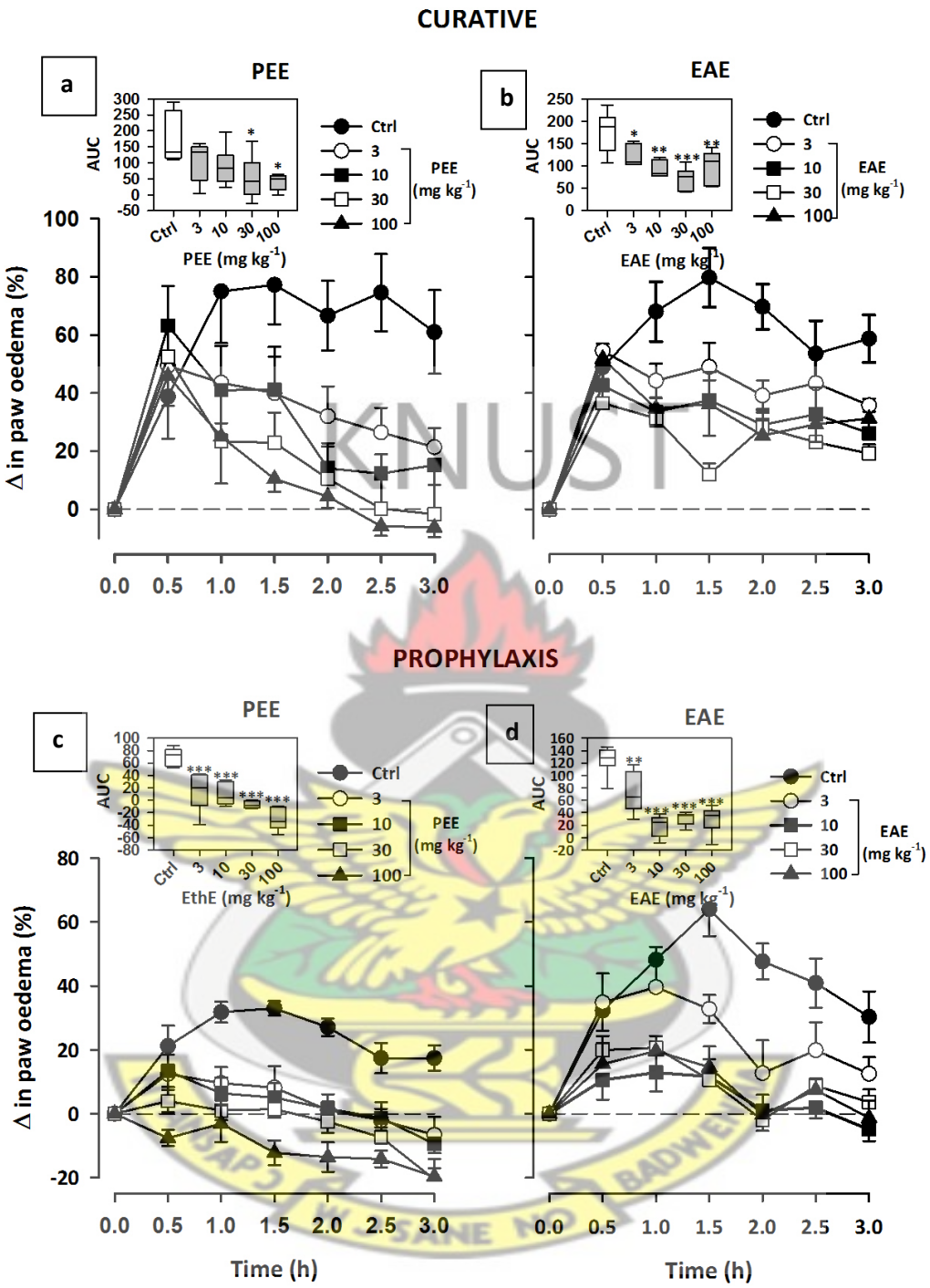


Figure 3.9: Effects of *Trichilia monadelpha* extracts on PGE_2-induced paw oedema
Time course curve effects of (a, c) PEE, and (b, d) EAE, for the curative and prophylactic processes, respectively. a – d insets are the boxplot derived from the time course curves, showing a dose-dependent total oedema amelioration for 3 h of each treatment [defined as the area under the time course curves (AUC)]. Each point and boxplot (showing the 1st quartile to the 3rd quartile. The vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± s.e.m (n=5). *** $P< 0.001$, ** $P< 0.01$: * $P< 0.05$, determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

3.3.2.4 Bradykinin (BK)-induced inflammation

Figures 3.10 (a, b) and (c, d) show the time course curves for effects of PEE and EAE on BK-induced oedema for the curative and prophylactic processes, respectively. One-way ANOVA (*control group* × *treatment groups*) revealed a significant effect (curative: $F_{4, 20}$=14.29 P<0.0001; $F_{4, 20}$=5.24 P<0.05; prophylactic: $F_{4, 20}$= 10.36 P<0.001; $F_{4, 20}$=4.86 P<0.01) of drug treatment for PE and EAE.

PEE (3-100 mg kg^{-1}, *p.o.*) significantly reduced foot oedema with maximal effect of 98 % at 100 mg kg^{-1} and 78 % at 100 mg kg^{-1} for curative and prophylactic treatments respectively. EAE (3-100 mg kg^{-1}, *p.o.*) also significantly reduced foot oedema with maximal effect of 49 % at 30 mgkg^{-1} and 62 % at 30 mgkg^{-1} for curative and prophylactic treatments respectively. (Figure 3.10 a - d insets)

Dose-response effects for the inhibition of foot oedema are shown in Figure 3.11 (c, d). PEE was less potent (curative: ED_{50}: 13.75±6.61 mg kg^{-1}, 4.66±4.62 mg kg^{-1}; prophylactic: ED_{50}: 15.05±10.77 mg kg^{-1}, 2.71±3.01 mg kg^{-1}, respectively) than EAE in both processes. However, PEE, in both processes was more efficacious (curative E_{max}: 113.4, 49.33; prophylaxis E_{max}: 96.41, 68.56, respectively) than EAE by possibly reducing the concentration of BK at the inflammatory site, observed as oedema.

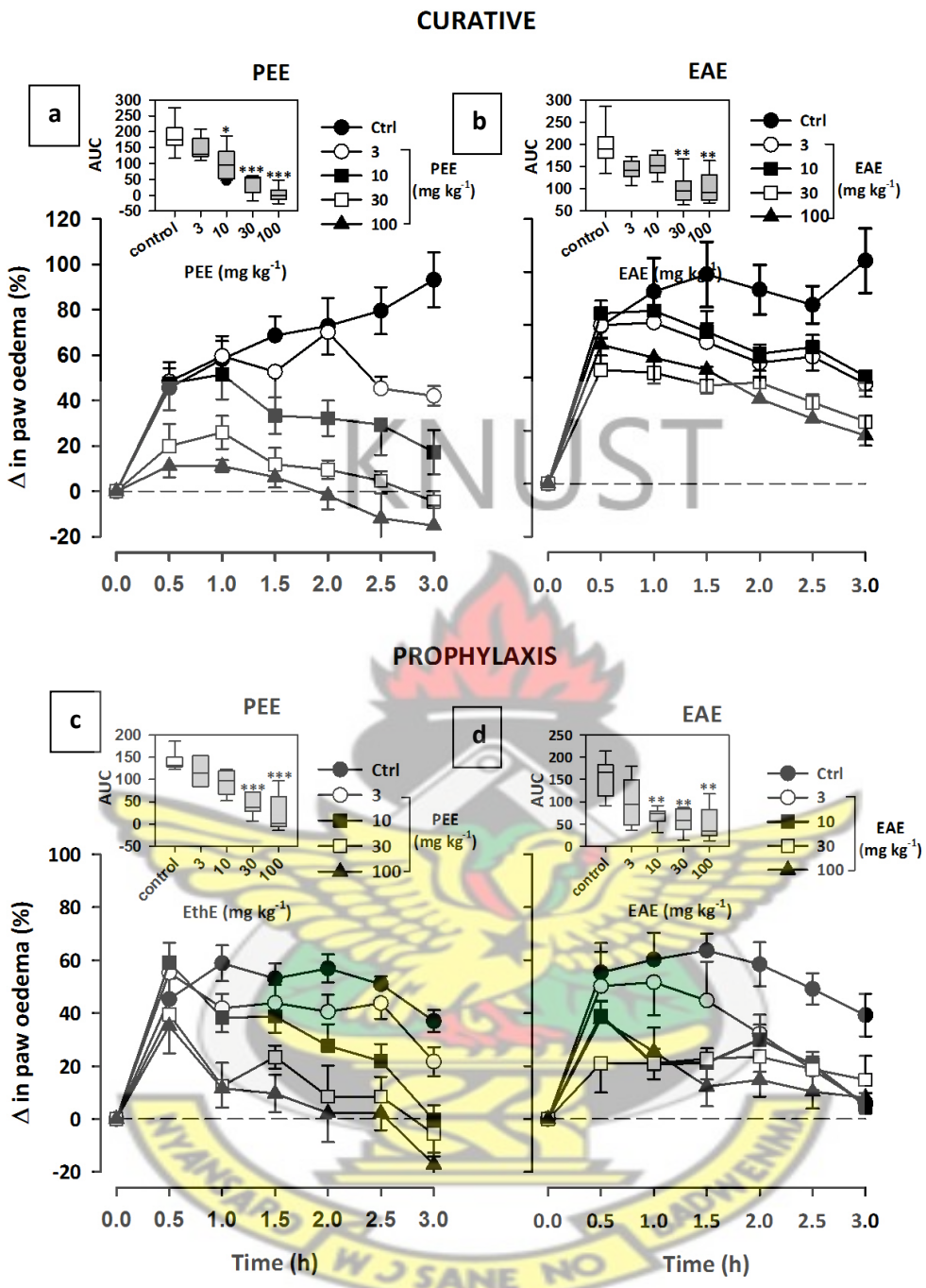


Figure 3.10: Effects of *Trichilia monadelpha* extracts on BK-induced paw oedema
Time course curve effects of (a, c) PEE, and (b, d) EAE, for the curative and prophylactic processes, respectively. a – d insets are the boxplot derived from the time course curves, showing a dose-dependent total oedema amelioration for 3 h of each treatment [defined as the area under the time course curves (AUC)]. Each point and boxplot (showing the 1st quartile to the 3rd quartile. The vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± s.e.m (n=5). *** $P< 0.001$, ** $P< 0.01$: * $P< 0.05$, determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

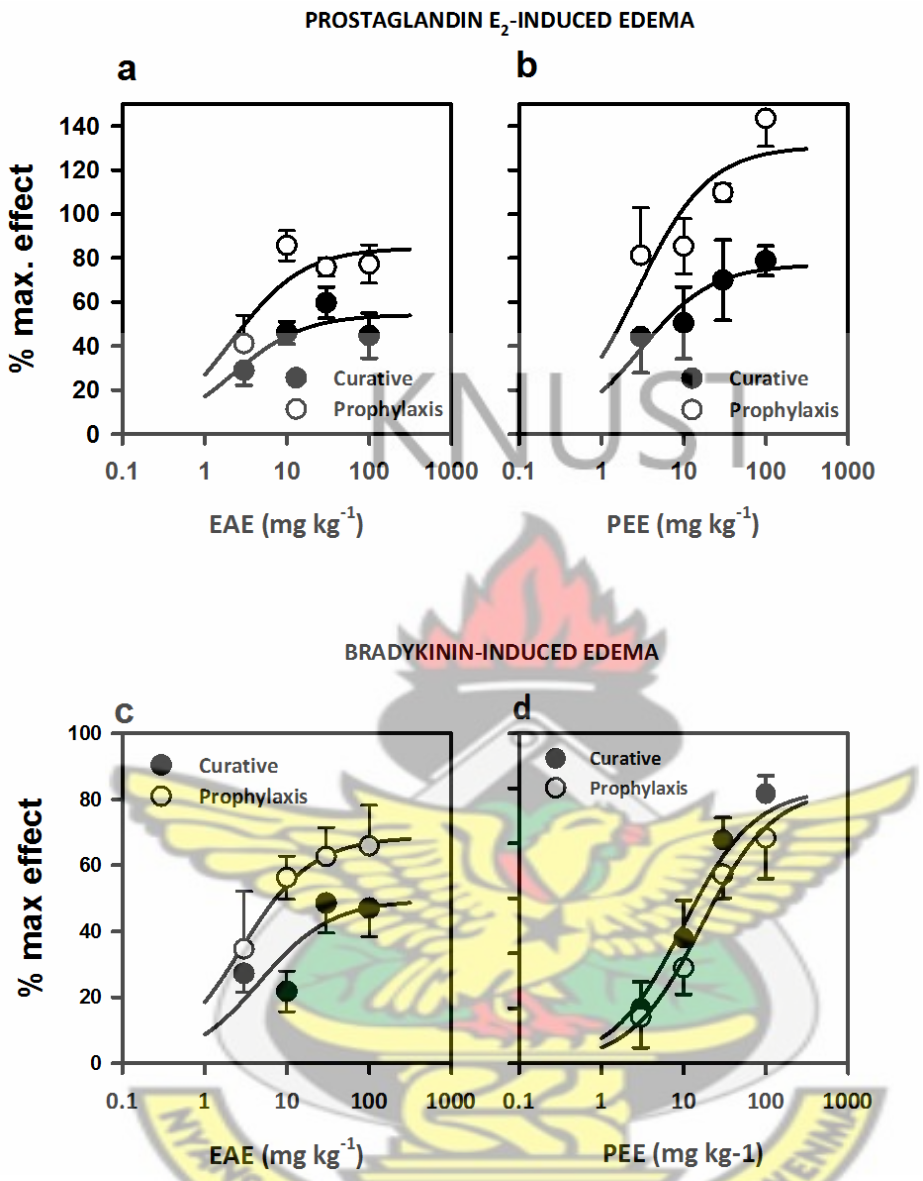


Figure 3.11: Dose-response curve of extracts of *Trichilia monadelpha* on PGE_2 and BK-induced paw oedema
Each extract exhibited different rate of potency and efficacy, observed as the high or low steepness of the hillslope of the curve. The data points reflect mean values of n= 4 repeated trials ± standard error. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

3.3.3.5 C-reactive protein, CRP, agglutination test

Preliminary study showed that serum from inflamed rats showed a positive result for the agglutination test with human CRP-latex kit. The preliminary study also showed that CRP levels are elevated at the third hour after induction of inflammation with carrageenan. These levels are sustained until the fifth hour. This shows the kit can be used for evaluating rat CRP levels.

The qualitative test for the inflamed rats treated with the extracts, PEE and EAE, showed no agglutination. To quantify this observation, semi-quantitative test was carried out and the results are shown in Figure 3.12 (a – c). PEE significantly ($F_{2,12}$= 6.67, P<0.01), and maximally (71.5 %) reduced CRP levels at 30 mg kg^{-1}. EAE also significantly ($F_{2,12}$= 6.33, P<0.01), and maximally (69.4 %) reduced CRP levels at 100 mg kg^{-1} in the serum of inflamed rats. Dexamethasone and diclofenac both significantly ($F_{2,12}$=7.56 P<0.01; $F_{2,12}$=7.45, P<0.01) reduced CRP levels at 1 and 30 mg kg^{-1} respectively.

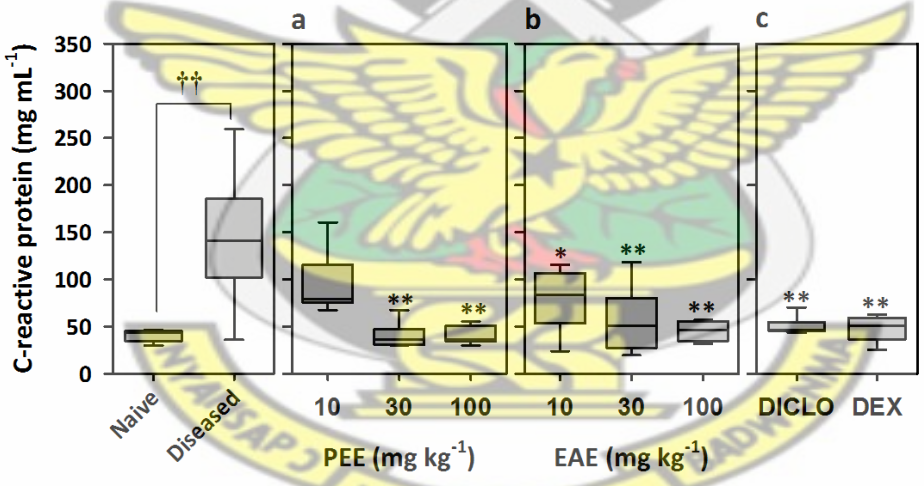


Figure 3.12: Boxplot of extracts of *Trichilia monadelpha*, diclofenac and dexamethasone on semi-quantitative CRP-latex agglutination test

The boxplot of (a) PEE, (b) EAE and (c) median doses of diclofenac and dexamethasone of the quantitative response for 6 h [defined as the area under the time course curves (AUC)]. Each point and boxplot (Showing the 1st quartile to the 3rd quartile. The vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± s.e.m (n=5). *** P< 0.001, ** P< 0.01: * P< 0.05 compares mean±SEM (n=5) of treated groups. †† P< 0.01 compares mean±SEM (n=5) of naïve control and disease control, using one-way ANOVA and Holm-Sidak's *post hoc* test.

3.4 DISCUSSION

Carrageenan (sulfated polyanionic polysaccharide)-induced inflammation in the rat paw represents a conventional model of oedema, extensively used in the development of NSAIDs and selective COX-2 inhibitors and also the screening of potential anti-inflammatory agents (Ichitani *et al*., 1997). The carrageenan model is a two-phase inflammatory response resulting in oedema formation (Vinegar *et al*., 1969). Beginning immediately after the subcutaneous/sub-plantar injection of carrageenan and lasting for the first two hours, is the first phase which involves the release of histamine and serotonin (vasoactive amines) mostly inhibited by NSAIDs such as diclofenac and aspirin (Crunkhon *et al*., 1971; Vinegar *et al*., 1969). These vasoactive amines are released following the mast cell degranulation by number of inflammatory mediators including substances P, IL-1. The release of these vasoactive amines likely induces the release of neuropeptide as well as release of prostaglandins and monohydroxy eicosatetranoic acid from endothelial cell leading to pro-inflammatory effects (Amann *et al*., 1995; Dray, 1995). The second phase, which begins at the end of the first phase and remains through the third hour up to the fifth/sixth hour, is due to the release of prostaglandins (PGE_2), protease and lysosome (Crunkhon *et al*., 1971; Suralkar *et al*., 2008; Vinegar *et al*., 1969). This is due to the rapid induction of COX-2 by the carrageenan, in the spinal cord and other region of the CNS (Ichitani *et al*., 1997). All these mediators, especially histamine, serotonin, PGE_2, and bradykinin, produce inflammation when injected sub-plantar into the rat paw (Vogel *et al*., 1997), thus swelling occurs due to the action of these mediators.

The results presented shows that PEE, EthE and EAE exhibited anti-inflammatory effects in carrageenan-induced oedema model in rats. PEE and EAE were more potent and efficacious than EthE. This observation could be due, partly, to the bioactive metabolites (Ben, 2013 #531) present in each of the extracts and could also be due to different mechanism by which each extract could be acting to elicit its effect.

One of the common symptoms of inflammation aside oedema, the febrile index was investigated. Evidence of the involvement of prostaglandins in the genesis of fever has led to the use of NSAIDs (e.g. aspirin, diclofenac) as antipyretics after it became known that the action of these drugs depend on their ability to inhibit COX-1 and or COX-2 activity (Vane *et al*., 1998). Drugs which block prostaglandins synthesis could effectively inhibit the febrile response (Zeisberger, 1999). When a pyrogenic agent, such as LPS, enters the body through a break in natural barriers, it will interact with immune cells, and promote the synthesis and release of endogenous mediators, such as cytokines (*e.g*. TNF-α, IL-1b, IL-6), PGs and endothelins (Fabricio *et al*., 2005; Kluger, 1991; Romanovsky *et al*., 2005). In the preoptic area of the anterior hypothalamus, PGE_2 seems to be crucial for the induction of fever, at least to LPS (Romanovsky, *et al*., 2005). Extracts (PEE, EthE and EAE) significantly reduced the febrile response to LPS, dose-dependently. It is possible that these extracts, at varying degree, possess antipyretic activity as can be observed with the different levels of potency and efficacy of the extracts. This effectiveness of the extracts in reducing febrile response could be due to inhibition of other mediators such as IL-1 (α, β), IL-6 and chemokines or pathways, which do not depend on PGE_2 synthesis to produce fever in LPS-induced pyrexia, such as endothelin-1 and macrophage inflammatory protein-1a (Fabricio *et al*., 2005; Fabricio *et al*., 2006; Malvar *et al*., 2011).

Managing inflammatory disease conditions mostly involves the curative treatment process to block the effects of released mediators followed by prophylactic treatment process to stabilize and prevent the further release of mediators. This treatment processes are thus employed in the total cure and prevention of further disease conditions or complications that could prove to be fatal. Some anti-inflammatory drugs have curative effects but not prophylactic effects. On this premise was the experiments conducted, to investigate, pathologically, if the extracts of *Trichilia monadelpha* would be able to block the effects of the released mediators and if the extracts possess properties of stabilizing the system through blocking the release of mediators. The three extracts were able to ameliorate inflammation induced by carrageenan. However the effect observed with EAE was relatively close to that exhibited by PEE. With this in mind,

the possible mechanism of action of PEE and EAE on mediators, were investigated. This sought to determine if the extracts that showed more potent and efficacious anti-inflammatory effects against carrageenan-induced model, are able to block the effects of mediators released or stabilize and prevent the release of mediators that could lead to fatal disease condition.

Vasoactive amines (histamine and serotonin) are important inflammatory mediators and they serve as potent vasodilators and increase the vascular permeability (Peter, 2010; Rang *et al.*, 2007; Suleyman *et al.*, 1999; Swingle *et al.*, 1972; Vinay *et al.*, 2005). Both extracts (PEE and EAE) were effective in ameliorating vasoactive amines-induced oedema. The receptor, 5-HT_3 is found mainly in the peripheral nervous system particularly on nociceptive sensory neurons. This receptor, blocked by a specific antagonist, ondansetron, is induced when serotonin is injected locally (*i.v.*) eliciting a fine display of autonomic reflexes which evokes pain (Fantone *et al.*, 1999; Majno *et al.*, 2004; Peter, 2010; Porth *et al.*, 2003; Rang *et al.*, 2007).

The effects of histamine-induced oedema are outward passage of plasma protein and fluid into the extracellular spaces, an increase in the flow of lymph and protein content and formation of oedema. Increased permeability results mainly from actions of histamine on postcapillary venules, where histamine causes the endothelial cells to contract and separate at their boundaries by mediating mostly the H_1 receptors that is specifically blocked by chlorpheniramine (Al-Haboubi *et al.*, 1983; Goodman *et al.*, 2003; Rang *et al.*, 2007; Vinay *et al.*, 2005). From the result, it is possible that the extracts were able to block the release of these vasoactive amines by possibly stabilizing and preventing the H_1 and 5-HT_3-mediated vasoconstriction and vasodilation which could lead to the microvascular permeability and stimulation of sensory nerve terminals that mediate arachidonic acid metabolism and kallirein-kinin cascade. PEE was the most efficacious for both models however it seems to be more anti-histaminic than anti-serotonergic, possibly mediating its effect more through a histaminic pathways and slightly through the serotonergic pathway.

Studies show that COX-2 levels are elevated in paw tissues and in the CNS following carrageenan-induced inflammation (Ibuki *et al.*, 2003; Nantel *et al.*, 1999; Seibert *et al.*, 1994). COX-2 and microsomal PGE_2 Synthase-1 (mPGES-1) have been observed to accumulate at the protein level in paws at the later time, after carrageenan-induced inflammation, during the maintenance of inflammation as PGE_2 levels increased (Guay *et al.*, 2004). Indicating the modest increase of PGE_2 could primarily be COX-2 –dependent and also elevated levels of COX-2 and mPGES-1 contribute to the sustained production and accumulation of PGE_2 at the inflammation site (Guay *et al.*, 2004). The pain response observed in carrageenan-induced inflammation is due to the increased levels of PGE_2 and moderate increase in PGD_2 and $PGF_{2\alpha}$ (Guay *et al.*, 2004). Thus agents that inhibit COX-2 will invariably inhibit the synthesis of prostaglandins, prostacyclin and thromboxane, most especially PGE_2 production associated with selective up-regulation of mPGES-1 (Guay *et al.*, 2004). The extracts (PEE and EAE) were able to ameliorate inflammation induced by PGE_2. The anti-inflammatory effect of the extracts against carrageenan-induced and PGE_2-induced inflammation is possibly through the inhibition and reduction of COX-2 levels in the paws.

Neutrophil extravasation results in the migration of phygocytic cells (polymorphonuclear leukocytes) followed and replaced by migration mononuclear cells which differentiate into macrophages that dominate the inflammatory site (Tomlinson *et al.*, 1994; Willis *et al.*, 1996). Inflammatory mediators are synthesized and released by these cells with the kallirein-kinin system and arachidonic acid metabolism playing a pivotal role (Takeshita *et al.*, 2004). COX-2, kallirein-kinin and bradykinin inhibitors can block neutrophil extravasation or phygocytic cells migration, *in vivo* (Dozen *et al.*, 1989; Katori *et al.*, 1978). A major product of the kallirein-kinin system is bradykinin. The synthesis of bradykinin potentiates the secretion of COX-2 which mediates the synthesis of PGE_2 and these are responsible for the oedema formation and also for the pain that accompanies the inflammatory reaction (both bradykinin and PGE_2 are able to sensitize primary afferent neurons) (Calixto *et al.*, 2004; Calixto *et al.*, 2003; Levant *et al.*, 2006; Linhart *et al.*, 2003). Studies show that captopril increases concentration of endogenous BK through the activation of B_2 receptors and blocking of angiotensin-1converting enzyme

(ACE) or kininase II, which potentiate paw oedema (Coelho dos Santos *et al*., 2010; Levant *et al*., 2006; Souza *et al*., 2004). Result shows that the extracts of *Trichilia monadelpha* were able to inhibit bradykinin-induced oedema. The inhibition of BK-induced oedema by the extracts could be possible through reducing the concentration of BK in the system. Yet to be proven is if the extracts are eliciting its effects through deactivation of B_1 or B_2 receptors.

Inflammatory responses play significant roles in the onset, development, and evolution of so many pathological diseases such as cardiac disorders (Libby *et al*., 1997; Maseri, 1997; Pasceri *et al*., 1999). Acute-phase proteins are released by cytokines in response to inflammation. High levels of acute-phase proteins, especially, C-rective protein (CRP), has been linked with increased risk of pathological disease conditions especially cardiac disorders (Kaneko *et al*., 1999; Ridker *et al*., 2001; Ueda *et al*., 1996). CRP is also a marker of inflammation (Ikeda *et al*., 2002). Modulating role of CRP include directly increasing the expression of adhesion molecules and chemokines in human endothelial cells (Pasceri *et al*., 2001; Pasceri *et al*., 2000) and stimulating iNOS expression and NO production in rat and murine macrophages (Arcoleo *et al*., 1997; Ratnam *et al*., 1998). Thus on this premise CRP levels, as marker of acute phase response, was measured and from the result, it can be seen that PEE and EAE was observed to significantly reduce the levels of CRP. It can, therefore be said that these extracts were significant in managing the inflammatory situation from progressing further to chronic state. Though yet to be studied, is if this reduction in CRP levels could be through the direct decrease of expression of adhesion molecules and chemokines in endothelial cells (Pasceri *et al*., 2001; Pasceri *et al*., 2000) and down-regulating iNOS expression and NO production in rat macrophages (Arcoleo *et al*., 1997; Ratnam *et al*., 1998).

This study therefore, show that the extracts particularly PEE effectively suppressed the oedema produced by carrageenan, and its associate early (histamine and 5-HT) and late (PGE_2 and Bradykinin) phases mediators. The extracts were also able to regulate systemic

inflammatory response by ameliorating fever and managing process that could lead to chronic inflammatory condition.

3.5 CONCLUSION

Extracts (petroleum ether, ethyl acetate and ethanolic extracts) of *Trichilia monadelpha* are effective against mediators of inflammation.

Chapter 4

EFFECTS OF EXTRACTS OF *TRICHILIA MONADELPHA* ON CHRONIC INFLAMMATION

4.1 INTRODUCTION

Recent studies showed that extracts of *Trichilia monadelpha* have anti-inflammatory effect against adjuvant-induced arthritis (Ainooson *et al*., 2012). This chapter would give insight into the effects of the plant extracts on some factors that contribute to chronic inflammatory response observed in Adjuvant-induced arthritis (AIA). This is a model of chronic inflammatory disease in rats, induced by heat-killed cells of *Mycobacterium tuberculosis*. Characteristics of the model is infiltration of the synovial membrane associated with destruction of the joints, resembling rheumatoid arthritis in humans (Behar *et al*., 1995; Kumar *et al*., 2002). This model is widely used for therapeutic and pathogenetic studies of chronic forms of arthritis (Pearson, 1963; Pearson *et al*., 1963). Experimental arthritis in animals have some of clinical and biochemical features of patients with polyarthritic diseases such as rheumatoid arthritis (Halim *et al*., 2007).

This model is associated with weight loss or cachexia due to systemic or local actions of cytokines such as IL-6 and TNF-α (Chamundeeswari *et al*., 2003). Another feature is anaemia (reduction in haemoglobin levels) (Glen *et al*., 1977). Pathological features (oedema, infiltration into the joint of mononuclear and polymorphonuclear cells, pannus formation, periostitis and erosion of cartilage and bone (Williams, 1998) are observed. Oxidative stress (Federico *et al*., 2007; Marnett *et al*., 2003) are also observed. The present study seeks to evaluate the effects of the extracts on bone morphology and histology as well as the effect on biomarkers of oxidative stress—superoxide dismutase (SOD), myeloperoxidase (MPO) and levels of malondialdehyde (MDA).

4.2 EXPERIMENTAL PROCEDURE

4.2.1 Chronic Anti-inflammatory Effect

4.2.1.1 Animals

Male Sprague-Dawley rats (150 – 200 g) were bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, and kept in the animal house of the Department of Pharmacology, KNUST, Kumasi, Ghana. Animals were housed in aluminum cages, with rat diet (GHAFCO, Tema, Ghana) and water available, *ad libitum*. Sample size of 8 animals per group was utilised throughout the study. Guidelines on animal use was observed as instructed by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised 1985) and was approved by the Departmental Ethics Committee.

4.2.1.2 Drugs

The extracts were triturated with Tween-80 (3 drops) in normal saline (NS), and administered orally to rats at doses ranging from 10 - 100 mg kg^{-1}. Heat killed *Mycobacterium tuberculosis* (strains C, DT and PN, mixed) was bought from the Ministry of Agriculture, Fisheries and Food, U.K. A list of chemicals used for the antioxidant assay is provided in Appendix. All chemicals listed for the antioxidant assay were bought from Sigma-Aldrich, Inc. St. Louis MO USA. Quantikine rat IL-6 and TNF-α Immunoassay kit (R&D Systems, Inc., Minneapolis, USA) were used to assay IL-6 and TNF-α levels. The reference drugs used were; dexamethasone (Wuhan Grand, China) and methotrexate (Dabur Pharma, India). Animals of the extract-treated groups received ≤1 ml of extract solution. Animals of the standard drugs-treated groups received ≤1 ml of drug solution. Control group received only intraplantar injection of CFA suspension and were given normal saline. All drugs were freshly prepared.

4.2.1.3 CFA-induced Arthritis

Animals were assigned to groups of eight animals per group. Adjuvant arthritis was induced as previously described by Pearson (1956) and Wooley (1991) with modifications (Woode *et al*., 2008). Briefly, CFA was prepared from a suspension of 5 mg ml^{-1} of heat killed *Mycobacterium tuberculosis* in paraffin oil. Animals were injected intraplantar with 0.1 ml CFA into the right hind paw of rat. Arthritic control group received only sub-plantar injection of CFA, whiles non-arthritic control/IFA group received only subplantar injection of 0.1 ml Incomplete Freund's Adjuvant (IFA) (sterile paraffin oil). *Trichilia monadelpha* extracts (10, 30, and 100 mg kg^{-1} *p.o.*), dexamethasone (0.3 - 3.0 mg kg^{-1} *i.p.*) and methotrexate (0.1 – 1.0 mg kg^{-1} *i.p.*) were administered to rats in the various groups respectively.

Foot volume was measured using a water displacement plethysmometer (IITC Life Science Equipment, Woodland Hills, USA) for both the ipsilateral (injected paw) and the contralateral paw (non-injected paw). This was before subplantar injection of CFA (day 0) and every other day (day 2, 4, 6, 8,….28) after subplantar injection of CFA and IFA. The oedema component of inflammation was quantified by measuring the difference in foot volume between day 0 and the various days (Fereidoni *et al*., 2000).

The initial body weight of rats was taken on day 0 after grouping and every other day for 28 days of the experiment. This was followed by subplantar injection of 0.1 ml CFA. Drugs were administered on day 9 with the onset of arthritis.

The animals were grouped as follows:

Group 1	Non-arthritic control/IFA (sub-plantar injection of 0.1 ml IFA)
Group 2	Arthritic control/CFA (sub-plantar injection of 0.1 ml of CFA)

Group 3-5 Treated with dexamethasone (0.3, 1.0, 3.0 mg kg^{-1} *i.p.*) from day 9 and administered every other day.

Group 6-8 Treated with methotrexate (0.1, 0.3, 1.0 mg kg^{-1} *i.p.*) from day 9 and administered every 4 days.

Group 9-17 Treated with extracts (10, 30 and 100 mg kg^{-1} *p.o.*) from day 9 and administered every day.

Raw scores for ipsilateral and contralateral paw volumes were individually recorded as percentage of change from their values at day 0 and then averaged for each treatment group. Data was presented as the effect of drugs on the time course and the total oedema response of adjuvant-induced arthritis for the 28 days period. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (Kaneko *et al.*) and to determine the percentage inhibition for each treatment, the following equation was used.

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

Differences in AUCs were analysed by one-way analysis of variance (1-way ANOVA) and Holm-Sidak's *post-hoc test* using SigmaPlot version 12.3 (Systat Software Inc. Chicago USA, webmaster@cranessoftware.com).

4.2.1.4 Arthritic score

Articular index (AI) scores were recorded for each hind joint and the tail by a consistent observer blinded to the treatment received by the animal. Scoring was performed on a 0–5 scale where:

0 = no swelling or erythema,

1 = slight swelling and/or erythema,

2 = low to moderate oedema,

3 = pronounced oedema with limited joint use,

4 = excess oedema with joint rigidity,

5 = moderate to pronounced oedema on tail and/or erythema.

Pictures of the affected hindlimbs were taken on day 28 using a digital camera (Sony digital camera DCR-DVD 705E).

4.2.1.5 X-ray radiography

On day 28, the animals were anaesthetised intraperitoneally with 20 mg kg^{-1} pentobarbitone. The hindlimbs were subjected to radiographic analysis using a Faxitron X-ray machine (Hewlett-Packard, Buffalo Grove, IL) with a 0.5-mm focal spot, beryllium window, and X OMAT TL (onscreen) film. The focal film distance was 61 cm, and exposures were made over 30 s at 45 kVp and 3 mA. Radiographs were analysed by a board-certified radiologist who was blinded to the treatment groups. Quantitative scores were generated for radiographic changes in the joints in the following areas: soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes. The values were based on increasing severity in the paws (Fletcher *et al*., 1998) as follows

0 = no degenerative joint changes,

1 = slight soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes,

2 = low to moderate soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes,

3 = pronounced soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes,

4 = excess soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes.

4.2.1.6 Haematology Screening

Haemoglobin (Hb) content, Red blood cell (RBC), White blood cell (WBC) counts, Lymphocytes, Neutrophils counts, Platelet counts and Haematocrit amount or Packed cell volume (PCV) were determined using an Automated Haematology Analyser (Hardware-Wholesale, Hardware Industry Community, China).

4.2.1.7 Histology

After the X-ray rats were sacrificed by ether narcosis; hindlimbs were removed and fixed in 10 % buffered formalin. The limbs were decalcified in 5 % formic acid, processed for paraffin embedding, sectioned at 5 µm thickness, and later stained with haematoxylin and eosin for examination under a light microscope. The histopathological change of joints was blindly graded by board certified pathologists and assigned a score of 0–3 based on the following:

0 – absence of synovial hyperplasia, pannus, bone erosion, fibrosis or presence of inflammatory cells as observed as neutrophils

1 – Minimal presence of synovial hyperplasia, pannus, bone erosion, fibrosis or presence of inflammatory cells as observed as neutrophils

2 – mild/moderate presence of synovial hyperplasia, pannus, bone erosion, fibrosis or presence of inflammatory cells as observed as neutrophils

3 – More intense presence of synovial hyperplasia, pannus, bone erosion, fibrosis or presence of inflammatory cells as observed as neutrophils

The histological scores were computed and differences in AUCs were analysed by one-way analysis of variance (1-way ANOVA) and Holm-Sidak's *post-hoc-test* using SigmaPlot version 12.3 (Systat Software Inc. Chicago USA, webmaster@cranessoftware.com).

4.2.2 Determination of oxidative stress parameters

The levels of some biochemical markers of oxidative stress (MPO, MDA and SOD) were determined at site of inflammation. Paws of animals were detached from animals, rinsed and weighed, put into tubes with 5 ml of 0.01 M PBS: 1 g of paw weight. These were freeze-thawed using liquid nitrogen and tissue samples were homogenised for 10 minutes. The centrifugation to separate the various supernatant for the various assay (Ormrod, 1987; McCord, 1969; Reilly, 2001; Alberts, 2002) is describe in Figure 4.1. All centrifugation was carried out at 4 ^{0}C.

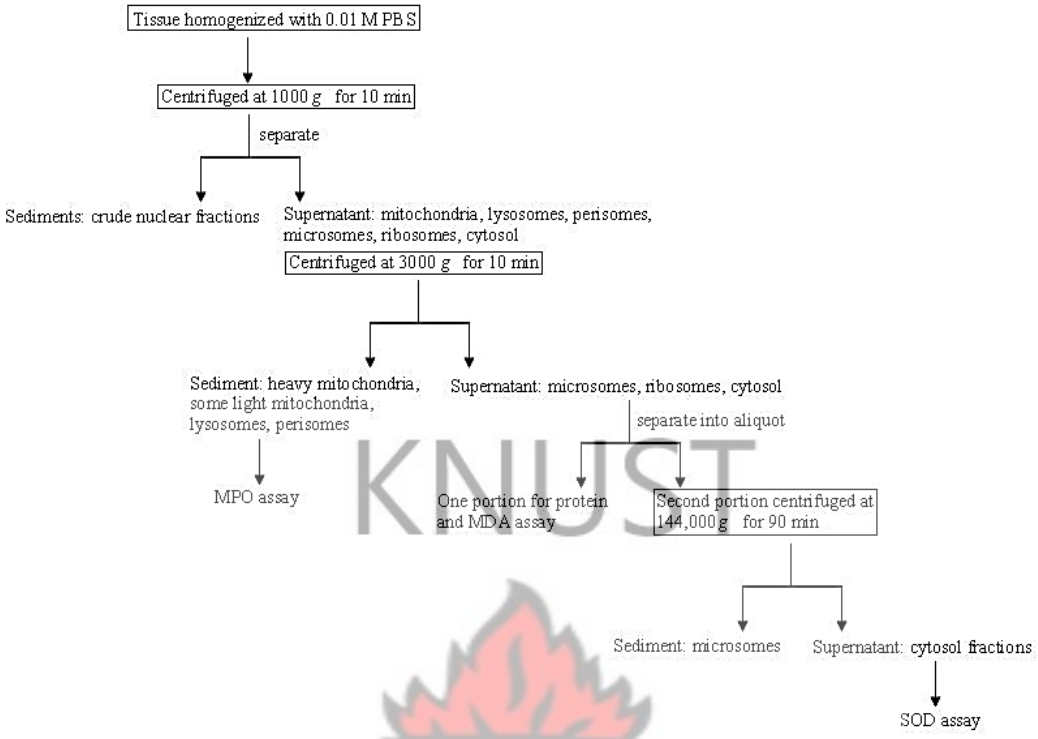


Figure 4.1: Schematic diagram of tissue preparation for evaluation of some biochemical markers of oxidative stress.

4.2.2.1 Protein Assay

The protein concentration of the tissue samples was measured at an absorbance of 700 nm using UV mini-1240 single beam spectrophotometer (Shimadzu Scientific Instruments (SSI), Kyoto, Japan). Modified Lowry's protein assay method (Lowry *et al.*, 1951; Stoscheck, 1990) was used for protein assay (see Appendix). The protein equivalent (mg) in tissue, extrapolated from standard (BSA) was used in calculations of the biochemical markers of oxidative stress evaluated.

4.2.2.2 Measurement of tissue MDA levels

Malondialdehyde (MDA) was measured according to procedure described by Reilly, 2001. A 1× working solution of TBA/TCA/HCl reagent was prepared by diluting the stock solution 4-fold in water. While stirring the solution with a magnetic stir bar, BHT was added to a final concentration of 0.03 %. The supernatant (described in Figure 4.1) and blank

(without sample) was combined with the TBA/TCA/HCl reagent at a reagent/sample ratio of 2:1 (v/v). This was mixed thoroughly, and placed in a boiling water bath for 15 min. The mixture was allowed to cool to room temperature and then centrifuged at 1000 *g* for 10 min at room temperature. The absorbance of the solution was read at 535 nm against the blank using UV mini-1240 single beam spectrophotometer (Shimadzu Scientific Instruments (SSI), Kyoto, Japan). The concentration of MDA was calculated as specific activity (U m g^{-1}) using the extinction coefficient of 1.56 × 105M^{-1} cm^{-1} (Wills, 1969), as follows:

$$volume\ activity(\ Uml^{-1}) = \frac{\Delta A_{535} \times V_t}{V_s \times \varepsilon} \times dilution\ factor\ - 1$$

$$weight\ activity(\ Umg^{-1}) = \frac{volume\ activity(\ Uml^{-1})}{mg\ material\ (tissue)(ml^{-1})}$$

$$specific\ activity(\ Umg^{-1}) = \frac{weight\ activity(\ Umg^{-1})}{mg\ protein}$$

4.2.2.3 Measurement of tissue MPO levels

The assay and Units of myeloperoxidase (MPO) activity, a constituent of neutrophil granules were performed and calculated as described by Ormrod *et al.* (1987). This involves the hydrolysis of H_2O_2. Briefly, Separated sediment (Figure 4.1) was suspended in 2 ml 0.05 M PBS (pH 6.0) containing 0.5 % c-tab or centrimide. The suspension was freeze-thawed three times and finally centrifuged at 1000 *g* for 10 minutes at room temperature. A 1 cm path length cuvette contained 873 µl of 0.05 M PBS (pH 6.0), 30 µl of undiluted sample suspension, 100 µl of *o*-dianisidine dihydrochloride (ODA) and 10 µl aminotriazole (AMT) (see Appendix for c-tab, ODA, H_2O_2 and AMT preparation). This was allowed to incubate at room temperature for 3 minutes so AMT could inhibit eosinophil peroxidase (EPO) present. Finally 10 µl of 0.5 mM H_2O_2 was added to the cuvette and change in absorbance was read at 460 nm every 5 seconds for 3 minutes. Measurements were done in duplicate. MPO was expressed as units where 1 unit is defined as that degrading 1 µmol of peroxide/min at 25 ^{0}C using the absorbance index of H_2O_2 of 11.3 (µ mol $ml^{-1})^{-1}$ cm^{-1}. Calculated as follows:

$$MPO\ total\ OD\ sec^{-1} = (\Delta MPO\ OD\ sec^{-1}) \times \frac{(1\ + weight\ of\ sample)}{0.03}$$

$$MPO\ total\ units\ = \frac{MPO\ total\ OD\ sec^{-1}}{11.3}$$

$$MPO/mg\ of protein = \frac{MPO\ total\ units}{mg\ protein}$$

4.2.2.4 Measurement of total tissue SOD activity

The total superoxide dismutase (SOD) activity was measured using the xanthine oxidase/xanthine/ cytochrome *c* method (McCord *et al.,* 1969). This method involves the competition for xanthine and xanthine oxidase generated superoxide radical between SOD and cytochrome c. The action of SOD produces a colour complex that was measured at 550 nm for 5 minutes at 1-minute interval to obtain $\Delta A_{550\ nm}$/min. SOD reaction cocktail and xanthine oxidase (XOD) solution were prepared as described in Appendix. The assay medium consisted of a reaction mixture of 2.8 ml of SOD reaction cocktail, 0.1 ml XOD solution and 0.1 ml sample in a 3 ml cuvette of 1 cm path length. The blank consisted of 2.8 ml of SOD reaction cocktail, 0.1 ml XOD solution and 0.1 ml distilled water in a 3 ml cuvette of 1 cm path length. The SOD activity was expressed as U/mg protein. The unit definition is that one unit will inhibit the rate of reduction of cytochrome c by 50 % in a coupled system using xanthine and xanthine oxidase at pH 7.8 at 25 ^{0}C in a 3 ml reaction volume. The SOD activity was calculated as follows:

$$\%\ inhibition = \frac{\Delta A_{550}\ test\ -\ \Delta A_{550}\ control}{\Delta A_{550}\ control\ -\ \Delta A_{550}\ blank} \times 100$$

$$volume\ activity\ \rightarrow\ units/ml\ enzyme = \frac{\%\ inhibition\ \times df}{50\ \%\ \times 0.1}$$

$$specific\ activity\ \rightarrow\ units/mg\ protein = \frac{units/ml\ enzyme}{mg\ protein/ml\ enzyme}$$

Where df – dilution factor of sample

50 % - inhibition of the rate of cytochrome c reduction as per the unit definition

0.1 – Volume (ml) of enzyme (XOD) used.

4.2.3 Effects on Serum Cytokine levels

The cytokines analysed were TNF-α and IL-6. Both pro-inflammatory cytokine mediators were analysed using the serum of rats induced with arthritis. Blood was obtained from rat through cardiac puncture and allowed to clot for 2 hours at room temperature. This was centrifuged at 1000 g for 20 minutes at room temperature and the serum was removed and assayed for the cytokines.

Assays were carried out as described in the kit manual. The assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α and IL-6 has been pre-coated on to a microplate. Standards, control and samples were pipetted into the wells and any rat TNF-α and IL-6 present are bound by the immobilised antibody. The wells were washed to remove any unbound substances. After the washing an enzyme-linked polyclonal antibody specific for these cytokines were added into the wells. A substrate solution was added to the wells after washing the wells to remove any unbound antibody-enzyme reagent. The enzyme reaction yielded a blue product that turned yellow when a Stop solution was added. The intensity of the colour was measured at 450 nm using Sunrise microplate reader XREAD PLUS version: V4.30 (Tecan Inc., Switzerland) powered by Smart Magellan data analysis software. Absorbance is equivalent to the amount of cytokines in standard. The sample values were read off the standard curve.

4.2.4 Statistical Analysis

The ED_{50} is the dose responsible for 50 % of the maximal effect, used as a measure of the drug's potency. E_{max} is maximal effectiveness of the drug define as the efficacy of the drug. The inhibitory effects of drugs were analysed by using an iterative computer least squares method.

This was done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) with the following nonlinear regression (three-parameter logistic equation) of a sigmoid dose-response model.

$$Y = \frac{a + (a - b)}{1 + 10^{((Log\,ED_{50} - X)}}$$

Where, X is log concentration. Y is response starting at ***a***, and goes to ***b*** with a sigmoid shape.

The fitted midpoints (ED_{50}s) of the curves were compared statistically using *F* test (Motulsky *et al*., 2003). Levels of significance were determined by one-way analysis of variance (1-way ANOVA) and Holm-Sidak's *post hoc-test* using SigmaPlot version 12.3 (Systat Software Inc. Chicago USA, webmaster@cranessoftware.com). All values were expressed as mean ± SEM. $P < 0.05$ and higher *F* values ($F > 4.0$) were considered significant.

The maximal effects of the treatment given were calculated according to the formula below, using the means caluculated through column statistics:

$$\%\ \text{maximal effect} = [Mean_{control} - Mean_{treatment}) / Mean_{control}] \times 100\ \%$$

4.3 RESULTS

4.3.1 Chronic Anti-inflammatory Effect

4.3.1.1 CFA-induced Arthritis

CFA injection into the paws of rats produced a biphasic response observed as paw swelling or oedema of the ipsilateral and contralateral paws. The first phase is the acute phase (day 0 -10 post CFA inoculation), characterised by unilateral oedema of the ipsilateral paws. This

phase peaked on day 9. The subsequent phase, polyarthritic/chronic phase (day 10-28 post CFA inoculation), is characterised by oedema of the contralateral paws. All arthritic control animals showed acute inflammatory oedema at the ipsilateral (injected paw) around days 9-10 followed by chronic polyarthritic phase which begins around day 10-12 as previously described by Pearson (1989) and Woode *et al.* (2009) . Throughout the 28-day experiment, there was no significant change in the paw volume of the non-inflamed control groups injected IFA.

ACUTE PHASE

PEE, EthE, EAE, dexamethasone and methotrexate significantly ($F_{3, 28}$=42.93 P<0.0001; $F_{3, 28}$=5.79 P<0.01; $F_{3, 28}$=14.24 P<0.0001; $F_{3, 28}$=47.42 P<0.0001; $F_{3, 28}$=60.53 P<0.0001, respectively) reduced acute phase (Figures 4.2 a, c, e; 4.3 a, c). PEE and EAE ameliorated the oedema in the ipsilateral paw, with maximal effect of 92.7 % and 76.2 % at 100 mg kg^{-1}, respectively. EthE was not significant (38.8 % at 100 mg kg^{-1}); (Figure 4.3 b, d, f). Dexamethasone and methotrexate, with maximal effect of 100.0 % at 3 mg kg^{-1} and 80.0 % at 1 mg kg^{-1} respectively, were significant in ameliorating the oedema in the ipsilateral paw (Figure 4.3 b, d).

Comparing potency of inhibition of paw oedema, PEE was the less potent (ED_{50}: 12.05±3.48 mg kg^{-1}), but most efficacious (E_{max}: 97.89). EAE was the most potent (ED_{50}: 8.45±3.60 mg kg^{-1}), and had an efficacy (E_{max}: 77.05) relatively close to PEE. EthE was the least potent (ED_{50}: 18.28±10.52 mg kg^{-1}), and the least efficacious (E_{max}: 39.08). Dexamethasone and methotrexate had very high potency (ED_{50}: 0.14±0.03 mg kg^{-1}, ED_{50}: 0.01±0.01 mg kg^{-1}, respectively) and high efficacy (E_{max}: 100.00, E_{max}: 78.46, respectively) (Figure 4.4).

POLYARTHRITIC PHASE

PEE, EthE, EAE dexamethasone and methotrexate significantly ($F_{3, 28}$=13.37 P<0.0001; $F_{3, 28}$=5.57 P<0.01; $F_{3, 28}$=5.62 P<0.01; $F_{3, 28}$=57.76 P<0.0001; $F_{3, 28}$=24.27 P<0.0001, respectively) reduced polyarthritic/chronic phase (Figure 4.2 a, c, e insets; 4.3 a, c insets). PEE, EAE, dexamethasone and methotrexate also significantly ($F_{3, 42}$=11.31 P<0.0001; $F_{3, 42}$=4.69 P<0.01; $F_{3, 42}$=10.08 P <0.0001; $F_{3, 42}$=10.43 P<0.0001, respectively) minimized the progression of the

inflammation from the acute to the polyarthritic phase, (Figure 4.2 b, f; 4.3 b, d). PEE, EthE and EAE ameliorated the oedema in the contralateral paw, with maximal effect of 98.0, 69.1, 70.8 %, respectively, at the highest doses of each extract (Figure 4.2 b, d, f). Dexamethasone and methotrexate also with maximal effect of 125.3 % at 3 mg kg^{-1} and 94.7 % at 1 mg kg^{-1}, respectively were significant in ameliorating the oedema in the contralateral paws (Figure 4.3 b, d).

Comparing potency of inhibition of paw oedema, PEE was the most potent (ED_{50}: 11.36±5.74 mg kg^{-1}), and most efficacious (E_{max}: 100.00). EAE was the least potent (ED_{50}: 28.05±11.64 mg kg^{-1}), and close efficacy (E_{max}: 101.4) to PEE. EthE, potent (ED_{50}: 15.85±42.40 mg kg^{-1}), was the least efficacious (E_{max}: 77.96). Dexamethasone and methotrexate had very high potency (ED_{50}: 0.08±0.02 mg kg^{-1}, ED_{50}: 0.02±0.03 mg kg^{-1}, respectively) and high efficacy (E_{max}: 127.1, E_{max}: 98.59, respectively) (Figure 4.4).

Plates 4.1 and 4.2 show photographs of experimental groups of rats.

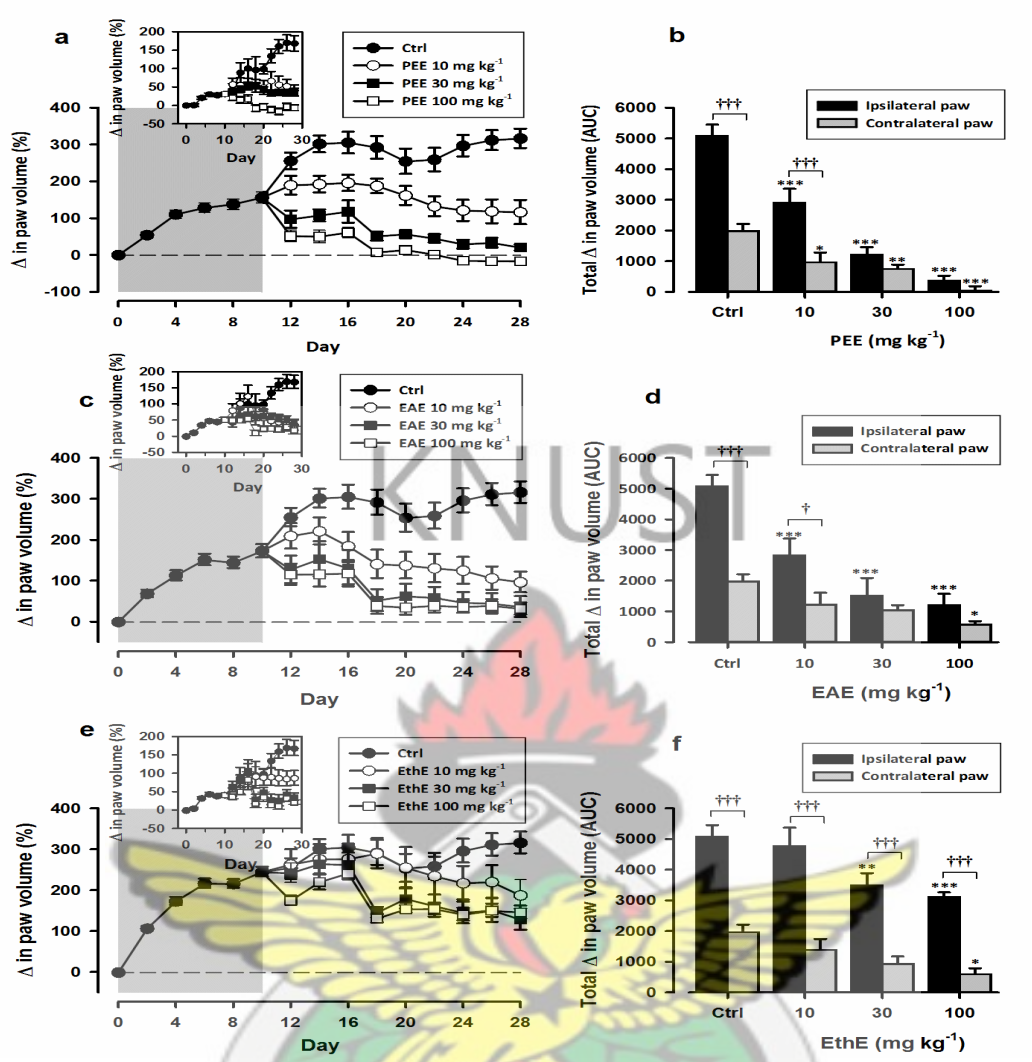


Figure 4.2: Effects of PEE, EAE and EthE, on CFA-induced arthritis
Time course curves shows effects of (a) PEE, (c) EAE and (e) EthE, (shaded and unshaded portions show acute and polyarthritic phases, respectively). Each bar represents mean± SEM (n=8). *** $P< 0.001$, ** $P< 0.01$: * $P< 0.05$. ††† $P< 0.001$, †† $P< 0.01$: † $P< 0.05$ compares mean± SEM using One-way (*control group* × *treatment groups*) and two-way (*ipsilateral* x *contralateral*) ANOVA of total change in paw oedema (AUC), respectively. All comparison of variances was done using Holm-Sidak's *post hoc* test.

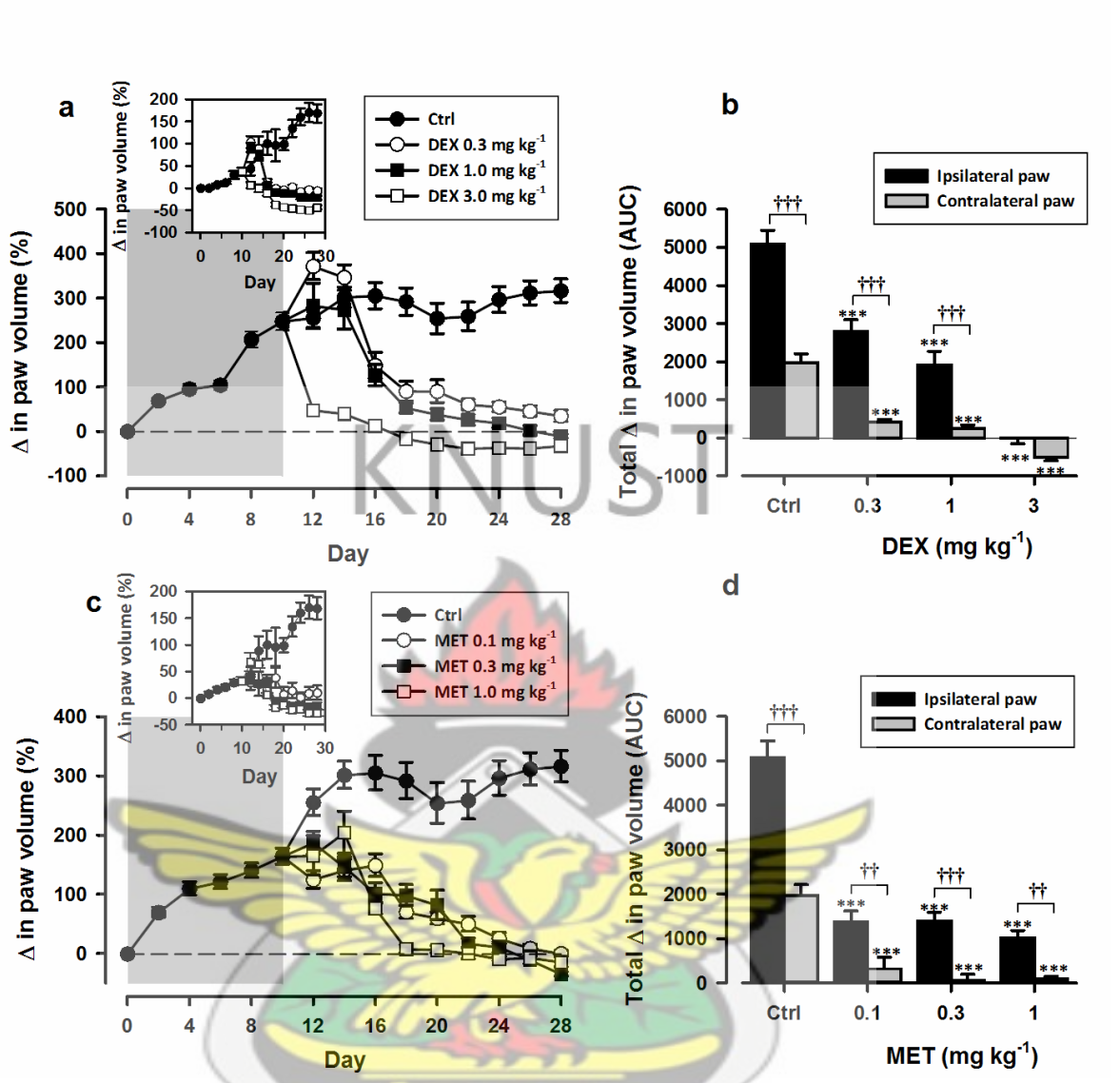


Figure 4.3: Effects of dexamethasone and methotrexate, on CFA-induced arthritis
Time course curves of (a) dexamethasone and (c) methotrexate (shaded portion shows actue phase). Each bar represents mean± SEM (n=8). *** *P*< 0.001, ** *P*< 0.01: * *P*< 0.05. †††*P*< 0.001, ††*P*< 0.01: †*P*< 0.05 compares mean± SEM using One-way (*control group* × *treatment groups*) and two-way (*ipsilateral* x *contralateral*) ANOVA of the total change in paw oedema (AUC), respectively. All comparison of variances was done using Holm-Sidak's *post hoc* test.

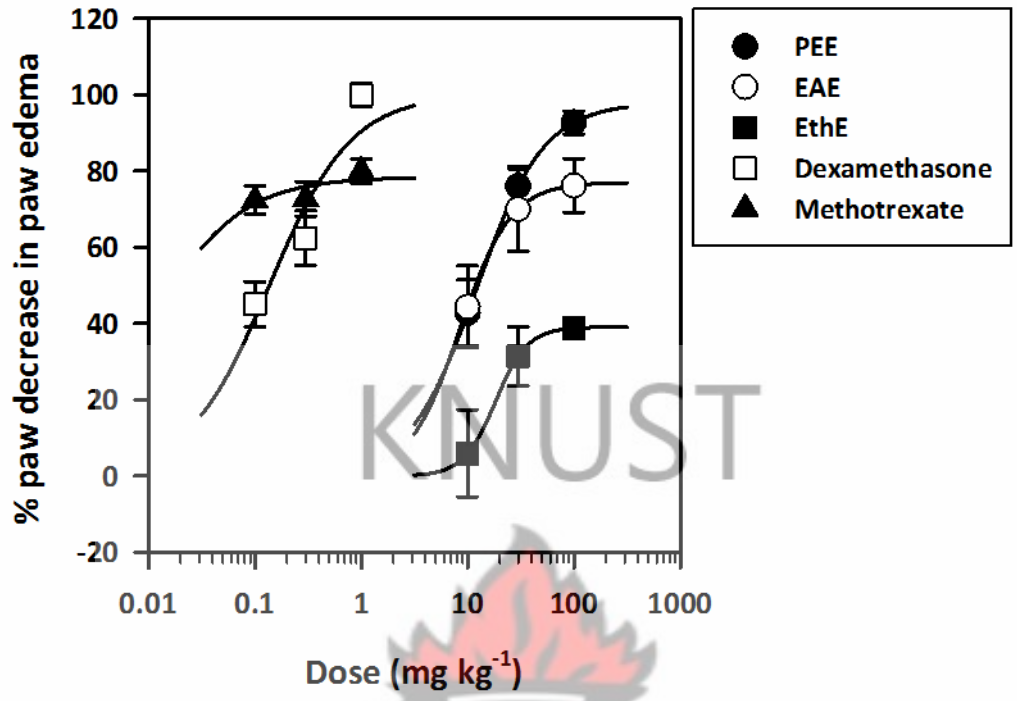


Figure 4.4: Dose-response curve of extracts of *Trichilia monadelpha*, dexamethasone and methotrexate on CFA-induced arthritis

Each extract exhibited different potency and efficacy, observed as the high or low steepness of the Hillslope of the curve. The data points reflect mean values of $n=3$ repeated trials ± s.e.m. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

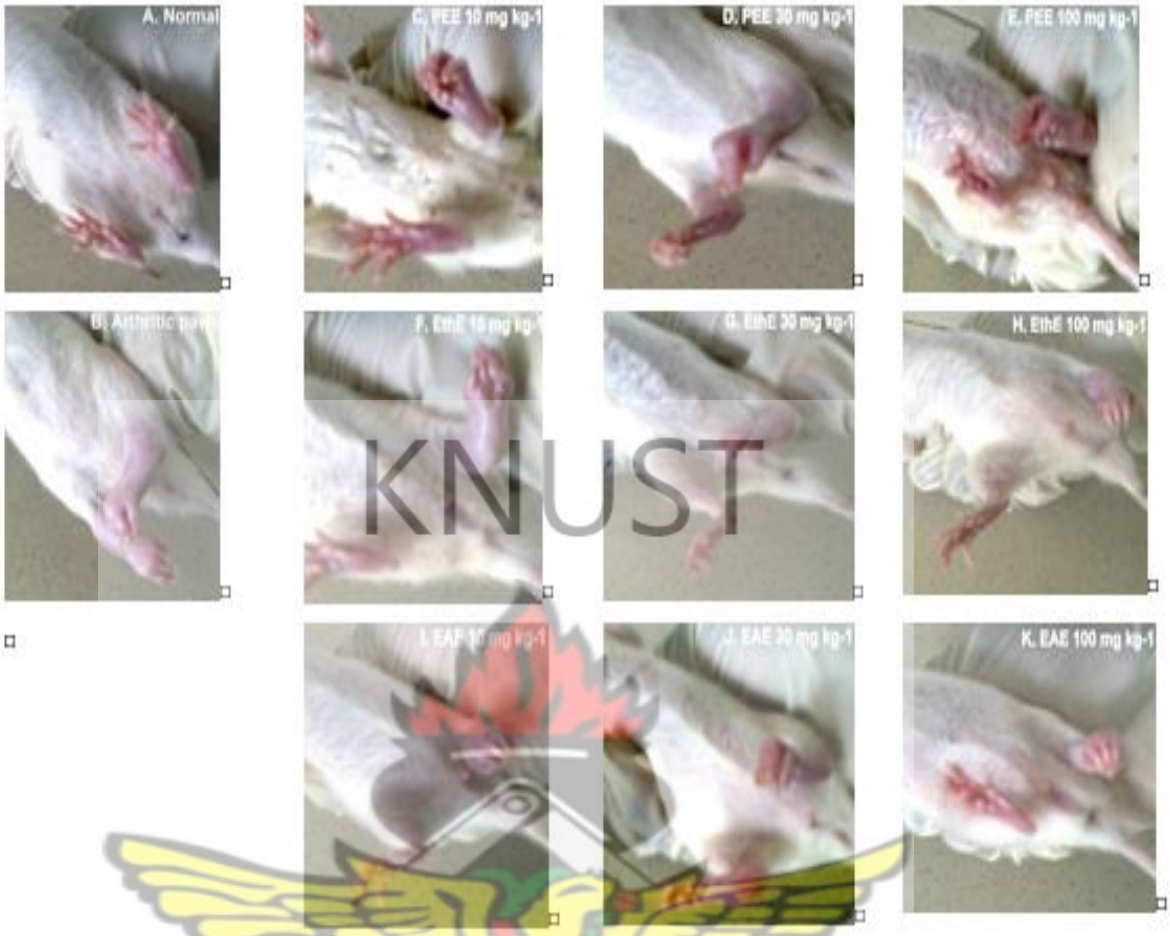


Plate 4.1: Photographs of representative rats from (B) CFA/arthritic control, (A) IFA/ non arthritic control and extracts-treated groups (10, 30 and 100 mg kg^{-1}); PEE (C, D and E), EthE (F, G and H) and EAE (I, J and K) respectively. (The alphabets represent the respective doses).

Plate 4.2: Photographs of rats treated with dexamethasone (0.3, 1.0, and 3.0 mg kg^{-1}) (A, B and C) and methotrexate (0.1, 0.3 and 1.0 mg kg^{-1}) (D, E and F). (The alphabets represent the respective doses).

4.3.1.2 Body weight

The CFA group experienced weight loss with excess swelling, erythema and joint rigidity in both ipsilateral and contralateral paws (Plate 4.1). EthE, dexamethasone and methotrexate significantly ($F_{3, 28}$=5.01 P<0.01; $F_{3, 28}$=4.28 P<0.05; $F_{3, 28}$=17.32 P<0.0001) improved the body weights of the rats (Figure 4.5 c – e). PEE and EAE did not significantly improve the body weight of the arthritic rats. (Figure 4.5 a and b).

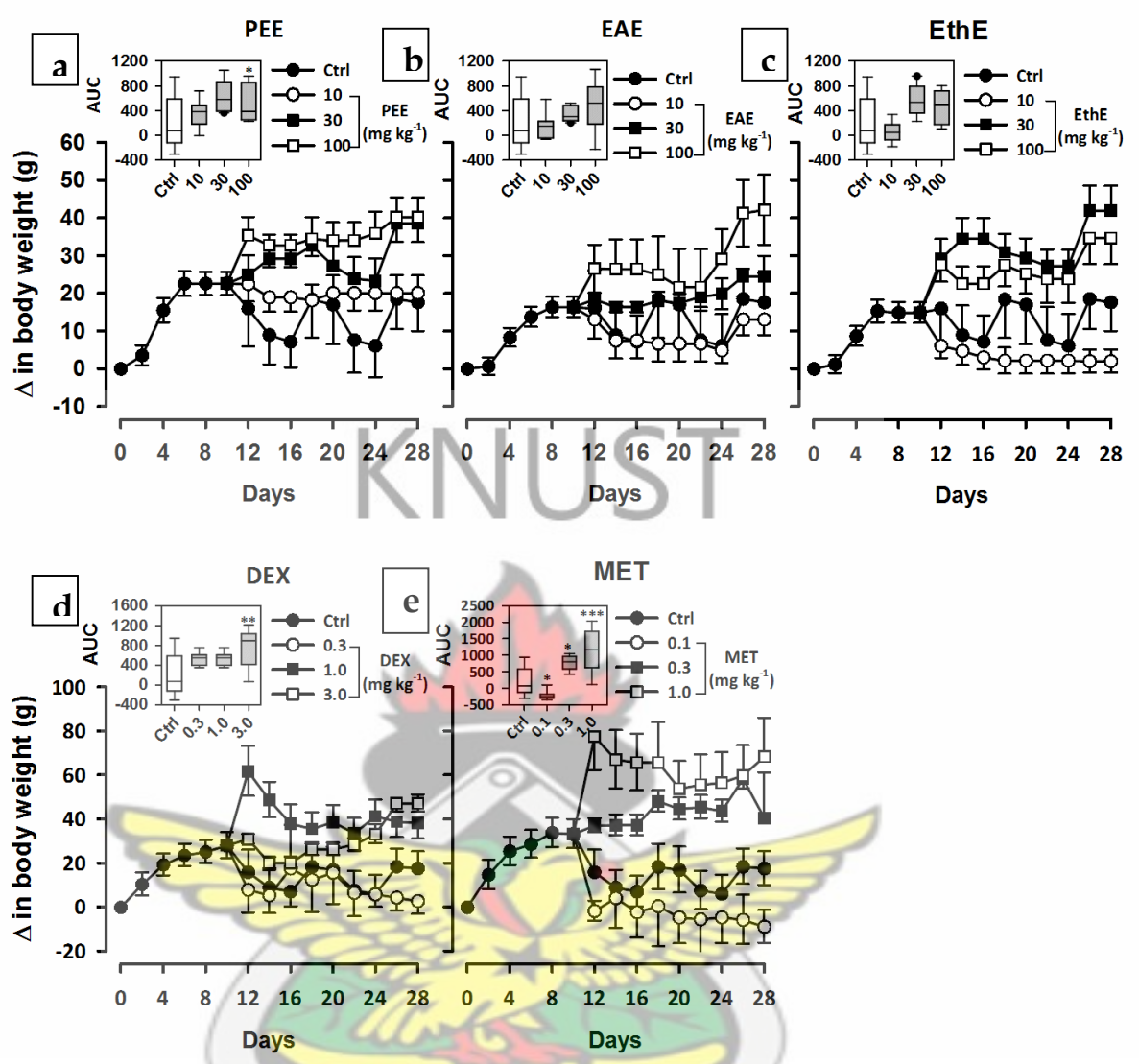


Figure 4.5: Effects of *Trichilia monadelpha* extracts, dexamethasone and methotrexate on body weight of CFA-induced arthritis rats.

Time course curve effects of (a) PEE, (b) EAE, (c) EthE, (d) dexamethasone and (e) methotrexate and boxplot (a – e) derived from the time course curves for 28 days [defined as the area under the time course curves (AUC). Each point and boxplot, (showing the 1st quartile to the 3rd quartile; the vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± SEM (n=5). *** $P< 0.001$, ** $P< 0.01$: * $P< 0.05$ compares mean ± SEM, determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

4.3.1.3 Arthritic Score

PEE, EthE and EAE, significantly ($F_{3, 28}$=5.77 P<0.01; $F_{3, 28}$=3.84 P<0.05; $F_{3, 28}$=3.77 P<0.05, respectively) reduced arthritic score similar to dexamethasone and methotrexate ($F_{3, 28}$=32.16 P<0.0001; $F_{3, 28}$=13.34 P<0.0001, respectively) (Figure 4.6 a – e; Plate 4.1, 4.2).

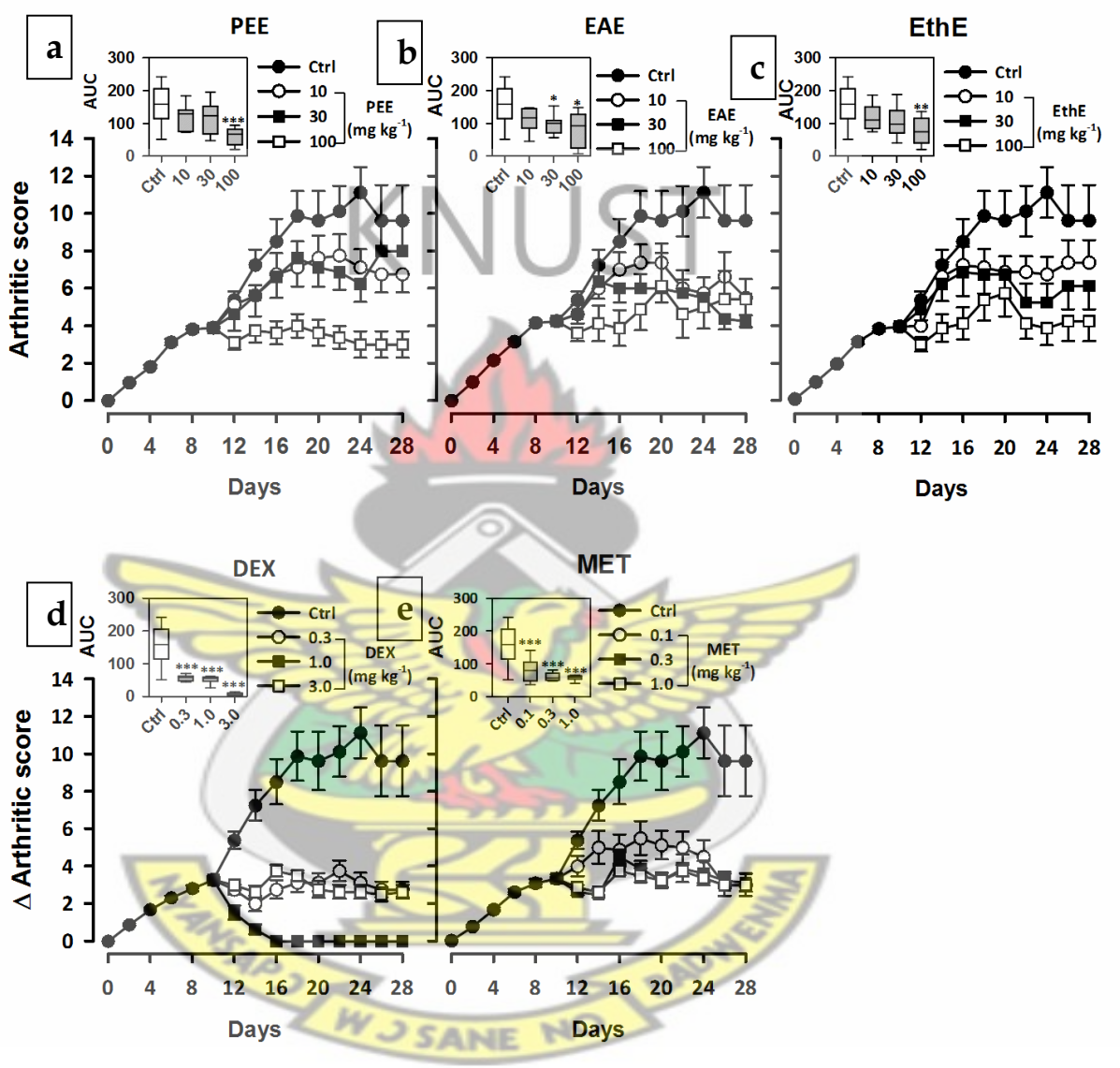


Figure 4.6: Effects of *Trichilia monadelpha* extracts, dexamethasone and methotrexate on score of CFA-induced arthritis rats.

Time course curve effects of (a) PEE, (b) EAE, (c) EthE, (d) dexamethasone and (e) methotrexate and boxplot (a – e) derived from the time course curves, showing the total oedema response for 28 days [defined as the area under the time course curves (AUC)]. Each point and boxplot, (showing the 1st quartile to the 3rd quartile; the vertical line is drawn at the 2nd quartile which is the median of the data) represents the mean ± SEM (n=5). *** P< 0.001, ** P< 0.01: * P< 0.05 compares mean ± SEM determined using one-way ANOVA and Holm-Sidak's *post hoc* test.

4.2.1.5 X-ray radiography

Radiographs of representative rats from CFA group displayed arthritic changes in both primary and secondary paws compared with IFA group (Plate 4.3). These arthritic changes were characterised by soft tissue swelling and evidence of bone changes with the most obvious damage taking place in the tibiotarsals joint. The radiographs were scored for the extent of bone damage at the joints as shown in Tables 4.1. CFA group had severe bone enlargement with active osteophytosis in the bone metaphysis, osteolysis, focal areas of excessive bone resorption, subchondal erosion, and subluxation. The bones of the IFA/non arthritic control group were intact.

PEE, EthE, EAE, dexamethasone and methotrexate-treated groups showed suppressed radiological features (Plate 4.3 c – k; Plate 4.4 a – f). The radiological scores of PEE, EthE, EAE, dexamethasone and methotrexate are shown in Table 4.1.

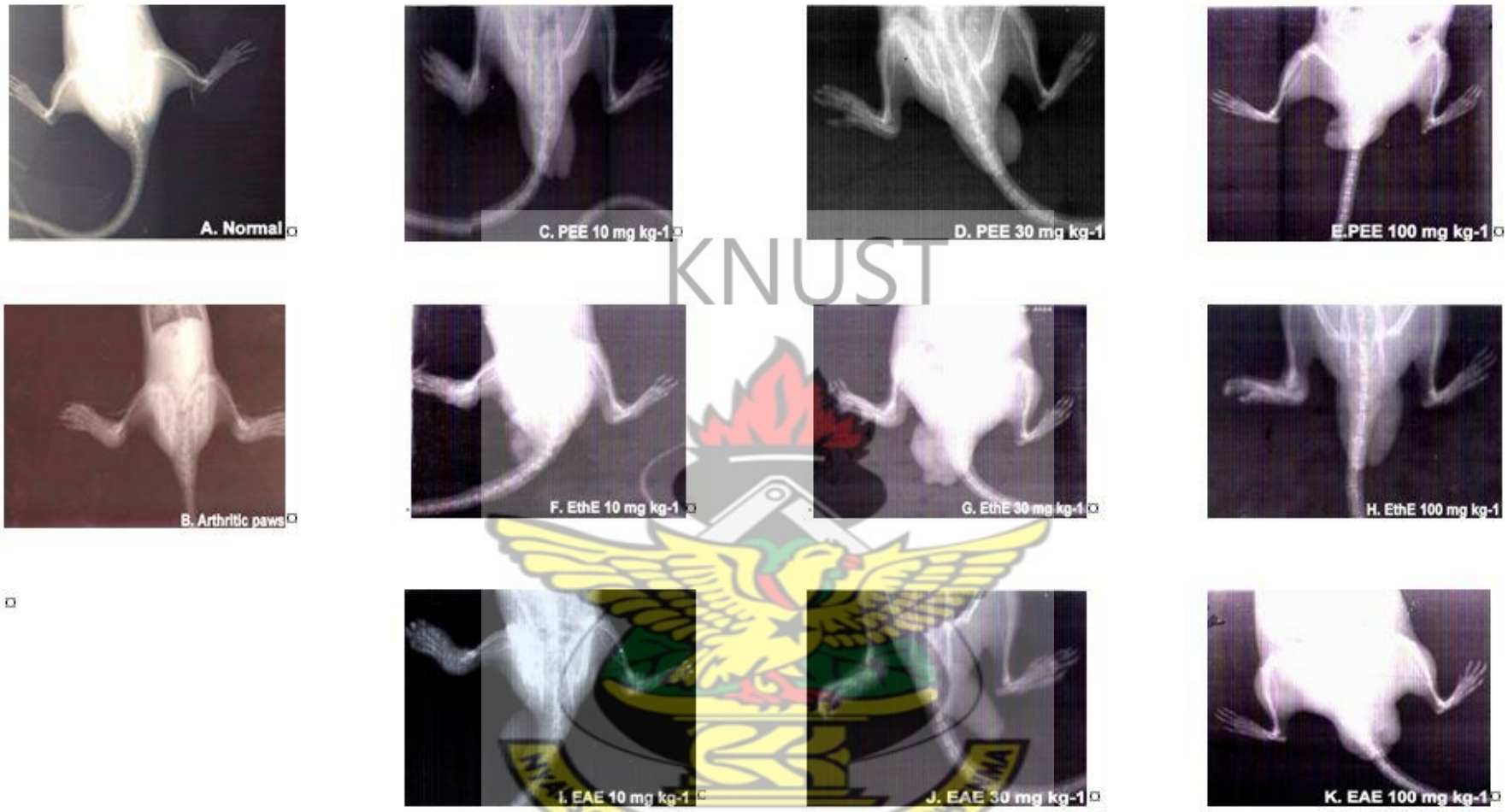


Plate 4.3: Radiographs of representative rats from (A) CFA/arthritic control, (B) IFA/ non arthritic (B) control and extracts-treated groups (10, 30 and 100 mg kg^{-1}); PEE (C, D and E), EthE (F, G and H) and EAE (I, J and K) respectively. (The alphabets represent the respective doses).

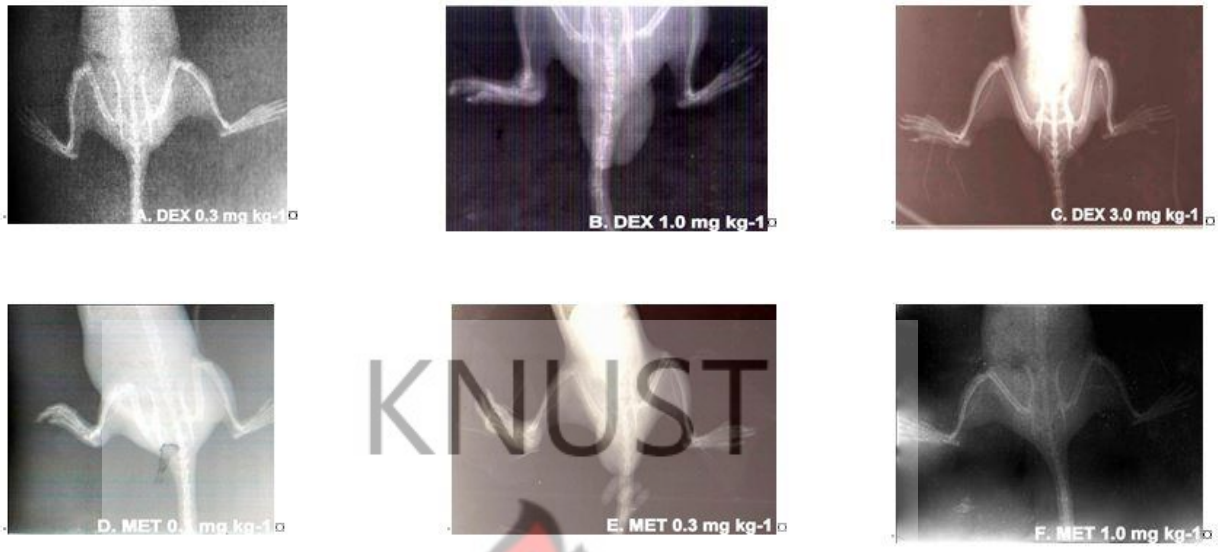


Plate 4.4: Radiographs of rats treated with dexamethasone (0.3, 1.0, and 3.0 mg kg^{-1}) **(A, B and C) and methotrexate (0.1, 0.3 and 1.0 mg kg^{-1}) (D, E and F). (The alphabets represent the respective doses).**

Table 4.1: Arthritic scores of arthritic paws (ipsilateral and contralateral) obtained from X-ray radiography.

	Group	X-ray score	
		Ipsilateral paws	Contralateral paws
	IFA	0**	0**
	CFA	4±0.3	4±0.3
PEE	10 mg kg^{-1}	2±0.9	2±0.3
	30 mg kg^{-1}	2±0.7	0.7±0.7
	100 mg kg^{-1}	1±0.6	1±0.3
EthE	10 mg kg^{-1}	3±0.6	2±0.3
	30 mg kg^{-1}	2±0.9	2
	100 mg kg^{-1}	4±0.3	2±0.9
EAE	10 mg kg^{-1}	3±0.3	2±1.0
	30 mg kg^{-1}	3±0.6	1±0.7
	100 mg kg^{-1}	2±0.9	2
Dexamethasone	0.3 mg kg^{-1}	1±0.9	0.7±0.7
	1.0 mg kg^{-1}	0**	0.3±0.3*
	3.0 mg kg^{-1}	0**	0**
Methotrexate	0.1 mg kg^{-1}	0.7±0.3	0.3±0.3*
	0.3 mg kg^{-1}	1±0.6	0.7±0.7
	1.0 mg kg^{-1}	0.3±0.3*	0**

4.2.1.6. Haematology Screening

Table 4.2 shows the haematological parameters such as Hb, RBC count, percentage Lymphocytes and Neutrophil count, PCV, WBC count, platelet count, of the normal and experimental groups of rats. A significant decrease in the level of Hb, RBC count, and PCV were observed in arthritic rats (CFA) when compared with normal rats (IFA). Administration of the drugs and extracts to arthritic rats resulted in an increase of Hb, RBC count, and PCV to near normal levels. The increased levels of WBC, platelets were significantly (P<0.05) suppressed by extracts (PEE, EthE and EAE), dexamethasone and methotrexate.

Table 4.2: Haematological parameters profile of *Trichilia monadelpha* extracts and reference drugs in normal and experimental rats.
***P < 0.001; ** P < 0.01; *P < 0.05 compared to Arthritic group (CFA)

	WBC (µl)	RBC (µl)	Hb (g dl^{-1})	PCV (%)	PLT (µl)	LYM (%)	NEUT (%)
IFA	$5.40\pm1.10\times10^3$	$7.77\pm0.21\times10^6$	13.40±0.00	45.55±0.95	$778.0\pm13.00\times10^3$	31.75±3.15	27.55±3.65
CFA	$19.10\pm1.70\times10^3$	$5.00\pm0.10\times10^6$	6.55±0.35	24.10±3.50	$3070\pm499.5\times10^3$	89.55±8.95	70.85±7.45
PEE 10 mg kg^{-1}	$13.20\pm1.30\times10^3$	$8.55\pm0.71\times10^{6***}$	14.20±0.20***	49.90±1.90**	$681.5\pm116.5\times10^{3***}$	60.15±11.75	41.90±11.20
30 mg kg^{-1}	$12.80\pm2.10\times10^3$	$7.50\pm0.16\times10^{6**}$	11.90±0.50***	42.10±1.40	$949.0\pm267.0\times10^{3***}$	57.20±9.70	38.45±9.75
100 mg kg^{-1}	$14.25\pm0.95\times10^3$	$8.00\pm0.11\times10^{6**}$	12.90±0.20***	45.05±0.85*	$737.0\pm29.0\times10^{3***}$	71.60±5.40	29.35±3.75**
EthE 10 mg kg^{-1}	$10.25\pm2.05\times10^3$	$7.92\pm0.17\times10^{6**}$	13.05±0.75***	46.15±1.75*	$683.0\pm244.0\times10^{3***}$	69.50±1.60	53.95±2.75
30 mg kg^{-1}	$10.55\pm2.25\times10^3$	$7.50\pm0.28\times10^{6**}$	12.90±0.30***	44.50±01.60*	$789.0\pm192.0\times10^{3***}$	68.50±6.10	57.75±0.95
100 mg kg^{-1}	$9.65\pm3.85\times10^3$	$7.80\pm0.07\times10^{6**}$	12.70±0.50***	43.60±1.70*	$767.5\pm87.5\times10^{3***}$	77.25±11.25	51.50±2.80
EAE 10 mg kg^{-1}	$12.85\pm1.45\times10^3$	$7.89\pm0.27\times10^{6**}$	14.90±0.40***	47.15±1.95**	$794.5\pm24.50\times10^{3***}$	52.60±3.80	58.15±4.95
30 mg kg^{-1}	$8.90\pm1.30\times10^3$	$8.74\pm0.48\times10^{6***}$	15.95±0.35***	50.20±1.50**	$601.5\pm57.50\times10^{3***}$	42.60±8.60	55.20±5.10
100 mg kg^{-1}	$9.35\pm1.95\times10^3$	$7.63\pm0.03\times10^{6**}$	14.60±0.10***	46.20±0.90*	$567.5\pm206.5\times10^{3***}$	56.25±13.05	59.05±0.95
DEX 0.3 mg kg^{-1}	$9.70\pm4.70\times10^3$	$8.01\pm0.33\times10^{6**}$	14.55±0.05***	50.65±0.25**	$787.5\pm71.50\times10^{3***}$	44.70±10.40	57.40±8.6
1.0 mg kg^{-1}	$5.00\pm0.00\times10^{3}$*	$8.64\pm0.07\times10^{6***}$	14.75±0.35***	49.85±3.25**	$730.5\pm56.50\times10^{3***}$	35.55±3.45*	34.85±1.15*
3.0 mg kg^{-1}	$4.65\pm0.35\times10^{3}$*	$8.80\pm0.20\times10^{6***}$	15.50±0.70***	57.70±1.00***	$720.5\pm266.50\times10^{3***}$	33.30±0.50*	30.10±0.50**
MET 0.1 mg kg^{-1}	$8.45\pm0.25\times10^3$	$8.45\pm0.25\times10^{6***}$	12.35±0.55***	43.05±1.85	$937.5\pm482.5\times10^{3***}$	62.15±11.85	50.85±5.95
0.3 mg kg^{-1}	$5.15\pm0.15\times10^{3}$*	$9.20\pm0.30\times10^{6***}$	14.80±0.70***	57.20±9.70***	$625.0\pm95.0\times10^{3***}$	38.15±3.65*	37.15±3.55
1.0 mg kg^{-1}	$5.25\pm1.15\times10^{3}$*	$9.60\pm0.80\times10^{6***}$	13.05±0.65***	51.05±4.55**	$758.5\pm227.5\times10^{3***}$	31.75±1.45**	31.95±2.15*

4.2.1.7 Histology

Arthritis induced in rats resulted in synovial hyperplasia, pannus formation, exudation of inflammatory cells, observed as accumulation of abundant monomorphonuclear and polymorphonuclear cells into the joint space, and erosion of bone and cartilage (Plate 4.5). PEE, EthE and EAE-treated group showed a dose-dependent reduction ($F_{4,9}$ =65.14 P<0.001; $F_{4,9}$ =19.10 P<0.01; $F_{4,9}$ =6.99 P<0.05, respectively) of inflammation with the morphology of the synovium looking normal synovium (Figures 4.7 a – b; Plate 4.5 e - f). Dexamethasone and methotrexate improved the morphology of the tissue histology significantly ($F_{4,9}$ =17.75 P<0.01; $F_{4,9}$ =18.75 P<0.01, respectively) (Figures 4.7 d and e; Plate 4.6 c and d).

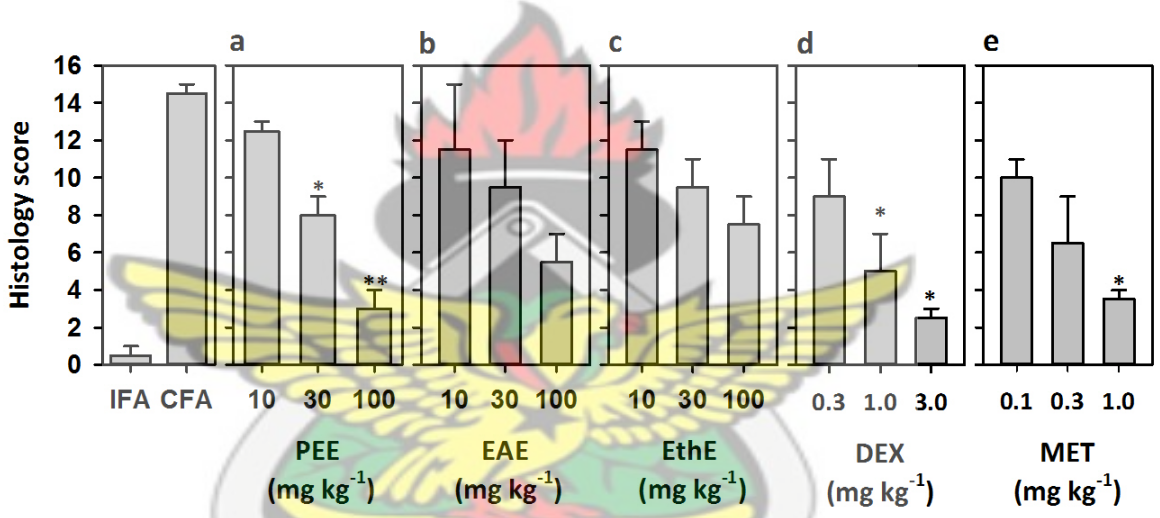


Figure 4.7: Histological score of the representative rat paw synovium
Histological score for bone erosion, inflammatory cell infiltration, pannus formation, synovial hyperplasia and fibrosis. Column bars represent mean score Values ± SEM. (n = 8). ***P < 0.001; ** P < 0.01; *P < 0.05 compared to CFA group. All comparison of variances was done using Holm-Sidak's *post hoc* test.

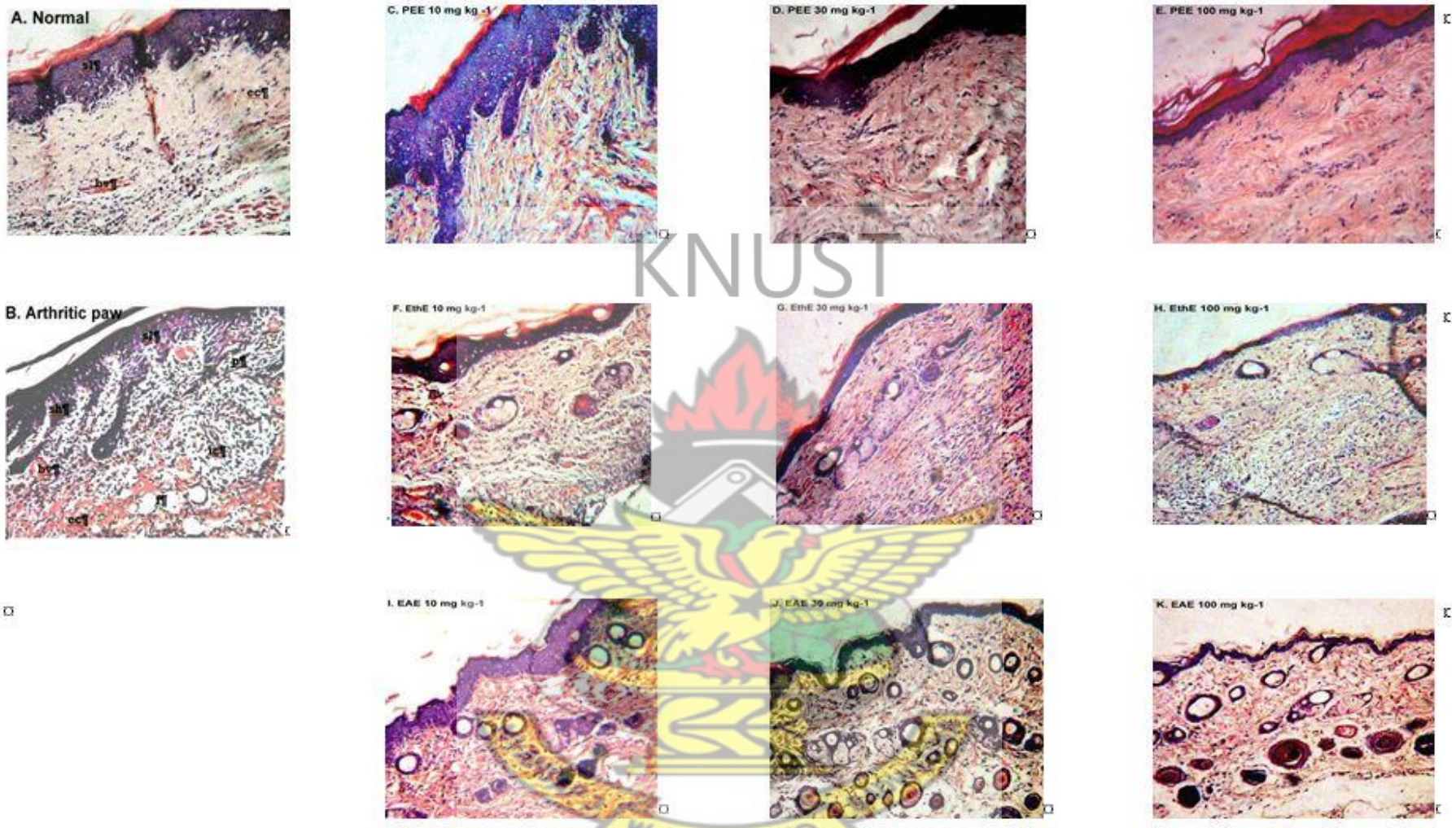


Plate 4.5: Histology of the representative rat paw synovium of control groups and extracts-treated groups

Histology of the representative synovium of control and extracts-treated rat paws showing H&E staining of the synovium revealing degree of inflammatory cell infiltration (magnification x400). A – IFA. B – CFA. C – K represents synovium of various doses of extracts as indicated. sl- synovial lining. ec- endothelial cells. bv- blood vessels. p- pannus. f- fibrosis. ic- inflammatory cells. sh-synovial hyperplasia.

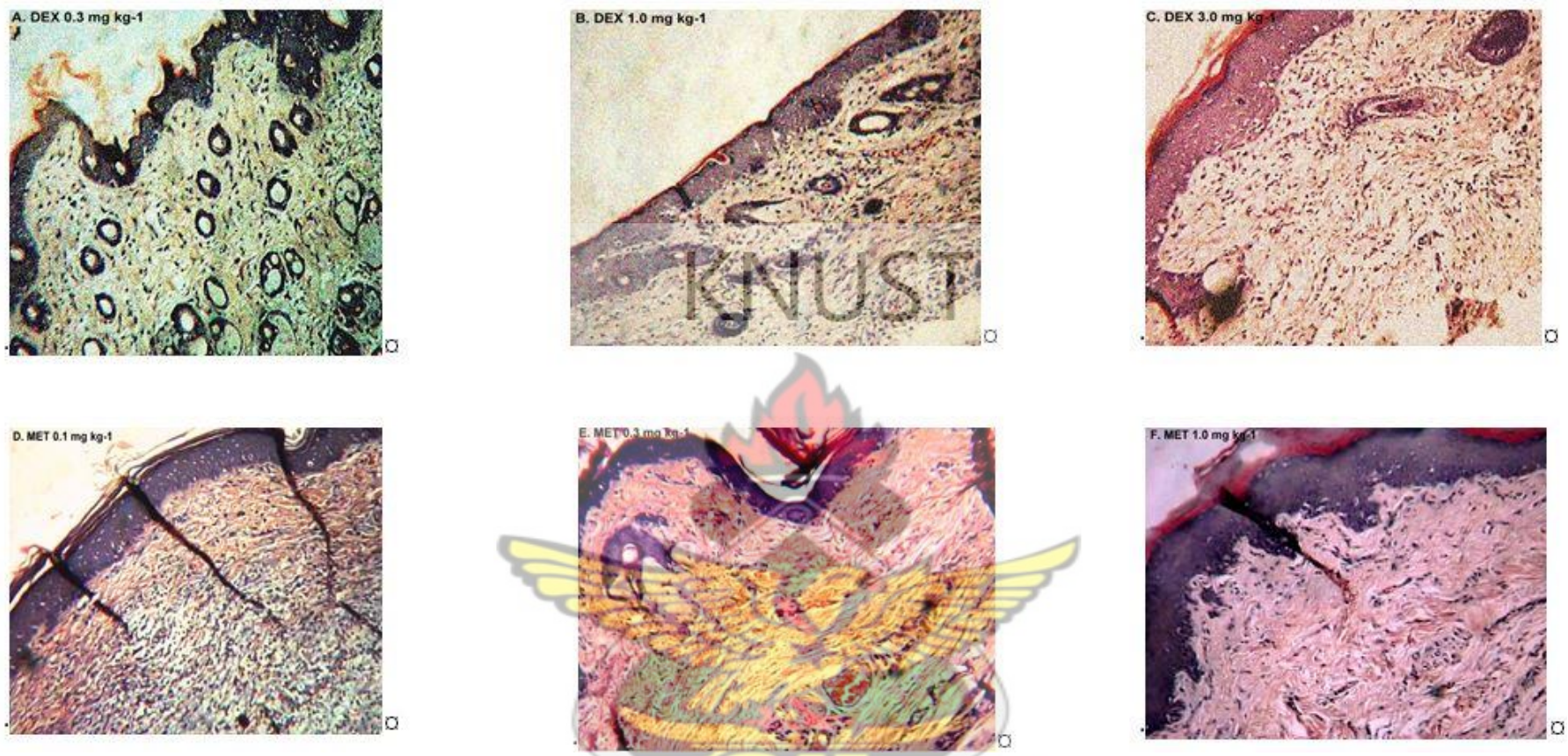


Plate 4.6: Histology of the representative synovium of rat paw treated with reference drugs
Histology of the representative synovium of dexamethasone and methotrexate-treated groups showing H&E staining of the synovium revealing a dose-dependent reduction in degree of inflammatory cell infiltration (magnification x400). A –f represents the doses of dexamethasone and methotrexate as indicated.

4.3.2 Levels of oxidative stress parameters

4.3.2.1 Tissue MDA levels

It was observed CFA group showed a significant high-level of MDA activity. The administered doses of the extracts PEE, EAE and EthE, had significant ($F_{4, 16}$=810.2 P<0.0001; $F_{4, 16}$=8572.0 P<0.0001; $F_{4, 16}$=3971.0 P<0.0001, respectively; Figure 4.8 a - c) dose-dependent effects in reducing MDA levels in the first inflammatory response phase. Dexamethasone and methotrexate also had significant ($F_{4, 16}$=289.0 P<0.0001; $F_{4, 16}$=8975.0 P<0.0001, respectively; Figure 4.8 d, e) dose-dependent effects in reducing MDA levels in the first inflammatory response phase.

The extracts PEE, EAE and EthE, showed significant ($F_{1, 4}$=211.5 P<0.001; $F_{1,4}$=404.8 P<0.0001; $F_{1, 4}$=625.8 P<0.0001, respectively; Figure 4.8 a - c) effects in reducing MDA levels in the ipsilateral paws and the contralateral paws in the second phase of inflammation. Dexamethasone and methotrexate also had significant ($F_{1, 4}$=119.0 P<0.0001; $F_{1, 4}$=1099.0 P<0.0001, respectively; Figure 4.8 d, e) effects in reducing MDA levels in the ipsilateral paws and the contralateral paws in the second phase

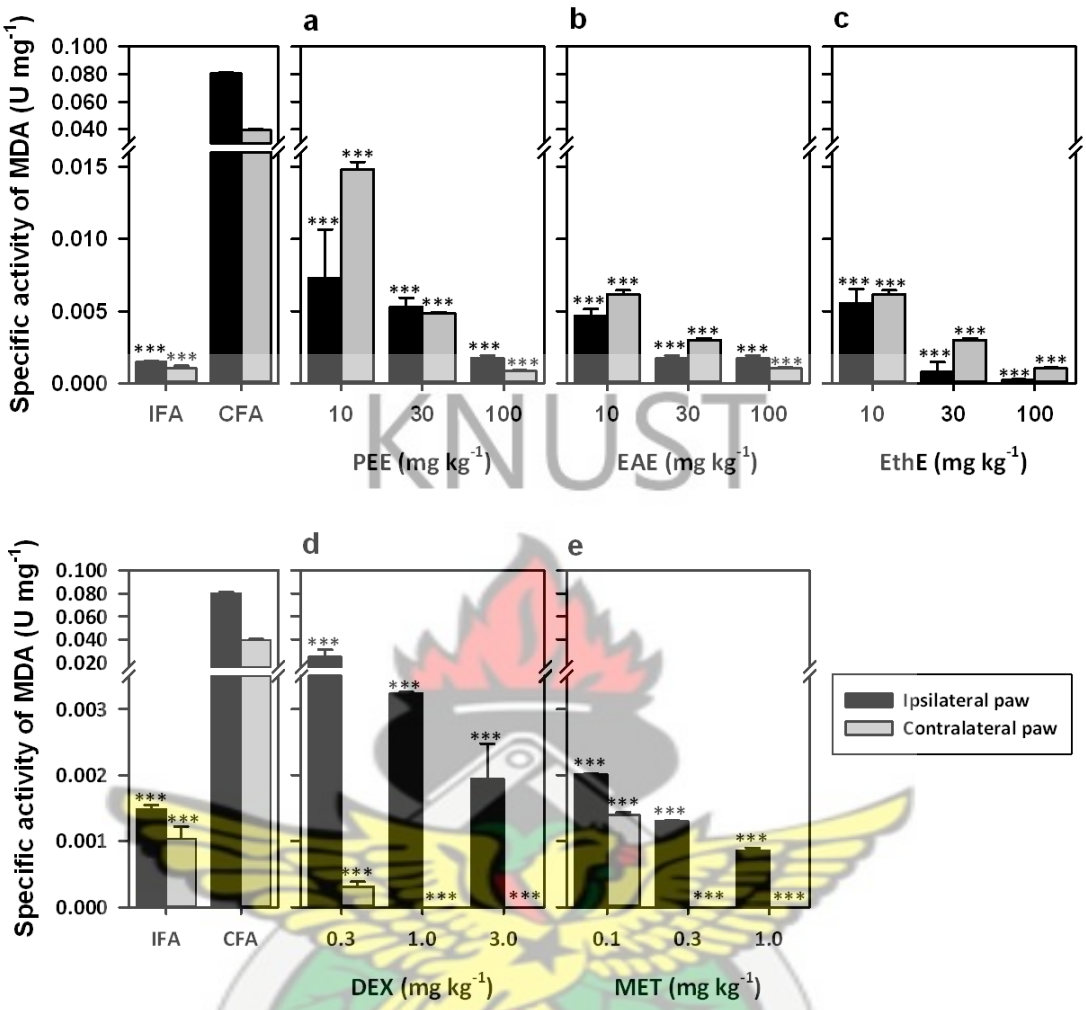


Figure 4.8: Tissue MDA (malondialdehyde) levels in Adjuvant-induced arthritis in rats.
The tissue MDA levels were markedly reduced in (a) PEE, (b) EAE, (c) EthE-treated groups and the reference drugs (d) dexamethasone and (e) methotrexate-treated groups for both the ipsilateral and contralateral paws of the arthritic rats. Values are mean ± SEM. (n = 8). ***P < 0.001; ** P < 0.01; *P <0.05 compared to CFA group. All comparison of variances was done using Holm-Sidak's *post hoc* test.

4.3.2.2 Tissue MPO levels

CFA group showed a significant high-level of MPO activity. The administered doses of the extracts PEE, EAE and EthE, had significant ($F_{4, 16}$=111.5 P<0.0001; $F_{4, 16}$=103.7 P<0.0001; $F_{4, 16}$=110.1 P<0.0001, respectively; Figure 4.9 a - c) dose-dependent effects in reducing MPO levels in the first inflammatory response phase. Dexamethasone and methotrexate also had significant ($F_{4, 16}$=100.9 P<0.0001; $F_{4, 16}$=114.5 P<0.0001, respectively; Figure 4.9 d, e) dose effects in reducing MPO levels in the first inflammatory response phase.

PEE, EAE and EthE significantly ($F_{1, 4}$=0.08 P>0.05; $F_{1, 4}$=0.0009 P>0.05; $F_{1, 4}$=0.12 P>0.05, respectively; Figure 4.9 a - c) reduce MPO levels in the ipsilateral paws and the contralateral paw of phase two. Dexamethasone and methotrexate also was significant ($F_{1, 4}$=0.06 P>0.05; $F_{1, 4}$=0.04 P>0.05, respectively; Figure 4.9 d, e) in reducing MPO levels in the ipsilateral paws and the contralateral paws in the second phase.

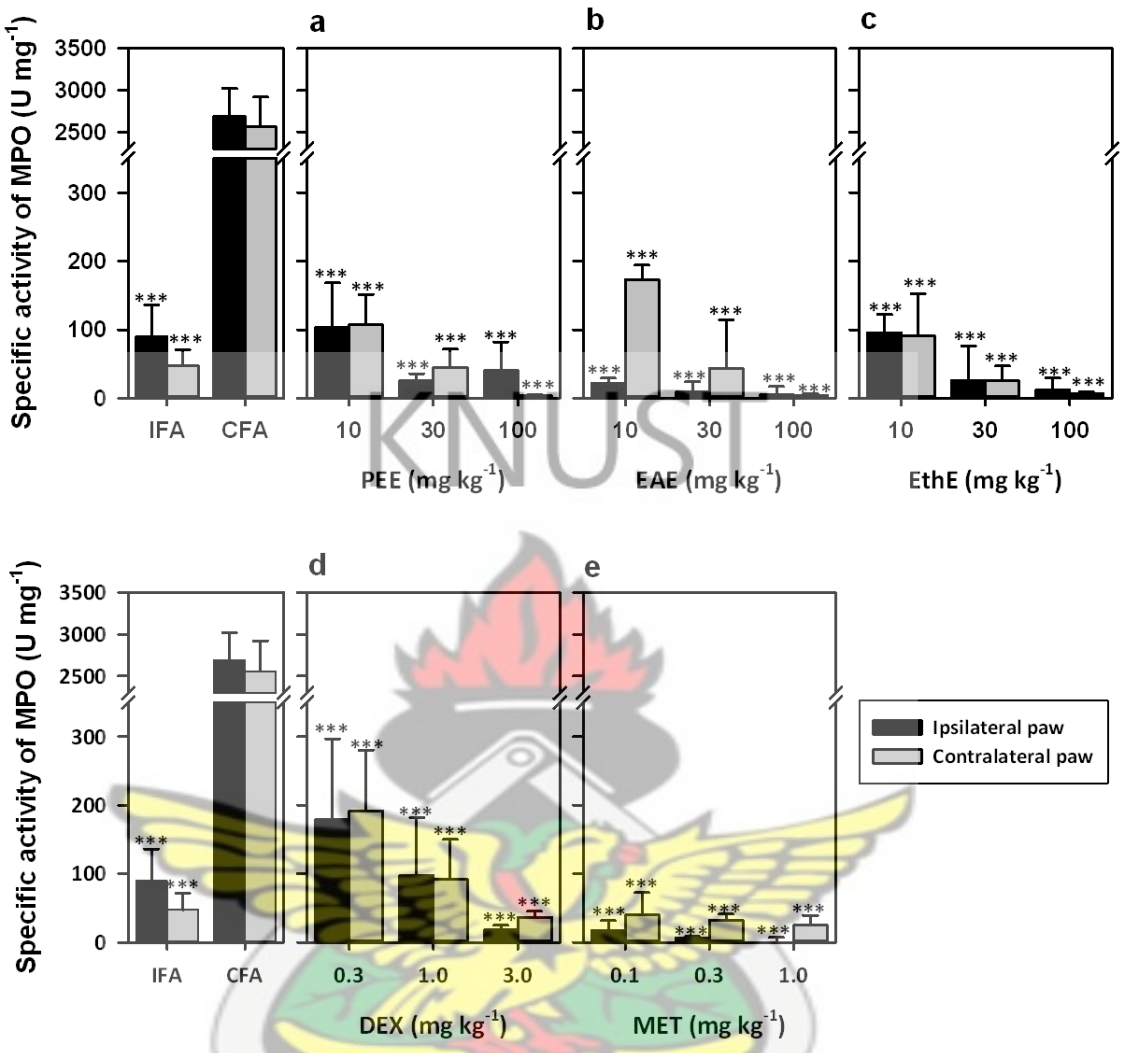


Figure 4.9: Tissue MPO (myeloperoxidase) levels in Adjuvant-induced arthritis in rats.
The tissue MPO levels were markedly reduced in (a) PEE, (b) EAE, (c) EthE-treated groups and the reference drugs (d) dexamethasone and (e) methotrexate-treated groups for both the ipsilateral and contralateral paws of the arthritic rats. Values are mean ± SEM. (n = 8). ***P < 0.001; ** P < 0.01; *P <0.05 compared to CFA group. All comparison of variances was done using Holm-Sidak's multiple comparison *post hoc* test.

4.3.2.3 Total tissue SOD activity

The rate of inhibition of xanthine generated superoxide, expressed as SOD activity was low in CFA. Administered doses of the extracts PEE, EAE and EthE, significantly ($F_{4, 10}$=52.79 P<0.0001; $F_{4, 10}$=41.45.00 P<0.0001; $F_{4, 10}$=55.61 P<0.0001, respectively; Figure 4.10 a - c) and dose-dependently increased SOD activity in the first phase of inflammation. Dexamethasone and methotrexate also had significant ($F_{4, 10}$=40.97 P<0.0001; $F_{4, 10}$=26.69 P<0.0001, respectively; Figure 4.10 d, e) dose-dependent effects in increasing SOD activity in the first phase of inflammation.

PEE, EAE and EthE significantly ($F_{1, 4}$=0.26 P>0.05; $F_{1, 4}$=2.29 P>0.05; $F_{1, 4}$=3.36 P>0.05, respectively; Figure 4.10 a - c) increase SOD activity in the second phase. Dexamethasone and methotrexate also was significant ($F_{1, 4}$=1.51 P>0.05; $F_{1, 4}$=2.19 P>0.05, respectively; Figure 4.10 d, e) in increasing SOD activity in the ipsilateral paws and the contralateral paws in the second phase.

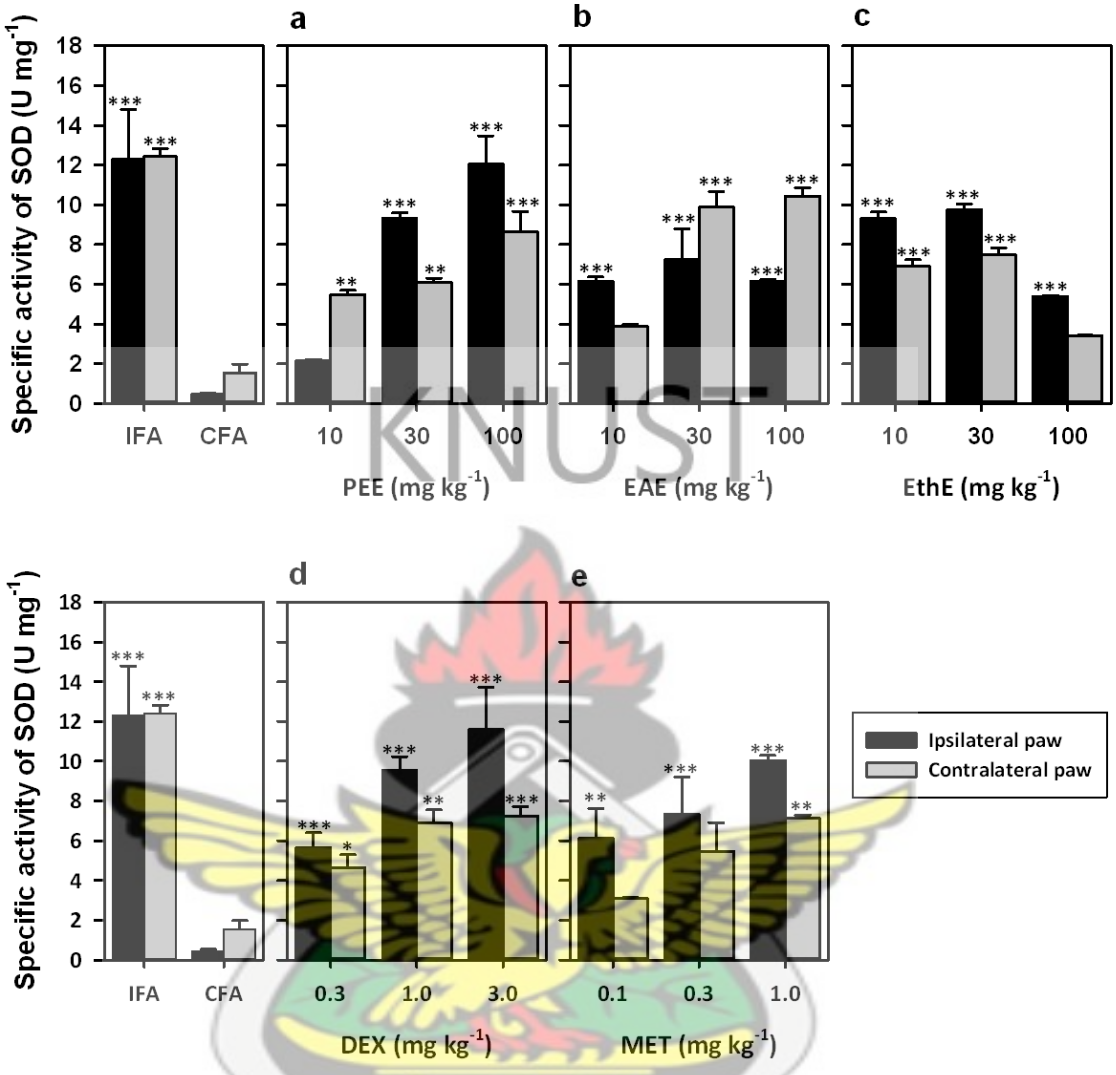


Figure 4.10: Tissue SOD (superoxide dismutase) levels in Adjuvant-induced arthritis in rats.
The tissue SOD activity were markedly increased in (a) PEE, (b) EAE, (c) EthE-treated groups and the reference drugs (d) dexamethasone and (e) methotrexate-treated groups for both the ipsilateral and contralateral paws of the arthritic rats. Values are mean ± S.E.M. (n = 8). ***P < 0.001; ** P < 0.01; *P <0.05 compared to CFA group. All comparison of variances was done using Holm-Sidak's *post hoc* test.

4.3.3 Effects on Cytokine levels

The result of effects of extracts and reference drugs on cytokine levels are shown in Figure 4.11. PEE, EthE and EAE were effective ($F_{3,8}$=20.86 P<0.001; $F_{3,8}$=21.67 P<0.001; $F_{3,8}$=23.53 P<0.001, respectively) in reducing TNF-α levels. These showed maximal effects of 48.1 % at 100 mg kg^{-1}, 47.2 % at 30 mg kg^{-1} and 50.3 % at 30 mg kg^{-1}, respectively. Dexamethasone and methotrexate dose-dependently ($F_{3,8}$=19.15 P<0.001 $F_{3,8}$=14.50 P<0.01, respectively) reduced TNF-α production in arthritic group treated with these drugs. These showed maximal effect of 50.2 % at 3.0 mg kg^{-1} and 51.7 % at 1.0 mg kg^{-1}, respectively.

PEE, EthE and EAE were effective ($F_{3,8}$=4.39 P<0.05; $F_{3,8}$=5.61 P<0.05; $F_{3,8}$=9.56 P<0.01, respectively) in reducing the production of IL-6. These showed maximal effects of 61.0 % at 100 mg kg^{-1}, 64.0 % at 10 mg kg^{-1} and 71.9 % at 100 mg kg^{-1}, respectively. Dexamethasone and methotrexate dose-dependently reduced ($F_{3,8}$=6.47 P<0.05; $F_{3,8}$=7.57 P<0.05, respectively) IL-6 production in arthritic group treated with these drugs. These showed maximal effect of 78.1 % at 3.0 mg kg^{-1} and 72.9 % at 1.0 mg kg^{-1}, respectively.

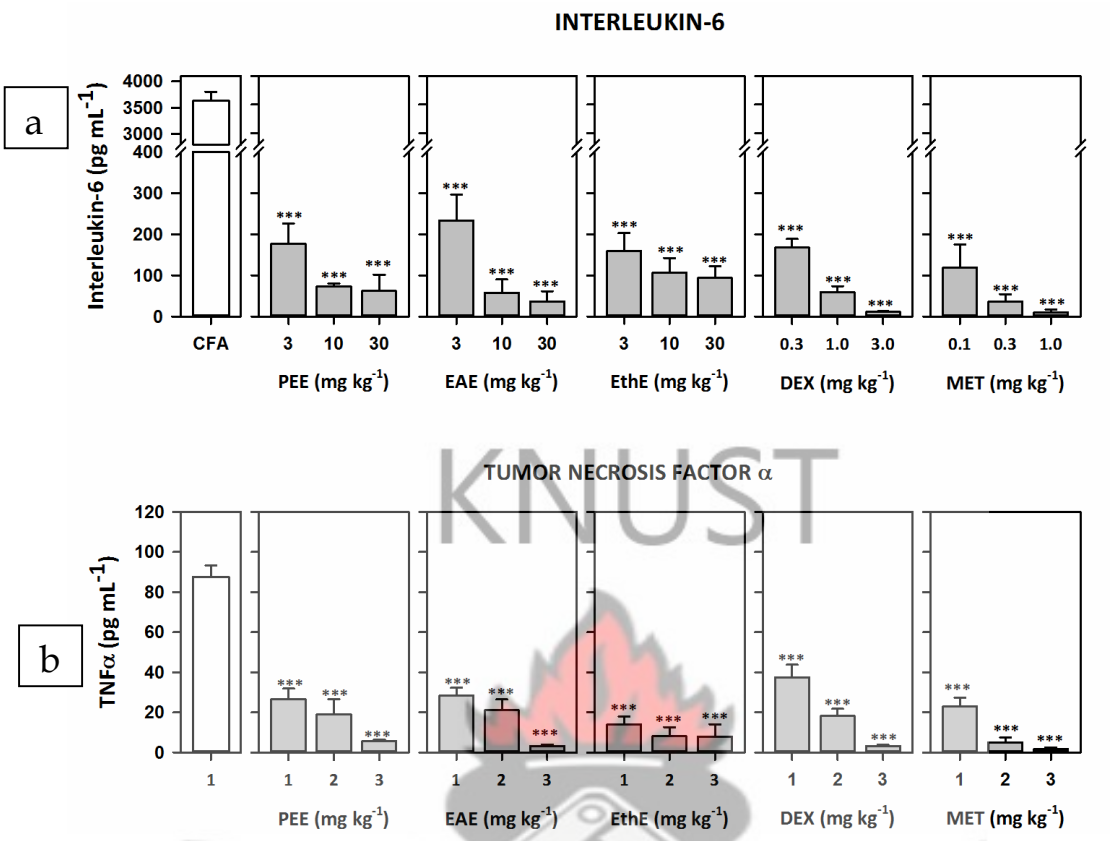


Figure 4.11: Tissue TNF-α andIL-6 levels in CFA-induced arthritis in rats.

The tissue (a) TNF-α and (b) IL-6 production were markedly reduced in PEE, EthE and EAE, dexamethasone and methtrexate-treated groups of the arthritic rats. Values are mean ± S.E.M. (n = 8). ***P < 0.001; ** P < 0.01; *P <0.05 compared to CFA group. All comparison of variances was done using Holm-Sidak's *post hoc* test.

4.4 DISCUSSION

Adjuvant-induced arthritis (AIA) in rats is a model of chronic inflammatory disease, induced by heat-killed cells of *Mycobacterium tuberculosis*. This is characterised by infiltration of the synovial membrane and associated with destruction of the joints and resembles rheumatoid arthritis in humans (Behar *et al*., 1995; Kumar *et al*., 2002). This model is widely used as a model for therapeutic and pathogenetic studies of chronic forms of arthritis (Pearson, 1963; Pearson *et al*., 1963). Experimental arthritis in animals has some of clinical and biochemical features similar to patients with polyarthritic diseases or RA (Halim *et al*., 2007). This condition is associated with weight loss due to systemic or local actions of cytokines such as IL-6 and TNF-α (Chamundeeswari *et al*., 2003). Pathological features (oedema, infiltration into the joint of mononuclear and polymorphonuclear cells, pannus formation, periostitis and erosion of cartilage and bone (Williams, 1998) are observed. Anaemia (reduction in haemoglobin levels) (Glen *et al*., 1977) and oxidative stress (Federico, 2007 ; Marnett *et al*., 2003) are also observed. Increased levels cytokines such as TNF-α, interferon c (INFc), IL-1, IL-6 and IL-17A mRNA have been detected in lymph nodes and/or inflamed joints of rats with AIA (Ayer *et al*., 2000; Bush *et al*., 2001). Blockade of these cytokines ameliorates the disease, indicating that these cytokines contribute to the pathology in this model (Bush *et al*., 2002; Feige *et al*., 2000; Young *et al*., 2007).

In this study adjuvant-injected paw was typified by a rapid onset of inflammation evident within 24 h of adjuvant injection and continued to increase up to day 21. This allowed the study of the acute inflammatory reaction in the local area as well as the immunological reaction that develops approximately 9 days later in the contralateral paw and various organs (Klareskog, 1989). The arthritic rats showed soft tissue swelling around the ankle joints during arthritis, and it was considered as oedema of the particular tissues. As the disease progressed a more diffused demineralization developed in extremities (Begum *et al*., 1988). Secondary lesions of adjuvant arthritis occurred after a delay of approximately 10 days and were characterized by inflammation of non-injected sites (right hind legs, ears, tail) and further increases in the volume of the injected hind leg.

A therapeutic treatment regimen was followed in this research by initiating treatment from day 9 till day 28. All treatments with extracts, especially PEE and EAE, were effective in reducing this primary oedema by day 18 compared with arthritic controls. The non-injected paw developed secondary lesion by day 14 post-adjuvant injection as a result of the immune response to the bacterial adjuvant (Pearson, 1956). Treatment of adjuvant-injected rats with the extracts showed a significant reduction of secondary paw inflammation. Reduction of paw swelling from the third week onwards may have been due to immunological protection rendered by the plant extracts, preventing the systemic spread and ultimately reducing the destruction of joints as seen in the scores for the photographs and the radiographs.

Reduced bone structure and increased resorption is responsible for bone loss in adjuvant-induced arthritis in rats (Aota *et al.*, 1996; Findlay *et al.*, 2005; Makinen *et al.*, 2007). Results of radiographic scores clearly showed increased bone loss in arthritic groups. Decreased bone loss in extracts and reference drug-treated groups shows that the extracts, especially PEE, conformed to one of the therapeutic strategy of managing arthritis. This is suppressing the inflammation, synovitis and protecting bone structure especially joint protection (Atzeni *et al.*, 2007; Hoffmann *et al.*, 1997; Sharma *et al.*, 2004).

Changes in body weight or cachexia have also been used to assess the course of the disease and the response to therapy of anti-inflammatory drugs (Winder *et al.*, 1996). Extracts significantly improved body weight of arthritic rats as compared with arthritic group. This may be due to the systemic or local action of cytokines resulting from chronic inflammation (Chamundeeswari *et al.*, 2003).

The major target for inflammatory process in adjuvant-induced arthritis is the synovium which results in tissue inflammation as a result of infiltration of the tissue with multiple immune cells and cytokines (Cantley *et al.*, 2009). This is observed as expansion of the synovial tissue and pannus formation that invades the bone and cartilage destroying the tissue as it proceeds (Walsh *et al.*, 2005). This process promotes osteoclastogenesis that leads to focal articular bone erosion at the site of pannus formation, as well as systemic bone loss similar to osteoporosis (Romas *et al.*, 2006; Walsh *et al.*, 2005). Inflammatory tissue invasion, into the subchondal bone, results in the involvement of many cell types such as fibroblasts, lymphocytes and monocytes (Cantley *et al.*, 2009). Monocytes are the precursors of

osteoclasts which bring about the resorption of bone through the acidic disillusion of bone mineral and enzymatic destruction of bone matrix. This resorption of the bone by osteoclasts is due to the synthesis of proteases by the synovial fibrobalsts, neutrophils and the chondrocytes. Studies have shown that at sites of bone erosion, large multinucleated osteoclastic cells resorb subchondral bone. Osteoclasts formation is as a result of exposure of inflammatory cytokines present in synovial tissue (Cantley *et al*., 2009). The extracts were able to reduce cell infiltration thereby ameliorating tissue inflammation in the rat synovial tissue.

The anaemic condition, measured as RBC and Hb concentration, observed in arthritic condition could be due to erythrocyte deformability that leads to shortening life span of erythrocytes (Allard *et al*., 1977). Low RBC and Hb concentration of blood of arthritic group confirms with studies that anaemia is associated with arthritis-induced rats and even in patients with RA (Mowat, 1971). The extracts were able to significantly increase the levels of RBC and Hb to normal thus improving the anaemic state of the arthritic rats. Of the cells involved in inflammation, some (vascular endothelial cells, mast cells and tissue macrophages) are normally present in tissues, while others (platelets and leucocytes) gain access from the blood, marked by the movement of phagocytic white blood cells (leucocytes) into the area of injury (Maria *et al*., 1983). The WBC count increases in arthritic rats to destroy invading pathogenic microorganisms and bring about resolution of inflammation (Ramesh *et al*., 2003). Induration (increased thickness of soft tissue) is due to the accumulation of leucocytes (mostly neutrophils and lymphocytes) and oedema fluid (Maria *et al*., 1983). Reduction of the WBC levels to normal levels, by the extracts could be attributed to the resolution of the inflammatory response. In the present study the migration of leukocytes into the inflamed area was significantly suppressed by the extracts especially PEE at the highest dose. PEE also significantly decreased WBC count, platelet count and percentage of lymphocytes and neutrophil.

Bone resorption, due to differentiation and activation of osteoclasts, involves a number of cytokines such as TNF-α, IL-1, IL-6. Activated macrophages as well as synoviocytes in the inflamed synovial tissue produces TNF-α which directly and indirectly induces osteoclast formation forming a link between the immune and bone system (Cantley *et al*., 2009). From

the study the extracts were able to significantly reduce the levels of TNF-α and IL-6 expression. Pro-inflammatory properties of TNF-α include stimulating the production of collagenase and PGE_2 by synovial cells and thus contribute to joint damage. On the other hand IL-6 acts as a marker for systemic activation of proinflammatory cytokines. It has both proinflammatory and anti-inflammatory properties. Its proinflammatory property is seen as it stimulating the synthesis of acute phase proteins. The anti-inflammatory property of IL-6 is seen as down-regulating the synthesis of IL-1 and TNF-α and inducing the synthesis of glucocorticoids that trigger the synthesis of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) (Chamundeeswari *et al.*, 2003).

Lipid peroxidation is considered a critical mechanism of injury that occurs during arthritis. An indicative method extensively used in evaluating lipid peroxidation is analysis of tissue malondaldehyde (MDA) (Reilly, 2001). The extracts were able to significantly reduce MDA levels in the arthritic tissue comparable to the elevated levels observed in arthritic group. Myeloperoxidase (MPO) is a constituent of neutrophil granules, according to Ormrod *et al.* (1987) and is a marker of infiltration of polymorphonuclear cells which was observed histologically as infiltration of inflammatory cells in the synovial tissue (Federico *et al.*, 2007) of arthritic group in the study. The study showed that the extracts were able to decrease this infiltration by reducing levels of MPO. The superoxide radicals are the first product of molecular oxygen reduction. The enzyme, superoxide dismutase (SOD) acts as a catalyst for dismutation of superoxide radicals into H_2O_2 and into molecular oxygen to protect cells and tissues from superoxide radicals and other peroxides such as lipid peroxides *in vivo* (Marnett *et al.*, 2003). In the arthritic group, SOD activity was markedly reduced. This decrease can be as a result of inhibition of the enzyme by hydrogen peroxide, indicating a high degree of superoxide anion production. High levels of superoxide anion in the tissue suggest increased hydrogen peroxide liberation through dismutation reaction. Increased in enzyme activity observed in the extracts-treated groups suggest that there was an adaptive response of the animal against possible damage caused by oxygen free radicals. The extracts can thus be said to inhibit lipid peroxidation through the decrease in neutrophil accumulation and decrease in the chemotactic reduction of peroxide.

4.5 CONCLUSION

This study has demonstrated that the petroleum ether, ethyl acetate and ethanol extracts of the stem bark of *Trichilia monadelpha* has anti-arthritic property and hence would be useful in managing arthritic disorders.

Chapter 5

EFFECTS OF EXTRACTS OF *TRICHILIA MONADELPHA* ON ANAPHYLAXIS

5.1 INTRODUCTION

The previous chapters have shown that *Trichilia monadelpha* (Thonn.) J. J. de Wilde (*Meliaceae*) has anti-inflammatory effects. This was observed as amelioration of inflammatory response induced by carrageenan and arthritis induced by *Mycobacterium tuberculosis*. The plant extracts also ameliorated vasoactive amines (histamine and serotonin)-induced inflammation. This observation provokes a question, could the stem bark extracts also have anti-allergy or anti-anaphylactic properties since it was able to inhibit oedema induced by vasoactive amines? Vasoactive amines (histamine and serotonin) are released when mast cells and platelets degranulate. They have complex effects on the vasculature, causing increased vascular permeability and vasodilation, or vasoconstriction, depending on the context. The immediate effects of their release by mast cells can be harmful in sensitised organisms, resulting in vascular and respiratory collapse during anaphylactic shock (Medzhitov, 2008; Ruslan, 2008). Mast cells are important components in various biological processes of allergic diseases and anaphylaxis (Sang-Hyun *et al*., 2005).

Mast cell degranulation is due to cross-linking of surface bound IgE resulting in the immediate release of granule contents, including histamine, and more gradual elaboration of other pro-inflammatory mediators. The degranulation can bring about clinical manifestations ranging from seasonal allergic rhinitis to life-threatening anaphylaxis (Nigrovic *et al*., 2005). Mucosal and epithelial surfaces have served as cluster sites and contact to the external surroundings for mast cells (Metcalfe *et al*., 1997). Mast cells are found near linings of peritoneum, pleural spaces, synovial cavity as well as blood vessels and skin. They portray surveillance characteristics in which they detect pathogens and initiate inflammatory response, accumulating in chronic inflamed joints (Galli *et al*., 1999; Nigrovic *et al*., 2005).

Prominent mediators found in mast cells are histamine and tryptase. These are present at high concentrations in synovial fluid of inflamed joints (Buckley *et al*., 1997; Frewin *et al*., 1986; Lavery *et al*., 1994; Malone *et al*., 1986; Partsch *et al*., 1982). The pathogenesis of RA is associated with immune complexes (IgG immune complexes), complement activation, TLR ligands and microbial agents stimulated pathways that trigger mast cells degranulation. These immune complexes and complement activation are found in serum, synovial fluid, synovium and cartilage of patients with RA (Firestein, 2003; Monach *et al*., 2004; Pekin *et al*., 1965; Ruddy *et al*., 1969; Schur *et al*., 1975).

Mast cell degranulation can be stimulated in laboratory rats and mice by subcutaneous or intradermal injection of compound 48/80, a synthetic substance and polymers of basic amino acids (Ennis *et al*., 1980). A high concentration of compound 48/80 induces an about 90 % release of histamine from mast cells, in comparison to a natural process (Ennis *et al*., 1980). As a result, an appropriate amount of compound 48/80 is used as a direct and convenient reagent to study allergy and anaphylaxis (active and passive processes) (Allansmith *et al*., 1989). Mast cells are found in the skin and in all mucosal tissues at homeostasis. These are elevated in asthmatics lungs (Amin *et al*., 2000) gastrointestinal tract of inflammatory bowel disease (Amin, 2012). Therefore, murine mast cell is a good experimental model for the study of compound 48/80-induced histamine release because of its association with anaphylactic symptoms and mast cells degranulation (Choi *et al*., 2010).

Compound 48/80 can also be used to induce passive cutaneous anaphylaxis, PCA (Choi *et al*., 2010). PCA is induced in rat skin by challenging the rat skin with compound 48/80. This forms an antigen-antibody-like complex in the mast cells, inducing the release of mediators, resulting in vasodilation, increase in permeability of the vessel walls and leakage of the plasma (Choi *et al*., 2010). Evans blue dye is administered with the compound to make the allergic reaction visible. The Evans blue dye gets attached to the albumin fraction of the plasma, producing a blue spot, an indication that anaphylactic reaction has occurred in the skin (Choi *et al*., 2010). Histological examination of this anaphylactic reaction was carried out using toluidine blue dye since mast cells granules are metachromatic (Crookham *et al*., 1991; Luna, 1968; Sheehan *et al*., 1980).

5.2 EXPERIMENTAL PROCEDURE

5.2.1 Animals

Male Sprague-Dawley rats (150 – 200 g) and C57BL/6 mice (20 – 25 g) were bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, and kept in the animal house of the Department of Pharmacology, KNUST, Kumasi, Ghana. Animals were housed in aluminium cages, with standard diet and water available, *ad libitum*. Sample size of 10 animals per group was utilised throughout the study. Guidelines on animal handling was followed as instructed by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised 1985) and was approved by the Departmental Ethics Committee.

5.2.2 Drugs

The extracts were triturated with Tween-80 (3 drops) in normal saline and administered orally to rats at doses ranging from 10 to 1000 mg kg^{-1}. Compound 48/80 (Sigma-Aldrich, Inc. St. Louis MO USA) was used to induce systemic anaphylaxis and passive cutaneous anaphylaxis. Evans blue dye and toluidine blue dye, disodium chromoglycate, (DSCG), formamide, formalin, alcohol and sodium chloride were bought from Sigma-Aldrich, Inc. St. Louis MO USA. Quantikine rat IL-6 and TNF-α Immunoassay kit (R&D Systems, Inc., Minneapolis, USA) were used to assay IL-6 and TNF-α level. Animals of the extract-treated groups received ≤1 ml of extract solution. Animals of the standard drugs-treated groups received ≤1 ml of drug solution. Control group received only normal saline. All drugs were freshly prepared.

5.2.2 Compound 48/80-induced systemic anaphylaxis

Compound 48/80-induced systemic reaction was performed as described in literature (Kim *et al.*, 2005). Briefly, the extracts (100, 300 and 1000 mg kg^{-1} *p.o.*) and DSCG (2.5 – 250 µg kg^{-1} *i.p.*), were first administered to the mice orally, one hour before the induction of anaphylactic reaction. Mice (n = 10) were intraperitoneally injected with the mast cell degranulator, compound 48/80 (8 mg kg^{-1}). Mortality measured was based on control mice that had been

killed in 15 min by compound 48/80. Mortality was monitored for 1 h after induction of anaphylactic shock.

The results were plotted as percentage survival against time, expressed as mean ±SEM for the number of experiments. One-way ANOVA, Multiple Comparison Procedures (Holm-Sidak's method) was used to express the difference from the control group. This was done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and SigmaPlot version 12.3 (Systat Software Inc. Chicago USA,webmaster@cranessoftware.com).

The percentage mortality and percentage protection were calculated as follows;

$$\%\ Mortality\ = \frac{number\ of\ animals\ dead}{total\ number\ of\ animals} \times 100$$

$$\%\ Protection\ = \%\ mortality_{control}\ -\ \%\ mortality_{extract}$$

The *P-value, F-value and* χ^2 which are statistical measure of significant levels and survival trend, was analysed using Kaplan-Meier Survival Log-Rank Analysis.

5.2.3 Compound 48/80-induced passive cutaneous-like anaphylactic reaction

The experiment was done as described by Choi *et al* (2010). Extracts (10, 30, 100 mg kg^{-1} *p.o.*) and DSCG (2.5 – 250 µg kg^{-1} *i.p.*) were administered to each rat 1 hour before injecting compound 48/80, intradermally (0.25 µg/50 µL) into the dorsal skin. Evans blue solution (1 %) was later intravenously injected into the penile vein of each rat and 30 minutes after the injection, the rats were sacrificed. Tissue sections around the intradermal injection site was excised and weighed, followed by extraction of extravasated Evans blue dye by incubation of biopsies in 1 mL formamide at 55 °C for 24 hours (Agnieszka *et al*., 2010). Absorbance was measured at 620 nm with UV mini-1240 single beam spectrophotometer (Shimadzu Scientific Instruments (SSI), Kyoto, Japan). Tissue Evans blue concentrations was quantified by interpolation on a standard curve of dye concentrations in the range of 0.01 to 30 µg mL^{-1}.

5.2.4 Histological evaluation

To evaluate the effects of extracts on the histology of mast cells the process for PCA was repeated. Lesion portion of the skin was cut out and fixed in 10 % formalin and cut into sections of 4 µ. A toluidine blue working solution of 5 ml of toluidine stock solution (1 g of toluidine/ 100 ml of 70 % alcohol) and 45 ml of 1 % sodium chloride solution was prepared. The staining procedure was carried out by first deparaffinising and hydrating tissues in distilled water. This was placed in the toluidine blue working solution for 1-2 minutes, rinsed in distilled water 3 times and dehydrated quickly through 95 % and absolute alcohol. This was cleared in xylene, covered with coverslip and viewed under a light microscope. The histopathological features were blindly graded by board certified pathologists and assigned a score of 0–4 based on the following:

0 – absence of tissue damage, granulation and degranulation

1 – minimal presence of either tissue damage, granulation and degranulation

2 – mild/moderate presence of tissue damage, granulation and degranulation

3 – intense presence of either tissue damage, granulation and degranulation

4 – extensive tissue damage, granulation and degranulation

The histological scores were computed and differences in AUCs were analysed by ANOVA followed by Bonferroni's *post hoc* test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

5.2.5 Assay of TNF-α and IL-6 secretion

The cytokines analysed were TNF-α and IL-6. Both proinflammatory cytokine mediators were analysed using the serum of anaphylactic rats (induced with compound 48/80). Blood was taken from rat through cardiac puncture and allowed to clot for 2 hours at room temperature. This was centrifuged at 1000 *g* for 20 minutes at room temperature and the serum was removed and assayed for the cytokines. Assays were as described in the kit manual. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α and IL-6 has been pre-coated onto a microplate. Standard, control and

samples were pipetted into the wells where any rat TNF-α and IL-6 present were bound by the immobilised antibody. The wells were washed to remove any unbound substances and after the washing an enzyme-linked polyclonal antibody specific for these cytokines were added into the wells. A substrate solution was added to the wells after washing the wells to remove any unbound antibody-enzyme reagent. The enzyme reaction yielded a blue product that turned yellow when a Stop solution was added. The intensity of the colour was measured at 450 nm using Sunrise microplate reader XREAD PLUS version: V4.30 (Tecan Inc., Switzerland) powered by Smart Magellan data analysis software. Absorbance measured were equivalent to the cytokines in the standard. The sample values were read off the standard curve and computed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and SigmaPlot version 12.3 (Systat Software Inc. Chicago USA, webmaster@cranessoftware.com).

The maximal effects of the treatment given were calculated according to the formula below, using the means caluculated through column statistics:

% maximal effect = $[Mean_{control} - Mean_{treatment}) / Mean_{control}] \times 100$ %

5.3 RESULTS

5.3.1 Compound 48/80-induced systemic anaphylaxis

In control group, injection of compound 48/80 elicited anaphylactic responses such as tremors and eventual death, observed as a drop in a step drop in the survival curve. PEE significantly ($F_{3, 17}$=5.15 P<0.05; Figure 5.1 a) delayed onset of anaphylactic responses. EAE did not produce a significant ($F_{3, 17}$= 0.31 P>0.05; Figure 5.1 b) delay of onset of anaphylactic responses. DSCG also did not produce a significant ($F_{3, 17}$= 2.36 P>0.05; Figure 5.1 c) delay of onset of anaphylactic responses. On improving the chance of survival of animals after induction of anaphylaxis, PEE significantly (P< 0.0001 χ^2 (df =3) = 41.70) improved survival with a significant (P< 0.0001 χ^2 (df =1) = 22.79) trend of the treatment groups on median survival. EAE also significantly (P< 0.05 χ^2 (df =3) = 8.75) improved survival of the animals

after induction of anaphylaxis, showing a significant ($P< 0.01$ χ^2 (df =1) = 7.15) trend of the treatment groups on median survival. DSCG also improved, significantly ($P< 0.0001$ χ^2 (df =3) =53.34) survival of the animals after induction of anaphylaxis with a significant ($P< 0.0001$ χ^2 (df =1) = 23.63) trend of the treatment groups on median survival. Thus there was a dose-dependent increase in probability of survival with time, with PEE considered the more significant extract in inhibiting compound 48/80-induced systemic anaphylaxis.

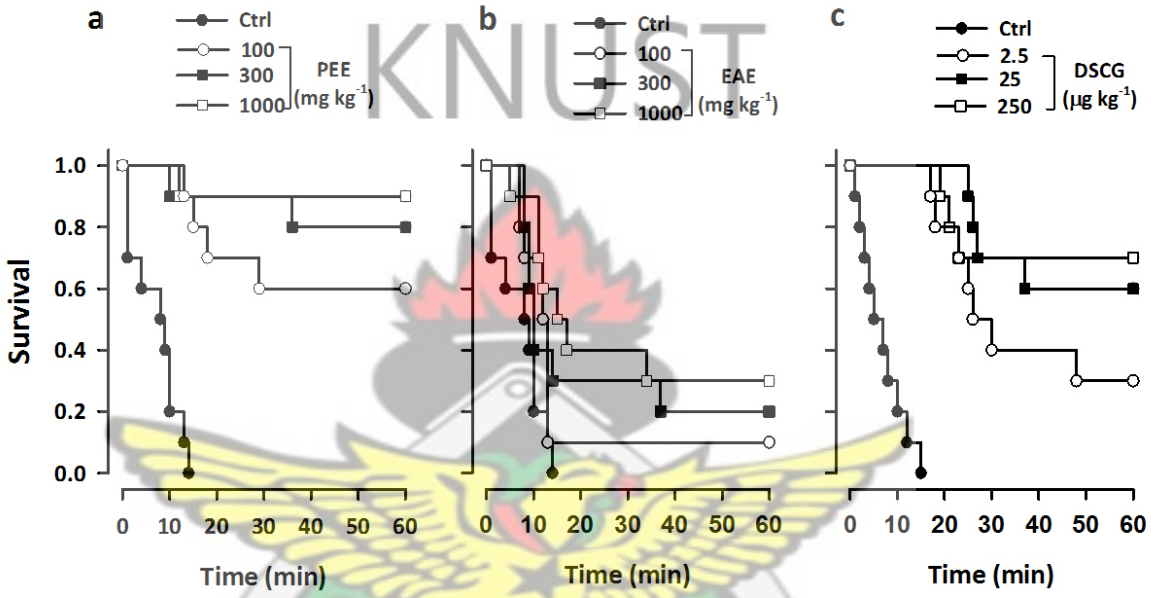


Figure 5.1: Survival plot of percentage survival against time for (a) PEE, (b) EAE and (c) DSCG.

Staircase with ticks starting at 100 %, represent percentage survival against time. Each death is observed as a drop in a step drop in survival.

5.2.2 Compound 48/80-induced passive cutaneous-like anaphylactic reaction

Compound 48/80 induced extravasation of Evans blue in the anaphylactic control groups (Figure 5.2). PEE significantly ($F_{3,8}$= 159.9, $P< 0.0001$; Figure 5.2 a inset) inhibited PCA reactions dose-dependently. EAE also produced a significant ($F_{3,8}$= 194.4, $P< 0.0001$; Figure 5.2 b inset) inhibition of PCA reactions dose-dependently. DSCG also significantly ($F_{3,8}$= 408.1, $P< 0.0001$; Figure 5.2 c inset) inhibited PCA reactions dose-dependently.

PEE was efficacious and potent (ED_{50}= 7.89±2.28, E_{max}= 66.08; Figure 5.3) exhibiting maximal inhibitory effect of 58.2 % at dose 100mg kg^{-1}. EAE was also potent and efficacious (ED_{50}= 8.06±1.03, E_{max}= 77.62; Figure 5.3) with maximal inhibitory effect of 72.8 % at dose 100 mg kg^{-}

[1]. DSCG also showed a dose-dependent inhibition (ED_{50}= 0.56±0.14, E_{max}= 92.36; Figure 5.3) of the PCA reaction with maximal inhibitory effect of 95.8 % at 250 µg kg^{-1}.

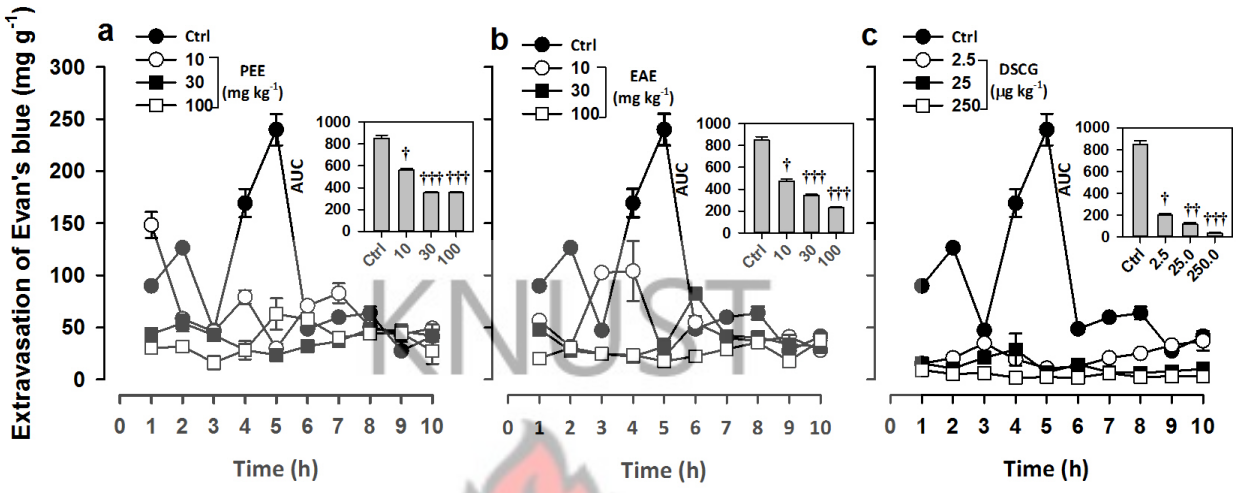


Figure 5.2: Inhibitory effects of extracts and DSCG on the compound 48/80-induced PCA-like anaphylactic reaction.

Time course curves shows effects of (a) PEE, (b) EAE and (c) DSCG on the extravasations of Evans Blue dye. Each bar represents mean± s.e.m (n=8). ††† $P< 0.001$, †† $P< 0.01$: † $P< 0.05$ compares mean±SEM using One-way ANOVA (*control group* × *treatment groups*). All comparison of variances was done using Holm-Sidak's *post hoc* test.

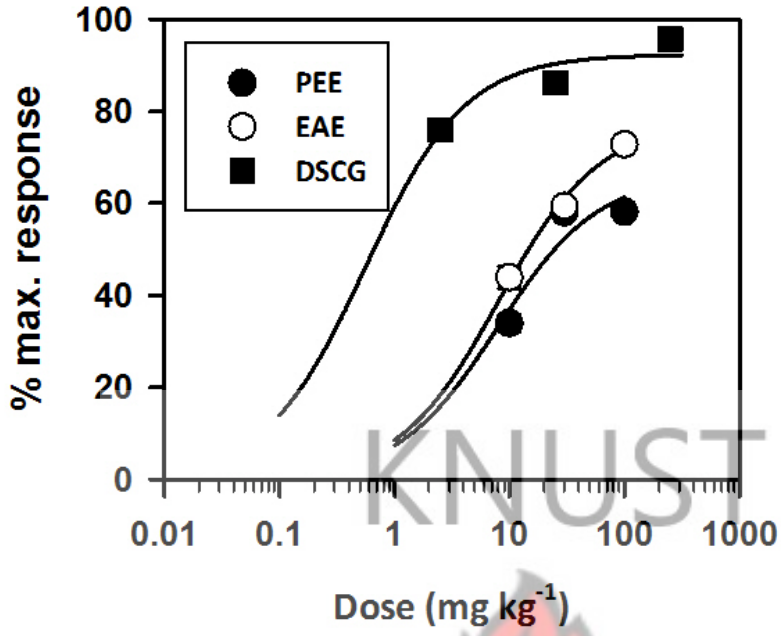


Figure 5.3: Dose-response curve of extracts of *Trichilia monadelpha*, and DSCG on compound 48/80-induced PCA-like reaction.
The data points reflect mean values of n= 3 repeated trials ± s.e.m. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

5.2.3 Histological evaluation

The histology of the anaphylactic control group (Plate 5.1 A and B) showed an intense degranulation of the mast cells. This resulted in intense tissue damage compared to the normal rat skin control (Plate 5.1 C and D). However treatment of animals with PEE and EAE showed minimal degranulation of the mast cells and little or no tissue damage. PEE-treated group showed a dose-dependent and significant ($F_{4,5}$ = 65.14, P<0.001; Figure 5.4 a; Plate 5.2 A-F) minimal evidence of inflammation with the morphology of the skin looking like the normal rat skin biopsy. This showed maximal effect of 61.7 % at 100 mg kg^{-1}. EAE-treated group showed a dose-dependent and significant ($F_{4,5}$ = 15.70, P<0.0001; Figure 5.4 b; Plate 5.3A-F) minimal evidence of inflammation with maximal effect of 61.7 % at 100 mg kg^{-1}. DSCG-treated group also showed a dose-dependent and significant ($F_{4,5}$ = 9.83, P<0.001; Figure 5.4 c; Plate 5.4 A-F) minimal evidence of inflammation. The morphology of the skin looked like the normal skin with maximal effect of 51.1 % at 250 µg kg^{-1}.

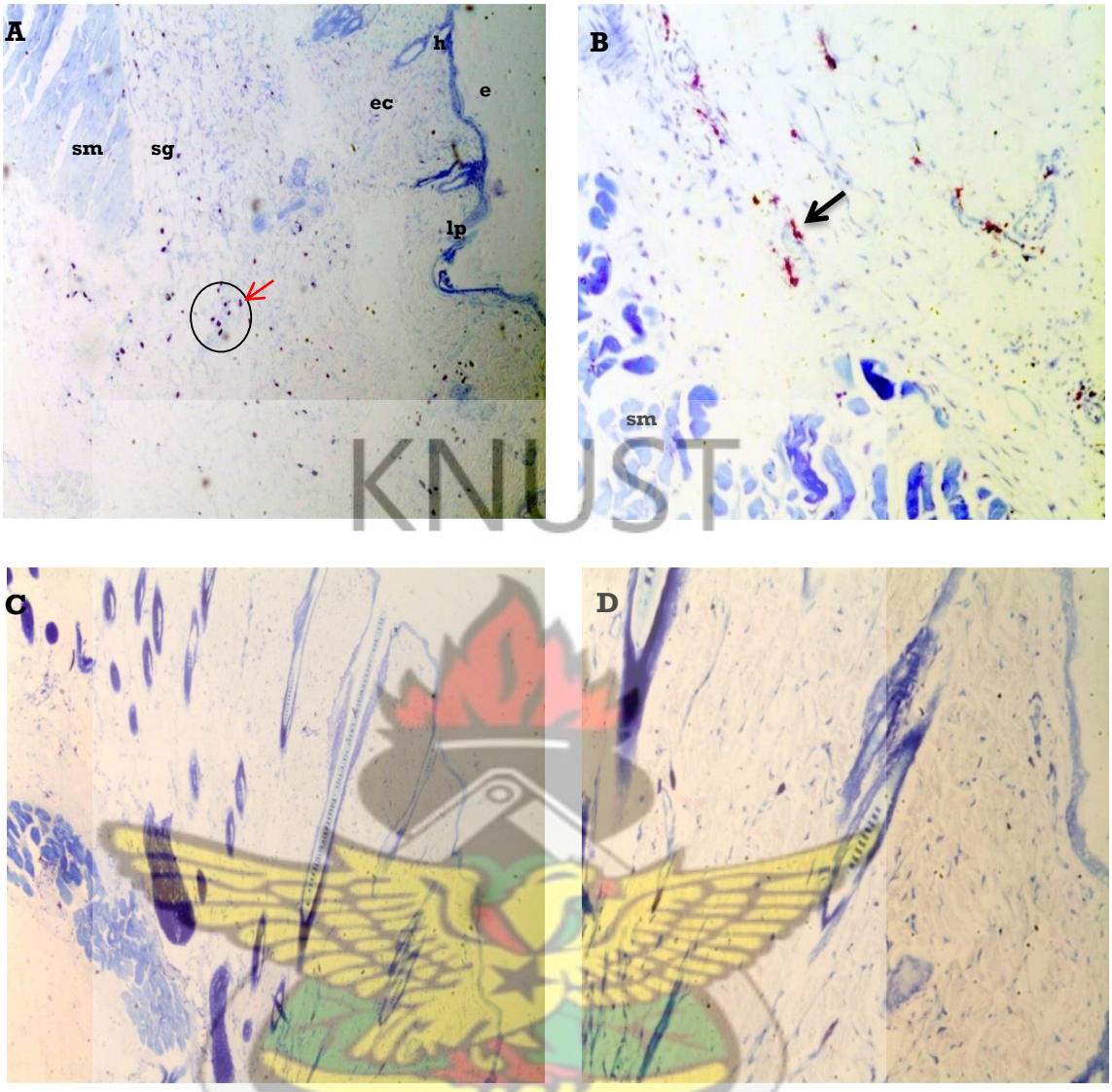


Plate 5.1: Histology of representative rat skin stained with toluidine blue.
A. control skin showing intense granulation of mast cells (magnification x100). Degranulation and intense tissue damage of the endothelial cells is observed in B (magnification x400). C (magnification x100) and D (magnification x400) shows the morphology of a normal rat skin. Features shown are e- epidermis, lp – lamina propia, sm- subcutaneous muscular layer, sg – sebecious gland, ec – epithelial cells. The red arrow shows cluster of mast cells. Black arrow shows degranulated mast cell.

Plate 5.2: Histology of representative rat skin for PEE-treated group
Magnification x100 (A, C, and E) and magnification x400 (B, D, and F) show representative rat skin of PEE-treated groups (10, 30 and 100 mg kg^{-1}, respectively).

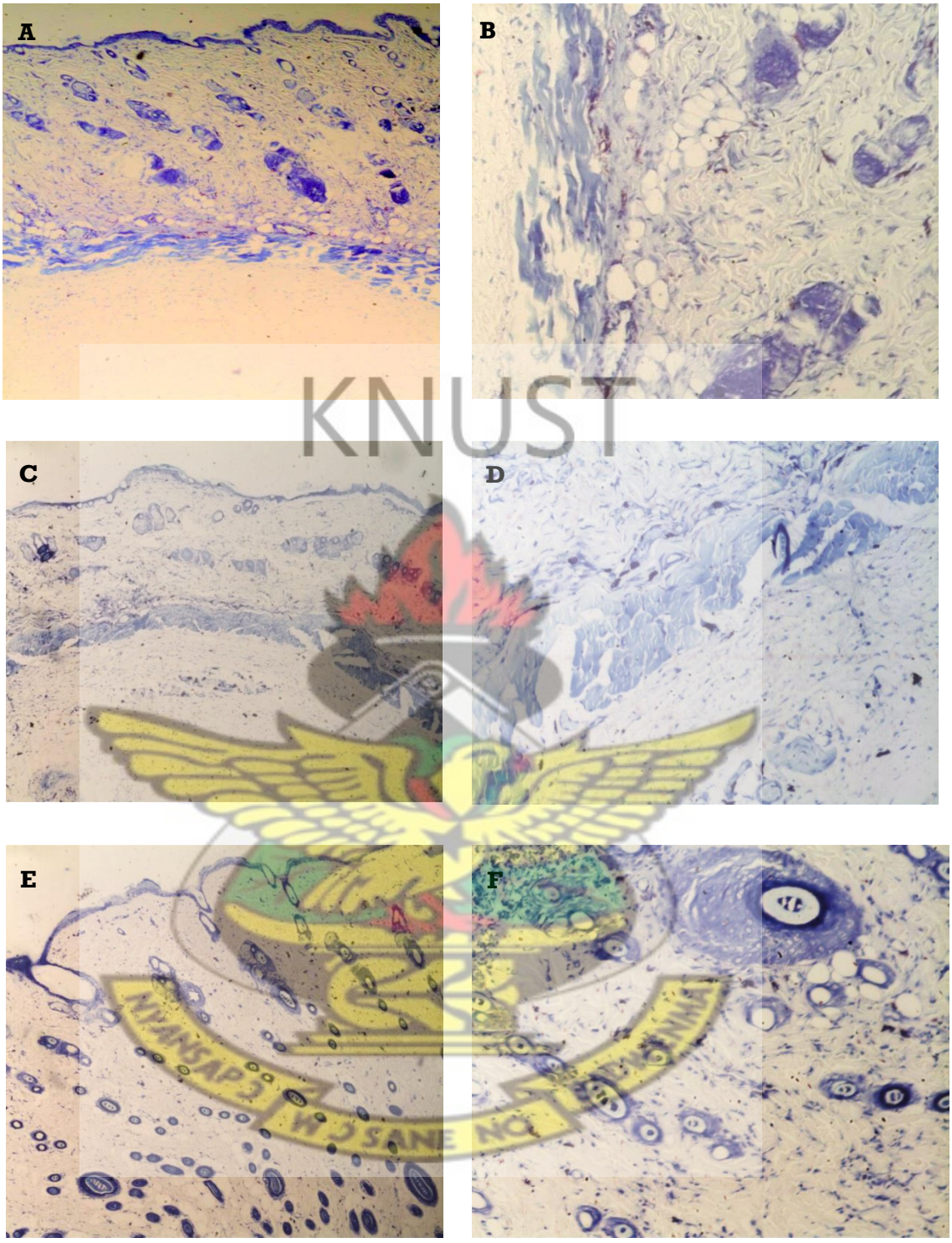


Plate 5.3: Histology of representative rat skin for EAE-treated group
Magnification x100 (A, C, and E) and magnification x400 (B, D, and F) show representative rat skin of EAE-treated groups (10, 30 and 100 mg kg^{-1}, respectively).

Plate 5.4: Histology of representative rat skin for DSCG-treated group
Magnification x100 (A, C, and E) and magnification x400 (B, D, and F) show representative rat skin of DSCG - treated groups (2.5, 25 and 250 µg kg^{-1}, respectively).

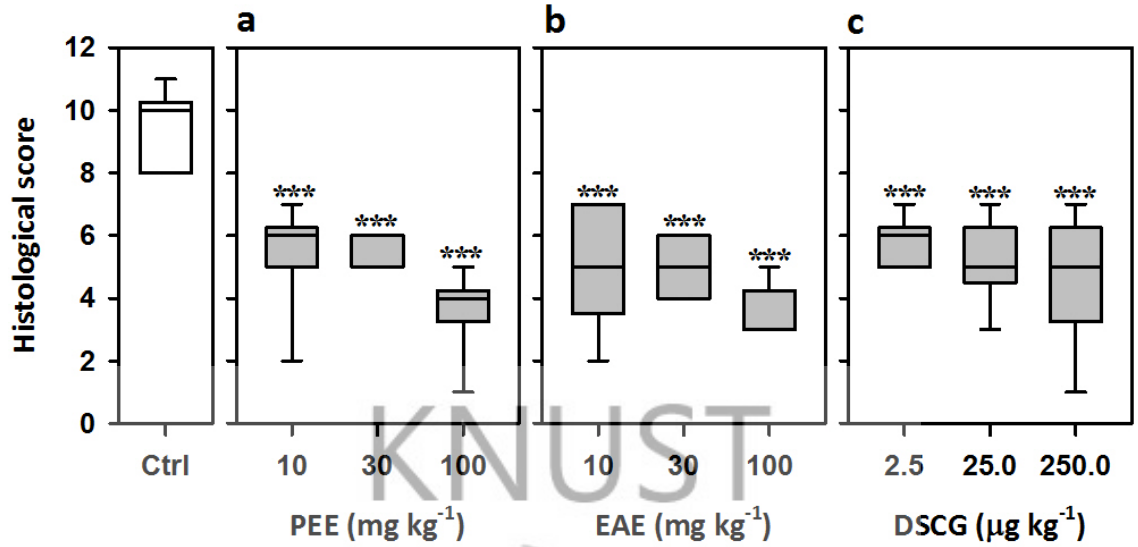


Figure 5.4: Histological score of extracts and DSCG-treated groups.
The extracts (a) PEE, (b) EAE) and (c) DSCG, reduced granulation, degranulation and tissue damage. Column bar represent mean±SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All comparison of variances was done using Holm-Sidak's *post hoc* test.

5.2.5 Effects of extracts on TNF-α and IL-6 secretion from mast cells

The results on the effects of extracts and reference drug on cytokine secretion from mast cells is shown in Figure 5.5. PEE and EAE were significant ($F_{3,8}$=25.17 P<0.001; $F_{3,8}$=13.21 P<0.01, respectively) in reducing production of TNF-α. PEE showed maximal effects of 95.4 % at 100 mg kg^{-1} and EAE was 92.7 % at 100 mg kg^{-1}. DSCG dose-dependently and significantly ($F_{3,8}$ = 32.59 P<0.0001) reduced TNF-α production in anaphylactic group, with maximal effect of 93.2 % at 250 µg kg^{-1}.

PEE and EAE were significant ($F_{3,8}$=26.59 P<0.001; $F_{3,8}$=32.74, P<0.0001, respectively) in reducing the production of IL-6 also. PEE showed maximal effects of 98.5 % at 100 mg kg^{-1}, and EAE was 94.1 % at 100 mg kg^{-1}. DSCG dose-dependently and significantly ($F_{3,8}$ = 46.05 P<0.0001) reduced IL-6 production in animal groups treated with these substances, with maximal effect of 99.5 % at 250 µg kg^{-1}.

Doses of PEE on TNF-α levels (ED_{50}= 0.65±0.23, E_{max}= 100; Figure 5.6 b) and IL-6 levels (ED_{50}= 1.71±0.80, E_{max}= 100; Figure 5.6), was observed to be potent and efficacious. Doses of EAE

on TNF-α levels (ED_{50}= 2.75±0.08, E_{max}= 100; Figure 5.6) and IL-6 levels (ED_{50}= 0.54±0.31, E_{max}= 100; Figure 5.6), was also observed to be potent and efficacious. Doses of DSCG on TNF-α levels (ED_{50}= 0.00031±0.00015, E_{max}= 100; Figure 5.6) and IL-6 levels (ED_{50}= $9.80e^{-005}$±0.0002, E_{max}= 100; Figure 5.6), was also potent and efficacious.

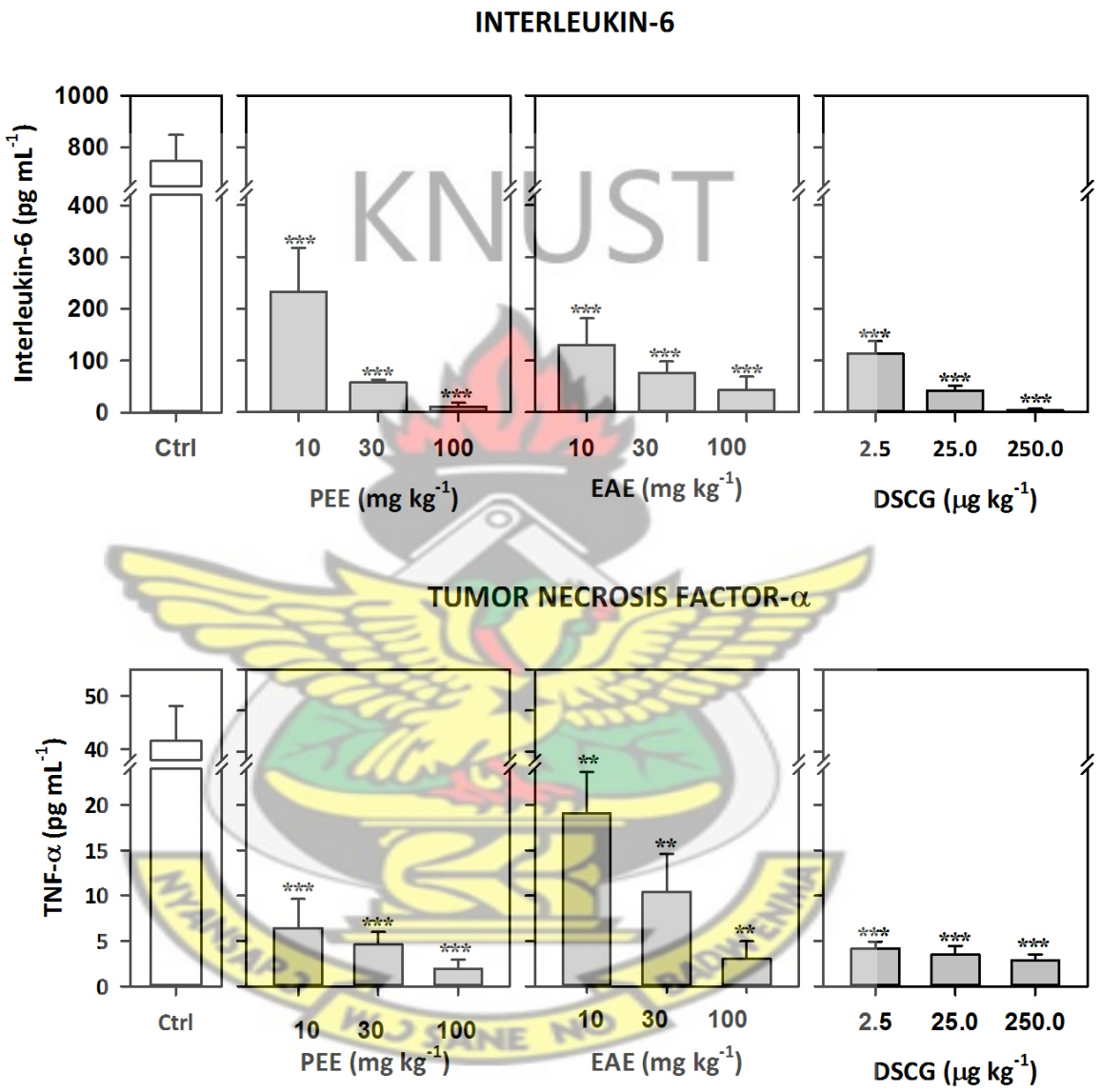


Figure 5.5 Effects of extracts and DSCG on IL-6 and TNF-α secretion.

Column bar represent mean±SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All comparison of variances was done using Holm-Sidak's *post hoc* test.

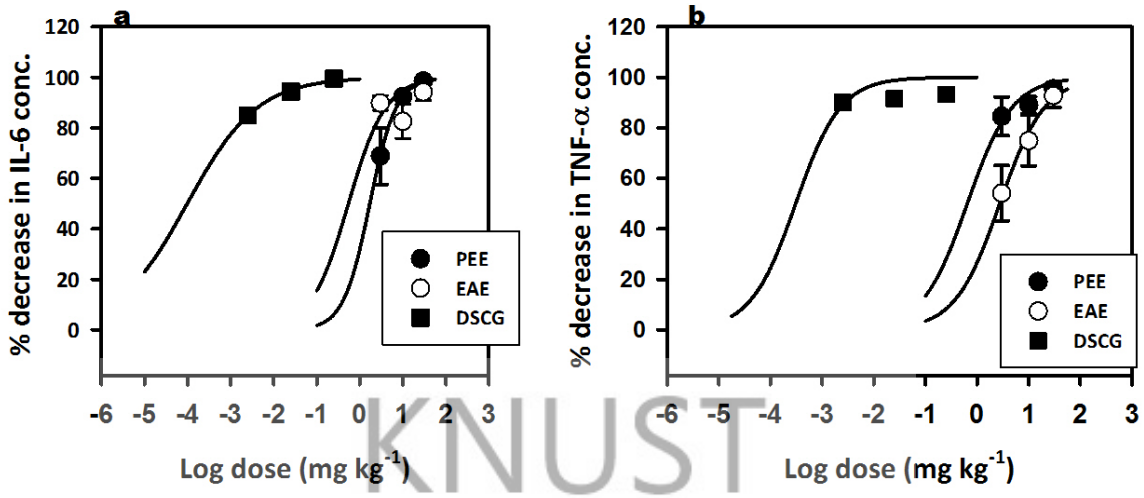


Figure 5.6: Dose-response curve of extracts of *Trichilia monadelpha*, and DSCG on IL-6 and TNF-α secretion. Each extract exhibited different potency and efficacy, observed as the high or low steepness of the hillslope of the curve. The data points reflect mean values of $n = 3$ repeated trials ± s.e.m. Logistic curve fitting was performed using SigmaPlot and the EC_{50} and E_{max} were determined from the curve for each treatment.

5.4 DISCUSSION

Mast cell degranulation is stimulated by subcutaneous or intradermal injection of compound 48/80, a synthetic substance and polymers of basic amino acids (Ennis, 1980 #4382). High concentration of compound 48/80 induces about 90 % release of histamine from mast cells, in comparison to a natural process (Choi *et al.*, 2010; Ennis *et al.*, 1980). As a result, an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to investigate the mechanisms of allergy and anaphylaxis (active and passive processes) (Allansmith *et al.*, 1989).

Anaphylaxis is an acute systemic allergic reaction which is mediated by the release of histamine (stimulated by agents such as compound 48/80) in response to the antigen cross-linking of immunoglobulin E (IgE) bound to the Fcε receptor I (FcεI) on the mast cells. The mast cells start the process of degranulation, after activation via the FcεI, which results in release of a wide variety of other inflammatory mediators such eicosanoids, proteoglycans, proteases and several proinflammatory and chemotactic cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-4, IL-6, IL-8 and IL-13 and transforming growth factor-β (Artuc *et al.*, 1999; Royer *et al.*, 2001; Royer *et al.*, 2001; Stassen *et al.*, 2001). Recent studies have demonstrated that IL-6 up-regulates histamine production rather than

increases its storage and is an important inducing factor for the expression of IgE FcεI (Conti *et al*., 2002). The release of these mediators starts up the allergic reactions observed, in anaphylactic reaction, as sneezing, bronchoconstriction, tarchycardia, tremor and in severe cases, death or mortality.

The present study seems to give an answer to the question; could stem bark extracts of the plant possess anti-anaphylactic properties?. This was observed as extracts of *Trichilia monadelpha* inhibited or reduced compound 48/80-induced mast cells degranulation and tissue damage. Compound 48/80 triggers mast cells degranulation by the aggregation of high affinity receptor for the Fc region of IgE (FcεI) caused by cross linking of IgE by polyvalent antigens. This cross linking leads to activation of PL_C (Fukugasako *et al*., 2003). PL_C activation in mast cells results in hydrolysis of phosphatidyl inositol 4, 5-biphosphate (PIP_2) producing IP_3 and DAG. IP_3 binds to its receptors on the intracellular calcium ions $[Ca^{++}]i$ storage site to release Ca^{++}, while DAG activates PK_C. The increase $[Ca^{++}]i$ and activation of PK_C lead to degranulation, which are required to release histamine (Ohmori *et al*., 2000). During degranulation, storage granules are transformed into secreting granules; the early histological sign of this change is the granule matrix disorganization (Fukugasako *et al*., 2003). The disorganization of the granule matrix releases majorly, histamine and cytokines that destroy the connective tissue of the cell, observed as tissue damage. The histological result showed that skin biopsies of rats treated with extracts of *Trichilia monadelpha* had intact tissue structure and intact mast cells granules. Thus the mast granules and tissue were protected from disorganization and damage.

The extracts significantly reduced or inhibited secretion of TNF-α and IL-6 (pro-inflammatory cytokines) which would lead to inhibition or damage to tissue accompanying anaphylactic reactions. The mediators released from activated and degranulated mast cells increases vascular permeability, with TNF-α, IL-1, and histamine stimulating the expression of the adhesion molecules P-selectin, E-selectin, ICAM-1, and VCAM-1 on the endothelial surface (Iriti *et al*., 2010; Kusano *et al*., 2011). Circulating leucocytes accompany this response. The pro-inflammatory cytokines trigger and sustain the allergic inflammatory response in mast cells (Conti *et al*., 2002), through 'inside-out' regulation, recruiting circulating leucocytes along gradients of chemotactic mast cell products (leukotriene B4, monocyte chemoattractant protein-1, tryptases (for example mouse mast cell protease 6, mMCP6), and

IL-8). These products bring about activation of resident tissue macrophages, arriving monocytes and neutrophils by interferon-γ, IL-6 and TNF-α, amplifying leucocyte recruitment and an improved output of pro-inflammatory cytokines (Nigrovic *et al*., 2005). Documented is IL-6 up-regulation of histamine production which is an important inducing factor for the expression of IgE FcεI receptor (Conti *et al*., 2002) and its local accumulation is associated with PCA reaction (Stockigt *et al*., 2010).

When compound 48/80 is injected subcutaneously into rats or mice, it produces symptoms observed as anaphylactic shock or tremors, tarchycardia, bronchoconstriction, and eventually death. This occurs as compound 48/80 activates and degranulates mast cells and the release of mediators and pro-inflammatory cytokines as earlier discussed. From the result it was observed that the extracts significantly delayed the onset of these symptoms or anaphylactic responses. The extracts also improved and increased survival time with significant trend in treatment of mice with extracts. These altogether resulted in the low mortality and mortality rate observed.

Per the findings, this study has provided evidence that *Trichilia monadelpha* extracts are able to inhibit mast cell-mediated anaphylactic reactions and inflammatory cytokines secretion. The probable mechanism of activity being inhibition of mast cells tissue damage and degranulation by stabilizing and strengthening mast cell membrane and pregranular membrane. Thus *Trichilia monadelpha* would have clinical applicability to allergic disorders and anaphylaxis.

5.5 CONCLUSION

The petroleum ether and ethanolic extracts of *Trichilia monadelpha* stem bark has anti-anaphylactic effect on compound 48/80-induced systemic and passive anaphylaxis in Sprague Dawley rats and C57BL/6 mice.

Chapter 6

ANTIOXIDANT PROPERTIES (*IN VITRO*) OF EXTRACTS OF *TRICHILIA MONADELPHA*

6.1 INTRODUCTION

The damaging effects of reactive oxygen species (ROS) (such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite) on cells are shown to be abrogated by plants shown to possess antioxidant compounds (Dasgupta *et al.*, 2007). Research has shown the regular consumption of antioxidant rich plants or vegetables and the use of medicinal plants have ameliorating effects on human diseases (Rice-evans *et al.*, 1995). Thus, the study of plants as a source of medicine has become essential as oxidative stress is one of the major causes of health hazards (Rice-evans *et al.*, 1995).

The aim of this study is to show the plant, *Trichilia monadelpha* contains antioxidant properties, serving as a natural source of antioxidant. This looked at the reducing power, phenolic content and free radical-scavenging property of the plant. The study will also seek to determine which of the solvent extracts had phytochemicals with strong or high antioxidant potential.

6.2 EXPERIMENTAL PROCEDURE

6.2.1 Reducing power

The reducing power of the three extracts (0.1, 0.3, 1, 3 mg ml^{-1}, in methanol), were determined according to the method of Oyaizu (1986), with modifications. Tannins (0.1, 0.3, 1, 3 mg ml^{-1}, in methanol) a physiological antioxidant as was the reference antioxidant. The method is based on the chemical reaction of Fe^{3+} reduction to Fe^{2+} (Oyaizu, 1986). Absorbance of reaction mixture, using three replicates for each concentration was read at 700 nm. Increased absorbance indicated increased reducing power.

Principle:

The method is based on the ability of a test compound to reduce Fe^{3+} in ferric chloride to Fe^{2+}. The resultant Fe^{2+} then reacts with ferricyanide ion to form a Prussian blue complex with maximum absorbance at 700 nm as shown in the equation of reaction below:

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

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

Higher absorbance due to increase in the intensity of the blue complex shows a greater reducing power.

Procedure:

The reference antioxidant/extract (1 ml) was mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide solution ($K_3Fe[CN]_{6(aq)}$) in a test tube. The mixture was incubated at 50 °C for 20 min. Following this, 1.5 ml of 10 % trichloroacetic acid solution (TCA) (w/v) was added to the incubated mixture, and centrifuged at 1000 *g* for 10 min at room temperature. The supernatant (2.5 ml) was then mixed with 2.5 ml distilled water and 0.5 ml of 0.1 % ferric chloride solution ($FeCl_{3(aq)}$) in a test tube. The absorbance was measured at 700 nm using UV mini-1240 single beam spectrophotometer (Shimadzu Scientific Instruments (SSI), Kyoto, Japan). The blank was prepared by adding distilled water (1-ml) to 2.5 ml sodium phosphate buffer and 2.5 ml 1 % potassium ferricyanide ($K_3Fe[CN]_6$) in a test tube.

Three replicates were used. Data was presented as concentration-absorbance curves. EC_{50} value (mg extract/ml), was measured. This is effective concentration with absorbance of 0.5 for reducing power or the concentration that gives 50 % of maximal response. This was obtained by interpolation from linear regression analysis, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

6.2.2 Total phenolics

Phenolics content present in three extracts (0.3, 1, 3 mg ml^{-1} in triplicates) was quantified using the Folin-Ciocalteu's phenol reagent (Singleton, *et al*., 1965) with some modifications. Tannic acid (0.01, 0.03, 0.1, 0.3 mg ml^{-1}) was used as standard.

Principle:

Phosphomolybdate-phosphotungstate salts of Folin-Ciocalteau's reagent are reduced by phenolic compounds in alkaline medium giving a blue coloration. The intensity is quantified spectrophotometrically at 760 nm. Absorbance increases with increasing phenolic content.

Procedure:

The extracts (1 ml) were added to 1 ml Folin-Ciocalteu's reagent (diluted ten-fold in distilled water) in separate test tubes. The content of each test tube was mixed and allowed to stand for five minutes at 25 ˚C in an incubator. One millilitre (1 ml) of 2 % sodium carbonate solution (Na_2CO_3) was added to the mixture. This was allowed to stand for 2 hours at 25 ˚C in an incubator and centrifuged at 1000 *x g* for 10 min at room temperature to get a clear solution. The absorbance of the supernatant was then determined at 760 nm using UV mini-1240 single beam spectrophotometer (Shimadzu Scientific Instruments (SSI), Kyoto, Japan). Distilled water (1 ml) was added to 1 ml Folin-Ciocalteu's reagent (diluted ten-fold in distilled water) and processed the same way as the test drugs and used as blank.

All measurements were done in triplicate. Data was presented as linear regression of concentration of tannic acid against absorbance and EC_{50} determined using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

6.2.3 DPPH Scavenging Activity

The scavenging of the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples, including plant extracts (Chang, 2002). The experiment was carried out as described in literature (Blois, 1958) with a few modifications.

Principle:

The 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) a stable radical with characteristic violet colour (and maximum absorption at 517 nm). It accepts an electron or hydrogen in the presence of a suitable free radical scavenger (reducing agent) to form reduced 2, 2-diphenyl-1-picrylhydrazyl, which is yellow The residual DPPH was determined at 517 nm in a spectrophotometer. The absorbance decreases with increasing free radical scavenging ability.

The percentage (%) reduction of DPPH radical, as the free radical scavenging activity (% antiradical activity), was calculated using the equation:

$$\% \text{ Antiradical Activity} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} \times 100$$

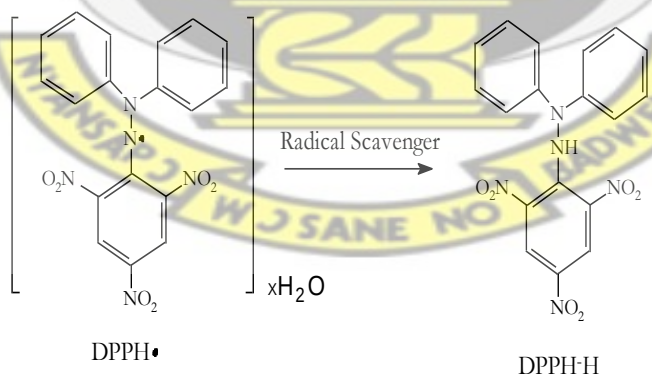


Figure 6.1: DPPH molecular structure and its reduced form.

The absorbance decreases with increasing free radical scavenging ability.

Procedure:

The extract (0.1, 0.3, 1, 3 mg ml^{-1} in methanol) was compared to *n*-propyl gallate (0.01, 0.03, 0.1, 0.3 mg ml^{-1} in methanol) as standard free radical scavengers.

The extracts (1 ml) were added to 3 ml methanolic solution of DPPH (20 mg l^{-1}) in a test tube. The reaction mixture was kept at 25 ˚C for 1 h in an orbital shaker (BoroLabs, Aldermaston Berkshire, UK.). The absorbance of the residual DPPH was determined at 517 nm in UV mini-1240 Single beam Spectrophotometer (Shimadzu Scientific Instruments (SSI), Kyoto, Japan). Methanol (99.8 %) (1 ml) was added to 3 ml DPPH solution, incubated at 25 ˚C for 1 h and used as control and Methanol (99.8 %) was used as blank.

Each experiment was carried out in triplicates and results were recorded as mean % antiradical activity against concentration. Results were recorded as mean % DPPH scavenged ± SD and EC_{50} determined. All data was analysed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

6.3 RESULTS

6.3.1 Reducing power

The absorbance of the extracts and tannic acid were normalised and expressed as EC_{50} values. The effectiveness in reducing power correlated inversely, with the EC_{50} values in ascending order, PEE >EthE> EAE > tannic acid (Table 6.1). The reducing ability (Fe^{3+} to Fe^{2+}, with increase in absorbance) was found greatest in EAE (EC_{50} 0.87±0.11), indicating a maximum reducing power. However, there was a statistically similar reductive capacities observed in PEE and EthE (Figure 6.2), this also indicating a significant antioxidant potential in its reducing ability, comparable to tannic acid.

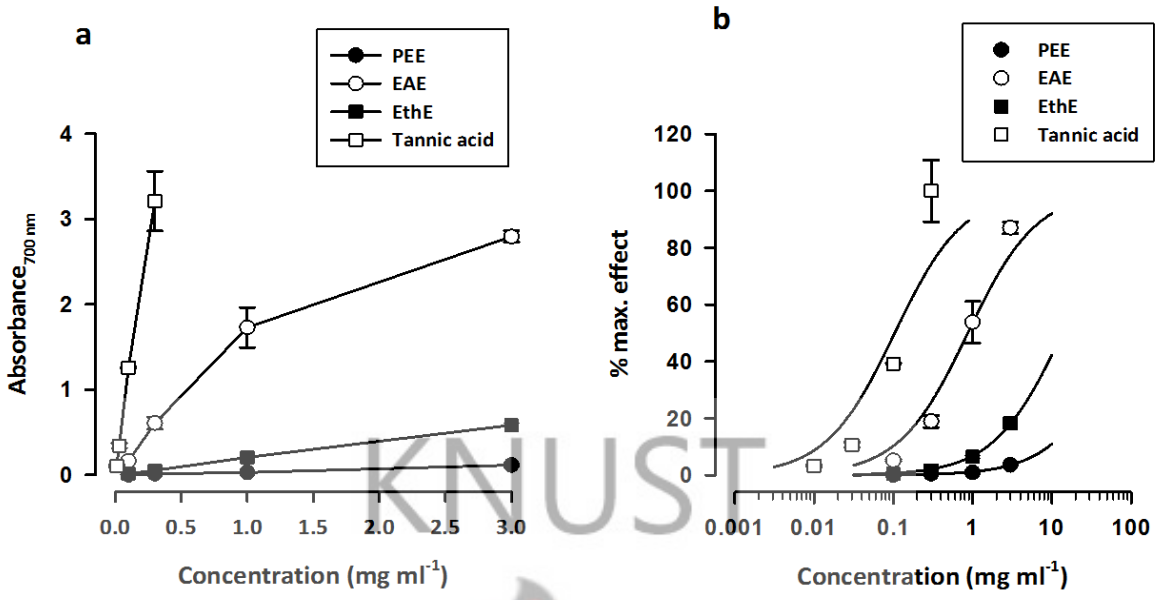


Figure 6.2: Change in (a) Absorbance and (b) maximal reducing power of the three extracts (0.1-3 mg ml^{-1}) of *Trichilia monadelpha* compared to tannic acid (0.1-3 mg ml^{-1}).
Each point represents the mean ± SEM. (n= 4).

Table 6.1: Antioxidant tests showing the EC_{50}

	Reducing Power	Phenolics	DPPH scavenging activity
PEE	81.06 ± 4.35	13.95 ± 0.61	0.24 ± 0.04
EthE	13.63 ± 0.38	1.91 ± 0.19	0.08 ± 0.01
EAE	0.87 ± 0.11	0.36 ± 0.05	0.04 ± 0.04
tannic acid	1.04 ± 0.26	0.06 ± 0.01	-
***n*-proplygallate**	-		0.02 ± 0.01

6.3.2 Total phenolics

Phenolic content of tannic acid (0.01-0.3 mg ml^{-1}) increased with increasing concentration (Figure 6.3). The result for the extracts is showed in Figure 6.3. All the extracts (0.1-1.0 mg ml^{-1}) contained a notable amount of total phenolics content with the EAE having significantly the highest phenolics content than PEE and EthE, for all the concentrations used for the assay (Table 6.2). Comparing the total phenolics content of the standard, tannic acid, to the three extracts, PEE was about 5 folds lower than tannic acid and EthE was about 2 folds lower. EAE had almost the same amount of phenolics content as tannic acid.

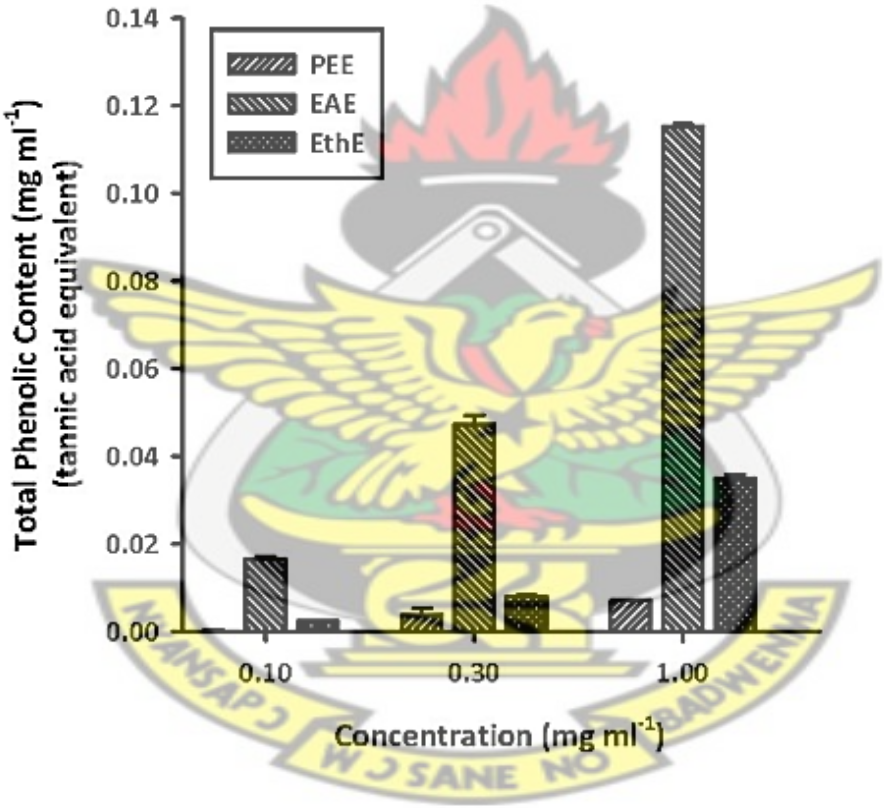


Figure 6.3: Total phenolics (expressed as tannic acid equivalents) present in various concentrations of PEE (0.1-1 mg ml-1), EthE (0.1-1 mg ml-1) and EAE (0.1-1 mg ml-1).
Each column represents the mean ± SEM. (n= 3).

Table 6.2: Total phenol content of *Trichilia monadelpha* extracts expressed as milligram tannic acid equivalent per gram of extracts

Extracts	Total Phenol content (mg TAE/g of extract)
PEE	7.51 ± 0.87
EthE	34.14 ± 0.78
EAE	119.30 ± 3.20

6.3.3 DPPH scavenging activity

The percentage of DPPH scavenged is reported in Figure 6.4 and Table 6.1. EAE scavenged more DPPH than the other extracts, dose-dependently

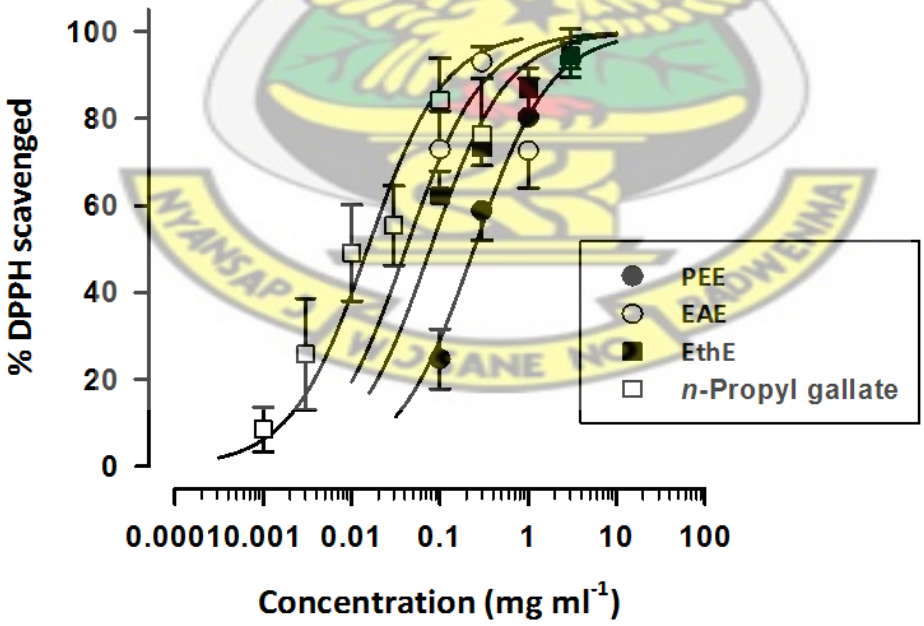


Figure 6.4: Free radical scavenging ability of extracts
Free radical scavenging ability of the extracts, PEE, EthE, EAE (0.1-3 mg ml^{-1}) compared to *n*-propyl gallate (0.001-0.3 mg ml^{-1}) in the DPPH radical assay. Each point represents the mean ± s.e.m (n = 3).

6.4 DISCUSSION

The antioxidant tests conducted seek to show the plant contains antioxidant properties, possibly serving as a natural source of antioxidant. The tests conducted were, reducing power, total phenolics and radical scavenging activity.

Reducing power assay is a convenient and rapid screening method for measuring the antioxidant potential (Meir *et al*., 1995) of a substance. From the result, there was significant antioxidant potential of the extracts compared with tannic acid. This shows the plant has significant antioxidant potential in its reducing capacity, in the plant.

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralising free radicals, quenching singlet and triplet oxygen or breaking up peroxides (Osawa, 1994). Polyphenolic compounds like flavonoids and phenolic acids commonly found in plants have multiple biological effects, including an antioxidant activity (Gil *et al*., 1999; Vinson *et al*., 1995). The notable amount of phenolics content observed in the three extracts shows the stem bark of *Trichilia monadelpha*, as a whole, possesses antioxidant properties and may contribute to the antioxidative action of the plant.

The effects of antioxidants on DPPH, is due to their hydrogen donating ability (Baumann *et al*., 1979). The potency of antioxidants in extracts was determined by EC_{50}, as shown in table 6.2. A low EC_{50} value indicates strong antioxidant activity in the extract. The DPPH radical scavenging ability of EAE was significantly as potent as that of the standard, n-propyl gallate, but that of PEE and EthE was far or less potent in scavenging the DPPH radical. The DPPH scavenging ability of EAE shows some proton-donating ability. This extract could serve as a free radical inhibitor or scavenger, acting possibly as a primary antioxidant. This shows the stem bark of *Trichilia monadelpha* possesses radical inhibiting or scavenging potential.

EAE was the extract that showed the highest levels of phenolics, reducing power and scavenged the highest percentage of the DPPH radical. However this partly had anti-inflammatory effects. It showed a considerable anti-inflammatory effect but was however,

not as efficacious as PEE, which rather showed lowest levels for all the antioxidant (*in vitro*) assay carried out. This shows that natural antioxidant effects of the plant alone are not enough prove of its anti-inflammatory effects. Other non-antioxidant secondary metabolites are responsible for the anti-inflammatory effects of the plant. These findings make *Trichilia monadelpha* a fertile ground to be exploited on.

6.5 CONCLUSION

Extracts of *Trichilia monadelpha* showed promising antioxidant and radical scavenging activities and the difference in their antioxidant activities can be attributed to their difference in phenolic content. It can by hypothesised the stem bark of *Trichilia monadelpha* is a good source of natural antioxidants and might be useful in treating the diseases associated with oxidative stress.

Chapter 7

GENERAL DISCUSSION

This study considered the anti-inflammatory effect of the plant, *Trichilia monadelpha*. The study focused on finding out the possible mechanism of action of *Trichilia monadelpha* as an anti-inflammatory agent. Areas covered in this study was first, effect of the plant on mediators synthesised and released in the 2-phase reaction of carrageenan-induced inflammation and expression of acute phase proteins specifically C-reactive protein (CRP). The second was the effect of the plant on the histopathology of the rat bone, oxidative stress biomarkers (MDA, MPO and SOD) observed during adjuvant-induced arthritis. Antioxidant property of plant was studied. The plant extracts, especially PEE was anti-histaminic. This led to study on anti-anaphylactic effect of the plant since histamine has been shown to be a major mediator expressed during anaphylactic reaction.

Compounds in the plant were extracted by three solvents. The phytochemical study on these three solvent extracts confirmed with previous studies (Ainooson *et al*., 2012; Iwalewa *et al*., 2007). This showed the plant constituted of important metabolites such as alkaloids, flavonoids, glycosides, terpenoids and steroids. Also anthroquinones, reducing sugars, cardiac glycosides, coumarins, triterpenoids, steroidal compounds and phenolic compounds were found to be present in the plant.

The results showed clearly that *Trichilia monadelpha* affected the release of inflammatory mediators which resulted in an improvement of inflammation. This effectiveness could be due to biochemical components present in the plant which were extracted by three solvents. This property lies more in PEE and EAE, although EthE also showed some effectiveness. This shows phytoconstituents in the plant contribute possibly synergically to the effectiveness of the plant as a whole. Compared with control group, treatment with extracts of *Trichilia monadelpha* significantly reduced inflammatory and anaphylactic macroscopic features and histological damage dose-dependently. This dose-dependent response is mostly observed in PEE and EAE. The determination of paw swelling is an obviously simple, sensitive and quick procedure for evaluating the degree of inflammation and the therapeutic efficacy of drugs. Carrageenan and adjuvant (*Mycobacterium tuberculosis*) are model agents used to induce acute and chronic inflammation to investigate drug efficacy

and potency (Brand, 2005; Escandell *et al*., 2007; Hughes *et al*., 1989; Wang *et al*., 2008; Winter *et al*., 1962). Carrageenan-induced inflammation and adjuvant-induced arthritis were adopted to see the effects of extracts because these models are reproducible and have similar morphological similarities to human disease features and a quantitative scoring system. These models are characterised by oxidative stress, infiltration of inflammatory cells, synthesis and release of mediators, activation of proinflammatory cytokines such as TNF-α and IL-6 and immunological reactions observed as anaphylactic reactions that cause cell injury and tissue damage.

Administration of extracts of *Trichilia monadelpha* showed a significant inhibition of paw oedema volume. Characteristics of inflammatory process are the release of mediators and release of acute phase proteins. These mediators (histamine, serotonin, bradykinin, PGE_2 and pro-inflammatory cytokines such as TNF-α and IL-6) infiltrate into the system activating innate immune response and initiating adaptive immune responses. This infiltration seeks to phagocytize foreign particles or microorganisms and clears the system or cell of invading pathogens and dead cells. In this process the system could lose its ability to distinguish self from non-self, resulting in immune reactions against one's own tissues and cells (Cantley *et al*., 2009). This in turn results in tissue damage and cell injury as observed in autoimmunity. Classical example of autoimmunity is rheumatoid arthritis which is a chronic systemic disorder with symmetrical, inflammatory polyarthritis that may produce progressive joint damage. Inflammation of the joint tissues is associated with the release of toxic substances in the synovium that lead to bone and cartilage destruction (Cantley *et al*., 2009).

Management of Inflammation therefore, targets two major aspects of the inflammatory processes. The first is reducing inflammation and relieving pain which are induced and mediated by the release of mediators and pro-inflammatory cytokines. This seeks to block pathway or inhibit specific enzymes or proteins expression that express or lead to the release or synthesis of these mediators. The second aspect is the control and suppression of inflammatory-induced tissue damage such as joint inflammation and bone erosion. Inflammatory-induced tissue damage is observed as the initiation of oxidative stress which leads to the increase expression of MDA, MPO and suppression of SOD (Atzeni *et al*., 2007; Hoffmann *et al*., 1997; Sharma *et al*., 2004). Severity of this process can lead to immune response observed as the involvement of mast cell degranulation. This releases components

of the mast cells (majorly histamine and heparin) that initiates hypersensitivity of the immune system resulting in physiological features and biochemical processes observed in anaphylaxis.

This study has shown that these two major targets of inflammatory therapy were accomplished by the administration of the plant extracts. The plant extracts reduced or ameliorated oedema induced by carrageenan and specific mediators (histamine, 5-HT, bradykinin, and PGE_2) in rats. This was possibly blocking or inhibiting specific enzymes or protein expression that express or lead to the release or synthesis of these mediators. The plant also suppressed inflammatory-induced tissue damage such as joint inflammation and bone erosion. This was achieved by the suppression of oxidative stress and synthesis of pro-inflammatory cytokines that contribute to the initiation of oxidative stress. The plant extracts also minimize immune responses that initiate hypersensitivity of the immune system, thus inhibiting anaphylactic reaction.

Specific effects of the plant on acute phase proteins such as CRP levels show the plant extracts have a possible positive effect against cardiovascular-related diseases. The anti-anaphylactic effect of the plant also shows the plant possesses a possible anti-allergic effect. These activities are possible due to the presence of important secondary metabolites that are present in the plant. This shows *Trichilia monadelpha* is a promising therapeutic agent for clinical studies. Possible pathways blocked by the plant extracts are illustrated in Figure 7.1.

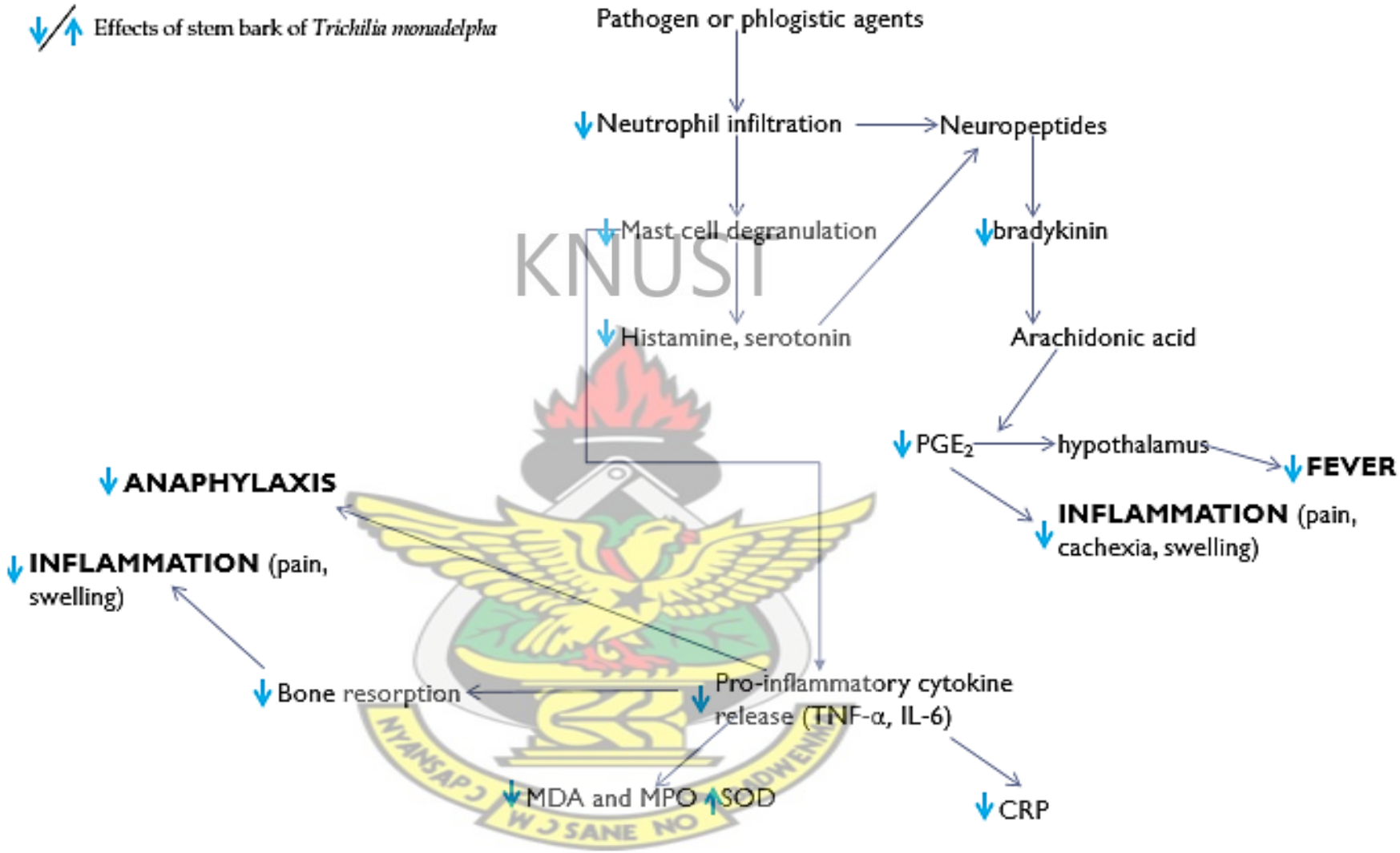


Figure 7.1: ***Trichilia monadelpha*** **extracts ameliorate important mediators and features of inflammation and anaphylaxis.**

Chapter 8

SUMMARY OF FINDINGS AND CONCLUSIONS

Trichilia monadelpha is a promising therapeutic agent, since it was proven to show the following effects;

- It ameliorated acute inflammation by blocking the release of vasoactive amines such as histamine and serotonin. This possibly stabilised and prevented the H_1 and 5-HT_3-mediated vasoconstriction and vasodilation. Preventing microvascular permeability and stimulation of sensory nerve terminals that mediate arachidonic acid metabolism and kallirein-kinin cascade.

- The anti-inflammatory effect of the extracts against carrageenan-induced inflammation was possible through also the inhibition and reduction of PGE_2 – mediated oedema in the paws

- It inhibited neutrophil extravasation induced by bradykinin. The synthesis of bradykinin potentiates the secretion of COX-2. This mediates the synthesis of PGE_2 which are responsible for the oedema formation and pain that accompanies the inflammatory reaction. Both bradykinin and PGE_2 can sensitise primary afferent neurons (Calixto *et al.*, 2004; Calixto *et al.*, 2003; Levant *et al.*, 2006; Linhart *et al.*, 2003).

- The plant reduced symptoms of systemic manifestation of inflammation by reducing febrile response induced by LPS. LPS, through a break in its natural barriers, would interact with immune cells, and promote the synthesis and release of endogenous mediators, such as cytokines (*e.g.* TNF-α, IL-1b, IL-6), PGs and endothelins (Fabricio *et al.*, 2005; Kluger, 1991; Romanovsky *et al.*, 2005).

- It reduced the levels of acute phase proteins such as CRP which are released by cytokines in response to inflammation. These are linked with increased risk of pathological disease conditions especially cardiac disorders (Kaneko *et al.*, 1999;

Ridker *et al.*, 2001; Ueda *et al.*, 1996) and serves as a marker of inflammation (Ikeda *et al.*, 2002).

- The plant was also able to suppress the inflammation, synovitis and protect bone structure especially joint protection. It also improved anaemic state associated with rheumatoid arthritis.

- The plant inhibited lipid peroxidation through the reduction of peroxide by reducing MDA and MPO levels and improving or increasing SOD levels.

- The plant prevented compound 48/80-induced perturbation, causing stability in the lipid bilayer membrane. This is observed as reduction in compound 48/80-induced mortality rate and reduction of degranulation and tissue damage in the histopathology of the tissue, stabilising membrane fluidity. Also the extracts protected animals from compound 48/80-mediated PCA, an important *in vivo* model of anaphylaxis in local allergic reaction suggesting its usefulness in treating allergic skin reactions.

- The extracts reduced levels of proinflammatory cytokines, TNF-α and IL-6. This shows that reduction of proinflammatory cytokines is essential for reduced allergic reaction and reduced bone reabsorption due to infiltration of the synovium with inflammatory cells. The extracts significantly reduced or inhibited secretion of these proinflammatory cytokines in compound 48/80-induced cytokine secretion and secretion associated with adjuvant-induced arthritis.

- Finally the plant possess free radical inhibiting or scavenging potential, showing the plant is a natural antioxidant agent which contributes to the antioxidative action of the plant.

These findings have in fact created an opportunity of providing a new and promising therapeutic agent for further research. Areas for further studies are;

- Isolation and identification of specific biochemical in the stem bark of the plant.

- Determination of the specific receptors the plant might be acting on to elicit anti-inflammatory effects.

- Effects of plant on cell proliferation can also be explored since most agents that are anti-inflammatory could possess antiangiogenic and anticancer properties.

- Another area could be plant effects on functions of macrophages and neutrophils, evaluating its relevance to its anti-inflammatory response.

REFERENCES

(2009). "***Airway hyperresponsiveness and airway inflammation in elite swimmers***." Clin Respir J. **3**(1): 62.

Abbas, Lichtman and Pillai. (2007). "***Cellular and molecular immunology***." **6th edition,** Saunders Elsevier India.

Abotsi, W. K. M., Woode, E., Ainooson, G. K., Amo-Barimah, A. K. and Boakye-Gyasi, E. (2010). "***Antiarthritic and antioxidant effects of the leaf extract of Ficus exasperata P. Beauv.(Moraceae)***." Pharmacognosy Research. **2**(2): 89.

Addo-Fordjour, P., Anning, K. A., Belford, E. J. D. and Akonnor, D. (2008). "***Diversity and conservation of medicinal plants in the Bomaa community of the Brong Ahafo region, Ghana***." Journal of Medicinal Plants Research. **Vol. 2**(9): 226-233.

Adler, M., Schulz, S. and Spengler, M. (2009). ***"Cytokine Quantification in Drug Development: A comparison of sensitive immunoassay platforms"***. Chimera Biotech. (Report). Retrieved 26 January 2010.

Agnieszka, K., Carlos, A., Maria Jose, P. and Yasmina, M. (2010) "***Evaluation of Evans Blue extravasation as a measure of peripheral inflammation.***" Protocol Exchange (2010). Nature Publishing Group. http://dx.doi.org/10.1038/protex.2010.209.

Ahmed, S., Anuntiyo, J., Malemud, C. J. and Haqqi, T. M. (2005). "***Biological Basis for the Use of Botanicals in Osteoarthritis and Rheumatoid Arthritis: A Review***." eCAM. **2**: 301-308.

Ainooson, G. K., Owusu, G., Woode, E., Ansah, C. and Annan, K. (2012). "***Trichilia monadelpha bark extracts inhibit carrageenan-induced foot-oedema in the 7-day old chick and the oedema associated with adjuvant-induced arthritis in rats***." Afr J Tradit Complement Altern Med. **9**(1): 8 - 16.

Akama, H. (2007). "***[Antibody to IL-1 or IL-15]***." Nippon Rinsho. **65**(7): 1227-1230.

Akinlolu, A. A., Otulana, J. O., Olatunde, O., Adebayo, B. E. and Akinola, O. B. (2007). "***The antispermatogenic and antifertility effects of arthemether on the testis of adult wistar rats***." Pak. J. Pathol. **18**(2): 64-67.

Al-Haboubi, H. A. and Zeitlin, I. J. (1983). "***Re-appraisal of the role of histamine in carrageenan-induced paw oedema***." Eur J Pharmacol. **88**(2-3): 169-176.

Aladesanmi, A. J. and Odediran, S. A. (2000). "***Anti-microbial activity of Trichilia heudelotii leaves***." Fitoterapia. **71**(2): 179-182.

Albrich, J. M. and Hurst, J. K. (1982). "***Oxidative inactivation of Escherichia coli by hypochlorous acid. Rates and differentiation of respiratory from other reaction sites***." FEBS Lett. **144**: 157.

Allansmith, M. R., Baird, R. S., Ross, R. N., Barney, N. P. and Bloch, K. J. (1989). "***Ocular anaphylaxis induced in the rat by topical application of compound 48/80. Dose response and time course study***." Acta Ophthalmol Suppl. **192**: 145-153.

Allard, S., O'Driscoll, J. and Laurie, A. (1977). "***Salmonella osteomyelitis in aplastic anaemia after anti-lymphocytic globulin and steroid treatment***." J Clin Pathol. **2**: 174–175.

Alm, P. E. (1984). "***Modulation of mast cell cAMP levels. A regulatory function of calmodulin***." Int Arch Allergy Appl Immunol. **75**: 375 - 378.

Aloe, L. and Levi-Montalcini, R. (1977). "***Mast cells increase in tissues of neonatal rats injected with the nerve growth factor***." Brain Res. **133**: 358–366.

Alon, R., Hammer, D. A. and Springer, T. A. (1995). "***Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow***." Nature. **374**: 539–542.

Amann, R., Schuligoi, R., Lanz, I. and Donnerer, J. (1995). "***Histamine induced edema in the rat paw-effect of capsaicin denervation and a cgrp receptor antagonist***." Europian Journal of Pharmacology. **279**: 227-231.

Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1993). "***Oxidants, antioxidants and the degenerative diseases of aging***." Proceed. Nat. Acad. Sci. **90**: 7915-7922.

Amin, K. (2012). "***The role of mast cells in allergic inflammation***." Respir Med. **106**(1): 9-14.

Amin, K., Lúdvíksdóttir, D., Janson, C., Nettelbladt, O., Bjórnsson, E., Roomans, G. M., Boman, G., Sevéus, L. and Venge, P. (2000). "***Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma***." Am J Respir Crit Care Med. **162**(6): 2295-2301.

Anthony, D. S. and Peter, L. (1986). "***A Comparison of Air pouch, sponge and pleurisy models of acute carrageenan inflammation in rat***." Agents Actions. **18**: 3/4.

Aota, S., Nakamura, T., Suzuki, K., Tanaka, Y., Okazaki, Y., Segawa, Y., Miura, M. and Kikuchi, S. (1996). "***Effects of indomethacin administration on bone turnover and bone mass in adjuvant-induced arthritis in rats***." Calcif Tissue Int. **59**(5): 385-391.

Araico, A., Terencio, M. C., Alcaraz, M. J., Dominguez, J. N., Leon, C. and Ferrandiz, M. L. (2007). "***Evaluation of the anti-inflammatory and analgesic activity of Me-UCH9, a dual cyclooxygenase-2/5-lipoxygenase inhibitor***." Life Sci, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17490689

Arcoleo, F., Milano, S. and D'Agostino, P. (1997). "***Effect of partially modified retro-inverso analogues derived from C-reactive protein on the induction of nitric oxide synthesis in peritoneal macrophages***." Br J Pharmacol. **120**: 1383–1389.

Arnett, H. A. and Viney, J. L. (2010). "***Gatekeepers of intestinal inflammation***." Inflamm Res. **59**: 1-14.

Artuc, M., Hermes, B., Steckelings, U. M., Grutzkau, A. and Henz, B. M. (1999). "***mast cells and their mediators in cutaneous wound healing-active participants or innocent bystanders?***" Exp Dermatol. **8**: 1 -16.

Asongalem, E. A., Foyet, H. S., Ekobo, S., Dimo, T. and Kamtchouing, P. (2004). "***Antiinflammatory, lack of central analgesia and antipyrectic properties of Acanthus montanus (Ness) T. Anderson***." Journal of Ethnopharmacology. **95**(2004): 63-68.

Atindehou, K. K., Schmid, C., Brun, R., Koné, M. W. and Traore, D. (2004). " ***Antitrypanosomal and antiplasmodial activity of medicinal plants from Côte d'Ivoire***." J. Ethnopharmacol. **90**: 221-227.

Atzeni, F. and Sarzi-Puttini, P. (2007). "***[Early rheumatoid arthritis]***." Reumatismo. **59**(2): 100-117.

Auddy, B., Ferreira, M., Blasina, F., Lafon, L., Arredondo, F., Dajas, F., Tripathi, P. C., Seal, T. and Mukherjee, B. (2003). "***Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases***." J Ethnopharmacol. **84**(2-3): 131-138.

Aust, S. D. (1994)."***Thiobarbituric acid assay reactants***."In Methods in Toxicology. **Tyson, C. A. and Frazier, J. M.** Academic Press, San Diego. **1B:** 367-376.

Ayer, L. M., Issekutz, A. C., Waterhouse, C. C. and Stadnyk, A. W. (2000). "***Cytokine mRNA in the joints and draining lymph nodes of rats with adjuvant arthritis and effects of cyclosporine A***." Inflammation. **24**: 447–461.

Azzolina, A., Bongiovanni, A. and Lampiasi, N. (2003). "***Substance P induces TNF-alpha and IL-6 production through NF kappa B in peritoneal mast cells.***" Biochem Biophys Acta. **1643**: 75 - 83.

Baeza, M. L. and Zubeldia, J. M. (2007). "***Immunology of anaphylaxis: lessons from murine models***." Curr Allergy Asthma Rep. **7**: 49–55.

Bainton, D. F., Ullyot, J. L. and Farquhar, M. G. (1971). "***The development of neutrophilic polymorphonuclear leukocytes in human bone marrow***." J Exp Med. **134**: 907.

Bajtner, E., Nandakumar, K. S., Engstrom, A. and Holmdahl, R. (2005). "***Chronic development of collagen-induced arthritis is associated with arthritogenic antibodies against specific epitopes on type II collagen***." Arthritis Res Ther. **7**(5): R1148-1157.

Baliga, M. S., Jagetia, G. C., Ulloor, J. N., Baliga, M. P., Venkatesh, P., Reddy, R., Rao, K. V., Baliga, B. S., Devi, S., Raju, S. K., Veeresh, V., Reddy, T. K. and Bairy, K. L. (2004).

"***The evaluation of the acute toxicity and long term safety of hydroalcoholic extract of Sapthaparna (Alstonia scholaris) in mice and rats***." Toxicol Lett. **151**(2): 317-326.

Banniste, J., Bannister, W. and Rotilio, G. (1987). "***Aspects of the structure, function, and applications of superoxide dismutase***." CRC Crit Rev Biochem. **22**: 111-180.

Bao, L., Zhu, Y., Elhassan, A. M., Wu, Q., Xiao, B., Zhu, J. and Lindgren, J. U. (2001). "***Adjuvant-induced arthritis: IL-1 beta, IL-6 and TNF-alpha are up-regulated in the spinal cord***." Neuroreport. **12**(18): 3905-3908.

Barreiro, O., Yanez-Mo, M., Serrador, J. M., Montoya, M. C., Vicente-Manzanares, M., Tejedor, R., Furthmayr, H. and Sanchez-Madrid, F. (2002). "***Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes***." J Cell Biol. **157**(7): 1233-1245.

Baumann, H. and Gauldie, J. (1994). "***The acute phase response***." Immunol Today. **15**: 74-80.

Baumann, H., Richard, C. and Gauldie, J. (1987). "***Interaction among hepatocytestimulating factors, interleukin 1, and glucocorticoids for regulation of acute phase plasma proteins in human hepatoma (HepG2) cells***." J Immunol. **139**: 4122-4128.

Baumann, J., Wurn and Bruchlausen, F. V. (1979)."***Prostaglandin synthetase inhibiting O-2 radical scavenging properties of some flavonoids and related phenolic compounds***."In Deutsche Pharmakologische Gesellschaft Abstracts of the 20th Spring Meeting, Naunyn-Schmiedebergs. Abstract No: R27. Arch Pharmacol**307:** R1–R77.

Bedard, K. and Krause, K. H. (2007). "***The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology***." Physiological Reviews. **87**(1): 245–313.

Begum, V. H. and Sadique, J. (1988). "***Long term effect of herbal drug Withania 98 NARENDHIRAKANNAN ET AL. somnifera on adjuvant induced arthritis in rats***." Indian J Exp Biol. **26**: 877–882.

Behar, S. M. and Porcelli, S. A. (1995). "***Mechanisms of autoimmune disease induction. The role of the immune response to microbial pathogens***." Arthritis Rheum. **38**(4): 458-476.

Benklebia, N. (2005). "***Free-radical scavenging capacity and antioxidant properties of some selected onions (Allium cepa L.) and Garlic (Allium sativum L.) extracts.***" Braz. arch. biol. technol. **48**: 753-759.

Berlin, C., Bargatze, R. F., Campbell, J. J., von Andrian, U. H., Szabo, M. C., Hasslen, S. R., Nelson, R. D., Berg, E. L., Erlandsen, S. L. and Butcher, E. C. (1995). "***α4 integrins mediate lymphocyte attachment and rolling under physiologic flow***." Cell. **80**(3): 413–422.

Bersani-Amado, C. A., Barbuto, J. A. and Jancar, S. (1990). "***Comparative study of adjuvant induced arthritis in susceptible and resistant strains of rats. I. Effect of cyclophosphamide***." J Rheumatol. **17**(2): 149-152.

Bianchi, M. E. (2007). "***DAMPs, PAMPs and alarmins: all we need to know about danger***." J Leukoc Biol. **81**(1): 1-5.

Bienenstock, J., Tomioka, M., Matsuda, H., Stead, R. H., Quinonez, G., Simon, G. T., Coughlin, M. D. and Denburg, J. A. (1987). "***The role of mast cells in inflammatory processes: evidence for nerve mast cell interactions***." Int. Arch. Allergy Appl. Immunol. **82**: 238–243.

Blank, U. and Rivera, J. (2004). "***The ins and outs of IgE-dependent mast-cell exocytosis***." Trends Immunol. **25**: 266–273.

Blatteis, C. M. and Sehic, E. (1997). "***Fever: how may circulating cytokines signal the brain?***" News in Physiological Sciences. **12**: 1-9.

Blaydon, D. C., Biancheri, P., Di, W.-L., Plagnol, V., Cabral, R. M., Brooke, M. A., van Heel, D. A., Ruschendorf, F., Toynbee, M. and Walne, A. (2011). "***Inflammatory skin and bowel disease linked to ADAM17 deletion***." New England Journal of Medicine. **365**(16): 1502-1508.

Borish, L. and Joseph, B. Z. (1992). "***Inflammation and the allergic response***." Medical Clinics of North America. **76**(4): 765-787.

Botana, L. M. and macGlashan, D. W. (1994). "***Differential effects of cAMP-elevating drugs on stimulus-induced cytosolic calcium changes in human basophils***." J Leukoc Biol. **55**: 789 - 804.

Brain, K. R. and Turner, T. D. (1975). "***The practical evaluation of phytochemicals.***" Wright Science Technical Bristol. 56-64.

Brand, D. D. (2005). "***Rodent models of rheumatoid arthritis***." Comp Med. **55**: 114-122.

Branen, A. L. (1975). "***Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene***." Journal of the American Oil Chemists' Society. **52**(2): 59-63.

Brewer, J. M., Pollock, K. G., Tetley, L. and Russell, D. G. (2004). "***Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles***." J. Immunol. **173**: 6143–6150.

Brigelius-Flohe, R. (1999). "***Tissue-specific functions of individual glutathione peroxidases***." Free Radic Biol Med. **27**: 951-965.

Brown, J. E. and Rice-Evan, C. A. (1998). "***Luteolin-rich Artichoke extract protects low density lipoprotein from oxidation in vitro***." Free Radic Res. **29**: 247-255.

Buckley, M. G., Walters, C., Wong, W. M., Cawley, M. I., Ren, S., Schwartz, L. B. and Walls, A. F. (1997). "***Mast cell activation in arthritis: detection of α- and β-tryptase, histamine and eosinophil cationic protein in synovial fluid***." Clin Sci (Lond). **93**: 363-370.

Burkill, H. M. (1985). "***Entry for Trichilia monadelpha (Thonn.) JJ de Wilde [family MELIACEAE]***." Burkill, H.M. 1985. The useful plants of west tropical Africa. **Vol 4**.

Bush, K. A., Walker, J. S., Lee, C. S. and Kirkham, B. W. (2001). "***Cytokine expression and synovial pathology in the initiation and spontaneous resolution phases of adjuvant arthritis: interleukin-17 expression is upregulated in early disease***." Clin Exp Immunol. **123**: 487–495.

Bush, K. A., Farmer, K. M., Walker, J. S. and Kirkham, B. W. (2002). "***Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein***." Arthritis Rheum. **46**: 802–805.

Busia, K. (2007). "***Ghana Herbal Pharmacopoeia***." Science and Technology Policy Research Institute (STEPRI) Council for Scientific and Industrial Research (CSIR), Cantonments. Accra-Ghana.

Butcher, E. C. (1991). "***Leukocyte–endothelial cell recognition: three (or more) steps to specificity and diversity***." Cell. **67**: 1033–1036.

Calixto, J. B., Otuki, M. F. and Santos, A. R. S. (2003). "***Anti-inflammatory compounds of plant origin, Part 1. Action on arachidonic acid pathway, nitric oxide and nuclear factor kB (NF-kB)***." Planta Med. **69**: 973—983.

Calixto, J. B., Campos, M. M., Otuki, M. F. and Santos, A. R. S. (2004). "***Anti-Inflammatory Compounds of Plant Origin. Part II. Modulation of Pro-Inflammatory cytokines, Chemokines and Adhesion Molecules***." Planta Med. **70**: 93-103.

Campos, S. P., Wang, Y., Koj, A. and Baumann, H. (1994). "***Insulin cooperates with IL-1 in regulating expression of a1-acid glycoprotein gene in rat hepatoma cells***." Cytokine. **6**: 485-492.

Cantley, M. D., Smith, M. D. and Haynes, D. R. (2009). "***Pathogenic bone loss in Rheumatoid arthritis: mechanisms and therepeutic approaches***." Int. J. Clin. Rheumatol. **4**(5): 561-582.

Cao, C., Matsumura, K., Yamagata, K. and Watanabe, Y. (1995). "***Induction by lipopolysaccharide of cyclooxygenase-2 mRNA in rat brain; its possible role in the febrile response***." Brain Research. **697**: 187-196.

Carman, C. V. and Springer, T. A. (2004). "***A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them***." J. Cell Biol. **167**: 377–388.

Carraway, R., Cochrane, D. E., Lansman, J. B., Leeman, S. E., Paterson, B. M. and Welch, H. J. (1982). "***Neurotensin stimulates exocytotic histamine secretion from rat mast cells and elevates plasma histamine levels***." J Physiol. **323**: 403-414.

Casey, R., Newcombe, J., McFadden, J. and Bodman-Smith, K. B. (2008). "***The acute-phase reactant C-reactive protein binds to phosphorylcholine-expressing Neisseria meningitidis and increases uptake by human phagocytes***." Infect Immun. **76**(3): 1298-1304.

Ceciliani, F., Giordano, A. and Spagnolo, V. (2002). "***The systemic reaction during inflammation: The acute phase proteins***." **9**: 211-223.

Chamundeeswari, D., Vasantha, J., Gopalakrishnan, S. and Sukumar, E. (2003). "***Free radical scavenging activity of the alcoholic extract of Trewia polycarpa roots in arthritic rats***." J Ethnopharmacol. **88**: 51–56.

Chan, C.-C., Panneton, M., Taylor, A. M., Therien, M. and Rodgers, I. W. (1997). "***A selective inhibitor of cyclooxygenase-2 reverses endotoxin-induced pyretic responses in non-human primates***." Eur. J. Pharmacol. **327**: 221–225.

Chandrasoma, P. and Taylor, C. R. (1998). "***Concise pathology***." **3rd,** CT: Appleton & Lange Stamford. pp. 31–92

Chelikani, P., Fita, I. and Loewen, P. C. (2004). "***Diversity of structures and properties among catalases***." Cell Mol Life Sci. **61**: 192-208.

Chen, C. C., Grimbaldeston, M. A., Tsai, M., Weissman, I. L. and Galli, S. J. (2005). "***Identification of mast cell progenitors in adult mice***." Proc. Natl Acad. Sci. USA. **102**: 11408–11413.

Chen, Q. and Wei, W. (2005). "***New therapeutic approaches for rheumatoid arthritis***." Assay Drug Dev Technol. **3**(3): 329-337.

Chen, X., Oppenheim, J. J. and Howard, O. M. (2004). "***Chemokines and chemokine receptors as novel therapeutic targets in rheumatoid arthritis (RA): inhibitory effects of traditional Chinese medicinal components***." Cell Mol Immunol. **1**(5): 336-342.

Chew, Y. L., Goh, J. K. and Lim, Y. Y. (2009). "***Assessment of in vitro antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia*** " Food Chem. **119**: 373–378.

Choi, C. W., Kim, S. C., Hwang, S. S., Choi, B. K., Ahn, H. J., Lee, M. Y., Park, S. H. and Kim, S. K. (2002). "***Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison***." Plant Sci. **163**: 1161-1168.

Choi, J. Y., Kim, H., Choi, Y. J., Ishihara, A., Back, K. and Lee, S. G. (2010). "***Cytoprotective activities of hydroxycinnamic acid amides of serotonin against oxidative stress-induced damage in HepG2 and HaCaT cells***." Fitoterapia. **81**(8): 1134-1141.

Choi, Y. H., Chai, O. H., Han, E. H., Choi, S. Y., Kim, H. T. and Song, C. H. (2010). "***Lipoic acid suppresses compound 48/80-induced anaphylaxis-like reaction***." Anat Cell Biol. **43**(4): 317-324.

Christensen, A. C. (1975)."***Leydig Cells***."In Handbook of Physiology. **Greep, P. O. and Astwoods, E. B.** Am. Physiol. Soc, Washington D.C. 165-172.

Christie, M. J., Connor, M., Vaughan, C. W., Ingram, S. L. and Bagley, E. E. (2000). "***Cellular actions of opioids and other analgesics: Implications for synergism in pain relief***." Clinical and experimental pharmacology and physiology. **27**: 520-523.

Chu, Y. F., Sun, J., Wu, X. and Liu, R. H. (2002). "***Antioxidant and antiproliferative activities of common vegetables***." J. Agric. Food Chem. **50**: 6910-6916.

Coceani, F., Lees, J. and Bishai, I. (1988). "***Further evidence implicating prostaglandin E2 in the genesis of pyrogen fever***." American Journal of Physiology. **254**: R463-R469.

Coelho dos Santos, J. S., Menezes, C. A., Villani, F. N., Magalhaes, L. M., Scharfstein, J., Gollob, K. J. and Dutra, W. O. (2010). "***Captopril increases the intensity of monocyte infection by Trypanosoma cruzi and induces human T helper type 17 cells***." Clin Exp Immunol. **162**(3): 528-536.

Coleman, J. W. (2002). "***Nitric oxide: a regulator of mast cell activation and mast cell-mediated inflammation***." Clin. Exp. Immunol. **129**: 4-10.

Conti, B., Tabarean, I., Andrei, C. and Bartfai, T. (2004). "***Cytokines and fever***." Front Biosci. **9**: 1433-1449.

Conti, P., Kempuraj, D., Di Gioacchino, M., Boucher, W., Letourneau, R., Kandere, K., Barbacane, R. C., Reale, M., Felaco, M., Frydas, S. and Theoharides, T. C. (2002). "***Interleukin-6 and mast cells***." Allergy Asthma Proc. **23**: 331 - 335.

Conti, S., Costa, B., Colleoni, M., Parolaro, D. and Giagnoni, G. (2002). "***Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat***." Br J Pharmacol. **135**(1): 181-187.

Corpas, F. J., Barroso, J. B. and del Río, L. A. (2001). "***Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells***." Trends Plant Sci. **6**: 145-150.

Corpas, F. J., Fernández-Ocaña, A., Carreras, A., Valderrama, R., Luque, F. and Esteban, F. J. (2006). "***The expression of different superoxide dismutase forms is cell-type dependent in olive (Olea europaea L.) leaves***." Plant Cell Physiol. **47**: 984-994.

Costa-Lotufo, L. V., de Lucena, D. F., Andrade-Neto, M., Bezerra, J. N., Leal, L. K., de Sousa, F. C. and Viana, G. S. (2004). "***Analgesic, antiinflammatory and central depressor effects of the hydroalcoholic extract and fractions from Aeolanthus suaveolens***." Biol Pharm Bull. **27**(6): 821-824.

Crofford, L. J., Sano, H., Karalis, K., Webster, E. L., Goldmuntz, E. A., Chrousos, G. P. and Wilder, R. L. (1992). "***Local secretion of corticotropin-releasing hormone in the joints of Lewis rats with inflammatory arthritis***." J Clin Invest. **90**: 2555-2564.

Cronstein, B. N. (1995). "***The antirheumatic agents sulfasalazine and methotrexate share an anti-inflammatory mechanism***." Br. J. Rheumatol. **34**(Suppl. 2): 30–32.

Crookham, J. and Dapson, R. (1991). "***Hazardous Chemicals in the Histopathology Laboratory***." **2nd ed,** Anatech

Crunkhon, P. and Meacock, S. C. (1971). "***Mediators of the inflammation induced in the rat paw by carrageenan***." British Journal of Pharmacology. **42**: 392-402.

Cui, K., Lou, X., Xu, K. and Ven Murthy, M. R. (2004). "***Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants***." Progress in Neuro-Psychopharmacology & Biological Psychiatry. **28**: 771-799.

Cypcar, D., Sorkness, R., Sedgwick, J., Nagata, M., Clough, J., Kaplan, M. and Lemanske, R. F., Jr. (1996). "***Rat eosinophils: isolation and characterization of superoxide production***." J Leukoc Biol. **60**(1): 101-105.

Dai, Y., Hou, L. F., Chan, Y. P., Cheng, L. and But, P. P. (2004). "***Inhibition of immediate allergic reactions by ethanol extract from Plumbago zeylanica stems***." Biol Pharm Bull. **27**(3): 429-432.

Dalgleish, A. G. and O'Byrne, K. J. (2002). "***Chronic immune activation and inflammation in the pathogenesis of AIDS and cancer***." Adv. Cancer Res. **84**: 231-276.

Damas, J. (1980). "***[Kinins-formation, hypotensive and oedematogen activities of four carrageenans (author's transl)]***." Pathol Biol (Paris). **28**(5): 287-292.

Day, S. M., Lockhart, J. C., Ferrell, W. R. and McLean, J. S. (2004). "***Divergent roles of nitrergic and prostanoid pathways in chronic joint inflammation***." Ann Rheum Dis. **63**(12): 1564-1570.

De Simone, R., Alleva, E., Tirassa, P. and Aloe, L. (1990). "***Nerve growth factor released into the bloodstream following intraspecific fighting induces mast cell degranulation in adult male mice***." Brain Behav Immun. **4**(1): 74-81.

Demiry¨urek, A. T. and Wadsworth, R. M. (1999). "***Superoxide in the pulmonary circulation***." Pharmacology and Therapeutics. **84**(3): 355–365.

Dennis, F. (2002). "***Manual for the propagation of medicinal plants in Ghana. Darwin Initiative for the Survival of Species.***".

Desai, A. K. and Grossberg, G. T. (2003). "***Herbals and botanicals in geriatric psychiatry***." Am J Geriatr Psychiatry. **11**: 498-506.

Devakumar, C. and Sukh, D. (1996)."***Chemistry***."In. **Randhawa, N. S. and Parmar, B. S.:** 63—99.

Di Rosa, M., Giroud, J. P. and Willoughby, D. A. (1971). "***Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine***." J Pathol. **104**(1): 15 - 29.

Dick, W. C. (1972). "***An Introduction to Clinical Rheumatology***." Churchill-Livingstone Edinburgh.

Dinarello, C. A. (1996). "***Biologic basis for interleukin-1 in disease***." Blood. **87**: 2095-2147.

Dinarello, C. A. (1997)."***Cytokines as endogenous pyrogens***."In Fever: basic mechanisms and management. **Mackowiak, P. A.** Lippincott-Raven, Philadelphia. 87-116.

Donadieu, J., Leblanc, T., Bader Meunier, B., Barkaoui, M., Fenneteau, O., Bertrand, Y., Maier-Redelsperger, M., Micheau, M., Stephan, J. L., Phillipe, N., Bordigoni, P., Babin-Boilletot, A., Bensaid, P., Manel, A. M., Vilmer, E., Thuret, I., Blanche, S., Gluckman, E., Fischer, A., Mechinaud, F., Joly, B., Lamy, T., Hermine, O., Cassinat, B., Bellanne-Chantelot, C. and Chomienne, C. (2005). "***Analysis of risk factors for myelodysplasias, leukemias and death from infection among patients with congenital neutropenia. Experience of the French Severe Chronic Neutropenia Study Group***." Haematologica. **90**(1): 45-53.

Doshi, N. and Mitragotri, S. (2010). "***Macrophages recognize size and shape of their targets***." PLoS ONE. **5**: e10051.

Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T. and Tschopp, J. (2008). "***Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica***." Science. **320**(5876): 674-677.

Dozen, M., Yamaki, K. and Oh-Ishi, S. (1989). "***Captopril uncovers kinin-dependent release of arachidonic acid metabolites in carrageenin-induced rat pleurisy***." Jpn J Pharmacol. **51**(1): 101-105.

Dray, A. (1995). "***Inflammatory mediators of pain***." British Journal of Anesthesia. **75**: 25-131.

Dubreuil, P., Letard, S. and Ciufolini, M. (2009). "***Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT***." PLoS One. **4**: e7258.

Dubuisson, D. and Dennis, S. G. (1977). "***The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats***." Pain. **4**(2): 161-174.

Dubuisson, D. and Dennis, S. G. (1977). "***The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats***." Pain. **4**(2): 161-174.

Dunford, H. B. (1987). "***Free radicals in iron-containing systems***." Free Radic Biol Med. **3**: 405.

Duwiejua, M., Zeitlin, I. J., Waterman, P. G. and Gray, A. I. (1994). "***Anti-inflammatory activity of Polygonum bistorta, Guaiacum officinale and Hamamelis virginiana in rats***." J Pharm Pharmacol. **46**(4): 286-290.

Egbunike, G. N., Branscheid, W., Pfisterer, J. and Holtz, W. (1986). "***Changes in porcine sperm lactate dehydrogenase isoenzymes during sperm maturation***." Andrology. **18**: 108-113.

Ejebe, D. E., Ojieh, A. E., Ovuakporaye, S. I., Odion-Obomhense, H. K., Adegor, E. C., Amadi, C. N., Nwadito, C., Emudainohwo, J. O. T. and Ozoko, T. C. (2008). "***Effects of anti-malarial alkaloids on the sperm properties and blood levels of reproductive hormones of adult men***." Afr. J. Biotech. **7**(19): 3395-3400.

El-Tawil, B. D. H. (1983). "***Chemical constituents of indigenous plants used in Native Medicines of Saudi Arabia, II***." Arab Gulf J Sci Res. **A1**(12): 395 -410.

Elhrlich, P. (1878). "***Beitrage zur theorie und praxis der histologischen färbung***." Thesis, University of Leipzig. Leipzig, Germany.

Elmquist, J. K., Breder, C. D., Sherin, J. E., Scammel, T. E., Hickey, W. F., Dewitt, D. and Saper, C. B. (1997). "***Intravenous lipopolysaccharide induces cyclooxygenase 2-like immunoreactivity in rat brain perivascular microglia and meningeal macrophages***." Journal of Comparative Neurology. **381**: 119-129.

Ennis, M., Pearce, F. L. and Weston, P. M. (1980). "***Some studies on the release of histamine from mast cells stimulated with polysine***." Br J Pharmacol. **70**: 329 - 334.

Escandell, J. M., Recio, M. C., Manez, S., Giner, R. M., Cerda-Nicolas, M. and Rios, J. L. (2007). "***Cucurbitacin R reduces the inflammation and bone damage associated with adjuvant arthritis in lewis rats by suppression of tumor necrosis factor-alpha in T lymphocytes and macrophages***." J Pharmacol Exp Ther. **320**(2): 581-590.

Fabricio, A. S. C., Rae, G. A., D'Orleans-Juste, P. and Souza, G. E. (2005). "***Endothelin-1 as a central mediator of LPS-induced fever in rats***." Brain Res. **1066**: 92-100.

Fabricio, A. S. C., Veiga, F. H., Cristofoletti, R., Navarra, P. and Souza, G. E. P. (2005). "***The effects of selective and non-selective cyclooxygenase inhibitors on endothelin-1-induced fever in rats***." Am. J. Physiol. Regul. Integr. Comp. Physiol. **288**: R671—R677.

Fabricio, A. S. C., Rae, G. A., Zampronio, A. R., D'Orleans-Juste, P. and Souza, G. E. P. (2006). "***Central endothelin ETB receptors mediate IL-1-dependent fever induced by preformed pyrogenic factor and corticotropin-releasing factor in the rat***." Am. J. Physiol. Regul. Integr. Comp. Physiol. **290**: R164—R171.

Fairchild, K. D., Singh, I. S., Patel, S., Drysdale, B. E., Viscardi, R. M., Hester, L., Lazusky, H. M. and Hasday, J. D. (2004). "***Hypothermia prolongs activation of NF-kappaB and augments generation of inflammatory cytokines***." Am J Physiol Cell Physiol. **287**(2): C422-431.

Fantone, J. C. and Ward, P. A., (1999). "***Inflammation***." Pathology **Rubin, E. and Farber, J. L.** Lippincott Williams & Wilkins., Philadelphia.

Fattori, E., Cappelletti, M. and Costa, P. (1994). "***Defective inflammatory response in interleukin 6-deficient mice***." J Exp Med. **180**: 1243-1250.

Fauna and Flora. (2011). "***Fauna and Flora of Liberia: Trichilia monadelpha (Meliaceae)***." Accessed in April, 2012, from http://www.liberianfaunaflora.org.

Federico, A., Morgillo, F., Tuccillo, C., Ciardiello, F. and Loguercio, C. (2007). "***Chronic inflammation and oxidative stress in human carcinogenesis***." International Journal of Cancer. **121**(11): 2381–2386.

Federico, A., Morgillo, F., Tuccillo, C., Ciardiello, F. and Loguercio, C. (2007). "***Chronic inflammation and oxidative stress in human carcinogenesis***." International Journal of Cancer **121** (11): 2381–2386.

Feige, U., Hu, Y. L., Gasser, J., Campagnolo, G., Munyakazi, L. and Bolon, B. (2000). "***Anti-interleukin-1 and anti-tumor necrosis factor-a synergistically inhibit adjuvant arthritis in Lewis rats***." Cell Mol Life Sci. **57**: 1457–1470.

Feldmann, M., Brennan, F. M. and Maini, R. N. (1996). "***Role of cytokines in rheumatoid arthritis***." Annu Rev Immunol. **14**: 397-440.

Fereidoni, M., Ahmadiani, A., Semnanian, S. and Javan, M. (2000). "***An accurate and simple method for measurement of paw edema***." J Pharmacol Toxicol Methods. **43**(1): 11-14.

Fiedorczyk, M., Klimiuk, P. A., Sierakowski, S. and Kita, K. (2006). "***[Matrix metalloproteinases and tissue inhibitors of metalloproteinases in the pathogenesis of rheumatoid arthritis]***." Pol Merkur Lekarski. **20**(116): 228-231.

Finckh, A. (2009). "***Early inflammatory arthritis versus rheumatoid arthritis***." Current opinion in rheumatology. **21**: 118-123.

Findlay, D. M. and Haynes, D. R. (2005). "***Mechanisms of bone loss in rheumatoid arthritis***." Mod Rheumatol. **15**(4): 232-240.

Finkel, R., Clark, M. A. and Cubeddu, L. X., (2009). "***Lippincott's Illustrated Reviews: Pharmacology***." **Richard, A., Harvey, P. and Champe, C.** Lippincott Williams & Wilkins,

Finkelman, F. D. (2007). "***Anaphylaxis: lessons from mouse models***." J Allergy Clin Immunol. **120**: 506–515.

Finkelman, F. D., Rothenberg, M. E. and Brandt, E. B. (2005). "***Molecular mechanisms of anaphylaxis: lessons from studies with murine models***." J Allergy Clin Immunol. **115**: 449–457.

Firestein, G. S. (2003). "***Evolving concepts of rheumatoid arthritis***." Nature. **423**: 356-361.

Fletcher, D. S., Widmer, W. R., Luell, S., Christen, A., Orevillo, C., Shah, S. and Visco, D. (1998). "***Therapeutic administration of a selective inhibitor of nitric oxide synthase does not ameliorate the chronic inflammation and tissue damage associated with adjuvant-induced arthritis in rats***." J Pharmacol Exp Ther. **284**(2): 714-721.

Foreman, J. C. (1987). "***Peptides and neurogenic inflammation***." Brain Res. Bull. **43**: 386–398.

Frenzel, L. and Hermine, O. (2012). "***Mast cells and inflammation***." Joint Bone Spine http://dx.doi.org/10.1016/j.jbspin.2012.08.013(0).

Frewin, D. B., Cleland, L. G., Jonsson, J. R. and Robertson, P. W. (1986). "***Histamine levels in human synovial fluid***." J Rheumatol. **13**: 13-14.

Fries, J. F., Williams, C. A., Morfeld, D., Singh, G. and Sibley, J. (1996). "***Reduction in long-term disability in patients with rheumatoid arthritis by disease-modifying antirheumatic drug-based treatment strategies***." Arthritis Rheum. **39**(4): 616-622.

Fukugasako, S., Ito, S. and Ikemoto, Y. (2003). "***Effect of P-hydroxybenzoate (Methyl Paraben) on Ca2+ concentration and histamine release in rat peritoneal mast cells***." Br J Pharmacol. **139**: 381–387.

Fulton, C. C. (1932). "***The Identification of Alkaloids by Precipitation***." Journal of the Association of Official: 497.

Furst, D. E. and Munster, T. (2001)."***Nonsteroidal Anti-Inflammatory Drugs, Disease-Modifying Antirheumatic Drugs, Nonopioid Analgesics, & Drugs Used in Gout***."In Basic & Clinical Pharmacology. **Katzung, B. G. e.** McGraw-Hill Companies**:** 596-623.

Gabay, C. and Kushner, I. (1999). "***Acute-Phase Proteins and Other Systemic Responses to Inflammation***." New England Journal of Medicine. **340**(6): 448-454.

Galli, S. J. and Kitamura, Y. (1987). "***Genetically mast-cell-deficient W/Wv and Sl/Sld mice. Their value for the analysis of the roles of mast cells in biologic responses in vivo***." Am J Pathol. **127**: 191–198.

Galli, S. J., Maurer, M. and Lantz, C. S. (1999). "***Mast cells as sentinels of innate immunity***." Curr Opin Immunol. **11**: 53-59.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. and Baumann, H. (1987). "***Interferon b2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells***." Proc Natl Acad Sci U S A. **84**: 7251-7255.

Gil, M. I., Ferreres, F. and Tomas-Barberan, F. A. (1999). "***Effect of Postharvest storage and processing on the antioxidant constituents (Flavonoids and Vitamin C) of fresh-cut spinach***." J Agric Food Chem **47**: 2213-2217.

Glen, E. M., Bowman, B. J., Ronloff, N. A. and Seely, R. J. (1977). "***A major contributory cause of arthritis in adjuvant inoculated rats. Granulocytes***." Agents Actions. **7**: 265–282.

Goetzl, E. J., Cheng, P. P. J., Hassner, A., Adelman, D. C., Frick, O. L. and Speedharan, S. P. (1990). "***Neuropeptides, mast cells and allergy: novel mechanisms and therapeutic possibilities***." Clin. Exp. Allergy. **20**: 3–7.

Goldblatt, F. and Isenberg, D. A. (2005). "***New therapies for rheumatoid arthritis***." Clin Exp Immunol. **140**(2): 195-204.

Gonda, T. A., Tu, S. and Wang, T. C. (2009). "***Chronic inflammation, the tumor micro-environment and carcinogenesis***." Cell cycle. **8**: 2005-2013.

Goodman, R. B., Pugin, J., Lee, J. S. and Matthay, M. A. (2003). "***Cytokine-mediated inflammation in acute lung injury***." Cytokine Growth Factor Rev. **14**(6): 523-535.

Govindarajan, R., Rastogi, S., Vijayakumar, M., Shirwaikar, A., Rawat, A. K. S., Mehrotra, S. and Pushpangadan, P. (2003). "***Studies on the Antioxidant Activities of Desmodium gangeticum***." Biol. Pharm. Bull. **26**: 1424-1427.

Grice, H. C. (1986). "***Safety evaluation of butylated hydroxytoluene (BHT) in liver, lung and gastrointestinal tract***." Food Chem. Toxicol. **24**: 1127-1130.

Griffiths, M. M., Cannon, G. W., Corsi, T., Reese, V. and Kunzler, K. (2007). "***Collagen-induced arthritis in rats***." Methods Mol Med. **136**: 201-214.

Grimbaldeston, M. A., Chen, C. C., Piliponsky, A. M., Tsai, M., Tam, S. Y. and Galli, S. J. (2005). "***Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo***." Am. J. Pathol. **167**: 835–848.

Guay, J., Bateman, K., Gordon, R., Mancini, J. and Riendeau, D. (2004). "***Carrageenan-induced paw edema in rat elicits a predominant prostaglandin E2 (PGE2) response in the central nervous system associated with the induction of microsomal PGE2 synthase-1***." J Biol Chem. **279**(23): 24866-24872.

Gupta, M., Mazumder, U. K., Kumar, R. S., Gomathi, P., Rajeshwar, Y., Kakoti, B. B. and Selven, V. T. (2005). "***Anti-inflammatory, analgesic and antipyretic effects of methanol extract from Bauhinia racemosa stem bark in animal models***." J Ethnopharmacol. **98**(3): 267-273.

Halayko, A. J. and Amrani, Y. (2003). "***Mechanisms of inflammation-mediated airway smooth muscle plasticity and airways remodeling in asthma***." Respir Physiol Neurobiol. **137**(2-3): 209-222.

Halim, T. Y., Song, K. W., Barnett, M. J., Forrest, D. L., Hogge, D. E., Nantel, S. H., Nevill, T. J., Shepherd, J. D., Smith, C. A., Sutherland, H. J., Toze, C. L. and Lavoie, J. C. (2007). "***Positive impact of selective outpatient management of high-risk acute myelogenous leukemia on the incidence of septicemia***." Ann Oncol. **18**(7): 1246-1252.

Hallmann, R., Horn, N., Selg, M., Wendler, O., Pausch, F. and Sorokin, L. M. (2005). "***Expression and function of laminins in the embryonic and mature vasculature***." Physiol Rev. **85**(3): 979-1000.

Han, X., Shen, T. and Lou, H. (2007). Int J Mol Sci. **8**: 950-988.

Harborne, J. B. (1998). "***Phytochemical Methods—A Guide to Modern Techniques of Plant Analysis***." Chapman and Hall; London, UK.

Harbourne, J. B. (1984). "***Phytochemical Methods: A Guide to Modern Technique of Plant Analysis, 2nd ed. Chapman & Hall, London***." 282.

Harrison, J. E., Shultz, J. . (1976). "***Studies on the chlorinating activity of myeloperoxidase*** " J Biol Chem **251**: 371

Hayward, M. A., Howard, G. A., Neuman, R. G., Wood, D. D., Weichman, B. M. and Van Sickle, D. C. (1989). "***Prostaglandins in inflammatory bone pathology: mechanism and therapeutic benefit of etodolac***." Agents Actions. **26**(3-4): 310-318.

Heinecke, J. W., Li, W., Daehnke, H. L. and Goldstein, J. A. (1993). "***Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages***." J Biol Chem. **268**: 4069.

Herrera, B., Fernandez, M., Alvarez, A. M., Roncero, C., Benito, M., Gil, J. and Fabregat, I. (2001). "***Activation of caspases occurs downstream from radical oxygen species production, Bcl-xL down-regulation, and early cytochrome C release in apoptosis induced by transforming growth-factor β in rat fetal hepatocytes***." Hepatology. **34**: 548–556.

Higgs, G. A. (1989)."***Use of Inplanted sponges to Study the Acute Inflammatory Response***."In Pharmacological Methods in the Control of Inflammation. **Chang, J. Y. and Lewis, A. J.** Alan R. Liss, Inc, New York. 151-171.

Higgs, G. A., Moncada, S. and Vane, J. R. (1984). "***Eicosanoids in inflammation***." Ann Clin Res. **16**(5-6): 287-299.

Hochberg, M. C., Altman, R. D., Brandt, K. D., Clark, B. M., Dieppe, P. A., Griffin, M. R., Moskowitz, R. W. and Schnitzer, T. J. (1995). "***Guidelines for the medical management of osteoarthritis. Part I. Osteoarthritis of the hip.American College of Rheumatology***." Arthritis Rheum. **38**(11): 1535-1540.

Hoffmann, J. C., Herklotz, C., Zeidler, H., Bayer, B. and Westermann, J. (1997). "***Anti-CD2 (OX34) MoAb treatment of adjuvant arthritic rats: attenuation of established arthritis,***

selective depletion of CD4+ T cells, and CD2 down-modulation." Clin Exp Immunol. **110**(1): 63-71.

Hoffmann, J. C., Herklotz, C., Zeidler, H., Bayer, B., Rosenthal, H. and Westermann, J. (1997). "***Initiation and perpetuation of rat adjuvant arthritis is inhibited by the anti-CD2 monoclonal antibody (mAb) OX34***." Ann Rheum Dis. **56**(12): 716-722.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., Fitzgerald, K. A. and Latz, E. (2008). "***Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization***." Nat Immunol. **9**(8): 847-856.

Huang, A. J., Manning, J. E., Bandak, T. M., Ratau, M. C., Hanser, K. R. and Silverstein, S. C. (1993). "***Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells***." J Cell Biol. **120**(6): 1371-1380.

Hughes, P., DeVirgilio, J., Humber, L. G., Chau, T., Weichman, B. and Neuman, G. (1989). "***Synthesis and biological evaluation of 4,6-diethyl-1,3,4,5-tetrahydropyrano[4,3-b]indole-4-acetic acid, an isomer of etodolac***." J Med Chem. **32**(9): 2134-2137.

Hundley, T. R., Gilfillan, A. M., Tkaczyk, C., Andrade, M. V., Metcalfe, D. D. and Beaven, M. A. (2004). "***Kit and FcepsilonRI mediate unique and convergent signals for release of inflammatory mediators from human mast cells***." Blood. **104**: 2410–2417.

Hunskaar, S. and Hole, K. (1987). "***The formalin test in mice: dissociation between inflammatory and non-inflammatory pain***." Pain. **30**(1): 103-114.

Huo, Y., Schober, A., Forlow, S. B., Smith, D., R., Hyman, M. C., Jung, S., Littman, D. R., Weber, C. and Ley, K. (2003). "***Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E***." Nature Med. **9**: 61–67.

Hurley, J. V. (1972). "***Acute inflammation***." Churchill Livingstone Edinburgh, London.

Hurst, J. K., (1991). "***Myeloperoxidase: active site structure and catalytic mechanisms***." Peroxidases in Chemistry and Biology. **Everse J, E. K., Grisham MB**. Boca Raton, FL, CRC.

Ibuki, T., Matsumura, K., Yamazaki, Y., Nozaki, T., Tanaka, Y. and Kobayashi, S. (2003). J. Neurochem. **86**: 318–328.

Ichitani, Y., Shi, T. J., Haeggstrom, J. Z., Samuelsson, B. and Hokfelt, T. (1997). "***Increased levels of cyclooxygenase-2 mRNA in the rat spinal cord after peripheral inflammation: an in situ hybridization study***." NEUROREPORT. **8**(13): 2949-2952.

Ikeda, U., Maeda, Y., Yamamoto, K. and Shimada, K. (2002). "***C-Reactive protein augments inducible nitric oxide synthase expression in cytokine-stimulated cardiac myocytes***." Cardiovasc Res. **56**(1): 86-92.

Imhof, B. A. and Aurrand-Lions, M. (2004). "***Adhesion mechanisms regulating the migration of monocytes***." Nature Rev. Immunol. **4**: 432–444.

Imida, K., Fukushima, S., Shival, T., Ohtani, M., Nakanishi, K. and Ito, N. (1983). "***Promoting activities of BHA and BHT on 2-stage urinary bladder carcinogenesis and inhibition of α-glutamyl transpeptidase-positive foci development in the liver of rats***." Carcinogenesis. **4**: 885-889.

Ip, W. K., Takahashi, K., Moore, K. J., Stuart, L. M. and Ezekowitz, R. A. (2008). "***Mannose-binding lectin enhances Toll-like receptors 2 and 6 signaling from the phagosome***." J. Exp. Med. **205**: 169–181.

Iriti, M., Varoni, E. M. and Vitalini, S. (2010). "***Melatonin in traditional Mediterranean diets***." J Pineal Res. **49**(2): 101-105.

Ishikawa, T., Nishigaki, F., Miyata, S., Hirayama, Y., Minoura, K., Imanishi, J., Neya, M., Mizutani, T., Imamura, Y., Ohkubo, Y. and Mutoh, S. (2005). "***Prevention of progressive joint destruction in adjuvant induced arthritis in rats by a novel matrix metalloproteinase inhibitor, FR217840***." Eur J Pharmacol. **508**(1-3): 239-247.

Issekutz, C. A. and Issekutz, T. B. (1989)."***Quantitation of Blood Cell Accumulation and Vascular Responses in Inflammatory Reactions***."In Pharmacological Methods in the Control of Inflammation. **Chang, J. Y. and Lewis, A. J.** Alan R. Liss, Inc., New York. 129-150.

Issuree, P. D. A., Maretzky, T., McIlwain, D. R., Monette, S., xE, bastien, Qing, X., Lang, P. A., Swendeman, S. L., Park-Min, K.-H., Binder, N., Kalliolias, G. D., Yarilina, A., Horiuchi, K., Ivashkiv, L. B., Mak, T. W., Salmon, J. E. and Blobel, C. P. (2013). "***iRHOM2 is a critical pathogenic mediator of inflammatory arthritis***." The Journal of Clinical Investigation. **123**(2): 928-932.

Ito, N., Fukushima, S., Hagiwara, A., Shibata, M. and Ogiso, T. (1983). "***Carcinogenicity of butylated hydroxyanisole in F344 rats***." J Natl Cancer Inst. **70**(2): 343-352.

Iwalewa, E. O., McGaw, L. J., Naidoo, V. and Eloff, J. N. (2007). "***Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions***." Afr J Biotechnol. **6**: 2868–2885.

Iyer, G. Y. N., Islam, M. F. and Quastel, J. H. (1961). "***Biochemical aspects of phagocytosis*** " Nature **192**: 535

Janiszewski, J., Bienenstock, J. and Blennerhassett, M. G. (1994). "***Picomolar doses of substance P trigger electrical responses in mast cells without degranulation***." Am. J. Physiol. **267**: C138-C145.

Janský, L., Vybiral, S., Pospíšilová, D., Roth, J., Dornand, J., Zeisberger, E. and Kaminkova, J. (2008). "***Production of systemic and hypothalamic cytokines during the early phase of endotoxin fever***." Neuroendocrinology. **62**(1): 55-61.

Jeon, H. J., Kang, H. J., Jung, H. J., Kang, Y. S., Lim, C. J., Kim, Y. M. and Park, E. H. (2008). "***Anti-inflammatory activity of Taraxacum officinale***." J Ethnopharmacol. **115**(1): 82-88.

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., Prestwich, G. D., Mascarenhas, M. M., Garg, H. G., Quinn, D. A., Homer, R. J., Goldstein, D. R., Bucala, R., Lee, P. J., Medzhitov, R. and Noble, P. W. (2005). "***Regulation of lung injury and repair by Toll-like receptors and hyaluronan***." Nat Med. **11**(11): 1173-1179.

Johnson, K., Kotiesh, A., Boitnott, J. K. and Torbenson, M. (2007). "***Histology of symptomatic acute hepatitis C infection in immunocompetent adults***." Am J Surg Pathol. **31**(11): 1754-1758.

Kaneko, K., Kanda, T. and Yamauchi, Y. (1999). "***C-reactive protein in dilated cardiomyopathy***." Cardiology. **91**: 215–219.

Kang, J. S., Yoon, W. K., Youm, J. K., Jeong, S. K., Park, B. D., Han, M. H., Lee, H., Moon, E. Y., Han, S. B., Lee, C. W., Lee, K., Park, S. K., Yang, K. H. and Kim, H. M. (2008). "***Inhibition of atopic dermatitis-like skin lesions by topical application of a novel ceramide derivative, K6PC-9p, in NC/Nga mice***." Exp Dermatol. **17**(11): 958-964.

Kannan, K., Ortmann, R. A. and Kimpel, D. (2005). "***Animal models of rheumatoid arthritis and their relevance to human disease***." Pathophysiology. **12**(3): 167-181.

Kao, P. C., Shiesh, S. C. and Wu, T. J. (2006). "***Serum C-reactive protein as a marker for wellness assessment***." Ann Clin Lab Sci. **36**: 163–169.

Katori, M., Ikeda, K., Harada, Y., Uchida, Y., Tanaka, K. and Oh-Ishi, S. (1978). "***A possible role of prostaglandins and bradykinin as a trigger of exudation in carrageenin-induced rat pleurisy***." Agents Actions. **8**(1-2): 108-112.

Kerfoot, S. M. and Kubes, P. (2002). "***Overlapping roles of P-selectin and α4 integrin to recruit leukocytes to the central nervous system in experimental autoimmune encephalomyelitis***." J. Immunol. **169**: 1000–1006.

Kettle, A. J. and Winterbourn, C. C. (1997). "***Myeloperoxidase: A key regulator of neutrophil oxidant production*** " Redox Rep **3**: 3

Khan, F. A. and Khan, M. F. (2010). "***Inflammation and Acute phase response***." International Journal of Applied Biology and Pharmaceutical Technology. **I**(2): 312-321.

Khanna, N. and Sharma, S. B. (2001). "***Anti-inflammatory and analgesic effect of herbal preparation: septilin***." Indian J Med Sci. **55**(4): 195-202.

Kikuchi, T., Ishida, S., Kinoshita, T., Sakuma, S., Sugawara, N., Yamashita, T. and Koike, K. (2002). "***IL-6 enhances IgE-dependent histamine release from human peripheral blood-derived cultured mast cells***." Cytokine. **20**(5): 200-209.

Killedar, S. G. and More, H. N. (2010). J Pharm Res. **3**(3): 554-556.

Kim, S. H., Choi, C. H., Kim, S. Y., Eun, J. S. and Shin, T. Y. (2005). "***Anti-allergic effects of Artemisra twayomogi on mast cell mediated allergy model***." Exp Biol Med (Maywood). **230**: 82 - 88.

Kim, S. H., Choi, C. H., Kim, S. Y., Eun, J. S. and Shin, T. Y. (2005). "***Anti-allergic effects of Artemisia iwayomogi on mast cell-mediated allergy model***." Exp Biol Med (Maywood). **230**(1): 82-88.

Kinashi, T. (2005). "***Intracellular signalling controlling integrin activation in lymphocytes***." Nature Rev. Immunol. **5**: 546–559.

Kindt, Goldsby and Osborne. (2004). "***Kuby Immunology***." **6th edition,** W.H Freemann and Company New York.

Kirwan, J. R. (1995). "***The effect of glucocorticoids on joint destruction in rheumatoid arthritis. The Arthritis and Rheumatism Council Low-Dose Glucocorticoid Study Group***." N Engl J Med. **333**(3): 142-146.

Kirwan, J. R., Minnock, P., Adebajo, A., Bresnihan, B., Choy, E., de Wit, M., Hazes, M., Richards, P., Saag, K., Suarez-Almazor, M., Wells, G. and Hewlett, S. (2007). "***Patient perspective: fatigue as a recommended patient centered outcome measure in rheumatoid arthritis***." J Rheumatol. **34**(5): 1174-1177.

Kitamura, Y. and Ito, A. (2005). "***Mast cell-committed progenitors***." Proc. Natl Acad. Sci. USA. **102**: 11129–11130.

Kitamura, Y., Go, S. and Hatanaka, K. (1978). "***Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation***." Blood. **52**: 447–452.

Klareskog, L. (1989). "***What can we learn about rheumatoid arthritis from animal models?***" Springers Semin Immunopathol. **11**: 315–377.

Klebanoff, S. J. (1968). "***Myeloperoxidase-halide-hydrogen peroxide antibacterial system*** " J Bacteriol **95**: 2131

Klebanoff, S. J., (1992). "***Phagocytic cells: Products of oxygen metabolism***." Inflammation: Basic Principles and Clinical Correlates. **Gallin, J. I., Goldstein, I. M. and Snyderman, R.**, New York, NY, Raven.

Klebanoff, S. J. (2005). "***Myeloperoxidase: friend and foe***." Journal of Leukocyte Biology. **77**(5): 598–625.

Kluger, M. J. (1991). "***Fever role of pyrogens and cryogens*** " Physiological Reviews **71**: 93-127.

Kneilling, M. and Rocken, M. (2009). "***Mast cells: novel clinical perspectives from recent insights***." Exp. Dermatol. **18**: 488–496.

Kokate, C. K. (2005). "***A Textbook for Practical Pharmacognosy***." **5th Ed,** 107-111.

Kostopanagiotou, G., Kalimeris, K., Mourouzis, I., Costopanagiotou, C., Arkadopoulos, N., Panagopoulos, D., Papoutsidakis, N., Chranioti, A., Pafiti, A., Spanou, D., Smyrniotis, V. and Pantos, C. (2009). "***Thyroid hormones alterations during acute liver failure: possible underlying mechanisms and consequences***." Endocrine. **36**(2): 198-204.

Kraft, S. and Kinet, J. P. (2007). "***New developments in FcepsilonRI regulation, function and inhibition***." Nat. Rev. Immunol. **7**: 365–378.

Kröncke, K. D. (2003). "***Nitrosative stress and transcription***." Biol Chem. **384**: 1365–1377.

Kumar, D. A., Manikandan, P., Sumitra, M., Raju, K. V., Gayathri, C., Arutselvan, N. and Puvanakrishnan, R. (2002). "***A novel peptide derivative exhibits anti inflammatory and antioxidant activity in adjuvant induced arthritis in rats***." Mol Cell Biochem. **229**(1-2): 9-17.

Kusano, M., Tabuchi, M., Fukushima, A., Funayama, K., Diaz, C., Kobayashi, M., Hayashi, N., Tsuchiya, Y. N., Takahashi, H., Kamata, A., Yamaya, T. and Saito, K. (2011). "***Metabolomics data reveal a crucial role of cytosolic glutamine synthetase 1;1 in coordinating metabolic balance in rice***." Plant J. **66**(3): 456-466.

Kushner, I. (1993). "***Regulation of the acute phase response by cytokines***." Perspect Biol Med. **36**(4): 611-622.

Langford, M. P., Orillac, R., Chen, D. and Texada, D. (2003). "***Systemic and ocular antibody responses to inactivated acute hemorrhagic conjunctivitis (AHC) virus; enterovirus 70 (EV70)***." Ocul Immunol Inflamm. **11**(3): 197-209.

Laudanna, C., Kim, J. Y., Constantin, G. and Butcher, E. C. (2002). "***Rapid leukocyte integrin activation by chemokines***." Immunol. Rev. **186**: 37–46.

Lavery, J. P. and Lisse, J. R. (1994). "***Preliminary study of the tryptase levels in the synovial fluid of patients with inflammatory arthritis***." Ann Allergy. **72**: 425-427.

Le Bars, D., Gozariu, M. and Cadden, S. W. (2001). "***Animal models of nociception***." Pharmacol Rev. **53**(4): 597-652.

Lee, D. M., Friend, D. S., Gurish, M. F., Benoist, C., Mathis, D. and Brenner, M. B. (2002). "***Mast Cells: A Cellular Link Between Autoantibodies and Inflammatory Arthritis***." Science. **297**(5587): 1689-1692.

Lee, E. O., Kim, J. I., Davis, A. H. and Kim, I. (2006). "***Effects of regular exercise on pain, fatigue, and disability in patients with rheumatoid arthritis***." Fam Community Health. **29**(4): 320-327.

LeMay, L. G., Vander, A. J. and Kluger, M. J. (1990). "***Role of interleukin-6 in fever in the rat***." American Journal of Physiology **258**: R798-R803.

Lemmens, R. H. M. J. (2008). "***Trichilia monadelpha (Thonn.) J.J.de Wilde***." In: Louppe, D., Oteng-Amoako, A.A. & Brink, M. (Editors). **Prota 7(1): Timbers/Bois d'œuvre 1. [CD-Rom].**

Leng, S., McElhaney, J., Walston, J., Xie, D., Fedarko, N. and Kuchel, G. (2008). "***Elisa and Multiplex Technologies for Cytokine Measurement in Inflammation and Aging Research***." J Gerontol a Biol Sci Med Sci. **63**(8): 879–884.

Lequin, R. (2005). "***Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA)***." Clin. Chem. **51**(12): 2415–2418.

Levant, A., Levy, E., Argaman, M. and Fleisher-Berkovich, S. (2006). "***Kinins and neuroinflammation: dual effect on prostaglandin synthesis***." Eur J Pharmacol. **546**(1-3): 197-200.

Ley, K., Laudanna, C., Cybulsky, I. M. and Nourshargh, S. (2007). "***Getting to the site of inflammation: the leukocyte adhesion cascade updated***." Nature Reviews - Immunology. **7**: 678-689.

Libby, P., Geng, Y. J., Sukhova, G. K., Simon, D. I. and Lee, R. T. (1997). "***Molecular determinants of atherosclerotic plaque vulnerability***." Ann NY Acad Sci. **811**: 134–145.

Lima, L. M., Perazzo, F. F., Tavares Carvalho, J. C. and Bastos, J. K. (2007). "***Anti-inflammatory and analgesic activities of the ethanolic extracts from Zanthoxylum riedelianum (Rutaceae) leaves and stem bark***." J Pharm Pharmacol. **59**(8): 1151-1158.

Linhart, O., Obreja, O. and Kress, M. (2003). "***The inflammatory mediators serotonin, prostaglandin E2 and bradykinin evoke calcium influx in rat sensory neurons***." Neuroscience. **118**(1): 69-74.

Liu, H. C., Yang, Y. Y., Chou, Y. M., Chen, K. P., Shen, W. W. and Leu, S. J. (2004). "***Immunologic variables in acute mania of bipolar disorder***." J Neuroimmunol. **150**(1-2): 116-122.

Liu, K. D., Glidden, D. V., Eisner, M. D., Parsons, P. E., Ware, L. B., Wheeler, A., Korpak, A., Thompson, B. T., Chertow, G. M. and Matthay, M. A. (2007). "***Predictive and pathogenetic value of plasma biomarkers for acute kidney injury in patients with acute lung injury***." Crit Care Med. **35**(12): 2755-2761.

Low, J. M. and Moore, T. L. (2005). "***A role for the complement system in rheumatoid arthritis***." Curr Pharm Des. **11**(5): 655-670.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). "***Protein measurement with the Folin phenol reagent***." J Biol Chem. **193**(1): 265-275.

Loyer, P., Ilyin, G. and Abdel Razzak, Z. (1993). "***Interleukin 4 inhibits the production of some acute-phase proteins by human hepatocytes in primary culture***." FEBS Lett. **336**: 215-220.

Lucas, R., Giannini, C., D'Auria M, V. and Paya, M. (2003). "***Modulatory effect of bolinaquinone, a marine sesquiterpenoid, on acute and chronic inflammatory processes***." J Pharmacol Exp Ther. **304**(3): 1172-1180.

Luna, L. (1968). "***Manual Of Histologic Staining Methods from the AFIP***." **3rd Ed,** McGraw-Hill NY. 162-163.

Lundberg, A. M. and Hansson, G. K. (2010). "***Innate immune signals in atherosclerosis***." Clin Immunol. **134**: 5-24.

Lyoussi, B., Hilaly, J. E. and Israili, Z. H. (2004). "***Acute and chronic toxicological studies of Ajuva iva in experimental animals***." Journal of Ethnopharmacology. **91**(2004): 43-50.

Mackiewicz, A., Speroff, T., Ganapathi, M. K. and Kushner, I. (1991). "***Effects of cytokine combinations on acute phase protein production in two human hepatoma cell lines***." J Immunol. **146**: 3032-3037.

Mackiewicz, A., Shooltink, H., Heinrich, P. C. and Rose-John, S. (1992). "***Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins***." J Immunol. **149**: 2021-2027.

Maeda, H. and Akaike, T. (1998). "***Nitric oxide and oxygen radicals in infection, inflammation, and cancer***." Biochemistry (Mosc). **63**(7): 854-865.

Majno, G. and Joris, I. (2004). "***Cells, Tissues and Disease***." **2nd,** (Oxford Univ. Press,) New York.

Makinen, H., Kautiainen, H., Hannonen, P., Mottonen, T., Leirisalo-Repo, M., Laasonen, L., Korpela, M., Blafield, H., Hakola, M. and Sokka, T. (2007). "***Sustained remission and reduced radiographic progression with combination disease modifying antirheumatic drugs in early rheumatoid arthritis***." J Rheumatol. **34**(2): 316-321.

Male, D., Brostoff, J. and Roitt, B. D. (2006). "***Immunology***." **7th edition,** Mosby Elsevier Canada.

Malmberg, A. B. and Yaksh, T. L. (1992). "***Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat***." J Pharmacol Exp Ther. **263**(1): 136-146.

Malone, D. G., Irani, A. M., Schwartz, L. B., Barrett, K. E. and Metcalfe, D. D. (1986). "***Mast cell numbers and histamine levels in synovial fluids from patients with diverse arthritides***." Arthritis Rheum. **29**: 956-963.

Malvar, D. C., Soares, D. M., Fabrício, A. S. C., Alexandre, K., Machado, R. R., Figueiredo, M. J., Rae, G. A. and Souza, G. E. P. (2011). "***The antipyretic effect of dipyrone is unrelated to inhibition of PGE_2 synthesis in the hypothalamus***." British Journal of Pharmacology. **162**(6): 1401-1409.

Mamta, S. and Jyoti, S. (2012). "***Phytochemical Screening of Acorus calamus and Lantana camara***." Int Res J Pharm. **3**(5): 324-326.

Maria, M., Engeniusz, M., Miroslaw, K., Maria, K. and Iwona, P. (1983). "***Adjuvant induced disease in rats, clinical findings and morphological and biochemical changes in the blood histological changes in internal organs***." Rheumatology. **2**: 231–245.

Marnett, L. J., Riggins, J. N. and West, J. D. (2003). "***Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein***." J Clin Invest. **111**: 583–593.

Marquez, L. A. and Dunford, H. B. K. (1996). "***Kinetics of oxidation of tyrosine and dityrosine by myeloperoxidase compounds I and II***." J Biol Chem. **270**: 30434.

Maseri, A. (1997). "***Inflammation, atherosclerosis, and ischemic events: exploring the hidden side of the moon***." New Engl J Med. **336**: 1014–1016.

Matsuda, H., Kawakita, K., Kiso, Y., Nakano, T. and Kitamura, Y. (1989). "***Substance P induces granulocyte infiltration through degranulation of mast cells***." J Immunol. **142**(3): 927-931.

McCord, J. M., Keele, B. B. and Fridovich, I. (1971). "***An enzyme based theory of obligate anaerobiosis: the physiological function of superoxide dismutase*** " Proc. Natl. Acad. Sci. USA. **68**: 1024–1027.

Mckellar, G., Madhok, R. and Singh, G. (2007). "***The problem with NSAIDs: what data to believe?***" Current Pain Headache Reports. **11**: 423–427.

Medzhitov, R. (2008). "***Origin and physiological roles of inflammation***." Nature. **454(24)**: 428-435.

Medzhitov, R. and Janeway, C. A., Jr. (1997). "***Innate immunity: the virtues of a nonclonal system of recognition***." Cell. **91**(3): 295-298.

Meir, S., Kanner, J., Akiri, B. and Hadas, S. P. (1995). "***Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves***." Journal of Agricultural and Food Chemistry. **43**: 1813.

Melgar, S., Bjursell, M., Gerdin, A. K., Svensson, L., Michaelsson, E. and Bohlooly, Y. M. (2007). "***Mice with experimental colitis show an altered metabolism with decreased metabolic rate***." Am J Physiol Gastrointest Liver Physiol. **292**(1): G165-172.

Metcalfe, D. D., Kaliner, M. and Donlon, M. A. (1981). "***The mast cell***." Crit Rev Immunol. **3**(1): 23-74.

Metcalfe, D. D., Baram, D. and Mekori, Y. A. (1997). "***Mast cells***." Physiol Rev. **77**: 1033-1079.

Milanino, R., Moretti, U., Concari, E., Marrella, M. and Velo, G. P. (1988). "***Copper and zinc status in adjuvant-arthritic rat: studies on blood, liver, kidneys, spleen and inflamed paws***." Agents Actions. **24**(3-4): 365-376.

Milton, A. S. (1982)."***Prostaglandins in fever and the mode of action of antipyretic drugs.***"In Pyretics and antipyretics **Milton, A. S.** Berlin: Springer**:** 259–267.

Mino, J., Moscatelli, V., Hnatyszyn, O., Gorzalczany, S., Acevedo, C. and Ferraro, G. (2004). "***Antinociceptive and antiinflammatory activities of Artemisia copa extracts***." Pharmacol Res. **50**(1): 59-63.

Mitchell, R. N. and Cotran, R. S. (2003)."***Acute and Chronic Inflammation.***"In Basic Pathology **Kumar, V., Cotran, R. S. and Robbins, S.** W.B. Saunders., Philadelphia.

.

Moldawer, L. L. and Copeland, E. M. (1997). "***Proinflammatory cytokines, nutritional support, and the cachexia syndrome: interactions and therapeutic options***." Cancer. **79**: 1828-1839.

Molla, M. and Panes, J. (2007). "***Radiation-induced intestinal inflammation***." World Journal of Gastroenterology. **13**: 3031–3032.

Monach, P. A., Benoist, C. and Mathis, D. (2004). "***The role of antibodies in mouse models of rheumatoid arthritis, and relevance to human disease***." Adv Immunol. **82**: 217-248.

Moon, T. C., St Laurent, C. D. and Morris, K. E. (2010). "***Advances in mast cell biology: new understanding of heterogeneity and function***." Mucosal Immunol. **3**: 111–128.

Mooradan, A. D., Morley, J. E. and Koreman, S. G. (1987). "***Biological actions of androgens***." Endo Rev. **8**: 1-28.

Morakinyo, A. O., Oludare, G. O., Ojulari, S. and Afolabi, A. O. (2009). "***Effect of short term administration of Artemether-Lumefantrine on testicular functions and antioxidant defence in the rat***." Res. J. Med. Med. Sci. **4**(2): 165-170.

Moreland, L. (2005). "***Unmet needs in rheumatoid arthritis***." Arthritis Res Ther. **7 Suppl 3**: S2-8.

Moshage, H. (1997). "***Cytokines and the hepatic acute phase response***." Journal of Pathology. **181**: 257-266.

Motulsky, H. J. and Christopoulos, A. (2003). "***Fitting model to biological data using linear and nonlinear regression. A practical guide to curve fitting***." GraphPad Software Inc. San Diego, CA.

Motulsky, H. J. and Christopoulos, A. (2003)."***How does a treatment change the curve?***"In Fitting models to biological data using linear and non-linear regression. A practical guide to curve fitting. **Motulsky, H. J. and Christopoulos, A.**, San Diego, CA. **GraphPad Software Inc:** 160 - 187.

Mowat, G. (1971). "***Haematological abnormalities in rheumatoid arthritis***." Semin Arthritis Rheum. **1**: 195–199.

Mshana, N. R., Abbiw, K., Addae-Mensah, I., Adjanohoun, E., Ahyi, M.R.A., Ekpere, J.A., Enow-Orock, E.G.,Gbile, Z.O., Naomesi, G.K., Odei, M.A., Odunlami, H., Oten-Yeboah, A.A., Sarpong, K., Sofowora, A. and Tackie, A.N. (2000). "***Traditional medicine and Pharmacopoeia. Contribution to the Revision of Ethnobotanical and Floristic studies***

in Ghana." Accra: Organization of African Unity/ Scientific, Technical and research commission.

Muller, W. A. (2003). "***Leukocyte–endothelial-cell interactions in leukocyte transmigration and the inflammatory response***." Trends Immunol. **24**: 327–334.

Mythilypriya, R., Shanthi, P. and Sachdanandam, P. (2008). "***Synergistic Effect of Kalpaamruthaa on Antiarthritic and Antiinflammatory Properties—Its Mechanism of Action***." Inflammation. **31**: 391-398.

Nairn, R. and Helbert, M. (2002). "***Antigens and antibody structure***." Immunology for medical students. St. Louis: Mosby: 23-37.

Nakamura, Y., Torikai, K., Ohto, Y., Murakami, A., Tanaka, T. and Ohigashi, H. (2000). "***A simple phenolic antioxidant protocatechuic acid enhances tumor promotion and oxidative stress in female ICR mouse skin: dose- and timing-dependent enhancement and involvement of bioactivation by tyrosinase***." Oxford Journals Life Sciences & & MedicineCarcinogenesis. **21**(10): 1899-1907.

Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitamura, Y. and Galli, S. J. (1985). "***Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/Wv mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells***." J Exp Med. **162**: 1025–1043.

Nantel, F., Denis, D., Gordon, R., Northey, A., Cirino, M., Metters, K. M. and Chan, C. C. (1999). "***Distribution and Regulation of Cyclooxygenase-2 in carrageenan-induced inflammation***." Br. J. Pharmacol. **128**: 853—859.

Nathan, C. (1992). "***Nitric oxide as a secretory product of mammalian cells***." FASEB J. **6**(12): 3051-3064.

Nathan, C. (2003). "***Specificity of a third kind: reactive oxygen and nitrogen intermediates in cell signalling***." J Clin Invest. **111**: 769–778.

Nauseef, W. M. (2001). "***Contributions of myeloperoxidase to proinflammatory events:more than an antimicrobial system***." International Journal of Hematology. **74**(2): 125–133.

Nauta, A., Knippels, L., Garssen, J. and Redegeld, F. (2007). "***Animal models of anaphylaxis***." Curr Opin Allergy Clin Immunol. **7**: 355–359.

Nautiyal, K. M., Ribeiro, A. C., Pfaff, D. W. and Silver, R. (2008). "***Brain mast cells link the immune system to anxiety-like behavior***." Proc. Natl Acad. Sci. USA. **105**: 18053–18057.

Nigrovic, P. A. and Lee, D. M. (2005). "***Mast cells in inflammatory arthritis***." Arthritis Research and Therapy. **7**(1): 1–11.

O'Byrne, K. J. and Dalgleish, A. G. (2001). "***Chronic immune activation and inflammation as the cause of malignancy***." Br. J. Cancer. **85**: 473-483.

Odugbemi, T. and Odunayo, A. (2008). "***Medicinal Plants According to Family Names***." Outlines and Pictures of Medicinal Plants from Nigeria. **T. Odugbemi** (Lagos, University of Lagos Press): 117-146.

Odum, L., Petersen, L. J., Skov, P. S. and Ebskov, L. B. (1998). "***Pituitary adenylate cyclase activating polypeptide (PACAP) is localized in human dermal neurons and causes histamine release from skin mast cells***." Inflamm Res. **47**(12): 488-492.

Ogawa, Y. and Grant, J. A. (2007). "***Mediators of anaphylaxis***." Immunol Allergy Clin North Am. **27**: 249–260.

Oh-ishi, S. (1997). "***Analysis of chemical mediators involved in acute inflammatory reaction with the rat pleurisy model***." Nippon Yakurigaku Zasshi. **110**: 59-68.

Ohmori, H., Egusa, H., Ueura, H., Matsumoto, Y., Kanayama, N. and Hikida, M. (2000). "***Selective augmenting effects of nitric oxide on antigen-specific IgE response in mice***." Immunopharmacology. **46**: 55–63.

Okwu, D. E. (2001). Global J Pure Appl Sci. **B-8**: 455-459.

Okwu, D. E. (2001). "***Improving the nutrition value of cassava tapioca meal and local species***." Nutraceutical, Functional and Medicinal Food. **A-3**: 43-51.

Ormrod, D. J., Harrison, G. L. and Miller, T. E. (1987). "***Inhibition of neutrophil myeloperoxidase activity by selected tissues***." J. Pharmacol. Methods. **18**: 137-142.

Osawa, T., (1994). "***Novel natural antioxidants for utilization in food and biological system.***" Post harvest biochemistry of plant food-materials in the tropics. **Uritani, I., Garcia, V. V. and Mendoza, E. M.** Japan Scientific Societies Press, Japan.

Oyaizu, M. (1986). "***Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine***." Japanese Journal of Nutrition. **44**: 307–315.

Oyelowo, O. T., Bolarinwa, O. L. and Morenikeji, O. A. (2011). "***Assessment of sperm indices and testosterone level on the effect of Trichilia monadelpha extract in male albino rats***." African Journal of Pharmacy and Pharmacology **5**(16): 1956-1958.

Ozgová, Š., Heřmánek, J. and Gut, I. (2003). "***Different antioxidant effects of polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems***." Biochemical Pharmacology. **66**(7): 1127-1137.

Palmer, H. S., Kelso, E. B. and Lockhart, J. C. (2007). "***Protease-activated receptor 2 mediates the pro inflammatory effect of synovial mast cells***." Arthritis Rheum. **56**: 3532–3540.

Panthong, A., Witthawaskul, P., Kanjanapothi, D., Taesothikul, T. and Lertprasertsuke, N. (2003). "***Acute and subacute toxicities of the saponin mixture isolated from Schefflera leucantha Viguier***." Journal of Ethnopharmacology. **89**: 115-121.

Papas, A. M. (1999). "***Diet and antioxidant status*** " Food Chem Toxicol **37** 999-1007.

Partsch, G., Schwagerl, W. and Eberl, R. (1982). "***Histamine in rheumatic diseases***." Z Rheumatol. **41**: 19-22.

Pasceri, V. and Yeh, E. T. (1999). "***A tale of two diseases: atherosclerosis and rheumatoid arthritis***." Circulation. **100**: 2124–2126.

Pasceri, V., Willerson, J. T. and Yeh, E. T. (2000). "***Direct proinflammatory effect of C-reactive protein on human endothelial cells***." Circulation. **102**: 2165–2168.

Pasceri, V., Chang, J., Willerson, J. T. and Yeh, E. T. (2001). "***Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs***." Circulation. **103**: 2531–2534.

Paul, C., Sans, B. and Suarez, F. (2010). "***Masitinib for the treatment of systemic and cutaneous mastocytosis with handicap: a phase 2a study***." Am J Hematol. **85**: 921–925.

Paul, W. E. (2008). "***Fundamental Immunology***." **6th edition,** Lippincott Williams and Wilkins Philadelphia.

Pearson, C. M. (1956). "***Development of arthritis, periarthritis and periostitis in rats given adjuvants.***" Proc Soc Exp Biol. **91**(1): 95-101.

Pearson, C. M. (1963). "***Experimental Joint Disease Observations on Adjuvant-Induced Arthritis***." J Chronic Dis. **16**: 863-874.

Pearson, C. M. and Wood, F. D. (1963). "***Studies of arthritis and other lesions induced in rats by the injection of mycobacterial adjuvant. VII. Pathologic details of the arthritis and spondylitis***." Am J Pathol. **42**: 73-95.

Peavy, R. D. and Metcalfe, D. D. (2008). "***Understanding the mechanisms of anaphylaxis***." Curr Opin Allergy Clin Immunol. **8**(4): 310-315.

Pekin, T. J. J. and Zvaifler, N. J. (1965). "***Hemolytic complement in synovial fluid***." J Clin Invest. **43**: 1372-1382.

Pepys, M. B. and Hirschfield, G. M. (2003). "***C-reactive protein: a critical update***." J Clin Invest. **111**: 1805–1812.

Peter, A. W. (2010)."***Acute and Chronic Inflammation***."In Fundamentals of Inflammation. **Charles, N. S., Peter, A. W. and Derek, W. G.** CAMBRIDGE UNIVERSITY PRESS, Cambridge. www.cambridge.org www.cambridge.org/9780521887298**:** 1 - 457.

Phillipson, M., Heit, B., Colarusso, P., Liu, L., Ballantyne, C. M. and Kubes, P. (2006). "***Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade***." J Exp Med. **203**(12): 2569-2575.

Porth, C. and Caspard, K. J. (2003). "***Essentials of Pathophysiology***." **Bk & CD-ROM** Lippincott Williams & Wilkins 150 - 167.

Pritsos, C. A. (2000). "***Cellular distribution, metabolism and regulation of the xanthine oxidoreductase enzyme system***." Chemico-Biological Interactions. **129**(1-2): 195–208.

Pulsatelli, L., Dolzani, P., Silvestri, T., De Giorgio, R., Salvarani, C., Macchioni, P., Frizziero, L. and Meliconi, R. (2006). "***Synovial expression of vasoactive intestinal peptide in polymyalgia rheumatica***." Clin Exp Rheumatol. **24**(5): 562-566.

Puratchikody, A., Yasodha, A., Kumar, S. A. and Hari, V. B. N. (2011). "***Preliminary Phytochemical and Anti-arthritic Activity of an Ayuverdic Formulation Yogaraja Gulgulu***." J Phytol. **3**: 31-36.

Puri, K. D., Dogget, T. A., Haung, C. Y., Douangpanya, J., Hayflick, J. S., Turner, M., Penninger, J. and Diacovo, T. G. (2005). "***The role of endothelial PI3Kγ activity in neutrophil trafficking***." Blood. **106**(1): 150–157.

Quinton, L. J., Jones, M. R., Robson, B. E. and Mizgerd, J. P. (2009). "***Mechaninsm of hepatic acute phase response during bacterial Pneumonia***." Infec Imm. **77**: 2417-2426.

Rada, B. and Leto, T. (2008). "***Oxidative innate immune defenses by Nox/Duox Family NADPH oxidases***." Contributions to Microbiology. **15**: 164–187.

Raji, Y. and Bolarinwa, A. F. (1997). "***Antifertility activity of Quassia amara in male rats- in vivo study***." Life Sci. **64**: 1067-1074.

Ramesh, B. A. and Vinod, D. R. (2003). "***Phytochemical investigation and evaluation of anti-inflammatory and anti-arthritic activities of essential oil of Strobilanthus ixiocephala Benth***." Indian J Exp Biol. **41**: 890–894.

Ramprasath, V. R., Shanthi, P. and Sachdanandam, P. (2006). "***Curative effect of Semecarpus anacardium Linn. nut milk extract against adjuvant arthritis -- with special reference to bone metabolism***." Chem Biol Interact. **160**(3): 183-192.

Rang, H. P., Dale, M. M., Ritter, J. M. and Flower, R. J. (2007). "***Rang and Dale Pharmacology***." **6th Edition,** Elsevier's Health Sciences 131 - 265.

Ratnam, S. and Mookerjea, S. (1998). "***The regulation of superoxide generation and nitric oxide synthesis by C-reactive protein***." Immunology. **94**: 560–568.

Redegeld, F. A. and Nijkamp, F. P. (2005). "***Immunoglobulin free light chains and mast cells: pivotal role in T-cell-mediated immune reactions?***" Trends Immunol. **24**: 181–185.

Redegeld, F. A., van der Heijden, M. W., Kool, M., Heijdra, B. M., Garssen, J., Kraneveld, A. D., Van Loveren, H., Roholl, P., Saito, T., Verbeek, J. S., Claassens, J., Koster, A. S. and Nijkamp, F. P. (2002). "***Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses***." Nat Med. **8**(7): 694-701.

Reiter, C. D. and Gladwin, M. T. (2003). "***An emerging role for nitric oxide in sickle cell disease vascular homeostasis and therapy***." Curr Opin Hematol. **10**(2): 99-107.

Rich, R. R. (2008). "***Clinical Immunology***." **3rd edition,** Mosby Elsevier China.

Richards, C. D., Langdon, C., Pennica, D. and Gauldie, J. (1996). "***Murine cardiotrophin-1 stimulates the acute-phase response in rat hepatocytes and H35 hepatoma cells***." J Interferon Cytokine Res. **16**: 69-75.

Ridker, P. M., Rifai, N. and Clearfield, M. (2001). "***Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events***." New Engl J Med. **344**: 1959–1965.

Rios, J.-L. (2008)."***Mechanism of action of Anti-inflammatory phytochemicals***."In Botanical Medicine In Clinical Practice. **Watson, R. R. and Preedy, V. R.** CAD International, Washington D. C. 524-534.

Rizki, T. M. and Rizki, R. M. (1992). "***Lamellocyte differentiation in Drosophila larvae parasitized by Leptopilina***." Dev Comp Immunol. **16**(2-3): 103-110.

Rock, K. L. and Kono, H. (2008). "***The inflammatory response to cell death***." Annu Rev Pathol. **3**: 99-126.

Rödel, F., Keilholz, L., Herrmann, M., Sauer, R. and Hildebrandt, G. (2007). "***Radiobiological mechanisms in inflammatory diseases of low-dose radiation therapy***." International Journal of Radiation Biology. **83**: 357–366.

Rojkind, M., Dom´ınguez-Rosales, J. A., Nieto, N. and Greenwel, P. (2002). "***Role of hydrogen peroxide and oxidative stress in healing responses***." Cellular and Molecular Life Sciences. **59**(11): 1872–1891.

Romanovsky, A. A., Almeida, M. C., Aronoff, D. M., Ivanov, A. I., Konsman, J. P., Steiner, A. A. and Turek, V. F. (2005). "***Fever and Hypothermia in systemic inflammation: recent discoveries and revisions***." Frontiers Biosci. **10**: 2193—2216.

Romas, E. and Gillespie, M. T. (2006). "***Inflammation-induced bone loss: can it be prevented?***" Rheum. Dis. Clin. North Am. **32**: 759-773.

Rosas, M. (2008). "***The induction of inflammation by dectin-1 in vivo is dependent on myeloid cell programming and the progression of phagocytosis***." J. Immunol. **181**: 3549–3557.

Roth, J. and de Souza, G. E. P. (2001). "***Fever induction pathways: evidence from responses to systemic or local cytokine formation***." Brazilian Journal of Medicinal and Biological Research **34** (3): 301-314

Roth, J., Conn, C. A., Kluger, M. J. and Zeisberger, E. (1993). "***Kinetics of systemic and intrahypothalamic IL-6 and tumor necrosis factor during endotoxin fever in the guinea pig*** " American Journal of Physiology **265** R653-R658.

Roy, M. K., Kobori, M., Takenaka, M., Nakahara, K., Shinmoto, H. and Tsushida, T. (2006). "***Inhibition of colon cancer (HT-29) cell proliferation by a triterpenoid isolated from Azadirachta indica is accompanied by cell cycle arrest and up-regulation of p21***." Planta Med. **72**(10): 917-923.

Royer, B., Varadaradjalou, S., Saas, P., Guillosson, J. J., Kantelip, J. P. and Arock, M. (2001). "***Inhibition of IgE-induced activation of human mast cells by IL-10***." Clin Exp Allergy. **31**: 694 - 704.

Royer, B., Varadaradjalou, S., Saas, P., Gabiot, A. C., Kanelip, B., Feger, F., Guillosson, J. J., Kantelip, J. P. and Arock, M. (2001). "***Autocrine regulation of cord blood-derived human mast cell activation by IL-10***." J Allergy Clin Immunol. **108**: 80 - 86.

Ruddy, S., Britton, M. C., Schur, P. H. and Austen, K. F. (1969). "***Complement components in synovial fluid: activation and fixation in seropositive rheumatoid arthritis***." Ann NY Acad Sci. **168**: 161-172.

Ruslan, M. (2008). "***Origin and physiological roles of inflammation***." NATURE. **Vol 454**(24): 428 - 435.

Salah, W., Miller, N., Paguaga, G., Tybury, G., Bolwell, E., Rice, E. and Evans, C. (1995). Arch Biochem. **2**: 239-346.

Sang-Hyun, K. and Tae-Yong, S. (2005). "***Amomum xanthiodes Inhibits Mast Cell-Mediated Allergic Reactions through the Inhibition of Histamine Release and Inflammatory Cytokine Production***." Experimental Biology and Medicine. **230**: 681 - 687.

Santhi, R., Lakshmi, G., Priyadharshini, A. M. and Anandaraj, L. (2011). Int Res J Pharm. **2**: 131-135.

Santos-Beulga, C. and Scalbert, A. (2000). "***Proanthocyanidins tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health***." J Sci Food Agric. **80**(7): 1094-1117.

Saper, C. B. and Breder, C. D. (1994). "***The neurologic basis of fever***." N Engl J Med. **330**(26): 1880-1886.

Sauter, B., Albert, M. L., Francisco, L., Larsson, M., Somersan, S. and Bhardwaj, N. (2000). "***Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells***." J Exp Med. **191**(3): 423-434.

Savithramma, N., M., L. R. and Ankanna, S. (2012). "***Preliminary Phytochemical Analysis of Traditionally used Medicinal Plants***." Res J Pharm Bol Chem Sci. **3**(1): 308-314.

Sayed, B. A., Christy, A. L., Walker, M. E. and Brown, M. A. (2010). "***Meningeal mast cells affect early T cell central nervous system infiltration and blood–brain barrier integrity through TNF: a role for neutrophil recruitment?***" J. Immunol. **184**: 6891–6900.

Scheibmeir, H. D., Christensen, K., Whitaker, S. H., Jegaethesan, J., Clancy, R. and Pierce, J. D. (2005). "***A review of free radicals and antioxidants for critical care nurses***." Intensive care and critical care nursing. **21**: 24-28.

Schenkel, A. R., Mamdouh, Z. and Muller, W. A. (2004). "***Locomotion of monocytes on endothelium is a critical step during extravasation***." Nature Immunol. **5**: 393–400.

Schmid-Schönbein, G. W. (2006). "***Analysis of inflammation***." Annu Rev Biomed Eng. **8**: 93-131.

Schur, P. H., Britton, M. C., Franco, A. E., Corson, J. M., Sosman, J. L. and Ruddy, S. (1975). "***Rheumatoid synovitis: complement and immune complexes***." Rheumatology. **6**: 34-42.

Schwab, J. M. and Serhan, C. N. (2006). "***Lipoxins and new lipid mediators in the resolution of inflammation***." Curr Opin Pharmacol. **6**(4): 414-420.

Scott, P., Ma, H., Viriyakosol, S., Terkeltaub, R. and Liu-Bryan, R. (2006). "***Engagement of CD14 mediates the inflammatory potential of monosodium urate crystals***." J Immunol. **177**(9): 6370-6378.

Seebeck, J., Kruse, M. L., Schmidt-Choudhury, A. and Schmidt, W. E. (1998). "***Pituitary adenylate cyclase activating polypeptide induces degranulation of rat peritoneal mast cells via high-affinity PACAP receptor-independent activation of G proteins***." Ann. NY Acad. Sci. **865**: 141–146.

Segawa, Y., Yamaura, M., Aota, S., Omata, T., Tuzuike, N., Itokazu, Y., Oka, H., Tamaki, H. and Nakamura, T. (1997). "***Methotrexate maintains bone mass by preventing both a decrease in bone formation and an increase in bone resorption in adjuvant-induced arthritic rats***." Bone. **20**(5): 457-464.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L. and Isakson, P. (1994). "***Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain***." Proc Natl Acad Sci U S A. **91**(25): 12013-12017.

Serhan, C. N. (2007). "***Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways***." Annu Rev Immunol. **25**: 101-137.

Sharma, P. K., Hota, D. and Pandhi, P. (2004). "***Biologics in rheumatoid arthritis***." J Assoc Physicians India. **52**: 231-236.

Shaw, S. K., Bamba, P. S., Perkins, B. N. and Luscinskas, F. W. (2001). "***Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium***." J. Immunol. **167**: 2323–2330.

Sheehan, D. and Hrapchak, B. (1980). "***Theory and practice of Histotechnology***." **2nd Ed,** Battelle Press Ohio. 282.

Shen, Y., Xu, S., Wei, W., Sun, X., Yang, J., Liu, L. and Dong, C. (2002). "***Melatonin reduces memory changes and neural oxidative damage in mice treated with D-galactose***." Journal of Pineal Research. **32**(3): 173–178.

Shi, J., Arunasalam, K., Yeung, D., Kakuda, Y., Mittal, G. and Jiang, Y. (2004). J Med Food. **7**: 67-78.

Shin, K., Nigrovic, P. A. and Crish, J. (2009). "***Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes***." J Immunol. **182**: 647–656.

Shivkar, Y. M. and Kumar, V. L. (2003). "***Histamine mediates the pro-inflammatory effect of latex of Calotropis procera in rats***." Mediators Inflamm. **12**(5): 299-302.

Simons, F. E., Frew, A. J. and Ansotegui, I. J. (2007). "***Risk assessment in anaphylaxis: current and future approaches***." J Allergy Clin Immunol. **120**: S2–24.

Singh, H., Kumar, S., Dewan, S. and Kumar, V. L. (2000). "***Inflammation induced by latex of Calotropis procera--a new model to evaluate anti-inflammatory drugs***." J Pharmacol Toxicol Methods. **43**(3): 219-224.

Singh, S., Taneja, M. and Majumdar, D. K. (2007). "***Biological activities of Ocimum sanctum L. fixed oil--an overview***." Indian J Exp Biol. **45**(5): 403-412.

Siraganian, R. P. (2003). "***Mast cell signal transduction from the high-affinity IgE receptor***." Curr. Opin. Immunol. **15**: 639–646.

Smolen, J. S., Aletaha, D. and Keystone, E. (2005). "***Superior efficacy of combination therapy for rheumatoid arthritis: fact or fiction?***" Arthritis Rheum. **52**(10): 2975-2983.

Sofowora, A. (1993). "***Phytochemical screening of medicinal plants and traditional medicine in Africa 2nd Edition Spectrum Books Limited, Nigeria;***." 150-156.

Somchit, M. N. and Nur, S. M. H. (2003). "***Antiinflammatory property of ethanolic and aqueousextracts of Zingiber zerumbet***." Indian J Pharmacol **35**: 181-183.

Sonis, S. T. (2002). "***The biologic role for nuclear factor-kappaB in disease and its potential involvement in mucosal injury associated with anti-neoplastic therapy***." Critical Reviews in Oral Biology and Medicine. **13**: 380–389.

Souza, G. E., Cunha, F. Q., Mello, R. and Ferreira, S. H. (1988). "***Neutrophil migration induced by inflammatory stimuli is reduced by macrophage depletion***." Agents Actions. **24**(3-4): 377-380.

Souza, P. E., Rocha, M. O., Rocha-Vieira, E., Menezes, C. A., Chaves, A. C., Gollob, K. J. and Dutra, W. O. (2004). "***Monocytes from patients with indeterminate and cardiac forms of Chagas' disease display distinct phenotypic and functional characteristics associated with morbidity***." Infect Immun. **72**(9): 5283-5291.

Spector, W. G. and Willoughby, D. A. (2009)."***The Inflammatory Response***."In Inflammation Protocols. **Winyard, P. G. and Willoughby, D. A.** Humana Press Inc., Totowa, NJ, NJ. **225** 118-149.

Springer, T. A. (1995). "***Traffic signals on endothelium forblymphocyte recirculation and leukocyte emigration***." Annu. Rev. Physiol. **57**: 827–872.

Srinivas, K. V. N. S., Koteswara Rao, Y., Mahender, I., Das, B., Rama Krishna, K. V. S., Hara Kishore, K. and Murty, U. S. N. (2003). "***Flavonoids from Caesalpinia pulcherrima*** " Phytochem. **63**(7): 789–793

Stassen, M., Muller, C., Arnold, M., Hultner, L., Klein-Hessing, S., Neudorfl, C., Reineke, T., Serfling, E. and Schmitt, E. (2001). "***IL-9 and IL-13 production by activated mast cells is strongly enhanced in the presence of lipopolysaccharide: NF-kappa B is decisively involved in the expression of IL-9***." J Immunol. **166**: 4391 - 4398.

State Geographer, U. S. (2012). **Google earth**. **6.2.2.6613**. kh.google.com.

Stitt, J. T. (1986). "***Prostaglandin E as the neural mediator of the febrile response***." Yale J Biol Med. **59**: 137–149.

Stockigt, J., Hammes, B. and Ruppert, M. (2010). "***Construction and expression of a dual vector for chemo-enzymatic synthesis of plant indole alkaloids in Escherichia coli***." Nat Prod Res. **24**(8): 759-766.

Stoscheck, C. M. (1990). "***Quantitation of Protein***." Methods in Enzymology. **182**: 50-69.

Strait, R. T., Morris, S. C. and Yang, M. (2002). "***Pathways of anaphylaxis in the mouse***." J Allergy Clin Immunol. **109**: 658–668.

Subramanian, S., Tovey, M., Afentoulis, M., Krogstad, A., Vandenbark, A. A. and Offner, H. (2005). "***Ethinyl estradiol treats collagen-induced arthritis in DBA/1LacJ mice by inhibiting the production of TNF-alpha and IL-1beta***." Clin Immunol. **115**(2): 162-172.

Suleyman, H., Demirezer, L.O., Kuuruuzum, A., Banog, Z. N., and Gocer, F., Ozbair, G. . (1999). J. Ethanopharmacol. **65**: 141-146

Suralkar, A. A., Sarda, P. S., Ghaisas, M. M., Thakare, V. N. and Deshpande, A. D. (2008) "***In-vivo animal models for evaluation of anti-inflammatory activity.***" pharmoinfor.net. copyright 2005-2011.

Suurmond, J., Schuerwegh, A. J. M. and Toes, R. E. M. (2011). "***Anti-citrullinated protein antibodies in rheumatoid arthritis: a functional role of mast cells and basophils?***" Ann Rheum Dis. **70**: 55–58.

Swingle, K. F. and Shideman, F. E. (1972). J. Pharmacol. Exp. Ther. **183**: 226-234

Takeshita, K., Bacon, K. B. and Gantner, F. (2004). "***Critical role of L-selectin and histamine H4 receptor in zymosan-induced neutrophil recruitment from the bone marrow: comparison with carrageenan***." J Pharmacol Exp Ther. **310**(1): 272-280.

Tal, M. and Liberman, R. (1997). "***Local injection of nerve growth factor (NGF) triggers degranulation of mast cells in rat paw***." Neurosci Lett. **221**(2-3): 129-132.

Tam, S. Y., Tsai, M., Yamaguchi, M., Yano, K., Butterfield, J. H. and Galli, S. J. (1997). "***Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells***." Blood. **90**: 1807–1820.

Tanzola, M. B., Robbie-Ryan, M., Gutekunst, C. A. and Brown, M. A. (2003). "***Mast cells exert effects outside the central nervous system to influence experimental allergic encephalomyelitis disease course***." J. Immunol. **171**: 4385–4391.

Teather, L. A., Magnusson, J. E. and Wurtman, R. J. (2002). "***Platelet-activating factor antagonists decrease the inflammatory nociceptive response in rats***." Psychopharmacology (Berl). **163**(3-4): 430-433.

Theoharides, T. C. (1996). "***The mast cell: a neuroimmunoendocrine master player***." Int J Tissue React. **18**(1): 1-21.

Theoharides, T. C. and Cochrane, D. E. (2004). "***Critical role of mast cells in inflammatory diseases and the effect of acute stress***." J Neuroimmunol. **146**(1-2): 1-12.

Tillett, W. S. and Francis, T. (1930). "***Serological reactions in Pneumonia with a non-protein somatic fraction of Pneumococcus***." J Exp Med. **52**(4): 561-571.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G. and Kaur, H. (2011). "***Phytochemical screening and Extraction: A Review***." Internationale Pharmaceutica Sciencia. **1**(1): 98 - 106.

Tlustochowicz, W. (2006). "***[Rational therapeutic approach in rheumatoid arthritis]***." Ann Acad Med Stetin. **52 Suppl 2**: 5-10.

Tofovic, S. P. and Jackson, E. K. (1999). "***Effects of long-term caffeine consumption on renal function in spontaneously hypertensive heart failure prone rats***." J Cardiovasc Pharmacol. **33**(3): 360-366.

Tomlinson, A., Appleton, I., Moore, A. R., Gilroy, D. W., Willis, D., Mitchell, J. A. and Willoughby, D. A. (1994). "***Cyclo-oxygenase and nitric oxide synthase isoforms in rat carrageenin-induced pleurisy***." Br J Pharmacol. **113**(3): 693-698.

Torchinsky, M. B., Garaude, J. and Blander, J. M. (2010). "***Infection and apoptosis as a combined inflammatory trigger***." Curr. Opin. Immunol. **22**: 55–62.

Trease, G. E. and Evans, W. C. (1989). "***Textbook of Pharmacognosy***." **12th ed. ,** Balliere; Tindall, London, UK 57–59, 343–383.

Tseng, A. (1991). Proc Am Assoc Cancer Res. **32**: 2257.

Ueda, S., Ikeda, U. and Yamamoto, K. (1996). "***C-reactive protein as a predictor of cardiac rupture after acute myocardial infarction***." Am Heart J. **131**: 857–860.

Underhill, D. M. *et al.* (1999). "***The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens***." Nature. **401:** 811–815.

Underhill, M. D. and Goodridge, S. H. (2012). "***Information processing during phagocytosis***." NATURE REVIEWS: IMMUNOLOGY. **12**: 492-502.

Ushiyama, S., Yamada, T., Murakami, Y., Kumakura, S., Inoue, S., Suzuki, K., Nakao, A., Kawara, A. and Kimura, T. (2008). "***Preclinical pharmacology profile of CS-706, a novel cyclooxygenase-2 selective inhibitor, with potent antinociceptive and anti-inflammatory effects***." Eur J Pharmacol. **578**(1): 76-86.

Vajkoczy, P., Laschinger, M. and Engelhardt, B. (2001). "***α4-integrin–VCAM-1 binding mediates G proteinindependent capture of encephalitogenic T cell blasts to CNS white matter microvessels***." J. Clin. Invest. **108**: 557–565.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. and Telser, J. (2007). "***Free radicals and antioxidants in normal physiological functions and human disease***." Int J Biochem Cell Biol. **39**(1): 44-84.

Van Wagoner, N. J., Oh, J.-W., Repovic, P. and Benveniste, E. N. (1999). "***Interleukin-6 (IL-6) production by astrocytes: autocrine regulation by IL-6 and the soluble IL-6 receptor***." The Journal of neuroscience. **19**(13): 5236-5244.

van Zee, K. J., DeForge, L. E., Fischer, E., Marano, M. A., Kenney, J. S., Remick, D. G., Lowry, S. F. and Moldawer, L. L. (1991). "***IL-8 in septic shock, endotoxemia, and after IL-1 administration***." Journal of Immunology. **146**: 3478-3482.

Vane, J. R., Bakhle, Y. S. and Botting, R. M. (1998). "***Cyclooxygenases 1 and 2***." Annu Rev Pharmacol Toxicol. **38**: 97-120.

Vasconcelos, S. M., Reboucas Oliveira, G., Mohana de Carvalho, M., Rodrigues, A. C., Rocha Silveira, E., Maria Franca Fonteles, M., Florenco Sousa, F. C. and Barros Viana, G. S. (2003). "***Antinociceptive activities of the hydroalcoholic extracts from Erythrina velutina and Erythrina mulungu in mice***." Biol Pharm Bull. **26**(7): 946-949.

Vasudevan, M., Gunnam, K. K. and Parle, M. (2007). "***Antinociceptive and anti-inflammatory effects of Thespesia populnea bark extract***." J Ethnopharmacol. **109**(2): 264-270.

Verpoorte, R. (1999). "***Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development***." Drug Discov Today. **3:** 232–238.

Vinay, K., Abul, A. and Nelson, F., (2005). "***Robbins and Cotran PATHOLOGIC BASIS OF DISEASE***." **Vinay, K., Abul, K., Abbas and Nelson, F.** Elsevier's Health Sciences Rights Department in Philadelphia, PA, USA, The Curtis Center 170 S Independence Mall W 300E Philadelphia, Pennsylvania 19106.

Vinegar, R., Schreiber, W. and Hugo, R. (1969). "***Biphasic development of carrageenan oedema in rats***." Journal of Pharmacological Experimental Therapeutics. **66**: 96-103.

Vinson, J. A., Dabbagh, Y. A., Serry, M. M. and Jang, J. (1995). " ***Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease***." J Agric Food Chem. **43**: 2800-2802.

Vogel, G. H. and Vogel, W. H. (1997). "***Anti-Inflammatory activity***." Drug Discovery and Evaluation. Pharmacological Assays. **New York, NY: Springer**: 406 - 409.

von Hundelshausen, P., Weber, K. S. C., Hou, Y., Proudfoot, A. E. I., Nelson, P. J. and Weber, C. (2001). "***RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium***." Circulation. **103**: 1772–1777.

Wachulec, M., Peloso, E. and Satinoff, E. (1997). "***Individual differences in response to LPS and psychological stress in aged rats*** " Am J Physiol **272**: R1252–R1257.

Wall, M. E., Krider, M. M., Krewson, C. F., Eddy, C. R., Willaman, J. J., Corell, D. S. and Gentry, H. S. (1954). "***Steroidal Sapogenins. XII. Survey of Plants for Steroidal Sapogenins and other Constituents***." J. Am Pharm Asso Am Pharm Assoc (Baltim). **43**(8): 503-505.

Walsh, N. C., Crotti, T. N., Goldring, S. R. and Gravallese, E. M. (2005). "***Rheumatoid disease: the effects of inflammation on bone***." Immuno Rev. **208**: 228-251.

Wang, L., Gu, Q., Xu, Y., Li, S., Gui, J., Yang, J., Yao, Q. and Ji, Y. (2008). "***Effects of Yunke (technetium-99 conjugated with methylene diphosphonate; (99)Tc-MDP) and/or colloidal chromic phosphate phosphonium-32, alone and in combination, in rats with adjuvant arthritis***." Clin Exp Pharmacol Physiol. **35**(1): 23-28.

Wang, X. N., Fan, C. Q., Yin, S., Gan, L. S. and Yue, J. M. (2008). "***Structural elucidation of limonoids and steroids from Trichilia connaroides***." Phytochemistry. **69**(6): 1319-1327.

Weisburger, J. H. (2002). "***Lifestyle, health and disease prevention: the underlying mechanisms***." Eur. J. Cancer Prev. **11**(2): S1–S7.

Wells, G., Li, T., Maxwell, L., Maclean, R. and Tugwell, P. (2008). "***Responsiveness of patient reported outcomes including fatigue, sleep quality, activity limitation, and quality of life following treatment with abatacept for rheumatoid arthritis***." Ann Rheum Dis. **67**(2): 260-265.

West, A. P., Brodsky, I. E., Rahner, C., Woo, D. K., Erdjument-Bromage, H., Paul Tempst, P., Walsh, M. C., Choi, Y., Shadel, G. S. and Ghosh, S. (2011). "***TLR signalling augments macrophage bactericidal activity through mitochondrial ROS***." Nature. **472**: 476–480.

Weydert, C. J. and Cullen, J. J. (2010). "***Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue***." Nature Protocols. **5**: 51 - 66.

Wheeler-Aceto, H., Porreca, F. and Cowan, A. (1990). "***The rat paw formalin test: comparison of noxious agents***." Pain. **40**(2): 229-238.

WHO. (2002). "***Traditional medicine-growing needs and potential***." WHO Policy Perspectives Med. **2**: 1-6.

Wigmore, S. J., Fearon, K. C. H., Maingay, J. P., Lai, P. B. S. and Ross, J. A. (1997). "***Interleukin-8 can mediate acute-phase protein production by isolated human hepatocytes***." Am J Physiol. **273**: E720-E726.

Wilks, C. and Issembé, Y. (2000). "***Les arbres de la Guinée Equatoriale***." CUREF, http://http://plants.jstor.org/ Jstor (ex Aluka); http://www.metafro.be/ Metafro; http://www.theplantlist.org/ The Plant list 546.

Willey, J. M., Sherwood, L. M. and Woolverton, C. J. (2008). "***Prescott, Harley and Klein's Microbiology***." **7th edition,** McGrawHill Company New York, America. 578.

Williams, R. O. (1998). "***Rodent models of arthritis: relevance for human disease***." Clin Exp Immunol. **114**(3): 330–332.

Williams, R. O. (2007). "***Collagen-induced arthritis in mice: a major role for tumor necrosis factor-alpha***." Methods Mol Biol. **361**: 265-284.

Willis, D., Moore, A. R., Frederick, R. and Willoughby, D. A. (1996). "***Heme oxygenase: a novel target for the modulation of the inflammatory response***." Nat Med. **2**(1): 87-90.

Winder, C. V., Lambke, L. A. and Stephens, M. D. (1996). "***Comparative bioassay of drugs in adjuvant-induced arthritis in rats; flufenamic acid, mefenamic acid and phenyl butazone***." Arthritis Rheum. **12**: 472.

Winter, C. A., Risley, E. A. and Nuss, G. W. (1962). "***Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs***." Proc Soc Exp Biol Med. **111**: 544-547.

Wittmann, C., Chockley, P., Singh, S. K., Pase, L., Lieschke, G. J. and Grabher, C. (2012). "***Hydrogen Peroxide in Inflammation: Messenger, Guide, and Assassin***." Advances in Hematology. **Volume 2012**: 1-6.

Woode, E., Boakye-Gyasi, E., AmaningDanquah, C., Ansah, C. and Duwiejua, M. (2009). "***Anti-Arthritic Effects of Palisotahirsuta K. Schum. Leaf Extract in Freund's Adjuvant-Induced Arthritis in Rats***." Int. J. Pharmacol. **5**(3): 181-190.

Woode, E., Ainooson, G. K., Boakye-Gyasi, E., Ansah, C., Obiri, D. D., Koffour, G. A., Mensah, A. Y. and Duwiejua, M. (2008). "***Anti-arthritic and antioxidant properties of the ethanolic stem bark extract of Newbouldia laevis (P. Beauv.) Seaman ex Bureau (Bignoniaceae)***." Journal of Medicinal Plants Research. **2**(8): 180-188.

Wuerges, J., Lee, J. W., Yim, Y. I., Yim, H. S., Kang, S. O. and Djinovic Carugo, K. (2004). "***Crystal structure of nickel-containing superoxide dismutase reveals another type of active site***." Proc Natl Acad Sci. **101**: 8569-8574.

Xing, Z., Gauldie, J. and Cox, G. (1998). "***IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses***." J Clin Invest. **101**: 311-320.

Yen, G. C., Duh, P. D. and Tsai, C. L. (1993). "***Relationship between antioxidant activity and maturity of peanut hulls***." J Agric Food Chem. **41**: 67–70.

Yirmiya, R., Rosen, H., Donchin, O. and Ovadia, H. (1994). "***Behavioral effects of lipopolysaccharide in rats: Involvement of endogenous opioids*** " Brain Res **648**: 80–86.

Young, D. A., Hegen, M., Ma, H. L., Whitters, M. J., Albert, L. M. and Lowe, L. (2007). "***Blockade of the interleukin-21/interleukin-21 receptor pathway ameliorates disease in animal models of rheumatoid arthritis***." Arthritis Rheum. **56**: 1152–1163.

Zakaria, Z. A., Wen, L. Y., Abdul Rahman, N. I., Abdul Ayub, A. H., Sulaiman, M. R. and Gopalan, H. K. (2007). "***Antinociceptive, anti-inflammatory and antipyretic properties of***

the aqueous extract of Bauhinia purpurea leaves in experimental animals." Med Princ Pract. **16**(6): 443-449.

Zeisberger, E. (1999). "***From humoral fever to neuroimmunological control of fever***." Journal of Thermal Biology. **24**: 287-326.

Zelko, I., Mariani, T. and Folz, R. (2002). "***Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression***." Free Radic Biol Med. **33**: 337-349.

Zhang, Y., Lu, L., Furlonger, C., Wu, G. E. and Paige, C. J. (2000). "***Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis***." Nat Immunol. **1**(5): 392-397.

Zheng, H., Fletcher, D. and Kozak, W. (1995). "***Resistance to fever induction and impaired acute-phase response in interleukin-1 b-deficient mice***." Immunity. **3**: 9-19.

APPENDIX

PHARMACOLOGICAL METHODS

PREPARATION OF CARRAGEENAN SUSPENSION

A 0.5 % carrageenan suspension was prepared by sprinkling small amounts of the powder (50 mg) evenly over the surface of 10 ml of 0.9 % NaCl solution and left to soak between additions. This was left for 2-3 hours before use.

PREPARATION OF COMPLETE FREUND'S ADJUVANT (CFA)

100 mg heat-killed *Mycobacterium tuberculosis* [strains C, DT and PN (mixed) obtained from the Ministry of Agriculture, Fisheries and Food, U.K] was finely grounded in a mortar using a pestle. Liquid paraffin was added gradually to make 20 ml of 5 mg ml^{-1} suspension.

PREPARATION OF PHOSPHATE BUFFER

PBS tablets were dissolved in distilled water to a concentration of 0.01 M, pH 7.2 and used to homogenize tissue samples.

FOOT VOLUME MEASUREMENT

A liquid column containing water was placed on a balance. When an object is immersed, the liquid applies a force F to attempt its expulsion. Physically, F is the weight (W) of the volume of liquid displaced by that part of the object inserted into the water. A balance was used to measure this force (F=W). Volume of inflamed foot of rat can be calculated. This is done using the specific gravity of the immersion liquid, at equilibrium mass/specific gravity. Since water was used as the immersion liquid in this case, the mass or weight of the foot inserted in the water will be the same as its volume. The extent of oedema at time *t* will be *v1 – v0*. The foot measured was kept from touching the wall of the column containing the water while value on the balance was read.

CALCULATION

Percentage increase in foot volume = $(v_t - v_o) / P_t X 100$

Where, P_t is the foot volume at time t (after injection).

V_o is the foot volume before injection. (0 h)

PROCEDURE AND CACULATIONS FOR BIOMARKERS OF OXIDATIVE STRESS

Procedure - modified Lowry (room temperature)

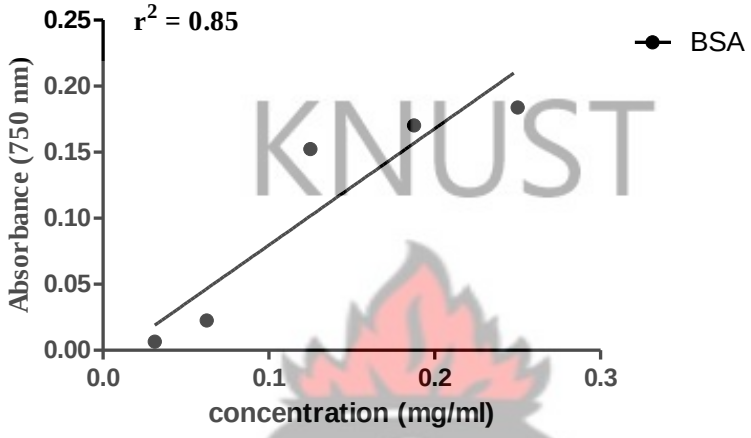
Reagents Preparation

1. Sodium carbonate (20 g) was dissolved in 260 ml water, 0.4 g cupric sulphate (5x hydrated) in 20 ml water, and 0.2 g sodium potassium tartrate in 20 ml water. All three solutions were mixed to prepare the copper reagent.
2. A 100 ml of 1 % solution (1 g/100 ml) of sodium dodecyl sulphate (SDS) was prepared.
3. A 1 M solution of NaOH (4 g/100 ml) was prepared.
4. For the 2x Lowry concentrate 3 parts copper reagent was mixed with 1 part SDS and 1 part NaOH. Solution is stable for 2-3 weeks. The solution was warmed to 37 ^{0}C at the formation of white precipitate. These three stock solutions were kept and only mixed just before use
5. A 0.2 N Folin reagent was prepared by mixing 10 ml 2 N Folin reagents with 90 ml water and kept in an amber bottle.

Assay

1. Dilute samples of an estimated 0.025-0.25 mg ml^{-1} with buffer was prepared. Each dilution was prepared at 400 µl. samples were prepared in duplicates.
2. A blank of 400 µl buffer was prepared. Standards from 0.25 mg ml^{-1} bovine serum albumin (BSA) was prepared by adding 40-400 µl. to 13 x 100 mm tubes + buffer to bring volume to 400 µl./tube.
3. 400 µl of 2x Lowry concentrate was mix thoroughly and incubated at room temperature 10 minutes.
4. 200 µl 0.2 N Folin reagent was added, quickly, and vortexed immediately. This was incubated at room temperature for 30 minutes. Complete mixing of the reagent must

be accomplished quickly to avoid decomposition of the reagent before it reacts with protein. Incubate for 30 min. more at room temperature. Absorbance was taken at 750 nm. A linear curve was obtained by plotting various concentrations versus absorbance to determine the protein concentration in sample as BSA equivalent. The linear curve is shown below:



MEASUREMENT OF MALONDIALDEHYDE

Materials

Supernatant of tissue homogenate separated for MDA assay (Figure 4.1)

4× TBA/TCA/HCl reagent (Prepared by adding 150 g TCA, 500 ml distilled water, 20.8 ml 0.2 N HCl and 3.7 g TBA into a tube. This was made up to one litre with distilled water. This preparation was heated to 70 ^{0}C to dissolve the TBA)

2 % (w/v) butylated hydroxytoluene (BHT) in ethanol (store protected from light up to 1 month at room temperature)

Boiling water bath

Spectrophotometer or spectrofluorometer

MEASUREMENT OF MYLOPEROXIDASE (MPO)

Reagents and Solutions

Aminotriazole (AMT)

3-Amino-1, 2, 4 triazole was dissolved at 42 mg ml^{-1} water (final concentration of 0.5 M).

Cetrimide

Cetrimide (Hexadecyltrimethylammonium bromide) was prepared by adding 0.5 g to 100 ml of 0.05M PBS, pH 6.0.

H_2O_2, 0.5 mM

On the day of the assay, 8.5µl of 30 % H_2O_2 was added to 4.99 ml 0.05 M PBS, pH 6.0. This was kept on ice throughout the assay

ODA

This was prepared by dissolving 1.67 mg ml^{-1} *o*-dianisidine dihydrochloride in water.

MEASUREMENT OF TOTAL SUPEROXIDE DISMUTASE (SOD) LEVEL

Reagents and Solutions

Cytochrome c stock solution, 1 mM

1 mM ferricytochrome *c* (from horse heart, minimum 95 %) was prepared in 0.05 M PBS, pH 7.8.

Xanthine oxidase solution

Purified bovine buttermilk xanthine oxidase (grade III) was dissolved at ~0.3 IU ml^{-1} in 0.05 M PBS, pH 7.8/0.1mM EDTA.

Xanthine stock solution

Prepare 1 mM xanthine (sodium salt) was prepared by dissolving the salt in 0.05 M PBS, pH 7.8. This was heated to near boiling to assure complete dissolution

SOD assay cocktail

To 141 ml of 0.05 M potassium phosphate buffer, pH 7.8/0.1 mM EDTA,

7.5 ml of 1 mM xanthine stock solution was added. To this mixture, 1.5 ml of 1 mM Cytochrome *c* solution was added.