KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

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ANTI-OEDEMIC, ANTIPYRETIC AND ANTINOCICEPTIVE EFFECTS OF THE ETHANOL ROOT EXTRACT OF *ALBIZIA ZYGIA* (DC.) J.F. MACBR (LEGUMINOSAE-MIMOSOIDEAE)

by

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

Medications for inflammation, pain and fever are the most frequently patronized over-thecounter drugs. Research into these medicines is nevertheless unending, due to the need for newer improved alternatives. *Albizia zygia* (DC.) J.F. Macbr is such an alternative; it is used traditionally in pain and fever therapy. The plant is widespread in forest zones of West and East Africa, and Asia. In this study, the actions of the ethanol extract of the roots of *Albizia zygia* (AZE) on inflammation (oedema and oxidative imbalance), fever and pain were investigated. Animal models of acute oral toxicity, acute inflammation, pyrexia and nociception were employed. The primary phytochemical assay of AZE disclosed the constituents: saponins, tannins, alkaloids, flavonoids, terpenoids and glycosides. There was no death recorded after acute oral toxicity studies conducted; LD_{50} of AZE was estimated to be greater than 5000 mg/kg. AZE (30-300 mg/kg; *p.o.*) and diclofenac administrations in the prophylactic and therapeutic protocols of carrageenan-induced pedal oedema significantly attenuated pedal swelling in the ipsilateral paw by the highest doses [AZE: 57.65±6.70 % (pre-emptive) and 55.41±7.37 % (curative); Diclofenac: 77.08±3.80 % (preemptive) and 75.87±4.00 % (curative)]. Evaluation of the extract in Baker's yeast-induced pyrexia displayed significant inhibition of pyrexia after 4 h of sustained pyrexia. Maximal attenuation of antipyrexia was achieved by AZE at dose 300 mg/kg ($F_{4, 24}$=18.39; P<0.0001) and highest dose of paracetamol ($F_{4, 25}$=15.73; P<0.0001). In the *in vivo* assay of antioxidant activity, pre-treatment of the rats with AZE significantly elevated the endogenous expressions of SOD, CAT and GSH at the inflamed site. AZE also reduced the formation of MDA and the pro-inflammatory enzyme MPO. AZE ($F_{3, 14}$=9.27; P=0.0012) and morphine ($F_{3, 15}$=12.91; P=0.0002) significantly augmented the time of reaction to heat (tail immersion test). Mechanical hyperalgesia, initiated with the phlogistic agent carrageenan, was attenuated by AZE ($F_{3, 16}$=11.90; P=0.0002), and the standards diclofenac ($F_{3, 16}$=12.52; P=0.0002) and morphine ($F_{3, 16}$=39.51; P<0.0001). The total number of writhes were maximally inhibited by AZE (85.85±3.96 %) and diclofenac (99.65±0.35 %) at their highest doses. AZE ameliorated formalin-induced nociception in the timedependent neurogenic and inflammatory phases. Significant analgesic effect of AZE was antagonised by atropine and naloxone pre-administration suggesting that AZE possibly acts via the muscarinic and opioidergic signalling pathways. No negative neurological deficits were observed in the rotarod test, thus, confirming the true analgesic effect of AZE. In all, these results confirm the propensity of the ethanol extract of *Albizia zygia* in ameliorating oedema, oxidative imbalance, pain and pyrexia.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AP-1	Activator protein 1
cAMP	Cyclic adenosine monophosphate
CFA	Complete Freund's adjuvant
DAMP	Damage-associated molecular pattern
GPCR	G-protein coupled receptor
IASP	International Association for the Study of Pain
IL	Interleukin
NF-κB	Nuclear factor kappa B
NK	Neurokinin
NMDA	N-methyl-D-Aspartate
p.o.	Per os
RPM	Revolution per minute
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Chapter 1

INTRODUCTION

1.1 COMPREHENSIVE OVERVIEW

Inflammation, fever and pain are general signals of diseases and infections. Health management provides relief, preserve and restore health, and improve quality of life of patients suffering from these inflammatory conditions (Fields and Martin, 2005). Conventional analgesics, antipyretics and anti-inflammatory agents provide rapid relief of these symptoms. Nonetheless, there is still continuous research into finding new and safer alternative agents for these conditions.

The plant kingdom affords us new chemical entities which provide leads for drug research. Their dosage formulations are presented as crude drugs in the forms of powders, tinctures, poultices and in other herbal formulations (Butler, 2004; Reichert, 2003; Balunas and Kinghorn, 2005). Herbal medications are now acceptable in national health ministries. Despite the user-experienced efficacy of herbals, there is little or no scientific proof giving the credibility of their use. Also, the diagnosis for which these drugs are prescribed are more often vague, hence resulting in an even inexact dosing regimen (Sofowora, 1996). Like all other herbals, *Albizia zygia* is traditionally used in arthritis, fever and pain but with little scientific credence. The quest for scientific accreditation of the anti-inflammatory, antipyretic and analgesic uses of *Albizia zygia* directed the course of this project.

1.2 THE PLANT

1.2.1 Plant Vignette

Albizia zygia (D.C.) Macbr (Leguminosae-Mimosoideae). It is also referred to as Red Nango or West African Walnut (Ashton *et al*., 1975). It is an indigenous plant of tropical Africa with distribution in evergreen and semi-deciduous forest zones of East and West Africa, India and Australia. It is locally called "Okuro" (Ghana), "Nyie avu" (Igbo), "Ayinre weere" (Yoruba), "Red Nongo" (Uganda) and "Nongo" (Swahili). It is a deciduous tree with a spreading canopy which can grow up to 30 m (fig. 1.1). The leaflets are glabrous and bipinnate. The bark is smooth with grey colour. The flowers are spiked and subsessile. The fruit pods are flat with reddish-brown colour and the seeds are roundshaped. The fruits ripen between November and April (Orwa *et al*., 2009; Kokila *et al*., 2013; Anim-Kwapong and Teklehaimanot, 1995).

1.2.2 Ethnomedicinal and Ethnobotanical Values

1.2.2.1 Medicinal

- **Inflammatory Pain:** The bark and leaf decoctions are used in fever and waist pain management (Arbonnier, 2004; Ndjakou Lenta *et al.*, 2007; Apetorgbor, 2007).
- **Pathogenic Infections:** The stem bark extract is very active in malaria and sleeping sickness (Ndjakou Lenta *et al.*, 2007; Abdalla and Laatsch, 2012). The leaf extract possesses molluscicidal properties. The roots are used in the management of

tuberculosis in Lake Victoria Region, Kenya (Abere *et al.*, 2014). Rasped bark is administered topically in the management of wounds, eczema, toothache and yaws. Decoctions of the bark is administered as purgative, vermifuge, stomachic and as an antidote. Ophthalmia and bronchial infections are treated with the sap. Leaf decoctions are used in diarrhoea treatment. Ground roots are incorporated in foods as expectorant (Apetorgbor, 2007).

- **Sexually Transmitted Infections and Sterility:** Root bark formulations are used to treat venereal diseases (Ndjakou Lenta *et al.*, 2007). Also formulations of the bark are consumed as aphrodisiacs, and treatment of female sterility (Apetorgbor, 2007).

Figure 1.1 *Albizia zygia* plant

1.2.2.2 Non-medicinal

The tree is a lesser-used timber species with the trade name “Okuro”. It is a source of fuel (firewood or charcoal) in Ghanaian communities. The canopy suffices as windbreak and provides shade for cocoa plantations. The plant is an ornamental for avenues, boulevards and recreational sites. It is a rich source of nitrogen for soil enrichment when used as mulch (Orwa *et al*., 2009; Anim-Kwapong and Teklehaimanot, 1995). The gum mucilage (obtained from the incised trunk) is a good stabilizer in ice cream. The gum is used as a thickener, suspending agent and drug coating (Femi-Oyewo *et al*., 2007; Mital *et al*., 1978).

1.2.3 Previous Studies on A. zygia

The methanol stem bark extract possesses analgesic effects in rodents (Abere *et al.*, 2014). *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum* K1 strain were found to be susceptible to the plant extract (Ndjakou Lenta *et al.*, 2007). Flavonoids isolated from Sudanese *A. zygia* demonstrated high antimalarial potency (Abdalla and Laatsch, 2012). *A. zygia* exhibits cytotoxicity against human T-lymphoblast-like leukaemia, prostate and breast cancer cell lines (Appiah-Opong *et al*., 2016). Physical properties evaluation shows the gum exhibits non-Newtonian flow with thixotropy (Mital *et al*., 1978; Ashton *et al*., 1975).

1.3 INFLAMMATION

Inflammation is a biological response of an organism's immune system to cellular and vascularized tissue damage (Nathan, 2002). It presents with the cardinal signs of pain (*dolor*), swelling (*tubor*), redness (*rubor*), heat (*calor*) and impaired functionality (*functio laesa*) of the affected area (Holdcroft and Jaggar, 2005). It is a beneficial healing mechanism. Inflammation presents with a delicate balance between protection and harm. Inflammation when gone awry, initiates an innocent bystander attack on the host cells. The discharge of toxic metabolites and enzymes by inflamed cells destroy the uninjured tissue in the vicinity (Stahel *et al*., 2007).

1.3.1 The Response to Tissue Injury

1.3.1.1 Acute Inflammatory Response

Danger-associated molecular patterns (DAMPs) or "alarmins" from traumatic insult interact with TLRs on cell surfaces of the sentinel cells (Bianchi, 2007). Immediately, intermediary pro-inflammatory agents- cytokines (TNF-α, IL-1), chemokines, histamine and prostaglandins- are secreted by the sentinel cells (mast cells, macrophages and dendritic cells). The complement system and neutrophil chemoattractant trigger histamine release via mast cell degranulation. The chemokines and cytokines cause the expression of adhesion molecules (integrins) which bind neutrophils to E-selectin on the intimal surface of the endothelium (Rhen and Cidlowski, 2005; Griffiths *et al*., 2009). The activated leukocytes gravitate along the chemotactic gradient out of the vasculature to the infection site. This confines the injury and promotes healing, thus reducing further damage (Lippross *et al*., 2012; Griffiths *et al*., 2009; Mackay *et al*., 2000). Prostaglandins (PGE_2 and PGI_2)

and histamine dilate arterioles, venules and capillaries. This leads to increased circulation to the inflamed site. Blood then accumulates with increased post-capillary and venular permeability causing fluid and plasma protein exudation (seen as oedema) (Neher *et al*., 2011) (fig. 1.2).

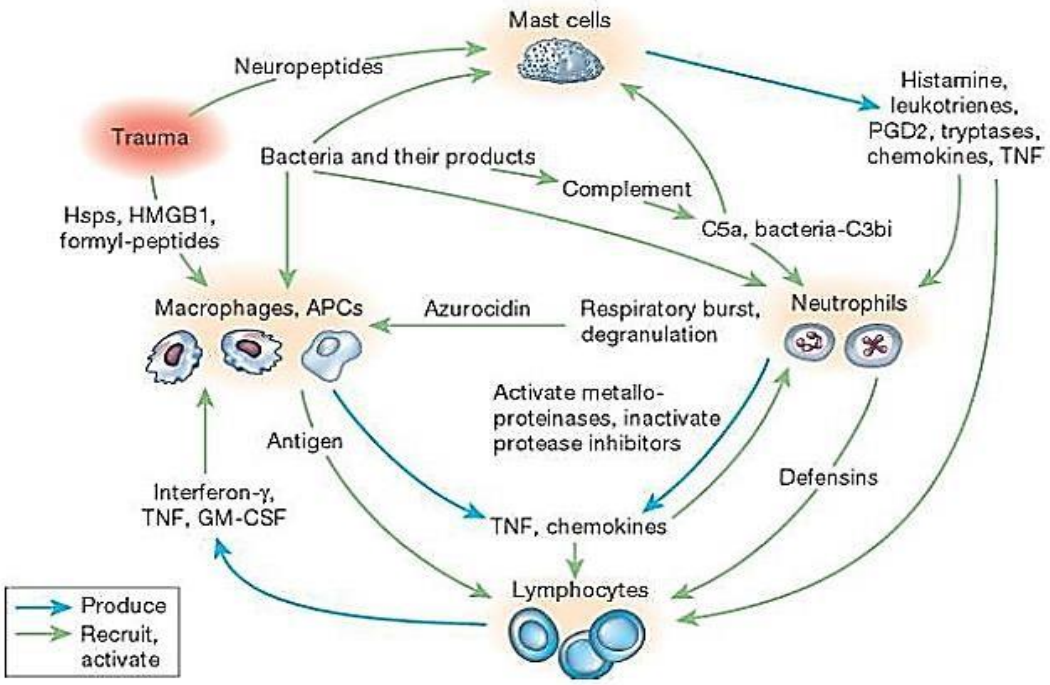


Figure 1.2 Pathways traversed after acute inflammation during mild trauma and infection. Injury and infection initiate recruitment and interplay of pro-inflammatory mediators and activation of inflammatory cascade (*adapted from Nathan, 2002*).

1.3.1.2 Reactive Oxygen Species (ROS)

Tissue injury generates ROS by releasing transition metal ions from damaged cells, activating phagocytes or produce nitric oxide ($NO^{\cdot}$) to kill pathogens. ROS also participate in signal transduction (Devasagayam *et al*., 2004). Their overproduction can damage healthy cells yielding highly cytotoxic substances (fig. 1.3). Antioxidants augment in

delaying or inhibiting the oxidation of substrates. Superoxide dismutase (SOD), glutathione (GSH), catalases (CAT), and glutathione peroxidase (GPx) are endogenous whilst ascorbic acid, α-tocopherol and flavonoids are exogenous antioxidants (Halliwell, 2012; Zhu *et al*., 2008).

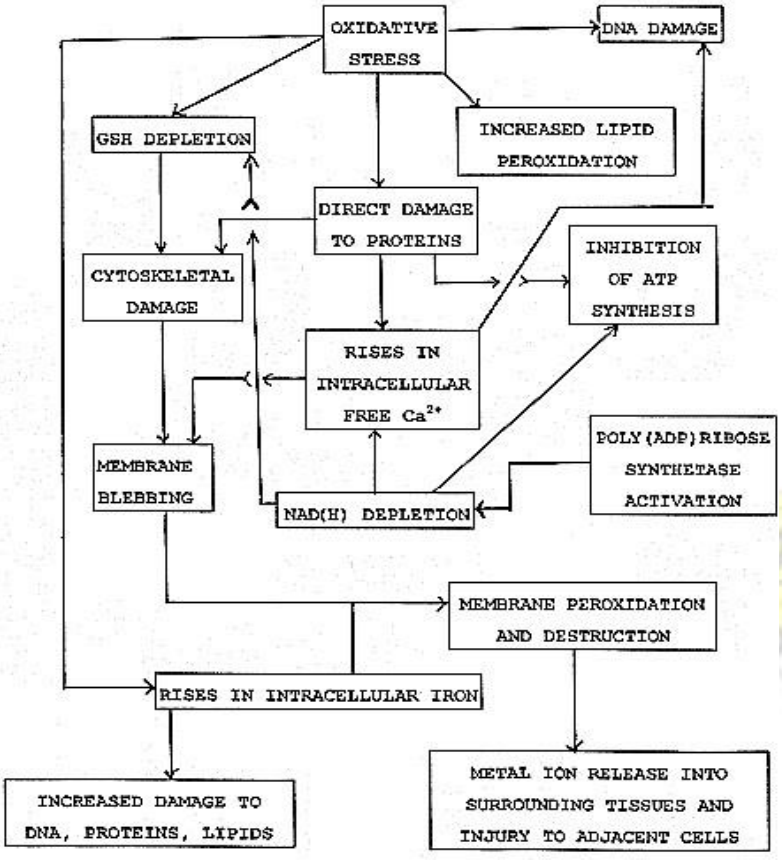


Figure 1.3 Mechanisms and related damaged products of oxidative stress (*adapted from Aruoma, 1998*)

1.3.1.3 Chronic Inflammatory Response

Acute inflammatory conditions proceed to chronicity when the endogenous antiinflammatory pathways fail to terminate the inflammation. Chronic inflammation can also be an anterograde effect following slow, insidious, asymptomatic inflammatory

reaction. Such conditions include painful enfeebling diseases such as rheumatoid arthritis, multiple sclerosis, atherosclerosis, tuberculosis, chronic lung diseases, chronic granulomatous disease (CGD) and cancer (Nathan, 2002). The development of chronic inflammation depends largely on cytokines and chemokines persisting at the inflammatory site. Chronic inflammation, unlike acute inflammation, manifests as a pertinacious reaction to injury (Cotran *et al*., 1999; Gabay, 2006).

1.4 FEVER (SYSTEMIC INFLAMMATION)

Infection, inflammation, tissue damage, non-infectious immune challenges, graft rejection and other diseases present with fever. A healthy adult body temperature > 37.7 ^{o}C (>100 ^{o}F) at any time during the day, or an equivalent rectal temperature of ≥ 38 ^{o}C (100.4 ^{o}F) or axillary temperatures of ≥ 37.5 ^{o}C (99.5 ^{o}F) is considered fever (Ogoina, 2011). Fever is induced when exogenous pyrogens such as toxins, microbes and bacterial products e.g. lipopolysaccharide and peptidoglycan present in plasma stimulate white blood cells (monocytes/macrophages and neutrophils) to synthesize endogenous pyrogens (TNF-α, IL1, IL-6 and interferons). These pyrogens are then transported to the Organum vasculosum laminae terminalis (OVLT) (Ryan and Levy, 2003; Boulant, 2000). The OVLT synthesizes PGE_2, which directly acts on the pre-optic nucleus to restrict the firing rate of warm sensitive neurons thereby increasing body temperature. Fever tends to inhibit bacterial growth and increase bactericidal activities of neutrophils and macrophages. To meet the new balance point, the consequent febrile response initiates body heat loss reducing mechanisms (Dinarello and Gelfand, 2005; Legget, 2008).

1.5 PAIN

Pain is explained as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP). Pain is subjective and influenced by plasticity. Pain perception is always displeasing, hence it is also an emotional experience (Merskey *et al*., 1979). Pain is essential in that the incidence of impending tissue damage warns the individual to protect himself. When tissue damage however occurs, the pain informs the individual to protect that site of the body from further injury. It also informs society of potential injury as soon as an individual is injured (Le Bars *et al*., 2001).

1.5.1 Neurobiology of Nociceptive Pain

The neural proceedings involved in encoding and processing noxious stimulus constitute nociception. Modulation of nociceptive impulses occurs at every level of the nervous system (Riedel and Neeck, 2001). Nociception is initiated when chemical entities bind with nociceptors on primary (1°) afferent neurons innervating peripheral tissues. 1^{o} afferent nociceptors in the periphery react to mechanical, thermal or chemical stimuli. 1^{o} afferent neurons are classified into two distinct categories, namely A- and C-fibres. A-fibres transmit a phasic pain with sharp, prickling and aching sensation. C-fibres transmit tonic pain characterized by a burning, itching and aching sensation (Clark and Treisman, 2006; Costigan and Woolf, 2000). Pain transmission travels along the nerve fibres, synapses in the spinal dorsal horn (DH) and continue to brain cortical regions (where pain is perceived). The inflamed damaged tissues release algogens, recruit inflammatory cells and release

more mediators such as serotonin (5-HT), histamine, bradykinin, PGs, $NO^{\cdot}$, H^{+}, K^{+}, cytokines and growth factors (fig. 1.4). They either directly activate the nociceptors or mostly, indirectly sensitize them to enhance their activation by ensuing stimuli resulting in hyperalgesia and allodynia (Yaksh, 1999; Fitzgerald and Beggs, 2001; Woolf and Costigan, 1999).

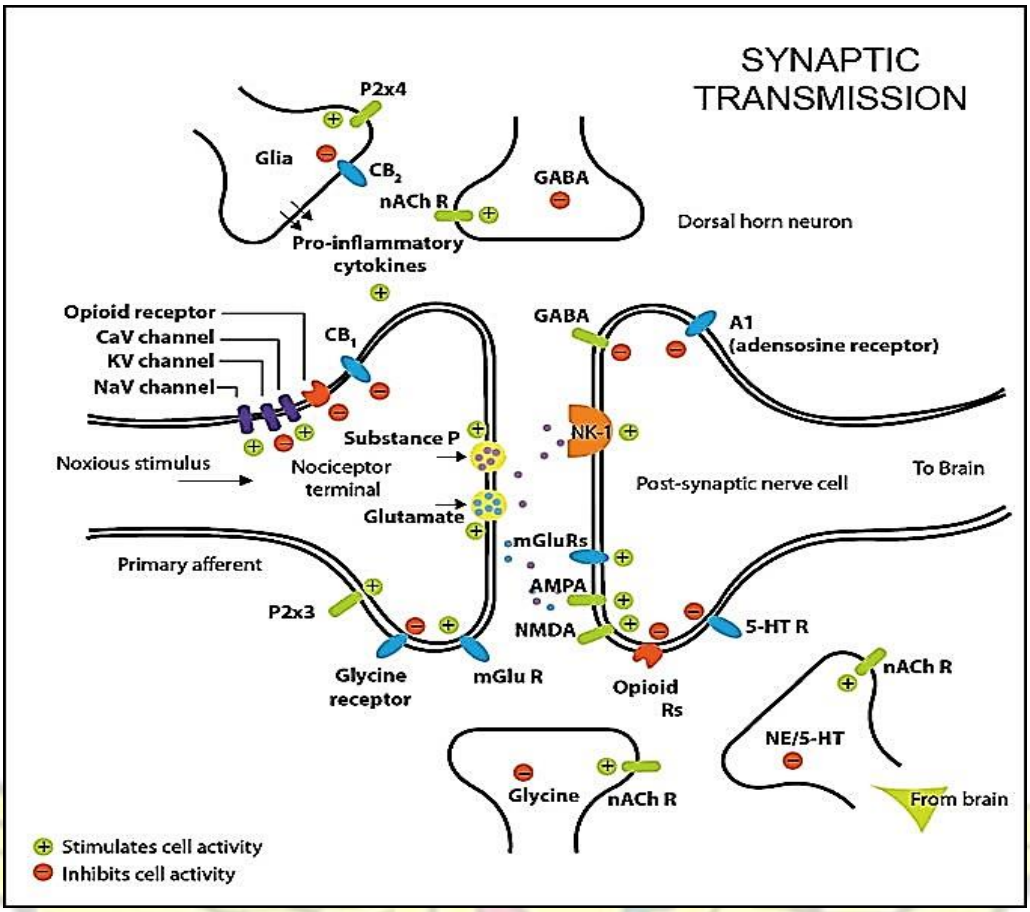


Figure 1.4 Schematic diagram of nociceptive transmission and modulation at the synapse (photo credit: projects.hsl.wisc.edu)

1.6 CLINICAL MANAGEMENT OF INFLAMMATION, PYREXIA AND PAIN

The conventional agents entail the non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatoid drugs (DMARDs) and corticosteroids. NSAIDs provide symptomatic relief in pain, pyrexia and inflammation (at higher doses) but do not slow down disease progression. Their inhibition of COX-2 provides the analgesic, antipyretic and anti-inflammatory actions. Diclofenac, ketorolac, mefenamic acid, naproxen,

ibuprofen and indomethacin, celecoxib, rofecoxib and meloxicam are some examples of NSAIDs (Vane and Botting, 1998; Mitchell and Warner, 1999).

DMARDs alter the underlying disease by slowing the progression of joint damage in rheumatoid arthritis. They possess no acute symptomatic anti-inflammatory and analgesic effects, but, eventually reduce swelling, stiffness and joint pain over a period of weeks or months. Methotrexate, ciclosporin, cyclophosphamide, hydroxychloroquine, sulfasalazine and gold are examples of conventional DMARDs. Biologics such as abatacept, rituximab, adalimumab, etanercept and infliximab are prescribed for consumers who relapse of are intolerable to DMARDs. Biologics target specific mediators of inflammation. They work faster than conventional DMARDs.

The corticosteroids suppress generalized flares of synovitis and also help to manage the disease progression. They exert pleiotropic anti-inflammatory and immunosuppressive effects. Dexamethasone, prednisolone, methylprednisolone and triamcinolone are examples of corticosteroids (Rhen and Cidlowski, 2005). Opioids and paracetamol are also used in pain management (Botting, 2000). Opioids are preferred in moderate and severe pain conditions such as post-operative and musculoskeletal pain (Fields and Martin, 2005). Some examples of opioids are morphine, pethidine, fentanyl, codeine, buprenorphine and tramadol.

1.7 EXPERIMENTAL MODELS

1.7.1 Inflammation

These models mimic the attributes of vascular permeability, local pain, leukocyte migration and hyperthermia. They provide insight and mechanisms into human pain conditions (Barbosa-Filho *et al*., 2006). The phlogistic agents used include carrageenan, zymosan, mustard oil, formalin, complete Freund's adjuvant, capsaicin, acidic saline, bee venom, lipopolysaccharide, sodium urate crystals and cytokines. The design of chronic models investigates the effects of test drugs on disease progression. The more widely used models of chronic pain are rheumatoid arthritis and osteoarthritis (Boyce-Rustay *et al*., 2010).

1.7.2 Nociception

1.7.2.1 Acute Pain Models

Pain is examined via animal behavioural responses to nociceptive stimuli. Phasic acute pain models measure the response time to a nociceptive threshold of a short duration stimulus. The target site of stimulation is the somatic region. Examples of this model based on the source of stimulus are electrical [Tail, Limb (paw) and Dental pulp stimulation], thermal (Hot plate test, Tail flick test, Tail immersion test, Hargreaves thermal hyperalgesia test) and mechanical (Paw pressure test, Pinprick test, Von Frey monofilaments test). Tonic acute pain models mimic pain arising from deep tissues and the visceral. Pain is induced by an irritant algonic chemical over a period of tens of minutes. Examples of tonic pain assays are intradermal (formalin test), intraperitoneal (i.p.; acetic acid challenge) and intradental tests (bradykinin test) (Le Bars et al., 2001; Xie, 2011).

1.7.2.2 Chronic Pain Models

Some examples of these models are carrageenan, bee venom, complete Freund's adjuvant, urate crystal and burn injury-induced models. Chronic neuropathic pain models include total sciatic nerve transection (neuroma model), sciatic cryoneurolysis, chronic constriction injury (CCI or Bennett model), streptozocin-induced diabetic neuropathy and vincristineinduced or taxol-induced peripheral neuropathy (Chemotherapy-induced model) (Wang and Wang, 2003).

1.8 JUSTIFICATION, AIM AND OBJECTIVES OF THE STUDY

1.8.1 Justification

There are several synthetic agents for inflammation, pyrexia and algesia available on the market. Nevertheless, there is incessant research into finding new and alternative agents for clinical usage. The current available remedies are very potent and effective, but however do come with considerable side effects. NSAIDs present with gastric irritation, bleeding and renal impairment. The corticosteroids cause adrenal suppression and increased susceptibility to infections. Paracetamol at high doses causes liver impairment. Respiratory depression, constipation, sedation, pruritis, nausea and vomiting are some of the associated side-effects of opioids (Fields and Martin, 2005; Rhen and Cidlowski, 2005). Moreover, most sufferers of inflammatory disorders are also discontent with their care treatments with respect to drug cost, accessibility, safety, and insufficient pain control leading to pain chronicity, functional disability and reduced quality of life (Clark and Treisman, 2006). This therefore augments the need for the development of newer and more effective agents with improved safety.

Traditional medicine still remains an important avenue for new drug sources (Butler, 2004; Newman *et al*., 2003). Disorders of inflammation, pain and pyrexia as well as peripheral oxidative imbalance are traditionally managed with plants such as *Albizia zygia*. Hence, scientific evaluation and development of plants traditionally used in inflammatory disorders is prudent. The merits of herbal medicine are as follows: (a) they are relatively cheaper with greater availability than orthodox medicine in Africa; (b) herbal remedies being natural, are well accepted by Africans; (c) they also provide cheap prototypical

products for drug synthesis (Sofowora, 1996). Based on the above reasons, *A. zygia* was chosen for the study. *A. zygia* has scanty scientific documentation on its efficacy and safety. The study is therefore aimed at scientifically establishing *Albizia zygia* folkloric use in management of inflammation, pain and pyrexia.

1.8.2 Aim and Objectives

The aim of the study is to evaluate the anti-inflammatory, antipyretic and analgesic properties of the ethanol root extract of *Albizia zygia* in animal models.

The specific objectives of the study therefore entail evaluating the extract for its:

1. Phytochemical constituents using models by Evans (2002) and Sofowora (1993)
2. Acute oral toxicity, observed using the Irwin's test
3. Acute anti-inflammatory activity during oedema
4. Antioxidant properties via *in vivo* analysis
5. Antipyretic properties in rodents (rat)
6. Analgesic properties, and its possible mechanisms using divers pain stimuli models

Chapter 2
MATERIALS AND METHODS

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Collection of Root Sample

Roots of *A. zygia* were sampled from the campus (6°40′31.8″N 1°34′44.1″W) of Kwame Nkrumah University of Science and Technology, Kumasi (KNUST), in January, 2015 and authenticated (via visual inspection) by Dr. George Henry Sam of the Department of Herbal Medicine, KNUST. A voucher specimen (no: KNUST/H/M/2016/R001) was preserved in the Department's herbarium.

2.1.2 Preparation of Extract

Root sample was room dried for a fortnight, pulverized and the powder was cold macerated with ethyl alcohol 70 % ($^{v}/_{v}$) for 5 days. The collected supernatant was concentrated in a rotavapor (R-210, BUCHI, Switzerland) at 60 ^{o}C. It yielded 9.03 % ($^{w}/_{w}$) of a browncoloured concentrate. The concentrate is here then referred to as *Albizia zygia* extract (AZE) or extract.

2.2 ANIMALS

Gallus gallus cockerels (30-50g) of a day old, purchased from Akropong Farms (Kumasi) were grouped 12-14 chicks per stainless steel cage. The chicks had free access to feed and water (kept in 1-qt gravity-fed feeders and waterers). The chicks were acclimatized to the laboratory environment and tested on 7^{th} day.

Sprague-Dawley rats (150-200 g) and ICR mice (20-30 g) of both sexes acquired from Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana, Accra were grouped (5 or 6) in stainless steel colony cages. Animals had free access to chow (Agricare Ltd, Kumasi, Ghana), and water *ad lib*. The animals were humanely handled in all experiments according to the internationally accepted principles concerning animal protection in experimental protocols (EU Directive of 2010; 2010/63/EU) (Hartung, 2010). In addition, ethical approval was granted by the Ethics Committee of the Department.

2.3 DRUGS

These enlisted materials were used: λ-carrageenan, atropine, naloxone, trichloroacetic acid (TCA), thiobarbituric acid (TBA), potassium dichromate, Tris(hydroxymethyl) aminomethane HCl, Triton X-100, ethylenediaminetetraacetic acid (EDTA), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), sodium bicarbonate (Sigma-Aldrich Inc., St. Louis, MO, USA); diclofenac sodium (Troge, Hamburg, Germany); paracetamol , morphine HCl

(Phyto-Riker, Accra, Ghana); sodium dihydrogen orthophosphate monohydrate (Hopkins & Williams Ltd, Swansea, Wales); formalin, acetic acid, theophylline, analytical grade glacial acetic acid, ethanol, hydrogen peroxide, chloroform, disodium hydrogen phosphate (BDH, Poole, England); Complete Protease Inhibitor Cocktail Tablet, EDTA-free (Santa Cruz Biotechnology, Dallas, TX, USA); baker yeast (*S. cerevisiae;* Saf do Brasil Produtos limenticios Ltd, Brazil).

2.4 PHYTOCHEMICAL SCREENING

2.4.1 Saponins (Frothing test)

An amount of 0.2 g of AZE was added to a test tube containing 4 ml of water, and vigorously agitated to froth. The mixture was monitored for at least 5 min to determine if frothing persists (Sofowora, 1993).

2.4.2 Tannins (Ferric chloride test)

About 0.2 g of AZE was added to 4 ml of water and brought to boil in a water bath. It was then cooled under running water and filtered. To 1 ml of the filtrate was added 2 ml H_2O and 5 drops of 1 % $FeCl_3$ solution and monitored for a blue-black or olive-green precipitation (Evans, 2002).

2.4.3 Flavonoids (Ammonia test)

About 5 ml of H_2O was mixed with 0.2 g of AZE and then filtered. 5 ml dilute NH_3 solution and 5 ml conc H_2SO_4 were dissolved with 1 ml aq. filtrate and monitored for a yellow colouration (Ayoola *et al*., 2008).

2.4.4 Alkaloids (Dragendorff's test)

To a 5 ml quantity of dilute HCl was added 0.2 g AZE and brought to boil for 5 min, and then cooled and filtered. To 1 ml filtrate, 3 drops of Dragendorff's reagent was introduced and monitored for reddish-orange precipitate (Sofowora, 1993).

2.4.5 General test for Glycosides (Reducing Sugars)

A mixture of 4 ml dilute H_2SO_4 and 0.2 g AZE was brought to boil for 2 min, cooled and 5 drops of 20 % NaOH was added. 1 ml of Fehling's A solution and 1 ml Fehling's B solution were then dissolved in it. It was heated for 2 min and monitored for precipitation of brick-red of cuprous oxide (Evans, 2002).

2.4.6 Phytosterols (Liebermann-Burchard's test)

An amount of 0.2 g AZE was dissolved in a mixture of 4 ml chloroform and 2 ml acetic anhydride in a test tube. About 1 ml of conc H_2SO_4 was carefully introduced along the inner tube walls and observed for the formation of a blue colour at the interface (Sofowora, 1993).

2.4.7 Terpenoids (Salkowski test)

A mixture of 4 ml chloroform and 0.5 g AZE was prepared in a test tube. About 1 ml conc H_2SO_4 was carefully streamed down the inner tube walls. The mixture was observed for the formation of a reddish-brown coloration at the interface (Sofowora, 1993).

2.5 ACUTE TOXICITY STUDIES (IRWIN'S TEST)

Five groups (n=5) of ICR mice (20-25 g) of both sexes were kept in the vivarium to acclimatize for a day. Animals were denied food overnight, but had no restraint to water.

Mice were assessed for their basal behavioural functions on the test day (0 h) before the oral administration of AZE (0.3, 1, 3 and 5 g/kg) or vehicle (10 ml/kg of normal saline). Animals were then monitored for gross behavioural and physiological functions (Irwin, 1968). They were monitored at 0, 15, 30, 60, 120 and 180 min, and also at 24 h post-AZE administration. Characteristic behaviours with respect to CNS stimulation, CNS depression, autonomic functions and also death were assessed. The subjects were also monitored daily for up to 14 days to detect any delayed deaths.

2.6 ANTI-OEDEMIC AND ANTIOXIDANT ACTIVITIES

2.6.1 Carrageenan-induced oedema test

The acute anti-oedemic effect of AZE was investigated in the carrageenan-induced oedema model in chicks using a modification of the method by Roach and Sufka (2003).

A volume of 10 µl carrageenan suspension (2 %, $^{w}/_{v}$) was administered into the chicks' right footpads. Dorso-ventral ipsilateral pedal thickness was measured initially (0 h) and at every hour post-carrageenan administration for 5 h (using electronic callipers Z22855, Milomex Ltd, Bedfordshire, UK) (Murayama *et al*., 1991). Oedema was calculated as the normalized percentage change in foot thickness from time 0 h using the equation:

$$\%\ change\ in\ pedal\ thickness = \left(\frac{V_t - V_o}{V_o}\right) \times 100$$

Where, V_o is carrageenan pre-administration pedal thickness (0 h)

V_t is carrageenan post-administration pedal thickness (at time *t*)

Two sets of experiment, prophylactic and curative, were performed. Drug administrations were made 30 min (intraperitoneal; i.p) or 1 h (oral) pre-carrageenan challenge in the prophylactic experiment; or 1 h post-carrageenan in the curative protocol. Chicks were grouped according to their drug treatments at n=6 (prophylactic treatment) and n=7 (curative treatment). The treatments groups were: vehicle control (10 ml/kg of 2 % tragacanth; *p.o.*), AZE suspended in 2 % tragacanth (30-300 mg/kg; *p.o.*) or diclofenac (3-30 mg/kg; i.p.).

2.6.2 Antioxidant Assay

The assay of endogenous antioxidants was carried out according to the protocol prescribed by Halici *et al*. (2007). Intraplantar carrageenan administration was made into the subcutaneous paw tissues of rats, which subsequently induced oxidative stress at the inflamed site. Rats were assigned into eight groups (n=3) with respective drug treatments as follows: experimental naïve control (normal saline; 10 ml/kg; i.p.), vehicle control (tragacanth; 10 ml/kg 2 % $^{w}/_{v}$; *p.o.*), AZE suspended in 2 % tragacanth (30-300 mg/kg; *p.o.*) or diclofenac (3-30 mg/kg; i.p.). General intraplantar introduction of 100 µl carrageenan (1 % $^{w}/_{v}$), either 30 min (i.p.) or 1 h (*p.o*) post-drug administration, induced oedema in the right hind paw.

2.6.2.1 Biochemical Investigations

Five (5) hours after carrageenan administration, rats were sacrificed by cervical dislocation. Oedematous paw tissues were harvested and immediately stored at -80 ^{o}C for subsequent enzyme analysis. A volume of 4.5 ml of TNG buffer at pH 7.4 (150 mM Tris HCl, 150 mM NaCl, 1 % Triton X 100, 10 % glycerol and protease inhibitor cocktail) was added to each sample, and homogenized on ice using a homogenizer (Ultra-Turrax T-25;

IKALabortechnik, Staufen, Germany) at 24000 $\times g$ for 15 min. Each sample was centrifuged at 4000 $\times g$ for 20 min, decanted and preserved for enzyme analysis. Sample protein content was calculated using the Bradford method (Bradford, 1976). All microtiter plate readings were carried out in triplicates.

2.6.2.1.1 Superoxide Dismutase (SOD) Assay

Adrenaline autoxidation to adrenochrome is prevented in the presence of SOD (Misra and Fridovich, 1972). A 20 min centrifugation of a mixture of 500 µl homogenate, 150 µl of ice-cold chloroform and 750 µl ethanol (96 % $^{v}/_{v}$) was carried out at 2000 $\times g$. Successive dissolutions of 1 ml carbonate buffer (0.1 M; pH 10.2) and 0.5 ml EDTA (0.6 mM) into 500 µl of the supernatant were made. A 1.3 mM adrenaline solution of volume 0.05 ml was then added to initiate adrenochrome formation. A blank solution containing all reagents (except tissue homogenate) was processed in a similar manner. A pipetted volume of 150 µl was then dispensed into a 96-well plate. Absorbance was read spectrophotometrically at 480 nm using Synergy H1 Multi-mode Reader (BioTek Technologies, Winooski, VT, USA). The percentage inhibition of adrenaline autoxidation was calculated as:

$$\%\ inhibition = \left(\frac{Absorbance_{\ test} - Absorbance_{\ blank}}{Absorbance_{test}}\right) \times 100$$

Specific SOD activity was expressed in units per mg protein, where 1 unit is the enzyme quantity needed to prevent 50 % of the autoxidation of adrenaline at 25 °C; calculated using the formula:

$$Units\ of\ \ SOD\ activity/mg\ protein = \left(\frac{\%\ inhibition}{50\ \times wt\ of\ protein}\right)$$

2.6.2.1.2 Catalase (CAT) Assay

The test principle is based on the propensity of CAT to hydrolyse H_2O_2, thereby inhibiting the dichromate in acetic acid reduction to chromic acetate by H_2O_2 (Sinha, 1972). Successive additions of 0.4 ml H_2O_2 (1.18 M) and 1 ml phosphate buffer (0.01 M; pH 7.0) were made to 0.1 ml of homogenate and incubated for 5 min (25 ^oC). A 2 ml dichromateacetic acid mixture (containing 3 parts glacial acetic acid and 1 part 5 % potassium dichromate) was then added to terminate the reaction. A volume of 150 µl of the mixture was pipetted into a 96-well plate. Chromogenate absorbance was calorimetrically read at

620 nm.

Specific CAT activity was expressed as units per mg protein based on the molar extinction coefficient of H_2O_2, 39.4 M-1 cm-1 at 620 nm. One unit is the enzyme quantity needed to hydrolyse 1 mmol of H_2O_2/min in a neutral pH (25 °C), i.e.

$$mUnit\ of\ CAT\ activity/mg\ protein = \left(\frac{Absorbance_{620\ nm}}{39.4\ \times wt\ of\ protein}\right) \times 1000$$

2.6.2.1.3 Reduced Glutathione (GSH) Assay

The concentration of GSH in inflamed paw was measured by the procedure stated by Ellman (1959). To 100 µl of the homogenate was added 2.4 ml 0.02 M EDTA; solution was then cooled at 4 °C for 10 min. Additions of 2 ml H_2O and 0.5 ml of 50 % $^w/_v$ TCA were made to the mixture and centrifuged at 3000 $\times g$ for 5 min. About 50 µl of 10 mM DTNB solution and 2 ml of Tris buffer (0.4 M; pH 8.9) were then thoroughly mixed with 1 ml of the supernatant and the reaction incubated for 5 min (25 ^oC). A reaction mixture was repeated also for the blank. The wells of 96-well plate were filled with 150 µl of

mixture and absorbance was spectrophotometrically read at 412 nm. GSH concentration was expressed in µmol per mg protein, and determined using the curve $y = 0.0004\,x + 0.0026$.

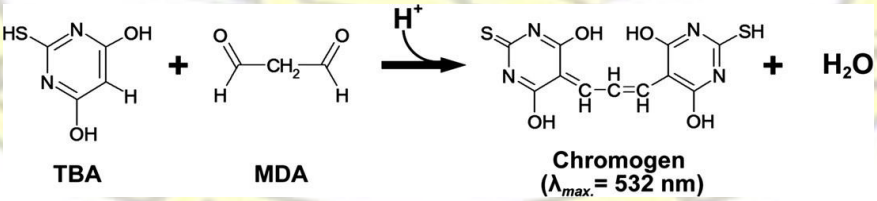
2.6.2.1.4 Myeloperoxidase (MPO) Assay

Enzyme concentration was determined spectrophotometrically by a modified o-dianisidine method by Şenoğlu et al. (2009). An assay mixture- consisting of 5 ml 0.02 M *o*dianisidine + 3 ml H_2O_2 (0.01 M) + 3 ml phosphate buffer (0.1 M; pH 6.0) and made up to 30 ml- was freshly prepared. About 125 µl aliquot of it was dispensed into a 96-well microtiter plate and topped up with 0.01 ml of tissue homogenate. The absorbance increase was read pronto at 460 nm in 60 s cycles for 600 s. MPO specific activity was expressed in units per mg protein, where 1 unit increases absorbance by 0.001 per 60 s.

$$Unit\ of\ MPO/mg\ protein = \left(\frac{Absorbance_{460\ nm}/min}{0.001\ \times (total\ protein/10\ \mu l\ aliquot)}\right)$$

2.6.2.1.5 Lipid Peroxidation Assay

Polyunsaturated fatty acid (PUFA) degradation is measured by the amount of malondialdehyde (MDA) formed. It is the commonly used indicator of PUFA oxidative damage in the thiobarbituric acid (TBA) assay due to the chromogenic complex formed.



The level of MDA formation was determined by a modified Heath and Packer (1968) protocol. A 1 ml volume of homogenate was added to 3 ml of the mixture (3 ml 20 % TCA

containing 0.5 % TBA) in a test tube. It was heated at 95 °C for 30 min, cooled immediately and then centrifuged at 5000 $\times g$ for 10 min. Absorbance was initially read at 532 nm and then read again at 600 nm to correct for nonspecific absorbance. The molar extinction coefficient of MDA-TBA abduct, 155 $mM^{-1}cm^{-1}$, was used to determine the levels of MDA from the equation:

$$nmol\ MDA/mg\ protein = \frac{Absorbance_{532\ nm} - Absorbance_{600\ nm}}{155 \times total\ protein} \times 10^6$$

2.7 ANTIPYRETIC ACTIVITY

2.7.1 Baker's Yeast-Induced Pyrexia

The method described by Tomazetti *et al*. (2005) was employed. A total of 48 Sprague Dawley rats in 8 groups (n=6) were fasted overnight and during the experiment, but were unrestrained from drinking water. A 2-day habituation session was conducted for the subjects i.e. a lubricated digital thermometer was used to measure their rectal temperature

(T_R). On day 3, basal T_R was checked before administration of 0.135 g/kg baker's yeast (i.p). Change in T_R was monitored hourly for 8 h. At 4 h, individual rat groups were administered with their respective drugs: vehicle control (2 % tragacanth solution 10 ml/kg; *p.o*); AZE (30-300 mg/kg; *p.o*.) or paracetamol (15, 50, 150 mg/kg; *p.o*.); naïve control were given 0.5 ml (i.p.) of normal saline. The final T_R were calculated as normalized readings from their respective 0 h values.

2.8 ANTI-NOCICEPTIVE ACTIVITY

2.8.1 Acetic Acid-Induced Writhing Test

Acetic acid (10 ml/kg of 0.6 % $^{v}/_{v}$; i.p.) administration to mice induces nociception in the peritoneum (Koster *et al.*, 1959) which is depicted by abdominal contractions called writhing. Writhing is portrayed by abdominal stretching coupled with hind limb extension. Drug administrations were made 30 min (intraperitoneal) or 1 h (oral) prior to acetic acid injection; the mice (n=5) were administered vehicle (10 ml/kg, *p.o.*), or AZE (30-300 mg/kg; *p.o.*) or diclofenac (3-30 mg/kg; i.p.). Mice were placed (1 per cell) in a perspex chamber of cell dimensions 15 cm × 15 cm × 15 cm. A camcorder (Everio^{TM}, model GZMG1300) placed opposite a mirror situated obliquely beneath the chamber captured the panorama of all the mice. The total number of writhes was captured for 30 min (5 min blocks). JWatcherTM Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia) was used to track the nociceptive behaviour (starting on the 5th min) for anti-nociceptive analysis.

2.8.2 Formalin test

The right hind paw of mice were subcutaneously injected with 10 µl of 5 % formalin (an algogen) which resulted in the characteristic nociceptive behaviour of licking, biting, or lifting or shaking of the ipsilateral paw (Dubuisson and Dennis, 1977; Abbott et al., 1995).

A habituation session of 2 h in the test chamber was undertaken for all mice. Drug administrations were made 30 min (intraperitoneal) or 1 h (oral) prior to formalin injection; the mice (n=5) were pre-administered with vehicle (10 ml/kg, *p.o.*), or AZE (30, 100, 300 mg/kg; *p.o.*) or morphine (1, 3, 10 mg/kg; i.p.). After which each subject was injected with

formalin and nociceptive behaviour was tracked for 1 h (similar to section 2.8.1). Total nociception was computed as the product of the length of time and frequency of licking/biting of the ipsilateral paw. The scores were presented as mean ± SEM in divisions of the initial (0-10 min) and later (10-60 min) phases.

2.8.3 Tail Immersion Test

The test principle involves measuring the tail withdrawal threshold i.e. the time (s) a rat takes to withdraw the tail from the water bath (49±0.5 ºC) (Janssen *et al.*, 1963). The maximum allowable time for retraction of tail was set at 10 s to prevent tail damage.

Baseline withdrawal threshold was determined for all rats (n=5) before drug treatments: vehicle control (10 ml/kg; *p.o.*), or AZE (30-300 mg/k; *p.o.*) or morphine (1, 3 and 10 mg/kg; i.p.). Each treatment group was then experimented on at 0.5, 1, 2, 3, 4 and 5 h posttreatment. Increase in tail retraction time was reckoned as antinociceptive activity. Analgesic effect was expressed as percentage maximal possible effect (MPE), as determined by the equation:

$$\% MPE = \frac{T_2 - T_1}{T_o - T_1} \times 100$$

Where T_o is the cut-off time; T_1, T_2 are respective pre- and post- treatment retraction times.

2.8.4 Carrageenan-induced Mechanical Hyperalgesia

The effect of AZE on mechanical hyperalgesia was determined using a modified rat paw pressure test (Randall, 1957; Villetti *et al.*, 2003). In this test the blunt perspex cone of an analgesimeter (Model No. 15776, Ugo Basile) impresses a linearly-increasing pressure to the dorsal surface of the paw till the rat withdraws its tail. The pressure (grams) required to

evoke a withdrawal was adjudged the paw withdrawal threshold (PWT). The maximum allowable pressure for paw retraction was 250 g to avoid damage to the paw.

A total of 10 animal groupings (n=5) were used for the experiment: vehicle control (2 % tragacanth solution; 10 ml/kg; *p.o.*), AZE (30-300 mg/kg, *p.o*), diclofenac (3-30 mg/kg, i.p.) or morphine (1-10 mg/kg, i.p.). The experimentally naïve mice were conditioned to the experimental process for 2 prior days. Baseline PWT was determined again on the test day which was followed by the administration of 100 µl of a 2 % $^{w}/_{v}$ carrageenan suspension into the right hind paw. Induction of mechanical hyperalgesia was confirmed by measuring the PWT on the ipsilateral paw at 2.5 h. Rats received their respective treatments and PWT determinations were made at 3.5, 4, 4.5, 5, 5.5 and 6 h. Decrease in hyperalgesia was calculated as % MPE.

2.8.5 Exploration of Possible Anti-nociceptive Mechanisms of AZE

Nociceptive transmission involves several mediators acting in several pathways. The formalin test affords the evaluation of the involvement of such nociceptive pathways. The possible anti-nociceptive mechanisms of AZE were investigated by pre-emptive blocking of nociceptive pathways with different antagonists. Antagonist doses were extracted from experimental literature obtained from the Department library.

2.8.5.1 The Opioidergic Pathway

The opioidergic pathway was antagonized in mice (n=5) by pre-administering naloxone (2 mg/kg; i.p.). After 15 min the various groups received their respective treatments and the formalin test was performed (section 2.8.2).

2.8.5.2 The Adenosinergic Pathway

The adenosinergic pathway was antagonized in mice (n=5) by pre-administering theophylline (10 mg/kg; i.p.). After 15 min the various groups received their respective treatments and the formalin test was performed (section 2.8.2).

2.8.5.3 The Muscarinic Cholinergic Pathway

The muscarinic cholinergic pathway was antagonized in mice (n=5) by pre-administering atropine (5 mg/kg; i.p.). After 15 min the various groups received their respective treatments and the formalin test was performed (section 2.8.2).

2.8.6 Rotarod test

A sample of 30 experimentally naïve mice (n=5) were habituated to the experimental process for 3 successive days by conditioning them to walk on a rota-rod (Ugo Basile, model 7600, Comerio, Varese, Italy) revolving 20 times per min for 3 min. A cut-off latency of 180 s was set as the maximum limit. The mice were placed on the rod for a minimum of 10 s (at 0 rpm) to acclimatize to the environment. Mice which failed to learn the test or fell off the rod before the 180 s cut-off limit were excluded from the study. Judgment of neurological deficit was based on the time of fall from the rotating rod within the test period (Dunham and Miya, 1957). On day 4, the animals were administered their respective drugs: AZE (30-300 mg/kg; *p.o*), *d*-tubocurarine (0.1 mg/kg; i.p.), diazepam (8 mg/kg; *p.o*) and normal saline (10 ml/kg; *p.o.*). The mice were made to walk at 0, 0.5, 1.0, 1.5 and 2 h post drug administration to determine their performance.

2.9 ANALYSIS OF DATA

Statistical analyses and ED_{50} determinations were done using GraphPad Prism for Windows version 6 (GraphPad Software, San Diego, CA, USA). The time course curves were analyzed by two way repeated measures analysis of variance (ANOVA). The mean treatment differences at each time period were compared by Tukey *post hoc* test. Subsequent area under the curves (AUCs) were then calculated (arbitrary units) to determine the holistic treatment effect. One-way ANOVA was used to analyse the AUC mean differences; pairwise comparisons were done using Tukey *post hoc* test. $P<0.05$ between treatments was accepted as statistically significant. The percentage attenuation of effect with respect to the control was determined by the following equation:

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

F-test analysis was done for the ED_{50}s (Motulsky and Christopoulos, 2004). The nonlinear regression equation below was used to determine the ED_{50}s of each treatment with the aid of an iterative computer least squares configuration.

$$Y = \frac{a+(b-a)}{(1+10^{(LogED50-X)})}$$

Where, X = Log [Dose] and Y = response. Y commences from the minimum (a) and ends at the maximum (b) in a sigmoidal form.

Chapter 3

RESULTS

3.1 PHYTOCHEMICAL SCREENING

Saponins, flavonoids, alkaloids, condensed tannins, triterpenoids and glycosides were found to be present in AZE after the phytoconstituent analysis (Table 3.1).

Table 3.1 Primary phytochemical screening of AZE

TEST	PHYTOCHEMICAL	OBSERVATION	INFERENCE
Frothing	Saponins	Persistent frothing	++
Ferric chloride	Tannins	Olive-green precipitate	++
Ammonia	Flavonoids	Yellow coloration	++
Dragendorff's	Alkaloids	Orange-red precipitate	+
Fehling's	Glycosides	Brick-red precipitate	+
Lieberman-Burchard's	Sterols	No blue coloration	-
Salkowski	Terpenoids	Reddish-brown coloration	+

-: Not detected; +: Low quantity; ++: High quantity

3.2 ACUTE TOXICITY STUDIES (IRWIN'S TEST)

There was no lethality observed in all the animals after 2 weeks. There was positive effect for analgesia when tested at all dosages of AZE. No adverse event was observed after 300 mg/kg oral administration. Doses of 1000-5000 mg/kg elicited sedation whilst doses greater than 1000 mg/kg demonstrated marked urination (muscarinic effects).

Table 3.2 Gross behavioural changes after AZE administration in Irwin's test

Dose (mg/kg)	Deaths	Physiological signs
0	0/5	-
300	0/5	Analgesia
1000	0/5	Sedation, analgesia
3000	0/5	Sedation, analgesia, urination
5000	0/5	Sedation, analgesia, urination

3.3 ANTI-OEDEMIC AND ANTI-OXIDANT ACTIVITIES

3.3.1 Chick Carrageenan Challenge

The introduction of 10 µl of 2 % carrageenan suspension into pedal tissues of 7-day old chicks generates an acute local oedema. Oedema peaked at 2-3 h in all paws (fig. 3.1 and

3.2), correlating with the findings of treatments across the time courses of AZE (30-300 mg/kg; *p.o.*) and diclofenac (3-30 mg/kg: i.p.) (Roach and Sufka, 2003). Figures 3.1 (a, c) (pre-emptive) and 3.2 (a, c) (curative) represent the time course of effects of the respective treatments. The column graphs of diclofenac and AZE are represented in fig. 3.1 (b, d) for preemptive and fig. 3.2 (b, d) for curative treatments respectively.

Analysis of the curves with respect to the vehicle control group shows a significant reduction in the mean maximal paw oedema at 2-3 h by AZE (prophylaxis: $F_{3, 20}$=10.87, P<0.0001; therapeutic: $F_{3, 24}$=6.107, P= 0.0031) and diclofenac (prophylaxis: $F_{3, 99}$=97.64, P<0.0001; therapeutic: $F_{3, 24}$=20.32, P<0.0001). Total oedema was significantly reduced by the drug treatments except AZE 30 mg/kg (*i.p.*). AZE demonstrated prominent inhibition of mean total oedema by 57.65±6.70 % (pre-emptive) and 55.41±7.37 % (curative) at dose

300 mg/kg. Diclofenac also similarly inhibited the mean total oedema by 77.08±3.80 % (pre-emptive) and 75.87±4.00 % (curative) at 30 mg/kg.

Figure 3.3 compares the dose-response relationships of AZE and diclofenac. The ED_{50} values of AZE and diclofenac (obtained by non-linear regression) were 195.2±63.98 mg/kg (pre-emptive); 1650±6.63 mg/kg (curative) and 1.43±0.69 mg/kg (pre-emptive);

38.05±1.49 mg/kg (curative) respectively (Table 3.3).

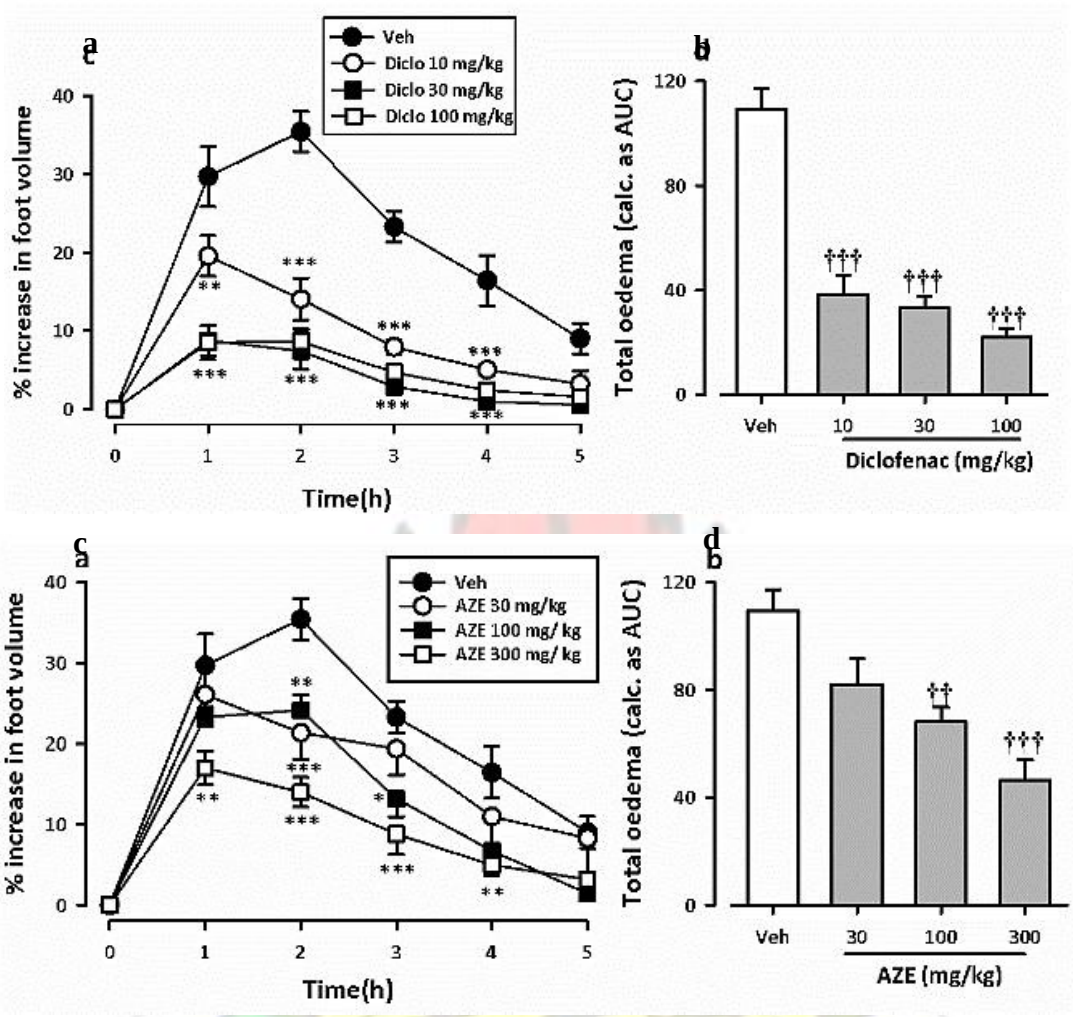


Figure 3.1 Prophylactic reduction of pedal oedema (mean ± S.E.M.; n=6) by diclofenac (3-30 mg/kg, i.p.) (**a** and **b**) and AZE (30-300 mg/kg; *p.o.*) (**c** and **d**) after intraplantar carrageenan injection in chicks. Left panels show the time course of effects over the 5 h period and the right panels show the total oedema score calculated from AUCs over the 5 h. The administration of 0.01 ml of 20 mg/ml carrageenan suspension induced acute pedal oedema over 5 h.

*/**/*** P<0.05/<0.01/<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test).

††/††† P<0.01/<0.001 versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

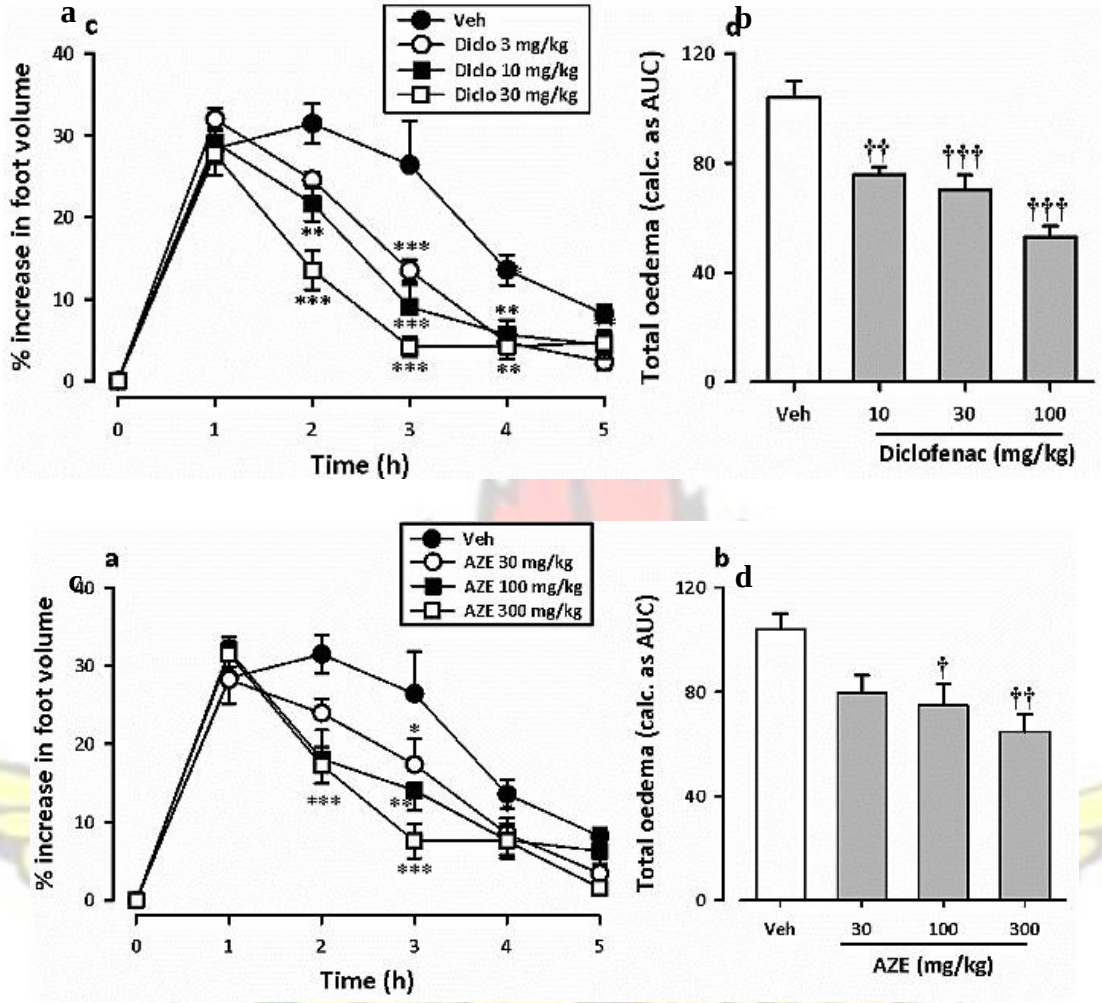


Figure 3.2 Therapeutic reduction of pedal oedema (mean ± S.E.M.; n=6) by diclofenac (3-30 mg/kg, i.p.) (**a** and **b**) and AZE (30-300 mg/kg; *p.o.*) (**c** and **d**) after intraplantar carrageenan injection in chicks. Left panels show the time course of effects over the 5 h period and the right panels show the total oedema score calculated from AUCs over the 5 h. The administration of 0.01 ml of 20 mg/ml carrageenan suspension induced acute pedal oedema over 5 h. $^{*/**/***}P$<0.05/<0.01/<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test). $^{\dagger/\dagger\dagger/\dagger\dagger\dagger}P$<0.05/<0.01/<0.001 versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

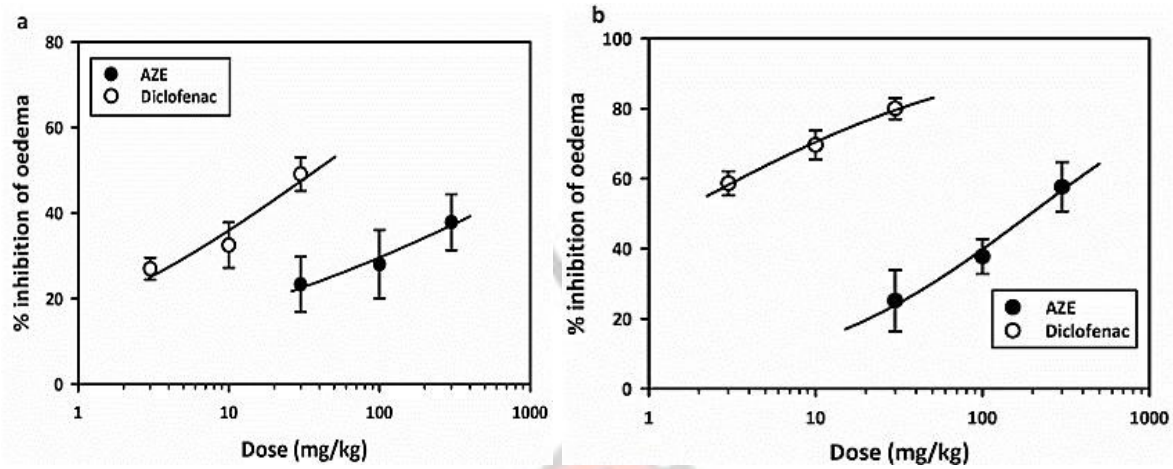


Figure 3.3 Comparison of dose-response relationship of diclofenac (3-30 mg/kg; i.p.) and AZE (10-300 mg/kg; *p.o*) in the therapeutic (a) and prophylactic (b) assays of oedema induced by carrageenan in chicks.

Table 3.3 ED_{50} values obtained from dose-response curves of the chick carrageenan challenge

Drugs	**ED_{50} (mg/kg)**	
	Prophylaxis	Therapeutic
Diclofenac	1.43±0.69	38.05±1.49
AZE	195.2±63.98	1650±6.63

3.3.2 Antioxidant Activity

3.3.2.1 Catalase Assay

The intraplantar carrageenan administration induced an acute local inflammation in the rat paw with resultant oxidative stress. The oxidative stress caused a marked reduction in CAT levels in negative control group compared to the naïve group (fig. 3.4a). On the other hand, AZE (100 and 300 mg/kg; *p.o.*) and diclofenac (3-30 mg/kg; i.p.) increased CAT activity ($F_{7, 16}$=16.24, P<0.0001). The lowest dose of AZE produced no significant change in CAT activity but a dose-dependent effect was seen with diclofenac. Antioxidant levels after drug administration are relatively higher than that of naïve rats due to increased expression so as to overcome excess ROS generated and to establish eventual oxidative balance in peripheral tissues.

3.3.2.2 Superoxide Dismutase Assay

The vehicle-treated group showed a marked reduction in SOD concentration at the inflamed site compared to that in the naïve subjects (fig. 3.4b). AZE (100 and 300 mg/kg; *p.o.*)-treated rats had a significant upregulation of SOD activity ($F_{4, 10}$=13.91, P=0.0004). However, a dose-dependent effect was obtained with diclofenac (3-30 mg/kg; i.p.) ($F_{4, 10}$=21.71, P<0.0001).

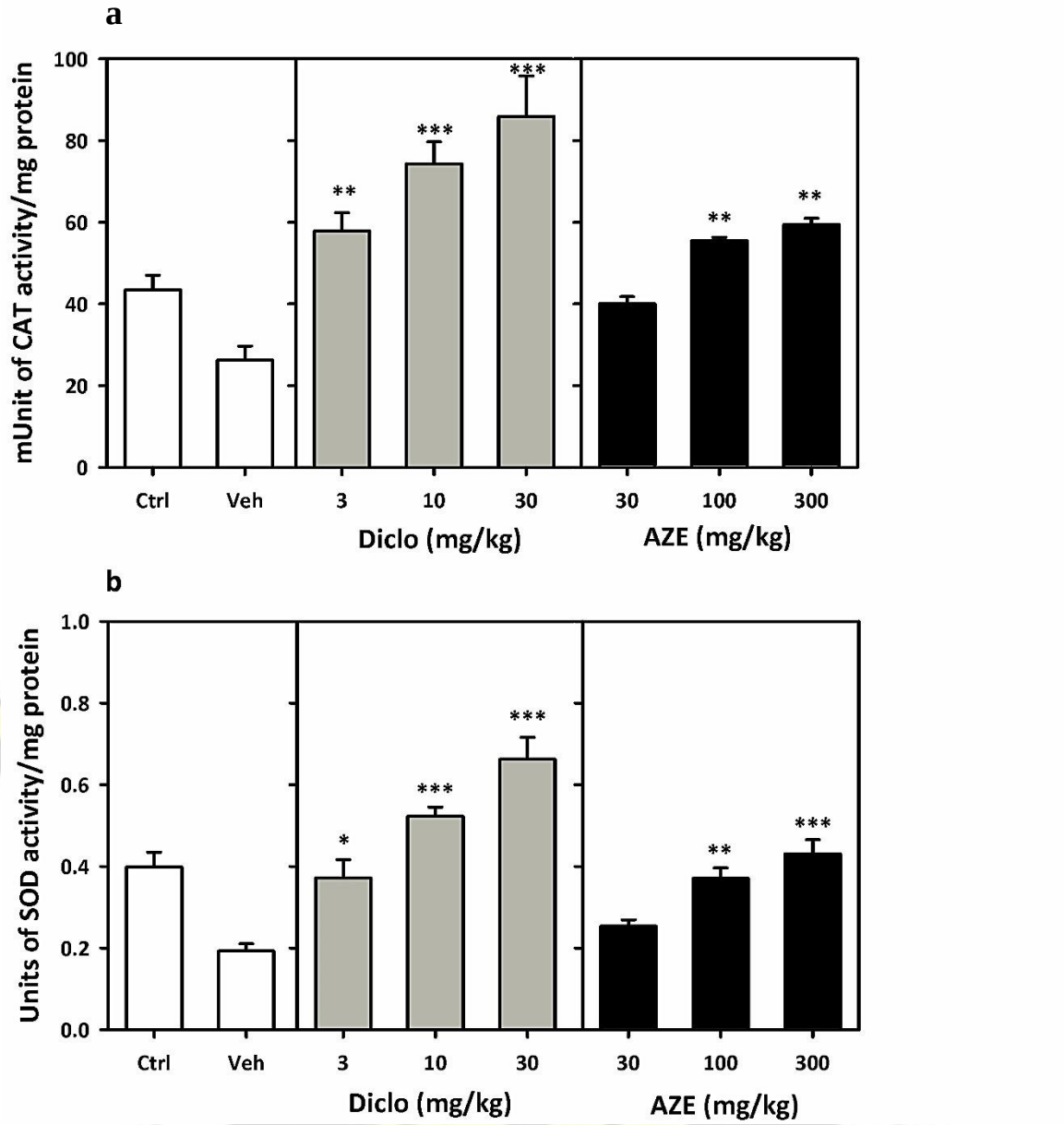


Figure 3.4 Increased expression of CAT (a) and SOD (b) (mean ± S.E.M.; n=3) by AZE (30-300 mg/kg; p.o.) and diclofenac (3-30 mg/kg, i.p.) in carrageenan-induced peripheral oxidative stress in rats. The administration of 100 µl of carrageenan suspension (10 mg/ml) induced acute pedal oedema and oxidative imbalance over 5 h. $^{*/**/***}P<0.05/<0.01/<0.001$ versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

3.3.2.3 Reduced Glutathione Assay

GSH is a cofactor needed in the degradation of H_2O_2 by glutathione peroxidase (GPx) and detoxification of hydroperoxides by glutathione s-transferase (GST). Increased oxidative imbalance significantly reduced GSH concentration (fig. 3.5a). Preemptive administration of AZE (300 mg/kg; *p.o.*) and diclofenac (10 and 30 mg/kg; i.p.) however, significantly increased GSH expression in the ($F_{7, 16}$=16.81, P<0.0001).

3.3.2.4 Myeloperoxidase Assay

Increased neutrophil activity proportionally amplified MPO activity in the negative control group relative to naïve group (fig. 3.5b). AZE and diclofenac pre-treatments significantly reversed the increased MPO concentration in their respective groups ($F_{5, 11}$=3.221, P=0.0493). The significant effects were seen in only AZE 100 mg/kg (*p.o.*) and diclofenac 30 mg/kg (i.p.)

3.3.2.5 Lipid Peroxidation Assay

Oxidative stress increases lipid peroxidation with a resultant increase in malondialdehyde generated from the decomposition of lipid hydroperoxides. The measurement of MDA levels revealed an increased concentration in negative control group relative to the naïve group (fig. 3.6). The administration of AZE (30-300 mg/kg; *p.o.*) and diclofenac (3-30 mg/kg; i.p.) significantly and dose-dependently reduced MDA levels ($F_{7, 16}$=10.47, P<0.0001).

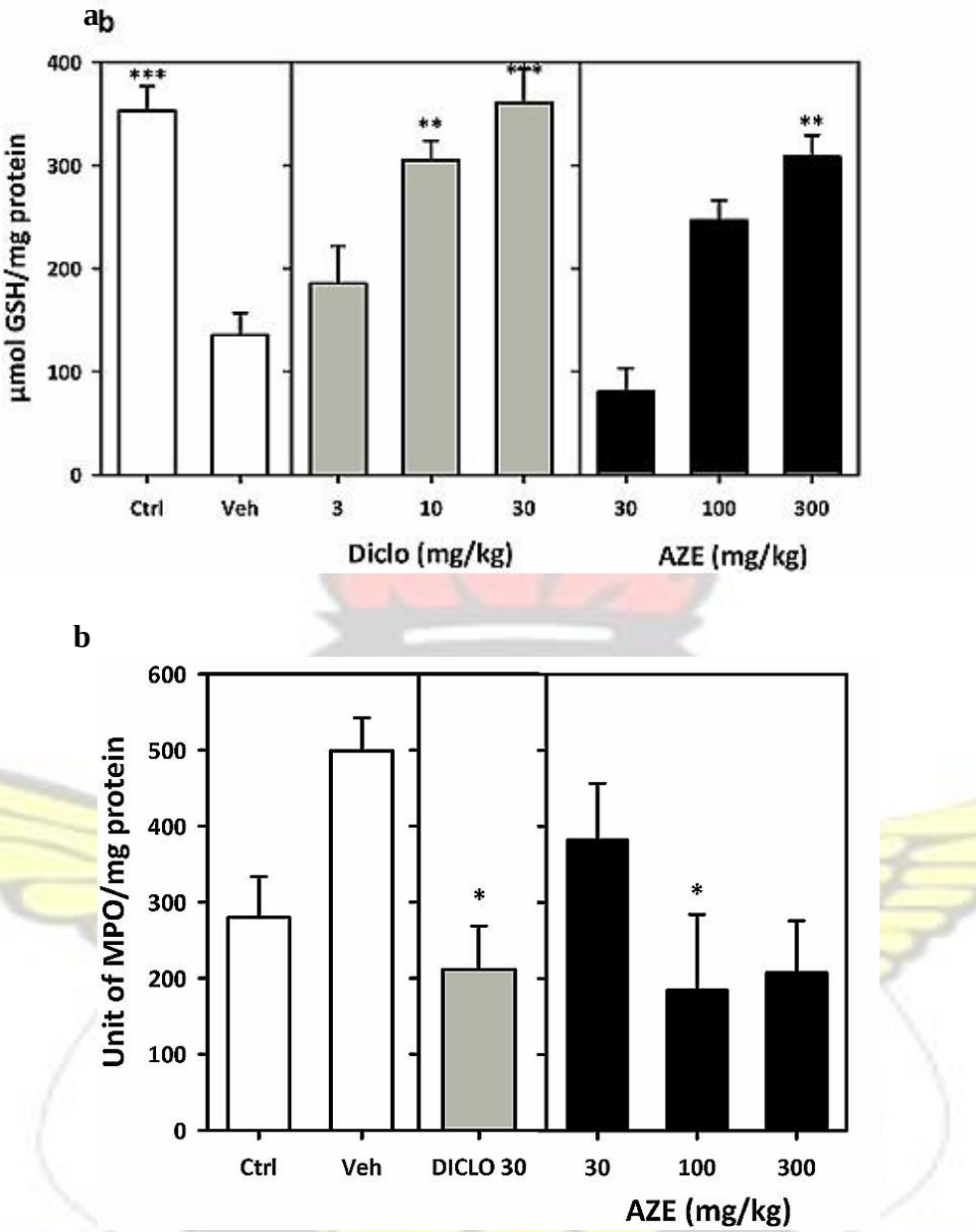


Figure 3.5 Activity of AZE (30-300 mg/kg; p.o.) and diclofenac (3-30 mg/kg, i.p.) in carrageenan-induced peripheral oxidative changes (mean ± S.E.M.; n=3) in GSH (a) and MPO (b) levels in rats. The administration of 100 µl of carrageenan suspension (10 mg/ml) induced acute pedal oedema and oxidative imbalance over 5 h. $^{*/**/***}P<0.05/<0.01/<0.001$ versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

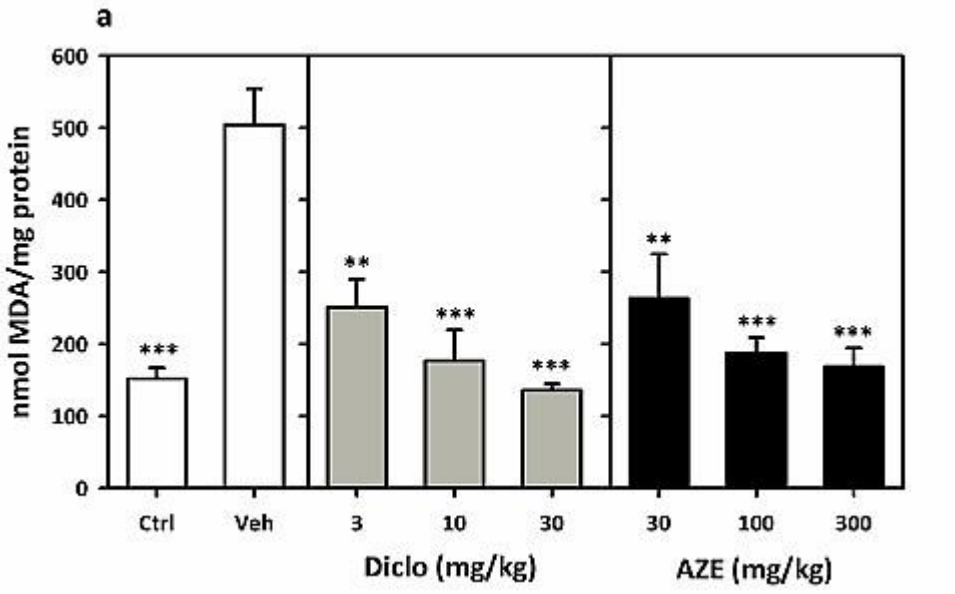


Figure 3.6 Reduction of MDA levels (mean ± S.E.M.; n=3) by AZE (30-300 mg/kg; p.o.) and diclofenac (3-30 mg/kg, i.p.) in carrageenan-induced peripheral oxidative stress in rats. The administration of 100 µl of carrageenan suspension (10 mg/ml) induced acute pedal oedema and oxidative imbalance over 5 h. $^{**/***}P<0.01/<0.001$ versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

3.4 ANTIPYRETIC ACTIVITY

3.4.1 Intraperitoneal Baker's Yeast Challenge in Rats

Pyrexia was prominently manifested in rats after Baker's yeast (0.135 g/kg; i.p) administration, typifying the observations indicated by Tomazetti *et al*. (2005). Pyrexia peaked after the 4 h (except the experimentally naïve subjects), and was sustained till the 8 h in all groups. Analysis of the curves with respect to the vehicle treatment group shows marked reduction in elevated T_R by AZE (30-300 mg/kg; *p.o.*) ($F_{4, 24}$=18.39; P<0.0001; fig 3.7c) and paracetamol (15-150 mg/kg; *p.o.*)($F_{4, 25}$=15.73; P<0.0001; fig. 3.7a).

From the AUCs, AZE dose-dependently and significantly reduced the elevated rectal temperature ($F_{4, 24}$=16.04; P<0.0001) (fig. 3.7d). Also, paracetamol, the positive control, significantly and dose-dependently attenuated the induced rectal hyperthermia ($F_{4, 25}$=17.99; P<0.0001) as depicted in fig. 3.7b.

Figure 3.8 compares the dose-response relationships of AZE and paracetamol. The ED_{50} values obtained by non-linear regression were 48.59±2.59 mg/kg and 26.19±1.33 mg/kg for AZE and paracetamol respectively. Thus, paracetamol was twice as potent as AZE (Table 3.4).

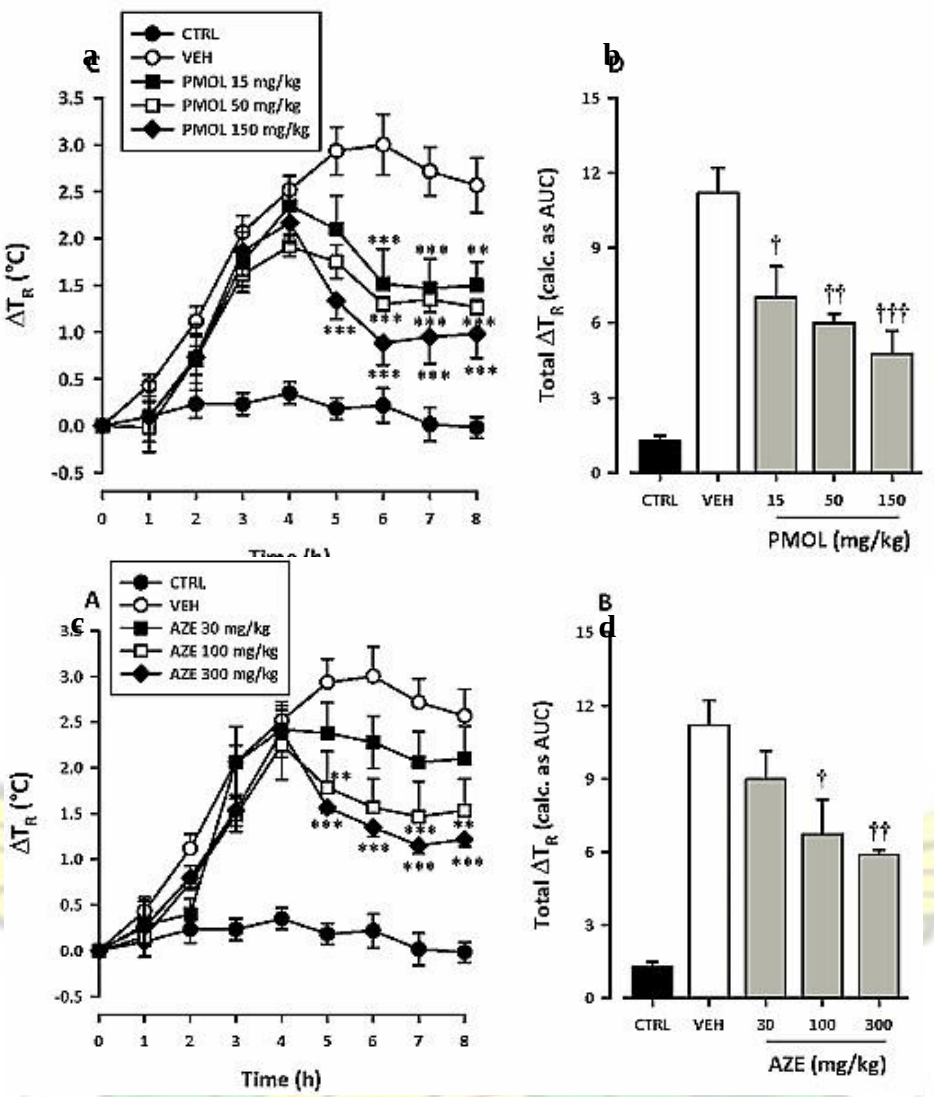


Figure 3.7 Reduction in rectal temperature (T_R) (mean ± S.E.M.; n=6) by paracetamol (15-150 mg/kg; *p.o.*) (**a** and **b**) and AZE (30-300 mg/kg; *p.o.*) (**c** and **d**) after intraperitoneal baker's yeast administration in rats. Left panels show the time course of effects over the 8 h period and the right panels show the total pyretic score calculated from AUCs over the 8 h. The administration of baker's yeast (0.135 g/kg; i.p.) induced fever over 4 h. $^{**/***}P$<0.01/<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test). $^{\dagger/\dagger\dagger/\dagger\dagger\dagger}P$<0.05/<0.01/<0.001 versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

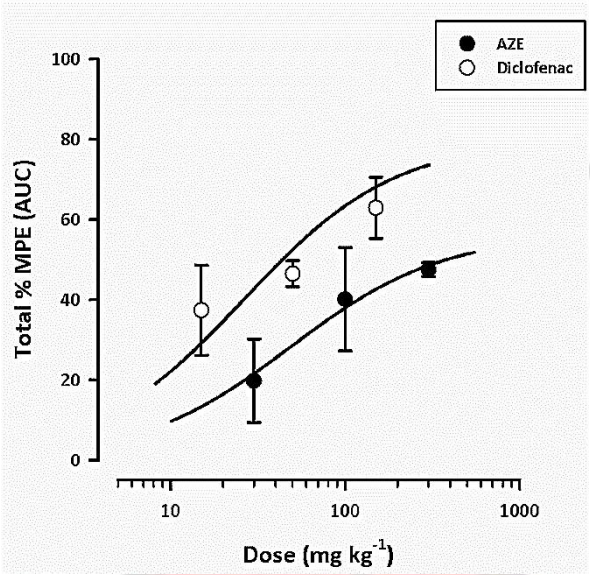


Figure 3.8 Comparison of dose response relationship of paracetamol (15-150 mg/kg; *p.o*) and AZE (10-300 mg/kg; *p.o*) on T_R changes during the baker's yeast-induced pyrexia in rats.

Table 3.4 ED_{50} values obtained from dose-response curves in baker's yeast-induced pyrexia in rats

Drugs	**ED_{50} (mg/kg)**
Paracetamol	26.19±1.33 %
AZE	48.59±2.59 %

3.5 ANTI-NOCICEPTIVE ACTIVITY

3.5.1 Acetic Acid Challenge

Vehicle-treated control group exhibited abdominal stretching, coupled with the extension of the hind limbs. Nociceptive scoring begun after the 5 min of acetic acid administration.

Analysis of the curves reveal marked attenuation of mean number of writhes by both drugs [(diclofenac: $F_{3, 84}$=54.86; P<0.0001) and AZE: $F_{3, 84}$=22.74.; P<0.0001); fig. 3.9a, c]. AZE demonstrated a significant inhibition of 85.85±3.96 % (fig. 3.9d) only at the dose of 300 mg/kg ($F_{3, 14}$=7.08; P=0.004). Diclofenac demonstrated a significant dose-dependent effect. Diclofenac almost completely diminished writhing in the mice (fig 3.9b) with a maximum of 99.65±0.35 % at dose 30 mg/kg ($F_{3, 14}$=14.7; P=0.0002).

Figure 3.10 compares the dose-response relationships of AZE and diclofenac. The ED_{50} values of AZE and diclofenac obtained by non-linear regression were 109.6±1.99 mg/kg and 3.80±1.49 mg/kg respectively.

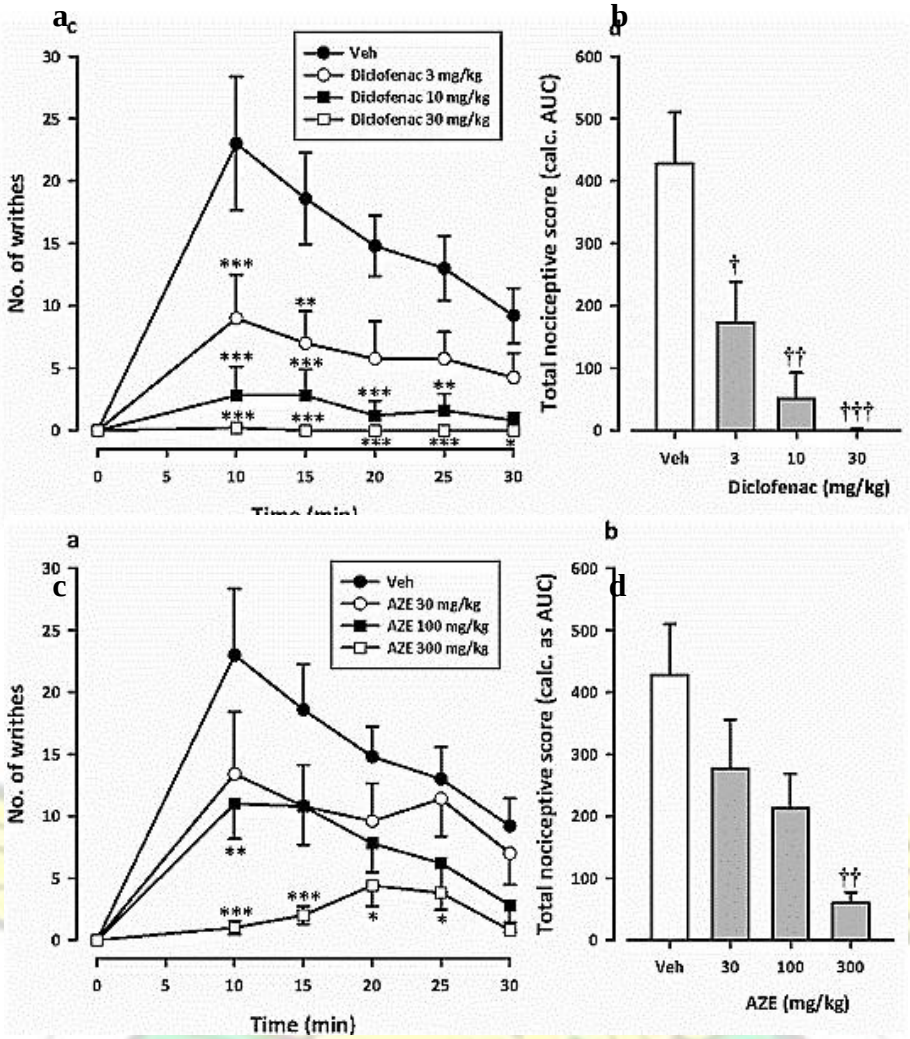


Figure 3.9 Reduction of writhes (mean ± S.E.M.; n=5) by diclofenac (3-30 mg/kg; i.p.) (**a** and **b**) and AZE (30-300 mg/kg; *p.o.*) (**c** and **d**) in the mice writhing assay. Left panels show the time course of effects over the 30 min period and the right panels show the total nociceptive score calculated from AUCs over the 30 min. Frequency of writhing in mice was captured after the 5th min of 10 ml/kg acetic acid (0.6% $^{v}/_{v}$; i.p.) administration. $^{*/**/***}P$<0.05/<0.01/<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test). $^{\dagger/\dagger\dagger/\dagger\dagger\dagger}P$<0.05/<0.01/<0.001 versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

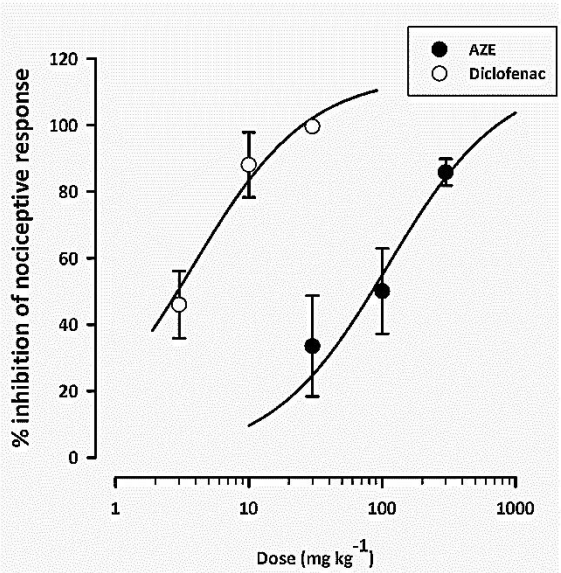


Figure 3.10 Comparison of dose response relationship of diclofenac (3-30 mg/kg; i.p.) and AZE (10-300 mg/kg; *p.o*) in the mice after intraperitoneal acetic acid challenge.

3.5.2 Formalin-induced pain in mice

The algogen, 5 % formalin, induced the fingerprint nociceptive behaviour of licking, biting, and paw lifting or shaking of the ipsilateral paw in mice (Abbott *et al*., 1995). Nociceptive behaviour occurs in two phases: an initial (neurogenic) phase which diminishes after the 10th min of formalin injection; and a late (inflammatory) phase immediately following, peaking between 20-30 min, and lasting till the 60th min (Hunskaar *et al*., 1985).

Time course nociceptive evaluation shows both agents significantly diminished licking/biting in their respective groups (morphine: $F_{3, 208}$=170.60; P<0.0001; AZE: $F_{3, 208}$=95.03; P<0.0001; fig. 3.11a, c). AZE (30-300 mg/kg; *p.o*) diminished total nociception in both neurogenic ($F_{3, 16}$=24.20; P<0.0001) and inflammatory ($F_{3, 16}$=51.76; P<0.0001) phases with respective maximal inhibitions of 79.44±8.51 % and 97.20±1.58 % at the highest dose (fig. 3.11d). Morphine (1-10 mg/kg) showed a similar pattern in both

neurogenic ($F_{3, 16}$=48.86; P<0.0001) and inflammatory ($F_{3, 16}$=59.27, P<0.0001) phases. However, morphine showed a much more significant inhibition of the first phase than AZE; 99.81±0.13 % (neurogenic) and 96.78±1.31 % (inflammatory) (fig. 3.11b).

The IC_{50} values of AZE are approximately 26.71±6.5 mg/kg (neurogenic) and 40.46±6.23 mg/kg (inflammatory) and that of morphine was 0.23±0.06 mg/kg and 0.20±0.04 mg/kg respectively (fig. 3.12).

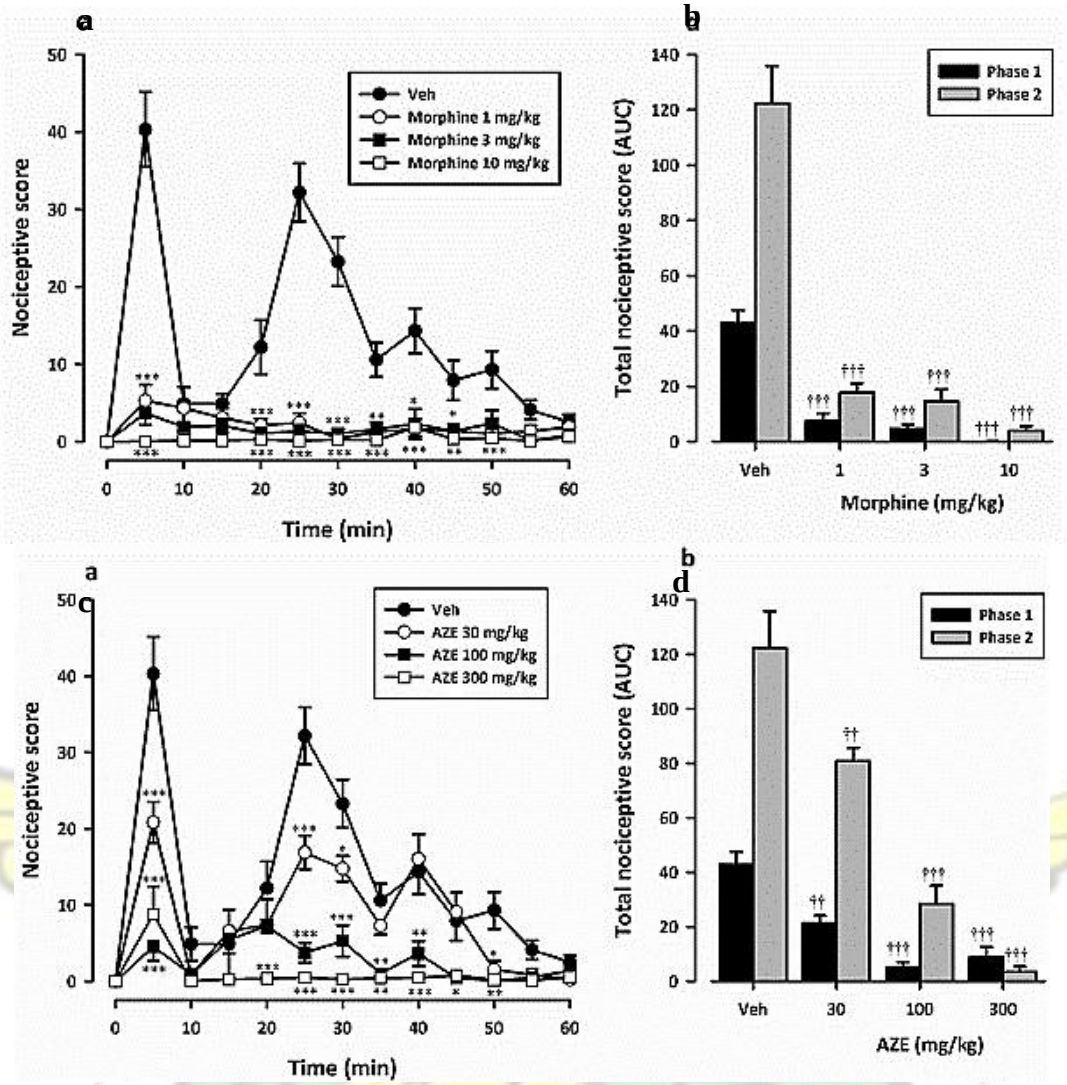


Figure 3.11 Inhibition of formalin-induced nociception (mean ± S.E.M.; n=5) by morphine (1-10 mg/kg; i.p.) (**a** and **b**) and AZE (30-300 mg/kg; *p.o.*) (**c** and **d**) in mice. Left panels show the time course of effects over the 60 min period and the right panels show the total nociceptive score calculated from AUCs over the 60 min. The intraplantar administration of 5% formalin induced the nociceptive behaviour of licking or biting of ipsilateral paw. Nociceptive score was calculated as the product of the frequency and length of time of paw licking/biting.

$^{*/**/***}P$<0.05/<0.01/<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test).

$^{\dagger/\dagger\dagger/\dagger\dagger\dagger}P$<0.05/<0.01/<0.001 versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

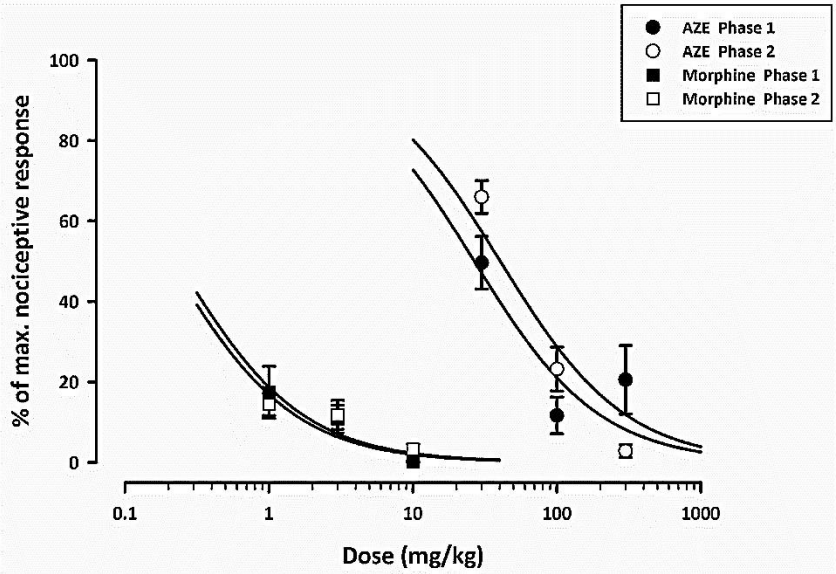


Figure 3.12 Comparison of dose response relationship of morphine (1-10 mg/kg; i.p) and AZE (10-300 mg/kg; *p.o*) in mice after intraplantar formalin challenge.

3.5.3 Tail Immersion Test

Rat tail withdrawal latency- determined by the time (s) a rat takes to retract its tail from the water bath- was increased by all test drugs and extracts.

The prophylactic administration of the drugs generally increased withdrawal latency across all groups. Only doses of AZE 100 and 300 (mg/kg; *p.o.*) significantly augmented the tail retraction time ($F_{3, 98}$=18.85; P<0.0001; fig. 3.13c). A more pronounced effect was seen with morphine (1–10 mg/kg; i.p.) ($F_{3, 97}$=23.70; P<0.0001; fig 3.13a). AZE ($F_{3, 14}$=9.27; P=0.0012; fig 3.13d) and morphine ($F_{3, 15=12.91}$; P=0.0002; fig 3.13b) pre-administration increased latency time as seen in the total anti-nociceptive effect.

Figure 3.14 compares the dose-response relationships of AZE and morphine. The ED_{50} values of AZE and morphine obtained by non-linear regression were 82.59±3.2 mg/kg and 1.84±0.11 mg/kg respectively.

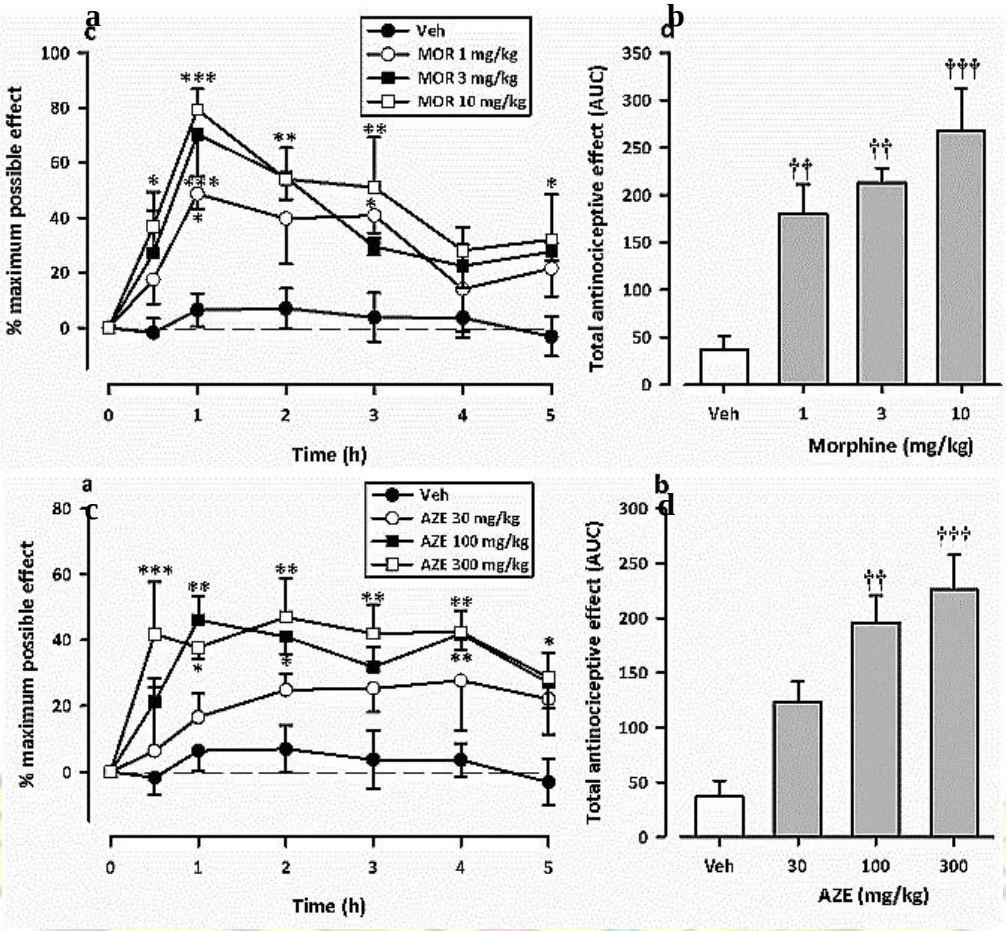


Figure 3.13 Maximum possible effect (mean ± S.E.M.; n=5) of morphine (1-10 mg/kg; i.p.) (**a** and **b**) and AZE (30-300 mg/kg; *p.o.*) (**c** and **d**) in rats in tail immersion test. Left panels show the time course of effects over the 5 h period and the right panels show the total nociceptive score calculated from AUCs over the 5 h. Tail retraction is calculated as the time (s) a rat takes to retract its tail from the water (49±0.5 ºC). $^{*/**/***}P$<0.05/<0.01/<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test). $^{\dagger\dagger/\dagger\dagger\dagger}P$<0.01/<0.001 versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

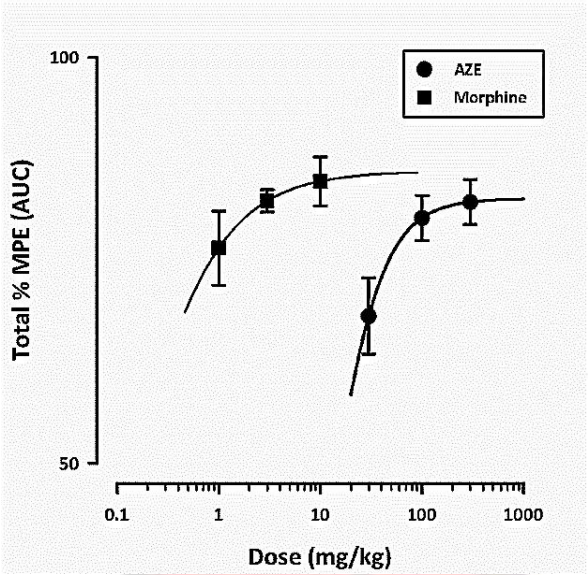


Figure 3.14 Comparison of dose response relationship of morphine (1-10 mg/kg; i.p.) and AZE (10-300 mg/kg; *p.o*) in the tail immersion assay in rats.

3.5.4 Carrageenan-induced Mechanical Hyperalgesia

After 2 prior days of experimental conditioning of rats, they averaged with a baseline withdrawal threshold ranging from 70 to 150 g. Intraplantar carrageenan administration induced general mechanical hyperalgesia 2.5 h post-administration. Baseline withdrawal threshold was reduced to 45-80 g. Hyperalgesia was sustained for at least 3 h. There was significant increase in tail retraction time by AZE (30–300 mg/kg; *p.o*.), diclofenac (3–30 mg/kg) and morphine (1-10 mg/kg). Mechanical anti-nociception was most significant in all the highest doses [(diclofenac: $F_{3,\ 16}$=13.27; P=0.0001; morphine: $F_{3,\ 16}$=43.29; P<0.000; AZE: $F_{3,\ 16}$=11.75; P=0.0003) (fig. 3.15a, c, e)]. The initial dose of each drug was insignificant in the total effect (diclofenac: $F_{3,\ 16}$=12.52; P=0.0002; morphine: $F_{3,\ 16}$=39.51; P<0.0001; AZE: $F_{3,\ 16}$=11.90; P=0.0002) (fig. 3.15b, d, f).

Figure 3.16 compares the dose-response effects of AZE, diclofenac and morphine. The ED_{50}s obtained by non-linear regression are 547.2±106.57 mg/kg (AZE), 59.46±10.30 mg/kg (diclofenac) and 12.22±1.61 mg/kg (morphine). **a**

b

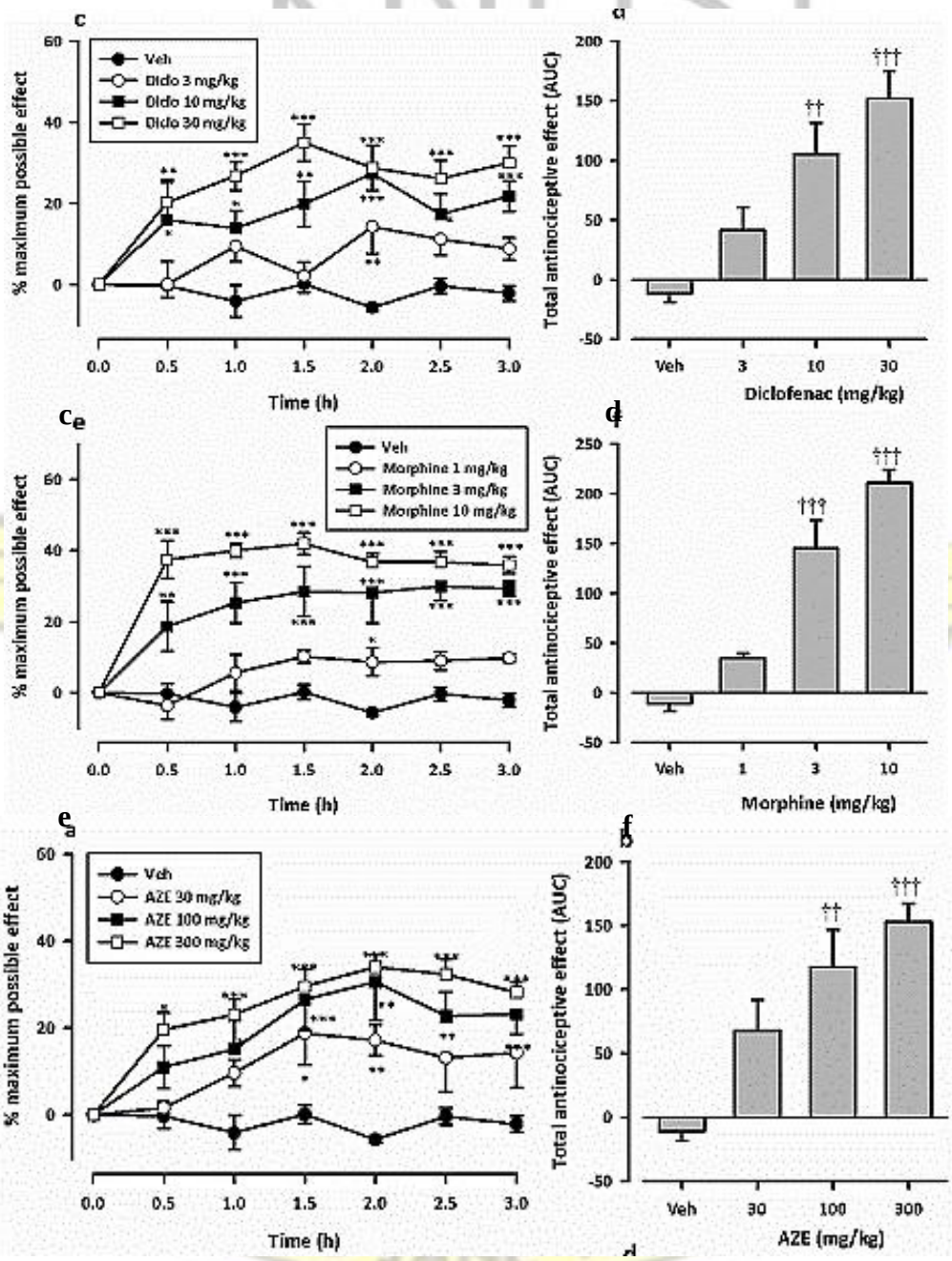


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Figure 3.15 Reversal of hyperalgesia (mean ± S.E.M.; n=5) in the rat paw pressure test by diclofenac (3-30 mg/kg; i.p.) (**a** and **b**), morphine (1-10 mg/kg; i.p.) (**c** and **d**) and AZE (30-300 mg/kg; *p.o.*) (**e** and **f**) after carrageenan administration. Left panels show the time course of effects over the 3 h period and the right panels show the total nociceptive score calculated from AUCs over the 3 h. Intraplantar administration of 100 µl of 20 mg/ml carrageenan suspension induced hyperalgesia after 2.5 h. $^{*/**/***}P<0.05/<0.01/<0.001$ versus vehicle control (Two-way ANOVA; Tukey *post hoc* test). $^{\dagger\dagger/\dagger\dagger\dagger}P<0.01/<0.001$ versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

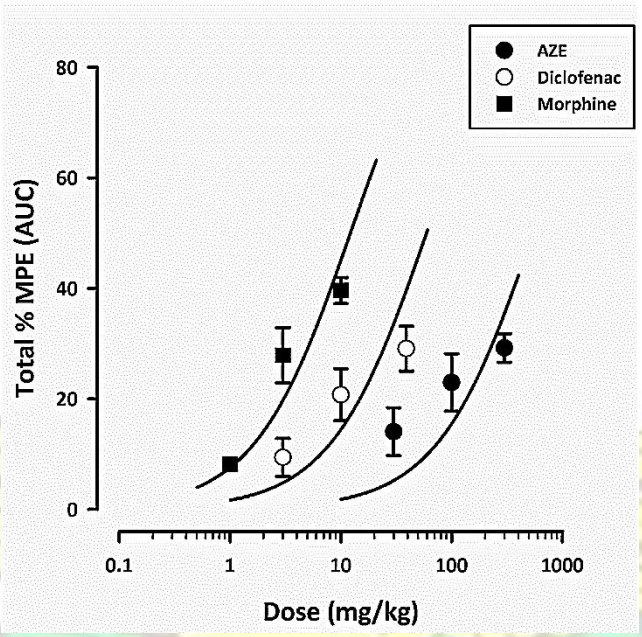

Figure 3.16 Dose response relationship of the activity of diclofenac (3-30 mg/kg; i.p.), morphine (1-10 mg/kg; i.p.) and AZE (10-300 mg/kg; *p.o*) against hyperalgesia induced by intraplantar carrageenan administration in rats.

3.5.5 Exploration of Possible Anti-nociceptive Mechanisms of AZE

The pathways of activity of AZE (100 mg/kg; *p.o.*) was investigated using the antagonists: naloxone (2 mg/kg; i.p.), theophylline (10 mg/kg, *i.p.*) and atropine (5 mg/kg; i.p.) (fig. 3.17). Similarly, the morphine treated groups were treated with these 3 antagonists since

antagonism of morphine by these drugs is a well-known observation. It also validates the method of receptor antagonism using the formalin test.

Naloxone pre-treatment partially antagonized AZE anti-nociception. Naloxone is a nonspecific opioid receptor antagonist. Opioidergic pathway antagonism was manifested in both neurogenic and inflammatory phases (fig. 3.17c, d). A similar result was seen in the morphine treated group (3 mg/kg; i.p.) with a more prominent antagonism occurring in the 2nd phase (fig. 3.17a, b).

Antagonism of the adenosinergic anti-nociceptive pathway was investigated using theophylline, a non-selective P1 receptor antagonist. AZE anti-nociceptive effect was not blocked by theophylline as seen in Fig. 3.17c, d. The inhibitory effect of morphine in both nociceptive phases was blocked by the pre-administration of theophylline (fig. 3.17a, b).

Atropine non-selectively antagonizes acetylcholine at muscarinic receptors. The preadministration of atropine showed a general inhibitory effect across both drug treatments. Atropine reversal of anti-nociception of AZE occurred in both phases, whilst the pathway inhibition of the morphine treated group was manifested in only the 2nd phase (fig. 3.17).

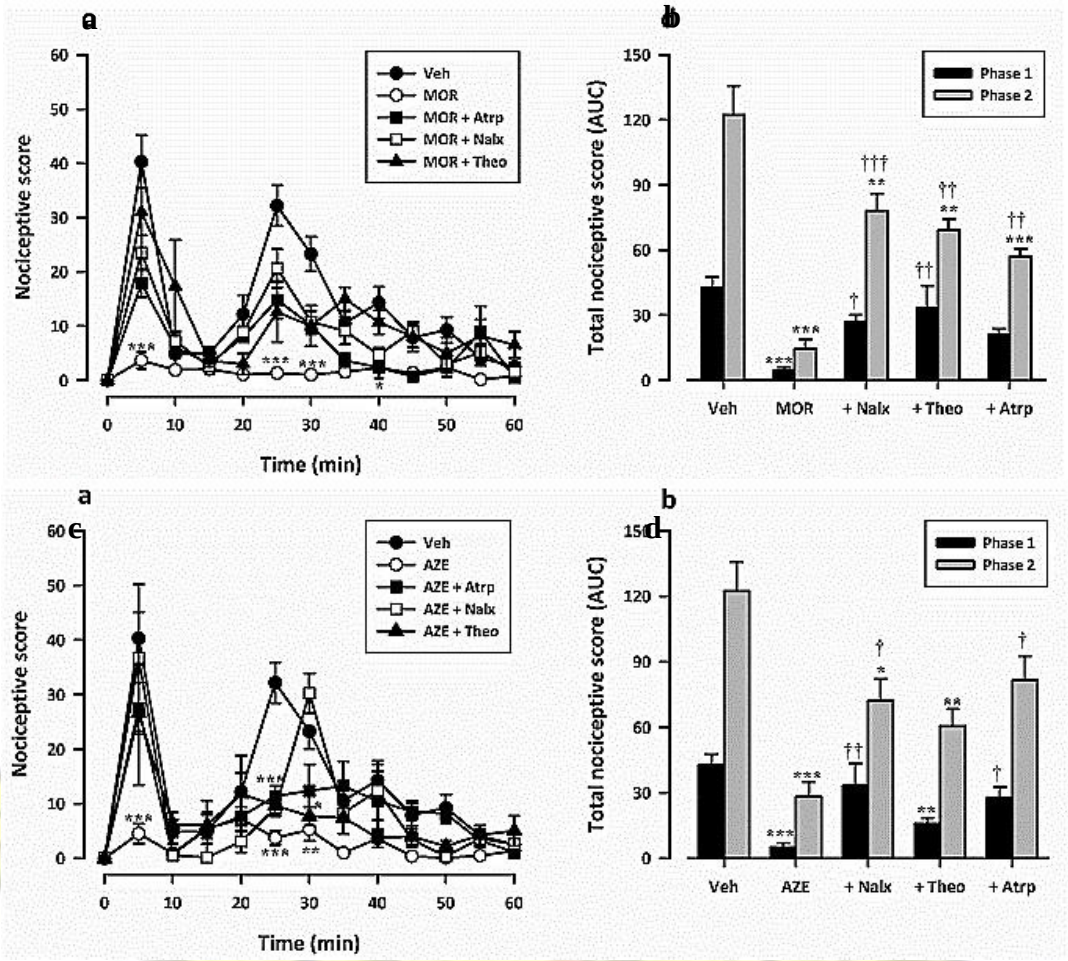

Figure 3.17 Reversal of the anti-nociception (mean ± S.E.M.; n=5) of morphine (3 mg/kg; i.p.) (**a** and **b**) and AZE (100 mg/kg; *p.o.*) (**c** and **d**) after pre-treatment of mice with naloxone (Nalx; 2mg/kg; i.p.), theophylline (Theo; 10 mg/kg; i.p.) and atropine (Atrp; 5 mg/kg; i.p.) in the formalin nociception test. Left panels show the time course of effects over the 60 min period and the right panels show the total nociceptive score calculated from AUCs over the 60 min. $^{*/**/***}P$<0.05/<0.01/<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test). $^{\dagger/\dagger\dagger/\dagger\dagger\dagger}P$<0.05/<0.01/<0.001 versus respective drug-treated group (One-way ANOVA; Tukey *post hoc* test).

3.5.6 Rotarod test

The results obtained revealed no significant impairment of neuromuscular coordination by AZE ($F_{3, 24}$=0.4158, P=0.7432) at all doses as compared to vehicle treatment group. There

was, however, significant impairment in motor coordination on the rotating rod when d-tc and diazepam were administered ($F_{2, 18}$ = 26.71, P<0.0001) (fig 3.18).

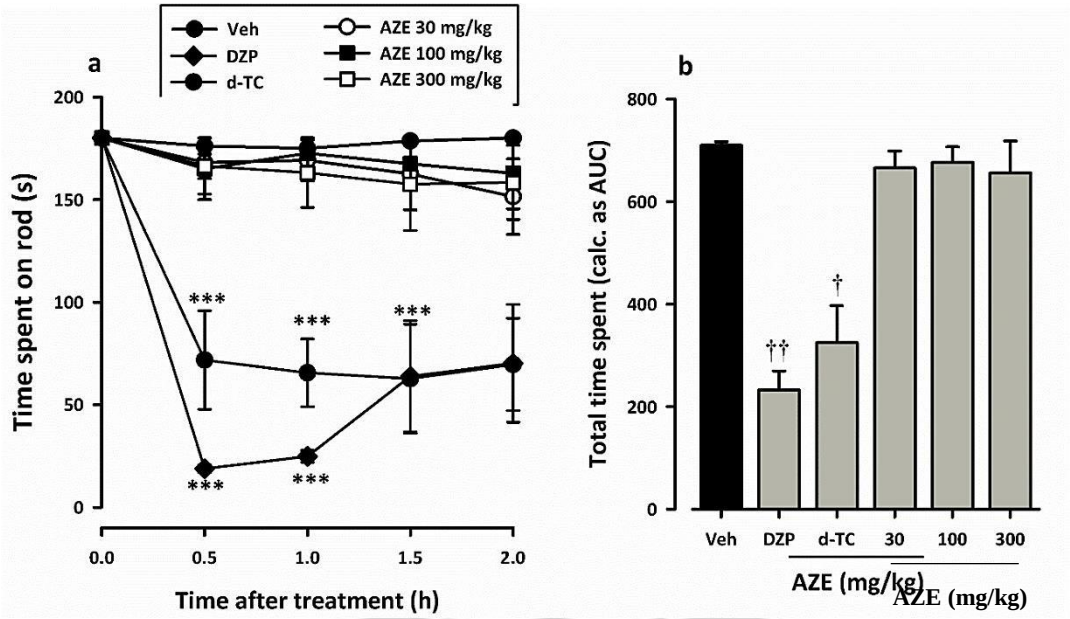

Figure 3.18 Assessment of motor coordination in mice (mean ± S.E.M; n=5) pre-administered with *d*-tc (0.1 mg/kg; i.p.), diazepam (8 mg/kg; i.p.) and AZE (30-300 mg/kg; *p.o.*). Determination of endurance limit was tracked for 3 min over a course of 2 h (a) and the total endurance score was calculated from AUCs over the 2 h (b). $^{***}P$<0.001 versus vehicle control (Two-way ANOVA; Tukey *post hoc* test). $^{\dagger/\dagger\dagger}P$<0.05/<0.01 versus vehicle control (One-way ANOVA; Tukey *post hoc* test).

Chapter 4

DISCUSSION

Divers parts of *A. zygia* plant are traditionally used in the management of pain and arthritis. Thus, an investigation was undertaken to assess the activity of AZE in animal models of inflammation (oedema and oxidative stress), pyrexia and pain in order to establish the purported effect.

The pharmacological activity of plant extracts has been accredited to the constituent presence of several phyto-constituents such as alkaloids, flavonoids, sterols, etc. Serafini *et al*, (2010) reported that some flavonoids induce their anti-inflammatory effect either by inhibiting pro-inflammatory enzymes, inhibiting NF-κB and AP-1 or activating antioxidant detoxifying enzymes. Also some classes of saponins, terpenoids, tannins and glycosides have been identified as anti-inflammatory agents (Darshan and Doreswamy, 2004). Furthermore, Barbosa-Filho et al. (2006) confirmed the observation by publishing the activity of the alkaloids aconitine, isoquinoline, indole and diterpene alkaloids in pain and arthritis. The identified phyto-constituents of AZE can therefore be adjudged to be liable for the observed pharmacological effects of *A. zygia* in traditional medicine.

The results of preliminary phytochemical analysis of AZE confirm observations from earlier works done on the plant. Abere *et al*. (2014) indicated alkaloids, tannins, flavonoids saponins and cardiac glycosides to be present in the methanol stem bark extract. The *Albizia* genus contains flavonoids, saponins, terpenes, alkaloids and glycosides (Kokila *et al*., 2013). Also Abdalla and Laatsch (2012) isolated the flavonoids 3-O-methylfisetin (3',4',7-trihydroxy-3-methoxyflavone), 3',4',7- trihydroxy-flavone and 4',7dihydroxyflavanone from Sudanese *A. zygia*.

Abere *et al* (2014) conducted acute toxicity studies on the methanol stem bark extract of *A. zygia*. This study, however, focused on determining the acute oral toxicity of the ethanol extract of the roots. Animal mortality was absent after 24 h of observation. The LD_{50} of AZE can therefore be estimated as greater than 5000 mg/kg. The highest dose of AZE used in all experiments, 300 mg/kg, displayed no observable signs of adverse effects. Doses greater than 300 mg/kg, presented with sedation—mainly reduced activity and reactivity

to touch—as well as cholinergic effects such as urination. These effects were all reversed after

24 h of observation. The results of the test were similar to that obtained by Abere *et al*, 2014. In that experiment, doses up to 5000 mg/kg (*p.o.*) manifested no significant change in behaviour, stool nature and frequency, mood, posture, motor activity, and no signs of convulsion nor writhing. The LD_{50} was greater than 5000 mg/kg.

The European Economic Community (EEC) classification criteria of acute oral toxicity states that substances with LD_{50} greater than 2000 mg/kg are relatively harmless (Yam *et al*., 1991). This therefore justifies that AZE is acutely non-toxic at all experimental doses in mice. The acute toxicity study assesses the human risk at such a time as when patients will consume large quantities of the drug (overdose) (Robinson *et al*., 2008). The results of the testing may: (a) enable drug classification and labelling, (b) predict dose selection of a repeated-dose tests, (c) help predict the LD_{50}, and (d) suggest the particular target organs susceptible (Shetty Akhila and Alwar, 2007; Langley, 2005). On the other hand, the test provides inconclusive data in extrapolating animal effects to humans. The variations are due to differences in sensitivity to the toxic agents. Animals and humans also differ with respect to adsorption, distribution, metabolism and excretion (Langley, 2005).

The outcome of the acute oral toxicity testing enabled the selection of doses for the pharmacological assays. A step down of the 300 mg/kg dose by a factor of 3 resulted in the doses 300, 100 and 30 mg/kg.

In analysing the anti-inflammatory activity, pedal oedema induction was done using a simple, reproducible and a highly reliable method. Overdependence on rodent models was curtailed by replacing rodents with a less sentient species, chicks (Fereidoni *et al*., 2000).

Subcutaneous injection of carrageenan induces a biphasic inflammation characterized by the symptoms oedema, erythema and pain. Pro-inflammatory mediators including histamine, serotonin, tachykinins, bradykinin, ROS and complement proteins are responsible for the induction (Morris, 2003). Prostaglandins then initiate the last phase of the oedema via the action of COX-2 together with inducible nitric oxide synthase (iNOS) (Posadas *et al*., 2004). These mediators increase vascular permeability causing fluid and protein exudation out of the vessels- manifested as the oedema (Giroud and Willoughby, 1970). Therapeutic doses of anti-inflammatory agents ameliorate the oedema induced by this phlogistic agent (carrageenan). Pre-emptive and curative administration of AZE in the chicks significantly suppressed the acute local oedema in both treatment protocols. It can therefore be postulated that since AZE inhibited total pedal oedema in the chicks, it is possibly inhibiting pro-inflammatory mediators of oedema synthesis, or their release and/or activity at target sites. The precise mechanism by which AZE suppresses inflammatory however needs to be established. AZE was compared to the standard anti-inflammatory analgesic, diclofenac, a non-selective COX inhibitor which prevents the synthesis of the arachidonic acid metabolites such as prostaglandins, thromboxanes and leukotrienes (Mitchell and Warner, 1999). Diclofenac similarly and dose-dependently suppressed the pedal oedema.

Oxidative imbalance abounds in the pathophysiology of tissue inflammation after trauma (Wang *et al*., 2004). Free radicals possess short half-lives and are less concentrated. As such oxidative damage is best assessed by measuring the amount of products and enzymes produced (Hovatta *et al*., 2010). $O_2^{\cdot -}$ is converted by SOD to H_2O_2, with either CAT or GPx eventually converting the H_2O_2 to oxygen and water. GSH and its congeners detoxify

peroxynitrites, hydroperoxides and electrophiles such as reactive aldehydes (malondialdehyde). MPO is used by neutrophils to synthesize HOCl during phagocytic lysis of foreign bodies (Halliwell, 2012; Zhu *et al*., 2008; Şenoğlu *et al*., 2009). Histamine, serotonin and eicosanoids mediate the initial phase of oedema after carrageenan paw challenge whilst polymorphonuclear infiltration is responsible for the delayed inflammatory phase. Neutrophil-derived $OH^{\cdot}$, H_2O_2 and $O_2^{\cdot -}$, MPO and MDA concentrations are augmented whilst antioxidant levels are relatively diminished in the paw tissue thereby increasing cellular susceptibility to oxidative damage (Halici et al., 2007, Bilici et al., 2002). Pre-treatment of the rats with AZE significantly elevated the peripheral concentrations of the preventive enzyme antioxidants (SOD and CAT) and the scavenger, chain breaking or repair antioxidant, GSH. AZE also reduced the formation of MDA and the pro-inflammatory enzyme MPO. Similar results were obtained for the positive control, diclofenac. The anti-inflammatory effect of AZE may be partly due to its prevention/inhibition of oxidative damage. AZE may also be demonstrating its antinociceptive effect via the inhibition of oxidative damage. Valerio *et al*. (2009) reported that induced oxidative imbalance enhances hyperalgesia during inflammatory pain. Free radicals such as peroxynitrites induce nociception when injected into peripheral paw tissues. Hence, the peripheral or central inhibition of oxidative imbalance attenuates hypernociception.

The pyrexia model (Tomazetti *et al.*, 2005) induces pronounced fever which is characteristically regressed to normal body temperature by conventional antipyretics. PGE_2 is the ultimate mediator for fever production. Since AZE ameliorated the fever induced in the rats, it is probable that AZE exerts its antipyretic activity by inhibiting the biochemical

processes involved in PGE_2 synthesis; or by antagonizing either directly or as a functional antagonist at PGE_2 receptors. Medicinal therapy for management of fever is often not necessary; however, pharmacological and mechanical (tepid sponging) antipyretics improve the invalid's discomfort (Anochie, 2013).

The analgesic activity of AZE was holistically assessed using different types of pain stimuli. In chemical stimulations- formalin and acetic acid-induced writhing- the algogen induces a slow, progressive stimulus over a specified period of time. The stretching, cramping, abdominal constriction or writhing assay is a commonly used model. It is easy to perform, simple and highly replicable (Verri *et al*., 2006). The test imitates pain as a result of cutaneous incision. Intraperitoneal acetic acid injection wields a response involving the generation of pro-inflammatory cytokines such as TNF-α, IL-1β, chemokines and eicosanoids to activate the nociceptors (Ribeiro *et al*., 2000; Santos *et al*., 1998). Antinociceptive effect however, cannot be easily distinguished from anti-inflammatory effect

(Collier *et al*., 1968). AZE and diclofenac significantly inhibited the abdominal writhes. This therefore, establishes a plausible anti-nociceptive and anti-inflammatory effect of AZE since AZE possibly inhibited mediators release and/or their sensitization effect.

The formalin test, unlike the writhing assay, is of more significance and predictability. The test procedure is the most congruent in representation of tissue injury-induced acute clinical pain. The commencement and conclusion of analgesic activity are best assessed in tonic pain tests (Dubuisson and Dennis, 1978; Tjølsen *et al*., 1992). The characteristic first phase of nociception is a neurogenic pain resulting from direct C-fibre activation of nociceptors.

Transient subsidence of the neurogenic phase was possibly due to induction of auto analgesia (Abbott *et al*., 1995). The 2nd phase consists of an enduring acute inflammatory pain coupled with dorsal horn operative changes. The two-phased process affords the study in variations of the time and site of action of analgesics. The initial phase processes involve the activity of Substance P (SP) and bradykinin whilst the 2nd phase is mediated by histamine, serotonin, prostaglandin and bradykinin (Shibata *et al*., 1989; Tjølsen *et al*., 1992; Dubuisson and Dennis, 1978). AZE significantly inhibited both phases. Opioid agonists (morphine and congeners) are centrally acting analgesics, and therefore ameliorate perception of nociception in both pain periods. AZE is therefore portraying a central action of analgesic activity. On the other hand, steroidal anti-inflammatory agents and some NSAIDs only attenuate nociception in the late phase. Such kinds of analgesics act peripherally (Shibata *et al*., 1989; Hunskaar and Hole, 1987). AZE inhibiting the second phase is suggestive of a possible peripheral and anti-inflammatory effect. This observation further substantiates the earlier stated positive activity of AZE in inflammation (carrageenan-induced oedema).

The tail-immersion experiment employs the use of a noxious thermal stimulus. The temperature of the noxious stimulus tends to affect analgesic outcome (Ramabadran *et al*., 1989). Tail-immersion test is sensitive to centrally acting analgesics, with specific effect to pure narcotic analgesics, as well as partial agonist e.g. nalorphine, pentazocine and cyclazocine in mice (Sewell and Spencer, 1976). Peripherally acting agents such as paracetamol and aspirin appear to be active at temperatures of 45 ^{o}C and below (Luttinger, 1985). The pre-treatment of the rats with AZE and morphine significantly alleviated thermal nociception. Animal response to the thermal stimulus is a spinal motor reflex action

with minimum cognitive processing (Ramabadran et al., 1989). Therefore, AZE attenuating thermal nociception is suggestive of a probable anti-nociceptive activity mediated by central (spinal) mechanisms.

The intraplantar injection of the carrageenan into tissues induces local oedema and hyperalgesia in the ipsilateral paw (Radhakrishnan *et al*., 2003). The application of the analgesimeter stimulus activates low-threshold mechanoreceptors in the ipsilateral paw (Sluka *et al*., 2007; Lewin and Moshourab, 2004). AZE dose-dependently inhibited hyperalgesia induced by intraplantar carrageenan challenge. The standard drugs, diclofenac and morphine, potently and dose-dependently exhibited anti-hyperalgesic effects. Hyperalgesia is divided into primary and secondary hyperalgesia. Peripheral nociceptor sensitization (by inflammatory mediators) largely results in high mechanical and thermal stimuli sensitization (primary hyperalgesia) at the injury site. The surrounding uninjured zone of the injury site becomes sensitized, and exhibits secondary hyperalgesia and allodynia.

The continuous barrage of nociceptive firing from the afferent nociceptors significantly increases dorsal horn neuronal activity. This is due to changes in central neural plasticity in the spinal cord thereby modifying CNS responsiveness to future stimuli (Fitzgerald and Beggs, 2001; Holdcroft and Jaggar, 2005). The responsiveness of silent nociceptors is also revealed in the inflammation. This therefore augments the firing of afferent nociceptive impulses to the spinal cord. Glutamate (NMDA receptors) and SP (NK-1 receptors) mediate secondary hyperalgesia in DH (Holdcroft and Jaggar, 2005).

The writhing test is highly unspecific. This is because adrenergic blockers, muscle relaxants, antipyretics, antimuscarinics, neuroleptics, antihistamines, opioid antagonist, and sympathomimetics produce false positive antinociceptive effect in this test (Collier *et al*., 1968; Hendershot and Forsaith, 1959). An inherent muscle relaxant property of AZE can therefore invalidate a verdict on any true antinociceptive effect derived from the writhing assay. Due to this possibility, AZE was investigated for neuromuscular or sensory blockage effect.

The true analgesic property of AZE was corroborated in the rota-rod test. It eliminated any probable neuromuscular relaxant tendencies of AZE. Rota-rod test evaluates the combined action of fore- and hind limb functions of mice (Baskin *et al*., 2003). The sensitivity of the test procedure was increased by using a faster rod speed i.e. 20 rpm. Also stress induced by animal handling was reduced to the barest minimum by the 3 prior days of habituation (Rozas *et al*., 1997). The activity of AZE was compared to diazepam (a CNS depressant with muscle relaxant effects) and d-tubocurarine (a non-depolarizing neuromuscular blocker). Pre-treatment of mice with all doses of AZE displayed no significant impairment of neuromuscular performance on the rotating rod. On the other hand, d-tubocurarine and diazepam significantly reduced mouse motor performance.

Based on the positive effect seen with AZE in the three different stimuli models of nociception, further studies into its possible mechanisms of anti-nociception was carried out. Nociceptive modulation and transmission involves several mediators along the pathway i.e. adenosine, NO, acetylcholine, endogenous opioids, adrenergic agonists, serotonin, etc. Some of these mediator pathways were antagonized to characterize the

possible anti-nociceptive effect of AZE. The formalin test, being more specific for site and mechanism of action, was employed for this evaluation.

Naloxone pre-administration markedly antagonized the opioidergic pathway after administration of morphine and AZE. Both the first and second phases of nociception where reversed. This is suggestive of a possible activity along the opioidergic pathway with antagonism at μ-, κ-, and δ-receptors. Opioids act by directly inhibiting pain transmission and by activating pain-inhibitory neurons in the CNS. They act pre-synaptically to reduce cAMP and post-synaptically to increase potassium influx resulting in membrane hyperpolarization (Holdcroft and Jaggar, 2005).

There is credible data confirming the antinociceptive effect of systemically administered cholinomimetics (Wess et al., 2003; Petersson et al., 1986; Yaksh et al., 1985). The muscarinic receptors, M_2 and M_4, are involved in mediating muscarinic agonist-dependent analgesia. The antinociceptive effect of AZE was significantly attenuated by atropine. The activity of AZE may therefore be mediated via the cholinergic pathway of nociception at M_2 and M_4 receptors. Atropine antagonizing morphine confirmed the observation by Dirksen and Nijhuis (1983) i.e. morphine analgesia is potentiated by physostigmine and ameliorated by atropine.

The influence of nociceptive transmission by adenosine occurs at the spine and periphery. Adenosine modifies cell function by binding to GPCRs A_1, A_{2A}, A_{2B} and A_3. A_1 receptor activation by adenosine decreases cAMP at nerve terminals, yielding an antinociceptive effect. A_2 and A_3 receptor activation enhance pain transmission (Sawynok, 1998; Ribeiro *et al*., 2002). The antagonism of AZE with theophylline (non-selective P1 receptor blocker)

produced no significant inhibition of nociception. Adenosine is spinally released by morphine, hence theophylline being a phosphodiesterase inhibitor attenuated the analgesic effect of morphine by antagonizing adenosine at P1 receptors (Contreras *et al*., 1990; Ahlijanian and Takemori, 1985; Sweeney *et al*., 1987).

Chapter 5

CONCLUSION & RECOMMENDATIONS

5.1 CONCLUSION

Preliminary phytochemical constituent analysis of AZE indicate tannins, flavonoids, saponins, alkaloids, terpenoids and glycosides to be present. The ethanol extract of the roots of *Albizia zygia* is of low toxicity in mice after acute oral administration since the LD_{50} is estimated to be greater than 5000 mg/kg. The ethanol extract of the roots of *Albizia* zygia demonstrated acute anti-oedemic effect. AZE also possesses antioxidant activity in peripheral injury conditions, and this may contribute to its anti-inflammatory propensity. AZE exhibited potent anti-pyretic effect in rats. It also possesses peripheral and central anti-nociceptive activity in rodents, which is possibly mediated via the opioidergic and cholinergic muscarinic pathways. These results validate the folkloric use of *A. zygia* in the treatment of fever, pain and inflammatory disorders.

5.2 RECOMMENDATIONS

The findings in this study confirm the folkloric use of the plant. However, further experimental work with high conclusiveness need to be undertaken; this will facilitate drug extrapolation to human consumption.

The following are therefore recommended:

- The precise mechanism(s) of the identified pharmacological effects should be investigated. Pathways such as arachidonic acid metabolism, inflammatory markers expression and signalling processes should be investigated.

- The effect of AZE in other inflammatory pain models such as CFA-induced arthritis, and also chronic pain models of neuropathy should be investigated.

- Specific receptors involved in drug interactions should be examined via receptor binding studies to further characterize AZE mechanism of action.

- The crude extract should be fractionated and the active constituent(s) responsible for the observed effects in this study be isolated and characterized.

- A more comprehensive safety assessment including subacute and chronic toxicity studies needs to be undertaken.

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APPENDIX

PHARMACOLOGICAL METHODS

Drug Preparation and Administration

2 g tragacanth powder was triturated with normal saline, and the solution made up to 100 ml to make a 2 % $^{w}/_{v}$ solution. The various -concentrations of *Albizia zygia* extract were prepared by triturating weighed quantities with the tragacanth solution. Lower concentrations of AZE were prepared by serial dilutions. The standard drugs were formulated by dissolving the powder in or diluting the stock solution with normal saline.

The maximum equivalent volumes of drugs administered were 0.5 ml (i.p.) and 1 ml (*p.o.*)

TNG Buffer Preparation

About 30 ml distilled water was used to dissolve 0.8766 g NaCl and 0.605 g Tris Base in a 100 ml volumetric flask. 10 ml glycerol and 1 ml Triton X-100 were dissolved in the mixture and the pH of the solution made up to 7.4. Two tablets of complete protease inhibitor cocktail were dropped in and solution was made up to 100 ml.

Phosphate Buffer Preparation

To prepare 0.1 M phosphate buffer, about 30 ml distilled water was used to dissolve 1.09 g of disodium hydrogen phosphate and 0.31 g of sodium dihydrogen orthophosphate monohydrate in a 100 ml volumetric flask. The pH was adjusted to 6 and solution was made up to 100 ml.