UNIVERSITY OF CAPE COAST

PHARMACOLOGICAL EVALUATION OF EXTRACT AND ISOLATED COMPOUNDS FROM THE ROOT BARK OF *ZIZIPHUS ABYSSINICA* HOCHST EX. A RICH (RHAMNACEAE)

ISAAC TABIRI HENNEH

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BY

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

SEPTEMBER, 2019

DECLARATION

Candidate's Declaration

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

Candidate's Signature:..... Date:.....

Name: Isaac Tabiri Henneh

Supervisors' Declaration

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

Principal Supervisor's Signature:..... Date:.....

Name: Prof. Martins Ekor

Co-Supervisor's Signature: ..... Date:.....

Name: Dr. Francis Ackah Armah

ABSTRACT

The diversity offered by natural products has timelessly positioned it as a good source for novel therapeutics for the management of various medical conditions, including pain and CNS disorders. This study evaluated hydro-ethanolic root bark extract (ZAE) and isolated compounds from *Ziziphus abyssinica* for analgesic, anti-inflammatory, anxiolytic and antidepressant properties. Established *in vitro* and *in vivo* experimental models were adopted in assessing ZAE and the isolated compounds for these pharmacological properties. Elucidation of structure of the isolated compounds was carried out using infra-red spectroscopy, mass spectrometry, nuclear magnetic resonance and X-ray crystallography. Two pentacyclic triterpenes, β-amyrin and polpunonic acid, were for the first time isolated from the plant. This is also the first time the crystal structure of polpunonic acid is reported. ZAE was found to be relatively safe in acute and sub-chronic toxicity studies in rats. ZAE exhibited analgesic effect that is possibly mediated via opioidergic, ATP-sensitive potassium channels and nitric oxide - cyclic guanosine monophosphate pathways. ZAE also exhibited anti-inflammatory activity via membrane stabilisattion as well as inhibition of protein denaturation, neutrophil degranulation and activity of inflammatory mediators (TNF-α, IL-1β, prostaglandin E_2 and bradykinin). The isolated compounds, β-amyrin and polpunonic acid, also exhibited analgesic, anti-inflammatory (anti-arthritic), anxiolytic and antidepressant properties in murine models. It is concluded that ZAE, β-amyrin and polpunonic acid possess analgesic, anti-inflammatory, anxiolytic and antidepressant properties.

KEYWORDS

β-Amyrin

Biological activity

Drug discovery

Herbal medicine

Phytochemistry

Polpunonic acid

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DEDICATION

This thesis is dedicated to the loving memory of my dear mum: Maame Akua Tabuaa.

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LIST OF ACRONYMS

ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
AST	Aspartate Aminotransferase
BA	β-amyrin or Beta-amyrin
BSA	Bovine Serum Albumin
CFA	Complete Freund's Adjuvant
CINC-1	Cytokine-induced Neutrophil Chemoattractant-1
COSY	Correlation Spectroscopy
COX	Cyclooxygenase
D-BIL	Direct Bilirubin
DEPT	Distortionless Enhancement by Polarization Transfer
DHET	Dihydroxyeicosatrienoic Acids
DPPH	Diphenylpicrylhydrazyl
EDTA	Ethylenediaminetetraacetic Acid
EETs	Epoxyeicosatrienoic Acids
ERK	Extracellular Signal–regulated Kinases
FST	Forced Swimming Test
GPCRs	G-protein Coupled Receptors
HCT	Haematocrit
HETE	Hydroxy Eicosatetraenoic Acids
HMBC	Heteronuclear Multiple Bond Correlation
HRBC	Human Red Blood Cells

HRMS	High Resolution Mass Spectroscopy
HRMS	High Resolution Mass Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
IASP	International Society for the Study of Pain
ICR	Imprinting Control Region
IFA	Incomplete Freund‘s Adjuvant
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IR	Infra-red
JNK	c-Jun N-terminal kinases
KATP	Activating Adenosine Triphosphate-sensitive Potassium
KC	keratinocyte-derived Chemokine
L-NAME	N^G-L-Nitro-Arginine Methyl Ester
LOX	Lipoxygenase
MAPK	Mitogen-activated protein kinases
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCP-1	Monocyte chemoattractant protein-1
MCV	Mean Corpuscular Volume
mPGES-1	Microsomal Prostaglandin E synthase-1
MPV	Mean Platelet Volume
NF-κB	Nuclear Factor kappa B
NGF	Nerve Growth Factors
NMR	Nuclear Magnetic Resonance
NMR	Nuclear Magnetic Spectroscopy

NSAID	Non-steroidal Anti-inflammatory Drug
PA	Polpunonic Acid
PBS	Phosphate-buffered Saline
PG	Prostaglandin
PLT	Platelets
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
RDW	Red Cell Distribution Width
ROS	Reactive Oxygen Species
SA	Sympathetic Amines
T-BIL	Total Serum Bilirubin
THC	Tetrahydrocannabinol
TLR2	Toll-like Receptor 2
TMS	Tetramethylsilane
TNF-α	Tumour Necrosis Factor-Alpha
tNSAIDS	traditional Non-steroidal Anti-inflammatory Drugs
TRPV	Transient Receptor Potential Vanilloid
TST	Tail Suspension Test
TXA	Thromboxane
UHPLC	Ultra-High Performance Liquid Chromatography
WBC	White Blood Cells
ZAE	Hydro-ethanolic root bark extract of *Ziziphus abyssinica*

CHAPTER ONE

INTRODUCTION

Background to the Study

Chronic pain, depression and anxiety disorder are known to be multiple expressions of a common clinical and pathophysiological core which is linked to abnormal inflammatory responses (Arango-Dávila & Rincón-Hoyos, 2018). Inflammation is a complex and multifaceted biological process involving the body's response to cell and tissue injuries caused by chemical (alkalis and acids,), physical (ultrasonic waves, ionizing radiation, magnetic field, etc), and biological factors (exotoxins,viruses, bacteria, fungi, and endotoxins) (Headland & Norling, 2015). A cardinal sign of inflammation is pain which is an abnormal sensory or emotional feedback accompanying actual or potential tissue damage possibly as a result of nerve damage, inflammation, muscle spasm, tumour, exposure to noxious chemical, thermal or mechanical stimuli (Morrison & Morrison, 2006). Pain accounts for the most frequent reason why people seek medical attention and also the most common reason for absenteeism, unemployment and underperformance in the workplace (Corrigan, Desnick, Marshall, Bentov, & Rosenblatt, 2011). The severe pain associated with chronic inflammatory conditions has been shown to cause clinically diagnosable depressive and anxiety disorders (Baigi & Stewart, 2015).

Whereas depression is a common mental disorder that presents with depressed mood, feelings of guilt or low self-worth, loss of interest or pleasure, disturbed sleep or appetite, low energy, and poor concentration (Kumar, Srivastava, Paswan, & Dutta, 2012), anxiety is an adaptive response

to stress or threat. It is characterized by nervousness, irritability and an exacerbated reaction to danger (American Psychiatric Association, 2013). There is ample evidence that implicate inflammatory mediators in the pathogenesis of mood disorders (anxiety and depression) such that the activation of the inflammatory system reduces the clinical therapeutic benefit of antidepressant and anxiolytic drugs (Carvalho *et al*., 2013). This has called for the search for newer and more effective drugs that could target the co-existing conditions and plants cannot be left out.

This is because plant-derived bioactive compounds have contributed significantly to the management of inflammation, pain, anxiety disorders and depression (Calixto, Scheidt, Otuki, & Santos, 2001, Uddin et al., 2018). Clinically relevant anti-inflammatory and analgesic drugs such as morphine, salicylic acid and capsaicin were obtained from plants such as *Papaver somniferum, Salix species and Capsicum species* respectively, a testimony of how plant-derived compounds have translated into effective medicines. Consequently, over 25% of drugs prescribed worldwide are derivatives from plants (Wachtel-Galor & Benzie, 2011).

Ziziphus abyssinica, a member of the Rhamnaceae family, is a well-known medicinal plant widely distributed in the tropics and warm temperate regions (Kaleem, Muhammad, Khan, & Rauf, 2014). It is commonly called 'larukluror' (Sisaala, Ghana,) and catch thorn (English) (Humphrey Morrison Burkill, 1994; Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009). Previous studies have demonstrated extracts from various parts of the plant to possess antioxidant, antibacterial and antifungal activities (Gundidza & Sibanda, 1991; Nyaberi et al., 2010a; Wagate *et al*., 2010a), antiplasmodial (Muthaura *et al*.,

2015), anti-ulcerogenic (Ugwah, Etuk, Bello, Aliero, & Ugwah-Oguejiofor, 2013) and anti-diarrhoeal (Ugwah-Oguejiofor, Alkali, Ugwah, Abubakar, & Ugwah-Oguejiofor, 2011) properties. The phytochemical composition of *Ziziphus abyssinica* and the anti-nociceptive activity of the hydro-ethanolic leaf extract shown to be mediated via inhibition of TNF-α, IL-1β, bradykinin and prostaglandin E_2 have been reported (Boakye-Gyasi, Henneh, Abotsi, Ameyaw, & Woode, 2017a, 2017b).

However, the anti-inflammatory, analgesic, antidepressant and anxiolytic effect of the root bark extract of the plant have not been reported. Also, very few bioactive phytocompounds have been isolated from the plant which include abyssenine-A, abyssenine-B, abyssenine-C**,** mucronine-A, mucronine-B and mucronine-C (Kaleem *et al*., 2014). Considering the unmet need for novel therapeutics with improved safety and clinical efficacy against pain, inflammation, anxiety and depression, further studies have become necessary in order to fully characterise these effects in the crude extract and track down the constituents responsible.

Statement of the Problem and Justification

As it is well-recognised, the search for therapeutic agents that target depression-anxiety-chronic pain/inflammation comorbidity is needed(Arango-Dávila & Rincón-Hoyos, 2018). Though inflammatory responses are the body's defence mechanism to many harmful stimuli, its dysregulation is known to trigger various disease complications such as neurodegenerative and neuropsychiatric diseases (depression and anxiety), among others (Ricciotti & FitzGerald, 2011; Vodovotz, Csete, Bartels, Chang, & An, 2008). Arthritis, mostly accompanied with neuropsychiatric complications, constitutes a

primary source of debility of many people around the world and has often been referred to as the 'king of human miseries' (Kamble, Khan, Khan, Mular, & Sohai, 2017). Another important complication of inflammatory diseases is pain which is associated with many hospital visits and decreases productivity (Corrigan *et al*., 2011). Consequently, pain associated with chronic inflammatory diseases such as those experienced in arthritis has a negative affective component which is closely related to anxiety and depression (Baigi & Stewart, 2015). It is also worth-noting that more than 50% of patients who suffer from chronic pain/inflammation also express clinically diagnosable symptoms of depression (Dworkin & Gitlin, 1991).

Anxiety disorder has been shown to be the most predominant comorbid condition of depression (Kessler, 2007). The coexistence of the two conditions in addition to pain/inflammation makes them extremely debilitating with increased suicidal predisposition, decreased quality of life and reduced response to medications (de Heer *et al*., 2014). This calls for more specific treatment and accurate treatment options (Hou & Ng, 2014; Ohayon & Roberts, 2014; Arango-Dávila & Rincón-Hoyos, 2018). However, most of the currently available analgesic and anti-inflammatory drugs are devoid of antidepressant and anxiolytic action (Hamann *et al.*, 2016). Also, current treatment for several types of pain and inflammation are often inadequate and additionally pose several side effects (i.e. addiction, respiratory depression, peptic ulcers and renal impairment) to users such that recovery is mostly impaired. These reasons have thus, necessitated the need for inclusion of antidepressants and anxiolytics in pain management (Gilron, 2016). The

search, therefore, for novel therapeutics for the management of inflammatory conditions, pain, depression and anxiety comorbidity remains relevant.

In the past decades, herbal medicine has attracted worldwide attention as a versatile source for pharmacological agents with few adverse effects (Hou & Ng, 2014). Due to their diverse nature, many herbal preparations have been prescribed for the treatment of inflammation, pain and depression in the literature of alternative medicine (Uddin *et al.*, 2018). Consequently, patients with inflammatory disorders as well as acute or chronic pain are prone to seek alternative methods for relief and are among the highest users of herbal medicines and other complementary and alternative medicines (Singh, Nair, & Gupta, 2011; Simpson, 2015). The factors responsible for the continual and widespread usage of these herbal remedies are their availability, effectiveness, inexpensiveness, comparatively less toxic effects and insufficiency of practitioners of modern medicine in rural areas (Agrawal *et al*., 2011). It is against this backdrop that efforts have been made to integrate herbal medicine practice into the already established orthodox medicine in some countries with Ghanaian hospitals, not an exception.

One of the medicinal plants which has been used in folk medicine to treat pain, inflammation and depression in many African countries is *Ziziphus abyssinica*. It belongs to the Rhamnaceae family together with over 900 other species majority of which are known medicinal plants and widely distributed in many parts of the world especially the tropics and warm temperate regions (Kaleem *et al*., 2014). Though, the analgesic properties of the leaf extract of the plant has been previously reported (Boakye-Gyasi *et al.*, 2017a, 2017b), further investigation into the analgesic properties of the root bark extract is

still relevant since different plant parts are known to exhibit varying degrees of pharmacological effects (Al-Khazraji, Al-Shamaony, & Twaij, 1993; Darabpour, Bavi, Motamedi, & Nejad, 2011). Additionally, there are no scientific reports in the literature on the anti-inflammatory, anxiolytic and anti-depressant effect of the plant hence the need for the current research.

Hypothesis

Extract and isolated compounds from *Ziziphus abyssinica* root bark have direct effect on pain, inflammation, anxiety and depression.

General Objective

To evaluate extract and isolated compounds from *Ziziphus abyssinica* root bark for analgesic, anti-inflammatory, anxiolytic and antidepressant frre

Specific objectives

a. To assess the hydro-ethanolic root bark extract of *Ziziphus abyssinica* (ZAE) for acute and sub-chronic toxicity.
b. To evaluate ZAE for analgesic property in murine models of thermal, chemical and musculoskeletal pain and its probable mechanism(s) of action.
c. To investigate ZAE for anti-inflammatory property in acute inflammatory models.
d. To assess ZAE for anxiolytic and antidepressant effects.
e. To isolate compounds from the root bark of *Ziziphus abyssinica* and evaluate them for analgesic, anti-inflammatory, anxiolytic and antidepressant effects.

Significance of the Study

The role of inflammation in the pathogenesis of several diseases including chronic pain, anxiety disorders and depression is well established (Arango-Dávila & Rincón-Hoyos, 2018). Currently, there is no single agent which combines useful pharmacological properties relevant for effective treatment/management of comorbidity of pain, anxiety disorder and depression. Existing treatment strategies combine different pharmacological agents to control the various components of the comorbidity. This research project is contributing to the search for novel compounds or agents which combine anti-inflammatory/analgesic effects with anxiolytic and antidepressant properties for effective treatment of comorbidity of pain, anxiety disorder and depression.

Since the selection of *Ziziphus abyssinica* was based on its ethnobotanical use in traditional medicine, this study will also confirm the folkloric claim of its anti-inflammatory effect and provide scientific evidence to explain the pharmacological basis for the folklore use. Also, since the study of medicinal properties of *Ziziphus abyssinica* is based on extending and augmenting knowledge of traditional healing practices that is already ingrained in many localities across Africa and beyond, it constitutes an effective way of improving access to treatment for ordinary people.

Traditional medicine has a strong presence in Ghana, and the local culture holds medicinal herbs and traditional treatments in high regard. Therefore, this project will help make good use of indigenous plants to develop effective plant-based medicines. This will be done by extracting and further investigating compounds obtained from a local plant that is used to

treat pain, inflammation and CNS disorders in traditional Ghanaian medicine. This project will also contribute to the development of more effective drugs for the treatment of those prevalent conditions. As such, data from this study will provide a foundation for further development of bioactive small molecules from the plant into drugs.

Furthermore, despite the widespread use of the roots of *Ziziphus abyssinica* for the treatment of various ailments, there is a dearth of information and scientifically verified evidence to ascertain its safety on either acute or long-term exposure The acute and sub-chronic toxicity evaluation of the root bark extract of *Ziziphus abyssinica* in this present study will provide information regarding its safety and will be useful to guide dose selection in traditional medicine practice.

The significance of the current research is amplified by the fact that the plant under investigation has been reported to possesses anti-ulcerogenic and gastroprotective properties (Ugwah *et al*., 2013; Yau, Abdulazeez, Anigo, & Garba, 2017) These pharmacological properties are regarded as antidote to the most predominant adverse effect caused by already existing anti-inflammatory drugs. As such, it is expected that the medicinal product developed from this plant, though it may have an anti-inflammatory effect, might not cause gastrointestinal bleeding which characterise steroidal and non-steroidal anti-inflammatory agents.

Limitation of the Study

The present study is not without some limitations. First, due to ethical consideration, five animals were used per group for the *in vivo* aspects of study except the antidepressant evaluation where seven mice per group were

used. The strength of data interpretation and statistical significance may be influenced by this sample size, although reliance on statistical significance in biological experiments remains controversial.

Secondly, the *in vivo* aspect of the study was limited to only two mammalian species (Sprague Dawley rats and ICR mice). It was therefore extremely difficult to mimic the human clinical co-morbid condition of pain, inflammation, anxiety and depression in a single rodent without producing false negative or positive results. As such each of the conditions was investigated separately instead of an ideal experimental co-morbid condition.

Definition of terms

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

Pain: an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (as defined by the International Society for the Study of Pain [IASP]).

Inflammation: an immediate or delayed reaction to injury characterised by changes in blood flow, increased permeability of blood vessels and migration of fluids, proteins and leukocytes from blood vessels into injury sites.

Depression: a common mental disorder that presents with depressed mood, feelings of guilt or low self-worth, loss of interest or pleasure, disturbed sleep or appetite, low energy, and poor concentration.

Structure elucidation: the process of determining the chemical structure of a compound which involves the use of nuclear magnetic

resonance (NMR) spectroscopy, mass spectrometry, infrared spectroscopy and X-ray crystallography.

Nociception: a signal arriving at the central nervous system as a result of stimulation of specialised sensory receptors in the peripheral nervous system called nociceptors.

Histopathology: examination of tissues from an organism under a microscope to spot the signs and characteristics of a disease.

The Organisation of the Study

The thesis is presented in five chapters. The first chapter covers the introduction of the study. It comprises the background to the study, statement of the problem, the purpose of the study, research objectives and significance of the study. The chapter also looks at the delimitations, limitations and the operational definition of terms as well as the organisation of the thesis.

Chapter two comprises the literature review. It describes inflammation, types of inflammation, mediators and mechanisms of inflammation, rheumatoid arthritis, models of acute and chronic inflammation and anti-inflammatory drugs. Also, the link between inflammation and pain, inflammatory pain mediators and models, and the involvement of cytokines in hyperalgesia. Included in the chapter is the involvement of inflammation in depression. Also, antidepressant drugs and models of depression are discussed. Furthermore, anxiety and its experimental models are discussed. Finally, medicinal plants as sources of drugs, toxicity of medicinal plants and its products, the family Rhamnaceae, the genus *Ziziphus* and the plant *Ziziphus abyssinica*. Descriptions of the plant included taxonomy, geographical

location, pharmacognistic properties, phytochemical properties, traditional and reported medicinal properties of the plant.

Chapter 3 outlines the materials and methods used in the study which include plant material collection, preparation of the extract, phytochemical screening as well as biological assay of the extract in models of toxicity, pain, inflammation, anxiety and depression. Isolation and identification of compounds from the plant and biological activity of the isolated compounds were assessed. The chapter ends with statiscal tools employed in the analysis of the data.

Chapter four covers the results of the study. This includes results from the phytochemical screening, acute and sub-chronic toxicity studies on the extract, analgesic, anti-inflammatory, anxiolytic and antidepressant property of the crude extract, isolation and identification compounds from the plant. The chapter also outlines results from the biological activity of the isolated compounds.

Chapter five covers the discussion of the results from the study, summary of findings, conclusions and recommendations.

CHAPTER TWO

LITERATURE REVIEW

Introduction

In this chapter, inflammation, pain, anxiety and depression are described. Also, the relationship between chronic pain/inflammation and depression/anxiety have been described as bidirectional with the presence of each predicting the others (de Heer *et al*., 2014). Inflammation has been shown to be closely associated with each of the conditions through the elevation in the levels of inflammatory markers. With plants known to produce diverse medicinal properties, the possible role of *Ziziphus abyssinica* in mitigating these complex comorbid conditions has also been discussed.

Inflammation

It is an immediate reaction to infection and injury by the body's immune system characterised by changes in blood flow, increased permeability of blood vessels and migration of fluids, proteins and leukocytes from blood vessels into injury sites (Cai *et al*., 2014). Inflammation is one of the body's own carefully coordinated mechanism for the elimination of foreign materials and toxic substances. However, this rather good mechanism of the body when not properly controlled could result in tissue damage which is a contributory factor in the development of a wide range of inflammatory diseases (Muller, 2013).

Acute and Chronic Inflammation

There are two main types of inflammation - acute and chronic inflammation. If the swelling winds up in less than 48 hours, it is acute inflammation (e.g., abscess) and if it persists for more than 48 hours (i.e.,

weeks, months, or years), then it is chronic inflammation (Ansar & Ghosh, 2016).

Acute inflammation

The acute inflammatory response consists of two components. These are innate non-adaptive response and the adaptive immune response. These responses are generally protective but if improperly deployed could become deleterious (Rang, Ritter, Flower, & Henderson, 2014). The acute vascular reaction usually occurs within seconds after tissue injury and can persist for some minutes. The acute response occurs as a result of vasodilation and an increase in capillary permeability due to alterations in the vascular endothelium, leading to increased blood flow (hyperaemia), which causes redness (erythema) and the entry of fluid in tissues (oedema). If the injury is not sufficient, the inflammatory response terminates after tens of minutes. Irrespective of the initiating stimulus, the classical signs of inflammation are calor (heat), dolor (pain), rubor (redness), tumor (swelling) and functio laesa (loss of function) (Kumar, Abbas, Fausto, & Aster, 2014). Neutrophils are the major cell types in acute inflammation, and the outcomes of acute inflammation are resolution, abscess, and ulcer (fistula, sinus) which may expand to chronic inflammation (Ansar & Ghosh, 2016).

Chronic inflammation

Chronic inflammation results from acute inflammation or occurs without an acute phase (Sochocka, Diniz, & Leszek, 2017). As such, in chronic inflammation, an irritant either induces an acute inflammatory response which fails to resolve on its own, or the insult is generated continuously locally. Different types of inflammatory mediators and growth

factors are produced locally depending on the nature of the irritant, giving rise to different morphological patterns of chronic inflammation (Wakefield & Kumar, 2001). The body receives a signal of health hazard and switches from the acute to a chronic inflammatory state if the activity of the stimulating factor is persistent and there is dysregulation in the mechanisms of proper development of inflammation (Franceschi & Campisi, 2014). This results in an imbalance in the immune system and a subsequent dysregulation in inflammatory markers. Chronic inflammation consequently leads to tissue degeneration and the development of autoimmune or circulatory system diseases, arthritis, cancers, and central nervous system disorders (Maskrey, Megson, Whitfield, & Rossi, 2011; Sochocka, Diniz, & Leszek, 2017). In this type of inflammation, mononuclear cells (mostly lymphocytes, macrophages, and plasma cells) are the major cell types, and the outcomes are usually tissue destruction, fibrosis and necrosis (Ansar & Ghosh, 2016).

Mediators of Inflammation

The enzymes of the arachidonic acid cascade

Arachidonic acid is acted on by three key oxidative enzymes. These are cyclooxygenases, lipoxygenases and cytochrome P_{450} epoxygenases. Inflammatory mediators of the arachidonic acid cascade, notably, from lipoxygenase (LOX) and cyclooxygenase (COX) pathways are primarily responsible for many diseases that affect human beings (Jacob, Manju, Ethiraj, & Elias, 2018).

Cyclooxygenases (COXs)

The COXs enzymes catalyse the first two critical steps leading to the synthesis of prostaglandins (PGs, i.e. PGE_2, PGD_2, $PGF_{2\alpha}$), thromboxane

(TXA2) and PGI_2. COX-1 and COX-2 are the two main isoforms of COX that have been identified (Vane, Bakhle & Botting, 1998). The traditional, non-selective non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both isoforms of the enzyme, and this is responsible for their roles in pain, fever and inflammation (Sala, Proschak, Steinhilber, & Rovati, 2018). COX-1 is expressed constitutively in many cells; hence the generally acknowledged postulate that the prostaglandins produced by COX-1 are homeostatic. Metabolites derived from COX-2 enzymes are said to be inducible and as such exert a wide range of physiological and pathophysiological roles through their interaction with specific G-protein coupled receptors (GPCRs) (Smith, Urade, & Jakobsson, 2011). Microsomal PGE synthase-1 (mPGES-1) is one of the downstream secondary enzymes of COX activity, and it is the crucial isoform which converts PGH_2 into PGE_2 in an inflammatory cascade. This characterises an essential target for the development of novel anti-inflammatory drugs and has received significant interest in recent years (Koeberle & Werz, 2015). COX-2 synthesis is mostly lacking or low in healthy individuals but upregulated by proinflammatory cytokines such as TNF-a and IL-1 in response to inflammatory disease or infection (Dinarello, 2010).

Lipoxygenases

Lipoxygenases (LOXs) belong a group of iron-containing (non-haeme) deoxygenates which catalyse molecular oxygen insertion into polyunsaturated fatty acid, including arachidonic acid, with at least two isolated cis-double bonds (Sala *et al.*, 2018). The primary oxygenated products of LOXs on arachidonic acids are several hydroperoxy-eicosatetraenoic acids (HpETEs),

which then reduce into monohydroxy eicosatetraenoic acids (such as 5-HETE, 12-HETE or 15-HETE) by peroxidases, or transformed into biologically active compounds such as leukotrienes (LTs), lipoxins and hepoxilins. The mediators produced by 5-LOX, 12-LOX and 15-LOX are linked to allergic reactions, increased proliferation of cancer cells and pathogenesis of atherosclerosis respectively (Jacob et al., 2018). Inhibitors of 5-LO activity binding molecules preventing its translocation to nuclear membrane in response to increased intracellular [Ca^{2+}] are still being developed for the management of asthma (Bruno *et al*., 2018).

Cytochrome P_{450} epoxygenases

Epoxyeicosatrienoic acids (EETs), produced by cytochrome P_{450} epoxygenases, belong to the CYP_{450} 2C and 2J subfamilies of the CYP2 monooxygenase family that preferentially use arachidonic acid as substrate (Sala *et al*., 2018). The EETs produced from arachidonic acids exhibit a wide range of potent cardiovascular protective effects which include regulation of vascular tone and homeostasis. Additionally, they stimulate endothelial cells proliferation and angiogenesis and exert anti-inflammatory effects particularly in the cardiovascular system (Spector & Kim, 2015). Activation of cAMP/protein kinase A signaling pathway is involved in EET dependent vasodilation and other EET-dependent functions mediate the activation of different kinase pathways. However, EETs may also enter cells and can interact directly with intracellular effectors like PPARγ, cardiac KATP channels and TRPV4 Ca^{2+} channels (Spector & Kim, 2015). According to Sala *et al*., (2018), an essential pathway of catabolism phospholipids is the hydrolysis by soluble epoxide hydrolase (sEH) which converts EETs to their

corresponding dihydroxyeicosatrienoic acids (DHET). sEH inhibition stabilises EETs, increases their incorporation into phospholipids and other metabolites, and enhances their functional responses which is associated with potential therapeutic benefits (Sala *et al.*, 2018).

Rheumatoid Arthritis

Rheumatoid arthritis is the commonest inflammatory arthritis and a key cause of disability (Firestein, 2003). It is a chronic inflammatory disease that involves a network complex inflammatory cytokines and activated immune cells leading to impairment of the cartilage, synovial membrane and bone (Jabeen, Mesaik, Simjee, Bano, & Faizi, 2016). Though its aetiology is not entirely understood, the activation of both T and B cells have been implicated in the initial phase of RA (Brennan *et al.*, 2002; Dörner & Burmester, 2003). Pro-inflammatory cytokines such as TNFα, IL-1, IL-17 have been shown to stimulate inflammation and destruction of bone and cartilage, and as such, they have been employed to induce experimental arthritis (Mateen, Zafar, Moin, Khan, & Zubair, 2016). Activation of transcription factors such as AP-1 and NF-κB by proinflammatory cytokines is known to cause hyper-proliferation of synovial fibroblasts (Yang, Chen, Chi, Lin, & Hsiao, 2017b).

NF-κB plays an essential role in regulating the transcription of many genes involved in cell growth, immune responses and inflammation. It controls the synthesis of pro-inflammatory cytokines, iNOS and COX-2 and is implicated in LPS-induced signalling pathways. Targeting NF-κB is therefore found to be effective in many models of arthritis and provides a direct link between inflammation and hyperplasia in arthritic joint (Firestein, 2003). The

transcription factor AP-1 mediates the modulation of many genes in RA including TNF-α, IL-1 and metalloproteinases and its component c-Jun and c-Fos which are highly expressed in RA synovium (Yang, Chen, Chi, Lin, & Hsiao, 2017). Additionally, AP-1 mediates most activities of IL-1 (Firestein & Manning, 1999). The MAPKs, being the regulators of cytokines and metalloproteinase production, provide a good target in RA. In the pathogenesis of rheumatoid arthritis, all the three members of MAPKs including ERK, JNK and p38 are extremely activated in synovial cells (Paul, Gohil, & Bhutani, 2006). Increased ERK activation was detected in synovial fibroblasts, and its inhibition was found to be beneficial. The inhibitors of p38 and JNK were found to reduce inflammation and provide protection against bone and cartilage destruction (Paul, Gohil, & Bhutani, 2006).

Models of Inflammation

Models of acute inflammation

Carrageenan-induced acute foot oedema in laboratory animals is one of the models used for screen anti-inflammatory effect of test drugs (Winter, Risley, & Nuss, 1962). Carrageenan, a polysaccharide derivative of Irish sea moss, is a phlogistic agent of choice for testing anti-inflammatory drugs as it is known to be antigenic and is devoid of apparent systemic effect (Kaur, Hamid, Ali, Alam, & Athar, 2004). Morris (2003) reported that intraplantar injection of carrageenan induces a biphasic inflammation in which the first phase occurs mostly one hour post-carrageenan injection, and it is characterised by symptoms such as oedema, erythema and pain. The induction results in a subsequent release of pro-inflammatory mediators including histamine, serotonin, tachykinins, bradykinin, reactive oxygen species (ROS) and complement proteins (Morris, 2003). Prostaglandins are known to mediate the

late phase of oedema via the action of cyclooxygenase-2 (COX-2) together with inducible nitric oxide synthase (iNOS) (Posadas *et al.*, 2004). During the inflammatory phase, IL-6, IL-1β, TNF-α, and MCP-1 levels are also enhanced (Fulgenzi *et al*., 2005).

Acute inflammatory responses (swelling, redness, heat, pain) may be induced by injection of inflammatory agents such as killed bacteria (e.g. *E. coli*), polymorphonuclear leucocytes, chemotactic factors, vasoactive agents (e.g. PAF and histamine), arachidonic acid (in acetone), carrageenan, zymosan, formalin and monosodium urate crystals into various parts of the body (Issekutz & Issekutz, 1989). The effect can be monitored by responses such as increase in foot volume produced by oedema (e.g. in rat's paw), detection of plasma markers in skin, local rise in temperature, measurement of inflammatory mediators in plasma exudates, hyperaemia, polymorphonuclear leucocyte accumulation, lymphocyte accumulation, monocytes infiltration, quantisation of haemorrhage, platelet deposition and thrombosis using diverse techniques (Issekutz & Issekutz, 1989).

Models of arthritis (Chronic inflammatory models)

Collagen-induced arthritis

This model of arthritis imitates human rheumatoid arthritis (Hablot *et al*., 2017). It is marked by the production of auto-antibodies toward self which is similar to what happens in human RA hence, a gold standard *in vivo* model for rheumatoid arthritis studies (Asquith, Miller, McInnes, & Liew, 2009). It can be applied to non-human primates, rabbit, mouse and rat and can generate polyarthritis involving T-cell response and antibodies. The intensity of the disease peaks around day 35. The animals then enter healing stages which is characterized by upregulation of serum IL-10 and a subsequent decrease in

pro-inflammatory Th1 cytokines (Mauri, Williams, Walmsley, & Feldmann, 1996).

Zymosan-induced arthritis

Zymosan is isolated from the polysaccharides from *Saccharomyces cerevisiae* cell wall. It has recurring glucose units which are connected by β-1,3-glycosidic linkages. The model has an advantage of being able to be induce different strains of rodents. Zymosan causes RA via binding to toll-like receptor 2 leading to protein phosphorylation, generation of proinflammatory cytokines, arachidonic acid release and activation of the complement *via* the alternative pathway. Intra-articularly injection of zymosan into the knee joints of mice therefore leads to proliferative inflammatory arthritis with pannus formation, synovial hypertrophy as well as mononuclear cell infiltration. The disease reaches its climax at about day 3 and subsides by 7. It rather exhibits a biphasic activity as relapse has been shown to occur mostly on the 25th day (Frasnelli, Tarussio, Chobaz-Péclat, Busso, & So, 2005; Keystone, Schorlemmer, Pope, & Allison, 1977).

Antigen-induced arthritis

Various strains of mice develop inflammatory arthritis when primed with an antigen (*e.g.* methylated BSA in complete Freund's adjuvant) and subsequently challenged by intra-articular injection of the same antigen (Brackertz, Mitchell, & Mackay, 1977; Brackertz, Mitchell, Vadas, Mackay, & Miller, 1977). Such models are useful in that mice of several strains can be investigated to establish a hierarchical role for given factors in adaptive immune-mediated articular damage. Subsequent pathology comprises immune complex-mediated inflammation followed by articular T-cell-mediated responses. The model does not, however, recapitulate the endogenous breach

of tolerance that is typical of RA pathogenesis and as such the model has limitations in applicability to RA. Further development of this model comprises of prior adoptive transfer of transgenic ovalbumin-specific T cells followed by ovalbumin priming and later intra-articular challenge (Maffia *et al*., 2004). The recipient mice develop arthritis, which is followed by the emergence of auto-reactivity to collagen, and the presence over time of rheumatoid factors. This model has the advantage of facilitating imaging of the pathogenic T cells that in turn promote a breach of self-tolerance to articular antigens (Nickdel *et al.*, 2009).

Pristane-induced arthritis

A single subcutaneous injection of small amounts of pristane leads to the development of a severe acute inflammation followed by a chronic relapsing phase in rats and mice (Wooley, Seibold, Whalen, & Chapdelaine, 1989). The model is mostly T-cell dependent, and the main pathological features include oedema accompanied by an acute phase response, infiltration into the joint of mononuclear and polymorphonuclear cells, pannus formation and the erosion of cartilage and bone (Asquith, 2009).

Pharmacological management of inflammation

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs constitute the mainstay of pharmacotherapy for many inflammatory conditions. They comprise of a chemically heterogeneous group of drugs with anti-inflammatory, analgesic, and antipyretic properties and their primary mechanism of action being the inhibition of prostanoids biosynthesis through cyclooxygenase (COX) pathway (Vane, 1971).

Aside from COX inhibition which is known to be the primary mechanism of action of NSAIDs, other mechanisms that are not dependent on

COX have been suggested in the literature. These include membrane stabilisation, suppression of reactive oxygen species (ROS), activator protein-1 (AP-1), nuclear factor (NF)-kappa B, which modulates the expression of pro-inflammatory mediators such as cytokines, chemokines and adhesion molecules (Tegeder *et al*., 2001).

They are used in short-term treatments for pain as well as in long-term management of chronic inflammatory diseases such as osteoarthritis and rheumatoid arthritis (Pereira, Nunes, Jamal, Cuccovia, & Reis, 2017). Traditional NSAIDs (tNSAIDs) that are nonselective prostaglandin inhibitors are associated with a broad spectrum of adverse effects such as gastrointestinal bleeding (Wolfe, Lichtenstein, & Singh, 1999), stroke and renal failure (Jacob *et al.*, 2018). COX-2 selective inhibitors (Coxibs) were introduced with an aim to overcome NSAID related GI toxicity. However, these agents had enhanced cardiovascular risk due to a reduction in endothelial prostaglandin I2 (PGI2) and increased levels of platelet aggregator thromboxane A2 (TXA2) (Dogné, Supuran, & Pratico, 2005). Some coxibs such as valdecoxib had to be withdrawn from the market due to their adverse cardiovascular side effects. Both tNSAIDs and coxibs are coupled with higher risk of cardiovascular problems and stroke (Harirforoosh, Asghar, & Jamali, 2014).

Steroidal anti-inflammatory drugs

Gluccorcorticoids such as dexamethasone, betamethasone, prednisolone etc. have been used extensively in the treatment of several inflammatory diseases including asthma and rheumatoid arthritis (Rang et al., 2014). They have been categorised as natural (hydrocortisone, cortisone, corticosterone) or synthetic (dexamethasone, betamethasone, prednisolone, triamcinolone) steroids. The anti-inflammatory effect of corticosteroids has

been shown to be mediated via the inhibition of phospholipase A2 activity which is associated with the conversion of membrane-released phospholipids to arachidonic acid (Lorenz *et al*., 2010). Despite their positive effects on arthritis and other inflammatory diseases, complications such as immunosuppression, osteoporotic bone fracture, cataract and renal impairment (Dima et al., 2017).

Disease-modifying antirheumatic drugs (DMARDs)

They are a group of medications used for the management of rheumatoid arthritis. They have immunosuppressive effect and are designed to minimise the damage done to joints, and they can induce or maintain remission, reduce the frequency of flare-ups, and allow for tapering of steroids while sustaining disease control (Abbasi *et al.*, 2019). They can also be used to in the treatment of other autoimmune disorders such as vasculitis, scleroderma, inflammatory myositis, spondyloarthritis, systemic lupus erythematosus, inflammatory bowel disease, and some types of cancers (Benjamin & Lappin, 2018). The two main types of DMARDs are traditional and biologics.

Traditional DMARDs

These comprise of leflunomide, methotrexate, sulfasalazine hydroxychloroquine, cyclophosphamide and tacrolimus (FK506). These drugs act via several mechanisms as they mediatecertain important pathways in the inflammatory cascade. For instance, methotrexate, stimulates adenosine release from fibroblasts, reduces neutrophil adhesion, inhibits leukotriene B4 synthesis by neutrophils, inhibits local IL-1 production, reduces levels of IL-6 and IL-8, suppresses cell-mediated immunity, and inhibits synovial

collagenase gene expression (Benjamin & Lappin, 2018). Other medications in this class inhibit proliferation or cause dysfunction of lymphocytes.

Biologics DMARDs

They are generally recommended after evidence of disease progression and structural joint damage despite treatment with steroids and conventional therapy. Some biologic agents include etanercept, abatacept, infliximab, tocilizumab and rituximab. These drugs are made of monoclonal antibodies, chimeric fusions antibodies, whereas others are receptors that have been fused to another part of human immunoglobulins. Their design is highly specific and targeted to affect immune function (Benjamin & Lappin, 2018). These type of antirhematoid drugs are very selective in their mechanism of action. Their main mode of action include interfering with cytokine function or production, inhibiting the "second signal" required for T-cell activation, and depleting B-cells or inhibiting factors that activates B-cells (Aletaha & Smolen, 2018).

Inflammatory Pain

Pain is defined by the International Society for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Merskey & Bugduk, 1994). Inflammatory pain, however, is a type of pain which mostly occurs as a result of excessive sensitivity to pain due to injury. A feature of this type of pain is the development of hypersensitivity such that stimuli, which generally would not produce pain or which would usually be innocuous, begins to do so resulting in the production of a broad range of inflammatory mediators by inflammatory cells. These mediators act on and alter the properties of high-threshold primary-sensory neurons. The three significant features underlying this type of pain are peripheral sensitisation

(the peripheral sensitivity of high-threshold nociceptors is altered), phenotypic switch (changes in properties and function of the chemical makeup of neurons) and central sensitisation (up-regulation in the sensitivity of the neurons in the central nervous system) (Woolf, 2004).

Inflammatory mediators of pain

Inflammatory mediators such as prostaglandins, bradykinins, endothelins, substance P, sympathetic amines and nerve growth factors (NGF) are marked sensitisers of nociceptors. These mediators facilitate the electrical activity of the neuronal membrane by acting on neuronal receptors directly resulting in the activation of some molecular mechanisms. It is generally recognised that the trigger of G-protein coupled receptors activates the enzyme adenylate cyclase by the inflammatory mediators which in turn lead to the production of cAMP. A group of protein kinases A and C are subsequently initiated by the activation of cAMP leading to the phosphorylation of ion channels in the membrane. This facilitates inward sodium current by tetrodotoxin-resistant Na^+ channels and inward Ca^{++} currents whereas outward K^+ currents are inhibited. This is a primary peripheral mechanism that may account for the hyperalgesia induced by the stimulation of subthreshold chemical, thermal, or mechanical stimuli (Linley, Rose, Ooi, & Gamper, 2010; Schaible, Ebersberger, & Natura, 2011; Verri Jr, Cunha, Poole, Ferreira, & Cunha, 2007).

The role of cytokines in hyperalgesia

Cytokines are essential mediators of peripheral sensitisation. Intradermal administration of cytokines such as keratinocyte-derived chemokine (KC), TNF-α, interleukins IL-1β, IL-8, IL-12, IL-15 and IL-18

have produced intense and sustained mechanical sensitisation and hyperalgesia in rodents (Stein *et al*., 2009).

Previous studies led to the proposal of the following sequence of events in hyperalgesia. In the first place, TNF-α is stimulated by carrageenan, lipopolysaccharide or the antigen itself which, in turn, induces IL-1β and IL-6, thus activating the synthesis of COX products (PGE_2). Again, TNF-α can induce another cytokine, IL-8, thus stimulating the local production of sympathetic amines which subsequently produces hyperalgesia. Also, endothelin-1 can be activated by IL-18 and IL-12 resulting in hyperalgesia (Ferreira, Lorenzetti, & Poole, 1993; Nakamura, Fujita, & Shiomi, 1996). Figure 1 shows that mechanical hyperalgesia in rats is mediated by inflammatory stimulus-induced cytokine cascades.

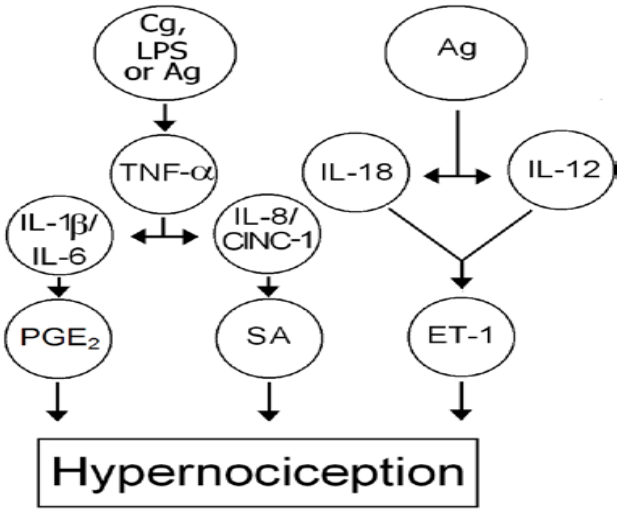

Figure 1: Inflammatory mediators coordinated release initiated by different stimuli. Cg (carrageenan), Ag (antigen), CINC-1 (cytokine-induced neutrophil chemoattractant-1), IL (Interleukin), ET-1 (endothelin-1), LPS (lipopolysaccharide), SA (sympathetic amines), PGE_2 (prostaglandin E_2) adapted from (Verri Jr et al., 2007).

Inflammatory pain models

The objective of these models is to induce a painful condition that mimics clinical inflammatory pain. Pain originating from acute inflammation act as a physiological function to prevent further tissue damage and it mostly ceases after removal of the noxious stimulus. Chronic inflammatory pain, however, occurs when healing persists beyond the expected time, due to an ongoing inflammatory process. These models have helped researchers to understand the underlying mechanism of inflammatory pain and in developing potential treatments. To induce inflammation, irritating substances or the inflammatory mediators such as beer yeast, croton oil, complete Freund's adjuvant (CFA), formalin, or carrageenan is injected into the body part of an animal such as the hind paws (Gilfoil, Klavins, & Grumbach, 1963; Winter & Flataker, 1965).

After the induction of inflammation the measurement of pain responses, such as withdrawal latency and tail-flick latency, using analgesiometry is performed over time (hours to days depending on the lasting effect of a specific substance) compared to baseline values obtained preceding the substance administration (Randall, 1957). Generally, tissue inflammation lowers the nociceptive threshold and reduces the latency period of paw withdrawal.

Inflammation and Depression

Accumulated evidence suggests that increased hyperactivity and inflammation of the hypothalamic–pituitary–adrenal axis are two of the most consistent postulates linked to the pathogenesis of major depression (Pariante, 2017). There is ample evidence that suggests the involvement of

inflammatory mediators in mood disorders and that the activation of the inflammatory system reduces the clinical therapeutic benefit of many antidepressants (Carvalho *et al*., 2013). Also, other investigations have reported elevated levels of inflammatory markers (IL-6, IL-10, and CRP) in patients with major depression (Seidel et al., 1995). Interestingly, relief of depressive symptoms is reported in patients with concurrent depression and inflammatory disease following administration of anti-cytokines (Lotrich, 2015). Pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-α) affect synaptic plasticity leading to the establishment of depression-like behaviours and mood disorders (Khairova, Machado-Vieira, Du, & Manji, 2009).

Antidepressant drugs

It is projected that about 50% of depressed patients are unresponsive to currently available antidepressant drugs (Ménard, Hodes, & Russo, 2016; Rush et al., 2006). With that notwithstanding, the following classes of antidepressant drugs are clinically available (Abdel-Salam, Nofal, & El-Shenawy, 2003; Fasipe, 2019; McIntyre, Suppes, Tandon, & Ostacher, 2017; Sacre, Medghalchi, Gregory, Brennan, & Williams, 2010).

1. Tricyclic antidepressants (TCAs) which include amitriptyline, nortriptyline, clomipramine, trimipramine, protriptyline desipramine, imipramine, trimipramine, doxepin.
2. Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, sertraline, paroxetine, citalopram, escitalopram, and fluvoxamine.
3. Norepinephrine reuptake inhibitor with serotonin receptors antagonism (NRISA) such as maprotiline

4. Monoamine oxidase inhibitors (MAOIs) such as phenelzine, nialamide, isocarboxazid, hydracarbazine, tranylcypromine, moclobemide, bifemelane, pirlindole, toloxatone, selegiline, rasagiline and safinamide.
5. Serotonin receptors antagonist with serotonin reuptake inhibition (SARI) such as trazodone, nefazodone, and vortioxetine.
6. Serotonin-norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine, desvenlafaxine, duloxetine, ansofaxine, nefopam and levomilnacipran.
7. Norepinephrine-dopamine reuptake inhibitor (NDRI) such as bupropion.
8. Selective norepinephrine reuptake inhibitors (NRIs) such as Reboxetine, viloxazine, teniloxazine (also known as sulfoxazine or sufoxazine), and atomoxetine.
9. Serotonin 5-HT_{1A} autoreceptor partial agonist with serotonin reuptake inhibition (SPARI) such as vilazodone.
10. Serotonin-norepinephrine reuptake inhibitor and serotonin receptors antagonism antidepressant with potent antipsychotic D_2receptor blockade/antagonism (SNRISA with potent antipsychotic D_2 receptor blockade/antagonism) such as amoxapine.
11. Noradrenergic α_2 -receptor antagonist with specific serotonergicreceptors-2 and -3 antagonism (NASSA) such as mirtazapine and mianserin.
12. Atypical antipsychotics that exhibit weak D_2 receptor antagonism with potently strong 5-$HT_{2A/2C}$ receptor blockade such as olanzapine, quetiapine, risperidone, lurasidone, aripiprazole and brexpiprazole
13. NMDA-glutamatergic ionoceptor blockers that exhibit a direct action on the excitatory glutamatergic neurotransmission system such as ketamine,

CP-101,606 (traxoprodil), GLYX-13 (rapastinel), NRX-1074 (Apimostinel) and Riluzole.

Animal models of depression

Modelling human depression in animals is quite complex bearing in mind the nonexistence of objective biomarkers coupled with the biased nature of the multiple psychological and physiological symptoms of the disease (Ménard *et al.*, 2016). However, the forced swim test (FST) and the tail suspension test are two most widely used models to study the antidepressant effects of test drugs.

The forced swimming test

Porsolt *et al.*, (1977) initially described the forced swimming test (FST), which is now the most widely used laboratory experiment to assess the antidepressant potential of drugs and medicinal compounds (Cryan, Markou, & Lucki, 2002; 2005). This has been primarily attributed to its reliability across laboratories, ease of use, ability to detect a broad spectrum of antidepressants, and its capacity to meet the high throughput demands of the pharmaceutical industry (Borsini & Meli, 1988; Rupniak, 2003). The immobility response elicited when rodents are placed in an inescapable cylinder of water is known to mimic the cessation of persistent escape-directed behaviour that serves various adaptive functions in response to stress (Cryan &. Mombereau, 2004; Detke, Johnson, & Lucki, 1997). The ability of test compounds to reduce the immobility times has been designated as antidepressant property.

Tail Suspension Test

This test is based on the fact that rodents subjected to the short-term, inescapable stress will develop an immobile posture (Cryan & Mombereau,

2004). The stressful situation, in this test, involves the haemodynamic stress of being hung uncontrollably by their tail (Thierry, Steru, Simon, & Porsolt, 1986).

In the TST, the tails of the mice are attached and suspended by an adhesive tape. The time the animals spend being immobile during the six minutes is interpreted as a measure of depressive-like behaviour. Various antidepressant medications reverse this immobility and promote the occurrence of escape-related behaviours (Abelaira *et al.*, 2013). A distinct advantage of this test is its ability to detect a broad spectrum of antidepressants irrespective of their underlying mechanism; it is inexpensive, methodologically unsophisticated and readily amenable to automation.

Anxiety

Anxiety is an adaptive response to stress or threat. It is characterized by nervousness, irritability and an exacerbated reaction to danger (American Psychiatric Association, 2013). This emotional-behavioral state may occur in response to exteroceptive visual, auditory, olfactory, or somatosensory stimuli or to interoceptive input through the viscera and the endocrine, and autonomic nervous systems (Charney & Drevets, 2002). Anxiety disorders are among the most common psychiatric disorders that affect all age groups of the general population which include panic attack, agoraphobia, specific phobia, separation anxiety disorder, social anxiety disorder (social phobia) and selective mutism (American Psychiatric Association, 2013). Epdemiological studies have revealed that between 5–30% of people are affected by an anxiety disorder at some point in their life whereas about 12% are affected in a given year (Craske & Stein, 2016; Kessler, Ruscio, Shear, & Wittchen, 2009).

Benzodiazepines such as diazepam have also been used for the treatment of anxiety. However, these compounds have a number of harmful effects including psychomotor impairment, sedation, myorelaxation, hepatotoxicity, and dependence among others (Lader & Mortan, 1991). Other pharmacological treatments employed in the treatment of anxiety disorders include selective serotonin-reuptake inhibitors and serotonin–noradrenaline-reuptake inhibitors, tricyclic antidepressants (Craske & Stein, 2016).

It is well-known that high comorbidity exists between depression and anxiety; two mental diseases that reduce quality of life of patients (de Heer *et al*., 2014), .

Animal models of anxiety

The elevated plus maze

The elevated plus maze is a simple method for assessing anxiety responses in mice/rats and this was first described by Pellow and co-workers (Pellow, Chopin, File, & Briley, 1985) and later reviewed by Walfe and Frye (2007)). Briefly, it consists of two closed arms and two open arms arranged to form a plus shape. The test is grounded on the observation that naturally, rodents display an aversive behaviour in response to exposure to novel open spaces and fear of balancing on a relatively narrow, raised platform. The assessment of anxiety behaviour of rodents is done by using the ratio of time spent on the open arms to the time spent on the closed arms. In the test, mice or rats are placed at the junction of the four arms of the maze, facing an open arm, and frequency of entries/duration in each arm are recorded by a video-tracking system and observed for 5 minutes. Other ethiological parameters (i.e. head dips, rearing, and stretch-attend postures) are used to determine the anxiolytic effect of test compounds (Walf & Frye, 2007).

The open field test

The test provides a comprehensive assessment of the motor as well as behavioural activities of mice. It is commonly used to measure anxiety-related behaviours in rodents (Prut & Belzung, 2003). The procedure consists of subjecting an animal, usually a rodent, to an unknown environment from which escape is prevented by surrounding walls (Walsh & Cummins, 1976). In fact, anxiety behavior in the open field is triggered by two factors which include the stress imposed by isolating the animal from its social group (testing animals on individual bases) and the exposure of the animal to a relatively large, well-illuminated and novel arena compared to the animal's breeding or natural environment (i.e. inducing agoraphobia). The open field test is designed to study exploratory activity of rodents in such new environments (Crawley, 1985).

Medicinal plants as sources of modern drugs

Nature has been a source of many medicinal products as many useful drugs have been developed from plants and other natural sources (Cragg & Newman, 2013). For instance, verapamil which is currently used as an antihypertensive was derived from papaverine obtained from *Papaver somniferum* (Fabricant & Farnsworth, 2001). Also, sodium cromoglycate used as a bronchodilator were obtained from structural modification of khellin, a compound isolated from *Ammi visnaga* (L) Lamk. (Fabricant & Farnsworth, 2001). Metformin and other bisguanidines used as antidiabetic drugs were derived from galegine, a compound isolated from *Galega officinalis* L. (Fabricant & Farnsworth, 2001). Quinine, obtained from the bark of *Cinchona officinalis* formed the basis for the synthesis of antimalarial drugs, chloroquine and mefloquine (Cragg & Newman, 2013). Furthermore, artemisinin and its

analogues obtained from *Artemisia annua* have been very useful in the treatment of malaria (Miller & Su, 2011). Reserpine, an antihypertensive agent, was isolated from *Rauwolfia serpentina* (Cragg & Newman, 2013).

Ephedra sinica, which has long used in traditional Chinese medicine, formed the basis for the isolation of ephedrine and the synthesis of the anti-asthma agents (beta agonists) such as salbutamol and salmeterol (Buss, Cox, & Waigh, 2003). Also, *Chondrodendron* and *Curarea* species used by indigenous groups in the Amazon as an arrow poison formed the basis for the isolation of tubocurarine, a muscle relaxant agent (Buss *et al*., 2003).

Of the plant-derived anticancer drugs in clinical use, the best known are the so-called vinca alkaloids, vinblastine and vincristine isolated from the Madagascar periwinkle, *Catharanthus roseus* (Cragg & Newman, 2013), together with the two clinically-active agents, etoposide and teniposide, which are semisynthetic derivatives of the natural product epipodophyllotoxin (Cragg, Kingston, & Newman, 2011)

Paclitaxel, an exciting plant-derived anticancer occurs along with several vital precursors (the baccatins) in the leaves of various *Taxus* species (Newman, Cragg, & Kingston, 2005).

Toxicity of Herbal Medicine

Medicinal plants play an essential role in health management in Africa and the world as a whole (Mahomoodally, 2013). They are reliable sources of raw materials for both traditional and modern medicines (Cragg & Newman, 2013). However, to ascertain the safety of new medicinal products, toxicological studies are needed as they give information on therapeutic and toxic indices of drugs and xenobiotics (Rahman *et al.*, 2014). It is also an

ethical, regulatory and scientific requirement that the safety of any new potential medicinal product must be ascertained in animal studies to define safe human doses before it can be administered to humans (Al-Afifi, Alabsi, Bakri, & Ramanathan, 2018). Even though herbal-based medicinal products are generally regarded as natural, time-tested, and therefore safe compared with synthetic drugs (Schachter, 2009), other reports suggest such medicines have the potential of causing life-threatening side effects and even death when used for a long time or at high doses in a similar manner as synthetic drugs (Adongo *et al*., 2018). Tremendously toxic substances like the digitoxins, cyanogenic glycosides and strychnine were obtained from plants. It is only after careful investigations that the safety of any particular plant species can be assured (Araújo *et al*., 2017).

It is therefore vital to establish their safety through the use of validated and well-controlled scientific toxicity studies or protocols (Bhushan, Sardana, & Bansal, 2014). Acute toxicity is one of the methods used to estimate the safety of new compounds which takes into consideration unwanted effects that occur either immediately or at a short time interval after a single or multiple administration of substances within 24 hours (Chinedu, Arome, & Ameh, 2013). The test is also useful in the estimation of the lethal dose, LD_{50} (i.e. the dose that kills 50% of the population), the onset of action, duration and degree of recovery of survived animals and other biological effects. On the other hand, repeated-dose (sub-acute, sub-chronic and chronic) toxicity studies provide data for predicting the maximum tolerable levels for the species during potential lifetime exposure (De Jong, Carraway, & Geertsma, 2012). Subacute and sub-chronic toxicity differ in duration of exposure. Whereas

subacute systemic toxicity is defined as adverse effects occurring after multiple or continuous exposure between 24 h and 28 days, sub-chronic systemic toxicity is defined as adverse effects arising after a continuous or repeated administration of a test sample for up to 90 days or not exceeding 10% of the animal's lifespan (De Jong, Carraway, & Geertsma, 2012).

The Family Rhamnaceae

The family *Rhamnaceae* belongs to the order *Rhamnales* comprising of over 50 genera and more than 600 species (Sheng & Shen, 2011). Among the several genera in this family are *Adolphia* Meisn. (prickbush), *Alphitonia* Reissek ex Endl. (alphitonia), *Auerodendron* Urb. (auerodendron), *Berchemia* Neck. ex DC. (supplejack), *Ceanothus* L. (ceanothus), *Colubrina* Rich. ex Brongn. (nakedwood), *Condalia* Cav. (snakewood), *Rhamnus* L. (buckthorn), *Ventilago* Gaertn. (ventilago), *Hovenia* Thunb. (hovenia), *Sageretia* Brongn. (mock buckthorn), *Gouania* Jacq. (chewstick), *Karwinskia* Zucc. (karwinskia), *Maesopsis* Engl. (umbrella-tree), *Paliurus* Mill. (Jerusalem thorn) and *Ziziphus* Mill. (jujube) (United States Department of Agriculture, 2019).

Genus *Ziziphus*

. The generic name *Ziziphus* was derived from the latinized version of the Arabic vernacular name ‘zizouf’ which means ‘from Ethiopia’ (Orwa et al., 2009). This is suggestive of the African origin and wide distribution of the plant in Africa. Over a hundred species of plants are found in this genera, and most of them are known to contain medicinal properties (Kaleem *et al*., 2014).

Phytochemicals from the Genus

Alkaloids from Ziziphus genus

The genus Zizphus is rich in cyclopeptide alkaloids constituting about 50 % of 170 cyclopeptides isolated from plants. Among these alkaloids isolated from the various species of Ziziphus are abyssenine-A (**1**), abyssenine-B (**2**), abyssenine-C (**3),** mucronine-A (**4**), mucronine-B (**5**), mucronine-C (**6),** Zizyphine-D (**7**), Zizyphine-E (**8**), Lotusanine-A (**9**), Oxyphylline-A (**10),** Amphibine-A (**11**), Xylopyrine-A (**12**) among others (Kaleem et al, 2014). Their chemical structures have been drawn as shown in Figure 2.

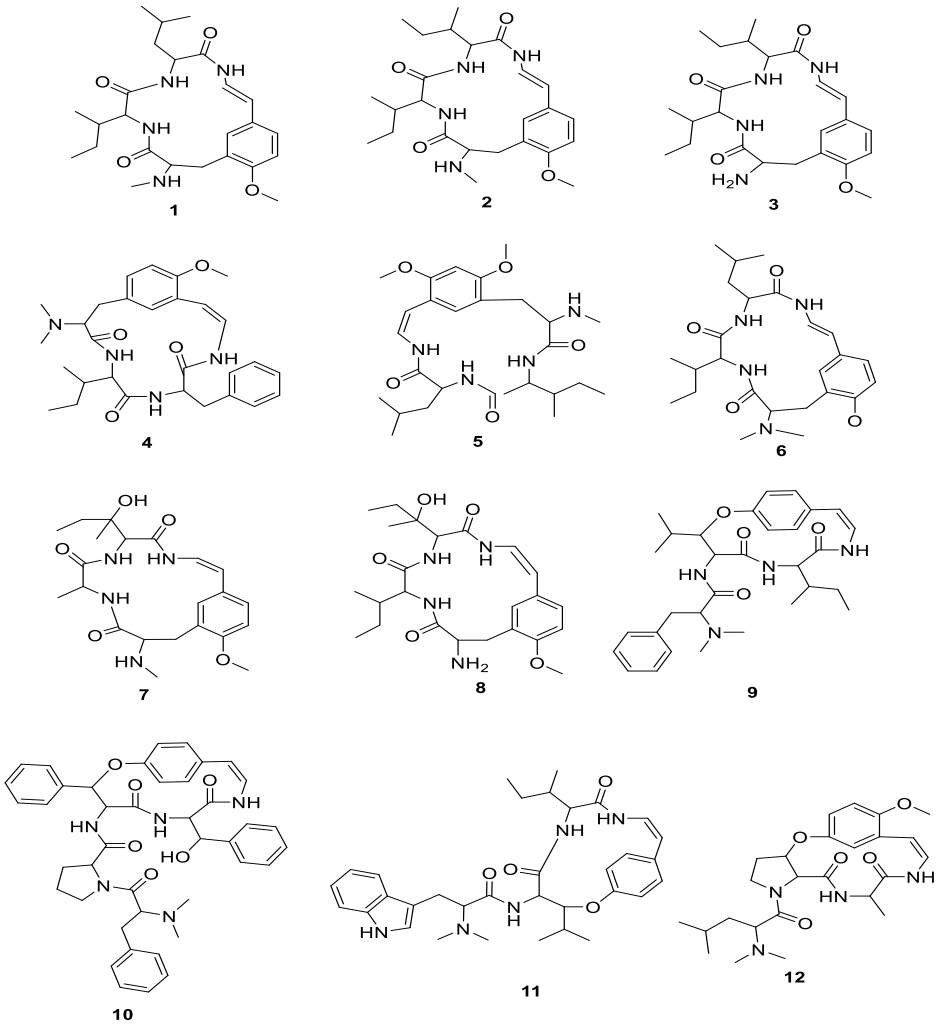

Figure 2: Cyclopeptide alkaloids isolated from various species of Ziziphus

Triterpenoids from Ziziphus genus

The *Ziziphus* genus is rich in triterpenoic acids as shown in Figure 3. 3-O-cis-p-coumaroyl-alphitolic acid (**13**), zizyberenalic acid (**14**), alphitolic acid (**15**), 3-O-cis-p-coumaroylmaslinic acid (**16**), betulinic acid (**17**), betulonic acid (**18**), oleanolic acid (**19**), and oleanonic acid (**20**) are among triterpeonoids that have been isolated from the fruits of *Z. jujube* (Lee, Min, Lee, Kim, & Kho, 2003). These triterpenes have also been isolated from the roots of *Z. mauritiana* (Mahajan & Chopda, 2009). Also, lupeol (**21**), α-amyrin (**22**) and β-amyrin (**23**) have been reported in *Ziziphus mauritiana (Mohammad et al*., 2015).

Flavonoids from Ziziphus genus

As presented in Figure 4, flavonoids such as kaempferol (**24)**, quercetin (**25**), and phloretin (**26**) have been isolated from the fruits of *Ziziphus jujube* Miller and *Ziziphus spina-christi* (Pawlowska, Camangi, Bader, & Braca, 2009).

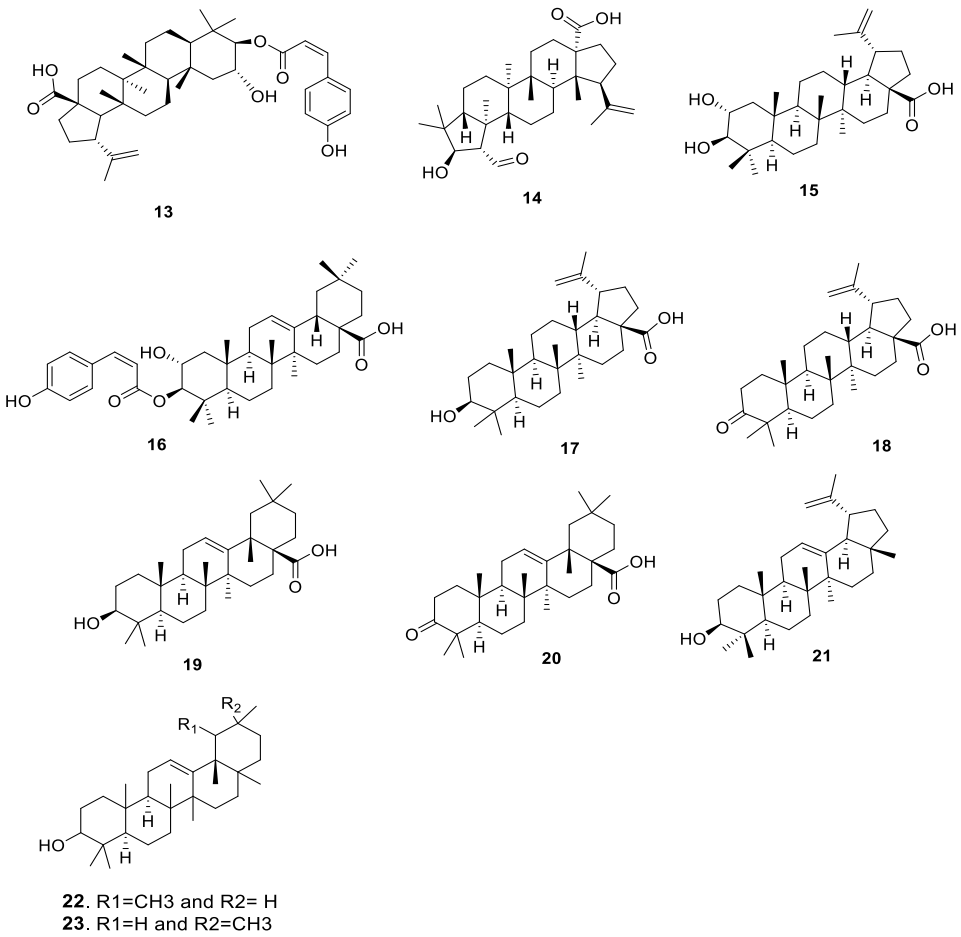

Figure 3: Triterpenoids that have been isolated from various species of *Ziziphus*

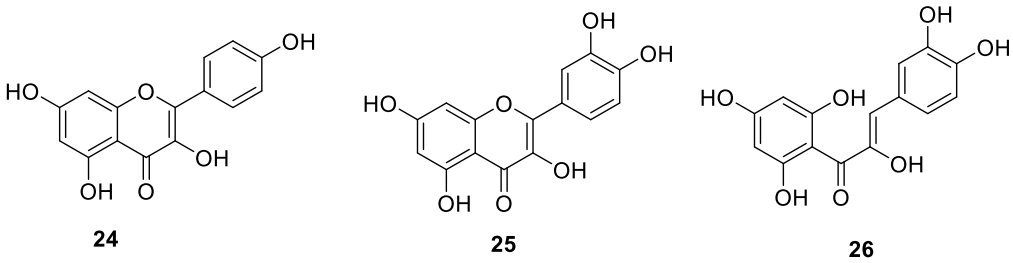

Figure 4: Flavonoids that have been isolated from various species of *Ziziphus*

Other phytochemicals from Ziziphus genus

Phytosterols such as β-sitosterol and stigmasterol have been isolated from the leaves of *Ziziphus mauritiana* (Mohammad *et al*., 2015). Saponin glycosides such as jujuboside B and jujubogenin glycosides have been isolated from *Zizphus lotus* (Maciuk *et al*., 2004). Also, water soluble polysaccharide

(ZSP3) has been isolated from *Ziziphus jujuba* cv. Jinsixiaozao (Kaleem *et al.*, 2014).

Pharmacological effect of Ziziphus genus

Analgesic and anti-inflammatory effect

Aqueous fraction of *Zizyphus lotus* given intraperitoneally produced dose-dependent analgesic and anti-inflammatory effect in rodents (Borgi, Ghedira, & Chouchane, 2007). Fruit extract of *Ziziphus jujube* demonstrated a potent anti-inflammatory effect in carrageenan-induced paw oedema in rats and this effect was partially attributed to the presence of betulinic acid and quercetin (Mesaik, Poh, Bin, Elawad, & Alsayed, 2018). Also, crude methanolic extract of *Ziziphus oxyphylla* exhibited potent analgesic and anti-inflammatory effect in murine models (Nisar *et al*., 2007). Antinociceptive activity of *Zizyphus spina-christi* root bark extract has also been reported (Adzu, Amos, Wambebe, & Gamaniel, 2001).

Central nervous system effect

Ethanolic extract, ethyl acetate fractions and ethanolic extract of *Zizyphus xylopyrus* exerted antidepressant effect in two behavioral models, forced swimming and tail suspension tests (Sharma, Chauhan, Lodhi, & Singhai, 2009). *Zizyphus spina-christi* leaf extract was also found to have neuroprotective and therapeutic roles against pentylenetetrazol convulsant effect (Waggas & Al-Hasani, 2010). The aqueous extract of the same plant also exhibited central nervous activity in exploratory behavior, spontaneous motor activity and pentobarbitone induced sleeping time (Adzu, Amos, Dzarma, Wambebe, & Gamaniel, 2002). Sanjoinine A, one of the major alkaloid compounds in *Ziiyphus Spinosi* Semen, was found to exhibit

anxiolytic effect possibly through the involvement of GABAergic transmission (Han *et al*., 2009).

Gastro-intestinal tract effect

Roots, leaves and stem extract of *Ziziphus lotus* produced anti-ulcer effect in HCl/ethanol induced ulcer (Wahida, Abderrahman, & Nabil, 2007). The methanol extract of *Ziziphus lotus* showed a significant reduction of gastric juice secretion and total acidity and an increase in pH value in pylorus-ligature model in rats similar to positive control drug (Bakhtaoui, Lakmichi, Megraud, Chait, & Gadhi, 2014). *Zizphus abyssinica* has also been shown to exhibit anti-ulcerogenic effect (Ugwah et al., 2013). Extractives from the leaves of *Ziziphus mauritiana* showed antidirrhoeal effect (Mohammad et al., 2015).

Ziziphus abyssinica

Though the official scientific name of the plant is *Ziziphus abyssinica* (Hochst ex A. Rich), it has also been identified with other names such as *Ziziphus mauritiana* var. abyssinica (Hochst. ex A. Rich.) and *Ziziphus atacorensis* A.Chev (theplantlist.org). Table 1 contains the local names of the plant in different parts of the word. Also, the taxonomy of the plant has been described in Table 2.

Table 1: List of local names for *Ziziphus abyssinica* in different parts of the world

No.	Locality/language	Local name	Reference
1.	Ghana (Sisala)	Larukluror	(Burkil, 1985; Orwa *et al*., 2009).
2	French	Jujubier sauvage	(Burkil, 1985; Orwa *et al*., 2009).
3	Nigeria (Hausa)	Magariya	(Burkil, 1985; Orwa *et al*., 2009).
4	English	Catch thorn	(Burkil, 1985; Orwa *et al*., 2009).
5	Kenya (West Pokot)	Angau	(Nyaberi *et. al*. 2010)
6	Ethiopia (Dek Island)	Kurkura	(Teklehaymanot, 2009)
7	Machakos and Kitui District	Kiae	(Wagate *et al*. 2010)

Table 2: Taxonomy of *Ziziphus abyssinica*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order:	Rhamnales
Family	Rhamnaceae
Genus:	*Ziziphus*
Species	*abyssinica*

Plant description

Ziziphus abyssinica is a small half-evergreen tree or spiny shrub of up to 12 meters tall with a straight but sometimes crooked single central stem. It has spreading branches which hang down to form a dense, rounded and untidy crown. The leaves of the plant are ovate with three prominent veins and a broadly tapering apex arranged alternately along the stem. The leaves also have finely toothed margins with prominent asymmetrically lobed base and a 1.2 cm long petiole as shown in Figure 5.

Fresh bark of the tree has creamy to greyish brown appearance, fissured longitudinally and becoming rough in older plants. The branches are almost zigzag with single or paired thorns of up to 12 mm. Flowers look creamy to yellowish-green, star-shaped and smallish with an unpleasant sharp smell. The stalk is 1-2 cm long beside the leaves. The plant has smooth shiny red or reddish-brown spherical fruits which are about 2-3 cm in diameter when mature. The fruits have an inner stone which contains 1 or 2 light brown glossy seeds (Nyaberi *et al*., 2010a; Orwa, *et al.*, 2009; Ibrahim *et. al.*, 2015).

Figure 5: *Ziziphus abyssinica* tree, leaves and fruits (source: field)

Geographical distribution of the plant

The plant is native to Ghana, Mozambique, Botswana, Ethiopia, Djibouti, Eritrea, Kenya, Democratic Republic of Congo, Somalia, Zambia, Tanzania, Uganda, Swaziland, South Africa and Lesotho. It is also indigenous to India and as an exotic plant in the United States of America (Nyaberi *et al*., 2010; Orwa et al., 2009). The availability of the plant in Nigeria has also been well documented. The map in Figure 6 shows the geographical location of the plant.

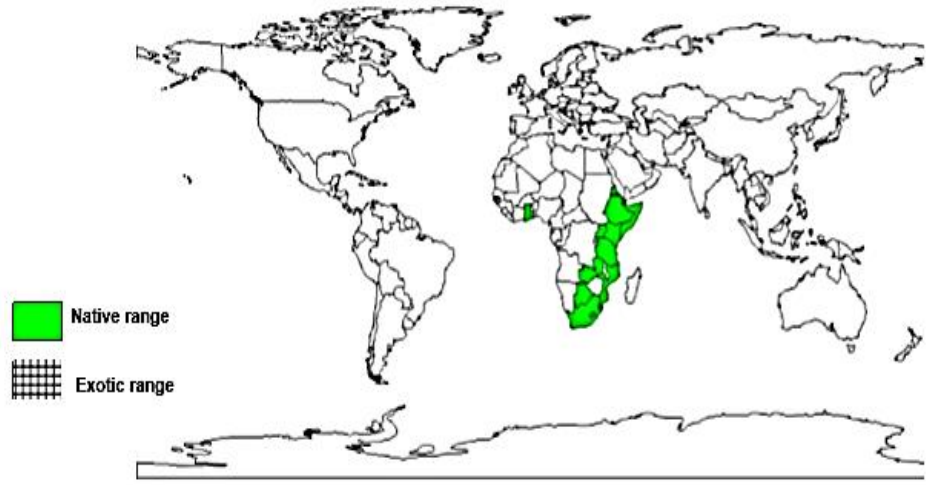

Figure 6: The map above shows countries with the documented species distribution of *Ziziphus abyssinica* (Orwa *et al*., 2009)

Phytochemicals screening of the plant

Qualitative phytochemical investigations performed on the aqueous and methanol fruit extracts of *Ziziphus abyssinica* have revealed the presence of saponins, tannins, sterols and steroids, alkaloids, flavonoids and reducing compounds (Nyaberi *et al.*, 2010). In another study, even though flavonoids were absent, carbohydrates, alkaloids, saponins, tannins, glycosides, anthraquinones and steroids were detected in aqueous root extract of the plant collected from Sokoto region in Nigeria in December (Ugwah *et al.*, 2013). It has also been reported that the hydro-ethanolic leaf extract of the plant collected from Ejura (Ghana) revealed the presence of tannins, phenols, alkaloids, triterpenes, flavonoids, phytosterols reducing sugars, tannins, flavonoids, proteins and amino acids (Boakye-Gyasi *et al*., 2017a). A very comprehensive phytochemical screening on the plant was done by Ibrahim *et al*. (2015). Per their report, the leaves of the plant contained carbohydrates (monosaccharides, reducing sugars, ketosis and pentose), tannins, cardiac

glycosides, saponins, flavonoids and indole alkaloids whiles anthraquinone glycosides, tropane and isoquinoline alkaloid were absent.

Isolated compounds from Zizyphus abyssinica

The root and stem bark of *Ziziphus abyssinica* has been reported to contain six cyclopeptide alkaloids. These are abyssenine-A (**1**), abyssenine-B (**2**), abyssenine-C (**3**), mucronine-A (**4**), mucronine-B (**5**), and mucronine-C(**6**) as shown in Figure 2 (Kaleem et al., 2014; Tschesche et al., 1974).

Traditional uses of *Ziziphus abyssinica*

Medicinal uses of the plant

A poultice made from steaming hot leaves of the plant soaked in boiling water is applied on the chest to treat people with pneumonia. Similarly, ash obtained from the burnt leaves is mixed with salt and used to treat tonsillitis (Orwa et al., 2009). Also, ashes from burnt leaves of *Ziziphus abyssinica* mixed with other recipes have been used to treat snakebite, cuts, wounds and stings from other venomous animals (Martine Baerts & Lehmann, 1991). Newcastle disease is treated elsewhere with crushed stalks and leaves of the plant mixed with drinking water (Lans & Tonya, 1999). In Ethiopia, the plant's fresh roots and leaves are grounded and mixed with water and administered orally to treat jaundice and liver diseases (Lulekal, Kelbessa, Bekele, & Yineger, 2008; Teklehaymanot, 2009). Also, the leaves have been used in Burundi to treat tachycardia and pectoral pain (Baerts & Lehmann, 1989). The root barks of *Ziziphus abyssinica* are used to relieve headaches and migraines (Baerts & Lehmann, 1989). The pounded root barks are used to treat burns (Lulekal *et al.*, 2008). The roots have also been used as pain-killers, anti-diarrhoeal, abortifacients, emetics and for nasopharyngeal infections (Burkill, 1985). The dried powdered barks of stems, branches and trunks are

used to relieve constipation, abdominal pains and gastric ulcer. The barks of roots and stems have been used to treat burns (Okello, Nyunja, Netondo, & Onyango, 2010). The roots of the plant are also used to treat diarrhoea in Nigeria (Adamu *et al*., 2005; Etuk, Ugwah, Ajagbonna, & Onyeyili, 2009). The use of the roots and stem barks of the plant for the treatment of kidney and stomach ailments have been documented *(Wagate et al*., 2010). The pastoral community of West Pokot also use this herb to treat ailments like diarrhoea and various stomach infections (Nyaberi *et al*., 2010b). It has also been documented that the herbalists in the northern community in Nigeria employ the decoction of the root in the management of gastric ulcer and mental disorders (Ugwah *et al*., 2013). Fresh leaves are chewed and applied directly to wounds, boils and sores to reduce pain and inflammation and promote healing (Orwa *et al*., 2009)

Non-medicinal uses of the plant

Fresh leaves of the plant are eaten as a vegetable after cooking whiles the fruits are sweet and edible. In the Democratic Republic of Congo, it is cultivated as a fodder crop as it is browsed by livestock despite its thorns. Due to the availability of pollen and nectar, the plant is excellent for bees and honey production. It is a reliable source of firewood and charcoal production. The wood is durable, dense and termite-resistant hence used for furniture and fencing. Also, the bark produces cinnamon-like coloured dye which has been used as dyes, stains, tattoos, inks and mordants (Orwa *et al*., 2009). The pastoralists in West Pokot, Kenya, apply the leaves of *Ziziphus abyssinica* to preserve meat for hundreds of years (Mureithi, 1996; Nyaberi *et al*., 2010b).

The agro-residues (inner stone) of the plant have been considered as an alternative source of bioethanol production (Wayah & Abdallah, 2014).

Pharmacological investigations on the plant

Antimicrobial activity

Gundiza *et al*. (1991) tested for the anti-microbial activity of various extracts of the barks and leaves of *Ziziphus abyssinica* against *Staphylococcus aureus, Escherichia coli* and *Candida albicans* using the whole plate diffusion and the test tube dilution methods. The results indicated that plant extract showed significant activity against *S. aureus* and *C. albicans*. Three years later, Beetjie, (1994) confirmed the results in a study that evaluated the anti-microbial effect of aqueous extract of *Ziziphus abyssinica* against *Staphylococcus aureus* and *Candida albicans*. Also, Adamu *et al*. (2005) screened an aqueous extract of the plant against *Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli* using the agar well diffusion method. From the results obtained, the extract was active against all the microbes except *Staphylococcus aureus. Bacillus cereus* unlike *Pseudomonas aeruginosa, Escherichia coli* and *Micrococcus lutea* have been reported to be active against methanolic stem bark extract of the plant (Wagate *et al.*, 2010).

The methanol extracts of *Ziziphus abyssinica* (Hochst ex A. Rich) found in Bauchi State, Nigeria, showed antifungal activity when tested by the agar diffusion method against dermatophytes, *Trichophyton rubrum, T. mentagaphytes, Microsporum canis* and *Aspergillus fumigatus* (Adamu, Abayeh, Ibok, & Kafu, 2006).

Anti-parasitic effect

The antiplasmodial effect of the methanolic leaf extract of the plant was validated *in vitro* against *P. falciparum* (strain) with an IC_{50} of 17.5 µg/mL. Also, the molluscicidal activity of the plant has been reported in laboratory-grown Limnaids (*Lymnaea natalensis* Krauss) (Kela, Ogunsusi, Ogbogu, & Nwude, 1989).

Anti-diarrhoeal effect

An aqueous root extract of *Ziziphus abyssinica* A. Rich (200, 400 and 600 mg/kg, *p.o.*), significantly ($p<0.05$) and dose-dependently inhibited castor oil-induced diarrhoea and reduced gastrointestinal motility in charcoal meal test in rats. This suggests that the plant may possess anti-diarrhoeal properties (Ugwah-Oguejiofor *et al.*, 2011).

Anti-ulcer effect

An aqueous root extract of *Ziziphus abyssinica* (300 mg/kg) ameliorated both ethanol and indomethacin-induced gastric lesions in Wistar rats (Ugwah *et al.*, 2013). Also, the gastro-protective effect of the aqueous, methanol and hexane root extracts of *Ziziphus abyssinica* on ethanol-induced gastric ulcer in Wistar rats as models (Yau *et al*., 2017). According to the study, the aqueous extract was more effective than the others and also it was found out that pretreatment with aqueous extract resulted in the preservation of the functional cytoarchitecture of the entire mucosa with little pathological changes, compared to other extracts.

Analgesic effect

The use of the leaves of the plant for the treatment of various forms of pain has been validated (Boakye-Gyasi et al., 2017a). According to the study, hydro-ethanolic leaf extract of the plant dose-dependently and significantly

inhibited chemical-induced nociception in the acetic acid, formalin (phase 1 and 2) and glutamate tests. The extract also dose-dependently and significantly increased reaction times in both tail-immersion and carrageenan-induced hypernociceptive tests. Follow-Up research on mechanism(s) that mediated the analgesic activity of the plant revealed the involvement of opioidergic, adenosinergic, ATP-sensitive potassium channels and nitric oxide-cyclic GMP pathways (Boakye-Gyasi *et al*., 2017b). Additionally, the plant's extract also inhibited hypernociception induced by tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), bradykinin and prostaglandin E_2.

Anti-oxidant effect

It has been reported that the fruit extracts of *Ziziphus abyssinica* exhibited a very potent antioxidant activity compared to the reference drug, sodium metabisulphite, in the stable radical 2, 2 diphenylpicrylhydrazyl (DPPH) test (Nyaberi et al., 2010).

Acute toxicity study

A hydroalcoholic leaf extract of the plant at doses of (30, 100, 300, 1000, 3000 and 5000 mg/kg, *p.o.*) did not produce any observed toxic effects in ICR mice for up to 14 days following drug administration (Boakye-Gyasi *et al.*, 2017a).

CHAPTER THREE

MATERIALS AND METHODS

Collection of Plant Material

Fresh root bark of *Ziziphus abyssinica* was collected from Ejura (7°23′00.16″N, 1°22′00.00″W) in the Ashanti Region of Ghana in September 2017. It was authenticated by Mr. Clifford Asare, a botanist at the Herbarium Unit of the Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST). It was compared to a voucher specimen (KNUST/HM/2016/R003) which had already been deposited at the herbarium.

Preparation of Plant Extract

Fresh root bark of *Ziziphus abyssinica* was air-dried at room temperature for twenty-one (21) days and pulverised into a fine powder with the aid of a hammer mill. A portion (800 g) of the powdered roots was extracted with 2 L of 70% v/v ethanol for 48 h using soxhlet extraction apparatus (Aldrich®, St. Louis, MO, USA). The extract obtained was subsequently concentrated using a rotary evaporator (Rotavapor R-215 model, BÜCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure and temperature (50°C), labelled as ZAE and preserved in a desiccator containing activated silica until it was ready for use. The yield obtained was 13.8 % w/w.

Phytochemical Tests

Qualitative phytochemical analysis was conducted on *Ziziphus abyssinica* root extract to determine the presence of the various phytoconstituents. Phytochemical analysis was carried out by a procedure

based on previous reports (Evans, 2009; Tiwari, Kumar, Kaur, Kaur, & Kaur, 2011).

Alkaloids (Dragendroff's test):

ZAE (0.5 g) was dissolved in 5 mL dilute HCl and filtered. To two (2) mL of the filtrate, 1 mL of potassium bismuth iodide solution (Dragendroff's reagent) was added drop by drop. Formation of an orange red precipitate indicated the presence of alkaloids.

Triterpenoids (Salkowski test)

The extract (0.5 g) was dissolved in 5 mL chloroform. The chloroform solution was carefully treated with 3 mL of concentrated sulphuric acid and mixed well. A reddish-brown colouration of the interface indicated the presence of triterpenoids.

Flavonoids (Lead Acetate test):

The extract (0.5 g) was dissolved in water and filtered. To two (2) mL of the filtrate, 1 mL of lead acetate solution was added and observed for yellow coloured precipitate.

Tannins

To 2 mL of the extract, two drops of the following reagents were added. The presence of tannins was indicated deep blue-black colouration was formed on addition of 0.1% $FeCl_3$ solution.

Saponins (Foam test)

ZAE (0.5 g) was shaken with 2 mL of water in a test tube. Persistence of foam produced for ten minutes indicated the presence of saponins.

Steroids (Lieberman Burchardt's test)

ZAE (0.5 g) was extracted with chloroform and treated with few drops of acetic anhydride. The mixture was heated on water bath for 30 min and allowed to cool. Concentrated H_2SO_4 (1 mL) was carefully added, shaken and allowed to stand. The presence of steroids was confirmed by the appearance of bluish green colour.

Biological Activity of the Extract

Animals

Male ICR mice (20-25 g) and Sprague – Dawley rats (170-250 g) were bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon-Ghana. They were kept in stainless cages (34 × 47 × 18) cm^3 in groups of six rats at the animal house facility of the Department of Biomedical Sciences, University of Cape Coast, Ghana. They were fed with normal commercial diet bought from Flour Mills of Ghana Limited, Tema, Ghana and water was given *ad libitum.* The protocols used in this study are in line with the "Principles of Laboratory Animal Care" (NIH Publication No. 85-23, Revised 1985) and established public health guidelines in 'Guide for Care and Use of Laboratory Animals'(National Research Council, 2010).

Ethical approval for the study was obtained from the University of Cape Coast Institutional Review Board (UCCIRB). ID: UCCIRD/CHAS/ 2016/13. University of Cape Coast, Cape Coast, Ghana.

Drugs and Chemicals

The following chemicals and drugs were used in the study: formalin, theophylline (British Drug House, Poole, England); glibenclamide (Sanofi-Aventis, Guildford, UK); yohimbine hydrochloride (Walter Ritter GmbH +

Co. KG, Germany); atropine sulphate, naloxone hydrochloride, morphine sulphate (Duopharma (M) Sdn Bhd, Malasia); nifedipine, diclofenac sodium (Denk Pharma, Germany); fluoxetine, bradykinin acetate salt, and murine recombinant tumour necrosis factor-alpha (TNF-α) and L-glutamic acid, N^G-L-nitro-arginine methyl ester (L-NAME) dexamethasone, carrageenan, bovine serum albumin and other reagents used were of analytical grade and purchased from Sigma-Aldrich Inc, St. Louis, MO, USA.

Toxicity Studies

Acute toxicity

Thirty-five male Sprague-Dawley rats were randomy selected and divided into seven groups of five rats in each group. ZAE (30, 100, 300, 1000, 3000 and 5000 mg/kg, *p.o.*) was orally administered to one group of animals respectively. Control group rats received oral administration of distilled water (10 mL/kg). Animals were observed over 24 h after treatment for changes in behaviour or death. Using the Irwin's test (Irwin, 1968), the rats were observed at 0, 15, 30, 60, 120 and 180 min, and 24 h after treatment for behaviours specifically related to central nervous system stimulation (hypersensitivity to external stimuli, excitation, stereotypies, jumping, aggressive behaviour and straub tail), central nervous system depression (hyposensitivity to external stimuli, decreased muscle tone, loss of traction, hypothermia, loss of balance, sedation, motor incoordination, rolling gait, akinesia, and catalepsy,) neurotoxicity (tremor and convulsions), and effects on autonomic functions, such as respiration, body temperature, salivation, urination and defecation, were also noted. The rats were then observed daily for 14 days for any delayed toxicity.

Sub-chronic toxicity

Four groups of rats (n=5) were used for this study and were treated as follows; Group A (control) received distilled water (10 mL/kg *p.o.*) daily for 90 days: Groups B, C and D were treated daily with ZAE (30, 100 and 500 mg/kg *p.o.*) respectively for 90 consecutive days. Animals were monitored closely for signs of toxicity. Animals were sacrificed on the 91st day under light anaesthesia using chloroform and blood obtained by cardiac puncture. Blood sample (1.5 mL) was collected into a test tube containing 2.5 µg of ethylenediaminetetraacetic acid (EDTA) for haematological assay. Also, 3.5 mL of blood was collected into Vacutainer™ tubes containing separating gel. Serum was collected and stored at – 80 ºC until ready for use for assay of biochemical parameters. Organs (liver, kidney, spleen, heart, lungs and testes) were harvested, weighed and processed for histology.

Haematological parameters assessment

Haematological parameters were assessed using automatic analyser (CELL-DYN 1700, Abbot Diagnostics Division, Illinois, USA). The parameters assessed include white blood cells (WBC), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), haematocrit (HCT), mean corpuscular haemoglobin concentration (MCHC), platelets (PLT), mean platelet volume (MPV) and red cell distribution width (RDW). Also, differential white cell count comprising of eosinophil %, neutrophil %, basophil % and lymphocytes % were also determined.

Serum biochemical parameters assessment

Activities of the liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP)] and levels of

total serum bilirubin (T-BIL), direct bilirubin (D-BIL), total protein, albumin, blood urea nitrogen (BUN) and creatinine were determined. These tests were performed using automatic analyser ATAC 8000 Random Access Chemistry System (Elan Diagnostics, Smithfield, RI, USA).

Organ weight assessment

Organs (liver, kidney, spleen, heart, lungs and testes) were quickly excised, trimmed of fat and connective tissue and weighed. Body weight of rats was measured on the 91st day following an overnight fast. The relative organ weight (ROW%) was calculated as

$$\text{ROW\%} = \frac{\text{Organ weight of animal}}{\text{Body weight of animal}} \times 100$$

Histological examination

The liver, kidney, spleen, heart, lungs and testes were harvested for histological examination. Tissues were fixed in 10% buffered formalin (pH 7.2). Dehydration was done with a series of ascending grade of ethanol solutions, cleared with xylene, embedded in paraffin wax and finally processed into tissue blocks for sectioning. Sections (5 µm thick) were then cut with a rotary microtome and stained using routine haematoxylin and eosin (H & E). The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (CCD) camera. The histological evaluation was then performed for any possible pathological defect.

Anti-nociceptive Effect of the Extract

Tail – immersion test

The test was performed as previously described (Janssen, 1963; Sewell & Spencer, 1976). Seven groups of Sprague-Dawley rats (n = 5) were allowed

to adapt to the environmental conditions in the laboratory for seven days before the experiment. The lower portion of the tail of each rat was marked and later immersed into a water bath at 55 ± 0.5°C. The rats reacted by flicking or withdrawing their tail, and this was measured as the reaction time. Animals were tested before and at 1, 2, 3, 4 and 5 hours after administration of ZAE (30, 100 and 300 mg/kg, *p.o.*), morphine (3 mg/kg, *p.o.*) or distilled water (10 mL/kg). Animals in the control group were given vehicle (distilled water, 10 mL/kg, *p.o.*). The cut-off time for tail immersion was set at 15 seconds to prevent any tissue injuries.

The percentage maximum possible effect (%MPE) was calculated using the formula below:

$$\%\text{MPE} = \left(\frac{L_2 - L_1}{L_0 - L_1}\right) \times 100$$

Where L_1 is the pre-drug latency, L_2 is the post-drug latency and L_0 is the cut-off latency.

Acetic acid-induced writhing test

A previously described method was adopted (Ezeja, Omeh, Ezeigbo, & Ekechukwu, 2011; Koster, Anderson, & De Beer, 1959) with some modifications. Mice (n = 5) were administered with either vehicle (Distilled water, 10 mL/kg, *p.o.*), ZAE (30, 100, 300 mg/kg *p.o.*) or morphine (3 mg/kg, *p.o.*). After 1 hour, they received an intraperitoneal injection of acetic acid (10 mL/kg of 0.6% $^{v}/_{v}$). The mice were then individually placed in testing chambers (a Perspex chamber of 15 x 15 x 15 cm^3). The nociceptive behaviours of the mice were then captured with a camcorder (Sony-Handycam, model: HDRCX675/B, Tokyo, Japan) for 30 min and later tracked using a a public domain software, JWatcher™ software Version 1.0, available

at http://www.jwatcher.ucla.edu/ (designed by University of California, Los Angeles, USA and Macquarie University, Sydney, Australia). The total number of writhes per every five minutes time bloc was computed, and this was used to plot time-course curves from which the areas under the curves (AUCs) were determined.

Formalin test

This test was carried out using an earlier established protocol (Hunskaar, Fasmer, & Hole, 1985; Woode & Abotsi, 2011). Five groups (n = 5) of male ICR mice were used for the test. For them to acclimatise to their new environment, each mouse was placed in one of the twenty Perspex chambers (15×15×15 cm^3) for one hour to acclimatise before the test. Each group of mice received either vehicle (distilled water, 10 mL/kg, *p.o.*), ZAE (30, 100 and 300 mg/kg, *p.o.*) or morphine (3 mg/kg, *p.o.*) one hour before intraplantar injection of formalin (5% $^{v}/_{v,}$ 10 µL/paw). Mice were transferred instantly into the transparent testing chambers and were their nocifensive behaviours were captured with the aid of a camcorder for one hour as described above. A nociceptive score for every 5 min time bloc was determined as the product of duration and frequency of licking/biting of injected paws. The results obtained were considered as either neurogenic/early phase (0 – 10 min) or inflammatory/late (10 – 60 min) phase from which time-course curves were plotted and the areas under the curves for each phase and each treatment determined.

Effect of the extract in acute and chronic musculokeletal pain

Carrageenan-induced acute musculoskeletal pain

The test was performed as described earlier (Radhakrishnan, Moore, & Sluka, 2003). Acute muscle hyperalgesia was induced in rats by injecting 100 µL of 3% carrageenan percutaneously into their right gastrocnemius muscle. Acute muscle pain was developed within 12 hours after injection which was confirmed in all the rats using an analgesimeter (IITC Life Science Inc. Model 2888, Woodland Hills, CA, USA) as described earlier (Randall & Selitto, 1957). Baseline reading was taken before induction of hyperalgesia. Rats were then given ZAE (30, 100 and 300 mg/kg, *p.o.*), morphine (3 mg/kg) and normal saline (10 mg/kg). The pain thresholds were measured again hourly for 5 hours and the change in pain threshold resulting from the treatment of *Ziziphus abyssinica* and morphine was recorded. Paw withdrawal latencies were again measured hourly for 5 hours after the various drug treatments to determine the effects of the various treatments on pain threshold. Percentage maximum possible effect (% MPE) was calculated as follows:

$$\%\text{MPE} = \frac{(\text{postdrug treatment latency} - \text{predrug treatment latency})}{(\text{Cutoff latency} - \text{predrug treatment latency})} \times 100$$

Carrageenan induced chronic musculoskeletal pain

Similarly, chronic musculoskeletal pain was induced in rats by injecting them with 100 µL of 3% carrageenan percutaneously into the right gastrocnemius muscles. The inflammation induced by the carrageenan was allowed to progress for ten days to allow the development of chronic pain as validated earlier (Radhakrishnan *et al.*, 2003). Chronic hyperalgesia was confirmed in all the rats by measuring the paw withdrawal latency for the right

paw of the animals in the Randall-Selitto test. Briefly, an analgesimeter (IITC Life Science Inc. Model 2888, Woodland Hills, CA, USA) was used to apply a linearly increasing pressure on the paw of each rat until it vocalized or forcefully withdrew its limbs (Randall & Selitto, 1957). The weight that produced limb withdrawal or vocalization was recorded. A cut-off weight of 250 g was used to avoid tissue damage. Rats were then given ZAE (30, 100 and 300 mg/kg, *p.o.*), morphine (3 mg/kg) and normal saline (10 mg/kg). Paw withdrawal latencies were again measured hourly for 5 hours after the various drug treatments to determine the effects of the various treatments on pain threshold.

Mechanism of analgesic action of the extract

Involvement of nociceptive pathways

To investigate the involvement of various nociceptive pathways of nociception in the analgesic activity of the extract, six groups of mice (n=5) were pretreated orally with naloxone (2 mg/kg, i.p.; a nonselective opioid receptor antagonist), theophylline (5 mg/kg, *p.o.*; a nonselective adenosine receptor antagonist), glibenclamide (8 mg/kg, *p.o.*; an ATP-sensitive K channel inhibitor), L-NAME, (10 mg/k g, i.p.; a nitric oxide synthase inhibitor), yohimbine (3 mg/kg, *p.o.*; an α_2 receptor antagonist) atropine (3 mg/kg, i.p., nonselective muscarinic antagonist), or nifedipine (10 mg/kg, *p.o.*; L-type voltage-gated calcium channel blocker). After 60 min. (*p.o.*) or 30 min. (i.p.) of post-antagonist treatment, mice were given oral administration of 100 mg/kg ZAE. One-hour after ZAE treatments, nociception was induced with 10 µL of 5% formalin in all the groups, and the nociceptive score was measured for 1 h and analysed as described above. The above procedure was repeated in

another set of six groups of mice that received of 3 mg/kg morphine (*p.o.)* one hour before the test. The negative control group received only distilled water (10 mL/kg, *p.o.*), whereas ZAE- and morphine-treated controls received pretreatment with vehicle (10 mL/kg, *p.o.*), and further treatment with 100 mg/kg ZAE or 3 mg/kg morphine respectively.

Tumour necrosis factor-alpha (TNF-α)-induced hyperalgesia

The test was performed using an earlier described method (Vale *et al.*, 2004). Mechanical hyperalgesia was induced with TNF-α irritant (2.5 pg/paw; 20 µL) into the plantar regions of rats' right hind paws. This was preceded by pretreatment of rats with either ZAE (30, 100 and 300 mg/kg, *p.o.*) or morphine (3 mg/kg, *p.o.*) to separate groups of rats (n=5) for an hour. Rats in the control group were given vehicle (10 mL/kg, *p.o.*). Intraplantar injection of TNF-α into the right hind paws produced hyperalgesia which was measured at times 0, 1, 2, 3, 4 and 5 using an analgesimeter (Ugo Basile, Comerio, Varese, Italy) as has been described previously (Randall & Selitto, 1957).

Percentage maximum possible effect was calculated using the formula:

$$\%\text{MPE} = \left(\frac{\text{PWTt}-\text{PWTo}}{250\text{g}-\text{PWTo}}\right) \times 100$$

where PWT*t* is the paw withdrawal threshold at time t and PWT*o* is the paw withdrawal threshold at time zero (0). The cut-off latency was set at 250 g.

Interleukin-1β -induced hyperalgesia

The effect of ZAE on IL-1β-induced hyperalgesia was assessed in rats using a previously described method (Vale *et al*., 2004). Rats were pre-treated with ZAE (30, 100 and 300 mg/kg, *p.o.*), vehicle (10 mL/kg, *p.o.*), or morphine (3 mg/kg, *p.o.*) for 1 h before intraplantar injection of IL-1β irritant (1 pg/paw; 20 µL) into their right hind footpads. Hyperalgesia was measured

in the injected paws at 1, 3 and 5 h post IL-1β injection using an analgesimeter as described above.

Bradykinin-induced hyperalgesia

To evaluate the effect of ZAE pretreatment on mechanical hyperalgesia induced by bradykinin in rats, a previously described method was used (Boakye-Gyasi *et al*., 2017b). Rats were pre-treated with ZAE (30, 100 and 300 mg/kg, *p.o.*), vehicle (10 mL/kg, *p.o.*), or morphine (3 mg/kg, *p.o.*) for 1 h before intraplantar injection of bradykinin (500 ng/paw; 20 µL) into their right hind paws. Hyperalgesia was measured in the injected paws at 1, 3 and 5 h post bradykinin injection using an analgesimeter as described earlier. Rats were pre-treated with captopril (5 mg/kg, s.c.) 1 h before the experiment to avoid break down of bradykinin by angiotensin converting enzyme.

Prostaglandin E_2 - induced hyperalgesia

The test was performed as described earlier (Boakye-Gyasi *et al*., 2017b; Vale *et al*, 2004). Hyperalgesia was induced with prostaglandin E_2 (PGE_2) irritant (100 ng/paw; 20 µL) into the plantar regions of rats' right hind paws. This was preceded by pretreatment of rats with either ZAE (30, 100 and 300 mg/kg, *p.o.*) morphine (3 mg/kg, *p.o.*) or vehicle (distilled water, 10 mL/kg, *p.o.*) for an hour. Anti-nociceptive effect of ZAE was assessed as previously described.

Anti-inflammatory Property of the Extract

Carrageenan – induced paw oedema in rats

Acute anti-inflammatory activity of the extract was evaluated in rats using a method described previously (Neha Mohan, Suganthi, & Gowri, 2013). Five groups of male Sprague-Dawley rats (n = 5) were used for the

study. Paw oedema was induced by the administration of 0.1 mL of 1 % suspension of carrageenan in 0.9 % sterile saline solution into the planter region of the rats' right hind paw. This was preceded by pretreatment of different groups with ZAE (30, 100, 300 mg/kg, *p.o.*), diclofenac (10 mg/kg, *p.o.*) or normal saline (10 mL/kg, *p.o.*) 1 h before paw oedema was induced. Rat's paw thickness was measured using Starrett 798A – 6 / 150 Electronic Digital Callipers before intraplantar injection of carrageenan and at hourly intervals for 5 h post oedematous injury. Raw scores of foot oedema were individually normalized as percentage of change in their paw diameter at time 0 and then averaged for each treatment group. This was used to plot a time course curve for the 5 h period. Total oedema response for each treatment was then calculated as area under the time course curves (AUC). The effect of the drugs was evaluated using percentage inhibition of oedema calculated as:

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

Formalin-induced paw oedema in rats

The test was performed as described previously (Choudhary, Kumar, Gupta, & Singh, 2014) with some modifications. Five groups of male Sprague-Dawley rats (n=5) were used for the study. Inflammation of the right hind paws was induced by intraplantar injection 0. 1 mL of formaldehyde (2% $^{v}/_{v}$) in normal saline. The point of injection was marked in order to maintain consistency in measurement of the paw circumference. All animals received treatment via oral gavage. One hour post formalin injection, rats in groups I, II and III received ZAE at doses of 30, 100 and 300 mg/kg *p.o.* respectively. Group IV rats received diclofenac (10 mg/kg, *p.o.*) whereas group V rats received distilled water (10 mL/kg *p.o.*). Paw oedema was measured with

electronic digital callipers (Starrett 798A – 6 / 150) once every day for ten days, starting from day one, after induction of inflammation. The treatment also continued once daily for the entire duration of the experiment. Paw oedema response was calculated from the difference between final and basal average paw diameters at different time intervals. Percent inhibition of oedema was calculated using the formula:

$$\% \text{ Inhibition} = \left(\frac{\text{Paw diameter at tme t}}{\text{Paw diameter at time 0}}\right) \times 100$$

Carrageenan-induced peritonitis

A method previously described by Nonato *et al*., (2012) was used to assess the effect of ZAE against carrageenan-induced peritonitis in rats. Thirty (30) Sprague-Dawley rats were used for this study. The rats were assigned to 6 groups of five rats per group. Three groups out of the 5 served as the experimental groups with the remaining three representing the positive, negative and normal control groups respectively. Animals in the three experimental groups were pre-treated with ZAE (30, 100 and 300 mg/kg *p.o.*) 1 h before intraperitoneal injection of 1 mL of 1% $^{w}/_{v}$ carrageenan. Positive control rats were pre-treated with dexamethasone (5 mg/kg, *p.o.*) while those in the negative and normal control groups were given normal saline (*p.o.*) before i.p. injection of 1% carrageenan (500 µg/mL). All drugs were administered at a reference volume dose of 10 mL/kg.

Five hours after induction of inflammation, the rats were euthanized under chloroform anaesthesia and peritoneal fluids were collected by abdominal laparoscopy. Five (5) mL of phosphate-buffered saline (PBS, pH 7.4) was injected into the peritoneal cavity of the rats. The abdomen was carefully massaged for approximately 10 - 15 s. A total of 3 mL fluid was

withdrawn from the peritoneal cavity of each animal and centrifuged at 1000 rpm for 5 min. The resulting cell pallet was gently suspended in 1.0 mL of phosphate-buffered saline (PBS, pH 7.4). Total leukocytes count was assayed using the 1.0 mL cell suspension and determined using Neubauer's chamber. Cells in each square corner of the chamber were counted and their average calculated. For differential cell counts, Hema^3 stain was used to stain the cytospin preparations. Differential cell counts were then performed by counting the cells and they were classified as either mononuclear or polymorphonuclear cells, based on conventional morphological criteria.

TNF-α–induced paw oedema

Paw oedema was induced in Sprague-Dawley rats following intraplantar injection of TNF-α (2.5 pg/paw; 20 µL) into rats' right hind paws as described previously (Vale *et al*., 2004). The effect of the vehicle (distilled water, 10 mL/kg, *p.o.*), ZAE (30, 100 and 300 mg/kg, *p.o*.) or diclofenac (10 mg/kg, *p.o*.) on inflammation induced by TNF- α was assessed by measuring paw diameter before and at 1, 2 and 3-hour post TNF- α injection using Starrett 798A – 6 / 150 electronic digital calipers. Time-course curves were plotted from which areas under the curves were obtained.

After the 3 hours, three (3) rats from each group were randomLy selected and euthanized. Excised tissues from the right hind paws were fixed in 10% phosphate buffered formalin. They were subsequently dehydrated, cleared and embedded in paraffin wax to form tissue blocks. Serial sections were taken using a rotary microtome and stained using routine haematoxylin and eosin (H&E) and later analysed for leucocytic cell infiltrates, vasodilation and congestion.

Interleukin-1β–induced paw oedema

A method previously described method (Vale *et al.*, 2004) was adopted. The effect of vehicle (10 mL/kg, *p.o.*), ZAE (30, 100 and 300 mg/kg, *p.o.*) or diclofenac (10 mg/kg, *p.o.*) following intraplantar administration of IL-1β (1 pg/paw; 20 µL) was assessed using similar methods as described above.

Prostaglandin E_2–induced paw oedema

The effect of pretreatment of rats with ZAE on prostaglandin E_2-induced paw oedema was evaluated as described previously (Vale *et al.*, 2004). Rats (n = 5) were pretreated with either vehicle (10 mL/kg, *p.o.*), ZAE (30, 100 and 300 mg/kg, *p.o.*) or diclofenac (10 mg/kg, *p.o.*) before intraplantar injection of PGE_2 (100 ng/paw; 20 µL) into their right hind paw. Paw oedema was measured before and at 1, 2 and 3-hour post PGE_2 injection using similar methods as described earlier.

Bradykinin-induced paw oedema

To evaluate the effect of ZAE pretreatment on paw oedema induced by bradykinin in rats, rats were pre-treated with vehicle (10 mL/kg, *p.o.*), ZAE (30, 100 and 300 mg/kg, *p.o.*) or diclofenac sodium (10 mg/kg, *p.o.*) for 1 hour before intraplantar injection of bradykinin (500 ng/paw; 20 µL) into their right hind paws. Hyperalgesia was measured in the injected paws at 1, 3 and 5 h post bradykinin injection using an analgesimeter in a similar manner as described above. Rats were pre-treated with captopril (5 mg/kg, s.c.) 1 hour before the experiment to avoid break down of bradykinin by angiotensin converting enzyme.

Antidepressant Effect of the Extract

Forced swimming test

The forced swimming test was carried out using an earlier described model (Porsolt, Bertin, & Jalfre, 1977). Seven groups of male ICR mice (n=7) received either distilled water 10 mL/kg, ZAE (30, 100 and 300 mg/kg, *p.o.*) or fluoxetine (3, 10, 30 mg/kg, *p.o.*). Behavioural experiments were conducted 60 min after drug treatments. Briefly, mice were gently placed in similar cylindrical plastic tanks (25 cm high, 12 cm internal diameter and depth of 15 cm) containing water (25 ± 0.5°C). They were allowed to swim for 6 min and recorded with a camera suspended 80 cm above the tanks. Duration of escape-oriented behaviours (climbing—vertically-directed movement with forepaws along the side of swim tanks; swimming—horizontal movement throughout the swim tank) and immobility over the last four min of the test were quantified using the public domain software JWatcher™ Version 1.0.

Tail suspension test

Tail suspension test was performed as previously described (Steru, Chermat, Thierry, & Simon, 1985). Mice were randomLy divided to eight groups (n=7) and were given either ZAE (30, 100 and 300 mg/kg, *p.o.*), fluoxetine (3, 10, 30 mg/kg) or vehicle (10 mL/kg, *p.o.*). After 60 min, the mice were individually suspended at their tail (1 cm from the tip) with an adhesive tape on a horizontal bar raised 52 cm from the top of a laboratory bench. Duration of escape-oriented behaviours (pedalling, curling and swinging) and immobility were recorded with a camera for 6 min and quantified with JWatcher™. Version 1.0.

Anxiolytic Effect of the Extract

Elevated plus maze

The test was performed following a previously method (Pellow et al., 1985) with some modifications. The elevated plus maze consisted of two opposite open arms (15 cm × 5 cm) without side walls and two enclosed arms (15 cm × 5 cm ×30 cm), extending from a common central square platform (5×5 cm). A rim of Plexiglas (0.5 cm in height) surrounded the perimeter of the open arms to provide additional grip and prevent the mice from falling off. The EPM was raised 50 cm above the floor. The mice were divided into ten groups of seven mice each, and pre-treated with doses of ZAE (30, 100 and 300 mg/kg, *p.o.*) or reference drugs diazepam (0.3, 1 and 3 mg/kg, *p.o.*). Control group animals were pre-treated with vehicle (distilled water, 10 mL/kg; *p.o*). The mice were placed individually in the center of the maze, with their heads facing toward open arm at the start of the experiment and their behaviour was videotaped for five minutes using a digital camera placed 75 cm above the maze and tracked time spent in either open or closed arm using a JWatcher software.

Open field test

The test was conducted as described previously (Nagaraja, Mahmood, Krishna, Thippeswamy, & Veerapur, 2012). The apparatus consisted of a wooden box (60 × 60 × 60 cm). The arena of the open field was divided into 16 squares (15 × 15 cm), the four inner squares in the center, 8 squares in the periphery along the walls and 4 squares at the corner. The open field arena was well illuminated. After 60 min of oral treatment with vehicle (distilled water, 10 mL/kg; *p.o*), diazepam (0.3, 1 and 3 mg/kg, *p.o.*) or ZAE (30, 100

and 300 mg/kg, *p.o.*) the mice were placed individually in one of the corner squares and time spent within each of the three categories of squares videotaped for 5 min and tracked for duration in either the corner, periphery or centre squares of the field using a JWatcher software.

Extraction and Isolation of Compounds

A portion (800 g) of the powdered roots of *Ziziphus abyssinica* was sequentially extracted with 2 L of petroleum ether, chloroform and 70% w/w methanol using Soxhlet Apparatus (Aldrich®, St. Louis, MO, USA) at 50°C. The filtrates obtained were separately concentrated using a rotary evaporator (Rotavapor R-215 model, BÜCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure and temperature (50°C). They were further dried at room temperature and preserved in a desiccator containing activated silica. The yield obtained were 2.8% $^{w}/_{w,}$ 5.8 % $^{w}/_{w}$ and 7.4% $^{w}/_{w}$ for the chloroformic and methanolic fractions respectively.

The activity of the three fractions were tested using the carrageenan-induced paw oedema model in rats (Neha Mohan, Suganthi, & Gowri, 2013). The results revealed that the chloroform fraction was more potent than the hydro-alcoholic portion (Appendix D). A portion of the chloroform fraction (35 g) was loaded onto a glass column (60 cm × 3 cm) packed with silica gel (70-230 mesh). It was then eluted with gradient mixtures of petroleum ether and ethyl acetate. A total of 225 fractions were collected in 60 mL aliquots and bulked together into five sub-fractions based on their TLC profiles. Two fractions (21-27 and 125-150) were found to be more active following carrageenan-induced paw oedema test (Appedix D). They were subsequently purified further by washing repeatedly with petroleum ether. After obtaining

single spots on TLC, the compounds were recrystallised using acetone. This resulted in whitish needle-like (1.030 g) and shiny (1.642 g) crystals and were labelled CP **1** and CP **2** respectively (Figure 7).

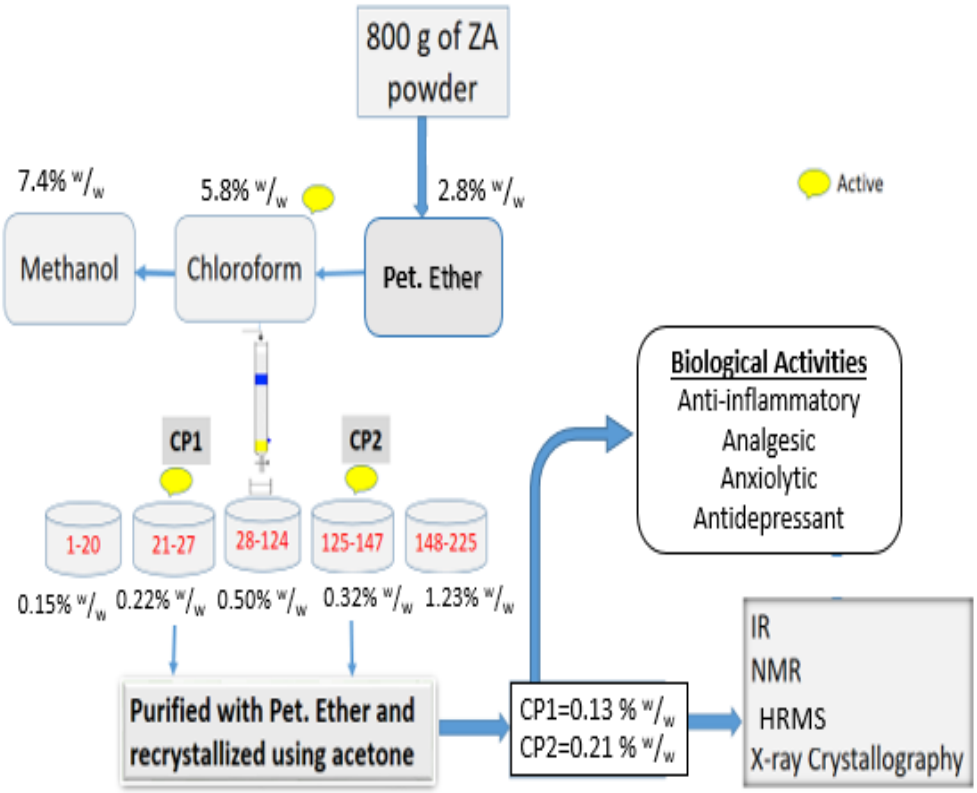

Figure 7: Schematic diagram for the isolation of bioactive compounds

Melting point determination

Capillary tubes were filled with powdered samples of CP 1 and CP 2 to about 3 mm high. The crystals were forced to slide to the bottom of the capillary tubes by dropping the capillary tubes through a 3 foot glass tubing. The capillary tubes were then placed in a Thermoscientific Electrothermal 9100 equipment. The tubes were observed for temperature ranges at which the samples melted. This procedure was repeated for three times for each sample and the melting point determined for each sample.

Characterisation of compounds 1 and 2 (CP 1 and CP 2)

^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), Distortionless Enhancement by Polarization Transfer (DEPT) NMR as well as 2D-NMRs such as Correlated Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra were obtained for both compounds at ambient temperature with tetramethylsilane (TMS) as the internal standard on a Bruker Ultrashield 400 Plus spectrometer (Bruker, Germany). The COSY experiment was used to determine homonuclear ^{1}H connectivities. HSQC and HMBC were used to determine one bond as well as two or three ^{1}H-^{13}C bonds respectively. High resolution mass spectroscopy (HRMS) spectra were acquired from a PerkinElmer Flexar UHPLC with AxION 2 Time of Flight (TOF) Mass Spectrometer (PerkinElmer, USA). Infrared (IR) spectroscopy were recorded on a Nicolet iS10 FTIR Instrument (Thermo Scientific, USA). Specific rotation ([α]) analysis was performed on a JASCO P-2000 digital polarimeter (JASCO, USA).

X-ray Crystallography of compound 2 (CP 2)

For the crystal structure determination, single crystals of the Compound **2** mounted on MiTeGen Microloop were used for data collection on XtaLAB MM007-HF system (Cu Ka radiation, l = 1.54184) coupled with a Hybrid Photon Counting detector (Dectris Eiger 4M) at room temperature. *CrysAlis PRO* software (Rigaku, 2015) was used for data collection, data processing, cell refinement and data reduction. The structures were solved with ShelXT (Sheldrick, 2014) using Intrinsic Phasing, and refined with the olex2-refine (Bourhis, Dolomanov, Gildea, Howard, & Puschmann, 2015;

Dolomanov, Bourhis, Gildea, & Howard, 2009) refinement package using Gauss-Newton minimisation. H atoms were determined from a difference Fourier synthesis. The final difference Fourier maps showed no peaks of chemical significance.

A single crystal with approximate dimensions of 0.215 mm x 0.258 mm 0.324 mm was used for diffraction data collection. Crystal belonged to the monoclinic C2 space group with cell dimensions: a = 13.33700(7) Å, b = 6.31317(4) Å, c = 30.75651(15) Å, β = 100.3632(5)^o, Z = 4, volume = 2547.41(2) Å^3. Integration of the data yielded a total of 44153 reflections to a maximum θ angle of 67.05^o (0.84Å resolution), of which 4335 were independent (completeness = 95.0%, Rint = 2.28%, Rsig = 0.0093%), and 4331 were greater than 2σ(F2). The final data were corrected for absorption effects using the multi-scan method (SADABS). The final anisotropic full-matrix least-squares refinement on F2 with 318 variable converged at R1 = 2.67%, for the observed data and R2 = 7.22% for all data. The goodness-of-fit was 1.041. The largest peak in the final difference electron density synthesis was 0.13 e$^-$/ Å^3 and the largest hole was -0.19 e$^-$/Å^3. On the basis of the final model, the calculated density was 1.2377 g/cm^3 and F(000), 1051.0 e$^-$

Biological Activity of the Isolated Compounds

***In vivo* anti-arthritic activity:**

Forty-five Sprague-Dawley rats (170-250 g) (n=5) of both sexes were obtained from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon-Ghana. The animals were kept under normal laboratory conditions with regards to room temperature, humidity, food and water. All protocols used in the study were in accordance with the NIH Guidelines for

the Care and Use of Laboratory Animals and were approved by the Department of Biomedical Sciences. Effects of test compounds, β-amyrin and polpunonic acid, in chronic inflammation were evaluated using the adjuvant-induced arthritis animal model as previously described (Pearson & Wood, 1959). Complete Freund's Adjuvant (CFA, 0.1 mL) was intraplantarly injected into the right hind paws of rats. The arthritic control group received only intraplantar injection of CFA, while non-arthritic control (IFA group) received only intraplantar injection of 0.1 mL of Incomplete Freund's Adjuvant (IFA) (i.e sterile paraffin oil). Test groups received dexamethasone (3 mg/kg, *p.o.*), CP 1 (3, 10, 30 mg/kg, *p.o.*) and CP 2 (3, 10, 30 mg/kg, *p.o.*) from day 14 to day 28.

Change in body weight

Changes in body weight were determined every other day in all treatment groups till day 28. Percentage change in weights was calculated for each animal from which time-course curves were plotted. The total areas under these curves were estimated and plotted.

Change in paw thickness

Rat's paw oedema was measured using Starrett 798A – 6/150 Electronic Digital Calipers in both the ipsilateral (injected hind paw) and the contralateral paw (non-injected hind paw) before intraplantar injection of CFA (day 0) and on every other day up to the 28th day. Raw scores for ipsilateral and contralateral foot volumes were normalised individually as a percentage of change from their values at day 0 and then averaged for each treatment group. Percentage change in paw oedema was calculated using the formula:

$$\%\ Change\ in\ paw\ oedema = \left(\frac{Dt - Do}{Do}\right) \times 100$$

where D_t is the paw diameter at time T, and D_0 is the paw diameter before adjuvant injection, Data was presented as the effect of drugs on the time course curves and the total oedema response calculated in arbitrary units as the area under the curve (AUC) for 28 days.

Arthritic Index

On day 29, the severity of arthritis was graded according to the extent of erythema and oedema of the periarticular tissues as described previously (Zhao *et al*., 2000), using a scale of 0-4 per limb, where 0 = no inflammation, 1 = unequivocal inflammation of 1 joint of the paw, 2 = unequivocal inflammation of at least 2 joints of the paw or moderate inflammation of 1 joint; 3 = severe inflammation of 1 or more joints; and 4 = maximum inflammation of 1 or more joints in the paw. The scores for each paw were then added to get the total arthritis score (maximum possible score 16 per animal). The mean score for each treatment group was then designated as the arthritic index.

Photographic assessment

Photographs of the affected hind limbs were taken on day 29 using a digital camera (FE-5050, OLYMPUS, Tokyo, Japan).

Radiographic assessment

Measurement of paw or joint swelling only indicates oedematous changes in these regions whereas the most apparent damage takes place in the tibiotarsal joint itself. Hence radiographs of the hind limbs are essential. Rats were anaesthetised by intraperitoneal injection of 50 mg/kg pentobarbitone sodium. On the 29th day, radiographs were taken with X-ray apparatus (Softex,

Tokyo, Japan) and industrial X-ray film (Fuji Photo Film, Tokyo, Japan).operated at a voltage of 55 kV against 3.2 mA s^{-1} with a tube-to-film distance of 110 cm for lateral projection.

Haematological assessment

On the 29th day, blood samples were withdrawn from each animal via cardiac puncture into a test tube containing anticoagulant (5% EDTA). Blood samples were then analysed by a haem automated analyser (Abbott Laboratories, IL, USA) for total blood count and specific white blood cells differentials.

Biochemical analysis

Collected blood samples were allowed to clot for 30 minutes at room temperature and centrifuged at 1000 rpm for 10 minutes. The sera obtained were stored at -20°C until biochemical analysis was carried out. Serum indices were analysed by an automated analyser (ATAC 8000, Elan Diagnostics, CA, USA) and estimations for total protein, total bilirubin, direct bilirubin, indirect bilirubin, AST, ALT, ALP, Creatinine and BUN.

Histological Analysis

On the 29th day, the animals were humanely sacrificed via cervical dislocation, and right hind paws were excised for histopathological evaluations. The samples were fixed for 24 h in 10% formalin. After decalcification in 5% formic acid, processed for paraffin embedding, tissue sections (5 μm thick) were stained with haematoxylin and eosin. Portions of the tissues from the hind paws were used for the histopathological examination. Tissues were fixed in 10% neutral buffered formalin (pH 7.2) and were dehydrated through a series of ethanol solutions, embedded in

paraffin and routinely processed for histological analysis. Sections of 2 µm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through a microscope and photographed. A blinded and experienced pathologist evaluated the slides under light microscope.

Analgesic effect of the isolated compounds

The tail immersion test was adopted to test for the analgesic effect of the two isolated compounds from the plant, CP 1 (3, 10 and 30 mg/kg) and CP 2 (3, 10 and 30 mg/kg) using the method described earlier in this thesis (pages 62-63). ICR mice (20-25 g) with six animals in each group were used for the test.

Antidepressant effect of the isolated compounds

The forced swimming and tail suspension tests (as described earlier on pages 72-73) were employed to test the antidepressant-like effect of the isolated compounds, CP 1 (3, 10 and 30 mg/kg, *p.o*) and CP 2 (3, 10 and 30 mg/kg, *p*.o.) with fluoxetine (3, 10 and 30 mg/kg, *p.o*) as positive control as described for ZAE earlier.

Anxiolytic Effect of the isolated compounds

The anxiolytic effect of CP 1 (3, 10 and 30 mg/kg; *p.o*) and CP 2 (3, 10 and 30 mg/kg; *p.o.*) were assessed using the elevated plus maze and open field tests as described earlier in pages 74-75. Diazepam (0.3, 1 and 3 mg/kg, *p.o.*) was used as the reference drug and the negative control group animals received vehicle (distilled water, 10 mL/kg; *p.o*).

Statistical analysis

A sample size of five or seven rats per group was used in the *in vivo* test. Mean ± standard error of mean (SEM) were used in presenting all data. Time-course curves were subjected to two-way (*treatment x time*) analysis of variance (ANOVA) with Dunnet's post *hoc*. One - way ANOVA with Tukey's post *hoc* test was used to determine differences between treatments groups (Areas Under the Curves). Graphpad® Prism Version 7.0 (GraphPad Software, San Diego, CA, USA) for Windows were used to perform all statistical analysis with $P<0.05$ considered statistically significant for all tests.

Doses for 50% of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

$$Y = \frac{a + (b - a)}{\left(1 + 10^{(LogED_{50} - X)}\right)}$$

Where, X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

CHAPTER FOUR

RESULTS

Phytochemical screening

Alkaloids, triterpenes, flavonoids, tannins and steroids were detected in the root bark of *Ziziphus abyssinica.* Saponins were not detected. (Table 3).

Table 3: Results from phytochemical screening

Phytochemical	Results
Alkaloids	Detected
Triterpenes	Detected
Flavonoids	Detected
Tannins	Detected
Saponins	Not detected
Steroids	Detected

Toxicity Studies on the Extract

Acute Toxicity

Animals treated with ZAE (100-5000 mg/kg) exhibited analgesia when their tails were pinched. Also, sedation was observed at doses of 300-5000 mg/kg. Moreover, toxic signs such as disordered gait, convulsion, respiratory depression or death were not observed even after 14 days of drug administration suggesting an LD_{50} value greater than 5000 mg/kg (Table 4).

Table 4: The effect of varying doses of ZAE in an acute toxicity study

Dose (mg/kg)	Mortality Death/Total No. of Animals	Effects
0	0/5	No change
30	0/5	No change
100	0/5	Analgesia
300	0/5	Analgesia, Sedation
1000	0/5	Analgesia, Sedation
3000	0/5	Analgesia, Sedation
5000	0/5	Analgesia, Sedation

Sub-chronic toxicity studies

Total body weights of animals

The rats in the control and ZAE-treated groups exhibited a gradual increase in total body weights throughout the 90-day treatment period. Also, there were no significant differences in total body weight between treated and control groups (Figure 8)

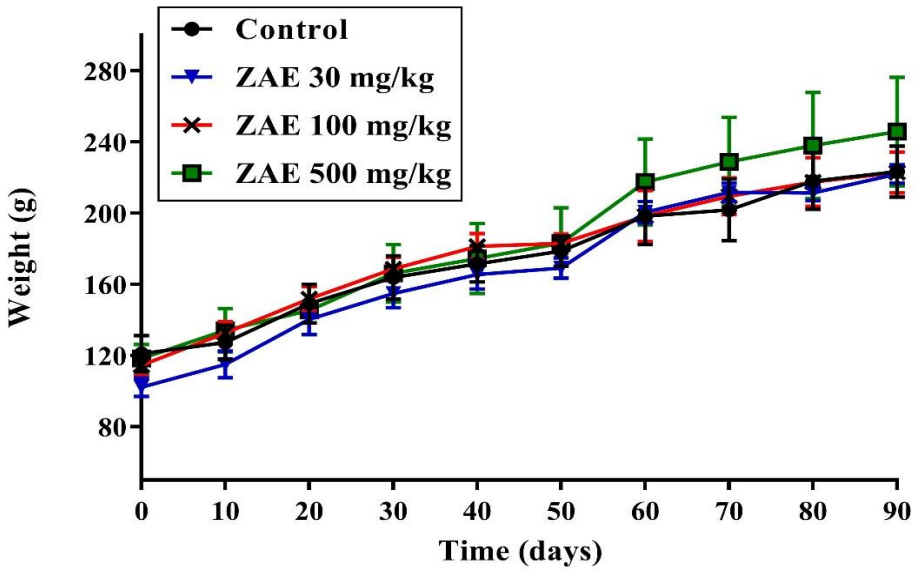

Figure 8: Weight of rats within the 90-day following treatment with ZAE (30, 100 and 500 mg/kg, *p.o.*) and distilled water. Data are presented as mean ± SEM. Treated groups were compared to a distilled water-treated control group using a one-way ANOVA.

Relative organ weights of animals

ZAE did not produce any significant changes in the weight of vital organs such as liver, kidney, spleen, heart, lungs and testes of the rats compared to the vehicle-treated control group (Figure 9).

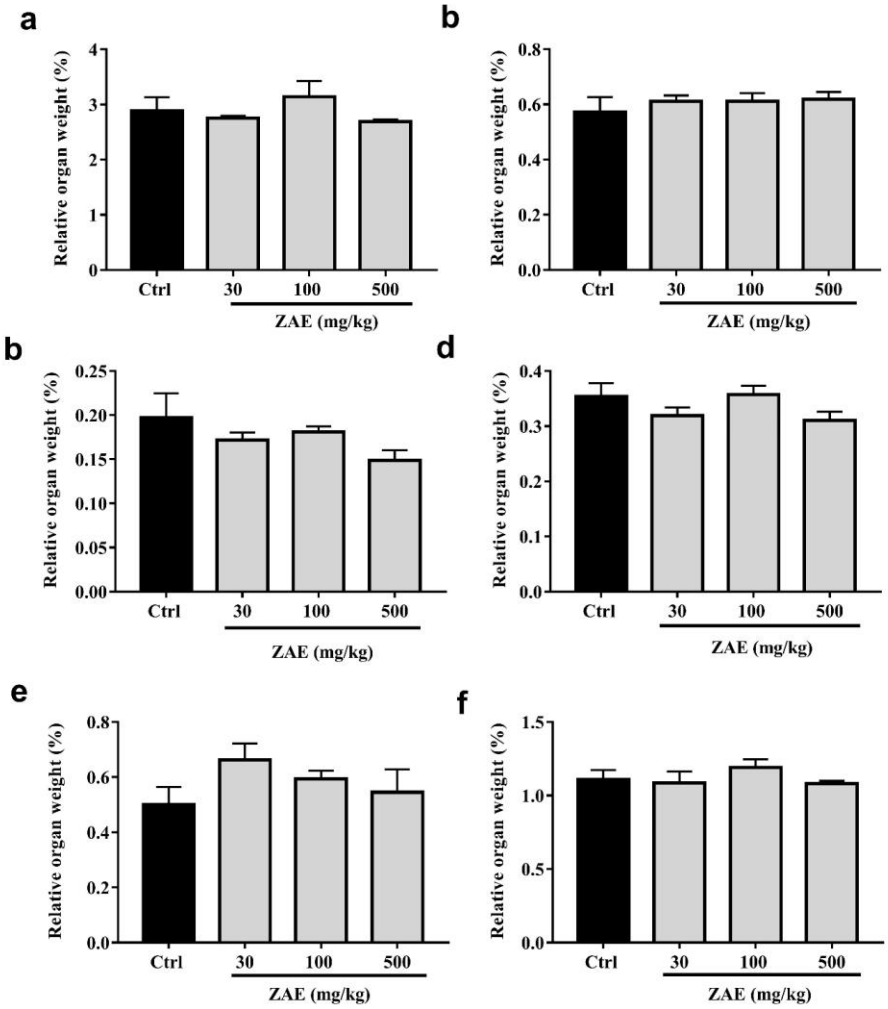

Figure 9: Effect of oral administration of ZAE (30, 100 and 500 mg/kg, *p.o.*) and distilled water (ctrl) on the relative organ weights of: (a) liver (b), kidney (c) spleen (d) heart (e) lungs and (f) testes. Data is presented as mean ± SEM. (n=5). ZAE treated groups were compared to control group (ctrl) using a one-way ANOVA.

Haematological parameters

There were no significant changes between the control group and ZAE-treated groups with P>0.05 in all the haematological parameters (Figures 10, 11 and 12).

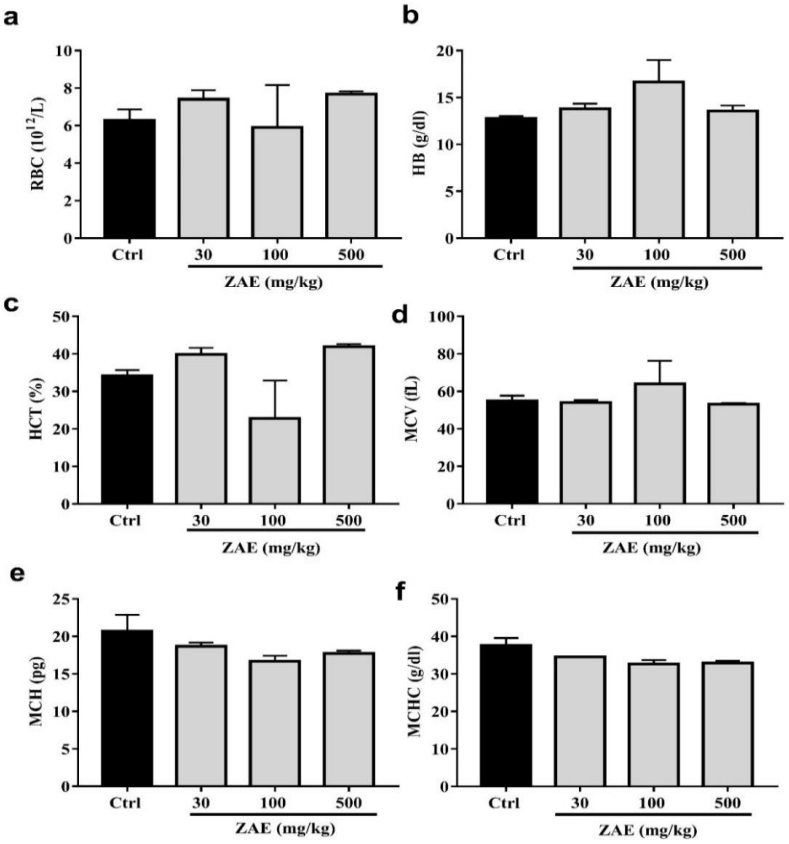

Figure 10: The effect of sub-chronic administration of ZAE (30, 100 and 500 mg/kg, *p.o.*) and distilled water (ctrl) to rats on (a) red blood cells (RBC), (b) haemoglobin (HB), (c) haematocrit (HCT), (d) mean corpuscular volume (MCV), (e) mean corpuscular haemoglobin (MCH), and (f) mean corpuscular haemoglobin concentration (MCHC). Data are presented as mean ± SEM. (n=5). Treated groups were compared to the control group (ctrl) using a one - way ANOVA.

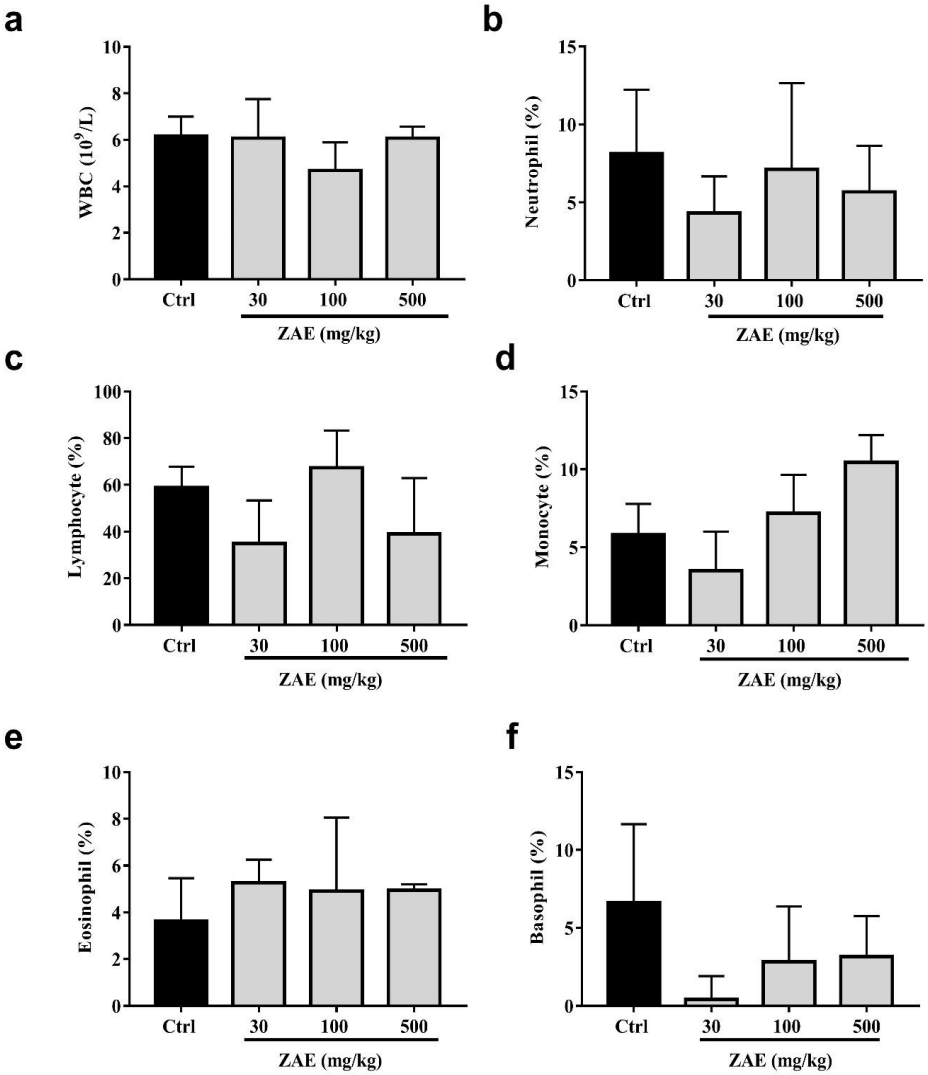

Figure 11: The effect of sub-chronic administration of ZAE (30, 100 and 500 mg/kg, *p.o.*) and distilled water (ctrl) to rats on (a) white blood cells count, (b) neutrophil (%) (c) lymphocyte (%), (d) monocyte (%), (e) eosinophil (%) and (f) basophil (%). Data are presented as mean ± SEM. (n=5). Treated groups were compared to a control group (ctrl) using a one-way ANOVA.

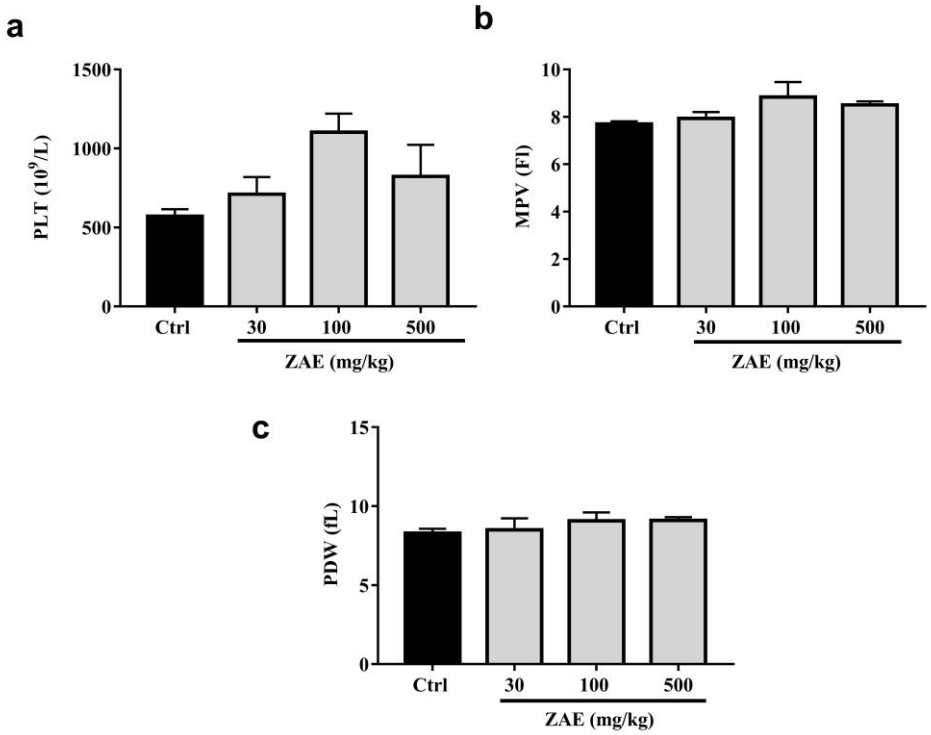

Figure 12: The effect of sub-chronic administration of ZAE (30, 100 and 500 mg/kg, *p.o.*) and distilled water (ctrl) to rats on (a) platelets (PLT), (b) mean platelet volume (MPV) and (c) red cell distribution width (RDW). Data are presented as mean ± SEM. (n=5). Treated groups were compared to a control group (ctrl) using a one -way ANOVA.

Serum biochemical parameters

The data on biochemical parameters in control and ZAE treated groups of sub-chronic oral toxicity (Figures 13, 14 and 15). There was no significant difference in the biochemical parameters between the ZAE-treated groups and control concerning serum proteins, liver enzymes and kidney function parameters.

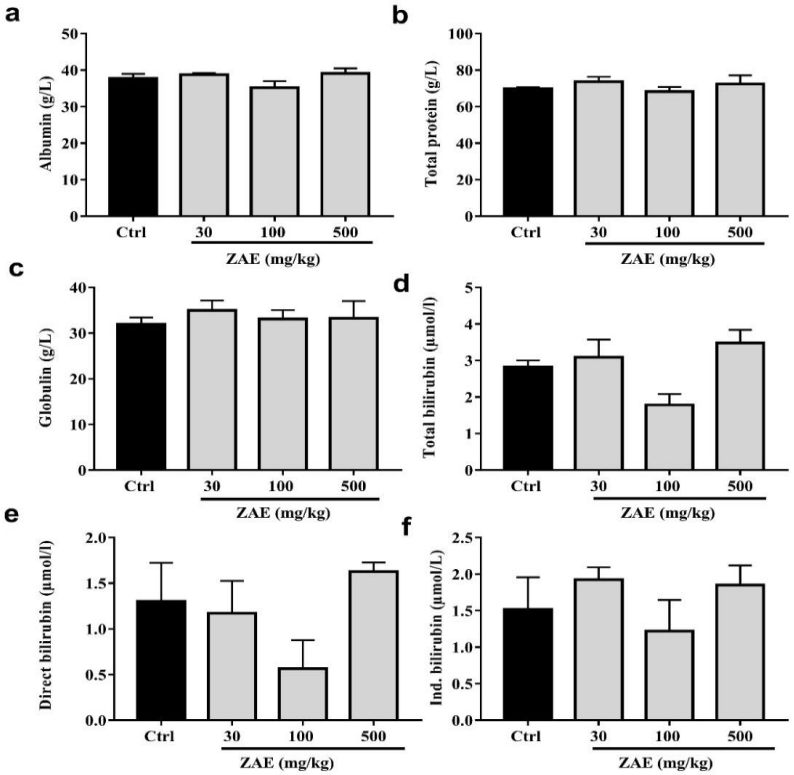

Figure 13: Effect of oral administration of ZAE on the serum proteins of rats (a) albumin (b), total protein (c) globulin (d) total bilirubin (e) direct bilirubin and (f) indirect bilirubin. Data are presented as mean ± SEM. (n=5). Treated groups were compared to a control group (ctrl) using a one-way ANOVA.

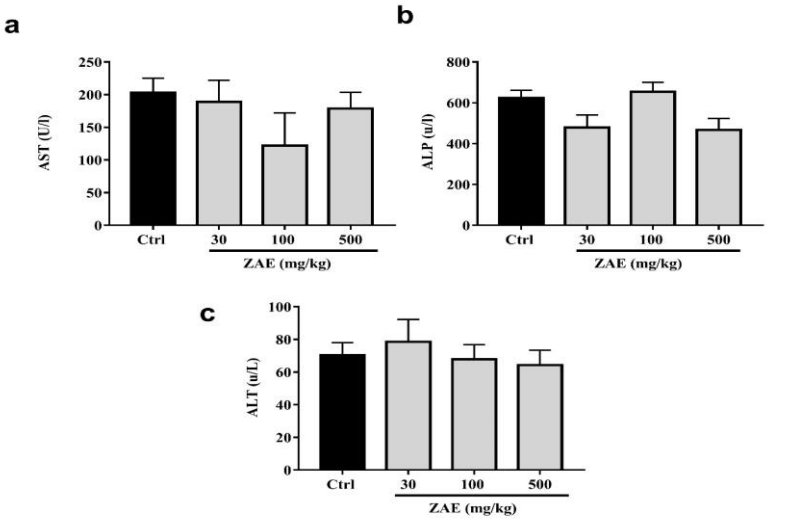

Figure 14: Effect of oral administration of ZAE on the liver enzymes of rats: (a) aspartate aminotransferase (AST), (b) alkaline phosphatase (ALP) and (c) alanine aminotransferase (ALT). Data are presented as mean ± SEM. (n=5). Treated groups were compared to a control group (ctrl) using a one-way ANOVA followed by Tukey's post *hoc* test.

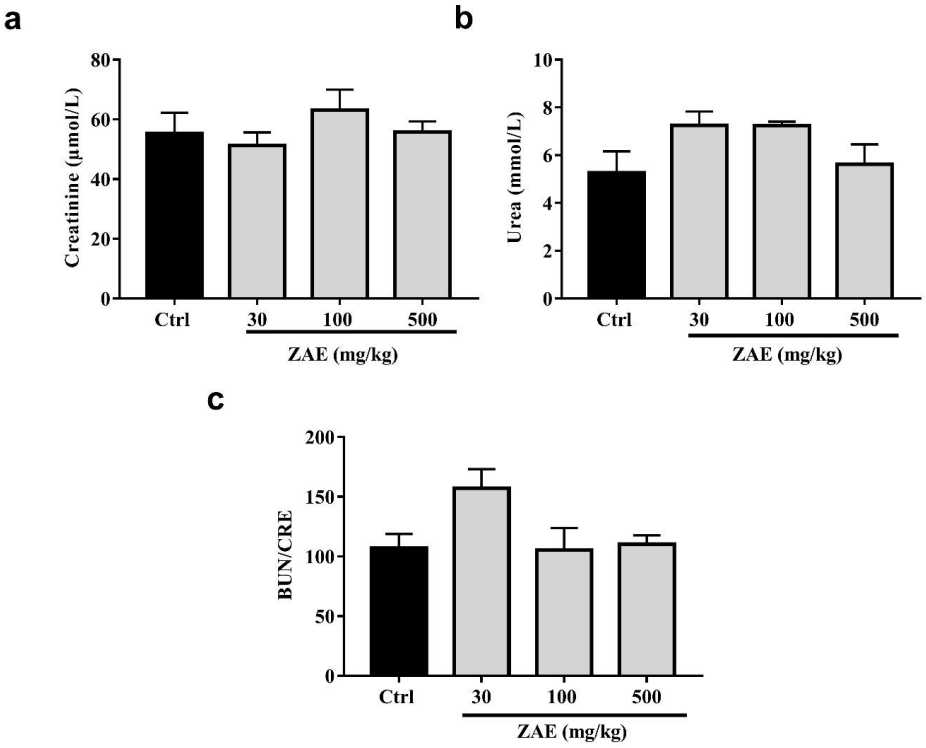

Figure 15: Effect of oral administration of ZAE on the kidney parameter of rats: (a) creatinine, (b) urea and (c) BUN/CRE. Data are presented as mean ± SEM. (n=5). Treated groups were compared to a control group (ctrl) using a one-way ANOVA.

Sperm analysis and fasting blood sugar

Results presented in Figure 16 (a) show the effect of sub-chronic oral administration of ZAE (30, 100 and 500 mg/kg) on the sperm count of rats. Though, there were no statistical difference between ZAE-treated groups and control, elevations in sperm count from an average of 6.4 million cells to 7.9, 8.4 and 8.4 million cells respectively for 30, 100 and 500 mg/kg of ZAE were observed.

Also, there were no statistical differences between ZAE-treated groups and control groups with respect to fasting blood glucose determined at the end of the 90-day treatment (Figure 16 b).

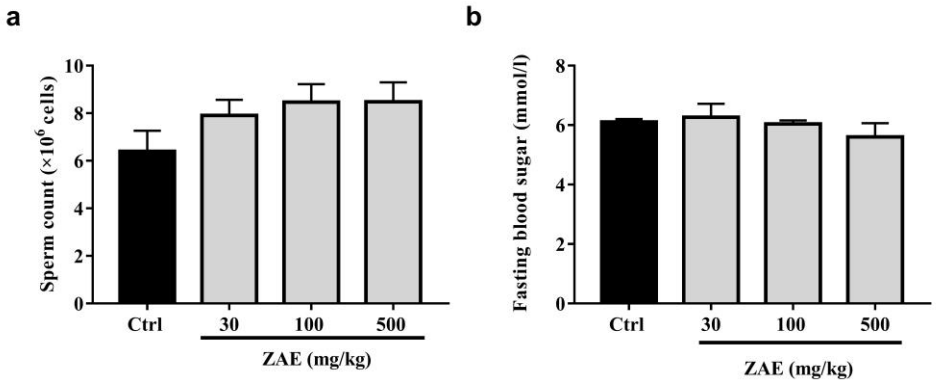

Figure 16: Effect of oral administration of ZAE on the (a) sperm count and (b) fasting blood sugar (FBS). Data are presented as mean ± SEM. (n=5). Treated groups were compared to a control group (ctrl) using a one-way ANOVA.

Histological results

Photomicrographs of representative samples from the heart, testis, kidney, liver and spleen of rats which received distilled water and ZAE (30, 100 and 500 mg/kg) (Figures 17-21).

Histology of the heart

A section through the heart of normal control rats revealed a normal heart with branched cardiomyocytes (Figure 17 A). Also, a section through the heart of rats that received 30 mg/kg of ZAE showing reduced eosinophilia, but viable cardiomyocytes (B). Rats that received 100 mg/kg of ZAE showed a normal ventricle, with focal areas of mild congestion (blue arrows) and viable cardiomyocytes (C) whereas a section through the heart of rats that received ZAE (500 mg/kg, *p.o.*) depicted normal ventricles with very few vessel congestion (blue arrows) (D).

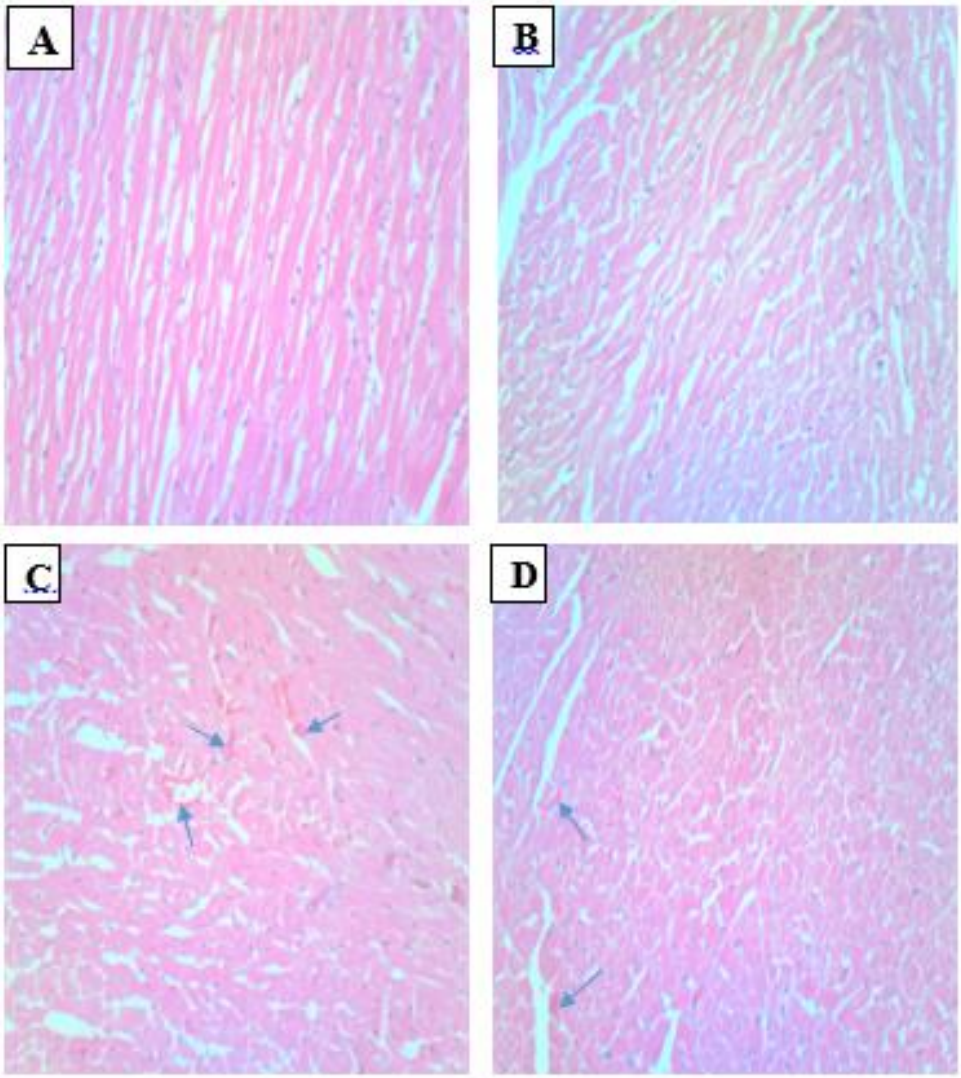

Figure 17: Photomicrograph of the sections of the heart of rats treated orally with (A) distilled water and (B) ZAE 30 mg/kg, (C) ZAE 100 m/kg and D 300 mg/kg for 90 days in sub-chronic toxicity study (H & E, ×100).

Histology of the testis

Sections through the testis of normal control rats which revealed clear and wide lumen of normal seminiferous tubule (blue arrows), with few spermatozoa (Figure 18 A). In the rats that received ZAE (30 mg/kg), there was an increase in size of fibrous connective tissue (black arrowhead) surrounding seminiferous tubules, accompanied by eosinophilic deposition within tubules (blue arrow) which depicted high spermatozoa content. Rats that received ZAE 100 mg/kg (C) and 500 mg/kg (D) revealed an increased

intra-tubular eosinophilic depositions (blue arrow), accompanied by an increase in fibrous connective tissue around tubules (black arrowhead) as compared with the normal control group (Figure 18).

Histology of kidneys

Sections from the control (A), 30 mg/kg (B) and 100 mg/kg (C) groups, showed normal histological architecture of the renal corpuscles (blue arrow) and the surrounding proximal and distal convoluted tubules. Animals treated with 500 mg/kg of ZAE (D), however, showed a mild form of distortion of the macula densa (black arrow) and the renal corpuscles (blue arrow) (Figure 19).

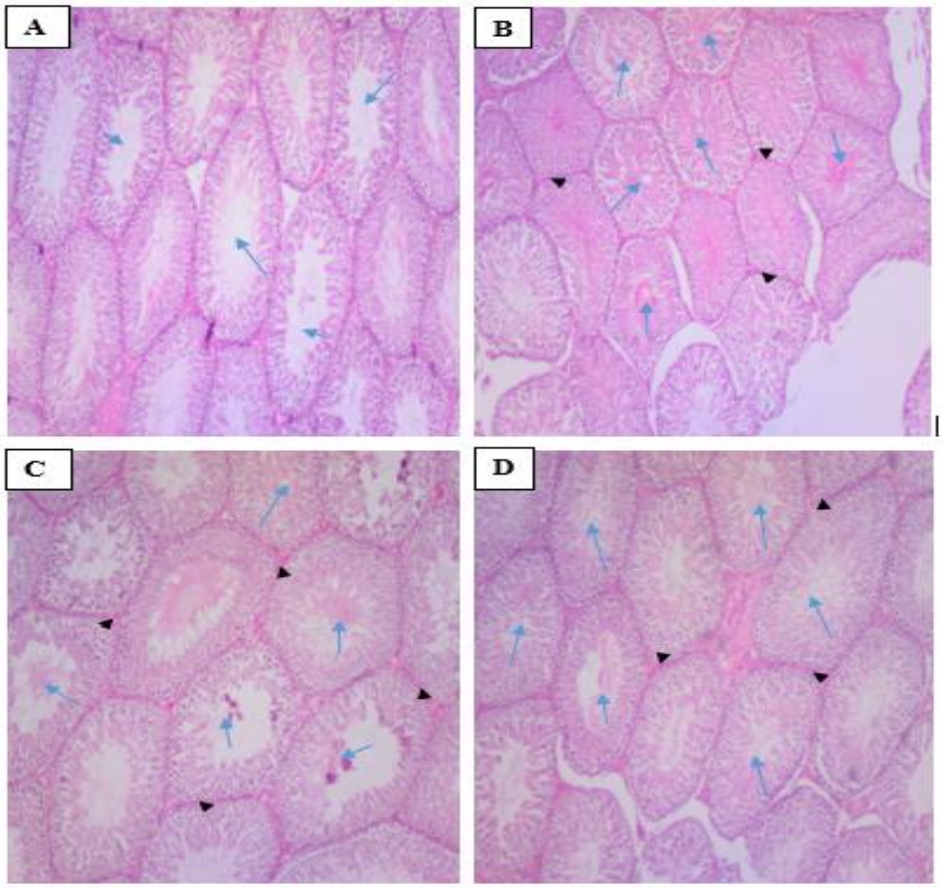

Figure 18: Photomicrograph of the sections of the testes of rats treated orally with (A) distilled water, (B) ZAE 30 mg/kg, (C) ZAE 100 m/kg and (D) 300 mg/kg for 90 days in sub-chronic toxicity study (H & E, ×100).

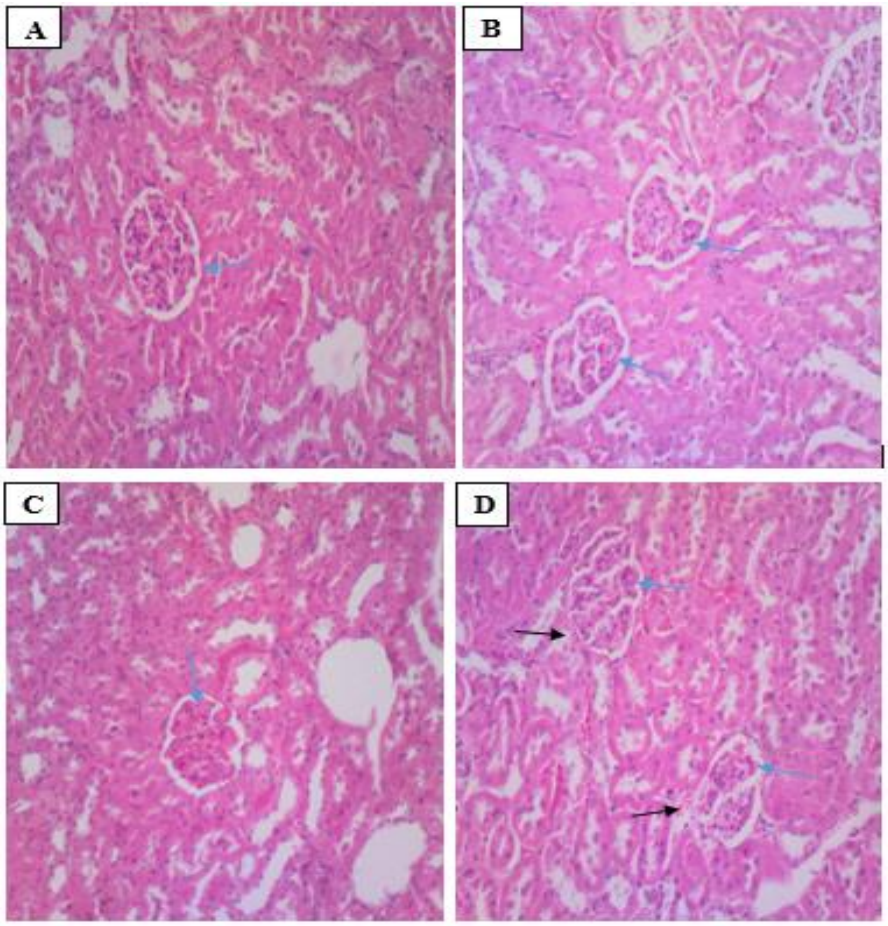

Figure 19: Photomicrograph of the sections of the kidney of rats treated orally with (A) distilled water, (B) ZAE 30 mg/kg, (C) ZAE 100 m/kg and (D) 300 mg/kg for 90 days in sub-chronic toxicity study (H & E, ×100).

Histology of liver

Histology of sections of the liver (Figure 20) of rats in the normal control group (A) depicted a normal central vein (CV) with surrounding viable hepatocytes. Rats that received ZAE 30 mg/kg also showed liver parenchymal cells and vessels (sinusoids) that could be likened to that of the normal control group (B). Rats that received 100 mg/kg of ZAE showed a partial congestion in CV (black arrow), accompanied by surrounding mild perivenular congestion in sinusoids (blue arrow) whereas D (ZAE 500 mg/kg) showed mild sinusoidal congestion (blue arrow) and leucocytic infiltrate.

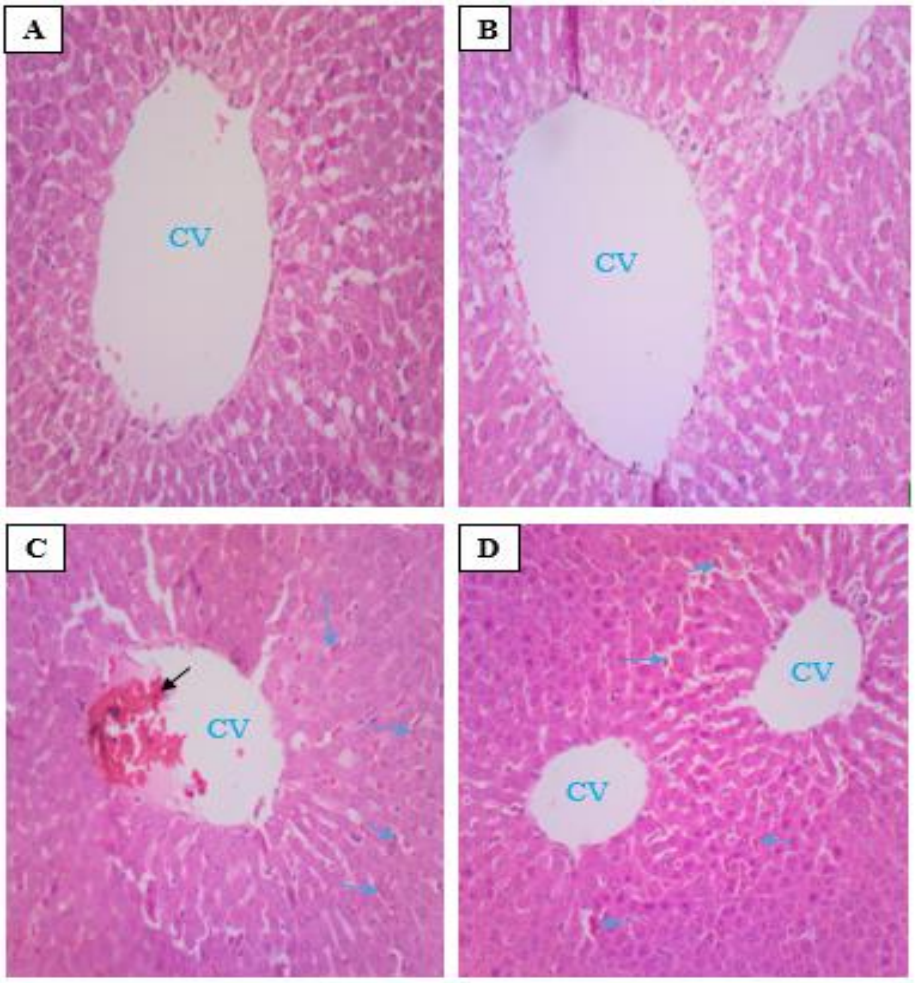

Figure 20: Photomicrograph of the sections of the liver of rats treated orally with (A) distilled water, (B) ZAE 30 mg/kg, (C) ZAE 100 m/kg and (D) 300 mg/kg for 90 days in sub-chronic toxicity study (H & E, ×100).

Histology of the spleen

Photomicrographs shown in Figure 21 indicate that the control group revealed normal histology of the white pulp (blue arrow) (A). All the other treatment groups as shown on A-D (ZAE 30-500 mg/kg) revealed a reduced eosinophilia of the white (blue arrows) and red (black arrows) pulp compared to the control group.

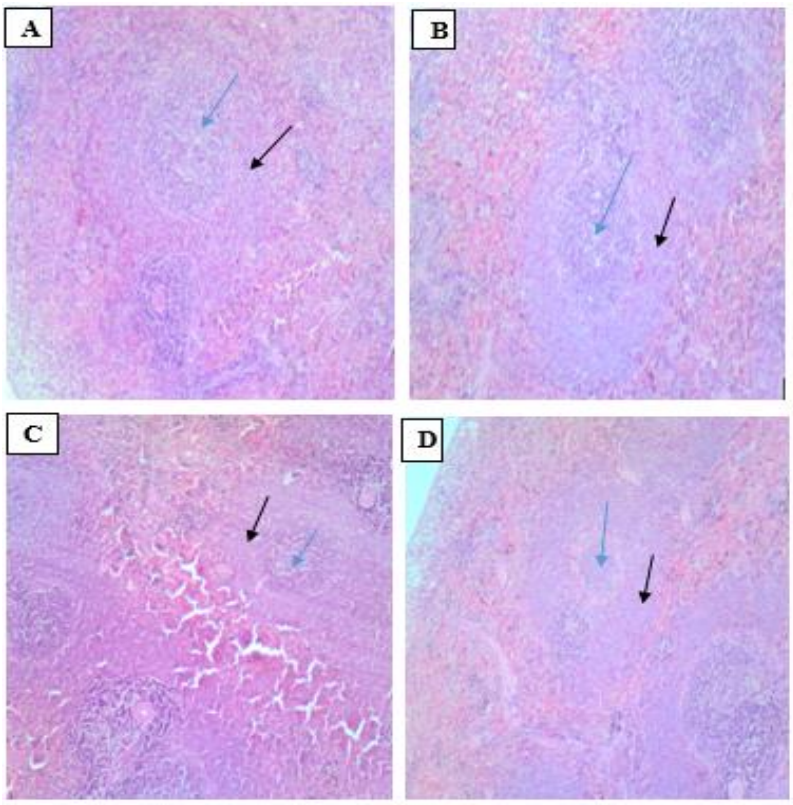

Figure 21: Photomicrograph of the sections of the Spleen of rats treated orally with (A) distilled water, (B) ZAE 30 mg/kg, (C) ZAE 100 m/kg and (D) 300 mg/kg for 90 days in sub-chronic toxicity study (H & E, ×100).

Anti-nociceptive tests

Tail immersion test

All the three dose levels of ZAE (30, 100 and 300 mg/kg, *p.o.*) showed significant ($F_{(4, 20)}$ = 13.94, P = 0.0020) inhibition compared to the control (vehicle). The positive control, morphine (3 mg/kg, *p.o.*), also demonstrated significant inhibition compared to control (Figure 22).

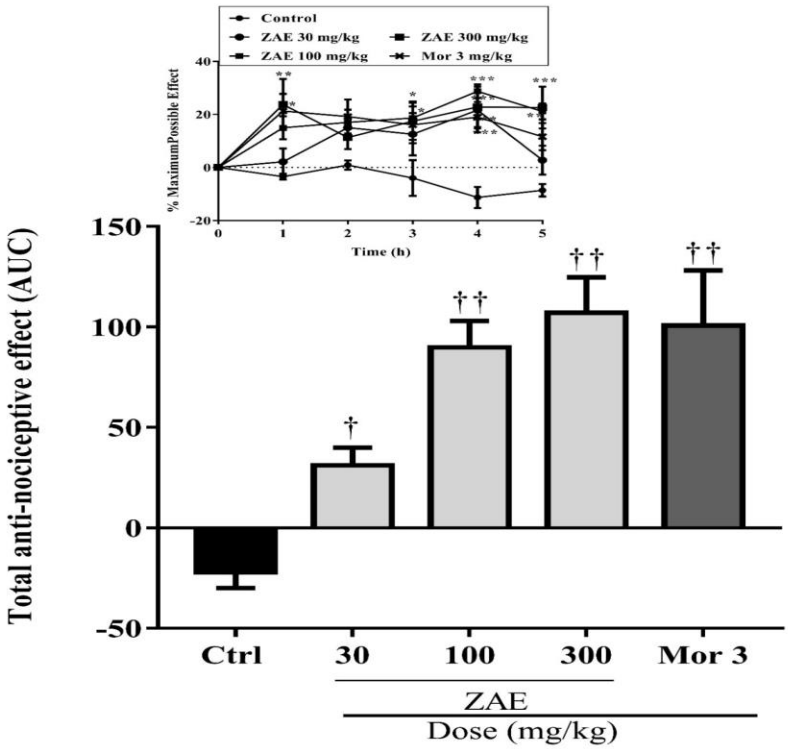

Figure 22: The total anti-nociceptive effect of ZAE (30, 100 and 300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.) presented as area under the curve AUC in tail-immersion test in mice. Data are presented as mean ± SEM. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the vehicle-treated group (one-way ANOVA followed by Tukey's post *hoc* test for AUC). Insert: The time course curves of the various treatments over 5 hours. *P<0.05, **P<0.01 and ***P<0.001 compared with the vehicle-treated group (two-way ANOVA followed by Dunnett's post *hoc* test).

Acetic acid-induced nociception

Administration of ZAE significantly ($F_{(4, 20)} = 8.211$, $P = 0.0010$; Figure 23) reduced the nociceptive effect produced by intraplantar injection of acetic acid. ZAE (30, 100 and 300 mg/kg) treatment produced a mean percentage inhibition of nociception of 10.68, 57.82 and 76.94% respectively. Morphine inhibited the writhing response by 94.21%.

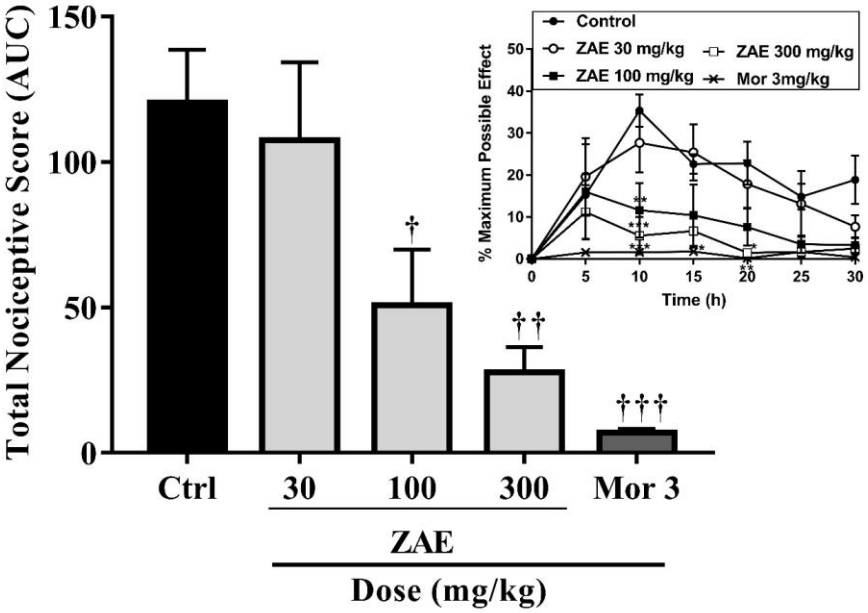

Figure 23: Effect of ZAE (30-300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.) on the total nociceptive score of the acetic acid test presented as area under the curve (AUC). Data are presented as mean ± SEM. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared with the vehicle-treated group (one-way ANOVA followed by Tukey's *post hoc* test for AUC). Insert: The time-course curves of nociception for the various treatment groups for 30 minutes. *P<0.05, **P<0.01 and ***P<0.001 compared with the vehicle-treated group (two-way ANOVA followed by Dunnet's post *hoc* test).

Formalin-induced nociception

The administration of formalin into the paws of the mice produced a typical bi-phasic nociceptive response consisting of an initial, rapid-licking acute neurogenic phase (phase I) within 10 min, followed by a slowly rising and long-lived 10-60 min inflammatory phase (phase II) (Figure 24). In the neurogenic phase, administration of 30, 100 and 300 mg/kg of the extract to the mice produced an ameliorative effect with a mean percentage inhibition of 44.11%, 69.50% and 78.43% respectively with morphine (3 mg/kg) producing 85.48%. In the inflammatory phase, ZAE 30, 100 and 300 mg/kg reversed formalin-induced nociception with mean percentage inhibitions of 43.16%,

61.82% and 83.36%. This was comparable to the effect of morphine (3 mg/kg) which produced a percentage inhibition of 90.96%.

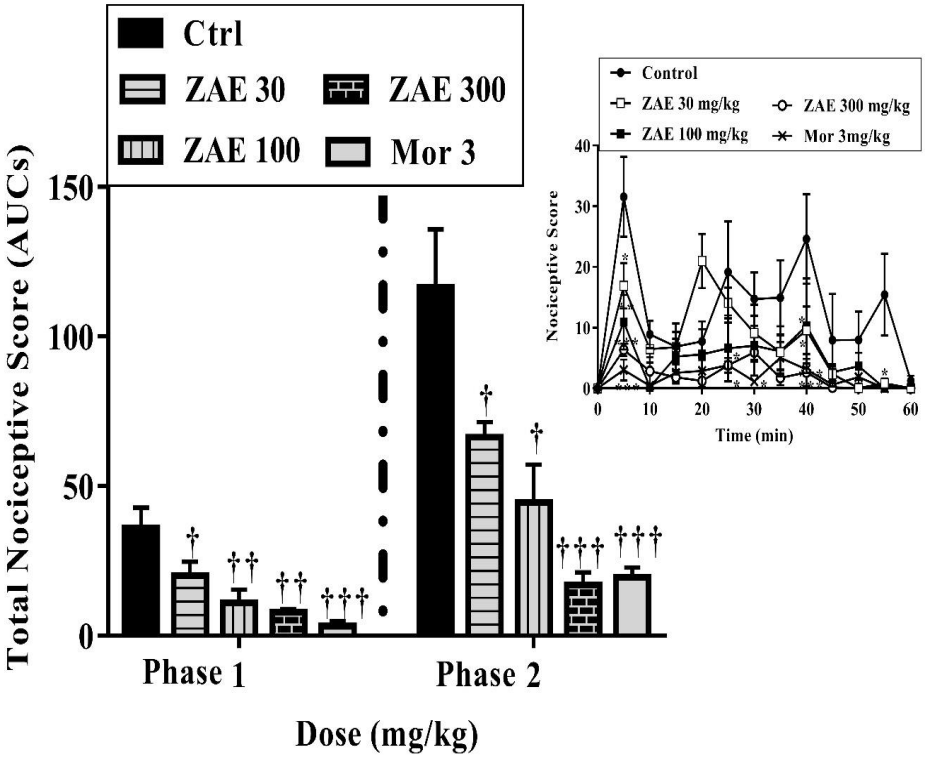

Figure 24: The effect of ZAE (30- 300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.) on the total nociceptive score of the formalin test presented as area under the curve (AUC). Data is presented as mean ± SEM. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared with the vehicle-treated group (one-way ANOVA followed by Tukey's *post hoc* test for AUC). Insert: The time-course curves of nociception in the various treatment groups for 60 minutes. *P<0.05, **P<0.01 and ***P<0.001 compared with the vehicle-treated group (two-way ANOVA followed by Dunnett's post *hoc* test).

Acute and Chronic Musculoskeletal pain

The time-course curves of nociception as well as total anti-nociceotive scores of ZAE, morphine and vehicle treated groups in carrageenan-induced acute musculoskeletal pain model in rats were analysed {Figure 25 (a and b)}. Acute muscle hyperalgesia was measured 12 hours after injection of carrageenan. *Ziziphus abyssinica* (30, 100 and 300 mg/kg, *p.o*) significantly

(P< 0.05) and dose-dependently inhibited the carrageenan induced acute muscle hyperalgesia in the time-course curve (Figure 25 a) in the ipsilateral limb compared to normal saline treated group. Morphine (3 mg/kg *i.p*) used as positive control also significantly (*P*<0.001) inhibited acute muscle hyperalgesia induced by carrageenan.

Similarly, there was a significant (P<0.05, P<0.01) and dose-related increase in the total antinociceptive effect of the ZAE (100 and 300 mg/kg respectively) in the carrageenan induced chronic muscle pain in rats (Figure 26). This was comparable to the effect produced by the positive control drug, morphine 3 mg/kg.

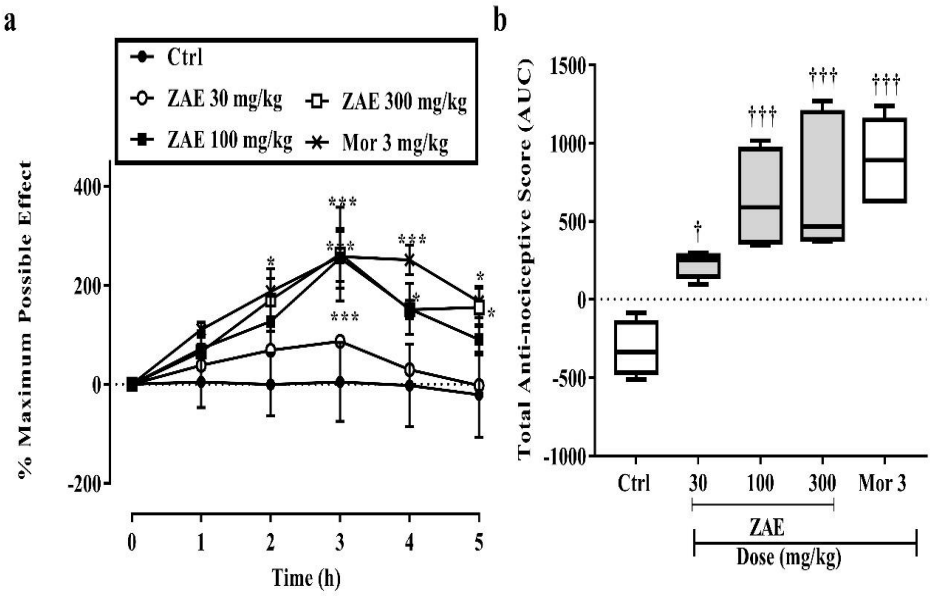

Figure 25: Effect of ZAE (30, 100 and 300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.) on the (a) nociceptive score (presented as percentage maximum possible effect) and (b) total anti-nociceptive effect in acute musculoskeletal pain in rats. Data are presented as mean ± S.E.M. ***p < 0.001; ** p < 0.01; * p < 0.05, compared to vehicle-treated group (Ctrl) (Two-way ANOVA followed by Dunnet's multiple comparison test). $^{\dagger}P$ < 0.05, $^{\dagger\dagger}P$ <0.01, $^{\dagger\dagger\dagger}P$ <0.001 compared to vehicle-treated group (One-way ANOVA followed by Tukey's multiple comparison test).

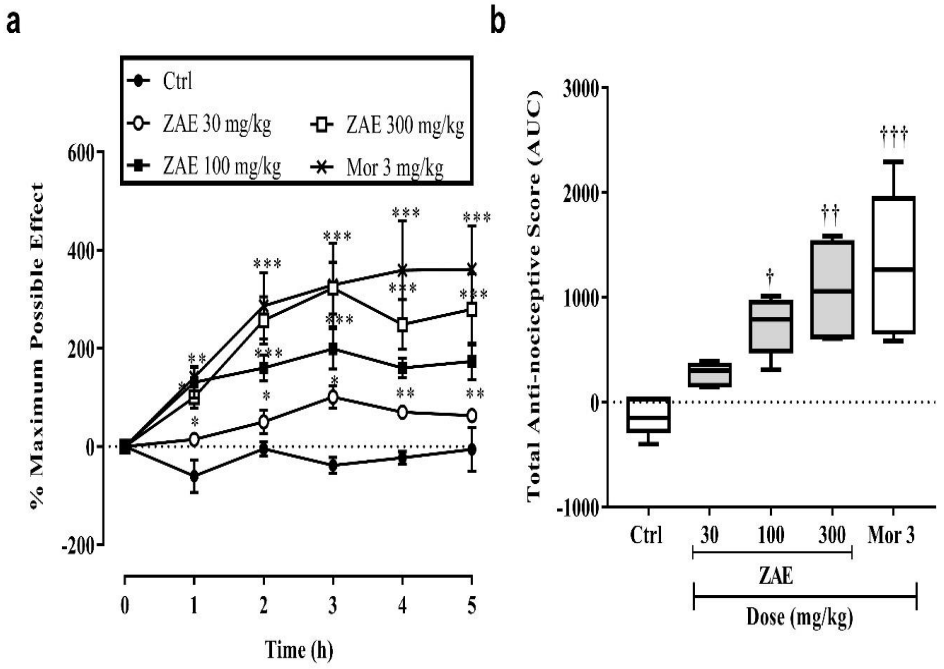

Figure 26: Effect of ZAE (30, 100 and 300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.) on the (a) nociceptive score (presented as percentage maximum possible effect and (b) total anti-nociceptive effect in chronic musculoskeletal pain in rats. Data are presented as mean ± S.E.M. ***P< 0.001; **P< 0.01; *P< 0.05, compared to vehicle-treated group (Ctrl) (Two-way ANOVA followed by Dunnet's multiple comparison test). †P < 0.05, ††P <0.01, †††P <0.001 compared to vehicle-treated group (One-way ANOVA followed by Tukey's multiple comparison test).

Mechanisms of Antinociceptive action of the extract

Assessment of involvement of nociceptive pathways using the formalin test

The pathways mediating the anti-nociceptive activities of ZAE and morphine were analysed (Figures 27-29). The anti-nociceptive effect of ZAE was significantly reversed by pretreatment of mice with naloxone in both phases of the formalin test whereas L-NAME and glibenclamide reversed only the inflammatory phase of the formalin test. The anti-nociceptive effect of morphine, on the other hand, was significantly diminished by yohimbine, nifedipine, naloxone, atropine and L-NAME. Glibenclamide, however,

significantly reversed the pain in the inflammatory but not the neurogenic phase of the test.

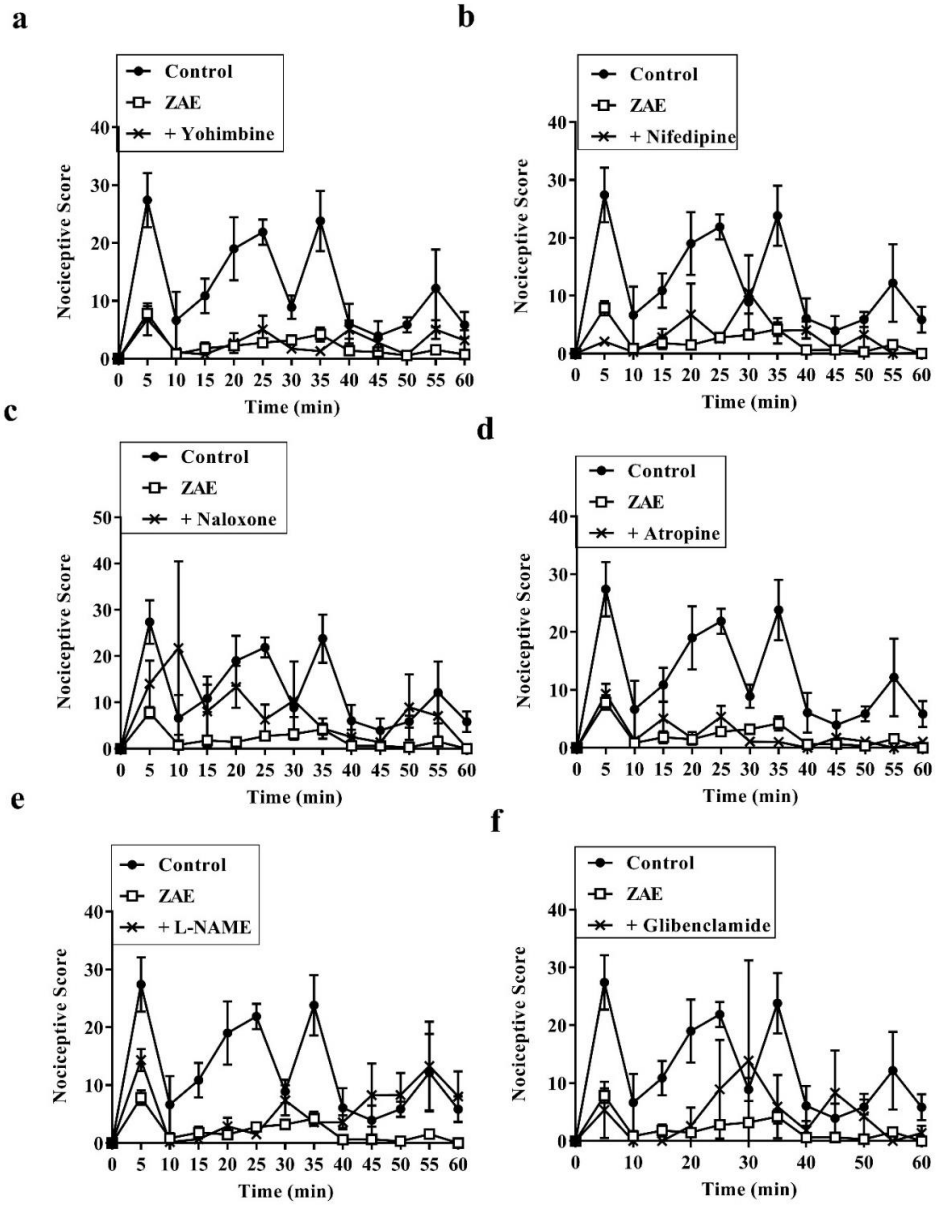

Figure 27: Effect of pretreatment of mice with (a) yohimbine (3 mg/kg, *p.o.*), (b) nifedipine (10 mg/kg, *p.o.),* (c) naloxone (2 mg/kg i.p.), (d) atropine (5 mg/kg, i.p.), (e) L- NAME (10 mg/kg, i.p) and (f) glibenclamide (8 mg/kg, *p.o.*) on the anti-nociceptive profile of ZAE, (100 mg/kg, *p.o.*) in phase 1 and phase 2 of formalin-induced nociception. Each column represents the mean of 5 animals and the error bars indicate S.E.M.

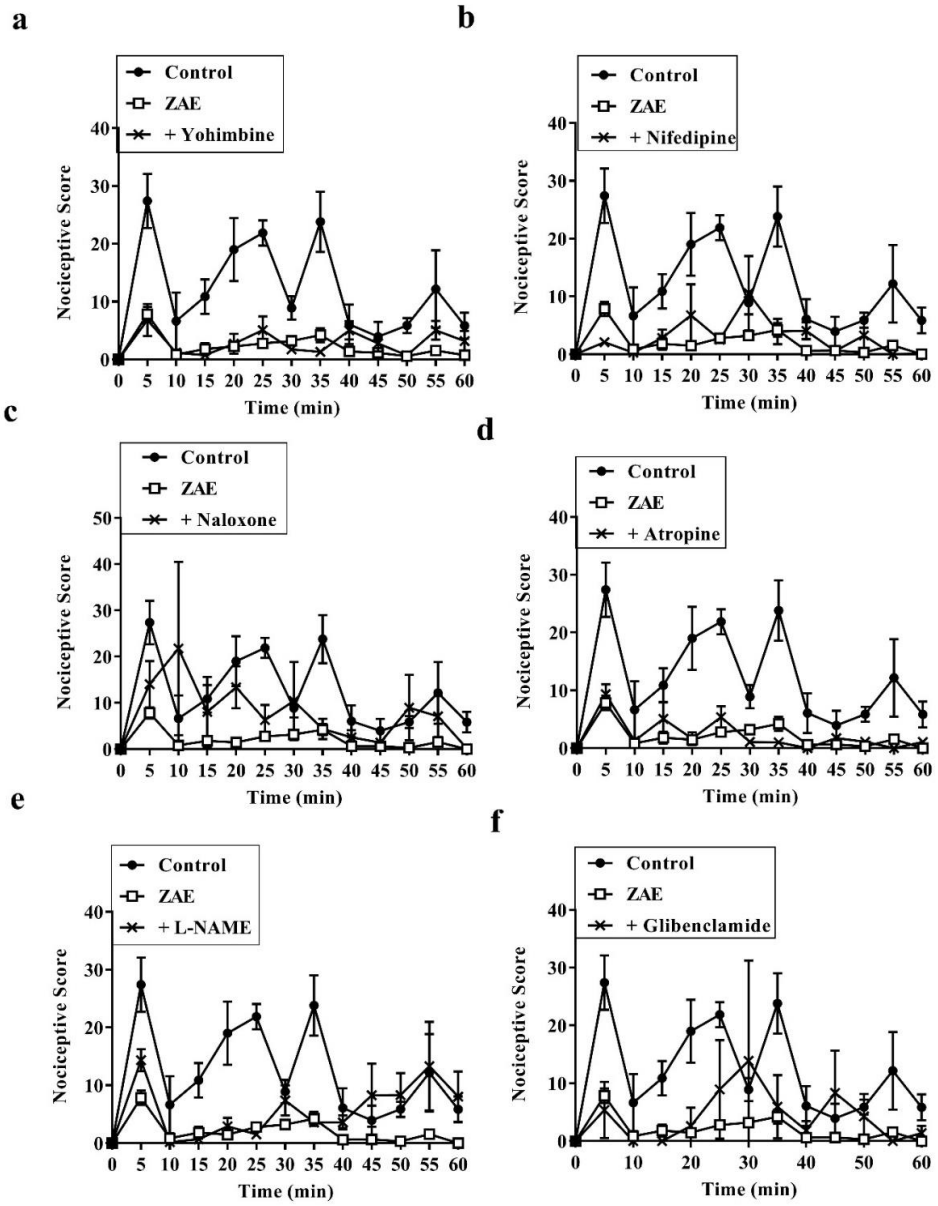

Figure 28: Effect of pretreatment of mice with (a) yohimbine (3 mg/kg, *p.o.*), (b) nifedipine (10 mg/kg, *p.o.)*, (c) naloxone (2 mg/kg i.p.), (d) atropine (5 mg/kg, i.p.), (e) L- NAME (10 mg/kg, i.p) and (f) glibenclamide (8 mg/kg, *p.o.*) on the anti-nociceptive profile of morphine (3 mg/kg, *p.o.*) in phase 1 and phase 2 of formalin-induced nociception. Each column represents the mean of 5 animals and the error bars indicate S.E.M.

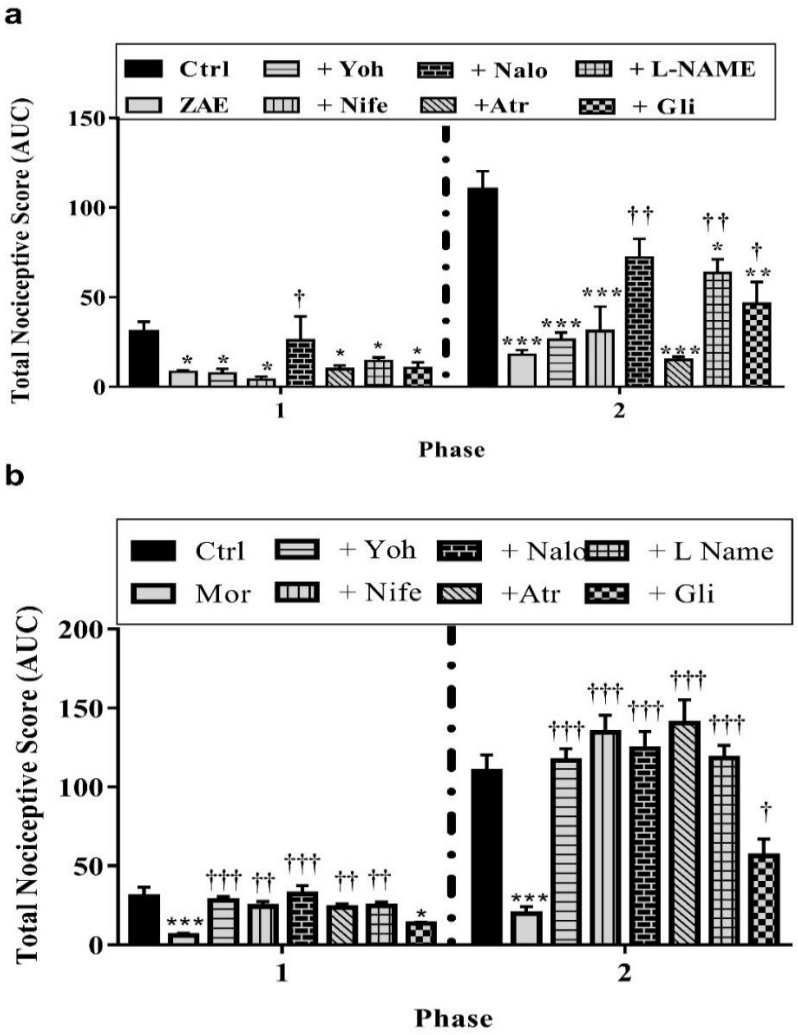

Figure 29: Effect of pretreatment of mice with yohimbine (3 mg/kg, *p.o.*), nifedipine (10 mg/kg, *p.o.*), naloxone (2 mg/kg i.p.), atropine (5 mg/kg, i.p.), L- NAME (10 mg/kg, i.p) and glibenclamide (8 mg/kg, *p.o.*) on the anti-nociceptive profile of (a) ZAE (300 mg/kg, *p.o.*) and (b) morphine (3 mg/kg, i.p.) in phase 1 and phase 2 of formalin-induced nociception. Each column represents the mean of 5 animals and the error bars indicate S.E.M. ***P<0.001 compared to respective vehicle-treated controls; $^{\dagger\dagger\dagger}P$ < 0.001, $^{\dagger\dagger}P$ < 0.01 and $^{\dagger}P$ < 0.05 compared to either ZAE 100 mg/kg or morphine 3 mg/kg (all one-way ANOVA followed by Tukey's post *hoc* test).

TNF-alpha and Interleukin 1β induced hyperalgesia

Mechanical hyperalgesia was produced in all the animals treated with TNF-alpha, and this effect was persistent in the negative control group throughout the entire duration of the test {Figure 30 (a and b)}. Administration of the extract of *Ziziphus abysinnica* significantly (P<0.001) reversed TNF-α

induced hyperalgesia in rats at all doses. Morphine (3 mg/kg) used as a reference drug in the TNF-α induced nociception test produced significant inhibition of the hyperalgesia.

Similarly, intraplantar administration of IL-1β induced a nocifensive response in the rats, and this was significantly (P<0.01) reduced by ZAE 100 and 300 mg/kg as well as morphine 3 mg/kg (Figure 30 c and d).

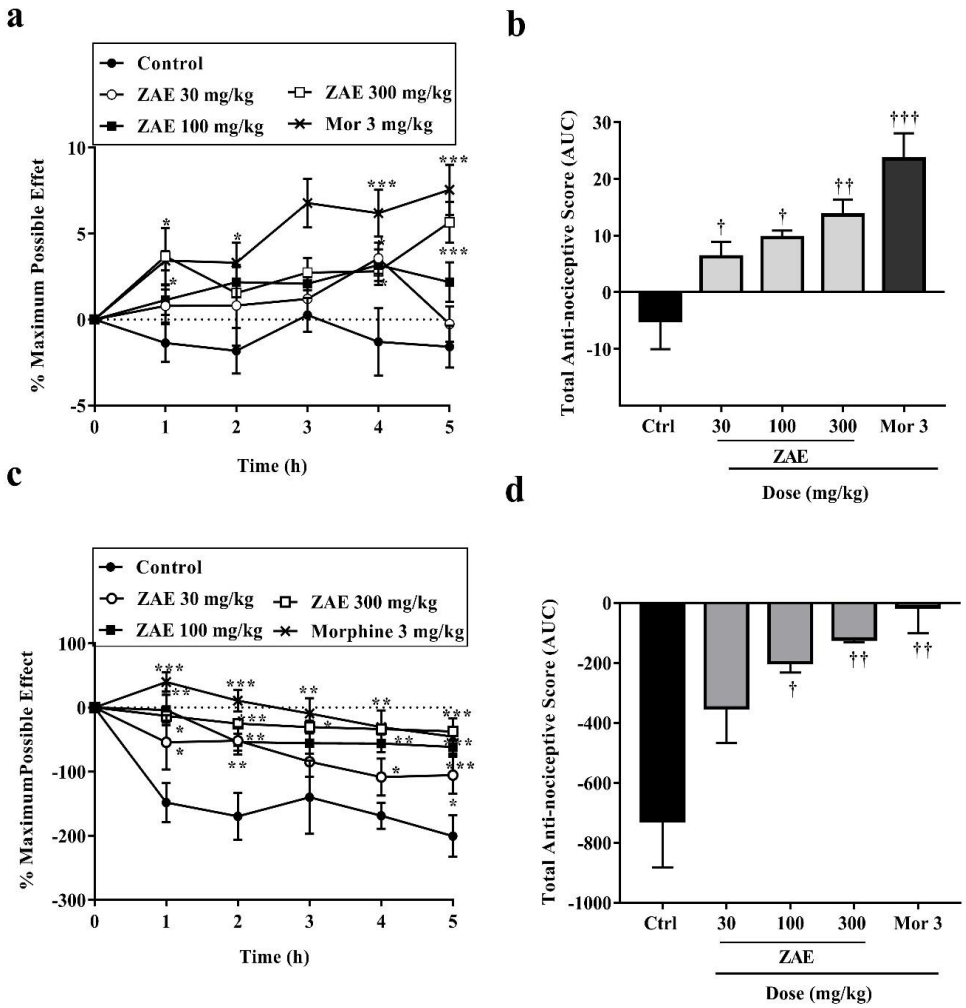

Figure 30: Effect of pretreatment of rats with ZAE (30-300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.) on TNF-α (a and b) and IL-1β (c and d) - induced hyperalgesia. Each data represents a mean of 5 (±S.E.M). Data (a and c) represent the time-course curves of nociception ***P < 0.001, **P < 0.01, *P < 0.05 (two-way ANOVA followed by Dunnet's post *hoc* test compared to the control group); whereas (b and d) represent total anti-nociceptive effects (AUC) $^{\dagger\dagger}P$ < 0.01 and $^{\dagger}P$ < 0.05 (one - way ANOVA followed by Tukey's post *hoc* test compared to the control (Ctrl) group).

Bradykinin and prostaglandin E_2-induced hyperalgesia

Mechanical pressure applied to the right hind paws of rats after intraplantar injection of bradykinin caused a decrease in paw withdrawal latencies. However, administration of the extract of *Ziziphus abysinnica* significantly ($P<0.001$; Figure 31 a and b) reversed the hyperalgesia in rats that received 30, 100 and 300 mg/kg ZAE. Morphine (3 mg/kg), used as the reference drug, also significantly reversed the hyperalgesia comparable to that of ZAE 300 mg/kg.

Also, prostaglandin E_2 irritant-induced hyperalgesia and this was significantly ($P<0.01$) reversed by oral administration of 100 and 300 mg/kg of ZAE and morphine 3 mg/kg (Figure 31 c and d).

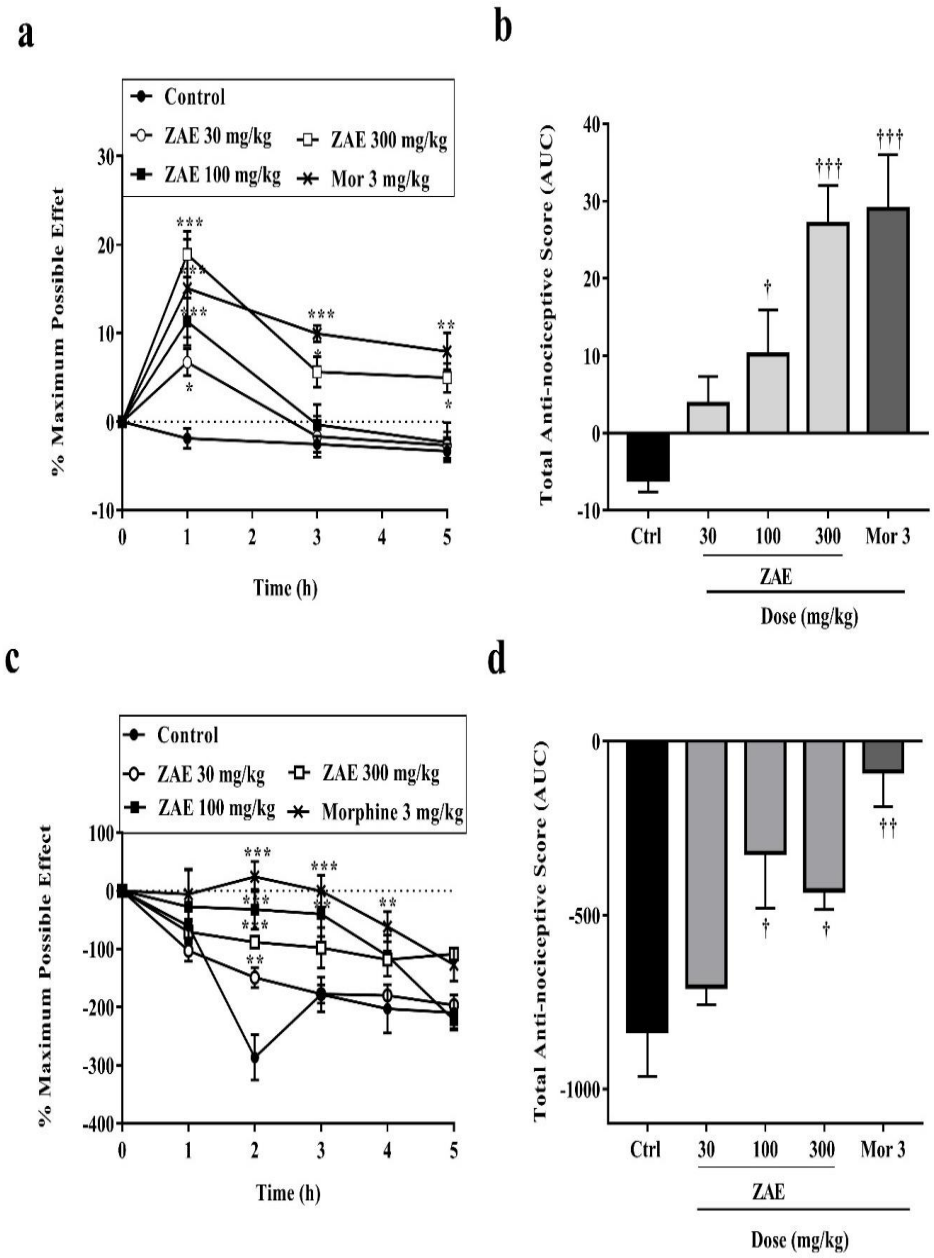

Figure 31: Effect of pretreatment of rats with ZAE (30, 100 and 300 mg/kg, *p.o.*) and morphine (3 mg/kg, i.p.) on bradykinin (a and b) and prostaglandin E_2 (c and d) - induced hyperalgesia. Each data represents a mean of 5 (±S.E.M). Data (a and c) represent the time-course curves, *P < 0.05, **P < 0.01 and ***P < 0.001 (two-way ANOVA followed by Dunnet's post *hoc* test compared to the control group); whereas (b and d) represent total anti-nociceptive effects (AUC) $^{\dagger\dagger}P$ < 0.01 and $^{\dagger}P$ < 0.05 (one - way ANOVA followed by Tukey's post *hoc* test compared to the control (Ctrl) group).

Anti-inflammatory Effect of Extract

In vitro anti-inflammatory test

Hypotonic solution-induced haemolysis of human red blood cells

ZAE significantly ($P<0.010$) and concentration-dependently inhibited HRBC haemolysis in a hypotonic solution. The percentage inhibitions increased from 42.98, 81.58 to 93.86% at a concentration of 100, 300 and 1000 µg/mL respectively. The percentage inhibitions were comparable with diclofenac which were 50.95, 68.89 and 86.73% at concentrations of 100, 300 and 1000 µg/mL respectively (Figure 32 a).

Heat-induced haemolysis of human red blood cells

ZAE also inhibited heat-induced haemolysis by 61.8%, 65.3% and 85.2% at 100, 300 and 1000 µg/mL respectively. The effect was similar to diclofenac which gave percentage inhibitions of 66.03, 68.33 and 86.49% at concentrations of 100, 300 and 1000 µg/mL respectively (Figure 32 b).

Egg albumin denaturation assay

A concentration-dependent inhibition of protein (albumin) denaturation was produced by ZAE which were 43.5, 52.7 and 65.9% at concentration 100, 300 and 1000 µg/mL respectively. This effect was comparable to diclofenac which produced 50.08, 54.48 and 72.23 percentage inhibitions at concentrations of 100, 300 and 1000 µg/mL respectively (Figure 32 c).

Bovine serum albumin denaturation assay

Results obtained shows the inhibitory effect of ZAE and diclofenac on heat-induced bovine serum albumin denaturation. At concentrations of 100, 300 and 1000 µg/mL of ZAE, the mean percentage inhibitions were 63, 70 and 97.15% respectively. Diclofenac similarly, showed concentration-dependent

inhibition of protein denaturation of 56.2, 73.77 and 99.02% at 100, 300 and 1000 µg/mL (Figure 32 d).

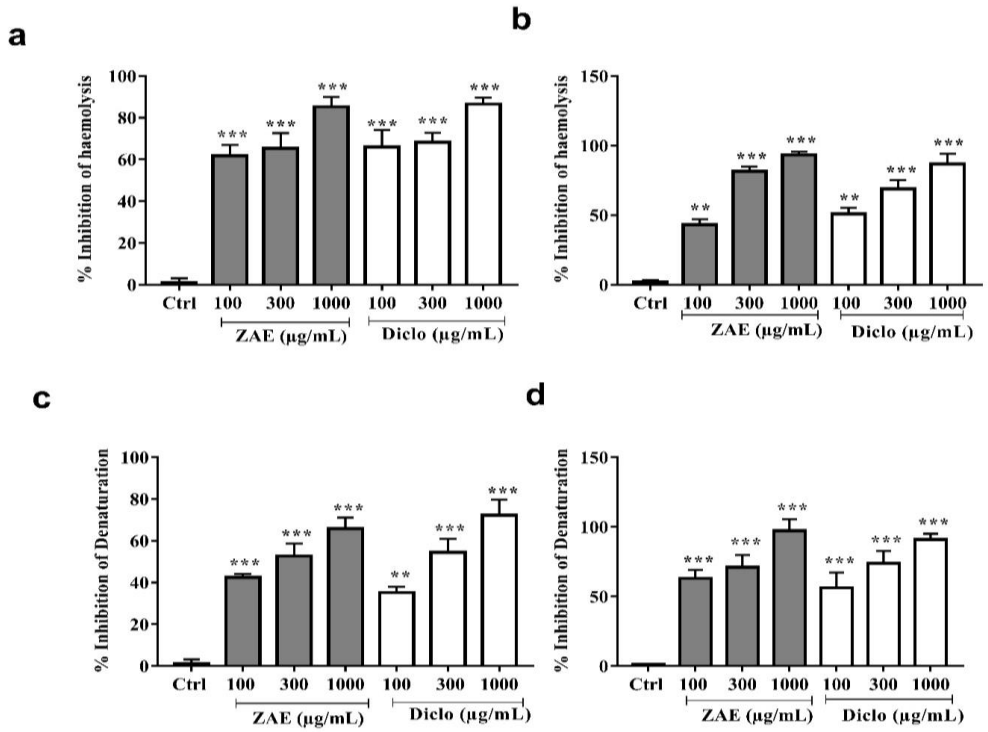

Figure 32: Effect of *Ziziphus abyssinica* root bark extract (ZAE) on (a) hypotonic solution-induced hemolysis (b) heat-induced hemolysis (c) egg albumin and (d) bovine serum albumin denaturation assay. Data is presented as mean ± standard error of mean (n = 3). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control group (one-way ANOVA followed by Tukey's post *hoc*). ZAE: *Ziziphus abyssinica* extract, Diclo: diclofenac.

In vivo anti-inflammatory effect

Carrageenan-induced paw oedema

Time course curves [two-way ANOVA (treatment x time)] revealed a significant effect of drug treatments on the percentage change in paw oedema ($P<0.001$, Figure 33). ZAE (300 mg/kg, *p.o.*) significantly ($P<0.05$) reduced paw oedema with a maximum percentage inhibition of 40.8 ± 6.8%. Diclofenac (10 mg/kg, p.o.) also produced significant ($P<0.01$) decrease in paw oedema (54.81 ± 3.74).

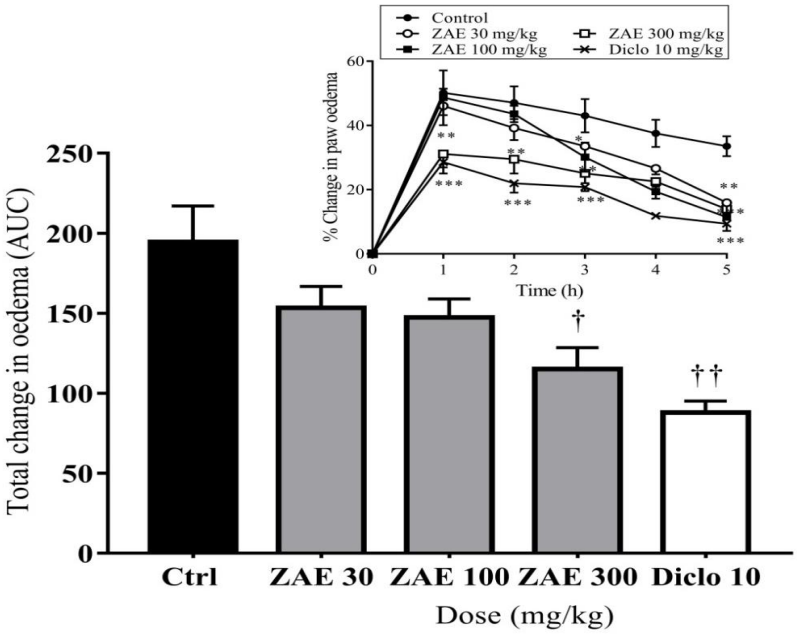

Figure 33: The effects of ZAE (30 - 300 mg/kg, *p.o.*) and diclofenac (Diclo 10 mg/kg, i.p) on the total change in paw oedema (calculated as AUCs) in carrageenan-induced paw oedema test in rats. † P < 0.05, †† P < 0.01 and ††† P < 0.001 compared to control (ctrl) group (one-way ANOVA followed by Tukey's *post hoc*). Insert: Percentage change in paw oedema and over 5 h peroid. Each data represents mean ± standard error of mean, n = 5: *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control group (two – way ANOVA followed by Dunnet's post *hoc*).

Formalin-induced inflammation

Intraplantar injection of formaldehyde into the right hind paws of the rats produced a prominent increase in paw oedema beginning in the first hour of injection (Figure 34). This effect was sustained throughout the entire duration of the experiment in the vehicle-treated group. The mean total anti-oedematous effect (calculated as areas under the curve in Figure 34 insert) obtained for ZAE (30, 100 and 300 mg/kg *p.o.*) were 151.8±35.72, 250±15.35 and 539±12.15 respectively. Diclofenac (10 mg/kg *p.o.*) produced a mean anti-oedematous effect of 637.2±16.4 whereas the negative control group had 123.9±11.2 The percentage inhibitions calculated from the total anti-

oedematous effect of ZAE (30, 100, 300 mg/kg) and diclofenac were 3.31±22.12, 49.89±2.98 and 76.98±0.50 and 80.51±0.53% respectively.

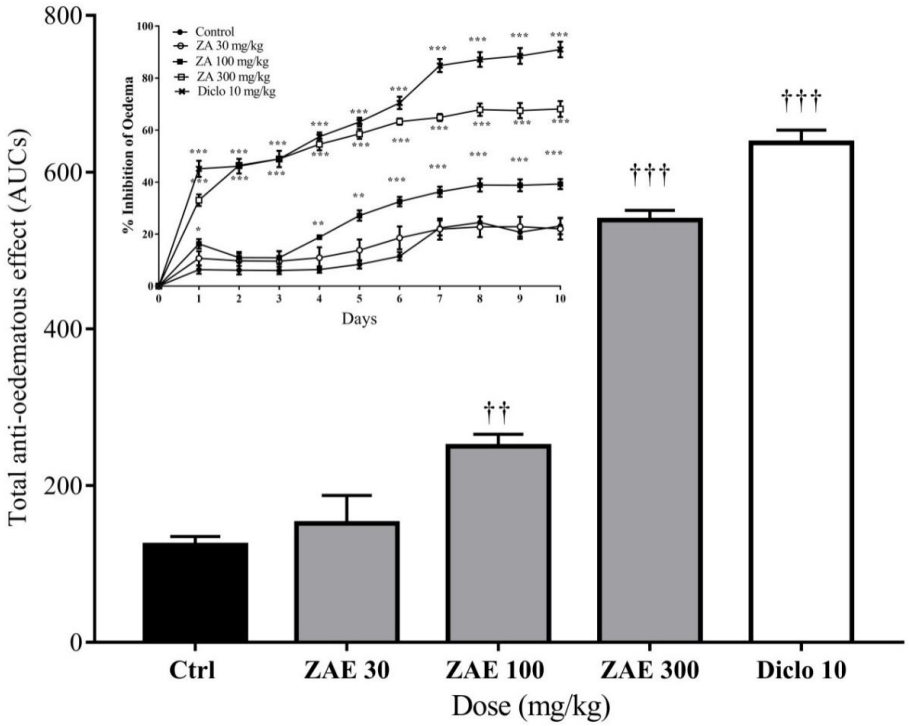

Figure 34: The effects of ZAE (30 - 300 mg/kg, *p.o.*) and diclofenac (Diclo 10 mg/kg, i.p) on (A) percentage inhibition of paw oedema and (B) total anti-oedematous effect (calculated as AUCs) in formalin-induced paw oedema test in rats. Each data represents mean ± standard error of mean, n = 5: † P < 0.05, †† P < 0.01 and ††† P < 0.001 compared to control group (two – way ANOVA followed by Dunnet's *post hoc*). P < 0.05 compared to the control (ctrl) group (one-way ANOVA followed by Tukey's post *hoc*).

Carrageenan-induced peritonitis

Intense inflammation was provoked in the rats by the injection of 1% carrageenan characterised by massive recruitment of leukocytes (mainly neutrophils) into the peritoneal cavity of the rats. ZAE (30, 100 and 300 mg/kg, *p.o.*) and dexamethasone (5 mg/kg, *p.o.*) showed a significant (P<0.01) reduction in the total number of cells compared to the saline-treated group (Figure 35). The differential count was performed using basic cell morphology

to differentiate between mast cells, neutrophils, macrophages, lymphocytes and basophils. Pretreatment of the rats with ZAE (30, 100 and 300 mg/kg) also caused marked ($P<0.001$) reduction in the number of neutrophils recruited to the peritoneal cavity compared to saline-treated rats (Figure 36). There was also a significant ($P<0.05$) decrease in the number of mononuclear cells in the peritoneal cavity of the animals that received ZAE (300 mg/kg) and dexamethasone (5 mg/kg).

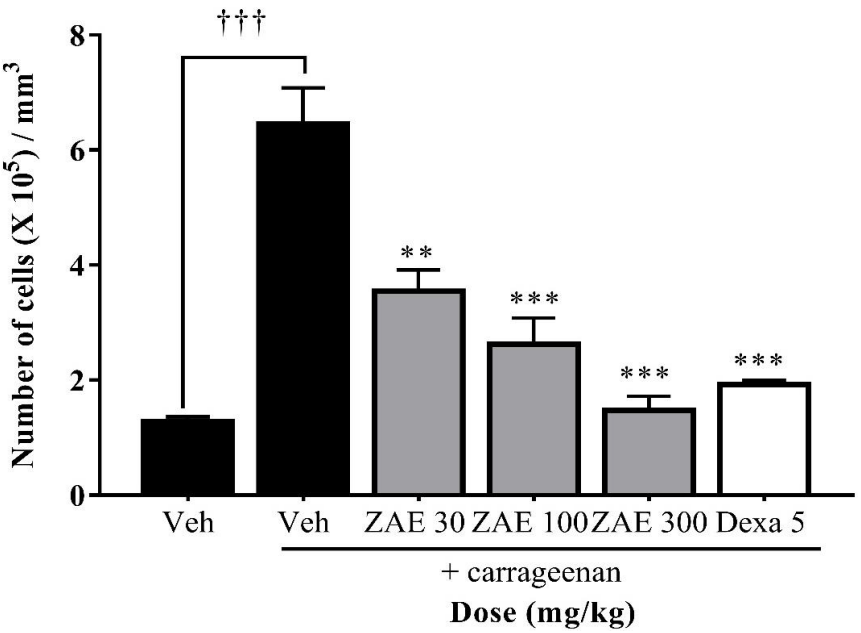

Figure 35: Effects of the administration of *Ziziphus abyssinica* root bark extract (ZAE, 30 - 300 mg/kg), Dexamethasone (5 mg/kg) or vehicle (Negative control) on acute carrageenan-induced peritonitis, measured by the number of cells in the peritoneal exudate. Results are presented as mean ± S.E.M. of cells/peritoneal cavity for n=5 rats. ††† or ***$p < 0.001$ and **$p < 0.01$ when compared with negative control (NC) group respectively (one-way ANOVA followed by Tukey's post *hoc*).

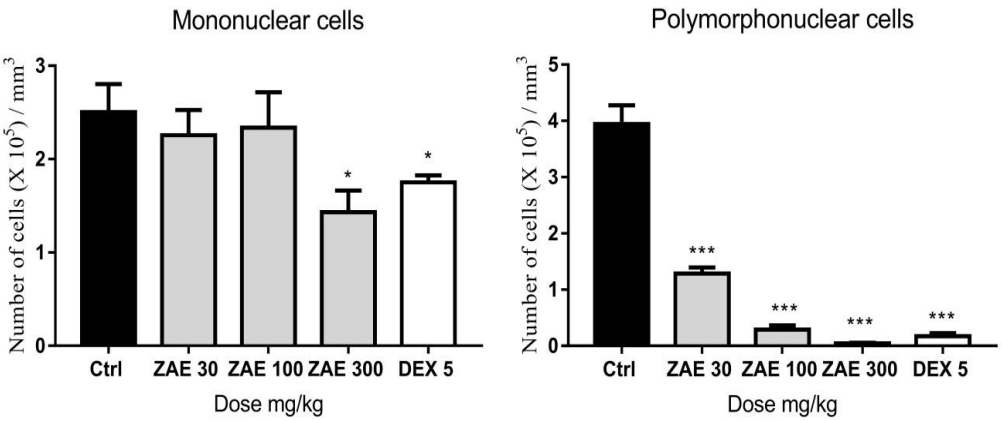

Figure 36: Differential leukocyte counts in the peritoneal cavity of rats pretreated with vehicle, (ZAE) or dexamethasone in the peritonitis model induced by carrageenan. Results are presented as mean ± S.E.M. of cells/peritoneal cavity for n=5 rats. Where presents *p < 0.001, *** represents p < 0.001 compared to negative control (ctrl) group (one-way ANOVA followed by Tukey's post *hoc*).

Mechanism of anti-inflammatory effect of ZAE

TNF-α – induced inflammation

Pretreatment of rats with ZAE (30, 100 and 300 mg/kg, *p.o.*) and diclofenac 10 mg/kg significantly (P<0.05) reduced TNF-alpha - induced paw oedema (Figure 37). The results was confirmed by histopathological assessment of the injected paws (Figure 38). Naïve control group (A) received only distilled water without TNF-α injection and there were no leukocytes present, and tissue structure appeared compact and normal. The disease control group which received an intraplantar injection of TNF-α and distilled water orally were characterised by severe leukocytosis, and the tissues appeared loose with lots of clear pockets showing a high level of oedema (B). There were however, negligible leukocytosis and tissue appeared loose with fewer spaces of oedema in the rats treated with diclofenac (C). The rats pretreated with ZAE 30 mg/kg (D) showed severe leucocytosis with congested

blood vessels and moderate spaces of oedema and tissues were moderately affected. Rats treated with a ZAE 100 mg/kg (E) showed low leukocytosis with moderately damaged tissue. In rats treated with highest dose of ZAE (300 mg/kg), there were very low leucocytosis with mild vascular congestion and the tissue appeared fairly compact/ normal (F).

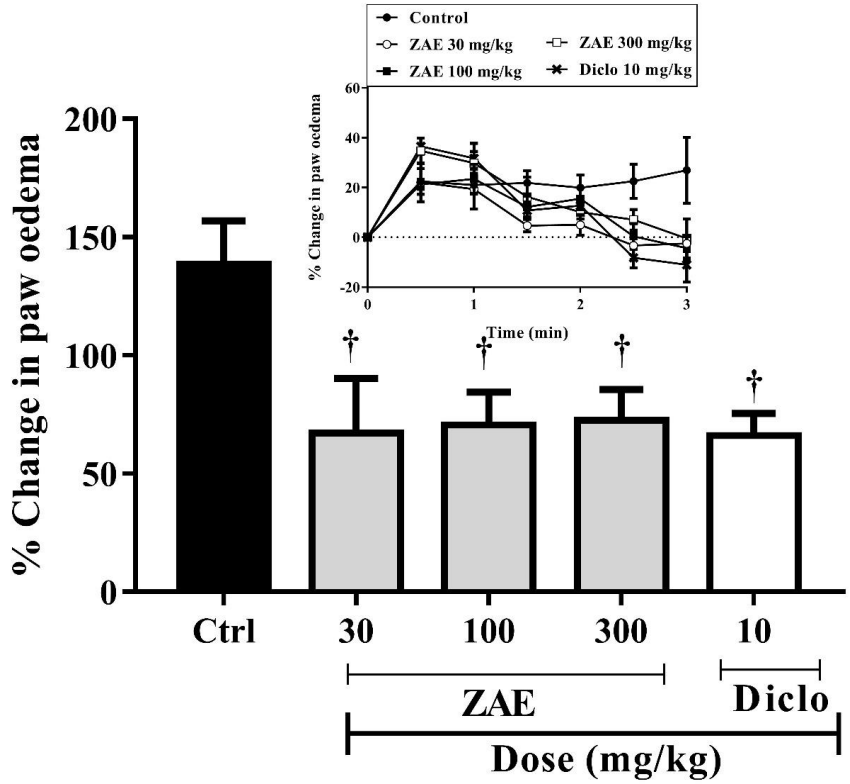

Figure 37: Effect of pretreatment of rats with ZAE (30 - 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, i.p.) on TNF-alpha – induced paw oedema in rats. Data is presented as mean±SEM. Data (a) represents the time-course curves **P < 0.01, *P < 0.05 (two-way ANOVA followed by Dunnet's post *hoc* test); whereas (b) represents total anti-nociceptive effects (AUC) ††P < 0.01 and †P < 0.05 (one - way ANOVA followed by Tukey's post *hoc* test).

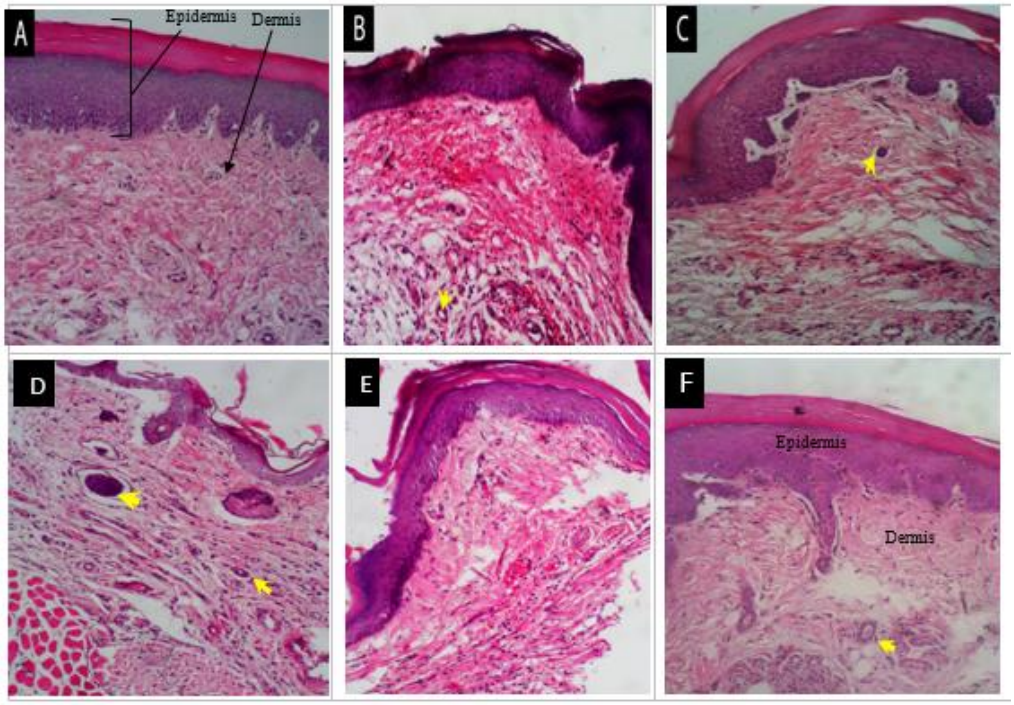

Figure 38: Effect of treatment of rats orally with (B) distilled water, (C) diclofenac 10 mg/kg, (D) ZAE 30 mg/kg, (E) 100 mg/kg, and (F) 300 mg/kg on leukocyte aggregation and tissue damage in TNF-α – induced inflammation in the skin tissue of the right hind paws of rats. Naïve control animals received distilled water only without TNF-α (A) (H&E ×100 magnification).

Interleukin-1β - induced paw oedema

Intraplantar administration of interleukin-1β caused an increased paw oedema which was sustained in the disease control group throughout the entire duration of the experiment. However, pretreatment with ZAE 100, 300 mg/kg and diclofenac 10 mg/kg significantly ($P<0.05$) ameliorated this inflammatory effect (Figure 39). Photomicrographs of the injected paws is presented in Figure 40. Naïve control group which received neither drug treatment nor intraplantar injection of IL-1β showed no leukocytes and tissue structure appeared compact and normal (A). Photomicrograph B represents the negative control which received pretreated with distilled water in addition to intraplantar injection of IL-1β. Leucocytosis was severe and tissues showed lots of spaces of oedema. The diclofenac 10 mg/kg treatment group (C)

showed mild leukocytosis and the tissue structure appeared to have been slightly affected. Animals in groups D, E and F received 30, 100 and 300 mg/kg of ZAE respectively in addition to intraplantar injection of IL-1β. In D, leucocytosis was high but significantly lower than the negative control group (B) with blood vessels highly congested. For the middle dose treatment group (E), leucocytosis was fairly low, and the tissue appeared compact and normal whereas in the highest dose group (F), the level of leukocytes was insignificant compared to the disease control group.

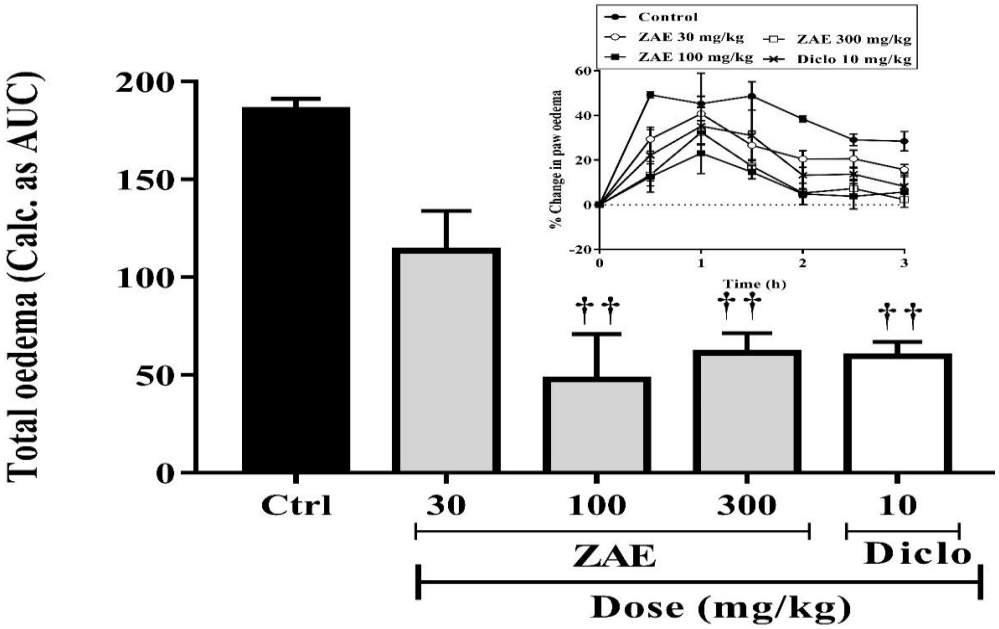

Figure 39: Effect of pretreatment of rats with ZAE (30 - 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, i.p.) on Interleukin-1β – induced paw oedema in rats. Data is presented as mean±SEM. Data (a) represents the time-course curves **P < 0.01, *P < 0.05 (two-way ANOVA followed by Dunnet's post *hoc* test); whereas (b) represents total anti-nociceptive effects (AUC) ††P < 0.01 and †P < 0.05 (one - way ANOVA followed by Tukey's post *hoc* test).

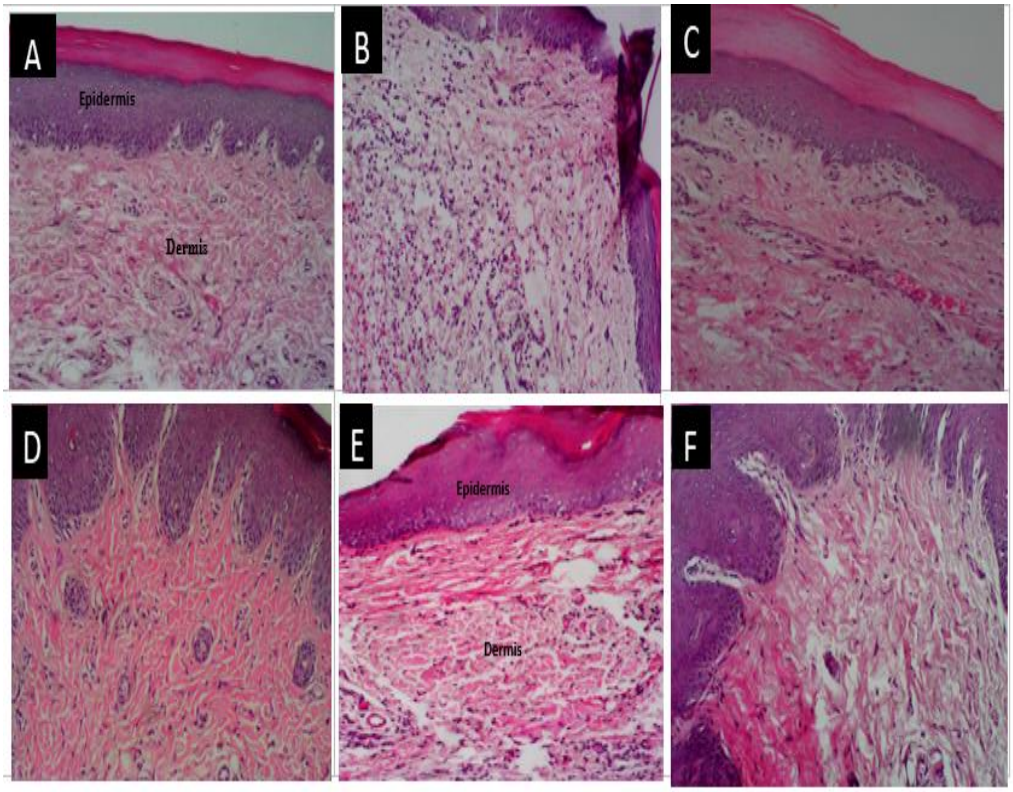

Figure 40: Effect of treatment of rats orally with (B) distilled water, (C) diclofenac 10 mg/kg, (D) ZAE 30 mg/kg, (E) 100 mg/kg, and (F) 300 mg/kg on leukocyte aggregation and tissue damage in IL-1β – induced inflammation in the skin tissue of the right hind paws of rats. Naïve control animals received distilled water only without IL-1β intraplantar injection (A) (H&E ×100 magnification).

Prostaglandin E_2-induced paw oedema

Intraplantar injection of prostaglandin E_2 caused increased oedema within thirty minutes, and this effect was sustained in the negative control group throughout the experimental period (Figure 41). However, pretreatment with ZAE 100 and 300 mg/kg orally significantly ($P<0.05$) reduced the paw oedema with percentage inhibitions of 72.87±20.92 and 85.88±15.48% respectively. Diclofenac reduced oedema by 37.39±11.65% although this effect was not significant when compared with control. The photomicrographs of the paws (Figure 42) depicted that in the naive control group (A), the dermis appeared normal. However, in the disease control group (B), there were severe leukocyte infiltration, dilation and congestion of vessels.

Treatment with diclofenac 10 mg/kg (C) resulted in a very mild levels of leukocytic infiltrate, accompanied by oedema. Animals in group D which represents treatment with ZAE 30 mg/kg showed section with moderate leukocytosis whereas ZAE 100 mg/kg (E) depicts leukocytosis and mild oedema. Animals treated with ZAE 300 mg/kg showed mild oedema with very low leukocytosis and tissue consistency is fairly normal (F).

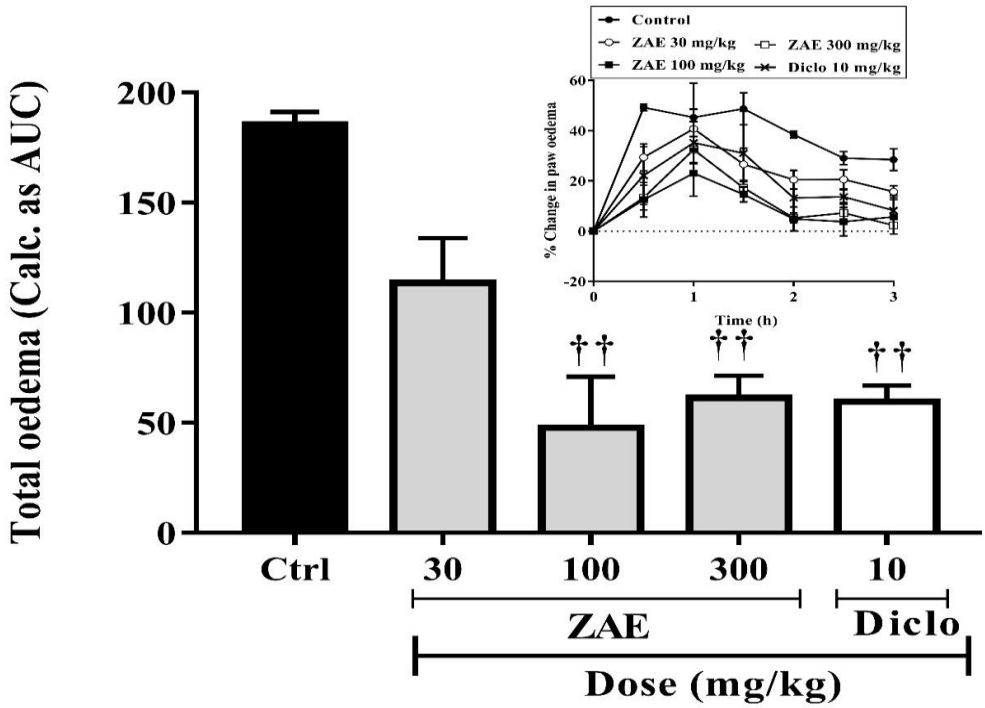

Figure 41: Effect of pretreatment of rats with ZAE (30 - 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, i.p.) on prostaglandin E2 – induced paw oedema in rats. Data is presented as mean±SEM. Data (a) represents the time-course curves (oedema score) **P < 0.01, *P < 0.05 compared to control (two-way ANOVA followed by Dunnet's post *hoc* test); whereas (b) represents total anti-nociceptive effects (AUC) ††P < 0.01 and †P < 0.05 compared to respective control group (one - way ANOVA followed by Tukey's post *hoc* test).

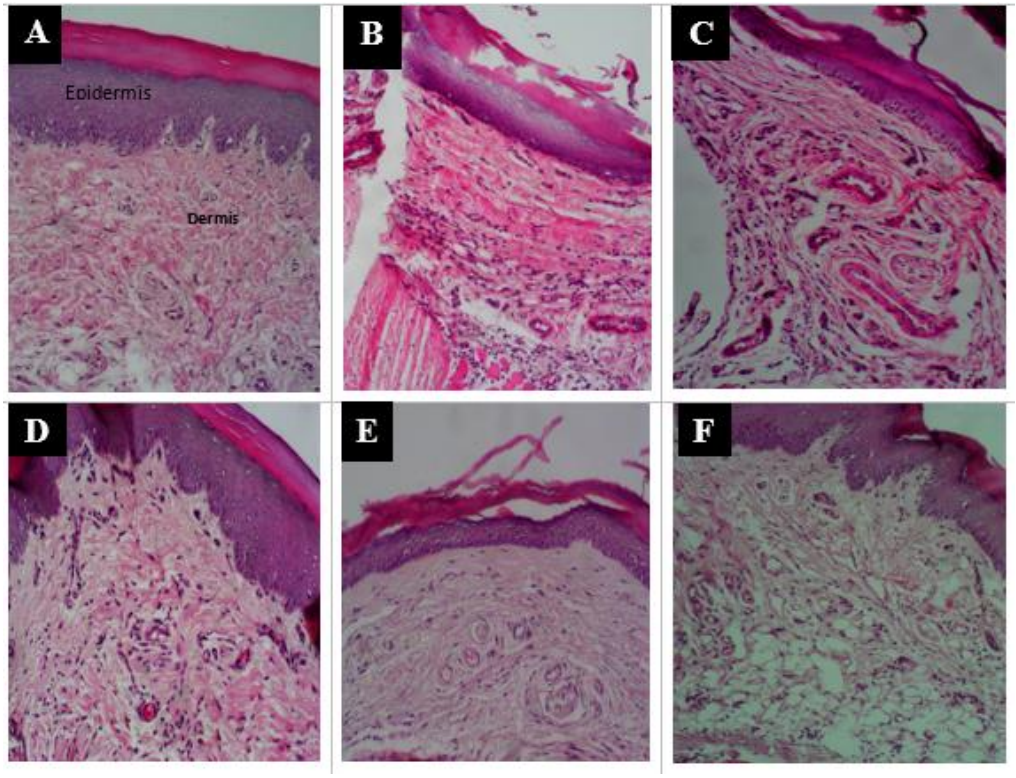

Figure 42: Effect of treatment of rats orally with (B) distilled water, (C) diclofenac 10 mg/kg, (D) ZAE 30 mg/kg, (E) 100 mg/kg, and (F) 300 mg/kg in Prostaglandin E_2 – induced inflammation in the right hind paws. Naïve control animals received distilled water only without Prostaglandin E_2 intraplantar injection (A) (H&E ×100 magnification).

Bradykinin-induced paw oedema

Pretreatment of rats with ZAE 100 and 300 mg/kg as well as diclofenac 10 mg/kg orally significantly ($P<0.001$) reduced paw oedema produced by intraplantar injection of bradykinin irritant by 62.24±16.08, 70.93±5.82 and 90.4±30.19% respectively (Figure 43). From the histological report (Figure 44), the normal control rats showed the dermis appears normal (A). However, in the negative control group (B), there were severe leukocyte infiltration, dilation and congestion of vessels (C). Positive control group show very mild levels of leukocytic infiltrate, accompanied by oe oedema (D). Treatment with ZAE 30, 100 and 300 mg/kg depicted sections with moderate leukocytosis and mild oedema and tissue consistency was fairly normal (E, F and G)

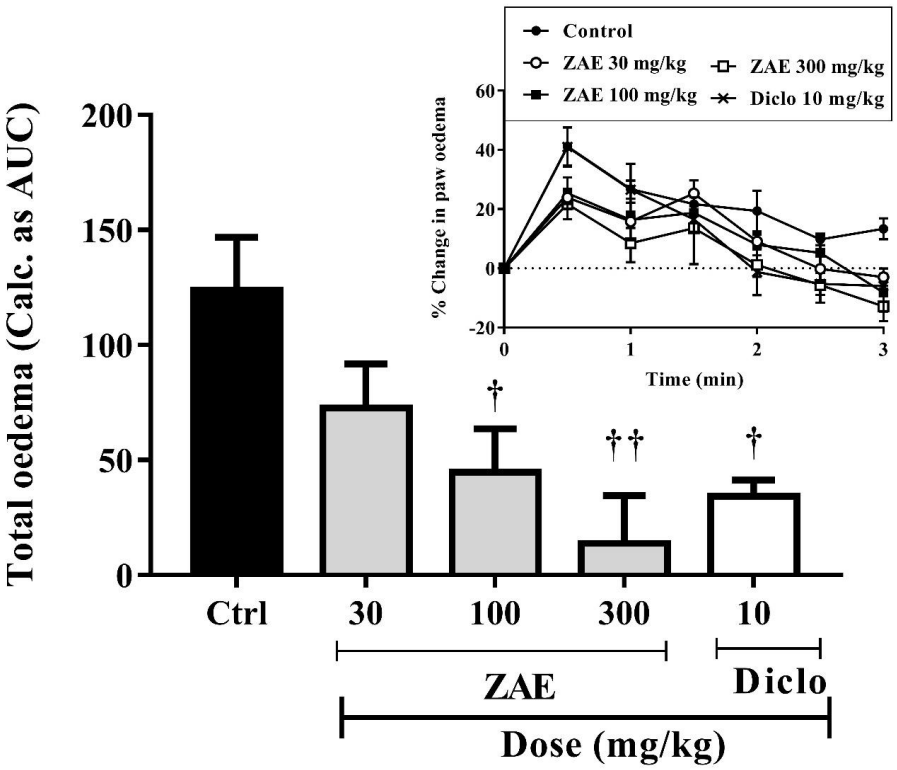

Figure 43: Effect of pretreatment of rats with ZAE (30 - 300 mg/kg, p.o.) and diclofenac (10 mg/kg, i.p.) on bradykinnin – induced paw oedema in rats. Data is presented as mean±SEM. Data (a) represents the time-course curves **P < 0.01, *P < 0.05 (two-way ANOVA followed by Tukey's post hoc test); whereas (b) represents total anti-nociceptive effects (AUC) ††P < 0.01 and †P < 0.05 (one - way ANOVA followed by Newman-Keuls' post hoc test)

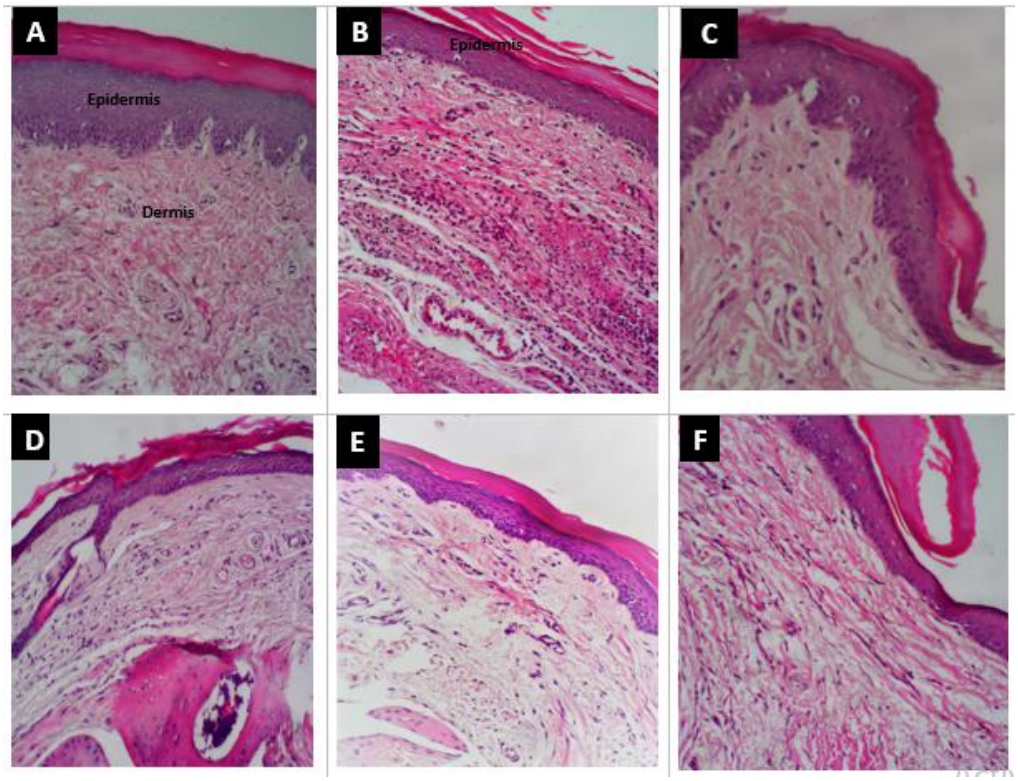

Figure 44: Effect of treatment of rats orally with (B) distilled water, (C) diclofenac 10 mg/kg, (D) ZAE 30 mg/kg, (E) 100 mg/kg, and (F) 300 mg/kg in bradykinin – induced inflammation in the right hind paws. Naïve control animals received distilled water only without bradykinin intraplantar injection (A) (H&E, ×100 magnification).

Antidepressant Effect of the Extract

Forced swimminga test (FST)

In the FST, immobility time was decreased significantly ($P<0.001$; Figure 45 a) by the 100 and 300 mg/kg dose of ZAE. Fluoxetine showed a similar significant decrease ($P<0.01$; Figure 45 b) at the 30 mg/kg dose. However, the frequency of immobility was not significantly decreased ($P> 0.05$; Figure 45 c*)* by any of the doses of the extract, unlike the fluoxetine which was significantly ($P<0.05$; Figure 45 d) decreased it at 30 mg/kg dose. Also, mobility times spent on swimming was significantly higher than that

spent on climbing in mice treated with ZAE (100 and 300 mg/kg), and fluoxetine (3 and 10 mg/kg) (Figure 46 a and b).

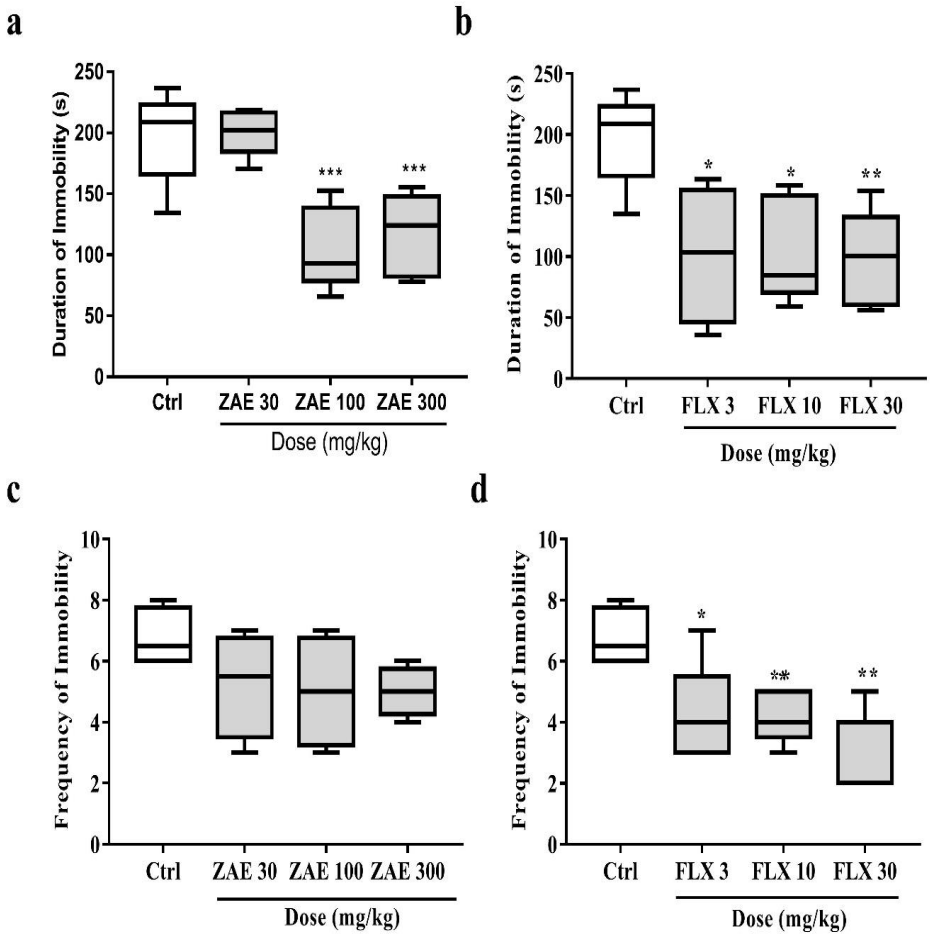

Figure 45: Effects of acute administration of ZAE (30, 100 and 300 mg/kg, *p.o.*) and fluoxetine (3, 10 and 30 mg/kg, *p.o.*) treatment on immobility duration (a, b) and frequency (c, d) of immobility in FST. Data are presented as group means with minimum to maximum points. Significant differences from control are denoted: *P<0.05, **P<0.01 (one –way ANOVA followed by Tukey's post *hoc* test).

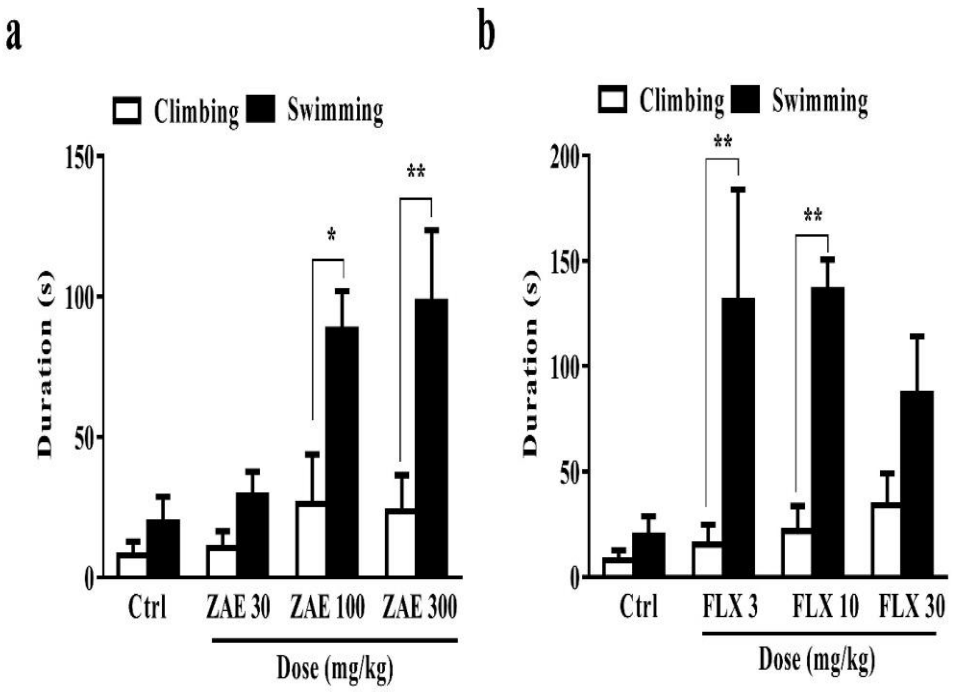

Figure 46: Effects of acute administration of (a) ZAE (30, 100 and 300 mg/kg, *p.o.*) and (b) fluoxetine (3, 10 and 30 mg/kg, *p.o.*) treatment on duration of swimming and climbing in FST. Data are presented as group means (±SEM). Significant differences from control are denoted: *P<0.05, **P<0.01 (one – way ANOVA followed by Tukey's post *hoc* test).

Tail suspension test

Results presented below indicate that ZAE at the doses of 100 and 300 mg/kg significantly (P<0.01; Figure 47 a) decreased immobility duration of mice. Fluoxetine (30 mg/kg) also significantly (P<0.001; Figure 47 b) decreased duration of immobility in the forced swimming test. Similarly, the frequency of immobility in the tail suspension test was significantly reduced by ZAE (10 mg/kg) and fluoxetine 10 and 30 mg/kg.

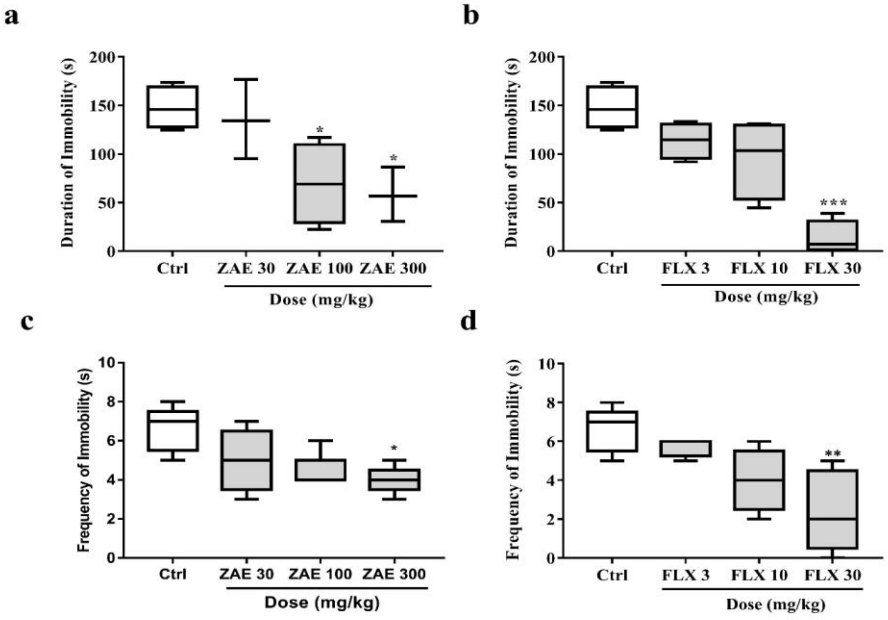

Figure 47: Effects of acute ZAE (30, 100 and 300 mg/kg, *p.o.*) and fluoxetine (3, 10 and 30 mg/kg, *p.o.*) treatment on immobility duration and frequency in TST. Data are presented as group means with minimum to maximum points. Significant differences from control are denoted: *P<0.05, **P<0.01 (one – way ANOVA followed by Tukey's *post hoc* test).

Anxiolytic effect of the extract and isolated compounds

In elevated plus maze, the vehicle treated group (distilled water, 10 mL/kg, *p.o.*) spent more time in the closed arm compared to the open arm of the maze within the five minutes. On the other hand, mice treated with diazepam (0.3, 1 and 3 mg/kg, *p.o.*) significantly spent more time in the open arm of the maze compared to the closed arm. Treatment with ZAE (100 and 300 mg/kg, *p.o.*; P<0.05) significantly increased the time spent in the open arms of the maze compared to the control mice (Figure 48 a and b).

In the open field test, diazepam-treated mice significantly (P<0.5) spent more time in the four centre squares of the open field compared to the vehicle-treated control mice. Similarly, ZAE (100 and 300 mg/kg, *p.o.*; P<0.05) significantly increased the time spent in the centre of the maze within the five minutes of the test (Figure 48 c and d).

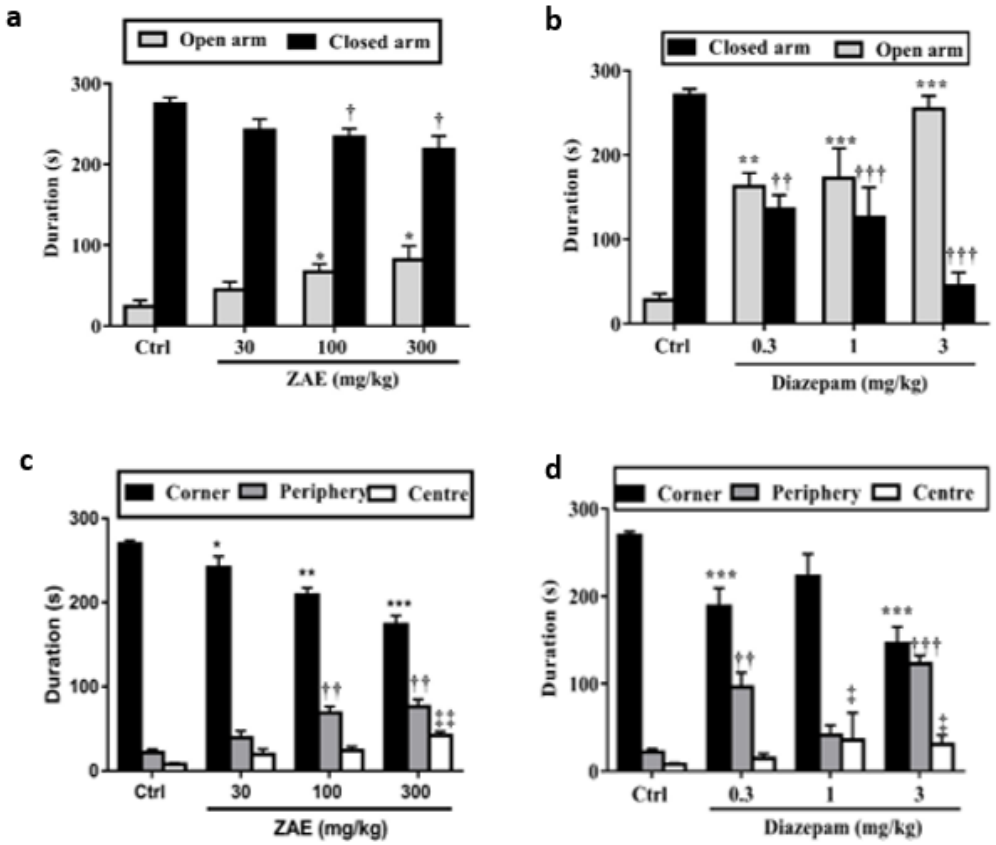

Figure 48: Effects of acute ZAE (30, 100 and 300 mg/kg, *p.o.*) and diazepam (3, 10 and 30 mg/kg, *p.o.*) treatment on (a and b) open and closed arm duration in the elevated plus maze as well as (c and d) duration in corner, peripheral and central arena duration in the open field test. Data are presented as group means ± SEM. Significant differences from control are denoted: *P<0.05, **P<0.01 (one –way ANOVA followed by Tukey's *post hoc* test).

Structural Elucidation of Isolated Compounds

Structural elucidation of compound 1

Compound **1** was obtained as whitish needle-like crystals with melting point of 202.6-204.2 ˚C. The IR spectrum showed a characteristic broad absorption band at 3253 cm^{-1}, indicating the presence of a hydroxy functional group [Appendix A(vi)]. HRMS sodium adduct ions at *m/z* 449.3800 $[M + Na]^+$ (calculated for $C_{30}H_{50}ONa$, 449.3759) and 875.7696 $[2M+Na]^+$ (calculated for $C_{60}H_{100}O_2Na$, 875.7621), corresponding to 6 indices of hydrogen deficiency (Appendix A-vii). ^{13}C NMR signals at δ 145.35 and 121.89 suggested the existence of an olefinic motif (Table 5 and Appendix Aii). It can also be

observed in the ^{1}H NMR spectra of compound **1** that there were 8 methyl groups in this compound [Appendix A(i)]. These signals were at δ 0.79, 0.83, 0.94, 0.97, 1.00, and 1.14 (each 3H, s), and 0.87 (3H × 2, s). Additionally, there was one triplet peak at δ 5.18 (J = 3.56 Hz, 1H) and one doublet of doublets peak at δ 3.22 (J_1= 10.84 Hz, J_2= 4.56 Hz, 1H) in the spectra [Appendix A (i and ii)]. Analysis of DEPT 135 NMR data yielded a compound with 10 methylene (CH_2) groups [Appendix A (i and ii)].Through comparison with spectroscopic data reported in the literature (Okoye et al., 2014), compound **1** was identified as β-amyrin (Figure 50 a). The ^{13}C NMR assignments showed 30 carbon signals, which were generally consistent with reported values (Okoye et al., 2014), are summarised in Table 5. This was also confirmed by the data obtained from 2D NMR spectra (COSY, HMBC and HSQC) as shown in Appendix A (iv and v).

Structural elucidation of compound 2

Compound **2** was isolated as shiny crystals with melting point of 282.2-283.6 and a specific rotation of $[\alpha]^{24}_{D}$−38.45 (*c* 0.07, $CHCl_3$). The molecular formula of compound **2** was determined to be $C_{30}H_{48}O_3$ by the HRMS ions at *m/z* 479.3512 $[M + Na]^+$ (calculated for $C_{30}H_{48}O_3Na$, 479.3501) and 935.7116 $[2M+Na]^+$ (calculated for $C_{60}H_{96}O_6Na$, 935.7105) [Appendix B (vi)] and from the ^{13}C NMR spectrum which showed 30 carbon signals as shown in Table 5 and Appendix B (ii). The IR spectrum as presented in Appendix B (v) exhibited two separate bands at 3532 and 3435 $cm^{-1}$and a sharp band at 1663 cm^{-1}, together with a broad absorption band between 3300 and 2500 cm^{-1}, suggesting the presence of a carboxylic acid group with hydrogen bonding. A sharp absorption band at 1726 cm^{-1} seen in the IR

spectrum indicated the presence of a ketone group. The functional groups were confirmed by ^{13}C NMR signals at δ 184.12 and 213.21. Molecular formula of compound **2** (Figure 50 b) implies 7 indices of hydrogen deficiency. X-ray crystal structure of compound **2** {Figure 50 and Appendices C (i and ii)} is in accord with its molecular formula. As can also be seen from the X-ray crystal structure, the carboxylic acid group formed a hydrogen bond with an additional water molecule. Upon literature investigations, compound **2** was elucidated to be a known triterpene derivative, polpunonic acid (maytenonic acid, 3-oxofriedelan-29-oic acid). This is the first time that the absolute configuration of polpunonic acid was determined by X-ray diffraction analysis. The ^{1}H NMR (assignments of the seven distinctive methyl groups for compound **2** were at δ 0.72, 0.86, 1.00, 1.10, and 1.26 (each 3H, s), and 0.88 (3H × 2, s), which are in general consistent with those reported in the literature (Itokawa *et al*., 1991) (Table 5). DEPT 135 analysis also revealed 11 methylene (CH_2) [Appendix B (ii and iii)]. Further assignment of all NMR signals and the structure came from 2D NMR studies (COSY, HSQC, HMBC) as shown in Appendices B (iii and iv).

The crystal structure of compound 2 with atom labelling and polymeric structure are shown in Figure 49. The C-C distances in five cyclohexane ring are within normal range of 1.52-1.57Å. The other geometric parameters of compound 2 are shown in Tables 6-9. These five cyclohexane rings have seven methyl, one carbonyl and one carboxylic group substitutes. Carboxylic group is making strong hydrogen bond interactions with a water molecule, found in the crystal lattice. However, the oxygen atom of the carbonyl group is not involved in any hydrogen bond interaction. Water-mediated three H-bonds

interactions (O24-H24 … O34 (1.8449(1) Å, O34-H … $O25^{i}$ (1.9657(1) Å (symmetry code: 1/2-x, 1/2+y, 1-z) and O34-Ha … $O25^{ii}$ (2.0843(1) (symmetry code: +x, 1+y, +z)) leading to the formation of a polymeric structure along the b axis. The H-bonds have a contribution in the formation of a stable structure (Figure 47). Hydrogen-bond geometry is shown in Table 9. Packing diagram of the crystal structure is shown in Appendix C (i).

Table 5: ^{13}C NMR assignments for compounds 1 **and** 2

Carbon number	**Compound 1**	B-amyrin (Okoye et al., 2014)	**Compound 2**	Polpunonic acid (Itokawa et al., 1991)
1	38.75	38.79	22.29	22.30
2	27.10	27.44	41.53	41.53
3	79.19	79.24	213.21	213.16
4	38.94	38.99	58.29	58.30
5	55.34	55.37	42.07	42.08
6	18.54	18.58	41.37	41.37
7	32.82	32.85	18.26	18.27
8	41.89	40.21	50.75	50.75
9	47.80	47.43	37.47	37.49
10	37.12	37.15	59.83	59.80
11	23.85	23.75	36.18	35.35
12	121.89	121.93	29.57	30.32
13	145.35	145.41	39.26	39.24
14	39.96	41.92	39.20	39.28
15	28.26	26.36	29.50	29.63
16	26.32	27.14	36.65	36.19
17	32.65	32.70	30.13	30.15
18	47.40	47.84	44.26	44.29
19	47.00	47.03	29.40	29.54
20	31.24	31.30	40.44	40.42
21	34.90	34.94	30.29	29.43
22	37.31	15.71	35.33	36.68
23	28.56	28.31	6.81	6.81
24	15.65	15.71	14.67	14.68
25	15.74	15.80	18.42	18.10
26	16.97	17.01	16.35	18.43
27	26.15	26.21	18.11	16.40
28	27.40	28.62	31.84	31.85
29	33.49	33.56	184.12	183.45
30	23.69	23.91	31.53	31.54

^{13}C NMR spectra were obtained at 100 MHz, and recorded in $CDCl_3$ at room temperature.

Table 6: Crystal data and structure refinement for Compound 2.

Empirical formula	$C_{30}H_{50}O_4$
Formula weight	474.73
Temperature/K	100.00(10)
Crystal system	monoclinic
Space group	C2
a/Å	13.33700(7)
b/Å	6.31317(4)
c/Å	30.75651(15)
α/°	90
β/°	100.3632(5)
γ/°	90
Volume/Å^3	2547.41(2)
Z	4
ρ_{calc}g/cm^3	1.2377
μ/mm^{-1}	0.620
F(000)	1051.0
Crystal size/mm^3	0.501 × 0.375 × 0.155
Radiation	Cu Kα (λ = 1.54184)
2Θ range for data collection/°	5.84 to 134.1
Index ranges	$-15 \leq h \leq 15$, $-6 \leq k \leq 7$, $-36 \leq l \leq 36$
Reflections collected	44153
Independent reflections	4335 [R_{int} = 0.0228, R_{sigma} = 0.0093]
Data/restraints/parameters	4335/1/318
Goodness-of-fit on F^2	1.041
Final R indexes [I>=2σ (I)]	R_1 = 0.0267, wR_2 = 0.0722
Final R indexes [all data]	R_1 = 0.0267, wR_2 = 0.0722
Largest diff. peak/hole / e Å^{-3}	0.13/-0.19
Flack parameter	0.26(14)

Table 7: For compound 2 Bond lengths

Atom	Atom	Length/Å	Atom	Atom	Length/Å
O25	C23	1.2233(15)	C10	C9	1.5382(15)
O24	C23	1.3142(16)	C16	C17	1.5460(16)
O30	C7	1.2094(15)	C11	C32	1.5466(17)
C23	C20	1.5276(15)	C21	C20	1.5508(15)
C15	C16	1.5463(15)	C5	C4	1.5426(15)
C15	C1	1.5517(15)	C5	C33	1.5368(17)
C2	C11	1.5632(15)	C5	C6	1.5690(17)
C2	C3	1.5405(14)	C27	C1	1.5455(17)
C2	C1	1.5642(15)	C4	C3	1.5273(16)
C22	C14	1.5653(15)	C17	C29	1.5478(17)
C22	C21	1.5483(14)	C17	C18	1.5436(15)
C22	C17	1.5908(14)	C7	C6	1.5193(16)
C26	C14	1.5505(18)	C7	C8	1.5103(16)
C14	C13	1.5435(15)	C19	C20	1.5657(15)
C14	C1	1.5735(14)	C19	C18	1.5264(16)
C12	C11	1.5453(14)	C28	C20	1.5214(17)
C12	C13	1.5331(15)	C6	C31	1.5282(15)
C10	C11	1.5709(16)	C9	C8	1.5324(18)
C10	C5	1.5654(14)			

Table 8: Bond Angles for compound 2

Atom	Atom	Atom	Angle/°	Atom	Atom	Atom	Angle/°
O24	C23	O25	123.22(11)	C6	C5	C33	108.68(9)
C20	C23	O25	124.40(11)	C3	C4	C5	113.54(10)
C20	C23	O24	112.37(10)	C16	C17	C22	113.25(9)
C1	C15	C16	116.05(9)	C29	C17	C22	111.67(10)
C3	C2	C11	110.21(9)	C29	C17	C16	108.15(9)
C1	C2	C11	116.80(9)	C18	C17	C22	109.31(9)
C1	C2	C3	114.45(9)	C18	C17	C16	106.96(10)
C21	C22	C14	110.65(9)	C18	C17	C29	107.23(9)
C17	C22	C14	114.23(9)	C6	C7	O30	122.93(11)
C17	C22	C21	111.39(9)	C8	C7	O30	122.00(11)
C26	C14	C22	110.31(9)	C8	C7	C6	115.07(10)
C13	C14	C22	110.45(9)	C18	C19	C20	112.24(9)
C13	C14	C26	106.82(9)	C12	C13	C14	113.38(9)
C1	C14	C22	108.78(9)	C4	C3	C2	109.92(9)
C1	C14	C26	111.91(9)	C7	C6	C5	110.17(10)
C1	C14	C13	108.55(9)	C31	C6	C5	115.89(10)
C13	C12	C11	113.80(9)	C31	C6	C7	110.97(10)
C5	C10	C11	116.03(9)	C8	C9	C10	111.39(10)
C9	C10	C11	113.83(9)	C2	C1	C15	109.14(9)
C9	C10	C5	110.91(9)	C14	C1	C15	108.90(9)
C17	C16	C15	118.24(10)	C14	C1	C2	109.80(9)
C12	C11	C2	107.42(9)	C27	C1	C15	107.80(9)
C10	C11	C2	106.07(9)	C27	C1	C2	109.21(9)
C10	C11	C12	109.05(9)	C27	C1	C14	111.94(9)
C32	C11	C2	115.43(9)	C21	C20	C23	106.08(9)
C32	C11	C12	107.09(9)	C19	C20	C23	108.51(9)
C32	C11	C10	111.59(9)	C19	C20	C21	109.90(9)
C20	C21	C22	114.93(9)	C28	C20	C23	109.12(10)
C4	C5	C10	108.54(8)	C28	C20	C21	111.47(10)
C33	C5	C10	114.08(10)	C28	C20	C19	111.57(10)
C33	C5	C4	109.73(10)	C19	C18	C17	112.71(10)
C6	C5	C10	107.55(9)	C9	C8	C7	111.81(10)
C6	C5	C4	108.09(10)				

Table 9: Hydrogen-bond geometry (Å, º) for compound 2

Compound	D – H --- A	D – H	H --- A	D --- A	D – H --- A
2	O24 – H24 --- O34	0.82	1.8449(1)	2.6013(1)	152.7852(1)
	O34 – H --- O25^{i}	0.85	1.9657(1)	2.8033(1)	168.2605(1)
	O34 – Ha --- Cl1^{ii}	0.85	2.0843(1)	2.9311(1)	174.0969(1)

Symmetry Codes: (i) 1/2-x, 1/2+y, 1-z; (ii) +x, 1+y, +z.

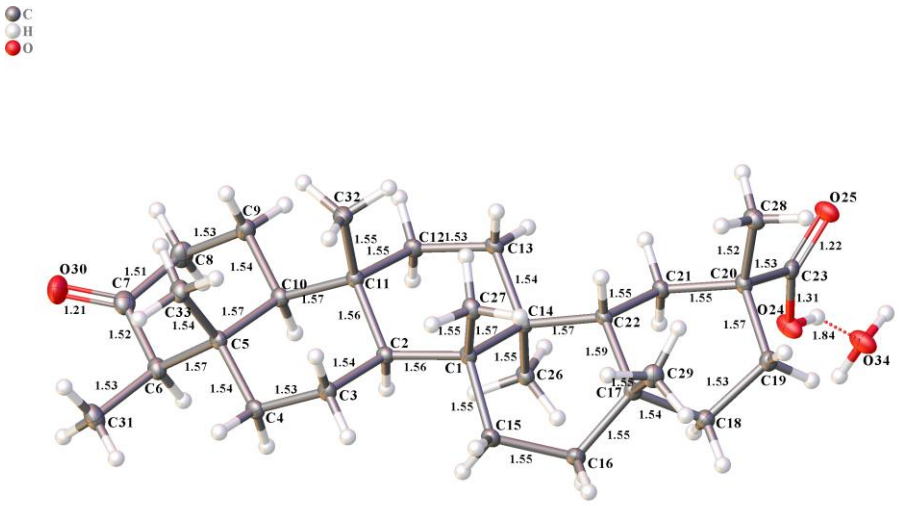

Figure 49: The molecular structure of compound 2, showing the atom-labeling scheme. Displacement ellipsoids are drawn at the 40% probability level.

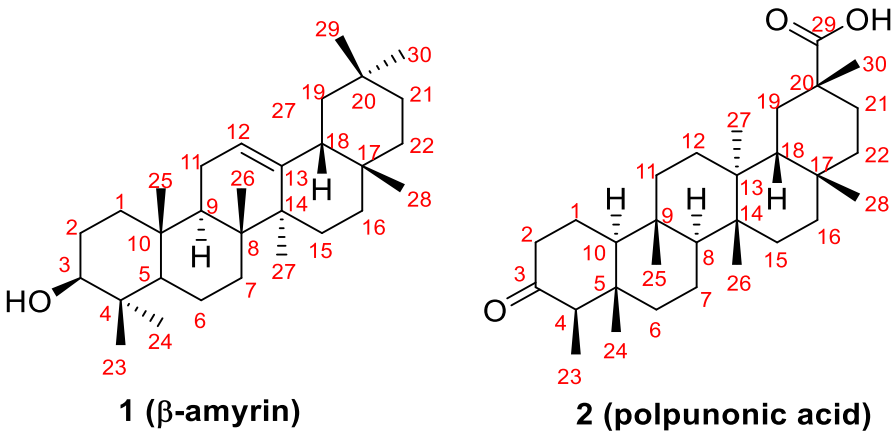

Figure 50: Chemical structures of compounds 1 and 2

Anti-arthritic Effect of the Isolated Compounds

Total body weight

From the results obtained, except for the CFA only treated group, all the other rats in the various treatment groups gained weight in the course of the 28 days (Figure 51). The total body weight of the rats expressed as areas under the curves were significantly ($P<0.05$) higher than the arthritic control group.

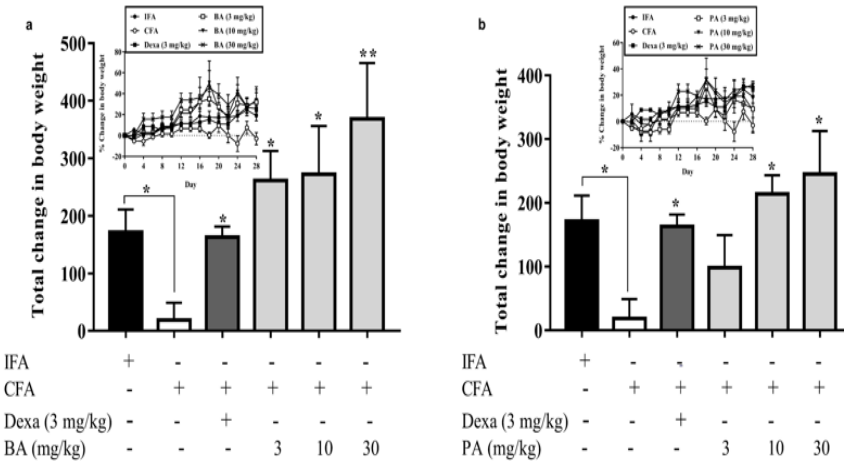

Figure 51: Effect of dexamethasone (Dexa), β-amyrin (BA) and polpunonic acid (PA) administration on the body weights of rats in Complete Freund's adjuvant (CFA) - induced arthritis in Sprague-Dawley rats. Data is presented as Mean ± SEM. (n = 5). The total body weights of the treatment groups were compared to the arthritic control group (CFA) with *P < 0.05, **P <0.01 (One-way ANOVA followed by Tukeys post hoc test). Insert: Time-course curves of the percentage change in body weights of the various treatments.

Paw thickness

Drug treatment with β-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*), dexamethasone (3 mg/kg) and normal saline (10 mL/kg) started on day 14 following the onset of the polyarthritic phase of the chronic inflammation (Figure 52). Paw swelling was measured every other day over 28 days. The progress of inflammatory oedema in the contralateral

(non-injected) limb was evident from day 12 in the CFA control and drug-treated rats. This is indicative of a systemic spread of the inflammation. β-amyrin (3, 10 and 30 mg/kg, *p.o.*) and polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) significantly (P<0.05, at all doses) altered the time course of the oedema progression in both paws and showed a marked decrease in the total change in paw oedema in both ipsilateral and contralateral paws

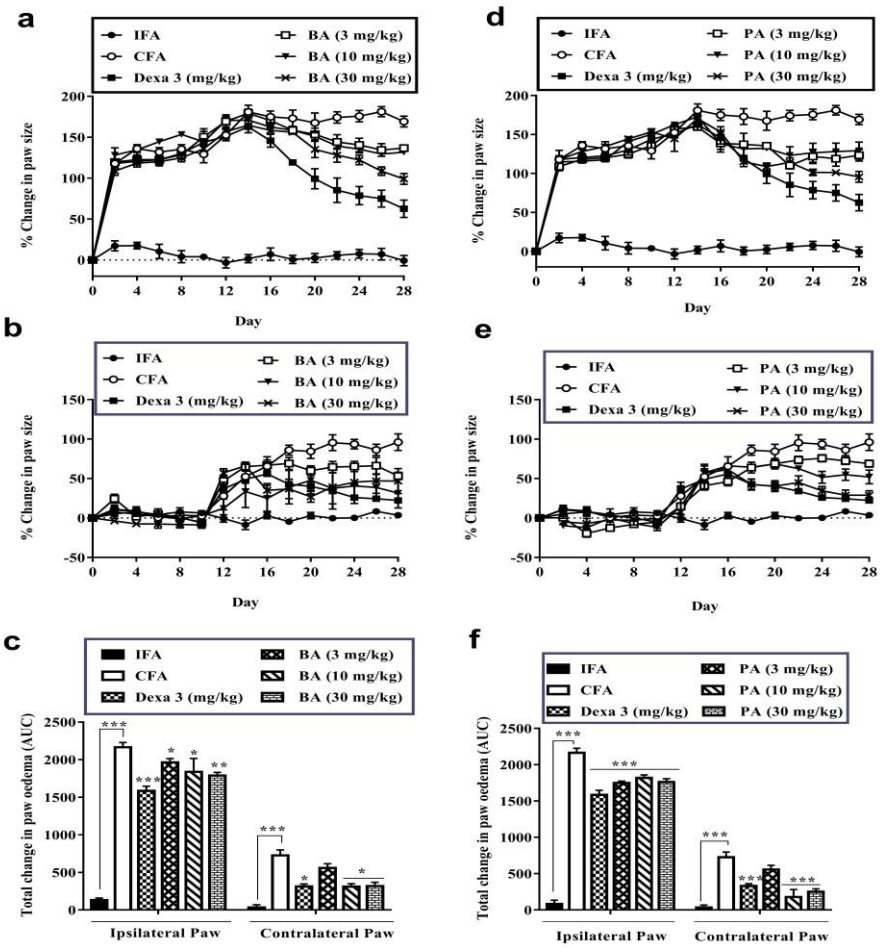

Figure 52: Effect of β-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*), dexamethasone (3 mg/kg) and normal saline (10 mL/kg) administration on Complete Freund's adjuvant (CFA) -induced arthritis in Sprague-Dawley rats. The oedema component of inflammation was monitored as the percentage change in paw oedema (a and b) and (d and e) for BA and PA respectively in both paws. Total oedema induced during the acute and polyarthritis phases was calculated as area under the time course curves, AUC (c and f). Data is presented as Mean ± SEM. (n = 5). The total anti-oedematous effect of treatment groups were, compared to the arthritic (CFA) control group with *P < 0.05, P <0.01, ***P <0.001 (One-way ANOVA followed by Tukeys post hoc test).

Arthritic index

The compounds beta-amyrin (3, 10 and 30 mg/kg, *p.o.*) and polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) significantly reduced the arthritic score of rats previously treated with CFA in adjuvant-induced arthritis model (Figure 54). This effect was comparable to the dexamethasone-treated group. The reduction in arthritis score was associated with reduced erythema and oedema in one or more paws.

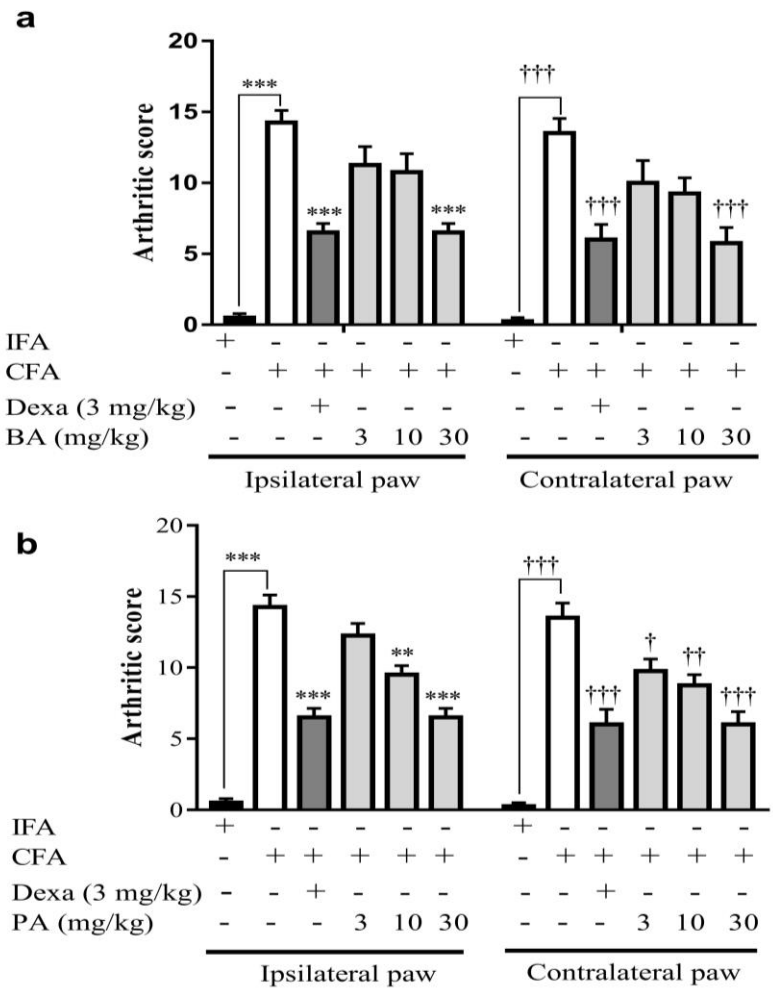

Figure 53: Effect of beta-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*), dexamethasone (3 mg/kg) and normal saline (10 mL/kg) administration on arthritic score of CFA -induced arthritis in Sprague-Dawley rats. Data is presented as Mean ± SEM, (n = 5). The total anti-oedematous effect of treatment groups were, compared to CFA treated control group with *P < 0.05, P <0.01, ***P <0.001 (One-way ANOVA followed by Tukeys post hoc test).

Photographs of the paws

Photographs (Figure 54) of the paws of the animals showed no signs of erythema and oedema in the non-arthritic (IFA) group (A). Arthritic (CFA) control group (B), however, exhibited massive erythema and swelling in both ipsilateral and contralateral hind limbs. The oral administration of dexamethasone 3 mg/kg (C) caused a reduction in swelling in both ipsilateral and contralateral limbs. Treatment with the various doses of beta-amyrin (D, E and F) and polpunonic acid (G, H and I) resulted in a reduced erythema and swelling with the effect of the highest doses of both compounds comparable to that the reference drug, dexamethasone.

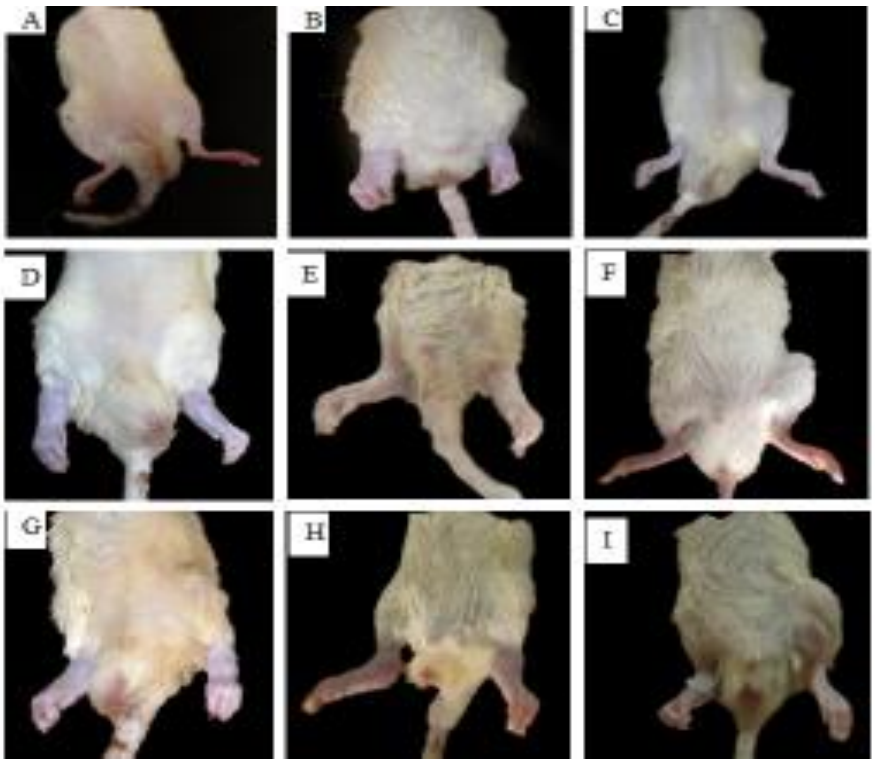

Figure 54: Photographs showing the curative effect of beta-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) and dexamethasone (3 mg/kg) in adjuvant-induced arthritis in rats. Sprague-Dawley rats (200 – 250 g) were injected intraplantarly with 100 µL of IFA or CFA into the right hind paw and monitored for 28 days. Treatment were as follows: (A) IFA/non-arthritic control, (B) CFA/arthritic control, (C) Dexamethasone, (D) BA 3 mg/kg, (E) BA 10 mg/kg, (F) BA 30 mg/kg, (G) PA, 3 mg/kg, (H) PA, 10 mg/kg and (I) PA,`30 mg/kg.

Radiography of the paws

Radiographs from the non-arthritic (IFA) control group (A) showed no lesions and joint deformation as expected. The arthritic (CFA) control group (B), however, showed noticeable signs of inflammation in both the ipsilateral and contralateral hind limbs including the tissues surrounding the bones of the foot. There were also noticeable signs of inflammation at the metatarso-phalangeal joint. Bone enlargement with active osteophytosis, characterised by very thin trabeculae increasing from bone to connective tissue was observed. The osteophytosis was marked on bone metaphysis and linked with lacunae. There were no observable joint spaces, and some bone islets arose in the connective tissues which were thickened and significantly enlarged. Erosion of the phalangeal bone was observed which was absent in the IFA group. Inflammation spread to the forelimbs and slightly affected the carpals. Treatment with the highest dose of both beta-amyrin (30 mg/kg, F) and polpunonic acid (30 mg/kg, I), as well as dexamethasone (3 mg/kg, C) resulted in the reduction of inflammation and the development of arthritic joints. Inflammation did not significantly spread to the affected connective tissues and bones significantly. At 3 and 10 mg/kg of beta-amyrin (D and E) and polpunonic acid (G and H), there were mild inflammation and osteophytosis compared to the CFA control group (Figure 55).

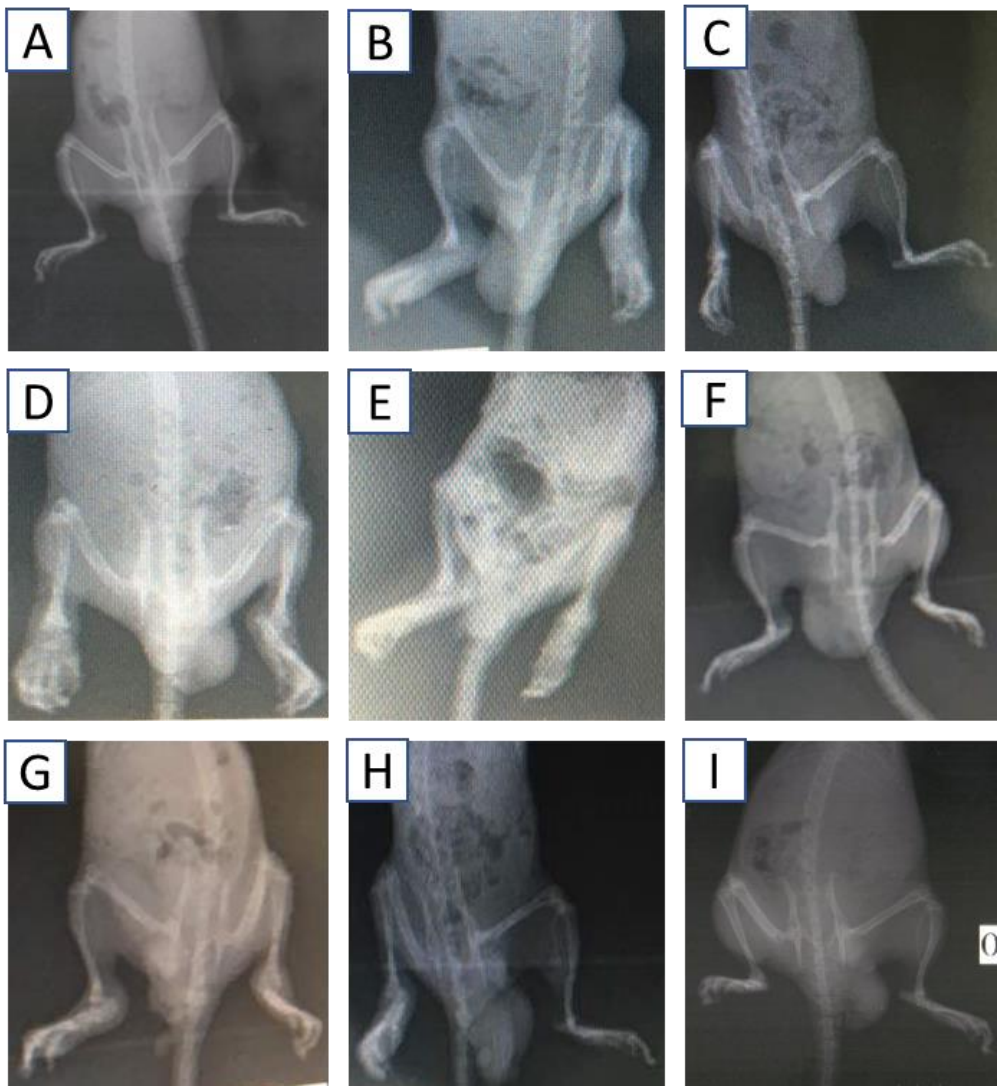

Figure 55: Radiographs showing the curative effect of beta-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) and dexamethasone (3 mg/kg) in adjuvant-induced arthritis in rats Treatment were as follows: (A) IFA/non-arthritic control, (B) CFA/arthritic control, (C) Dexamethasone, 3 mg/kg, Beta-amyrin (D) BA 3 mg/kg, (E) BA 10 mg/kg, (F) BA 30 mg/kg, and polpunonic acid (G) PA, 3 mg/kg, (H) PA, 10 mg/kg and (I) PA,`30 mg/kg.

Histology of the paws

Histopathological examination of articular cartilage of rat joints was examined (Figure 56). The severity pathological features comprising of cartilage erosion (white arrow), subchondral cyst (yellow arrow) and Weichselbaum's lacunae (red arrow) were examined in the treatment groups.

The normal control (IFA) group showed an intact articular cartilage with no sign of cartilage erosion, weichselbaum's lacunae and no sign of pannus invasion leading to the formation of subchondral cyst (A). In contrast, the negative control group which received CFA showed increased number of subchondral cysts, and weichselbaum's lacunae with erosion of articular cartilage (B). The positive control group (C) treated with dexamethasone 3 mg/kg showed no sign of pannus invasion and formation of subchondral cyst as there was an extensive erosion and loss of articular cartilage. The treatment groups showed reduction in these features. Rats treated with BA 3, 10 and 30 mg/kg, (D, E and F respectively) as well as PA 3, 10 and 30 mg/kg (G, H and I) showed high resolution of pannus invasion preventing the formation of subchondral cyst, fewer number of weichselbaum's lacunae with less articular erosion compared to the negative control (IFA) groups.

Also, the disease severity and the effect of β-amyrin and polpunonic acid on joint bone histology were illustrated by photomicrographs of sections stained with H&E (Figure 58). Histological features with evidence of bone remodeling and tissue infiltration of mononuclear inflammatory cells and inflammatory were assessed. The normal control (IFA) group showed an intact architecture of bone structure with no evidence of osteoclast activity and infiltration inflammatory cells (A). In contrast, the negative control (CFA) group showed extensive inflammation, blood vessel congestion with increased infiltration of inflammatory cells, increased bone cavitation with increased osteoclast activity (B). The results of the histological analyses indicated that inflammation as well as bone structure destruction were significantly reduced after treatment with dexamethasone (C). There was reduced osteoclast activity

with reduced bone cavitation. Treatment of rats with either of the compounds ameliorated bone destruction with a much improved tissue architecture, evidences of bone remodeling and reduced bone cavitation in each treatment group compared to the disease control group were observed (Figure 57).

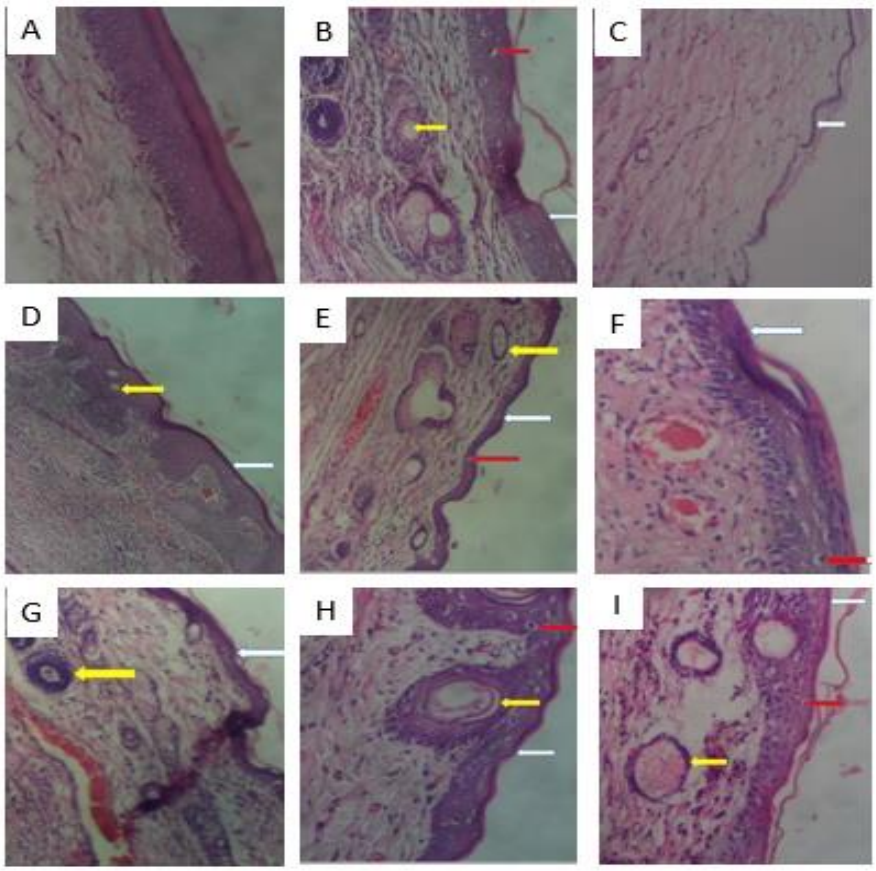

Figure 56: Histopathological examination of rat joints showing the articular cartilage stained with haematoxylin and eosin under a light microscope (× 100) in adjuvant-induced arthritis in rats. Treatment were as follows: (A) IFA/non-arthritic control, (B) CFA/arthritic control, (C) Dexamethasone, 3 mg/kg, Beta-amyrin (D) BA 3 mg/kg, (E) BA 10 mg/kg, (F) BA 30 mg/kg, and polpunonic acid (G) PA, 3 mg/kg, (H) PA, 10 mg/kg and (I) PA,`30 mg/kg.

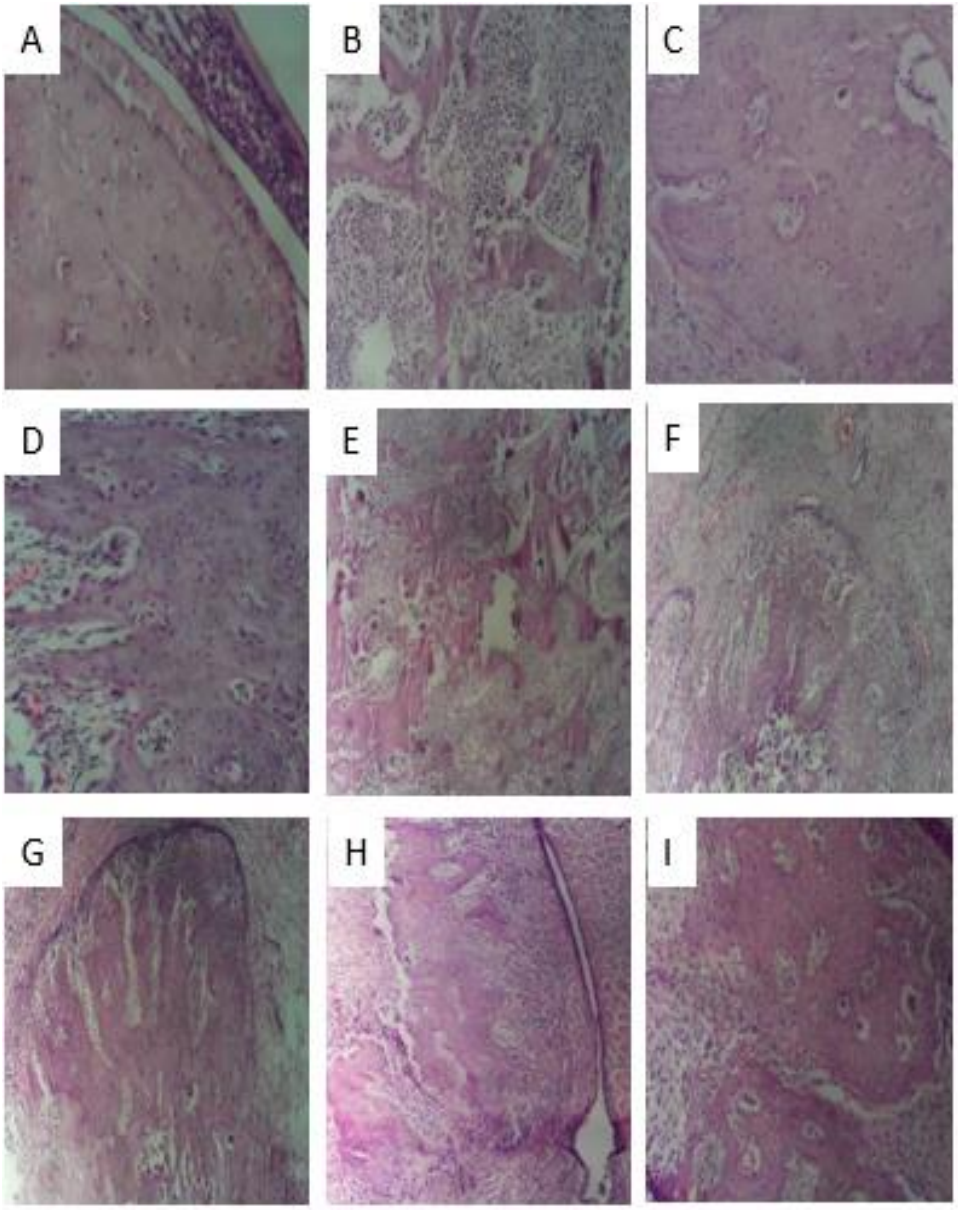

Figure 57: Histopathological examination of rat joints showing the articular bone structure stained with haematoxylin and eosin under a light microscope in adjuvant-induced arthritis in rats. Treatment were as follows: (A) IFA/non-arthritic control, (B) CFA/arthritic control, (C) Dexamethasone, 3 mg/kg, Beta-amyrin (D) BA 3 mg/kg, (E) BA 10 mg/kg, (F) BA 30 mg/kg, and polpunonic acid (G) PA, 3 mg/kg, (H) PA, 10 mg/kg and (I) PA,`30 mg/kg.

Haematological parameters

There were no significant differences between naïve control and CFA-treated (with or without drug treatment) rats with respect to almost all the haematological parameters. However, there was a significant difference

($P<0.01$) between CFA-treated group and naïve control with respect to white blood cell count. This increase in white blood cell count was significantly ($P<0.05$) reduced by treatment with dexamethasone (3 mg/kg), β-amyrin (30 mg/kg) and all doses of polpunonic acid (Figure 58). Also, there was a reduction in red blood cell count, haemaoglobin and haematocrit concentrations in the group of rats treated with only CFA when compared with the IFA treatment group, though not significantly (Figure 59).

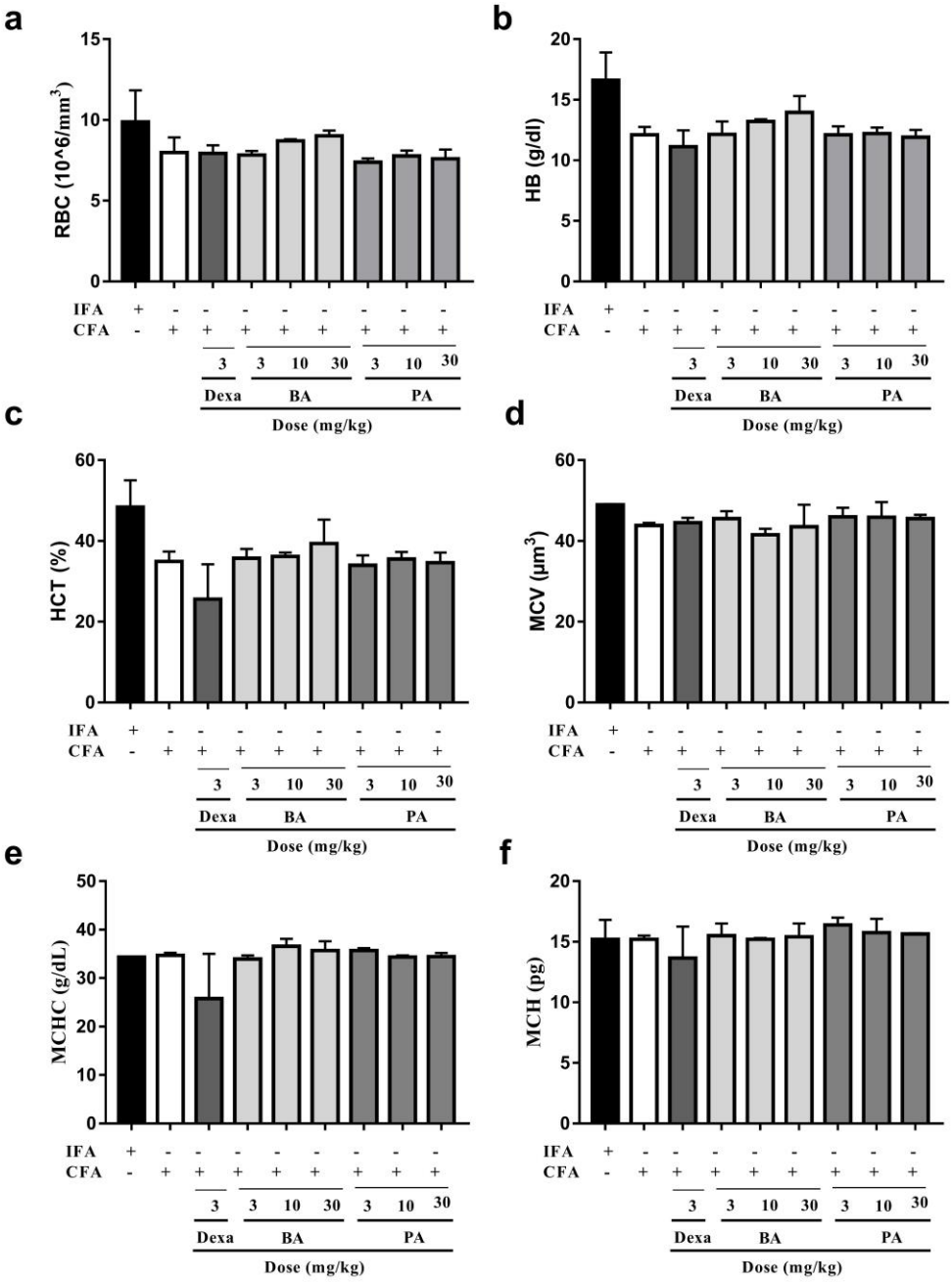

Figure 58: Effect of dexamethasone, β-amyrin (BA) and polpunonic acid (PA) administration on the haematological parameters in CFA-induced arthritis in Sprague-Dawley rats. Data presented as Mean ± SEM. (n = 5). There were no significant differences between arthritic control and treatment groups in any of the red blood cell counts and its related parameters assessed (One-way ANOVA).

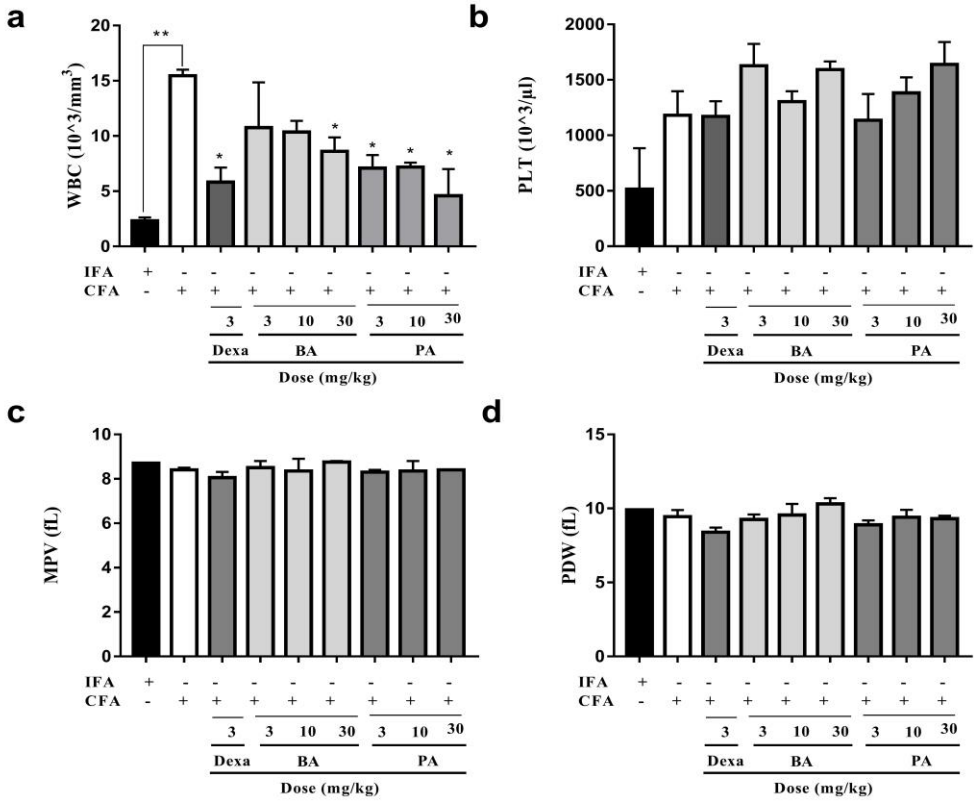

Figure 59: Effect of dexamethasone, β-amyrin (BA) and polpunonic acid (PA) administration on the haematological parameters in CFA-induced arthritis in Sprague-Dawley rats. Data presented as Mean ± SEM. (n = 5). *P<0.05 and **P<0.01, treatment groups compared to CFA-treated group (One-way ANOVA followed by Tukeys post hoc test)

Serum biochemistry

Serum biochemical parameters were not significantly affected by either the CFA alone or in combination with treatment with dexamethasone, β-amyrin or polpunonic acid. However, the levels of both total and indirect bilirubin were all elevated in the disease control groups, though not significant. Treatments with the test compounds also did not significantly affect the bilirubin levels (Figure 60).

Figure 60: Effect of dexamethasone, β-amyrin and polpunonic acid administration on the serum biochemical parameters in CFA-induced arthritis

in Sprague-Dawley rats. Data presented as Mean ± SEM. (n = 5). There were no significant differences between arthritic control and treatment groups in any of the biochemical parameters assessed (One-way ANOVA followed by Tukey's post *hoc* test).

Analgesic Effect of the Isolated Compounds

β-amyrin, polpunonic acid and morphine at all tested doses exhibited significant anti-nociceptive effects. Dose-response curves plotted revealed that morphine was the most potent followed by polpunonic acid and β-amyrin with ED_{50s} of 0.92, 3.70 and 5.51 mg/kg (Figures 61 and 62).

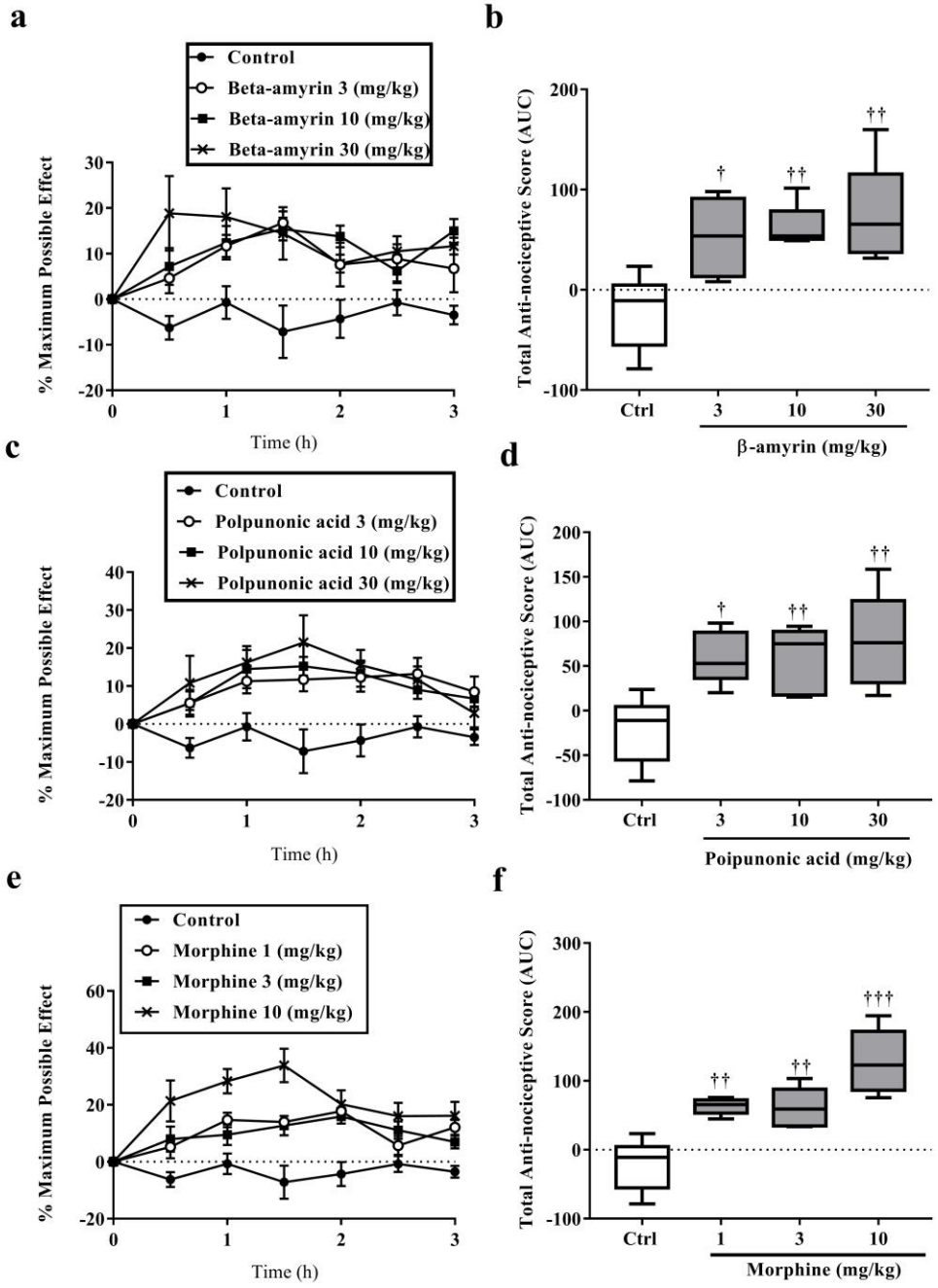

Figure 61: The percentage maximum possible effect (a, c and e) and total anti-nociceptive effect (b, d and f) of β-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) and morphine (1, 3 and 10 mg/kg, *p.o.*) presented as area under the curve (AUC) in tail-immersion test in mice. Data are presented as mean ± SEM. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the vehicle-treated group (one-way ANOVA followed by Tukey's post *hoc* test for AUC). The time course curves of the various treatments over 5 hours.

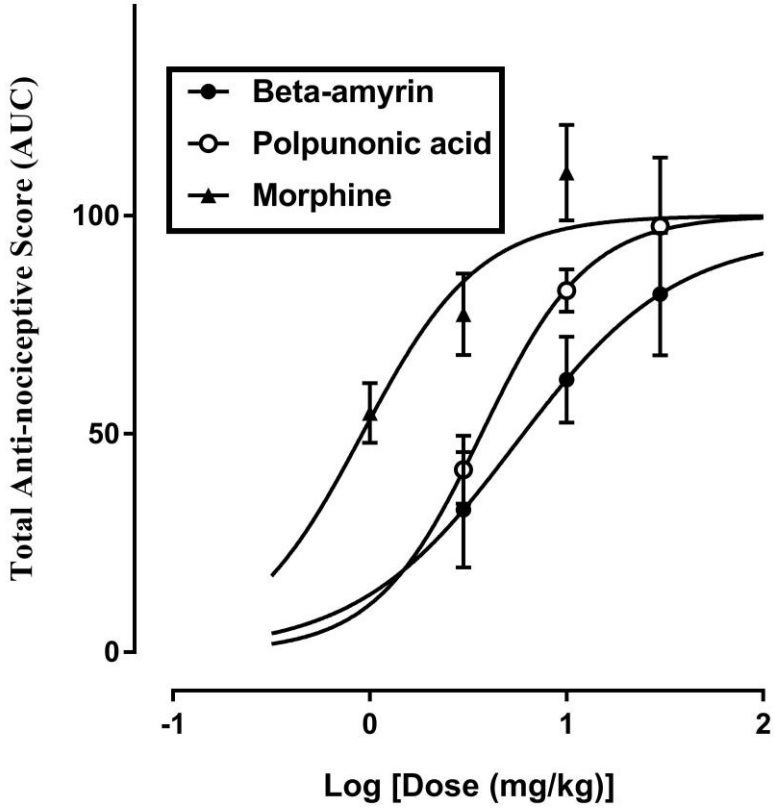

Figure 62: Dose-response curves of β-amyrin, polpunonic acid and morphine

Antidepressant Effect of Isolated Compounds

Forced swimming test

β-amyrin, polpunonic acid and fluoxetine exhibited significant antidepressant activities in the forced swimming test. Despite the fact that all doses of both compounds significantly reduced durations of immobility in the forced swim test, only polpunonic acid (30 mg/kg) could significantly ($P<0.01$) reduce frequency of immobility (Figure 63). On the durations of mobility, it was realised that rats that took either beta-amyrin, polpunonic acid or fluoxetine significantly ($P< 0.01$ for all doses) spent more time swimming than climbing (Figure 64).

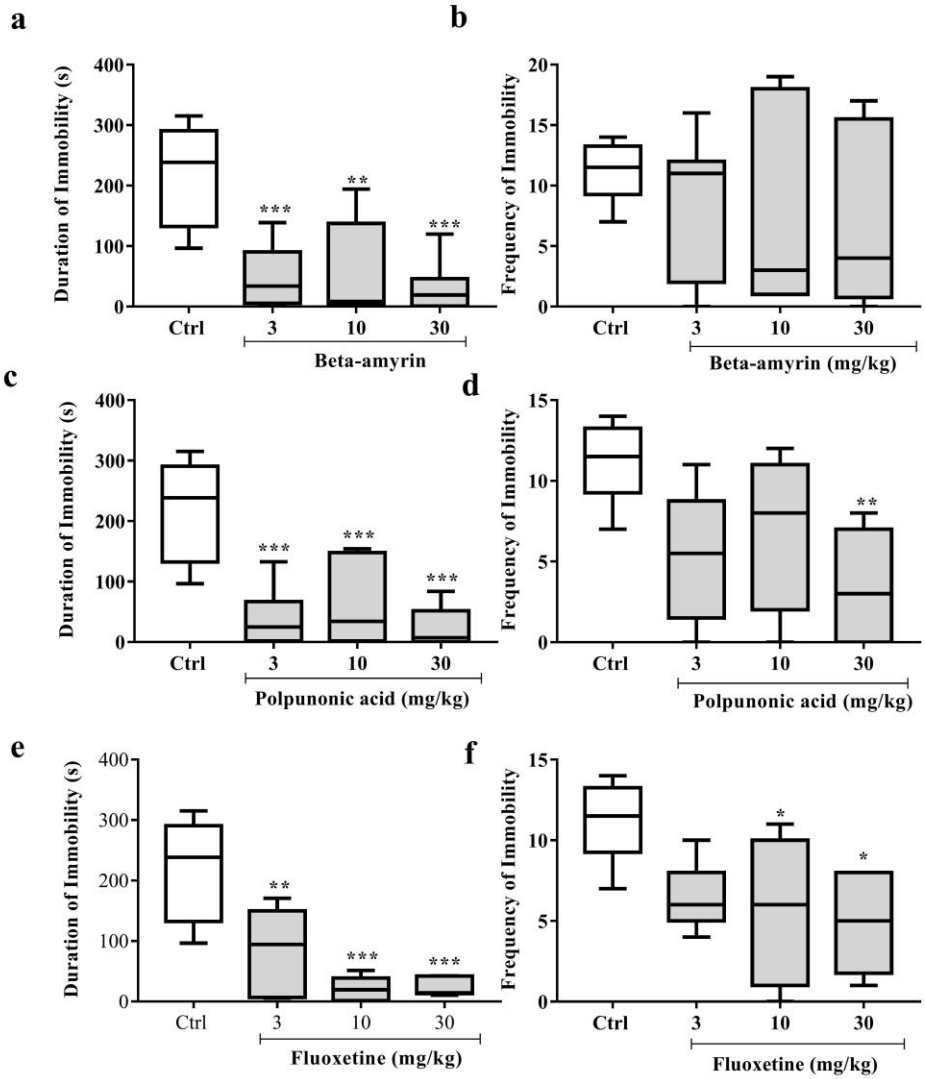

Figure 63: Effects of acute administration of β-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) and fluoxetine (3, 10 and 30 mg/kg, *p.o.*) treatment on duration (a, c and e) and frequency (b, d and f) of immobility in FST. Data are presented as group means with minimum to maximum points. Significant differences from control are denoted: *P<0.05, **P<0.01 (one –way ANOVA followed by Tukey's post hoc test).

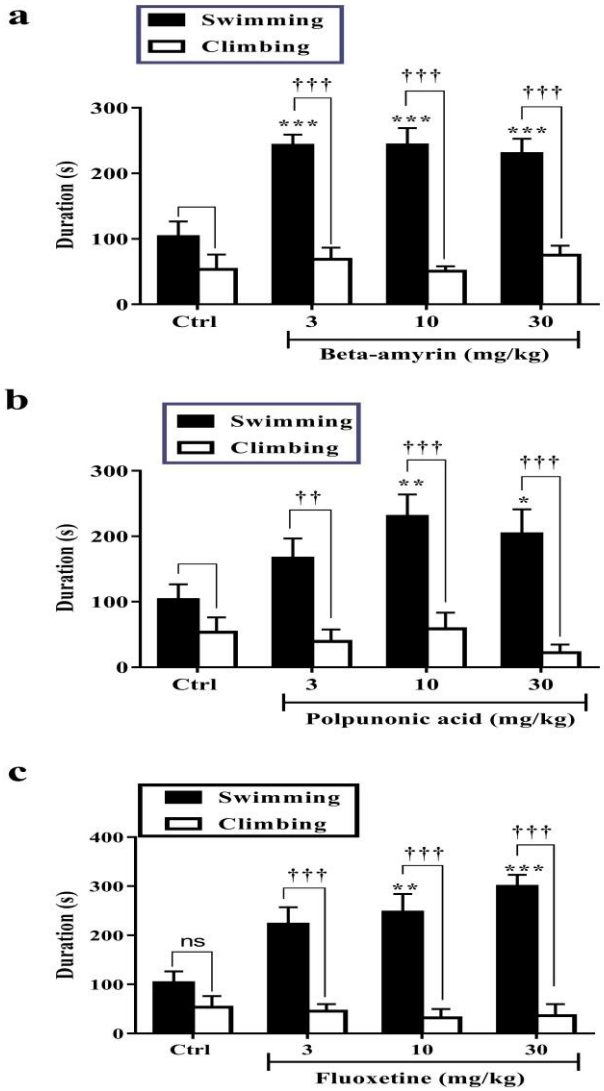

Figure 64: Effects of acute administration of beta-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) and fluoxetine (3, 10 and 30 mg/kg, *p.o.*) treatment on duration of swimming and climbing in FST. Data are presented as group means (±SEM). Significant differences from control are denoted: *P<0.05, **P<0.01 (one –way ANOVA followed by Tukey's post *hoc* test).

Tail suspension test

From the results presented in Figure 65, only the least doses (3 mg/kg) of both β-amyrin and pulpononic acid could significantly (P<0.05) reduce immobility durations. However, 10 and 30 mg/kg of fluoxetine significantly decreased immobility duration and frequency.

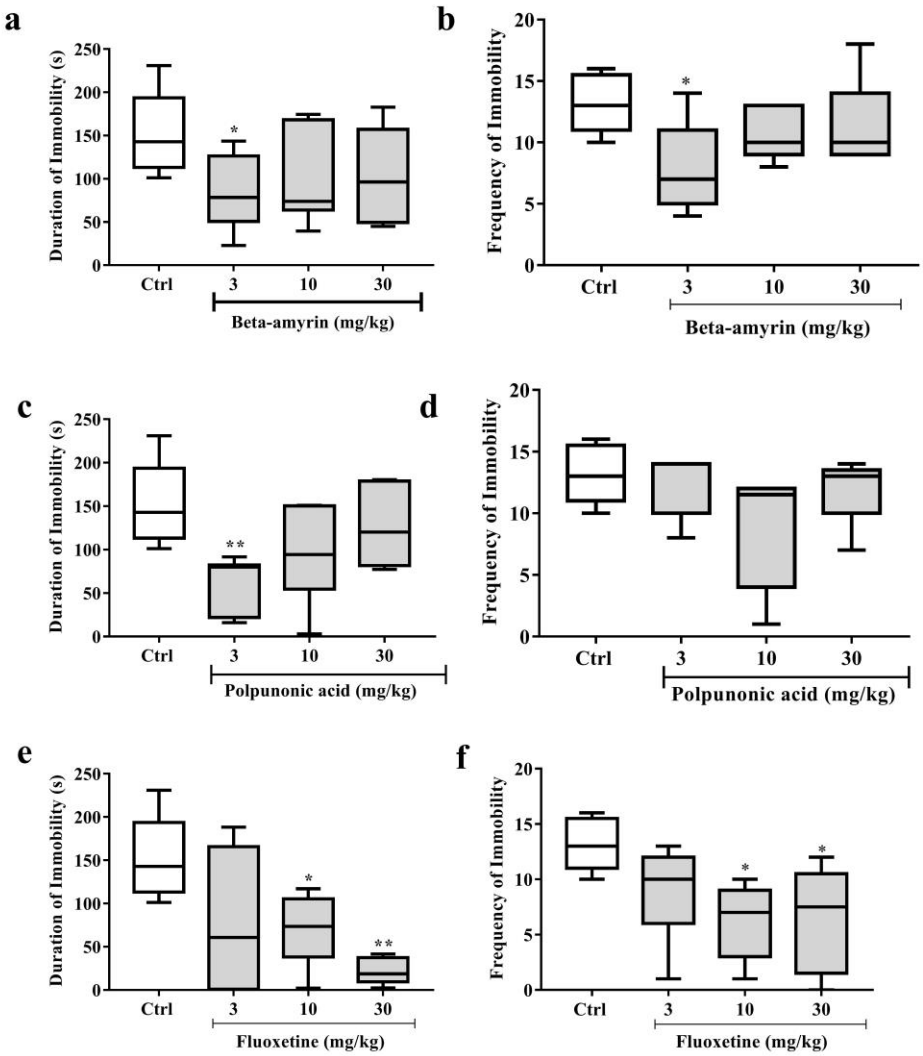

Figure 65: Effects of acute administration of β-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) and fluoxetine (3, 10 and 30 mg/kg, *p.o.*) treatment on immobility duration and frequency in TST. Data are presented as group means with minimum to maximum points. Significant differences from control are denoted: *P<0.05, **P<0.01 (one –way ANOVA followed by Tukey's post *hoc* test).

Anxiolytic Effect of the Isolated Compounds

Elevated plus maze and open field test

In the elevated plus maze, treatment mice with isolated compounds β-amyrin (30 mg/kg, *p.o.;* P<0.05) and polpunonic acid (10 and 30 mg/kg, *p.o.*; P<0.01) significantly increased the time spent in the open arms of the maze compared to the control group which received distilled water, 10 mL/kg.

Similarly, mice treated with diazepam (0.3, 1 and 3 mg/kg, *p.o.*) significantly spent more time in the open arm of the maze compared to the closed arm (Figure 66 a, b and c).

Also, in the open field test, treated mice diazepam significantly (P<0.5) spent more time in the four centre squares of the open field compared to the vehicle-treated control mice. Similarly, β-amyrin (10 and 30 mg/kg, *p.o.*; P<0.05) and polpunonic (30 mg/kg, *p.o.*; P<0.05) significantly increased the time spent in the central arena of the maze compared to the control group (Figure 66 c, d and e).

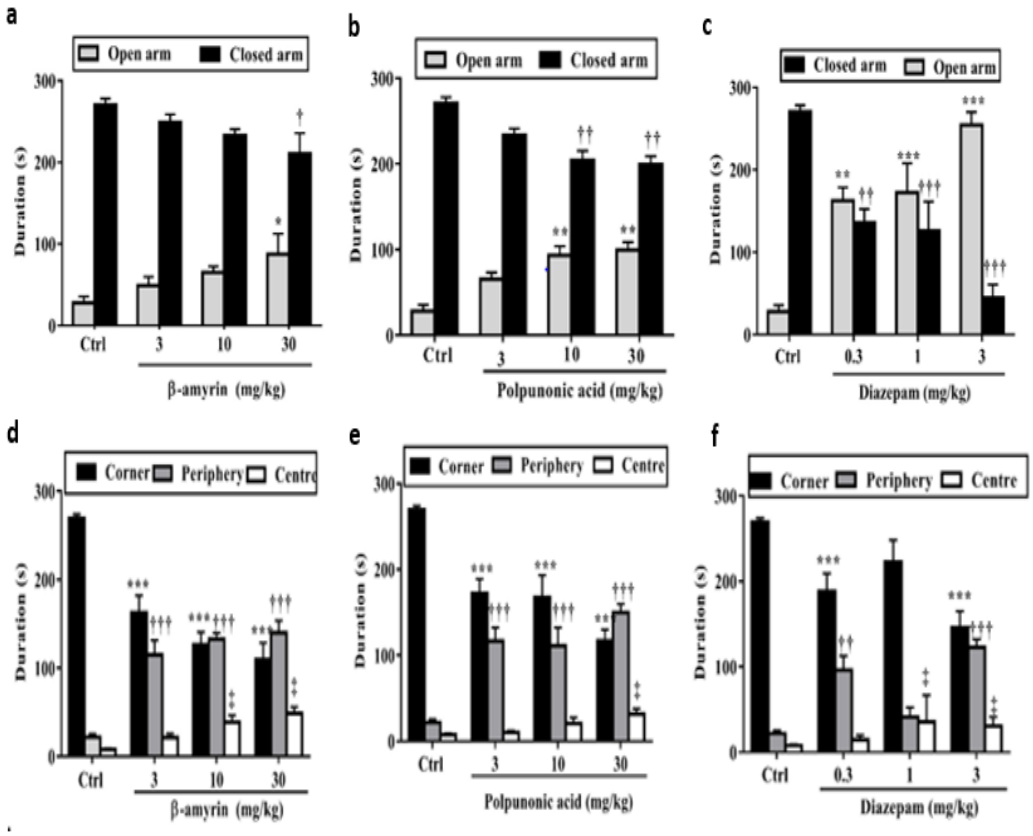

Figure 66: Effects of acute administration of β-amyrin (3, 10 and 30 mg/kg, *p.o.*), polpunonic acid (3, 10 and 30 mg/kg, *p.o.*) and diazepam (0.3, 1 and 3 mg/kg, *p.o.*) treatment on (a, band c) duration in open and closed arms in the elevated plus maze test as well as (d, e and f) duration in corner, periphery and centre of the open field test. data are presented as group means with minimum to maximum points. significant differences from control are denoted: *p<0.05, **p<0.01, ***p<0.001 (one –way anova followed by tukey's post *hoc* test compared to respective controls).

CHAPTER FIVE

DISCUSSION, SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Discussion

With many flowering plant species known to be used in folk medicine, it is expected that more new molecular entities from plants would be utilised in modern medicine. However, their widespread usage of plants in folk medicine has still not translated into the discovery of a corresponding number of orthodox drugs due to the lack of adequate scientific research (Mokgolodi, Hu, Shi, & Liu, 2011). Undoubtedly, *Ziziphus abyssinica* is one of the least researched species of over 100 species within the *Ziziphus* genus for their chemical and therapeutic properties despite their widespread usage in managing pain, inflammation and CNS disorders in traditional medicine (Orwa *et al*., 2009). This study evaluated the root bark of *Ziziphus abyssinica* for analgesic, anti-inflammatory, anxiolytic and antidepressant activities and isolated the compounds that may be responsible for its medicinal effect.

The assessment of the hydro-ethanolic root bark extract of the plant begun with phytochemical screening which revealed the presence of triterpenes, alkaloids, phytosterols, tannins and flavonoids. The results obtained were similar to those obtained in earlier studies either on the leaves, fruits and root bark of the plant *(*Boakye-Gyasi *et al.*, 2017a; Nyaberi *et al*., 2010; Ugwah *et al*., 2013). This suggests different parts of the plant possess similar phytochemicals. Also the presence of these secondary metabolites has been associated with the pharmacological effects of medicinal plants (Adedapo *et al*., 2008; Guzik, Korbut, & Adamek-Guzik, 2003; Kumar &

Pandey, 2013; Tiwari *et al*., 2011). These secondary metabolites also account for the toxic effects of plants, hence, bringing into the forefront issues relating to safety which have become major public health concerns (Neergheen-Bhujun, 2013). This underscored the need to investigate the root bark of *Ziziphis abyssinica* for any deleterious effect.

The Irwin's (1968) test, which involves a systematic observational method for assessing the effects of drugs on the behaviour and physiology of rodents was first adopted. It is a useful test in toxicological evaluation of test drugs as it helps in detecting potential adverse effects of drugs on the central nervous system (CNS) prior to clinical testing and may also be helpful in revealing novel therapeutic agents (Irwin, 1968; Porsolt, Lemaire, Durmuller, & Roux, 2002; Williams, Porsolt, & Lacroix, 2007). ZAE (300-5000 mg/kg) exhibited potential analgesic and sedative effect whereas 100 mg/kg of ZAE produced only analgesic effect. This calls for further evaluation of the plant as an analgesic agent with significant CNS modulatory property. Also, all the animals survived the various doses even after 14 days of delayed toxicity observations which suggests the LD_{50} of the plant may be higher than 5000 mg/kg. Other toxic signs such as respiratory depression, disordered gait and convulsions were also not observed in all the groups. With this, the plant could be said to be safe upon acute administration at high doses.

Despite the fact that medicinal agents could be safe on acute exposure, prolonged use of therapeutic doses could accumulate and cause deleterious effects to several organs. With this, another set of animals were subjected to a sub-chronic toxicity test to evaluate the continuous administration of relatively smaller doses over a long period. From the results obtained, there were no

significant changes in the overall body weights of the animals during the 90-day study period. Also, there were no significant changes in the relative organ weights of the testes, heart, lungs, spleen, liver and kidney. Evaluation of organ weights in toxicological studies constitute an integral element in the assessment of chemical and pharmaceutical agents as established by the Society of Toxicologic Pathology (STP) (Sellers *et al.*, 2007). It is worth noting that the consumption of toxic substances significantly cause changes in the relative weights of vital internal organs and the total body weight of animals (Owusu, Antwi-Adjei, & Henneh, 2018). Hydro-ethanolic root bark extracts of *Ziziphus abyssinica* could be said to be safe as it did not cause any significant alterations in either the body weight or relative organ weights of the animals. This, however, does not rule out possible toxicity as the STP also suggested that relative organ weights should always be interpreted alongside histologic studies (Sellers *et al*., 2007).

Histologic evaluation of the heart, kidney, testes, spleen and liver of the rats following a 90-day treatment with ZAE showed that the extract did not exhibit any serious toxic effect. Thus, the effect was subtle, not grave enough to evoke severe inflammatory or deleterious effect on the parenchyma of the various organs. In the spleen, reduced cellularity of leucocytes within the white pulp in the treated groups was presented as reduced eosinophilia. This agrees with haematological assessment which resulted in a reduction (though not statistically significant) in the levels of circulating lymphocytes and the predominant white blood cells, neutrophils, in the treated groups as compared to the control group. In contrast, beneficial effect of the extract on the testis was observed which happens to be the induction of high spermatogenesis in

the seminiferous tubules as compared to the control groups. This can be seen in the histological sections of the testes of the experimental groups which also corroborates the sperm count of the various treatment groups.

On the other hand, the haematological results obtained from the study suggested the various doses of the extract did not significantly alter the levels of both red blood cells and white blood cells compared to the control group. This showed the extract may not pose any haematological risk when consumed at those doses for three months. Haematological studies are useful indicators in the diagnosis of many diseases as well as investigating the extent of damage to the blood and other internal organs. This is because alterations in blood parameters are essential indicators which reflect the state or condition of subjects exposed to toxic substances (Etim, Williams, Akpabio, & Offiong, 2014). The principal roles of the white blood cells (WBC) and its differentials are to combat infections, guard the body against assault by foreign organisms through phagocytosis and to produce, transport or distribute antibodies in immune response. As such, abnormally low WBC count correlates with prolonged exposure to infection and/or suppression of the immune system. On the other hand, abnormally high counts of WBCs corresponds to an increased capability of generating antibodies in the process of phagocytosis, hence an increased resistance to infections. It also suggests the exposure to foreign agents is recent (Etim *et al*., 2014; Soetan, Akinrinde, & Ajibade, 2013).

Another important parameter in the assessment of sub-chronic toxicity is hepatic function. This has been monitored via the evaluation of the serum activities of alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) in conjunction with levels of other biochemical

analytes such as cholesterol, creatine kinase and creatinine (Oloyede, Okpuzor, Omidiji, & Odeigah, 2011). Aspartate aminotransferase (AST) is involved in the transfer of an amino group between aspartate and α-keto acids. Though elevated serum levels are non-specific indicators because of their wider distribution in cardiac tissues, liver and skeletal muscles, with minute amounts found in the kidney, pancreas, and erythrocytes, such elevated levels may be attributed to drug-induced hepatocellular damage causing both cytosolic and mitochondrial seeping of AST into the plasma (Gaw, Murphy, Srivastava, Cowan, & O'Reilly, 2013; Giannini, Testa, & Savarino, 2005). ALT, on the other hand, catalyses explicitly the transfer of an amino acid group from alkaline alanine to α-ketoglutarate with the formation of glutamate and pyruvate. This enzyme is also distributed in many tissues, but higher levels are present in the liver. Elevated serum levels of ALT are found in hepatocellular disorders than in intrahepatic or extrahepatic cholestasis disorders. ALT levels are mostly higher than AST levels in acute inflammation of the liver (Bishop, Fody, & Schoeff, 2013). Alkaline phosphatase, on the other hand, belongs to a group of enzymes that catalyse the hydrolysis of various phosphor-monoesters at an alkaline pH. ALP is significantly distributed in the intestines, liver, bone, spleen, placenta, and kidney. Elevated serum activities an indication of a hepatobiliary disorder caused by either intrahepatic or extrahepatic cholestasis (Bishop *et al*., 2013). The results obtained indicate there were statistically insignificant ($P>0.05$) differences in the in the activities of the hepatic enzymes (AST, ALT and ALP) between the ZAE-treated and control rats and this may suggest that the plant extract may not be exhibiting notable hepatotoxicity at the doses and

duration administered. This is also confirmed by the histological report on the liver which showed normal hepatocytes and sinusoids with only the group that received ZAE (100 mg/kg) showing moderate congestion in the central veins.

Aside the liver, the kidney is another important organ in the body. Renal function parameters such as creatinine, urea and blood urea nitrogen /creatinine ratio give an indication of the integrity of the kidney (Gaw *et al.*, 2013). Creatinine is nearly an ideal substance for measurement of clearance. It is an exogenous metabolic product synthesised at a constant rate for a given organism and cleared essentially only by glomerular filtration. It is not reabsorbed but slightly secreted by the proximal tubule. Urea is the principal nitrogenous waste product of amino acid metabolism. They are highly toxic, and hence they are freely filtered at the glomerulus, but the tubules reabsorb approximately 40%. Blood urea nitrogen (BUN) is the measure of serum urea and nitrogen levels. However, elevated levels of these parameters do not necessarily indicate an increased volume of distribution from metabolism but a reduced clearance rate due to reduced renal function. Also, dehydration causes an elevation in serum creatinine and urea levels (Bishop *et al.*, 2013). From the results obtained from the study, no significant alterations in the kidney function parameters were observed suggesting the plant extract may not have any deleterious effect on the integrity and function of the kidney when administered at those doses for that duration. This is also confirmed by the histopathological data on the kidneys of the rats.

Since the plant extract was found to be safe in acute and sub-chronic toxicity studies, it was expedient to progress to test for its efficacy in the management of pain, inflammation and CNS disorders as informed by its

folklore usage. Importantly, the extract tested positive for analgesia in the Irwin's test which also authenticates to some extent the folklore usage of the plant in the treatment of pain. It is therefore necessary to further investigate the analgesic properties of the extract and the possible mechanism(s) through which it exerts this analgesic effect.

The tail immersion test was first employed to assess the analgesic effect of the extract. The test utilises thermal stimuli and the ability to increase the reaction time of rodents in this model is primarily considered as an essential index for evaluating the central and peripheral analgesic activity of medicinal agents depending on the temperature (Abdelhalim *et al.*, 2015). ZAE significantly increased the reaction time of mice in withdrawing their tail from the hot water (55°C). This property of medicinal agents have been correlated with central anti-nociceptive mechanisms which are associated with the activation of opioidergic receptors (Hasan *et al.*, 2018). Specifically, thermal stimuli affect the μ_2/δ -opioid receptors as well as μ_1/μ_2-opioid receptors which mediate the spinal and supraspinal reflex mechanisms respectively (Moniruzzaman & Imam, 2014; Turner, 2013).

Since pain is quite complex and involves several pathways, the tail immersion test alone is not enough to completely assess the analgesic property of medicinal agents. The acetic-acid induced writhing test was therefore employed to further evaluate the analgesic effect of ZAE. This model is known to implicate both visceral and inflammatory pain (Agarwal & Kansal, 2018). Intraperitoneal injection of acetic-acid activates pain sensation through a localised inflammatory response leading to the release of free arachidonic acid from tissue phospholipids and the subsequent production of

prostaglandins (PGE_2 and PGF_2) in peritoneal fluids via the activities of cyclooxygenase (Uddin *et al*., 2018). Other related studies have linked IL-1β, IL-8 and tumour necrosis factor–alpha (TNF-α) from mast cells and resident macrophages within the peritoneum as contributing to the nociception caused by acetic acid (Ribeiro *et al.*, 2000). ZAE significantly decreased the writhing response induced by intraperitoneal injection of acetic acid, and this is suggestive of its analgesic effect possibly through the inhibition or down-regulation of inflammatory mediators and cytokines such as IL-1β, IL-8 and tumour necrosis factor–alpha (TNF-α) from mast cells and resident macrophages within the peritoneum.

Though the acetic-acid induced writhing test is simple, sensitive and particularly suitable for detecting even weaker analgesics, it is not a selective pain test as it gives false positive results for some non-analgesics such as muscle relaxants and sedatives (Le Bars, Gozariu, & Cadden, 2001). Therefore, to further confirm the analgesic action of ZAE, the formalin test was used. This test is beneficial for the assessment of novel analgesic drugs due to its highly predictive nature and its ability to mimic both acute and chronic pain. It also encompasses neurogenic, inflammatory and central mechanisms of nociception (Le Bars *et al.*, 2001; Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). The biphasic response of the formalin test involves neurogenic (0-10 min) and inflammatory (10-60 min) phases with opioidergic agonists known to inhibit both phases (Le Bars *et al*., 2001). In this study, ZAE inhibited both phases of the test similar to morphine which was used as the reference analgesic drug. The neurogenic (early) phase is as a result of direct stimulation of nerve endings leading to the peripheral release of

substance P and bradykinin. The late phase, also termed as inflammatory phase, is mediated by peripheral effect through the release of some inflammatory mediators such as histamine, serotonin, bradykinin and prostaglandins which to some extent can also cause sensitisation of central nociceptive neurons (Uddin *et al*., 2018). The inhibitory effect of ZAE in both phases indicates that the extract had central as well as peripheral analgesic properties. It also confirms the central analgesic properties observed in the tail suspension test. Furthermore, the inhibition of the inflammatory phase by the extract suggests the plant may possess anti-inflammatory properties which needs to be further investigated.

Though the extract exhibited potent analgesic properties in several acute models of pain, it was still necessary to assess its effect in acute and chronic pain models of musculoskeletal pain which underlines several pathological conditions (Nielsen & Henriksson, 2007). It is worth-noting that some medicinal plant extracts and isolates have been shown to attenuate both acute and chronic musculoskeletal pain (Ameyaw *et al*., 2014). Oral administration of ZAE (30, 100 and 300 mg/kg) exhibited analgesic properties by ameliorating acute and chronic muscle pain induced by injecting 3% carrageenan into the gastrocnemius muscles rats. Acute and chronic pain were assessed using the Randall Sellito test after 12 hours and 10 days respectively in separate tests. The type of musculoskeletal pain induced in this study is known to be closely related to fibromyalgia which is a generalized type of muscle pain experienced in humans (Skyba, Radhakrishnan, & Sluka, 2005). The acute muscle hyperalgesia is largely mediated by neutrophils which is accompanied by the local release of noxious chemicals such as glutamate,

prostaglandins, histamine and serotonin (Radhakrishnan *et al*., 2003). In this test also, secondary hyperalgesia has been shown to occur as a result of central sensitisation emanating from the activation of dorsal horn neurons, together with the increased sensitivity of the peripheral nociceptors (Sluka & Westlund, 1993). Both acute and chronic hyperalgesia resulting from the musculoskeletal pain were ameliorated significantly by the extract. The inhibitory effect of ZAE on neutrophil degranulation as well as prostaglandins as reported in this study could be possible mechanisms behind its analgesic/anti-inflammatory effect and relief of musculoskeletal pain.

Further investigations into the mode of analgesic activity using the formalin test revealed that the anti-nociceptive effect of ZAE was mitigated by naloxone, an opioid antagonist, in both phases of the formalin test. Previous studies have implicated the opioidergic pathway in the analgesic effect of the leaf extract of *Ziziphus abyssinica* (Boakye-Gyasi *et al*., 2017b). It is not surprising that the root bark extract also exerts similar effect suggesting the analgesic effect of the plant is partly mediated through the activation of opioidergic receptors or up-regulation of endogenous opioidergic agonists. Binding studies are therefore recommended to confirm this effect.

Also, it could be deduced from the results that the extract possibly mediated its analgesic activity partly through the nitric oxide-cyclic GMP pathway. This is because the anti-nociceptive activity of ZAE was reversed upon pretreatment of rats with L-arginine methyl ester, a nitric oxide synthase inhibitor. Nitric oxide, synthesised from L-arginine by nitric oxide synthase (NOS) enzyme, is known to play a complex and diverse role in the transmission of nociceptive signals peripherally and centrally (Cury, Picolo,

Gutierrez, & Ferreira, 2011; Galdino, Duarte, & Perez, 2015). The extract, therefore, may be exhibiting its analgesic activity by activating or increasing the release of nitric oxide synthase, an enzyme required for the production of nitric oxide. Increased production of nitric oxide then inhibits nociception in the peripheral as well as the central nervous system.

The results from the study also showed that ZAE possibly mediates its activity through the activation of the ATP sensitive potassium channels. It is worth noting that a series of investigations have highlighted a marked implication of the K^+ channels in nociceptive processing, particularly, in determining peripheral hyperexcitability. Pharmacologically active compounds such as diazoxide which act by opening potassium channels are known to exhibit anti-nociceptive effects (Alves *et al*., 2004). ATP sensitive potassium channel opening has been linked to the anti-nociception produced by systemic treatment with morphine, NSAIDs and even gabapentin (Alves *et al*., 2004; Tsantoulas & McMahon, 2014). ZAE therefore acts possibly through the activation of ATP sensitive potassium channels, thus a reversal of its anti-nociceptive effect upon pretreatment with glibenclamide.

The study has demonstrated that the possible involvement of the opioidergic pathway in the analgesic effect of ZAE may be linked to its interaction with ATP-sensitive potassium channels and nitric oxide (NO)-cyclic guanosine monophosphate pathways. This agrees with an earlier study which implicates the same pathways in the anti-nociceptive effect of tramadol, another important opioidergic agonist (Isiordia-Espinoza, Pozos-Guillén, Pérez-Urizar, & Chavarría-Bolaños, 2014). Also, nitric oxide is known to control ATP-sensitive potassium channels by increasing intracellular cGMP,

which regulates various physiological processes including anti-nociception (Romero & Duarte, 2009).

Additionally, ZAE reversed hyperalgesia induced by the intraplantar injection of TNF-α, IL-1β, prostaglandin E_2 and bradykinin in rats. This pathway follows an earlier assertion that intraplantar injection of TNF- α irritant triggers IL-1β production, thus inducing the production of cyclooxygenase products such as prostaglandin E_2 which subsequently causes hyperalgesia through sensitisation of nociceptors by bradykinin and serotonin (Verri Jr *et al*., 2007). This was supported by the ability of ZAE to inhibit hyperalgesia induced by TNF-α and the other downstream mediators, IL-1β, prostaglandin E_2 and bradykinin. The analgesic effect of the extract in both acetic-acid – induced writhing and formalin-induced nociception in mice as reported in this study could be attributed to its inhibitory effect on these inflammatory mediators and cytokines.

The inhibitory effect of the extract on inflammatory pain induced by formalin coupled with its ameliorative effect against hyperalgesia induced by inflammatory mediators and cytokines suggests that anti-inflammatory mechanisms may underpin the analgesic activity of ZA. This necessitates further research on the anti-inflammatory effect of the root extract of *Ziziphus abyssinica* and the possible involvement of membrane stabilisation, inhibition of protein denaturation and neutrophil degranulation, pro-inflammatory cytokines and mediators in its mode of action.

Considering the fact that research involving animal studies are fraught with ethical challenges particularly when there are available and appropriate *in vitro* models (Chandra, Chatterjee, Dey, & Bhattacharya, 2012), it was

advantageous for the anti-inflammatory activity of ZAE to be first assessed using *in vitro* models. As such, the human red blood cell membrane stabilisation models were employed due to the fact that the erythrocyte membrane is analogous to the lysosomal membrane hence the stability of the erythrocyte membrane could be extrapolated to the stabilisation of lysosomal membrane (Omale & Okafor, 2008). The haemolytic effect of hypotonic solution is related to the excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. This form of injury causes secondary damage through free radical-induced lipid peroxidation (Augusto, Kunze, & de Montellano, 1982; Ferrali, Signorini, Ciccoli, & Comporti, 1992). NSAIDs are known to exert their anti-inflammatory activities partly by stabilising lysosomal membrane to prevent the release of enzymes into the extracellular matrix (H. Rahman, Eswaraiah, & Dutta, 2015). In the hypotonic solution and heat-induced haemolysis test, ZAE and the standard drug showed dose-dependent stabilisation of red blood cells.

Another well-recognised cause of arthritis and inflammatory diseases is the denaturation of tissue proteins. Production of auto-antigens in certain arthritic diseases may be due to denaturation of proteins (Chandra *et al*., 2012; Opie, 1962). The antigenic property of the denatured proteins leads to diseases such as rheumatoid arthritis, glomerulonephritis, serum sickness and systemic lupus erythematosus (Duganath, Kumar, Kumanan, & Jayaveera, 2010). NSAIDs, in addition to their inhibitory effect on prostaglandin synthesis by blocking the cyclooxygenase pathway, also have the ability to prevent protein denaturation which contributes to their anti-inflammatory effects (Krishnaraju, Rao, Rao, Reddy, & Trimurtulu, 2009). This gives an indication that plant

extracts which have an inhibitory effect on protein denaturation may possess anti-inflammatory effects. In this present study, ZAE exhibited anti-denaturation property in both egg albumin and BSA-induced denaturation assays, and this is an indication of anti-inflammatory and anti-arthritic property.

Though *in vitro* studies are good predictors of anti-inflammatory properties of medicinal agents, it has been argued that cells in the body do not live in isolation and that other factors *in vivo* could affect the responses obtained. In order to be sure that the observed *in vitro* anti-inflammatory activity of ZAE is applicable *in vivo*, the root bark extract of the plant was further assessed using the carrageenan-induced rat paw oedema test in rats. It is a suitable and most widely used model for evaluating the anti-inflammatory effects of plant extracts and their possible underpinning mechanisms (Okpuzor & Oloyede, 2009). Intraplantar injection of carrageenan induces a biphasic inflammation in which the first phase occurs mostly one-hour post-carrageenan injection, and it is characterised by symptoms such as oedema, erythema and pain. The induction results in a subsequent release of pro-inflammatory mediators including histamine, serotonin, tachykinins, bradykinin, reactive oxygen species (ROS) and complement proteins (Morris, 2003). Prostaglandins are known to mediate the late phase of oedema via the action of cyclooxygenase-2 (COX-2) together with inducible nitric oxide synthase (iNOS) (Posadas et al., 2004). During the late phase also, IL-6, IL-1β, TNF-α, and MCP-1 levels are also enhanced (Fulgenzi *et al*., 2005). Oral administration of the *Ziziphus abyssinica* extract suppressed the oedematous response one hour after carrageenan injection, and this effect was sustained

throughout the entire 5 h duration of the experiment in a dose-related manner. This suggests a possible inhibitory effect of the extract on those inflammatory mediators.

With the carrageenan-induced model being general and basic anti-inflammatory screening model, the anti-inflammatory potential of ZAE was further investigated using formalin - induced paw oedema model, which is one of the most common methods for screening of agents with anti-arthritic properties (Choudhary *et al*., 2014; Nair, Singh, & Gupta, 2011). In the present study, ZAE at 100 and 300 mg/kg markedly decreased the paw oedema similar to diclofenac 10 mg/kg. The observed activity of ZAE may be due to alterations in the inflammatory response comparable with the mechanism of the standard drug diclofenac which exerts anti-arthritic potential through the inhibition of inducible COX-2.

Additionally, the carrageenan-induced peritonitis study was adopted to investigate the inflammatory cells recruited and the effect of the extract on these cells. It was observed that the anti-inflammatory property of ZAE was mediated through the inhibition of neutrophils recruitment to the site of inflammation. This is because carrageenan is known to induce neutrophil migration into the peritoneal cavity through an indirect mechanism that involves the activation of macrophages and the release of cytokines into the peritoneal cavity (de Brito *et al.*, 2013). The plant extract may therefore have inhibited carrageenan activation of macrophages leading to down-regulation of IL-1β and subsequent inhibition of neutrophil recruitment.

Since pro-inflammatory mediators and cytokines have been implicated in the inflammatory mechanism of carrageenan and formalin (de Brito *et al*.,

2013), the protective effect of the extract against intraplantar injection of murine recombinant pro-inflammatory cytokines, tumour necrosis factor-α and interleukin-1β, was assessed as described previously (Boakye-Gyasi *et al.*, 2017b; Vale *et al.*, 2004). Results obtained from the study show that treatment with ZAE and diclofenac significantly reduced paw oedema induce by these inflammatory insults in the rats. This was confirmed by the histopathological report where rats treated with ZAE or diclofenac showed a decreased leukocyte infiltration with mild dilation and congestion of vessels compared to the untreated negative control group.

From the study also, intraplantar injection of prostaglandin E_2 (PGE_2) resulted in paw oedema in the animals which was sustained throughout the study in the negative control group. This was significantly ($P<0.05$) reversed by ZAE (100 and 300 mg/kg) but not diclofenac. It is possible that mechanisms other than just inhibition of cyclo-oxygenase activity accounted for this anti-inflammatory effect of the extract. This possibly may account for the ineffectiveness of diclofenac in ameliorating the anti-inflammatory effect of PGE_2 - one of the most usual lipid mediators derived from arachidonic acid metabolism by cyclooxygenase (COX). As the rate-limiting enzyme, COX is known to produce its various activities comprising of inflammation, pyrexia and pain sensation by acting on four kinds of receptor subtypes (EP_1-EP_4) (Kawahara, Hohjoh, Inazumi, Tsuchiya, & Sugimoto, 2015). Other studies have reported that PGE_2 initiates mast cell activation via an EP3–Gi/o–Ca^{2+} influx/PI3K pathway, and this mechanism underlies PGE_2-induced vascular permeability and consequent oedema formation (Morimoto *et al.*, 2014). Even though diclofenac could not significantly reduce PGE_2-induced paw oedema,

the histopathology report suggested otherwise as a reduced cellular infiltration and inflammation was observed in the paw tissues. This could be explained by the diclofenac's ability to prevent further breakdown of arachidonic acid to prostaglandins as a result of its inhibitory effect on cyclooxygenase activities, despite its minimal activity on the already injected prostaglandins.

Again, intraplantar injection of bradykinin irritant has been linked to its role in the release of inflammatory mediators leading to dilation of arterioles and venules and increased vascular permeability (Yoke *et al.*, 2011). The extract and diclofenac significantly reduced this paw inflammation. This effect was also seen in the photomicrographs of tissues from the paw where treatments with both drugs resulted in reduced inflammatory cells infiltrations. The inhibitory effect of ZAE on the above pro-inflammatory mediators (prostaglandin E_2 and bradykinin) and cytokines (TNF-α and IL-Iβ) could be the mechanisms underlying its anti-inflammatory effect in the carrageenan and formalin-induced paw oedema, as well as, carrageenan-induced peritonitis as reported in this study.

With chronic pain and inflammation known to be causally linked to a depressive-like behaviour (Hamann *et al.*, 2016), it was expedient to explore the possible antidepressant-like effect of the hydro-ethanolic root bark extract of *Ziziphus abyssinica*. It has been shown from previous studies that pro-inflammatory cytokines such as interleukin 1 (IL-1) and TNF-α affect synaptic plasticity leading to the establishment of depression-like behaviours and mood disorders (Khairova *et al*., 2009). Interestingly, relief of depressive symptoms is reported in patients with concurrent depression and inflammatory disease following administration of anti-cytokines (Lotrich, 2015). Chronic pain, on

the other hand, has been reported in clinical studies to be a major cause of depression with up to 85% of patients with chronic pain also affected by major depression (Sheng, Liu, Wang, Cui, & Zhang, 2017; L. Williams *et al.*, 2003). This underscores the deep relationship between inflammatory conditions, pain and depression.

To assess the antidepressant effect of the extract as well as the isolated compounds, the tail suspension and the forced swimming tests were therefore employed: both being despair-based rodent models widely used for screening antidepressant drugs in mice. The two models are sensitive to all classes of antidepressant drugs including monoamine oxidase inhibitor, tricyclic antidepressants and selective serotonin reuptake inhibitors (Cryan, Athina Markou, & Irwin, 2002). The immobility displayed by rodents when subjected to aversive stimuli such as being suspended by the tail or forced to swim in a confined cylinder is thought to reflect a state of despair or lowered mood – all being signs of depression (Fekadu, Shibeshi, & Engidawork, 2016). From the study, the duration and frequency of immobility were reduced by the acute treatment with ZAE, and a significant correlation of their potential antidepressant effect can be drawn from this. This effect was also seen in mice that received the reference drug, fluoxetine, indicating the experimental procedure is valid enough to reveal the possible antidepressant effect of the ZAE. Also, it was observed that the ZAE treated mice exhibited more swimming than climbing behaviour. This is suggestive of an involvement of serotonergic pathways in the possible mechanism of action of ZAE. This is because it has been recognised that treatment with drugs that increase serotonin neurotransmission boost swimming behaviour whereas

norepinephrine-targeting antidepressants selectively enhance climbing in the FST (Bogdanova, Kanekar, D'Anci & Renshaw, 2013). Again, it was observed that ZAE-treated mice exhibited more immobility times in the FST than the TST models. This could be explained by the fact that TST is more susceptible to the sedative effects of 5-HT_{1A} agonist hence produces little reduction in immobility as compared to the forced swim test (Castagné, Porsolt, & Moser, 2009).

To test for the anxiolytic effect of the extract, the elevated plus maze model was adopted. Important advantages of this test procedure are that it is simple, less time consuming, highly predictable and reliable and for the fact no prior training or noxious stimuli (sound or light) is required. Compounds with anxiolytic effects result in an increased time in the open arm (Latha, Rammohan, Sunanda, Maheswari, & Mohan, 2015; Pellow, Chopin, File, & Briley, 1985). In the test, ZAE (100 and 300 mg/kg) and diazepam (0.3-3 mg/kg) significantly increased time spent in the open arm whiles decreasing time spent in the closed arm compared to control mice. This was an indication that the extract from *Ziziphus abyssinica* possessed anxiolytic effect.

This anxiolytic effect subsequently confirmed in the open field test. In this test, outcomes such as defecation, duration in the center time, and activity within the first five minutes are used to determine the emotional state of rodents including anxiety (Gould, Dao, & Kovacsics, 2009). Exposure of the rodents to a relatively large, well-illuminated and novel arena compared to their breeding or natural environment induces agoraphobia and the ability of test compounds to increase the time spent in the centre but not the corners has been ascribed as anxiolytic effect. In the test, ZAE and diazepam significantly

decreased the time spent in the corners whiles increasing time spent in the central arena of the open field. This confirms the anxiolytic effect of the extract from the root bark of *Ziziphus abyssinica*.

With the extract exhibiting pronounced anti-inflammatory properties and the preliminary studies on the isolated bioactive compounds showing pronounced anti-inflammatory property in carrageenan-induced paw oedema test, it was expedient to test the compounds in a chronic disease model of inflammation. The Complete Freund's Adjuvant (CFA) - induced arthritis in rats was chosen since it is a well-established animal model that mimics the human rheumatoid arthritis, comprising of joint swelling, loss of joint function, cartilage degradation and pain (Shen *et al*., 2017). One of the essential features of the model is chronic synovitis, which includes the infiltration of inflammatory cells and synovial hyperplasia (Ashraf, Mapp, & Walsh, 2010). As such, CFA-induced arthritis in rats has been employed frequently to assess the possible medicinal effects of test agents in the treatment of rheumatoid, a chronic inflammatory condition (Asquith *et al*., 2009).

The model is known to encompass both primary and secondary arthritis phases. In the primary phase, there is pronounced swelling of the ipsilateral paw, mediated mainly by prostaglandins and its related inflammatory mediators (Choudhary et al., 2014). This is followed by inflammation in the contralateral and front paws (secondary chronic arthritis) in which autoantibodies are generated to mediate the process of inflammation (Patil, Kandhare, & Bhise, 2012). It has been shown that various pro-inflammatory mediators such as tumour necrosis factor (TNF-α), interleukin-1

beta (IL-1β), platelet derived growth factor (PDGF) reactive oxygen species (ROS) and activated CD^{4+} play important roles in the pathogenesis of rheumatoid arthritis (Jabeen et al., 2016). A good anti-arthritic agent should, therefore, be capable of suppressing at least one of these phases or mediators (Patil *et al*., 2012).

In this study, paw swelling in both ipsilateral and contralateral hind limbs, and arthritic scores were employed as indices of measuring the anti-arthritic activity of the isolated bioactive compounds which are now known to be β-amyrin and polpunonic acid respectively. It was realised that both compounds at the various doses significantly reduced the primary, as well as the secondary paw swelling and erythema in the rats. The plant's anti-inflammatory effect supports this result as its hydro-ethanolic root bark extract has been shown to possess significant anti-inflammatory property in this very study. The extract was also found in this study to mediate its anti-inflammatory activity through its inhibitory effect on inflammatory mediators such as bradykinin, prostaglandin, TNF-alpha and interleukin-1β.

In order to determine true remission of the disease and for accurate evaluation of the arthritis status, radiographic techniques were employed (Kitamura *et al*., 2007). Quantitative radiographic assessment in individuals with suspected arthritis is valuable when determining treatment strategies and designing clinical trials (Kinds *et al.*, 2012). Structural changes, which can be visualised by conventional radiography or other imaging techniques, help in distinguishing rheumatoid arthritis from other arthritic disorders, and this is also of value when alternative means of assessment of rheumatoid arthritis (RA) is not conclusive (Aletaha *et al.*, 2010; Bohndorf & Schalm, 1996).

Results obtained from our study show that β-amyrin and polpunonic acid reduced inflammation at the metatorso-phalangeal joints when compared to the CFA control group. Also, erosion of the phalangeal bone was observed in the CFA control group but this was reduced in the groups that received β-amyrin and polpunonic acid, as well as the group that took dexamethasone. The radiographic results support the findings on the ameliorative effect of the two compounds on paw oedema and arthritic score in the adjuvant-induced arthritis.

Augmenting the above results is the histological examination of the hind paws of the rats which further demonstrated the beneficial effects of the compounds as they significantly attenuated cartilage erosion, subchondral cyst and Weichselbaum's lacunae formation as compared to the arthritic control group. Also, evidence of bone remodelling was observed in all groups treated with β-amyrin, polpunonic acid as well as dexamethasone compared to the arthritic control group.

Although liver enzymes and markers of liver toxicity such as AST, urea, total serum proteins among others were not significantly affected by either CFA alone or when combined with the bioactive compounds, it is well reported that arthritis is not only confined to the limbs but also affects the liver resulting in hepatomegaly (Bendele, 2001; Tuncel *et al*., 2016). It is, therefore, possible that this effect could have been seen if the study had persisted for longer duration.

It was not surprising that both β-amyrin and polpunonic acid, pentacyclic triterpenes, exhibited potent anti-arthritic effect in the CFA induced rat model of arthritis. This is because β-amyrin is already known to

exhibit pronounced anti-inflammatory effects despite the fact that its effect in arthritis has not been previously investigated. It has been reported to mediate its anti-inflammatory activities through membrane stabilisation and inhibition of leukocyte migration (Okoye *et al*., 2014). Also, inhibition of nuclear factor kappa-B, reduction of COX-2 expression and cAMP response element-binding protein activation have been implicated in its mechanism of action (Vitor *et al*., 2009). Polpunonic acid, on the other hand, has been reported to exhibit anti-inflammatory activity as it counteracted lipopolysaccharide (LPS) effects on iNOS expression and pro-inflammatory cytokines mRNA levels in Bv-2 microglial cells (Villar-Lorenzo *et al.*, 2016). It is therefore possible that these triterpenes contributed to the anti-inflammatory effect of the extract as reported in this study.

With inflammatory processes strongly implicated in pain, coupled with the fact that the analgesic effect of the extract from the plant has already been well-established in several models of pain, the isolated compounds were also screened for analgesic effect in the tail-immersion test. β-amyrin and polpunonic acid, similar to the extract, also exhibited significant analgesic effect with ED_{50s} comparable to that of morphine. This gives an indication that further development of the two compounds could lead to the discovery of potent and efficacious analgesics to complement already existing ones.

With inflammation and pain known to underpin depression and anxiety disorders (Arango-Davila & Rincon-Hoyos, 2018). β-amyrin and polpunonic acid were tested in murine models of anxiety and depression. Similar to the extract, the isolated compounds also produced a prominent antidepressant effect in both forced swim and tail suspension tests (Latha, *et al*., 2015;

Pellow, Chopin, *et al.*, 1985). Also, the test compounds significantly increased the time spent in the open arm of the elevated plus maze as well as time mice spent in the central arena of the open field test. reported that beta-amyrin palmitate, a palmitic acid ester of beta-amyrin, exerts its antidepressant effect through the release of norepinephrine from newly synthesised pools, and thus, activation of noradrenergic activity (Martins & Brijesh, 2018; Subarnas *et al*., 1993). Polpunonic acid, on the other hand, has been shown to possess several pharmacological activities including antidiabetic (Ardiles *et al.*, 2012), anti-inflammatory (Villar-Lorenzo *et al.*, 2016), inhibitory effects on the proliferation of human breast cancer cells MCF-7 (Fan, Parhira, Zhu, Jiang, & Bai, 2016) and anti-ulcerogenic effects (Andrade *et al*., 2006). This is, however, the first report of its anxiolytic and anti-depressant effect.

With the isolated compounds from *Ziziphus abyssinica*, β-amyrin and polpunonic acid, exhibiting a pronounced analgesic, anti-inflammatory, anxiolytic and antidepressant effect in murine models, they might have contributed significantly to the similar effect observed in the extract of the plant. The isolated compounds as well as the extract are therefore good candidates that could be evaluated further for the management of the co-morbid condition of chronic pain, inflammation, anxiety and depression.

Summary

The study evaluated the pharmacological effect of the extract and isolated bioactive compounds from the root bark of *Ziziphus abyssinica* for the management of inflammatory conditions.

The extract was found to contain phytochemicals such as alkaloids, triterpenes, phytosterols, flavonoids and tannins and these may account for its medicinal properties.

Acute toxicity evaluation of the hydro-ethanolic root bark extract of the plant (ZAE) revealed that the extract is relatively safe with LD_{50} of more than 5000 mg/kg. Sub-chronic administration of the extract over 90 days revealed no organ damage or death. Also, biochemical and haematological parameters were not significantly different from the naïve control animals suggesting the extract may be safe upon sub-chronic exposure.

The hydro-ethanolic extract of the plant produced an analgesic effect in tail-immersion, acetic acid and formalin tests. The proposed mechanism was found to be mediated by the opioidergic, ATP-sensitive potassium channels and nitric oxide cyclic GMP pathways. The extract, additionally, ameliorated hyperalgesia produced by the intraplantar injection of TNF-α, IL-1β, prostaglandin E_2 and bradykinin.

On its anti-inflammatory effect, the extract was found to inhibit heat as well as hypotonic solution induced haemolysis, and egg albumin, as well as bovine serum albumin, induced protein denaturation. Also, the extract produced anti-inflammatory properties in carrageenan-induced paw oedema, formalin-induced paw inflammation and carrageenan-induced peritonitis in rats. The inflammation produced by intraplantar injection of TNF-α, IL-1β, prostaglandin E_2 and bradykinin were also ameliorated by the extract.

Since inflammatory processes are known to underpin neuropsychiatric conditions such as anxiety and depression, the extract was tested in murine

models of anxiety and depression. The extract significantly produced anxiolytic and antidepressant effects in all the models.

An activity guided isolation of the plant was subsequently conducted which yielded two pentacyclic triterpenes, β-amyrin and polpunonic acid.

The isolated bioactive compounds exhibited pronounced anti-inflammatory property in CFA-induced arthritis in rats. Also, the isolated compounds produce analgesic property in the tail-immersion test. Furthermore, beta-amyrin and polpunonic acid exhibited pronounced anxiolytic and antidepressant effect.

Conclusions

From all the results of this study, it can be concluded that:

1. ZAE is relatively safe following acute and sub-chronic administration in rats.
2. ZAE possesses analgesic effect that is possibly mediated through opioidergic, ATP-sensitive potassium channels and nitric oxide cyclic GMP pathways.
3. ZAE possesses anti-inflammatory properties that are possibly mediated via membrane stabilisation, inhibition of protein denaturation, neutrophil degranulation and inflammatory mediators such as TNF-α, IL-1β, prostaglandin E_2 and bradykinin.
4. ZAE produced a prominent anxiolytic and antidepressant effect
5. β-amyrin and polpunonic acid were isolated for the first time from *Ziziphus abyssinica* plant.

6. The isolated bioactive compounds from the plant, β-amyrin and polpunonic acid, exhibited anti-inflammatory properties in CFA-induced arthritis model.
7. The extract, β-amyrin and polpunonic acid exhibited antidepressant and anxiolytic effects in murine models.

Recommendations

Based on the findings from this study it is recommended that:

- drug discovery companies and bodies should consider further clinical assessment of the compounds β-amyrin and polpunonic acid as new drugs for the management of inflammatory diseases and depression.
- further studies should consider isolation of other compounds which may similarly account for the medicinal effect of the plant.
- further studies should consider structural modification of the isolated compounds for an optimal activity.
- future research should consider molecular mechanisms that may mediate the pharmacological effects of the extracts and isolated bioactive compounds reported in this study.

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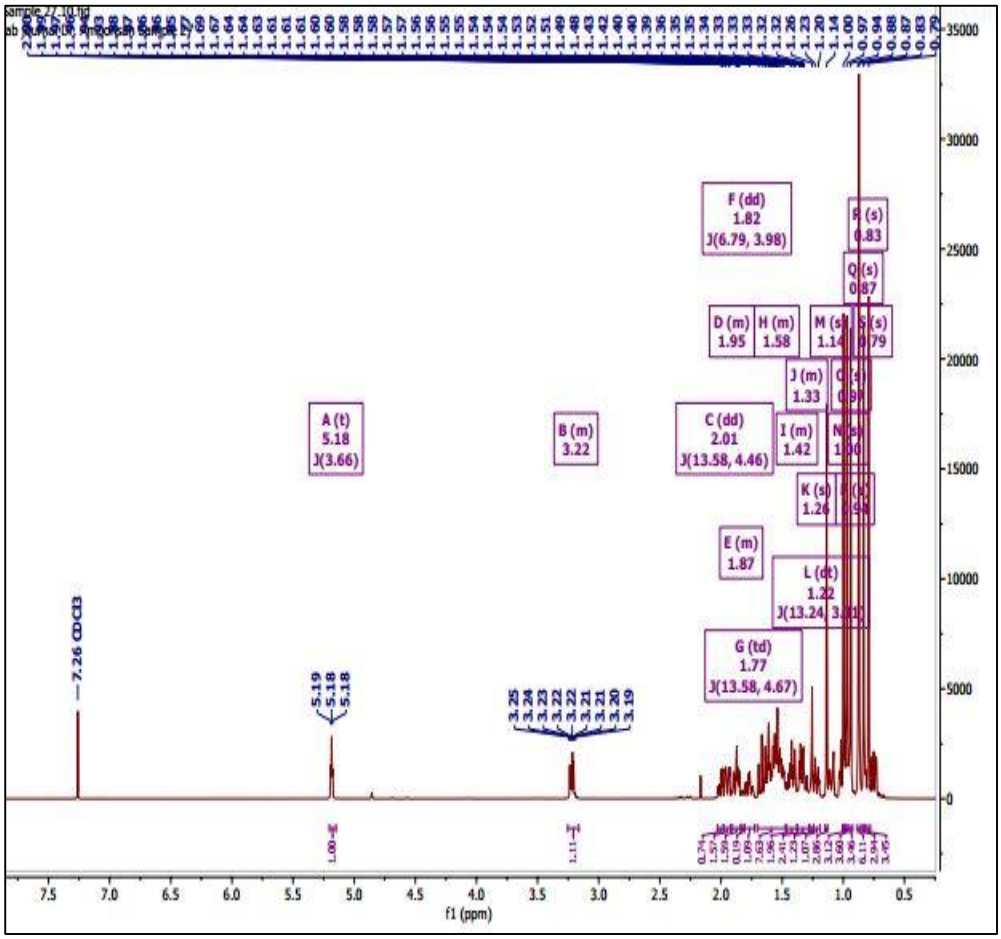
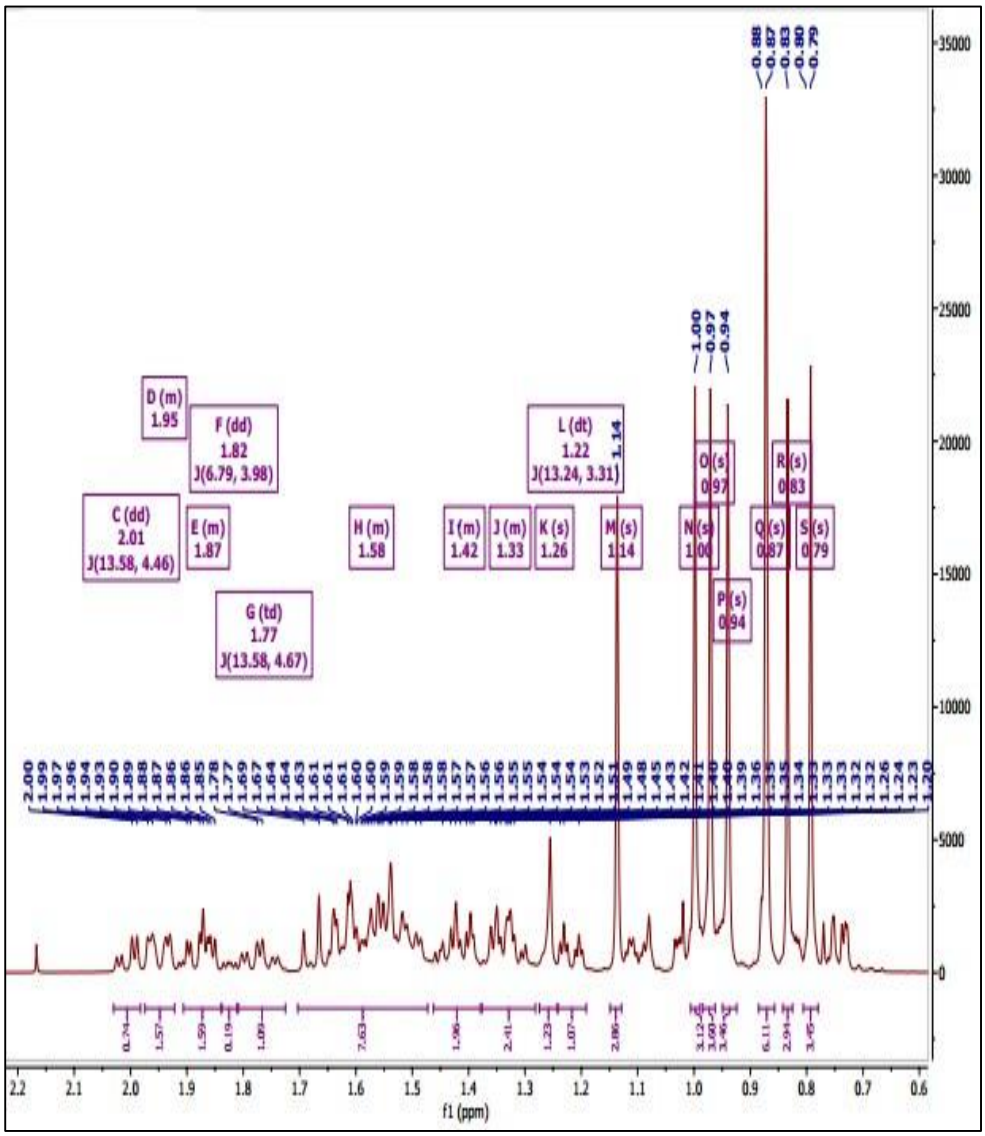
APPENDICES

Appendix A: Spectra Data of B-Amyrin

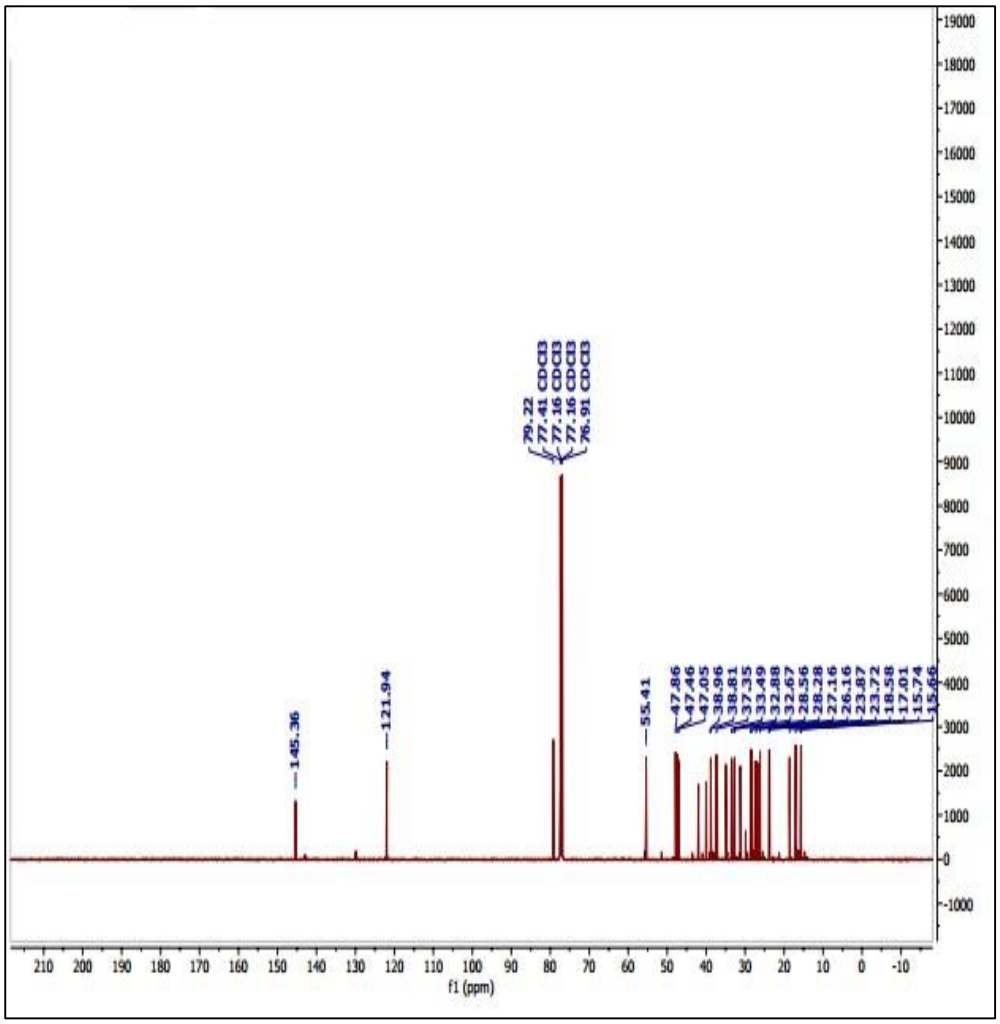
Appendix A(i): ^{1}H-NMR Spectra of β-amyrin



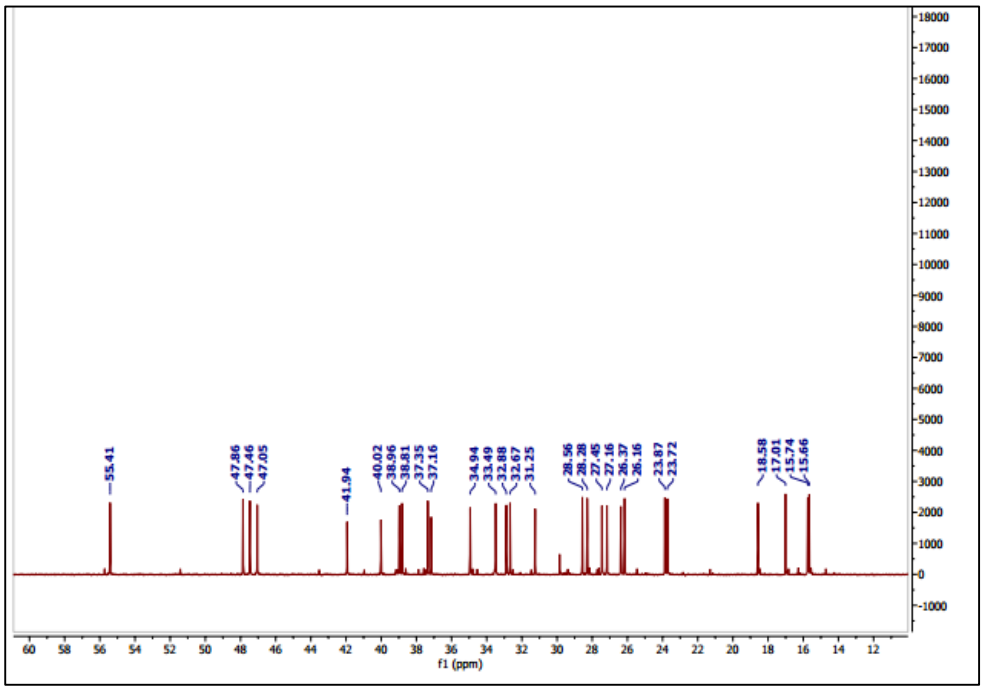
Appendix A (i cont'd): ^{1}H-NMR Spectra of β-amyrin



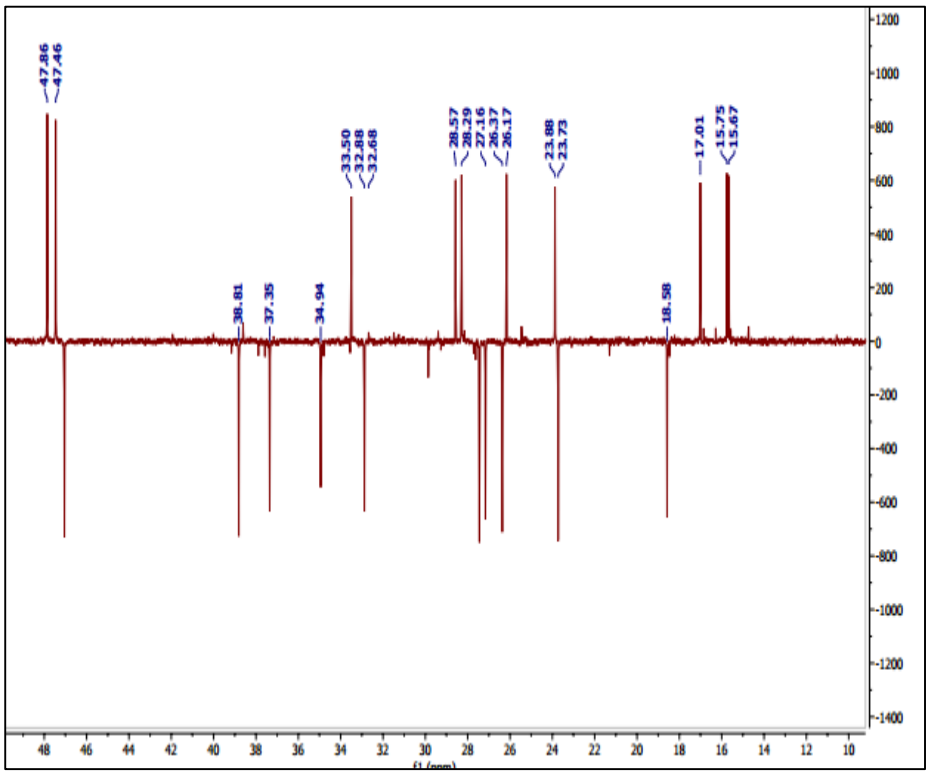
Appendix A (ii): ^{13}C-NMR Spectra of β-amyrin



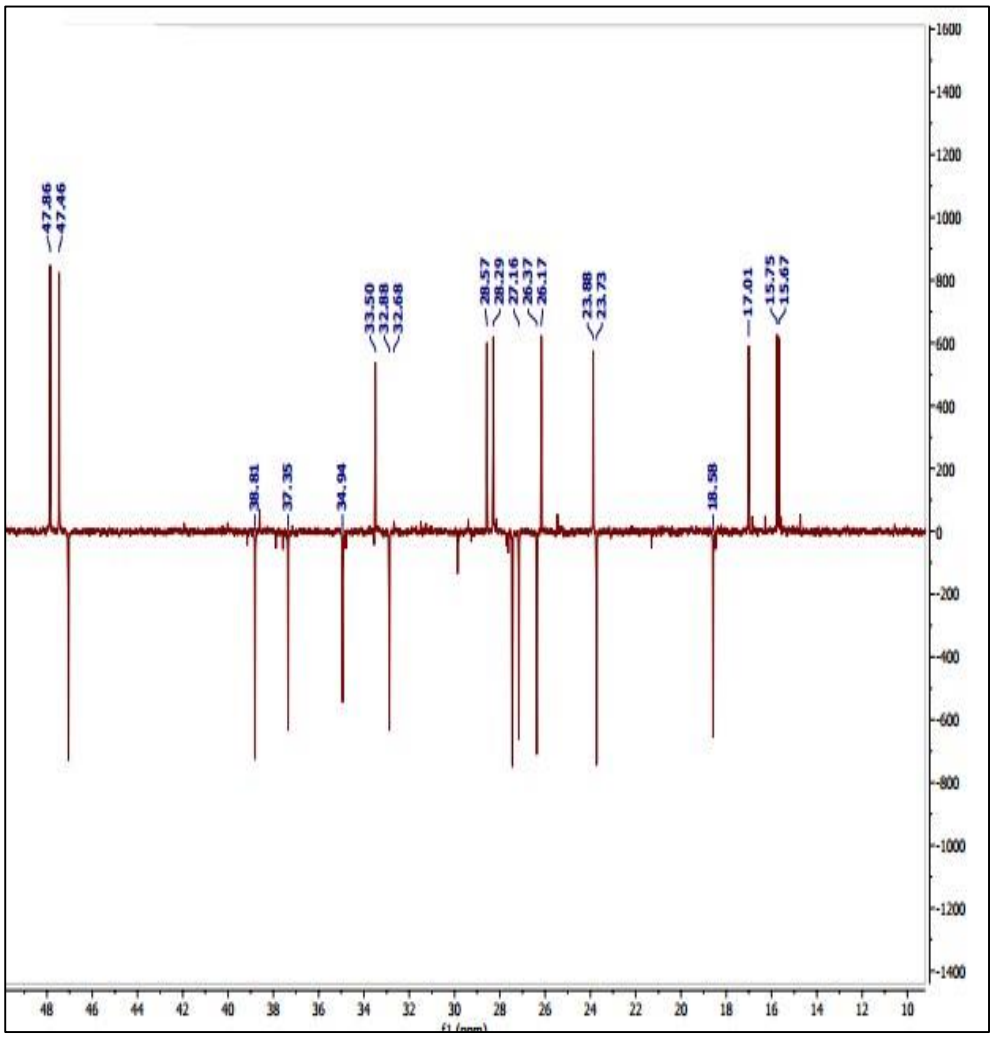
Appendix A (ii cont'd): ^{13}C-NMR Spectra of β-amyrin



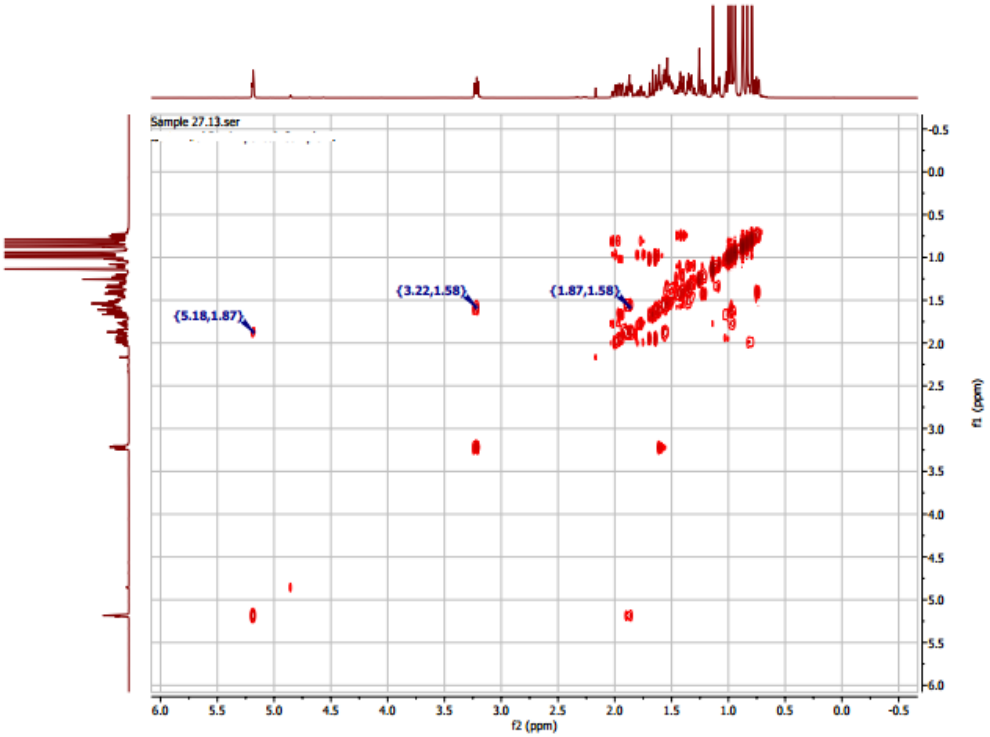
Appendix A (iii): Distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR Spectra of β-amyrin



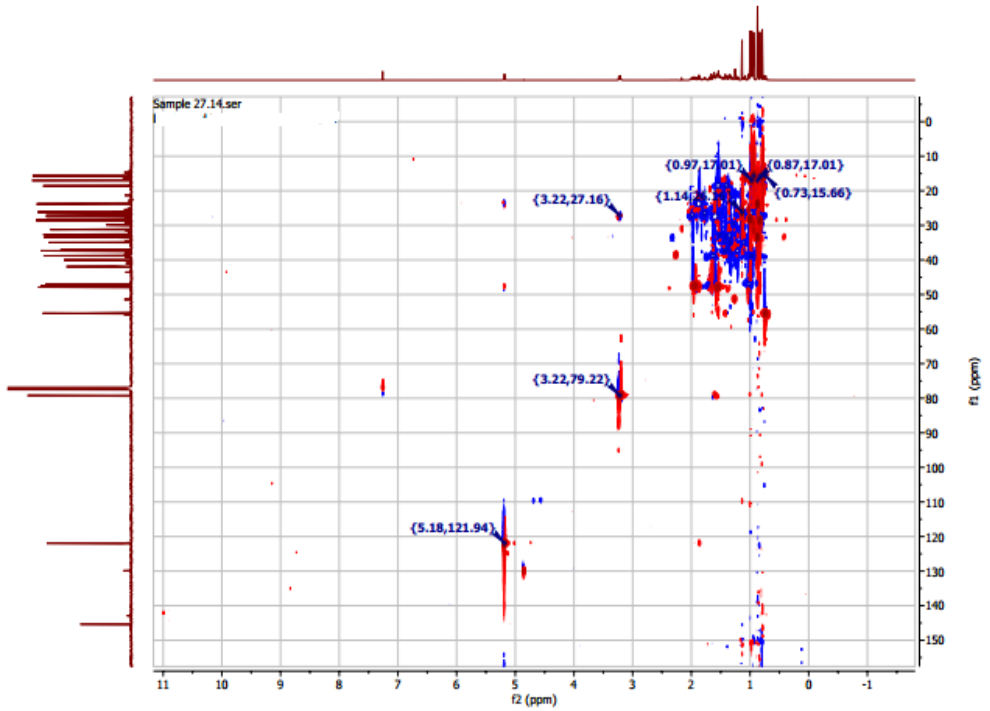
Appendix A (iii cont'd): DEPT 13C-NMR Spectra of β-amyrin



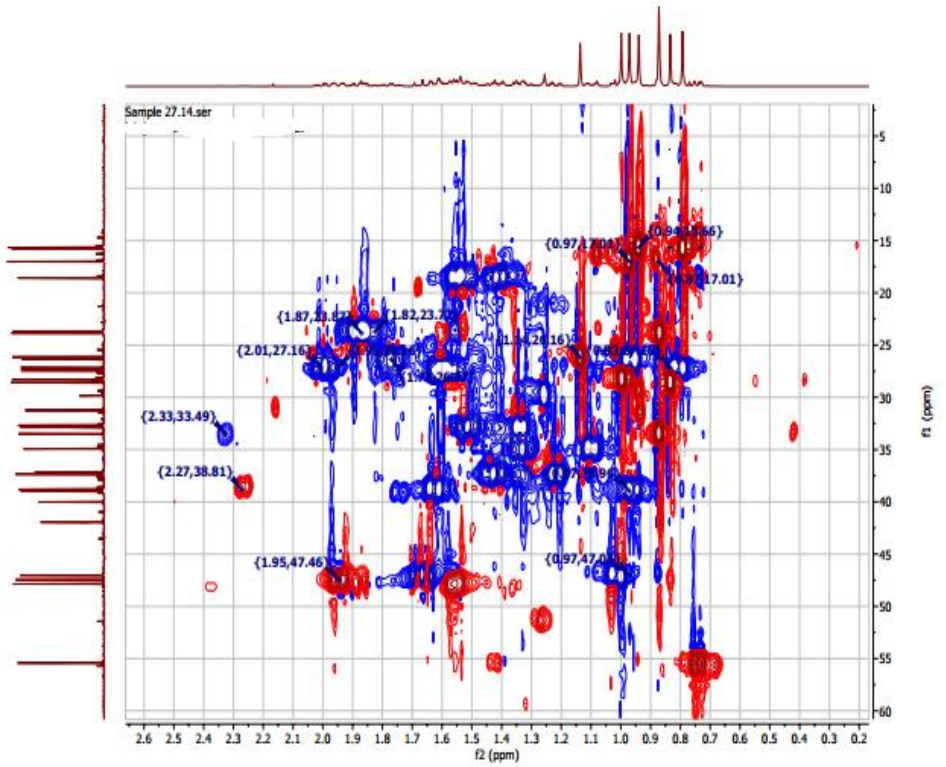
Appendix A (iv) ^{1}H-^{1}H Correlated Spectroscopy (COSY) of β-amyrin



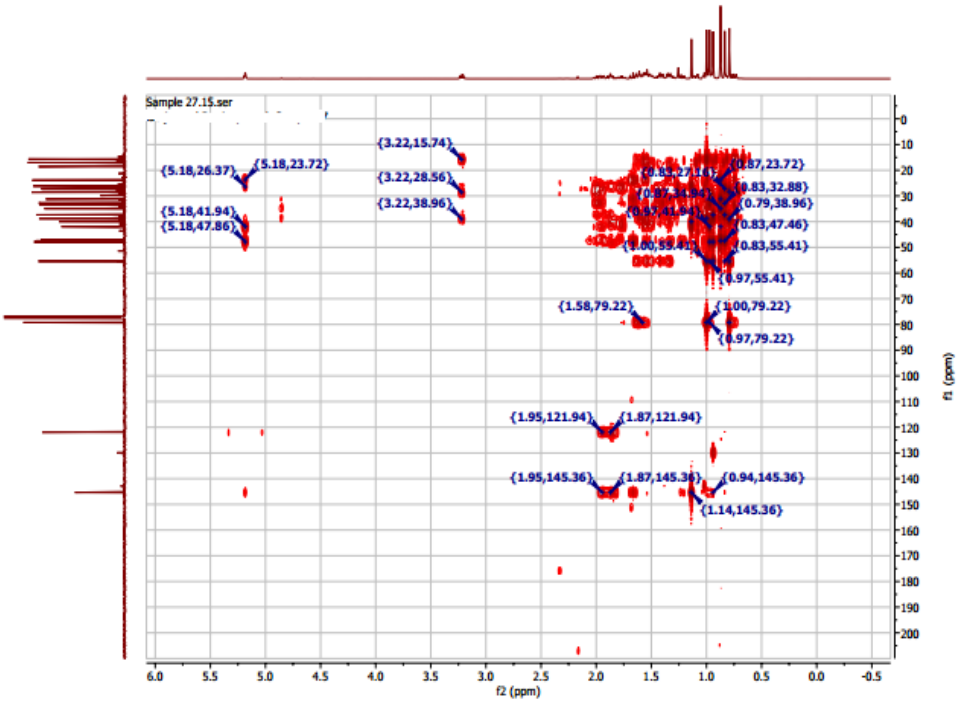
Appendix A (v) Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) Spectra of β-amyrin



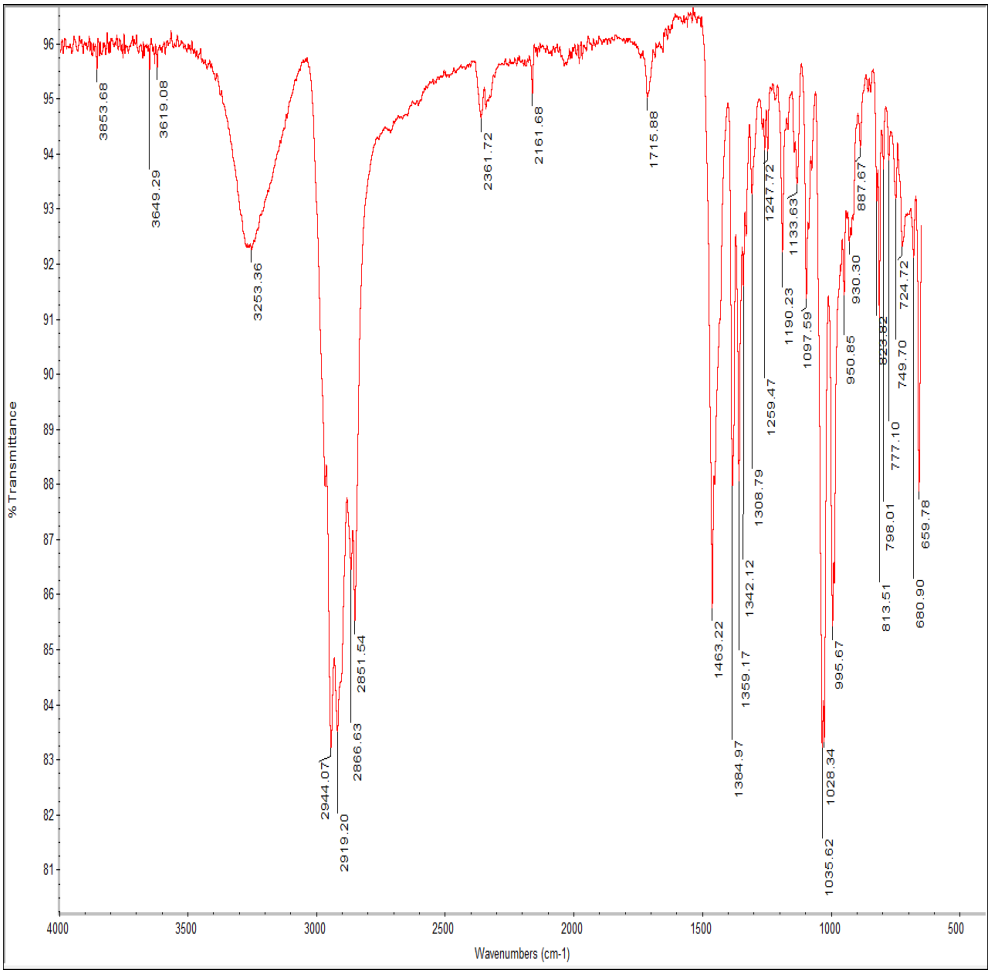
Appendix A (v cont'd)



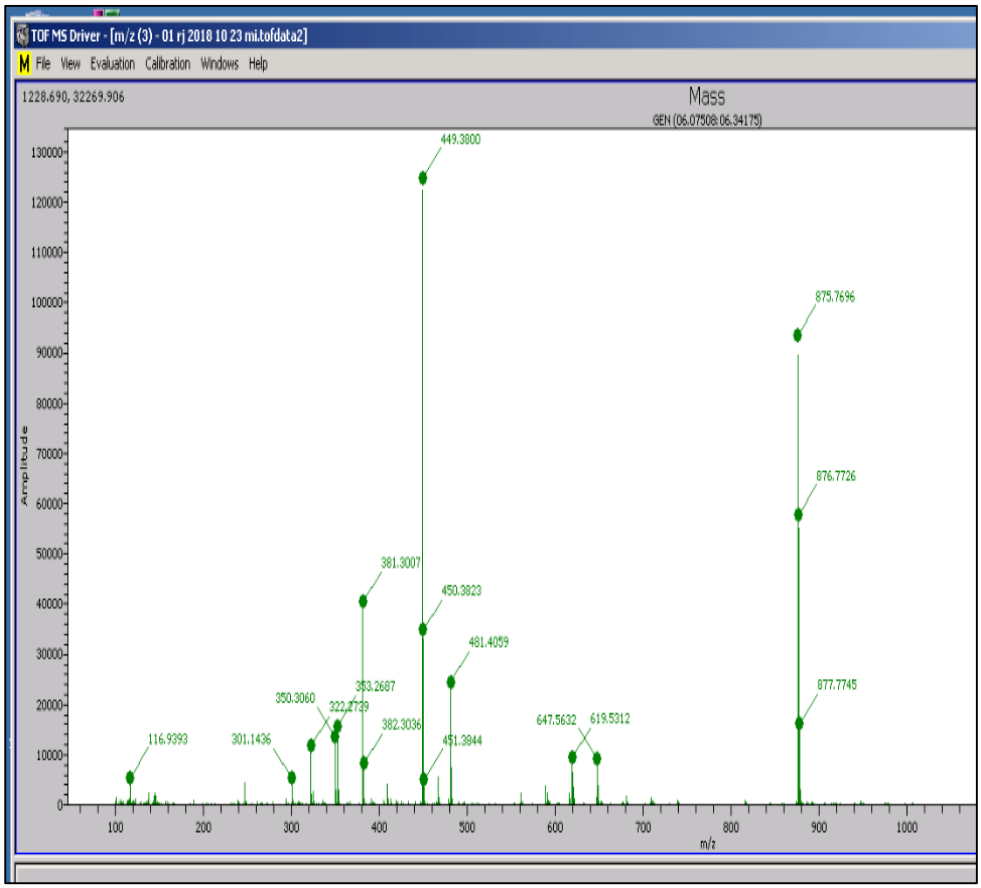
Appendix A (v cont'd)



Appendix A (vi): Infra-red spectra of β-amyrin

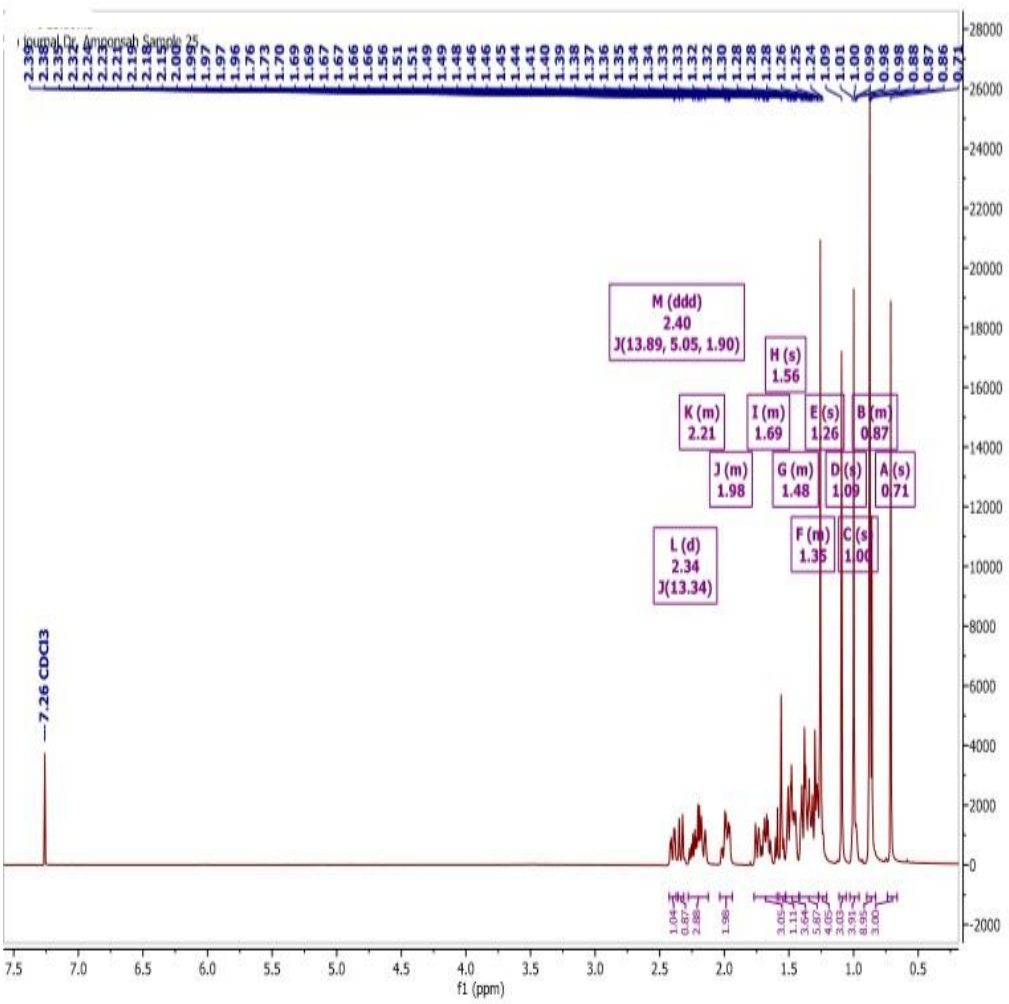


Appendix A (vi) Mass Spectrum of β-amyrin

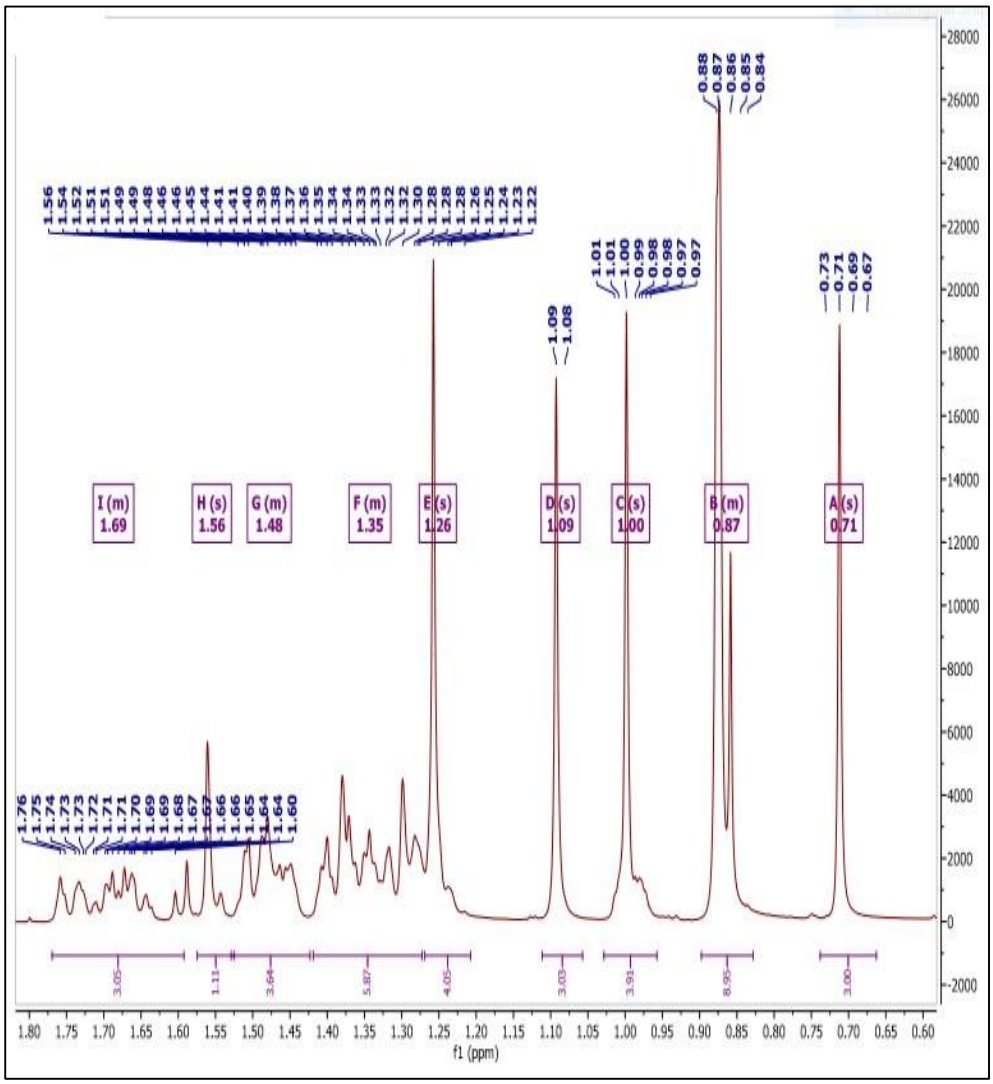


Appendix B: Spectra Data of Polpunonic Acid

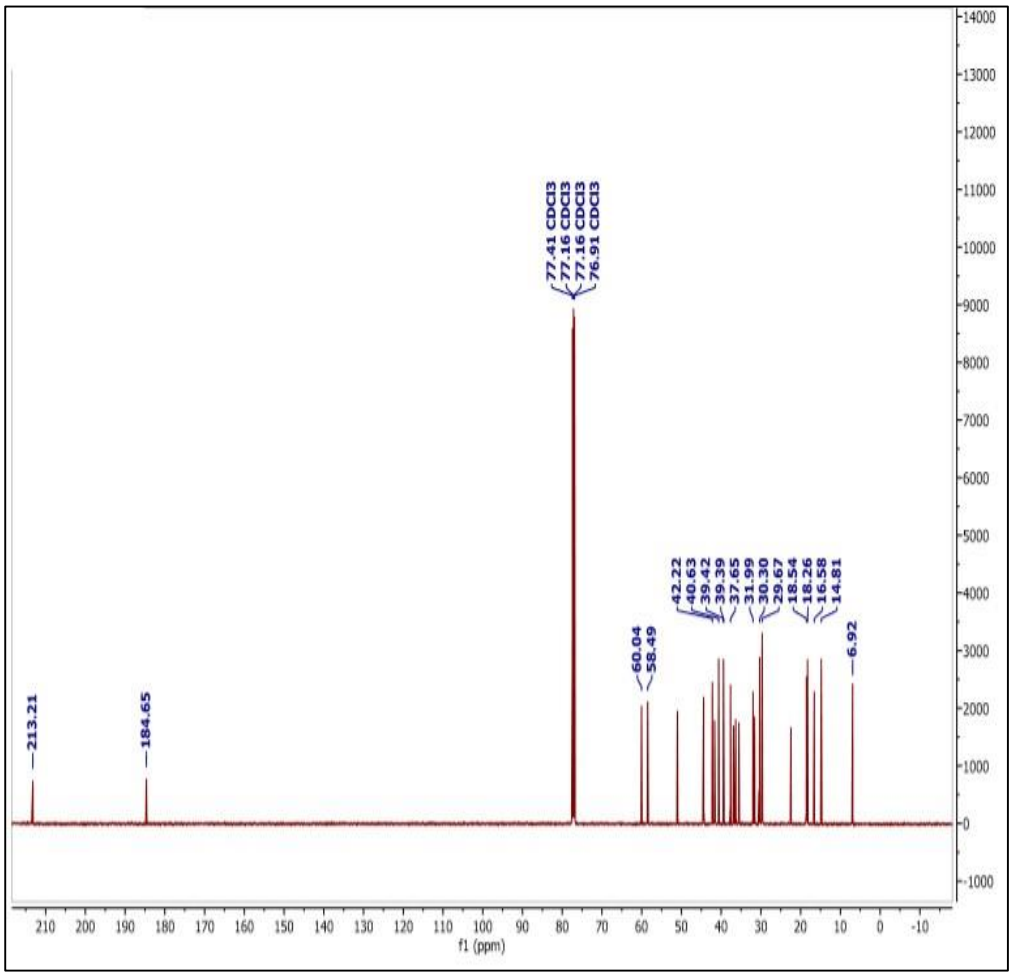
Appendix B (i): ^{1}H-NMR Spectra of Polpunonic Acid



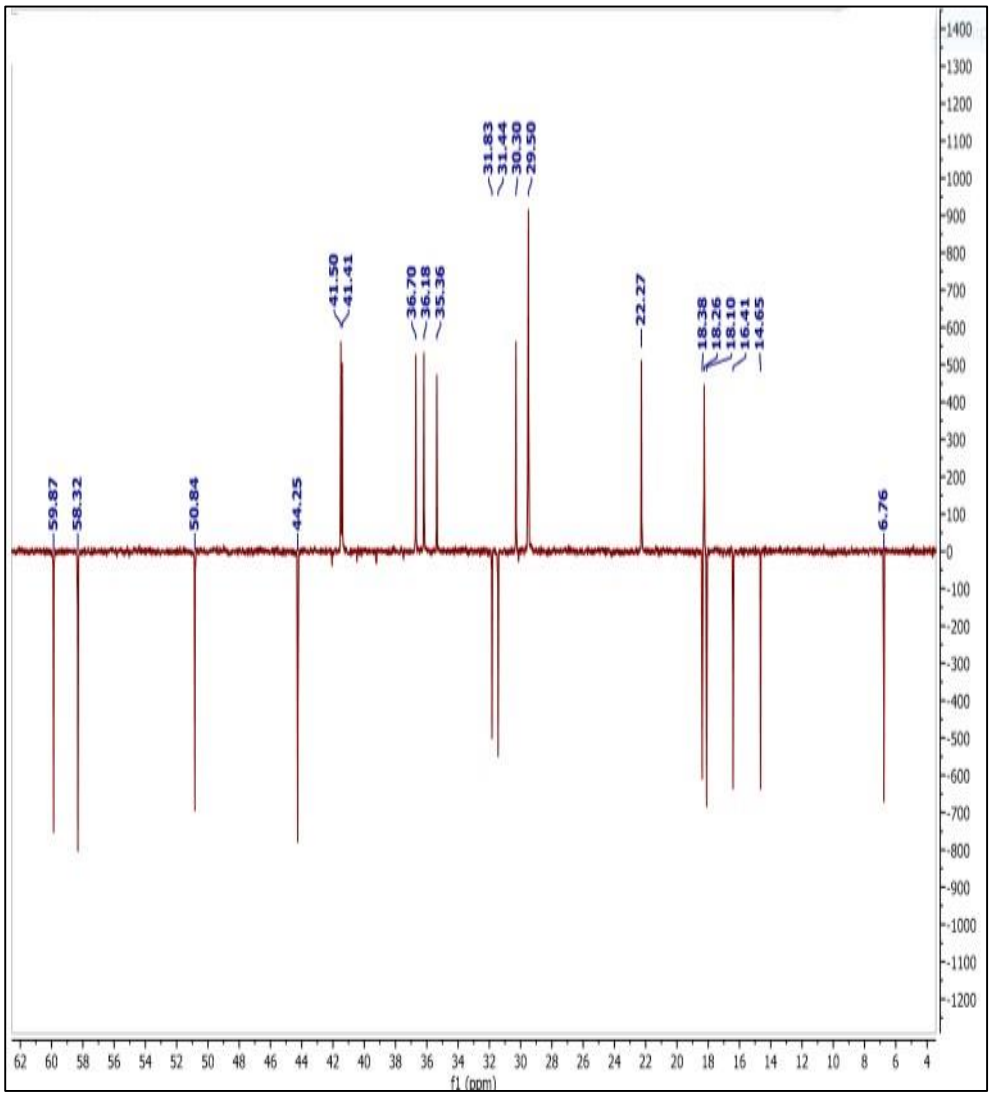
Appendix B (I cont'd): ^{1}H-NMR Spectra of Polpunonic Acid



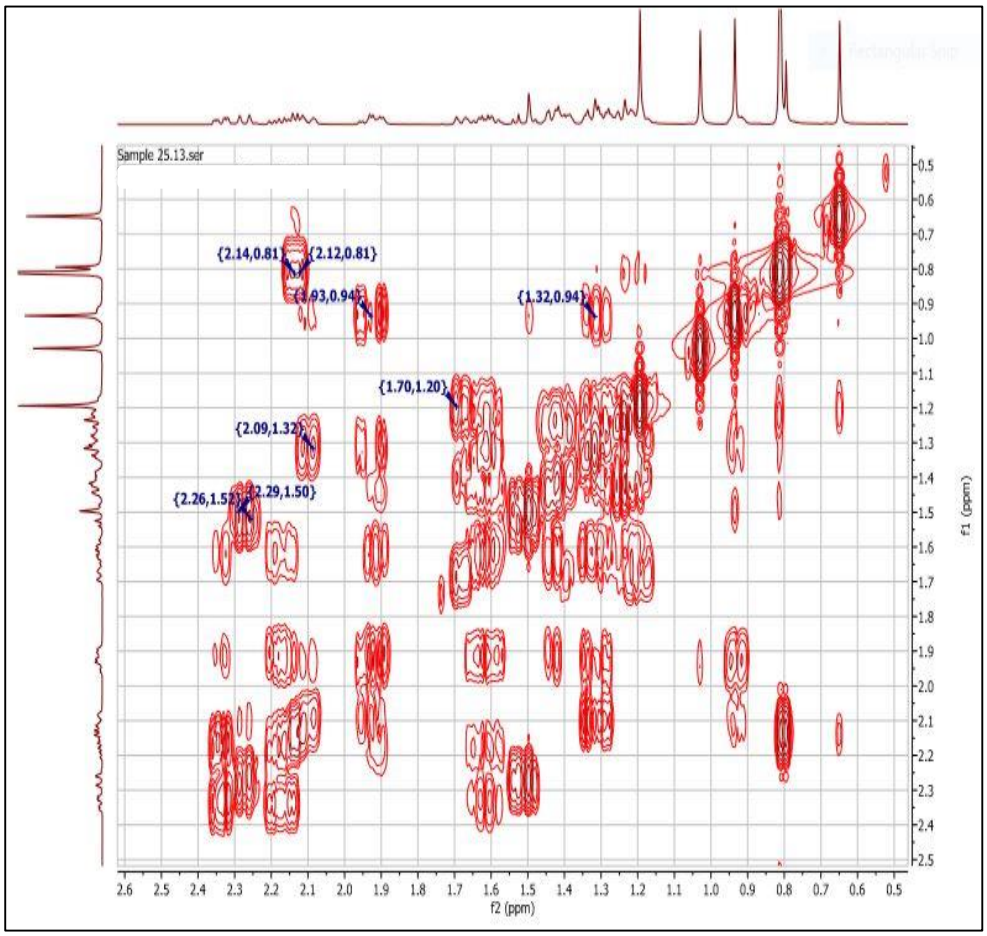
Appendix B (ii): ^{13}C-NMR Spectra of Polpunonic Acid



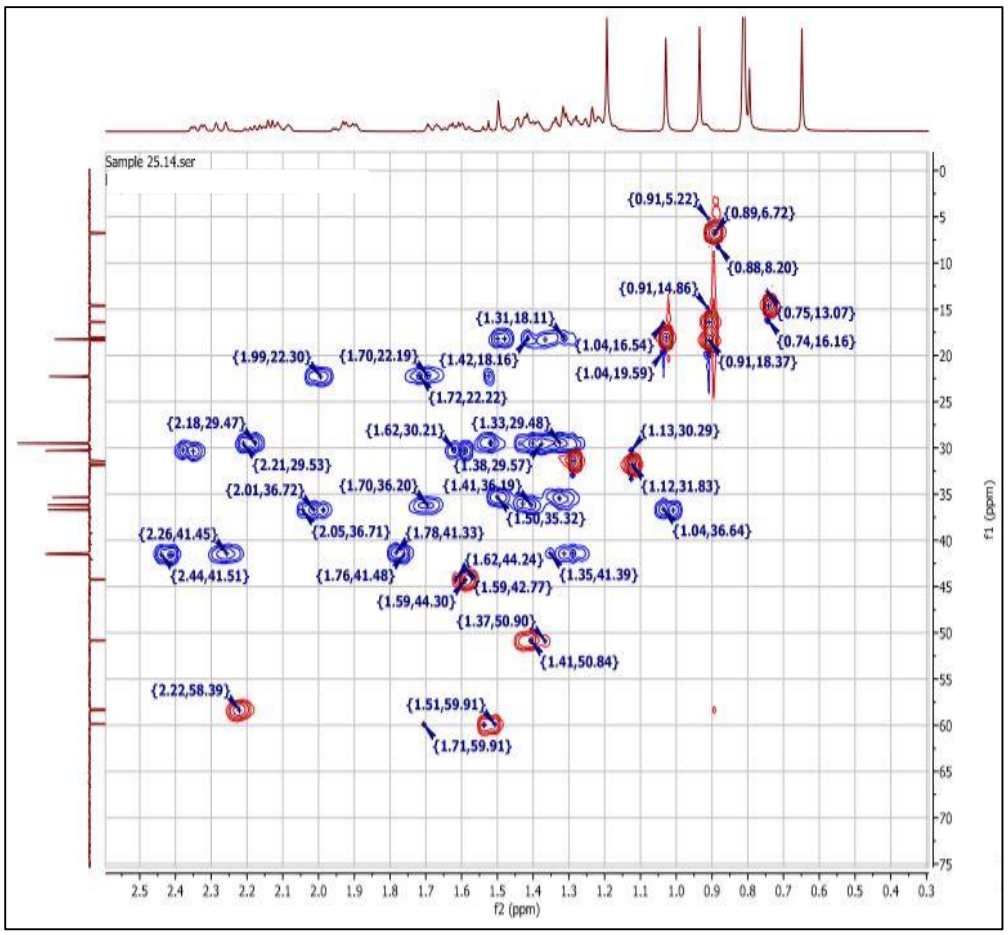
Appendix B (iii): DEPT ^{13}C-NMR Spectra of Polpunonic Acid



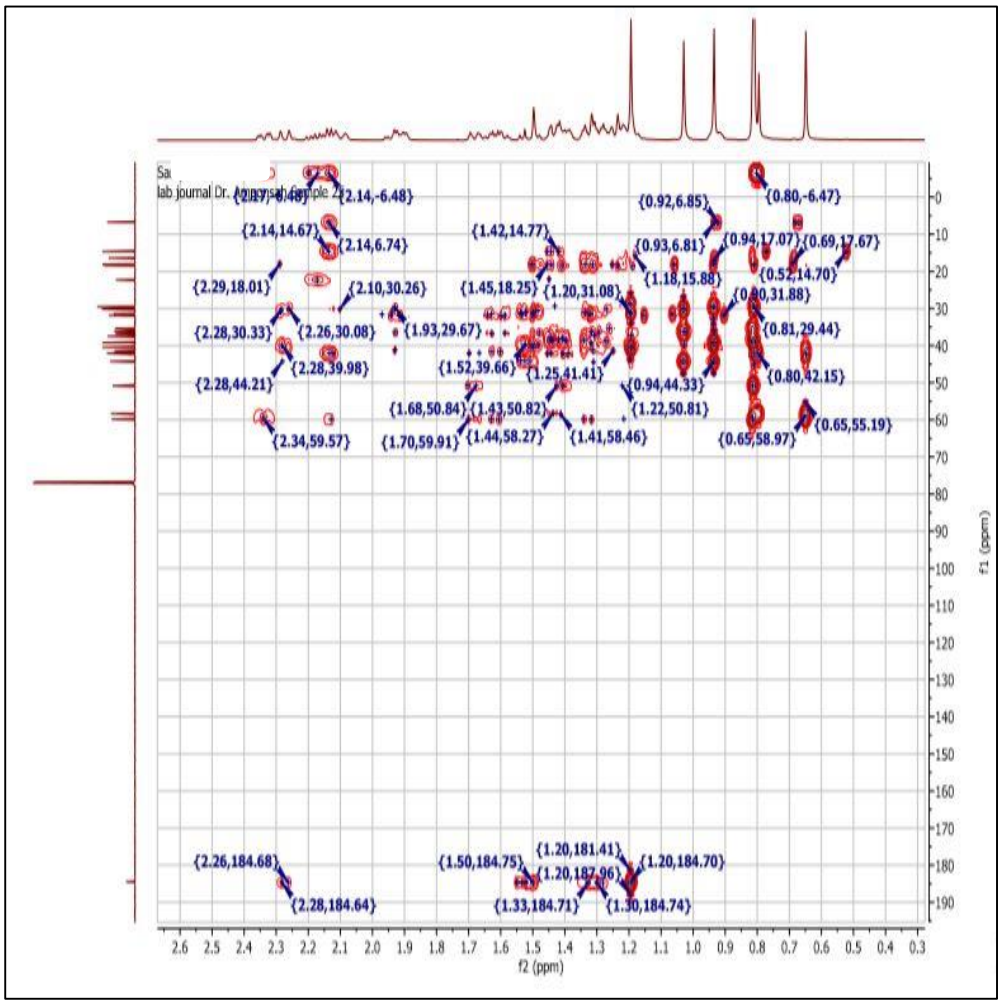
Appendix B (iii): ^{1}H-^{1}H Correlated Spectroscopy (COSY) of β-amyrin



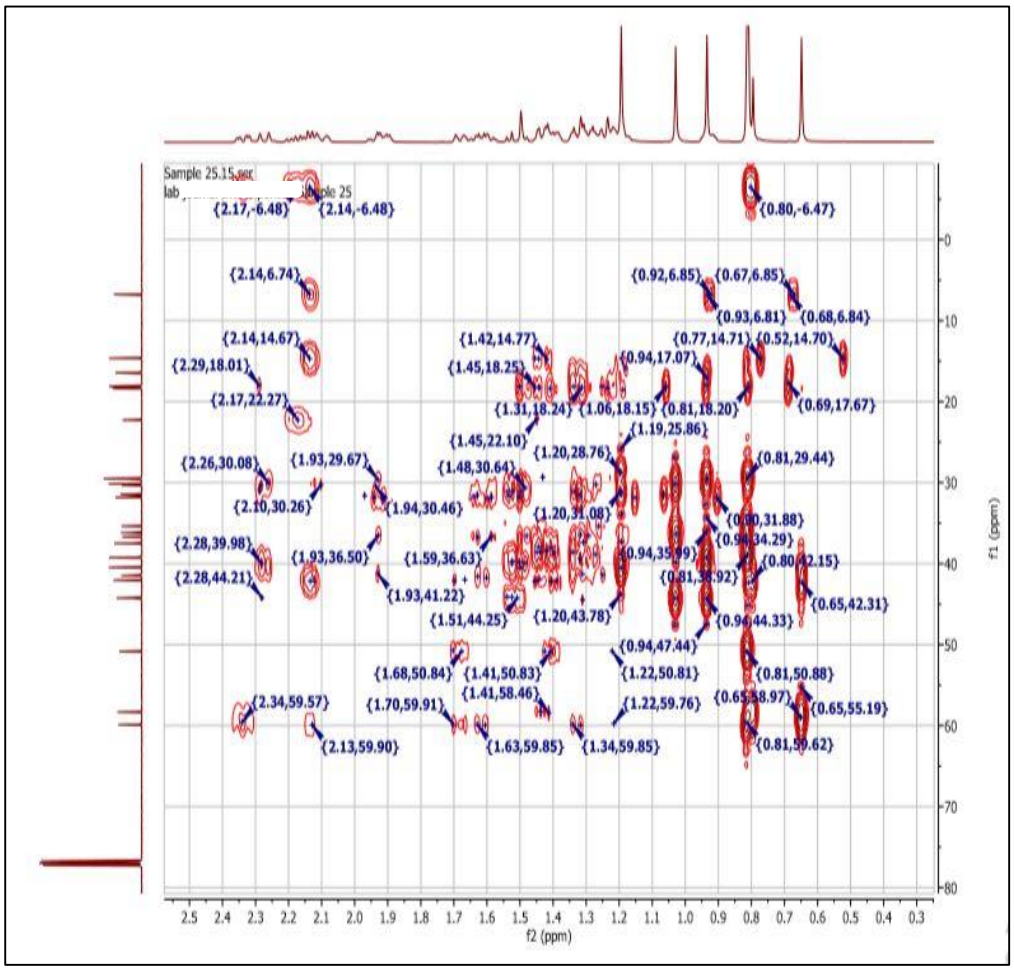
Appendix B (iv): Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) Spectra of Polpunonic Acid



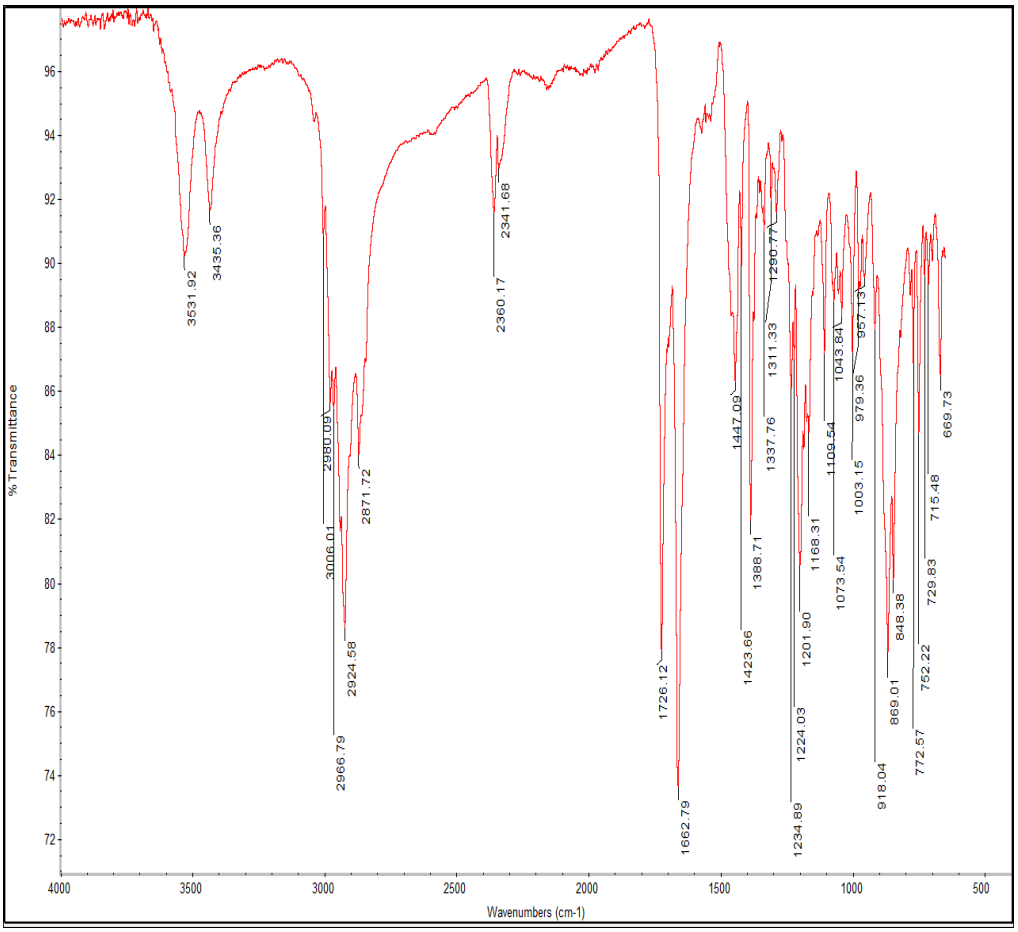
Appendix B (iv cont'd)



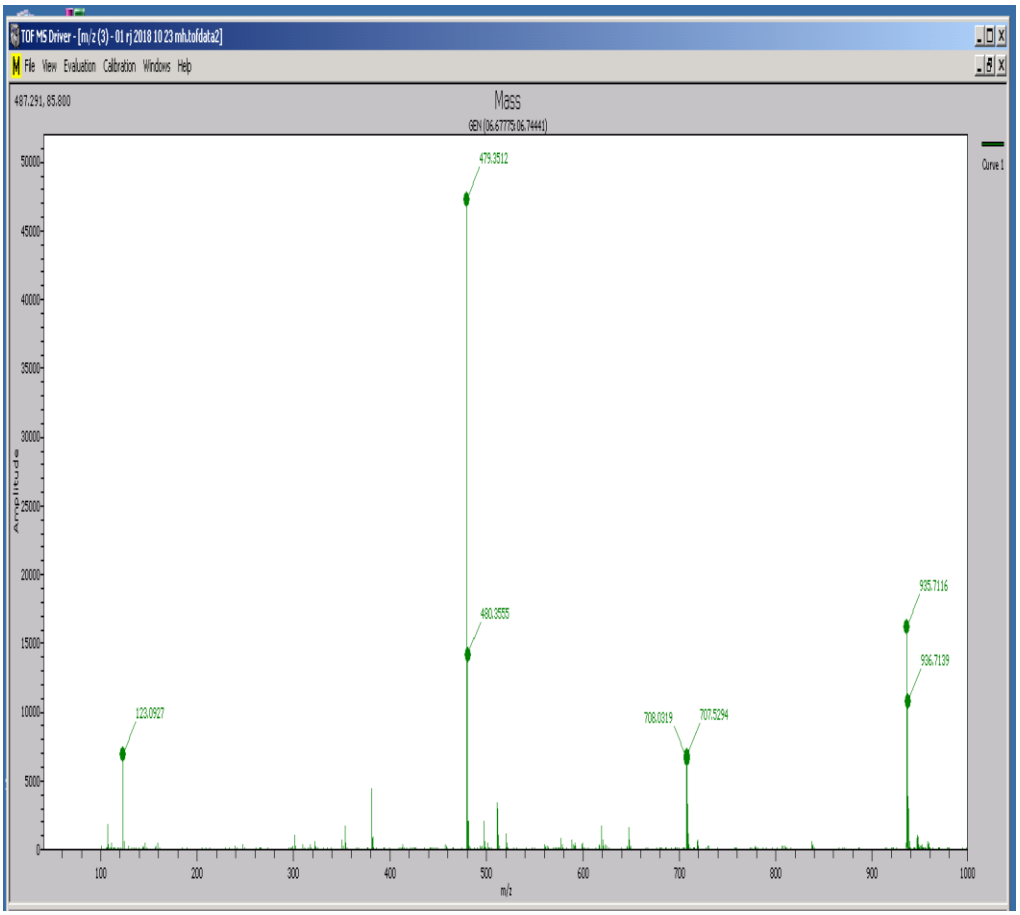
Appendix B (iv cont'd)



Appendix B (v): Infra-red Spectroscopy of Polpunonic Acid

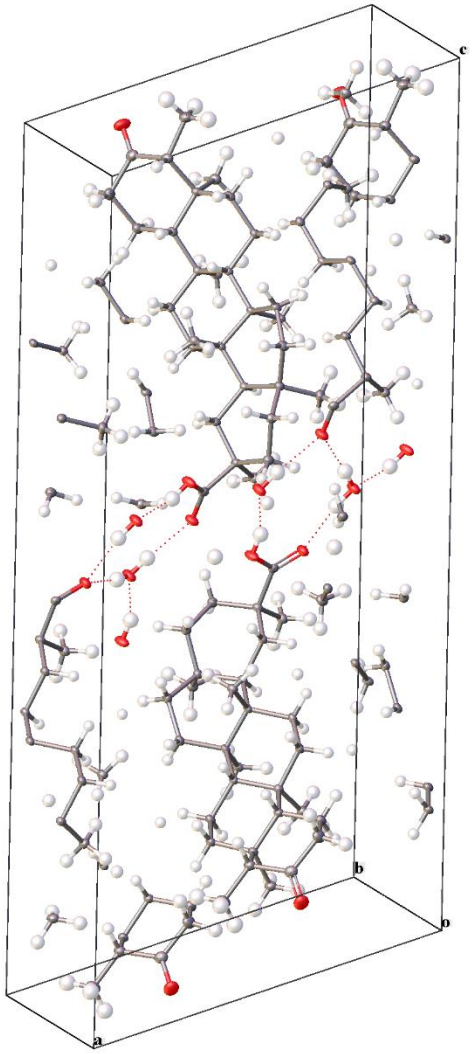


Appendix B (vi): Mass Spectrum of Polpunonic Acid

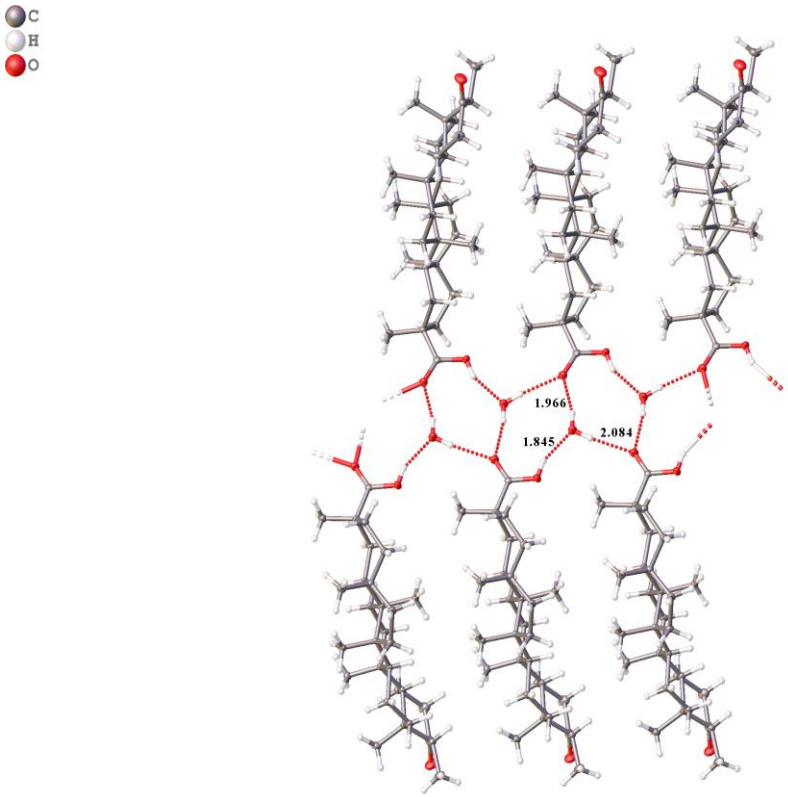


Appendix C: Crystal Structure of Polpunonic Acid

Appendix C (i): The molecular packing in compound 2. Hydrogen bonding is shown as dashed lines.



Appendix C (ii): Detail of solid state inter-molecular H-bonding along b axis of Polpunonic Acid.



Appendix D: Results from activity-guided isolation of compounds from the plant.

Activity-guided isolation PART 1

Fraction	% Inhibition
Pet. Ether (300 mg/kg)	45.4
Chloroform (300 mg/kg)	78.2
Methanol (300 mg/kg)	55.9
Diclofenc (10 mg/kg)	85.7

Activity-guided isolation PART 2

Fraction	% Inhibition
1-20 (30 mg/kg)	36.7
21-27 (30 mg/kg)	75.3
28-124 (30 mg/kg)	45.8
125-147 (30 mg/kg)	83.5
185-225 (30 mg/kg)	47.8
Diclofenc (10 mg/kg)	89.2