PHYTOCHEMICAL ANALYSIS, ANTI-INFLAMMATORY ACTIVITY, ANTIOXIDANT ACTIVITY AND TOXIC EFFECTS OF AQUEOUS ROOT EXTRACT OF *Launaea cornuta* (Hochst. Ex Oliv. & Hiern.)

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (MEDICAL BIOCHEMISTRY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

JANUARY, 2022

DECLARATION

I Akimat Kapanat Evans, duly declare that this Thesis is my original work and has not been presented for a degree in any other University.

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To God for un-ending love, protection, strength, faithfulness and His guidance throughout my studies.

My lecturers/supervisors, friends and family for their great support, encouragement and their prayers

ACKNOWLEDGEMENT

It is not an easy task to undertake scientific research without guidance and support. It is for this reason that I wish to acknowledge the following for their continued support in the development of my project proposal.

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LIST OF ABBREVIATIONS AND ACRONYMS

COX	Cyclooxygenase
DPPH	2,2-Dipheny-l-picrylhydrazyl
HCL	Hydrochloric Acid
HRBC	Human Red Blood Cells
IL-1	Interleukin-1
LPS	Lipopolysaccharide
NACOSTI	National Council of Science Technology and Innovation
NO_S	Nitric Oxides
NSAIDs	Nonsteroidal Anti-Inflammatory drugs
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin I_2
ROs	Reactive Oxygen species
ROIs	Reactive Oxygen Intermediates
RNs	Reactive Nitrogen species
RNIs	Reactive Nitrogen Intermediates
TCA	Trichloroacetic Acid
TNF	Tumor Necrosis Factor
TXA_2	Thromboxane A_2
UDP	Up-and-Down Procedure

ABSTRACT

Anti-inflammatory, anti-nociceptive and antioxidant drugs have alleviated the agony of millions of people, especially in the developing countries where management of inflammation remains a big challenge. Nonetheless, despite being beneficial, these drugs are now seriously jeopardized by the adverse side effects associated with these synthetic compounds which include heart attack, stomach ulcers, liver and kidney diseases. In most African countries, the anti-inflammatory agents are limited and expensive. Therefore, scientists are tasked to generate new ideas of alternative and novel drugs. The root extracts of *Launaea cornuta* have been locally used in traditional medicine for decades to manage inflammatory conditions and other oxidative-stress-related syndromes; however, their pharmacologic efficacy has not been scientifically investigated and validated. Hence, we investigated the *in vitro* antioxidant activity, anti-inflammatory (*in vitro, ex vivo* and *in vivo*) efficacy, acute oral toxicity, and qualitative phytochemical composition of the aqueous root extract of *L. cornuta*. The ferric-reducing antioxidant power (FRAP) and the 2,2-diphenyl-2-pycrylhydrazyl (DPPH) methods were used to determine the studied extract's antioxidant activity. Besides, the anti-inflammatory efficacy of the studied plant extract was investigated using *in vitro* (anti-proteinase and protein denaturation), *ex vivo* (membrane stabilization), and *in vivo* (carrageenan-induced paw oedema in Swiss albino mice) methods. OECD guidelines were used to conduct acute oral toxicity test using mice model. The studied plant extract demonstrated significant *in vitro* antioxidant effects, that were evidenced by higher DPPH radical scavenging (53.30% to 86.84%) and FRAP activities (0.56 to 0.74 absorbance), which were in a concentration-dependent manner ($p<0.05$). Generally, the studied plant extract exhibited significant *in vitro, ex vivo,* and *in vivo* anti-inflammatory efficacy, respectively, and in a concentration/dose-dependent manner, compared with respective controls ($p<0.05$). For instance, the extract dose of 250 mg/kgbw had higher potency than the standard drug Dexamethasone in dose and time dependent manner in all mice used. Moreover, the studied plant extract did not cause any observable signs of acute oral toxicity even at the highest dose of 2000 mg/Kg BW (LD_{50} >2000 mg/Kg BW). Additionally, the qualitative phytochemistry revealed the presence of tannins, cardiac glycosides, anthraquinones, alkaloids, steroids, terpenoids, phenols and flavonoids. Some of these phytochemicals like flavonoids are antioxidant- and anti-inflammatory-associated phytochemicals and were deemed responsible for the reported pharmacologic efficacy. Therefore, aqueous root extract of *L. cornuta* has *in vitro, ex vivo* and *in vivo* anti-inflammatory, and *in vitro* antioxidant activity thus further studies to characterise bioactive molecules and their mode(s) of pharmacologic efficacy are encouraged.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Inflammation is a critical component of host response to infection and injury required for the maintenance of health. However, prolonged excessive inflammation can be injurious leading to the pathological process of certain ailments like rheumatoid arthritis, asthma, diabetes, inflammatory bowel disease, atherosclerosis, mental disorders like Parkinson's disease among others (Salvemini, 2003; Aggarwal *et al*., 2006; Gürkan, 2008; Tang *et al*., 2012).

The four known evidences/signs of inflammation, following immediate response to stimuli, are pain (algesia), edema(swelling), redness, and heat which are caused by vascular changes in vessel caliber (Nathan, 2002). During inflammation, endothelial cells are activated leading to vasodilation, which increases pore size in endothelial walls of blood vessels leading to leakage/movement of fluid and plasma proteins from the circulation and emigration of leukocytes through the vessel wall. Macrophages and neutrophils are the major leukocytes entangled in acute inflammation. On the other hand, during chronic inflammation, mononuclear cells like macrophages and lymphocytes release inflammatory mediators (Kumar *et al*., 2004).

Numerous mediators (prostaglandins like PGE_2, cytokines, nitric oxides, interleukins (IL-1) among others) are secreted from macrophages in response to invading pathogenic microbes. Excessive secretion of pro-inflammatory mediators cause tissue damage which

is evident in various inflammatory mediated diseases (Allam & Anders, 2008). Therefore, pharmacological intervention targeting the suppression of pro-inflammatory mediators offers an auspicious therapeutic tactic to curb the detrimental activity resulting from pro-inflammatory mediators like prostaglandins (Lin *et al*., 2007). Presently, several anti-inflammatory medications are used for chronic inflammation management, however, they are associated with adverse side effects and have comparatively reduced efficacy (Johnston *et al*., 2008).

Free radicals produced during inflammation, attack membranes and biomolecules thereby intensifying inflammatory process. Free radicals encompass highly reactive molecules containing charged nitrogen and oxygen ions. They include peroxyls and hydroxyls (OH, -OOH and ROO-) which contribute to oxidative stress if not well regulated (Rahman *et al*., 2012). They also participate in the initiation, development and aggravation of neurodegenerative ailments (such as Alzheimer's disease) and other ailments like cardiovascular diseases, aging, Alzheimer, cancer and cataracts among others (Zuo *et al*., 2015). As a remedy to ward off oxidative stress, antioxidants are used to scavenge free radicals thus protecting cells/tissues against oxidative attack. For that effect they can be used as chemotherapeutic agents for management of elevated levels of free radicals (Liu *et al*., 2018).

Plants have for a long period provided mankind with varied medicines for managing many diseases (Inta *et al*., 2013). In developing countries, herbs play a pivotal task in meeting the basic health care necessities in treatment of varied range of illnesses such as

immune system abnormalities, rheumatism, mental disorders, inflammation, circulatory disorders, asthma, bronchitis and epilepsy (Srivastava *et al*., 2011). This is owed to the fact that medicinal plants possess various classes of phytochemical agents that exhibit antioxidative, anticarcinogenic, antimutagenic and immunomodulatory properties which can be attributed to their potential value in treating associated diseases (Punturee *et al*., 2004). Through research, important phytochemicals can be analyzed and isolated so as to be used as chemotherapeutic agents for certain ailments.

Toxicity can be defined as an expression of being poisonous/noxious to mammalian cells. Toxicants interaction with cells and other biomolecules in the body elicit toxic reactions that may impair functioning of organs (kidney and the liver) (Jothy *et al*., 2011). Despite most plant species being used to manage diseases, some species are harmful when consumed as medicine. Some phytochemicals are toxic to body cells, for instance, Some alkaloids have been reported to interfere with neurotransmitter systems (Ifeoma & Oluwakanyinsol, 2013). Besides, some terpenes (lipid soluble terpenes) inhibit mammalian cholinesterase (Savelev *et al*., 2004). Therefore, there is need to assess safety levels of herbal products and validate their use.

Launaea cornuta is traditionally used in African countries like Kenya to treat swollen testicles, ear-ache and joint pains/algesia (Masinde, 2010; Wambugu *et al* ., 2011). The Embu and Pokot people use *L cornuta* to treat/manage inflammatory conditions. In addition, it is also thought to contain ascorbic acid (vitamin C) hence considered antioxidant (Schippers, 2004). However, no scientific research has been conducted on this plant/herb to confirm its anti-inflammatory effects, antioxidant activity, safety levels

and phytochemical composition. It is, therefore, imperative to perform research on medicinal plants like *L. cornuta* based on their ethno-medical usage towards identifying novel molecules with elevated efficacy and reduced adverse reactions for the management of inflammatory-allied ailments (Islam *et al*., 2012). This research, therefore, is intended to find out the anti-inflammatory activity, antioxidant activity, toxic effects and phytochemical composition of aqueous root extract of *Launea cornuta*.

1.2 The Statement of the Problem

Prolonged inflammation like in the case of chronic inflammation can be injurious and life threatening as involved in pathologies such as rheumatoid arthritis, asthma, diabetes among other diseases (Salvemini, 2003). Furthermore, management of inflammation-associated pathologies is an up-hill task since the available conventional drugs have negative impacts/side effects. The drugs used include corticosteroids, Nonsteroidal anti-inflammatory drugs (NSAIDs), opiates and antioxidant supplements such as Vitamin C.

1.3 Justification

Regardless of the healing capabilities of the anti-inflammatory drugs, they have adverse side effect including heart attack, stomach ulcers, liver and kidney diseases (Sun *et al*., 2007). The continuous use of NSAIDS result in the suppression of the cyclooxygenase (COX) enzyme and production of prostaglandins. This leads to gastric erosion that causes ulcers and upper gastrointestinal bleeding, and later may result in death in some individuals (Derle *et al*., 2006). In addition, these synthetic molecules are expensive to purchase and also need qualified medical personnel who are always not readily available

to prescribe them.

To manage detrimental effects of inflammation and evade the negative outcomes of NSAIDs, scientists are tasked/obligated to search for safe anti-inflammatory chemotherapeutic agents from medicinal herbs. In Kenya and the world at large, there are immeasurable varieties of medicinal plants whose active agents manage/treat many diseases of any nature (inflammatory diseases).

Therefore, this study was aimed at confirming the use of *Launaea cornuta* in management of inflammation and oxidative stress as well as assessing its safety levels and phytochemical composition. This study is important in development of alternate anti-inflammatory drugs/agents that have no or less side-effects.

1.4 Research Questions

i. What are the *ex vivo, in vitro* and *in vivo* anti-inflammatory activities of aqueous root extract of *L. cornuta*?

ii. What are the *in vitro* antioxidant activities of aqueous root extract of *L. cornuta*?

iii. Does aqueous root extract of *L. cornuta* have acute oral toxicity effect in mice?

iv. What is the qualitative phytochemical profile of aqueous root extract of *L. cornuta*?

1.5 Objectives

1.5.1 General Objectives

To determine anti-inflammatory activity, antioxidant activity, toxicity effects and qualitative phytochemical analysis of aqueous root extract of *Launaea cornuta*.

1.5.2 Specific Objectives

i. To evaluate *ex vivo*, *in vitro* and *in vivo* anti-inflammatory activities of aqueous root extract of *L. cornuta.*

ii. To determine the *in vitro* antioxidant effects of aqueous root extract of *L. cornuta*.

iii. To evaluate acute oral toxicity of aqueous extract root of *L. cornuta*.

iv. To determine qualitative phytochemical profile of aqueous root extract of *L. cornuta*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Immune Response

Immunity is the body's defense mechanism against potentially harmful substances by recognizing and responding to surface molecules of cells such as viruses, fungi and bacteria (Kumar *et al*., 2004; Leelaprakash *et al*., 2011). Immune system also recognizes and responds to harmful chemicals, toxic substances, drugs, and other foreign particles like allergens (Islam *et al*., 2012).

2.2 Inflammation

Inflammation is a complex immune process derived from a latin term *inflammare* whose meaning is to set on fire (to burn) (Abdulkhaleq *et al*., 2018). The body, through inflammation, attempts to protect itself against deleterious stimuli (Perretti & Montero, 2017). The final result is elimination of the stimuli (which could be pathogen, irritants or damaged cells), healing and repair of the affected tissue (Medzhitov, 2008).

Inflammation entails cells of the host, proteins, blood vessels and among other mediators which together co-ordinate to eradicate the stimuli that initiated cell injury. It is however notable that excessive or dysregulated inflammation is harmful as involved in the progression most diseases like malignant tumors/cancer, stroke, arthritis, cardiovascular and neurodegenerative conditions among others (Iwalewa *et al*., 2007; Ricciotti and Fitzgerald, 2011).

2.2.1 Phases of Inflammation

Inflammation manifest itself in two critical stages; acute phase and chronic phase

(Chatterjee *et al*., 2015).

The Acute Phase

Acute phase has instant onset that lasts for a short duration. It is characterized by rapid influx of monocytes (Monocytes form inflammatory macrophages) and inflammatory neutrophils (Amin and Hussain, 2015). The assembly of various cell types of immune system and a large supply of proteins at the site of inflammation initiate and drive the immune response.

The proliferated macrophages and other cells release chemical mediators which interfere with the functioning of cells/tissue leading to the fundamental manifestation of inflammation, which include: dolor (pain), function failure, rubor (redness), tumor (swelling) and color (heat) (Nathan, 2002; Rhen & Cidlowski, 2005; Chatterjee *et al*., 2015). These chief local manifestations signs results from increased blood flow and dilation of blood vessels followed by extravasation of plasma proteins and fluid, and finally accumulation of white blood cells in affected site (Nathan, 2002; Amin and Hussain, 2015)

The redness and warmth in and around the inflamed area result from the increased influx of blood to the affected tissue. The swelling/edema is due to the increased amount of proteins and blood present in this site (Figure 2.1). The pain experienced is as a result of tissue expanding and exerting mechanical pressure on neurones, and the presence of mediators of pain (Amin & Hussain, 2015; Of & Bark, 2016). Increased temperature shifts balance of chemical reactions to favour host's metabolic reactions hence protects body against pathogens. Once an inflammatory process has set in, it lasts until the agent that has stimulated it has been removed (Ricciotti & Fitzgerald, 2011).

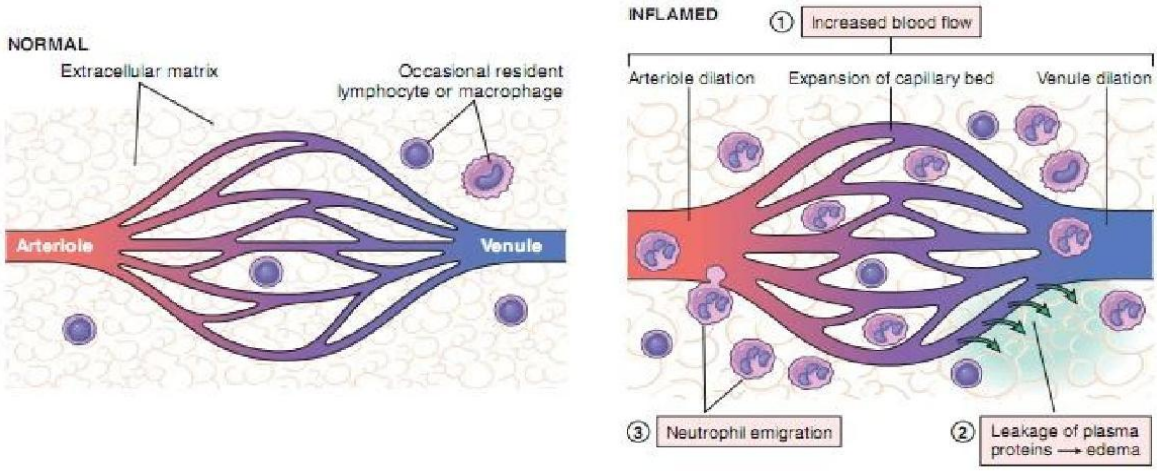

Figure 2.1: Edema due to increased blood and proteins (Adopted from Amin & Hussain, 2015)

Fig 2.1 shows dilated blood vessel, the intention is to increase blood flow to the injured tissue. Leukocytes are assembled at the site of injury so as to engulf pathogens and other foreign noxious stimuli. Pus results from consumption of dead cells during initiation of healing process (Amin and Hussain, 2015). Granulocytes are removed during the resolution process. Lymphocytes and macrophages are reduced to their normal numbers as in pre-inflammatory state. Subsequently acute inflammation resolves successfully with complete repair of damaged tissue (Ricciotti & Fitzgerald, 2011).

Chronic inflammation

This phase lasts for a longer period (days, weeks to years) and histologically manifests by tissues having macrophages and lymphocytes (Amin and Hussain, 2015). It is characterized by persistent aggravation of uncontrolled inflammatory response that might eventually trigger cell death, tissue destruction and organ failure. Therefore, it can be deduced that failure of the body to resolve first phase (acute inflammation) predispose the

host to chronic dysplastic inflammation, autoimmunity and enormous tissue damage (Ricciotti and Fitzgerald, 2011)

Exposure of tissues to inflammation that lasts for an extended duration aggravates degenerative diseases such as acquired immunodeficiency syndrome, atherosclerosis, cancer, alzheimer, rheumatoid arthritis, multiple sclerosis, infections, congestive heart failure, aging, diabetes and heart diseases. All of these are characterized by immuno-pathological processes which are of importance in the initiation and progression of these conditions (Ghai, 2004; Iwalewa *et al*., 2007)

2.2.2 Chemical Mediators of Inflammation (Proinflammatory Mediators)

Stimuli (infections, toxins, trauma) induce the production of chemical factors that induce changes in cells and blood vessels during inflammation (Igbe *et al*, 2010; Newton and Dixit, 2012). Mediators are produced in two ways. They are released, locally, by cells located in affected tissue or by inactive precursors circulating with blood (Medzhitov, 2008). These precursors are activated when they reach the site of insult (Table 2.1) (Ramashish *et al*., 2012).

Chemical mediators of inflammation are excreted primarily from immune cells (nerutrophils, monocytes, basophils, mast cells, platelets, endothelial cells in walls of blood vessels and macrophages) as well as from resident damaged tissue cells (Lu *et al*., 2017). Mediators that originate from cells (cell-derived) are located in granules inside cells and are released rapidly when the cells are activated, for example, histamine release from mast cells (Amin and Hussain, 2015). Alternatively, mediators can be synthesized, *de novo*, in response to stimuli. For example, prostaglandins and nitric oxides synthesis

from white blood cells/leukocytes (Figure 2.2) (Yilmaz, 2001; Newton and Dixit, 2012). Proinflammatory mediators, after secretion, may bind to specific receptors present on cells of interest leading different end consequences depending on the cell type targeted (Iwalewa *et al*., 2007). One cell or more cells can be targeted by a particular mediator with diverse actions. Other mediators like lysosomal proteases employ toxic and enzymatic activities thus do not necessitate binding to specific receptors.

The actions of most mediators are tightly regulated and short-lived (Amin and Hussain, 2015). Once the injurious substance is eliminated, mediators are quickly broken down and degraded. For instance, arachidonic acid metabolites are inactivated by enzymes like kinases.

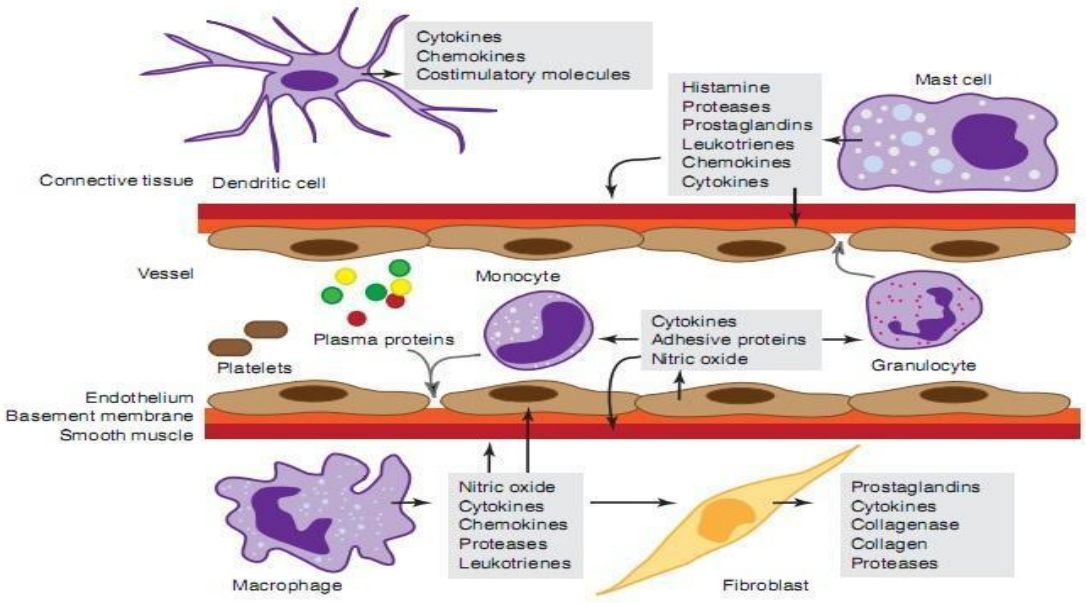

Figure 2.2: Cells and mediators of the inflammation

In response to chemical, physical or infectious stimuli, cells and proteins stimulate inflammation by inducing vascular activation and migration (*Adopted from* Newton and Dixit, 2012)

Table 2.1: Principal mediators of the inflammation and their effects

Mediator	Source(s)	Effects
Histamine	Basophils, Platelets, Mast cells	Blood vessels permeability Endothelial activation Vasodilation
Serotonin	Platelets	Constriction of blood vessels
Leukotrienes	Leukocytes, Mast cells	Chemotaxis, Leukocyte adhesion and activation Vascular permeability
Prostaglandins	Leukocytes, Mast cells	Pain, Vasodilation, fever
Platelet-activating factor	Leukocytes, Mast cells	Leukocyte adhesion, Degranulation, Vasodilation, Vascular permeability, Oxidative burst
Nitric oxide	Macrophages, Endothelium	Killing microbes, Relaxation of vascular muscle
Chemokines	Activated macrophages, Leukocytes	Leukocyte activation, chemotaxis
Reactive oxygen species	Leukocytes	Tissue damage, killing bacteria and other microbes
Cytokines (IL-1,IL-6, TNF)	Mast cells, Edothelium, Macrophages cells	Activation of endothelium, stimulate production of activation molecuels Systemic – metabolic abnormalities, fever, low blood pressure

(Adopted from R. Kumar *et al*., 2012)

2.3.1.1 Prostaglandins

Prostaglandin, a lipid mediator, is an eicosanoid synthesized from arachidonic acid (Ricciotti and Fitzgerald, 2011). Eicosanoids (Prostaglandins (PG), thromboxanes (TXs) and prostacyclins (PGI)) are structurally similar and belong to same family of lipid mediators (Kubata *et al*., 2007). PGs induces pain, vasodilation and platelet aggregation among other tissue-dependent functions (Yilmaz, 2001).

Eicosanoids originate from phospholipids break down as illustrated in figure 2.3 (eicosanoids pathway). When cells are stimulated, membranes (nuclear membranes and

endoplasmic reticular) containing phospholipids are broken down by action of phopholipase A_2 to release arachidonic acid. Arachidonic acid is further broken down by cyclo-oxygenase (I or II or both) enzymes to produce endoperoxides (PGH_2). The enzyme, cyclooxygenase (COX) involved in cyclooxygenation reactions also takes part in the reduction of a hydroperoxyl in PGG_2 to a hydroxyl generating PGH_2 (Simmons *et al*., 2004). PGH_2 is finally converted to (i) prostaglandins (PGE_2, PGD_2, PGF_{2a}) by prostaglandin isomerase, (ii) thromboxane-A_2 (TXA_2) by thromboxane synthase and (iii) Prostacyclin (PGI_2) (Murakami and Kudo, 2004; Kubata *et al*., 2007; Ricciotti & Fitzgerald, 2011).

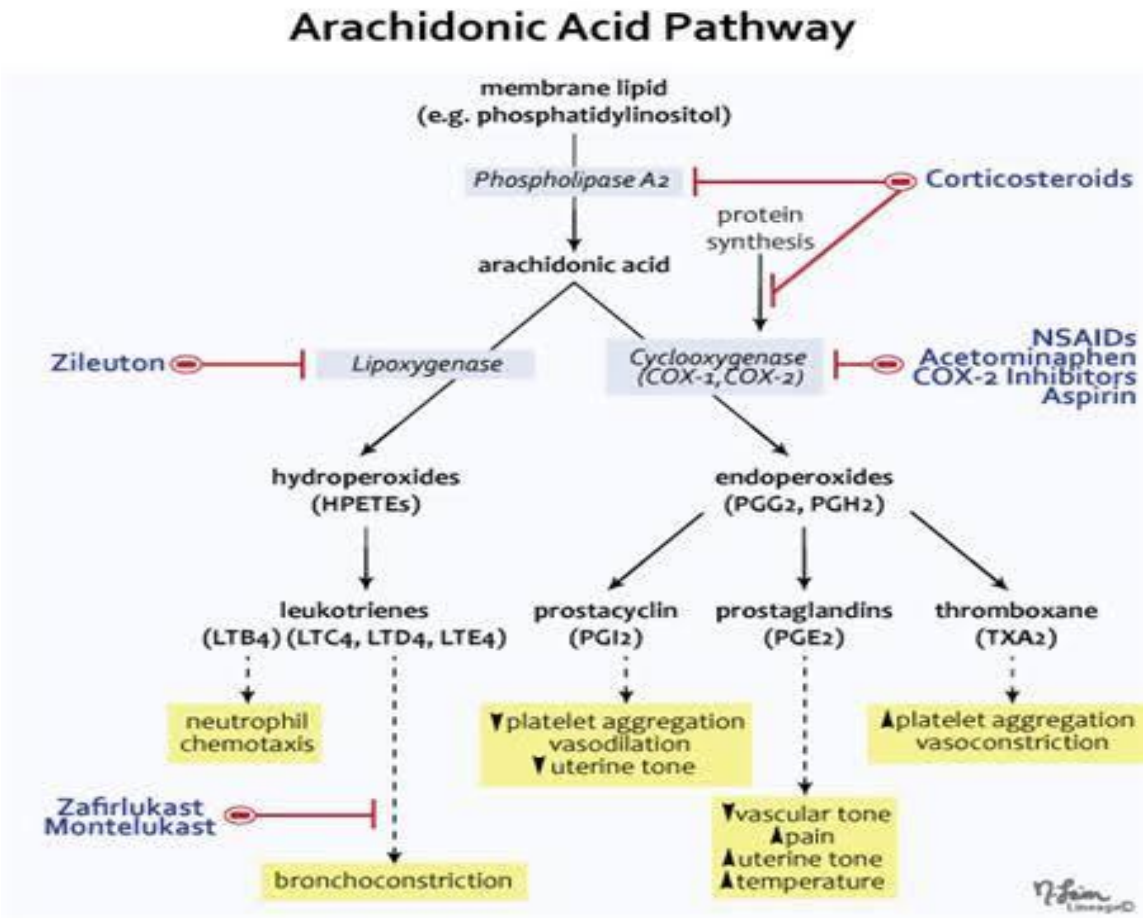

Figure 2.3: Eicosanoids pathways (Adopted from https://step1.medbullets.com/msk/112051/arachidonic-acid-products)

PGE_2 has been shown to regulate various functions of immune cells including being one of the key mediators that induce changes in blood vessels and blood flow. These vascular changes cause usual signs of inflammation which include redness, swelling, pain, fever and if it persists overtime may lead to loss of function. Swelling/edema and redness are attributed to increased permeability of blood vessels and increased blood flow to affected/inflame tissue. Pain is cause by increased pressure to never ending by dilated blood vessels and also by action of Prostaglandin ($PGE_{2)}$ on nervous system (peripheral

sensory neurons and central nervous system) (Ricciotti and Fitzgerald, 2011). Despite the fact that it a mediator of local inflammation through stimulating vasodilatation, local attraction and immune cells (neutrophils, macrophages, and mast cells) activation at early inflammatory stages, PGE_2 can also trigger the influx of mast cells and macrophages that affect the functions of different innate effector cells (Kalinski, 2012)

Increased levels of PGE_2 is stimulated by cytokines (produced by activated immune cells) or by pyrogens like lipopolysaccharides (LPS). Selective suppression of COX-2 gene alleviates fever and reduces PGE_2. The activity of both COX (COX-1 and COX-2) can be disrupted by NSAIDs like indomethacin (Murakami and Kudo, 2004; Ricciotti and Fitzgerald, 2011).

COX-1 is has advantageous roles in the body and is present in most tissues since it is important for housekeeping roles for normal body physiological functions such as platelets aggregation and intestinal walls protection (Park *et al*., 2006). COX-2, however, under normal conditions cannot be detected in tissues, but detectable during inflammation following its stimulation by cytokines and other mediators. COX-2 controls cell growth and inflammation, therefore, agents that hinder the its secretion or activity might probable targets against inflammatory conditions and cancer (Hong *et al*., 2002).

2.3 Reactive Oxygen/Nitrogen Species

These are substances possessing unpaired electrons in their chemical structure; unpaired electrons could be one or more (Gürkan, 2008). They can also be molecules that are able to disintegrate and produce more free radicals (Kunwar and Priyadarsini, 2011). Free

radicals produced during chronic inflammation destabilizes biomolecules and membranes hence furthering inflammation (Nemudzivhadi & Masoko, 2014).

2.3.1 Sources of Reactive Oxygen Species

Reactive oxygen species arise from both normal cellular metabolism processes and exogenous sources (Fig 2.4) (Lai *et al*, 2011) . Imbalance of radicals and antioxidants in the body leads to increased production of more free radicals by redox reactions in organs and tissues. Some of the oxygen's free radicals produced from redox mechanisms include superoxides, hydrogen peroxide, nitric oxide, hydroxyls and peroxides (Rahman *et al*., 2012). For instance, in electron transfer chain (ETC) oxygen takes up electron to form superoxide radical which again takes up electrons to form hydrogen peroxide and later hydrogen peroxide is converted to hydroxyl radical. (Pizzino *et al*., 2017).

In addition, free radicals in the body are generated through respiratory burst. Respiratory burst is an inflammatory response for body's defense against harmful substance, it occurs when immune cells (mononuclear cells and polymorphonuclear cells) such as phagocytes and macrophages are stimulated to release various proinflammatory mediators and noxious molecules such as hypochlorous acid, superoxides and hydrogen peroxides in order to eradicate invading harmful microorganisms (Biller-Takahashi *et al*., 2013). It is worth noting that non-immune cells can also be induced by cytokines to produce large amounts of NO that affect the neighbouring cells (Miguel, 2010). For instance, cytokine-activated endothelial cells have been demonstrated to lyse tumour cells and induce NOs (Förstermann & Sessa, 2012).

Other source of the free radical species include the external environment such as smoke,

ultraviolet radiation, pesticides and air-pollution (Gürkan, 2008).

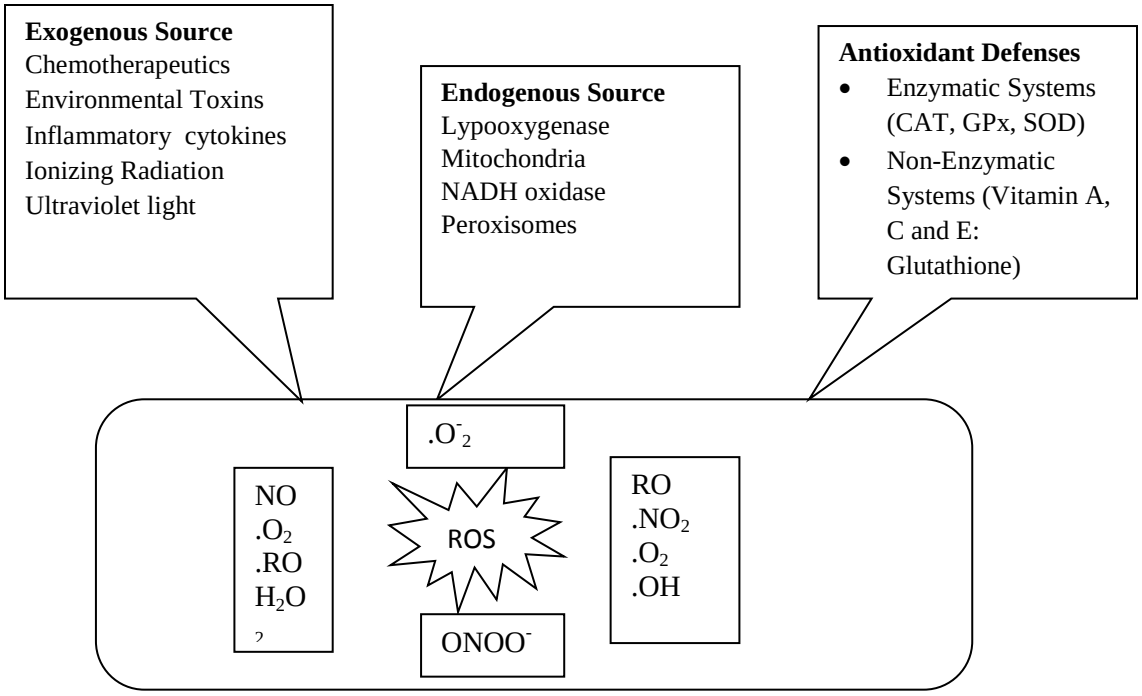

Figure 2.4: Flow chart diagrams showing the body sources of free radicals

(adopted from Rahman *et al.*, 2012) ($ONOO^-$ = Peroxy nitrite ion, H_2O_2 = Hydrogen Peroxide, .RO = Alkoxy radical, $.O_2$ = Oxygen radical, NO = Nitric Oxide, .OH = Hydroxyl radical, $.NO_2$ = Nitrogen dioxide, $.O^-_2$ = Superoxide anion radical)

2.3.2 Role of Reactive Oxygen Species/Free Radicals

Normally regulated physiological and biological cellular processes, that are vital to human beings, constantly produce normal levels of free radicals (Pizzino *et al.*, 2017). These intermediates have great importance in most vital processes (biological) like neurotransmission, vasodilation, inflammatory regulation, bronchodilation and defense mechanism against micro-organism (Alderton *et al*., 2001). For instance, Nitric oxide

(NO) impact on immune defense as microbicidal chemical. Besides, NO regulates vascular tone, nerve transmission and inflammatory processes in the host. In addition, superoxide is used as by-product of cellular processes (Hamilton *et al*., 2004).

Free radicals, apart from destroying invading pathogens, are signaling molecules that impact the cell proliferation and differentiation (Lu *et al*., 2017). Additionally, they do regulate existence and functioning of immune cells hence by so doing also regulates reactions that are harmful to cells and tissues. In certain forms of inflammation, reactive oxygen species are generated at high levels in order to counter deleterious effect of noxious stimuli (Tripathi *et al*., 2007). However, high levels of free radicals is disastrous as in the case of chronic inflammation; this is one of the basis of most pathological conditions (Loscalzoand Welch, 1995).

2.3.3 Oxidative Stress

Increased rate of dysregulated oxidation produces high amounts of free radicals that amounts to oxidative stress (free radicals and antioxidants imbalance) (Bulbul *et al*., 2011). High levels of free radicals (Oxidative stress) is usually harmful since it causes cell and tissue damage as illustrated in Fig 2.5 (Bellance *et al*., 2009; Lu *et al*., 2017; Pizzino *et al*., 2017). The mechanism of actions of these damaging substances (free radicals) is attributed to existence of unpaired electron in their outer shell which makes them highly reactive. They obtain stability by taking electrons from biomolecules in the body (Bengal, 2010).

Oxidation results in removal of electrons from biomolecules by unstable compounds, subsequently biomolecules are disrupted and therefore may lead to loss of structure

which finally impairs normal functioning of the affected biomolecules/structures. For instance, if the reaction involves DNA and proteins the end result is formation of adducts and cross-likages which may lead to loss of function. For example, NO directly disrupt DNA of target cells causing strand breaks and fragmentation, while, Peroxynitrite reacts with lipid and protein molecules producing lipid peroxides which through chain reaction produce in cascade more free radicals (Sakat *et al*., 2010). It is believed that a combination of these effects forms the basis of pathogenesis of most serious and life-threatening conditions (diseases) (Kunwar and Priyadarsini, 2011).

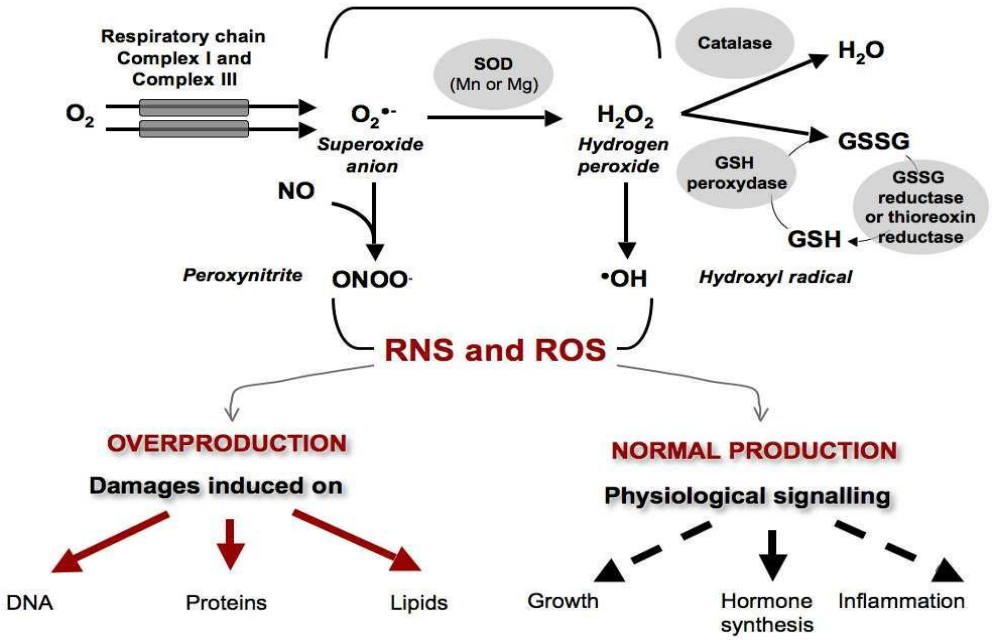

Figure 2.5 Generation and effects of reactive oxygen species. (Adopted from Bellance *et al*., 2009.)

2.3.4 Oxidative Stress and Human Disease

Oxidative stress and nitro active stress implicate negatively on metabolic

processes/events at cellular and extracellular level (Di Meo *et al*., 2016). Free radicals are of great importance to body's normal functioning when maintain in normal levels. They required for muscle contraction and signaling, however, overproduction is injurious since it can lead to disastrous interaction with proteins, DNA and lipids (Bellance *et al*., 2009).

Oxidative attack is one of the major drivers of that contribute to aggravation of almost all known chronic inflammatory-associated ailments (cancer, arthritis, atherosclerosis, chronic fatigue syndrome and malaria) (Lobo *et al*., 2010; Rahman *et al*., 2012; Lu *et al*., 2017). For instance *Helicobacter pylori* bacteria partakes in production of superfluous free radicals in the stomach therefore increases the risk of developing cancer (gastric cancer) (Brenneisen and Reichert, 2018). In addition, development and exacerbation of Alzheimer disease and some age-associated neurodegenerative conditions are attributed to products of lipid peroxidation such as monialdehyde, 4-hydroxy-2,3-noneal (HNE), f2-isoprostanes and acrolein (Bulbul *et al*., 2011; Förstermann & Sessa, 2012).

Since oxidative stress contributes immensely to inflammation, reducing levels of free radicals is one of the appropriate ways of preventing continuous and unending inflammation in chronic conditions/diseases. Reports have shown that antioxidants can reduce or rather get rid of oxidative stress in the body (Prasad and Ramakrishnan, 2012). Therefore, scholars for decades have been working on various studies and research work on herbal plants in order to discover novel agents to counteract effects of free radicals and prevent damages caused. Plants (vegetables and fruits) possess phytochemicals and other bioactive components that counter and neutralize free radicals (Kopaei *et al*., 2013).

Not only do plants have antioxidants, they also contain other compounds that protect and cure our bodies against various diseases. This has boosted the need to search for natural/novel anti-oxidants.

2.4 Antioxidants (Regulation of Reactive Oxygen Species)

These are molecules with capability of scavenging/neutralizing free radicals to maintain their normal levels and prevent oxidative stress from building up. They are chemicals whose functions is to counter free radicals in order to prevent oxidation of biomolecules (Charles, 2013). Antioxidants express their activity in various ways; they inhibit oxidation of biomolecules, act as reductants, scavenge free radicals and stimulate endogenous enzymes (Bulbul *et al*., 2011; Banjarnahor & Artanti, 2014).

Antioxidants are of two groups: those that are produced by the body's complex mechanisms (endogenous antioxidants) to quench free radicals and exogenous antioxidants that are mostly obtained from diet like some vegetables and fruits are rich in antioxidants such as vitamins E and C (Prasad & Ramakrishnan, 2012; Starlin & Gopalakrishnan, 2013; Banjarnahor & Artanti, 2014).

Our bodies have complex mechanisms that produce antioxidants for protecting cells and tissues from reactive oxygen species during oxidative stress, these include enzymatic and non-enzymatic reactions. Cytosolic enzymes involved in regulation of ROs include Catalase enzyme, Superoxide Dismutase enzyme and Glutathione (reduced) enzyme (Rahman *et al*., 2012). Glutathione (GSH), selenium, urate, plasma protein thiols, dihydrolipoic acid, transferrin/ferritn (iron binding proteins), histidine peptides and

melatonin are examples of non-enzymatic substances that quench ROS by donating an electron (Prasad & Ramakrishnan, 2012; Starlin & Gopalakrishnan, 2013). Nonetheless, body's mechanisms can be overpowered especially in chronic conditions that produces excess free radicals perpetually (Patel *et al*., 2010). Failure of endogenous antioxidant systems, to quench free radicals, causes oxidative stress which subsequently aggravate disease. (Duganath *et al*., 2010). This calls for reinforcement with exogenous antioxidants.

Exogenous antioxidants in plant extracts (flavonoids) also could reinforce endogenous antioxidants when the endogenous mechanisms are overwhelmed due to build-up of oxidative stress (Banjarnahor & Artanti, 2014). Reinforcing the body with exogenous supplements boosts antioxidants levels in the cells which together with endogenous sources work to reduce increased levels of free radicals in the cells. Therefore, external antioxidants are crucial in restoring oxidative balance in the body.

2.5 Safety Concerns of Herbal Drugs

There is increasing demand and use of herbal medicine all over the world, however, little is known on toxic composition of majority of these plant products. Therefore, there is need to assess safety levels of herbal products and validate their use. Conducting toxicity assays exposes toxic effects of herbs so as to avoid their harmful effects when consumed as medicine.

Some plants defend themselves against insects and herbivorous by producing phytochemicals that are harmful. The same phytochemicals are as well toxic to humans

since biomolecules (nucleic acids) of targeted organisms have some degree of resemblances to human biomolecules (Ifeoma and Oluwakanyinsol, 2013).

Some alkaloids have been reported to interfere with neurotransmitter systems (Ifeoma and Oluwakanyinsol, 2013). Some terpenes (lipid soluble terpenes) inhibit mammalian cholinesterase (Savelev *et al*., 2004). Saponins have been found to be potent surfactants which have the ability to disrupt cellular membranes that are rich in lipids such as those of human erythrocytes (Ifeoma & Oluwakanyinsol, 2013). Some terpenoids increase the activity of inflammatory-associated mediators such as the NO radical instead of inhibiting it (Gouveia *et al*., 2011). Presence of heavy metals (lead, mercury, cadmium and arsenic) and toxic minerals render some herbs toxic (Dwivedi & Dey, 2002). Therefore, it is prudent to conduct safety biossays on herbal medicine like *Launaea cornuta* to validate their use in traditional medicine.

2.6 Conventional Management of Inflammation and Oxidative Stress

The currently available agents for inflammatory diseases, exert their effects by mainly inhibiting the synthesis and activity of major mediators that participate in inflammation (Tung *et al.*, 2008). Corticosteroids, narcotic and NSAIDs include current mainstay for countering inflammation. These available agents show decreased efficacy on diseases (inflammatory) due to their serious adverse reactions. Steroidal drugs have specific mechanisms of action hence are used as anti-inflammatory agents. However, steroidal anti-inflammatory drugs inhibit critical basal physiological functions like inhibition of leukotriene hence may cause high blood pressure, potassium loss among other side-effects. In addition, side-effects caused may be due to similarity of the steroid hormones

to steroidal drugs. On the other hand, NSAIDs have relatively fewer and less adverse effects than the steroidal counterparts, the side effects of NSAIDs are impaired blood clotting process and bleeding in gut among others (Burk *et al.,* 2010)

Therefore, there is a need for treatment options that have less or no side effects and have high potency. The selective inhibition of COX-2 can offer a possible solution to the anti-inflammatory agent that has these characteristics. Selective inhibition of COX2 activity averts inflammation without interfering with the normal functioning of COX 1 (Miguel, 2010). Celecoxib and roxecib are currently available agents/drugs capable of inhibiting COX-2 selectively. (Kaur *et al*., 2010). These clinical drugs have been found to be effective selective COX-2 inhibitors. However, they are, to some extent, toxic to the heart, kidney and liver because of preventing release of COX-2 to low levels that cannot support normal physiological processes (Kaur *et al*., 2010). Such disasterous effects triggered banning of some remedies/drugs such as rofecoxib and valdecoxid drugs (Sun *et al*., 2007).

Currently, there are no specific antioxidant drugs prescribed for inflammation, however, antioxidants such as Vitamin E are used as supplements to manage inflammation. Akkol *et al.* (2012) demonstrated that Vitamin E is potent COX-2 inhibitor. Synthetic antioxidant (butylated hydroxyanisole and butylated hydoxytoluene) are used as supplements (additives) to remove free radicals from food, however, they are carcinogenic and toxic to body organs (Seo *et al*., 2012).

Since the available conventional antioxidant and anti-inflammatory agents have exhibit

negative outcomes, there is need to search for alternate remedies that are effective and with almost zero or no side effects. So many plants are utilized in herbal medicine to manage inflammation, exploring their healing capabilities through research may lead to discovery of safe agents. This will alleviate pain from millions of people ailing from chronic and degenerative diseases like arthritis (Boukhatem *et al*., 2013).

2.7 Ethnopharmacological Management of Inflammation

A large percentage of the developing countries population depend on traditional herbs to manage health issues including inflammatory illnesses (Matthew *et al*., 2013). Some of the plants used in the treatment of inflammation are *Combretum zeyheri*, *Lippia javanica*, *Cassica abbreviate*, and *Diospyros mespliformis* among others (Zoysac, 1985). The plants used are from across the plant kingdom, however, some families and genera of plant species are more effective than others in treating diseases (Maroyi, 2013). Herbs are considered to be of greater benefit as compared to artificial drugs since they are easily biodegradable, non-narcotic in nature, have minimal environmental hazards and adverse effects and relative affordability (Umapathy *et al*., 2010). Furthermore, herbs are accessible whereas the synthetic drugs and conventional drugs have relatively higher cost (Saleem *et al*., 2011).

Medicinal plants have ability to produce compounds or secondary metabolites (alkaloids, tannins, flavonoids and phenolic compounds) that exhibit therapeutic properties in the body (Chandhur *et al*., 2011). These substances are synthesized from relatively simple molecules such as glucose and water (Ramakrishna & Ravishankar, 2011). These compounds could be lead chemicals to develop effective and safe medicine. Since they

are biological in nature, they are easily metabolized in the body hence have less or no toxic effects as compared to synthetic alternatives (Kaur *et al*., 2010). The consumption of plant products (fruits) have been reported to alleviate inflammatory-associated ailments like diabetes mellitus, arthritis and arteriosclerosis (Iwalewa *et al*., 2007). Several phytoconstituents have been reported to suppress COX-2 (Miguel, 2010). Hence, the healing capabilities of plants should not be underestimated.

Bioactive substances in plants such as vitamins, tannins, flavonoid, carotenoids among others have described to possess antioxidant and anti-inflammatory potency thus justifying usage of some plants to cure illnesses by herbal medicine. Terpenoids have been shown to inhibit COX activity and scavenge free radicals, while phenols interfere with NFKβ gene transcription and prevent lipid peroxidation (Salminen *et al*., 2008). Combination of various phytochemicals confers efficient relief from inflammation due to ability of various phytochemicals to act on more than one target in an attempt to alleviate inflammation.

2.8 Ethnobotanical Description of *Launaea cornuta*

Launaea cornuta (Figure 2.7) belongs to the family Asteraceae (Compositae)(Faith and Jonathan, 2016). The centre of stem, of this perennial herb, is hollow with milky juice and the leaves are alternate, sessile and deeply divided. The highest it can grow to is 1.5m high above the ground. The plant is indigenous in Africa and sometimes called bitter lettuce. It is locally known as, mchunga in Swahili, Muthunga (Meru, Kikuyu and Embu), Mnyinya in Taita, Talwa in Pokot and Achak in Luo. It grows on alluvial type of soils and mostly found in tilled land, including, bush vegetation, road-sides, and river banks. It

spreads by rhizomes and covers a large area. It is the common *Launaea* species in Kenya (Maundu *et al*., 1999).

Figure 2.6: *Launaea cornuta*

2.8.1 Ethnomedical uses of *Launaea cornuta*

Launaea cornuta has diverse uses ranging from being consumed (as a vegetable) in some communities in Africa, for example in some parts of Nigeria and Kenya. It is also thought to contain ascorbic acid (vitamin C) hence antioxidant (Schippers, 2004). A decoction prepared from this plant is used to treat typhoid, gonorrhea, ascariasis, stomach pains and swollen testicles (Masinde, 2010). Moreover, the juice of the leaves is used to relief ear-ache while the roots are traditionally used in managing pain in joints and also in treating warts (Wambugu *et a*l., 2011).

2.9 Inflammation Assays/Techniques

Oedema/swelling is one of the key manifestations of inflammation (Amin and Hussain, 2015). The principle of commonly used *in vivo* anti-inflammatory assays involve use of substances capable of inducing oedema that can be quantified in experimental animals. Carrageenan, formalin and acetic acid are examples of most preferred chemicals in stimulation of oedema (Stark, *et al*., 2013). Carrageenan and formalin induce swelling when injected in sub-plantar tissue of rats and mice while acetic acid induce swelling when smeared on ear pinna of mice and rats.

In this study, carrageenan-induced edema technique was adopted to assess anti-inflammatory effects (*in vivo*) of aqueous *L. cornuta* extract. This technique was considered since it induces swelling effectively. Furthermore, the method is simple, highly sensitive and reproducible (Dzoyem *et al*., 2017). Carrageenan is a carbohydrate (polysaccharide) extracted from a seaweed whose name is *Chrondus crispus.* In Ireland the seaweed is called carraigin while in England it is called Carrageen Moss or Irish Moss (Necas & Bartosikova, 2013).

Once in the tissue of the experimental animal, carrageenan acts as thermal/mechanical stimuli thereby stimulating inflammatory response that eventually amounts to development of edema (Barth *et al*., 2016). The swelling occurs in two phases; each of these phases is characterized by different inflammatory mediators. For instance, detectable mediators in the first phase are serotonin, bradykinin and histamine whereas PGDs are released in the second phase (Chatterjee *et al*., 2015).

Ex vivo anti-inflammatory assays include membrane stabilization techniques (heat-induced hemolysis and hypotonicity-induced hemolysis) while *in vivo* assays antiproteinase technique and protein denaturation inhibition assay. During inflammatory response biological membranes lyse/rapture to release mediators of inflammation, proteins disintegrate eliciting inflammatory response and some enzymes (proteinases) may stimulate inflammatory reactions that intensifies inflammatory process (Sakat *et al*., 2010; Gunathilake *et al*., 2018). Aqueous *L. cornuta* extract was therefore evaluated for its ability to stabilize biological membranes, inhibit protein denaturation and inhibit proteinase enzymes.

2.10 Antioxidant Assays/Techniques

Antioxidants have the ability to neutralize/quench free radicals (Duganath *et al*., 2010). Some of the commonly used assays are 2,2-diphenyl-picrylhydrazyl assay (DPPH), Ferric-reducing antioxidant power (FRAP), metal chelating, H_2O_2 scavenging activities among others (Rameshrad *et al*., 2015; Fathiazad *et al*., 2017). FRAP and DPPH assays were used to test for antioxidant activity/effects.

DPPH molecule (radical form) has an absorbance at 517 nm which disappears when DPPH is mixed with an antioxidant compound. DPPH accepts an electron from antioxidant to form stable diamagnetic molecule (Adjimani, 2015). On the other hand, FRAP assay entails reaction of potassium ferrocyanide with Fe^{3+} ion to yield Fe^{3+} - Fe^{2+} (ferric-ferrous) compound that has absorbance at 700 nm (Adjimani, 2015; Assumpta, *et al*., 2019). Antioxidant reduce formation of ferric-ferrous complex hence the absorbance increases with increase of antioxidant concentration.

2.11 Acute Oral Toxicity Assays/Techniques

Toxicity can be defined as an expression of being poisonous/noxious to mammalian cells. Toxicants interaction with cells and other biomolecules in the body elicit toxic reactions that may impair functioning of organs (kidney and the liver) (Jothy *et al*., 2011). According to OECD, (2008), Acute Toxicity test examines response/sensitivity of the experimental animals when exposed to the test substance for some time. Most preferred experimental organisms are rat, mice, brine shrimp, rabbits and guinea pigs. Administration route, of the test substance, may be intravenous, intramuscular, intraperitoneal, inhalation or by oral gavage. After administration, animals are subjected to strict surveillance for toxicological responses within the first 30 mins and daily (24hrs) for 14 days to cater for delayed toxicities (Oecd, 2008).

2.12 Phytochemical Assays/Techniques

Qualitative phytochemical assessment is aimed at identifying presence or absence of certain phytochemical constituent in plant extracts. The principle of most of the phytochemicals is based on their ability to react with some chemicals/reagents to produce distinct chromophore/colour that is specific to some known phytochemical. Some phytochemicals upon mixing with reagents form froath/foam, emulsions, air bubbles among other observable changes. For example, Saponins form persistent foam when persistent solution is vigorously shaked/agitated (Bibi *et al*., 2012). In addition, reaction of Alkaloids, 1% HCl and Dragendorff's reagent produce orange precipitate (Jared *et al*., 2018). The specific observational changes indicate presence of particular phytochemical.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection and Preparation

Fresh roots of *L. cornuta* were collected, with help of an herbalist, from Irangi forest located in eastern side of Mt. Kenya, Embu County in Kenya (S 0° 20' 51.4716", E 37° 27' 4.32"). The plant was first identified by local herbalist then later authenticated by a taxonomist (Voucher number: GM-005-2017) in National Museum of Kenya. Herbarium samples were deposited at Kenyatta University, Department of Plant Science.

The obtained plant samples were cleaned with water to remove dirt/soil particles and air-dried, under shade, at 25ºC ± 1 for ten days. The samples were grabbled frequently to aid even drying. Finally, the dried roots were milled (using electric-plant mill), put in khaki envelopes and stored (at RT 25ºC ± 1.) awaiting aqueous extraction.

3.2 Preparation of Aqueous Extract

The powdered sample (50 g) of *L. cornuta* roots was heated in 200 ml distilled water for about 5 minutes, cooled to RT (25ºC ± 1), decanted, filtered (Whatman No. 1 filter paper; vacuum pump), lyophilized (freeze-dried) for 48 hours and transferred to clean-dry pre-weighed universal bottle which were then weighed.

The %yield was obtained by the formula (Eq. 1) stipulated by Bibi *et al*. (2012);

$$Wt\ of\ extract = Wt\ of\ the\ bottle\ with\ the\ extract - wt\ of\ empty\ bottle$$

$$\%\ Yield = \frac{Wt\ of\ the\ extract}{Wt\ of\ the\ macerated\ sample}\ x\ 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(Eq. 1)}$$

Where wt represent weight.

Air-tight sealed universal glass bottle containing the extract were then stored in refrigerator (4^oC) awaiting bioassays.

3.3 Determination of *ex vivo* Anti-Inflammatory Effects

3.3.1 Determination of Membrane Stabilization Activity

The human erythrocyte membrane stabilization test employed protocols of Oyedapo & Famurewa (1995) and Sakat *et al*. (2010) with modifications in concentrations.

3.3.2 Preparation of Extract Concentrations

Four experimental concentrations were used in this study; (1000, 100, 10 and 1 µg/ml). To obtain a 1000 µg/ml extract concentration, 0.01 g of the extract (*L. cornuta*) was put in 10 ml normal saline. The rest of 100, 10 and 1 µg/ml extract concentrations were obtained by 10-fold serial dilutions of the solutions with 1000 µg/ml extract concentration. The same protocol was applied when preparing standard drug (Indomethacin) concentrations.

3.3.3 Preparation of Human Red Blood Cells (HRBCs)

HBRCs samples were acquired from the National blood bank, at Kenyatta National Hospital, Nairobi, Kenya. About 10 ml blood and 10 ml Alseiver's solution (aseptic; anticoagulant) were mixed together and then subjected to centrifugation (3000 rpm; 10 min). The obtained packed RBCs were rinsed (3 times) using isosaline (0.9 % NaCl, pH 7.0). The 10 % (v/v) erythrocytes suspension was prepared in isosaline for the HRBC stabilization tests.

3.3.4 Hypotonicity-Induced Haemolysis

This was done as follows; About 1 ml of the four concentrations of *L. cornuta* aqueous extracts (1000, 100, 10 and 1 µg/ml) were separately dissolved in Phosphate buffer (`1ml, 150 mM, pH 7.4), HRBCs suspension (0.5 ml) and 2 ml hyposaline (0.36 % KCl). For positive control, four concentrations (1000, 100, 10 and 1 µg/ml) of the reference drug, Indomethacin, were also treated the same way as the extract concentrations. The obtained solutions went through incubation process (37^{o}C; 30 min) and eventually centrifuged (3000 rpm) (Microprocessor based centrifuge) for 5 minutes. The supernatant was aspirated and the haemoglobin's absorbance obtained at 560 nm (Microprocessor Ultraviolet-Visible spectrophotometer). Negative control comprised 1 ml normal saline, 1 ml phosphate buffer, 0.5 ml HRBCs suspension and 2 ml hyposaline. This experiment was conducted in triplicates. The % of RBC membrane protection/stabilization was obtained using the formula (Eq. 2) described by Oyedapo & Famurewa (1995) and Sakat *et al*. (2010);

$$\%\ stabilization = 100 - \left[\frac{Abs\ of\ Sample}{Abs\ of\ control} X100\right] \ldots\ldots\ldots\ldots\ldots (\text{Eq. } 2)$$

3.3.5 Heat-induced Haemolysis

This was done as follows; About 1 ml of the four concentrations of *L. cornuta* aqueous extracts (1000, 100, 10 and 1 µg/ml) were separately mixed with 1 ml 10% RBCs suspension. For positive control, four concentrations (1000, 100, 10 and 1 µg/ml) of the reference drug, Indomethacin, were also separately mixed with 1 ml 10% RBCs suspension. The obtained solutions were subjected to high temperatures of 56ºC for a period of 30 minutes, followed by cooling and centrifugation for 5 min (2500 rpm). The

absorbance/turbidity was obtained at 560 nanometers (Microprocessor Ultraviolet-Visible spectrophotometer). Negative control had 1 ml normal saline and 1 ml 10% RBCs suspension. This experiment was conducted in triplicates. Percentage protection or stabilization was obtained using the formula(Eq. 3) stipulated by Oyedapo & Famurewa (1995) and Sakat *et al*. (2010);

$$\%\ stabilization = 100 - \left[\frac{Abs\ of\ Sample}{Abs\ of\ control} X100\right] \ldots\ldots\ldots\ldots\ldots (\text{Eq. } 3)$$

3.4 Determination of *in vitro* anti-inflammatory activities

3.4.1 Determination of antiproteinases activity

This was also carried out according to Oyedapo & Famurewa (1995) and Sakat *et al*. (2010) protocols. About 1 ml of the four concentrations of *L. cornuta* aqueous extracts (1000, 100, 10 and 1 µg/ml) were separately mixed with TrisHCl buffer (20 mM, 1ml, pH 7.4) and trypsin (0.06 ml, 10 µg/ml). Same set-up was used for the four different concentrations (1000, 100, 10 and 1 µg/ml) of indomethacin. Obtained mixtures went through incubation (37^{o}C; 5 min) after which casein (0.8 % w/v, 1 ml) was added followed by further incubation for 20 min at 37^{o}C and eventually addition of perchloric acid (2ml, 70%) followed to stop the reaction. Negative control had normal saline (1 ml), Tris HCl buffer (1 ml), 0.06 ml trypsin, 1ml casein and 2 ml perchloric acid. Centrifugation was done and absorbance of supernatant obtained at 210 nm (Microprocessor UV-VIS Spectrophotometer; Double beam). This experiment was conducted in triplicates. Percentage antiproteinase activity was obtained using the formula (Eq. 4) stipulated by Oyedapo & Famurewa (1995) and Sakat *et al*. (2010);

$$\%\ antiproteinase\ activity\ of\ trypsin = \left[\frac{Abs\ of\ Control - Abs\ of\ Sample}{Abs\ of\ control} X100\right] \ldots\ldots\ldots\ldots\ldots (\text{Eq. } 4)$$

3.4.2 Inhibition of Albumin Denaturation

Bovine albumin denaturation assay adopted Mizushima and Kobayashi (1968) and Sakat *et al*. (2010) protocols. Briefly, about 1.5 ml of the four concentrations of *L. cornuta* aqueous extracts (1000, 100, 10 and 1 µg/ml) were separately mixed with 1 % bovine albumin (1.5 ml) aqueous solution. Just like the extract, four concentrations (1000, 100, 10 and 1 µg/ml) of positive control (Indomethacin) were mixed with 1.5 ml of 1 % bovine albumin aqueous solution. Mixture of 1 ml normal saline and 1.5 ml albumin solution were used as negative control. Resultant mixtures were then put in water bath (37 ºC) for 20 min, then subjected to high temperatures (51ºC) for 20 min and left to cool to RT. Finally, turbidity of the mixture was determined using Microprocessor UV-VIS Spectrophotometer (Double beam) at 660 nm. The assay was conducted in triplicates; % protein denaturation was obtained using the formula (Eq. 2) described by Oyedapo & Famurewa (1995) and Sakat *et al*. (2010);

$$\%\ inhibition\ of\ protein\ denaturation = \left[\frac{Abs\ of\ Control - Abs\ of\ Sample}{Abs\ of\ control} X100\right] \ldots\ldots\ldots\ldots\ldots (\text{Eq. } 5)$$

3.5 Evaluation of *in vivo* Anti-Inflammatory Activities

3.5.1 Experimental Animals

Swiss-Albino mice (4 - 5 weeks old, 25 ± 2 g bw) were used to determine in vivo anti-inflammatory activity and acute oral toxicity effects of aqueous root extract of *L. cornuta*. These animals were obtained from research center (Kenya Medical Research Institute) and kept in propylene cages in animal handling facility in Kenyatta University. Wood shavings were put in the cages to act as bedding. The animals received water (*ad-libitum*) and food (pellets diet). They were exposed to natural day/night cycle, 65% humidity, room temperature and 360 lux lighting (natural light conditions). They were kept to acclimatize for five days prior to subjecting them to the study. The use of animals was permitted (NACOSTI/P/21/9936) by National Commission of Science, Technology and Innovation in Nairobi, Kenya. Humane management and international standard ethical guidelines by American Institute of Laboratory Animals Resources, Kenyatta University and Public Health Service (PHS) Policy on Humane Care and Use of Laboratory animals (IAUC) were observed.

3.5.2 Induction/Stimulation of Inflammation

Paw edema was induced in Swiss albino mice in accordance with protocols described by Mujumdar and Misar (2004), Boukhatem *et al*. (2013) and Rahman, (2013). carrageenan (1%; 100 µl) was introduced into the subplantar tissue via injection in order to induce oedema/swelling; oedema represented inflammation.

3.5.3 Experimental Design and Bioassay

This study adopted a completely-controlled-randomized study design, from which experimental design (repeated measures/within groups experimental design) was adopted. Animals were selected on random basis and placed in 7 groups each consisting of 5 mice. The decision to use 7 groups was based on piloting study that was conducted prior to performing actual study, and was also based on previous similar research. At the onset, group I and II (normal and negative control groups) received normal saline 0.9 % NCl (10 ml/kg b.w) *p.o,* group II (positive control animals) received dexamethasone (4.00 mg/kg bw) *p.o* while test groups IV to VII received extract doses of 31.25, 62.50, 125.00 and 250.00 mg/kg bw *p.o* respectively. After half an hour, oedema was induced in all mice except in normal control group (Table 3.1).

Table 3.1: Experimental design for *In vivo* antiinflammatory activity determination

	T
	; b.w) *p.o*
	: (10 ml/Kg b.w) *p.o* + Carrageenan (100 µl)
	ne (4.00 mg/Kg b.w) *p.o* + Carrageenan (100 µl)
	; mg/Kg b.w) *p.o* + Carrageenan (100 µl)
	) mg/Kg b.w) *p.o* + Carrageenan (100 µl)
	)0 mg/Kg b.w) *p.o* + Carrageenan (100 µl)
	)0 mg/Kg b.w) *p.o* + Carrageenan (100 µl)

Extract: aqueous *L. cornuta* root extract. Gp = Group, NS = Normal Saline

The change in paw size was measured immediately after inducing inflammation (0 hr) and periodically at 1, 2, 3, and 4 hrs, in that order. Vernier caliper was used in measuring

paw volume/paw size. Percentage (%) change in paw size (paw diameter) was obtained using the formula (Eq. 6) stipulated by Rahman *et al.* (2013);

$$\%\ edema\ inhibition = \left[\frac{Vc - Vt}{Vc}\right] X100 \quad \ldots\ldots\ldots\ldots\ldots (\text{Eq. } 6)$$

Where

Vc represent mean paw size/volume (control)

Vt represent mean paw size/volume (treatment)

3.6 Determination of Acute Toxicity

This was done following the Up-and-Down-Procedure (UDP) stipulated by OECD (2008). The experimental animals were not supplied with food (fasted) for four hours prior to subjecting them to study. The animals were orally administered with respective aqueous root extract of *L. cornuta*, after which, they were observed for 14 days for any sign of toxicity. The wellness parameters that were monitored include appearance of skin fur, salivation, mucous membrane, lethargy, eyes, convulsions, diarrhea, coma, tremors, sleep, mortality and body weight (OECD, 2008).

The first set of animals, consisting of five mice, were orally administered with 175 mg/kgbw of aqueous root extract of *L. cornuta* and were observed for 14 days. OECD guidelines state that when the lethality of the extract is not known the default starting dose is 175 mg/kgbw. First group appeared normal with no sign of toxicity, hence, the extract dose was increased to 550 mg/kgbw for the subsequent group of animals. The last group received dose 2000mg/kgbw after the second group showed no sign of toxicity. Normal control animals at each dose level received normal saline (10 ml/kg bw) orally.

3.7 *In vitro* antioxidant assays

3.7.1 In vitro 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

This was done following protocols stated by Ruiz *et al.* (2008) and modified by Jared *et al*., (2018) was adopted. DPPH is a free radical that has dark-purple colour and has absorbance at 517 nm which decreases when DPPH is reduced to non-radical form by accepting electrons (Ilhami *et al*., 2005). Therefore, free radical scavenging activity can be established as absorption decreases. When DPPH is reduced, the colour changes from dark purple to a colourless solution.

This assay was conducted as follows; About 1.5 ml of the six concentrations of *L. cornuta* aqueous extracts (1000, 100, 10, 1, 0.1 and 0.01 µg/ml) were separately added to DPPH solution (1.5 ml; 0.3 mM). The same amount of DPPH was separately added to 1500 µl of the six concentrations of the positive control (Ascorbic Acid; 1000, 100, 10, 1, 0.1 and 0.01 µg/ml). Resultant mixtures were placed in the dark for 30 min and absorbance was spectrophotometrically (Microprocessor UV-VIS Spectrophotometer; Double beam) determined at 517 nm against negative control which comprised normal saline (1.5 ml) and DPPH (1.5 ml). This assay was executed in triplicates. The % radical scavenging/neutralizing activity (RSA) was obtained through the formula (Eq.7) described by Jared *et al*. (2018);

$$\% \, RSA = \left(\frac{(AO - A)1}{A0} \right) \times 100 \, \ldots\ldots\ldots\ldots\ldots \, (\text{Eq. } 7)$$

where

A_1 = Standard or extract absorbance

A_0 = Control absorbance

3.7.2 *In vitro* Ferric Reducing Power

The principle behind this assay is that antioxidants reacts with $K_3[Fe(CN)_6]$ to form $K_4[Fe(CN)_6].3H_2O$, which later reacts with $FeCl_3$ to produce Prussian blue (ferric-ferrocyanide complex), that is, a blue substance that has absorbance at 700 nm (Assumpta *et al*., 2019).

Protocols postulated by Benzie & Strain (1999) were adopted in this assay. About 1.5 ml of the six concentrations of *L. cornuta* aqueous extracts (1000, 100, 10, 1, 0.1 and 0.01 µg/ml) were separately mixed with 30 mM Potassium Ferricyanide (2500 µl) and Phosphate buffer (pH 6.6, 2500 µl, 200 mM). Same set up was used for the positive control (L-ascorbic acid) of 1000, 100, 10, 1, 0.1 and 0.01 µg/ml concentrations. The obtained mixtures were put in water bath (50°C) for about 20 min then later 600 mM Trichloroacetic Acid (2.5 ml) was added and finally subjected to centrifugation (3000 rmp) for about 10 min. About 2500 µl of the resultant supernatant, 2500 µl distilled water and 500 µl of 6 mM $FeCl_3$ were mixed and their absorbance recorded at 700 nm using Microprocessor spectrophotometer. The negative control had 1500 µl normal saline, 2.5ml phosphate buffer, 2500 µl Potassium Ferricyanide, 2500 µl Trichloroacetic Acid, 2500 µl distilled water and 500 µl $FeCl_3$. The assay was done in triplicates.

3.8 Qualitative Phytochemical Analysis

The aqueous root extract of *L. cornuta* was analyzed as per the following standard protocols.

3.8.1 Alkaloids Assay

About 0.1 g of aqueous root extract of *L. cornuta* and 1% HCl (5 ml) were mixed, gently warmed for 3 minutes in water bath, filtered and subjected to Dragendorff's test and

Mayer's test . In the first test, 100 µl of Dragendorff's reagent were put in 2ml of filtrate. Reddish -brown/orange suspension signaled the existence of alkaloids. In the second test, 100 µl of Mayer's reagent were added to 2ml of filtrate. Emergence of cream precipitate signaled the existence of alkaloids (Usman *et al*., 2009; Jared *et al*., 2018).

3.8.2 Tannins Assay

Nearly 0.5 g of *L. cornuta* extract was mixed with 5 ml of normal saline in a test tube, heated in a water bath and filtered. Thereafter, 150 µl of $FeCl_3$ (0.1 %) solution was added to the filtrate. Blue-green color indicated the existence of tannins (Ayoola *et al*., 2008; Bibi *et al*., 2012; Snehlata *et al*., 2018)

3.8.3 Phenols Assay

The 0.1 g of *L. cornuta* extract was boiled with 70% ethanol (10 ml) in a test tube for a period of 5 minutes in water bath, then filtered and cooled. Two ml was drawn from the filtrate and mixed with 1.5 ml (5 drops) of 5% Ferric chloride. A green suspension was an indication of phenols' presence (Jared *et al*., 2018).

3.8.4 Saponins Assay

Approximately 0.5 g of *L. cornuta* extract was mixed with normal saline (5 ml) in a test tube. The resultant solution was heated in water bath, cooled and shaken vigorously. Persistent frothing that would take >2 minutes indicated presence of saponins (Bibi *et al*., 2012; Jared *et al*., 2018; Snehlata *et al*., 2018).

3.8.5 Coumarins Assay

The aqueous crude extract of *L. cornuta* (0.2 g) and normal saline (2 ml) were mixed in water bath. Resultant solution was then treated with NH_4OH (10%) solution and covered with filter paper (Whatman filter paper) for five mutes. The filter paper was removed and

subjected to UV light (365 nm). Yellow fluorescence would suggest presence of coumarins (Soni and Sosa, 2013).

3.8.6 Glycosides Assay

Three tests were adopted:

(i) **Keller-Killiani Test**. Approximately 0.2 g of aqueous root extract of of *L. cornuta* was mixed with 5 ml of chloroform in a test tube and evaporated to dryness. Glacial acetic acid (0.4 ml; with trace amount of $FeCl_3$) and concentrated sulphuric acid (0.5 ml) were added respectively. Blue acetic layer indicated the presence of cardiac glycosides (Snehlata *et al*., 2018).

(ii) **Borntrager's Test**. About 0.1 g of aqueous root extract of *L. cornuta* was mixed with 1ml of H_2SO_4 acid and boiled for 5 minutes in a test tube, filtered, cooled and mixed with equal amount of chloroform. Emergent chloroform layers were separated and mixed with dilute ammonia while shaking. The color of ammoniacal layer was keenly observed. A rose-pink to red colour showed the presence of anthraquinones glycosides (Jared *et al*., 2018).

(iii) **Modified Borntrager's Test**. Solution was made from about 0.1 g of aqueous root extract of *L. cornuta* in a test tube, 100 µl of Kedde reagent were then added. Appearance of purple color would indicate presence of anthraquinones glycosides (Jared *et al*., 2018).

3.8.7 Flavonoids Assay

Nearly 0.5g of aqueous root extract of *L. cornuta* was dissolved in ethanol; 250 µl of concentrated HCl was then added. Instant emergence of red colour would indicate flavonoid presence. Other method was also employed to confirm presence of flavonoids. Solution of aqueous root extract of *L. cornuta* was mixed witt H_2SO_4 acid (10%) followed

by addition of NaOH (10%) solution. Appearance of yellow colour indicated the flavonoid's presence (Usman *et al*., 2009).

3.8.8 Terpenoids Assay

The extract solution (2 ml) was placed in a test tube. Acetic acid anhydride (5 drops) was added. Thereafter, concentrated H_2SO_4 acid (5 drops) was added carefully. Development of blue-green ring would indicate terpenoids presence (Usman *et al*., 2009).

3.8.9 Steroids Assay

Three drops of the Liebermann-Burchard reagent was added to 1 ml solution of *L cornuta* extract. Reddish-purple colour would indicate existence of steroids (Usman *et al*., 2009).

3.9 Data Management and Statistical Analysis

The yield data of aqueous *L. cornuta* extract was tabulated and expressed as % of total crude extract. Antioxidant and anti-inflammatory effects data were tabulated in Excel spreadsheet; descriptive statistics were performed and data expressed as mean ± SEM. Unpaired student t-test and One-Way Analysis of Variance (ANOVA) were used where appropriate. Fisher's LSD was used for pairwise separation and comparison of means. $p<0.05$ was considered significantly different. Results were presented in graphs and tables.

CHAPTER FOUR

RESULTS

4.1 Percentage yield of aqueous root extract of *Launea cornuta*

After extraction process, light brown powdered extract was produced. The percentage yield of the extract was found to be 13%.

4.2 Ex vivo anti-inflammatory activity of the aqueous root extract of *L. cornuta*

Ex vivo anti-inflammatory effects of the studied plant were evaluated through human erythrocyte membrane stability assays.

4.2.1 Membrane stabilization test

The red blood cell membrane lysis technique was employed to determine the ability of the aqueous root extract of *L. cornuta* to protect HRBCs against hemolysis induced by hypotonic medium and heat.

The ability of the *L. cornuta* aqueous root extract to stabilize the human erythrocyte cell membrane and inhibit hemolysis in a hypotonic medium was investigated. It was observed that, at all concentrations, *L. cornuta* extract and standard drug (Indomethacin) protected the human red blood cells membranes against hypotonicity-induced lysis. The extract exhibited varied degrees of membrane stabilization ranging from 13.18 % to 82.19 %. At the concentration of 1 µg/ml the extract had a significantly lower percentage membrane stabilization activity compared to the standard. However, the 10-1000 µg/ml extract concentrations were potent in protecting the HRBC membrane from lysis with values ranging from 50.22 % to 82.19 %. These values were significantly higher than those of the standard drug, indomethacin, whose values ranged from 43.90 % to 74.95 % at the same concentrations ($p<0.05$; Table 4.1).

Table 4.1: Percentage HRBC membrane stabilization effect of aqueous root extract of *L. cornuta* against hypotonicity induced haemolysis

Concentration (µg/ml)	Membrane stabilization effect (Mean±SEM)	
	Extract (Launaea cornuta)	**Standard (Indomethacin)**
1	**13.18±1.15^{b}**	**35.91±0.26^{a}**
10	**50.22±1.89^{a}**	**43.90±0.73^{b}**
100	**71.40±0.23^{a}**	**56.68±0.52^{b}**
1000	**82.19±1.06^{a}**	**74.95±0.38^{b}**

Different superscript letters within a given row indicate significant difference (Unpaired student t-test, $p<0.05$)

It was also observed that the % membrane stabilization effects of *L. cornuta* extracts was directly proportional to the extract concentrations and that there was significant difference at each concentration level ($p<0.05$) (Figure 4.1).

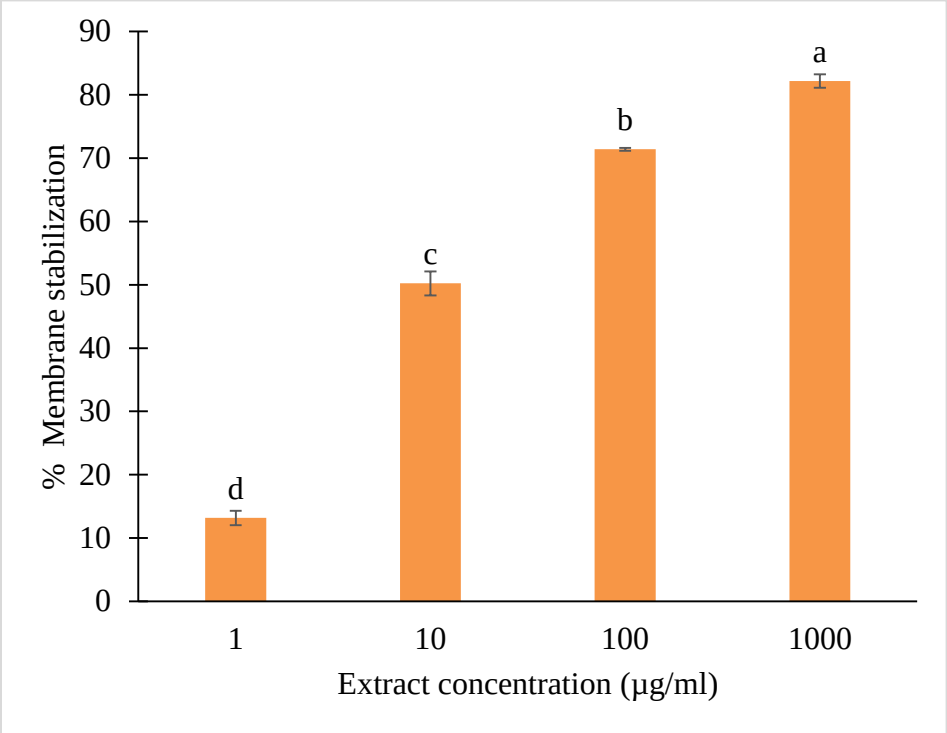

Figure 4.1: Percentage membrane stabilization activity of the aqueous root extract of *L. cornuta* against hypotonicity-induced haemolysis

Bars that don't share letters indicate significant difference ($p<0.05$) (ANOVA; Fisher's LSD)

For the heat induced hemolysis test, the extract exhibited significantly higher membrane stabilization potency than the standard drug (Indomethacin) at 1-100 µg/ml extract concentrations. However, at the concentration of 1000 µg/ml, the extract showed a significantly lower percentage membrane stabilization effect compared to that of standard drug at same concentration ($p<0.05$) (Table 4.2).

Table 4.2: Percentage HRBCs stabilization effect of aqueous root extract of *L. cornuta* against heat induced haemolysis

Concentration (µg/ml)	**% Membrane stabilization effect (Mean±SEM)**	
	Extract (*Launaea cornuta*)	**Standard (Indomethacin)**
1	76.65±0.69[a]	60.58±0.093[b]
10	81.16±0.37[a]	68.44±0.45[b]
100	81.79±0.55[a]	75.83±0.17[b]
1000	82.24±0.43[b]	85.91±0.46[a]

Different superscript letters within a given row indicate significant difference (Unpaired student t-test, $p<0.05$)

It was also noted that the membrane stabilization effects of extract concentrations 10, 100 and 1000 µg/ml had no significance differences among themselves ($p>0.05$; Figure 4.2). However, the effects of the three extract concentrations (10, 100 and 1000 µg/ml) were significantly higher than the effect of 1 µg/ml extract concentration ($p<0.05$; Figure 4.2).

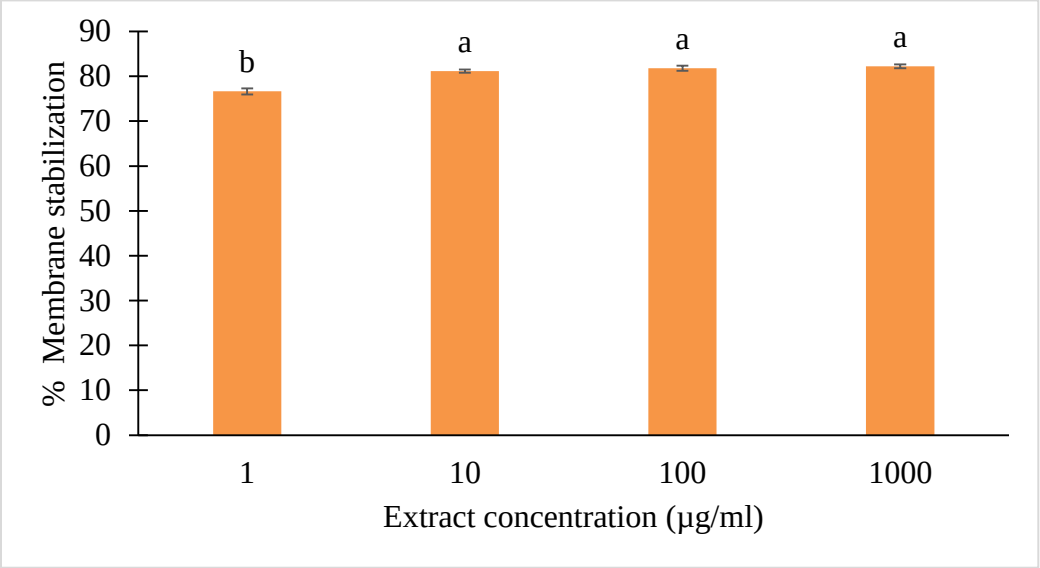

Figure 4.2: Percentage membrane stabilization activity of the aqueous root extract of *L. cornuta* against heat-induced haemolysis.

Bars that don't share letters indicate significant difference ($p<0.05$) (ANOVA; Fisher's LSD)

4.3 In vitro anti-inflammatory activity of the aqueous root extract of *L. cornuta*

In vitro anti-inflammatory extract (*L. cornuta*) effects were evaluated through antiproteinase activity and inhibition of protein denaturation assays.

For the antiproteinase activity, it was observed that there was significantly higher proteinase inhibitory activity at 100 and 10 µg/ml extract concentrations compared to Indomethacin ($p<0.05$) (Table 4.3). However, at 1 µg/ml and 1000 µg/ml extract concentrations there was no significant protein inhibitory activity compared to indomethacin (standard drug) ($p>0.05$) (Table 4.3). It was noted that both the extract and the standard drug, indomethacin, showed a low inhibitory effect on trypsin action, for

instance, at the highest concentration (1000 µg/ml) *L. cornuta* extract and indomethacin gave percentage antiproteinase activity values of 21.424 % and 20.979 % respectively.

Table 4.3: Percentage antiproteinase activity of aqueous root extract of *L. cornuta*

Concentration (µg/ml)	**% Antiproteanase activity (Mean±SEM)**	
	Extract (*Launaea cornuta*)	**Standard (Indomethacin)**
1	10.69±0.42[a]	10.40±0.20[a]
10	15.23±0.18[a]	14.56±0.08[b]
100	20.27±0.39[a]	14.91±0.31[b]
1000	21.42±0.47[a]	20.98±0.41[a]

Different superscript letters within a given row indicate significant difference (Unpaired student t-test, $p<0.05$)

ANOVA and Fisher's LSD analysis revealed that the antiproteinase activities of the extracts 100 and 1000 µg/ml had no significant difference ($p>0.05$) (Figure 4.3). However, they registered significantly higher effects than those of extracts 1 and 10 µg/ml.

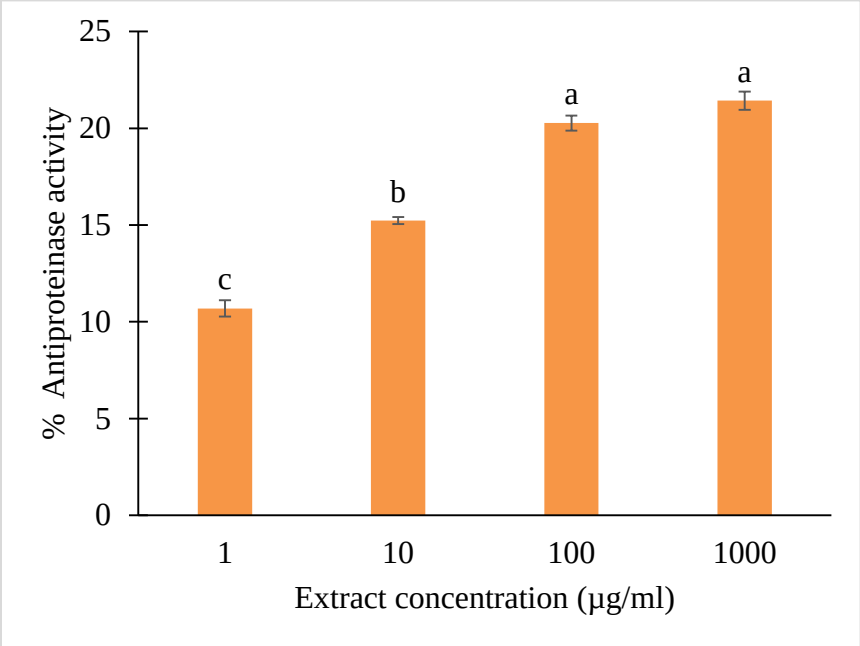

Figure 4.3: Percentage antiproteinase activity of the aqueous root extract of *L. cornuta*.

Bars that don't share letters indicate significant difference ($p<0.05$) (ANOVA; Fisher's LSD)

For the inhibition/prevention of denaturation of proteins, the results showed that the aqueous root extract of *L. cornuta*, in all concentrations, produced significantly higher % protection of albumin denaturation compared to the % protection caused by the standard at the similar concentrations ($p<0.05$; Table 4.4). It was also observed that at concentration 1 µg/ml, the *L. cornuta* extract % inhibition (76.216 %) was significantly more pronounced than the effect of the standard drug ($p<0.05$; Table 4.4).

Table 4.4: Percentage inhibition of protein denaturation of aqueous root extract of *L. cornuta*

Concentration (µg/ml)	**% Inhibition of protein denaturation (Mean±SEM)**	
	Extract (*Launaea cornuta*)	**Standard (Indomethacin)**
1	76.22±0.55[a]	40.31±0.13[b]
10	88.43±0.21[a]	76.28±0.24[b]
100	90.66±0.36[a]	80.67±0.41[b]
1000	93.11±0.15[a]	91.91±0.28[b]

Different superscript letters within a given row indicate significant difference (Unpaired student t-test, $p<0.05$)

In addition, it was noted that effect of *L. cornuta* extract to inhibit protein denaturation was concentration dependent and that there were significant differences among all concentrations ($p<0.05$; Figure 4.4).

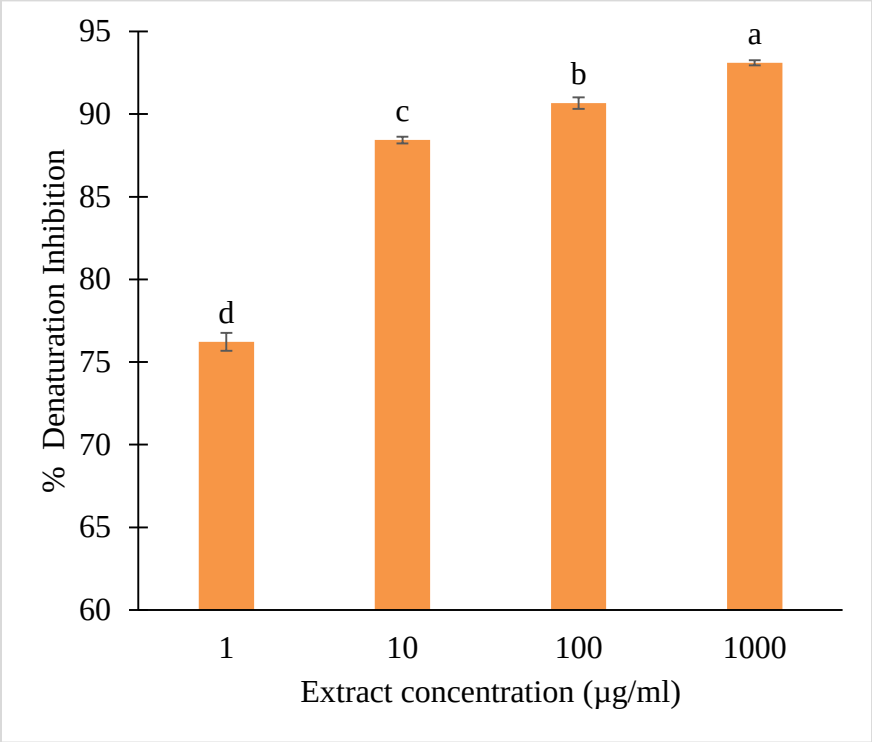

Figure 4.4: Percentage inhibition of protein denaturation of the aqueous root extract of *L. cornuta*.

Bars that don't share letters shows significant difference ($p<0.05$) (ANOVA; Fisher's LSD)

4.4 In vivo anti-inflammatory activity of aqueous root extract of *Launea cornuta*

In this study, the aqueous root extract of *L. cornuta* significantly reduced carrageenan-induced paw edema (inflammation) in a dose-dependent manner. For instance, in the first hour, the percentage edema reduction of the four extract doses (31.25, 62.5 125.0 and 250.0 mg/kg bw) were 2.58%, 7.63%, 10.81% and 15.27% respectively (Table 4.5).

Generally, the effects of the positive control (Dexamethasone) were significantly higher

than effects of doses 31.25, 62.5 and 125.0 mg/kg bw. However, the extract dose of 250 mg/kg bw showed remarkably significant reduction of paw edema (84.729 % to 61.176 %) compared to the standard drug (92.535 % to 67.324 %) at each time point ($p<0.05$; Table 4.5). The difference of the effect of dose 62.5mg/kg bw and Dexamethasone were insignificant at 1st hour while effects of dose 125 mg/kg bw and Dexamethasone were also insignificant in the 3rd and 4th hour ($p>0.05$; table 4.5).

Table 4.5: *In vivo* anti-inflammatory activity of aqueous root extract of *L. cornuta* on carrageenan-induced paw oedema

Group	treatment	percentage change in paw size				
		0 Hr	**1 Hr**	**2 Hr**	**3 Hr**	**4 Hr**
normal control	normal saline mg/kgbw	100.00±0.00	100.19±0.10[b] (-0.19)	99.39±0.18[b] (0.61)	99.60±0.10[b] (0.40)	99.91±0.062[b] (0.09)
negative control	carrageenan + normal saline	100.00±0.00	123.33±0.14[a] (-23.33)	124.50±0.22[a] (-24.50)	125.02±0.32[a] (-25.02)	126.61±0.17[a] (-26.61)
positive control	carrageenan + 4.00 mg/kgbw	100.00±0.00	92.54±0.22[d] (7.47)	83.79±0.21[f] (16.21)	74.22±0.20[e] (25.78)	67.32±0.21[e] (32.68)
lc root extract	carrageenan + 31.25 mg/kgbw	100.00±0.00	97.42±0.19[c] (2.58)	95.48±0.14[c] (4.52)	93.35±0.18[c] (6.65)	87.38±0.18[c] (12.62)
	carrageenan + 62.50 mg/kgbw	100.00±0.00	92.37±0.24[d] (7.63)	90.28±0.21[d] (9.72)	89.63±0.17[d] (10.37)	81.40±0.18[d] (18.60)
	carrageenan + 125.00 mg/kgbw	100.00±0.00	89.19±0.11[e] (10.81)	86.19±0.10[e] (13.81)	74.81±0.66[e] (25.19)	67.36±0.13[e] (32.64)
	carrageenan + 250.00 mg/kgbw	100.00±0.00	84.73±0.10[f] (15.27)	80.75±0.10[g] (19.25)	68.93±0.17[f] (31.07)	61.18±0.13[f] (38.82)

Values were expressed as Mean±SEM. Means with different subscript letters within a given column are significantly different (p<0.05) (One-way ANOVA; Fisher's LSD). The values in brackets represent percentage edema reduction.

Hourly comparison of the effects of each of the four *L. cornuta* extract doses revealed that the extract doses of 31.25, 125.00 and 250 mg/kg bw had significant time-dependent effects, that is, they demonstrated significant reduction of the edema in all hours (p<0.05; Figure 4.5). However, extract dose 62.5 mg/kg bw had insignificant edema reduction between 2nd and 3rd hour (p>0.05; Figure 4.5).

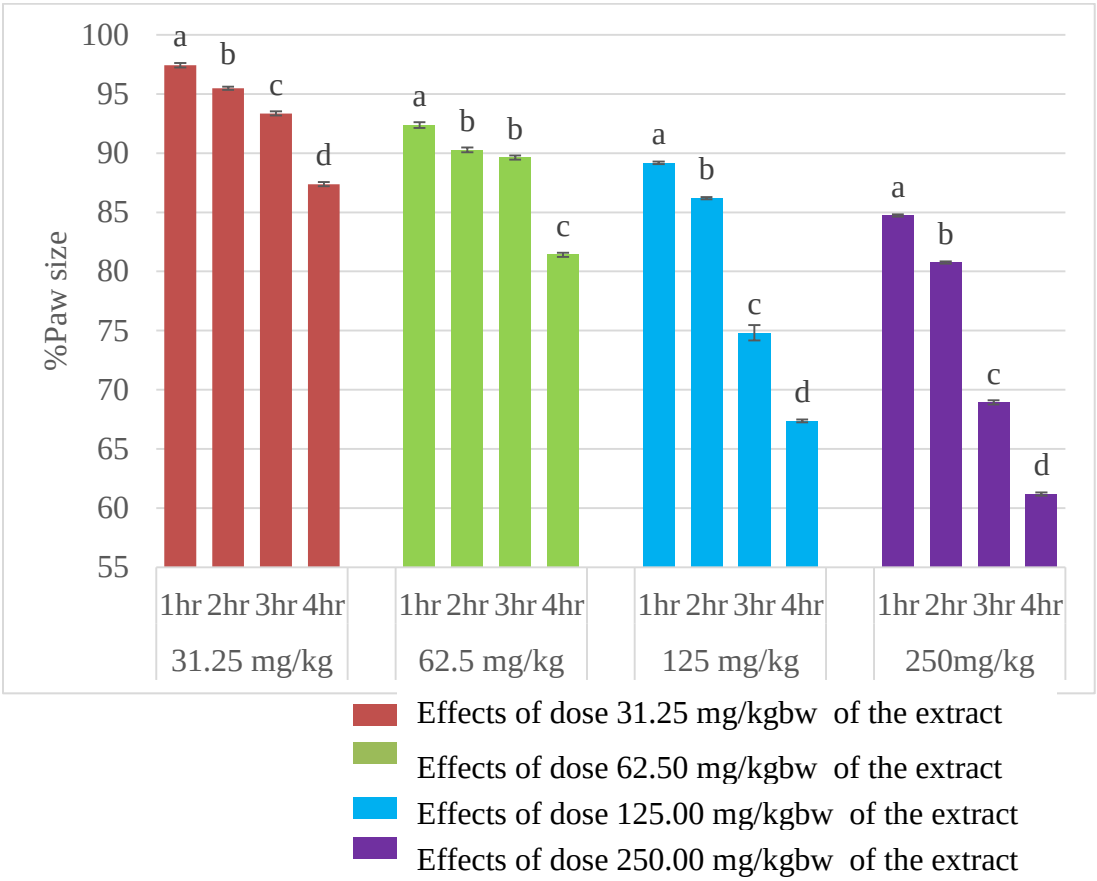

Figure 4.5: Percentage change in paw size. Bars that don't share letters shows significant difference (p<0.05) (ANOVA; Fisher's LSD)

4.5 Determination of *in vitro* antioxidant activities of *L. cornuta* extract

The results showed that the standard (Ascorbic Acid) had significantly higher free DPPH radical scavenging activity compared to the extract ($p<0.05$; Table 4.6). When the extract was mixed with DPPH, the dark purple colour turned colourless especially at 1000 µg/ml extract concentration. This showed that the extract possesses antioxidant compounds that were able to neutralize the stable DPPH radical (dark purple).

Table 4.6: DPPH radical scavenging activity of aqueous root extract of *L. cornuta.*

Concentration (µg/ml)	Percentage free radical scavenging activity % RSA (Mean±SEM)	
	Extract (*Launaea cornuta*)	**Standard (Ascorbic Acid)**
0.01	53.30 ± 0.55^{b}	60.52 ± 0.38^{a}
0.1	60.28 ± 0.91^{b}	71.49 ± 0.88^{a}
1	61.05 ± 0.50^{b}	79.32 ± 2.50^{a}
10	62.28 ± 1.26^{b}	99.01 ± 0.010^{a}
100	66.72 ± 1.78^{b}	99.08 ± 0.017^{a}
1000	83.84 ± 0.75^{b}	99.11 ± 0.01^{a}

Different superscript letters within a given row indicate significant difference (Unpaired student t-test, $p<0.05$)

ANOVA and Fisher's analysis showed that the extract concentration 1000 µg/ml had significantly higher DPPH radical scavenging activity than the other extract concentrations ($p<0.05$; Figure 4.6). However, there was no significant difference in

DPPH radical scavenging activity among concentrations 0.1 to 10 µg/ml and between concentrations 10 and 100 µg/ml ($p>0.05$; Figure 4.6).

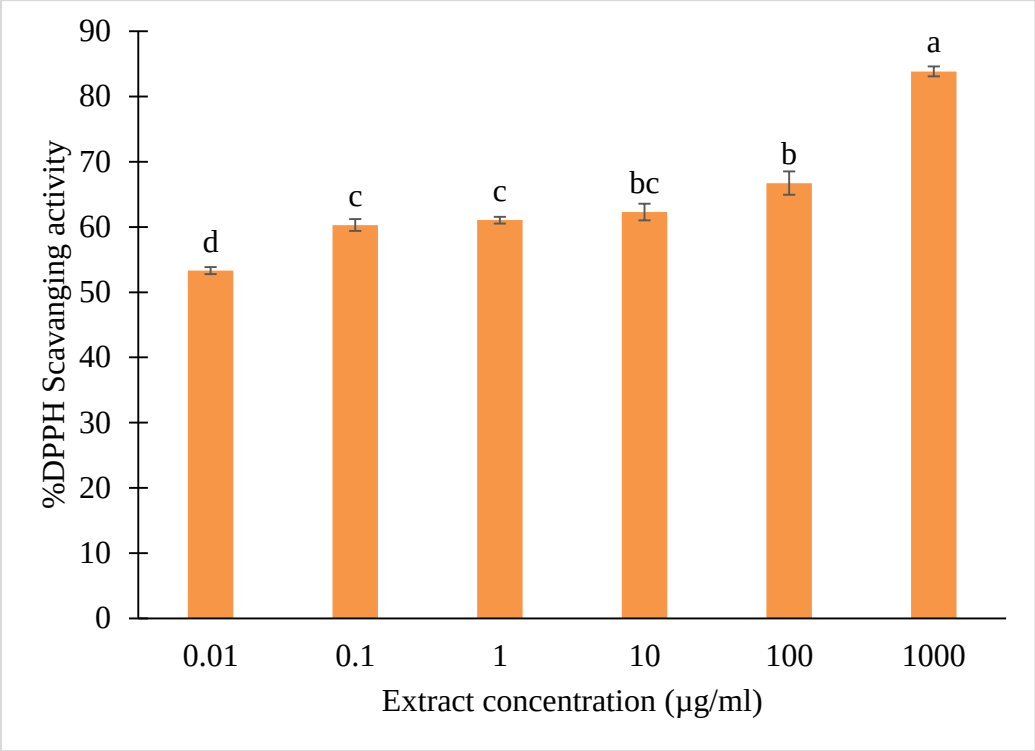

Figure 4.6: Percentage DPPH scavenging activity of the aqueous root extract of *L. cornuta*. Bars that don't share letters indicate significant difference ($p<0.05$) (ANOVA, Fisher's LSD)

For Ferric Reducing Antioxidant power test, the ascorbic acid (standard) showed significantly higher Free radical reducing power than the *L. cornuta* extract ($p<0.05$; Table 4.7).

Table 4.7: Ferric antioxidant reducing power of aqueous root extract of *L.* cornuta.

Concentration (µg/ml)	Absorbance (Mean±SEM)	
	Extract (*Launaea cornuta*)	**Standard (Ascorbic Acid)**
0.01	0.56 ± 0.06^{b}	2.31 ± 0.08^{a}
0.1	0.66 ± 0.01^{b}	2.48 ± 0.02^{a}
1	0.68 ± 0.00^{b}	2.54 ± 0.01^{a}
10	0.70 ± 0.01^{b}	2.61 ± 0.04^{a}
100	0.72 ± 0.01^{b}	2.65 ± 0.02^{a}
1000	0.74 ± 0.01^{b}	2.65 ± 0.06^{a}

Different superscript letters within a given row shows significant difference (Unpaired student t-test, $p<0.05$)

It was also observed that extract concentrations 0.1 to 1000 µg/ml had no significant Ferric reducing power among themselves ($p>0.05$; Figure 4.7).

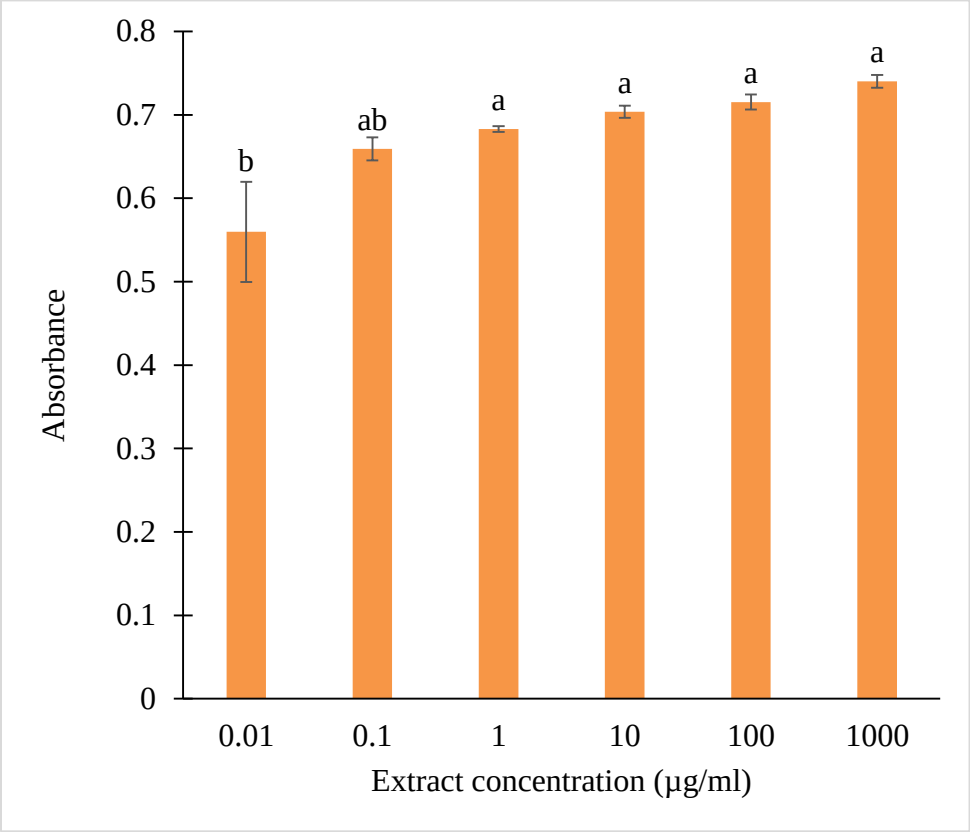

Figure 4.7: Ferric reducing power of the aqueous root extract of *L. cornuta*. Bars that don't share letters shows significant difference ($p<0.05$) (ANOVA; Fisher's LSD)

4.6 Determination of acute toxicity of *L. cornuta* extracts

Acute toxicity using Swiss albino mice was performed to determine the safety profile of the *L. cornuta* extract. The acute toxicity effect of the aqueous extract was determined as per the Up and Down procedures in OECD guideline 425 (OECD, 2008).

No mortality was observed after oral administration of aqueous root extract of *L. cornuta* at the doses of 175, 550 and 2000 $mgkg^{-1}$ bw. The behavioral parameters that show toxicity such as appearance of skin fur, salivation, mucous membrane, lethargy, eyes,

convulsions, diarrhea, coma, tremors, sleep, mortality and body weight were observed for fourteen days. No deaths and signs of toxicity observed in the animals (Table 4.8). All mice exhibited normal consumption of food and water.

Table 4.8: General behavioral parameters observed.

Observation	**Control**	**175 mg/kg bw**	**550 mg/kg bw**	**2000 mg/kg bw**
Convulsions	Not present	Not present	Not present	Not present
Salivation	Normal	Normal	Normal	Normal
Coma	Not present	Not present	Not present	Not present
Lethargy	Normal	Normal	Normal	Normal
Food intake	Normal	Normal	Normal	Normal
Water consumption	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Diarrhea	Not present	Not present	Not present	Not present
Mucous membrane	Normal	Normal	Normal	Normal
Tremors	Not present	Not present	Not present	Not present
Sleep	Normal	Normal	Normal	Normal
Death	Alive	Alive	Alive	Alive

4.7 Qualitative phytochemical screening

As the table 4.9 shows, qualitative phytochemical screening of aqueous root extract of *L. cornuta* revealed presence of tannins, cardiac glycosides, anthraquinones, alkaloids, steroids, terpenoids, phenols and flavonoids. However, saponins and coumarins were found to be absent or were negligibly present.

Table 4.9: The phytochemical assessment of *L. cornuta*

Phytochemical	Presence/absence
Phenols	+
Tannins	+
Alkaloids	+
Flavonoids	+
Steroids	+
Terpenoids	+
Cardiac glycosides	+
Anthraquinones gylcosides	+
Saponins	-
Coumarins	-

(+) implies presence of respective phytochemicals while (-) indicates their absence.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Medicinal plants/herbs have been used since time immemorial for the curing and management of various diseases as they are considered a healthy source of medicines. The use of medical plants is advantageous as they are 100% natural hence largely considered safe with minimal adverse side effects (Sofowora, 1993; Srivastava *et al*., 2011; Inta *et al*., 2013) Since antiquity, humans have been using plant decoctions against infectious diseases and even long before the discovery of existence of pathogenic microorganisms, the notion that particular plant parts conferred healing properties was well accepted (Sofowara, 1993; Snehlata *et al*., 2018; Larayetan *et al*., 2019).

It is now known that the plants' medicinal benefit is facilitated by some chemical substances produced by the plant that have a effective physiological activity on our body's processes (Sokmen *et al*., 2004). Among the many substances produced by plants are like coumarins, alkaloids, flavonoids, glycosides, tannins and phenolic compounds are responsible for pharmacological bioactivity (Inta *et al*., 2013; Snehlata *et al*., 2018; Larayetan *et al*., 2019).

Inflammation aids protection against stimuli (infections, allergens and other stimuli) in the body. An uncontrolled and persistent inflammation has been established as a crucial etiologic factor for great number of chronic ailments. In spite of inflammation being a body's defense response, its persistence lead to extremely elevated levels of mediators

posing threat to body's normal functioning (Kumar *et al*., 2004). The current conventional anti-inflammatory drugs have proved unsatisfactory with enormous side effects. Therefore, the development potent, affordable and safe anti-inflammatory drugs need no further emphasis.

The potential for medicinal plants to provide novel anti-inflammatory agents is immense as they produce a diverse range of novel phytocompounds that are biologically active. Plants have served as templates for anti-inflammatory agents since the advent of the first synthetic anti-inflammatory drug, aspirin. Aspirin was developed from salicylic acid, a metabolite of salicin which was isolated from the *Salix* species (Shi *et al*., 1999). The *Salix* species was used by Native Americans as a herbal remedy to treat pain, fever and inflammation (Ahmad *et al*., 2007).

Plant phytocompounds and their various metabolites, from many herbs, have been demonstrated to possess anti-inflammatory activities. Some African communities use *Lauanae cornuta* to manage pain and inflammation. For instance, in Tanzania and Malawi root decoctions of *Launaea cornuta* are used to treat stomachache, pain emanating from spleen and ear-ache. In Kenya, boiled chopped portions of the plant are taken to remedy/treat soar throat, swollen testicles and joint pains, among other conditions perceived to be of inflammatory nature and origin (Kokwaro, 2009; Musila, 2012; Wambugu *et al*., 2011; Karau *et al*., 2014).

The present study was conducted to determine *in vitro*, *ex vivo* and *in vivo* anti-

inflammatory activity, *in vitro* antioxidant activity, acute oral toxicity effects and qualitative phytochemical profile of the aqueous root extract of *L. cornuta*. For anti-inflammatory effect, I started with *in vitro* assay and when the extract showed activity I was curious and wanted to see whether the plant also exhibit *ex vivo* and *in vivo* anti-inflammatory activity.

Antiproteinase effects, albumin denaturation inhibition and membrane stabilization assays were used to determine the potential *in vitro* and *ex vivo* anti-inflammatory activities of the aqueous extract of *L. cornuta*. Membrane stabilization process involves keeping the biological membranes intact against lysis upon their exposure to high temperatures or hypotonic medium (Assumpta *et al*., 2019; Jitta *et al*., 2019). This technique was considered in this study because it is simple and reproducible. Human red blood cells were used because the lysis of erythrocytes' membranes when subjected to heat or hypotonic medium, mimics to great extent the lysis of the lysosomal membrane (Lavanya *et al*., 2010; Labu, 2015).

The lysosomal enzymes (bactericidal protease) released following rapture of lysosomal membranes upon stimulation by inflammatory event are considered key drivers in some disorders such as diabetes mellitus (Govindappa *et al*., 2011; Rahman *et al*., 2012). Therefore, stabilizing lysosomal membranes of activated neutrophils prevents release of proteases and bacterial enzymes that would aggravate the inflammatory process (Sakat *et al*., 2010; Labu, 2015). The NSAIDs work by either inhibiting the activity of these enzymes or they can prevent lysis of the lysosomal membranes so as to block release of

the proteases (Yoganandam *et al*., 2010 *et al*., 2010; Kosala *et al*., 2018).

In this study, the *L. cornuta* aqueous root extract exerted significant membrane stabilization activity both in hypotonic and heat induced lysis. This suggests that some *L. cornuta* phytochemicals were able to stabilize erythrocytes membranes. Some bioactive phytochemicals bind to erythrocytes, thereby causing alterations of charges on cell surfaces. Changes in the surface charges may hinder interaction with lysis-causing agents or literary repel like charges that would cause hemolysis. Flavonoids and tannins have been greatly demonstrated to stabilize lysosomal membranes. Tannins have the ability to bind negatively charged ions thereby stabilizing red blood cells' membranes and other biological membranes (Assumpta *et al*., 2019).

Reports demonstrate that the primary cause of biological membrane lysis is oxidative stress. Oxidative attack weakens the ability of HRBCs to endure heat and osmotic stress. The existence of antioxidant phytochemicals like tannins and flavonoids in *L. cornuta* extract neutralized reactive oxygen/nitrogen species. Decoctions from parts this of plant (*L. cornuta*) are reportedly used to treat swollen testicles, joint pains and wounds (Kokwaro, 2009; Wambugu *et al*., 2011). The stabilization/protection of the membranes of lysosomes is the probable mechanism of action of *L. cornuta* extracts when used to decrease inflammation during wound healing since it aids release of less tissue-damaging enzymes to the inflamed site.

Leukocyte proteinases in neutrophils, when released, cause tissue damage (Gunathilake *et*

al., 2018). Inhibition of proteinases can provide significant protection against inflammatory response (Truong *et al*., 2019) The mechanism of denaturation of serine proteinases may be associated with an alteration of bonds like hydrogen bonds in their structure. Protein denaturation entails destruction or disruption of tertiary, secondary or quaternary structures of proteins and nucleic acids leading to loss of bioactivity. Denaturation occurs following subjection of proteins to extremes of pH (strong acids and bases), high temperatures, organic solvents or highly concentrated inorganic salts (Leelaprakash *et al*., 2011).

Most biological proteins, as in the case of chronic inflammation, lose their biological function when denatured leading to a myriad of disease conditions associated with inflammation (Govindappa *et al*., 2016). When proteins are heated, they undergo denaturation leading to exposure of antigens that resemble the antigens produced during chronic inflammation like in rheumatoid arthritis (Duganath *et al*., 2010). The NSAIDs, apart from suppressing prostaglandins synthesis, have been found to protect proteins from undergoing denaturation. (Umapathy *et al*., 2010).

In this present study, the low inhibitory activity of trypsin by the *L. cornuta* aqueous extract and standard drug could perhaps mean that the tested extract is not too helpful in averting tissue injury caused by proteinase activity following hemolysis of the lysosomes. This means that the potency of the extract is more based on inhibition of release of tissue-damaging enzymes by stabilizing lysosomal membranes and inhibiting protein denaturation as observed in effective inhibition of albumin denaturation. Similar results

were obtained by Govindappa *et al*. (2011). The phytocompounds present in the *L. cornuta* extracts were possibly able to prevent albumin (protein) from getting denatured by reacting with the amino acids that are exposed due to heat. Interaction with amino acids like lysine and threonine could lead to the prevention of protein precipitation (Duganath *et al*., 2010). Therefore, it can be deduced that prevention of protein denaturation could be one of the mechanisms of action the of *L. cornuta* extract that is traditionally used to treat inflammatory conditions. Similar *ex vivo* anti-inflammatory results were observed by Leelaprakash *et al*. (2011).

Carrageenan-induced paw edema/swelling on mice model was adopted in evaluating *in vivo* anti-inflammatory activity of the plant extract. Carrageenan, a polysaccharide, is extracted from algae (*Chondrus crispus*) (Rosa, 1972; Barth *et al*., 2016). It is capable of inducing inflammation in a highly sensitive and reproducible way (Zhao *et al*., 2012; Dzoyem *et al*., 2017). Upon introduction of carrageenan into mice's hind paw, it acts as a thermal and mechanical stimulus thus provoking the mice immune defense mechanism to respond in order to destroy and clear the stimulus (Barth *et al*., 2016). In the process, complex mechanisms illicit development of oedema which, therefore, indicates physical manifestation of inflammation (Salvemini *et al*., 1996).

The development of the swelling (oedema) in the subplantar tissue of the mice after injection of carrageenan is caused by release of serotonin, prostaglandin, brandykinin, histamine among other mediators (Rosa, 1972; Formagio *et al*., 2019). The swelling is known to occur in two phases (biphasic); the first phase (0-1h) of the swelling is due to

production of hydroxytryptamine, histamine, brandykinin and serotonin while the second phase (1-4 h) is contributed by release of prostaglandin-like mediators, free radicals and Cox-2 activation (Necas and Bartosikova, 2013; Chatterjee *et al*., 2015; Fathiazad *et al*., 2017; Jitta *et al.,* 2019).

In this study, the standard drug (Dexamethazone) was used as positive control, this drug inhibited inflammation at all hours (1-4 Hrs; both phases) of the experiment. Dexamethozone is a steroidal (synthetic glucocorticoid) anti-inflammatory agent. Steroids exert their anti-inflammatory activity by inhibiting phospholipase A_2 enzyme whose function is to cleave phospholipids in order to liberate arachidonic acid hence hindering prostaglandins synthesis. In addition, Dexamethasone inhibits synthesis of proteins (expression of inflammatory meditors) that would initiate and maintain inflammatory process (Rhen & Cidlowski, 2005; Amin and Hussain, 2015).

The extract generally reduced swelling at all hours (1-4hrs; both phases) at all administered doses. The same trend was observed by Ilavarasan *et al* (2006), Fathiazad *et al* (2017) and Demsie & Yimer (2019). These scientists, using extracts of *Ricinus communis, Marrubium vulgarae and Cucumis ficifolius* respectfully, observed increased attenuation of oedema in both phases of carrageenan-induced oedema. In this study, aqueous root extract of *L. cornuta* had maximum reduction of the swelling at the last hour (4^{th} hour). Demsie & Yimer (2019) attested that the late phase is more sensitive to conventionally used agents, therefore, supporting this observation. The observed extract potency against inflammation, in both phases (1-4hrs), may be owed to existence of

bioactive constituents in *L. cornuta* root extract that are capable of attenuating secretion of amines (histamine) and peptides (kinins) in the first phase as well as eicosanoids (prostaglandins and COX) and nitrogen/oxygen species which are released in the second phase of inflammation.

It was also observed that extract dose 250 mg/kg, registered greater potency than dexamethasone and lower doses of the extract, perhaps due to higher concentrations of bioactive substances present at this dose level. Phytochemical assessment of *L. cornuta* root extract revealed presence of steroids, phenols, tannins, flavonoids, alkaloids and anthraquinone glycosides. These compounds may have enabled *L. cornuta* to inhibit synthesis or action of mediators of inflammation. Previous research shows that flavonoids have anti-inflammatory activity (Kariawasam *et al*., 2017). Flavonoids inhibit prostaglandin synthetase (endoperoxidase) (Chatterjee *et al*., 2015). Ilavarasan (2006) demonstrated that flavonoids are potent agents for antiarthritic activity. Furthermore, flavonoids have antioxidant activity hence could have neutralized free radicals released in the second phase of inflammation. Terpenoids inhibits crucial steps in nuclear factor – kappa B (NF-kB) signaling pathway, thereby down-regulating production of some inflammatory-mediators like cytokines. Steroids have been reported to inhibit secretion of anti-inflammatory mediators (Kosala *et al*., 2018)

Natural products from plants are regarded, especially in developing countries, as safe and are consumed with no regard on their toxic effects on human health hence assessing safety is crucial or rather necessary (Jothy *et al*., 2011). Most of the herbs used have not

been proved and validated scientifically to be fit for human consumption (Kifayatullah *et al*., 2015). *L. cornuta* is used in African communities to treat some ailments such as arthritis hence oral acute toxicity was included in this study to determine safe doses. Reports indicate mice, just like humans, are sensitive to toxic compounds in plant extracts this is due mice's biological activities having closely resemblance to that of humans (Parra *et al*., 2001). Mice were used instead of rats because mice data could be more suitable or rather appropriate to anticipate same toxic effects in humans (Saleem *et al*., 2017).

In this study, no mortality or abnormal behavior was observed; this indicated that the extract is safe at 2000 mg kg^{-1} and its LD_{50} is greater than 2000 mg kg^{-1}. Substances or drugs with LD_{50} >1000 mg kg^{-1}, taken orally, are viewed as being practically low toxic or safe (Kifayatullah *et al*., 2015). On the other hand, according to LD_{50} classification by Loomis and Hayes (1996), LD_{50} of 2000 mg kg^{-1} bw falls under slightly toxic category. Almost similar results were obtained for single-oral dose (2000 mg/kg bw) administration of *C. fistula* extracts in mice (Jothy *et al*., 2011).

Inflammation produces free radicals which exacerbate inflammatory process by attacking cellular components as witnessed in some diseases like arthritis, arteriosclerosis among others (Ilhami, 2005). Oxidative stress lead to destruction of biomolecules in cells (Ilavarasan *et al*., 2006; Zhao *et al*., 2008; Adjimani, 2015). Antioxidants exert their quenching effects by either preventing synthesis of free radicals or by inactivating these damaging agents. Endogenous body systems produce antioxidants to counteract effects of

radicals produced during inflammation. However, the amount of free radicals produced may be too much for the body's antioxidants to avert hence need to supplement antioxidants in diet to aid in fighting oxidative stress (Patel *et al*., 2010; Adjimani, 2015).

In this study, the DPPH radical scavenging and Ferric reducing power assays were employed in determining antioxidant activity of aqueous root extract of *L. cornuta*. Based on the FRAP and DPPH data, *L. cornuta* aqueous extract is a powerful free radical neutralizer and can be a good antioxidant in treating chronic inflammatory conditions. Research has shown that polyphenols like flavonoids have profound antioxidant activity (Ilavarasan *et al*., 2006; Sakat *et al*., 2010). Tannins and flavonoids could be the phenolic compounds that contributed to the results obtained. These phenols provided electrons required to stabilize or scavenge free radicals (Salminen *et al*., 2008). Presence of hydroxyl groups and conjugated structures render polyphenols excellent antioxidants. They scavenge radicals through hydrogenation; they break free radical and donate hydrogen atom (Madoui *et al*., 2018; Assumpta *et al*., 2019). The fact that aqueous root extract of *L. cornuta* possess antioxidants explains the anti-inflammatory effect of the plant. This is because free radicals are inflammatory mediators that propagate inflammation.

5.2 Conclusions

The following conclusions were drawn from this study;

i. The aqueous root extract possesses anti-inflammatory activity due to its ability inhibit carrageenan-induced oedema, albumin denaturation, proteinase activity and maintain integrity of red blood cells.

ii. The aqueous root extract of *L. cornuta* also possess antioxidant activity and can serve as free radical scavenger.

iii. The aqueous root extract of *L. cornuta* has no oral acute toxicity effects.

iv. The aqueous root extract of *L. cornuta* possess tannins, cardiac glycosides, anthraquinones, alkaloids, steroids, terpenoids, phenols and flavonoids. These phytochemicals could be responsible for the antioxidant and anti-inflammatory activities of aqueous root extract of *L. cornuta*.

This study, therefore, affirmatively answers the research questions drawn in this study.

5.3 Recommendations

i. The aqueous extract of *L. cornuta* can be a good candidate to be subjected to higher tests to elucidate if the plant could be used to develop affordable and safe novel antiiflammatory agents.

ii. The extract could also serve as alternative antioxidant supplement.

iii. The extract dose of 250 mg/kg bw is appropriate for anti-inflammatory activity in mice models.

iv. The extract appears to be safe, however, histopathological and biochemical effects need to be assessed before validating its traditional use.

v. The aqueous extract of *L. cornuta* possess phytochemicals such has flavoids that have anti-inflammatory and antioxidant activities.

5.4 Suggestions for Further Study

(i) Extraction and isolation of specific active compounds responsible for antiinflammatory and antioxidant activities.

(ii) Incorporate leaves and stem to identify potency intensity of bioactive compounds in different parts of the plant as far as antioxidant and anti-inflammatory effect is concerned.

(iii) Bio-screening of organic solvent extracts of *L. cornuta* so as to compare their antioxidant and anti-inflammatory activities with aqueous extracts.

(iv) Quantitative analysis of phytochemicals present in aqueous root extract of *L. cornuta*.

(v) Cellular level quantitative analysis of antioxidant and anti-inflammatory activities of *L. cornuta*.

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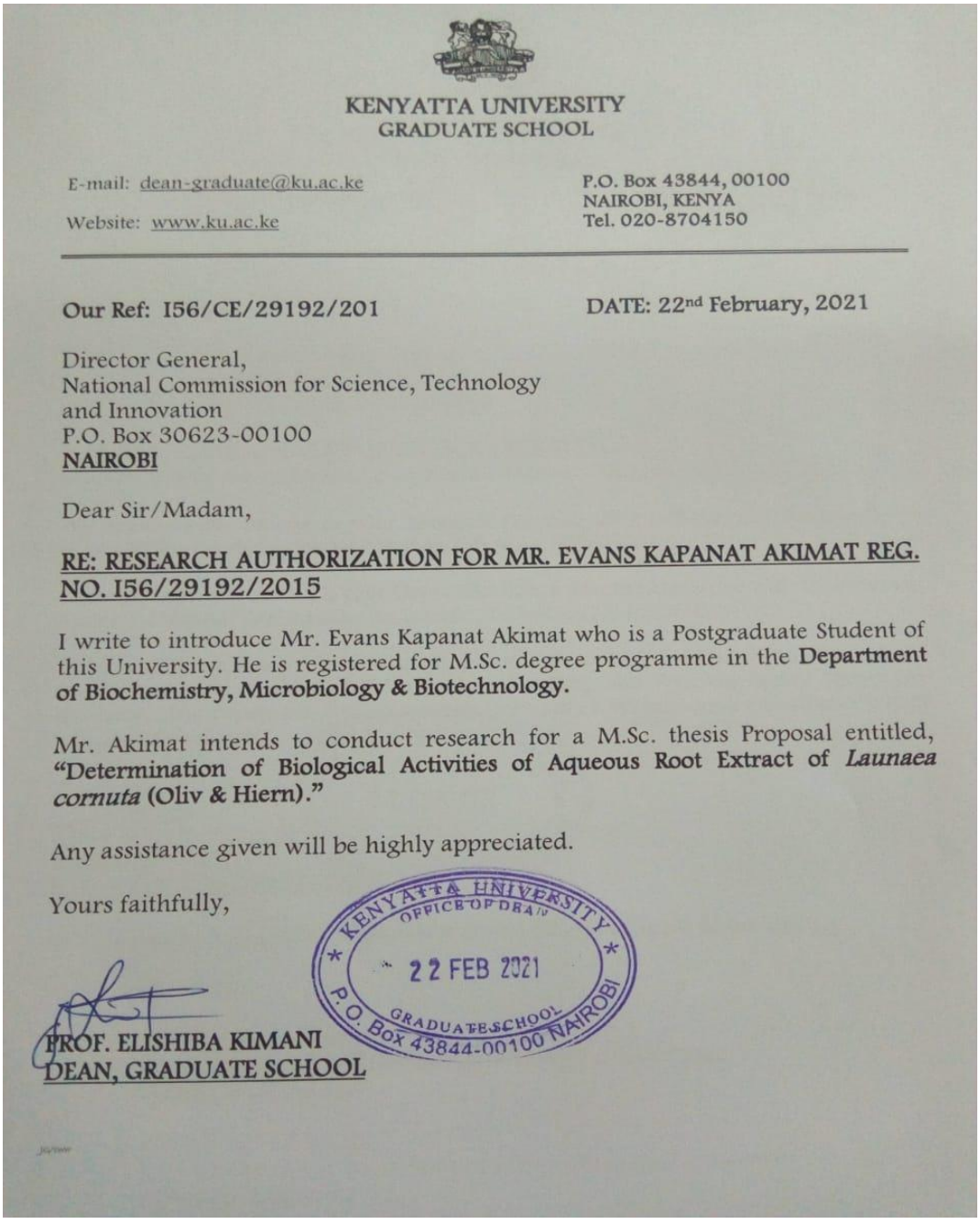
APPENDICES

Appendix I: Kenyatta university Research Authorization letter



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P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Our Ref: I56/CE/29192/201

DATE: 22nd February, 2021

Director General,
National Commission for Science, Technology
and Innovation
P.O. Box 30623-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR MR. EVANS KAPANAT AKIMAT REG. NO. I56/29192/2015

I write to introduce Mr. Evans Kapanat Akimat who is a Postgraduate Student of this University. He is registered for M.Sc. degree programme in the **Department of Biochemistry, Microbiology & Biotechnology.**

Mr. Akimat intends to conduct research for a M.Sc. thesis Proposal entitled, **"Determination of Biological Activities of Aqueous Root Extract of *Launaea cornuta* (Oliv & Hiern)."**

Any assistance given will be highly appreciated.

Yours faithfully,

KENYATTA UNIVERSITY
OFFICE OF DEAN
22 FEB 2021
GRADUATE SCHOOL
P.O. Box 43844-00100 NAIROBI

PROF. ELISHIBA KIMANI
DEAN, GRADUATE SCHOOL

Appendix II: National Commission for Science Technology and Innovation Approval letter

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **507304**

Date of Issue: **16/April/2021**

RESEARCH LICENSE

This is to Certify that Mr.. Evans Akimat of Kenyatta University, has been licensed to conduct research in Nairobi on the topic: DETERMINATION OF BIOLOGICAL ACTIVITY OF AQUEOUS ROOT EXTRACT OF Launaea cornuta (Hochst. Ex Oliv. and Hiern.) for the period ending : 16/April/2022.

License No: **NACOSTI/P/21/9936**

507304

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

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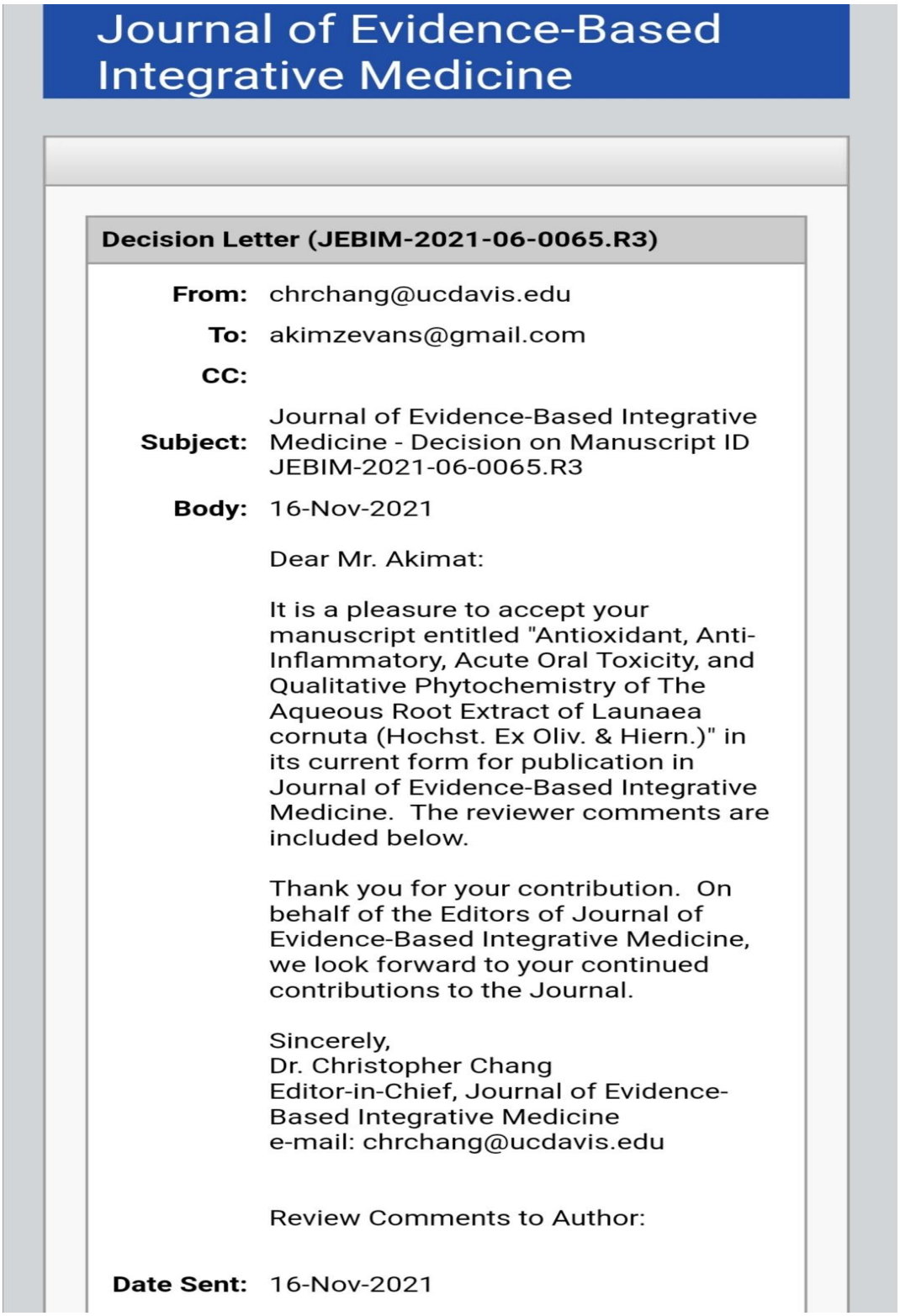
Appendix III: Publication Acceptance letter



Journal of Evidence-Based Integrative Medicine

Decision Letter (JEBIM-2021-06-0065.R3)

From: chrchang@ucdavis.edu

To: akimzevans@gmail.com

CC:

Subject: Journal of Evidence-Based Integrative Medicine - Decision on Manuscript ID JEBIM-2021-06-0065.R3

Body: 16-Nov-2021

Dear Mr. Akimat:

It is a pleasure to accept your manuscript entitled "Antioxidant, Anti-Inflammatory, Acute Oral Toxicity, and Qualitative Phytochemistry of The Aqueous Root Extract of Launaea cornuta (Hochst. Ex Oliv. & Hiern.)" in its current form for publication in Journal of Evidence-Based Integrative Medicine. The reviewer comments are included below.

Thank you for your contribution. On behalf of the Editors of Journal of Evidence-Based Integrative Medicine, we look forward to your continued contributions to the Journal.

Sincerely,
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