PHYTOCHEMICAL PROFILE, ANTIPYRETIC, ANALGESIC AND ANTI-INFLAMMATORY POTENTIAL OF DICHLOROMETHANE LEAF EXTRACTS OF *Eucalyptus globulus* (LABILL) and *Senna didymobotrya* (FRESENIUS)

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (MEDICAL BIOCHEMISTRY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

MAY, 2021

DECLARATION

I, Joseph Kiambi Mworia, duly declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

This thesis is dedicated to; Esther Kinya Kiambi (My Lovely Wife) and Zipporah Mworia (my mum) for their immense support and contribution towards my education.

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ABBREVIATIONS AND ACRONYMS

AAIN	Acetic Acid-induced Writhing
ACC	Anterior Cingulate Cortex
ANOVA	Analysis of Variance.
BW	Body weight
CB1	Cannabinoid Receptor Type 1
CNS	Central Nervous System
COX	Cyclooxygenase Enzyme
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
FIP	Formalin Induced Pain
GC-MS	Gas chromatography-Mass spectrophotometry
IASP	International Association for the Study of Pain
IP	Intraperitoneal
IV	Intravenous
KG	Kilogram
MF	Molecular Formula
Mg	Milligram
MS Excel	Microsoft excel
Ng	Nanogram
NIST	National institute of standards and Technology
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OVLT	Organum vasculosum laminae terminalis
PAG	Periaqueductal Gray
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PH	Hydrogen potential
PNS	Peripheral nervous system
RT	Retention Time
SEM	Standard Error of Mean
TAHM	Traditional African Herbal Medicine
TENS	Transcutaneous Electrical Nerve Stimulation
THC	Tetrahydro-Cannibinol
TM	Traditional Medicine
TNF	Tumor Necrosis Factor
TRPA 1	Transient Receptor Potential Ankyrin Subtype 1receptors
TRPV 1	Transient Receptor Potential Vanniloid Receptors
WHO	World Health Organization
NMDA	N-methyl-D-aspartate
MRSA	Methicillin-resistant *Staphylococcus aureus*

ABSTRACT

Pain,fever and inflammation are managed using synthetic drugs, which are associated with many undesirable effects. Herbal medicines form an alternative therapy to synthetic drugs since they possess fewer side effects. *Eucalyptus globulus* and *Senna didymobotrya* are traditionally used by the Embu and Mbeere communities of Kenya in the management and/or treatment of many ailments including pain, fever and inflammation. Empirical data on their effects against pain, fever and inflammation is, however, lacking. This study, therefore, sought to determine the quantitative phytochemical profiles, as well as *in vivo* antipyretic, analgesic and anti-inflammatory effects of dichloromethane (DCM) extracts of *E. globulus* and *S. didymobotrya.* The plant samples were obtained from Mbeere North Sub-County, Kenya. Three grams of leaf extracts of *E. globulus* and *S. didymobotrya* samples were obtained and analyzed to determine quantitative phytochemical composition using GC-MS. Swiss albino mice were used in the bioscreening of analgesic and anti-inflammation activities, while albino rats were used in the antipyretic activity. In each test group, nine groups of five experimental animals were used: Positive, normal, negative control and six experimental groups. Turpentine, Formalin and Carrageenan were used for induction of fever, pain and inflammation respectively. Aspirin was used as a reference antipyretic drug while diclofenac was used as reference analgesic and anti-inflammatory drug. Six extract doses of each studied plant were tested for antipyretic, antinociceptive and anti-inflammatory activities (25, 50, 100, 150, 200 and 250mg/kgbw). The GC-MS results revealed 25 and 10 phytocompounds for *E.globulus* and *S.didymobotrya* respectively. Results of antipyretic, antinociceptive and anti-inflammatory assays in animal models showed that the two leaf extracts have potential antipyretic, analgesic and anti-inflammatory activities attributed to the constituent phytocompounds namely alpha-pinene,borneol,limonene among others. *E. globulus* extract at 250mg kg bw reduced pyrexia by 2.29%, 3.27%, 3.59% and 4.83% while *S. dymobotrya* reduced fever by 1.31%, 2.24%, 3.08% and 3.97% in the 1^{st} , 2^{nd} , 3^{rd} and 4^{th} hours respectively. For pain bioscreening, *E. globulus* and *S. didymobotrya* at 250 mg/kgbw reduced the paw licking time in the late phase by 98.52% and 96.82%, respectively. *E. globulus* extract at 250mg kg bw reduced inflamed paw in the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} hours, by 2.27, 6.52, 9.09 and 10.90% respectively while *S. didymobotrya* at similar doses, reduced paw by 2.41, 5.43, 8.31 and 9.05% respectively. The administered extract doses (200, 250 mg) are appropriate in the bioassay of antipyretic, analgesic and anti-inflammatory activity in animal models. Therefore, this study, confirms and supports the use of studied plant extracts as alternative and/or complementary remedies against pain, fever and inflammation. It also sets pace for further studies to develop plant-derived drug compounds for treatment of pain, fever and inflammation perhaps by fractionating, isolating, purifying and bioassaying phytocompounds.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Fever refers to an increase in body temperature beyond the regulatory set point of 36.5 - 37.5^{o}C (98- 100^{o} F) (Kumar and Pathak, 2019). This increase in temperature triggers muscle tone and shivering. Fever signifies several illnesses. Symptoms of fever include sweating, chills, a sensation of cold and other subjective sensations. The absence of these symptoms when the temperature is high can be a pointer to a serious illness (Székely and Garai, 2018). Fever may be caused by infections caused by parasites, viruses, bacteria and immune reactions (including defects in collagen, immunological abnormalities and acquired immunodeficiency (Tohidpour *et al*., 2017). Fever can also be a result of the destruction of tissues during trauma, local necrosis (infarction), an inflammatory reaction in tissues and vessels (flebitis, arthritis), pulmonary infarction, and rhabdomyolysis (Anochie, 2013).

In humans, temperature regulation is controlled by the thermoregulatory center in the hypothalamus (Barbi *et al*., 2017). The input to the hypothalamus comes from peripheral as well as central thermo-receptors. Research has revealed neural substrates for thermoregulatory control (Cabral *et al*., 2012). The peripheral and central thermo-receptors situated beneath the skin are of two subtypes namely those that respond to cold and those responding to warmth (Ward and Linden, 2017). The warm central thermo-

receptors are located in the hypothalamus, spinal cord, viscera, and great veins, which are more numerous than cold thermo-receptors (Patel *et al*., 2016).

Pain is an unpleasant sensory affliction and emotional experience usually associated with actual or potential tissue damage or described in terms of such damage (Srilakshmi and Sachchidananda, 2018). Pain serves as a warning signal against disturbances. Pain is aimed at protecting the organism but often leads to discomfort (Singh *et al*., 2014). We have two types of pain nociceptive and pathological pain. Nociceptive pain is also known as acute pain, which usually accompanies noxious stimuli warning of impending tissue damage (Rio *et al*., 2014). Pathological pain, according to its cause, is divided into inflammatory pain and neuropathic pain, both belong to chronic pain (Díaz-Rodríguez *et al*., 2012). Chronic pain completes the list of largest medical health problems in countries with many low-income cadres of people (Gelband *et al*., 2016). Treatment of pain and related problems calls for a good understanding of how pain signals are initially interpreted and subsequently transmitted and perpetuated (Dimitroulas *et al*., 2014).

Antinociceptives such as opiates and non-steroidal anti-inflammatory drugs (NSAIDs) have many side effects (Hogans and Barreveld, 2019). Pain studies, treatment and management have made important progress now, but remain underestimated and poorly managed mostly in developing countries (Porta-Sales *et al*., 2015). This leads to pain among hospitalized patients is extensive, and significantly more (Franceschi *et al*., 2015).

Inflammation is a major cause of morbidity today. If poorly managed, inflammation may become complicated leading to the development of rheumatoid arthritis, diabetes, cancer, Alzheimer's disease, and atherosclerosis along with pulmonary, autoimmune and cardiovascular diseases (Patil *et al*., 2019). Inflammation is a response of living tissue to injury (Eming *et al*., 2017). Inflammation may be referred to as innate immunity. It may appear due to microbial infections, physical factors (trauma, radiation, temperature), chemical substances (irritant and corrosive chemicals), as well as tissue necrosis and hypersensitivity reactions (Silva, 2015). It is didactically characterized by redness, heat, swelling, pain and dysfunction of the organs involved (Actor and Smith, 2019).

The search for new drugs is on going. Research on plant-based drugs used as alternative and complementary medicine is of great interest because they are cheap, have fewer side effects and are readily available (Reid *et al*., 2018). Although a considerable number of drugs are available for the treatment of fever, pain and inflammation, there is a continuous search for new compounds as therapeutic alternatives, because these drugs exert a wide range of side effects and low efficacy, especially for chronic diseases (Calogero *et al*., 2017).

Pain, fever and inflammation are managed using conventional drugs such as diclofenac, Ibuprofen, morphine, acetylsalicylic acid (ASA) and acetaminophen (Díaz-Rodríguez *et al*., 2012). Non-steroidal anti-inflammatory drugs are frequently prescribed analgesics (Abdulla *et al*., 2013). For they are a highly effective drug for the management of fever,

pain and inflammation; however, NSAIDs are known to have multiple adverse effects, including gastrointestinal bleeding, cardiovascular side effects, and NSAID induced nephrotoxicity (Wongrakpanich *et al*., 2018). NSAIDs reduce pain by inhibiting COX enzymes and reducing prostaglandin synthesis (Wongrakpanich *et al*., 2018). Natural products have been one of the most successful sources of new therapeutic agents (David *et al*., 2015). Alternative and complementary therapy form a better option for they are cheap and readily available. Medicinal plants formed an integral part of human society to combat different diseases at the beginning of human civilization (Kooti *et al*., 2014). Herbal plants are a good source of phytochemicals that have many therapeutic potentials.

Medicinal plants contain pharmacologically active chemicals that can be used to cure diseases (Daniel, 2016). For instance, nicotine is from the tobacco plant while cocaine is extracted from the coca plant (*Erythroxylum coca*) while (*Nicotiana tabacum*), 9-tetrahydro- cannibinol (THC) comes from the marijuana plant (*Cannabis sativa*), similarly, the well-known pain killer morphine is obtained from opium poppy plant (*Papaver somniferum*), the precursor for the synthesis of aspirin salicylic acid is extracted from the bark of the willow tree (*Salix nigra*) and (*Salix alba*) (Rai and Tewari, 2018). Therefore, the drawbacks associated with these antipyretic, analgesic and anti-inflammatory conventional drugs have led to alternative therapy, that is, the use of herbal medicines (Kaur *et al*., 2016).

Medicinal plants form an integral part of human society to combat different diseases at the beginning of human civilization (Kooti *et al*., 2014). Herbal plants are a good source of many types of phytochemicals which have many therapeutic potentials. The term phytochemical has its origin in the Greek word phyto, which means plant (Fazilah *et al*., 2018). The secondary metabolites are biologically active compounds (Fiorito *et al*., 2017). Medicinal plants are useful and many contain phytocompounds that can be used to cure diseases (Daniel, 2016). For instance, nicotine is from the tobacco plant while cocaine is extracted from the coca plant (*Erythroxylum coca*) while (*Nicotiana tabacum*), 9-tetrahydro- cannibinol (THC) comes from the marijuana plant (*Cannabis sativa*), similarly, the well-known pain killer morphine is obtained from opium poppy plant (*Papaver somniferum*), the precursor for the synthesis of aspirin salicylic acid is extracted from the bark of the willow tree (*Salix nigra*) and (*Salix alba*) (Rai and Tewari, 2018).

Phytochemicals are found in plants leaves, stems, barks, flowers, fruits and roots. Phytochemicals are categorized into two main groups namely primary and secondary metabolites based on the kind of function they have in plant metabolism (Jain *et al*., 2019). Proteins, amino acids, pyrimidines, purines, Chlorophyll, and Sugars form the primary metabolites while terpenes, phenolics, alkaloids, steroids, saponins, flavonoids and glycosides form the secondary metabolites (Alamgir, 2018).

Phytochemicals protect plants from harsh environmental hazards like predation, stress, drought, UV exposure and attack by pathogens (Das *et al*., 2016). They form an

important source of information that is useful in drug development in pharmaceutical industries (Böttger *et al*., 2018). Studies have shown that terpenoids have several pharmacological benefits that are useful in the treatment of inflammatory, cancer, malaria, viral infections and many bacterial infections (Kabera *et al*., 2014). Phenolics are the most abundant group of phytochemicals in medicinal plants (Zaynab *et al*., 2018).

Dietary phenolics constitute flavonoids, phenolic acids, and polyphenols. A diet that is rich in Polyphenol possesses several health benefits namely improvement of cardiac function, decrease anginas and lowers cholesterol levels (Baião *et al*., 2017). Flavonoids also reduce the production of pathogenic thrombosis in mice models. Alkaloids are used as analgesic agents and are found in medicinal plants (Dembitsky, 2014).

In Embu County, Kenya, several medicinal plants are used in the management of fever, pain and inflammation. These include *Eucalyptus globulus* (Labill) and *Senna didymobotrya* (Fresenius). *E. globulus* is used in the treatment of bronchitis, cancer, arthritis, asthma, boils, cold, cough, diabetes, dysentery, dyspepsia, malaria, sore throat, tuberculosis, vaginitis and wounds (Dixit *et al*., 2012) while *S. didymobotrya* pharmacological activities include treatment of skin infections, malaria, ringworm, jaundice, intestinal worm, bacterial infections, fungal, sickle cell anemia, haemorrhoids and hypertension (Maobe, 2014). However, there is no scientific information available in the literature to validate their biological activities. Therefore, this study seeks to carry out

quantitative phytochemical screening, antipyretic, analgesic and anti-inflammatory potential of leaf extracts of *E. globulus* and *S. didymobotrya*.

1.2 Statement of the problem

Fever, pain and inflammation are primary indicators of many clinical conditions that present a maojor problem world over (Wong, 2014). They cause suffering and discomfort among the victims and are a serious healthcare concern in the world. Pain ,fever and inflammation lower the productivity of the victims and make the victims live life that is meaningless and continue to make life intolerable (Egoscue and Gittines, 2014). These conditions greatly compromise the productivity of the victims which in turn affects the quality of life and the country's gross domestic product.

1.3 Justification

World Health Organization (WHO) estimated that 80% of the population of developing countries relies on phytomedicine for their primary health care needs. Currently, management of fever, pain and inflammation is mainly through the use of conventional medicines such as NSAIDs. However, NSAIDs are known to have multiple adverse effects, including gastrointestinal bleeding, cardiovascular side effects,pump inhibitors, renal toxicity, allergy, hypertension, tolerance addiction, dependence, nephrotoxicity among others (Wongrakpanich *et al*., 2018). Herbal medicines are now emerging as important alternative therapeutic drugs as they are cheap, readily available, and are arguably assumed to have fewer side effects (Amoah, 2018). The increased use of herbal medicine in management of pain, fever and inflammation has necessitated demand for evidence on the safety, efficacy and quality of medicinal plants products. On the other

hand, herbal medicines forms the basis of modern drug discovery by the pharmaceutical industry.

The *E. globulus* and *S. didymobotrya* are traditionally used in the management of fever, pain and inflammation among communities living in Embu, County, Kenya (Kareru *et al*., 2007). However, no empirical data is available to support these claims. This study, therefore, was designed to determine the phytochemical composition, antipyretic, antinociceptive and anti-inflammatory potential of leaf extracts of *E. globulus* and *S. didymobotrya*. This preliminary information, therefore, forms the basis for the development of effective plant-derived antipyretic, antinociceptive and anti-inflammatory drugs.

1.4 Research questions

i. Do the dichloromethane leaf extracts of *E. globulus* and *S. didymobotrya* have antipyretic effects in rat models?
ii. Do the dichloromethane leaf extracts of *E. globulus* and *S. didymobotrya* have analgesic effects in mice models?
iii. Do the dichloromethane leaf extracts of *E. globulus* and *S. didymobotrya* have anti-inflammatory effects in mice models?
iv. What is the quantitative phytochemical composition of dichloromethane leaf extracts of *E. globulus* and *S. didymobotrya* ?

1.5 Objectives

1.5.1 General objective

To bio-screen dichloromethane leaf extracts of *E. globulus* and *S. didymobotrya* for antipyretic, analgesic, and anti-inflammatory potential in animal models.

1.5.2 Specific objectives

i. To investigate the phytochemical composition of DCM leaf extracts *E. globulus* and *S. didymobotrya* using gas chromatography-mass spectrometry.

ii. To investigate *in vivo* antipyretic potential of DCM leaf extracts *E. globulus* and *S. didymobotrya* on turpentine-induced fever in Rats.

iii. To investigate *in vivo* analgesic activities of DCM leaf extracts of *E. globulus* and *S. didymobotrya* on formalin-induced pain in mice.

iv. To investigate *in vivo* anti-inflammatory potential of DCM leaf extracts of *E. globulus* and *S. didymobotrya* on Carrageenan-induced inflammation in mice

CHAPTER TWO

LITERATURE REVIEW

2.1 Fever

Fever is a Latin word meaning heat or burning while pyrexia is a Greek word that refers to fire. The terms "fever" and "pyrexia" have been used interchangeably, although some use the term 'fever' to mean increased body temperature which is generated as a result of thermoregulatory pyrogens response on the hypothalamus; for example, in inflammatory conditions and sepsis (Hamlin, 2014). Human beings are homoiothermic organisms and as such their body temperature is maintained at a relatively constant 37°C. However, this temperature may differ by up to one degree Celsius in healthy individuals (Houdas and Ring, 2013). Fever commonly occurs in diseased individuals. Nosocomial fever is estimated to occur in approximately one-third of hospitalized patients (Skenderi *et al*., 2018).

As reported by the American College of Critical Care Medicine, the Infectious Diseases Society of America and the International Statistical Classification of Diseases, pyrexia is defined as temperature that is higher than the human core temperature or the upper limit of a normal human body temperature of 38.3°C, regardless of the causative agent (Willoughby, 2009).

Hyperthermia is defined as the temperature that is above 38.2°C. Others have defined hyperthermia as an elevated body's temperature above which is set by the hypothalamus,

thus excluding conditions where fever is caused by pyrogens (Szota *et al*., 2013). Normal body temperatures may vary depending on various factors such as ambient temperature, level of activity, time of the day, age, among others. Elevated body temperature is not always pyrexia. For instance, the body temperature of a healthy individual may increase during exercise, but this is not considered pyrexia, as the setpoint remains normal (Charkoudian *et al*., 2017).

Hyperthermia is normally cytotoxic, and thus it affects the stability of the cell membrane and trans-membrane transport protein function. As a result, the transport of ions is disrupted which in turn leads to an increase in the accumulation of sodium and calcium ions in the cells (Le Renard, 2011).

At lower temperatures, the nuclear matrix usually shows injury compared to the other cell's parts. It shows significant endothermic changes when the temperature is at 40°C (Walter *et al*., 2016). Causes of pyrexia include malignancy, the impact of secondary infection among other diseased states. Pyrexia is the natural body defense mechanism that is aimed at creating an environment that is not conducive for the infectious agent or damaged tissue survival (Maharaj, 2007).

Fever can also occur because of tissue injury or via infection caused by pyrogens produced by micro-organisms. Pyrogens act on white blood cells, which in turn synthesize endogenous toxins that affect the hypothalamus anterior raising the human

body temperature to above 37.5°C, a phenomenon that is referred to as fever (Mbiri, 2017). When the body temperature increases, an individual experiences cold, which stimulates shivering with muscle fasciculation creating heat, after which a new higher temperature is attained, and sweating ensues to lose the excess heat (Collins, 1992).

The infected tissue triggers the generations of pro-inflammatory mediators like cytokines (interleukin-1β and tumor necrosis factor-alpha (TNF-α). These cytokines stimulate increased prostaglandin E_2 synthesis near the preoptic region of the hypothalamus which then initiates the hypothalamus to raise human body temperature (Oka, 2004). Fever is a natural response that occurs as a result of several diseases (Patel and Gallagher, 2010). In most cases, the lack of natural response is even a bigger problem than the induction of fever. There are several signs of fever namely sweating, cold sensation and other subjective sensations (Hamlin, 2014). If any of these symptoms miss during high temperatures then one is seriously ill. Fever can be caused by infections from viruses, parasites, bacteria, chlamydia, rickettsia and immune reactions such as immunological abnormalities, collagen defects and acquired immune deficiency Fever may also occur as a result of tissue destruction like infarction (local necrosis), trauma and inflammatory reaction in the blood vessels and tissues (Hamlin, 2014).

Hyperthermia is an increased human body temperature beyond the hypothalamus thermoregulatory set-point, as a result of insufficient thermoregulation and/or excessive heat production (Anochie, 2013). The nervous feedback mechanism usually regulates the

human body temperature. When the human body temperature rises, the nervous feedback mechanism widens the blood vessels resulting in increased sweating which in turn reduces the temperature of the body (Tansey and Johnson, 2015). On the other hand, when the temperature of the human body reduces, the hypothalamus protects the internal temperature through vasoconstriction. According to Begum *et al*. (2011), high fever frequently increases illness development by increasing tissue dehydration and catabolism (Tansey and Johnson, 2015).

The majority of anti-inflammatory drugs possess the antipyretic potential, for instance, the NSAIDs (non-steroidal anti-inflammatory drugs) (Day and Graham, 2015). The term NSAIDs refers to a class of drugs whose major therapeutic effects are pain suppression (analgesia), reduction in elevated body temperature (antipyresis), and decreased inflammatory signs (anti-inflammatory effect). It has been observed that PGE (prostaglandin) mediates pyrogenic fever. The ability of NSAIDs to suppress PEG synthesis helps in the explanation of antipyretic activity (Day and Graham, 2015).

Studies have shown that a rise in body temperature results in fever-induced stroke, metabolic acidosis and dehydration in children (Rao and Sailaja, 2015). Therefore, measurement of body temperature is a key clinical procedure in hospitals because it is a primary indicator of many clinical conditions. Fever is recognized as a sign in diagnosing and treating diseases and provides essential information on the well-being of an individual (Nascimento, 2012).

2.2 Pathogenesis of fever

The regulation of the body temperature requires an equilibrium between generation and loss of heat in the hypothalamus (Dulceata, 2014). Studies have revealed that many mediators induce pyrexia. Polypeptide cytokines are important “endogenous pyrogens” involved in generating a highly coordinated inflammatory reaction to tissue infection and damage (Grodzinsky and Levander, 2020). Pyrogenic cytokines, like tumor TNF-α and interleukins (Iβ and 6) act directly on the hypothalamus to induce fever reaction (Roth and Blatteis, 2011).

Microbial surface components that are endogenous pyrogens induce pyrexia via pyrogenic cytokines stimulation. Lipopolysaccharides (endotoxins) from Gram-negative bacteria outer membrane can function at the level of the hypothalamus similarly as IL-1β (Roth and Blatteis, 2011).

2.3 Experimental models of fever

Pyrogens are substances that cause fever. Two types of pyrogens exist; endogenous and exogenous pyrogens. Endogenous pyrogens are produced by the body’s cells in a reaction to an outside stimulus, while exogenous pyrogens originate from the outside the body like toxins from bacteria (Borton and Coleman, 2018). A good example of exogenous pyrogen is a substance known as lipopolysaccharide (LPS) which is found in some bacterial cell walls (Kluger and Opp, 2016). According to Roth and Blatteis (2011), various models on pyrexia have been developed to mimic natural pyrexia in laboratory animals.

Pyrexia is a common symptom of inflammation or infection but also arise from non-infectious causes (Roth and Blatteis, 2011). Lipopolysaccharide is the most widely used pyrexia-inducing agent in research studies to characterize the physiological, immunological and neuroendocrine processes and the identification of the neuronal circuits that are fundamental in the manifestation of fever response (Harden *et al*., 2015). Pyrexia can be induced experimentally on laboratory animals using several pyrogens. These include brewer's yeast, turpentine and lipopolysaccharides among others (Kamau *et al*., 2016).

2.3.1 Brewer's yeast test

This method involves the subcutaneous or intraperitoneal injection of 20% of brewer's yeast in rats or mice (10ml/kg body weight). The animals are then fasted and their rectal temperature is taken by inserting a digital thermometer in the rectum after 18 hours. Rats whose rectal temperatures rise by 1°C above normal are considered pyretic (Nisar *et al*., 2008; Mahendran and Narmatha, 2013).

2.3.2 Lipopolysaccharide-induced fever

Lipopolysaccharide is also a common pyrogen that induces pyrexia in animal models. The animals (rats) are normally injected intramuscularly with 50mg/kg body of lipopolysaccharide dissolved in normal saline. The rat's rectal temperature is then scored using a digital thermometer and recorded (Yao *et al*., 2012).

2.3.3 Turpentine fever induction

This test model involves the injection of turpentine (20mg/kg body weight) in rats intraperitoneally. The rats are then allowed to stay for one hour to develop the fever. The lectal temperatures of rats under experimentation is measured one hour after turpentine administration for fours in a row. The mean magnitude of pyrexia which occurs as a result of intraperitoneal injection of turpentine after one hour is considered one hundred percent fever response (Annan *et al*., 2013).

2.4 Inflammation

Inflammation is the body's normal physiological reaction to stimuli, including tissue damage and infections (Chovatiya and Medzhitov, 2014). However, persistent and extreme inflammation may result in a variety of pathological conditions (Stankov, 2012). Inflammation is the body's physiological reaction that arises due to injurious agents such as physical trauma, chemicals, bacterial infection among other phenomena (Rea *et al*., 2018).

The process of inflammation is vital for immune vigilance, maximum tissue repair, and tissue regeneration. The process of inflammation protects the human body from illness by discharging mediators and cells that prevent infection and combat foreign substances (Akram *et al*., 2017). The skin provides an immediate barrier against microbial pathogens and traumatic damage. Besides, the skin has many active defense systems and coordination of these systems is vital, as misdirected or inappropriate immune response

may be involved in the development of many inflammatory skin disorders (Uzal *et al*., 2016).

Edema is a physiological response that is mediated by signaling molecules synthesized by the mast cells, leucocytes, and macrophages undergoing different cellular reactions including phagocytic uptake and generation of inflammatory mediators like interleukins (1β and 6), nitric oxide (NO), PGE_2, and TNF-α (Delves *et al*., 2017). These inflammatory mediators initiate the formation of inflammation due to the extravasation of proteins and fluid and aggregation of leucocytes at the region of tissue damage (Vendramini-Costa and Carvalho, 2012). Besides, it has been conceptualized that cytokine generated by the central nervous system or immune cells may stimulate the peripheral nociceptors directly (Staud, 2015).

Inflammation is a protective reaction that generates several inflammatory mediators due to infection, irritation or tissue damage to eliminate the microbe or irritant and stimulate tissue repair. Inflammation can also cause various degrees of tissue damage that can last beyond acute inflammatory responses such as effusion, allergic reactions, scarring and edema ((Bergenholtz *et al*., 2013).

The inflammatory process normally occurs in two phases namely acute and chronic. Both phases have complex processes that are induced by various classes of chemical mediators. These chemical mediators include leukotrienes, platelet-activating factor and

prostaglandins (Mizejewski, 2015). The acute inflammatory reaction phase is distinguished by a rise in blood vessels permeability and infiltration of cells which results in edema while chronic inflammation is a result of the reaction which arises if the acute inflammation is insufficient in eliminating causative agents of inflammation (Munn, 2017).

Chronic inflammation is associated with fibroblast proliferation and infiltration of neutrophils with fluid exudation. Chronic inflammation occurs through proliferative cell development which can either form granuloma or spread (Fassbender, 2013). Chronic inflammation can also be caused by factors such as antigen or persistence of infection, failure of endogenous anti-inflammatory mechanisms, or recurring tissue injury (Fassbender, 2013; Edlmann *et al*., 2017).

Macrophages regulate various immunopathological phenomena as well as the excess production of pro-inflammatory cytokines and inflammatory mediators (Ren *et al*., 2014). Adhesion molecules activating signals stimulate immune cells during the process of inflammation to intensify inflamed tissue migration capacity and form clusters of a heterotypic cell between the inflamed, immune and endothelial cells (Hua, 2013).

2.5 Experimental induction of inflammation

Generally, several agents can be used to induce inflammation in animal models. Some of these agents include; carrageenan, xylene, histamine, turpentine, among others (Niu *et al*., 2014).

2.5.1 Carrageenan-induced paw edema test

Carrageenan is a polysaccharide with gel-forming and viscosifying properties and is recovered through the extraction of specific red seaweed species that belong to the class *Rhodophyceae* (Necas and Bartosikova, 2013). These red seaweed species are commonly found in Britain, Europe and North America (Pereira, 2018). Carrageenan has nutritional value although it is commonly utilized in the preparation of food due to its thickening, emulsifying and gel-forming properties. It is mostly used in pharmaceutical applications and experimental medicine as an anti-inflammatory inducing agent (Pereira, 2018).

Carrageenan is a sulfated polygalactan containing about fifteen 15-40 % ester-sulfate. It has a relative molecular mass higher than one hundred kDa. It has alternate units of 3, 6-anhydro-Galactose (linked by β-1, 4-glycoside and α-1,3-glycoside linkages) and D-galactose. It is classified in different types such as μ, λ, ε, κ, and ι (Elmajdoub *et al*., 2015). These carrageenan types contain 22 to 35% of sulfate groups. This classification is derived as per the solubility of carrageenan in the solution of potassium chloride. The initial variation which controls the type of carrageenan properties is the content of 3, 6-anhydro-Galactose and the position and number of ester sulfate groups (Li *et al*., 2014).

The chemical reaction of carrageenan is mainly attributed to the groups of half-ester sulfate. These half-ester groups are strongly anionic and are comparable to inorganic sulfate. Commercially available carrageenan are stable salts of calcium, sodium and potassium (Necas and Bartosikova, 2013). For edema induction, subcutaneous injection

of 1% carrageenan (0.1ml) on the left hind paw region of mice or rats respectively is effected to induced inflammation. After one hour, the animals receive plant extracts of known doses. The efficacy of these plant extract in the reduction of the inflamed paw is determined by measuring the paw diameter using digital calipers which are then compared to that of the reference drug in every hour interval for 4 hours (Saravanan *et al*., 2014).

2.5.2 Histamine-induced paw edema model

It is a model where the pain is induced through the injection of histamine. Twenty microliters of histamine (2µg/paw) are usually injected into the left hind paw sub-plantar tissue. Thirty minutes later the animal is then administered intraperitoneally with a known dosage of the plant extract or the standard drug and then paw edema reduction is recorded and tabulated after every hour intervals for 2 hours (Silva *et al*., 2015).

2.5.3 Xylene-Induced Ear Edema

This method involves xylene topical application, on the posterior (20 mL) and anterior (20 mL) surfaces of the right ear. Two hours before xylene application, dexamethasone (5 mg/kg, intraperitoneal) can be used as a control on the left ear. Experimental animals are sacrificed and circular sections of 8mm of their both ears cut with a puncher after one hour of edema induction. The difference between the weights of the left and right ears is used to compute the extent of edema (Basting *et al*., 2014).

2.6 Pain

Pain refers to whatever the experience an individual says it is and exists whenever the individual says it does (Pesut and McDonald, 2007). Pain is an unpleasant sensory and

emotional experience linked with potential or actual tissue injury as defined by the International Association for the Study of Pain (Alcock, 2017). Recently, there has been a change in pain perception that has extremely affected pain research as well as treatment (Clauw, 2015). According to Swan and Hamilton (2019), pain is viewed as a disease rather than a symptom. The occurrence, duration, response to treatment, disabling consequences and severity of pain differ from one individual to another. Since pain has extreme emotional and cognitive effects, it is, therefore, more than a biological phenomenon just like other diseases (Thorn, 2017).

Pain is an overall effect of a complex interaction of the ascending and descending nervous systems that involve biochemical, physiological, psychological, and neocortical processes (Swan and Hamilton, 2016). Pain affects all sorts of human life including emotions, daily activities, sleep, and thoughts. Flor and Turk (2015), stated that pain can be classified as acute or chronic pain.

Pain is a sensation that cannot be objectively measured and its intensity is not consistently a direct reflection of the nociceptive inputs provoking it. Pain is a common experience and cannot be avoided by humans (Turk and Wilson, 2012). Pain poses a protective role that motivates individuals to withdraw from a potentially harmful situation and prevent the same situations from occurring in the future. Also, pain protects damaged tissue during healing (Butler and Moseley, 2013).

2.7 Physiology of pain

Pain occurs when sensory nerve endings called nociceptors come into contact with a painful or noxious stimulus. The capacity to feel pain upon harmful external stimulation or bodily harm is the most important gift for mankind (Butler and Moseley, 2013). Generally, pain arises when a harmful signal sends an impulse via the spinal cord to the brain (Shah and Thaker, 2015). The brain usually interprets the meaning of the signal and sends back instructions to the body on how to respond. The efficacy and sensitivity of the brain circuits influence how much an individual feels and how an individual handles the pain although this system is similar in humans (Lebedev and Nicolelis,2017).

2.8 Management of pain

Both non-pharmacological and pharmacological approaches are used in pain management. The non-pharmacological approaches include cognitive behavioral therapy, massage, reflexology, relaxation, acupuncture and transcutaneous electrical nerve stimulation (TENS) (Bogduk and Merskey, 1994). Two main types of pain exist; fast or rapid pain and slow pain. Rapid or fast pain is experienced after a stimulant strike in one or more seconds and then its severity increased slowly after several seconds or even minutes (Vaso *et al.,* 2014). Rapid pain is described by some names such as tingling, stabbing, acute and electrical pain (Bahmani *et al*., 2014). Slow pain has various names like burning, throbbing, unclear and chronic pain. Slow pain is usually accompanied by tissue damage and may result in unbearable pain. It can be created in both the skin and tissue of an organism (Bahmani *et al*., 2014). Rapid pain is usually created by heat or

mechanical stimuli while slow pain is stimulated by painful chemical stimulants (Debbag and Khidhir, 2016). Pain falls under five critical signs that numerous negative outcomes are created if it is not noticed (Larbi *et al*., 2016).

Once the pain is perceived, the brain usually sends a message downward to affect the nerve's behavior and sensitivity. Neuromodulators such as serotonin, gamma amino-butyric acid, norepinephrine and endogenous opioids (enkephalins and endorphins) are then discharged by the body. These chemicals inhibit pain transmission and promote the production of an analgesic effect. The pain impulse inhibition is referred to as pain modulation. The descending efferent fibers paths extend from the cortex down to the spinal cord and can affect pain impulses at the spinal cord level. This system provides an essential survival function, because it manages fear and anxiety, allowing the alteration of pain experience according to the situation rather than pain domination (Swan and Hamilton, 2016).

2.8.1 The gate control theory of pain

This theory was developed by Melzack and Wall in the year 1965. The gate control theory of pain hypothesized that the dorsal horn of the spinal cord possesses tiny neural networks that are in charge of relieving pain when an intense tactile stimulation is applied in a specific location of the body (Chakravarthy *et al*., 2018). This phenomenon is experienced when rubbing a spot where tissue damage has just occurred (Livingston, 2012). The axons of first-order afferent nociceptors and low-threshold afferent mechanoreceptors converge to the same neurons in the SG (substantia gelatinosa) of the

spinal cord dorsal horn, where nociceptive signals are blocked by inhibitory interneurons on their way to the brain (Guo and Hu, 2014). Mechanoreceptors usually generate high-rate action potential since they are low-threshold and their axons are myelinated. Contrary, since nociceptive stimuli are transmitted via non-myelinated axons, they are less intense in terms of the transmission rate (Cordero-Erausquin *et al*., 2016).

2.8.2 Mechanisms of pain

Secondary hyperalgesia refers to sensitization that arises within the central nervous system (CNS) (Terman *et al*., 2001). Central sensitization is usually achieved through repeated recruitment of C-fibers following tissue damage which in turn results in changes in the response properties of secondary neurons membranes. This may cause an increased firing rate, a phenomenon referred to as windup (Gao and Ji, 2010).

The C-fibers high-frequency recruitment is either by a tonic stimulation or increased repetitive stimuli may induce an increased perceived pain, even though the stimulation intensity remains constant (Granot *et al*., 2006). The spinal sensitization may persist for minutes, hours or even days (Gao and Ji, 2010).

The prolonged N-methyl-D-aspartate (NMDA) receptors activation can lead to rapidly expressed genes (c-jun, c-fos) transcription, resulting in the sensitization of nociceptors. The neuronal plasticity of the secondary neuron can lead to decreased secondary neuron recruitment threshold in the spinal cord and generate allodynic and hyperalgesic reactions

that may last even after tissue injury healing. Early and aggressive pain management may help in the avoidance of ongoing chronic pain (Roeckel *et al*., 2016).

2.8.3 Pain pathways

The spinothalamic and the spinoreticular tracts are the main pathways that secondary neuron travels to superior centers. The former usually sends the afferents to the thalamus lateral nuclei while the latter usually sends afferents to the brainstem nuclei, including medial thalamus, Periaqueductal Gray (PAG) and N-methyl-D-aspartate (MNDA), the two nuclei involved in the descending pain modulation (Willis, 1985). According to Palecek and Willis (2003), another pathway, from the lemniscal (medial dorsal cord) is linked with non-nociceptive afference and also conducts nociceptive afference from the viscera.

When nociceptive afference reaches the cortex, pain is usually perceived. The term nociception, therefore, is used to describe the signal resulting from a tissue injury while pain is a complex perception that requires the activity of the CNS. Activation of a complex network of cortical structures is usually essential during pain perception (Baliki and áVania, 2015). The cortex is categorized into structures that are associated with either the sensory or the affective components of pain.

According to Coghill *et al*. (1994), four cortical structures are important for pain perception and have been identified using brain imaging. These include the somatosensory cortex found in the postcentral circumvolution of the parietal lobe, the

secondary somatosensory cortex found in the parietal operculum, the insular cortex under the frontal and the temporal lobes at the level of the Sylvian fissure and the anterior cingulate cortex above the corpus callosum circumvolution. The somatosensory cortex (SI) and the secondary somatosensory cortex (SII) structures are majorly linked with the pain sensory discriminative aspect, while the insular cortex (IC) and anterior cingulate cortex (ACC) structures are linked with the pain affective component (Granot *et al*., 2006).

2.9 Experimental induction of pain

The analgesic effects of plant extracts and various drugs can be analyzed experimentally by the use of several methods namely; Glutamate-induced nociception**,** writhing tests, formalin test, tail immersion tests, tail-flick test, Haffner's tail clip method and hot plate test.

2.9.1 Glutamate-induced nociception

The method involves oral treatment of rodents with various extracts dosages and normal saline (control, 10 mL/kg). After one hour, the animals are injected with 0.2mL glutamate (30 mmol/paw in normal saline) in the right hind paw sub-plantar tissue. The rodents are then observed for fifteen minutes, and the time spent in licking is recorded and considered as an indication of nociception (Beirith *et al*., 2002).

2.9.2 Writhing test

This test model induces peripheral pain. 0.6% acetic acid (15mL/kg) is usually injected intraperitoneally. Nociception is determined by the number of counts of abdominal constrictions in fifteen minutes after the injection of acetic acid. This method is highly

sensitive but it is not selective due to false positives that occur with muscle relaxants and sedatives (Pinto *et al*., 2015).

2.9.3 Formalin test

Formalin activates primary afferent sensory neurons through specific and direct action on TRPA1, a member of the transient receptor potential family of cation channels that is highly expressed by a subset of C-fibre nociceptors.TRPA1 is activated by several irritants that cause pain (Taylor-Clark and Undem, 2016). This test consists of two phases: the early and late phases that reflect distinct pain types. The early phase reflects the direct formalin effect (non-inflammatory pain) on nociceptors, while the late phase reflects the inflammatory pain (Hunskaar and Hole, 1987). Thirty microliters of one percent formalin are usually injected in the right hind paw sub-plantar tissue. The time taken in biting and/or licking is recorded as the pain response indicator (Agbaje *et al*., 2008).

Compounds like lidocaine (anesthetics) affect the first phase of pain (Milnes and Wilson, 2015). The second phase has been conceptualized to result from activity-dependent sensitization of central nervous system neurons within the dorsal horn (Woolf, 1983). Numerous analgesics, as well as the intrathecal NSAIDs, gabapentin, morphine and NMDA antagonists only inhibit the second phase responses of pain (Chincholkar, 2018). The advantages of this test model include; observation of spontaneous pain responses in an unrestrained rodent, no additional stimulus is required to induce nociceptive behaviors, and nociceptive behaviors can be scored for a longer period. However, despite

the widespread use of this model, the C-fiber activation mechanism remains unknown and is attributed to tissue injury (McNamara *et al*., 2007).

2.9.4 Haffner's Tail clip model

The model involves application of a metal clip at the base of the artery in the tail of the rodent to apply pressure. The time taken to dislodge the tail is then recorded and scored. This test model does not require sophisticated equipment. The Tail-clip test is commonly used in elucidating centrally mediated analgesic responses, which focus on changes above the level of the spinal cord. This test does not detect the peripheral analgesics of the salicylate type (Dzoyen *et al*., 2017).

2.9.5 Manual von Frey method

The model was developed by physiologist Maximilian von Frey. The model evaluates mechanical allodynia in rodents. This model remains the gold standard for evaluating mechanical thresholds in rodents despite the development of the electronic Von Frey test. In this model, the rodent paw is removed either during stimulus application or immediately after the filament. The dorsal surface of the hind paw or the abdomen can be used for testing although the plantar surface of the hind paw is the most commonly used area (Minett *et al*., 2014).

2.9.6 Thermal probe test

According to Deuis and Vetter (2016), this model is used to quantify heat thresholds in mice. It is based on the application of a thermal probe (2 mm) on the hind paw and can be performed using the same mouse enclosures as the electronic von Frey test. Specific temperature can be recorded as the handle rotates for a given time (Deuis *et al*., 2016).

2.9.7 Acetone evaporation test

This model measures the aversive behaviors caused by evaporative cooling. It is used to measure cold allodynia. This model is performed on a mesh floor and acetone is sprayed or dabbed on the hind paw plantar surface, eliciting cooling of the skin to innocuous temperatures of between 15 to 21°C (Harriott *et al*., 2019).

2.9.8 Cold plantar assay

A clean glass enclosure is used in this model. A cut off syringe filled with wet ice (17°C) or dry ice (5 to 12°C) is applied onto the glass underneath the paw of an animal for cold stimulation (Colburn *et al*., 2014). Glass cooling result in paw unilateral exposure to a cooled surface, the temperature of which can be measured by attaching a thermocouple probe to the skin or glass. To quantify cold allodynia and hyperalgesia, the latency to paw withdrawal is measured and recorded. However, to achieve efficient temperature transfer, the paw being tested must remain in contact with the cooled glass (Brenner *et al*., 2015).

2.9.9 Hot-plate and electrical stimulation test

This method is used in many scientific research on central pain and not peripheral pain. Hot-plate involves placing an experimental animal (rat or mouse) at 52.5°C on an enclosed hot plate. The latency to jump or lick a hind paw when the animal is put on the hot plate is then counted and scored. Low-intensity hot plates are suitable and sensitive to analgesic drugs. This method causes false positives with sedatives and muscle relaxants. Mixed opiate agonists-antagonists also give unreliable results (Singh *et al*., 2018).

2.9.10 Tail-flick

The rats are manually restrained using a perforated Perspex tube exposing the tail of the rat towards the operator on and a light beam (375-w) is focused onto the rat's tail with the help of a condenser lens. The time the rodent takes before the withdrawal of the tail is scored and recorded (Campos *et al*., 2006).

2.9.11 Tail-immersion test

This test model is used to test for acute pain. The test is helpful in the differentiation of central opioid analgesics from peripheral analgesics (Singh *et al*., 2018). The method involves immersion of the lower five centimeters section of the rodent tail into a beaker with hot water maintained at 55±0.5 degrees Celsius. The reaction time is measured thirty minutes before and after the administration of extracts dosages. The time spent by the rodent to withdraw its tail from hot water is recorded as the reaction time; with a cut-off time set at ten seconds (Gupta *et al*., 2015).

2.10 Conventional management of pain

Conventionally, pain, is managed using analgesics. Analgesics are either classified as opioids or non-opioid drugs. The opioid drugs are those that adhere to special opioid receptors located in the CNS and among other tissues (Kapur *et al*., 2014). The commonly used analgesics included; NSAIDs such as aspirin, anti-inflammatory steroids such as cortisone and μ-opioid agonists such as morphine. These drugs are not uniformly effective, and their undesirable effects often limit their use. For a long time, the focus on drug discovery has been the search for novel analgesics (Negus *et al*., 2006).

The specific non-opioid drugs include paracetamol and cyclooxygenase inhibitors like NSAIDs (Paul and Chauhan 2005; Labianca *et al*., 2012). The non-specific non-opioid drugs consist of drugs with analgesic effects that are mostly utilized for other purposes, for example in the treatment of depression or epilepsy (Van Hout *et al*., 2014). Opioids are usually prescribed in the management of moderate to strong pain. They can be classified as synthetic substances, for instance, pentazocine, fentanyl and methadone, or naturally occurring, for example, morphine (Pasternak *et al*., 2013). Opioids usually bind to opioid receptors belonging to the G-protein-coupled receptors family in the body. Many opioids demonstrate their analgesic activities by binding to μ-receptor in the CNS (central nervous system). Also, they may bind to peripheral opioid receptors when they are present (Hutchinson *et al*., 2011).

Different effects of opioids drugs can be caused by binding to the various opioid receptors. Long-term use of opioid drugs may not be well tolerated on one-third of individuals with chronic pain (Mercadante and Bruera, 2016). The benefits of analgesic opioids are weighed on the risk of abuse in susceptible individuals, costs, and severe effects like nausea, constipation, respiratory sedation, depression, hyperalgesia and death (McCleane and Smith, 2007). During chronic opioid therapy, some changes may lead to abnormal behaviors in some patients. It has been established that patients who are under opioid therapy tend to increase dosages dramatically. This creates a potential conundrum while medical practitioners pursue an ever-moving target for adequate pain relief (Schneider and Kirsh, 2010).

The NSAIDs are cyclooxygenases 1 and 2 (COX-1 and COX-2) inhibitors. The suppression of the cyclooxygenases leads to inhibition of thromboxanes and prostaglandins syntheses. It is generally postulated that inhibition of cyclooxygenase-2 brings about analgesic effects (Harirforoosh *et al*., 2014). The NSAIDs which inhibit COX-1, such as aspirin, may bring about problems such as ulcers and gastrointestinal bleeding (Alfonso *et al*., 2014). Meta-analysis has confirmed that acetaminophen is effective postoperative analgesia and the addition of codeine (60mg) produced a worthwhile pain relief upon administration of a single dose orally (Au *et al*., 2015).

2.11 Alternative and complementary medicines in the treatment of pain, fever and inflammation.

Natural products from minerals, animals and plants have been the basis of the treatment of human diseases and disorders (Lahlou, 2013). Currently, it is estimated that about eighty percent of people living in developing nations still relays on traditional medicine for their primary health care. Verma and Singh (2008), noted that herbal medicine plays a vital role in the development of potent therapeutic agents. Therefore, the observed drawbacks observed with the availability and use of conventional drugs used in the treatment of pain, fever and inflammation lead to seeking alternative methods including herbal remedies.

Scopolamine is extracted from *Hyoscyamus niger* and it is used as a narcotic and sedative for treatment of motion sickness (Shakeran *et al*., 2014). Quinine is extracted from *Cinchona officinalis* and is used as an antimalarial especially in the treatment of resistant

strains such as *Plasmodium falciparum* (Gurung and De, 2017). Sanguinarine is extracted from *Eschscholzia californica* and studies have shown that it has antibacterial and antiplaque properties. Sanguinarine is used in oral rinses and toothpaste (Ncube and Van Staden, 2015). Emetine is obtained from *Uragoga ipecacuanha* and possesses amoebicide properties toothpaste (Ncube and Van Staden, 2015) Morphine is extracted from (*Papaver somniferum*)and is used as a powerful narcotic analgesic in strong pain management especially for cancer patients (Booth, 2013). *Harpagophytum procumbens* (Devil's Claw) has been used in Africa for the management of osteoarthritis, fever, skin conditions, rheumatoid arthritis and pain (Abhishek *et al*., 2016). Harpagoside one of the compounds of *Harpagophytum procumbens* has been shown to suppress the synthesis of COX-2 through inactivation of nuclear factor κB and inhibition of lipopolysaccharide-induced inducible nitric oxide synthase (Chung *et al*., 2018). Other sources of anti-pain remedies include the Cannabis plant. It contains at least sixty different cannabinoids, many of which possess pharmacological bioactive compounds. The crucial active component of *Cannabis* (THC) has been shown to possess analgesic activities in experimental animals (Andre *et al*., 2016). Leroux in 1829 isolated salicin from the willow bark, and later in 1838 salicylic acid was discovered (Rainsford, 2016).

The activities observed with medicinal plants have been attributed to the presence of the secondary metabolites known as phytochemicals terpenoids. Terpenoids are groups of natural products that are widespread and chemically diverse. Terpenoids are flammable unsaturated hydrocarbon which exists in the form of liquid and there are commonly

found in resins, oleoresins and essential oils (Doughari, 2012). They include hydrocarbons with a general formula of $(C_5H_8)n$ and depending on the number of hydrocarbons they are classified as monoterpenoids, diterpenoids, triterpenoids and sesquiterpenoids (Sheehama, 2017). Examples of monoterpenoids include thujone, camphor, terpinen-4-ol, menthol and eugenol. Taxol and resin are some of the important diterpenes (C20) and they are considered to possess anticancer properties. Some of the important triterpenes (C30) include cardiac glycosides, sterols, steroids, and which possess sedative, anti-inflammatory, and insecticidal effects. Other triterpenes include amyrins, ursolic acid, and oleanolic acid sesquiterpene (C15) which are crucial essential oil constituents (Duke *et al.,* 2016).

The sesquiterpene functions as irritants when externally applied and when consumed internally their action looks like those irritants of the gastrointestinal tract. Antioxidants prevent cells from damage caused by oxygen free radicals like singlet oxygen, peroxynitrite, peroxyl radicals, superoxide and hydroxyl radicals which causes cellular damage due to oxidative stress (Doughari, 2012).

Natural antioxidants are used in management and prevention of degenerative and chronic ailments such as neurodegenerative disorders, atherosclerosis, carcinogenesis, cardiac and cerebral ischemia, DNA damage and aging, diabetic pregnancy and rheumatic disorder (Bisht and Dada, 2017).

2.12 Methods of analysis of phytochemicals in plant extracts

Generally, several methods can be used to analyze phytochemical compounds in a sample namely; qualitative and quantitative phytochemical analysis (Luthria *et al*., 2015). The choice of the test model depends on the compounds that the scientist targets and whether one wants the actual amount or if one wants to confirm their presence or absence (Atanasov *et al*., 2015).

2.12.1 Qualitative phytochemical screening

This test method is carried out to ascertain the presence or absence of phytochemicals. It is normally carried out using various chemical reagents and the investigator observes color changes from which a conclusion is made. Normally the investigator follows a given protocol (Momin *et al*., 2014).

2.12.2 Quantitative phytochemical profiling using GC-MS

The GS-MS (gas chromatography-mass spectrometry) test model is the most preferred test for analyzing organic compounds. It is a suitable tool for testing and profiling quantitative phytochemicals in the plant extracts. The GC-MS test is more effective in the chemical analysis because it takes a longer time to analyze and has a higher resolution power compared to LC-MS (liquid chromatography-mass spectrometry). The results obtained are useful in providing a classical spectral output of the total compounds that undergo separation in a given sample (Asuzu, 2019).

The process is done by first injecting the sample into the injectable part of the gas chromatography device to vaporize the sample. This is followed by the separation and

analysis of different components in the sample. Every component generates a certain spectral peak that is electronically recorded on a paper chart. Retention time refers to the time taken between injections and elution of a sample. The peak is calibrated from the bottom to the peak tip (Asuzu, 2019).

2.12.3 Quantitative phytochemical profiling using LC-MS

Liquid chromatography-mass spectrometry (LC-MS) is an investigative method that couple liquid chromatography physical separation abilities with mass spectrometry. The test model generally separates mixtures that have multiple components. It is the most suitable method in the analysis of polar, non-volatile and unstable ionic compounds (Wolfender *et al*., 2018).

2.12.4 Agro-ecological parameters

Mbeere North Sub-County receives annual rainfall ranges between 640 to 1,100 millimeters with most parts of the area receiving an average of 550 mm of rainfall per year. The temperature ranges between 25-33ºC with hot and dry semi-arid climatic conditions. The residents practice mixed farming with crops cultivated being maize, beans, pigeon peas, green grams, cowpeas and sorghum. The soils are generally sandy, blackish-grey, or reddish-brown however some places have brown soil. The Forest occupies about 2000 Ha of a predominantly indigenous forest, with less than 5% exotic plantations mainly found at the foot and top of the hill. There are also pockets of exotic plantations which include *E. globulus*, *P. patula* and *C. lusitanica* among others (Mugatha, 2004).

2.13 Plants used in this study

2.13.1 *Eucalyptus globulus*

E. globulus belongs to the family Myrtaceae (Yong *et al*., 2019). The plant grows well in many countries, for example, in India, it grows at an altitude of 5,000-8,300ft above sea level. Other places where the plant is cultivated include Kullu, Ranikhet, Chamba and Kangra. *E. globulus* is a huge tree with a height of 300 ft or more. Under forested conditions, *E. globulus* grows in a clean straight trunk but tends to branch freely in an open field (Mousa *et al*., 2019). Junevile leaves of *E. globulus* are opposite, cordate-ovate, sessile and covered with bluish-white bloom. Adult leaves are lanceolate, alternate, 1-2 inches broad and 6-12 inches long. The coppice shoots and seedlings stem appears quadrangular (Dixit *et al*., 2012). *E. globulus* is an evergreen tree with a straight trunk of about 0.6 to 2 meters diameter and a height of 40-70 m tall. It has well spread deep roots and smooth, brownish, greenish, mottled gray and long peeling bark (Mishra *et al*., 2010).

E. globulus is a plant that possesses many pharmacological and medicinal properties. This is because the plant has many phytochemicals and other volatile components. Some of the ailments and conditions treated using the *E. globulus* include antiphlogistic, antiperiodic, antiseptic, deodorant, astringent, anthelmintic, expectorant, inhalant, diaphoretic, insect repellant, sedative, rubefacient, vermifuge and suppurative (Shaffique *et al*., 2018). Besides, *E. globulus* is utilized in the management of abscess, asthma, burns, sore throat, bronchitis, colds, cancer, cough, diphtheria, diabetes, arthritis, malaria,

dyspepsia, dysentery, miasma, boils, tuberculosis, spasms, wounds, worms and vaginitis (Ray *et al*., 2015).

Research studies have shown that Eucalyptus oil possesses antiseptic effects *in vitro*. Besides, Eucalyptus oil has been demonstrated to possess potent mucolytic and expectorant effects, which stimulates the epithelium of the bronchial. Further, Eucalyptus oil is considered to decongest the upper respiratory tract during the common cold (Ray *et al*., 2015).

Figure 2. 1: Photograph of *Eucalyptus globulus* (captured in 2016)

2.13.2 *Senna didymobotrya*

S. didymobotrya is a flowering plant that belongs to the legume family. The plant grows to a maximum height of about 30-90cm. The roots extracts of *S. didymobotrya* is utilized in the management of intestinal worms and malaria. The aqueous root or leaf extract is used in the treatment of skeletal muscle and venereal diseases. The stems, leaves and roots extract of the plant have been reported to cure fungal, bacterial, parasitic infections, hypertension, sickle cell anemia, fibroids and backache (Ngule and Swamy, 2013).

The medicinal values of *S. didymobotrya* have been explored by herbalists in various regions of the world. In Burundi Congo, Kenya, Rwanda, Tanzania and Uganda, the aqueous root extract of *S. didymobotrya* is used in the management of various diseases which include jaundice, ringworm, intestinal worm and malaria (Nyamwamu *et al*., 2015). The plant is also an effective medicine in the treatment of various ailments such as fungal, hypertension, sickle cell anemia, bacterial infections and a range of diseases affecting women like fallopian tubes inflammation, backache, fibroids, uterine wall contraction and stimulation of milk in lactating mothers (Nyamwamu *et al*., 2015). In Kenya, for example, the Kipsigis community has for many years used the plant as an anti-malarial drug. Many pastoralists in West Pokot use the plant bark in the preservation of milk (Tabuti, 2007). In Embu County, this plant is used in the treatment of colds, flu, pain, fever and inflammation (Kareru *et al*., 2007).

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Figure 2.2: Photograph of *Senna didymobotrya* (captured in 2016)

CHAPTER THREE

QUANTITATIVE PHYTOCHEMICAL COMPOSITION OF *Eucalyptus globulus* AND *Senna didymobotrya* USING GC-MS

3.1 Introduction

Phytochemicals are found in varying quantities in plants depending on the geographical location, ecological zone, where the plant grows and the amount of rainfall. For example, plants that grow in arid areas have high concentrations of phytochemicals that are crucial in surviving adverse weather conditions (Gahukar, 2014), while after predation, plants produce secondary metabolites like tannins, which make them less palatable for a while (Coley and Barone,1996).

Phytochemicals serve as effective chemotherapeutic agents against several human diseases (Iqbal *et al*., 2017). Phytochemicals are arguably more efficacious, less toxic, highly biodegradable and easily accessible alternatives to conventional medicine (Kuma and Sharma, 2018). Interestingly, many conventional drugs in current use are plant-derived. For example, Cocaine, a well-known alkaloid, is obtained from the leaves of *Erythroxylon coca*, while nicotine is obtained from tobacco plant (*Nicotiana tabacum*), 9-tetrahydro cannabinol (THC) comes from marijuana (*Cannabis sativa*). (Bharate *et al*., 2018).

The pharmacological efficacy of phytochemicals may be due to the synergistic effects (Malongane *et al*., 2017). Due to biocompatibility and overlapping mechanisms of action, proper analysis of the active agents is necessary (Verri *et al*., 2012).

E. globulus and *S. didymobotrya* are used in Embu County, Kenya in the management of pain, fever and inflammation (Kareru *et al*., 2007). It is postulated that the biological activities observed with these plants are due to the presence of phytochemicals. However, there is no scientific study that has been carried out to validate and or confirm this.

Therefore, this study was carried out to elucidate the quantitative details of the phytochemical composition of the leaf extracts of *E. globulus* and *S. didymobotrya* using GC-MS. It is envisaged that the identity of phytochemicals in the two plant extracts will widen the understanding of their reported antipyretic, analgesic and anti-inflammatory activities.

In this chapter, quantitative phytochemical screening of leaf extracts of *E. globulus* and *S. didymobotrya* using GC-MS is described. The two medicinal plants were later bio-screened for their antipyretic, antinociceptive and anti-inflammatory potential in animal models. These are described in Chapters 4, 5 and 6.

3.2 Materials and methods

3.2.1 Collection and preparation of plant materials

After an extensive literature review on the medicinal uses of these plants and with bio-conservation considerations in mind, fresh leaves of *E. globulus* and *S. didymobotrya* were collected from Makunguru village, Nthawa location, Siakago division, Mbeere North Subcounty in Embu County, Kenya during a dry season on 16th of August in the year 2016. All the samples were collected once the same day and month. The GPS location for *E. globulus* and *S. didymobotrya* specimens were 0º35'12."S, 37º38'32"E and 0º35'28."S, 37º38'25"E respectively. The collection of these samples was done onc based on ethnobotanical information availed by local herbalists in the area. These plants are in traditional use against pain, fever and inflammation.

The samples were cleaned to remove dirt and other contaminants. They were wrapped in cotton bags and transported to Kenyatta University in the Department of Biochemistry, Microbiology and Biotechnology for further processing. The samples were identified, authenticated and taxonomically assigned voucher specimen numbers by an acknowledged taxonomist. *E. globulus* was assigned voucher specimen no (JKM001) while *S. didymobotrya* was assigned (JKM002). Voucher specimens were deposited at the Plant Sciences departmental Herbarium of Kenyatta University for future reference.

The leaf samples of the two plants were air-dried at a temperature of 25 ºC in the Biochemistry laboratory of Kenyatta University for two weeks. They were then ground

using an electric mill into a fine powder. They were stored at room in well-labeled airtight khaki papers until use in extraction. This study was undertaken in the animal breeding and experimentation laboratory of Kenyatta University, Kenya.

3.2.2 Extraction

A mass of five 500g of the powder of each plant was each weighed and put into separate conical flasks and labeled. A volume of 1500 ml of dichloromethane was then put into each conical flask and corked. The mixture was left to stand for twenty-four hours. Filtration of the extracts was then done in separate well labeled conical flasks using Whatman No.1 filter papers. 500ml of dichloromethane was added to each remnant and allowed to stand for twenty-four hours, and then the second filtration was done. This procedure was repeated until the DCM remained clear. A rotary evaporator was used to concentrate the extract at 40ºC until dry. The extracts were then put in open 100ml beakers for five days to allow any remaining organic solvent to evaporate until a sticky solid was formed, which was then stored at -4°C awaiting use in the analysis.

3.2.3 Gas Chromatography-Mass Spectrometry analysis

Three grams of the leaf extracts of *E. globulus* and *S. didymobotrya* were separately obtained and analyzed to determine their quantitative phytochemical composition at ICIPE (International Centre of Insect Physiology and Ecology) laboratory. The protocol followed for analyzing the samples was reviewed.

A mass of 1.1 mg leaf extract of *E. globulus* and 1.2mg *S.didymobotrya* were weighed and diluted in respective volumes by partitioning between hexane and methanol. The mixture was then vortexed and centrifuged.

The samples were analyzed on an Agilent Gas Chromatograph 7890A/5975C Mass Spectrometer in full scan mode with the following specifications; gas chromatography Column (HP-5 MS low bleed capillary column (30 m by 0.25 mm i.d., 0.25 μm) (J&W, Folsom, California, United States of America)), flow rate, (Helium) (1.25 ml/min, constant flow mode), injection split mode, the temperature of oven (35°C) for initial five minutes and then elevated by 10°C per minute to 280°C Celsius for 10.5 minutes; run time 70 minutes) (Chen *et al*., 2019).

The hexane layer was then dried by passing through anhydrous Na_2SO_4 and analyzed by GC-MS. The peak area was used for quantification. Serial dilutions of authentic standard (1,8-cineole; 99%, Gillingham, Dorset, England) (50ng/µl, 150ng/µl, 250ng/µl, 350 ng/µl and 550 ng/µl) were prepared and analyzed by GC-MS, whose area was used for quantification purposes.

Gas chromatography linked with mass spectrometry is a technique of choice that is used in the analysis of phytochemicals. It has a mobile phase where carrier inert helium or other inert gases such as nitrogen are used. It has a microscopic layer, which is a stationary phase consisting of a polymer or liquid on an inert solid support, inside a

column. A stationary phase that is usually fine solid support coated with a nonvolatile liquid is found in the capillary column. A stream of helium gas is used to sweep the sample through the column (Tang *et al*., 2015).

The separation of each of the sample components from each other is based on the time taken to pass through the column because the time taken by each component is different. Mass Spectrometry (MS) is the detector for gas chromatography. The sample is usually fragmented by gas chromatography-mass spectrometry as it exits the end of the gas chromatography column. Based on volatility, the gas chromatography separates various components in the sample into pulses of pure chemicals (McEwen and McKay, 2005).

3.2.4 Data management and statistical analysis

The leaf extract of *E. globulus* and *S.didymobotrya* phytocompounds identities were carried out based on their fragmentation pattern and the reference spectra published by library-mass spectrometry databases of the National Institute of Standards and Technology (NIST). Identification of the phytocompounds was done using NIST'08, 05, Adams and chemical mass spectral databases. The retention indices of these compounds were determined using the C5-C32 hydrocarbons range. Identification spectra above 60% of the library match were required for the identification of phytocompounds. The compound molecular weight, name, the chemical class and the structural formulae were identified. The relative amounts of each component were expressed as a percentage with peak-area normalization (Ibrahim *et al*., 2015). The percentage abundance of each phytochemical identified by GC-MS was computed using the formula below;

$$Percentage\ Abundance = \frac{\text{Concentration of each Phytochemical}}{\text{Total Concentration of all Phytochemicals}} \times 100$$

3.3 Results

3.3.1 Quantitative phytochemical composition of leaf extracts of *E. globulus* and *S. didymobotrya*

The GC-MS analysis of the leaf extract of *E. globulus* revealed the presence of twenty-five phytocompounds (Table: 3.1). The major compounds were α-Deudesmol**,** α-phellandrene and β-Pinene with percentage abundances of 14.81%,12.73% and 10.68% respectively. The compounds with the least percentage yield were Tridecane with percentage abundances and 4- terpineol with percentage abundances of 0.61% and 0.74% respectively. The other compounds were: α-pinene , Camphene, Myrcene , *p*-Cymene, Limonene, γ-terpinene, Terpinolene, Undecane, Endofenchol, Camphor , Borneol, α-terpineol, α-Copaene, α-Gurjunene, (*E*) Caryophyllene, Aromadendrene, α-selinene, δ-armophene and Globulol with percentage abundances of 5.73%, 2.01%, 0.75%, 7.18%, 10.44%, 2.18%, 0.93%, 1.70%, 1.15%,4.03%, 0.90%, 1.06%, 1.5%, 7%, 3.74%, 1.95%, 2.75%, 1.70 % and 2.30% respectively (Table 3.1).

RT (mins)	Compound name	Molecular Formula	Chemical class	Concentration (µg/mg)	% Abundance
9.78	α-pinene	(C10H16)	Mono terpenoids	17.9	5.73
10.11	Camphene	(C10H16)	Mono terpenoids	6.3	2.01
10.7	β-Pinene	(C10H16)	Mono terpenoids	33.4	10.68
11.01	Myrcene	(C10H16)	Mono terpenoids	2.4	0.75
11.26	α-phellandrene	(C10H16)	Mono terpenoids	39.8	12.73
11.64	p-Cymene	(C10H14)	Mono terpenoids s	24.4	7.81
11.71	Limonene	(C10H16)	Mono terpenoids	31.4	10.44
12.27	γ – terpinene	(C10H16)	Mono terpenoids	6.8	2.18
12.8	Terpinolene	(C10H16)	Mono terpenoids	2.9	0.93
12.98	Undecane	(C11H24)	Fatty acids derivatives	5.3	1.7
13.25	Endo-fenchol	(C10H18O)	Mono terpenoids	3.6	1.15
13.74	Camphor	(C10H18O)	Mono terpenoids	12.6	4.03
14.12	Borneol	(C10H18O)	Mono terpenoids	2.8	0.9
14.33	4- terpineol	(C10H18O)	Mono terpenoids	2.3	0.74
14.57	α-terpineol	(C10H18O)	Mono terpenoids	3.3	1.06
15.94	Tridecane	(C13H28)	Fatty acids derivatives	1.9	0.61
17.17	α- Copaene	(C15H24)	Mono terpenoids	4.9	1.57
17.64	α-Gurjunene	(C15H24)	Mono terpenoids	6.9	2.21
17.77	(E) – Caryophyllene	(C15H24)	Mono terpenoids	11.7	3.74
18.04	Aromadendrene	(C15H24)	Mono terpenoids	6.1	1.95
18.31	Allo-aromadendrene	(C15H24)	Mono terpenoids	18.5	5.92
18.76	α-selinene	(C15H24)	Mono terpenoids	8.6	2.75
19.05	δ- armophene	(C15H24)	Mono terpenoids	5.3	1.7
19.84	Globulol	(C15H26O)	Sesquiterpenoids	7.2	2.3

20.64	α- Eudesmol	(C15H26O)	Mono terpenoids	46.3	14.81

Table 3. 1 Quantitative phytochemical composition of leaf extract of *E. globulus*

The GC-MS chromatogram of leaf extract of *E. globulus* is shown in figure 3.1.

```
File       :C:\COMMERCIAL_2017\June 2017\BSB\RK20170702A-EG.D
Operator   : [BSB3]OK
Acquired   :  2 Jul 2017    5:09     using AcqMethod HEX VOLATILES 35-280 XTD 70MINUTES .M
Instrument :    ICIPE MSD
Sample Name: E. globulus
Misc Info  : E. globulus
Vial Number: 8
```

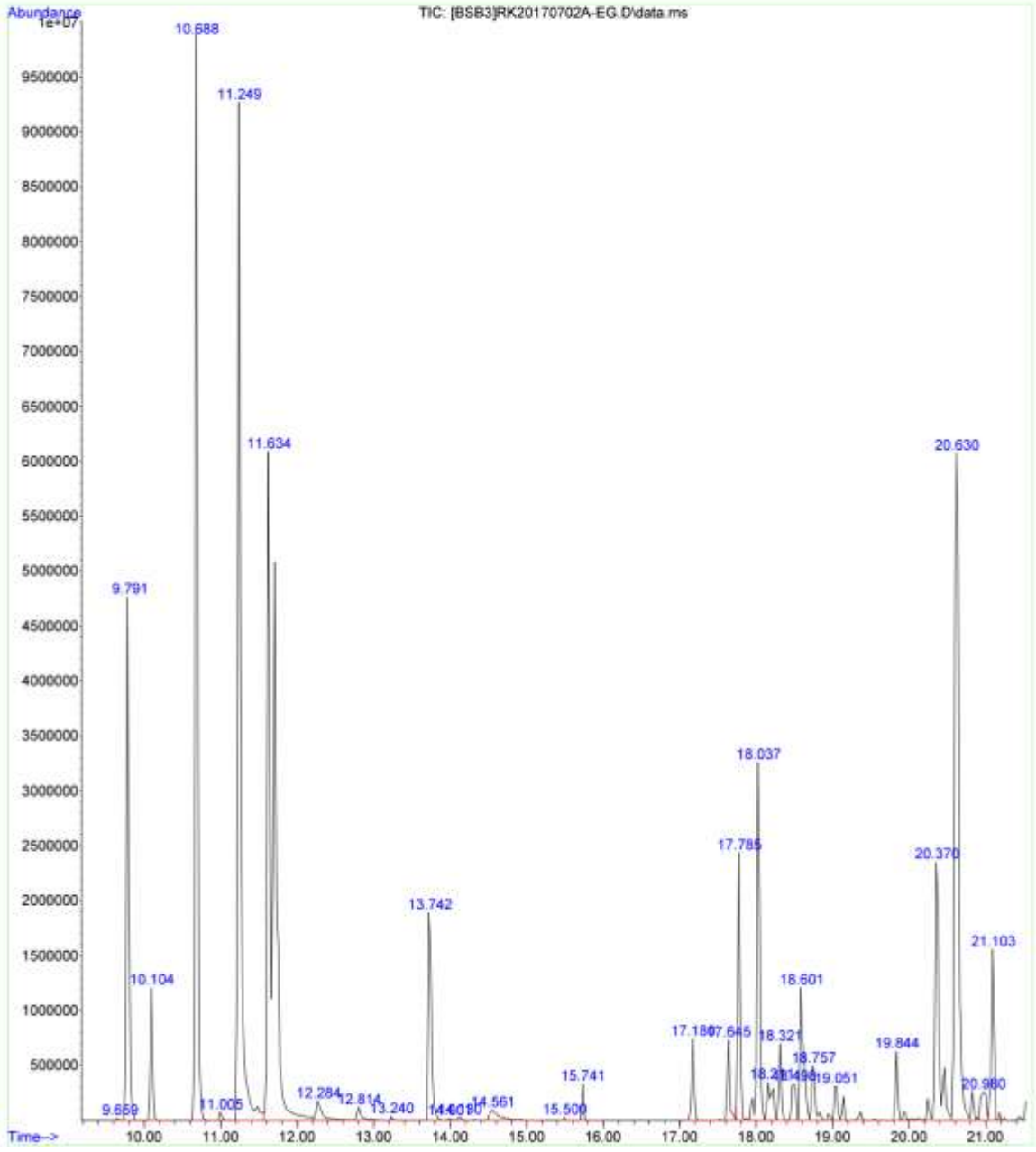

Figure 3. 1: Representative total ion chromatogram of the leaf extract of *E. globulus* with retention time.

On the other hand, the GC-MS analysis of the leaf extract of *S. didymobotrya* revealed that it had ten compounds all of which were terpenoids. The most abundant compound was camphor followed by Limonene with percentage abundances of 34.01% and 24.90% respectively, while Cumene had the lowest percentage abundance of 3.23%. The percentage abundances for the other compounds were as follows; α-pinene, Camphene, β-pinene, α-phellandrene, γ-terpinene,α-terpinene and Terpinolene with percentage abundances of (5.47%), (9.11%), (3.85%), (3.24%), (3.24%), (8.30%) (4.66%) respectively (Table 3.2; Figure 3.2).

Table 3.2: Quantitative phytochemical compositions of leaf extract of *S. didymobotrya*

RT (mins)	**Compound name**	**Formula**	**Chemical class**	**Concentration (µg/mg)**	**% Abundance**
9.68	Cumene	(C_9H_{12})	Mono terpenoids	1.6	3.23
9.80	α-pinene	($C_{10}H_{16}$)	Mono terpenoids	2.7	5.47
10.11	Camphene	($C_{10}H_{16}$)	Mono terpenoids	4.5	9.11
10.72	β-pinene	($C_{10}H_{16}$)	Mono terpenoids	1.9	3.85
11.26	A-phellandrene	($C_{10}H_{16}$)	Mono terpenoids	1.6	3.24
11.37	α-terpinene	($C_{10}H_{16}$)	Mono terpenoids	1.6	3.24
11.73	Limonene	($C_{10}H_{16}$)	Mono terpenoids	12.3	24.90
12.26	γ – terpinene	($C_{10}H_{16}$)	Mono terpenoids	4.1	8.30
12.82	Terpinolene	($C_{10}H_{16}$)	Mono terpenoids	2.3	4.66
13.74	Camphor	($C_{10}H_{18}O$)	Mono terpenoids	16.8	34.01

```
File       :C:\COMMERCIAL_2017\June 2017\BSB\RK20170702A-SENNA.D
Operator   : [BSB1]OK
Acquired   :  2 Jul 2017    6:11     using AcqMethod HEX VOLATILES 35-280 XTD 70MINUTES .M
Instrument :   ICIPE MSD
Sample Name: Senna
Misc Info  : Senna
Vial Number: 9
```

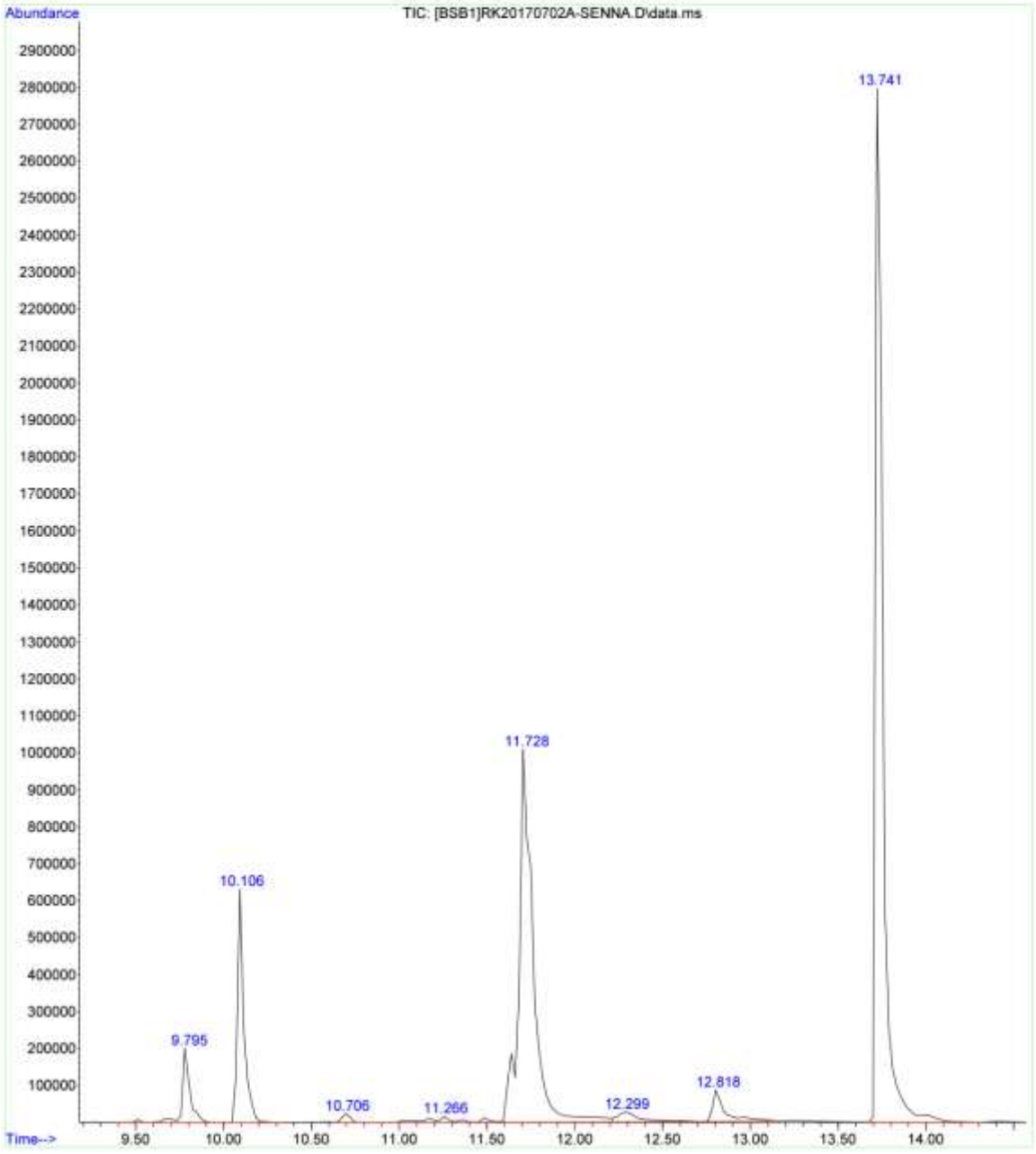

Figure 3.2: Representative total ion chromatogram of the DCM leaf extract of *S. didymobotrya* with RT.

The principles identified in the leaf extract of these plants belonged to Mono terpenoids, Fatty acids derivatives and sesquiterpenoids. Mono terpenoids include;terpenes, monoterpene, myrcene, limonene, camphor, camphene. alpha-phellandrene, *p*-Cymene, γ-terpinene 4-terpineol, α-terpineol, Terpinolene, α-Eudesmol, Borneol and α-terpineol. The second category are Fatty acids derivatives, which include Undecane and Tridecane and sesquiterpenoids such as Globulol (Figure 3.1 and 3.2).

After analysis by GC-MS, it was observed that the *E. globulus* leaf extract had a higher percentage of phytochemical agents than the *S. didymobotrya* extract. However, seven phytochemicals were found in both plant extracts but varying concentrations (Figure 3.3).

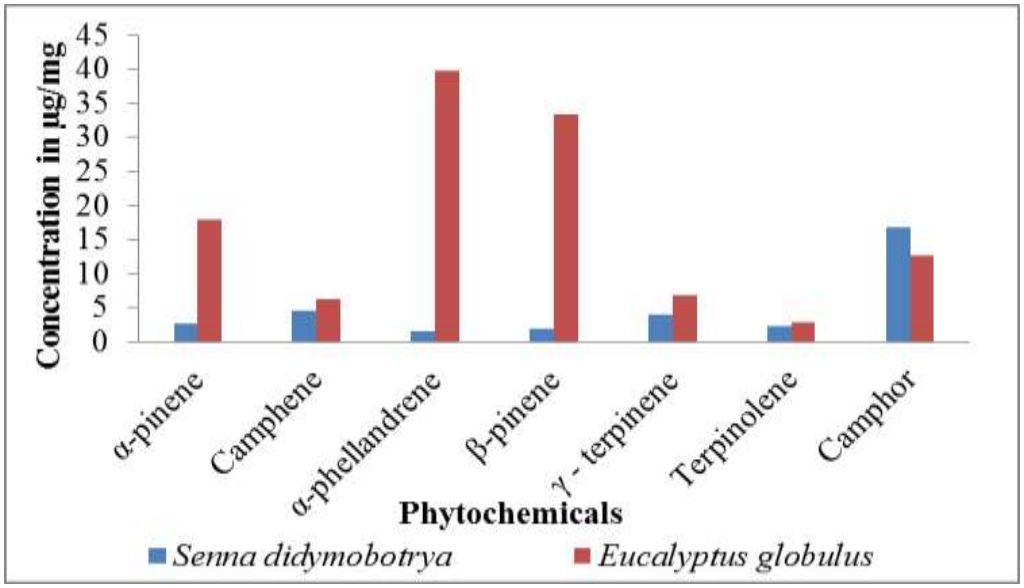

Figure 3. 3: Comparison of the concentration of phytochemicals present in the leaf extracts of *E. globulus* and *S.didymobotrya*.

3.4 Discussion

Gas chromatography coupled with mass spectrometry (GC-MS) is a suitable technique for the separation of volatile organic compounds based on their polarity (Li *et al*., 2015). A basic track recorder prints the gas chromatogram with each peak on the chromatogram representing a compound. The larger peak corresponds to the major product while the smaller peak shows a minor product. In this study, phytochemical profiles of the leaf extracts of *E. globulus* and *S. didymobotrya* were identified by GC-MS analysis. The gas chromatogram revealed relative concentrations of different phytocompounds eluted at various retention times (Figures 3.1 and 3.2). Based on the GC-MS results, most of the total phytochemicals characterized in the two plants belonged to terpenoids. The terpenoids form a major component of the primary constituents of many types of essential oils among various plant species (Keilwagen *et al*., 2017).

The results of GC-MS analysis also revealed the presence of terpenoids. Animal studies have shown that terpenoids possess anti-diabetic, anti-cancer, anti-hypertriglyceridemia properties, anti-hypercholesterolemia and memory enhancement properties (Vijayan *et al*., 2017).

Among the terpenoids identified in the *E. globulus* extract was Borneol ($C_{10}H_{18}O$), bi-cyclic mono-terpenoid alcohol. It is a very useful bioactive compound that has its hydroxyl group attached at the *endo* position. Borneol is

used as folk medicine in China and India in the management of pain, itching and hemorrhoids. Borneol shrinks hemorrhoidal tissue thus relieving the burning sensation. It is useful in providing relief of anorectal discomforts and reduces inflammatory activity by protecting the anorectal surface thus making bowel movements smooth and less painful (Mohammadhosseini *et al*., 2017).

In vivo experiments carried out in mice and rabbits have revealed that Borneol is a novel agent because it boosts drug delivery to the central nervous system (CNS) by enhancing the permeability of the blood-brain barrier (Zhang *et al*., 2017). Borneol has a wide range of other pharmacological uses namely; it improves circulation and tones the heart, it stimulates the production of gastric juices, it is used in the treatment of bronchitis, coughs and colds, it is a good pain reliever caused by rheumatic diseases, it is an agent of choice for the management of inflammation, it is a good agent that relieves stress and it can be used as a tonic to promote relaxation and reduce exhaustion. It is also used as an insect repellant agent (Juhás *et al*., 2008).

Globulol, was also found in the leaf extract of *E. globulus*. Globulol belongs to a group of hydrocarbons referred to as 5, 10-cycloaromadendrane sesquiterpenoids. They are aromadendrane sesquiterpenoids that arise from the C5-C10 cyclization of the aromadendrane skeleton. Globulol exists as a solid and is a non-polar compound, which is relatively neutral. It is located in the membrane. Studies have

revealed that Globulol obtained from *E. globulus* has antimicrobial potential (Al-Snafi, 2017). According to a study carried out by Carson *et al*. (2006) on antimicrobial activity of *Melaleuca alternifolia* (Tea Tree) Oil on *Escherichia coli*, *Haemophilus influenzae*, *Streptococcus pyogenes* and *Streptococcus pneumonia* revealed that Globulol is effective in killing bacteria by lysis of the cell membrane, loss of membrane integrity, leakage of ions and inhibition of respiration were demonstrated (Yadav *et al*., 2017).

E. globulus leaf extract had Endo-fenchol, which is extensively utilized in flavors with lemon, herbal, pine, or floral note. Endo-fenchol can also be esterified by various types of organic acids to widen its use in fragrance and flavor (Aprotosoaie *et al*., 2017).

The leaf extract of *E. globulus* and *S. didymobotrya* revealed the presence of Terpineols. Terpineols exert a broad range of various biological actions on plants, animals and humans. The α-terpineol possesses a wide range of medicinal properties which include, antioxidant, anticancer, anti-inflammatory, antiulcer, antihypertensive and antinociceptive (Khaleel *et al*., 2018).

Further, the GC-MS analysis of *E. globulus* leaf extract revealed the existence of α-Eudesmol, which is a component of essential oil possessing antimicrobial, antinociceptive and anti-inflammatory activities (Mohammadhosseini *et al*.,

2019). The compound α-Eudesmol has potent cytotoxic activity against cancerous cells in the liver in that it reduces the proliferation and causes the death of tumor cells by caspase-mediated apoptosis (Russo and Marcu, 2017).

A study by Majid Mohammadhosseini (2017) on the Chemical composition of the essential oils and extracts of Achillea species and their biological activities of Asteraceae (Compositae) found in Iran, Turkey and Serbia, revealed that α-Eudesmol possesses antimicrobial, antinociceptive and anti-inflammatory activities.

The leaf extracts of the two plants also revealed the presence of α-pinenes. α -pinenes have several medicinal uses that include antibacterial, antifungal, anti-cancer, antinociceptive, anti-inflammatory and bronchodilator. They have broad-spectrum antibiotics effective against Methicillin-resistant *Staphylococcus aureus* (MRSA) (Kumar *et al*., 2017).

Working in synergy with the cannabinoids CBD and CBN, α-pinenes increase alertness and counteract some of the ill-effects of THC such as anxiety, work to improve benefits with the entourage effect on cannabinoids like THC (Rufino *et al*., 2014).

Asgari (2013), in his study on the analgesic effect of ethanolic extract of *Tanacetum parthenium* in acetic acid writhing test in mice, revealed that α-pinenes possess analgesic activity in mice. Asgari (2013) used 100 male mice which were obtained randomly and administered with normal saline intraperitoneally, ibrufen 100mg/kgbw, morphine 0.5mg/kg bw and plant extracts at dose levels of 10, 20, 30 and 40 mg/kgbw. The pain was then induced by the injection of 0.9% acetic acid IP. Fifteen minutes after each treatment analgesic activity was scored by counting the number of abdominal constrictions for 30 minutes. The findings from his study indicated that the group that received a dose level of 40 mg/kgbw of the plant extract showed a significant analgesic effect than the group that received the reference drug, ibuprofen (Asgari and Parvin, 2013).

Him *et al*. (2008) using the tail-flick test model reported that the antinociceptive potential of *Foeniculum vulgare* was due to the presence of alpha-pinene. It was also observed that the leaf extracts of *E. globulus* and *S. didymobotrya* contain Camphene, a monoterpene of the carbide family. Camphene is present in numerous plant species. It has a waxy smell of pine, which is used as a fragrance in the preparation of perfumes, and as a flavoring. The curative value of camphene includes use as a bronchial stimulant and an antispasmodic. It also lowers cholesterol levels and triglycerides in the blood, which in turn helps to reduce the risk of heart disease (Zachariah and Leela, 2018).

Besides, the leaf extracts of *E. globulus* and *S. didymobotrya* were found to contain terpinolene, a bioactive agent that possesses anti-inflammatory and antimicrobial, antifungal and anticancer activities. It also reduces anxiety and depression (Wendschuh *et al*., 2016).

Finally, analysis of the leaf extracts of *E. globulus* and *S. didymobotrya* also revealed the presence of Camphor. Camphor oil is a good agent in pain management and it also helps in itching reduction. On the skin, it helps in the reduction of irritation, thus it is used topically to relieve pain and inflammation (Beerling *et al*., 2002). Camphor oil relieves pain, inflammation and prevents skin redness by causing numbness of sensory nerve endings of skin (Martin *et al*., 2004). Camphor oil has been reported to have a calming effect on the mind and brings a good night's sleep. Camphor can be used as an anti-cough agent hence it is used in the management of cold, cough and throat decongestants (Worwood, 2016). In conclusion, this study has revealed that these plants are endowed with the many bioactive compounds which are used in the management of various ailments. Many phytochemicals present in these plants are effective in the management of pain, fever and inflammation.

CHAPTER FOUR

ANTIPYRETIC POTENTIAL OF DCM LEAF EXTRACTS

4.1 Introduction

Fever manifests in an individual when someone begins to look for a warmer environment or starts dressing heavily. Physiological fever manifestation includes shivering, cutaneous vasoconstriction and non-shivering generation of heat via the enhanced release of glucocorticoids, catecholamines and thyroid hormones (Gelb, 2014). Fever comes with metabolic disturbances such as an increase in pulse rate, respiration rate, blood pressure and cardiac output among others hence the need to manage it using antipyretic agents (Tripathi, 2013).

The general symptoms of fever include increased sweating, chills and a sensation of cold. Lack of any of the said symptoms indicates serious illness. Fever has several causes such as infection by richettsia, chlamydia, parasites, bacteria and viruses. Other causes of fever include immune reaction and tissue destruction such as local necrosis (infarction), trauma, inflammatory reaction in the blood vessels and tissues (arthritis, flebitis), rhabdomyolysis and pulmonary infarction (Anochie, 2013).

Fever is managed using antipyretic agents such as diclofenac, aspirin and paracetamol among others. Other non-conventional interventions employed in fever management include removal of clothing from the patients to expose the body to lose heat into the environment. Besides, the patient can undergo massage

using a sponge that has been dipped in warm water. This helps in conductive heat loss (Scaravilli *et al*., 2011). Medicinal plants also form an integral part of the management of fever. A wide variety of these medicinal plants are currently used in the management of fever namely *Acacia hockii* and *Kigelia africana* (Kamau *et al*., 2016), *Cissus quadrangularis* (Vijay and Vijayvergia, 2010), *Urtica diocia* (Safari *et al*., 2016) among others.

E. globulus and *S. didymobotrya* are used by people in Embu County in the management of pain, fever and inflammation, where the leaves are boiled in water and then they bath with this water or mixing the powdered leaves in alcohol and drink the concoction; however, no preliminary scientific research has been done to the bio-screen antipyretic potential of these plants. This chapter, therefore, details the antipyretic potential of the leaf extract of *E. globulus* and *S. didymobotrya* in rats.

4.2 Materials and methods

4.2.1 Plant samples collection, preparation and extraction

The collection and preparation of plant samples were done as detailed in Chapter Three Section 3.2 Subsections 3.2.1 and 3.2.2.

4.2.2 Preparation of treatment doses

The choice of doses used in this study was arrived at after extensive literature review for they are commonly used doses by many researchers and through experimentation in the laboratory. The different treatment doses used in this study

were prepared as follows, to prepare 100 ml of normal saline; 0.85 g of sodium chloride was dissolved in 100ml of distilled water. To prepare 25mg/kg body weight dose level, 0.005g of the extract was dissolved in 0.3ml of 3% DMSO and 0.7ml of normal saline was added. The dose of 50mg/kg body weight was prepared by dissolving 0.01g of the plant extract was dissolved in 0.3ml 3% DMSO to which 0.7ml of normal saline was added. A 100mg/kg body weight dose level was prepared by dissolving 0.02g of the extract which was dissolved in 0.3ml of 3% DMSO and mixed with 0.7ml of normal saline to make 1ml of the drug. The 150mg/kg body weight dose level extract was prepared by dissolving 0.03g of the plant extract in 0.3ml of 3% DMSO and mixed with 0.7ml of normal saline.

The 200mg/kgbw dose level was prepared by dissolving 0.04g of the plant extract in 0.3ml of 3% DMSO and mixed with 0.7ml of normal saline while 250 mg /kg body weight dose level was prepared by dissolving 0.05g of the plant extract in 0.3ml of 3% DMSO and then 0.7ml of normal saline. To prepare 3% DMSO 3ml of DMSO was dissolved in 97 ml of normal saline. To prepare 100mg/kg body weight aspirin each rat needed 13mg of the drug dissolved in 0.5ml of normal saline. Therefore, to prepare a larger volume of the drug, 0.5 g of aspirin was dissolved in 19.23ml of normal saline. All the extracts and solutions administered were freshly prepared.

4.2.3 Experimental animals

Male Swiss albino rats were used in this study. The rats were aged between 2 and 3 months and with an average weight of 150 grams. The animals were obtained and bred at Kenyatta University animal breeding and research facility. They were kept in approved polyethylene cages at room temperature (25±2°C) with 40 to 60 % humidity and 12h dark hours and 12h light cycle. They were provided with standard diet *ad libitum* and water (Vogel, 2002).

The authors obtained approval for the use of animals from the National Commission for Science, Technology and Innovation (NACOSTI/P/16/6765/14525). The animals were cared for and handled according to the ethical guidelines and procedures for handling animals stipulated in the American Institute of Laboratory Animal Resources and Kenyatta University. Development of the experimental protocols and procedures were performed under the guidance of the Veterinarian, who is a member of the Kenya Veterinary Board (KVB). All procedures were carried out following the Public Health Service (PHS) Policy on Humane Care and Use Committee (IACUC) (Section 8.3.2) and KVB (Sikes, 2016).

4.2.4 Experimental design

This study adopted a completely randomized experimental design, where each rat from the selected pool was given an equal chance to be part of the experimental group (Clewer and Scarisbrick, 2013). The principle of randomization involves

the allocation of treatment to experimental units at random to avoid any bias in the experiment resulting from the influence of some extraneous unknown factor that may affect the experiment (Barr *et al*., 2013). Experimental rats were split into nine groups of five animals each (n = 5).

Group one (normal control) comprised normal rats that were administered with 3% DMSO. Group two (negative control) comprised rats that had been induced with pyrexia using 20% turpentine. They were administered with 3% DMSO. Group three (positive control) comprised turpentine-induced pyretic rats that were administered with aspirin (100mg/kg bw).

Group four comprised of turpentine induced pyretic rats that were administered with extract dose of 25mg/kg bw. Group five comprised turpentine-induced-pyretic rats that were administered with extract dose of 50mg/kg bw. Group six comprised turpentine-induced pyretic rats that were administered with an extract dose of 100mg/kg bw. Group seven comprised of turpentine induced pyretic rats that were administered with extract dose of 150mg/kg bw. Group eight comprised turpentine induced pyretic rats that were administered with extract dose of 200mg/kg bw while group nine comprised of turpentine induced pyretic rats that were administered with extract dose of 250mg/kg body weight. This experimental design is summarized in Table 4.1.

Table 4.1: Antipyretic test of leaf extracts of *E. globulus* and *S. didymobotrya* on turpentine-induced pyrexia in rats.

Groups	**Treatment dose**
Group1	3%DMSO
Group II	Turpentine + Normal saline
Grou111	Turpentine + 100mg/kg bw asprin
Group 1V	Turpentine + 25mg/kg bw extract
Group V	Turpentine + 50mg/kg bw extract
Group V1	Turpentine + 100mg/kg bw extract
Group V11	Turpentine + 150mg/kg bw extract
Group VIII	Turpentine + 200mg/kg bw extract
Group IX	Turpentine + 250mg/kg bw extract

The body temperature of rats in all the groups was taken after fever induction and at hourly intervals following administration of treatments for four hours (Wan *et al*., 2013). Approximately 3cm of a well-lubricated digital thermometer (thermistor probe®) was inserted into the anal region of the rats to measure the rectal temperature (Nthiga *et al*., 2016). The thermistor animals in the experimental group were taken using both types of thermometers and compared. The thermostor probe® was first quantified against a mercury thermometer, where temperatures of the animals in the experimental groups were recorded using both thermometers and compared. The baseline/initial mean rectal temperature was calculated by measuring the rectal temperature of rats at fifteen minutes intervals for 1 hour before the induction of fever.

The rectal temperatures of rats were measured and recorded at hourly intervals for 4 hours after the administration of different treatments. The rats whose rectal

temperatures rose by one degree Celsius one hour after intraperitoneal injection of turpentine (20mg/kg bw) were termed pyretic and were used for the studies (Nthiga *et al*., 2016). The difference in rectal temperatures before and after treatments was obtained and the % inhibition in the rectal temperature computed according to the formula as described by Hukkeri *et al.*, 2006; Yemitan and Adeyemi, 2017).

$$\% \text{ Inhibition of Pyrexia} = \frac{B - Cn}{B} X\ 100$$

Where,

B - Rectal temperature at one hour following turpentine injection

C_n - Rectal temperature after treatments.

4.2.5 Data management and statistical analysis

Data on pyrexia was obtained, recorded into a spreadsheet. Descriptive statistics was then done and the data expressed as mean ± SEM. Inferential statistics were done using one-way ANOVA followed by Tukey's post hoc test for pairwise separation and comparison of means. An unpaired student t-test was used to compare the antipyretic effects of the two plant extracts. The confidence level was set at 99.5% ($p \leq 0.005$). Statistical analysis was done using Minitab statistical software (version 17). Data was presented in form of tables and graphs.

4.3 Results

4.3.1 Antipyretic activity of DCM leaf extract of *E. globulus* in Swiss albino rats.

The leaf extract of *E. globulus* generally exhibited *in vivo* antipyretic activities in rats, which was evidenced by a reduction in rectal temperature against turpentine-induced fever (Table 4.2). After one hour of treatment, the groups of Swiss albino rats that received aspirin (100mg/kg body weight) and the extract doses of 25, 50, 100, 150, 200 and 250 mg/kg bw lowered the rectal temperature to 98.07%, 98.96%, 98.53%, 98.49%, 98.13%, 97.57% and 97.71% respectively (Table 4.2). The *E. globulus* extract dose of 200 caused the highest antipyretic activity, which reduced pyrexia by 2.43% in the first hour. This change was higher than that caused by the reference drug, aspirin, which reduced pyrexia by 1.93%. However, the effect of aspirin was comparable to that of extracts dose levels of 50, 100, 150, 200 and 250 ($p > 0.005$).

In the 2nd hour, the *E. globulus* leaf extract reduced the elevated rectal temperature in a dose-dependent fashion. At doses of 25, 50, 100, 150, 200 and 250, the extract lowered the raised rectal temperature to 98.18%, 97.91%, 97.44%, 96.94%, 96.80% and 96.73% respectively (Table 4.2). The antipyretic activities of the leaf extract doses of 25, 50, 100, 150 and 200 were statistically similar and comparable to that of aspirin ($p < 0.005$; Table 4.2).

In the 3rd hour post-treatment, the leaf extract doses of 25, 50 , 100, 150, 200 and 250 lowered the elevated rectal temperature in rats to 97.46%, 97.28%, 96.60%, 97.37%, 96.31% and 96.41% respectively (Table 4.2). Similarly, at this hour the extract showed a dose-independent antipyretic potential. The rats that received the *E. globulus* extract at doses of 25, 50, 100, 150, 200 and 250 exhibited antipyretic activities that were significantly different ($p < 0.005$; Table 4.2). However, the antipyretic activity of aspirin was statistically similar compared to that of the extract at all tested dose levels ($p > 0.005$; Table 4.2).

In the 4th hour, the *E. globulus* leaf extract reduced raised rectal temperature in a dose-dependent manner. The extract of *E. globulus* doses of 25, 50, 100, 150, 200 and 250 reduced pyrexia to 97.04%, 96.28%, 95.98%, 95.53%, 95.20% and 95.17%, respectively (Table 4.2). At this hour, the group that received leaf extract of *E. globulus* at a dose of 250 recorded the highest antipyretic effects, which was higher than that of aspirin (Table 4.2). The antipyretic effects of the extract doses of 100, 150, 200 and 250 were not significantly different from each other and were comparable to that of the reference drug, aspirin ($p > 0.005$; Table 4.2).

Table 4. 2: Antipyretic effects of DCM leaf extract of *E. globulus* on turpentine-induced pyrexia in rat

Group	Treatment	Percentage change in rectal temperatures (ºC)				
		0hr	1hr	2hr	3hr	4hr
Normal control	3% DMSO	100.00±0.00	99.89±0.13^{a} (0.11)	100.16±0.22^{a} (-0.16)	99.78±0.13^{a} (0.22)	99.89±0.18^{a} (0.11)
Negative Control	Turpentine + DMSO	100.00±0.00	100.42±0.06^{a} (-0.42)	100.16±0.10^{a} (-0.57)	100.68±0.18^{b} (-0.68)	100.52±0.12^{a} (-0.52)
Positive Control	Turpentine + Aspirin	100.00±0.00	98.07±0.07^{cd} (1.93)	97.55±0.15^{bc} (2.45)	96.82±0.12^{cde} (3.18)	95.31±0.16^{d} (4.69)
DCM: leaf Extract	Turpentine + 25 mg/kg bw	100.00±0.00	98.96±0.08^{b} (1.04)	98.18±0.23^{b} (1.82)	97.46±0.25^{c} (2.54)	97.04±0.26^{b} (2.96)
	Turpentine + 50 mg/kg bw	100.00±0.00	98.53±0.06^{bc} (1.47)	97.91±0.17^{b} (2.09)	97.28±0.07^{cd} (2.72)	96.28±0.13^{bc} (3.72)
	Turpentine + 100 mg/kg bw	100.00±0.00	98.49±0.10^{bc} (1.51)	97.44±0.05^{bcd} (2.56)	96.60±0.01^{de} (3.40)	95.98±0.10^{cd} (4.02)
	Turpentine + 150 mg/kg bw	100.00±0.00	98.13±0.15^{cd} (1.87)	96.94±0.05^{cd} (3.06)	96.37±0.09^{e} (3.63)	95.53±0.07^{cd} (4.47)
	Turpentine + 200 mg/kg bw	100.00±0.00	97.57±0.10^{d} (2.43)	96.80±0.06^{cd} (3.20)	96.13±0.08^{e} (3.87)	95.20±0.07^{d} (4.80)
	Turpentine + 250mg/kg bw	100.00±0.00	97.71±0.05^{d} (2.29)	96.73±0.07^{d} (3.27)	96.41±0.05^{e} (3.59)	95.17±0.10^{d} (4.83)

Descriptive statistics are expressed as mean ± SEM for 5 rats per group. Values with different superscript letters are statistically significant ($p \leq 0.005$) along the same column. The figures in brackets represent mean % inhibition.

Notably, the animals in the normal control group showed no remarkable change in rectal temperature from zero to the fourth hour ($p>0.005$; Appendix v). However, the animals in the negative control group had a significant increase in rectal temperature from hour zero to hour three ($p<0.005$; Appendix v). On the other hand, there was a significant reduction in rectal temperature of rats that were treated with aspirin and *E. globulus* extract at all the doses tested from hour zero to the fourth hour ($p<0.005$; Appendix v).

4.3.2 Antipyretic effects of DCM leaf extract of *S. didymobotrya* (Fresenius) in rats

The leaf extract of *S. didymobotrya* equally showed *in vivo* antipyretic potential in rats. This was exhibited by a decrease in previously raised rectal on turpentine-induced fever in rats (Table 4.3).

After the first hour of treatment, the groups of rats that received the reference drug aspirin at 100mg/kg body weight and leaf extract of *S. didymobotrya* at the doses of 25, 50, 100, 150, 200 and 250 mg/kg body weight lowered the raised rectal temperature to 98.75%, 99.48%, 98.97%, 98.86%, 99.00%, 98.70% and 98.69% respectively (Table 4.3). At this hour, 250mg/kg body weight dose recorded the highest antipyretic effect with a 1.31% reduction compared to aspirin, which recorded a 2.24% reduction. The antipyretic effect of *S. didymobotrya* extract doses exhibited no significant differences and was comparable to the effect of the aspirin at this hour ($p > 0.005$; Table 4.3). The effect of the extract at this hour was dose-independent.

In the second hour, the leaf extract of *S. didymobotrya* at the doses of 25, 50, 100, 150, 200 and 250 mg/kgbw, lowered the elevated rectal temperatures to 98.79%, 97.83%, 97.56%, 98.05%, 97.60% and 97.76% respectively (Table 4.3). The reference drug reduced the raised rectal temperature to 97.45% (Table 4.3). The 100mg/kg body weight dose recorded the highest antipyretic activity with a 2.44% reduction (Table 4.3). There was no significant difference in the antipyretic activities of the *S. didymobotrya* extract doses of 50, 100, 150, 200 and 250 mg/kg bw. Their effects were comparable with that of aspirin ($p > 0.005$; Table 4.3).

In the third hour, the *S. didymobotrya* leaf extract doses of 25, 50, 100, 150, 200 and 250 mg/kg bw lowered the elevated rectal temperature to 98.27%, 97.47%, 97.40%, 97.36%, 96.77% and 96.92% respectively (Table 4.3). At this hour, the extract showed a dose-independent antipyretic potential. At 200mg/kg bw dose, the extract revealed the highest antipyretic activity with a fever reduction of 3.23% (Table 4.3). The antipyretic activities of the *S. didymobotrya* at doses of 100, 200 and 250 mg/kg bw revealed no significant difference ($p > 0.005$; Table 4.3). Further, the effect of aspirin was comparable to that of the extract doses of 150, 200 and 250mg/kg bw ($p > 0.005$; Table 4.3).

In the fourth hour, the leaf extract also reduced the raised rectal temperature in pyretic rats in a dose-independent response. The leaf extract of *S. didymobotrya*,

at doses of 25, 50, 100, 150, 200 and 250 mg/kg bw, lowered rectal temperatures to 97.64%, 97.01%, 96.36%, 96.67%, 96.25% and 96.04% respectively (Table 4.3). There was no significant variation in the antipyretic activities among the extract at the doses of 100, 150, 200 and 250 mg/kg bw ($p > 0.005$; Table 4.3). Similarly, the effect of the reference drug aspirin was not significantly different from the effect of the extract doses of 100, 200 and 250mg/kg bw ($p > 0.005$; Table 4.3).

Table 4. 3: Antipyretic effects of DCM leaf extracts of *S. didymobotrya* on turpentine-induced pyrexia in rats

Group	Treatment	Percentage change in rectal temperatures (ºC)				
		0h	**1h**	**2h**	**3h**	**4h**
Normal control	3% DMSO	100.00±0.00	99.73±0.12^{b} (0.27)	99.95±0.21^{b} (0.05)	100.11±0.20^{a} (-0.11)	100.00±0.19^{b} (-0.00)
Negative Control	Turpentine + DMSO	100.00±0.00	100.52±0.08^{a} (-0.52)	100.67±0.06^{a} (-0.67)	100.67±0.06^{a} (-0.67)	100.72±0.05^{a} (-0.72)
Positive Control	Turpentine + Aspirin	100.00±0.00	98.75±0.15^{d} (1.25)	97.45±0.09^{d} (2.55)	96.67±0.05^{e} (3.33)	95.89±0.13^{f} (4.11)
DCM: leaf Extract	Turpentine + 25mg/kg bw	100.00±0.00	99.48±0.08^{bc} (0.52)	98.79±0.16^{c} (1.21)	98.27±0.23^{b} (1.73)	97.64±0.16^{c} (2.36)
	Turpentine + 50mg/kg bw	100.00±0.00	98.97±0.08^{cd} (1.03)	97.83±0.14^{d} (2.17)	97.47±0.04^{c} (2.53)	97.01±0.05^{cd} (2.99)
	Turpentine + 100mg/kg bw	100.00±0.00	98.86±0.13^{cd} (1.14)	97.56±0.06^{d} (2.44)	97.40±0.01^{cd} (2.60)	96.36±0.01^{def} (3.64)
	Turpentine + 150mg/kg bw	100.00±0.00	99.00±0.15^{cd} (1.00)	98.05±0.07^{d} (1.95)	97.36±0.01^{cde} (2.64)	96.67±0.10^{de} (3.33)
	Turpentine + 200mg/kg bw	100.00±0.00	98.70±0.12^{d} (1.30)	97.60±0.09^{d} (2.40)	96.77±0.06^{de} (3.23)	96.25±0.07^{ef} (3.75)
	Turpentine + 250mg/kg bw	100.00±0.00	98.69±0.09^{d} (1.31)	97.76±0.11^{d} (2.24)	96.92±0.14^{cde} (3.08)	96.04±0.08^{ef} (3.97)

Descriptive statistics are expressed as mean ± SEM for 5 rats per group. Values with a different superscript letter are statistically significant ($p \leq 0.005$) along the same column by one-way ANOVA followed by Tukey's post hoc test. Aspirin = 100 mg/kgbw; DMSO = 3%; Turpentine = 20%; bw = body weight. The figures in brackets represent mean % inhibition.

There was no significant hourly change in rectal temperature in rats in the normal control group ($p>0.005$; Appendix vi). However, the rectal temperature in rats in the negative control group increased significantly in the 1st, 2nd and 3rd hours compared to the zero hour ($p<0.005$; Appendix vi). Further, the animals that were administered with the extract at the six doses and reference drug, aspirin, significantly reduced the rectal temperatures from hour zero to the fourth hour ($p<0.005$; Appendix vi).

4.3.3 Comparision of antipyretic activities of DCM extracts of *E. globulus* and *S. didymobotrya*

In comparison, the antipyretic activity of *E. globulus* extract was significantly higher compared to *S. didymobotrya* dose of 25 in the first hour ($p<0.005$; Figure 4.1). However, the antipyretic effects of the DCM leaf extracts of *E. globulus* and *S. didymobotrya* in rats, at the dose of 25, were not significantly different in the 2nd, 3rd and 4th hours ($p>0.005$; Figure 4.1).

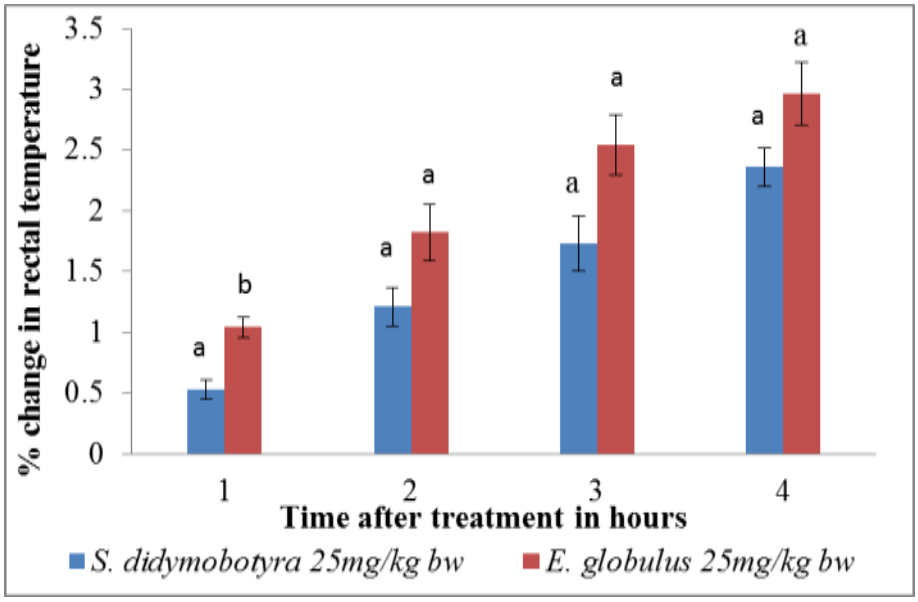

Figure 4. 1: Comparison of antipyretic effects of leaf extracts of *E. globulus* and *S. didymobotyra* at the dose of 25mg/kg bw.

At the dose of 50mg/kg bw, the antipyretic effect of the *E. globulus* was significantly higher compared to that of *S. didymobotrya* in the first and fourth hours ($p<0.005$; Figure 4.2). In contrast, the antipyretic activities of the leaf extracts of *S. didymobotrya* and *E. globulus* exhibited no significant difference in the second and third hours ($p>0.005$; Figure 4.2).

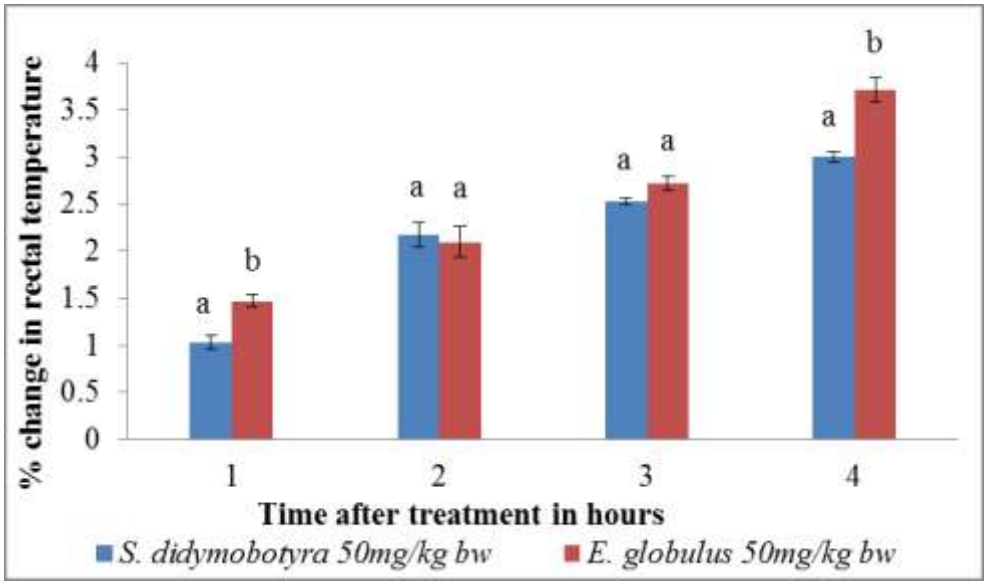

Figure 4. 2: Comparison of antipyretic effects of leaf extracts of *E. globulus* and *S. didymobotyra* at the dose of 50mg/kg bw.

At the extract dose of 100, the antipyretic effects of the leaf extracts of *S. didymobotrya* and *E. globulus* exhibited no significant differences in the first, second and fourth hours ($p>0.005$; Figure 4.3). However, the antipyretic effect of *E. globulus* was significantly higher compared to that of the *S. didymobotrya* extract in the third hour ($p < 0.005$; Figure 4.3).

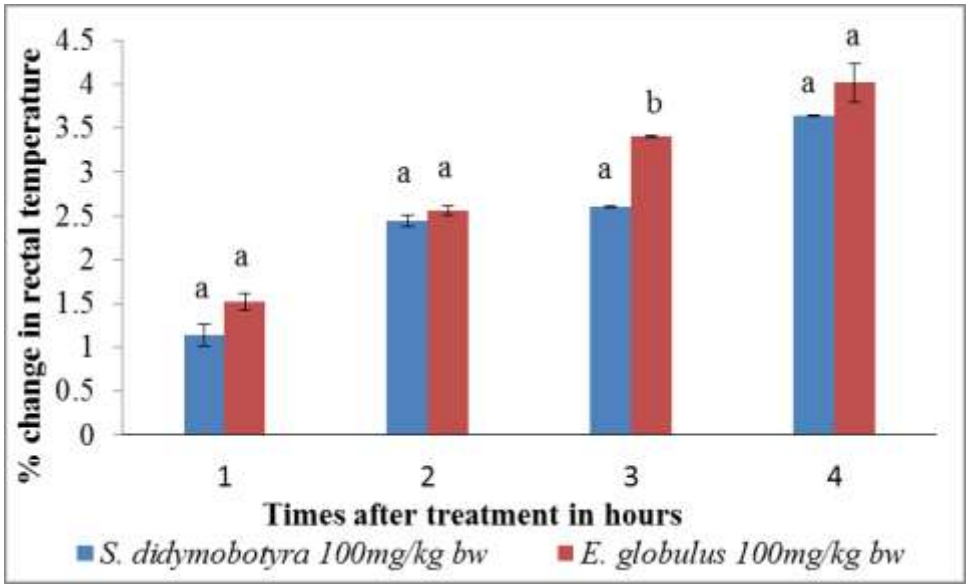

Figure 4. 3: Comparison of antipyretic effects of leaf extracts of *E. globulus* and *S. didymobotyra* at the dose of 100mg/kg bw.

At the dose of 150, the antipyretic activity of the leaf extract of *E. globulus* was significantly higher compared to that of *S. didymobotrya* in the first, second, third and fourth hours ($p \leq 0.005$; Figure 4.4).

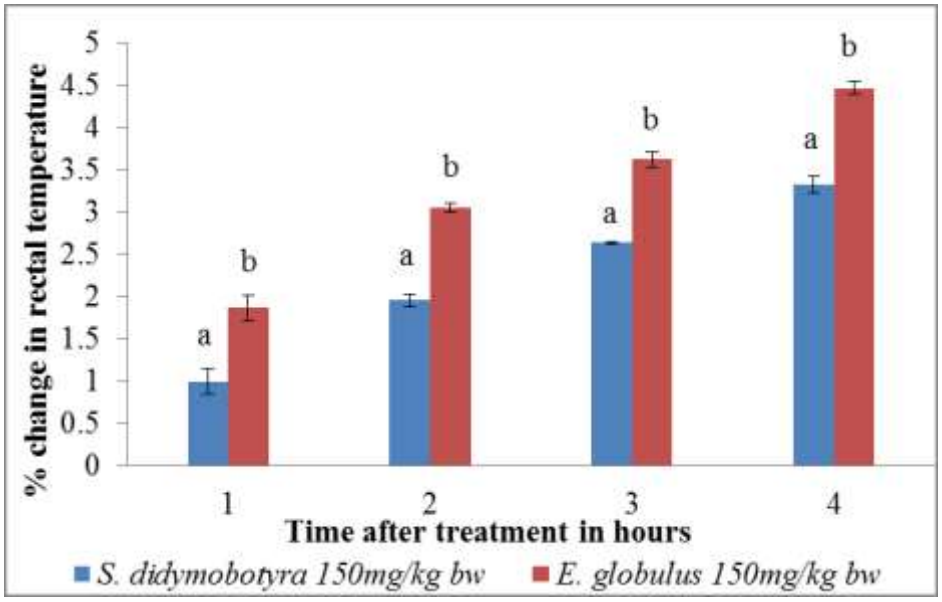

Figure 4. 4: Comparison of antipyretic effects of leaf extracts of *E. globulus* and *S.didymobotyra* at the dose of 150mg/kg bw.

The antipyretic activity of *E. globulus* was significantly higher than that of *S. didymobotrya* at the dose of 200 at the 1st, 2nd, 3rd, and 4th hours ($p < 0.005$; Figure 4.5).

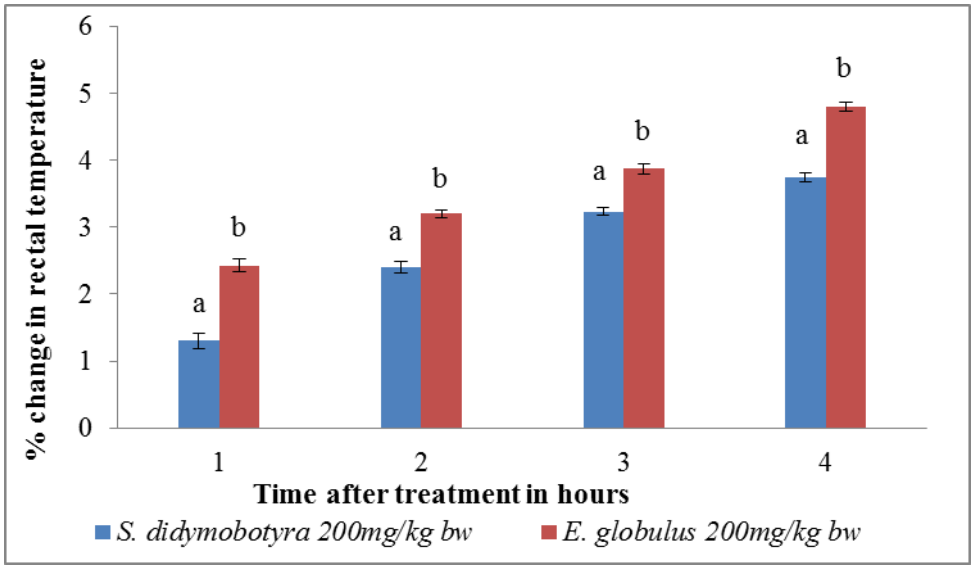

Figure 4. 5: Comparison of antipyretic effects of leaf extracts of *E. globulus* and *S. didymobotyra* at a dose of 200mg/kg bw.

At extract dose 250, the antipyretic activity of leaf *E. globulus* was significantly higher compared to that of *S. didymobotrya* in the 1st, 2nd, and 4th in rats ($p<0.005$; Figure 4.6). In contrast, the antipyretic effects of *E. globulus* and *S.didymobotrya* extracts were not significantly different in the third hour ($p > 0.005$; Figure 4.6).

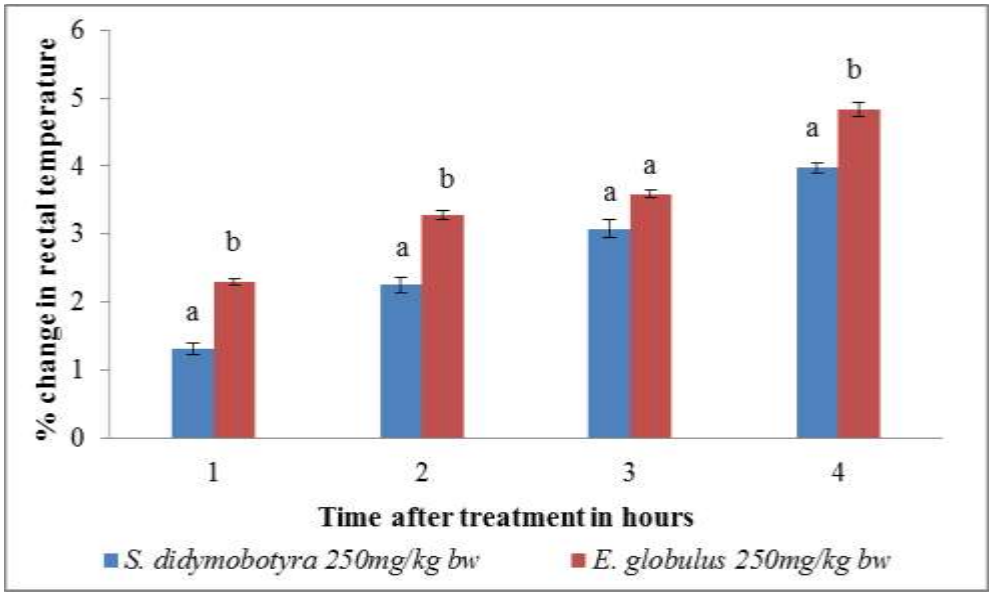

Figure 4. 6: Comparison of antipyretic effects of leaf extracts of *E. globulus* and *S. didymobotyra* at a dose of 250mg/kg bw.

4.4 Discussion

Turpentine induced pyrexia is a preferred model for antipyretic studies for its quick and reliable way of testing for fever, for example, as opposed to yeast induced fever model which takes 18-24 hours after administration for pyrexia to be induced (Vanitha, 2008). The current study aimed at evaluating the antipyretic activities of *E. globulus* and *S. didymobotyra* on turpentine-induced fever in Swiss albino rats.

Upon bio-screening for the antipyretic activities of leaf extracts of *E. globulus* and *S. didymobotra* on turpentine-induced fever in Wistar rats, the results showed that fever was remarkably reduced at all extract doses. The leaf extracts of *E. globulus* and *S. didymobotra*, at the doses of 250mg/kg bw, exhibited the highest rectal temperature reduction which compared well with the reference drug, aspirin. The lower doses of 25, 50 100 and 150mg/kgbw were not effective compared to those of higher doses of 200 and 250. This could be due to the rapid metabolism, clearance and inactivation of the lower concentration of the bioactive agents or chemical modification in the body (biotransformation) where the drug loses biological activity (Yang *et al*., 2014).

The findings from the present study are in agreement with other studies on the antipyretic potential of medicinal plants in animal models. A similar work by Saptarini and Deswati (2015), showed that ethanol extract of *Ceiba pentandra*

demonstrated antipyretic activity on yeast-induced pyrexia in mice. Besides, Safari *et al*. (2016), showed that an aqueous leaf extract of *Urtica dioica* (L.) has an antipyretic effect against brewer's yeast-induced fever in mice. Similarly, Rauf *et al*. (2014), revealed that methanol extract of *Diospyros lotus* L. has an antipyretic effect in albino mice on yeast-induced pyrexia. A study by Thirumal *et al*. (2013) reported that aqueous leaf extract of *Clerodendrum inerme* (L.) Gaertn possesses antipyretic activity on milk induced pyrexia in rabbits.

Generally, the NSAIDs produce their antipyretic action via prostaglandin biosynthesis inhibition within the hypothalamic pre-optic region (Habib and Waheed, 2013). It has been conceptualized that a leak in the blood-brain barrier at the level of the OVLT permits the CNS to sense the presence of endogenous pyrogens (Dantzer, 2017). Other mechanisms that have been conceptualized include active transport of cytokines into the OVLT or cytokine receptors activation in endothelial cells of the neural vasculature, which then transduce signals to the brain (Yeo *et al*., 2014). It is, therefore, possible that the leaf extracts of *E. globulus* and *S. didymobotra* have a similar mechanism of action to that of aspirin through prostaglandin biosynthesis inhibition in the hypothalamus.

The results in this study indicated that varying doses have different antipyretic potentials. The present study used dose ranges of 25, 50, 100, 150, 200 and 250 mg/kg bw. Research studies have used similar dose ranges while evaluating for

antipyretic activities in medicinal plants. Akuodorl *et al*. (2013), in his study on the antipyretic activity of *Pseudocedrela kotschyi* ethanolic leaf extract, used dose ranges of 50, 100 and 150mg/kg bw while Muhammad *et al*. (2012) used dose ranges of 100, 200 and 300mg/kg bw while studying the effects of the methanol extract of *Viola betonicifolia* on brewer's yeast induced fever in mice.

The choice of dose ranges used in bio-screening of medicinal plants can be attributed to the ecological location of the medicinal plant, temperatures and type of soils. For example, plants that grow in extreme weather conditions of high or very low temperatures have a high concentration of phytochemicals, thus a low dose is effective in giving desired results, on the other hand, plants that grow in well-watered areas have low concentrations of phytochemicals because these plants experience less harsh climatic conditions. A high extract dose is needed in plants growing in well water areas to give similar results with the one growing in extreme conditions (Brito *et al*., 2019). *E. globulus* and *S. didymobotra* found in mbeere a semi arid place where the plants experience extreme wheather conditions.

The leaf extracts of *E. globulus* and *S. didymobotra* at all the tested doses of 25, 50, 100, 150, 200 and 250, never lowered rectal temperatures in the 1^{st} and 2^{nd} hours as effectively as in the 3^{rd} and 4^{th} hours. These findings could be due to the biotransformation of active agents in the extract to become antipyretic. The

intraperitoneal route of drug administration provides a relatively slow onset of drug effects because the drug takes time to diffuse across the membrane and interact with the drug receptors. On the other hand, most of the absorbed drug agents enter the portal circulation and there may be significant inactivation of the active compounds before they reach the desired sites of action (Djouahri *et al*., 2013; Al-Salihi, 2016; Fanun, 2016).

The *E. globulus* extract dose of 200 was marginally more effective than aspirin, while *S. didymobotra* extract dose of 250 was equally effective as aspirin. These findings suggest a better or a similar prostaglandin synthesis inhibition by the active components in the plant extracts. There is, therefore, the possibility of the plant extracts working effectively by blocking alternative mechanisms of cox-2 or prostaglandin E-2 synthesis during fever inhibition.

The anti-pyretic effects of *E. globulus* and *S. didymobotra* extracts may be due to their phytochemicals observed in chapter three section 3.3 subsection 3.3.1. The GC-MS analysis of the two leaf extracts demonstrated the presence of several bioactive compounds like terpinolene, alpha-pinene, globulol, borneol and essential oils. Several studies have associated these compounds with antipyretic activities, which confirm the observed antipyretic activities of these plant extracts. The fact that the results of GC-MS show that the *E. globulus* extract had more compounds than *S. didymobotra* is a possible explanation for their differences in

antipyretic activities. The GC-MS analysis showed the presence of globulol. Globulol is a compound that comprises 90% of essential oil. Studies have shown that essential oil possesses potential antipyretic effects. Limberger *et al*. (2001), who investigated antipyretic activities of *Pergularia daemia* and *Carissa carandas* in Brewer's yeast-induced pyrexia in rat models, revealed that essential oils have antipyretic activity.

The GC-MS results revealed the presence of terpineols that form components of essential oils. Moran *et al*. (1989), revealed that terpineols isolated from Artemisia caerulescens subsp Gallica possess antipyretic activity in animal models. The GC-MS revealed the presence of borneol. Kumar *et al*. (2018) in a study on the chemical composition of rhizome oleoresin, revealed that borneol has potential antipyretic activity against turpentine-induced fever in mice. The GC-MS results also revealed the presence of alpha-pinene which possesses antipyretic activity in animal models. Khan (2017), in his study, revealed that alpha-pinene isolated from Black cumin seed had significant antipyretic effects against turpentine induced pyrexia in mice.

In conclusion, this study has revealed that these two plants are endowed with many bioactive compounds such as Terpinolene, Alpha-pinene, Borneol, Globulol and Terpineols which exhibit antipyretic activity in rats. The extracts showed a dose-independent response with a dose level of 250mg/kg bw having the highest

antipyretic activity. The present study supports the traditional use of these plants in the management of fever.

CHAPTER FIVE

ANALGESIC ACTIVITY OF LEAF EXTRACTS IN MICE

5.1 Introduction

Pain is an emotional experience and unpleasant sensory that is associated with a potential or actual tissue injury (Hughes, 2008). Pain is vital in the body's defense mechanism. Its major role is to provide a speedy warning to the nervous system so that they can initiate motor responses that will lead to the minimization of physical harm (Underwood *et al*., 2013). Disease and tissue damage are often associated with pain. In the management of pain, nonsteroidal anti-inflammatory drugs (NSAIDs) are highly prescribed (Altman *et al*., 2015). However, many conventional drugs have many side effects such as gastrointestinal complications namely peptic ulcers bleeding; obstructions and perforation, and therefore, their clinical uses have been limited in pain management (Ofman *et al*., 2002).

Selective cyclooxygenase-2 inhibitors have some benefits in preventing such side effects, while the risk of cardiovascular adverse events demands important consideration (Hippisley Cox and Coupland, 2005; Lenzer, 2005). Narcotics are used in the management of pain. Narcotics refer to opium, opium derivatives, and their semi-synthetic or fully synthetic substitutes as well as cocaine and coca leaves (Rai and Tewari, 2018). The community abuse and additional side effects such as addiction, respiratory depression, tolerance, constipation, psychological dependency and sedation linked with narcotic analgesics are the major setbacks in

the chronic pain management as well their inadequacy (Benyamin *et al*., 2008: Bell-Sharp *et al*., 2013).

Pain is also managed using medicinal plants such as *C. spinarum, C.edulis, E. globulus* and *S. didymobotrya* among others. In Embu County, Kenya the traditional practitioners use *E. globulus* and *S. didymobotrya* in the management of pain (Kareru *et al*., 2007). However, there is no scientific data to support this biological activity. Therefore, this study was designed to determine the analgesic potential of DCM leaf extracts of *E. globulus* and *S. didymobotrya* in mice.

5.2 Materials and methods

5.2.1 Plant sample collection, preparation and extraction

The collection, preparation and extraction of plant samples was done as detailed in Chapter Three Section 3.2 Subsections 3.2.1 and 3.2.2.

5.2.2 Preparation of treatment doses

The preparation of extract doses was done following the procedure described in chapter four, section 4.2.2. To prepare 2.5% formalin, 97.5 ml of distilled water was added to 2.5 ml of formalin. To prepare diclofenac sodium for 40 mice, 42.8ml of diclofenac sodium was dissolved in 4ml of normal saline.

5.2.3 Experimental animals

Swiss albino mice of both sexes aging between 5-6 weeks of approximately 20g were used to assess for the analgesic activities of the two extracts. The authors obtained approval for using animals from the National Commission for Science,

Technology, and Innovation (NACOSTI/P/16/6765/14525). The animals were cared for and handled according to the ethical guidelines and procedures for handling animals for Kenyatta University. They were selected twenty-four hours before experimentation based on their normal response to sensorimotor testing. The sensorimotor test model was carried out by pulling-up of the animal. This helped to ensure that all experimented animals were healthy. This was done by holding the mice in a fully extended and inverted position one hour after administration of controls and dosages.

At the end of the experimentation, the mice were attempting to gain an upright position and touched the hand fingers of the research using both forepaws within a period of between 1 to 15 seconds simultaneously (Okindo, 2014; Gad, 2019). Swiss albino mice were randomly allocated to nine groups of 5 mice (n=5) and treated as follows. The animals were handled following the protocol described in Chapter Four Subsection 4.2.3.

5.2.4 Experimental design

A completely randomized experimental design was adopted in this study as described in chapter four section 4.2.4. Each mouse received treatment as follows; group one (normal control group) comprised normal mice that received 0.01ml of 2.5% formalin. Group two (negative control) received 3% DMSO. Group three (positive control) received 0.1ml of diclofenac at 15mg/kg body weight and after

thirty minutes were administered with 2.5% of 0.01ml formalin as the pain-inducing agent.

Group four comprised mice that received 25mg/kg body weight of the plant extract and thirty minutes later administered with 2.5 % formalin. Group five comprised of mice that received 50 mg/kg body weight of the plant extract and thirty minutes later administered with 2.5% formalin. Group six comprised mice that received 100mg/kg body weight of the plant extract and thirty minutes later administered with 2.5% formalin. Group seven comprised mice that received 150mg/kg body weight of the plant extract and thirty minutes later administered with 2.5 % formalin. Group eight comprised mice that received 200mg/kg body weight of the plant extract and thirty minutes later administered with 2.5% formalin and Group nine comprised of mice that received 250 mg/kg body weight of the plant extract and thirty minutes later administered with 2.5% formalin. The experimental design is summarised in Table 5.1

Table 5. 1: Analgesic activity of leaf extracts of *E. globulus* and *S. didymobotrya*

Animal group	Treatment
I	3% DMSO
II	3% DMSO+ Formalin
III	15mg/kg bw diclofenac + Formalin
IV	25mg/kg bw extract + Formalin
V	50mg/kg bw extract + Formalin
VI	100mg/kg bw extract + Formalin
VII	150mg/kg bw extract + Formalin
VIII	200mg/kg bw extract + Formalin
IX	250mg/kg bw extract + Formalin

Bw = body weight; DMSO = Dimethylsulphoxide;

The formalin-induced pain was carried out as described by Hunskaar and Hole (1985), where all the animals received 0.1ml of treatments intraperitoneally and 30 minutes later injected with 0.01ml of formalin (2.5%) in the left hind paw to generate pain behavior of shaking, licking, biting and lifting (Tjolsen *et al*., 1992; Da Silva *et al*., 2010).

The time taken a licking, shaking, biting or lifting of hind paw induced with pain was measured and recorded (Tjolsen *et al*., 1992). The experimentation of Swiss albino mice was done inside a transparent Plexiglas chamber with a mirror put at the side of the chamber to provide a clear observation of the animals being experimented. Two phases of intensive pain behaviors were determined and recorded singly. The early phase was measured and recorded between zero and the fifth minute while the second phase (late phase) measured and recorded

between the fifteenth and thirtieth minute. The percentage of pain inhibition was computed utilizing the following formula.

$$\text{Percentage pain inhibition} = \frac{C - T}{C} \times 100$$

Where,

C = Each phase vehicle control group value

T = Each phase treated group value

5.2.4 Data management and statistical analysis

Data on pain was obtained, recorded and entered into Microsoft Excel broadsheet. It was cleaned and then transferred for statistical analysis in Minitab statistical software (version 17.0). The data were subjected to descriptive statistics and expressed as mean ± SEM. An inferential statistic one-way ANOVA was applied to analyze for statistical variation among various sets of treatment groups accompanied by Tukey's post hoc test for mean separations and comparison. Antinociceptive effects of the two plant extracts were carried out using unpaired student t-tested. The confidence level was set at 99.5% ($p \leq 0.005$).

5.4 Results

5.4.1 Analgesic activities of DCM leaf extracts of *E. globulus* and *S. didymobotrya* in mice.

Two phases were used to assess the antinociceptive activity of *E. globulus* and *S. didymobotrya* leaf extracts on formalin-induced nociception in Swiss albino mice. They include the early phase which persisted for the first 5 minutes and the late phase which endured between the fifteenth to the thirtieth minute after injection of formalin. The animals that received leaf extract of *E. globulus* revealed analgesic activity on the formalin-induced nociception in the two phases. This was evident by a decline in paw shaking, biting, licking and lifting time (Table 5.2).

The Swiss albino mice that received an extract of *E. globulus* at the dose levels of 25, 50, 100, 150, 200 and 250 mg/kgbw as well as diclofenac (15mg/kg body weight), decreased the paw licking time by 8.29%, 31.87%, 19.84%, 31.71%, 29.76%, 30.57% and 31.87% respectively in the early phase (Table 5.2). The analgesic activity of *E. globulus* extract at the six dosages exhibited a significant difference in the early phase (p <0.005; Table 5.2). However, the analgesic effect of diclofenac was statistically insignificant compared to the effect of *E. globulus* at dosages of 50, 150, 200 and 250 in the early phase (p <0.005; Table 5.2). The antinociceptive effect of the leaf extract of *E. globulus* showed a dose-independent response in the early phase (Table 5.2).

The mice that were administered with leaves extracts of *E. globulus* at the dose levels of 25, 50, 100, 150, 200 and 250 including the aspirin lowered the paw licking time by 34.03%, 60.79%, 84.33 %, 90.65%, 94.49%, 98.52% and 98.32% respectively in the late phase (Table 5.2). The analgesic activity of the leaf extract at the six dosages revealed a significant difference in the late phase ($p < 0.005$; Table 5.2). However, the analgesic activity of the diclofenac was comparable to that of leaf extract of *E. globulus* at a dose of 250 in the late phase ($p > 0.005$; Table 5.2). The analgesic effect of the leaf extract of *E. globulus* showed a dose-dependent response in the late phase (Table 5.2). In comparison, the analgesic effect of extract of *E. globulus* at all the six dose levels was significantly effective at the late phase compared to the early phase in mice ($p < 0.005$; Figure 5.1).

Table 5.2: Analgesic activity of *E. globulus* leaf extract on formalin-induced pain in mice

Group	Treatment	Early Phase (1-5 min)	Late Phase (15-30 min)
Baseline	3% DMSO	0.00±0.00^{e} (0.00)	0.00 ±0.00^{h} (0.00)
Negative control	DMSO + Formalin	123.00±1.55^{a} (100.00)	297.40±9.58^{a} (100.00)
Positive control	Diclofenac 15mg/kg bw + Formalin	83.80± 1.39^{d} (31.87)	5.00±0.89^{g} (98.32)
DCM leaf extract of *E. globulus*	25mg/kg bw + Formalin	112.80±1.24^{b} (8.29)	196.20±1.05^{b} (34.03)
	50mg/kg bw + Formalin	83.80±1.62^{d} (31.87)	116.80±0.93^{c} (60.79)
	100mg/kg bw + Formalin	98.60±1.08 ^{c} (19.84)	46.60±0.93^{d} (84.33)
	150mg/kg bw + Formalin	84.00±0.55^{d} (31.71)	27.80±1.16^{e} (90.65)
	200mg/kg bw + Formalin	86.40±1.96^{d} (29.76)	16.40±1.03^{f} (94.49)
	250mg/kg bw + Formalin	85.40±1.78^{d} (30.57)	4.40±0.75^{g} (98.52)

Descriptive statistics are expressed as mean ± SEM for 5 mice. Values with a different superscript letter are statistically significant along the same column by one-way ANOVA followed by Tukey's post hoc test ($p \leq 0.005$). The values in brackets represent % pain inhibition.

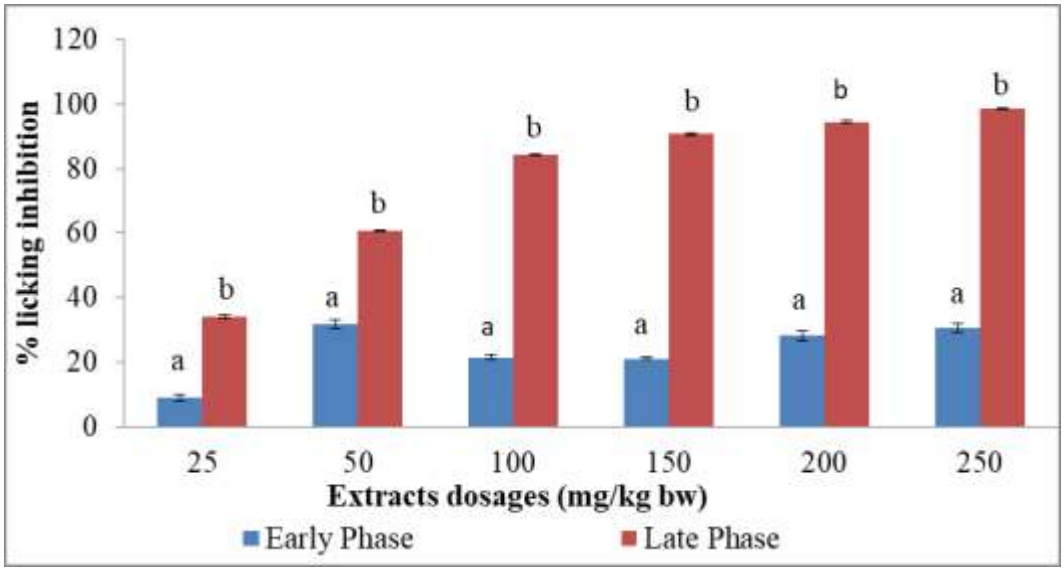

Figure 5. 1: Comparison of the analgesic activity of leaf extract of *E. globulus* in early and late phases.

On the other hand, the mice that received leaf extract of *S. didymobotrya* (Fresenius) showed antinociceptive activity on formalin-induced pain that was indicated by reduced paw time licking in both early and late phases (Table 5.3). The mice that received leaf extract of *S. didymobotrya* at the dosages of 50, 100, 150, 200 and 250 as well as the reference drug (diclofenac) reduced paw licking by 0.43%, 1.61%, 5.81%, 7.20%, 7.96%, and 38.17% respectively in the early phase (Table 5.3). However, the plant extract at 25mg/kg body weight dose level never showed any analgesic effect in the early phase as shown in Table 5.3. The analgesic effect of *S. didymobotrya* at the six dose levels exhibited a significant difference in the early phase ($p < 0.005$; Table 5.3). The analgesic activity of the diclofenac was significantly higher compared to that of *S. didymobotrya* extract at all dose levels in the early phase ($p < 0.005$; Table 5.3). The antinociceptive

effect of *S. didymobotrya* demonstrated a dose-dependent response in the early phase whereby increase dosage lead to increased analgesic activity of the administered extract (Table 5.3).

The leaf extracts *S. didymobotrya* at the dose levels of 25, 50, 100, 150, 200 and 250 including diclofenac lowered the paw licking time by 26.48%, 32.96%, 87.04%, 91.27%, 93.40%, 90.97% and 96.82% respectively in the late phase (Table 5.3). The analgesic activity of the leaf extract at the six doses was statistically significant in the late phase ($p < 0.005$; Table 5.3). The effect of diclofenac was significantly higher compared to those of extract at the six doses in the late phase ($p < 0.005$; Table 5.3). The analgesic effect of *S. didymobotrya* had a dose-independent response in the late phase where an increase in the dose that was administered did not lead to increased activity of the extract (Table 5.3).

Table 5. 3: Analgesic activity of leaf extract of *S. didymobotrya* on formalin-induced pain in mice

Animal Group	**Treatments**	**Early Phase (1-5 min)**	**Late Phase (15-30 min)**
baseline	3% DMSO only	0.00 ± 0.00^{e} (0.00)	0.00 ± 0.00^{h} (0.00)
Negative control	DMSO + Formalin	186.00 ± 0.01^{a} (100.00)	321.00 ± 0.01^{a} (100.00)
Positive control	Diclofenac 15mg/kg bw + Formalin	115.00 ± 3.29^{d} (38.17)	10.20 ± 0.49^{g} (96.82)
DCM leaf extract of *S. didymobotrya*	25mg/kg bw + Formalin	189.60 ± 0.93^{a} (-1.94)	236.00 ± 1.70^{b} (26.48)
	50mg/kg bw + Formalin	185.20 ± 1.69^{a} (0.43)	215.20 ± 0.97^{c} (32.96)
	100mg/kg bw + Formalin	183.00 ± 1.22^{ab} (1.61)	41.60 ± 1.8^{d} (87.04)
	150mg/kg bw+ Formalin	175.20 ± 1.39^{bc} (5.81)	28.20 ± 0.97^{e} (91.27)
	200mg/kg bw + Formalin	172.00 ± 0.93^{c} (7.20)	21.20 ± 1.07^{f} (93.40)
	250mg/kg bw + Formalin	171.20 ± 1.77^{c} (7.96)	29.00 ± 1.26^{e} (90.97)

Descriptive statistics are expressed as mean ± SEM for 5 mice in every group. Values with a different superscript letter are statistically significant ($p\leq0.005$) along the same column by one-way ANOVA accompanied by Tukey's post hoc test. The values in brackets represent % pain inhibition.

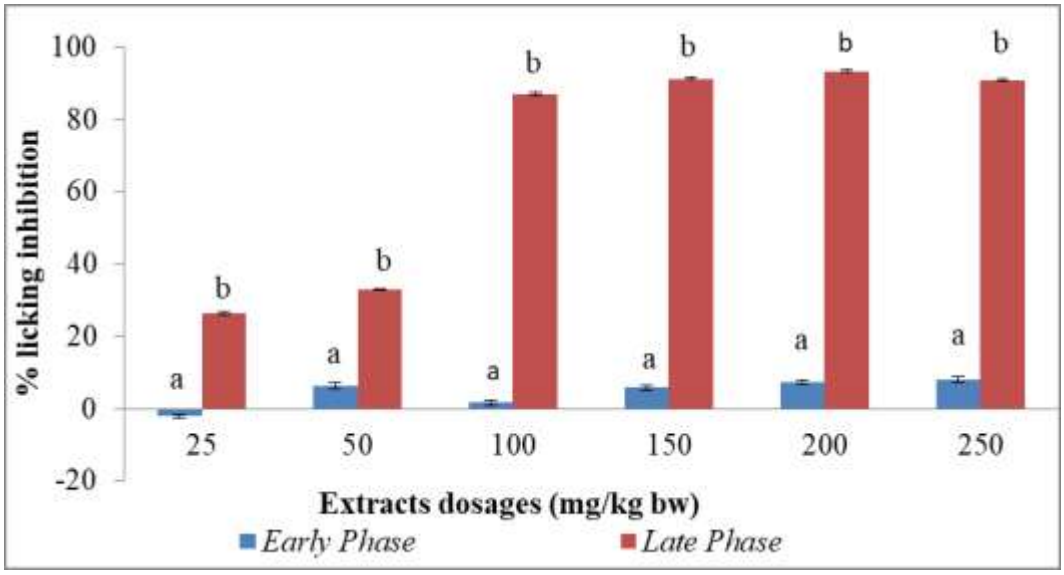

Figure 5.2: Comparison of analgesic activity of leaf extract of *S. didymobotrya* in early and late phase.

In comparison, the analgesic activity of leaf extract of *E. globulus* was significantly higher compared to *S. didymobotrya* at the same dose levels in the early phase ($p < 0.005$, Figure 5.3).

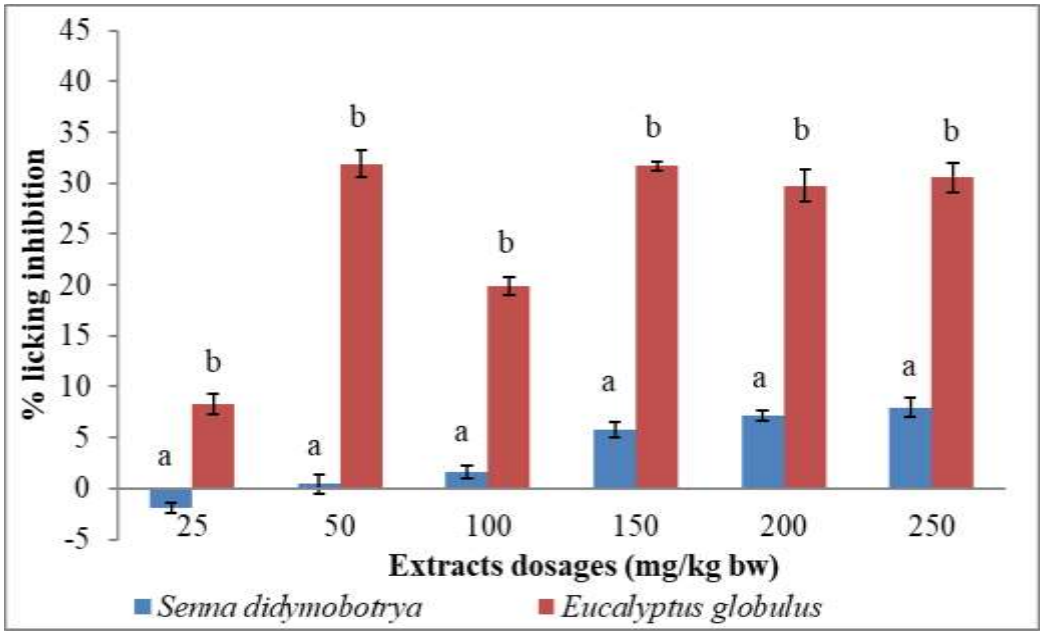

Figure 5. 3: Comparison of analgesic activities of leaf extracts of *E. globulus* and *S. didymobotrya* in the early phase.

The analgesic activities of the leaf extract of *S. didymobotrya* and *E. globulus* exhibited no significant difference at the doses of 100, 150 and 200 in the late phase ($p > 0.005$; Figure 5.4). However, at the same dosages of 25, 50 and 250; the analgesic activity of the *E. globulus* was significantly higher compared to that of *S. didymobotrya* in the late phase ($p < 0.005$; Figure 5.4).

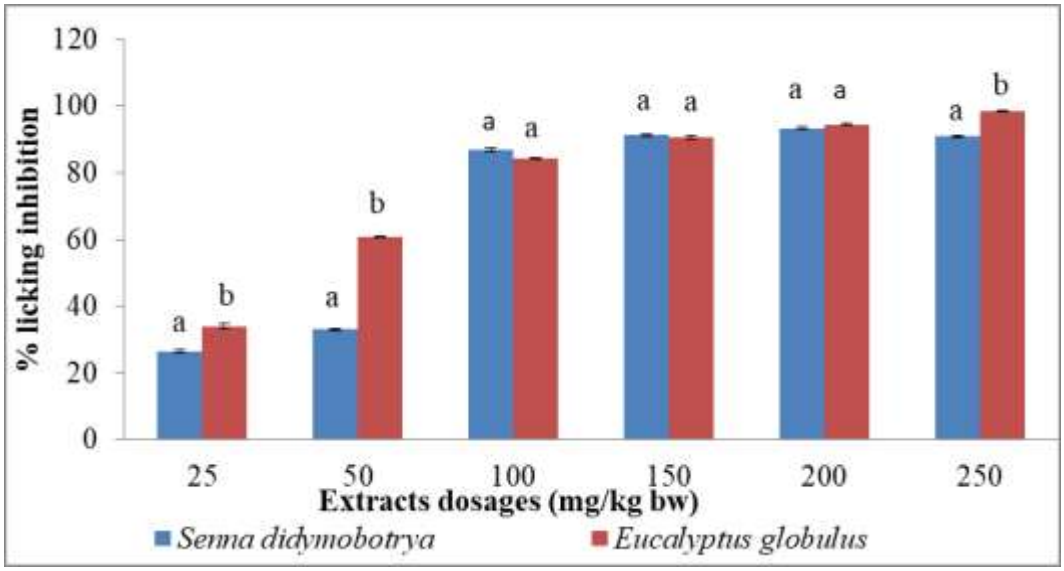

Figure 5. 4: Comparison of analgesic activities of leaf extracts of *E. globulus* and *S. didymobotrya* in the late phase in mice.

5.5 Discussion

The formalin test is the most commonly used method in the assessment of mild analgesic activity of drugs that work through the central and peripheral analgesic activities (Shang *et al*., 2015). Injection of formalin in the left hind paw of mice results in two distinct phases of pain. The first phase is also known as the neurogenic phase (0-5 minutes) develops as a result of chemical stimulation that releases substance P and bradykinin. The second phase (15–30 minutes) usually arises as a result of the release of inflammatory mediators such as prostaglandin and histamine (Hung *et al*., 2012).

The leaf extracts of *E. globulus* and *S. didymobotrya* inhibited pain in both phases. However, pain inhibition was more prominent in the late phase. It is postulated that the two plant extracts exhibited both peripheral and central antinociceptive activities (Sofidiya *et al*., 2014). The central analgesic activity could have been due to inhibition of the nociceptive effects of noradrenaline, bradykinin, prostaglandins, adrenaline, adenosine, serotonin, and acetylcholine (Ness, 2001; Geppetti *et al*., 2005; Zakaria *et al*., 2018).

On the other hand, the peripheral analgesic effect could be attributed to inhibition of the discharge of endogenous pain mediators like PGE_2 and PGE_2-α in peritoneal fluids including lipooxygenase, which triggers the nociceptive neurons (Liang *et al*., 2004). Sensory nerve terminals exposure to algogenic substances

and mediators discharged at the injury site results in peripheral sensitization, where there is an increased HT peripheral sensory neurons sensitivity (Chapman and Vierck, 2017).

Management of pain prevents the release of different inflammatory mediators and neurotransmitters, which sensitize the peripheral nociceptors (Benoliel *et al*., 2012). Through the reduction of prostaglandin synthesis, cyclo-oxygenase inhibitors usually inhibit the nociceptive response in the injured tissues (González-Hernández *et al*., 2018).

The two plant extracts exhibited analgesic effects by decreasing paw licking time in early and late phases on formalin-induced nociception in mice. These results were in agreement with other studies carried out on analgesic activities of other medicinal plants. Gitahi *et al*. (2015), in a study on the DCM: methanol extract of *Carissa edulis* revealed the analgesic effect by decreasing paw licking time in both phases of formalin-induced pain in mice.

The dose ranges used in the present study were within the dose ranges utilized by similar research studies on analgesic effects of medicinal plants. Hossain *et al*. (2014), evaluating for the analgesic activity of leaves and stems methanol extracts of *Alternanthera sessislis* (L.) on acetic-induced writhing in mice used dose ranges of 50, 100, 200 and 400. The choice of the dose ranges used in the present

study was based on literature review and titration of extracts in the laboratory to establish the appropriate dose ranges that can be adopted.

The significant antinociceptive effects of leaf extract of the two studied plants in Swiss albino mice could be attributed to the existence of analgesic components that acts by blocking the prostaglandin pathways (Akumu, 2018). The two extracts mechanism of action can be postulated to be similar to that of NSAIDs like diclofenac and ibuprofen. These drugs block the synthesis of prostaglandins by truncating the cyclooxygenase pathway (Duffy, 2015). This inhibition lowers the peripheral nervous tissue sensitization leading to less nerve stimulation and pain reduction (Scarpignato, 2013) acid-induced writhing in mice.

GC-MS results revealed α-pinenes, which belongs to a class of compounds known as monoterpenoids and forms about 90% of essential oils. Li *et al*. (2016), in their study, reported that α-pinenes possess significant analgesic activity on formalin-induced pain in mice. In another study by Him *et al*. (2008) also revealed that α-pinenes significantly reduced tail flick-induced pain in mice.

In this study, the findings of GC-MS demonstrated that the leaf extracts were endowed with several phytochemicals agents observed in Chapter Three that possess antinociceptive activity. For example, *E. globulus* had 25 phytochemical agents which were categorized into terpenoids, essential oils and flavonoids

(Table 3.1) while *S. didymobotrya* had 10 phytocompounds which were all terpenoids (Table 3.2). Generally, *E. globulus* exhibited higher analgesic potential compared with *S. didymobotrya*. This could be attributed to the higher number of phytochemicals that were observed in the quantitative phytochemical screening of the leaf extract of *E. globulus*.

The analysis of GC-MS also revealed; Endo-fenchol, Camphor, Borneol, 4-terpineol and α-terpineol. Fontaine *et al*. (2013), reported that camphor possesses antinociceptive activity. A study by Radulović *et al* (2013), reported analgesic activity of *F. ovina* on acetic acid abdominal constrictions, hot plate, tail immersion and dynamic hot plate tests in mice at doses 50, 100 and 200 mg/kgbw. Dagne *et al*. (2000), showed that α-Eudesmol also possesses analgesic activity by reducing acetic acid-induced writhing in mice. Similarly, Radulović *et al.* (2013), demonstrated that Endo-fenchol induces hyperalgesia in mice by reducing acetic acid abdominal writhing, latency time response against tail immersion test and formalin-induced paw licking test.

The GC-MS analysis also revealed Limonene, a bioactive agent in the essential oils of aromatic plants (Radulović *et al*., 2013). Erasto and Viljoen (2008), in a review on biosynthetic, ecological and pharmacological relevance that limonene reported it possesses analgesic activity. A study by Paula-Freire *et al*. (2016) found out that essential oil possess antihypernociceptive activity in neuropathic

pain models by reducing latency time response in the hot plate test model in rodents.

The analysis of leaf extracts revealed the presence of Myrcene. Shah *et al*. (2012), reported that myrcene possesses antinociceptive activity by reducing the acetic acid writhing in rodents. Studies reported by Paula-Freire *et al* (2016), on antihypernociceptive activity of *O.gratissimum* essential oil, indicated that myrcene had significant antinociceptive activity against von Frey and hot plate tests models in mice. De Sousa (2011), showed that α-pinenes possess significant analgesic activity in mice. Quintans (2013) and Santana *et al*. (2011), reported that p-cymene enhance by inclusion in β-cyclodextrin possess antinociceptive activity that significantly reduced the number of writhings in rodent models. Rao *et al*. (1990), on myrcene isolated from lemongrass oil (*Cymbopogon citratus*) exhibited an antinociceptive effect in mice against acetic acid-induced writhing and hot plate methods.

Alamgir (2017), reported that Borneol is used as folk medicine in China and India in the management of pain. The mechanism of pain inhibition by Borneol is by shrinking hemorrhoidal tissue thus relieving the burning sensation. It is useful in providing pain relief (Otuki *et al*., 2001). Alpha-Terpineol, a monoterpenoid alcohol is found in essential oils of several species of Eucalyptus. Its analgesic activity is through central and peripheral pathways (Ludwiczuk *et al*., 2017).

Alpha-terpineol also monoterpenoid alcohol is a component of essential oil. Saleh-e-in *et al*. (2018), reported that alpha-terpineol has analgesic activity in rodents by reducing formalin, acetic acid-induced writhing, glutamate and capsaicin-induced nociception in animal models. Further, the GC-MS analysis of *E. globulus* leaf extract revealed the presence of α-Eudesmol, (Quintans-Júnior *et al*., 2011), reported that α-Eudesmol possesses analgesic activity.

In conclusion, this study revealed that *E. globulus* and *S. didymobotrya* have analgesic potential against formalin-induced pain in mice. The study also reveals that DCM leaf extracts of *E. globulus* and *S. didymobotrya* contain potent chemical agents effective in the management of pain. These findings support the traditional use of *E. globulus* and *S. didymobotrya* in the management of pain.

CHAPTER SIX

ANTI-INFLAMMATORY ACTIVITY OF LEAF EXTRACTS IN MICE

6.1 Introduction

Inflammation is an immune system response that triggers cellular and enzymatic processes that safeguard the body against any kind of trauma (Cadirci *et al*., 2016). This process is usually associated with pain which is caused by the afferent nerve fibers stimulation by inflammatory chemical mediators, whose initial role is to protect the organism (Batista *et al*., 2010). Inflammation generates a wide range of inflammatory mediators to tissue injury, irritation, or infection to eliminate the microbes or irritants and stimulate repair of the damaged tissue (Ricciotti and FitzGerald, 2011; Sajid *et al*., 2017).

The process of inflammation is a complex biological reaction of the vascular tissues to noxious stimuli like damaged cells, irritants and pathogens. Even though inflammation is a body defense mechanism, the inflammatory mediators and complex events that participate in the inflammatory response can lead to the development of many ailments (Gupta *et al*., 2006; Bullon *et al*., 2014).

Inflammation is managed using anti-inflammatory drugs such as aspirin, diclofenac, paracetamol among others. Some of these conventional drugs have side effects (Moore *et al*., 2015).

Medicinal plants such as *Carissa spinarum, Ximenia americana* and *Clutia abyssinica* form a better alternative therapy for they have fewer side effects and readily available (Fenetahun and Eshetu, 2017). This study seeks to explore the anti-inflammatory potential of *E. globulus* and *S. dimobotrya* in mice.

6.2 Materials and methods

6.2.1 Plant samples collection, preparation and extraction

Collection, preparation and extraction of medicinal plant material was executed as described in Chapter Three Section 3.2 Subsection 3.2.1.

6.2.2 Preparation of experimental doses

The experimental doses were prepared as outlined in chapter four section 4.2.2**.** To prepare carrageenan**,** a quantity of 1g of freshly obtained carrageenan powder was dissolved in 100 ml of normal saline to make a 1% suspension.

6.2.3 Experimental animals

Swiss albino mice of both sexes were used in this study. The animals were aged 5-6 weeks and weighing between 20-25grams. The animals were cared for as described in Chapter 4 Subsection 4.2.3.

6.2.4 Induction of inflammation

To induce inflammation each mouse was treated with a 1% carrageenan solution through injection with 0.1ml of this solution in the left hind paw. The mice would then wait for one hour after which treatment with either diclofenac for positive control group or extracts dosages, the paw edema circumference was measured (Fotso *et al*.,2014).

6.2.5 Experimental design

This study adopted a completely Randomized Controlled Study Design, from which an experimental design was drawn as described in chapter four section 4.2.4. The experimental mice were categorized into nine groups of five animals each (n=5). The animals received treatments as follows; Group I (normal control) mice were treated with DMSO only. (Group II) (Negative control group) mice were treated with 1% carrageenan only. Group III (Positive control) mice were treated with 1% carrageenan to induce inflammation and then treated with the reference drug 15 mg/kg bw diclofenac sodium.

Groups IV, V, VI, VII, VII and IX mice were induced with inflammation and then treated with extracts at dose levels of 25, 50, 100, 150 and 250 mg/kg bw respectively after one hour of carrageenan administration. The summary of this design is detailed in Table 6.1.

Table 6. 1: Anti-inflammatory effects of *E. globulus* and *S. didymobotrya* leaf extracts in mice.

Group	Treatment
I	3% DMSO only
II	Carrageenan + DMSO
III	Carrageenan + DMSO + 15mg/kgbw diclofenac
IV	Carrageenan + 25mg/kg bw extract
V	Carrageenan + 50mg/kg bw extract
VI	Carrageenan + 100mg/kg bw extract
VII	Carrageenan +150mg/kg bw extract
IX	Carrageenan + 200mg/kg bw extract
X	Carrageenan + 250mg/kg bw extract

DMSO = Dimethyl sulphoxide

The paw circumference was then determined for four hours at an hourly interval (Bamgbose and Noamesi, 1981). The paw circumference was measured before the induction of inflammation and compared with the paw circumference after induction of inflammation. The paw circumference was measured using cotton thread and then transferred to the ruler to obtain the reading in millimeters (da Silva *et al*., 2014). The percentage % inhibition of edema of the two extracts was computed using the formula as described by Umamageswari and Kudagi (2015).

$$\%\ edema\ inhibition = \frac{Vc - Vt}{Vc} X100$$

Where Vc is the Mean edema volume in the control group and Vt is the Mean edema volume in the treated group.

6.2.6 Data management and statistical analysis

The paw edema data was obtained, recorded and entered into a Microsoft Excel spreadsheet. Descriptive statistics were computed and the data expressed as mean± SEM. The inferential statistic was done using one-way ANOVA followed by Tukey's post hoc test for pairwise separation and comparison of means. An unpaired student t-test was used to compare the anti-inflammatory effects of the two plant extracts. The confidence level was set at 99.5% ($p \leq 0.005$). Results were presented in form of graphs and tables.

6.3 Results

6.3.1 Anti-inflammatory effects of *E. globulus* and *S. didymobotrya* leaf extracts on carrageenan-induced inflammation in mice

Generally, the leaf extract of *E. globulus* at the six dose levels (25, 50, 100, 150, 200 and 250) reduced carrageenan-induced edema in Swiss albino mice. This was revealed by a decrease in the circumference of the inflamed paw after administration of DCM leaf extract of *E. globulus* at various doses as shown in Table 6.2.

In the 1st hour, the dichloromethane leaf extract of *E. globulus* at the five doses of 50, 100, 150, 200 and 250 reduced the inflamed paw in a dose-related manner by 0.85 %, 1.45 %, 1.41 %, 2.02 % and 2.27 % respectively (Table 6.2). However, the extract dose of 25 did not reduce inflammation (Table 6.2). At this hour, the anti-inflammatory activities of *E. globulus* extract at doses of 50, 100, 150, 200 and 250 were statistically similar and comparable to the activity of diclofenac as presented in Table 6.2 ($p > 0.005$).

In the second hour, the *E. globulus* extract, at the doses of 50, 100, 150, 200 and 250 reduced the paw edema in mice by 1.87%, 3.86%, 4.70%, 5.90% and 6.52% respectively (Table 6.2). However, at this hour, the *E. globulus* extract at the dose of 25 exhibited no anti-inflammatory effects. There was a significant variation in the anti-inflammatory effect of *E. globulus* extract at the six doses ($p < 0.005$; Table 6.2). However, the anti-inflammatory activity of diclofenac was not

significantly different compared to that of the extract of *E. globulus* at the dose of 200 and 250 in mice as presented in Table 6.2 ($p > 0.005$).

In the 3rd hour, the *E. globulus* extract at the dose of 25, 50, 100, 150, 200 and 250 reduced the inflamed paw of animals by 1.28%, 3.22%, 5.80%, 7.35%, 8.70% and 9.09% respectively (Table 6.2). The anti-inflammatory effect of *E. globulus* leaf extract at the six doses exhibited significant differences in mice ($p < 0.005$; Table 6.2). However, the anti-inflammatory effect of the reference drug was comparable to those of leaf extract of the *E. globulus* at the doses of 150, 200 and 250 as shown in Table 6.2 ($p > 0.005$).

In the 4th hour, the leaf extract of *E. globulus* at 25, 50, 100, 150, 200 and 250 doses decreased the inflamed paw circumference of experimental animals by 2.72%, 3.89%, 6.13%, 8.60%, 9.94% and 10.90% respectively (Table 6.2). There was a significant variation in the anti-inflammatory effect of *E. globulus* leaf extract at all the tested doses in mice ($p < 0.005$; Table 6.2). However, the anti-inflammatory effect of diclofenac was statistically similar to those of extract of *E. globulus* at 150, 200 and 250 doses ($p > 0.005$; Table 6.2). Generally, the *E. globulus* extract exhibited a dose-dependent response at all the hours tested (Table 6.2). Notably, the percentage paw circumference in the negative control group mice was significantly higher compared to those of extract-treated rats as well as

those of rats in the positive control and normal control groups at all the tested hours ($p < 0.005$; Table 6.2).

There was an insignificant change in paw circumference of the mice in the normal control group at all the test hours ($p>0.005$; Appendix VII). In contrast, the paw circumference of mice in the negative control group significantly increased from the zero hours to the 4^{th} hour (Appendix VII). Further, the paw circumference of animals that were treated with the 25mg/kgbw extract dose of *E. globulus* significantly reduced in the fourth hour compared to zero, first and second hours ($p<0.005$; Appendix VII). The paw circumference of animals that were administered with the 50mg/kg bw extract dose at the zero and first hours were significantly higher than that in the second, third and fourth hours ($p<0.005$; Appendix VII). The paw circumference of animals that received diclofenac and *E. globulus* extract at 150, 200 and 250 doses significantly reduced from zero to the fourth hours ($p <0.005$; Appendix VII).

Table 6.2: Anti-inflammatory effects of leaf extract of *E. globulus* on carrageenan-induced inflammation in Swiss albino mice.

Group	**Treatment**	**Percentage change in paw circumference (mm)**				
		0h	**1h**	**2h**	**3h**	**4h**
Normal control	3% DMSO	100.00±0.00	100.00±0.00^{bc} (0.00)	100.00±0.00^{b} (0.00)	100.00±0.00^{b} (0.00)	100.00±0.00^{b} (0.00)
Negative control	Carrageenan + DMSO only	100.00±0.00	103.06±0.44^{a} (-3.06)	106.65±0.42^{a} (-6.65)	108.18±0.36^{a} (-8.18)	109.11±0.39^{a} (-9.11)
Positive control	Carrageenan + Diclofenac 15mg/kg bw	100.00±0.00	98.23±0.15^{d} (1.77)	93.57±0.23^{f} (6.43)	92.11±0.32^{ef} (7.89)	90.03±0.17^{ef} (9.97)
DCM leaf extract	Carrageenan + 25mg/kg bw	100.00±0.00	100.81±0.50^{b} (-0.81)	100.15±0.31^{b} (-0.15)	98.72±0.20^{b} (1.28)	97.28±0.15^{c} (2.72)
	Carrageenan + 50mg/kg bw	100.00±0.00	99.15±0.01^{cd} (0.85)	98.13±0.18^{c} (1.87)	96.78±0.17^{c} (3.22)	96.11±0.30^{c} (3.89)
	Carrageenan + 100mg/kg bw	100.00±0.00	98.55±0.16^{cd} (1.45)	96.14±0.27^{d} (3.86)	94.20±0.34^{d} (5.80)	93.87±0.19^{d} (6.13)
	Carrageenan + 150mg/kg bw	100.00±0.00	98.59±0.15^{cd} (1.41)	95.30±0.04^{de} (4.70)	92.65±0.17^{e} (7.35)	91.40±0.44^{e} (8.60)
	Carrageenan + 200mg/kg bw	100.00±0.00	97.98±0.18^{d} (2.02)	94.10±0.22^{ef} (5.90)	91.30±0.18^{ef} (8.70)	90.06±0.32^{ef} (9.94)
	Carrageenan + 250mg/kg bw	100.00±0.00	97.73±0.23^{d} (2.27)	93.48±0.20^{f} (6.52)	90.91±0.34^{f} (9.09)	89.10±0.19^{f} (10.90)

Descriptive statistics are expressed as mean ± SEM. Values with a different superscript letters are statistically significant along the column by one-way ANOVA accompanied by Tukey's post hoc test ($p \leq 0.005$).The values in brackets represent % inflammation inhibition.

On the other hand, the dichloromethane leaf extracts of *S. didymobotrya* at the six tested doses demonstrated an anti-inflammatory effect on carrageenan-induced inflammation in Swiss albino mice. This was revealed by a decrease in inflamed paw oedema after mice received the leaf extracts of *S. didymobotrya* (Table 6.3). In the first hour, the extract of *S. didymobotrya* at the doses of 100, 150, 200 and 250 decreased the inflamed paw circumference in mice by 1.11%, 1.40%, 2.14% and 2.41% respectively. However, the extract never exhibited an anti-inflammatory effect at 25 and 50 doses (Table 6.3). The anti-inflammatory activities of leaf extract *S. didymobotrya* at the doses of 100, 150, 200 and 250 exhibited no significant difference ($p > 0.005$; Table 6.3). Similarly, the anti-inflammatory activity of the reference drug (diclofenac) was comparable to those of *S. didymobotrya* extract at the doses of 150, 200 and 250 as presented in Table 6.3 ($p > 0.005$).

In the 2nd hour, the leaf extract of *S. didymobotrya*, at doses of 50, 100, 150, 200 and 250 decreased the inflamed paw circumference in mice by 0.50%, 2.54%, 3.71%, 4.12% and 5.13% respectively (Table 6.3). However, the extract at a dose of 25 never revealed anti-inflammatory activity (Table 6.3). The anti-inflammatory activity of *S. didymobotrya* at the tested dose levels exhibited a significant difference in mice ($p<0.005$; Table 6.3). However, the anti-inflammatory activity of diclofenac was statistically insignificant compared to that of *S. didymobotrya* at the doses of 200 and 250 ($p > 0.005$; Table 6.3).

In the 3rd hour, the *S. didymobotrya* extract at doses of 25, 50, 100, 150, 200 and 250 reduced the inflamed paw circumference by 1.13%, 1.99%, 3.81%, 5.11%, 5.50% and 8.31% respectively (Table 6.3). The anti-inflammatory effects of *S. didymobotrya* extract exhibited a significant difference at the six tested doses in mice at this hour ($p<0.005$; Table 6.3). However, the effect of the diclofenac was comparable to that of the extract of *S. didymobotrya* at 250mg/kg bw in Swiss albino mice ($p > 0.005$; Table 6.3).

The leaf extract of *S. didymobotrya* at dosages of 25, 50, 100, 150, 200 and 250 reduced the inflamed paw circumference by 2.59%, 3.16%, 4.61%, 6.34%, 7.03% and 9.05% respectively in the fourth hour, (Table 6.3). At this hour, there was a significant variation in the anti-inflammatory effect of *S. didymobotrya* extract at the six tested doses in mice ($p<0.005$; Table 6.3). However, the anti-inflammatory effect of the reference drug, diclofenac was comparable to that of extract at 250 doses in mice ($p >0.005$; Table 6.3). Generally, the percentage paw circumference in the negative control group mice was significantly higher compared to extract-treated rats as well as rats in the positive and normal control groups at all the tested hours ($p < 0.005$; Table 6.2).

The normal control group mice exhibited an insignificant change in the paw circumference in the test period ($p>0.005$; Appendix VIII). However, the paw

circumference of animals in the negative control group increased significantly from the zero to the fourth hour ($p<0.005$; Appendix VIII). Further, the paw circumference of animals that received extract at the doses of 25 and 50 was significantly lower in the fourth compared to the other hours ($p<0.005$; Appendix VIII). On the other hand, the paw circumference in animals treated with extract at the doses of 100, 150, 200 and 250, as well as diclofenac significantly reduced from zero to the fourth hour ($p<0.005$; Appendix VIII).

Table 6.3: Anti-inflammatory effects of leaf extract of *S. didymobotrya* on carrageenan-induced inflammation in Swiss albino mice

Group	**Treatment**	**Percentage change in paw circumference (mm)**				
		0h	**1h**	**2h**	**3h**	**4h**
Normal control	3% DMSO	100.00±0.00	100.00±0.00^{b} (0.00)	100±0.00^{b} (0.00)	100.00±0.00^{b} (0.00)	100.00±0.00^{b} (0.00)
Negative Control	Carrageenan + DMSO	100.00±0.00	102.86±0.41^{a} (-2.86)	106.54±0.42^{a} (-6.54)	108.40±0.29^{a} (-8.40)	109.51±0.36^{a} (-9.51)
Positive Control	Carrageenan + Diclofenac + DMSO	100.00±0.00	97.27±0.0.21^{e} (2.73)	94.53±0.41^{d} (5.47)	92.95±0.21^{f} (7.05)	91.98±0.16^{fg} (8.02)
DCM: leaf Extract	Carrageenan + 25 mg/kgbw	100.00±0.00	101.30±0.0.33^{b} (-1.30)	100.50±0.20^{b} (-0.50)	98.87±0.20^{bc} (1.13)	97.41±0.19^{c} (2.59)
	Carrageenan + 50 mg/kgbw	100.00±0.00	100.50±0.20^{b} (-0.50)	99.50±0.20^{b} (0.50)	98.01±0.20^{c} (1.99)	96.84±0.16^{c} (3.16)
	Carrageenan + 100 mg/kgbw	100.00±0.00	99.89±0.20^{cd} (1.11)	97.46±0.15^{c} (2.54)	96.19±0.29^{d} (3.81)	95.39±0.16^{d} (4.61)
	Carrageenan + 150 mg/kgbw	100.00±0.00	98.60±0.15^{de} (1.40)	96.29±0.15^{c} (3.71)	94.89±0.21^{de} (5.11)	93.66±0.27^{e} (6.34)
	Carrageenan + 200 mg/kgbw	100.00±0.00	97.86±0.16^{de} (2.14)	95.88±0.38^{cd} (4.12)	94.50±0.13^{e} (5.50)	92.97±0.24^{ef} (7.03)
	Carrageenan + 250mg/kgbw	100.00±0.00	97.59±0.13^{de} (2.41)	94.57±0.27^{d} (5.43)	91.69±0.41^{f} (8.31)	90.95±0.32^{g} (9.05)

Descriptive statistics are expressed as mean ± SEM. Values with different superscript letters are statistically significant along the column by one-way ANOVA accompanied by Tukey's post hoc test ($p \leq 0.005$). The values in brackets represent % inflammation inhibition.

In comparison, the anti-inflammatory activities of the leaf extracts of *E. globulus* and *S. didymobotrya* at the dose of 25 were not significantly different at the four hours of the test period in mice ($p > 0.005$; Figure 6.1).

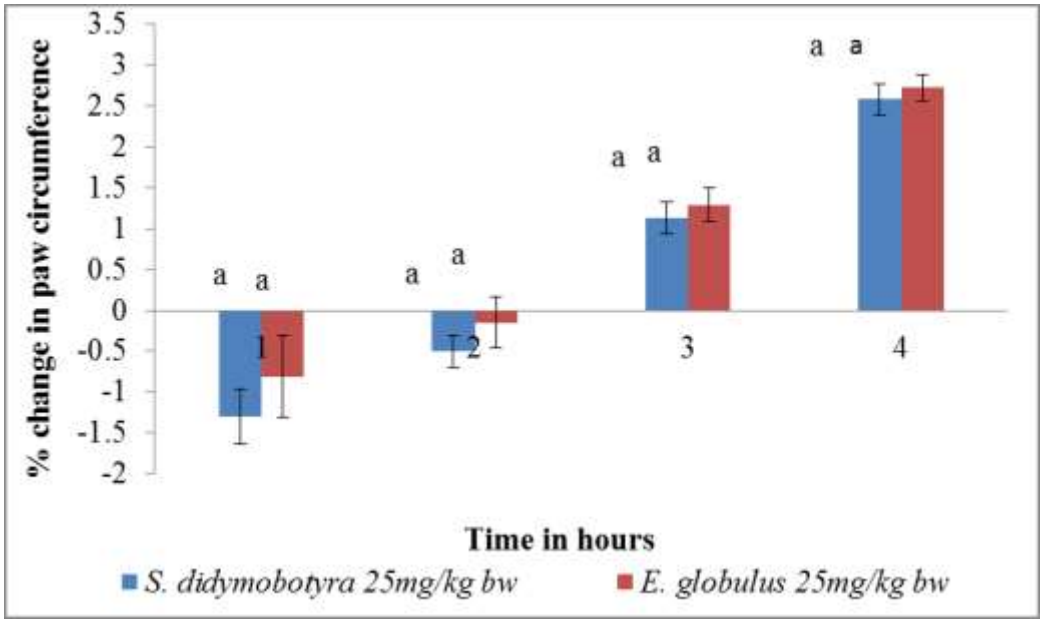

Figure 6. 1: Comparison of anti-inflammatory effects of *E. globulus* and *S. didymobotyra* leaf extracts at 25mg/kgbw on carrageenan-induced inflammation.

Comparatively, the anti-inflammatory activity of *E. globulus* extract at 50 dose was significantly higher compared to that of *S. didymobotyra* in the first, second and third hours ($p<0.005$; Figure 6.2). However, at the same dose, the anti-inflammatory effects of *E. globulus* and *S. didymobotyra* extracts showed no significant difference in the fourth hour in mice ($p > 0.005$; Figure 6.2).

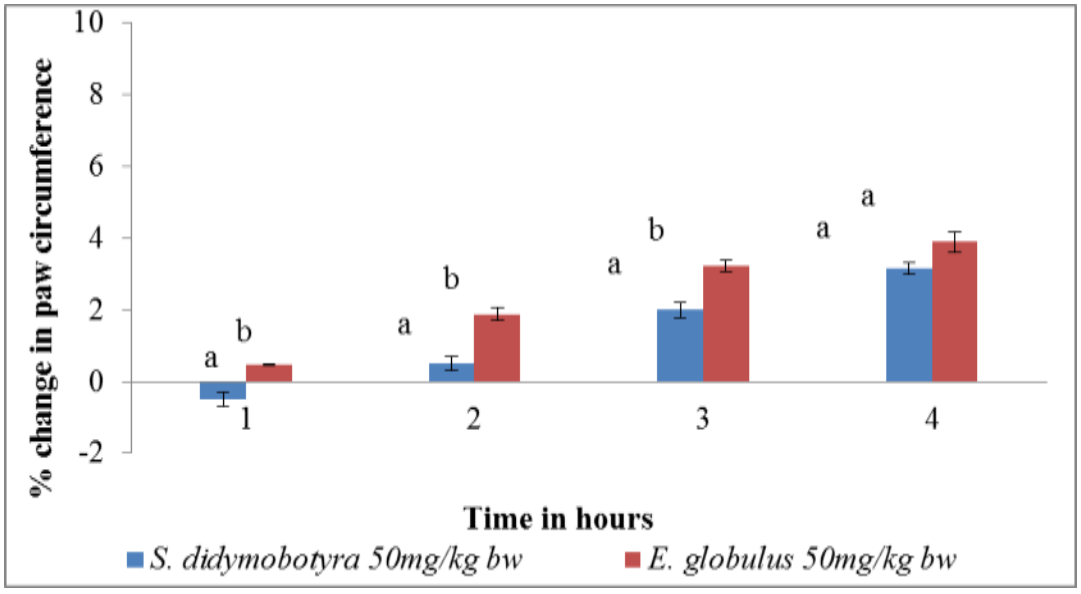

Figure 6.2: Comparison of anti-inflammatory activities of *E.globulus* and *S. didymobotyra* leaf extracts at 50mg/kg bw on carrageenan-induced inflammation.

At a dose of 100, the anti-inflammatory effects of leaf extracts of *E. globulus* and *S. didymobotyra* were not significantly different in the first hour ($p > 0.005$; Figure 6.3). In contrast, the anti-inflammatory effect of *E. globulus* extract was significantly higher compared to that of *S. didymobotyra* at the same dose in the second, third and fourth hours ($p \leq 0.005$; Figure 6.3).

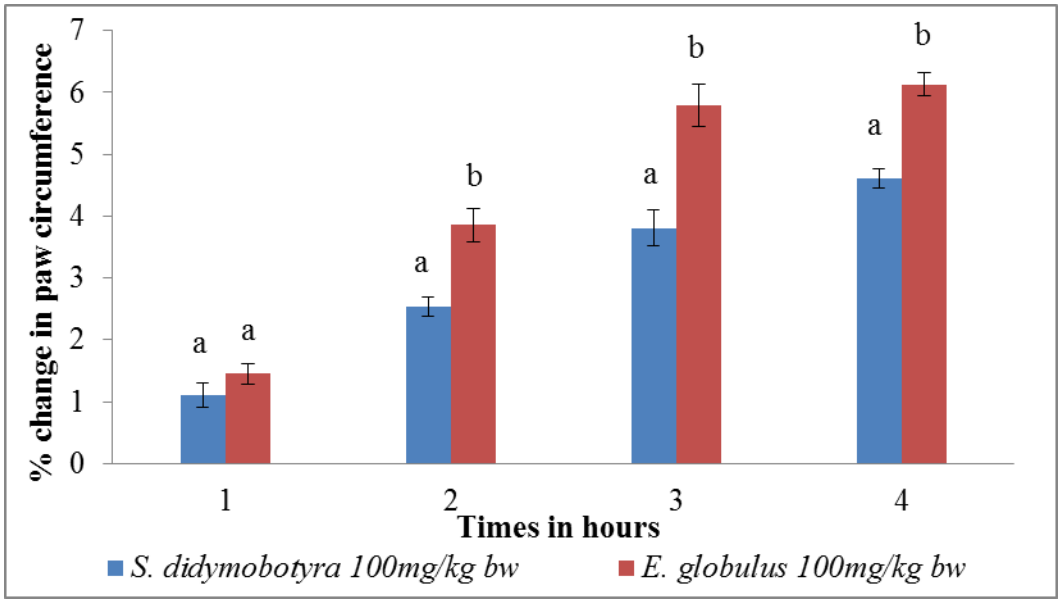

Figure 6.3: Comparison of anti-inflammatory effects of *E. globulus* and *S. didymobotyra* at 100mg/kgbw.

The anti-inflammatory effects of *E. globulus* and *S. didymobotyra* extract dose of 150 revealed no significant difference in mice ($p > 0.005$; Figure 6.4). However, the anti-inflammatory activity of *E. globulus* extract in the same dose was significantly higher compared to that of *S. didymobotyra* in the second, third and fourth hours ($p \leq 0.005$; Figure 6.4).

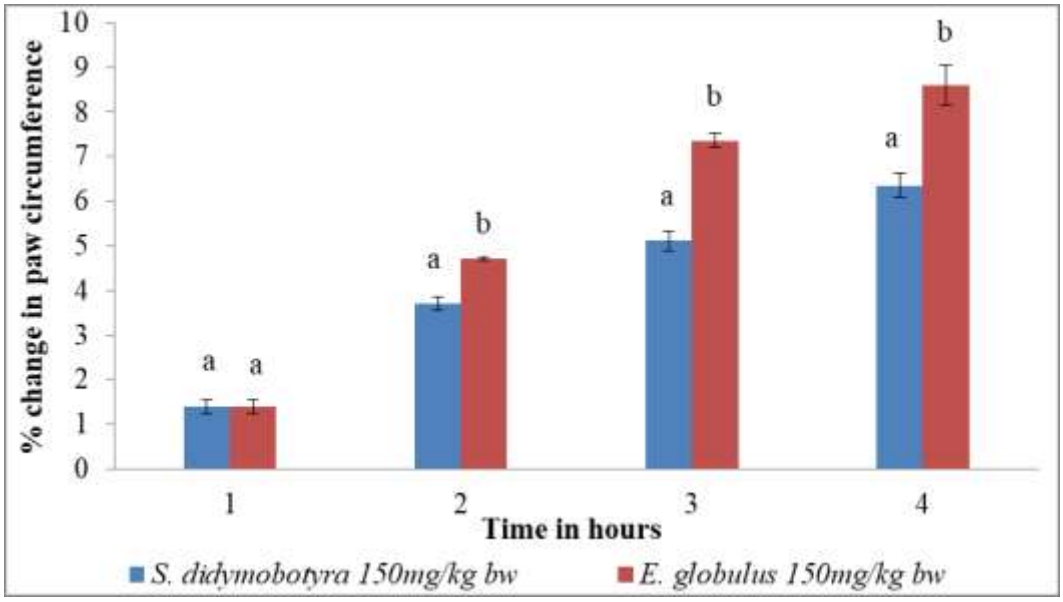

Figure 6.4: Comparison of anti-inflammatory effects of *E. globulus* and *S. didymobotyra* at 150mg/kgbw.

There was insignificant variation in the anti-inflammatory effects of *E. globulus* and *S. didymobotyra* extracts dose of 200 in mice in the first and second hours ($p > 0.005$; Figure 6.5). However, the anti-inflammatory effects of the *E. globulus* extract at the same dose were significantly higher than that of *S. didymobotyra* in the third and fourth hours ($p< 0.005$; Figure 6.5).

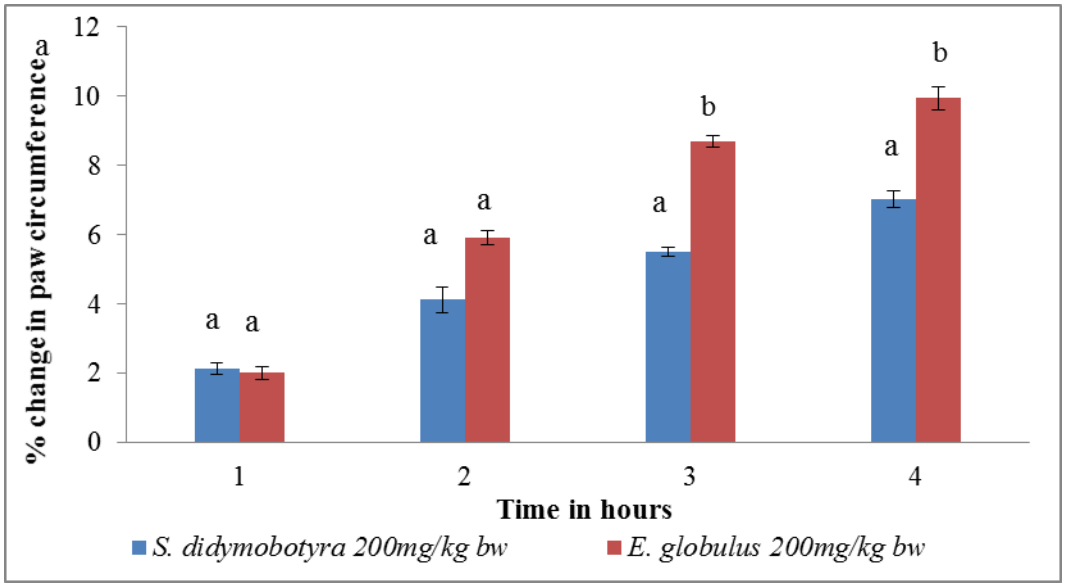

Figure 6.5: Comparison of anti-inflammatory effects of *E. globulus* and *S.didymobotyra* leaf extracts at 200mg/kgbw.

At a dose of 250, the anti-inflammatory effects of leaf extracts of *E. globulus* and *S. didymobotyra* in mice were statistically similar in the first, second and third hours ($p > 0.005$; Figure 6.6). However, in the fourth hour, there was an insignificant variation in the anti-inflammatory effects of the two plant extracts ($p<0.005$; Figure 6.6).

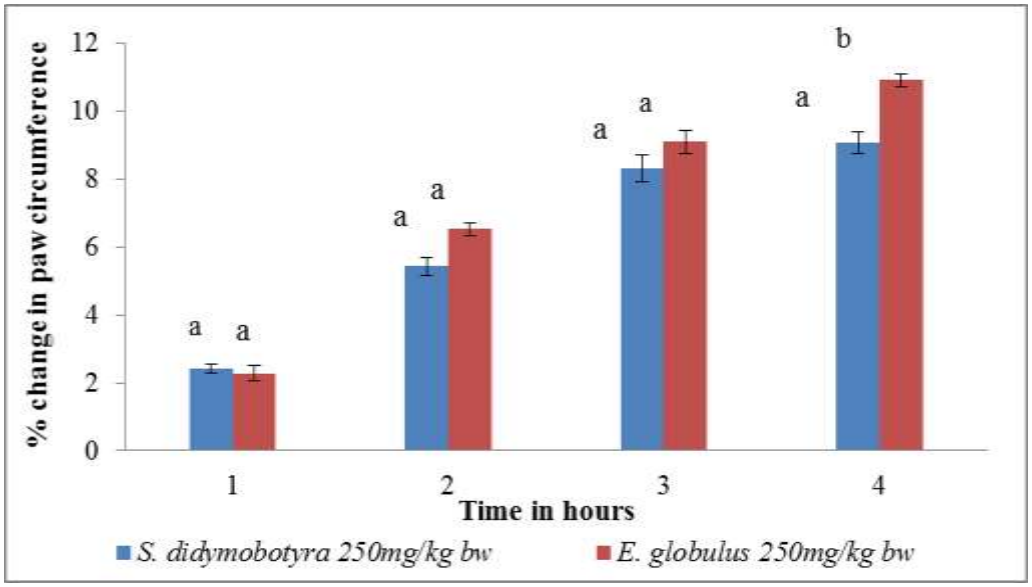

Figure 6. 6: Comparison of anti-inflammatory effects of *E. globulus* and *S. didymobotyra* leaf extracts 250mg/kgbw.

6.4 Discussion

Inflammation is a complex protective reaction of the body against noxious agents like microorganisms or injured cells (Pombeiro-Sponchiado *et al*., 2017). Carrageenan-induced paw inflammation is the most suitable model in the determination of the anti-inflammatory activities of different agents that act by blocking the acute inflammatory mediators *in vivo* (Huang *et al*., 2012). Carrageenan-induced inflammation is a biphasic phenomenon (Pundarikakshudu *et al*., 2016). The early phase, which occurs between 90 to one 180 minutes of inflammation, occurs as a result of the discharge of serotonin, histamine among other substances while the late, phase which occurs between 270 to 360 minutes is *et* postulated to be linked to the activation of lysosome, prostaglandins and 62 proteases (Ashraf *al*., 2016). In this study inflammation bioscreening was carriedout between first and fourth hour.This is because beyond this time the extract administered will have under gone degradation in the liver hence will have lost its effectiveness hence bioavailability wound be low.

The findings of this study revealed that the *E. globulus* and *S. didymobotyra* leaf extracts possess anti-inflammatory effects, which were evident by the reduction of inflamed paw circumference in mice after treatment. These findings are consistent with earlier research work on the anti-inflammatory effects of other herbal plants in experimental animals. Matthew *et al*. (2013), who carried out studies on anti-inflammatory activities of ethanol and aqueous extracts of dried stem of plant

Kalanchoe pinnata (Lam.) Pers against carrageenan induced inflammation in rats revealed that this plant has potent anti-inflammatory potential.

This study used diclofenac which belongs to a group of drugs known as NSAIDs as reference drug. The NSAIDs like diclofenac reduce edema by blocking enzyme cyclooxygenase (COX) which catalyzes prostaglandin biosynthesis (Ahuja *et al*., 2008). Cyclooxygenase enzymes exist in two forms; cyclooxygenase-1 and cyclooxygenase-2. These two enzymes catalyze the synthesis of prostaglandins which stimulate inflammation. The NSAIDs blocks the cyclooxygenase enzymes and decrease prostaglandins in the entire body leading to a reduction of inflammation (Harris *et al*., 2014).

The phytochemical composition of these plants revealed that these plants are endowed with many bioactive agents that possess anti-inflammatory effects. The anti-inflammatory effects of these plants are attributed to phytochemicals mainly terpenoids (chapter three of this study). *E. globulus* showed higher anti-inflammatory activity than *S. didymobtrya* in almost all the doses.

Several studies have shown that most of the phytochemical agents present in the two plant extract have anti-inflammatory activity. Kim *et al*. (2015), demonstrated that α-Pinene exhibits anti-inflammatory activity through the suppression of mitogen-activated protein kinases in mice. According to Kaushik *et al*. (2012),

who studied Carrageenan-induced paw edema and Cotton Pellet granuloma activity of petroleum ether extract of *Pinus roxburghii* Sarg, revealed that camphene possesses anti-inflammatory activity in rats. Almeida *et al*. (2013), in a study on anti-inflammatory effects of borneol on the leukocyte migration after carrageenan injection in mice, revealed that borneol possesses significant anti-inflammatory effects. The results of GC-MS revealed the presence of Alpha-terpineol. Hajhashemi *et al*. (2003), reported anti-inflammatory effects of hydroalcoholic leaves to extract of *Lavandula angustifolia* Mill. *Lamiaceae* on carrageenan-induced inflammation in rats revealed that Alpha-terpineol has potent anti-inflammatory activity.

The GC-MS results revealed the presence of terpinolene, A study by Usman *et al*. (2010), showed anti-inflammatory activity of aqueous leaf extract of *Chenopodium album* in male swiss mice. Further analysis of GC-MS showed the presence of globulol, Esteves *et al*. (2005), reported that globulol extracted from aqueous leaf extracts of *Casearia sylvestris* possesses anti-inflammatory activity on carrageenan-induced paw edema in rats. The results of GC-MS revealed the presence of α-phellandrene. Silva *et al*. (2015), in a study on topical and oral anti-inflammatory activity of the LEO oil ear edema model in mice, revealed that α-phellandrene possesses significant anti-inflammatory activity. In another study by Quintans *et al*. (2013), reported that p-cymene possesses anti-inflammatory activity on carrageenan-induced inflammation in mice. Research by Kummer *et*

al. (2013), has shown that Limonene also possesses anti-inflammatory activity against carrageenan induced-inflammation in rats. Salleh *et al*. (2015), reported anti-inflammatory activities of the essential oils by measurement of the mushroom tyrosinase enzyme, AChE enzymes, and lipoxygenase assay, revealed that aromadendrene possesses an anti-inflammatory effect.

In conclusion, this study revealed that the DCM leaf extracts of *E. globulus* and *S. didymobotrya* have anti-inflammatory activity in mice. The anti-inflammatory activity could be associated with the presence of bioactive compounds. The present study, therefore, supports the traditional use of these plants in the management of inflammation.

CHAPTER SEVEN

GENERAL SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

7.1 General Summary

This study aimed at evaluating the phytochemical profile of the leaf extracts of *E. globulus* and *S. didymobotrya*. Besides, it focused on the determination of the antipyretic, antinociceptive and anti-inflammatory potential of *E. globulus* and *S. didymobotrya* leaf extracts in animal models.

The results on the quantitative phytochemical composition revealed that the *E. globulus* leaf extract had 25 phytochemical agents which include α-Deudesmol, α-Phellandrene, β-Pinene, Tridecane and 4-Terpineol, α-Pinene, Camphene, Myrcene, *p*-Cymene, Limonene, γ-Terpinene, Terpinolene, Undecane, Endofenchol, Camphor, Borneol, α-Terpineol, α-Copaene, α-Gurjunene, (*E*)-Caryophyllene, Aromadendrene, α-Selinene, δ-Armophene and Globulol, while *S. didymobotrya* (Fresenius) extract had ten phytocompounds namely camphor, Limonene, Cumene, γ-terpinene, Camphene, α-pinene, β-Pinene, α-Phellandrene, α-Terpinene and Terpinolene. From previous studies, these bioactive phytocompounds are linked with antipyretic, analgesic and anti-inflammatory effects in animal models through inhibition of cyclooxygenase (COX) enzymatic activity and therefore, resulting in the reduction of the levels of PGE_2 biosynthesis which is the key inducer of pain, fever and inflammation.

This study revealed that the *E. globulus* and *S. didymobotrya* leaf extracts possess potent antipyretic, analgesic and anti-inflammatory activities. The study also revealed that the effects of two plant extract at doses of 200 and 250mg/kg bw were comparable with that of the reference drugs, aspirin and diclofenac. The synergistic and additive effects of the bioactive constituents increase their bioavailability and action on multiple molecular targets thus, the observed activities of *E. globulus* and *S. didymobotrya* leaf extracts are attributed to secondary metabolites they contain. This study, therefore, confirms and supports the use of the studied plant extracts as an alternative and/or complementary remedy against pain, fever and inflammation. The study also sets the pace for further studies in an effort to develop plant-derived drug compounds for the treatment of pain, fever and inflammation.

7.2 Conclusions

From this study, it is concluded that;

i. The DCM leaf extracts of *E. globulus* and *S. didymobotrya* have several phytochemical agents associated with antipyretic activity in rats.
ii. The DCM leaf extracts of *E. globulus* and *S. didymobotrya* contain phytochemical compounds associated with antinociceptive potential in mice.
iii. The DCM leaf extracts of *E. globulus* and *S. didymobotrya* contain phytochemical compounds associated with anti-inflammatory activity in mice.

Therefore, the research questions formulated in this study were answered in the affirmative and the study objectives were successfully executed and achieved.

7.3 Recommendations

7.3.1 Recommendations from the study

i. The leaf extract of *E. globulus* and *S.didymobotrya* can be used as complementary and alternative remedies against fever after comprehensive toxicological studies.

ii. The leaf extract of *E. globulus* and *S.didymobotrya* can be used as complementary and alternative remedies against pain.

iii. The leaf extract of *E. globulus* and *S. didymobotrya* can be used as complementary and alternative remedies against inflammation.

iv. The administered extract doses (200, 250 mg) are appropriate in the bioassay of antipyretic,analgesic and anti-inflammatory activity in animal models.

7.3.2 Recommendations for further studies

For further studies, this study recommends;

i. Elucidation of the mechanisms of action for the two plant leaf extracts.

ii. Evaluation of other plant parts for antipyretic, anti-nociceptive and anti-inflammatory activities.

iii. Isolation of pure compounds for subtractive analysis for antipyretic, anti-nociceptive and anti-inflammatory activities.

iv. Toxicity studies to be done to assess the safety of the plant extracts.

v. Evaluation of the plants' biological activities using different solvents of different polarities than that of dichloromethane.

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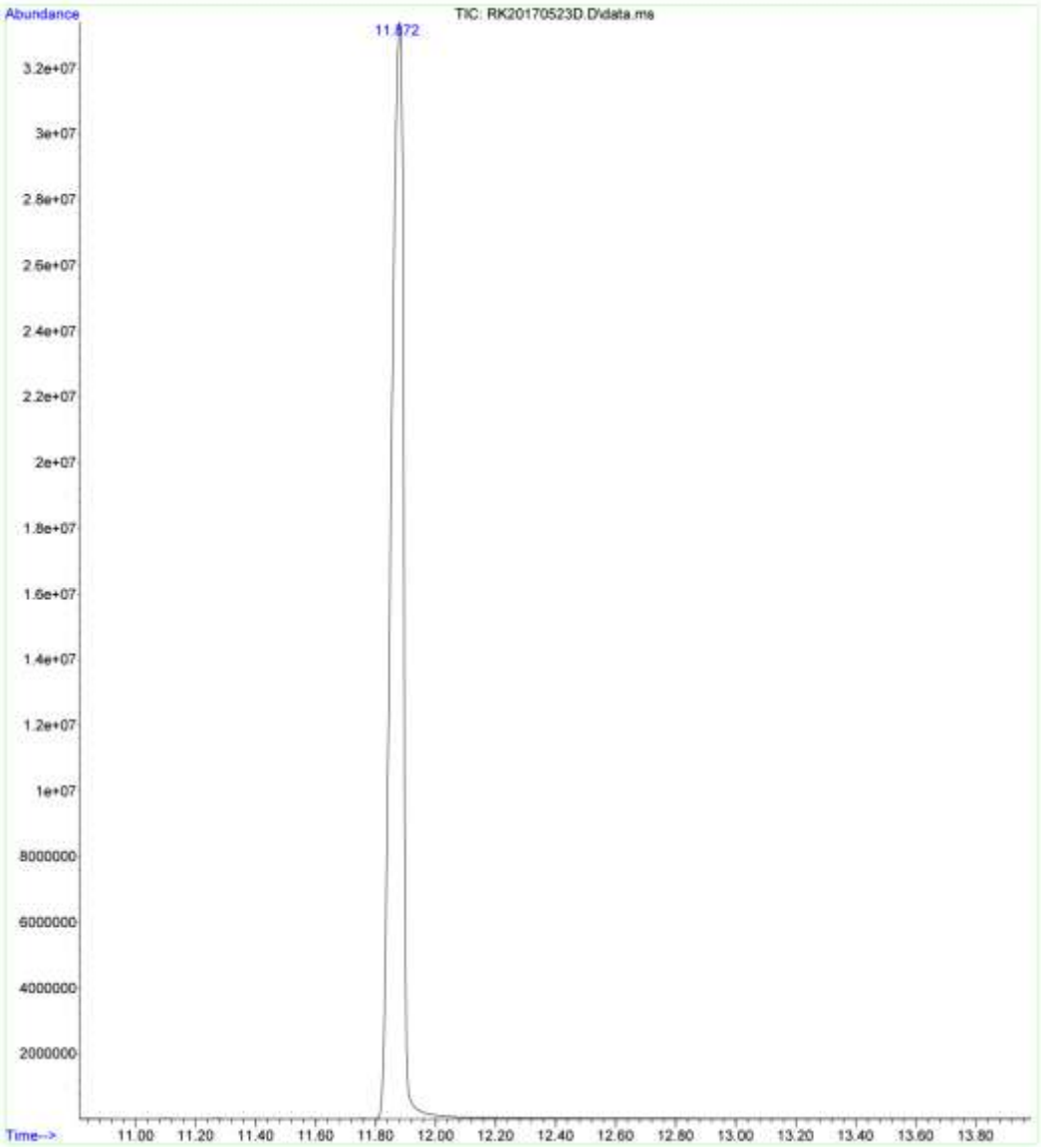
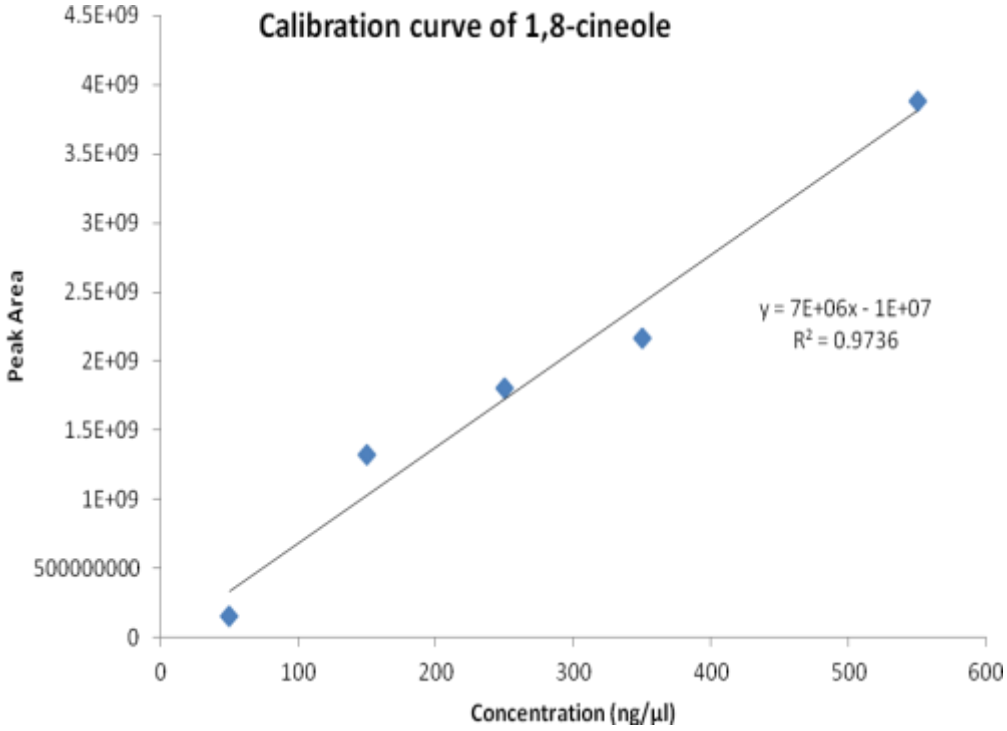
APPENDICES

Appendix I Representative total ion chromatogram of 1,8-cineole of *Eucalyptus globulus*



Appendix II *Eucalyptus globulus* calibration curve of 1,8-cineole (peak area vs. concentration) with the following equation; [y=7E+06-1E+07] which served as the basis for the external quantification of the target compound



Appendix III Representative total ion chromatogram of *Eucalyptus globulus* leaf extract with no RT

```
File       :C:\COMMERCIAL_2017\June 2017\BSB\RK20170702A-SENNA.D
Operator   : [BSB1]OK
Acquired   :  2 Jul 2017    6:11     using AcqMethod HEX VOLATILES 35-280 XTD 70MINUTES .M
Instrument :   ICIPE MSD
Sample Name: Senna
Misc Info  : Senna
Vial Number: 9
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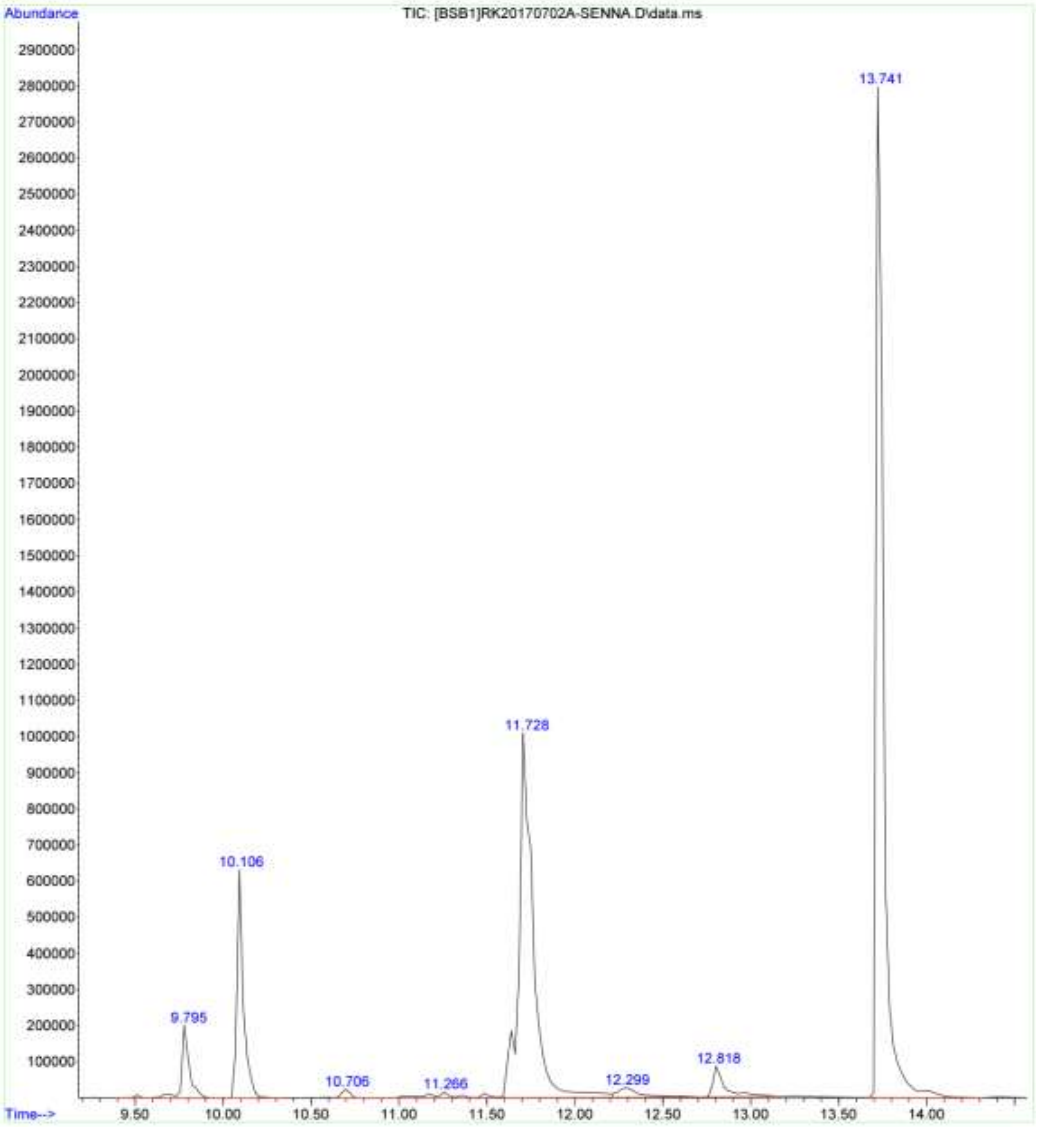

Appendix IV Representative total ion chromatogram of *Senna didymobotrya* leaf extract with no RT

```
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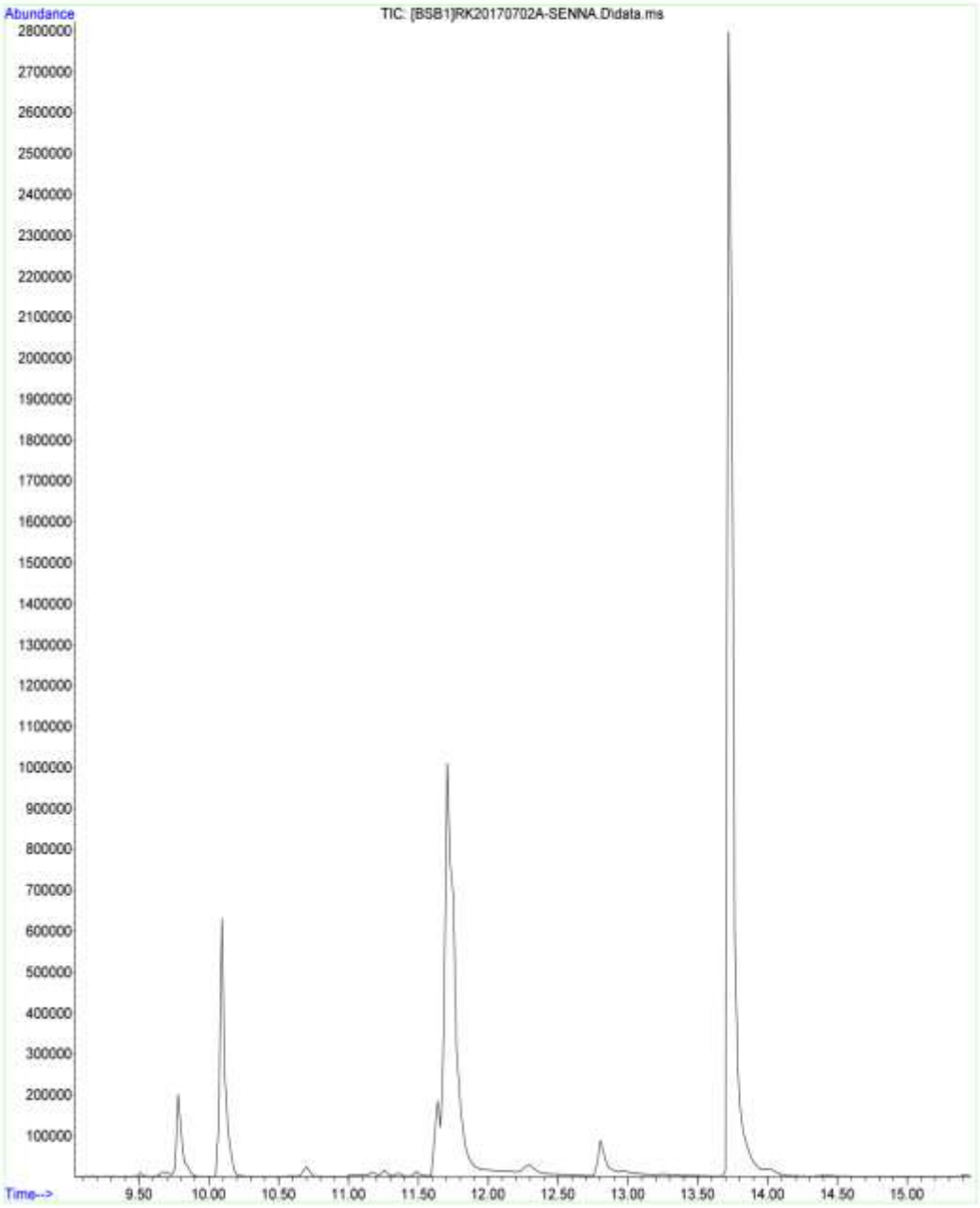
Operator   : [BSB1]OK
Acquired   :  2 Jul 2017    6:11      using AcqMethod HEX VOLATILES 35-280 XTD 70MINUTES .M
Instrument :    ICIPE MSD
Sample Name: Senna
Misc Info  : Senna
Vial Number: 9
```



Appendix V Comparision percent change of hourly dosages of *E. globulus* on turpentine induced fever

Treatment	Percentage change in rectal temperatures (ºC)				
	0hr	1hr	2hr	3hr	4hr
None	100.00 ± 0.00^{A}	99.89 ± 0.13^{A}	100.16 ± 0.22^{A}	99.78 ± 0.13^{A}	99.89 ± 0.18^{A}
Turpentine + DMSO	100.00 ± 0.00^{B}	100.42 ± 0.06^{AB}	100.16 ± 0.10^{AB}	100.68 ± 0.18^{A}	100.52 ± 0.12^{AB}
Turpentine + Aspirin + DMSO	100.00 ± 0.00^{A}	98.07 ± 0.07^{B}	97.55 ± 0.15^{B}	96.82 ± 0.12^{C}	95.31 ± 0.16^{D}
Turpentine + 25 mg/kg bw	100.00 ± 0.00^{A}	98.96 ± 0.08^{AB}	98.18 ± 0.23^{BC}	97.46 ± 0.25^{CD}	97.04 ± 0.26^{D}
Turpentine + 50 mg/kg bw	100.00 ± 0.00^{A}	98.53 ± 0.06^{B}	97.91 ± 0.17^{C}	97.28 ± 0.07^{D}	96.28 ± 0.13^{E}
Turpentine + 100 mg/kg bw	100.00 ± 0.00^{A}	98.49 ± 0.10^{B}	97.44 ± 0.05^{C}	96.60 ± 0.01^{D}	95.98 ± 0.10^{E}
Turpentine + 150 mg/kg bw	100.00 ± 0.00^{A}	98.13 ± 0.15^{B}	96.94 ± 0.05^{C}	96.37 ± 0.09^{D}	95.53 ± 0.07^{E}
Turpentine + 200 mg/kg bw	100.00 ± 0.00^{A}	97.57 ± 0.10^{B}	96.80 ± 0.06^{C}	96.13 ± 0.08^{D}	95.20 ± 0.07^{E}
Turpentine + 250mg/kg bw	100.00 ± 0.00^{A}	97.71 ± 0.05^{B}	96.73 ± 0.07^{C}	96.41 ± 0.05^{C}	95.17 ± 0.10^{D}

Results are expressed as Means ± SD for five rats per group. means within respective rows followed by superscript of similar upper case letters are not significantly different at $p\leq0.01$; analyzed by ANOVA followed by Tukey's post hoc test for multiple comparison.

Appendix VI Comparision percent change of hourly dosages of *S. didymobotrya* on turpentine induced fever

Treatment	Percentage change in rectal temperatures (ºC)				
	0h	**1h**	**2h**	**3h**	**4h**
None	100.00 ± 0.00^{A}	99.73 ± 0.12^{A}	99.95 ± 0.21^{A}	100.11 ± 0.20^{A}	100.00 ± 0.19^{A}
Turpentine + DMSO	100.00 ± 0.00^{B}	100.52 ± 0.08^{A}	100.67 ± 0.06^{A}	100.67 ± 0.06^{A}	100.72 ± 0.05^{A}
Turpentine + Aspirin + DMSO	100.00 ± 0.00^{A}	98.75 ± 0.15^{B}	97.45 ± 0.09^{C}	96.67 ± 0.05^{D}	95.89 ± 0.13^{E}
Turpentine + 25mg/kg bw	100.00 ± 0.00^{A}	99.48 ± 0.08^{AB}	98.79 ± 0.16^{BC}	98.27 ± 0.23^{CD}	97.64 ± 0.16^{D}
Turpentine + 50mg/kg bw	100.00 ± 0.00^{A}	98.97 ± 0.08^{B}	97.83 ± 0.14^{C}	97.47 ± 0.04^{C}	97.01 ± 0.05^{D}
Turpentine + 100mg/kg bw	100.00 ± 0.00^{A}	98.86 ± 0.13^{B}	97.56 ± 0.06^{C}	97.40 ± 0.01^{C}	96.36 ± 0.01^{D}
Turpentine + 150mg/kg bw	100.00 ± 0.00^{A}	99.00 ± 0.15^{B}	98.05 ± 0.07^{C}	97.36 ± 0.01^{D}	96.67 ± 0.10^{E}
Turpentine + 200mg/kg bw	100.00 ± 0.00^{A}	98.70 ± 0.12^{B}	97.60 ± 0.09^{C}	96.77 ± 0.06^{D}	96.25 ± 0.07^{D}
Turpentine + 250mg/kg bw	100.00 ± 0.00^{A}	98.69 ± 0.09^{B}	97.76 ± 0.11^{C}	96.92 ± 0.14^{D}	96.04 ± 0.08^{E}

Results are expressed as Means ± SD for five rats per group. means within respective rows followed by superscript of similar upper case letters are not significantly different at $p\leq0.01$; analyzed by ANOVA followed by Tukey's post hoc test for multiple comparison.

Appendix VII Comparision percent change of hourly dosages of *E. globulus* on carrageenan induced inflammation

Treatment	Percentage change in paw circumference (mm)				
	0h	**1h**	**2h**	**3h**	**4h**
None	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}
Carrageenan + DMSO	100.00 ± 0.00^{D}	103.06 ± 0.44^{C}	106.65 ± 0.42^{B}	108.18 ± 0.36^{AB}	109.11 ± 0.39^{A}
Carrageenan + Diclofenac 15mg/kg + DMSO	100.00 ± 0.00^{A}	98.23 ± 0.15^{B}	93.57 ± 0.23^{C}	92.11 ± 0.32^{D}	90.03 ± 0.17^{E}
Carrageenan + 25 mg/kg	100.00 ± 0.00^{AB}	100.81 ± 0.50^{A}	100.15 ± 0.31^{AB}	98.72 ± 0.20^{BC}	97.28 ± 0.15^{C}
Carrageenan + 50 mg/kg	100.00 ± 0.00^{A}	99.15 ± 0.01^{A}	98.13 ± 0.18^{B}	96.78 ± 0.17^{C}	96.11 ± 0.30^{C}
Carrageenan + 100 mg/kg	100.00 ± 0.00^{A}	98.55 ± 0.16^{B}	96.14 ± 0.27^{C}	94.20 ± 0.34^{D}	93.87 ± 0.19^{D}
Carrageenan + 150 mg/kg	100.00 ± 0.00^{A}	98.59 ± 0.15^{B}	95.30 ± 0.04^{C}	92.65 ± 0.17^{D}	91.40 ± 0.44^{D}
Carrageenan + 200 mg/kg	100.00 ± 0.00^{A}	97.98 ± 0.18^{B}	94.10 ± 0.22^{C}	91.30 ± 0.18^{D}	90.06 ± 0.32^{E}
Carrageenan + 250mg/kg	100.00 ± 0.00^{A}	97.73 ± 0.23^{B}	93.48 ± 0.20^{C}	90.91 ± 0.34^{D}	89.10 ± 0.19^{E}

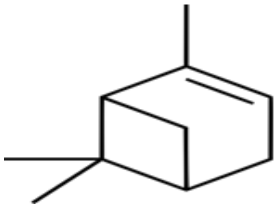
Results are expressed as Means ± SD for five rats per group. Means within respective rows followed by superscript of similar upper case letters are not significantly different at $p\leq0.01$; analyzed by ANOVA followed by Tukey's post hoc test for multiple comparison.

Appendix VIII Comparision percent change of hourly dosages of *senna didymobotrya* on carragenaan induced inflammation

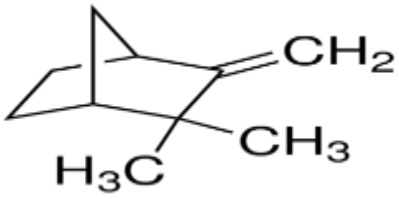
Treatment	**Percentage change in paw circumference (mm)**				
	0h	**1h**	**2h**	**3h**	**4h**
None	100.00±0.00^{A}	100.00±0.00^{A}	100±0.00^{A}	100.00±0.00^{A}	100.00±0.00^{A}
Carrageenan + DMSO	100.00±0.00^{D}	102.86±0.41^{C}	106.54±0.42^{B}	108.40±0.29^{AB}	109.51±0.36^{A}
Carrageenan + Diclofenac + DMSO	100.00±0.00^{A}	97.27±0.0.21^{B}	94.53±0.41^{C}	92.95±0.21^{D}	91.98±0.16^{D}
Carrageenan + 25 mg/kg	100.00±0.00^{BC}	101.30±0.0.33^{A}	100.50±0.20^{AB}	98.87±0.20^{C}	97.41±0.19^{D}
Carrageenan + 50 mg/kg	100.00±0.00^{AB}	100.50±0.20^{A}	99.50±0.20^{B}	98.01±0.20^{C}	96.84±0.16^{D}
Carrageenan + 100 mg/kg	100.00±0.00^{A}	99.89±0.20^{B}	97.46±0.15^{C}	96.19±0.29^{D}	95.39±0.16^{D}
Carrageenan + 150 mg/kg	100.00±0.00^{A}	98.60±0.15^{B}	96.29±0.15^{C}	94.89±0.21^{D}	93.66±0.27^{E}
Carrageenan + 200 mg/kg	100.00±0.00^{A}	97.86±0.16^{B}	95.88±0.38^{C}	94.50±0.13^{D}	92.97±0.24^{E}
Carrageenan + 250mg/kg	100.00±0.00^{A}	97.59±0.13^{B}	94.57±0.27^{C}	91.69±0.41^{D}	90.95±0.32^{D}

Results are expressed as Means ± SD for five rats per group. means within respective rows followed by superscript of similar upper case letters are not significantly different at $p \leq 0.01$; analyzed by ANOVA followed by Tukey's post hoc test for multiple comparison.

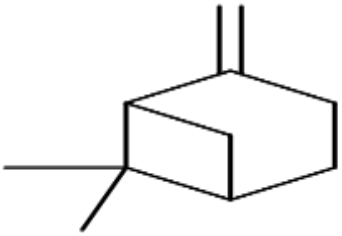
Appendix IX The structural formula of compounds identified by GC-MS analysis of the leaf extract of *E. globulus* and *S. didymobotrya*



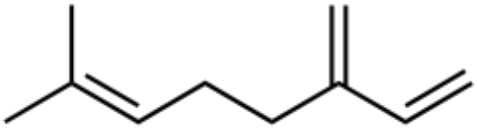
α-pinene ($C_{10}H_{16}$)



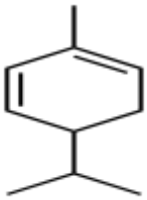
Camphene ($C_{10}H_{16}$)



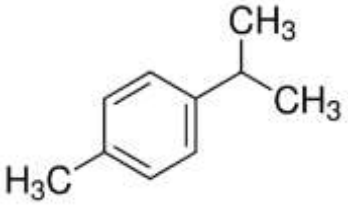
β-pinene ($C_{10}H_{16}$)



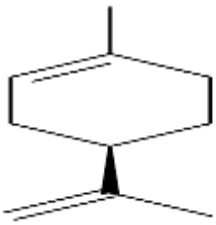
Myrcene ($C_{10}H_{16}$)



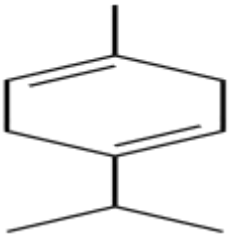
α-phellandrene ($C_{10}H_{16}$)



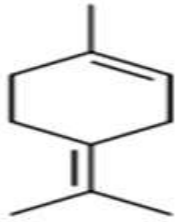
p-Cymene ($C_{10}H_{14}$)



Limonene ($C_{10}H_{16}$)

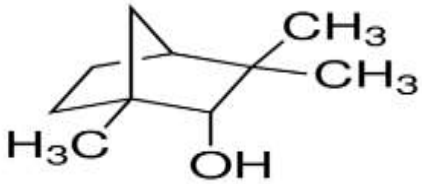


γ – terpinene ($C_{10}H_{16}$)

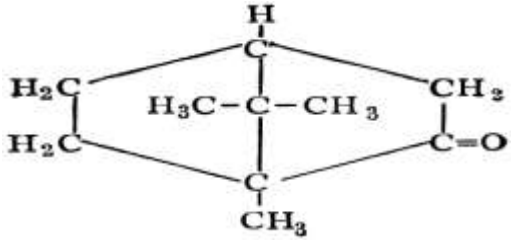


Terpinolene ($C_{10}H_{16}$)

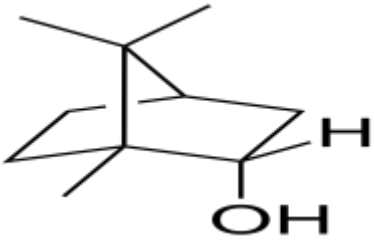
Undecane ($C_{11}H_{24}$)



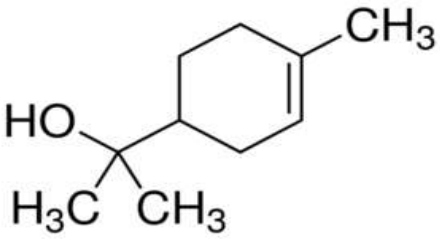
Endo-fenchol ($\underline{C_{10}H_{18}O}$)



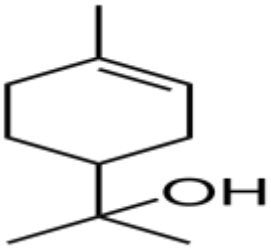
Camphor ($C_{10}H_{18}O$)



Borneol ($C_{10}H_{18}O$)

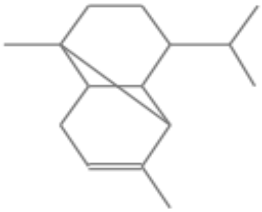


4- terpineol ($C_{10}H_{18}O$)

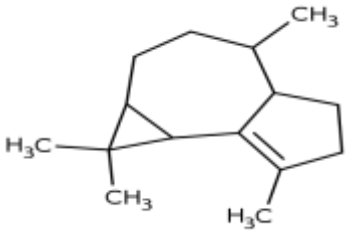


α-terpineol ($C_{10}H_{18}O$)

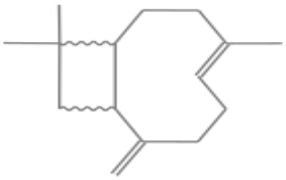
Tridecane ($C_{13}H_{28}$)



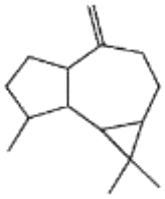
α- Copaene ($C_{15}H_{24}$)



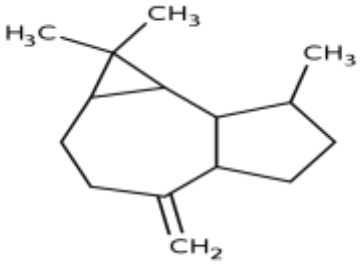
α-Gurjunene ($C_{15}H_{24}$)



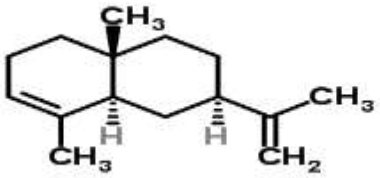
(*E*) – Caryophyllene ($C_{15}H_{24}$)



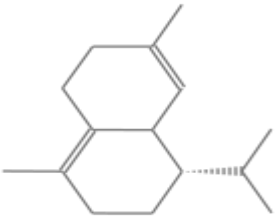
Aromadendrene ($C_{15}H_{24}$)



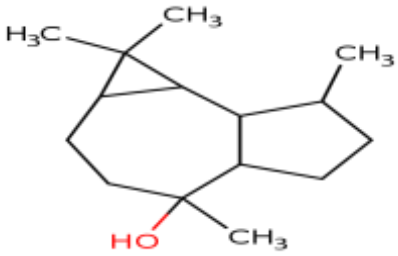
Allo-aromadendrene ($C_{15}H_{24}$)



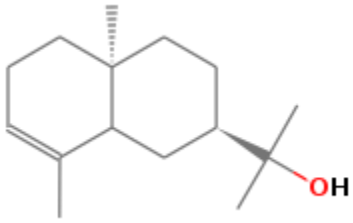
α-selinene ($C_{15}H_{24}$)



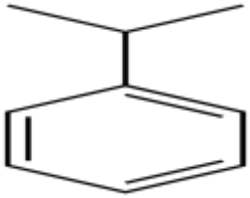
δ- armophene ($C_{15}H_{24}$)



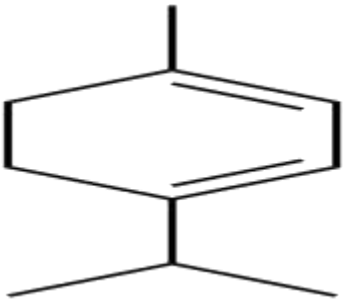
Globulol ($C_{15}H_{26}O$)



α- Eudesmol ($C_{15}H_{26}O$)



Cumene (C_9H_{12})



α-terpinene ($C_{10}H_{16}$)

Appendix X Raw data for evaluation of analgesic activity of DCM extract of *E. globulus*

Treatments Groups	**phase 1 (sec)**	**phase 2 (sec)**
Normal control		
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
Negative control		
1	120	260
2	128	300
3	122	310
4	125	305
5	120	312
Positive control		
1	87	3
2	85	4
3	86	8
4	80	6
5	81	4
25mg/kg bw		
1	110	196
2	115	200
3	110	190
4	116	200
5	113	195
50mg/kg bw		
1	88	115
2	86	115
3	85	120
4	80	117
5	80	116
100mg/kg bw		
1	100	45
2	97	46
3	102	50
4	96	45
5	98	47

150mg/kg bw		
1	84	30
2	85	26
3	85	31
4	84	27
5	82	25
200mg/kg bw		
1	91	20
2	90	15
3	86	14
4	80	16
5	85	17
250mg/kg bw		
1	80	2
2	88	6
3	83	4
4	90	6
5	86	4

Appendix XI Raw data for evaluation of analgesic activity of DCM extract of *S. didymobotrya*

Treatments Groups	**phase 1 (sec)**	**phase 2 (sec)**
Normal control		
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
Negative control		
1	180	325
2	189	300
3	185	330
4	186	320
5	190	330
Positive control		
1	106	9
2	120	10
3	108	12
4	120	10
5	121	10
25mg/kg bw		
1	190	230
2	187	237
3	192	240
4	191	235
5	188	238
50mg/kg bw		
1	179	218
2	189	216
3	185	215
4	186	212
5	187	215
100mg/kg bw		
1	181	36
2	187	40
3	184	45
4	183	41
5	180	46

150mg/kg bw		
1	172	30
2	180	25
3	176	27
4	173	29
5	175	30
200mg/kg bw		
1	170	20
2	174	22
3	171	25
4	173	19
5	175	20
250mg/kg bw		
1	170	26
2	165	26
3	172	32
4	175	30
5	174	31

Appendix XII Raw data (in °C) for evaluation of antipyretic activity of DCM extract of *E. globulus*

Treatments Groups	**0hour**	**1hour**	**2hour**	**3hour**	**4hour**	**5hour**
Normal control						
1	37.1	37.3	37.1	37.6	37.2	37.1
2	36.8	36.9	36.9	37	36.8	37
3	37.2	37.2	37.1	37	37	37
4	36.5	37	37.1	37	37.1	37
5	36.7	36.5	36.5	36.6	36.4	36.6
Negative control						
1	37.4	38.4	38.6	38.7	38.9	38.7
2	37.3	38.3	38.5	38.5	38.6	38.5
3	37.1	38.5	38.6	38.8	38.7	38.6
4	36.9	38.5	38.7	38.6	38.7	38.8
5	37.1	38.5	38.6	38.7	38.6	38.6
Positive control						
1	37.3	38.5	37.8	37.5	37.2	36.6
2	37.4	38	37.2	37.2	36.9	36.4
3	37.1	38.4	37.7	37.6	37.3	36.7
4	37.4	38.5	37.7	37.4	37.2	36.6
5	37	38.3	37.6	37.3	37	36.4
25mg/kg bw						
1	37.1	38.6	38.3	38	37.4	37.3
2	37.2	38.6	38.2	37.6	37.5	37.4
3	36.7	38.6	38.2	37.8	37.5	37.2
4	37.1	38.3	37.8	37.7	37.5	37.4
5	37.4	38.4	38	37.9	37.7	37.5
50mg/kg bw						
1	37.4	38.3	37.8	37.6	37.3	36.9
2	37.2	38.3	37.7	37.4	37.2	36.9
3	36.5	38	37.4	37.4	36.9	36.4
4	37.3	38.1	37.6	37.2	37.1	36.8
5	37.3	38.2	37.6	37.3	37.2	36.8
100mg/kg bw						
1	37	38.2	37.7	37.3	36.9	36.7
2	37.4	38.2	37.6	37.2	36.9	36.6
3	36.6	38.5	37.9	37.5	37.2	36.9
4	37.3	38.4	37.7	37.4	37.1	36.8
5	37.4	38	37.5	37	36.7	36.6

150mg/kg bw						
1	37.3	38.6	37.8	37.4	37.2	36.9
2	37.4	38.9	38.4	37.7	37.5	37.2
3	37.3	38.6	37.8	37.4	37.2	36.9
4	36.8	38.3	37.5	37.2	36.8	36.6
5	37.5	38.5	37.8	37.3	37.2	36.68
200mg/kg bw						
1	37.2	39	38	37.7	37.4	37.2
2	37.1	38.6	37.6	37.4	37.1	36.7
3	36.9	38.6	37.6	37.4	37.1	36.7
4	37.4	38.9	38	37.6	37.5	37
5	37.1	38.5	37.7	37.3	37	36.7
250mg/kg bw						
1	36.8	38.7	37.8	37.5	37.3	36.8
2	37.1	38.6	37.8	37.3	37.2	36.6
3	37.2	38.2	37.3	36.9	36.8	36.4
4	37.4	38.4	37.5	37.1	37.1	36.6
5	37.3	38.5	37.6	37.3	37.1	36.7

Appendix XIII Raw data (°C) for evaluation of antipyretic activity of DCM extract of *S. didymobotrya*

Treatments Groups	0hour	1hour	2hour	3hour	4hour	5hour
Normal control						
1	36.8	36.8	36.8	36.9	36.8	36.8
2	36.7	36.6	36.6	36.7	36.8	36.8
3	37.4	37.3	37.2	37.2	37.3	37.2
4	37.7	37.7	37.5	37.4	37.5	37.5
5	37.4	37.1	36.9	37.2	37.3	37.2
Negative control						
1	37.5	38.6	38.9	38.9	38.8	38.9
2	37.2	38.8	39	39	39	39
3	37	38.7	38.9	39	39	39
4	37.5	38.8	38.9	39	39.1	39.1
5	37.2	38.9	39.1	39.2	39.2	39.2
Positive control						
1	37.4	38	37.7	37.1	36.7	36.5
2	38.3	39	38.6	37.9	37.7	37.4
3	37.6	38.2	37.6	37.2	37	36.7
4	37.6	39	38.4	38	37.7	37.2
5	37.5	38.1	37.6	37.2	36.8	36.6
25mg/kg bw						
1	36.8	38.2	38	37.8	37.6	37.2
2	37.9	38.2	38	37.9	37.8	37.2
3	37.2	38	37.9	37.6	37.4	37.2
4	37.5	38	37.8	37.4	37.1	37
5	37.1	38	37.7	37.4	37.2	37.3
50mg/kg bw						
1	37.7	38.6	38.1	37.8	37.6	37.4
2	37.8	38.6	38.3	37.9	37.6	37.4
3	37.8	39.7	39.3	38.8	38.7	38.5
4	37.8	38.1	37.7	37.1	37.2	37
5	37.8	38.6	38.2	37.8	37.6	37.5
100mg/kg bw						
1	37.7	38.3	37.9	37.4	37.3	36.9
2	37.9	38.5	38	37.5	37.5	37.1
3	37.7	38.6	38.2	37.7	37.6	37.2
4	37.6	38.6	38	37.7	37.6	37.2
5	38.6	38.4	38.1	37.4	37.4	37

150mg/kg bw						
1	36.4	37.8	37.6	37	36.8	36.6
2	36.5	37.8	37.3	37	36.8	36.5
3	36.8	37.7	37.2	37	36.7	36.5
4	36.8	37.7	37.4	37	36.7	36.5
5	37.5	38.3	37.9	37.6	37.3	36.9
200mg/kg bw						
1	37.5	38.1	37.7	37.3	36.8	36.7
2	37.7	38.1	37.5	37.2	36.9	36.6
3	37.3	38.1	37.6	37.1	36.9	36.7
4	38.3	39	38.6	38	37.7	37.6
5	37.5	38.5	37.9	37.6	37.3	37
250mg/kg bw						
1	37.4	38.1	37.6	37.1	37	36.7
2	37.7	38	37.4	37.2	36.8	36.5
3	37.7	38	37.5	37.1	36.9	36.5
4	37.4	38.7	38.2	37.9	37.6	37.1
5	37.6	38.8	38.4	38	37.4	37.2

Appendix XIV Raw data (in mm) for evaluation of anti-inflammatory activity of DCM extract of *E. globulus*

Treatments Groups	**0hour**	**1hour**	**2hours**	**3hours**	**4hours**	**5hour**
Normal control						
1	10.5	10.5	10.5	10.5	10.5	10.5
2	10.5	10.5	10.5	10.5	10.5	10.5
3	10.8	10.8	10.8	10.8	10.8	10.8
4	11.5	11.5	11.5	11.5	11.5	11.5
5	10.3	10.3	10.3	10.3	10.3	10.3
Negative control						
1	11.3	12.5	12.8	13.4	13.5	13.6
2	11.8	12.2	12.5	13.1	13.3	13.4
3	12.3	13.4	13.7	14.1	14.5	14.5
4	12.5	13.7	14.3	14.7	14.9	15.1
5	12.2	13	13.5	13.8	13.9	14.1
Positive control						
1	9.8	11.8	11.6	11	10.9	10.6
2	10.4	12.2	12	11.5	11.1	11
3	10.2	12.7	12.5	11.9	11.7	11.4
4	10.6	12.8	12.5	12	11.9	11.6
5	10.8	12.7	12.5	11.8	11.7	11.4
25mg/kg bw						
1	10.2	12.8	13	12.9	12.7	12.5
2	10.4	12.9	13.1	12.9	12.7	12.5
3	10.4	13.1	13	13.1	12.9	12.7
4	9.8	11.9	12.1	12	11.8	11.6
5	9.5	11.6	11.6	11.5	11.4	11.3
50mg/kg bw						
1	9.5	11.5	11.4	11.3	11.1	11.1
2	9.8	11.6	11.5	11.3	11.2	11.2
3	10.5	12.1	12	11.9	11.7	11.6
4	9.8	11.7	11.6	11.5	11.4	11.3
5	9.7	12.1	12	11.9	11.7	11.5
100mg/kg bw						
1	9.5	12.1	12	11.7	11.4	11.3
2	9.8	12.4	12.2	12	11.7	11.7
3	10.2	12.9	12.7	12.3	12	12.1
4	9.5	12.2	12	11.7	11.5	11.5
5	9.6	12.4	12.2	11.9	11.8	11.6

150mg/kg bw						
1	9.8	12.7	12.5	12.1	11.8	11.8
2	9.5	12.8	12.6	12.2	11.9	11.7
3	10	13.2	13	12.6	12.2	11.9
4	9.8	12.6	12.5	12	11.7	11.5
5	9.4	12.6	12.4	12	11.6	11.5
200mg/kg bw						
1	10.2	12.9	12.7	12.2	11.8	11.7
2	10.4	13.1	12.8	12.4	12	11.8
3	10	12.8	12.5	12	11.6	11.4
4	10.2	13	12.7	12.2	11.9	11.8
5	9.2	12.6	12.4	11.8	11.5	11.3
250mg/kg bw						
1	10.5	13.1	12.9	12.3	11.8	11.7
2	9.5	12.6	12.3	11.7	11.5	11.3
3	10.4	13.2	12.9	12.4	11.9	11.7
4	10.6	13.4	13	12.5	12.3	11.9
5	10.7	13.7	13.4	12.8	12.5	12.2

Appendix XV Raw data (in mm) for evaluation of anti-inflammatory activity of DCM extract of *S. didymobotrya*

Treatments Groups	0hr	1hour	2hour	3hour	4hour	5hour
Normal control						
1	10.8	10.8	10.8	10.8	10.8	10.8
2	10.5	10.5	10.5	10.5	10.5	10.5
3	10.2	10.2	10.2	10.2	10.2	10.2
4	11.2	11.2	11.2	11.2	11.2	11.2
5	10.8	10.8	10.8	10.8	10.8	10.8
Negative control						
1	11.2	12.3	12.5	13.2	13.3	13.5
2	11.6	12	12.3	12.9	13.1	13.2
3	12	13.2	13.6	13.9	14.3	14.4
4	12.3	13.5	13.9	14.3	14.7	14.9
5	10.2	12	12.5	12.8	12.9	13
Positive control						
1	10	11.9	11.6	11.2	11.1	11
2	10.2	12.2	11.8	11.5	11.4	11.2
3	10.5	12.9	12.6	12.4	11.9	11.8
4	10.6	12.8	12.5	12	11.9	11.8
5	10.8	12.5	12.1	11.8	11.6	11.5
25mg/kg bw						
1	10	12.9	12.9	12.9	12.7	12.6
2	10.2	12.9	13.1	12.9	12.8	12.6
3	10.8	12.5	12.7	12.6	12.4	12.2
4	10.2	12.1	12.3	12.2	11.9	11.7
5	9.8	11.7	11.9	11.8	11.6	11.4
50mg/kg bw						
1	10	12.1	12.1	12	11.9	11.7
2	10.2	12.1	12.2	12.1	11.9	11.7
3	10.2	12	12.1	11.9	11.7	11.7
4	10.2	12.1	12.1	12	11.8	11.7
5	9.6	11.9	12	11.9	11.7	11.5
100mg/kg bw						
1	9.8	12.5	12.4	12.2	11.9	11.9
2	10	12.3	12.1	12	11.9	11.7
3	9.8	12.8	12.6	12.4	12.3	12.2
4	10	12.8	12.7	12.5	12.3	12.2
5	9.5	12.5	12.4	12.2	12.1	12

150mg/kg bw						
1	10	12.9	12.7	12.4	12.2	12
2	9.8	13	12.8	12.5	12.3	12.1
3	10.2	13.5	13.3	13	12.9	12.7
4	9.6	12.7	12.6	12.3	12	11.9
5	9.5	12.5	12.3	12	11.9	11.8
200mg/kg bw						
1	10	13.2	12.9	12.8	12.5	12.2
2	10.5	13.3	13.1	12.6	12.5	12.3
3	10.2	13.1	12.8	12.5	12.4	12.2
4	10.2	13	12.7	12.5	12.3	12.1
5	9.4	12.8	12.5	12.3	12.1	12
250mg/kg bw						
1	10.8	13.5	13.2	12.9	12.5	12.3
2	9.5	12.6	12.3	11.9	11.4	11.5
3	10	13	12.7	12.3	11.9	11.7
4	10.8	13.7	13.3	12.9	12.5	12.4
5	10.5	13.5	13.2	12.7	12.5	12.4

Appendix XVI National Commission for Science Technology and Innovation Approval letter

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone: +254-20-2213471, 2241349,3310571,2219420
Fax: +254-20-318245,318249
Email: dg@nacosti.go.ke
Website: www.nacosti.go.ke
when replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref. No. NACOSTI/P/16/6765/14525

Date: 9th November, 2016

Kiambi Joseph Mworia
Kenyatta University
P.O. Box 43844-00100
NAIROBI

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Antipyretic, antinociceptive and anti-inflamatory potential of dichloromethane leaf extracts of eucalyptus globules (Labill) and senna didymobotrya (Fresenius) in animal models,"*** I am pleased to inform you that you have been authorized to undertake research in **Nairobi County** for the period ending **8th November, 2017.**

You are advised to report to **the County Commissioner and the County Director of Education, Nairobi County** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

BONIFACE WANYAMA
FOR: DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Nairobi County.

COUNTY COMMISSIONER
NAIROBI COUNTY
P. O. Box 30186-00100, NBI
TEL: 341558

The County Director of Education
Nairobi County.

11 NOV 2016

National Commission for Science, Technology and Innovation is ISO 9001:2008 Certified

THIS IS TO CERTIFY THAT:
MR. KIAMBI JOSEPH MWORIA
of KENYATTA UNIVERSITY, 0-60200 Meru,has been permitted to conduct research in *Nairobi County*

on the topic:
ANTIPYRETIC,ANTINOCICEPTIVE AND ANTI-INFLAMATORY POTENTIAL OF DICHLOROMETHANE LEAF EXTRACTS OF EUCALYPTUS GLOBULES (LABILL) AND SENNA DIDYMOBOTRYA (FRESENIUS) IN ANIMAL MODELS

for the period ending:
8th November,2017

Permit No : NACOSTI/P/16/6765/14525
Date Of Issue : 9th November,2016
Fee Recieved :ksh 2000

.....
Applicant's Signature

.....
Director General
National Commission for Science, Technology & Innovation

Appendix XVII Publications

1. Mworia, J. K., Kibiti, C. M., **Ngugi, M. P.,** & Ngeranwa, J. N. (2019). Antipyretic Potential of Dichloromethane Leaf Extract of *Eucalyptus globulus* (Labill) and *Senna didymobotrya* (Fresenius) in Rats Models. *Heliyon, 5*(12), e02924. doi:10.1016/j.heliyon.2019.e02924
2. Joseph KM, Cromwell MK, Joseph NN, **Mathew PN** (2020). Analgesic potential of dichloromethane leaf extracts of *Eucalyptus globulus* (Labill) and *Senna didymobotrya* (Fresenius) in mice models. *Journal of Herbmed Pharmacology*, 9(4):391-399. doi: 10.34172/jhp.2020.49
3. Mworia JK, Kibiti CM, Ngeranwa JJN and **Ngugi MP** (2021): Anti-inflammatory potential of dichloromethane leaf extracts of *Eucalyptus globulus* (Labill) and *Senna didymobotrya* (Fresenius) in mice. *Africa Health Sciences, 21(1).* DOI: 10.4314/ahs.v21i1.50