EVALUATION OF THE ANTINOCICEPTIVE, ANTI-INFLAMMATORY AND ANTIPYRETIC ACTIVITIES OF *RUTA GRAVEOLENS* L. IN MICE AND RATS

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Pharmaceuticae in the School of Pharmacy, University of the Western Cape

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DECLARATION

I declare that the thesis, Evaluation of the antinociceptive, anti-inflammatory and antipyretic activities of *Ruta graveolens* L. in mice and rats, is my own work, that it has not been submitted before for any degree examination in any other University and that all the sources I have used or quoted have been indicated and acknowledged by complete reference.

Firdows Loonat November 2012

Signed……..

DEDICATION

I dedicate this thesis to my loving parents, Rashard and Yasmin Loonat, for their sacrifices and endless love, care and support that has got me to where I am today. Thank you for believing in me and encouraging me to be the best that I can be.

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KEYWORDS

Ruta graveolens

Antinociceptive activity

Anti-inflammatory activity

Antipyretic activity

Traditional medicine

Phytochemical analysis

Acute toxicity

HPLC analysis

Mice

Rats

ABBREVIATIONS

WHO	World Health Organization
HPLC	High Performance Liquid Chromatography
PC	Paper Chromatography
TLC	Thin Layer Chromatography
GLC	Gas Liquid Chromatography
PMNs	Polymodal nociceptors
COX	cyclo-oxygenase
IL	interleukin
TNF-α	tumor necrosis factor-α
LPS	lipopolysaccharide
E. coli	*Escherichia coli*
i.p.	intraperitoneally
i.m.	intramuscularly

ABSTRACT

Evaluation of the antinociceptive, anti-inflammatory and antipyretic activities of *Ruta graveolens* L. in mice and rats

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Ruta graveolens (Rutaceae) L. is a medicinal plant that is commonly used to manage and treat essential events such as pain, inflammation and fever. Despite its popularity, particularly as a medicinal plant in the Calvinia district and Bredasdorp region of South Africa, scientific data to substantiate its widespread traditional use and the possible mechanisms of action for this plant species is lacking. Therefore, the objectives of this study were: to scientifically evaluate and validate the antinociceptive, anti-inflammatory and antipyretic activities of *Ruta graveolens* using the acetic-acid writhing test and hot-plate test, the carrageenan rat paw oedema test, and the *E. coli*-induced pyrexia test, respectively; to investigate the possible mechanisms of the antinociceptive, anti-inflammatory and antipyretic activities of the plant using interaction studies; to determine some secondary metabolites present in the plant species using standard phytochemical analytical procedures; to characterise the plant species using HPLC techniques; and to determine the safety profile of the plant species using an acute toxicity study.

Three percent (3 %) acetic acid (0.25 ml, i.p.) produced a substantial number of writhes in mice. The leaf methanol extract of *Ruta graveolens* (100 mg/kg, i.p.) significantly reduced the number of writhes induced by 3 % acetic acid (0.25 ml, i.p.). *R. graveolens* (100 mg/kg, i.p.) produced 54 % inhibition of 3 % acetic acid-induced writhes. Indomethacin (20 mg/kg,

i.p.) and paracetamol (500 mg/kg, i.p.) significantly reduced the number of 3 % acetic acid-induced writhes. Indomethacin (20 mg/kg, i.p.) and paracetamol (500 mg/kg, i.p.) produced 57 % and 80 % inhibition of 3 % acetic acid-induced writhes, respectively. *R. graveolens* (25 – 50 mg/kg, i.p. and 200 – 400 mg/kg, i.p.) and indomethacin (10 mg/kg, i.p.) did not significantly reduce the number of writhes induced by 3 % acetic acid. However, combined therapy of the leaf methanol extract of *R. graveolens* (25 mg/kg, i.p.) and indomethacin (10 mg/kg, i.p.) significantly reduced the number of 3 % acetic acid-induced writhes. The combined therapy of the lowest and sub-effective doses of the leaf methanol extract of *R. graveolens* (25 mg/kg, i.p.) and indomethacin (10 mg/kg, i.p.) produced 59 % inhibition of the writhes elicited by 3 % acetic acid. The leaf methanol extract of *R. graveolens* (50 – 400 mg/kg, i.p.) greatly delayed the reaction time in mice to thermal stimulation produced with hot-plate. 50 – 400 mg/kg (i.p.) of the leaf methanol extract of *R. graveolens* significantly antagonised rat paw oedema induced by 1 % carrageenan (0.1 ml, subplantar) over the 4 h period of testing. In addition, indomethacin (10 mg/kg, i.p.) significantly antagonised 1 % carrageenan-induced rat paw oedema. *R. graveolens* (25 mg/kg, i.p.) and indomethacin (2 mg/kg, i.p.) given separately did not significantly alter rat paw oedema induced by 1 % carrageenan. However, combined therapy of the leaf methanol extract of *R. graveolens* (25 mg/kg, i.p.) and indomethacin (2 mg/kg, i.p.) significantly reduced 1 % carrageenan-induced rat paw oedema. The leaf methanol extract of *R. graveolens* (400 mg/kg, i.p.) significantly reduced the mean rectal temperature of normothermic rats. *Ruta graveolens* (100 – 400 mg/kg, i.p.) significantly reduced pyrexia induced by *E. coli* (50 μg/kg, i.m.) over the 5 h period of testing. In addition, pentoxifylline (50 mg/kg, i.p.) significantly reduced *E. coli*-induced pyrexia. *Ruta graveolens* (25 – 50 mg/kg, i.p.), paracetamol (500 mg/kg, i.p.) and pentoxifylline (10 mg/kg, i.p.) did not significantly reduce pyrexia induced by *E. coli*. However, combined therapy of the leaf methanol extract of *R. graveolens* (25 mg/kg, i.p.)

and pentoxifylline (10 mg/kg, i.p.) significantly reduced *E. coli* (50 µg/kg, i.m.)-induced pyrexia.

The phytochemical studies of the powdered leaves of *Ruta graveolens* indicated the presence of alkaloids, cardiac glycosides, flavonoids, saponins, tannins and triterpene steroids. The HPLC fingerprint indicated characteristic peaks at the following retention times; 1.654 min, 2.271 min, 2.403 min, 4.705 min and 7.691 min. The LD_{50} obtained for *Ruta graveolens* after oral administration was probably greater than 4000 mg/kg which shows that the plant extract is non-toxic to mice.

In conclusion, the data obtained indicate that *Ruta graveolens* possesses antinociceptive, anti-inflammatory and antipyretic activities. Since prostaglandins have been shown to mediate acetic acid-induced writhes, prostaglandins, histamine, serotonin, capsaicin and bradykinin implicated in carrageenan-induced rat paw oedema, and tumor necrosis factor-α (TNF-α) implicated in *E.coli*-induced pyrexia, it is possible that *R. graveolens* may be producing its antinociceptive, anti-inflammatory and antipyretic activities by affecting these chemical mediators. The data obtained also justify the use of the plant species by traditional medicine practitioners for the treatment of painful and inflammatory conditions, and pyrexia.

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CHAPTER 1

INTRODUCTION

For centuries traditional medicine was the only system available for health care in the diagnosis, prevention and treatment of illnesses (WHO, 2003). Increasing in popularity and acceptance in African communities, traditional medicine continues to grow (Cohen, 2003; WHO, 2003). The World Health Organisation (WHO) defines traditional medicine as the total combination of knowledge and practices, whether explicable or not, used in diagnosing, preventing or eliminating physical, mental or social diseases and which may rely exclusively on past experience and observation handed down from generation to generation, verbally or in writing (WHO, 2003).

Traditional medicine is a comprehensive term that broadly encompasses traditional medicine systems such as Chinese medicine, Indian ayurveda and Arabic unani medicine, and various forms of indigenous medicine. Traditional medicine varies from country to country and region to region (WHO, 2012; Sofowora, 1982). African traditional medicine systems are mainly passed on from generation to generation via word of mouth. However, valuable traditional knowledge is lost or has been lost as a result of this practice (Light et al., 2005; Williamson et al., 1996). Literature indicates though that over the last three hundred years, references to indigenous materia medica were found in early European and other travellers' accounts in southern Africa (Light et al., 2005; van Wyk et al., 1997). The most important of these documentations is that of Watt and Breyer-Brandwijk (1962) first published in 1932. This indigenous botanical medicine manuscript provides comprehensive data on medicinal plants of the southern and eastern African regions, and comprises of valuable reference material (Light et al., 2005).

Rich in plant diversity, Southern Africa accounts for almost 10 % of the world's higher plants. Out of the 30 000 species of higher plants, 3000 of these have been used across the country as traditional medicine (Thring et al., 2005; van Wyk et al., 1997). Despite the use of orthodox medicine in the treatment and management of diseases, an estimated 70% of the South African population in both rural and urban areas rely on the use of plants for medicinal purposes (Light et al., 2005; Tabuti et al., 2003; Amabeoku et al., 1998). One of such plants is *Ruta graveolens* L.

R. graveolens has been used to treat many conditions ranging from central nervous system disorders to autoimmune diseases (van Wyk et al., 1997). *R. graveolens* is one of many plants used in the treatment of pain, inflammation and fever due to underlying conditions such as toothache and earache, autoimmune diseases and infections (Raghav et al., 2006; van Wyk et al., 1997). The use of the plant species, among other medicinal plants, as an effective and potent medicine requires evaluation by standard scientific methods (Williamson et al., 1996).

Therefore, *R. graveolens* was selected for comprehensive research to determine its phytochemical components, evaluate pharmacological activities, obtain an HPLC fingerprint, and perform toxicological studies to determine the safety profile of the plant species. Growth in scientific documentation is required to preserve knowledge of indigenous plant use for generations to come (van Wyk et al., 1997).

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

2.1.1. Overview on Traditional Medicine

During the years of early human existence, many plants were used to manage and treat diseases either by instinct, intuition, or trial and error. Humans sought to manage and treat disease with assistance, inspiration and guidance from their natural surroundings. Ancient cultures and communities developed their own materia medica as ideas of different tribes permeated each other, occasionally bringing together religion, rituals and magic (van Wyk et al., 1997). One of the earliest records of the use of herbal medicine is that of the *Hydnocarpus Gaertn* species. The Chaulmoogra oil derived from the plant species was effective in the treatment of leprosy, and this was recorded between 2730 and 3000 BC. Similarly, the castor oil seed (*Ricinus communis* L.) and the seed of opium poppy (*Papaver somniferum* L.) were unearthed from ancient Egyptian tombs, thereby indicating their use in Africa as far back as 1500 BC. In addition, records found in Ebers papyrus also confirm the use of medicinal plants in Egypt at the time (Sofowora, 1982).

The use of herbal medicine continued to grow for centuries, with many physicians recording and describing their herbal practices. However, it was not until 1785 that the leaves of foxglove (*Digitalis purpurea* L.) were found to be the active ingredient in a potion that was used for centuries to treat Dropsy – a condition of the heart that results in fluid retention and general swelling of the body. Since then the plant was thoroughly investigated, isolating the two active constituents digoxin and digitoxin, both official drugs in pharmacopoeia. These

are merely two of the many drugs used in the present day that have been derived from traditional plants (Sofowora, 1982).

The system of traditional medicine is based on past experience and cultural beliefs and practices that have been handed down from generation to generation, verbally or in writing (WHO, 2012). Traditional medicine practitioners are people recognised by their communities as competent health care providers that use vegetable, animal, mineral substances and other methods taking inspiration from social, cultural, and religious backgrounds (WHO, 2012; Jäger et al., 2004; van Wyk et al., 2000; Sofowora, 1982). Traditional medicine practitioners are commonly known by the Zulu people as *isangomas* and *inyangas* (Perrott et al., 2003).

Traditional medicine practitioners play an essential role in the health of millions of people. The exact number of these practitioners is difficult to assess due to them being unregulated, however, it is estimated that 250 000 practitioners provide health care to the South African population (Kayne, 2010; Perrott et al., 2003). In addition, the work force represented by traditional medicine practitioners is an essential resource for health-care coverage (Springfield et al., 2005). The socio-economic status of developing countries, the magnitude of health problems, and the few resources available make traditional medicine an essential matter to be viewed. Hence, there are many advantages to using traditional medicine: (1) it is cheaper than orthodox medicine, (2) it is more accessible to most of the population, (3) it is also more acceptable. Traditional medicine is also a potential source of new drugs or a cheap source of known drugs such as *Montanoa tomentosa* Cerv. used as a contraceptive. Also, obstacles such as time-consuming processes like compounding substances in a laboratory, are avoided in traditional medicine (Sofowora, 1982).

Despite these advantages, traditional medicine still faces many shortcomings, some of which include: (1) imprecise diagnosis often given by the practitioners, (2) lack of precise dosages of medicaments used, (3) criticism that traditional medicine practitioners are not hygienic in their practices, and witchcraft, and (4) evil aspects of traditional medicine can also be criticised. However, the greatest argument against traditional medicine is the lack of scientific proof of its efficacy (Springfield et al., 2005; Sofowora, 1982).

In developing countries more than one third of the population lacks access to essential medicines (Kayne, 2010). In Africa, 80% of the population rely on traditional medicine to meet their primary health care needs (Kayne, 2010; Springfield et al., 2005). Presently, Africa comprises of approximately 52 states of which 46 of these states maintain vegetation ranging from lowland rain forest to semi-desert scrubland (Kayne, 2010). However, only a few African indigenous remedies have found their way into western pharmacopoeias. A pharmaceutical monograph for the African plant species belonging to the Rutaceae family appears in current and earlier editions of the British Pharmacopoeia, British Pharmaceutical Codex and European Pharmacopoeia (Kayne, 2010). Despite this, safety and efficacy data available for plants used in South Africa are few (Springfield et al., 2005).

In order to enhance the safety and efficacy of plant medicines, there is the need for characterisation and standardisation of the plant materials. This can be achieved through phytochemical qualitative analysis, HPLC fingerprint and toxicity studies (Springfield et al., 2005).

2.2. Phytochemical analysis

Phytochemical tests aid in the screening of plants by employing methods for isolating and identifying constituents present in a plant species. In addition, the characterisation of an active constituent responsible for toxic or beneficial effects can be useful when using the plant extract in living systems. Methods used aid in identifying alkaloids, quinines, terpenoids, and so forth known as secondary metabolites that may contribute immensely to the effectiveness of the plant materials in therapy (Harborne, 1984).

2.3. High performance liquid chromatography

Chromatography may be defined as the separation of solutes in a mixture. These solutes are separated according to their affinity to the stationary phase, through which the mobile phase moves. Separation may occur through adsorption, partition, molecular diffusion or electrophoresis, or a combination of these (Williamson et al., 1996; Harborne, 1984). Chromatographic techniques include paper chromatography (PC), thin layer chromatography (TLC), gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC).

High accuracy and precision, ease of use, easy sample preparation, rapid analysis and high sensitivity make HPLC the fastest growing analytical technique. The solubility and volatility properties of compounds to be separated should be considered when choosing an appropriate chromatographic technique for the sample (Harborne, 1984). The HPLC technique may be used for less volatile constituents that need to be separated, example terpenoids, alkaloids and all types of phenolics (Harborne, 1984). In addition, HPLC with UV-detector is most useful when the solutes have pronounced ultraviolet absorption (Williamson et al., 1996). The successful separation of compounds is indicated by characteristic peaks at specific retention times. A chromatographic fingerprint presents the chemical characteristics of herbal

medicines. Samples with similar chromatographic fingerprints have similar properties (Fan et al., 2006). Chemical fingerprints allow phytochemical constituents of the plant species to be identified. (Bombardelli, 2001).

2.4. Toxicity studies

Therapeutic and toxic effects are determined by measuring the safety margin of compounds (Rang et al., 2012). Known as the therapeutic index (TI) or therapeutic ratio, it is a comparison dosage range between the dose producing the desired effect and the dose producing a lethal one (Rang et al., 2012; Loomis and Hayes, 1996). The TI is obtained experimentally from two dose-response curves using mice or rats. The maximum no-observed-adverse-effect-level (NOAEL) is established using the dose-response curve. This provides a level of assurance to the researcher and regulatory authority on the safety of the test compound (Poole and Leslie, 1989). In addition, the LD_{50} can be obtained. The LD_{50} is the dose at which mortality of 50 % of the test population occurs.

2.5. Analgesia

2.5.1. Pain and Nociception

Pain is considered to be an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage, or described in terms of such damage. Pain is often classified as acute or chronic. The nature of pain also varies and may be described as pricking, stabbing, burning, throbbing, aching, and so forth. Pain is associated with impulse activity in the primary afferent fibres of peripheral nerves. These nerves have sensory endings in peripheral tissues that are activated by a variety of stimuli such as mechanical, thermal, and chemical (Rang et al., 2012).

Pain and nociception differ in that pain is a subjective experience that includes a strong emotional (affective) component, whereas nociception is the perception of noxious stimuli. It is the mechanism whereby noxious peripheral stimuli are transmitted to the central nervous system. There are many factors that contribute to the amount of pain that a particular stimulus produces (Rang et al., 2012; Webster and Jordan, 1989). A stabbing sensation in the chest will cause much more pain if it spontaneously occurs in an individual than if it occurs as a result of poking an individual in the ribs with a stick. The affective component differs to that of the nociceptive component (Rang et al., 2012). It is recognised clinically that many analgesics, particularly those of the morphine type, can reduce the distress associated with pain even though the patient reports no change in the intensity of the sensation.

2.5.2. Pain Transmissions

Pain is associated with impulse activity via small-diameter myelinated Aδ fibres and unmyelinated C-fibres and of peripheral nerves. Myelinated Aδ fibres convey sharp sensations of pain, whereas unmyelinated C-fibres convey a dull, diffuse, burning pain. These C-fibres are associated with polymodal nociceptive endings. Nociceptive information is conveyed via Aδ and C and fibres from muscle, viscera, and skin (Rang et al., 2012). Receptors found in the skin and subcutaneous tissue respond to stimuli such as touch, pressure, heat, cold, and pain. Nociceptors are sensory receptor neurons sensitive to noxious stimuli or tissue-damaging stimuli (Kierszenbaum, 2007). There are three categories of nociceptors namely; thermal, mechanical, and polymodal. Thermal receptors respond to temperatures less than 5ºC and higher than 45ºC. Mechanical nociceptors respond to tissue-damaging mechanical stimuli, example a needle or tissue injury that is an immediate cause of pain (Kierszenbaum, 2007; Martin, 2012). Polymodal nociceptors respond to noxious thermal or mechanical stimuli (Kierszenbaum, 2007).

Tissue injury is the immediate cause of pain. Tissue injury results in the local release of chemicals such as bradykinin and kallidin that act on nerve terminals, either activating them or enhancing their sensitivity to other forms of stimulation. Prostaglandins are responsible for enhancing the pain-producing effect of an agent like bradykinin (Rang et al., 2012). Pain is transmitted from free nerve endings via a transduction process, synapsing in the spinal cord via myelinated Aδ fibres and the unmyelinated C-fibres. These free bared nerve endings, innervate various tissues as a result of their branched tree-like structures (Kierszenbaum, 2007).

Polymodal nociceptors (PMNs) are the main category of peripheral sensory neuron that responds to noxious stimuli. Most PMNs are unmyelinated C-fibres with nerve endings that respond to thermal, mechanical and chemical stimuli (Rang et al., 2012). In the dorsal root ganglia lie the cell bodies of spinal nociceptive afferent fibres. These fibres enter the spinal cord via the dorsal roots, ending in the grey matter of the dorsal horn. Most of these nociceptive afferent fibres terminate in the superficial region of the dorsal horn, resulting in the formation of synaptic connections with transmission neurons running to the thalamus (Rang et al., 2012; Williamson et al., 1996). Glutamate, a fast transmitter and various peptides such as substance P are released by PMN neurons, acting as slow transmitters. Peptides are released peripherally and contribute to neurogenic inflammation (Rang et al., 2012).

Sensitisation of PMNs to prostaglandins, elicits the mechanism of action commonly used by pain killers and non-steroidal inflammatory drugs (Rang et al., 2012). These drugs act by blocking the action of cyclo-oxygenase which converts arachidonic acid to prostaglandins,

thereby preventing the sensitisation of receptors to alogenic substances (Webster and Jordan, 1989).

Various animal models for studying nociceptive activity exist (Williamson et al., 1996). These include: (1) Acetic acid writhing test used to study the peripheral effects of drugs acting against nociception; (2) Hot plate test and tail-flick test used to study the central antinociceptive effects of drugs.

2.5.3. Pain management

Standard analgesic drugs used in the management and treatment of pain fall into three categories namely; opioids, non-opioid analgesics, and non-steroidal anti-inflammatory drugs (Portenoy, 2000; SAMF, 2012).

2.5.3.1. Opioid analgesics

The term ‘opioid’ refers to any substance, whether endogenous or synthetic, that produces a morphine-like effect. Opioid analgesics may be classified as low-efficacy opioids, high-efficacy opioids and intermediate-efficacy opioids, and are used for the relief of mild to moderate pain or severe chronic pain (Rang et al., 2012; SAMF, 2012). Examples of low-efficacy opioids include codeine used to relieve mild to moderate pain, and dextropropoxyphene which has been withdrawn from the market as a result of increased risk to adverse cardiovascular events. Examples of high-efficacy opioids include morphine, a valuable opioid analgesic used to relieve severe painful conditions and methadone which has a long duration of action and may be used in the management of a non-productive cough in lung cancer (SAMF, 2012).

Opioids act as agonists by inhibiting the transmission of nociceptive impulses through the dorsal horn, subsequently suppressing nociceptive spinal reflexes. This occurs at specific opiate receptor binding sites in the central nervous system and other tissues (McCarver, 2001). Opioid receptors are widely distributed in the limbic system, thalamus, striatum, hypothalamus, midbrain and the spinal cord (Rang et al., 2012; McCarver, 2001).

2.5.3.2. Non-opioid analgesics

Acetaminophen, classified as a non-opioid analgesic and also known as paracetamol, is one of the most commonly used analgesic-antipyretic agents with weak anti-inflammatory effects (Rang et al., 2012). Paracetamol is used for the relief of mild to moderate pain, or pyrexia. It is safe to use in pregnancy, and only interacts with oral anticoagulants such as warfarin (SAMF, 2012). According to Graham and Scott (2003) and Rang et al., (2012), Paracetamol is a weak inhibitor of cyclo-oxygenase (COX)-1 and COX-2 (Rang et al., 2012; Graham and Scott, 2003). Paracetamol is also a potent inhibitor of prostaglandin synthesis in intact cells (Graham and Scott, 2003). This is in the presence of low arachidonic acid concentrations. Despite the mechanism of action of paracetamol not being completely established, it is known to inhibit prostaglandin synthesis in the brain, but not peripherally, thereby explaining the lack of anti-inflammatory activity (SAMF, 2012). In addition, Snipes et al., (2005) proposed that an alternative COX isoform in dog brain, COX-3 and renamed COX-1b, exists. Furthermore, Ayoub et al., (2006) hypothesised that COX-1b was involved in the antinociceptive activity of paracetamol in mice.

2.5.3.3. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are characteristic to aspirin with regard to analgesic, anti-inflammatory and antipyretic activities (SAMF, 2012). NSAIDs may be used for the symptomatic relief of mild to moderate pain and inflammation. Some examples of NSAIDs include aspirin, ibuprofen, and indomethacin which can be used in rheumatological disorders (Rang et al., 2012; SAMF, 2012). NSAIDs inhibit cyclo-oxygenase, the enzyme responsible for prostaglandin synthesis. By competing for the active sites of cyclo-oxygenases with arachidonic acid, NSAIDs inhibit the action of COX-1 and COX-2 (Katz, 2002; Lee et al., 2005; Pinardi et al., 2005; SAMF, 2012). The analgesic activity of NSAIDs allow for the prevention of nociceptive nerve ending sensitisation to allogenic substances, thus producing analgesic activity (Webster and Jordan, 1989; Rang et al., 2012).

2.6. Inflammation

2.6.1. Introduction

Inflammation occurs due to a characteristic series of changes that follow a damaged area after injury. The reaction may last for hours or even days (Hurley, 1972). There are five cardinal signs of inflammation that manifest themselves as Rubor (redness), Tumour (swelling), Calor (heat), Dolor (pain), and function laesa (loss of function). Appearances of inflammation that occur in living transparent tissues (injured) include changes in vascular calibre and flow, increased vascular permeability leading to the formation of protein-rich exudates and local oedema, and escape of leucocytes from circulating blood into the extravascular tissues (Hurley, 1972). The inflammatory response occurs in tissues subsequent to exposure to a pathogen or other noxious substance. Responses are usually protective. Mediators generated by cells or plasma modify and regulate the magnitude of the response (Rang et al., 2012; Kumar and Clark, 2009).

2.6.2. Inflammatory Process/Mediators

The inflammatory process is initiated by the release of chemical mediators into extracellular fluid (Hoffman, 2003). Mediators are molecules that promote the enhancement of blood flow, increase vessel permeability, and induce emigration of inflammatory cells from blood into extravascular tissues. Once molecules have reached the site of injury, the cells are stimulated to phagocytise bacteria and debris. An inflammatory mediator is a chemical messenger that acts on blood vessels and/or cells to contribute to an inflammatory response, and can be classified as either an exogenous or an endogenous mediator. Mediators include histamine, serotonin, bradykinin, prostaglandin E2, leukotriene B4, platelet-activating factor, interleukin-1 (IL-1), and so forth (Larsen et al., 1983).

There are various animal models for inflammation which can be used to study the effect of drugs. These include Rat hind paw oedema test, Inflammation of Rodent Ear and Adjuvant Arthritis in rat and mouse (Williamson et al., 1996).

2.6.3. Anti-inflammatory Drugs

All NSAIDs are characteristic of the prototype aspirin, having analgesic, anti-inflammatory and antipyretic activities. They may be used in the treatment of dental and bone pain, dysmenorrhoea and headaches (SAMF, 2012). NSAIDs include aspirin, ibuprofen, diclofenac, indomethacin and so on. Indomethacin may be used for the relief of pain and inflammation in rheumatological conditions, especially in patients that do not respond well to alternatives available (SAMF, 2012). NSAID's have the ability to inhibit cyclo-oxygenase (COX), the enzyme responsible for prostaglandin synthesis (SAMF, 2005). NSAIDs reduce components of the inflammatory and immune response in which prostaglandins play a significant role. This includes vasodilatation and oedema. COX inhibitors suppress

prostanoid synthesis in inflammatory cells through inhibition of COX-2 isoform of the arachidonic acid. COX-2 inhibitors include celecoxib and etoricoxib (Rang et al., 2012). Celecoxib may be used in the symptomatic relief of pain and inflammation in rheumatoid arthritis (SAMF, 2012). Furthermore, anti-inflammatory drugs include disease-modifying antirheumatic drugs such as auranofin, penicillamine and chloroquine. Auranofin inhibits the induction of IL-1 and tumour necrosis factor alpha (TNF-α), while the precise mechanism of action of penicillamine is still a matter of speculation. However, penicillamine is thought to modify rheumatic disease by decreasing the immune response, IL-1 generation, and preventing the maturation of newly synthesised collagen by affecting the synthesis of collagen (Rang et al., 2012).

2.7. Pyrexia

2.7.1. Introduction

Pyrexia, also known as fever, is a physiological mechanism that is beneficially effective in fighting infections. This is the body's way of retarding the growth and reproduction of bacteria and viruses, enhancing neutrophil production and T-lymphocyte proliferation, and aiding in the acute-phase reaction (Sullivan et al., 2011). Normal body temperature is regulated by the thermoregulatory centre in the anterior hypothalamus by controlling the balance between heat loss and heat production (Rang et al., 2012; Kumar and Clark, 2009). A disturbance in the hypothalamic 'thermostat' results in a fever, which leads to a rise in normal body temperature (Rang et al., 2012). Gram-negative bacteria may cause this rise in temperature. Toll-like receptors generate signals that lead to the formation of inflammatory cytokines IL-1, -6, -12, TNF-α and many others. These cytokines act on the thermoregulatory centre, thereby increasing prostaglandin-E_2 synthesis (Kumar and Clark, 2009).

In animal models, pyrexia can be induced using Breuer's yeast and lipopolysaccharides, bacterial endotoxins. Prostaglandins have been implicated in Breuer's yeast-induced pyrexia while mediators such as TNF-α have been implicated in lipopolysaccharide-induced pyrexia (Santos and Rao, 1998; Williamson et al., 1996).

2.7.2. Antipyretic drugs

NSAIDs and the non-opioid analgesic, paracetamol, may be used in the treatment of pyrexia due to a mediator such as the prostaglandins. NSAIDs exercise their antipyretic action by inhibiting prostaglandin production in the hypothalamus. This inhibits the E-type prostaglandins from elevating the body's temperature. COX inhibitors control pyrexia by inhibiting IL-1 from releasing prostaglandins in the central nervous system, thereby preventing an elevation in body temperature (Rang et al., 2012).

Pain, inflammation and fever have been well managed and treated with the numerous effective orthodox medicines available. Despite this, plant medicines have also been used by traditional medicine practitioners especially in rural communities and also in urban and peri-urban areas of South Africa to manage and treat these conditions. One such plant medicine widely used to treat pain, inflammation and fever is *Ruta graveolens* L.

2.8. Description of the plant

2.8.1. Introduction

Ruta graveolens L, also known as Rue or the Herb of Grace in English, belongs to the Rutaceae family. It is native to Europe, but commonly grown in South Africa. It is commonly known in Afrikaans as "wynruit or binnewortel." Ruta is a woody, evergreen shrub of up to a metre in height with a characteristic aromatic scent. The plant has yellow flowers that are

made up of 4 petals each. The leaves are irregularly divided into hairless leaflets that have translucent glands (van Wyk et al., 1997). These leaves can be used fresh or dried for medicinal purposes.

Parts of the plant that are used include the leaves and twigs. Leaf infusions are taken for fever, epilepsy and hysteria. Alcoholic tinctures have been used for respiratory problems and heart diseases. Bruised leaves are used for toothache and earache, while decoctions have been used to ease childbirth (van Wyk et al., 1997; Watt and Breyer-Brandwijk, 1962).

Several plants of the Rutaceae family are used in traditional medicine worldwide for several therapeutic purposes (Raghav et al., 2006). In Italian traditional medicine Rutaceae is among the most used plant species, showing abundant use in ancient systematic record of medical practice, the *Corpus Hippocraticum* (Polio et al., 2008). A study conducted in Jordan on the effects of some medicinal plant extracts found that *R. graveolens* was one of the plant species used in Jordanian traditional medicine, showing anti-inflammatory activities (Atta et al., 1998). Rue is a remedy for deep aching pain, rheumatism, headaches, dizziness, and fever (Cartaxo et al., 2010; Raghav et al., 2006). A study conducted in the Southern Overberg Bredasdorp region of South Africa, found that *R. graveolens* was one of the most commonly used plants by community members to treat ailments such as headaches, arthritis, fever, convulsions, and so forth (Thring et al., 2006).

2.9. Objective of the study

Even though various reports and studies have shown that *R. graveolens* has been widely used by traditional medicine practitioners and homeopaths to effectively treat headaches and other painful conditions, arthritis, fever among other ailments (Cartaxo et al., 2010; Thring et al., 2006; Raghav et al., 2006; Atta et al., 1998; van Wyk et al., 1997, Watt and Breyer-

Brandwijk, 1962), no literature exists on any study on the possible mechanisms of action of the plant species in painful conditions, inflammation and fever. The study intended, therefore, to:

1. Verify the antinociceptive, anti-inflammatory and the antipyretic activities of *R. graveolens* by investigating the effects of the plant species against nociception induced by acetic acid and thermal method, inflammation induced by carrageenan and pyrexia induced by lipopolysaccharide, a bacterial endotoxin, respectively.
2. Investigate the possible mechanisms of the antinociceptive, anti-inflammatory and antipyretic activities using interaction studies.
3. Carry out phytochemical qualitative analysis to determine some of the secondary metabolites present in the plant species.
4. Carry out an HPLC study in order to characterise the plant species.
5. Carry out an acute toxicity study in an attempt to establish the safety profile of *R. graveolens*.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant materials

3.1.1. Selection, collection and identification of plant material

R. graveolens L. was selected based on its therapeutic claims by traditional medicine practitioners for its useful antinociceptive, anti-inflammatory and antipyretic activities. Freshly picked leaves and twigs of the plant species were bought from Herb Organic Gardens, Robertson, Western Cape, Republic of South Africa. The plant species was authenticated by a taxonomist in the Department of Biodiversity and Conservative Biology at the University of the Western Cape. A voucher specimen of *R. graveolens* (UWC 8968) was deposited in the Herbarium of the University.

3.1.2. Preparation of leaf methanol extract of *R. graveolens*

The fresh leaves and twigs of the plant species were weighed (2.5885 kg) and then dried in the oven at 35°C to 40°C for 2 days. The dried leaves and twigs were then weighed (1.0163 kg) and ground to a fine powder using a laboratory miller. The fine powder obtained weighed 968.2 g. A weighed quantity 50.0 g of the powder was extracted in a soxhlet extractor using methanol 500 ml as a solvent for 24 h. The methanol filtrate was evaporated to dryness using a Buchi RE II rotavapor and Buchi 461 water bath. The resultant semi-solid substrate was then frozen at - 80°C and freeze-dried (LSL Secfroid SR, Model 3021, Switzerland) for 5 days. A yield of 9.4 g of dried leaf methanol extract was obtained and stored in a desiccator until further use. Fresh solution of the crude methanol extract was prepared on each day of the experiment by dissolving a given quantity of the methanol extract in a small volume of dimethylsulfoxide (DMSO) and made up to the appropriate volume with physiological saline.

The plant extract solution was administered in a volume of 1 ml/ 100g of the body weight of animals.

3.2. Phytochemical analysis of *R. graveolens*

The dried powdered leaf of the plant species was analysed for various chemical compounds using standard protocols of Ikhiri et al. (1992) and Harborne (1984).

3.2.1. Alkaloids

Powdered *R. graveolens* (0.5 g) was boiled with 10 ml of dilute hydrochloric acid (alcoholic) in a test tube for 5 min. The mixture was cooled and the debris was allowed to settle. The supernatant liquid was filtered and 1 ml of the filtrate was taken into another test tube to which 3 drops of Dragendorff's reagent (potassium bismuth iodide solution) was added, shaken and observed for the appearance of an orange-red spot and a precipitate formation.

3.2.2. Cardiac glycosides

Powdered *R. graveolens* (0.5 g) was boiled in 5 ml of 70 % ethyl alcohol for 2 min. The mixture was filtered and 10 ml of water and 5 ml of chloroform were added to the filtrate. It was then shaken. The lower chloroform layer was separated off and evaporated to dryness in a water bath. The cooled chloroform residue was dissolved in 3 ml of glacial acetic acid containing 0.1 ml of ferric chloride. The solution was carefully transferred to the surface of 2 ml of sulphuric acid and observed for a reddish-brown layer that was formed at the interface and whether the upper layer gradually acquired a bluish-green colour.

3.2.3. Flavonoids

Powdered *R. graveolens* (10 g) was boiled for 2 - 3 min in 100 ml of water in a water bath. To 3 ml of the filtrate, 3ml of acetic acid-alcohol (Ethanol:Water:Concentrated hydrochloric

acid in a ratio of 1:1:1), solid magnesium (1 cm) and 1 ml of t-amyl-alcohol were added. The mixture was then observed for a rose-orange or violet colour change.

3.2.4. Quinones

Powdered *R. graveolens* (0.1 g) was shaken in 10 ml of 15 % ferric chloride solution and 5 ml of hydrochloric acid, and immersed in a water bath for 10 min. The mixture was filtered immediately. The filtrate was cooled and extracted with 10 ml of carbon tetrachloride. The carbon tetrachloride layer was separated, washed with 5 ml of water and shaken with 5 ml of dilute ammonia solution. The resultant mixture was observed for a rose pink to cherry red colour in the ammoniacal layer.

3.2.5. Saponins

Powdered *R. graveolens* (0.2 g) was shaken with water and the mixture was observed for a persistent froth.

3.2.6. Tannins

Powdered *R. graveolens* (0.2 g) was boiled in 5 ml of water. The mixture was cooled and filtered. To the filtrate, 3 drops of 5 % ferric chloride solution were added to the filtrate and observed for a blue-black precipitate formation.

3.2.7. Triterpine steroids

Powdered *R. graveolens* (1 g) was extracted for 24 hrs in ether. The filtrate (1 ml) was evaporated to dryness and the residue re-dissolved in several drops of acetic anhydride and then several drops of sulphuric acid were added to solution. The mixture was then observed for a green colour change.

3.3. Experimental animals

Male and female albino mice and rats bred in the Animal House of the Discipline of Pharmacology, University of the Western Cape, Bellville, South Africa were used in this study. Mice weighing between 18 – 30 g, and rats between 180 – 210 g were used. Mice were used in groups of eight and rats in groups of six for the experiments. All the animals had access to food and water *ad libitum*. Prior to commencement of the experiments, the animals were fasted for 16 h but still had access to water. The laboratory in which experiments were performed was maintained at an ambient temperature of 22 ± 2°C. Alternate 12 h light and 12 h dark cycle was also maintained.

3.4. Drugs and chemicals

Acetic acid (Merck) was dissolved in physiological saline to an appropriate volume. Indomethacin (Sigma Chemical Co.) was dissolved in a minimum amount of dimethylsulfoxide (DMSO, Sigma Chemical Co.) and made up to an appropriate volume with physiological saline. Carrageenan (Sigma Chemical Co.) was dissolved in physiological saline to an appropriate volume. Paracetamol (4-acetamidophenol, Sigma Chemical Co.) was dissolved in a minimum volume of propylene glycol and made up to an appropriate volume with physiological saline. *Escherichia coli* (*E. coli*, Sigma Chemical Co.) and pentoxifylline (Sigma Chemical Co.) were dissolved in physiological saline to appropriate volumes. DMSO solution was prepared by dissolving the same volume used to dilute the plant material, in physiological saline to an appropriate volume.

Acetic acid, indomethacin, paracetamol and DMSO were administered intraperitoneally (i.p.) to all mice. Acetic acid was administered in a volume of 0.25 ml to mice. Indomethacin, paracetamol, pentoxifylline and DMSO were administered intraperitoneally to all the rats.

Carrageenan was injected into the subplantar surface of the right hind paws of the rats. *E. coli*, lipopolysaccharide, was administered intramuscularly (i.m.) into the thighs of rats. Fresh drug solutions were prepared each morning of the experiment. All drugs were administered in a volume of 1 ml/100 g of the body weight of animals. Control animals received equal volume injections of the appropriate vehicle. The doses and pre-treatment times of the plant extract and standard drugs were obtained from preliminary studies conducted in our laboratory. The pre-treatment times prior to the administration of 3 % acetic acid were 15 min (plant extract), 15 min (paracetamol) and 30 min (indomethacin). The pre-treatment times prior to the administration of carrageenan were 15 min (plant extract) and 30 min (indomethacin). The pre-treatment times prior to the administration of *E. coli* were 15 min (plant extract), 15 min (paracetamol) and 15 min (pentoxifylline). The pre-treatment time prior to the administration of either acetic acid, carrageenan or *E. coli* was 15 min (DMSO).

3.5. Pharmacological screening

3.5.1. Assessment of the Antinociceptive activity of *R. graveolens*

3.5.1.1. Acetic-acid writhing test

The methods of Garcia et al. (2004); Williamson et al. (1996) and Koster et al. (1959) were used to assess the antinociceptive activity of *R. graveolens*. Mice were used in groups of eight per dose of plant extract, standard drugs, physiological saline or DMSO. The animals were kept individually in transparent Perspex mouse cages before the commencement of the experiment. Control mice were pre-treated with physiological saline and after 15 min each mouse was injected intraperitoneally with 0.25 ml of 3 % acetic acid. 5 min after the administration of acetic acid, the animals were observed and writhes were counted for 30 min. The experiment was repeated using other groups of animals which were pre-treated for 15 min with graded doses of plant extract, paracetamol or indomethacin prior to injecting them with 0.25 ml of 3 % acetic acid. All the experiments were performed in the pharmacology laboratory between 09h00 and 17h00. The ability of the plant extract to significantly reduce the number of acetic acid-induced writhes was taken as antinociceptive activity.

3.5.1.2. Hot-plate test

The methods of Williamson et al. (1996) and Eddy and Leimback (1953) were used. Mice were used in groups of eight per dose of plant extract, physiological saline or DMSO. The animals were kept individually in transparent Perspex mouse cages before the commencement of the experiment. Control mice were pre-treated with physiological saline and after 15 min each mouse was placed in an analgysiometer (TIIC, USA) maintained at 55°C. The pain threshold is considered to be reached when the animals lift and lick their hind paws or attempt to jump out of the beaker. The time taken for animals to exhibit these

characteristics was noted by means of the 'STOP' button on the analgysiometer. The animals were tested before and then 15 min, 30 min, 45 min and 60 min after intraperitoneal administration of physiological saline. The experiment was repeated using other groups of animals which were tested before and then 15 min, 30 min, 45 min and 60 min after the administration of graded doses of plant extract. All the experiments were performed in the pharmacology laboratory between 09h00 and 17h00. The ability of the plant extract to delay the reaction time was taken as antinociceptive activity.

3.5.2. Assessment of the Anti-inflammatory activity of *R. graveolens*

3.5.2.1. Rat paw oedema test

The modified method of Williamson et al. (1996) was used to assess the anti-inflammatory activity of *R. graveolens*. Rats were used in groups of 6 per dose of plant extract, standard drug, physiological saline, or DMSO. The animals were kept individually in transparent Perspex rat cages before the commencement of the experiment. Control animals were pre-treated for 15 min with 0.25 ml (i.p.) of physiological saline. Acute inflammation or oedema was induced by injecting 0.1 ml of 1 % carrageenan into the subplantar surface of the right hind paw of the rat. Inflammation or oedema following carrageenan injection was noticeable within 30 min. The volume of the right hind paw was measured before and then after the injection of carrageenan at 30 min intervals for 4 h by volume displacement method using plethysmometer (TIIC, USA). The experiment was repeated using other groups of animals which were pre-treated with graded doses of plant extract, standard drug or DMSO. The volumes of the untreated rats' right hind paws were also measured at 30 min intervals for 4 h. Acute inflammation or oedema was expressed as a mean increase in paw volume with respect to physiological saline control. All the experiments were performed in the pharmacology laboratory between 09h00 and 17h00. The ability of the plant extract to attenuate the paw

oedema was taken as an indication of anti-inflammatory activity. Inhibition was expressed as a percentage increase or decrease in oedema volume (Williamson et al., 1996).

3.5.3. Assessment of the Antipyretic activity of *R. graveolens*

3.5.3.1. *E. coli*-induced pyrexia test

The method of Santos and Rao (1998) was modified for the antipyretic activity assessment of *R. graveolens*. Rats were used in groups of six per dose of plant extract, standard drug, physiological saline or DMSO. The animals were kept individually in transparent Perspex rat cages before the commencement of the experiment. Control animals were pre-treated for 15 min with 0.25 ml (i.p.) of physiological saline. Pyrexia was induced with 50 µg/kg of *E. coli* administered intramuscularly (i.m.) into the thighs of the animals. The rectal temperature was measured before and after the administration of *E. coli* at 1 h intervals for a period of 5 h with a digital thermometer (TIIC, USA), inserted 2.3 cm into the rectum of the rat. The experiment was repeated using other groups of animals which were pre-treated with graded doses of plant extract, standard drugs or DMSO. The rectal temperatures of the untreated rats were also measured at 1 h intervals for 5 h. The increase of at least 1°C above the normal temperature after the administration of *E. coli* was taken as pyrexia (Amabeoku et al., 2010; Santos and Rao, 1998). The rectal temperatures of normothermic rats were also measured at 1 h intervals for 5 h. The effects of the different doses of plant extracts used were also evaluated alone on the rectal temperature of normothermic rats. All the experiments were performed in the pharmacology laboratory between 09h00 and 17h00. The ability of the plant extract to attenuate *E.coli*-induced pyrexia was taken as an indication of antipyretic activity.

3.6. HPLC analysis

Chromatographic system: Beckman HPLC system consisting of a double pump Programmable Solvent Module model 126; Diode Array detector module model 160; Samsung computer 386 with management System Gold (V601) software applied by Beckman; column. C18 Bondapak 5 µm and dimensions (250 x 4.6 mm^2).

Chromatographic conditions: Mobile phase, solvent A: 1 % acetic acid; solvent B: methanol, Mode: gradient flow rate, 1 min/min; injection volume, 10 µl; detector, UV at 350 nm. The HPLC operating conditions were programmed to give the following: 0 min, solvent B: 20 %; 5 min, solvent B: 40 %; 15 min, solvent B: 60 %; 20 min, solvent B: 80 % and 27 min. The run rate was 30 min.

3.7. Acute toxicity

The methods described by Lorke (1983) and modified by Ojewole (2006) were used to determine the median (LD_{50}) of the plant extract. Mice were fasted for 16 hrs and then randomly divided into groups of eight per cage. Graded doses of the plant extract (100, 400, 800, 1200, 1600, 2000, 2400, 2800, 3200, 3600, 4000 kg-1) were separately administered orally to mice in each test group by means of a bulbed steal needle. Control mice were pre-treated orally with 0.25 ml of physiological saline by means of a bulbed steel needle. The mice were allowed free access to food and water and observed for 5 days for signs of acute toxicity or death. If death was recorded within the period of observation, log dose-response curves would be constructed for the plant extract from which the median lethal dose would be calculated.

3.8. Statistical analysis

The data obtained for the antinociceptive, anti-inflammatory and antipyretic activities were analysed using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (GraphPad Prism, version 5.0, GraphPad Software, Inc., San Diego CA 92130, USA) and presented as Mean ± Standard Error Mean (SEM). *P* values of less than 5 % ($P < 0.05$) were considered to be significant.

3.9. Ethical considerations

All experimental protocol used in this study were approved (12/2/23), by the University of the Western Cape Ethics Committee, Bellville 7535, South Africa and conformed to the University's Regulation Act concerning animal experiments.

CHAPTER 4

RESULTS

4.1. Phytochemical analysis

Data obtained from the phytochemical analysis of *R. graveolens* indicated the presence of the following chemical components: alkaloids, cardiac glycosides, flavonoids, saponins, tannins, and triterpene steroids. However, the test performed showed that the leaves of *R. graveolens* did not contain quinones (Table 1).

Table 1. Phytochemical analysis of *R. graveolens*

Chemical Components	Results
Alkaloids	+
Cardiac glycosides	+
Flavonoids	+
Quinones	-
Saponins	+
Tannins	+
Triterpene steroids	+

Key

+ (Positive) means present

- (Negative) means absent

4.2. Pharmacological screening

4.2.1. Antinociceptive activity

4.2.1.1. Acetic acid writhing test

Three percent (3 %) acetic acid (0.25 ml) produced a significant number of writhes in control mice that were pre-treated with physiological saline (0.25 ml, i.p.). The leaf methanol extract of *R. graveolens* (100 mg/kg, i.p.) significantly reduced the number of acetic acid-induced writhes by 54 %. Similarly, indomethacin (20 mg/kg, i.p.) and paracetamol (500 mg/kg, i.p.) profoundly reduced the number of writhes induced by 0.25 ml (i.p.) of 3 % acetic acid by 57 % and 80 %, respectively. The plant extract (25, 50, 200 and 400 mg/kg, i.p.), 10 mg/kg (i.p.) of indomethacin and DMSO (0.25 ml, i.p.) did not significantly alter the writhes induced by 0.25 ml (i.p.) of 3 % acetic acid. *R. graveolens* (25 mg/kg, i.p.) produced 39 % inhibition of 3 % acetic acid-induced writhes whereas 50, 200 and 400 mg/kg (i.p.) of the plant extract, indomethacin (10 mg/kg, i.p.) and DMSO (0.25 ml, i.p.) produced 40 %, 31 %, 32 %, 20 % and 1 % inhibition of the acetic acid-induced writhes respectively. When combined, the lowest and sub-effective dose of the leaf methanol extract (25 mg/kg, i.p.) and the lowest and sub-effective dose of indomethacin (10 mg/kg, i.p.) significantly reduced the writhes induced by 0.25 ml of 3 % acetic acid in mice despite the individual doses having no significant effect. Together, both sub-effective doses produced 59 % inhibition of 3 % acetic acid-induced writhes in mice (Table 2).

Table 2. Effect of *R. graveolens* on acetic acid-induced writhing in mice

Treatment Groups	Dose (mg/kg)	Number of Writhes Mean ±	SEM	Percentage inhibition (%)
PS	-	26.0	1.94	-
R. graveolens	25	15.75	3.16	39
	50	15.5	0.5	40
	100	12.0*	1.94	54
	200	18.0	2.58	31
	400	17.75	3.20	32
Indomethacin	10	20.75	2.29	20
	20	11.25*	2.94	57
R. graveolens	25			
+ Indomethacin	10	10.75*	2.48	59
Paracetamol	500	5.13**	2.45	80
DMSO	-	25.63	4.80	1

*p<0.05, **p<0.001 compared to 3% acetic acid control (0.25 ml, i.p.), ANOVA (n = 8).

Writhes are expressed as number of counts per 30 minutes.

PS: Physiological saline

DMSO: Dimethylsulfoxide

4.2.1.2. Hot-plate test

Animals reacting to hot-plate thermal stimulation at 55°C were observed using parameters such as licking and/or lifting of hind paws, or attempting to jump out of the beaker. Mice pre-treated with physiological saline reacted to hot-plate thermal stimulation. This occurred within 8.58 ± 0.73 sec in the first 15 min after administration of physiological saline (0.25 ml, i.p.) and within 6.85 ± 1.74 sec, 60 min later. The leaf methanol extract of *R. graveolens* (50 mg/kg, i.p.) significantly delayed the reaction time of mice to hot-plate thermal stimulation 30 min after treatment. *R. graveolens* (100 mg/kg, i.p.) significantly delayed the reaction time of mice to thermal stimulation 15, 30, and 60 min after treatment. *R. graveolens* (200 mg/kg, i.p.) significantly delayed the reaction time of mice 60 min after treatment. *R. graveolens* (400 mg/kg, i.p.) significantly delayed the reaction time in mice to thermal stimulation 15, 30, 45, and 60 min after treatment. The plant extract (25 mg/kg, i.p.) and DMSO (0.25 ml, i.p.) did not significantly affect the reaction time of mice to hot-plate thermal stimulation (Table 3).

Table 3. Effect of *R. graveolens* on hot-plate induced nociception in mice

Treatment groups	Dose (mg/kg)	Reaction time (s) 0 min	15 min	30 min	45 min	60 min
PS	0.25 ml	8.48±0.32	8.58±0.73	8.48±0.64	10.50±1.81	6.85±1.74
R. graveolens	25	6.85±0.95	9.70±0.41	10.39±0.87	10.89±0.33	9.19±0.52
	50	8.85±0.38	9.76±0.54	16.18±0.80**	8.77±0.67	7.26±0.51
	100	9.19±0.72	12.88±0.62*	14.22±0.26**	9.94±0.39	11.25±0.25*
	200	6.85±1.00	9.84±0.78	9.90±0.69	9.85±0.44	11.79±0.66**
	400	8.48±0.11	13.93±0.68**	14.50±0.51**	15.58±0.35*	12.05±0.35**
DMSO	0.25 ml	9.77±1.12	10.54±1.51	10.06±1.09	8.16±1.60	7.66±0.36

*$p<0.05$, **$p<0.025$ compared to physiological saline control, ANOVA (n = 8).

The reaction time in seconds is expressed as Mean±SEM.

PS: Physiological saline

DMSO: Dimethylsulfoxide

4.3. Anti-inflammatory activity

4.3.1. Rat paw oedema test

One percent (1 %) carrageenan injected into the subplantar of the right hind paws of the rats pre-treated with physiological saline induced acute inflammation or oedema in the paws within 30 min which was also the peak time of the oedema. The leaf methanol extract of *R. graveolens* (50 – 400 mg/kg, i.p.) significantly reduced the carrageenan-induced oedema over the 4 h period of testing. Indomethacin (10 mg/kg, i.p.) significantly reduced the carrageenan-induced oedema over the 4 h period of testing. The plant extract (25 mg/kg, i.p.) and indomethacin 2 mg/kg (i.p.) did not significantly affect the oedema elicited by 1 % carrageenan in the rats' right hind paws. However, when combined, the lowest dose of *R. graveolens* (25 mg/kg, i.p.) and the lowest dose of indomethacin (2 mg/kg, i.p.) produced a significant reduction in carrageenan-induced oedema over the 4 h period of testing. DMSO (0.25 ml, i.p.) did not significantly affect the carrageenan-induced oedema in rats (Table 4).

Table 4. Effect of *R. graveolens* on carrageenan-induced oedema in the right hind paw of rat

Treatment group	Dose (mg/kg)	Paw volume (ml)(Mean ± SEM) 0 (min)	30	60	90	120	150	180	210	240
UR	-	0.65±0.02	0.52±0.02	0.58±0.05	0.62±0.03	0.62±0.03	0.68±0.05	0.72±0.06	0.72±0.10	0.75±0.03
PS	0.25 ml	0.72±0.10	1.53±0.27	1.28±0.18	1.52±0.16	1.35±0.10	1.27±0.11	1.28±0.15	1.32±0.80	1.30±0.65
R. graveolens	25	0.75±0.07	1.47±0.11	1.12±0.20	1.45±0.08	1.13±0.16	1.23±0.10	1.31±0.14	1.28±0.16	1.25±0.08
	50	0.68±0.05	0.92±0.06*	0.80±0.07	0.90±0.10*	0.73±0.05*	0.73±0.04*	0.77±0.07*	0.68±0.07*	0.63±0.06*
	100	0.62±0.03	0.72±0.06*	0.75±0.03*	0.70±0.05*	0.70±0.06*	0.63±0.02*	0.67±0.07*	0.53±0.04*	0.57±0.03*
	200	0.58±0.05	0.68±0.09*	0.72±0.03*	0.70±0.07*	0.63±0.04*	0.60±0.02*	0.63±0.08*	0.63±0.09*	0.57±0.05*
	400	0.33±0.02	0.42±0.03*	0.45±0.03*	0.42±0.03*	0.35±0.02*	0.35±0.02*	0.37±0.03*	0.33±0.06*	0.33±0.03*
Indomethacin	2	0.62±0.03	1.28±0.03	1.15±0.09	1.90±0.04	1.25±0.08	1.28±0.08	1.21±0.07	1.25±0.05	1.30±0.07
	10	0.53±0.02	0.58±0.05*	0.68±0.05*	0.68±0.06*	0.62±0.03*	0.60±0*	0.57±0.03*	0.53±0.05*	0.52±0.04*
R. graveolens + Indomethacin	25 2	0.63±0.02	0.88±0.04*	0.87±0.02	0.70±0.05*	0.55±0.06*	0.45±0.03*	0.47±0.05*	0.47±0.07*	0.40±0.03*
DMSO	0.25 ml	0.62±0.10	1.52±0.12	1.30±0.07	1.53±0.09	1.35±0.08	1.28±0.09	1.27±0.11	1.27±0.13	1.35±0.03

*p<0.05 compared to physiological saline control, ANOVA (n = 6)

UR: Untreated rats

PS: Physiological saline

DMSO: Dimethylsulfoxide

4.4. Antipyretic activity

4.4.1. *E. coli*-induced pyrexia

Normothermic rats showed mean rectal temperatures ranging from 35.2±0.35 ºC within the 1st hour of measurement up to 36.0±0.43 ºC at the 5th hour of measurement. *E. coli* (50 µg/kg, i.m.) produced a time-dependent increase in the mean rectal temperature in rats pre-treated with physiological saline. *R. graveolens* (100 – 400 mg/kg, i.p.) significantly reduced *E. coli*-induced pyrexia over the 5 h period of testing. Pentoxifylline (50 mg/kg, i.p.) also significantly reduced *E. coli*-induced pyrexia over the 5 h period of testing. *R. graveolens* (25 mg/kg, i.p.) and pentoxifylline (10 mg/kg, i.p.) did not significantly alter the mean rectal temperatures of rats over the 5 h period of measurement. When combined, the lowest dose of *R. graveolens* (25 mg/kg, i.p.) and the lowest dose of pentoxifylline (10 mg/kg, i.p.) produced a significant reduction in pyrexia induced by *E. coli* (50 µg/kg, i.m.) over the 5 h period of measurement. Paracetamol (500 mg/kg, i.p.) and DMSO (0.25 ml, i.p.) did not significantly affect *E. coli*-induced pyrexia in rats over the 5 h period of measurement (Figure 1). The leaf methanol extract of *R. graveolens* (400 mg/kg, i.p.) significantly reduced the mean rectal temperature in normothermic rats over the 5 h period of measurement. 25 – 200 mg/kg (i.p.) of *R. graveolens* did not affect the mean rectal temperatures of normothermic rats over the 5 h period of measurement (Figure 2).

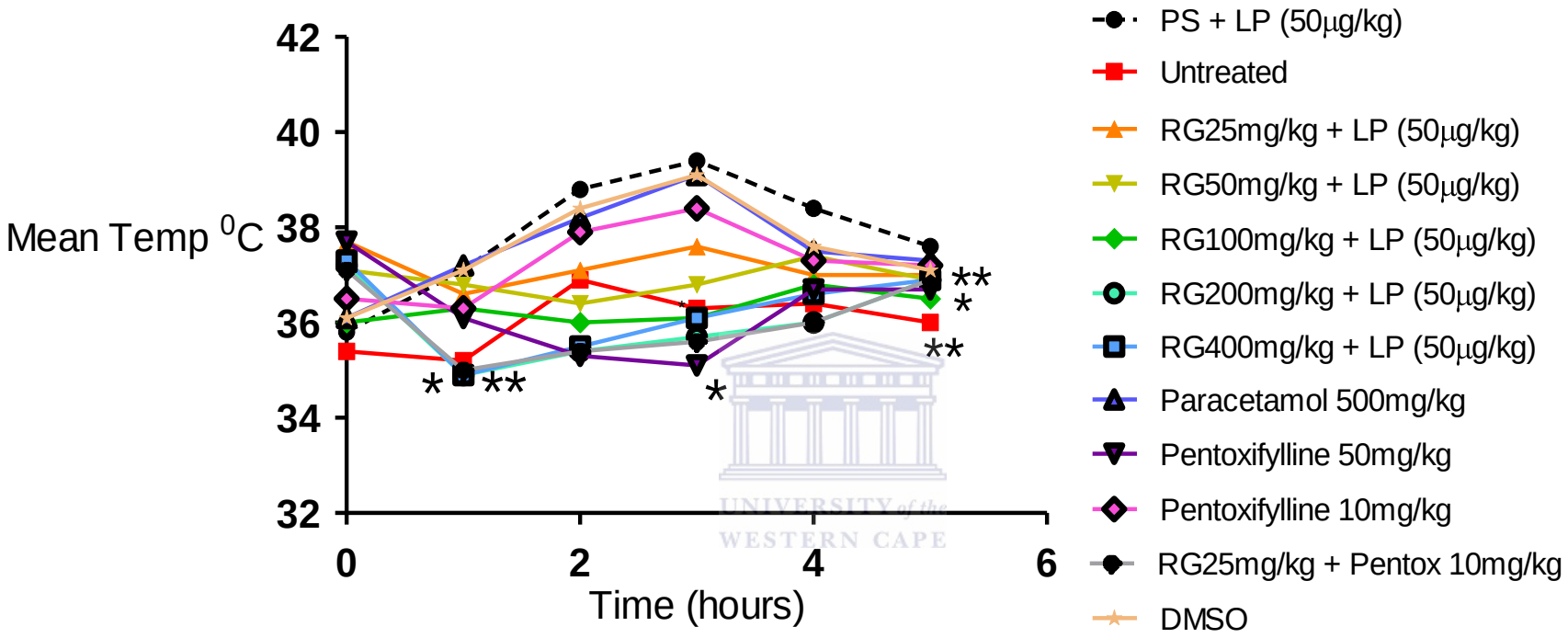

Figure 1. Effect of *R. graveolens*, paracetamol, pentoxifylline on lipopolysaccharide (*E. coli*)-induced pyrexia in rats

PS: Physiological saline

LP: Lipopolysaccharide

RG: *R. graveolens*

DMSO: Dimethylsulfoxide

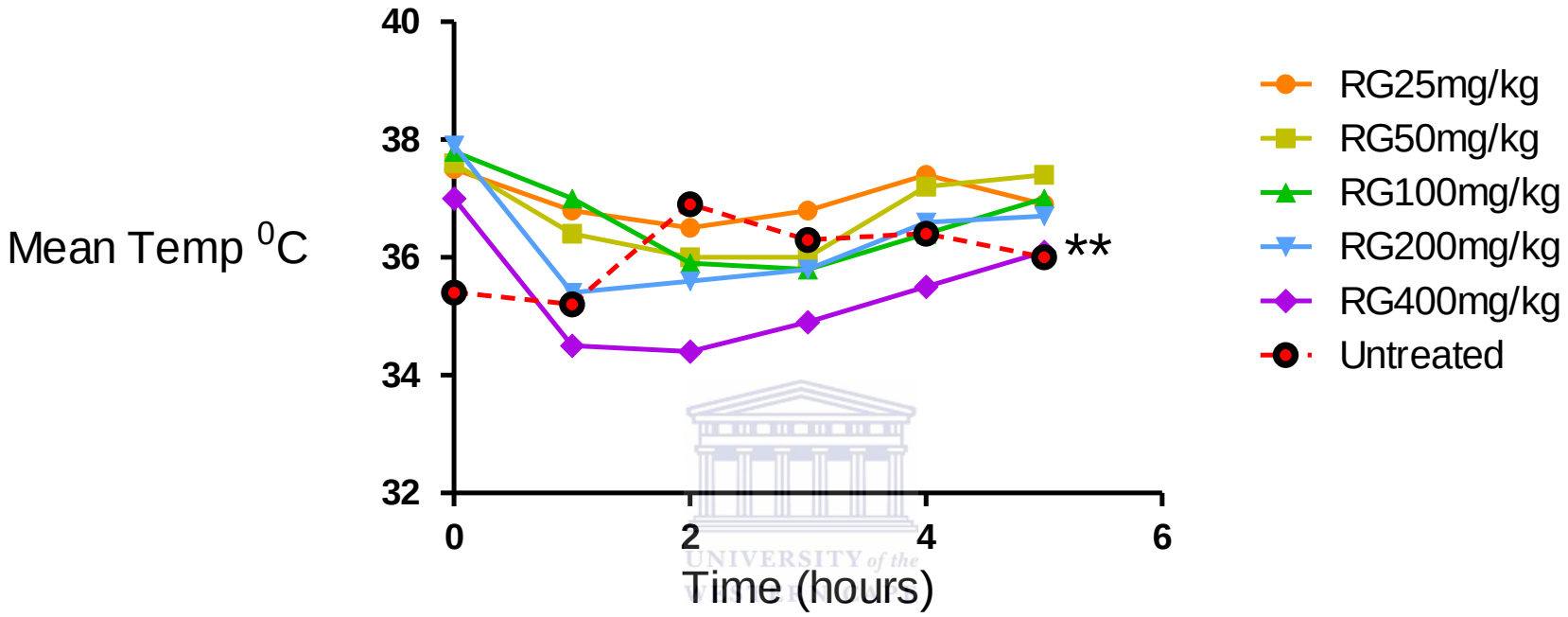

Figure. 2. Effect of *R. graveolens* on the rectal temperature of normothermic rats

RG: *R. graveolens*

4.5. Acute toxicity study

The acute toxicity study did not show any deaths or signs of acute toxicity in mice following oral administration of 100 – 4000 mg/kg of the leaf extract of *R. graveolens*. The 4000 mg/kg (p.o.) dose of the plant extract was therefore, the no-adverse-effect-level (NOAEL). The LD_{50} obtained for the plant species may probably be greater than 4000 mg/kg (p.o.) in mice.

4.6. HPLC analysis

The HPLC fingerprint of the plant species obtained revealed major characteristic peaks at the following retention times (minutes): 1.654, 2.271, 2.403, 4.705 and 7.691 (Figure 3).

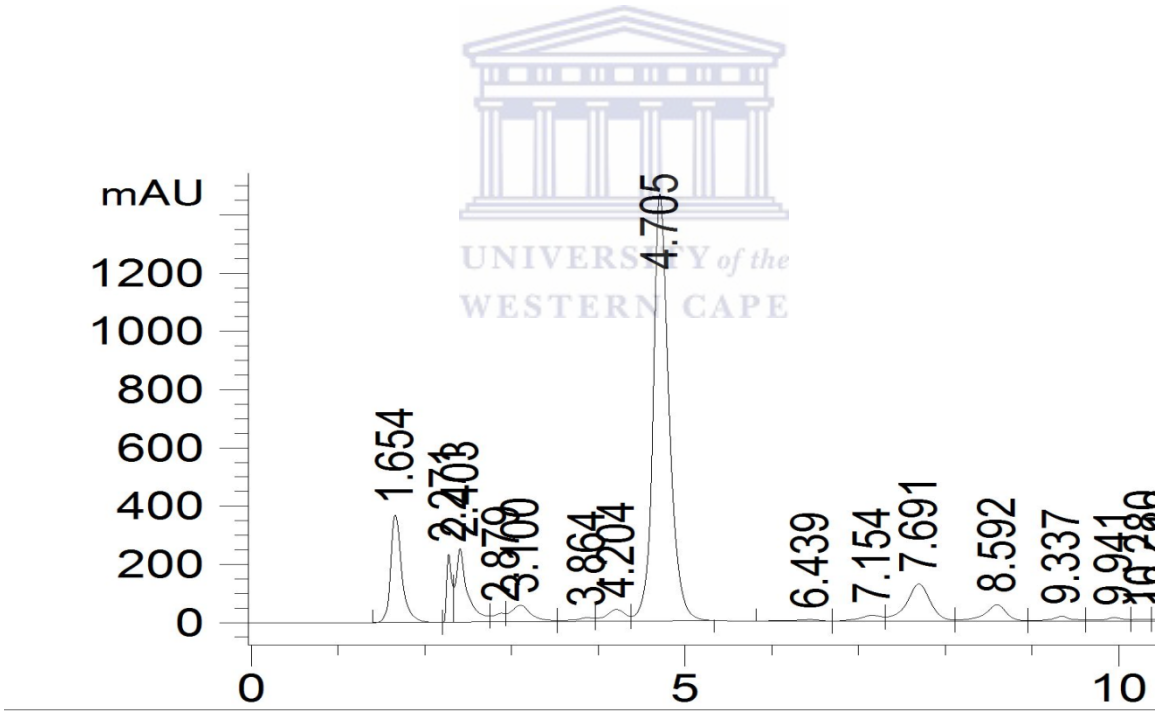

Figure 3. HPLC fingerprint of *R. graveolens*

CHAPTER 5

DISCUSSION

The results of the study indicate that *R. graveolens* has antinociceptive, anti-inflammatory and antipyretic activities. Two models namely, the acetic acid writhing test and the hot-plate test, were used to investigate the peripheral and central antinociceptive activities of *R. graveolens,* respectively, in mice. The carrageenan-induced rat paw oedema test was used to investigate anti-inflammatory activity of the plant extract in rats. *E.coli*-induced pyrexia was used to investigate the antipyretic activity of the plant extract in rats.

In this study, the leaf methanol extract of *R. graveolens* significantly antagonised the acetic acid-induced writhing in mice. Similarly, indomethacin and paracetamol significantly antagonised acetic acid writhing in mice. In addition, sub-effective doses of *R. graveolens* and indomethacin combined significantly antagonised acetic acid-induced writhing in mice. Satyanaraya et al. (2004) has shown that acetic acid induces writhing by stimulating the production of prostaglandins. Standard analgesic drugs, indomethacin and paracetamol, have been shown to inhibit prostaglandin synthesis in the brain (Rang et al., 2012; Flower and Vane, 1972). Therefore, it is not surprising that both indomethacin and paracetamol significantly antagonised acetic acid-induced writhing. Since *R. graveolens* and also the combined therapy of sub-effective doses of *R. graveolens* and indomethacin antagonised acetic acid-induced writhes, it is possible to suggest that the plant extract may be producing antinociceptive activity through the manipulation of the prostaglandin system.

The leaf methanol extract of *R. graveolens* significantly delayed the reaction time of mice to thermal stimulation produced by the hot-plate. Morphine, a standard centrally acting analgesic drug, is known to attenuate nociception produced by the hot-plate (Amabeoku et al., 2012). It is therefore, possible that *R. graveolens* may be acting through certain central pain receptors to delay nociception produced by hot-plate in the study. Williamson et al. (1996), Koster et al. (1959), and Eddy and Leimback (1953), stipulated that the acetic acid writhing and hot-plate tests are used to evaluate peripherally and centrally acting analgesic drugs. Since *R. graveolens* significantly antagonised both acetic acid-induced writhing and thermal stimulation produced by hot-plate, it is tempting to suggest that the plant species has both peripheral and central antinociceptive activities.

In the present study, the leaf methanol extract of *R. graveolens* profoundly reduced carrageenan-induced rat paw oedema. Indomethacin also antagonised carrageenan-induced rat paw oedema. In addition, sub-effective doses of *R. graveolens* and indomethacin combined significantly antagonised carrageenan-induced rat paw oedema. Carrageenan induces rat paw oedema due to histamine, serotonin, bradykinin and prostaglandin like substances (Vinegar et al., 1969). Perianayagam et al. (2006), Adedapo et al. (2008) and Swingle (1974), have shown that mediators such as prsotaglandins, bradykinin, histamine and serotonin are implicated at different phases of carrageenan-induced rat paw oedema. Capasso et al. (1975) and Di Rosa et al. (1971) have also reported the implication of histamine, 5-hydroxytryptamine, bradykinin, nitric acid, and prostaglandins in carrageenan-induced rat paw oedema. The carrageenan-induced rat paw oedema test is known to be sensitive to cyclo-oxygenase (COX) inhibitors, and may also be used to evaluate the effect of NSAIDs (Rao et al., 2005). COX – 1 and COX – 2 enzymes interact to

form prostaglandins. In inflamed tissue, COX – 2 is unregulated. This may be responsible for the enhanced production of prostaglandins (Ratheesh et al., 2009). In a study conducted by Ratheesh et al. (2009), the reduction of rat paw oedema and the reduced activity of COX – 2 indicated anti-inflammatory effects. Amabeoku et al. (2012), reported that *C. orbiculata* reduced carrageenan-induced rat paw oedema, and suggested that the plant may have affected inflammatory mediators to produce its anti-inflammatory activity. Also, indomethacin produces anti-inflammatory effects by inhibiting COX, thus inhibiting prostaglandin synthesis (Rang et al., 2012). The data on the anti-inflammatory activity of *R. graveolens* in the present study are in agreement with those of Amabeoku et al. (2012).

In the present study, the leaf methanol extract of *R. graveolens* alone significantly reduced the rectal temperature in normothermic rats. *R. graveolens* also significantly reduced pyrexia induced by the lipopolysaccharide (LPS) or bacterial endotoxin, *E. coli*. In addition, pentoxifylline significantly reduced *E. coli*-induced pyrexia in rats. Furthermore, combined therapy of sub-effective doses of *R. graveolens* and pentoxifylline antagonised *E. coli*-induced pyrexia. According to Kluger (1991), lipopolysaccharide can induce fever in rats. This may be due to prostaglandins and cytokines such as interleukin (IL) - 1β, IL - 6 and tumor necrosis factor (TNF) – α. Flower and Vane (1972), found that the antipyretic activity of paracetamol is produced by the inhibition of prostaglandin synthetase. This results in the blockade of prostaglandin synthesis in the brain. In this study, paracetamol, an analgesic and antipyretic drug, did not significantly affect *E. coli*-induced pyrexia in rats. While it is believed that prostaglandins regulate body temperature (Dascombe, 1985), studies suggest that pyrexia produced by LPS in rats, guinea-pigs and rabbits implicated the stimulation of TNF – α (Roth

and Zeisberger, 1995; Kluger, 1991; LeMay et al., 1990). This may explain why paracetamol did not significantly affect *E. coli*-induced pyrexia in rats. Pentoxifylline is a TNF–α antagonist (Marcinkiewicz et al., 2000; LeMay et al., 1990). It is not surprising therefore, that pentoxifylline antagonised pyrexia induced by LPS in this study. This is in agreement with the findings of Santos and Rao (1998), which show that pentoxifylline reduced pyrexia induced by LPS. Since *R. graveolens* and also the combined therapy of sub-effective doses of *R. graveolens* and pentoxifylline antagonised *E. coli*-induced pyrexia, it is probable that TNF-α inhibition may underpin the antipyretic activity of the plant species.

The phytochemical tests carried out on the powdered leaf of *R. graveolens* indicated the presence of alkaloids, cardiac glycosides, flavonoids, saponins, tannins, and triterpine steroids. Israili (2009) has reported that saponins and flavonoids have analgesic and antipyretic activity. Bruneton (1995) reported that saponins have analgesic and anti-inflammatory activities. Pendota et al. (2009), conducted a study on the use of *H. pauciflorus* in inflammation, pain and fever and suggested that these activities may be due to the presence of tannins, flavonoids, steroids and/or terpenes. Another study by Ojewole (2005), speculated that the presence of flavonoids and triterpenoids in *B. pinnatum* may account for the plant species' antinociceptive and anti-inflammatory activities. According to Kotb (1985) and Benazir et al. (2011), *R. graveolens* contains volatile oils, resins and flavonoids. Flavonoids isolated from other plant extracts have proven to possess analgesic activity. Since plant samples with similar HPLC fingerprints have similar properties (Fan et al., 2006), the presence of flavonoids found in the leaf methanol extract of *R. graveolens* may be suggestive that the antinociceptive activity produced is due to the content of flavonoids. Also, since *R. graveolens* contains other secondary metabolites such as

saponins and triterpene steroids, it is possible that these metabolites may be contributing to the antinociceptive, anti-inflammatory and antipyretic activities of the plant species in this study.

The HPLC fingerprint obtained for *R. graveolens* revealed characteristic peaks at the following retention times (minutes): 1.654 min, 2.271 min, 2.403 min, 4.705 min and 7.691 min. The acute toxicity test carried out revealed that the LD_{50} value obtained for the plant species after oral administration may be greater than 4000mg/kg. This high LD_{50} indicates that *R. graveolens* is non-toxic and/or safe in mice.

CHAPTER 6

CONCLUSION

The data obtained in this study indicate that *R. graveolens* has antinociceptive, anti-inflammatory and antipyretic activity which may be due to the plant species inhibiting mediators implicated in pain, inflammation and fever. The role of saponins, flavonoids and triterpene steroids suggest that these secondary metabolites may contribute to the antinociceptive, anti-inflammatory and antipyretic activities of *R. graveolens*. The relatively high LD_{50} value of 4000 mg/kg (p.o.) obtained for the plant species indicates that it may be non-toxic to/or safe in mice. The results obtained substantiate the use of the plant species by traditional medicine practitioners in South Africa for the management and treatment of essential events such as pain, inflammation and fever. Further studies are needed to elucidate the full mechanisms of antinociceptive, anti-inflammatory and antipyretic activities of *R. graveolens*. In addition, further toxicological studies are needed to determine the safety profile of *R. graveolens*.

REFERENCES

Adedapo, A.A., Sofidiya, M.O., Masika, P.J. and Afolayan, A.J. 2008. Anti-inflammatory and analgesic activities of the aqueous extract of Acacia karroo stem bark in experimental animals. *Basic and Clinical Pharmacology and Toxicology,* **103** (5): 397-400.

Amabeoku, G.J. and Kabatende, J. 2012. Antinociceptive and anti-inflammatory activities of leaf methanol extract of cotyledon orbiculata L. (Crassulaceae). *Advances in Pharmacological Sciences,* doi: 10.1153/2012/862625, 2012: 1-6.

Amabeoku, G.J., Leng, M.J. and Syce, J.A. 1998. Antimicrobial and anticonvulsant activities of Viscum capense. *Journal of Ethnopharmacology,* **61** (3): 237-241.

Atta, A.H. and Alkofahi, A. 1998. Anti-nociceptive and anti-inflammatory effects of some Jordanian medicinal plant extracts. *Journal of Ethnopharmacology*, **60** (2): 117-124.

Ayoub, S.S., Colville-Nash, P.R., Willoughby, D.A. and Botting, R.M. 2006. The involvement of a cyclooxygenase 1 gene-derived protein in the antinociceptive action of paracetamol in mice. *European journal of pharmacology,* **538** (1-3): 57-65.

Benazir, J., Suganthi, R., Renjini Devi, M., Suganya, K., Monisha, K., Nizar Ahamed, K. and Santhi, R. 2011. Phytochemical profiling, antimicrobial and cytotoxicity studies of methanolic extracts from Ruta graveolens. *Journal of Pharmacy Research*, **4** (5): 1407-1409.

Bombardelli, E. 2001. Approaches to the quality characteristics of medicinal plant derivatives. *European Phytojournal,* **1**: 30-33.

Bruneton, J. 1995. Pharmacognosy, phytochemistry, medicinal plants. Intercept. Hampsphire pp. 371-733.

Capasso, F., Balestrieri, B., Di Rosa, M., Persico, P. and Sorrentino, L. 1975. Enhancement of carrageenan foot edema by 1,10-phenanthroline and evidence for bradykinin as endogenous mediator. Agents Actions, **5** (4): 359–363.

Cartaxo, S.L., De Almeida Souza, M.M. and De Albuquerque, U.P. 2010. Medicinal plants with bioprospecting potential used in semi-arid northeastern Brazil. *Journal of Ethnopharmacology,* **131** (2): 326-342.

Cohen, M.H. 2003. Regulation, religious experience, and epilepsy: A lens on complementary therapies. *Epilepsy and Behavior,* **4** (6): 602-606.

Dascombe, M.J. 1985. The pharmacology of fever. *Progress in Neurobiology,* **25** (4): 327-373.

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

Eddy, N.B. and Leimback, D. 1953. Synthetic analgesics. II. Dithienylbutenyl-and dithienylbutylamines. *Journal of Pharmacology and Experimental Therapeutics,* **107** (3): 385-393.

Fan, X.-., Cheng, Y.-., Ye, Z.-., Lin, R.-. and Qian, Z. 2006. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. *Analytica Chimica Acta*, **555** (2): 217-224.

Flower, R.J. and Vane, J.R. 1972. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature*, **240** (5381): 410-411.

García, M.D., Fernández, M.A., Alvarez, A. and Saenz, M.T. 2004. Antinociceptive and anti-inflammatory effect of the aqueous extract from leaves of Pimenta racemosa var. ozua (Mirtaceae). *Journal of Ethnopharmacology*, **91** (1): 69-73.

Graham, G.G. and Scott, K.F. 2003. Mechanisms of action of paracetamol and related analgesics. *Inflammopharmacology*, **11** (4-6): 401-413.

Harborne, J.B. 1984. Phytochemical methods a guide to modern techniques of plant analysis. Springer, pp. 125-171.

Hurley, J.V. 1972. Acute Inflammation. Churchill Livingstone, London, pp. 1-6.

Hutchings, A. 1996. Zulu medicinal plants: an inventory. University of Natal Press, University of Zululand, pp. 151.

Ikhiri, K., Boureima, D. and Dan-Koulodo, D. 1992. Chemical screening of medicinal plants used in the traditional pharmacopoeia of Niger. *International Journal of Pharmacognosy*, **30** (4): 251-262.

Israili, Z.H. and Lyoussi, B. 2009. Ethnopharmacology of the plants of genus Ajuga. *Pakistan Journal of Pharmaceutical Sciences,* **22** (4): 425-462.

Jäger, A.K., Mohoto, S.P., Van Heerden, F.R. and Viljoen, A.M. 2005. Activity of a traditional South African epilepsy remedy in the GABA-benzodiazepine receptor assay. *Journal of Ethnopharmacology,* **96** (3): 603-606.

Katz, N. 2002. Coxibs: Evolving role in pain management. *Seminars in arthritis and rheumatism,* **32** (3 SUPPL. 1): 15-24.

Kayne, S.B. 2010. Traditional Medicine. Pharmaceutical Press, London, pp. 1, pp. 82-85.

Kluger, M.J. 1991. Fever: Role of pyrogens and cryogens. *Physiological Reviews,* **71** (1): 93-127.

Kierszenbaum, A.L. 2007. Histology and Cell Biology: An Introduction to Pathology. 2nd ed. Mosby Elsevier, Philadelphia, pp. 340-343.

Koster, R., Anderson, M. and De Beer, E. 1959. Acetic acid for analgesic screening. *Federation Proceedings*, pp. 412.

Kotb, F.T. 1985. *Medicinal plants in Libya.* Arab encyclopedia house.

Kumar, P and Clark, M. 2009. Clinical Medicine. 7th ed. Elsevier Limited, London, pp. 86-87.

Larsen, G.L. and Henson, P.M. 1983. Mediators of Inflammation. *Immunology*, 1: 335-59.

Lee, Y., Rodriguez, C. and Dionne, R. 2005. The role of COX-2 in acute pain and the use of selective COX-2 inhibitors for acute pain relief. *Current pharmaceutical design,* **11** (14): 1737-1755.

LeMay, L.G., Vander, A.J. and Kluger, M.J. 1990. The effects of pentoxifylline on lipopolysaccharide (LPS) fever, plasma interleukin 6 (IL 6), and tumor necrosis factor (TNF) in the rat. *Cytokine,* **2** (4): 300-306.

Light, M.E., Sparg, S.G., Stafford, G.I. and Van Staden, J. 2005. Riding the wave: South Africa's contribution to ethnopharmacological research over the last 25 years. *Journal of Ethnopharmacology,* **100** (1-2): 127-130.

Loomis, T.A. and Hayes, A.W. 1996. Loomi's essentials of toxicology. 4th ed. Academic Press, California, pp. 208-245.

Lorke, D. 1983. A new approach to practical acute toxicity testing. *Archives of Toxicology,* **54** (4): 275-287.

Martin, J.H. 2012. Neuroanatomy Text and Atlas. 4th ed. McGraw Hill, New York, pp. 111-113.

Marcinkiewicz, J., Grabowska, A., Lauterbach, R. and Bobek, M. 2000. Differential effects of pentoxifylline, a non-specific phosphodiesterase inhibitor, on the production of IL-10, IL-12 p40 and p35 subunits by murine peritoneal macrophages. *Immunopharmacology,* **49** (3): 335-343.

McCarver, B.M. 2001. Essential Psychopharmacology: Neuroscientific Basis and Practical Applications. *Primary Care Companion to The Journal of Clinical Psychiatry,* **3** (6): 269.

Ojewole, J.A.O. 2006. Antinociceptive, anti-inflammatory and antidiabetic properties of Hypoxis hemerocallidea Fisch. & C.A. Mey. (Hypoxidaceae) corm ['African Potato'] aqueous extract in mice and rats. *Journal of Ethnopharmacology,* **103** (1): 126-134.

Ojewole, J.A.O. 2005. Antinociceptive, anti-inflammatory and antidiabetic effects of Bryophyllum pinnatum (Crassulaceae) leaf aqueous extract. *Journal of Ethnopharmacology*, **99** (1): 13-19.

Pendota, S.C., Yakubu, M.T., Grierson, D.S. and Afolayan, A.J. 2009. Anti-inflammatory, analgesic and antipyretic activities of the aqueous extract of Hippobromus pauciflorus (L.f) Radlk leaves in male Wistar rats. *African Journal of Biotechnology,* **8** (10): 2036-2041.

Perianayagam, J.B., Sharma, S.K. and Pillai, K.K. 2006. Anti-inflammatory activity of Trichodesma indicum root extract in experimental animals. *Journal of Ethnopharmacology,* **104** (3): 410-414.

Perrott, C.A. 2003. Emergency medicine in South Africa: A personal perspective. *Journal of Emergency Medicine*, **25** (3): 325-328.

Pinardi, G., Prieto, J.C. and Miranda, H.F. 2005. Analgesic synergism between intrathecal morphine and cyclooxygenase-2 inhibitors in mice. *Pharmacology Biochemistry and Behavior,* **82** (1): 120-124.

Pollio, A., De Natale, A., Appetiti, E., Aliotta, G. and Touwaide, A. 2008. Continuity and change in the Mediterranean medical tradition: Ruta spp. (rutaceae) in Hippocratic medicine and present practices. *Journal of Ethnopharmacology*, **116** (3): 469-482.

Poole, A. and Leslie, G.B. 1989. A practical approach to toxicological investigations. 1st ed. Cambridge University Press, London, pp. 2-117.

Portenoy, R.K. 2000. Current pharmacotherapy of chronic pain. *Journal of pain and symptom management,* **19** (1 SUPPL. 1): 16-20.

Raghav, S.K., Gupta, B., Agrawal, C., Goswami, K. and Das, H.R. 2006. Anti-inflammatory effect of Ruta graveolens L. in murine macrophage cells. *Journal of Ethnopharmacology,* **104** (1-2): 234-239.

Rang, H.P., Dale, M.M., Ritter, J.M., Flower, R.J. and Henderson, G. 2012. Pharmacology. 7th ed. Edinburgh, Churchill Livingstone, pp. 503-511.

Rao, C.V., Kartik, R., Ojha S.K., Amresh, G., Rao, G.M.M. 2005. Anti-inflammatory and antinociceptive activity of stem juice powder of *Tinospora cordifolia* Miers. in experimental animals. Hamdard Medicus XLVIII: 102–106.

Ratheesh, M., Shyni, G.L. and Helen, A. 2009. Methanolic extract of Ruta graveolens L. inhibits inflammation and oxidative stress in adjuvant induced model of arthritis in rats. *Inflammopharmacology,* **17** (2): 100-105.

Rossiter, D. 2012. *South African medicines formulary* (SAMF). 10th ed. Health and Medical Publishing Group, Cape Town, pp. 389-395, 429-437.

Roth, J. and Zeisberger, E. 1995. Endotoxin tolerance alters thermal response of guinea pigs to systemic infusions of tumor necrosis factor-α. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology,* **268** (2 37-2): 514-519.

Santos, F.A. and Rao, V.S. 1998. A study of the anti-pyretic effect of quinine, an alkaloid effective against cerebral malaria, on fever induced by bacterial endotoxin and yeast in rats. *Journal of Pharm Pharmacology,* **50** (2): 225-229.

Satyanarayana, P.S.V., Jain, N.K., Singh, A. and Kulkarni, S.K. 2004. Isobolographic analysis of interaction between cyclooxygenase inhibitors and tramadol in acetic acid-induced writhing in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* **28** (4): 641-649.

Snipes, J.A., Kis, B., Shelness, G.S., Hewett, J.A. and Busija, D.W. 2005. Cloning and characterization of cyclooxygenase-1b (putative cyclooxygenase-3) in rat. *Journal of Pharmacology and Experimental Therapeutics,* **313** (2): 668-676.

Sofowora, A. 1982. Medicinal plants and traditional medicine in Africa. John Wiley and Sons Ltd, pp. 1-103.

Springfield, E.P., Eagles, P.K.F. and Scott, G. 2005. Quality assessment of South African herbal medicines by means of HPLC fingerprinting. *Journal of Ethnopharmacology,* **101** (1-3): 75-83.

Swingle, K.F. 1974. Evaluation for anti-inflammatory activity. *Antiinflammatory Agents: Chemistry Pharmacology,* **2**: 33-122.

Tabuti, J.R.S., Dhillion, S.S. and Lye, K.A. 2003. Traditional medicine in Bulamogi county, Uganda: Its practitioners, users and viability. *Journal of Ethnopharmacology,* **85** (1): 119-129.

Thring, T.S.A. and Weitz, F.M. 2006. Medicinal plant use in the Bredasdorp/Elim region of the Southern Overberg in the Western Cape Province of South Africa. *Journal of Ethnopharmacology,* **103** (2): 261-275.

Vinegar, R., Schreiber, W. and Hugo, R. 1969. Biphasic development of carrageenin edema in rats. *Journal of Pharmacology and Experimental Therapeutics*, **166** (1): 96-103.

Watt, J.M. and Breyer-Brandwijk, M.G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2nd ed. E and S Livingstone Ltd, London, pp. 1457.

Webster, R.A. and Jordan, C. 1989. Neurotransmitters, drugs and disease.1st ed. Blackwell Scientific Publications, Lodon, pp. 301-344.

World Health Organization. 2012. Traditional medicine. http://www.who.int/medicines/areas/traditional/definitions/en/index.html. (accessed May 2012)

World Health Organization. 2003. The African Health Monitor: *Traditional medicine*. WHO Regional Office for Africa, pp. 1-2.

Williamson, E.M., Okpako, D.T. and Evans, F.J. 1996. Pharmacological Methods in Phytotherapy Research: Selection, Preparation and Pharmacological Evaluation of Plant Material. John Wiley and Sons, New York, pp. 1-23.

Wyk, B.E., Oudtshoorn, B. and Gericke, N. 1997. Medicinal plants of South Africa. Briza, pp. 220.