Comparison of flavonoid profile and respiratory smooth muscle relaxant effects of *Artemisia afra versus Leonotis leonurus.*

Tjokosela Tikiso

A thesis submitted in fulfilment of the requirements for the degree of *Magister Scientiae* (Pharmaceutical Sciences) in the Discipline of Pharmacology at the University of the Western Cape, Bellville, South Africa.

UNIVERSITY *of the*
WESTERN CAPE

Supervisor: Prof. James Syce

Co-supervisor: Dr Kenechukwu Obikeze

September 2015

Comparison of flavonoid profile and respiratory smooth muscle relaxant effects of *Artemisia afra versus Leonotis leonurus*.

Tjokosela Tikiso

Key words

Artemisia afra

Leonotis leonurus

Freeze dried aqueous extract

Trachea smooth muscle relaxant

Isolated guinea-pig trachea

Flavonoids

Luteolin

HPLC

Summary

Leonotis leonurus (*L. leonurus*) and *Artemisia afra* (*A. afra*) are two of the most commonly used medicinal plants in South Africa traditionally advocated for use in asthma. However, proper scientific studies to validate these claimed uses are lacking and little is known about the mechanisms for this effect. These plants contain flavonoids, which are reported to have smooth muscle relaxant activity and may be responsible for the activity of these two plants.

The objectives of this study were to: (1) determine and compare the flavonoid profiles and levels in *A. afra* and *L. leonurus,* (2) compare the respiratory smooth muscle relaxant effects of freeze-dried aqueous extracts of *A. afra* and *L. leonurus* and (3) investigate whether K^+ - channel activation (i.e. K_{ATP} channel) is one possible mechanism of action that can explain the effect obtained in traditional use of these two plants. It was hypothesized that: (1) the flavonoid levels and profile of *A. afra* would be greater than the flavonoid levels and profile of *L. leonurus*, (2) *A. afra* would have a more potent respiratory muscle relaxant effect than *L. leonurus* and (3) *A. afra* and *L. leonurus* will inhibit K^+ - induced contractions in a superior manner than carbachol and histamine - induced contractions.

To realize these objectives, freeze-dried aqueous extracts (FDAE) of the dried leaves of the two plants were prepared. A validated HPLC assay was developed and used to identify and determine the levels of luteolin in the plant preparations. Solutions of the plant extracts were studied in the isolated guinea-pig trachea tissue preparation in the presence of carbachol, histamine and KCL. The possible mechanism of action of the two plants was determined by cumulative log dose-response curves (LDRC) for carbachol, histamine and KCL in the absence and presence of 1, 30 and 100 mg/ml solutions of the plant extracts.

The flavonoid profile of un-hydrolyzed and hydrolyzed *L. leonurus* was greater than that of un-hydrolyzed and hydrolyzed *A. afra.* The levels of free and total luteolin in *A. afra* FDAE (8.977 ± 0.73 µg/ml and 16.394 ± 0.884 µg/ml, respectively) were significantly ($p < 0.001$) higher than that in *L. leonurus* FDAE (0.929 ± 0.066 µg/ml and 3.093 ± 0.531 µg/ml, respectively). *L. leonurus* and *A. afra* relaxed tracheal smooth muscles contracted with

iii

histamine, KCL and carbachol in a dose dependent manner. The degree of relaxant activity of *L. leonurus versus* the three inducers of contraction (agonists) could be classified as KCL > carbachol > histamine, with EC_{50} values of 9.87, 29.34 and 94.76 mg/ml, respectively. The *A. afra* tracheal smooth muscle relaxant activity was categorized as carbachol > histamine > KCL, with EC_{50} values of 13.93, 15.47 and 19.88 mg/ml, respectively. Overall, *A. afra* which contained the higher levels of luteolin, was more potent at relaxing the guinea pig tracheal smooth muscle than *L. leonurus*.

Collectively, the results confirm that aqueous solutions of *A. afra* and *L. leonurus* as used in local traditional practice have potent but different degrees of bronchodilator activities that could be useful in the treatment of asthma, and that these actions may be related to each plant's luteolin (or flavonoid) levels. Moreover it is very unlikely that K_{ATP} channels are primarily responsible for the actions of *A. afra* and *L. leonurus,* but rather that more than one mechanism of action is involved in the tracheal smooth muscle relaxant effects of these two plants.

UNIVERSITY *of the*
WESTERN CAPE

Declaration

I declare that the thesis **Comparison of flavonoid profile and respiratory smooth muscle relaxant effects of *Artemisia afra versus Leonotis leonurus*** is my own work, that it has not been submitted before for any degree or examination in any other University and that all the sources I have used or quoted have been indicated and acknowledged by means of complete referencing.

Tjokosela Tikiso
September, 2015

Signed: UWC, Bellville

Dedication

I dedicate this master's thesis to my family, for their unending love, support and constant encouragement.

Acknowledgement

I would like to sincerely acknowledge the following:

Prof. J.A. Syce my supervisor for his guidance, support and expert supervision throughout the project.

Dr Kenechukwu Obikeze my co-supervisor who was such a great mentor, his contribution towards the completion of this thesis was invaluable.

Mr Andre Braaf for his assistance with the freeze drying techniques.

Dr Edith Antunes for the wholesome training and assistance in the use of HPLC.

Mrs Oluchi Mbamalu for her constant help and assistance throughout the project.

Staff and colleagues at the School of Pharmacy and SASHMI at UWC for their presence and assistance at various stages of this research project.

My colleagues and friends at the School of Pharmacy and in the Plant Medicine Research Group for their support and encouragement that made the completion of this thesis possible.

Finally, to God Almighty for giving me the strength and ability to carry out this project to completion.

Table of contents

List of Figures

List of Tables

UNIVERSITY *of the*
WESTERN CAPE

List of Appendices

List of abbreviations and units

Artemisia afra	*A. afra*
Adenosine triphosphate	ATP
Cyclic adenosine monophosphate	cAMP
Freeze-dried aqueous extracts	FDAE
Global Initiative for Asthma	GINA
High-performance liquid chromatography	HPLC
Leonotis leonurus	*L. leonurus*
Log dose-response curves	LDRC
Long acting β_2 adrenoceptor agonists	LABAs
Phosphodiesterase	PDE
Short acting β_2 adrenoceptor agonists	SABAs
World Health Organization	WHO
eta	β
Degrees Celsius	°C
microgram	μg
micrometer	μm
microliter	μl
Milligram	mg
Millilitre	ml
Minute	min
Millimola	mM

Chapter 1

Introduction

Asthma is a chronic inflammatory condition of the airway which leads to hyper-secretion of the mucus glands, bronchoconstriction, and increased hyper-responsiveness of the airways, with involvement of many cells in particular mast cells, eosinophils and T lymphocytes. This chronic inflammation leads to recurrent episodes of breathlessness, reversible airflow obstruction, wheezing, chest tightness and coughing, particularly at night or in the early morning (GINA, 2015). It is estimated to affect over 300 million individuals worldwide, with approximately 250,000 annual deaths (Bousquet et al., 2000; Silva et al., 2015). South Africa is ranked 25th in the world in the prevalence of asthma, estimated to affect 8.1 % of the population. Shockingly, it was ranked fourth in highest asthma death rate in the World among 5 to 35-year-olds in 2012 (Bateman et al., 2008). It is estimated that the number of individuals with asthma worldwide will increase by more than 100 million by the year 2025 (Masoli et al., 2004).

Current asthma treatment involves the usage of compounds such as corticosteroid anti-inflammatories, β_2 - adrenoceptor agonists, anti-leukotrienes, and anti-cholinergics (Rang and Dale, 2003). Despite the availability (and widespread use) of these anti-asthmatic drugs, the substantial economic load associated with disease control and morbidity continues to intensify. Moreover, the anti-asthmatic drugs do not always give the required therapeutic outcome (Barnes, 2012). They do not appear to modify the course of the disease significantly, and they are not curative (Barnes, 2012). Asthma symptoms and inflammation rapidly recur when the treatment is discontinued. All currently available inhaled corticosteroids are absorbed from the lungs and thus have the potential for systemic side effects (Barnes, 2012). There is thus a strong need for additional therapies especially alternative treatment such as herbal medicines to be explored.

Traditional medicine in particular herbal medicine is a commonly practiced form of medicine in Africa with an estimated usage by 80 % of the population (WHO, 2002). About 27 million South Africans rely on traditional herbal medicine as a source of health, and because the use of herbal medicine forms a fundamental part of South African culture, this reliance on herbal

medicine is unlikely to change (Fennell et al., 2004). Medicinal plants are also gaining popularity for reasons such as fewer side effects, low cost and high accessibility and acceptability due to long history of use (Thomas, 2002; Vermani and Garg, 2002). There is, therefore, a need for data related to the efficacy of these medicinal plants.

Leonotis leonurus and *Artemisia afra* are two of the most commonly used medicinal plants in South Africa traditionally advocated for use in asthma (Suliman, 2011). In the Eastern Cape Province of South Africa infusions of leaves of *A. afra* are used in the treatment of asthma (Thring and Weitz 2006). The leaves are also heated and the vapours inhaled to alleviate symptoms of asthma (Roberts 1990). Some tribes also commonly smoke the leaves to help relieve an acute attack (Roberts 1990). On the other hand, the leaf infusion of *L. leonurus* has also been used for asthma and headaches (Van Wyk and Gericke, 2000). The infusion is taken on a regular basis to prevent attacks from occurring (Taylor et al., 2001). But it is not known whether or not this herb is helpful when administered during an actual asthma attack. Despite the vast traditional use of these two plants, very little is however known about the constituents/compounds responsible for, or the mechanism(s) involved in, producing the claimed anti-asthmatic effect.

There are various physiological mechanisms by which current anti-asthmatic drugs produce their anti-asthmatic effect. The most effective and widely used agents generally produce anti-inflammatory and smooth muscle relaxant effects, while some are mast cells stabilizers (Rang et al., 2003). The smooth muscle relaxant effect is particularly useful in acute attacks of asthma (Rang et al., 2003). The way the medicinal plants are used traditionally, strongly suggest that, if effective, they might be so because of a high potential of producing a smooth muscle relaxation. Now the crucial question will be which constituents in the plants are responsible for such smooth muscle relaxant effect.

L. leonurus and *A. afra* contain a variety of constituents of which flavonoids are probably the most likely constituents known to be implicated in smooth muscle relaxation. *A. afra* contains flavonoids such as luteolin, kaempferol, apigenin, hesperetin, chrysoeriol, acacetin, genkwanin and quercetin (Muganga, 2004) with luteolin and quercetin present in higher levels than the others (Mulubwe, N.2007). Harris (2002) found that aqueous extracts of *A. afra* demonstrated a significant amount of smooth muscle relaxant effect, and also noted that flavonoid-rich fractions showed a greater bronchodilator effect. Similar to *A. afra, L.*

leonurus also contains a variety of flavonoids of which luteolin and hesperetin are the most abundant (Mulubwe, 2007).

Several mechanisms have been investigated for the way in which flavonoids may be able to produce smooth muscle relaxation. They have been documented to block calcium ion channels, activate cGMP/PKG pathway, inhibit the release of histamine, xanthine oxidase, protein kinase C and phosphodiesterase (Yu and Kuo, 1995; Hayashi et al., 1988; Ferriola, 1989; Kuppusamy and Das, 1992). Another mechanism may involve potassium channels, specifically K_{ATP} and BK_{ca} channels. For instance, Liu et al., (2008) demonstrated that isoliquiritigenin relaxes tracheal smooth muscle by stimulation of the cGMP/ PKG signalling cascade, resulting in the opening of the above mentioned potassium channels thus leading to smooth muscle relaxation.

 Given the above arguments, the overall aim of this study was to determine whether *A. afra* and *L. leonurus* produced their anti-asthmatic effects in and by similar means. The specific objectives were therefore to compare the respiratory smooth muscle relaxant effects of freeze-dried aqueous extracts of *A. afra* and *L. leonurus* on tracheal smooth muscle contracted with mediators of asthma (i.e. histamine, carbachol and potassium chloride). It was hypothesized that the flavonoid levels and profile of *A. afra* would be greater than and different to the flavonoid levels and profile of *L. leonurus*, *A. afra* would have a more potent respiratory muscle relaxant effect than *L. leonurus* and that *A. afra* and *L. leonurus* will inhibit K^+ -induced (i.e. K_{ATP} channel mediated) contractions in a superior manner than carbachol and histamine induced contractions.

.

Chapter 2

Literature review

2.1   Introduction

In this chapter a brief description of the selected plant species *Artemisia afra,* and *Leonotis leonurus* is given as well as the definition of asthma, current treatment and challenges of asthma therapy. In addition this chapter also discusses the chemistry, classification, biological activity and the analytical techniques suitable for the determination of flavonoids. It includes a brief description of the flavonoid luteolin with focus on its biological activities, pharmacokinetics and possible mechanisms of action as well as the rational for the use of isolated guinea pig trachea method for the investigation of *A. afra* and *L. leonurus* respiratory smooth muscle relaxant effects.

2.2   Asthma

2.2.1   Definition of asthma

Asthma is defined based on its pathological, clinical and physiological characteristics with emphasis placed on chronic inflammation and hyper-responsiveness (Harkness et al., 2015; Murdoch and Lloyd, 2010). According to Global Initiative for Asthma (GINA), asthma is a chronic inflammatory condition of the airway which leads to hyper-secretion of the mucus glands, bronchoconstriction, and increased hyper-responsiveness of the airways, with involvement of many cells in particular mast cells, eosinophils and T lymphocytes. This chronic inflammation leads to recurrent episodes of breathlessness, reversible airflow obstruction, wheezing, chest tightness and coughing, particularly at night or in the early morning (GINA, 2015).

2.2.2   Aetiology of asthma

Asthma pathogenesis develops as a consequence of complex interactions of genetic susceptibility and environmental influences i.e. timing and dose of allergen and co-exposure to infection. This ends in an inappropriate inflammatory response from a wide variety of inflammatory cells and mediators (Bateman et al., 2008; Murdoch and Lloyd, 2010). Asthmatic attack consists of two phases, the immediate and the late (delayed) phase. The immediate phase of allergic asthma mainly results in bronchial spasms, mucous hyper-secretion and mucosal oedema. During this phase, re-exposure to an antigen leads to antigen-antibody interaction with antigen presenting cells (APC), dendritic cells, macrophages and mast cells on airway epithelial cells leading to release and synthesis of various mediators such as histamine, leukotrines (C_4 and D_4), prostaglandins D_2 and tryptase (Maddox and Schwartz, 2002; Robinson, 2010). The late phase occurs 2-8 hours after allergen inhalation and lasts up to 24 hours. It is associated with cellular infiltration (predominantly eosinophils and lymphocytes) and the progressive interaction of these cells with local cells (e.g., mast cells, macrophages, epithelial cells, endothelial cells) creates a cascade of events that contributes to the chronic inflammation and clinical manifestations associated with asthma (Holgate, 2011; Khan et al., 2008; Maddox and Schwartz, 2002). Mediators produced by this interaction are cytokines characteristically produced by T helper lymphocytes type 2 (Th2), particularly interleukins 4, 5, 9 and 13, eosinophils and the chemokine IL-8. In turn, these mediators attract other inflammatory cells, thereby contributing to further inflammation, sustained bronchoconstriction and mucus hyper-secretion (Maddox and Schwartz, 2002; Pelaia et al., 2015). Asthma due to antigen challenge is very common, but in some instances bronchoconstriction can be caused by non-antigenic stimuli such as exercise, distilled water, cold water and sulphur dioxide. This is called non-specific bronchial hyper reactivity to distinguish it from bronchial responsiveness to specific antigens (Maddox and Schwartz, 2002; Rolph et al., 2006; Szczeklik and Stevenson, 2003).

2.3   Treatment of asthma

The main aim of asthma therapy is to control current asthma impairment and reduce future exacerbations. Anti-asthmatic drugs are grouped into two categories, anti-inflammatories (inhibits   and   prevents   inflammatory   process)   and   bronchodilators   (reverse

bronchoconstriction) (Barnes, 2006). Within the current treatment guidelines, seven classes of drugs have been adopted, these include β_2-adrenoceptor agonists (sympathomimetics), corticosteroids, selective muscarinic antagonists (anti-cholinergics), cromones (cromoglycate and necrodomil), xanthines (including theophylline - a precursor to the search for other phosphodiesterase (PDE) inhibitors, particularly of PDE4), cysteinyl leukotriene antagonists and the anti-IgE monoclonal antibody (Bateman et al., 2008). Drugs in common use are adrenoceptor agonists (used as relievers or bronchodilators) and inhaled corticosteroids (used as controllers or anti-inflammatory agents) (Barnes, 2006; GINA,2015).

2.3.1 Bronchodilators

Drugs falling under this category include β_2 - adrenoceptor agonists (sympathomimetic), xanthines, muscarinic receptor antagonists and cysteinyl-leukotriene receptor antagonists.

2.3.1.1 B$_2$-adrenoceptor agonists

The β_2-adrenoceptor agonists are the most used bronchodilators for the treatment of asthma. They cause airway smooth muscle relaxation by acting on β_2-adrenoceptors thereby stimulating adenylyl cyclase and increasing the formation of cAMP. They also inhibit tumor necrosis factor (TNF-α) and release of mediators from mast cells. Moreover, they increase mucus clearance by cilia action (Rang and Dale, 2015). There are different routes of administration for adrenoceptor agonists (i.e. oral, parenteral and inhalation) but delivery by inhalation is the preferred route due to it having the least systemic toxicity and the greatest local effect on airway smooth muscle. B$_2$-adrenoceptor agonists cause tachycardia and skeletal muscle tremor as toxic effects (Barnes, 2006; Pera and Penn, 2014; Rang and Dale, 2015).

The β_2-adrenoceptor agonists can be divided into short acting and long acting β_2 adrenoceptor agonists (SABAs and LABAs, respectively). SABAs are given by inhalation and used on an 'as needed' basis to control symptoms. Their effect occurs within 30 minutes and lasts for duration of 4-6 hours. Some degree of tolerance can occur if used for more than 2-3 weeks, but it can be reversed by parenteral steroids. Similar to SABAs, Long acting agents are also

given by inhalation, but due to their high lipid solubility which allows them to dissolve in smooth muscle cell membrane in high concentrations, the duration of action is 12 hours. These drugs interact with corticosteroids to improve asthma control. Unlike SABAs, long acting agonists are not used as needed, but are given regularly, twice daily as adjunctive therapy in patients whose asthma is inadequately controlled by glucocorticoids (Barnes, 2006; Bateman et al., 2008; Rang and Dale, 2015).

2.3.1.2 Muscarinic receptor antagonists

The cholinergic system is regarded to play a significant role in asthma. The binding of acetylcholine on muscarinic receptors in the airways induces a conformational change on the receptors which activates a cascade of events that leads to stimulation of airway smooth muscle and mucus glands, with subsequent bronchoconstriction and mucus secretion (Bateman et al., 2008). Muscarinic antagonists efficiently block the contraction and increased mucus secretion caused by vagal activity. They do not discriminate between muscarinic receptor subtypes, and it is likely that their blockade of M_2-autoreceptors on the cholinergic nerves increases acetylcholine release and decreases the efficiency of their antagonism at the M_3- receptors on the smooth muscle (Bateman et al., 2008; Rang and Dale, 2015).

2.3.1.3 Methylxanthines

Methylxanthines are an exceptional class of drugs for treatment of asthma. They demonstrate efficacy in reducing the three principal features of asthma (reversible airflow obstruction, airway hyper-responsiveness, and airway inflammation). Bronchodilatory effects of methylxanthines are identified at doses at which toxic side effects are sometimes observed (Page and Barnes, 2004). At lower concentrations, methylxanthines are weak bronchodilators but retain their capacity as immune-modulators, anti-inflammatory, and broncho-protective drugs (Barnes, 2010). Several mechanisms of action have been suggested for the detected bronchodilatory and immunomodulatory effects of methylxanthines in the asthmatic airway. Phosphodiesterase (PDE) inhibition and adenosine receptor antagonism are the most established. Effects on mucociliary transport, inhibition of immune cell activation and proliferation and reduction in pro-inflammatory gene expression via the induction of histone deacetylase (HDAC) activity have also been reported (Rang and Dale, 2015; Tilley, 2011).

7

2.3.1.4 Cysteinyl-leukotriene receptor antagonists

Cysteinyl-leukotriene receptor antagonists (LTRAs) are a fairly new form of asthma treatment that exerts bronchodilation and anti-inflammatory effects by blocking cysLT1 receptors (Korenblat, 2000). Cysteinyl-leukotrienes (cysLTs) are lipid mediators synthesized from arachidonic acid by various cells, mostly eosinophils, mast cells, basophils and macrophages (Mastalerz and Kumik, 2010; O'Byrne et al., 2009). They trigger contractile and inflammatory processes through the specific interaction with cysLT1 receptors on target cell surfaces. They are also responsible for mucous hyper-secretion, micro-vascular leakage, eosinophil chemotaxis and airway remodelling. They are involved in both immediate and late asthmatic responses induced by allergen exposure (Niimi, 2013). This class of antagonist drugs that blocks the binding of leukotrienes to $cysLT_1$ receptors, has been given the generic suffix –lukast, and drugs based on these are known as leukotriene receptor antagonists. Three of these (i.e. montelukast, pranlukast and zafirlukast), together with 1 leukotriene synthesis inhibitor (zileuton), have been approved for use in the treatment of asthma (Korenblat, 2000; O'Byrne et al., 2009). LTRAs are utilized as second- to third-line controller medication of persistent asthma and are highly safe and can be taken orally.
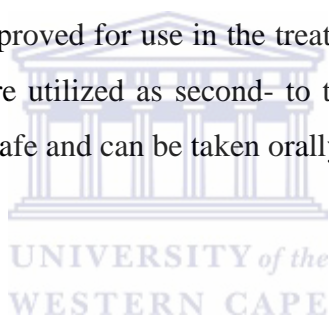
2.3.2 Anti-inflammatory

There are two different types of anti-inflammatories used in the treatment of asthma. Glucocorticoids which are used mainly in chronic conditions and sodium cromoglycate which are thought to affect their action by reducing bronchial hyper-reactivity.

2.3.2.1 Glucocorticoids

Glucocorticoids have become the predominant form of therapy for mild, moderate and severe persistent asthma due to their widespread anti-inflammatory properties. They mimic the action of the endogenous hormones (e.g. cortisol) that are involved in the regulation of the inflammatory response in the airways (Barnes, 2000; Rang and Dale, 2015).

Glucocorticoids bind to the glucocorticoid receptor (GR) on target cells, and then the corticosteroid-receptor complex is rapidly transported to the cell nucleus where it alters the transcription of proteins associated with inflammation. Glucocorticoids also block production

or release of cytokines (in particular the Th2 that recruit and activate eosinophils), decrease the expression of adhesion molecules and influence the presence of cells that mediate inflammation. In terms of response, after the corticosteroid molecule enters the cell, hours or even days may elapse before significant quantities of new proteins are produced. This explains the delay in detecting the beneficial action of systemic corticosteroids (Barnes, 2010; Bateman et al., 2008). Glucocorticoids are not effective in the treatment of the immediate response to the eliciting agent and do not reverse airway smooth muscle directly. However, they do reduce bronchial reactivity, frequency of asthma exacerbations, asthma symptoms, improve health-related quality of life, improve lung function, and decrease airways hyper-responsiveness (AHR) (Barnes, 2010; Bateman et al., 2008; Rang and Dale, 2015). In the management of chronic asthma, in which there is predominant inflammatory component, their efficacy is unequivocal.

2.3.2.2 Sodium cromoglycate and nedocromil

They are not bronchodilators, they do not have any effect on smooth muscle nor do they inhibit any of the known smooth muscle stimulants. If given prophylactically they can reduce both the immediate and the late phase asthmatic responses and reduce bronchial hyper-reactivity (Bateman et al., 2008). They are effective in antigen, exercise, and irritant induced asthma. Though not all asthmatic subjects respond and it is not possible to predict which patients will benefit. Children are more likely to respond than adults. Therefore these agents have become the anti-inflammatory drugs of first choice in children (Barnes, 2006). Pre-treatment with one of the agents before exposure to an eliciting stimulus may be very effective in young patients. Cromolyn and nedocromil differ structurally but are thought to share a common mechanism of action, an alteration in the function of delayed chloride channels in the cell membrane inhibiting cellular activation. Nedocromil's action on the airway nerves is thought to be responsible for inhibition of cough, on mast cells inhibition of early response to antigen challenge and on eosinophils for inhibition of the inflammatory response to inhalation to allergens, the inhibitory effect on mast cell seem to be specific to cell type.

2.4   Challenges of asthma therapy

Current management of asthma is aimed at controlling disease symptoms and for the majority of asthmatics inhaled corticosteroids (ICS) with or without long-acting β$_2$-agonists in combination are effective. However, even when taken regularly, ICS do not appear to modify the course of the disease significantly, and they are not curative. Asthma symptoms and inflammation rapidly recur when the treatment is discontinued. All currently available ICS are absorbed from the lungs and thus have the potential for systemic side effects. This has led to a concerted effort to find safer ICS, with reduced oral bioavailability and reduced absorption from the lungs or inactivation in the circulation. Concern has also been expressed about the long-term safety of LABA, although when administered in combination with ICS, this does not seem to be a problem (Barnes, 2012). But this approach requires lifetime therapy, and a subset of patients remains symptomatic despite optimal treatment. Furthermore, over 80 % of patients have poor adherence with regular inhaled therapy. A major unmet need in asthma is thus the development of more effective treatment, especially for patients with severe asthma who are relatively corticosteroid-resistant. The other unmet need in asthma is to develop effective oral therapy for patients with mild and moderate disease (Quirce et al., 2012).
.
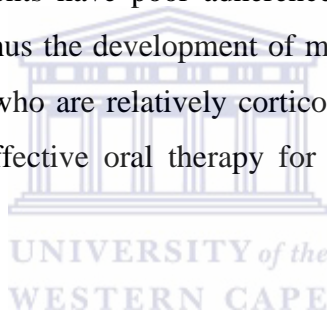
2.5   The need for new therapies

A major problem facing new drug development is that existing therapies for asthma, particularly combination inhalers, are highly effective, relatively inexpensive and safe, and there is a strong scientific rationale for this approach to asthma therapy (Page and Barnes, 2004). This poses an enormous challenge in discovering drugs that improve on existing therapy. Another problem is that small animal models of asthma are poorly predictive of efficacy in asthma. Most drugs that are effective in animal models have failed in clinical trials, and drugs that might be effective would not be identified by these models. However, there are still concerns about the current treatment (Barnes, 2012). The types of new drugs needed for asthma include new classes that are effective in severe poorly controlled asthma, an oral treatment that is as effective as inhaled corticosteroids but without any side effects, and drugs that modify the course or even cure the disease. The approaches that have usually

been taken are to improve existing treatments, such as ICS and LABA, or to find drugs against novel targets identified through better understanding of the disease process, such as cytokine blockers. An oral therapy might have an advantage in that it would treat not only asthma but also rhinitis and atopic dermatitis, which are commonly associated, although oral therapies have a much greater risk of side effects (Weatherall et al., 2010).

Many new treatments are specific, targeting a single mediator or receptor, and are unlikely to have a major clinical effect, although they might be effective in specific asthma phenotypes. Drugs with more widespread effects, such as kinase inhibitors, might be more effective, but have a greater risk of side effects (Barnes, 2010). New treatments targeting the underlying allergic/immune process would treat concomitant allergic diseases. Improved immunotherapy approaches have the prospect of disease modification, although prospects for a cure seem remote until the molecular and genetic causes of asthma are better understood. It has proved difficult to discover novel classes of therapy for asthma, despite intense effort and investment. Asthma is a very complex disease so it is unlikely that targeting a single receptor or mediator will be greatly effective (Barnes, 2010). The chronic nature of asthma and lack of preventive and curative therapy is leading researchers and asthma patients to seek alternative medicine treatment.
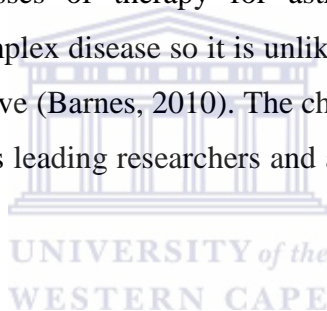
2.6   Herbal medicine

The World Health Organization (WHO) estimates that herbal medicines provide primary healthcare for approximately 3.5 to 4 billion people worldwide. Today, approximately 80 % of antimicrobial, cardiovascular, immunosuppressive, and anticancer drugs are of plant origin, their sales exceeded US$ 65 billion in 2003 (Gordaliza, 2009). It is widely accepted that more than 80 % of drug substances are either directly derived from natural products or developed from a natural compound (Dias et al., 2012). During the period 1981 to 2006, 47.1 % of a total of 155 clinically approved anticancer drugs were derived from nature in North America, Europe, and Japan market (Newman and Cragg, 2007). In China, at least 130‑140 new drugs, either single chemical entities extracted from herbal medicines or synthetically modified compounds, are currently in clinical use (Pan et al., 2013). Some examples are

anisodamine (Poupko et al., 2007) indirubin (Suzuki et al., 2005), huperzine (Desilets et al., 2009), and bicyclol (Pan et al., 2013).

The historical importance of herbal medicine in the treatment of asthma is indisputable. Four of the five classes of drugs currently used to treat asthma namely, β_2-agonists, anti-cholinergics, methylxanthines and cromones have origins in herbal treatments going back at least 5000 years (Bielory and Lupoli, 1999). The development of modern medicine still draws on clinical experiences from traditional medicines and therapies. In general, herbal medicines with a long history and theory of use in clinical settings should be more promising candidates for drug discovery (Pan et al., 2013).

Based on their historical pattern of use and claimed efficacy, we suspect that *A. afra* and *L. leonurus* have good potential to possess anti-asthmatic activity. These two plants have an apparent widespread traditional use for bronchial conditions, a vast pattern of distribution and are easily obtainable by most people. These studies are necessary prerequisites for benefit/risk balance assessment of traditional practices and for formulation of standardized anti-asthmatic herbal drugs (WHO, 2002).
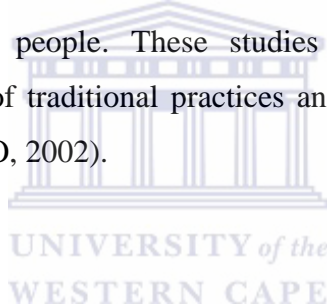
2.7   Study of *Artemisia afra* and *Leonotis leonurus*

2.7.1   *Artemisia afra*

Artemisia afra is one of the oldest, best known and most widely used of all the indigenous medicines in Southern Africa (Thring and Weitz, 2006). The different or common local names describing this plant may be attributed to the extensive use by different ethnic groups (Watt and Breyer-Brandwijk, 1962). In the Xhosa it is known as "Umhlonyane", in Zulu "Mhlonyane", in Sotho "Lanyana", Tswana "Lengana", in English "African wormwood" and in Afrikaans "Wilde als".

Figure 2.1: *Artemisia afra* **in its natural habitat at Montague gardens,**
          South Africa

2.7.1.1 Botanical description

A. afra is a highly aromatic plant. It is an erect multi-stemmed perennial shrub which grows up to two metres in height (Van Wyk et al., 1997). The feathery leaves are of soft texture, dark green on the adaxial surface and a lighter green on the abaxial surface, reaching a length of 8 cm and a width of 4 cm. *A. afra* blossoms from January to June, producing yellow, butter-coloured flowers with abundant bracts. In winter the branches die but rapidly regenerate from the base (Hilliard, 1977; Van Wyk et al., 1997).

2.7.1.2 Taxonomy

A. afra is classified into the Kingdom: Plantae, Division: Mannoliphyta, Class: Magnoliopsida, Sub-class: Asteridae, Order: Asterales, Family: Asteraceae, Genus: *Artemisia* and Species: *Artemisia afra* (Jackson, 1990).

2.7.1.3 Distribution

A. afra is widely distributed in Southern Africa, (i.e. South Africa, Namibia, and Zimbabwe). In South Africa, it grows in the northern provinces of Gauteng and Limpopo along the eastern parts of South Africa, including Swaziland and Lesotho, to the Western Cape in the south. It

is also abundantly found in KwaZulu-Natal province from the coast to the Drakensberg (Van Wyk et al., 1997; Hilliard, 1977) at altitudes of 20 to 2440 m on damp slopes, along streamside's and forest margins.

Figure 2.2: Geographical distribution of *A. afra* in South Africa (Adapted from SATMERG Pharmacopoeia, Monographs Project, 1999).

2.7.1.4 Medicinal uses

A. afra is traditionally widely used for numerous ailments including colds, coughs, malaria, convulsions, headache, inflammation, stomach disorders, whooping cough, colic, heartburn, diabetes mellitus, bronchitis and asthma among others (Roberts, 1990; Thring and Weitz, 2006; Van Wyk et al., 2000; Watt and Breyer-Brandwijk, 1962).

Many different preparations of this plant are employed to treat the various symptoms and ailments. Syrup prepared from *A. afra* is used for bronchial troubles, while infusions or decoctions can be applied as a lotion to bathe haemorrhoids and for earache. An infusion of leaves or roots of this species is also used for the treatment of diabetes and respiratory problems in the Eastern Cape Province of South Africa. Respiratory infections are normally treated through inhaling the vapour from boiling leaves and this vapour is also used to treat menstrual chill. Fresh tips are inserted into the nose for colds and headaches and into hollow teeth to treat toothache (Erasto et al., 2005; Mahop and Mayet, 2007).

2.7.2   *Leonotis leonurus*

L. leonurus is known under different vernacular names such as wild dagga; wilde dagga; lebake; umfincafincane and umhlahlampetu in English, Afrikaans, Zulu, Sotho, Xhosa and Shona, respectively.

Figure 2.3: *L. leonurus* **in its natural habitat at Montague gardens, South Africa.**

2.7.2.1   Botanical description

This attractive plant species is a shrub of two to five meters in height with thick, woody base and pale brown branches. All parts of the plant have a strong smell. The leaves are opposite each other on the stems that are long and narrow, toothed in the upper half and distinctly hairy. *L. leonurus* has bright orange, tubular flowers that are borne in characteristic rounded groups and are neatly arranged along the branch ends. The hairy flowers resemble lion's ears, hence the name 'leonurus' which means lion's ears (Van Wyk et al., 2000).

2.7.2.2   Taxonomy

Leonotis leonurus is classified into Kingdom: Plantae, Order: Lamiales, Family: Lamiaceae Genus: *Leonotis*, Species: *L. leonurus* (Van Wyk et al., 2000).

2.7.2.3   Distribution

L. leonurus has a wide natural distribution over large parts of South Africa and has become a popular garden plant (Van Wyk et al., 2000).

2.7.2.4   Medicinal uses

Numerous traditional uses have been reported for *L. leonurus*. It is used to treat asthma, colds, influenza, bronchitis, high blood pressure, headache and viral hepatitis. In addition, it is used to treat boils, eczema, skin diseases, itching, muscular cramps, snakebite, other bites and stings (Van Wyk and Gericke, 2000). *L. leonurus* leaves and stems are mainly used, but sometimes also the roots. Decoctions of *L. leonurus* are used externally for skin problems and internally, either by mouth or as an enema, to treat coughs, colds, fever, headaches and high blood pressure. In addition, leaf infusions have been used for asthma and viral hepatitis (Thring et al., 2006; Van Wyk et al., 2000). Because it is only mildly narcotic, there are reports of the plant being smoked as a substitute for dagga. It has, however, been smoked for the relief of epilepsy. In Europe, this traditional phytomedicine is used for the symptomatic treatment of coughs in acute bronchial disease (Van Wyk and Gericke, 2000).
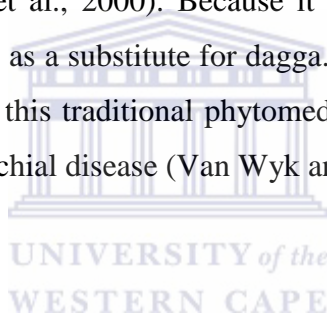
2.8   Common features of *Artemisia afra* and *Leonotis leonurus*

A. afra and *L. leonurus* are amongst the most popular used medicinal plants in South Africa. They are traditionally used to treat many conditions such as asthma (Thring and Weitz, 2006). However, proper scientific studies to validate these claimed uses are lacking. The few studies conducted on *A. afra* have reported it to possess smooth muscle relaxant properties (Harris, 2002).

For these plants to be developed into new drugs that meet the criteria of modern medicine, they have to be thoroughly investigated. For effective research on these plants to be conducted, various systems such as animals, tissue culture techniques and cell lines need to be used.

2.9   Evaluation of asthma medication using animal models

Asthma is a uniquely human disease and, with the exception of perhaps cats (eosinophilic bronchitis) (Reinero, 2011; Venema and Patterson, 2010) and horses (heaves) (Herszberg et al., 2006), no animals, including those commonly used to study asthma (mice, rats, guinea pigs and rabbits) naturally exhibits an asthma like syndrome that is similar to the disease in humans. The utility of animal models of asthma to understand human disease and to develop therapeutics is the topic of considerable debate (Holmes et al., 2011; Persson et al., 1997). Several species and study designs have been used to try and model human asthma, suggesting that there is no generally accepted model of the human disease. Species and strain differences, variations in sensitisation and challenge schedules, and the method by which drugs are delivered to the lungs (e.g. intranasal or intra-tracheal instillation of solutions, nebulised compounds, or dry powders) are all parameters that can influence the outcome of a study. The extent to which these factors affect study outcomes, and the potential impact that they have on the relevance of these models to humans, is difficult to determine. Little information exists in the publically available literature, suggesting that further research to better characterise these parameters is required. Nonetheless, for obvious ethical reasons the types of experiments required to dissect accurately the mechanisms that are involved at cellular and molecular levels are not possible in humans.

Animal models of asthma have been in use for more than 100 years (Karol, 1994). While, as with any model of disease, the link between the processes observed in the animal and humans needs to be carefully considered, they are an ideal vehicle for identifying and testing mechanisms linked to the development of the asthmatic phenotype. The species chosen and method used dependents highly on the specific aspect of the disease that is of interest. Some species or strains are better for modelling particular characteristics of asthma than others. Out of all the species, mice, rats as well as guinea pigs are the most commonly employed models for studying the effect of drugs on respiratory smooth muscle (Brewer et al., 1999; Zhang, 2008).

2.9.1 Mouse model of asthma

Mouse models of allergic airway disease offer numerous advantages when compared to the use of other animals. Immunoglobulin E (IgE) is the primary allergic antibody in mice, making this species appropriate for investigation of the role of humoral immune factors in allergic airway disease. Further, mouse models offer the opportunity to explore detailed mechanisms of allergic reactions because of the availability of numerous immunological reagents such as antibodies against cytokines, growth factors, and cell surface markers (Shapiro, 2008). Moreover, the emerging technologies involving gene manipulation in animals are well-advanced in mice. The ease of breeding and short gestational period is an additional advantage. Accordingly, major advances in the understanding of the disease concept, asthma as Th2 dominant disease emerged from studies in mice. A disadvantage with mouse models is the considerable physiologic differences that exist between mice & humans (Canning and Chou, 2008; Shapiro, 2008).

2.9.2 Rat models of asthma

Rats are also popular as models of allergic airways disease. Like mice, they are relatively cheap, which allows large scale studies on multiple outcomes to be conducted. Historically, as experimental models, rats were more popular than mice, but have been overtaken in recent years due to the rapid expansion of genetic technologies associated with mice (Holmes et al., 2011). Rats represent a significant advantage over mice due to their larger size. This makes it easier to measure the classic characteristics of allergic airways disease such as airway and systemic markers of inflammation due to an increase in the volume of serum and bronchoalveolar lavage (BAL) that can be obtained (Holmes et al., 2011; Karol, 1994). This larger size and their increased stability under anaesthesia may also be seen as an advantage in terms of measuring physiological outcomes such as airway hyper- responsiveness and acute responses to allergen inhalation. Rats are often used as a standard model for testing new drug therapies; in particular, the efficacy and toxicity of new drugs are often tested in rats before proceeding with clinical trials. However, it should be highlighted that rats have lower sensitivity to bronchoconstrictors (i.e. more agonist is necessary to produce a narrowing of the airways) than guinea pigs (Hylkema et al., 2002; Karol, 1994; Martin and Tamaoka, 2006).

2.9.3 Guinea pig model of asthma

The guinea pig model was the first described model of asthma and contributed greatly to the development of receptor agonist therapies. Many fundamental processes, mediators and regulators of airway disease pathogenesis were discovered or demonstrated first in guinea pigs, including the Schultz–Dale (immediate type hypersensitivity) reaction, the actions of histamine, the cysteinyl-leukotrienes and their two receptors, beta adrenoceptor subtypes, thromboxane, alveolar macrophage derived neutrophil chemotactic factor(s) and the roles of cyclic adenosine monophosphate (cAMP) and inositol triphosphate in signal transduction (Jones et al., 1988; Suckow et al., 2012). Receptor pharmacology in guinea pigs more closely matches that of human receptor pharmacology than most other commonly used species (Canning and Chou, 2008; Muccitelli et al., 1987; Ressmeyer et al., 2006). Several breakthroughs in measuring lung mechanics were first made in studies using this species, while models of the late phase response following an allergen challenge have been perfected in guinea pigs (Amdur and Mead, 1958; Meurs et al., 2006). However, the emergence of transgenic mouse studies has and will continue to result in the diminished use of guinea pigs for modelling airways disease. This is unfortunate, as for many endpoints guinea pigs are superior to mice for studies of processes related to asthma (Canning and Chou, 2008; Meurs et al., 2006; Persson et al., 1997).

2.10 Techniques to measure the effect of plants in asthma

For evaluations of pharmacological effect of plants with anti-asthmatic effect, three types of methods can be used, i.e. *in-silico, in vivo* and *in vitro* methods.

2.10.1 *In-silico* models

Computer simulations used to generate virtual ligand-receptor interactions, cells, respiratory tracts, and patients are the newest trend in modelling allergic asthma. *In-silico* screening to identify novel drugs might become a useful tool and can be divided into virtual drug discovery, virtual organs and virtual patients.

2.10.1.1 Virtual drug discovery

An example of virtual drug discovery in allergic asthma is a computational screening of ligand-receptor interactions based on 3-D structure of the X-ray analysis of a particular binding site (Patel, 2014). This creates a 3-D 'pharmacophore' model, which generates a pattern of spatial and chemical constraints of the ligand. This approach was used to produce non-peptidic antagonists of Very Late Antigen-4 (VLA-4), an adhesion molecule involved in cell-cell interactions (Patel et al, 2014).

2.10.1.2 Virtual organs

A variety of computer simulations of lungs have led to the so called, 'lung physiome' or 'virtual lung'. These simulations provide physiological measurements of healthy and asthmatic lungs, and can be used for testing drug efficacy. One example is the simulation of a 3-D human respiratory tract using magnetic resonance imaging (MRI) and a single-photon emission computed tomography (SPECT) images from lungs and thorax. The virtual respiratory tract enables one to evaluate lumen diameter based on bronchoconstriction, inflammation and mucosal thickness and has been used to predict the efficiency of aerosol drug delivery in healthy versus asthmatic subjects (Patel et al , 2014).

2.10.1.3 Virtual patients

The virtual allergic asthmatic patient promises to be the next generation of *in-silico* model. One example is the virtual rat lung model developed to determine the effects of environmental pollution on lung function. A second example is a computer-based mathematical model of airway structure and function, inflammation, and immune responses to create virtual asthmatic patients. A major aim of the virtual patient model is to reduce the cost of getting new drugs to market (Patel et al, 2014).

2.10.2 *In vitro* methods

There are several *in vitro* methods that are used that are based on the measurement of:

2.10.2.1 Reactivity of the isolated perfused trachea

The method is used to evaluate the effect of the epithelium on trachea muscle reactivity. Contractile agonists are either added on the serosal or mucosal surface in stepwise increasing cumulative concentrations. The trachea from the animal is removed, attached to a stainless steel perfusion holder and placed in an organ chamber filled with Krebs solution. The solution is maintained at 37 °C and aerated with 95 % O_2 plus 5 % CO_2. The solution is also pumped at a constant rate through the mucosal lumen. The inlet and outlet catheters are connected to the positive and negative sides, respectively, of a force transducer. Responses are quantified as ΔP in centimetres of H_2O. Responses of the musculature are obtained by measuring changes in inlet-outlet ΔP between side holes of indwelling catheters. Geometric EC_{50} values are determined from least square analysis of a logit model and are presented along with 95 % confidence intervals (Fedan et al., 2001; Vogel, 2002).

2.10.2.2 Bronchial perfusion of isolated lung

This method has been used to evaluate pharmacological reactions of sympathomimetic drugs on bronchiolar muscle. The trachea and lungs of the animal are removed and the trachea is attached to a cannula of a perfusion apparatus which pumps the solution at a constant rate in a monometric tube connected with the perfused organ. Only one lung is perfused, the other is tied off. The lower part of the lower lobe is cut off and the rest of the lung surface is scratched deeply assuring maximum pre-medication flow. Liquid is poured down the trachea through the bronchi and escapes the lung through the alveoli. Resistance to flow (bronchoconstriction) results in an increase in the height of the column of fluid in the manometer. The intensity of the bronchodilator effect is measured by the fall of the column in the manometer. (Vogel, 2002)

2.10.2.3 Vascular and airway responses in the isolated lung

The isolated perfused rat lung method allows the simultaneous registration of pulmonary, vascular and airway responses to various drugs. Pulmonary arterial perfusion pressure, airway pressure, and reservoir blood level are continuously monitored, electronically averaged and recorded. The lungs are ventilated by a certain amount of negative pressure and a tidal volume. Artificial thorax chamber pressure is measured with a differential pressure transducer, and air flow velocity with a pneumotachograph tube connected to a differential pressure transducer. The arterial pressure is continuously monitored by means of a pressure transducer which is connected with the cannula ending in the pulmonary artery. All data is transmitted and analysed on a computer (Fedan et al., 2001; Vogel, 2002).

2.10.2.4 Spasmolytic activity in isolated trachea

The isolated trachea model is used to evaluate the compounds ability to induce bronchodilation or prevent bronchoconstriction. Tracheas from the animals are dissected and prepared free of adhering fat and connective tissue. These prepared trachea tissues are mounted in organ baths filled with Krebs solution. The solution is maintained at 37 °C and aerated with 95 % O_2 plus 5 % CO_2. To achieve steady spontaneous tone level, an initial tension of 1-2 g is applied for at least 1 hour with washing at 15-minute intervals. Bronchospasm is induced on the respiratory smooth muscle. If the compound induces relaxation, bronchospasm of the respiratory smooth muscle will reduce upon administration of the compound. When an increase in concentration produces no further increment in response, the tissue is washed to re-establish the baseline tension. Cumulative concentration curves are constructed and percentage inhibition of the spasmogens induced contractions is calculated. From dose-response curves EC_{50} values are calculated (Blume and Davies, 2013; Englert et al., 1992; Vogel, 2002).

2.10.3 *In vivo* methods

2.10.3.1 Broncho-spasmolytic activity in anesthetized guinea pigs (Konzett-Rössler method)

The method is based on registration of air volume changes of a living animal in a closed system consisting of the respiration pump of the trachea and the bronchi as well as of a reservoir permitting measurement of volume or pressure of excess air. Administration of spasmogens like acetylcholine, histamine, and bradykinin results in contraction of bronchial smooth muscle, bronchospasm decreases the volume of inspired air and increases the volume of excess air. Thus, the degree of bronchospasm can be quantified by recording the volume of excess air. This method permits the evaluation of a drug's bronchospasmolytic effect by measuring the volume of air, which is not taken up by the lungs after bronchospasm. The results from the bronchial constriction caused by spasmogens are expressed as percentage inhibition of induced bronchospasm over the control agonistic responses. From this, the ED_{50} value is calculated (Blume and Davies, 2013; Bonfield, 2012).

2.10.3.2. Bronchial hyper reactivity method

In this method guinea pigs are introduced to bronchospasm inducing agents such as histamine through aerosols produced by an ultra-sound nebulizer so as to mimic anaphylactic convulsions resembling bronchial asthma in patients. The animal is placed in an inhalation cage to which the test drug or substance is introduced using an ultra-sound nebulizer. Alternatively the animal is treated orally or subcutaneously with the test drug or substance. The animal is then passed to another inhalation cage where it is exposed to bronchospasm inducing agents and time until appearance of anaphylactic convulsions is measured. Symptoms first observed include increased breathing frequency, forced inspiration, and finally anaphylactic convulsions. The introduction of antagonistic drugs can then delay the occurrence of these symptoms. Evaluation is determined from the percentage increase of pre-convulsion time versus controls and ED_{50} values can be calculated, i.e. 50 % increase of pre-convulsion time (Blume and Davies, 2013).

2.10.3.3. Pneumotachography in anesthetized guinea pigs method

The use of a pneumotachograph is based on the principle of the Fleisch – tube and of additional pressure transducers. It allows simultaneous measurements of pulmonary mechanics, respiratory parameters and circulatory parameters in anesthetized guinea pigs (De la Motta, 1991). A cannula is inserted to the trachea then connected with pieces of tubing to a Fleisch tube (pneumotachograph). The Fleisch tube is connected with sensitive differential pressure transducers, both of which are connected to a pre-amplifier. The signals for airflow are monitored at the output of the pre-amplifier with a digital 2-channel oscilloscope. The signals are presented at the output of the instrument as analogue electrical signals on a computer. For each individual experiment, data of the last 5 minutes before the first substance application are averaged and used as controls. The response values after substance application are then expressed as percentages of the controls. For an analysis of the results the response values are averaged over certain time intervals (Glaab et al., 2007; Vogel and Vogel, 2013).

2.10.3.4. Airway micro-vascular leakage method

In this method Evans Blue dye and radio labelled albumin are used to determine plasma exudation in guinea-pig airways. This method can be used to study the antagonism against bradykinin- and platelet-activating factor-induced airway micro-vascular leakage and vagal stimulation-induced airway responses (Rogers et al., 1989; Vogel, 2002). The animal is anesthetized then a tracheal cannula inserted in the lumen of the cervical trachea, a catheter into the left carotid artery to monitor blood pressure and heart rate and another catheter into the external jugular vein for administration of drugs. The test compound (antagonist) is given intravenously. Ten minutes later Evans Blue dye is injected intravenously for 1 minute. Bronchoconstriction and micro-vascular leakage is induced by inhalation or injection of bradykinin. The thoracic cavity is then opened 6 minutes later, and a cannula inserted in the aorta through a ventriculotomy in order to remove the intravascular dye from the systemic circulation. The lungs are removed, blotted dry and Evans Blue dye extracted. Between the treated and control groups, Evans Blue dye concentration as well as lung resistance are compared by statistical means (unpaired Student's t-test or Mann- Whitney U test).

2.10.3.5. The effects of respiratory depressant drugs in conscious rats method

This is a non-invasive method for distinguishing central from peripheral nervous system effects of respiratory depressant drugs in conscious rats using gas and sodium cyanide exposures and carotid body denervation. Group means and standard errors of the (Fedan et al., 2001) means (SEM) are presented for all quantitative data. Mean values of treated groups are considered to be statistically different from the control group if the calculated P value is < 0.05 (Vogel, 2002).

In silico, *in vitro* and *in vivo* methods all have their various advantages and disadvantages. In this study an *in vitro* spasmolytic activity in isolated trachea method was used.

2.11   Trachea preparations

Although there are marked differences between species, with regard to innervating, receptors and neuromodulatory mechanisms, dissected guinea pig trachea tissue immersed or superfused by isolated tissue bath techniques provides important ways to study the effects of drugs on the neural pathways, or on the smooth muscle directly. The guinea pig trachea is generally accepted as a relevant and sensitive model of human large and central airways, which enable the screening of new compounds with potential therapeutic effect. There are two main techniques in which the trachea can be prepared for testing of smooth muscle activities, immersion techniques and superfusion techniques.

2.11.1   Immersion techniques

The isolated trachea tissue is immersed in an isolated tissue bath warmed to 37 °C from a constant temperature circulator. The tissue is suspended in Krebs-Henseleit (KH) solution and fixed to the bottom of the bath chamber on one side, and on the other side to an isometric force transducer for measurement of change in force as the tissues contract. A mixture of 95 % O_2 and 5 % CO_2 is continuously bubbled from the bottom of the bath chamber for oxygenation, mixing and pH maintenance (Uhlig and Taylor, 2012). Some examples of immersion techniques include tracheal chain, spirally cut trachea, the zig-zag trachea strip and trachea tube preparation.

2.11.1.1   Trachea chain

Trachea chain preparation was introduced by Castillo de Beer and initially used to study the relaxant effects of relaxant drugs, especially those acting on the β_2-adrenoceptors. The guinea pig trachea is dissected and then divided into four to six trachea rings, which are tied together by a cotton thread. These chains are then immersed in the isolated tissue bath as described above. Contractile agents (i.e. histamine, acetylcholine, carbochol, KCL) or relaxant drugs (i.e. β_2-adrenoceptor agonists, methylxanthines, papaverine, sodium nitoprusside), are introduced in the tissue bath in 0.5 log unit increments, and from the isomeric tension changes, cumulative concentration response curves are generated. Contractile or relaxant responses are expressed as percentage values of the maximum response caused by a reference compound. Disadvantages of the trachea chain preparation are the tedious process of tying the trachea chains together and the small maximum responses recorded, compared to that observed with other trachea preparations. Advantages are that the system is sensitive to changes in response (Castillo and De Beer, 1947; Uhlig and Taylor, 2012).

2.11.1.2   Spirally cut trachea

This is a changed and enhanced technique, which disposes of the time intensive procedure of entwining the tracheal rings and which has the extra advantage of creating more prominent force. The trachea is sliced diagonally to deliver a few fragments, each containing up to five trachea rings. By means of stainless steel wire introduced into the tracheal lumen, spirally structured strips can be formed briefly by cutting the connective tissue to separate the rings in a way that they are still held together by a little portion of the connective ligament between every single ring. After mounting the twisting strips in tissue baths containing KH solution under resting tension of (e.g. 1-2 g) and development of unconstrained tone, isometric compressions to cumulative added spasmogens, can reproducibly be measured for 6 hours. Advantages of this method are the faster rate of preparation and the significant improvement of force. Anderson and Lee showed the resistance of bronchodilator medications *in vitro* by utilizing the spirally cut trachea (Uhlig et al., 1998).

2.11.1.3 Zig-zag trachea strip

A comparative preparation to that of a spirally cut trachea is the instantly prepared zig-zag strip portrayed by Emmerson and Mackay. The trachea is cut longitudinally, by slicing through the cartilage rings opposite the trachealis muscle, and after that, traverse slits are cut into the connective tissue at equally spaced interval, first on one side of the strip, then on the other. The spiralling tissue is cut into required number of lengths (2-4) of equivalent size needed for the test. Strings are tired to every end of the tissue, which are set up in the isolated tissue bath(s) as previously portrayed under a resting tension of 1-2 g (Allegra et al., 1993; Uhlig and Taylor, 2012).

Further advantages other than the pace of preparation, is that the zig-zag trachea strip preparation has been utilized in different applications, bringing about the determination that predominantly cAMP and less cyclic guanosine monophosphate (cGMP), play a critical part in the regulation in guinea pig tracheal smooth muscle tone. β_2 -adrenoceptor agonists cause relaxation by stimulating cAMP, and agents that inhibit cyclic nucleotide phosphodiesterase (PDE) have been predicted to apply a greater portion of their relaxant impact by increasing intracellular cAMP level (Uhlig and Taylor, 2012).

The electrically stimulated version of the zig-zag strip has often been used to investigate the nonadrenergic, noncholinergic (NANC) innervation and neurotransmission of the guinea-pig trachea. In other cases the electric field stimulated trachea has frequently been used to study indirectly the modulation of neurotransmitter release from airway nerves, by measuring post-junctional response. It can in this manner be seen that a zigzag strip is an important mechanism with a wide application bringing about the determination of imperative mechanisms happening in smooth muscle (Uhlig and Taylor, 2012).

2.11.1.4 Trachea tube preparations

These include:

(i)   Un-stimulated perfused trachea tube: dissected trachea preparation suspended in organ bath, but lack lumen-epithelium interface.

(ii)   Transmural stimulation: another version of the intact trachea tube that is not perfused, but suspended, and subjected to electric field stimulation.

(iii)   Sympathetic and vagus nerve stimulation: the trachea is removed with sympathetic and vagus nerves attached and cannulated at both ends, and mounted horizontally in an organ bath (Uhlig et al., 1998)

.

2.11.2   Superfusion techniques

This is another method, used to a lesser extent, but sometimes more appropriate, e.g. to perform a bioassay of perfusates and evaluation of potency of labile substances or those of extremely low quality. An apparent beneficial effect of this method is the ability to rapidly remove potentially toxic metabolites, preventing them from accumulating in the tissue, and thereby reducing the washing time. Techniques employed under this method include, electrically stimulated zigzag trachea strip and epithelium-denuded trachea (Uhlig et al., 1998).
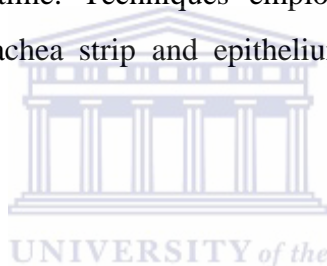
2.12   Contraction and relaxation of smooth muscle.

As described by Webb (2003), various agonists (neurotransmitters, hormones, etc.) bind to specific receptors to activate contraction in smooth muscle. Subsequent to this binding, the prototypical response of the cell is to increase phospholipase C activity via coupling through a G protein. Phospholipase C produces two potent second messengers from the membrane lipid phosphatidylinositol 4, 5-bisphosphate: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP3). IP3 binds to specific receptors on the sarcoplasmic reticulum, causing release of activator calcium (Ca^{2+}). DG, along with Ca^{2+}, activates Protein kinase C (PKC), which phosphorylates specific target proteins. In most smooth muscles, PKC has contraction-promoting effects such as phosphorylation of Ca^{2+} channels or other proteins that regulate cross-bridge cycling. Activator Ca^{2+} binds to calmodulin, leading to activation of myosin light chain kinase (MLC kinase). This kinase phosphorylates the light chain of myosin, and, in conjunction with actin, cross-bridge cycling occurs, initiating shortening of the smooth muscle cell. However, the elevation in Ca^{2+} concentration within the cell is transient, and the

contractile response is maintained by a Ca^{2+} sensitizing mechanism brought about by the inhibition of myosin phosphatase activity by Rho kinase. This Ca^{2+} sensitizing mechanism is initiated at the same time that phospholipase C is activated, and it involves the activation of the small GTP-binding protein RhoA. The precise nature of the activation of RhoA by the G protein-coupled receptor is not entirely clear but involves a guanine nucleotide exchange factor (RhoGEF) and migration of RhoA to the plasma membrane. Upon activation, RhoA increases Rho kinase activity, leading to inhibition of myosin phosphatase. This promotes the contractile state, since the light chain of myosin cannot be dephosphorylated (Webb, 2003).

Smooth muscle relaxation occurs either as a result of removal of the contractile stimulus or by the direct action of a substance that stimulates inhibition of the contractile mechanism. Regardless, the process of relaxation requires a decreased intracellular Ca^{2+} concentration and increased MLC phosphatase activity. The sarcoplasmic reticulum and the plasma membrane contain Ca,Mg-ATPases that remove Ca^{2+} from the cytosol. Na^{+}/Ca^{2+} exchangers are also located on the plasma membrane and aid in decreasing intracellular Ca^{2+}. During relaxation, receptor- and voltage-operated Ca^{2+} channels in the plasma membrane close resulting in a reduced Ca^{2+} entry into the cell (Webb, 2003). In the case of K_{ATP} channels induced relaxation in the airway smooth muscle, several endogenous agonists (e.g. intracellular nucleotides and calcitonin-gene related peptide) activate K_{ATP} channels resulting in hyperpolarization and relaxation (Sutovska et al., 2007).

The compounds in plant products such as in *A. afra* and *L. leonurus* could probably affect the smooth muscle in asthma by blocking anti-muscarinic receptors, anti-histaminergic receptors and activating K_{ATP} channels.

2.13   Flavonoids

Flavonoids are a diverse group of low molecular weight secondary plant phytochemicals. They are important compounds in the human diet and are widely distributed in the plant kingdom as intrinsic components of fruits, vegetables and beverages. They play a vital role in

plant growth and development, and in plant defence against microorganisms. Over 4,000 flavonoid compounds have been identified (Havsteen, 2002).
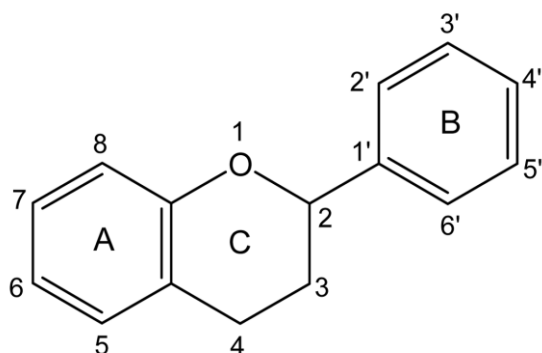
Figure 2.4: General structural formula of flavonoids.

2.13.1 Chemistry and classification

Flavonoids are recognized by the presence of a C6-C3-C6 phenyl-benzopyran skeleton comprising of two aromatic rings (A and C) connected by a three carbon unit which could possibly structure a third ring (B) (Grotewold, 2006). The position of the phenyl ring in connection to the benzopyran moiety permits division of these compounds into flavonoids (2-phenyl-benzopyrans), isoflavonoids (3-phenyl-benzopyrans) and neoflavonoids (4-phenyl-benzopyrans), they can further be partitioned into subclasses based on substitution patterns of ring C, the oxidation state of the heterocyclic ring and the position of ring B (Mukinda et al., 2010). These basic variations may explain the observed contrasts in bioactivity identified with these compounds. The most well-known sub-classes are the flavones, flavonols, flavanones, catechins, isoflavones and anthocyanidins, which represent around 80 % of flavonoids (Aherne et al., 2007). Flavones and flavonols have been identified in almost all plants. The ones found most frequently being those with B-ring hydroxylation in the C-3 and C-4 positions (Havsteen, 2002). Flavonoid molecules not attached to sugar moieties are referred to as the aglycones, whereas flavonoid molecules with sugar moieties are called flavonoid glycosides (Markham, 1982).

The chemical nature of the flavonoids depends on structural class, degree of hydroxylation, other substitutions, conjugations, and degree of polymerization (Harborne and Williams,

2000). In plants, the flavonoids are relatively resistant to heat, oxygen, dryness and moderate degrees of acidity.

Flavonoids containing a pyran ring i.e. a hydroxyl group in position C3 of the C ring are classified as 3-hydroxyflavonoids (i.e. the flavonols, anthocyanidins and catechins) and those lacking a hydroxyl group in position C3 as 3-desoxyflavonoids i.e. the flavanones (e.g. hesperetin, naringenin) and flavones (e.g. luteolin, apigenin) (Markham, 1982).
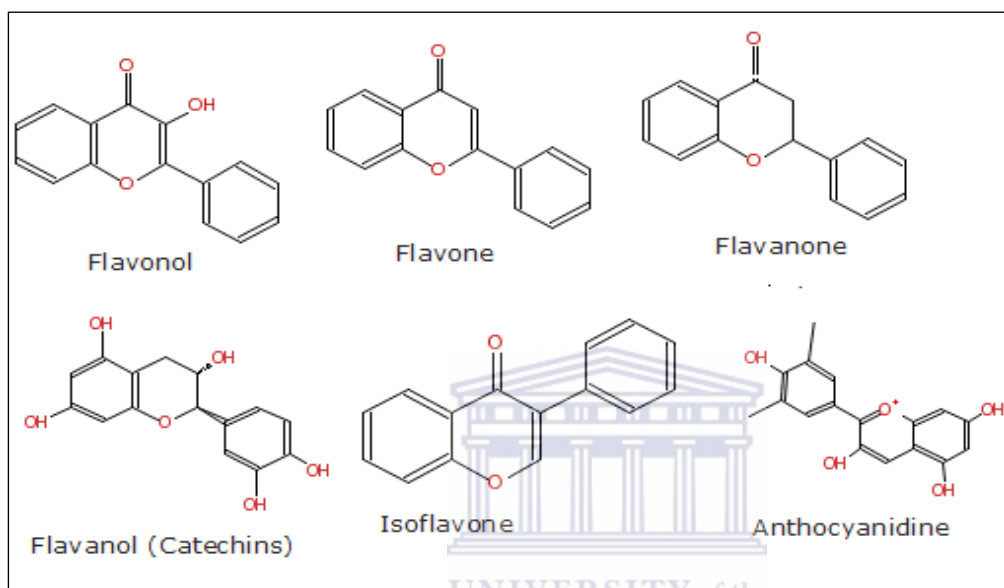
Figure 2.5: Structures of the main classes of flavonoids.

2.13.2   Biological activities

Flavonoids have a wide range of biological or pharmacological activities. These include anti-oxidation, anti-inflammation, anti-platelet, anti-tumour, anti-angiogenic, antiviral anti-thrombotic action, anti-allergic effects and smooth muscle relaxation. Furthermore, they inhibit enzymes such as cyclooxygenase, lypoxygenase, prostaglandin synthase, xanthine oxidase, protein kinase c and phosphodiesterase (Ko et al., 2004). Within the last decade, flavonoids have emerged as potential alternatives for treating diabetes, hyperlipidemia and oxidative stress, involving multiple signalling pathways (Heim et al., 2002). Luteolin (the main flavonoid of interest in this study) has been reported to relax tracheal smooth muscle (de Rojas et al., 1995; Ko et al., 2004).

2.14. Luteolin

Luteolin is one of the most common flavonoids present in edible plants and in plants used in traditional medicine to treat a wide variety of ailments. It is most often found in leaves, barks, clover blossom, rag weed and pollen.

Luteolin is present as an aglycone (molecule without any sugars bound to it) in some plants such as perilla seeds and as glycosides (molecule with one or several sugars bound to it) in celery, green pepper, perilla leave, chamomile tea, etc (Shimoi et al., 1998). Most glycosides of luteolin are O-glycosides, i.e., the sugar moieties are bound to the aglycone through one or more of the four free hydroxyl (OH) groups (Figure 2.3). These glycosides usually have sugar moieties at position 5, 7, 3' and 4', as luteolin is 5, 7, 3', 4'- tetrahydroxyflavone (Lopez-Lazaro, 2009; Shimoi et al., 2001).
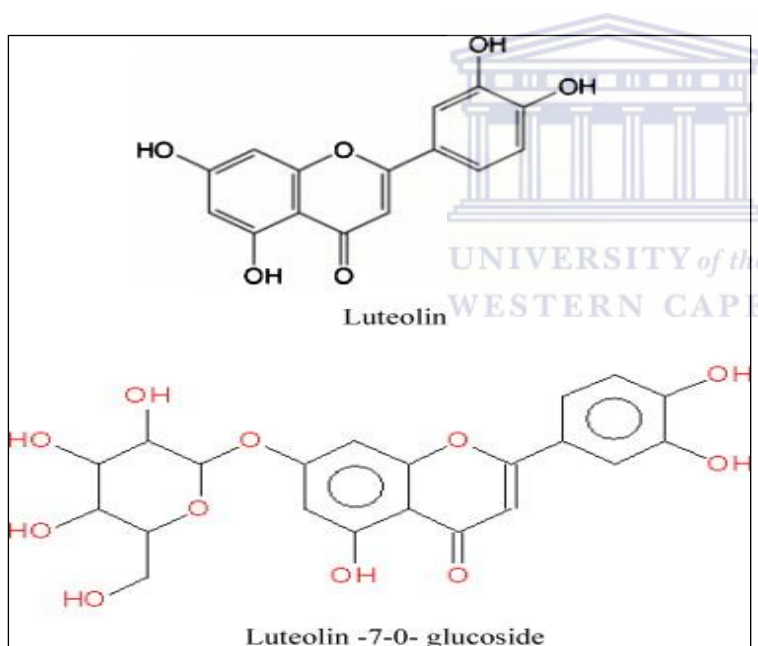
Figure 2.6: The structure of Luteolin and Luteolin-7-0-glucoside.

2.14.1 Biological activities of luteolin

Luteolin may be a significant, accessible developing pharmacological agent because of its numerous advantageous properties. It has been shown that luteolin has anti-inflammatory and antispasmodic action (Lopez-Lazaro, 2009), cancer prevention activity (Areias et al., 2001),

anti-allergic and immune-modulatory properties to suppress hyperactive immune system (Shimoi et al., 1998). Moreover, luteolin has been shown to be a developing anti-cancer agent that totally represses the catalytic action of eukaryotic DNA topoisomerase I (Chowdhury et al., 2002; Gálvez et al., 2003). It additionally helps in the prevention of the neo-vascular infection of the eye by repressing corneal angiogenesis *in vivo* (Joussen et al., 2000). More importantly, because of its anti-allergic, anti-inflammatory and smooth muscle relaxing properties, luteolin has potential in the prevention and treatment of numerous respiratory conditions including asthma and chronic bronchitis (Havsteen, 2002; De Rojas et al., 1995; Sadzuka et al., 1997). The smooth muscle relaxant effect of luteolin is possibly mediated via inhibition of cyclic adenosine monophosphate (cAMP) - and cyclic guanosine monophosphate (cGMP)-phosphodiesterases (Ko et al., 2005). Phosphodiesterase inhibition has also been implicated in trachea smooth muscle relaxation of the flavonoid luteolin (Ko et al., 2005).

2.15. Analytical techniques suitable for the determination of flavonoids

Several techniques can be used to separate, identify and quantitate flavonoids. They include gas chromatography (GC), high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Among these (HPLC) appears to be the most popular and applicable technique for the analysis of flavonoids in plant extracts, because it is a sensitive, rapid, and economical technique, which can be used in the separation, identification and quantification of flavonoids in plant extracts (Kalili and de Villiers, 2011; Khoddami et al., 2013; Stalikas, 2007).

HPLC is essentially a manifestation of column chromatography that uses a high-pressure pump to create rapid flow of eluent, and therefore radically enhancing analysis time. Various components influence HPLC analysis of flavonoids, including sample purification, mobile phase, column types and detectors (Picó, 2012; Stalikas, 2007). All in all, flavonoids are essayed with a HPLC instrument utilizing a reversed phase C18 column (RP-C18), photo diode array detector (PDA) and polar acidified organic solvents (Merken and Beecher, 2000; Naczk and Shahidi, 2004; Zarena and Sankar, 2012). Generally, acetonitrile and methanol, or

their aqueous forms, are the prevailing mobile phases used in HPLC evaluation of phenolics (Khoddami et al., 2013; Stalikas, 2007; Zarena and Sankar, 2012).

Chapter 3

Plan of work

3.1 Introduction

In this chapter the objectives set, hypotheses to be tested and the study approach followed to compare the flavonoid profile and respiratory smooth muscle relaxant effects of *Artemisia afra versus Leonotis leonurus* are presented.

3.2 Objectives

The overall aim of this study was to determine whether *Artemisia afra* and *Leonotis leonurus* produce their anti-asthmatic effects in and by similar means and the specific objectives were to:

- Determine and compare the flavonoid profiles and levels in *Artemisia afra* and *Leonotis leonurus* (as traditionally used),
- Compare the respiratory smooth muscle relaxant effects of freeze-dried aqueous extracts of *Artemisia afra* and *Leonotis leonurus* and
- Investigate whether K^+ - channel activation (i.e. K_{ATP} channel) was one possible mechanism of action that can explain the effect obtained in traditional use of these 2 plants.

To realize these objectives it was decided to determine the effects that the plants and select flavonoids may have on contraction induced by agonists (carbachol, histamine and KCl) known to be mediators of bronchoconstriction. And, to determine the flavonoid profiles of hydrolysed and un-hydrolysed aqueous plant extracts a HPLC assay was used.

3.3 Hypothesis

We hypothesized that: (i) *A. afra* has greater respiratory muscle relaxant effect than *L. leonurus,* (ii) *A. afra* and *L. leonurus* will inhibit K^+ -induced (i.e. K_{ATP} channel mediated)

contractions in a superior way than carbachol and histamine induced contractions, and (iii) that the freeze-dried aqueous extract of *A. afra* has a different flavonoid profile and greater flavonoid levels than *L. leonurus* extract.

3.4 Study approach

To realize the above objectives, freeze-dried aqueous extracts were prepared, an appropriate method for determining smooth muscle relaxant effect selected and used, and a sensitive assay procedure employed to determine the profiles and levels of free and total flavonoid forms in the plant materials. Motivation for this approach is as follows:

3.4.1 Form of *A. afra* and *L. leonurus* used

Traditionally aqueous extracts of *A. afra* and *L. leonurus* are used and have been claimed to have smooth muscle relaxant activity (Harris, 2002). In this study freeze dried aqueous extracts of *A. afra* and *L. leonurus* were to be used. These aqueous extracts are relatively easy to prepare, convenient to store and stable (and thus consistent in chemical composition) if properly stored, and would mimic the dry form of the traditionally used tea dosage form.

3.4.2 Method for determination of smooth muscle relaxant effect of *A. afra* and *L. leonurus*

For this study the isolated guinea pig tracheal smooth muscle model was used. It is a well-established, standard and commonly used tool in the preclinical research phase of anti-asthma drug discovery, especially because guinea pigs are particularly sensitive to the general triggers that initiate asthma in humans (Canning and Chou, 2008). Moreover the model is particularly useful for evaluation of the mechanism of action of such drugs and has previously been used in the testing of a herbal product, viz. *A. afra* (Harris, 2002). The zig-zag tracheal strip method was chosen due to its rapid and easy mode of preparation, and the tissue can be used for up to 10 hours. From the knowledge of the known mediators of bronchoconstriction in allergic inflammatory respiratory conditions, it was decided to focus on histamine, carbachol and potassium channel mediated constriction in this study since these mediators are commonly focused on in preclinical testing of anti-asthmatic drugs. The

isolated zig-zag cut guinea pig trachea muscle model was thus used to compare the degrees of smooth muscle relaxation of *A. afra versus L. leonurus* and to determine whether the relaxation caused by the two plants were more K^+ channel mediated. The focus on the K^+ channel (i.e. K_{ATP} channel mediated) mechanism was motivated by K_{ATP} channel openers that have been shown to have airway smooth muscle relaxation activity, and more importantly, because this mechanism has never been studied in these two plants.

3.4.3. The focus on flavonoid profile & luteolin levels in *A. afra* and *L. leonurus*

Flavonoids have been identified in both *A. afra* and *L. leonurus* crude extracts (Harris, 2002; Muganga, 2004). Some studies have shown flavonoids (particularly luteolin and quercetin) to induce smooth muscle relaxation (Ko et al., 2004; Polya, 2003). Consequently, it was decided to focus on flavonoids and, primarily, luteolin, as the marker flavonoid compound for this study. Both *A. afra* and *L. leonurus* are expected to contain luteolin, but little has been scientifically reported on the effects of luteolin-containing *A. afra* and *L. leonurus* on respiratory smooth muscle relaxation. At the same time, luteolin might also serve as an effective marker compound for the flavonoid group as a whole. To determine the flavonoid profile and luteolin levels a HPLC assay method was employed because it is a sensitive, rapid and economical technique. It was of particular interest to determine if *A. afra* had greater luteolin levels than *L. leonurus* and whether such a difference would match with the difference in relaxant activity.

Chapter 4

Determination of flavonoid profiles and luteolin levels *in A. afra* and *L. leonurus*

4.1   Introduction

In this chapter the materials, reagents, equipment, methods and procedures used to prepare the freeze dried aqueous extract (FDAE) of *A. afra* and *L. leonurus* for analyses are discussed. The development and validation of the HPLC assay used for quantitation of luteolin in *A. afra* and *L. leonurus* is described, the determination of flavonoid profiles and levels presented, and the results obtained discussed.

4.2.   Chemicals and materials

4.2.1   Chemicals

Acetonitrile, methanol (HPLC grade), ethyl acetate, acetic acid, hydrochloric acid and acetone were all solvents purchased from Merck (Darmstadt, Germany). Luteolin standard was purchased from Sigma (St. Louis, USA). Distilled water used was prepared in the pharmacology laboratory in the School of Pharmacy and purified using the Analyst HP water purifier (Oxon, England).

4.2.2   Equipment

Analytical balance (*Mettler®, model PE 6000*), a scientific balance (*Ohaus®, model GA 110*), a freeze- dryer (*Virtis ®, Freeze mobile model 125L*), -85°C freezer (*Lozone CFC Freezer, Model U855360, New Brunswick Scientific, USA*), a water bath (*Labcon®, model CDH 110 Maraisburg, SA*), filtration system (SUPELCO) connected to vacuum pump (*Medi-Pump Model 1132-2, Thomas Industries, Inc., USA*), filter paper (*Whatman No. 41 & Whatman No.1, Whatman, England*), oven (*Model Memmet 854 Schwabach, West Germany*),

38

a centrifuge (*Multex MSE®,* England), desiccators, a vortex mixer (Vortex-2, G-560E, Scientific Industries, Inc. Bohemia, N.Y. 11716 USA), test sieve shaker (*Endecott sieve shaker, E.F.L. 1MK11, Endecotts (test sieve) LTD, London, England*), micropipettes, Agilent 1200 Series HPLC system equipped with a quaternary pump (*model G1311A*), photodiode array detector (PDA) (*model G1315B*), in-line degasser (*model G1322A*), column oven (*G1329A*) and PC with ChemStation software (*Chemetrics, South Africa*), reverse phase C-18 column (*Phenomenex, USA*) with a 5 μm particle size, and column length of 4.6 mm x 250 mm.

4.2.3   Plant materials

A. afra and *L. leonurus* leaves were purchased from a local industrial herbal supplier, Grassroot Group (Pty) Limited (*Groenvlei farm, South Africa*), placed in plastic bags and stored in a cool place at room temperature away from sunlight until use.

4.3   Methods and procedures

4.3.1   Preparation of *A. afra* and *L. leonurus* extracts

To mimic the form used in traditional medicine, *A. afra* and *L. leonurus* were dried and extracted as described in literature by herbal practitioners. In traditional use, a quarter cup quantity of medicinal plant leaves is allowed to stand in a cup of boiling water for 10 minutes (Roberts, 1990). However, in this study, the dried leaves were suspended in distilled water (100 g of dried leaves in 3.5 L of distilled water) and the mixture boiled for 30 minutes. After boiling, the decoction obtained was left to cool before it was filtered using Whatman no. 1 filter paper, the filtrate transferred to a round bottom flask and frozen at -85 °C in a freezer. The frozen extract was then dried under vacuum using the Virtis™ mobile freeze-dryer. The resultant extract powder was analysed for its organoleptic features and weighed to determine the percentage yield. Thereafter, the freeze-dried aqueous extract (FDAE) powders were placed in stoppered amber glass bottles and stored at room temperature in desiccators until used.

4.3.2   HPLC method development and validation

4.3.2.1   Method development

The method described by Dube (2006), with some modifications, was used. Luteolin was selected as a marker for the comparison of the level of active compounds in *A. afra* and *L. leonurus*. Separation was effected on a Phenomenex Luna C-18 column with 5 μm particle size and a column length of 250 x 4.60 mm and the column was kept at 25 °C. The mobile phase consisted of 0.1% formic acid in distilled water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The elution gradient was: 0-22 min 15% B; 22-27 min 40% B; 27-28 min 100% B and 28-33 min 15% B. The solvents were filtered and degassed by membrane filtration prior to use. The eluent was pumped at a flow rate of 0.8 ml/min, with injection volume of 20 μl. The luteolin peaks were detected at 350 nm.

4.3.2.2   Preparation of calibration standards

A stock solution of luteolin (1 mg/ml) was prepared by weighing out a certain amount of luteolin standard and dissolving it in methanol. Standard solutions were prepared on the day of use by diluting the stock solution with methanol to concentrations of 0.5, 1, 2, 4, 8 and 16 μg/ml.

4.3.2.3   Validation of the HLPC assay

The validation of the method for suitability and effectiveness is the most important part in method development. Method development is only complete when acceptable analytical performance has been demonstrated (Bräter et al., 1994). The validation tests conducted included determination of calibration curve linearity, assay specificity, accuracy and precision and limits of detection and quantitation.

4.3.2.3.1 Linearity (calibration curve)

For each luteolin calibration sample mentioned in section 4.3.2.2, 20 μl was injected onto the column. The areas obtained in the chromatogram for the luteolin peaks were noted and plotted against their concentrations to obtain a calibration curve. The linearity of the curve

over the concentrations studied was determined by linear regression analysis using the least square regression method.

4.3.2.3.2   Detection and quantitation limits (sensitivity)

The limit of detection (LOD) and quantitation (LOQ) for the luteolin reference standard was determined by the signal-to-noise ratio method (Shah et al., 2010) using peak area as the analyte response. LOD was defined as the lowest concentration level resulting in a peak area of three times (3:1) the baseline while LOQ was defined as the lowest concentration level that provided a peak area with signal to noise ratio of higher than ten (10:1). Both limits were calculated from the relative standard deviation (RSD) and slope(s) of the calibration curves using equations LOD = 3.3 (RSD/S) and LOQ = 10 (RSD/S).

4.3.2.3.3 Accuracy and precision

The precision of the HPLC method was determined by calculating the repeatability (intra-day) and intermediate precision (inter-day). For this, 3 replicates of samples containing high (16 ug/ml), intermediate (4 ug/ml) and low (0.5 ug/ml) concentrations of luteolin were assayed each day on 3 consecutive days and on each occasion the average, standard deviation and % relative standard deviation (RSD) calculated and compared to determine the intra-day and inter-day precision. The accuracy of the method was determined by the mean concentrations obtained from the replicates and the percentage difference.

4.3.2.3.4   Specificity and stability

To determine the specificity of the assay, a series of luteolin samples were subjected to a variety of conditions (listed below) and thereafter injected onto the HPLC system to assess the analytes in the presence of degradants/impurities which may arise.

To determine the effect of acid hydrolysis, 100 μl of concentrated HCl was added to an 8 μg/ml solution of luteolin. The samples were then heated at 60 $^{\circ}$C for 1 hour, cooled and injected onto the HPLC column. To determine the effect of heat a solution of 8 μg/ml luteolin was heated at 60 $^{\circ}$C for 3 hours in a water bath. The sample was then cooled and 20 μl

injected onto the HPLC column. In each case the chromatograms were analysed for peak area, purity and presence of additional peaks.

4.3.3   Quantification of luteolin in the plant preparations

A. afra and *L. leonurus* preparations were assayed in two ways, viz. as hydrolysed and un-hydrolysed samples. From the latter the level of free flavonoids and the former the level of total flavonoid in the plant extracts were determined.

4.3.3.1   Determination of free flavonoid levels

For this, 10 mg FDAE *A. afra* and *L. Leonurus* were each weighed in labelled centrifuge tubes, 1 ml of distilled water added, the tubes shaken, 5 ml of ethyl acetate added the mixture vortexed for 1 minute and centrifuged for 10 minutes at 3000 rpm. Thereafter, the top layer of ethyl acetate was removed and transferred to a clean tube using a disposable plastic pipette and evaporated to dryness under a gentle stream of nitrogen. To the residue, 1 ml of mobile phase (acetonitrile: water 50:50) was added, the tube gently vortexed for 20 seconds and the solution drawn up into a 2 ml syringe (without needle). A 0.22 μm membrane syringe filter was then attached to the syringe and the solution filtered and transferred to a labelled HPLC vial. Twenty microliters of each sample was then injected onto the HPLC column for analysis.

4.3.3.2   Determination of total flavonoid levels

For this, 10 mg FDAE *A. afra* and *L. leonurus* were each weighed in labelled centrifuge tubes. One millilitre of distilled water was added and the mixture vortex-mixed for 20 seconds. To hydrolyse the samples, 2 ml of 2 M hydrochloric acid was added to the plant solutions and the mixture heated at 80 °C for 40 minutes in a water bath. Thereafter the tubes were removed, allowed to cool for 10 minutes, 5 ml ethyl acetate added and the samples mixed with a vortex mixer for 1 minute and centrifuged for 10 minutes at 3000 rpm. The ethyl acetate top layer was then transferred to a clean tube using a disposable plastic pipette and evaporated to dryness under a gentle stream of nitrogen. To the residue 1 ml of mobile phase (acetonitrile : water 50:50) was added, the tube gently vortexed for 20 seconds, the

solution then drawn into a 2 ml syringe (without needle), filtered (using 0.22 μm membrane syringe filter) and transferred to a labelled HPLC vial. Twenty microliters of each sample was then injected onto the HPLC column for analysis.

To identify luteolin in the plant samples, the retention time and UV-spectra of the possible luteolin peaks were identified and compared with the UV-spectrum and the retention time obtained in the chromatograms of the standard reference luteolin sample.

4.4 Results and discussion

4.4.1 Organoleptic properties of freeze-dried aqueous extracts (FDAE)

The *A. afra* extract was a light brown, brittle and hygroscopic powder with a characteristic and aromatic odour. The extract of *L. Leonurus* produced was brown, brittle and hygroscopic and also had a characteristic odour. The samples of extract produced provided sufficient material for the entire study. The average yield for the freeze dried aqueous extract was 21.8 % and 19.5 % for *A. afra* and *L. leonurus,* respectively. These yields were very close to those obtained by previous investigators, e.g. 19.9 % by Komperlla (2005) and 21.96 % by Dube (2006) for *A. afra*, and 18.42 % by Ma (2006) for *L. leonurus.*

4.4.2. Validation of HPLC assay

Under the HPLC conditions used in this study, symmetrical chromatographic peaks of luteolin were obtained at 23.14 ± 0.255 minutes (figure 4.1). In this study, the absorbance of luteolin was read at 350 nm, which differed slightly from the 349 nm maximum wavelength for luteolin recommended by Markham (Markham, 1982; Muganga, 2005) and the 340 nm used by Waithaka for luteolin from *A. afra* (Waithaka, 2004).

43
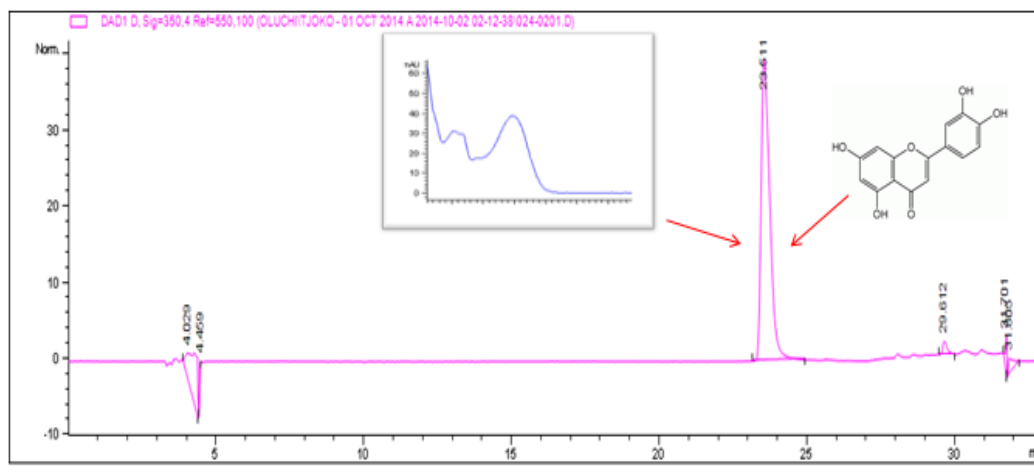
Figure 4.1: HPLC chromatogram for the luteolin reference standard at 350 nm. Insert: UV/V spectrum for the reference compound with peak at 23.5 min. (n=3)

4.4.2.1 Linearity and range

The standard curve of peak area *versus* luteolin was found to be linear ($r^2 = 0.9998$) over the concentration ranges studied (0.5 to 16 µg/ml) (figure 4.2). Linearity in this range was sufficient to provide a highly accurate value for the luteolin content in the plant samples. The standard curve was described by the equation y = 131. 27x-22.759 (where Y= peak area ratio, X = luteolin concentration in µg/ml). Using the established calibration curve, the content of luteolin in the plant extracts was quantified. Linearity, linear regression equation and correlation coefficients of the concentration ranges for the reference compound are given in figure 4.2 and table 4.1.
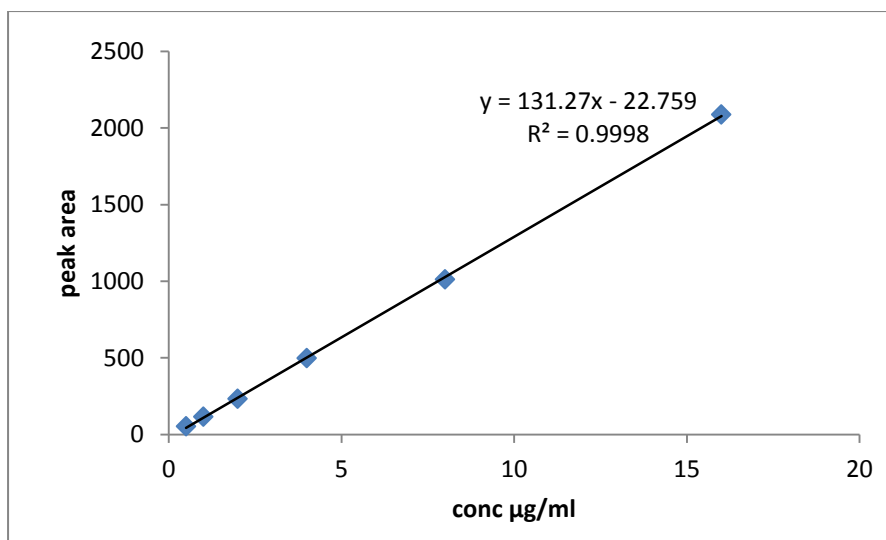
Figure 4.2: Calibration curve of mean peak area against the concentration of replicate samples of luteolin at 350 nm.

Table 4.1: Concentration *versus* peak area of luteolin monitored at 350 nm

Concentration (µg/mL)	Mean peak area (mAU) (n = 3)	%RSD
0.5	54.0	10.1
1	115.6	6.6
2	233.4	7.5
4	497.2	2.9
8	1011.5	5.6
16	2086.9	3.9

4.4.2.2 Accuracy and precision

Accuracy and precision values calculated during the intra- and inter-day assay runs are given in tables 4.2 and 4.3

Table 4.2: Intra-day variations of HPLC method for determination of luteolin

Concentration (ug/ml)	Mean peak area (mAU) (n=3)	Standard deviation	%RSD	Mean Concentration found
0.5	54.333	3.459	6.366	0.597
4	472.224	22.312	4.723	3.875
16	2021.210	48.707	2.409	16.029

Table 4.3: Inter-day variations of HPLC method for determination of luteolin

Concentration (ug/ml)	Mean peak area (mAU) (n=9)	Standard deviation	% RSD	Mean Concentration found (ug/ml)	% recovery
0.5	59.544	6.068	10.191	0.554	110.801
4	508.911	44.235	8.692	3.929	98.230
16	2117.744	110.207	5.204	16.015	100.090

Method precision had a relative standard deviation (RSD) below 10 % for repeatability and 7 % for intermediate precision. In both cases, percent RSD values were found well within 10 %, suggesting that the method provided highly reproducible results. The accuracy results were expressed as percent recoveries, and these fell within the range of 98.23 to 110.80, indicating good accuracy of the proposed HPLC method.

4.4.2.3 Detection and quantitation limits.

The minimum concentration levels at which the analytes could be detected (LOD) was 0.159 µg/ml and quantified (LOQ) was 0.482 µg/ml (Table 4.4).

Table 4.4: Assay validation parameters for luteolin quantification in *A. afra* and *L. leonurus* preparations

Validation parameters	Parameter value
Retention time of luteolin (min)	23. 140
Linearity-Regression of coefficient (R^2)	0.9998
Inter-assay precision (RSD in 3 days) (%)	8.029
LOD (µg/ml)	0.159
LOQ (µg/ml)	0.482

4.4.2.4 Specificity and stability

A series of samples were subjected to a variety of conditions and injected onto the HPLC system to assess the analytes in the presence of degradants which may emerge, Figures 4.3 and 4.4 show chromatograms of luteolin samples after acid and heat treatment, respectively. The luteolin peak remained symmetrical in shape and the peak retention time was maintained. The luteolin peak areas were not significantly affected by acid ($p = 0.0896$) and heat ($p = 0.2428$) treatment. The UV/Vis spectrum was unchanged and no evidence of extra peak(s) of degradation products was evident. The results obtained from the HPLC system software peak purity tool demonstrated that the peak of luteolin was pure and the purity factor was within the calculated threshold in all cases, thus confirming the absence of other substances in the same retention time, suggesting that, under these conditions, luteolin was stable. This HPLC assay was very specific for un-hydrolysed and hydrolysed *A. afra* as well as un-hydrolysed *L. leonurus* (figures 4.5, 4.6 and 4.7, respectively), but it was not specific for hydrolysed *L. leonurus* samples as it did not completely manage to separate luteolin from some peaks close to or interfering with the luteolin peak (fig 4.8).
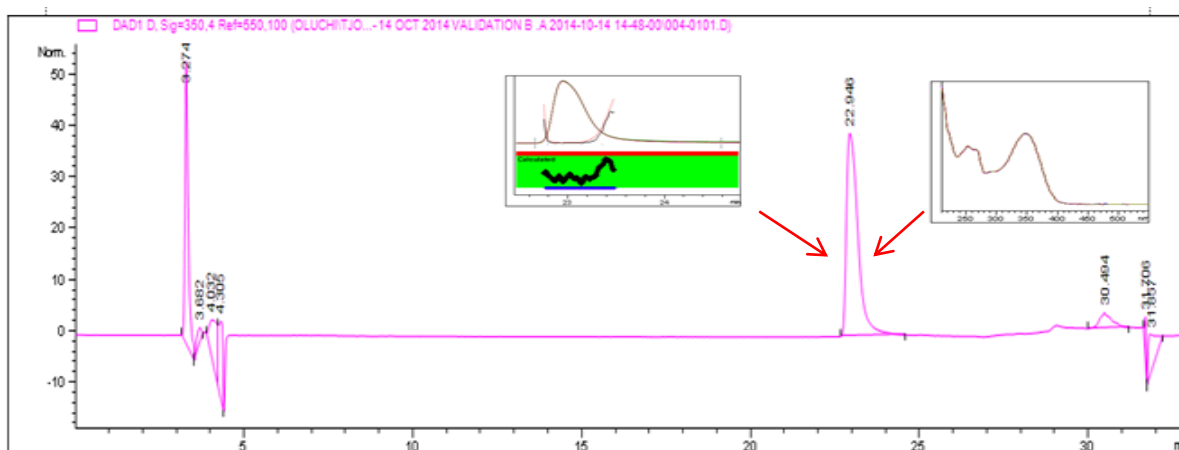
Figure 4.3: HPLC chromatogram for the luteolin reference standard at 350 nm after the addition of acid (HCl) and heating at 60 °C for 1 hour. The inserted diagrams indicate the peak purity and UV spectrum. (n=3)
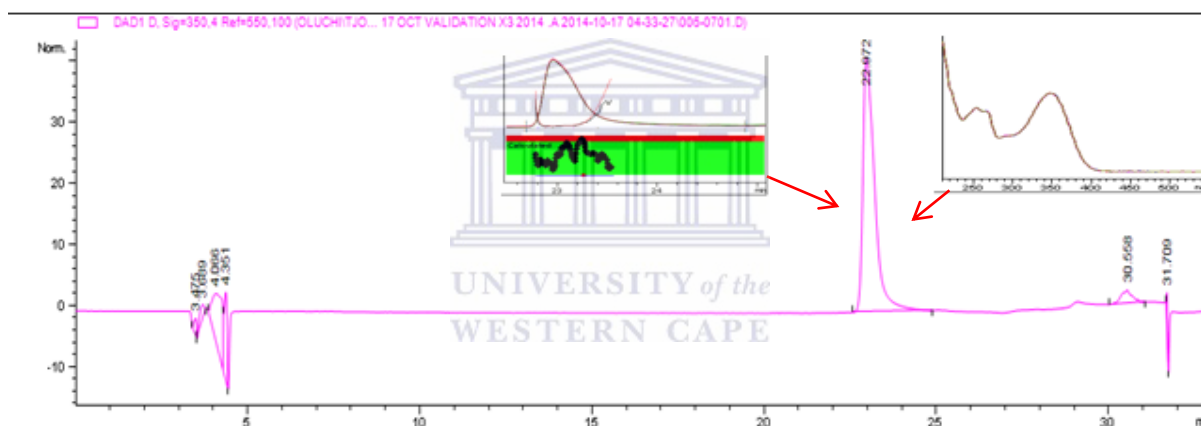
Figure 4.4: HPLC chromatogram for the luteolin reference standard at 350 nm after subjection to heating at 60 °C for 3 hours. The inserted diagrams indicate the peak purity and UV spectrum. (n=3)

4.4.3   Identification and level of luteolin in *A. afra* and *L. leonurus* preparations

The above-mentioned validated HPLC assay was used to identify and quantitate the levels of luteolin in the un-hydrolysed and hydrolysed *A. afra and L. leonurus* plant preparations. The hydrolysed and un-hydrolysed samples of plant extracts were analysed and representative chromatograms obtained for each plant are given in figures 4.5 to 4.8.

48
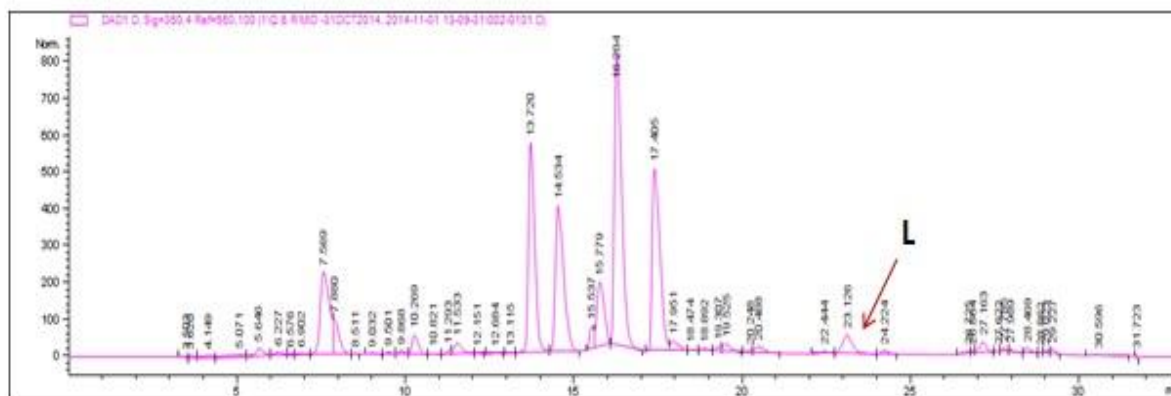
Figure 4.5: HPLC chromatogram of un-hydrolyzed A. *afra*. The retention time of luteolin (L) is 23.1 min.
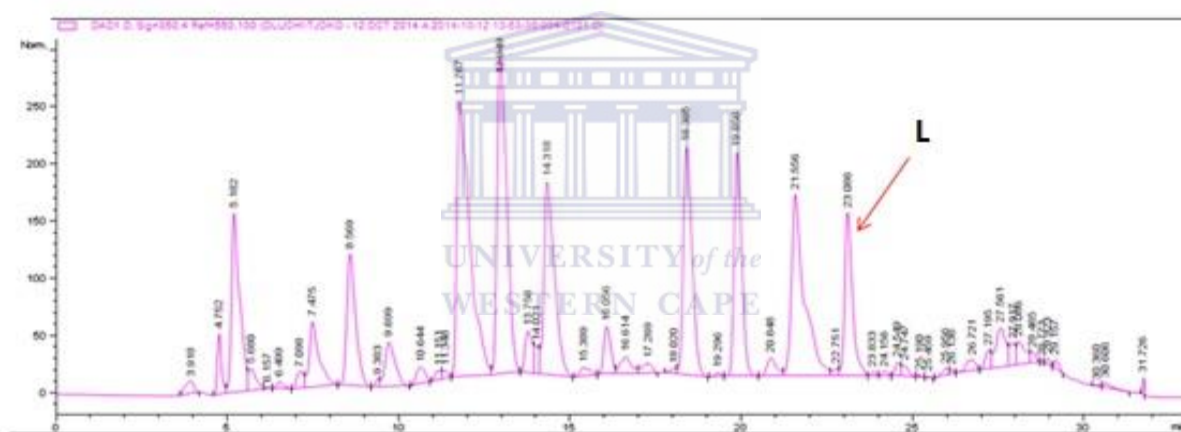
Figure 4.6: HPLC chromatogram of acid hydrolysed A. *afra*. The retention time of luteolin (L) is 23.0 min.
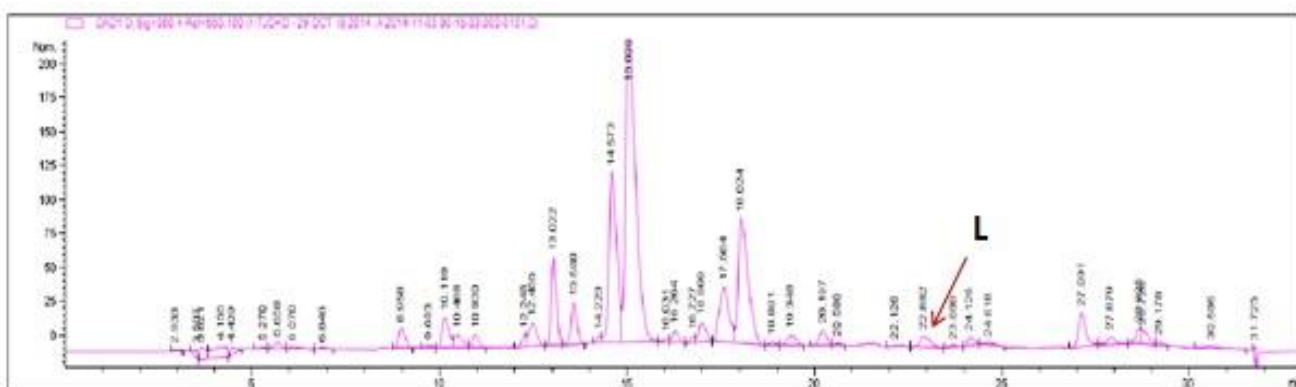
Figure 4.7: HPLC chromatogram of un-hydrolyzed *L. leonurus in which the* retention time of luteolin (L) is 22.9 min
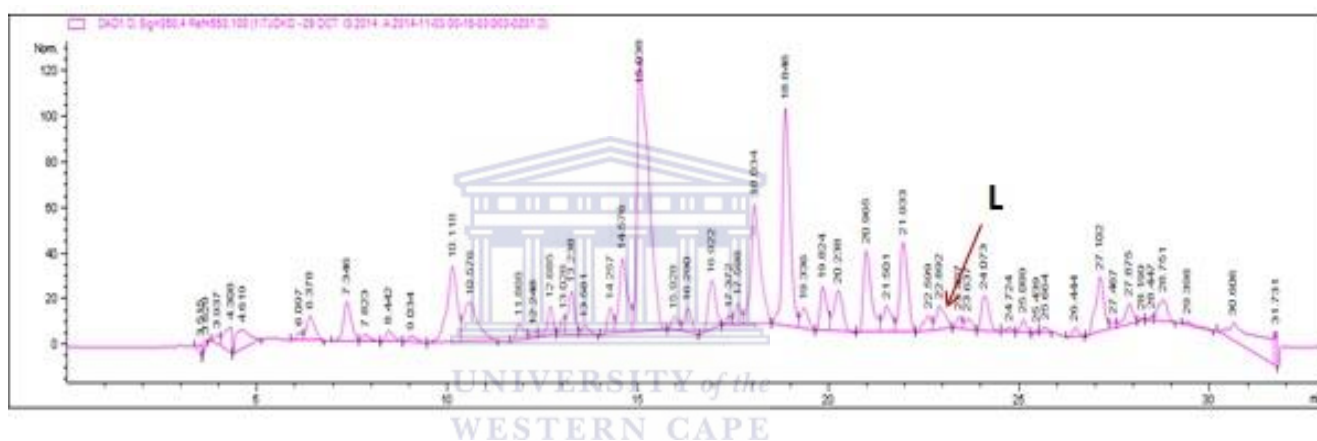
Figure 4.8: **HPLC chromatogram of acid hydrolysed *A. afra*. The retention time of luteolin (L) is 22.9 min**

Table 4.5: Levels of luteolin in *A. afra* and *L. leonurus* plant preparations

Plant samples	Luteolin concentration (µg/mg of plant extract) Mean ± SD (n=3)		Conjugated luteolin (%)
	Free luteolin	**Total luteolin**	
A. afra	8.977 ± 0.73	16.394 ± 0.884	45
L. leonurus	0.929 ± 0.066	3.093 ± 0.531	69

Collectively, the results confirmed the presence of quantifiable amounts of luteolin in *A. afra* and *L. leonurus* extracts. To the best of my knowledge, studies concentrating on the presence of luteolin in *L. leonurus* have not been done previously. Other researchers have, however, previously reported on the presence of luteolin in *A. afra* (Muganga, 2004; Waithaka, 2004).

Chromatograms of the hydrolysed and un-hydrolysed *A. afra* and *L. leonurus* samples showed peaks of several other compounds, of which most had a UV/Vis spectrum similar to that of flavonoids. The luteolin peak (retention time 23.14 ± 0.255 min) was identified by spiking samples and by UV spectral analysis done using the diode array detector.

The flavonoid profile of un-hydrolysed *A. afra* showed roughly 14 distinctive (above height of 6) peaks, and 10 of the peaks had UV/Vis spectrum similar to that of flavonoids. The un-hydrolysed *L. leonurus* on the other hand displayed around 19 distinctive peaks with 13 peaks exhibiting UV/Vis spectrum similar to that of flavonoids. Extra peaks were evident from both plants after acid hydrolysis. In general, *L. leonurus* had a greater flavonoid profile than *A. afra.*

The luteolin peak height in the un-hydrolysed *A. afra* was 51.7 mAU, but after hydrolysis it had increased to 143 mAU. Similar, but to a lesser extent the luteolin peak height of un-hydrolysed *L. leonurus* was 5.4 mAU but after hydrolysis it was 7.8 mAU. *A. afra* contained 8.977 ± 0.73 µg free luteolin/mg while *L. leonurus* contained only 0.929 ± 0.066 µg free luteolin/mg (Table 4.5). *A. afra* contained four to five times as much free luteolin than *L. leonurus* ($p = 0.0026$). On the other hand, *A. afra* contained 16.394 ± 0.884 µg total luteolin/mg FDAE whereas *L. leonurus* contained 3.093 ± 0.531 µg total luteolin/mg FDAE. After acid hydrolysis, luteolin levels in *A. afra* ($p = 0.0004$) and *L. leonurus* ($p = 0.018$) were significantly increased in comparison with the un-hydrolysed plant samples (Table 4.5). Acid hydrolysis presumably leads to increase in peak height of aglycones as a result of flavonoid glycoside hydrolysis to flavonoid aglycones (Baranowska and Magiera, 2011). About two thirds of the luteolin from both plants was in the glycoside form. *L. leonurus* had a substantially greater percentage of glycosides (79 %) than *A. afra* (45 %), meaning that although *L. leonurus* has a lower load of luteolin it has more of the conjugated form. Compared to *L. leonurus*, *A. afra* contained very little acid hydrolysable (i.e. conjugated) luteolin glycosides (Table 4.5).

4.4.4. Conclusion

Overall, from the results acquired the following conclusions could be drawn.

Moderate to good yields of 21.8 % and 19.5 % were obtained for FDAE *A. afra* and *L. leonurus,* respectively. The HPLC method used for quantitative analysis of luteolin proved to be suitable for fingerprinting of luteolin and was sufficiently accurate and reproducible for the quantitative analysis of luteolin in FDAE *A. afra* and *L. leonurus*. Finally, *A. afra* had significantly higher levels of total and free luteolin than *L. leonurus*, with relatively low values of total and free luteolin observed in *L. leonurus*. However, *L. leonurus* contained a greater percentage of its luteolin in the conjugated form than *A. afra*.

UNIVERSITY *of the*
WESTERN CAPE

Chapter 5

Respiratory smooth muscle relaxant effects of *A. afra* and *L. leonurus*

5.1   Introduction

One specific objective of this study was to compare the respiratory smooth muscle relaxant effects of freeze-dried aqueous extracts of *A. afra* and *L. leonurus* and to determine whether K_{ATP} − channel activation is one possible mechanism of action that can explain the effect obtained with the traditional use of these two plants. Other researchers have found *Artemisia afra* to possess smooth muscle relaxant effects, we thus hypothesized that: (i) *Artemisia afra* would have a greater respiratory muscle relaxant effect than *Leonotis leonurus,* and (ii) that *A. afra* and *L. leonurus* will inhibit K^+ -induced (i.e. K_{ATP} channel mediated) contractions in a superior manner than carbachol and histamine induced contractions. In this chapter, the chemicals, materials and methods used to establish and compare the tracheal smooth muscle relaxant effects of the 2 plants and their possible mechanisms of action are reported, and the results obtained are presented and discussed.

5.2   Chemicals, materials and equipment,

5.2.1   Chemicals

Carbachol, histamine diphosphate salt, isoproterenol hydrochloride (isoprenaline), rutin and quercetin were all obtained from Sigma-Aldrich, S.A. (Pty) Ltd. Sodium pentobarbitone 6 % injection was purchased from Kryon Lab (Pty) Ltd (South Africa). All drugs and reagents were dissolved in distilled water, unless noted otherwise. Composition of Krebs-Henseleit solution (mM) was: NaCl 118, KCl 4.77, $CaCl_2$ 2.0, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 11. All these chemicals were obtained from Merck Laboratory and were of analytical reagent (AR) grade.

5.2.1.1   Preparation of stock solutions

Stock solutions of histamine (1 mM), carbachol (1 µM) and potassium chloride (100 mM) in the Krebs-Henseleit solution (KH) were freshly prepared 24 hours before use. The KH stock solution (minus glucose) was freshly prepared and refrigerated at 2^oC until use when glucose was added. The KH stock solution was discarded 10 days after preparation.

5.2.2   Materials

Surgical equipment was purchased from Lasec (Pty) Ltd, South Africa. The PowerLab$^®$ data acquisition system controlled by the LabChart$^®$ version 7 data analysis software was supplied by AD Instruments (Australia). *A. afra* and *L. leonurus* extracts, as described in chapter 4, were used in this set of experiments.

5.2.3   Equipment

5.2.3.1   The organ bath system

The system used is shown in figures 5.1 and 5.2 and consisted of a 15-ml water jacketed glass organ bath (A) that was supplied with perfusion medium (KH) from a 2 L reservoir (B). On its way from the reservoir the perfusion fluid passed in tubing through a condenser (C) and the perfusion flow was controlled via two taps (D). Fluid was drained via another tap (E) and outlet tubing from the organ bath. Perfusion medium in the bath was kept at 37 ^{o}C by circulating water through the jackets of the condenser and the organ bath with the aid of a thermo-stated water bath (F). The perfusion medium in the tissue bath was aerated with a mixture of 5 % CO_2 and 95 % O_2 from a cylinder and via a 5 ml syringe needle inserted in the top of the bath. To measure contractile responses, the tissue was held at a fixed position with the aid of cotton threads tied to stainless steel tissue holder (K) located at the bottom of the bath and to a force transducer (G). Contraction of the trachea created tension on the force transducer, and this was converted into a voltage signal at the pre-amplifier. Signals from the pre-amplifiers were channelled to an analog to digital converter (H) connected to a desktop computer (J) running the Labchart$^®$ 7 software (I) that allowed continuous recording of tension throughout the experiment. Two of the above organ bath systems (figure 5.1) were

connected in series to allow the simultaneous execution of two replicates of the experiment (A).
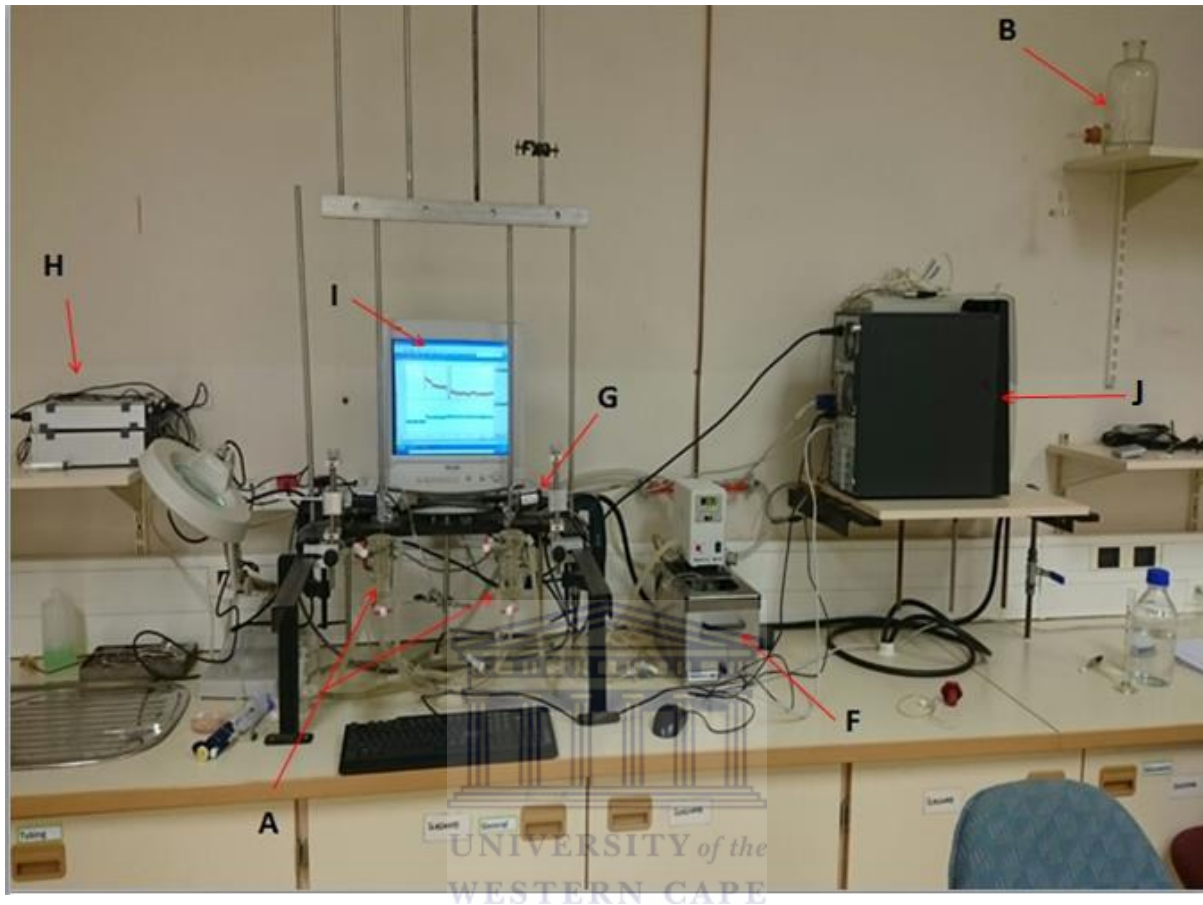
Figure 5.1: The isolated organ bath system used for the bronchodilator experiments in the laboratory.
A = Two organ bath systems; B = 2 Litre reservoir; C = Condenser; D = Taps controlling perfusion flow; E = Taps draining perfusion fluid; F = Thermo-stated water bath; G = Force transducer; H = Powerlab®, I = Labchart® 7 software; J = Desktop computer.
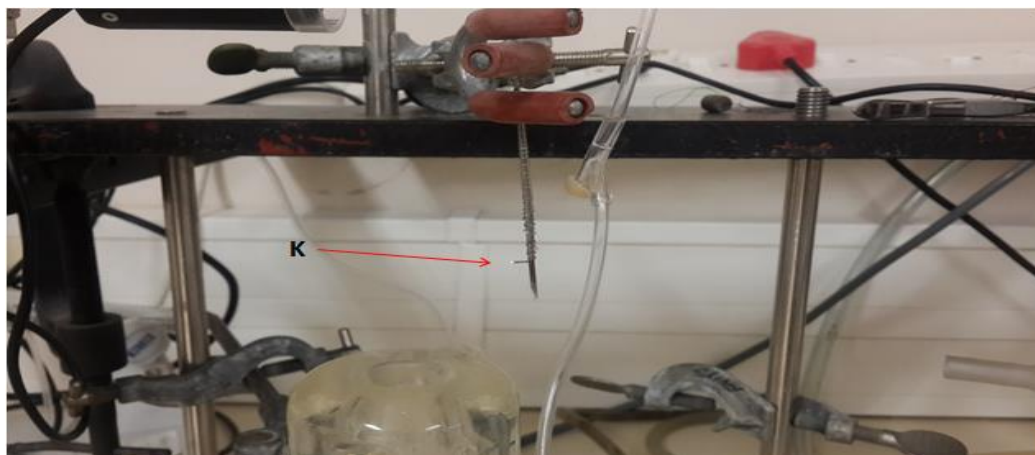
Figure 5.2: Part of the organ bath system showing a stainless steel hook (K) used in the bronchodilator experiments in the laboratory.
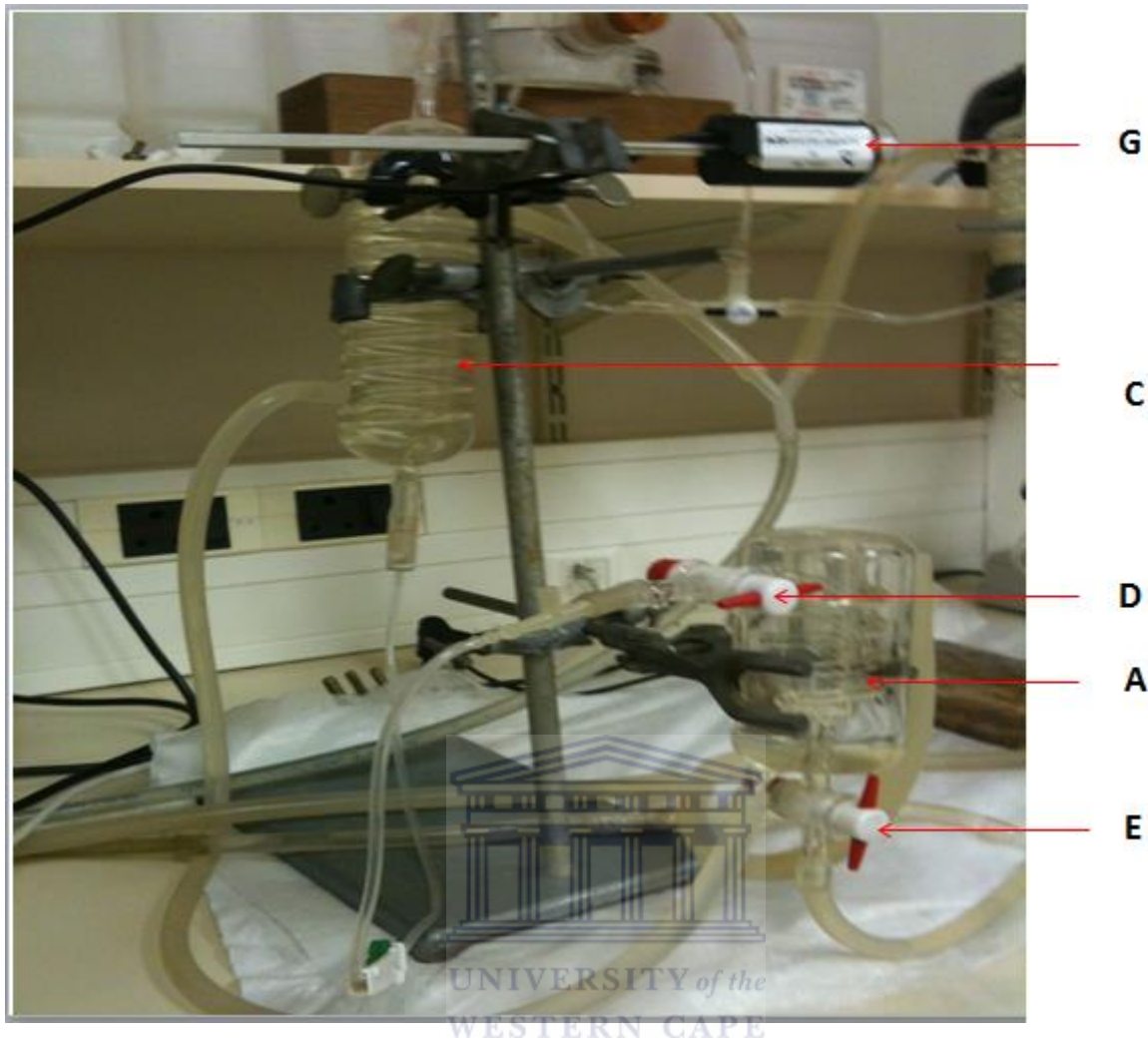
Figure 5.3: A close-up of an isolated organ bath showing a condenser and a force transducer to which a zigzag trachea tissue is fixed. A = organ bath systems; C = Condenser; D = Tap controlling perfusion flow; E = Tap draining perfusion fluid; G = Force transducer

To quantify the contractile and relaxant responses of the tracheal muscle the force transducers, amplifiers and recorders were calibrated as follows. First, the reading on the Labchart® 7 was set to zero then low weights (0.5 to 5 g) were attached to the transducer and the tensions recorded. Finally, a plot of mass (force) *versus* recorder reading was established and this calibration was done before each series of experiments.

5.3   Methods

5.3.1   Measurement of contractile force in isolated tracheal preparation

5.3.1.1 Animals

Male and female pathogen-free Dunkin Hartley guinea-pigs (350–460 g) were purchased from the Centre for Animal Studies (North West University). Before they were used they were housed at the School of Pharmacy (UWC) animal room under controlled conditions, with lighting regulated to produce day and night conditions (12-hour cycle). They were allowed free access to water and commercial chow. The experiments were approved and carried out in accordance with the ethical requirements of the University of The Western Cape Animal Research Ethics Committee (AREC).

5.3.1.2 Tissue preparations

For each experiment, one guinea pig was weighed and anaesthetized with sodium pentobarbital (50 mg/kg) intra-peritoneally. The throat and chest of the animal was cut open from under the chin to the thorax and the trachea exposed and quickly excised by cutting below the bronchial bifurcation and above the pharynx. The excised tissue was rinsed in chilled KH solution and transferred to a dissecting dish also containing chilled KH solution. During cleaning, the trachea was held at the pharynx or below the bifurcation so as to prevent applying the forceps directly on the trachea itself. Surgical scissors were used to trim off any adhering fat and connective tissue. The trachea was then cut open on the cartilage ring side with surgical scissors, flattened and pinned below the bifurcation and above the pharynx on the dissecting dish with disposable syringe needles. Thereafter the opened trachea was cut parallel to the cartilage rings to form zigzag strips of equal widths i.e. one cartilage ring per segment. Each cut was made through three-quarters of the tissue width before rotating the tissue 180° and then cutting through three-quarters from the opposite side. This procedure was done for the entire length of the tissue and the tissue was gently spiralled to its full length. The ends of the zigzag tracheal strip were then cut below the pharynx and above the bronchial bifurcation and the remaining zigzag strip cut into two equal pieces. On each end of the two pieces, a cotton thread was fixed by passing a surgical needle with thread through the cartilage material taking care not to damage the muscle in the process. The tissues were

thereafter mounted in the isolated organ bath filled with 15 ml of KH solution at 37 ^{o}C. One end of the tissue was attached to the stainless steel hook (k) and the opposite end to the force displacement transducer (G). A 2 gram resting tension was applied to the tissue and the tissue was then allowed to equilibrate for 1 to 2 hours with washing at 15 min intervals with KH before it was exposed to various concentrations of the broncho-constricting mediators and plant extracts.

5.3.2 Protocols

5.3.2.1 Establishing concentration of agonist to use.

To establish the concentration of agonist to use in this study, increasing serial concentrations of each agonist were injected into the bath. From the results a log dose response curve was obtained and from such curve the concentration of agonist giving responses of $> 50 \% < 100 \%$ was selected. Concentrations in the results section are those obtained in the 15 ml organ bath after injection of 0.1 ml of the solutions.

5.3.2.2 Determining the relaxant effect of the plants on agonist-induced contraction

To quantitate the tracheal smooth muscle relaxant effect of the two plant extracts, the trachea was contracted by injection of 0.1 ml volumes of histamine (1 mM), carbachol (1 μM) or potassium chloride (100 mM) into the bath. When stable contractions were achieved, 0.1 ml volumes of increasing concentrations of aqueous extracts of *A. afra* and *L. leonurus* (1, 3, 10, 30 and 100 mg/ml) were added to the bath without washing in between injections, followed by a final 0.1 ml volume of isoprenaline (6.67×10^{-6}). The magnitude of response on each injection was measured, recorded and displayed with the LabChart$^{®}$ 7 software. The degree of relaxation produced by each of the plant extract samples were calculated as a percentage of the full relaxation response obtained with the isoprenaline. A decrease in tone was considered to be a relaxant (bronchodilatory) effect and expressed as a positive percentage change. An increase in tone was considered as a contractile (bronchoconstriction) effect which was expressed as negative percentage change (Martin et al., 1994). For each plant, responses to four replicates of each concentration were obtained. No correction for the 15 ml bath volume

was made and relaxation *versu*s concentration curves were elaborated and analysed to assess the smooth muscle relaxant effects of the two plant extracts.

5.3.2.3   Establishing possible mechanism involved in the smooth muscle relaxant effect of both plants

To determine the possible mechanism involved in the smooth muscle relaxant effect of both plants, the same procedure described in section 5.3.2.2 was followed, i.e. 0.1 ml solutions of aqueous *A. afra* and *L. leonurus* extracts (1- 30 mg/ml) were injected in the bath and left for 20 min before cumulative concentrations of the agonist were introduced. The responses obtained after each injection were expressed as a percentage of the maximum contraction obtained per tissue and used to generate a dose response curve. For each plant four replicate dose response curves were obtained.

5.4   Data analysis

The experiments were done in triplicate (n=3). The plant concentration *versus* % relaxation curves were analysed using GraphPad$^®$ Prism. The EC_{50} values were calculated by linear regression and compared to each other. All response data was expressed as mean $\pm$ S.E.M. The difference between means was determined by use of unpaired student- t test. Values were considered significant if the p value was less than 0.05.

5.5   Results and Discussion

5.5.1   Effect of *L. leonurus* and *A. afra* on trachea constricted with carbachol, KCL and histamine

To determine whether *A. afra* and *L. leonurus* directly relaxes the pre-contracted airway smooth muscle, the isolated guinea-pig trachea was perfused with various concentrations (1 - 100 mg/ml) of the aqueous extracts. *L. leonurus* and *A. afra* relaxed tracheal tissue pre-

contracted with carbachol (1 µM), high potassium Krebs Henseleit solution (K^{+} 100 mM) and histamine (1 mM) in a dose-dependent manner (figure 5.4 and figure 5.5).

The 100 mg/ml solutions of *A. afra* caused 93.8 ± 2.39 %, 91.5 ± 0.31 % and 88.5 ± 4.20 % relaxation of carbachol*,* histamine and KCL pre-contracted airway smooth muscle, respectively, while the 1 mg/ml solutions produced 1.25 ± 0.95 %, 4.0 ± 3.72 % and 8.4 ± 4.61 % relaxation of carbachol, histamine and KCL induced contractions (figure 5.4).
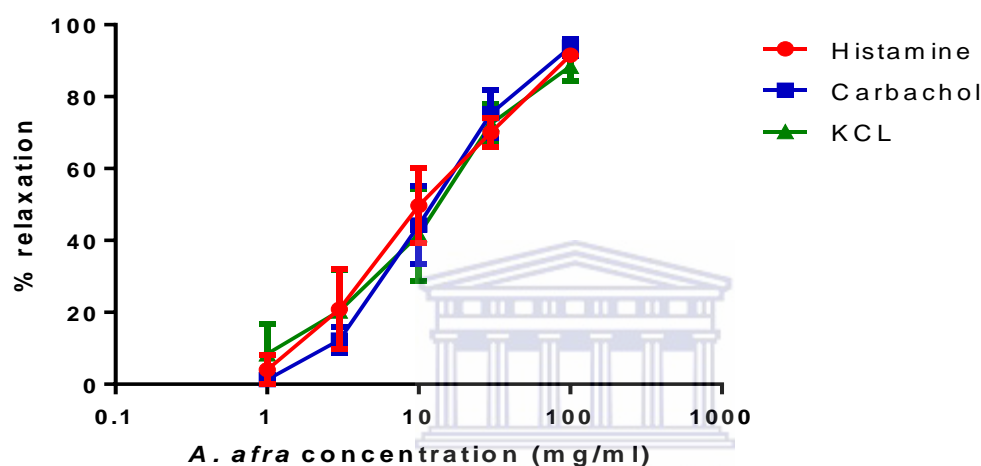
Figure 5.4: Dose response curves of relaxation (%) caused by cumulative concentrations of
$\quad$ *A. afra* (1-100 mg/ml) on isolated guinea pig trachea constricted with carbachol
$\quad$ (1 µM), KCL (100 mM) and histamine (1 mM) (n=4)

For *A. afra*, the dose dependency of the bronchodilatory effect was similar for all three mediators of contraction. To compare the potency of *A. afra* on the 3 different mediators, the EC_{50} was used. EC_{50} is the concentration inducing 50 % relaxation of pre-contracted guinea pig tracheal muscle. The EC_{50} value of *A. afra* for relaxing carbachol, KCL and histamine induced contractions were 13.93, 19.88 and 15.47 mg/ml, respectively. Although the induced relaxations were very similar, *A. afra* was more effective in relaxing carbachol induced contractions compared to KCL ($P = 0.0223$) and histamine induced contraction ($P = 0.0928$), suggesting that relaxant activity of *A. afra versus* the three agonists could be categorized as follows: carbachol > histamine > KCL (i.e. 1 : 1. 11 : 1.43 EC_{50} value order).

Similar to *A. afra,* the bronchodilatory effect of *L. leonurus* was also dose dependant. The 100 mg/ml solutions of *L. leonurus* induced 93.5 ± 2.18 % and 95.8 ± 2.93 % relaxation of the airway smooth muscle pre-contracted with carbachol and KCL, respectively, while the 1 mg/ml and 3 mg/ml solutions caused 5.25 ± 2.10 % and 4 ± 2.03 % relaxation, respectively. On the histamine pre-contracted tissue, the 100 mg/ml solutions of *L. leonurus* caused 55.1 ± 4.28 % and the 3 mg/ml solutions 2.29 ± 1.67 % relaxation of the pre-contracted trachea tissue while the 1 mg/ml dose produced 0 % relaxation (figure 5.5). The dose dependant effect of *L. leonurus* on carbachol and KCL induced contractions thus appeared to be quantitatively similar but substantially stronger than that against the histamine induced contractions.
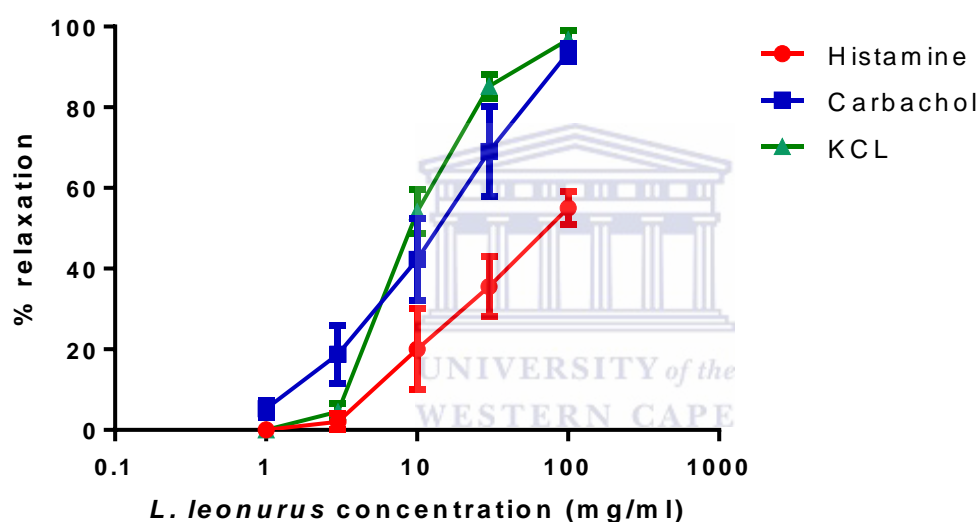
Figure 5.5: Dose response curves of the relaxation (%) caused by cumulative concentrations of *L. leonurus* (1-100 mg/ml) on isolated guinea pig trachea contracted with carbachol (1 μM), KCL (100 mM) and histamine (1 mM) (n=4)

To compare the potency of *L. leonurus* on the different mediators, EC_{50} was calculated (table 5.1). *L. leonurus* was significantly more effective in relaxing KCL induced contractions (EC_{50} value of 9.877 mg/ml), compared to carbachol (EC_{50} value of 29.74 mg/ml; $P = 0.0010$) and histamine-induced contractions (EC_{50} value of 94.76 mg/ml; $P = 0.0002$). Overall, based on EC_{50} values, the relaxant activity of *L. leonurus versus* the three inducers of contraction (agonists) appear to be in 1:3:10 order or classified as follows: KCL > carbachol >> histamine.

Table 5.1: EC$_{50}$ values of *A. afra* and *L. leonurus* induced relaxation of KCL, histamine and carbachol – induced contractions of the guinea pig tracheal muscle.

Mediators	*A. afra*	*L. leonurus*
	EC50 (mg/ml)	EC50 (mg/ml)
KCL	19.88	9.87
Carbachol	13.93	29.34
Histamine	15.47	94.76

Interestingly, when the tissue was exposed to carbachol, a stable contraction obtained, thoroughly washed (to remove any traces of the agonist), and then exposed to doses of *L. leonurus* plant extract in the absence of carbachol, the muscle showed a complex reaction. At 1 and 3 mg/ml *L. leonurus* produced, instead of the expected no response, a pronounced slow contractile response (figure 5.6, A). However, doses ≥ 10 mg/ml *L. leonurus* injected immediately post carbachol washout produced no such contractile response (figure 5.6, B). When the 1 and 3 mg/ml doses of *L. leonurus* were followed by doses ≥ 10 mg/ml (figure 5.6, C) the contractile responses were however antagonised. Contrary to these *L. leonurus* effects, *A. afra* extract at all concentrations injected (i.e. 1 to 100 mg/ml) elicited no response (i.e. no contraction) of the washed carbachol-exposed tissue. The reason(s) for these differences in effects between *L. leonurus* and *A. afra* is not clear but other researchers have observed similar biphasic effects e.g. with methanolic extract of *Dichrostachys cinerea* and aqueous extract of *A. afra* (Aworet-Samseny et al., 2011; Harris, 2002). It is possible that *L. leonurus* contains both active contractile and relaxant compounds.
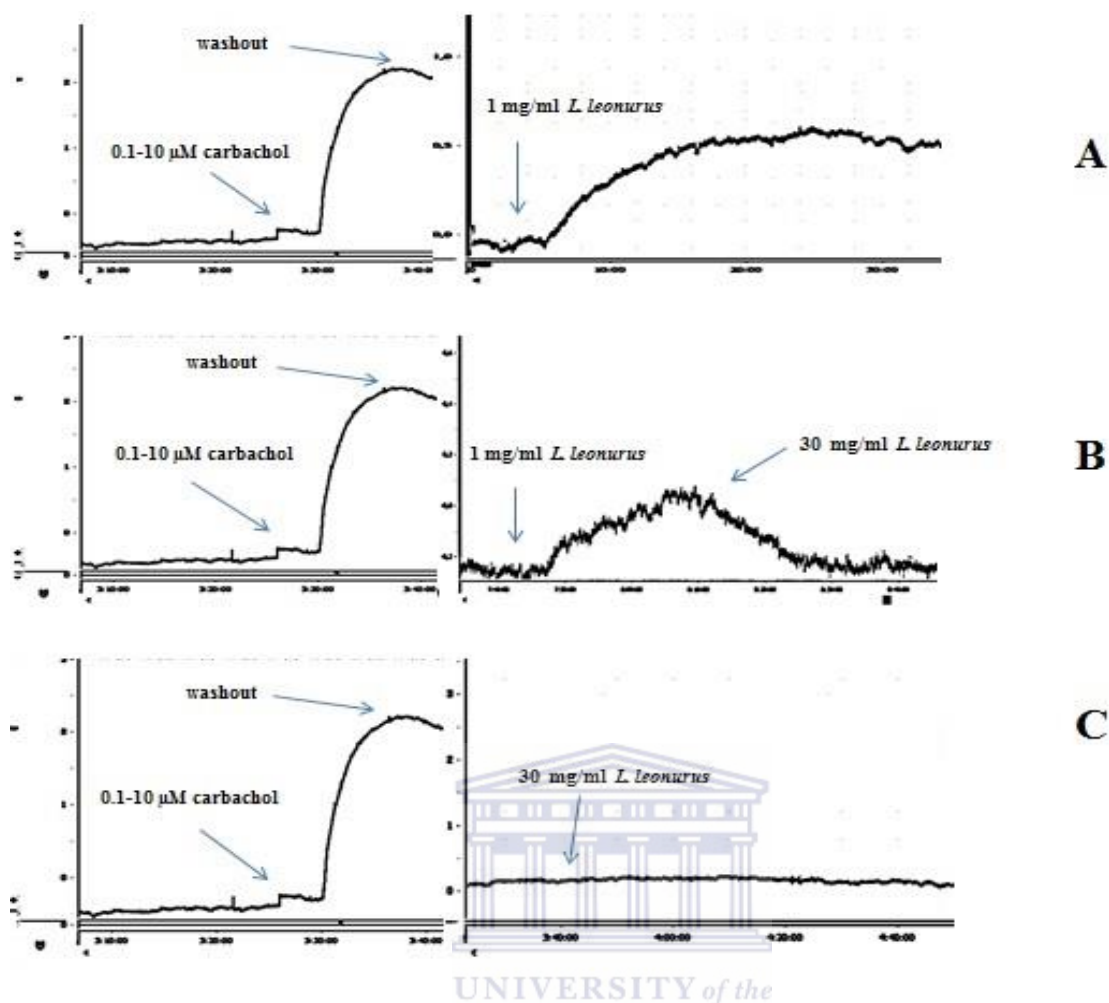
Figure 5.6: Contraction induced by *L. leonurus* (1 mg/ml) on isolated guinea pig trachea after carbachol was thoroughly washed out, with full antagonism by 30 mg/ml *L. leonurus*.

In summary, both *A. afra* and *L. leonurus* were clearly able to relax cholinergic, histaminergic and KCL induced contractions in a dose dependent manner. There were however subtle differences. For instance, at the doses studied, *L. leonurus* was more effective in inducing guinea pig trachea relaxation against KCL induced contractions (EC$_{50}$ value 9.87 mg/ml) than *A. afra* (EC$_{50}$ value 19.88 mg/ml). On the other hand *A. afra* showed far more significant relaxant activity against carbachol (EC$_{50}$ value of 13.93 mg/ml) and histamine (EC$_{50}$ value of 15.47 mg/ml) than *L. leonurus* on carbachol (EC$_{50}$ value 29.34 mg/ml) and histamine (EC$_{50}$ value 94.76 mg/ml) induced tracheal relaxations, respectively. Nevertheless, in general, both *L. leonurus* and *A. afra* were effective antagonists against all three agonists which play a major part in asthma and thus have good potential as bronchodilators. There is

however also no other previous studies on these plants with which this present findings can be compared.

It is possible that the similarity and subtle differences in the potencies of *A. afra* and *L. leonurus versus* the three broncho-constrictors used may be due to differences in chemical composition. In this study the flavonoid, especially luteolin content of the two extracts was quantified (Chapter 4). *A. afra* was found to contain significantly more (16.394 ± 0.884 µg total luteolin/mg FDAE) luteolin than *L.* leonurus (3.093 ± 0.531 µg total luteolin/mg FDAE). Despite the vast difference in luteolin levels between *A. afra* and *L. leonurus*, the bronchodilatory effects concerning the two were not significantly different with exception to histamine. An explanation could be that luteolin if responsible for the bronchodilatory effects is not the main constituent responsible for the observed relaxations. It is more likely that flavonoids found in the plants work in synergy to produce the observed responses i.e. luteolin and quercetin both found in *A. afra* have been implicated in trachea smooth muscle relaxation (Ko et al., 2004). In addition, synergy between flavonoids and other chemical compounds also cannot be excluded as a further explanation for the present finding. Clearly a more detailed chemical profile of the extracts will be required to fully answer this question.

5.5.2 Identification of possible mechanism(s) of action involved in the inhibitory effects of *L. leonurus* and *A. afra.*

To determine the possible or predominant mechanisms involved, the tissue was exposed to cumulative doses of carbachol (0.1-10 µM), histamine (0.1-10 mM) and KCL (20-100 mM) in the absence and presence of *L. leonurus* and *A. afra* (1, 30 and 100 mg/ml). The results obtained are summarised in figures 5.7 and 5.8 below.
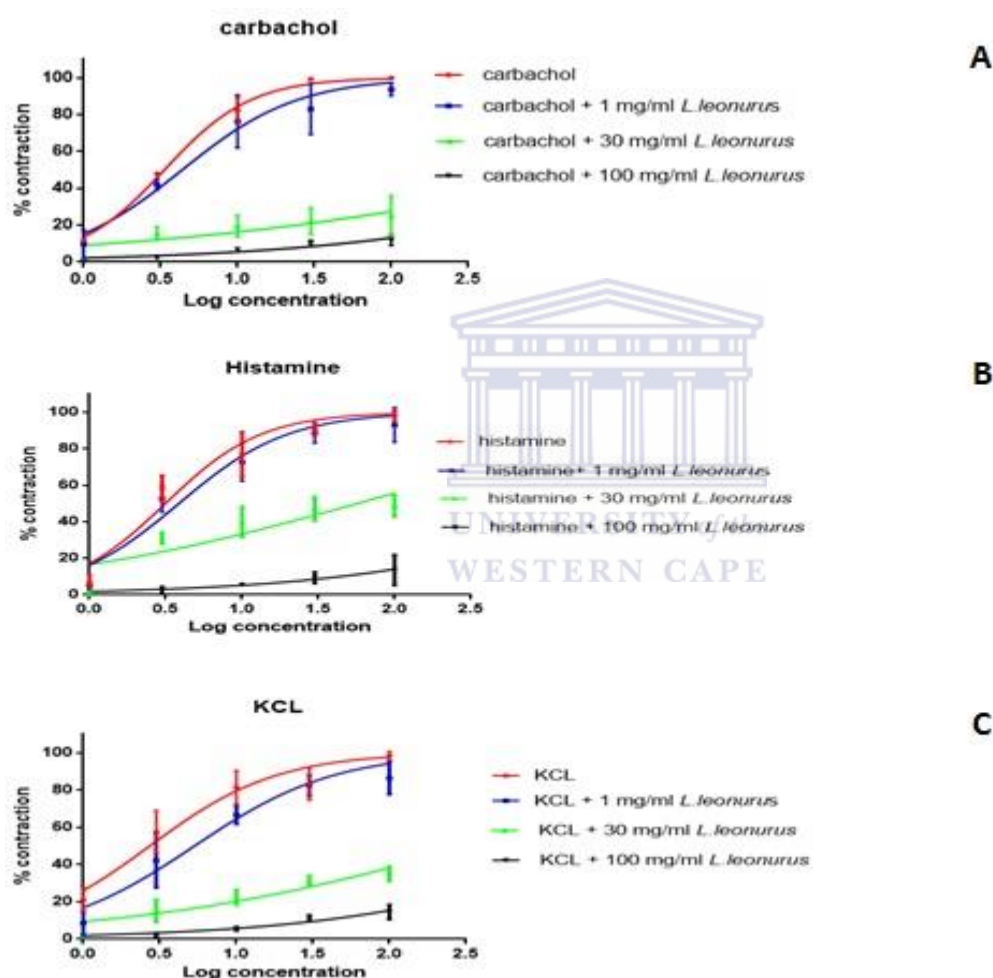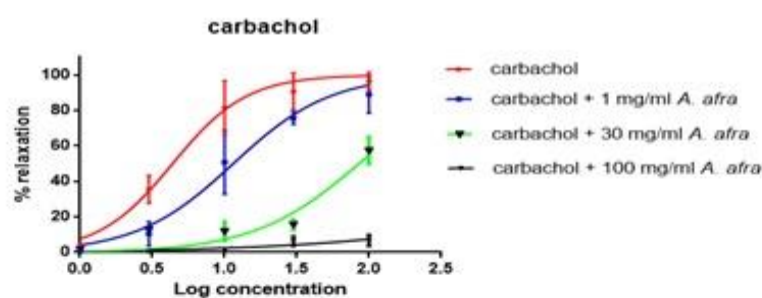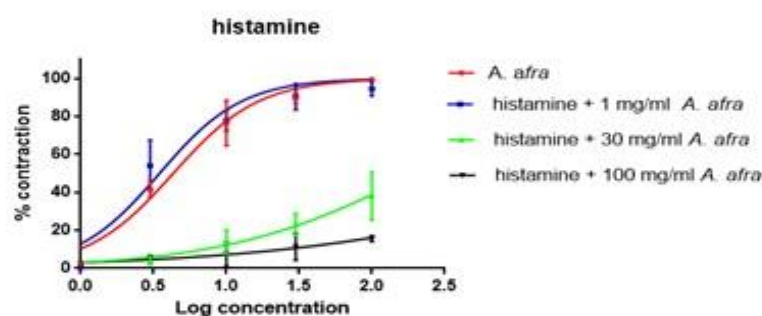

Figure 5.7: Carbachol (A), histamine (B) and KCL (C) induced contraction (%) *versus* **log concentration curves in the absence and presence of 1, 30 and 100 mg/ml** *L. leonurus.* **(n=4)**
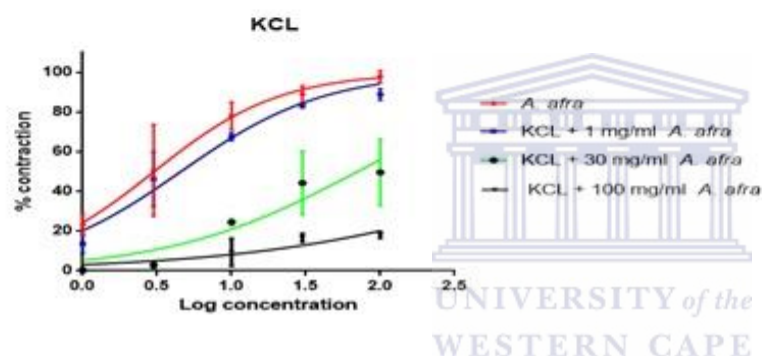
Figure 5.8: Carbachol (A), histamine (B) and KCL (C) induced contraction (%) *versus* log concentration curves in the absence and presence of 1, 30 and 100 mg/ml *A. afra.* (n=4)

At the doses used, *L. leonurus* inhibited the contractions of all three constrictors. At 1 mg/ml its action appeared to be competitive i.e. the carbachol, histamine and KCL graphs were displaced to the right and magnitudes of maximal response as well as the concentrations producing maximal response were not altered (Gilani et al., 2008). At doses of 30 and 100 mg/ml its action appeared to be non-competitive i.e. there was a non-parallel shift of the carbachol, histamine and KCL graphs with suppression of the maximum effect (Gilani et al., 2008; Khan et al., 2008). At a dose of 100 mg/ml *L. leonurus*, it was impossible to recreate the LDRC's using very high doses of carbachol, histamine and KCL. Collectively, these results clearly indicate that *L. leonurus* affected the mediator induced contractions in similar

ways that and do not suggest a more prominent involvement of K^+ channels, above histaminic and cholinergic receptors, in the relaxant effect of *L. leonurus*.

Likewise, *A. afra* also antagonised the contractions induced by all three constrictors in a similar manner. In the presence of 1 mg/ml *A. afra*, carbachol and KCL log dose response curves were displaced to the right with no alteration of concentration and maximal response (Figure 5.8). However, there was no rightward shift (instead an evident slight parallel leftward shift) of the histamine LDRC in the presence of 1 mg/ml *A. afra*. The mechanism involved in the inhibition of histamine-induced contractions was thus most likely non-competitive although a competitive histamine receptor mediated mechanism cannot be ruled out. The 100 mg/ml dose of *A. afra* however caused significant non-parallel shifts with suppression of the maximum effect of the LDRC for all three agonists, strongly suggesting a non-competitive inhibitory mechanism for the plant. As for *A. afra*, these results also suggest a possible involvement of K^+ channels, histaminic as well as cholinergic receptors in the relaxant effect of *A. afra*, and also do not suggest a more prominent involvement of K^+ channels, above histaminic and cholinergic receptors, in the relaxant effect of this plant.
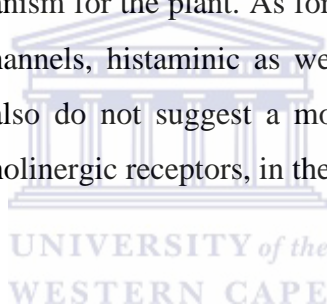
It was hypothesised that K_{ATP} channels would be the significant mechanism responsible for the smooth muscle relaxant actions of both *A. afra* and *L. leonurus*. K_{ATP} channel activators, as typified by cromakalim exert their actions by binding to the (sulphonylurea) receptor subunit inducing a conformational change in this subunit, leading to an increased open probability of the K^+ channel pore. By virtue of such a mechanism, these drugs are able to hyperpolarize the smooth muscle and cause a relaxant response (Pelaia et al., 2002). However, based on the above results, this hypothesis looks very unlikely since K_{ATP} channel activators are known to also show a poor inhibitory effect on contractile responses induced by cholinergic agonists in guinea-pig trachea (Pelaia et al., 2002) and in the present study *A. afra* and *L. leonurus* showed good inhibitory effects against the cholinergic agonist (carbachol) used, strongly suggesting that it is very unlikely that K_{ATP} channels are the predominant mechanism responsible for the actions of *A. afra* and *L. Leonurus*. Unfortunately, specific inhibitors (e.g. glibenclamide) of such channels were not used in this study to fully challenge this hypothesis.

Although not focussed on in this study phosphodiesterase inhibition may also possibly play a prominent role in the inhibitory effects of *A. afra* and *L. leonurus* on the agonist- induced contractions. Histamine, and to a lesser extent carbachol, stimulate cyclic nucleotide phosphodiesterase which catalyse the hydrolysis and inactivation of cAMP leading to contraction of tracheal smooth muscle (Krymskaya and Panettieri, 2007; Turner et al., 1994). Phosphodiesterase (PDE) 3 and 4 inhibitors are potent relaxants of guinea pig trachea pre-contracted with histamine and carbachol (Krymskaya and Panettieri, 2007). Luteolin (contained in both plants) inhibits cyclic adenosine monophosphate (cAMP) - and cyclic guanosine monophosphate (cGMP)-phosphodiesterases (PDE), with the subsequent increase of these two cyclic nucleotides which causes smooth muscle relaxation (Ko et al., 2005). In addition, quercetin inhibits phosphodiesterase 4 (specifically PDE4D) as well as phospholipase Cβ (or PLCβ), another PDE enzyme, preventing the degradation of cAMP which in turn binds to and activate protein kinase A (PKA) which in turn induces smooth relaxation by (1) increasing Ca^{2+} -sequestration or extraction, (2) preventing Ca^{2+} influx, (3) hyperpolarizing cell membranes by activating Na^+, K^+ - ATPase or opening of large conductance K^+ channels, or (4) direct modulation of contractile protein (Ko et al., 2004; Spina et al., 1998; Townsend and Emala, 2013). Therefore phosphodiesterase inhibition (instead of K_{ATP} channels mediation) may possibly better explain the inhibitory effects of *A. afra* and *L. leonurus* on agonist- induced contractions in the present study. Finally, differences in the variety plus levels of other compounds (i.e. besides flavonoids) also present in the two plant extracts would also need to be elucidated if a correct full final explanation of the present data is to be made.

5.6   Conclusion

Overall, the findings obtained indicate that *A. afra* and *L. leonurus* were able to relax cholinergic, histaminergic as well as KCL induced contractions in a dose dependent manner. Maximal or near maximal reversal of the agonist induced contractions were obtained with the plant concentrations used in this study. In general *A. afra* was a stronger tracheal smooth muscle relaxant, it effectively relaxed KCL and histamine induced tracheal contractions more than *L. leonurus.* The mechanism for the antagonism was complex, being competitive at low doses of the plant extracts but non- competitive at high doses of the extracts. K_{ATP} channel

activation clearly did not appear to be a predominant mechanism involved in the relaxant activities of both *A. afra* and *L. leonurus*, rather more than one mechanism of action possibly reflective of differences in active ingredients seems to be involved.

Chapter 6

Conclusions and Recommendations

The overall aim of this study was to determine whether *Artemisia afra* and *Leonotis leonurus* produce their anti-asthmatic effects in and by similar means, and the specific objectives were to determine and compare the profiles and levels of flavonoids in *Artemisia afra* and *Leonotis leonurus*, compare the respiratory smooth muscle relaxant effects of freeze-dried aqueous extracts of *Artemisia afra* and *Leonotis leonurus* and investigate whether K_{ATP} - channel activation is one possible mechanism of action that can explain the effect obtained during traditional use of these two plants. It was hypothesized that the flavonoid profile and levels of *Artemisia afra* would be greater than that of *Leonotis leonurus, Artemisia afra* would be more potent i.e. have greater respiratory muscle relaxant effect than *Leonotis leonurus* and, finally, that *A. afra* and *L. leonurus* will inhibit K^+ -induced (i.e. K_{ATP} channel mediated) contractions in a superior manner than carbachol and histamine induced contractions.

From the results obtained the following conclusions could be drawn:

1. The freeze-dried aqueous extract of *A. afra* and *L. leonurus* are different in flavonoid profile and levels. *A. afra* had higher levels of the marker luteolin whereas *L. leonurus* contained greater number of flavonoid profile and higher percentage of luteolin conjugates.

2. *A. afra* is more potent at relaxing guinea pig trachea smooth muscle than *L. leonurus.* Although *L. leonurus* was more effective at relaxing KCL induced contractions, *A. afra* showed a greater relaxation on carbachol and histamine induced contractions of the tracheal smooth muscle. It is likely that more than one active compound is responsible or a common pathway shared by the agonists (carbachol, histamine and KCL) used is involved in the trachea smooth muscle relaxation of the two plants.

3. Both *A. afra* and *L. leonurus* relaxed KCL induced contractions, but they also relaxed histamine and carbachol. Thus inhibition via K_{ATP} channels is likely not a prominent mechanism of relaxation for both *A. afra* and *L. leonurus* FDAE.

Overall the results obtained confirmed that, aqueous solutions of *A. afra* and *L. leonurus,* as used in local traditional practice, have potent bronchodilator activity that could be of use in the treatment of asthma.

The study had a few limitations. Unfortunately, K_{ATP} channel inhibitors such as glibenclamide were not used in this study to fully investigate K_{ATP} channel involvement in the smooth muscle relaxant effect of the two plants. Moreover, it would have been worthwhile to assess luteolin relaxant effect on the guinea pig trachea. This would have given a much more elaborate indication of whether luteolin is indeed involved in smooth muscle relaxation induced by *A. afra* and *L. leonurus.*

The results also highlighted several areas for further research. Firstly, further studies are required to exactly identify the compounds responsible for the relaxant activities of the two plants. It may be useful to focus on flavonoids present in the plants for the first investigations. But at least greater detail on the variety plus levels of different individual flavonoids might prove useful. Secondly, other mechanism(s) of action should also be investigated. For instance, it would be interesting to ascertain the role, if any, of PDE 4 inhibitory activity, which has already been suggested for *L. leonurus* (Mulubwe, 2004), intracellular Ca^+ regulation and the Ca^+ activated potassium channels. Definitive studies on the mechanism would however be best explored after active compounds have been identified and isolated. Lastly, an actual assessment of the relative chemical safety the two plants, a prelude to chemical efficacy study in asthmatics may be well warranted. From such studies, more effective anti-asthma treatments may arise and it may also guide to more effective utilization of these plants.

References

Aherne, S.A., Kerry, J.P., O'Brien, N.M., 2007. Effects of plant extracts on antioxidant status and oxidant-induced stress in Caco-2 cells. *Br. J. Nutr*. 97, 321.

Allegra, L., Braga, P.C., Negro, R., 1993. Methods in Asthmology. Springer Science & Business Media, Springer Verlag, Berlin, Heidelberg.

Amdur, M.O., Mead, J., 1958. Mechanics of respiration in unanesthetized guinea pigs. *Am. J .Physiol*. 192, 364–368.

Areias, F.M., Rego, A.C., Oliveira, C.R., Seabra, R.M., 2001. Antioxidant effect of flavonoids after ascorbate/Fe2+-induced oxidative stress in cultured retinal cells1. *Biochem. Pharmacol*. 62, 111–118.

Aworet-Samseny, R.R., Souza, A., Kpahé, F., Konaté, K., Datté, J.Y., 2011. *Dichrostachys cinerea* hydro-alcoholic extract action on the contractility of tracheal smooth muscle isolated from guinea-pig. *BMC Complement. Altern. Med*. 11, 23.

Baranowska, I., Magiera, S., 2011. Analysis of isoflavones and flavonoids in human urine by UHPLC. *Anal. Bioanal. Chem*. 399, 3211–3219.

Barnes, P.J., 2012. Corticosteroid therapy for asthma. *Pulmão. RJ*. 21, 53–59.

Barnes, P.J., 2010. New therapies for asthma: is there any progress? *Trends Pharmacol. Sci.* 31, 335–343.

Barnes, P.J., 2006. Drugs for asthma. *Br. J. Pharmacol*. 147, 297–303.

Barnes, P.J., 2000. New treatments for asthma. *Eur. J. Intern. Med*. 11, 9–20.

Bateman, E.D., Hurd, S.S., Barnes, P.J., Bousquet, J., Drazen, J.M., FitzGerald, M., Gibson, P., Ohta, K., O'Byrne, P., Pedersen, S.E., Pizzichini, E., Sullivan, S.D., Wenzel, S.E., Zar, H.J., 2008. Global strategy for asthma management and prevention: GINA executive summary. *Eur. Respir. J.* 31, 143–178.

Bielory, L., Lupoli, K., 1999. Herbal interventions in asthma and allergy. *J. Asthma Off. J. Assoc. Care Asthma.* 36, 1–65.

Blume, C., Davies, D.E., 2013. In vitro and ex vivo models of human asthma. Biological Barriers A Need for Novel Tools in Nanotoxicology and Nanomedicine The 8th International Conference and Workshop - Biological Barriers - in vitro Tools in Nanotoxicology and Nanomedicine. *Eur. J. Pharm. Biopharm.* 84, 394–400.

Bonfield, T.L., 2012. In vivo models of lung disease. *Lung Dis. State Art Rev. InTech.* 407–428.
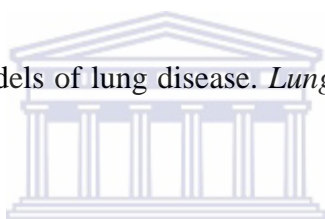
Bousquet, J., Jeffery, P.K., Busse, W.W., Johnson, M., Vignola, A.M., 2000. *Asthma. Am. J. Respir. Crit. Care Med.* 161, 1720–1745.

Bräter, P., Ribas, B., Schramel, P., 1994. Trace Element Analytical Chemistryin Medicine and Biology. *Editorial CSIC - CSIC Press.*

Brewer, J.P., Kisselgof, A.B., Martin, T.R., 1999. Genetic variability in pulmonary physiological, cellular, and antibody responses to antigen in mice. *Am. J. Respir. Crit. Care Med.* 160, 1150–1156.

Canning, B.J., Chou, Y., 2008. Using guinea pigs in studies relevant to asthma and COPD. *Pulm. Pharmacol. Ther.* 21, 702–720.

Castillo, J.C., De Beer, E.J., 1947. The tracheal chain; a preparation for the study of antispasmodics with particular reference to bronchodilator drugs. *J. Pharmacol. Exp. Ther*. 90, 104–109.

Chowdhury, A.R., Sharma, S., Mandal, S., Goswami, A., Mukhopadhyay, S., Majumder, H.K., 2002. Luteolin, an emerging anti-cancer flavonoid, poisons eukaryotic DNA topoisomerase *I. Biochem. J*. 366, 653–661.

De la Motta, S., 1991. Simultaneous measurement of respiratory and circulatory parameters on anesthetized guinea pigs. Seventh Freiburg Focus on Biomeasurement (FFB7) Publ. by Biomesstechnik Verlag, 79232 March, Germany. B IV, pp 45–65

De Rojas, V.R.S., Ortega, T., Villar, A., 1995. Inhibitory effects of Cistus populifolius on contractile responses in the isolated rat duodenum. *J. Ethnopharmacol*. 46, 59–62.

Desilets, A.R., Gickas, J.J., Dunican, K.C., 2009. Role of huperzine a in the treatment of Alzheimer's disease. *Ann. Pharmacother*. 43, 514–518.

Dias, D.A., Urban, S., Roessner, U., 2012. A Historical Overview of Natural Products in Drug Discovery. *Metabolites*. 2, 303–336.

Dube, A. 2006. The design, preparation and evaluation of *Artemisia afra* and placebos in tea bag dosage form suitable for use in clinical trials. A Master's degree, University of the Western Cape, Bellville.

Englert, H.C., Wirth, K., Gehring, D., Fürst, U., Albus, U., Scholz, W., Rosenkranz, B., Schölkens, B.A., 1992. Airway pharmacology of the potassium channel opener, HOE 234, in guinea pigs: in vitro and in vivo studies. *Eur. J. Pharmacol*. 210, 69–75.

Erasto, P., Adebola, P. O., Grierson, D. S. & Afolayan, A. J. 2005. An ethnobotanical study of plants used for the treatment of diabetes in the Eastern Cape Province, South Africa. *Afr. J. Biotechnol.* 4**,** 1458-1460.

Fedan, J.S., Van Scott, M.R., Johnston, R.A., 2001. Pharmacological techniques for the in vitro study of airways. *J. Pharmacol. Toxicol*. Methods 45, 159–174.

Fennell, C.W., Light, M.E., Sparg, S.G., Stafford, G.I., van Staden, J., 2004. Assessing African medicinal plants for efficacy and safety: agricultural and storage practices. *J. Ethnopharmacol*. 95, 113–121.

Ferriola P.C., Cody V., Middleton Jr E., 1989. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *Biochem. Pharmacol.* 38, 1617-24.

Gálvez, M., Martin-Cordero, C., López-Lázaro, M., Cortés, F., Ayuso, M.J., 2003. Cytotoxic effect of Plantago spp. on cancer cell lines. *J. Ethnopharmacol*. 88, 125–130.

Gilani, A.H., Khan, A., Ali, T., Ajmal, S., 2008. Mechanisms underlying the antispasmodic and bronchodilatory properties of Terminalia bellerica fruit. *J. Ethnopharmacol*. 116, 528–538.

GINA_Report_2015.pdf [WWW Document], n.d. URL http://www.ginasthma.org/local/uploads/files/GINA_Report_2015.pdf (accessed 5.14.15).
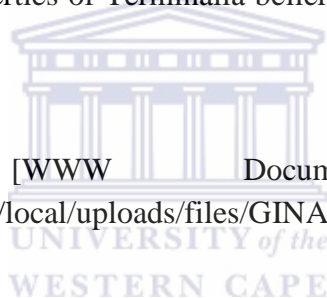
Glaab, T., Taube, C., Braun, A., Mitzner, W., 2007. Invasive and noninvasive methods for studying pulmonary function in mice. *Respir. Res*. 8, 63.

Gordaliza, M., 2009. Terpenyl-Purines from the Sea. *Mar. Drugs*. 7, 833–849.

Grotewold, E.d., 2006. The science of flavonoids. Springer, New York, NY.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. *Phytochemistry* 55, 481–504.

Harkness, L.M., Ashton, A.W., Burgess, J.K., 2015. Asthma is not only an airway disease, but also a vascular disease. *Pharmacol. Ther*. 148, 17–33.

75

Harris, L., 2002. An evaluation of the bronchodilator properties of *Mentha longifolia* and *Artemisia afra*, traditional medicinal plants used in the Western Cape. A Master's thesis. University of the Western Cape, Bellville.

Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther*. 96, 67–202.

Hayashi T., Sawa K., Kawasaki M., Arisawa M., Shimizu M., Morita N., 1988. Inhibition of cow's milk xanthine oxidase by flavonoids. *J. Nat. Prod*. 51, 345-8.

Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem*. 13, 572–584.

Herszberg, B., Ramos-Barbón, D., Tamaoka, M., Martin, J.G., Lavoie, J.P., 2006. Heaves, an asthma-like equine disease, involves airway smooth muscle remodeling. *J. Allergy Clin. Immunol.* 118, 382–388.
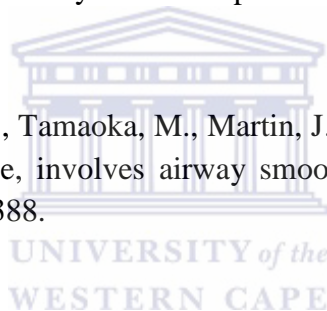
Hilliard, O. M. 1977. Compositae in Natal. Pietermaritzburg: University of Natal Press.

Holgate, S.T., 2011. Pathophysiology of asthma: What has our current understanding taught us about new therapeutic approaches? *J. Allergy Clin. Immunol*. 128, 495–505.

Holmes, A.M., Solari, R., Holgate, S.T., 2011. Animal models of asthma: value, limitations and opportunities for alternative approaches. *Drug Discovery Today*.16, 659–670.

Hylkema, M.N., Hoekstra, M.O., Luinge, M., Timens, W., 2002. The strength of the OVA-induced airway inflammation in rats is strain dependent. *Clin. Exp. Immunol*. 129, 390–396.

Jackson, W.P.U., 1990. Origins and meanings of names of South African plant genera. U.C.T. Printing Dept., Cape Town.

Jones, T.R., Charette, L., Denis, D., 1988. Antigen-induced contraction of guinea-pig isolated trachea: studies with novel inhibitors and antagonists of arachidonic acid metabolites. *Br. J. Pharmacol.* 95, 309–321.

Joussen, A.M., Rohrschneider, K., Reichling, J., Kirchhof, B., Kruse, F.E., 2000. Treatment of Corneal Neovascularization with Dietary Isoflavonoids and Flavonoids. *Exp. Eye Res.* 71, 483–487.

Kalili, K.M., de Villiers, A., 2011. Recent developments in the HPLC separation of phenolic compounds. *J. Sep. Sci.* 34, 854–876.

Karol, M.H., 1994. Animal models of occupational asthma. *Eur. Respir. J.* 7, 555–568.

Khan, A., Gilani, A.H., 2009. Antispasmodic and bronchodilator activities of Artemisia vulgaris are mediated through dual blockade of muscarinic receptors and calcium influx. *J. Ethnopharmacol.* 126, 480–486.

Khan, S., Park, S., Sirajuddin, I., Grayson, M., 2008. Respiratory virus and asthma: The role of immunoglobulin. *E. Clin. Ther.* 30, 1017–1024.

Khoddami, A., Wilkes, M., Roberts, T., 2013. Techniques for Analysis of Plant Phenolic Compounds. *Molecules.* 18, 2328–2375.

Komperlla, M. K. 2005. The formulation, manufacture and evaluation of rapid release tablets made from Artemisia afra plant material. A Master's thesis, University of the Western Cape, Bellville.

Korenblat, P.E., 2000. The role of cysteinyl leukotriene receptor antagonists in asthma therapy. *Hosp. Physician.* 36, 50–66.

Ko, W.C., Shih, C.M., Lai, Y.H., Chen, J.H., Huang, H.L., 2004. Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure–activity relationships. *Biochem. Pharmacol.* 68, 2087–2094.

Ko, W.C., Shih, C.M., Leu, I.J., Chen, T.T., Chang, J.P., 2005. Mechanisms of Relaxant Action of Luteolin in Isolated Guinea Pig Trachea. *Planta. Med.* 71, 406–411.

Krymskaya, V.P., Panettieri, R.A., 2007. Phosphodiesterases Regulate Airway Smooth Muscle Function in Health and Disease, in: Current Topics in Developmental Biology. *Elsevier*, 38. 61–74.

Kuppusamy U.R., Das N.P., 1992. Effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes. *Biochem. Pharmacol*. 44, 1307-15.

Lopez-Lazaro, M., 2009. Distribution and biological activities of the flavonoid luteolin. *Mini Rev. Med. Chem.* 9, 31–59.

Ma, H. (2006). The formulation, manufacture and evaluation of capsules containing freeze-dried aqueous extracts of *Leonotis leonorus* and *Mentha longifolia*. A Master's thesis, University of the Western Cape, Bellville.

Maddox, L., Schwartz, D.A., 2002. The pathophysiology of asthma. *Annu. Rev. Med.* 53, 477–498.

Markham, K.R., 1982. Techniques of flavonoid identification. Academic Press. pp.1-60.

Martin, J.G., Tamaoka, M., 2006. Rat models of asthma and chronic obstructive lung disease. *Pulm. Pharmacol. Ther*. 19, 377–385.

Mahop, T. M. & Mayet, M. 2007. Enroute to biopiracy? Ethnobotanical research on antidiabetic medicinal plants in the Eastern Cape Province, South Africa. *Afr. J. Biotechnol,* 6**,** 2945-2952.

Masoli, M., Fabian, D., Holt, S., Beasley, R., Global Initiative for Asthma (GINA) Program, 2004. The global burden of asthma: executive summary of the GINA Dissemination Committee Report. Allergy 59, 469–478.

Mastalerz, L., Kumik, J., 2010. Antileukotriene drugs in the treatment of asthma. *Pol. Arch Med. Wewn.* 120, 103–108.

Merken, H.M., Beecher, G.R., 2000. Measurement of Food Flavonoids by High-Performance Liquid Chromatography: A Review. *J. Agric. Food Chem.* 48, 577–599.

Meurs, H., Santing, R.E., Remie, R., van der Mark, T.W., Westerhof, F.J., Zuidhof, A.B., Bos, I.S.T., Zaagsma, J., 2006. A guinea pig model of acute and chronic asthma using permanently instrumented and unrestrained animals. *Nat. Protoc.* 1, 840–847.

Muccitelli, R.M., Tucker, S.S., Hay, D.W., Torphy, T.J., Wasserman, M.A., 1987. Is the guinea pig trachea a good in vitro model of human large and central airways? Comparison on leukotriene-, methacholine-, histamine- and antigen-induced contractions. *J. Pharmacol. Exp. Ther*. 243, 467–473.

Muganga, R. 2004. Luteoln levels in selected folkloric preparations and the bioavalablity of luteolin from *Artemisia afra* acqueous extract in velvet monkey. A Master's thesis, University of Western Cape, Bellville.

Mukinda, J.T., Syce, J.A., Fisher, D., Meyer, M., 2010. Effect of the Plant Matrix on the Uptake of Luteolin Derivatives-containing Artemisia afra Aqueous-extract in Caco-2 cells. *J. Ethnopharmacol*. 130, 439–449.

Mulubwe, N., 2007. A comparative study of the phosphodiesterase 4 inhibitory activity of *Artemisia afra, Leonotis leonorus* and *Mentha longifolia* plant medicines. A Master's thesis, University of the Western Cape, Bellville.

Murdoch, J.R., Lloyd, C.M., 2010. Chronic inflammation and asthma. *Mutat. Res. Mol. Mech. Mutagen.* 690, 24–39.

Naczk, M., Shahidi, F., 2004. Extraction and analysis of phenolics in food. *J. Chromatogr. A* 1054, 95–111.

Newman, D.J., Cragg, G.M., 2007. Natural Products as Sources of New Drugs over the Last 25 Years. *J. Nat. Prod.* 70, 461–477.

Niimi, A., 2013. Cough, asthma, and cysteinyl-leukotrienes. *Pulm. Pharmacol. Ther.* 26, 514–519.

O'Byrne, P.M., Gauvreau, G.M., Murphy, D.M., 2009. Efficacy of leukotriene receptor antagonists and synthesis inhibitors in asthma. *J. Allergy Clin. Immunol.* 124, 397–403.
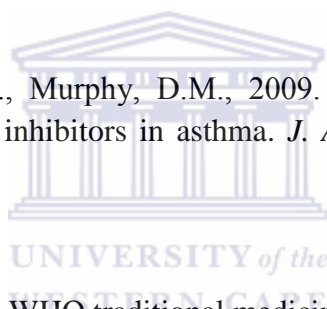
Organization, W.H., others, 2002. WHO traditional medicine strategy 2002-2005.

Page, C.P., Barnes, P.J., 2004. Pharmacology and Therapeutics of Asthma and COPD, *Handbook of Experimental Pharmacology*. Springer-Verlag Berlin Heidelberg.

Pan, S.-Y., Zhou, S.-F., Gao, S.-H., Yu, Z.-L., Zhang, S.-F., Tang, M.-K., Sun, J.-N., Ma, D.-L., Han, Y.-F., Fong, W.-F., Ko, K.-M., 2013. New Perspectives on How to Discover Drugs from Herbal Medicines: CAM's Outstanding Contribution to Modern Therapeutics. *Evid. Based Complement. Alternat. Med.* 1–25.

Patel, K.N., others, 2014. Animal models of asthma. *J. Pharm. Res. Opin.* 1.

Pelaia, G., Gallelli, L., Vatrella, A., Grembiale, R.D., Maselli, R., De Sarro, G.B., Marsico, S.A., 2002. Potential role of potassium channel openers in the treatment of asthma and chronic obstructive pulmonary disease. *Life Sci.* 70, 977–990.

Pelaia, G., Vatrella, A., Busceti, M.T., Gallelli, L., Calabrese, C., Terracciano, R., Maselli, R., 2015. Cellular Mechanisms Underlying Eosinophilic and Neutrophilic Airway Inflammation in Asthma. *Mediators. Inflamm.* vol. 2015,1-8.

Pera, T., Penn, R.B., 2014. Crosstalk between beta-2-adrenoceptor and muscarinic acetylcholine receptors in the airway. *Curr. Opin. Pharmacol., Respiratory Musculoskeletal* 16, 72–81.

Persson, C.G., Erjefält, J.S., Korsgren, M., Sundler, F., 1997. The mouse trap. *Trends. Pharmacol. Sci.* 18, 465–467.

Picó, Y., 2012. Chemical Analysis of Food: Techniques and Applications: Techniques and Applications. Academic Press. University of Valencia.

Polya, G., 2003. Biochemical Targets of Plant Bioactive Compounds: A Pharmacological Reference Guide to Sites of Action and Biological Effects. CRC Press. University, Melbourne, Australia.

Poupko, J.M., Baskin, S.I., Moore, E., 2007. The pharmacological properties of anisodamine. *J. Appl. Toxicol.* 27, 116–121.

Quirce, S., Bobolea, I., Barranco, P., 2012. Emerging drugs for asthma. *Expert Opin. Emerg. Drugs* 17, 219–237.

Rang, H.P., Dale, M., 2015. *Pharmacology*. Churchill Livingstone. New York.

Reinero, C.R., 2011. Advances in the understanding of pathogenesis, and diagnostics and therapeutics for feline allergic asthma. *Vet. J*. 190, 28–33.

Ressmeyer, A.R., Larsson, A.K., Vollmer, E., Dahlen, S.E., Uhlig, S., Martin, C., 2006. Characterisation of guinea pig precision-cut lung slices: comparison with human tissues. *Eur. Respir. J.* 28, 603–611.

Robinson, D.S., 2010. The role of the T cell in asthma. *J. Allergy Clin. Immunol.* 126, 1081–1091.

Roberts, M., 1990. Indigenous Healing Plants. Southern Book Publishers, Halfway House, South Africa.

Rogers, D.F., Boschetto, P., Barnes, P.J., 1989. Correlation between evans blue dye and radiolabeled albumin in guinea pig airways in vivo. *J. Pharmacol. Methods* 21, 309–315.

Rolph, M.S., Sisavanh, M., Liu, S.M., Mackay, C.R., 2006. Clues to asthma pathogenesis from microarray expression studies. *Pharmacol. Ther*. 109, 284–294.

Sadzuka, Y., Sugiyama, T., Shimoi, K., Kinae, N., Hirota, S., 1997. Protective effect of flavonoids on doxorubicin-induced cardiotoxicity. *Toxicol. Lett*. 92, 1–7.

Shah, N., Cynkar, W., Smith, P., Cozzolino, D., 2010. Use of Attenuated Total Reflectance Midinfrared for Rapid and Real-Time Analysis of Compositional Parameters in Commercial White Grape Juice. *J. Agric. Food Chem.* 58, 3279–3283.

SATMERG, 1999. http://www.sahealthinfo.org/traditionalmeds/htm.

Shapiro, S.D., 2008. The use of transgenic mice for modeling airways disease. *Pulm. Pharmacol. Ther*. 21, 699–701.

Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M., Hara, Y., Yamamoto, H., Kinae, N., 1998. Intestinal absorption of luteolin and luteolin 7-O-β-glucoside in rats and humans. *FEBS Lett. 438, 220–224.*

Shimoi, K., Saka, N., Nozawa, R., Sato, M., Amano, I., Nakayama, T., Kinae, N., 2001. Deglucuronidation of a Flavonoid, Luteolin Monoglucuronide, during Inflammation. *Drug Metab. Dispos.* 29, 1521–1524.

Silva, R.C., Couceiro, J.N., Câmara, F.P., Valle, S., Santos, N., 2015. Asthma exacerbation and viral infection in adult patients, Brazil. *Braz. J. Infect. Dis.* 15,1413-8670.

Stalikas, C.D., 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. *J. Sep. Sci*. 30, 3268–3295.

Suckow, M.A., Stevens, K.A., Wilson, R.P., 2012. The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents. *Academic Press*. London, UK.

Suliman, S., 2011. Antimicrobial interactions of *Artemisia afra* used in African traditional medicine. Faculty of Health Sciences, University of the Witwatersrand.

Sutovska, M., Nosalova, G., Franova, S., 2007. The role of potassium ion channels in cough and other reflexes of the airways. *J. Physiol. Pharmacol*. 58, 673–683.

Suzuki, K., Adachi, R., Hirayama, A., Watanabe, H., Otani, S., Watanabe, Y., Kasahara, T., 2005. Indirubin, a Chinese anti-leukaemia drug, promotes neutrophilic differentiation of human myelocytic leukaemia HL-60 cells. *Br. J. Haematol.* 130, 681–690.

Szczeklik, A., Stevenson, D.D., 2003. Aspirin-induced asthma: Advances in pathogenesis, diagnosis, and management. *J. Allergy Clin. Immunol.* 111, 913–921.

Taylor, J.L.S., Van Staden, J., 2001. COX-1 inhibitory activity in extracts from *Eucomis* L"Herit species. *J. Ethnopharmacol*. 75, 257-265.

Thomas, C., 2002. The Role of Ayurvedic Therapies for HIV/AIDS Care: A Comprehensive Review of the Literature. *J. Glob. Health Perspect*. 6.120-135.

Thring, T.S.A., Weitz, F.M., 2006. Medicinal plant use in the Bredasdorp/Elim region of the Southern Overberg in the Western Cape Province of South Africa. *J. Ethnopharmacol*. 103, 261–275.

Tilley, S., 2011. Methylxanthines in Asthma. *Handbook of Experimental Pharmacology*. Springer Berlin Heidelberg, pp. 439–456.

Townsend, E.A., Emala, C.W., 2013. Quercetin acutely relaxes airway smooth muscle and potentiates β-agonist-induced relaxation via dual phosphodiesterase inhibition of PLCβ and PDE4. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 305, L396–L403.

Turner, N.C., Lamb, J., Worby, A., Murray, K.J., 1994. Relaxation of guinea-pig trachea by cyclic AMP phosphodiesterase inhibitors and their enhancement by sodium nitroprusside. *Br. J. Pharmacol*. 111, 1047–1052.

Uhlig, S., Taylor, A.E., 2012. *Methods in Pulmonary Research*. Birkhäuser Basel.

Uhlig, S., Uhlig, S., Taylor, A.E., 1998. *Methods in Pulmonary Research. Springer Science & Business Media*. Birkhäuser Basel

Van Wyk, B., Oudshoorn, V., Gericke, N., 1997. Medicinal Plants of South Africa. first ed. Pretoria: Briza Publications, Pretoria

Van Wyk, B. E. & Gerike, N. 2000. A Guide to Useful Plants of Southern Africa. Briza Publications, Pretoria.

Venema, C.M., Patterson, C.C., 2010. Feline asthma: what's new and where might clinical practice be heading? *J. Feline Med. Surg*. 12, 681–692.

Vermani, K., Garg, S., 2002. Herbal medicines for sexually transmitted diseases and AIDS. *J. Ethnopharmacol*. 80, 49–66.

Vogel, H.G., 2002. Drug Discovery and Evaluation: Pharmacological Assays ; Including a CD-ROM. Springer Science & Business Media. Berlin Heidelberg.

Vogel, H.G., Vogel, W.H., 2013. Drug Discovery and Evaluation: Pharmacological Assays. Springer Science & Business Media. Berlin Heidelberg.

Waithaka, J. 2004. The Evaluation of Markers for Quality Control Studies of Flavonoid–Containing Medicinal Preparations. A Masters thesis, University of the Western Cape.

Watt, J. M. & Breyer-Brandwijk, M. G. 1962. Medicinal and Poisonous Plants of Southern and Eastern Africa. *E & S*, Livingstone Ltd.: Edimburgh & London

Weatherall, M., Wijesinghe, M., Perrin, K., Harwood, M., Beasley, R., 2010. Meta-analysis of the risk of mortality with salmeterol and the effect of concomitant inhaled corticosteroid therapy. *Thorax*. 65, 39–43.

Webb, R.C., 2003. Smooth Muscle Contraction and Relaxation. *Adv. Physiol. Educ*. 27, 201–206.

WHO, Press Release, 2002. Who launches the first global strategy on traditional and alternative medicine. Pg 38.

Yu, S.M., Kuo, S.C., 1995. Vasorelaxant effect of isoliquiritigenin, a novel soluble guanylate cyclase activator, in rat aorta. *Br. J. Pharmacol*. 114, 1587 – 1594.
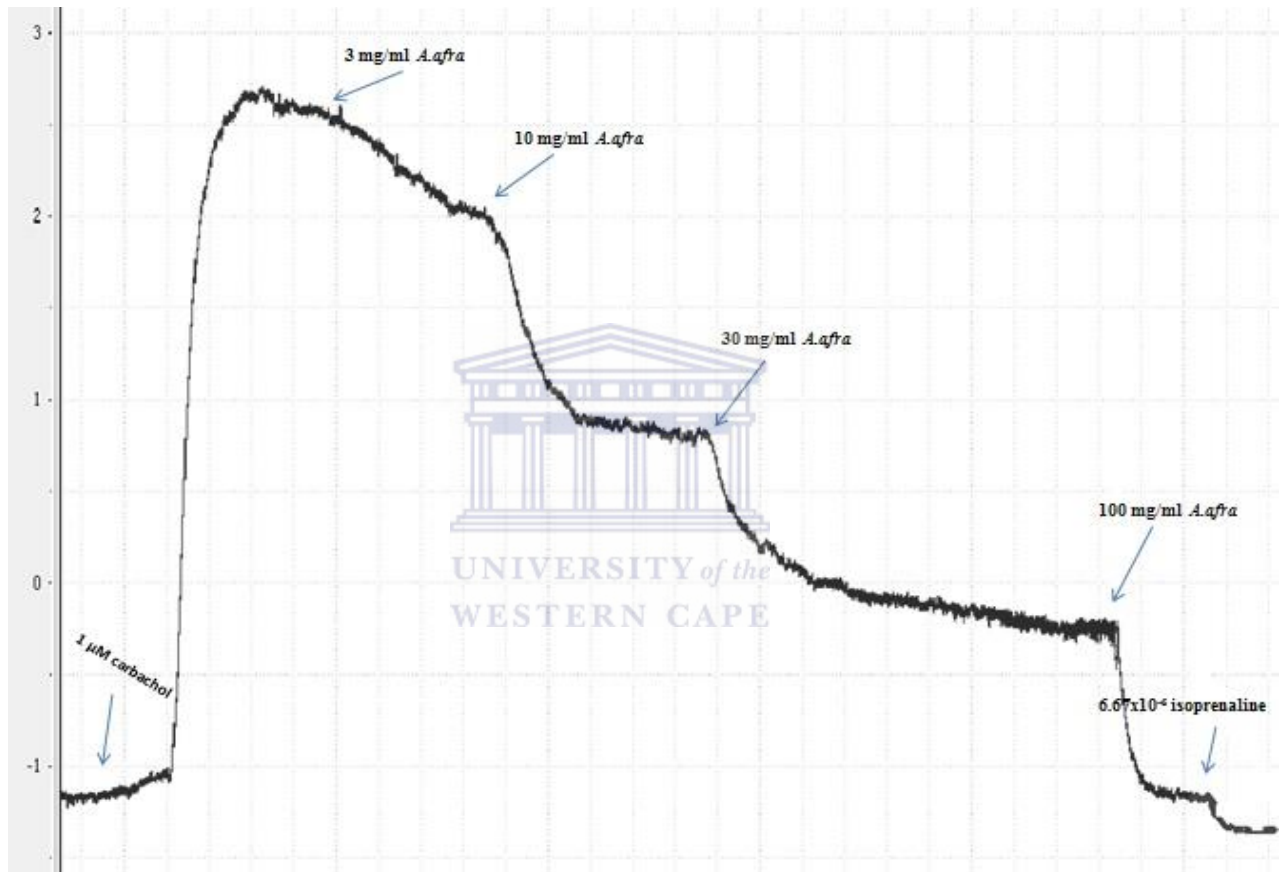
Zarena, A. s., Sankar, K.U., 2012. Phenolic Acids, Flavonoid Profile and Antioxidant Activity in Mangosteen (garcinia Mangostana L.) Pericarp. *J. Food Biochem*. 36, 627–633.

Zhang, B., 2008. Anti-inflammatory effects of inhibitors of the NF-kB pathway in the mouse asthma model. *Pharmacol Exp Ther*. 317, 1285-94.
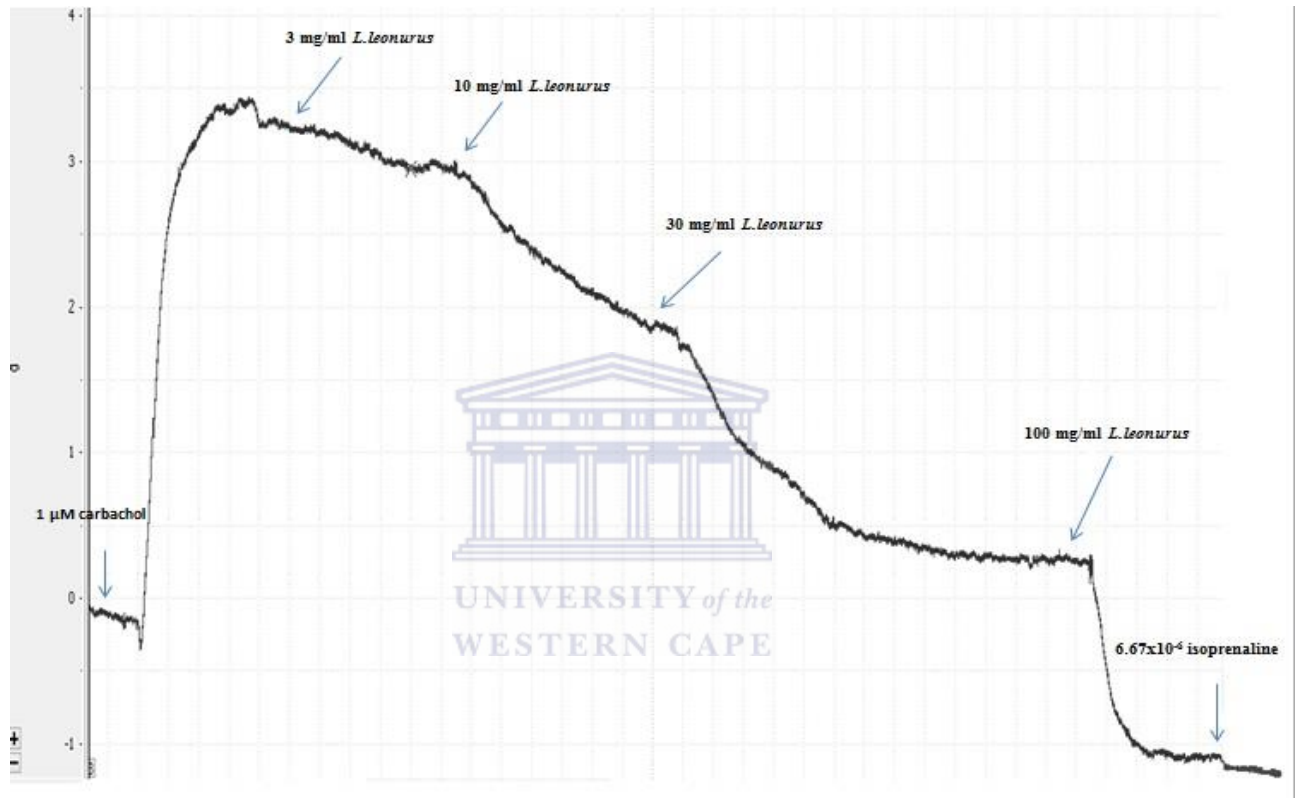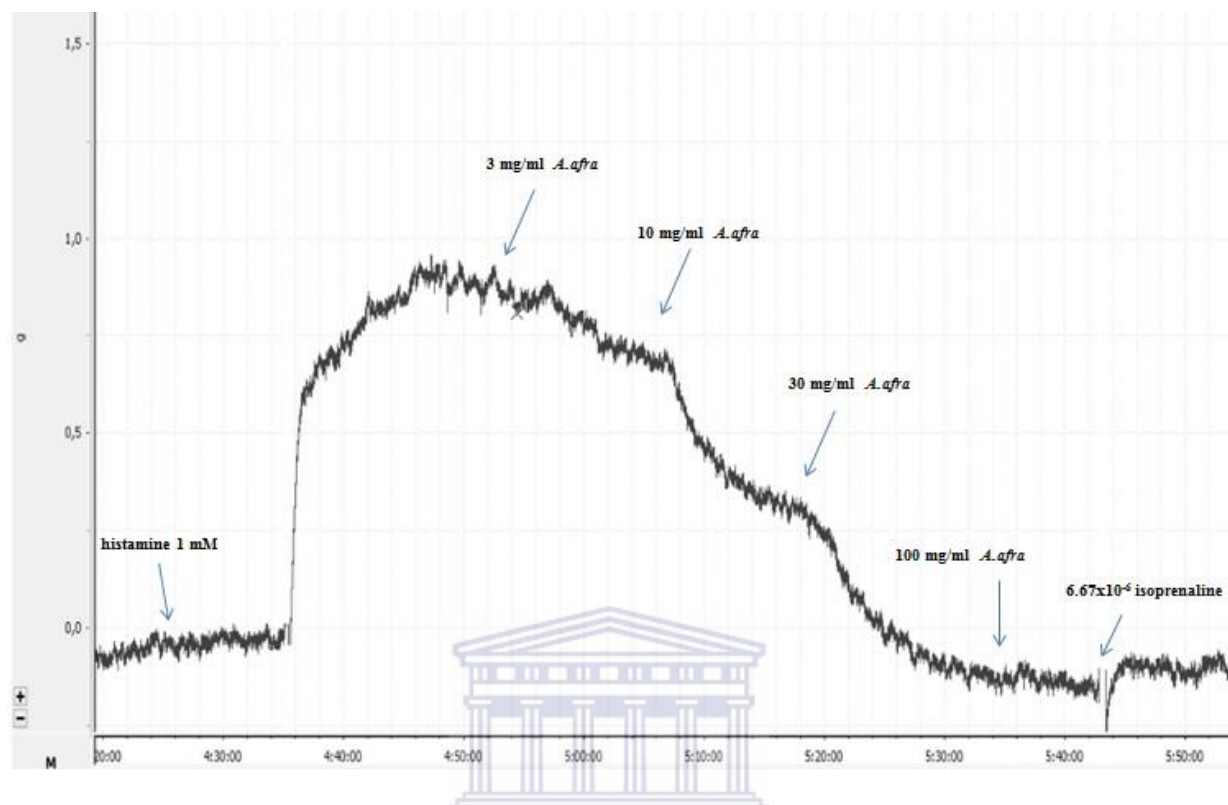
Appendices

Appendix 1

Appendix 1: Trace of relaxation (g force) caused by cumulative concentrations of *A. afra* on isolated guinea pig trachea constricted with carbachol.
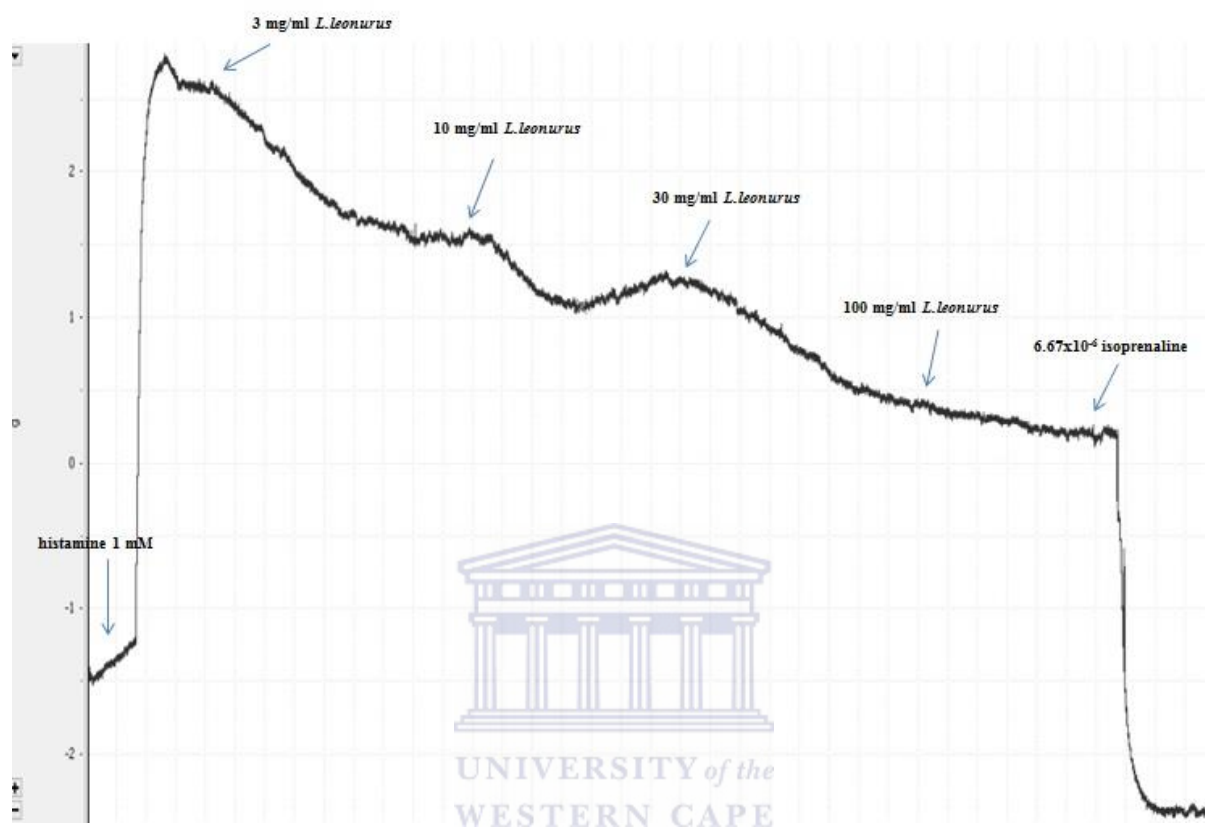
Appendix 2

Appendix 2: Trace of relaxation (g force) caused by cumulative concentrations of *L. leonurus* on isolated guinea pig trachea constricted with carbachol.

Appendix 3

Appendix 3: Trace of relaxation (g force) caused by cumulative concentrations of *A. afra* on isolated guinea pig trachea constricted with histamine.
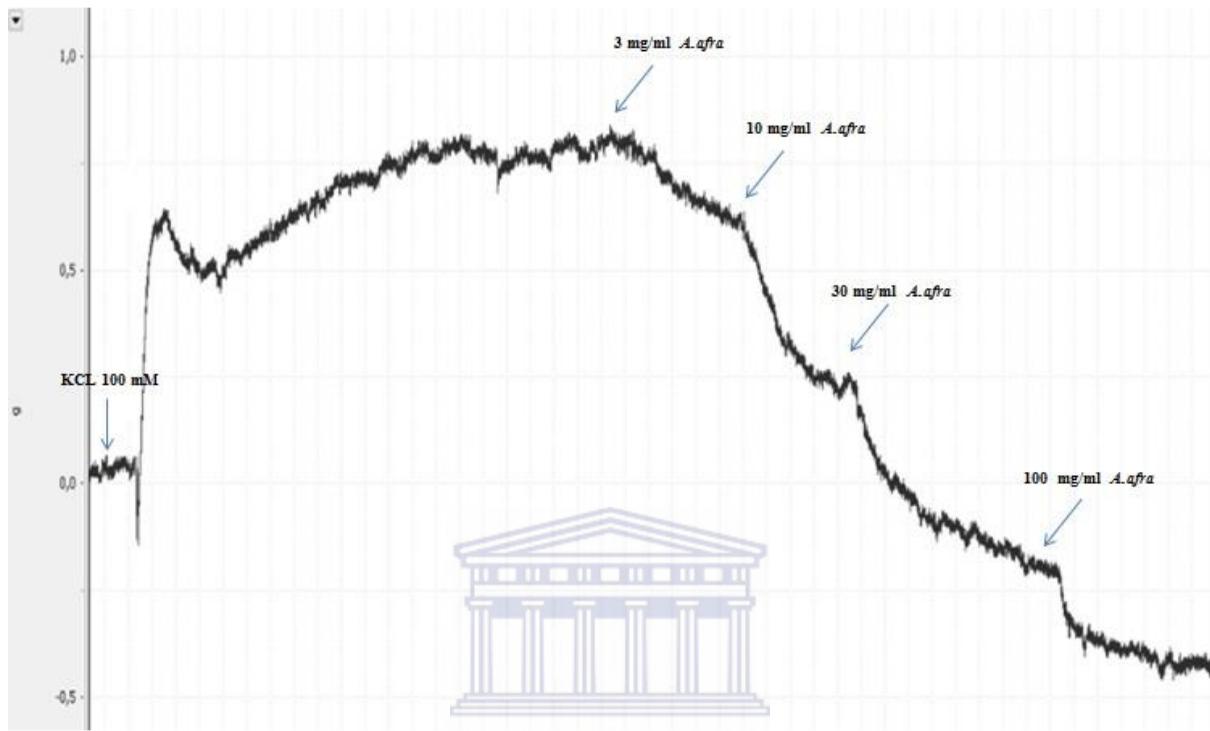
Appendix 4

Appendix 4: Trace of relaxation (g force) caused by cumulative concentrations of *L. leonurus* on isolated guinea pig trachea constricted with histamine.
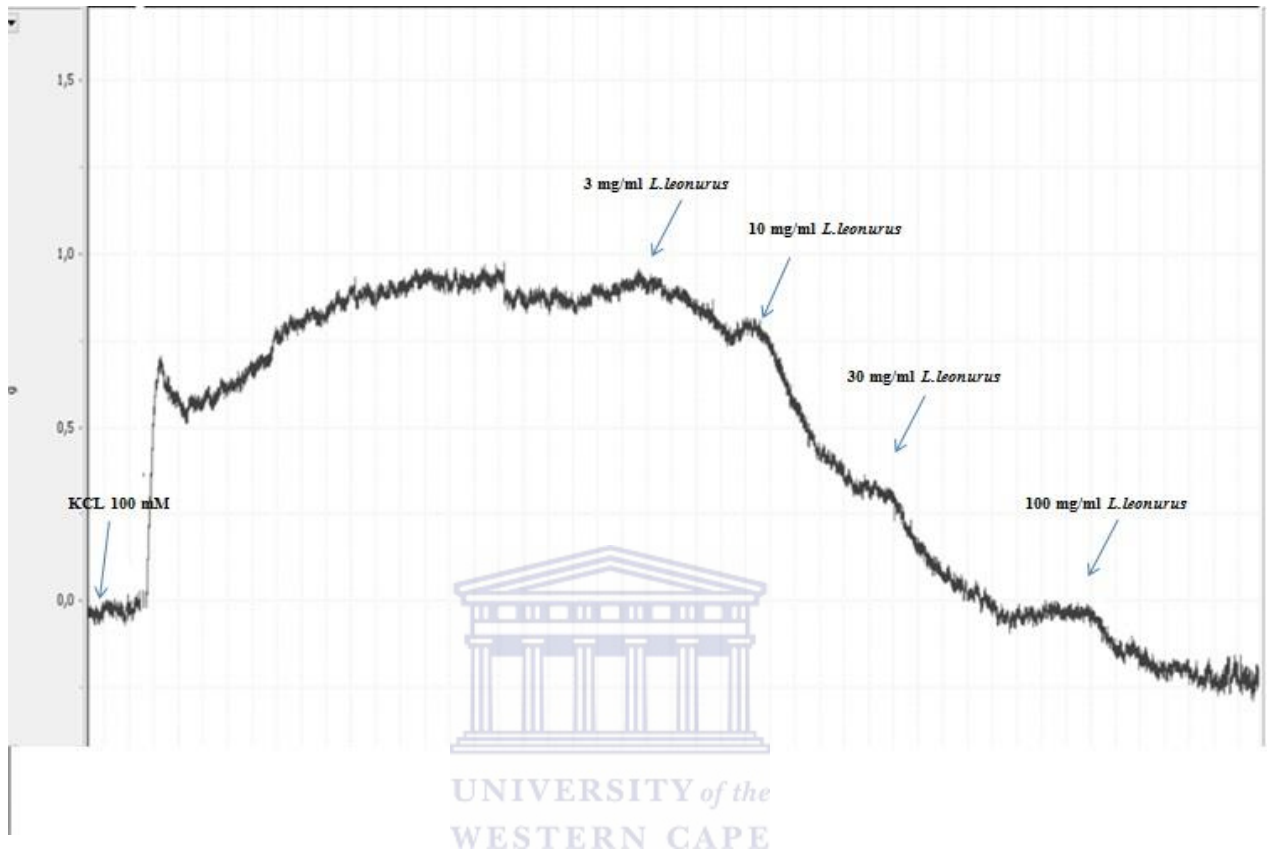
Appendix 5

Appendix 5: Trace of relaxation (g force) caused by cumulative concentrations of *A .afra* on isolated guinea pig trachea constricted with KCL.

Appendix 6: Trace of relaxation (g force) caused by cumulative concentrations of *L. leonurus* on isolated guinea pig trachea constricted with KCL.

Appendix 7