Evaluation of the pulmonary effects of the traditionally prepared *Artemisia afra* steam inhalation and nebulized aqueous extract and the possible involvement of flavonoid luteolin

Sizwe Joel Mjiqiza

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

Supervisor: Prof J. A. Syce
School of Pharmacy
University of the Western Cape
Bellville
South Africa

October 2006

Parts of this study were presented and awarded a third prize during the Young Scientist Competition at the 4th International Congress of Pharmaceutical and Pharmacological Sciences (ICPPS) held in Vanderbiljpark, Gauteng, South Africa in 20-23 September 2006

Evaluation of the pulmonary effects of the traditionally prepared *Artemisia afra* steam inhalation and nebulized aqueous extract and the possible involvement of flavonoid luteolin

Keywords

Pulmonary effects

Artemisia afra

Aqueous extract

Luteolin

HPLC assay

Isolated perfused lung (IPL)

Steam inhalation

Nebulization

Tidal volume (TV)

Lung compliance (CL)

Lung resistance (RL)

DECLARATION

I, Sizwe Joel Mjiqiza, declare that "**Evaluation of the pulmonary effects of traditionally prepared *Artemisia afra* steam inhalation and nebulized aqueous extract and the possible involvement of flavonoid luteolin**" is my own work, that it has not been submitted for any degree or examination to any other University, and that all my sources that I have used or quoted have been indicated and acknowledged by means of complete references.

Signature: **October 2006**

DEDICATION

To my late mother, Nomthandazo Mjiqiza, for bringing me up the way you did and making me the person I am today, for believing in me and supporting me all these years. "*Ndiyabonga Tshawekazi*"

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the following people and institutions for their contribution:

The Almighty, for giving me and my family, the strength through all these years.

Prof. J. A. Syce, my supervisor, for his inspiration, participation, guidance, advice, and the supervision throughout this research project.

Mr. Vinesh Jeaven, for the technical support, advice and always making sure that I have the animals, chemicals etc.

My family, April, Jabane, and Nghona families, for all their support, encouragement and believing in me. Without you all, I wouldn't be here and this wouldn't be possible.

The Eastern Cape Department of Health for the financial assistance.

Dr. Nelson Mandela, Jakes Gerwel, Rejoice Ngcongo, Shaun Johnson, Steve Thobela, Nomonde Mbulawa, Ruth Andrews and the entire Mandela Rhodes Foundation family, thank you for believing in me, for the encouragement, financial support and making sure that I don't lose the mark.

NRF for the financial support

My colleagues, Ian Backman, Admire Dube, Haiqiu Ma, Ojangole Max, Caroline Kinyua, Lebogang Manthata, James Mukinda, Kenechukwu Obikeze, Doris Tshambuluka, Pule Lesego, Solange Malubwe for their support, assistance and for just being themselves. I thank you.

The staff of the School of Pharmacy (UWC) and to all those who participated either directly or indirectly, thank you for the support.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CL	Lung compliance
HPLC	High performance liquid chromatography
IPL	Isolated perfused lung
IS	Internal standard
RL	Lung resistance
TCM	Timer Counter Module
TV	Tidal volume
UV	Ultra violet
VCM	Ventilation control module

SUMMARY

Artemisia afra is a popular medicinal plant traditionally used to treat respiratory conditions such as asthma, bronchitis etc., most frequently as a steam inhalation. The effectiveness of this steam inhalation, its active constituent(s), and the disposition of the latter has not yet been evaluated. The plant, however, contains appreciable amounts of flavonoids, such as luteolin, that are potential markers for use in the evaluation of the pulmonary effects of the plant, in a model such as the isolated perfused rat lung (IPL).

The objectives of this study were, firstly to compare the luteolin content in dried crude *A. afra* leaves and an aqueous extract of *A. afra*; secondly, to compare the pulmonary effects of the traditionally-prepared *A. afra* steam inhalation and nebulized *A. afra* aqueous extract and luteolin solutions; and thirdly, to determine the pulmonary disposition and effect of intravenously administered luteolin.

To realize these objectives, aqueous extract of *A. afra* was prepared according to the traditional healers method. Luteolin contents of the plant solutions and perfusion medium were determined using HPLC. The IPL model was adapted to allow administration via steam inhalation and nebulization, as well as the monitoring of pulmonary function tidal volume (TV), respiratory resistance (RL) and compliance (CL). To determine the pulmonary disposition, luteolin was administered in the perfusion medium, perfusate samples taken over an hour and half, and assessed for luteolin content.

The traditionally prepared *A. afra* extract contained significantly ($p<0.001$) higher luteolin levels (49. 554 ± 0.4021µg/25mg, $n=5$) than the *A. afra* crude dried leaves (15. 939 ± 1.1829 µg/25mg, $n=5$) indicating that the aqueous extraction process concentrated the plant's active constituents. Lung preparations remained stable during the equilibration period, for example, CL increased at a steady rate of 0.00118± 0.00149 and 0.00155±0.00128 ml/cmH_2O per min ($n=4$) for the steamed and nebulized saline group of lungs respectively indicating that the IPL was successfully

adapted for this study, and the isolated lungs remained suitably stable and sensitive for the measurement of lung function.

Inhaled steam of *A. afra* (10 mg/ml) changed lung function drastically, for example, a dose of 10 mg/ml increased TV significantly *($p<0.001$)* by 3.56 %, CL significantly *($p<0.05$)* by 3.42 %, and decreased RL significantly *($p<0.05$)* by 9.72 % immediately after inhalation while inhaled saline did not affect lung function. Higher doses of the plant extract (50 mg/ml) produced even higher changes in lung function (TV and CL increased by 7.59% and 11.76%, while RL decreased by 10.98%) indicating that the steam inhalation of *A. afra* produced significant *($p<0.05$)* dose-dependent improvement in lung function.

Administration of nebulized luteolin improved pulmonary function, TV and CL increased significantly *($p<0.01$)* by 10.96% and by 36.42% *($p<0.001$)* respectively while RL significantly *($p<0.001$)* decreased by 19.73 %. Nebulized saline, however, had negligible effect on these parameters. Nebulized aqueous extract produced even better pulmonary function improvement with TV and CL, which increased by 17.96% and 43.43% respectively while RL decreased by 8.47%. The results obtained in this study clearly indicate that the nebulized *A. afra* aqueous extract had better and sustainable pulmonary effects than nebulized luteolin even though they were equivalent in pre-calculated luteolin doses. Furthermore, higher luteolin levels were recovered from the aerosolized mist collected during the three-minute nebulization of the aqueous extract than during luteolin nebulization, viz. 21.54 ± 0.815 and 8.992 ± 0.403µg/ml, respectively.

The study demonstrated that the intravenously administered luteolin improved lung function (i.e. confirmed by stabilized TV, increased CL and decreased RL at low doses). Luteolin seemed to particularly increase the lung compliance, further confirming its possible bronchodilator activities.

The results obtained indicate that the inhalation of *A. afra* steam improved pulmonary function, therefore, supporting the use of such steam against pulmonary ailment, and

this effect seem to improve with higher plant's extract dose. Further, nebulized luteolin and aqueous extract produced even better pulmonary effects suggesting that these substances could be used alternatively to steam inhalation, provided that other factors (i.e. convenience, availability of nebulizers etc.) are considered.

CHAPTER ONE

INTRODUCTION

In man's quest for food during the early nomadic period of his existence, he would have most certainly encountered some plants that were poisonous, others that would serve as adequate food, and still others that produced bizarre unusual effects by altering his consciousness. Among this latter group were those that would simultaneously relieve pain and disease, and these experiences would have been passed from one generation to another (Springfield *et al.*, 2005). Over the past decade, traditional medicines have become a topic of augmented global importance, having impacted on the world health and trade. In terms of the world health, traditional medicines play a central role in healthcare systems of large proportions of the world's population (Stafford *et al.*, 2005). Furthermore, they are an important part of culture and tradition of African and Asian people. Apart from their cultural significance, medicinal plants tend to be more accessible and affordable. As a consequence, there is an increasing trend world-wide, to integrate traditional medicines with primary health care (Fennell *et al.*, 2004).

Today in South Africa, most of the populations in urban, as well as smaller rural communities are still reliant on medicinal plants for their health care needs (Fennell *et al.,* 2004). One of the medicinal plants used in South Africa is *Artemisia afra,* a multi-stemmed perennial shrub with greyish-green feathery leaves. Most commonly, the roots, stems and leaves of *Artemisia afra* are used medicinally. They are taken as enemas, poultices, infusions, body washes, lotions, smoked, snuffed, inhaled or drunk as tea (Van Wyk, and Gericke, 2000; Roberts, 1990). Traditionally, *A. afra* is used as a steam inhalation for the treatment of respiratory disorders such as coughs, colds, bronchitis, blocked sinuses and tight-chest or asthma. Usually, 2-3 cups of dried leaves are boiled in sufficient boiling water in a pot. The resultant vapour is inhaled while the head and the bowl are covered by a towel, and this is done until the mixture has cooled down (Komperlla, 2005). However, not much has been done to evaluate the effectiveness of this inhaled steam, even though it is still widely practiced today. Furthermore, there is no scientific proof for the efficiency of this inhalation method

so often employed by traditional healers, and therefore, it is necessary that the pulmonary effectiveness of the traditional steam inhalation is evaluated.

Artemisia afra has been shown to possess various biological and pharmacological activities. In our laboratory, it has been found that the aqueous extract of *A. afra* relaxes respiratory smooth muscles that had been contracted by various known bronchoconstrictors (Harris, 2002). This provides experimental support for the empiric ethno-pharmacological use of this plant in traditional medicines (Mukinda, 2005). However, the active ingredients responsible for the therapeutic activities of the plant are not yet known. Scientifically, it could be assumed that the activities of this plant are due to the bio-active ingredients it contains (Muganga, 2004), and the aqueous extract of *A. afra* has been shown to contain flavonoids among other constituents (Waithaka, 2004).

Flavonoids are polyphenolic compounds that occur ubiquitously in plants and are members of a class of natural substances that has recently been subject of considerable scientific and therapeutic scrutiny (Havsteen, 2002). They have most likely in ancient times played a major role in successful medical use of plants, and their uses have persevered up to now. They are divided into various classes based on their chemistry (Scalbert and Williamson, 2000). Luteolin, a flavone belonging to one of the subclasses of flavonoids, has been found in high concentrations in aqueous extract of *Artemisia afra.* It is easily extractable from various preparations of *A. afra* (e.g. dried crude leaves, aqueous extract, tablets, etc). It seems stable in the raw material under various processing conditions (Markham, 1982), and can be selectively quantified using HPLC methods (Harborne and Williams, 2000), all qualities or attributes that may make it suitable as a marker compound to use in the evaluation of *A. afra.* In addition, flavonoids, including luteolin, are biologically active. In fact, luteolin has been shown to have muscle relaxant activity (Harris, 2002; Das *et al.*, 2003). Luteolin could thus be an ideal marker compound for evaluating the pulmonary effects of *Artemisia afra.* However, for *A. afra* to be effective for its

claimed traditional respiratory use, its actives, including luteolin, must also have a favorable disposition profile in the lungs.

To investigate the above hypotheses, a suitable model that would allow simultaneously determination of lung function as well as determination of absorption, distribution and metabolism of the active constituents of *Artemisia afra* such as luteolin in the lung, would be needed, and the isolated perfused lung (IPL) model could serve this function. It offers several advantages such as being an organ model with all its physiological cell-to-cell contacts intact, a model in which respiratory mechanics such as tidal volume, lung compliance, and resistance as well as edema formation could be continuously and simultaneously monitored (something which is not possible with *in vivo* cell culture models). Furthermore, the IPL could be adapted to make administration of plant extract/medicine via inhalation, instillation, bolus injection or continuous perfusion (Syce and Parkar, 2004; Uhlig and Taylor., 1998) possible. In short, the IPL appeared to be ideal to study the pulmonary effects of *A. afra*.

Given the above arguments, the aims of this study were: (1) To compare the luteolin content in dried crude *A. afra* leaves and an aqueous extract of *A. afra*. (2) To compare the pulmonary effects of the traditionally-prepared *A. afra* steam inhalation and nebulized *A. afra* aqueous extract and luteolin solutions. (3) To determine the pulmonary disposition and effect of intravenously administered luteolin.

CHAPTER 2
LITERATURE REVIEW

2.1 Medicinal plants

A medicinal plant is a plant in which one or more of its parts contains substances that can be used for therapeutic purposes, or which is a precursor for the synthesis of a direct therapeutic agent. Medicinal plant's products in various forms have for many years been available to treat diseases in both developing and developed countries, and are an important part of the culture and tradition of African people. Besides their cultural significance, medicinal plants are more accessible and affordable (Fennell *et al.*, 2004). They have been used by traditional practitioners for ages in localities where scientifically trained medical staff is not readily available. Their knowledge is based on experience passed from one generation to another, or common sense. Some of the actions of these medicinal plants often discovered as the results of trial and error could be effective. Therefore, medicinal plants deserve to be examined by modern scientific methods (Havsteen, 2002; Mukinda, 2005; Waithaka, 2004).

Medicinal plants have large numbers of compounds that have their potential medicinal activities influenced by the presence of other compounds, making it difficult to obtain the similar action when the suspected compounds are isolated from the plant material. In traditional medicine, a complex mixture of extracts is therefore, used to treat certain ailments. However, often only one of these ingredients is responsible for the possible therapeutic activity (Barton and Ollis, 1986), and some of these compounds could be harmful thus minimizing the interest in traditional medicinal plants. As a consequence, the use of natural products has declined due to the development of drugs with enhanced selectivity, potency and fewer side-effects (Waithaka, 2004). Hence, semi- or synthetic substances from plants are often used, e.g. "Artemether" synthesized from *Artemisia* species is used to treat malaria (Rang *et al.*, 1999) and more than 25% of the prescribed medicines today are of plant origin. Today most of the people in urban South Africa, as well as smaller rural communities are still reliant on medicinal plants for their health care needs. Although continuously

used, only a small portion of the traditional medicines have been scientifically evaluated especially in South Africa (Springfield *et al.*, 2005), because of various reasons including the challenge of acquiring well documented plant materials. Furthermore, traditional therapies are often based on faith, mainly due to the non-scientific process by which these systems operate (Waithaka, 2004). Plant constituents which have potential medicinal activity also vary, depending on environmental and geographical conditions such as soil type, time of harvest and storage (Silva *et al.*, 1998). Therefore, based on this knowledge, traditional medicines require thorough scientific analysis to ensure their effective, optimal and safe use. The quantity and quality of the safety and efficacy data of traditional medicines are far from sufficient to meet the criteria needed to support their use worldwide. This is due to lack of adequate or acceptable research methodology for evaluating these traditional medicines (Springfield *et al.*, 2005). However, with the recent advancement in research technology, the evaluation of these medicinal plants is therefore, a necessity.

2.1.1 Steam inhalation of medicinal plants

Medicinal plants, especially those known to contain essential oils, have for many years been used to treat respiratory conditions such as common cold, bronchitis and asthma. Plant's parts, often leaves, are boiled in a pot with the resultant vapour inhaled while the head and the bowl are covered with a towel, and this is done until the mixture cools down (Komperlla, 2005). However, not much has been done to evaluate the effectiveness of such steam inhalation. Despite this lack of scientific data for this route so often used by traditional healers, steam inhalation is still popularly practiced today. As a result, there are commercially available steam inhalers being marketed around Europe (www.mypharmacy.co.uk/health).

Lately, there has been an increasing trend world-wide of isolating plant constituents thought to be responsible for the therapeutic actions of steam inhalation. Essential oils e.g. lavender, eucalyptus are thought to be responsible for the therapeutic actions of steam inhalation. These oils are isolated from plants by steam distillation, and are

incorporated in commercially available products used to treat respiratory conditions (American Family Physician, 1991).

Despite the continued use, there is no scientific evidence supporting the traditional claims for the use of aromatherapy or the traditional steam inhalation. However, there's been a recent interest world-wide of evaluating the effectiveness of steam inhalation, and a few *in vivo* studies have been conducted. In a double-blind, randomized study conducted in America for evaluating the efficacy of steam inhalation in treating common cold symptoms, found no noted significant differences in the symptom scores and the nasal resistance measurement between the patients who received active treatment and those who received the placebo. Symptoms seemed to abate more rapidly in the placebo group than the treatment group. Even-though the study findings demonstrated no benefits of the steam inhalation in alleviating common cold symptoms, the majority of patients in both groups stated that they would recommend the treatment to others (American Family Physician, 1991). In another study, steam inhalation alleviated cold symptoms and increased nasal patency in a significantly higher percentage of patients in the actively treated group than in the placebo-treated group (Ophir and Eladin, 1987). The results of both studies contradicted, hence, further studies were required to provide more information about the effectiveness of steam inhalation, and to the best of our knowledge, no *in vitro* studies were conducted yet. Therefore, the evaluation of traditionally prepared steam inhalation *in vitro* was necessary to provide new and more accurate scientific information.

2.2 *Artemisia afra*-plant used in the study

2.2.1 Vernacular names and taxonomy

Artemisia afra is one of the oldest and best known medicinal plants, and it is still effectively used today in South Africa by people of different cultures. It is popularly known as African wormwood in English, "wildeals" in Afrikaans, "umhlonyane" in Xhosa, 'mhlonyane" in Zulu and "lengana" in Sotho. Due to colonization, the plant

was made popular because of its resemblance to European wormwood (*Artemisia vulgaris*). *Artemisia afra* belongs to:

- Division : Magnoliphyta
- Class : Magnoliopsida
- Subclass : Asteridae
- Order : Asterales
- Family : Asteraceae
- Genus : *Artemisia*
- Species : *A. afra*

(Van Wyk, and Gericke, 2000; Roberts, 1990)

2.2.2 Botanical description

Artemisia afra, named after the Greek hunting goddess Artemis, grows in a thick, bushy, slightly untidy clump. It has tall stems that are thick, woody at the base and become thinner and softer towards the top. These stems are ribbed with strong swollen lines that run all the way up, and can grow up to 2m high. *Artemisia afra* is easily identified by its characteristic strong, sticky and sweet aromatic odor that exudes when touched or cut. Traditionally, it is the fresh and dried leaves that are commonly used for medicinal purposes. These leaves are finely divided, almost fern-like and ovate (egg-shaped) and grow up to 8cm long and 4cm wide. The upper surface of the leaves is dark-green whereas the undersides and the stems are covered with small white hairs, which give the shrub the characteristic overall grey color. *Artemisia afra* flowers in late summer. The individual creamy, yellow flowers are small (3-4mm in diameter), nodding and crowded at the tips of the branch. Lastly, the fruits are about 1mm long, 3-angled, slightly curved with a silvery-white coating (Van Wyk and Gericke, 2000; Roberts, 1990).

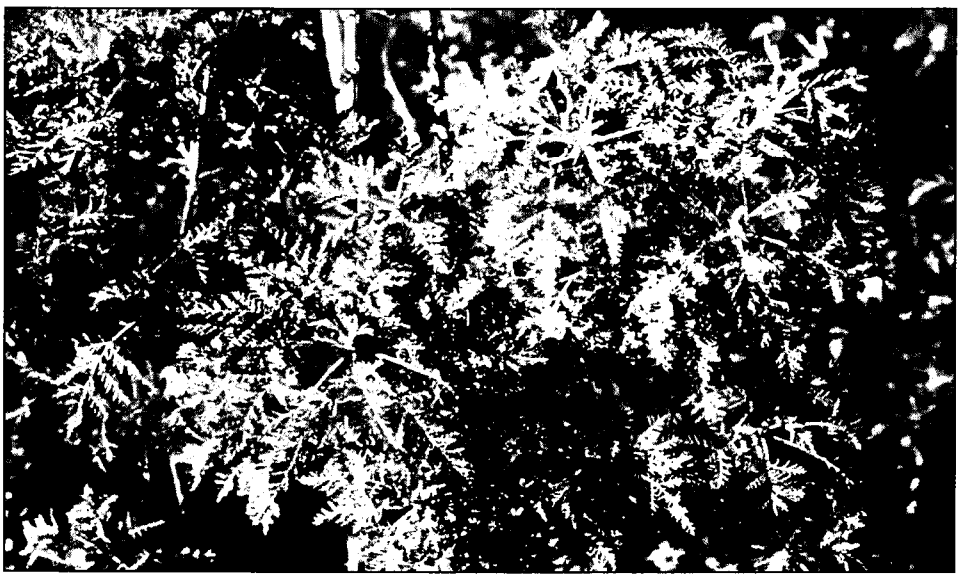


Figure 1: ***Artemisia afra (A. afra)*** **adapted from pharmacopoeia monographs project, 1999**

2.2.3 Distribution

Artemisia afra is a common species in South Africa with a wide distribution from the Cederberg Mountains in the Cape, northwards to tropical East Africa and stretching as far as Ethiopia. It is widespread in all provinces in South Africa, except the Northern Cape. In the wild, *Artemisia afra* grows at altitudes between 20-2440m on damp slopes, along the stream-sides and forest margins (Van Wyk and Gericke, 2000; Roberts, 1990).

2.2.4 Uses, pharmacology and phytochemistry of *Artemisia afra*

Artemisia afra is one of the most popularly used medicinal plants in Southern African traditional medicine. It is known as "cure all" because of its use to treat a wide range of ailments, including respiratory disorders such as coughs, colds, bronchitis, blocked sinuses and tight-chest or asthma. It is also used as a bitter tonic and purgative for gastro-intestinal conditions such as dyspepsia and other disturbances, and has been used as an analgesic for headaches, earaches and toothache. Additionally, it has been used to treat malaria, intestinal worms and skin conditions such as mumps and lesions (Van Wyk and Gericke, 2000; Roberts, 1990; Hutchings, and van Staden, 1994).

The roots, stems and leaves of *Artemisia afra* are commonly used, and are taken as enemas, poultices, infusions, body washes, lotions, smoked, snuffed, inhaled or drunk as tea. *A. afra* has a very bitter taste and it is usually sweetened with sugar or honey when drunk, or can be drunk as brandy popularly known as "wildeals" brandy (Van Wyk, and Gericke, 2000; Roberts, 1990). *Artemisia afra* is used in many different ways for treating different ailments, including the insertion of fresh leaves into the nostrils to clear blocked nasal passages (Van Wyk, and Gericke, 2000) or placement of leaves inside the socks to alleviate sweaty feet (Roberts, 1990; Watt and Breyer-Brandwijk, 1962). Traditionally, it is used as a steam inhalation for treatment of asthma whereby 2-3 cups of dried leaves are boiled in sufficient boiling water in a pot, with the resultant vapour inhaled, while the head and the bowl are covered by a towel (Komperlla, 2005).

Artemisia afra has been found to contain volatile oils such as α- and β- thujone, 1, 8-cineol, camphor and borneol that showed definite antimicrobial and anti-oxidative properties (Van Wyk and Gericke, 2000), and might be responsible for at least some of the activities of the plant, especially since volatile, they could be inhaled during *A. afra* steam inhalation (Roberts, 1990). The plant is also associated with toxic hallucinations thought to be caused by thujone and overdose or continued use of *Artemisia afra* over long periods, could therefore, be potentially harmful (Mukinda, 2005).

In our laboratories, *Artemisia afra* has been shown to relax respiratory smooth muscles contracted by various known bronchoconstrictors (Harris, 2002). However, the chemical constituents responsible for this action are not yet known. But scientifically, it could be assumed that the activities of *A. afra* are due to the active ingredients that it contains (Muganga, 2004). The aqueous extract of *A. afra* has been shown to contain flavonoids such as luteolin, quercetin, apigenin and kaempferol among other constituents. Luteolin and quercetin are present at higher levels than the other flavonoids. These two flavonoids have also been shown to be stable during storage, and can be assayed by simple extraction and HPLC analytical procedures

(Waithaka, 2004), suggesting that flavonoids could be used as potential markers for evaluating the pulmonary effects of *A. afra.*

2.3 Flavonoids

Flavonoids are polyphenolic compounds that occur ubiquitously in plants and are members of a class of natural compounds that has been recently subject of considerable scientific and therapeutic interest. They are mainly found in leaves and the outer parts of the plants, are responsible the plant coloration. About 2% of photosensitized carbon in plants is converted into flavonoids or closely related compounds (Markam, 1982; Nuurtila *et al.*, 2002; Scalbert *et al.*, 2002).

2.3.1 Classification and chemistry of flavonoids

There are over 4000 different types of flavonoids that have been described and they exist in a wide range of chemical structures (Hollman and Katan, 1999). The basis of such great variability among flavonoids is due to:

(a) the differences in the ring structure of the aglycone and its state of oxidation/reduction;

(b) differences in extent of hydroxylation of the aglycone and the positions of the hydroxyl groups, and;

(c) differences in derivatization of the hydroxyl groups e.g. with methyl groups, carbohydrates, etc (Havsteen, 2002,).

However, all flavonoids arise from an initial reaction that involves condensation of a cinnamic acid catalyzed by the chalcone synthetase enzyme. The chalcone formed in this initial reaction is then converted rapidly into a phenylbenzopyran, and further modifications lead to the formation of the different sub-classes within the flavonoid family (Markham, 1982). Flavonoids are therefore, divided into five main groups based on molecular structure, degree of hydroxylation or polymerization and other substitutions or conjugations on the parent nucleus, namely:

- Flavonols e.g. quercetin and kaempferol,
- Anthocyanidinis e.g. anthocyanins,

- ❖ Flavanol e.g. catechins,
- ❖ Flavonones e.g. hesperitin, and:
- ❖ Flavones e.g. apigenin and luteolin.

Structurally, flavonoids contain fifteen-carbon atoms in their basic nucleus arranged in a C_6-C_3- C_6 configuration that consists of two benzene rings A and B connected by a heterocyclic ring C containing oxygen (see figure 2 below)(Markam, 1982; Muganga, 2004, Waithaka, 2004).

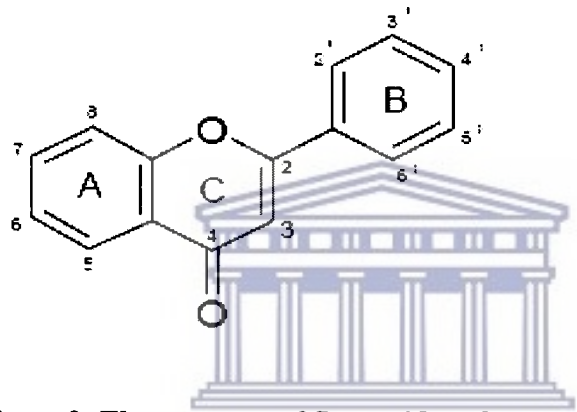


Figure 2: The structure of flavonoid nucleus

2.3.2 Types of flavonoids

Flavonoids are found mainly in two forms, namely the aglycone form where there is no sugar moiety attached to the parent structure, and the glycoside form where they are bound with a sugar moiety. In the glycoside form, sugars normally straight or branched (mono-, di- or tri-saccharides) are linked to the C-ring forming the acid resistant flavonoid C-glycoside or O-glycoside where one or more of the flavonoid hydroxyl group is bound to a sugar(s) via an acid-labile bond (Markam, 1982; Paganga and Rice-Evans, 1997). Flavonoids are mainly found as glycosides in plants, whereas the aglycone form is less frequently found, with the O-glycosides being the most prevalent form obtained in plants (Manach *et al.*, 1998). Although less prevalent, the aglycones are known to exhibit pharmacological activities more than the glycoside form (Shimoi *et al.*, 1998). The quantitative determination of the individual glycosides in plant material is difficult due to the large number of

flavonoids obtained in plant material, flavonoid glycosides are, therefore, hydrolyzed into corresponding aglycones which are then identified and quantified (Nuurtila *et al.*, 2002).

2.3.3 Pharmacological effects of flavonoids

Flavonoids have been associated with variety of biological effects in numerous mammalian cell systems *in vitro* as well as *in vivo* (Hollman and Katan, 1999). They are linked to pharmacological activities such as anti-microbial and anti- inflammatory activities (Hai Qui Tang, 1999; Rotteli *et al.*, 2003). Certain studies suggested that they act as anti-oxidants (Burns *et al.*, 2000; Harborne and Williams, 2000; Kaneko & Baba, 1999; Manach *et al.*, 1998; Shimoi *et al.*, 1996), and there is some evidence from epidemiological and *in vivo* studies that their consumption is associated with the reduced risk of certain cancers and cardiovascular diseases (Hertog *et al.*, 1993; Knert *et al.*, 1996). Literature reveals that these compounds have anti-allergic, antiviral, anti-thrombotic, and anti-turmorogenic activities (Benavente-Garcia *et al.*, 1997; Harborne and Williamson, 2000; Hollman and Katan, 1999; Wang *et al.*, 2003).

2.4 Luteolin

Luteolin could be used as a potential marker to evaluate the pulmonary effects of *A. afra* and its possible involvement in the effects associated with the plant. Luteolin is a yellowish, well-known flavone which has been shown to be the major flavonoid found in the aqueous extract of *A. afra* and other medicinal plants which have potential pharmacological activities. It may be present in plants as luteolin-7- O-glucuronide, luteolin-5-O-glucoside, luteolin-3'- glucuronide forms with the aglycone form possibly present in smaller quantities than the glycoside forms (Grayer *et al*, 2002; Heitz *et al.*, 2000; Waithaka, 2004).

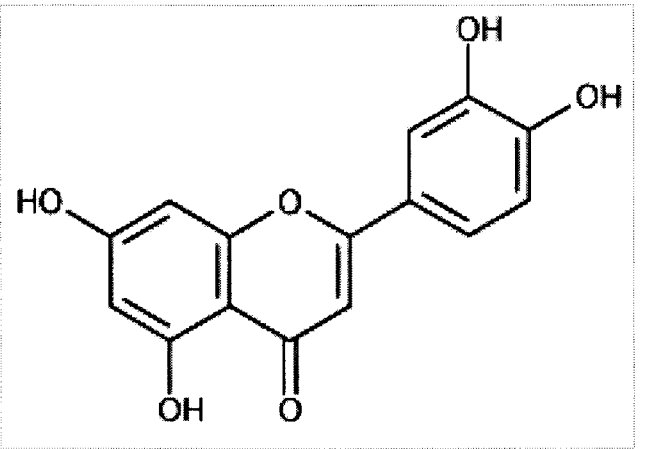


Figure 3: Chemical structure of luteolin

2.4.1 Pharmacological actions of luteolin

Luteolin has been specifically found to have anti-inflammatory, antispasmodic (Yamazaki *et al.*, 2002), anti- tumorigenic (Ueda *et al.*, 2003), anti-oxidation, anti-bacterial, anti-cholesterol activities (Harborne and Williamson., 2000; Rice-Evans *et al.*, 1997; Shimoi *et al.*, 1998). It is also an effective smooth muscle relaxant (Sanchez de Rojas *et al.*, 1995) and alleviates broncho-constriction and airway hyper-reactivity in ovalbumin-sensitized mice (Das *et al.*, 2003). This might explain the traditional use of luteolin-containing plants such as *Artemisia afra,* in treating respiratory conditions, including asthma.

2.4.2 Analytical methods suitable for luteolin analysis

Luteolin and other flavonoids are phenolic compounds and are therefore, very challenging to analyze because of their reactivity. However, various solvents such as alcohols (i.e. methanol), water or the mixture of these two are most frequently used for the extraction of flavonoids from the plant material. Organic solvents such as ethyl acetate, chloroform or hexane are commonly used to extract the aglycone form of flavonoids. Traditionally, water is commonly used whereby plant parts such as leaves, are boiled for sufficient time (± 30 minutes) to improve flavonoid extraction (Markam, 1982; Muganga, 2004).

The complete analysis of the absolute structure and configuration of flavonoids is usually a complicated task which requires the application of advanced techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy, X-ray diffraction, optical rotary dispersion and mass spectrophotometry. Since only a few laboratories are equipped and staffed to make all these expensive methods available, simpler approaches to flavonoids characterization are often desired. Therefore, modern chromatographic techniques such as Thin Layer Chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) are often used. Some of these techniques are normally coupled with different detectors including the fluorescence, UV spectrophotometry (i.e. most commonly used), photo diode array (Havsteen, 2002; Hollman and Katan, 1999; Muganga, 2004). The identification and quantification of flavonoids from different matrixes (e.g. plasma, urine) is therefore, possible (Harborne and Williamson, 2000).

The HPLC is the mostly commonly used method, and has become the standard equipment for analysis of flavonoids. It yields excellent resolution and retention times that are extremely reliable in identification of the individual flavonoids. It has several advantages such as simplicity, sensitivity, specificity and better separation when using smaller sample quantities (Waithaka, 2004). Flavonoids are very soluble in polar solvents. Therefore, reversed-phase HPLC is often employed for their analysis, with the octadecylsilane (ODS, C-18, RP-18) commonly used as the stationary phase. Eluting solvents are usually a mixture of polar, water-miscible organic solvents (e.g. methanol, acetonitrile) and water. Also, pH modifiers such as acetic acid or formic acid are normally used to reduce presence of the ionized form of phenolic compounds (such as flavonoids), thus improving their retention time (Muganga, 2004). Therefore, the availability and sensitivity of these analytical methods provides the possibility of studying the pharmacokinetics and bioavailability of flavonoids.

2.4.3 Pharmacokinetics and bioavailability of flavonoids

The pharmacokinetics and bioavailability of flavonoids, especially dietary ones, has been investigated a lot recently. Their absorption and metabolism has been studied

using *in vitro* methods such as human intestinal Caco-2 cells (Shimoi *et al.*, 1998) and rat liver microsome (Gradolatto *et al.*, 2004). Flavonoids have been shown to be absorbed *in vivo,* and studies have showed that luteolin-O-β-D-glucoside and apigenin-O-β-*D*-glucoside were not detected in dog plasma after they were orally administered. However, the aglycone form and its metabolites were detected (Liping *et al.*, 2004). This suggested that the flavonoid glycosides are bio-transformed into a corresponding aglycone form by intestinal microorganisms or hydrolytic enzymes such as *β*-glucoronidase (Shimoi *et al.*, 1998; Spencer *et al.*, 1999), while the aglycones are capable of being absorbed freely by passive diffusion across the cells (Scalbert *et al.*, 2000).

Studies showed that, once flavonoids were inside the cells, they were rapidly metabolized into methylated, glucorindated or sulfated metabolites. These metabolites are polar and water-soluble thus circulated in the blood, and were excreted into the bile and urine (Manach *et al.*, 1998; Scalbert *et al.*, 2002). The HPLC / mass spectrophotometry analysis of the rat plasma after administration of luteolin, the major flavonoid of *Artemisia afra*, showed that the main metabolite obtained was luteolin mono-glucorinide, and that free luteolin (i.e. aglycone) was also present (Shimoi *et al.*, 1998; Spencer *et al.*, 1999). However, in another study, quercetin, another major flavonoid of *A. afra*, was not detected in its aglycone form in human plasma after the administration of 200mg as tea, but only its glucuronide could be detected (Wittermer and Veit, 2003). In another study, it was found that the levels of aglycone luteolin in the plasma were small relative to the amount of total luteolin glucorinide recovered after intestinal perfusion with luteolin aglycone and glucuronides (Spencer *et al.*, 1999; Muganga, 2004). These studies suggest glucorunidation, a Phase II, metabolism as the major flavonoid metabolic pathway (Wang *et al.*, 2003).

Glucorunidation is a very important process for detoxification of xenobiotics (Manach *et al.*, 1998), and it has been unclear whether flavonoids glucorunides can function *in vivo* like their corresponding flavonoid aglycones. In fact, the

glucorunides have been found to have less antioxidant activity than aglycones and it is not clear whether they entered cells or not (Manach *et al.*, 1998). Deglucorunidation of flavonoids has been reported to occur in the human large intestine by β-glucoronidase and arylsulphatase enzymes (Shimoi *et al.*, 1998). However, β-glucoronidase, which is the lysosomal enzyme present in mammalian cells and is known to be released from neutrophils and eosinophils during inflammation (Marshall *et al.*, 1988), and therefore, these enzymes may affect disposition of flavonoids across the cells.

In general, the bioavailability of flavonoids although having high lipophilicity, is relatively low due to limited absorption and rapid elimination. Furthermore, flavonoids contained in traditional medicines and other natural drugs are rapidly and extensively metabolized, and the biological activities of their metabolites are not always the same as those of parent compounds (Liping *et al.*, 2004). Various routes have been used to study the pharmacokinetics and bioavailability of luteolin. They include the isolated rat intestine model to study luteolin aglycone absorption (Spencer *et al.*, 1999), Caco-2 cells (Shimoi *et al.*, 1998) and intra-peritoneal injection (Lui *et al.*, 2005), but the pulmonary route has not yet been used.

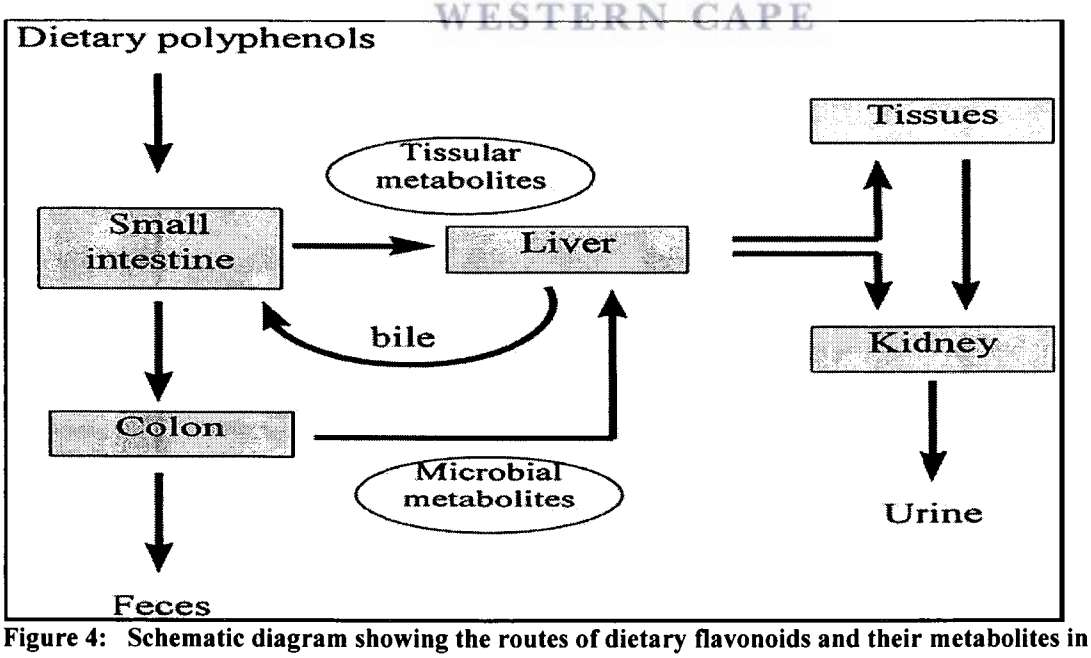


Figure 4: Schematic diagram showing the routes of dietary flavonoids and their metabolites in humans (adapted from Scalbert *et al.*, 2002).

2.5 Aspects of pulmonary absorption and disposition of drugs

Pulmonary assessment for drug absorption and disposition is becoming increasingly essential to provide information in drug development. This information can be used to maximize pulmonary selectivity of drugs, screen drugs and to help evaluate bioequivalence of inhaled products (Mobley and Hochhaus, 2001). Lungs are pharmacologically active organs that can take up, retain, metabolize or delay release of many drugs and compounds that have diverse chemical structures and pharmacological actions (Boer, 2003). Lungs could therefore, affect blood concentrations of aerosolized or intravenously administered drugs.

Anatomically, lungs are complex and heterogeneous organs that are characterized by the high relative perfusion, high surface area (i.e. in-contact with blood and respiratory gases) and small diffusion distances. This anatomy and physiology can influence drug kinetics such as the uptake, distribution, metabolism (Mobley and Hochhaus, 2001). Assessment of lung deposition and absorption of drugs provides important information for drug candidates, and is essential for development of pulmonary inhalation products. The amount of drug that enters the lungs, deposition site, residence time at the deposition site and absorption into the systematic circulation, can all provide crucial knowledge for determining pulmonary selectivity or bioavailability of systematically acting compounds (Mobley and Hochhaus, 2001). Therefore, for *Artemisia afra* to be effective for traditional claims especially its use against respiratory conditions, its constituents, including luteolin, should at least be absorbed and disposition in lung tissues.

2.5.1 Experimental methods for studying pulmonary disposition and absorption of drugs

Historically, the pharmacokinetic methods used to evaluate the pulmonary disposition of drugs have lacked accuracy due to extremely low concentrations present in systematic circulation at any present time. However, recent advances in analytical methodology allow the accurate determination of pulmonary disposition of drugs after inhalation (Derendorf *et al.*, 2001). Lung uptake can be studied both in the

laboratory as well as at bedside with several methods being available to investigate pulmonary drug disposition, and are divided into three categories, namely: *in vivo*, *in vitro* and perfused lung models (Boer, 2003).

2.5.1.1 *In vivo* methods

In vivo methods are designed to quantify first pass uptake of drugs into the lung after a bolus injection or continuous perfusion. A bolus drug is often injected via a catheter into pulmonary or right artery, and then, arterial blood sampled using a different catheter. Other studies involve sampling of venous blood after inhalation of drugs in human subjects (Upton and Doolette, 1999). However, when using *in vivo* methods, various approaches are normally put in place to block oral absorption of the inhaled drug, and activated charcoal is often used to minimize gut absorption of inhaled drugs (Derendorf *et al.*, 2001).

2.5.1.2 *In vitro* methods

In vitro methods often employ cell cultures derived from the airway epithelial cells that are developed from several species including rats, guinea pigs, rabbits and humans. Alveolar cell cultures prepared from alveolar type II cells can also be used, and these cells are often used to assess drug transport. However, cell cultures have several disadvantages, such as the absence of drug clearance mechanisms, i.e. phagocytosis and mucociliary transport. It is also difficult to mimic the air-water interface and change in permeability during breathing (Mobley and Hochhaus, 2001).

2.6 Isolated perfused lung method

The isolated perfused lung (IPL) model is the most common perfused lung method employed, and has been used by many researchers to investigate various physiological, biochemical and metabolic aspects of lungs (Seeger *et al.*, 1999). It could be a good model to use for investigating the pulmonary effects and disposition of flavonoids contained in medicinal plants such as. *A. afra* than afore-mentioned methods, because it offers solutions to problems already mentioned. It is the transitional model from *in vitro* experiments to intact animal models. Thus, IPL

represents a system that is much less complicated than the whole animal, while preserving the integrity of the lungs. Therefore, IPL stands between *in vivo* experiments with whole animal and *in vitro* experiments with cultured cells (Wang, 1998). The perfused lungs are maintained in their "normal" anatomical and physiological conditions, are not fragmented or dispersed, and the transcellular transport and diffusion of agents are probably not altered. Furthermore, there are very few transected cells leaking their contents into the medium of the IPL compared to tissue slices. There is also no dilution of intracellular co-factors, especially when using blood as a perfusion medium, as this usually occurs in homogenate experiments (Niemeier, 1984).

The IPL offers the following advantages:

- lung physiological regulations are maintained, thus studies are performed in an intact organ with physiological cell-to-cell contacts, native extra-cellular matrix and cell polarities;
- influence of hormones, mediators found in plasma and the circulating blood can be controlled;
- respiratory mechanics such as tidal volume, lung compliance, and resistance as well as edema formation can be studied simultaneously, which is yet not possible with *in vivo* cell cultures;
- IPL can be adapted to make administration of plant medicine via inhalation, instillation, bolus injection or continuous perfusion possible, and;
- animal suffering is minimal (Foth, 1995; Uhlig and Taylor, 1998)

However, the IPL has several disadvantages such as the deterioration of lung mechanics with time limiting the duration of experiments to about three hours (Uhlig and Wollin, 1994). Furthermore, one lung represents one data point, and because of the time required to accumulate data; this makes the process very slow and expensive. There is also the absence of nervous regulation and lymph drainage, and even with lungs with stable baseline conditions and physiological variables within their normal

range, the activation of biochemical and metabolic pathways caused by isolation and perfusion procedure cannot be fully excluded. (Uhlig and Taylor, 1998; Wang, 1998)

Despite these limitations, IPL offers a direct and practical measurement of drug pulmonary disposition because the lung effluent (i.e. leaving lung from the venous side) can be continuously collected and analyzed (Liu *et al.*, 2005). This data can be transformed into plots that show the fraction of the dose absorbed over time, thus permitting determination of absorption rate and pulmonary residence time. Such systems were used for the characterization of polypeptide absorption, and for detailed investigations on the dissolution and absorption behavior of drugs (Mobley and Hochhaus, 2001).

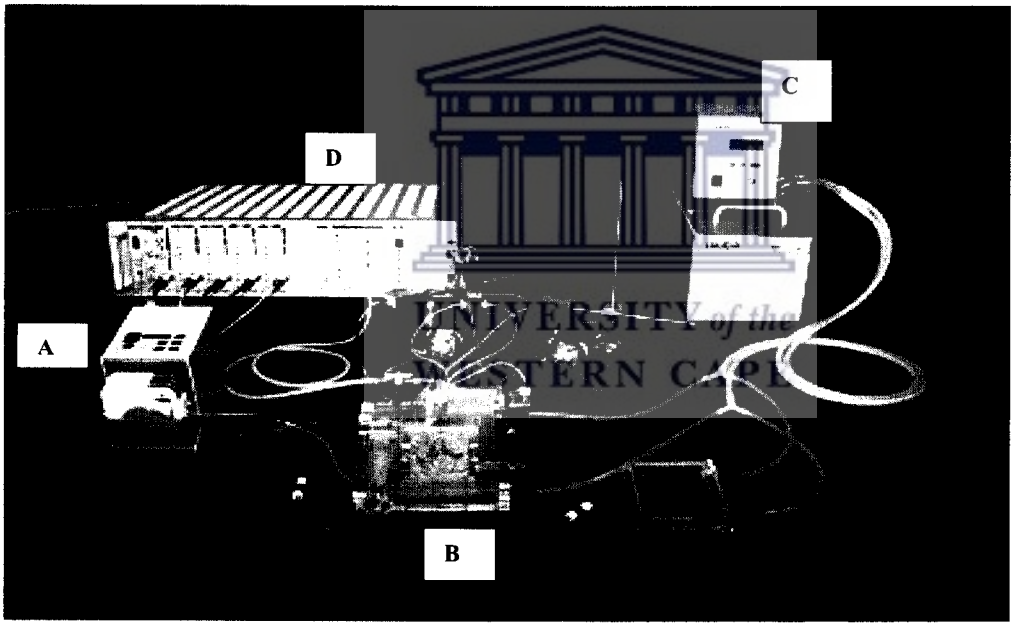


Figure 5: Typical basic components of the Isolated perfused lung system: (A) The peristaltic pump used to pump the perfusion medium into the system; (B) Artificial thorax chamber used to accommodate the lung thus mimics the normal animal thorax; (C) Thermostatted water reservoir used to keep the perfusate and system at constant temperature (usually 37 °C); (D) The ventilation control module (VCM) that ventilates the lung using either positive or negative pressure (adapted from Huggo Sachs)

The IPL has been used in various studies for pulmonary disposition of drugs including the study of the distribution of ciprofloxacin (Raquel *et al.*, 1999), determination of pharmacokinetics of an alkaloid sinomenine (Liu *et al.*, 2005), and

the study of the pharmacokinetics of polypeptides such as inhaled insulin (Pang *et al.*, 2005). This indicates that the IPL could, therefore, be an ideal model for the investigation of the effects of nebulized aqueous extract, or pulmonary disposition of luteolin administered as bolus dose or via continuous infusion. IPL model also allows the use of mouse, rabbit, guinea-pig or rat lungs. However rat lungs are often preferred because they are readily available, more robust and sturdy than guinea-pig or mouse lungs during surgery. They are also easier to control, and keep viable in the IPL system. Rat lungs have been used previously in IPL set up to be used for this study (Wang, 1998).

2.6.1 Methods of administrating compounds into IPL

Delivery of drugs via the pulmonary route is a well-establish means for treatment of respiratory diseases such as asthma, and has gained increased attention during the last decade for trans-mucosal delivery of drugs into systematic circulation. The IPL has contributed a lot in the evaluation of compounds on pulmonary function, and in this model, drugs can be administered by pulmonary route utilizing two techniques, namely intra-tracheal instillation and aerosol inhalation (Tronde, 2002).

2.6.1.1 Intra-tracheal instillation

This entails administration of chemicals directly into the trachea via endotracheal tube under positive ventilation. This offers rapid delivery of defined doses to small animals. However, it results in central patchy and un-homogenous distribution of drugs (Tronde, 2002). Therefore, to obtain a more reliable dosing, the solution must be carefully instilled such that it does not cause airway obstruction or disturb normal breathing pattern. Another concern with instillation method is the effect of vehicle used to dissolve or suspend the test drug because, should the vehicle alter the physico-chemical nature of the drug, the effect of the drug on lung tissues may also be altered. Furthermore, lung physiological barriers might also be altered. Despite these drawbacks, instillation method is most commonly employed, and has been applied in many pharmacokinetic and pharmacodynamic studies (Byron, 2004; Tronde, 2002).

2.6.1.2 Aerosol administration

Administration of aerosolized drugs into the lungs has been employed for many years to treat diseases localized within the bronchi. This results in a more uniform lung distribution of drugs than intra-tracheal instillation, and has more pronounced distribution into the alveolar region. However, the large proportion of the aerosolized drug is not delivered to the lungs, and it is also very difficult to measure the exact dose taken by the lungs. Therefore, when using smaller doses, longer exposure periods are often required, making the method more expensive than the instillation method. Despite these drawbacks, the use of pulmonary delivery method that mimics the methods used in clinical therapy is important for preclinical investigation of pulmonary drug function (Tronde, 2002; Zheng *et al.*, 1995), and hence aerosolization of drugs is often employed.

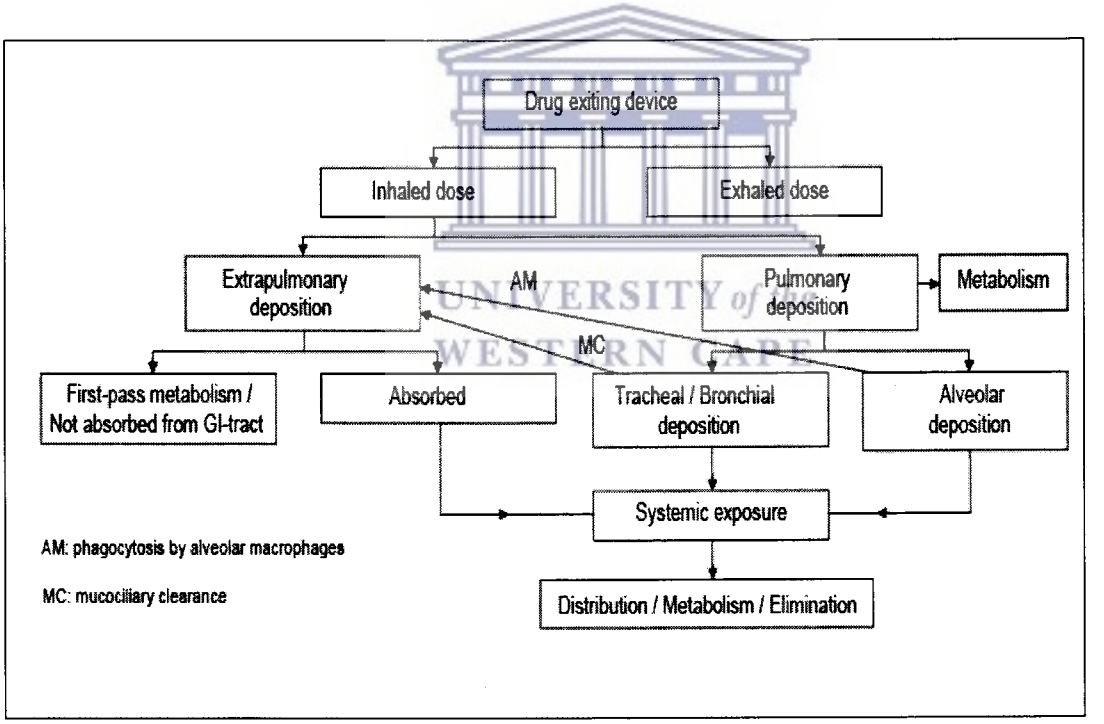


Figure 6: Schematic illustration of the fate of an inhaled drug (adapted from Tronde, 2002)

There are three clinically used devices that can be employed to administer pharmaceutical aerosols in IPL, and these include nebulizers, pressurized metered dose inhalers (pMDI's) and dry powder inhalers (DPI's). However, the use of pMDI's and DPI's is limited due to the very limited quantities of the drugs being tested.

Therefore, the nebulized aerosols are predominantly used (Tronde, 2002), and there are two types of nebulizers that can be used in IPL, namely the jet or ultrasonic nebulizers.

Most jet nebulizer designs force pressurized gas, usually air, from a nozzle (or jet) at high velocity past a liquid feed tube such that the nebulized solution is atomized at the capillary exit. The bulk of the aerosol mist (which may be traveling at up to sonic velocity), imparts against the baffle, drains back into the base of the reservoir and re-circulates. Only very small droplets (i.e. >5μm) escape the baffle and are available for inhalation, therefore, making them suitable when using lungs from small animals, including rats, due to better deposition and distribution of drug particles. Jet nebulizers are cheaper, can work well to create less than 5μm, highly respirable aerosols and can be used for aqueous solution or suspension formulations (Byron, 2004).

Ultrasonic nebulizers, unlike jets, are unable to nebulize suspension formulations. Despite this, their use produce deposition of large drug particles, but the distribution of the deposited drug particles may, however, be much more restrictive compared to that obtained with jet nebulizers, and there is also no high gas flow required for aerosolization (Byron, 2004). The advantage of ultrasonic nebulizers is that they can be weighed before and after nebulization in order to determine the mass of the aerosolized drug (Tronde, 2002).

Therefore, with all these possibilities, the IPL should be an ideal model for the proposed study of evaluating the effects produced by traditionally prepared *Artemisia afra* steam inhalation on lung function as well as the comparison of the effects produced by nebulized luteolin vs. aqueous extract on lung function, and the determination of the disposition profile of IV administered luteolin.

2.6.2 Pulmonary function parameters

Besides the above mentioned advantages and disadvantages, the IPL is a good model for the simultaneous evaluation of the effects that various chemicals and compounds might have on lung parameters such as tidal volume (TV), lung resistance (RL) & compliance (CL). Svens and Rvrfeldt (2001) used the IPL to determine acute impairment of lung function by amitriptylline, while Goggel *et al.* (2003) used it to determine acute pulmonary toxicity of lamp oil aspiration. In both studies, the results were related to the effect of the tested compounds obtained in humans. The following lung function parameters obtained in IPL are often employed for determination of effects of certain drugs and chemicals on lung function.

2.6.2.1 Tidal volume (TV)

The tidal volume is the volume of gas inspired or expired during each respiratory cycle, and is usually measured in milliliters (ml). Conventionally, expired volume is measured as TV and usually in human beings, the simple spirometry is used for TV measurement. TV has been found to decrease during bronchoconstriction, while it increased during bronchodilation, and is often affected during pulmonary disorders such as asthma, COPD (Ruppel, 2004).

2.6.2.2 Lung compliance (CL)

Compliance is the volume change per unit of pressure change for lungs and is measured in millimeters per centimeter of water (ml/cmH_2O) (Ruppel, 2004). CL is an index of functional stiffness of the lung, and it will decrease if lung parenchyma becomes stiffer through mechanisms such as constriction of alveolar duct smooth muscle, acute cellular infiltration. Furthermore, airway closure which is thought to occur predominantly in small airways will also make the lung functionally stiffer by reducing the amount of parenchyma available to accept inspired gas. These alterations represent changes in small airways, thus making compliance a useful test to indicate changes in periphery of the lung (Drazen, 1984). Therefore, measurement of compliance determines the elasticity or ease with which the lungs are stretched out. CL varies with lung volume at the end-expiratory level (FRC), thus to compare the

compliance of diseased lungs with that of normal lungs, FRC in each case should be known. CL is normally decreased in pulmonary edema or congestion, atelectasis, pneumonia and restrictive diseases such as pulmonary fibrosis, asthma. Known bronchodilators usually increase the lung compliance (Ruppel, 2004).

2.6.2.3 Airway resistance (RL)

Airway resistance is defined as the pressure difference required for a unit flow change, usually measured in centimeters of water per milliliter per second. Basically, it is the amount of pressure gradient needed to stretch out the alveoli to a given volume of air. This pressure change is created by the friction of flowing molecules coming into contact with the conducting airways. RL is the measurement of clinical and physiological interest, and is useful in evaluating the respiratory effects of bronchodilatory or broncho-constrictive drugs, as well as airborne contaminants and naturally occurring particulates. Increased resistance is clinically related to an assortment of respiratory diseases such as asthma, bronchitis. This increase is due to narrowing and collapse of airways, and as the resistance increases its harder to inflate the lungs and the RL is often decreased by increase in lung volume (Lausted and Johnson, 1999; Ruppel, 2004).

CHAPTER THREE
PLAN OF WORK

3.1 Introduction

This chapter describes the objectives, hypothesis and study approach proposed for the study.

3.2 Objectives

The objectives of this study were:

1) to compare the luteolin content in dried crude *A afra* leaves and an aqueous extract of *A. afra,*
2) to compare the pulmonary effects of traditionally-prepared *A. afra* steam inhalation and nebulized *A. afra* aqueous extract and luteolin solutions, and
3) to determine the pulmonary disposition and effect of intravenously administered luteolin.

3.3 Hypothesis

It was hypothesized that:

(i) The traditionally-prepared aqueous extract of *A. afra* contained higher luteolin levels than the equivalent amount of crude dried leaves of *A. afra,*

(ii) the inhaled steam of *A. afra* would improve lung function of the isolated perfused rat lung by increasing both the tidal volume and respiratory compliance, and by decreasing lung resistance,

(iii) The nebulized solution of the aqueous extract of *A. afra* would improve lung function better than an equivalent dose of luteolin solution (i.e. luteolin is better absorbed from plant dosage form),

(iv) Intravenously administered luteolin would cause smaller changes in lung compliance and resistance compared to inhaled steam inhalation of *A. afra,* and that

(v) Lungs rapidly absorb and metabolize luteolin administered via the pulmonary vasculature.

3.4 Study approach

The above objectives and hypotheses were realized using the following approach:

3.4.1 Why *Artemisia afra*?

Several factors strongly supported the use of *Artemisia afra* in this study. Firstly, *Artemisia afra* is commonly and traditionally used in the form of a steam inhalation to treat asthma and other respiratory ailments. However, there was no scientific evidence on the pulmonary effect produced by such steam inhalation. Secondly, *Artemisia afra* formed part of the research program of the School of Pharmacy that focused on the manufacture and evaluation of aqueous extracts and other improved dosage forms from medicinal plants. Finally, earlier data from the School of Pharmacy had found that the aqueous extract of *A. afra* relaxed respiratory muscles that had been contracted by various known bronchoconstrictors, an activity that raised our interest in the pulmonary effects of the plant.

3.4.2 Why luteolin?

There was a strong suspicion that flavonoids could contribute to the pharmacological effect of *A. afra* and luteolin was one of the major flavonoids found in appreciable amounts in the aqueous extract of *Artemisia afra*. Furthermore, this flavonoid had previously been shown to have bronchodilatory activity, an effect that might link luteolin intimately to the pulmonary effects of *A. afra*. Luteolin also seemed to be easily extractable from plant material, stable in the various plant samples under various processing conditions and could easily be assayed using a simple extraction and HPLC analytical procedure.

3.4.3 Why the evaluation of steam inhalation?

Traditionally, inhalation of steam obtained by boiling either plant material or essential oils, has been continuously used world-wide. Basically, the dried leaves of the plant are boiled in sufficient boiling water in a pot to produce steam that is inhaled while the head and the bowl are covered by a towel, and the inhalation continued until the mixture is cooled down. Despite this widely used mode of administration, there was,

however, no scientific evidence on the effectiveness of this mode of administration. It was therefore thought necessary to evaluate the pulmonary effects of the traditionally prepared steam inhalation of *A. afra* and particularly its effect on pulmonary functions.

3.4.4 Why the pulmonary disposition of luteolin?

A. afra is traditionally used to treat respiratory conditions. However for it to be active, its constituents, including luteolin, should have optimal lung disposition i.e. be optimally bio-available and not too quickly metabolized. The bioavailability and disposition of flavonoids in the gut have, in recent times, been extensively investigated, but their pulmonary disposition has specifically not yet been elucidated.

3.4.4 Why the use of isolated perfused lung model (IPL)?

To evaluate the effectiveness of the steam inhalation mode of administration and the pulmonary effects of *Artemisia afra*, a suitable experimental model was needed. The isolated perfused lung model has been used by several investigators interested in the physiological, biochemical and metabolic aspects of drugs in the lungs. It was thought that it might also be a good model for this study because it was a model that offered various advantages such as being an organ model with all its physiological cell-to-cell contacts intact, a model in which respiratory mechanics such as tidal volume, lung compliance, and resistance as well as edema formation could be continuously and simultaneously monitored (something which is not possible with *in vivo* cell culture models). Furthermore, the IPL could be adapted to allow the administration of plant medicine via inhalation, instillation, bolus injection or continuous perfusion, while experiments in the IPL system could be stable for at least three hours, giving enough time for the evaluation of the pulmonary effects of the inhaled steam of *A. afra*

3.4.5 Why use of HPLC for quantification?

Finally, investigating the objectives of this study necessitated the quantification of very low concentrations of the flavonoid luteolin in both plant materials and biological samples (i.e. lung homogenates and perfusion medium). In recent times,

HPLC has become the standard method for the analysis of flavonoids because it yields excellent resolution and retention times that are extremely reliable in the identification of individual flavonoids. It also offers several advantages such as being a simple analytical technique that is quite specific, sensitive and reproducible in compound separation when using smaller sample quantities. In addition, a validated HPLC assay could generate reproducible and interpretable data (e.g. chromatographic finger print) that could be useful for quality control purposes. These were all attributes that suggested the HPLC could be effectively applied for the present investigation.

CHAPTER FOUR
MATERIALS AND METHODS

The following drugs, chemicals, reagents, instruments, animals, experimental systems and procedures were used in this investigation.

4 .1 Chemicals and reagents

The following chemicals and reagents were used in the preparation of the plant materials, the assay of the plant materials, lung and perfusate samples and the isolated perfused lung experiments:

Sodium chloride; potassium chloride; calcium chloride 2-hydrate; potassium dihydrogen phosphate; magnesium chloride 6-hydrate; sodium hydrogen carbonate and glucose, all of analytical grade (Holpro Analytics (Pty) Ltd. Johannesburg, South Africa); bovine serum albumin (Fraction V), luteolin (97%) and dimethyl sulfoxide (Sigma-Aldrich South Africa, Vorna Valley, South Africa); heparin sodium injections (5000units/ml) (Fresenius Kabi (Pty) Ltd., Cape Town, South Africa); sodium pentobarbitone (200mg/ml) 10ml vials (Kyron Laboratories (Pty) Ltd., Johannesburg, South Africa); carbon dioxide gas, helium gas and nitrogen gas (all from Afrox Ltd Epping, South Africa); concentrated hydrochloric acid, methanol, and acetone (all analar grade, KIMIX Chemicals & laboratory suppliers, USA); acetonitrile (HPLC grade, ACS specifications and manufactured in the USA by Allied Signal Inc.; formaldehyde and ethyl acetate (AR Saarchem Merck Chemicals (PTY) LTD).

4.2 Equipment and instruments

4.2.1 Isolated Perfused Lung (IPL) System

The IPL consisted of the following parts:

Small operating table, tracheal cannula, pulmonary artery cannula and left atrial cannula (Medical Biosciences, Kent, U.K.); roller pump, model MS-4 REGLO/8-100; PLUGSYS modular central electronics system, type 603;

ventilation control module (VCM), type 681; timer counter module (TCM), type 868; external input module (EIM), TYPE 673; analog digital convector (ADC), type 663; Validyne® pressure transducer, model SP 2040 D; HSE DC bridge amplifier; glass artificial thorax chamber along with perplex lid; perfusate reservoir; pneumotachometer attached to a Carrier Frequency Bridge Amplifier (CFBA), and the PULMODYN® software and PC Analog digital card (Hugo Sachs Eletronik (HSE), March-Hugstettem, Germany); Sanpo display monitor, model number (BDS/14735) (Sanpo Corporation, Taiwan); vacuum pressure gauge, model 7769 (Control instruments products, Johannesburg, South Africa); thermostatted circulation bath, model 02 ph623 (Heto (Pty) Ltd., Denmark); SP Validyne® Differential pressure transducer, model P/N0004-150 (UTAH Medical Products, USA); Universal pressure transducer readout, model SC1001(Gould Statham Company, Oxnard, CA., USA); Carrier Demodulator model CD12 (Validyne® Engineering Corp. North Ridge, CA., USA); Digital pH meter, model PHM82 (Radiometer, Copenhagen, Denmark); jet and ultrasonic nebulizers (Omron Tateisi Electronics Co., Tokyo, Japan).

4.2.2 High Performance Liquid Chromatography (HPLC) Systems

Two HPLC systems that were used in this study:

System one consisted of a pump (Model 110A, Beckman), injector (Model, Beckman), UV-VIS detector (Shimadzu Co-operation, Japan), chart recorder (model R-03, Rikadenki Koygo Co. Ltd., Tokyo, Japan)

System two was a Beckman System Gold HPLC system with a 32-Karat ™ software package (Fullerton CA, USA), consisting of a Beckman Gold Module 126 series programmable binary gradient pump, a Beckman Gold Module 507 series auto sampler with a Beckman Gold Module 168 series diode array detector.

The column used in both these systems was the Luna ® C-18 reverse phase column (Phenomenex, USA) having 5µm particle size and a column length of 250 x 4.60 mm.

4.2.3 Other instruments

In addition to the above the following instruments were also used:

Millipore Filter Unit (Cameo 25 AS, DDA 02025So MSI: Micro separation Inc., USA); Vortex-2 (G-560E, manufactured by Scientific Industries, Inc. Bohemia, N.Y. 11716 USA); Centrifuge (Labofuge 200, Made in Germany); pH meter (Basic 20 Crison Instruments, S.A.); Micropipette100µl and 1000µl (Gilson Medical Electronics (France). S.A); Oven (Memmert 854 Schwabach Made in Germany); Balance 1 (Wirsam Scientific & Precision Equipment (PTY) Ltd. Model GA 110 Made in Germany); Balance 2 (Mettler Pe 6000 Mettler Instrumente Ag Ch-8606 Greifensee- Zurich Made in Switzerland); Hot Plate (Type Rct13 Kika-Werke Gmbh&Co.Kg D-79219 Staufen Made in Germany); -85 Freezer (Lozone Cfc Freezer Model U855360, New Brunswick Scientific, USA); Freeze-Dryer (Model Virtis Freeze Mobile 72sl, The Virtis Company Gardner, New York, USA); Filtration System (Supelco, South Africa); Watch Glass: (8cm Diameter) Vacuum Pump (Medi-Pump Model 1132-2, Thomas Industries, Inc., USA); Water Bath (Cph110, Manufactured By Lab design Engineering Pty Ltd); Fi-streem 4 litre water distiller (Fisons, Germany)

4.3 Animals

For this investigation, male Wistar rats (300-400g) obtained from University of Cape Town (UCT) and Medical Research Council (MRC), South Africa were used. These experimental animals were kept on a 12-hour light/12-hour darkness cycle in a well ventilated animal room in the School of Pharmacy: Discipline of Pharmacology at the University of the Western Cape (UWC). The animals had free access to water and chow until taken for surgery. The experiments were performed in accordance to the

requirements of the protocol approved in April 2005 by the Animal Ethics Committee of the MRC and UWC Senate Research Committee.

4.4 EXPERIMENTAL METHODS AND PROCEDURES

4.4.1 Collection, identification and preparation of plant material

The *Artemisia afra* plant material used in this study was obtained from Montague Museum (Montague, Western Cape Province, South Africa) during the summer of 2005. Samples of the collected materials were verified by a botanist at the University of the Western Cape (UWC), and a voucher specimen (6735) was deposited in the Herbarium at UWC. The fresh leaves of the *A. afra* were removed, weighed and then dried at 30°C in a well-ventilated oven for 72 hours until a constant weight was obtained. Thereafter, the dried leaves were weighed, ground with an electric blender, packed into a container, sealed and stored in a dark cupboard at room temperature until further use.

4.4.1.1 Extraction of plant material

In this investigation, only plant materials that closely resembled the traditional plant products were used. Thus, the dried *A. afra* leaves were, as soon as possible, extracted with water to isolate flavonoids present in cell vacuole and to prevent enzymatic oxidation or hydrolysis of flavonoids in the dried, ground plant material. Aqueous extraction was preferred since this most closely mimicked the traditionally-prepared aqueous preparations of *Artemisia afra*. Therefore, 100g of dried ground plant leaves were boiled in 1000ml of distilled water for 30 minutes. The resultant infusion was filtered, cooled and transferred into round bottom flasks. These flasks were put into a freezer at -4°C for 48 hours and the frozen infusions freeze-dried under vacuum to produce a dry flaky brownish extract. The resultant aqueous extract was weighed immediately, transferred into amber bottles, sealed tightly and stored in a dessiccator until further use.

4.4.1.2 Preparation of plant material for HPLC assay

To help standardize the study material, quantification of luteolin levels in *A. afra* plant material was done using HPLC analysis. For this, solutions of un-hydrolyzed and hydrolyzed plant samples, both the aqueous extract and the crude dried leaves, were prepared to determine the level of free (aglycone) and total luteolin (aglycone + glycosides), respectively. To 25mg of aqueous extract and 100mg dried leaves, 300µl and 1ml of distilled water were respectively added to solubilize the plant material. One hundred microliters of morin solution (300µg/ml) were added in each sample and the contents thoroughly vortex-mixed for one minute. Thereafter, the un-hydrolyzed samples were directly extracted with 5ml of ethyl-acetate, while for the hydrolyzed samples 4ml of 2N HCl was added, this acid/water mixture heated in a water-bath at 80°C for 40 minutes, cooled at room temperature, and only then extracted with 5ml of ethyl-acetate. In all samples, the ethyl-acetate extracts were evaporated to dryness under a gentle stream of nitrogen gas, the residue re-dissolved into 500µl of HPLC mobile phase and 100µl of the solution injected unto the HPLC column.

4.5 HPLC assay for luteolin levels determination

The HPLC assay was developed and validated for the qualitative or quantitative determination luteolin in samples of the aqueous extract and dried leaves of *A. afra*. In this section, only the final procedures used are reported, the development aspects are reported in section 5.3.1.

4.5.1 General HPLC procedure

First, stock solutions of luteolin (1mg/ml) and morin (3mg/ml) were made up in dimethyl sulfoxide (DMSO), stored, protected against light and refrigerated at -20°C, and only thawed just prior to use. Working solutions for luteolin (100µg/ml) and morin (300µg/ml) were made by diluting aliquots of the stock solutions with methanol. Each day standard solutions of luteolin in the concentration range of 5 - 30µg/ml in water, were prepared from the luteolin working solution. These were then spiked with 30µg morin (IS), acid hydrolyzed and extracted as described above in

section 4.4.1.2 before 100 µl aliquots were injected onto column. The samples were then eluted using a mobile phase consisting of 30% acetonitrile/ 70% phosphate buffer (100mM, pH 2) isocratically pumped through a Luna ® C-18 (250 x 4.6mm, 5µm) at a flow rate of 1ml/min. The peaks produced during analysis were monitored at λ 349 nm.

4.5.2 Identification of luteolin peaks

To establish the retention time for the luteolin standards, 100µl aliquots of standard solutions were injected six times onto the HPLC column. The retention time of the peak produced was noted and the average and standard deviation (ave ± SD) determined. To identify the luteolin peak in the plant material, the retention times of the peaks obtained after injection of the luteolin standard samples were compared to those obtained when plant material was subjected to analysis under similar HPLC conditions. Furthermore, the plant samples were spiked with luteolin to see changes in the height of the suspected peak. To further identify the luteolin peak, the diode array detector was used to scan the peaks found for the standard solution and that for the plant.

4.5.3 Validation of the HPLC assays

The HPLC assays used in the study were all validated for: separation and specificity, the inter- and intra-day variability, recovery, linearity and lowest limit of detection. The separation and specificity of the assay was assessed by determining the absence of peaks of endogenous compounds interfering with the luteolin and morin peaks both, in the plant material and the perfusate. To check for inter-and intra- day reproducibility of the assay, five different concentrations of luteolin were analyzed several times and on different days and the percentage coefficient of variation calculated for each individual concentration. Assay recovery was determined by interpolating the ratio of the luteolin to internal standard peak heights obtained after the hydrolysis, extraction and chromatography of six samples of the same concentration from the standard curve. The linearity of the standard curve for luteolin was assessed by subjecting the luteolin to morin peak height ratio *versus*

concentration data obtained, for standard solution of luteolin in both plant material and perfusate, to linear regression. Lastly, the lowest limit of detection was taken to be the lowest luteolin concentration which under the described HPLC conditions produced a peak height at least three times higher than the base line noise. At least four replications were used for each concentration. The results obtained are presented and discussed in the next chapter.

4.6 Experiments to assess the pulmonary effect of steam inhalation, nebulized extract, and luteolin; and the pulmonary disposition of luteolin after intravenously administration of luteolin.

To determine the effects of luteolin, the extract and steam of crude dried leaves of *A. afra* on lung function, as well as the pulmonary disposition of luteolin, the isolated perfused lung system was used.

4.6.1 Isolated Perfused Lung System

The isolated perfused lung (IPL) system used in this study was a modification of that used by Uhlig and Taylor. (1998) and could be divided into four sections, namely: (a) the negative pressure chamber with the lung; (b) the perfusion apparatus; (c) the ventilation system, and (d) the data capture system. A schematic diagram of the full system is shown in figure 7.

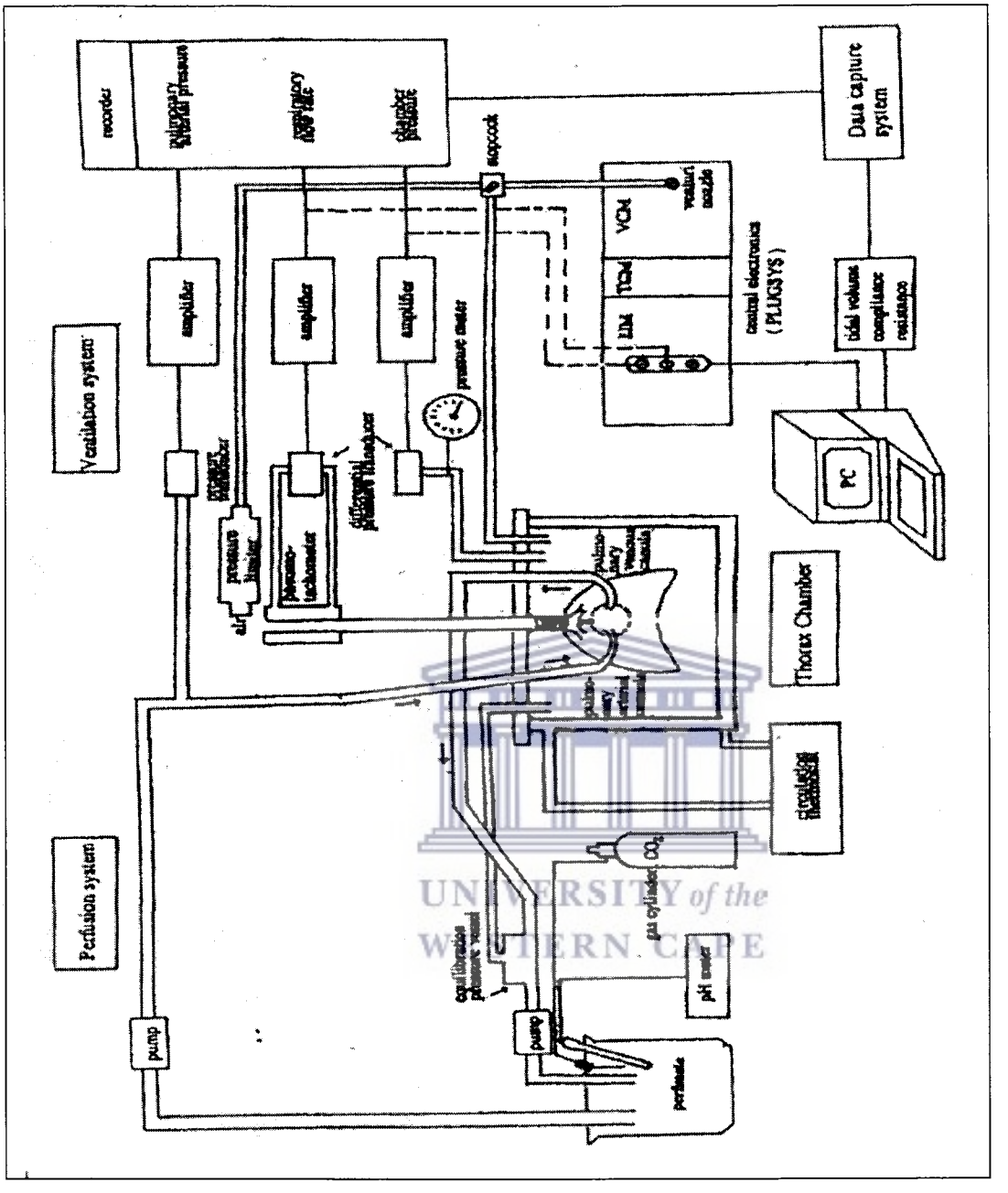


Figure 7: Typical set-up of the isolated perfused rat lung

4.6.1.1 The artificial thorax chamber with the lung

In this system, the lung was placed in a water-jacketed artificial glass thorax chamber (A in fig 8) fitted with a Plexiglas lid (B). This chamber had a central opening (organ holder) to which a pneumotachometer (C) was connected, and two side holes through which two glass tubes penetrated. The lung was connected to the organ holder via a tracheal cannula (D). The pulmonary cannula was connected to one of the two side

glass tubes fitted in the two side holes of the chamber lid. The perfusate was pumped into the lung via this pulmonary arterial cannula (E). The other glass tube was connected to a pulmonary venous cannula (F) which was placed into the left ventricle to discharge the perfusate coming from the lungs (effluent perfusate). The lung chamber had the following additional connections: one for negative pressure venture; another linked to a chamber pressure transducer for measuring trans-pulmonary pressure (chamber pressure) and two other connections for the circulation of thermostatted water (37°C). There was a small volume of water in the bottom of the lung chamber (G) to humidify the air. Lastly, there was a drainage outlet blocked with a plastic stopper (H) for draining excess fluid inside the chamber and during cleaning.

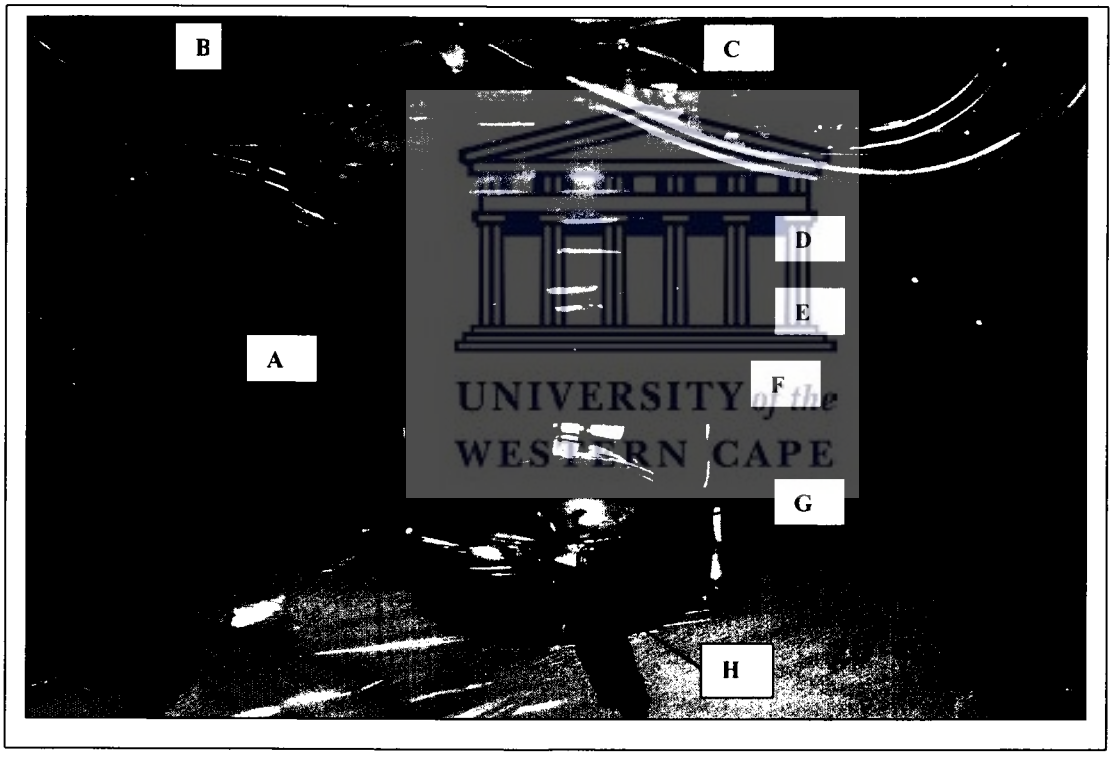


Figure 8: **Design of the artificial thorax chamber. (A): Canulated isolated rat lung; (B): Plexiglass lid Bubble trap connected to the arterial cannula (influent perfusate); (C): Pneumotachometer connected to tracheal cannula and organ holder; (D): Trachea connected via tracheal cannula; (E) Arterial cannula with influent perfusate; (F): Venous cannula with effluent perfusate; (G) Small volume of water used to humidify the chamber; (H): Drain connected to a stopper.**

4.6.1.2 The perfusion apparatus

The lung was supplied with the required nutrients via the perfusion medium (see section 4.6.2 below) to maintain its working conditions in the chamber. The peristaltic roller pump (A in figure 9) was employed to continuously drive the perfusate (maintained at 37°C) from a 500ml water-jacketed reservoir (B) through a short perfusion line to and through the lung vasculature. The perfusate entered the lung via the pulmonary artery (i.e. arterial cannula) and left it via the left ventricle (i.e. venous cannula) back to the reservoir. The bubble trap was placed before the pulmonary arterial cannula prior to the point where the perfusion line entered the inside of the thorax chamber. This bubble trap also had a small opening through which bolus drug administration could be made. Further, connected (via a thin tubing) inside the bubble trap was a pressure transducer used for the continuous measurement of pulmonary arterial pressure (PAP). Before returning to the reservoir, the perfusate passed through a pressure equilibration vessel which was located at the level of the left atrium and was responsible for keeping the trans-mural pressure measurement.

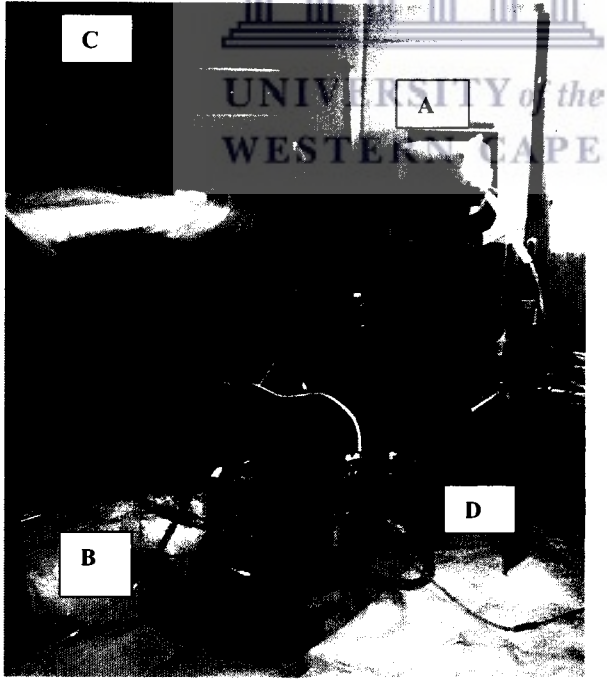


Figure 9: Some of the IPL components: (A) Peristaltic roller pump used to drive the perfusate to and from the lungs; (B) the water-jacketed perfusate reservoir containing 100ml perfusate; (C) The pH meter (D) pH electrode immersed in the perfusion medium.

4.6.1.3 The ventilation system

The ventilation system was needed to maintain the respiratory function of the lungs inside the chamber. This system consisted of several parts coordinated through a central electronics modular system, the PLUGSYS ™. The main component of this system was the ventilation control module (VCM) which was connected to the trachea via a tube and there was a change-over valve in-between the lung and the VCM. The VCM was used to ventilate the lungs with atmospheric air and generated fluctuating negative or positive pressure ventilation inside the chamber. For positive pressure ventilation, air was pumped directly from the VCM into the lungs while switching of the change-over valve allowed the change of direction of the airflow through a venturi gauge thus transforming positive pump pressure and creating negative pressure (with respect to ambient atmosphere) ventilation. This venturi gauge was mounted within the chamber wall. The respiratory rate and the level of the end-expiratory and end-aspiratory pressures were controlled by the VCM settings while the frequency of deep breaths ("sighs") was adjusted through the Timer Counter Module (TCM) of the PLUGSYS ™. The pneumotachometer connected to the trachea via a central opening in the lid perspex was used for measurement of the airflow passing through the lungs. The signal from this pneumotachometer was passed via a differential pressure transducer (model SP 2040D), the amplifier and External Input Module (EIM) of the PLUGSYS™ electronic unit to the computer. The respiratory volume was derived by the computer program (PULMODYN ™) from the flow signal. To measure the trans-pulmonary pressure, the two arms of a differential pressure transducer (Validyne ®, model DP45-24) were connected to the inside and the outside of the chamber and the signal similarly amplified and passed onto the computer. The negative pressure meter or gauge was connected to the chamber and continuously displayed the chamber pressure.

4.6.1.4. The data sampling and capturing system

Several measurements were continuously recorded while the lung was being perfused and ventilated. The respiratory mechanics such as tidal volume, lung resistance, lung compliance and mean pulmonary pressure were monitored. In addition to these

parameters, airflow rate and trans-pulmonary pressure were also measured. The signals for these parameters were fed into a computer via a PLUGSYS ™ and an analogue digital (A/D).card. The PULMODYN ™ computer software was used for data collection and manipulation, real time data display and storage. At the end of each experiment, a hard copy of data was saved onto a stiffy disk and then printed.

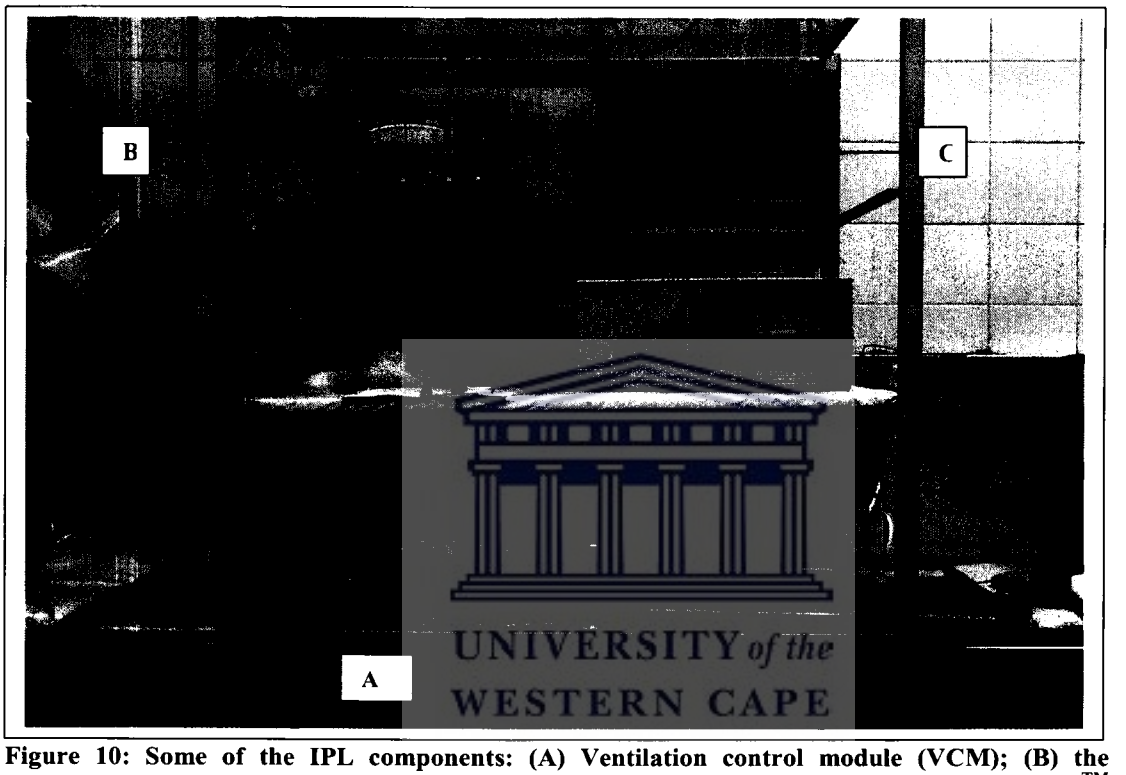


Figure 10: Some of the IPL components: (A) Ventilation control module (VCM); (B) the Validyne ® Carrier Demodulator; (C) The computer with the PULMODYN ™ software for data acquisition.

4.6.2 The perfusion medium

The perfusion medium used for each experiment consisted of 200ml of Krebs-Henseleit buffer containing 2% bovine serum albumin (BSA), 0.1% glucose and the salts given in table 1. The perfusion medium was always freshly prepared on the day of use. After addition of all the components, the perfusion medium was filtered before use. The medium was also heated to 37°C in a water-jacketed glass reservoir before and during experiments, and throughout the experiments and kept at a pH between 7.30 -7.40 by sparingly bubbling with carbon dioxide (CO_2) when needed.

Table 1: The composition (Mm) of Krebs-Henseleit buffer solution

Chemical	Quantity (mM)
Calcium chloride	2.50
Magnesium sulphate	1.20
Sodium chloride	118
Potassium chloride	4.70
Potassium dihydrogen phosphate	1.20

4.6.3 Surgical removal of the lung

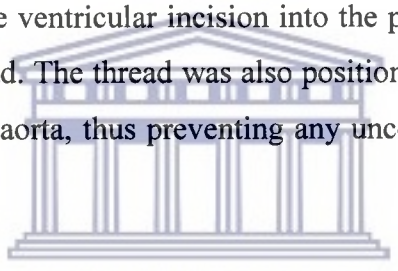
The male Wistar rats (300-400g) were anaesthetized with sodium pentobarbitone at a dose of 100mg/kg intra-peritoneally. When optimum depth of anaesthesia was reached which was determined by testing for the absence of pain reflex (e.g. briefly clamping the animal front paw with forceps), the animal was placed onto the operating table in the ventral position (on its back). The rat was fixed onto this table by tying its paws with elastic bands to the sides of the table (see A in fig. 11). Using scissors, the skin of the fully anaesthetized rat was incised ventrally in the median line from the upper abdomen to the neck and then separated from the muscles of the thoracic wall (see B below).

The trachea was exposed using blunt dissection and a surgical cotton thread drawn through underneath the trachea which was then loosely looped around it. A curved forceps was placed underneath the trachea such that the cannula doesn't slip out, and it was left there until the lung was completely isolated (see C below). The trachea was cut about two thirds through its width with a small spring scissors and then the tracheal cannula was inserted and tied in place with the thread placed around it (see D below). The cannula was connected to the organ holder of the chamber to which the pressure limiter was mounted beforehand (see B below).

Immediately thereafter, the ventilation control module (VCM) was switched on and the lung ventilated at positive pressures of about 10cm H_2O and 80 breaths /minute with no sighs (i.e. no periodic deep breathing). Afterwards, the two femoral arteries were cut with a surgical blade to exsanguinate the animal. The abdominal wall was

then incised from the upper abdomen to the apex of the *manubruim sterni* with the incision extending bilaterally along the rib bows of both sides. The diaphragm was carefully resected from the thorax wall, then the thorax opened by median sternotomy and both halves of the thorax partially cut off so as to appropriately expose the lung.

The thymus was removed around the heart and then heparin sodium (2000IU/kg) was injected into the right ventricle to prevent blood clotting. The heart was lifted and curved forceps were inserted between the left heart auricle and the left ventricle just behind the aorta and the pulmonary trunk. A cotton thread was then drawn through behind the two large vessels and a loose ligature placed around it. Using small scissors the right ventricle was incised about 5mm from the start of the pulmonary trunk in the region of the outflow path. The arterial cannula, pre-filled with the perfusate, was inserted through the ventricular incision into the pulmonary trunk and then tied with the pre-looped thread. The thread was also positioned in such a way so that it simultaneously tied off the aorta, thus preventing any uncontrolled outflow of the perfusate (see E below).



The apex of the heart was raised with forceps and then another loose thread ligature was placed around the middle part of the heart, a longitudinal incision was made in the left ventricle close to the heart apex and parallel to the inter-ventricular septum. The pulmonary venous cannula was inserted through the ventricular incision into the left atrium. Care was taken to only apply the appropriate force needed to pass the cannula through the mitral valve without damaging the thin pulmonary vein. The ventricular cannula was then tied with the pre-looped thread but not too tightly (see F below). The roller pump was immediately switched on to initially perfuse the lung at 5ml/min. At this stage, the inferior vena cava, thoracic aorta, the two vagal trunks, oesophagus and the upper part of the trachea were carefully removed (see G below). The lower part of the lung and the trachea were dissected free by cutting step-by-step in the cranio-caudal direction. The isolated lung was then tied to the lid via the tracheal cannula and the two blood vessel cannulae were lifted free from the animal

residues and placed into the artificial thorax chamber with the lid secured in place (see H below).

When the lung was placed inside the chamber (see I below) the positive pressure ventilation was stopped and changed to negative pressure ventilation by switching the changeover stopper. The pressure in the chamber now fluctuated between an end-inspiratory pressure of -10 to -16cm H_2O and end-expiratory pressure of -2cm H_2O. At this point the flow rate was increased to 25ml/min by adjusting the roller pump. The pressure limiter was then removed from the central opening of the chamber lid and a pneumotachometer installed in this opening to measure the airflow rate. Finally, the Timer Counter Module (TCM) of the PLUGSYS ^{TM} electronics system was set to produce a hyperinflation (-16cm H_2O) every 2 minutes (see J below). After the lung was in the chamber, the perfusate was pumped using single pass perfusion for 5 minutes to clear the cell debris before changing to recirculation mode for the rest of the experiment to mimic the normal body circulation.

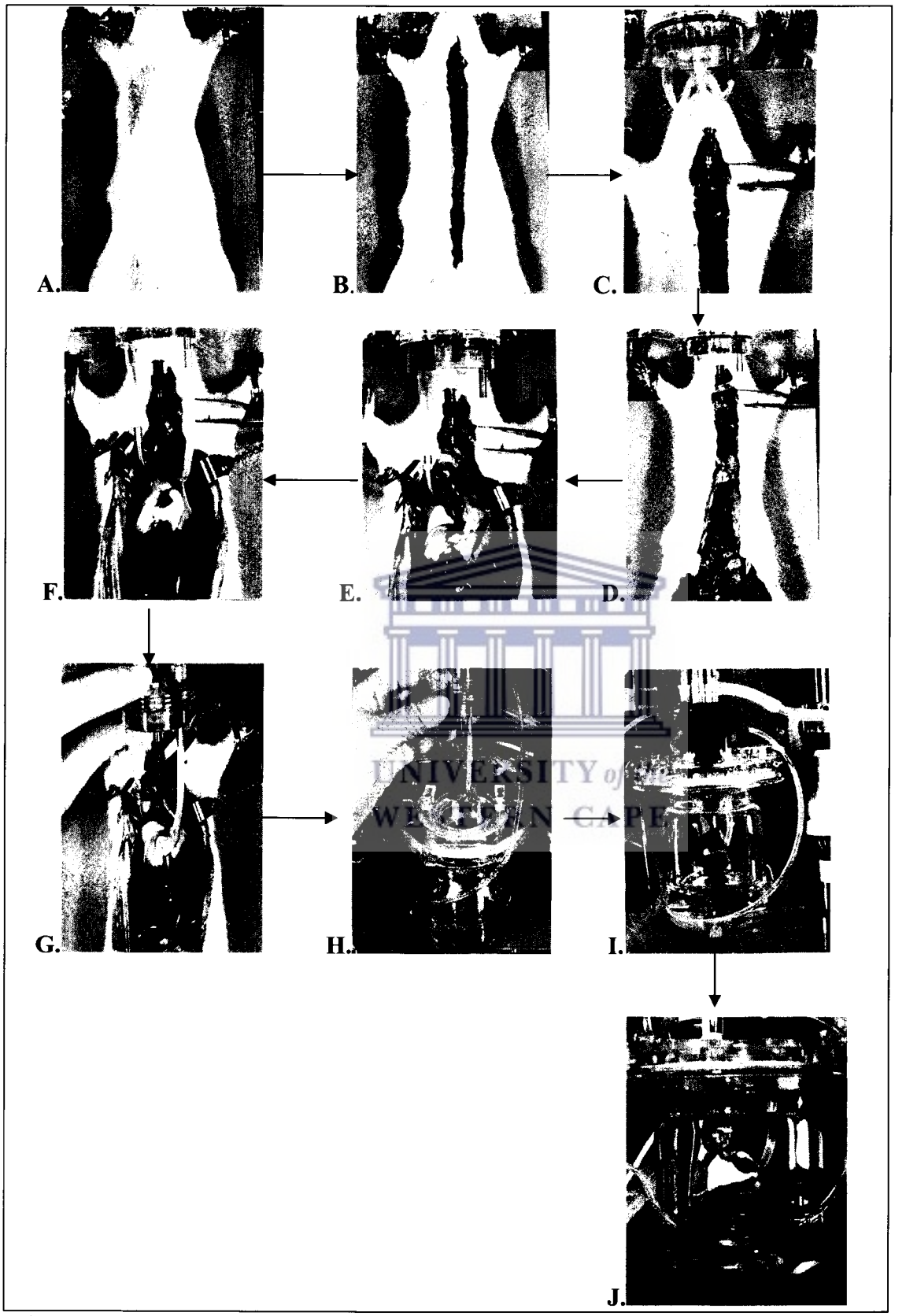


Figure 11: The schematic diagrams showing the isolation of the lung in IPL adapted from the Hugo-Sacks manual.

4.6.4 Administration methods (Protocols)

The study was divided into three study protocols. For all experiments lungs were allowed to equilibrate and stabilize for 20 minutes before treatment with test compounds. The following protocols were implemented.

4.6.4.1 Study protocol one: To determine the effect of traditionally prepared *Artemisia afra* steam inhalation on lung function

To administer steam into the IPL, a 500ml conical flask (A) placed on top of a heater was used to boil the dried leaves of *Artemisia afra* as it is normally done traditionally. This flask was sealed with a rubber stopper that had a centered hole into which a glass tube was inserted. Attached to this glass tube was a three-way perspex valve connected to compressed air cylinder that was used to drive a stream of the resultant vapour into the direction of the lung, and this was further linked to a 2nd flask via teflon tubing. The second flask (B) used to trap condensate was sealed with a rubber stopper that had two holes in which two glass tubes were then inserted, one tube was used to link the conical flask (A) while the other was connected to the 3rd conical flask (C) that was also used to trap condensate and allow the steam to cool. The third conical flask was connected to a round perspex steam feeder used to administer the steam into the lung. This feeder had two outlets, one to be inserted into the IPL for steam administration while the other was used to allow steam to escape.

For each experiment, dried leaves were boiled for 5 minutes before the resultant steam was pumped via compressed air to create a stream of vapor into the direction of the lung. During administration the pneumotachometer was not in place i.e. removed from the artificial thorax chamber of the IPL (i.e. lung function was not recorded). Then, the outlet of the steam feeder was immediately inserted, and the lungs were allowed to inhale the resultant steam for 3 minutes under negative ventilation. Thereafter, the pneumotachometer was immediately re-inserted into the chamber to monitor lung function for thirty minutes after inhalation. During steam inhalation, the outlet of the steam-feeder was immersed in 1 ml of distilled water to trap aerosolized

compounds. These samples were subjected to HPLC analysis to identify and quantify the amount of luteolin (if any) trapped during the steam inhalation period.

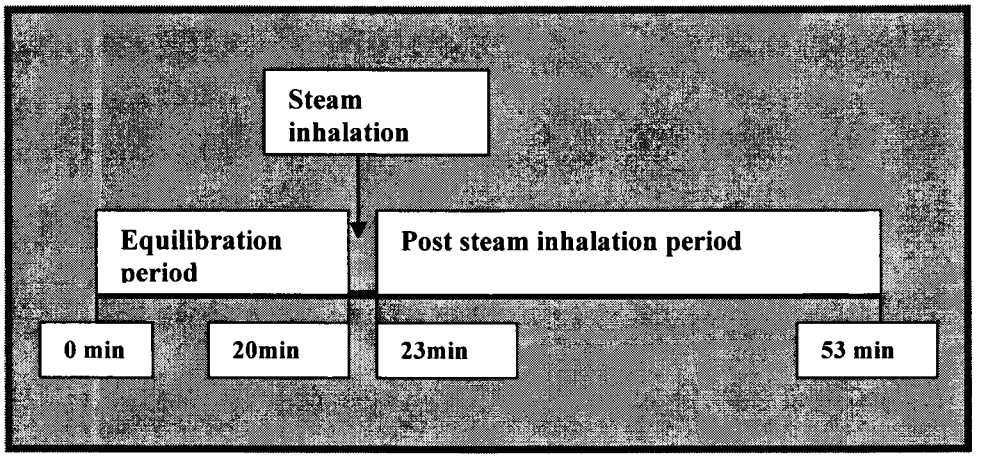


Figure 12: Schematic diagram showing the protocol for steam inhalation study design. Lungs were allowed to stabilize and equilibrate for 20 minutes (0-20min), and then steamed with either saline or *Artemisia afra* (50 or 10mg extract per ml of saline). Thereafter lung function was monitored for 30 minutes (23 -53 min) after start of each experiment.

With this design, the following protocol was used:

(a) **Control group**: The lungs were allowed to inhale steam for three minutes that had been produced by boiling 100ml of saline.

(b) **Test group:** The test group of lungs was divided into two, each for using a different dose of *A. afra* leaves, viz.

1) **10mg/ml group**: These lungs were allowed for three minutes to inhale steam that had been produced by boiling 1g of *Artemisia afra* leaves in 100ml of saline.
2) **50mg/ml group**: The lungs were allowed for three minutes to inhale steam that had been produced by boiling 5g of *Artemisia afra* leaves in 100ml of saline.

4.6.6.2 Study protocol two: To determine the effect of nebulized luteolin and aqueous extract on lung function

To determine the effects of nebulized luteolin and traditionally prepared aqueous extract on lung function relative to saline, a jet nebulizer was used to generate the

aerosols. The nebulizer was linked via thin silicone tubing with a 5mm internal diameter (i.d) to a feeder used as in the case of the steam inhalation. The generated aerosol was pumped through tubing into the round perspex feeder from where it flowed either into the lung via the tracheal cannula or to the outlet tubing which was immersed in 1ml of water to trap the contents of the mist. After the equilibration period (t = 20 min), the pneumotachometer was removed from the thorax chamber, and therefore, lung function monitoring stopped. The lungs were nebulized for three minutes under negative ventilation either with saline, luteolin or extract. While this was done, the pneumotachometer was immediately re-installed to allow the monitor of lung function for 30 minutes post nebulization.

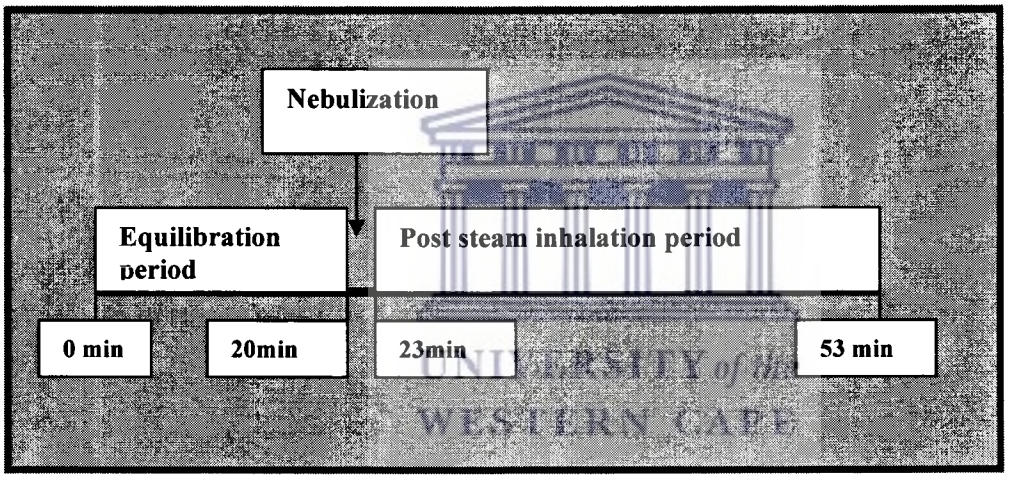


Figure 13: Schematic diagram showing the protocol for nebulization study design. Lungs were allowed to stabilize and equilibrate for 20 minutes (0-20 min), and then nebulized with either saline or *Artemisia afra* aqueous extract or luteolin. Thereafter lung function was monitored for 30 minutes (23 -53 min) after start of each experiment.

The following protocol was used:

(a) **Control group**: The lungs were nebulized for three minutes with 4ml of saline.

(b) **Luteolin group:** The lungs were nebulized with 4ml saline containing 250µg luteolin per ml of saline for three minutes

(c) **Aqueous extract group**: The lungs were nebulized for three minutes with 4 ml saline containing 100mg *Artemisia afra* aqueous extract per ml of saline.

During the nebulization, the outlet of the nebulizer feeder was immersed in 1 ml of distilled water to trap any of the aerosolized compounds. These one milliliter samples were then analyzed via HPLC to identify and quantify the amount of luteolin (if any) trapped during the nebulization period. This was done as explained previously.

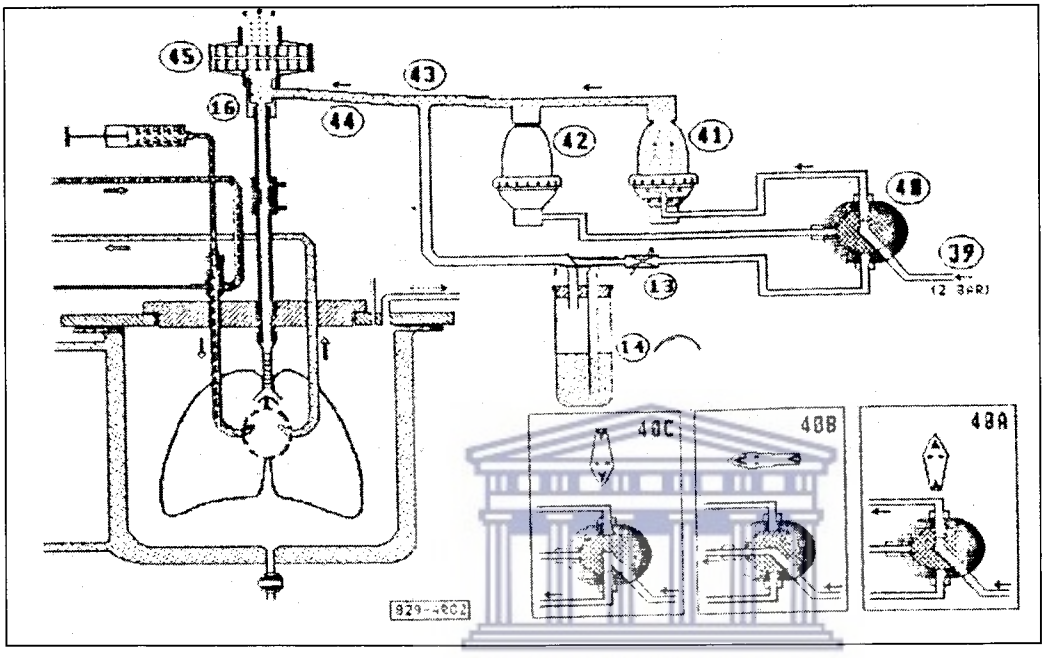


Figure 14: The schematic diagram showing the administration of compounds via a jet nebulizer into the isolated perfused lung.

4.6.4.3 Study protocol three: Disposition and effect of IV administered luteolin in IPL

Firstly, a suitable HPLC assay was required for the determination of the disposition profile of intravenously administered luteolin in IPL. The assay was similarly designed, developed and validated as for the plant materials. In fact, the analytical standard solutions which were prepared by spiking blank perfusate with internal standard (i.e. 10µg in each sample) and luteolin such that the concentration range of 2.5 to15 µg/ml were the only differences. These perfusate analytical standard solutions were used to validate the assay using the same procedures and the same HPLC conditions described in section 4.4.1.2.

added to each sample. The latter mixture was heated in a water bath at 80°C for 40 minutes, cooled at room temperature, and only then extracted with 5ml of ethyl acetate. In all samples, the ethyl acetate extracts were evaporated to dryness under a stream of nitrogen gas, the residue re-dissolved into 500µl of HPLC mobile phase, and 100µl of the solution injected into HPLC column.

4.6.5 Post experiments procedure

At the end of each experiment, the lungs were assessed for edema formation and this was done by visual inspection of any signs of edema such as presence of foamy liquid emerging from trachea, or presence of translucent spots. Thereafter, the lung was separated from the trachea and the heart before being weighed on an electronic balance. The lungs were then preserved in 10% formalin in a glass container for further use, if required.

4.7 Data analysis

To determine the changes in lung mechanics after treatment with the different plant compounds, the following parameters were noted at 10 second intervals: tidal volume, lung compliance and resistance using the PULMODYN™ Software in the IPL. This data (collected in Dbase) was transferred to Microsoft excel ™ to calculate the mean values at each specified time interval. The mean values were then transferred to Graphpad Prism ™ for the plotting of graphs and statistical analysis. The mean values were subjected to two-way ANOVA analysis followed by the Bonferroni post test or *t*-test analysis and the level of significance for differences was divided into four categories namely:

- P>0.05: Not significant (NS)
- P<0.05: Significant (*)
- P<0.01: Very significant (**)
- P<0.001: Highly significant (***)

To obtain the levels of luteolin in the effluent perfusate and the plant material, the luteolin and morin peak heights were obtained from the HPLC 32-Karat ™ software

package generated data, and the luteolin to morin peak height ratios calculated using Excel ™. The concentration vs. ratios data were then subjected to linear regression analysis, and used for construction of the standard curve and the interpolation of the luteolin concentration from the same standard curve using Graphpad Prism ™ software.

CHAPTER FIVE
RESULTS AND DISCUSSION

5.1 Introduction

This chapter reports the results obtained during, (1) the preparation of the *A. afra* material and subsequent preparation of the plant samples for HPLC analysis, (2) the development and validation of a suitable HPLC assay for the analysis of luteolin, (3) the determination of the luteolin levels contained in the plant material, (4) the establishment of the effects of the traditionally prepared steam inhalation (5) nebulized extract and luteolin on lung function, and (6) the determination of the disposition profile and lung effect of the intravenously administered luteolin.

5.2 Collection, identification and preparation of plant material

After the collection of *Artemisia afra* from Montague gardens, a botanist from the University of the Western Cape herbarium identified the plant, and a voucher specimen (6735) was deposited. Upon drying for 72 hours in a well ventilated oven at 30°C, the weight of the plant leaves decreased by 22.07% from 2496.6g (wet) to 1945.4g (dry leaves). In the literature, extraction of flavonoids from plant material is normally done with organic solvents such as methanol. However in this study, water was used to mimic the traditional way of preparing *A. afra* for medicinal purposes, 100g of dried leaves of *A. afra* were extracted with 1000ml of distilled boiling water for 30 minutes. After freeze-drying the resulting infusion, the yield of dried extract obtained was 24.7%. Figure 16 shows pictures of the dried leaves (a) and aqueous extract (b). While the dried *A. afra* leaves were dark green, the dried aqueous extract was brownish, flaky and very hydroscopic, but both materials retained the characteristic odor of the plant.

(a)

(b)

Figure 16: The dried leaves (a) and aqueous extract (b) of *Artemisia afra*

5.3 Quantification of luteolin in the plant material

5.3.1 Development of HPLC assay

To quantify the luteolin levels in both hydrolyzed and un-hydrolyzed (i.e. aglycone) *Artemisia afra* aqueous extract and crude dried leaves a suitable internal standard (IS) was required. Several compounds including p-coumaric acid, mefenamic acid, morin, cinnamic acid and salicylic acid were all tested for suitability as internal standard. In the end, morin was selected as internal standard because it is (1) also a flavonoid, (2) absent in *A. afra*, (3) extractable in ethyl acetate, (4) absorbs at 349 nm, and (5) had a chromatographic retention time that did not interfere with other peaks in the chromatograms of the samples.

Different HPLC conditions were tested for the separation of the luteolin peak from that of other compounds found in the chromatograms of both the aqueous extract and dried leaves of *Artemisia afra*. Firstly, a Silica C-18 (250 x 4.6mm, 5µm), Luna ® C-18 (150 x 4.60mm, 5µm), or Luna® C-18 (250 x 4.6mm, 5µm), and a C-8 (250 x 4.60mm, 5µm) analytical columns were used. Different mobile phases consisting of either methanol or acetonitrile with a mixture of acetic acid or formic acid in water or phosphate buffer at different concentrations to control the pH and suppression of tailoring were also tested. The best separation of *Artemisia afra* plant material was obtained when a mixture of acetonitrile (30%) and phosphate buffer (100mM, pH 2) (70%) was pumped isocratically through a Luna ® C-18 (250 x 4.6mm, 5um) at a flow rate of 1ml/min. Under these conditions, the retention time for morin and luteolin were 10.903 ± 0.0734 *(n=6)* and 14.430 ± 0.298 *(n=6)* minutes, respectively. The luteolin peaks were easily detected at the wavelength of 349 nm.

5.3.2 Validation of the HPLC assay.

The HPLC assay for the analysis of the luteolin levels in the *Artemisia afra* aqueous extract and crude dried leaves was validated for specificity, separation, linearity, low limit of detection, and inter- and intra-day variability. The results obtained are summarized in table 2 and figure 17.

Table 2: Validation results obtained for luteolin quantification in aqueous extract and crude dried leaves of *Artemisia afra* using HPLC analysis.

Slope	0.02526 ± 0.00065
Intercept	0.07150 ± 0.01174
Correlation coefficient (r^2)	0.9916
Intra-day variability (CV %)	0.474%
Inter-day variability (CV %)	1.926%
Low limit of detection	70ng

The developed HPLC assay was fairly robust, linear over the concentration range of 5 to 30 µg of luteolin/ml solution (shown in fig 17) with a correlation coefficient (r^2) of 0.9916. The assay had high assay reproducibility (i.e. CV below 3%), was quite

sensitive with a LLQ of 70ng on column, and was, therefore deemed suitable for the study.

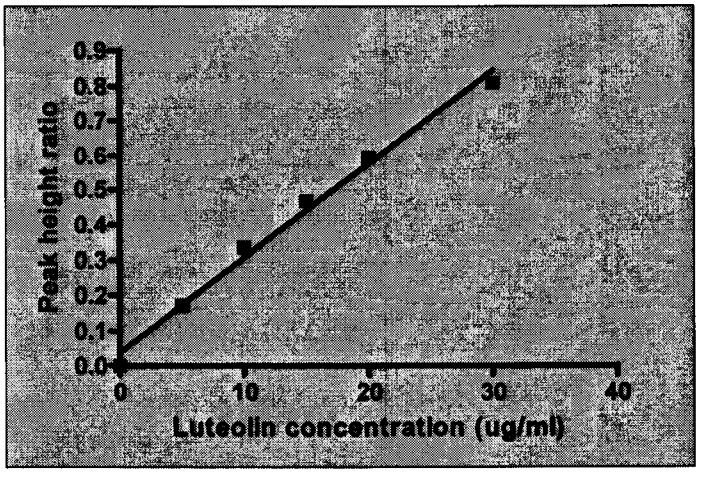


Figure 17: Standard curve for determination of luteolin levels from the aqueous extract & dried leaves of *Artemisia afra*. Samples of luteolin were subjected to methanol-acid treatment, extracted with ethyl acetate, separated by HPLC and detected by UV absorption at 349nm. Each data point represents mean (± SEM) of 6 replications.

5.3.3 Identification and quantification of luteolin in the plant material

Both un- and hydrolyzed forms of the aqueous extract and dried leaves of *A. afra* were analyzed by HPLC assay. Examples of representative chromatograms obtained are given in figures 18 to 21 below. Symmetrical peaks were found for both morin (i.e. the internal standard) and luteolin, with retention times of 10.903 ± 0.0734 and 14.430 ± 0.298 minutes (*n=6*), respectively. While the hydrolyzed plant extract and leaves (figure 18 and 20), and the un-hydrolyzed plant materials (not shown) had no peak at the retention time for morin, each contained easily quantifiable amounts of luteolin. Additional peaks with much shorter retention times than luteolin and morin were evident in the chromatograms, with the peaks from the solutions of the hydrolyzed extract being more prominent than that of the hydrolyzed leaves.

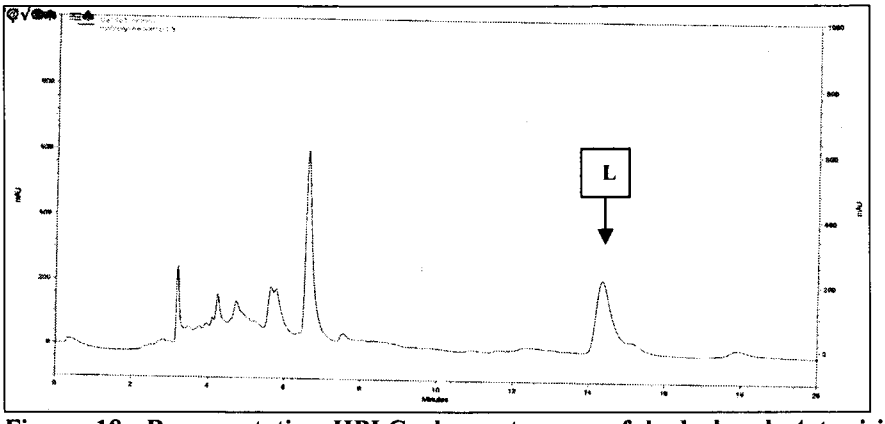


Figure 18: Representative HPLC chromatogram of hydrolyzed *Artemisia afra* leaves without (IS). 100 mg of the dried leaves were subjected to acid hydrolysis, extracted with ethyl acetate, separated by HPLC and detected by UV absorption at 349nm. The retention time of luteolin peak (L) was 14.45min.

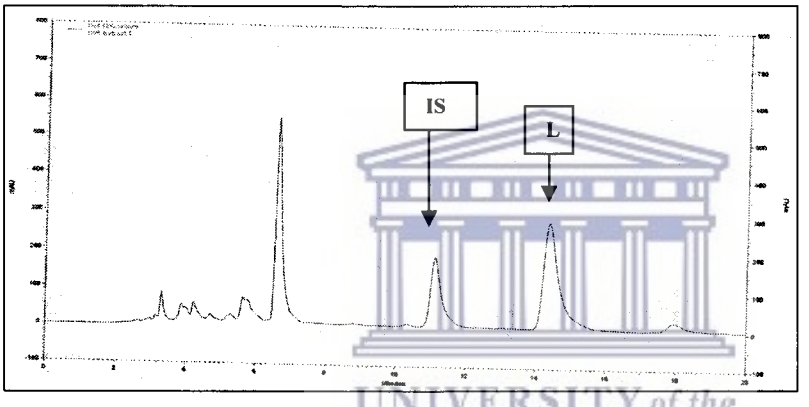


Figure 19: Representative HPLC chromatogram of hydrolyzed *Artemisia afra* crude leaves with morin (IS). 100mg of the dried leaves were subjected to acid hydrolysis, extracted with ethyl acetate, separated by HPLC and detected by UV absorption at 349nm. The retention time of (IS) and luteolin (L) peaks were 10.9 and 14.5 min, respectively.

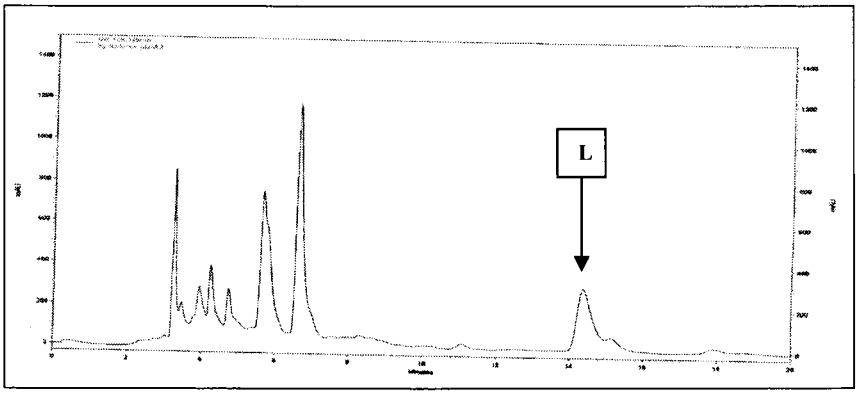


Figure 20: Representative chromatogram of hydrolyzed *Artemisia afra* extract without morin (IS). 25 mg of the extract was treated with HCL, extracted with ethyl acetate, separated by HPLC and detected by UV absorption at 349nm. The retention time of luteolin peak (L) was 14.33 min.

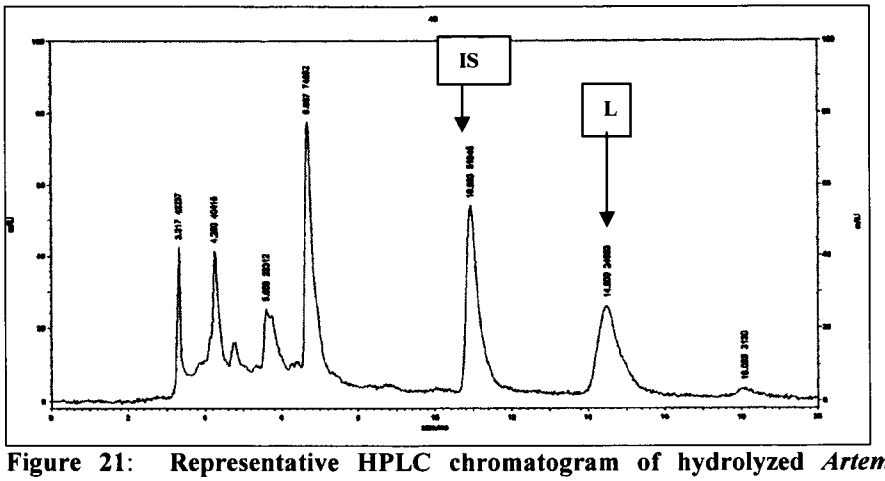


Figure 21: Representative HPLC chromatogram of hydrolyzed *Artemisia afra* aqueous extract with morin (IS). 25 mg of the extract was extracted with ethyl acetate, separated by HPLC and detected by UV absorption at 349nm. The retention time of (IS) and luteolin (L) peaks were 11.05 and 14.56 min, respectively.

The actual levels of luteolin found in the different plant materials are given in table 3.

Table 3: Concentration (µg/25mg) of luteolin obtained in plant extract and dried leaves. Aliquots of plant material were subjected to either acid or no acid treatment, extracted with ethyl acetate and analyzed with HPLC using UV detection. Each data point represents average (± SEM) of 6 replications

***Artemisia afra* material**	**Luteolin concentration (µg/25mg) ± SEM**
Hydrolyzed aqueous extract	49. 554 ± 0.4021
Un-hydrolyzed aqueous extract	25. 818 ± 0.1289
Hydrolyzed dried leaves	15. 939 ± 1.1829
Un-hydrolyzed dried leaves	12. 419 ± 0.257

Generally, the traditionally prepared aqueous extract had higher (> 2 fold) content of luteolin (per 25mg plant material) of which approximately 50% was in acid-hydrolysable, presumably glycoside form, while the leaves tended to contain less luteolin in hydrolysable form, suggesting that the extraction process perhaps concentrated the plant constituents as previously reported (Markam, 1982). In fact, luteolin levels could possibly be even higher in the plant materials than that obtained because the water used in this study is not the best of solvents for the extraction of flavonoids (Muganga, 2004). Overall, the results confirmed the presence of appreciable amounts luteolin in the *A. afra* plant material, amounts that were comparable to those found by other researchers (Muganga, 2004; Waithaka, 2004),

clearly indicating that the leaves and extracts contained reasonable amounts of luteolin which could be expected to have an effect on the lung.

5.4 The effects of traditionally prepared steam inhalation and nebulized extract of *A. afra*, and luteolin on lung function.

The isolated perfused lung (IPL) used in this study has been previously used before to evaluate effect of inhaled compounds on lung function. In untreated rat lungs, the tidal volume (TV), lung compliance (CL) and resistance (RL) normally range between (0 -3ml), (2 - 4 ml/cmH_2O) and (2 - 5 cmH_2O.s/ml), respectively. However, for this study, the IPL was set at higher values in order to evaluate even a small change that might occur in these lung parameters. In the end, the present the study was conducted using three study designs, and the results obtained are presented and discussed below as they were derived with the various study designs.

5.4.1 The effect of traditionally prepared *Artemisia afra* steam inhalation on lung function (Study design 1)

Traditionally, water is used to boil dry *Artemisia afra* leaves for inhalation of the resultant steam. However, in this study, saline was used instead to minimize lung edema caused by changes in osmolality. Further, saline was used for the validation of the IPL and also as one of the controls in the study.

5.4.1.1 Effect *Artemisia afra* steam inhalation on tidal volume

The results depicting the effect which the inhalation of steam produced by boiling saline (i.e. the vehicle), and 1g and 5g quantities of dry *Artemisia afra* leaves in 100ml saline, had on tidal volume, are summarized in figure 22 and tables 4. In these experiments, lungs were equilibrated for 20 minutes, exposed to the steam inhalation for 3 minutes and the respiratory mechanics monitored for 30 minutes (i.e. post steam inhalation period).

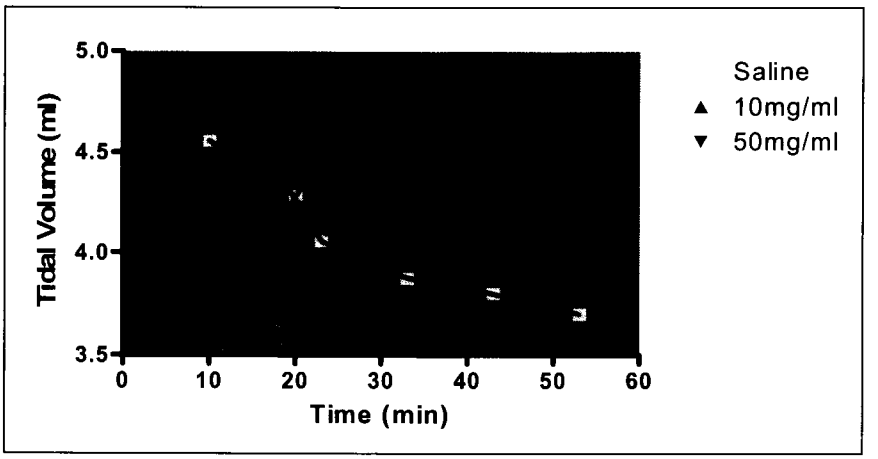


Figure 22: Graph showing effect of steam inhalation of saline and *Artemisia afra* (10 & 50mg/ml) on tidal volume. The lungs *(n=6* for each*)* were allowed to stabilize and equilibrate for 20 minutes and then steamed (IN) for three minutes thereafter the tidal volume was monitored for 30 minutes. Results of *t-test* analysis for post steam inhalation times vs. 20min time point (* p<0.05), (p<0.01), (***p<0.001.**

During the equilibration period, the tidal volume decreased at a steady rate of 0.0303 ± 0.0034ml/min for the saline group of lungs (*n=4* lungs) (i.e. green line in figure 22 above). Immediately after exposure to steamed saline (i.e. at 23 min), the tidal volume had decreased to 4.058 ± 0.0968ml (*n=4*) which was significantly (*p<0.001*) different from the pre-inhalation (i.e. at 20 min) volume of 4.285 ± 0.0529ml (*n=4*); and continued to decrease at a steady rate of 0.0132 ± 0.0029ml/min over the following 30 minutes. The post inhalation rate of decrease was not significantly different (*p>0.05*) from the pre-inhalation rate, indicating that saline only had a brief insignificant negative effect on tidal volume.

In the lungs (*n=6*) exposed to steam produced from the 10 mg/ml *Artemisia afra* in saline solution, the tidal volume (TV) decreased at a rate of 0.0306 ± 0.0059ml/min in the equilibration period, a rate similar to that for the saline group, but immediately (t = 23 min) post steam inhalation of the plant the TV had significantly (*p<0.001*) increased by 3.56 % to 4.340 ± 0.140ml from the pre-inhalation volume of 4.191 ± 0.129ml. After this initial improvement, there was, however, a decrease in the TV at a steady rate of 0.0144 ± 0.0042ml/min (*n=6*), a rate which was similar to the post inhalation rate of decrease in the saline group of lungs. In fact 7 minutes post inhalation, the TV was still at the same level as the pre-inhalation values, indicating

that the inhalation of *A. afra* low dose solution for a short period of time (i.e. 3 min) caused a significant increase in TV, which, however, also lasted only for a short period of time. The inhalation with the higher 50mg/ml *A. afra* dose, caused a post inhalation improvement that was even higher, viz. 7.59% above the pre-inhalation value (i.e. from 4.223 ± 0.120ml to 4.543 ± 0.0879ml ($n=6$) post steam inhalation). In this case the improved TV was maintained for a longer period (i.e. about 16 minutes) before it started to decrease at a similar steady rate as that for saline group of lungs. This indicated that the *A. afra* steam inhalation produced a dose-dependent increase in tidal volume that may be long-lasting at higher plant doses.

Table 4: Effect the steam inhalation of saline and *Artemisia afra* (10 or 50mg/ml) had on tidal volume. Lungs were allowed to equilibrate for twenty minutes (0-20 min) then exposed for three minutes (20-23 min) to the steam produced by boiling 100ml saline (control) or either 1g or 5g *A. afra* dried leaves in 100ml saline. Tidal volume was then monitored for 30 minutes (23-53 min) post steam inhalation and compared to that obtained at 20 minutes before inhalation

Time (min)	Tidal volume (ml) Mean SEM	% Change (vs. 20 min)	P value	Level of Significance
Saline steam				
10	4.558 ±0.0487			
20	4.285±0.0529			
23	4.058±0.0968	↓ 5.29	P<0.001	***
33	3.875±0.0901	↓ 9.57	P<0.001	***
43	3.801±0.0711	↓ 11.29	P<0.001	***
53	3.702±0.0828	↓ 13.61	P<0.001	***
10mg/ml *A. afra* steam				
10	4.496 ±0.147			
20	4.191 ±0.129			
23	4.340 ±0.140	↑ 3.56	P<0.001	***
33	4.106 ±0.121	↓ 2.03	P<0.01	**
43	3.985 ±0.122	↓ 4.92	P<0.001	***
53	3.864 ±0.136	↓ 7.80	P<0.001	***
50mg/ml *A. afra* steam				
10	4.354±0.1352			
20	4.223±0.1201			
23	4.543±0.0879	↑ 7.590	P<0.001	***
33	4.403±0.1112	↑ 4.275	P<0.01	**
43	3.999±0.0671	↓ 5.284	P<0.01	**
53	3.909±0.0807	↓7.415	P<0.001	***

5.4.1.2 Effect *Artemisia afra* steam inhalation on lung compliance

The results depicting the effect of the inhalation of steam produced by boiling saline (i.e., the vehicle), and 1g and 5g quantities of dry *Artemisia afra* leaves each in 100ml saline, on lung compliance are summarized in figure 23 and table 5. In these experiments lungs were equilibrated for 20 minutes, exposed to the steam inhalation for 3 minutes and the respiratory mechanics monitored for 30 minutes.

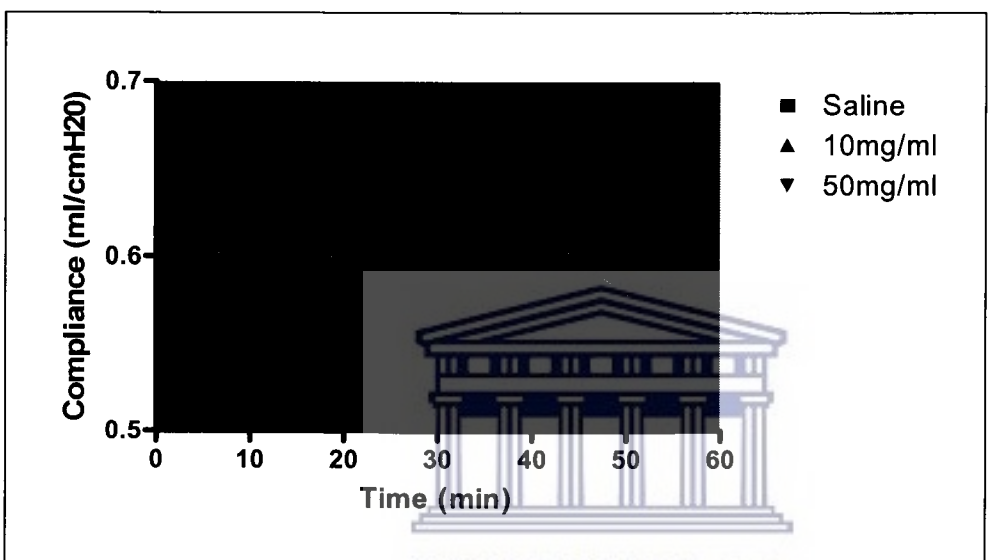


Figure 23: Graph showing effect of steam inhalation of saline and *Artemisia afra* (10 & 50mg/ml) on lung compliance. The lungs were allowed to stabilize and equilibrate for 20 minutes and then steamed (IN) for three minutes and compliance monitored for 30 minutes afterwards. Results of *t-test* analysis for post steam inhalation times vs. 20min time point. (* p<0.05), (p<0.01), (***p<0.001)**

During the equilibration period, the lung compliance in the saline steam group of lungs (*n=4* lungs) increased at a steady rate of 0.00118 ± 0.00149 ml/cmH_2O per min. Immediately after exposure to the steamed saline (i.e. at 23 min) the lung compliance had increased to 0.606 ± 0.0058 ml/cmH_2O (*n=4*) which was not significantly (*p>0.05*) different from the pre-inhalation (i.e. at 20min) compliance of 0.605±0.0081 ml/cmH_2O (*n=4*). Only at around 30 minutes (i.e. 10 min post steam inhalation) did the lung compliance begin to decrease at a slow rate of 0.00057 ± 0.00061 ml/cmH_2O per min. This data probably suggest that saline steam inhalation had no effect on lung compliance.

Table 5: Effect of steam inhalation of saline and *Artemisia afra* (10 or 50mg/ml) on lung compliance. Lungs were allowed to equilibrate for twenty minutes (0-20 min) then exposed for three minutes (20-23 min) to steam produced by boiling 100ml saline (control) or either 1g or 5g *A. afra* dried leaves in 100ml saline. Lung compliance was then monitored for 30 minutes (23-53 min) post steam inhalation and compared to that obtained at 20 minutes pre-inhalation

Time (min)	Lung compliance (cmH_2O/ml) *Mean ± SEM*	% Change (vs. 20 min)	P value	Level of Significance
Nebulized saline				
10	0.602±0.0190			
20	0.605±0.0081			
23	0.606±0.0058	↑ 0.17	P > 0.05	NS
33	0.610±0.0104	↑ 0.83	P > 0.05	NS
43	0.597±0.0200	↓ 1.32	P > 0.05	NS
53	0.596±0.0138	↓ 1.49	P > 0.05	NS
10mg/ml *A. afra* steam				
10	0.619±0.0169			
20	0.615±0.0223			
23	0.636±0.0191	↑ 3.42	P < 0.05	*
33	0.631±0.0121	↑ 2.60	P < 0.05	*
43	0.622±0.0198	↓ 1.14	P >0.05	NS
53	0.583±0.0196	↓ 5.20	P<0.01	**
50mg/ml *A. afra* steam				
10	0.606±0.0126			
20	0.587±0.0264			
23	0.656±0.0269	↑ 11.76	P<0.001	***
33	0.646±0.0166	↑ 10.05	P<0.001	***
43	0.662±0.0169	↑ 12.78	P<0.001	***
53	0.665±0.0183	↑ 13.29	P<0.001	***

In the lungs (*n=6*) exposed to steam produced from the 10 mg/ml *Artemisia afra* in saline, there was in the equilibration period, a slight decrease in the lung compliance over time of 0.00053 ± 0.00105 ml/cmH_2O per min. Immediately (i.e. t = 23 min) post inhalation of the plant, the CL, however, increased significantly *($p<0.05$)* by 3.42 % to 0.6360 ± 0.0191 ml/cmH_2O from the pre-inhalation compliance of 0.6150 ± 0.0223 ml/cmH_2O. This improvement lasted for at least 20 minutes before slow reversal of lung compliance became apparent. The inhalation of low dose *A. afra* solution for a short period of time (i.e. 3 min) thus caused a significant increase in CL which, however, only lasted for a short period of time. Inhalation with the higher 50mg/ml *A. afra* dose, however, effected a significantly (*$p<0.001$*) bigger immediate improvement of 11.76% in CL (i.e. from the pre-inhalation value of 0.587 ± 0.0264

ml/cmH_2O to 0.656 ± 0.0269 ml/cmH_2O post steam inhalation). In this case, the improved CL was evident for the entire post inhalation monitoring period. Collectively, these results suggested that *A. afra* steam inhalation produced a dose-dependent, long-lasting improvement in lung compliance.

5.4.1.3 The effect *Artemisia afra* steam inhalation on lung resistance

The results depicting the effect the inhalation of steam (produced by boiling saline) , and 1g and 5g quantities of dry *Artemisia afra* leaves in 100ml saline, had on lung resistance are summarized in figure 24 and table 6. In these experiments, the isolated lungs were equilibrated for 20 minutes, exposed to the steam inhalation for 3 minutes and the respiratory mechanics monitored for 30 minutes.

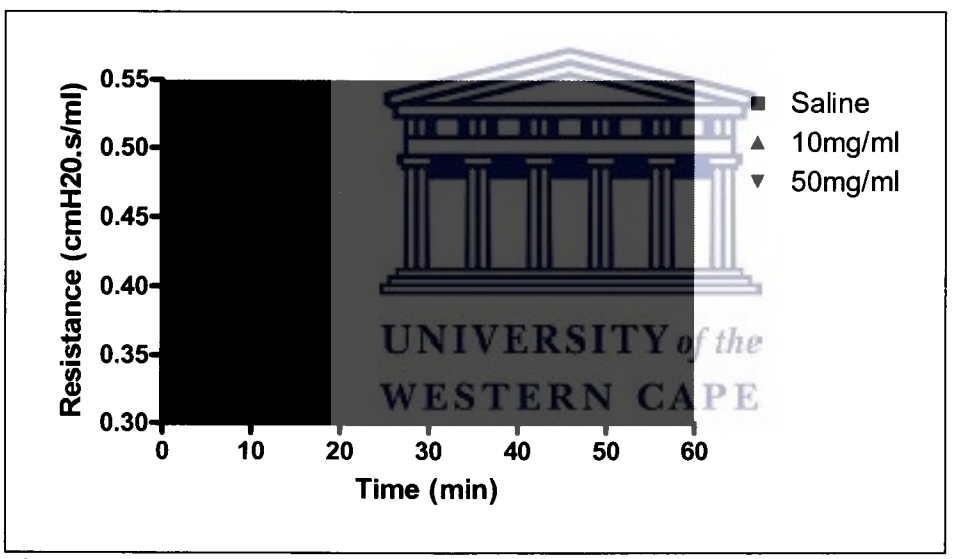


Figure 24: Graph showing effect of steam inhalation of saline and *Artemisia afra* (10 & 50mg/ml) on lung resistance. The lungs *(n=6* for each*)* were allowed to stabilize and equilibrate for 20 minutes and then steamed (IN) for three minutes and resistance monitored for 30 minutes afterwards. Results of *t-test* analysis for post steam inhalation times vs. 20min time point. (* p<0.05), (p<0.01), (***p<0.001)**

During the equilibration period, the lung resistance in the saline steam group of lungs (*n=4* lungs) increased at a steady rate of 0.00333 ± 0.00488 cmH_2O.s/ml per min. Immediately after exposure to the steamed saline (i.e. at 23 min), the resistance had increased to 0.423 ± 0.0268 cmH_2O.s/ml (*n=4*), a level which was significantly (*p<0.05*) different from the pre-inhalation (i.e. at 20min) resistance of 0.397 ± 0.0233 cmH_2O.s/ml. This increased level was maintained for the whole post inhalation

period, suggesting that saline steam inhalation might have a negative effect on lung resistance.

Table 6: Effect of steam inhalation of saline and *Artemisia afra* (10 or 50mg/ml) on lung resistance. Lungs were allowed to equilibrate for twenty minutes (0-20 min) then exposed for three minutes (20-23 min) to steam produced by boiling 100ml saline (control) or either 1g or 5g *A. afra* dried leaves in 100ml saline. Lung resistance was then monitored for 30 minutes (23-53 min) post steam inhalation and compared to that obtained at 20 minutes before inhalation.

Time (min)	Lung resistance ($cmH_2O.s/ml$) *Mean ± SEM*	% Change (vs. 20 min)	P value	Level of Significance
Saline				
10	0.364±0.0147			
20	0.397±0.0233			
23	0.423±0.0268	↑ 6.55	P < 0.05	*
33	0.426±0.0212	↑ 7.31	P < 0.05	*
43	0.398±0.0136	↑ 0.25	P > 0.05	NS
53	0.414±0.0196	↑ 4.28	P >0.05	NS
10mg/ml *A. afra* steam				
10	0.372±0.0232			
20	0.391±0.0183			
23	0.353±0.0268	↓ 9.72	P< 0.05	*
33	0.413±0.0304	↑ 5.63	P> 0.05	NS
43	0.426±0.0263	↑ 8.95	P> 0.05	NS
53	0.446±0.0426	↑ 14.07	P<0.01	**
50mg/ml *A. afra* steam				
10	0.364±0.0153			
20	0.364±0.0039			
23	0.324±0.0071	↓ 10.98	P< 0.05	*
33	0.352±0.0087	↓ 3.30	P>0.05	NS
43	0.444±0.0242	↑ 21.98	P<0.01	**
53	0.500±0.0262	↑ 37.36	P<0.001	***

In the lungs (*n=6*) exposed to the steam produced from the 10 mg/ml *Artemisia afra* in saline, there was a slow steady increase in the lung resistance during the equilibration period (at a rate of 0.00203 ± 0.00124 $cmH_2O.s/ml$ per min), followed by a significant *($p<0.05$)* 9.72 % decrease in resistance immediately (i.e. t=23 min) after inhalation of the plant extract (i.e. from 0.391 ± 0.0183 $cmH_2O.s/ml$ to 0.353 ± 0.0268 $cmH_2O.s/ml$). After this decrease, the RL however, increased at a steady rate of 0.00311 ± 0.00148 $cmH_2O.s/ml$ per min (*n=6*), which was similar to the pre-inhalation rate of increase in the same group of lungs. In fact, 6 minutes post inhalation the RL was still at the same levels as the pre-inhalation values. The

inhalation of a low dose *A. afra* solution for a short period of time (i.e. 3 min) thus caused a significant decrease in RL which was, however, also short lasting. With the higher 50mg/ml *A. afra* dose, the post inhalation improvement was even higher, viz. 10.98% (from the pre-inhalation value of 0.364 ± 0.0039 $cmH_2O.s/ml$ to 0.324 ± 0.0071 $cmH_2O.s/ml$ post steam inhalation). Again after the initial decrease in the RL there was a steady increase (at a rate of 0.00584 ± 0.00096 $cmH_2O.s/ml$ per minute, (*n=6*) over the 30 minutes post inhalation period. This rate of increase was higher than that for the post inhalation rate of decrease induced by the 10mg/ml dose. In fact 13 minutes post inhalation, the RL level in the high dose lungs was still comparable to the pre-inhalation values. Collectively, this data suggest that the inhalation of *A. afra* produced a dose-dependent lowering of lung resistance which was, however, not sustained.

In summary, the above findings indicated that the *A. afra* steam inhalation had a dose-dependent effect on lung function (i.e. confirmed by increased TV and CL, and decreased RL) that may be long-lasting at higher plant doses. Firstly, the effects observed with inhalation of saline steam appeared to be negligible and, therefore, the saline contribution to the pulmonary effects seen with *A. afra* steam inhalation was minimal. Secondly, the effect produced by *A. afra* steam inhalation on lung function was similar to that obtained with known bronchodilators (Svens and Rvrfeldt, 2001) indicating that the plant perhaps contained compounds that had bronchodilator activities. In fact luteolin, a major flavonoid contained in *A. a*fra had been associated with bronchodilator activities before (Das *et al.*, 2003), and luteolin could therefore, be involved in the pulmonary effects produced by the steam inhalation of the plant. However, luteolin was not detected in the one milliliter water samples used to trap vaporized compounds produced in the line (post the lung) during the three-minute administration of the steam inhalation. Perhaps, the concentration of the luteolin in the steam inhalation was below the low limit of detection of the HPLC assay used in this study. Alternatively, the effects produced by the steam inhalation of *A. afra* might have been caused by other plant constituents such as the volatile oils (i.e. thujone) (Roberts, 1990). Clearly, *A. afra* steam inhalation affected lung function, but to

establish whether luteolin or other compounds were responsible for this effects, testing whether inhaled luteolin given by itself had an effect on lung function was needed. Therefore, determination of the effect of nebulized luteolin was necessary.

5.5 Effect of nebulized luteolin (250µg/ml) and *Artemisia afra* aqueous extract on lung mechanics (Study design two)

One of the aims of this project was to determine whether the nebulized freeze-dried aqueous extract of *A. afra* could affect lung mechanics, and whether luteolin could contribute to such effects. To realize these objectives, ultrasonic and jet nebulizers were first tested as possible devices for generating and delivering the aerosolized plant solutions. In a previous study in our laboratory (Syce and Parkar, 2004) it had been found that pulmonary surfactant had greater effect on lung function when delivered by ultrasonic nebulization. However, in the preliminary experiments in the present study, nebulization of the *A. afra* aqueous extract using the ultrasonic nebulizer caused excessive frothing and not a deliverable aerosol. This occurred even when low concentrations (10mg/ml) of the extract were nebulized and was most likely due to the small solution volumes (>3ml) used in the ultrasonic nebulizer and possible surfactant materials in the extract. With the jet nebulizer, frothing, however, only occurred when high solution volumes (over 4ml) were used. Therefore, the jet nebulizer was chosen as mode of nebulization for the study.

Secondly, saline was used as vehicle of choice because it has previously been used in the IPL system, and also dissolved both the luteolin and the aqueous extract easily. Thirdly, it was found that three minutes was sufficient to nebulize solutions (i.e. 4ml) of saline, luteolin and extract. Fourthly, the effect of the vehicle on lung function was determined before it was then decided to use it as negative control. Lastly, it was decided that highest aqueous extract dose (100mg/ml) that did not froth during preliminary studies would therefore be used to assess the effect on lung parameters, and an equivalent luteolin (250µg/ml) dose was then determined based on levels of luteolin contained in the 100mg of *Artemisia afra* aqueous extract.

5.5.1 The effect of nebulized solutions on tidal volume

The results depicting the effect of the nebulized saline (i.e. the vehicle), luteolin (250µg/ml) and *Artemisia afra* aqueous extract (100mg/ml) had on tidal volume are summarized in figure 25 and table 7. In these experiments lungs were equilibrated for 20 minutes then exposed to the nebulized solutions for 3 minutes and the respiratory mechanics monitored for 30 minutes (post nebulization period).

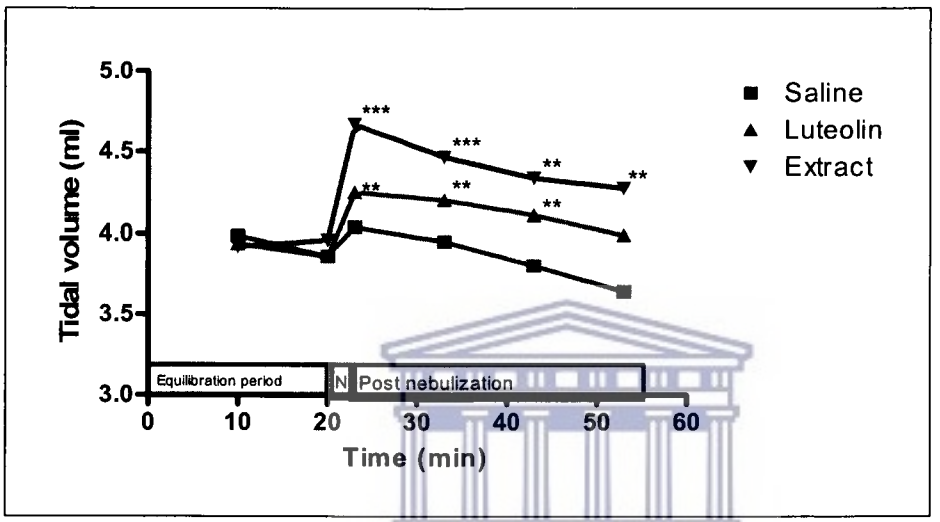


Figure 25: Graph showing the effects of nebulized saline, luteolin (25µg/ml) and *Artemisia afra* extract (100mg/ml) on tidal volume. Lungs were allowed to equilibrate for 20 minutes (0-20 min) and then nebulized (IN) for three minutes (20-23min). Post nebulization tidal volume was monitored for 30 minutes (23-53 min) afterwards. Results of *t-test* analysis for post steam inhalation times vs. 20min time point (* p<0.05), (p<0.01), (***p<0.001)**

During the equilibration period the tidal volume decreased at a steady rate of 0.01258 ± 0.00391ml/min in the nebulized saline group of lungs (*n=4* lungs) (i.e. green line in figure 25). Immediately after exposure to nebulized saline (i.e. at 23 min), the tidal volume had increased insignificantly by 4.64% to 4.041 ± 0.08025ml from the pre-inhalation (i.e. at 20min) volume of 3.862 ± 0.11245ml (*n=4*). However, after this initial improvement, the TV continued to decrease at a steady rate (i.e. 0.0133 ± 0.00144 ml/min) similar to that obtained during pre-nebulization period, indicating that the nebulized saline caused a small insignificant increase in TV that was short lasting.

Table 7: The effect that nebulized saline, luteolin (250µg/ml) and *Artemisia afra* (100mg/ml) had on the tidal volume. Lungs were allowed to equilibrate for twenty minutes (0-20 min) then nebulized for three minutes (20-23 min) with saline (control), luteolin (250µg/ml) or *Artemisia afra* (100mg/ml. Tidal volume was then monitored for 30 minutes (23-53 min) post steam inhalation and compared to that obtained at 20 minutes before inhalation

Time (min)	Tidal volume (ml) *Mean ±SEM*	% Change (vs. 20 min)	P value	Level of Significance
Nebulized saline				
10	3.988±0.09369			
20	3.862±0.11245			
23	4.041±0.08025	↑4.64	P > 0.05	NS
33	3.953±0.06236	↑2.36	P > 0.05	NS
43	3.802±0.05943	↓1.55	P > 0.05	NS
53	3.641±0.11217	↓5.72	P > 0.05	NS
Nebulized luteolin (250µg/ml)				
10	3.937±0.0571			
20	3.858±0.0778			
23	4.251±0.1893	↑10.96	P<0.01	**
33	4.205±0.1580	↑8.99	P<0.01	**
43	4.113±0.1695	↑6.61	P<0.01	**
53	3.990±0.1726	↑3.42	P > 0.05	NS
Nebulized *A. afra* extract (100mg/ml)				
10	3.920±0.0835			
20	3.959±0.0546			
23	4.670±0.1563	↑17.96	P<0.001	***
33	4.466±0.1304	↑12.81	P<0.001	***
43	4.339±0.0796	↑9.60	P<0.01	**
53	4.278±0.0862	↑8.06	P<0.01	**

In the lungs (*n=5*) nebulized with luteolin, the tidal volume decreased in the equilibration at a rate of 0.00786 ± 0.00326ml/min, a rate similar to that obtained in the nebulized saline group of lungs. Immediately post nebulization of the luteolin, the TV increased significantly *(p<0.01)* by 10.96% to 4.251 ± 0.1893ml from the pre-inhalation volume of 3.858 ± 0.0778ml. Thereafter, the improved TV was sustained at a steady rate (i.e. 0.00868 ± 0.00463ml/min, *n=5*) for the entire thirty minutes post nebulization period. Nebulized luteolin (250µg/ml) administered for a short period of time (i.e. 3 min) thus caused a long lasting, significant increase in TV. However, with the nebulized *A. afra* aqueous extract the post nebulization improvement was even better, viz. 17.96% to 4.670 ± 0.1563ml from the pre-nebulization volume of 3.959 ± 0.546, indicating that the nebulized *A. afra* extract produced a highly significant, long-lasting increase in tidal volume.

5.5.2 The effect of nebulized solutions on lung compliance

The results depicting the effect of nebulized saline, luteolin and *A. afra* extract on lung compliance are summarized in figure 26 and table 8. In these experiments the lungs were equilibrated for 20 minutes then exposed to the steam inhalation for 3 minutes, and the respiratory mechanics monitored for 30 minutes.

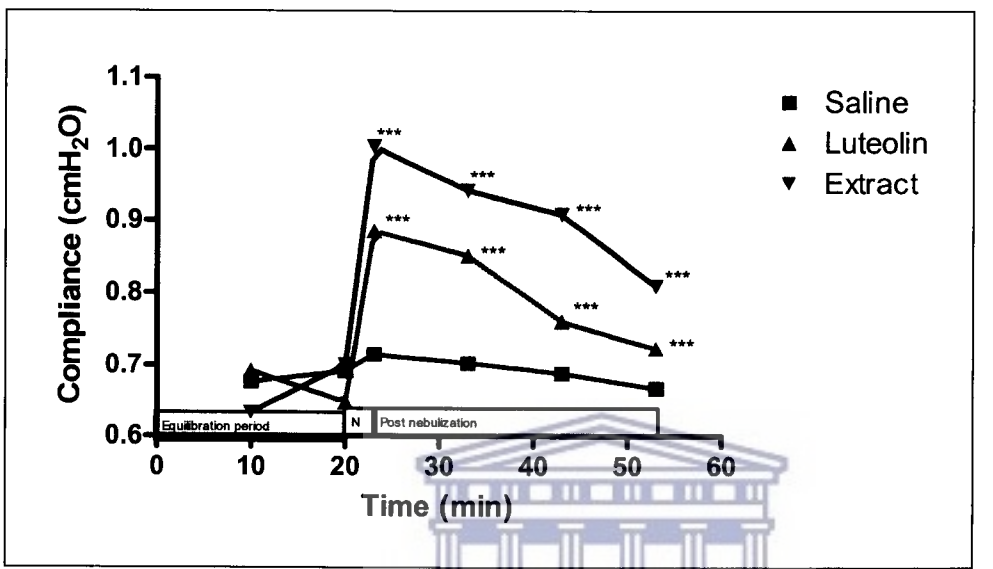


Figure 26: Graph showing the effect of nebulized saline, luteolin (25µg/ml) and *Artemisia afra* extract (100mg/ml) on lung compliance. Lungs were allowed to stabilize and equilibrate for 20 minutes (0-20 min) and then nebulized for three minutes (20-23min) with saline, luteolin or *A. afra* extract (IN). Post nebulization compliance was monitored for 30 minutes (23-53 min) afterwards. Results of *t-test* analysis for post steam inhalation times vs. 20min time point (* p<0.05), (p<0.01) & (***p<0.001)**

During the equilibration period in the nebulized saline group of lungs (*n=4)*, lung compliance increased at a steady rate of 0.00155 ± 0.00128 ml/cmH_2O per minute. Immediately after nebulization with saline (i.e. at 23 min), the lung compliance had increased to 0.715 ± 0.0301 ml/cmH_2O (*n=4*) which was not significantly (*p>0.05*) different from the pre-inhalation (i.e. at 20min) lung compliance of 0.692 ± 0.0315 ml/cmH_2O (*n=4*). Only at around 43 minutes (i.e. 20 min post steam inhalation) did the lung compliance begin to decrease at a slow rate of 0.002 ± 0.0006 ml/cmH_2O per min. This data suggest that nebulized saline has a negligible effect on lung compliance.

Table 8: The effect that nebulized saline, luteolin (250µg/ml) and *Artemisia afra* (100mg/ml) had on lung compliance. Lungs were allowed to equilibrate for twenty minutes (0-20 min) then nebulized for three minutes (20-23 min) with saline (control), luteolin (250µg/ml) or *Artemisia afra* (100mg/ml. Lung compliance was then monitored for 30 minutes (23-53 min) post steam inhalation and compared to that obtained at 20 minutes before inhalation

Time (min)	Lung compliance (cmH_20/ml) *Mean ± SEM*	% Change (vs. 20 min)	P value	Level of Significance
Nebulized saline				
10	0.677±0.0226			
20	0.692±0.0315			
23	0.715±0.0301	↑3.32	P.>0.05	NS
33	0.703±0.0295	↑1.59	P.>0.05	NS
43	0.688±0.0303	↓0.58	P.>0.05	NS
53	0.667±0.0312	↓3.61	P.>0.05	NS
Nebulized luteolin				
10	0.692±0.0202			
20	0.648±0.0121			
23	0.884±0.0187	↑36.42	P<0.001	***
33	0.851±0.0210	↑31.33	P<0.001	***
43	0.760±0.0264	↑17.28	P<0.001	***
53	0.723±0.0420	↑11.57	P<0.001	***
Nebulized *A. afra* extract				
10	0.633±0.0156			
20	0.700±0.0293			
23	1.004±0.0425	↑43.43	P<0.001	***
33	0.941±0.0379	↑34.43	P<0.001	***
43	0.908±0.0462	↑29.71	P<0.001	***
53	0.809±0.0546	↑15.57	P<0.001	***

In the lungs (*n=5*) nebulized with luteolin, the lung compliance decreased at a steady rate of 0.00446 ± 0.00175 ml/cmH_2O per min during the equilibration period (comparable to that found in the saline group). Immediately (i.e. t =23 min) after nebulization with luteolin the CL, however, increased significantly *(p<0.001)* by 36.42% to 0.884 ± 0.0187 ml/cmH_2O from the pre-inhalation compliance of 0.648 ± 0.0121 ml/cmH_2O at 20 minutes. This improvement was sustained for the entire post nebulization monitoring period of 30 minutes. Nebulization with luteolin (250µg/ml) for a short period of time (i.e. 3 min) thus caused a highly significant and sustainable increase in CL. When the lungs were, however, nebulized with *A. afra* extract containing an amount of luteolin equivalent to that used in the luteolin nebulization, the post inhalation improvement was even higher, viz. 43.43% to 1.004 ± 0.0425

ml/cmH_2O from pre-inhalation lung compliance of 0.700 ± 0.0293 ml/cmH_2O, indicating that the nebulized *A. afra* (100mg/ml) administered for a short period of time (3 min) caused a greater improvement in lung compliance that an equivalent dose of luteolin, and strongly suggesting that something additional to luteolin was responsible for the effect of the nebulized *A afra* on lung compliance.

5.5.3 The effect of nebulized solutions on lung resistance

The results depicting the effect of nebulized saline, luteolin and *Artemisia afra* aqueous extract on lung resistance are summarized in figure 27 and table 9. In these experiments the lungs were equilibrated for 20 minutes, exposed to the steam inhalation for 3 minutes and then the respiratory mechanics monitored for 30 minutes

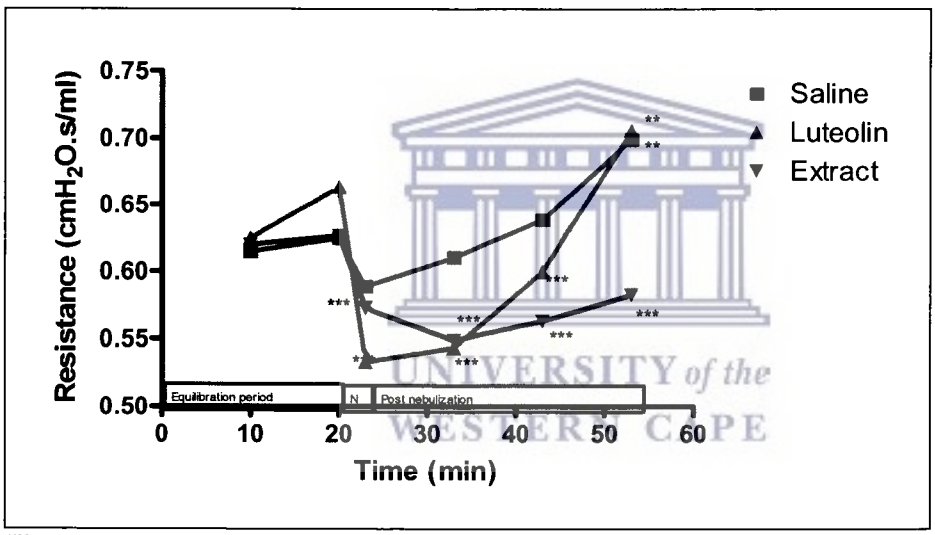


Figure 27: Graph showing effect of nebulized of saline, luteolin (25µg/ml) and *Artemisia afra* extract (100mg/ml) on lung resistance. Lungs were allowed to stabilize and equilibrate for 20 minutes (0-20 min) and then nebulized for three minutes (20-23min) with saline, luteolin or *A. afra* extract (IN). Post nebulization resistance was monitored for 30 minutes (23-53 min) afterwards. Results of *t-test* analysis for post steam inhalation times vs. 20min time point (* p<0.05), (p<0.01), (***p<0.001)**

During the equilibration period in the nebulized saline group of lungs (*n=4* lungs), the lung resistance increased at a slow steady rate of 0.0001 ± 0.00204cmH_2O.s/ml per min. Immediately after the nebulized saline (i.e. at 23 min) the resistance decreased to 0.589 ± 0.0425 cmH_2O.s/ml (*n=4*) which was not significantly (*p>0.05*) different from the pre-nebulization (i.e. resistance of 0.625 ± 0.0294 cmH_2O.s/ml at 20min). This decrease was, however, followed by a steady increase in RL at a rate of

0.00284 ± 0.00056 $cmH_2O.s/ml$ per min (*n=4)* over the 30 minutes post nebulization. Nebulized saline administered for a short period of time (i.e. 3 min) thus caused an initial insignificant decrease in RL which was also short-lasted.

Table 9: The effect the nebulized saline, luteolin (250µg/ml) and *Artemisia afra* (100mg/ml) had on the lung resistance. Lungs were allowed to equilibrate for twenty minutes (0-20 min) then exposed for three minutes (20-23 min) to nebulized saline (control), luteolin (250µg/ml) and *Artemisia afra* (100mg/ml). Lung resistance was then monitored for 30 minutes (23-53 min) post steam inhalation and compared to that obtained at 20 minutes before inhalation

Time (min)	Lung resistance ($cmH_2O.s/ml$) *Mean ± SEM*	% Change (vs. 20 min)	P value	Level of Significance
Nebulized saline				
10	0.615±0.0184			
20	0.625±0.0294			
23	0.589±0.0425	↓5.76	P > 0.05	NS
33	0.610±0.0304	↓2.40	P > 0.05	NS
43	0.639±0.0402	↑2.24	P > 0.05	NS
53	0.699±0.0497	↑11.84	P<0.01	**
Nebulized luteolin				
10	0.624±0.0078			
20	0.664±0.0140			
23	0.533±0.0139	↓19.73	P<0.001	***
33	0.543±0.0128	↓18.22	P<0.001	***
43	0.599±0.0116	↓14.74	P<0.001	***
53	0.705±0.0224	↑6.18	P<0.01	**
Nebulized *A. afra* extract				
10	0.620±0.0137			
20	0.626±0.0132			
23	0.573±0.0381	↓8.47	P<0.001	***
33	0.549±0.0239	↓12.30	P<0.001	***
43	0.563±0.0254	↓10.06	P<0.001	***
53	0.582±0.0165	↓7.03	P<0.001	***

In the lungs (*n=6*) nebulized with luteolin, the lung resistance increased at a rate of 0.0039 ± 0.00186 $cmH_2O.s/ml$ per min in the 20 min equilibration period, but immediately after (i.e. t = 23 min) post luteolin nebulization, the RL had significantly *(p<0.001)* decreased by 19.73% to 0.533 ± 0.0139 $cmH_2O.s/ml$ from the pre-nebulization resistance of 0.664 ± 0.0140 $cmH_2O.s/ml$. However, thereafter the RL again increased at a steady rate of 0.00574 ± 0.0009 $cmH_2O.s/ml$ per min (*n=6*) similar to the post-nebulization rate of increase in the saline group of lungs. In fact, 26 minutes post-nebulization, the RL was still similar to the pre-nebulization values

indicating that nebulization with luteolin for a short period of time (i.e. 3 min) caused an initial, significant decrease in RL which lasted for quite some time (i.e. at least 26 min) before being reversed. On the other hand, for the nebulized *A. afra* extract the post nebulization decrease in RL was smaller, viz. 8.47% (i.e. from pre-inhalation value of 0.626 ± 0.0132cmH_2O.s/ml to 0.573 ±0.0381cmH_2O.s/ml post nebulization). This decrease was, however, sustained during the entire post-nebulization period indicating that nebulizing with the *A. afra* extract, even for a short period of time (i.e. 3 min), produced a highly significant and sustainable decrease in the lung resistance. It was clearly different from that caused by nebulization with a pre-calculated equivalent amount of luteolin.

In summary, the above findings clearly showed that the nebulized luteolin and extract improved lung function. The saline aerosol seemed to have negligible effect on lung function (i.e. no drastic changes in TV, CL and RL), indicating that the contribution of the vehicle to the pulmonary effects produced by luteolin or extract was perhaps minimal. On the other hand, the aerosol administration of luteolin (250µg/ml) or *A. afra* extract (100mg/ml) caused significant improvements in lung function (i.e. increased TV, CL and decreased RL), an action similar to that produced by known bronchodilators (Sven and Rvrfeldt, 2001) on lung function, thus indicating that nebulized luteolin and extract seem to have bronchodilator activities. Luteolin has been associated with bronchodilatory effects (Das *et al.*, 2003), and since it was present in appreciable amounts in the plant extract, it could therefore, be responsible for the pulmonary effects produced by the plant. Furthermore, luteolin was recovered from the aerosolized mist during the three-minute nebulization with both the extract and the luteolin.

The results also clearly indicated that the nebulized *A. afra* aqueous extract had better and sustainable pulmonary effects than the nebulized luteolin even though they were equivalent in pre-calculated luteolin dose. There could be a variety of reasons for such effects, including the possible involvement of other active constituents (i.e. flavonoids such as quercetin, etc) in the plant or that the luteolin perhaps, could be

solely responsible for such pulmonary effects but were more bio-available from the aerosolized plant extract. In fact, higher luteolin levels were recovered from the aerosolized mist collected in the line exposed to the lung during the three-minute nebulization of the extract than during the luteolin nebulization, viz. 21.54 ± 0.815 and 8.992 ± 0.403µg/ml, respectively. Clearly, luteolin was being delivered (in the inhaled air) to the lung but at different levels from the two solutions (i.e. luteolin and *A. afra* aqueous extract solutions) that were supposedly equivalent in total luteolin content. For some reason, more bio-available luteolin was derived from the nebulized *A. afra* aqueous extract. This latter deduction is in agreement with the facts that luteolin showed better release when contained in the plant material than in its aglycone form (Markham, 1982), and was more bio-available in the vervet monkey after oral ingestion of the plant extract compared to the aglycone luteolin (Muganga, 2004).

If the nebulized *A. afra* delivered more luteolin to lung to produce the greater pulmonary effects, the luteolin might also have a favorable lung disposition profile that could be reflected in the lung perfusate (i.e. perfusion medium) levels. However, no luteolin could be detected in the perfusate after the inhalation of the nebulized luteolin or *A. afra* aqueous extract solutions. Perhaps, its levels were below the low limit of detection (i.e. LLQ) of the HPLC assay used in this study, but there could also be a variety of others reasons e.g. only a small portion of the nebulized dose actually reaches the lung vasculature (Tronde, 2001), and/or it may be rapidly metabolized (i.e. conjugated), etc. Further studies are required to substantiate these speculations, for instance, it could be useful to at least determine the disposition profile and see whether, and at what levels, intravenously administered luteolin produced comparable pulmonary effects. Nevertheless, although not detected in the perfusate, luteolin still seemed to be involved in the pulmonary effects produced by nebulized *A. afra* extract, because similar effects were obtained when it was administered as a nebulized solution, or was contained in appreciable levels in the *A. afra* extract and recoverable, also in appreciable amounts, from the mist of the aerosolized extract.

5.6 The effect and disposition of intravenously administered luteolin in the IPL

For *Artemisia afra* to be effective for the traditional claims especially its use in treatment of respiratory conditions, plant constituents including luteolin should have a favorable (i.e. uptake and low metabolism) disposition in lung tissues. Therefore, one of the aims of this study was to determine the pulmonary disposition and effects of luteolin administered via the perfusion medium. To realize these objectives, it was decided to continuously perfuse the lungs with 100ml perfusate containing luteolin (2 or 10 μg/ml) in a re-circulated mode while effluent (i.e. perfusion medium exiting the lungs) luteolin levels were determined from samples collected at selected time intervals using the HPLC assay. Initially, the lungs were perfused with drug free perfusate (i.e. 100ml) for 20 minutes (i.e. equilibration period) before changing to luteolin-containing perfusate (i.e. post treatment phase). During preliminary studies, it was found that 30 seconds was sufficient to change the drug-free perfusate to a drug-containing perfusate. Also, it was found that two minutes was sufficient to clear drug free perfusate out of the IPL system hence it was decided that the first effluent sample be taken at three minutes after change-over. The results depicting the effect produced by the luteolin administered into the perfusion medium and the effluent luteolin levels obtained after recirculation through the lungs with either 2 or 10 μg luteolin per ml of perfusate are discussed further in this chapter.

5.6.1 The effect of IV administered luteolin on lung function

It was felt necessary to determine the effect of luteolin administered via the perfusion medium on lung function because if luteolin was involved in the pulmonary effects produced by *A. afra*, it should, at least have similar effects when administered intravenously. To realize this objective, lungs were initially perfused with drug-free perfusate for 20 minutes (i.e. pre-treatment period) and then for 90 minutes with 100ml perfusate containing either 200μg or 1mg luteolin (i.e. post-treatment period), changes in lung function monitored throughout and the function during the post treatment phase compared to the pre-treatment values (i.e. at 20 minutes).

5.6.1.1 The effect IV administered luteolin on tidal volume

The results depicting the effect of intravenously administered luteolin on tidal volume are summarized in figure 28 and tables in APPENDICES VI & VII. In these experiments, lungs were initially perfused with drug-free perfusate for 20 minutes (i.e. pre-treatment period) then immediately perfused with luteolin-containing perfusate for 90 minutes (i.e. post treatment period).

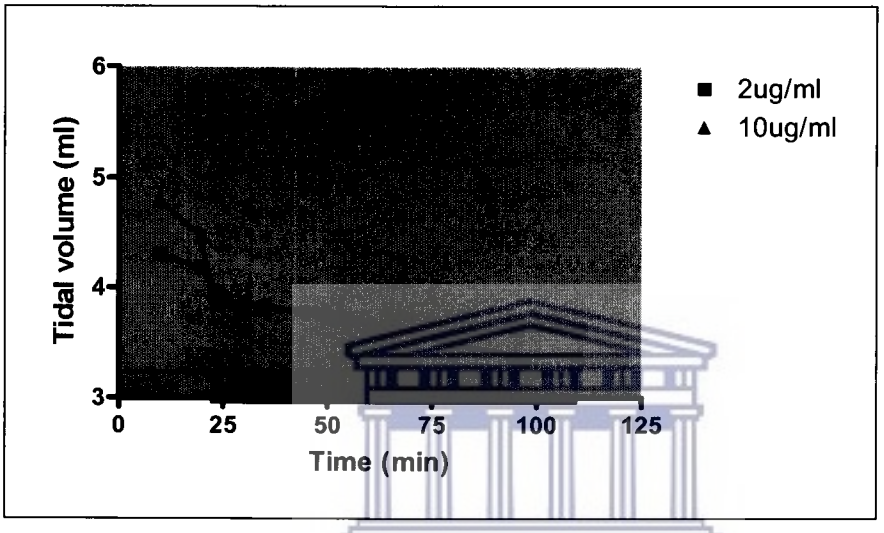


Figure 28: The effect of intravenously administered luteolin (2 or 10μg/ml) on tidal volume. The lungs were allowed to equilibrate for 20 minutes in a closed system with luteolin-free perfusate, then at 20 minutes perfusate was changed 100ml perfusate containing either 2 or 10μg luteolin per ml. Tidal volume was immediately recorded. Each data point represents mean (± SEM) for 5 experiments.

During the equilibration (i.e. luteolin-free) period, the tidal volume decreased at a steady rate of 0.05252 ± 0.006042 ml/min in the 2μg/ml group of lungs ($n=5$ lungs). Immediately after exposure to the circulating luteolin (i.e. at 23 min) the tidal volume had decreased to 3.947 ± 0.29728 ml ($n=5$), which was not significantly ($p>0.05$) different from the pre-treatment (i.e. at 20min) volume of 4.182 ± 0.26581ml ($n=5$), and continued to decrease at a steady rate of 0.00389 ± 0.00270ml/min over the following 87 minutes. The post treatment rate of decrease in TV was significantly ($p < 0.05$) less (i.e. 12.85 times) than the pre-treatment rate suggesting some stabilization of the tidal volume by this low dose of luteolin. With the higher 10μg/ml luteolin dose, the post treatment change in TV was steady at a rate of 0.0053 ±

0.00106 ml/min, which was also significantly ($p < 0.05$) less (i.e. 4.79 times) than the pre-treatment rate (i.e. 0.02539 ± 0.00846 ml/min), further indicating that indeed luteolin administered into the perfusion media stabilized the tidal volume.

5.6.1.2 The effect of IV administered luteolin on lung compliance

The results depicting the effect that luteolin administered intravenously via re-circulating perfusion medium (i.e. 100ml perfusate containing either 2 or 10µg luteolin per ml of perfusate) had on lung compliance are summarized in figure 29 below and tables in APPENDICES VI & VII.

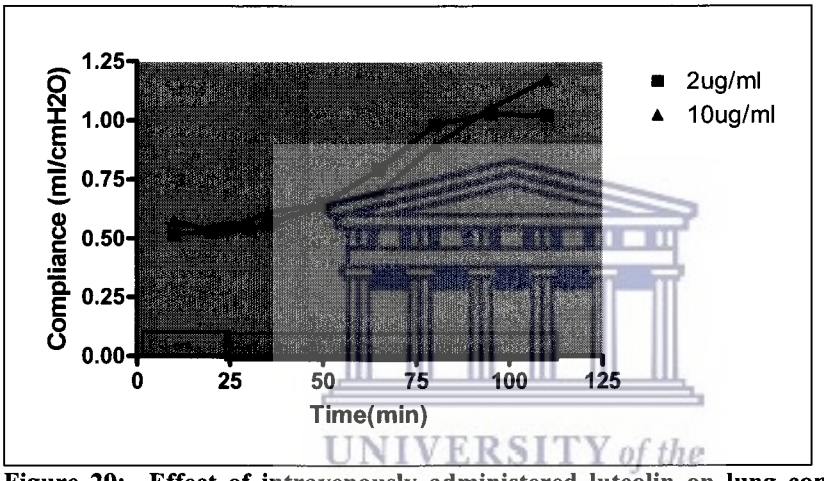


Figure 29: Effect of intravenously administered luteolin on lung compliance. The lungs were allowed to equilibrate for 20 minutes in a closed system with luteolin-free perfusate, then at 20 minutes perfusate was changed 100ml perfusate containing either 2µg or 10µg luteolin per ml. Lung compliance was immediately recorded. Each data point represent mean (± SEM) for 5 experiments ($n=5$).

During the equilibration period, lung compliance increased at a steady rate of 0.00134 ± 0.00135 ml/cmH_2O per minute, but immediately after the lungs were perfused with 2µg/ml luteolin (i.e. at 23 min), the lung compliance increased to 0.5116 ± 0.04699 ml/cmH_2O ($n=5$), a level which was not significantly ($p>0.05$) different from the pre-treatment (at 20min) compliance of 0.5068± 0.03887 ml/cmH_2O ($n=5$). The lung compliance, however, continued to increase at a steady rate of 0.00558 ± 0.00289 (ml/cmH_2O) per minute over the entire post treatment period (i.e the following 87 minutes), with the highest significant ($p<0.05$) increase of 98.48% in lung compliance being obtained 75 minutes after the start of the treatment. With the higher

luteolin (10µg/ml) dose, the post treatment improvement in lung compliance was 1.87 % from the pre-inhalation value of 0.535 ± 0.0339 (ml/cmH_2O) to 0.545 ± 0.039 (ml/cmH_2O) immediately (at 23 minutes) post treatment. In this case, the improvement in CL was also sustained at a steady rate of 0.00721 ± 0.00113 (ml/cmH_2O) per minute for entire post treatment period. In fact, this rate of increase was similar to that of low luteolin dose (i.e. 2µg/ml), both sets of results thus indicating that luteolin administered into the perfusion media produced a significant improvement in lung compliance, which was long–lasting.

5.6.1.3 Effect of IV administered luteolin on lung resistance

The results depicting the effect intravenously administered luteolin had on lung resistance are summarized in figure 30 and tables in APPENDICES VI &VII. In these experiments, the lungs were initially perfused with drug-free perfusate for 20 minutes (i.e. pre-treatment period) then perfused, in a re-circulating manner, with luteolin containing perfusate for 90 minutes (i.e. post treatment).

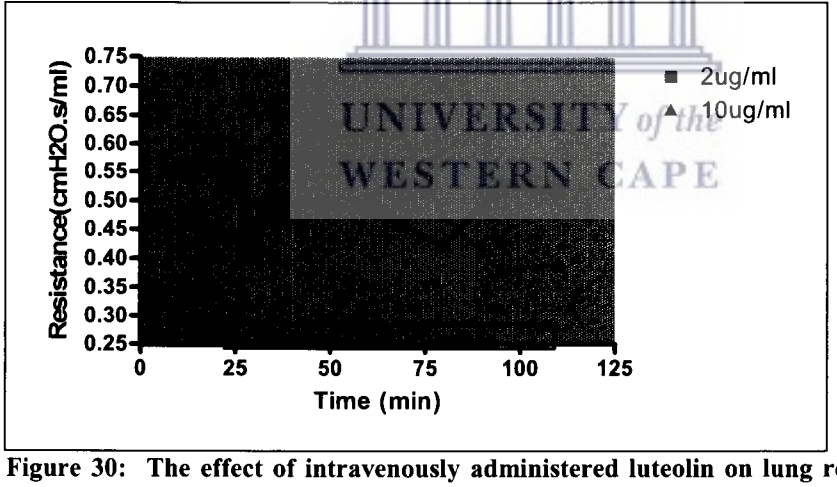


Figure 30: The effect of intravenously administered luteolin on lung resistance. The lungs were allowed to equilibrate for 20 minutes in a closed system with luteolin-free perfusate, then at 20 minutes perfusate was changed 100ml perfusate containing 2µg luteolin per ml. Lung resistance was immediately recorded. Each data point represent mean (± SEM) for 5 experiments (*n=5*).

During the equilibration period, lung resistance increased at a steady rate of 0.01032 ± 0.00429 cmH_2O.s/ml per min in the lungs (*n=5*) that were eventually dosed with the 2µg/ml luteolin dose. Immediately after the start of perfusion with the luteolin-

containing perfusate (i.e. at 25 min), the resistance had decreased to 0.5462 ± 0.06529 $cmH_2O.s/ml$ ($n=5$) which was not significantly ($p>0.05$) different from the pre-treatment (at 20min) resistance of 0.5576 ± 0.06529 $cmH_2O.s/m$ but at 30 minutes, it had decreased to 0.4060 ± 0.07716 before it increased to 0.4940 ± 0.04360 (at 45 min), and essentially stayed at levels ranging between 0.4922 ± 0.08966 and 5288 ± 0.05848 until the end of the monitoring period. Levels which were significantly lower than the pre-treatment RL. Although fluctuating, the low luteolin dose thus produced a sustainable decreased in lung resistance. The lungs that were eventually exposed to the higher luteolin dose appeared to be more unstable as far as RL was concerned, with the RL increasing at a rate of 0.0103 ± 0.0280$cmH_2O.s/min$ during the equilibration period. After the administration of the higher luteolin dose (10µg/ml), there was an immediate, though statistically significant ($p<0.05$), increase in RL to 0.564 ± 0.0634 $cmH_2O.s/ml$ from the pretreatment resistance of 0.413 ± 0.0519 $cmH_2O.s/ml$ at 20 minutes, followed by a brief, sharp drop to 0.4060 ± 0.07716 ($p<0.001$) in RL (at 30 min) and a subsequent steady increase in RL to levels that remained significantly higher than the pretreatment RL levels, for the entire post treatment period. Overall, the effect of luteolin thus appeared to be that of a sustained increase in the RL, although a brief decrease was evident immediately after the introduction of the luteolin. The reason for this RL-effects-profile is not immediately clear, but may be the result of a complex dose-dependent mechanism of action for luteolin e.g. muscle relaxing at low levels and RL-promoting effects at higher concentrations. Before this hypothesis can be explored, it would, however, be prudent to ascertain the actual levels of luteolin attained within the lung.

In summary, this study for the first time, demonstrated that intravenously administered luteolin improved lung function (i.e. confirmed by stabilized TV, increased CL and decreased RL at low dose), further confirming its possible bronchodilator activities. In fact, luteolin seems to particularly increase the lung compliance, an effect that might be caused by a variety of factors, including an increased lung elasticity, smooth muscle relaxation or improved activity of the pulmonary surfactant (Niemeier, 1984). Unfortunately, the objective of this study was

not to determine how luteolin acted on the lungs. But, the above results clearly indicated that luteolin has pulmonary effects that are similar to that produced by inhaled *A. afra.*

5.6.2 The disposition profile of intravenously administered luteolin in the IPL

The last objective of this study was to determine the lung disposition profile (i.e. up-take and metabolism) of luteolin when given intravenously. To realize this objective, a suitable HPLC assay was required for the determination of luteolin levels from the perfusate.

5.6.2.1 HPLC assay of luteolin in perfusate

5.6.2.1.1 Development of the HPLC assay

The HPLC system and conditions employed to quantify the levels of free (aglycone) and total luteolin (aglycone and conjugated) in the perfusate after the administration of luteolin via the perfusion media into the lung vasculature were similar to that described in section 4.5.2 with morin being used as the internal standard. The latter did not interfere with any endogenous substances in the perfusate and the employed HPLC assay provided well separated peaks for both luteolin and the internal standard.

5.6.2.1.2 Validation of the HPLC assay

The HPLC assay for the quantification of perfusate luteolin levels was validated for specificity, separation, linearity, low limit of detection, inter- and intra-day variability and the results obtained are summarized in table 10 and figure 31. The retention times obtained for the internal standard and luteolin were 10.840 ± 0.0919 and14.228 ± 0.1363 (*n=6*) minutes, respectively, and were similar to those obtained with the plant material assay. The assay had good reproducibility shown by low coefficient of variation (0.5 to 1.18%), and was linear over the concentration ranges of 2.5-15µg luteolin/ml of perfusate. The luteolin could be extracted from perfusate samples with 94.1382 ± 0.5917% efficiency, and had a low limit of detection of 70ng on column, a

sensitivity similar to that obtained with the plant material assay. In fact, the perfusate had no peaks at the retention time for morin and luteolin as shown in figure 32-34.

Table 10: The validation results obtained for luteolin quantification in perfusate using HPLC analysis.

Slope	0.2101 ± 0.001510
Intercept	0.2086 ± 0.09886
Correlation coefficient (r^2)	0.9998
Intra-day variability (CV %)	0.5%
Inter-day variability (CV %)	1.18%
Low limit of detection	70ng
Recovery	94.1382 ± 0.5917%

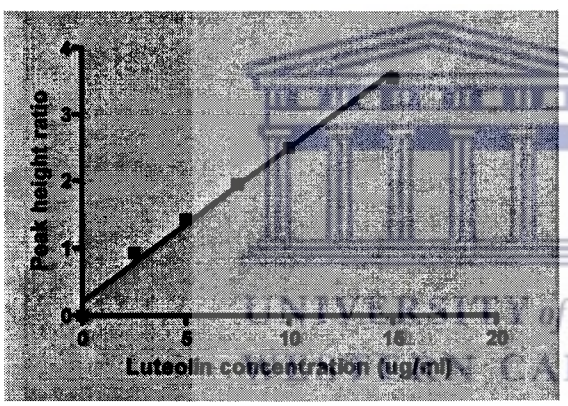


Figure 31: The standard curve for quantification of luteolin from perfusate samples obtained in IPL. Blank samples of the perfusate were spiked with luteolin and morin (IS) were subjected to methanol-acid treatment, extracted with ethyl acetate, separated by HPLC and detected by UV absorption at 349nm. Each data point represent average ratio of luteolin peak height: morin peak height for 6 replications at each concentration.

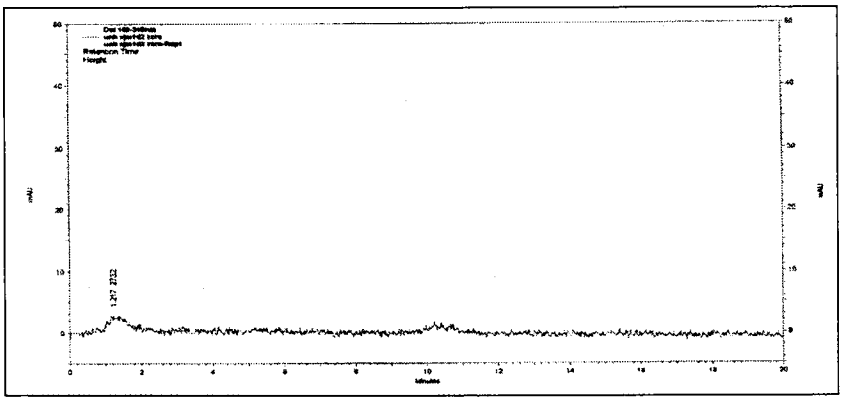


Figure 32: Representative HPLC chromatogram of an extracted blank perfusate

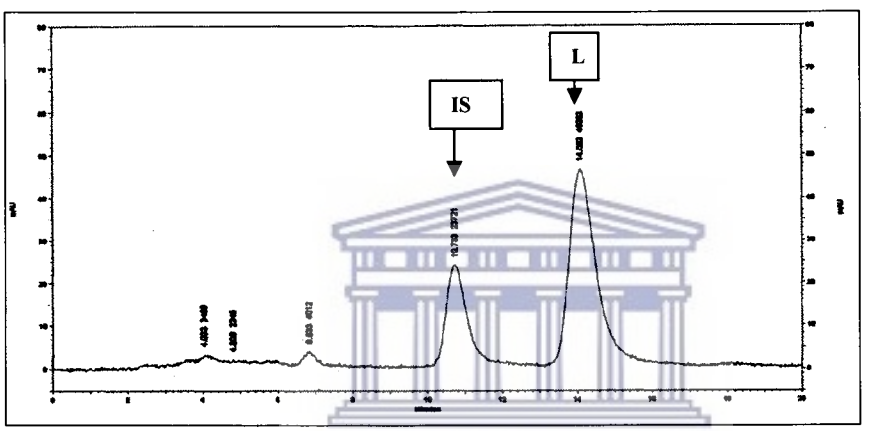


Figure 33: Representative HPLC chromatogram of a hydrolyzed perfusate sample collected 75 minutes after continuous perfusion with luteolin-containing medium in IPL. The retention times for IS and luteolin (L) were 10.733 and 14.083 min respectively.

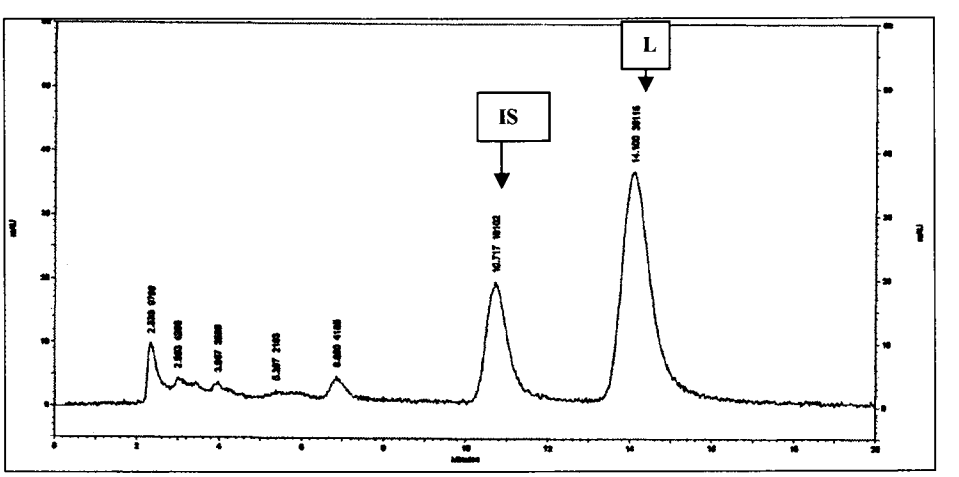


Figure 34: Representative HPLC chromatogram of an unhydrolysed perfusate sample collected 75 minutes after continuous perfusion with luteolin-containing medium in IPL. The retention times for IS and luteolin (L) were 10.717 and 14.100 min respectively.

5.6.3 The levels of luteolin in the perfusate

The levels of luteolin in each perfusate sample were easily determined by interpolation of the luteolin peak height to internal standard peak height ratio vs. luteolin concentration from the standard curve. For each collected perfusate sample (1ml), the level of free (i.e. aglycone) luteolin was obtained by extracting half of each sample (i.e. 500µl) with ethyl-acetate, while the other half was acid-hydrolyzed before ethyl-acetate extraction to obtain the total (aglycone + conjugated) luteolin levels. The levels of conjugated luteolin were calculated as the difference between total and aglycone luteolin levels of the same sample. Before the administration of the drug-containing perfusate into IPL, an initial sample of the perfusate was taken in each experiment to determine the initial luteolin level (i.e. dose) before administration into the lung via recirculated perfusion. In the lungs ($n=5$) given 2µg/ml luteolin the initial free luteolin level (i.e. un-hydrolyzed) obtained was 1.957 ± 0.0143µg/ml, while a comparable 1.978 ± 0.0074µg/ml was obtained after acid hydrolysis indicating that there were similar luteolin levels obtained for both hydrolyzed and un-hydrolyzed perfusate from each sample taken just before administration into the lung vasculature. Even for the higher dose (10µg/ml) the initial aglycone luteolin level was 9.870 ± 0.0098µg/ml and similar to the 9.857 ± 0.0933µg/ml obtained for total luteolin, strongly confirming that no alteration in luteolin levels occurred in the albumin containing perfusion medium. The results depicting the perfusate luteolin levels obtained after perfusion with 100ml perfusate containing 200µg or 1mg luteolin per ml of perfusate are shown in figures 35-36 and tables in Appendix IV.

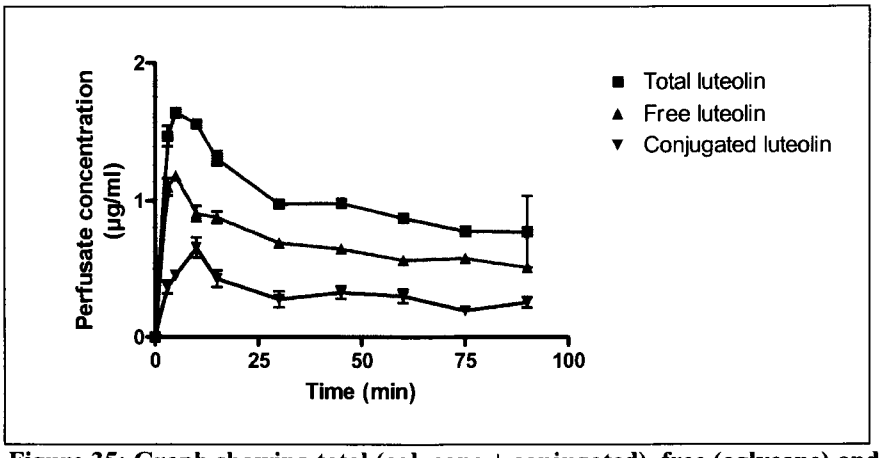


Figure 35: Graph showing total (aglycone + conjugated), free (aglycone) and conjugated (metabolized) luteolin levels after continuous perfusion with 100ml perfusate containing 2µg luteolin per ml of perfusate in a closed system using IPL. Each data point represents a mean (± SEM) for 5 experiments.

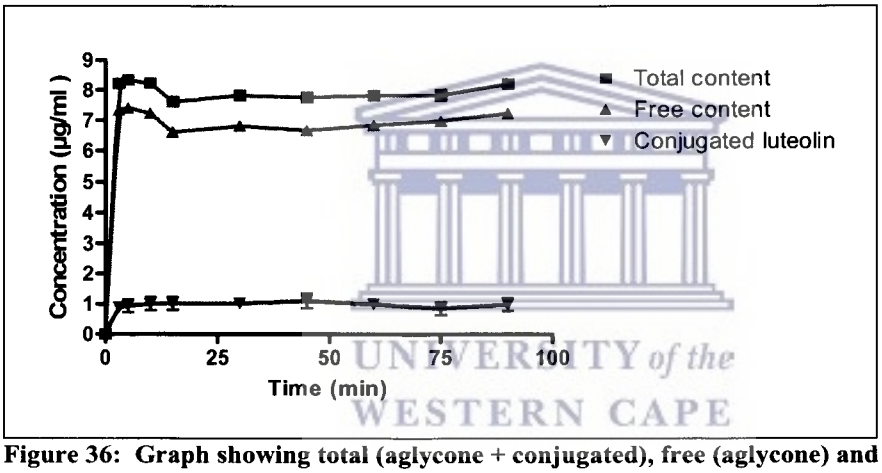


Figure 36: Graph showing total (aglycone + conjugated), free (aglycone) and conjugated (metabolized) luteolin levels after continuous perfusion with re-circulated 100ml perfusate containing 10µg luteolin per ml in IPL. Each data point represents the mean (± SEM) for 5 experiments

Table 11: The data showing the maximum concentration and time at which maximum concentration were obtained as well as the biphasic rates of decrease in luteolin levels in the perfusate after perfusing lungs with either 2 or 10 µg/ml

Luteolin dose	C_{max} (µg/ml)	T_{max} (min)	Rate of decrease (µg/ml.min^{-1})	
			First phase	Second Phase
10 (µg/ml) group				
Total levels	8.328 ± 0.2658	5	0.508 ± 0.00093	0.390 ± 0.01605
Free levels	7.400 ± 0.1647	5	0.594 ± 0.00190	0.423 ± 0.01667
Conjugated levels	1.094 ± 0.5244	45	0.085 ± 0.08120	0.032 ± 0.08063
2(µg/ml) group				
Total levels	1.648 ± 0.0423	5	0.202 ± 0.000874	0.669 ± 0.003867
Free levels	1.183 ± 0.0461	5	0.179 ± 0.002086	0.487 ± 0.003674
Conjugated levels	0.662 ± 0.1629	10	0.022 ± 0.005910	0.174 ± 0.001773

The levels of free luteolin obtained immediately (i.e. 3 min) after continuous perfusion of the lungs ($n=5$) with the low dose (2μg/ml) of luteolin were 1.101 ± 0.1460 μg/ml, followed by a slight increase to 1.183 ± 0.0461 μg/ml (i.e. at 5 min) (figure 35), suggesting a short period of distribution of the luteolin into the pulmonary vasculature and extracellular lung volume. The perfusate levels then decreased rapidly at a rate of 0.179 ± 0.002086μg/ml per minute to 0.696 ± 0.0515 μg/ml at 30 minutes, followed by a slower decrease of a significantly different ($p<0.005$) steady rate of 0.487 ± 0.003674 μg/ml/min to a level of 0.517 ± 0.0508μg/ml at the end of the treatment period (i.e. 90 min). The first order rate constant and half-life for the terminal phase were 8.73×10^{-3} min^{-1} and 79.381 min, respectively. Clearly, luteolin was avidly taken up into the lung. The total luteolin levels in the perfusate also seemed, after the initial distribution phase, to follow the same above biphasic trend of an initial rapid decline followed by a slower decrease, confirming the biphasic luteolin disappearance from the perfusate. Presumably, the faster initial phase may be combination of uptake and conversion of the luteolin, while the slower later phase reflect conversion of luteolin to some products not recoverable from the perfusate with the assay method (acid hydrolysis) used in this study.

For the higher luteolin dose (10μg/ml), the free luteolin perfusate levels increased to 7.400 ± 0.1647 μg/ml at 5 min (i.e. during the distribution/equilibration phase), then, over the next 10 minutes, decreased at a steady rate of 0.594 ± 0.0019 μg/ml/min until 15 minutes after the continuous perfusion. Thereafter, the levels essentially remained stable (increased steadily at a very low rate of 0.423 ± 0. 01667μg/ml/min) until the end of the perfusion period (i.e. 90 min). The first order rate constant and half-life for the terminal phase were 2.501×10^{-4} min^{-1} and 2770.89 min, respectively. The total luteolin levels in the perfusate followed the same trend, suggesting that after the immediate distribution or mixing phase (i.e. first 5 minutes), the lungs rapidly absorbed and conjugated luteolin into hydrolysable conjugates. However, the higher luteolin dose had a higher half-life indicating the possible saturation kinetics of luteolin (i.e. lungs possibly saturated by small amounts of luteolin). Unfortunately, it was not the purpose of this study to determine how the lungs absorbed and

metabolized luteolin, the site of conjugation or even types of the resultant metabolites and/or the kinetics of these processes. However, the results clearly provide evidence that luteolin was rapidly absorbed and metabolized, and this might be the reason why luteolin was not detected in the perfusate samples after the lungs were nebulized with luteolin or the extract.

When the luteolin perfusate levels and lung effects were compared over time, as for example in figures 37 and 38, the results showed that as the perfusate levels of luteolin decreased over time (which is indicative of luteolin uptake and/or metabolism by the lung), the CL increased steadily over time. This was evident following the administration of both luteolin doses (2 or 10 µg/ml), and especially after the faster initial phase of luteolin disappearance in the perfusate had passed. Collectively, the data seemed to suggest that the observed CL could have been due to accumulating levels of luteolin over time in the lung.

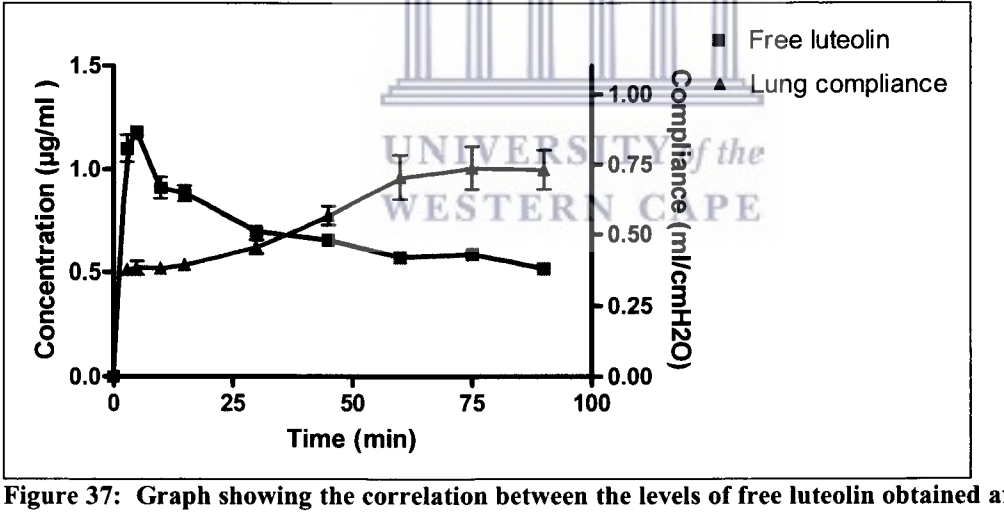


Figure 37: Graph showing the correlation between the levels of free luteolin obtained after continuous perfusion with 100ml perfusate containing 2µg luteolin per ml of perfusate in a closed system and the resultant effect produced on lung compliance. Each data point represents the mean (± SEM) for 5 experiments

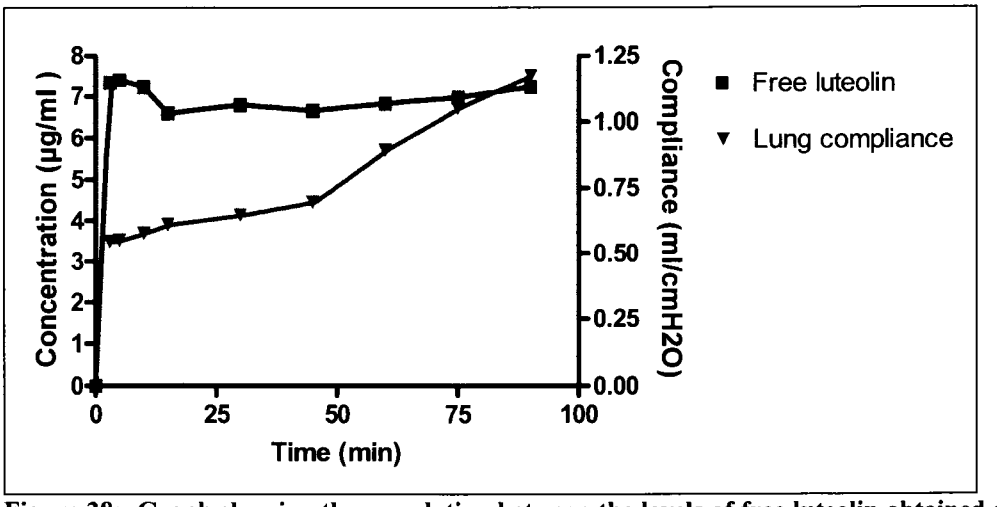


Figure 38: Graph showing the correlation between the levels of free luteolin obtained after continuous perfusion with 100ml perfusate containing 10µg luteolin per ml of perfusate in a closed system and the resultant effect produced on lung compliance. Each data point represents the mean (± SEM) for 5 experiments

Clearly, luteolin had an effect on pulmonary function as seen in the change in lung compliance (and the other parameters). The pulmonary effect might possibly be caused by the smooth muscle relaxation effect of the luteolin (Das *et al.*, 2003) and the data obtained in this study might, therefore, further confirm the possible bronchodilator activities of luteolin. Further studies comparing the luteolin perfusate levels and its lung effects must however, be done before one can truly confirm such hypothesis. Nevertheless, the preliminary data already obtained strongly suggest that luteolin could be a worthy marker to monitor, establish and quantitate the pulmonary effects of *A. afra*.

Overall, the results obtained in this study indicate that inhalation of *A. afra* steam and nebulized *A. afra* extract and luteolin and intravenously-administered luteolin affected lung function. Firstly, the inhalation of saline steam or nebulized saline appeared to produce negligible effects and transient changes in lung the parameters. These changes were similar in magnitude to those previously observed using the same IPL system (Syce and Parker, 2004). The contribution of saline to the pulmonary effects produced by *A. afra* steam or extract nebulization was therefore minimal.

Secondly, the inhalation of *A. afra* steam and the nebulized aqueous *A. afra* extract and luteolin produced improvements in lung function (i.e. all the doses increased TV,

CL and decreased RL), an effect similar to that obtained with known bronchodilators (Svens and Rvrfeldt, 2001). The *A. afra* steam inhalation and nebulized *A. afra* extract and luteolin thus seem to have bronchodilator activities. Luteolin has been associated with bronchodilator activities before (Das *et al.*, 2003), and this study provided further evidence on such activities. Since *A. afra* contained luteolin (with its bronchodilator activities) in appreciable amounts, it is not surprising that the steamed or nebulized plant extract produced pulmonary effects similar in magnitude to that of luteolin, suggesting that luteolin could be an important contributor to the pulmonary effects of by *A. afra*.

Thirdly, the results clearly showed that *A. afra,* when steamed or nebulized, produced particularly significant improvements in CL, an effect that was similar to that produced with nebulized or IV administered luteolin, providing more evidence about the possible association of luteolin with pulmonary effects produced by *A. afra*. An increase in CL can be caused by a variety of factors including an increase in lung elasticity, smooth muscle relaxation, and improved activity of the pulmonary surfactant (Niemeier, 1984). It was not the objective of this study to determine how luteolin or *A. afra* acted on the lungs, but it could be speculated that the luteolin effects might occur via smooth muscle relaxation, since luteolin has been found to have this effect (Das *et al.*, 2003). Flavonoids (luteolin) can perhaps trigger the release of pulmonary surfactants which in turn reduce the surface tension in the lung tissues, increasing the elasticity of the lung and the increased CL. While the above results clearly indicate that *A. afra* improved lung function in a similar way to luteolin, and that luteolin could therefore, be associated with the pulmonary effects produced by *A. afra,* the mechanism for this effect i.e via smooth muscle relaxation and or changes in pulmonary surfactant activity, must still be established.

Lastly, the overall results showed that nebulization produced better and sustainable pulmonary effects than steam inhalation. There may be a variety of reasons for this, including that nebulization provided better deposition of plant actives in the lung than the steam inhalation. The administration of nebulized *A. afra* extract could thus be a

better alternative to the traditionally-used steam inhalation for the treatment of respiratory conditions, provided that other factors (i.e. convenience, availability of nebulizer e.t.c.) are also considered and dealt with.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

The objectives of this study were, firstly to compare the luteolin content in crude dried *Artemisia afra* leaves and an aqueous extract of *A. afra*; secondly, to evaluate and compare the pulmonary effects of the traditionally-prepared *A. afra* steam inhalation and nebulized *A. afra* aqueous extract and luteolin solutions; and thirdly, to determine the pulmonary disposition and effect of intravenously administered luteolin.

To realize these objectives, the aqueous extract of *A afra* was prepared according to the traditional method, the luteolin content in plant solutions and perfusion medium were determined using HPLC, the isolated perfused lung (IPL) model adapted to allow administration of materials via steam inhalation and nebulization, as well as the monitoring of pulmonary functions such as tidal volume (TV), respiratory resistance (RL) and compliance (CL). To determine the pulmonary disposition, luteolin was administered in the perfusion medium, perfusate samples taken over an hour-and-a-half and assessed for luteolin content. The methods and procedures employed in this study worked well, and from the obtained results the following conclusions can be drawn:

1) Traditionally prepared *A. afra* aqueous extract contained higher content of luteolin, of which approximately 50% was in acid-hydrolysable, presumably glycoside form, while the crude dried leaves contained less luteolin in hydrolysable form.
2) The IPL can be adapted for the evaluation of traditionally prepared steam inhalation dosage forms with stable, valid and reproducible results. *A. afra* steam inhalation has a dose-dependent effect on lung function (i.e. confirmed by increased TV and CL and decreased RL) that may be long-lasting at higher plant doses.
3) Nebulized aqueous extract had much better pulmonary effects than an equivalent dose of aglycone luteolin as confirmed by higher and sustainable

improvement in lung parameters. This could be caused by (a) possible involvement of other plant constituents present in the aerosolized mist, and (b) luteolin was better released from the nebulized extract than with aglycone luteolin (i.e. higher levels obtained from aerosolized extract mist).

4) Luteolin is implicated in the pulmonary effects produced by inhaled *Artemisia afra* as it produced similar effects when nebulized, and was obtained from the aerosolized mist of the *A. afra* aqeous extract.
5) Intravenously administered luteolin is taken–up and/or metabolized in the lungs, as confirmed by conjugation in the IPL, explaining why luteolin was not detected from perfusate samples after the lungs were nebulized with extract and luteolin.
6) Intravenously administered luteolin stabilizes lung function with time as confirmed by improved lung compliance and stabilized tidal volume, further confirming luteolin involvement in the pulmonary effects of *A. afra.*

Collectively, the results of this study indicated that the traditionally prepared *A. afra* steam inhalation improves lung function and could, therefore, alleviate some of the respiratory conditions as traditionally claimed. Luteolin is contained in appreciable amounts in traditionally prepared aqueous extract of *A. afra,* and has comparable improvement in lung function to that of the extract when nebulized. Therefore, luteolin could be implicated in the pulmonary effects of the *A. afra,* since the lungs can also absorb it.

In addition to the above conclusions, the following recommendations were made:

a) Traditionally, steam inhalation is used until the boiled mixture has cooled down. However, in this study, lungs were only exposed for a short period (i.e. lung parameters deteriorate with time in the IPL). Further studies are therefore required to determine the effect of steam inhalation after long exposure, as it is normally done traditionally.
b) Although steam inhalation with *A. afra* improved lung function, the active constituents responsible for such action are not yet known. Therefore, it might

be necessary to determine the plant's constituents responsible for this action, although luteolin could be implicated.

c) Steam inhalation improved lung function. However, its mechanism of action is not yet known. Therefore, future studies are required to determine how the steam affects lung parameters, particularly lung compliance.
d) Lungs can take up and conjugate luteolin. However, the site of conjugation, types of conjugates and the effects these conjugates might have on lung function, is not yet known. Therefore, future studies are required to provide more evidence and clarity, and
e) Further studies are required to correlate or determine the relationship between the exact amounts of luteolin contained in the lungs and the obtained effect pulmonary function.

Overall, the results of this study provide valuable information about the use and effects of the traditionally prepared *A. afra* steam inhalation against pulmonary conditions, thereby providing experimental support for the empiric ethno-pharmacological use of this plant in traditional medicines.

LIST OF REFERENCES

American Family Physician (1991) Volume 264, pg 989.

Barton, D. and Ollis W. D., (1986): *Advances in medical Phytochemistry*. John Libbey, Eurotext: 47-61.

Benavente-Garcia, O., Castillo, J., Marin, F. R., Ortuno, A., Del Rio, J. A. (1997): *Uses and properties of citrus flavonoids*. J. Agricultural and Food Chemistry, (45): 4506-4515.

Boer, F., (2003): *Drug handling by the lung.* British journal of anaesthesia. 91 (1), 50-60.

Burns, J., Gardner, P. T., O'Neil, J., Crawford, S., Morecroft, I., Mcphail, D. B., Lister, C., Matthews, D., Maclean, M. R., Lean, M. E., Duthie, G. G., Crozier, A., (2000): *Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines*. Journal of Agricultural and Food Chemistry, (32) 274-276.

Byron, P., (2004): *Drug delivery devices*. Proceedings of the American Thoracic Society, (1) 321-328.

Das, M., Ram, A., Ghosh, B., (2003): *Luteolin alleviates bronchoconstriction and airway hyper reactivity in ovalbumin sensitized mice.* Inflammation Research, (52): 101-106.

Derendorf, H., Hochhaus, G., Mollmann, H., (2001): *Evaluation of pulmonary absorption using pharmacokinetic methods. J. Aerosol Medicine* (14): 1, 9-17.

Drazen, J. M., (1984): *Physiological basis and interpretation of indices of pulmonary mechanics*. Environmental Health Perspectives (56): 3-9.

Fennell, C.W.; Lindsey, K. L.; Mcgraw L. J.; Sparg S. G.; Stafford, G. I.; Elgorashi, E. E.; Grace, O. M.; Van Staden, J.(2004). *Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology.* J. Ethnopharmacology, 94: 207-217.

Foth, H., (1995): *Role of the lung in the accumulation and metabolism of xenobiotic compounds- implication for chemically induced toxicity.* Crit. Rev. Toxicol, (25): 165-205.

Gradolatto, A., Canivennc-Lavier, J.P., Basly, J.P., Siess, M.H., Teyssier, C. (2004): *Drug metabolism and disposition* (32) 58-65.

Grayer, R. J., Kite, G. C., Veitch, N. C., Eckert, M. R., Marin, P. D., Senanayake, P. Paton, A. J. (2002*)*: *Leaf flavonoid glycosides as chemosystematic characters in Ocimum.* J. Biochemical Systematics and Ecology, (30): 327-342.

Goggel, R., Pitterman., W., Aulman, W., Uhlig, S. (2003): *Assessment of the acute pulmonary toxicity of lamp oil aspiration in the isolated perfused rat lung.* J. Food and Chemical toxicity, (41), 1029-1033.

Hai Qiu Tang (1999): *Terpenoids and Flavonoids from Artemisia species.* J. Planta Med, (66): 391.

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

Harris, L. (2002): *An evaluation of the bronchodilatory properties of Mentha longifolia and Artemisia afra.* Dissertation for a degree of Master's in Pharmacy at the University Of The Western Cape, Bellville.

Havsteen, B.H. (2002): *The biochemistry and medical significance of the flavonoids*: Review article. Pharmacology and Therapeutics, volume 96 issue 2-3, 67-202.

Hertog, M. G., Hollman, P. C. H., & Venema, D. P., (1993): *Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits*. Journal of Agricultural and Food Chemistry, (40): 2379-2383.

Heitz, A., Carnat, A., Fraisse, D., Carnat, AP., Lamaison, JL. (2000): *Luteolin 3' – glucuronide, the major flavonoid from Melisa officinalis subsp. Officinalis.* J. Fitoterapia, (71): 201-202.

Hollman, P.C.H., Katan, M. B. (1999): *Dietary Flavonoids: Intake, Health Effects and Bioavailability.* J. Food and Chemical Toxicology, (37): 937-942.

Hutchings, A. & Van Staden, J. (1994): *Plants used for stress related ailments in traditional Zulu, Xhosa and Sotho medicine. Part 1: Plants used for headaches.* J. Ethnopharmacol, (43): 89-124.

Kaneko, T. & Baba, N., (1999) *Protective effect of flavonoids on endothelial cells against linoleic acid hydroperoxide-induced toxicity.* Biosciences, Biotechnology & biochemistry, (63): 323-328.

Knekt, P., Jarvinen, R., Reunanen, A., and Maatela, J.M (1996): *Flavonoid intake and coronary mortality in Finland: a cohort study*. British Medical Journal, (312): 478-481.

Komperlla, M.K., (2005): *The formulation, manufacture and evaluation of rapid release tablets made from Artemisia afra plant material*. Dissertation for a degree of Master's in Pharmacy at the University Of The Western Cape, Bellville.

Lausted, C. G., Johnson, A. (1999): *Respiratory resistance measurement by an airflow pertubation device,* Physio. Meas, (20): 21-35.

Liping, L., Huidi, J., Wu, H., Zeng, S., (2004): *Simultaneous determination of luteolin and apigenin in dog plasma by RP-HPLC*. J. of Pharmaceutical and Biomedical Analysis, (37): 615-620.

Lui, Z., Chan, K., Hua, Z., Jiang, Z., Wong, Y., Xu, H. and Liang, L., (2005): *Pharmacokinetics and tissue distribution of sinomenine in rats and its tissue binding activity in vitro*. J. Life Sciences, (77): 3197-3209.

Manach, C., Morand, C., Crespy, V., Demigne, C., Texier, O., Remesy, C. (1998): *Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties*. Federation of European Biochemical Societies Letters, (426): 331-336.

Markam, K. R. (1982): *Techniques of flavonoid identification.* London, New York, Paris: Academic press: 1-16.

Muganga, R., (2004): *Luteolin levels in selected folkloric preparations and the bioavailability of luteolin from Artemisia afra aqueous extract in the vervet monkey.* Dissertation for a degree of Master's in Pharmacy at the University Of The Western Cape, Bellville.

Mukinda, J.T., (2005): *Acute and chronic toxicity of the flavonoids-containing plant Artemisia afra in rodents*. Dissertation for a degree of Master's in Pharmacy at the University Of The Western Cape, Bellville.

Mobley, C. and Hochhaus, G. (2001): *Methods used to assess pulmonary disposition and absorption of drugs*. DDT Vol. 6, No. 7.

Nuurtila, A.M., Kammiovirta, K. and Oksman-Caldentey, K.M. (2002): *Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis*. J. Food chemistry, 76, 519-525.

Niemeier, R., W., (1984): *The isolated perfused lung*. Environmental Health Perspective. (56): 35-41.

Ophir, D., Elad, Y. (1987): *Effects of steam inhalation on nasal patency and nasal symptoms in patients with the common cold*. AM. J. Otolaryngol, (3): 149 -153.

Paganga, G., Rice-Evans, C.A. (1997): *The identification of flavonoids as glycosides in human plasma.* J. Federation of European Biochemical Societies Letters. (401):78-82.

Pang, Y., Sakagami, M., Byron, P.R. (2005): *The pharmacokinetics of pulmonary insulin in the in vitro isolated perfused rat lung: Implications of metabolism and regional deposition*. J. Pharmaceutical Sciences, DTD.

Rang, H.P., Dale, M.M., Ritter, J.M. (1999): *Pharmacology 4th edition*. Churchill Livingstone pg 734.

Raquel, F.R., Sanchez-Navarro, A., Lanao, J.M. (1999): *Distribution of ciprofloxacin in the isolated rat lung in the presence and absence of tissue oedema*. European journal of Pharmaceutical Sciences (8) 203-209.

Rice-Evans, C.A., Miller, N. J., Paganga, G. (1997). *Antioxidant properties of phenolic compounds.* J. Trends in Plant Science, 97 (2): 152- 159.

Roberts. M. (1990). *Indigenous Healing Plants*. Southern Book Publishers (Pty) Ltd, Halfway House: 226-228.

Rotteli, A.E.; Guardia, T., Juarez, A.O. Rocha, N.E. AND Pelzer, L.E. (2003): *Comparative study of flavonoids in experimental models of inflammation.* J. Pharmacological Research, (48): 601-606.

Ruppel, G. (2004): *Manual of Pulmonary Function Testing,* eight edition, Mosby, pg 17-46.

Sanchez de Rojas, V.R., Ortega, T., Villar, A., (1995): *Inhibitory effect of Cistus Populifolius on contractile responses in the isolated rat duodenum.* J. of Ethnopharamacology (46) 59-62.

Scalbert, A. Morannd, C., Manach,C., Rmesy, C. (2002): *Absorption and metabolism of polyphenols in the gut and impact on health.* J. Biomedicine and pharmacotherapy, (56): 276-282.

Scalbert, A., Williamson, G., (2000): *Dietary intake and bioavailability of polyphenols in Symposium: Chocolate: Modern science investigates an ancient medicine.* J. Nutrition supplement, (130): 2073-2085.

Seeger, W., Walmarth, D., Grimminger, F., Rosseau, D., (1999): *Adult respiratory distress syndrome: model systems using isolated perfused rabbit lungs.* Methods in Enzymologist, (233): 549-58.

Shimoi, K., Masuda, S., Shen, B., Furugori, M., Kinae, N. (1996): *Radioprotective effects of antioxidative plant flavonoids in mice.* J. Mutation Research, (350): 153 - 161.

Shimoi, K., Okada, H., Hara, Y., Hiroyo, Y., Kinae, N., Goda, T., Furugori, M., Takase, S. (1998): *Intestinal absorption of luteolin 7- O -β - glucoside in rats and humans.* J. FEBS Letters, 438 (1998): 220-224.

Silva, L.G., Lee, I., Kinghohrn, A.D. (1998): *Special problems with the extraction of plants in, Methods in Biotechnology: Natural products isolation* (Cannell JP, ed.). Totowa, New Jersey, Human Press: 343.

Spencer, J.P.E, Chowrimooto, G., Choudhary, R., Debnam, E.S., Srai, S.K., Rice-evans, C., (1999): *The small intestine can both absorb and glucuronidate luminal flavonoids*. FEBS Letters (468) 224-230.

Springfield, E.P.; Eagles, P. K. F.; Scott, G. (2005). *Quality assessment of South African Herbal medicines by means of HPLC fingerprinting.* J. Ethnopharmacology, 101: 75-83.

Stafford, G.I., Jager, A. K., van Staden, J. (2005). *Effect of storage on chemical composition and biological activity of several South African medicinal plants.* J. Ethnopharmacology, 97: 107-115.

Svens, K., Rvrfeldt, A. (2001): *A study of mechanisms underlying amitriptylline-induced acute lung function impairment.* J. of Toxicology and Pharmacology, (177), 179-187.

Syce, J., and Parkar, S. (2004): *The effect of jet versus ultrasonic nebulized pulmonary surfactant on the respiratory mechanics of detergent-damaged isolated perfused rat lung.* (poster).The 38th Congress of the Southern African Pharmacology Society on 24-27th October 2004 The President Hotel, Naval Hill, Bloemfontein, South Africa.

Tronde, A., (2002): *In vitro and in vivo investigations of drug absorption across the lung barrier and its relation to drug physicochemical properties.* Dissertation for the degree of Doctor of Philosophy in Biopharmaceutics at Uppsala University.

Ueda, H., Yamazaki, C., Yamazaki, M. (2003): *Inhibitory Effect of Perilla Leaf Extract and Luteolin on Mouse Shin Tumor Promotion.* J. Biol. Pharm. Bull, 26(4): 560-563.

Uhlig, S., Taylor, A.E. (1998): *Methods in Pulmonary Research.* Deutsche Bibliothek Cataloging-in-Publication Data, Berlin Germany: 29-55

Uhlig, S., Wollin, L., (1994): *An improved set-up for the isolated perfused rat lung.* J. of Pharmacology and Toxicological Methods, (31), 85-94

Upton, R.N., Doolette, D.J. (1999): *Kinetic aspects of drug disposition in the lung.* J. Clinical and Experimental Pharmacology and Physiology, (26), 381-391.

Van Wyk, B.E. & Gerike, N. (2000): *Medicinal Plants of South Africa.* 2nd edition. Pretoria: Briza Publications: 44-45.

Van Wyk, B.E. & Gerike, N. (2000): *People's plants, First edition.* Pretoria: Briza Publications: 142, 143, 205 and 216.

Waithaka, J. (2004): T*he Evaluation of Markers for Quality Control Studies of Flavonoid – Containing Medicinal Preparations*. Dissertation for the degree of Master's in Pharmacy at the University Of The Western Cape, Bellville.

Wang, F.M., Yao, T.W., Zeng, S. (2003): *Determination of quercetin and kaempferol in human urine after orally administrated tablet of ginkgo biloba extract by HPLC.* J. Pharmaceutical and Biomedical Analysis, (33). 317 - 321.

Wang, W., (1998): *Effect of inhaled cationic polypeptides on respiratory mechanics in the isolated perfused rat lungs*. Dissertation for a degree of Master's in Pharmacy at the University Of The Western Cape, Bellville.

Watt, J. M. & Breyer-Brandwijk, M.G. (1962): *The Medicinal and Poisonous Plants of Southern and Eastern Africa*, 2nd edition. Livingstone, London: 199-202.

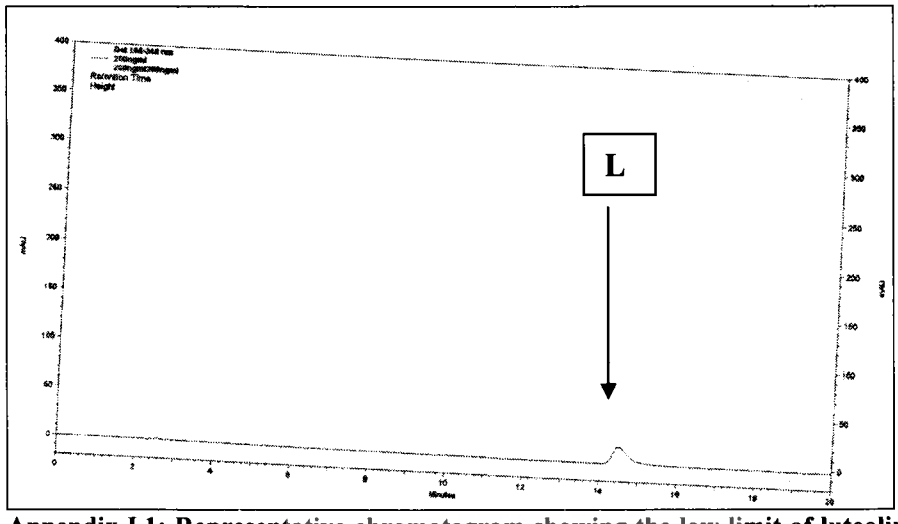
Wittemer, S. M. & Veit, M. (2003): *Validated method for the determination of six metabolites derived from artichoke leaf extract in human plasma by high performance liquid chromatography- coulometric-array detection.* J. Chromatography B, (793): 367-375.

Yamazaki, M., Ueda, H., Yamazaki, C. (2002): *Luteolin as an Anti-inflammatory and Anti-allergic Constituent of Perilla frutescens.* J. Biol. Pharm. Bull, 25(9): 1197-1202.

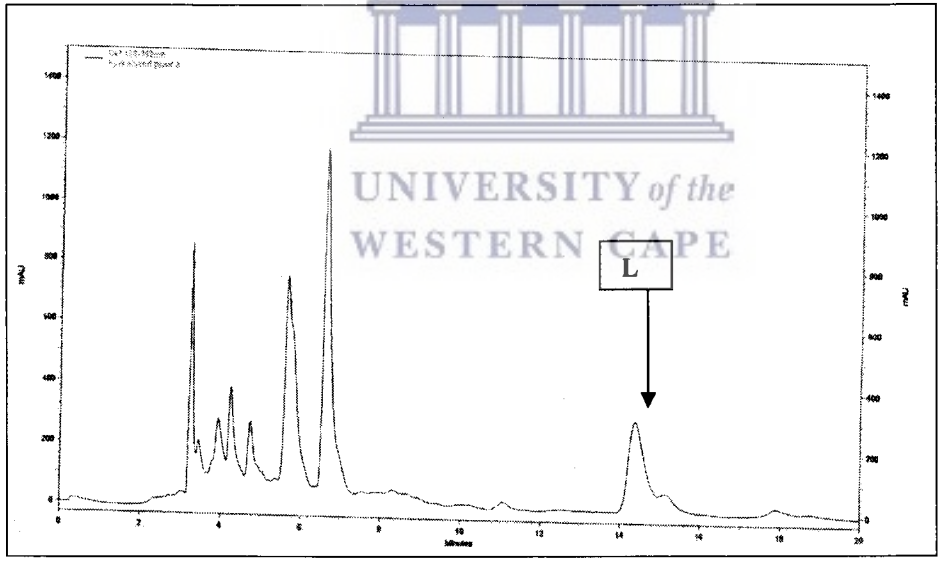
Zheng, X. M., Martin, G. P., Marriot, C., (1995): *The controlled delivery of drugs to the lung*. International Journal of Pharmaceutics, (124), 149-164.

(www.mypharmacy.co.uk/health).

APPENDIX I

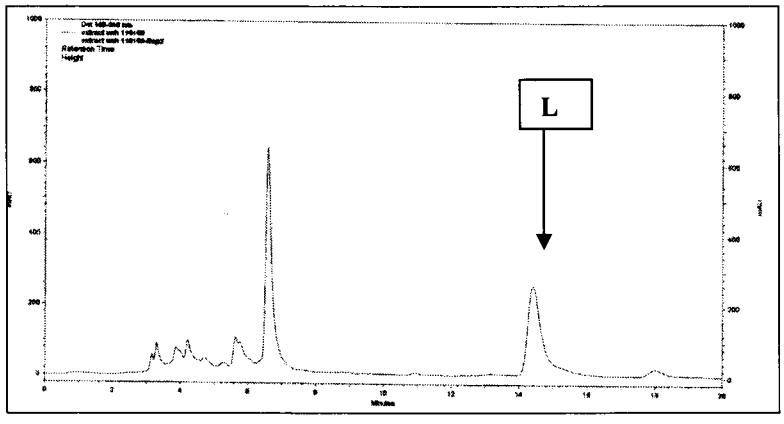


Appendix I.1: Representative chromatogram showing the low limit of luteolin diction (70ng on column)

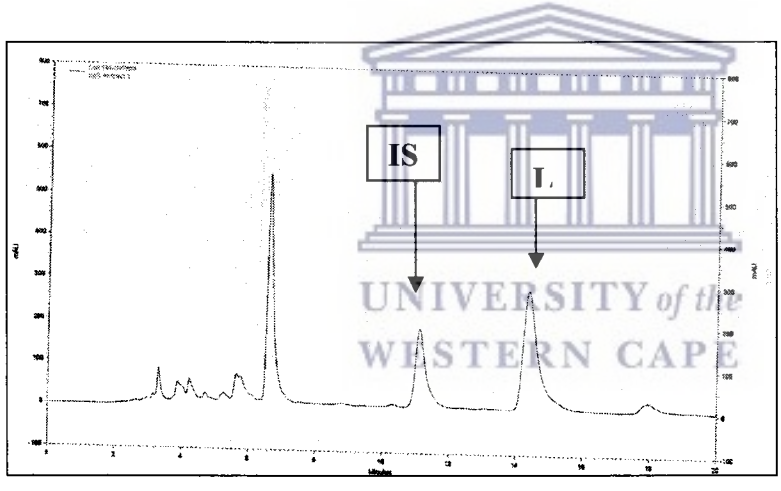


Appendix I.2: Representative chromatogram of unhydrolyzed extract without morin (IS)

APPENDIX II



Appendix II.1: Representative chromatogram of unhydrolyzed dried leaves without morin (IS)



Appendix II.2: Representative chromatogram of unhydrolysed dried leaves with morin (IS)

APPENDIX III

The effect of steam inhalation on tidal volume

Appendix III.1: The effect of steam inhalation on tidal volume. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then exposed to steam produced by boiled 100ml of saline. Tidal volume was monitored for 30 minutes afterwards

Time (min)	Tidal volume (ml) obtained after 4 experiments (n=4)						% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	Mean	SEM			
10	4.40	4.60	4.700	4.533	4.558	0.0487			
20	4.10	4.30	4.430	4.310	4.285	0.0529			
23	3.80	3.90	4.200	4.333	4.058	0.0968	↓ 5.29	P<0.001	***
33	3.60	3.80	3.950	4.150	3.875	0.0901	↓ 9.57	P<0.001	***
43	3.60	3.70	4.000	3.905	3.801	0.0711	↓ 11.29	P<0.001	***
53	3.40	3.70	3.850	3.857	3.702	0.0828	↓ 13.61	P<0.001	***

Appendix III.2: The effect of steam inhalation on tidal volume. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then exposed to steam produced by boiled 10mg *Artemisia afra* dried leaves per ml of saline. Tidal volume was monitored for 30 minutes afterwards

Time (min)	Tidal volume (ml)								% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	5	6	Mean	SEM			
10	4.600	4.69	4.500	4.947	3.690	4.551	4.496	0.147			
20	4.200	4.21	4.416	4.562	3.480	4.277	4.191	0.129			
23	4.400	4.52	4.440	4.564	3.483	4.361	4.340	0.140	↑ 3.56	P<0.001	***
33	4.300	3.58	4.429	4.361	3.763	4.203	4.106	0.121	↓ 2.03	P<0.01	**
43	4.000	3.50	4.390	4.247	3.623	4.141	3.985	0.122	↓ 4.92	P<0.001	***
53	4.000	3.48	4.212	4.175	3.260	4.046	3.864	0.136	↓ 7.80	P<0.001	***

Appendix III.3: The effect of steam inhalation on tidal volume. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then exposed to steam produced by boiled 50mg *Artemisia afra* dried leaves per ml of saline. Tidal volume was monitored for 30 minutes afterwards

Time (min)	Tidal volume (ml)								% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	5	6	Mean	SEM			
10	4.133	4.00	5.00	4.567	4.425	4.00	4.354	0.1352			
20	3.946	3.90	4.80	4.423	4.267	4.00	4.223	0.1201			
23	4.533	4.30	4.80	4.817	4.624	4.20	4.543	0.0879	↑ 7.590	P<0.001	***
33	4.237	4.50	4.40	4.869	4.514	3.90	4.403	0.1112	↑ 4.275	P<0.01	**
43	3.902	3.92	4.20	4.086	4.189	3.70	3.999	0.0671	↓ 5.284	P<0.01	**
53	3.764	4.00	4.10	4.082	4.012	3.50	3.909	0.0807	↓7.415	P<0.001	***

APPENDIX IV

The effect of steam inhalation on lung compliance

Appendix IV.1: The effect of steam inhalation on lung compliance. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then steamed for three minutes with normal saline steam. Lung compliance was monitored for 30 minutes afterwards

Time (min)	Tidal volume (ml) obtained after 4 experiments (n=4)						% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	Mean	SEM			
10	0.604	0.632	0.620	0.524	0.602	0.0190			
20	0.611	0.617	0.594	0.571	0.605	0.0081			
23	0.585	0.593	0.619	0.599	0.606	0.0058	↑ 0.17	P > 0.05	NS
33	0.641	0.582	0.602	0.588	0.610	0.0104	↑ 0.83	P > 0.05	NS
43	0.613	0.610	0.612	0.509	0.597	0.0200	↓ 1.32	P > 0.05	NS
53	0.592	0.602	0.603	0.529	0.596	0.0138	↓ 1.49	P > 0.05	NS

Appendix IV.2: The effect of steam inhalation on tidal volume. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then steamed for three minutes with normal saline steam. Tidal volume was monitored for 30 minutes afterwards

Time (min)	Compliance (cmH_20/ml)								% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	5	6	Mean	SEM			
10	0.596	0.550	0.636	0.673	0.549	0.614	0.619	0.0169			
20	0.592	0.510	0.670	0.651	0.536	0.631	0.615	0.0223			
23	0.640	0.530	0.575	0.675	0.641	0.653	0.636	0.0191	↑ 3.42	P < 0.05	*
33	0.647	0.620	0.619	0.634	0.616	0.548	0.631	0.0121	↑ 2.60	P < 0.05	*
43	0.712	0.606	0.592	0.606	0.555	0.559	0.622	0.0198	↓ 1.14	P >0.05	NS
53	0.606	0.506	0.519	0.653	0.576	0.537	0.583	0.0196	↓ 5.20	P<0.01	**

Appendix IV.3: The effect of steam inhalation on lung compliance. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then steamed for three minutes with 50mg/ml *Artemisia afra* steam. Lung compliance was monitored for 30 minutes afterwards

Time (min)	Compliance (cmH_20/ml)								% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	5	6	Mean	SEM			
10	0.557	0.628	0.642	0.593	0.574	0.642	0.606	0.0126			
20	0.540	0.634	0.659	0.587	0.459	0.643	0.587	0.0264			
23	0.628	0.754	0.667	0.691	0.519	0.660	0.656	0.0269	↑ 11.76	P<0.001	***
33	0.630	0.714	0.662	0.672	0.581	0.607	0.646	0.0166	↑ 10.05	P<0.001	***
43	0.603	0.708	0.700	0.656	0.702	0.604	0.662	0.0169	↑ 12.78	P<0.001	***
53	0.600	0.717	0.694	0.659	0.716	0.605	0.665	0.0183	↑ 13.29	P<0.001	***

APPENDIX V

The effect of steam inhalation on lung resistance

Appendix V.1: The effect of steam inhalation on lung compliance. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then steamed for three minutes with normal saline steam. Lung compliance was monitored for 30 minutes afterwards

Time (min)	Lung resistance (cmH2O.s/ml)						% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	Mean	SEM			
10	0.393	0.316	0.350	0.395	0.364	0.0147			
20	0.365	0.472	0.415	0.335	0.397	0.0233			
23	0.478	0.439	0.453	0.322	0.423	0.0268	↑ 6.55	P < 0.05	*
33	0.466	0.460	0.430	0.347	0.426	0.0212	↑ 7.31	P < 0.05	*
43	0.439	0.405	0.392	0.354	0.398	0.0136	↑ 0.25	P > 0.05	NS
53	0.450	0.464	0.375	0.365	0.414	0.0196	↑ 4.28	P >0.05	NS

Appendix V.2: The graph showing the effect of steam inhalation on lung resistance. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then steamed (S) for three minutes with 10mg/ml *Artemisia afra* steam. Lung resistance was monitored for 30 minutes afterwards.

Time (min)	Resistance (cmH20.s/ml)								% Change vs. 20 min	P value	Level of Significance
	1	2	3	4	5	6	Mean	SEM			
10	0.375	0.382	0.499	0.329	0.329	0.321	0.372	0.0232			
20	0.377	0.417	0.477	0.347	0.397	0.330	0.391	0.0183			
23	0.312	0.576	0.413	0.294	0.271	0.249	0.353	0.0426	↓ 9.72	P < 0.05	*
33	0.439	0.531	0.490	0.335	0.310	0.373	0.413	0.0304	↑ 5.63	P > 0.05	NS
43	0.428	0.495	0.538	0.342	0.375	0.381	0.426	0.0263	↑ 8.95	P > 0.05	NS
53	0.435	0.506	0.660	0.320	0.349	0.405	0.446	0.0426	↑ 14.07	P<0.01	**

Appendix V.3: The graph showing the effect of steam inhalation on lung resistance. The lungs *(n=6)* were allowed to equilibrate for 20 minutes then steamed (S) for three minutes with 50mg/ml *Artemisia afra* steam. Lung resistance was monitored for 30 minutes afterwards.

Time (min)	Resistance (cmH20.s/ml)								% Change vs. 20 min	P value	Level of Significance
	1	2	3	4	5	6	Mean	SEM			
10	0.349	0.332	0.451	0.341	0.354	0.343	0.364	0.0153			
20	0.362	0.369	0.378	0.366	0.363	0.344	0.364	0.0039			
23	0.341	0.322	0.349	0.332	0.304	0.297	0.324	0.0071	↓ 10.98	P< 0.05	*
33	0.364	0.372	0.312	0.368	0.367	0.329	0.352	0.0087	↓ 3.30	P>0.05	NS
43	0.491	0.512	0.343	0.502	0.380	0.437	0.444	0.0242	↑ 21.98	P<0.01	**
53	0.562	0.560	0.454	0.561	0.376	0.484	0.500	0.0262	↑ 37.36	P<0.001	***

APPENDIX VI

The effect of intravenously administered luteolin (2µg/ml) on lung function

Appendix VI.1: The effect of intravenously administered luteolin on tidal volume. The lungs were allowed to equilibrate for 20 minutes in a closed system with luteolin-free perfusate, then at 20 minutes perfusate was changed 100ml perfusate containing 2µg luteolin per ml. Tidal volume was immediately recorded. Each data point represent Mean and SEM for 5 experiments (*n=5*)

Time (min)	Tidal volume (ml)							% Change (vs. 20 min)	P value	Level of significance
	1	2	3	4	5	Mean	SEM			
10	4.770	4.370	3.365	3.32	5.42	4.306	0.30892			
20	4.436	4.23	3.654	3.36	5.23	4.182	0.26581			
23	3.917	3.83	3.37	3.3	5.32	3.947	0.29728	↓ 5.61	P > 0.05	NS
25	4.200	3.75	3.25	3.17	5.05	3.884	0.28220	↓ 7.12	P > 0.05	NS
30	3.933	3.85	3.36	3.30	4.87	3.863	0.23015	↓ 7.64	P > 0.05	NS
35	4.083	3.77	3.45	3.23	4.63	3.833	0.20099	↓ 8.36	P < 0.05	*
50	4.100	3.47	3.417	3.28	4.35	3.723	0.17216	↓ 10.97	P<0.001	***
65	3.833	3.38	3.367	23.43	4.21	3.644	0.13519	↓ 12.87	P<0.001	***
80	3.867	3.2	3.35	3.30	4.03	3.549	0.13612	↓ 15.13	P<0.001	***
95	3.850	3.32	3.36	3.18	4.43	3.628	0.18796	↓ 13.25	P<0.001	***
110	3.860	3.35	3.355	3.36	4.12	3.609	0.13137	↓ 13.70	P<0.001	***

Appendix VI.2: The effect of intravenously administered 2µg luteolin per ml on lung compliance.

Time (min)	Compliance (ml/cmH_2O)							% Change (vs. 20 min)	P value	Level of significance
	1	2	3	4	5	Mean	SEM			
10	0.389	0.531	0.570	0.542	0.435	0.4934	0.03461			
20	0,426	0.558	0.576	0.575	0.399	0.5068	0.03887			
23	0.544	0.557	0.535	0.594	0.328	0.5116	0.04699	↑ 0.95	P > 0.05	NS
25	0.681	0.554	0.52	0.587	0.262	0.5208	0.07005	↑ 2.76	P > 0.05	NS
30	0.646	0.547	0.518	0.581	0.299	0.5182	0.05880	↑ 2.25	P > 0.05	NS
35	0.701	0.551	0.52	0.595	0.308	0.5349	0.06447	↑ 5.56	P > 0.05	NS
50	0.902	0.574	0.559	0.611	0.458	0.6208	0.07423	↑ 22.49	P > 0.05	NS
65	1.118	0.593	0.577	0.826	0.737	0.7702	0.09847	↑ 51.97	P < 0.05	*
80	1.912	0.667	0.588	0.646	0.979	0.9584	0.24794	↑ 89.11	P < 0.05	*
95	1.655	0.628	0.576	0.665	1.506	1.0059	0.23607	↑ 98.48	P<0.001	***
110	1.488	0.672	0.569	0.693	1.565	0.9974	0.21736	↑ 96.73	P<0.001	***

Appendix VI.3: The effect of intravenously administered 2µg luteolin per ml on lung resistance.

Time (min)	Resistance (cmH_2O.s/ml)							% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	5	Mean	SEM			
10	0.667	0.204	0.535	0.673	0.648	0.5454	0.08895			
20	0.570	0.310	0.589	0.753	0.566	0.5576	0.07095			
23	0.577	0.492	0.638	0.747	0.650	0.6074	0.03693	↑ 8.93	P < 0.05	*
25	0.770	0.361	0.52	0.548	0.532	0.5462	0.06529	↓ 2.05	P > 0.05	NS
30	0.356	0.574	0.231	0.602	0.267	0.4060	0.07716	↓ 27.19	P<0.001	***
35	0.615	0.486	0.469	0.547	0.353	0.4940	0.04360	↓ 11.41	P < 0.05	*
50	0.714	0.232	0.566	0.612	0.337	0.4922	0.08966	↓ 11.73	P<0.01	**
65	0.506	0.238	0.591	0.743	0.292	0.4740	0.09378	↓ 14.99	P<0.01	**
80	0.542	0.167	0.585	0.628	0.181	0.4206	0.10161	↓ 24.57	P<0.001	***
95	0.554	0.300	0.592	0.571	0.627	0.5288	0.05848	↓ 5.170	P > 0.05	NS
110	0.565	0.424	0.557	0.635	0.396	0.5154	0.04534	↓ 7.57	P > 0.05	NS

APPENDIXVII

The effect of intravenously administered luteolin (10µg/ml) on lung function

Appendix VII.1: The effect of intravenously administered luteolin on tidal volume. The lungs were allowed to equilibrate for 20 minutes in a closed system with luteolin-free perfusate, then at 20 minutes perfusate was changed 100ml perfusate containing 10µg luteolin per ml. Tidal volume was immediately recorded. Each data point represent Mean and SEM for 5 experiments (*n=5*)

Time (min)	Experiments							% Change (vs. 20 min)	P value	Level of significance
	1	2	3	4	5	Mean	SEM			
10	5.240	4.410	4.733	4.754	4.794	4.786	0.10823			
20	4.864	4.121	4.255	4.670	4.413	4.465	0.11053			
23	4.350	3.580	3.300	4.300	3.743	3.855	0.16725	↓ 13.66	P<0.001	***
25	4.500	3.317	3.383	4.583	3.733	3.903	0.22071	↓ 12.59	P<0.001	***
30	4.400	3.280	3.050	4.433	3.577	3.748	0.23309	↓ 16.06	P<0.001	***
35	4.383	3.330	3.117	4.583	3.610	3.805	0.23639	↓ 14.78	P<0.001	***
50	4.171	3.514	3.100	4.617	3.595	3.799	0.21748	↓ 14.92	P<0.001	***
65	4.117	3.167	3.233	4.217	3.506	3.648	0.17959	↓ 18.30	P<0.001	***
80	3.917	3.133	2.983	4.100	3.344	3.496	0.17889	↓ 21.70	P<0.001	***
95	3.683	3.050	2.817	4.183	3.183	3.383	0.20009	↓ 24.23	P<0.001	***
110	3.600	3.120	2.920	4.114	3.213	3.393	0.17255	↓ 24.01	P<0.001	***

Appendix VII.2: The effect of intravenously administered 10µg luteolin per ml on lung compliance.

Time (min)	Compliance (ml/cmH_2O)							% Change (vs. 20 min)	P value	Level of significance
	1	2	3	4	5	Mean	SEM			
10	0.491	0.575	0.508	0.768	0.525	0.573	0.0414			
20	0.464	0.552	0.472	0.689	0.496	0.535	0.0339			
23	0.468	0.576	0.460	0.719	0.501	0.545	0.039	↑ 1.87	P > 0.05	NS
25	0.467	0.606	0.462	0.704	0.512	0.550	0.0378	↑ 2.80	P > 0.05	NS
30	0.484	0.615	0.537	0.708	0.545	0.578	0.0316	↑ 8.04	P > 0.05	NS
35	0.508	0.658	0.587	0.712	0.584	0.610	0.0285	↑ 14.02	P<0.001	***
50	0.539	0.699	0.639	0.729	0.626	0.646	0.0268	↑ 20.75	P<0.001	***
65	0.615	0.769	0.661	0.754	0.683	0.696	0.0236	↑ 30.09	P<0.001	***
80	0.855	0.911	0.974	0.807	0.913	0.892	0.0232	↑ 66.73	P<0.001	***
95	1.139	1.026	1.135	0.859	1.100	1.052	0.0427	↑ 96.64	P<0.001	***
110	1.211	1.204	1.256	0.968	1.224	1.172	0.0424	↑ 119.07	P<0.001	***

Appendix VII.2: The effect of intravenously administered 10µg luteolin per ml on lung resistance.

Time (min)	Resistance (cmH_2O.s/ml)							% Change (vs. 20 min)	P value	Level of Significance
	1	2	3	4	5	Mean	SEM			
10	0.320	0.244	0.195	0.571	0.218	0.310	0.0560			
20	0.562	0.377	0.244	0.557	0.324	0.413	0.0519			
23	0.681	0.616	0.412	0.354	0.756	0.564	0.0634	↑ 32.2	P<0.001	***
25	0.851	0.617	0.671	0.512	0.330	0.596	0.0705	↑ 44.31	P<0.001	***
30	0.621	0.417	0.333	0.602	0.298	0.454	0.0548	↑ 9.93	P < 0.05	*
35	0.775	0.592	0.499	0.339	0.501	0.541	0.0582	↑ 30.99	P<0.001	***
50	0.611	0.657	0.456	0.513	0.904	0.628	0.0633	↑ 52.06	P<0.001	***
65	0.688	0.624	0.763	0.863	0.422	0.672	0.0605	↑ 62.71	P<0.001	***
80	0.631	0.513	0.468	0.323	0.441	0.475	0.0409	↑ 15.01	P<0.01	**
95	0.472	0.369	0.265	0.421	0.371	0.380	0.0280	↓ 7.99	P > 0.05	NS
110	0.441	0.361	0.264	0.513	0.377	0.391	0.0340	↓ 5.33	P > 0.05	NS

APPENDIX VIII

Percentage changes after administration of luteolin intravenously

Appendix VIII.1: The percentage changes in lung function after perfusing lungs with 100ml perfusate containing either 2 or 10 µg per ml of perfusate in IPL. Each data point was recorded at the same time as when the luteolin levels were determined.

Time (min)	Tidal volume (ml)		Compliance (ml/cmH_2O)		Resistance ($cmH_2O.s/ml$)	
	2µg/ml	10µg/ml	2µg/ml	10µg/ml	2µg/ml	10µg/ml
10						
20						
23	↓ 5.61	↓ 13.66	↑ 0.95	↑ 1.87	↑ 8.93	↑ 32.2
25	↓ 7.12	↓ 12.59	↑ 2.76	↑ 2.80	↓ 2.05	↑ 44.31
30	↓ 7.64	↓ 16.06	↑ 2.25	↑ 8.04	↓ 27.19	↑ 9.93
35	↓ 8.36	↓ 14.78	↑ 5.56	↑ 14.02	↓ 11.41	↑ 30.99
50	↓ 10.97	↓ 14.92	↑ 22.49	↑ 20.75	↓ 11.73	↑ 52.06
65	↓ 12.87	↓ 18.30	↑ 51.97	↑ 30.09	↓ 14.99	↑ 62.71
80	↓ 15.13	↓ 21.70	↑ 89.11	↑ 66.73	↓ 24.57	↑ 15.01
95	↓ 13.25	↓ 24.23	↑ 98.48	↑ 96.64	↓ 5.170	↓ 7.99
110	↓ 13.70	↓ 24.01	↑ 96.73	↑ 119.07	↓ 7.57	↓ 5.33

Appendix VIII.1: Data showing the total luteolin (free + conjugated) , free and conjugated levels of luteolin after continuous perfusion with a 100ml perfusate containing 2µg/ml luteolin for 5 experiments.

Time (min)	Total levels (µg/ml) (free + conjugated) Mean± SEM	Free levels (µg/ml) (aglycone) Mean± SEM	Conjugated (µg/ml) (total-free) Mean± SEM
0	0.000± 0.0000	0.000± 0.0000	0.000±0.0000
3	1.473±0.1720	1.101±0.1460	0.372±0.1058
5	1.648±0.0423	1.183±0.0461	0.457±0.0671
10	1.568±0.0263	0.906±0.1221	0.662±0.1629
15	1.312±0.1180	0.878±0.0906	0.434±0.1381
30	0.979±0.0798	0.696±0.0515	0.283±0.1332
45	0.985±0.0689	0.653±0.0545	0.333±0.1053
60	0.876±0.0820	0.570±0.0323	0.306±0.1123
75	0.782±0.0702	0.584±0.0420	0.198±0.0783
90	0.777±0.5780	0.517±0.0508	0.261±0.0905

Appendix VIII.3: Data showing the total luteolin (free + conjugated) , free and conjugated levels of luteolin after continuous perfusion with a 100ml perfusate containing 2µg/ml luteolin for 5 experiments.

Time (min)	Total levels (µg/ml) (free + conjugated) Mean± SEM	Free levels (µg/ml) (aglycone) Mean± SEM	Conjugated (µg/ml) (total-free) Mean± SEM
0	0.000±0.0000	0.000±0.0000	0.000±0.0000
3	8.215±0.1164	7.335±0.1665	0.881±0.2603
5	8.328±0.2658	7.400±0.1647	0.928±0.4599
10	8.224±0.2709	7.227±0.1651	0.997±0.4628
15	7.610±0.1730	6.601±0.2239	1.009±0.4580
30	7.820±0.2681	6.806±0.1288	1.013±0.3772
45	7.758±0.2640	6.664±0.3274	1.094±0.5244
60	7.829±0.2643	6.839±0.2627	0.991±0.3501
75	7.831±0.4037	6.980±0.2197	0.851±0.4741
90	8.210±0.3464	7.229±0.1889	0.981±0.4599

APPENDIX IX

The luteolin levels obtained in the perfusate after IV administered luteolin

Appendix IX.1: **Average levels of total luteolin in re-circulated perfusion medium in the isolated perfused rat lung after the administration 100ml perfusion medium containing 2ug luteolin/ml solution.**

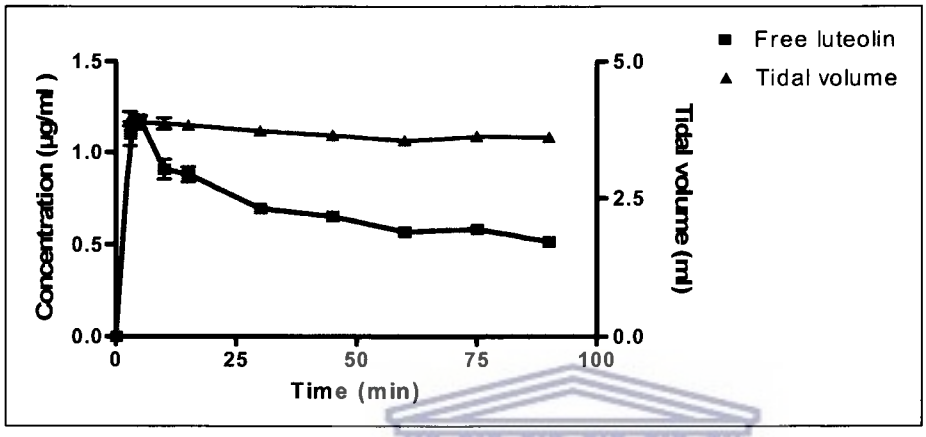
Time (min)	1	2	3	4	5	Mean	SEM
3	0.645000	1.759880	1.591105	1.774469	1.593369	1.472765	0.171970
5	1.567000	1.615210	1.763460	1.518767	1.774469	1.647781	0.042294
10	1.459200	1.658127	1.558663	1.593369	1.569209	1.567714	0.026263
15	1.398700	0.913779	1.156239	1.788450	1.301683	1.311770	0.117965
30	1.259000	1.120285	0.689642	0.925119	0.902890	0.979387	0.079746
45	1.076000	1.168047	0.714317	0.865862	1.099351	0.984716	0.068878
60	0.978900	1.176815	0.784133	0.572822	0.865862	0.875707	0.081963
75	0.987500	0.852634	0.707730	0.487260	0.872605	0.781546	0.070194
90	0.986500	0.893669	0.710441	0.595592	0.699413	0.777123	0.057983

Appendix IX.2: **The free (un-conjugated) luteolin levels obtained after the administration of 100ml perfusion medium containing 2ug/ml luteolin in the isolated perfused lung. The level of aglycone luteolin in each sample (unhydrolysed & extracted in ethyl acetate) was determined by HPLC assay.**

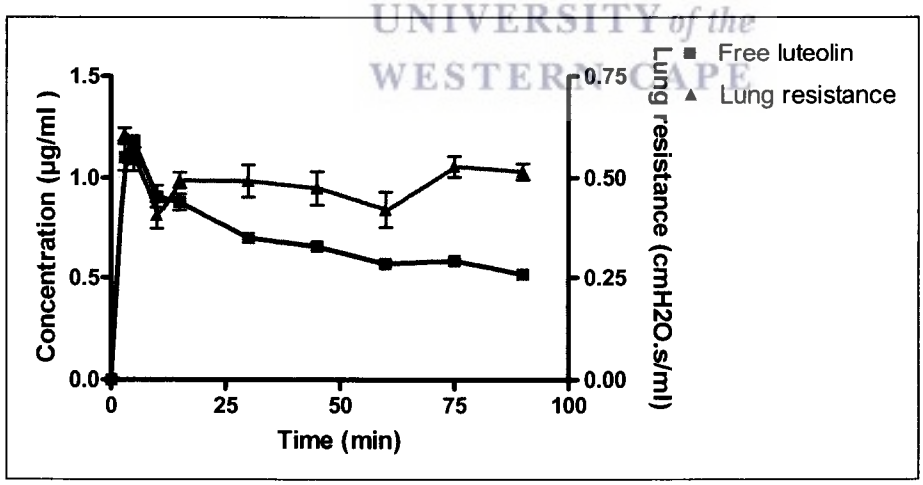
Time (min)	1	2	3	4	5	Mean	SEM
3	0.456000	1.448620	1.202440	1.005890	1.390180	1.100626	0.146039
5	1.032000	1.194351	1.090425	1.248675	1.350276	1.183145	0.046114
10	0.896400	0.449081	0.729959	1.187488	1.264396	0.905465	0.122139
15	0.723400	0.721235	0.678739	1.014396	1.251193	0.877792	0.090565
30	0.476700	0.786645	0.631672	0.806609	0.779406	0.696207	0.051523
45	0.453000	0.861171	0.709292	0.617878	0.617878	0.651844	0.054456
60	0.456800	0.589367	0.701236	0.539543	0.563252	0.570040	0.032345
75	0.534400	0.586555	0.699978	0.417429	0.680597	0.583792	0.042029
90	0.601300	0.352634	0.672740	0.388468	0.567568	0.516542	0.050811

APPENDIX X

Correlation between the disappearance over time of 2µg luteolin in the perfusate and the resultant effect on lung parameters



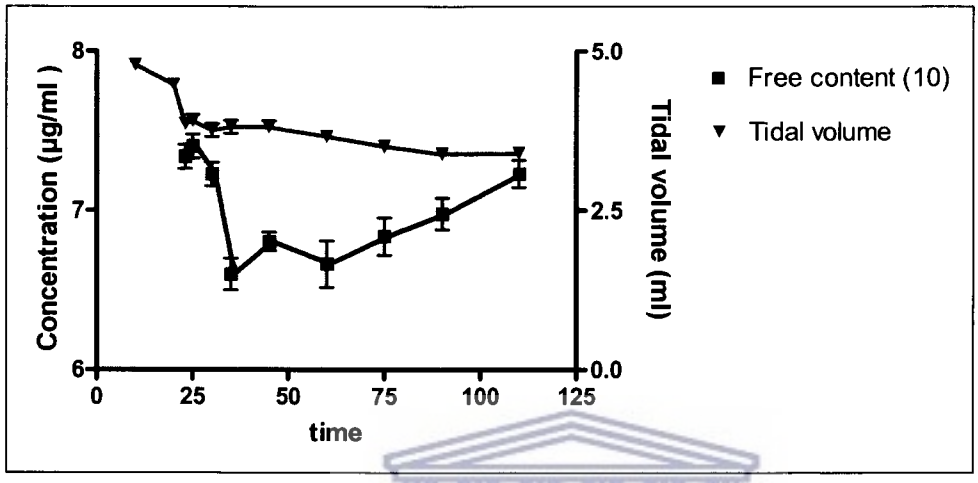
Appendix X.1: Graph showing the correlation between the levels of free luteolin obtained after continuous perfusion with 100ml perfusate containing 2µg luteolin per ml of perfusate in a closed system and the resultant effect produced on tidal volume. Each data point represents the mean ± SEM for 5 experiments



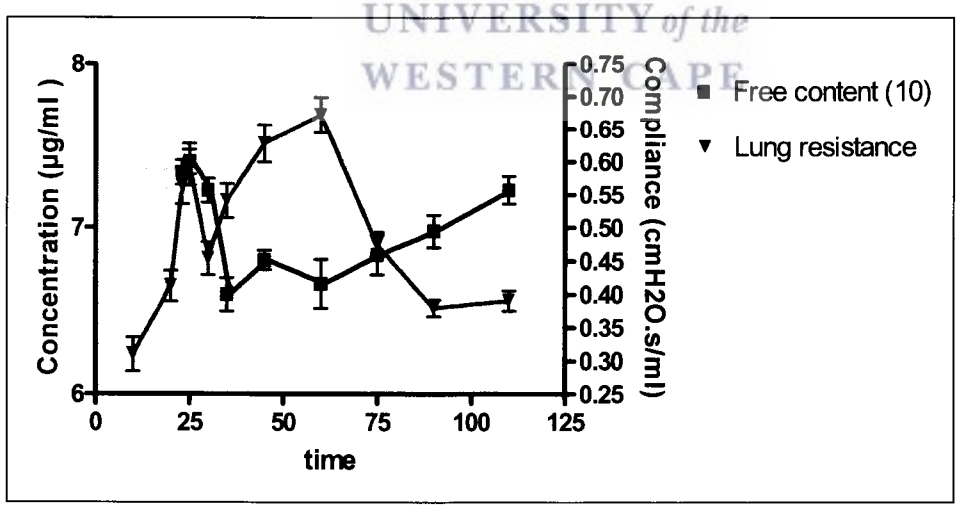
Appendix X.1: Graph showing the correlation between the levels of free luteolin obtained after continuous perfusion with 100ml perfusate containing 2µg luteolin per ml of perfusate in a closed system and the resultant effect produced on lung resistance. Each data point represents the mean ± SEM for 5 experiments

APPENDIX XI

Correlation between the disappearance over time of 10µg luteolin in the perfusate and the resultant effect on lung parameters

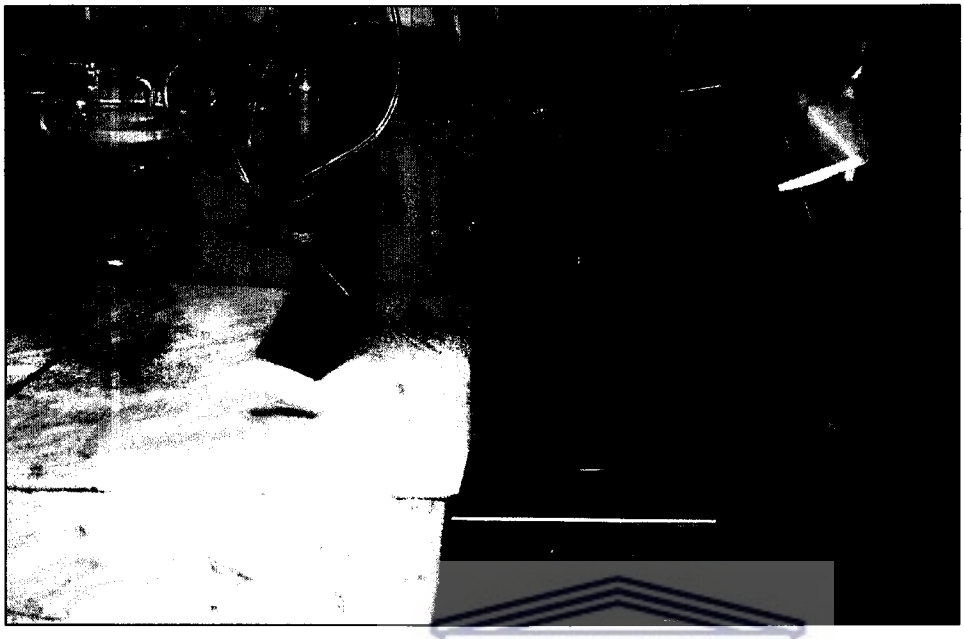


Appendix XI.1: Graph showing the correlation between the levels of free luteolin obtained after continuous perfusion with 100ml perfusate containing 2µg luteolin per ml of perfusate in a closed system and the resultant effect produced on tidal volume. Each data point represents the mean ± SEM for 5 experiments

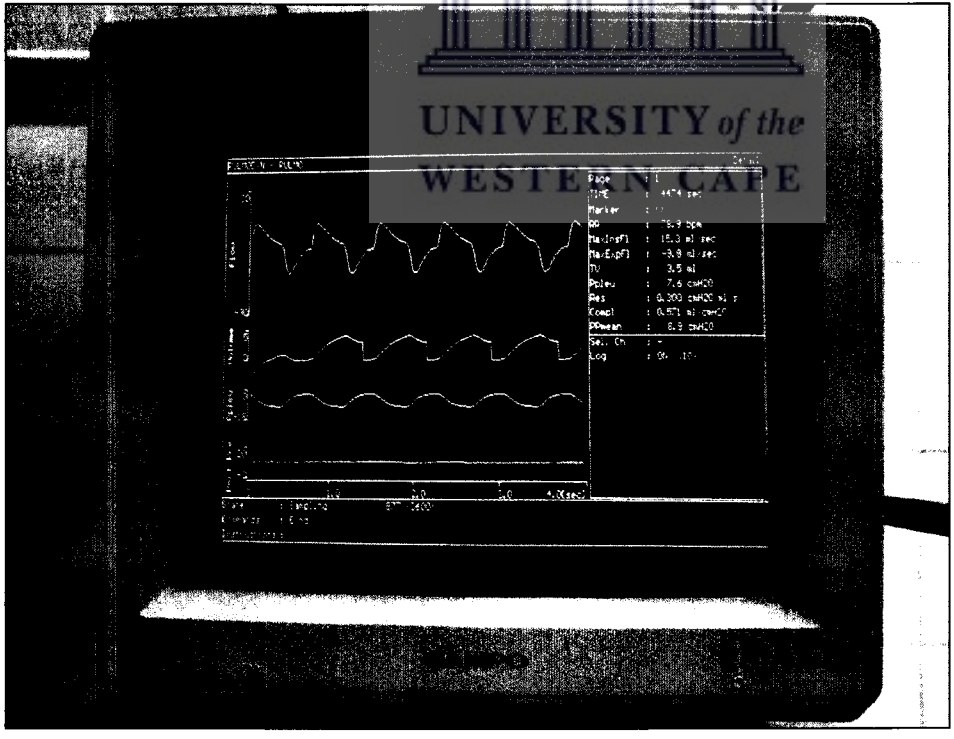


Appendix XI.2: Graph showing the correlation between the levels of free luteolin obtained after continuous perfusion with 100ml perfusate containing 2µg luteolin per ml of perfusate in a closed system and the resultant effect produced on lung resistance. Each data point represents the mean ± SEM for 5 experiments

APPENDIX XII



Appendix XII.1: Diagram showing the traditionally prepared *A. afra* steam



Appendix XII.2: Typical IPL computer showing the typical lung parameters monitored during

Brief introduction of the author:

Sizwe Joel Mjiqiza was born in Queenstown, Eastern Cape (RSA) and studied at Nkwanca Senior Secondary School until matriculation. He then enrolled for the Bachelor of Pharmacy degree at the University of the Western Cape (UWC) in 2001 and graduated in 2004. In 2005 he was among the first ever recipients of the Mandela Rhodes Scholarship and enrolled for M.Pharm as part of his academic internship.