# A COMPARATIVE STUDY OF THE PHOSPHODIESTERASE 4 INHIBITORY ACTIVITY OF *ARTEMISIA AFRA, LEONOTIS LEONORUS* AND *MENTHA LONGIFOLIA* PLANT MEDICINES

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**A thesis submitted in partial fulfillment of the requirements for the degree of Magister Pharmaceuticiae in the Discipline of Pharmacology at the University of the Western Cape, Bellville, South Africa.**

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**November 2007**

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## Key words

***Artemisia afra***

***Leonotis leonorus***

***Mentha longifolia***

**Phosphodiesterase**

**Cyclic adenosine monophosphate**

**Flavonoid**

**Luteolin**

**Hesperetin**

**Phenolic compound**

**Asthma**

# SUMMARY

Although *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia* have been shown to possess respiratory smooth muscle relaxant properties, little is known about the mechanisms for this effect. These plants contain flavonoids, which are reported to have phosphodiesterase (PDE), particularly PDE 4, inhibitory activity that can contribute to the plant actions. The smooth muscle relaxant activity of *Artemisia afra*, *L. leonorus* and *M. longifolia* may thus, be mediated via inhibition of cyclic adenosine monophosphate (cAMP) phosphodiesterase by, among others, the flavonoids.

The specific objectives of this study were to investigate whether *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia* have PDE 4 inhibitory activity, to determine and compare the levels of the total phenolic compounds, total and individual flavonoids, especially luteolin and hesperetin, in the three plants and, finally, to determine if there was a correlation between the PDE inhibitory activity and the levels of flavonoids or phenolic compounds in the plants. It was hypothesized that the plants with higher levels of total flavonoid and/or aglycone luteolin and/or hesperetin had higher PDE 4 inhibitory activity.

To realize the above objectives, freeze-dried aqueous extracts of the three plants were prepared, the PDE inhibitory activity of desalinated aqueous solutions of the extracts determined using the Biomol Green $^{TM}$ Phosphodiesterase Assay and the total flavonoid content (TFC), total phenolic compound content (TPC) and individual flavonoid marker levels of the extracts determined by the aluminium chloride, Folin Ciocalteu and an HPLC assay methods, respectively.

The desalinated aqueous solutions of all three species dose dependently inhibited PDE 4. The $IC_{50}$ for PDE-4 inhibition were 161.7 ± 0.01, 212.1 ± 0.04 and 337.1 ± 0.04 µg/ml for the desalinated unhydrolyzed extracts of *A. afra*, *L. leonorus* and *M. longifolia*, respectively, and 144 ± 0.358, 167.3 ± 0.037 and 280.2 ± 0.0329 µg/ml, respectively for the hydrolyzed extracts. Thus, the PDE 4 inhibitory potency order was *A. afra* > *L.*

*leonorus* > *M. longifolia*. Before desalination with Amberlite MB-1 ion exchanger resin, the unhydrolyzed *A. afra* extracts contained the highest levels of TFC (84.70 ± 3.88µg/mg), followed by *L. leonorus* (40.89 ± 0.02 µg/mg) and finally *M. longifolia* (30.4 ±1.08 µg/mg). After acid hydrolysis, the TFC for *L leonorus* & *M. longifolia* were significantly increased (92.60 ± 9.94 and 94.12 ± 3.94µg/mg, respectively) compared to the levels in the unhydrolyzed extracts. The highest TPC was registered in *M. longifolia* (672.66 ± 0 µg/mg) followed by *A. afra* (357.18 ± 8.99 µg/mg) and finally *L leonorus* (347.36 ± 3.04 µg/mg). On the other hand, acid-hydrolysis significantly increased the levels of TPC in all three plant extracts (489.36 ± 6.21, 371.72 ±11.5, and 833.41 ± 9.29 µg/mg) and treatment the Amberlite MB-1 decreased the plant extracts TPC. The unhydrolyzed and hydrolyzed aqueous extracts of *A. afra* and *L. leonorus* contained 0.85 ± 0.42 µg/mg, 0.92 ± 0.105 µg/mg and 0.427 ± 0.032 µg/mg, 0.559 ± 0.075 µg/mg, respectively. *M. longifolia* aqueous extract contained 0.147µg/mg of hesperetin which was not detected in the hydrolyzed extracts. Desalination treatment with Amberlite MB-1 resin caused a decrease in the levels of luteolin in *A. afra* and *L. leonorus* and hesperetin in *M. longifolia* extracts. There was no correlation between the TFC and the PDE 4 $IC_{50}$ of the plant extracts (r = - 0.18, p value = 0.46), but a significant correlation between the TPC, the individual flavonoid markers, luteolin and hesperetin, levels and the plants PDE 4 $IC_{50}$ ( r = 0.82, p value < 0.0001, - 0.724, p value = 0.023, respectively) suggesting that flavonoids and phenolic compounds may possibly contribute to the plant activity.

In summary, the desalinated aqueous solutions of *A. afra*, *L. leonorus* and *M. longifolia* have PDE 4 inhibitory activity and, flavonoids and phenolic compounds are likely to be involved in this activity of the plants.

# DEDICATION

**In memory of my deceased mother Henriette Kibwalwe for her love and support.**

**To my father Ernest Mulubwe for his love and encouragement.**

**To my brothers and sisters for their support and encouragement.**

# DECLARATION

I declare that the thesis A comparative study of the phosphodiesterase 4 inhibitory activity of *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia* plant medicines is my own work, that it has not been submitted before for any degree or examination in any other University and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

N. Mulubwe November 2007

Signed: UWC, Bellville

## ACKNOWLEDGEMENTS

I wish to gratefully acknowledge the following people:

- Prof J.A. Syce for his guidance, advice and expert supervision throughout the project which enabled me to perform the project.
- Mr. V. Jeaven and Mr. Y. Alexander for their assistance with laboratory equipment and materials.
- Mr. Timothy Lesch in the department of chemistry, for his assistance with HPLC experimental work
- Mr. Patrick Nawej and Mrs Mamitchou Nawej for their support and encouragement.
- Mr. Lucien Kasongo for his support and encouragement.
- All the staff in the Pharmacy school at UWC for their encouragement.
- My colleagues and friends; Max Ojangole, Pule Mothobi, James Mukinda, Caroline Kinyua, Anne Ngetish, Haiqiu Ma, Admire Dube, Sizwe Mjiqiza, Kenechukwu Obikeze, Esraa Taha, , Genine Stone, Nathalie Tendeshi and Christelle Kabeja for their friendship and support that made the completion of this thesis possible.

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# CHAPTER 1

## INTRODUCTION

African people have been using medicinal plants for thousands of years and it is estimated that 80% of South African population still use them for their health care. Despite their popularity and use over many centuries, only a few plants have been scientifically validated in South Africa (Springfield et al., 2005). There is, therefore, a need for data related to the quality, safety and efficacy of South African medicinal plants. In addition, the knowledge of these plants may allow the discovery of new alternatives for the treatment of many diseases. In fact, with the view to guarantee an acceptable and affordable health care to all countries, the World Health Organization (WHO) has encouraged the rational use of traditional medicines (Springfield et al., 2005). Since the world is technically well equipped today and has a better understanding of the physiology of the human body, it is easier and more convenient to elucidate the scientific rationale that may underpin the effectiveness of some of these plant medicines.



*Artemisia afra, Leonotis leonorus* and *Mentha longifolia* are among the most popular medicinal plants used in South African traditional medicine (Thring and Weitz, 2006). These plant species are traditionally used for the treatment of multiple and diverse ailments. However, they do have many common features such as some of their uses, claimed activities and constituents. For instance, all three species are traditionally used for asthma, and have been shown to possess respiratory smooth muscle relaxant properties. However, little is known about the mechanisms through which they produce this effect and the plant chemical constituents possibly involved. These three plant species contain flavonoids that are naturally occurring polyphenolic compounds and have a wide distribution in the plant kingdom (Ko et al., 2005). One of the flavones common to these plant species, viz. luteolin has been shown to possess tracheal muscle relaxant effect that may be mediated via inhibition of cAMP-phosphodiesterase (PDE) (Ko et al., 2005. Since these plants contain flavonoids, which are reported to inhibit the activity of phosphodiesterases, PDE inhibition could be one of the mechanisms by which they

produce their anti-asthmatic properties. However, this mechanism has not yet been investigated for these three species.

The major objective of this study was, therefore, to investigate whether *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia* have PDE inhibitory activity, and specifically the ability to inhibit PDE 4, which is predominant in asthma. The second objective was to determine the levels of the phenolic compounds, the total flavonoids and particularly luteolin or hesperetin that may have PDE 4 inhibitory activity and contribute to the plant activity. The final objective was to compare the PDE inhibitory activity of these three plant species and correlate it to the levels of phenolic compounds and flavonoids in the plants.

# CHAPTER 2

## LITERATURE REVIEW

In this chapter a brief description of the selected plant species *Artemisia afra, Leonotis leonorus* and *Mentha longifolia* is given as well as the chemistry, classification, biological activity and the analytical techniques suitable for the determination of flavonoids and total phenolic compound levels in plant materials are discussed. In addition, the definition and the current treatment of asthma and an overview of the molecular mechanism of action of cyclic nucleotides, the classification and structural biology of phosphodiesterases and the method to determine the phosphodiesterase inhibitory activity of compounds are presented.

### 2.1. *Artemisia afra, Leonotis leonorus* and *Mentha longifolia*

#### 2.1.1. *Artemisia afra* Jacq.

*Artemisia afra* (*A. afra*) belongs to the family Asteraceae and is known as African wormwood in English; als, alsem or wilde als in Afrikaans; umlhonyane in Xhosa and Zulu and lengana in Sotho and Tswana (Van Wyk et al., 2000).

*Artemisia afra* is a very common species and easily recognised medicinal plant in Southern Africa. It is an erect multi-stemmed perennial shrub of up to two meters in height. It has feathery leaves that are finely divided and usually have a greyish-green colour. Flowers that are pale yellowish are borne along the branch ends (Van Wyk et al., 2000). The plant's natural distribution stretches from the southern regions of Africa northwards into tropical east Africa, as far north as Ethiopia.

**Figure 2.1:** *Artemisia afra* (adapted from *Artemisia afra* Herba, 1999.)



Limited parts and only a few dosage forms of *Artemisia afra* are generally used. The leaves are mainly used but sometimes the roots are also used to treat the above-mentioned ailments. An infusion or decoction of *A. afra* leaves is generally prepared, often made syrupy with the addition of honey or sugar to mask the plant's natural bitter taste. Plugs of fresh leaves may also be inserted in the nostrils to act as decongestant, or the leaves may be boiled in water and the fumes inhaled (Thring and Weitz, 2006; Van Wyk et al., 2000).

*Artemisia afra* is also one of the oldest, best known and most widely used of all the indigenous medicines in Southern Africa (Thring and Weitz, 2006). It has diverse and multiple uses, being mainly used for the treatment of coughs, colds, influenza, asthma, pneumonia as well as fever, loss of appetite, colic, headache, earache, malaria and intestinal worms (Thring and Weitz, 2006; Van Wyk et al., 2000).

Indeed, *Artemisia afra* may have many useful pharmacological activities. Studies conducted on aqueous extracts of the plant have shown that it possesses bronchodilator

and anti-inflammatory activity and flavonoids are said to be responsible for some of these pharmacological activities of *A. afra* (Harris, 2002). In addition, *A. afra* may have narcotic analgesic and antihistaminic activities and the decongestant and antibacterial effects of its volatile oil are reputedly well known (Van Wyk et al., 2000).

Finally, little information is available on the chemical constituents of *Artemisia afra.* It contains many phenolic compounds, which are mostly dominated by flavonoids. For instance, luteolin, kaempferol, apigenin, hesperetin, and quercetin have been detected in aqueous extracts of *Artemisia afra* (Muganga, 2004). In addition, *A. afra* contains volatile oil that mainly consists of 1, 8-cineole, α-thujone, camphor and borneol (Van Wyk et al., 2000).

### 2.1.2. *Leonotis leonorus*

*Leonotis leonorus (L. leonorus)* belongs to the family of Lamiaceae and is known under different vernacular names such as wild dagga; Wilde dagga; lebake; umfincafincane and umhlahlampetu in English, Afrikaans, Zulu, Sotho, Xhosa and Shona, respectively.



**Figure 2. 2:** *Leonotis leonorus* (hhtp://www.plantzafrica.com/plantklm/leonotisleon.htm)

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

*L. leonorus* has a wide natural distribution over large parts of South Africa and has become a popular garden plant.

Numerous traditional uses have been reported for *L. leonorus*. It is used to treat colds, influenza, bronchitis, high blood pressure, headache, asthma and viral hepatitis. In addition, it is used to treat boils, eczema, skin diseases, itching, muscular cramps, snakebite, other bites and stings (Van Wyk and Gericke, 2000).

For *L. leonorus*, the leaves and stems are mainly used but sometimes also the roots. Decoctions of *Leonotis leonorus* are used externally for skin problems and internally, either by mouth or as an enema, to treat coughs, colds, fever, headaches and high blood pressure. In addition, leaf infusions have been used for asthma and viral hepatitis (Van Wyk and Gericke, 2000; Van Wyk et al., 2000; Thring and Weitz, 2006). Because it is only mildly narcotic, there are some doubts about early reports of the plant being smoked as a substitute for dagga. It has, however, been smoked for the relief of epilepsy. In Europe, this traditional phytomedicine is used for the symptomatic treatment of coughs in acute bronchial disease (Van Wyk and Gericke, 2000).

In addition to volatile oil and flavonoids, *Leonotis* species contain several unusual diterpenoids (labdane type lactones). A typical example of these is marrubiin, which has been isolated from *Leonotis leonorus* (Van Wyk et al., 2000).

### 2.1.3. *Mentha longifolia*

*Mentha longifolia* (*M. longifolia*) belongs to the family of Lamiaceae and is known as wild mint in English; ballerja and kruisement in Afrikaans; ufuthane lomhlange in Zulu; koena- ya- thaba in Sotho and inixina in Xhosa.

**Figure 2. 3:** *Mentha longifolia* (adapted from *Mentha longifolia* Herba, 1999.)

*Mentha longifolia* is a perennial herb with creeping rhizomes below the ground and erect flowering stems of up to 0.8 meter in height. All parts of the plant are highly aromatic with a strong typical mint smell. The leaves appear opposite each other in pairs along the stems, which are square in cross-section. Small white or pale purple flowers are borne in elongated clusters on the tips of the stems. Several different subspecies of *M. longifolia* are known, of which the best known one in South Africa is the subspecies *capensis* (Van Wyk et al., 2000).

*M. longifolia* is widely distributed in South Africa but is only found in wet places.

*M. longifolia* is used for many different ailments, mainly for coughs, colds, asthma, and other respiratory ailments. It has also been used to treat headaches, fevers, indigestion,

flatulences, and hysteria, painful menstruations, delayed pregnancy and for urinary tract infections. Externally, it has been used to treat wounds and swollen glands (Van Wyk et al., 2000).

*M. longifolia* has been reported to be a diaphoretic and antispasmodic. Its volatile oils possess decongestive, antibacterial and antiseptic effects (Van Wyk et al., 2000).

The leaves are mostly used but sometimes the stems and the rhizomes are also used. Infusions or decoctions of the leaves of *M. longifolia* are drunk or administrated as enemas. In addition, crushed leaves may be inserted in the nostrils for the relief of headache or placed under the bedding to reduce breathing problems (Van Wyk and Gericke, 2000; Van Wyk et al., 2000).

The composition of the active ingredients of the plant is known to vary considerably at different localities but no information appears to be available on South African plants (Van Wyk et al., 2000). The species contains hesperidin and several other flavonoids such as the glycosides of tricetin, vicenin, luteolin and isoorientin as well as aglycones such as luteolin (Sharaf et al., 1999; Ghoulami et al., 2001).

### 2.1.4. Common features of the three plants

*Artemisia afra, Leonotis leonorus* and *Mentha longifolia* are among the most widely used medicinal plants in South African traditional medicine for the treatment of many diseases. One claimed use they have in common is that of the treatment of asthma (Thring and Weitz, 2006). Appropriate validation of these claimed uses, including the treatment of asthma, is however lacking and requires the proper scientific study of these medicinal plants.

Studies conducted on *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia* have reported that they possess smooth muscle relaxant properties (Harris, 2002; Erasmus, 2004). Luteolin, one of the flavones found in these plants, has been shown to possess a

tracheal muscle relaxant effect that is not mediated via any of the following mechanisms: activation of β-adrenoceptor, activation of adenylate cyclase or guanylate cyclase nor the opening of adenosine triphosphate-sensitive potassium channels. The effect is also unrelated to the nitric oxide formation or the neuropeptides (Ko et al., 2005). The relaxant effect of luteolin may rather be mediated via the inhibition of cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-phosphodiesterases, and the subsequent increase of these two cyclic nucleotides (Ko et al., 2005). Because they contain luteolin and/or other flavonoids, the anti-asthmatic effect of *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia* may therefore be mediated via phosphodiesterase inhibition.

## 2.2. Asthma and Phosphodiesterases

### 2.2.1. Asthma

Asthma is an inflammatory disease of the airways characterized by recurrent reversible airway obstruction and hyperresponsiveness to non-specific stimuli such as air pollutants, cold air, exercise, allergens and infections (Das, 2003). Each of these stimuli evokes airway narrowing indirectly through the release of mediators from a variety of effector cells, including inflammatory cells and epithelial cells (Wong and Koh, 2000; Chung, 2006).

Some of the different inflammatory cells involved in asthma are predominant in asthmatic inflammation. Cumulative findings support the notion that T- helper 2 (Th 2) cells, B cells, mast cells and eosinophils contribute to the chronic inflammation of the airways and that mediators such as histamine, prostaglandins and leucotrienes contract airway smooth muscle, increase microvascular leakage, increase airway mucus secretion and attract other inflammatory cells (Chung, 2006; Sun et al., 2006). Asthma is also a chronic inflammatory disease, with inflammation persisting over many years in most patients.

Inhaled $\beta_2$ adrenergic receptor agonists and corticosteroids have represented the mainstay of the therapeutic management of asthma for at least 25 years. Beta-2 adrenoceptor agonists, which inhibit bronchoconstriction, would provide little more than symptomatic relief, while the anti-inflammatory effect of glucocorticoids may affect disease progression (Wenzel, 1998; Piaz and Giovannoni, 2000).

#### 2.2.1.1. Current treatment of asthma

There are two main categories of anti asthma drugs: the bronchodilators and the anti - inflammatory agents.

##### 2.2.1.1.1. Bronchodilators drugs

Three types of bronchodilators are used to treat asthma, namely the $\beta_2$- adrenoceptor agonists, the xanthines and the muscarinic receptor antagonists.

Drugs that act as selective $\beta_2$- adrenoceptor agonists are the first line bronchodilator agents for the treatment of asthma. The main actions of $\beta_2$- adrenoceptor agonists are bronchial smooth muscle relaxation, inhibition of mediators released from inflammatory cells, inhibition of cholinergic neurotransmission, reduced vascular permeability and increased mucociliary clearance (Rang et al., 2003). Considering their duration of action following inhalation of conventional doses, $\beta_2$- agonists can be divided into three broad groups: (a) the catecholamines, such as rimiterol, which have a very short action of 1-2 h; (b) those described as short acting, such as salbutamol and terbutaline, which are active for 3-6 h, although fenoterol may be slightly shorter acting and (c) the long-acting β-agonists salmeterol and formoterol, which cause bronchodilation for at least 12 h (Chung and Barnes, 1993). Finally, the $\beta_2$- adrenoceptor agonists are usually given by inhalation of aerosol, powder or nebulised solution, but some may also be given orally or by injection (Rang et al., 2003).

The xanthine bronchodilator group constitutes three pharmacologically active, naturally occurring methylxanthines, viz. theophylline, theobromine and caffeine. Theophylline (1, 3-dimethylxantine) is the most frequently employed in clinical medicine and can also be used as theophylline ethylenediamine, known as aminophylline. Theophylline has bronchodilator action, but it is rather less effective than the $\beta_2$- adrenoceptor agonists. Despite their widespread use, the mechanisms of action of the xanthine drugs in asthma are still unclear. The relaxant effect on bronchial smooth muscle is attributed to the inhibition of the phosphodiesterase (PDE) isoenzymes, with a resultant increase in cAMP. However, the concentrations necessary to inhibit the isolated enzyme greatly exceed the therapeutic range (Rang et al., 2003). Finally, the xanthine drugs are usually given orally in sustained-release preparations. Aminophylline can also be given by slow intravenous injection of a loading dose followed by intravenous infusion (Rang et al., 2003).

Finally, the main muscarinic receptor antagonist that is specifically used in asthma is ipratropium bromide. It relaxes bronchial constriction caused by parasympathetic stimulation, which occurs particularly in asthma induced by irritant stimuli and in allergic asthma (Rang et al., 2003). Ipratropium bromide inhibits the augmentation of mucus secretion that occurs in asthma and may increase the mucociliary clearance of bronchial secretions. Generally, ipratropium bromide is given by aerosol inhalation.

#### 2.2.1.1.2 Anti-inflammatory agents

Two different types of anti-inflammatory drugs are used in the treatment of asthma: the glucocorticoids that are mainly used in chronic conditions and sodium cromoglycate, which is thought to reduce bronchial hyper-reactivity.

Steroids are the most effective therapy currently available for asthma and inhaled glucocorticoids have become the mainstay of therapy for patients with chronic disease. Glucocorticoids are highly effective because they block many of the inflammatory pathways activated in asthma (Rang et al., 2003). They exert their effects by binding to

glucocorticoids receptors (GRs), which are localized in the cytoplasm of target cells and therefore may control inflammation by inhibiting many aspects of the inflammatory process. The main glucocorticoid compounds used are beclomethasone dipropionate, budesonide and fluticasone propionate, which are given by inhalation with a metered-dose inhaler, the full effect being attained only after several days of therapy (Rang et al., 2003).

Cromoglicate and the related drug nedocromil sodium are not bronchodilators. They do not have any indirect effects on smooth muscle, nor do they inhibit the actions of any of the known smooth muscle stimulants. If given prophylactically, they can reduce both the immediate and the late-phase asthmatic responses and reduce bronchial hyper-reactivity. They are effective in antigen-induced, exercise-induced and irritant-induced asthma. Cromoglicate is only given by inhalation, either as an aerosol, as a nebulised solution or in powder form (Rang et al., 2003).

#### 2.2.1.1.3 Other drugs



Leukotriene inhibitors and phosphodiesterase 4 inhibitors are the new classes of drugs for the treatment of asthma.

Leukotriene modifiers are an entirely new class of asthma treatment, which have entered clinical practice in 1996-7 in several countries including Britain, Japan, and the United States (Sampson and Holgate, 1998). Leukotriene synthesis inhibitors and cysteinyl leukotriene receptor antagonists constitute the two types of leukotriene modifier (Sampson and Holgate, 1998; Scow et al., 2007). Both are used to block the bronchoconstrictor and pro-inflammatory activity of cysteinyl leukotrienes within the asthmatic airway (Sampson and Holgate, 1998). The leukotriene receptor antagonists include zafirlukast and montelukast ; zileuton is the only leukotriene synthesis inhibitor (Scow et al., 2007). In the treatment of asthma, randomized controlled trials have shown leukotriene inhibitors to be more effective than placebo but less effective than inhaled corticosteroids or long-acting $beta_2$ agonists (Scow et al., 2007). Although their anti-inflammatory effects are likely to be less pronounced than those of high dose

corticosteroids, their excellent side effect profile and their availability as oral drugs are likely to ensure that compliance with treatment is substantially better than for inhaled corticosteroids (Sampson and Holgate, 1998). While interrupting the leukotriene pathway offers a new opportunity for treating asthma, the position of such drugs in the asthma armamentarium has not yet been firmly established. Further effectiveness studies are needed to determine the true value of this oral anti-asthma treatment (Sampson and Holgate, 1998).

Over the last decade, considerable attention has focused on cyclic nucleotide phosphodiesterase isozymes 4 as a molecular target for drugs useful for the treatment of pulmonary diseases (Torphy et al., 1999). From the beginning of 1990s, growing interest was devoted to identifying selective PDE 4 inhibitors and more recently, the critical role of various signal transduction pathways in mediating inflammatory cell responses has been confirmed. In general, an increase in the level of the intracellular second messenger cAMP is usually associated with the suppression of immune and inflammatory cells (Piaz and Giovannoni, 2000).



The type 4 cAMP specific isoenzyme, which was identified in 1985, is particularly abundant in the brain and immunocompetent cells. In the eosinophils, which are considered the effectors "par excellence" of asthma, PDE 4 is the only isoform present (Piaz and Giovannoni, 2000).

Inhibition of PDE 4 in all these cells produces a marked reduction of inflammatory immunomodulatory response. Moreover, *in vi*vo PDE 4 inhibitors reduce the tone of bronchial smooth muscle and produce bronchodilation induced by a variety of agents such as histamine, leukotrienes D4 (LTD 4), carbachol or methacholine. Taken together these data suggest a possible therapeutic utility of PDE 4 inhibitors as anti-inflammatory and immunomodulatory agents (Piaz and Giovannoni, 2000).

Traditionally, PDE 4 inhibitors are classified into three main chemical classes:

1. Catechol ethers, in which a wide variety of flexible molecules of inhibitors structurally related to rolipram are grouped;
2. Quinazolinediones, in which are classified the PDE 4 inhibitors structurally related to nitraquazone;
3. Xanthines, to which theophylline belongs.

The growing interest in the development of antiasthma drugs displaying both bronchodilatory and anti-inflammatory activity is motivated by reasons such as the adverse effects arising with the chronic administration of glucocorticoids, the systemic side effects such as tachycardia, palpitation and headache observed with inhaled β2-adrenoceptors and, finally, a possible reduced inhaled drug delivery due to airway obstruction subsequent to disease progression. There is a need for novel drugs that are able to compete with and, perhaps replace corticosteroids and $\beta_2$-adrenoceptor agonists in the therapeutic management of asthma (Piaz and Giovannoni, 2000). Since inflammation and bronchoconstriction constitute the major pathological features of asthma, the inhibition of PDE enzymes represents a promising therapeutic target (Lacombe et al., 2006).



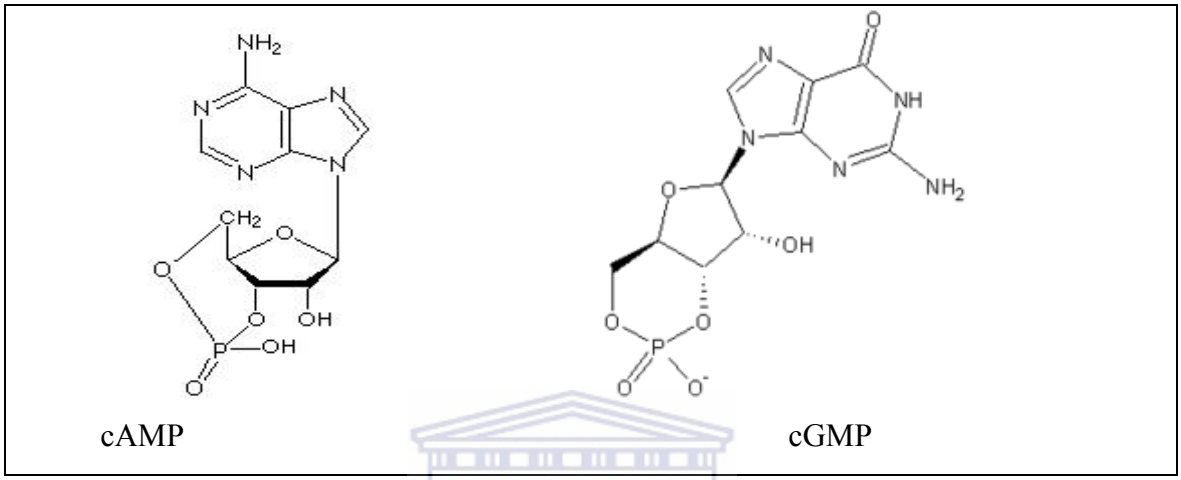
### 2.2.2. Phosphodiesterases (PDEs)

The phosphodiesterases (PDEs) are a superfamily of metalloenzymes which inactivate the intracellular second messenger cyclic nucleotides 3', 5'-cyclic adenosine monophosphate (cAMP) and 3', 5'-cyclic guanosine monophosphate (cGMP) (see figure 2.4) by catalyzing the hydrolytic cleavage of the 3'-phosphoester bond to form the corresponding 5'-nucleotide inactive product (Chung and Barnes, 1993; Piaz and Giovannoni, 2000; Chung, 2006). Inhibition of these enzymes leads to an increase in the intracellular level of cAMP and cGMP.

The understanding of PDE inhibition began with a series of publications in the late 1950s by Sutherland and Rall who described the properties of a cyclic adenine ribonucleotide later called cAMP (Essayan, 1999). More recently, many studies on the physiological

role of cAMP and cGMP have described their mechanism in the regulation of important cellular functions such as secretion and contraction (Piaz and Giovannoni, 2000).

### 2.2.2.1. Molecular mechanisms of cyclic nucleotides action




cAMP cGMP

**Figure 2.4:** chemical structures of the cyclic nucleotides c AMP and c GMP

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are ubiquitous intracellular second messengers that mediate the physiologic response to a host of hormones, neurotransmitters, autocoids and drugs. Cyclic nucleotides, especially cAMP, have important regulatory roles in virtually all cell types involved in the pathophysiology of asthma, as shown in figure 2.5 (Torphy, 1998; Essayan, 1999; Piaz and Giovannoni, 2000).

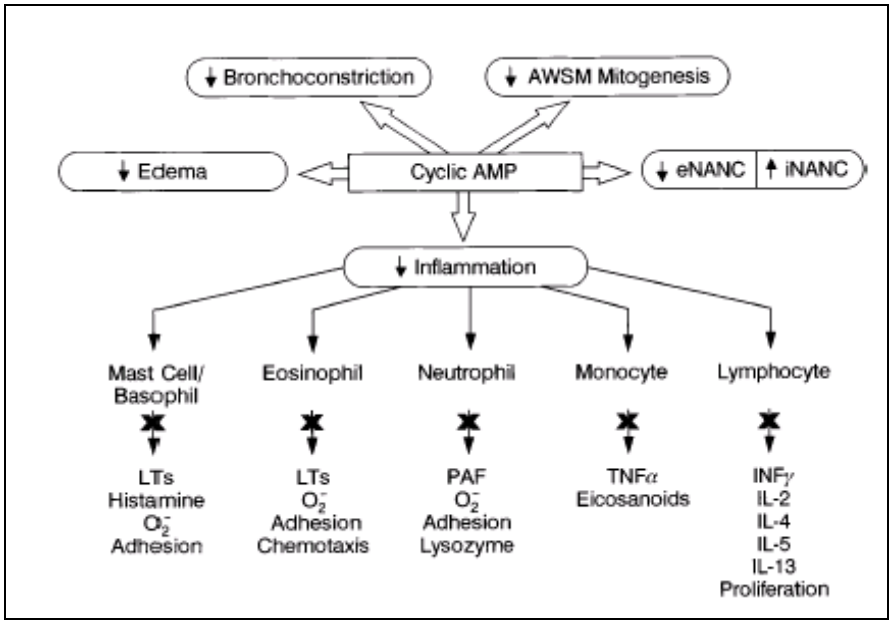



**Figure 2.5:** Proposed beneficial actions of cyclic AMP. The downward-pointing arrows represent inhibition and the upward-pointing arrow represents stimulation. Listed at the bottom of the figure are specific inflammatory cell functions inhibited by cyclic AMP. *Abbreviations*: AWSM *5* airway smooth muscle; eNANC *5* excitatory nonadrenergic noncholinergic neurotransmission; iNANC *5* inhibitory nonadrenergic noncholinergic neurotransmission; IL *5* interleukin; LT *5* leukotriene; IFN*g 5* interferon-gamma; O2 *2 5* superoxideanion; PAF *5* platelet-activating factor. (Torphy et al., 1999)

Cyclic AMP broadly suppresses the activity of virtually all immunocompetent cells. In addition, it induces airway smooth muscle relaxation, suppresses smooth muscle mitogenesis and has beneficial modulatory effects on the activity of pulmonary nerves (De Gonzales et al., 2004).

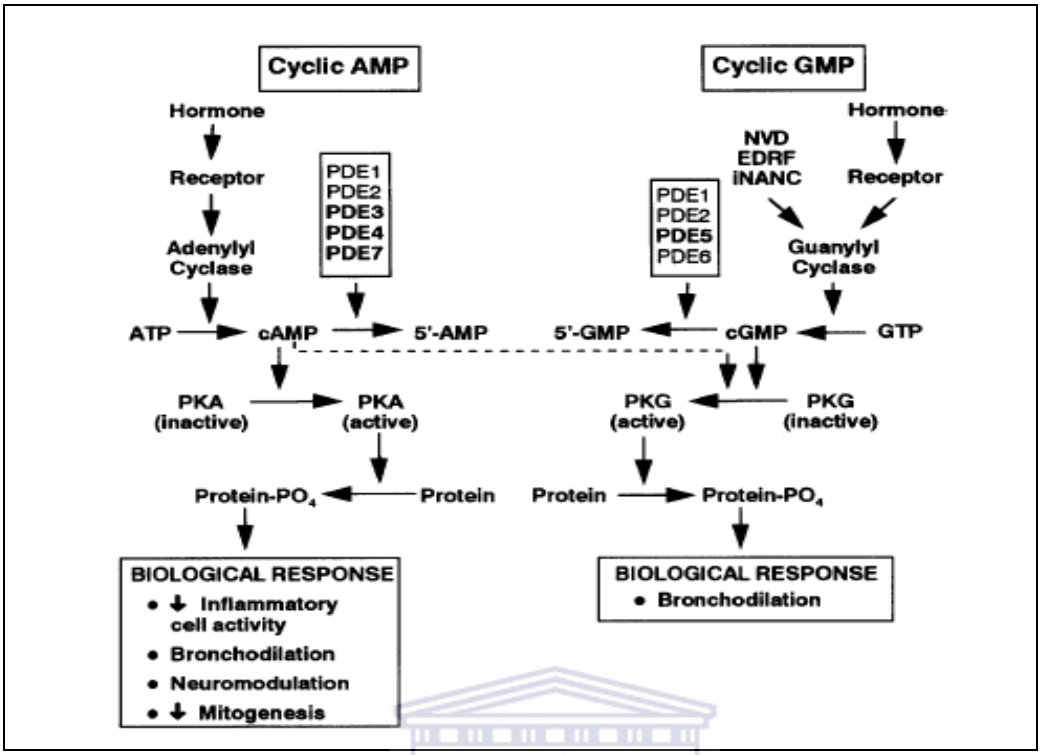



**Figure 2.6:** Cyclic AMP versus cyclic GMP second messenger pathways. Key PDE isozymes responsible for regulating cyclic AMP or cyclic GMP content in immunocompetent cells and airway smooth muscle are in *bold face*. *Abbreviations*: cAMP *5* cyclic AMP; cGMP *5* cyclic GMP; EDRF *5* endothelium-derived relaxant factor (i.e., NO); iNANC *5* inhibitory nonadrenergic noncholinergic nerves; PKA *5* cAMP-dependent protein kinase; PKG *5* cGMPdependent protein kinase; NVD *5* nitrovasodilators (Torphy, 1998)

Cyclic AMP and cyclic GMP are formed from ATP and GMP by the catalytic action of adenylyl cyclase and guanylyl cyclase, respectively (Torphy, 1998; Essayan, 1999). As the intracellular concentrations of the cyclic nucleotides rise, they bind to and activate their target enzymes, protein kinase A (PKA) and protein kinase G (PKG). These protein kinases phosphorylate substances (e.g., ion channels, contractile proteins, transcription factors) that regulate key cellular functions. Phosphorylation alters the activity of these substrates, and thus changes cellular activity, as shown in figures 2.6 and 2.7 (Chung and Barnes, 1993; Torphy, 1998).

Studies with airway smooth muscle suggest that activation of cAMP -dependent protein kinase can induce relaxation by (1) increasing $Ca^{2+}$-sequestration or extraction, (2)

preventing Ca $^{2+}$ influx, (3) hyperpolarizing cell membranes by activating $Na^+$, $K^+$-ATPase or opening large-conductance $K^+$ channels, or (4) direct modulation of contractile protein (Spina et al., 1998). On the other hand, inhibition of inflammatory cell activity following activation of cAMP-dependent protein kinase may be mediated via (1) inhibition of $Ca^{2+}$ influx and/or release, (2) stimulation of $Ca^{2+}$ extrusion, (3) direct inhibition of mediator release by a mechanism that is independent of changes in $Ca^{2+}$ levels, and (4) by interference with the production of certain cytokines. Moreover, it is unlikely that the functional response of any individual cell to an increase in cAMP accumulation is the result of a single biochemical mechanism. Instead, cell activation is likely to be regulated by the simultaneous or sequential activation of several cAMP – dependent pathways (Chung and Barnes, 1993).

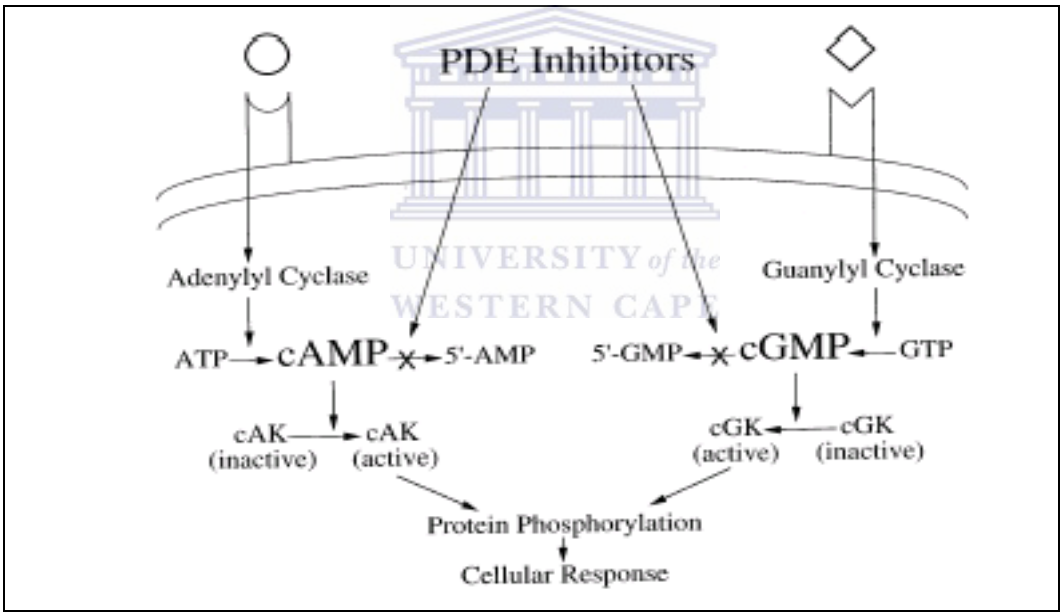



**Figure 2.7:** Cyclic nucleotide homeostasis. Abbreviations: cAK, cAMP-dependent protein kinase; and cGK, cGMP-dependent protein kinase (Essayan, 1999)

The mechanism(s) by which cGMP relaxes airway smooth muscle is thought to be analogous to that of cAMP. There is a corresponding decrease in protein kinase activity as cAMP or cGMP are inactivated by PDEs. In addition, phosphoprotein phosphatases dephosphorylate substrates and cellular activity return to normal. Obviously, altering the rate of cyclic nucleotides formation or degradation will thus change the activation state of these pathways (Chung and Barnes, 1993; Essayan, 1999). The elevation of the

intracellular levels of cyclic nucleotides by inhibition of phosphodiesterase isozymes therefore represents a useful strategy for eliciting a variety of pharmacological effects (Piaz and Giovannoni, 2000).

#### 2.2.2.2 Phosphodiesterase isozymes

As already mentioned, the understanding of PDE inhibition began with a series of publications in the late 1950s by Sutherland and Rall (Essayan, 2000). The anti-inflammatory potential of PDE inhibitors was first clearly demonstrated in a series of reports by Lichtenstein and colleagues in the early 1970s (Essayan, 2000). These findings indicated a presence of a complex family of PDE enzymes and suggested the potential utility of selectivity in a design of PDE inhibitors (Essayan, 2000).

It is now recognized that 11 families of PDEs exist, the major families being designated by Arabic numerals (Barber, 2004). Most of the families include more than one product as well as multiple splice variants. The GenBank nomenclature for PDEs includes the species and gene family designation (e.g. HSPDE4 for Homo sapien PDE isozyme 4) followed by the gene product or "subtype" (e.g. HSPDE4D) and the splice variant (e.g., HSPDE4D3) (Torphy, 1998). The families are also named according to the identity of the inhibitors (e.g., cyclic AMP) or the endogenous activators such as calmodulin (Torphy, 1998).

For some isoenzymes, endogenous regulators such as $Ca^{2+}$ and calmodulin, co-factors such as $Mg^{2+}$ and possibly $Zn^{2+}$ and synthetic selective inhibitors such as milrinone, siquazodan selective inhibitors of PDE 3, rolipram for PDE 4, zaprinast and sildenafil for PDE 5, are known (Piaz and Giovannoni, 2000).

Each family of phosphodiesterases is encoded by distinct genes (e.g., A, B, C or D for PDE 4) and within each of them, there are multiple isoforms expressed. There are at least 44 distinct human PDEs (Piaz and Giovannoni, 2000).

Amongst the PDEs, with respect to substrate, PDE 4, PDE 7 and PDE 8 are specific for cAMP, whereas PDEs 5, 6, 9 are selective for cGMP. The isoenzyme PDE 3 binds cAMP and cGMP with similar affinity, but it hydrolyses cGMP relatively poorly. At the same time, PDEs 1 and 2 hydrolyze both cyclic nucleotides, although with PDE 1, the relative efficiencies vary with isozymes subtypes (Perry and Higgs, 1998).

Over the last decade, compounds that display a marked degree of selectivity for one PDE isozyme over another have become available and have fuelled the hypothesis that PDE inhibitors could have therapeutic potential in a number of inflammatory diseases including asthma (Kelly et al., 1996). Furthermore, isoenzyme selective PDE inhibitors might be associated with reduced incidence of side effects compared to that of the non-selective drugs (e.g. theophylline) that indiscriminately inhibit all PDE isoenzymes (Kelly et al., 1996, Beasley et al., 1998). Indeed a vigorous pursuit of selective PDE 4 inhibitors has resulted.



#### 2.2.2.2.1 The PDE 4 Family

The PDE 4 family is currently the largest and four genes encode the PDE 4 isoenzyme. This group of isoenzymes is widely expressed in many tissues. However, very little information has been published to show exactly which gene is expressed in which tissue, or the localization of the splice variant. A number of tissues and cell types have received particular attention recently, including the lung lymphocytes. PDE 4 is likely to play a particular important role in inflammatory and immunomodulatory cells (Beavo, 1995). In addition, it is the predominant PDE in inflammatory cells, including mast cells, eosinophils, neutrophils, T cells, macrophages and in structural cells such as sensory nerves and epithelial cells relevant to the pathogenesis of asthma. PDE 4 inhibitors block the hydrolysis of cAMP, leading to elevation of intracellular cAMP levels and subsequent suppression of inflammatory activity and decrease of mediator release from these inflammatory cells (Ko et al., 2004; Chung, 2006).

#### 2.2.2.2.2. Methods to measure the PDE inhibitory activity of compounds

Several methods are mentioned in the literature for the measurement of the PDE – inhibitory activity of compounds. These include the BIOMOL GREEN™ phosphodiesterase assay, the method of Cook et al. (1995), the High Efficiency Fluorescence Polarization and the Phosphodiesterase on Flash Plate® from Perkin Elmer. These methods are discussed below.

##### 2.2.2.2.2.1. The BIOMOL GREEN ™ Phosphodiesterase assay

The BIOMOL Cyclic Nucleotide Phosphodiesterase (PDE) Assay is a colorimetric, non-radioactive assay designed in a microplate format that may be used to screen inhibitors and modulators of cyclic nucleotides (www.biomol.com).

The basis for the assay is the cleavage of cAMP or cGMP by a cyclic nucleotide phosphodiesterase. The 5'-nucleotide released is further cleaved into the nucleoside and phosphate by the enzyme 5'- nucleotidase. The phosphate released due to enzymatic cleavage is quantified using BIOMOL GREEN™ reagent, which is made of ammonium molybdate and malachite green.

The phosphate released reacts with ammonium molybdate in an acidic solution to form the phosphomolybdic acid. The molybdenum formed is then reduced by the malachite green in the phosphomolybdate to give a blue colour (Cogan et al., 1999; Wong, 2000). Using a spectrophotometer or a micro-titer plate reader the absorbance (at wavelength 620 to 670 nm) of this complex can easily be quantified (Simonovic et al., 2004). The amount of phosphate detected is indirectly proportional to the PDE inhibitory activity of the compound tested.

The BIOMOL GREEN™ phosphodiesterase assay is a non-radioactive highly sensitive method. It uses a 1-step reagent for phosphate detection and is convenient and suitable for microplate or cuvette assay.
Since the BIOMOL GREEN™ is a highly sensitive phosphate detection method, the effective removal of phosphate from the extracts to be tested and/or the labware and reagents solutions used is recommended to avoid any increase in the background absorbance of the assay (www.biomol.com).

#### 2.2.2.2.2.2. Method of Cook et al. (1995)

Homogeneous radiometric assays, such as scintillation proximity assays (SPA, Amersham Biosciences) and Flashplate technology (NEN/Perkin Elmer) enable the direct detection of [$^{125}$I]-labelled cAMP or [$^{3}$H] –labelled cAMP once it is in close proximity to a solid scintillant surface.

The method of Cook et al. (1995) is based on the breakdown of [$^{3}$H] cAMP by PDE to the corresponding labelled nucleoside 5´-monophosphate, which is subsequently dephosphorylated by alkaline phosphatase to give the labelled nucleoside (Ko et al, 2005). In tests of enzyme inhibition, the reaction mixture contains various concentrations of the inhibitor and the radioactivity of the labelled nucleoside is determined by liquid-scintillation counting in ACS II scintillant (Amersham).

These radiometric technologies are to some extent presently being superseded by safer non-radiometric read-outs, which might be cheaper and more readily miniaturized.

#### 2.2.2.2.2.3. Phosphodiesterase Assays on FlashPlate® Microplates

The Phosphodiesterase Assays on FlashPlate® Microplates method involves adding a source of phosphodiesterase and [$^{125}$I]-labelled cAMP or [$^{125}$I]-labelled cGMP to a FlashPlate well that contains an antibody to either cAMP or cGMP. The phosphodiesterase will catalyze the breakdown of the [$^{125}$I]-labelled cAMP or [$^{125}$I]-

labelled cGMP to their corresponding 5'nucleotides. The antibody will not bind to the catalyzed product, therefore the decrease in the amount of the [$^{125}$I] cAMP or [$^{125}$I] cGMP will be detected (Kasila, 2000).
To screen for inhibitors of phosphodiesterase, a source of phosphodiesterase, [$^{125}$I] cAMP or [$^{125}$I] cGMP, and the test compounds are added to a FlashPlate well as explained above. If the compound is found to be an inhibitor of phosphodiesterase, the catalyzed breakdown of cAMP or cGMP will not occur, therefore, a decrease in the amount of [$^{125}$I] cAMP or [$^{125}$I] cGMP will not occur (Kasila, 2000).

The Flashplate technology offers distinct advantages over more traditional methods in terms of convenience (stimulation and detection can be carried out in the same well), time and reproducibility. Although advances are still being made in this area for example, with the development of Red-Shifted Plates that aim to minimize compound artifacts, the radiometric technologies are however to some extent being superseded by safer non-radiometric read-outs, as mentioned above in the method of Cook, et al., (De Jong et al., 2005).



#### 2.2.2.2.2.4. The High-Efficiency Fluorescence Polarization (HEFP$^{TM}$) Phosphodiesterase assay

The fluorescence polarization (FP) measures the change in rotational speed of a ligand during its excited lifetime upon binding to its receptor. In the (HEFP$^{TM}$) PDE assay a fluorescent-labeled ligand is excited by polarized light and the polarization of the emitted light determined. The emitted light is largely depolarized in the presence of a small and therefore rapidly rotating ligand, while rotational speed and thus depolarization is significantly reduced when the ligand is bound to a receptor (De Jong et al., 2005).

The HEFP Phosphodiesterase assay measures the activity of PDEs that use cAMP or cGMP as substrates. The fluorescein (fluorophore)-labeled derivatives of c AMP (FL-cAMP) and cGMP (FL-cGMP) are hydrolyzed by PDE, and bind to the Binding Reagent. In this bound form, the fluorescence is highly polarized. This assay is designed to

determine an increase in activity of cyclic nucleotide-dependent PDE in solution (www.moleculardevices.com).

The HEFP Phosphodiesterase assay is a sensitive, non-radioactive, homogenous and rapid assay and requires 1 labelling step. However, the FP technique displays a number of disadvantages, like the potential for compound interference. Data from previous studies indicate that some artifacts, at least up to a final assay concentration of 10 μM, were observed using the fluorescein-labelled cAMP and that there was a lack of precision at low nanomolar concentrations. As for all other similar technologies, the sensitivity of FP is dependent on the affinity of the ligand, the quantity of the receptor and the intensity of the fluorophore (www.moleculardevices.com).

#### 2.2.2.2.3. Current problems associated with PDE 4 inhibitors

One of the major issues associated with the development of PDE 4 inhibitors has been their propensity to induce emesis. This observation has been reported on several prototypical compounds (Lacombe et al., 2006). Rolipram, a highly selective first generation PDE 4 inhibitor caused high levels of nausea and vomiting. This side effect is an important cause for drug unacceptability amongst patients, particularly for a medication that has to be taken on a long -term basis. There is, therefore, a need to develop PDE 4 inhibitors devoid of these side effects.

*A. afra*, *L. leonorus* and *M. longifolia* contain flavonoids that have been reported to inhibit the PDEs and to have mild, if any, side-effects when consumed medicinally (Chi et al., 2001). By containing such flavonoids these plant species may thus, also possess a PDE inhibitory activity and be devoid of the above-mentioned side-effects.

### 2.3. Flavonoids

Flavonoids are naturally occurring polyphenolic compounds with a wide distribution in the plant kingdom. They are found in regularly consumed foods (e.g. vegetables and

fruits) and beverages like tea and red wine. Many of the 4000 different flavonoids known to date are part of the regular human diet (Havsteen, 2002).

Flavonoids are composed of two aromatic rings linked through three carbon atoms that form an oxygenated heterocyclic ring. Variations on the basic structure of flavonoids yield different classes of flavonoids. They are usually subdivided according to their chemical structure into flavanols, anthocyanidins, flavones, flavanones and chalcones (Orallo et al., 2004).

### 2.3.1 Chemistry and classification

Flavonoids are a group of low-molecular-weight polyphenolic substances. They are formed from the combination of derivatives synthesized from phenylalanine (via the shikimic acid pathway) and acetic acid.

The structure of the flavonoids is based on the flavonoid nucleus (Fig. 2.8), which consists of three phenolic rings referred to as the A, B, and C rings. The benzene ring (A) is condensed with a six-member ring (C), which in the 2-position carries a phenyl benzene ring (B) as a substituent. Ring C may be a heterocyclic pyran, which yields flavanols (catechins), and anthocyanidins, or pyrone, which yields flavonols, flavones, and flavanones. The term *4-oxo-flavonoid* is often used to describe flavonoids, such as flavanols (catechins), flavanones, flavonols and flavones, which carry a carbonyl group on C-4 of ring C (figure 2.8). The chemical nature of the flavonoids depends on structural class, degree of hydroxylation, other substitutions, conjugations, and degree of polymerization. In plants, the flavonoids are relatively resistant to heat, oxygen, dryness and moderate degrees of acidity, the molecule depends on the nature of the hydroxyl group attached to C-3 of ring C. The absence or glycosylation of this hydroxyl group results in high photostability of the molecule (Aisling and O'Brian, 2002).

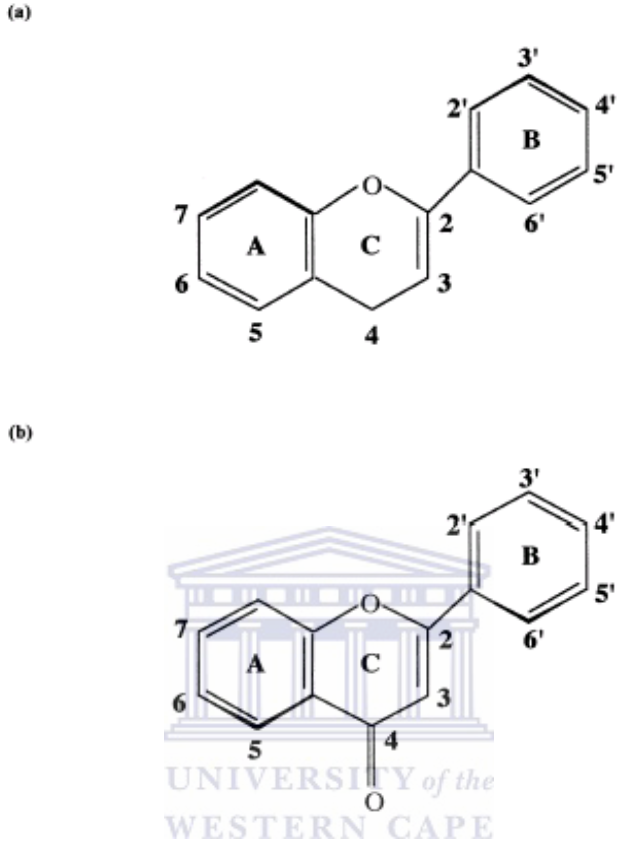




**Figure 2. 8:** (a) Flavan nucleus and (b) 4-oxo-flavonoid nucleus (Aisling and O'Brian, 2002)

The basic structure of the flavonoid nucleus allows for a multitude of substitution patterns in the A, B, and C rings, resulting in various subgroups. The flavonoids are divided into classes according to their oxidation level on the C-ring, which include anthocyanidins, flavanols (catechins), flavones, flavonols, flavanones, and isoflavonoids, 4, 5 among others. Flavones and flavonols have been identified in almost all plants; the ones found most frequently being those with B-ring hydroxylation in the C-3 and C-4 positions (Havsteen, 2002).

### 2.3.2 Flavonoid Glycosides

The structures of flavonoids differ greatly within the major classifications and substitutions include glycosylation, hydrogenation, hydroxylation, malonylation, methylation, and sulfation. Flavonoid molecules not attached to sugar moieties are referred to as the aglycone form, whereas flavonoid molecules with sugar moieties are called flavonoid glycosides (Markham, 1982).

Except for catechins, flavonoids rarely occur in plants as aglycones, the most frequently occurring forms are the glycoside derivatives in plants. Glycosylation at positions 5, 7 and 9 increases the polarity of the flavonoid molecule, which is necessary for storage in plant cell vacuoles. Flavonols and flavones occur in food usually as *o*-glycosides. D-glucose is the most usual sugar residue, but other carbohydrate substitutions include arabinose, galactose, glucorhamnose, lignin, L-rhamnose, and xylose (Markham, 1982).

### 2.3.3 Biological activities of flavonoids

Flavonoids possess various biological/pharmacological activities including antioxidant, antitumour, antiangiogenic, anti-inflammatory, antiallergic and antiviral properties. Flavonoids have also been reported to inhibit xanthine oxidase, protein kinase C and PDE (Ko et al., 2004; Havsteen, 2002). Within the last decade, reports on flavonoid activities have been predominantly associated with anti-proliferative activity and enzyme inhibition or induction.



### 2.3.4 Analytical techniques suitable for the determination of flavonoid levels in plant material

Several methods are used to separate, purify, identify and quantitate flavonoids in plant material. The methods include many steps such as separation, purification and identification of different constituents that may be present in the plants. Depending on the form of flavonoids (aglycone or glycoside), different solvents can be used to extract them. Flavonoids possessing a number of un-substituted hydroxyl groups or sugar are polar and generally soluble in polar solvents such as ethanol, methanol, dimethyl

sulphoxide and water. Less polar aglycones such as flavonols and flavones are more soluble in non-polar solvents such as ether and chloroform (Markam, 1982).

Some colorimetric methods are used to determine the total flavonoids contents in plants. For instance, the aluminium chloride method that is based on the formation of aluminium chloride-flavonoid aglycone complex is commonly used to determine the total flavonoid levels in plants.

High performance liquid chromatography (HPLC) is being increasingly utilized for the screening of drugs, vitamins, and natural products. HPLC is a sensitive, rapid, and economical technique, which can be used in the separation, identification and quantification of flavonoids in plant extracts (Springfield et al., 2005). In HPLC assays, various proportions of solvents such as water-methanol, water-methanol-acetic acid or formic acid and water - acetonitrile have been reported for the elution of flavonoids such as luteolin and hesperetin (Markham, 1980).



### 2.3.5 Examples of flavonoids in *Artemisia afra, Leonotis leonorus* and *Mentha longifolia*

*Artemisia afra, Leonotis leonorus* and *Mentha longifolia* contain several flavonoids such as luteolin, apigenin, hesperetin, hesperidin, and quercetin. The flavonoids most likely to be found in *Artemisia afra, Leonotis leonorus* and *Mentha longifolia* are luteolin and hesperetin. These two compounds are thus ideal candidates as marker compounds for studies to assess their PDE-inhibitory activity.

#### 2.3.5.1. Luteolin

Luteolin (5,7,3', 4'tetrahydrooxy flavone) is a flavone widely found in glycoside form in some plants, such as celery, green pepper, perilla leave, chamomile tea, and as an aglycone in perilla seeds (Ko et al., 2005).

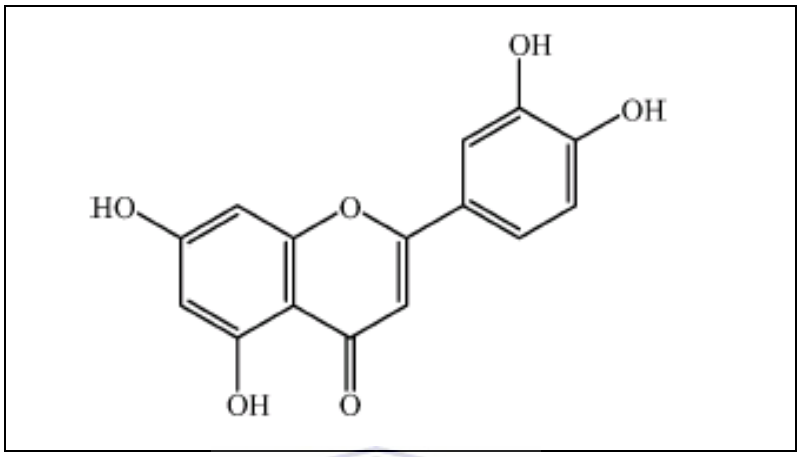



**Figure 2. 9:** Chemical structure of luteolin (Ko et al., 2005)

Luteolin has been attributed with anti-inflammatory, anti-allergic**,** antimutagenic, antiplatelet aggregation, anticancer and antioxidant properties (Kim et al., 2002; Das, 2003).



The anti-inflammatory and anti-allergic properties of luteolin are attributed to the attenuation of tumor necrosis factor (TNF)-α production and intracellular adhesion molecule-1 expression and the abolition of infiltration of leukocytes in the lung and liver of lipopolysaccaride – treated mice. Earlier studies showed that luteolin was effective in reducing the release of histamine, leukotrienes, prostaglandins $D_2$ and other mediators from various effector cells including mast cells *in vitro* (Das et al., 2003). In addition, Ko et al. (2005) have demonstrated that luteolin possess an effective inhibitory activity on PDE 1 to 5. Luteolin has also been shown to possess a tracheal muscle relaxant effect that may be due to its inhibitory effect on cyclic adenosine monophosphate (c-AMP) phosphodiesterase (Ko et al., 2004).

Because of this profile of action, luteolin could be used as a lead molecule to identify an effective anti-asthma therapy or as a means to identify novel anti-asthma targets (Ko et al., 2004).

### 2.3.5.2 Hesperetin

Hesperetin (3', 5, 7-trihydroxy-flavanone) is mainly present in citrus fruits and also in many plants.

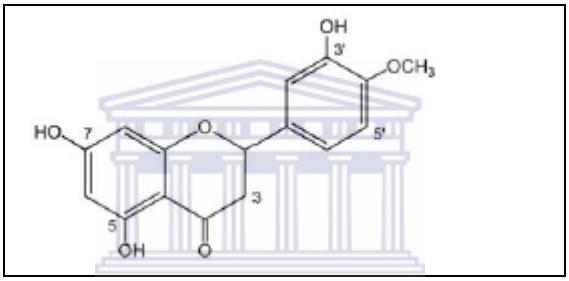



**Figure 2.10:** Chemical structure of hesperetin (Orallo et al., 2004)



Hesperetin displays a number of biological effects. It has been shown to inhibit chemically induced mammary, urinary bladder, and colon carcinogenesis in laboratory animals. Hesperetin also possesses some antioxidant activities, although this activity is poorer compared with many other polyphenols. Other possible effects of hesperetin are on lipid metabolism. Hesperetin has been reported to regulate apolipoprotein B secretion by HepG2 cells, possibly through inhibition of cholesterol ester synthesis, and to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acyl coenzyme A: cholesterol *O*-acyltransferase in rats (Erlund, 2004). In addition, hesperetin has been shown to possess inhibitory effect on PDE 4 activity that plays an important role in asthma. Hesperetin may therefore reduce the bronchoconstriction and airways hyperreactivity by affecting a number of pathways involved in asthma pathogenesis (Ko et al., 2004; Orallo et al., 2004).

# CHAPTER 3

# WORK PLAN

## 3.1 Objectives

The specific objectives for this study were to:

(i) Investigate whether *Artemisia afra, Leonotis leonorus* and *Mentha longifolia* have PDE-4 inhibitory activity,

(ii) Determine and compare the levels of the total phenolic compounds, the total flavonoids, especially luteolin and hesperetin, in the 3 plant species, and

(iii) Determine if there was a correlation between the PDE inhibitory activity and the levels of flavonoids or phenolic compounds in the plants.



## 3.2 Hypothesis

It was hypothesized that the plants with higher levels of total flavonoid and/or aglycone luteolin and/or hesperetin had higher PDE - 4 inhibitory activity.

## 3.3 Study approach

To realize the above objectives suitable preparations of the three plants (viz. freeze-dried aqueous extracts) were to be prepared, a suitable method to determine PDE inhibition selected and sensitive assay procedures used to determine the levels of total flavonoid and the aglycone forms of luteolin and/or hesperetin in the plant materials.

### 3.3.1 Why *Artemisia afra, Leonotis leonorus* and *Mentha longifolia* and their aqueous extracts?

*Artemisia afra, Leonotis leonorus* and *Mentha longifolia* are, in South African traditional medicine, among the most popular medicinal plants advocated for the treatment of respiratory diseases such as asthma (Thring and Weitz, 2006). These plant species have been shown to possess respiratory smooth muscle relaxant properties, although little is known about the mechanisms for this effect. Nevertheless, they do contain flavonoids, and possibly other chemicals, which in turn have been linked to smooth muscle relaxant effects (Ko et al., 2004).

In traditional practice, these plants are mainly used as an infusion or decoction of the loose leaves in hot water (Dube, 2006; Ma, 2006). Therefore, as per traditional method of preparation, aqueous extracts of *Artemisia afra, Leonotis leonorus* and *Mentha longifolia* leaves would be the most appropriate form of the plant to use in an investigation of the mechanism that may underpin the bronchial smooth muscle relaxation properties of these plant medicines and the plant chemical constituents possibly involved. The aqueous extracts are relatively easy to prepare, convenient to store and relatively stable (and thus consistent in chemical composition) if properly stored.



### 3.3.2 Why the PDE 4 inhibitory activity and the BIOMOL GREEN™ method?

PDE 4 isozyme plays a particular important role in inflammatory and immunomodulatory cells. Inhibition of PDE 4 in all these cells produces a marked reduction of inflammatory immunomodulatory response. Moreover *in vivo* PDE 4 inhibitors reduce the tone of bronchial smooth muscle and produce bronchodilation. Over the last decade considerable attention has focused on cyclic nucleotide phosphodiesterase isozymes 4 as a molecular target for drugs for the treatment of pulmonary diseases. In addition, some studies have shown that luteolin and apigenin, which are found in *Artemisia afra, Leonotis leonorus* and *Mentha longifolia,* inhibit the activity of PDE 4 (Ko et al., 2005). Taken together these data suggest that PDE 4 inhibition may be one of the mechanisms through which these plants produce the relaxation of the tracheal smooth muscle in the treatment of asthma.

Several methods may be used to determine PDE inhibitory activity. For this study, the BIOMOL GREEN™ Phosphodiesterase Assay Quantizyme was selected because it offered many advantages such as compatibility with the plant material, high assay sensitivity, convenience, rapidity, non-radioactivity and suitability for microplate or cuvette assay. In addition, this phosphate detection method has a moderate cost and uses a one-reagent step for phosphate detection. The other methods have disadvantages such as high cost and high potential for compound interference.

### 3.3.3 Why Luteolin or hesperetin?

Some studies have shown that the flavonoid luteolin possesses a tracheal muscle relaxant effect that may be mediated via inhibition of cAMP and/or cGMP phosphodiesterase (PDE), and that luteolin and hesperetin inhibit the activity of PDE 1 to 5 (Ko et al, 2004). In addition, luteolin has been found abundantly in *Artemisia afra* and *Leonotis leonorus* and hesperetin in *Mentha longifolia*. These two flavonoids may thus contribute to any PDE inhibition these plants may produce. At the same time, these two aglycones could also serve as marker compounds for the flavonoid group as a whole. Furthermore, their levels in the aqueous extracts of the plants could be easily quantified by HPLC assay because it is a sensitive, rapid and economical technique, while the total flavonoid levels and the total phenolic content in the plants could be measured by rapid, cheap, sensitive colorimetric methods viz the aluminium chloride method and the Folin-Ciocalteu method, respectively (Meda et al., 2004).

# CHAPTER 4

# METHODS AND MATERIALS

In this chapter, the materials, reagents, equipment, methods and procedures used to, (1) prepare the various plant materials, (2) determine the PDE 4 inhibitory activity of the plants and (3) quantify the levels of luteolin and hesperetin, as well as the total flavonoids and the total phenolic compounds in the plant extracts, are presented.

## 4.1. Chemicals, reagents, materials and equipment

### 4.1.1 Chemicals

Acetonitrile and methanol were purchased from Burdick & Jackson (USA); ethyl acetate, dimethylsulfoxide (DMSO), ethanol, malachite green, aluminium chloride from Merck (Darmstardt, Germany); formic acid, ammonium molybdate, potassium acetate, sodium carbonate from BDH Chemicals Ltd (England); hydrochloric acid from Kimix (USA), Amberlite MB-1 (desalting resin), trizma base from Sigma Aldrich (St Louis, USA); potassium dihydrogen phosphate from Riede de Haen (Germany), while the distilled water used was purified using the Analyst HP water purifier.



### 4.1.2 Reagents

Phosphodiesterase 4 (PDE 4), cyclic adenosine monophosphate (cAMP), isobutyl methyl xanthine (IBMX) were purchased from Sigma-Aldrich (St Louis, USA) and 5'-nucleotidase from Biomol $^{TM}$ (USA). All these reagents were used as per the Biomol Green$^{TM}$ PDE inhibitory assay and were stored in a freezer at – 20 or – 70 °C. Luteolin, hesperetin, morin, rutin and quercetin and the Folin Ciocalteu reagent were purchased from Sigma –Aldrich (Darmstardt, Germany).

### 4.1.3 Materials

*A. afra* and *L. leonorus* leaves were collected from Cogmanskloof in Montague in the Western Cape Province of South Africa, and *M. longifolia* leaves from Kirstenbosch National Botanical Garden in Cape Town, South Africa. In addition, sterile 96-well polystyrene microplates with lid (B&M Scientific, Cape Town, South Africa), 0.45µm Millipore syringe filters (Separations, (PTY) Ltd, Cape Town, South Africa*)* were used.

### 4.1.4 Equipment

The equipment used included an oven Memmert 854 schwabach (West Germany); a balance 1 Model GA 110(Wirsam scientific & Precision Equipment (PTY) Ltd., West Germany); a balance 2 Mettler Pe 6000 (Mettler instrumente Ag Ch-8606 Greifensee-Zurich, Switzerland); a hot plate Type Rct 13 (Kika-Werke Gmbh & Co. KgD-79219 Staufen, Germany); a spectrophotometer Beckman DU 640 ( USA); a microplate reader Labsystems Multiskan®, Bichromatic; an ice-bucket; a -70°C freezer Maldon$^{R}$, model CFC U 85360 (England), a -26 °C freezer; a refrigerator; a freeze-dryer Model Virtis Freeze Mobile 72sl ( the Virtis company Gardner, New York, USA); a filtration system Supelco; a vacuum pump Medi-pump Model 1132-2 (Thomas industries, Inc, USA);a dessicator; a water bath Cph 110 ( Labdesign Engineering Pty Ltd.); 9.0 cm filter papers Watman® (England); a centrifuge Labofuge 200 (Germany); a vortex mixer G-560E (Scientific industries, Inc. Bohemia, NY. 11716 USA); a pH meter Basic20 (Crison Instruments, S.A.); micropipettes Gilson (Medical Electronics (France) S.A.); an Agilent$^{TM}$ 1200 HPLC system, an HPLC system consisting of an auto sampler Beckman Gold Module 507, a programmable binary gradient pump Beckman Gold Module 126 series, diode array detector Beckman Gold Module 168 series and PC system monitored and controlled via a Beckman System Gold 32-Karat$^{TM}$ HPLC software system Beckman (Fullerton CA,USA); an hydro-reverse phase column Phenomenex (USA) having 4µm particle size and a column length of 250 x 4.60mm.

## 4.2 Methods and procedures

### 4.2.1 Preparation of freeze - dried aqueous extracts of *A. afra, L. leonorus* and *M. longifolia*

The fresh leaves of *A. afra* were collected in Montagu in the Western Cape Province of South Africa in February 2005. A voucher specimen (voucher No: 6735) was deposited in the herbarium, Botany Department, University of the Western Cape. The leaves were washed with water and dried in the oven at 60°C for 3 days under strict hygienic conditions.

The fresh leaves of *L. leonorus* were collected from Cogmanskloof in Montagu in the Western Cape Province of South Africa on 7th March 2005. A voucher specimen (voucher No: 6736) was deposited in the herbarium, Botany Department, University of the Western Cape. These leaves were also washed with water and dried in the oven at 60°C for 3 days under strict hygienic conditions.

Finally, the fresh leaves of *Mentha longifolia* were collected from Kirstenbosch Garden Western Cape Province of South Africa on 21st March 2004. A specimen of the collected plant (voucher No: 6635) was deposited in the herbarium, Botany Department, University of the Western Cape. These leaves were treated as for the *L. leonorus* and *A. afra*.

For all three plants the dried plant material were packed in plastic bags which were then put in brown paper bags and stored at room temperature until they were extracted.

The aqueous extracts of the dried plant materials were prepared using a procedure mimicking the methods used by the traditional healers who usually take 1l of boiling water to extract one teaspoon of dried *Artemisia afra*, *Leonotis leonorus* or *Mentha longifolia* leaf powder (i.e. about 2.5g). Thus, in this study, 100g dried *Artemisia afra* leaves were boiled in 3500ml of distilled water for 10 minutes, cooled, the solution filtered and the filtrate frozen at -26°C and finally vacuum freeze-dried over 3 days at – 45 °C. Once it was dried, it was collected, placed in an amber bottle and kept in a

desiccator until use. The same procedure was used for the preparation of the freeze-dried *L. leonorus* and *M. longifolia* aqueous extracts.

### 4.2.2 Determination of the PDE inhibitory activity of the plant extracts.

The method used to evaluate the PDE-inhibitory activity of the plant extracts was the Biomol Green™ Phosphodiesterase assay method (www.biomol.com). To use the Biomol Green™ method to assess the PDE–inhibitory activity of the plants, aqueous solutions of the plant extracts first had to be desalinated before the PDE-activity could be measured and for this, a suitable protocol had to be developed.

#### 4.2.2.1 Desalination of plant extracts

The Malachite Green reagent used in the Biomol Green™ Phosphodiesterase assay is a highly sensitive phosphate detection solution that changes in colour from yellow to green in the presence of free phosphate (e.g. any phosphate present on labware, in the reagent solutions, etc.). When 100 µl of Malachite Green solution was added to 50 µl of 0.5 mg/ml aqueous solutions of each plant extract the solution turned green indicating the presence of phosphate in these solutions. To remove the excess phosphate, aqueous solutions of the plant extracts were first desalted using Amberlite MB-1, an ion-exchanger resin.

For the desalination process, 10 g of the resin (Amberlite MB-1), placed in a 500ml beaker, was rehydrated by adding 250ml of phosphate free deionized water, and stirred for 4 hours. Thereafter, the water was carefully decanted and the rehydrated resin put into a 5ml plastic syringe to obtain a 5ml settled-bed volume column of resin. The water was allowed to drain by gravity, the column equilibrated by adding 4ml of 10 mM Tris-HCl buffer at pH 7, 4 and this was also allowed to drain by gravity.

To desalinate, 2 ml samples of the unhydrolyzed plant solutions or a 10 µg/ml phosphate solution (as control) was added to the top of the column, allowed to drain by gravity and

the effluent collected. To test for the effective quantitative removal of phosphate from the extract or phosphate solutions, 100µl of Malachite Green reagent was added to 50µl of extract, phosphate solution (both non-desalinated), a separate sample of deionized water (blank) or samples of the extract and phosphate solution effluents (i.e. that collected after passing through resin) in a 96-well microplate. Twenty minutes later the absorbance of the contents of each well was measured at 620nm using a microplate reader. The effective removal of the phosphates by desalination was assessed by comparing the optical density (OD) of the pre- and the post-desalinated plant solutions with that of the phosphate-free deionized water. After this, the desalted extract solutions were immediately frozen and stored at - 26°C until tested for PDE inhibitory activity.

#### 4.2.2.2. Protocol for the PDE inhibitory assay

For the assay, several reagents needed to be prepared and the method validated by using different concentrations of a known PDE inhibitor, isobutyl methyl xanthine (IBMX), as a positive control. The procedure used to analyze the data obtained is also described below.



##### 4.2.2.2.1. Solutions:

Firstly, the following solutions were prepared:

a) 10 mM Tris-HCl, pH 7.4 assay buffer

b) 0.05 mU/ml PDE 4 in assay buffer

c) 1 mM of 3'5'cyclic- adenosine monophosphate (c-AMP) in assay buffer

d) Malachite Green solution (Harder et al., 1994).

#### 4.2.2.2.2. Validation of the method and assay for cAMP-PDE 4 inhibitory activity

The Biomol Green™ assay method is based on the cleavage of cAMP or cGMP by a cyclic nucleotide phosphodiesterase (www.biomol.com). The 5'-nucleotide released in the reaction is further cleaved to the nucleoside and phosphate by the enzyme 5'-nucleotidase and the phosphate released quantified using BIOMOL GREEN™ reagent. In the present study, cAMP was used as the substrate, and isobutyl methyl xanthine (IBMX), a well-known non-specific PDE inhibitor, as a positive control.

To validate the assay the PDE- inhibitory activities of various concentrations (200, 100, 50 and 25, 10 µM in assay buffer) of IBMX were first determined. An aqueous stock solution of 10mg/ml of each desalinated plant extract was diluted in deionized water to obtain 25, 50, 125, 250, 500, 1000 and 2000 µg/ml solutions of unhydrolyzed extracts, and 1ml of the 10mg/ml stock solution was hydrolyzed with 1ml of 2M HCl and neutralized with 1ml of 2M NaOH for the hydrolyzed extracts and then diluted to provide the above concentrations. Thereafter, the assay was carried out with seven different concentrations (25, 50, 125, 250, 500, 1000, 2000 µg/ml) of the unhydrolyzed and the hydrolyzed plant extracts. This was achieved by placing 10 µl of buffer into wells of a 96-microtiter plate and then successively adding 40 µl of the substrate (1 mM cAMP), 10 µl of IBMX or the unhydrolyzed or hydrolyzed plant extract sample, and 10 µl of 5-nucleotidase to each well. The volume was then adjusted to 80µl with deionized water if necessary. Finally, 20 µl of PDE (20 mU) was added to start the reaction and the microtiter plate placed in an incubator at 30 ˚C for 90 minutes. To terminate the reactions, 100 µl of Malachite Green solution was added and the plate gently agitated to mix the contents (taking care to avoid the production of air bubbles in the wells). The colour was allowed to develop for 20 minutes before reading on the microplate reader taking care to assure that the samples spend approximately the same time in contact with the reagent. Finally, the optical density at 620nm was read for each well on a microtiter-plate reader.

The net increase in optical density (OD) was calculated as the OD in the presence of enzymes and substrates (with and without test compound present) minus the OD obtained when the substrate and the compound tested were not present (background OD). The PDE inhibitory activity was assessed by calculating the percentage inhibition as follows:

$$\% \text{ Inhibition} = \left[ \frac{\text{Net OD without inhibitor-net OD with inhibitor}}{\text{Net OD without inhibitor}} \right] \times 100$$

Thereafter the plant extract solution concentration *versus* % inhibition data was entered into the Graphpad Prism 4 Program, sigmoidal concentration-response curves of *A. afra, L. leonorus* and *M. longifolia* extracts plotted and the concentrations inducing 50% of the maximum inhibition (i.e. $IC_{50}$) estimated. The latter parameter was subsequently used to compare the PDE inhibitory effects of the three plant species.

### 4.2.3. Determination of total flavonoid content in the plant material

The total flavonoid content of the plants was determined using the aluminium chloride colorimetric method (Meda et al., 2005) and quercetin was used to establish the calibration curve. For the latter purpose, 10 mg of quercetin were dissolved in 10ml of 80 % ethanol in water (v/v) and then diluted with 80 % ethanol to provide 25, 50, 100, 200 and 250 µg/ml solutions. To 0.5 ml of each standard solution 1.5 ml of 95 % ethanol (v/v), 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water was added and the contents of the tubes incubated at room temperature for 30 minutes, where after the absorbance of the reaction mixture was measured at 415 nm using a UV/VIS spectrophotometer. For the blank the amount of 10 % aluminium chloride was substituted by the same amount of distilled water in the blank. To establish the standard curve to be used in the quantification of the total flavonoids, the optical densities (OD) of quercetin were plotted against the corresponding concentrations.

The total flavonoid content in the plants was obtained as follows: 2.5 mg of each plant were weighed and placed in a screw-capped Kimax tube, then dissolved in 0.5 ml of

distilled water. To hydrolyze the sample, 1 ml of 2 M hydrochloric acid were added to the solution and the mixture heated at 80°C for 40 minutes in a water bath. The solution was allowed to cool and then neutralized with 1ml of a 2M NaOH solution. To determine the total flavonoid content in the unhydrolyzed and hydrolyzed samples, the solutions were reacted with aluminium chloride as described above. The OD of the mixture was read at 415nm and the quercetin standard curve used to determine the corresponding flavonoid concentrations in the plant extract (hydrolyzed and unhydrolyzed) samples. The total flavonoid levels in the desalinated plant extracts were obtained in a similar fashion.

### 4.2.4. Determination of the total phenolic content (TPC) in plant material

The total phenolic content of the plants was determined using the Folin Ciocalteu method (Meda et al., 2005). Rutin was used as the standard to establish the calibration curve. For the latter purpose, 10mg of rutin was dissolved in 10ml of water (v/v), and then diluted to provide 50, 100, 150, 200, 250 and 500 μg/ml solutions. To 0.5 ml of each standard solution, 2.5 ml of 0.2N Folin-Ciocalteu reagent was added and left to stand for 5 min. Then 2 ml of 75g/ml solution of sodium carbonate ($Na_2CO_3$) was added and the mixture incubated at room temperature for 2 hours. The absorbance of the reaction mixture was read at 765nm using a UV/VIS spectrophotometer, against a blank composed of distilled water and all the reactants except rutin. The absorbance readings obtained were plotted against the corresponding concentrations of rutin to establish the calibration curve for TPC determination in the plants.

To determine the TPC, a 1mg/ml solution of each plant was prepared in distilled water and 0.5 ml of this solution used to quantitate the TPC in each plant. To hydrolyze the plant extracts, 0.5 ml of the 1mg/ml solution were placed in a screw-capped Kimax tube, then hydrolyzed and neutralized as described in the determination of the TFC in section 4.2.3. The unhydrolyzed and hydrolyzed extracts of each plant were treated as described above and the Optic density (OD) of the mixture was read at 765nm. The rutin standard curve was then used to determine the corresponding phenolic concentrations expressed in

rutin equivalent per mg of plant extract. The TPC in the desalinated plant extracts were similarly obtained.

### 4.2.5. HPLC assay of luteolin or apigenin in the plant extracts

#### 4.2.5.1. Validation of the HPLC assay

Luteolin and hesperetin were selected as markers for the HPLC analysis of *A. afra, L. leonorus* and *M. longifolia*.

The luteolin levels in *A. afra* and in *L. leonorus* as well as the hesperetin level in *M. longifolia* were determined by HPLC using a reversed phase method. The mobile phases consisted of 30 % acetonitrile, 70 % $KH_2PO4$ buffer (pH 2) for luteolin and 45 % methanol, 55 % formic acid 0.1% for hesperetin. These eluents were pumped isocratically, at room temperature (20°C) through a $C_{18}$ reverse phase column (Phenomenex, 4μm particle size and 250 x 4.60 mm), at a flow rate of 1ml per minute. Morin was used as an internal standard in the HPLC assay for *A. afra*, luteolin and hesperetin as external standards for the HPLC assay of *L. leonorus* and *M. longifolia*, respectively.



Morin was chosen as an internal standard because it was not present in the above plant materials, absorbed strongly at the luteolin absorption wavelength (349nm), was extractable in ethyl acetate, had similar physiochemical characteristics to luteolin, and had a retention time that did not interfere with other peaks from *A. afra*. For all the plant runs the diode array detector, scanning between wavelengths 168 and 349 nm, were used to detect the flavonoids

To validate the HPLC assay, stock solutions of luteolin, hesperetin and morin (1mg/ml) were prepared in dimethyl sulphoxide (DMSO), stored at -20 °C in glass container wrapped in aluminium foil to protect it from light using aluminium foil. Standard solutions were prepared each day by diluting the stock solutions to 1, 2, 4, 8 and 16μg/ml of luteolin in distilled water, 5, 10, 15, 20, 25 μg/ml of hesperetin in distilled water and

100μg/ml of morin in distilled water. These concentrations were used to prepare the standard curve. When using the internal standard method 10 μg of morin was added to each concentration of luteolin. For each standard, 50 μl was injected onto the column and the luteolin and hesperetin peaks were detected at 349 nm and 280 nm, respectively. The peak height ratios between luteolin and morin were plotted against the corresponding concentrations of the injected standard for the internal standard method and the peak height of luteolin or hesperetin were plotted against corresponding concentrations of the injected luteolin or hesperetin for the external standard method.

To validate the assay, the linearity, lowest limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were determined. The Graphpad Prism 4 Program software was used to calculate the mean and percentage coefficient of variance & the linear regression coefficient.

#### 4.2.5.2. Quantification of luteolin or hesperetin in the plant extracts

The freeze-dried aqueous extracts of the plants, prepared as described in section 4.1.2.1, were assayed in two ways, viz. by hydrolyzed and unhydrolyzed methods. The latter provides the level of free flavonoid and the former the level of total flavonoid.

To determine the free flavonoid level 10mg of *Artemisia afra* aqueous extract, 25mg of *Leonotis leonorus* or 100 mg of *Mentha longifolia* was placed in a screw-capped Kimax tube and dissolved in 1ml of distilled water. Except for *Mentha longifolia* aqueous solution that was directly injected (25 μl) onto the HPLC column, 100 μl of morin solution (100 μg/ml) was then added as internal standard to the *A. afra* samples, followed by 5 ml of ethyl acetate to both *A. afra* and *L. leonorus* samples and the mixture vortex-mixed for 1 minute and centrifuged for 10 minutes at 3000 rpm. The top layer of ethyl acetate was removed to another clean tube using a disposable plastic pipette, evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted in 1 ml of mobile phase and filtered before 50 μl thereof was injected onto the HPLC column.

On the other hand the total flavonoid (free plus conjugated) levels were obtained by first hydrolyzing the plant extracts. For that purpose, 10mg of *Artemisia afra*, 25 mg of *Leonotis leonorus* or 100 mg of *Mentha longifolia* were weighed and placed in screw-capped Kimax tubes and dissolved in 1 ml of distilled water. One hundred microliter of morin solution (100 μg/ml) was added as internal standard to the *A. afra* samples. Then to hydrolyze the sample, 2 ml of 2 M hydrochloric acid were added to the solution and the mixture heated at 80°C for 40 minutes in a water bath. The solution was allowed to cool after the heating, 5 ml of ethyl acetate added and the mixture vortex-mixed for 1 minute and centrifuged for 10 minutes at 3000 rpm. The top layer of ethyl acetate was removed to another clean tube using a disposable plastic pipette, evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted in 1 ml of mobile phase before injection onto the HPLC column.

To identify the luteolin or hesperetin in the plants, the UV-spectra and the chromatographic retention times of the pure luteolin or pure hesperetin peaks were recorded after each run and compared to the UV-spectra and the retention times of luteolin or hesperetin peaks in the chromatograms of the plant samples.



To determine the level of luteolin or hesperetin in the desalinated samples, the plant extract samples were first desalinated as described in section 4.2.2.1 and thereafter prepared for HPLC assay using the above procedure.

### 4.2.6. Correlation between the flavonoid markers, total flavonoid and phenol concentrations and the PDE 4 inhibitory activity of the plants

To determine the relationship between the level of the total flavonoids, the total phenolic, or the luteolin or hesperetin levels in the plant and the PDE- 4 inhibitory activity of the plants ($IC_{50}$), the Pearson correlation coefficient for the regression of each of these parameters versus the PDE - 4 inhibitory activity was calculated using the Graphpad Prism 4 program (GraphPad Prism 4, 2003).

# CHAPTER 5
# RESULTS AND DISCUSSION

In this chapter, the results obtained during the preparation of freeze-dried aqueous extracts of *A. afra, L leonorus* and *M. longifolia* and the determination of their PDE 4 inhibitory activity, total flavonoid, total phenolic, luteolin and hesperetin contents and, finally, the possible correlation between the total flavonoid and total phenolic compound concentrations *versus* the PDE 4 inhibitory activity of the plants, are reported and discussed.

## 5.1. Preparation of freeze-dried aqueous extracts (FDAE)

The freeze-dried aqueous extracts of *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia* were prepared from the dried leaves as described in section 4.2.1 and the yields of freeze-dried aqueous extracts obtained were 21.5 %, 19.40 % and 15.4% for *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia*, respectively. These yields were very close to those obtained by previous investigators, viz 19.9 % by Komperlla (2005) and 21.96 % by Dube (2006) for *A. afra*, 18.42 % by Ma (2006) for *L. leonorus* and 12.42 % by Ma (2006) for *M. longifolia*. The appearance of the freeze-dried aqueous extracts of *Artemisia afra*, *Leonotis leonorus* and *Mentha longifoli*a are shown in Figures 5.1, 5.2 and 5.3. The *A. afra* extract was a light brown, brittle and hygroscopic powder with a characteristic and aromatic odour. The extract produced by *L. leonorus* was brown, brittle and hygroscopic and had a characteristic odour while that obtained from *M. longifolia* was yellow-brown, brittle and hygroscopic with a mint like aromatic odour.

**Figure 5.1:** The freeze-dried aqueous extract of *A. afra*


**Figure 5.2:** The freeze-dried aqueous extract of *L. leonorus*

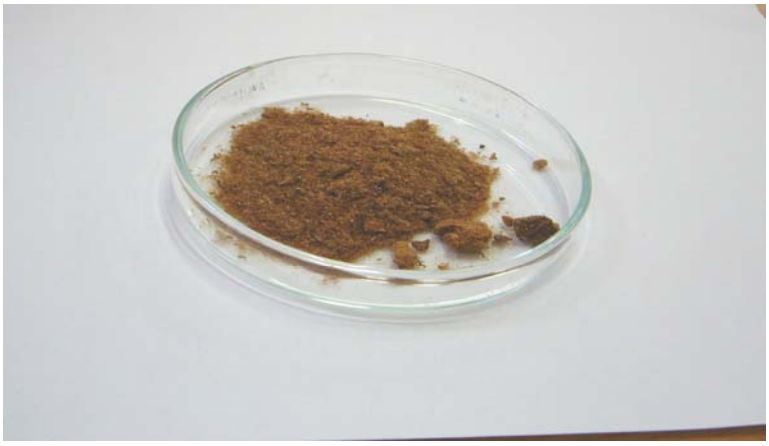


**Figure 5.3:** The freeze-dried aqueous extract of *M. longifolia*

## 5.2. PDE inhibitory activity of *Artemisia afra, Leonotis leonorus* and *Mentha longifolia*

The determination of the PDE inhibitory activity of the plants required desalination of the plant extracts, adaptation and validation of the PDE method and finally, use of this method to determine PDE 4 inhibitory activity.

### 5.2.1. Desalination of plant extracts.

For the PDE-inhibitory assay the plant extracts were desalinated using the double bed resin Amberlite MB-1 as described in section 4.2.2.1. First, a standard curve of various concentrations of phosphate in deionized water *vs* absorbance at 620nm that was linear over a range of 1 to 5 µg/ml ($r^2$: 0.982) and corresponding to the equation y = 0.1783x – 0.0058 where y = absorbance (620nm) and x = phosphate concentration (µg/ml), was set up. The amount of phosphates in the plant material before and after the desalination process was consequently determined from this standard curve and the UV absorbance of the aqueous solutions of the plant extracts, the amount of phosphates in the plants before and after the desalination process and percent phosphates removed through the desalination process are reported in Tables 5.1 and 5.2.



**Table 5.1:** UV absorbance and concentration of phosphates in deionized water and plant freeze-dried aqueous extracts solutions (50µg/ml), before and after desalination treatment

| **Samples** | **Absorbance at 620nm (AUFS), average ± SD (n=3)** | **Concentration of phosphate (µg /ml) of plant extracts solution (50 µg/ml), average ± SD (n=3)** |
|---|---|---|
| Deionized water | 0.067 ± 0.002 | 0.404 ± 0.012 |
| Non-desalinated *A. afra* extract | 0.760 ± 0.002 | 4.293 ± 0.011 |
| Desalinated *A. afra* extract | 0.069 ± 0.001 | 0.419 ± 0.003 |
| Non-desalinated *L. leonorus* extract | 0.227 ± 0.001 | 1.303 ± 0.006 |
| Desalinated *L. leonorus* extract | 0.068 ± 0.001 | 0.414 ± 0.003 |
| Non-desalinated *M. longifolia* extract | 0.652 ± 0.002 | 3.687 ± 0.011 |
| Desalinated *M. longifolia* extract | 0.067 ± 0.002 | 0.406 ± 0.010 |

**Table 5.2:** The percent phosphates removed through the desalination process from the plant extract solutions

| Plant samples | % phosphate removed |
|---|---|
| *A. afra* | 86.16±1.50 |
| *L. leonorus* | 93.85±1.54 |
| *M. longifolia* | 99.50±0.87 |

The non-desalinated *A. afra*, *L. leonorus* and *M. longifolia* extracts contained 4.293 ± 0.011, 1.303 ± 0.006 and 3.687 ± 0.011µg/ml (n=3) of phosphate, respectively, of which more than 85% was removed in the desalination process. The non-desalinated *L leonorus* contained significantly less (one-way analysis of variance (ANOVA), $p < 0.0001$) phosphate compared to the other two plants. The residual phosphate levels remaining after desalination were similar in the three plant solutions and comparable to the level in the distilled water (ANOVA, p value: 0.1575). Clearly the plant solutions contained significant amounts of phosphate which would have interfered with the PDE activity assay, but which was also appropriately removed by the desalination process illustrating the suitability of the Amberlite MB-1 used for the removal of the phosphate.



Many studies that have, however, used Amberlite MB-1 for plant material desalination, have also reported a loss in certain other constituents of the plants. For instance, Davies (1988) in a study conducted on the quantification of sugars in potato tuber extracts, reported a decrease in the sugar contents after Amberlite MB-1-treatment of the extracts to remove organic anions. In the present study, the desalination process did actually also lead to a decrease in flavonoids, as is shown later in table 5.7. Since flavonoids have been implicated in the phosphodiesterase inhibitory activity of plants, this loss of flavonoids from the *A. afra*, *L. leonorus* and *M. longifolia* extracts during the desalination step may have significantly influenced the plants biological activity. Fortunately, even though a great loss of flavonoids was registered after the Amberlite MB-1-treatment of the plant extracts, these extracts were still able to demonstrate a substantial PDE 4 inhibitory activity as reported in section 5.2.3.

Collectively, the above results indicate that the plant extracts contained significant amounts of phosphate, which would have interfered with the PDE activity assay and were appropriately removed through the desalination process using Amberlite MB-1. Although a great loss of flavonoids was registered after the desalination process, the plant extracts were able to substantially inhibit the PDE 4.

### 5.2.2. Validation of the PDE method

To validate the method, isobutyl methyl xanthine (IBMX), a non-specific cyclic nucleotide phosphodiesterase inhibitor, was used as a positive control. As negative control the assay buffer was used in-place of plant solutions or IBMX and caused no change in PDE activity. The PDE 4 inhibitory activity of 6 different concentrations (5, 10, 25, 50, 100,200 μM) of IBMX was evaluated and the results obtained are shown in table 5.3 and figure 5.4, the latter being a log concentration-response curve of the data fitted using a non-linear regression program. From this curve the concentration inducing 50 % of the maximum inhibition (i.e. $IC_{50}$) was estimated.



**Table 5. 3:** The percentage PDE 4 inhibition of IBMX

| IBMX (μM) | % PDE 4 inhibition ±SD (n=3) |
|---|---|
| 5 | 3.67 ± 0.58 |
| 10 | 9.31 ± 1.57 |
| 25 | 28.42 ± 3.15 |
| 50 | 39.04 ± 2.29 |
| 100 | 61.37 ± 1.52 |
| 200 | 64.33 ± 1.15 |

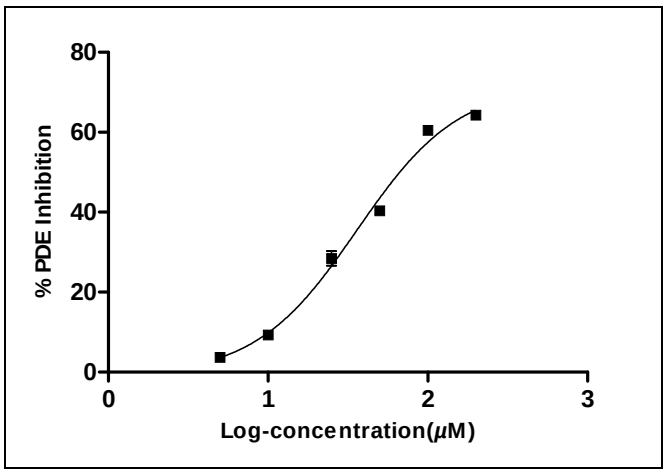



**Figure 5. 4:** Log concentration vs PDE-inhibitory response curve of IBMX. Each point is the mean of 3 replications.

Under the experimental conditions (i.e. enzyme levels etc.) used in this study (see section 4.2.2.2.2), IBMX showed a maximal % inhibition of approximately 60% and a PDE 4 $IC_{50}$ of 35.98 ± 0.035µM or 7.91 ± 0.008 µg/ml. These values were close to the PDE 4 $IC_{50}$ of approximately 25µM reported for IBMX in the Biomol Phosphodiesterase Assay (www.biomol.com) product brochure. Given these results the assay was thus deemed validated and used in this study.



### 5.2.3. PDE 4 inhibitory activity

The PDE 4 inhibitory activity obtained for the plants in this study are shown in tables 5.4, 5.5 and 5.6 and figures 5.5 and 5.6.

**Table 5.4:** The % PDE inhibition induced by different concentrations of *A. afra*, *L. leonorus* and *M. longifolia* unhydrolyzed aqueous extracts

| **Plant concentration (µg/ml)** | **% PDE inhibition (mean ± S.D, n=3)** | | |
|---|---|---|---|
| | ***A. afra*** | ***L. leonorus*** | ***M. longifolia*** |
| 25 | 9.1 ± 0.25 | 7.62 ± 2.61 | 18.15 ± 0.66 |
| 50 | 14.74 ± 0.92 | 15.23 ± 0.50 | 24.46 ± 1.72 |
| 125 | 35.60 ± 3.26 | 38.86 ± 5.05 | 41.36 ± 0 |
| 250 | 60.99 ± 2.15 | 49.70 ± 4.39 | 49.59 ± 0.29 |
| 500 | 73.7 ± 0.25 | 82.33 ± 0.47 | 75.83 ± 0.47 |
| 1000 | 79.85 ± 2.36 | 89.56 ± 0.94 | 97.39 ± 2.46 |
| 2000 | 84.39 ± 2.49 | 93.33 ± 1.03 | 100 ± 0 |

**Table 5.5:** The % PDE inhibition induced by different concentrations of *A. afra*, *L. leonorus* and *M. longifolia* hydrolyzed aqueous extracts

| **Plant concentration (µg/ml)** | **% PDE inhibition (mean ± S.D, n=3)** | | |
|---|---|---|---|
| | ***A. afra*** | ***L. leonorus*** | ***M. longifolia*** |
| 25 | 30.09 ± 2.52 | 19.50 ± 3.05 | 23.97 ± 3.21 |
| 50 | 51.75 ± 1.24 | 32.02 ± 0.00 | 30.84 ± 1.28 |
| 125 | 54.22 ± 2.99 | 51.49 ± 3.86 | 45.10 ± 4.37 |
| 250 | 72.70 ± 1.32 | 67.61 ± 2.73 | 53.98 ± 0.37 |
| 500 | 92.00 ± 0.00 | 95.23 ± 0.59 | 93.50 ± 0.71 |
| 1000 | 93.26 ± 0.91 | 97.50 ± 0.71 | 99.57 ± 0.33 |
| 2000 | 96.11 ± 1.45 | 99.19 ± 1.15 | 100.0 ± 0.00 |

**Table 5.6:** The PDE inhibition $IC_{50}$ values of IBMX and the unhydrolyzed and the hydrolyzed plants

| Sample | $IC_{50}$ (µg/ml) | |
|---|---|---|
| | Unhydrolyzed plant | Hydrolyzed plant |
| *A. afra* | 161.70 ± 1.806 | 144.0 ± 0.356 |
| *L. leonorus* | 212.12 ± 0.040 | 167.3 ± 0.037 |
| *M. longifolia* | 337.10 ± 0.042 | 280.2 ± 0.033 |
| IBMX | 7.91± 0.008 | |

All three plants demonstrated a dose-dependent PDE 4 inhibition with a maximal inhibition of 84.39 ± 2.49 % and 96.11 ± 1.45 %; 93.33 ± 1.03 % and 99.19 ± 1.15 %, and finally 100 ± 0 % and 100 ± 0 % for the unhydrolyzed and the hydrolyzed aqueous

extracts of *A. afra*, *L. leonorus* and *M. longifolia*, respectively. Interestingly, at concentrations above 500µg/ml all three plants showed higher maximal PDE inhibitory activity than the highest dose of IBMX (44.45 µg/ml) used.

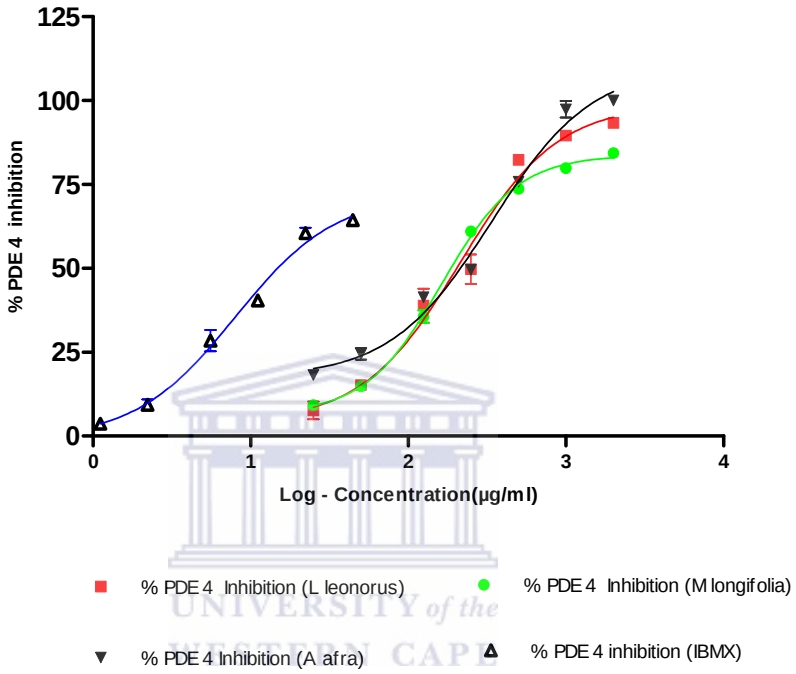



**Figure 5.5:** Log concentration - PDE-inhibitory response curve of unhydrolyzed plant extracts. Each point being the mean of 3 replications.

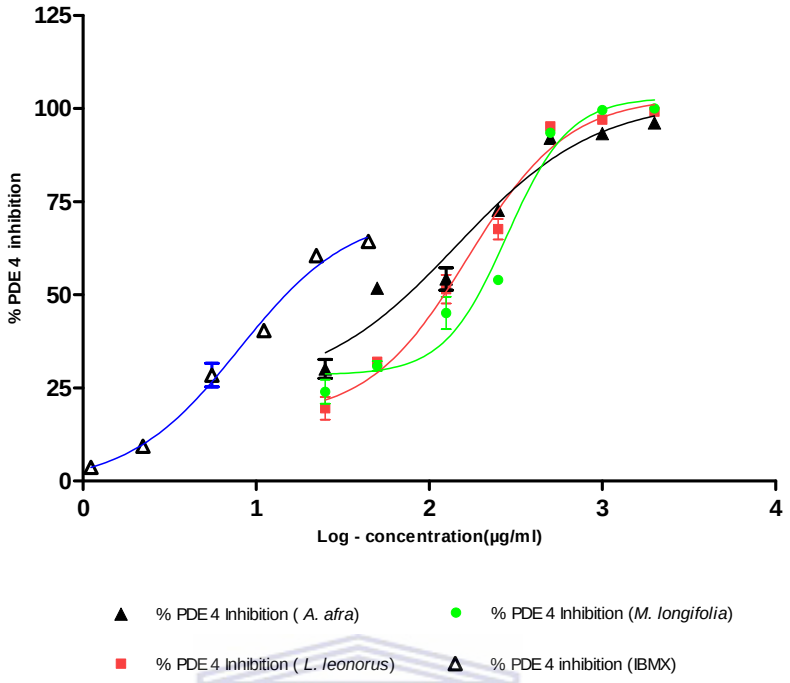



**Figure 5. 6:** Log concentration- PDE-inhibitory response curve of hydrolyzed plan extracts. Each point being the mean of 3 replications.

The PDE-4 inhibition $IC_{50}$ of each plant, estimated from the concentration-response curves were 161.7 ± 0.01µg/ml; 212.1 ± 0.04 µg/ml and 337.1 ± 0.04 µg/ml for the unhydrolyzed extracts of *A. afra, L. leonorus* and *M. longifolia*, respectively while that for the hydrolyzed extracts were 144 ± 0.358 µg/ml; 167.3 ± 0.037 µg/ml and 280.2 ± 0.0329 µg/ml, respectively. These PDE 4 $IC_{50}$ results showed, for both the unhydrolyzed and hydrolyzed extracts, that *A. afra* was more potent than *L. leonorus,* which in turn was more potent than *M. longifolia*. In addition, although their PDE 4 $IC_{50}$s were not statistically different (p value = 0.6232), the hydrolyzed extracts were more active than the unhydrolyzed extracts for all 3 plants i.e. there was a 10.95%, 21.12% and 16.87% decrease in the $IC_{50}$ values for the hydrolyzed *vs* the unhydrolyzed extracts of *A. afra, L. leonorus* and *M. longifolia*, respectively. It is possible that the hydrolysis of the flavonoid glycosides lead to increased free aglycone (and sugars) levels that resulted in a subsequent increase in the level of PDE 4 inhibitory activity of the plants. Such an interpretation is supported by the findings of Francisco Orallo et al. (2004) who studied the cyclic nucleotide phosphodiesterase-inhibitory effects of hesperetin and hesperidin.

They reported that the aglycone hesperetin inhibited the PDE 4 isolated from bovine aorta while its glycoside form, the 7-rhamnoglucoside (hesperidin) was inactive, the possible reason being that the glycosylation of the C7 atom produces a spatial configuration that does not favour interaction with the PDE 4 (Orallo et al., 2004). Ko et al. (2004) in their study on the inhibitory effects and structure–activity relationships of flavonoids on phosphodiesterase isozymes from guinea pig also reported that the aglycone luteolin was, in terms of PDE inhibition, more potent than its glycoside form, luteolin-7-glucoside, and probably because the bulky glycosyl residues at the 7- position may hinder its binding to the isozyme. The same study also reported that the structure of flavonoids determined their PDE inhibition. In fact, Ko et al. (2004) showed that, among others, (1) the C-3′ hydroxyl group of flavones was very important for PDE inhibition, as illustrated by luteolin being more potent than apigenin, and that (2) flavonols and isoflavones were more potent than flavones as illustrated by apigenin being less potent than genistein with regard to PDE 4 inhibition. Such differences in potency among the flavonoids based on their structure could partly explain the degree of potency of each of the presently studied plants.



Finally, despite being able to produce higher maximal PDE inhibitory activity, the PDE 4 $IC_{50}$ values of the plants were however significantly (Student's t-test, p value = 0.0118) higher than that for IBMX (7.91 ± 0.008 μg/ml) clearly suggesting a much lower selectivity of the plant extract as far as PDE-inhibitory action is concerned. Such lower selectivity may also be the result of the presence of many plant constituents, each with a differing $IC_{50}$ value, which may collectively contribute to the overall PDE – inhibitory activity of the plant extracts.

Collectively, the above results indicate that all three plants dose dependently inhibited PDE 4, had roughly the same potency but were significantly less potent than IBMX, the positive control. In addition, the hydrolyzed extracts of the plants were slightly (although not statistically significantly) more active than the unhydrolyzed extracts suggesting that the PDE 4 inhibitory activity may be linked to the presence of the aglycone forms of flavonoids in the plants.

## 5.3. Total flavonoid content (TFC) of in the plant materials

In this study the total flavonoid content (TFC) of the plant materials was determined using the aluminium chloride method. Quercetin was used to establish the calibration curve (figure 5.7), which was linear from 5 to 250 µg/ml ($r^2$ = 0.9938). The total flavonoid content of the plant materials, expressed as quercetin equivalent (QE), are shown in tables 5.7 and figures 5.8 and 5.9. In addition, the percent of flavonoids lost during the desalination process of plant extracts is given in table 5.8.

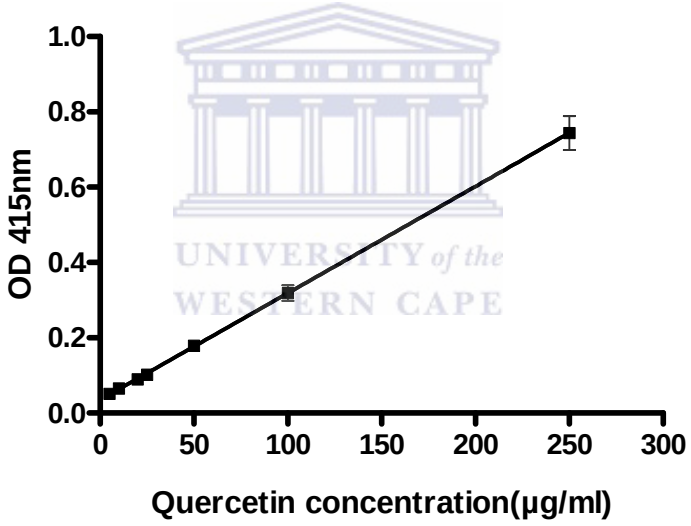



**Figure 5.7:** Standard curve of quercetin concentration versus absorbance at 415 nm used for the quantification of the total flavonoid contents in *A. afra, L leonorus* and *M. longifolia.* ($r^2$ = 0. 9938; slope = 0.0028 ± 0.00005447; y-intercept = 0. 03468 ± 0.002929)

**Table 5.7:** The total flavonoid content expressed in µg of quercetin equivalents (QE) per mg of unhydrolyzed and acid-hydrolyzed plant material, before and after the desalination process

| Plant | Desalination | Hydrolysis | TFC (µg of QE /mg ± SD, n=3) | T test, p value |
|---|---|---|---|---|
| *A. afra* | No | No | 84.70 ± 3.88 | |
| | No | Yes | 88.82 ± 3.56 | 0.4387; NS |
| | Yes | No | 20.61 ± 0.29 | |
| | Yes | Yes | 28.40 ± 5.36 | 0.2165; NS |
| *L. leonorus* | No | No | 40.89 ± 0.02 | |
| | No | Yes | 92.60 ± 9.94 | 0.0124; S |
| | Yes | No | 16.28 ± 0.36 | |
| | Yes | Yes | 70.76 ± 7.10 | 0.0058; S |
| *M. longifolia* | No | No | 30.40 ± 1.08 | |
| | No | Yes | 94.12 ± 3.94 | 0.0007; S |
| | Yes | No | 16.84 ± 1.20 | |
| | Yes | Yes | 50.80± 7.22 | 0.0121; S |

**Where S = p < 0.05 = statistically significant and NS = p > 0.05 = not statistically significant**

**Table 5.8:** Percentage of the total flavonoid content (TFC) lost from the plant materials during the Amberlite MB-1desalination treatment

| Plant | % of TFC removed by desalination |
|---|---|
| Unhydrolyzed A. *afra* | 75.67 |
| Hydrolyzed *A. afra* | 68.03 |
| Unhydrolyzed *L. leonorus* | 60.18 |
| Hydrolyzed *L. leonorus* | 23.58 |
| Unhydrolyzed *M. longifolia* | 44.61 |
| Hydrolyzed *M. longifolia* | 46.79 |

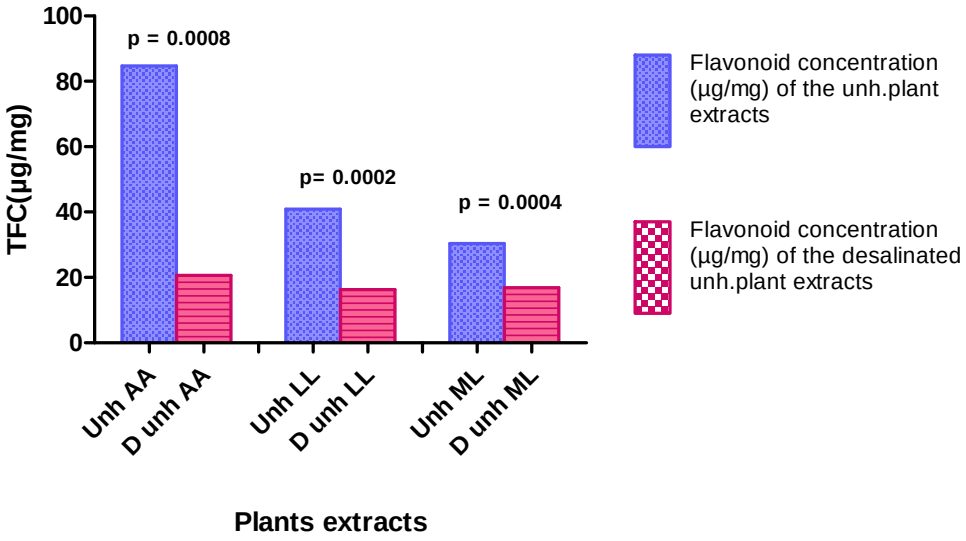



**Figure 5.8:** The total flavonoid content (µg/mg) of the non-desalinated unhydrolyzed extracts of *A. afra* (AA unh), *L. leonorus* (LL unh) and *M. longifolia* (ML unh) and the desalinated unhydrolyzed plants (D unh AA, D unh LL, D unh ML). The p-values are for t-test of TFC in unhydrolyzed plant levels *vs* that in desalinated unhydrolyzed plant.

Before the desalination treatment, the *A. afra* unhydrolyzed extracts clearly contained the highest levels of total flavonoids (84.70±3.88µg/mg), followed by *L. leonorus* (40.89 ± 0.02µg/mg) and finally *M. longifolia* (30.4±1.08 µg/mg) (table 5.7 & figure 5.8). After desalination these levels were significantly (T test, $p_{A\ afra} = 0.0008$, $p_{L\ leonorus} = 0.0002$, $p_{M\ longifolia} = 0.0004$) decreased, in all three extracts, (table 5.7 and figure 5.8) and, now, the TFC of the unhydrolyzed extracts of *A afra* was higher than those in the other plants which then were quite similar 16.28 ± 0.36µg/mg and 16.84 ± 1.20µg/mg (figure 5.8).

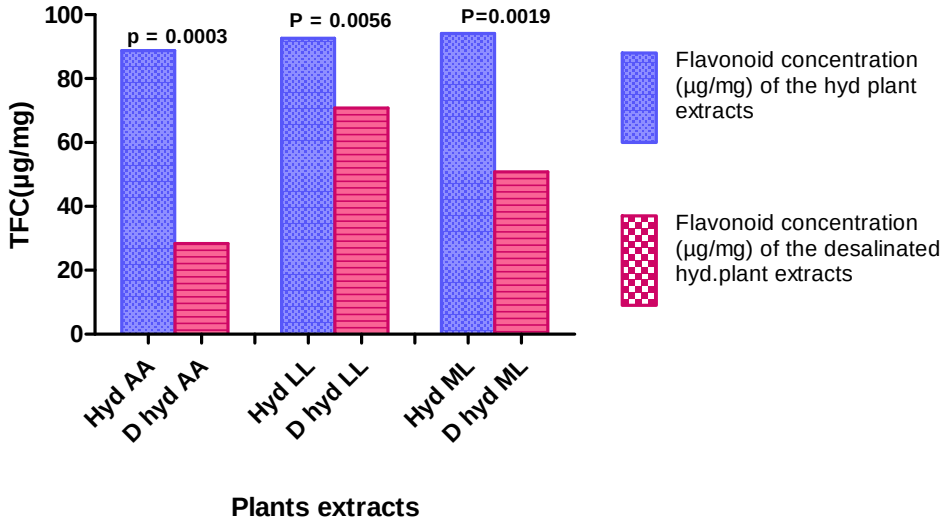



**Figure 5.9:** The total flavonoid content (µg/mg) of the non-desalinated hydrolyzed extracts of *A. afra* (AA hyd), *L. leonorus* (LL hyd) and *M. longifolia* (ML hyd) and the desalinated hydrolyzed plants (D hyd AA, D hyd LL, D hyd ML). The p-values are for t-test of TFC in hydrolyzed plant levels *vs* that in desalinated hydrolyzed plant.

When the plant extracts were subjected to acid hydrolysis the levels of TFC in all 3 extracts were relatively similar (ANOVA, p value = 0.624) and now the TFC for *L. leonorus* & *M. longifolia* were significantly increased compared to the levels in the unhydrolyzed samples (Student's t-test, $p_{L\ leonorus} = 0.00124$ and $p_{M\ longifolia} = 0.0007$). Further, the desalination treatment did not affect the *L. leonorus* acid-hydrolyzed extract much, only 23.58 % of the initial flavonoid content was removed, while significantly more i.e. 68.03 % and 46.79 % of TFCs was removed from the hydrolyzed *A. afra* and *M. longifolia* extracts, respectively.

The different plants and when they are subjected to hydrolysis and /or desalination with Amberlite thus produced several samples with different TFC levels. *A. afra, L. leonorus* and *M. longifolia* have all been reported to contain several flavonoids and because they are naturally different, their inherent flavonoid contents are different from each other. The increase in the total flavonoid content noticed after acid-hydrolysis can thus possibly be explained by the fact that the flavonoid glycosides present in the plant extracts broke down and produced aglycones that were able to complex with aluminium chloride

through binding at the *ortho-dihydroxy-*, *hydroxy-oxo-*, and *ortho-hydroxy-oxo-* groups (Smirnova et al., 1998). In the case of *A. afra* the increase was not significant probably due to the fact that the plant contained more aglycones than glycoside flavonoids, possibly because the extract absorbed some moisture during storage and was subject to hydrolysis.

The decreased in the level of flavonoids following the desalination of the plant extracts using Amberlite MB-1 was probably due to the fact that Amberlite, being a double bed resin, retained both anions and cations. Moreover, since Amberlite has been used in many studies to extract flavonoids for their quantification (D'Arcy, 2005), it was expected in this study that the desalination process might also decrease the levels of flavonoids in the plant extract solutions.

Many investigators have used the aluminium chloride method used in this study to determine the total flavonoids content of plants and foods. For instance, Meda et al. (2004) used this method to determine the TFC in Burkina Fasau honey and reported that this method is only specific for flavones and flavonols and tended to underestimate the real total flavonoid content. This underestimation of the TFC may have some implication on the correlation between total flavonoid concentrations and the PDE 4inhibitory activity of the plants.

Flavonoids play a crucial role in the phosphodiesterase inhibitory activity of many plants (Ko et al., 2004). And, as reported in section 5.2.3, the PDE 4 inhibitory activity of the plants investigated in the present study was dose-dependent (figures 5.5 and 5.6) suggesting that this activity may be related to the level of constituents such as flavonoids in the plants. Therefore, differences in the inherent levels of flavonoids in the plants or changes in the level of flavonoids as caused by the desalination or hydrolysis processes could thus be expected to possibly affect the plants activities.

Collectively, the above results showed that *A. afra, L. leonorus* and *M. longifolia* had different total flavonoid contents which were, for all three plants, decreased during the

desalination process. Hydrolysis significantly increased the TFC in the *L. leonorus* and *M. longifolia* extracts, but not in *A. afra* leading to relatively similar TFC in hydrolyzed extracts of all 3 plants. The difference in the TFC of *A. afra, L. leonorus* and *M. longifolia* could possibly affect the plants' activities.

## 5.4. Total phenolic compounds content (TPC) in the plant materials

The total phenolic compounds content of the plant extracts was determined by the Folin-Ciocalteu method using a rutin standard curve for quantification (Figure 5.10). The rutin standard curve was linear over a range of 50 to 500 µg/ml ($r^2$: 0. 9959) and corresponded to the equation: y = 0.001521x + 0.1271, where y = OD 765nm and x = rutin concentration (µg/ml). The total phenolic contents of the plant extracts, expressed as rutin equivalents (RE), are shown in table 5.9 and figures 5.11 and 5.12.



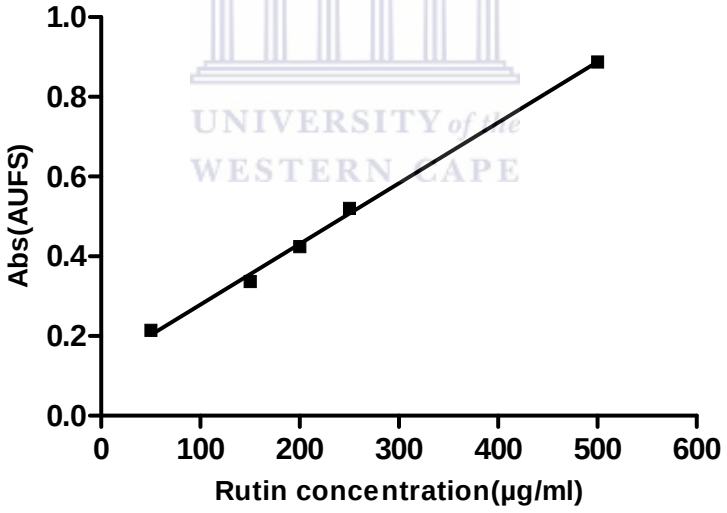



**Figure 5.10:** Standard curve of rutin for the quantification of the total phenolic contents of *A. afra, L. leonorus* and *M. longifolia.* ($r^2$ = 0. 9959; slope = 0.001521 ± 0.0002692; y-intercept = 0.1271 ± 0.007396)

**Table 5.9:** The total phenolic content, expressed in µg of rutin equivalents (RE) per mg of unhydrolyzed and hydrolyzed plant material, before and after the desalination process using Amberlite MB-1

| Plant | desalination | hydrolysis | TPC (µg of RE/mg ± SD, n=3) | T test, p value |
|---|---|---|---|---|
| *A.. afra* | No | No | 357.18 ± 8.99 | 0.0001; S |
| | No | Yes | 489.36 ± 6.21 | |
| | Yes | No | 111.57 ± 4.56 | 0.0001; S |
| | Yes | Yes | 121.64 ± 1.90 | |
| *L. leonorus* | No | No | 347.36 ± 3.04 | 0.0377; S |
| | No | Yes | 371.72 ± 11.5 | |
| | Yes | No | 71.24 ± 3.040 | 0.0012; S |
| | Yes | Yes | 96.66 ± 1.520 | |
| *M. longifolia* | No | No | 672.66 ± 0.00 | 0.0011; S |
| | No | Yes | 833.41 ± 9.29 | |
| | Yes | No | 253.56 ± 7.44 | 0.0128; S |
| | Yes | Yes | 277.24 ± 3.71 | |

**Where S = p < 0.05 = statistically significant and NS = p > 0.05 = not statistically significant**

**Table 5.10:** Percentage of the total phenolic content (TPC) lost from the plant materials after the desalination treatment using Amberlite MB-1

| Plant | % of TPC removed by the desalination process |
|---|---|
| Unhydrolyzed A. *afra* | 68.76 |
| Hydrolyzed *A. afra* | 75.14 |
| Unhydrolyzed *L. leonorus* | 79.49 |
| Hydrolyzed *L. leonorus* | 74.17 |
| Unhydrolyzed *M. longifolia* | 62.30 |
| Hydrolyzed *M. longifolia* | 66.73 |

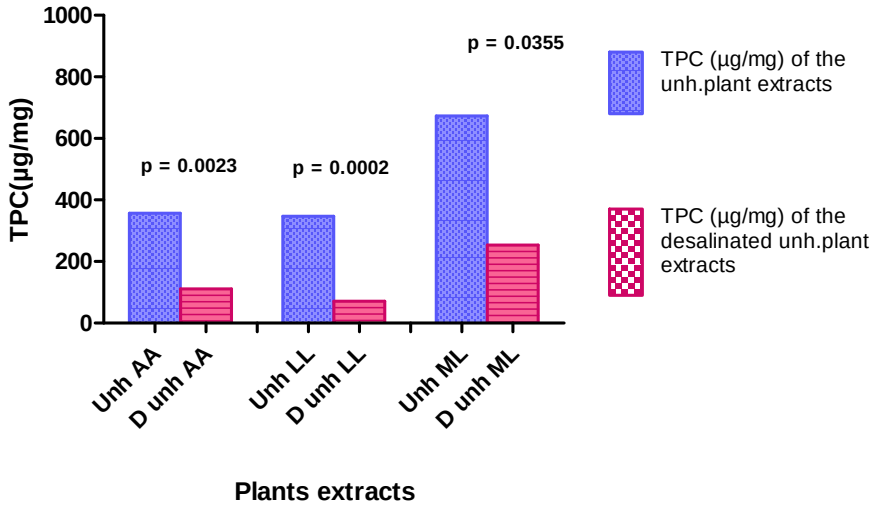



**Figure 5.11:** The total phenolic content (µg/mg) of the non-desalinated unhydrolyzed extracts of *A. afra* (AA unh), *L. leonorus* (LL unh) and *M. longifolia* (ML unh) and the desalinated unhydrolyzed plants (D unh AA, D unh LL, D unh ML). The p-values are for t-test of TPC in unhydrolyzed plant levels *vs* that in desalinated unhydrolyzed plant



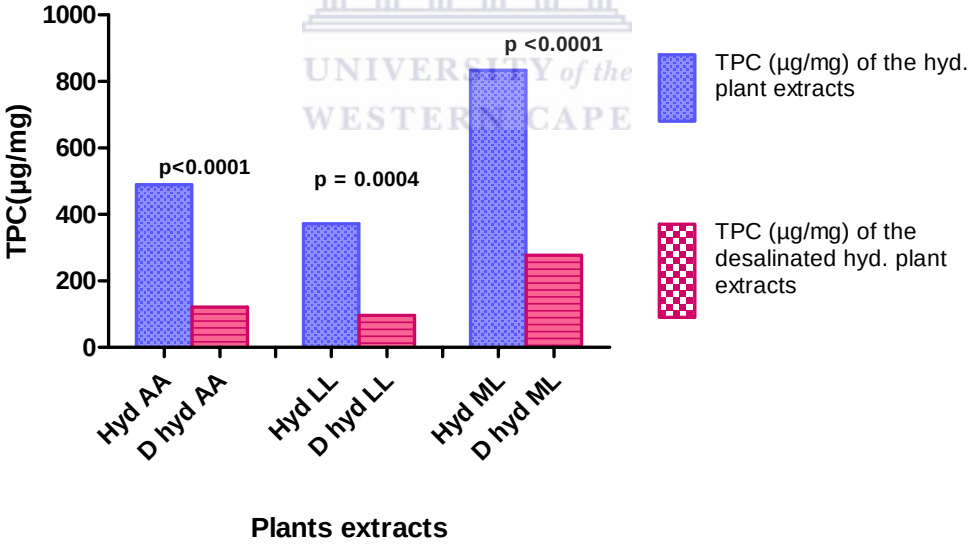



**Figure 5.12:** The total phenolic content (µg/mg) of the non-desalinated hydrolyzed extracts of *A. afra* (AA hyd), *L. leonorus* (LL hyd) and *M. longifolia* (ML hyd) and the desalinated hydrolyzed plants (D hyd AA, D hyd LL, D hyd ML). The p-values are for t-test of TPC in hydrolyzed plant levels *vs* that in desalinated hydrolyzed plant

Before the desalination treatment *M. longifolia* had the highest total phenolic compounds content (table 5.9 and figure 5.11) with the levels in *A. afra* and *L. leonorus* lower and quite similar (T test, p value = 0.1476). The desalination process removed more than 60 % of the phenolic compounds in all three plants (table 5.10) and the 3 desalinated extracts differed from each other in TPC levels (T test, $p < 0.0001$), the level being highest in the *M. longifolia* extract.

On the other hand, the acid-hydrolysis significantly increased the levels of TPC ($p<0.05$) in al three plant extracts (table 5.9 and figure 5.12). The highest total phenolic content was registered in *M. longifolia* (833.41± 9.29 µg/mg) followed by *A. afra* (489.36 ± 6.21µg/mg) and finally *L. leonorus* (371.72 ± 11.47µg/mg). However, more than 65 % of the TPC were removed from these hydrolyzed plant extracts during the desalination process (table 5.10). All three plants are reported in the literature to contain phenolic compounds (Muganga, 2004; Van Wyk et al., 2000; Sharaf et al., 2005) and the increase in the TPC after the hydrolysis process thus suggests that the acid hydrolysis may have caused the break down of glycosidic forms of phenolic compounds into aglycones, thus increasing their levels. Moreover, since flavonoids are the most abundant phenolic compounds in the plants, the loss of phenolic compounds from the plants during the desalination process probably reflected mainly flavonoid loss, which in turn could have affected the phosphodiesterase inhibitory activity of the different plant species.

Collectively, the above results indicate that *A. afra, L. leonorus* and *M. longifolia* extracts contained different levels of total phenolic compounds which however were not significant between *A. afra* and *L. leonorus.* These levels were increased by the hydrolysis process and decreased by the desalination process for all plant species. Finally, the difference in the TPC could affect the PDE 4 inhibitory activity of the plant species.

## 5.5. Quantification of luteolin or hesperetin in the plant extracts

### 5.5.1. Validation of the HPLC assay

The levels of luteolin or hesperetin in the plant materials were determined by a validated HPLC assay method. The results of the assay validation are summarized in table 5.11 and the linearity of the luteolin and hesperetin standard curves shown in figures 5.15 and 5.16, and sample chromatograms of standard solutions of luteolin and hesperetin and the plant materials shown in figures 5.13, 5.14,15 and 5.17 to 5.23

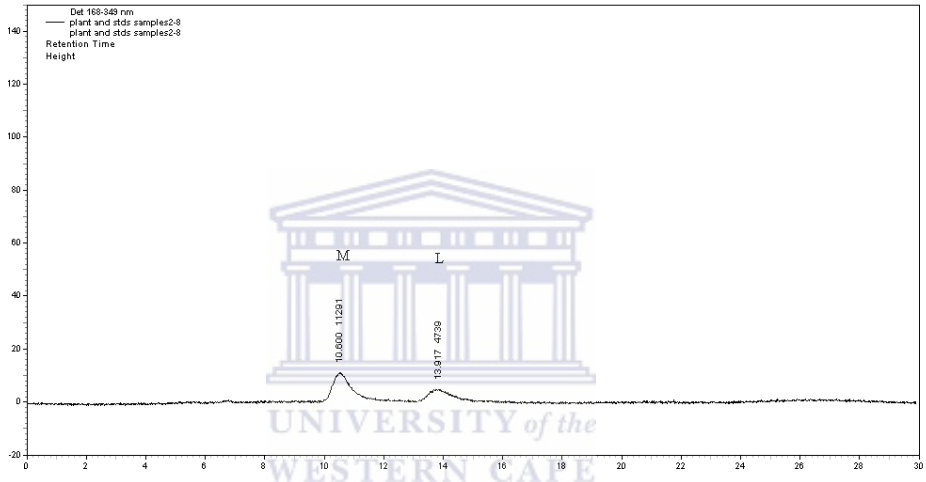



**Figure 5.13:** HPLC chromatogram showing the retention time of morin (10.600 min, peak M) and luteolin (13.917 min, peak L). Luteolin and morin were dissolved in deionized water and extracted with ethyl acetate. The chromatographic conditions are described in section 4.2.5.

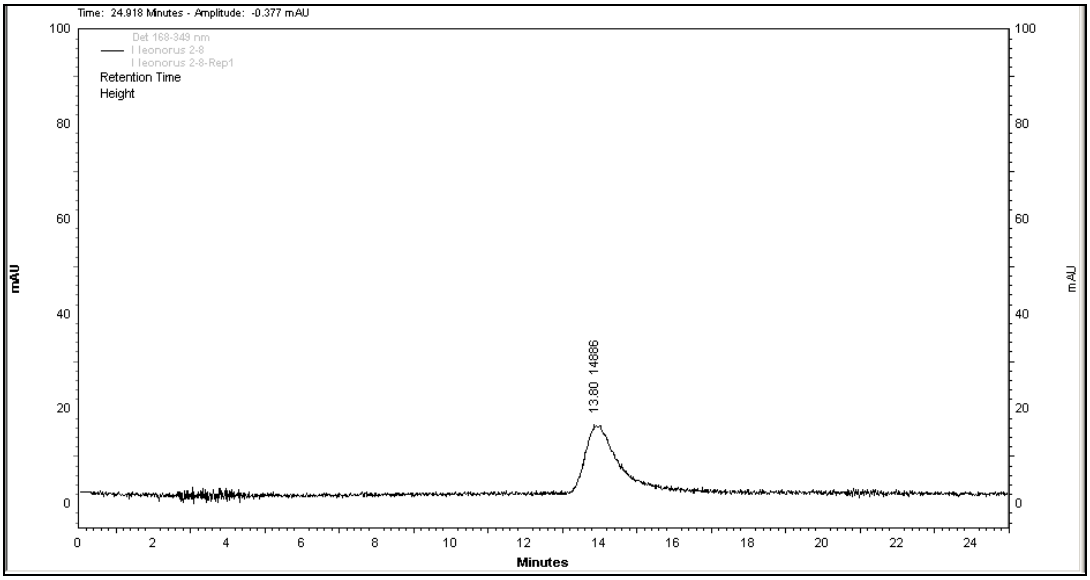



**Figure 5.14:** HPLC chromatogram showing the retention time of luteolin (13.800 min). Luteolin was dissolved in deionized water and extracted with ethyl acetate. The chromatographic conditions are described in section 4.2.5.



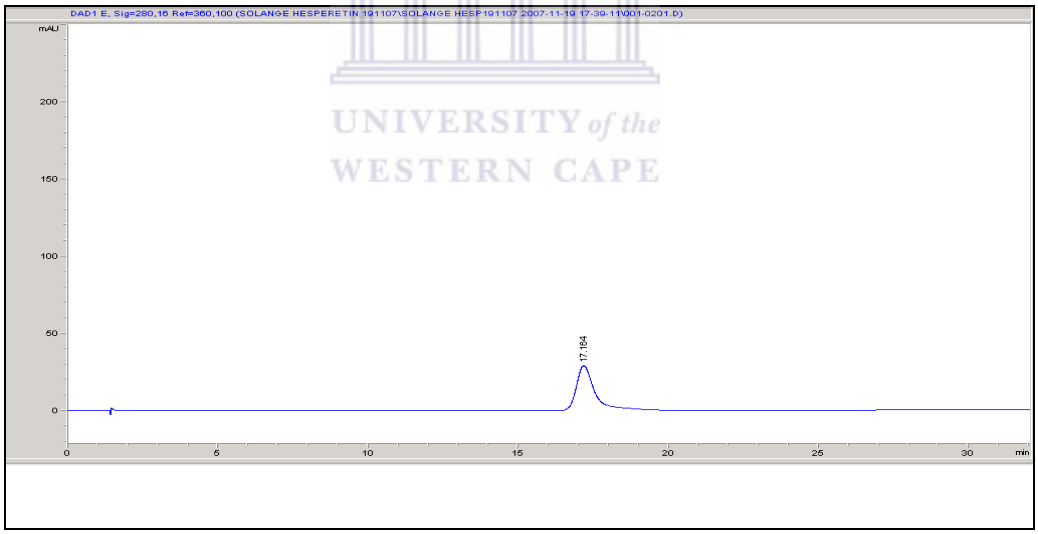



**Figure 5.15:** HPLC chromatogram showing the retention time of hesperetin (17.164 min). Hesperetin was dissolved in deionized water and the chromatographic conditions are described in section 4.2.4.

As described in section 4.2.5, the luteolin levels in *A. afra* and *L.* leonorus extracts and the hesperetin levels in *M. longifolia* extract were determined by HPLC using a reversed phase method. The mobile phases consisted of 30 % acetonitrile and 70 % $KH_2PO4$ buffer (pH 2) for luteolin assay and 45 % methanol and 55% formic acid for hesperetin

assay. These eluents were pumped isocratically, at room temperature (20°C) through a $C_{18}$ reverse phase column (Phenomenex, 4µm particle size and 250 x 4.60 mm), at a flow rate of 1ml per minute. Under the above conditions, the retention time of luteolin and morin, at 349 nm, varied between 13.133 minutes to 14.200 minutes (figure 5.13 and figure 5.14) and 9.17 to 10.922 minutes (figure 5.13), respectively. The retention time of hesperetin at 280 nm varied between 17.371 and 17.751min (figure 5.15). The peaks of luteolin, morin and hesperetin were symmetrical and the peak of morin was well separated from the luteolin peak when used as internal standard. While morin could have interfered with the other peaks in *L. leonorus* (figure 5.20) solutions or did not absorb at the wavelength used for hesperetin in the *M. longifolia* chromatogram, morin was well separated from and did not interfere with other peaks in *A. afra* (figure 5.19), indicating the suitability of morin as internal standard for the quantification of luteolin in *A. afra*. The retention times of luteolin and morin reported above, were comparable to those obtained by previous investigators (Dube, 2006 and Ma, 2006).



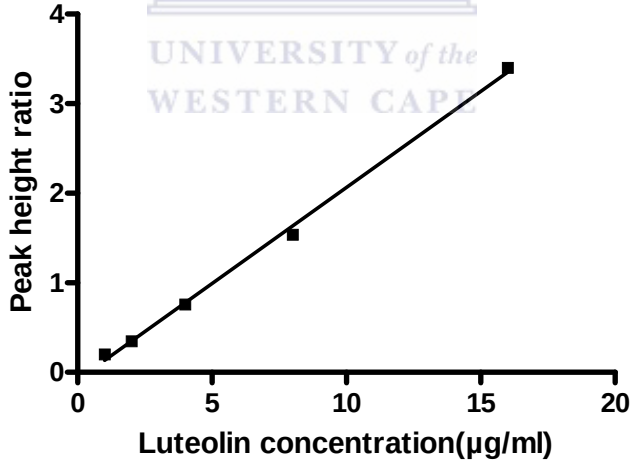



**Figure 5.16**: Luteolin standard curve for the quantification of luteolin in *A. afra* by HPLC assay. Linear regression equation: Peak height ratio = 0.5958 + 0.2144 x conc. (µg/ml); $r^2$=0.9976

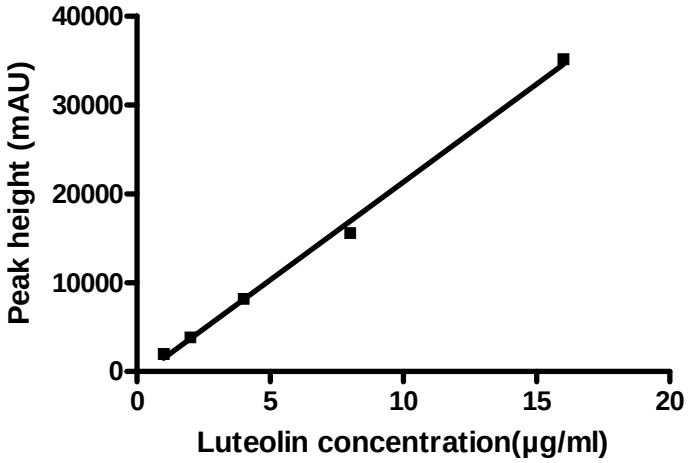



**Figure 5.17**: Luteolin standard curve for the quantification of luteolin in *L. leonorus* by HPLC assay. Linear regression equation: Absorbance (mAU) = - 713.4 + 2205 x conc. (µg/ml); $r^2$=0.9967



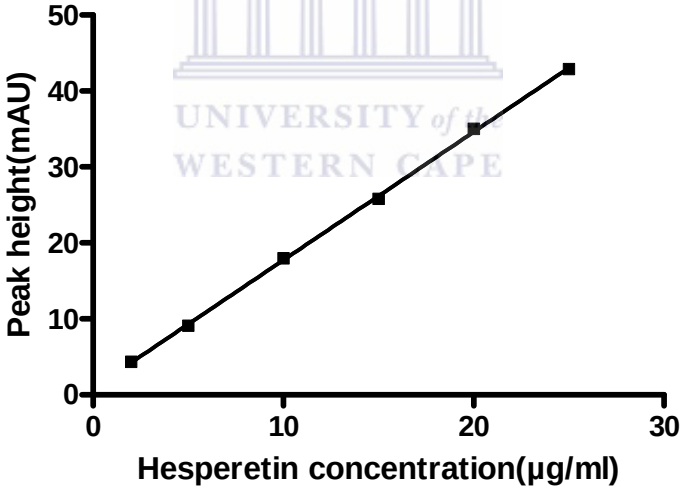



**Figure 5.18**: Hesperetin standard curve for the quantification of hesperetin in *M. longifolia* by HPLC assay. Linear regression equation: Absorbance (mAU) = 0.8598 + 1.688 x conc. (µg/ml); $r^2$=0.9991

**Table 5.11:** The validation results of the HPLC assay for luteolin and hesperetin quantification in plant extracts.

| **Validation parameter** | **Luteolin assay in *A. afra*** | **Luteolin assay in *L. leonorus*** | **Hesperetin assay in *M. longifolia*** |
|---|---|---|---|
| Limit of detection ng/ml (LOD) | 20 ng | 20 ng | 25 ng |
| Limit of quantification (LOQ) | 1µg/ml | 1µg/ml | 2 µg/ml |
| Linearity (correlation coefficient) | 0.9976 | 0.9967 | 0.9991 |
| Intra-assay reproducibility (% RSD) | 6.73 | 1.06 | 0.85 |
| Inter-assay reproducibility (% RSD) | 5.26 | 6.91 | 1.91 |

The results of the assays validation are summarized in table 5.11. The assays were linear over a range of 1 to16 µg/ml and had a regression coefficient of 0.9976 and 0.9967 for luteolin quantification in *A. afra* and *L. Leonotis*, respectively. The assay for hesperetin quantification in *M. longifolia* was linear over a range of 2 to 25µg/ml and had a regression coefficient of 0.9991. The LOD was 20ng for both luteolin assays in *A. afra* and *L. leonorus*, and 25 ng for the hesperetin assay, while the LOQ was 1µg/ml for the luteolin assays and 2 µg/ml for the hesperetin assay. The % RSD calculated to determine the intra assay reproducibility were 6.73; 1.06 and 0.85 % for the luteolin assay in *A. afra* and *L. leonorus* and the hesperetin assay in *M. longifolia*, respectively. In the same order, the inter-reproducibility was 5.26; 6.91 and 1.91 %.

In general the assays were specific and reproducible enough and linear over suitable concentration ranges to quantify luteolin in the *A. afra* and *L. leonorus* and hesperetin in the *M. longifolia* aqueous extracts.

### 5.5.2 Levels of luteolin or hesperetin in the plant extracts

With a view to correlate the level of luteolin and hesperetin to the PDE 4 inhibitory activity of the plant extracts**,** luteolin and hesperetin were quantified in the hydrolyzed and the unhydrolyzed plant extracts before and after the desalination process using the validated assay methods.

Using the retention time and the UV – spectra of the standard, luteolin, morin and hesperetin were identified in the plant materials as shown in figures (5.19, 5.20, 5.21, 5.22 and 5.23). The results indicating the quantitated levels of luteolin & hesperetin are reported in table 5.12 and the percent of flavonoid lost during the desalination process reported in table 5.13. The fingerprint and the desalinated plant chromatograms are shown in appendices 1 to 11.



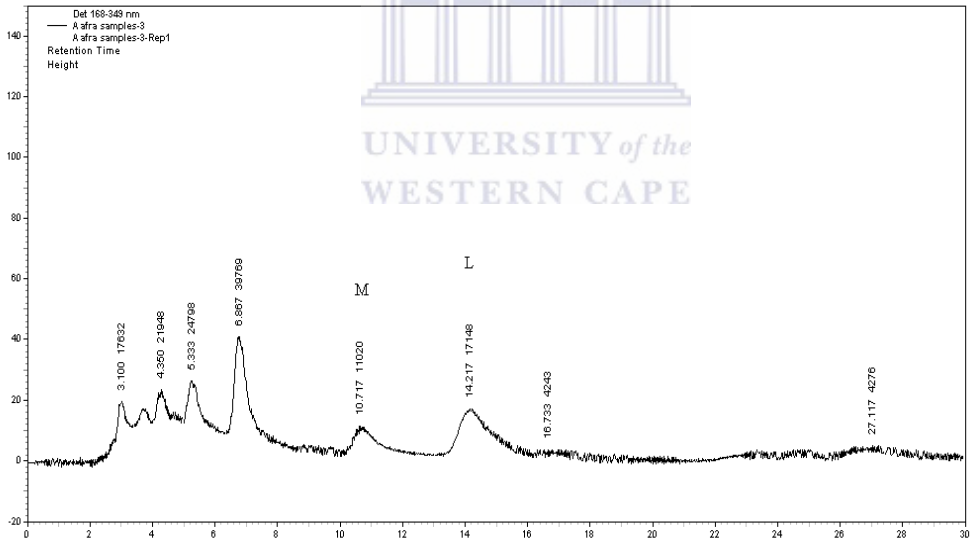



**Figure 5.19:** HPLC chromatogram of an unhydrolyzed extract of *A. afra.* Luteolin is represented by the peak L (retention time: 14.217 min) and morin by the peak M (retention time: 10.717 min). The chromatographic conditions are described in section 4.2.5.

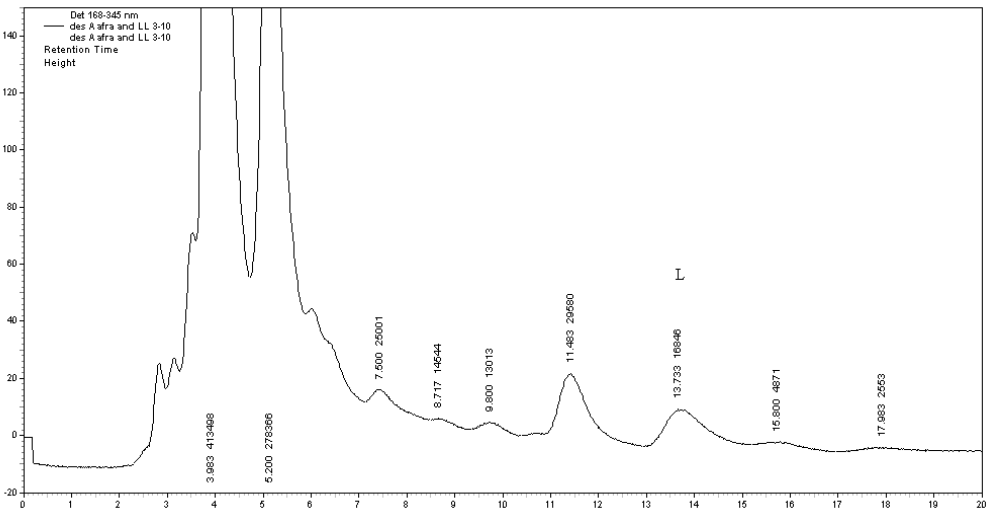



**Figure 5. 20:** HPLC chromatogram of an unhydrolyzed extract of *L leonorus.* Luteolin is represented by the peak L (retention time: 13.733 min). The chromatographic conditions are described in section 4.2.5.

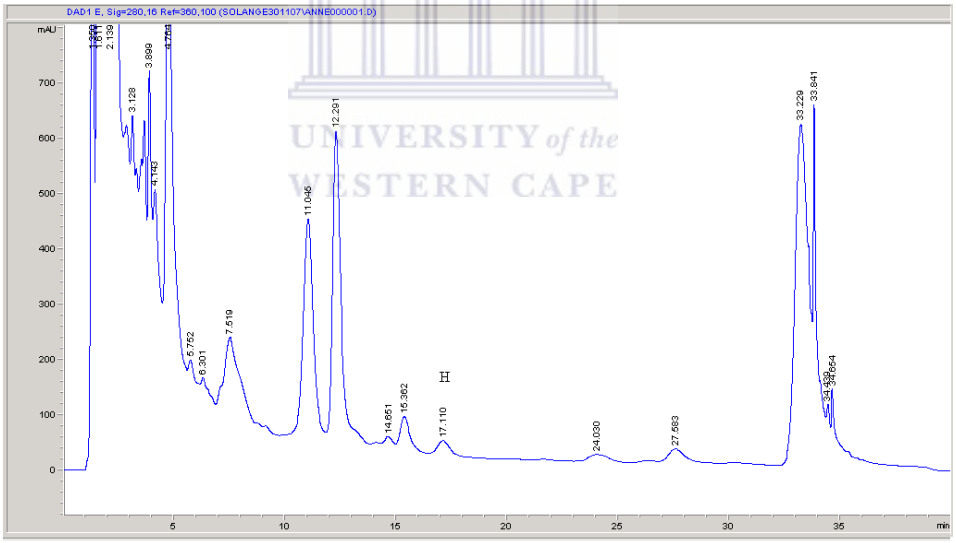



**Figure 5. 21:** HPLC chromatogram of an unhydrolyzed extract of *M.longifolia.* Hesperetin is represented by the peak H (retention time: 17.110 min). The chromatographic conditions are described in section 4.2.5.

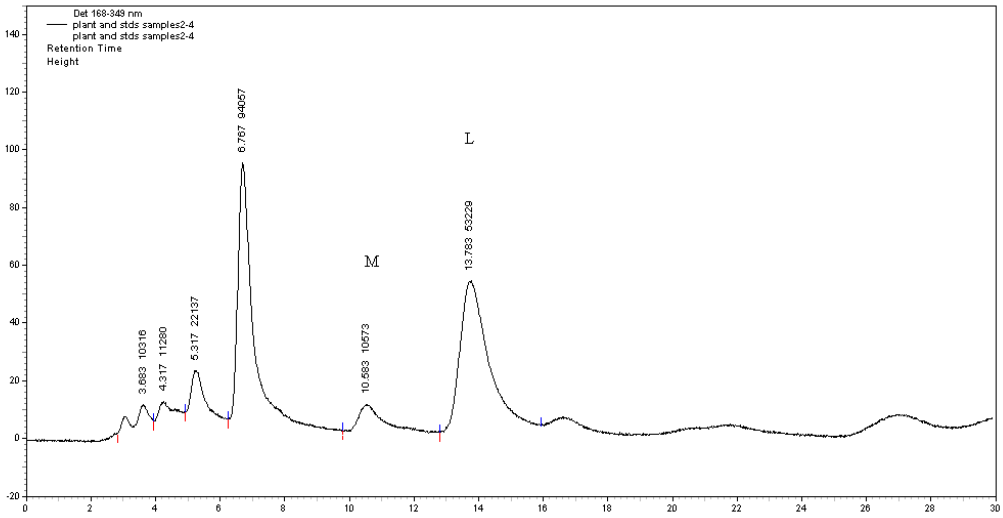



**Figure 5. 22:** HPLC chromatogram of a hydrolyzed extract of *A. afra.* Luteolin is represented by the peak L (retention time: 13.783 min) and morin by the peak M (retention time: 10.583 min). The chromatographic conditions are described in section 4.2.5.



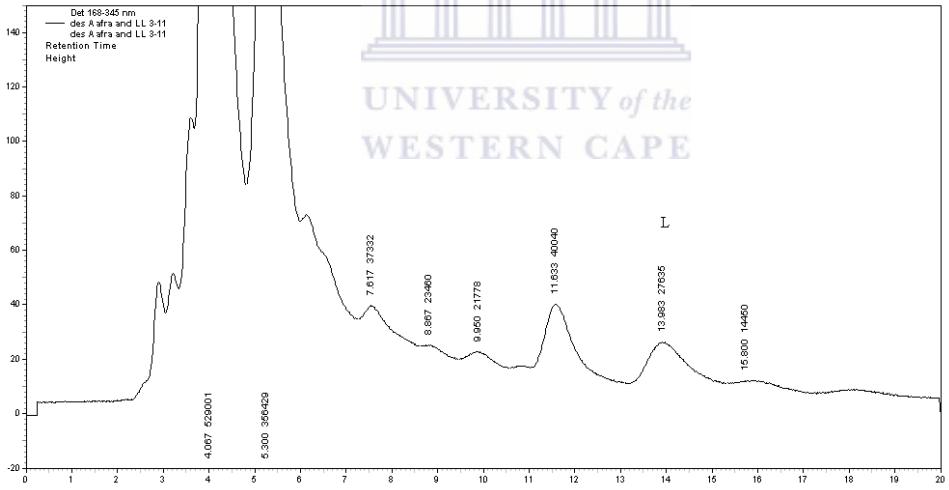



**Figure 5. 23:** HPLC chromatogram of a hydrolyzed extract of *L leonorus.* Luteolin is represented by the peak L (retention time: 13.983 min). The chromatographic conditions are described in section 4.2.5.

**Table 5.12:** Luteolin and hesperetin content (μg/mg of plant material) of the plant extracts

| Plant samples | Luteolin concentration (μg/mg of plant extract) Mean ± SD (n=6) | | Hesperetin concentration (μg/mg of plant extract) Mean ± SD (n=6) | |
|---|---|---|---|---|
| | Unhydrolyzed plant | hydrolyzed plant | Unhydrolyzed plant | Hydrolyzed plant |
| *A. afra* | 0.85 ± 0.042 | 0.92 ± 0.105 | - | - |
| *Desalinated A. afra* | 0.52 ± 0.046 | 0.83 ± 0.046 | - | - |
| *L. leonorus* | 0.427 ± 0.032 | 0.559 ± 0.075 | - | - |
| Desalinated *L. leonorus* | 0.126 ± 0.013 | 0.351 ± 0.040 | - | - |
| *M. Longifolia* | - | - | 0.147 ± 0.027 | ND |
| Desalinated *M. longifolia* | - | - | 0.082 ± 0.018 | ND |

Abbreviations: ND: not detected in the sample, -: not determined in the sample



**Table 5.13:** Percent loss in luteolin and hesperetin contents of the plant during the desalination process

| Plant | % of luteolin or hesperetin removed by the desalination process |
|---|---|
| Unhydrolyzed A. *afra* | 38.82 |
| Hydrolyzed *A. afra* | 9.78 |
| Unhydrolyzed *L. leonorus* | 70.49 |
| Hydrolyzed *L. leonorus* | 39.20 |
| Unhydrolyzed *M. longifolia* | 44.22 |
| Hydrolyzed *M. longifolia* | Not determined |

The unhydrolyzed and hydrolyzed aqueous extracts of *A. afra* and *L. leonorus* contained the following concentrations of luteolin: 0.85 ± 0.42 μg/mg, 0.92 ± 0.105 μg/mg and 0.427 ± 0.032 μg/mg, 0.559 ± 0.075 μg/mg, respectively (table 5.12). After the acid-hydrolysis, the levels of luteolin were slightly higher in the plant extracts (an increase of 8.23 % and 30.91 % was registered for *A. afra* and *L. leonorus*, respectively) although not statistically significant for *A. afra* (T test, p = 0.2350), when compared to those in the unhydrolyzed extracts. This finding suggests that the unhydrolyzed extracts contained

luteolin glycosylated forms, which after hydrolysis, broke down and liberated the luteolin aglycone, as previous investigators have reported (e.g. Muganga, 2004). In the case of *A. afra*, the increase was not significant probably due to the fact that the plant already contained more aglycones than glycoside flavonoids, possibly because the plant extract may have absorbed some moisture during the storage and this causing premature hydrolysis in the extract.

The levels of luteolin in the unhydrolyzed and the hydrolyzed extracts of *A. afra* were also comparable to those obtained in earlier studies by Muganga (2004) and Mukinda (2005), respectively, while the free luteolin level estimated in the unhydrolyzed extracts and the total luteolin level obtained after the hydrolysis of *L. leonorus* extracts were comparable to those reported by Ma (2006).

*M. longifolia* aqueous extract that was directly injected onto the column as described in section 4.2.5 contained 0.147µg/mg of hesperetin. However hesperetin was not detected in the hydrolyzed extracts probably because the hydrolysis process changed the nature of the compound. The levels of hesperetin found in the *M. longifolia* in the present studies were approximately 10 times higher than those obtained by Waithaka (2004). The difference in the time of harvest, the preparation of the plant extracts and the storage conditions may explain this variation in the plant composition (Croll and Cordes, 2006).

Desalination caused a loss of luteolin in both plants. In the unhydrolyzed extracts, the levels of luteolin decreased by 38.82 % and 9.78 % for *A. afra* and *L. leonorus*, respectively, while it decreased by 70.49 % and 39.2 % for the hydrolyzed plant extracts. The level of hesperetin in *M. longifolia* extract was decreased by 44.22 % (table 5.13). One might expect that these changes might be reflected in the PDE-inhibitory activity of the plant extract, but because the PDE inhibitory assay was not conducted on the non-desalinated plants, it is difficult, at this stage, to establish at which level the loss of total flavonoids or individual flavonoid marker (luteolin or hesperetin) during the desalination process could have affected the activity of each plant.

Finally, it must be remembered that the luteolin or hesperetin contents of these plants may have been higher if more appropriate solvents were used to prepare the plant extracts since water, which was used for the extraction of plant materials in this study, is not the best solvent for flavonoid extraction.

Collectively**,** the above results show that the levels of luteolin were different in *A. afra* and *L. leonorus* and were higher in the *A. afra* extracts than in *L. leonorus* extracts. The levels of luteolin increased in both plants after hydrolysis, and decreased after the desalination process. Hesperetin could only be quantified in the unhydrolyzed aqueous extracts of *M. longifolia* and was not detected in the hydrolyzed extracts. In addition, the level of hesperetin decreased after the desalination process.

### 5.6. Correlation between the flavonoid markers, total flavonoid and phenol concentrations and the PDE 4 inhibitory activity of the plants



Since flavonoids are reported to inhibit PDE 4, and variable total flavonoid and phenol concentrations and PDE-4 inhibitory activities were obtained for the 3 plants studied it was also of interest to determine whether an actual correlation existed between the flavonoid markers levels, the TFC and TPC of the three plants and their PDE 4 inhibitory activity. Only the TFC, the TPC and the individual flavonoid levels versus PDE 4 $IC_{50}$ data for the desalinated plant extracts were assessed for a correlation because the PDE 4 inhibitory activity was only determined on the desalinated extracts of the plant solutions and not on the non desalinated plant solutions which contained high level of phosphates which could have interfered with the Biomol PDE assay. The results of this analysis are summarized in table 5.14.

**Table 5.14**: Correlation of the TFC, TPC, luteolin and hesperetin levels of the plants with the PDE 4 inhibitory activity ($IC_{50}$)

| Unhydrolyzed plants | PDE 4 $IC_{50}$ (µg/ml) ± SD | TFC (µg of QE /mg) ± SD | Pearson correlation coefficient (r) | TPC (µg of RE/mg) ± SD | Pearson correlation coefficient (r) | Marker level (µg/mg) ± SD | Pearson correlation coefficient (r) |
|---|---|---|---|---|---|---|---|
| *A afra* | 161.70 ± 1.80 | 20.61 ± 0.29 | | 111.57 ± 4.56 | | 0.52 ± 0.05 | |
| *L leonorus* | 212.12 ± 0.04 | 16.28 ± 0.36 | - 0.618* | 71.24 ± 3.04 | 0.88** | 0.126 ± 0.02 | -0.71** |
| *M longifolia* | 337.10 ± 0.04 | 16.84 ± 1.20 | | 253.56 ± 7.44 | | 0.082± 0.02 | |
| **Hydrolyzed plants** | **PDE 4 $IC_{50}$ (µg / ml) ± SD** | **TFC (µg of QE / mg) ± SD** | **Pearson correlation coefficient (r)** | **TPC (µg of RE/mg)) ± SD** | **Pearson correlation coefficient (r)** | **Marker level (µg/mg) ± SD** | **Pearson correlation coefficient (r)** |
| *A afra* | 144.0 ± 0.36 | 28.40 ± 5.36 | | 121.64 ± 1.90 | | 0.83 0.05 | |
| *L leonorus* | 167.3 ± 0.04 | 70.76 ± 7.10 | 0.18* | 96.66 ± 1.52 | 0.96** | 0.351 0.04 | -0.98** |
| *M. longifolia* | 280.2 ± 0.03 | 50.80 ± 7.22 | | 277.24 ± 3.71 | | - | |
| Hydrolyzed and unhydrolyzed extracts | - | - | -0.18* | - | 0.82** | - | -0.72** |



Where * = p value > 0.05: not significant

** = p value <0.05: significant

There was no correlation observed between the TFC and the PDE $IC_{50}$ of the unhydrolyzed plants (r = - 0.61, p value = 0.0837), the hydrolyzed plants (r = 0.18, p value= 0.6367) and both unhydrolyzed and hydrolyzed extracts taken together (- 0.18, p value = 0.46). This lack of correlation between the TFC and the PDE $IC_{50}$ of the plants aqueous extracts might have been because either the content of flavonoids was underestimated by the aluminium chloride method which only quantified the flavones and flavonols, or because the hydrolysis step degraded other compounds also involved in the PDE inhibitory activity. For instance, Meda et al. (2004) used the same method and found a low correlation between the TFC of some Burkina Fasau honey samples and their antioxidant activity. However, the lack of correlation between the TFC and the

PDE 4 $IC_{50}$ of the plants could suggest that their PDE inhibitory activity was not only caused by flavonoids but other compounds may also be involved.

A significant correlation was, however, found between the TPC and the PDE $IC_{50}$ of the unhydrolyzed plants, the hydrolyzed plants and both hydrolyzed and unhydrolyzed extracts ($r = 0.88$, p value: 0.018, $r = 0.96$, p value $< 0.0001$, $r = 0.82$, p value $< 0.0001$, respectively) strongly suggesting that the phenolic compounds (e.g. flavonoids and phenolic acids) present in the plants may contribute to their PDE 4 inhibitory activity.

If one assumes that luteolin and hesperetin have approximately the same PDE 4 inhibition potency, a correlation may then be established between luteolin or hesperetin levels and the PDE 4 $IC_{50}$. A significant negative correlation was thus observed between luteolin or hesperetin levels and the PDE 4 $IC_{50}$ of the unhydrolyzed extracts, the hydrolyzed extracts and the hydrolyzed and unhydrolyzed extracts combined ($r = -0.706$, p value = 0.034, $r = -0.988$, p value = 0.0002, $r = -0.724$, p value = 0.023, respectively). These results suggest that flavonoids could possibly contribute to the plant activity. In addition, the data also suggest that luteolin could be a good marker for the PDE 4 inhibitory activity of *A. afra* and *L. leonorus*, but hesperetin may not for *M. longifolia* because it was not detected in the hydrolyzed extracts.

In summary, there was no correlation between the TFC and the PDE 4 $IC_{50}$ of the plant extracts, but a good correlation was established between the TPC and the flavonoid markers levels, luteolin and hesperetin levels, and the plants PDE $IC_{50}$ suggesting that flavonoids and phenolic compounds may possibly contribute to the PDE inhibitory activity of *A. afra*, *L. leonorus* and *M. longifolia*.

# CHAPTER 6
# CONCLUSIONS

The specific objective of this study was to investigate whether *Artemisia afra, Leonotis leonorus* and *Mentha longifolia* have PDE-4 inhibitory activity, to determine and compare the levels of the total phenolic compounds, the total flavonoids and especially luteolin and hesperetin, in the 3 plant species, and finally, to determine if there was a correlation between the PDE inhibitory activity and the levels of flavonoids or phenolic compounds in the plants.

It was hypothesized that the plants with higher levels of total flavonoid, total phenolic compounds or aglycone luteolin or hesperetin had higher PDE - 4 inhibitory activity.

From the results obtained in this study the following primary conclusions can be drawn:

1. Aqueous solutions of *A. afra*, *L. leonorus* and *M. longifolia* contain significant amounts of phosphate which can interfere with the Biomol Green $^{TM}$ PDE assay. Fortunately, however, the phosphate can also be appropriately removed by a desalination process using Amberlite MB-1, an ion exchanger resin, after which the Biomol Green TM PDE assay can indeed be effectively applied.

2. Aqueous solutions of the freeze-dried aqueous extracts of A. *afra*, *L. leonorus* and *M. longifolia* have PDE 4 inhibitory activity. The latter was dose dependent, higher for the hydrolyzed than the unhydrolyzed extracts of the plants and quite likely linked to the presence of the aglycone forms of flavonoids. Based on the $IC_{50}$ the order of PDE inhibition activity was *A. afra* > *L. leonorus* > *M. longifolia* but all less potent than isobutyl methyl xanthine (IBMX), a known PDE inhibitor.

3. *A. afra*, *L. leonorus* and *M. longifolia* contain total flavonoids and total phenolic compounds contents which were highest in hydrolyzed extracts of the plants and were substantially decreased by desalinating using Amberlite MB-1 ion

exchanger resin. The TFC and the TPC for the desalinated extracts were in order *A. afra* > *L. leonorus* > *M. longifolia* and *M. longifolia* > *A. afra*> *L. leonorus,* respectively

4. The freeze-dried aqueous extracts of *A. afra* contains higher levels of the marker luteolin than *L. leonorus* extracts, which increased in both plants after hydrolysis, and decreased after the desalination of the extracts with Amberlite MB-1 desalination process. On the other hand, hesperetin was only quantified in the unhydrolyzed extracts of *M. longifolia* where its level was also decreased after the desalination process using the ion exchanger resin Amberlite MB-1.

5. There was no apparent correlation between the TFC and the PDE 4 $IC_{50}$ of the plant extracts, but the correlation was significant between the TPC and the flavonoid markers luteolin and hesperetin contents and the plants PDE 4 $IC_{50}$ suggesting that flavonoids and phenolic compounds may possibly contribute to the plants PDE 4 inhibitory activity. In addition luteolin may be a good marker for the PDE 4 inhibitory activity of *A. afra* and *L. leonorus* but not hesperetin for *M. longifolia* because it was not detectable in the hydrolyzed extracts of this plant species.

In summary, *A. afra*, *L. leonorus* and *M. longifolia* possess PDE 4 inhibitory activity which may correlate with the TPC and the individual flavonoid markers, luteolin and hesperetin, levels of the plant species.

Further studies are required to investigate whether the *in vitro* PDE 4 inhibitory activity of the plant species also occur *in vivo*. A good marker for *M. longifolia* and a good assay method for its quantification need to be found and the same study should also be conducted on different batches of plant materials to further clarify the possible role of flavonoids in the PDE inhibitory activity of these plants. However, it is clear that *A. afra*, *L. leonorus* and *M. longifolia* at least do have PDE 4 inhibitory activity which can contribute to their effectiveness in the treatment of asthma.

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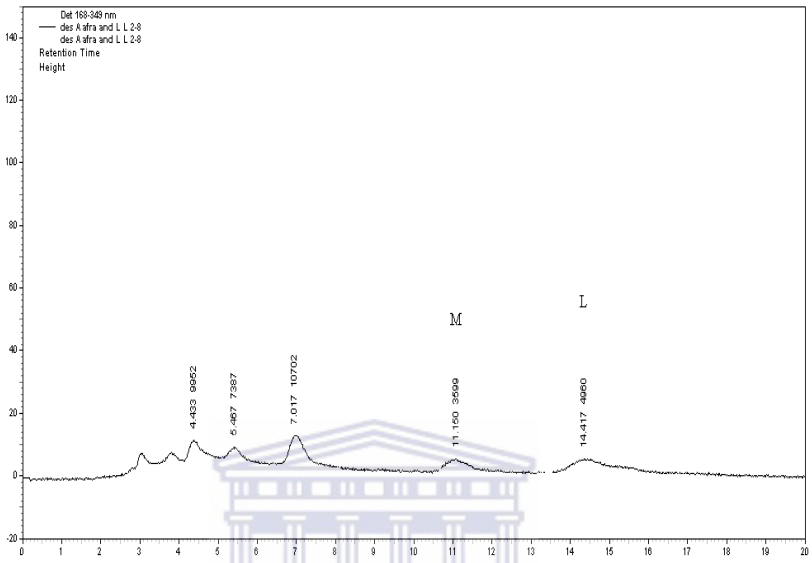
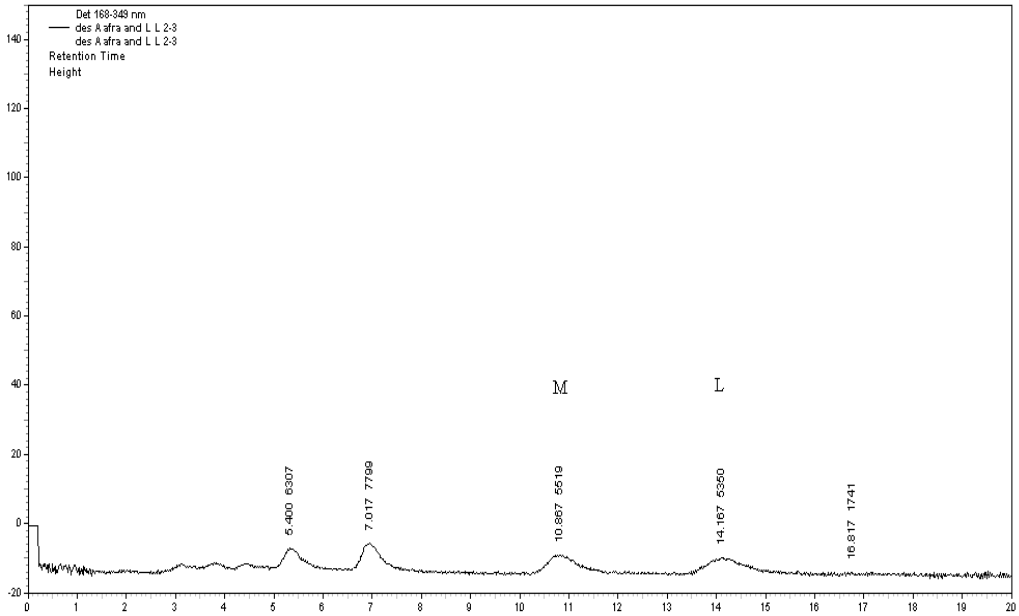
# APPENDICES

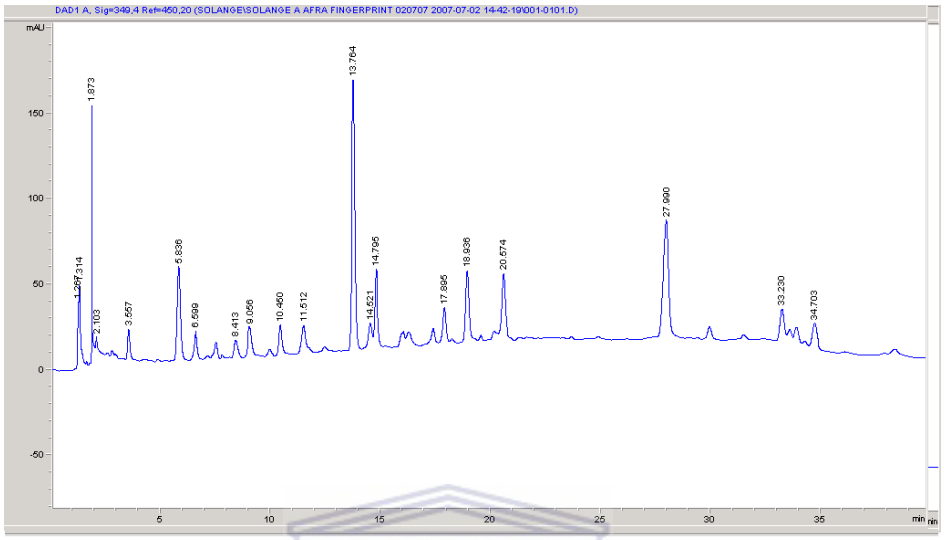
**Appendix 1:** HPLC chromatogram of a desalinated unhydrolyzed extract of *L leonorus.* Luteolin is represented by the peak L (retention time: 13.733 min). The chromatographic conditions are described in section 4.2.4.



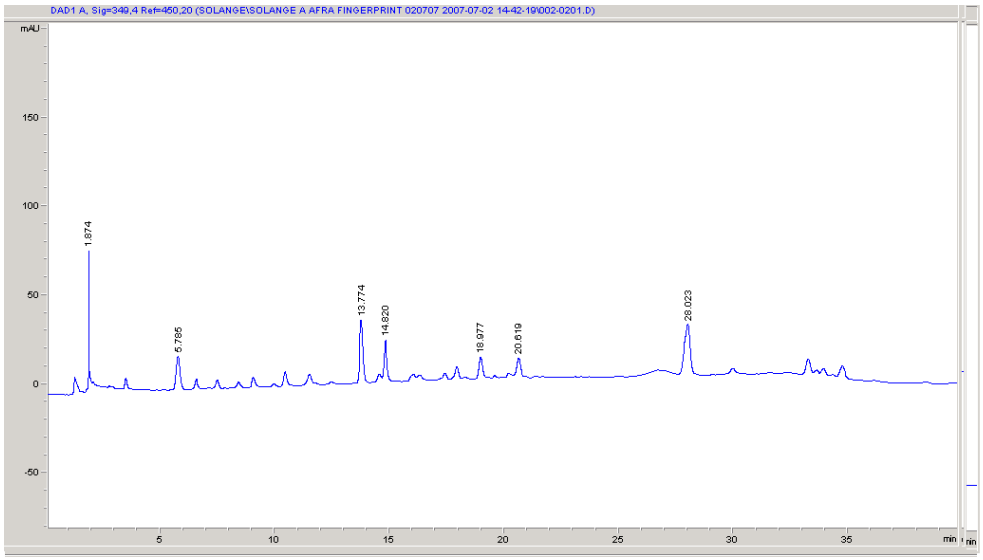

**Appendix 2:** HPLC chromatogram of a desalinated hydrolyzed extract of *A. afra.* Luteolin is represented by the peak L (retention time: 13.783 min) and morin by the peak M (retention time: 10.583 min). The chromatographic conditions are described in section 4.2.4.



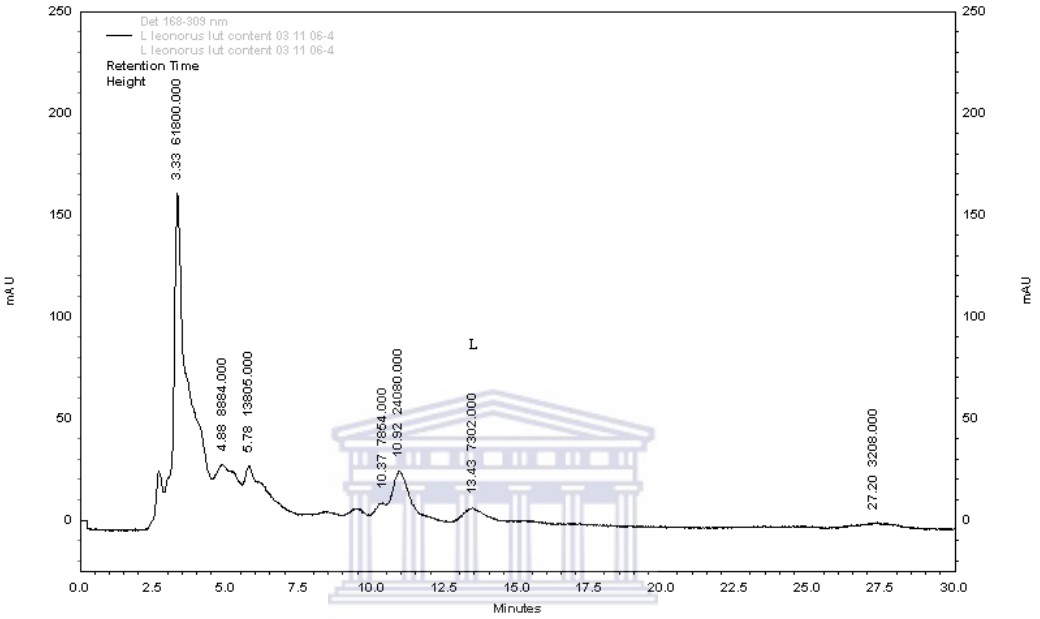
**Appendix 3:** Chromatographic fingerprint of *A. afra* aqueous extract (λ: 349 nm, mobile phase acetonitrile (solvent A) / 0.1% formic acid (solvent B), gradient elution: 95% B (2min), 90% B (3min), 85%B (5min), 80% B (8min), 75% B (22min), 20 % B (5min)




**Appendix 4:** Chromatographic fingerprint of the desalinated *A. afra* aqueous extract (λ: 349 nm, mobile phase acetonitrile (solvent A) / 0.1% formic acid (solvent B), gradient elution: 95% B (2min), 90% B (3min), 85%B (5min), 80% B (8min), 75% B (22min), 20 % B (5min)

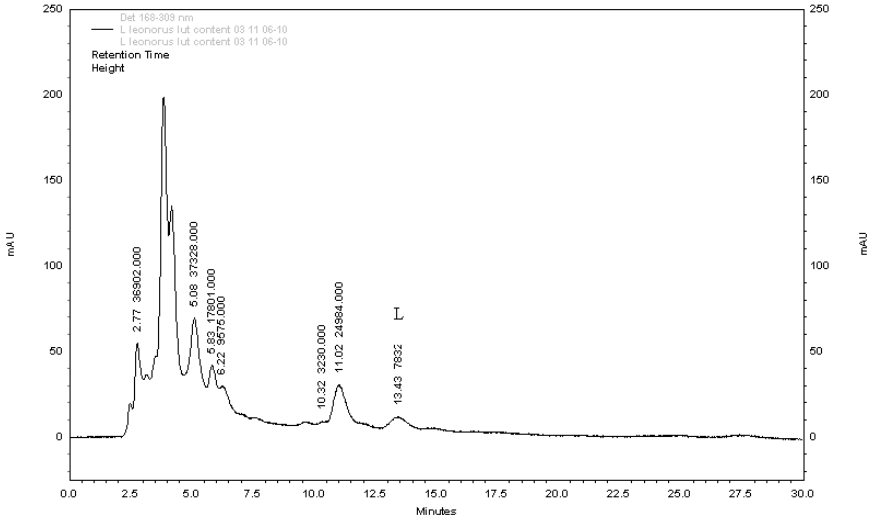


**Appendix 5:** HPLC chromatogram of a desalinated unhydrolyzed extract of *L. leonorus*. Luteolin is represented by the peak L (retention time: 13.783 min). The chromatographic conditions are described in section 4.2.4.

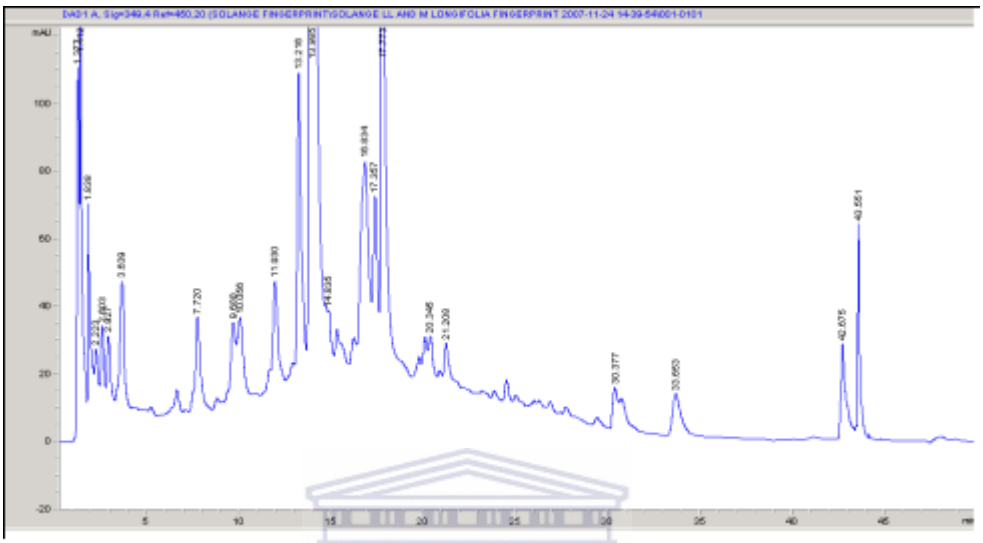




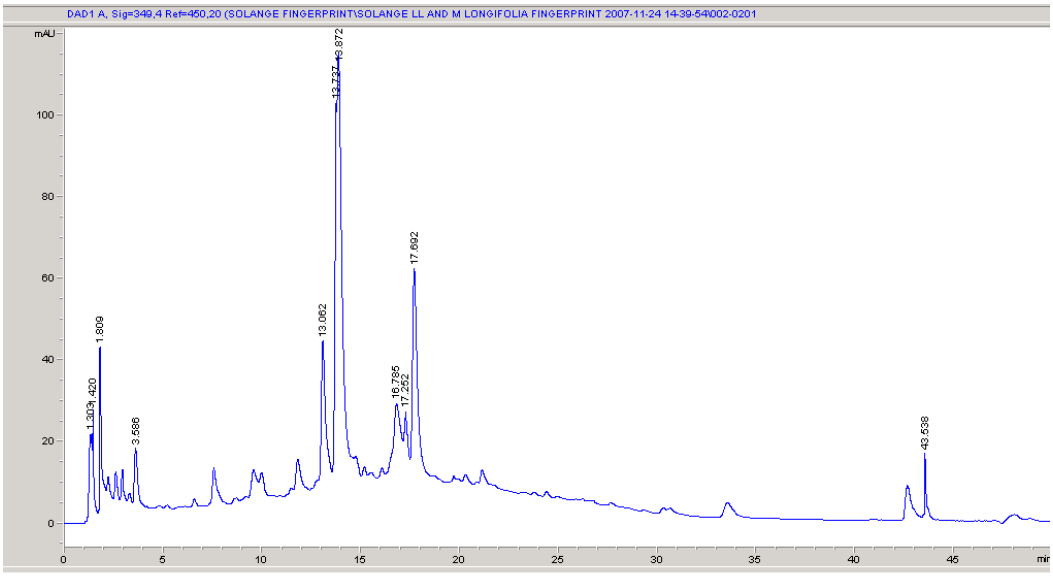

**Appendix 6:** HPLC chromatogram of a desalinated hydrolyzed extract of *L. leonorus*. Luteolin is represented by the peak L (retention time: 13.783 min). The chromatographic conditions are described in section 4.2.4.



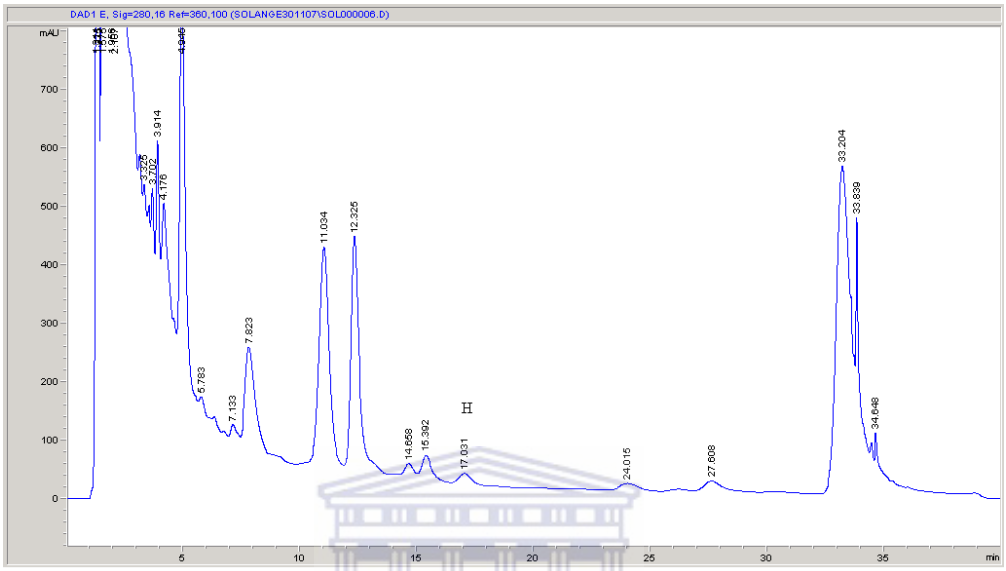
**Appendix 7:** Chromatographic fingerprint of *L. leonorus* aqueous extract (λ: 349 nm, mobile phase acetonitrile (solvent A) / 0.1% formic acid (solvent B), gradient elution: 95% B (2min), 90% B (3min), 85%B (5min), 80% B (8min), 75% B (22min), 20 % B (5min)



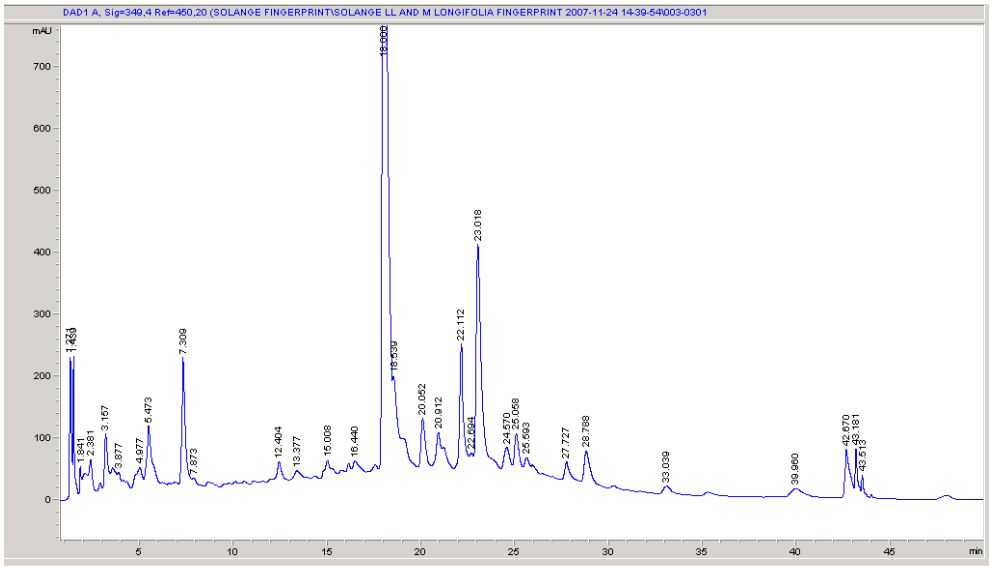
**Appendix 8:** Chromatographic fingerprint of the desalinated *L. leonorus* aqueous extract (λ: 349 nm, mobile phase acetonitrile (solvent A) / 0.1% formic acid (solvent B), gradient elution: 95% B (2min), 90% B (3min), 85%B (5min), 80% B (8min), 75% B (22min), 20 % B (5min)



**Appendix 9:** HPLC chromatogram of an unhydrolyzed extract of *M. longifolia.* Hesperetin is represented by the peak L (retention time: 13.783 min). The chromatographic conditions are described in section 4.2.4.




**Appendix 10:** Chromatographic fingerprint of the aqueous extract of *M. longifolia* (λ: 349 nm, mobile phase acetonitrile (solvent A) / 0.1% formic acid (solvent B), gradient elution: 95% B (2min), 90% B (3min), 85%B (5min), 80% B (8min), 75% B (22min), 20 % B (5min)



**Appendix 11:** Chromatographic fingerprint of the desalinated aqueous extract of *M. longifolia* (λ: 349 nm, mobile phase acetonitrile (solvent A)/ 0.1% formic acid (solvent B), gradient elution: 95% B (2min), 90% B (3min), 85%B (5min), 80% B (8min), 75% B (22min), 20 % B (5min)