TITLE:

An Evaluation of the Bronchodilator properties of *Mentha longifolia* and *Artemisia afra*, Traditional Medicinal Plants used in the Western Cape.

Lynne Harris

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Supervisor: Prof J. A. Syce

School of Pharmacy

University of the Western Cape

Bellville

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DEDICATION

In memory of my deceased parents for instilling confidence and a solid work ethic.

To my husband David Hitchman for all his support and encouragement.

To Jennifer and Christopher for your understanding and patience.

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SUMMARY

The overall objective of this study was to investigate the claims that *Mentha longifolia (ML)* and *Artemisia afra (AA)* have anti-asthmatic properties. To realize this objective we were to determine the effects that the plants may have on contractions induced by agonists (e.g. methacholine, histamine, and leukotriene D_4) and also to partially investigate the mechanism that may be involved. We hypothesized that extracts of *Mentha longifolia* and *Artemisia afra* would have respiratory airway smooth muscle relaxant properties and would be able to reverse methacholine and/or, histamine and/or leukotriene D_4-induced contractions.

Plants were collected from Kirstenbosch National Botanical Institute and aqueous extracts prepared. Solutions of plant extracts were injected into an organ bath containing a zigzag cut guinea pig tracheal strip that had been pre-contracted with methacholine, histamine or leukotriene D_4. The relaxant effects of the plants were expressed as a percentage of the maximal effect produced by isoprenaline ($6.67X10^{-5}M$). To determine the mechanism for the muscle relaxant effects of the plants, cumulative log dose-response curves (LDRC) for methacholine and histamine were obtained in the absence and presence of 2%, 10% and 20% solutions of the plant extracts. In addition the possible involvement of a β2-adrenoreceptor-mediated mechanism was assessed by determining the effects of increasing concentrations of *ML* and *AA* on methacholine-induced contractions in the absence and the presence of propranolol.

ML produced no direct contractile effect on the guinea pig tracheal muscle, but it relaxed methacholine ($6.67X10^{-8}M$)-induced contractions in a dose dependent manner. *ML* was able to fully reverse the methacholine-induced contraction, but the 2% and 10% solutions caused a non-parallel rightward shift in the LDR curves. In concentrations above 0.1% *ML* gave a 100% relaxation of histamine-induced ($6.67X10^{-6}M$) contractions. However, except for a slight leftward shift induced by 2% *ML*, the plant extract had no significant effect on the histamine LDRC. Concentrations of *ML* ≥ 5 % produced maximal relaxation of LTD_4–induced

($6.93X10^{-9}M$) contraction. Finally, in the presence of propranolol ($6.67X10^{-6}M$) the maximal relaxant effect of *ML* on methacholine ($6.67X10^{-5}M$)-induced contractions was reduced by approximately 20%.

AA produced no direct contractile effect on the guinea pig tracheal muscle, but on tracheal tissue not previously exposed to methacholine it produced a dose-dependent relaxation. When the tissue had, however, been exposed to methacholine and thoroughly washed before being exposed to doses of plant extract, 1% and 2 % *AA* solutions induced major contractile responses and displaced the LDRC upwards, while 10% to 30% *AA* solutions induced relaxation. These contractile responses were inhibited by ipratropium ($1.67X10^{-3}M$) and mepyramine ($4.13X10^{-9}M$). In the presence of 20% *AA* there was a pronounced non-parallel rightward shift of the LDRC of methacholine. *AA,* 1% to 30%, also caused a dose-dependent relaxation of histamine ($6.67X10^{-6}M$)- and LTD_4 ($6.93X10^{-9}M$)-induced contractions. Finally, in the presence of propranolol ($6.67X10^{-6}M$) the maximal relaxant effect of *AA* on methacholine ($6.67X10^{-5}M$)-induced contractions was reduced by approximately 27%.

Collectively these results indicate that aqueous extracts of *Mentha longifolia* and *Artemisia afra* have potent and qualitatively similar smooth muscle relaxant activity. These actions may be mediated via several receptor pathways and/or involve one or more common intermediate step (e.g. intracellular calcium flux) commonly involved in the mechanism of action of the agonists used. The results also very strongly suggest that the extracts may contain more than one active principle some of which may differ between the two plants. Overall the results confirm that aqueous solutions of *Mentha longifolia* and *Artemisia afra,* as used in local traditional practice, have potent bronchodilator activity that could be useful in the treatment of asthma.

CHAPTER 1: INTRODUCTION

Bronchial asthma is an old disease, but one whose incidence has been increasing alarmingly over the last few years.[1] Presently there is an epidemic of this chronic disease and the number of people with asthma has more than doubled from 1980 to 1996. In South Africa 10 - 15% of people have asthma and also here we have evidence of an increase in its incidence.[2]

Asthma is a complex disease for which several treatment options exist. It is primarily an inflammatory condition of the bronchial airways and manifests itself, in its various forms, as a syndrome of bronchial inflammation, hyperresponsiveness and airflow obstruction.[3] Current treatment of this condition involves the use of a wide range of bronchodilator agonists, and anti-inflammatory glucocorticoids, but often such treatment does not give the desired therapeutic outcome. The search for possible new treatments is thus an important ongoing pursuit and this includes exploring the potential of the herbal medicines advocated by traditional healers.

Traditional medicine, especially herbal medicine, is widely practiced in South Africa, but also has its limitations. It has been estimated that up to 80% of patients seen by medical practitioners also consult traditional healers.[4, 5] There is however little evidence in the available literature to substantiate the effectiveness of most traditional herbal treatments. In general there is indeed an urgent need for the scientific evaluation of the claimed effectiveness and safety of traditional herbal treatments. Such investigation may not only improve the existing use of herbal treatments, but could also lead to new drug discovery or advance the use of indigenous herbal medicines for orthodox treatment.

Several traditional medicinal plants have, over many years, been claimed to be effective and have actually been used to treat asthma and other respiratory airway diseases.[4, 5, 6, 7, 8, 9, 10] The traditional medicinal plants, *Mentha longifolia* and *Artemisia afra* have been used over many hundreds of years by the local people in the Western Cape especially for coughs, colds, and respiratory conditions.

According to researchers at the Montagu Museum numerous local traditional medicinal plants have been used for their anti-asthmatic properties, including: *Mentha longifolia* (wildekruisement) and *Artemisia afra* (wildeals).[9] Similar findings were recorded by Watt- and Breyer-Brandwijk.[10]

For a plant medicine to be effective it would need to able to reverse asthma pathology i.e. reverse or prevent the bronchial inflammation, and/or hyperresponsiveness and/or airflow obstruction. While plants have been shown to have bronchodilator and antimicrobial activity[7,8] that can be of use in respiratory disease, few of the local plant medicines, including *Mentha longifolia* and *Artemisia afra*, that may possibly be of use in asthma have, however, been scientifically validated for any of these asthma specific effects.

The primary objective of this investigation was therefore to evaluate the claims that *Mentha longifolia* and *Artemisia afra* had anti-asthmatic properties. When used for asthma or wheezing or chest tightness both *Mentha longifolia* and *Artemisia afra* were advocated to be used in the form of aqueous decoctions and drunk as tea. It was consequently suspected that aqueous extracts of these plants may have bronchodilatory effects. The specific objective of this investigation was therefore to determine the effect that crude aqueous extracts of these plants may have on airway smooth muscle that had been contracted by known bronchial constrictors and mediators of asthma, viz. methacholine, histamine, and leukotriene D_4. It was hypothesized that such crude aqueous plant extracts would contain bronchodilator substance(s) that could reverse the contraction of airway smooth muscle induced by the above-mentioned mediators. To realize this objective we consequently proposed to examine the effect that the extract(s) had on mediator-induced contraction of the guinea pig tracheal smooth muscle. Any relaxation of the contracted tracheal smooth muscle would indicate the presence of bronchodilator activity in the crude plant extracts.

Confirmation of the pharmacological effects of these plants under scientific conditions in the laboratory could lead to the plants being further investigated to identify the active ingredients.

CHAPTER 2: LITERATURE REVIEW

2.1 Asthma

In this literature review the following issues related to asthma are discussed, viz. a definition of asthma, its aetiology and its prevalence. Also included, are the current and possible future treatment methods as well as the potential of finding new treatments when researching the use of traditional medicinal plants, with emphasis on the two plants *Mentha longifolia* and *Artemisia afra*.

2.1.1 Definition of asthma

Asthma is a chronic, episodic disease of the airways with a wide spectrum of manifestations. In 1995, the National Heart, Lung and Blood Institute (NHLBI)[11] gave this working definition:

"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli."[12]

2.1.2 Aetiology of asthma

There is an emerging body of knowledge in asthma pathogenesis that implicates several different mediators in the asthma inflammatory cascade. (Figure 2.1) It appears that there are inflammatory cellular, epithelial, neurogenic and various different biochemical mediators that are important. It is likely that multiple cells and multiple mediators are involved in asthmatic responses in different individuals or even within a single asthmatic patient. A possible explanation for the inflammatory cascade is that a stimulus (either specific or non-specific) interacts with airway effector cells (such as mast cells or macrophages) to release a variety of preformed chemical mediators. These mediators (which may include histamine,

prostaglandins and leukotrienes) may produce the early asthmatic response (EAR) by immediate effects on target airway tissue, resulting in: airway smooth muscle constriction, mucous hypersecretion, and mucosal oedema.

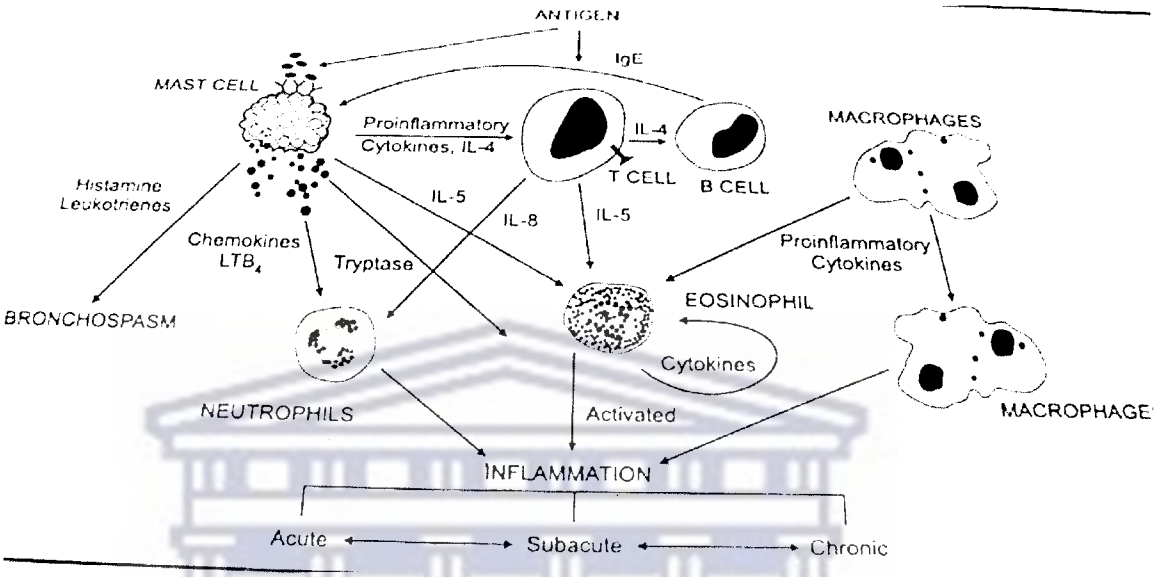


Figure 2.1 A summary of the proposed mechanisms in the inflammatory cascade in asthma.[12] (Abbreviations used: IgE, immunoglobulin E, IL-4 (-5,-8), interleukin 4 (5, 8), LTB_4 leukotriene B_4.)

Simultaneously, lymphokines and other chemotatic compounds may elicit a migration of lymphocytes, neutrophils and eosinophils to the site of degranulation and may activate the late asthmatic response (LAR) that may take hours to develop. These additional cells could subsequently produce mediators that may: damage the respiratory epithelium; perpetuate or amplify the inflammatory progress; stimulate afferent nerve endings and propagate a stimulus along other airways.[12]

Numerous studies[12] have recently advanced the notion that the T-lymphocyte plays a pivotal role in the regulation and expression of local eosinophilia and immunoglobulin E (IgE) production in both asthma and allergic disease. Whole blood from patients with atopic asthma reveals expression of CD_4-positive lymphocytes (T–helper cells). It appears that T-helper cells can further be

categorized as T_{H1} or T_{H2} cells based on the profile of cytokines these cells are capable of releasing. The T_{H1} cells produces interleukin 2 and 3 (IL-2 and IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon gamma (INF-γ), which leads to delayed hypersensitivity-type response. In contrast, T_{H2} lymphocytes mediate allergic inflammation in atopic asthmatics by a cytokine profile that involves IL-4 (which directs B-lymphocytes to synthesize IgE), IL-5 (which is essential for the maturation of eosinophils), along with IL-3 and GM-CSF. Therefore, preliminary evidence suggests that atopic asthma is regulated by activation of the T_{H2}-like T-cell population.[12]

The inflammatory cells that infiltrate the lungs during asthma produce a variety of mediators. These lipid mediators which are products of arachidonic acid metabolism have especially been implicated in the airway inflammation of asthma. Prostaglandins are generated by the cyclo-oxygenation of arachidonic acid, and leukotrienes are generated by the lipoxygenation of arachidonic acid. The proinflammatory prostaglandins (PGD_2, PGF_2, TXB_2) cause bronchoconstriction, whereas other prostaglandins are considered protective and may elicit bronchodilation (PGE_2 and PGI_2 or prostacyclin). Leukotrienes C_4, D_4 and E_4 comprise the compound slow –reacting substance of anaphylaxis (SRS-A), a potent stimulus of smooth muscle contraction and mucus secretion. LTB_4 is a highly potent chemotatic factor for both neutrophils and eosinophils. Pharmacologic antagonism of the action of these mediators has therefore been pursued in order to find new treatment for inflammation of asthma, and a new group of agents (anti-leukotrienes) that interfere with leukotriene action has recently been approved by the Food and Drug Administration (FDA) for use in asthma.[12]

2.1.3 Prevalence of asthma

Worldwide, and in South Africa, there is a marked increase in the prevalence of asthma. Asthma is an epidemic of a chronic disease, the number of people with asthma throughout the world have more than doubled from 1980 to 1996, with children under five experiencing the highest rate of increase.[11] The prevalence rate in South Africa is 10-15% of the population.[2]

2.2 Current treatments of asthma

Both the frequency and severity of asthma symptoms can be reduced by treatment with medications and reduction in exposure to environmental triggers. There are two main categories of anti-asthma drugs, viz. the bronchodilators and anti-inflammatory agents.

2.2.1 Bronchodilator drugs

There are three types of bronchodilators used to treat asthma, namely the β2-adrenoceptor agonists, the xanthines and the muscarinic receptor antagonists.

2.2.1.1 β2-adrenoceptor agonists

The β2-adrenoceptor agonists are the drugs of choice for the immediate phase of the asthmatic attack, and their action is two-fold. The main effect is to dilate the bronchi by a direct action on the β2-adrenoceptors on the smooth muscle. Being physiological antagonists, these drugs can relax the bronchial muscle irrespective of the spasmogen involved. β2-adrenoceptor agonists may also inhibit mediator release from mast cells, and inhibit the release of tumour necrosis factor (TNF-α) from monocytes, one of the primary mediators of inflammation. In addition they may enhance mucous clearance by an action on cilia.[13]

The β2-adrenoceptor agonists are usually given by inhalation in the form of an aerosol, powder or nebulised solution, but some may be given orally or parenterally. In the case of aerosol devices used by the elderly and children, spacer devices are used for ease and greater efficiency.[13]

There are two categories of β2-adrenoceptor agonists used in asthma, viz. the shorter acting and longer acting agents. The shorter acting agents include the two main drugs salbutamol and terbutaline. When administered by inhalation these agents start to act within a few minutes, with the maximum effect being reached within 30 minutes and the effects lasting for 4-6 hours. These β2-adrenoceptor agonist aerosols should only be used when necessary to control symptoms. Some degree of tolerance to their bronchodilator effect can develop if they are used for 2-3 weeks; but the decreased responsiveness can be reversed by parenteral steroids.[13] There are also

reports that a rebound bronchial hyper-reactivity may follow cessation of treatment after 2 weeks of continuous therapy. In severe attacks salbutamol and terbutaline may be given by the parenteral route.[13]

Longer-acting β2-adrenoceptor agonists include salmeterol and formoterol, which when given by inhalation produce effects for up to 12 hours. They are used twice daily regularly to control asthma, and are often used as adjuvant in patients whose asthma is inadequately controlled by glucocorticosteroids.[13]

2.2.1.2 Xanthine drugs

There are three pharmacologically active naturally-occurring methylxanthines: theophylline; theobromine and; caffeine. The xanthine usually employed in clinical medicine is theophylline (1,3-dimethylxanthine), which can be used also as theophylline-ethylenediamine, known as aminophylline. Caffeine and theophylline are constituents of coffee and tea, and theobromine is a constituent of cocoa. Both theophylline and aminophylline have bronchodilator action, though they are rather less effective in this regard than β-adrenoceptor agonists. Several clinical studies have shown that xanthines can be effective both in relieving the acute attack and in the treatment of chronic asthma. Actions in addition to bronchodilatation seem to be involved since there is some evidence that these agents can inhibit the late phase, as shown by measurement of FEV1, after bronchial allergen challenge. However, they do not appear to prevent bronchial hyper-responsiveness.[13]

The way in which this group of drugs produces its effects in asthma is still unclear. The relaxant effect on smooth muscle has been attributed to inhibition of phosphodiesterase (PDE) with resultant increase in cyclic AMP. An increase in cyclic AMP could also inhibit activation of inflammatory cells. However, the concentrations necessary to inhibit the isolated enzyme greatly exceed the therapeutic range. There is some evidence that the smooth muscle relaxation could be related to an effect on a cyclic GMP phosphodiesterase. When theophylline is used in asthma, most of its other effects, such as those on the CNS, cardiovascular system and gastrointestinal tract, are unwanted side effects. Furthermore, theophylline has a relatively low therapeutic index.[13]

2.2.1.3 Muscarinic-receptor antagonists

The compound of this class of agents which is specifically used as an anti-asthmatic is ipratropium bromide. This drug is not particularly effective against allergen challenge. It is only of real use in asthmatic attacks in which there is a distinct component of reflex bronchospasm mediated by parasympathetic nerves; particularly in asthma produced by irritant stimuli. It inhibits enhanced mucous secretion which occurs in asthma, and also increases ciliary clearance of mucous. It has no effect in the late inflammatory phase, but may be useful as an adjunct to other bronchodilator therapy, particularly in severe acute asthma. It is normally given in aerosol form where it acts on muscarinic receptors in the bronchi. The maximum effect occurs within 30 minutes and lasts for 3-5 hours. Finally, ipratropium bromide is well tolerated and can be used with β2 agonists.[13]

2.2.2 Histamine H1-receptor antagonists

Although mast cell mediators are thought to play a part in the immediate phase of allergic asthma and in exercise-induced asthma, histamine H1-receptor antagonists have had no place in therapy. However, clinical trials have recently shown that some newer non-sedating antihistamines such as loratidine are moderately effective in mild atopic asthma.[13]

2.2.3 Anti-inflammatory agents

There are two different types of anti-inflammatory drugs used in the treatment of asthma. viz., glucocorticoids, which are used mainly in chronic conditions for its anti-inflammatory action and sodium cromoglycate which is thought to effect their action by reducing bronchial hyper-reactivity.[13]

2.2.3.1 Glucocorticoids

Glucocorticoids are not bronchodilators and are not effective in the treatment of the immediate response to the eliciting agent. In the management of chronic asthma, which comprises predominantly an inflammatory component, their effect is unequalled. Glucocorticoids reduce the formation of cytokines, particularly Th2 cytokines that recruit and activate eosinophils and are responsible for promoting the production of IgE and the expression of the IgE receptors. Glucocorticoids inhibit

the generation of the vasodilators, PGE_2 and PGI_2, by inhibiting the induction of cyclo-oxygenase-2. In addition, by virtue of inducing lipocortin, they may inhibit production of the spasmogens, LTC_4 and LTD_4 and reduce the synthesis of the leukocyte chemotaxins, LTB_4 and platelet activating factor (PAF), thus reducing recruitment and activation of inflammatory cells. It has also been shown that the glucocorticoids can inhibit the allergen-induced influx of eosinophils into the lung. Furthermore, glucocorticoids can up-regulate β2-adrenoceptors, reduce microvascular permeability and decrease mediator release from eosinophils. The reduction in synthesis of IL-3 (the cytokine that regulates mast cell production) possibly explains why long-term steroid treatment leads to a reduction in the early-phase response to allergens, and prevents exercise induced asthma. [13]

The main examples of the glucocorticoids used are beclomethasone, budesonide, and fluticasone which are given by metered dose inhalation, the full effect being attained only after several days of therapy.

Glucocorticoids given by inhalation constitute a major advance in the overall management of asthma. They are able to control the disease without causing adverse systemic effects or adrenal suppression. For acute, severe or rapidly deteriorating asthma, a short course of an oral glucocorticoid (e.g. prednisolone) is indicated, combined with an inhaled steroid to reduce the oral dose required. Unwanted effects are uncommon with inhaled steroids, especially with the newer compounds. Oropharyngeal candidiasis can occur, as can dysphonia (voice problems), but these are less likely to occur if spacer devices are used.[13]

2.2.3.2 Sodium cromoglycate and nedocromil

These compounds are not bronchodilators and do not have any direct effects on smooth muscle, nor do they inhibit the actions of any of the known smooth muscle stimulants. Continuous treatment with cromoglycate or nedocromil is thought to result in a decrease in bronchial hyper-reactivity. Given prophylactically they can prevent both the immediate and the late-phase asthmatic responses. They are effective in antigen-induced, exercise-induced and irritant-induced asthma, though not all asthmatic subjects respond, and it is not possible to predict which patients will benefit. It is generally said that children are more likely to respond, and

therefore these agents have become the anti-inflammatory drugs of first choice in children. Pre-treatment with one of the agents before exposure to an eliciting stimulus may be very effective in many young patients.[13]

As far as the mechanism of action is concerned, it was originally thought to act by preventing mediator release from mast cells, but they are not very potent as mast cell stabilizing agents, being in fact much less effective than salbutamol in this respect.[13]

2.2.4 Severe acute asthma

Severe acute asthma is a medical emergency requiring hospitalization. Treatment includes oxygen, and bronchodilator drugs such as salbutamol in oxygen given by nebulizer and intravenous hydrocortisone, followed by a course of oral prednisolone. Additional measures include the use of nebulized ipratropium, intravenous salbutamol and antibiotics if bacterial infection is present.[13]

2.2.5 Possible future strategies for asthma therapy

Although there are several agents that can reverse the acute asthmatic attack and others which reduce the underlying inflammation and bronchial hyper-responsiveness, none is ideal in that they can actually 'cure' all patients of the disease. Many pharmaceutical firms are trying new approaches in attempts to develop more effective anti-asthma drugs, however, the cysteinyl leukotriene receptor antagonists (CysLTRAs) are the first new class of anti-asthma drugs for more than 20 years. [1, 13]

The development of agents affecting production or action of leukotrienes, offers an exciting new approach to the treatment of asthma. [1, 13] Two approaches to anti-leukotriene therapy have been developed: blocking their production by inhibiting the action of 5-lipoxygenase enzyme, or blocking the CysLTRl receptor. Both approaches have been tried in studies in asthma and overall results are encouraging, with decrease in both daytime and nocturnal symptoms, decrease in additional β2-adrenoceptor agonist usage, and improved lung function. Corticosteroids do not inhibit the production of cysteinyl leukotrienes *in vivo*, suggesting that CysLTRAs and corticosteroids affect different targets. Furthermore, the bronchodilator properties of CysLTRAs seem to be additive to those of β2-adrenoceptor agonists.

The anti-leukotrienes are intended for patients with persistent mild to moderate asthma. However, beneficial effects have been shown in the management of all grades of asthma severity. Furthermore, anti-leukotriene therapy has been shown to have an effect in specific types of asthma, viz., aspirin-sensitive and exercise induced asthma.[1, 13]

Longer term studies are needed in other areas such as severe asthma and chronic persistent asthma in both children and adults to provide evidence for the appropriate placement of anti-leukotriene treatment in current asthma guidelines, in comparison with other well established treatments. Further studies are also needed to determine long-term efficiency and tolerance to define their place in the strategy of asthma management.[1, 13]

The leukotriene receptor antagonists available are zafirlukast, which is indicated for the long term control of asthma, and is usually prescribed for children 12 years and over and also for adults. The normal dose is a twice daily dosage to be taken on an empty stomach.[1]
Montelukast is available in our country, and it has the advantage that it can be used for children as young as two years, prescribed as chew tablets to be taken once daily and given without regard to meals. Patients must be made aware that, as with inhaled corticosteroids, both zafirlukast and montelukast are not intended for acute asthma, and must be taken regularly to derive benefit, and that the response is obtained within 24 hours. [14]

Both zafirlukast and montelukast decrease circulating levels of eosinophils and could have other useful anti-inflammatory properties.[1, 13] They block the effects of cysteinyl leukotrienes in the airways. Administration of CysLTRAs allows doses of inhaled corticosteroids to be reduced. Currently available CysLTRAs are free of serious side effects and are available in oral forms. [14]

Other approaches and agents being explored for future asthma therapy are: bronchodilatation by selective potassium channel activators; new selective muscarinic M3 receptor antagonists and/or M2 receptor agonists; selective opioid

receptor agonists to inhibit release of sensory neuropeptides; selective H3-receptor agonists to inhibit acetylcholine release; inhibitors of phospholipase-A2; inhibitors of 5-lipoxygenase or 5-lipoxygenase activating protein (FLAP); selective phosphodiesterase inhibitors; anti-cytokines or anti-inflammatory cytokines (IL-10); immunomodulating agents e.g. cyclosporine; anti-chemokines or antibodies against adhesion molecules and; antagonists of tachykinins.[1, 13]

It can thus be seen that there still clearly is a need for new asthma treatments, which lead us to investigate the potential use of traditional medicinal plants that have been used for many hundreds of years by the traditional healers and generations of people as instructed by the older people in their communities.

2.3 Herbal treatments of asthma

Several local plants are routinely used for their anti-asthmatic properties. According to information collected by researchers of traditional medicinal plants and, researchers at the Montagu Museum over the last fifteen years the following plants have been used for their anti-asthmatic properties: *Mentha longifolia* (wildekruisement); *Samolus valerandii* (bronkhors); *Leysera gnaphalodes* (teringtee); *Salix mucronata* ssp. *capensis* (rivier wilger); *Artemisia afra* (wildeals); *Passerina obtusifolia* (bakkersbos); *Agathosma crenulata* (bergboegoe); *Salvia chamelaeagnea* (bloublomsalie); *Rafnia amplexicaulis* (boesmantee); *Helichrysum hamolosum* (sesemberbos); *Artemisia absinthium* (groenamara); *Helichrysum nudifolium* (kaffertee); *Portulaca oleracea* (misbredie); *Datura stramonium* (stinkblaar)**; *Oncosiphon suffruiticosum* (strinkkruid)**; *Plantago lanceolata* (lamb's tongue); *Ruta graveolens* (wynruit)[8,9]; *Helichrysum petiolare* (hottentot's bedding); and *Salvia Africana-lutea* (geelblomsalie)[15]. According to Watt- and Breyer-Brandwijk[10] the following plants are also used in asthma: *Osmitopsis asteriscoides* (belskruie); *Tarchonanthus camphoratus* (wildekanferbos); *Alepidea amatymbica* (kalmoes); *Catha edulis* (boesmanstee); *Viscum capense* (voëlent) and; *Warburgia salutaris* (peperbasboom).

The majority of the traditional medicinal plants used, are of the two families, *Asteraceae*, and *Lamiaceae*, but also to a lesser extent from other families; viz., *Salicaceae*, *Rutaceae*, *Apiaceae, Celastraceae, Viscaceae, Canellaceae,* and *Vistaceae*.[8,9,10,15]

In most cases it is the leaves of the traditional medicinal plants, listed above, that are used to make the infusions or decoctions. The tea is normally made by using 1 teaspoonful (5g) of the dried leaves in 1 cup (250ml) of boiling water and left to draw. All infusions must be made and stored in only glass or enamel containers, and then drunk cold; the normal dose being half a cup three times daily. Other methods of use of the leaves are as steam inhalations, plugs of leaves into nose and stuffing under bedding to clear colds and congestion.[8,9,10,15]

[** In the case of *Datura stramonium* (stinkblaar) and *Oncosiphon suffruiticosum* (strinkkruid), either whole leaves or leaves made into a wet poultice are bound to the chest. *Datura stramonium* is also used by steam inhalation and it is highly toxic when drunk.[9]]

Based on their pattern of use and claimed efficacy we suspect that *Mentha longifolia* (wildekruisement) and *Artemisia afra* (wildeals) have good potential to possess anti-asthmatic activity. These two plants have an apparent widespread use for bronchial conditions, a vast pattern of distribution and are easily obtainable by most people.

2.4 Practice in South Africa using traditional medicinal plants

Herbal medicine is currently enjoying a revival in popularity in the Western world, and in some parts of the world it remains the primary source of medicine.[16] Dependence on plants as the source of medicine is prevalent in developing countries where traditional medicine plays a major role in healthcare.[17] The WHO estimates that 80% of people living in developing countries almost exclusively use traditional medicine. Southern Africa contains approximately 10% of the world's

plant diversity, but relatively little research, about the chemical composition and activity of these constituents, has been done on these medicinal plants.[18]

The following examples of plants, given with indication of their uses, have been commonly employed for their medicinal properties: *Leyssera gnaphaloides* (teringteebos) is used for tuberculosis, influenza, colds, chest complaints, stomach ailments; *Sutherlandia frutescens* (kankerbossie) is used for cancer treatment, wounds, fever, blood purifier, stomach complaints; *Salix mucronata ssp, capensis* is used for backache, gout, rheumatism, kidney complaints, and lung problems; *Zantedeschia aethiopica* (Varkblaar) is used for inflamed chest by putting warmed leaves to chests and *Leonotis ocymifola* (klipdagga) is used for cancer, stroke, high blood pressure, anaemia, diabetes, and stomach complaints.[9]

These are only a few of the numerous commonly used plants and their uses. From the above examples it can however clearly be noted that each plant may be used for a wide range of medical conditions, possibly due to the mixture of active ingredients present in the plants, and/ or possibly due to an active ingredient having a broad spectrum of different activities. The same applies to the uses of *Mentha longifolia* and *Artemisia afra*, the two medicinal plants we have chosen for this study.

2.5 Study of the two plants: *Mentha longifolia* and *Artemisia afra*

2.5.1 *Mentha longifolia* Family: *Lamiaceae*

Botanical description

The plant, shown in Figure 2.2, is a perennial herb with creeping rhizomes below the ground, and erect flowering stems of up to 0.8m in height. All parts are highly aromatic with a strong typical mint smell. The leaves appear opposite each other in pairs on the stems, which are square in cross-section. Small white or pale purple clusters of flowers are borne in elongated clusters at the tips of the stems.[8]

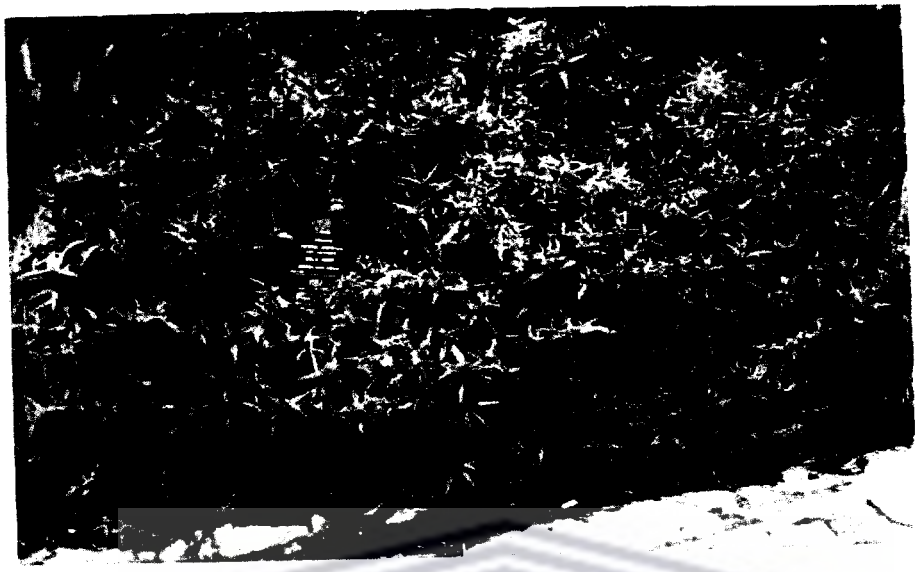


Figure 2.2 *Mentha longifolia* in the medicinal herb garden at Kirstenbosch National Botanical Institute

Distribution:

M. longifolia is widely distributed in South Africa, especially in wet places.[8]

Parts of the plants used:

The leaves are mostly used, sometimes also the stems and rhizomes.[8]

Preparation and dosage:

Infusions or decoctions of the leaves are drunk or administered as enemas. Crushed whole leaves may be inserted into nasal passages for relief of headache, or placed under bedding to reduce breathing problems.[8,9,10]

Medicinal Uses:

Wild mint is used for many different ailments. Its main use is for coughs, colds, asthma, and other respiratory conditions. It has also been used to treat headaches, fevers indigestion, flatulence, spastic colon, constipation, painful or delayed menstruation, delayed pregnancy, for urinary tract infections, for hysteria and epilepsy. It is also believed to reduce excessive sweating. Externally it has been used to treat wounds and swollen glands.[8,9,10]

2.5.2 *Artemisia afra* **Family:** *Asteraceae*

Botanical description

This highly aromatic plant, shown in Figure 2.3, is an erect multi-stemmed perennial shrub of up to two metres in height. The feathery leaves are finely divided and usually have greyish-green colour. Flowers are borne along the branch ends and they are pale yellowish and inconspicuous. In cold regions the branches die back in winter, but rapidly regenerate from the base.[8]

Figure 2.3 *Artemisia afra* in the medicinal herb garden at Kirstenbosch National Botanical Institute

Distribution

Artemisia afra is a very common species in South Africa and its natural distribution extends northwards into tropical east Africa, as far north as Ethiopia.[8]

Plant parts used:

The leaves are mainly used, but sometimes also the roots.[8]

Preparation and dosage:

An infusion or decoction is generally used, often sweetened by the addition of honey or sugar to mask the natural bitter taste. Plugs of fresh leaves are inserted

into the nose to act as decongestant, or the leaves may be boiled in water and steam inhaled.[8,9,10]

Medicinal Uses:

Artemisia afra is one of the most widely used traditional medicinal plants in South Africa. Numerous ailments are treated with it; mainly coughs, colds and influenza, but also fever, loss of appetite, colic gout, rheumatism, sweaty feet, headache, earache, malaria and intestinal worms. Fresh leaves are inserted into the nose as decongestant. The roots are used to treat colds and fever.[8,9,10]

Both *Artemisia afra* and *Mentha longifolia*, as traditional plant medicines, are advocated for several clinical uses, including asthma (see Section 2.3). Appropriate validation of these claimed uses are, however, lacking and requires the proper scientific study of the medicinal plants i.e., requires the use of correct and validated procedures for the collection, identification, preparation, chemical identification of active ingredients and the bioassay of the plant extracts.

2.6 Methods used in research on medicinal plants

Plants are considered to be medicinal if they possess pharmacological activities of possible therapeutic use. These activities are often known due to experiences gained over hundreds of years of use. For these plants to be developed into new drugs that meet the criteria of modern medicines, they have to be thoroughly investigated. The goals of research in this field are thus; firstly, to identify the active ingredients of the medicinal plants, and to determine whether the extracts are safe, effective, and of constant activity and; secondly, to isolate the active ingredients, and to determine their structure, so that they may be synthesized, structurally modified, or simply extracted efficiently.

The methods used in research into medicinal plants must be rigorous. If the research is to be effective it must be performed logically, systematically and by a multidisciplinary team. The members of the team need to include a pharmacognosist, a systemic botanist, a pharmaceutical chemist, and a pharmacologist. Each person contributes a different field of expertise to the total effectiveness and analytical function of the team. Throughout the project, each

person is to have a clearly defined protocol and responsibility in the team. The pharmacologist is often the team leader, since the pharmacological activity of the substance under investigation will determine the responsibilities of the other specialists.

The series of procedures fall into eight stages, which must be performed sequentially by the specialists listed. Initially, usage in traditional medicine has to be determined. This is done by the pharmacognosist interacting with traditional healers, extracting folk custom. Secondly, the harvesting of the correct plant family has to be ensured by the pharmacognosist and the systemic botanist. Thirdly, the plant substances are to be investigated for activity; the pharmacognosist, chemist and pharmacologist performing this stage. The fourth stage is the need for the positive identification of the plant by the systemic botanist. The fifth step is the investigation of the active ingredient(s) by the chemist, and pharmacologist. The sixth stage is the classical pharmacological investigation, performed by the pharmacologist. The seventh stage is the determination of chemical structure by the chemist. The final stage, the synthesis and structural modification performed by the chemist and pharmacologist. After completion of each stage, each member of the team must consult with the other members and then decide whether to continue the project.[19]

Plants remain an important reservoir of potential for the discovery of new substances with valuable pharmacological activities. These substances may be used in their natural state, or can serve as models for pharmaceutical chemists for the synthesis of new compounds of even greater therapeutic value. To find these substances, it is necessary to search and investigate rigorously and methodically, working in multidisciplinary teams.[19]

For research to be conducted, use is often made of various systems which may include; animals; tissue culture techniques, and cell lines.

2.7 Evaluation of asthma medication using animals

Animals are frequently used to evaluate the effectiveness of asthma medication. Both rats and guinea pigs are good substitutes for the study of the effect that agonists may have on bronchial tissue of humans. The rat produces an antibody

IgE, similar to that produced by human asthmatics. Rat lung is pharmacologically similar to the human lung in its response, but guinea-pig lung resembles it more closely histologically.[16]

The guinea-pig is commonly employed as a model for studying the effect of drugs on respiratory smooth muscle and airway dynamics, and shows broad pharmacological similarity to airway function in man. In both species two main neuronal mechanisms, i.e. cholinergic nerves using acetylcholine, and excitatory nonadrenergic, noncholinergic (e-NANC) nerves using tachykinins as neurotransmitters, are involved in contraction of smooth muscle. In guinea pigs both adrenergic nerves and inhibitory nonadrenergic, non-cholinergic (i-NANC) nerves are responsible for relaxation of airway smooth muscle. In human airway smooth muscle, it appears the only neuronal bronchodilator pathway consists of the i-NANC system, utilizing vasoactive intestinal peptide (VIP) and nitric oxide (NO) as neurotransmitters. The interaction between these separate neuronal pathways and the modulation of neurotransmitter release regulates an important mechanism for controlling airway caliber and function.[20]

To evaluate the pharmacological effect of plants with anti-asthmatic properties, two types of methods can be used, i.e. *in vivo* and *in vitro* methods.

2.7.1 Techniques to measure the effect of anti-asthma medication: in vivo methods

In live animals and humans the determination of lung function parameters, e.g. FEV_1 (Forced expiratory volume in one second) and PEF (Peak expiratory flow) etc., may be used to evaluate the effect of anti-asthma medication.[11] Many techniques may be used. For instance the Dixon Brodie Technique in which bronchoconstriction can be measured in an anaesthetized animal by connecting the trachea to a pressure transducer, and ventilating the animal artificially.[16] Drugs can be administered orally or injected and their effect on airflow monitored. Sensitized animals (e.g. sensitized with ovalbumin) may be used and bronchoconstriction induced by antigen stimulation. Here, bronchoconstriction starts approximately

one minute after antigen challenge, depending on the dose, and rapidly reaches a maximum.[16]

2.7.2 Techniques to measure the effect of anti-asthma medication: in vitro methods – tissues and cells

Examples of *in vitro* methods employed to test anti-asthma effects include the use of tracheal spirals and lung parenchyma strips. The tracheal spiral is often used to represent the large airways; it is a useful model though technically difficult to prepare. Lung parenchyma represents peripheral airways. These are normally taken from the same animal.[16] The aforementioned tissues are typically used as follows; histamine, methacholine, LTD_4, PAF and arachidonic acid are used to contract the tissue and then the effect of plant extracts are tested against these contractions.

Arachidonic acid-induced contractions are used to evaluate both cyclo-oygenase and 5-lipoxygenase modulators. In the presence of indomethacin, a cyclo-oxygenase inhibitor, the lipoxygenase pathway predominates and drugs which affect leukotriene-induced contractions can be assessed.[16]

Tissues from sensitized animals (e.g. sensitized with ovalbumin) may respond to other mediators, in addition to arachidonic acid metabolites. When such sensitized tissues are pre-treated with indomethacin, the cyclo-oxygenase pathway is suppressed, and with it the production of PGE_2. PGE_2 has bronchodilator activity and also inhibits further release of other mediators, including arachidonic acid. By removing the effect of PGE_2, there is an increased release of arachidonic acid, and amplified contractile responses.[16] In practice, when evaluating the lipoxygenase modulators, indomethacin is thus added to the perfusing fluid to enhance responses to histamine, methacholine, LTD_4, and PAF.

In the following summary, Table 2.1, a representation is given of the possible experimental design combinations of tissue, agonist and mediators that may be studied using the *in vitro* methods.

Table 2.1 The possible experimental design combinations of tissues, agonists and mediators as used *in vitro* methods.[16]

Tissue	**Agonist (constrictor)**	**Mediators**
Non-sensitized	Arachidonic acid	Mainly cyclo-oxygenase products
Non-sensitized + indomethacin	Arachidonic acid	5-Lipoxygenase products
Sensitized	Antigen (ovalbumin)	Arachidonic acid metabolites: Cyclo-oxygenase + 5-lipoxygenase products
Sensitized + indomethacin	Antigen	Enhanced response to LTD's, PAF, histamine

Other methods of research involve the use of tissues and cells. Because of the limited amount of smooth muscle tissue available from patients with respiratory disease, not much data has been published using human airway smooth muscle. The majority of published articles involve studies performed on animal smooth muscle tissue (e.g. guinea-pig, or bovine trachea) or more recently on airway myocytes in primary culture. Both of these systems, viz., the animal smooth muscle tissue, and the myocytes in primary culture, have potential drawbacks. Although animal airway smooth muscles tissue has many similarities to human tissue, there are slight differences, e.g. in the receptor subtype expression and also in the balance of the inflammatory mediators present in the preparation. Using cultured airway smooth muscle, where human tissue is utilized, a different series of problems arises, comprising the differentiation that occurs during cell culture. Due to these limiting factors to date the majority of published data, using cultured airway smooth muscle, has been done on cells, called airway myofibroblasts.[21]

In general, the isolated tracheal preparations of the guinea pig are employed as an accepted sensitive model for the study of the human lung.

2.8 Tracheal preparations

Although there are marked differences between species, with regard to innervation, receptors and neuromodulatory mechanisms, dissected guinea pig tracheal tissue immersed or superfused by isolated tissue bath techniques provides important ways to study the effects of drugs on the neuronal pathways, or on the smooth muscle directly. The guinea pig trachea is generally accepted as a relevant and sensitive model of human large and central airways, which enable the screening of new compounds with potential therapeutic effect.[20]

The following is a description of the most frequently employed types of methods of tracheal preparations. The possible advantages and limitations are discussed, but the method employed depends on the information required in a particular study. The types of methods employed are divided into immersion and superfusion techniques with a discussion of the various tracheal preparations employed by each.

2.8.1 Immersion techniques

The isolated tracheal tissue is immersed in an isolated tissue bath warmed to 37°C from a constant-temperature circulator. The tissue is suspended in Krebs-Henseleit (KH) solution and fixed to the bottom of the bath chamber on one side, and on the other side to an isometric transducer for measurement of change in force, as the tissues contract. A mixture of 95% O_2/5% CO_2 is continuously bubbled from the bottom of the bath chamber for oxygenation, mixing and pH maintenance.[20]

2.8.1.1 Tracheal chain

The tracheal chain preparation was introduced by Castillo de Beer[22] and initially used to study the effects of relaxant drugs, especially those acting on the β2-adrenoceptors. The trachea is dissected from a guinea pig, and then divided into rings. Four to six alternately allocated tracheal rings are tied together by means of cotton thread, forming up to six chains from one animal. These chains are then immersed in the isolated tissue bath as previously described. Cumulative concentration-response curves of isomeric tension changes, either to contractile agents (i.e. histamine, acetylcholine, carbochol, methacholine, LTD_4, serotonin,

KCl) or relaxant drugs (i.e. β2-adrenoceptor agonists, methylxanthines, papaverine, sodium nitoprusside), are obtained by increasing drug concentrations in 0.5 log unit increments. Contractile or relaxant responses are expressed as percentage values in comparison with the maximum response caused by a reference compound. i.e. KCl or by the agent alone, or in the case of relaxation, with that caused by isoprenaline or papaverine. Disadvantages of the tracheal chain preparation are the tedious process of tying the tracheal chains together and the small maximum responses recorded, compared to that observed with other tracheal preparations.[20]

Advantages are that the system is sensitive to changes in responses, and can be used to study relaxant effects of β2-adrenoceptor agonists with tone raised by carbachol or by KCl. The potencies of β2-agonists are significantly reduced in tissues pre-contracted with either carbachol or KCl. However, due to its lower receptor reserves an advantage of KCl-depolarised trachea is its ability to identify β2-agonists with low intrinsic activities.[23] Generally, for the study of β2-adrenoceptor interactions, ascorbic acid may be added to KH solution to prevent oxidation of added catecholamine, phentolamine to block α adrenergic activity, and cortisone to prevent extraneuronal uptake of catecholamines.[23, 24]

The guinea pig trachea often requires exceptionally long equilibrium times to permit optimal agonist-induced contractions. Inhibition of cyclo-oxygenase by the addition of indomethacin, which abolishes the spontaneous active tension generated, due to the decreased production of prostaglandin E_2, serves to reduce the time taken for the tissue to be stabilized and obtain reproducible and optimal contractile responses to constrictors.[20]

2.8.1.2 Spirally cut trachea

This is a modified and improved method, which eliminates the time consuming process of tying the tracheal rings together and which has the additional advantage of developing greater force. The trachea is cut diagonally to produce several segments each containing up to five rings. By means of stainless steel wire introduced into the tracheal lumen, spirally formed strips can be prepared within a short time by cutting the connective tissue to separate the rings in a manner so that

they are still held together by a small portion of the connective ligament between each single ring. After mounting the spiral strips in tissue baths containing KH solution under a resting tension (e.g. of 1 - 2g) and development of spontaneous tone, isometric contractions to cumulative added spasmogens, can reproducibly be measured for 6 hours. Advantages therefore are the greater speed of preparation and the greater development of force. Anderson and Lee[25] demonstrated tolerance to bronchodilator drugs *in vitro* by using the spirally cut trachea from chronically pretreated trachea. For testing contractile responses to LTC_4, l-serine borate, (the glutamyl transpeptidase inhibitor) is added to the nutrient solution to prevent conversion to LTD4.[26]

A further advantage is the way in which sensitized spirally cut tracheas are used to study smooth muscle contractile responses resulting from generation and release of a mixture of inflammatory mediators, prostaglandins and nitrous oxide. These sensitized tissues are obtained by active sensitization of the guinea pigs, on day 1 and 4, by injection of a particular antigen; often intraperitoneal injection of ovalbumin-saline. The animals are used only after day 25. Contractions of sensitized trachea to ovalbumin consist of two components, a rapid initial phase (within 2 minutes) and a later tonic phase (10-15 min), which have been correlated to the release of histamine, and then the process of eicosanoid synthesis and release. To study those effects derived from mediators generated by the 5-lipoxygenase pathway, indomethacin is added to exclude the cyclooxygenase pathway. By addition, of either a H1 antagonist i.e. blocking of phase 1; or leukotriene receptor antagonist, or lipoxygenase inhibitor, or a phospholipase-A_2 inhibitor, inhibiting the second phase of contraction, confirmation can be obtained, as to which mediators are responsible for each phase of the contraction.[27, 28] The selective PDE_4 inhibitor, markedly reduced the second phase of contraction, indicating these inhibitors are more effective in inhibiting the formation and release of newly formed lipid mediators (LTC_4 and PGD_2) than histamine release in airways.[29]

2.8.1.3 Zigzag tracheal strip

A similar preparation to the spirally cut trachea is the quickly prepared zigzag strip described by Emmerson and Mackay[30]. The trachea is cut open longitudinally, by

cutting through the cartilage rings, opposite the trachealis muscle, and then pinned onto a cork board to cut the traverse slits into the connective tissue at equally spaced intervals, first on one side of the preparation, then on the other. The tissue is spiraled out, cutting it into the required number lengths (2-4) of equal size, needed for the experiment. Threads are tied to each end of the tissue, which are set up in the isolated tissue bath(s) as previously described under a resting tension of 0.5 – 1.0g. Vitality of the tissue is tested by adding 50nM KCl for 10min, during which time approximately 1.8g of force should be maintained.

Further advantages beside the speed of preparation, is that the zigzag tracheal strip has been employed in various applications resulting in the determination that predominantly cAMP and less cGMP, play an important role in the regulation of guinea pig tracheal smooth muscle tone. β2-adrenoceptor agonists cause relaxation by stimulating the formation of cAMP, and agents that inhibit cyclic nucleotide phosphodiesterase (PDE) have been anticipated to exert a major part of their relaxant effect by increasing intracellular cAMP levels.

As a further example, the zigzag preparation has been used to demonstrate the participation of Ca^{2+}-activated K^+ channels (BK_{Ca}) in trachea relaxation by β2-adrenoceptor agonists, the mode of action which has until recently been thought to be solely due to cAMP-dependent increase in the sequestation of intracellular Ca^{2+} and/or extrusion from the cell.[31] Tracheal smooth muscle contains a variety of distinct K^+ channels, which include voltage-dependent K^+ channels, a high density of Ca^{2+}-activated K^+ channels, and possibly ATP-dependent K^+ channels which play an important role in regulating membrane potential and thus contractile state of airway smooth muscle.[31,32] Drugs such as cromakali, acting as openers of ATP-dependent K^+ channels, or isoprenaline and salbutamol, acting on the other more prevalent high-conductance Ca^{2+}-activated K^+ channel, relax airway smooth muscle via membrane hyper-polarization.[31] For studying these two latter K^+ channels, carbachol-pre-contracted zigzag strips are set up for isometric tension recording in the presence of indomethacin and exposed to increasing concentrations of salbutamol or theophylline to obtain cumulative concentration-

response curves which are repeated in the presence of the BK_{Ca} antagonists, charybdotoxin and iberiotoxin. These antagonists also produce a further contraction of the pre-contracted tracheal tissue by causing smooth muscle membrane depolarization via opening voltage-dependent Ca^{2+} channels and enhancement of Ca^{2+} influx [31]. It can thus be seen that the zigzag strip is a sensitive system with a wide application resulting in the determination of important mechanisms occurring in smooth muscle.[20]

The electrically stimulated version of the zigzag strip has often been used to investigate the nonadrenergic, noncholinergic (NANC) innervation and neurotransmission of the guinea-pig trachea. In other cases, the electric field stimulated trachea has frequently been used to study indirectly the modulation of neurotransmitter release from airway nerves, by measuring post-junctional response.[20]

2.8.1.4 Tracheal tube preparations

Unstimulated perfused tracheal tube: Dissected tracheal preparation suspended in organ bath, but lack lumen-epithelium interface.

Transmural stimulation: Another version of the intact tracheal tube that is not perfused, but suspended, and subjected to electric field stimulation.

Sympathetic and vagus nerve stimulation: The trachea is removed with sympathetic and vagus nerves attached and cannulated at both ends, and mounted horizontally in an organ bath.[20]

2.8.2 Superfusion techniques

Another method, used to a lesser extent, but sometimes more appropriate, e.g. to perform a bioassay of perfusates and evaluation of potency of labile substances or those of extremely low quantity. An apparent beneficial effect is the ability to rapidly remove potentially toxic metabolites, preventing them from accumulating in the tissue, and thereby reducing the washing time.

Methods employed are, electrically stimulated zigzag tracheal strip in a perfusion chamber. Other studies performed using superfusion techniques, is done by using epithelium-denuded trachea. [20]

As can be seen above, there are numerous techniques available, which can be employed in smooth muscle tracheal studies.

CHAPTER 3: WORK PLAN

3.1 Objectives

The overall objective of this project was to investigate the claims that *Mentha longifolia* (wildekruisement) and *Artemisia afra* (wildeals) have anti-asthmatic properties. To realize this objective it was decided to determine the effects that the plants may have on contractions induced by agonists (e.g. methacholine, histamine, and leukotriene D_4) known to be mediators of bronchoconstriction and also to partially investigate the mechanism which may be involved if the plant extracts had any muscle relaxant activity.

3.2 Hypothesis

We hypothesized that extracts of *Mentha longifolia* and *Artemisia afra* would have bronchial smooth muscle relaxant properties and were able to reverse methacholine and/or, histamine and/or leukotriene D_4-induced contractions by competitive antagonistic mechanisms.

3.3 Study approach

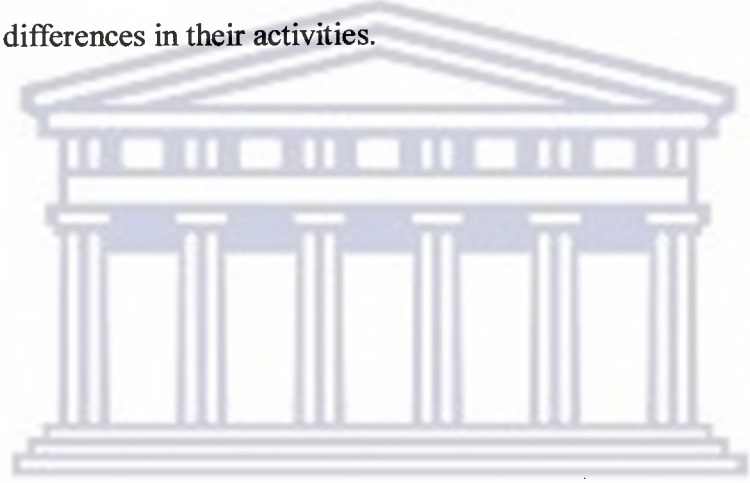
Firstly, aqueous extracts of the plants were used in the bioassays to adhere as closely as possible to traditional methods used by traditional healers and generations of local people.

Secondly, it was decided to use the guinea pig tracheal strip preparation for this study. The reason it was chosen is the fact that the guinea pig tracheal tissue resembles human lungs so closely histologically and it is generally accepted as a relevant and sensitive model of human large and central airways. The reasons we chose the zigzag tracheal strip method was the fact that it was a relatively fast and simple technique to prepare. The tissue once stabilized under the resting tension can be used for up to 10 hours. The system is sensitive with a wide range of applications allowing a vast amount of information to be collected. Results are relatively easy to interpret, and they are easily reproducible. It is a validated method, often used scientifically for this type of research.

Thirdly, the agonists, methacholine, histamine and leukotriene D_4, known mediators of bronchoconstriction in allergic inflammatory conditions of the lungs, were chosen to induce tracheal contraction.

Fourthly, to determine the possible mechanism for any relaxant effects of the plant extracts cumulative concentration curves of the three agonists were obtained in the absence and presence of various concentrations of the plant extracts. From this data log dose response curves could be plotted and analyzed to determine the possible mechanism (e.g. partial agonism, competitive or partial antagonism, etc) that may be involved.

Finally, the results obtained with both plants were to be compared to see if there were any differences in their activities.



CHAPTER 4: METHODOLOGY

4.1 Introduction

In this section a description is given of the equipment, materials and methods used in the collection and preparation of the aqueous extracts of *Mentha longifolia* and *Artemisia afra* and the screening of the plants for bronchodilator properties.

4.2 Collection and preparation of plant materials

4.2.1 Equipment

The following equipment was used during the preparation of the crude aqueous extracts:

A scientific balance (Ohaus model GA110), oven (Memmert model 854), Phillips electric grinder (model CG100 PK032/AD), heating plate (Labcon model HPE45), Endecotts test sieve, specification B.S. 200mm, aperture 850 micron and a freeze-dryer (Virtis freezemobile model 125L).

4.2.2 Collection of plants and preparation of the extracts

The plants were obtained from Kirstenbosch National Botanical Institute while they were flowering during the summer of 2001. A horticulturist working at the Institute identified the specimens and voucher specimens, voucher numbers 6634 and 6635, were deposited at the Herbarium at University of the Western Cape.

The freshly cut plants were collected in the morning weighed on the same day (i.e. green weight recorded) and were then kept at 2°C in a cold room for 12 days. Thereafter all the leaves were removed and dried in an oven at 30°C for 4 days and the dry weight recorded. The dried leaf material was stored in a dark cupboard at room temperature in brown paper bags until it was used for extraction. Some of the dried leaves were ground in an electric grinder to a powder, sieved through 850 mesh sieve and stored in sterile brown glass bottle, sealed with poly film, until needed for extraction.

After the first collection more plant material was needed, but this was collected within three days, included with the first collection and worked together as a single batch. The green and dry weights were used to calculate the percentage yield.

The dried leaves were extracted using distilled water. For each gram of dried material 2 ml of solvent was used. The dried material was weighed and the correct proportion of distilled water added and the mixture was boiled for 130min. The aqueous extracts were filtered through Whatman No 1 filter paper into a 1-litre volumetric flask and left to cool on the desk. The extraction process was repeated three times. The first filtrate was poured into a round-bottomed 1-litre boiling flask and frozen as a thin film on the inside of the flask. The process was repeated with the other two filtrates. The three frozen extracts, were kept frozen on dry ice and taken to the freeze-dryer, where the samples were put on to dry for 4 days. The dried flaky extracts were removed from the glass flasks, ground in a pestle and mortar to a homogenous mass, weighed, put into brown glass jars and sealed with poly film and then stored at room temperature in dark cupboard until needed for the bioassay process.

4.3 Bronchodilator studies

4.3.1 Materials, chemicals and experimental animals

The following drugs, chemicals and experimental animals were used in the isolated tracheal strip experiments.

The agonists used were acetylcholine chloride, acetyl-beta-methylcholine chloride (methacholine), histamine diphosphate salt, isoproterenol hydrochloride (isoprenaline), leukotriene D_4 free acid and propranolol hydrochloride, all obtained from Sigma-Aldrich, S.A. (Pty) Ltd. Potassium chloride (AR grade) was obtained from Merck Laboratory Supplies (Pty) Ltd.

The antagonists used were atropine sulphate and mepyramine maleate both supplied by Sigma-Aldrich, S.A. (Pty) LTD. Montelukast sodium was extracted with water from tablets (Singulair 10mg), containing 10mg free acid.

All the chemicals used for making the Krebs-Henseleit solution, listed below, were of AR grade, and obtained from Merck Laboratory Supplies (Pty) Ltd. The composition of the Krebs-Henseleit (Krebs bicarbonate solution) (KH solution) in mM was: NaCl 120mM, KCl 4.77mM, KH_2PO_4 1.2mM, $MgSO_4.7H_2O$ 1.2mM, $NaHCO_3$ 25mM, $CaCl_2.2H_2O$ 2.5mM and glucose 11mM. Indomethacin $1X10^{-6}$M was added to the KH buffer to give Krebs-Henseleit-indomethacin (KHI) solution.

The following stock solutions of methacholine (1M), histamine ($1x10^{-1}$M), atropine (1mM), potassium chloride (60mM) and propranolol ($1x10^{-3}$M), were made up in KHI. All these stock solutions were refrigerated at 2°C until shortly before use and were discarded within 10 days of preparation. KHI solution (minus the glucose) was freshly prepared, within 24 hours of use, and kept cold until just before use when the glucose was added. Serial dilutions of the stock solutions were made, and the log dose response curves of each agonist were generated. Where single doses of agonist or antagonist solutions were used the following working solutions were prepared: methacholine ($1X10^{-2}$M), histamine ($1X10^{-3}$M), atropine ($4.7X10^{-9}$M), potassium chloride (60mM) and propranolol ($1X10^{-3}$M). The concentrations used in the text in the results section were those in the bath obtained once 0.1ml single doses of the solutions were injected into the bath. On the other hand the concentrations used for the plant solutions (0.1% to 30%) were the uncorrected concentrations i.e. although 0.lml of each plant solution was injected into the 15ml bath no correction was made for the dilution factor. The freeze-dried crude extracts of *Mentha longifolia* and *Artemisia afra* that were used, as well as the other agonists, e.g. LTD_4 ($1.04X10^{-6}$M), montelukast ($1.6X10^{-5}$M, isoprenaline ($1X10^{-2}$M) and mepyramine ($6.2X10^{-8}$M), were all dissolved in the freshly prepared KHI on the day of the experiment. The LTD_4 and isoprenaline were in fact prepared or kept frozen until just before injection and no antioxidant was added to the isoprenaline solution.

In the experiments male and female guinea pigs (500 – 550g) were used. They were obtained from S. A. Vaccines Producers, Sandringham, Johannesburg, in South Africa and were bred under conventional conditions for research purposes. Before use they were kept in a well-ventilated animal room with free access to

food and water. The lighting in the animal room was regulated to reproduce day and night conditions (12-hr cycle). Ethics approval for use of the animals in the experiments was obtained from the UWC Senate Ethics committee.

4.3.2 The organ bath system

The various components of the isolated tissue bath system that were used are shown in figures 4.1, 4.2 and 4.3. Basically the system consisted of a 15-ml water-jacketed glass organ bath (A) that was supplied with perfusion medium from a 2-L reservoir (B). On its way from the reservoir the perfusion medium passed through a condenser (C) and the perfusion flow were controlled via 2 taps (D1 & D2). Fluid was drained from the organ bath via another tap (D3) and outlet tubing. The perfusion medium in the bath was kept at 37°C by circulating water through the jackets of the condenser and the organ bath with the aid of a thermostated-water bath (E). The perfusion medium in the tissue bath was aerated with carbogen mixture of 5% CO_2 and 95% O_2 from a cylinder (F) via outlets (G) in the bottom of stainless steel tissue holder (bubbler). To measure the contractile responses, the tissue was attached with the aid of cotton thread (H) to the bottom of the tissue holder and the hook of a force transducer (I). The latter was, in turn, attached to an

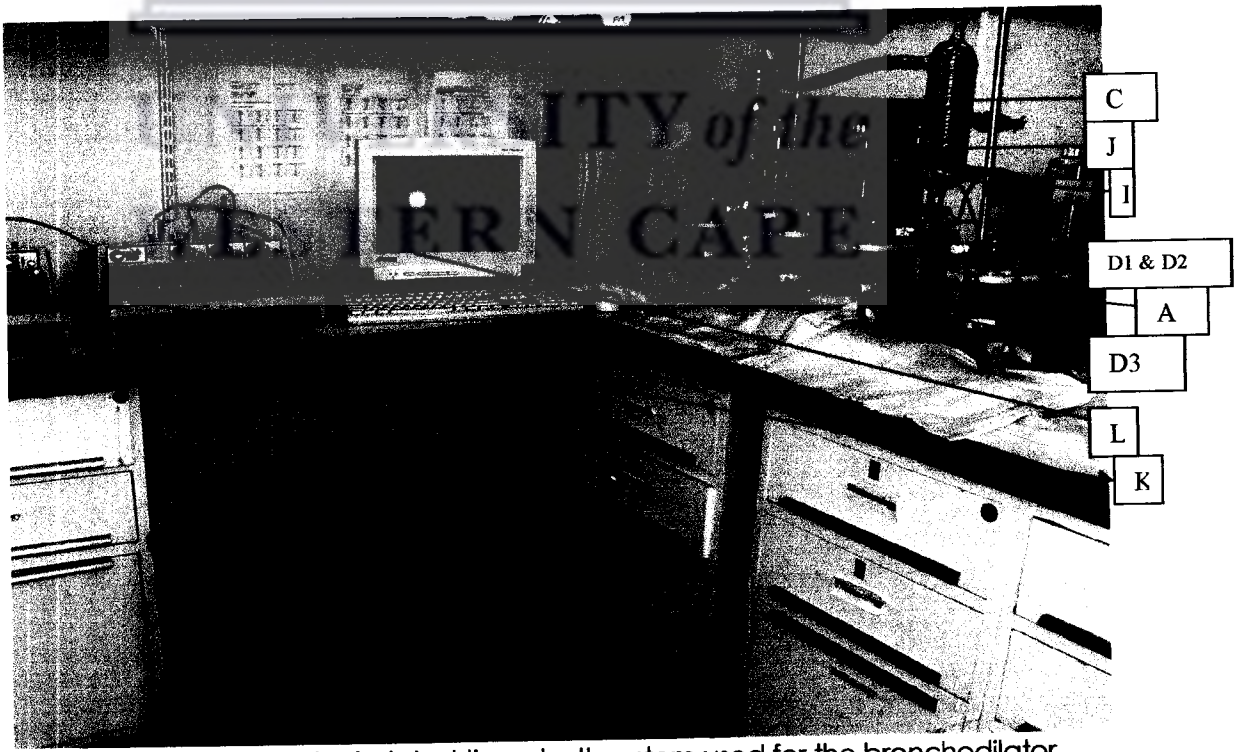


Figure 4.1 The isolated tissue bath system used for the bronchodilator experiments in the laboratory.

amplifier (J) that was further connected to the printer (K 4 channels Rikadenki) and a computer (L) where the responses were recorded. Four of the above organ bath systems were connected in series that allowed the simultaneous execution of 4 replicates of the experiment. The organ-baths, transducers and amplifiers were all manufactured by the Electronics and Instrument Workshop at the University of the Western Cape.

4.3.2.1 Calibration of the equipment

To quantify the contractile and relaxant responses of the tracheal muscle the force transducers, amplifiers and recorders were calibrated as follows. First, with no force on the hook of the transducer, the amplifier and recorder were set to zero. Then low weights (0.5 to 4g) were added to the hook and the responses on the transducer amplifier and the recorder measured. A plot of mass (force) versus amplifier (digital reading) or recorder reading (in millimeters) was found to be linear (r^2= 0.9909) and used to interconvert responses from force to recorder response, as required. A calibration was done before each series of experiments.

4.3.3 Animal procedures, tracheal strip preparations

The animal was weighed and then anaesthetized by injecting sodium pentobarbital (95mg/500g) intra-peritoneally. The anaesthetized animal was cut open from under the chin to the thorax; the trachea was exposed and surgically removed. After excess fatty connective tissue was carefully cut off the dissected tracheal tissue was rinsed in chilled KHI solution and transferred to a dissecting dish also containing chilled KHI solution. The tissue was examined to confirm which side of the trachea had the cartilage rings, and which side had the muscle. Throughout the tissue was handled with care to minimize any damage to this muscle. The trachea was cut open on the cartilage ring-side with a surgical scissors, and flattened and pinned onto cork surface inside the perti-dish using disposable syringe needles.

The opened trachea was then cut into a zigzag strip using a surgical blade. This was achieved by cutting the tissue parallel to the cartilage rings, trying to keep the cutting widths equal, i.e. having one cartilage ring per segment. Each cut was made through three-quarters of the tissue width before rotating the tissue 180° and

then cutting through three- quarters from this opposite side. This procedure was repeated for the entire length of the tissue and then the tissue was gently spiraled out to its full length. Finally the zigzag tissue strip was cut into four pieces of equal length. On each length of tissue two pieces of cotton thread were fixed, one on each end, through cartilage material taking care not to damage the muscle in the process.

The tissue was then suspended in the isolated organ bath containing aerated KHI at 37°C. One end of the tissue was tied to the oxygen bubbler and the opposite end attached to the force transducer. The screw on the force transducer was then turned to apply 2 gram of resting tension on the tissue. The tissue was then allowed to equilibrate and was washed at 10 to 15 min intervals with KHI over the next 1 to 2 hours before it was exposed to the drugs or plant extracts.

Figure 4.2 The isolated tissue bath system excluding recorders.

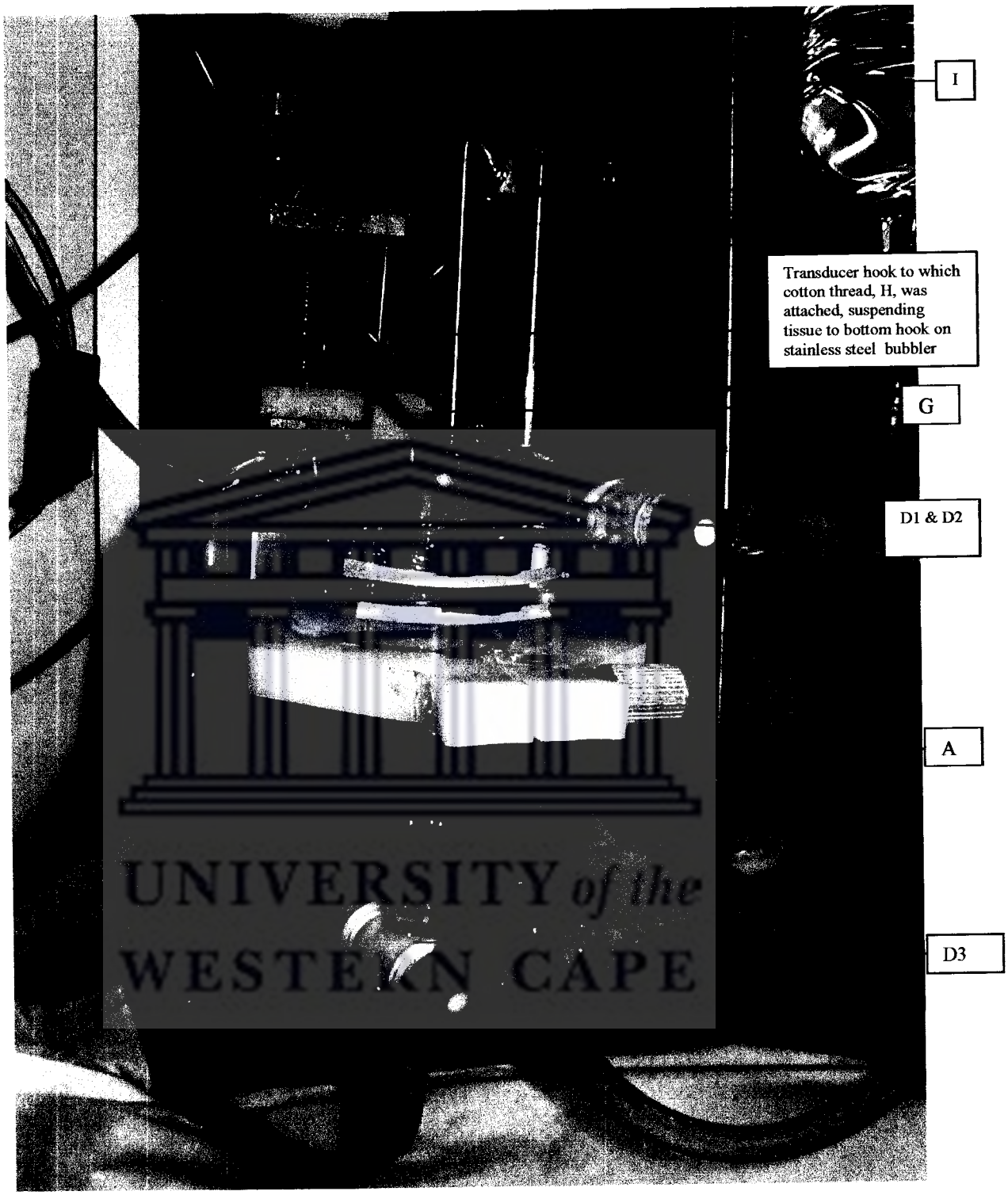


Figure 4.3 A close-up of an isolated tissue bath showing the hook at end of O_2 bubbler and transducer hook to which zigzag tracheal tissue is fixed.

4.3.4 Experimental protocol of tracheal strip bioassay

4.3.4.1 General protocol

Before each experiment the system was equilibrated for at least one hour. This was done by keeping the tissues at a resting tension of 2 gram and rinsing the tissues with fresh KHI solution at 15-minute intervals. During this period the tension of the tissue slowly decreased until it stabilized at about 1.5g tension. The tissue was then used without further adjustment of the tension. Further, to standardize the tissue responses, the tissues were depolarized by injecting two doses of 60mM KCl to determine if 2 reproducible responses were obtained. The KHI, of course, contained indomethacin ($1.5X10^{-4}$M to give $1.0X10^{-6}$M in bath) to eliminate any contractions due to mediators produced by the cyclo-oxygenase pathway. The system was now ready to add the drug or plant solutions and the latter were administered in 0.1 ml volumes using a 1 ml syringe and according to the protocols described below.

4.3.4.2 Protocol to establish effect of plant on agonist-induced contraction

To establish the concentration of agonist to use in this study, log dose response curves were obtained by injecting increasing concentrations of each agonist into the bath and measuring the response. From such curves the concentration of agonist giving responses of > 50% < 100% were selected. To assess the effect of the plant extracts on agonist-induced contractions, 0.1ml volumes of a fixed concentration of agonist, i.e. methacholine ($1X10^{-2}$M to give $6.67X10^{-5}$M in bath), histamine ($1X10^{-3}$M to give $6.67X10^{-6}$M in bath) or LTD_4 ($1.04X10^{-6}$M to give $6.93X10^{-9}$M in bath) were injected into the bath and left to react for 20 minutes. Once a stable contraction was observed, 0.1 ml volumes of increasing concentrations of the aqueous extracts of *ML* (1%, 2%, 5%, 10%, 20%, 30%) or *AA* (1%, 2%, 5%,10%, 20%, 30%) were added without washing the tissue. The responses were continuously recorded on the printer and the magnitude of the response to each addition was measured by determining the direction and degree of deflection of the printer pen once the response had stabilized after each injection. At the end of the experiment isoprenaline ($6.67X10^{-5}$M in bath) was added in a single (0.1ml) dose to obtain the maximal relaxation response for the

tissue and the plant extract-induced relaxant responses of the tissue were expressed as a percentage of this maximum relaxant response. In some cases fixed doses of the control antagonists i.e. atropine ($7X10^{-7}M$ to give $4.67X10^{-9}M$ in bath), mepyramine ($6.2X10^{-8}M$ to give $4.13X^{-10}M$ in bath) and montelukast ($1.6X10^{-5}M$ to give $1.1X10^{-7}M$ in bath) were added to the bath after contraction was induced with the agonist methacholine ($1X10^{-2}M$ to give $6.67X10^{-5}$ M in bath); histamine ($1X10^{-3}M$ to give $6.67X10^{-6}M$ in bath) and LTD_4 ($1.04X10^{-6}M$ to give $6.93X10^{-9}M$ in bath). These responses were also expressed as percentage of the maximum isoprenaline–induced ($6.67X10^{-5}M$ in bath) relaxation. The above protocol for injecting agonists and corresponding antagonists at the specific concentrations mentioned were used to quantitate the smooth muscle relaxant effect of the plant extracts.

4.3.4.3 Protocol for cumulative concentration response curves

The experiments listed under 4.3.4.2 indicated that both plants had smooth muscle relaxant effects and to determine whether a competitive mechanism was involved the following protocols were implemented. In these experiments log dose response curves (LDRC) were established by injecting cumulative concentrations (0.1ml) volumes of increasing concentrations of methacholine, histamine or LTD_4 into the bath. The tissue was not washed in between injections and the injections were made at 15 – 20 min intervals to allow sufficient time for maximum response to be achieved. To test the effect of the plant extracts on the agonist cumulative log dose response curve, 0.1ml solutions of crude aqueous extracts of *ML* (2%, 10%, or 20%), or *AA* (2% or 20%), were injected into the bath and left for 20 minutes before the cumulative concentrations of the agonist were injected. The responses obtained at each injection were expressed as a percentage of the maximum contraction obtained per tissue and used to generate log dose response curves.

4.3.4.4 Protocol to determine β-adrenergic activity

The following protocol was used to determine whether the smooth muscle relaxant effects of the plant extracts possibly involved β-adrenergic agonistic activity.

After the equilibration period, the response of the tissue was first assessed by injecting 0.1ml methacholine ($1X10^{-2}$M to give $6.67X10^{-5}$M in bath) into the tissue bath. At this concentration methacholine produced a large response that was easily measurable on the graph paper. The tissue was then washed and allowed to relax to its original position. Methacholine (0.1ml) was then injected again to check the reproducibility of the contractile response before increasing concentrations, (1%, 2%, 5%, 10%, 20%, 30%) of both plants *ML* and *AA* in volumes of 0.1ml, were injected in a cumulative manner (i.e. without washing in between). The resulting plant extract-induced relaxant responses were measured, and expressed as a percentage of the total relaxation produced by isoprenaline ($6.67X10^{-5}$M in bath) injected after the last dose of plant was injected in a sequence. The experiments were repeated, but now propranolol ($6.67X10^{-6}$M in bath) was first injected into the tissue bath, left for 20 minutes, before being followed by methacholine ($6.67X10^{-5}$M in bath) and the corresponding concentrations of plant extracts injected previously. The extent of relaxation was measured at each cumulative concentration of plant extract and expressed as a percentage of the total relaxation produced by isoprenaline injected at the end of the sequence of injections. Any decrease in response between the two runs was to be attributed to the β-adrenergic blocking effect of propranolol.

4.4 Data and statistical analysis

All responses of the tissue to the drugs or plant extracts were first measured and were expressed as a percentage of maximal responses, either as maximal contraction induced by the full agonists or maximal relaxation induced by isoprenaline. All experiments were performed in at least 4 replicates. The Excel™ software package was used to transform the raw data into percentages and to calculate the average ± SEM for each data point. Data was transferred from the Excel™ software package into the Graphpad Prism™ program.[33] The latter graphics program was used to plot log dose response curves and fit the data, using the non-linear sigmoidal dose-response curve facility. The program was also used derive the EC_{50} and Hill slope values and to do statistical analysis. The unpaired t-test (with unequal variances) was used to compare the results obtained in the

presence and the absence of the plant extracts or antagonists, with $p \leq 0.05$ set as the level of significance.

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Collection and preparation of plant materials

A sufficient amount of plant material was collected and identified by a horticulturist at the Kirstenbosch National Botanical Institute for this project and voucher specimens stored at the Herbarium at University of the Western Cape. The plants were then dried at 30°C for 4 days and the aqueous extractions were performed. These extracts were then freeze-dried and weighed to determine the yield. The extraction yields were reasonable and comparable to yields obtained in other similar studies.[34, 35]

The green weight of *Mentha longifolia* was 651.7g and the dried weight recorded was 198.10g which resulted in a 30.40 % yield. The leaves of *Mentha longifolia* when dried were a deep green colour, while the smells of the dried leaves were still a characteristic aromatic, pungent minty fragrance. The aqueous crude extract of *Mentha longifolia* had a caramel brown crystalline appearance when freeze-dried. The yield of freeze-dried powdered *Mentha longifolia* was 3.6g (10.61% yield).

The green weight of *Artemisia afra* was 853.8g and the dried weight recorded was 249.9g which resulted in a 29.27% yield. The leaves when dried were feathery greyish-green in colour and they retained a distinct aromatic smell. The freeze-dried aqueous extract of *Artemisia afra* had a greyish light brown crystalline feathery appearance and the yield of freeze-dried powdered extract of *Artemisia afra* was 6.5g (14.22% yield).

5.2 Bronchodilator studies

5.2.1 Effect of *Mentha longifolia* on agonist-induced contraction of the tracheal muscle.

Aqueous extracts of *Mentha longifolia* in concentrations of 0.1% to 30% w/v produced no direct contractile effect on the guinea pig tracheal muscle. However, when the tissue had been contracted with each of the three agonists used in this study, the plant extract had definite relaxant effects as described below.

5.2.1.1 Effect of *Mentha longifolia* on methacholine-induced contraction of the tracheal muscle.

To assess the effect that *Mentha longifolia* would have on methacholine-induced contractions, the tracheal muscle was contracted with a single dose (0.1ml) of methacholine ($6.67X10^{-8}M$) and the contracted tissue exposed to cumulative doses of plant extract.

Methacholine ($6.67X10^{-8}M$) caused a near maximal (98.16 ± 3.91%) contraction of the tracheal tissue, versus maximum contraction produced by $6.67X10^{-4}M$ methacholine, which was reversed by *Mentha longifolia (ML)* in a dose dependent manner (Figure 5.1). The 1%, 2%, 5% and 10% *ML* produced a (26.26 ± 14.93 %), (96.14 ± 2.39%), (96.82 ± 2.25%) and (97 ± 1.37%) relaxation of the

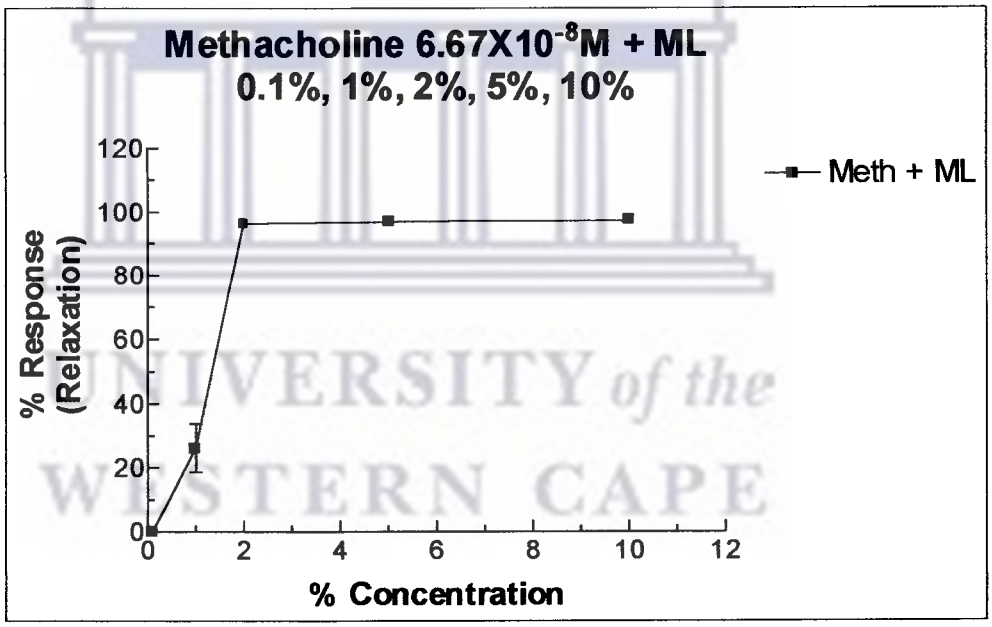


Figure 5.1 The relaxant effect of *Mentha longifolia* (0.1%, 1%, 2%, 5% and 10%) on methacholine ($6.67X10^{-8}M$)-induced contraction. (n = 4)

contracted tissue, respectively (Figure 5.1). These percentage relaxations were all relative to maximal relaxation induced by isoprenaline ($6.67X10^{-5}M$). Samples of the recorder tracings of methacholine-induced contraction showing the relaxation caused by *Mentha longifolia* can be seen in Diagram 5.1 in Appendix 1.

The results strongly suggest that the *Mentha longifolia* had significant bronchodilatory properties which may possibly be mediated via cholinergic receptors. Other researchers have used methacholine-induced contraction of tracheal tissue and tested other plant extracts e.g. aqueous extracts of *Nigella. sativa* to prove the bronchodilatory and anti-cholinergic effects of the plant extracts. [34]

To determine whether the mechanism may involve a competitive anti-cholinergic (anti-muscarinic) effect the tissue was exposed to cumulative doses of the agonist, in the absence and presence of two concentrations, 2% and 10%, of the plant, the log dose response curves (LDRC) were determined and analyzed. The LDRC is shown in Figure 5.2 and the results of the analysis in Table 5.1.

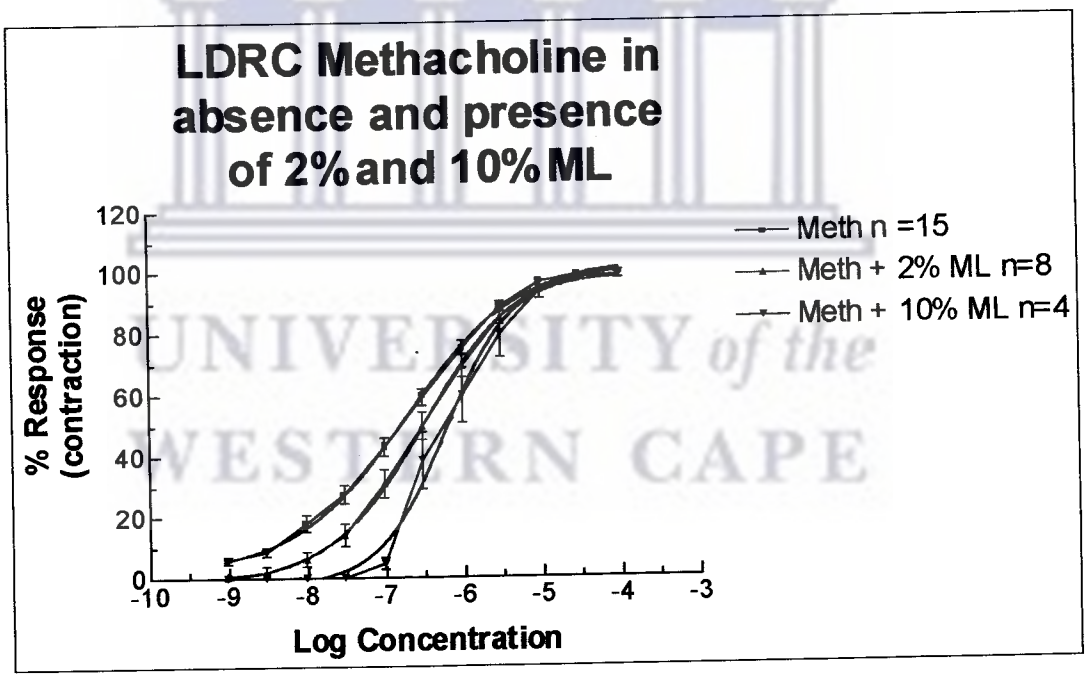


Figure 5.2 The cumulative log dose response curves of methacholine in the absence and the presence of 2% and 10% *Mentha longifolia*. The data was fitted using the non-linear sigmoidal dose response facility of Graph Pad Prism™.[33]

In the presence of the plant extracts there was a dose dependent rightward shift in the methacholine LDRC (Figure 5.2), especially at the lower concentrations of the

agonist. The magnitude of maximal response, as well as the concentration of methacholine ($9.63X10^{-5}M$) producing maximal response was unaltered.

Analysis of the LDRC clearly indicated differences in slope and elevation (Table 5.1). There was a significant increase in EC_{50} values and a significant change in mean Hill slope values going from 0 to 10% *ML*. The increasing EC_{50} values observed mean that increasing concentrations of the drug, methacholine, would be required to be added to remove the effect of the (competitive) antagonism of the *ML*.

Table 5.1 The EC_{50} and Hill slope values of LDRC of methacholine in the absence and the presence of 2% and 10% *Mentha longifolia*.

	Meth n= 15	**Meth + 2% ML** n=8	**Meth + 10% ML** n= 4
LOG EC_{50}	-6.740 ± 0.05672	-6.517 ± 0.05772*	-6.239 ± 0.07048*
HILL SLOPE	0.6022 ± 0.05063	0.7178 ± 0.06877	1.0250 ± 0.1540 **
EC_{50}	1.819e-007	3.044e-007	5.776e-007

Each value represents the mean ± SEM of 4 or more values as indicated, n=15; n=8; n=4.

*Significantly different from the mean log EC_{50} of methacholine ($p<0.05$)

**Significantly different from the mean Hill slope of methacholine ($p<0.05$)

Note a dose-dependent increase in Hill slope and EC_{50} values.

Although *Mentha longifolia* was able to fully reverse the methacholine-induced contraction, the non-parallel shift in the curves suggests that the mechanism was clearly not a fully competitive one, and/or one involving only the cholinergic receptors. The fact that the plant caused smooth muscle relaxation together with the effects described above implies that *ML* may have reversible anti-cholinergic activity which shows some competitive nature.[36, 37] Similar conclusions were drawn by other researchers when studying the bronchodilatory and anti-cholinergic effects on *Nigella sativa*.[34, 35]

To determine whether the tracheal smooth muscle relaxant activity of the plant was perhaps also mediated via β2-adrenergic mechanisms; these were tested for in *Mentha longifolia* in the following manner. The tracheal tissue was contracted with a single dose of methacholine ($6.67X10^{-5}M$) and increasing concentrations of the plant were added in the absence and presence of propranolol ($6.67x10^{-6}M$). The results for this part of the study are given in Figure 5.3.

In the absence of propranolol, *ML* produced a significant degree of relaxation, up to 74.70 ± 4.18 % of the maximum induced by isoprenaline (Figure 5.3). In the

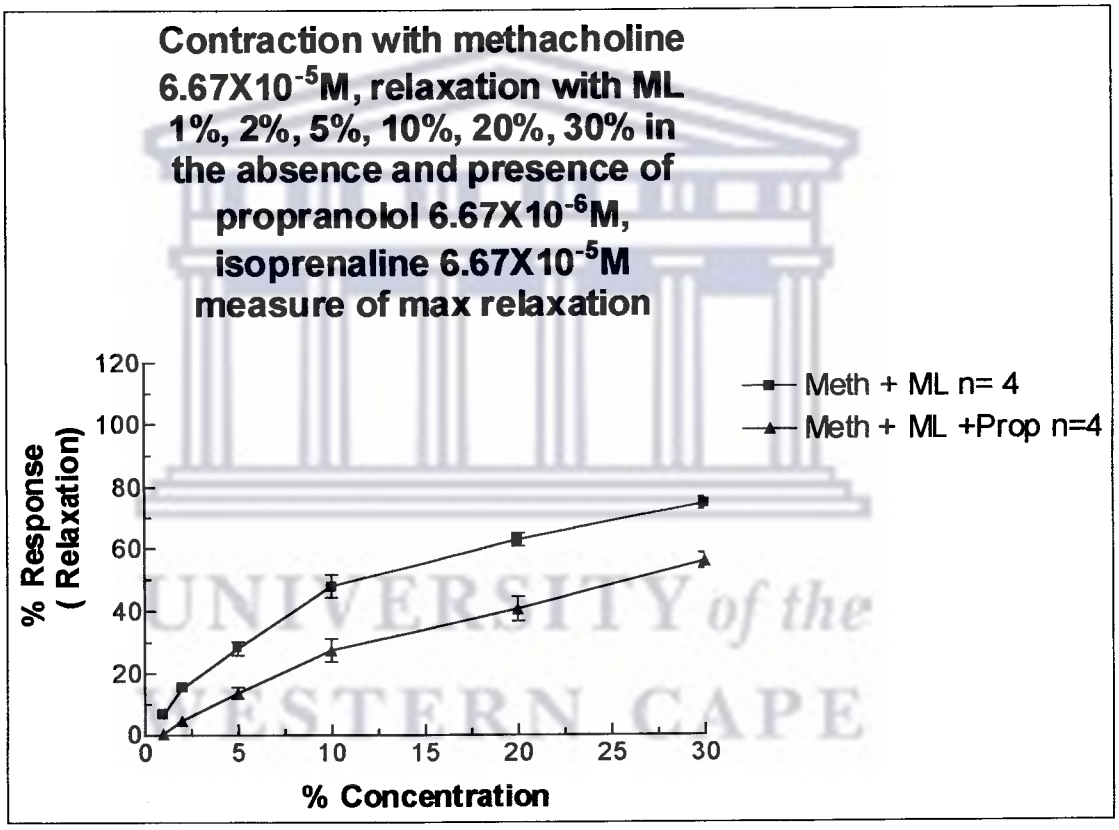


Figure 5.3 The effect of *Mentha longifolia*, added in increasing concentrations, on methacholine ($6.67X10^{-5}M$)-induced contraction in the absence and presence of propranolol ($6.67X10^{-6}M$).

presence of propranolol a marked decrease in relaxation was, however, noted and only 56.06 ± 4.72 % of the maximal relaxation was attained. The presence of the propranolol thus caused an approximately 20% reduction in the relaxation of the tissue. From Figure 5.3 it can also be seen that in the absence of propranolol

12.5% *ML* caused a 50% relaxation of the methacholine ($6.67X10^{-5}M$)-induced contraction, but in the presence of the propranolol it took 27.5% *ML* to give a 50% relaxation. This may suggest that some of the relaxant activity of the *Mentha longifolia* was blocked by the propranolol and thus possibly β2-adrenergic receptor mediated. Other researchers have also used propranolol to identify β2-adrenergic activity and have likewise found that there was a shift in EC_{50} values towards a higher concentration when the tissue was previously incubated with propranolol [38, 39, 40]. It may thus be assumed that a part (approximately 20%) of *Mentha longifolia's* smooth muscle relaxant effect may be mediated via the β2-adrenergic pathway, but certainly not all of the relaxation (i.e. *Mentha longifolia* also caused relaxation via other mechanisms).

5.2.1.2 Effect of *Mentha longifolia* on histamine-induced contraction of the tracheal muscle.

To assess the effect that *Mentha longifolia* would have on histamine-induced contractions, the tracheal muscle was contracted with a single dose (0.1ml) of histamine ($6.67X10^{-6}M$) and the contracted tissue exposed to cumulative doses of plant extract.

Histamine ($6.67X10^{-6}M$) caused 88.41 ± 6.77% contraction of the tracheal tissue relative to the maximum contraction caused by $6.67x10^{-5}M$ histamine. *ML* aqueous extract in concentrations above 0.1% gave a 100% relaxation (n=4) (relative to the maximum relaxation induced by isoprenaline ($6.67X10^{-5}M$)) of the histamine-induced contracted tissue (Figure 5.4). Samples of the recorder tracings of histamine-induced contractions showing relaxation caused by *Mentha longifolia* can be seen in Diagram 5.2 in Appendix 2.

The results strongly suggest that *Mentha longifolia* had significant smooth muscle relaxant activity mediated via the histaminic receptors. The fact that *ML* relaxed histamine-contracted tissue to such a major extent indicated that *ML* had significant bronchodilator properties, and the potential existence of potent anti-histaminic action. Other researchers have also used histamine-induced contraction

of tracheal tissue to prove that extracts of other plants have bronchodilator properties. [41]

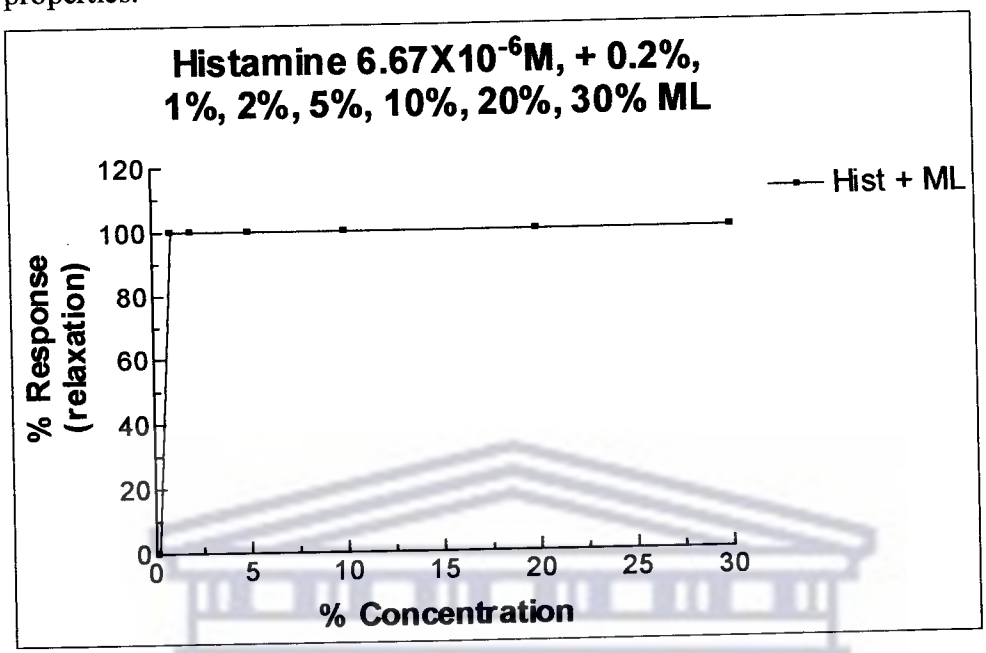


Figure 5.4 The relaxant effect of increasing concentrations of *Mentha longifolia* on histamine ($6.67X10^{-6}M$)-induced contraction. n=4

To determine whether the mechanism may involve a competitive anti-histaminic effect the tissue was exposed to cumulative doses of the agonist, in the absence and the presence of 2%, 10% and 20% of *Mentha longifolia*; the log dose response curves (LDRC) were determined and analyzed. The LDRC is shown in Figure 5.5 and the results of the analysis in Table 5.2.

In the presence of the higher concentrations of plant extracts (especially 20%) there was a slight dose dependent rightward shift in the histamine LDRC. At the low concentration (2%) of *ML* there was however a slight leftward displacement of the curve. The same maximum response (100%) was attained at the same concentration ($1.11X10^{-4}M$) of histamine with all the curves and histamine-induced auto-inhibition (seen as a decrease in response at high histamine concentrations) was unaffected by the plant. Analysis of the results (Table 5.2) indicated that, except for the slight leftward shift induced by 2% *ML* (evidenced by change in EC_{50}) the plant induced no other significant differences in the histamine

LDRC. [Note: Interestingly there is a suggestion that lower concentrations (< 2%) of plant may displace the curve even more to the left, but this was not pursued further]. Other researchers have found plant extracts to cause a dose dependent rightward shift in the LDRC histamine-induced contraction indicating reversible competitive antagonistic[36, 37] activity on the H1 receptor.[42, 43]

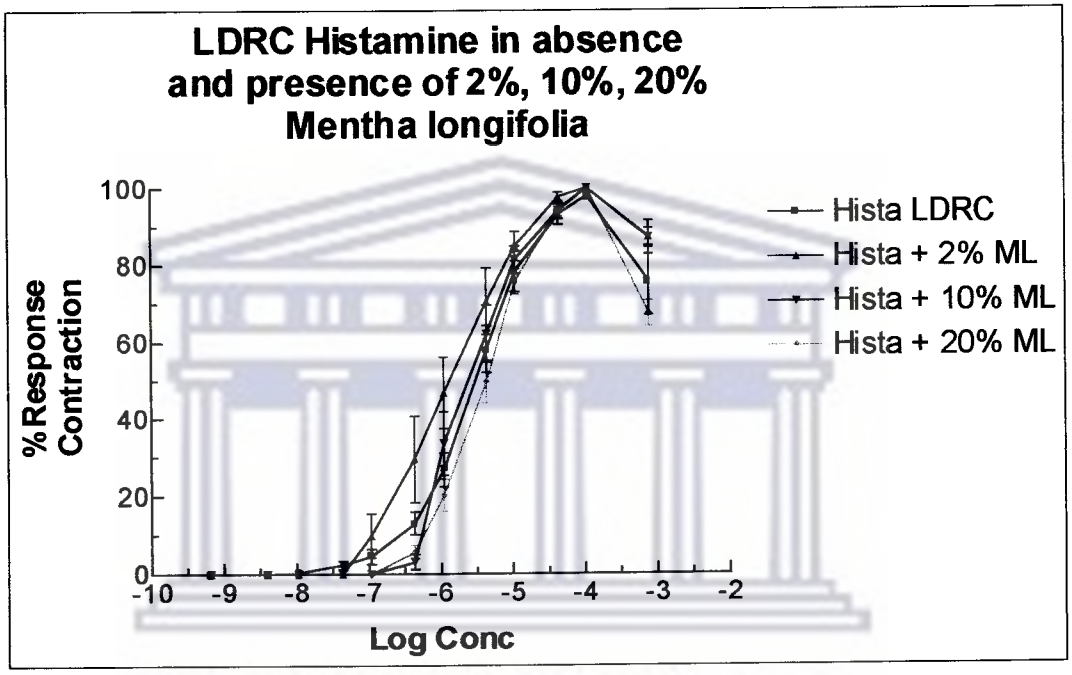


Figure 5.5 The cumulative log dose response curves of histamine in the absence and the presence of 2%, 10% and 20% *Mentha longifolia*. The data was fitted using the non-linear sigmoidal dose response facility of Graph Pad Prism™.[33]
Hista n= 16, Hista + 2% *ML* n = 4, Hista + 10%*ML* n = 4, Hista + 20% *ML* n =4

Overall the present results, however, appears to indicate that *ML* is not producing its reversal of histamine-induced contractions of the tracheal muscle via histaminergic receptors (i.e. it does not appear to be antihistaminic in action).

Table 5.2 The EC_{50} and Hill slope values of the LDRC of histamine in the absence and presence of 2%, 10% and 20% *Mentha longifolia*.

	Hista LDC n= 16	Hista + 2% ML n=4	Hista + 10% ML n=4	Hista + 20% ML n=4
LOG EC_{50}	-5.620±0.06309	-5.941±0.08845*	-5.644±0.05632	-5.495±0.06362
HILL SLOPE	1.206 ± 0.1671	0.9098 ± 0.1528	1.233 ± 0.1546	1.411 ± 0.2324
EC_{50}	2.399e-006	1.146e-006	2.267e-006	3.202e-006

Each value represents the mean ± SEM of 4 or more experiments as indicated; n= 16, n=4, n=4; n=4.

*Significantly different from the mean log EC_{50} of histamine ($p<0.05$)

5.2.1.3 Effect of *Mentha longifolia* on LTD_4-induced contraction of the tracheal muscle.

To determine the concentration of LTD_4 to use in the experiments assessing the effect of the plant extracts, the LDRC of LTD_4 was obtained.

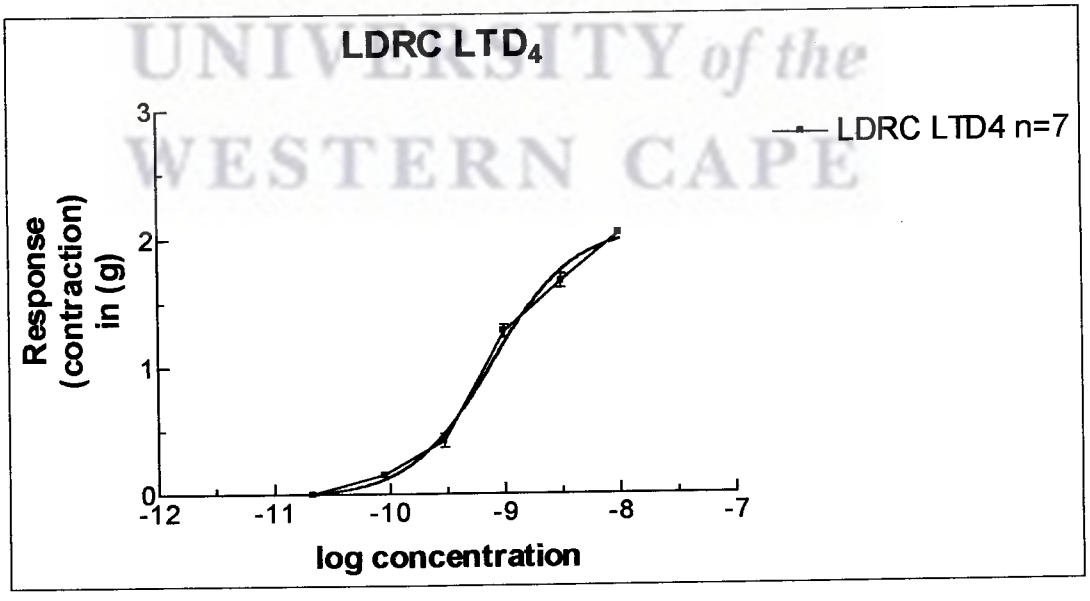


Figure 5.6 The cumulative log dose response curve of LTD_4, n = 7. The data was fitted using the non-linear sigmoidal dose response facility of Graph Pad Prism™ .[33]

The LDRC for LTD_4 obtained with doses up to $6.93X10^{-9}M$ is shown in Figure 5.6. The LDRC of LTD_4 showed contraction starting at concentration $3.11X10^{-11}M$ and the maximum concentration of LTD_4 obtained for use in the experiment was $6.93X10^{-9}M$, but as can be seen from the dose response curve this was not the maximum for the tissue. Therefore these results are reported in force contraction instead of percentage contraction.

To assess the effect that *Mentha longifolia* would have on leukotriene D_4-induced contractions, the tracheal muscle was contracted with a single dose (0.1ml) of LTD_4 ($6.93X10^{-9}M$) and the contracted tissue exposed to cumulative doses of plant extract. The results of this part of the study are given in Figure 5.7 and samples of the recorder tracings of the leukotrieneD_4-induced contraction showing relaxation caused by *Mentha longifolia* can be seen in Diagram 5.3 in Appendix 3.

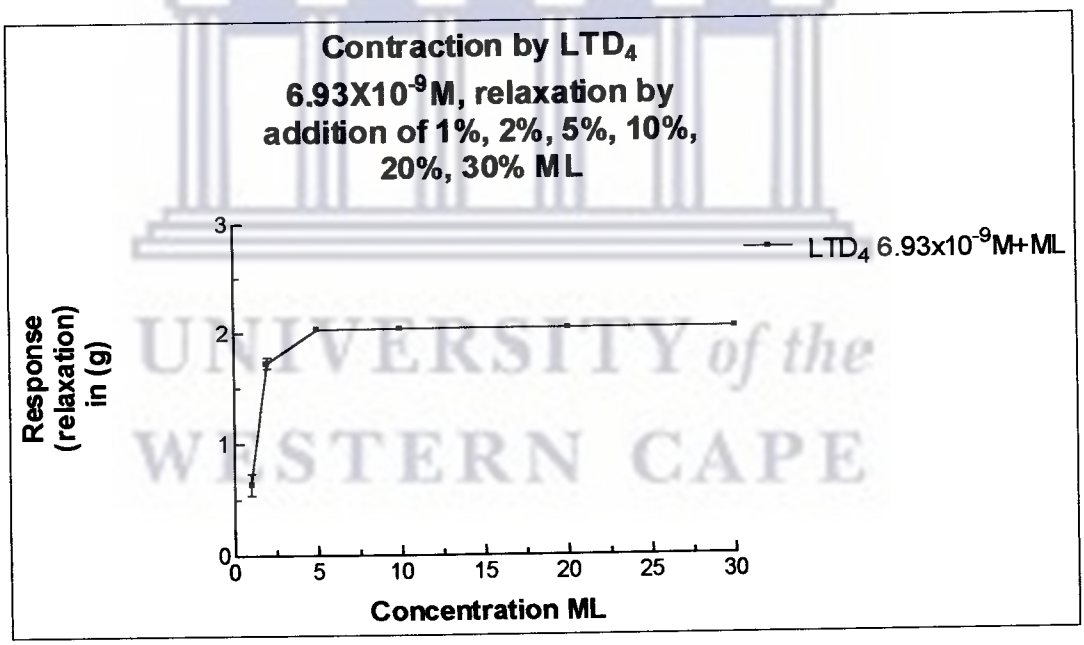


Figure 5.7 Effect of increasing concentrations (1 to 30%) of *Mentha longifolia* on LTD_4 ($6.93X10^{-9}M$)-induced contractions of the tracheal smooth muscle. (n = 4)

LeukotrieneD_4 ($6.93X10^{-9}M$) caused a significant contraction ($1.57 \pm 0.05g$) of the tracheal tissue which was relaxed by *Mentha longifolia* in a dose dependent manner (Figure 5.7). Concentrations of *ML* $\geq$ 5 % produced maximal relaxation of

the LTD_4–induced contraction. *ML* thus had a significant reversal effect on LTD_4 contractions again supporting its potential as a bronchodilator. Other researchers have also used LTD_4-induced contracted tracheal tissue to prove the existence of bronchodilator activity in compounds.[44, 45]

[NOTE: Our attempts by means of LDRC studies to determine whether a leukotriene D_4 receptor-mediated mechanism could be responsible for this potential bronchodilator effect of *ML* was, however, unsuccessful due to several logistical problems (i.e. the instability of LTD_4 in aerobic (oxygen) conditions and upon storage (despite efforts such as working under nitrogen atmosphere, silating the glassware, and storage below-20°C).

The overall conclusion from the LTD_4-induced studies is that *ML* has a major effect in reversing LTD_4-induced contractions, and therefore has great potential as bronchodilator and anti-asthmatic. However, it is unclear whether the mechanism is in fact anti-leukotriene in action.

5.2.1.4 Summary of the effects of *Mentha longifolia* on the agonists used in the studies

From the above results it was clear that *ML* was able to reverse cholinergic-, histaminergic- and leukotriene D_4- induced contractions. In Figure 5.8 the graphs and Table 5.3 depicting the comparative effects of a range of doses of the plant on the agonist-induced contractions are shown.

In cases where sub-maximal doses of agonists were used, full reversal of the agonist-induced contractions were achieved with relatively low concentrations (*) (<2%)* of *Mentha longifolia.* The EC_{50s} as deduced from the graph (Figure5.8) for the relaxant effects of *Mentha longifolia* on methacholine- ($6.67X10^{-8}M$), histamine- ($6.67X10^{-6}M$) and LTD_4-induced ($6.93X10^{-9}M$) contractions were at concentrations ca 1.7%*, ca 0.6%* and ca 1.7%* respectively. But, as would be expected in the presence of a higher dose of agonist, when the dose of agonist (e.g. methacholine) was increased to concentrations (i.e. $6.67X10^{-5}M$) producing maximal or near maximal contraction, the EC_{50} for the *ML* relaxant also increased,

viz. to concentration ca 10.5%*. *ML* was therefore a potent antagonist against concentrations of all three agonists that would be relevant in asthma and thus has good potential as a bronchodilator. *Mentha longifolia* possibly also has β2-adrenergic relaxant activity also showing some bronchodilator activity (ca 20%) via this mechanism.

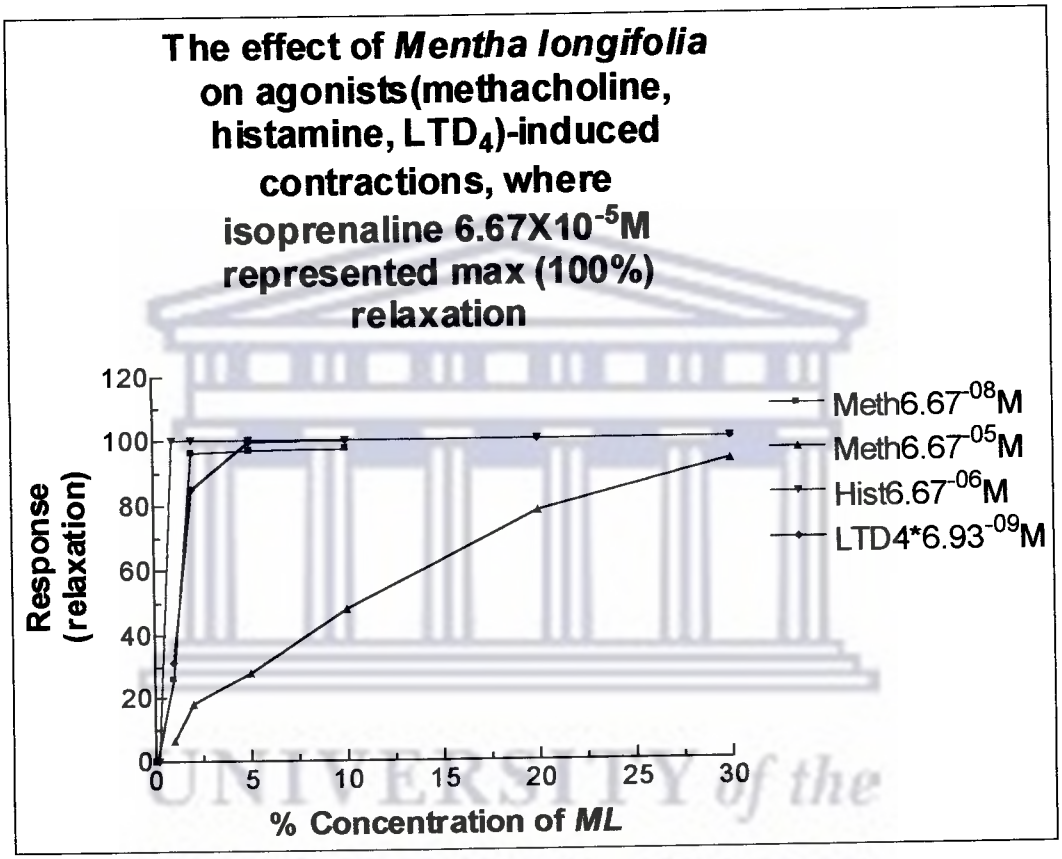


Figure 5.8 Relaxant effect of different doses of *Mentha longifolia* on the agonists, methacholine (6.67X10^{-8}M)-, methacholine (6.67X10^{-5}M)-, histamine (6.67X10^{-6}M)-, and LTD_4 (6.93X10^{-9}M)-induced contraction of the tracheal muscle. (n = 4 in all experiments)

The mechanisms responsible for *ML*'s antagonistic effect could, however, not be fully elucidated in this study. The relaxant effect of *ML* did not appear to involve histaminergic receptors as seen from LDRC of histamine (as there was no major right-ward shift in the curve, which would have indicated competitive action), but the involvement of cholinergic and β2-adrenergic receptors cannot, on the basis of the present data, be excluded. It is also quite possible that the plant may contain more than

one active constituent which may each individually act on different receptors and that the resultant relaxant effect of the *ML* extract is the sum of such interactions. For example, the role of L-menthol, a cyclic terpene alcohol present in the volatile oils of various species of *mentha* has been found to be specific, reversible and stereochemically selective in it regulating Ca^{++} channels situated on cell membranes of the guinea pig lung.[46] Another possibility is that the plant's relaxant effect, could be the result of the action of the active constituent(s) on a path (such as inhibition of intracellular calcium movements, or ion channels, etc) which is common to several receptor pathways.

On the other hand Hispidulin, a flavone, has been identified in several plant families, especially e.g. the *Asteraceae, Papilionaceae*, *Lamiaceae* and *Verbenaceae*.[41] The effect of hispidulin on isolated guinea-pig smooth muscle was investigated[41, 47] and it was found that the inhibitory action (i.e. smooth muscle relaxant effect) of hispidulin was mediated by its binding to intracellular Ca^{2+}-regulating proteins thereby preventing Ca^{2+} from binding to these proteins, resulting in the inhibition of the contractile force[47]. Such a type of compound and such a mechanism may possibly explain the inhibitory effects (smooth muscle relaxant effects) of *Mentha longifolia* on the agonist-induced actions found in the present study. *Mentha longifolia* is a member of the *Lamiaceae* family[8], and is known to contain several flavonoids[41, 48, 49] quite possibly also the flavonoid hispidulin[47] or a similarly acting one. [41] Identification of all the flavonoids to be found in *Mentha longifolia*, investigation of their activity as smooth relaxants[41] and the mechanism involved might therefore be a very worthy pursuit.

Table 5.3 Comparison of the degrees of smooth muscle relaxant effects produced by various concentrations of the plants versus agonist-induced contractions

Plant & Concentration	⁂ Degree of relaxation (in %) of contractions produced by		
	Methacholine ($6.67x\ 10^{-05}M$	**Histamine ($6.67 \times 10^{-06}M$)**	**LTD_4 (6.93×10^{-09} M)**
Mentha longifolia			
0.10%	-	-	-
0.20%	-	0	-
1%	6.52	100	31.33
2%	18.19	100	85.07
5%	28.13	100	99.51
10%	47.99	100	100
20%	78.05	100	100
30%	93.22	100	100
Artemisia afra			
0.10%	-	-	-
0.20%	-	0	-
1%	7.49	0	3.04
2%	17.95	6.62	7.68
5%	30.05	21.29	21.01
10%	52.38	47.61	57.88
20%	80.17	86.2	94.16
30%	92.81	100	99.27

⁂ Responses expressed as a % of the total relaxation produced by isoprenaline ($6.67X10^{-5}M$)

5.2.2. Effect of *Artemisia afra* on agonist-induced contraction of the tracheal muscle.

Aqueous extracts of *Artemisia afra (AA)* in concentrations of 0.1% to 30% w/v produced no direct contractile effect on the guinea pig tracheal muscle. However, when the tissue had been contracted with each of the three agonists used in this study, the plant extract had definite relaxant effects as described below.

5.2.2.1 Effect of *Artemisia afra* on methacholine-induced contractions of the tracheal muscle.

To assess the effect that *Artemisia afra* would have on methacholine-induced contractions, the tracheal muscle was contracted with a single dose (0.1ml) of methacholine ($6.67X10^{-5}M$) resulting in a 84.91 ± 12.43% contraction versus maximum contraction produced by $6.67X10^{-4}M$ methacholine, and the contracted tissue exposed to cumulative doses of plant extract. In the tissue that had not previously been exposed to cumulative doses of methacholine, the plant extracts produced a dose dependent relaxation of the contracted tissue (Figure 5.9), starting

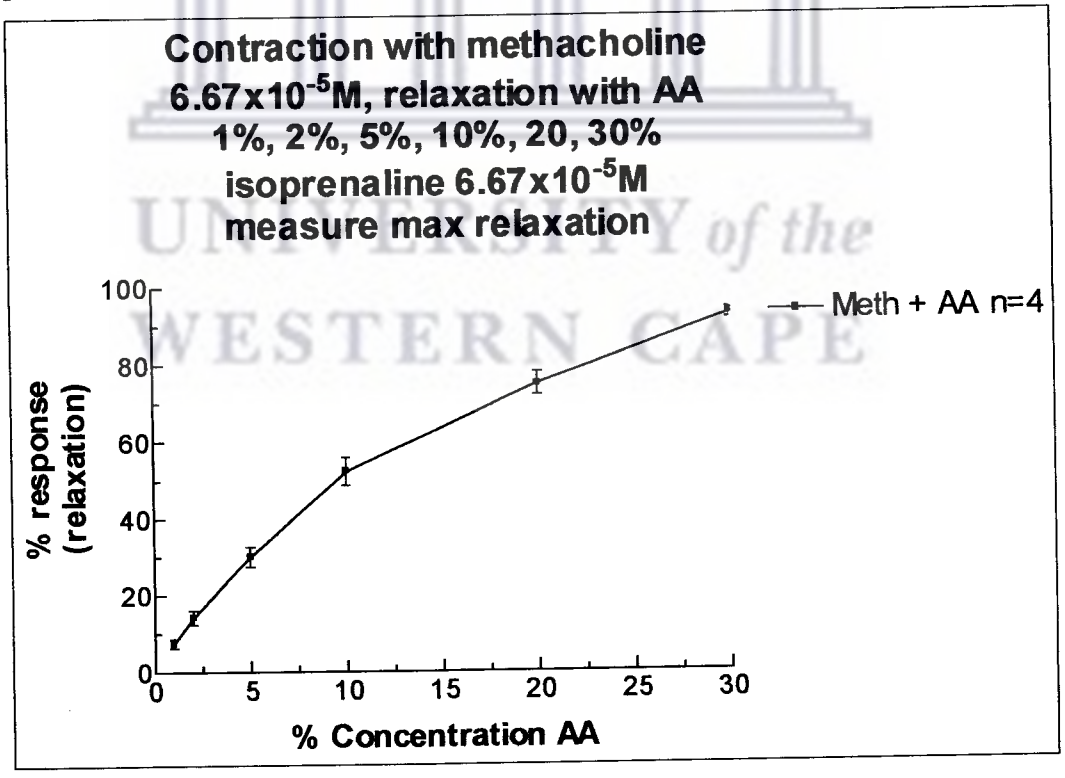


Figure 5.9 Relaxant effect of various doses (1 - 30%) of *Artemisia afra* on methacholine ($6.67x10^{-5}M$)-induced contraction of the tracheal muscle.

with 7.49 ± 2.36% relaxation with 1% *AA* and 92.81 ± 2.59% relaxation with 30 % *AA*. These percentage relaxations were all relative to the relaxation produced by $6.67X10^{-5}M$ isoprenaline. Samples of the recorder tracings of methacholine ($6.67X10^{-5}M$)-induced contraction showing relaxation caused by *Artemisia afra* can be seen in Diagram 5.4 in Appendix 4.

The findings suggested that *Artemisia afra* had potential bronchodilator activity which may possibly be mediated via cholinergic receptors. Other researchers have used methacholine-induced contraction of tracheal tissue and tested other plant extracts e.g. (aqueous extracts of *Nigella. sativa*).[34, 35] Relaxation of the contracted tissue by the plant extract was considered a bronchodilatory effect.

However, when the tissue had been exposed to methacholine (i.e. after obtaining a full cumulative LDRC of methacholine) and thoroughly washed to remove any traces of the agonist and was then exposed to doses of plant extract (in absence of added methacholine) a more complex picture of the plant's effects emerged (Figure 5.10). Concentrations of 1% and 2% *AA*, surprisingly, produced pronounced contractile responses (shown as negative responses) in order of -77.88 ± 18.66% and -78.56 ± 18.39%, relative to the maximum obtained for methacholine ($6.67X10^{-4}M$) in the previous LDRC, respectively. These contractile responses could be completely reversed by both ipratropium ($1.67X10^{-3}M$) and mepyramine ($4.13X10^{-10}M$). At concentrations of 10% and 20%, *AA*, however, again produced relaxation of the contraction that had been induced with the earlier lower concentrations of the plant extracts (1% or 2 %) of *AA*. These relaxation responses were of the order of 19.49 ± 3.58% and 54.02 ± 12.82% for 10% and 20 % *AA* (n=4), respectively. The magnitudes of these relaxations were expressed relative to the maximal relaxation induced by isoprenaline ($6.67X10^{-5}M$). It appeared that the higher doses of *AA* (10% and 20%) were thus able to antagonize the contractions induced by the lower concentrations of *AA* (1% and 2%), in these methacholine-sensitized tissue. Samples of the recorder tracings showing the contraction and relaxation of methacholine sensitized tissue can be seen in Diagram 5.5 in Appendix 5.

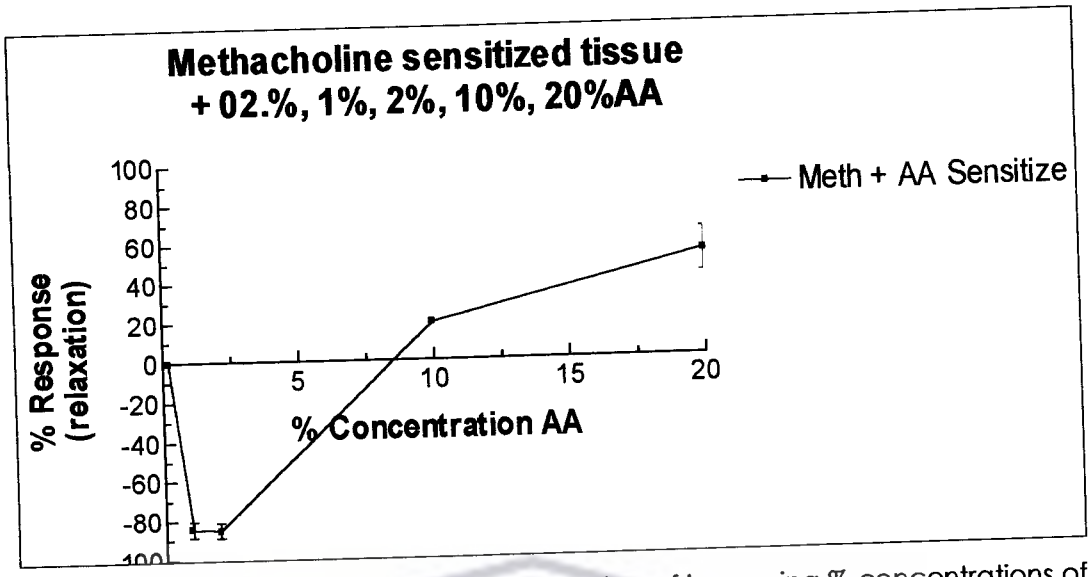


Figure 5.10 The effect of the addition of increasing % concentrations of *Artemisia afra* after the tissue was exposed to methacholine and washed 6 times over a 30 minute period. 1% and 2% AA caused major contractile responses (negative values), relaxation starting at 10% AA. (n = 4)

The fact that *Artemisia afra* relaxed methacholine-induced contracted tissue indicated that it had bronchodilatory properties. The contractile effects caused by the low concentrations of *AA,* in the methacholine-sensitized tissue, however, suggest that it may also be able to potentiate the bronchoconstrictor effects of methacholine. These results also strongly suggest that more than one active ingredient may be involved (or that the mechanism could be quite complex). More data and/or the isolation of such active constituents are, however, required to fully understand these results.

To determine whether the mechanism responsible for the relaxant effect of *AA* may involve a competitive anti-cholinergic (anti-muscarinic effect) the tissue was exposed to cumulative doses of methacholine, in the absence and presence of two concentrations, 2% and 20%, of *AA,* and the log dose response curves (LDRC) were determined and analyzed. The LDRC is shown in Figure 5.11 and the results of the analysis in Table 5.4.

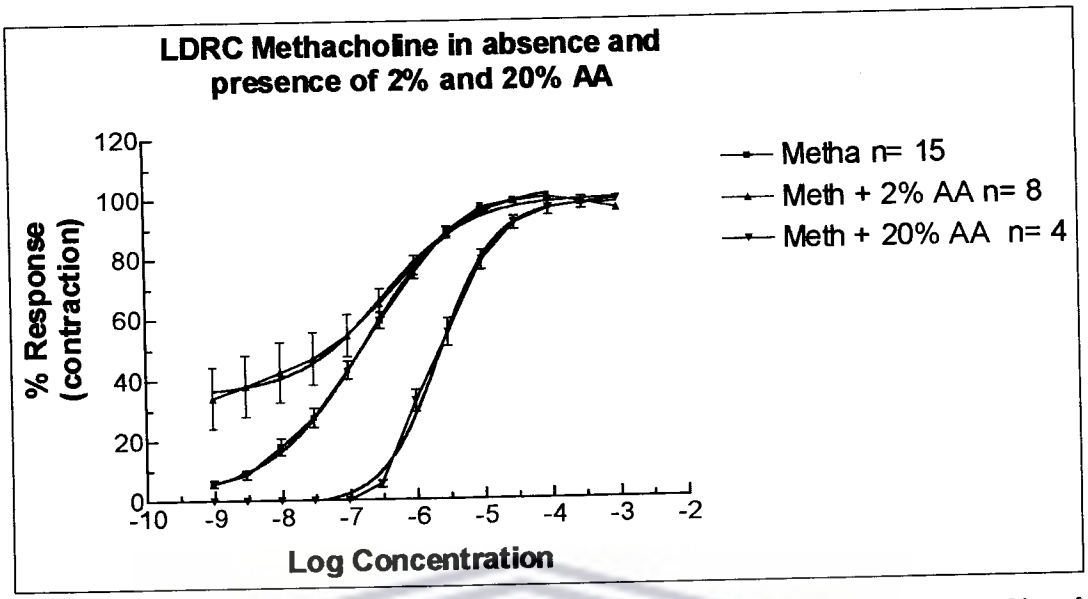


Figure 5.11 The cumulative log dose response curves (LDRC) of methacholine in the absence and the presence of 2% and 20% *Artemisia afra*. The data was fitted using the non-linear sigmoidal dose response facility of Graph Pad Prism™ .[33]

In the absence of plant, methacholine-induced contractions were discernible starting at concentration $9.60X10^{-10}M$ and reaching maximum contraction at concentration $9.63X10^{-5}M$. In the presence of 2% *AA* (left in contact with the tissues for 20 minutes) there was a major contractile response (in the absence of the methacholine) that resulted in an upward shift and leftward shift in the LDRC at lower concentrations (below $9.63\ X10^{-7}M$) of methacholine. However, the concentration ($9.60X10^{-10}M$) at which the methacholine-induced response started and the concentration ($9.63X10^{-5}M$) giving the maximal response were unchanged. Also there were no significant changes in the EC_{50} and Hill slope values (Table 5.4). At the lower concentration, the plant thus appeared to have a partial agonistic /dualistic effect.[36, 37]

In the presence of 20% *AA* there was, however, a pronounced non-parallel rightward shift of the LDRC of methacholine. Now the methacholine-induced contractions were only measurable from a higher concentration ($2.96X10^{-7}M$), and also reached maximum contraction at a higher concentration ($2.96X10^{-4}M$).

Table 5.4 The EC_{50} and Hill slope values of the LDRC of methacholine in the absence and the presence of 2% and 20% *Artemisia afra*.

	Meth n= 15	Meth + 2% AA n= 8	Meth + 20% AA n= 4
LOG EC_{50}	-6.740 ± 0.05672	-6.471 ± 0.2002	-5.658 ± 03245*
HILL SLOPE	0.6022 ±0.05063	0.6869 ± 0.2055	1.0290± 0.07061**
EC_{50}	1.819e-007	3.379e-007	2.199e-006

Each value represents the mean ± SEM of 4 or more experiments as indicated; n= 15, n=8, n=4.

* Significantly different from mean log EC_{50} obtained in absence of plant, ($p<0.05$)

** Significantly different from mean Hill slope obtained in absence of plant, ($p<0.05$)

Furthermore, there were statistically significant differences in the EC_{50} and Hill slope values obtained in the absence and presence of the 20% *AA* (Table 5.4). This result suggests that higher doses of *AA* can antagonize the methacholine-induced contraction, but the non-parallel shift in the curve suggests that the mechanism did not involve only cholinergic receptors and / or may not be one of full competitive antagonism[36, 37] (even though the antagonism was fully reversible).

Similar conclusions were drawn by researchers studying bronchodilatory and anti-cholinergic effects of *Nigella sativa*.[34, 35]

To determine whether the tracheal smooth muscle relaxant activity of the plant was perhaps also mediated via β2-adrenergic mechanisms; these were tested for in *Artemisia afra* in the following manner. The tracheal tissue was contracted with a single dose (0.1ml) of methacholine ($6.67X10^{-5}M$) and increasing concentrations of the plant were added in the absence and presence of propranolol ($6.67X10^{-6}M$). The results for this part of the study are given in Figure 5.12.

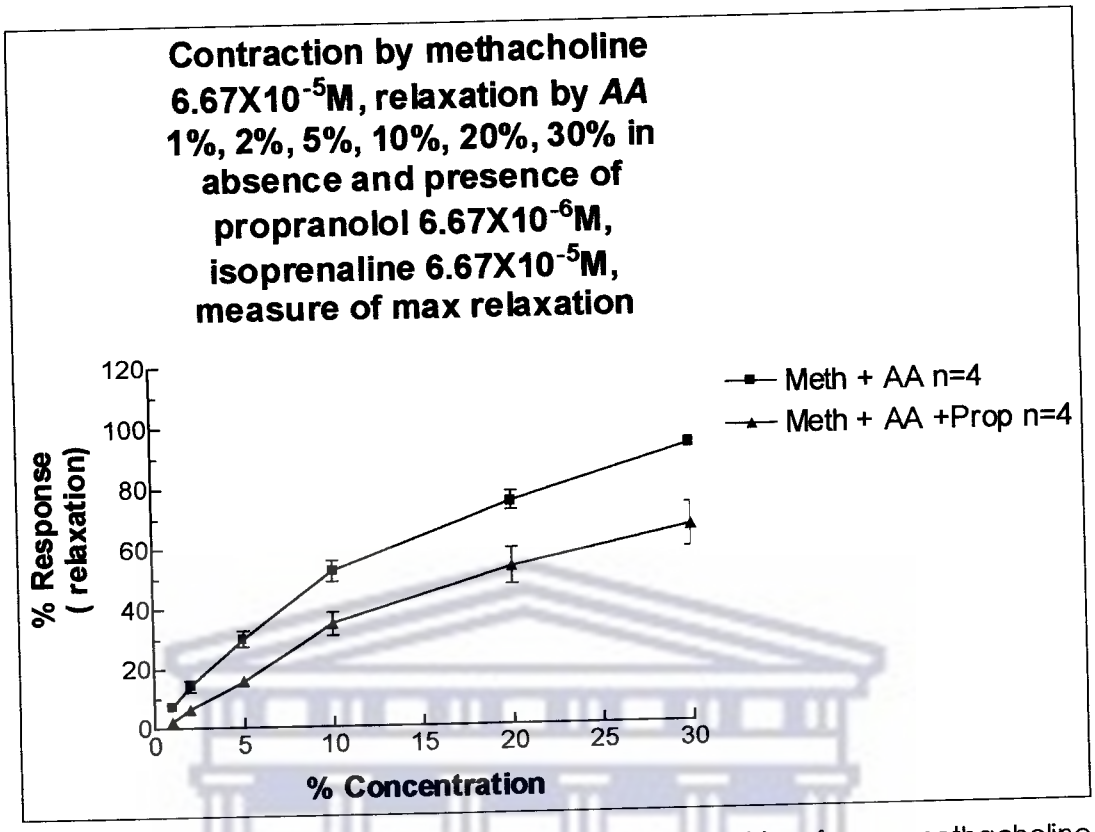


Figure 5.12 Effect of various doses of *Artemisia afra* on methacholine ($6.67X10^{-5}M$)-induced contractions of tracheal muscle, in the absence and presence of propranolol ($6.67X10^{-6}M$).

In the absence of propranolol, *AA* produced a significant degree of relaxation, up to 92.81 ± 2.59% of the maximum induced by isoprenaline ($6.67X10^{-5}M$). In the presence of propranolol a marked decrease in relaxation was, however, noted and now only 65.83 ± 14.58% of the maximal relaxation was attained. The presence of propranolol resulted in an approximately 27% reduction of relaxation induced by *AA*. From Figure 5.12 it can also be seen that in the absence of propranolol approximately 10% *AA* caused 50% relaxation of the methacholine ($6.67X10^{-5}M$)-induced contraction, but in the presence of the propranolol approximately 19% *AA* was needed to cause 50% relaxation of the contracted tissue. Similar to the case of *Mentha longifolia* (Figure 5.3) it is seen that some of the relaxant activity of *Artemisia afra* was also blocked by propranolol and thus possibly β2-adrenergic receptor mediated. The magnitude of the reduction in the relaxation (i.e. ca. 20% and 27%) caused by the plants were also relatively similar. Some of the relaxant effect of *Artemisia afra* can thus possibly be mediated via the β2-adrenergic

pathway, but certainly not all of it. (i.e. *Artemisia afra* also caused relaxation via other mechanisms).

Other researchers have also used propranolol to identify β2-adrenergic activity and have likewise found that there was a shift in EC_{50} values towards a higher concentration when the tissue was previously incubated with propranolol. [38]

5.2.2.2 Effect of *Artemisia afra* on histamine-induced contraction of the tracheal muscle.

To assess the effect which *Artemisia afra* would have on histamine-induced contractions, the tracheal muscle was contracted with a single dose (0.1ml) of histamine ($6.67X10^{-6}M$) and the contracted tissue exposed to cumulative doses of plant extract. The results of this part of the study are summarized in Figure 5.13.

Histamine ($6.67X10^{-6}M$) caused 92.30 ± 3.93% contraction (relative to the maximum contraction for $6.67X10^{-5}M$ histamine) of the tracheal tissue and *AA* caused a dose dependent inhibition of this contraction. While concentrations of 0.1% and 1% *AA* had no effect, 2%, 20% and 30% *AA* caused progressively higher degrees of relaxation, i.e. 6.62 ± 1.66%, 86.20 ± 10.14% and 100% (n = 4 in all experiments), (relative to the maximum relaxation produced by isoprenaline ($6.67X10^{-5}M$)), respectively. Samples of the recorder tracings of histamine-induced contractions showing relaxation caused by *Artemisia afra* can be seen in Diagram 5.6 in Appendix 6.

The results strongly suggest that *Artemisia afra* had significant smooth muscle relaxant activity mediated via the histaminic receptors. The result that *AA* relaxed histamine-contracted tissue to such a major extent indicated that *AA* had significant bronchodilator activity and the potential existence of major anti-histaminic action. Other researchers have used histamine-induced contraction of tracheal tissue to prove their plant extracts have bronchodilator activity.[38, 41]

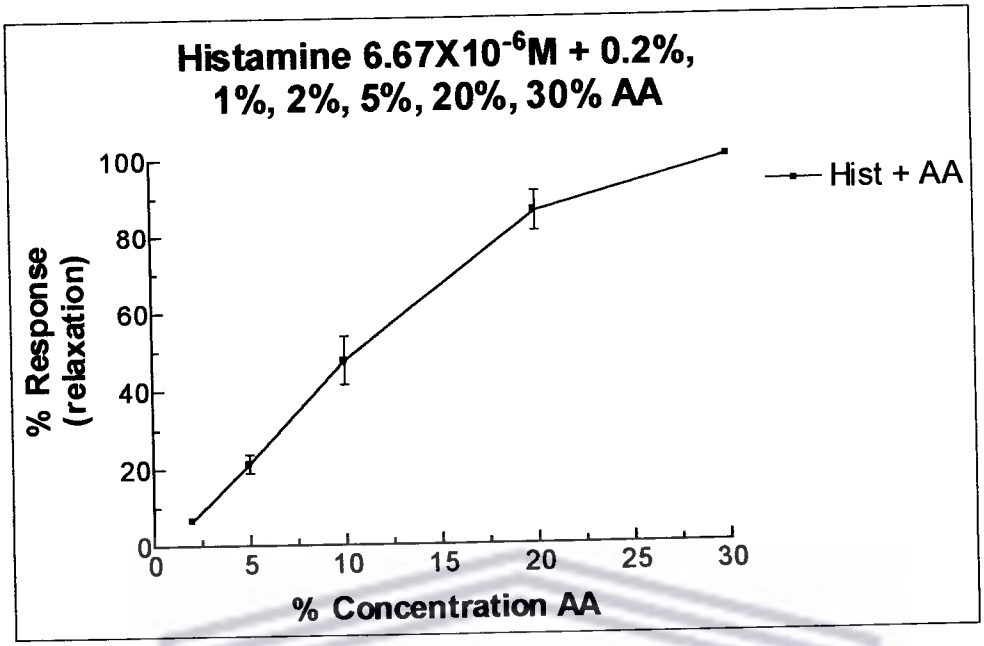


Figure 5.13 The relaxant effect of increasing concentrations of *Artemisia afra* on histamine ($6.67X10^{-6}M$) -induced contraction. (n = 4)

To determine whether the mechanism may involve a competitive anti-histaminic effect the tissue was exposed to cumulative doses of the agonist, in the absence and the presence of 2% and 20% of *Artemisia afra*; the log dose response curves (LDRC) were determined and analyzed. The LDRC is shown in Figure 5.14 and the results of the analysis in Table 5.5.

While there was little difference between the LDRC in the absence and presence of 2% *AA*, the LDRC obtained in the presence of the higher concentration (20%) of *AA* was significantly shifted to the right. The magnitude of the maximal response, as well as the concentration of the histamine ($1.11X10^{-4}M$) producing maximal response was unaltered. In all cases auto-inhibition was noted after reaching the maximum concentration. The shift in the LDRC in the presence of the 20% *AA* was confirmed by the significantly different EC_{50} and Hill slope values found (Table 5.5), suggesting the possible involvement of histaminergic receptors in the relaxant action of *AA*.

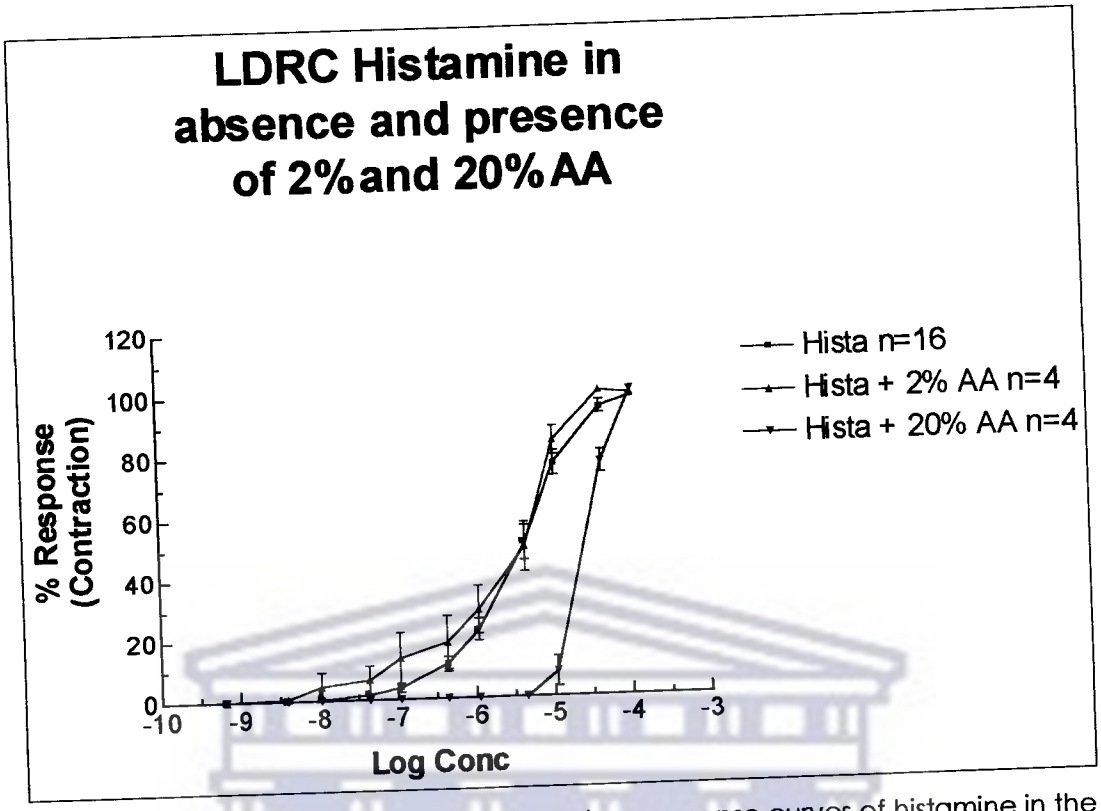


Figure 5.14 The cumulative log dose response curves of histamine in the absence and the presence of 2% and 20% *Artemisia afra*. The data was fitted using the non-linear sigmoidal dose response facility of Graph Pad Prism™.[33]

While the plant was able to fully reverse the action of the agonist, the rightward shift in the curve was, however, not fully parallel (especially at the higher concentrations) as would be required for fully competitive antagonism.[36,37] Nevertheless, from the present data we would be reluctant to exclude the possibility of some degree of competitive reversible antagonism[36,37] at the histaminergic receptor as a mechanism for the relaxant effect of this plant on histamine-induced contractions. Indeed, other researchers have interpreted the findings of plant extracts which caused a dose dependent rightward shift of the curve, without change in the maximum response, as indicating competitive reversible antagonist activity[36, 37] on the H1 receptor.[42]

Table 5.5 The EC_{50} and Hill slope values of the LDRC of histamine in the absence and presence of 2% and 20% *Artemisia afra*.

	Hista n=16	Hista + 2% AA n=4	Hista + 20% AA n=4
LOG EC_{50}	-5.375 ± 0.04773	-5.380 ± 0.1239	-4.534 ± 0.01960*
HILL SLOPE	0.9918 ± 0.09259	0.8666 ± 0.1786	2.553± 0.2032**
EC_{50}	4.214e-006	4.173e-006	2.922e-005

Each value represents the mean ± SEM of 4 or more experiments as indicated; n = 16, n = 4 and n = 4.

* Significantly different from the mean log EC_{50} of histamine ($p<0.05$)

** Significantly different from mean hillslope of histamine ($p<0.05$)

5.2.2.3 Effect of *Artemisia afra* on LTD_4-induced contraction of the tracheal muscle.

To assess the effect which *Artemisia afra* would have on leukotriene D_4-induced contractions, the tracheal muscle was contracted with a single dose (0.1ml) of LTD_4 ($6.93X10^{-9}M$) and the contracted tissue exposed to cumulative doses of the plant extract. The results of these experiments are depicted in Figure 5.15.

LTD_4 ($6.93X10^{-9}M$) produced a significant contraction (1.55 ± 0.30g) of the tracheal tissue and this was relaxed by *Artemisia afra* in a dose dependent manner. The relaxant activity of *AA* was a more gradual slower process than that seen with *ML*. Only slight relaxation was obtained with 1% and 2% *AA* while 5%, 10%, 20% and 30 % *AA* produced 0.43 ± 0.08g, 1.19 ± 0.15g, 1.93 ± 0.09g and 2.04 ± 0.00g relaxation, respectively. These gram force relaxations were relative to the maximal relaxation induced by isoprenaline ($6.67X10^{-5}M$). Samples of the recorder tracings of the leukotrieneD_4-induced contraction showing relaxation caused by *Artemisia afra* can be seen in Diagram 5.7 Appendix 7. This resultant smooth muscle relaxation further proved the bronchodilator effect of *AA*. Other researchers have also used LTD_4-induced contracted tracheal tissue to prove the existence of bronchodilator activity.[44]

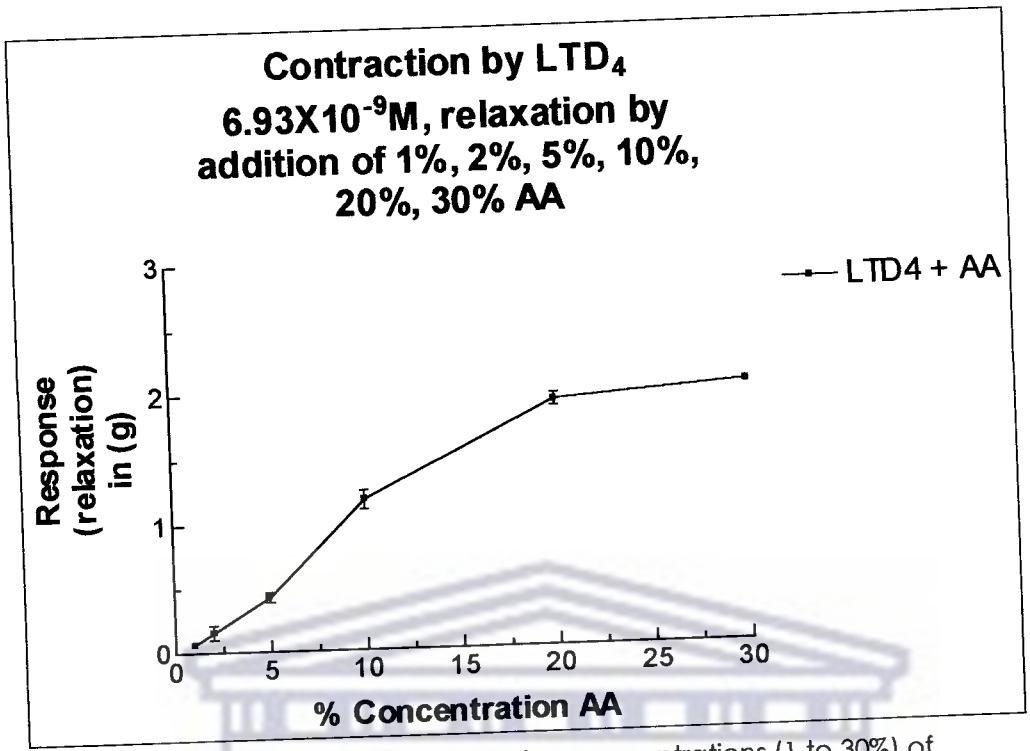


Figure 5.15 Effect of increasing concentrations (1 to 30%) of *Artemisia afra* on LTD_4 ($6.93X10^{-9}M$)-induced contractions of the tracheal smooth muscle. (n = 4)

To determine whether the mechanism may involve anti-leukotrieneD_4 effect we attempted to expose the tissue to cumulative doses of the agonist, in the absence and the presence of 2% and 20% *AA*, but were not able to obtain reproducible results.

We encountered similar problems as previously explained. Possible degradation of the product was suspected, and not tissue deterioration, as tissue viability was tested with two other agonists, methacholine and histamine and the tissue proved to be working optimally.

The overall conclusion from the LTD_4-induced studies is that *AA* has major effect in reversing LTD_4-induced contractions, and therefore has great potential as bronchodilator and anti-asthmatic. However, it is unclear whether the mechanism is in fact anti-leukotriene in action.

5.2.2.4 Summary of the effects of *Artemisia afra* on the agonists used in the studies

It was clear from all the afore-mention results that *AA* was able to reverse cholinergic, histaminergic and LTD_4-induced contractions of the tracheal muscle in a dose dependent manner. From Figure 5.16 it can be seen that the dose response relationships were fairly similar for each of the agonists (i.e. $EC_{50} \approx 10\%$ of *AA*, see Table 5.3) and that maximal or near maximal reversal of the agonist-induced contraction could be obtained with the plant concentrations used in this study. This serves to indicate the great potential which *AA* has as a bronchodilator.

The studies to determine the mechanisms involved in *Artemisia afra's* relaxant action was however less conclusive. From the present data we were able to suggest the following possible mechanisms;

Firstly, due to technical difficulties encountered in the attempt to ascertain whether the relaxant effect of *Artemisia afra* on LTD_4-induced contractions involved the LTD_4 receptor, no conclusions could be drawn with regard to leukotriene receptor mediated mechanism. Secondly, the mechanism by which *AA*, in high doses (30%), was able to fully inhibit histamine-induced contractions was most likely a non-competitive one, although a competitive histaminergic receptor mediated mechanism cannot be entirely excluded based on the present data. Thirdly, there is evidence that at least a part of the relaxant effect of *AA* may be mediated via the β2-adrenergic system. Finally, the results of the study on mechanism for the relaxant effect of *AA* on methacholine–induced contractions were the most complex. Again involvement of the cholinergic receptor, although not likely, cannot be excluded in the mechanism of action of the plant. What was, however, more significant were the stark differences in the (seemingly opposing) effects seen at low and high concentrations of the plant and whether the tissue had been sensitized with methacholine prior to exposure to the plant or not.

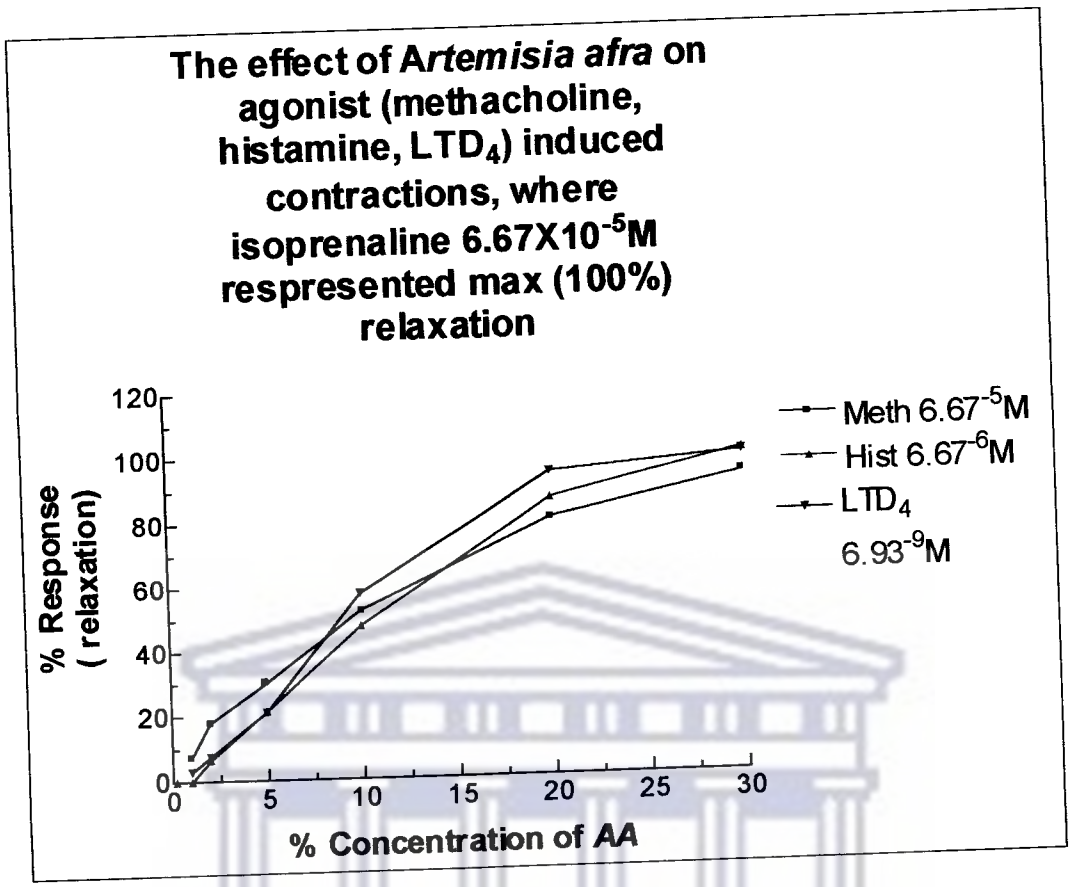


Figure 5.16 Relaxant effect of *Artemisia afra* on methacholine ($6.67X10^{-5}M$), histamine ($6.67X10^{-6}M$) and LTD_4 ($6.93X10^{-9}M$)-induced contraction. (n = 4)

This finding most clearly suggests that more than one active ingredient may be responsible for the overall relaxant activity of this plant, although it also does not exclude the involvement of a common pathway (such as inhibition of intracellular calcium movements, intracellular Ca^{2+} protein-binding, or ion channels) as was suggested for the effects of *Mentha longifolia*.

5.2.2.5 Summary - A comparison of *Mentha longifolia* and *Artemisia afra* in bronchodilator activity

Mentha longifolia was able to fully reverse the agonist-induced contractions in this study, and this was achieved with relatively low concentrations (< 2%) of *ML*. *Mentha longifolia* may also induce some of its bronchodilation (ca 20%) via β2-adrenoreceptors. *Artemisia afra* on the other hand, showed complex mixed dualism at low concentration at the muscarinic receptors. *AA* was able to reverse

cholinergic, histaminergic and LTD_4-induced contractions of the tracheal muscle in a dose dependent manner. At EC_{50} the dose-response relationships were similar for the agonists i.e. EC50 ≈ 10% *AA* for all agonists. *Artemisia afra* may also induce some of its bronchodilation via β2-adrenoreceptors, which was similar to that of *Mentha longifolia* in activity. (ca 27% *AA*, ca 20% *ML*)

Mentha longifolia exhibited potent bronchodilator activity. *Artemisia afra* also showed good bronchodilator activity, but the onset of action was more gradual than that of *Mentha longifolia.* Both plants could possibly be used for asthma treatment because of their good bronchodilator activities, mediated via so many different pathways as seen from our studies. Further isolation of their active ingredients and the pharmacological studies of these are warranted.

CHAPTER 6: CONCLUSIONS

The overall objective of this study was to investigate the claims that *Mentha longifolia* (wildekruisement) and *Artemisia afra* (wildeals) have anti-asthmatic properties. To realize this objective it was decided to determine the effects that the plant extracts may have on contractions induced by agonists (e.g. methacholine, histamine, and leukotriene D_4) known to be mediators of bronchoconstriction and also to partially investigate the mechanism which may be involved if the plants had any muscle relaxant activity. The primary hypothesis to be tested was that extracts of *Mentha longifolia* and *Artemisia afra* would have bronchial smooth muscle relaxant properties and were able to reverse methacholine and/or, histamine and/or leukotriene D_4-induced contractions by competitive antagonistic mechanisms. The following conclusions may be drawn from the results of this investigation:

1. *ML* is a potent relaxant of methacholine-, histamine- and LTD_4-induced contractions of the tracheal smooth muscle. It is however unlikely that a purely competitive receptor-mediated mechanism underpins this activity or that a beta-adrenergic mechanism fully accounts for the relaxant effect. More likely, more than one active principle in the aqueous extract is implicated and/ or a pathway (e.g. intracellular calcium ion fluxes, etc), which is common to the mechanisms of action of each of the agonists (methacholine-, histamine- and LTD_4-) used, is involved.

2. Similar to *ML*, *AA* is also a potent relaxant with possibly similar and only quantitatively different, mechanisms for its smooth muscle relaxant activity.

3. Apart from insignificant quantitative differences between the two plant extracts, *AA*, however, also had a very striking dose-dependent constrictor effect on previously methacholine-sensitized muscle tissue. This may indicate differences in the composition of active principles between the two

plants. It also suggests that more caution is required when considering the use of *AA*, than *ML*, for the treatment of asthma.

Overall, the results of this study confirm that aqueous solutions of *Mentha longifolia* and *Artemisia afra*, as used in local traditional practice, have potent bronchodilator activity that could be useful in the treatment of asthma.

Further studies on both plants however need to be pursued, especially to identify the active principles responsible for the complex activities displayed by the plants and/or to more fully elucidate the mechanism(s) of action involved. It may be particularly useful to initially focus on the flavonoids that may be present in the plants. Secondly, it may be interesting to more fully ascertain the role, if any, of intracellular Ca^{2+} regulation, the Ca^{2+}-activated potassium channels and/or β-adrenoceptors in the mechanism of action of the plant extracts. The studies on the mechanism would however best be explored after the active principles have been isolated. From such future studies prototypes for more effective anti-asthmatic treatments may arise and/or it may guide the more effective use of these plants, even in the traditional aqueous dosage forms currently used.

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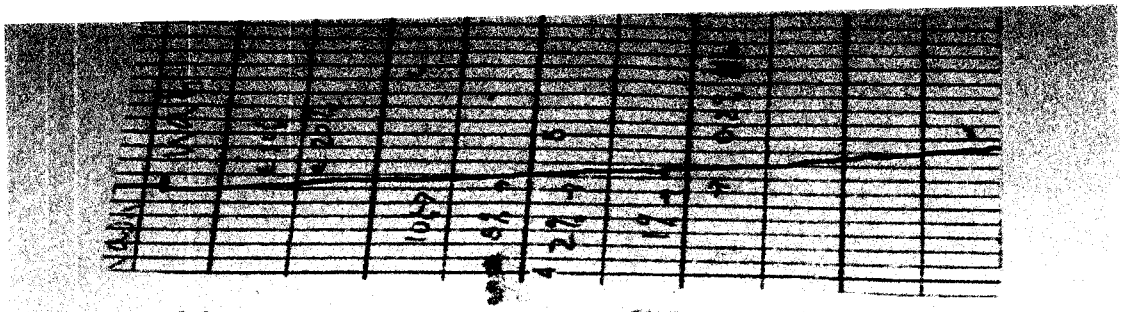
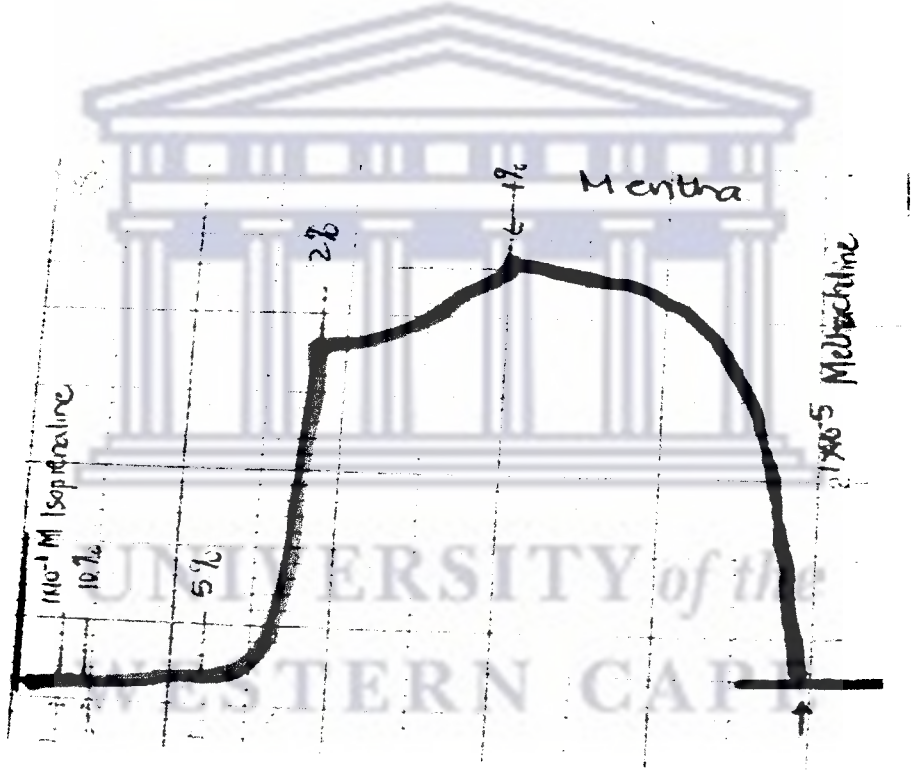
Appendix 1.



Effect of ML on trachael tissue
No response



Contraction by Methacholine 6,67 X10^{-5}M
relaxation by increasing % concentrations
of extract of ML

Diagram 5.1 Recorder tracings showing the relaxant effect of *Mentha longifolia* (0.1%, 1%, 2%, 5% and 10%) on methacholine (6.67X10^{-8}M)-induced contraction.

Appendix 2.

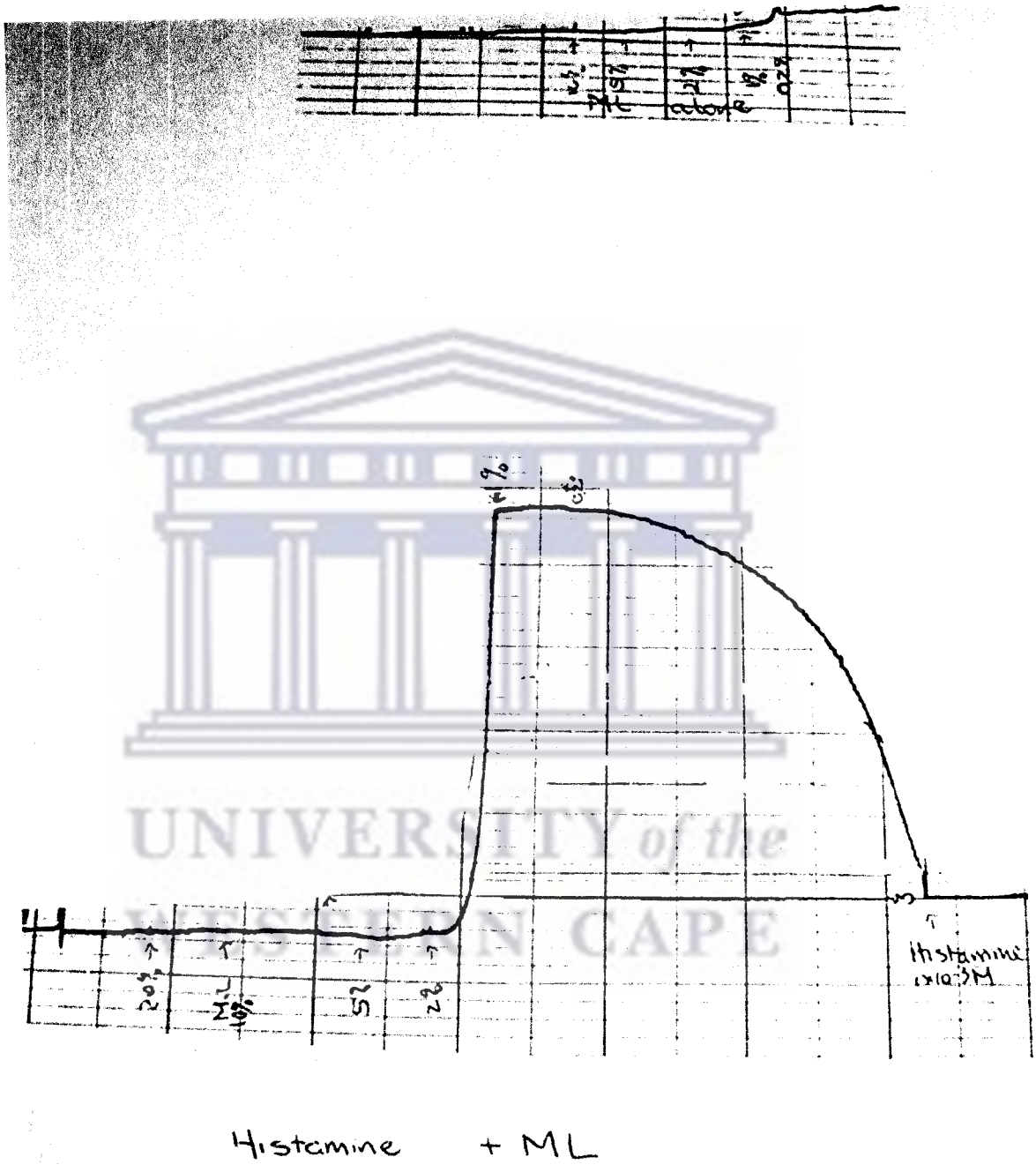


Diagram 5.2 Recorder tracings of histamine ($6.67X10^{-6}M$)-induced contraction showing *Mentha longifolia* extract in concentrations above 0.1% gave a 100% relaxation.

Appendix 3.

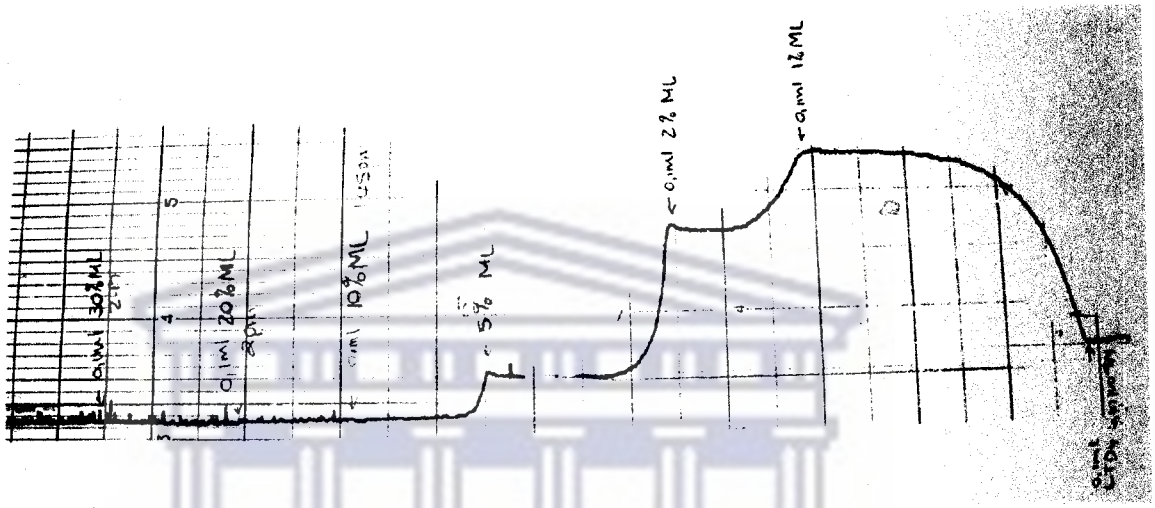


Diagram 5.3 Recorder tracings of LTD_4 ($6.93X10^{-9}M$)-induced contraction showing relaxation caused by 1%, 2%, 5%, 10%, 20%, and 30% *Mentha longifolia* extract.

Appendix 4.

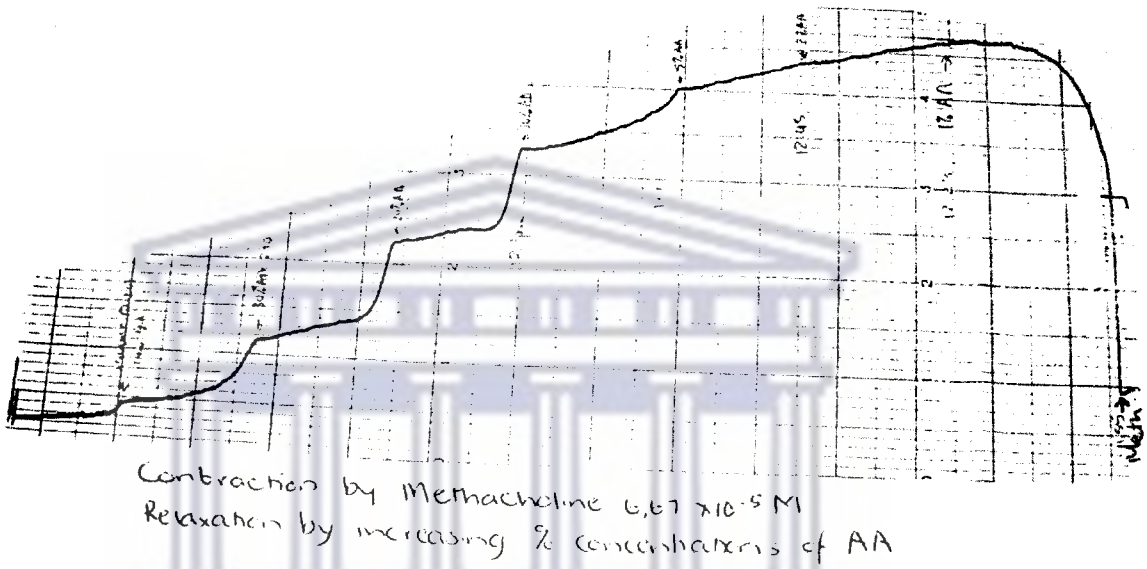


Diagram 5.4 Recorder tracings of methacholine (6.67X10^{-5}M)-induced contraction showing relaxation caused by 1%, 2%, 5%, 10%, 20%, and 30% *Artemisia afra* extract.

Appendix 5.

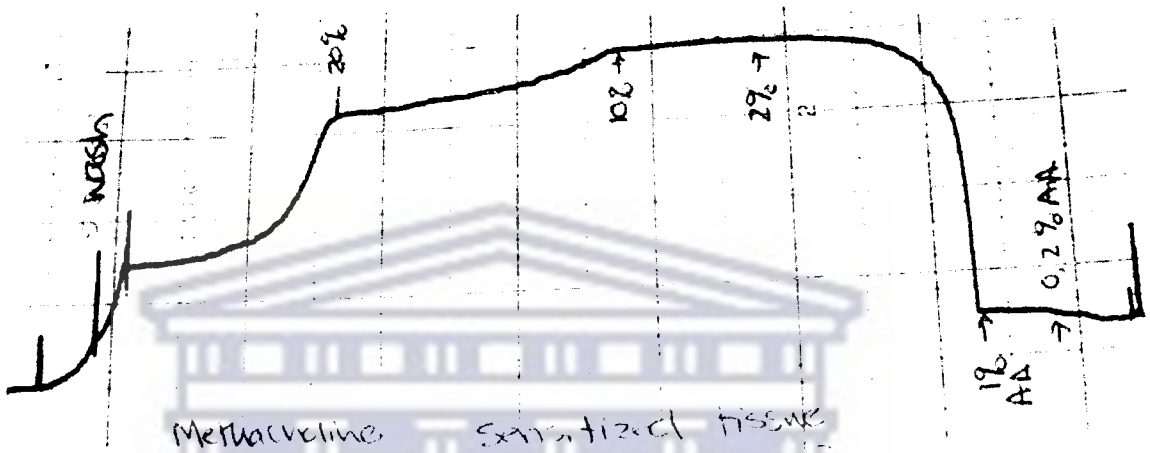


Diagram 5.5 Recorder tracings showing the contraction (1%, 2% AA) and relaxation of methacholine sensitized tissue caused by higher concentrations (10%, 20%) of *Artemisia afra* after the tissue was exposed to LDRC of methacholine and thoroughly washed.

Appendix 6.

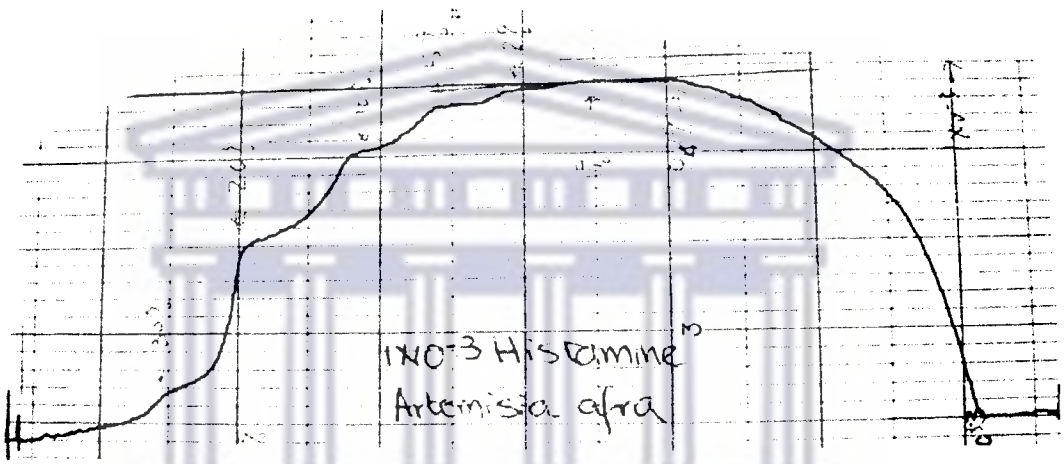


Diagram 5.6 Recorder tracings of histamine (6.67X10^{-6}M)-induced contractions showing relaxation caused by 0.1%, 0.2%, 1%, 2%, 5%, 10%, 20%, and 30% *Artemisia afra*.

Appendix 7.

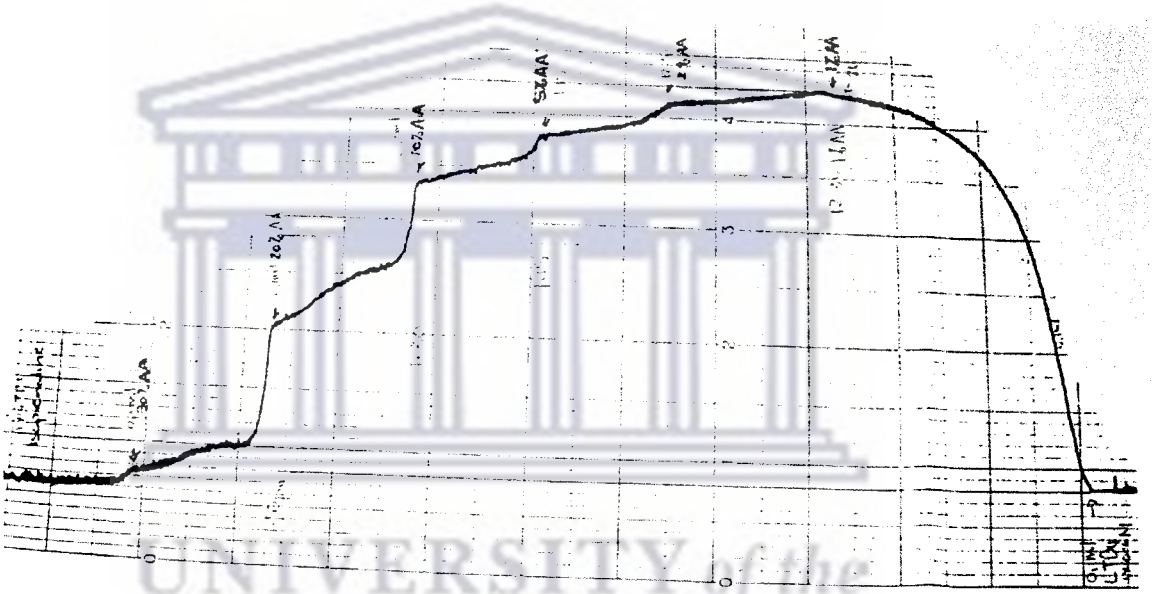


Diagram 5.7 Recorder tracings of the LTD_4 ($6.93X10^{-9}M$)-induced contraction showing relaxation caused by 1%, 2%, 5%, 10%, 20%, and 30% *Artemisia afra*.

CURRICULUM VITAE

NAME:	Lynne May Harris
SEX:	Female
DATE OF BIRTH:	08 February 1959
ADDRESS:	40 Molteno Road Oranjezicht 8001
TELEPHONE NUMBER:	021-4233583 (Home)
EMAIL	dghitch@mweb.co.za

QUALIFICATIONS:

Academic:	Matriculated from Huguenot High School Wellington (1977) Diploma in Pharmacy Cape Technikon 1983
Professional:	Pharmacist
Professional affiliations:	Registered with the South African Pharmacy Council
Papers delivered:	An Evaluation of the Bronchodilator properties of *Mentha longifolia* and *Artemisia afra* Indigenous Plant Use Forum (IPUF) Knysna, July 2002
Poster:	50th Annual Congress of Society for Medicinal Research held 8 - 12 September 2002, Barcelona, Spain Syce, J.A. and Harris, L.: Preliminary characterization of the effect of *Artemisia afra* on guinea pig airway muscle.