A LABORATORY MODEL FOR STUDYING INHALATION THERAPY IN TRADITIONAL HEALING RITES

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A research report submitted to the faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Pharmacotherapy).

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DECLARATION

I, Miles Charles Braithwaite, declare that this research report is my own work. It is being submitted in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Pharmacotherapy) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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DEDICATION

This research report is dedicated to those people in my life who have supported me in all my academic endeavours, and those loved ones who have always believed in my potential and supported me during my years of tertiary enlightenment.

ABSTRACT

The burning of selected indigenous plants and the inhalation of the smoke liberated from them has been a widely accepted and practised form of administration in traditional healing therapy dating back to as far as the Koi and San, and is a method still widely practised in South Africa today. Inhalation has various advantages as a method of administration in both allopathic and traditional practices. Not only is inhalation a highly effective mode of administration because of its direct and local effect on the lungs for the treatment of respiratory ailments, but also because of its ability to deliver drugs effectively systemically. This study elucidated the rationale behind this widely practised treatment by examining chromatographic and antimicrobial data. Five plants that are commonly administered traditionally through inhalation were chosen: *Heteropyxis natalensis, Myrothamnus flabellifolius, Artemisia afra, Pellaea calomelanos,* and *Tarchonanthus camphoratus.* An apparatus was designed and constructed and the burning process that occurs in the traditional setting was simulated with the selected plants. The induced volatile fraction (smoke) was captured for analysis. Control solvent extracts were made for each plant using conventional extraction solvents, methanol, acetone, water, and the essential oil of the aromatic plants was also investigated. Antimicrobial assays revealed that the extracts (smoke) obtained after burning had lower minimum inhibitory concentration (MIC) values than the corresponding solvent extracts in most cases. For *Klebsiella pneumoniae* all five inhalation samples were far more active than the conventional extracts. When tested against the pathogen *B. cereus, M. flabellifolius* and *P. calomelanos* inhalation samples proved to exhibit superior antimicrobial activity compared to the respective solvent extracts. *Pellaea calomelanos* inhalation extract had the lowest MIC values compared to the solvent extracts for all pathogens (*P. calomelanos* inhalation extract MIC values: 0.53; 1.00; 0.53; 0.53 mg/ml for *S. aureus, B. cereus, K. pneumoniae* and *C. neoformans* respectively). Inhalation extracts exhibited different chemical profiles from the solvent extracts of the same plant. For example, *A. afra* inhalation extract had an abundance of peaks at various retention times from 3.2 to 5.4 minutes, which were not present in the chromatograms of the acetone and methanol extracts of the same plant. These results, albeit preliminary, suggest that the chemistry and antimicrobial activity of plants are influenced by the combustion process which is often used in traditional healing rites.

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PREFACE

Medicinal plants have been used for their curative properties by traditional healers for centuries (Hutchings, 1996; van Wyk *et al*., 1997). The literature documents the use of plants for a range of conditions and, as a consequence, Western scientists have become increasingly interested in the rationale behind the use of traditional remedies. There are many ways in which medicinal plants may be administered. One of these is inhalation, which is widely practiced and is an area that warrants investigation.

Inhalation therapy often involves the burning of plant material and the smoke liberated during the process is either inhaled under covering or directed to a specific body part to aid in the healing process. Inhalation of smoke from burning plant matter is a unique method of administration and has been used traditionally to treat respiratory conditions such as asthma, bronchitis, influenza, respiratory infections and the common cold (Hutchings, 1996; van Wyk *et al*., 1997). Aerosol delivery of drugs is also well practised in allopathic medicine and has the advantage of being site specific thus enhancing the therapeutic ratio for respiratory ailments (Dipiro *et al*., 2002).

Generally, only the extracts or essential oils of the plant used in healing rituals described above have been investigated. In order to ensure a true investigation into inhalation therapy, the volatile fractions need to be captured and analysed, so as to determine whether the mode of administration, i.e. burning, has an impact on the pharmacological efficacy. It is hypothesised that burning liberates certain compounds (perhaps artefacts), which may affect the activity.

The objective of this study was to mimic the burning process in the laboratory, obtain extracts after burning, and compare these to extracts not subjected to burning. Bioassays facilitated a comparison of the antimicrobial activities of inhalation extracts compared to those of conventional solvent extracts. Chromatographic profiles of extracts before and after burning were also undertaken. Such comparisons of constituents before and after the burning process would provide valuable information on the change in chemical composition and biological activity occurring during combustion, and would give insight into the benefits of inhalation as a common mode of administration practised in traditional healing rites.

CHAPTER 1: INTRODUCTION

The delivery of drugs to the respiratory tract has become an increasingly important way of treatment in medical spheres. Inhalation of drugs has become invaluable therapeutically in the treatment of a variety of pulmonary disorders including asthma, bronchitis, pneumonia and cystic fibrosis (Suarez and Hickey, 2000).

It is common practice in traditional healing to burn plants and inhale the resultant smoke to treat respiratory complaints including coughs, colds, infections, influenza and asthma (Hutchings, 1996; van Wyk *et al*., 1997). Even though administration through inhalation dates back to the earliest records of ancient cultures and its techniques may be viewed as crude and unrefined, the advantages of this form of treatment have essentially remained the same. Several studies have shown the clinical efficacy of inhalation therapy for the treatments of lung disorders (Neville *et al.*, 1977; Clarke, 1972). The delivery of active compounds directly to the lungs provides a local treatment and effectively delivers the drug to the site of action. In this way, smaller doses are able to achieve a maximal therapeutic effect and have less risk of side-effects than those associated with larger doses. Secondly, the lower doses provide considerable cost savings and reduce systemic exposure to patients (Suarez and Hickey, 2000). In addition, it has also been noted that the lungs may provide a perfect organ system for systemic delivery of drugs to the body (Suarez and Hickey, 2000). Suarez and Hickey (2000) have cited studies that show that this mode of administration provides substantially greater bioavailability for macromolecules than any other site of entry to the human body.

Various inhalation delivery devices have been designed and patented in allopathic medicine, including metered dose inhalers, dry powder inhalers, nasal sprays and nebulizers. Novel respiratory drug delivery systems are indeed topical at the moment and the intra-nasal route, for example, has also been successfully exploited in the systemic delivery of many drugs. Miacalcic Nasal Spray 200IU produced and patented by Novartis is one such ideal delivery system to administer the hormone Calcitonin to the body through the respiratory system. This nasal spray has shown success in the reduction of new vertebral fractures in post-menopausal osteoporosis patients, which provides further evidence of the value of the respiratory system in effective drug delivery to the body.

Not only could traditional methods shed light on improved means of administering inhalation as an invaluable form of therapy, but could open up avenues for the development of antibiotic inhalant devices that could further exploit the efficacy of inhalation as a mode of treatment in the medical field.

Previous studies have investigated the antimicrobial activity of essential oil vapour obtained from aromatic plants (Inouye *et al.*, 2001). This study will for the first time investigate the antimicrobial activity and comparative chemistries of the smoke-derived fractions from ethnomedicinally selected plants.

Using two sources (Hutchings, 1996; van Wyk *et al*., 1997) a comprehensive literature review of South African medicinal plants revealed 34 plants that are burnt and the smoke inhaled (Table 1.1). This investigation focused on five such plants, namely: *Heteropyxis natalensis, Myrothamnus flabellifolius, Artemisia afra, Tarchonanthus camphoratus* and *Pellaea calomelanos* (bold text in Table 1.1).

Table 1.1: Medicinal plants traditionally administered by inhalation (Hutchings, 1996; van Wyk *et al*., 1997)

	Species / Family	**Plant part used**	**Treatment**
1	*Acalypha* sp. (Euphorbiaceae)	Twigs	Headaches.
2	*Alepidea amatymbica* (Apiaceae)	Rhizomes and roots	Colds, asthma, chest ailments, influenza, abdominal complaints. Smoke inhaled to induce mild sedation.
3	*Anemone caffra* (Ranunculaceae)	Roots	Smoke from smouldering roots inhaled to treat colds and headaches.
4	*Ansellia africana* (Orchidaceae)	Roots	Smoke from burning roots inhaled by the Zulu as an antidote to bad dreams.
5	***Artemisia afra*** (Asteracaea)	Leaves and sometimes roots	Colds, influenza, headache, earache, malaria.
6	*Becium grandiflorum* (Lamiaceae)	Leaves	Smoke inhaled to treat chest pains.
7	*Bridelia cathartica* (Euphorbiaceae)	Roots	Headaches, menorrhagia, sterility in men. Inhaled to treat epilepsy.
8	*Clausena anisata* (Rutaceae)	Wood and leaves	New-born babies held in the smoke to strengthen and clear the lungs and strengthen the heart.

	Species / Family	Plant part used	Treatment
9	*Clematis brachiata* (Ranunculaceae)	Leaves	In Botswana, smoke inhaled for blood problems associated with itchy sores.
10	*Croton gratissimus* (Euphorbiaceae)	Roots, leaves	Coughs, abdominal pains, and used as protective charms.
11	*Dombeya rotundifolia* (Sterculiaceae)	Bark and sometimes the roots and wood	Chest complaints, nausea in pregnant women. Powdered root burnt and smoke inhaled.
12	*Euphorbia ingens* (Euphorbiaceae)	The latex	Asthma, bronchitis.
13	*Gnidia anthylloides* (Thymelaeaceae)	Plants	Burning plants inhaled to treat fevers and bad dreams.
14	*Helichrysum nudifolium* (Asteraceae)	Leaves	Headaches.
15	*Helichrysum odoratissimum* (Asteraceae)	The whole plants	Inhaled as protective cleansers and to treat coughs and colds.
16	*Helichrysum* species (Asteraceae)	Leaves, twigs, sometimes the roots	Coughs, colds, fever, infections, headache, menstrual pain. Smoke from burning leaves inhaled for pain relief.
17	*Heteromorpha arborescens* (Apiaceae)	Roots and sometimes stem, bark and leaves	Headaches, abdominal pains, colic, fever, dyspnoea, asthma, coughs, illness and mental disorders. Inhaled smoke from burning plant to treat headaches.
18	***Heteropyxis natalensis*** (Myrtaceae)	Leaves, sometimes the roots	Treatment of colds.
19	*Knowltonia anemonoides* (Ranunculaceae)	Leaves	Smoke inhaled to treat headaches.
20	*Knowltonia vesicatoria* (Ranunculaceae)	Fresh leaves, sometimes the roots	Headaches, rheumatism, colds, influenza. Smoke from burning leaves inhaled for relief of headaches.
21	*Lippia javanica* (Verbenaceae)	Leaf infusions are inhaled	Coughs, colds.
22	***Myrothamnus flabellifolius*** (Myrothamnaceae)	Leaves, twigs, rarely the roots	Chest pains and asthma, respiratory ailments. Smoke from burning leaves inhaled.
23	*Ocotea bullata* (Lauraceae)	Stem bark	Ground bark inhaled to treat headaches. Also used for gastro-intestinal complaints, and as an emetic for nervous and emotional disorders.
24	*Oxygonum dregeanum*	Fruit	Smoke from burning fruit inhaled to stop

	Species / Family	Plant part used	Treatment
	(Polygonaceae)		nosebleeds.
25	***Pellaea calomelanos*** (Adiantaceae)	Leaves and rhizomes	Chest and head colds, asthma. Other fern species also inhaled.
26	*Protasparagus laricinus* (Asparagaceae)	The whole plant	Gynaecological problems. Smoke from burning plants used by the Tswana and Kwena for 'diseases of women'.
27	*Ranunculus multifidus* (Ranunculaceae)	The whole plants	Sotho inhale smoke from burning plants to treat headaches.
28	*Salvadora australis* (Salvadoraceae)	Leaves	Inhaled smoke is used to stop nosebleeds.
29	*Schotia brachypetala* (Caesalpinaceae)	Leaves	Nosebleeds.
30	*Solanum aculeatissimum* (Solanaceae)	Fruit	Smoke from burning fruit used to treat toothache.
31	*Sutera* sp. (Scrophulariaceae)	The whole plants	Smoke inhaled to treat mental patients.
32	***Tarchonanthus camphoratus*** (Asteraceae)	Leaves and twigs	Inhaled smoke from burning crushed leaves and branches used for treating asthma, headache and rheumatism.
33	*Veronia natalensis* (Asteraceae)	The whole plants	Smoke inhaled to treat headaches.
34	*Ximenia caffra* (Olacaceae)	Roots	Smoke from powdered burning roots inhaled by the Vhavenda to cease bleeding from the nose and mouth.

1.1 *Heteropyxis natalensis* (Myrtaceae)

Common names: Lavender tree (English), Laventelboom (Afrikaans), Inkunzi (Zulu)

1.1.1. Botanical description: Described as a small tree of not more than 10 metres in height (Palmer and Pitman, 1972; Coates Palgrave, 1977) consisting of a branched trunk, dense leafy branches and highly aromatic foliage. The bark has a distinctly mottled appearance and the leaves are simple, oblong and shiny green at maturity (van Wyk *et al.*, 1997). The small yellow flowers are inconspicuous and are followed by tiny dry capsules (van Wyk *et al.*, 1997). The blossoms and ground leaves have a strong odour of lavender and thus *H. natalensis* is named the "lavender tree" (Palmer and Pitman, 1972).

Figure 1.1: Leaves and buds of *H. natalensis.*

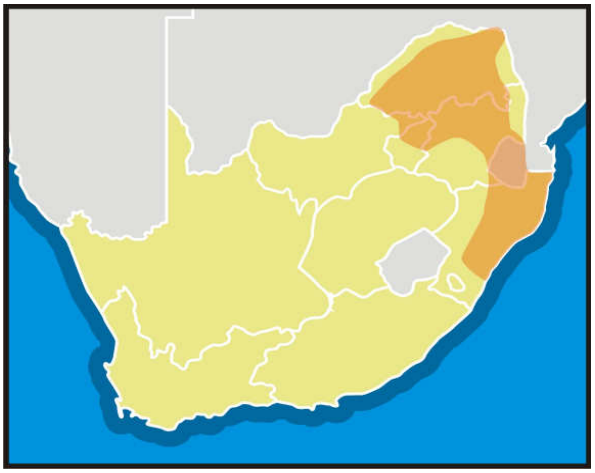

Figure 1.2: Geographical distribution of *H. natalensis* in South Africa.

1.1.2. Distribution: Common in the north-eastern parts of South Africa (van Wyk *et al*., 1997).

1.1.3. Medicinal uses and preparations: Infusions of the leaves are mainly used to treat colds, but root decoctions have also been used to treat menorrhagia (Hutchings, 1996; van Wyk *et al*., 1997). Powdered leaves form part of a traditional veterinary medicine, and a medicinal tea may be made from the leaves (Watt and Breyer-Brandwijk, 1962).

1.1.4. Previous work: The essential oil of the plant is highly aromatic and previous studies have shown antimicrobial activity against bacteria and fungi (Gundidza *et al*., 1993). The essential oil contains a wide range of monoterpenoids, mainly, 1,8-cineole, limonene, linalool, myrcene, and β-ocimene (Gundidza *et al*., 1993; Weyerstahl *et al*., 1992). According to van Wyk *et al.* (1997) the chemical basis for the reported haemostatic effect is not known.

1.2 *Myrothamnus flabellifolius* (Myrothamnaceae)

Common names: Resurrection plant (English), Bergboegoe (Afrikaans), Uvukwabafile (Zulu)

1.2.1. Botanical description: A small woody shrub of not more than 0, 4 meters tall, with tough, rigid branches (Weimarck, 1936). The leaves are toothed along their upper edges and are fan-shaped (van Wyk *et al*., 1997). The flowers are inconspicuous and are only borne in summer. A remarkable feature of the plant is its ability to transform its seemingly dead and

brown leaves to bright green when placed in water, hence its vernacular name: The resurrection bush/plant.

Figure 1.3: *M. flabellifolius* in habitat.

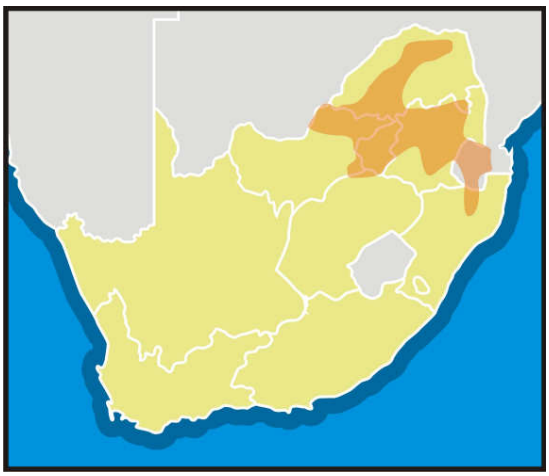

Figure 1.4: Geographical distribution of *M. flabellifolius* in South Africa.

1.2.2. Distribution: Found in the northern parts of South Africa, in exposed rocky places and dry mountain slopes (Weimarck, 1936).

1.2.3. Medicinal uses and preparation: Numerous traditional uses have been reported by utilising the leaves and twigs of the plant (Hutchings, 1996; van Wyk *et al*., 1997). *Myrothamnus flabellifolius* is usually taken orally as an infusion or a decoction, or the leaves are burnt and smoke inhaled (Hutchings, 1996). Chest pains and asthma have been treated by inhaling smoke from burning leaves (Hutchings, 1996; Watt and Breyer-Brandwijk, 1962). Infusions are taken orally to treat colds and respiratory ailments (van Wyk *et al*., 1997). Decoctions are used to treat various ailments, including menstrual pain, backache, kidney problems, and even haemorrhoids. Topical uses include the treatment of abrasions and using the dry and powdered leaves for dressing wounds and burns (van Wyk *et al*., 1997; von Koenen, 1996).

1.2.4. Previous work: According to van Wyk *et al.* (1997), camphor is the major constituent of the oil. There may also be small amounts of α-pinene and 1,8-cineole (van Wyk *et al*. 1997). Camphor and other monoterpenoids are known to have decongestant and antiseptic effects (Bruneton, 1995), and van Wyk *et al.* (1997) report that the pain-relieving activity must be related to these constituents. Later studies by Viljoen *et al.* (2002) have identified the major compounds in the essential oil to be pinocarvone, *trans*-pinocarveol, limonene, *trans*-*p*-menth-1-(7)-8-diene-2-ol, and *cis*-*p*-menth-1-(7)-8-diene-2-ol. The essential oil exhibited

antibacterial and antifungal activity against nine out of eleven pathogens tested in a previous study by Viljoen *et al.* (2002).

1.3 *Artemisia afra* (Asteracaea)

Common names: African Wormwood (English), Wildeals (Afrikaans), Umhlonyane (Zulu, Xhosa), Lengana (Sotho, Tswana)

1.3.1. Botanical description: An aromatic, erect, multi-stemmed, perennial shrub of not more than two metres in height. The small flowers are yellow and situated along the branch ends. The leaves are greyish-green in colour, finely divided, and feathery in texture (van Wyk *et al.*, 1997).

Figure 1.5: Leaves of *A. afra.*

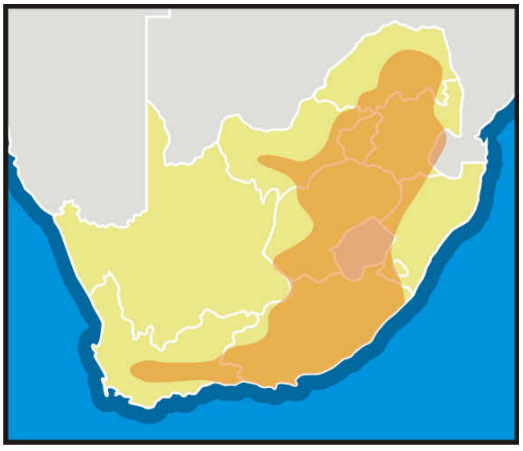

Figure 1.6: Geographical distribution of *A. afra* in South Africa.

1.3.2. Distribution: A very common species in South Africa and is naturally distributed northwards towards tropical east Africa as far as Ethiopia (van Wyk *et al.*, 1997).

1.3.3. Medicinal uses and preparation: *Artemisia afra* is one of the most widely used traditional medicines in South Africa (van Wyk *et al.*, 1997). Various illnesses are treated with this plant - mainly, colds, coughs and influenza (Rood, 1994; Watt and Breyer-Brandwijk, 1962; Dykman, 1891). Other symptoms and conditions reported to be treated with *Artemisia afra* are colic, fever, headache, earache, loss of appetite, intestinal helminthes, and malaria (Hutchings and van Staden, 1994; Watt and Breyer-Brandwijk, 1962). The leaves and sometimes the roots are used to make an infusion or a decoction that is sweetened to mask the bitter taste (Rood, 1994). It is also not uncommon for the leaves to be boiled and the fumes inhaled for therapeutic purposes (Bhat and Jacobs, 1995). The aerial parts of the plant are concentrated in essential oils and are often used as an inhalant (Bhat and Jacobs, 1995).

1.3.4. Previous work: Preliminary tests revealed analgesic, narcotic and antihistaminic activities of this plant (Hutchings, 1989). Work by Graven *et al.*, (1992) confirmed the antimicrobial and anti-oxidative properties of the volatile oil of *Artemisia afra.* Bruneton (1995) confirmed the decongestant and antibacterial effects of the oils of *Artemisia afra*. The oil contains 1,8-cineole, β-thujone, α-thujone, borneol, and camphor (Graven *et al*., 1992). Other constituents include terpenoids of the eudesmadien and germacrene types and coumarins and acetylenes, all of which have no known contribution to the biological activity of the plant. Antimicrobial activity of *A. afra* was reported in previous studies by Graven *et al.* (1992), and this was later validated by time-kill studies of the essential oil using the respiratory pathogens *Cryptococcus neoformans* and *Klebsiella pneumoniae* (Viljoen *et al.*, 2006).

1.4 *Tarchonanthus camphoratus* (Asteraceae)

Common names: Wild camphor bush (English), Vaalbos (Afrikaans), igqeba-elimhlophe, siduli-sehlathi (Zulu), Mathola (Xhosa), Mohata (Tswana)

1.4.1. Botanical description: A small tree or shrub of up to six meters tall with a greyish appearance (van Wyk *et al.*, 1997). The oblong leaves have a dark green and net-veined upper surface, and pale grey and velvety lower surfaces. The plant is adorned with small whitish flower heads and tiny woolly fruits (Palmer and Pitman, 1972).

Figure 1.7: Leaves of *T. camphoratus*.

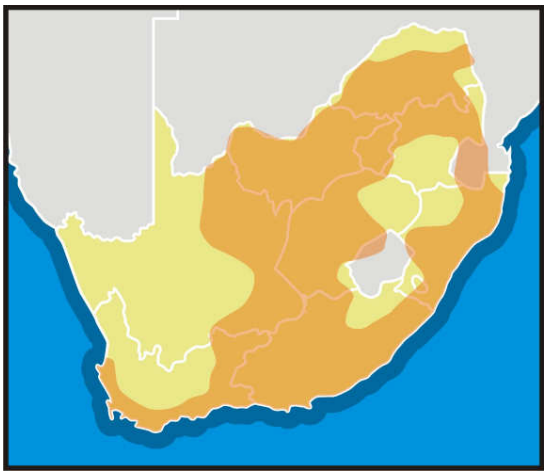

Figure 1.8: Geographical distribution of *T. camphoratus* in South Africa.

1.4.2. Distribution: Found in a wide variety of habitats in almost any part of South Africa (Coates Palgrave, 1977).

1.4.3. Medicinal uses and preparation: Fresh or dried plant leaves and branches are crushed and burnt and smoke is inhaled for asthma, rheumatism, and headaches. (Watt and Breyer-Brandwijk, 1962; Hutchings and van Staden, 1994). According to Watt and Breyer-Brandwijk (1962), the Koi and San smoke the dry leaves in the way tobacco is smoked and experience a type of narcotic effect. Van Wyk *et al.* (1997) reported the use of a hot poultice for treating bronchitis and inflammation, in addition to headaches and asthma. Leaves and twigs have also been used to prepare infusions and tinctures that are either taken orally or chewed to produce an effect. Infusions and tinctures are used traditionally to treat abdominal complaints, headaches, toothache, asthma, bronchitis and inflammation (van Wyk *et al.*, 1997; Hutchings and van Staden, 1994).

1.4.4. Previous work: The volatile oil has a characteristic camphor-like aroma and is the part of the plant said to be responsible for the reported analgesic, decongestant, diaphoretic and analgesic effects (Bruneton, 1995*)*. The plant actually contains only small amounts of camphor, with flavonoids and other constituents of the volatile oil being far more abundant (van Wyk *et al.*, 1997). Major active ingredients include α-fenchyl alcohol, 1,8-cineole, α-terpineol, and pinocembrin (van Wyk *et al.*, 1997).

1.5 *Pellaea calomelanos* (Adiantaceae)

Common names: Hard fern (English), Inkomankomo (Zulu), Iehorometso (Sotho)

1.5.1. Botanical description: Described in van Wyk *et al*. (1997) as a common and distinctive fern with an underground rootstock covered in small brown scales and about 6 mm in diameter. The tiny leaflets are blue-green in colour and triangular in shape, and join together to form large firm-textured leaves (Schelpe and Anthony, 1986; Jacobson, 1983). The leaves bear a distinct line of brown spore-producing bodies (sori) along their edges (Schelpe and Anthony, 1986).

Figure 1.9: *P. calomelanos* in habitat.

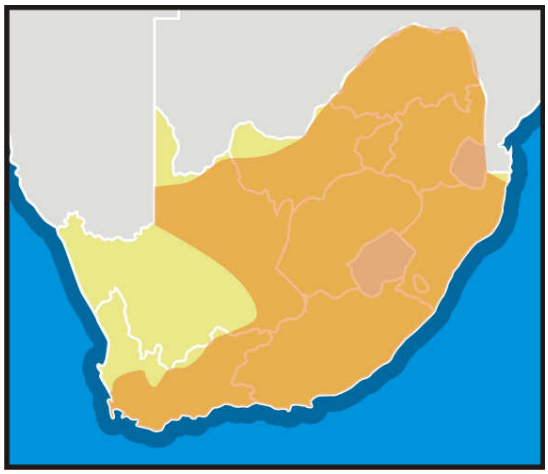

Figure 1.10: Geographical distribution of *P. calomelanos.*

1.5.2. Distribution: Is distributed widely across southern Africa, can withstand seasonal droughts and is therefore found even in extremely dry places (van Wyk *et al*., 1997; Jacobson, 1983).

1.5.3. Medicinal uses and preparations: The leaves are burnt and the smoke is inhaled to treat chest colds, head colds, and asthma (Watt and Breyer-Brandwijk, 1962; van Wyk *et al*., 1997; Hutchings and van Staden, 1994). An early Cape remedy was a tea made from this species that was used to treat chest complaints and colds (van Wyk *et al*., 1997). Decoctions have also been prepared from the rhizomes and applied externally to heal boils and abscesses and are taken internally to remedy intestinal parasites (van Wyk *et al*., 1997).

1.5.4. Previous work: Very little has been reported on the chemistry of *Pellaea* species (Dictionary of Natural Products on CD-ROM, release 4:2, Chapman & Hall, London, 1996). Many ferns do, however, contain triterpenoids of the hopane type such as adiantone, as well as a number of flavonoids. No relationship has been found between the chemistry of any fern leaves, including those of *Pellaea calomelanos*, and their therapeutic uses, but van Wyk *et al*. (1997) postulate that triterpenoid saponins play a role. It is worth noting that a few saponin-containing medications have known potential as anti-inflammatory and analgesic agents (Bruneton, 1995).

Study objectives:

- To simulate the burning process employed traditionally during inhalation therapy with selected plant species and to capture the volatile (smoke) fraction for analysis and biological testing;
- To determine and compare the antimicrobial activity of the inhalation extracts and conventionally prepared solvent extracts;
- To compare the chromatographic profiles of the solvent extracts to the smoke fraction; and
- To provide insight into inhalation therapy and emphasise the benefits of inhalation as a common mode of administration in traditional healing.

CHAPTER 2: MATERIALS AND METHODS

2.1. Preparation of samples for combustion experiment

Fresh plant material was collected from local sites and allowed to air dry. The selected plant parts, i.e. roots, stems, leaves, rhizomes and twigs, were prepared by cutting, grinding, and crushing the plant material. A standard dry weight of 20g of plant material was used in each of the combustion experiments.

Table 2.1: The origin and voucher details for plants used in this study.

Species/ Family	Locality	Voucher	Plant part used
Heteropyxis natalensis (Myrtaceae)	Johannesburg Botanical Gardens	SVV 954	Leaves
Artemisia afra (Asteracaea)	Klipriviersberg	SVV 935	Leaves
Myrothamnus flabellifolius (Myrothamnaceae)	Klipriviersberg	SVV 934	Leaves and twigs
Pellaea calomelanos (Adiantaceae)	Walter Sisulu Botanical Garden	AV 1280	Aerial parts
Tarchonanthus camphoratus (Asteraceae)	Walter Sisulu Botanical Garden	SVV 961	Leaves

The combustion apparatus depicted below (Figure 2.1) consisted of a modified pressure-cooker with an entry pipe and an exit pipe, a heating plate (maintained at 150°C), a condenser surrounding the exit pipe, a water bath, a round-bottomed flask, and a cryostat. Plant material (20g) was carefully placed in a foil dish and inserted into the pot and allowed to burn to produce smoke. A pump attached to the entry pipe maintained a positive pressure which directed the smoke through the tubing exit pipe and into a round-bottomed flask containing solvent. A mixture of a polar and a non-polar solvent was used in order to capture polar and non-polar compounds from the smoke fraction. The solvent was a 1:1 mixture of hexane and methanol (250ml).

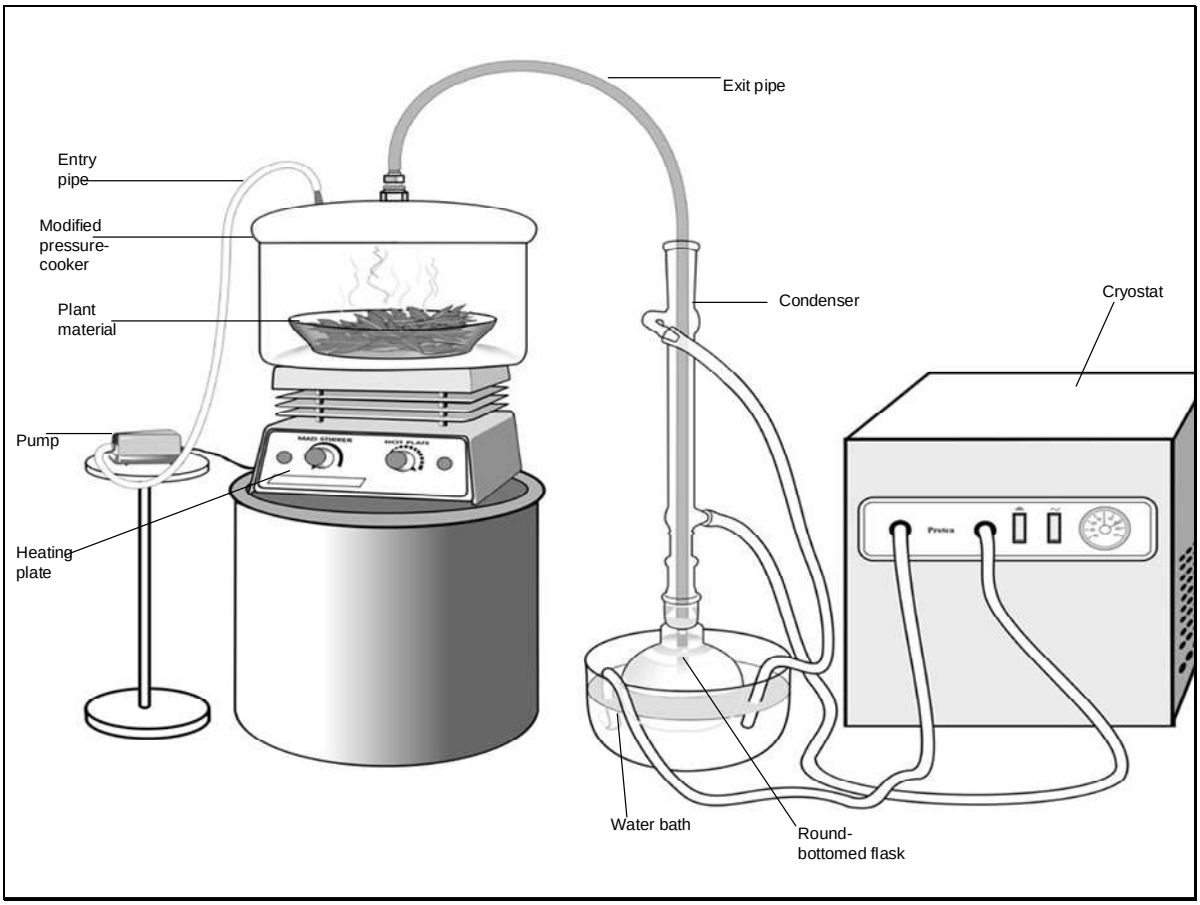

Figure 2.1: The combustion apparatus used in this study to mimic the inhalation process used in a traditional setting.

The plant material was burnt for a standard time of 30 minutes to simulate the average time a patient would be exposed to the therapy by a traditional healer. The smoke bubbled into the hexane-methanol mixture in the bottom of the flask. The round-bottomed flask was placed in a water bath containing water and polyethylene-glycol (anti-freeze). The water-polyethylene-glycol mixture was pumped through the water bath in which the round-bottomed flask was placed and also through the jacket of the condenser. The water bath-condenser-cryostat circuit cooled down the round-bottomed flask and minimised evaporation of the smoke fraction. The cryostat effectively maintained the water bath temperature at -8 °C.

Figure 2.2: Smoke captured in hexane-methanol.

Figure 2.3: Concentrating the smoke fraction.

After 30 minutes the hexane-methanol smoke fraction was concentrated using a rotavap. The inhalation samples/extracts were then removed from the rotavap and left to dry in a fume hood in order to produce dry extracts for analysis and microbiological testing.

2.2. Preparation of solvent (methanol and acetone) and aqueous extracts

Dried plant material (2.0g) was finely cut, ground, and extracted with 50ml of each solvent (water, methanol, and acetone). The plant extracts were macerated, extracted overnight, then filtered and allowed to dry in a fume hood and oven (at 40°C). Water extracts were freeze dried to accelerate the drying process. The residues were reconstituted with their respective solvents by adding 1ml of solvent. Essential oil samples of all of the selected plants with the exception of *Pellaea calomelanos* (which is not aromatic) were made available from previous studies.

2.3. Antimicrobial assays / minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration that prevents visible growth of an organism after incubation in a specified growth medium and was used to quantitatively measure the *in vitro* antimicrobial activity of test extracts before and after burning (Dipiro *et al*., 2002; van Vuuren, 2003). In this study, the MIC was determined by using the *p*-iodonitrotetrazolium violet (INT) microplate bioassay technique (Eloff, 1998a).

Antimicrobial assays were performed on the 24 samples, with the plant samples being tested against four micro-organisms. The prepared plant extracts were tested for activity against *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Klebsiella pneumoniae* (ATCC 9633), and *Cryptococcus neoformans* (ATCC 90112). MICs were performed at least in duplicate and in some instances, triplicate where MIC values were not congruent between repetitions.

The MIC microtitre plate method was employed, containing a standard inoculum of bacteria (1-5 x 10^5 colony forming units), liquid growth media and doubling serial dilutions of the antimicrobial substance of interest (inhalation extracts and laboratory extracts). The microtitre plates were then prepared aseptically by placing 100µl sterile water in all wells. 100µl of each sample/extract to be tested was placed in Row A, so that wells A1-AH contained 100µl of each sample. Serial dilutions were then carried out from wells A through to H. For example, 100µl of contents from well A1 was withdrawn (after its contents had been thoroughly mixed) and transferred to well B1. This procedure was then repeated from well C1 onwards to well H1, diluting by 100µl each time. The 100µl withdrawn from well H1 was discarded. Then 100µl of the specific cultured respiratory pathogen was added to all the wells. The plates were then incubated for 24 hours (bacterial pathogens) and for 48 hours (*Cryptococcus neoformans*) at a temperature of 37°C.

After incubation of the plates, 40µl of 0.02 mg/ml *p*-iodonitrotetrazolium violet (INT) solution was prepared and added to each well. Plates were then viewed after six hours and yeast plates were viewed after 24 hours. INT was used to indicate biological activity and showed a colour change in relation to the concentration of microbial growth. The first colourless well in a row of decreasing concentrations of a given extract is the MIC of that specific sample (Eloff, 1998a).

Positive and negative controls were included in the assay. The positive controls consisted of wells containing commercially used antibiotics, either ciprofloxacin (for bacteria) or amphotericin B (for the yeast *C. neoformans*). The positive controls gave an indication of the sensitivity of the micro-organisms tested. Negative controls consisted of broths that were incubated under the same conditions as the plates so that sterility of the nutrient media was ensured. Blank controls (using solvent) were also included to ascertain whether the diluents had any inherent activity. Blank controls were carried out for methanol, acetone and water.

Figure 2.4: Aseptic preparation of MIC
plates to record the antimicrobial activity of the extracts.

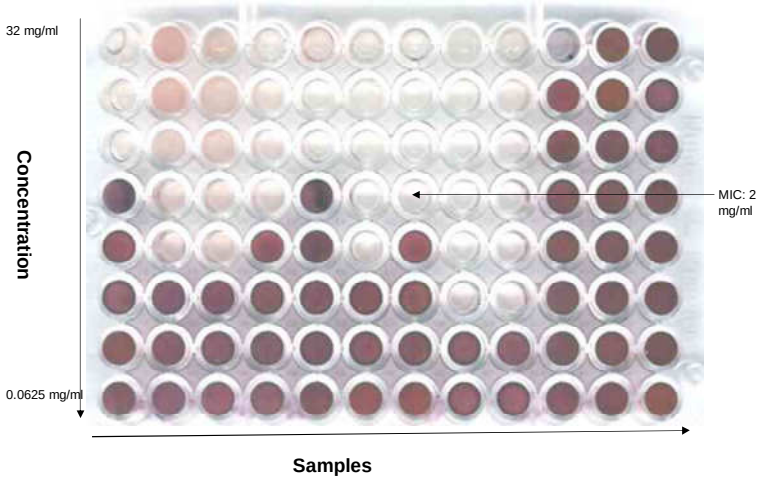

Figure 2.5: Example of a MIC plate showing the wells after INT was added (the red to pink colour indicates microbial growth and the colourless wells indicate no microbial growth due to antimicrobial activity of the samples).

2.4. Thin layer chromatography (TLC)

The chromatographic profiles of the extracts were initially examined by thin layer chromatography. The five samples obtained from the combustion experiment were spotted next to their respective methanol extracts (Figure 3.1).

The samples were dissolved in 1ml methanol to yield a concentration of 100 mg/ml of which 5µl was applied to a silica gel (Merck) TLC plate using a calibrated micro-capillary tube. The plates were developed in two mobile phases: 1. toluene:ethyl acetate (93:7), and 2. toluene:dioxane:acetic acid (90:25:10) for 10-20 minutes. The plates were visualised under UV light (365 and 254nm). The plates were sprayed with vanillin-sulphuric acid (1% ethanolic vanillin, 10% ethanolic sulphuric acid) spray reagent, and placed in an oven at 110°C for five minutes.

2.5. High performance liquid chromatography (HPLC)

The application of HPLC in pharmacognosy is well established and can be used for the separation and identification of alkaloids, glycosides, saponins, flavanoids and secondary metabolites (Skoog and Leary, 1992). HPLC is also widely used for the separation of complex organic and inorganic molecules such as antibiotics, steroids, analgesics, diuretics and anti-inflammatory compounds (Skoog and Leary, 1992; Hannah, 2001).

The methanol and acetone extracts and the samples obtained from the combustion experiment were analysed by HPLC-UV-MS (because of insufficient samples the water extract was not analysed by HPLC). Extracts were weighed and transferred to eppendorffs to which calculated concentrations of methanol or acetone was added to yield a final concentration of 50 mg/ml.

A Waters 2690 HPLC system (equipped with a Phenomenon Aqua C18 column, 250mm x 2.1mm column) equipped with a 996 photodiode array detector (PDA) and a thermabeam mass selective detector (TMD) was used to analyse the samples. The thermabeam generates classical electron impact spectra, which can be compared to commercial MS spectra libraries like NIST and WILEY. The injection of 10µl was done under the following conditions: flow rate was 0.2ml/min, gas flow in the nebulizer was 301/h, the nebulizer temperature was 80°C, the expansion region was 90°C, and the source of temperature was 225°C. The mobile phase started off with 10% acetonitrile, 90% water containing 100mM formic acid. The solvent ratio was changed through a linear gradient to 90% acetonitrile, 10% water and formic acid at 40 minutes. The ratio was maintained for 10 minutes, and then the solvent ratio was changed back to the initial starting conditions. The data was analysed using Empower software.

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Chemical comparison of extracts

3.1.1. Thin layer chromatography (TLC)

TLC was used to compare the chromatographic profiles of the various extracts. Figure 3.1 (A and B) shows the TLC profiles of all five inhalation extracts together with the respective methanol extracts developed in two different mobile phases. It was observed that the profiles of the inhalation sample compared to the methanol extract of the same plant were distinctively different. Track 1.2 represents the methanol extract of *H. natalensis* and shows a spot at Rf 0.2 which is absent in the sample produced by the combustion experiment (track 1.1). Similarly, plate B shows distinct differences between the two samples of *Pellaea calomelanos* (track 4.1 and 4.2).

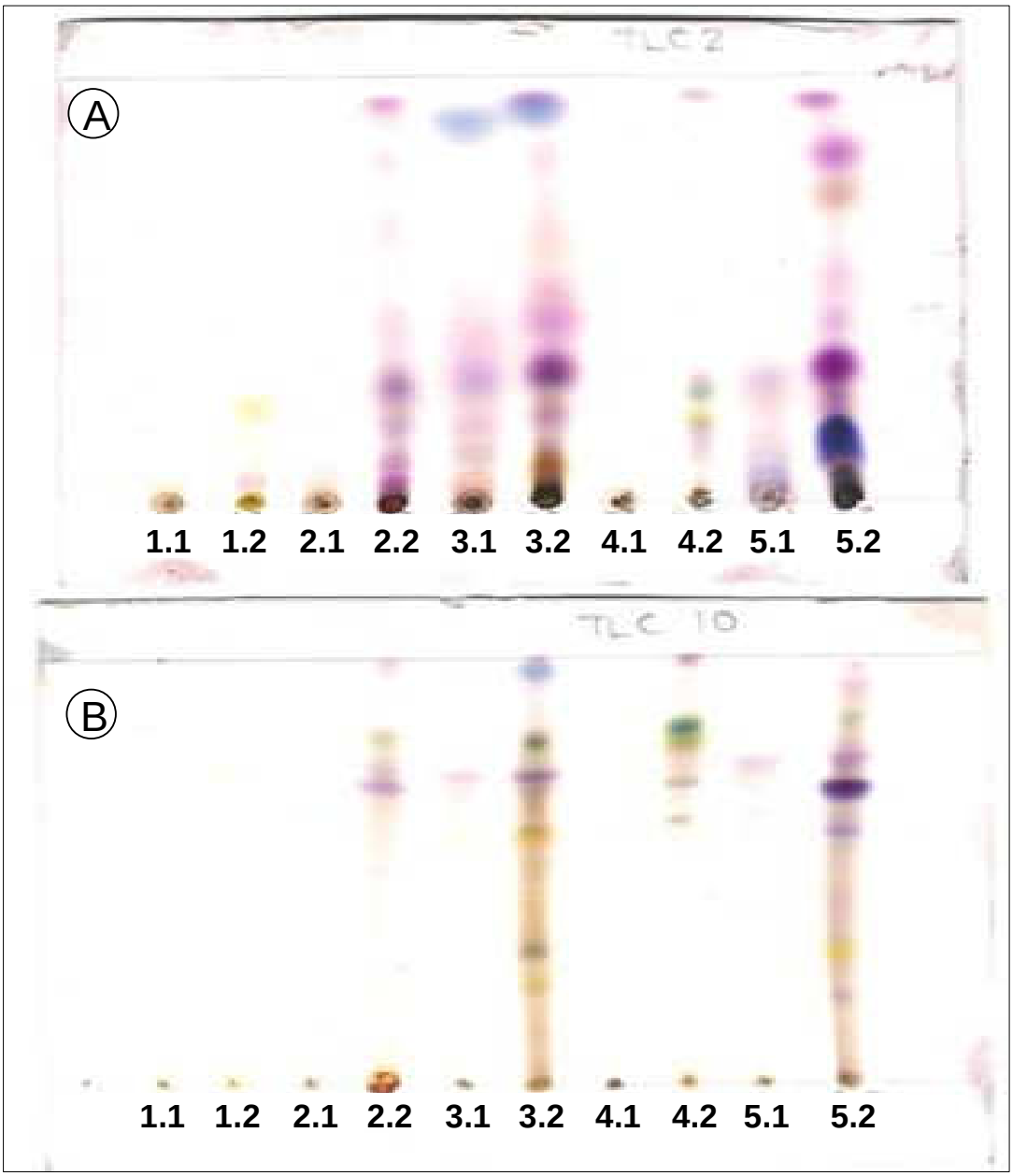

Figure 3.1: Thin layer chromatography performed on the inhalation and methanol extracts developed in two mobile phases: A = toluene:ethyl acetate (93:7), B = toluene:dioxane:acetic acid (90:25:10)

Tracks = 1.1. *H. natalensis* (inhalation), 1.2. *H. natalensis* (MeOH), 2.1 *M. flabellifolius* (inhalation), 2.2 *M. flabellifolius* (MeOH), 3.1 *A. afra* (inhalation), 3.2 *A. afra* (MeOH), 4.1 *P. calomelanos* (inhalation), 4.2 *P. calomelanos* (MeOH), 5.1 *T. camphoratus* (inhalation), 5.2 *T. camphoratus* (MeOH).

3.1.2. High performance liquid chromatography (HPLC)

Figures 3.2-3.27 represent the HPLC chromatograms of the methanol, acetone, and inhalation extracts. The first set of HPLC analyses (HPLC-UV) detected constituents in the extracts that have chromophores (absorb light within the UV-spectrum), and the second set of HPLC analyses (HPLC-MS) was coupled to mass spectroscopy and detected constituents in the extracts that may not have chromophores (do not absorb light in the UV-spectrum).

3.1.2.1. *Heteropyxis natalensis*

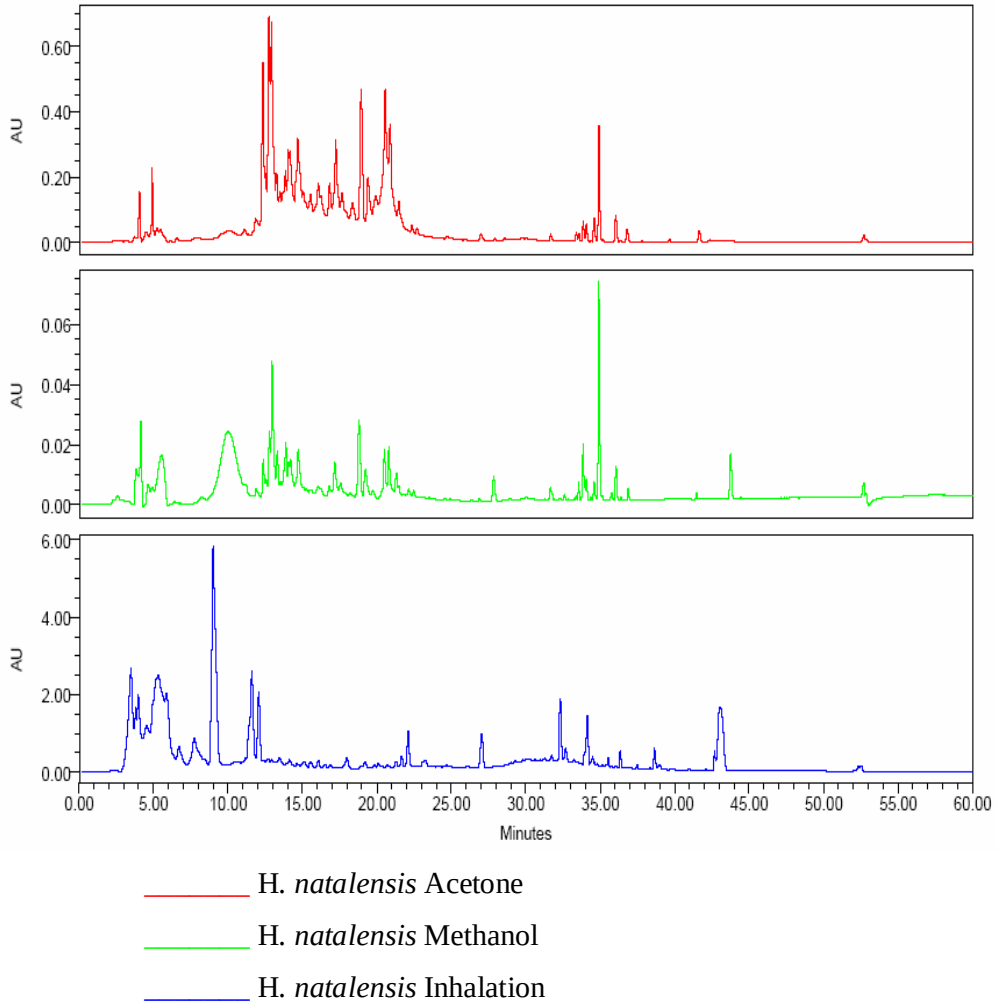

Figure 3.2: HPLC-UV chromatograms of the acetone, methanol and inhalation extracts of *Heteropyxis natalensis.*

The three HPLC-UV profiles for this plant were found to be quite similar, with the same compounds observed at retention times 3.53 minutes, 12.37 minutes, 34.92 minutes, and 36.33 minutes. The same compound is also observed in the methanol and acetone profiles at

retention time 12.96 minutes. In contrast, it is interesting to observe how few compounds are detected for the inhalation sample as opposed to the methanol and acetone samples over the retention times 13-22 minutes (Table 3.1). It is a possibility that the burning process destroyed certain constituents.

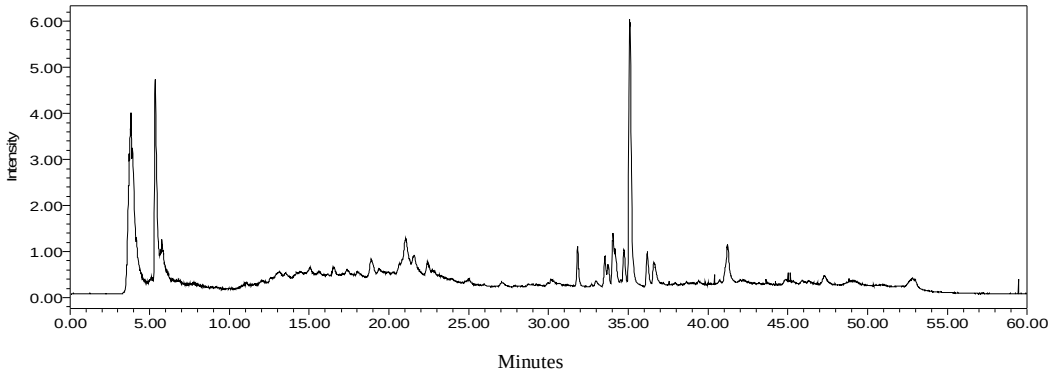

Figure 3.3: HPLC-MS chromatogram of *H. natalensis* acetone extract.

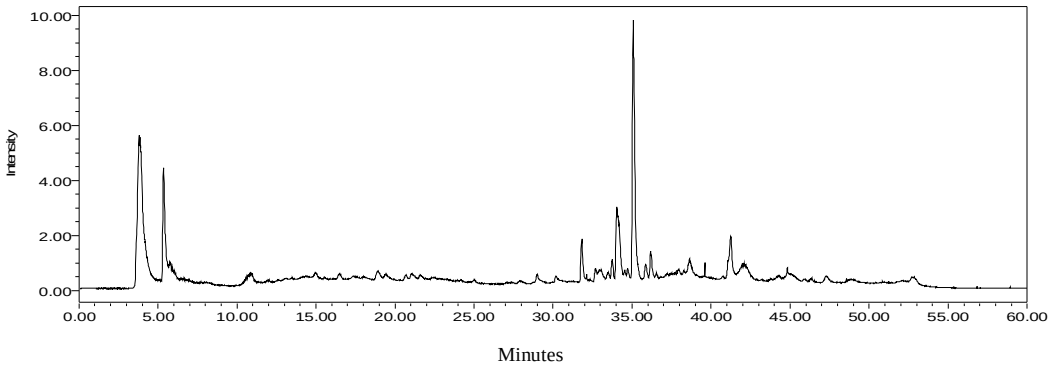

Figure 3.4: HPLC-MS chromatogram of *H. natalensis* methanol extract.

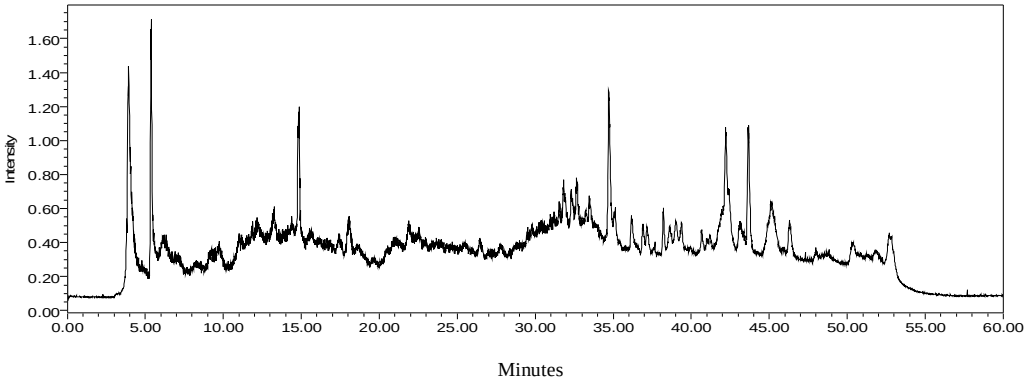

Figure 3.5: HPLC-MS chromatogram of *H. natalensis* inhalation extract.

The *H. natalensis* inhalation extract above (Figure 3.5) showed a dramatically different HPLC-MS chromatogram from the acetone and methanol chromatograms of the same plant (Figure 3.3 and Figure 3.4 respectively). There is a characteristic peak at retention time 14.82 minutes and at 45.15 minutes for the inhalation sample. It was also interesting to note that there were obvious similarities between the acetone and the methanol profiles above

(common compounds occur at retention times 3.91, 4.04, 5.32, 31.91, and 35.02 minutes), showing that the two solvents may extract with similar efficiency. The inhalation sample contained certain compounds also found in the conventional solvent extracts (compound at retention time 34.69 minutes is common to all three HPLC-MS profiles above).

3.1.2.2. *Myrothamnus flabellifolius*

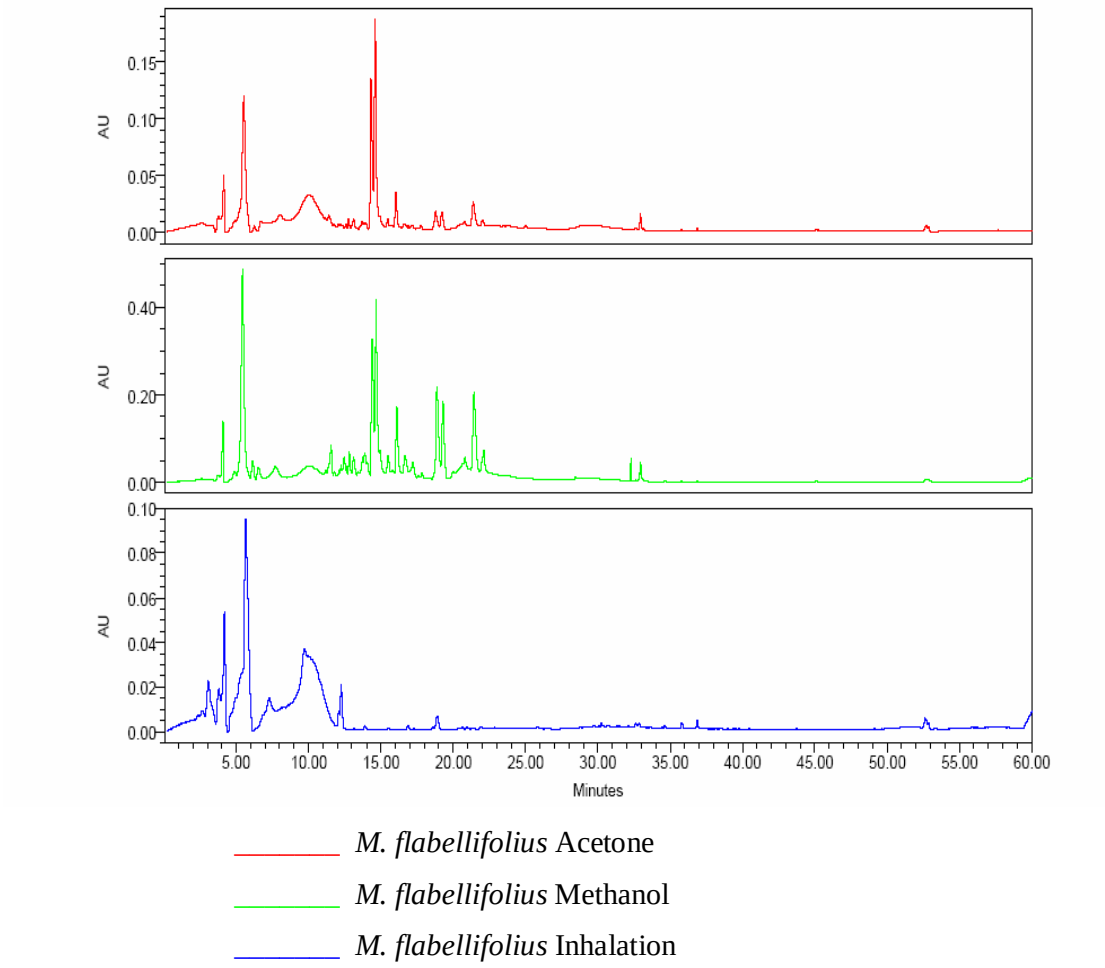

Figure 3.6: HPLC-UV chromatograms of the acetone, methanol and inhalation extracts of *Myrothamnus flabellifolius.*

The acetone and methanol extracts have similar HPLC-UV profiles, but the inhalation extract differs. The same large peak is seen in the methanol and acetone profiles at retention time 14.40 minutes and another compound is also common at retention time 21.44 minutes (Table 3.1). The grouping of characteristic 'polar' peaks early on in the inhalation sample chromatogram and again the lack of substantial peaks from retention time 13 minutes onwards was worth noting. The same two compounds are seen in all three profiles at retention times 4.67 minutes and 5.59 minutes (confirmed by UV data). However, the inhalation sample has peaks at 3.06 minutes and 9.72

minutes that are not present in the methanol and acetone extracts of the same plant (Table 3.1).

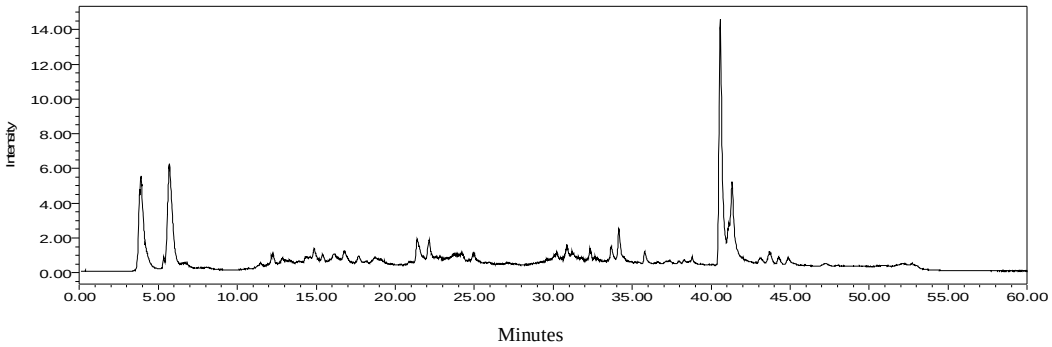

Figure 3.7: HPLC-MS chromatogram of *M. flabellifolius* acetone extract.

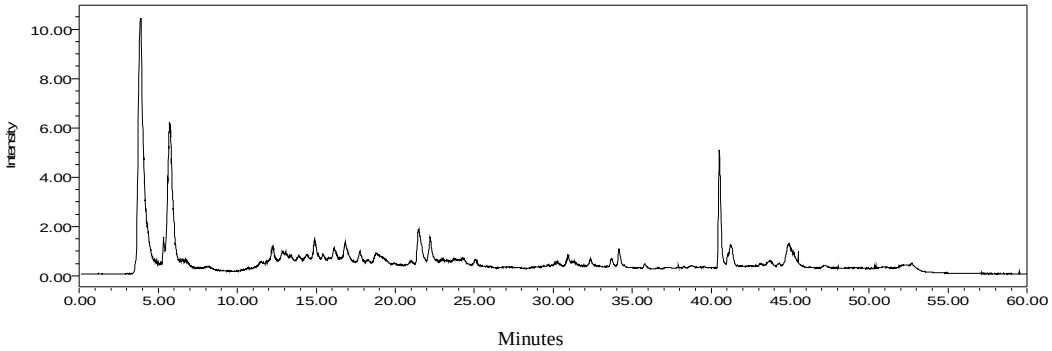

Figure3.8: HPLC-MS chromatogram of *M. flabellifolius* methanol extract.

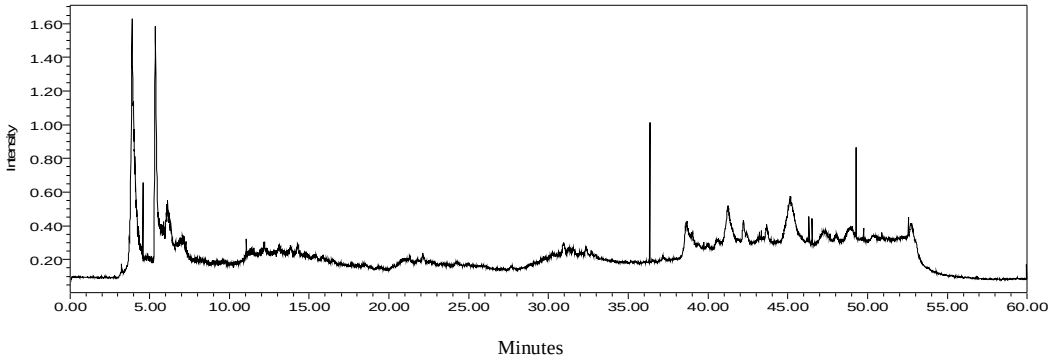

Figure 3.9: HPLC-MS chromatogram of *M. flabellifolius* inhalation extract.

The comparison of *M. flabellifolius* HPLC-MS chromatograms above again revealed comparable chromatograms between the conventional methanol and acetone extracts. The chromatogram of the inhalation extract is distinctively different from the chromatograms of the acetone and methanol extracts. Evident is the abundance of late eluting peaks in the inhalation chromatogram, which is absent in the other two analysed samples between retention times 46.05-52.43 minutes. In summary, the *M. flabellifolius* inhalation chromatogram showed characteristic UV absorbance peaks early on in the UV profile (Figure 3.6) and characteristic compounds eluting late in the HPLC-MS profile (Figure 3.9).

3.1.2.3. *Artemisia afra*

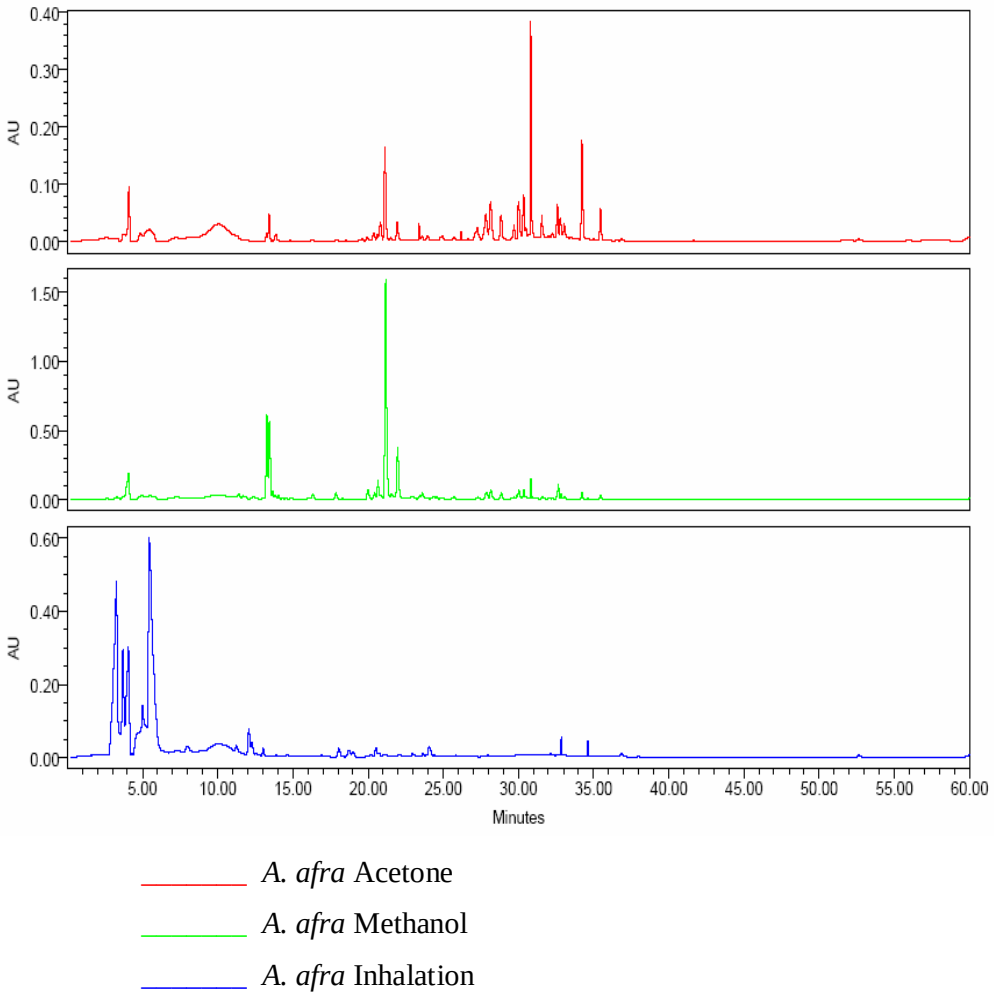

Figure 3.10: HPLC-UV chromatograms of the acetone, methanol and inhalation extracts of *Artemisia afra*.

Evident above is the presence of the same peak in all three extracts of *A. afra* at retention time 13.41 minutes (Table 3.1). Another compound is common to all three profiles at retention time 32.86 minutes (Table 3.1). It is also important to point out certain similarities in the profiles of the methanol and acetone profiles with the same peaks observed at retention times 21.13 minutes, 23.39 minutes, 28.18 minutes and 28.87 minutes (Table 3.1). There is also a marked dissimilarity between the acetone and the methanol extracts, with an abundance of peaks occurring late on in the acetone profile between retention times 25 minutes to 35 minutes (Table 3.1).

The HPLC-UV chromatogram of the inhalation sample, above, shows a few major peaks early on in the profile (at 3-6 minutes) that are not present in the acetone and methanol extracts (Table 3.1). Worth noting is the major peak in the inhalation sample at retention time 5.46 minutes with an integration area of 34.18%. This compound is absent in the methanol and acetone samples (Table 3.1).

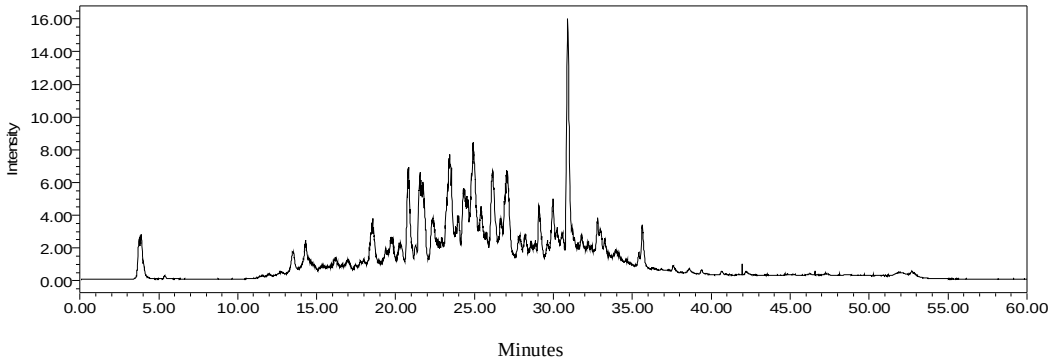

Figure 3.11: HPLC-MS chromatogram of *A. afra* acetone extract.

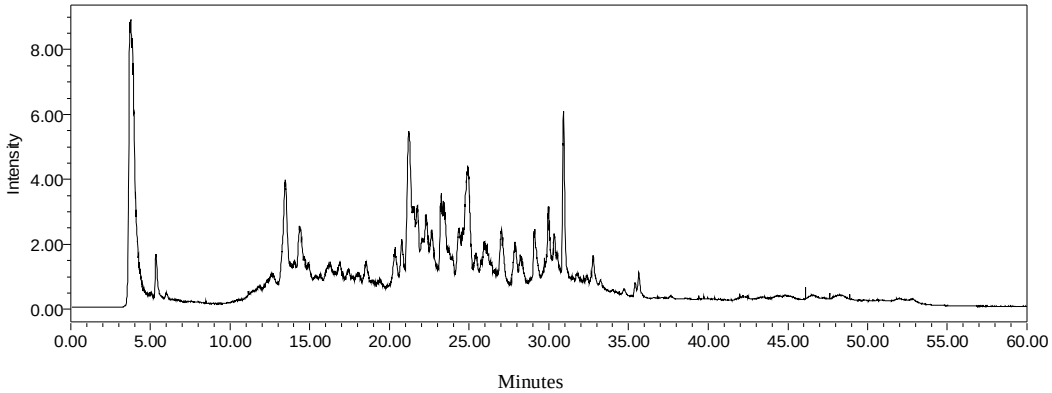

Figure 3.12: HPLC-MS chromatogram of *A. afra* methanol extract.

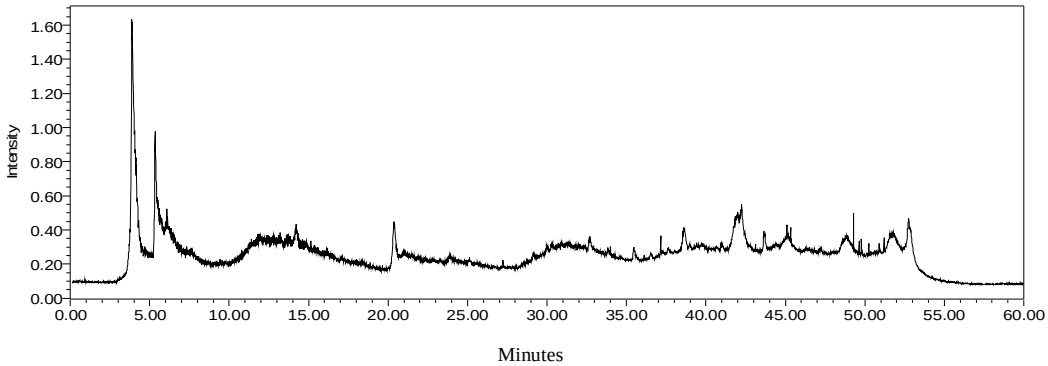

Figure 3.13: HPLC-MS chromatogram of *A. afra* inhalation extract.

In the *A. afra* HPLC-MS chromatograms the compounds present in the acetone and methanol samples are not as prominent in the inhalation extract, perhaps because of destruction during

combustion. There were distinct commonalities between the acetone and methanol extracts of *A. afra*. There were also a number of distinctive peaks late on in the inhalation chromatogram that was unique to the *A. afra* inhalation sample at retention times 35.98 to 52.77 minutes (Figure 3.13). The late peaks seen in Figure 3.13 and the distinctive early peaks seen in Figure 3.10 for the inhalation extract may elude to the differences observed in the microbiological results.

3.1.2.4. *Pellaea calomelanos*

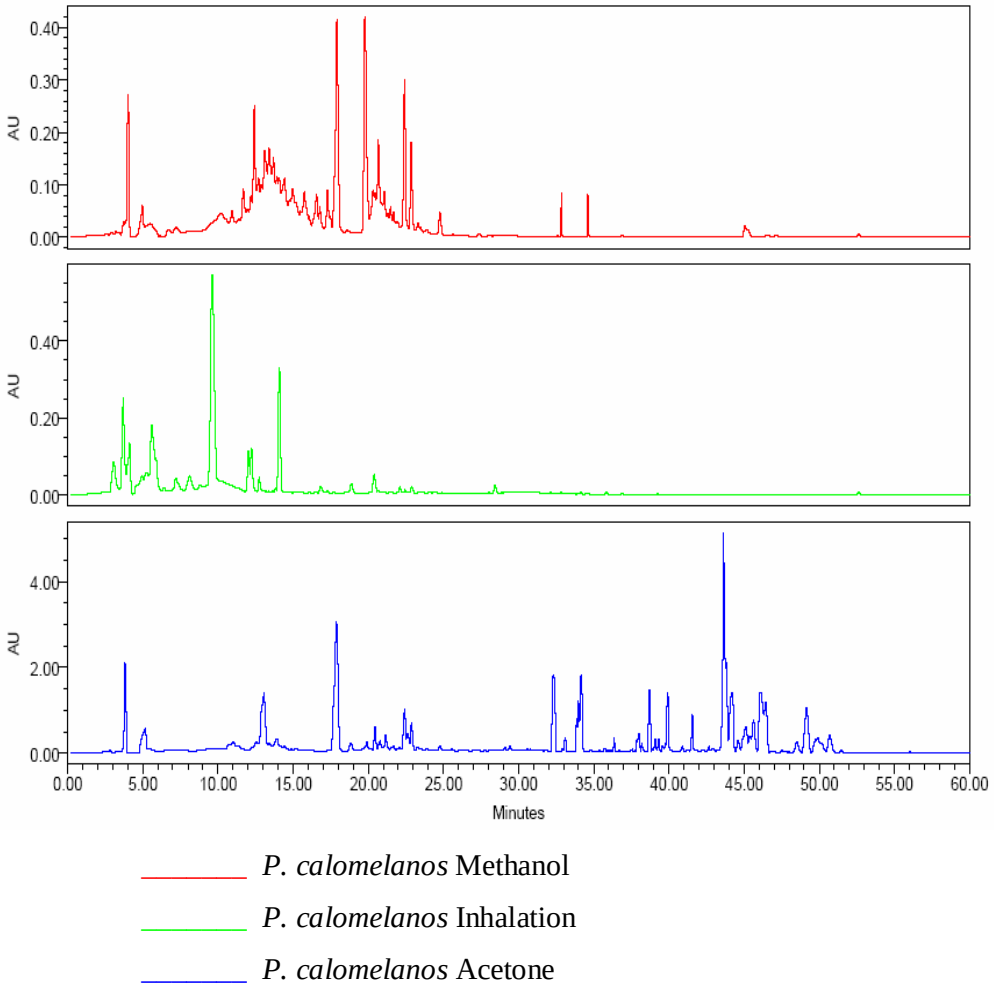

Figure 3.14: HPLC-UV chromatograms of the acetone, methanol and inhalation extracts of *Pellaea calomelanos*.

It is clear that the three profiles are very different. A major peak is evident in the inhalation extract at 9.62 minutes that is not seen in the methanol and acetone extracts (Table 3.1). This is a major peak with an integration area of 31.86%. This chemical difference occurs alongside

marked antimicrobial superiority of the *P. calomelanos* inhalation extract when tested against *S. aureus* and *B. cereus* (Table 3.2). It should also be mentioned at this point that it was the *P. calomelanos* inhalation extract that demonstrated greater antimicrobial activity than all solvent extracts and against all pathogens tested (Table 3.2). Another unique peak in the inhalation chromatogram occurs at retention time 14.08 minutes. Noticeable too is the cluster of late eluting peaks in the acetone extract (retention times: 39-51 minutes) which are absent in both the inhalation and the methanol profiles.

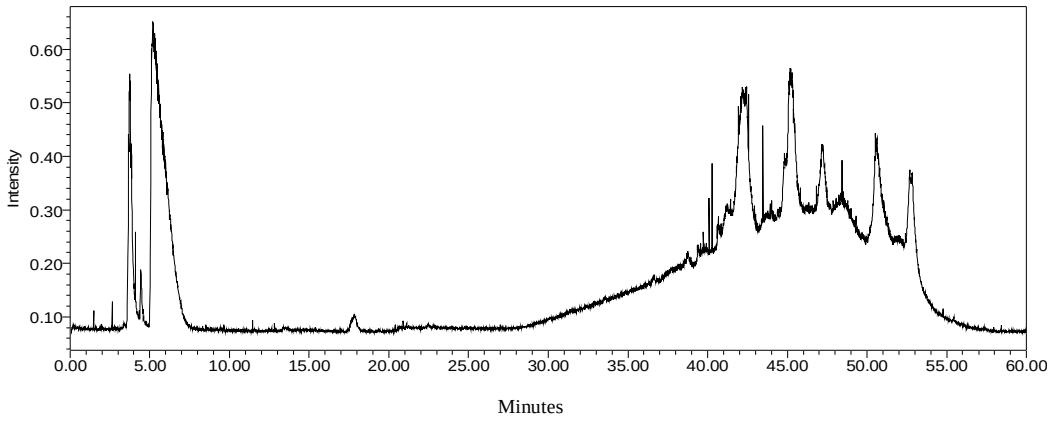

Figure 3.15: HPLC-MS chromatogram of *P. calomelanos* acetone extract.

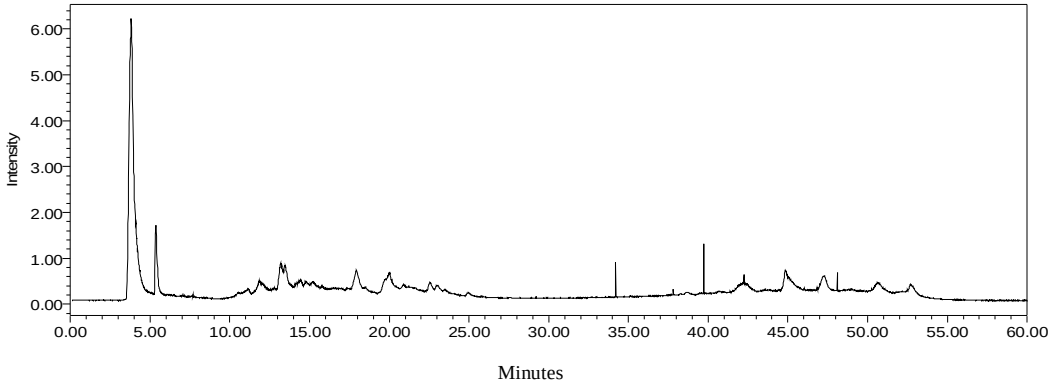

Figure 3.16: HPLC-MS chromatogram of *P. calomelanos* methanol extract.

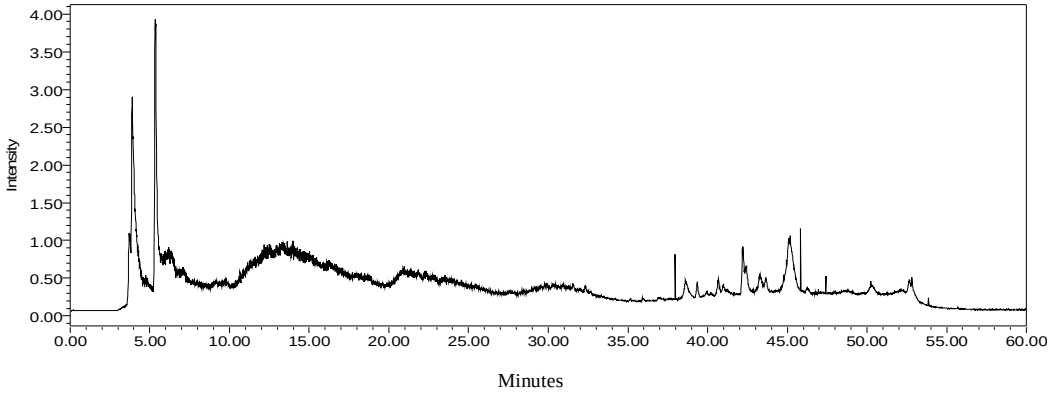

Figure 3.17: HPLC-MS chromatogram of *P. calomelanos* inhalation extract.

The chromatograms of the acetone and inhalation extracts of *Pellaea calomelanos* are alike and seem to contain similar constituents.

3.1.2.5. *Tarchonanthus camphoratus*

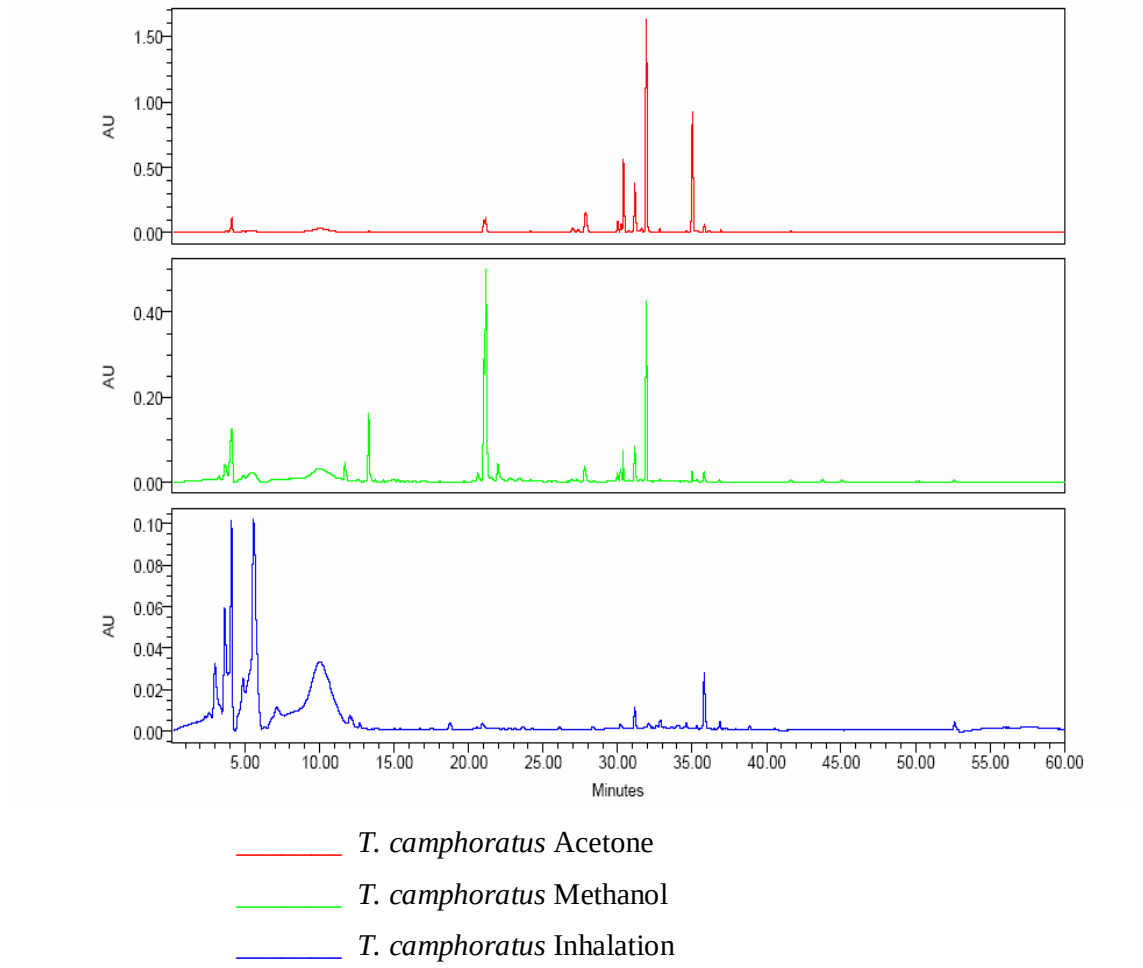

Figure 3.18: HPLC-UV chromatograms of the acetone, methanol and inhalation extracts of *Tarchonanthus camphoratus.*

The HPLC-UV chromatogram of the inhalation extract is different from the acetone and methanol profiles. A number of significant peaks are evident at the beginning of the inhalation chromatogram that are not present in the other profiles. The characteristic peak at 5.59 minutes for the inhalation sample has an integration area of 39.97%. The same compounds are evident in the methanol and acetone extracts at retention times 21.04 minutes and 30.41 minutes (Table 3.1). The same compounds are seen in all three profiles at retention times 31.16 minutes and 35.83 minutes (Table 3.1).

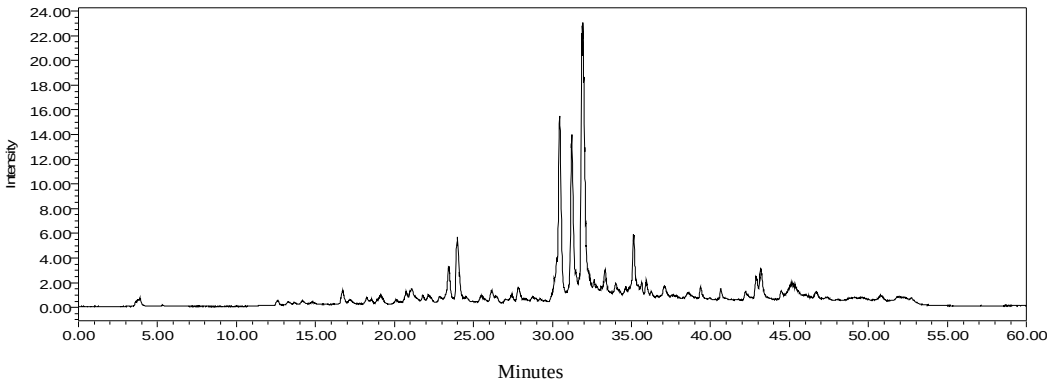

Figure 3.19: HPLC-MS chromatogram of *T. camphoratus* acetone extract.

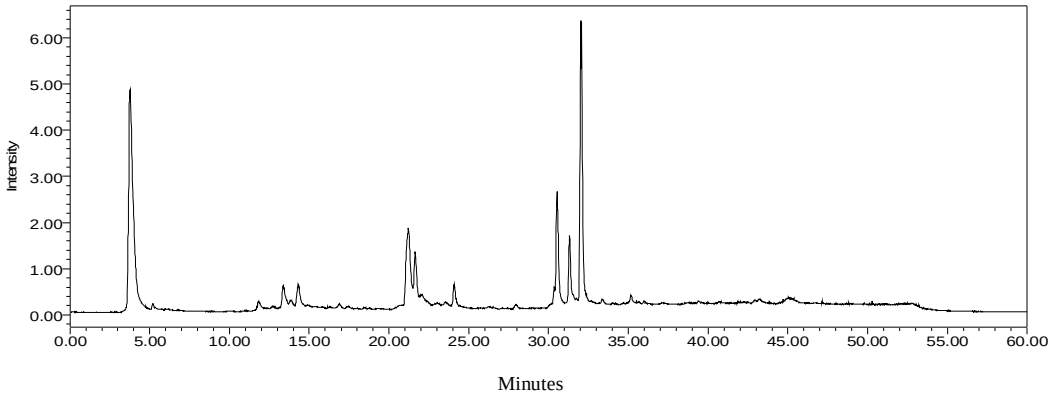

Figure 3.20: HPLC-MS chromatogram of *T. camphoratus* methanol extract.

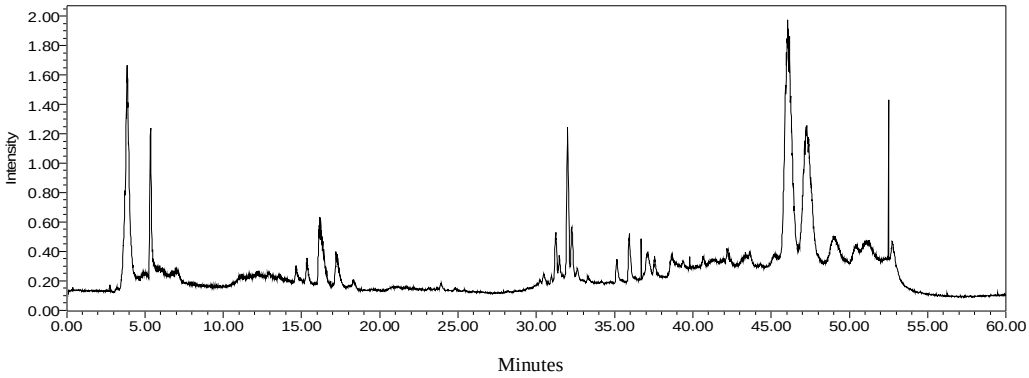

Figure 3.21: HPLC-MS chromatogram of *T. camphoratus* inhalation extract.

The *T. camphoratus* HPLC-MS chromatograms significantly supported the hypothesis of this study. The HPLC-MS chromatogram of the inhalation extracts shows several constituents not seen in the methanol and acetone extracts of the same plant. The *T. camphoratus* inhalation extract had unique compounds at retention times: 46.03, 46.05, 47.24, 47.31, 48.91, 48.96, and 52.43 minutes that did not occur in the methanol and acetone extracts of the same plant (Figures 3.19 and 3.20).

Table 3.1: Summary of HPLC-UV data: Showing the integration (%) for the most prominent peaks in the chromatograms. (derived from Figures 3.2 – 3.21).

Retention	*H. natalensis*			*M. flabellifolius*			*A. afra*			*P. calomelanos*			*T. camphoratus*		
Time (mins)	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone
2.98													7.54%		
3.06				2.48%											
3.07										5.22%					
3.27							24.15%								
3.53	11.67%	6.85%	4.65%												
3.67													11.68%	3.51%	
3.69										10.43%					
3.70							8.02%								
3.73											0.78%				
3.76						2.13%									
3.79				3.21%											
3.84		2.79%													
3.92	4.50%														
3.99												7.09%			
4.02											6.37%				
4.07			2.06%		2.66%		10.34%	5.61%							
4.08													15.04%		
4.09														10.63%	
4.10	1.29%								5.71%	6.09%					2.70%
4.13						4.38%									
4.16		4.74%													
4.18				8.27%											
4.59										0.44%					
4.63													1.41%		
4.65		1.89%													
4.67				1.13%	2.85%	6.09%	2.79%								
4.87													7.02%		
4.88												2.68%			
4.92			2.62%												

Table 3.1: Summary of HPLC-UV data: Showing the integration (%) for the most prominent peaks in the chromatograms. (derived from Figures 3.2 – 3.21).

Retention	*H. natalensis*			*M. flabellifolius*			*A. afra*			*P. calomelanos*			*T. camphoratus*		
Time (mins)	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone
4.96											0.88%				
4.99				2.93%											
5.01							7.06%								
5.37	14.83%														
5.46							34.18%								
5.52						17.44%									
5.55		9.58%													
5.59				26.31%	13.58%	7.51%				20.08%			39.57%		
6.13					1.33%										
6.54					1.19%										
7.14													1.30%		
7.28				1.69%											
7.74	2.54%														
8.11										2.23%					
9.05	21.79%														
9.62										31.86%					
9.72				43.15%											
9.93													8.90%		
10.01		22.50%				36.04%									
11.39								0.51%							
11.41						1.19%									
11.54	11.84%														
11.55					11.06%										
11.69											1.44%				
11.70														2.53%	
12.08	5.10%			0.67%			1.89%			3.52%			0.999%		
12.20											0.78%				
12.23										3.98%					
12.25				2.43%	0.21%										

Table 3.1: Summary of HPLC-UV data: Showing the integration (%) for the most prominent peaks in the chromatograms. (derived from Figures 3.2 – 3.21).

Retention	*H. natalensis*			*M. flabellifolius*			*A. afra*			*P. calomelanos*			*T. camphoratus*		
Time (mins)	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone
12.28							0.85%								
12.37	8.56%	1.60%	11.52%												
12.47					0.52%										
12.70													0.26%		
12.77		2.75%				0.47%									
12.82					1.05%										
12.96		7.92%	14.77%												
13.02							0.49%								
13.11											4.99%	4.01%			
13.29		2.23%	1.61%					11.40%						7.82%	
13.41							1.25%	11.06%	2.45%		4.68%				
13.69											3.09%				
13.74					0.53%										
13.83								0.65%							
13.89		3.50%	1.05%		0.78%										
13.93											1.71%				
14.06								0.70%							
14.08										11.00%					
14.17		2.09%	5.27%												
14.40					10.18%	12.25%									
14.42											2.46%				
14.69		3.08%	5.53%												
14.97											0.58%				
15.46															
15.50					1.07%										
15.75											0.77%				
16.04						2.52%									
16.07			2.56%												
16.10					4.54%										

Table 3.1: Summary of HPLC-UV data: Showing the integration (%) for the most prominent peaks in the chromatograms. (derived from Figures 3.2 – 3.21).

Retention	*H. natalensis*			*M. flabellifolius*			*A. afra*			*P. calomelanos*			*T. camphoratus*		
Time (mins)	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone
16.33								1.34%							
16.56											1.41%				
16.67					1.46%										
16.79											0.77%				
16.83			1.91%												
17.18		2.18%	4.84%												
17.29											2.71%				
17.89								1.22%							
17.92											15.46%	10.16%			
17.95	0.81%														
18.06							0.66%								
18.75													0.60%		
18.81		5.13%													
18.89				0.82%	8.42%	1.90%				0.90%					
18.95			10.22%												
19.22						1.70%									
19.24		2.29%													
19.30					6.42%										
19.41			2.56%												
19.78											14.98%				
19.99								1.86%							
20.40										1.75%	3.58%	1.29%			
20.45								1.00%							
20.54							0.94%								
20.56			8.38%												
20.63														1.17%	
20.67								3.44%							
20.69											5.43%				
20.81		2.89%													

Table 3.1: Summary of HPLC-UV data: Showing the integration (%) for the most prominent peaks in the chromatograms. (derived from Figures 3.2 – 3.21).

Retention	*H. natalensis*			*M. flabellifolius*			*A. afra*			*P. calomelanos*			*T. camphoratus*		
Time (mins)	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone
20.82					0.45%										
20.83									2.46%						
20.89			4.52%												
21.04														13.01%	2.18%
21.08											1.36%				
21.13								35.56%	11.97%						
21.16														29.38%	
21.31		1.09%													
21.44					7.64%	2.82%									
21.48											0.44%				
21.58								1.01%							
21.65	1.02%														
21.95															
21.97								9.25%	2.54%					2.06%	
22.08	2.84%														
22.11					1.89%										
22.41											7.04%	0.95%			
22.88											4.13%	0.50%			
23.39								0.39%	1.54%						
23.61								0.98%							
24.09							2.26%								
24.76											1.19%				
26.99															0.96%
27.00	2.77%														
27.80														2.60%	
27.84									3.76%						
27.86		1.51%													
28.18								1.72%	6.33%						
28.87								0.93%	3.57%						

Table 3.1: Summary of HPLC-UV data: Showing the integration (%) for the most prominent peaks in the chromatograms. (derived from Figures 3.2 – 3.21).

Retention	*H. natalensis*			*M. flabellifolius*			*A. afra*			*P. calomelanos*			*T. camphoratus*		
Time (mins)	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone
29.71									1.28%						
30.21														1.48%	
30.23															1.42%
30.41														1.93%	30.407%
30.83		1.20%													
31.16													1.13%	4.05%	8.85%
31.56															
31.61															0.76%
31.73	0.61%														
31.93															39.03%
32.32												22.02%			
32.33	3.06%														
32.59								2.13%	3.95%						
32.67	1.47%														
32.86							4.46%	0.88%	2.62%		0.95%		0.44%		0.88%
32.95					0.97%										
33.05									2.76%						
33.22	0.56%														
33.85		2.38%	1.86%												
33.95	1.57%														
34.09	3.41%														
34.16												5.87%			
34.24									11.84%						
34.59	0.32%		1.15%												
34.62											0.91%				
34.64							1.92%								
34.92	6.25%	8.17%	4.96%												
35.03														0.61%	19.48%
35.46									3.78%						

Table 3.1: Summary of HPLC-UV data: Showing the integration (%) for the most prominent peaks in the chromatograms. (derived from Figures 3.2 – 3.21).

Retention	*H. natalensis*			*M. flabellifolius*			*A. afra*			*P. calomelanos*			*T. camphoratus*		
Time (mins)	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone	Inhalation	Methanol	Acetone
35.53	0.28%														
35.73	0.31%														
35.83													3.17%	1.21%	1.54%
36.33	1.32%	1.35%	1.27%												
36.87													0.40%		
36.88				0.35%											
36.92	0.51%														
36.93															0.30%
37.47	0.78%														
38.62	1.93%														
39.91												2.75%			
39.92	0.32%														
41.55												0.26%			
42.14	0.99%														
42.67	1.57%														
43.16												5.28%			
43.63												12.42%			
43.77		2.19%													
43.83												1.60%			
44.14												5.90%			
45.06											1.11%				
46.07												7.09%			
46.35												2.79%			
49.10												2.87%			
52.71		1.93%													

3.1.3. HPLC-UV and HPLC-MS comparison of the five inhalation extracts

The chromatographic profiles of the five inhalation extracts were compared. This was done to ascertain whether or not combustion produced the same or similar compounds regardless of the particular plant burnt.

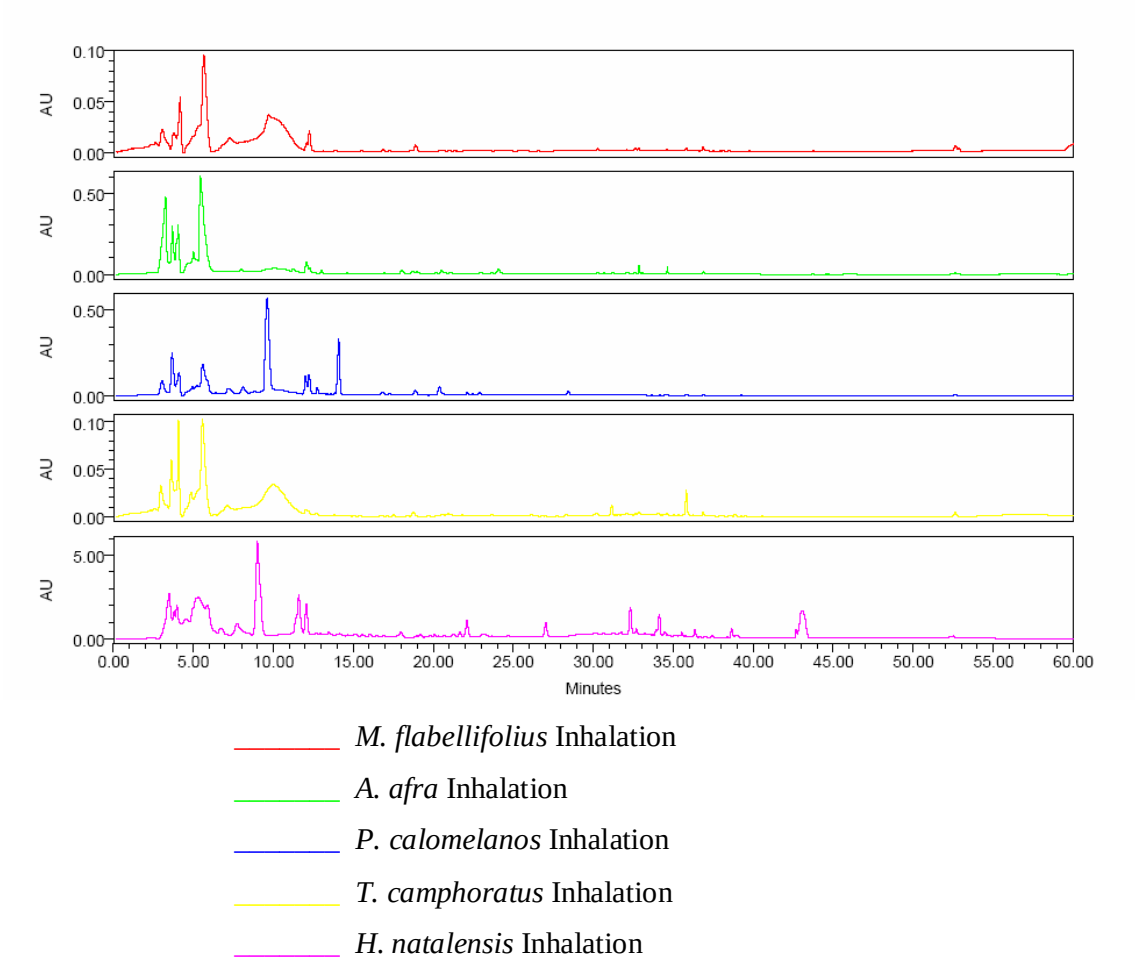

Figure 3.22: HPLC-UV chromatograms of all five inhalation samples i.e. *Myrothamnus flabellifolius, Artemisia afra, Pellaea calomelanos, Tarchonanthus camphoratus,* and *Heteropyxis natalensis.*

The HPLC-UV inhalation chromatograms (Figure 3.22) illustrate that although similar compounds do form during combustion of all five plants, their profiles are still significantly different and additional compounds are formed that are unique to each species studied. It is interesting to note that the *P. calomelanos* inhalation extract has a major peak at a retention time of 14.083 minutes which is absent in all other profiles (Figure 3.22 and Table 3.1). It should also be mentioned at this point that it was the *P. calomelanos* inhalation extract that

demonstrated greater antimicrobial activity than all of its solvent extracts against all pathogens tested (Table 3.2). A common compound is noted for the *H. natalensis* and *M. flabellifolius* inhalation extract at retention time 5.37 minutes (Table 3.1). A common compound is also seen in *A. afra* and *M. flabellifolius* inhalation extracts at retention time 4.67 minutes (Table 3.1). It is this same compound that is common in all three extracts (inhalation, methanol and acetone) of the plant *M. flabellifolius* at retention time 4.67 minutes and is clearly observed as a common peak in Figure 3.6. There is also a common compound seen in all five inhalation extracts at retention time 12.08 minutes (Table 3.1).

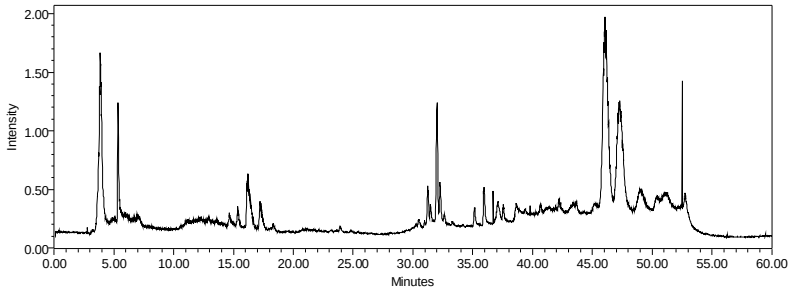

Figure 3.23: HPLC-MS chromatogram of *T. camphoratus* inhalation extract.

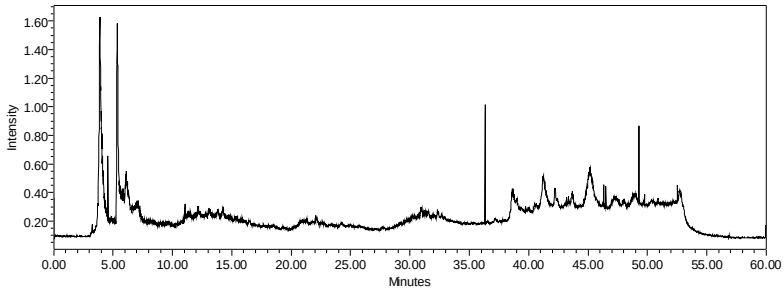

Figure 3.24: HPLC-MS chromatogram of *M. flabellifolius* inhalation extract.

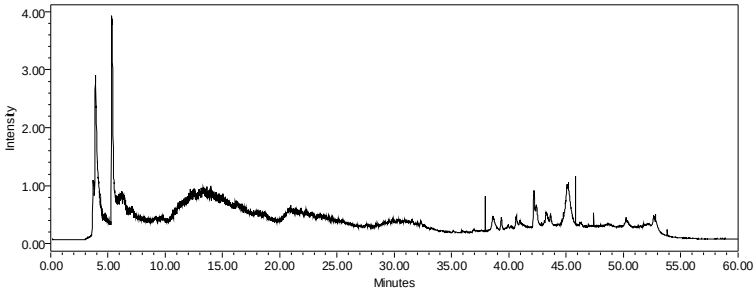

Figure 3.25: HPLC-MS chromatogram of *P. calomelanos* inhalation extract.

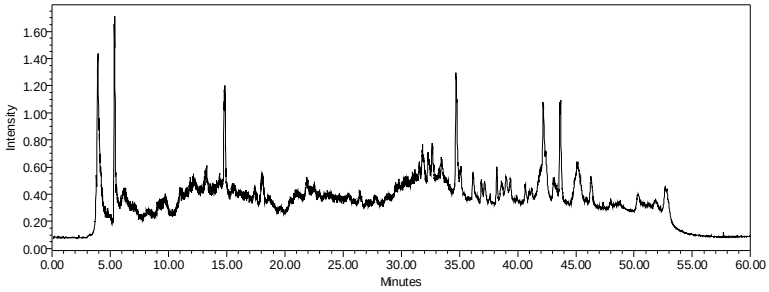

Figure 3.26: HPLC-MS chromatogram of *H. natalensis* inhalation extract.

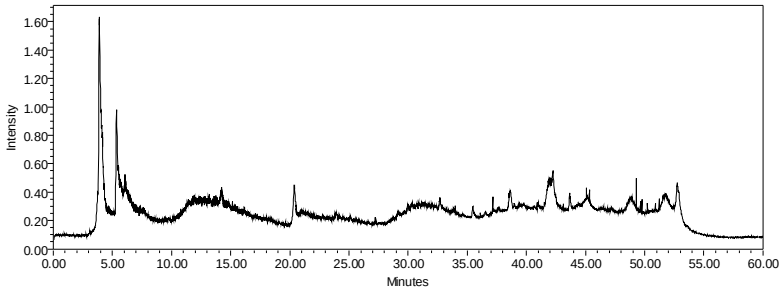

Figure 3.27: HPLC-MS chromatogram of *A. afra* inhalation extract.

A comparison of the HPLC-MS inhalation chromatograms revealed that combustion may produce some common compounds but each extract also has unique compounds. HPLC-MS chromatograms revealed six compounds that are common to all five of the inhalation samples, i.e. the compounds at retention times, 3.97, 5.38, 26.14, 31.95, 43.66, and 47.28 minutes. However, out of the 90 compounds found in the five inhalation samples during HPLC-MS analysis only the above six were common. Out of the 90 compounds found in the inhalation samples, 16 compounds are unique to a particular extract and are absent in all other samples. Ten unique compounds are only found in the *A. afra* inhalation extract and six unique compounds are only found in the *P. calomelanos* inhalation extract.

3.1.4. General discussion of the chemical composition

Chromatographic profiles provide a comparison of the various extracts/samples for each plant. It is interesting to note the differences in the chromatograms for the same plant. These differences highlight two important points: first, the solvent used for extraction has a major impact on the compounds extracted from the plant material; and, secondly, the process of burning has a profound impact on the chemistry. It is also clear that the inhalation samples produced very different chromatograms from the methanol and acetone extracts of the same

plant, especially evident when examining the HPLC-UV and HPLC-MS chromatograms of *Artemisia afra*, *Pellaea calomelanos*, and *Tarchonanthus camphoratus*.

Whether the burning of all plant material generates the same active compounds has been a topic of debate. Van Staden *et al*. (1995) reported that similar compounds are found in smoke extracts derived from different plants. A comparison allowed the identification of 12 compounds of which seven were common in the two extracts investigated by Van Staden *et al.* (1995). There were some common compounds found in this study as would be expected; e.g. *M. flabellifolius* and *P. calomelanos* inhalation extracts have two common compounds at retention times of 5.59 and 18.89 minutes observed from the HPLC-UV chromatograms (Table 3.1) and the same compounds were present on the HPLC-MS chromatograms at retention times 16.20 and 37.11 minutes (Figures 3.25 and 3.24). *A. afra* and *T. camphoratus* inhalation extracts have one common compound found at retention time 32.86 minutes (Table 3.1). *P. calomelanos* and *T. camphoratus* inhalation extracts also have one common compound at retention time 12.03 minutes (Table 3.1). However, there were no compounds found to be common to all five inhalation extracts when the HPLC-UV chromatographic profiles of the five inhalation samples were compared (Figure 3.22). The HPLC-UV analysis of the five inhalation extracts showed different chromatograms (Figure 3.22), indicating that burning does not produce the exact same compounds regardless of the plant, although certain common constituents are noted between one or two of the extracts. A total of 74 out of the 78 compounds found in the inhalation samples in the HPLC-UV analysis were unique and were absent in all other plant extracts. Minorsky (2002) noted that not all smoke derived from individual species is equally effective in promoting seed germination, thus there must be differences in chemical composition.

One interesting observation from the HPLC-MS analysis is the common compounds that occur in the *H. natalensis* inhalation sample and the *T. camphoratus* inhalation sample, i.e. at retention times: 3.92, 5.43, 14.82, 17.28, 18.14, 32.76, 35.02, 38.56, 41.24, 47.37 and 49.17 minutes (Figures 3.5 and 3.21). Common compounds are also found in the methanol and the acetone samples of the same two plants, namely: *H. natalensis* and *T. camphoratus* at retention times 3.93, 4.04, 5.32, 23.94, 27.07, 31.22, 43.15, 44.98 and 46.05 minutes (Figures 3.3, 3.4, 3.19, 3.20). These distinct structural similarities in the HPLC-MS chromatograms of the two plants, *H. natalensis* and *T. camphorates*, did not, however, correlate with any of the antimicrobial data.

Besides the extensive HPLC-UV and HPLC-MS analysis performed above, gas chromatography may have also been valuable in elucidating the compounds responsible for enhanced antimicrobial effects observed during antimicrobial testing. A few of the selected species in this investigation are rich in volatiles and would ideally have been analysed using gas chromatography (GC). However, because of the high carbon content and high molecular weights of the compounds formed during combustion, an initial attempt to achieve good GC profiles proved impossible. In addition, the compounds formed seemed to have extremely high boiling points and proved difficult to vaporise during a trial analysis of two of the inhalation samples. For the above mentioned reasons, however, gas chromatography was not used to analyse the samples under investigation.

3.2 Antimicrobial activity of extracts

The minimum inhibitory concentration (MIC) was used to quantitatively measure the *in vitro* antibacterial activity of the test extracts before and after burning. Bioassays facilitated an interesting comparison of the antimicrobial activities of inhalation extracts compared to those of the conventional solvent extracts of the same plants. Table 3.2 shows the results obtained from the antimicrobial testing.

Table 3.2: The MIC values for combustion, methanol, acetone and water extracts and isolated essential oils (mg/ml): (Ciprofloxacin was used as the control for the bacterial pathogens and amphotericin B was used as the control for *C. neoformans*).

Sample	***S. aureus*** (ATCC 25923)	***B. cereus*** (ATCC 11778)	***K. pneumoniae*** (ATCC 9633)	***C. neoformans*** (ATCC 90112)
H. natalensis				
Inhalation	1.86	0.35	0.70	0.93
Methanol	0.38	0.25	1.00	0.83
Acetone	0.38	0.25	2.00	1.50
Water	4.00	1.50	2.00	4.00
Essential oil	32.00	4.00	8.00	8.00
M. flabellifolius				
Inhalation	0.72	0.27	0.36	0.36
Methanol	4.00	3.00	3.00	1.00
Acetone	1.00	0.50	1.50	0.25
Water	4.00	8.00	6.00	0.75
Essential oil	6.00	2.00	3.00	3.30
A. afra				
Inhalation	0.52	0.26	0.52	0.52
Methanol	2.00	1.00	3.00	2.00
Acetone	0.25	0.25	2.00	0.75

Sample	*S. aureus* (ATCC 25923)	*B. cereus* (ATCC 11778)	*K. pneumoniae* (ATCC 9633)	*C. neoformans* (ATCC 90112)
Water	4.00	4.00	4.00	4.00
Essential oil	16.00	12.00	8.00	32.00
P. calomelanos				
Inhalation	0.53	1.00	0.53	0.53
Methanol	>16.00	>16.00	6.00	4.00
Acetone	2.00	4.00	1.50	>32.00
Water	>16.00	>16.00	16.00	4.00
T. camphoratus				
Inhalation	0.62	0.23	0.93	0.47
Methanol	2.00	1.00	3.00	1.00
Acetone	0.50	<0.13	1.50	0.38
Water	8.00	6.00	8.00	>32.00
Essential oil	4.00	0.75	6.00	6.60
Positive controls (commercial antibiotic)				
	0.5×10^{-3}	2.5×10^{-3}	0.8×10^{-3}	1.25×10^{-3}
Blank controls (solvent)				
Methanol	16.0	16.0	8.0	8.0
Acetone	No activity	No activity	No activity	No activity
Water	No activity	No activity	No activity	No activity

The results tabulated above are graphically represented for each species in Figures 3.28 – 3.32.

3.2.1. *Heteropyxis natalensis*

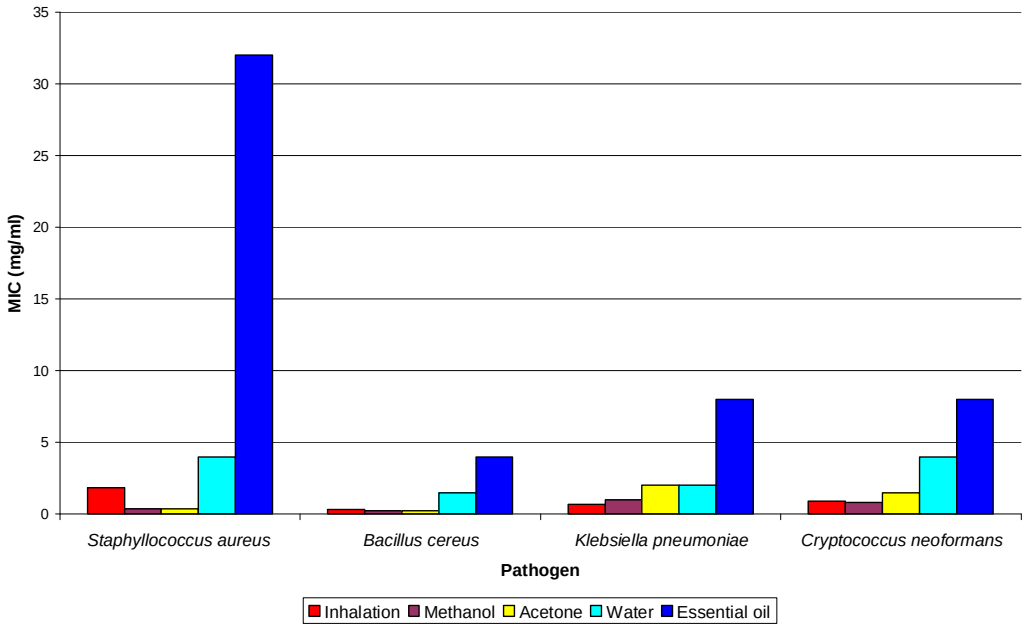

Figure 3.28: MIC values (mg/ml) for extracts and essential oils of *H. natalensis* tested against four pathogens.

The inhalation extract of *H. natalensis* exhibited MIC values lower than the essential oil and water extracts in all cases when antimicrobial testing was done against the above four pathogens. The inhalation extract of *H. natalensis* was a stronger antimicrobial than all other extracts of this plant when tested against *K. pneumoniae*, which is one of the bacterial pathogens isolated in patients with an acute exacerbation of chronic bronchitis and in bacterial pneumonia cases (Dipiro *et al.*, 2002). Bacterial pneumonia is indeed a respiratory condition suited for treatment through inhalation therapy. The inhalation extract demonstrated low MIC values against all pathogens (Figure 3.28 and Table 3.2). However, it was not as strong an antimicrobial as either the methanol or acetone extracts of the plant for the pathogens *S. aureus*, *B. cereus*, and *C. neoformans*. The MIC results for the acetone and methanol extracts also gave exactly the same MIC values for these two samples when tested against *S. aureus* and *B. cereus* (Table 3.2) and this is worth noting because there were obvious similarities between the acetone and the methanol chromatographic profiles (Figure 3.3 and Figure 3.4).

3.2.2. *Myrothamnus flabellifolius*

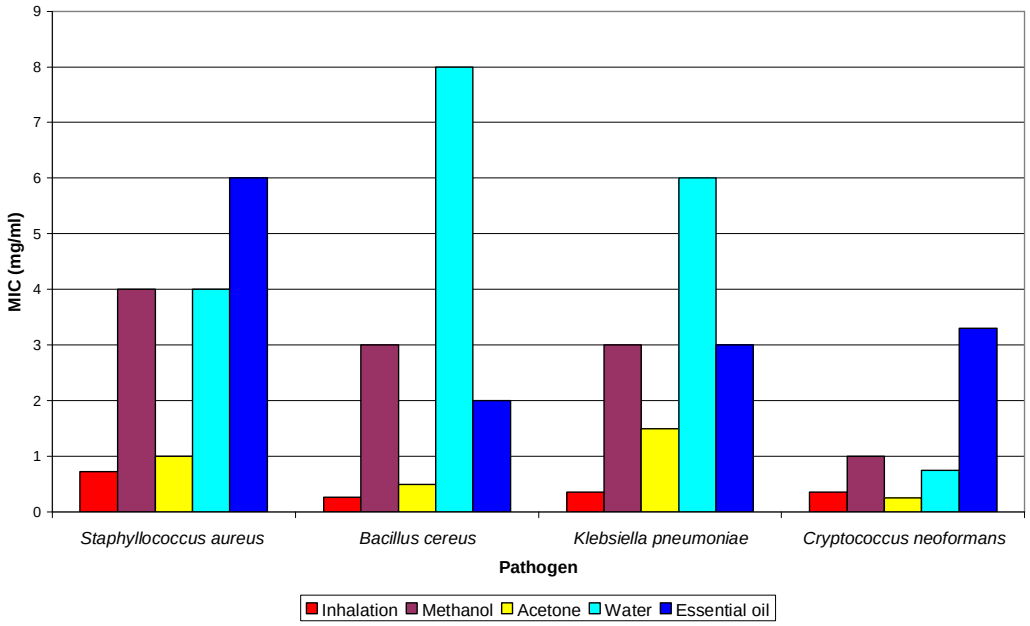

Figure 3.29: MIC values (mg/ml) for extracts and essential oils of *M. flabellifolius* tested against four pathogens.

The inhalation extract of *Myrothamnus flabellifolius* had lower MICs than the methanol, acetone, water, and essential oil extracts of the plant, when tested against all the pathogens

with the exception of *Cryptococcus neoformans* (Figure 3.29 and Table 3.2). The abundance of peaks late in the HPLC-MS chromatogram (Figure 3.9) may play a role in the enhanced activity observed for the *M. flabellifolius* inhalation extract.

Another interesting observation is the low MIC values obtained for all *Myrothamnus flabellifolius* extracts when tested against *Cryptococcus neoformans*. The average MIC value for all *M. flabellifolius* extracts is far lower against *C. neoformans* than against any of the other pathogens. It seems that *M. flabellifolius* is more active against the yeast than a bacterial pathogen. A *Cryptococcus neoformans* respiratory infection is a chronic infection which involves mainly the lungs and meninges, and traditional practices have seen *Myrothamnus flabellifolius* smoke inhaled to treat chest complaints and infections (Hutchings, 1996; van Wyk *et al.*, 1997). Strong antimicrobial activity was also found when *M. flabellifolius* was tested against yeast/fungal pathogens in a study by Viljoen *et al.* (2002). Another testament to the use of this plant for respiratory ailments is the current use of one of its essential oil components (*trans*-pinocarveol) in a commercial pharmaceutical preparation called Ozopulmin to treat respiratory conditions (Viljoen *et al.*, 2002).

3.2.3. *Artemisia afra*

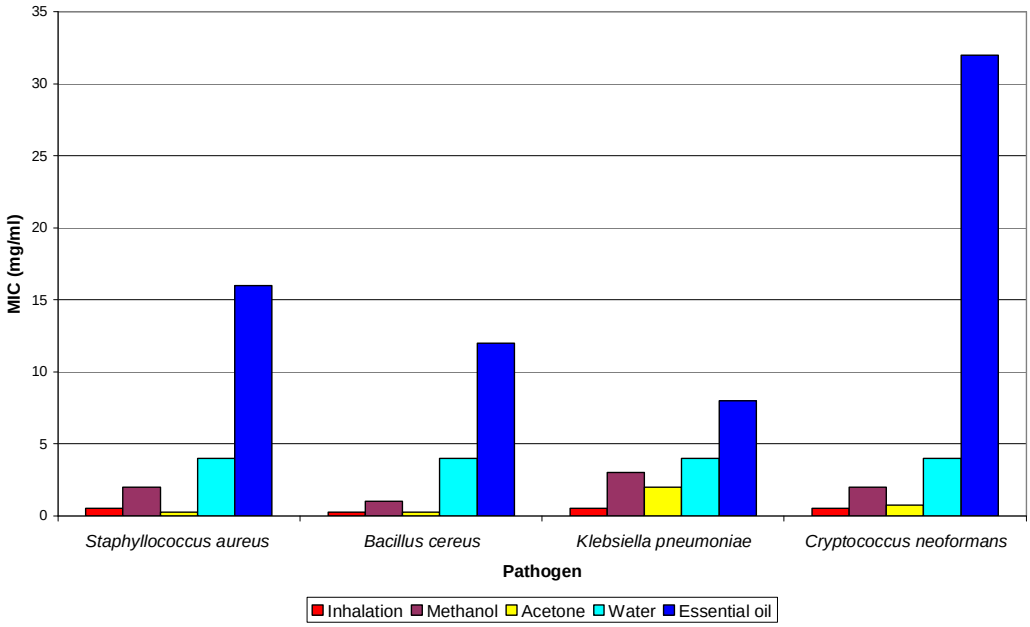

Figure 3.30: MIC values (mg/ml) for extracts and essential oils of *A. afra* tested against four pathogens.

The inhalation extract of *A. afra* had the lowest MIC when compared to the other extracts of the plant tested against *K. pneumoniae* and *C. neoformans* (Figure 3.30 and Table 3.2). *Artemisia afra* is also a plant that has been shown throughout the literature to demonstrate high antimycotic ('anti-fungal and anti-yeast') potency. It has been said that *A. afra* has greater activity as an antimycotic agent than as an antibacterial agent (Graven *et al.*, 1992). This antimycotic superiority over an antibacterial effect was not evident in this study for *Artemisia afra*, as the plant demonstrated an equally effective antimicrobial effect against the bacterial pathogens as it did against the yeast *C. neoformans*. A study done by Gundidza (1993) clearly indicated *Artemisia afra's* strong inhibitory effect on seven out of ten fungi tested in this earlier study. In addition, various studies note the susceptibility of the mycotoxigenic strain *Aspergillus ochraceus* to *A. afra* essential oil (Graven *et al.*, 1992; Gundidza, 1993). In contrast, this particular study demonstrated a very high MIC of 32.00 mg/ml for the essential oil of *A. afra* against the yeast *C. neoformans* (Figure 3.30 and Table 3.2).

It is clear that the inhalation extract of *A. afra* was a stronger antimicrobial than the other solvent extracts when tested against *C. neoformans* (MIC 0.52 mg/ml), and was also a more potent antibacterial agent than its conventional solvent extracts when tested against *K. pneumoniae* (MIC 0.52 mg/ml) (Table 3.2). The unique early eluting peaks and, in particular, the major peak observed in the inhalation sample (HPLC-UV) chromatogram at retention time 5.46 minutes (integration area of 34.18%) (Table 3.1 and Figure 3.10) may shed light on the MIC potency observed when the *A. afra* inhalation extract was tested against *K. pneumonia* and *C. neoformans.*

3.2.4. *Pellaea calomelanos*

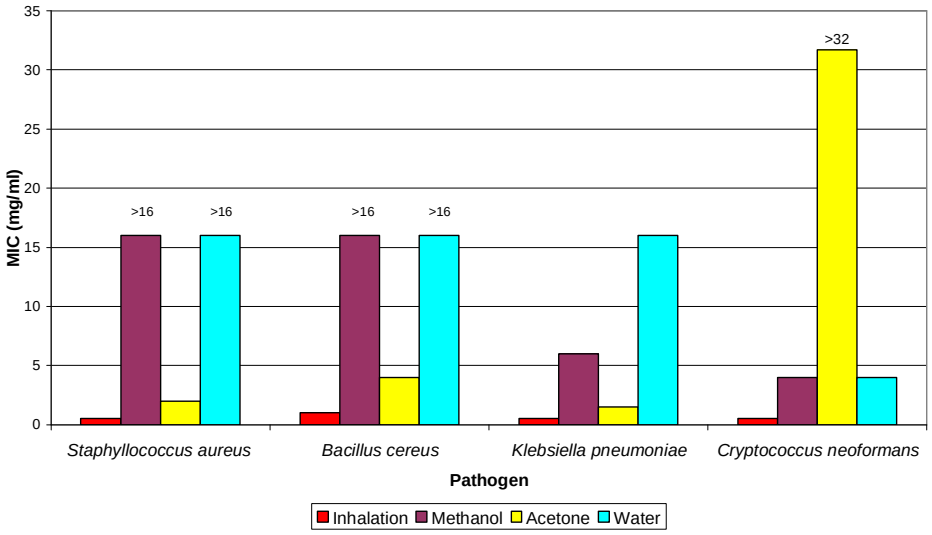

Figure 3.31: MIC values (mg/ml) for extracts of *P. calomelanos* tested against four pathogens

The inhalation extract of *P. calomelanos* had the lowest MIC results compared to its respective extracts for all pathogens tested (Figure 3.31 and Table 3.2). The superior MIC values obtained for *Pellaea calomelanos* may be linked to the presence of unique compounds found in the inhalation sample as seen in the chemical comparison of the extracts. In the HPLC-UV chromatogram (Figure 3.14), two unique peaks are observed at retention times 9.62 minutes and 14.08 minutes and are only present in the inhalation sample (Figure 3.14 and Table 3.1).

3.2.5. *Tarchonanthus camphoratus*

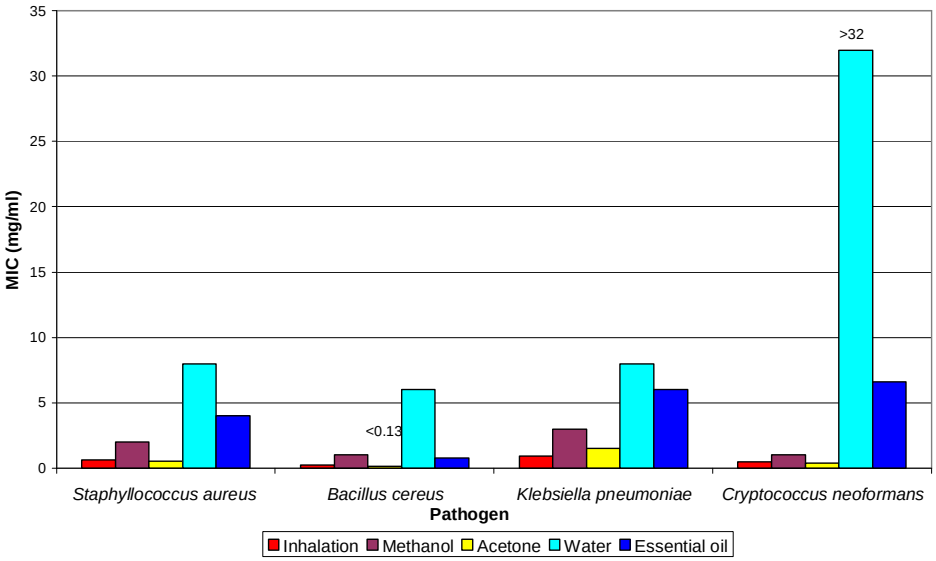

Figure 3.32 (13): MIC values (mg/ml) for extracts and essential oils of *T. camphoratus* tested against four pathogens.

The *Tarchonanthus camphoratus* inhalation extract had a lower MIC value compared to all other extracts of the plant when tested against *Klebsiella pneumoniae* (MIC 0.93 mg/ml). Generally, the inhalation extracts of this plant had low MIC values, only the acetone extracts showed a lower MIC than the inhalation sample for the other three pathogens (Figure 3.32 and Table 3.2). Significant peaks seen early in the HPLC-UV profile (Figure 3.18) of the inhalation extract were found to be unique to this extract. Noticeable peaks were also observed late in the HPLC-MS chromatogram (Figure 3.21). Perhaps these chemical differences observed in the UV chromatograms and the MS chromatograms of the various extracts may account for the enhanced antimicrobial activity recorded for the inhalation extract for *Klebsiella pneumoniae* (Figure3.32 and Table 3.2).

3.2.6. General discussion of antimicrobial activity

Many inhalation extracts had low MIC values, which highlighted their superior antimicrobial potency when compared to the solvent and aqueous extracts and the essential oils of the aromatic plants. The inhalation extract was more potent than any of its respective conventional extracts in a number of cases; *A. afra* tested against *K. pneumoniae* (0.52

mg/ml); *P. calomelanos* tested against *K. pneumoniae* (0.53 mg/ml); *A. afra* tested against *C. neoformans* (0.52 mg/ml); and *M. flabellifolius* tested against *B. cereus* (0.27 mg/ml).

A pronounced increase in antimicrobial activity is shown after combustion in a number of cases; e.g. *M. flabellifolius* samples tested against *S. aureus*; essential oil MIC (6.00 mg/ml), water MIC (4.00 mg/ml), methanol MIC (4.00 mg/ml), acetone MIC (1.00 mg/ml), and inhalation sample MIC (0.72 mg/ml). This increase in antimicrobial effect is observed also when *M. flabellifolius* was tested against all four pathogens, with the inhalation sample proving the most potent in all cases (except for *C. neoformans*). In a second instance, the inhalation extract of a plant proved more potent than any other sample of that same plant when tested against a particular pathogen; e.g. *H. natalensis* vs. *K. pneumoniae* MIC's: inhalation sample (0.70 mg/ml), methanol (1.00 mg/ml), water (2.00 mg/ml), acetone (2.00 mg/ml), essential oil (8.00 mg/ml). An important observation is noted for *P. calomelanos*, where the inhalation extract demonstrated greater antimicrobial activity than all of its solvent extracts against all 4 pathogens tested.

In the case of *Klebsiella pneumoniae*, all five inhalation extracts exhibit stronger antimicrobial activity than their respective conventional extracts: *H. natalensis* (0.70 mg/ml), *M. flabellifolius* (0.36 mg/ml), *A. afra* (0.52 mg/ml), *P. calomelanos* (0.53 mg/ml), and *T. camphoratus* (0.93 mg/ml). *Klebsiella pneumoniae* is one of the bacterial pathogens isolated in patients with an acute exacerbation of chronic bronchitis and in bacterial pneumonia cases. Enteric Gram-negative bacteria are the leading causes of nosocomial pneumonia according to Dipiro *et al.* (2002). It is relevant to note that *Klebsiella pneumoniae* is the most frequently encountered pathogen in this community-acquired enteric gram-negative pneumonia. This type of pneumonia is identified commonly among patients with chronic illness (diabetes mellitus and alcoholism), and is associated with high mortality (>50% in some cases). The potential of *K. pneumoniae* and other Gram-negative bacilli to produce extensive morbidity and mortality is enhanced by the emergence of highly resistant organisms (Dipiro *et al.*, 2002). The strong antimicrobial effect observed for the inhalation samples against this dangerous pathogen may be useful in drug development and in the quest to enhance treatment efficacy.

Testing against the pathogen *S. aureus* revealed that the *M. flabellifolius* and *P. calomelanos* inhalation extracts were the strongest antimicrobials when compared to all their respective laboratory control extracts. *M. flabellifolius* inhalation MIC was 0.72 mg/ml and *P.*

calomelanos inhalation MIC was 0.53 mg/ml. In the case of the results of *H. natalensis, A. afra*, and *T. camphoratus* obtained with *S. aureus*, only their acetone samples were superior in antimicrobial strength to the inhalation extracts.

Results obtained for *B. cereus* were similar to those recorded for *S. aureus*. The *M. flabellifolius* and *P. calomelanos* inhalation extracts had superior antimicrobial activity than their respective solvent extracts (*M. flabellifolius* inhalation MIC was 0.27 mg/ml and *P. calomelanos* inhalation MIC was 1.00 mg/ml). Only the acetone extracts were more active than the inhalation extracts in terms of antimicrobial potency for *H. natalensis, A. afra*, and *T. camphoratus.*

The results for *C. neoformans* demonstrated the lowest MIC values for the inhalation extracts of two plants, i.e. *A. afra* and *P. calomelanos,* when compared to their respective methanol, water, and acetone extracts, and compared to their essential oils. In other words, the inhalation extracts of *A. afra* and *P. calomelanos* were more potent than the plants' conventional solvent extracts with this particular pathogen. The acetone extracts demonstrated better MIC values than all others for *T. camphoratus* and *M. flabellifolius* when tested against *C. neoformans.* The methanol extract of *H. natalensis* was the most potent extract, followed by the inhalation sample (MIC 0.83 mg/ml and 0.93 mg/ml respectively) when tested against *Cryptococcus neoformans*.

On average, the conventional solvent extracts showed higher MIC values and were less potent than the inhalation extracts; e.g. average MIC for methanol, acetone, water and essential oil extracts of *M. flabellifolius* and *B. cereus* were 3.38 mg/ml compared to 0.27 mg/ml for the inhalation sample.

Generally, acetone extracts were shown to have extremely low MIC's. It seemed evident that inhalation and acetone extracts were comparable in terms of antimicrobial efficacy, yet there is a large quantitative difference between inhalation extracts and the other solvent extracts (methanol, water and essential oil). This is an interesting finding considering that previous work supports the theory that solvent extracts may actually be superior to traditionally prepared extracts (Jäger, 2003). The study by Jäger (2003) raised concerns about the traditional way of preparing plant extracts and concluded that the traditional means of preparation may not lead to very active extracts. In the current study, however, the inhalation

extracts (products of a crude method of traditional preparation of plant remedies) were in fact very active, with low MICs indicating that the active compounds were retained.

Rabe and van Staden (1997) reported that the majority of the antibacterial activity of plants was present in methanol extracts. Traditionally, however, plant extracts are prepared with water and it seems unlikely for traditional healers to extract those constituents that are responsible for activity in the methanol extracts (Rabe and van Staden, 1997).

It is also highly unlikely and impractical for traditional healers to extract plants with acetone. This study has shown strong antimicrobial activity with the acetone extracts and the inhalation extracts. Acetone is very efficient as a solvent for extracting compounds with high antimicrobial activity (Eloff, 1998b). Eloff (1998b) conducted a study to evaluate which extractant should be used for the screening and isolation of antimicrobial components from plants. That study concluded that acetone was by far the best and most useful solvent as it extracts polar and non-polar inhibitors and demonstrated the lowest MIC's. This present study has confirmed Eloff's (1998b) evaluation of acetone by also demonstrating high antimicrobial activity with the acetone extracts. It follows, therefore, that one should raise the question as to whether *in vitro* antimicrobial studies can be used to scientifically confirm theories about traditional practices, if traditional healers do not use 'superior' extractants like acetone.

The essential oils, together with the GC-MS profile for this study was obtained from the research collaborator Dr. van Vuuren (van Vuuren, 2007). The antimicrobial activity of essential oils was initially evaluated in the liquid phase until a systematic evaluation of the vapour activity was carried out by Maruzella *et al*. (1959) and Kienholz (1959), and then by Maruzella and Sircurella (1960). The role of essential oils in inhalation therapy was further investigated by Inouye *et al.* (2001), who derived a method to express the antimicrobial activity by gaseous contact. A parameter called the MID (minimal inhibitory dose per unit space) was introduced to compare the relative vapour activity of essential oils. In comparison, this study has successfully created a novel way to mimic more closely what happens in a traditional setting with the specially designed inhalation apparatus, but a similar indicator such as the MID used by Inouye *et al*. (2001) may be useful in further quantifying the antimicrobial strength of the plant inhalation extracts. Embarking on a similar study to that conducted by Inouye *et al*. (2001), where smoke instead of essential oil vapour is allowed to impregnate into agar which contains respiratory pathogens, may shed more light on the

antimicrobial effects of smoke-derived constituents. Also of interest would be the solubility/absorption into an aqueous medium such as agar.

Various factors may also influence the antibacterial effect of inhaled substances as a viable therapy. Time of exposure to the inhaled smoke, the concentration of 'gas' produced, the heat of combustion and the mode of burning (open fire, electric stove, gas stove) may all influence the effect achieved. Some studies on the inhalation of essential oils concluded that the antimicrobial action by gaseous contact is most efficient when the pathogen is exposed at high vapour concentration for a short time (Inouye *et al*., 2001). Various temperatures may produce different compounds with changing activities. Slow combustion of dry or green plant material gives rise to the active principles that are apparently produced around 160-200°C and are volatilised at higher temperatures (Minorsky, 2002).

The antimicrobial activity of all volatile compounds is a result of two factors: the direct vapour absorption on microorganisms; and an indirect effect through the medium that absorbed the vapour (Moleyar and Narasimham, 1986).

The MIC results indicate that active compounds are formed on combustion and these constituents, only present after burning, have enhanced antimicrobial activity. Similarly, Minorsky (2002) commented on smoke-derived germination cues having high activity at very low concentrations (e.g. *Nicotiana attenuata* was active at concentrations < 1pg/seed). A study done by van Staden *et al*. (2004) investigated the role of smoke-derived fractions as a germination stimulant. Plant-derived smoke does indeed contain several thousand different compounds and it is generally accepted that active constituents are produced directly by the combustion of the plant material (van Staden *et al.*, 2004). It may be postulated that the phytoconstituents formed on combustion serve to enhance the antimicrobial activity of the plant (a synergistic or additive effect), or that new antimicrobial compounds are formed (artefacts) that enhance activity. Smoke extracts have already found wide application as seed germination stimulants, in that they have been used as seed pretreatments for enhancing the conservation of threatened or rare species of plants (Minorsky, 2002). In addition, smoke-induced germination studies have shown a long-lasting ability of plant smoke to overcome seed dormancy. Seeds treated with smoke have retained an enhanced ability to germinate even after a year of storage (Minorsky, 2002). Perhaps the improved germination ability (Minorsky, 2002) is as a result of the enhanced antimicrobial activity found in the inhalation extracts in the current study (Table 3.2)

It has also been noted that the degradation of certain essential oil components (limonene and α-pinene) by oxidation may produce oxygenated products that exhibit better activity than the parent hydrocarbons (Inouye *et al*., 2001). Adriansz *et al*. (2000) identified 1,8-cineole as an active germination enhancer, and octanoic acid was identified as an active factor in smoke in another study (Sutcliffe and Whitehead, 1995). The exact compounds responsible for the antimicrobial activity would need to be identified and isolated in similar future studies. The promising MIC values documented after combustion and the known benefits of site-specific administration could explain the benefits of inhalation therapy in traditional medicine for treating respiratory ailments.

3.3. General discussion

Out of the 90 compounds found in the inhalation samples during HPLC-MS, 16 compounds are unique to a particular extract and do not occur in any other sample; ten compounds are found only in *A. afra* inhalation extract and six compounds are found only in *P. calomelanos* inhalation extract and no other extract. It could be postulated that it is these unique compounds that account for the antimicrobial potency of the *A. afra* inhalation extract when tested against *K. pneumoniae* and *C. neoformans*.

Another interesting finding was the occurrence of the same compounds in *M. flabellifolius* inhalation sample and *P. calomelanos* inhalation sample at retention times 16.20 and 37.11 minutes during HPLC-MS analysis. *M. flabellifolius* and *P. calomelanos* inhalation extracts also have two common compounds at retention times of 5.59 and 18.89 minutes (Table 3.1). These similarities are pertinent as the *M. flabellifolius* inhalation sample had the strongest antimicrobial effect when compared to its respective methanol, acetone, water and essential oil samples when tested against all pathogens except one (*C. neoformans*). As discussed previously, the *P. calomelanos* inhalation sample was also superior to all other conventional extracts of this plant when tested against all four pathogens. It is interesting that these two plants have shown higher antimicrobial potency in nearly all cases with the inhalation extracts compared to their respective solvent extracts (Table 3.2).

Despite the need for further investigation into isolating the exact constituents responsible for the low MIC values of the inhalation extracts, work also needs to be done on the possible role of a physical mechanism that occurs in a patient when inhaling a vapour. Investigation into

the exact physical mechanism of action of smoke-derived constituents may further explain the superiority of this mode of administration. Many inhaled medicines are used to treat colds and respiratory conditions, and essential oil inhalation has long been used to treat acute and chronic bronchitis, sinusitis, and other respiratory conditions (Inouye *et al.*, 2001). It has been said that the vapours of essential oils increase the outflow of respiratory tract fluid (Boyd and Sheppard, 1970), maintain the ventilation drainage of the sinuses (Burrow *et al*., 1983), and have an anti-inflammatory effect on the trachea (Shubina *et al*., 1990), in addition to their antibacterial activities. Perhaps smoke inhalation has a similar mechanical effect on the tissues, in addition to its enhanced antimicrobial effect.

In an age of modern advances in the medical field many rural folk still use inhalation as a means of treatment not only because they lack the finances to seek allopathic cures but because of their belief in its efficacy. Inhalation is a highly effective mode of administration not only because of its direct and local effect on the lungs for the treatment of respiratory afflictions, but also because of its ability to deliver drugs effectively to the rest of the body. In addition, the large surface area for absorption and the low metabolic activity of the lungs make this organ system a potential route for systemic delivery of drugs that cannot be administered by other means (Suarez and Hickey, 2000). A few studies have shown that the lungs provide a substantially greater bioavailability for macromolecules than any other port of entry to the body (Suarez and Hickey, 2000).

The need for further investigations into this interesting area of study is evident. An interesting area for future study into inhalation therapy might be an investigation of bacterial vs. fungal susceptibility towards inhalation extracts. Even work done with inhalation extracts concentrating only on fungal pathogens would be valuable. Further MICs, in conjunction with more advanced structural analysis and careful isolation of the active compounds in inhalation extracts, would be helpful in revealing the exact compounds responsible for the enhanced antimicrobial effects, and this would shed more light on inhalation as an effective and extensively utilised method of treatment in both western and traditional cultures.

CHAPTER 4: CONCLUSIONS

- The inhalation extracts had extremely low MIC values emphasising their antimicrobial potency against respiratory pathogens. The MIC's of the inhalation extracts ranged between 0.23 mg/ml (*T. camphoratus* for. *B. cereus*) to 1.86 mg/ml (*H. natalensis* for *S. aureus*). All inhalation extracts had MIC values that on average are lower than the average MIC obtained for the solvent extract.
- When comparing inhalation extracts to conventional solvent extracts, a dramatic change in antimicrobial activity and composition was evident. In specific cases the inhalation extract of a plant was stronger as an antimicrobial against certain pathogens than any of the other conventional solvent extracts of that same plant. This superior activity of the inhalation extract was observed for *M. flabellifolius* when tested against all pathogens (except for *C. neoformans*). *Pellaea calomelanos* inhalation extract had the lowest MIC values when compared to the solvent extracts for all pathogens.
- All five inhalation extracts demonstrated stronger antimicrobial activity than their respective solvent and aqueous extracts when tested against *Klebsiella pneumoniae*.
- Chromatographic profiles revealed a difference in composition between different extracts of the same plant.
- Inhalation extracts gave rise to a very different chemical profiles compared to the solvent extracts of the same plants, and inhalation samples contained a number of unique compounds not present in the other extracts. HPLC-MS profiles revealed that of the 90 compounds noted in the inhalation samples, 16 compounds are unique and do not occur in any other sample. It is clear that combustion process produces compounds not present in any of the solvent extracts.
- HPLC comparisons of the five inhalation extracts revealed that similar and different constituents are formed during the burning process and that combustion does not liberate the same compounds regardless of the plant material.
- The choice of solvent used for extraction and the burning process greatly influences the chemical composition and the observed antimicrobial activity.

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APPENDIX

A presentation was given at The Indigenous Plant Use Forum, Grahamstown, 27-30 June 2005 [Podium presentation] where some of the data on the plant *Pellaea calomelanos* was presented.

"Some variables to be considered in pharmacognostic studies using South African medicinal plants as a demonstration model"

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Many variables are involved in plant-based studies and microbiological assays are only as reliable and sensitive as the test system employed. Using South African medicinal plants, some aspects of Pharmacognosy studies will be highlighted to demonstrate the factors that may influence the results.

Joint assays may corroborate findings, as in the case of *Osmitopsis asteriscoides* where results obtained from disc diffusion, MIC, time-kill methodology and confocal membrane integrity studies are congruent. Studies on *Eriocephalus* spp. however, reveal that the choice of assay can change the whole focus of the results. Geographical and /or genetic factors may account for variation within and between plant populations as seen with antimicrobial data generated for *Eriocephalus aromaticus*. Seasonal variation may or may not impact on the results obtained, as noted in a study on *Heteropyxis natalensis*, where plants were harvested on a monthly basis for one year. The MIC values obtained from individual plants indicated little variation. However, the ratio of the two major compounds (1,8-cineole and limonene) showed some monthly fluctuation. The role these two compounds have on the overall antimicrobial activity was assessed and results indicate that in combination they show variable synergistic and antagonistic interaction. The traditional use of plants as a treatment for infectious diseases is not always restricted to a single plant part. Antimicrobial studies on *Croton gratissimus* show that not only do the leaf, bark and root extracts have higher sensitivities than the essential oils, but that in root/leaf combination, synergy is enhanced. Using a simple combustion experiment for plants of which the smoke is inhaled we have illustrated that the smoke fraction is antimicrobially more active than the solvent extracts. This clearly demonstrates the need to mimic the traditional mode of administration as closely as possible in the laboratory.