



Declaration

Identification of Commonly Used Traditional Medicines by Planar Chromatography for Quality Control Purposes

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Declaration

I declare that this dissertation is my own unaided work conducted under the supervision of Prof. J. N. Eloff. It is submitted to the Department of Pharmacology, Faculty of Health Sciences, University of Pretoria, Pretoria, for the Degree of Magister Scientiae. It has not been submitted before for any degree or examination in any other University.

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Abstract

South Africa contains more than 9% of the world higher plant species diversity, of which many are used to treat human and livestock health problems. Unfortunately, the motivation to put medicinal plants on the market is frequently not to provide an essential service but to make money. In some overseas countries falsification of selling plant materials adulterated with cheaper products by dishonest traders is a problem. In South Africa a number of patients have become ill or died after using wrong plant or dose. It is frequently difficult or impossible to identify a plant from the root, bulb or bark form sold in most markets. Thus far, not much has been done to address this problem. Quality control of medicinal plants is therefore an important topic wherever traditional or herbal medicines are used. Planar chromatography has been used widely to verify the identity of phytomedicines used in the western herbal industry. Thin layer chromatography (TLC) atlases are available for many herbal medicines. This study proposes to use TLC to verify the identity of plant species of the bark and bulb material sold in Pretoria informal market.

After interviewing herbal traders and healers, plants were selected on the basis of availability, cost, toxicity and accessibility. Reference plant materials based on the traditional name provided were obtained from the Pretoria National Botanical Garden and Agricultural Research Council. Powdered material of the market and reference species were extracted with three solvents (ethanol, acetone and hexane) and final crude extracts separated in three TLC systems (polar, intermediate and non-polar systems) and the compound composition was detected using three spray reagents. As an indication of biological activity, the antibacterial activity of the selected materials was determined using four bacteria by bioautography and minimum inhibitory concentration methods.

In general, the plant materials sold by different traders in Pretoria had a similar chemical profile to the reference samples, although there were variations in the chemical profile of the same species from different areas. Although TLC technique is useful in the identification of traditional plants, it is not able to differentiate closely related plant species due to the similarities in chemical compositions and slight variation. Environmental factors did not have a major impact on the on the chemical composition of *Artemisia afra*. In conclusion, identification of traditional medicines by planar chromatography is possible, although it may be complicated by chemical variation and geographic differences. Planar chromatography can also be used to determine the magnitude of adulteration in markets that sell African traditional medicines and to determine the identity of illegally collected over-

exploited plant medicines. It therefore appears that plants sold in the Pretoria Traditional medicine market are correctly identified.

Samevatting

Suid-Afrika beskik oor meer as 9% van die wêreld se hoërplante spesies. Baie van hierdie plante word gebruik vir behandeling van mens- en diersiektes. Dikwels is die doel van verskaffers van medisinale plante eerder om geld te maak en nie om 'n essensiële diens te lewer nie. Omdat dit prakties onmoontlik is om gemaalde plantprodukte te identifiseer, het dit dikwels in die buiteland gebeur dat die etiket van westerse kruiemedisyne nie ooreenstem met die plantmateriaal in die houër nie. In Suid-Afrika is pasiënte al vergiftig omdat die verkeerde plant of dosis gebruik is. Dit is dikwels onmoontlik om 'n plant van die wortel, bol of bas wat verkoop word, te identifiseer. Min is tot dusver gedoen om die omvang van die probleem aan te spreek. Gehaltebeheer van tradisionele medisinale plante is gevolglik 'n belangrik onderwerp. Dunlaag chromatografie [DLC] is al in die buiteland gebruik vir die identifisering van westerse kruiemedisyne en DLC atlasse is beskikbaar. In hierdie studie is beplan om DLC te gebruik om te bepaal tot watter mate die plante wat in Pretoria verkoop word, korrek geïdentifiseer is.

Na onderhoude met verkopers en gebruikers van tradisionele plantmedisyne, is plante uitgesoek op basis van beskikbaarheid, koste, toksisiteit en gemak om te versamel. Verwysings plantmateriaal is vanaf die Pretoria Nasionale Botaniese Tuin en Landbou-navorsingsraad verkry. Gemaalde materiaal is ge-ekstraheer met drie ekstraheermiddels met verskillende polariteite en geskei deur silika gel dunlaagchromatografie. As maatstaf van biologiese aktiwiteit is antibakteriese aktiwiteit bepaal deur minimum inhiberende konsentrasie te bepaal met vier bakterieë. Deur bio-outografie is die verskeidenheid en eienskappe van die inhiberende verbindings ook bepaal.

Oor die algemeen het die chemiese profiel en antibakteriese aktiwiteit van die materiaal wat verkoop is, ooreengestem met die verwysingsmateriaal. Hoewel DLC bruikbaar is in die identifikasie van verskillende plante werk dit nie goed vir na-verwante plante nie. Omgewings faktore het nie 'n baie groot invloed gehad op die chemiese samestelling van *Artemisia afra* nie. DLC was ook bruikbaar om plante wat bedreig is en onwettig versamel is te identifiseer vir moontlike vervolging van oortreders. Dit blyk dus dat plante wat op die Pretoria tradisionele medisyne mark verkoop word, korrek geïdentifiseer is.

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Abbreviations

µl:	Micro liter
A:	Agricultural Research Council sample
AA:	Acetone-acetone extract
ACBB:	<i>Acacia caffra</i> Pretoria National Botanical Garden sample
Ace:	Acetone
ACMB:	<i>Acacia caffra</i> market sample
AE:	ARC ethanol extract
AH:	ARC hexane extract
ArAg:	<i>Artemisia afra</i> private garden sample
ArAml:	<i>Artemisia afra</i> maize land sample
ArARC:	<i>Artemisia afra</i> Agricultural Research Council sample
ArAveld:	<i>Artemisia afra</i> veldt sample
ArBB:	<i>Artemisia afra</i> Pretoria National Botanical Garden sample
ArBG:	<i>Artemisia afra</i> Pretoria National Botanical Garden sample
ARC:	Agricultural Research Council
ArDg:	<i>Artemisia afra</i> market sample
B:	Pretoria National Botanical Garden sample
BA:	PNBG acetone extract
BAC:	PNBG <i>Acacia caffra</i>
BAK:	PNBG <i>Acacia karoo</i>
BAM:	PNBG <i>Acacia Montana</i>
BE:	PNBG ethanol extract
BEA:	Benzene, ethanol and ammonium in ratio of 9:1:0.1 respectively
BH:	PNBG hexane extract
BHAb:	<i>Boophane haemanthoides</i> Agricultural Research Council sample
BHMb:	<i>Boophane haemanthoides</i> market sample
BPA:	PNBG <i>Peltophorum africanum</i>
CEF:	Chloroform, ethyl acetate and formic acid in ratio of 5:4:1 respectively
CMM:	Raw Chinese Medicinal Material
COX-1:	Cyclo-oxygenase-1
CPM:	Chinese Proprietary Medicine
CSBB:	<i>Croton sylvaticus</i> Pretoria National Botanical Garden sample

CSMB:	<i>Croton sylvaticus</i> market sample
CTM:	Chinese Traditional Medicine
D:	Fertilized soil sample
<i>E. coli</i> :	<i>Escherichia coli</i> .
EA:	Ethanol-acetone extract
EE:	Ethanol-ethanol extract
EMW:	Ethyl acetate, methanol and water in ratio of 10:1.35:1 respectively
<i>Entero</i> :	<i>Enterococcus faecalis</i>
EtOH:	Ethanol
GC:	Gas chromatography
H:	Shade sample
HA:	Hexane-acetone extract
Hex/Hax:	Hexane
INT:	ρ -iodonitrotetralium violet
J:	Pyrethrum-treated sample
L:	Home garden sample
LC:	Liquid chromatography
M:	Maize land sample
MA:	Market acetone extracts
MAC:	Market <i>Acacia caffra</i>
ME:	Market ethanol extract
mg/ml:	Milligram per milliliter
MH:	Market hexane extract
MIC:	Minimum inhibitory concentration
MPA:	Market <i>Peltophorum africanum</i>
N:	Seed source sample
NP/PEG:	1% Diphenylboric acid –2 amino ethyl ester in methanol/ 5% Polyethylene glycol
PABB:	<i>Peltophorum africanum</i> Pretoria National Botanical Garden sample
PAMB:	<i>Peltophorum africanum</i> market sample
PNBG:	Pretoria National Botanical Garden
<i>Psuedo</i> :	<i>Psuedonomas aeruginosa</i>



RA:	Reference acetone extract
RE:	Reference ethanol extract
R _f value:	A ratio of the distance from the origin to the center of the separated zone divided by the distance from the origin to the solvent front.
RH:	Reference hexane extract
RP:	Reverse phase
rpm:	Revolutions per minute
S:	SeSotho
SATM:	South African Traditional Medicine
<i>Staph:</i>	<i>Staphylococcus aureus</i>
T:	Private garden sample
TLC:	Thin layer chromatography
UV:	Ultraviolet
V:	Veldt sample
WSBE:	<i>Warburgia salutaris</i> Pretoria National Botanical Garden sample
WSLE:	<i>Warburgia salutaris</i> Pretoria National Botanical Garden leaf sample
WSMB:	<i>Warburgia salutaris</i> market sample
Z:	IsiZulu

Conference presentations

1. J. Manana and J. N. Eloff [Poster] July 2000. Can planar chromatography be used to identify commonly used herbal medicines in the Pretoria area? Indigenous Plant Use Forum, Nelspruit, South Africa
2. J. Manana and J. N. Eloff [Paper] January 2001. The use of planar chromatography to identify commonly used herbal medicines in the Pretoria area. Conference South African Association of Botanists. Rand Afrikaans University, Johannesburg, South Africa.
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1 General Introduction

Plants are an important source of modern pharmaceuticals, some of which may be difficult or impossible to synthesize (Bateman *et al.*, 1998). South Africa is endowed with a large variety of plants, accounting approximately 10% of the world's flowering plant species (Kirsten and Reid, 1982). Many of these plants are used for medicinal purposes. South Africans have been using plants as sources of medicine for a long time, although the first records of Xhosa and Zulu medicinal plants were only published in 1885 (Hutchings, 1989). Watt and Breyer-Brandwijk (1962) initiated research on traditional medicines, which was later expanded by many other workers including Hutchings *et al.* (1989) and van Wyk *et al.* (1997). Nevertheless, it is still believed that many of such plants and knowledge are kept secret by traditional medicine practitioners. Not long ago, Wagner and Bladt (1996) made use of the technique 'Thin layer Chromatography (TLC)' in quality assessment of western medicine. This technique is now also used to assess traditional medicines, e.g. Chinese Traditional Medicines (CTM)

Currently, studies are conducted worldwide in many research areas to determine pharmacological effects of traditional medicine (Rabe and van Staden, 1997). The first efforts made to identify and isolate chemical constituents of plants medicine were performed in the nineteenth century; for example quinine, an anti-malaria agent was isolated from the bark of *Cinchona* species (Lambers *et al.*, 2002), morphine and codeine were obtained from the opium poppy (*Papaver somniferum*) (Fisher *et al.*, 1995) and atropine from deadly nightshade (*Atropa belladonna*) (Rothe *et al.*, 2001). In most instances, compounds isolated were found to be responsible for known uses of traditional medicines (Bell, 1993).

The main chemical difference between isolated pharmaceuticals and traditional medicines is that the former consists of one or more known compounds, whereas traditional remedies have many unknown compounds present in dilute solution (Robbers and Tyler, 2000).

Several methods have been designed to evaluate the biological activity (e.g. bioautography) and chemical composition (e.g. Thin Layer Chromatography) of plant medicines. When assessing plants for biological activity and chemical composition, it should be considered that both these parameters might vary according to the collection time, season, geographical location and the plant part analyzed. Traditional healers generally prefer to use stem bark because its availability is not seasonal, and it is easy to harvest (Tagwireyi *et al*, 2002). Studies have shown that there is a difference in chemical composition between different parts of a plant (Chakraborty and Brantner, 1999). The extensive use of traditional medicines, especially in Africa, has resulted in several problems. The problems include exploitation of bark materials, substitution of rare plant species with plant species looking similar to the rare species and poisoning. The next section deals with the implications of exploitation of such plant medicines.

1.1 Problems with traditional medicines

Over-exploitation of commonly used medicinal plants has become a major problem in the country. The economic exploitation of South Africa's rich natural plant resources is limited. Presently, the indigenous flower industry has relatively successfully established small and medium scale entrepreneurs (Mander *et al.*, 1995). The demand for traditional medicines as alternatives to orthodox medicines has increased. As a consequence of this, unsustainable rate of plant harvesting has frequently resulted in some wild species already being threatened by extinction (Anandhi *et al.*, 2002). The use of plants as medicines may result in extinction of parts

of the valuable flora of South Africa. The result is that popular plant species are becoming scarce to such a degree that the collectors turn to plants in conserved areas for supplies. Illegal and uncontrolled stripping of barks for medicinal purposes has affected the status of natural forests and the natural habitats of South Africa (Kareiva, 2001).

Conservation officials from these sectors are concerned about the uncontrolled utilization of medicinal plants in South Africa's parks and reserves. Certain plant species have become over-exploited resulting in a general shortage of supply. This in turn appears to have led to escalating cost. Plants in very high demand such as *Siphochilus aethiopicus* [wild ginger] might have to be produced commercially (Rhainds, *et al.* 2002). Another case involves *Ocotea bullata* (Stinkwood), which is an important traditional medicine in KwaZulu-Natal. This plant species has become over-exploited in the province and throughout the country (Zschocke *et al.*, 2000a).

People of South Africa have turned to *Cryptocarya* species namely *C. latifolia* and *C. myrtifolia* as a substitute for *O. bullata*. These species are in turn threatened as their demand increases (van Staden and Zschocke, 2000). Since the country's natural flora of popular plants is facing over-exploitation, the ability to identify popular medicinal plants in any form (i.e. root, bark) is important for enforcing conservation measures and developing policies.

Another problem faced with traditional medicines is poisoning. Toxicity related to traditional medicines and food plants has been a problem in the country for a long time (Savyah, *et al.* 2002). Brandt *et al.* (1995) reported poisoning incidents in patients admitted at Ga-Rankuwa Hospitals, in the province of Gauteng, South Africa, that resulted from ingestion of plant medicines. However, there are few incidents reported and recorded. Effects of plant toxins may only become clear after prolonged ingestion of a plant or plant products. Under such circumstances, it may not be easy to relate the physiological effect to the plant or, subsequently,

to identify the specific toxin or plant (Bell, 1993). The implication is that poisoning incidents may also be caused by insufficient ethnobotanical knowledge and law enforcement (Robbers and Tyler, 2000). Some communities have medicinal plants in their private gardens and the South African Government has implemented no restrictive regulations. This may lead to individuals with limited ethnobotanical training collecting plants with a similar appearance to those used for medicinal purposes. These plants may have completely different physiological effects from the desired plants or may even be toxic.

Use of herbal medicines in developed countries is also common (Edgar, *et al.* 2002). Poisoning incidents relating to herbs rarely occur in these countries, since their products undergo a degree of quality control and incidents of toxicity has to be reported.

Aristolochia species contain aristolochic acid, which is one of the natural products known to have potent carcinogenic effects (Ong and Woo, 2001; Stoborova *et al.*, 2001). Consumption of products with aristolochic acid resulted in several life-threatening adverse events e.g. two patients in the United States of America were reported to have developed end-stage renal disease because of the use of botanical preparations containing aristolochic acid (Huang *et al.*, 1997).

In South Africa's big cities like Pretoria, Durban and Cape Town, traditional medicines are openly sold in markets called Muthi markets in the form of bark, roots and leaves etc. These plant medicines do not undergo any quality assessment before commercialization, therefore, no one knows if the correct plant medicines are circulated from traders to consumers. It has been observed that plant medicines demanded by a large population become short in supply and consequently expensive with time. In general, these poisoning incidents were a result of either misidentification, ingestion of incorrectly prescribed medicines or deliberate substitution. There

is a need for a mechanism to determine the authenticity and quality of traditional medicine. This is one of the objectives of the present study.

Adulteration appears to be another problem with traditional medicines or herbal products (Bateman, *et al.*, 1998). “Adulteration” means to make something impure, to contaminate (Collins, 1993). Huang, *et al.* (1997) refers to adulterated products as medicines that do not contain chemical substances labeled as part of the contents.

Dishonest traditional healers and traders may substitute rare medicinal plants with other plant species with a similar appearance. The material used for substitution may contain toxic compounds that can result in fatality.

Chinese Traditional Medicine (CTM) has made a major impact on health issues in East Asia for over the last 5000 years. CTM are grouped into two categories namely: raw Chinese Medicinal Material (CMM) and Chinese Proprietary Medicine (CPM). CMM are medicines used in their natural form and are usually subjected to simple processing (cutting and drying) whereas CPM are plant medicines that have been formulated into tablets, pills, or mixtures (Li *et al.* 2003). The governments of Asia impose minimal quality control on CMM as compared to CPM because formulated medicines are often adulterated with western medicine or other ingredients (Tomlinson *et al.*, 2000).

Adulteration of CTM has been reported in various occasions and is a public health concern in Taiwan. In 1997, Huang *et al.* (1997) conducted a quality control study on 2609 samples collected from eight major general hospitals, following the established standard procedures. The results showed that 23% of the samples contained adulterants. Half of the adulterated samples contained two or more adulterants.

CTM are sometimes found to contain heavy metals or animal parts. This is due to manufacturing problems or to the expense of procuring these species and widespread cultural beliefs in the use of animal parts as tonics (Li *et al.* 2003).

Some traditional medicines are adulterated with undeclared pharmaceutical ingredients such as caffeine, acetaminophen, indomethacin, hydrochlorothiazide, and ephedrine (Koh and Woo, 2000). Adulteration with such ingredients may lead to serious physiological complications, for instance adulteration with mefenamic acid and cadmium is associated with renal failure (Hirshon, 2001).

Wagner and Bladt (1996) developed a thin layer chromatography (TLC) method for drug quality assessment. TLC was found to be useful for assessing CTM quality standards (Jork *et al.*, 1990). The British Herbal Pharmacopoeia approached adulteration and substitution of drugs by providing monographs of quality standards for 169 commonly used herbs in the United Kingdom for the preparation of botanical drugs. In this study, an attempt is made to assess the level of adulteration in traditional medicines sold in the Pretoria area using a TLC technique.

1.2 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a separation method in which uniform thin layers of sorbent or selected media are used as a carrier medium. The first reference to TLC was in 1938 in what was called a drop chromatography on horizontal thin layers (Sgoutas and Kummerow, 1963). Little notice was taken of the method until 10 years later when two American chemists described the separation of terpenes in essential oils by thin layer chromatography (Touchstone and Dobbins, 1983). However, the procedure was not generally accepted in its early years because of the lack of media and apparatus for coating plates (Ulrich, 1966). The effectiveness of

the technique for separation was publicized when Stahl (1969) described equipment and efficient sorbents for preparation of plates in his book “Thin Layer Chromatography”. Today, TLC is still one of the most popular and widely used separation techniques.

TLC uses sorbents such as silica gel, which is the most popular layer material and is slightly acidic in nature. A binding agent such as calcium sulfate hemihydrate is used to hold the silica gel (silicic acid) onto the support. Two ultraviolet (UV) indicators, zinc and sodium salts may be incorporated either singly or together with the silica gel to aid in the location of separated substances. Zinc silicate fluoresces when exposed to UV light of 254 nm wavelength, so that substances absorbing this wavelength such as aromatic compounds appear dark with extinguished greenish-yellow fluorescing background (Ziegler *et al.*, 2001). The sodium salts of hydroxypurene sulfonic acids fluoresces at 366 nm and provide a contrast background for substances that absorb at this frequency (Hahn-Deinstrop, 2000; Jork *at al.*, 1990).

Alumina (aluminum oxide) is also widely used as a sorbent and is chemically basic. Silica gel separates large quantities of material as compared to alumina. Alumina is more chemically reactive than silica gel, so care must be exercised with some compounds to avoid decomposition or rearrangement of these substances during sample application (Touchstone, 1983).

Kieselguhr is chemically neutral and does not separate compounds as well as alumina, although it is used mainly as the support for the stationary phase in partition chromatography. The sorbent cellulose is used in paper chromatographic separation. Table 1.1 lists the most common TLC sorbents, the typical compound application for each sorbent and the major chromatographic mechanism.

Table 1.1 Sorbent materials and mode of separation (Touchstone and Dobbins, 1983)

Sorbent	Chromatographic Mechanism	Typical application
Silica gel	Adsorption	Steroids, amino acids, alcohols, hydrocarbons, lipids, aflatoxins, bile acids, vitamins and alkaloids
Silica gel RP	Reverse phase	Fatty acids, vitamins, steroids, hormones and carotenoids
Cellulose, Kieselguhr	Partition	Carbohydrates, sugars, alcohols, amino acids, carboxylic acids and fatty acids
Aluminum oxide	Adsorption	Amines, alcohols, steroids, lipids, aflatoxins, bile acids, vitamins and pyrimidines
Magnesium silicate	Adsorption	Steroids, pesticides, lipids and alkaloids

Adsorption is used to separate highly non-polar, hydrophobic (fat soluble) substances with non-polar mobile phase solvents, whereas partition may be used for polar, hydrophilic (water soluble) substances with polar mobile phase solvents.

Several factors must be considered before development, for example the polarity of both the mobile phase and the sample. In addition, the environment where the experiment is conducted should not be humid or dusty because these will interfere with the development and the visualization of the plates. TLC plates should also be protected from solvent vapours in the laboratory environment (Jork *et al.*, 1990).

In practice, a sample to be separated is applied on the coating 1-2 cm from the one end of the plate. The edge of application is called the starting point or origin. Separation is achieved by passing a solvent, the mobile phase through the coat (layer). The layer, with the sample zone at the bottom is placed on a slight angle from the vertical into a closed tank containing a small

amount of the mobile phase. The nature of the mobile phase is determined by the type of substance to be separated and the type of sorbent to be used for the separation (Jork *et al.*, 1990). The composition of the mobile phase can be as simple as a single, pure solvent (e.g. benzene is used to separate dye on alumina) or as complex as three to four components mixture such as a 9:1:0.1 solution of benzene (B), ethanol (E) and ammonium (A) in the BEA system. The BEA system is used in the present study to separate non-polar compounds.

Capillary action initiates the movement of the mobile phase through the medium in a process called development. Ascending development is the most common, but horizontal, descending and centrifugal methods have been described. After the developed plate is dried, the spots can be visualized in a number of ways such as viewing under ultra violet (UV) light, or spraying with one of the wide variety of reagents. After visualization, many experiments can be considered complete, although other experiments such as bio-autography can be performed on the basis of TLC detection. The distance travelled by the compounds represented as a spot can be calculated in terms of the differential retention (R_f) value.

The R_f value is a convenient way of expressing the position of the substance on a developed chromatogram. It is calculated as the ratio that varies between 0 and 1.0 and it is constant under reproducible conditions. The R_f can be represented mathematically as follows:

$$R_f = \frac{\text{Distance of compound from origin}}{\text{Distance of solvent front from origin}} \quad (1.1)$$

The distance is measured to the centre of the eluted spot. There are factors affecting the R_f value that should be considered during the development process. For instance, the R_f value can change to a higher range if the chamber is not saturated and the solvent evaporates from the

plate. The concentration and the complexity of the solute applied can also affect the R_f value. Highly concentrated samples may make a smear not a clear spot without any separation. Therefore, the R_f value may become difficult to measure and would be worthless.

1.2.1 Advantages of TLC

TLC has many advantages over other chromatographic methods such as liquid chromatography (LC) and gas chromatography (GC). It uses less solvent for the development of plates compared to LC and the apparatus required are several orders of magnitude cheaper, which makes it a relatively cheap and low technology. The polarity of the solvent can be changed in about 5 minutes. TLC is the easiest chromatographic method to set up because of the short development time and easy change of mobile phase. The most advantageous feature of TLC as opposed to other chromatographic methods is the number of samples that can be handled simultaneously. GC and LC are limited to the analysis of a single sample at a time whereas as many as 22 samples can be applied to a single 20 x 20 cm TLC plate.

TLC is used in the pharmaceutical industry for the identification, purity testing and determination of the concentration of active ingredients, auxiliary substances and preservatives in drugs in synthetic manufacturing processes (Hahn-Deinstrop, 2000). In biochemistry, TLC is used to determine active substances and their metabolites in medical diagnosis. It is also used for the diagnosis of metabolic disorders such as cystinuria, a condition whereby large amounts of cystine are excreted in the urine (Wills, 1985). In cosmetology, TLC is used to analyze constituents of perfumes as well as separating crude material to yield isolated compounds. This technique is widely used for research purposes in food monitoring and environmental analysis (Hahn-Deinstrop, 2000). In environmental monitoring, groundwater and air are analyzed for

pollutants. Ntloedibe (2001) developed a relatively simple TLC technique that has been useful in distinguishing more than 80 western herbal medicines. The same technique will be applied in this study.

1.3 Chemotaxonomy and fingerprinting of plants

Chemotaxonomy of plants is the classification of plants based on their chemical constitution (Smith, 1976). Chemical variation studies were suggested to be one of the principle growing points in the field of taxonomy. There are many factors that give rise to differentiation in a plant's chemical composition. The number and composition of classes of compounds such as alkaloids, flavonoids, essential oils and isoprenoids vary in species and habitats. The variation thus allows the use of chemical composition as a tool for classification of plants to complement botanical classification based mainly on morphology. There are, however, factors that influence the accuracy of classification based on the chemistry of the plants namely: age of the plant, geographic location, ecological habitat and genotypic polymorphism. Influences of these factors on the chemistry of a plant are observed only if the concentration of chemical compounds is sufficient for detection. Plants contain thousands of compounds but the concentration and the polarity of the compounds influence the detection of these compounds by the applicable methods such as TLC (Robbers and Tyler, 2000).

TLC fingerprinting is a good tool for chemotaxonomic classification of plant species, although there are limitations, as some closely related species may be difficult to distinguish. In such cases, the classification based on morphological parameters may be used to differentiate related species. An example is aristolochic acid. Its presence in many plant species and its

toxicology has facilitated the classification of *Aristolochiaceae* species, as a family-characteristic metabolic end product (Hegnauer, 1986).

1.4 Problem statement and objectives

After conducting a literature review, it was established that a large section of South Africa's population depends on traditional medicine (Steyn and Muller, 2000) and that worldwide between 70 to 80% of people use herbs (Griffiths, 1999). This chapter also indicated that the use of traditional medicine is fraught with problems, such as misidentification, over-exploitation, adulteration and substitution of traditional medicines. Based on these problems, this study aims to:

- Identify commonly used traditional medicines in the Pretoria area.
- Develop techniques for authentication and quality assessment of at least 6 plant medicines commonly used in the Pretoria area.
- Determine the magnitude of adulteration and substitution in the Pretoria markets
- Determine the influence of environmental changes on the chemical composition of the *Artemisia afra* species grown in different areas that originated from the same seed source.
- Provide preliminary data on antimicrobial activity of these species
- Compile a TLC fingerprint of the bark samples of over-exploited traditional medicines.

The results of the objectives outlined above are described in Chapter 3 and the approaches followed to accomplish the investigations are given in Chapter 2.

1.5 Conclusion

After a literature review conducted as part of this study, it was found that the use of traditional medicines in South Africa has problems. These include incomplete database of traditional medicines in the Pretoria area, accidental poisoning associated with the intake of such medicines, over-exploitation and substitution of traditional medicines. The next chapter will introduce the methods followed to accomplish the objectives given in Section 1.4

2 Materials and methods

2.1 Introduction

As previously stated in Chapter 1, this study is interested on the identification of medicinal plants that are used in Pretoria area. The aim of this chapter is to give a detailed description of all the experiments followed in this thesis.

2.2 Selection of commonly used medicinal plants in Pretoria area

The information-gathering exercise was conducted by interviewing traditional healers and traders in markets that sell African traditional medicines around Bloed and van der Walt streets in the city of Pretoria. A meeting with a group of traditional healers, traders, collectors and Agricultural Research Council (ARC) conservation officials was attended in Roodeplaats in the year 2000. This assisted in obtaining further information on traditionally used medicines. This meeting discussed a collaborative project between ARC and the traditional healers. During these interviews, information on the identity, use, history of poisoning incidents, cost and plant parts often used, was gathered for plant species commonly used in the Pretoria area. A questionnaire, appearing in the appendix was used during these interviews.

Points were allocated to prioritize the traditionally used medicines. Plant species associated with poisoning or accidental deaths were categorized as most important in this investigation as they pose a threat to the community. These plants were allocated 10 points whereas those that had no such reports were allocated 5 points.

The more difficult it is to obtain a plant part, like digging roots and stripping bark from a tree, the more expensive the materials could be, compared to harvesting leaves. Therefore,

medicines in which the whole plant was used were allocated 10 points, whereas roots, bark, and leaves were allocated 10, 8 and 4 points, respectively.

Only bark materials of the plant samples identified as commonly used, were bought from the markets in the Pretoria city. Reference samples of plants bought from the market were collected from the Pretoria National Botanical Garden (PNBG) and Agricultural Research Council (ARC) in Roodeplaat. These plant samples were used in the laboratory for identification and further investigations.

2.3 Processing of plant materials

All the plant materials were bought around Central Pretoria in the month of May and August 2000 and once in the laboratory, the materials were processed in a similar manner. Fresh plant materials in the form of tree bark collected from the market, ARC and PNBG were cut into small pieces with a side cutter. The pieces were then dried at room temperature in the shade. The insect damaged and contaminated materials were excluded. The sample pieces were then ground to a fine powder in a mill (Kika-labortechnik, Janke and Kunkel). The weights of different materials were determined using a scale (Ohaus corporation) then stored in glass bottles in the dark until required. The powder of the samples was extracted to analyze its chemical composition.

2.4 Thin layer chromatography (TLC) analysis

The powder was divided into three (1.0 gram) and then extracted with three solvents of different polarities: ethanol (polar), acetone (intermediate polarity) and hexane (non-polar). Ten ml of each extracting solvent was added to the powder, which was shaken vigorously (Labotech-

shaking machine) for 5 minutes then centrifuged (Hettich zentifugen) at $\pm 3500 \times g$ for another 5 minutes. The supernatant was transferred to a clean weighed container. The extraction procedure was repeated two more times on the same plant materials and the marc was discarded.

The supernatant was dried in a laminar flow at room temperature. The weight of the crude extract was determined then redissolved in acetone (Merck) [since it dissolves both polar and non-polar compounds] or where necessary in absolute ethanol [Merck] to make up a final concentration of 10 mg/ml. These stock solutions were used for TLC analysis.

Three freshly made separation systems of various polarities were prepared in advance for use in TLC analysis as shown in Table 2.1 below.

Table 2.1 Separation systems used in TLC analysis

Separation system	Ratio
BEA: non-polar, basic	80 (benzene): 10 (ethanol): 1 (ammonium)
CEF: intermediate polarity, acidic	4 (chloroform): 3 (ethyl acetate): 1 (formic acid)
EMW: polar, neutral	10 (ethyl acetate): 1.35 (methanol): 1 (water)

Aliquots of 5 μl of concentrated extracts were spotted onto the silica gel plates (Macherey-Nagel, alugram/UV), 1 cm from the bottom of the plate. The plates were then allowed to develop in the TLC tanks until the mobile phase reached about 1 cm from the top of the plate. The plates were then taken out of the tanks, dried and then visualized under UV at 254 and 350. The visualized plates were then sprayed with one of several spray reagents shown in Table 2.2. In the case of autography method, once the plates were developed, and before they were sprayed with visualizing reagents, they were dried overnight and prepared as described in Section 2.5.

Table 2.2. The principles of detection reagents (Stahl, 1969)

Spray reagent	Method of preparation	Detectable compounds
Anisaldehyde-sulphuric acid (H ₂ SO ₄)	1 ml each of sulphuric acid and <i>p</i> -anisaldehyde in 18 ml of ethanol.	Sugars, steroids and terpenes
Natural (NP/PEG)		Flavonoids
Phosphoric acid (H ₂ PO ₄)	15 ml 85% phosphoric acid diluted to 100 ml with methanol.	Steroids
Trichloroacetic acid	25% trichloroacetic acid in chloroform.	Steroids
Toluene	20% solution of <i>p</i> -toluenesulphuric acid in chloroform.	Steroids, flavonoids and catechins
Vanillin phosphoric acid (H ₂ SO ₄)	1% solution of vanillin in 50% aqueous phosphoric acid.	Steroids
Vanillin sulphuric acid (H ₂ SO ₄)	1 ml sulphuric acid in a solution of 0.1 g vanillin in 28 ml of methanol.	High alcohols, phenols, steroids and essential oils

2.5 Bio-autography

Bio-autography is a method used to locate antibacterial activity on a chromatogram. This technique has been used widely in the search for new antibiotics (Hamburger and Cordell, 1987). Chemical compounds extracted from the powdered materials of plant species were separated on TLC plate in three mobile systems (see Section 2.4). Developed TLC plates were carefully dried under flow of air overnight for complete removal of the solvents. Any residual solvent from the plates may inhibit the growth of bacteria, therefore, leading to false results.

Internationally accepted strains of all bacteria in Table 2.3 were received from the Medical Microbiology Department at the University of Pretoria and then cultured in nutrient broth every two weeks.

Table 2.3 Details of microorganisms used to test the biological activity of medicines

Micro-organism	Bacterial type	Bacterial strain
<i>Enterococcus faecalis</i>	Gram positive	ATCC 29212
<i>Escherichia coli</i>	Gram negative	ATCC 25922
<i>Staphylococcus aureus</i>	Gram positive	ATCC 29213
<i>Psuedonomas aeruginosa</i>	Gram positive	ATCC 27853

Fresh cultures of the bacteria that were prepared by Laboratory Technician were centrifuged at $\pm 5300 \times g$ for 20 minutes to concentrate the bacteria. The supernatant was discarded and the bacterial cells re-suspended in fresh nutrient broth. The suspension was dispersed evenly over the TLC plates using a spray gun. The TLC plates were then incubated overnight in a 70% humid atmosphere at 37°C. Dehydrogenase-activity detecting ρ -iodonitrotetralium (INT) violet (Sigma-Aldrich) reagent (2 mg/ml) was evenly sprayed onto the plates and care was taken to minimize airborne contamination at all times. INT is a colourless tetrazolium salt that is converted into a coloured formazan by metabolically active bacteria (Hamburger and Cordell, 1987).

After one-hour incubation, plates sprayed with INT were scanned for later reference. Compounds with anti-bacterial activity appear as clear spots against a pink background.

2.6 Minimum inhibitory concentration (MIC) and total activity

Distilled water (100 μ l) was added into each well of the micro-plate, then 100 μ l of the extracts (10 mg/ml) was added into the first well and then mixed thoroughly with water. The concentration in these wells was half (5 mg/ml) the stock concentration. Half of the volume (100 μ l) from the first well was transferred to the next well and mixed thoroughly, giving it a

concentration of 2.5 mg/ml. The serial dilution was carried on across the plate until the final concentration was 0.04 mg/ml. The last extra volume of 100 μ l was discarded from the final well. A volume of 100 μ l of each strain of bacteria in Table 2.3 was added into each well, mixed and incubated at 37° C for overnight in a 100% relative humidity. Forty microliters of 0.2 mg/ml INT (Sigma-Aldrich) was added into each well the following day, incubated at 37°C for 30 minutes and then viewed at 30-minute intervals for 2 hours (Eloff, 1998). This experiment was carried in laminar flow cabinet and spills were cleaned with 70% ethanol.

The experimental procedure followed in this study is illustrated as shown in Figure 2.1. This figure gives the order in which the experiments were done. The experiments conducted were: the selection and collection of commonly used traditional medicines in the Pretoria areas; chemical processing of the selected materials; TLC analysis of extract; determination of MIC values and total activity; bio-autography analysis and documentation of results.

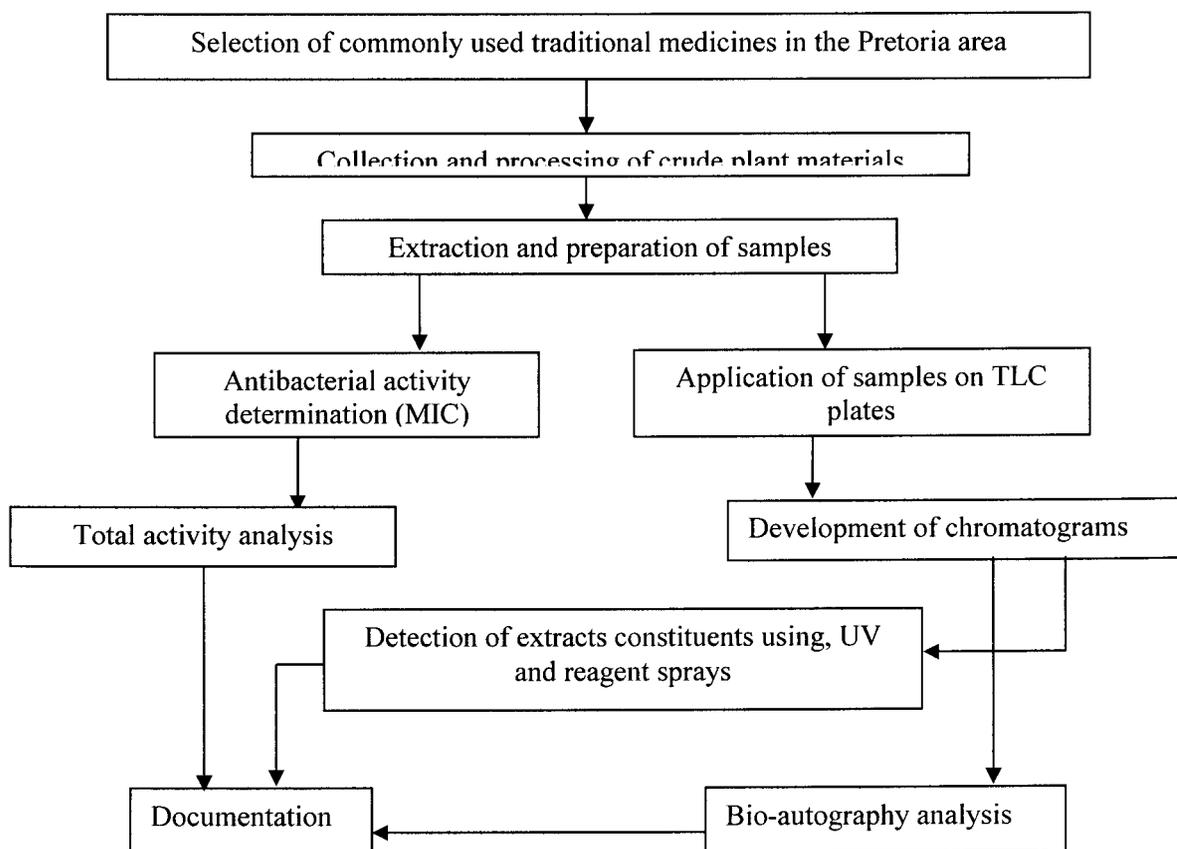


Figure 2.1. The schematic illustration of experimental procedures followed in this study.

2.7 Conclusion

In this chapter, the experiments used in this study are described. The order in which the methods were used is outlined. The use of the experiments was determined by the objectives of each investigation. The next chapters give the objectives and results of the investigations conducted.

3 Selection of commonly used traditional medicines in the Pretoria area

3.1 Introduction

The knowledge of traditional medicine in South Africa is passed orally from one generation to another (Bateman, *et. al.*, 1998). Different traditional plants are currently sold at the traditional medicine markets in South African towns and cities. These plants are used for physiological and spiritual disorders. Traditional medicinal plants in Pretoria markets are identified by African names mainly South seSotho and isiZulu and not by scientific names. In many cases, the same plant species are given more than one African name or different species identified by the same African name (Brandt *et al.*, 1995). The multiple naming of traditional medicines frequently leads to confusion.

Some of the traditional medicines in Pretoria street markets are collected from KwaZulu-Natal, Limpopo Province and the Cape by traders or by general collectors in the province of origin. There might be contamination or a mix up of species during their transportation from collector to trader or healer. After exhaustive literature review conducted as part of this study, it was found that studies on whether these medicinal plants are of the correct taxa have not been conducted. This research contributes to the literature by studying the chemical characteristics of traditional medicines so that this information can be used to assess the effect of the medicines and be used as package insert.

The aim of this chapter was to collect data on the state of commonly used traditional medicines in the Pretoria area (in order to record all popular traditional medicines in this area). This was achieved by obtaining African and scientific names of common traditional medicines,

their traditional use and geographical distributions in the country through interviewing traditional healers and traders and through published literature. This survey conducted concentrated on species that are in high demand by the community. This demand may cause suppliers to extract unfair profit by adulterating (see Section 1.1). The next section gives the results obtained from investigation conducted in a manner that was explained in Section 2.2.

3.2 Results and discussion

The outcome of the interviews conducted was a variety of 59 plant names; of which 38 were published in literature. The scientific names of the remaining 21, which are listed in Table 3.1 could not be established. Table 3.2 lists the African names of the 38 plant species, their use, the part of the plant often used, scientific name, family names and the total scores allocated to indicate their priority. These species are arranged from high score, indicating high priority to low score indicating low priority. This information has been collected from Hutchings (1989), van Wyk, *et al.* (1997) and van Wyk and van Wyk (1997).

The information in Table 3.1 was obtained from traditional traders and healers. It is possible that the botanical identity of these plant species is known although the African names were not found in available documents. The manner in which the medicines are sold makes it difficult if not impossible to distinguish amongst the materials traded, such that the same plant medicine can be misnamed without notice by the buyer.

Table 3.1 African names and medicinal uses of plant species that could not be identified from African name

No.	African name Sotho/Zulu	Toxicity	Plant part used	Medicinal uses
1	Bothobotlho (S)	No	Bark	love charm
2	Hlonya (S)	No	Bulb	constipation
3	Ibohlole- mandawu *	No	Bark	dream reminder, treat lung diseases
4	Igobinkosi (Z)	No	Root	good luck
5	Ihlunguhlungu (Z)	No	Bark	heart dysfunctions
6	Imbosisho*	Yes	Roots	diarrhoea
7	Isibhaxha (Z)		Bark	sore throat
8	Kgashi *	Yes	Bark	HIV/AIDS treatment
9	Lehabe (S)	No	Bark	dropsy, sores, HIV
10	Lethwele (S)	No		menstrual problems, drop, heart and lung problems
11	Loeto latlou (S)	Yes	Bulb	purgative
12	Makgurumetsa (S)	No	Bulb	menstrual problems
13	Maroke (S)	No	Bulb	luck charm
14	Mayime (S)	Yes	Bulbs	urinary problems, constipation administered through the anus, sexual stimulation, swelling and painful feet, erection, and constipation
15	Mokgaloane (S)e	No	Bark	drop and sexual discharge
16	Moruta tsusa (S)	No	Bark	coughs
17	Mpetswa (S)	No	Bulb	bad luck
18	Poha-yabashimana (S)	No	Bark	emetic
19	Sirukulu (S)	No	Root/bark	good luck
20	Umafekufeni (Z)	No	Bark	heart problems
21	Utshongwe (Z) Poo thetlha (S)	No	Bark	coughs

* Neither seSotho (S) nor isiZulu (Z)

Table 3.2 (continues). Traditional medicines commonly used in the Pretoria area

No.	Scientific name	Vernacular name	Family name	Plant part	Medicinal purpose	Total 40
19	<i>Dierama pendulum</i>	Undwendweni	Dierama	Bulb	mixtures for luck remedies.	19
20	<i>Achyroline stenoptera</i>	Imphepho	Asteraceae	Whole Plant	burnt and inhaled smoke, believed to relieve headaches and protect against evil spirits. Sold as s ritual incense.	27
21	<i>Urginea delagoensis</i>	Umahlanganisa	Hyacinthaceae	Bulb	impotency, tapeworms and roundworms. Reported to be a dangerous and poisonous. All parts of the plant are toxic and lethal, the flowering stems are more toxic than the leaves. (Hutchings <i>et al</i> , 1996)	26
22	<i>Hypoxis hemerocallidea</i>	Monna maledu inkomfe	Hypoxidaceae	Bulb	coughs, asthma, tuberculosis, prostate hypertrophy, anticancer, anti-HIV, anxiety, palpitation, rheumatoid arthritis, can be confused with <i>Hypoxis cochicifolia</i> .	26
23	<i>Glycyrrhiza glabra</i>	Mlomo-mnandi	Fabaceae	Roots	appendicitis, tuberculosis, spiritual power, antispasmodic, anti-inflammatory, ulcer and luck.	26
24	<i>Eucomis autumnalis</i>	Umathunga Mathuba difala	Hyacinthaceae	Bulb	wound, used after operations, to cleanse the digestive system, urinary diseases, stomach ache, fever, colic, flatulence, hangover, coughs, respiratory ailments and to facilitate childbirth.	26
25	<i>Elephantorrhiza lephantina</i>	Intolwane	Fabaceae	Bark	menstrual problems, oedema, diarrhea, dysentery, stomach, disorders, emetics, hemorrhoids and perforated peptic ulcer.	26
26	<i>Callilepsi laureola</i>	Impila	Asteraceae	Roots	cancer, sore throat, also used for luck, open wounds, tapeworms, infertility, snakebites, cough and traditional pregnancy.	26
27	<i>Bowiea volubilis</i>	Igibisila, sekgaga	Hyacinthaceae	Bark	headaches, oedema (dropsy), infertility, sore eyes, sterility, abortions, skin diseases and bladder complaints.	26
28	<i>Celosia trigyna L.</i>	Velabahleke	Celosia	Roots	luck, love charm, usually mixed with other love remedies.	25
29	<i>Bersama lucens</i>	Indiyaza	Melanthaceae	Bark Roots	menstrual pain, headache, stroke, nervous disorders, impotency and infertility.	25
30	<i>Myrothamnus f labellifolius</i>	Umafevuka	Myrothamnaceae	Leaves	respiratory ailment, backaches, kidney problems, hemorrhoids and painful menstruation.	24
31	<i>Vernonia natalensis</i>	Mokgalo	Asteraceae	Bark	prevent thunder damage and for luck.	23
32	<i>Lannea edulis</i>	Mophuroku	Anacardiaceae	Bark	sore eyes, boils and abscesses.	23
33	<i>Hippobromus pauciflorus</i>	Umfazi-thethayo	Sapindaceae	Bark	Luck charm	23
34	<i>Diospyros villosa</i>	Indodemnyama	Diospyros	Bark	love remedy.	23
35	<i>Dicoma anomala</i>	Thlonya	Asteraceae	Leaves	fever, influenza, high blood pressure, diarrhea, cancer, also as snuff for headaches and coughs.	23
36	<i>Harpephyllum caffrum</i>	Umgwenya	Anacardiaceae	Bark Roots	love charm, blood purification, emetics, acne and eczema. Roots are used to treat paralysis caused by poison.	22
37	<i>Allium dregeanum</i>	Uphunyuka-bemphethe	Allium	Bulb	protection against evil spirits, used also for luck and to provoke vomiting. Tastes bitter.	21
38	<i>Nelaginaceae selago</i>	Umhlabelo	Nidorella	Bark	fungal infections treatment.	20

Table 3.2 Traditional medicines commonly used in the Pretoria area

No.	Scientific name	Vernacular name	Family name	Plant part	Medicinal purpose	Total 40
1	<i>Croton sylvaticus</i>	Umahlabekufeni Umahlanganisa	Euphorbiaceae	Bark Leaves	stomachache, fever, epilepsy, toothache, and sore throat as mouthwash.	36
2	<i>Artemisia afra</i>	Lengana, wilde als, umhlonwane	Asteraceae	Leaves	fever, colds, flu, sore throat, coughs, asthma, pneumonia, headaches, gastritis, indigestion, poor appetite, flatulence, colic, earaches, malaria and intestinal worms. The roots are known as inyathelo.	33
3	<i>Warburgia salutaris</i>	Mlaka, Isibhaha	Canellaceae	Bark	diarrhea icough, ulcer, dyspepsia, diarrhea and diabetes	31
4	<i>Pentanisia prunelloides</i>	Icishamililo	Rutaceae	Root Leaves	burns, swellings, sore joints, fever, chest pain, rheumatism, heartburn, vomiting, toothache, tuberculosis, blood impurities, hemorrhoids and snake bites.	31
5	<i>Peltophorum africanum</i>	Mosetlha	Fabaceae	Bark	coughs, sore throat, fever, wounds, intestinal parasites, eye complains, dropsy, infertility, venereal diseases, abdominal pain and constipation.	31
6	<i>Alepidea amatymbica</i>	Lesokwana Ikhathazo	Apiaceae	Roots	coughs, tuberculosis bronchitis, asthma, fever, colds, influenza, sore eye, smoke is used as cigarette and bee repellent from hive.	31
7	<i>Acacia caffra</i>	Mosetlhana	Fabaceae	Leaves	cough, bronchitis, sores and asthma.	31
8	<i>Urginea sanguinea</i>	Skanama	Hyacinthaceae	Bulb	anticoagulant, stomach ulcer, hypoxia, asthma, rheumatic arthritis, cubes (kidney), skin complexion after aches, impotency. Bulb is boiled and the extract is used as a drink, overdose is toxic.	30
9	<i>Knowltonia vesicatonia</i>	Inkathazo	Ranunculaceae	Leaves	rheumatism, arthritis and gout.	30
10	<i>Boophane hymanthoides</i>	Legwama	Hypoxidaceae	Bulb	wounds, diarrhea, and skin diseases and as an anticoagulant.	30
11	<i>Zanthoxylum capense</i>	Monokwane	Rutaceae	Whole Plant	stomachache, fever, epilepsy, toothache, and sore throat as mouthwash.	27
12	<i>Vernonia adoensis</i>	Inyathelo	Asteraceae	Leaves	given to pregnant women for discomfort, used for abdominal pain, colic. This plant may be confused with <i>V. natalensis</i> .	27
13	<i>Turraea floribunda</i>	Umadlozana	Meliaceae	Leaves	heart failure, rheumatism and as an emetic. Can be confused with <i>obtusifolia</i> and <i>T. nicotica</i> .	27
14	<i>Scabiosa columbaria</i>	Ibheka	Dipsacaceae	Leaves	colic, sore throat and heartburn.	27
15	<i>Prunus africana</i>	Inyazangoma	Rosaceae	Bark Leaves	cough, bronchitis and asthma.	27
16	<i>Cotyledon orbiculata</i>	Intelezi	Crassulaceae	Leaves	earache, epilepsy and toothache.	27
17	<i>Calodendrum capense</i>	Umemezi omhlophe	Rutaceae	Bark Leaves	skin treatment.	27
18	<i>Aloe ferox</i>	Inhlaba	Asphodelaceae	Leaves	indigestion, heartburn nausea, colic, gout, boils, constipation, rheumatism, arthritis, wound, sores, rashes, burns and conjunctivitis.	27

In Table 3.2, the common names gathered from the interviews were matched with published names, and scientific identification of published common names was assumed to be of the investigated plant species. The plant species in Table 3.2 were compared to the traditional medicine recorded as commonly used in KwaZulu-Natal listed in Table 3.3 (Eloff, 1998a). Only 7 traditional medicines commonly used in both KwaZulu-Natal and Pretoria areas were identified from this comparison and they are: *Alepidea amatymbica*, *Bowiea volubilis*, *Harpephyllum caffra*, *Hippobromus pauciflorus*, *Pentanisia prunelloides*, *Urginea sp.* and *Warburgia salutaris*. This observation may, however, not represent the true common usage of traditional medicine within the provinces.

There are several possible explanations for this minor overlap between medicinal plants commonly used in these areas. For instance, according to the information on their distribution from the Pretoria National Botanical Institute, all these plant species are found in both Gauteng and KwaZulu-Natal. *Alepidea amatymbica* has two sub-species, one called ‘aquatia’ and is found in KwaZulu-Natal and another called ‘microbracteata’ is found in Transvaal.

Table 3.3 (continues). Medicinal plants used in kwaZulu-Natal for medicinal purposes (Eloff, 1998)

No.	Plant	Family	Use
25	<i>Ocotea bullata</i>	Lauraceae	urinary complaints, headaches
26	<i>Pentanisia prunelloides</i>	Rubiaceae	haemorrhoids, snakebite, rheumatism, burns, stomach pains, tuberculosis, swellings, sore joints, palpitations, boils
27	<i>Protorhus longifolius</i>	Anacardiaceae	emeticum, heartburn, bleeding from stomach, depilatory
28	<i>Rapanea melanophloeos</i>	Myrsinaceae	heart problems, palpitations, acidity, stomach and muscular pains, expectorant, emetic, enema
29	<i>Rhoicissus tridentata</i>	Vitaceae	renal complaints, sterility, epilepsy, stomach ailments, menorrhagia, indigestion
30	<i>Scilla natalensis</i>	Liliaceae	enemas, purgative, induce childbirth, boils, veld sores
31	<i>Sclerocarya birrea</i>	Anacardiaceae	malaria, diarrhea, heart problems, blood cleansing, abdominal pain, proctitis, fevers, headaches, ulcers, toothache, backache
32	<i>Senecio gregatus</i>	Compositae	enema, venereal diseases
33	<i>Senecio serratuloides</i>	Compositae	infections, sores, blood purifier, burns, swollen gums, chest pains
34	<i>Stangeria eriopus</i>	Cycadales	emetic, headaches, purgative, flatulence, blood pressure
35	<i>Trichilea dregeana & Trichilea emetica</i>	Meliaceae	stomach complaints, backache, kidney problems, blood cleanser, lumbago, intestinal worms, dysentery
36	<i>Turbina oblongata</i>	Convolvulaceae	arthritis, gout, spine pain, sores, abscesses, rheumatism
37	<i>Urginea sp.</i>	Liliaceae	rheumatism, gout, bronchitis, asthma, hoarseness, influenza, diuretics, abdominal pains, swellings, demulcent
38	<i>Warburgia salutaris</i>	Canellaceae	expectorant, rheumatism, malaria, sores, colds, coughs, venereal diseases, constipation, stomach ulcers
39	<i>Zanthoxylum davyi</i>	Rutaceae	coughs, colds, tonic, infected wounds, sore throats, mouth ulcers, boils, toothache, pleurisy, bilharsia

Table 3.3 (continues). Medicinal plants used in kwaZulu-Natal for medicinal purposes (Eloff, 1998)

No.	Plant	Family	Use
25	<i>Ocotea bullata</i>	Lauraceae	urinary complaints, headaches
26	<i>Pentanisia prunelloides</i>	Rubiaceae	haemorrhoids, snakebite, rheumatism, burns, stomach pains, tuberculosis, swellings, sore joints, palpitations, boils
27	<i>Protorhus longifolius</i>	Anacardiaceae	emeticum, heartburn, bleeding from stomach, depilatory
28	<i>Rapanea melanophloeos</i>	Myrsinaceae	heart problems, palpitations, acidity, stomach and muscular pains, expectorant, emetic, enema
29	<i>Rhoicissus tridentata</i>	Vitaceae	renal complaints, sterility, epilepsy, stomach ailments, menorrhagia, indigestion
30	<i>Scilla natalensis</i>	Liliaceae	enemas, purgative, induce childbirth, boils, veld sores
31	<i>Sclerocarya birrea</i>	Anacardiaceae	malaria, diarrhea, heart problems, blood cleansing, abdominal pain, proctitis, fevers, headaches, ulcers, toothache, backache
32	<i>Senecio gregatus</i>	Compositae	enema, venereal diseases
33	<i>Senecio serratuloides</i>	Compositae	infections, sores, blood purifier, burns, swollen gums, chest pains
34	<i>Stangeria eriopus</i>	Cycadales	emetic, headaches, purgative, flatulence, blood pressure
35	<i>Trichilea dregeana & Trichilea emetica</i>	Meliaceae	stomach complaints, backache, kidney problems, blood cleanser, lumbago, intestinal worms, dysentery
36	<i>Turbina oblongata</i>	Convolvulaceae	arthritis, gout, spine pain, sores, abscesses, rheumatism
37	<i>Urginea sp.</i>	Liliaceae	rheumatism, gout, bronchitis, asthma, hoarseness, influenza, diuretics, abdominal pains, swellings, demulcent
38	<i>Warburgia salutaris</i>	Canellaceae	expectorant, rheumatism, malaria, sores, colds, coughs, venereal diseases, constipation, stomach ulcers
39	<i>Zanthoxylum davyi</i>	Rutaceae	coughs, colds, tonic, infected wounds, sore throats, mouth ulcers, boils, toothache, pleurisy, bilharsia

Some of the species commonly used in KwaZulu-Natal are found all over the country for example *Scilla natalensis* (Arnold and de Wet, 1993) and are not listed in Table 3.2. Surprisingly, these plants could be listed under the unidentified species in Table 3.1 since they are not listed in Table 3.2. Some plant species like *Stangeria eriopus* are not found in a region formerly known as Transvaal but only in KwaZulu-Natal and South Western Cape. These two areas are also temperate compared to Transvaal since they are along the sea. Because of difference in temperatures, which is one of the variables that affect plant growth, some species will not grow at particular areas.

The plant species in Table 3.2 are organized according to their importance to society and traditional healers. This is based on their demand and unit cost. Plants that are trusted by many people for their purported efficacy might become endangered if action is not taken to preserve them. Plant species on demand may become expensive if their supply is limited. Therefore, these plant medicines have the highest possibility of being adulterated. This thesis, therefore, proposes that the plant species that are on demand should be examined. The characteristics of the selected medicines are explained in the next sections.

3.2.1 *Acacia caffra* (Fabaceae)

Common names: amaquasdoornboom (Afrikaans), hook-thorn (English), umkaya (Ndebele), muguhwa (Shona), morulthana (Tswana), umtoli (Xhosa) and umthole (Zulu)

Acacia caffra is a tree naturally occurring all over South Africa (Arnold and de Wet, 1993). This tree belongs to Group 4 of the acacias, which have hooked pair of thorns and inflorescence (McKinnon–Villa, 1996). The name *Acacia* was derived from "akis " meaning a point or barb and *caffra* means an epithet frequently bestowed on plants from the eastern parts of South Africa in previous centuries (Thomas, and Grant, 1998). *Caffra* in Hebrew means "person

living on the land" (Van Wyk *et al.*, 1997; Venter, 1996). In Zulu traditional medicine, the bark is used for blood cleansing, the root as love charm emetics and the leaf for infantile abdominal disorders. *Acacia caffra*'s young wilted twigs when removed from the tree by storm are associated with prussic acid poisoning. The bark of *Acacia caffra* is known to have tannins (Hutchings *et al.*, 1996) and is often confused with *A. ataxacantha*.

3.2.2 *Acacia karroo* (Fabaceae)

Common names: soetdoring (Afrikaans), sweet/white thorn (English), isingawa (Ndebele), mookana (Northern Sotho), munenje (Shona), moshaka (Tswana) and umunga (Zulu).

Acacia karroo belongs to the same genus as *Acacia caffra* and is the most widely distributed of all South African trees (van Wyk *et al.*, 1997). Its roots are nitrogen fixers and they make this tree ideal for planting on disturbed and poor soils (van Wyk and van Wyk, 1997). Bark decoctions are used as emetics for ailments believed to be caused by sorcery. The bark is also used to relieve stomach ache (Hutchings *et al.*, 1996). The whole tree (gum, bark, and leaves) is used for colds, diarrhoea, dysentery, conjunctivitis and haemorrhage. The chemical constituents of this tree are tannins and rhamnose (Hutchings *et al.*, 1996; van Wyk *et al.*, 1997).

3.2.3 *Artemisia afra* (Asteraceae)

Common names: wilde-als (Afrikaans), African wormwood (English) lengana (Sotho) and umhlonyane (Zulu)

The aromatic *Artemisia afra* is a very common species in South Africa and is used as a multi-purpose medicinal plant. It inherited its name from its ability to eradicate worms. The leaves are the only part mainly used, but the roots may also be used. Fresh leaves are used to treat blocked nose by insertion into the nostrils. Leaf concoctions are used for treatment of

headache, loss of appetite, colic, ear ache, malaria, intestinal worms, fever, cough, colds and influenza. This species is known to have antibacterial and anti-oxidative properties, exerted by the volatile oils [mainly 1, 8 – cineole, α - thujole, β - thujole, camphor and borneol]. The thujoles are associated with harmful effects such as hallucination, when concoctions are overdosed or used over a long period (van Wyk *et al*, 1997 and Hutchings *et al*, 1996).

3.2.4 *Boophane haemanthoides* (Amaryllidaceae)

Common names: Legwama (Sotho)

The *Boophane* genus is made up of geophytes with large bulbs that are thickly tunicated. The *Boophane* consist of six species that are distributed throughout South Africa (Du Plessis and Duncan, 1989). *Boophane disticha* is the most common of all species of this genus and is also known to be poisonous. *Boophane haemanthoides* has been shown to contain alkaloids of the lycorine and crinine type, haemanthine and distichamine (Hutchings *et al*, 1996). This species only occurs in the Cape and few studies have been reported on it (Arnold and de Wet, 1993). This bulb species is often confused with *Boophane disticha*, which is also a medicinal plant. *Boophane disticha* contain toxic alkaloids that have been isolated from the bulb. These alkaloids are: narcissine, boophanine that resemble hydrazine in pharmacological action and exhibit convulsive action similar to colchicines, and haemanthine, which is closely related to atropine (Chan *et al.*, 1994; Perharic *et al.*, 1994).

3.2.5 *Croton sylvaticus* (Euphorbiaceae)

Common names: koorsboom (Afrikaans), forest fever-berry (English) and umanhlanganisa or umahlabekufeni, (Zulu)

Croton sylvaticus share a common Zulu name with *C. gratissimus* and has more than eight Zulu names given to it (Hutchings, 1989). Therefore, possibilities of confusing these two species are high. The medicinal uses of this species include treating abdominal disorders, intestinal inflammations, dropsical swellings and uterine disorders. Its roots are used as purgatives, for pleurisy and indigestion. This species has been reported to be toxic to birds and fish. It is possible that it may be toxic to humans, mostly toxicity is related to dose. The tree bark is known to have tannins whereas the root contains croton (Hutchings et al, 1996). *Croton sylvaticus* is restricted to the East Coast, Mpumalanga and the Northern Province (van Wyk *et al*, 1997).

3.2.6 *Peltophorum africanum* (Rosaceae)

Common names: huilboom (Afrikaans) weeping wattle (English) and isikhaba-mkhombe (Zulu)

The name 'weeping wattle', is due to the activity of *Ptyelus grossus*, also known as "spittle bug", which occurs in large colonies on the branches of this tree sucking out the sap. They filter out the nutrients, excreting the excess water as a protective froth, which constantly drips to the ground, causing the tree to "weep" (Venter and van den Heever, 1998). This species is one of the versatile trees from South Africa with roots; bark and leaves used in traditional medicine for a variety of ailments e.g. abdominal and chest pain.

It belongs to a genus known to have potential toxins and has anti-inflammatory activity against carrageenan-induced edema in rats. The timber can also be used for furniture and fuel. The roots of this tree are occasionally mixed with other plant species (*Bridelia cathartica*) to make decoctions for treating infertility and backache (Hutchings *et al*. 1996; Wyk *et al.*, 1997).

3.2.7 *Warburgia salutaris* (Canellaceae)

Common names: koorsboom (Afrikaans), fever tree, pepper-bark tree (English) and isibhaha (Zulu, siSwati and Ndebele), muranga (Shona), or chibaha (Tsonga).

Warburgia salutaris is one of the most popular traditional medicines in South Africa and has become threatened as a result of over-harvesting. It is sold in urban marketplaces in Mozambique, Swaziland, South Africa, Lesotho and Zimbabwe (Low and Rebelo, 1996). This species is highly valued for its activity as commercial herbal medicine, probably due to biologically active drimane sesquiterpenoids, typically warburginal and mannitol, the latter being more widely used as a diuretic and to treat dyspepsia. The bark and roots of this species are the parts mostly used whereas the leaves are the least used. The bark is used as an emetic or purgative, for rheumatism, influenza, malaria, venereal diseases, headache, toothache and gastric ulcers (van Wyk *et al.* 1997).

This plant is often used in mixed remedies. The leaves are used for coughs and to make lotions used for urethral inflammations, sores and irritants. The bark preparations have been found to be harmful, especially if they are extracts of the inner bark. The bark is known to have tannins, mannitol, and sesquiterpenoid dialdehydes. The extracts of *Warburgia salutaris* have broad minimum inhibitory concentration [warburganal, muzugadial and polygodial with polygodial showing potent activity] against *Saccharomyces cerevisiae*, *Candida utilis* and *Sclerotonia libertiana* (Hutchings *et al.* 1996)

In Zimbabwe, *Warburgia salutaris* became locally scarce due to over-harvesting for medicinal purposes, resulting in bark supplies being brought into Zimbabwe from Mozambique. This destruction of *Warburgia salutaris* populations is a widespread problem for this taxon in

Africa (van Wyk *et al.*, 1997). Therefore, this species has a limited distribution in Southern Africa, where it is listed as a vulnerable species in the recent Red Data List for this region (Low and Rebelo, 1996).

3.3 Conclusion

This study has shown that collectors and traders are harvesting numerous plant species. This may lead to over-exploitation of these plant species. The comparison of plant medicines commonly used in KwaZulu-Natal to Pretoria areas has revealed common plant medicines. From the study conducted as part of this thesis, only 7 plant species are commonly used in KwaZulu-Natal and Pretoria.

Traditional medicines collected in Gauteng province were allocated 5 points while those collected elsewhere were allocated 10 points. Traditional medicines collected from KwaZulu-Natal, Northern Province and Cape Town are relatively costly. Expensive medicines were allocated 10 points since the cost is the most important factor in determining whether to replace plant species or not.

SeSotho and isiZulu African names of the traditional medicines traded in Pretoria market areas are the most common trade names. Upon obtaining the African names of the commonly used traditional medicines, the scientific names of those plant species were then retrieved from published documents. Six of the top 10 plant species were selected for TLC identification, based on their availability in Pretoria National Botanical Garden (PNBG) and Agricultural Research Council (ARC). From this chapter, it is recommended for further study that the degree in which plants sold in the market are labeled correctly, is established.

4 The use of Thin Layer Chromatography (TLC) to identify medicinal plants

4.1 Introduction

In Chapter 3, Table 3.2 shows a summary of commonly used traditional medicines in the Pretoria area. However, the identities of those plant species have not been verified experimentally. The documented names of medicinal plants found in literature, have not been verified scientifically. Therefore, there is a need to establish the connection between documented African names and their scientific names.

The objective of this section is to identify traditional medicines traded in the Pretoria markets in the form of barks by developing a comparative Thin Layer Chromatography (TLC) profile of both the market species and reference species from Pretoria National Botanical Garden (PNBG) and Agricultural Research Council (ARC). To do this the TLC method was used, as described in Section 2.3. First a standard of botanically verified samples of plant believed to be those on the market was developed. This research continues from the findings in Chapter 3. Ethical names turn to be generic rather than specific.

Amongst the 20 highly ranked commonly used medicines in the Pretoria area (Table 3.2), 6 were chosen for further identification and biological analysis. These medicinal plants were selected because their reference samples were available in the PNBG and ARC. The medicinal plants selected are *Warburgia salutaris* (Molaka), *Peltophorum africanum* (Mosetlha), *Croton sylvaticus* (Umahlanganis), *Boophane haemanthooids* (Legwama), *Artemisia afra* (Lengana) and *Acacia caffra* (Mosetlhana).

The developed TLC plates were visualized under ultra violet (UV) light and fluorescent spots marked by HB pencil and then sprayed separately with *p*-anisaldehyde, vanillin-sulphuric acid and vanillin-phosphoric acid reagents. The results are shown in Figure 4.1 through to 4.6. In all cases the reference sample (denoted with A for ARC, B for PNBG or E for J. N Eloff, depending on the supplier of the samples used) were analyzed alongside the suspected market sample (denoted M for market). The suspected market sample was retrieved by literature search using the African names as shown in Table 3.2. The results of each species are discussed in detail in Section 4.2.1.

4.2 Results and discussion

4.2.1 Thin Layer Chromatography Identification

4.2.1.1 *Warburgia salutaris* (Molaka)

Figure 4.1 shows the TLC profiles of Molaka (market) and *Warburgia salutaris* (PNBG) extracts detected with *p*-anisaldehyde, vanillin, sulfuric acid and vanillin phosphoric acid spray reagents. The chromatographic profile of the market species extracted with ethanol (ME), acetone (MA) and hexane (MH) was generally similar to that of the reference material (RE, RA and RH) from a tree in the garden of J. N. Eloff.

However, there are variations in the fluorescence detected between the market and the reference extracts. For instance, a compound only found in reference extracts showed a red fluorescence that was not present in the market extract. This compound has an R_f value of 0.92 in the CEF [chloroform (4): ethyl acetate (3): formic acid (1)] profile. Another compound was found, only in the market BEA [benzene (80): ethanol (10): ammonium (1)] extract that had an R_f of 0.62 with a white fluorescence.

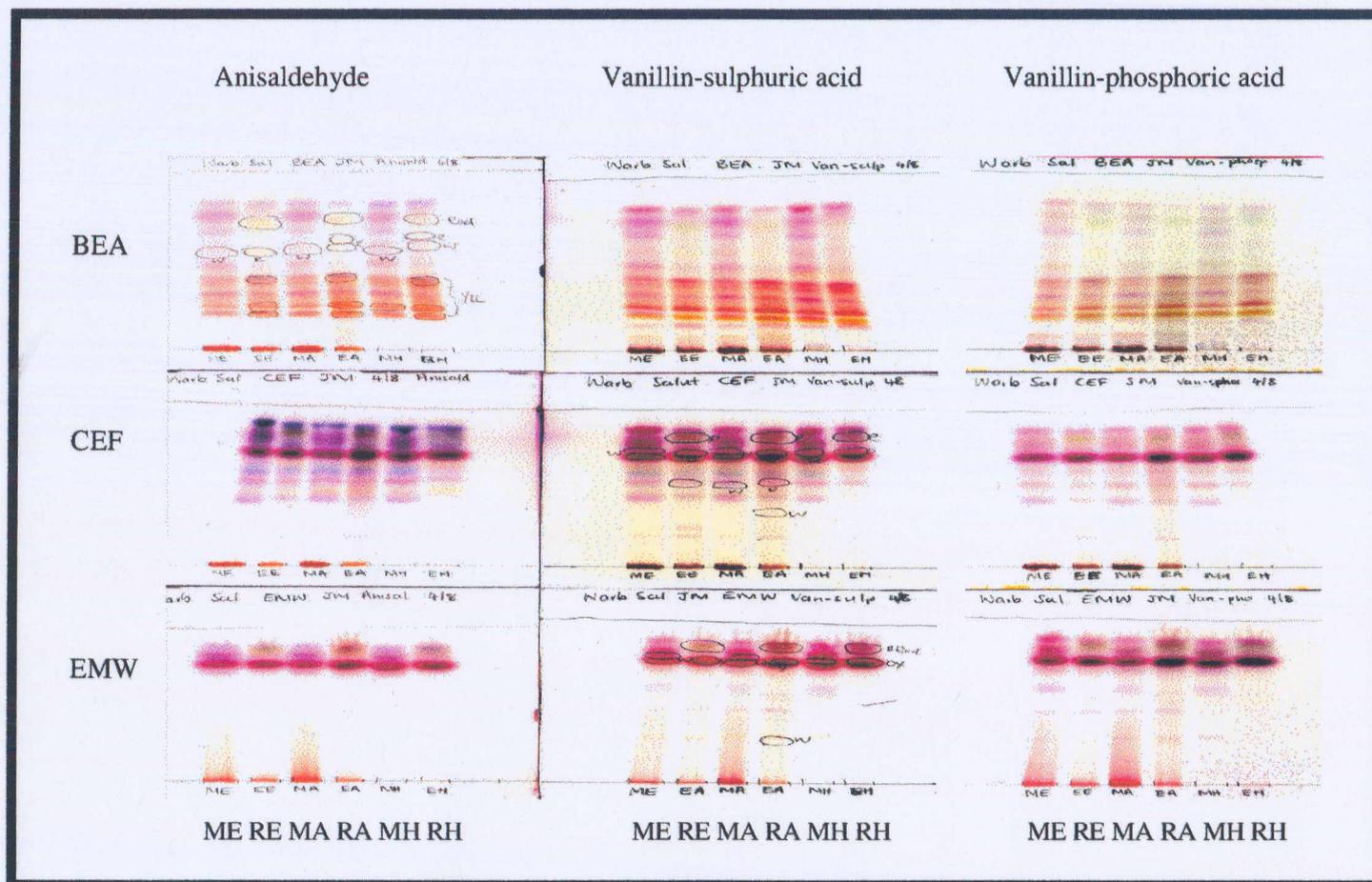


Figure 4.1 TLC profiles of Molaka (ME, MA and MH) and *Warburgia salutaris* (RE, RA and RH) showing chemical components sprayed with *p*-anisaldehyde, vanillin-sulphuric acid and vanillin-phosphoric acid respectively. Key: ME: market ethanol extract; RE: reference ethanol extract; MA: market acetone extracts; RA: reference acetone extract; MH: market hexane extract; RH: reference hexane extract; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

Fluorescence can be a very sensitive way of detecting compounds separated and may indicate the presence of compounds in low concentrations. There was no great difference amongst the compounds detected with the three spray reagents. However, the intensity differences of the colour implied varying concentrations. The BEA profile of vanillin-sulphuric acid reagent shows a major variation in the detection of compounds falling between the R_f value range of 0.44 and 0.81. This may be the result of genetic variation or environmental factors (Solomon, *et al.* 1999).

Vanillin-sulphuric acid appears to be the best reagent for profiling this plant species because some compounds are more intensely detected by it compared to the other reagents. *P*-anisaldehyde, vanillin-sulphuric acid and vanillin-phosphoric acid spray reagents detected some compounds coloured in purple–brown in Figure 4.1. This plant species has steroid-like compounds because all three-spray reagents are expected to detect steroid (Table 2.2). The BEA separation system shows better separation of compounds for this plant species than CEF and EMW [ethyl acetate (10): methanol (1.35): water (1)]. This could mean that *Warburgia salutaris* and Molaka have more non-polar compounds than intermediate and polar compounds.

Based of the foregoing and not withstanding the variations noted, the market species Molaka is similar to the reference species *Warburgia salutaris* from the J. N Eloffs' garden. Therefore, the market sample is *Warburgia salutaris*.

4.2.1.2 *Peltophorum africanum* (Mosetlha)

Figure 4.2 shows the TLC profile of Mosetlha (market) and *Peltophorum africanum* (PNBG) extracts detected with *p*-anisaldehyde, vanillin-sulphuric acid and vanillin-phosphoric acid spray reagents. The TLC profile of Mosetlha from the market (ME, MA and MH) and

fingerprints of *Peltophorum africanum* from the PNBG (RE, RA and RH) show similar compounds for all extracts separated by all three systems (BEA, CEF and EMW), which indicates that they are the same species.

Like *Warburgia salutaris*, this species also has a red fluorescing compound that is restricted to the reference extracts circled in BEA ($R_f = 0.72$), CEF ($R_f = 0.77$) and EMW ($R_f = 0.81$) TLC systems. In addition, a blue fluorescing compound with an R_f value of 0.62 separated by the EMW system of the ethanol and acetone extracts was detected in both samples (ME, MA, RE and RA). Furthermore, the hexane extracts showed a white compound of R_f value of 0.24, which was not detected with any of the chosen spray reagents. *P*-anisaldehyde seems to be the best reagent for profiling this plant species, since it detected the most compounds.

This species has polar compounds clearly shown as smears in the EMW profiles detected by all three reagents. These smears showed a blue fluorescing colour before the plates were sprayed with the detecting reagents. Therefore, the detected compound could be chromophores. It is also possible that some fluorescing compounds are present in low concentrations and may, therefore, not be significant for visualization. In conclusion, Mosetlha can be confirmed to be *Peltophorum africanum*.

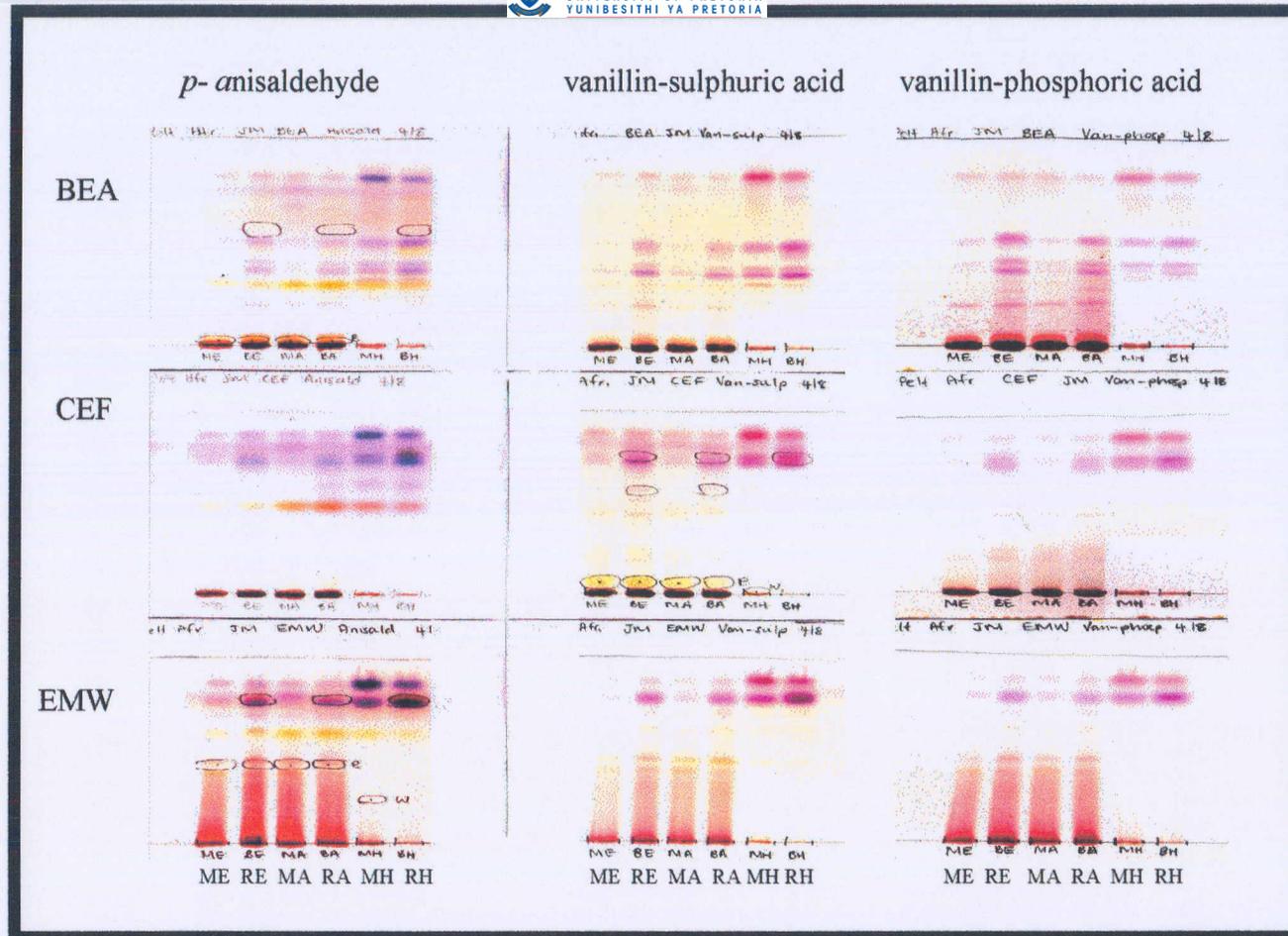


Figure 4.2 TLC profiles of Mosetlha (ME, MA and MH) and *Peltophorum africanum* (RE, RA and RH) extracts sprayed with *p*-anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid respectively. Key: ME: market ethanol extract; RE: reference ethanol extract; MA: market acetone extracts; RA: reference acetone extract; MH: market hexane extract; RH: reference hexane extract; .BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

4.2.1.3 *Croton sylvaticus* (Umahlanganisa)

Figure 4.3 show the TLC profiles of Umahlanganisa (market) and *Croton sylvaticus* (PNBG) extracts separated in the three systems described in Section 2.3. The TLC profiles for all the market extraction (ME, MA and MH) are completely different from the reference *Croton sylvaticus* (RE, RA and RH) extracts. The market extracts showed a blue colour under UV [R_f values 0.29 (BEA), 0.72 (CEF) and 0.8 (EMW)], which was not detected in the reference extracts. Furthermore, an orange-yellow compound was seen in the market (ME and MA) extracts [R_f values 0.16 (CEF) and 0.3 (EMW)] that was not detected in the reference sample (RE and RA) under UV and after spraying with spray reagents.

Overall, there are major differences in the chromatograms sprayed in the number of compounds separated, relative concentrations and R_f values. However, some compounds are present in both the market and the reference extracts and they were clearly detected with vanillin-sulphuric acid. These compounds have R_f values of 0.74, 0.29 and 0.26 in the BEA system: 0.87 and 0.83 in the CEF system and 0.9 in the EMW system. These results indicate that the market and reference samples belong to the same genus but are not of the same species or they may be totally different plant species with no relationship.

The traditional medicine traded as Umahlanganisa that was collected from the market is, therefore, not *Croton sylvaticus* as the African name would imply. Preliminary investigations conducted as part of this study indicated that the *Croton* commonly used is not *C. sylvaticus* but *C. gratissimus*. The experiments were conducted to determine the similarities in chemical profiles of Umahlanganisa and *Croton gratissimus*. The possible reason for the variations in the market and reference material profiling could, therefore, be because of the incorrectly labelled plant product that was received from the market.

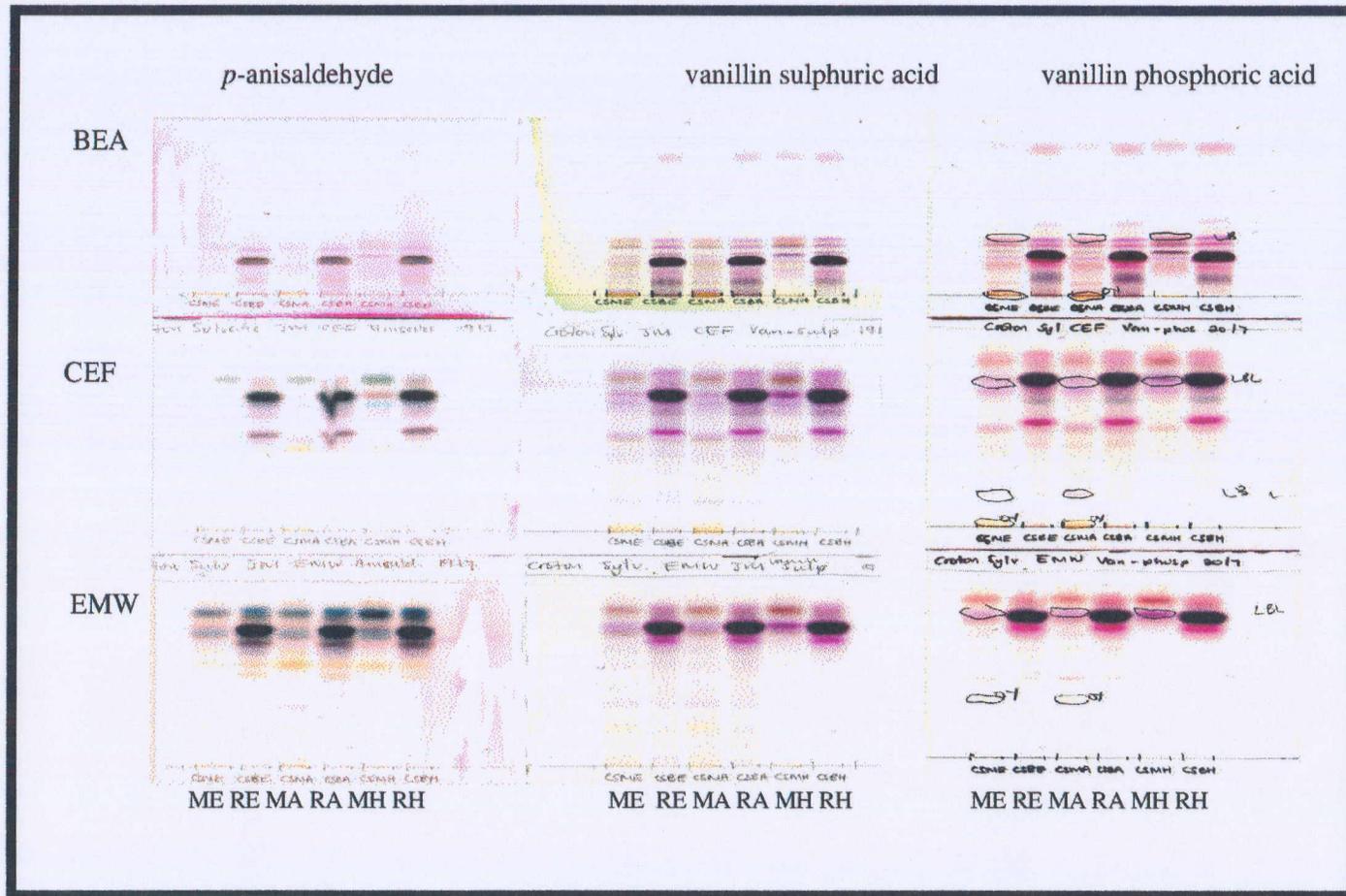


Figure 4.3 TLC profiles of market Umahlanganisa (ME, MA and MH) and reference *Croton sylvaticus* (RE, RA and RH) extracts sprayed with *p*-anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid spray reagents. Key: ME: market ethanol extract; RE: reference ethanol extract; MA: market acetone extracts; RA: reference acetone extract; MH: market hexane extract; RH: reference hexane extract; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

The other reason may be because different species may be identified by the same name. A comparison of the chemical fingerprints of different *Croton* species can be used to resolve this issue.

4.2.1.4 *Boophane haemanthoides* (Legwama)

Figure 4.4 shows the TLC profiles of Legwama (market) and *Boophane haemanthoides* (ARC) detected with *p*-anisaldehyde and vanillin-sulphuric acid spray reagents. These two samples have similar compounds as shown in Figure 4.4. However, they showed some degree of variation in the compound intensity.

Best separation of compounds in both the analyte and reference was achieved by the BEA system and best detected with *p*-anisaldehyde spray reagent. Consequently, it is probable but not definite that Legwama is *Boophane haemanthoides*. According to documentations, *B. haemanthoides* only grows in the Cape while *B. disticha* is found all over the country. The market plant species is likely to be *B. disticha*, but since *Boophane* species are closely related, they cannot only be distinguished from each other by Botanists and not by TLC profiles during the flowering season (Du Plessis and Duncan, 1989).

4.2.1.5 *Artemisia afra* (Lengana)

Figure 4.5 shows the TLC profiles collected from the market and two reference species of *Artemisia afra* from ARC and PNBG. The solvent extracts of all three species from market, ARC and PNBG have similar chemical compositions detected by all three-spray reagents, although the intensity of detected compounds varies amongst the species. It was concluded that Lengana was *Artemisia afra*.

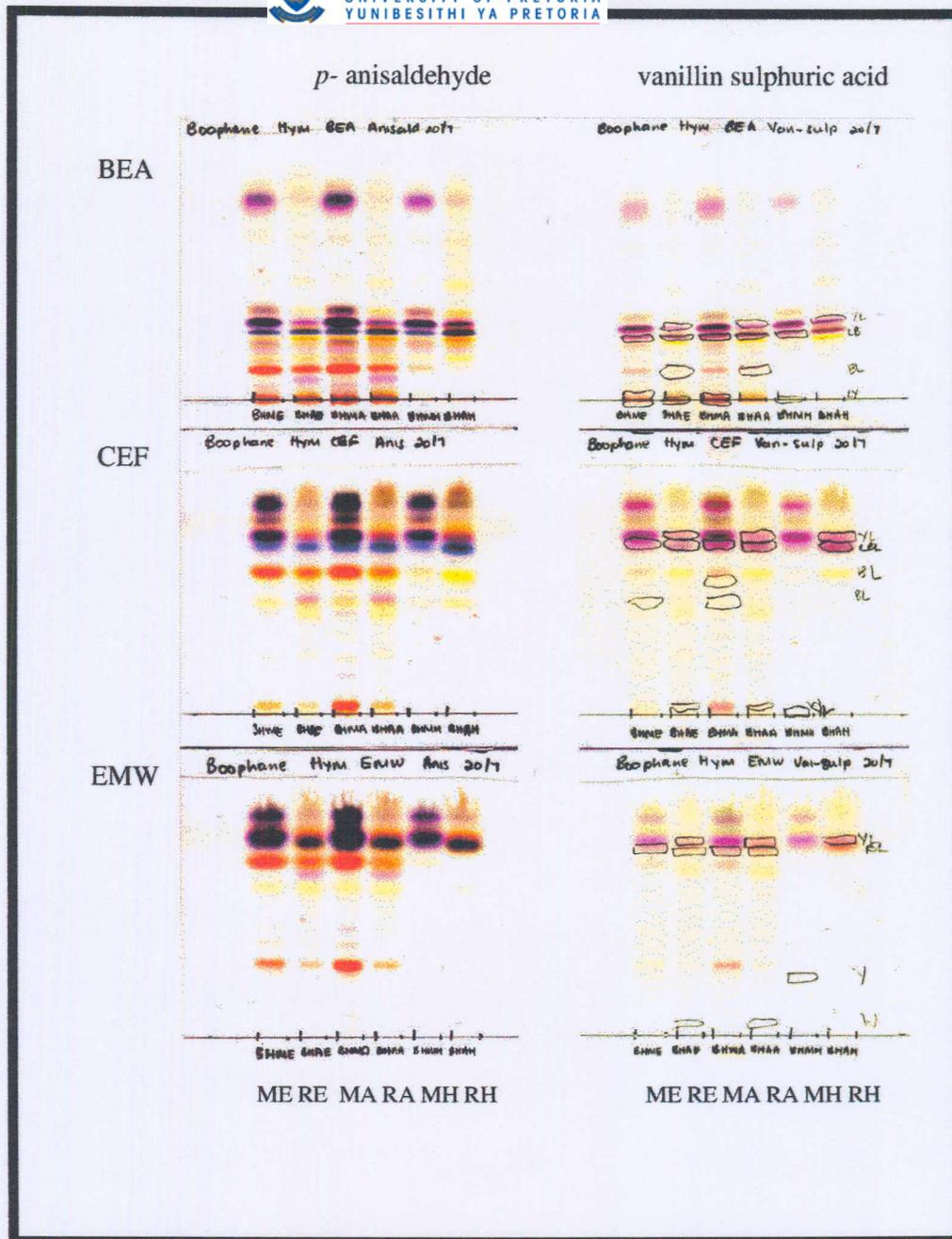


Figure 4.4 TLC profiles of Legwama and *Boophane haemanthooides* extracts sprayed with both *p*-anisaldehyde and vanillin-sulphuric acid. Key: ME: market ethanol extract; RE: reference ethanol extract; MA: market acetone extracts; RA: reference acetone extract; MH: market hexane extract; RH: reference hexane extract; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

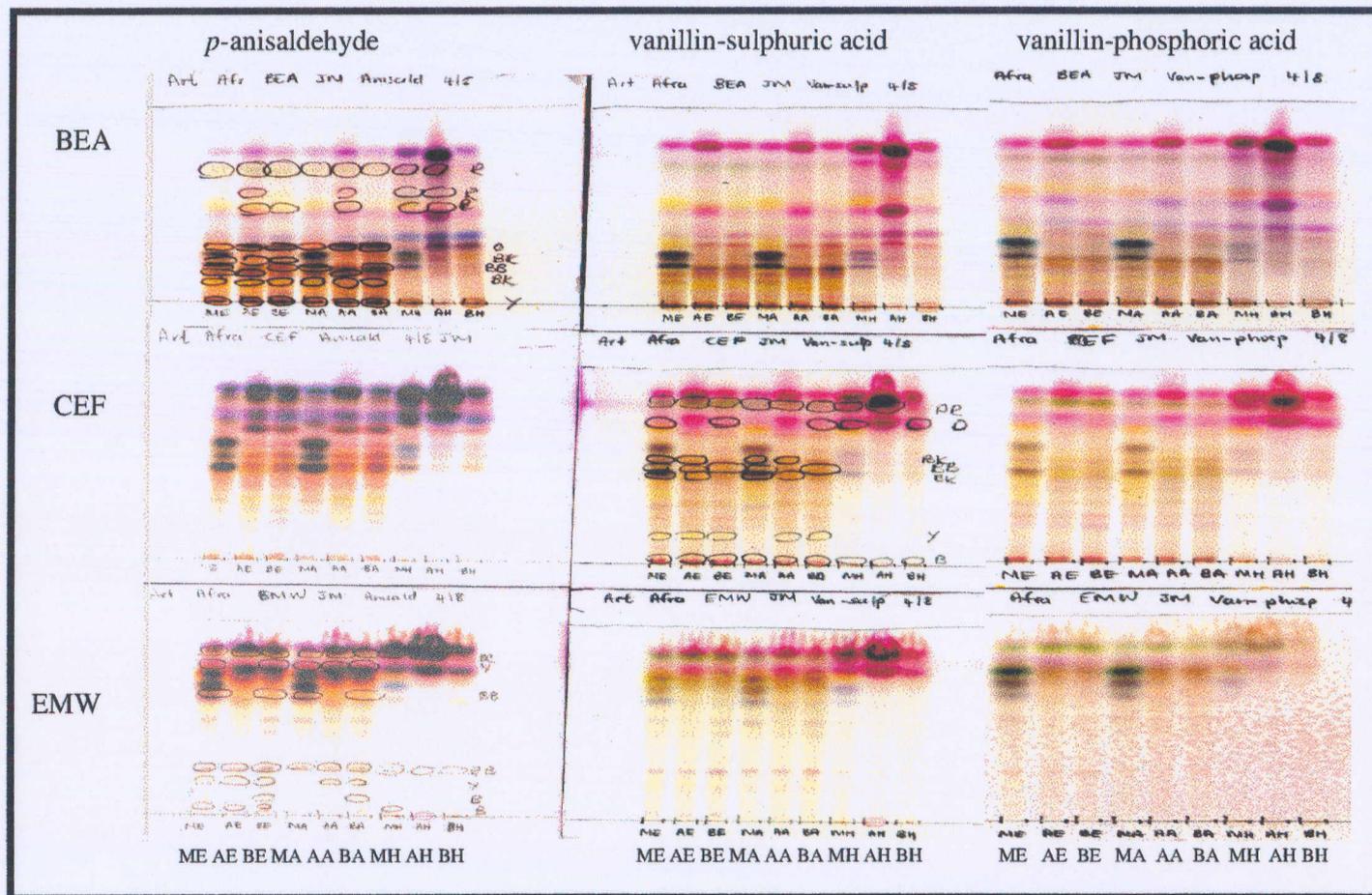


Figure 4.5 The TLC profiles of Lengana and *Artemisia afra* extracts sprayed with *p*-anisaldehyde, vanillin-sulphuric acid and vanillin-phosphoric acid respectively. Key: ME: market ethanol extract; AE: ARC ethanol extract; BE: PNBG ethanol extract; MA: market acetone extracts; AA: ARC acetone extract; BA: PNBG acetone extract; MH: market hexane extract; AH: ARC hexane extract; BH: PNBG hexane extract BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

4.2.1.6 *Acacia caffra* (Mosehlhana)

Figure 4.6 shows the TLC profiles of Mosehlhana (market) and *Acacia caffra* (PNBG) extracts that were detected with *p*-anisaldehyde, vanillin-sulphuric acid and vanillin-phosphoric acid spray reagents. The ethanol, acetone and hexane extracts reflect similar compound patterns for both species although there is a high variation in intensity amongst the extracts as indicated in Figure 4.6. However, it is clear that Mosehlhana and *Acacia caffra* have the same chemical profile and could be the same species.

In summary, this investigation showed that it is possible to use the chemical fingerprint of TLC as an identification tool for traditional medicines, although it may give equivocal results if closely related species are analyzed. Additionally, it was decided to investigate whether biological activity of species can be used to confirm the identity of market specimens. It was assumed that many, if not all, plant species traded in markets, as medicines are biologically active, although they do not undergo any assessment. Preliminary testing for biological activity was, consequently, undertaken. The bioassay chosen was for antibacterial activity. This was because the conditions for this assay had already been optimized in the laboratory where the experiment was conducted and because many plants have potential antibacterial activity and the assay is more convenient.

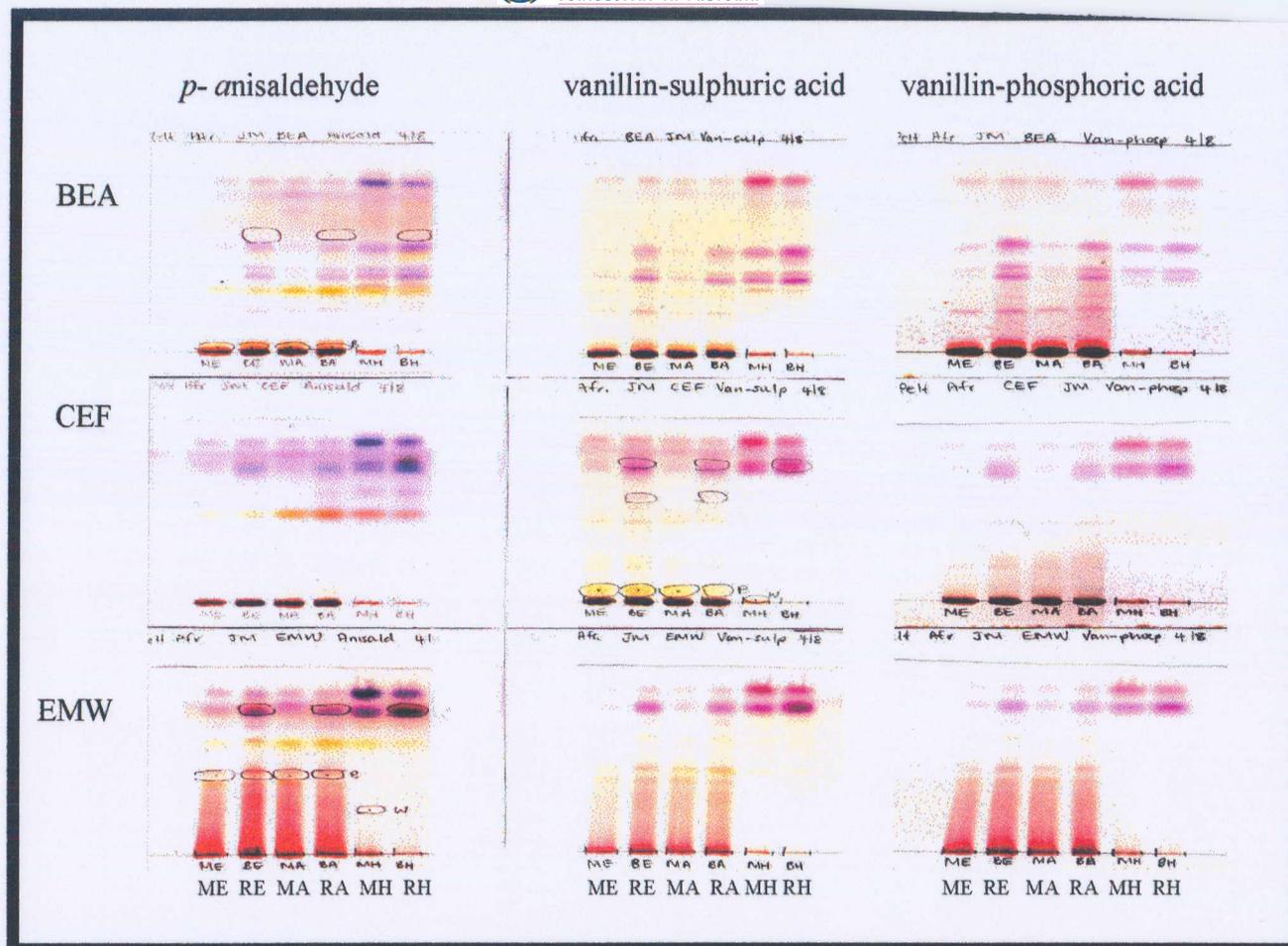


Figure 4.2 TLC profiles of Mosetlha (ME, MA and MH) and *Peltophorum africanum* (RE, RA and RH) extracts sprayed with *p*-anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid respectively. Key: ME: market ethanol extract; RE: reference ethanol extract; MA: market acetone extracts; RA: reference acetone extract; MH: market hexane extract; RH: reference hexane extract; .BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

4.2.2 Bio-autography

The method used is explained in detail in Section 2.5. The bacterial cultures used were *Enterococcus faecali* (*Entero*), *Staphylococcus aureus* (*Staph*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*Psuedo*). Not all bio-autography results of the plant species identified in Section 4.2.1.1 to 4.2.1.6 are shown. This is because antibacterial inhibition with some of the organisms e.g. *Escherichia coli* and *Pseudomonas aeruginosa* could not be visualized on the TLC plates due to low or no inhibition rates. Photographs of biauotgrams treated with *W. salutaris* and *Croton sylvaticus* are shown in the Figures 4.7 and 4.8 respectively. *W. salutaris* showed remarkable bacterial inhibition. Umahlanganisa varied in its activity with *C. sylvaticus*, this being a confirmation of the major variation in chemical profiles of these species in Section 4.2.1.

W. salutaris and Molaka are the only species that showed high bacterial inhibition, especially against *Staphylococcus aureus* as indicated in Figure 4.7. These species like the rest tested have very low anti-*Escherichia coli* activity. The similarities in bioassays of Molaka and *W. salutaris* is a further confirmation of the chemical profile obtained in Section 4.2.1

In Figure 4.8, the reference species *C. sylvaticus* from Pretoria National Botanical Garden (PNBG) shows biological activity against all three organisms that was not detected for the market Mosetlhana species. A possible explanation for this could be the fact that these are two different species, which are collected from different regions. It is certain that the environment where a plant is grown has an effect on its chemical profile and consequently its biological activity (Solomon et al. 1999). The biological activity, therefore, confirmed the results obtained through the chemical fingerprints using the specific solvent systems and spray reagents.

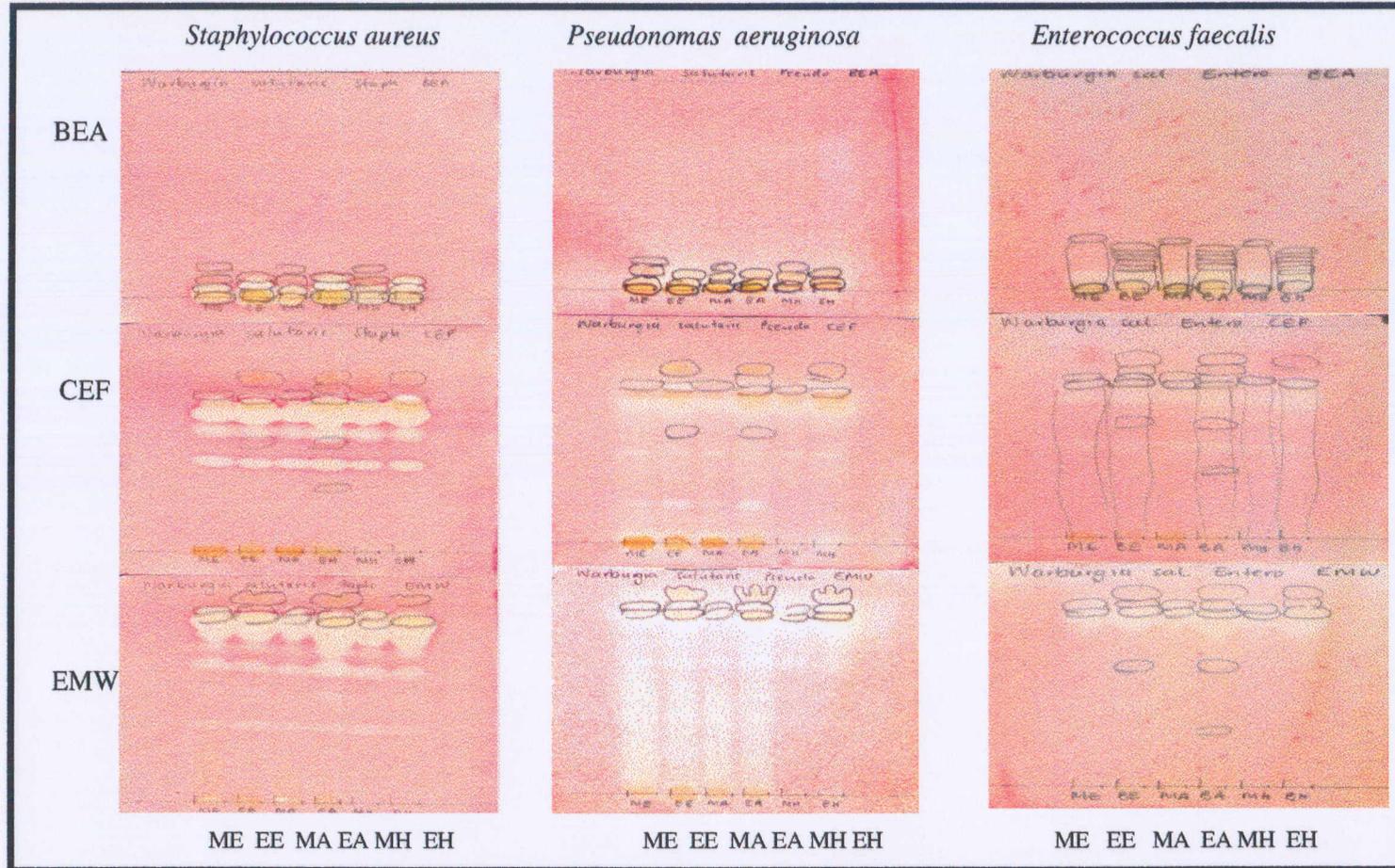


Figure 4.7 Bio-autograms of Molaka and *Warburgia salutaris* against *Staphylococcus aureus*, *Speudonomas aeruginosa* and *Enterococcus faecalis* respectively. Key: ME: market ethanol extract; EE: reference ethanol extract; MA: market acetone extracts; EA: reference acetone extract; MH: market hexane extract; EH: reference hexane extract; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

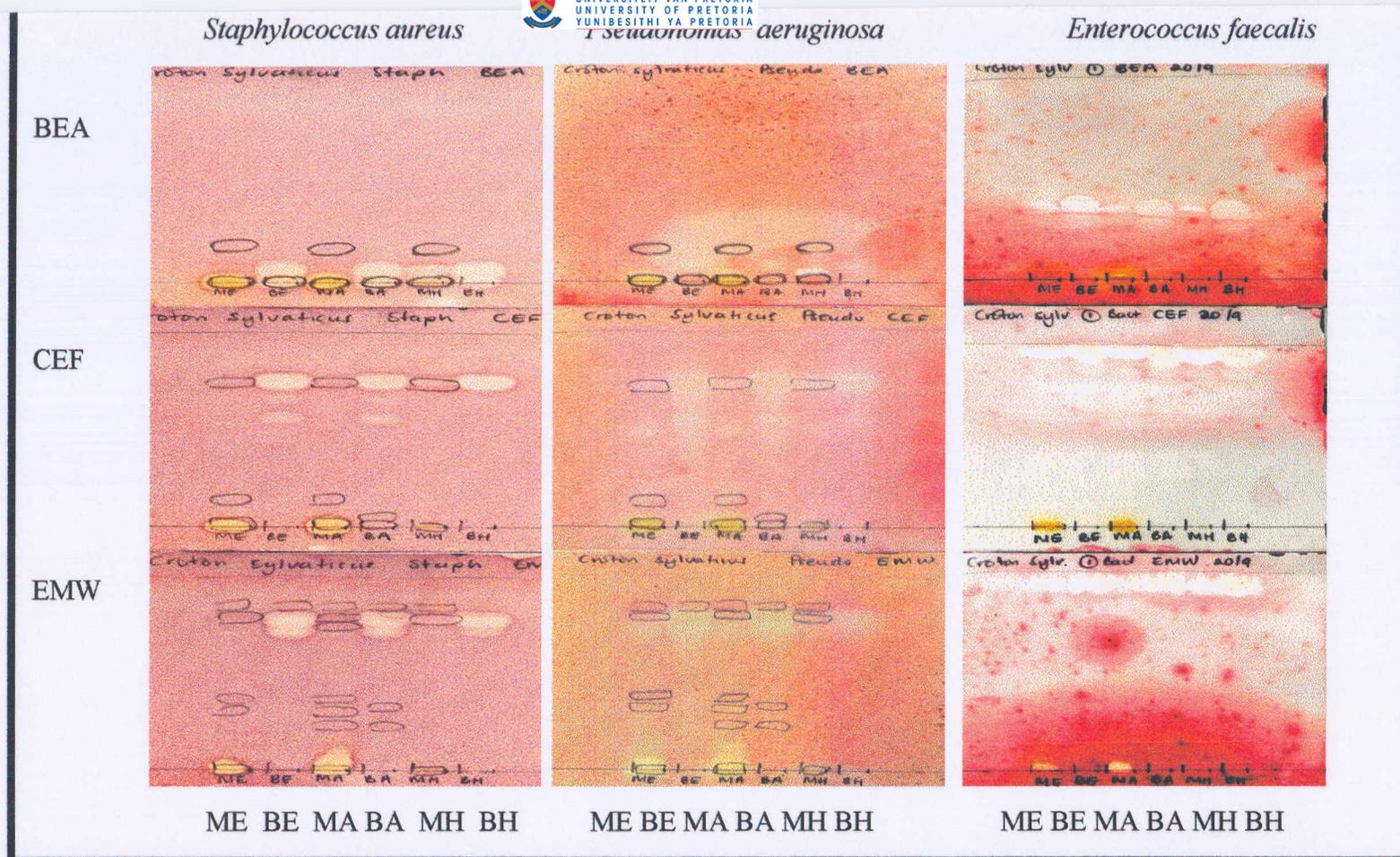


Figure 4.8 Bio-autograms of Moseithana and *Croton sylvaticus* against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* respectively. Key: ME: market ethanol extract; BE: reference (PNBG) ethanol extract; MA: market acetone extracts; BA: reference (PNBG) acetone extract; MH: market hexane extract; BH: reference (PNBG) hexane extract; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

4.2.3 Minimum Inhibitory Concentration and Total activity

The other test samples showed little or no activity in this bioassay. Their MIC values total activity which are shown in Table 4.1 and Figures 4.9, 4.10 and 4.11, show the graphical representation of the total activity.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of test sample dilution expressed in mg/L, which inhibited all visible bacterial growth on the plates. The MIC values for each sample and reference material were determined for all four organisms. In the analysis of MIC for all samples, it was found that *E. faecalis* is highly sensitive with the most frequent MIC of 0.0319mg/L. Most of the samples analyzed were not sensitive to *Escherichia coli* and *P. aeruginosa*. The total activity was calculated from MIC values obtained per 1-gram extract (i.e. quantity in mg extracted from 1 g/ MIC in mg/ml), indicated in Table 4.1 and Figure 4.9 through to Figure 4.12 according to Eloff (2000). This is an accurate measure of the total value of a plant because it takes into account the MIC and total quantity present in 1 g.

Table 4.1 MIC values and total activities (Eloff, 1998b) of identified plant species per 1-gram material

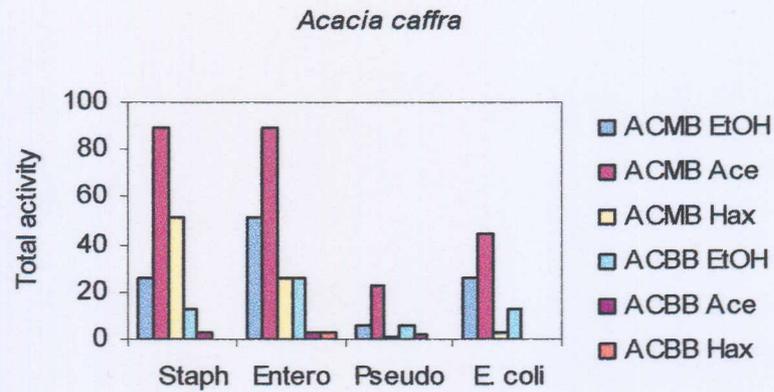
Extract code	Mass (mg)	Minimal inhibitory concentration (MIC)				Total Activity			
		<i>Staph.</i>	<i>Entero.</i>	<i>Pseudo.</i>	<i>E. coli</i>	<i>Staph.</i>	<i>Entero.</i>	<i>Pseudo.</i>	<i>E. coli</i>
<i>Croton sylvaticus</i> PNBG ethanol	60	0.156	0.156	5	5	384	384	12	12
<i>Croton sylvaticus</i> PNBG acetone	62	0.156	0.156	5	5	396.8	396.8	12.4	12.4
<i>Croton sylvaticus</i> PNBG hexane	52	0.156	0.039	5	5	332.8	1331	10.4	10.4
<i>Croton sylvaticus</i> market ethanol	14	0.078	0.156	0.625	0.156	179.2	89.6	22.4	89.6
<i>Croton sylvaticus</i> market acetone	12	0.078	0.313	0.625	0.313	153.6	38.4	19.2	38.4
<i>Croton sylvaticus</i> market hexane	14	1.25	0.625	5	5	11.2	22.4	2.8	2.8
<i>Acacia caffra</i> market ethanol	8	0.313	0.156	1.25	0.313	25.6	51.2	6.4	25.6
<i>Acacia caffra</i> market acetone	14	0.156	0.156	0.625	0.313	89.6	89.6	22.4	44.8
<i>Acacia caffra</i> market hexane	4	0.078	0.156	5	1.25	51.2	25.6	0.8	3.2
<i>Acacia caffra</i> PNBG ethanol	8	0.625	0.313	1.25	0.625	12.8	25.6	6.4	12.8
<i>Acacia caffra</i> PNBG acetone	2	0.625	0.625	1.25	5	3.2	3.2	1.6	0.4
<i>Acacia caffra</i> PNBG hexane	2	5	0.625	5	5	0.4	3.2	0.4	0.4
<i>Peltophorum africanum</i> market ethanol	70	0.156	0.078	0.625	0.313	448	896	112	224
<i>Peltophorum africanum</i> market acetone	90	0.016	0.016	0.313	0.313	5760	5760	288	288
<i>Peltophorum africanum</i> market hexane	6	0.625	0.313	1.25	5	9.6	19.2	4.8	1.2
<i>Peltophorum africanum</i> PNBG ethanol	46	0.156	0.078	0.313	0.156	294.4	588.8	147.2	294.4
<i>Peltophorum africanum</i> PNBG acetone	44	0.078	0.078	0.313	0.313	563.2	563.2	140.8	140.8
<i>Peltophorum africanum</i> PNBG hexane	8	0.313	0.078	5	5	25.6	102.4	1.6	1.6
<i>Boophane haemanthoides</i> ARC ethanol	6	5	0.156	5	0.156	1.2	38.4	1.2	38.4
<i>Boophane haemanthoides</i> ARC acetone	10	0.313	0.156	5	0.156	32	64	2	64
<i>Boophane haemanthoides</i> ARC hexane	6	0.156	0.078	5	2.5	38.4	76.8	1.2	2.4
<i>Boophane haemanthoides</i> market ethanol	6	0.625	5	5	5	9.6	1.2	1.2	1.2
<i>Boophane haemanthoides</i> market acetone	8	0.625	0.313	0.625	2.5	12.8	25.6	12.8	3.2
<i>Boophane haemanthoides</i> market hexane	12	0.625	1.25	5	2.5	19.2	9.6	2.4	4.8
<i>Warburgia salutaris</i> market ethanol	44	0.156	0.625	0.313	0.313	281.6	70.4	140.8	140.8
<i>Warburgia salutaris</i> market acetone	52	0.156	0.313	0.313	1.25	332.8	166.4	166.4	41.6
<i>Warburgia salutaris</i> market hexane	40	0.078	0.313	0.313	1.25	512	128	128	32

Table 4.1 (continues). MIC values and total activities (Eloff, 1998b) of identified plant species per 1-gram material

Sample code	Mass (mg)	Minimal inhibitory concentration (MIC)				Total Activity			
		<i>Staph.</i>	<i>Entero.</i>	<i>Pseudo.</i>	<i>E. coli</i>	<i>Staph.</i>	<i>Entero.</i>	<i>Pseudo.</i>	<i>E. coli</i>
<i>Warburgia salutaris</i> J. N. Eloff. ethanol	6	0.156	0.156	0.313	0.313	38.4	38.4	19.2	19.2
<i>Warburgia salutaris</i> J. N. Eloff acetone	5	0.078	0.156	0.156	0.078	64	32	32	64
<i>Warburgia salutaris</i> J. N. Eloff hexane	4	0.039	0.313	0.156	0.313	102.4	12.8	25.6	12.8
<i>Artemisia afra</i> market ethanol	13	0.313	0.078	0.625	1.25	41.6	166.4	20.8	10.4
<i>Artemisia afra</i> market acetone	15	0.313	0.313	0.313	0.625	48	48	48	24
<i>Artemisia afra</i> market hexane	4	0.078	0.156	0.313	0.625	51.2	25.6	12.8	6.4
<i>Artemisia afra</i> ARC ethanol	90	0.313	0.156	0.313	0.313	288	576	288	288
<i>Artemisia afra</i> ARC acetone	88	0.313	0.625	0.313	0.313	281.6	140.8	281.6	281.6
<i>Artemisia afra</i> ARC hexane	22	0.625	0.078	0.625	0.625	35.2	281.6	35.2	35.2
<i>Artemisia afra</i> PNBG ethanol	4.4	0.156	0.156	0.313	1.25	28.16	28.16	14.08	3.52
<i>Artemisia afra</i> PNBG acetone	6	0.313	0.313	0.313	0.313	19.2	19.2	19.2	19.2
<i>Artemisia afra</i> PNBG hexane	2	0.039	0.625	0.625	1.25	51.2	3.2	3.2	1.6

Key: *Staph.* : *Staphylococcus aureus*; *Pseudo.* : *Pseudomonas aeruginosa*; *Entero.* : *Enterococcus faecalis*; *E. coli* : *Escherichia coli*;
ARC: Agricultural Research Council; MIC: minimum inhibitory concentration; PNBG: Pretoria National Botanical Garden

A



B

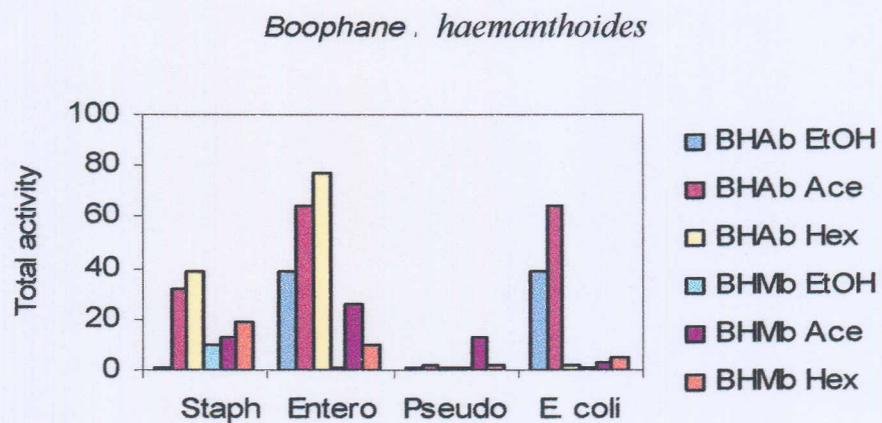
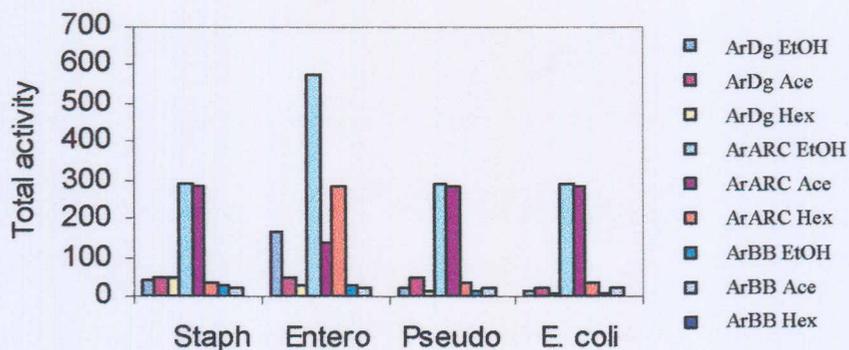


Figure 4.9 Total activities of *Acacia caffra* (A) and *Boophane haemanthoides* (B) tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*.
Key: ACMB: *Acacia caffra* market sample; ACBB: *Acacia caffra* Pretoria National Botanical Garden sample; BHAb: *Boophane haemanthoides* Agricultural Research Council sample; BHMb: *Boophane haemanthoides* market sample; EtOH: ethanol extract; Ace: acetone extract; Hex/Hax: hexane extract.

A

Artemisia afra



Croton sylvaticus

B

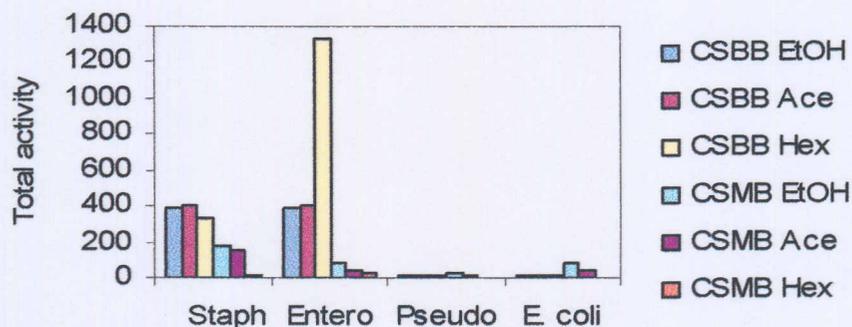


Figure 4.10 Total activities the *Artemisia afra* (A) and *Croton sylvaticus* (B) tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*. Key: ArDg: *Artemisia afra* market sample; ArARC: *Artemisia afra* Agricultural Research Council sample; ArBB: *Artemisia afra* Pretoria National Botanical Garden sample; CSBB: *Croton sylvaticus* Pretoria National Botanical Garden sample; CSMB: *Croton sylvaticus* market sample; EtOH: ethanol; Ace: acetone; Hex: hexane.

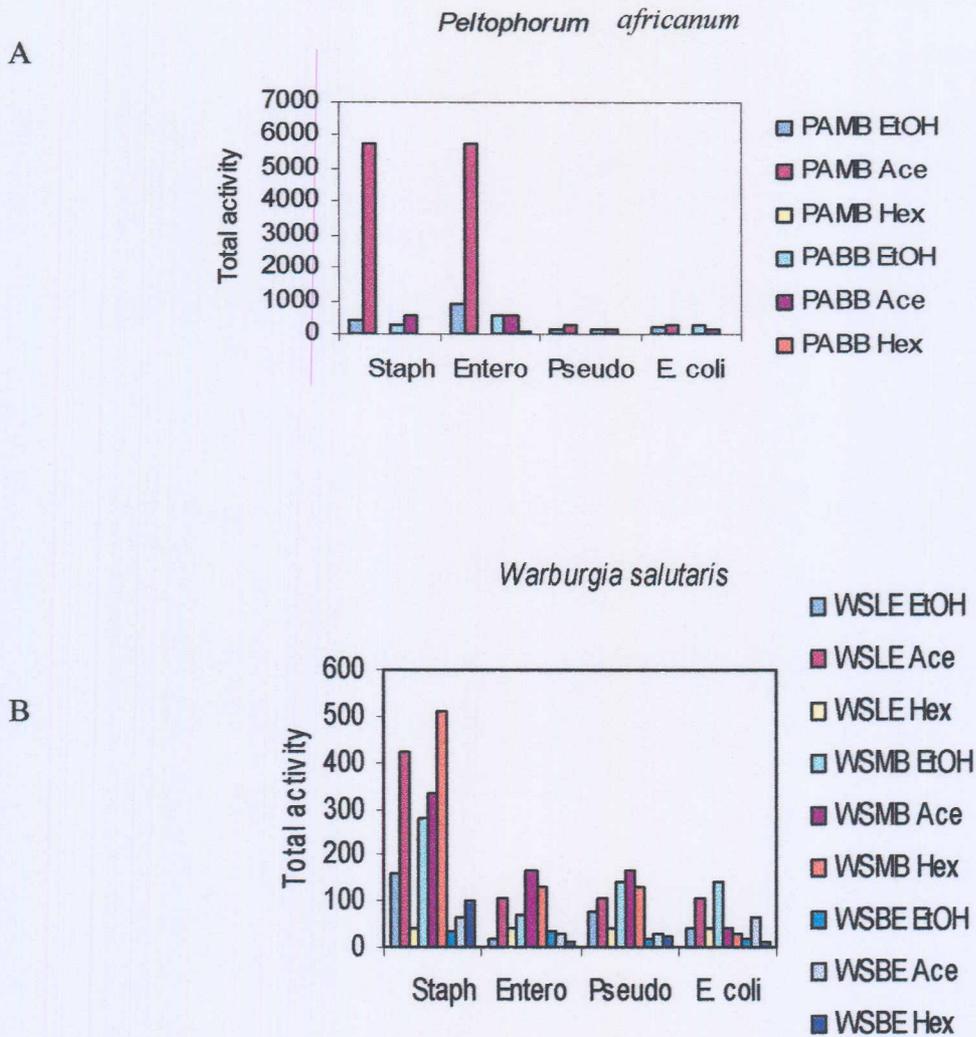


Figure 4.11 Total activities of *Peltophorum africanum* (A) and *Warburgia salutaris* (B) tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*. Key: PABB: *Peltophorum africanum* Pretoria National Botanical Garden sample; PAMB: *Peltophorum africanum* market sample; WSLE: *Warburgia salutaris* Pretoria National Botanical Garden leaf sample; WSBE: *Warburgia salutaris* Pretoria National Botanical Garden sample; WSMB: *Warburgia salutaris* market sample; EtOH: ethanol; Ace: acetone; Hex: hexane.

The total activity variation seen in Figures 4.9 through to Figure 4.11 gives similar indication as the observation in Section 4.2.1. On average, acetone extracts are the most active of all four organisms and this is shown in Figure 4.12.

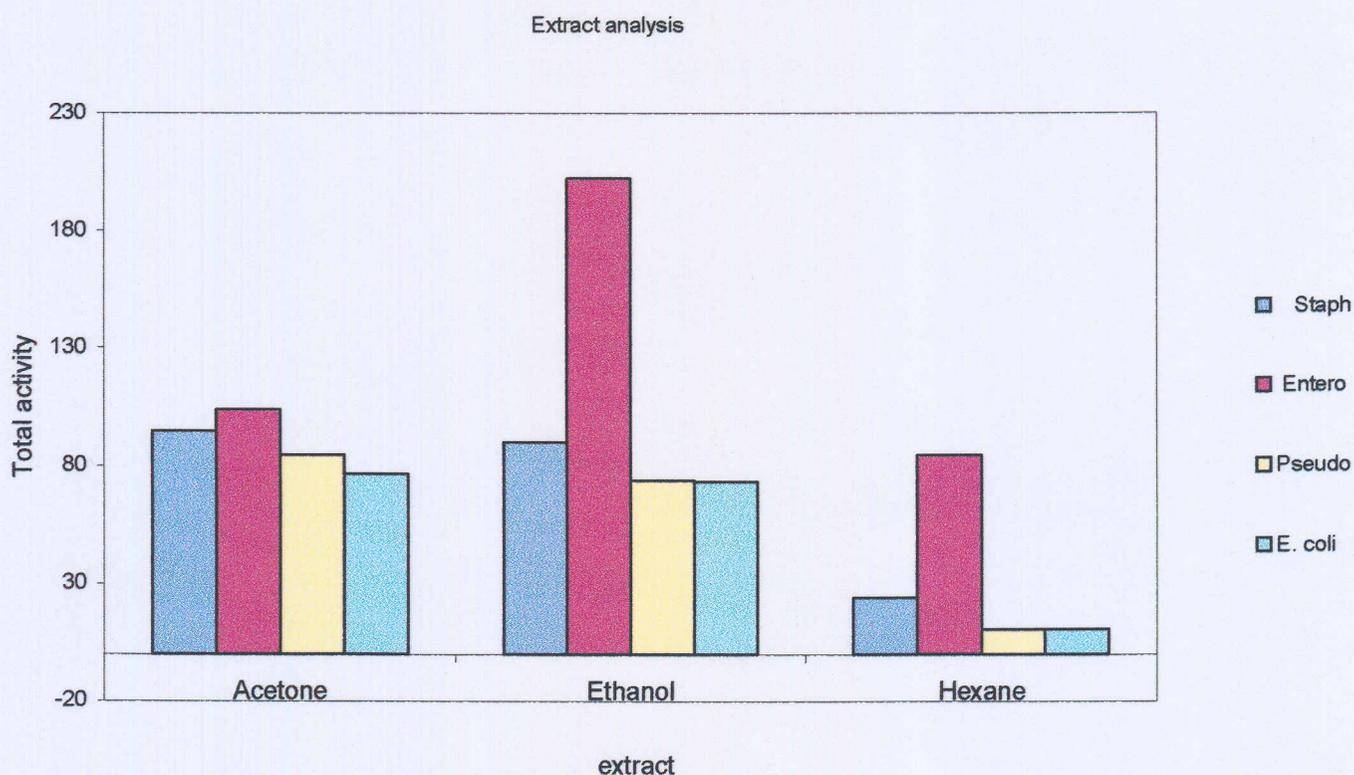


Figure 4.12. Total activities of ethanol, acetone or hexane extracts against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*.

On average, the ethanol extract was the best extraction solvent followed by acetone and then hexane. Figure 4.12 shows that using acetone as the intermediate polar solvent does not reflect the intermediate biological activity of both ethanol and hexane extract, however, it shows

a pattern close to the dominant extract. The biological activity of the samples was high in the ethanol extract against *Enterococcus faecalis* and was reduced in the acetone extract.

4.3 Conclusion

Some of the market extracts showed similar chemical profile to the reference materials namely; *W. salutaris*, *P. africana*, *B. hymanthoids*, *Art. afra* and *Ac. caffra* extract. This study indicated a high variation in the chemical composition within samples identified to be the same as the reference samples. However, Umahlanganisa showed a different compound profile to *C. sylvaticus* sample during detection with all reagents, therefore, Umahlanganisa collected from the market was not *Croton sylvaticus*.

Variation in the phytochemical constitution was detected also in the correctly identified market traditional medicines. The possible explanation for the compound variation could be the fact that the materials were of different ages and from different habitats. For instance, reference bark material of *Warburgia salutaris* used was still young and green, whereas the market barks was matured and brownish-black.

Differences in chromophores were also observed between the market and the reference materials. Some chemical compounds were detectable with all three reagents (anisaldehyde, vanillin-sulphuric acid and vanillin-phosphoric acid), which meant that these compounds could be steroids because these detection reagents commonly detect steroids. In conclusion, this investigation showed that the TLC method is a useful tool for the analysis of traditional medicines. However, the application of this tool in identification of closely related species is uncertain. The next chapter will apply the TLC method to identify closely related plant medicines, the magnitude of adulteration and the influence of environmental on plant medicines.

5 Application of Thin Layer Chromatography (TLC): Characterization of traditional medicines

5.1 Introduction

In Chapter 4, it was indicated that TLC can be used to identify traditional medicines, however, there is uncertainty if this technique could differentiate between closely related species. In this chapter, the application of the TLC method is expanded.

One of the aims of this chapter is to determine if the TLC method could differentiate between closely related plant medicines. This study was conducted in order to determine the effectiveness of this technique as a tool to identify unknown plant medicines. The Fabaceae family was chosen for this investigation, because of the availability of materials belonging to this plant family. The plants species studied were *Acacia caffra*, *Acacia karro*, *Acacia montana* and *Peltophorum africanum*.

Artemisia species are known to have highest variability amongst the species. *Artemisia afra* species has been used for centuries in traditional medicine for the treatment of fever and malaria (Kohler *et al.* 1997). Darwin's theory of evolution states that species undergo genetic variation with time to adapt to environmental changes (Solomon *et al.*, 1999). Therefore, the same species growing in widely different habitats may drift from the original genetic makeup as a mechanism of adaptation (Hegnauer, 1986). Therefore, same plant species that have been reproducing in different environments may have different chemical profiles. It is, therefore, hypothesized that the variations noted in the previous chapters may result from genetic variation that occurs as a result of environmental influence. *Artemisia afra* is one of the species with high genetic

variation (Balint, 2001). This chapter will also investigate the use TLC to determine the influence of the environment where a plant is grown on the chemical composition of the plant. Furthermore, an investigation of the accuracy of the technique in identifying plant species that were collected from plant is conducted.

Hahn-Deinstrop (2000) and many other workers have made use of TLC and declared this technique to be a good tool for the analysis of drugs substitution and adulteration. Research conducted thus far, has also shown TLC to be a good technique for analysis of botanical products in the investigation of additional substitutions (Ntloedibe, 2002). Little work has been done to develop and validate methods to determine adulteration in traditional medicine markets using TLC. This chapter also investigates the magnitude of adulteration and substitution of traditional medicines in the Pretoria market. Plant medicines chosen for this investigation are *Warburgia salutaris* and *Peltophorum africanum*, because they were traded by every vendor in the market.

5.2 Results and discussion

5.2.1 Application of TLC to differentiate Fabaceae species

The aim of this section was to determine whether the TLC technique can differentiate between closely related medicinal species of the same family. Bark material of species in question was collected from the same geographic area (PNBG). The samples were processed to fine powders as stated in Section 2.2 and then extracted with ethanol, acetone and hexane. The final extracts were separated using three systems: BEA [benzene (80): ethanol (10): ammonium (1)], CEF [chloroform (4): ethyl acetate (3): formic acid (1)] and EMW [ethyl acetate (10): methanol (1.35): water (1)].

The TLC plates analyzed were then sprayed with *p*- anisaldehyde and vanillin sulphuric acid spray reagents, as shown in Figure 5.1 and Figure 5.2, respectively. These chemical profiles indicate that *Acacia karoo* is very similar to *Acacia montana*. Therefore, there are complications that may be encountered when trying to identify plant species of the same family using the TLC method. This implies that when closely related species are sold it is not possible to confirm their identity without botanical evaluation.

5.2.2 Environmental influence on the chemical composition of *Artemisia afra* species

The aim of this section is to investigate the influence of the environment on the chemical profile of *Artemisia afra* species originating from the same seed source but growing in different environments. A farmer from the Delmas district, KwaZulu-Natal approached us to evaluate *Art. Afra* plants grown under widely different cultivation condition for him. This was confirmed by preparing TLC fingerprint of species collected from different geographic areas that originated from different seed sources and have been growing under different environments. These plant samples were collected from the market, Pretoria National Botanical Garden and Agricultural Research Council. The samples and their place of origin are listed in Table 5.1. The leaves were ground up, extracted with ethanol, acetone and hexane, and then developed as outlined in Section 2.3. The developed plates were sprayed with vanillin sulphuric acid, *p*-anisaldehyde and vanillin phosphoric acid spray reagents for detection of extract components.

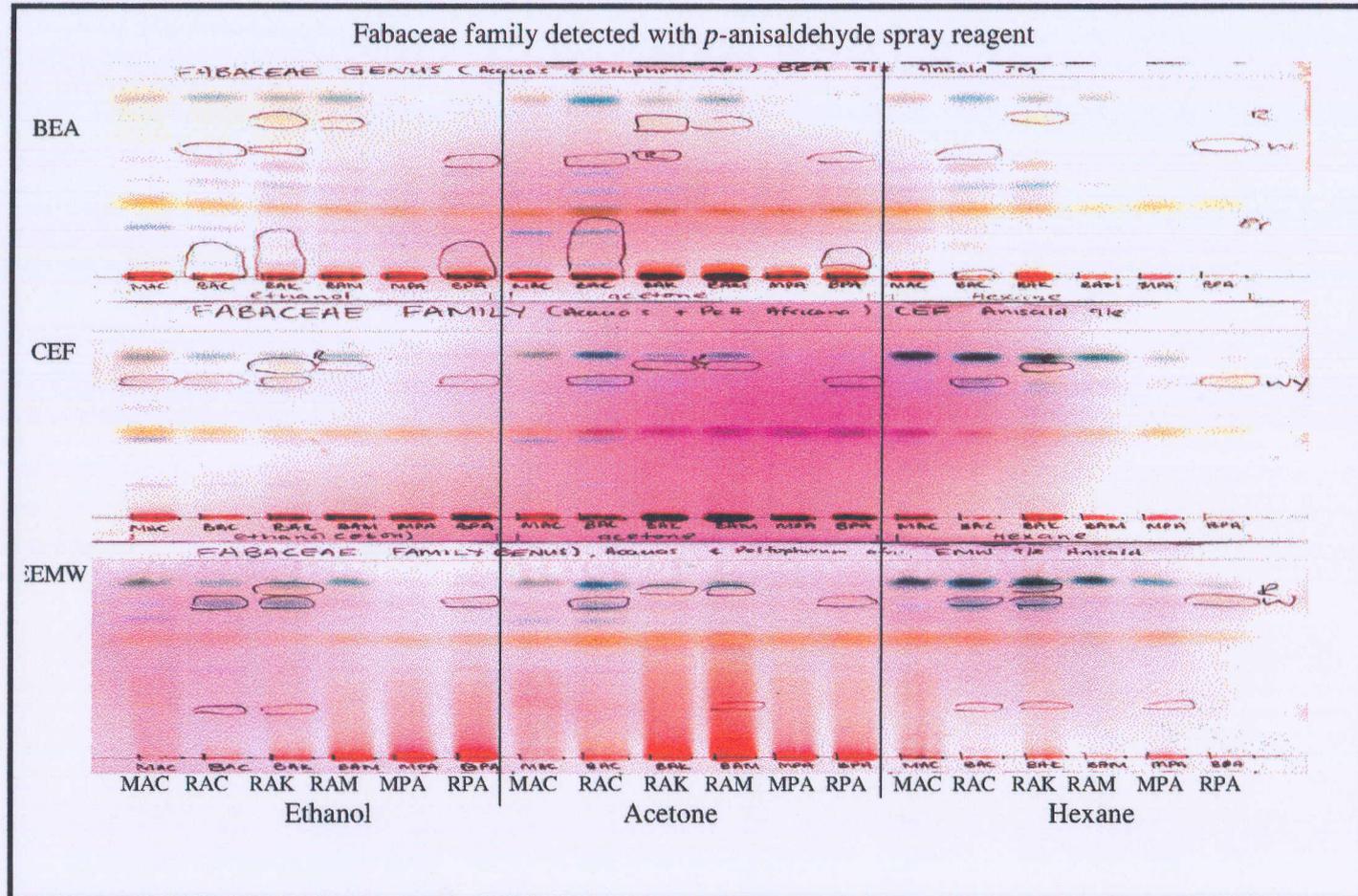


Figure 5.1 TLC profiles of Fabaceae species extracts, analyzed with *p*-anisaldehyde spray reagent. Key: MAC: Market *Acacia caffra*; BAC: PNBG *Acacia caffra*, BAK: PNBG *Acacia karoo*, BAM: PNBG *Acacia montana*, MPA: market *Peltophorum africanum* and RPA: PNBG *Peltophorum africanum*.

Fabaceae family detected with vanillin –sulphuric acid

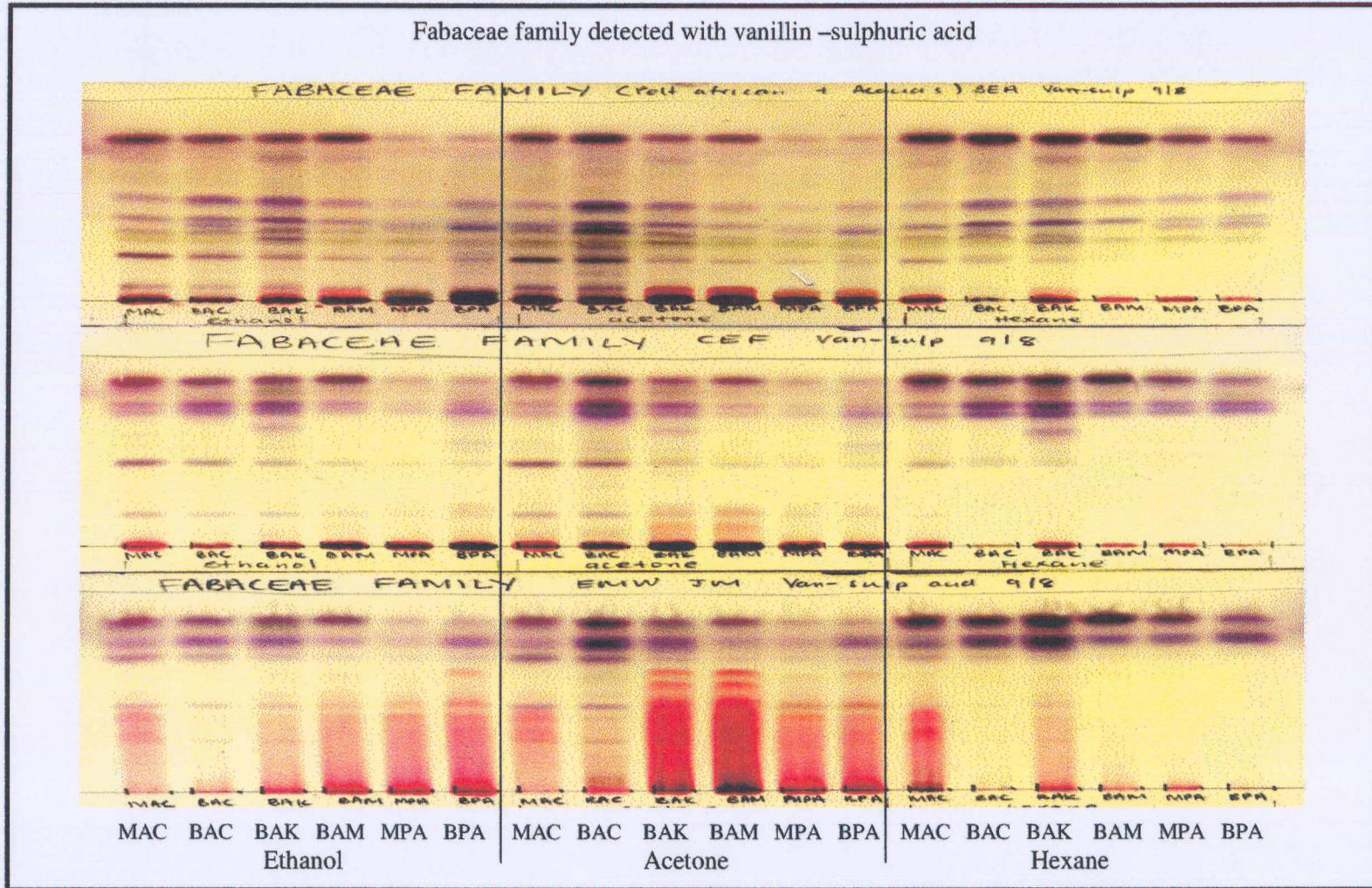


Figure 5.2 TLC profiles of Fabaceae species extracts of ethanol, acetone and hexane sprayed with vanillin sulphuric acid spray reagent. MAC: Market *Acacia caffra*, BAC: PNBG *Acacia caffra*, BAK: PNBG *Acacia karoo*, BAM: PNBG *Acacia montana*, MPA: market *Peltophorum africanum* and BPA: PNBG *Peltophorum africanum*.

Table 5.1. List of *Artemisia afra* species originating from different geographic areas and seed sources

Sample code	Environment
A	ARC Well cared for, given water and fertilizer
B	Pretoria National Botanical Garden Natural good soil and climate, naturally conserves environment
D	Wild, cold climate, Mpumalanga
M	Growing in a maize field, good soil, average climate
T	Well cared home garden, Gauteng
V	Wild, warm climate

Figure 5.3 shows the TLC profile of *Artemisia afra* species in Table 5.1 sprayed with anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid spray reagents. There was a distinct variation amongst the extracts indicating the difference in chemical composition. Variation in biological activity of these species can be expected, since variation in chemical composition was associated with variation in antibacterial activity (Section 3.2). Figure 5.4 presents the total activities of *Artemisia* species in Figure 5.3 against *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* micro-organisms.

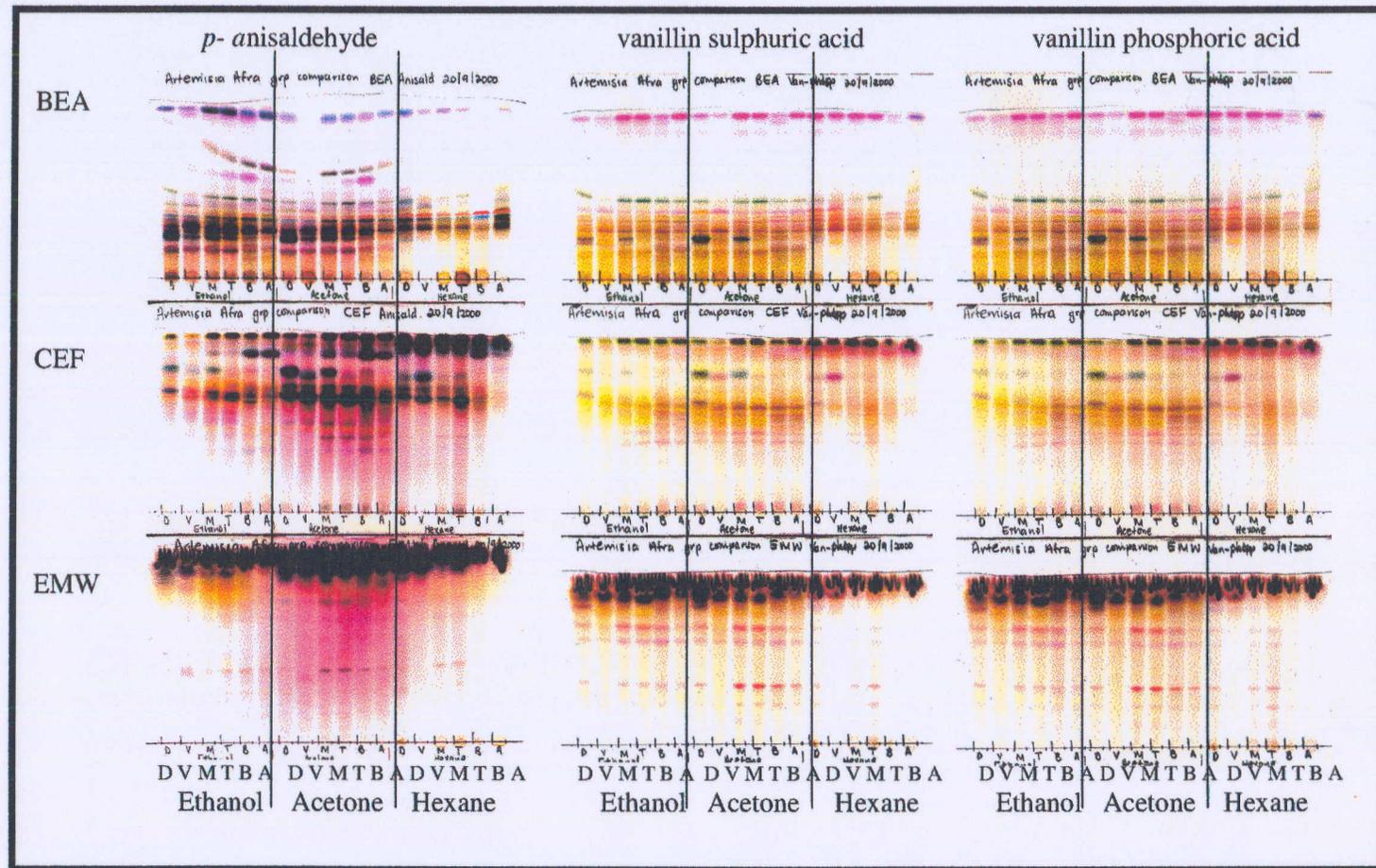


Figure 5.3 TLC profiles of *Artemisia afra* species originating from different geographic area and seed sources, sprayed with: *p*-anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid spray reagents. Key: D: market sample; V: veldt sample; M: maize land sample; T: private garden sample; B: Pretoria National Botanical Garden sample; A: Agricultural Research Council sample; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

Artemisia afra group analysis 1

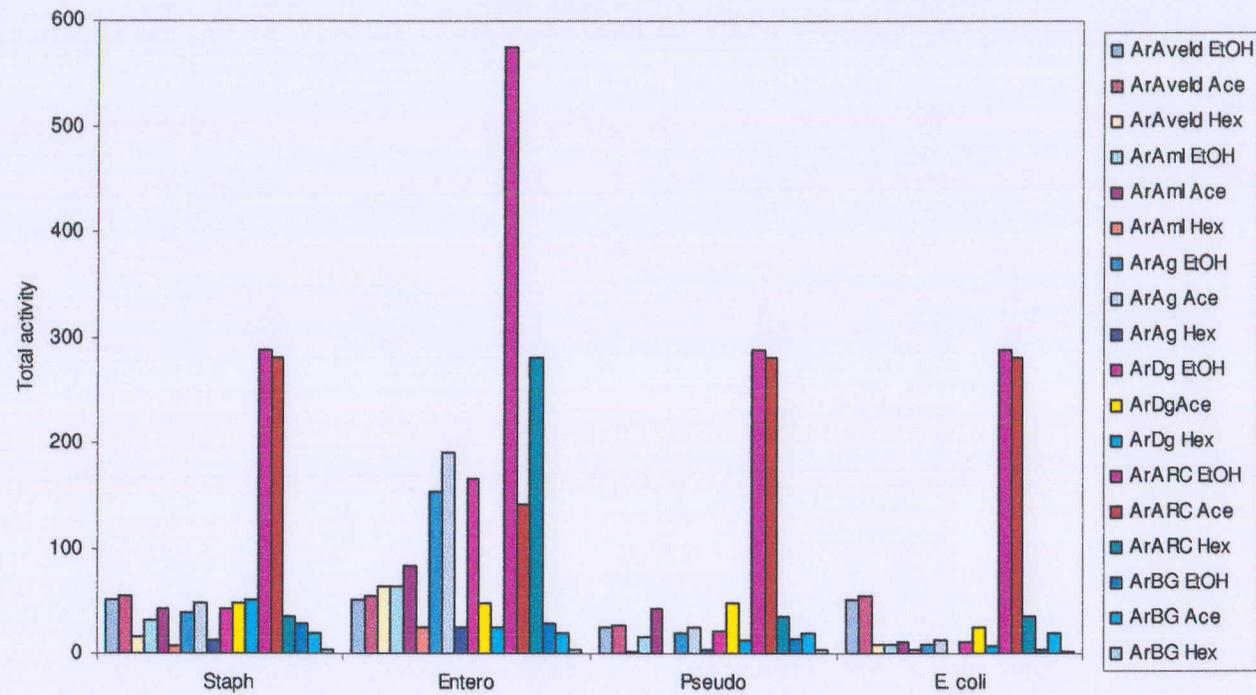


Figure 5.4 Total activities of *Artemisia afra* species tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* microorganisms. Key: ArAveId: *Artemisia afra* veldt sample; ArAml: *Artemisia afra* maize land sample; ArAg: *Artemisia afra* private garden sample; ArDg: *Artemisia afra* market sample; ArARC: *Artemisia afra* Agricultural Research Council Sample; ArBG: *Artemisia afra* Pretoria National Botanical Garden sample; EtOH: ethanol; Ace: acetone; Hex: hexane.

Variation observed may be due to genetic polymorphism, ecological and geographic differences (Balint, 2001). Figure 5.4 shows the total activity. These results correlate with the variation shown in Figure 5.3. This is because the total activity varies amongst the *Artemisia afra* species examined. A further investigation was done to determine the influence of the environment on the chemical composition of the same species originating from the same seed. The acetone and ethanol extract from ARC show high total activity against all four micro-organisms compared to the other extracts and this is indicated in Figure 5.4. This implies that the environment appears to be an important factor in determining both the chemical composition and biological activity.

Artemisia afra species originating from the same seed source were analyzed using the same experimental approach and then compared. The environments where these species were grown are shown in Table 5.2.

Table 5.2. *Artemisia afra* species originating for the same seed source that were grown in different environments

Sample code	Environment conditions
N	Seed source
A	Nutrient rich soil and watered by drop irrigation
D	Soil fertilized with KNO ₃
H	Grown under a tree, nutrients and water competition
J	Treated with pyrethrum, an insecticide
M	Grown in a maize field, average climate and soil
L	Grown in a well-cared garden, good soil and climate

The developed TLC plates were sprayed with *p*-anisaldehyde, vanillin sulphuric acid, methanol phosphoric acid, tri-chloroacetic acid (TCA) and toluene spraying reagents. TLC profile of TCA, methanol phosphoric acid and toluene spray reagents were not as good as those sprayed with as *p*-anisaldehyde, vanillin sulphuric acid spray reagents. These are shown in Figure 5.5. Total activities values of these *Artemisia afra* species are shown in Figure 5.6.

The TLC profile of these *Artemisia afra* species originating from the same seed source (N) but exposed to different environmental condition show variation in their chemical composition. Samples A and J are strikingly different from the other samples. N is the least complex showing the least compounds, while A was the most complex. This sample was grown in good soil and treated with drop irrigation regularly. When comparing sample A to N, it can be concluded that well cared plant species yield high quantity chemical components (Jork, *et. al.* 1990). H is open to speculations.

Sample J, treated with pyrethrum shows additional compounds well separated by the BEA system. This species has unique compounds of R_f values of 0.43 and 0.51. The additional compounds show that pyrethrum treatment may result in metabolic changes that result in the accumulation of certain compounds in the plant.

The total activities of the acetone extracts were determined and are represented by the graph in Figure 5.6 The acetone extracts were selected because they contain both the non-polar and polar compounds and showed good resolution. Samples N, A, D, H, J and L show some activity against *E. faecalis* but little activity against *S. aureu*, *P. aeruginosa* and *E. coli*. On average, *Artemisia afra* has low antibacterial activity.

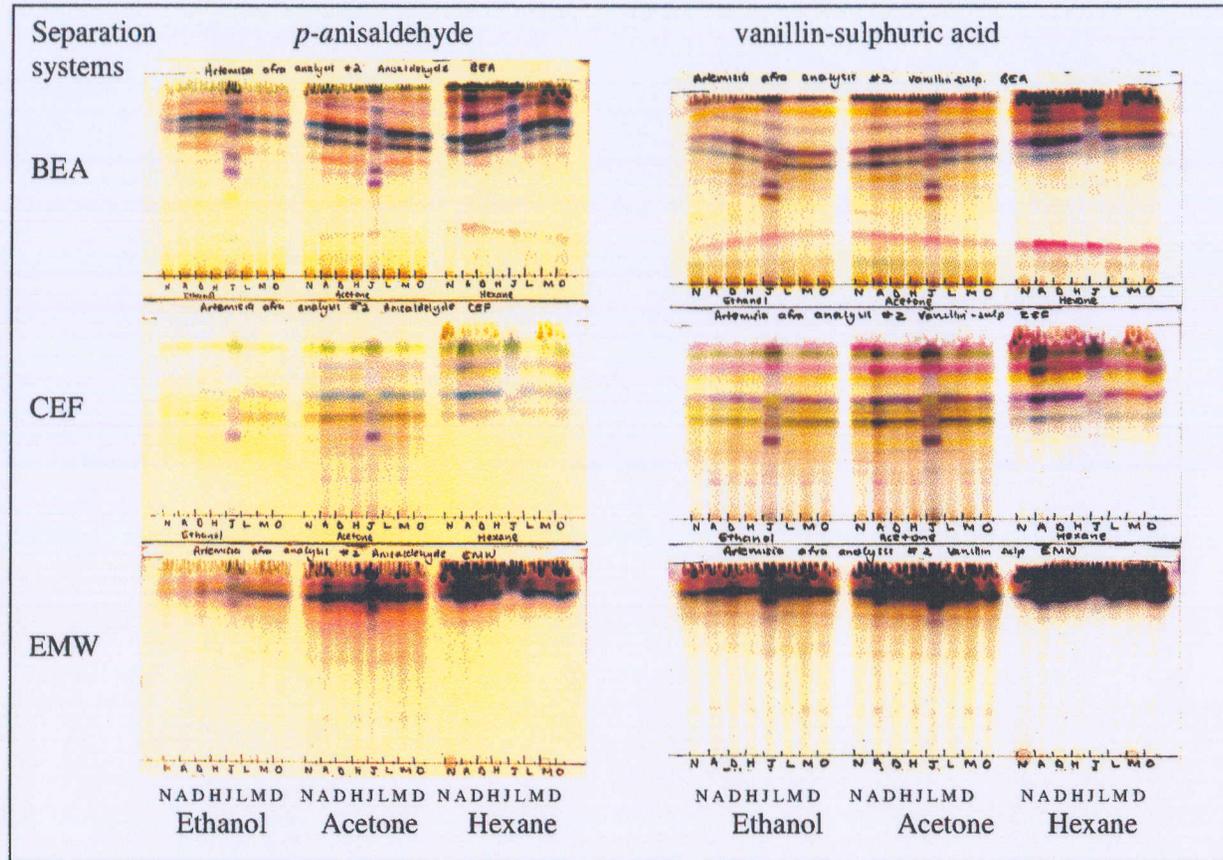


Figure 5.5 TLC Profiles of *Artemisia afra* species originating from the same seed source, sprayed with *p*-anisaldehyde and vanillin sulphuric acid spray reagents. Key: N: seed source sample; A: Agricultural Research Council sample; D: fertilized soil sample; H: shade sample; J: pyrethrum-treated sample; M: maize land sample; L: home garden sample; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

Artemisia afra group analysis 2

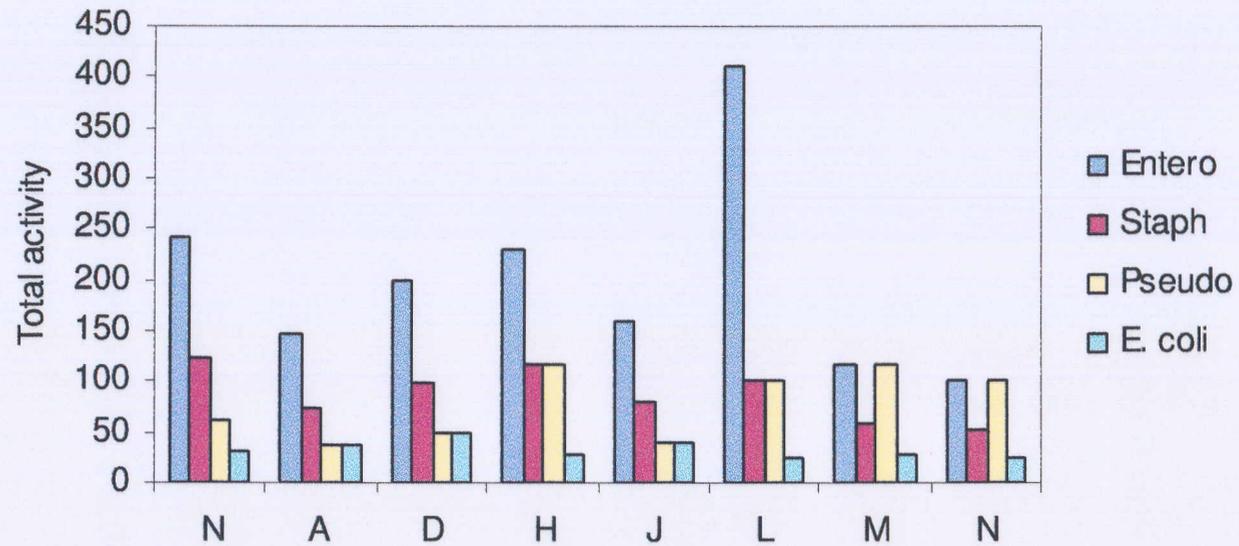


Figure 5.6 Total activities of acetone extracts of the *Artemisia afra* species tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* microorganisms. Key: N: seed source sample; A: Agricultural Research Council sample; D: fertilized soil sample; H: shade sample; J: pyrethrum-treated sample; M: maize land sample; L: home garden sample; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

Sample L, which was grown in a well-cared garden showed the highest activity against *E. faecalis*, *P. aeruginosa* and *E. coli*.

5.2.3 Determination of adulteration in traditional medicine markets

The aim of this section was to determine the magnitude of adulteration in traditional medicines traded in the market. The results of this investigation reflect the true extent of adulteration in Pretoria markets, which would have health implications.

A series of investigations were conducted to answer this question. Amongst plant species selected for identification in Section 3.1, *Warburgia salutaris* (Molaka) and *Peltophorum Africanum* (Mosetlha) were selected for this investigation since almost every trader in the market sold these species. Plant materials of these species were bought from at least four different traders situated at different outlets in Pretoria traditional medicine markets. It was assumed that the collectors are different. The weights of the materials received were determined (Table 5.3) and pictures of the bark samples were taken before the materials were processed and these are shown in Figure 5.7 and Figure 5.8.

Table 5.3. A list of *Warburgia salutaris* and *Peltophorum africanum* species bought from different traders for the same price (R5.00)

Trader's code	Plant species	Weight (gram)
Vendor A	<i>Warburgia salutaris</i>	28.76
	<i>Peltophorum africanum</i>	27.00
Vendor B	<i>Peltophorum africanum</i>	66.33
Vendor C	<i>Warburgia salutaris</i>	41.2
	<i>Peltophorum africanum</i>	111.29
Vendor D	<i>Warburgia salutaris</i>	17.01
	<i>Peltophorum africanum</i>	24.52
Vendor E	<i>Warburgia salutaris</i>	81.18
	<i>Peltophorum africanum</i>	48.17

The weight in Table 5.3 represents a wide variation in pricing. Vendor C gave more quantity of *P. africanum* than the other traders and Vendor E gave the most quantity of *Warburgia salutaris*. There is no pattern that can be deduced from these values. It is well known that traditional traders and healers don't often use measurements, as indicated by Figure 5.7 (*Warburgia salutaris*) and Figure 5.8 (*Peltophorum africanum*). The weight of the materials may not play a major role in identifying adulteration because it could reflect the water content of the plant.

Thus a fresh bark cannot be compared to a dry bark. In this study, all bark material received were dry, therefore, the weight of the materials could be compared in this case. The materials received from Vendor D have the lowest weight for both species. The significant variation in mass could be an indication of possible adulteration. Pricing variation may be a result of market force e.g. location, rental income of clientele and buying price.



Figure 5.7 Photographs of the bark of *Warburgia salutaris* bought from vendor A, C, D and E.

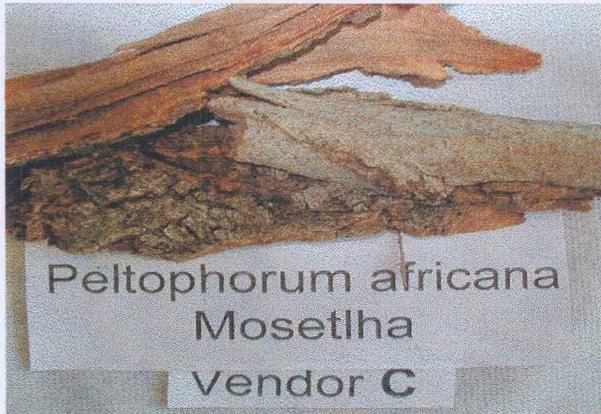
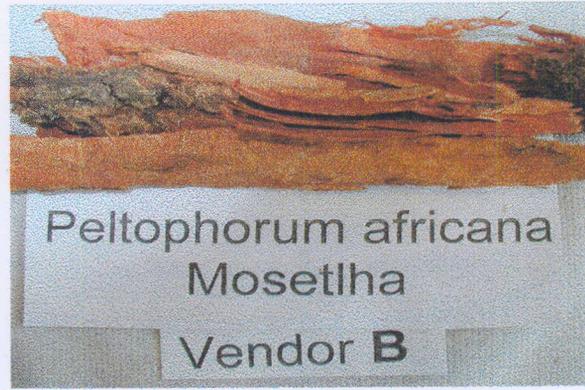
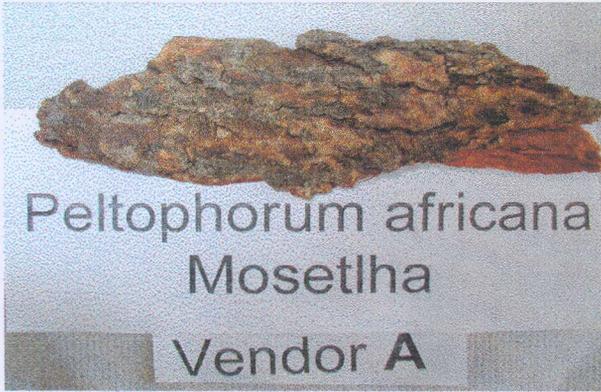


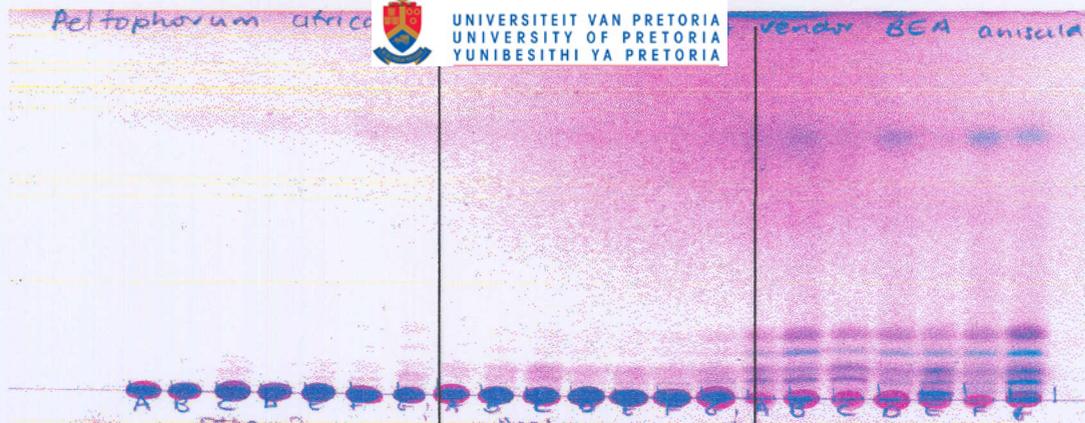
Figure 5.8 Photographs of the bark *Peltophorum africanum* (Moseitlha) bought from vendors A, B, C, D and E.

5.2.3.1 Determination of adulteration in *Peltophorum africanum* (Mosetlha) species

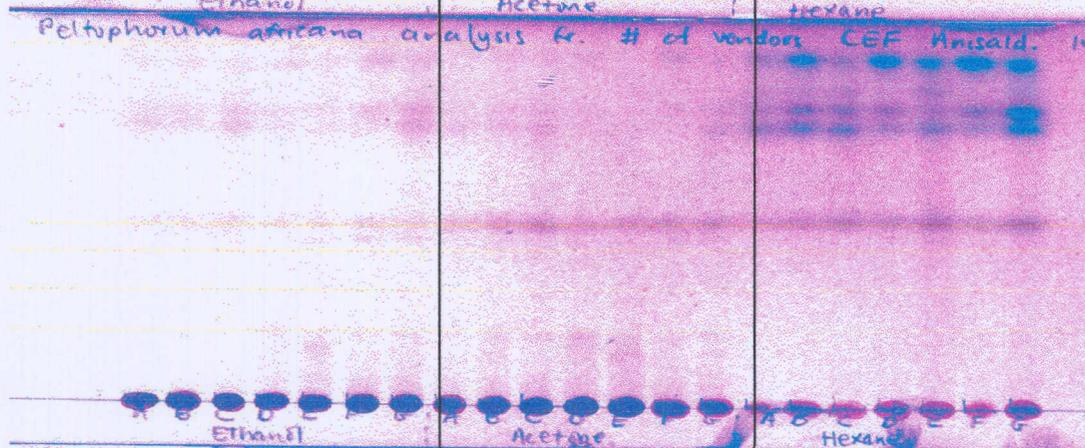
The bark material from five different traders was extracted with three solvents: ethanol, acetone and hexane. TLC plates loaded with the extracts were developed as before and then sprayed with *p*-anisaldehyde and vanillin sulphuric acid in Figures 5.9 and Figure 5.10, respectively. These figures show similarities in the extracts of all five samples of Vendors A to E. There is slight variation in the intensity of the bands detected, even though all samples have similar compounds. This appears to show variable concentration. The total activity results were also variable, confirming the results obtained in Chapter 4.



BEA



CEF



EMW

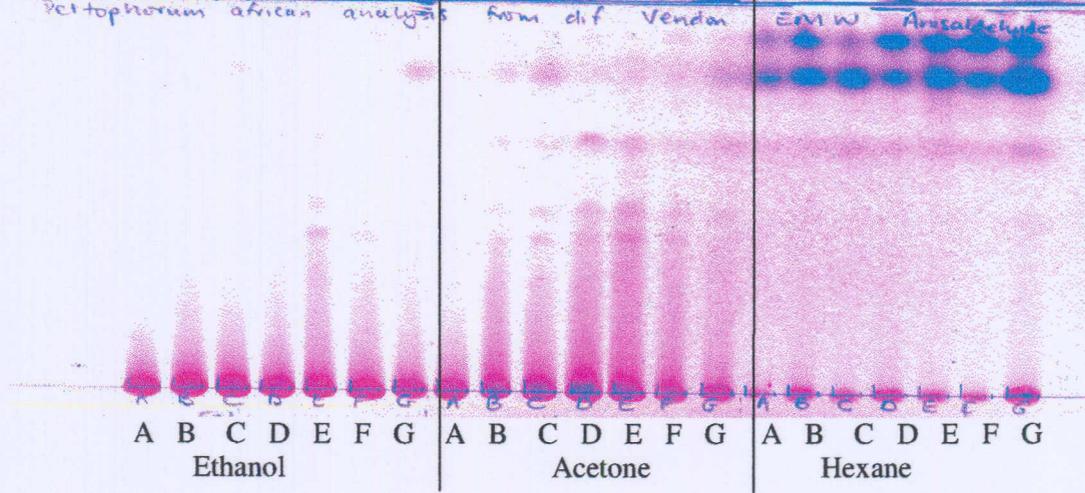


Figure 5.9 TLC profiles of *Peltophorum africanum* bark materials obtained from different vendors (A, B, C, D, E, F) and G extracted with ethanol, acetone and hexane and detected with *p*-anisaldehyde. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

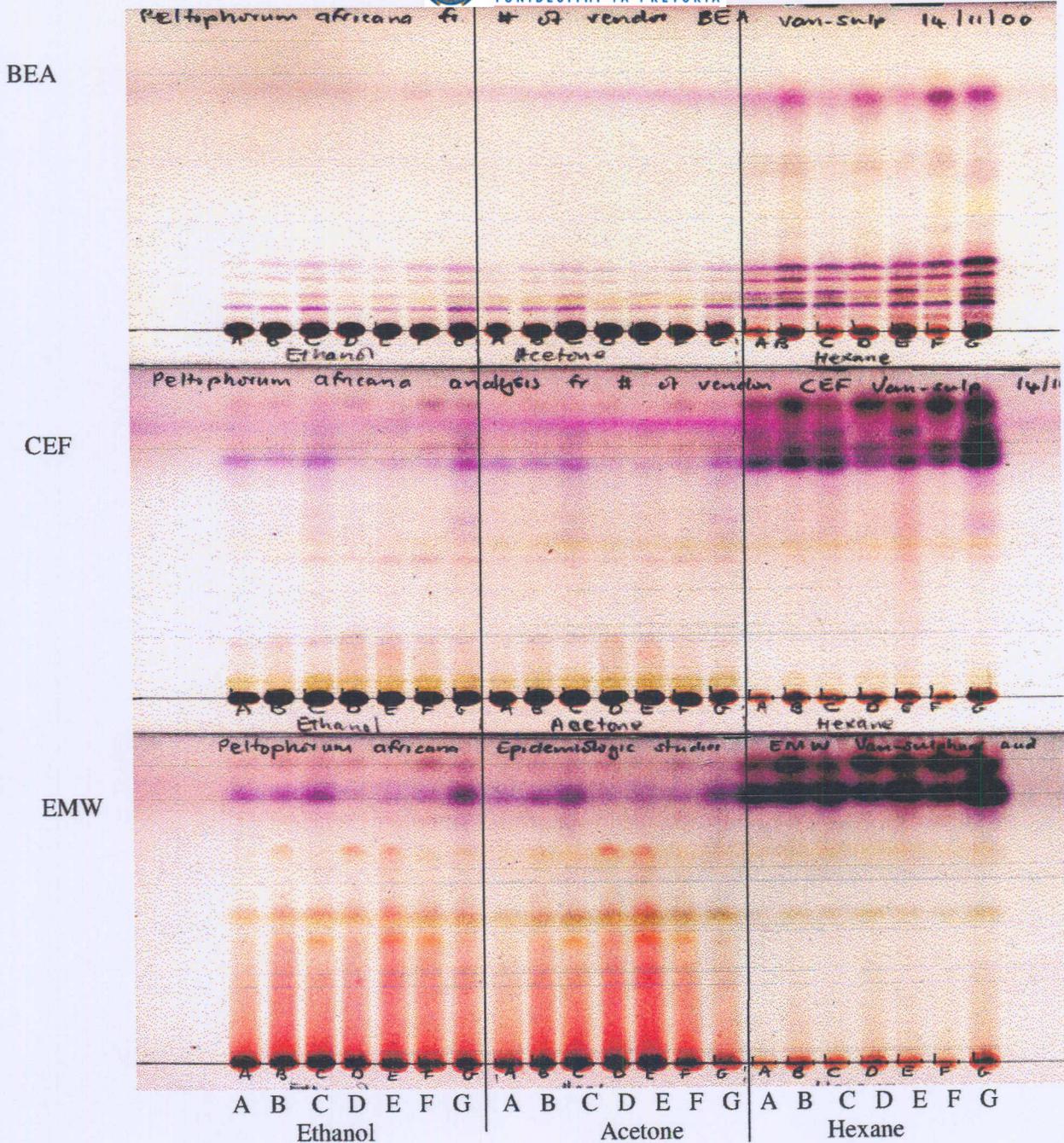


Figure 5.10 TLC profiles of *Peltophorum africanum* bark materials obtained from different vendors (A, B, C, D, E, F and G) extracted with ethanol, acetone and hexane and detected with vanillin sulphuric. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

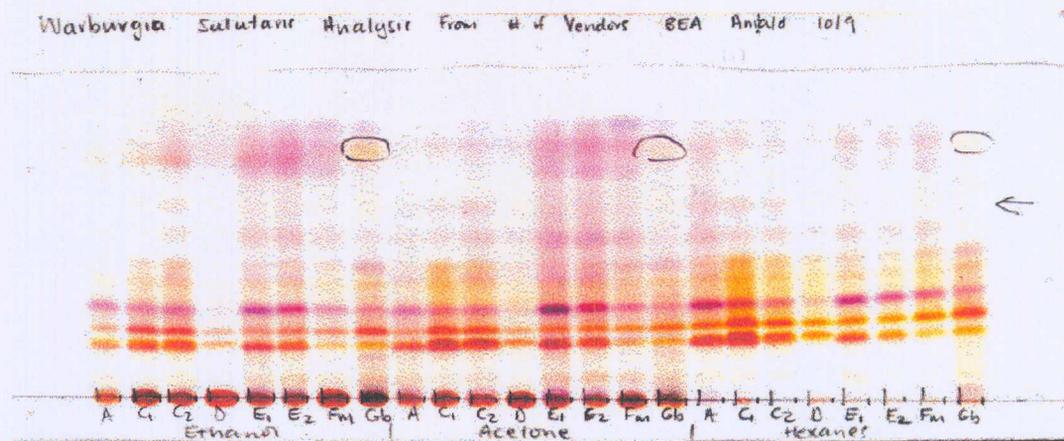
5.2.3.2 Determination of adulteration in *Warburgia salutaris* (Molaka) species

Bark material of *Warburgia salutaris* was bought from four vendors and the photographs of these materials are shown in Figure 5.7. The samples received from Vendor C and E were separated into two groups since the bark material did not look similar on visual examination. The samples were coded C₁, C₂, E₂₁ and E₂ respectively. Reference sample obtained from the Pretoria National Botanical Garden was also loaded as control.

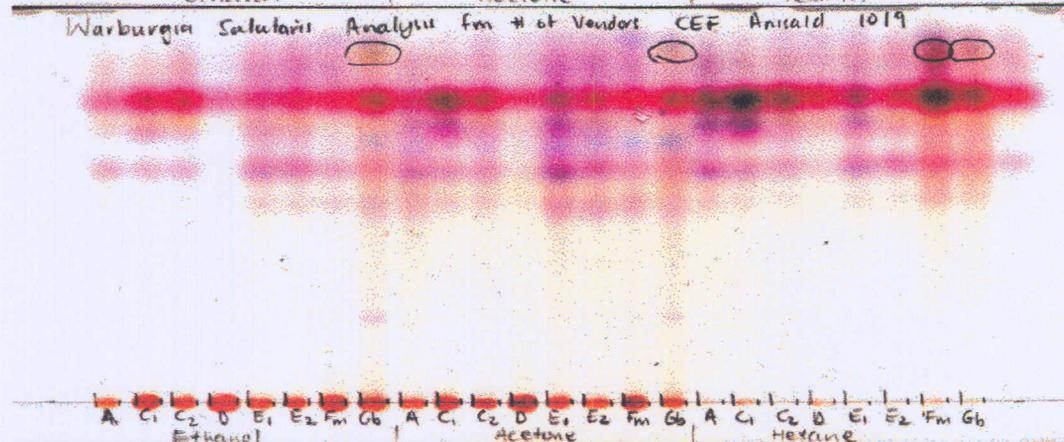
The bark was ground to fine powder extracted with ethanol, acetone and hexane and then separated using the three systems (BEA, CEF and EMW) outlined in Section 2.3. The developed TLC plates were sprayed with *p*-anisaldehyde and vanillin sulphuric acid and are shown in Figures 5.11 and 5.12, respectively.

There was no major difference between the samples analyzed, except the samples received from Vendor D. This sample lacks most of the compounds visualized in the other samples, although the hexane extracts were similar in all cases. The plate sprayed with *p*-anisaldehyde show this similarity better. It can, therefore, be concluded from this that Vendor D is not selling *W. salutaris*.

BEA



CEF



EMW

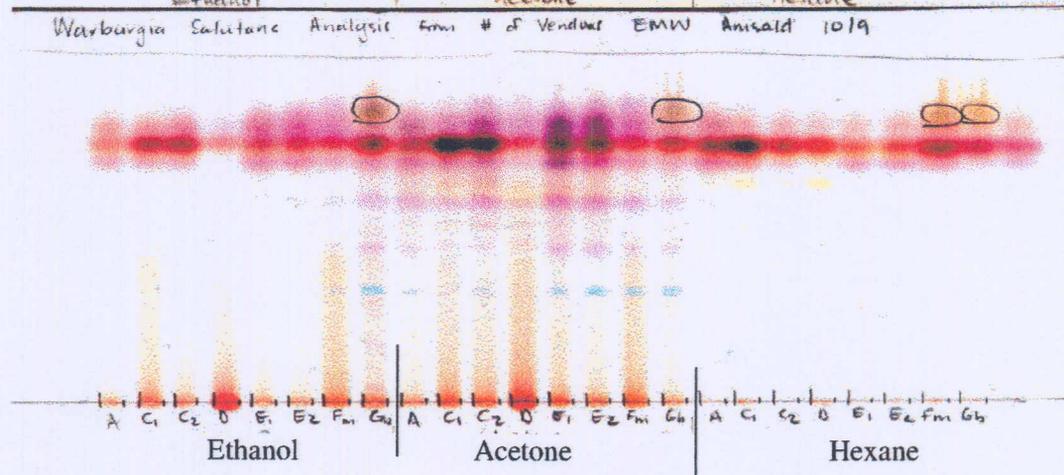
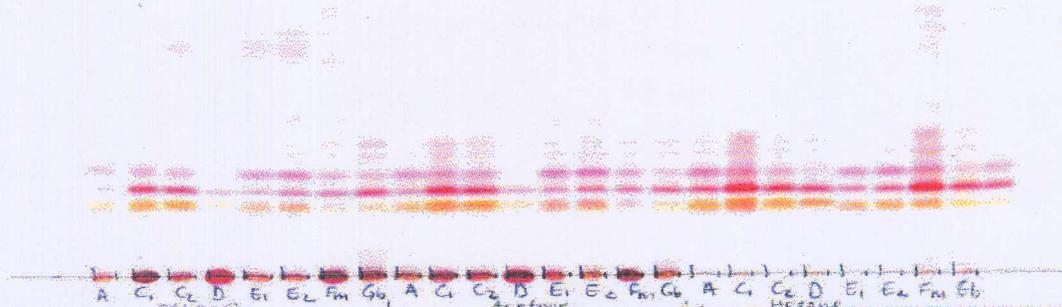


Figure 5.11 TLC profile of *Warburgia salutaris* species from different vendors (A, C, D, E and G) extracted with ethanol, acetone and hexane. The extracts' chemical components on the TLC plate were sprayed with *p*-anisaldehyde. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

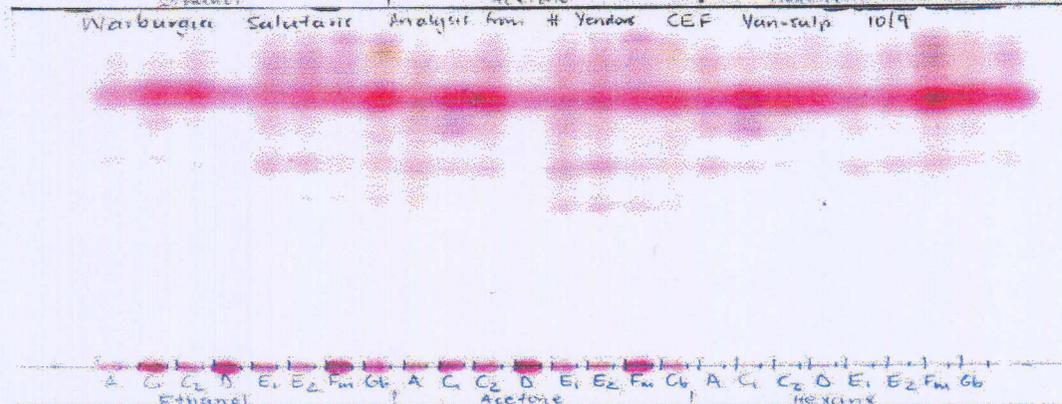
Warburgia salutaris Analysis from # of Vendors BEA Van-sulp 10/9

BEA



Warburgia salutaris Analysis from # of Vendors CEF Van-sulp 10/9

CEF



Warburgia salutaris Analysis from # of Vendors EMW Van-sulp 10/9

EMW

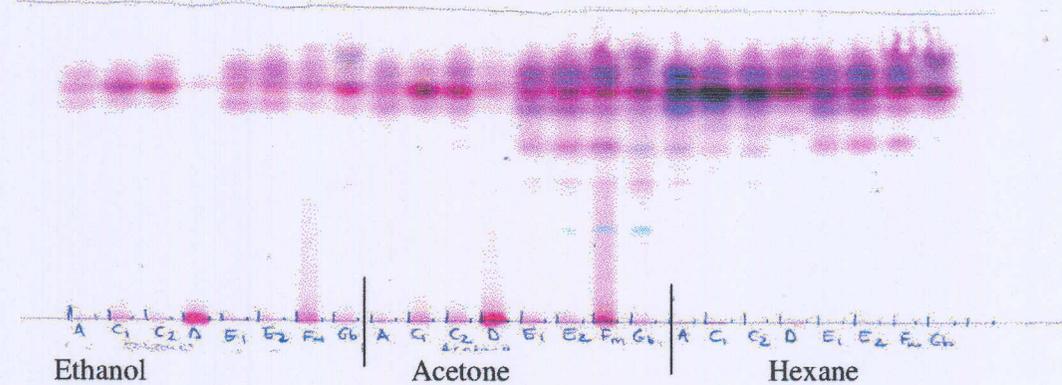


Figure 5.12 TLC profile of *Warburgia salutaris* species from different vendors (D, E, F and G) extracted with ethanol, acetone and hexane, sprayed with vanillin sulphuric acid spray reagent. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

5.3 Conclusion

The TLC technique is not suitable for differentiation between closely related species. Therefore, it can only be used to identify species belonging to different families and genera. It was indicated that similarities between closely related species may cause uncertainties when identifying these species by TLC, however, we need to expand this investigation by determining the environmental influence on the chemical profile. It is known that genetic mutations increase genetic diversity, however, the geographic influences may also affect the genotype of plant species over a period of time.

The environment has little effect on chemical composition of plants except if treated with insecticide. However, the genetic variation of a plant is more important. It has been shown here that TLC can be used in the identification of plant species sold in the market. However, it is still not known whether there is substitution or adulteration of popular plant medicines in traditional medicine markets. The next stage of this study is to determine the extent to which this happens. It would appear from the research conducted that the environment affects chemical composition in plants (N versus A). The extent to which this is important is not possible to ascertain. Genetic variation may be more important.

TLC fingerprints of *Warburgia salutaris* and *Peltophorum africanum* indicate that planar chromatography can also be used to analyze traditional material for adulteration assays.

The difference in extracts for *Warburgia salutaris* samples received from Vendor D could be an indication of contamination or factor of age. This chapter has indicated that TLC method can be used to determine the magnitude of adulteration, however, it cannot be used to differentiate closely related species. The next chapter will make use of TLC to fingerprint over-exploited medicinal plants.

6 The use of planar chromatography to identify over-exploited medicinal plants

6.1 Introduction

Uncontrolled utilization of bark for traditional medicine is of great concern to the conservation officials, researchers and traditional healers in South African. Conservation groups are currently conducting case studies to explore the extent of the threat posed to popular medicinal species in South Africa (Zschocke *et al.*, 2000b).

The most popular medicinal plants have been identified as *Cryptocarya woodii*, *Cryptocarya myrtifolia*, *Ocotea bullata*, *Prunus africana*, *Rapanea melanophloeas* and *Zanthoxylum dayvi*. *Prunus africana* (African Stinkwood) is of worldwide interest since it is heavily exploited for its bark for use in Benign Prostatic Hypertrophy (Traffic network, 2001 and Achieng, 1999). However, more plant species in Africa are endangered due to overuse such as *Warburgia salutaris* and Hypoxis. From the work reported in Section 5.2.1, it was concluded that TLC is limited when it comes to distinguishing closely related species. An attempt was made to use the method to profile medicinal plants that appear to be endangered.

Fresh bark samples of five over-exploited species were collected and are described in the next section. The bark material was ground up, extracted with ethanol, acetone and hexane and then separated on TLC plates in three systems (Chapter 2). Chemical components of the extracts were visualized with *p*-anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid spray reagents. Antibacterial activities of these species were

detected despite the fact that some species like *Ocotea bullata* and *Cryptocarya* are mainly used for headaches.

6.2 Results and discussion

6.2.1 TLC analysis of over-exploited traditional medicines

6.2.1.1 *Cryptocarya myrtifolia*

Common names: kanferboom (Afrikaans), camphor tree (English), igqeba or umkhondweni (Zulu)

The bark is used as a substitute for *Ocotea bullata* (Hutchings et al. 1996), however, the main constituents of *O. bullata* are not found in any of the *Cryptocarya* species (Zschocke et al., 2000a). Since *O. bullata* bark remedies are used for treating headaches, van Staden and Zschocke (2000) investigated the analgesic activity of *O. bullata* and *Cryptocarya* species. Their findings were similar for both plant species (van Staden and Zschocke, 2000). Figure 6.1 shows the TLC profile of *Cryptocarya myrtifolia* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid.

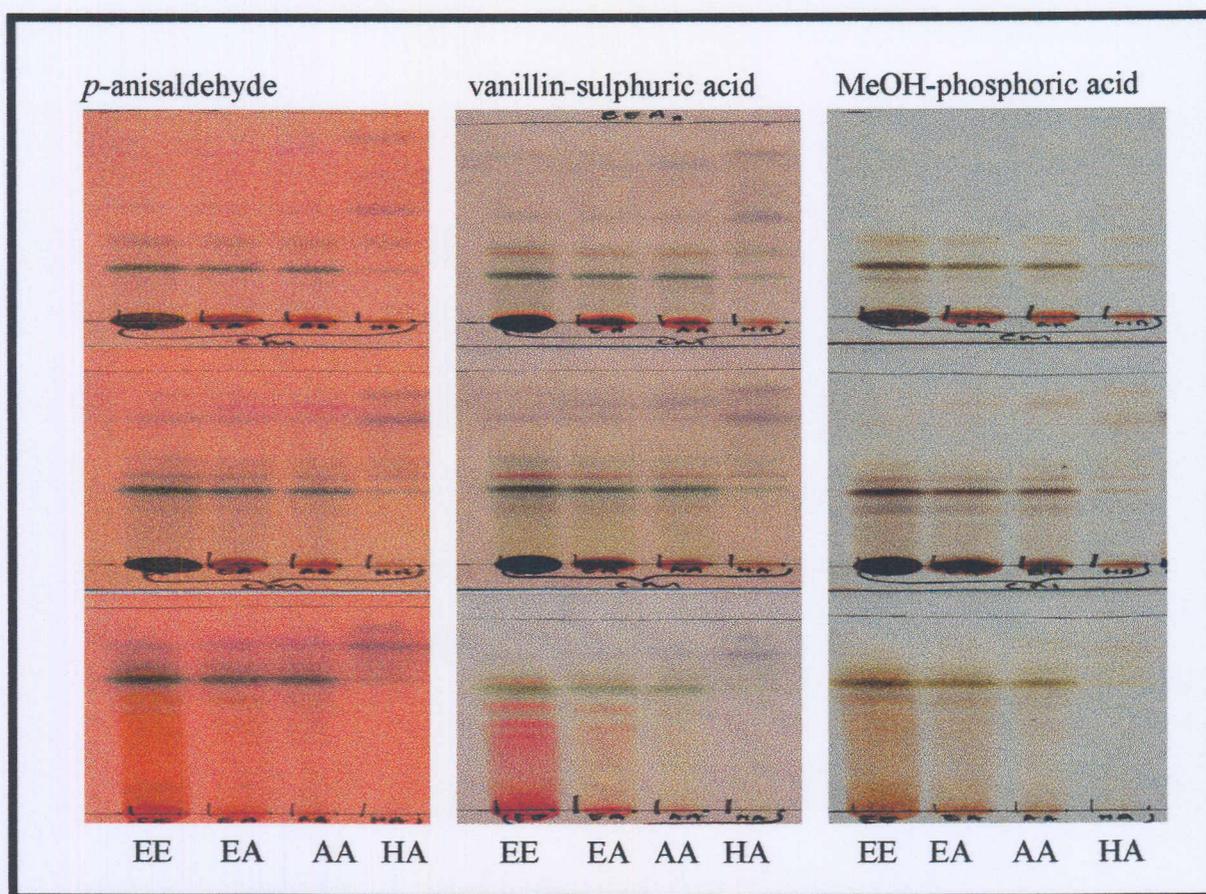


Figure 6.1. TLC profiles of *Cryptocarya myrtifolia* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid. Key: EE: ethanol-ethanol extract; EA: ethanol-acetone extract; AA: acetone-acetone extract and HA: hexane-acetone extract.

The ethanol-ethanol (EE) extract contain more compounds than the ethanol-acetone (EA) extract and acetone-acetone extract (AA). This is because, not all compounds extracted with ethanol can dissolve in acetone as these solvents have different polarities. Vanillin sulphuric acid spray reagent is a better detection reagent for this species and it detected compounds that were also detectable with *p*-anisaldehyde and methanol phosphoric acid spray reagents.

6.2.1.2 *Ocotea bullata*

Common names: stinkhout (Afrikaans) black stinkwood (English) and unukani (Zulu),

The stem bark of *Ocotea bullata* is one of the most frequently used traditional medicines in South Africa (Mander, 1997). The use of this plant species by traditional healers for a wide-range of ailments including headaches, back-ache, urinary tract problems and magical purposes, is well documented (Hutchings et al. 1996 and van Wyk et al. 1997). However, it has not been possible to associate any of the known compounds with specific biological activity such as cyclo-oxygenase-1 (COX-1) inhibitory activity (Jäger et al., 1996). It is also used for producing high quality furniture and its popularity has made it rare species.

This plant species is widely distributed in the forests along the southern and eastern parts of South Africa, from the Cape Peninsula eastwards to the Southern Cape, Eastern Cape, KwaZulu-Natal, Mpumalanga and the Limpopo Province (van Wyk et al. 1997). *O. bullata* is now protected in KwaZulu-Natal as it has become an endangered species. Its importance to the herbal medicine trade has attracted the attention of conservationist and natural products chemists. Currently, several new neolignans have been isolated from the bark of this species, e.g. ocobullenone (Zschocke et al., 2002). Its leaves are assumed to have unidentified volatile compounds such as monoterpenoids. The biological activity of this plant is suspected to result from the neolignans in the bark (van Wyk et al. 1997). Figure 6.2 illustrates the fingerprints of *Ocotea bullata* sprayed with *p*-anisaldehyde, vanillin sulphuric acid and methanol-phosphoric acid spray reagents.

6.2.1.3 *Rapanea melanophloeas*

Common names: isiqalaba-sehlathi (Xhosa and Zulu), isiqwane-sehlathi (Xhosa), umaphipha (Zulu), Cape beech (English) and Kaapse boekenhout (Afrikaans)

The tree grows naturally along the east coast of South Africa. Bark infusions are more frequently used than roots as expectorants, emetics and treatment of muscular pain, stomach and heart disorders. The bark and leaves are known to contain tannins and triterpenoid saponins, respectively. There is no readily available information about the pharmacological activity of this plant (Hutchings et al. 1996 and Wyk et al., 1997). Figure 6.3 shows the TLC profile of *Rapanea melanophloeas* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid spray reagents.

Lesser compounds were detected with the chosen sprays. Therefore, conclusions about its chemical composition cannot be drawn except that it contains the compounds detected by three spray reagents.

6.2.1.4 *Zanthoxylum davyi*

Common names: isimungumabele (Zulu) knobthorn (English), knoppiesdoring (Afrikaans)

Its bark is used for snakebites, chronic coughs, boils, toothache, pleurisy and as emetic; the leaves are used for chest pains and roots for sore throats, mouth ulcers, venereal diseases and aphrodisiacs. Figure 6.4 shows the TLC profile of *Zanthoxylum davyi* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid spray reagents.

6.3 Conclusion

In this chapter, TLC fingerprints of plant materials over-harvested were shown and may be used in future for adulteration investigation. In conclusion, planar chromatography can be used to compile fingerprints of traditional medicines as references. The next chapter gives a summary of all investigations conducted in this study and concludes by recommending future work.

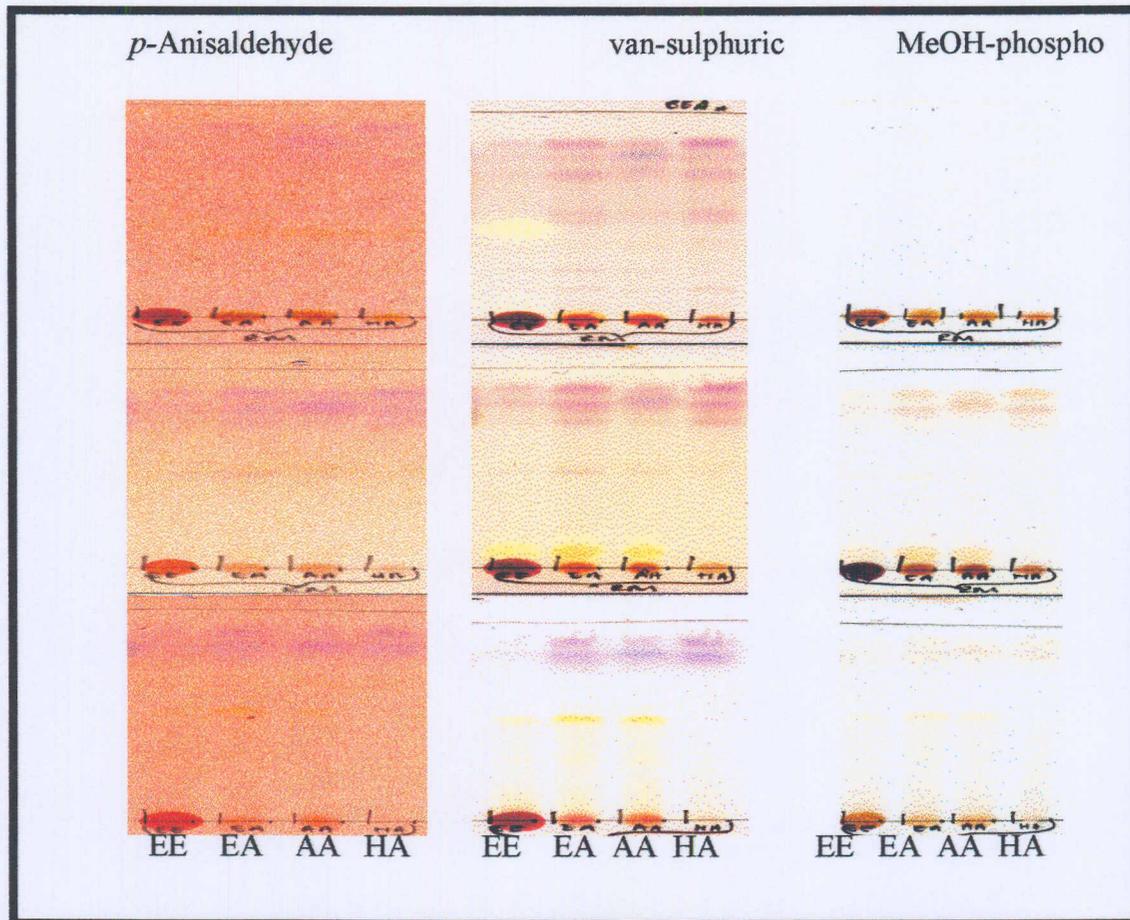


Figure 6.3 TLC profiles of *Rapanea melanophloeas* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid spray reagents. Key: EE: ethanol-ethanol extraction; EA: ethanol-acetone extract; AA: acetone-acetone extract and HA: hexane-acetone extract.

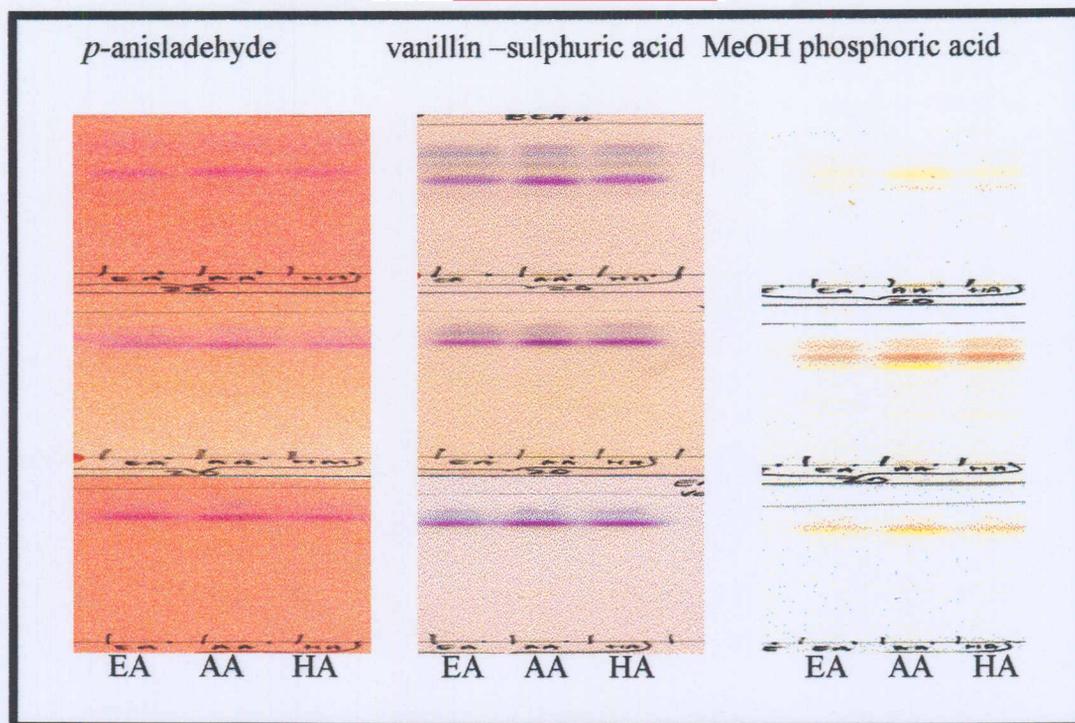


Figure 6.4 TLC profile of *Zanthoxylum davyi* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid spray reagents. Key: EA: ethanol-acetone extract; AA: acetone-acetone extract and HA: hexane-acetone extract.

The ethanol (EA), acetone (AA) and hexane (HA) extracts show similar chemical fingerprints for all extracts in Figure 6.4. This characteristic appears to be unique to this species when comparing it to all species analyzed. Like the rest of the over-exploited species analyzed in this chapter, it contains few compounds that are detectable using the spray reagents. The detected purple colour could be terpenoid.

6.2.1.5 *Prunus africana*

Common names: inyazangoma-elimnyama (Zulu), umkakase (Xhosa), rooistinkhout (Afrikaans) and red stinkwood (English)

The stem bark is the only plant part used for medicinal purposes such as chest pains (decoction) and benign prostate hypertrophy (lipid and phytosterol extracts). Previous investigations have revealed the presence of phytosterols (β -sitosterol and campesterol) and cyanogenic glycosides (amygdalin) from bark extracts. Pentacyclic triterpenoid esters and various linear aliphatic alcohols together with their ferulic acid esters were also observed in the extracts. Biological activity against prostatic adenoma has been reported as resulting from β -sitosterols. Other components may contribute to the beneficial biological activity. Its present exploitation has led to exceptional shortage in South Africa and Cameroon (Ndibi and Kay, 1997). Figure 6.5 shows the TLC profile of *Prunus africana* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid spray reagents.

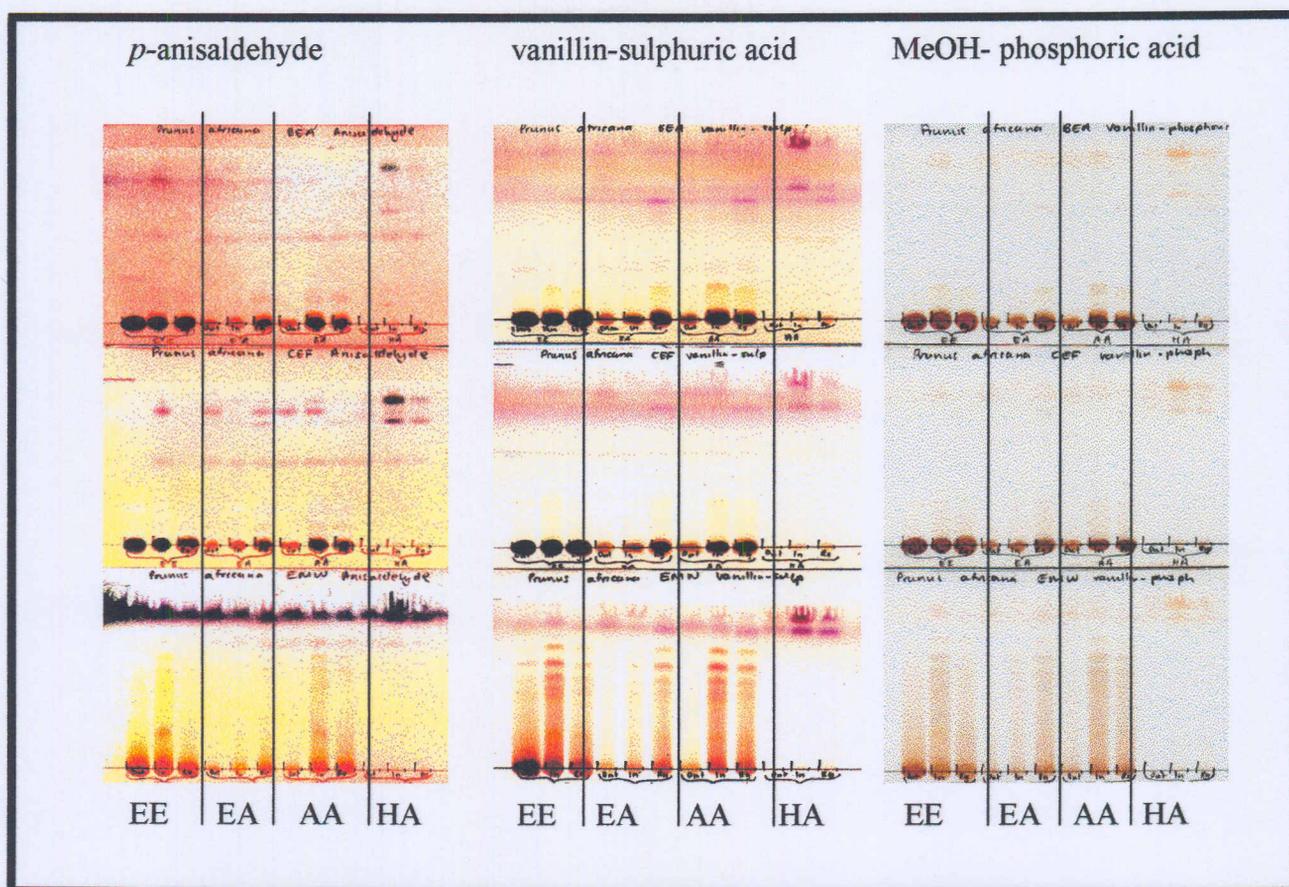


Figure 6.5 TLC profile of *Prunus africana* sprayed with *p*-anisaldehyde, vanillin-sulphuric and methanol-phosphoric acid spray reagents. Key: EE: ethanol-ethanol extraction; EA: ethanol-acetone extract; AA: acetone-acetone extract and HA: hexane-acetone extract.

The ethanol-ethanol (EE) extract of *Prunus africana* was similar to the acetone-acetone (AA) extract, whereas the ethanol-acetone (EA) extract showed little compound extraction. This observation could imply that compounds extracted with ethanol should be dissolved in ethanol not in acetone because they may appear insoluble in acetone.

6.2.2 Antibacterial activity analysis

Determination of the biological activity of the exploited species was conducted, because the techniques and material were available and also because many traditional medicines may have potential antibacterial activity. Figure 6.6 shows graphical illustrations of total activity of the plant species investigated in this section.

The MIC value for the ethanol-ethanol extract of *Rapanea melanophloeas* was 0.02 mg/ml. In this investigation we have compiled TLC fingerprints of over-exploited medicinal plants. *Cryptocarya myrtifolia* sample extracted with ethanol showed high sensitivity to *Staphylococcus aureus*. However, *Cryptocarya myrtifolia* was less sensitive to the other organisms. *Zanthoxylum dayvi* was less sensitive to all microorganisms. *Ocotea bullata* ethanol and acetone extracts were slightly sensitive to *Enterococcus faecalis*. In general, these species are vulnerable for over-exploitation and have shown poor antibacterial inhibition against the microorganisms used in the test.

During the extraction process it was noticed that the ethanol-extracted components were decreased drastically when the ethanol crude extract was dissolved in acetone. It was then decided to keep the extract compounds in ethanol although ethanol takes time to be absorbed into the silica plates.

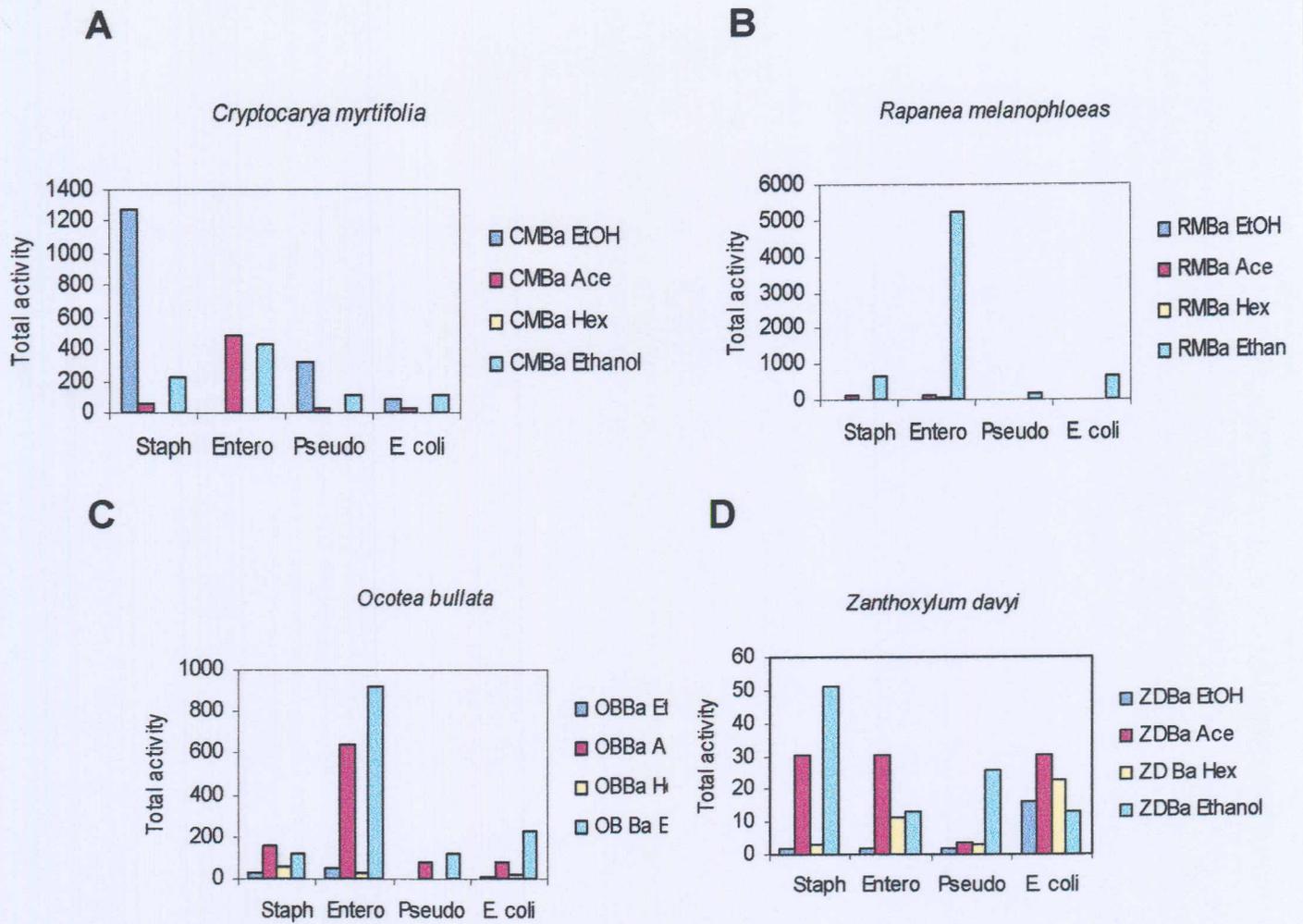


Figure 6.6 Total activity of the over-exploited species A: *Cryptocarya myrtifolia*, B: *Rapanea melanophloeas*, C: *Ocotea bullata*, and D: *Zanthoxylum davyi* tested against *E. faecalis* (Entero) *S. aureus* (Staph), *P. aeruginosa* (Pseudo) and *E. coli* microorganisms.

6.3 Conclusion

In this chapter, TLC fingerprints of plant materials over-harvested were shown and may be used in future for adulteration investigation. In conclusion, planar chromatography can be used to compile fingerprints of traditional medicines as references. The next chapter gives a summary of all investigations conducted in this study and concludes by recommending future work.

7 General Conclusions

The separation systems used BEA (benzene (8): ethanol (1): ammonium system ratio (0.1)), CEF (chloroform (4): ethyl acetate (3): formic acid system (1)) and EMW (ethyl acetate (10): methanol (1.35): water system (1)) were suitable for separating compound in all plant extracts. The study showed that planar chromatography could be used to identify traditional medicines by comparing chemical fingerprints of unknown plant species to reference species. However, this application did not appear suitable for identification of closely related plant species. The chemical fingerprint of the market species traded as isibhaha (IsiZulu), Mosetlha (SeSotho), Legwama (SeSotho), mosetlhana (SeSotho) and Lengana (SeSotho) were in agreement with those of *Warburgia salutaris*, *Peltophorum africanum*, *Boophane hymanthoides*, *Acacia caffra*, and *Artemisia afra*, respectively. However, the market species traded as umahlanganisa (Zulu) was identified as neither *Croton sylvaticus* nor *C. gratissimus*. It is possible that the *Croton* sample was substituted with a different species or that the Zulu name used for the medicinal plant refer to a different species. For future study, further identification of this species need to be confirmed.

Although the technique applied in this investigation was adequate for this study. It was shown that the Thin Layer Chromatography (TLC) method was unable to differentiate amongst closely related plant species of the Fabaceae family.

The application of TLC showed chemical and antibacterial activity variation amongst the samples analyzed. It was found that the environment where a plant is grown may influence its chemical composition, especially when treated with pesticides. This investigation confirmed that *Artemisia afra* has high diversity. Therefore, this study

indicated that the environment where the plant is grown play a minor role in changing the chemical composition and biological activity of the plant, unless treated with pesticides. This study has also shown that adulteration in the Pretoria market is not a problem. Planar chromatography can also be used to compile fingerprints of traditional medicines that can be used to identify plant species in poisoning incidences or during uncertainties in identifying plant part.

8 References

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9. Appendix

Questionnaire No. 1				
Assesement of the popularity of traditional medicines in Pretoria City				
Time		Date		
Vendor Name				
Market area				
Number	Medicine name	Plant part whole plant (10) root (10) bark (8) leaves (4)	Place of origin e.g KZN (10) local (5)	Medicinal use
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				