Storage of frequently used traditional South African medicinal plants

by

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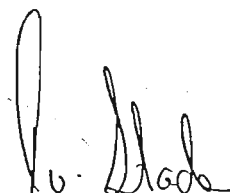
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DECLARATION

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal Pietermaritzburg, from April 2000 to November 2002, under the supervision of Professor A.K. Jäger and Professor J. van Staden.

These studies are the result of my own investigations, except where the work of others is acknowledged, and have not been submitted in any other form to another University.
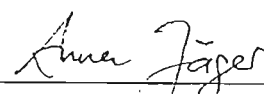
I declare the above statement to be true.

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ABSTRACT

The post-harvest physiology of nine frequently used indigenous southern African medicinal plants was investigated, in particular the effects of storage time and accelerated ageing on the biological activity and chemical constituents of these plants. Water, ethanol and hexane extracts of fresh plant material as well as material that had been stored in dry form in paper bags at room temperature for 90 days (short-term) were tested. Three bioassays, the COX-1 anti-inflammatory assay, nematode anthelmintic assay and minimum inhibitory concentration anti-bacterial assay, were used to determine biological activity. Thin layer chromatography of all the plant extracts were used to determine changes in chemical composition. The plants tested were *Alepidea amatymbica* Eckl. & Zeyh., *Leonotis leonurus* (L.) R. Br., *Drimia robusta* Bak., *Vernonia colorata* (Willd.) Drake, *Scilla natalensis* Planch., *Eucomis autumnalis* (Mill.) Chitt. subsp. *autumnalis*, *Bowiea volubilis* Harv. ex Hook. f., *Helichrysum cymosum* (L.) D. Don and *Siphonochilus aethiopicus* (Schweinf.) B. L. Burtt. Only those plants, which are known to exhibit a particular biological activity either traditionally or scientifically, were tested in the relevant bioassays. Of the plant extracts tested for anthelmintic activity only the water extracts showed activity and very little change in activity was observed after storage. Of the plant extracts tested for anti-inflammatory activity the ethanol extracts generally yielded highest activity. *S. natalensis* and *B. volubilis* both showed an increase in cyclooxygenase inhibition (anti-inflammatory) activity after storage whereas *S. aethiopicus, H. cymosum, D. robusta* and *V. colorata* showed a loss in activity after storage. The anti-inflammatory activity of *E. autumnalis* did not change. The water extracts of plants tested for antibacterial activity showed no activity, whereas the ethanol extracts generally showed an increase in activity. The TLC fingerprints indicated that there was chemical break-down during storage in certain species. These corresponded to the changes in biological activity.

Alepidea amatymbica, Eucomis autumnalis, Helichrysum cymosum, Leonotis leonurus, Siphonochilus aethiopicus and *Vernonia colorata* were investigated further as to the effect of one year's storage (long-term storage) on their chemical composition and biological activity. Similar trends to that of the 90-day storage were observed. Activity gained in plants that were stored for 90 days was retained after a year of storage.

Elevated temperature and humidity (55$\square$C and 100% relative humidity) were used to accelerate the ageing process of *Alepidea amatymbica, Leonotis leonurus* and *Vernonia colorata* plant material. Again changes in the chemical composition and biological activity were observed, and the extent of these changes was greater than those in the stored material. The compounds responsible for the cyclooxygenase

inhibition in the ethanolic extracts of *Alepidea amatymbica* leaf material appear to be stable and were not affected by the conditions of the accelerated ageing procedure (55□C and 100% humidity for seven days), but the root material lost activity, as did the leaf material of *Leonotis leonurus*. The leaf material of *Vernonia colorata* showed a slight (8%) increase in cyclooxygenase inhibition activity. The response of the plant material to accelerated ageing with respect to antibacterial activity varied with plant species. *Alepidea amatymbica* root material and *Vernonia colorata* leaf material appear to be stable whereas the other plant materials lost activity after prolonged (25 days) ageing.

CONFERENCE CONTRIBUTIONS
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26th Annual Congress of SAAB (South African Association of Botanists), Rand Afrikaans University, Johannesburg (South Africa):-

Poster: Shelf-life of South African medicinal plants.

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(G. I. Stafford, J. van Staden and A. K. Jäger)

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Poster: Effects of storage and accelerated ageing on the chemical composition and biological activities of frequently used South African medicinal plants.

(G. I. Stafford, J. van Staden and A. K. Jäger)

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LIST OF ABBREVIATIONS

AA	-	Arachidonic acid
dH2O	-	Distilled water
DMSO	-	Dimethylsulfoxide
EHEC	-	Enterohaemorrhagic Strains of *E. coli*
EtOH	-	Ethanol
GAP	-	Good Agricultural Practice
GMO	-	Genetically Modified Organism
GMP	-	Good Manufacturing Practice
Gram +ve	-	Gram positive
Gram -ve	-	Gram negative
Hex	-	*n*-Hexane
INT	-	*p*-Iodonitrotetrazolium violet
KZN	-	KwaZulu-Natal
MH	-	Mueller-Hinton
MIC	-	Minimum Inhibitor Concentration
NG	-	Nematode growth
OD_{600}	-	Optical density read at 600 nm
PG	-	Prostaglandin
R_f	-	Relative front
SOP	-	Standard Operating Procedures
TLC	-	Thin Layer Chromatography
UV	-	Ultra Violet
VIS	-	Visual
WHO	-	World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1. Role of plants and ethnobotanical research in health care and drug development

Living organisms are remarkable in their ability to produce a vast number of diverse metabolites ranging in chemical complexity and biological activity. Plants in particular because they are fixed in place and cannot flee injury, have evolved chemical defences that protect themselves. It is these 'defence and repair' chemicals that humankind has exploited for its own benefit. Humankind has relied on plants for medicinal purposes throughout its development, fossil records date human use of plants as medicines at least to the Middle Palaeolithic age some 60 000 years ago (SOLECKI and SHANIDAR, 1975). Botany and medicine were, for all practical purposes, synonymous fields of knowledge. Therefore, the traditional healer, usually an accomplished traditional botanist, represents, probably, the oldest professional man in the evolution of human culture (SCHULTES, 1967). The earliest known ethnobotanical work, *The Condition of the Flora of the southern Region,* was written at the end of the third century by Hi-Han and describes the Chinese introduction and use of numerous plants from Southeast Asia.

It is estimated that slightly less than 80% of the worlds population (6000 million people) live in developing countries and the World Health Organisation estimates that about 80% of these people rely almost exclusively on traditional medicine for their primary health care (PHC) needs. This equates to 65% of the world's population according to FARNSWORTH, AKERELE, BINGEL, SOEJARTO and GUO (1985).

Up until recently traditional medicine was not recognised by the South Africa authorities. Now however, the South African government recognises the importance of traditional medicine as a key provider of primary health care and is now promoting the integration of traditional healing in to the official health care system under the

Reconstruction and Development Plan (RDP) (HOLDSTOCK, 1979; DE VOS, 1988; GUMEDE, 189; PICK, 1992).

The number of higher plant species (Angiosperms and Gymnosperms) on this planet is estimated at 250 000, of these only about six percent have been screened for biological activity (VERPOORTE, 2000). The plant Kingdom has in the past been considered a valuable source of raw material for drug development, but pharmaceutical companies have generally been slow in taking up the challenge when you consider how long they have been in the game, especially those within the Third World. South Africa, as one of the more technologically advanced Third World countries with an extremely rich biodiversity is well placed to be leaders in plant drug development. As a starting point for plant-screening programmes, traditional knowledge has always been rewarding (FABRICANT and FARNSWORTH, 2001). There are three main collection methods used in sampling plant material for biologically active compounds (BALICK, 1990). Random sampling, taxonomically directed sampling and 'pre-screening' based on indigenous knowledge of plant use, or 'ethno-directed' sampling. It is the latter that appears to be the most rewarding. Ethnobotany is the study of the interactive relationships between non-industrial societies and their floral environment (COATS, 1970). Although ethnobotany is a relatively contemporary term, ethnobotanical research has a long and rich history.

According to FOURIE, SWART, and SNYCKERS (1992), research to date strongly suggests that the majority of the plants used in folk medicine in southern Africa do indeed possess some pharmacological activity'. The active ingredients in plants have potential value not only as medicines but also as new insecticides, molluscicides or flavourings. Of the plants FOURIE, SWART, and SNYCKERS (1992) tested, 81% showed biological activity. They believe that their findings, particularly in relation to plants with analgesic, anti-inflammatory, anti-microbial or anti-ulcer properties, 'give credibility to the practice of traditional medicine. The plant-derived pharmaceuticals that constitute about 30% of prescription drugs used in western medical systems are obtained from only about 120 plant species – a mere handful of the 250 000 or more known to Science.

The use of plant-based medications has become extremely popular in the First World, the use of herbal and dietry supplements in the United States increased by 380% between 1990 and 1997 (JACKSON, 2001) and it was estimated to be earning $1.5 billion per annum at the end of the nineteen eighties (ERNST, 1998). The European market is believed to be nearly tree time as large as the US market (ERNST, 1998).

1.2. Traditional medicine in South Africa

Southern Africa is exceptionally rich in plant diversity with some 30 000 species of flowering plants, that is almost one tenth of the world's higher plants. There are ten endemic families, while 80% of the species and 29% of the genera are endemic (GOLDBLATT, 1978). The utilization of plants in traditional medicine is an integral part of South African cultural life, a position that is unlikely to change to any considerable degree in years to come (CUNNINGHAM, 1988; BRANDT, OSUCH, MATHIBE and TSIPA, 1995; LEGGIADRO, 1995; MANDER, 1997). It is estimated that 27 million South Africans depend on traditional herbal medicines from as many as 700 indigenous plant species (DAUSKARDT, 1990; MEYER, AFOLAYAN, TAYLOR and ENGELBRECHT, 1996; WILLIAMS, 1996; MANDER, 1997). South Africa is faced with a lack of medical personnel in general but especially in rural areas. In 1982 it was estimated that there was in the region of one western medical practitioner for every 17 500 people (SAVAGE, 1985). This relative lack of medical personnel is likely to remain in the foreseeable future, and one question that often arises is how the health services for the entire population can be improved. Whatever is done in terms of the allocation of personnel and money is likely to fall far short of what is necessary, and drastic measures are called for. There are a number of these, but one possibility is the utilisation of the services of traditional healers who are indeed numerous and situated where they are required.

In 1998 the National Council of Provinces (NCOP) Select Committee on Social Services estimated that there are about 350 000 traditional practitioners in South Africa, providing their services to 60-80% of the population, yet there are only about 250 000 allopathic (modern) medical personnel in South Africa. This raises another

much asked question, can modern medicine and traditional indigenous medicine find common ground in the form of much needed primary health care?

One of the main hindrances for the improvement of traditional medicine, especially the medicinal plant aspect, in South Africa has been Clause 36 of the Medical, Dental and Supplementary Health Services Profession Act of 1974. This clause forbade any registered practitioner to practise in collaboration with a non-registered person, and for non-registered persons to perform acts pertaining to the medical or dental professions. The outcome of this legislation was the division of the traditional healers as a group in hundreds of secretive sub-groups. The trust between healers and allopathic practitioners and westerners in general was broken, making collaboration near impossible. Compounded by the over-sensationalism created by the media surrounding 'muthi' murders which has created the perception of traditional healers as being evil, we are left with lack of trust and unity amongst traditional healers which has proved to be the major stumbling block in the formation of a unified traditional healers' body. Until this happens traditional healers will not gain recognition from the Department of Health. Some progress has been made, in 1989, nine years after attempts were made by traditional healers to gain recognition; KwaZulu-Natal legislature changed their laws to allow 'black medicine-men', herbalists and midwives to practise, subject to certain provisions. This change in legislation lead to the establishment of a register of traditional healers aided by the longest standing non-governmental organisation in Zululand, the Inyangas' National Association (INA).

Despite these advancements no form of quality control exists amongst those who are registered and it had no recognisable code of ethics. In 1995 the KwaZulu-Natal Health Act proposed further investigations and found that the public called for more legal control. Again all efforts put in place to resolve the situation were hindered by the lack of unity between various healer groups. The University of Zululand under the guidance of Mrs. A. Hutchings undertook the task of explaining to traditional healers in KwaZulu-Natal the benefits of a unified traditional healers' council. Their success resulted in the formation of the KZN Traditional Healers' Council.

1.2.1. Witches, Doctors and 'Witch-Doctors'

The misunderstanding and ill stigma that is associated with traditional medicine in South Africa is largely due to the grouping of all traditional practitioners under the now taboo name of 'witch-doctors'. Traditionally the press has portrayed 'witch-doctors' as a group of evil, money hungry, con-artists who are responsible for the numerous 'muthi-killings' (the use of human remains). This is unfortunate as these exploits are most likely the work of *Umthakathi* whose intention is not to heal. The more socially acceptable term of 'Sintu Healer' has also been used as an Umbrella name. This refers to a person who treats patients with the intention to heal, based on his or her tradition and customs. Terms such as traditional healer, traditional medical practitioners and traditional herbalist are anglicised translations that are not entirely correct. The situation is a lot more complicated in Sintu culture with many specialized practitioners each with their own name. The terms 'inyanga' and 'izangoma' are often used to refer exclusively to the herbalist and diviner respectively, however today the distinction has become blurred in some cases, more often the diviners (izangoma) practises both arts (VAN WYK, VAN OUDSTHOORN and GERICKE, 1997)

Umthakathi

These persons, male or female, could be referred to as sorcerers. Umthakathi tend to go against social norms and the community does often not know their identity. If they are discovered they will be hunted and killed by the community, similar to that of 'witch-hunts' in medieval Europe. Umthakathi are believed to know which *umuthi* ingredients to mix in order to make *umuthi wakuthakatha* (witchcraft medicine). This is used for negative purposes such as to kill, cause disorder and stop childbirth. Umthakathi are reported to use specific human body parts to use in *umuthi wakuthakatha*, these are generally taken from young children. It should be stressed that these Umthakathi are not traditional healers and 'true traditional healers' (Izangoma and Inyanga) should not be painted with the same brush.

Inyanga

In western terms the Inyanga would be the medicine man, of which in South Africa the majority are male. The Izinyanga's craft is passed from father to son as opposed to that of Isangoma who are chosen by ancestral spirits (*amadlozi*). One may also

merely chose to be an Inyanga as a profession then be trained by a qualified Inyanga or several different Izinyanga's. An Inyanga trains for several years as an apprentice and focuses mainly on diagnosis of illnesses and the identification and use of plants in healing physical illness. An Izinyanga is often a very skilled botanist with a vast knowledge of the local flora. At the end of their training Izinyanga's can obtain a certificate from the Inyangas' National Association (INA) The Izinyanga specializes in different illness and it is not uncommon for this speciality for curing one particular illness or a group of illnesses to have run in the family for many generations.

Their mode of treatment generally starts with an interview where the patient is asked where they think they acquired the illness or symptoms in question. By combining this information with the observable symptoms, the Inyanga will prescribe *umuthi*. The *umuthi* usually consists of plant material, but may also contain animal parts, soils, and water from rivers, dams and seas. Coloured powders, which consist of soils, burnt animal hair and plant ash is sometimes added to the water (*Isiwasho*). Izinyanga also supply protective and good-luck charms that are used mostly for good intentions. The Inyanga is highly respected in the community for the skills that they posses.

Isangoma

Previously referred to as 'witch-doctors' but this term is no longer used due to its derogatory nature, Izangoma (plural) are traditionally considered to be diviners who consult with ancestral spirits (*amadlozi*). It however, appears as thought the role of the Isangoma has changed in recent years as many have similar 'herbalist' skills to those of the Inyanga. The majority of the Izangoma in South Africa are female although the gender inequality is not as definite as it is in the Izinyanga. Like the Izinyanga, Izangoma show specialization in various areas, for example an Isangoma that specializes in 'smelling-out' bad elements is referred to as an *Isanusi*. According to the way in which Izangoma divine they have been divided into three types, this is one form of classification and it is my understanding that there are many. The first is that of *Izangoma Sekhanda* who practices a form of divination that involves communication with the *amadlozi* or *ithango* (spirit) through a 'telepathic trance'. These trances are often induced through the inhalation of smoke from burning plant material, in particular *impepho* (*Helichrysum spp.*). The second type is the *Izangoma*

Samathambo. These Izangoma throw bones (*ukubhula*) and various other objects (e.g. shells and seed pods) and according to the position and arrangement of the objects various predictions can be made with the guidance of the *amadlozi*. The third, *Izangoma Sabalozi*, possibly the most mysterious of all, apparently asks questions and the amadlozi will answer in audible whistling which both patient and Isangoma can hear, these are then interpreted by the Isangoma who then informs the patient.

SWIFT and ASUNI (1975) suggest that healers, because of their knowledge of the ways of their people and the power conferred upon them by their people, can often provide peace of mind to the distressed and thus provide a feeling of protection to the threatened. LAING (1965) and others initiated a school of thought, much appalled by medical technocracy, that indigenous medicine is in many respects better, more holistic, than its modern western counterpart, that it is conducive with indigenous world-views and therefore more effective for indigenous patients than the biomedical approach (HAMMOND-TOOKE, 1989). They argue that traditional healers do not only know their patients as people, but also understand the social matrix in which they move and live and therefore are bound to be more effective.

1.2.2. Supply and demand for indigenous medicinal plants

In the past and to a large extent today the indigenous medicine market in South Africa is based on indigenous southern African plants, which are generally harvested from wild stocks in KwaZulu-Natal, neighbouring provinces and bordering countries. Unfortunately, the current demand for the most popular plant species used for indigenous medicine exceeds supply. This has resulted in the resent localised extinctions of several plant species outside protected areas. It was estimated that 19 500 tonnes of plant material are traded per annum in South Africa, with an estimated value of more than US$ 60 million (R420 million) (MANDER, 1997). It has long been realised that the decline in the availability of indigenous medicinal plants is potentially devastating to the health and economic welfare of the estimated 27 million indigenous medicine consumers in South Africa and the large support industry.

The increasing scarcity of many indigenous plants in the wild has already resulted in several concerns for people involved in the indigenous medicinal plant trade.

Indications of unsustainable use of wild plant populations is manifested in the notable decrease in size of bulbs, rhizomes and whole plants as well as the dosage prescribed, increase in the distances travelled by gatherers to stock populations (for example it was estimated that between 1988 and 1997 there has been a 45% increase in travel time between popular plants sources and the market (MANDER, 1997)), supply becoming increasingly irregular and/or some plants becoming unavailable in certain markets (MANDER, 1997). These current difficulties are compounded by the necessity for medicinal plant traders to increase the price of the products to compensate for the above-mentioned inconveniences.

The demand for medicinal plants is likely to remain vigorous in the future. Studies locally have indicated that current consumers do not anticipate any changes in their demand for indigenous medicine (MANDER, 1997). In first world countries there is a widespread trend of 'going back to nature' also known as the 'green wave' as the negative (or purported negative) effects of highly refined foods, pesticides, herbicides and synthetic drugs on humans and the environment is realised. Supporting this trend is the need for the development of new anti-microbial compounds for replacement of resistant organisms that have resulted from indiscriminate use of antibiotics. LEGGIADRO (1995) suggests that we may be entering a 'post-antibiotic' era where antibiotics are no longer effective. In addition to searching for anti-cancer and anti-AIDS/HIV drugs, investigating plants for effective anti-microbial components has high priority in First World countries. Thus the current trends being seen in the supply and demand of southern African medicinal plants would result in additional losses as potential income generating opportunities associated with a growing local and international demand are not realised.

As a result of the declining supply of medicinal plants and the localised extinctions that occurred, CUNNINGHAM (1988) and many others since then have recommended the cultivation of indigenous medicinal plants. Initially there was very little response to these recommendations mostly due to the lack of information on market dynamics and the cultivation of medicinal plants. Much of this has changed as a result of several research projects conducted by a wide variety of organisations and government bodies. Several areas of medicinal plant product development have been investigated such as the seasonal and clonal variation in the biological activity

of several commonly used medicinal plants (ZSCHOCKE, DREWES, PAULUS, BAUER and VAN STADEN, 2000).

The cultivation, management and enrichment planting of high value plants are an important strategy to meet consumer demand and to reduce the impact of the medicinal plant market on biodiversity. The success of cultivation trials undertaken to date have shown good potential for this strategy. Plant part substitution has also been investigated, it was assumed that the plant part used by the traditional healers has the highest activity but this has been shown not to be true in several cases (ZSCHOCKE, DREWES, PAULUS, BAUER and VAN STADEN, 2000; ZSCHOCKE, RABE, TAYLOR, JÄGER, and VAN STADEN, 2000). This knowledge to a large extent will determine what plant parts will be harvested and marketed. Obviously some plant parts such as leaves, fruit, flowers and to a certain extent stems are more beneficial to use if they show similar biological activity than others such as bark, roots and bulbs as the harvest of these organs are 'destructive' and result in the loss of the whole plant, the former plant parts can be regenerated by the plant without the need to propagate new plants.

1.2.3. Marketing of indigenous medicinal plant products

More than 1020 plant and 150 animal species are used for traditional medicine in KwaZulu-Natal, of which approximately 450 plant species are sold in large volumes in the markets. Nine plant species make up approximately one fifth of the market. The amount of plant material traded in KwaZulu-Natal is estimated at 4 500 tonnes per year (CUNNINGHAM, 1988; MEYER, AFOLAYAN, TAYLOR and ENGELBRECHT, 1996; MANDER, 1997). More than 500 species are reported to be traded in Witwatersrand markets (WILLIAMS, 1996). These independent studies and that of DAUSKARDT (1990) indicated that the situations in these two major regions are similar. While the mixing and prescription of plant products is generally sophisticated, the processing and (value-added) development of products is myopic compared to that of Chinese or Indian traditional medicine. **Table 1.1** illustrates the types and relative proportions of plant products sold in the street markets of KwaZulu-Natal.

Table 1.1: Medicinal plant products and parts that are sold in street markets in KwaZulu-Natal (MANDER, 1997).

Plant parts used	Relative proportion of the market	Form in which the product is sold	Preferred characteristics and desired qualities of the product
Bark	27%	Chunks (30cm-10cm long 10cm-3cm wide), chopped, or ground to a powder.	Thick bark originating from the main trunk of old trees. The age of the harvested product is not critical.
Roots	27%	Whole sections (40cm-10cm long depending on the species), or ground to a powder.	Roots which are mature but which can be easily chopped. Freshness is more important in roots.
Bulbs	14%	Whole (15cm-2.5cm in diameter depending on the species), sliced or chopped (2cm-5mm).	Larger bulbs are more desirable. The outer covering of the bulb should not be damaged.
Whole plants	13%	Plants should be alive and in relatively good condition for replanting or drying.	The fresher, healthier plants are most likely to sell. Mature plants are more highly sort after.
Leaves and stems	10%	Whole sections (40cm-10cm long), chopped (10mm-5mm long), or ground to a powder. In some instances whole sections are bound into bundles and dried.	The fresher the leaves and stems the more desirable. Younger stem before they turn woody are preferable.
Tubers	6%	Whole (40cm-10cm in diameter depending on species), chunks of the tuber, slices or chopped (2cm-5mm)	Tubers are preferred large, but not unmanageable. Fresh tubers are preferred.
Mixtures of various plant species	4%	A range of plant parts are usually chopped into 1cm to 3cm pieces and sold fresh (moist plant parts are not dried such as *intelezi*) or dried or bottled with various liquids.	Mixtures should be freshly prepared using plants which have the ideal characteristics indicated above.

In the informal street markets (**Plate 1.1**) and 'muthi shops' (**Plate 1.2, 1.3** and **1.4**) the plant material is sold in the raw form either fresh or dried, the most complex form is that of a mixture of ground plant parts. The plant products are marketed to consumers as self-medication or as a result of a traditional healers 'prescription'. The products are marketed in most instances from informal stands within residential areas dominated by black consumers or at transport nodes throughout urban and

rural areas (DAUSKARDT, 1990; WILLIAMS, 1996; MANDER, 1997). The conditions in the markets are generally poor, with most plant material displayed in the open. Under these conditions the material is exposed to microbial and insect attack as well as the effects of light, gases and temperature. Often these informal markets are situated close to both pedestrian and motor vehicle traffic, which places plant material in contact with all kinds of pollution. MANDER (1997) found that 84% of the black clinic patients he interviewed (n=100) indicated that they would prefer more hygienically packaged indigenous medicines, with most consumers indicating that they would prefer more modernised and hygienic trading sites.

The lack of storage facilities and trading infrastructure frequently results in the spoiling of plant materials, resulting in wastage and/or a decrease in product quality. It must be stressed that healers and consumers question the current marketing of indigenous medicine, as they are concerned about the quality of the products purchased in the markets.

At present in South Africa there is very little indigenous medicinal plant product development taking place, which supports the current market players (the traditional healers, gatherers and traders) and promotes the welfare of current users. This will inevitably lead to a situation where the knowledge of the current market players is being used to promote the interests of new and sophisticated entrants to the market, with little returns to the current market players (MANDER, 1998). There is an imbalance in support for indigenous medicine, with most investment being directed at seeking commercially useful chemicals within medicinal plants, while little or no investment is being directed to maintain or increase the benefits, which the current market is already delivering to millions of consumers.

Plate 1.1: **Photographs illustrating the informal nature of traditional medicinal plant street markets, Retief Street market, Pietermaritzburg, KwaZulu-Natal, South Africa.**

A. Retief Street in the eastern low-market section of Pietermaritzburg city centre

B. A family business: Mr Nkhize, his wife and son have traded in this street market for over five years

C. Plant material at street market; note how it is stored in large woven plastic sacks

D. Plant material at a street market; note the general disorganization and mixing of plant material. There are no labels on plant material at street markets

E. Entire street market on Retief Street. Most street markets in Pietermaritzburg are relatively small like this one as compared to the larger markets in Durban, such as the one a Warrick Junction
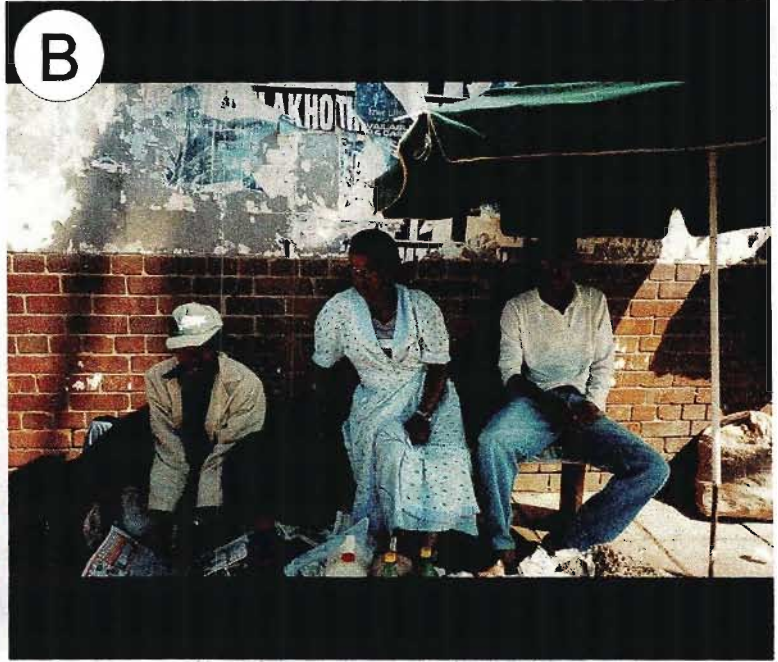
Plate 1.2: Photographs of a popular 'muthi' (traditional medicinal plant) shop in Retief Street, Pietermaritzburg, KwaZulu-Natal, South Africa.

A. Interior of a 'muthi' shop; note the close contact of customer and the plant material

B. Dried plant material that is hung from shelves or ceiling

C. Various colourful mixtures of finely chopped material mostly used in traditional medicine for cleansing the body and premises of 'evil spirits'

D. In the background are various bottled and package herbal preparations
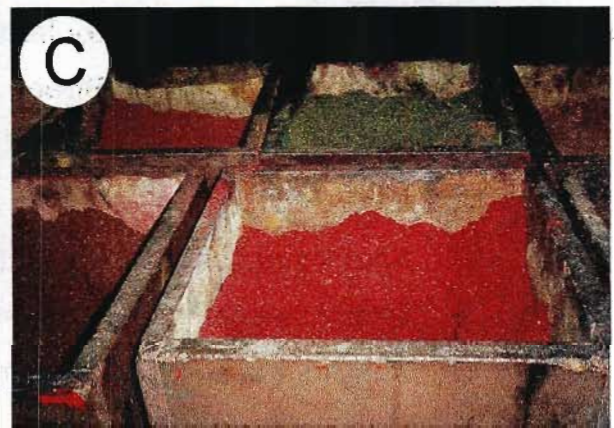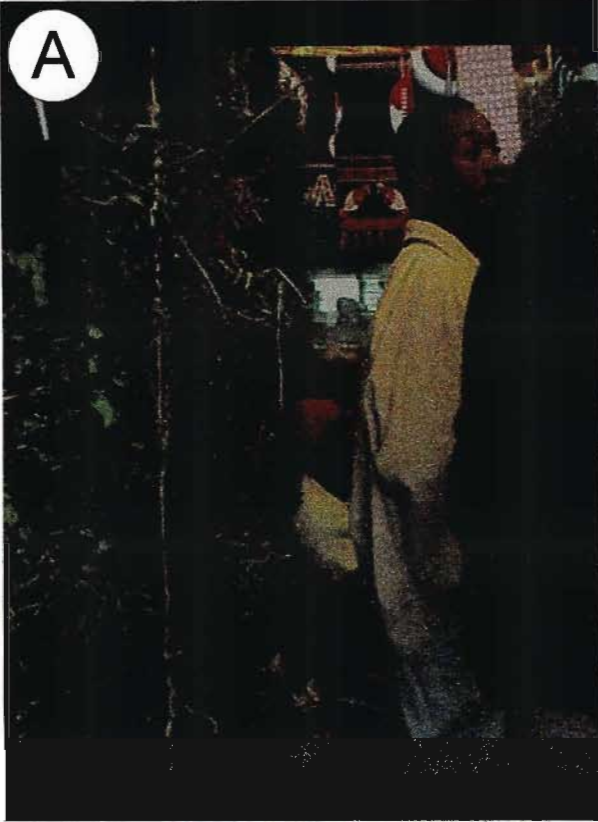
Plate 1.3: **Photographs of Kwa-Dabulamanzi 'muthi' (traditional medicinal plant) shop in Retief Street, Pietermaritzburg, KwaZulu-Natal, South Africa.**

A. Exterior of shop

B. Shop manager chopping material for customer

C. Shop manager dispensing powdered traditional medicine

D. A mixture of chopped plant material known as *intelezi*

E. Dried leaf material is hung from shelving

F. Bark material

G. Bottled and package herbal preparations used in traditional medicine

H. Shelves with various types of plant material

Plate 1.4: Photographs of Kwa-Nhlanhla 'muthi' (traditional medicinal plant) shop in Pietermaritz Street, Pietermaritzburg, KwaZulu-Natal, South Africa.

A. Exterior of 'muthi' shop

B. Interior of shop showing shelving that contains a variety of plant material; note the Hindu icons in the corner, this is not unusual as all the 'muthi' shop in Pietermaritzburg are owned by members of the local Indian community

C. Soil beds and plastic bowls filled with soil are used to keep plant material alive and fresh

D. Soil beds with live plant material

E. A close-up of a soil bed that contain several species of living bulbs

F. 'Pigeon-holes' which display many type of bark material; note that in this particular shop the material is labelled

At the extreme end of the spectrum VAN WYK (1998) has pointed out that some 90% of the natural medicines on offer in South African pharmacies are non-indigenous European formulations. There is no reason why the reverse cannot be true and that we cannot have 90% indigenous medicines. For every European medicinal plant there is a local equivalent which is sometimes even better than the imported item (VAN WYK, 1998).

1.3. Storage of indigenous medicinal plant material in South Africa

Medicinal plant gatherers usually build up stock at the rural homestead before an order can be supplied or before there is sufficient volume of material to justify a journey to the market (MANDER, 1997). The amount stored by gatherers is unknown as there are over 16 000 gatherers in KwaZulu-Natal alone. However, the volume is likely to reflect the total amount of material which the street traders retail (MANDER, 1997). For example, MANDER (1997) observed in Russell street (Durban) market that each trader had approximately 10 kg on display, with another 30 kg in storage. The total mass of plants stored in Russell street market was estimated to be 13.5 tonnes. The amount of material stored in the KwaZulu-Natal more formal traditional herbalist shops as opposed to the informal markets is variable but is believed to be higher than two tonnes in total.

Raw materials for trading, as opposed to those for retail, such as whole plants, plant parts and chopped material, are stored in recycled plastic woven sacks (50 kg capacity) (MANDER, 1997). This form of storage is most common and is used in all forms of the marketing channels. These sacks are generally stored in a dry place, preferably in the shade, to prevent the decomposition of the plant material. Shops and healers consulting rooms usually have an area dedicated to storing large numbers of these bags. Street traders store their bagged goods under plastic sheets in the street. There has been mention of some form of refrigerated storage being installed in the larger markets in Durban but this has not materialised. In some towns, street traders have managed to obtain a shipping container, which is used to store several traders' sacks overnight (MANDER, 1997).

Retail materials for sale directly to the consumers are stored in a variety of containers depending on the type of outlet. Street market traders display their smaller or partially processed (chopped) material either on plastic sheets or newspaper on the pavement or in plastic shopping bags. In healers' consulting rooms, products are usually stored in plastic containers with lids, on wooden shelves or in plastic shopping bags. In shops products are usually stored on shelves or in 'pigeon holes' where the wide array of plant material can be kept relatively neat and separate (MANDER, 1997). In one shop in particular in Pietermaritzburg materials were kept 'fresh' by replanting them in soil beds inside the shop itself **(Plate 1.4)**. This shop was popular with local traditional healer for this very reason (MTOLO, MKHIZE, MSANGO and SMITH *pers com.*). **Figure 1.5** illustrates the flow of plant material in the traditional South African medicinal plant trade.

Figure 1.5: **Schematic diagram illustrating the movement of plant material in the South African traditional medicinal plant trade.**

Plant material is collected/harvested by gatherers from various wild populations. This may involve travelling long distances and trips can last up to several months. The material is stored by the gatherer in large bags until just before the end of the month when the material taken into the populated area to street markets and 'muthi' shops. The material is stored there until it is bought by the consumer which may be right away, in the case of popular products, or up two five years. Once the material is purchased it is usually wrapped in recycled newspaper.

One to six months

Immediate to up to five years

'Muthi' shop

Gatherer

Street market

Packaged

Consumer-
Direct to patient
or through
healer

1.4. Current understanding of post-harvest physiology of medicinal plants

1.4.1. Shelf-life and its importance

The majority of the literature on medicinal and aromatic plants are concerned with questions pertaining to the botany, biological activity, chemistry or biotechnology of this valuable group of plants. Less is mentioned of the post-harvest physiology, not to speak of the storage and shelf-life. Shelf-life is a predetermined period of time for which a consumable can remain available to the consumer. It is a quality control process and ensures that the consumer receives a product that meets a specified standard. The shelf-life is determined by first defining the quality standard of the product you wish to market then to determine the rate of 'decay' of the product with time. Using these two values, how long the product can remain 'on the shelf' under specified conditions before it reaches a below standard state can be determined. At this point the product is said to have expired. Shelf life defines the point where a product is considered unable to meet the requirements it is used for and may be hazardous.

It is important to have such clearly defined concepts of what shelf-life is and what purpose it should serve. Although the shelf-life of a particular medicinal plant is extremely difficult to determine, as explained later, it serves the ultimate achievement in understanding the post-harvest physiology of a medicinal plant in order for it to meet the therapeutic needs of the consumer.

1.4.2. Product degradation and preservation methods

Due to the lack of literature with regard to the post-harvest physiology of medicinal plant material a large amount of insight into the topic is adapted from the food industry. Agriculture is called upon to ensure sufficient quantities of good-quality foodstuffs such as fruit, vegetables and other products, and to plan possible short-term storage of these products. Technologies and techniques of food preservation are required for the proper treatment of products obtained at the agricultural stage, to prevent 'spoilage' before the consumer uses the products. These technologies can in most instances be applied to that of medicinal plant production because the basic principles are essentially the same. In both instances the product is derived from

plant material and thus the same factors are involved in the 'spoilage' of the plant material. Possibly the major difference between the food industry and the medicinal plant industry is what is regarded as 'spoilage'. With regards to food products the commercial or sensory values such as appearances, odour and taste are most influential in determining its market value. Logically the most important consideration when it comes to the medicinal plant industry is the chemical constituents of the plant material as this determines the biological activity for which it is used as well as cytotoxicity of the material.

The conditions conducive to decomposition of plant material into simpler components, the most common form of 'spoilage', are governed by the composition of the plant material and by various environmental factors. At this point it should be mentioned that decomposition of medicinally active constituents is not necessarily a negative process as it has been shown in some instances that the breakdown product is more active than its precursor. Studies on the storage of *Malva passiflora* have shown that storage improved the antibacterial activity of the plant material (SHALE *per com.*).

It is understood that there are three main processes through which the quality of plant material can be degraded:

- Chemical breakdown and decomposition;
- Microbial attack; and
- Insect attack.

In this particular investigation microbial and insect attack were excluded as far as possible by drying the plant material (see Section 1.4.3) and therefore only the chemical decomposition or rather chemical changes were investigated. Chemical changes within the plant material are the changes that are going to affect the biological activity of the material the most. Like all chemical reactions the rate is determined by factors such as temperature, light, pH and enzymes (this wil be discussed in detail in CHAPTER 4). Endogenous deteriorating enzymes active in the course of drug production largely modulate these chemical changes, these are:

- Oxidases which cause the oxidation of phenolics and unsaturated fatty acids;

- Peroxidases which cause the oxidation of terpenes and terpenens;
- Hydrolases which cause the splitting up of the esteric and glycosidic bonds and breaking up of polysaccharides; and
- Isomerases that cause the isomerisation of ergot alkaloids and other optically active substances.

Most pre-storage processing of plant material, such as drying, heat, cooling and packaging, are means of preventing the degradation of the plant material by the above-mentioned processes during storage. **Table 1.2** shows the most common post-harvest preservation methods.

Non-microbial biochemical changes in plant material may be latent to the senses of a consumer, or they may be discernable by human senses. The consumer cannot perceive latent changes visually, olfactory or otherwise and there is little evidence to suggest it is any different for the traditional healers. Thus laboratory measurements are the only methods to detect them, yet it appears that very little scientific investigation or even consideration has been afforded to this idea.

MANDER (1997) found that the collectors or gatherers generally collect plant material at the beginning of the month, dry the material immediately and then store it in bags until the end of the month when it is sold to the market stall owners. Apparently the stall owners at the medicinal plant market like to buy stock just before the end of the month to ensure that they will have sufficient stock-material when the general public is most likely to purchase these plants, which is after they get paid at the end of the month. Thus one can assume that the majority of the more common plant species used for traditional medicinal purposes are stored for about a month before they are potentially being sold and/or being used. The more formal traditional herbalist shops on the other hand generally restock their dried plant material every three months (90 days) or when required or available.

Table 1.2: Most common methods of post-harvest preservation methods (After: COREY, 1989)

Method	Post-harvest application
Fresh • Refrigeration • Packaging[1] • • Modified Atmosphere •	Decrease metabolism; inhibit microbial growth Decrease water loss; possible modification of O_2-, CO_2- and C_2H_2- concentrations Decrease respiration; decrease C_2H_2- synthesis and action; inhibit microbial growth
Processed • Drying[2] • Freezing • Thermal • • Fermentation[3]	Stop metabolism; prevent microbial growth Decrease metabolism; prevent microbial growth Stop metabolism; stop microbial growth and contamination Stop metabolism; selective control of microbial growth
Fresh or Processed • Chemical	Inhibit or stop microbial growth

[1]Packaging; wrapping plant material in either newspaper, magazine paper or polyethylene bags occurs frequently in tradition medicinal practice in South Africa.

[2]Other that chopping and mixing plant material, drying is the only other post-harvest preparation that plant material is subjected to before storage. It is usually dried by being placed on drying racks in the sun.

[3]Fermentation does occur but is rare for example the use of *Sceletum sp.* and *Catha edulis* .

1.4.3. Preservation by drying

Preservation of foods and medicines by drying has been practised for centuries. Preservation is any purposeful treatment or processing of a plant material, which extends the keeping ability or shelf-life of a raw material for a period longer than its natural keeping ability. Most harvested plant material, other than some grains and bark which are sufficiently dry at harvest, contain enough moisture to permit action by their own enzymes and/or by microorganisms, so that in order to preserve them by dryness the removal or binding (e.g., by solutes) of moisture is necessary (FRAZIER, 1967). Moisture may be removed from plant material in a number of ways. From the ancient practice of sun drying to the modern artificial ones. FRAZIER (1967) states that most bacteria do not grow below 18 percent available moisture, yeast requires 20 percent or more and molds require 13 to 16 percent.

Sun drying is limited to climates with a hot sun and dry atmosphere and is the most common form of drying plant materials in Africa, although drying during smoking may also occur. The material is laid out on newspaper or plastic sheets on the ground, or in more advanced systems on racks and may be turned during drying. There is no control of temperatures, relative humidities or air velocities. A consideration of the proper control of dehydration includes the following factors (FRAZIER, 1967): (a) the temperature employed, which will vary with the type of material being dried and the method of drying; (b) the relative humidity of the surrounding environment, this too, is varied according to the plant material and the method of drying. It is usually higher at the start of drying than later in modern systems; (c) the velocity of the air passing over the plant material; (d) and lastly the duration of drying. Improper control of these factors may cause 'casehardening' due to more rapid evaporation of moisture from the surface than diffusion from the interior, with a resulting hard, impenetrable surface film that hinders further drying. If the drying process is too slow, that is the temperature is too low and the humidity too high, the material will start to decay. There is a gross lack of information pertaining to the effect of temperature, solar radiation, photo-oxidation and humidity on the biological activity of medicinal plants that calls for urgent investigation (SILVA, LEE and KINGHORN, 1998).

1.4.4. Chemical stability of pharmaceutical drugs

It is well understood (FLORENCE and ATTWOOD, 1988) that most drugs are subject to some form of chemical decomposition, particularly when formulated as liquid dosage forms. These degradations are manifested in loss of the desired pharmacological activity of the drug, discoloration that is usually associated with photo-degradation, or more seriously the production of harmful decomposition products. The latter however, is very fortunately the less common situation. The major distinction between pharmaceutical drugs and herbal medicines is that drugs contain relatively fewer chemical constituents than herbal preparations. Thus pharmaceutical preparations are relatively easy to work with, as their chemical composition is known and the proportions of those constituents is also known should there be more than one.

Studies of the chemical stability of drugs has provided useful information that may be relevant to the stability of herbal preparations but due to the lack of knowledge pertaining to the composition of herbal preparations this information is difficult, almost impossible to apply. There are many factors, other than the lack of knowledge pertaining to herbal preparations' chemical composition, such as the multitude of potential chemical interactions that could take place between these numerous chemical constituents, which could have an affect on the stability of herbal preparations. Other factors affecting stability than cannot be excluded in herbal preparations include enzyme-catalysed decomposition as mentioned earlier and growth of microorganisms that may be associated with the herbal preparation. Plant material is also not uniform in structure like solid dose forms of drugs and thus there exists a matrix of varying rates of chemical decomposition within plant material due to varying physical structure of the plant material. In light of this knowledge from single compound drug stability studies should only be used as a guide when applied to herbal preparations.

The two most common causes of the decomposition of drugs are hydrolysis and oxidation (FLORENCE and ATTWOOD, 1988). Drugs may also lose their activity due to isomerisation or photochemical decomposition.

Hydrolysis

According to FLORENCE and ATTWOOD (1988) the main classes of drugs that are susceptible to hydrolytic cleavage are the esters, amides and lactams. Hydrolysis is commonly catalysed by hydrogen ions (specific acid catalysis) or hydroxyl ions (specific base catalysis) or both which are commonly encountered as components of buffers (general acid-base catalysis). The most frequent type of ester hydrolysis is a bimolecular reaction involving acyl-oxygen cleavage.

Oxidation

Oxidative degradation is a major cause of drug instability but is not as well understood as hydrolysis. In many cases where hydrolytic and oxidative degradation occurs simultaneously, the oxidative component can be eliminated by storage under anaerobic conditions (FLORENCE and ATTWOOD, 1988) and thus investigation into the mechanism of oxidation was not required. Compounds predominantly affected by oxidation include phenolic compounds, catecholamines, steroids, antibiotics, vitamins, oils and lipids.

Oxidation involves the removal of an electropositive atom, radical or electron, or the addition of an electronegative atom or radical. Many pharmaceutical oxidations are chain reactions, which proceed relatively slowly under the influence of molecular oxygen. This process is referred to as autoxidation. In the oxidation of fats and oils (FARMER in FLORENCE and ATTWOOD, 1988) the initiation can be via free radicals formed from organic compounds by the action of light, heat or trace metals. The propagation stage of the reaction involves the combination of molecular oxygen with a free radical to form a peroxy radical $ROO\cdot$, which then removes a hydrogen from a molecule of the organic compound to form a hydroperoxide, ROOH, and in so doing creates a new free radical.

Initiation

$$R \cdot + R'-CH=CH-R'' \rightarrow R'-\dot{C}H-CH=CH-R'' + RH$$

Propagation

$$R'-\dot{C}H-CH=CH-R'' + O_2 \rightarrow R'-\overset{\overset{\displaystyle O-O\cdot}{|}}{C}H-CH=CH-R''$$

$$R'-\overset{\overset{\displaystyle O-O\cdot}{|}}{C}H-CH=CH-R'' + R'-CH_2-CH=CH-R''$$

$$\rightarrow R'-\overset{\overset{\displaystyle O-OH}{|}}{C}H-CH=CH-R'' + R'-\dot{C}H-CH=CH-R''$$
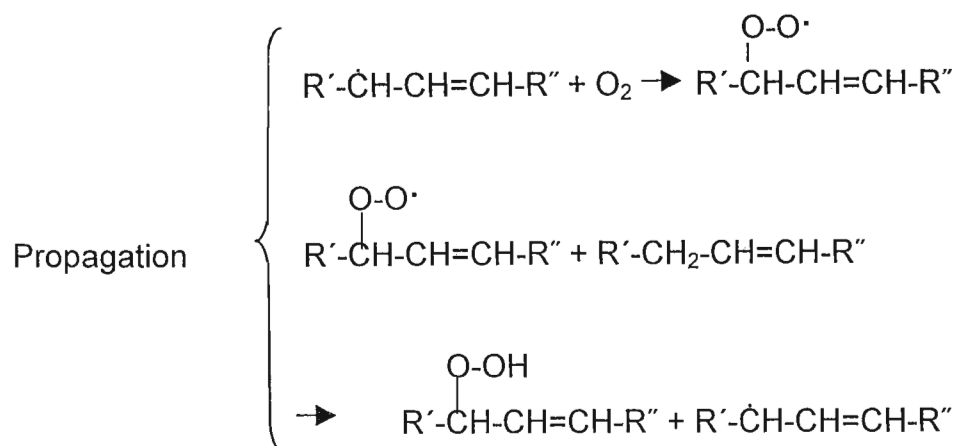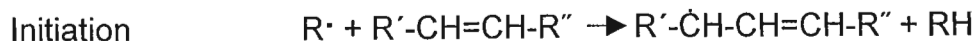
The reaction proceeds until the free radicals are destroyed by inhibitors or by side-chain reactions, which eventually break the chain. The rancid odour, which is a characteristic of oxidised fats and oils, is due to aldehydes, ketones and short-chain fatty acids, which are the breakdown products of the hydroperoxides.

FLORENCE and ATTWOOD (1988) suggest that stabilisation of drugs against oxidation will involve observing a number of precautions during manufacture and storage. The oxygen in pharmaceutical containers should be replaced with nitrogen or carbon dioxide; contact of the drug with heavy metal ions that catalyse oxidation should be avoided and storage at reduced temperature is advisable. It is very difficult to remove all of the oxygen from a storage vessel and often traces of oxygen are sufficient to initiate the oxidation chain. The propagation of the chain reaction may be prevented or hindered by adding low concentrations of compounds which act as inhibitors. These are 'free radical scavengers' commonly referred to as antioxidants; they work by interacting with the free radicals to produce a compound with insufficient reactivity to perpetuate the process. Examples of commercially used antioxidants are tocopherols, butylated hydroxyanisole, butylated hydroxytoluene, gallic acid and gallate derivatives. Reducing agents such as sodium metabisulphate may also be added to formulations to prevent oxidation. These compounds are more readily oxidised than the drug and so protect it from oxidation.

Isomerisation

Isomerisation is the process of conversion of a drug into its optical or geometric isomers. Since the various isomers of biologically active compounds are frequently not equally as potent such a conversion may be regarded as a form of degradation if it results in a drastic loss in therapeutic activity, as is often the case. *Cis-trans* isomerisation may be a cause of loss of potency of a drug if the two geometric isomers have different therapeutic activities. This phenomenon is observed in aqueous preparations of vitamin A, where the *cis* isomers at 2 and 6 positions lead to a decreased activity compared with the all-*trans* molecule (FLORENCE and ATTWOOD, 1988). Cis isomers of fatty acids are more effect as antibacterial agents than trans isomers (MARSHALL and BULLERMAN, 1994).

Photochemical decomposition

Despite the occurrence of photochemical decomposition in many pharmaceuticals few mechanisms have been fully elucidated. This is because these mechanisms are extremely complex. In most cases however the first step of degradation involves the loss of an electron to yield free radicals (FLORENCE and ATTWOOD, 1988).

1.4.5. Effect of storage of plant material and extracts

Despite the importance of understanding the effects of storage on the activity of medicinal plants very little work has been conducted in this area. ELOFF (1999) indicated that the antibacterial components of *Combretum erythrophyllum* are very stable in the dry state, as minimum inhibitor concentration (MIC) values remained constant for different plants over a number of years. In the same paper ELOFF (1999) showed that extracts from herbarium specimens of *Helichrysum pedunculatum*, which contain the fatty acids linoleic and oleic acids as the active antibacterial components, still had antibacterial activity. Thus, it was concluded that fatty acids appear to be stable in dry specimens stored under herbarium conditions. McGAW (2001) found the antibacterial activity of *Scotia brachypetala* leaf extracts to be unaffected by storage for 18 months. Again the compounds found to be responsible for the antibacterial activity was fatty acids. McGAW (2001) also concluded that these fatty acids are stable if the dried, powdered plant material is stored in the dark at constant temperature (temperature not given), and also extracts

stored at −15°C also did not show changes in chemical composition and activity. Samples of herbarium material have been shown to still contain stable compounds such as amino acids, flavonoids, volatile oils and alkaloids (PHILLIPSON, 1982).

Nineteen medicinal plants from Nepal were investigated for changes in anti-microbial activity after six years (GRIGGS, MANANDHAR, TOWERS and TAYLOR, 2001). They found that the changes in antibacterial and antifungal activity of the medicinal plants after the six-year storage period varied according to species. Of the 19 plants tested, three lost all activity, six retained all activity and the remainder retained only partial activity. GRIGGS, MANANDHAR, TOWERS and TAYLOR (2001) also found that the activity of some plants was enhanced by UV exposure. No investigation in the chemical changes that occurred as a result of storage were conducted in this investigation.

1.5. Aims and objects
1.5.1. Issues to be addressed

The current socio-economic and legislative environment in South Africa is ready to progress with the improvement of its extremely valuable yet under-developed and under-managed traditional healthcare system. The unification of traditional healer groups should allow the implementation of a code of ethics and quality control measures that will govern the practice and trade of traditional medicine. These developments are not intended to westernise traditional medicine but develop the indigenous knowledge surrounding traditional medicine and bring it up to date with the rest of the medical industry. One object is to develop a sound and sustainable supply of the plant material that is at the core of the traditional healthcare system. The introduction of medicinal plants as crops for small-scale rural farms has been one of the first initiatives in this regard. The next logical step will be to develop the post harvest aspects of the medicinal plant trade.

Most biologically active compounds, synthesised or natural, are subject to some form of chemical decomposition, particularly when formulated in liquid preparations. This knowledge is known from stability studies done on drugs and pharmaceuticals and the same can be assumed for plant materials used in traditional medicine. Some of

the consequences of degradation are that the aged medicinal preparation no longer has the desired pharmacological potency. It may also exhibit physical manifestations of decomposition such as discoloration that often follows photo-degradation. More seriously, but fortunately more rarely, it may result in harmful decomposition products. Little or no work has been conducted on the post-harvest physiology of South African traditional medicinal plants. This project aims to initiate the filling of this gap in the literature and thus aid in the development of traditional medicine.

The aim of this investigation more specifically is to gain further insight in what changes are occurring in frequently used plant material from its fresh form to when it is dried and stored for 90 days and one year, notably what changes occur in biological activity and chemical composition. Material from material stored for five years (*Leonotis leonurus*) will also be included in this investigation. Fingerprinting the chemical composition of the fresh and stored material and determining changes in biological activity using various bioassays will be used to show changes that are occurring. Bio-autographic antibacterial studies will be used to specifically indicate visually which compounds are being altered during storage. Accelerated aging studies will be employed to provide sufficient 'aged' material for further studies.

1.5.2. Potential application and impact

Surprisingly, very little work has been done on the post-harvest physiology of European medicinal herbs and aromatic plants despite the large effort that has been put into developing guidelines for Good Agricultural Practice (G.A.P.). Most of the work to date has focused on the shelf-life of essential oils and not on raw plant materials, which make up the bulk of the trade in medicinal plants for use in traditional medicine. Thus, we have a situation where this new knowledge developed from South Africa traditional medicine will be important to the medicinal and aromatic plant industry worldwide.

The knowledge gained from this project is intended to greatly benefit users of medicinal plant material. It will hopefully lead to the development of general concepts and understanding of the changes occurring during storage in medicinal plant

material that can be applied to the medicinal and aromatic plant industry. The knowledge gained from this project will benefit the South African medicinal plant market the most as the results will have specific relevance to popular plants used in South African traditional medicine. This project will also be an important step towards standardising traditional medicine and making the integration of western and traditional medicine that much easier and hopefully achievable.

CHAPTER 2

EFFECT OF SHORT-TERM STORAGE ON NINE POPULAR MEDICINAL PLANTS

2.1. Introduction

The rational behind conducting an investigation into the effects of short-terms storage lies in the observations made in the previous Chapter that the gathers tend to store plant material for short periods (one to six months) while they build up stock and await an opportune time to sell their material. The aim of this investigation was to gain some insight in what changes are occurring in plant material from its fresh form to when dried and stored for a relatively short period of 90 days, notably what changes occur in biological activity and chemical composition. Changes in chemical composition were observed visually using thin layer chromatography fingerprinting. Three aspects of biological activity were investigated, notably antibacterial, anti-inflammatory and anthelmintic activities.

The assays used in this investigation were chosen for their relative ease in performance and reliability of giving reproducible results. This is important in comparative analysis. They also assess the plant materials effectiveness at treating common or frequent occurring ailments in South Africa.

2.1.1. Extraction method

In order to produce meaningful comparable results between fresh and stored material all other factors, which may affect the chemical composition of the plant material, must be excluded. Thus, the plant collection, extraction method and analysis protocols should by identical for both the fresh and stored material. Any potentially 'destructive' methods of extraction, such as Soxhlet extraction, which cannot be used if the plant material contains thermolabile compounds (ELOFF, 1998c), were avoided. The use of a range of extracting solvents with differing polarity should be employed to ensure the extraction of both hydrophilic and lipophilic compounds.

It is advised (SHIMIZU, 1998) that methods, which interrupt the compartmentalization of macerated plant material, significantly improves the extraction of water-soluble compounds. This is because water-soluble compounds are mostly stored in protected states, such as binding to membranes, compartmentalization or protection by lipophilic materials. This protection can be overcome by freeze-thawing, freeze-drying, heating, enzyme digestion and sonication. The latter is most applicable in this investigation as it is less destructive and can be used for all solvents.

Traditional healers use both fresh and dried plant material and both have been shown to have activity by many researchers, but dried plant material is preferred as there are fewer problems encountered during extraction (ELOFF, 1998c). The major difficulties with using fresh material is that once it is harvested the endogenous enzymes begin breaking it down and it essentially starts to spoil altering its chemical composition. Often associated with this process is microbial growth, which too has an affect on the chemical composition. This means that fresh material has to be utilised as soon a possible, which is often not possible in research or industry. Secondly, differences in the water content of the fresh plant material may affect the solubility of compounds being extracted (ELOFF, 1998c).

A dilemma arose when deciding on a method of drying (see Section 1.4.3 for the effect of drying on plant material) the plant material, on the one hand it is desirable to dry the material as quickly as possible (FRAZIER, 1967) to prevent any spoiling but this generally requires heat which may effect thermolabile compounds. An option is freeze-drying but this was not possible at the time, so a compromise was made and a relatively low temperature at low humidity was used. SILVA, LEE and KINGHORN (1998) points out that plant material is best dried at room temperature or in an oven away from direct sunlight, because ultraviolet radiation may alter chemical composition. FRAZIER (1967) states that most bacteria do not grow below 18 percent available moisture; yeast requires 20 percent or more and molds require 13 to 16 percent, thus the material must be well ventilated.

2.1.2. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a rapid and valuable means of obtaining a characteristic analytical fingerprint of a plant extract (WAGNER and BLADT, 1996). When used correctly TLC-fingerprinting can be very sensitive to the point were it can show seasonal changes in chemical composition of plant material collected at different times of the year. It may also pick up any chemo-variation between different genetic lines of the same species. Although these factors pay tribute to the sensitivity of the TLC method they should also serve as a warning that if it is only chemical changes due to the effect of storage that one wants to observe then the effects of seasonal and genetic variation need to be eliminated. Thus, it was important to ensure that all plant material used in this investigation was obtained from the same genetic line and at the same time of year.

Another advantage of TLC-fingerprinting is that the results can be photographed providing a permanent record that can be used for authentification and comparison of various plant extracts (shown in **Appendix 1**).

2.1.3. Antibacterial assay

Bacterial infections are exceedingly common and cause substantial morbidity and mortality. Bacterial diarrhoea is a leading cause of infant mortality worldwide, and tuberculosis a very frequent cause of death due to infections (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997). Antibacterial drugs are amongst the most important therapeutic discoveries of the twentieth century and have dramatically changed the course of illness. Despite this there is need for the development of new anti-microbial compounds for replacement of existing ineffective drugs due to resistant organisms that have resulted from indiscriminate use of antibiotics. LEGGIADRO (1995) suggests that we may be entering a 'post-antibiotic' era where antibiotics are no longer effective. In addition to searching for anti-cancer and anti-AIDS/HIV drugs, investigating plants for effective anti-microbial components has high priority in First World Countries.

The most popular method of assessing antibacterial activity is to use agar diffusion assays, this works well with defined inhibitors (HEWITT and VINCENT, 1989 cited by ELOFF, 1998b). ELOFF (1998b) points out that when examining extracts containing unknown components, such as crude plant extracts as in this investigation, there are problems leading to false positive and false negative results. Thus it was decided that a quick, but more importantly, a sensitive assay was required and the scaled down serial dilution assay using a 96-well microplates developed by ELOFF (1998b) was suitable. This technique eliminates false positive and false negative results and could distinguish between bactericidal and bacteristatic effects. The minimum inhibitory concentration (MIC) could also be obtained which is more accurate that the minimum lethal concentration (MLC) and therefore more suitable to a comparative analysis such as this investigation.

2.1.4. Anti-inflammatory assay

Prostaglandins are involved in the complex processes of inflammation and are responsible for the sensation of pain (CAMPBELL, 1990). Most ailments that result in pain and inflammation are therefore a result of elevated levels of prostaglandins. The key enzyme in the synthesis of prostaglandin is cyclooxygenase (**Figure 2.1**). Inhibition of cyclooxygenase and hence the production of prostaglandin should result in the reduction of inflammation and to some extent pain relief (RANG and DALE, 1987). A convenient method of evaluating the effectiveness of anti-inflammatory treatments is the cyclooxygenase assay (COX-1 assay).
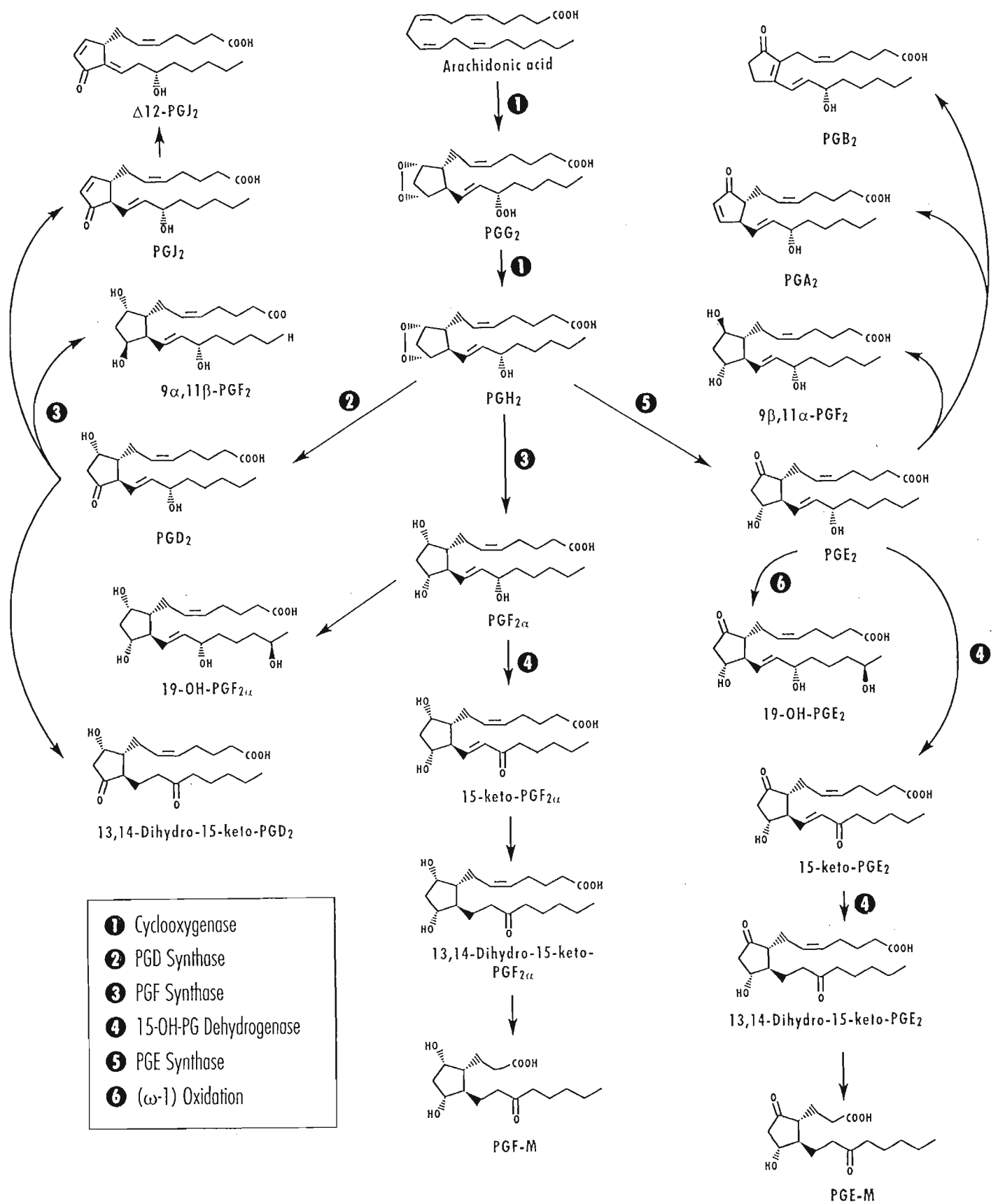
Figure 2.1: Cyclooxygenase pathway showing key enzymes involved in the biosynthesis of prostaglandins (After BIOMOL, 1999).

2.1.5. Anthelmintic assay

Gastrointestinal helminthiases are considered to be the most prevalent form of parasitic infection (BUNDY and COOPER, 1989). Nematodes and round worms are the most important group of parasites found to inhabit the intestines of humans and domestic animals. Many of the 'worms' that inhabit humans live inconspicuously in the intestine, with symptoms of their presence only manifesting themselves in cases of severe infection (LEWIS and ELVIN-LEWIS, 1977). The prevalence of helminthiasis is particularly high in children in developing countries with poor sanitation, a low standard of education and little health education (NOKES and BUNDY, 1992; ENWEREM, WAMBEBE, OKOGUN AKAH and GAMANIEL, 2001). Although the mortality rate associated with parasite infestations is naturally low, morbidity such as impaired physical and mental development is significant (TAYLOR, PILLAI and KVALSVIG, 1995). Helminth infections are a major contributing factor to the impairment of growth, nutrition, physical and learning performance of South African children (FINCHAM, MARKUS, APPLETON, EVANS, ARENDSE, DHANSAY and SCHOEMAN, 1998).

SIMPKIN and COLES (1981) has provided a review of a number of *in vitro* test protocols that use a variety of nematodes. The free-living nematode *Caenorhabditis elegans* (BRENNER, 1974; PLATZER, EBY and FRIEDMAN, 1977; SIMPKIN and COLES, 1981) was found to be a suitable test subject for an anthelmintic bioassay as much is known about it. SIMPKIN and COLES (1981) also advise that screening for anthelmintic compounds should be done using free-living nematodes, as the culture and subsequent screening are simple and do not require sophisticated or expensive equipment.

2.2. Selection of plants to be investigated

Early in the planning of this project it was decided that the plants used in the investigation should be selected according to two criteria. Firstly, they should be popular, that is, in frequent use and circulation in South African traditional medicine. Secondly, the plants should be easily available from a reliable source. No bark products were used in this investigation, as the type of bark used in traditional medicine is generally older bark, which is non-living and thus has been ageing on the tree sometime before harvesting.

Information pertaining to the popularity, use and any known chemical constituents of the plant material selected for this study was largely obtained from research done by WATT and BREYER-BRANDWIJK (1962), GELFAND, MAVI, DRUMMOND and NDEMERA (1985); MANDER, MANDER, CROUCH, McKEAN and NICHOLIS (1995), HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM (1996); VAN WYK, VAN OUDTSHOORN and GERICKE (1997); and MANDER (1997). The plants investigated were *Alepidea amatymbica* Eckl. & Zeyh., *Leonotis leonurus* (L.) R. Br., *Drimia robusta* Bak., *Vernonia colorata* (Willd.) Drake, *Scilla natalensis* Planch., *Eucomis autumnalis* (Mill.) Chitt. subsp. *autumnalis*, *Bowiea volubilis* Harv. ex Hook. f., *Helichrysum cymosum* (L.) D. Don and *Siphonochilus aethiopicus* (Schweinf.) B. L. Burtt. **Plate 2.2** shows the general morphology of the plant material used in this investigation.

Information pertaining to reported usage and work performed on biological activity and isolation of chemical constituents of the plants used in this investigation are given in **Table 2.1** and in the sections on each plant.

Plate 2.2: **General morphology of plant material from popular medicinal plants used to investigate the effect of storage on chemical composition and biological activity.**

(A) *Alepidea amatymbica* – rhizome
(B) *Bowiea volubilis* – bulb
(C) *Drimia robusta* – bulb
(D) *Eucomis autumnalis* – bulb
(E) *Helichrysum cymosum* – whole plant
(F) *Leonotis leonurus* – aerial parts
(G) *Scilla natalensis* – bulb
(H) *Siphonochilus aethiopicus* – corm
(I) *Vernonia colorata* – leaves

Picture A – H courtesy VAN WYK, OUDTSHOORN and GERICKE (1997)
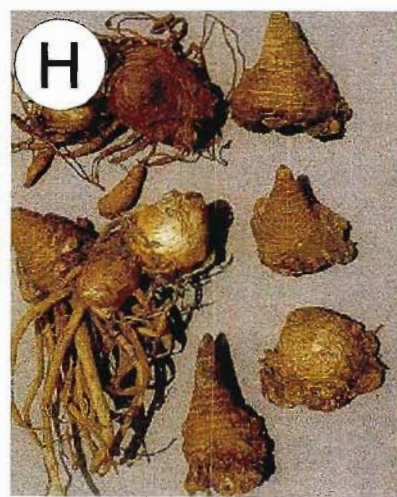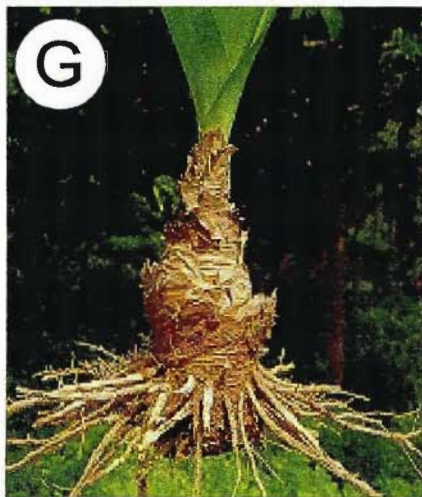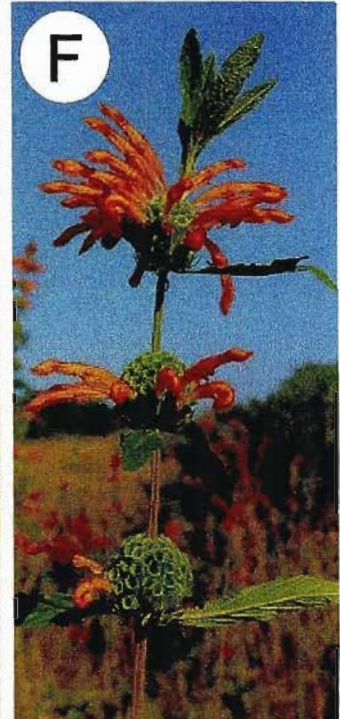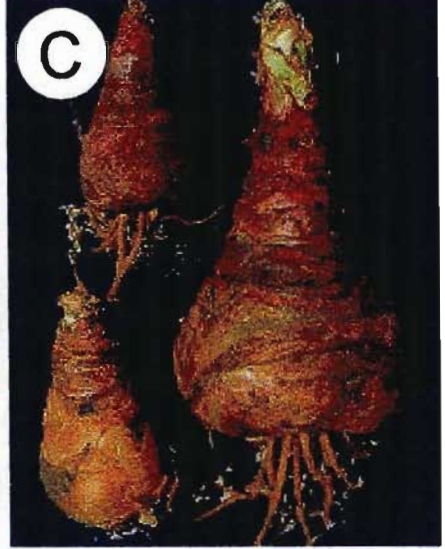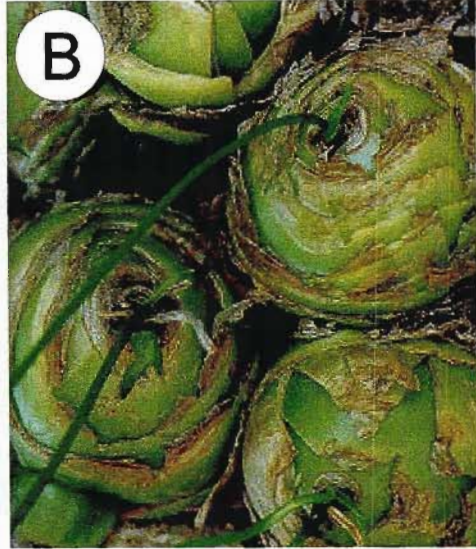
Table 2.1: Plant species used in this investigation and known medicinal properties

Botanical name	Plant material used	Medicinal properties	References
Alepidea amatymbica	Herb root	Anti-microbial, anti-hypertensive and diuretic activity	HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM (1996); SOMOVA, SHODE, MOODLEY and GOVENDER (2001)
Boweia volubilis	Bulb	Cardiac glycosides. Mild anti-inflammatory	WATT and BREYER-BRANDWIJK (1962); JÄGER, HUTCHINGS and VAN STADEN (1996); HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM (1996)
Drimia robusta	Bulb	Cardiac glycosides, mild anti-inflammatory	WATT and BREYER-BRANDWIJK (1962); LUYT, JÄGER and VAN STADEN (1999a and 1999b)
Eucomis autumnalis	Bulb	Anti-inflammatory and antispasmodic	JÄGER, HUTCHINGS and VAN STADEN (1996); TAYLOR (1999); ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN (2000)
Helichrysum cymosum	Whole plants	Pain relieving, anti-infective, anti-microbial and anti-inflammatory	WATT and BREYER-BRANDWIJK (1962); DILIKA, AFOLAYAN and MEYER (1996) and VAN WYK, VAN OUDTSHOORN and GERICKE (1997)
Leonotis leonurus	Leaves	Anti-inflammatory	WATT and BREYER-BRANDWIJK (1962); JÄGER, HUTCHINGS and VAN STADEN (1996); VAN WYK, VAN OUDTSHOORN and GERICKE (1997); KELMANSON, JÄGER and VAN STADEN (2000)
Scilla natalensis	Bulb	Antiseptic and anti-inflammatory	WATT and BREYER-BRANDWIJK (1962); JÄGER, HUTCHINGS and VAN STADEN (1996); VAN WYK, VAN OUDTSHOORN and GERICKE (1997); BANGANI; CROUCH and MULHOLLAND
Siphonochilus aethiopicus	Rhizome	Decongestant, anti-septic and diuretic	WATT and BREYER-BRANDWIJK (1962); ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN (2000), LIGHT *et al*, 2002
Vernonia colorata	Leaves	Anti-bacterial	ZDERO, BOHLMANN, WASSHAUSEN and MUNGAI (1991); KELMANSON, JÄGER and VAN STADEN (2000)

2.2.1. *Alepidea amatymbica*

Species of *Alepidea* F. Delaroche (Apiacea) are restricted to the African continent with approximately 28 species in South Africa (BURTT, 1991). *Alepidea amatymbica* is the only species that is traded commercially in South Africa.

Common name: kalmoes,

Zulu name: *ikhathazo*

Medicinal usage: Rhizomes and roots are widely used for colds and chest complaints (WATT and BREYER-BRANDWIJK, 1962; PUJOL, 1990), coughs and influenza (GERSTNER, 1939). They are also used for rheumatism, applied to wounds as styptics and chewed for sore throats (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). The rootstock is used for diarrhoea, abdominal pain, headaches and to repel bees (GELGAND, MAVI, DRUMMOND and NDEMERA, 1985). SOMOVA, SHODE, MOODLEY and GOVENDER (2001) demonstrated the effectiveness of *A. amatymbica* as a diuretic and having systemic hypotensive and coronary vasodilatory effects.

Known chemical constituents: Several terpenoid kaurene derivatives have been isolated from the roots and aerial parts and are shown in **Figure 2.3** (RUSTAIYAN and SADJADI, 1987; VAN WYK, VAN OUDTSHOORN and GERICKE, 1997, SHODE and RODGERS, 1998; SOMOVA, SHODE, MOODLEY and GOVENDER, 2001). Anti-hypertensive, anti-microbial and diuretic effects have been indicated in tests on animals (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). There appears to be very little work done on this extremely popular plant.

Ent-16-Kauren-19-oic acid

16α-Methoxy-ent-kaur-11-en-19-oic acid

11α-Acetoxy-ent-kaur-16-en-19-oic acid

Wedelia seco-kaurenolide
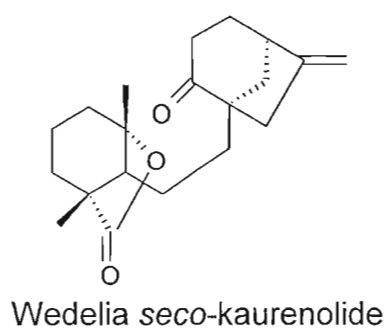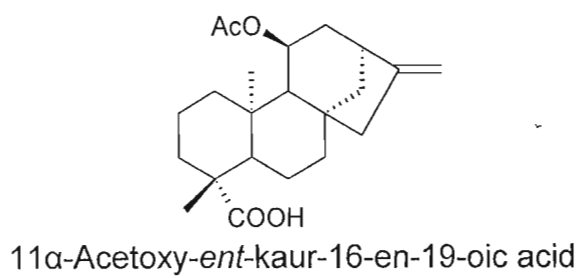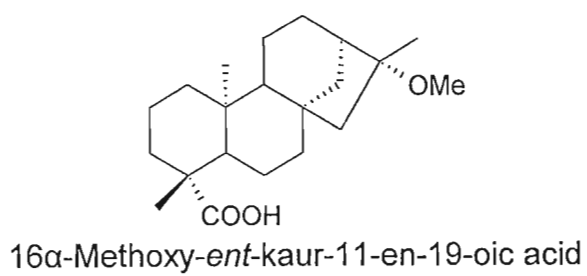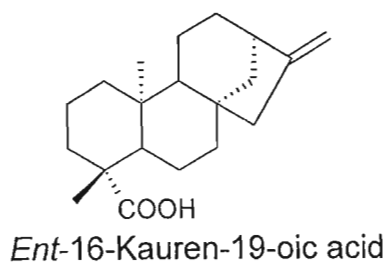
Figure 2.3: Terpenoid kaurene derivatives isolated from *A. amatymbica.*

2.2.2. *Bowiea volubilis*

Common names: knolklimop, climbing potato

Zulu names: *igibisila, iguleni, ugibisisila*

Medicinal usage: Bulbs are reported by GERSTNER (1941) to be used as ingredients in infusions taken during pregnancy to facilitate delivery. They are also used to procure abortions. Hot infusions of the outer bulb scales are used for dropsy (WATT and BREYER-BRANDWIJK, 1962). Lotions of bulb extracts are used to treat eye-sores and skin problems. Bulb decoctions are used to treat bladder pains associated with viral infection (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996).

Known chemical constituents: All parts of the plant are toxic and human and animal deaths have be recorded (WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). Bulbs are reported to produce haemolysis. Bulb sap causes skin irritation. Highly active cardiac glycosides of the scillaren type have been isolated (WATT and BREYER-BRANDWIJK, 1962).

2.2.3. *Drimia robusta*

Common name: brandui

Zulu names: *indongana-zibomvana, isiklenama*

Medicinal usage: Bulb scales are reported to be rubbed on the chest to relieve chest pain and as emetic for stomach ailments (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). WATT and BREYER-BRANDWIJK (1962) reported this plant to be used as expectorants and emetics.

Known chemical constituents: Bulbs and leaves contain oxalic acid crystals. LUYT, JÄGER and VAN STADEN (1999) have shown the presence of bufadienolides and isolated proscillaridin A.

2.2.4. *Eucomis autumnalis*

Common names: gifbol, pineapple flower/plant

Zulu names: *ukhokho, umakhandakantsele, umathunga*

Medicinal usage: CUNNINGHAM (1988) reported the bulbs as being widely used in decoctions administered as enemas. They are used to treat urinary disease, administered in small quantities as emetics and enemas for fevers (GERSTNER, 1941; BRYANT, 1966; HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996).

Known chemical constituents: Several homoisoflavones are known, such as eucomnalin and 3,9-dihydroeucomnalin (VAN WYK, VAN OUDTSHOORN and GERICKE, 1997). Flavonoids are known for their anti-inflammatory and anti-spasmodic action. Other constituents of this species include the benzopyrones autumnariol and autumnariniol, as well as some steroidal triterpenoids such as eucosterol (VAN WYK, VAN OUDTSHOORN and GERICKE, 1997). Triterpenoids are known to be beneficial in wound therapy. Extensive work on *Eucomis spp.* has been conducted by TAYLOR (1999). It was found that these plants all exhibited high levels of anti-inflammatory activity.

2.2.5. *Helichrysum cymosum*

Common names: everlastings, kooigoed

Zulu name: *imphepho*

Medicinal usage: Many ailments are treated with a wide variety of *Helichrysum* species, these popular medicinal plants are used to treat coughs, colds, fevers, infections, headaches and menstrual pain (WATT, and BREYER-BRANDWIJK, 1962; HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). It is a popular ingredient in wound dressings.

Known chemical constituents: This species is known to contain flavonoids, sesquiterpenoids and acylated phloroglucinols (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). Pain relieving, anti-infective and anti-inflammatory activity has been reported (JAKUPOVIC, KUHNKE, SCHUSTER, METWALLY and BOHLMANN, 1985).

2.2.6. *Leonotis leonurus*

Common names: Cape hemp, duiwelstabak, koppiesdagga, lion's ear, minaret flower, rooidagga, wild dagga. wildedagga..

Zulu names: *imunyamunya, umunyane (-omncane)*.

Medicinal usage: Water infusions of leaves are used to relieve feverish headaches (BRYANT, 1996), leaves and stems taken for dysentery and tapeworm or take orally for coughs and colds (GERSTNER, 1941; WATT and BREYER-BRANDWIJK, 1962).

Known Chemical constituents: Compounds extracted from leaves include two phenolic compounds and 19.8% resin (WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). The diterpene spiro ether, C-13 epimeric premarrubiin has also been isolated (LAONOGRO, LANZETTA, PARRILLI, ADINOLFI and MANGONI, 1979).

2.2.7. *Scilla natalensis*

Common names: blouberglelie, slangkop

Zulu names: *ichitha, imbiza-elukhulu, imbizankulu, inguduza, ubulika*

Medicinal usage: GERSTNER (1938) reported the use of bulb decoctions administered as enemas for cleansing. It is also used to treat sprains and fractures, possibly by reducing swelling, as a purgative, and in enemas administered for the treatment of internal tumours (WATT and BREYER-BRANDWIJK, 1962). The Swazis are reported to use *S. natalensis* to treat veld sores and boils (WATT and BREYER-BRANDWIJK, 1962).

Known chemical constituents: Shown to cause haemolysis in blood agar indicating the presence of saponins. Although extracts appear to possess antiseptic, anti-inflammatory and possibly other healing properties, it is relatively toxic and thus should only be used in topical applications (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). Poor antibacterial activity was shown against Gram-positive and Gram-negative bacteria, dichloromethane and hexane extracts showed good inhibition of COX-1 and COX-2 (SPARG, VAN STADEN and JÄGER, 2002). Ethanol extracts showed high activity against nematodes in the anthelmintic bioassay (SPARG, VAN STADEN and JÄGER, 2002). Aqueous extracts had good activity against *Schistosoma haematobium*, with a minimum inhibitory concentration of

0.4mgml⁻¹(SPARG, VAN STADEN and JÄGER, 2002). Phytochemical screening confirmed the presence of saponins and bufadienolides in bulb material (SPARG, VAN STADEN and JÄGER, 2002).

2.2.8. *Siphonochilus aethiopicus*

Common names: Natal ginger, wild ginger

Zulu names: *indungulo, isiphephetho*

Medicinal usage: Roots or rhizomes are used for coughs, colds, catarrh, influenza, sharp pain relief and hysteria (GERSTNER, 1938). *S. aethiopicus* is used by the Swazi for malaria and chewed by women during menstruation, possibly to relieve pain (WATT and BREYER-BRANDWIJK, 1962).

Known chemical constituents: The rhizome of *S. aethiopicus* yields 2% oil yet to be identified (WATT and BREYER-BRANDWIJK, 1962). Extracts were tested for a variety of pharmacological activity and screening showed antibacterial (ethanol and ethyl acetate extracts of all plant parts against Gram-positive bacteria) and anti-inflammatory activity, ethanolic leaf extracts inhibition of COX-1 and COX-2 (LIGHT, McGAW, RABE, SPARG, TAYLOR, ERASMUS, JÄGER and VAN STADEN, 2002). Aqueous extracts showed high levels of cytotoxicity.

2.2.9. *Vernonia colorata*

Common names: laeveldbittertee, lowveld bitter tea

Zulu names: *ibozane*

Medicinal usage: Roots are used as tonic and to cure boils (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM,1996), decoctions of leaves are used for fevers and as expectorants and laxatives (OLIVER-BEVER, 1986).

Known chemical constituents: Vernonin has been isolated from roots and a cardiac glycoside was isolated from stems, leaves and roots of plants from Nigeria (PATEL and ROWSON, 1964). Two sesquiterpenic lactones with *in vitro* cytotoxicity have been isolated by TOUBIANA (1969). Other compounds found in the leaves include alanine, phenylalanine, glycine, leucine, proline, serine, threonine, tyrosine,

2.3.2. Preparation of extracts

Only cold extractions were performed should any of the active compounds not be heat stable.

Fresh material

Immediately after harvesting the plant material was cleaned and approximately 15 g placed in a Waring blender with 100 ml of either distilled water, absolute ethanol or *n*-hexane and blended for 15 min. This was then filtered through Whatman No. 1 filter paper under vacuum and air-dried. The residues were dissolved in their initial extracting solvent (water, ethanol or hexane) resulting in a 100 mg residue/ml solvent extract. Air-dried residues were stored at -15°C to limit further chemical decomposition prior to redissolving which only took place just before the extract was required for a bioassay.

Dried and stored material

The plant material, once the portion required for the fresh extracts had been removed, was dried in a oven at 50°C (**Table 2.3**) and stored at room temperature (20°C) in brown paper bags until extraction The mass before and after drying was recorded and the percentage water lost calculated (**Table 2.3**). Dried material (5g) was ground and extracted with 100 ml of water, ethanol and hexane in an ultrasound bath for 15 min. The extracts were filtered through Whatman No. 1 filter paper under vacuum and air-dried. The residues were dissolved and stored in the same way as extracts prepared from fresh material.

Table 2.2: List of plant species used in this study and area of collection.

Botanical name	Collection site	Voucher specimen number
Alepidea amatymbica	Silverglen Nursery, Durban	*Stafford 1* NU
Boweia volubilis	Silverglen Nursery, Durban	*Stafford 3* NU
Eucomis autumnalis	University of Natal, Pietermaritzburg Botanical Gardens[*]	*Stafford 4* NU
Drimia robusta	University of Natal, Pietermaritzburg Botanical Gardens[*]	*Stafford 5* NU
Helichrysum cymosum	Silverglen Nursery, Durban	*Stafford 6* NU
Leonotis leonurus	University of Natal, Pietermaritzburg Botanical Gardens	*Stafford 2* NU
Scilla natalensis	University of Natal, Pietermaritzburg Botanical Gardens	*Stafford 7* NU
Siphonochilus aethiopicus	University of Natal, Pietermaritzburg Botanical Gardens[1]	*Stafford 8* NU
Vernonia colorata	University of Natal, Pietermaritzburg Botanical Gardens	*Stafford 9* NU

In **Table 2.2** there was reference made to plant material that was obtained from plants which were propagated *in vitro* via micropropagation techniques. Studies have shown (FINNIE, DREWES and VAN STADEN, 1994; LUYT, JÄGER and VAN STADEN, 1999) that tissue-cultured material does contain the active constituents for which it is used in traditional medicine.

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[*]Plant material taken from plants which were micropropagated through tissue culture and planted in the University of Natal, Pietermaritzburg Botanical Gardens.

Table 2.3: The duration of drying in a ventilated oven at 50°C required to dry to constant weight the plant material.

Plant species	Plant part	Initial mass (g)	Final mass (g)	Percentage water loss	Duration (days)
Alepidea amatymbica	Leaf	109.7	13.8	62	2
Alepidea amatymbica	Root		27.4		3
Boweia volubilis	Bulb	39.4	22.4	43	3
Eucomis autumnalis	Mature bulb	584.2	420.5	29	6
Drimia robusta	Bulb	439.8	279.7	36.5	7
Helichrysum cymosum	Whole plant	50.9	19.3	61	2
Leonotis leonurus	Leaves	99.5	30.1	70	2
Scilla natalensis	Mature bulb	773.6	484.7	38	7
Siphonochilus aethiopicus	Rhizome	444.1	353.8	20	4
Vernonia colorata	Leaves	101.2	38.5	62	2

2.3.3. TLC analysis

Plant extracts (0.5 mg each) were applied to TLC plates (Merck plastic sheets, 20 x 20 cm Silica gel 60 F_{254}) as 1 cm bands. The plates were developed over 7.5 cm. Solvent systems used were benzene:1,4 dioxane:glacial acetic acid (90:25:4 v/v/v) for *E. autumnalis*; *n*-hexane:ethyl acetate (2:1 v/v) for *A. amatybica, L. leonurus, S. aethiopicus* and *V. colorata,* chloroform:acetone (40:60 v/v) for *H. cymosum.*

Those plants which are known to contain cardiac glycosides, *Boweia volubilis, Drimia robusta* and *Scilla natalensis,* were developed in ethyl acetate:methanol:water (81:11:8 v/v/v) and stained by spraying with a 20% ethanolic solution of antimony-III-chloride and heated at 110°C for 6 min. This was then viewed under UV_{366} nm, cardiac glycosides should appear as red/orange bands. No bands are visible under UV_{254} nm. An additional plate with fresh dried material (4 days at 50°C) was produced to show any differences as a result of drying the plant material.

All plates (except those sprayed with antimony-III-chloride which was viewed under UV$_{366}$ nm only) were viewed under UV$_{254}$ nm and UV$_{366}$ nm and then sprayed with anisaldehyde stain and viewed under normal visual light (VIS). The anisaldehyde stain is made-up from 465 ml ethanol, 5 ml glacial acetic acid, 13 ml concentrated sulphuric acid and 13 ml para-anisaldehyde. After spraying the plates were developed for 5-10 min at 110°C. At TLC plates were photographed immediately after they were developed.

2.3.4. Anthelmintic bioassay

Culturing of *Caenorhabditis elegans*

The free-living nematode *Caenorhabditis elegans* var. Bristol (N2) nematodes were cultured on nematode growth (NG) agar (Appendix 2) seeded with *E. coli* according to the method of BRENNER (1974). Subculturing must be carried out in sterile conditions to prevent contamination. Fifty µl aliquots of *E. coli* culture grown overnight at 35°C was pipetted onto each NG agar plant and spread with a sterile glass spreader. Five ml of sterile M9 buffer (Appendix 2) was added to the initial nematode culture plate that is to be subcultured to wash off a portion of the nematodes. Aliquots (500 µl) of this nematode wash were pipetted onto each fresh plate with the *E. coli* inocula and incubated in the dark at 20°C.

Two hour mortality assay

A 1 mgml^{-1} concentration of ethanol and water extracts of fresh and stored material from *Alepidea amatymbica* leaves and roots, *Leonotis leonurus* leaves and *Vernonia colorata* leaves were tested for changes in anthelmintic activity after storage. The simple bioassay described by RASOANAIVO and RATSIMAMANGA-URVERG (1993), where nematode mortality after the addition of nematocidal compounds or plant extracts were assayed, with changes by McGAW, JÄGER and VAN STADEN (2000) was used to determine anthelmintic activity. In this assay, 500-1000 nematodes (take from seven to ten-day-old cultures and estimated by observing a sample under the dissecting microscope) in 30 µl M9 buffer (**Appendix 2**) were incubated with 4.5 ml of 1 mgml^{-1} plant extract (45 µl of 100 mgml^{-1} plant extract and 4455 µl dH$_2$0) in the dark at 25°C for two h. A standard of 5 µgml^{-1} levamisole was used as a control as well as a blank control consisting of nematodes incubated with

4.5 ml of dH_2O, no levamisole or plant extract. Using a dissecting microscope, the percentage of living nematodes was estimated, and their movement recorded and compared with the control.

2.3.5. Cyclooxygenase (COX-1) bioassay

The inhibition of prostaglandin biosynthesis by the plant extracts was investigated using the cyclooxygenase assay. The procedure was performed according to the method first described by WHITE and GLASSMAN (1974) and later modified by JÄGER, HUTCHINGS and VAN STADEN (1996).

Cyclooxygenase was prepared from sheep microsomal vesicles. Cyclooxygenase enzyme solution (0.4 µg protein) and co-factor solution (0.3 mgml^{-1} of each of L-adrenalin and reduced glutathione in 0.1 M Tris buffer, pH 8.2) were mixed 1:5 and incubated on ice for 15 min. Then 60 µl of the enzyme/co-factor solution to 20 µl of sample containing the specific amount of plant extract residue were dissolved in water or 5% aqueous ethanol depending on the extract. Only water and ethanol extracts were used. 20 µl of sample of ^{14}C-arachidonic acid (16 Cimol^{-1}, 3 mM) was added, and the assay mixture which was then removed from the ice and incubated at 37°C for10 min. The reaction was terminated by placing on ice and adding 10 µl 2N HCl. A background sample was kept on ice. Four µl of an unlabelled 0.2 mgml^{-1} carrier solution of prostaglandins (PGE$_2$:PGF$_2$ 1:1 v/v) were added.

The ^{14}C-labelled prostaglandins synthesised during the assay were separated from unmetabolized ^{15}C-arachidonic acid by column chromatography. Silica gel in eluent 1 (hexane:dioxane:acetic acid 350:150:1 v/v/v) was packed to a 3 cm height in Pasteur pipettes. One ml of eluent 1 was added to each of the assay samples and the mixtures applied to separate columns. The arachidonic acid was eluted from the column with 4 ml of eluent 1 and discarded. The radio labelled prostaglandins were eluted with 3 ml of eluent 2 (ethyl acetate:methanol 85:15 v/v) into scintillation vials. After mixing with Beckman's scintillation solution, the radioactivity in the samples was counted using a Beckman LS6000 scintillation counter. The percentage inhibition of the test solution was obtained by analysing the amount of radioactivity in these solutions relative to that present in the solvent blank.

In all the cyclooxygenase assays performed, a water and ethanol indomethacin standard solution was assayed together with the samples to verify the sensitivity of the assay. Fifty µg of plant residue was used in each assay, with a double determination for each. The ethanol and water extracts from fresh and stored plant material tested for changes in anti-inflammatory activity in the cyclooxygenase bioassay were taken from *Bowiea volubilis* bulbs, *Drimia robusta* bulbs, *Eucomis autumnalis* bulbs, *Helichrysum cymosum* plants, *Leonotis leonurus* and *Vernonia colorata* leaves.

2.3.6. Microtitre-plate minimum inhibitory concentration antibacterial bioassay

Two Gram-positive (Gram+ve) bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and two Gram-negative (Gram–ve) bacteria, *Escherichia coli* and *Klebsiella pneumoniae* were used to determine the minimum bacterial growth inhibition concentration of fresh and stored plant material. More information on the bacteria used in this bioassay is provided in **Table 2.4**. All the bacteria used have virtually identical growth curves and thus were treated similarly with respect to culturing and dilution.

Bacterial cultures were grown in 100 ml Mueller-Hinton (MH) broth overnight in a shaker at 37°C. In the morning the optical density (OD) at 600 nm for each bacterial broth was determined using a Varian Cary 50 Spectrophotometer (Table 6), an ideal OD_{600} is between 1.5 and 2.0. The bacterial culture was then diluted 1:100 with MH broth (approximately 10^6 bacteria ml^{-1}).

The micro plate method of ELOFF (1998b) was used with slight modifications to determine the MIC values for plant extracts with antibacterial activity. Ethanol and water extracts of fresh and stored material were redissolveed at 50 $mgml^{-1}$ with the extracting solvent. All extracts were tested initially at 12.5 $mgml^{-1}$ in 96-well microtitre plates and serially diluted two-fold to produce wells with the following concentrations: 12.5, 6.25, 3.13, 1,56, 0.78, 0.39, 0.20 and 0.1 $mgml^{-1}$. After the serial dilution 100 µl of diluted bacterial culture (approximately 10^6 bacteria ml^{-1}) were added to each well (**Table 2.4**).

Table 2.4: Bacterial cultures used in MIC microtitre plate bioassay.

Bacteria	ATCC numbers	Gram status	Growth description	Cell count for a saturated overnight culture (5 ml)	OD$_{600}$
Bacillus subtilis	6051	+	Colonial variation is observed. One type of colony is flat, irregular-shaped, and whitish in colour (opaque), the other is smaller, more circular and translucent, also flat/rough.	4.8×10^9 cell/ml	1.41
Staphylococcus aureus	12600	+	Colonies are entire, glistening, circular and smooth.	2.7×10^9 cell/ml	2.04
Escherichia coli	11775	-	Colonies are entire, glistening, smooth, translucent and 1,5-3 mm in diameter.	7.0×10^{10} cell/ml	1.65
Klebsiella pneumoniae	13883	-	Two colony types may be seen: predominantly entire, glistening, smooth, 2 mm in diameter, and opaque. The second is smaller, more translucent and varying in size.	2.5×10^9 cell/ml	1.77

The antibiotic neomycin, also in serial dilution, was included as a standard in each assay. Extract-free solution was used as a blank control. A serial dilution of ethanol starting at the same concentration as the strongest extract (i.e. 50% ethanol) is also required as a control to note where the ethanol in the extract no longer inhibits the bacterial growth and therefore any inhibition of that concentration is as a result of the plant material and not the ethanol. The micro plates were incubated overnight at 37°C. As an indicator of bacterial growth, 40 µl *p*-iodonitrotetrazolium violet (INT) (SIGMA) dissolved in dH$_2$O were added to the each well and incubated at 37°C for 30 min. MIC values were recorded as the lowest concentration of extract that completely inhibited bacterial growth. Since the colourless tetrazolium salt is reduced to a red-coloured product by biologically active organisms, the inhibition of growth can be detected when the solution in the well remains clear after incubation with INT.

2.4. Results

2.4.1. TLC analysis

Only significant differences between the TLC-fingerprints of the stored and fresh material will be described, they are too numerous to be characterized in detail.

Plate AP1.1 in Appendix 1 shows TLC plates for the fresh material that has not been dried, fresh dried material and stored material collected for *S. natalensis, D. robusta* and *B. volubilis*. The red-orange bands indicate the presence of cardiac glycosides as a result of the antimony-III-chloride spray reagent when viewed under UV_{365} nm. The hexane fraction of the three plants fresh and stored material appears to be similar. The ethanol and water fractions however, show signs of change in chemical composition notably in the water fraction. There were several bands in the fresh *S. natalensis* and *B. volubilis* water fractions that were not evident in the ones from stored material. There appears to be more compounds extracted as a result of the drying process. The dried fresh material and the dried stored material produce more bands especially in the ethanolic fractions than the fresh non-dried material. Although the orange bands in the stored ethanolic fractions did not appear as bold as the fresh bands in the photographic print they were visible on the actual plates.

The TLC-fingerprints of *A. amatymbica* (**Plate AP1.2 A** in Appendix) leaf and rhizome extracts of the fresh material have lower R_f-values than the stored material, that is the bands in the stored material appear to have travelled further along the plate than the fresh material. The fresh water leaf extract had four bands, which were not present in the stored water extract. The fresh and stored (90-day-old) material (**Plate AP1.2 C** in Appendix) were more similar.

The *E. autumnalis* (**Plate AP1.3 A**) fresh extracts appeared to be similar to the stored extracts. The orange bands in the anisaldehyde-treated plates were not as defined in the fresh extract as they did in the stored material. The two bands in the stored water extract detected in the anisaldehyde-treated plate did not appear on the plate with the fresh plant extract.

Under UV_{254} nm (**Plate AP1.3 B**) three bands were detected in the stored *H. cymosum* hexane and ethanol fractions that did not appear in the fresh material. These bands although not as pronounced, were also apparent under UV_{366} nm (R_f-

value 0.72). There was another band at R_f-value 0.2 in the hexane and ethanol fractions of the stored material that was not visible in the fresh material. In the plates treated with anisaldehyde acid the stored material had more pronounced bands.

A hexane extract for the *S. aethiopicus* fresh material was not obtained as not enough residue could be extracted from the fresh plant material. The fresh and stored ethanolic extracts appear to be similar in all respects except that there were three bands at R_f-values 0.22, 0.26 and 0.34 in the stored extract (noticeable on the anisaldehyde-treated plates) whereas there was only one band in the fresh extract (**Plate AP1.3 C**).

In the TLC-fingerprints of the *L. leonurus* (**Plate AP1.4 A**) there were considerable differences between the fresh and 90-day-old material especially in the hexane extracts. In (**Plate AP1.4 A**) The similarity between the ethanolic extracts is clearer, with the appearance of a new band at the top of the TLC plate in the stored material.

Plate AP1.5 A in Appendix 1 shows the TLC-fingerprints for fresh and stored material of *V. colorata*. It was difficult to determine what bands where present in the hexane extracts due to 'streaking' caused by the levels of oils (fatty acids) in the extract. It should be noted that this occurred more with the stored hexane extract than with the fresh one. The fingerprints of the ethanol extracts of the fresh and stored material were different in that the fresh extracts yielded higher R_f-values than the corresponding bands from the stored material.

2.4.2. Anthelmintic bioassay

From **Table 2.5** it is apparent that only the water extracts of *A. amatymbica* studied showed any anthelmintic activity. The root material having higher activity than the leaf material. There was no obvious difference between the activity from the fresh and stored material.

Table 2.5: Anthelmintic activity of extracts from fresh and stored plant material in the two-hour mortality assay.

Aqueous plant extracts			Ethanolic plant extracts		
Species	Fresh material score	Stored material score	Species	Fresh material score	Stored material score
A. amatymbica (Root)	8 7	6 7	A. amatymbica (Root)	2 1	1 1
A. amatymbica (Leaf)	3 4	3 4	A. amatymbica (Leaf)	1 1	1 2
L. leonurus	2 2	2 2	L. leonurus	1 2	1 1
V. colorata	2 2	2 1	V. colorata	2 1	2 1
STANDARD (5 µg/ml levamisole)	5 5		STANDARD (5 µg/ml levamisole)	5 5	
BLANK (30 µl dH₂0)	1 1		BLANK (30 µl ethanol)	1 1	

Key to the scoring system used in the table above.

Score	Percentage alive	Nematode activity
0	90 - 95	alive and moving vigorously
1	80 - 90	alive and moving vigorously
2	70 - 80	alive and moving vigorously
3	60 - 70	alive and moving fairly vigorously
4	50 - 60	alive and moving fairly vigorously
5	40 - 50	alive and moving fairly vigorously
6	30 - 40	alive and sluggish
7	20 - 30	alive and sluggish
8	10 - 20	alive and sluggish
9	0 - 10	dead

2.4.3. Cyclooxygenase (COX-1) bioassay

In general the ethanol extracts showed higher cyclooxygenase inhibition than the water extracts. *S. natalensis* (**Table 2.6**) had low anti-inflammatory activity in the fresh water extract but higher activity in the stored water extract. Of the plant extracts tested for anti-inflammatory activity the water extracts of *S. natalensis* and *B. volubilis* both showed an increase in anti-inflammatory activity after storage, as did their ethanol extracts. *S. aethiopicus, H. cymosum, D. robusta, L. leonurus* and *V. colorata* showed a loss in activity after storage in their water extracts. Of the ethanol extracts only *D. robusta* showed a loss in activity after storage. Although *S. aethiopicus* lost

activity in its water extracts the activity of the ethanol extract increased after storage. The ethanol extracts of *E. autumnalis*, *H. cymosum*, *L. leonurus* and *V. colorata* totally inhibited prostaglandin synthesis in both the fresh and stored extracts. The anti-inflammatory activity of *E. autumnalis* did not change.

Table 2.6: Percentage inhibition of prostaglandin synthesis by fresh and stored plant material extracts.

Plant species	Percentage inhibition			
	Water extracts		Ethanol extracts	
	Fresh material	Stored material	Fresh material	Stored material
S. natalensis	23	96	86	100
E. autumnalis	37	34	100	100
B. volubilis	32	73	97	100
S. aethiopicus	72	38	86	93
H. cymosum	52	17	100	100
L. leonurus	70	8	100	100
V. colorata	38	11	100	100
D. robusta	61	0	96	76
STANDARD	60	60	64	64

2.4.4. Microtitre-plate minimum inhibitory concentration anti-bacterial bioassay

Table 2.7 summarizes the results obtained from the MIC bioassays. The minimum inhibitory concentration is the lowest concentration, which totally inhibits bacterial growth and therefore the lower the value the more potent the extract. In general the stored ethanol extracts showed the highest activity (as they had the lowest MIC value). There was a definite increase in anti-bacterial activity after storage in most ethanol extracts (shown in bold in **Table 2.7**). *V. colorata* showed no change in antibacterial activity after 90 days storage whereas *S. natalensis* showed a change in activity (increases activity) against all bacterial tested.

Table 2.7 Minimum inhibitory values for ethanol extracts of fresh and stored (90 days) plant material tested against four bacteria.

Plant species	Minimum inhibitory values (mgml⁻¹)							
	B. subtilis		*S. aureus*		*E. coli*		*K. pneumoniae*	
	Fresh	Stored	Fresh	Stored	Fresh	Stored	Fresh	Stored
A. amatymbica (leaf)	1.56	1.56	1.56	1.56	**3.13**	**1.56**	1.56	1.56
A. amatymbica (rhizome)	**3.13**	**1.56**	1.56	1.56	1.56	1.56	1.56	1.56
B. volubilis	**6.25**	**3.13**	3.13	3.13	**3.13**	**1.56**	3.13	3.13
S. aethiopicus	**3.13**	**1.56**	**3.13**	**1.56**	1.56	1.56	**3.13**	**1.56**
H. cymosum	**1.56**	**0.78**	1.56	1.56	**1.56**	**0.78**	1.56	1.56
L. leonurus	**1.56**	**0.78**	**1.56**	**0.78**	1.56	1.56	**1.56**	**0.78**
V. colorata	0.78	0.78	1.56	1.56	1.56	1.56	0.78	0.78
S. natalensis	**6.25**	**3.13**	**6.25**	**3.13**	**6.25**	**3.13**	**6.25**	**3.13**

2.4.5 Summary of effect of short-term storage

Of the plant extracts tested for anthelmintic activity only the water extracts of *A. amatymbica* showed activity and very little change in activity was observed after storage. Of the plant extracts tested for anti-inflammatory activity the ethanol extracts generally yielded higher activity. *S. natalensis* and *B. volubilis* both showed an increase in anti-inflammatory activity after storage whereas *S. aethiopicus, H. cymosum, D. robusta* and *V. colorata* showed a loss in activity after storage. The anti-inflammatory activity of *E. autumnalis* did not change. The water extracts of

plants tested for antibacterial activity showed no activity, whereas the ethanol extracts generally showed an increase in activity with storage. The TLC fingerprints showed changes between the fresh and stored material in most plants. The least change occurred in *E. autumnalis.*

2.5. Discussion and conclusions

Changes in the chemical composition of the plant material during storage were made evident through the use of TLC-fingerprints. At this point all that can be deduced was that if there was a decrease in biological activity then the product of the chemical change (break-down product) is not active or less active than its precursor. If there was an increase in the biological activity, as was observed with the anti-bacterial activity, it can be deduced that the precursor is less active than the product of the chemical change. It may not be correct to assume than the product of the chemical change is a breakdown product, which implies that it is smaller than its precursor, when a polymerisation or esterification may have occurred resulting in a larger molecule being formed. However, the chemical degradation processes described in the introduction are most likely to be the cause of the major chemical changes.

Although only one plant extract, that of the water extract from *A. amatymbica,* showed an significant anthelmintic activity, this activity was the same in the fresh material and stored material. Whether all anthelmintic compounds are stable during storage under the conditions used in this investigation (in a brown paper bag at room temperature) remains to be shown.

Anti-bacterial compounds appear to be either stable or convert into more active compounds during storage. The MIC bioassay used in this study makes use of a two-fold serial dilution, that is the concentration of the extract from one well to the next is halved, resulting in a decrease in the difference between one concentration and the next (12.5, 6.25, 3.13, 1,56, 0.78, 0.39, 0.20 and 0.1 mgml^{-1}). Thus, in most instances where an increase in activity was observed, half the concentration of plant extract required from the fresh material was require from the stored material to inhibit bacterial growth. This means that the traditional healer should halve the dose he prescribes to his patient should he use 90-day-old material rather than fresh to achieve the same effect.

An interesting observation with regards to the anti-inflammatory bioassay results is that in most situations where the water extract showed an increase in activity after storage so did the ethanol extract. With *S. aethiopicus* however, this did not occur,

the water extract showed a loss in activity after storage and the ethanol extract showed an increase in activity after storage. This may indicate that there are different compounds in the water and ethanol extracts that are responsible for the anti-bacterial activity of *S. aethiopicus.*

TAYLOR (1999) conducted a investigation into the effect of storage on the anti-inflammatory activity of *E. autumnalis.* Extracts from material harvested and dried in 1996, but not extracted, as well as ethanolic extracts made in 1996 were tested in the COX-1 assay, and compared to fresh extracts made in 1999. No significant difference was observed in the levels of activity detected in the assay. These findings are consistent with those in this investigation as *E. autumnalis* showed little or no changes in biological activity. This corresponded with the lack obvious changes in chemical composition as indicated by the TLC-fingerprinting.

All conclusions made from this investigation cannot be attributed to storage alone as the drying process may also affect the chemical composition of the material. In hindsight, it was perhaps not wise to have used fresh material rather than newly dried material but this is the form in which it is usually preferred by traditional healers and is seldom tested in the laboratory. Ideally, the chemical composition of the material, as well as its biological activity should have also been determined directly after drying. Thus, the effect of drying plant material and the effect of storage on plant material should be separated. This is evident from the TLC-fingerprints of the plants that contain cardiac glycosides (**AP1.1** in Appendix 1). There appears to be more compounds extracted as a result of the drying process. The dried fresh material and the dried stored material produce more bands especially in the ethanolic fractions than the fresh non-dried material. This may be due to the rupturing and degradation of cell membranes during drying, which would result in a greater release of compounds during extraction.

Due to several factors such as time restrains and limited amount of plant material only two repeats of each bioassay were conducted on each plant extract, except for the anti-bacterial assay where the bioassay was performed only once. As a result the sample size was too small to conduct any meaningful statistics.

The aim of this investigation was to gain some insight in what changes are occurring between fresh material and stored material (90 days). Fingerprinting the chemical composition of the fresh and stored material and determining changes in biological activity using various bioassays were used to achieve this. It is very clear that the

chemical composition and activity of these medicinal plants are changing with time and storage conditions. It should be noted that these changes can not be detected by the human senses, thus consumers will not be able to determine the quality of these plant materials easily. These changes are not always negative but in fact the anti-bacterial activity increase with storage or ageing.

The implications of these findings could affect the way traditional healers, medicinal plant consumers and scientists working with medicinal plants treat their material. It is apparent from this investigation that the plants used for their anti-inflammatory activity may require special storage conditions or only be used when they are fresh. These measures particularly apply to water extracts of *D. robusta* where there was a total loss of anti-inflammatory activity after 90 days of storage. This may explain why this plant is not the most popular plant used to treat inflammatory complaints. The active ingredients in *E. autumnalis* appear to be stable and thus storing this plant in a dry form in the dark at room temperature should be sufficient to ensure little or no change in biological activity. SILVA, LEE and KINGHORN (1998) demonstrated that in some instances ultraviolet radiation may produce chemical reactions that give rise to compound artefacts. Plants used for their anti-bacterial properties seem not to be adversely affected by short-term (90 days) storage, and in fact may improve with age.

CHAPTER 3

EFFECT OF LONG-TERM STORAGE

3.1. Introduction

The motivation behind an investigation into the effects of long-terms storage follows from our understanding of the flow of plant material through the medicinal plant trade and that material is quite often stored for long periods especially in the 'muthi' shops. These establishments stock a wider variety of plant material than the informal street markets and thus some of the more rare, less frequently used products might be stored for up to 5 years (MTOLO, MKHIZE, MSANGO and SMITH *per com*). The aim of this investigation is to gain some insight in what changes are occurring in plant material of three medicinal plants from its fresh dried form and material that is stored for a relatively long period of one year, notably what changes occur in biological activity and chemical composition. Changes in chemical composition were observed visually using thin layer chromatography fingerprinting. Two aspects of biological activity were investigated, notably antibacterial, and anti-inflammatory.

The bioautographic method was incorporated into this investigation, as it is an excellent detection method for new or unidentified antibacterial compounds (RIOS, RECIO and VILLAR, 1988). The direct bioautographic assay was used as opposed to the agar-overlay method. In this assay a suspension of bacteria in liquid medium (MH broth) is sprayed uniformly on a developed TLC plate and incubated overnight. A solution of a tetrazolium salt such as *p*-iodonitrotetrazolium violet (INT) is then sprayed uniformly over the plate and incubated to detect areas of bacterial growth inhibition. This method excludes problems associated with differential diffusion of compounds through the agar (HAMBURGER and CORDELL, 1987). The advantage of bioautographic assays is that it allows the localization of activity, even in complex mixtures.

3.2. Materials and Methods

3.2.1. Plant material

Plants collected were initial chosen according to their popularity as a traditional medicine according to MANDER (1997) and then six plants, which have shown substantial changes as a result short-term storage in the pervious Chapter, were selected for long-term storage studies. *Alepidea amatymbica, Eucomis autumnalis, Helichrysum cymosum, Leonotis leonurus, Siphonochilus aethiopicus* and *Vernonia colorata* were selected as these plants not only showed interesting results due to short-term storage but also are easily obtainable. Only enough material of the *Alepidea amatymbica, Leonotis leonurus* and *Vernonia colorata* was available for the bioassays.

3.2.2. Preparation of extracts

It should be noted that only cold extractions as in the short-term storage investigation were performed should any of the active compounds not be heat stable. The fresh extract was produced from material dried immediately after harvesting and stored at -15°C to limit further chemical decomposition. Initially hexane, ethanol and aqueous extracts were prepared later only ethanol extracts were prepared for the bioassays as the hexane and aqueous extracts contained few or no active constituents.

Fresh material

Immediately after harvesting the plant material approximately 15 g of material was removed after cleaning and was dried in an oven at 50°C. The dried material (5g) was ground and extracted with 100 ml ethanol in an ultrasound bath for 15 min. This was then filtered through Whatman No. 1 filter paper under vacuum and air-dried. The residues were dissolved in their initial solvent resulting in a 100 mg residue/ml solvent extract. Air dried residues were stored at -15°C to limit further chemical decomposition prior to redissolving which only took place just before the extract was required for a bioassay.

Dried and stored material

The plant material was dried in an oven at 50°C and stored at room temperature (20°C) for one year in brown paper bags until extraction. In some instances 180-day old material extracts were made for TLC-fingerprinting. Dried material (5 g) was ground and extracted with 100 ml ethanol in an ultrasound bath for 15 min. The extracts were filtered through Whatman No. 1 filter paper under vacuum and air-dried. The residues were dissolved and stored in the same way as extracts prepared from fresh material. One hundred and eighty-day-old material of *Leonotis leonurus* and *Vernonia colorata* were used to produce ethanolic extracts, which were also fingerprinted.

Some five-year-old *L. leonurus* material that was collected by L. McGaw from the same plant at approximate the same time of year as the material in this investigation and thus could be used to compare with the fresh, 90 day-old and one-year-old material. This material was already ground and thus 5 g was extracted in 100 ml ethanol in an ultrasound bath for 15 min. The extract was filtered through Whatman No. 1 filter paper under vacuum and air-dried. The residue was dissolved and stored in the same way as extracts prepared from fresh material.

3.2.3. TLC preparation and bioassays methods

The TLC-fingerprints, the microtitre serial dilution MIC antibacterial bioassay and the cyclooxygenase assay were conducted as in the pervious chapter (CHAPTER 2, SECTIONS 2.2.3., 2.2.5. and 2.2.6. respectively). All TLC plates were run in a solvent mixture 2:1 hexane:ethyl acetate (v/v).

3.2.4. Antibacterial bioautographic method

An overnight culture of *S. aureus* in MH broth was prepared in a sterile centrifuge tube the previous day. The TLC plates were developed as described in the previous chapter (CHAPTER 2, SECTION 2.2.3). These were dried under a fume hood over night to ensure all solvents have evaporated. The overnight bacterial culture was centrifuged at 3 000 G for 10 min and the supernatant discarded. The bacterial pellet

was diluted with fresh sterile MH broth to give an optical density of approximately 0.8 at 600 nm. This was approximately 10 ml of MH broth per pellet. The TLC plate was sprayed with an even coat of bacterial suspension and incubated at 100% relative humidity at 38°C for 24 hr. A solution of a tetrazolium salt, p-iodonitrotetrazolium violet (INT), was then sprayed uniformly over the plate and incubated until red/pink develops where bacterial growth has occurred. Regions of no colour change indicate bacterial growth inhibition.

3.3. Results

3.3.1. TLC analysis

Alepidea amatymbica (Plate AP2 B and C)

The effect of one-year storage is quite different to that of 90-days, there are several more faint bands in the one-year-old material. These bands are hard to quantify exactly as they are not clear and are close to one another. The bands that reflect a vibrant red under UV $_{366}$ nm for the fresh leaf material of *A. amatymbica* do not appear to do so in the one-year old material. The root material showed a similar effect to 90-day storage when stored for one-year. Several bands that are visible in the fresh material (**Plate AP1.2 C**) under UV 254 nm are not visible in the stored material. Bands that are visible in the stored root materials under UV 366 nm are not as clearly defined in the fresh material. The TLC-fingerprints of the ethanolic extracts of fresh and one-year-old material in **Plate AP1.6** show a similar result to those in **Plate AP1.2 B**.

Eucomis autumnalis

From the TLC-fingerprints of the ethanolic fractions of fresh and one-year-old material of *Eucomis autumnalis* bulbs (**Plate AP1.6**) there appears to be little or no change in the chemical composition as a result of storage.

Helichrysum cymosum

Several bands visible in the fresh material TLC-fingerprint viewed under UV 366 nm are not visible in the one-year-old material. This indicates that changes in the chemical composition have occurred during one-years storage.

Leonotis leonurus

The TLC-fingerprints (**Plate1.4 B** and **C**) of the fresh and one-year-old material of *Leonotis leonurus* are similar and would suggest that little change in the chemical composition has occurred over this period. The five-year-old material's fingerprint is considerably different to those of the fresh and one-year-old material. The intense red bands at R_f 0.8 and R_f 0.6 in the fresh and one-year-old fingerprints viewed under UV $_{366}$ nm are very faint in the five-year-old material. **Plate AP 1.4 C,** which shows only the ethanolic extract fingerprints of fresh material and material stored for several different periods, illustrates well the changes in chemical composition with length of storage. The differences as a result of five-years storage are clear with the three bands between Rf 0.5 and Rf 0.7 being very faint in the five-year-old material but they have remained clear in the other material. The 90-day-old, 180-day-old and the one-year-old material have very similar TLC-fingerprints and only differ slightly from the fresh material.

Siphonochilus aethiopicus

From the TLC-fingerprints of the ethanolic fractions of fresh and one-year-old material of *Siphonochilus aethiopicus* rhizomes (**Plate AP1.7**) there appears to be little or no change in the chemical composition as a result of one year storage.

Vernonia colorata

The one-year-old material of *Vernonia colorata* produced a TLC-fingerprint considerably different to those of fresh, 90-day and 180-day-old material. The band indicated by the arrows (**Plates 1.5 B** and **C**) is very faint in the one-year-old material. The intensity of the red bands (R_f 0.5) is lost as a result of storage (**Plate 1.7**).

3.3.2. Cyclooxygenase (COX-1) bioassay

In both the *L. leonurus* and the *V. colorata* ethanol leaf extracts a small decrease in cyclooxygenase (COX-1) inhibition activity was observed (**Table 3.1**). It should be noted that the value obtained for the standard was also lower than that observed for the fresh and 90-day experiments. Only two repetitions for each experiment were

conducted and therefore it was not possible to determine if the differences were statistically significant.

Table 3.1: Percentage inhibition of prostaglandin synthesis by fresh and stored plant material extracts.

Plant species	Percentage inhibition		
	Fresh material	90-days	1 year
L. leonurus	100	100	95
V. colorata	100	100	92
STANDARD	64	64	55

3.3.3. Microtitre-plate minimum inhibitory concentration anti-bacterial bioassay

In the plant extracts tested no plants lost their antibacterial activity after either 90-days or one year of storage. In two instances (in bold in **Table 3.2**) *V. colorata* and *L. leonurus* against *E. coli* showed an increase in activity.

Table 3.2: Minimum inhibitory values for ethanol extracts of fresh and stored (90 days and one year) plant material tested against four bacteria.

Plant species	Minimum inhibitory values (mgml^{-1})											
	B. subtilis			*S. aureus*			*E. coli*			*K. pneumoniae*		
	Fresh	90 days	1 year	Fresh	90 days	1 year	Fresh	90 days	1 year	Fresh	90 days	1 year
A. amatymbica (leaf)	1.56	1.56	1.56	1.56	1.56	1.56	3.13	1.56	1.56	1.56	1.56	1.56
A. amatymbica (rhizome)	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
L. leonurus	1.56	0.78	0.78	1.56	0.78	0.78	**1.56**	**1.56**	**0.78**	1.56	0.78	0.78
V. colorata	0.78	0.78	0.78	1.56	1.56	1.56	**1.56**	**1.56**	**0.78**	0.78	0.78	0.78

The five-year-old *L. leonurus* material (**Table 3.3**) showed increased antibacterial activity against all bacteria except *K. pneumoniae* against which it appeared to have lost activity.

Table 3.3 Minimum inhibitory values for ethanol extracts of fresh and five-year-old *L. leonurus* leaf material tested against four bacteria.

Plant species	Minimum inhibitory values (mgml^{-1})							
	B. subtilis		*S. aureus*		*E. coli*		*K. pneumoniae*	
	Fresh	5 years	Fresh	5 years	Fresh	5 years	Fresh	5 years
L. leonurus	1.56	0.78	1.56	0.78	1.56	0.78	**1.56**	**3.13**

3.3.4. Bioautographic assay

Alepidea amatymbica

In the bio-autographic antibacterial plate (**Plate AP1.8 A**) it is clear to see that the anti-bacterial agents have changed as indicated by the arrows. This may explain the increase in the antibacterial activity of the stored extract that was observed in the micro-dilution assay (minimum inhibitory concentration changed from 3.1mg/ml to 0.8mg/ml). New compounds that were previously not present in the fresh extract have appeared in the extract that was stored for one year. The streaking that is observed is speculated to be due to the presence of fatty acids. These appear more abundant in the stored material.

Eucomis autumnalis

More streaking of the hexane extract is observed for the one-year-old material than the fresh (**Plate 1.9 A**). This resulted in a larger zone of bacterial growth inhibition. This may be caused by fatty acids or novel compounds formed as a result of storage. The reference plate viewed under UV $_{254}$ nm does not reveal and differences between the fresh and stored material that would cause the larger zone of bacterial growth inhibition.

Helichrysum cymosum

The upper antibacterial compounds (a) visible in the bioautographic plate of fresh and one-year-old material extracts of *Helichrysum cymosum* leaves appear to be stable as they are in both the fresh and stored extracts. The lower compounds (b) however, are not present in the stored extract suggesting possible break-down during storage. The reference plate confirms that these compounds, indicated by the arrows, are not present in the stored extract.

Leonotis leonurus

The major antibacterial compounds (upper zone of bacterial growth inhibition) appear to be stable as they are found in the fresh, one-year-old and five-year-old material (**Plate 1.10 A**). A second group of antibacterial compounds are observed in the five-year-old material but not in the fresh or one-year-old material. In the reference plate (**Plate 1.10 B**) bands that correspond to these areas of bacterial growth inhibition are

present in all extracts but are faintest in the fresh and one-year-old material. This would suggest that the quantities of these compounds might be increasing as a result of storage.

Vernonia colorata

The zones of antibacterial growth inhibition in the extracts made from fresh material are similar to those of the one-year-old material. This would suggest that the antibacterial compounds present in *Vernonia colorata* leaf material are stable.

3.3.5. Summary of effect of long-term (one and five years) storage

In many cases the were leaf material was used the red bands that are seen in the TLC-fingerprints of fresh material are not as clear or as red in the TLC-fingerprints of the stored material. These bands also become less noticeable the longer the storage period is. This is likely to be a result of the breakdown of chlorophyll which would cause the loss in colour. This change in chlorophyll composition over time could provide as useful indicator to measure the age of leaf material, that is leaf material's age could be estimated based on its chlorophyll content.

Different situations have been observed for each plant. Most extracts have compounds that are not affected by storage such as all of those in *Eucomis autumnalis*, which remained unchanged. There are those that appear to change over time, such as the *Vernonia colorata* in which the fresh, 90-day and 180-day-old material were similar but compounds were lost after one year. *Leonotis leonurus* too remained unchanged when stored for up to one year, but the five-year-material was considerably different. The changes may be due to the loss of compounds presumably as a result of that compound being broken-down. There are cases where there appears to be changes in the quantity of certain constituents. This is speculation but if the same amount (0.5 mg) of extract were placed onto the TLC plate for each storage regime one would expect the bands to be similar, unless the ratios of the constituents are different.

Only *Leonotis leonurus* and *Vernonia colorata* showed any change in biological activity after one-year storage compared to that shown for plant material stored for

90-days. They both showed an increase in activity against *E. coli* from an MIC of 1.56 to 0.78 mgml^{-1}. Any activity gained as a result of storage for 90-days was retained over a one-year period of storage. The five-year-old *Leonotis leonurus* material, which differed considerably in chemical composition from the fresh and 90-day-old material loss activity against *K. pneumoniae* but retained activity against the other three bacteria.

3.4. Discussion and conclusions

The changes in chemical composition, based on TLC-fingerprints, as a result of long-term storage were more pronounced than those of short-term storage. These changes however were not manifested in the biological activity except in two cases. *Leonotis leonurus* and *Vernonia colorata* showed a change in biological activity after one-year storage compared to that shown of plant material stored for 90-days. Both showed an increase in activity against *E. coli* from an MIC of 1.56 to 0.78 mgml^{-1}. The biological activity (both antibacterial and cyclooxygenase inhibition) was retained over one-year period. Antibacterial activity against all bacterial tested except *K. pneumoniae* was retained over a five-year storage period. The biologically active compounds in *Alepidea amatymbica* appear to be stable.

It is interesting again that in most instances antibacterial activity and with respect to *Alepidea amatymbica, Leonotis leonurus* and *Vernonia colorata* anti-inflammatory activity is not lost as a result storage. It should be noted that these plants were stored at in the dark and at room temperature. These conditions are somewhat different to those of a street market were temperatures fluctuate and the material is often displayed in direct sunlight. The amount of light exposure plant material receives in 'muthi' a shop varies according to its proximity to shop windows and entrances. Generally these shops are dark with minimal electrical lighting and light entering the windows is often obstructed.

The results obtained from this experiment provide important information not only to traditional healers and potential cultivators, but also have conservational implications. If a plant retains its activity for one to five years after harvesting, then this plant need not be discarded and fresh material harvested. The effect of storage

is species specific, as one would expect because the chemical composition of each species differs from the next. Thus, no general assumption can be made with respect to recommended shelf-lives of plant material. Each species will have to be investigated and this will be a time consuming and expensive undertaking.

It is speculated that leaf material generally has a shorter shelf-life than bark, roots and other underground storage organs. It is assumed that due to the fact that bark, roots and other underground storage organs store assimilates and secondary metabolites that these compounds are relatively stable and these organs due to their structure (i.e. lower surface area to volume ratio) are better suited to protecting compounds from degradation than are leaves. The traditional healers interviewed were of a similar opinion and preferred to purchase only freshly harvested leaf material but were not as selective when buying bark, roots and other underground storage organs (MTOLO, MKHIZE, MSANGO and SMITH *pers com.*).

CHAPTER 4

EFFECT OF ACCELERATED AGEING ON THE CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *A. AMATYMBICA, L. LEONURUS* AND *V. COLORATA*

4.1. Introduction

The application of elevated temperature and humidity has been used in the food industry for some time to accelerate the ageing process. This has enabled shelf-life studies of food products to be speed up considerably. The same concept is utilised in this investigation to simulate long-term storage in only a few days. The aim of this investigation was to age plant material rapidly using elevated temperature and humidity and determine the effect of this ageing on the chemical composition and biological activity.

4.1.1. Accelerated ageing

The effect of temperature on the rate of decomposition may be described by an empirical equation proposed by Arrhenius (in FLORENCE and ATTWOOD, 1988):

$$k = Ae^{-Ea/RT} \qquad\qquad \text{(equation 1)}$$

$$\text{or} \quad \log k = \log A - \frac{E_a}{2.303RT} \qquad\qquad \text{(equation 2)}$$

The pre-exponential factor A is often referred to as the frequency factor. E_a is the activation energy and is the energy, which must be exceeded if the collision of two reactant molecules is to lead to reaction. According to the later equation (equation 2) a plot of $\log k$ as a function of reciprocal temperature should be linear with a slope of $-E_a/2.303R$ from which E_a may be calculated.

Temperature increase often causes an appreciable increase of the decomposition rate. For many reactions the increase in the rate (k) is of the order of two to three times for a 10°C rise in temperature (Q_{10} values). This means that if the storage temperature is increased from 25°C to 55°C an increase in the rate of decomposition of eight to 27-fold can be expected. The above equations can be used effectively to calculate expiry dates of known compounds such as pharmaceuticals, but are of no use in complex, multi-components systems such as plant material. As mentioned earlier there are just to many additional factors determining the rate of chemical decomposition such as chemical interactions and the heterogeneous nature of plant structure. Moisture is known to significantly affect the kinetics of decomposition, KORNBLUM and SCIARRONE (1964) showed that added moisture decreased the lag time and increased the zero-order rate constant for the decomposition of aminosalicylic acid. A zero-order reaction is where decomposition proceeds at a constant rate and is independent of the concentration of any of the reactants. Many decomposition reactions in the solid phase (not in solution), or suspensions apparently follow zero-order kinetics (FLORENCE and ATTWOOD, 1988). FLORENCE and ATTWOOD (1988) presented several cases were there is a linear relationship between log k (logarithm of rate of decomposition) and water vapour pressure.

It is obvious from these findings that it is important to store plant material under cool dry conditions where possible if undesirable effects of ageing are to be avoided. However, for the purposes of studying the effects of ageing elevated temperatures and humidity provide the opportunity to rapidly age material thus saving time.

4.2. Selection of plants to be investigated

Plants collected were initial chosen according to their popularity as a traditional medicine according to MANDER (1997) and then three of these plants, which have shown substantial changes as a result of short-term storage in the pervious Chapters, were selected for accelerated ageing studies. *Alepidea amatymbica, Leonotis leonurus* and *Vernonia colorata* were selected as these plants not only were affected by short-term storage but also are easily obtainable. Plant material was collected from two locations (**Table 2.2**) and voucher specimens were deposited

at the Herbarium, School of Botany and Zoology, University of Natal, Pietermaritzburg.

4.3. Materials and methods

4.3.1. Plant material

Immediately after harvesting the plant material was cleaned and approximately 500 g of material was dried in an oven at 50°C. Twenty five g was used of initial accelerated ageing studies and the remainder was stored at -15°C for bioassay guided fractionation of aged material.

4.3.2. Accelerated ageing method

Once the plant material was dried and ground to a fine powder in a miniature mill 25 g was placed into a weighing boat. The plant material was then stored at 55°C and 100% humidity. This was achieved by placing the plant material in an airtight 5 l plastic jar with 100 ml of distilled water. The jar was laid on its side and the plant material in the weighing boat is suspended above the water by an Eppendorf rack. The jar and its contents were placed in an oven set at 55°C.

Initially plant material was removed at five-day intervals for 25 days and ethanol extracts as described in the next Section were made. For the vacuum liquid chromatography extract the ground plant material was aged for seven days.

4.3.3. Preparation of extracts

Five g of plant material was removed from the oven at five-day intervals for 25 days and extracted with 100 ml ethanol in an ultrasound bath for 15 min. The extracts were filtered through Whatman No. 1 filter paper under vacuum and air-dried. The residues were dissolved in their initial solvent resulting in a 100 mg residue/ml solvent extract. Air dried residues were stored at -15°C to limit further chemical decomposition prior to redissolving which only took place just before the extract was required for a bioassay.

4.3.4. TLC fingerprinting and bioassays performed

The TLC-fingerprints, the microtitre serial dilution MIC antibacterial bioassay and the cyclooxygenase assay were conducted as in the pervious chapter (CHAPTER 2, SECTIONS 2.2.3., 2.2.5. and 2.2.6. respectively). TLC fingerprints were performed on fresh and aged leaf material from *L. leonurus* and *V. colorata*. All plates were run in a 2:1 hexane:ethyl acetate (v/v) system.

4.3.5. Bioassay-guided fractionation for the isolation of antibacterial compounds

Bioassay-guided fractionation using vacuum liquid chromatography (VLC) and antibacterial bioautographic techniques was performed on ethanol extracts fresh and accelerated aged leaf material of *L. leonurus* and *V. colorata*.

4.3.5.1. Vacuum liquid chromatography

Leonotis leonurus

The ethanolic extract of fresh dried material (147.2 g which yielded 9.70 g crude extract) and aged dried material (99.4 g which yielded 9.57 g crude extract) at 55°C and 100% humidity for seven days was separated by VLC over silica gel (Merck 230-400 mesh) using a hexane:ethyl acetate gradient solvent system of increasing polarity. A large column (40 cm in length and 5 cm in diameter) was packed with 150 g silica. The solvent gradient system used was hexane:ethyl acetate which increased by 5% increments from 85% hexane to 60% hexane. Subsequently the ratio increased by 10% increments to 100% ethyl acetate. Two methanol washes (400 ml methanol) were included to recover any remaining extract. At each concentration 400 ml solvent was flushed under vacuum through the column. The bioautographic antibacterial assay was used to determine the active fractions.

Vernonia colorata

The ethanolic extract of fresh (97.5 g which yielded 9.63 g crude extract) and material aged (101.2 g which yielded 9.56 g crude extract) at 55°C and 100% humidity for seven days was separated by VLC over silica gel (Merck 230-400 mesh) using a

hexane:ethyl acetate gradient solvent system of increasing polarity. A large column (40 cm in length and 5 cm in diameter) was packed with 150 g silica. The solvent gradient system used was hexane:ethyl acetate which increased by 5% increments from 100% hexane to 60% hexane. Subsequently the ratio increased by 10% increments to 100% ethyl acetate. Two methanol washes (400 ml methanol) were included to recover any remaining extract. At each concentration 400 ml solvent was flushed under vacuum through the column. The bioautographic antibacterial assay was used to determine the active fractions.

4.4. Results

4.4.1. TLC analysis

Leonotis leonurus

The effect of accelerated ageing on the chemical composition of ethanolic extracts of aged *Leonotis leonurus* leaf material is clearly evident after just five days of ageing. The TLC-fingerprints (**Plate AP1.12 A**) show that four bands present in the fresh material are either not present or are not as bold after five days accelerated ageing. In the fingerprint corresponding to material aged for five days there are two bands that do not appear in either the fresh material or the material aged for longer periods.

Vernonia colorata

The TLC-fingerprints (**Plate AP1.12 B**) show that three bands present in the fresh material are either not present or are not as bold after five days accelerated ageing. Unlike *Leonotis leonurus,* no bands, which were present in the aged material and not in the fresh material, were observed.

4.4.2. Cyclooxygenase (COX-1) bioassay

Alepidea amatymbica

The rhizome material showed no significant change in cyclooxygenase inhibition for ethanolic extracts of fresh and aged material for up to 20 days (**Figure 4.1**). Material aged at 55°C and 100% humidity for 25 days appear to lose cyclooxygenase inhibition activity dropping from 94% inhibition to 77%. Whether there is a statistically significant difference cannot be determined as only two replicates were performed.
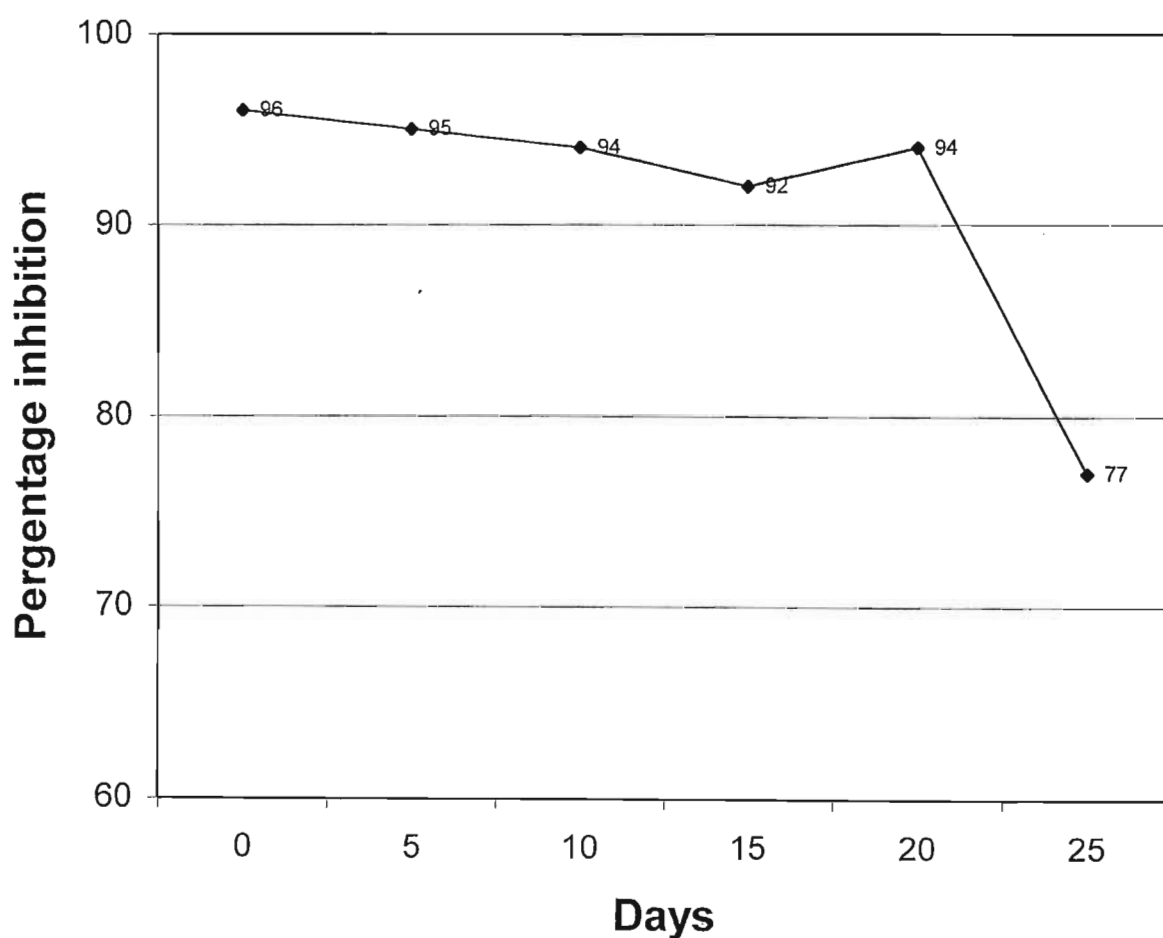
Figure 4.1: Percentage prostaglandin synthesis inhibition (COX-1 bioassay) for an ethanol extract of *A. amatymbica* rhizome after five-day periods of accelerated ageing (55°C and 100% humidity).

The compounds responsible for the cyclooxygenase inhibition in the ethanolic extracts of *Alepidea amatymbica* leaf material appear to be stable and were not affected by the conditions of the accelerated ageing procedure (**Figure 4.2**).
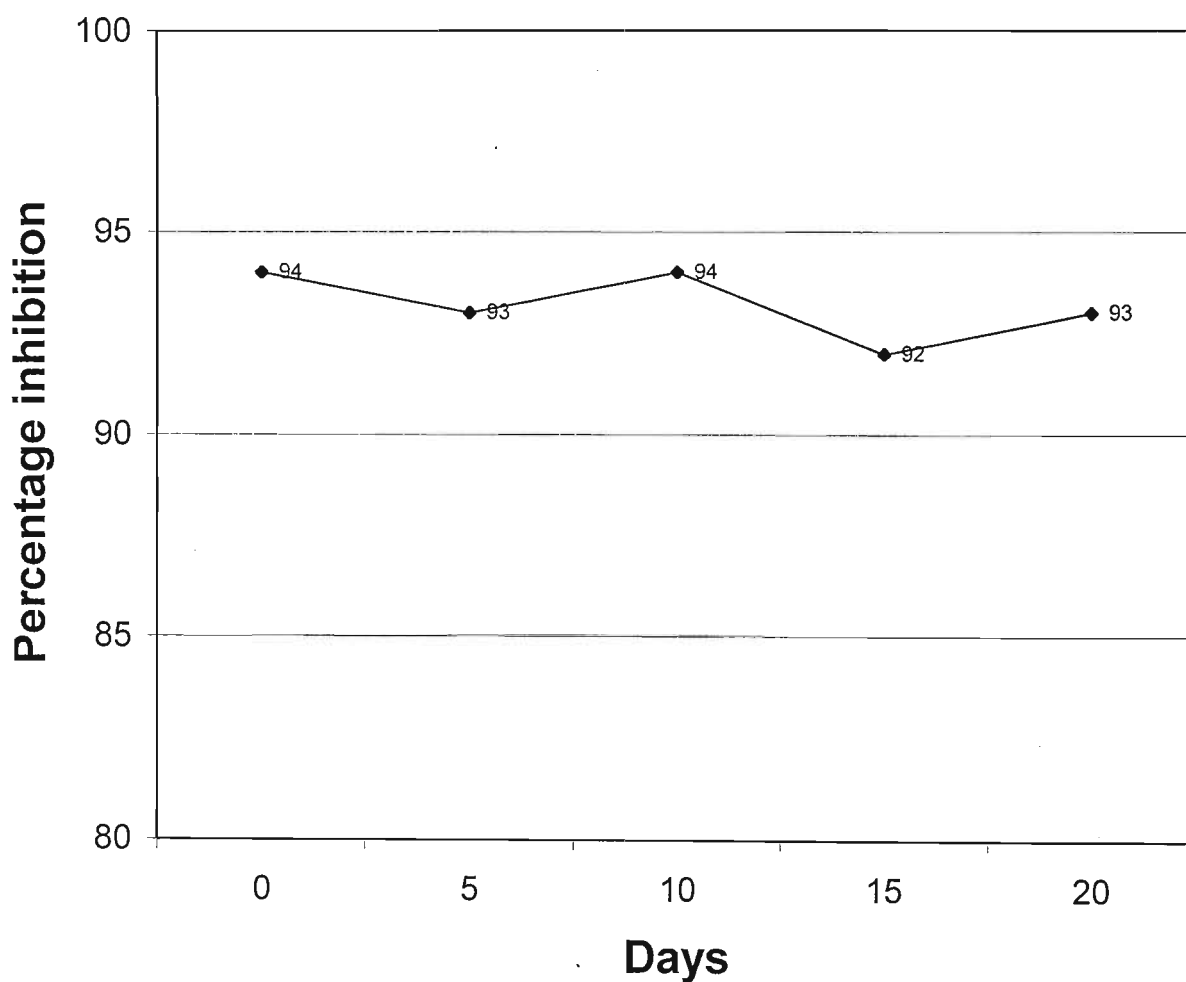
Figure 4.2: Percentage prostaglandin synthesis inhibition (COX-1 bioassay) for an ethanol extract of *A. amatymbica* leaf after five-day periods of accelerated ageing (55°C and 100% humidity).

Leonotis leonurus

The compounds responsible for the cyclooxygenase inhibition in the ethanolic extracts of *Leonotis leonurus* leaf material appear to be stable and were not affected by 20 days under conditions of the accelerated ageing procedure (**Figure 4.3**). However after 25 days of accelerated ageing the cyclooxygenase inhibition activity dropped from 96% to 85%. Again whether there is a statistically significant difference cannot be determined as only two replicates were performed.
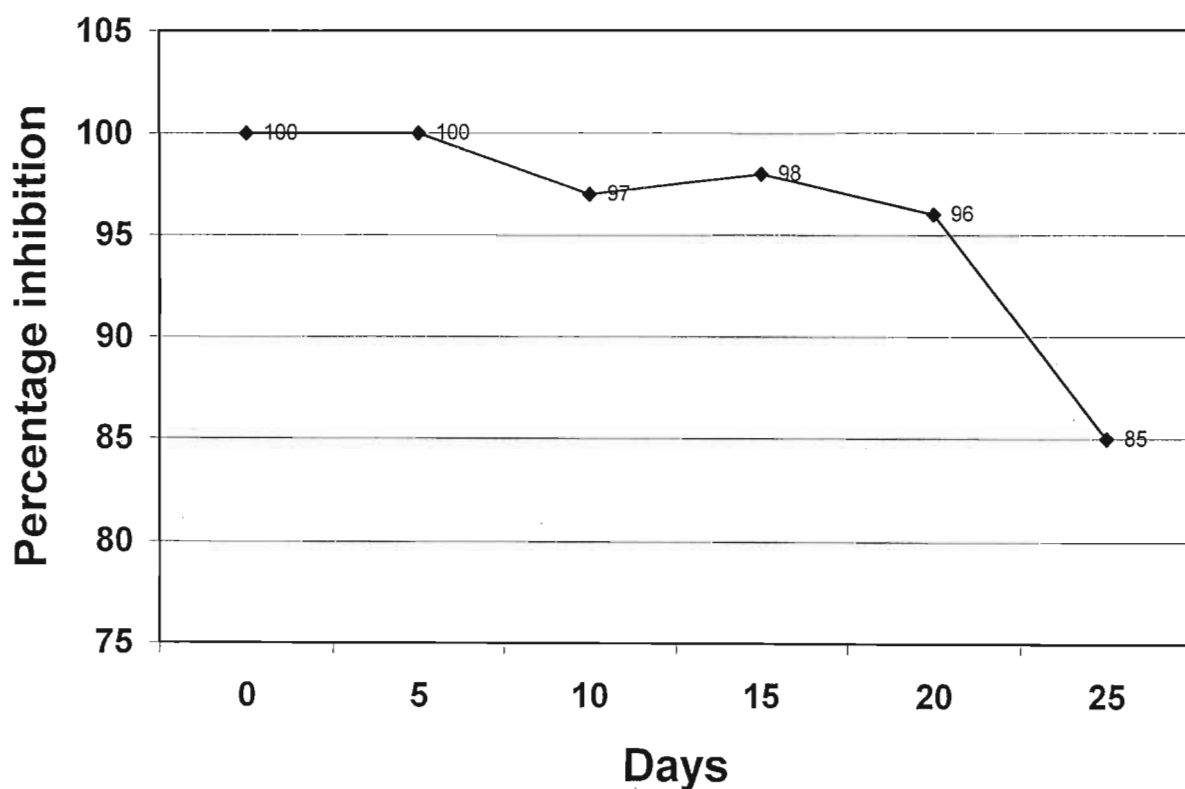
Figure 4.3: Percentage prostaglandin synthesis inhibition (COX-1 bioassay) for an ethanol extract of *L. leonurus* leaf after five-day periods of accelerated ageing (55°C and 100% humidity).

Vernonia colorata

Interestingly, unlike *Alepidea amatymbica* and *Leonotis leonurus* the ethanolic extracts of aged material of *Vernonia colorata* showed improved cyclooxygenase inhibition activity as a result of the accelerated ageing procedure, notably after 15 days or more (**Figure 4.4**).

Whether there is a statistically significant change in activity cannot be determined as only two replicates were performed. The changing in activity is relatively small with an increase in activity from 90% in the fresh material to 98% in material aged for 15 days or longer.
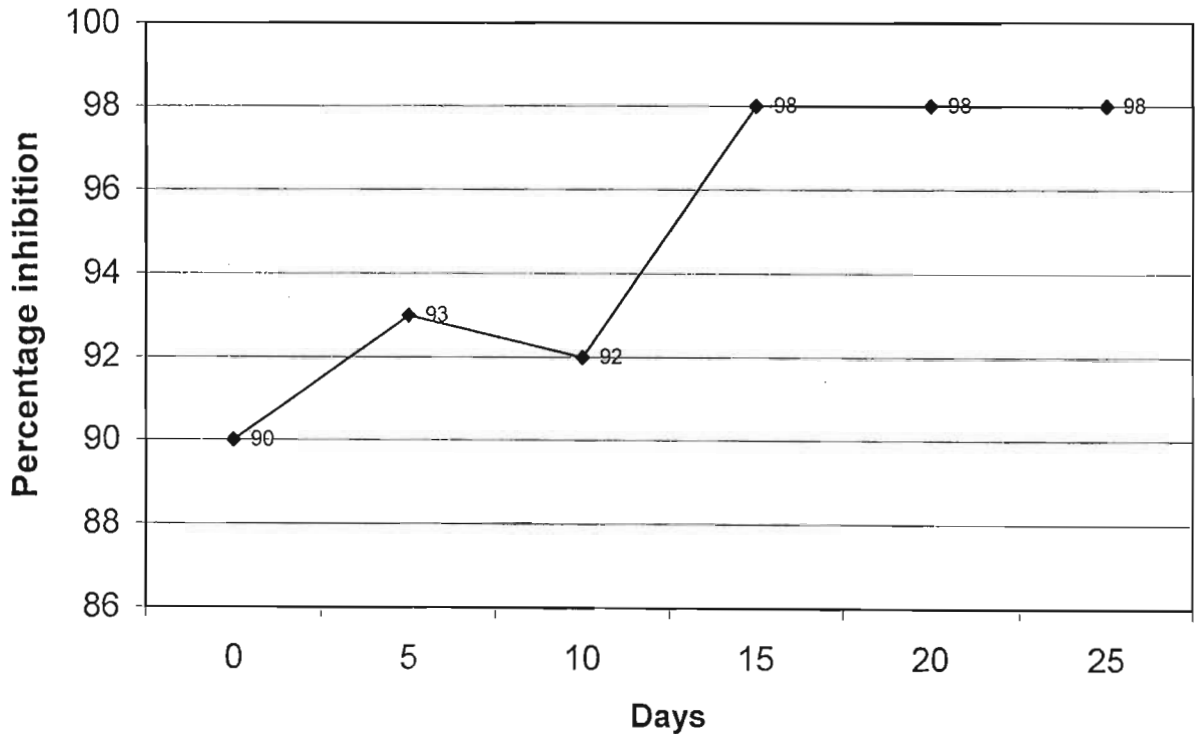
Figure 4.4: Percentage prostaglandin synthesis inhibition (COX-1 bioassay) for an ethanol extract of *V. colorata* leaf after five-day periods of accelerated ageing (55°C and 100% humidity).

4.4.3. Microtitre-plate minimum inhibitory concentration anti-bacterial bioassay

The minimum inhibitory concentrations (mgml^{-1}) against *Staphylococcus aureus* of fresh and aged ethanolic extracts of *Alepidea amatymbica* (leaf and rhizome material), *Leonotis leonurus* (leaf material) and *Vernonia colorata* (leaf material) are presented in **Table 4.1**. The lower the minimum inhibitory concentrations the higher the antibacterial activity exhibited by the extract.

Alepidea amatymbica

The compounds responsible for the antibacterial activity in the ethanolic leaf extracts of *Alepidea amatymbica* appear to be stable after 10 days ageing under 100% humidity at 55°C. After 15 days ageing activity is lost with a doubling in the minimum inhibitory concentration.

The ethanolic rhizome extracts of fresh and aged material of *Alepidea amatymbica* showed similar minimum inhibitory concentrations. This suggests that the compounds responsible for the antibacterial activity are stable under the ageing conditions.

Leonotis leonurus

Leonotis leonurus showed an increase in antibacterial activity as a result of five days ageing. This increase in activity was maintained after ten days ageing but was lost after 15 days ageing. The antibacterial activity of the material aged for 15 days or longer was less than that of the fresh material.

Vernonia colorata

Similar to *Leonotis leonurus*, *Vernonia colorata* showed an increase in antibacterial activity as a result of five days ageing but unlike *Leonotis leonurus* this improved activity was retained for all longer periods of ageing.

Table 4.1: Minimum inhibitory values for ethanol extracts of fresh and accelerated aged plant material of 5, 10, 15, 20 and 25 days tested against *Staphylococcus aureus*.

Plant species	Minimum inhibitory concentration (mgml^{-1})					
	Fresh	5 days	10 days	15 days	20 days	25 days
A. amatymbica (leaf)	1.56	1.56	1.56	3.13	3.13	Not enough material
A. amatymbica (rhizome)	1.56	1.56	1.56	1.56	1.56	1.56
L. leonurus	1.56	0.78	0.78	3.13	3.13	3.13
V. colorata	1.56	0.78	0.78	0.78	0.78	0.78

4.4.4. Bioassay guide fractionation of fresh and aged material of *Leonotis leonurus* and *Vernonia colorata*

4.4.4.1. Vacuum liquid chromatography

Vacuum liquid chromatography (VLC) of the ethanol extracts of fresh and aged material of *Leonotis leonurus* and *Vernonia colorata* resulted in good separation of compounds. Photographs of the TLC plates prepared from these fractions are shown in **Plates AP1.13** and **Plate AP1.14**. Also represented in these plates are the bioautographic antibacterial plates of the VLC fractions.

Leonotis leonurus

Table 4.2 (fresh material) and **Table 4.3** (aged material) show the mass and percentage of each fraction obtained from the VLC of *Leonotis leonurus*. The fractions which showed antibacterial activity are those in bold text.

Table 4.2: Solvent gradient and mass of each fraction obtained from VLC of **ethanolic extract of fresh *Leonotis leonurus* leaf material.** Bold fractions indicate antibacterial activity as revealed by bioautographic bioassay.

Fraction	Hexane (ml)	Ethyl actate (ml)	Mass of dried fraction (g)	Percentage of total mass of extract
A	400 (100%)	0	0.104	1.0
B	**340 (85%)**	**60**	**0.649**	**6.0**
C	320 (80%)	80	1.067	9.9
D	300 (75%)	100	2.672	24.8
E	280 (70%)	120	1.495	13.9
F	260 (65%)	140	0.423	3.9
G	240 (60%)	160	0.528	4.9
H	200 (50%)	200	1.731	16.1
I	**160 (40%)**	**240**	**0.941**	**8.7**
J	120 (30%)	280	0.501	4.7
K	80 (20%)	320	0.362	3.4
L	40 (10%)	360	0.09	0.8
M	0 (0%)	400	0.073	0.7
N	Methanol wash		0.079	0.7
O			0.44	0.4

Table 4.3: Solvent gradient and mass of each fraction obtained from VLC of ethanolic extract of aged *Leonotis leonurus* leaf material. Bold fractions indicate antibacterial activity as revealed by bioautographic bioassay.

Fraction	Hexane (ml)	Ethyl actate (ml)	Mass of dried fraction (g)	Percentage of total mass of extract
A	400 (100%)	0	0.033	0.5
B	340 (85%)	60	0.605	8.5
C	**320 (80%)**	**80**	**1.097**	**15.5**
D	300 (75%)	100	0.942	13.3
E	280 (70%)	120	1.082	15.3
F	260 (65%)	140	0.321	4.5
G	240 (60%)	160	0.367	5.2
H	200 (50%)	200	0.486	6.9
I	160 (40%)	240	0.86	12.1
J	120 (30%)	280	0.651	9.2
K	80 (20%)	320	0.199	2.8
L	40 (10%)	360	0.183	2.6
M	0 (0%)	400	0.062	0.9
N	Methanol wash		0.115	1.6
O			0.085	1.2

TLC plates of fractions from a VLC separation of ethanolic fractions of fresh and accelerated aged *Leonotis leonurus* leaf material are shown in **Plate AP1.13**. Plates shown are viewed under UV $_{254}$ nm (A) and UV $_{366}$ nm (B). The result of bioautographic antibacterial (*S. aureus*) assay is also shown (C). The antibacterial compounds (a and b) in the fresh and aged material appear to be similar. The other antibacterial compounds however, appear to be different with more defined groups in the aged material.

Vernonia colorata

Table 4.4 (fresh material) and **Table 4.5** (aged material) show the mass and percentage of each fraction obtained from the VLC of *Vernonia colorata*. The fractions that showed antibacterial activity are those in bold text.

Table 4.4: **Solvent gradient and mass of each fraction obtained from VLC of ethanolic extract of fresh *Vernonia colorata* leaf material.** Bold fractions indicate antibacterial activity as revealed by bioautographic bioassay.

Fraction	Hexane (ml)	Ethyl actate (ml)	Mass of dried fraction (g)	Percentage of total mass of extract
A	400 (100%)	0	0.083	1.1
B	340 (85%)	60	0.667	8.8
C	320 (80%)	80	0.383	5.1
D	300 (75%)	100	0.193	2.6
E	**280 (70%)**	**120**	**0.104**	**1.4**
F	**260 (65%)**	**140**	**0.097**	**1.3**
G	**240 (60%)**	**160**	**0.081**	**1.1**
H	**200 (50%)**	**200**	**0.101**	**1.3**
I	**160 (40%)**	**240**	**0.56**	**7.4**
J	**120 (30%)**	**280**	**1.556**	**20.6**
K	**80 (20%)**	**320**	**0.442**	**5.9**
L	**40 (10%)**	**360**	**0.815**	**10.8**
M	**0 (0%)**	**400**	**0.149**	**2.0**
N	Methanol wash		1.416	18.8
O			0.897	11.9

Table 4.5: Solvent gradient and mass of each fraction obtained from VLC of ethanolic extract of aged *Vernonia colorata* leaf material. Bold fractions indicate antibacterial activity as revealed by bioautographic bioassay.

Fraction	Hexane (ml)	Ethyl actate (ml)	Mass of dried fraction (g)	Percentage of total mass of extract
A	400 (100%)	0	0.018	0.1
B	340 (85%)	60	0.961	7.0
C	320 (80%)	80	0.353	2.6
D	**300 (75%)**	**100**	**0.358**	**2.6**
E	**280 (70%)**	**120**	**0.221**	**1.6**
F	**260 (65%)**	**140**	**0.135**	**1.0**
G	**240 (60%)**	**160**	**0.112**	**0.8**
H	**200 (50%)**	**200**	**0.3**	**2.2**
I	**160 (40%)**	**240**	**0.459**	**3.3**
J	**120 (30%)**	**280**	**0.317**	**2.3**
K	**80 (20%)**	**320**	**0.186**	**1.4**
L	**40 (10%)**	**360**	**0.251**	**1.8**
M	**0 (0%)**	**400**	**0.196**	**1.4**
N	Methanol wash		1.522	11.1
O			1.416	10.3

TLC plates of fractions from a VLC separation of ethanolic fractions of fresh and accelerated aged *Vernonia colorata* leaf material are shown in **Plate AP1.14**. The plate (A) shown has been treated with anisaldehyde/sulphuric acid spray reagent. The result of bioautographic antibacterial (*S. aureus*) assay is also shown (B). The antibacterial compounds (a and b) in the fresh and aged material appear to be similar. Several new antibacterial compounds (c), which show the distinctive streaking nature of fatty acids, appear in the aged material but not in the fresh. These compounds also stain a blue colour with anisaldehyde/sulphuric acid spray reagent.

4.5. Discussion

4.5.1. Fatty acids

Several of the TLC-fingerprints of the stored and aged material in which novel antibacterial compounds have appeared, such as *Alepidea amatymbica* (**Plate AP1.8**), *Eucomis autumnalis* (**Plate AP1.9 A**), *Helichysum cymosum* (**Plate AP1.9 B**) and *Vernonia colorata* (**Plate AP1.13**), has lead to speculation that these compounds may be fatty acids. The antimicrobial effects of fatty acids and their derivatives have been known for many years (KNAPP and MELLY, 1986). Fatty acids can inhibit the growth of numerous types of bacteria, as well as protozoa, viruses and fungi (NIEMAN, 1954; KNAPP and MELLY, 1986). The discovery of fatty acids as the primary antibacterial agents in plant material has often been meet with disappointment, as researchers would have far rather discovered a novel compound.

4.5.1.1. Occurrence of fatty acids in plants and their biological activity

Lipids, the bound form of fatty acids, comprise up to 7% of the dry weight in leaves in higher plants. They are esterified to glycerol and are important membrane constituents in chloroplasts and mitochondria (HARBORNE and BAXTER, 1993). Plant seeds contain a wide range of fatty acids, which provide a storage form of energy to use during germination, whereas those from leaf material are remarkably constant from species to species (HARWOOD, 1980). Quantitatively, the major fatty acids are palmitic, linoleic and in particular α-linolenic acids (HARWOOD, 1980).

Fatty acids do not generally exist as free carboxylic acids because of their affinity for many proteins (GURR and JAMES, 1980). Due to their affinity for proteins one would expect fatty acids to inhibit enzyme activity and this has been reported (GURR and JAMES, 1980). The antimicrobial effects of fatty acids and their derivatives has been recognized for several years, and there have been various proposals as to the mechanism of their action (NIEMAN, 1954; KNAPP and MELLY, 1986). Fatty acid sensitivity is thought to generally be a characteristic of Gram-positive bacteria with only a few Gram-negative bacterial species being affected (KNAPP and MELLY, 1986). There exists a close relationship between the structure of fatty acids and their

antimicrobial activity. Yeasts are affected to a larger extent by fatty acids containing 10-12 carbons, while slightly longer chain lengths mostly affect Gram-positive bacteria. Gram-negative bacteria are in most cases affected by very short chain fatty acids, that is C_6 or less (KABARA, 1980).

4.5.1.2. Effect of storage on plant tissue fatty acid composition

Although the new compounds that arise as a result of storage and ageing have not been identified yet they are suspected to be fatty acids due to their nature and appearance on the TLC plate. This is not entirely surprising as plant material is rich in lipids (7% of the dry weight in leaves in higher plants) and they contain lipases which can break these lipids down to produce fatty acids. Lipid biochemists are well aware of the high activities of lipolytic enzymes in many plants (GALLIARD, 1980). Extraction and analysis of lipids from plant material are frequently severely affected by the presence of endogenous lipolytic activity (GALLIARD, 1980). Our knowledge of lipolytic enzymes in plants is meagre. This is surprising considering the commercial importance of seed oils and other plant lipids. In addition to the obvious importance of lipid metabolism in oil seed crops, the involvement of storage and membrane lipid metabolism in plant biochemistry and its applications should be backed by a better understanding of lipid catabolism. The surface lipids of all plants have almost totally been neglected in this respect except for work by KOLATTUKUDY (1980). Very little is known about the catabolic aspects of lipid turnover in plants. Most of our knowledge arises from studies on materials where a net breakdown or loss of lipid occurs. Examples of such processes are germination of seeds utilizing storage lipids, senescence of plant tissues, and lipid degradation caused by cell damage through wounding or infection (GALLIARD, 1980). However, in most cases where lipolytic enzymes have been characterised, their physiological significance is not understood.

Lypolytic enzymes are usually considered hydrolyases using water as a co-substrate. This may explain the increased fatty acid production at higher humidity levels. Considerable confusion arises from ambiguous use of the term 'lipase'. It is loosely used to describe any enzyme activity causing the hydrolysis of lipids and long-chain

acyl esters. True lipases are enzymes that attack triacylglycerols and only act at an oil-water interface (GALLIARD, 1980).

Lipid acyl hydrolase in leaves

Since the proposed accumulation of fatty acids was observed in the stored and aged leaf material of *Helichysum cymosum* (**Plate AP1.9 B**) and *Vernonia colorata* (**Plate AP1.13**) the question arose; is this process know to occur naturally in leaves? The accumulation of free fatty acids and their inhibitory effects on chloroplasts have been ascribed to the hydrolysis of endogenous galactolipids (McCARTY and JAGENDORF, 1965; FRIEDLANDER and NEUMANN, 1968). Although this work is relatively old it does confirm that there are enzymes in leaves that are capable of breaking down lipids to produce free fatty acids. The harvesting and subsequent storage of plant material will allow the release of these lypolytic enzymes (as mention in Section1.4.2.) leading to the accumulation of fatty acids.

4.6.   Conclusion

Material was successfully aged rapidly using elevated temperature and humidity. A temperature of 55°C and 100% humidity achieved the desired effect. Perhaps a logical progression would be to test various temperature and humidity rations and determine how this effects the degradation of the plant material. Most medicinal plant material traded in traditional African markets are exposed to high temperatures and some areas such as coastal regions (e.g. Durban which has one of South Africa's largest markets) experience high humidity levels. This in itself should be cause enough for further investigations into the effects of temperature and humidity on stored medicinal plant material in southern and central Africa. As shown from these findings the effect of storage at elevated temperatures and humidity is not always negative. Some compounds in plant material are stable and others are not, but what is very interesting is that in some cases the activity improved. This leads to the question, can elevated temperature and humidity be used to 'cure' certain plant material to improve its desired activity as is done in the tobacco industry? This increase in activity may be the result of the action of endogenous lipases on the plants lipids resulting in the release of fatty acids, which are known to possess anti-microbial activities.

Another concern with storing material in an environment which experiences relatively high temperatures and humidity is whether this promotes growth of the existing microflora associated with the plant material (see Section 5.3.). Fortunately no visual signs of excessive growth were observed on the plant material investigated at 55°C and 100% humidity.

The effects of the elevated temperature and humidity observed were initially similar in the case of each plant to those observed due to short-term (90-days) and long-term (one-year) storage. This does support the idea that by increasing the temperature and relative humidity the storage environment one can in a much shorter time period achieve the same affect as storing plant material for long periods. The relationship between the magnitude of temperature and humidity elevation and the rate of plant material degradation is not known. It will be difficult or futile to attempt to estimate it based on Arrhenius's equations (see Section 4.1.1.) because these are based on pure compounds. The situation is different in plant material as this a complex system with many other physical and chemical interactions to consider. Thus, it is unlikely that accelerate ageing studies can be used to determine the shelf-life of herbal medicines but it can be used to determine if the active compounds are stable or not.

The effect of prolonged accelerated ageing of 15 days in *Leonotis leonurus* and 10 days in *Vernonia colorata* show a different trend to that observed in the stored material (i.e. the loss of the activity that was initially gained by five days ageing). Whether this is an artefact of the accelerated ageing process or if material was stored for longer periods a similar trend will be observed as in the accelerated ageing process. In support of the latter, that is prolonged accelerated (more than 15 days) ageing simulates prolonged storage (longer than one year) is that the *Leonotis leonurus* material that was stored for five years lost antibacterial activity against *K. pneumoniae*. It did however retained activity against the other three bacteria.

One trend that has now been made quite clear by these and the previous findings is that each plant behaves differently to storage, as one would expect because each plant contains different chemical constituents and these would have different stabilities. Unless a group of plants are known to contain similar compounds

responsible for their biological activity a general 'shelf-life' or prediction as to how the material will age can not be made. This would mean then that each plant has to be investigated individually and from these findings a 'shelf-life' for that plant be determined. The 'shelf-life' of North American herbal medicines, more recently referred to as dietary supplements, seems to be set arbitrarily (GRIGGS, MANANDHAR, TOWERS and TAYLOR, 2001). Batches of plant material are not individually tested for their stability, instead more general assessment is conducted and applied to several batches. The expiration dates for these batches are typically between 2 and 3 years (GRIGGS, MANANDHAR, TOWERS and TAYLOR, 2001), in contrast to allopathic medicines that are all labelled with more accurate expiration dates based on product composition. These are generally set at between 1-1 ½ years after manufacture.

The concept of attributing a shelf-life to a particular plant based on its ability to treat ailments after various periods of storage appears to be foreign to traditional healers in southern Africa. As mentioned in Chapter 1 traditional healers tend to assign 'shelf-life' arbitrarily to some of their plants. They do appear to be more concerned about freshness when dealing with leaf materials.

The testing of plants to determine the loss of biological activity over time will allow traditional healers and consumers to make informed decisions with regard to the use of stored material. The disposal of ineffective or potential unsafe plant material will contribute to the efficacy of traditional medicine. On the other hand plants with stable active ingredients can safely be stored for longer periods. This will benefit the traditional healer as he will not have to buy material as often and less material in theory will be harvested from the wild. This information will also be of great importance when considering medicinal plants as potential crops as discussed in the next chapter.

CHAPTER 5

CONCLUSIONS

5.1 Introduction

The use of plant material in traditional medicine is an important part of life to the majority of southern Africans, yet very little is known about the post-harvest physiology of these plants. Traditional healers (MKHIZE, MSANGO and SMITH *pers. com.*) are aware that certain plant materials (mostly leaf material) are best used fresh as they lose their potency over time, but healers seldom collect their own material and thus no longer know the age of the plant material they use. With the relatively recent introduction of gatherers, informal markets and 'muthi' shops the storage of plant material has become a necessity. Gatherers, street traders and shop owners exhibit little concern as to the storage conditions and period of their plant material. This is likely due to the lack of knowledge and understanding of the causes of plant product degradation and the lack of capital to upgrade storage conditions and facilities, such as adequate packaging and refrigerators. The consumers on the other hand have expressed in MANDER's (1997) report that they would prefer improved conditions.

The cultivation of medicinal plants in the near future is inevitable if the trade in several endangered or vulnerable species are to continue being traded (JÄGER and VAN STADEN, 2002). Thus, it is important that guidelines to aid potential growers be developed and this area is discussed in more detail in Chapter 6.

5.2. Effect of storage and accelerated ageing on medicinal plant material

The research presented thus far has investigated the effect of storage period in the dark at room temperature (± 20°C) on *Alepidea amatymbica* Eckl. & Zeyh., *Leonotis leonurus* (L.) R. Br., *Drimia robusta* Bak., *Vernonia colorata* (Willd.) Drake, *Scilla natalensis* Planch., *Eucomis autumnalis* (Mill.) Chitt. subsp. *autumnalis*, *Bowiea*

volubilis Harv. ex Hook. f., *Helichrysum cymosum* (L.) D. Don and *Siphonochilus aethiopicus* (Schweinf.) B. L. Burtt. The effect of accelerated ageing conditions of elevated temperature and relative humidity (55°C and 100% relative humidity) on plant material from *A. amatymbica*, *L. leonurus* and *V. colorata*. The effects of storage were assessed using TLC-fingerprinting to visualise changes in chemical composition and bioassays to monitor changes in biological activity.

All plants showed changes in chemical composition after only 90-days storage with the exception of *Eucomis autumnalis.* The most notable changes were in leaf material with the decomposition of chlorophyll in the stored and aged material.

The only plant to exhibit anthelmintic activity was *Alepidea amatymbica* aqueous root extracts, which showed no change in activity after 90-days storage. The effect of storage and accelerated ageing on antibacterial and cyclooxygenase (COX-1) inhibition activity varied according to species. Several species showed increased biological activity, this speculated to be due to the effect of increased levels of fatty acids as a result of lipid decomposition. Fatty acids are known to inhibit enzyme activity as they readily bind to proteins and exhibit antimicrobial activity.

Accelerated ageing provided a system whereby plant material can be rapidly aged making the study of the effects of storage on plant material easier and less time consuming. Analysis of the bioassay guided fractionation of fresh and aged material will hopefully provide confirmation of the role of fatty acid production in stored plant material.

This investigation has shown that the chemical composition of medicinal plant material once harvested is not static, but is dynamic and this often results in changes in the biological activity of this plant material. These changes are influenced by several intrinsic and extrinsic factors. These factors are numerous and summarized below:

- the nature of the plant;
- structure of the plant/plant part;
- plant's chemical composition;
- intracellular microbial contaminations;

- climate and humidity in particular;
- location/position of plant material from soil;
- harvesting methods;
- post-harvest treatment;
- packaging and storage conditions;
- preparation technique and physical state of the material (fresh or dried); and
- exogenous microbial contamination.

Although some of the roles of these factors in the changes in chemical composition of medicinal plant material have been investigated very few are well understood and thus much research in this area is still required. Two areas requiring urgent attention are the effects of photo-degradation and microbial contamination on plant material. The latter is discussed in more detail in Chapter 6.

5.3. Implications of findings for southern African traditional medicine

The findings obtained from this project indicate that no general storage measures for all plant material can be developed. It would be unwise to recommend that measures to reduce the temperature and relative humidity of the storage environment to moderate chemical decomposition, as does not always produce the desired affect. As shown in several plants chemical decomposition may result in increased biological activity. Whether a similar trend is observed as a result of varied UV light exposure and microbial load is not known. As mention already, SILVA, LEE and KINGHORN (1998) demonstrated that in some instances UV radiation may produce chemical reactions that give rise to compound artefacts.

Plant which have been shown to be either stable or increase in biological activity (summarized in **Table 5.1**) need not be discarded and can be stored for long periods. Those that have exhibited an increase in activity (**Table 5.1**) could be used more sparing as they mature as less older material will be required to obtain a similar response. It is important to mention here that in the assay used to determine changes in antibacterial activity was only repeated twice and therefore the significance of the differences (from a two-fold serial dilution) could not be determined. That is to say in some cases were there was an increase or decrease because the MIC doubled or halved due to one well being read or not read might not

be significant. A fourfold increase or decrease may be significant. Many more determinations (at least six) would be required to allow for statistical analysis. Perhaps an investigation in the amount of fresh and aged plant material required to produce a similar level of biological activity would have been useful here. The aqueous extracts of *D. robusta*, *H. cymosum* and *V. colorata* lost COX-1 inhibition activity after 90-days storage. The ethanolic extract of *D. robusta* also lost COX-1 inhibition activity after 90-days storage.

Table 5.1: Medicinal plants investigated which showed no change or an increase in antibacterial and COX-1 inhibition activity after storage.

Antibacterial activity		Anti-inflammatory activity (COX-1 inhibition)	
Stable	**Increased activity**	**Stable**	**Increased activity**
A. amatymbica leaf and rhizome (after one year)	*B. volubilis* (after 90 days)	*A. amatymbica* leaf (aged material)	*B. volubilis* (after 90 days)
	H. cymosum (after 90 days)	*E. autumanalis* (after 90 days)	*H. cymosum* ethanol extract only (after 90 days)
	L. leonurus (after five years)	*L. leonurus* (after one year)	*S. natalensis* (after 90 days)
	S. aethiopicus (after 90 days)	*S. aethiopicus* (after 90 days)	*V. colorata* (aged material)
	S. natalensis (after 90 days)		
	V. colorata (after one year)		

5.4. General conclusions

The results obtained from this investigation provide important information not only to traditional healers and consumers, but also in designing sustainable harvesting methods of these plants. For example, plants that can effectively be used in the treatment of microbial infection and as anti-inflammatories after many years of storage need not be discarded as readily as they are now. Thus these plants may not need to be harvested as frequently. With improved knowledge of the 'shelf-life' of plants used in traditional medicine better sustainable harvesting measures can be implemented.

Investigations that are conducted on herbarium plant specimens or any material that had been stored for several years may not yield an accurate reflection of the situation that occurs with respect to fresh material, or the material used by the healer or consumers. This investigation alluded to the fact that other post-harvest treatments of plant material used by scientist who work on medicinal plants may have an affect on the chemical composition and possibly the biological activity of the material. Thus the treatment, such as storage period and conditions should be noted and accompany any results published. Common practices such as drying and re-dissolving plant extracts, sonication, various filtering methods, application of heat for various reasons and the use of liquid nitrogen to grind plant material may all have adverse or beneficial effects on the chemical composition of the plant material and thus require careful consideration. The endogenous enzymes of the plant material should also be taken into account when working with plant material. Perhaps, these should be deactivated to prevent artefacts such as fatty acids being formed during storage and the preparation of extracts.

Much work remains if we are to completely understand the processes and effects of post-harvest plant physiology. It is hoped that this project has highlighted the importance of this knowledge to traditional healers and consumers as well as its role in the development of medicinal plant research and cultivation.

CHAPTER 6

DISCUSSION AND RECOMMENDATIONS FOR IMPLEMENTATION OF GOOD AGRICULTURAL PRACTICES (GAP) IN SOUTHERN AFRICA

6.1. Introduction

The exponential growth of the South African population in the latter half of the twentieth century has led to an almost exponential increase in the demand for medicinal plants used in local traditional medicine (CUNNINGHAM, 1993). Surveys conducted in the mid-nineteen nineties (MANDER, 1997) found that approximately 20 000 tonnes of plant materials were sold annually in South Africa's traditional medicinal plant trade. The majority of these plants are harvested from wild populations within South Africa and it neighbouring countries, with an insignificant amount being cultivated (*Agathosma betulina* and *Harpagophytum procumbens*). To add to this problem of overexploitation, a large proportion (±85%) of the plant material traded consists of non-renewable material, material which when harvested either severely damages the plant or kills it, such as bulbs, rhizomes and bark (MANDER, 1997). In some instances the entire plant is harvested. This material is harvested from the wild by gatherers who are extremely marginalized and thus, sustainable harvesting methods are not a priority for them. The short-term goal of meagre financial gain for the plants that they have harvested is far more important than the long-term benefit to all in conserving wild plant populations.

This excessive pressure on wild plant populations has resulted in many species becoming extremely rare and even nonexistent outside protected areas. Heavy exploitation of *Warburgia salutaris*, *Cassine transvaalensis*, *Alepidea amatymbica* and *Erythrophleum lasianthum* were recorded as early as 1938 (GERSTNER, 1941). It is clear that with the existing demand for medicinal plants sustainable harvesting is not feasible. If the future demand is to be met, it is essential that many of the popular species utilized in traditional medicine be developed for commercial cultivation.

There are two potential solutions, the production of plant materials by community nurseries and small-scale rural farmers and large-scale production by commercial farmers. However, before either the rural or commercial farms can venture into the production of southern African medicinal plants the cultivation, handling and processing protocols have to be developed. A major problem at present is the availability of seed stocks, harvesting from the wild will only enhance the strain on these populations. Simple, low-cost and reliable germination procedures will have to be established. Cultivation protocols with information pertaining to soil preference, water requirements, fertilizer regimes, light preferences and planting densities are also required by potential propagators. The expected time period from planting to harvest, expected yield and projected financial return would also be necessary to entice growers. Lastly, an important factor, which this project focuses on, is the storage requirements and 'shelf-life' of these plant materials. Thus understanding the post-harvest physiology of medicinal plants will benefit the current traders and consumers and will play an important role in the development of a more sustainable agricultural based future.

Until such necessary information is available, it would be unpractical to ask small-scale and commercial farmers to venture into such a 'high-risk' venture. Should this information be made available a more sound transition from conventional cash crops, such as sugarcane and maize, to more 'environmental friendly' crops with potentially higher returns can be advocated.

Much work has been done on the popular medicinal plants with respect to their micropropagation (APPELTON and VAN STADEN, 1995; McCARTAN and VAN STADEN, 1999; RABE and VAN STADEN, 1999; ZSCHOCKE and VAN STADEN, 2000) at the University of Natal. At present field trials on *Siphonochilus aethiopicus* (LIGHT pers. comm.; LIGHT, McGAW, RABE, SPARG, TAYLOR, ERASMUS, JÄGER and VAN STADEN, 2002) and *Scilla natalensis* (SPARG pers. comm.; SPARG, VAN STADEN, and JÄGER, 2002) are being conducted.

This project is the first to investigate the effects of storage on the post-harvest physiology of southern African medicinal plants and serves as an initial glance at the complexity of chemical changes and the subsequent changes in biological activity

during storage. These findings will have implications in the way traditional healers should handle and store various plant materials. It will also be relevant to the development and commercialisation of the medicinal plant trade in southern Africa.

6.2. Good Agricultural Practice (G.A.P.) of Medicinal and Aromatic Plants: lessons from Europe and their potential application in Africa.

6.2.1. Introduction

Storage regimes and standards are one part of the primary processing of medicinal plants. The guidelines for the Good Agricultural Practice of Medicinal and Aromatic (largely culinary) herbs is intended to apply to the growing and primary processing of all such plants traded and used in the European Union (FRANZ, 1989, SCHILCHER, 1989, TĒTĒNYI, 1989). Thus, it applies to the production of all plant materials used in the food, feed, medicinal, flavouring and perfume industries. It also applies to all methods of production including organic production in accordance with the European regulations. In southern Africa as mentioned in Chapter 1 there is little regulation and development of the medicinal plant trade despite its economic, conservational and cultural importance. If the production of South African traditional medicinal plants is to be commercialised similar guidelines need to be developed.

Growers involved in the production of herbs must ensure that they avoid damage to existing wildlife habitats, and that they make efforts to maintain and to enhance the biodiversity of their farms.

The present European Good Agricultural Practice Guidelines provide additional standards for the production and processing of raw materials insofar as they mainly focus on identifying those critical production steps (measures) that are needed to comply with good quality. A main aim is to ensure that the plant raw material meets the demands of the consumer and as such the standards of high quality. Especially important aspects are that they:

- are produced hygienically, in order to reduce microbiological load to a minimum; and

- are produced with care, so that the negative impacts affecting plants during cultivation, processing and storage can be limited.

As in the course of the production processed medicinal and aromatic plants and their products are exposed to a large number of both microbiological and other contaminants. The main aim of this Section is to, in light of the European Good Agricultural Practice Guidelines and current understanding of the medicinal plant trade in South Africa present guidelines for producers to reduce plant (raw material) contamination and decomposition (spoilage) to the greatest extent.

In Europe all participants of the production process (from primary producers to traders) are required to comply with these guidelines voluntarily and to elaborate practical measures in order to realize them. It would be useful to look at these guidelines and see if there are any that are applicable to medicinal plant trade in southern Africa. Although the situation in southern Africa is very different to that in Europe in that it is still very informal and underdeveloped it is necessary that some form of improvement, especially the commercialisation and cultivation of the plants, be implemented in the near future.

6.2.2. Principles and guidelines of the European system of Good Agricultural Practice (G.A.P.)

The World Health Organization (WHO), in the mid-eighties, initiated Good Manufacturing Practice (GMP) activities with the ultimate aim of ensuring the continuous high quality production of products for human consumption. Plant based products therefore must also comply with the unified standards (i.e. the 'EEC Guides to Good Manufacturing Practice for Medicinal Products). This has lead to the development of elaborate guidelines for the entire agricultural production process supplying raw materials to the plant-based pharmaceutical industry (round-table discussion at the 4th ISHS Symposium, in Angers, 1983) (PANK, FRANZ and HERBST, 1991). These guidelines are known as GAP and were initiated by three scientists, Franz (Austria), Schilcher (Germany) and Tétényi (Hungary). The results of more than a decade's research and debate are briefly presented in order to understand the current direction adopted in Europe.

Seeds and propagation material

Seeding materials are to be identified botanically, indicating plant variety, cultivar, chemotype and origin'. The material used should be 100 % traceable. The same applies to vegetatively propagated starting material. Starting material used in organic production has to be certified organic. Starting material should meet the requirements/standards concerning purity and germination (wherever available: certified seed/propagation material should be used). The starting material should be as free as possible of pests and diseases in order to guarantee healthy plant growth. When resistant or tolerant species or origins are available, they should be preferred. The occurrence of not species/variety-identical plants and parts of plants has to be controlled in the course of the entire production process (cultivation, harvest, drying, packaging). Such impurities would have to be eliminated promptly. Plant material or seeds derived from or comprising Genetically Modified Organisms (GMO) have to be in accordance with national and European regulations. In Africa very little is known about the genetic diversity of seed, as this will be obtained from wild populations. Much genetic and molecular research will have to be conducted to reach this objective.

Cultivation

Depending on the mode of cultivation e.g. conventional or organic, growers should be allowed to follow different standard operating procedures (SOP) for cultivation, these need to be developed for most African medicinal plants. Some cultivation procedures have been developed such as those for *Siphonochilus aethiopicus* (Natal Wild Ginger) (GORDON-GRAY, CUNNINGHAM and NICHOLS, 1989; CROUCH, LOTTER, KRYNAUW and POTTAS-BIRCHER, 2000). In general, care should be taken to avoid environmental disturbances. The principles of good crop husbandry must be followed including an appropriate rotation of crops.

Medicinal and aromatic plants cannot be grown in soils that are contaminated by sludge. Heavy metals, residues of plant protection products and other not naturally occurring chemicals should not contaminate soils. For this reason, minimum effective chemical input should be achieved. The manure applied should be void of human faeces and prior to application it should be thoroughly composted. All other fertilizing agents should be applied sparingly and in accordance with the demands of the plant

and the particular species (including application between harvests). The use of fertilizers should be in accordance with efforts to minimise leaching.

Irrigation should be minimized as much as possible and applied according to the needs of the plant. Irrigation-water should be in accordance with national quality standards and should be as free as possible of contaminants, such as faeces, heavy metals, pesticides, herbicides and toxicologically hazardous substances. Pesticide and herbicide application should be avoided as far as possible. When necessary they should be carried out using the minimum effective rates of approved plant protection products. Products for chemical plant protection have to conform to the European Union's maximum residue limits (EUROPEAN PHARMACOPOEIA, 2000). Application and storage of plant protection products has to be in accordance with the recommendations of manufacturers and the authorities.

Only qualified staff using approved equipment should carry out the application. Application should precede the harvest by a period either defined by the buyer or indicated by the producer of the plant protection product. The use of pesticides and herbicides has to be documented.

All measures regarding nutrient supply and chemical plant protection, should secure the marketability of the product. It is obligatory that the buyer of the batch be informed of the brand, quantity and date of pesticide use in a written form.

If plants are to be cultivated for the South African traditional market then several cultural aspects have to be taken into consideration. Conservative traditional healers believe that plants grown under western agricultural conditions (i.e. with fertilizers and in straight lines) will not have the same medicinal properties as those harvested from the wild. This is a 'supernatural' phenomenon and therefore scientific validation of the biological activity of cultivated plants will not sway traditional healers beliefs. However, the dwindling plant numbers coupled with the increased demand for these plants has resulted in large price increases and limited supplies of certain species. This has meant that most healers are willing to accept that cultivation is the only solution (MKHIZE, MSANGO and SMITH *pers com.*).

Harvesting

The harvest should take place when the plants are of the best possible quality according to the different utilizations. Work has been conducted on the seasonal variation in biological activity of several popular South African medical plants (TAYLOR and VAN STADEN, 2001; LIGHT, McGAW, RABE, SPARG, TAYLOR, ERASMUS, JÄGER, and VAN STADEN, 2002; TAYLOR and VAN STADEN, 2002). This information will prove very valuable in determining harvesting times. Harvest should preferably take place under the best possible conditions (wet soils, dew, rain or exceptionally high air humidity can be unfavourable as these conditions favour microbial growth). If harvest is performed under wet conditions, extra care should be taken in order to avoid the unfavourable influence of moisture. Certain South African species such as *Alepidea amatymbica* are traditionally harvested only in wet, misty, overcast conditions (SPRING *pers. com.*). Whether there is a valid scientific explanation for this is not known and should be investigated. It may be because the plant is not drought tolerant and should the plants be disturbed during harvesting those remaining will have an increased chance of survival under these conditions. A large number of plants are harvested traditionally only when there is a full moon. It would be interesting to determine if there is a link between lunar cycles and secondary metabolite accumulation.

Equipment should be both clean and technically in perfect working order. Those machine parts including their housings that come into direct contact with the harvested crop should be regularly cleaned and kept free of oil and other contamination.

Cutting devices of harvesters must be adjusted so that the collection of soil particles can be reduced to a minimum. In the course of harvest, care should be taken to ensure that no toxic weeds could mix with the harvested crop. Damaged and perished plant parts must be promptly eliminated.

All containers used in the harvest must be clean and must be kept free of the remnants of previous crops; containers out of use must also be preserved in a dry condition, free of pests and inaccessible to mice/rodents as well as livestock and domestic animals. The harvested crop should not be exposed to direct contact with

the soil. It must be promptly collected and under dry, clean conditions (e.g. sacks, baskets, trailers and containers, etc.) submitted to transport.

Mechanical damage and compacting of the crop that would result in undesirable quality changes must be avoided. In this respect, attention must be paid to:

- avoiding the overfilling of the sacks;
- the stacking up of sacks should not result in thickening of the crop; and
- the harvested crop should be transported and kept in containers or bags in such way that the occurrence of heating is prevented.

Delivery of freshly harvested plant material must occur as quickly as possible to the processing facility in order to prevent heating. The harvested crop must be protected from pests, mice/rodents and domestic animals. Pest control measures should be documented as part of GAP. KNEIFEL, CZECH and KOPP (2002), suggest that harvesting is a process that has a high microbial contamination risk. This is discussed in more detail in Section 6.3.

Primary processing

Primary processing includes steps of processing such as washing, freezing, distilling and drying. All these processes whether for food or medicinal use must conform to European and national regulations. None of these procedures other than sun-drying are implemented at present in South Africa due to the informal nature of the medicinal plant trade industry. The material that is found in informal markets and 'muthi' shops is seldom washed.

According to the guidelines for GAP, once arriving at the processing facility the harvested crop has to be promptly unloaded and unpacked. Prior to processing the material should not be exposed directly to the sun (except in case there is a specific need e.g. for distillation) and it must be protected from rainfall.

Buildings used in the processing of harvested crops must be clean, as well as thoroughly aerated and must never be used for housing livestock. Buildings must be constructed so as to provide protection for the harvested crop against birds, insects, rodents as well as domestic animals. In all storage (including packaging stores) and processing areas suitable pest control measures such as baits and electric insect

killing machines must be operated and maintained by professionally qualified staff or contractors. Processing equipment must be maintained clean and must be regularly serviced.

In the case of natural open air-drying, the crop must be spread out in a thin layer. In order to secure unlimited air circulation, the drying frames must be located at a sufficient distance from the ground. Attempts must be made to achieve uniform drying of the crop and as a consequence to avoid mould formation. Microbial contamination of medicinal plant material is dealt with in more detail in Section 5.3. When drying with oil, the exhaust fumes should not be reused for drying. Direct drying should not be allowed except with butane, propane, or natural gas. Most plant material that is dried for the traditional plant markets in South Africa is dried by being placed on either newspaper or plastic sheets on the ground. This is not ideal as the material will not dry uniformly and thus fungal growth often occurs. This has been observed on plant material in 'muthi' shops and the traditional healers did not seam too concerned about purchasing material with fungal growth on it (MKHIZE, MSANGO and SMITH *pers com.*). Drying directly on the ground or under direct exposure to the sunlight should be avoided unless it is required for a particular plant. All material must be inspected or sieved in order to eliminate sub-standard products and foreign bodies. Sieves must be maintained in a clean state and should be serviced regularly. Clearly marked waste-bins should be kept ready, emptied daily and cleaned. In order to protect it and to reduce the risk of pest attacks, the product should be promptly packaged.

Except in the case of natural open air-drying, the conditions (e.g. temperature, duration, etc.) must be selected taking into consideration the type (e.g. root, leaf or flower) and active substance content (e.g. essential oils and others) of the crude drug to be produced. Drying conditions should be documented.

Packaging

After the repeated control and eventual elimination of low-quality materials and foreign bodies, the product should be packaged in clean and dry, preferably new sacks, bags or cases. The label must be clear, permanently fixed and made from

non-toxic material. Information must conform to the European and national labelling regulations.

Packaging materials should be stored in a clean and dry place that has to be free of pests and inaccessible for livestock and domestic animals. It must be guaranteed that no contamination of the product takes place by the use of packaging material, particularly in the case of fibre bags. Reusable packaging materials should be well cleaned and perfectly dried prior to their usage. It must be guaranteed that no contamination takes place by reusing bags.

MANDER (1997) found that 84% of the black clinic patients he interviewed (n=100) indicated that they would prefer more hygienically packaged indigenous medicines, with most consumers indicating that they would prefer more modernised and hygienic trading sites.

Storage and Transport

Packaged dried materials and essential oils should be stored in a dry, well aerated building, in which the daily temperature fluctuations are limited and good aeration is given. Fresh products should be stored between 1°C and 5°C while frozen products should be stored below -18°C (or below -20°C for longer term storage). Essential oil storage must conform to the appropriate chemical storage standards.

As a protection against pests, birds, rodents and domestic animals, the window and door openings are to be protected, e.g. by wire netting. It is recommended that the packaged dry crop will be stored:

- in buildings with concrete or similar easy to clean floors;
- on pallets;
- with a sufficient distance to the wall; and
- thoroughly separated from other crops to avoid cross-contamination.

Organic products must be stored separately.

Traditional gathers transport the harvested material from the site of harvest to their homesteads on foot in sacks. Several harvesting trips are made before sufficient material has been collected to warrant a trip to the market. Material is generally

transported to the markets by public transport, either in the vehicle or in sacks tied to the roof (MANDER, 1997).

In South Africa raw materials for trading, as opposed to those for retail, such as whole plants, plant parts and chopped material, are stored in recycled plastic woven sacks (50 kg capacity) (MANDER, 1997). This form of storage is most common and is used in all forms of the marketing channels. These sacks are generally stored in a dry place, preferably in the shade, to prevent the decomposition of the plant material. Shops and healers consulting rooms usually have an area dedicated to storing large numbers of these bags. Street traders store their bagged goods under plastic sheets in the street. There has been mention of some form of refrigerated storage being installed in the larger markets in Durban but this has not materialised. In some towns, street traders have managed to obtain a shipping container, which is used to store several traders' sacks overnight (MANDER, 1997).

Retail materials for sale directly to the consumers are stored in a variety of containers depending on the type of outlet. Street market traders display their smaller or partially processed (chopped) material either on plastic sheets or newspaper on the pavement or in plastic shopping bags. In healers' consulting rooms, products are usually stored in plastic containers with lids, on wooden shelves or in plastic shopping bags. In shops products are usually stored on shelves or in 'pigeon holes' where the wide array of plant material can be kept relatively neat and separate (MANDER, 1997).

In the case of bulk transport, it is important to secure dry conditions and furthermore, in order to reduce the risk of mould formation or fermentation, it is extremely advisable to use aerated containers. As a substitute, the use of sufficiently aerated transport vehicles and other aerated facilities is recommended. Essential oil transport must conform to appropriate regulations. National and European regulations on transport have to be respected.

Fumigation against pest attack should be carried out only in the case of necessity and exclusively licensed personnel must carry it out. Only registered chemicals must be used. Any fumigation against pest attack should be reported in the

documentation. For fumigation of warehouses, only permitted substances should be used, according to European or national regulations. When frozen storage or saturated steam is used for pest control, humidity of the material must be controlled after treatment. Pests are a common problem for traditional plant traders and fumigation does occur in 'Muthi' shops (Anonymous shop owner, *pers. com.*). There appears to be no concern on the part of shop owners as to the effects of the pesticide residues on plant material. It is quiet possible that the chemicals used to treat premises and plant materials are not safe registered products as these are often costly, but rather a cheaper potentially hazardous one is used. No studies as to the levels of such compounds in traditional medicines in South African traditional medicines have been conducted.

Equipment

Equipment used in plant cultivation and processing should be easy to clean, in order to eliminate the risk of contamination. All machinery should be mounted in an easily accessible way. They must be well serviced and regularly cleaned. Fertilizer and pesticide application machinery must be regularly calibrated. Preferably non-wooden equipment should be used unless tradition demands wooden material. When wooden equipment (such as e.g. pallets, hoppers, etc.) is used, it should not come into direct contact with chemicals and contaminated/infected materials, so that infection of the plant material can be prevented.

Personnel and Facilities

Personnel should receive adequate botanical education before performing tasks that require this knowledge. Personnel entrusted with the plant material should be required to have a high degree of personal hygiene (including personnel working in the fields) and have received adequate training regarding their hygiene responsibilities.

The buildings where the plant processing is carried out have to be provided with changing facilities as well as toilets including hand-washing facilities, according to the respective regulations. The conditions in the South African markets are generally poor, with most plant material displayed in the open. Under these conditions the material is exposed to microbial and insect attack as well as the effects of light,

moisture, motor vehicle emissions and temperature fluctuations. Often these informal markets are situated close to both pedestrian and motor vehicle traffic, which places plant material in contact with all kinds of pollution.

Persons suffering from known infectious diseases transmittable via food, including diarrhoea, or being transmitters of such diseases, must be suspended from areas where they are in contact with the plant material, according to the respective regulations.

Persons with open wounds, inflammations and skin-infections should be suspended from the areas where plant-processing takes place, or have to wear appropriate protecting clothing or gloves, until their complete recuperation. Personnel should be protected from contact with toxic or potentially allergenic plant materials by means of adequate protective clothing. Many medicinal plants traded in South Africa are toxic and prolonged harvesting of plants such as *Scilla natalensis* or *Bowiea volubilis* without adequate protection could result in poisoning.

Documentation

All starting materials and processing steps have to be documented including the location of cultivation. All growers should maintain Field records showing previous cropping and inputs. All batches from coherent areas should be unambiguously and unmistakably labelled (e.g. by the application of a batch number). This should take place as early as possible.

Batches from differing areas shall be mixed only if it is guaranteed that the mixture in itself will be homogenous. Such mixing procedures should also be documented.

It is essential to document the type, quantity and the date of harvest of the crop, as well as the chemicals and other substances (e.g. fertilizers, pesticides and herbicides, growth regulators, etc.) used during production.

The application of the fumigation agents such as phosphin must be entered into batch documentation. All processes and procedures that could impact on the quality of the product must be entered into the batch documentation. All agreements

(production guidelines, contracts, etc.) between producer and buyer should be fixed in a written form.

It should be documented in a Way Bill (batch documentation) that cultivation, harvesting and production have been performed in accordance with the GAP Guidelines. Minimum information included in the Way Bill should cover geographical definition of growth place, country of origin and responsible producer.

Special circumstances during the growth period, which may influence the chemical composition, like extreme weather conditions. Pests, particularly in the harvest period, should be documented as these may have an affect on the quality and yield of the plant material.

Education

It is extremely advisable to educate all personnel dealing with the crop or those engaged in the direction of the production regarding production techniques and the appropriate use of herbicides and pesticides. One aspect of scientific research in southern Africa, particularly in the field of ethnobotany, which draws criticism, is the relatively low rate at which applicable findings of research are disseminated back to the very people who use the traditional medicine. This is not entirely the fault of the scientific community, as they often do not have access to these communities. Other institutions and governing bodies such as the Department of Agriculture and Environmental Affairs are better equipped to ensure the education, training and development of rural communities and therefore they need to work closely with the scientific community on such projects.

Quality Assurance

Agreements between producers and buyers of medicinal and aromatic plants, with regard to quality questions, e.g. active principles and other characteristic ingredients, optical and sensoric properties, limit values of germ numbers, plant protection chemical residues and heavy metals, must be based on internationally recognized or national specifications and should be laid down in a written form. Marketing surveys will have to be conducted to determine, firstly, the acceptability of cultivated crops

over wild harvested crops by the South African consumers. Secondly, other factors such as preferred age and size of plant material would need to be determined.

The lack of storage facilities and trading infrastructure frequently results in the spoiling of plant materials, resulting in wastage and/or a decrease in product quality. It must be stressed that healers and consumers question the current marketing of indigenous medicine, as they are concerned about the quality of the products purchased in the markets (MANDER, 1997).

6.2.3. GAP for southern Africa

Presently in southern Africa there is inadequately indigenous medicinal plant product development taking place that supports the current market players (traditional healers, gatherers and traders) and promotes the welfare of current users. There is an imbalance in support for indigenous medicine, with most investment being directed at seeking commercially useful chemicals within medicinal plants, while little or no investment is being directed to maintain or increase the benefits, which the current market is already delivering to millions of consumers. As described in Section 5.1., the cultivation of plants either by small-scale rural farmers or on a larger more commercial system is inevitable if the supply of traditional medicinal plants is to be maintained. Before the cultivation of many of these traditionally used medicinal plants can be realised several areas outlined in the guidelines for GAP require urgent attention.

The major limiting factor with the implementation of GAP for medicinal plant production in southern Africa is money as it is mostly small-scale rural farmers who will be the key players in its development (JÄGER and VAN STADEN, 2002). Thus, the major focus in the development of a South African version of the guidelines for GAP must be for low cost and low technology applications. Several tertiary education institutions as well as the KwaZulu-Natal Department of Agriculture and Environmental Affairs are working on various aspects that can be consider part of GAP, but there appears to be no consolidation of these ideas. It is important that future research be conducted under a framework such as the European guidelines for GAP so as to avoid replication and to provide common direction for all parties

involved. The situation in Europe is very different from those in southern Africa and thus application in Europe may not be suitable for the cultivation and development of herbal preparations in South Africa, but the underlying principles are the same. These guidelines also need to be considered if southern African plants or plant products are to be exported to Europe.

Time is not on our side and it must be stressed again that the situation with respect to the decline in wild populations of many plant species is serious. It is therefore imperative that current research on the cultivation of medicinal plants has a structure such as the guidelines outlined in GAP. This would provide direction for the various research bodies involved in this area.

Due to southern Africa's high biodiversity it is well positioned as a region for the development of potential pharmaceuticals and herbal medicines. From a pharmaceutical discovery and plant-drug development standpoint cultivation of the source plant is the only way to proceed. McCHESNEY (2001) stressed that a very important consideration, perhaps the one that most limits interest in plant-derived natural products for pharmaceutical discovery and development, has been concern over the availability of quantities of quality plant material. International market demand for the isolated product can reach hundreds of kilograms per annum scale (McCHESNEY, 2001). Most complex natural product active principals are not economically assessable by total synthesis. Alternatively, preparation by semi-synthesis, that is conversion by defined and straightforward chemical transformations of a more abundant, often simpler, related natural product into the desired active principle might well be feasible (McCHESNEY, 2001). This approach provides the anti-inflammatory steroid derivatives from bile acid salts and plant steroids, natural products in abundant supply as by-products of other industries. Wild populations of plants will not be a reliable source for isolation of drug or drug precursors at a commercial scale. Their harvest may be counter-productive to the development of a reliable, cost-effective, long-term production system of the agent.

6.3. Effect of microbial contamination of medicinal plant material

The microbial content of the plant material has not been considered in this investigation and has been excluded where possible. However it is an important factor in the degradation of plant material and therefore must be mentioned. It is to be expected that plant material harvested from wild populations and sold in informal street markets and 'muthi' shops will be host to a wide spectrum of microorganisms. Each microorganism is likely to have individual properties with significant diversity with regards to qualitative and quantitative aspects. Among these organisms, pathogens may occur and this at worst limits the utilisation of the plant material or may cause quality loss due to microbially induced spoilage (CZECH, KNEIFEL and KOPP, 2001). Bacterial endospores and fungal spores can be regarded as the two dominating groups of contaminants associated with medicinal plants, a broad diversity of bacterial, fungal cells and viruses can be found either in or on the plant material (McKEE, 1995 and KNEIFEL, CZECH and KOPP, 2002). Extraneous matter and filth material originating from rodents, insects and inorganic sources may be present in some preparations and cannot be overlooked (McKEE, 1995 and KNEIFEL, CZECH and KOPP, 2002).

It has been suggested that the degree of contamination found associated with plant material usually depends on the distance from the soil the material has been grown (ALONZO, MONFORTE, TUMINO, RAGUSA and BISIGNANO, 1994). Thus, one would expect underground organs such as bulbs and roots to have a high microbial content than leaves and other aerial parts. Certain plants produce natural barriers and antimicrobial compounds, which inhibit any microbial growth. These substances may have brought about the use of these plants in traditional medicine. It has been estimated that around 1400 herbs and spices in common use may possess antimicrobial activity (KNEIFEL, CZECH and KOPP, 2002).

The microbial load of plants is the result of a series of influences caused by animate and inanimate sources, and microbial contaminants are easily transferred via air- and soil-borne vectors as illustrated below (**Figure 6.1**).
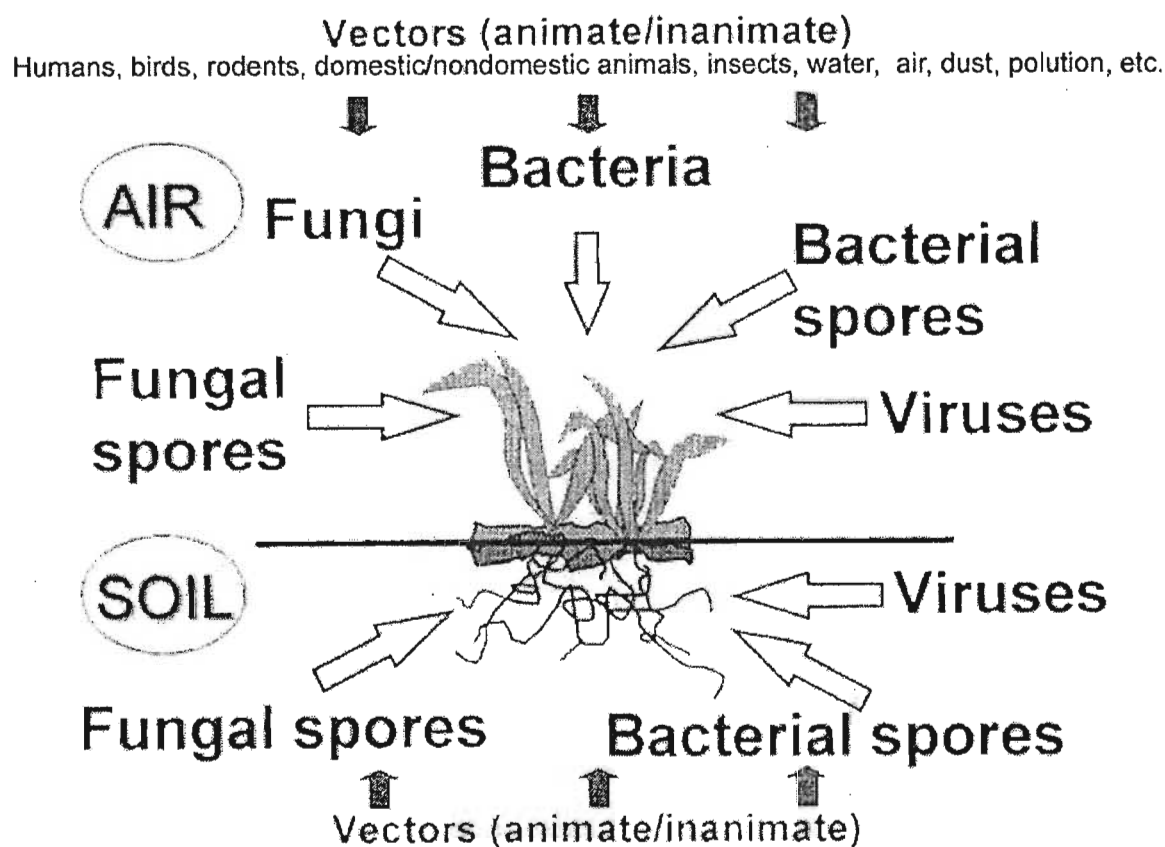
Figure 6.1: Schematic diagram of possible pathways of microbial contamination of medicinal plant material prior to harvesting (From KNEIFEL, CZECH and KOPP, 2002).

KNEIFEL, CZECH and KOPP (2002) have suggested that the persistence and resistance properties of the plant material's microflora are controlled by intrinsic such as:

- the nature of the plant and natural barriers;
- structure of the plant/plant part;
- plant's chemical composition (antimicrobial compounds); and
- intracellular microbial contaminations.

As well as extrinsic factors such as:

- climate and humidity in particular;
- location/position of plant material from soil;
- harvesting methods;

- post-harvest treatment;
- packaging and storage conditions;
- preparation technique and physical state of the material (fresh or dried); and
- exogenous microbial contamination.

No formal monitoring of the occurrence of microflora in medicinal plant material in the informal or formal sectors South Africa has been conducted. In Europe the evaluation of microbial contamination of medicinal plants has increasingly become an integral part of GAP and Hazard Analysis and Critical Control Point (HACCP) concepts. KNEIFEL, CZECH and KOPP (2002) have identified the microbial risk inherent to herbal plants at different stages of the industrial production line (**Table 6.1**) in order to develop a systematic strategy of quality assurance. To these observations have been added the proposed areas of risk in the current traditional medicinal plant market in South Africa based on MANDER's (1997) report on the state of KwaZulu-Natal's traditional medicinal plant market. There is no pre-cultivation step at present and the cultivation of medicinal plants in South Africa is negligible as compared to the volume of plant material harvested from the wild. Risk of contamination in wild harvested plants as opposed to cultivated plants would be higher as there is no control over the conditions in which wild plants grow. The risk of contamination during the harvesting of wild plants by gatherers is potentially very high as blunt unclean instruments are often used and the plant material is damaged introducing microbial infections. The gatherer stores the plant material for several weeks (MANDER, 1997) before it is taken to the market, during this period it may be exposed to warm temperatures and humidity, which will favour bacterial growth, and the material is often left exposed to air-borne microbes. The material is transported loosely packaged in woven plastic sacks, which are not airtight increasing the risk of contamination. The plant material that is sold in the traditional markets is often not washed, as there are seldom facilities to do so. Material is dried in sunlight on the ground, which may be covered by newspaper. This is a slow process and may not sufficiently eliminate or reduce microbial growth. Material is seldom packaged and is merely wrapped in recycled paper, which offers little protection from microbial contamination.

Table 6.1: Evaluation of microbiological contamination risk at different stages during the production of medicinal plant material in the formal (industrial/western) market and the informal (traditional) market (After KNEIFEL, CZECH and KOPP, 2002).

Explaination of ranking: 0 usually no risk, 1 no to low risk, 2 low to medium risk, 3 high risk.

Production steps	Risk level – formal market	Risk level – informal market
Pre-cultivation	1	-
Field/wild cultivation	3	3
Harvest	3	3
Intermediate storage (by cultivator or gatherer)	2	3
Transportation	1	2
Treatments (cleaning/cutting/drying)	2	3
Final product (packaged/stored)	0	3

No investigations into the quality and quantity of microbial loads in/on South African traditional medicinal plants have been conducted. The examination of the microbiological quality of European plant products are commonly based on general and specific evaluation criteria, each of them having a more or less pronounced indicative meaning, which enables the development of practical conclusions (**Table 6.2** from KNEIFEL, CZECH and KOPP, 2002).

Table 6.2: Indicative meaning derived from groups of microbes associated with plant material examined in routine European microbiological control of medicinal herbal plants (From KNEIFEL, CZECH and KOPP, 2002).

Group	Indication
Total aerobic mesophilic count	General hygiene and quality parameter
Enterobacteria	General hygiene and quality parameter, indicator of faecal contamination, but also ubiquitously present
Coliforms	Indicator of faecal contamination, but also ubiquitously present
Enterococci	Indicator of faecal contamination, but also ubiquitously present
Pseudo- and aeromonades	Indicator for enhanced spoilage potential, mainly from water-borne sources
Coagulase-positive staphylococci	Indicator for pathogens of human origin
Aerobic and anaerobic spore-forming bacteria	Bacteria, typical soil microflora
Lactobacilli	Possible indicator of spoiled plant material
Yeasts and moulds	Ubiquitously present microorganisms, in part indicators of possible mycotoxigenic potential
Pathogens	Occurrence bears high health risk, to be avoided/prevented (*Salmonella spp*, *Listeria monocytogenes*, *E. coli*, enterohaemorrhagic strains of *E. coli* (EHEC), *Campylobacter*, *Yersinia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, mycotoxin producers

There are few reports of pathogenic microorganisms in/on herbal plants (KNEIFEL, CZECH and KOPP, 2002). However, contaminations with pathogens should not be overlooked, the situation in South Africa may be very different to those in Europe. CZECH, KNEIFEL and KOPP (2001) have screened for a broad spectrum of pathogens associated with European medicinal herbs and found that these

microorganisms are not common. Exceptions were *Bacillus cereus* and *Clostridium perfringens*, however, these two spore-forming microbes usually do not appear in magnitudes great enough to cause a real toxicity potential. Herbal plants are known to frequently carry a considerable amount of moulds, which may result in the production of mycotoxins. Mould and fungal growth can often be observed if cold-water macerations of plant material are left at room temperature for several days. HITOKOTO, MONOZUMI, WAUKE, SAKAI and KURATA (1978) have shown that moulds like *Penicillium, Aspergillus, Rhizopus, Mucor, Cladoaporium* and *Aureobasidium spp.* can be found frequently in association with herbal drugs, but mycotoxin producers were only present around the level of 2%. KUMAR and ROY (1993), to the contrary detected considerable risk levels of aflatoxins in several herbal medicinal samples of different taxa. These findings would suggest that climate, humidity and hygiene largely contribute to the mycotoxin problem, which again reiterates the need for microbial load studies on southern African medicinal plants.

Various preparation techniques are likely to have an effect on the microbial load of the plant product that is administered. The application of hot aqueous infusions (e.g. herbal teas) would be expected to reduce the microbial load and also inactivate possible pathogens (KNEIFEL, CZECH and KOPP, 2002). Cold-water extractions may host a considerable amount of microbes and extractions performed at room temperature may promote microbial multiplication. Storage of cold-water extractions/macerations almost always results in prolific microbial growth. Not all traditional healer have access to clean water and therefore use non-sterile water which would introduce potentially hazardous microbes.

Tinctures, which are made by ethanolic extraction, would reduce the microbial load of the plant material to be administered but this will be dependent on the alcoholic concentration used (KNEIFEL, CZECH and KOPP, 2002). Snuffs are preparations of finely powdered, dried medicinal plants that can be drawn up into the nostrils through inhalation. This form of administration is not as common as the others mentioned. The microbial load of such a preparation will depend largely on the hygiene during preparation. The grinding of the material into a fine powder will increase the surface area of the plant material increasing the risk of microbial contamination.

Often in southern African traditional medicine the plant material is administered by inhaling the smoke of burning plant material. This would eliminate any risk purposed by microbial contamination.

6.4. Conclusions

The southern African medicinal plant industry is very different from that in Europe, and it is unlikely that the two will ever be similar. Africa has a very unique and firmly entrenched culture of utilizing plant materials for therapeutic purposes. The ancient origins of this culture are clearly evident in the very nature of the current trade of medicinal plants with very little evidence to suggest that much development has occurred. The 'the underground' or 'black-market' atmosphere, a result of traditional medicine being illegal during the apartheid era, is still noticeable in many aspect of the traditional trade. At present there appears to be a lot of interest by industry and agriculture in the medicinal plant trade as it is potentially a very lucrative area, but it is also a relatively unknown area and thus perhaps to risky to attract large investment.

If the development of the medicinal plant industry in southern Africa is to occur it is important that sound guidelines are developed that can be implemented from the onset. Some common ground between those currently used in Europe and the rest of the world and those to be implemented in Africa should be reached, particularly where export potential is envisaged.

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APPENDIX 1

TLC-FINGERPRINTS

Plate AP1.1: **TLC-fingerprints of bulb extracts from fresh (upper plate), fresh dried (middle) and stored material (lower plate) of *S. natalensis* (S), *D. robusta* (D) and *B. volubilis* (B) in hexane (H), ethanol (E) and water (W) under UV365 nm after staining with 20% solution of antimony-III-chloride.** The red-orange bands indicate the presence of cardiac glycosides as a result of the antimony-III-chloride spray reagent when viewed under UV_{365} nm. Note that these orange bands are present in both fresh, fresh dried and stored material; cardiac glycosides are known to be relatively stable. The hexane fraction of the three plants' fresh, fresh dried and stored material appears to be similar. The ethanol and water fractions however, show signs of change in chemical composition, notably in the water fraction. There are several bands in the fresh *S. natalensis* and *B. volubilis* water fractions that are not evident in the stored material.

Plate AP1.2: TLC-fingerprints of leaf and rhizome extracts of *Alepidea amatymbica* showing changes in chemical composition after storage.

(A)   TLC-fingerprint of *Alepidea amatymbica* leaf and rhizome extracts taken from fresh (left) and stored (right) material viewed under VIS after treatment with anisaldehyde/sulphuric acid spray reagent (upper pictures), UV_{254} nm (centre pictures), and UV_{365} nm (lower pictures)

(B)   Fresh and one-year-old material viewed under UV_{254} nm (upper pictures), and UV_{365} nm (lower pictures)

(C)   Ethanolic extracts of fresh, 90-day and one-year-old material of *Alepidea amatymbica* leaf and rhizome material viewed under UV_{254} nm (upper pictures), and UV_{365} nm (lower pictures).

Fresh material

Fresh dried material

Stored material

SH DH BH SE DE BE SW DW BW
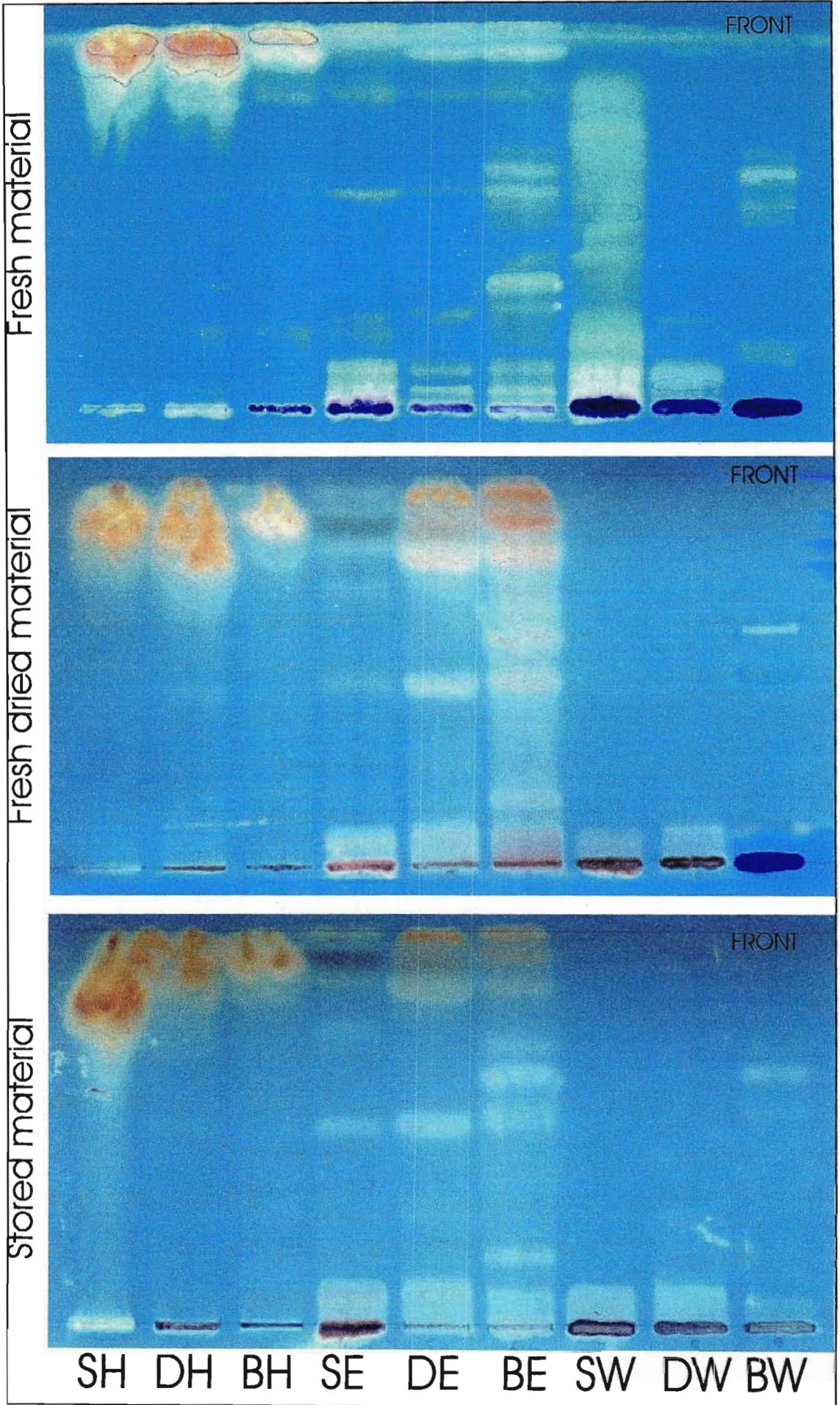
FRONT

FRONT

FRONT

Plate AP1.3: TLC-fingerprints of bulb extracts of *Eucomis autumnalis* (A); whole plant extracts of *Helichrysum cymosum* (B); and rhizome extracts of *Siphonochilus aethiopicus* (C) showing changes in chemical composition after storage.

(A) TLC-fingerprint of *Eucomis autumnalis* bulb extracts taken from fresh (left) and stored (right) material under VIS after treatment with anisaldehyde/sulphuric acid spray reagent (upper pictures); $UV_{254\,nm}$ (centre pictures); and $UV_{365\,nm}$ (lower pictures)

(B) TLC-fingerprint of *Helichrysum cymosum* whole plant extracts taken from fresh (left) and stored (right) material under VIS after treatment with anisaldehyde/sulphuric acid spray reagent (upper pictures); $UV_{254\,nm}$ (centre pictures); and $UV_{365\,nm}$ (lower pictures)

(C) TLC-fingerprint of *Siphonochilus aethiopicus* rhizome extracts taken from fresh (left) and stored (right) material under VIS after treatment with anisaldehyde/sulphuric acid spray reagent (upper pictures); $UV_{254\,nm}$ (centre pictures); and $UV_{365\,nm}$ (lower pictures)
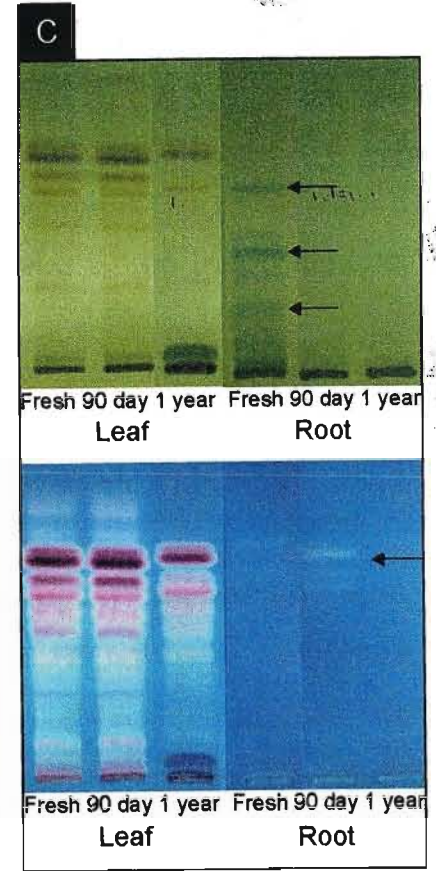
A

Hex | EtOH | H₂O | EtOH | H₂O | Hex | EtOH | H₂O | Hex | EtOH | H₂O
Fresh Root | Fresh Leaf | 90 day Root | 90 day Leaf

Hex | EtOH | H₂O | EtOH | H₂O | Hex | EtOH | H₂O | Hex | EtOH | H₂O
Fresh Root | Fresh Leaf | 90 day Root | 90 day Leaf

Hex | EtOH | H₂O | EtOH | H₂O | Hex | EtOH | H₂O | Hex | EtOH | H₂O
Fresh Root | Fresh Leaf | 90 day Root | 90 day Leaf

B

Hex | EtOH | H₂O | Hex | EtOH | H₂O | Hex | EtOH | H₂O | Hex | EtOH | H₂O
Fresh Leaf | 1 year Leaf | Fresh Root | 1 year Root

Hex | EtOH | H₂O | Hex | EtOH | H₂O | Hex | EtOH | H₂O | Hex | EtOH | H₂O
Fresh Leaf | 1 year Leaf | Fresh Root | 1 year Root

C

Fresh | 90 day | 1 year | Fresh | 90 day | 1 year
Leaf | Root

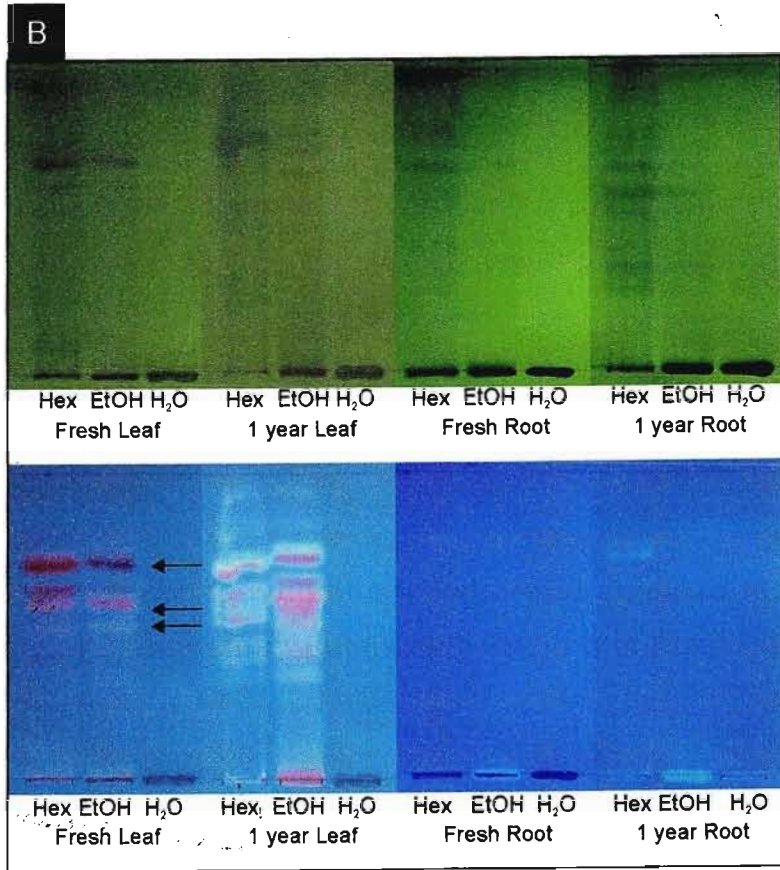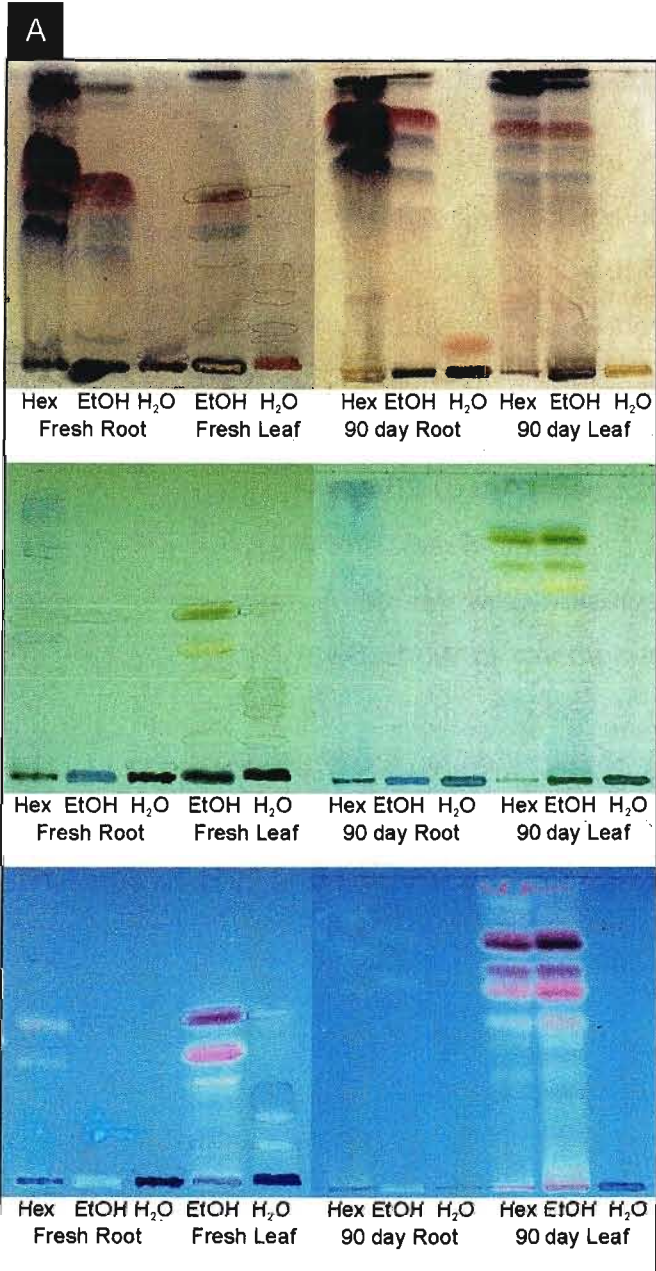Fresh | 90 day | 1 year | Fresh | 90 day | 1 year
Leaf | Root

Plate AP1.14: TLC plates of fractions from a VLC separation of ethanolic fractions of fresh and accelerated aged *Vernonia colorata* leaf material. The plate (A) shown here has been treated with anisaldehyde/sulphuric acid spray reagent. The result of bioautographic antibacterial (*S. aureus*) assay is also shown (B).

Several new antibacterial compounds (c), which show the distinctive streaking nature of fatty acids, appear in the aged material but not in the fresh.
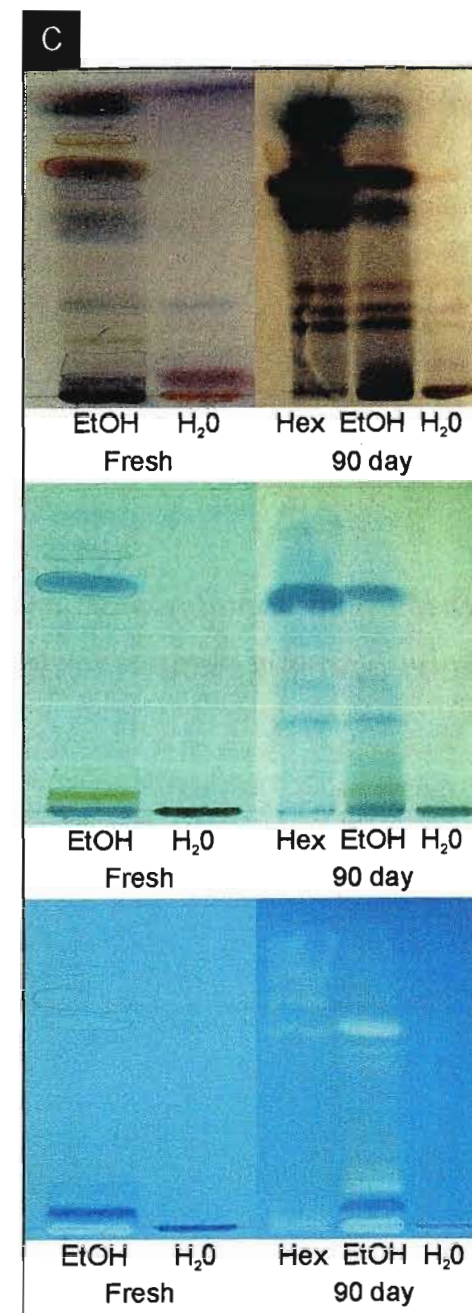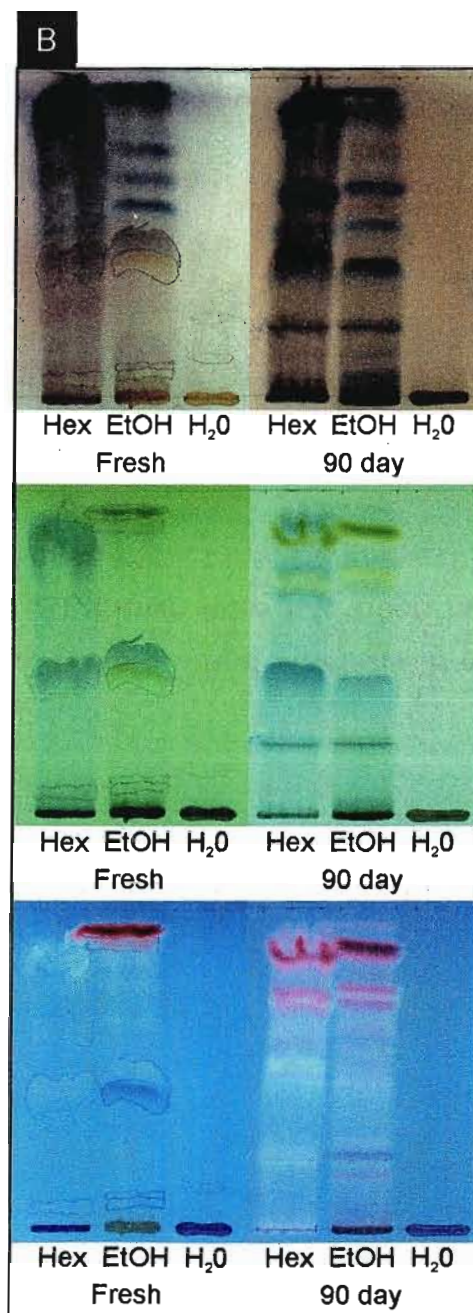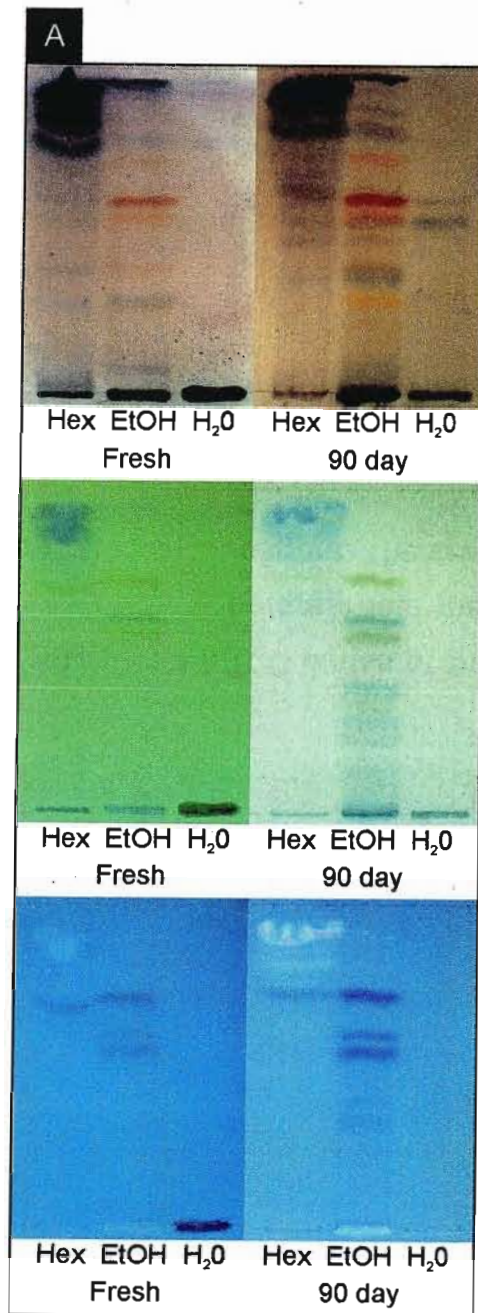
A							B							C						
Hex	EtOH	H_2O	Hex	EtOH	H_2O		Hex	EtOH	H_2O	Hex	EtOH	H_2O		EtOH		H_2O	Hex	EtOH	H_2O
Fresh			90 day				Fresh			90 day				Fresh			90 day		

Hex	EtOH	H_2O	Hex	EtOH	H_2O		Hex	EtOH	H_2O	Hex	EtOH	H_2O		EtOH		H_2O	Hex	EtOH	H_2O
Fresh			90 day				Fresh			90 day				Fresh			90 day		

Hex	EtOH	H_2O	Hex	EtOH	H_2O		Hex	EtOH	H_2O	Hex	EtOH	H_2O		EtOH		H_2O	Hex	EtOH	H_2O
Fresh			90 day				Fresh			90 day				Fresh			90 day		

Plate AP1.5: TLC-fingerprints of leaf extracts of *Vernonia colorata* showing changes in chemical composition after storage.

(A) TLC-fingerprint of *Vernonia colorata* leaf extracts taken from fresh (left) and stored (right) material viewed under VIS after treatment with anisaldehyde/sulphuric acid spray reagent (upper pictures), UV_{254} nm (centre pictures), and UV_{365} nm (lower pictures)
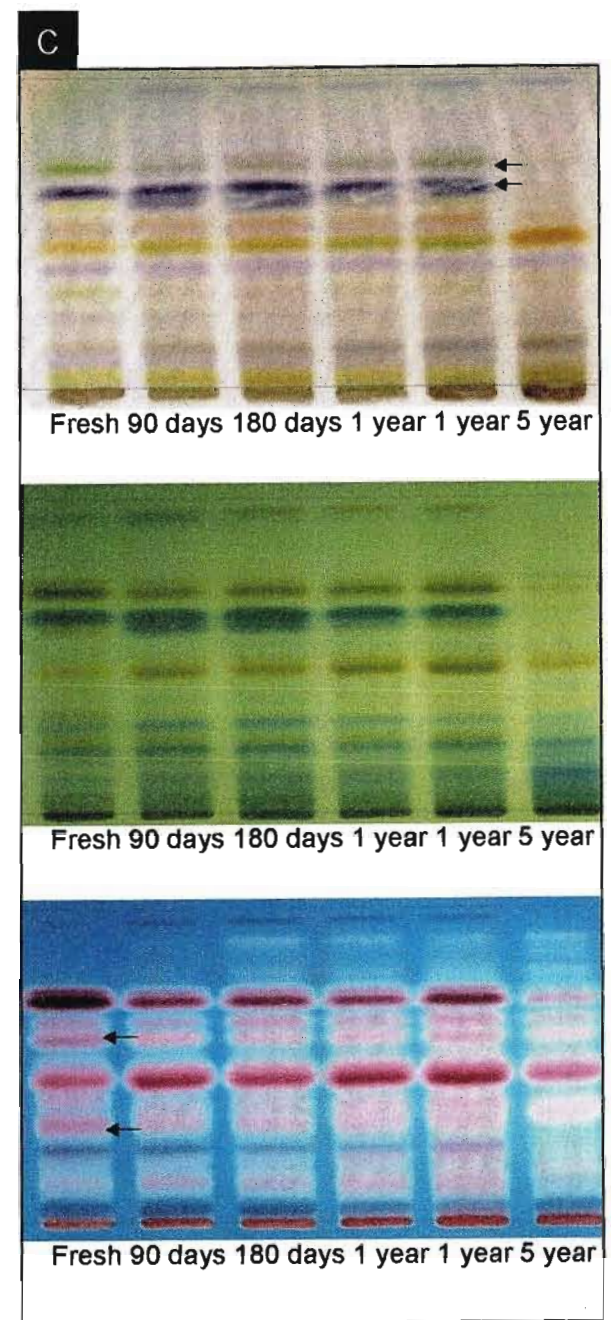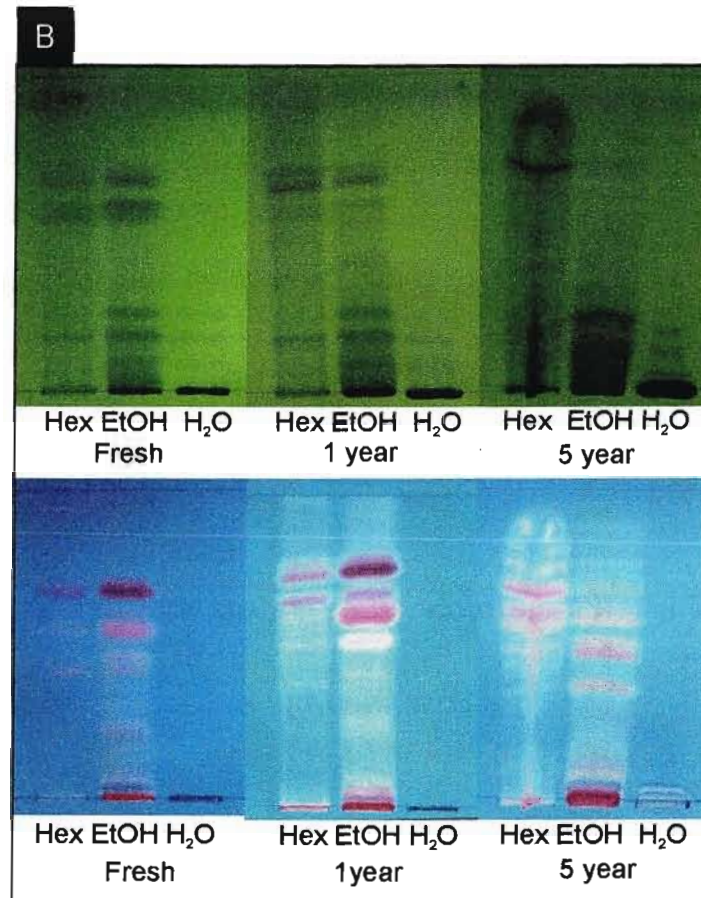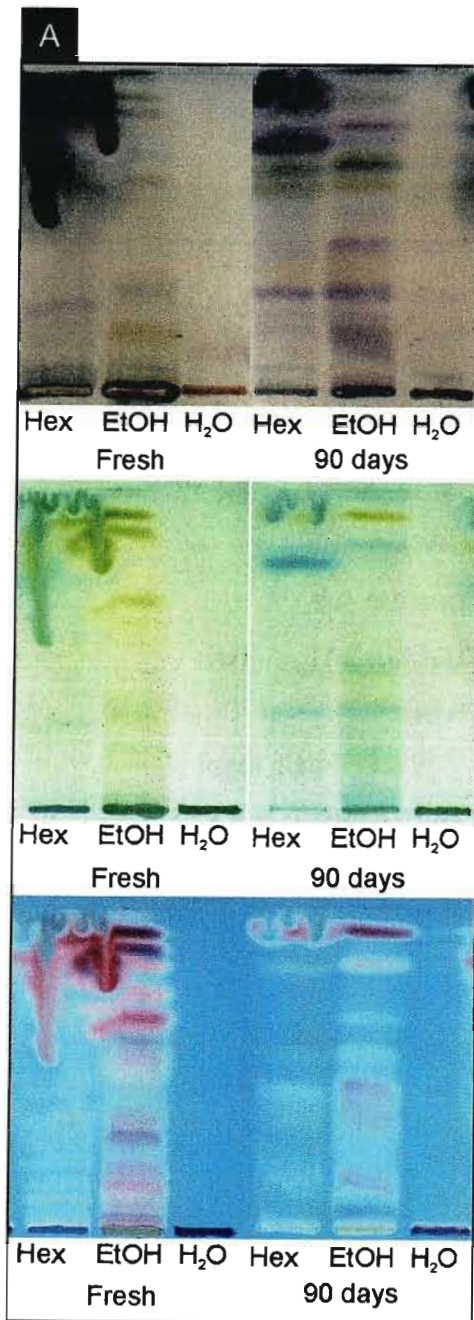
(B) Fresh and one-year-old material viewed under UV_{254} nm (upper pictures), and UV_{365} nm (lower pictures)

(C) Ethanolic extracts of fresh, 90-day, 180-day and one-year-old material of *Vernonia colorata* leaf material viewed under VIS after treatment with anisaldehyde/sulphuric acid spray reagent (upper pictures), UV_{254} nm (centre pictures), and UV_{365} nm (lower pictures)

A

Hex EtOH H₂O Hex EtOH H₂O
Fresh   90 days

Hex EtOH H₂O Hex EtOH H₂O
Fresh   90 days

Hex EtOH H₂O Hex EtOH H₂O
Fresh   90 days

B

Hex EtOH H₂O   Hex EtOH H₂O   Hex EtOH H₂O
Fresh   1 year   5 year

Hex EtOH H₂O   Hex EtOH H₂O   Hex EtOH H₂O
Fresh   1year   5 year

C

Fresh 90 days 180 days 1 year 1 year 5 year

Fresh 90 days 180 days 1 year 1 year 5 year

Fresh 90 days 180 days 1 year 1 year 5 year

Plate AP1.6: TLC-fingerprints of ethanolic extracts from fresh and one-year-old plant material of *Alepidea amatymbica* (leaf and root material), *Helichrysum cymosum* (whole plant), *Eucomis autumnalis* (bulb) and *Leonotis leonurus* (leaf). The first plate shown for each plant is viewed under VIS after treatment with anisaldehyde/sulphuric acid spray reagent, the second viewed under UV $_{254}$ nm and the third viewed under UV $_{366}$ nm.
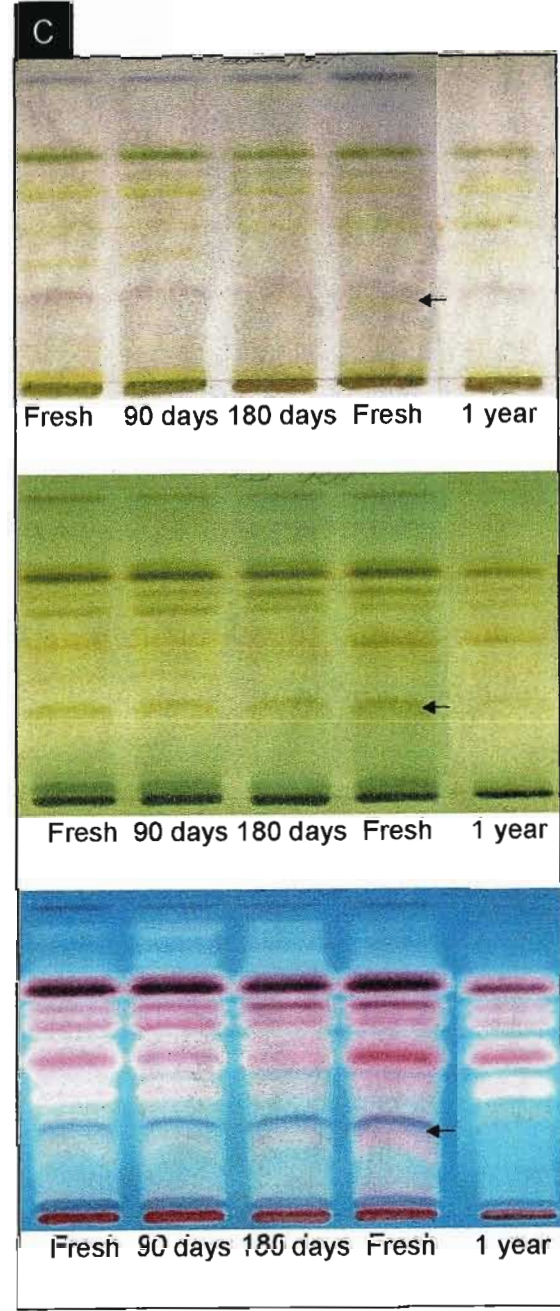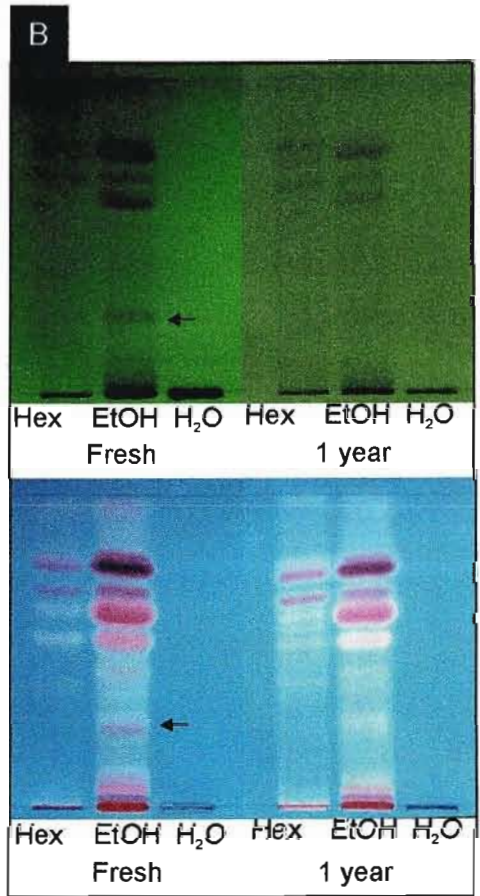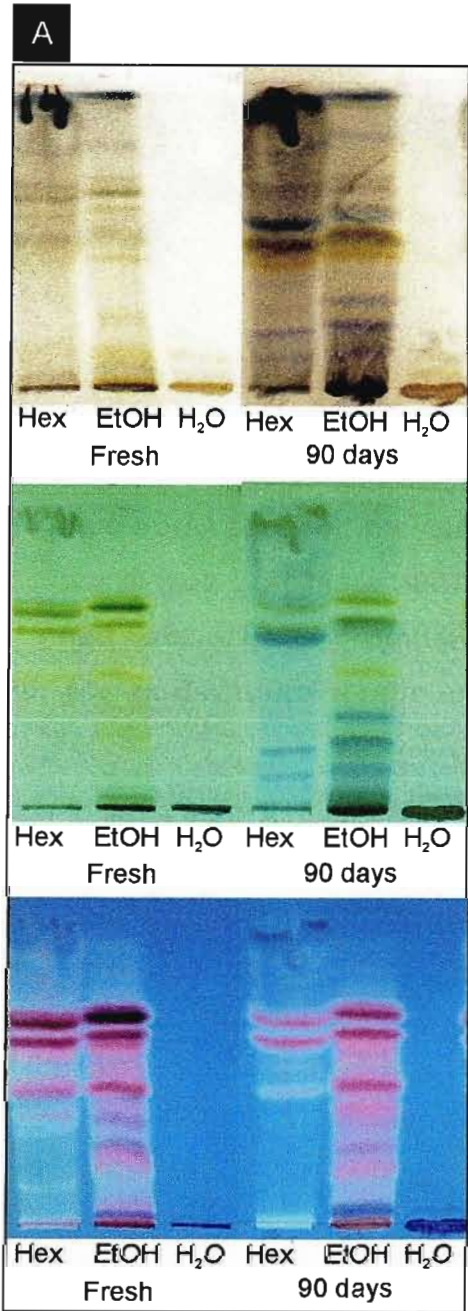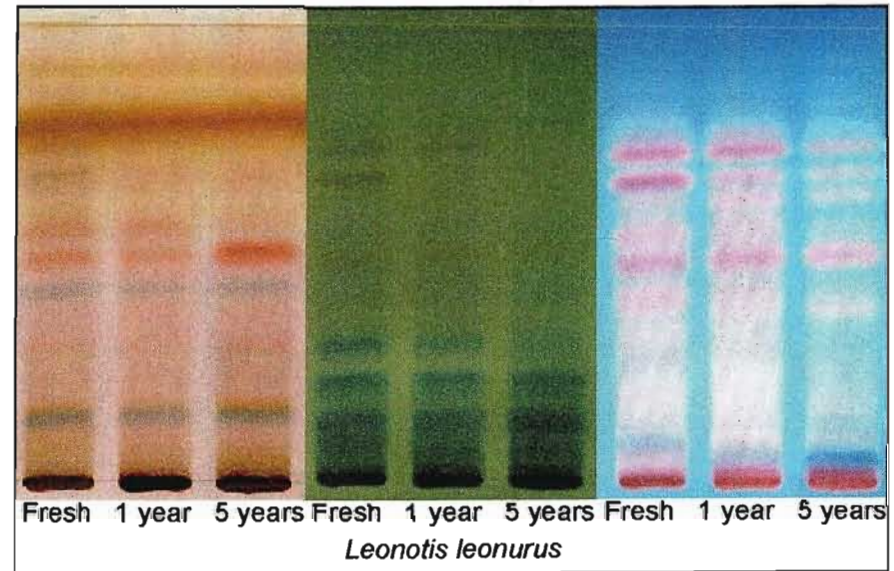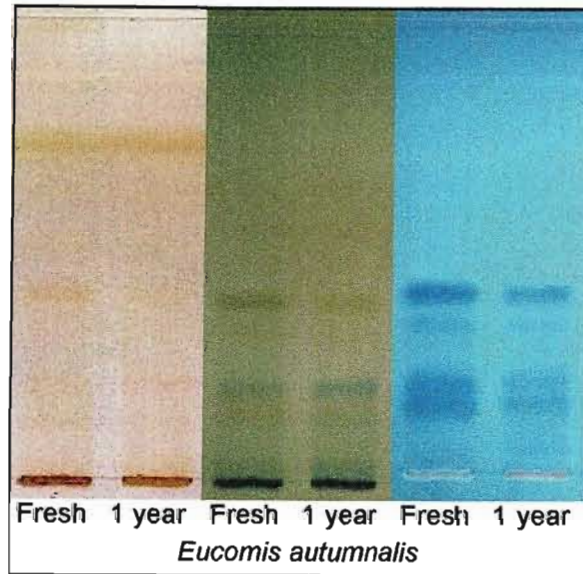
A

Hex EtOH H₂O Hex EtOH H₂O
Fresh 90 days

Hex EtOH H₂O Hex EtOH H₂O
Fresh 90 days

Hex EtOH H₂O Hex EtOH H₂O
Fresh 90 days

B

Hex EtOH H₂O Hex EtOH H₂O
Fresh 1 year

Hex EtOH H₂O Hex EtOH H₂O
Fresh 1 year

Hex EtOH H₂O Hex EtOH H₂O
Fresh 1 year

C

Fresh 90 days 180 days Fresh 1 year

Fresh 90 days 180 days Fresh 1 year

Fresh 90 days 180 days Fresh 1 year

Plate AP1.7: TLC-fingerprints of ethanolic extracts from fresh and one-year-old plant material of *Siphonochilus aethiopicus* (bulb) and *Vernonia colorata* (leaf). The first plate shown for each plant is viewed under VIS after treatment with anisaldehyde/sulphuric acid spray reagent, the second viewed under UV $_{254}$ nm and the third viewed under UV $_{366}$ nm.
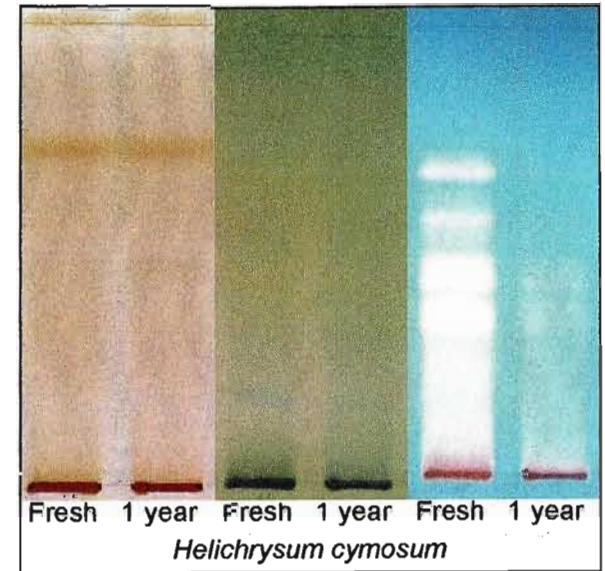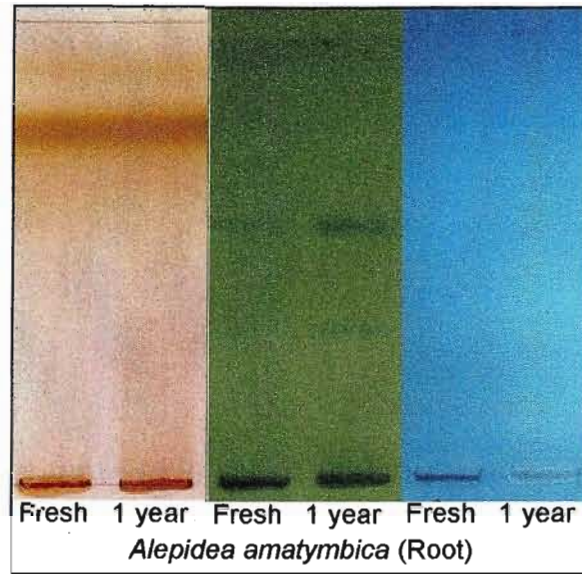
Fresh | 1 year | Fresh | 1 year | Fresh | 1 year
Alepidea amatymbica (Leaf)

Fresh | 1 year | Fresh | 1 year | Fresh | 1 year
Alepidea amatymbica (Root)

Fresh | 1 year | Fresh | 1 year | Fresh | 1 year
Helichrysum cymosum

Fresh | 1 year | Fresh | 1 year | Fresh | 1 year
Eucomis autumnalis

Fresh | 1 year | 5 years | Fresh | 1 year | 5 years | Fresh | 1 year | 5 years
Leonotis leonurus
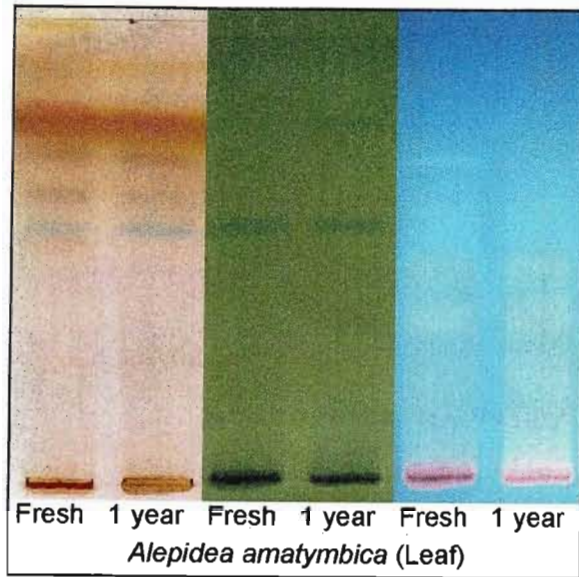
Plate AP1.8: **Bio-autographic plate showing the antibacterial (S. aureus) activity of fresh and stored material of Alepidea amatymbica leaves and rhizomes (A). Reference plate (B) viewed at UV $_{254}$ nm.**

In the bio-autographic antibacterial plate (A) it is clear to see that the anti-bacterial agents have changed as indicated by the arrows. This may explain the increase in the antibacterial activity of the stored extract that was observed in the micro-dilution assay (minimum inhibitory concentration changed from 3.1mg/ml to 0.8mg/ml). New compounds that were previously not present in the fresh extract are now present in the extract that was stored for one year. The streaking that is observed is speculated to be due to the presence of fatty acids. These appear to be more abundant in the stored material.
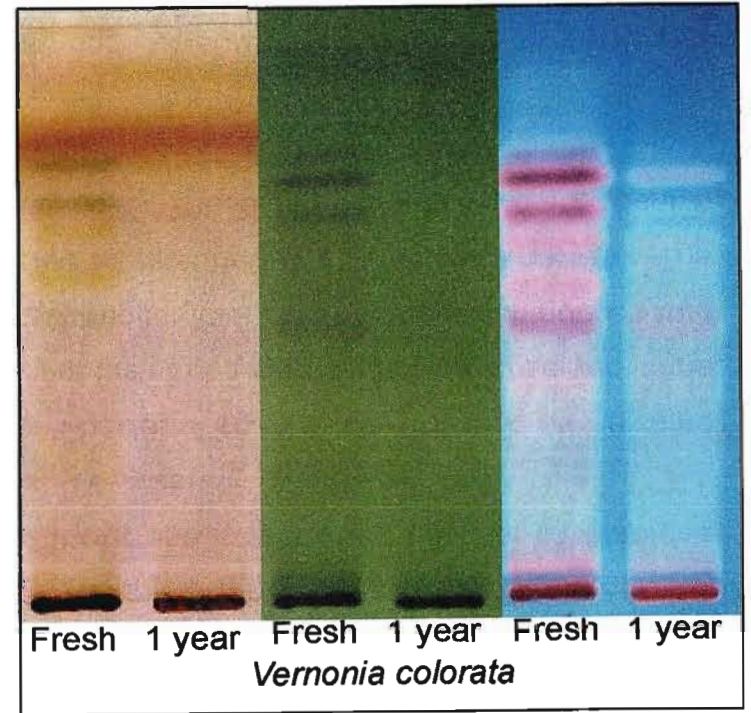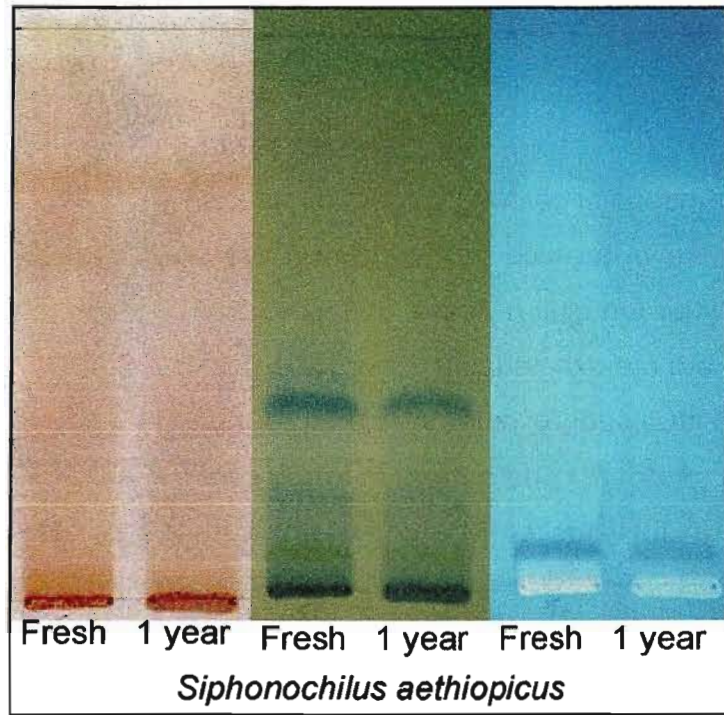
Fresh 1 year Fresh 1 year Fresh 1 year

Siphonochilus aethiopicus

Fresh 1 year Fresh 1 year Fresh 1 year

Vernonia colorata

Plate AP1.9: Bio-autographic plate showing the anti-bacterial (S. aureus) activity of fresh and stored (one-year-old) material of *Eucomis autumnalis* bulbs (A) and *Helichysum cymosum* (B) whole plants.

In the hexane extract of the stored material of *Eucomis* autumnalis there appears to be more activity in the region where the extract has 'streaked', this may be due to fatty acids. In the *Helichrysum cymosum* bio-autographic anti-bacterial plate (B) it is clear to see that the lower compound (b) that appears in the fresh extracts is not present in the stored and that the upper compound (a) has increased in the stored extracts. This may explain the increase in the anti-bacterial activity of the stored extract that was observed in the micro-dilution assay (minimum lethal concentration changed from 1.56mg/ml to 0.8mg/ml).
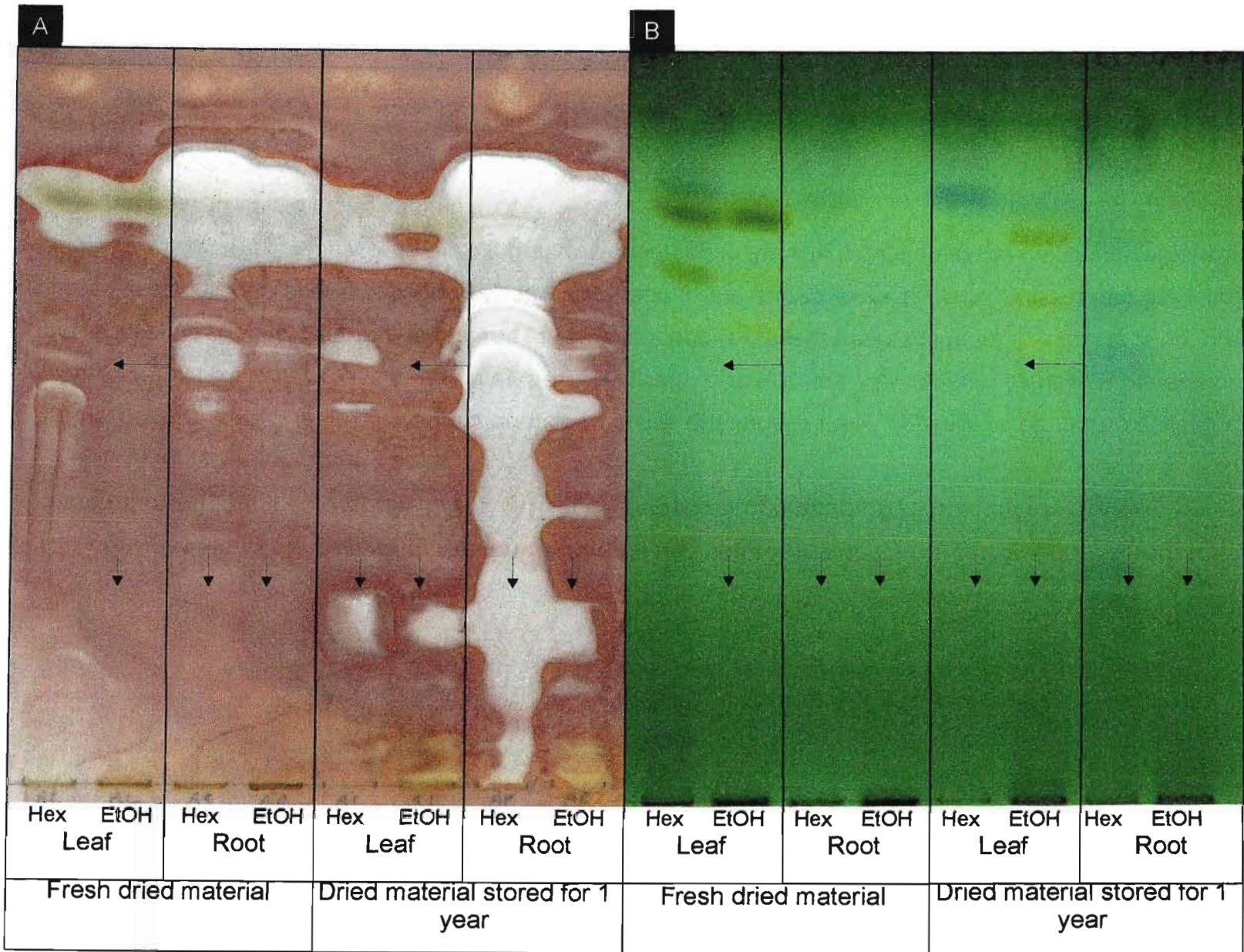
Hex	EtOH	Hex	EtOH	Hex	EtOH	Hex	EtOH	Hex	EtOH	Hex	EtOH	Hex	EtOH	Hex	EtOH
Leaf		Root		Leaf		Root		Leaf		Root		Leaf		Root	
Fresh dried material				Dried material stored for 1 year				Fresh dried material				Dried material stored for 1 year			

Plate AP1.10: Bio-autographic plate showing the antibacterial (S. aureus) activity of fresh and stored (one-year-old) material of *Leonotis leonurus* leaf material.

In the bio-autographic antibacterial plate it is clear to see that the anti-bacterial agents have changed after five years storage as indicated by the arrows. *Leonotis leonurus* also showed a two-fold increase in antibacterial activity after storage.
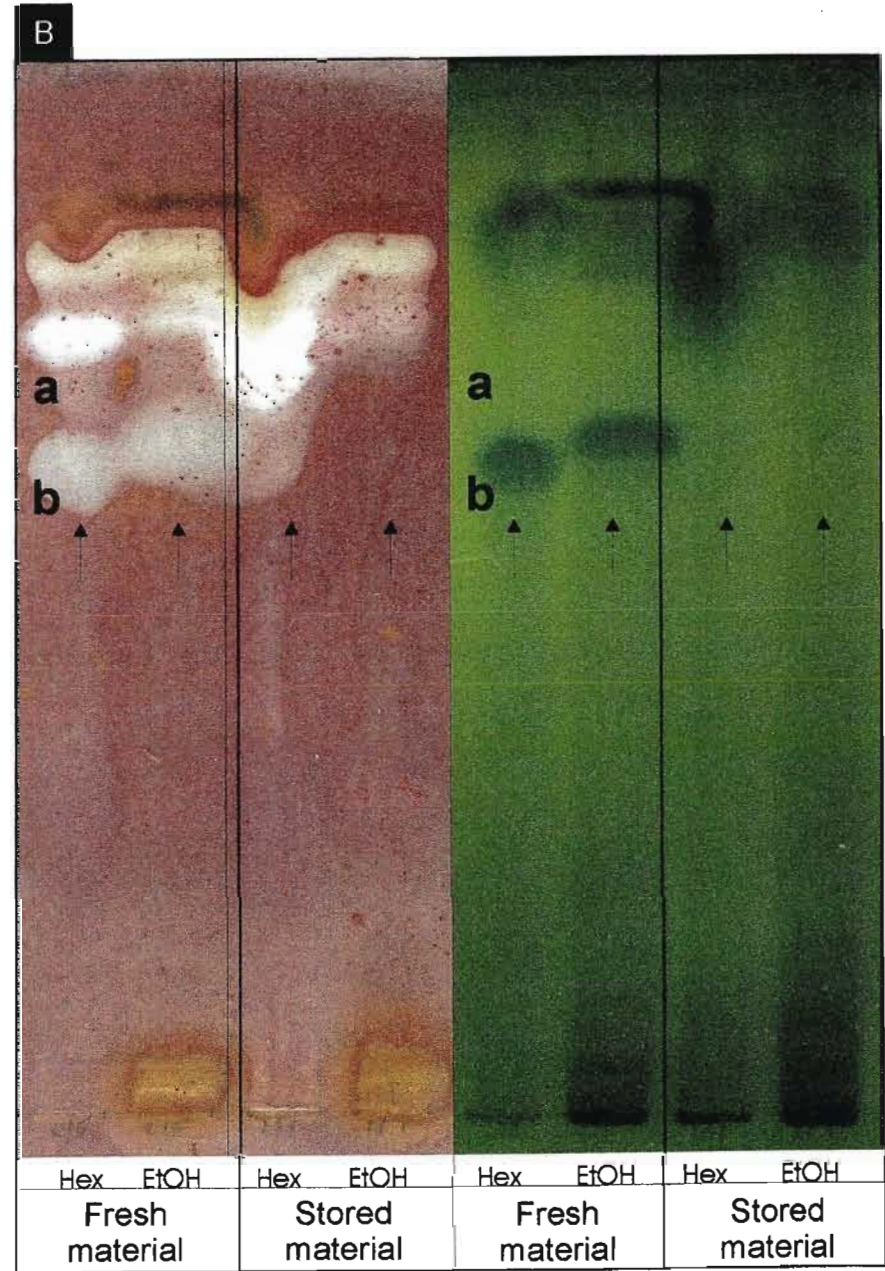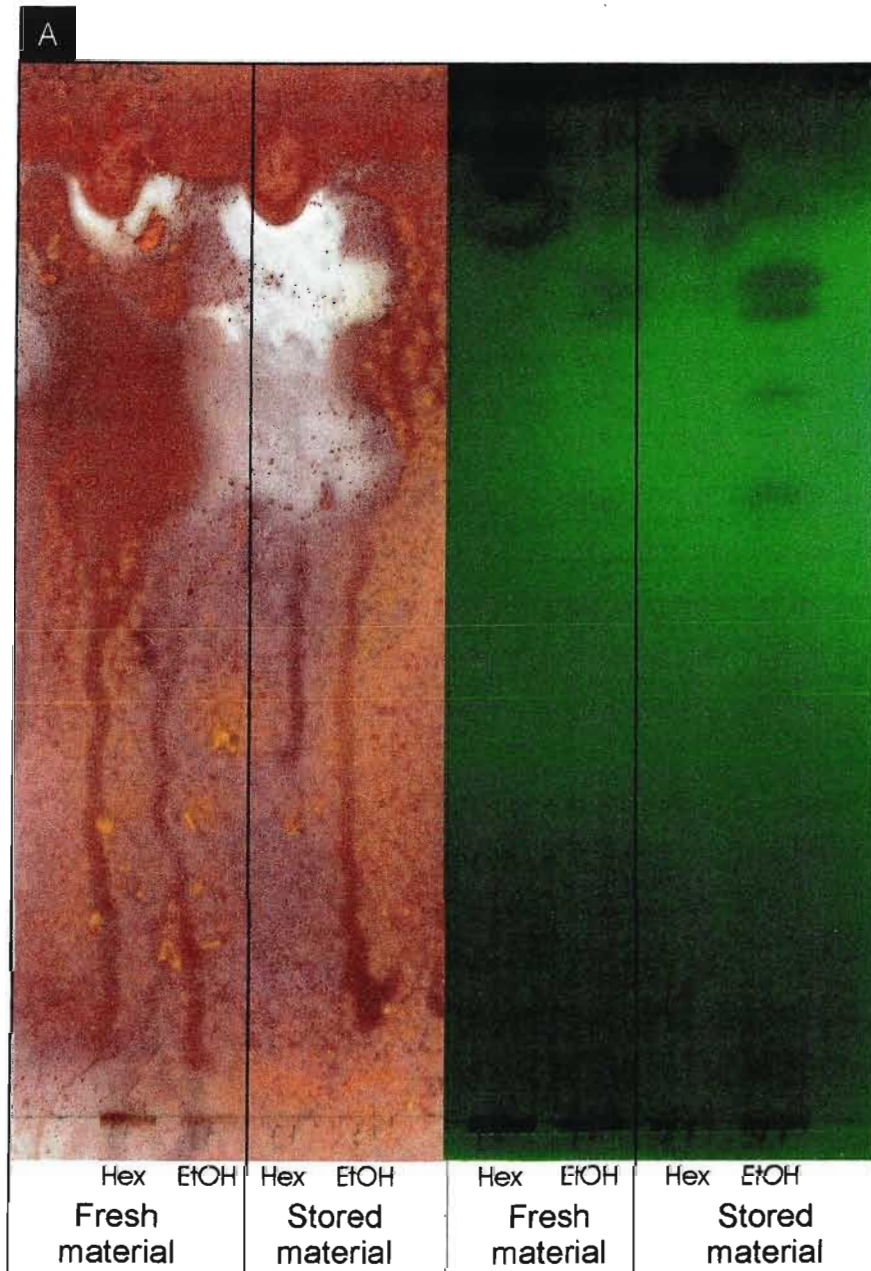
A

| Hex | EtOH | Hex | EtOH | Hex | EtOH | Hex | EtOH |
| Fresh material | | Stored material | | Fresh material | | Stored material | |

B

| Hex | EtOH | Hex | EtOH | Hex | EtOH | Hex | EtOH |
| Fresh material | | Stored material | | Fresh material | | Stored material | |

Plate AP1.11: Bio-autographic plate showing the antibacterial (*S. aureus*) activity of fresh and stored (one-year-old) material of *Vernonia colotara* leaf material.

In the bio-autographic antibacterial plate little change is visible as a result of one-year storage
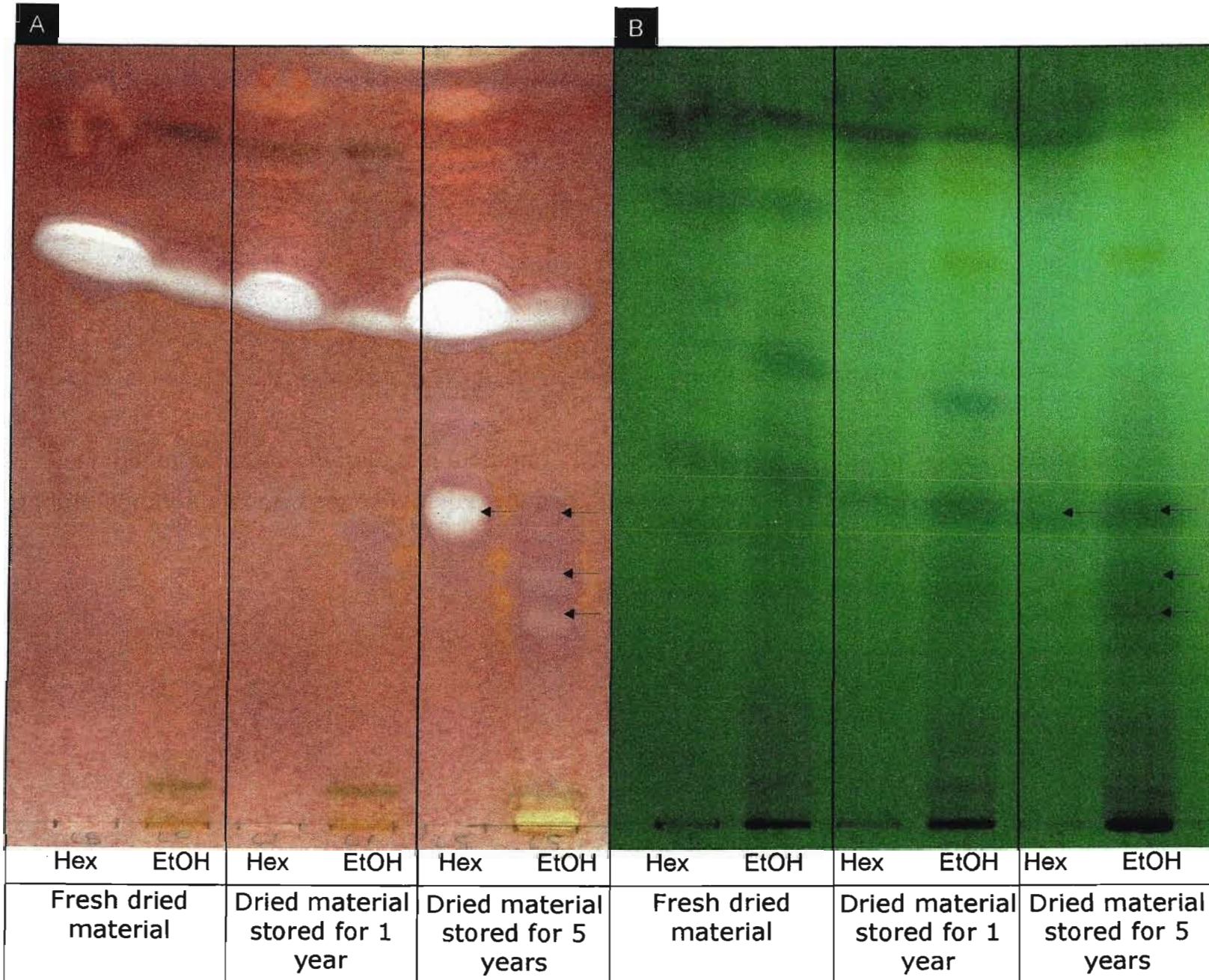
A					B				
Hex	EtOH	Hex	EtOH	Hex	EtOH	Hex	EtOH	Hex	EtOH
Fresh dried material		Dried material stored for 1 year		Dried material stored for 5 years		Fresh dried material		Dried material stored for 1 year	Dried material stored for 5 years

Plate AP1.12: TLC-fingerprints of ethanolic extracts of fresh and accelerated aged material of *Leonotus leonurus* (A) and *Vernonia colorata* (B) leaf material.

Ethanolic extracts of material aged for 5, 10, 15, 20 and 25 days were used and are shown here viewed under UV $_{254}$ nm and UV $_{366}$ nm. It is clear that some compounds are stable while others are not. Five days of ageing appears sufficient to bring about several changes in chemical composition. From 10 to 25 days of ageing there appears to be little change in chemical composition of the plant material.

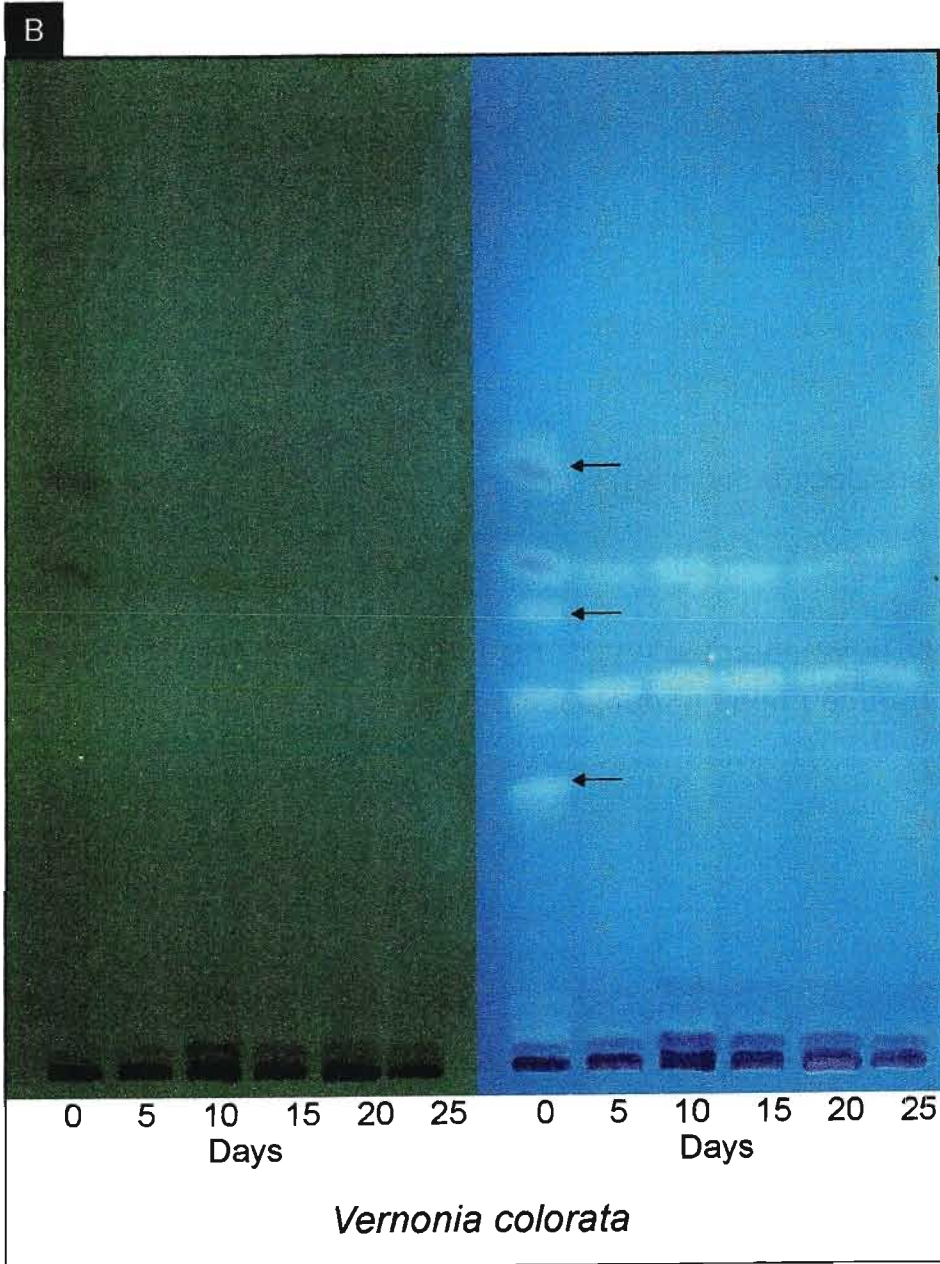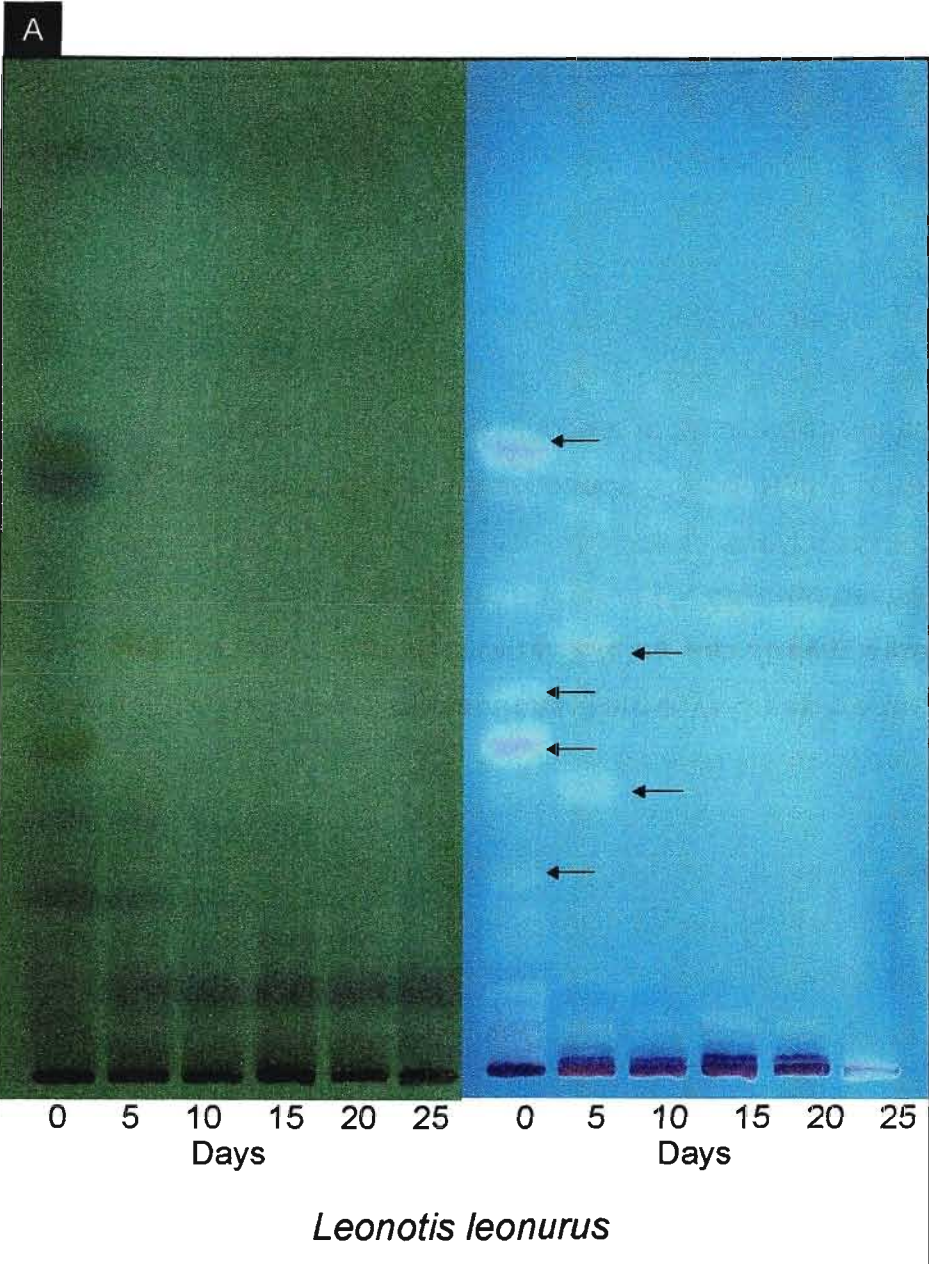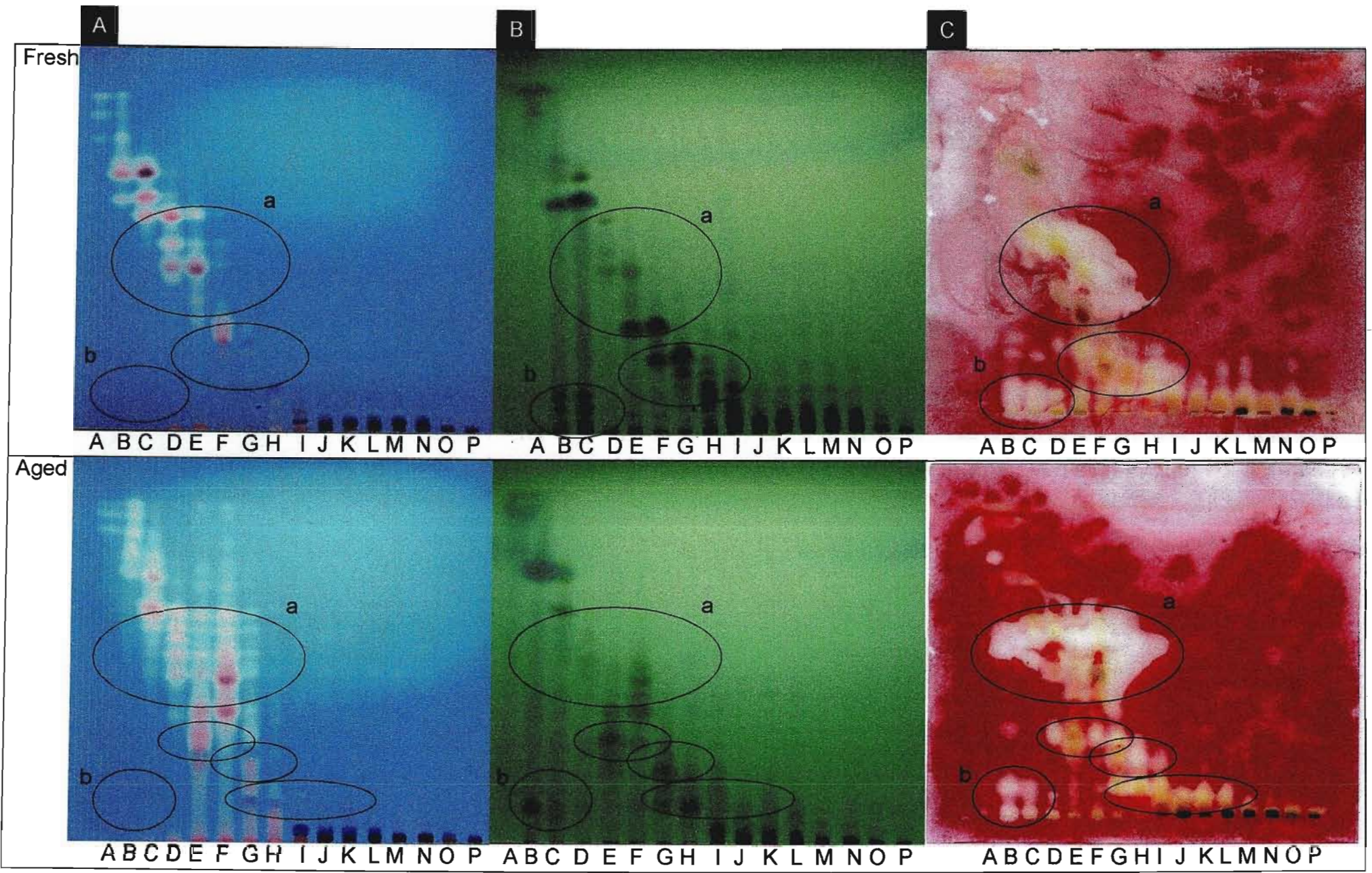| A			B			C		
Hex EtOH	Hex EtOH		Hex EtOH	Hex EtOH		Hex EtOH	Hex EtOH	
Fresh material	Stored material		Fresh material	Stored material		Fresh material	Stored material	
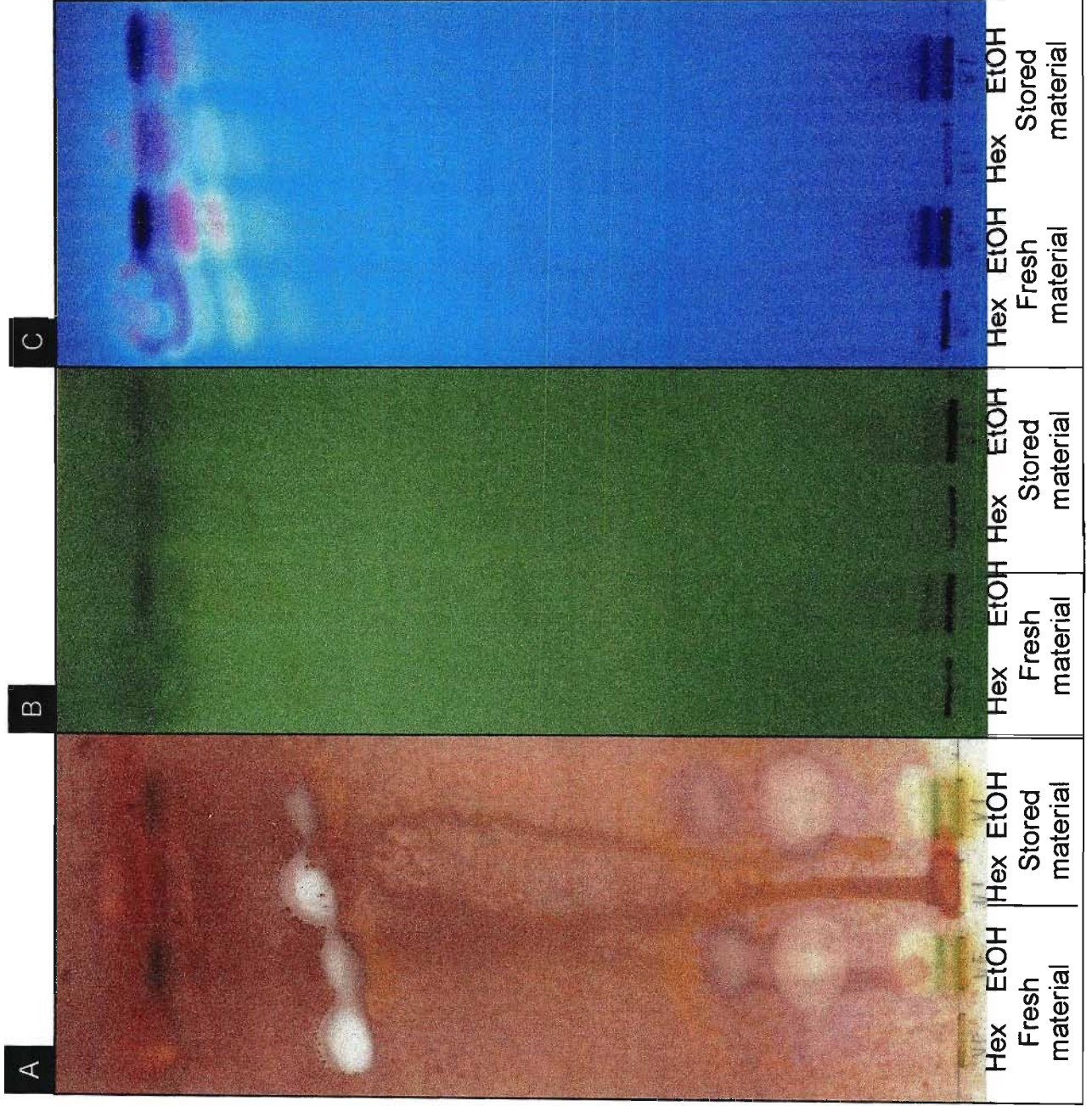
Plate AP1.13: **TLC plates of fractions from a VLC separation of ethanolic fractions of fresh and accelerated aged *Leonotis leonurus* leaf material. Plates are shown here viewed under UV $_{254}$ nm (A) and UV $_{366}$ nm (B). The result of bioautographic antibacterial (*S. aureus*) assay is also shown (C).**

The antibacterial compounds (a and b) in the fresh and aged material appear to be similar. The other antibacterial compounds however, appear to be different with more defined groups in the aged material.
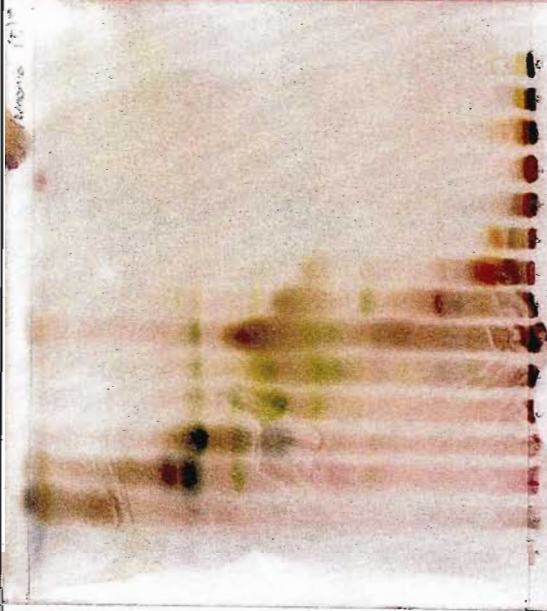
A

| 0 | 5 | 10 | 15 | 20 | 25 | 0 | 5 | 10 | 15 | 20 | 25 |
Days

Leonotis leonurus

B

| 0 | 5 | 10 | 15 | 20 | 25 | 0 | 5 | 10 | 15 | 20 | 25 |
Days

Vernonia colorata

Plate AP1.14: TLC plates of fractions from a VLC separation of ethanolic fractions of fresh and accelerated aged *Vernonia colorata* leaf material. The plate (A) shown here has been treated with anisaldehyde/sulphuric acid spray reagent. The result of bioautographic antibacterial (*S. aureus*) assay is also shown (B).

Several new antibacterial compounds (c), which show the distinctive streaking nature of fatty acids, appear in the aged material but not in the fresh.
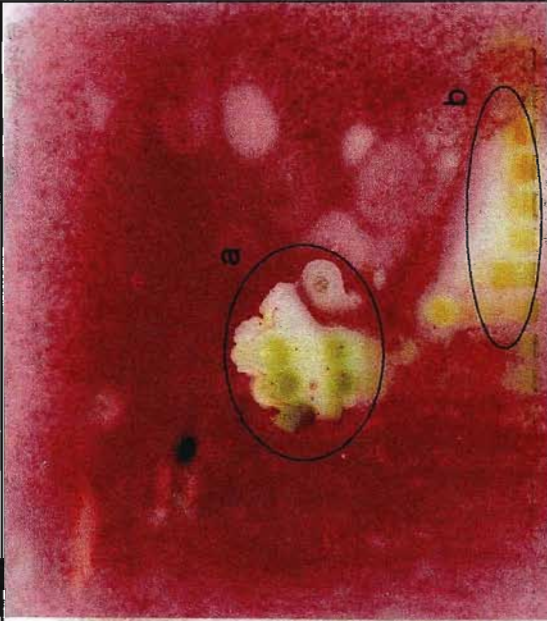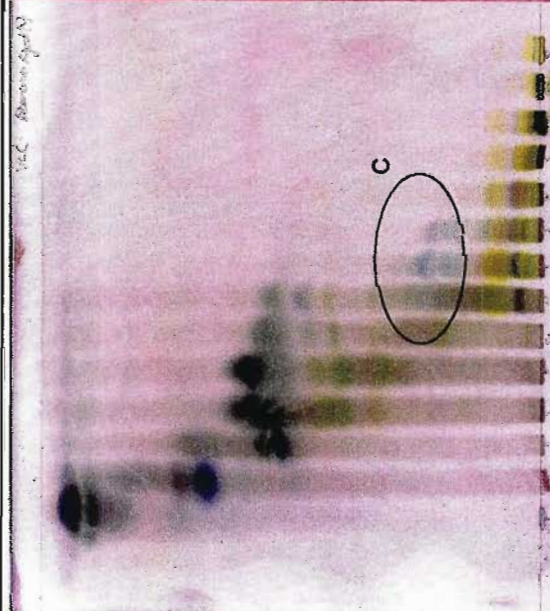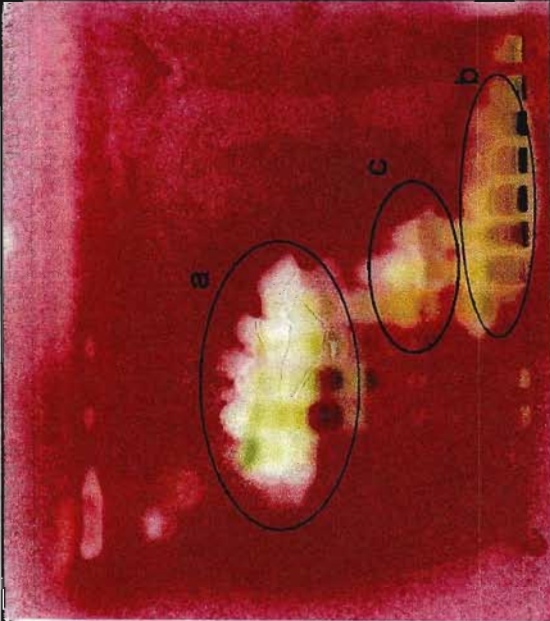
A B C D E F G H I J K L M N O P

Fresh

Aged

Fresh

A | B | ABCDEFGHIJKLMNO | ABCDEFGHIJKLMNO

Aged

ABCDEFGHIJKLMNO | ABCDEFGHIJKLMNO

APPENDIX 2

NEMATODE GROWTH AGAR (BRENNER, 1974):

Make up 3 g NaCl, 2.5 g peptone and 17g agar dissolved in 975 ml distilled water.
Autoclave for 20 min then add the following in order:

　　　1 ml cholesterol in ethanol (5 mgml^{-1})

　　　1 ml of 1M CaCl$_2$

　　　1 ml of 1M MgSO$_4$

　　　25 ml of 1M potassium phosphate buffer (pH 6.0)

POTASSIUM PHOSPHATE BUFFER (pH 6.0) (BRENNER, 1974):

Dissolve 12.014 g KH$_2$PO$_4$ and K$_2$HPO$_4$ in 100 ml distilled water.

M9 BUFFER (BRENNER, 1974):

In 1000 ml dissolve 6 g Na$_2$HPO$_4$, 3 g KH$_2$PO$_4$, 5 g NaCl and MgSO$_4$.7H$_2$0.