ANTI-BACTERIAL AND ANTI-INFLAMMATORY ACTIVITY OF MEDICINAL PLANTS USED TRADITIONALLY IN LESOTHO

by

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DECLARATION

The experimental work described in this research was carried out in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal Pietermaritzburg. The work was supervised by Professor J. van Staden and Doctor W. Stirk from January 2000 to February 2003.

The studies presented are the results of my own investigations, except where the work of others is acknowledged, and have not been submitted in any form to another Institute.

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I declare the above statement to be true.

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In loving memory of my mother, Mathato Beauty Shale

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ABSTRACT

A significant potion of the population in Lesotho relies on traditional medicine to meet its health care requirements. Traditional healers and herbalists were interviewed from Qacha's Nek (Highlands) and Mohale's Hoek (Lowlands) districts in Lesotho on plants used by the Basotho in traditional remedies. Fifteen plants were reported to be used for bacterial infections while thirteen plants were used for diseases associated with inflammation. Plant roots were most often used to make water extracts. Mainly high altitude plants are used with lowland healers obtaining most of their plant material from the highlands, either by collecting them or buying them from highland gatherers.

Leaves and roots of plants used to treat bacterial infections were extracted with hexane, methanol and water and the respective extracts screened at 100 mg ml^{-1} for anti-bacterial activity using the disc diffusion bioassay. Seven species displayed very high anti-bacterial activity against both Gram-positive and Gram-negative bacteria. A number of plant extracts had medium inhibitory activity, mostly against Gram-positive bacteria. This activity was mainly found in the root extracts. Six of the thirteen plants screened for anti-inflammatory activity using the cyclooxygenase-1 (COX-1) bioassay had activity above 90%. Hexane and methanol extracts were the most active while water extracts usually had lower activity.

Malva parviflora, Eriocephalus punctulatus and *Asparagus microraphis* exhibited high anti-inflammatory activity from hexane, methanol and water extracts made from leaf and root material. High anti-bacterial activity was also recorded from *M.*

parviflora and *E. punctulatus* hexane, methanol and water extracts. An investigation on seasonal variation and plant part substitution in medicinal activities for these plants was carried out.

Extracts of *M. parviflora* collected between June 1999 and July 2001 showed variation in anti-bacterial activity. Extracts made from leaves and roots inhibited the growth of both Gram-positive and Gram-negative bacteria. More bacterial strains were inhibited by extracts made from roots collected in cooler months. However, a trend in seasonal activity was not evident for either the roots or leaves because there was no detection of activity in some of the extracts made within the same months or seasons of the adjacent years. Variation in anti-inflammatory was detected for *M. parviflora* extracts. *E. punctulatus* leaf extracts did not exhibit any seasonal variation in anti-bacterial activity. Anti-inflammatory activity of *E. punctulatus* showed seasonal variation with the highest activity noted when material was collected during the cooler months and a decline in activity when collections were made during the warmer months. Hexane, methanol and water extracts made from leaves and roots of *A. microraphis* did not show any seasonal variation in anti-inflammatory activity. Thus, *M. parviflora* and *E. punctulatus* should be collected during the cooler months while *A. microraphis* can be collected throughout the year. Traditional healers, herbalists and vendors need to be encouraged to use aerial parts in substitution of ground parts which are reported to be highly utilized.

Effect of storage on anti-bacterial and anti-inflammatory activities of *M. parviflora, E. punctulatus* and *A. microraphis* were monitored. Dried, ground leaf and root material of the three plants was stored in a cold room, at room temperature and in the

Botanical Garden where the material was exposed to high and large changes in temperature. Dried hexane and methanol extracts made from leaves and roots of these plants were stored in a cold room and at room temperature. Initially, storage of the plant material under the three storage conditions caused an increase in anti-bacterial activity of the hexane, methanol and water extracts made from leaf and root material of *M. parviflora* and *E. punctulatus*. Storage for a longer period resulted in a decrease in inhibitory activity. TLC fingerprints developed from hexane and methanol extracts made from *M. parviflora* and *E. punctulatus* stored in a cold room and at room temperature showed a consistent number and colour of spots during the initial storage period. Prolonged storage resulted in a decline in the number and colour of detected spots. The stored hexane and methanol extracts made from leaves and roots showed a similar trend of increases and decreases in anti-bacterial activity as well as changes in spots with the storage of the extracts.

Testing of the effect of storage on anti-inflammatory activity of hexane, methanol and water extracts made from leaves and roots of *M. parviflora, E. punctulatus* and *A. microraphis* showed no change in inhibitory activity of hexane extracts obtained from the material and the extracts stored at the three storage conditions. Methanol and water extracts made from leaves and roots exhibited an increase in activity with prolonged storage. Generally, the stability of the inhibitory activity was longer for the stored dried plant material than the plant extracts.

Isolation of biological active compounds from *M. parviflora* was not successful due to loss in anti-bacterial activity as a result of collection of plant material from a different locality. Anti-inflammatory compounds could not be isolated due to insufficient

amount and the synergistic effect of the active compounds. The purified compounds exhibited loss of activity following HPLC purification which then re-appeared upon re-combining the fractions. A number of compounds were detected from essential oils of *E. punctulatus* using GC. Fractions containing these compounds gave positive anti-bacterial activity in the disc-diffusion, bioautographic and MIC bioassays as well as high anti-inflammatory activity with COX-1 and COX-2 anti-inflammatory bioassays. No anti-inflammatory compounds were isolated from *A. microraphis.*

PUBLICATIONS AND CONFERENCE PROCEEDINGS

A) *Oral papers*

T.L Shale, W.A Stirk and J. van Staden (1998). Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. 4th Annual Symposium on Indigenous Plant Use Research in Kwazulu-Natal (SIPUR). Department of Botany, University of Natal Pietermaritzburg.

T.L Shale, W.A Stirk and J. van Staden (1999). Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. South African Association of Botanists (SAAB) 25th Annual Congress. University of Transkei, Umtata.

Shale, T.L, W.A Stirk and J. van Staden (2000). Anti-bacterial and anti-inflammatory activity of *Malva parviflora*. South African Association of Botanists (SAAB) 26th Annual Congress. University of Potschefstroom.

Shale, T.L, W.A Stirk and J. van Staden (2001). Collection and storage of *Malva parviflora* and *Asparagus microraphis* for medicinal uses. South African Association of Botanists (SAAB) 27th Annual Congress. Rand Afrikaans University

B) *Posters*

Shale, T.L, W.A Stirk and J. van Staden (1999). Anti-bacterial and anti-inflammatory activities of medicinal plants used traditionally in Lesotho. 2000 Years of Natural

Products Research – Past, Present and Future. 5th Joint Meeting of the American Society of Pharmacognosy, Association Francaise pou 1' Enseignement et la Recherche en Pharmacognosie, Gesellschaft für Arzneipflanzenforschung and the Phytochemical Society of Europe. Amsterdam, The Netherlands.

c) Published papers

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d) Papers in preparation

T.L Shale, W.A Stirk and J. van Staden. Seasonal variation in medicinal activities and plant part substitution for *Malva parviflora, Eriocephalus punctulatus* and *Asparagus microraphis.* (In prep)

T.L Shale, W.A Stirk and J. van Staden. Effects of storage on anti-bacterial and anti-inflammatory activity of *M. parviflora, E. punctulatus* and *A. microraphis.* (In prep)

T.L Shale, W.A Stirk and J. van Staden. Variation in anti-bacterial and anti-inflammatory activity of *M. parviflora* collected at different localities. (In prep)

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CHAPTER 1

LITERATURE REVIEW

1.1 Plants as sources of medicine

Plants have long provided mankind with a source of medicinal agents, with natural products once serving as the source of all drugs (BALANDRIN, KINGHORN and FARNSWORTH, 1993). Utilization of plants as medicine has been in existence for hundreds of years before the development and spread of modern Scientific medicine (AKERELE, 1991 A). As early as 2000 B.C, the Chinese were using moulds to treat festering ulcers and the ancient Egyptians are known to have applied mouldy bread to open wounds. It is only relatively recently with the advent of modern technology and synthetic chemistry that we have been able to reduce our almost total dependence on the Plant Kingdom as a source of medicine (PLOTKIN, 1991). Today, higher plants continue to retain their historical significance as important sources of novel compounds, used directly as medicinal agents, as model compounds for synthetic or semi-synthetic structure modifications, and/or as sources of inspiration for the generation of synthetic organic medicinal chemicals (BALANDRIN, KINGHORN and FARNSWORTH, 1993; FARNSWORTH 1994).

1.2 Traditional medicine in developing countries

In developing countries where traditional medicine plays a major role in health care, people take recourse to herbal drugs for a variety of reasons. A large number of people depend on medicinal plants, mainly because they have no access to modern medicine (ELMI, 1991). The rural population of a country or people in peripheral slums of big cities are disposed to traditional ways of treatment because of its easy availability and reduced costs (ELMI, 1991; NKUNYA, WEENEN and BRAY, 1991; BANQUAR, 1993). A large number of rural people do not always utilize existing official health care services, as most of them are suspicious of Western medicine. This is partly because Western medicine does not encourage local beliefs and the behaviour related to health matters (SIDINGA, 1995). Moreover, Western medicine in many countries came in the wake of Colonialism and is considered an alien system imposed on the local people (ANAND and NITYANAND, 1984).

The World Health Organization estimates that approximately 80% of the developing world's population in rural areas, especially in Asia, Latin America and Africa meet their primary health care needs through traditional medicine (BANQUAR, 1993; FARNSWORTH, 1994; SRIVASTAVA, LAMBERT and VIETMEYER, 1996). For example, in China, plant based medicine is the backbone of the health care for perhaps a billion people with plants being used for the primary health care needs of 40% of China's urban patients and over 90% of its rural patients (SRIVASTAVA, LAMBERT and VIETMEYER, 1996). For more than two decades now, the World Health Organization has encouraged the use of traditional medicine, especially in

developing countries by promoting the incorporation of its useful elements into national health care systems (SIDINGA, 1995).

1.3 Traditional medicine in Western countries

In industrialised countries, about 25% of all prescription drugs contain active principles derived from natural products. These are still extracted from higher plants and the situation has persisted for at least the last 25 years (FARNSWORTH, 1994). Many of these drugs were discovered by following leads provided from knowledge of indigenous people (COX, 1994). These drugs are being used in modern Western medicine, but not necessarily for the same purpose as used by the native cultures (PRANCE, 1984).

A distinct trend is noticeable towards the use of plant drugs, perhaps due to reports of the occurrence of estrogenic diseases caused by some synthetic drugs and antibodies (ANAND and NITYANAND, 1984). The demand is being sustained by outbursts of consumer's interest in products that are "all-natural" as well as by aggressive marketing of herbal remedies (SRIVASTAVA, LAMBERT and VIETMEYER, 1996). For example, in 1980, American consumers paid $8 billion for prescription drugs derived solely from higher plant sources. From 1959-1980, drugs derived from higher plants represented a constant 25% of all new and refilled prescriptions dispensed from community pharmacies America (BALANDRIN, KINGHORN and FARNSWORTH, 1993).

1.4 Traditional folklore leads in plant derived drugs

On numerous occasions, the folklore records of many different cultures have provided leads to plants with useful medicinal properties. Plants used as toxins and hunting poisons in their native habitats have yielded numerous purified compounds which have proven to be indispensable in the practice of modern medicine (BALANDRIN, KINGHORN and FARNSWORTH, 1993). For example, the curare alkaloids were obtained from South American vines which had long been used to make arrow poison. Also, African *Strophanthus* species and calabar beans, which were originally used as arrow and ordeal poisons yielded medicinally useful cardiac glycosides (BALANDRIN, KINGHORN and FARNSWORTH, 1993).

Until the twenth century, most drugs were discovered from folk knowledge that was disseminated through indigenous cultures, either orally or though written form known as herbals (COX, 1995). Such folk knowledge was of two forms: specialist knowledge held by a few individuals within the society and generalist knowledge available to all members of a society (COX, 1995). The idea of developing drugs using plants as a starting point has always been highly regarded in developing countries as most of these countries have abundant starting material in their natural flora and scientific expertise is usually available to implement this type of a program (FARNSWORTH, 1994).

Even though the first chemical substance to be isolated from plants was benzoic acid, discovered in 1560, the search for useful drugs of known structures from the Plant Kingdom did not really begin until 1775 when WILLIAM WITHERING

discovered digitoxigin, a cardiotonic drug from *Digitalis purpurea*, using the ethnobotanical approach (COX, 1994). In 1804 morphine was separated from the dried latex of *Papaver somniferum* L. (opium) (COX, 1994; FARNSWORTH, 1994). Historically, ethnobotanical leads have resulted in three different types of drug development. These are: (1) unmodified natural plant products where ethnomedicinal use suggested clinical efficacy (e.g. Digitalis), (2) unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use (e.g. vincristine), and (3) modified natural or synthetic substances based on a natural products used in folk medicine (e.g. aspirin) (COX, 1994).

In some cases, biologically active substances derived from plants resulted in a poor pharmacological/toxicological profile for the purpose of using them as drugs in humans. Such compounds have, however, served as templates for designing and synthesising new drug entities or tools used in studying biological systems and disease processes (BALANDRIN, KINGHORN and FARNSWORTH, 1993; FARNSWORTH, 1994). For example, the alkaloids such as atropine, quinine and cocaine found in plants, have served as models for the design and synthesis of anticholinergics, antimalarial drugs and other local, anaesthetics such as the analgesics pentazocine (talwin) (BALANDRIN, KINGHORN, and FARNSWORTH, 1993).

1.5 Factors that contribute to the decline of traditional health care practices

Many medicinal plants have not been well studied, tested or documented. Most of the information is still in the hands of traditional healers. There is, therefore, a real danger that such knowledge will be lost when the healers pass away unless the information is carefully recorded (NYAMWAYA, 1992). Unfortunately, traditional knowledge of these medicine men or women is not being passed on to the next generation. The advent of Western medicine in some of the remote areas has caused young members of communities to show little interest in learning the traditional ethnomedicinal lore (PLOTKIN, 1991). In some cases, traditional healers make it difficult for Scientists to obtain detailed information from them. They consider such information their source of income and are therefore unwilling to part with it freely (KOFI-TSEKPO, 1991). From the viewpoint of many traditional healers, certain specialist knowledge should not be made public, even within their own societies. Much of this knowledge is kept private through ritual and taboo (CUNNINGHAM, 1991). Knowledgeable individuals, mainly elders who do not practice traditional healing are much more willing to impart information on plants used in traditional medicines. These people find themselves duty bound to treat a disease, or to solve a health problem in a community (KOFI-TSEKPO, 1991).

Despite their importance, medicinal plants are seldom handled within an organised, regulated sector. Plants are being lost at an ever-increasing rate, faster by orders of magnitude than rates of evolutionary replacement. Most plants are over-exploited with little or no regard to the future (MAHUNNAH and MSHIU, 1991; SRIVASTAVA,

LAMBERT and VIETMEYER, 1996). For instance, the discovery of taxol in the Pacific yew tree caused it to become endangered until the active ingredient could be synthesized from a precursor found in common yew species. *Prunus africana* from the forests of Africa is being eliminated because of the commercial exploitation of the bark to treat prostate problems (PRANCE, 1994; SRIVASTAVA, LAMBERT, and VIETMEYER, 1996). The indiscriminate harvesting of wild plants damages both ecosystems and biodiversity. The damage is especially serious when the bark, seeds and flowers, all essential for species survival, are removed (SRIVASTAVA, LAMBERT, and VIETMEYER, 1996).

Concern is growing that many medicinal plants are on the verge of extinction and there is an urgent need to protect rare and threatened species (SRIVASTAVA, LAMBERT, and VIETMEYER, 1996). PRINCIPE (1991) and BALADRIN, KINGHORN and FARNSWORTH (1993) reported that the earth is on the brink of experiencing an extinction of species unparalleled in human history. The most widely accepted estimate by the International Union for Conservation of Nature and Natural Resources (IUCN) and the World Wide Fund for Nature (WWF), is that 60 000 higher plant species could become extinct or near extinct by the middle of the twenty first century if the current trends of destruction of tropical forest habitats continue at current rates. Samples collected today may in future be found to combat dreaded diseases but there is no guaratee that the plants will then still exist. Many significant opportunities for successful drug development will also be lost (BALADRIN, KINGHORN and FARNSWORTH, 1993; SRIVASTAVA, LAMBERT, and VIETMEYER, 1996). Despite these problems, enthusiasm remains high among the people involved in the discovery of new biologically active natural products from

plants and other sources. The inherent interest have shown the existence of great opportunity to discover chemical entities that may cure some of the most threatening forms of diseases known to mankind (NIGG and SEIGLER, 1992).

It is therefore imperative that endangered, fragile and over-exploited genetic resources be preserved to the greatest extent possible for future generations. Such generations may have at their disposal the technical and intellectual tools necessary to successfully exploit and manage these species intelligently (BALADRIN, KINGHORN and FARNSWORTH, 1993). Ethnobotanical exploration should be linked to the urgent need for sustainable conservation strategies for medicinal plants, otherwise the human expansionist demands can be expected to bring environmental deterioration and biotic destruction (MAHUNNAH and MSHIU, 1991). However, ethnobotanical leads, biological indicators and other signs of medicinal potential tend to take few measures to protect plant species and their original habitats from destruction. This results from the race to uncover valuable compounds before they disappear and in the process causing plant species to experience an unprecedented rate of extinction (SHELDON, BALICK, and LAIRD, 1997).

1.6 The health care system in Lesotho

Lesotho is a country in southern Africa where the majority of the population are Sotho speaking. It is situated between 28 – 30 ^{0} South and 27 – 30 ^{0} East. According to a population census carried out in 1996, Lesotho's population is estimated at 1.8 million with an annual growth rate of 3.0%. The country has a land area of 30555 km^2 which is divided into 4 zones: a mountain zone comprising 65% of the land area,

a lowland area with an altitude below 1860 m (19%), foothills (7%) and the Senqu valley (8%). Fifteen percent of the population live in the lowland urban areas and the remainder in rural villages (GUILLARMOD, 1971; MARSHALL, 1998).

The government health service in Lesotho is based on Western medical principles. However, a significant portion of the population relies on traditional medicine to meet its health care requirements (MARSHALL, 1998). Traditional knowledge on utilisation of medicinal plants is passed on by traditional healers and herbalists (CHAPTER 3).

Prior to independence, traditional medicine was discouraged and legislated against. Following independence in 1966, national drug legislation was formulated for the control of some traditional remedies. The proposed regulation led to the registration of traditional medicines for an initial period of 8-10 years, based on safety as the sole criterion (AKERELE, 1991 B; MARSHALL, 1998). Legal regulations for traditional medicine have been in existence since 1969 in Legal Notice Number 23 of Laws of Lesotho. These were amended in 1976 and 1978. Act no. 19 of 1978 allowed the establishment of the Lesotho Universal Medicine Men and Herbalists Council (LUMHC). Members are appointed by the Ministry of Health and the Institution functions in both the Council which has a voice in government policy discussions and as a professional association (MARSHALL, 1998). This association currently includes 23% of all licensed Traditional Medical Practitioners (TMP's) as members. There are no other healer's associations which are allowed to operate legally in the country. While the remaining unregistered TMP's may practice, they have no voice in medicinal politics (MARSHALL, 1998). The present record shows that 1275 traditional healers and herbalists have been registered by the Ministry of Health

during 1995-1999, compared to 150 registered modern Western doctors (41 private practitioners, 95 in government hospitals and 19 in church/mission hospitals) (LESOTHO MEDICAL, DENTAL and PHARMACY COUNCIL, 1998/99). A record of traditional practitioners is kept by the Ministry of Health in Lesotho.

1.7 Inflammatory diseases

Inflammatory diseases are among the ailments treated traditionally using medicinal plants. The occurrence of some diseases resulting from inflammation are believed to be caused by the production of prostaglandin in the body (ZURIER, 1982). Prostaglandins are a family of C_{20} fatty acids widely distributed in almost all living animal cells, tissues and glands (HICKOCK, ALOSIO and BOCKMAN, 1985; RANG and DALE, 1987). They are formed through the action of a membrane-bound enzyme complex, prostaglandin synthase (HICKOCK, ALOSIO and BOCKMAN, 1985). The principal substrate for prostaglandin is arachidonic acid which is found as a constituent of phospholipids. Arachidonic acid is released from phospholipids by hydrolytic action of a class of membrane-associated enzymes referred to as phospholipases (HALL, and BEHRMAN, 1982). The acid is degraded by the so-called arachidonic "cascade" which comprises of two distinct pathways (Figure 1.1) (RANG and DALE, 1987).

Arachidonic acid may be oxidised by the enzyme cyclooxygenase (COX) to prostaglandin or converted by the enzyme 5-lipoxygenase to leukotrienes. The two products are believed to be mediators in causing inflammatory symptoms (RANG and DALE, 1987). COX exists in at least two isomers (COX-1 and 2) with similar

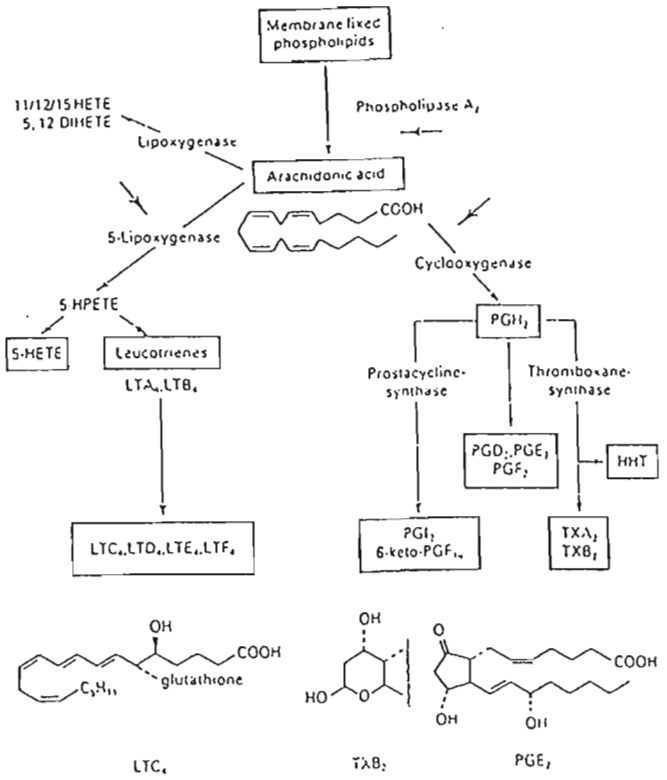


Figure 1.1 Metabolism of arachidonic acid (WAGNER and JURCIC, 1991)

molecular weights (approximately 70 kDa). COX-1 is expressed constitutively in many tissues and was first characterised, purified and cloned from sheep vesicular glands. COX-2 is induced in cells exposed to proinflammatory agents, including cytokines, mitogenes and endotoxin (MITCHELL, AKARASEREENONT, THIEMERMANN and FLOWER, 1994; TAKETO, 1998).

1.7.1 Effects of prostaglandins

Prostaglandins induce redness (erythema) and swelling (edema) associated with heat and pain. These result in the development of inflammatory diseases such as

asthma, articular rheumatism and psoriasis. The effects are due to the ability of prostaglandins to cause dilation of blood vessels (vasodilation) and increase vascular permeability, thus resulting in acute inflammation which leads to swelling (ZURIER, 1982). The vasodilation increases the flow of blood through the inflammed tissues and this augments the adema caused by agents such as brandykinin and histamine which increase vascular permeability (VANE and BOTTING, 1996).

1.7.2 Inhibition of prostaglandin synthesis

The inhibition of prostaglandin synthesis has been reported from drugs such as non-steroid anti-inflammatory drugs (NSAIDs). These include aspirin, indomethacin and other substituted arylacetic acids such as ibuprofen, which have the ability to inhibit cyclooxygenase activity in prostaglandin synthesis (HALL and BEHRMAN, 1982). Steroidal anti-inflammatory agents such as glucocortids act by blocking the catalytic degradation of membrane phospholipids by phospholipase, thus inhibiting the release of arachidonic acid (HALL and BEHRMAN, 1982). Compounds extracted and isolated from higher plants have shown potential to inhibit prostaglandin synthesis. For instance, ethanolic extracts of *Haphophyllum hispanicum* Spach, a perennial herb endemic to the Mediterranean area showed 50% reduction In the ear edema assay (PRITEO, RECIO, GINER, MANEZ and MASSMANIAN, 1996). Other reports indicate that *Bidens campylotheca* (Compositae) is used as a traditional remedy in Hawaiian folk medicine for anti-inflammatory and anti-asthmatic purposes (REDL, BREW, DAVIS and BAVER, 1994).

1.8 Infectious diseases

Other diseases commonly treated by traditional healers and herbalists using traditional medicinal plants are those that result from microbial infections. Infectious diseases caused by pathogenic bacteria and fungi have increased in terms of annual prevalence of morbidity and mortality. The factors enhancing infectious disease prevalence include inadequate sanitation, poor hygiene and overcrowded living conditions (RASOANAIVO and RATSIMANGA-URVERG, 1993; KERR and LACEY, 1995). Given the living conditions of the majority of people in developing countries, sanitation and hygiene levels are not comparable to those of first world countries. This exposes people in developing countries to a wider array of microbial pathogens, increasing their susceptibility to bacterial or infectious-type diseases, thus causing high mortality rate and low life expectancy in most developing countries (RASOANAIVO and RATSIMANGA-URVERG, 1993; TAYLOR, RABE, MCGAW, JÄGER and VAN STADEN, 2001). The spread of diseases associated with microbial infections also occurs in developed countries, with anti-microbial agents being the second most frequently prescribed class of drugs (KERR and LACEY, 1995).

A number of bacteria enter the body by penetrating the mucosa lining, the lesioned skin or through hair follicles and provoke bacterial infections. The diseases may occur as local purulence (e.g. furuncles, ulcers, phlegmous, inflammation of the oparynx and tonsilitis) which can subsequently become generalised as a blood infection (BRANTER and GREIN, 1994). In the past, antibiotics were used for therapy but many of the pathogenic bacteria are now resistant to these antibiotics. Natural products of higher plants have also received much attention as potential

useful sources of antibacterial agents for external use, e.g. compresses, cataplasm and ointments (RASOAVANAIVO and RATSIMANGA-URVERG, 1993; BRANTER and GREIN, 1994).

The discovery and development of a useful anti-microbial agent depends to a great extent on the search for analogues of a lead compound (CHU and SHEN, 1995). For many years, new anti-microbial lead structures have originated from microbiological fermentation or isolation of natural products from plant extracts (CHU and SHEN, 1995). For example, mashed leaves of *Helichrysum pedunculatum* (Billiard and Burtt) are used among Xhosa speaking tribes in South Africa to bandage the wound caused by circumcision. Water extracts of the leaves of this plant showed positive antibacterial activity *in vitro* against bacterial strains used in the test (DILIKA, NIKOLOVA and JACOBS, 1996).

1.9 Aims of the study were:

1. To investigate the role played by traditional healers and herbalists in the health care system in Lesotho (chapter 3);
2. To screen medicinal plants used by the Basotho people for anti-bacterial and anti-inflammatory activities (chapter 3);
3. To determine the seasonal variation in anti-bacterial activity of *Malva parviflora* and *Eriocephalus punctulatus* (chapter 4);
4. To determine the seasonal variation in anti-inflammatory activity of *M. parviflora*, *E. punctulatus* and *Asparagus microraphis* (chapter 4);

5. To determine if different plant parts (leaves and roots) could be substituted in traditional remedies to minimise destructive harvesting (chapter 4);

6. To determine the effects of storage on anti-bacterial activity of dried *M. parviflora* and *E. punctulatus* material and stored extracts (chapter 5);

7. To determine the effects of storage on anti-inflammatory activity of *M. parviflora*, *E. punctulatus* and *A. microraphis* material and stored extracts (chapter 5);

8. To extract, isolate and identify anti-bacterial and anti-inflammatory compounds from *M. parviflora* (chapter 6);

8. To extract, isolate and identify anti-bacterial and anti-inflammatory compounds from *E. punctulatus* (chapter 7) and

9. To extract, isolate and identify anti-inflammatory compounds from *A. microraphis* (chapter 8).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Preparation of plant extracts

Plant material was dried at 50 ^{0}C, finely ground and stored at room temperature. Extracts were prepared using organic and non-organic solvents. Dried organic solvent extracts were redissolved in the same extracting solvents. Extracts made using water were redissolved in water at the required concentrations.

Often plant extracts were first tested in the anti-bacterial bioassays and then dried down and redissolved in ethanol or water at the required concentration to be tested in the anti-inflammatory bioassays.

2.2 Anti-bacterial activity

2.2.1 Disc-diffusion bioassay

The disc-diffusion bioassay as described by RASOAVANAIVO and RATSIMANGA-URVERG (1993) and RABE and VAN STADEN (1997) was used to determine the growth inhibition of bacteria by plant extracts. The test bacteria used were the Gram-positive *(Micrococcus luteus, Bacillus subtilis, Staphylococcus aureus, S.epidermis)* and the Gram-negative *(Escherichia coli, Psuedomonas aeruginosa* and *Klebsiella*

pneumoniae) bacteria. Bacterial stock was obtained from the department of microbiology at the University of Natal Pietermaritzburg. These were maintained at 4 ^{0}C on molten Mueller-Hinton (MH) agar and were sub-cultured monthly.

Ten ml of MH agar (38 g in 1000 ml of distilled water) were poured into sterile petri-dishes (9 cm) and allowed to set for the preparation of base plates. Molten MH agar, held at 48 ^{0}C, was inoculated with a broth culture ($10^{6} - 10^{8}$ bacteria ml^{-1}) of test bacteria and poured over the base plates to form a homogenous top layer. Ten µl of the plant extracts being tested were applied to a filter paper disc made from Whatman No.3 filter paper (6 mm diameter). The discs were dried in a stream of air on a sterile laminar flow bench and placed on top of the agar plates. Each extract was tested in quadruplicate (4 discs/plate) and a neomycin (200-500 µg ml^{-1}) disc was used as a positive control. The plates were incubated at 37 ^{0}C for 18 hours after which the clear ring around each disc was measured. This was called the zone of inhibition.

Anti-bacterial activity of plant extracts was determined by the following equation:

$$\text{Inhibition activity} = \frac{\text{diameter of plant extracts zone of inhibition (mm)}}{\text{diameter of control zone of inhibition (mm)}}$$

2.2.2 Bioautographic bioassay

The bioautographic bioassay as described by HAMBURGER and CORDELL (1987) and SLUSARENKO, LONGLAND and WHITEHEAD (1989) was used to determine the growth inhibition of bacteria by plant extracts

Overnight cultures of *B.subtilis, S. aureus* and *E. coli* were prepared in 20 ml of MH broth in sterile centrifuge tubes at 37 ^{0}C. The following day (18 hours later), the cultures were centrifuged using a J_4 centrifuge at 3000 rpm for 10 min at 20 ^{0}C. The supernatant was decanted off and the pellet redissolved in 10 ml of fresh MH broth.

Dried plant extracts were redissolved in the solvent used for the extraction at a concentration of 50 mg ml^{-1}. Ten µl of the extracts were spotted onto glass TLC plates (TLC plate 20 X 20 cm, silica gel 60 F_{254}) and developed in a previously determined solvent system that gave the best separation for the extract. Plates were dried in a fume hood for 10 minutes.

Dried TLC plates were sprayed with the previously described dense bacterial suspension in a spray hood and incubated under 100% relative humidity at 38 ^{0}C for 24 hours. Plates were then sprayed with iodonitrotetrazolium chloride (INT) solution at 2 mg ml^{-1} and incubated for a further hour. The inhibition of bacterial growth was indicated by clear zones against the red colour on the TLC plate. The red colour resulted from the reaction of the INT with the bacteria.

2.2.3 Minimum inhibition concentration (MIC) microplate dilution method

The microplate bioassay as described by ELOFF (1998) was used to determine the minimal inhibitory concentration (MIC) values of the plant extracts with anti-bacterial activity.

Overnight cultures of *B.subtilis and S. aureus* were prepared in 5 ml of MH broth in sterile 20 ml McCartney bottles and incubated at 37 ^{0}C. Absorbancy reading of the bacteria was measured at the wavelength of 600 nm using a Varian Cary 50 Conc, UV – visible spectrophotometer before the tested bacteria were diluted. Bacterial cultures were then diluted at the ratio of 1:100 with MH broth (200 µl of the bacteria in 20 ml MH broth).

Hundred µl of sterile distilled water were pipetted into each of the 96-wells of the microplates and the plates labelled appropriately based on the test bacteria. The extracts were redissolved in ethanol at 50 mg ml^{-1} and 100 µl of the extracts were added into the wells at row A. Hundred µl of the extract were pipetted from well A to well B and mixed. The 2-fold serial dilution continued until well H was reached (final concentration of the extract 0.195 mg ml^{-1}) and 100 µl of this extract were then discarded. Hundred µl of the diluted bacterial cultures were added to each well.

The control included the following standards:

1) MH broth (or water) with no bacteria in order to test that the INT (a stain which turns red when reacting with bacteria) does not change colour;

2) The highest concentration of the solvent used (e.g. ethanol) to ensure that the solvent does not inhibit bacterial growth;

3) The test bacteria without addition of the extracts; and

4) Neomycin used as a positive control where 100 µl of neomycin (10 mg ml^{-1}) were serially diluted in the same way as the extracts.

Microplates were covered with parafilm and incubated at 37^0C overnight. Forty µl of INT (0.2 mg ml^{-1}) were added to every well and the plates incubated at 37^0C for a further 30 minutes. Inhibition of bacterial growth was indicated by wells which remained clear after the addition of INT.

2.3 Anti-inflammatory activity

2.3.1 Cyclooxygenase-1 (COX-1) bioassay

The active prostaglandin-synthesis inhibition compounds from plant extracts were investigated using the modified cyclooxygenase-1 (COX-1) assay as described by JÄGER, HUTCHINGS and VAN STADEN (1996).

Ten µl of sheep seminal vesicle microsomes (Appendix 1) and 50 µl co-factor solution (3 mg ml^{-1} adrenalin and 3 mg ml^{-1} reduced glutathione in 0.1M Tris buffer, pH 8.2) were incubated in an ice bath for 15 minutes. Twenty µl of aqueous plant extracts or 2.5 µl of the ethanolic extracts and 17.5 µl water were added to 20 µl ^{14}C-arachidonic acid and 60 µl of the enzyme/cofactor solution. This was incubated at 37

^{0}C for 8-10 minutes depending on the batch of enzyme used. The reaction was terminate by the addition of 10 µl 2N HCl.

Background and control samples were included where 2.5 µl ethanol and 17.5 µl water were used. For a background reaction, the HCl was added prior to the arachidonic acid solution. Indomethacin (20 µM), a commercially available anti-inflammatory drug was used as a standard.

After incubation, 4 µl prostaglandin carrier solution was added. Prostaglandins were separated from the unmetabolised arachidonic acid by silica gel column chromatography. The samples were loaded onto individual columns (silica gel 60, particle size 0.063-0.200 mm, Merck). Four ml of hexane:dioxane:acetic acid (350:150:1 v:v) mixture were added, one ml at a time, to the column to elute the arachidonic acid. The prostaglandins were then eluted by the addition of 3 ml ethyl acetate:methanol (425:75 v:v) mixture. Four ml scintillation solution were added to each vial containing the prostaglandins and radioactivity was measured using a Beckman LS 6000LL scintillation counter. The assay was performed in duplicate.

The percentage inhibition of the assays was determined by the following equation:

$$\text{Inhibition (\%)} = 1-\left[\frac{\text{Dpm sample} - \text{Dpm background}}{\text{Dpm control} - \text{Dpm background}}\right] \times 100$$

2.3.2 Indomethancin standard curve

Indomenthacin is a commercially available Non-Steroid anti-inflammatory drug (NSAID) which has the ability to inhibit the action of cyclooxygenase enzyme during the synthesis of prostaglandins from arachidonic acid (HALL and BEHRMAN, 1982). Indomenthacin (20 µM) was included in the COX-1 and 2 bioassays as a standard. A standard cure was also prepared. Indomethacin was serially diluted to concentrations 0.001, 0.01, 0.1, 1, 2.5, 7.5, 20 and 100 µM respectively. The dilutions were assayed using the COX-1 anti-inflammatory bioassay as described in Section 2.3.1 (Appendix 2).

2.3.3 Cyclooxygenase-2 (COX-2) bioassay

The assay was performed as described by NOREEN, RINGBOM, PERERA, DANIELSON and BOHLIN (1998).

Commercially available COX-2 enzyme prepared from sheep placenta was used for the assay. The procedure used to carry out the COX-2 bioassay was similar to the one used for the COX-1 bioassay with modifications which involved the addition of 100 µl haematin to 10 ml cofactor solution (6 mg adrenaline and 3 mg reduced glutathione in 0.1 M Tris buffer, pH 8.0). Also, 250 µl of the enzyme and 1250 µl of cofactor solution were incubated on ice for 5 minutes. In addition to indomenthacin (200 µM), nimesulide (200 µM) was also used as a standard.

2.4 TLC Fingerprinting

TLC fingerprinting was carried out using organic solvent extracts. Aqueous extracts were not tested. Leaf and root extracts of the same plant were developed using the same solvent system for comparison of compound spots. Aqueous extracts had the tendency of not separating well when run using the solvents used for the organic solvent extracts.

Dried organic solvent extracts were redissolved in organic solvent at 50 mg ml^{-1}. Ten µl of each extract were spotted onto a plastic TLC plate (TLC plastic sheets 20 X 20 cm, silica gel 60 F_{254}, Merck). These were cut to the size that accommodated the spotted extracts. TLC plates were developed in 20 ml of the various solvent systems to determine the solvent system which gave the best separation of the compounds.

After the separation was completed, the TLC plates were dried in a fume hood for 10 minutes and viewed under UV light at 254 nm and 366 nm. The plates were then stained with anisaldehyde (Appendix 3), dried for 5 minutes at 110 ^{0}C and observed under normal light. Photographs were taken both under UV and normal light.

CHAPTER 3

ETHNOBOTANICAL STUDIES AND SCREENING OF MEDICINAL PLANTS FOR ANTI-BACTERIAL AND ANTI-INFLAMMATORY ACTIVITIES

3.1 Introduction

Herbal medicine is the oldest form of therapy practiced by mankind, and much of this medicinal use of plants seems to have been on a highly developed "dowsing" instinct, which lead the healer of the tribe to the right plant and taught him or her its use (GRIGGS, 1981). To a modern mind, the idea may seem bizarre, but wild animals certainly posses such an instinct seeking out plants which will supply the nutrients they need and unerringly avoiding those that will poison them (GRIGGS, 1981). These dowsing powers would explain the astonishing continuity of medicinal plant usage in the days before there were written records, or in tribes who have never known the medicinal plants. The chain of oral tradition must have been broken over and over again by death and the scattering or obliteration of tribes (GRIGGS, 1981). Herbal drugs are neither the medicine of the poor alone nor the remedies for nostalgic people, but are actually an important tool for the treatment of millions of people of different cultures, social class and status throughout the world (ELMI, 1991).

Traditional medicine is a priceless heritage created in the historical course of prevention and treatment of diseases over a long period (MAHUNNAH and MSHIU, 1991. The usage of herbal medicines offer tremendous economic potential, not only as an export product, but as the resources for developing "locally controlled industries", which can substitute for costly pharmaceutical imports. Such developments are taking place in Thailand, Turkey, the Philippines and China, where herbal medicines constitute big business (MSHIU, 1991).

In most markets in underdeveloped countries one does not fail to find a corner which could be considered an "open pharmacy" and where medicinal plant preparations are spread out to attract the attention of customers. Vendors do not usually prescribe as the customers are quite knowledgeable about the type of plant they wish to purchase (BAGALE, NOAMESI and DAGNE, 1991). Although the usage of most of marketed traditional plants does not require special knowledge, there are instances where overdosing leads to toxic effects, particularly in the use of the anthelmintics (BAGALE, NOAMESI and DAGNE, 1991). The use of such medicines in their crude forms without establishing scientifically their efficacy and safety, could be detrimental to health (NKUNYA, WEENEN and BRAY, 1991).

Therefore, there is an urgent need to carry out scientific evaluations of traditional medicines worldwide. Apart from efficacy and safety of traditional medicines, scientific evaluation may lead to the isolation of a pure active ingredient which otherwise occurs in minute quantities in the crude plant extracts (NKUNYA, WEENEN and BRAY, 1991). Pharmacological studies not only help to determine efficacy of these traditional preparations, but also to establish the required dosage

(BAGALE, NOAMESI and DAGNE, 1991). With advances in plant molecular biology, new cell culture techniques, new bioassays and availability of new and precise analytical methods for screening plants, there is a possibility of expansion as far as the discovery of natural products is concerned (MSHIU, 1991). A 1988 consultancy report by a United Kingdom firm McAlping and Warrier indicated that the market potential for sophisticated herbal drugs in the Western world could range from $ 4.9 billion to $ 47 billion by the year 2000, especially if the AIDS epidemic continued unchecked (MSHIU, 1991).

3.2 Aims

The aims of this part of the study were:

1) To investigate the role played by traditional healers and herbalists in the health care system of Lesotho; and
2) To screen medicinal plants used by the Basotho people for anti-bacterial and anti-inflammatory diseases.

3.3 Materials and Methods

3.3.1 Collection of ethnobotanical data

Twenty Basotho traditional healers and herbalists were interviewed by questionnaire (Appendix 4) in March 2000. Ten were based in Mohale's Hoek district, a lowland area (altitude of 1860 m) and ten were from Qacha's Nek district, a highland area

(altitude of 2500 m). The questions asked ranged from the plants used for treating infections and inflammatory diseases, plant parts used, methods of extraction, extracting solvents and if the extraction used for the treatment of a particular disease was done from one plant or from a mixture of several plant extracts. Forms and dosage of medications, the storage and cultivation of plants for future use were also considered. Traditional healers and herbalists were asked on ways in which plants are collected and areas rich in plant materials, measures taken if a patient reacted negatively to the prescribed medication and if there is any follow up on the improvement of the treated patients. Other aspects covered in the questionnaire included training and acquisition of knowledge, the period of study and training, number of patients treated per month, cost of treatment and the extent of cooperation with other traditional healers and herbalists as well as Western doctors.

3.3.2 Plant material

Twenty-four plants used for the treatment of inflammation and bacterial infections were collected from Mohale's Hoek and Qacha's Nek districts in Lesotho during March 1998. The interviewed traditional healers and herbalists assisted in the plant collection and information on plant usage (Table 3.1). Voucher specimens of the collected plants were deposited at the Herbarium at the Natal University, Pietermaritzburg. Plant material was dried at 50 ^{0}C, ground to a fine powder and stored at room temperature until use.

Table 3.1: Medicinal plants used traditionally by the Basotho people to treat diseases caused by bacterial infections and inflammation

Family	Species name / voucher specimen	Plant parts used	Traditional uses
AMARYLLIDACEAE	*Boophane distica* (L.f) Herb. (SHALE 11 UN)	Bulb	Infusion used on external sores and wounds. The application also relieves rheumatic pain and draws out pus and pain
ASPARAGACEAE	*Asparagus microraphis* (Kunth) Oberm. (SHALE 15 UN)	Roots	Decoction drunk for rheumatism and menstrual pains
ASTERAEAE	*Aster bakeranus* Burtt Davy & C.A Sm (SHALE 6 UN)	Roots	Decoction drunk to treat syphilis and used to bathe syphilitic sores. Powder snuffed for headache
	Aster sp. (SHALE 22 UN)	Whole plant	Infusion used to treat wounds and sores
	Dicoma anomala Sond. (SHALE 10 UN)	Roots	Decoction used a remedy for diarrhoea, stomach cramps and skin lesions. Dried roasted and ground plant material is mixed with fat and used as ointment for wounds and sores
	Eriocephalus punctulatus L. (SHALE 25 UN)	Leaves	Used to fumigate the hut of a person suffering from diarrhoea, lice and bugs. An infusion is drunk for stomach-ache and as a cleanser
	Haplocarpha scaposa Harv. (SHALE 2 UN)	Roots	Decoction drunk for internal sores and paste applied to infected ear sores
	Schkuhria pinnata (Lam.) Cabr. (SHALE 23 UN)	Leaves	Infusion drunk to treat stomach-aches
	Senecio sp. (SHALE 3 UN)	Whole plant	An infusion used as a gargle for throats and mouth sores. Decoction is used in hot baths for treatment of rheumatic arthritic joints
	Xanthium spinosum L. (SHALE 17 UN)	Whole plant	Decoction is used to treat gonorrhoea and syphilis
EUPHORBIACEAE	*Euphorbia clavarioides* Boiss (E. basutica Marl.) (SHALE 9 UN)	Roots	Infusion used externally to treat body rash, sores and wounds

GERANIACEAE	*Monsonia brevirostrata* Knuth (SHALE 16 UN)	Whole plant	Powdered plant material applied is onto sores resulting from sexually transmitted diseases (e.g gonorrhoea)
	Pelargonium alchemilloides (L.) L' Herit (SHALE 1 UN)	Roots	Dried powder mixed with fat and applied to sore ears
HYPOXIDACEAE	*Hypoxis rigidula* Bak. Var. rigidula (SHALE 13 UN)	Bulb	Infusion used for treating wounds and itching rash. Decoction drunk for asthma and Arthritis
LAMIACEAE	*Salvia repens* Burch. ex Benth. (SHALE 19 UN	Whole plant	Used for stomach-aches and appendix problems
LEGUMINOSAE	*Rhynchosia adenoides* E. & Z. (SHALE 12 UN)	Roots	Decoction used to treat rheumatic pains, menstrual pains and dysentery
MALVACEAE	*Malva parviflora* L. (SHALE 21 UN)	Whole plant	Infusion used as a lotion for bathing bruised or broken limbs. Dried powder is applied onto clean wounds
PERIPLOCACEAE	*Pachycarpus rigidius* E. Mey. (SHALE 4 UN)	Roots	Powder snuffed for the relief of headaches. Decoction drunk for colds and stomach-Ache
POACEAE	*Festuca caprina* Nees. (SHALE 7 UN)	Roots	Infusion used for stomach-ache
POLYGONACEAE	*Rumex acetosella* L. (SHALE 14 UN)	Roots	Decoction used to bathe wounds and bruises
SOLANACEAE	*Solanum aculeatissimum* Jacq (SHALE 8 UN)	Whole plant	Decoction given after miscarriage for internal cleaning. Dry powder rubbed into wounds or placed on painful teeth
SOLANACEAE	*Solanum nigrum* L. (SHALE 18 UN)	Whole plant	Decoction drunk for body pains, heart problems and to treat rash
-	*Cheilanthes* sp. (SHALE 24 UN)	Whole plant	Slightly roasted powdered plant material applied to wounds
-	*Chenopodium* sp. (SHALE 20 UN)	Whole plant	Decoction used to treat wounds and sores

Voucher specimens : UN, Herbarium of the University of Natal Pietermaritzburg

3.3.3 Anti-bacterial activity

Fifteen medicinal plants were collected on the basis of their usage for the treatment of bacterial infections (Table 3.1). Leaves and roots (2 g) were extracted with 20 ml hexane, methanol or water respectively for 30 minutes in an ultrasound bath. The plant extracts were left to stand at room temperature overnight, filtered through Whatman No. 1 filter paper and the filtrates dried in a stream of air at room temperature. The residues were redissolved in hexane, methanol and water at 100 mg ml^{-1} and tested for anti-bacterial activity using the disc-diffusion bioassay (Section 2.2.1).

3.3.4 Anti-inflammatory activity

Thirteen medicinal plants were recorded to be used for diseases associated with inflammation (Table 3.1). Leaves and roots (500 mg) were extracted with 5 ml hexane, methanol or water respectively for 30 minutes in an ultrasound bath. The extracts were centrifuged at 3000 rpm using a J_4 Centrifuge for 10 minutes at 20 ^{0}C. Supernatants were decanted and dried in a stream of air. Extracts made using organic solvents were redissolved in ethanol at 8 mg ml^{-1} and water extracts were redissolved in water at 1 mg ml^{-1}. The extracts were tested for anti-inflammatory activity using the cyclooxygenase-1 (COX-1) bioassay (Section 2.3.1)

3.4 Results

3.4.1 Traditional healers and herbalists' responses to the questionnaire

The healers and herbalists indicated that plant roots are the most commonly used part of the plant. Water is mostly used to extract the active compounds because of its availability. In most cases, single plants were reported to be active against the treated diseases, while in a few instances different plant extracts are mixed for particular ailments. The dosage of the prescribed medication depends on the strength of the medication and age of the patient. Orally taken medication ranges from four teaspoons per day for children to four cups per day for adults. Other means of administration are indicated in Table 3.1.

Plants are normally air dried at room temperature and stored indoors for up to 5 years. Attempts to cultivate medicinal plants have been made. Some plants are easily cultivated while others are unable to survive outside their natural habitat. The main drawback with the cultivation of medicinal plants is that people steal them. Mainly highland plants are used in traditional remedies. Lowland healers obtain most of their plant material from highland areas, either by collecting them or buying them from highland gatherers.

Traditional healers acquire their knowledge from their ancestors/gods who are believed to convey the healing knowledge to the trainee through experienced healers. The training may last for up to ten years depending on the individual. Herbalists acquire their healing knowledge through experience, which involves

meetings and discussions with other herbalists and healers. Their knowledge acquisition is a continuous, learning process.

An experienced healer and herbalist may attend up to 100 patients per month. Charges range from R10.00 to a cow (live animal or money equivalent to the cost of a cow) depending on the extent of the treatment. Cases of patients reacting negatively to the prescribed medication have been encountered and in such situations, the medication is changed. Traditional healers and herbalists do sometimes refer their patients to Western doctors as with bone fracture, tuberculosis and extreme sexual diseases. However, traditional healers and herbalists have indicated that Western doctors do not refer patients to them.

3.4.2 Anti-bacterial activity

Extracts were considered highly active if their inhibition zone was between 0.7-1.0 mm, medium inhibitory activity with an inhibition zone between 0.3-0.7 mm and low or no inhibitory activity with an inhibition zone between 0.0-0.3 mm.

Extracts from *Aster bakeranus* (roots), *Eriocephalus punctulatus* (leaves), *Haplocarpha scaposa* (leaves and roots), *Malva parviflora* (roots), *Rumex acetosella* (leaves), *Solanum aculeatissimum* (leaves) and *Chenopodium* sp. (roots) had high inhibitory activity (Table 3.2). Methanol and water extracts were active against both Gram-positive and Gram-negative bacteria. Hexane extracts inhibited few bacteria. *Malva parviflora* (roots) inhibited most of the tested bacteria including *Escherichia coli*. This bacterium had the greatest resistance to most of the tested extracts. Water

extracts from *Chenopodium* sp. (roots) also showed high inhibitory activity against *E. coli.*

Aster sp., *Cheilanthes* sp., *Pelargonium alchemilloides, Senecio* sp. and *Xanthium spinosum* extracts showed medium inhibitory activity. The activity was mainly from root extracts and was most active against Gram-positive bacteria. *Euphorbia clavarioides* and *Hypoxis rigidula* extracts did not exhibit inhibitory activity against most bacteria. However, low inhibition against *Bacillus subtilis* and *Staphylococcus epidermis* resulted from root and bulb extracts. *Monsonia brevirostrata* showed no anti-bacterial activity in any of the tested extracts.

3.4.3 Anti-inflammatory activity

Six of the 13 plants screened had anti-inflammatory activity above 90% (Table 3.3). Hexane and methanol extracts were the most active, while water extracts usually had lower activity. There were a few exceptional cases where water extracts showed high inhibitory activity e.g. *Asparagus microraphis, Rhynchosia adenoides* and *Watsonia* sp. Both leaf and root extracts showed high inhibitory activity. Most of the other plant extracts had inhibitory activity between 60 and 90%, except for *Senecio* sp., which gave low activity with all the extracts tested. *Asparagus microraphis, Eriocephalus punctulatus* and *Malva parviflora* were selected for further analysis for the extraction and isolation of the active anti-bacterial and anti-inflammatory compound(s).

Table 3.2: Anti-bacterial activity of hexane, methanol and water extracts obtained from medicinal plants used by traditional healers and herbalists in Lesotho for treatment of bacterial infections

Plant name	Plant parts and Extracting solvent		Micro organisms						
			Gram-positive				Gram-negative		
			M.l	B.s	S.a	S.e	E .c	P.a	K.p
Aster bakeranus	Leaves	Hexane	-	-	-	-	0.3	-	-
		MeOH	-	0.5	-	-	0.5	-	-
		Water	-	-	0.5	0.6	-	0.6	0.5
	Roots	Hexane	-	0.3	0.6	0.8	-	0.3	0.4
		MeOH	-	0.3	0.6	0.8	-	0.3	0.4
		Water	0.4	0.4	0.7	-	0.5	-	-
Eriocephalus punctulatus	Leaves	Hexane	-	0.8	0.6	-	-	-	-
		MeOH	-	0.4	0.3	-	-	-	-
		Water	-	-	-	-	-	-	-
	Roots	Hexane	-	0.1	-	-	-	-	-
		MeOH	-	-	-	-	-	-	-
		Water	-	-	-	-	-	-	-
Haplocarpha scaposa	Leaves	Hexane	-	-	0.3	0.4	0.5	0.8	0.3
		MeOH	0.3	-	0.5	0.7	-	0.4	0.6
		Water	0.6	0.4	-	0.8	-	0.5	0.3
	Roots	Hexane	-	-	0.3	0.4	0.5	0.8	0.3
		MeOH	-	0.5	0.4	0.6	-	0.6	-
		Water	-	0.6	0.3	-	0.3	0.4	-
Xanthium spinosum	Leaves	Hexane	-	-	-	-	-	0.3	
		MeOH	-	0.3	-	-	-	-	-
		Water	-	0.4	0.4	0.3	-	0.4	0.4
	Roots	Hexane	-	-	-	-	-	-	0.3
		MeOH	0.4	0.1	0.5	-	-	-	-

		Water	0.2	0.1	0.4	-	0.7	0.5	0.4
Euphorbia clavarioides	Leaves	Hexane	-	-	-	-	-	-	-
		MeOH	-	-	-	-	-	-	-
		Water	-	-	-	-	-	-	-
	Roots	Hexane	-	0.4	-	-	-	-	-
		MeOH	0.2	-	-	0.3	-	0.2	-
		Water	-	-	-	-	-	0.1	-
Monsonia brevirostrate	Leaves	Hexane	-	-	-	-	-	-	-
		MeOH	-	-	-	-	-	-	-
		Water	-	-	-	-	-	-	-
Pelargonium alchemilloides	Leaves	Hexane	-	-	0.3	-	-	-	0.3
		MeOH	-	-	-	-	-	-	-
		Water	-	0.3	-	-	-	-	-
	Roots	Hexane	0.3	0.2	-	-	0.4	-	-
		MeOH	-	-	-	-	-	-	-
		Water	-	-	-	-	-	-	-
Hypoxis rigidula	Leaves	Hexane	-	-	-	-	-	-	-
		MeOH	-	-	-	-	-	-	-
		Water	-	-	-	-	-	-	-
	Roots	Hexane	-	0.4	-	-	-	-	-
		MeOH	0.2-	-	-	0.3	-	0.2	-
		Water	-	-	-	-	-	-	-
Malva parviflora	Leaves	Hexane	-	-	-	-	-	-	-
		MeOH	-	-	0.3	-	0.4	-	-
		Water	-	-	-	-	-	-	-
	Roots	Hexane	-	0.5	0.9	-	-	0.6	0.6
		MeOH	-	0.5	-	-	-	0.6	0.6
		Water	0.4	0.8	0.6	-	0.7	0.7	0.4
Rumex acetosella	Leaves	Hexane	0.2	0.1	0.3	0.6	0.1	0.2	0.3
		MeOH	-	0.3	0.4	0.3	0.5	0.8	0.3

		Water	-	0.2	0.3	0.5	0.2	0.7	0.3
	Roots	Hexane	-	0.2	0.7	0.5	0.6	-	-
		MeOH	-	-	-	-	-	-	-
		Water	-	-	-	-	-	-	-
Solanum aceleatissimum	Leaves	Hexane	-	0.4	-	-	-	-	-
		MeOH	-	0.3	-	0.8	-	0.6	-
		Water	-	0.3	-	0.8	-	0.6	-
	Roots	Hexane	-	-	-	-	-	0.4	0.3
		MeOH	0.2	-	0.2	0.7	0.4	0.4	-
	Fruits	Hexane	0.5	-	-	-	0.6	-	-
		MeOH	0.4	0.4	-	-	0.6	0.3	-
		Water	-	0.5	-	0.5	0.5	-	-
Cheilanthes sp.	Whole plant	Hexane	-	-	-	-	-	-	-
		MeOH	-	-	-	-	-	-	-
		Water	-	0.6	-	-	0.7	-	-
Chenopodium sp.	Leaves	Hexane	-	-	-	-	-	-	-
		MeOH	0.6	-	-	-	-	0.3	-
		Water	-	-	-	-	-	0.5	-
	Roots	Hexane	-	-	-	-	-	-	0.3
		MeOH	-	-	-	-	-	-	-
		Water	0.6	0.4	-	0.8	0.8	-	-
Aster sp.	Leaves	Hexane	-	-	-	-	-	-	-
		MeOH	0.6	-	-	-	-	-	-
		Water	-	-	-	-	0.5	-	-
	Roots	Hexane	-	0.4	-	-	0.3	-	-
		MeOH	-	-	0.3	0.8	0.3	-	-
Senecio sp.	Leaves	Hexane	-	-	-	-	-	-	-
		MeOH	-	-	0.3	-	-	-	-
		Water	-	0.3	-	0.5	-	-	-
	Roots	Hexane	0.4	-	-	0.5	0.3	-	0.7

	MeOH	0.6	0.3	-	0.4	0.7	-	-
	Water	-	-	-	0.6	-	-	-

Bacteria tested: M.l, *Micrococcus luteus;* B.s, *Bacillus subtilis;* S.a, *Staphylococcus aureus;* S.e, *Staphylococcus epidermis;* E.c, *Escherichia coli;* P.a, *Pseudomonas aeruginosa* and K.p, *Klebesiella pneumoniae.*

3.5 Discussion

The screening of plants for anti-bacterial and anti-inflammatory activity indicated that most of the plants tested were highly active. This supports their use in traditional remedies. The screening in both assays (Tables 3.2 and 3.3) showed that most inhibitory activity was recorded with root extracts. This confirmed the information from the interviewed healers and herbalists who use roots in preference to leaves. Plant extracts which showed medium or no anti-bacterial activity in the disc-diffusion bioassay may be active against other bacteria, which were not tested. The anti-bacterial activity of some plant families screened in this study have been reported to be active against some Gram-negative and Gram-positive bacteria in other studies. In particular, extracts from Asteraceae and Euphorbiaceae resulted in the inhibition of *Bacillus subtilis* and *Escherichia coli* (NICK, RALI and STICHER, 1995; VALSARAJ, PUSHPANGADAN, SMITT, ADSERSEN and NYMAN, 1997).

Plant extracts which showed moderate or low anti-inflammatory activity in the cyclooxygenase bioassay may contain active compounds but probably in smaller

Table 3.3: Anti-inflammatory activity of hexane, methanol and water extracts obtained from medicinal plants used traditionally in Lesotho for diseases associated with inflammation

Plant name	Parts used	Inhibition (%)		
		Hexane	Methanol	Water
Boophane distica	Leaves	55	62	65
Asparagus microraphis	Leaves	89	97	73
	Roots	97	69	73
Dicoma anomala	Leaves	86	85	5
	Roots	79	27	7
Eriocephalus punctulatus	Leaves	89	81	18
Festuca caprina	Leaves	78	85	69
	Roots	85	64	53
Malva parviflora	Leaves	62	57	23
	Roots	98	40	28
Pachycarpus rigidus	Leaves	85	91	41
	Roots	90	41	32
Rhynchosia adenoides	Leaves	95	89	84
	Roots	83	66	88
Salvia repens	Leaves	0	83	47
	Roots	45	30	44
Schkuhria pinnata	Leaves	93	62	49
	Roots	80	79	45
Solanum nigrum	Leaves	83	81	35
	Roots	92	76	9
Senecio sp.	Leaves	20	52	44
	Roots	29	23	32
Watsonia sp.	Leaves	83	74	72
	Roots	54	56	83
Indomethacin (20 µM)		87		

amounts and/or the screened crude extracts could yield more potent compounds if they had undergone some purification (FABRY, OKEMO and ANSORG, 1998). TAYLOR, RABE, MCGAW, JÄGER and VAN STADEN (2001) indicated that lack of a positive result in a screening assay does not always mean the absence of bioactive constituents. The active principle(s) may be present in insufficient quantities in the extracts to show activity in the dose levels employed. Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the active principles during the assay.

Although water was reported by the traditional healers and herbalists to be the most commonly used solvent to extract the active compounds, anti-bacterial and anti-inflammatory screening of the plants (Tables 3.2 and 3.3) generally resulted in higher inhibitory activity being detected with hexane and methanol extracts compared to the water extracts. This suggests that water is not the most effective solvent for extracting the active compounds from plants. However, considering the prescribed dosage which may be as much as four cups per day per adult, water extracts can still be considered as an appropriate extracting solvent for traditional remedies. The same dosage from hexane and methanol extracts would be more concentrated, potentially becoming dangerous to the patients unless smaller dosages were prescribed. Hexane and methanol may also extract other compounds from the plants in higher concentrations which could cause the crude extracts to become toxic.

The results indicated that a large number of plants used traditionally for remedies belong to the Asteraceae. Species from this family contain polyacetylene derivatives

which have potent insecticidal effects when sensitised by near-ultraviolet light (phototoxicity) due to the production of activated oxygen species or other radicals that damage the lipid membranes (CHRISTENSEN and LAM, 1990; GUILLET, CHAURET and ARNASON, 1997). This phototoxicity may also affect the results presented in this work but the same effects would be encountered by the traditional healers using these plants.

Another aspect to consider when promoting the use of traditional plant remedies is that plant extracts may be contaminated with non-plant material such as pesticides. This can occur in rural areas where the plants grow alongside commercial crops (STEWART, STEENKAMP and ZUCKERMAN, 1998). Toxicity can result from ingestion of plants that appear similar to a commonly used species but which otherwise contain a toxic component. For example, the forcible movement of a Tribe in Zambia from a flooded valley to an adjacent one led to an outbreak of poisoning as the variety of tuber in the second location was toxic when prepared in the same manner as the species safely used at the original site for many years. *Senecio* sp. are difficult to differentiate, even by experienced botanists; and so a toxic species may be inadvertently picked and used (STEWART, STEENKAMP and ZUCKERMAN, 1998).

Although traditional healers and herbalists refer patients to Western doctors on certain occasions, the referral is one way as Western doctors do not send patients to traditional healers and herbalists. There is thus little co-operation between Western doctors and traditional healers and herbalists. The incorporation of traditional healing in health care could improve the way of life for many people, especially those in rural

areas. Traditional healers and herbalists need to be educated about certain Western diseases such as cancer and cardiovascular illness which are unlikely to be recognised by indigenous people (COX, 1994). The diseases that are likely to be recognised by indigenous people include gastrointestinal maladies, inflammations, skin infections and certain viral diseases (COX, 1994).

Lesotho is surrounded by South Africa. This means that plants which are found in Lesotho are likely to occur in South Africa. Some plants used traditionally in South Africa by Zulu and Xhosa people for bacterial infections and inflammations are used for similar ailments by traditional healers in Lesotho. These include species such as *Boophane distica, Cheilanthes eckloniana, Rhynchosia curibaea* and *Rumex sagitatus* used by the Zulu for anti-inflammation (HUTCHINGS, 1992). Screening of these plants for anti-inflammatory activity using the cyclooxygenase bioassay resulted in detection of inhibitory activity (JÄGER, HUTCHINGS and VAN STADEN, 1996).

The knowledge of the traditional healers and herbalists should not to be underestimated or considered inferior to Western methods of treatment (BANQUAR, 1993). Incorporation of traditional healing into the health care system may promote the useful elements of traditional knowledge of the healers and herbalists. This can make significant contributions towards the implementation of an improved programme for health (MAHUNNAH and MSHIU, 1991; SINDIGA, 1995). This preliminary screening of crude extracts made from plants used by traditional healers and herbalists in Lesotho showed that most of the screened plants are potentially a rich source of anti-bacterial and anti-inflammatory agents. It demonstrates their

importance in traditional remedies in rural populations where Western medicine is not readily available. The healers and herbalists' traditional knowledge is thus a valuable guide in the selection of plants which can be used to isolate and identify active compounds.

CHAPTER 4

SEASONAL VARIATION IN MEDICINAL ACTIVITIES AND PLANT PART SUBSTITUTION FOR *MALVA PARVIFLORA, ERIOCEPHALUS PUNCTULATUS* AND *ASPARAGUS MICRORAPHIS*

4.1 Introduction

4.1.1 Seasonal variation in medicinal properties of plants used in traditional remedies

Many traditional herbal remedies consist of a mixture of several plants. Combining different plant species sometimes inactivates the unwanted compounds, allowing the useful active compound to be more effective (SPARG, VAN STADEN and JÄGER 2000). Although this mixing of plants may seem an effective way of administering traditional remedies used for curing ailments and diseases, it also encourages destructive collection as many plant species are used without considering the possibility of habitat depletion. This could lead to extinction of some endangered medicinal plants.

An important factor to consider in the use of medicinal plants by traditional healers is that the amount of the active constituents in different plant material varies in quantity depending on habitat, weather, time of harvest, plant part, plant age and the way the plant is harvested and handled (AFIFI and ABU-IRMAILEH, 2000; LIGHT, MCGAW, RABE, SPARG, TAYLOR, ERASMUS, JÄGER and VAN STADEN 2002). Studies have shown that the therapeutic products from field collected plants frequently differ from those obtained from a nursery. A compound from a chemical reactor inside a factory is identical batch by batch, but the amount of products in living plants vary within the same plant species due to the effects of sunlight, temperature, humidity, plant husbandry and packaging (SRIVASTAVA, LAMBERT and VIETMEYER, 1996).

Time of harvest may affect composition of secondary metabolites in different plant species. An investigation on seasonal variation in essential oil of *Virola surinamensis* leaves showed a constant (0.5%) oil in different seasons but the relative composition of compound classes or individual components varied quite sharply from plant material collected during February, June and October 1996 (LOPES, KATO, ANDRADE, MAIA and YOSHIDA, 1997). Seasonal effects on concentration of plant products was also observed from hypericin content of *Hypericum perforatum* L. This increased rapidly to a peak in early summer. A decrease in total hypericin was observed towards the end of summer followed by a steady decline during autumn and minimum levels were detected in winter (SOUTHWELL and BOURKE, 2001).

Variation in plant products can affect the medicinal activity of plants. MAKINDE, AWE and SALAKO (1994) noted seasonal variation in antimalarial activity of *Morinda lucida* on *Plasmodium berghei berghei* in mice. Various samples of *M. lucida* leaf

extracts administered orally (6.25-50g/kg/day) produced 16-81% chemosuppressive activity from the March samples, 24-59% was observed with the June samples, 24-58% with the September samples while 18-38% was noted with the December samples. Different seasons contribute to developmental stages of plants, thus resulting in variation in yield of natural products produced. Studies on chemical composition of essential oil of *Tagetes minuta* at different growth stages resulted in the detection of high percentage of oils from young plant leaves before flowering while stems and roots contained no oil. Similarly, the content of davanone, the major constituent of davana oil found in *Artimisia pallens* Wall. decreased drastically from the stage of flower head emergence stage (67%) to the initiation of a seed set stage (22.9–23.8%) (CHALCHAT, GARRY and MUHAYIMANA, 1995; MALLAVARAPU, KULKARNI, BASKARAN, RAO and RAMESH, 1999).

4.1.2 Plant part substitution

Plant species which are slow-growing and slow-reproducing are specially vulnerable to excessive collection. The destructive harvesting of the underground parts of these plants or even the plant as a whole, leads to scarcity of many species. This problem can be solved by encouraging healers to collect and use alternative plant parts such as leaves and twigs instead of underground parts and bark. However, Scientists need to evaluate the differences and similarities between various parts of the same plant with respect to chemical composition and pharmacological properties as one of the prerequisites prior to the encouragement of a policy on alternative use of plant parts (ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN 2000).

Utilisation of medicinal plants was formerly a specialist activity of traditional practitioners, who had little effect on plant resources, since small quantities of herbs were utilised (CUNNINGHAM, 1991; RAI, PRASAD and SHARMA, 2000). The advent of urbanization and the consequent commercialisation of traditional health care in recent years have caused an increase in the demand for medicinal herbs. Gathers/traders who are the main suppliers of traditional medicinal plants to urban areas have disastrous effects on many popular species. Usually, collectors get low prices for wild-harvested plant material and this may lead to over-harvesting (CUNNINGHAM, 1991; RAI, PRASAD and SHARMA, 2000; WILLIAMS, BALKWILL and WITKOWSKI 2000).

The majority of gatherers/traders collect wild medicinal plants through untrained and unskilled labourers. This is generally in the form of collection of the entire plant, roots, rhizomes, tubers and bulbs before seed set (RAI, PRASAD and SHARMA, 2000). Studies on analysis of plant parts traded at Witwaterstrand in South Africa showed roots as the most traded plant part (38.4%), followed by bark (26.6%), leaves/stems (13.5%) and bulbs (10.8%). The damage caused by partial removal of the roots drastically reduces water uptake and increases the susceptibility to fungal diseases, which can lead to mortality. The harvesting of aerial parts does not always result in mortality because leaves and fruits are considered renewable (WILLIAMS, BALKWILL and WITKOWSKI 2000).

4.1.3 Biological properties of *Malva parviflora, Eriocephalus punctulatus* and *Asparagus microraphis*

M. parviflora, E. punctulatus and *A. microraphis* (Figure 4.1) are medicinal plants used in traditional remedies for diseases caused by bacterial infection and inflammation. High anti-bacterial and anti-inflammatory activity detected from these plants (CHAPTER 3) resulted in the investigation on seasonal variation of their inhibitory activities and the possibility of plant part substitution as a means of conservation.

Malva parviflora

Malva parviflora L. belongs to the family Malvaceae which comprises trees, shrubs and herbs. Plants from this family are noted for their economic, horticultural and medicinal importance. The family includes genera such as *Gossypium* (cotton), *Althaea* (hollyhocks) and *Abutilous* (Chinese lanterns) (GUILLARMOD, 1971; BEER and HOWIE, 1985).

Malva parviflora originated in Europe but is now found worldwide. It is an annual herb with pale flowers 4-5 mm in diameter. The flowers are broadly ovate calyx-lobes, glabrous petals-claws with a spreading calyx. It is a trailing plant with a stem which is slightly hairy or nearly glabrous. Leaves are orbicular, slightly 3-5 lobed, cordata at the base, crenate-dentate and the petiole is 76-102 mm long. Carpels are rugose, hairy or smooth (ANDREWS, 1952; CLAPHAM, TUTIN and WARBURG, 1962; GUILLARMOD, 1971).

Eriocephalus punctulatus

Eriocephalus punctulatus L. is an attractive scented woody, sclerophyllous shrub that reaches a height of about 750-1000 mm. It is indigenous to certain high-lying mountainous areas of Southern Africa. It is a flowering perennial that begins flowering in Spring until mid-Summer after which it continues to grow vegetatively throughout Summer. If it grows unchecked for a number of seasons, the resultant growth is a round bush with a radius of about one metre. The stems are long, woody and brittle with leaves on the terminal ends of the young branches. Research conducted on farming of *E. punctulatus* for commercial oil at the University of Fort Hare, Alice, South Africa have indicated that *E. punctulatus* is not suited to the oakleaf soils with high pH, the plant survive well when planted on a heavy doleritic soil with a relatively low pH (WEBBER, MAGWA and VAN STADEN, 1999; WEBBER, MAGWA and VAN STADEN, 2000 B).

Asparagus microraphis

Asparagus microraphis (Kunth) Oberm. belongs to the family Asparagaceae. The plant is an evergreen, dome-shaped shrub which can grow up to 1 m high. It grows at the foot of cliffs and on steep rocky slopes up to an altitude of 2400 m. It flowers from October to December bearing cream or pinkish flowers possessing a green central stripe. Needle shaped leaves form dense clusters along spreading branches of the erect stems (HILLIARD and BURTT, 1987; POOLEY, 1998).

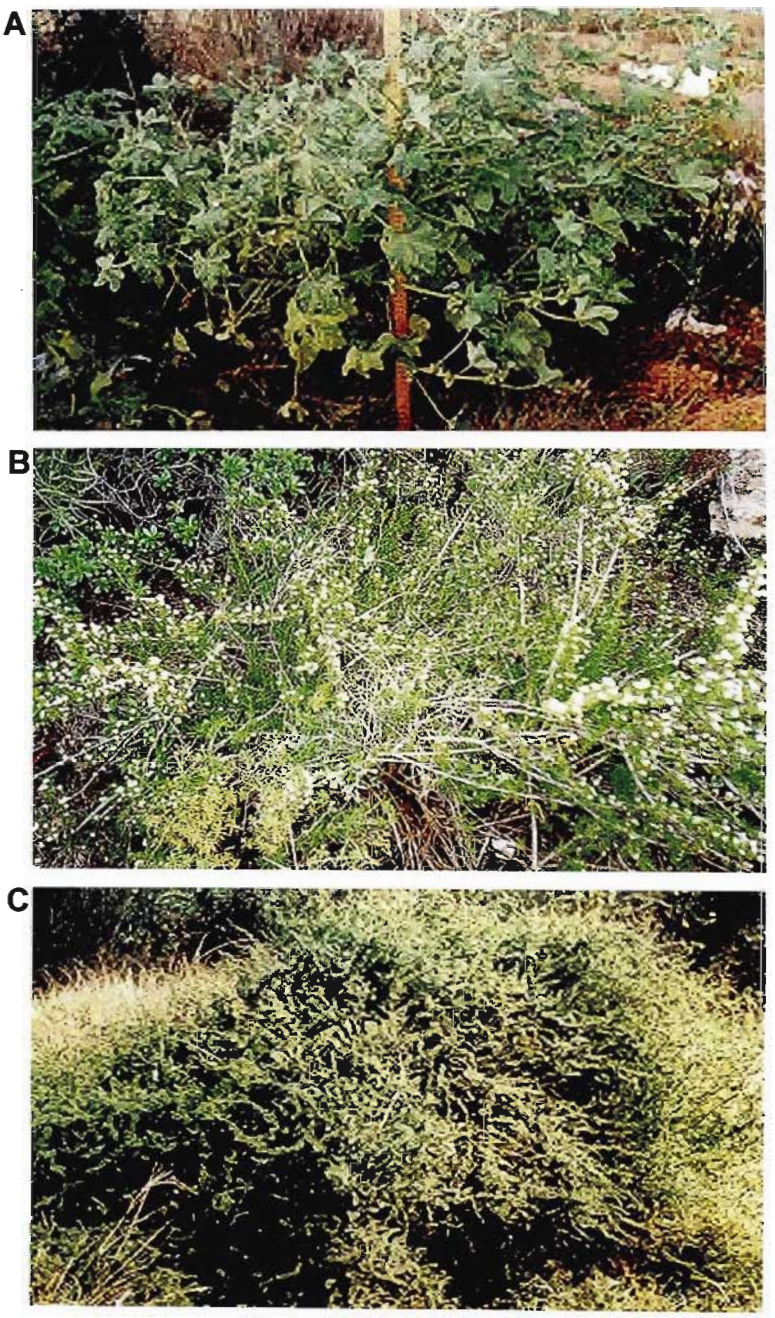


Figure 4.1: Photographs of *M. parviflora*, B) *E. punctulatus* and C) *A. microraphis*

4.2 Aims

The aims of this part of the study were:

1) To determine seasonal variation in anti-bacterial activity of *M. parviflora* and *E. punctulatus*;

2) To determine seasonal variation in anti-inflammatory activity of *M. parviflora, E. punctulatus* and *A. microraphis*; and

3) To determine if different plant parts (roots and leaves) could be substituted in traditional remedies to minimise destructive harvesting.

4.3 Materials and Methods

4.3.1 Plant material

M. parviflora, E. punctulatus and *A. microraphis* were collected from Qacha's Nek district in Lesotho at different times of the year between June 1999 and July 2001 (Table 4.1). The location where these plants were collected is in the most mountainous and high altitude part of the country (CHAPTER 3). Collection site experience early and long winter periods (May – September depending on years) with temperatures ranging from –5 to 28 ^{0}C and the occurrence of snow is frequent in winter. The collected plant material was dried at 50 ^{0}C, ground to a powder and immediately tested for anti-bacterial and anti-inflammatory activities in order to serve as a basis for the determination of storage effects on the biological activity of the plant material.

Table 4.1: Collection times of *M. parviflora, E. punctulatus* and *A. microraphis*

Plant collected	Parts used	Time of collection		
		1999	2000	2001
M. parviflora	Leaves & roots	June,July, Oct.	Jan, Feb, Aug, Dec	Jan, Feb, April, July
E. punctulatus	Leaves	Not collected	May, Aug, Dec	Jan, April, July
A. microraphis	Leaves & roots	Not collected	Jan, Feb, Aug, Dec	Jan, Feb, April, July

4.3.2 Testing for seasonal variation in anti-bacterial activity

Dried, ground *M. parviflora* and *E. punctulatus* were extracted with hexane, methanol or water as described in Section 3.3.3. Dried extracts were redissolved at a concentration of 100 mg ml^{-1} and tested for anti-bacterial activity using the disc-diffusion bioassay (Section 2.2.1).

4.3.3 Testing for seasonal variation in anti-inflammatory activity

M. parviflora and *E. punctulatus* hexane, methanol and water extracts tested for anti-bacterial activity (Section 4.3.2) were subsequently dried in a stream of air. *A. microraphis* was extracted with hexane, methanol or water as described in Section 3.3.4. All three dried extracts were then redissolved at a concentration of 8 mg ml^{-1} (organic solvent extracts) and 1 mg ml^{-1} (water extracts) and tested for anti-inflammatory activity using the COX–1 bioassay (Section 2.3.1).

4.4 Results

4.4.1 Seasonal variation in medicinal activity of *M. parviflora*

Anti-bacterial activity

Hexane, methanol and water extracts obtained from leaves and roots of *M. parviflora* were tested for seasonal variation in anti-bacterial activity. Extracts of *M. parviflora* collected between June 1999 and July 2001 showed variation in antibacterial activity. Generally the water extracts made from the leaves showed the most antibacterial activity against two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and one Gram-negative bacteria (*Pseudomonas aeruginosa*). This inhibitory activity was similar throughout the year. Hexane and methanol leaf extracts only inhibited bacterial growth on one occasion each (Table 4.2). Conversely, no inhibitory activity was measured in the water extracts made from the roots whereas the hexane and methanol root extracts inhibited bacterial growth. Generally the root material collected in the cooler months (June-September) inhibited more bacteria (both Gram-positive and Gram-negative) whereas roots collected in the warmer months (December-February) inhibited fewer bacterial strains (Table 4.2). However, a trend in seasonal activity is not evident for either the roots or leaves as on six collections (Table 4.1), no activity was detected in any of the extracts

Anti-inflammatory activity

Both the leaf and root material showed inhibitory activity in the Cox-1 assay with the hexane extract made from the leaves being the most effective. Hexane and methanol extracts made from leaves had higher anti-inflammatory activity than the

Table 4.2: Seasonal variation in anti-bacterial activity of *M. parviflora*. Results from extracts with little or no inhibitory activity (0.0 - 0.2 mm) were omitted

Collection time		Extracting solvent	Micro organisms						
			Gram-positive				Gram-negative		
			M.l	B.s	S.a	S.e	E.c	P.a	K.p
LEAF EXTRACTS									
1999	June	Water	-	0.30	-	-	-	0.25	-
	July	Water	-	-	0.22	-	-	0.33	-
2000	February	Methanol	-	-	-	-	-	0.28	-
		Water	-	0.28	-	-	-	0.30	-
	August	Hexane	-	0.44	-	-	-	-	-
2001	February	Hexane	-	-	0.25	-	-	-	-
	July	Water	-	-	0.22	-	-	0.28	-
ROOT EXTRACTS									
1999	June	Methanol	0.30	-	-	-	0.17	0.28	0.44
	July	Methanol	-	0.14	-	-	-	0.22	-
	September	Methanol	-	0.28	-	0.25	-	-	-
2000	January	Hexane	-	-	-	-	-	0.22	-
		Methanol	-	-	-	-	-	0.30	-
	August	Hexane	-	-	-	-	-	0.25	-
	December	Methanol	-	0.22	-	-	-	0.25	-
2001	January	Hexane	-	-	0.25	-	-	-	-
		Methanol	-	-	0.30	-	-	0.25	-
	February	Hexane	-	-	0.22	-	-	-	-
		Methanol	-	-	0.25	-	-	-	-

M.l *Micrococcus luteus*; B.s *Bacillus subtilis*; *S.a Staphylococcus aureus*; S.e *Staphylococcus epidermis*; E.c *Escherichia coli*; P.a *Pseudomonas aeruginosa* and K.p *Klebesiella pneumoniae.*

water extracts (Figure 4.2A). The hexane extracts of the root material exhibited high inhibitory activity while the methanol and water extracts had little inhibitory activity (Figure 4.2B). Although variation in anti-inflammatory activity was observed between plants collected at different times, no clear trend in seasonal biological activity was observed.

4.4.2 Seasonal variation in medicinal activity of *E. punctulatus*

Anti-bacterial activity

Seasonal variation in anti-bacterial activity of *E. punctulatus* was tested for hexane, methanol and water extracts obtained from leaf material. Roots were not tested for seasonal variation in inhibitory activity because during the initial screening of *E. punctulatus*, extracts made from root material did not exhibit any such activity. Also, healers and herbalists reported that they do not use the roots in traditional remedies (CHAPTER 3).

Hexane and methanol extracts made from the plant material collected between March 2000 and July 2001 only inhibited the growth of *B. subtilis and S. aureus* (Gram-positive bacteria). The growth of *M. luteus* was inhibited by hexane extracts made from the plant material collected in December 2000 and July 2001. Water extracts did not yield any anti-bacterial activity against the tested bacteria. The Gram-negative bacteria (*E. coli, P. aeruginosa* and *K. pneumoniae*) were not inhibited by hexane, methanol or water extracts made from leaf material for all the collections made. The results showed no seasonal variation in anti-bacterial activity of *E. punctulatus* (Table 4.3).

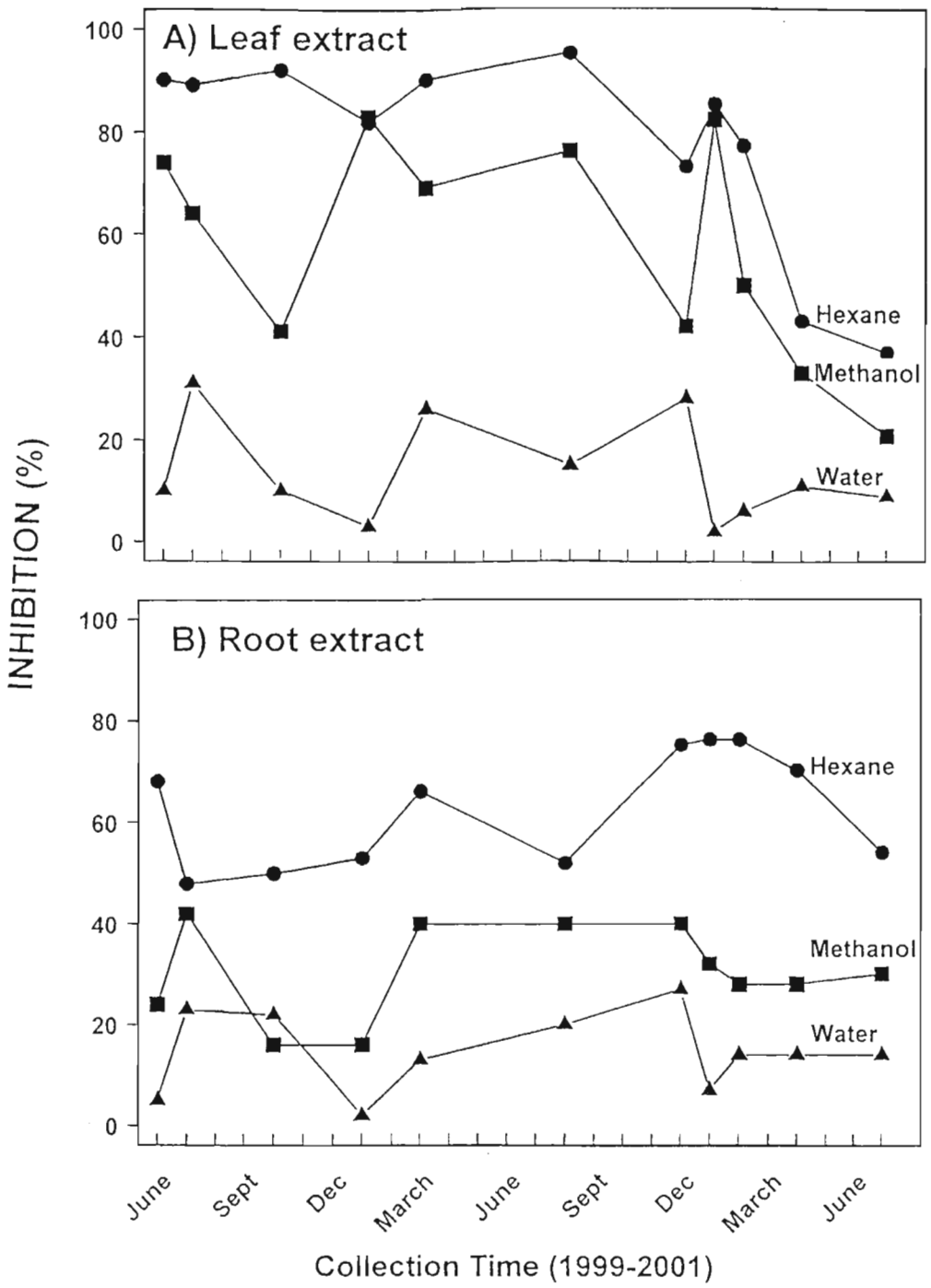


Figure 4.2: Seasonal variation in anti-inflammatory activity of leaf (A) and root (B) material of *M. parviflora*. Indomethacin standard (50 μM ml^{-1}) inhibition (%) = 64 ± 5%.

Table 4.3: Seasonal variation in anti-bacterial activity of *E. punctulatus*

Collection time		Parts used	Extracting solvent	Micro organisms						
				Gram-positive			Gram-negative			
				M.l	B.s	S.a	S.e	E.c	P.a	K.p
2000	March	Leaves	Hexane	-	0.1	0.4	-	-	-	-
			Methanol	-	0.4	0.4	-	-	-	-
			Water	-	-	-	-	-	-	-
	May	Leaves	Hexane	-	0.2	0.2	-	-	-	-
			Methanol	-	0.4	0.5	-	-	-	-
			Water	-	-	-	-	-	-	-
	Aug	Leaves	Hexane	-	0.2	0.5	-	-	-	-
			Methanol	-	0.3	0.4	-	-	-	-
			Water	-	-	-	-	-	-	-
	Dec	Leaves	Hexane	0.3	0.2	0.2	-	-	-	-
			Methanol	-	0.3	0.1	-	-	-	
			Water	-	-	-	-	-	-	-
2001	Jan	Leaves	Hexane	-	-	0.2	-	-	-	-
			Methanol	-	0.2	0.3	-	-	-	-
			Water	-	-	-	-	-	-	-
	April	Leaves	Hexane	-	0.1	0.3	-	-	-	-
			Methanol	-	0.3	0.4	-	-	-	-
			Water	-	-	-	-	-	-	-
	July	Leaves	Hexane	0.3	0.3	0.2	-	-	-	-
			Methanol	-	0.4	0.3	-	-	-	-
			Water	-	-	-	-	-	-	-

M.l *Micrococcus luteus*; B.s *Bacillus subtilis*; *S.a Staphylococcus aureus*; S.e *Staphylococcus epidermis*; E.c *Escherichia coli*; P.a *Pseudomonas aeruginosa* and K.p *Klebesiella pneumoniae*.

Anti-inflammatory activity

Seasonal variation in anti-inflammatory activity of *E. punctulatus* was tested for hexane, methanol and water extracts made from leaf material. Seven collections made between March 2000 and July 2001 exhibited seasonal variation in anti-inflammatory activity of hexane and methanol extracts made from leaves. High anti-inflammatory activity resulted from the collections made during the cooler months (May - August 2000, winter) with a decline in activity during the warmer months (September 2000 - March 2001, spring, summer and autumn). An increase in activity was again observed when collections were made in autumn and winter (May - July 2001). Water extracts made from leaf material collected between summer and winter (January - July 2001) exhibited anti-inflammatory activity below 50% (Figure 4.3).

4.4.3 Seasonal variation in medicinal activity of *A. microraphis*

Anti-inflammatory activity

Hexane, methanol and water extracts obtained from leaves and roots of *A. microraphis* were tested for seasonal variation in anti-inflammatory activity. Eight collections made between January 2000 and July 2001 showed high anti-inflammatory activity with hexane and methanol extracts made from both leaf and root material. Water extracts resulted in lower activity (Figure 4.4 A and B).

There was no seasonal variation in anti-inflammatory activity of the hexane and methanol extracts made from leaves and roots collected throughout the investigation period. Variation in anti-inflammatory activity of water extracts made from leaves and roots was observed and the highest inhibition (40%) was noted when the plant

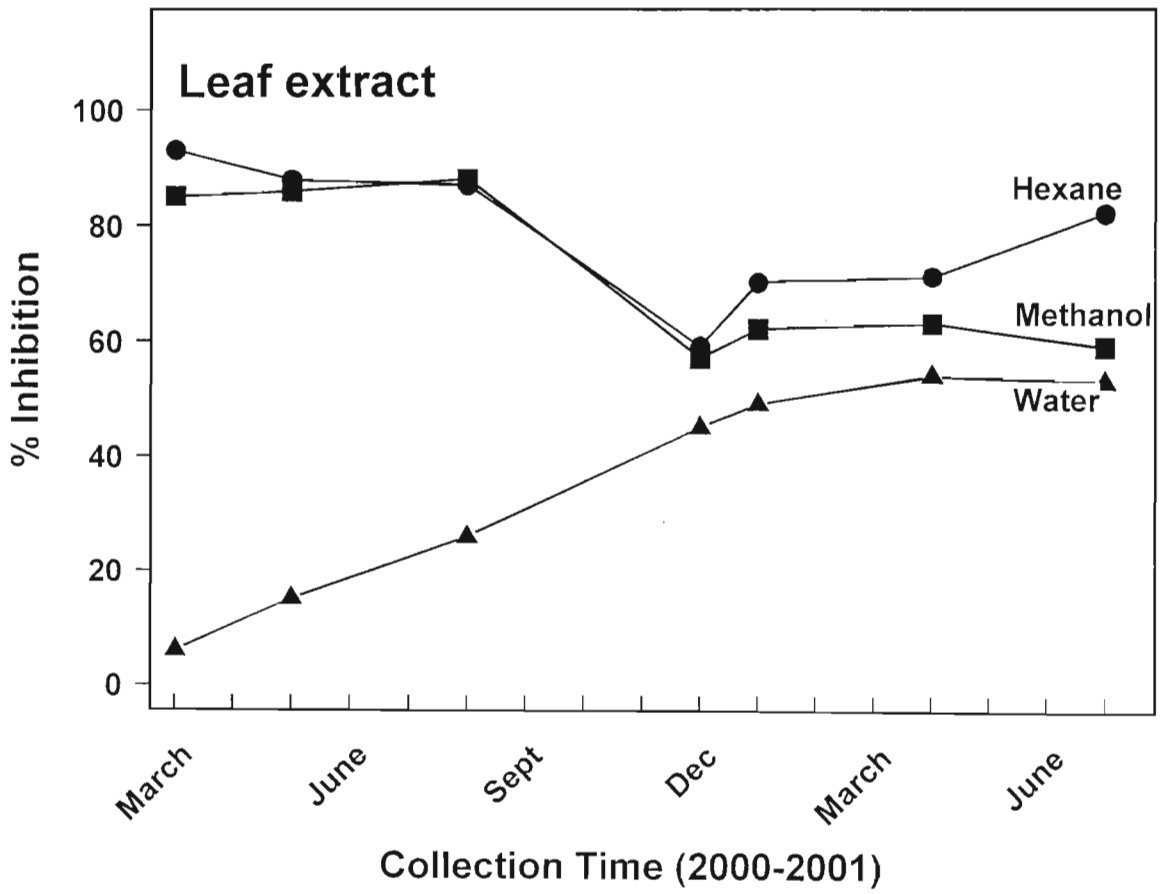


Figure 4.3: Seasonal variation in anti-inflammatory activity of leaf material of *E. punctulatus*. Indomethacin standard (50 μM ml^{-1}) inhibition (%) = 73 ± 7%.

material was collected in February 2001. However, the variation in the activity did not show any trend in seasonal variation (Figure 4.4).

4.5 Discussion

Seasonal variation in anti-bacterial and anti-inflammatory activity of *M. parviflora, E. punctulatus* and *A. microraphis* was investigated in order to determine the best time to collect medicinal plants in order to ensure high medicinal properties. Biological activity of the different plant parts was also investigated in order to determine if less destructive harvesting (aerial parts of the plant as opposed to the roots) could be encouraged amongst traditional healers. This followed the leads from the interviewed traditional healers and herbalists from Lesotho, who use mainly underground parts (roots) for preparation of water extracts in traditional remedies.

Anti-bacterial activity of root extract of *M. parviflora* (Table 4.2 and Figure 4.1) and anti-inflammatory activity of leaf extracts of *E. punctulatus* (Figure 4.3) exhibited seasonal variation from plant material collected at different seasons. The highest inhibitory activity for both plants was detected with the material collected during the cooler months, with a decline in activity when collections were made during the warmer months. Owing to the geographic location and climate of Lesotho, cooler weather is experienced during May to September, although this varies from year to year. Increased antibacterial activity of *M. parviflora* roots (Table 4.2 and Figure 4.2) and *E. punctulatus* (Figure 4.3) and anti-inflammatory activity of leaves was detected from collections made between May and September. For traditional healers,

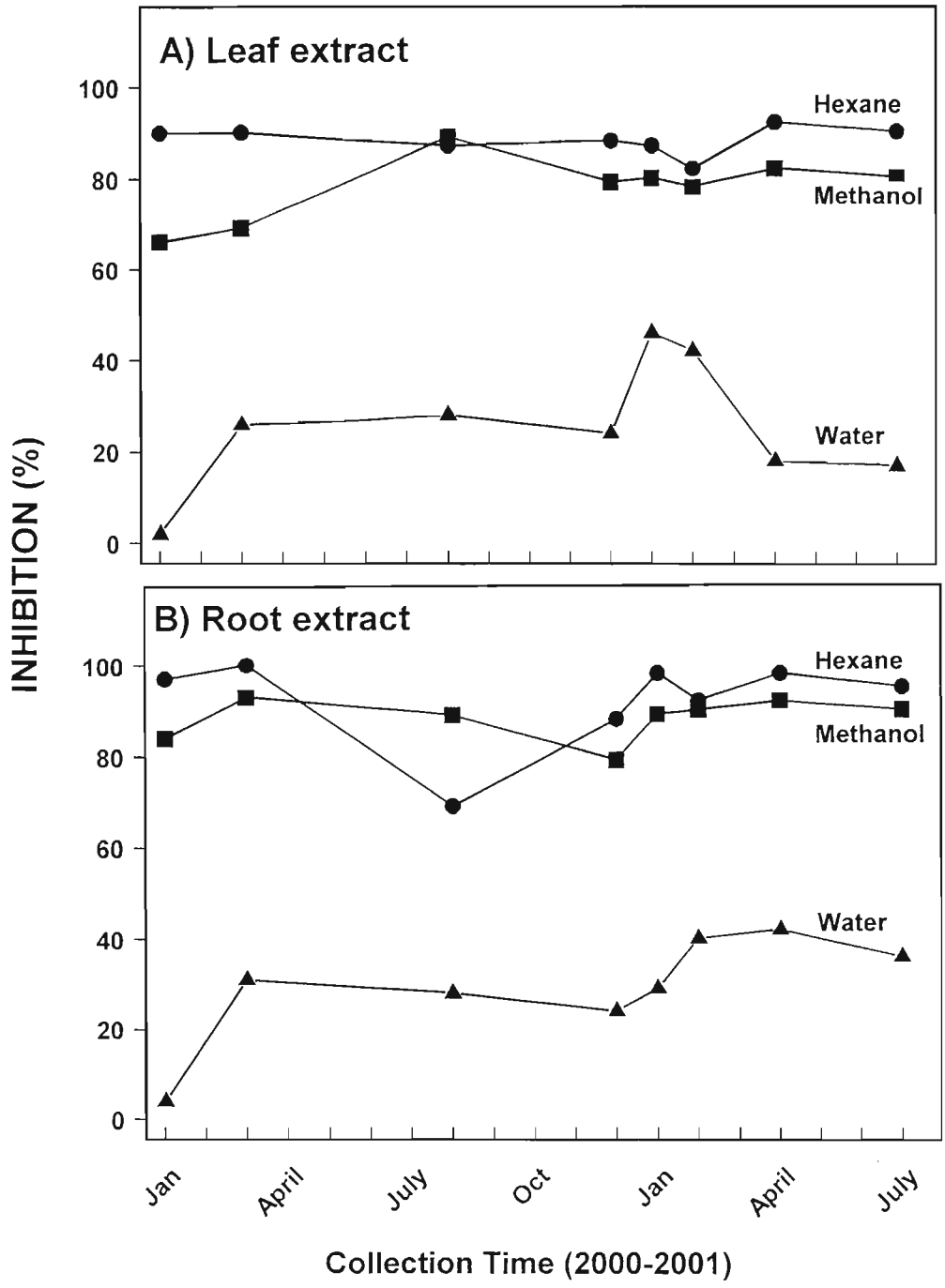


Figure 4.4: Seasonal variation in anti-inflammatory activity of leaf (A) and root (B) material of *A. microraphis*. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 73 ± 7%.

herbalists and gatherers to obtain the most effective *M. parviflora* and *E . punctulatus* plant material, plant harvesting need to be encouraged during the cooler months.

M. parviflora is considered a weed. It occurs abundantly close to villages. Since both its aerial and underground parts are utilised in traditional remedies, and these parts are potentially active, harvesting of either leaves or roots will not be detrimental to the sustainability of this species. In contrast, *E. punctulatus* grows in remote areas and is scarce. Since aerial parts are used traditionally and have tested active, its harvesting is also not too much of a threat to its continued growth in the wild. *A. microraphis* did not exhibit seasonal variation in anti-inflammatory activity of water extracts made from leaf and root material. However highest activity was noted from collections made in February 2001 (Figure 4.4). Since both leaves and roots are traditionally utilised, it is therefore advisable that traditional healers and herbalists should be encouraged to collect leaves rather than roots. This could help to conserve this highly exploited plant.

Variation in anti-bacterial and anti-inflammatory activity as a result of season has also been observed for *Siphonochilus aethiopicus.* Leaf extracts gave a minimum inhibitory concentration (MIC) of 0.2 mg ml^{-1} before senescence and 3.13 mg ml^{-1} after senescence, indicating a loss in anti-bacterial activity. Extracts prepared from roots gave MIC values of 3.13 and 0.1 mg ml^{-1} before and after senescence, indicating an increase in activity. High levels of anti-inflammatory activity resulted from leaves prior to senescence (LIGHT, MCGAW, RABE, SPARG, TAYLOR, ERASMUS, JÄGER and VAN STADEN, 2002).

MENKOVIC, SAVIKIN-FODULOVIC and SAVIN (2000) proposed that seasons and developmental stages in the plant caused variation in amounts of secondary compounds in certain plants. This was based on seasonal variation in levels of mangiferin and isorientin in *Gentiana lutea*. These compounds reached their maximum level between June and July (winter), coinciding with the flowering stage. A rapid decrease in the level of these compounds was recorded in May and August. These observations correlate with the results on essential oils of *E. punctulatus* (WEBBER, MAGWA and VAN STADEN, 2000 B). The highest level of these oils was recorded during late flowering/early seed development. A decrease in oil was noted at the bud initiation stage in spring. However, these findings do not correspond with the results obtained on determination of seasonal variation in medicinal activity of *E. punctulatus*. Anti-inflammatory activity of this plant was high during winter, the period which does not correspond with flowering and seed development when high level of essential oils was recorded. In summer and autumn, anti-inflammatory activity declined (Figure 4.3) whereas the anti-bacterial activity was not affected by seasonal changes (Table 4.3).

Although anti-bacterial activity of *M. parviflora* varies seasonally, fluctuation in the detected activity was also observed from year to year. ELGORASHI, DREWES and VAN STADEN (2002) observed a similar situation in levels of alkaloids found in C*rinum moorei* collected at different times. Plant material collected in 1998/1999 resulted in the highest total alkaloids in bulbs harvested during winter while leaves and roots harvested in summer had the highest content of alkaloids. In the second year (1999/2000), bulbs had the highest quantities of total alkaloids in summer whereas the highest concentration of the total alkaloids detected in the leaves and

roots was in spring and winter respectively. There can therefore be much variation in secondary product levels within plants or plant parts related to growing season.

Depending on the plant and the ailment and disease it is used for, the time of harvest and state of the material for extraction may only have minimal influence on the degree of the activity (LIGHT, MCGAW, RABE, SPARG, TAYLOR, ERASMUS, JÄGER and VAN STADEN, 2002). The results from this work suggest that *A. microraphis* can be collected throughout the year since no seasonal variation was noted in the anti-inflammatory activity of the extracts. A similar situation where the activity of the extracts was not affected by seasonal times was observed in the action of Brazilian propolis which did not show any seasonal anti-bacterial effects against *S. aureus, P. aeruginosa, E. coli* and *Salmonella typhimurium* from the extracts/material collected in spring, summer, autumn and winter (SFORCIN, FERNANDES, LOPES, BANKOVA and FUNARI, 2000). However, both *M. parviflora* and *E. punctulatus* showed an increase in pharmacological activity when collected during the cooler months and thus should only be collected during the cooler months and stored for future use.

Despite the fact that many plant species are threatened, few governments seem aware or show any concern about the problem of over harvesting. Data is not gathered and often the consequences are not obvious until the damage has been caused or particular species are threatened with imminent local extinction. It is therefore advisable that, in addition to associations/councils such as the Traditional Medical Practitioners (TMP`s) and the Lesotho Universal Medicine Men and Herbalists Council (LUMHC) formed in Lesotho (MARSHALL, 1998) to control the

traditional healers/herbalists' activities such as the collection of wild medicinal plants. Strict laws should be enforced for an effective control of people (healers/gatherers/traders) involved in the destructive and over harvesting of medicinal plants. For example, the illegal destruction of habitats has been reported in the Sikkim Himalayan region with the extraction of wild medicinal plants, deforestation, and excessive grazing pressure on pasture. Absence of officials at the remote sites allowed collectors and animals to enter protected sites (RAI, PRASAD and SHARMA, 2000) a situation not unlike what pertains in Lesotho.

Good collaboration with traditional healers and herbalists in the health care system and a proper education on conservation of endangered and over-exploited plants need to be emphasised. These could include methods such as propagation of medicinal plants for small scale farming (BANQUAR, 1993; COX, 1994). These may reduce the illegal destruction of natural habitats in Countries where the use of medicinal plants to cure certain ailments and diseases is actively practiced.

In Countries like South Africa, efforts are in progress to rationalise conservation policies regarding the gathering of herbs for medicinal purposes. Organisations such as the Kwazulu-Natal Bureau of Natural Resources, Natal Parks Board, and the Durban Municipality in conjunction with the Institute of Natural Resources (University of Natal, Pietermaritzburg), are investigating methods to overcome exploitation of flora and fauna for medicinal purposes, without inhibiting cultural practices. Commercial cultivation and inclusion of medicinal herbs as part of an annual crop by subsistence farmers have been suggested as a means of counteracting this problem (BYE and DUTTON, 1991).

Rules and regulations governing the utilisation of medicinal plants in Lesotho have been formulated (AKERELE, 1991 B; MARSHALL, 1998) but strict measures on people who continuously deplete natural habitats of most plant species are not taken. However, a number of traditional healers and herbalists are aware of the declining medicinal plants and are involved in cultivation of some medicinal plants at their living places. Theft, however, has become a limiting factor (CHAPTER 3). Rural community empowering projects under the Lesotho Highlands Water Project (LHDP), e.g the project on species conservation at Mohale catchment, LHDP Phase 1B, encourages the participation of community members to establish nurseries for conservation of medicinal plants in future (UNPUBLISHED).

CHAPTER 5

EFFECT OF STORAGE ON ANTI-BACTERIAL AND ANTI-INFLAMMATORY ACTIVITIES OF *MALVA PARVIFLORA*, *ERIOCEPHALUS PUNCTULATUS* AND *ASPARAGUS MICRORAPHIS*

5.1 Introduction

Medicinal plants used traditionally for treatment of diseases are often collected from areas distant to the people who utilise them. In Lesotho, the highest distribution of medicinal plants occurs in the highlands area (mountain zone) of the country where there is a rich diversity of indigenous plants with medicinal value. Traditional healers and herbalists obtain plants used in traditional remedies mainly from the highland areas (CHAPTER 3) while the vast majority of Lesotho's population live in the lowland area.

The major active ingredients that contribute to the medicinal value of the plants are affected by factors such as cultivar, species, number of years of cultivation, growing conditions and/or environment, methods of processing and harvesting techniques (LI and WARDLE, 2001). For example, harvesting of herbs that contain essential oils is better done when plants first begins to blossom because at that time the oil content is at its highest (MILO, 1952). The temperature during drying is also critical because

if it rises too high, volatile constituents, such as essential oils will be depleted or lost (WHITTEN, 1997). After harvesting, some plants tend to have a high moisture content of approximately 50 to 70% such as the fresh roots of *Echinacea*. Such plants need to be dried as soon as possible before storage in order to protect loss of active ingredients and prevent microorganism contamination (LI and WARDLE, 2001). Adequate drying of herbs before storage prevents moisture migration that may arise within the stored herbs and teas. If a herb goes into storage with a borderline moisture content of around 13%, temperature fluctuation may cause the moisture to move from the warm part of the container and condense in the cold part. This results in mould growth in the damp portion (WHITTEN, 1997).

Storage of medicinal plants and herbal teas under unsuitable conditions may cause plants to be infested by insects and mites (KALINOVIC and ROZMAN, 2000). An investigation on harmful insects and mites that occur in stored medicinal herbs and teas showed that *Althaea officinalis* (Malvaceae) is amongst the plants attacked by pests. The most frequent insects to attack the plants were the psocids (75%). These are mycophagous insects, which feed on microorganisms such as fungi and bacteria. All pests that infested the plants were found in older, damp and mouldy medicinal plants and teas. Other reports indicate that the contamination of medicinal plants by mould during wet periods or after inappropriate or prolonged storage causes poisoning of plant preparations used in herbal remedies (STEWART, STEENKAMP AND ZUCKERMAN, 1998; KALINOVIC and ROZMAN, 2000).

5.2 Aims

The aims of this part of the study were:

1) To determine the effects of storage on anti-bacterial activity of stored *M.parviflora* and *E. punctulatus* material and extracts; and

2) To determine the effects of storage on anti-inflammatory activity of stored *M. parviflora, E. punctulatus* and *A. microraphis* material and extracts.

5.3 Materials and Methods

5.3.1 Plant material

Leaves and roots of *M. parviflora* and *A. microraphis* were collected in January 2000 and leaves of *E. punctulatus* in May 2000 from Qacha's Nek district in Lesotho. The collected plant material was dried at 50 ^{0}C and finely ground.

5.3.2 Storage of *M. parviflora, E. punctulatus and A. microraphis*

The control experiment of *M. parviflora, E. punctulatus* and *A. microraphis* was carried out at the time of collection (0 months). Dried, ground material (2 g) was immediately extracted with 20 ml hexane, methanol or water in an ultrasound bath as described in Section 3.3.3. *M. parviflora* and *E. punctulatus* extracts were redissolved at 100 mg ml^{-1} and tested for anti-bacterial activity using the disc-diffusion bioassay (Section 2.2.1). The extracts were then dried in a stream of air.

Next, all the plant extracts were redissolved at 8 mg ml^{-1} (organic solvent extracts) and 1 mg ml^{-1} (water extracts) and tested in the COX–1 anti-inflammatory bioassay (Section 2.3.1).

The remaining dried ground plant material of all the three species were kept in airtight glass containers. The plant material was stored in a cold room (10 ^{0}C), at room temperature (25 ^{0}C) and on the roof of a garden shed in the Botanical Gardens where it was exposed to direct sunlight and large changes in temperature. Dried hexane and methanol extracts obtained from the leaves and roots of the plant material were kept in Schott bottles. These extracts were stored in a cold room (10 ^{0}C) and at room temperature (25 ^{0}C) only.

The stored dried material and extracts were bioassayed every three months to determine both anti-bacterial and anti-inflammatory activity as described above. The experiment ran for twelve months.

5.3.3 TLC Fingerprinting

In conjunction with testing the extracts for inhibitory activity in the two bioassays, TLC fingerprinting was carried out at the same time on all the hexane and methanol extract. TLC fingerprinting was not carried out on water extracts because these extracts did not give good separation on TLC plates.

Dried extracts were redissolved at 50 mg ml^{-1} and 10 µl of each extract were spotted onto plastic TLC plates as described in Section 2.4. The extracts were

developed using various solvent systems as shown in Table 5.1. These were derived at after exhaustive experimentation to determine the best solvent for each extract. Photographs of the TLC plates were taken at UV_{254} and UV_{366}. The anisaldehyde stained plates were also photographed.

Table 5.1: List of solvent systems used to develop TLC plates, which were spotted with *M. parviflora, E. punctulatus* and *A. microraphis* hexane and methanol extracts

Plant name	Extracts	Solvent systems (V:V)
M. parviflora	Hexane	Toluene : ethyl acetate (9:1)
	Methanol	Toluene : ethyl acetate (7:3)
E. punctulatus	Hexane	Benzene : ethyl acetate (7:3)
	Methanol	Benzene : ethyl acetate (8.5:1.5)
A. microraphis	Hexane	Chloroform : methanol (8.5:1.5)
	methanol	Toluene:ethyl acetate:acetic acid (6.5:3:0.5)

5.4 Results

5.4.1 Effect of storage on biological activity of *M. parviflora*

Anti-bacterial activity

Effect of storage on anti-bacterial activity of dried *M. parviflora* plant material and organic extracts was tested for hexane, methanol and water extracts made from leaves and roots.

At the time of collection, hexane and methanol extracts made from leaves showed anti-bacterial activity against *S.epidermis* (Gram-positive) and *P. aeruginosa* (Gram-negative) (Figures 5.1 A to 5.1 D). Water extracts had no activity against the tested bacteria (Figure 5.1 E).

Storage of the plant material in a cold room caused an increase in anti-bacterial activity of the hexane and methanol extracts of leaf material against the two bacteria investigated for up to six months of storage. Storage for longer than six months resulted in a decrease in activity. No activity was detected in any of water extracts throughout the storage period of twelve months (Figures 5.1 A, C and E).

Leaf material stored at room temperature (Figure 5.2) and in the Botanical Garden (Figure 5.3) showed a similar trend as those stored in a cold room with an in increase in anti-bacterial activity of the hexane and methanol extracts made from leaves after three months storage against both Gram-positive *(S. aureus, B. subtilis and S. epidermis)* and Gram-negative *(P. aeruginosa)* bacteria. The activity decreased after six months storage. Water extracts never showed any activity (Figures 5.2 A, C, E and 5.3 A, B, C).

Storage of hexane and methanol leaf extracts in a cold room and at room temperature showed fluctuations in anti-bacterial activity throughout the storage period. The activity against both Gram-positive (*B. subtilis and S. epidermis)* and Gram-negative *(P. aeruginosa)* bacteria decreased after three months storage followed by an increase in activity after six months storage. A decline in activity was

again observed after nine and twelve months storage (Figures 5.1 B, D and 5.2 B, D).

Hexane and methanol extracts obtained from root material had no anti-bacterial activity at the time of collection while the water extract exhibited high anti-bacterial activity against Gram-positive (*B. subtilis and S. epidermis)* bacteria (Figures 5.4 A to 5.4 E). Storage of the root material in a cold room (Figure 5.4), at room temperature (Figure 5.5) and in the Botanical garden (Figure 5.6) resulted in a slight increase in anti-bacterial activity against Gram-positive (*B. subtilis and S. epidermis)* bacteria from the hexane and methanol extracts after six and nine months storage. The activity decreased after twelve months. Water extracts made from the same stored root material showed a decrease in anti-bacterial activity against *B. subtilis* and *S. epidermis* after three months storage for the three storage conditions. (Figures 5.4 E, 5.5 E and 5.6 C).

The stored hexane and methanol extracts made from the roots of *M. parviflora* exhibited an increase in anti-bacterial activity against *B. subtilis* after 6 and 12 months storage in a cold room and at room temperature (Figures 5.4 B, D and 5.5 B, D)

Anti-inflammatory activity

Effect of storage on anti-inflammatory activity of dried *M. parviflora* material and extracts was tested for hexane, methanol and water extracts made from leaf and root material.

At the time of collection (0 months), hexane and methanol extracts made from leaves yielded high anti-inflammatory activity compared to the water extract. Storage in a cold room, at room temperature and in a Botanical Garden did not cause any change in anti-inflammatory activity of the hexane extract made from leaves throughout the twelve months storage (Figure 5.7 A). The methanol extract obtained from leaves showed a decrease in activity after three months storage regardless of the storage conditions. The activity then increased after six to twelve months storage (Figure 5.7 B). The water extract made from leaves also showed an increase in anti-inflammatory activity after three months storage, mainly from the material which was stored at room temperature (Figure 5.7 C).

Hexane, methanol and water extracts made from roots resulted in an increase in anti-inflammatory activity after three months storage with more activity detected in the extracts obtained from the plant material stored at room temperature (Figure 5.8).

Storage of hexane extracts made from leaves in a cold room did not cause any change in anti-inflammatory activity up to nine months storage. The activity began to decrease after twelve months storage (Figure 5.9 A). The hexane extract stored at room temperature exhibited a decrease in activity after six months (Figure 5.9 A). A decrease in activity was also detected from methanol extracts made from leaves stored in the cold room and at room temperature after nine months storage (Figure 5.9 B).

The hexane extract made from roots and stored in a cold room did not exhibit any change in activity throughout the twelve months storage period, while the hexane extract stored at room temperature resulted in a decrease in activity after nine months (Figure 5.10 A). The methanol extract made from roots showed an increase in activity after three months when stored in both a cold room and at room temperature (Figure 5. 10 B).

TLC fingerprinting

TLC fingerprinting was carried out using hexane and methanol extracts obtained from leaves and roots of *M. parviflora* material stored in a cold room, at room temperature and in the Botanical Garden. Hexane and methanol extracts made from leaves and roots and stored in a cold room and at room temperature were also separated by TLC.

Immediately after collection, hexane and methanol extracts made from leaf material separated into several spots when TLC plates were viewed under UV light at 254 and 366 nm and after staining with anisaldehyde. Six spots that separated from the methanol extract made from leaf material fluorescenced red while the hexane extract yielded two red spots when viewed at 366 nm. R_f values of the red spots are shown on Figure 5.11. Hexane and methanol extracts made from root material yielded three spots when viewed under both UV lights and after staining with anisaldehyde. They were no red fluorescence spots from the root extracts (Figure 5.11)

Storage of plant material for up to six months in a cold room, at room temperature and in the Botanical Garden did not cause any change in the number and colour of the spots that were detected in the hexane extracts made from leaves and roots (Figures 5.12 to 5.5.13). Prolonged storage (nine to twelve months) caused a decrease in the number of spots and the disappearance of the red fluorescence colour from the leaf hexane extracts obtained from plant material stored in the three storage conditions (Figures 5.14–5.15).

Methanol extracts obtained from leaf and root material stored at the three storage conditions exhibited a similar trend up to six months storage and the TLC profiles remained the same (Figures 5.16–5.17). Storage for longer than nine months resulted in a decrease in the number of spots (Figure 5.19).

Hexane and methanol extracts made from leaves and stored for three months in a cold room and at room temperature resulted in a decrease in the number of spots detected and disappearance of the red fluorescence spots. Hexane and methanol extracts made from roots and stored at the two storage conditions showed a similar trend after three months storage (Figure 5.20–5.23).

5.4.2 Effects of storage on biological activity of *E. punctulatus*

Anti-bacterial activity

Effect of storage on anti-bacterial activity of dried *E. punctulatus* plant material and organic extracts was tested for hexane, methanol and water extracts made from leaves.

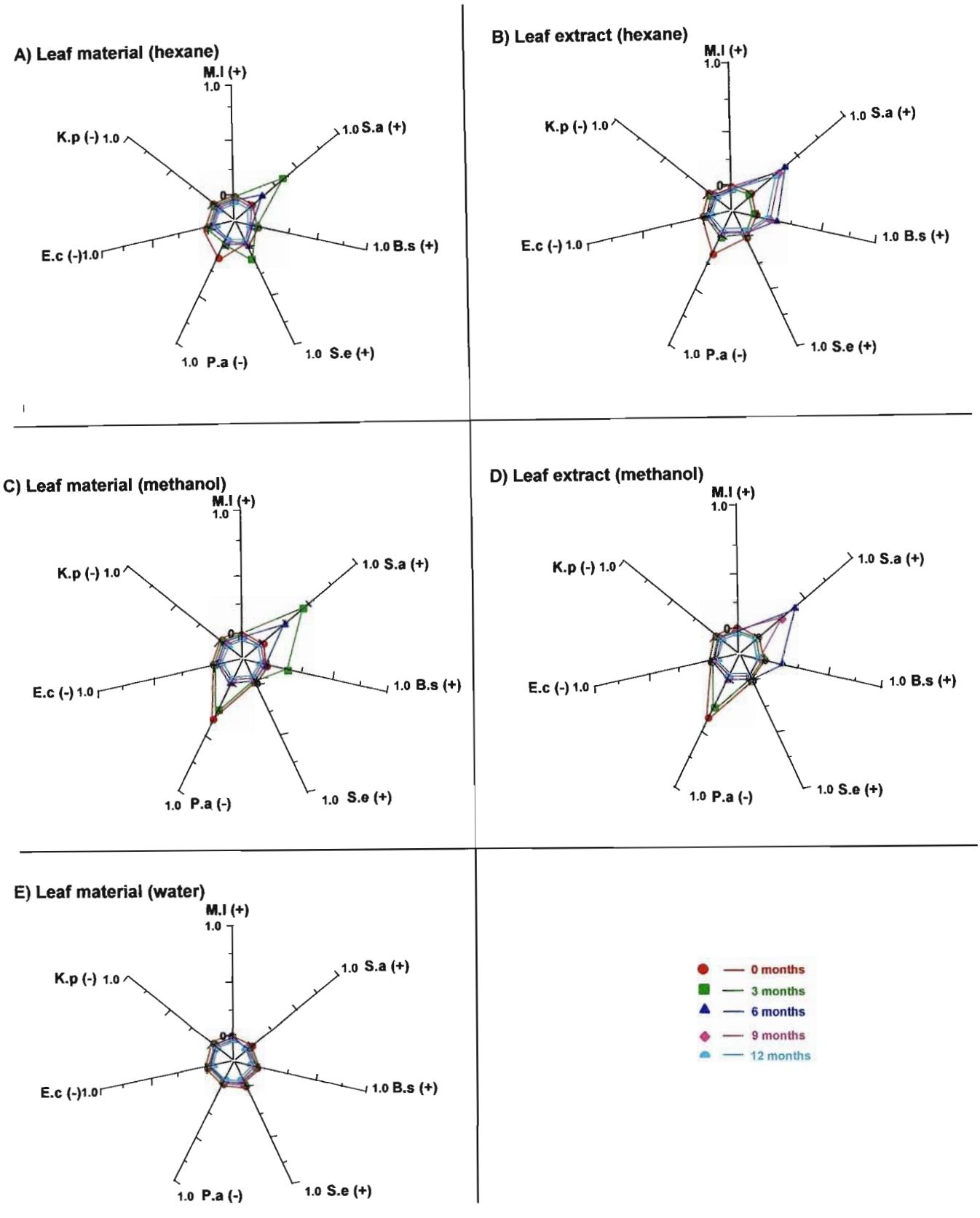


Figure 5.1: Effect of storing dried leaf material and extracts of *M. parviflora* in a cold room (10 °C) on anti-bacterial activity. A) and B) hexane, C) and D) methanol and E) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

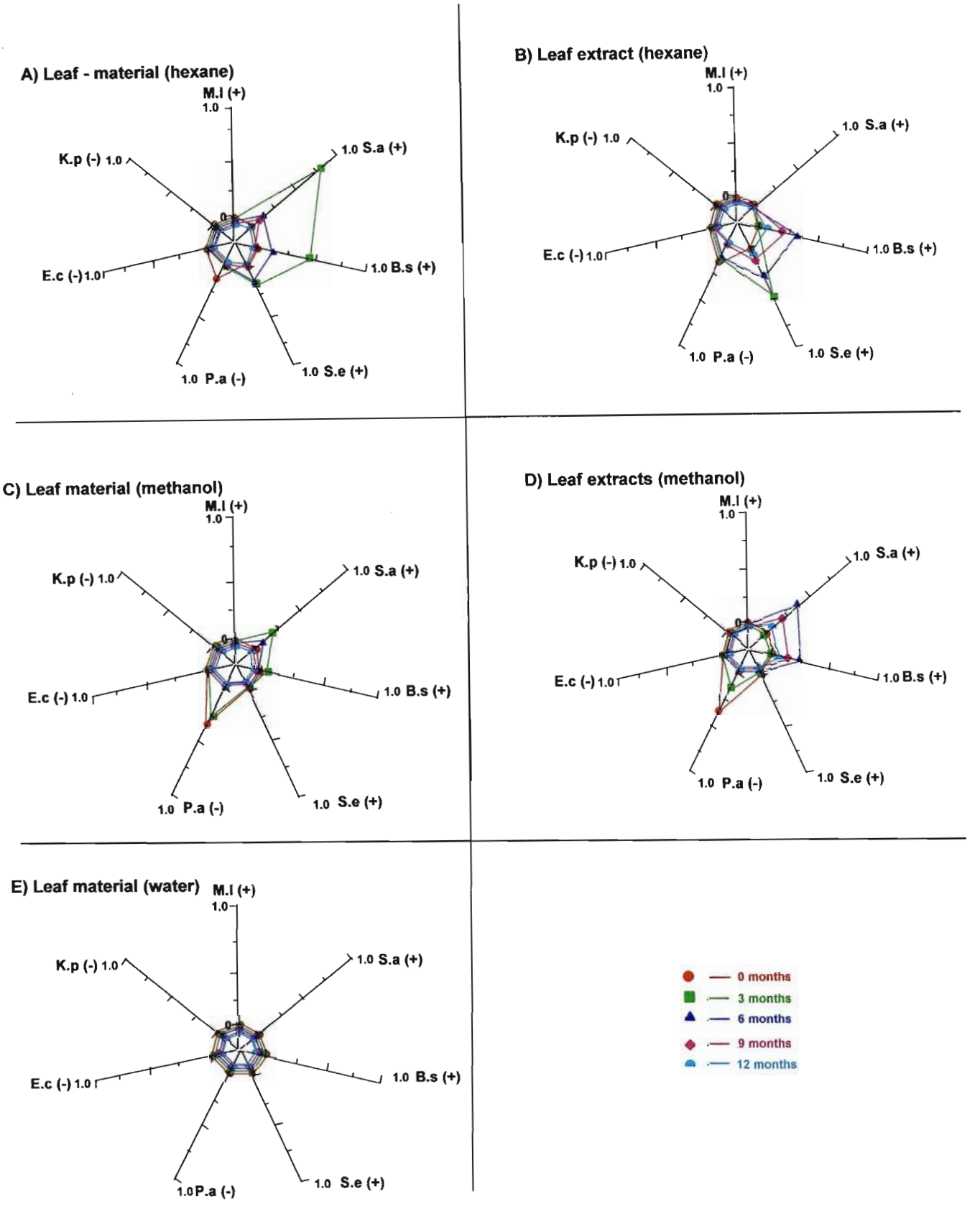


Figure 5.2: Effect of storing dried leaf material and extracts of *M. parviflora* at room temperature on anti-bacterial activity. A) and B) hexane, C) and D) methanol and E) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

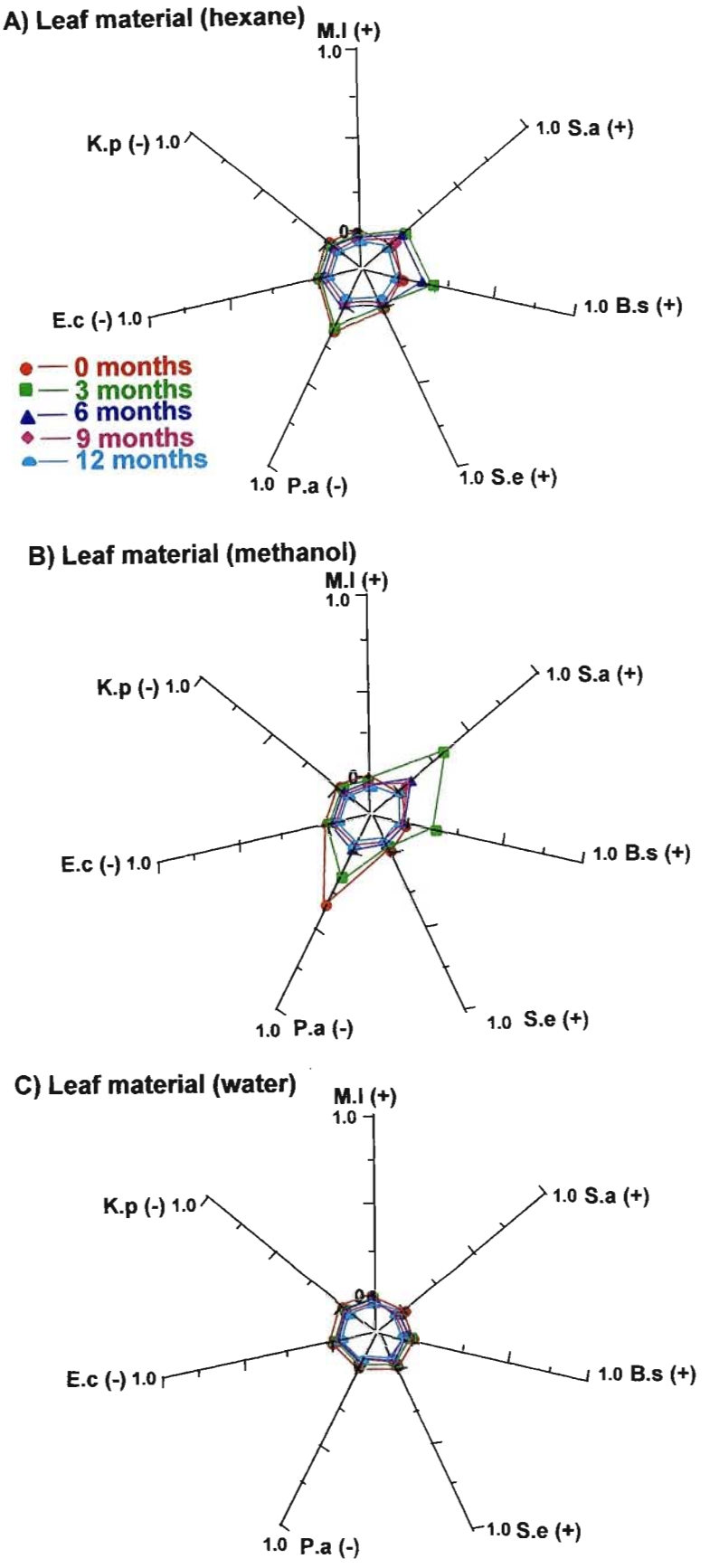


Figure 5.3: Effect of storing dried leaf material and extracts of *M. parviflora* in the Botanical Garden on anti-bacterial activity. A) hexane, B) methanol and C) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

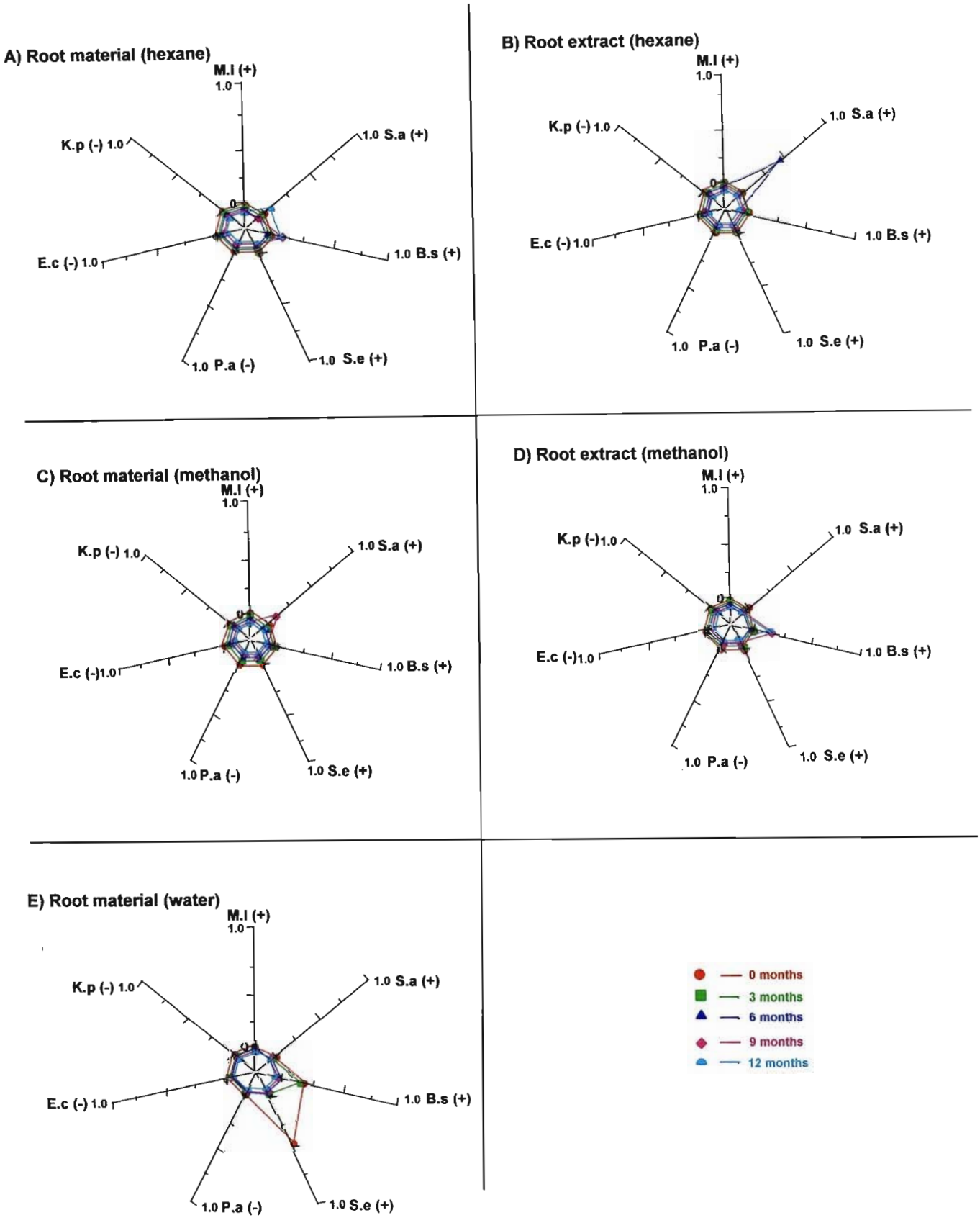


Figure 5.4: Effect of storing dried root material and extracts of *M. parviflora* in a cold room (10 °C) on anti-bacterial activity. A) and B) hexane, C) and D) methanol and E) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

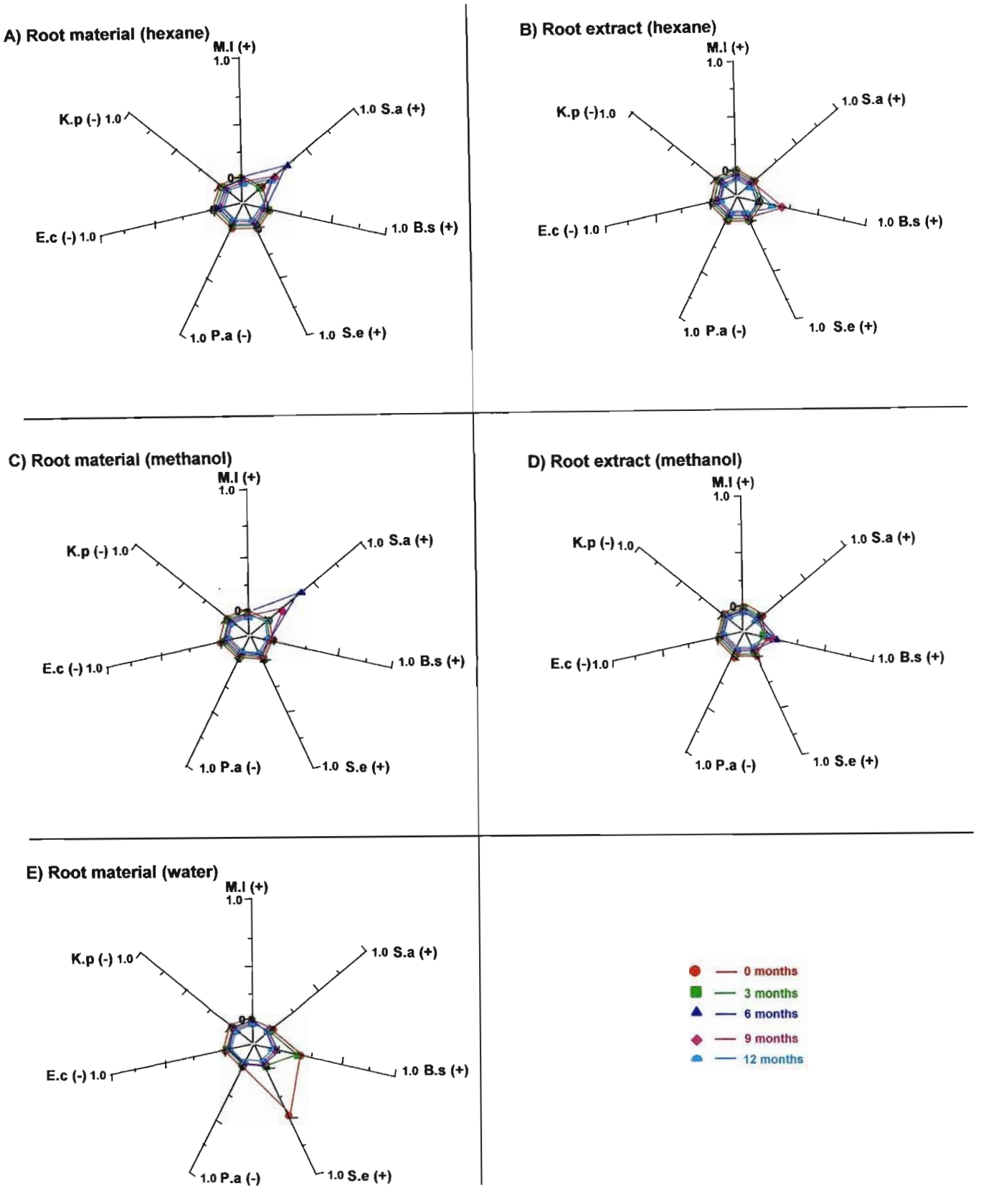


Figure 5.5: Effect of storing dried root material and extracts of *M. parviflora* in at room temperature on anti-bacterial activity. A) and B) hexane, C) and D) methanol and E) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

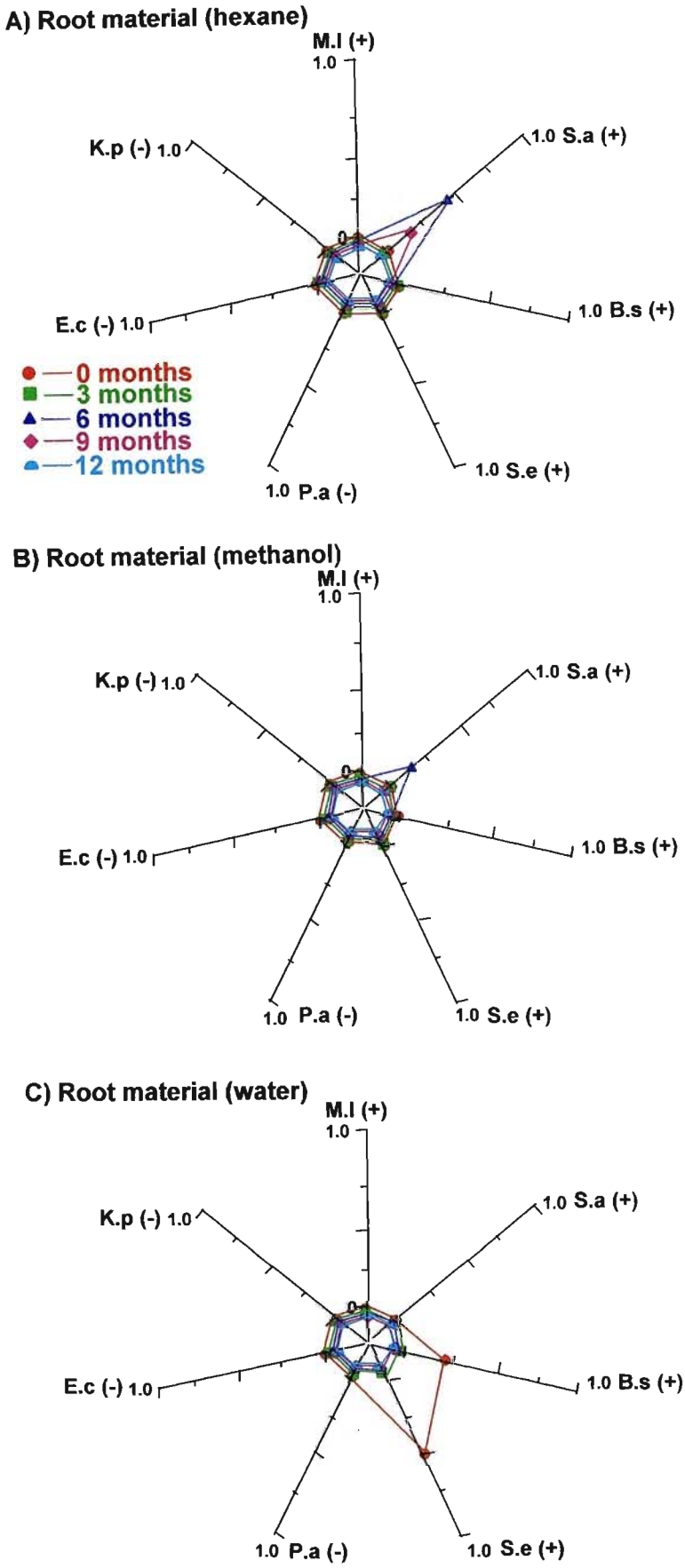


Figure 5.6: Effect of storing dried root material and extracts of *M. parviflora* in the Botanical Garden on anti-bacterial activity. A) hexane, B) methanol and C) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

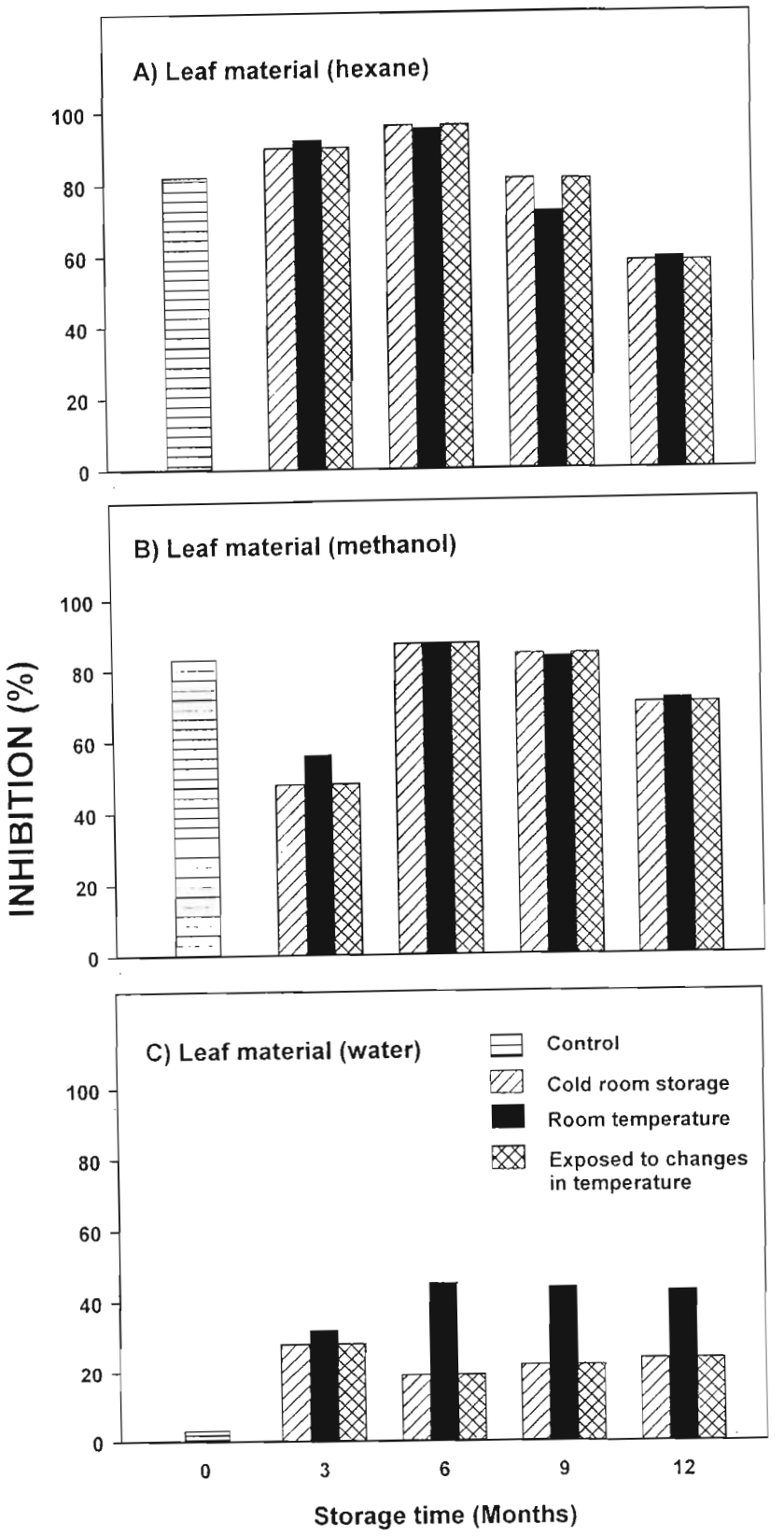


Figure 5.7: Effect of storage on anti-inflammatory activity of A) hexane, B) methanol and C) water extracts made from dried leaf material of *M. parviflora* stored in a cold room (10 ^{0}C), at room temperature and in the Botanical Garden. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 73 ± 7%.

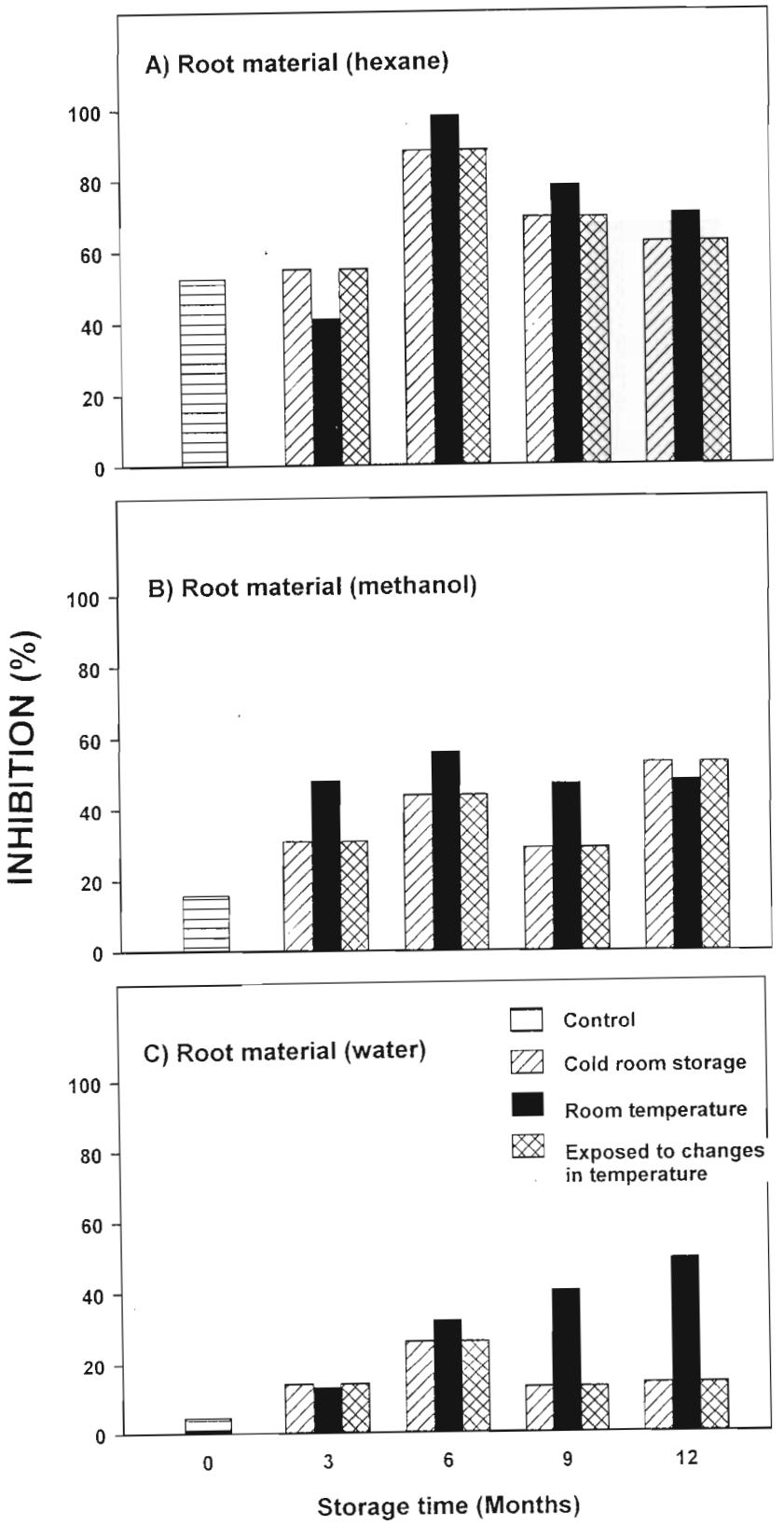


Figure 5.8: Effect of storage on anti-inflammatory activity of A) hexane, B) methanol and C) water extracts made from dried root material of *M. parviflora* stored in a cold room (10 ^{0}C), at room temperature and in the Botanical Garden. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 73 ± 7%.

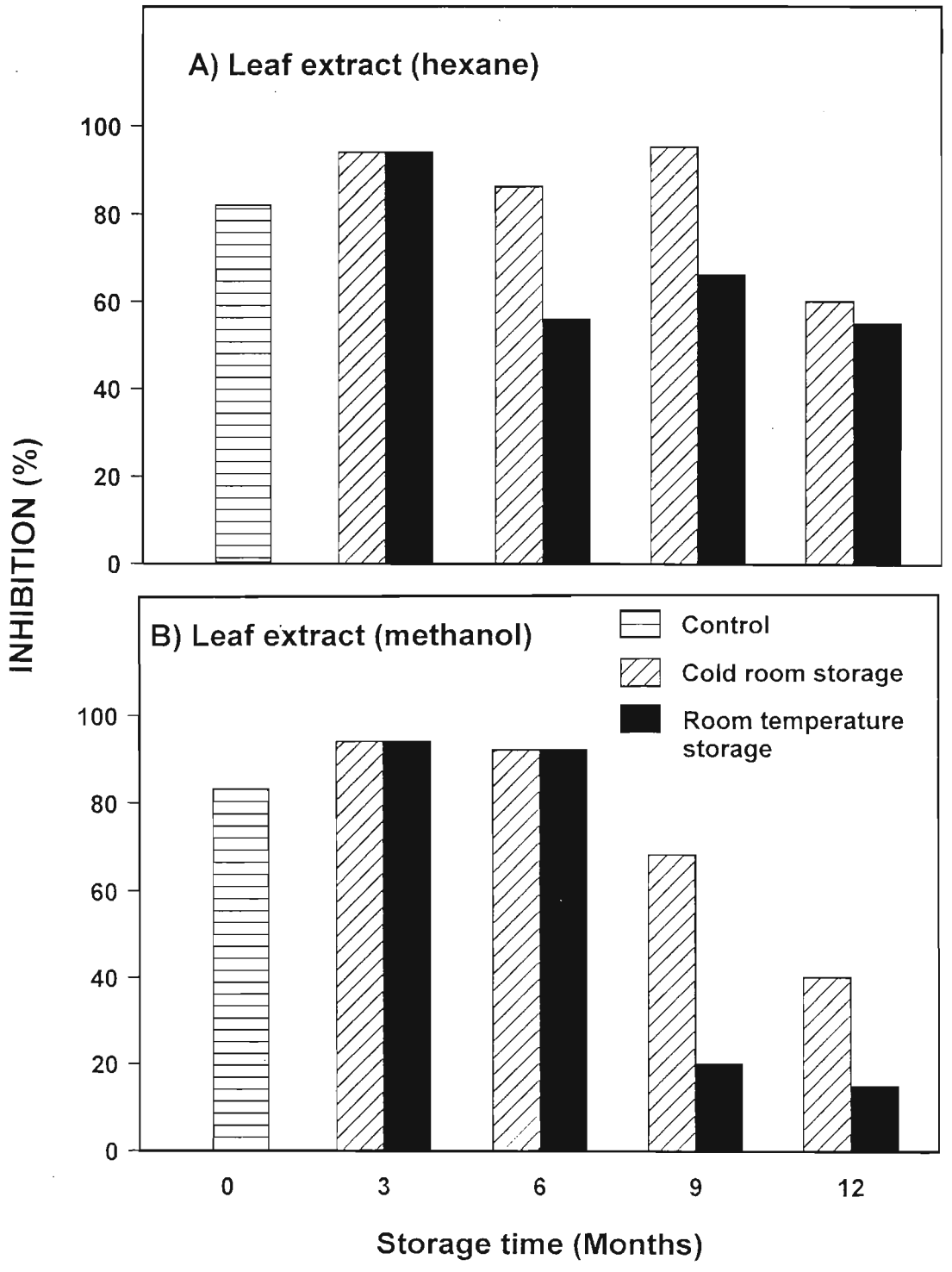


Figure 5.9: Effect of storage on anti-inflammatory activity of dried A) hexane and B) methanol extracts made from leaves of *M. parviflora* and stored in a cold room and at room temperature. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 73 ± 7%.

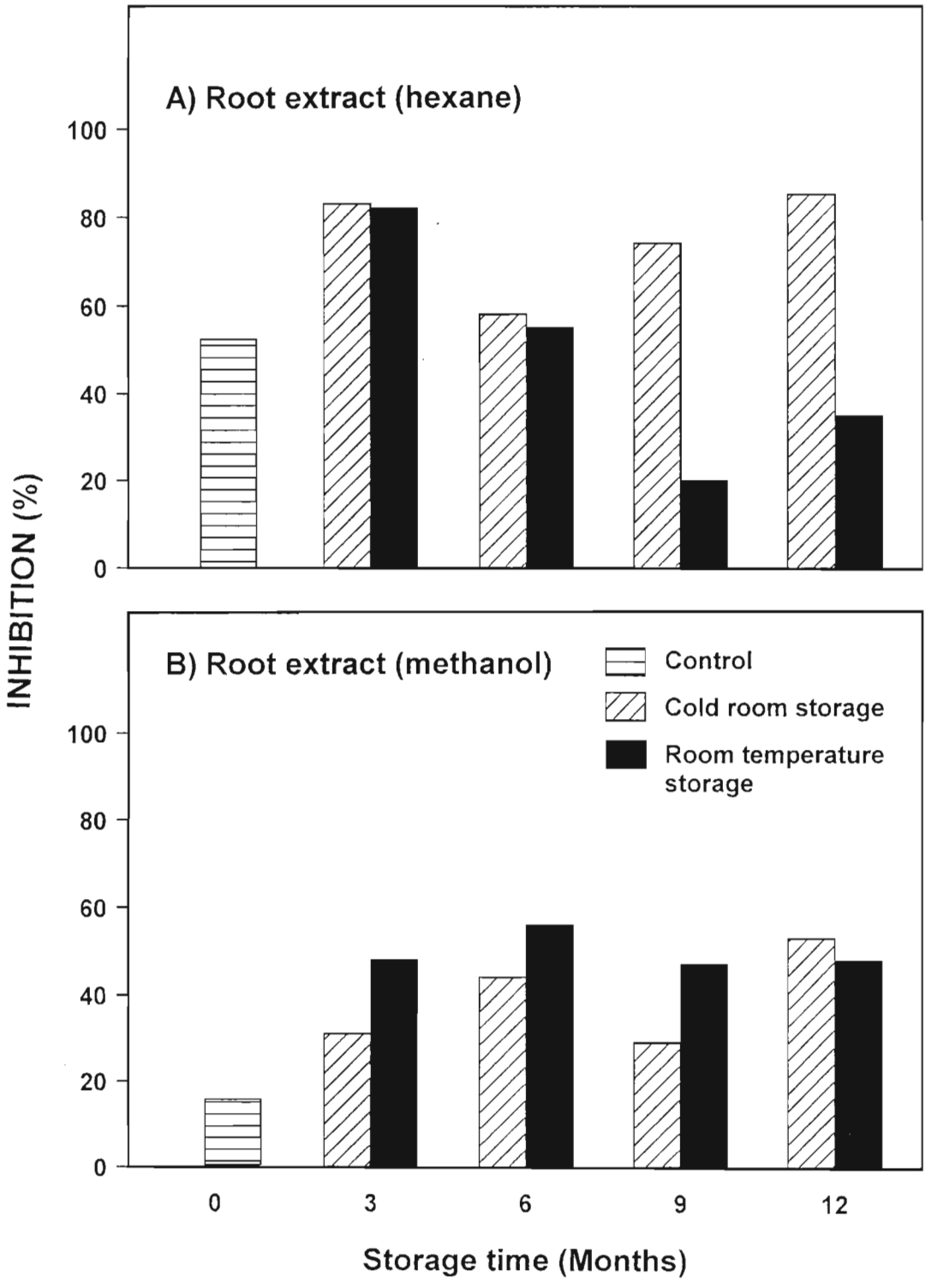


Figure 5.10: Effect of storage on anti-inflammatory activity of dried A) hexane and B) methanol extracts made from roots of *M. parviflora* and stored in a cold room and at room temperature. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 73 ± 7%.

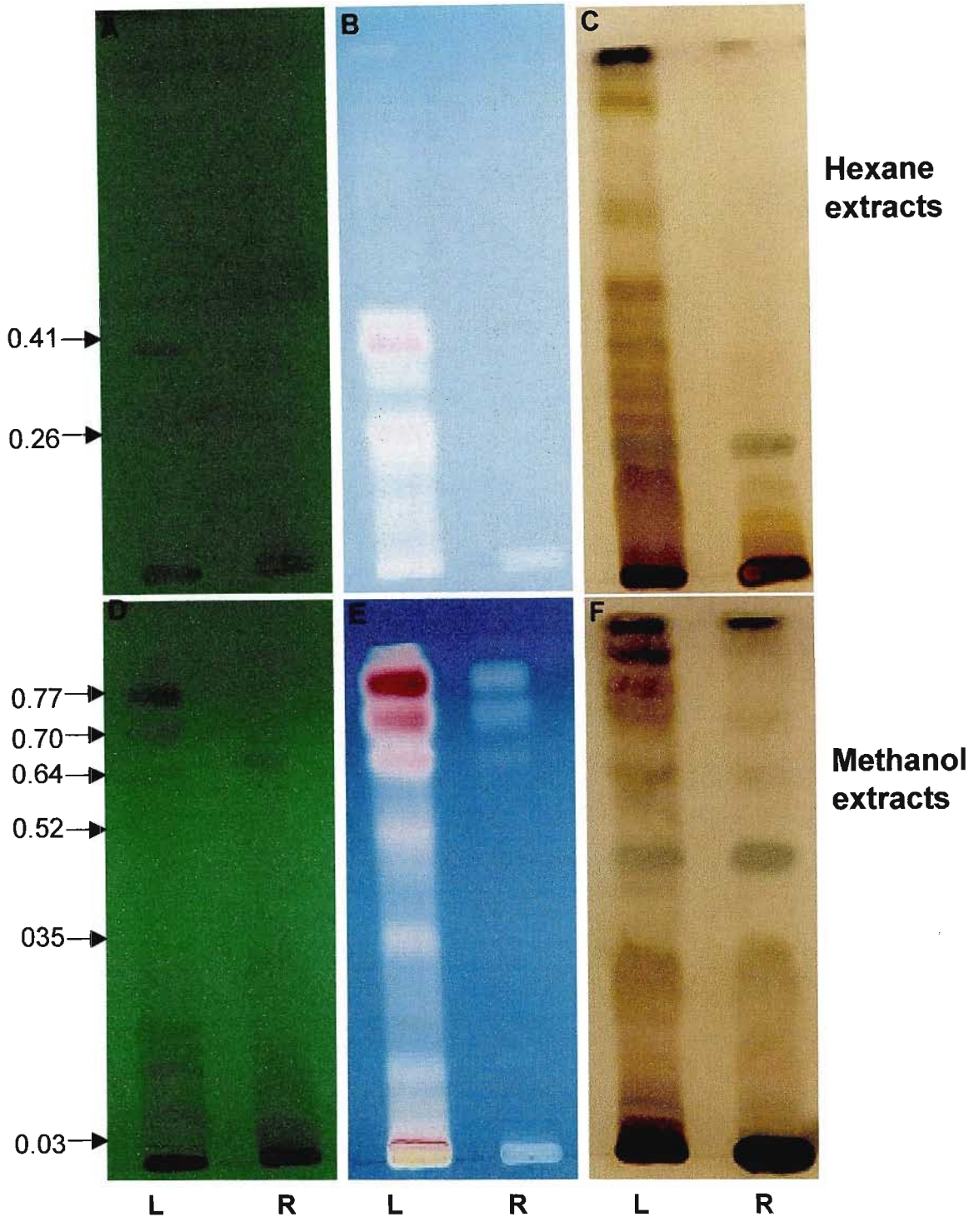


Figure 5.11: TLC fingerprints of hexane and methanol extracts made from leaf (L) and root (R) material of *M. parviflora.* TLC plates were run immediately after collection in January 2001 (0 months). These were viewed at 254 nm (A and D), 366 nm (B and E) and stained with anisaldehyde (C and F). Numbers refer to R_f values of the red spots.

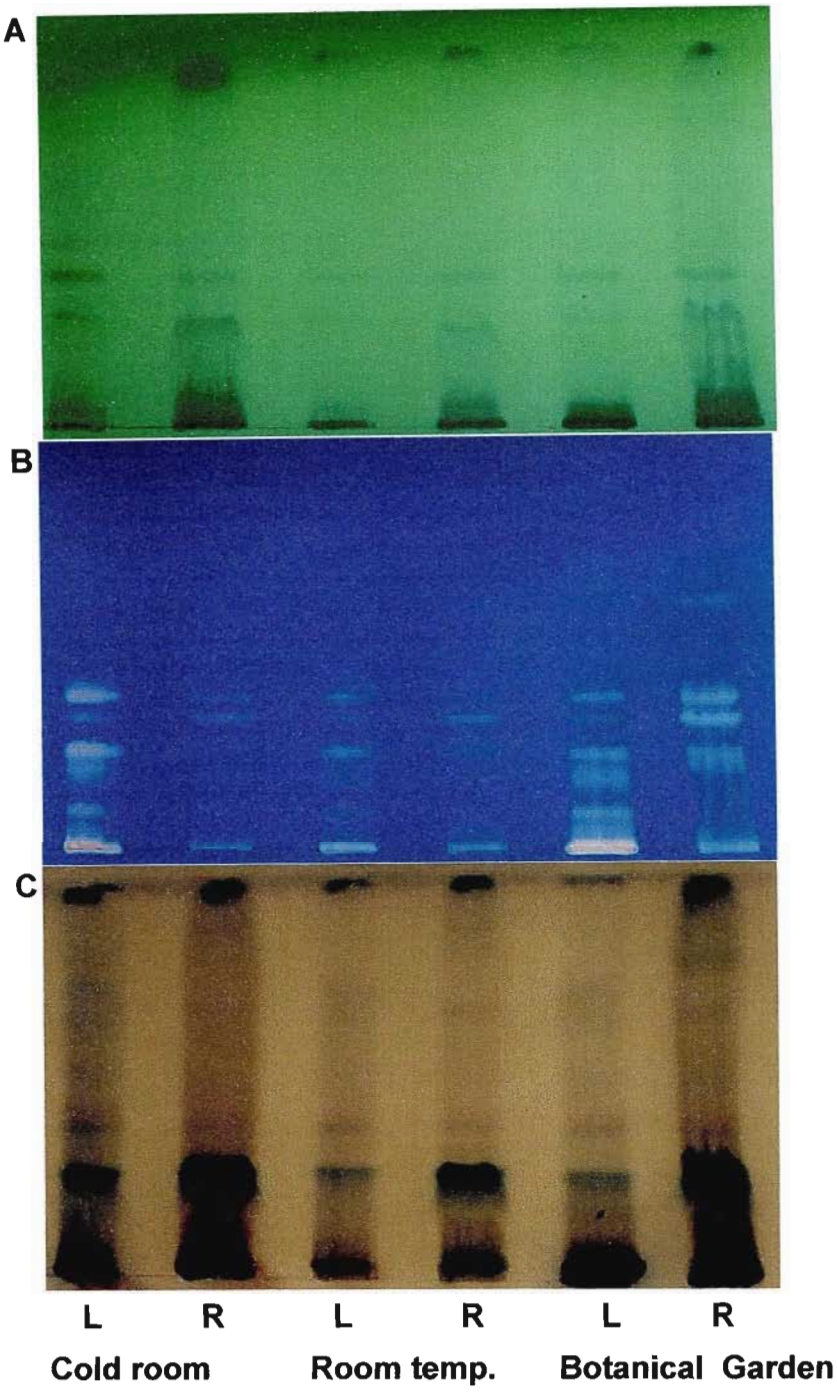


Figure 5.12: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for three months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

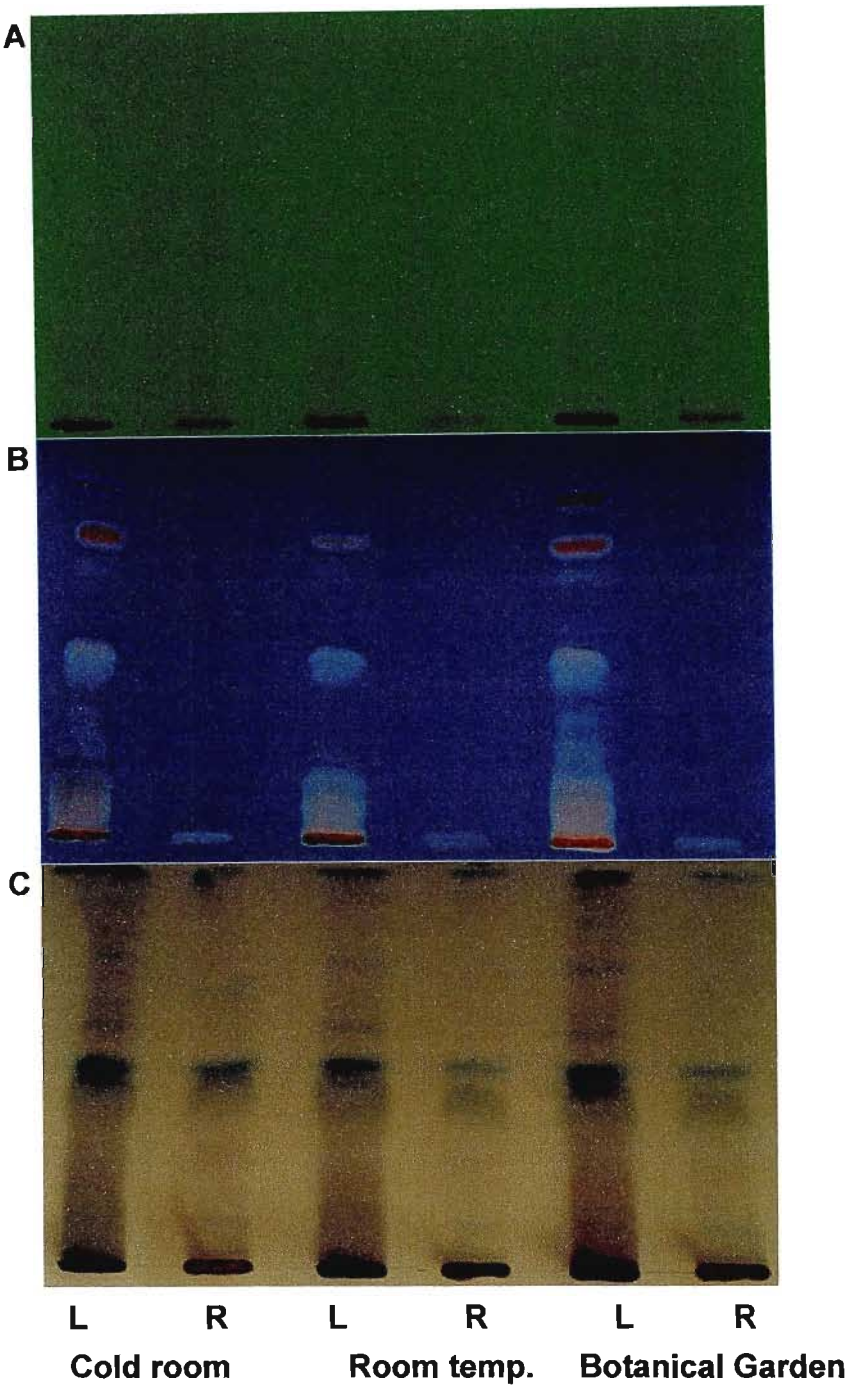


Figure 5.13: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for six months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

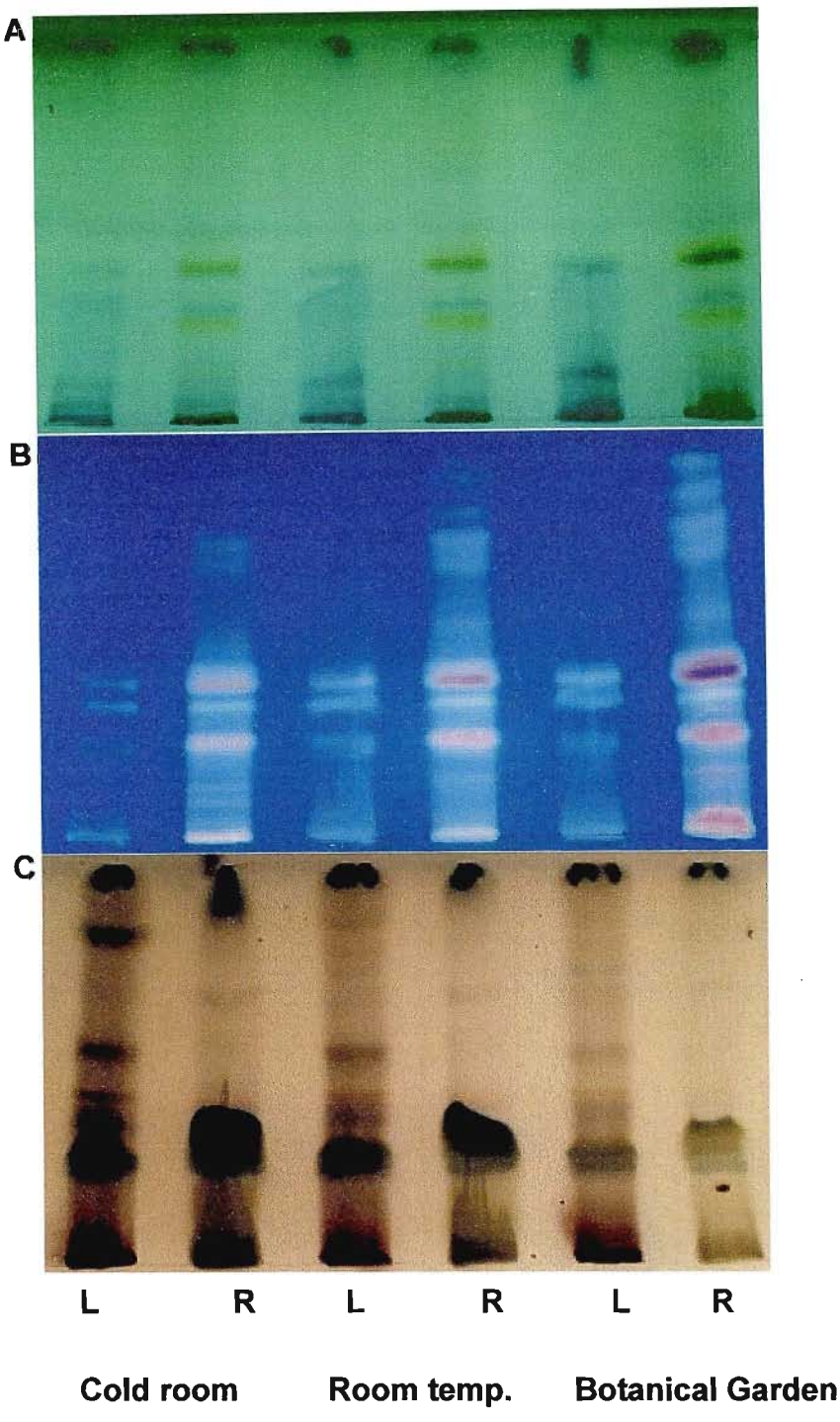


Figure 5.14: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for nine months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

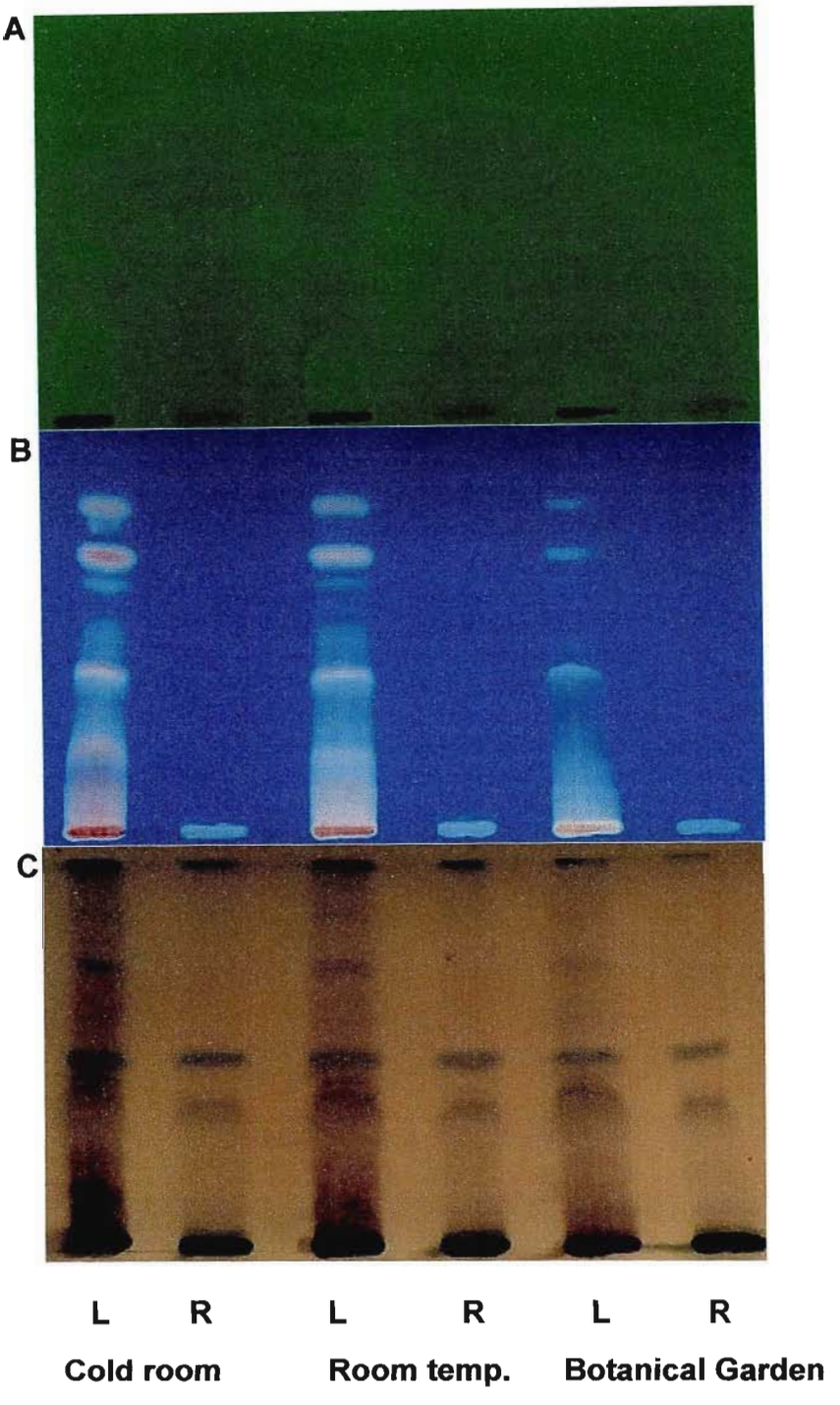


Figure 5.15: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for twelve months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

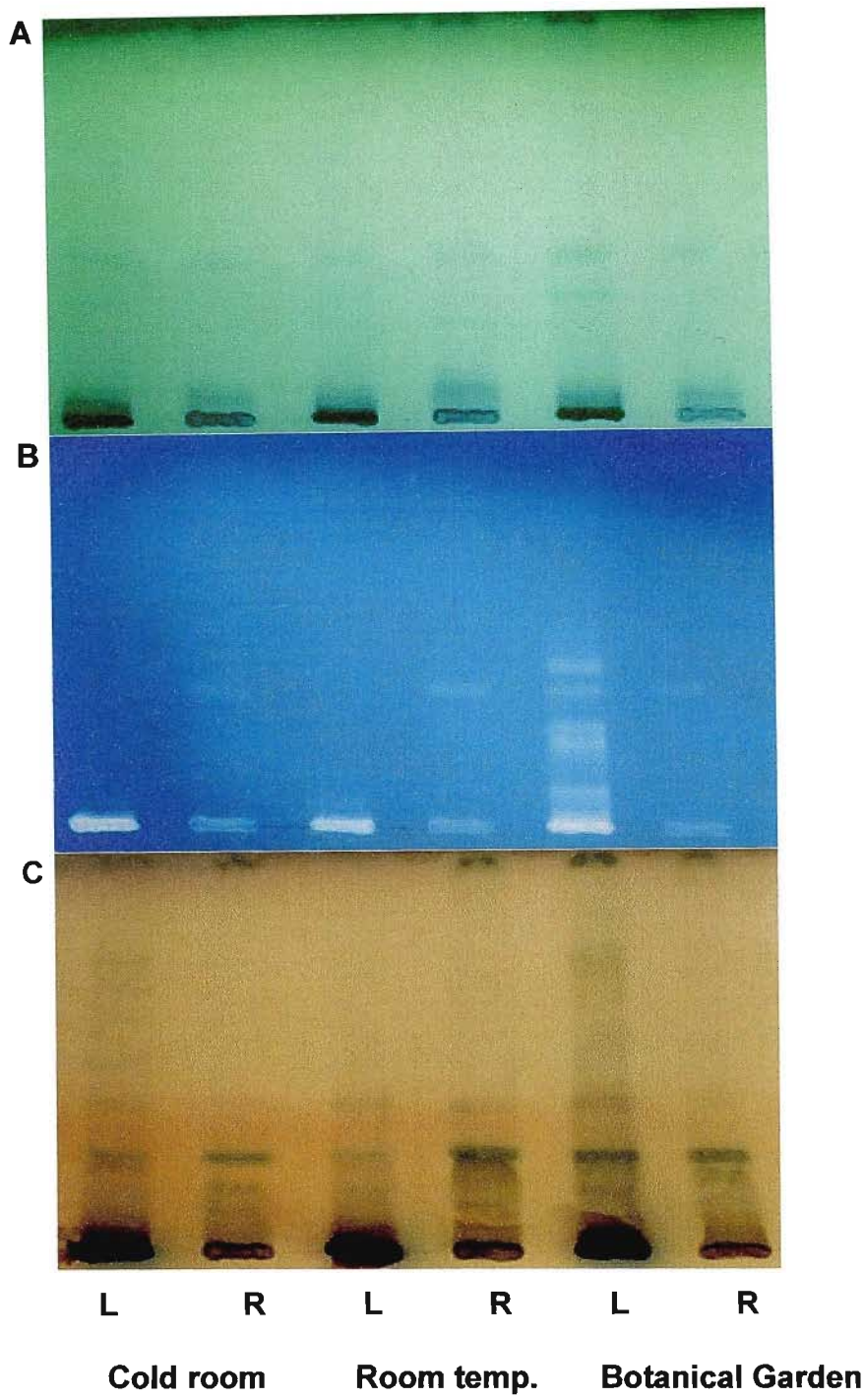


Figure 5.16: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for three months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

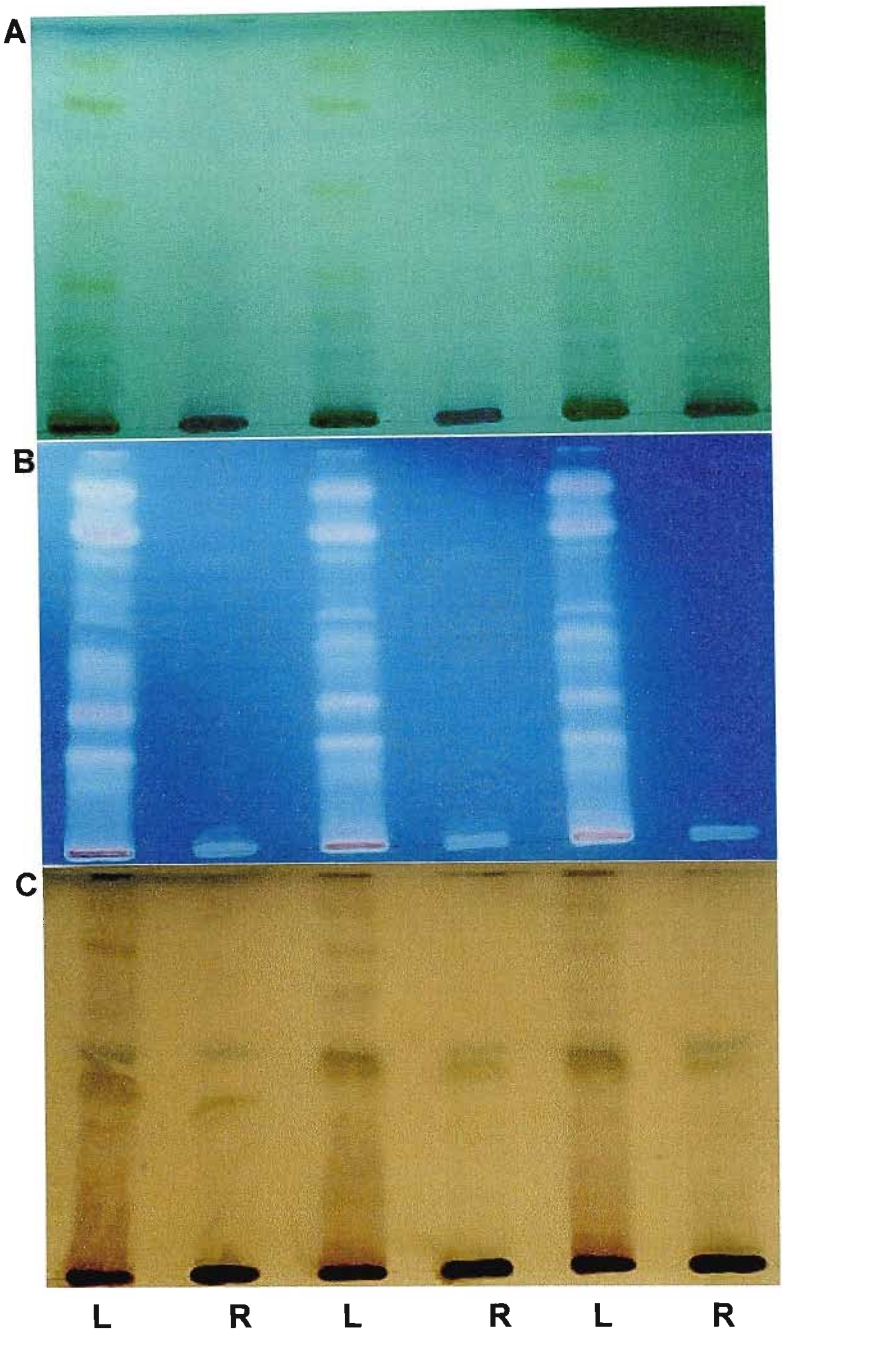


Figure 5.17: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for six months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

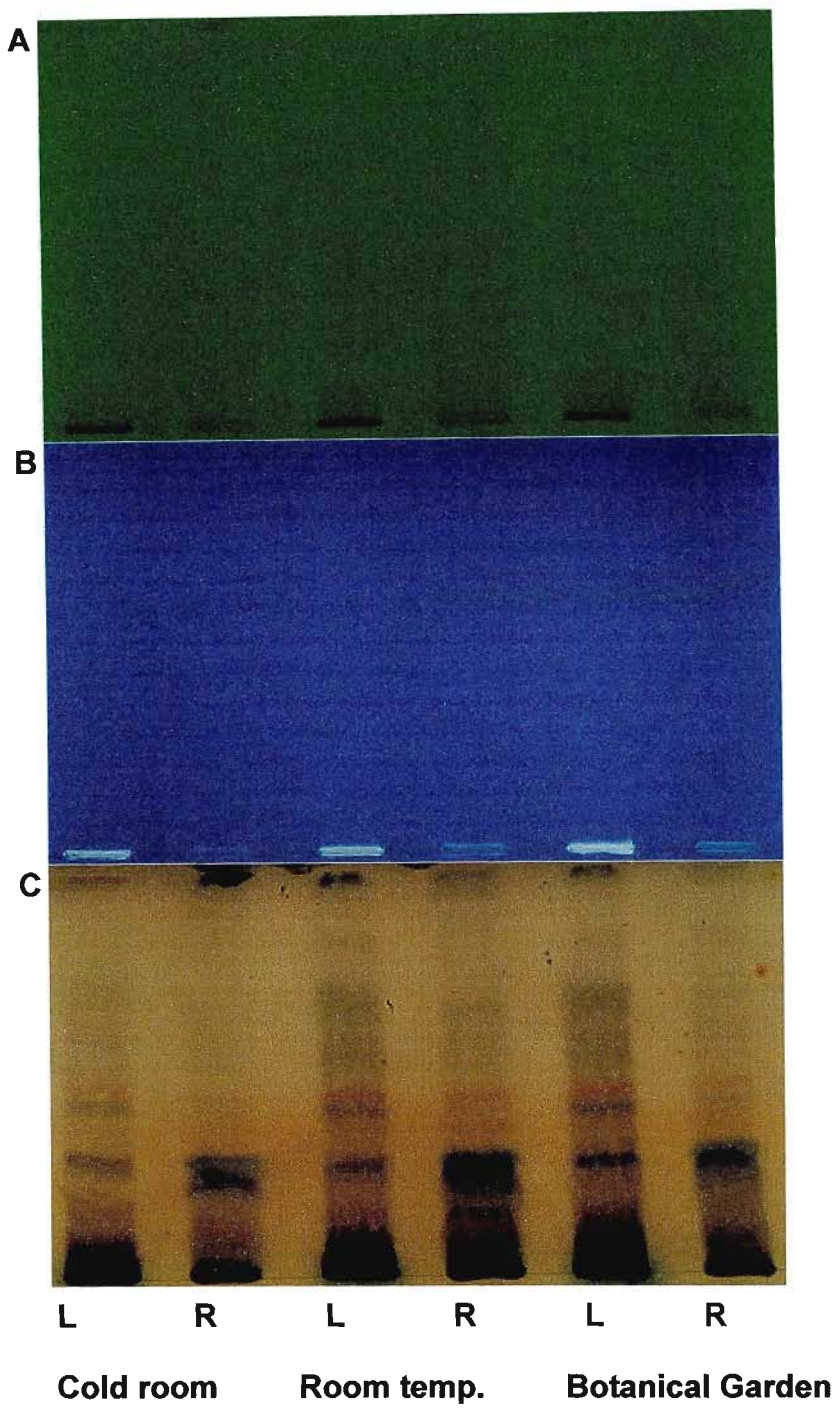


Figure 5.18: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for nine months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

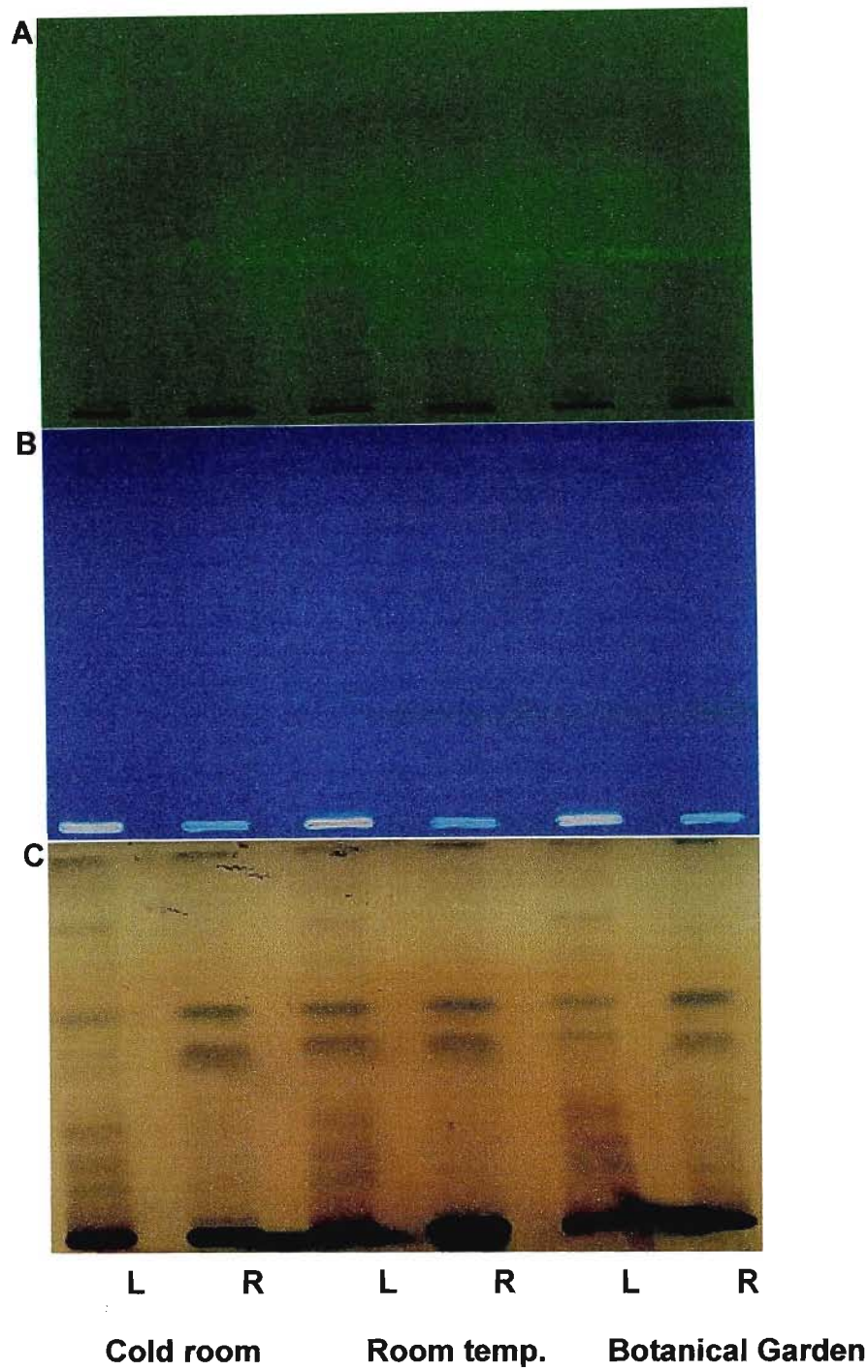


Figure 5.19: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *M. parviflora* stored in a cold room, at room temperature and in the Botanical Garden for twelve months. TLC plates were viewed at A) 254; B) 366 nm and C) stained with anisaldehyde

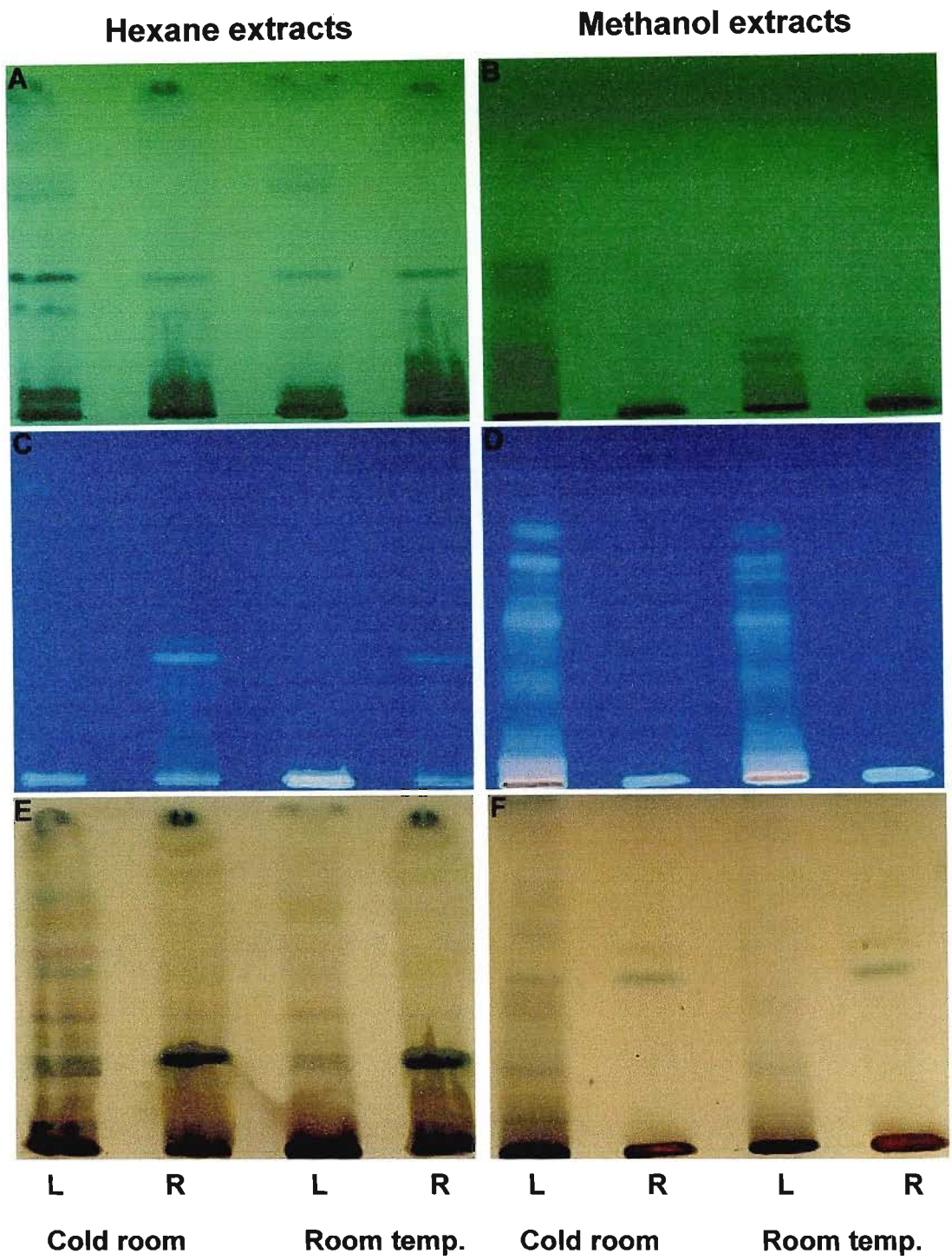


Figure 5.20: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of *M.* parviflora and stored in a cold room and at room temperature for three months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

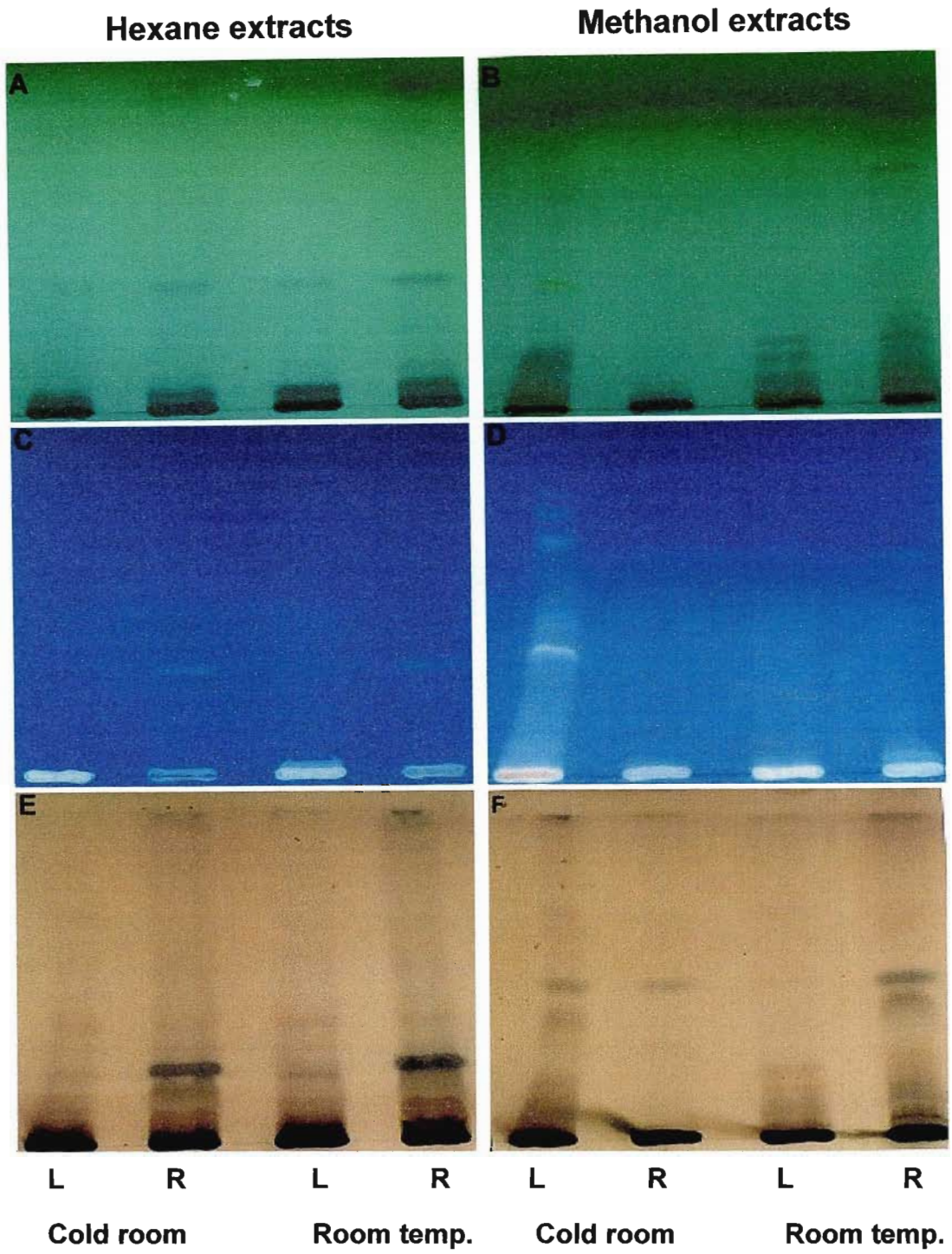


Figure 5.21: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of *M. parviflora* and stored in a cold room and at room temperature for six months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

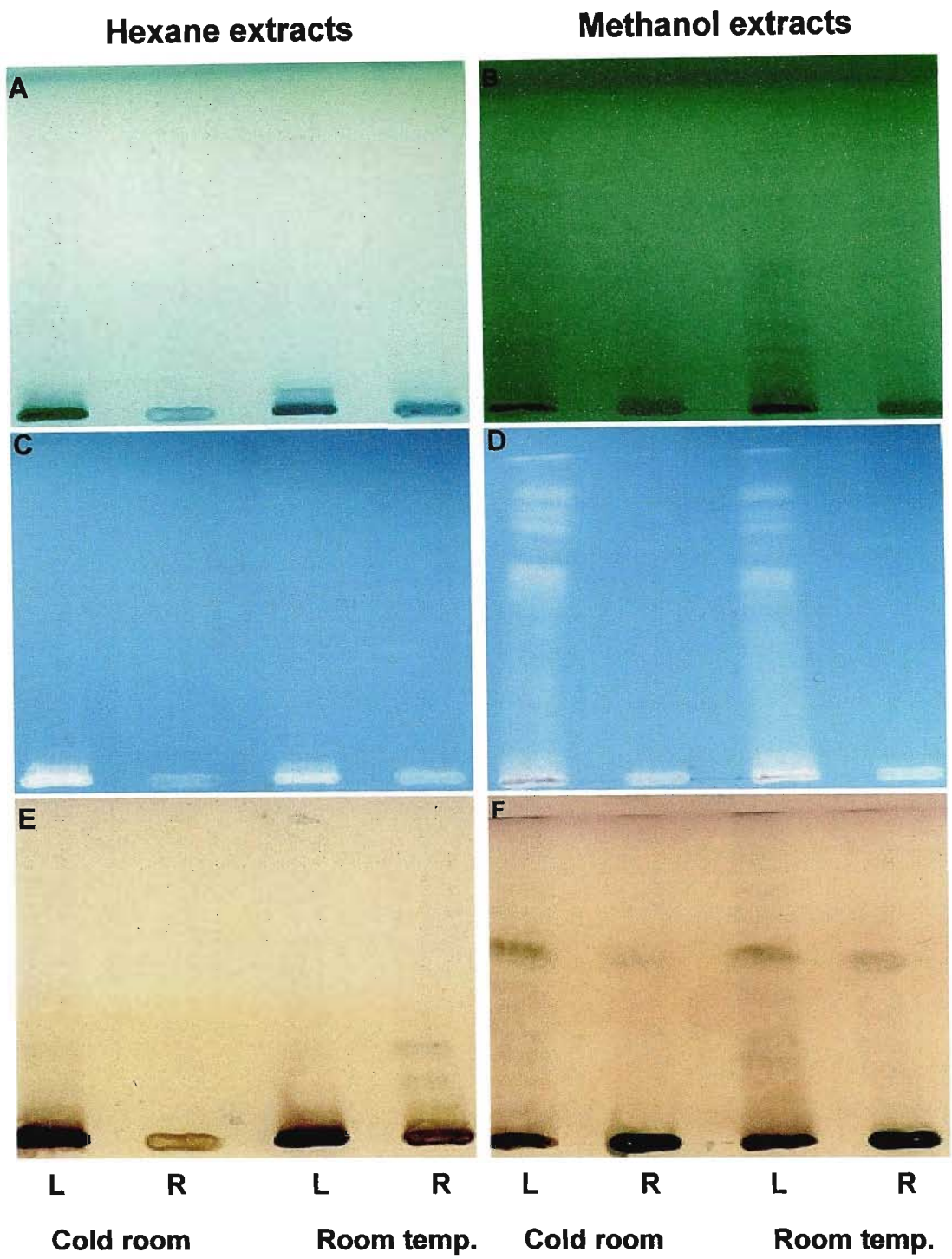


Figure 5.22: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of *M.* parviflora and stored in a cold room and at room temperature for nine months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

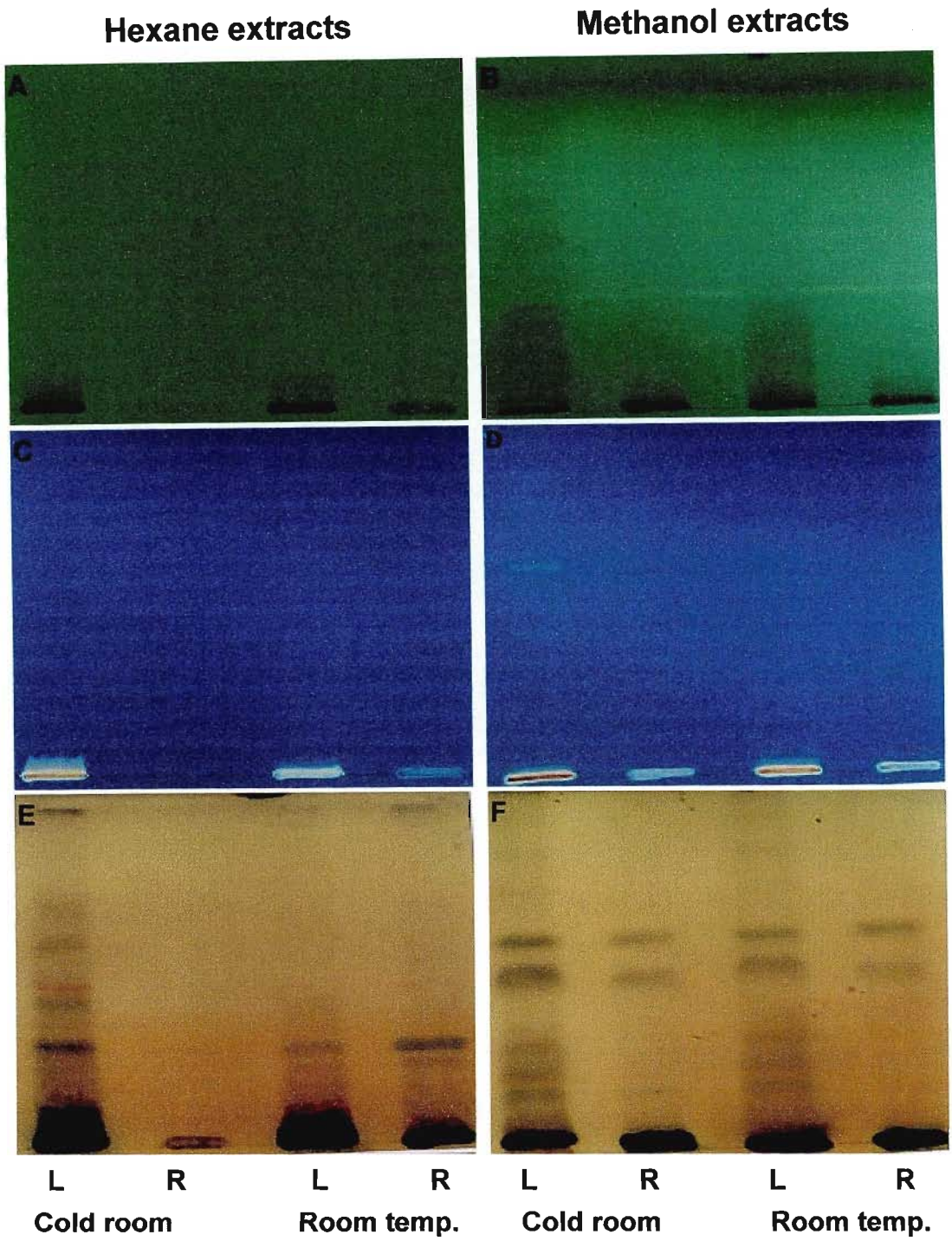


Figure 5.23: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of M. parviflora and stored in a cold room and at room temperature for twelve months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

At the time of collection, hexane and methanol extracts made from leaves of *E. punctulatus* exhibited anti-bacterial activity against Gram-positive (*B. subtilis and S. epidermis)* bacteria. Water extracts did not have any activity against the tested bacteria. Storage of the plant material in a cold room, at room temperature and outside in the Botanical Garden resulted in an increase in anti-bacteria activity of the hexane and methanol extracts after three months storage. Storage for longer than 3 months showed a decrease in activity. No activity was detected in any of the water extracts over the course of the experiment (Figure 5.24–5.26).

The hexane and methanol extracts made from leaves and stored in a cold room and at room temperature showed a similar trend. There was an increase in anti-bacterial activity against Gram-positive (*B. subtilis and S. epidermis)* bacteria after three months storage followed by a steady decrease in activity there after (Figure 5.24 and 5.25).

Anti-inflammatory activity

Effect of storage on anti-inflammatory activity of dried leaf material and extracts of *E. punctulatus* was tested for hexane, methanol and water extracts. At the time of collection, the hexane and methanol extracts made from leaves exhibited high anti-inflammatory activity compared to the water extract. Storage in a cold room, at room temperature and in the Botanical Garden did not cause any change in anti-inflammatory activity of hexane extracts made from leaves throughout the twelve months storage period (Figure 5.27 A). However, the methanol extract obtained from leaf material stored in the three storage conditions exhibited a decrease in anti-inflammatory activity if stored for longer than six months (Figure 5.27 B). Water

extracts made from leaf material showed an increase in activity after three months storage from the material stored in the three storage conditions. The activity declined to the original level after six and nine months storage (Figure 5.27 C).

Storage of hexane extract made from leaves of *E. punctulatus* and stored in a cold room showed no change in activity throughout the twelve months storage period. The hexane extract stored at room temperature exhibited a decrease in activity after nine months (Figure 5.28 A). Methanol extracts made from leaves and stored in a cold room and at room temperature resulted in a decrease in activity after nine months. The activity subsequently increased after twelve months storage (Figure 5.28 B).

TLC fingerprinting

TLC fingerprinting was carried out using hexane and methanol extracts made from leaves of *E. punctulatus*.

At the time of collection, hexane extracts made from leaf material separated into five spots, one of which fluorescenced red at 366 nm. Methanol extracts obtained from leaves separated into eleven spots of which one fluorescenced red at 366 nm. R_f values of red spots are shown on Figure 5.29.

Storage of leaf material for up to six months in a cold room, at room temperature and in the Botanical Garden did not cause any change in the number and colour of spots from the hexane extracts observed at UV light 254 and 366 nm and after staining with anisaldehyde (Figures 5.30 and 5.31). Prolonged storage (nine months and

more) caused an increase in the number of spots (Figures 5.32 and 5.33). Methanol extracts obtained from leaf material stored at the three storage conditions for up to twelve months, did not exhibit any change in the number nor the colour of spots when viewed under both UV wavelengths and after staining with anisaldehyde throughout the experiment (Figures 5.30-33).

When the hexane extract made from leaves was stored in a cold room, there was a decrease in the number of spots after 6 months storage (Figure 5.35). Storage for longer than 6 months resulted in an increase in the number of spots (Figure 5.36). The hexane extract stored at room temperature did not exhibit any change in number and colour of spots throughout the storage period (Figure 5.34 to 5.37). The methanol extract made from leaves stored in the cold room and at room temperature did not show any change in number and colour of compound spots throughout the experiment (Figure 5.34–5.37).

5.4.3 Effects of storage on biological activity of *A. microraphis*

Anti-inflammatory activity

The effect of storage on anti-inflammatory activity of dried *A.microraphis* plant material and organic extracts was tested for hexane, methanol and water extracts made from leaves and roots.

At the time of collection, both hexane and methanol extracts made from leaves and roots had high anti-inflammatory activity compared to the water extracts. Storage of

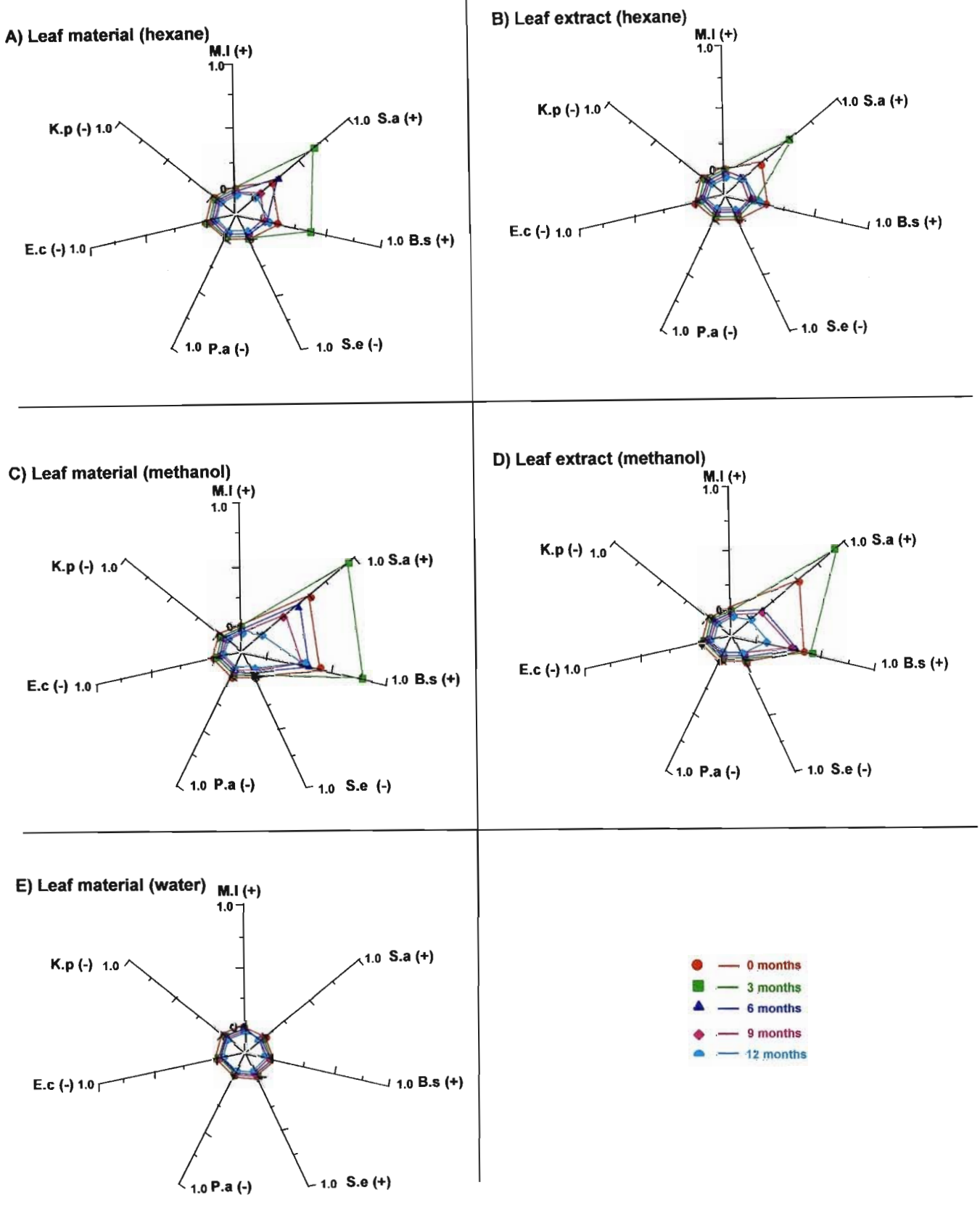


Figure 5.24: Effect of storing dried leaf material and extracts of *E. punctulatus* in a cold room on anti-bacterial activity. A) and B) hexane, C) and D) methanol and E) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

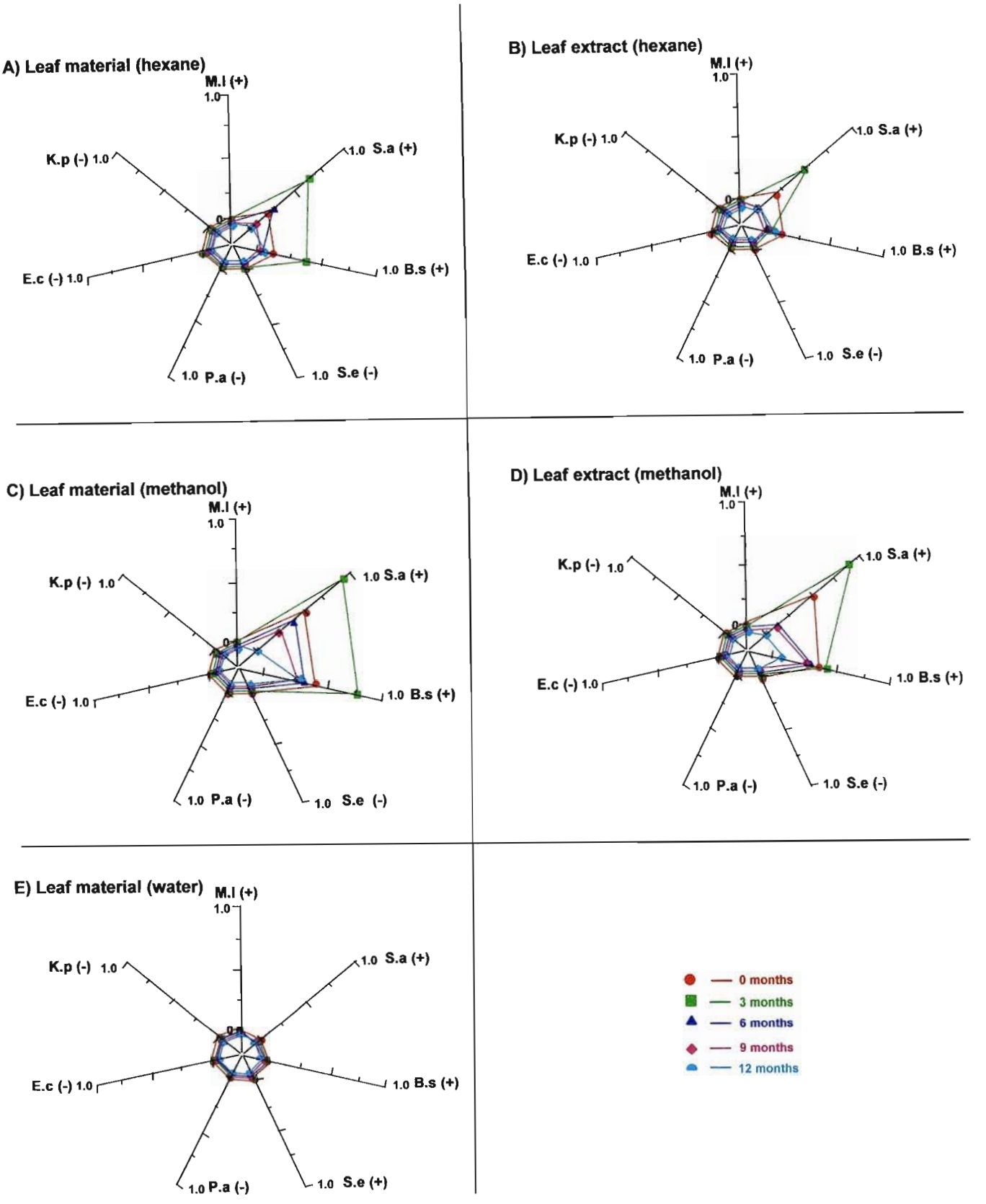


Figure 5.25: Effect of storing dried leaf material and extracts of *E. punctulatus* at room temperature on anti-bacterial activity. A) and B) hexane, C) and D) methanol and E) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

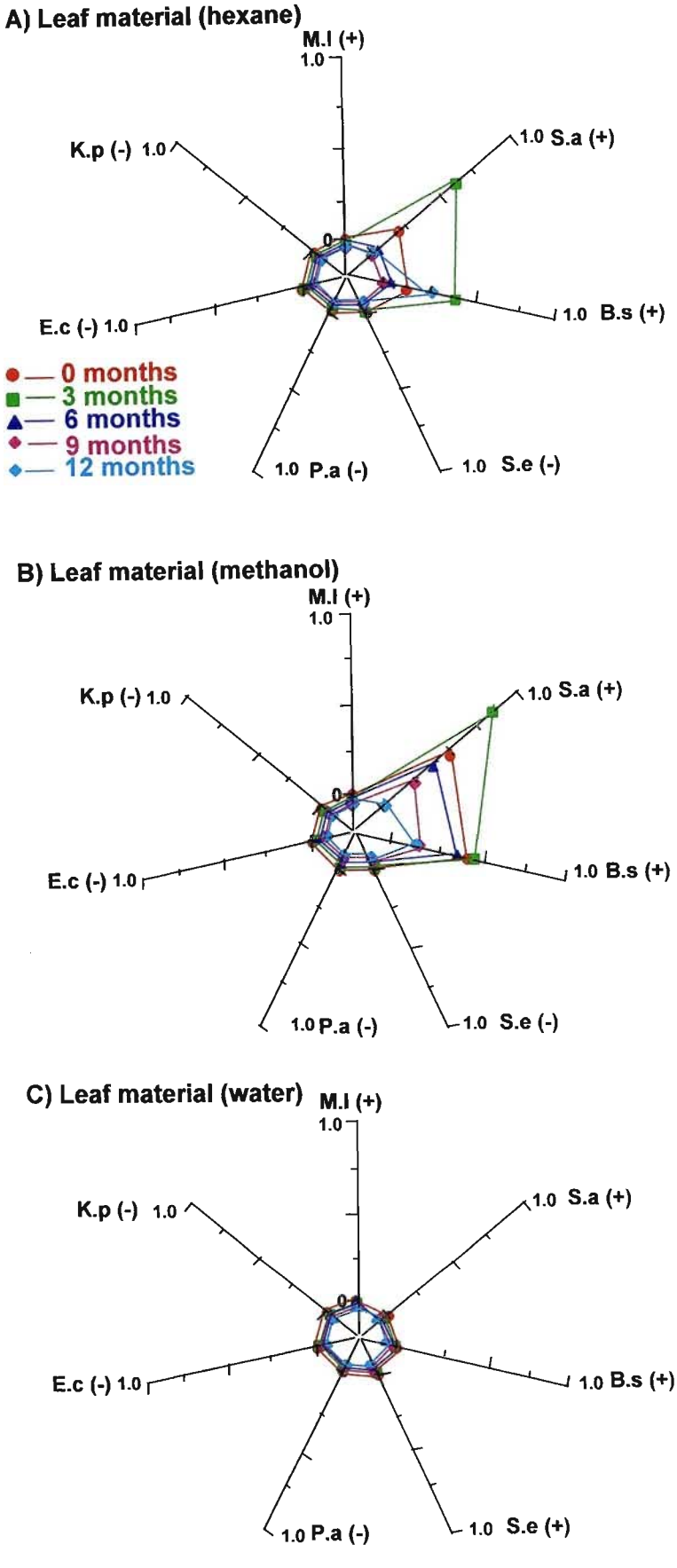


Figure 5. 26: Effect of storing dried leaf material and extracts of *E. punctulatus* in the Botanical Garden on anti-bacterial activity. A) and B) hexane, C) and D) methanol and E) water extract. The axes show the zone of inhibition determined as the ratio of the clear zone around the plant extract to the neomycin control.

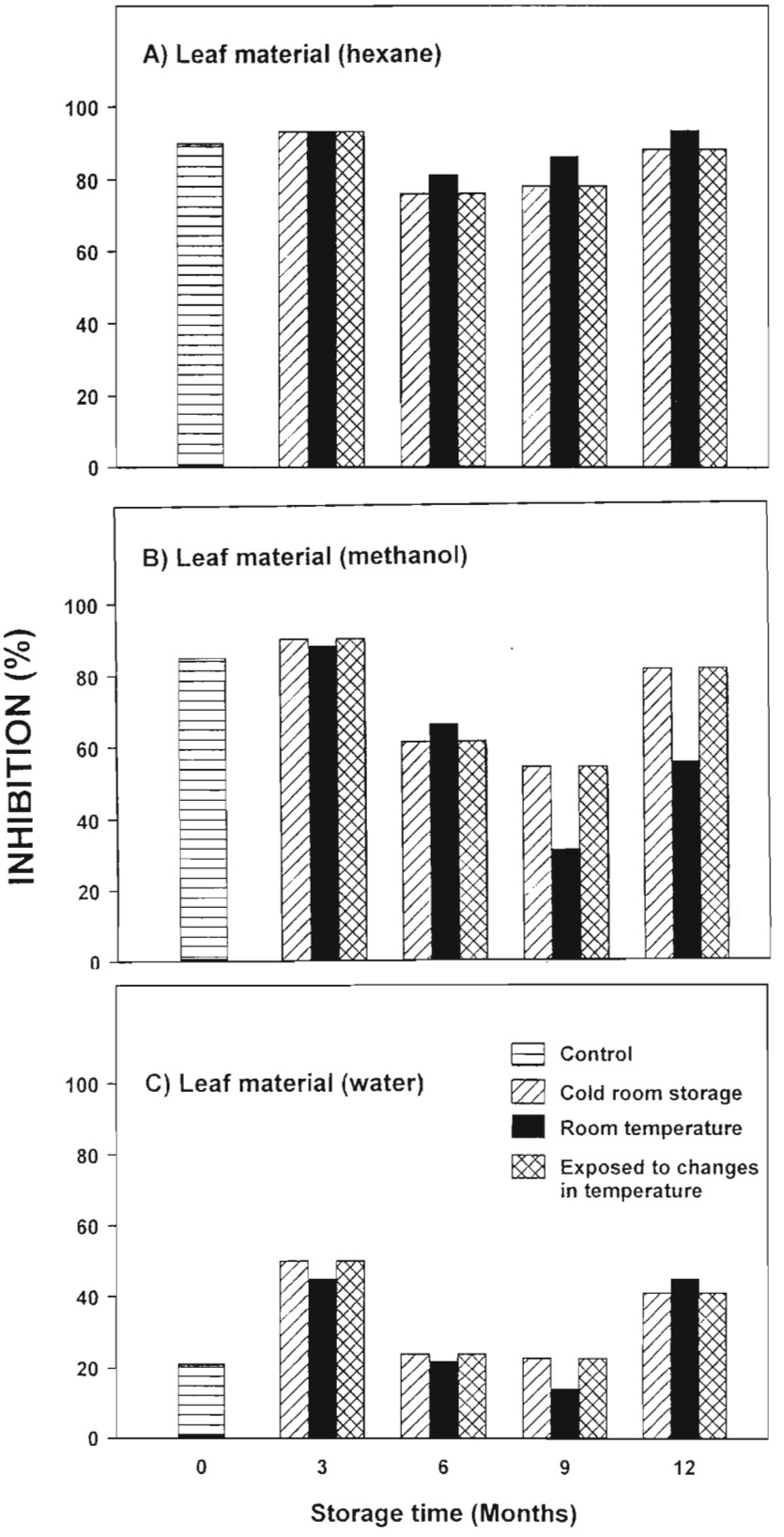


Figure 5.27: Effect of storage on anti-inflammatory activity of A) hexane, B) methanol and C) water extracts made from dried leaf material of *E. punctulatus* stored in a cold room (10 ^{0}C), at room temperature and in the Botanical Garden. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 63 ± 4%.

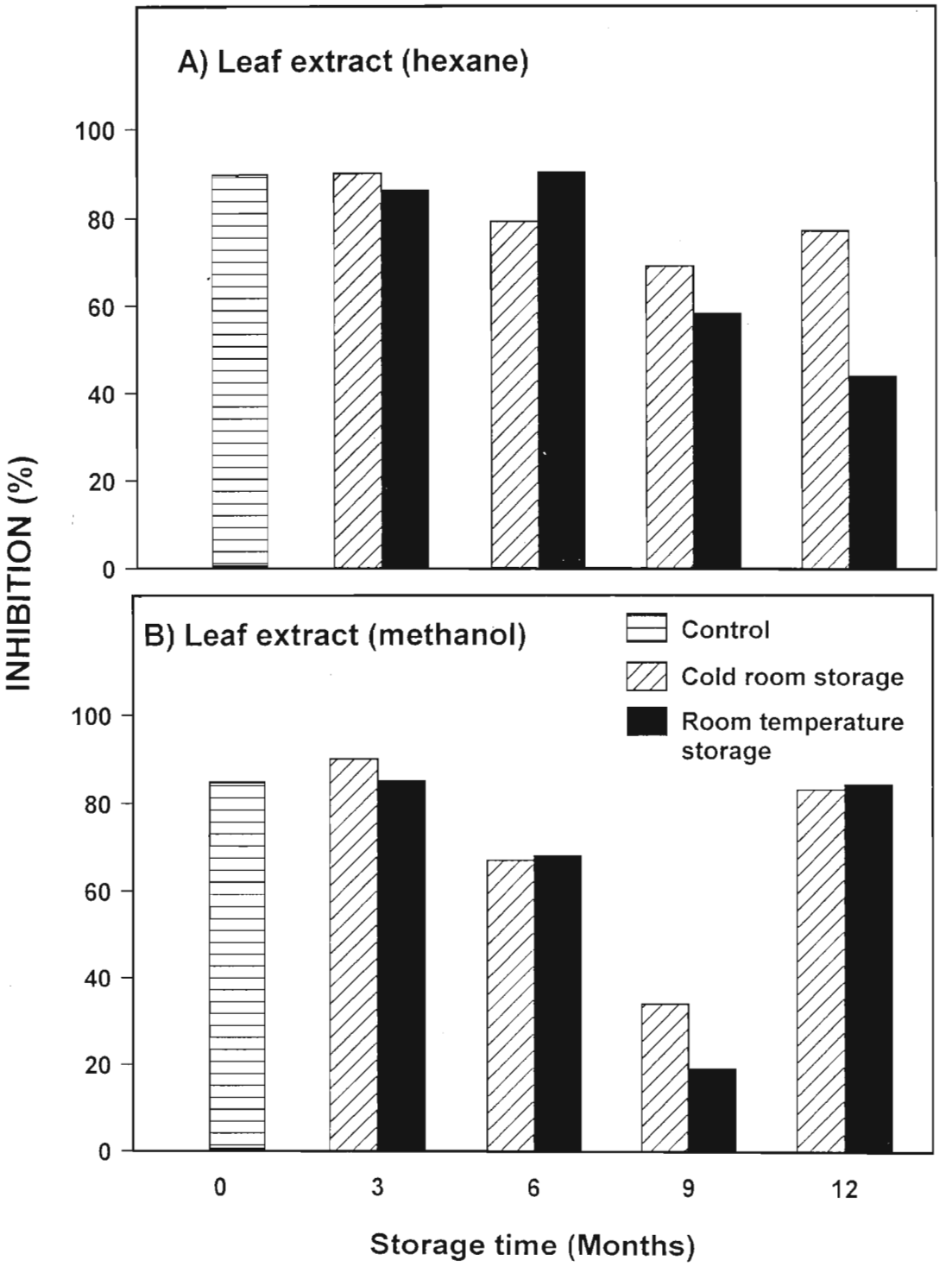


Figure 5.28: Effect of storage on anti-inflammatory activity of dried A) hexane and B) methanol extracts made from leaves of *E. punctulatus* and stored in a cold room and at room temperature. Indomethacin standard (50 μM ml^{-1}) inhibition (%) = 63 ± 4%.

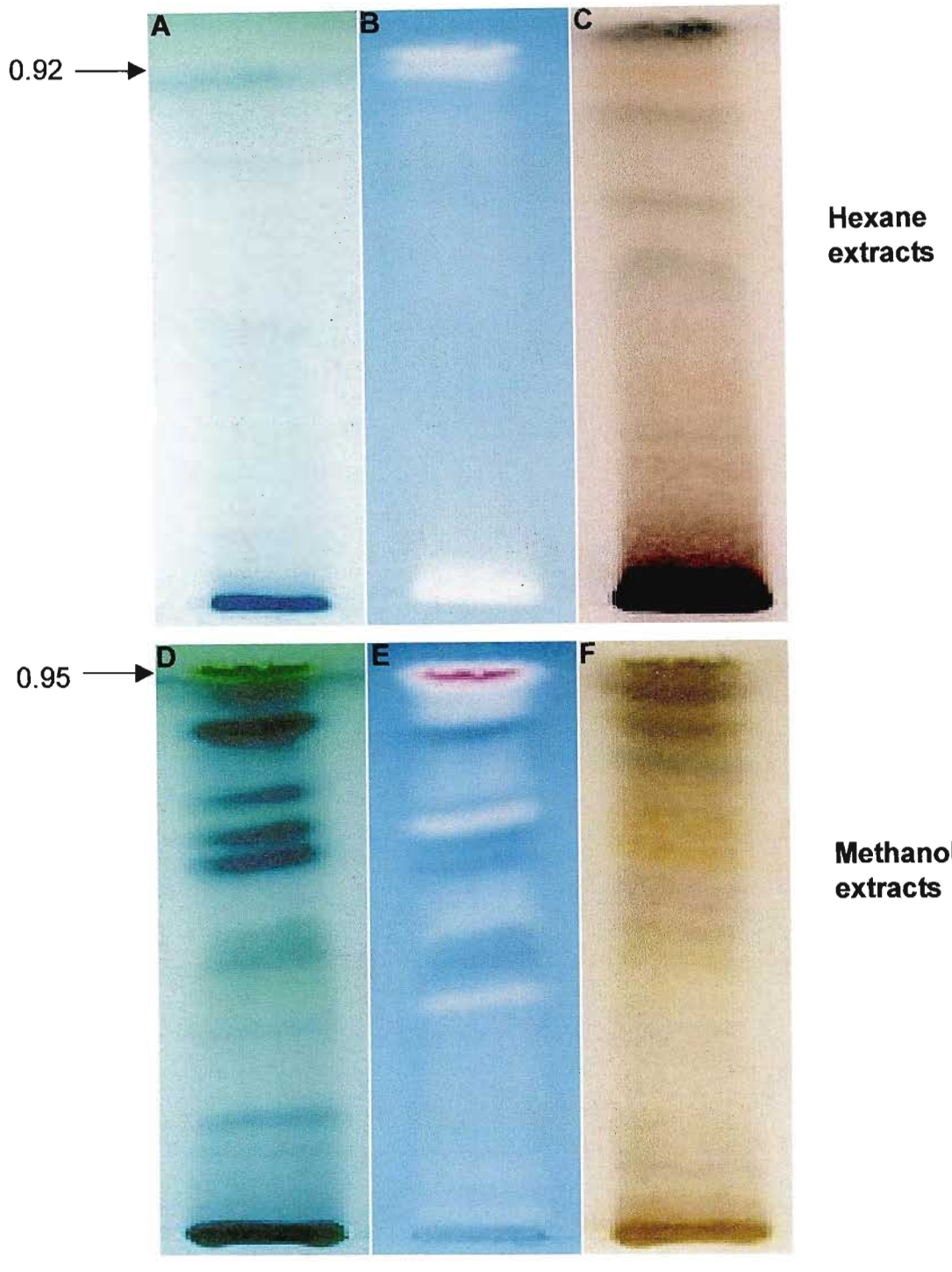


Figure 5.29: TLC fingerprints of hexane and methanol extracts made from leaf material of*E. punctulatus.* TLC plates were run immediately after collection in May 2001 (0 months). These were viewed at 254 nm (A and D), 366 nm (B and E) and stained with anisaldehyde (C and F). The numbers refer to R_f values of the red spots.

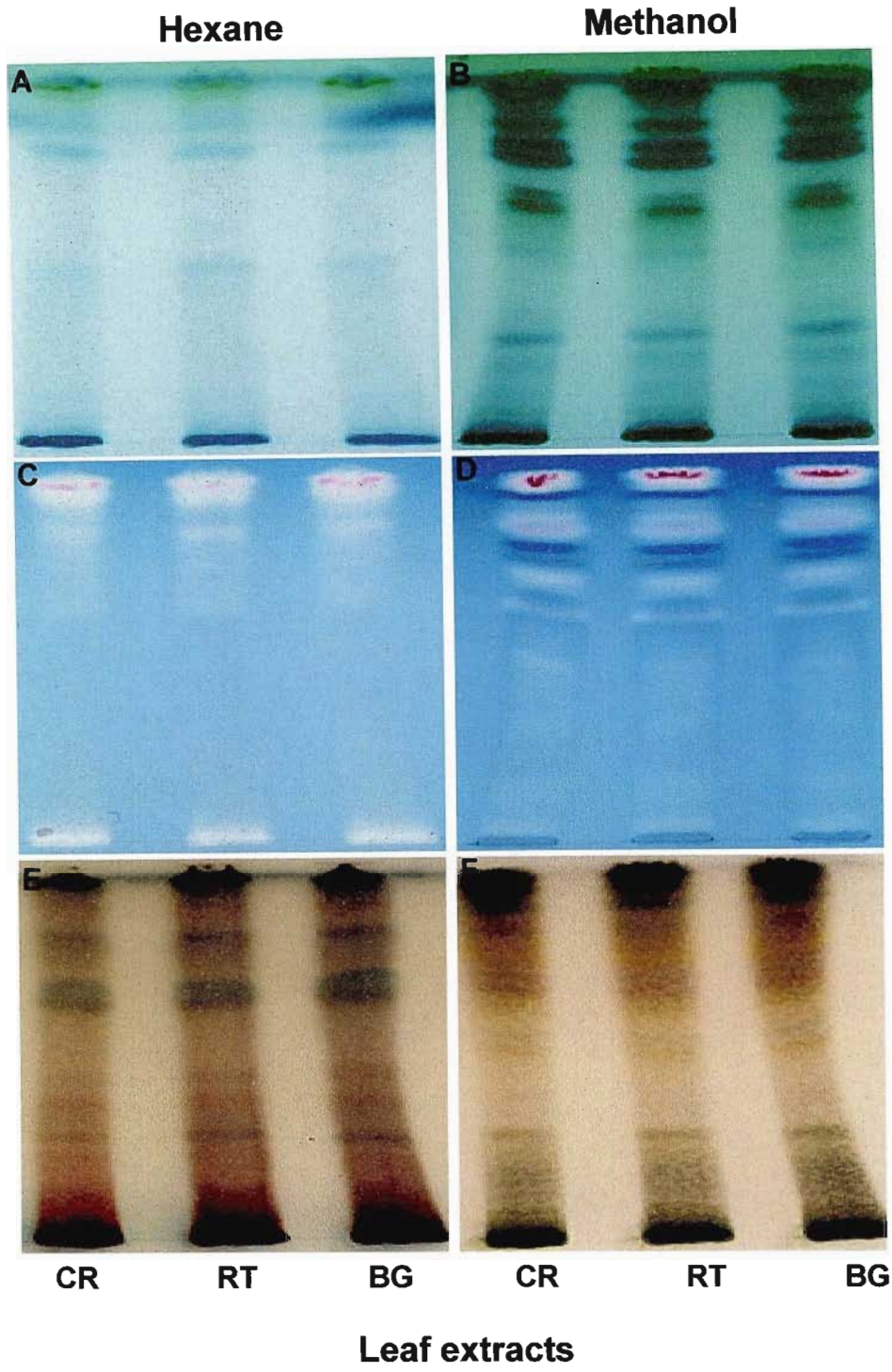


Figure 5.30: TLC fingerprints of hexane and methanol extracts made from leaf material of *E. punctulatus* and stored in a cold room (CR), at room temperature (RT) and in the Botanical Garden (BG) for three months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

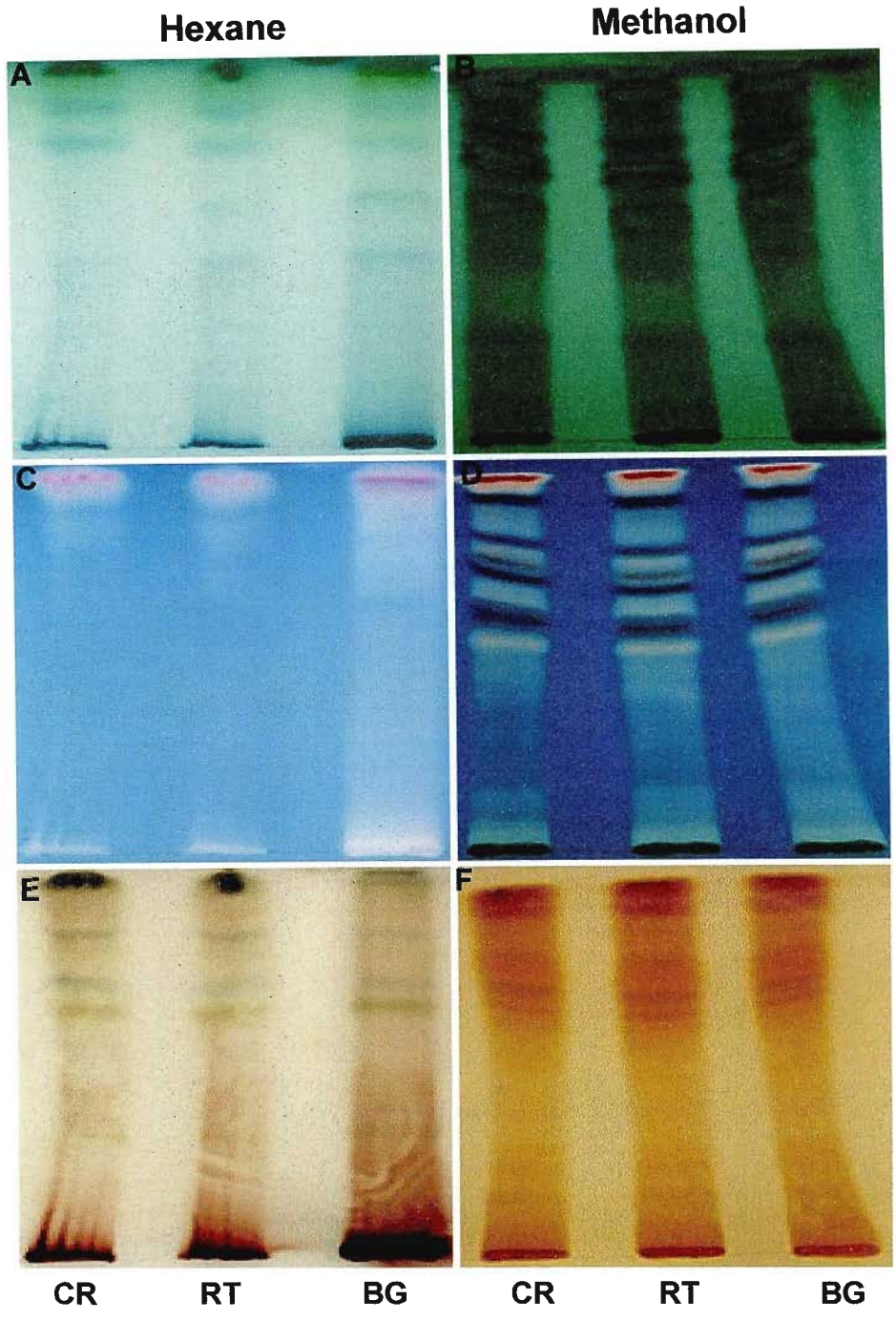


Figure 5.31: TLC fingerprints of hexane and methanol extracts made from leaf material of *E. punctulatus* and stored in a cold room (CR), at room temperature (RT) and in the Botanical Garden (BG) for six months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

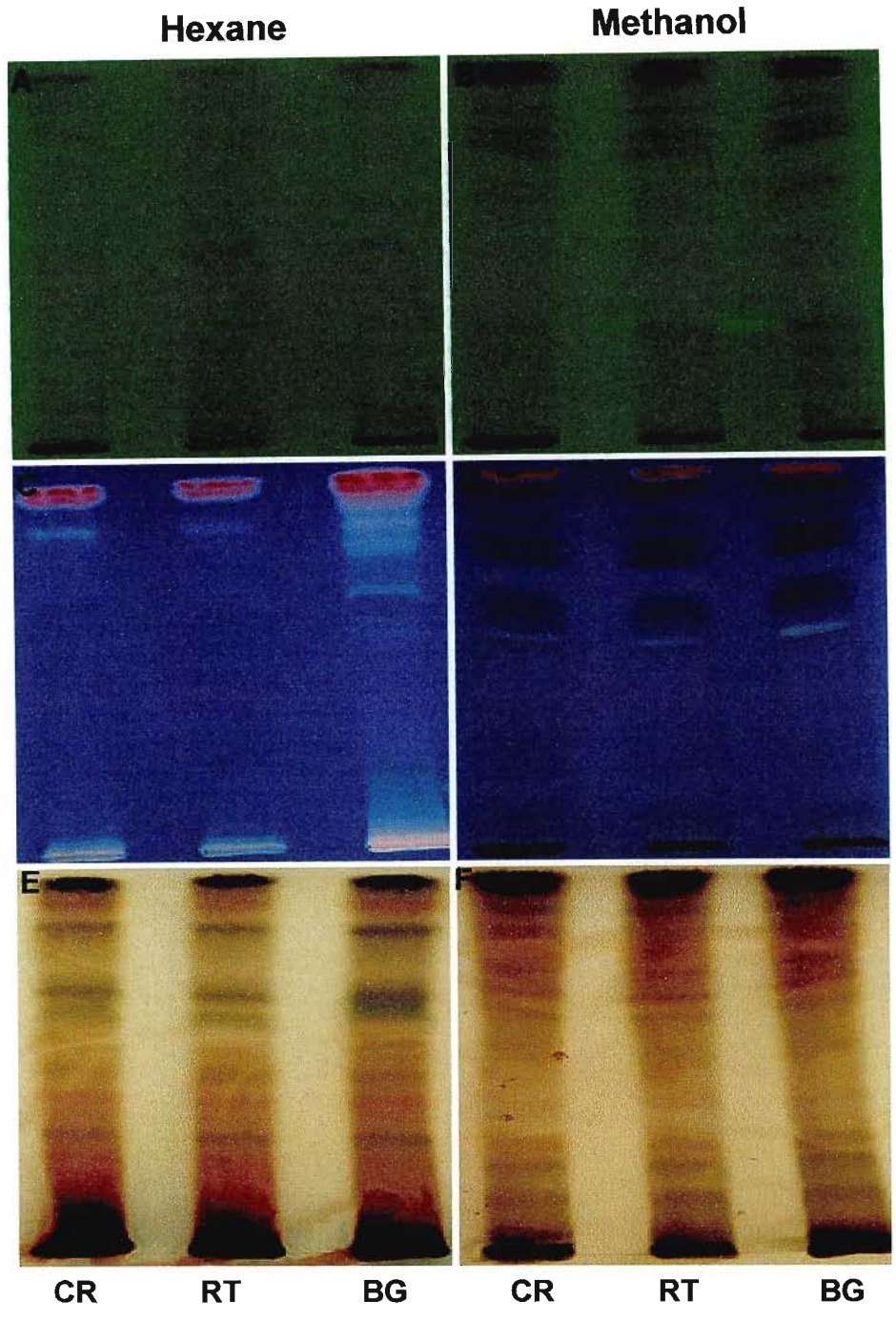


Figure 5.32: TLC fingerprints of hexane and methanol extracts made from leaf material of *E. punctulatus* and stored in a cold room (CR), at room temperature (RT) and in the Botanical Garden (BG) for nine months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

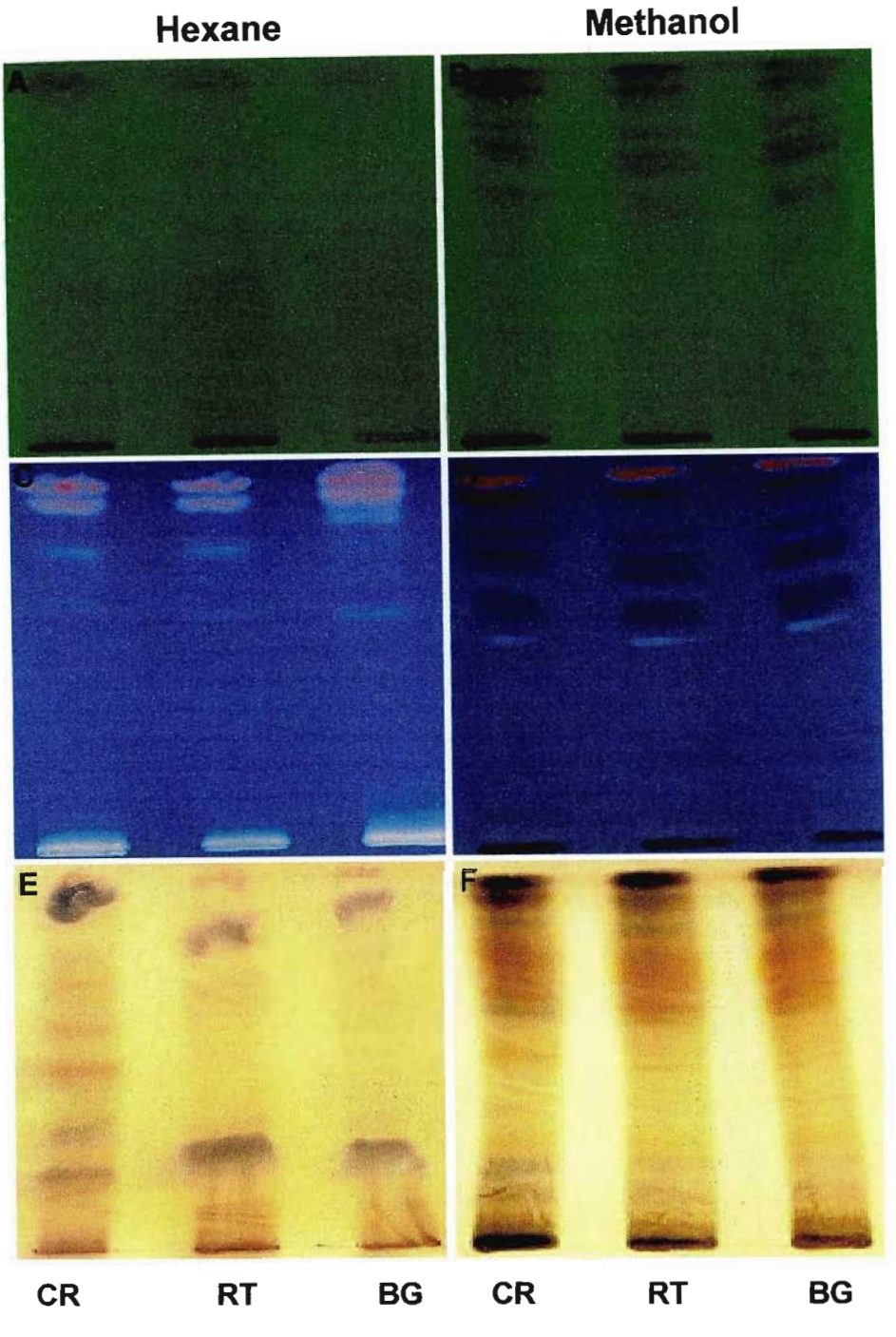


Figure 5.33: TLC fingerprints of hexane and methanol extracts made from leaf material of *E. punctulatus* and stored in a cold room (CR), at room temperature (RT) and in the Botanical Garden (BG) for twelve months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

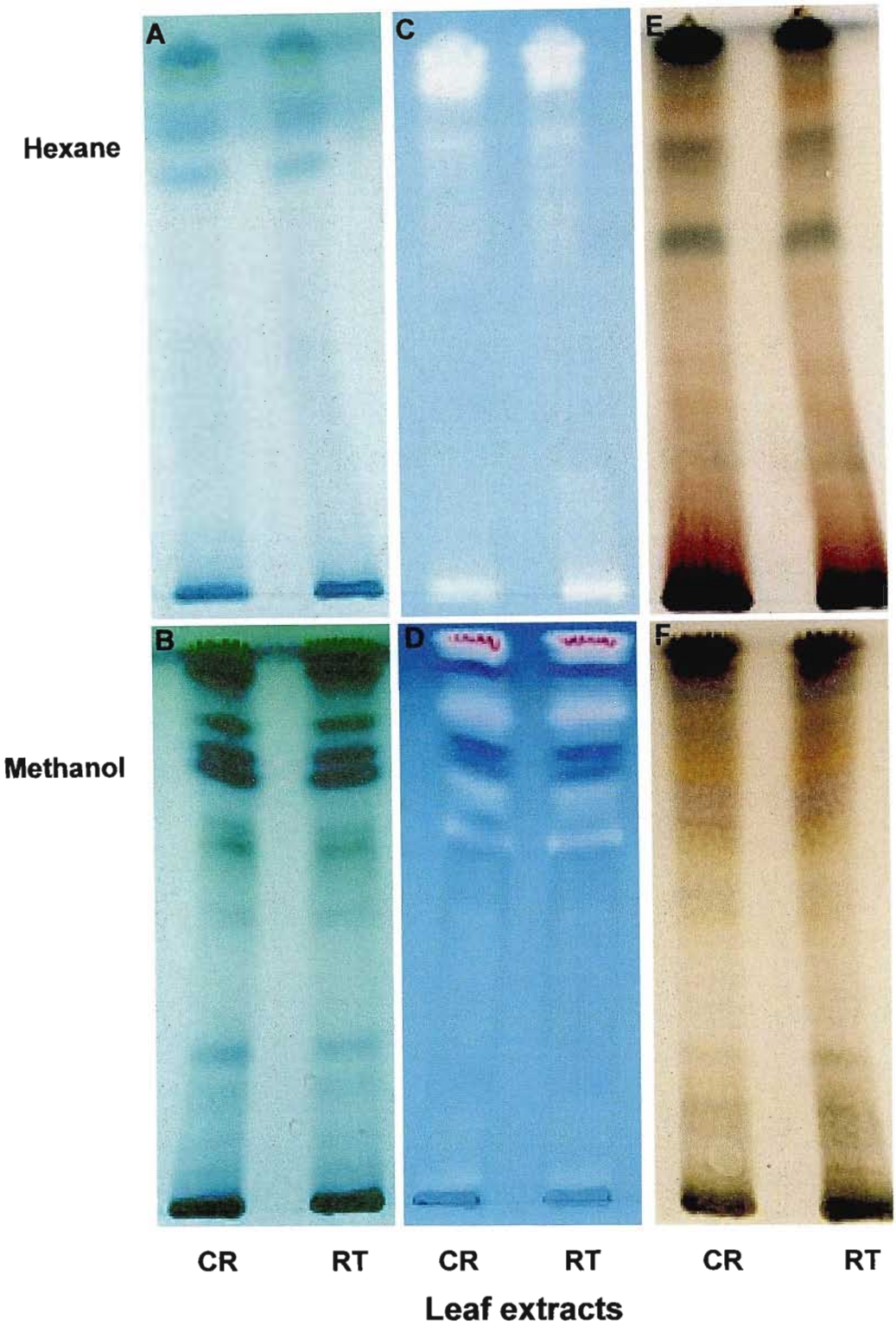


Figure 5.34: Fingerprint of hexane and methanol extracts made from leaves of *E. punctulatus* and stored in a cold room (CR) and at room temperature (RT) for three months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

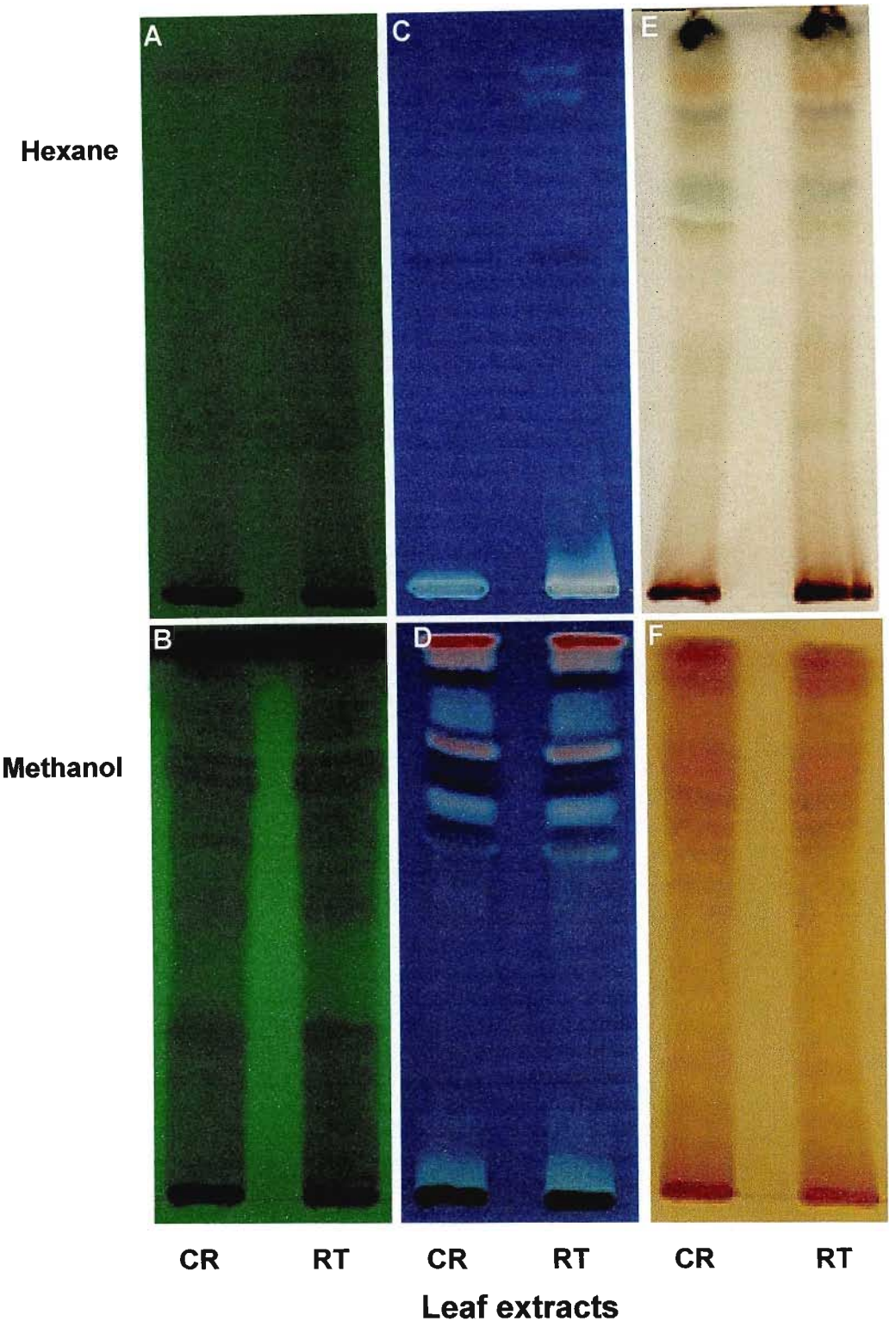


Figure 5.35: Fingerprint of hexane and methanol extracts made from leaves of *E. punctulatus* and stored in a cold room (CR) and at room temperature (RT) for six months. TLC plates were viewed under UV light at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

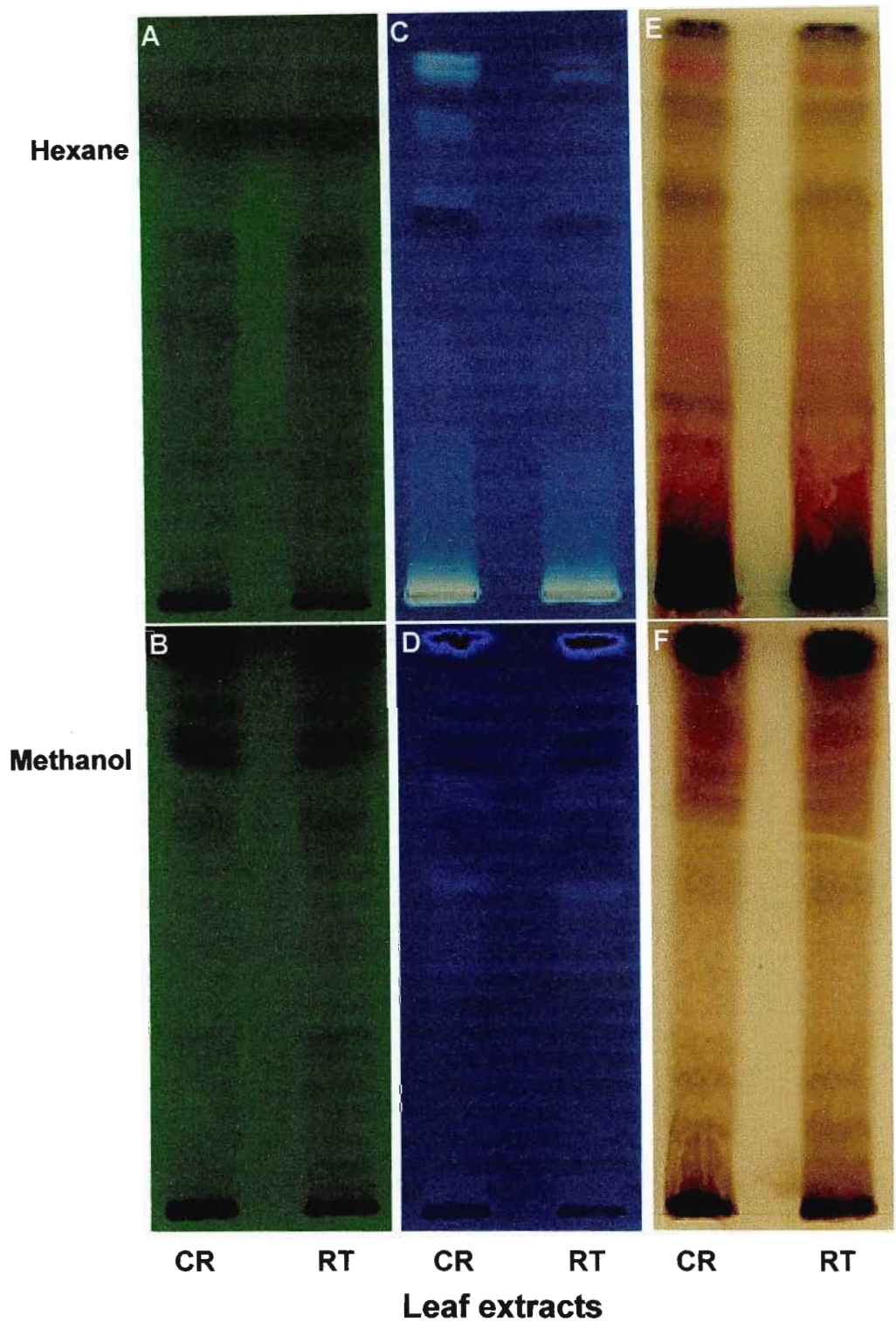


Figure 5.36: Fingerprint of hexane and methanol extracts made from leaves of *E. punctulatus* and stored in a cold room (CR) and at room temperature (RT) for nine months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

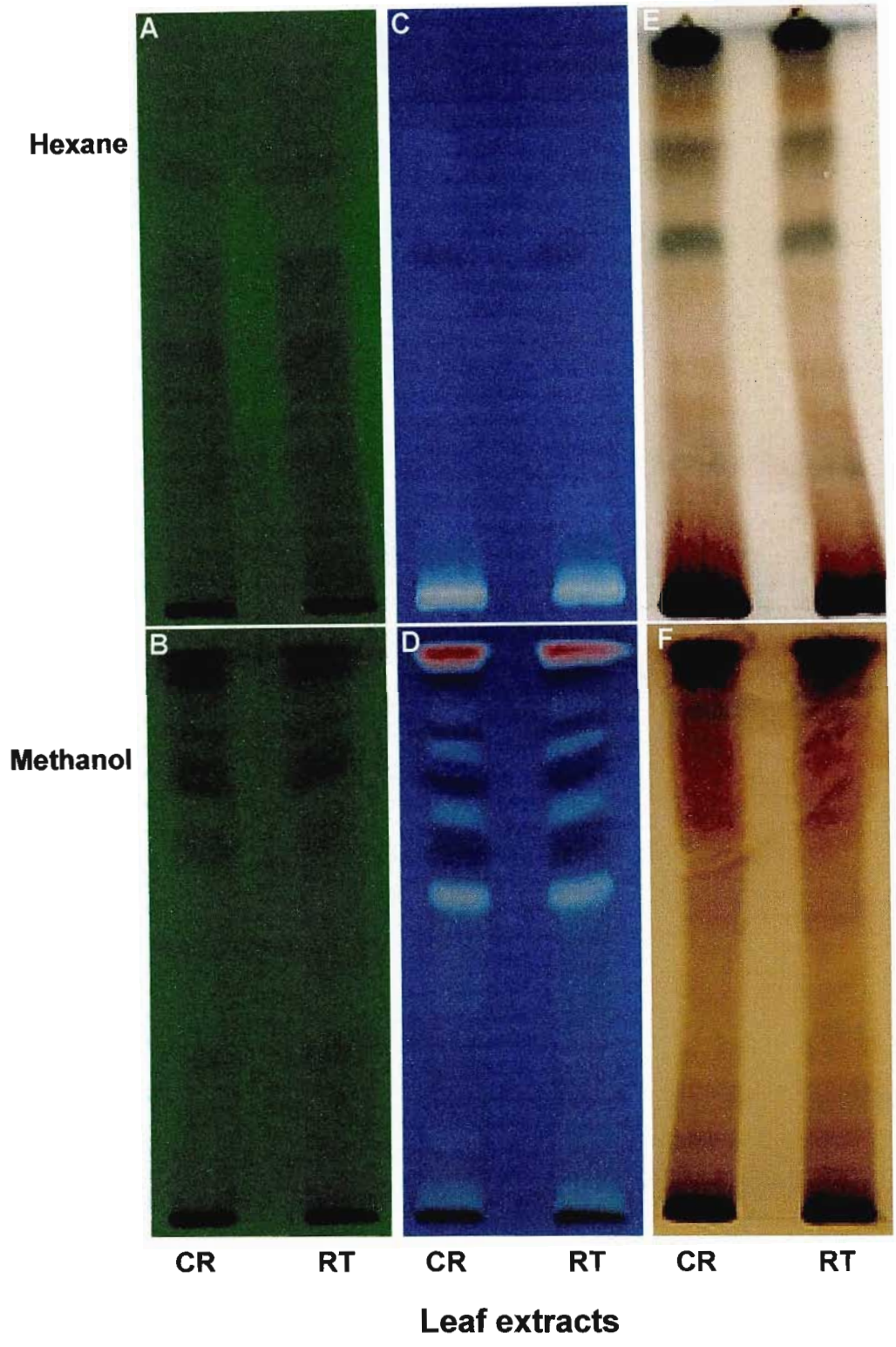


Figure 5.37: Fingerprint of hexane and methanol extracts made from leaves of *E. punctulatus* and stored in a cold room (CR) and at room temperature (RT) for twelve months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

the plant material in a cold room, at room temperature and in the Botanical Garden did not cause any change in anti-inflammatory activity of hexane and methanol extracts obtained from leaves and roots during the twelve month storage period. The water extracts made from leaves and roots showed an increase in activity after three months from the material stored under the three storage conditions (Figures 5.38 and 5.39).

Anti-inflammatory activity of the stored hexane extracts made from leaves stored in a cold room and at room temperature decreased after twelve months storage while the activity of the methanol extracts showed no change over this period (Figure 5.40).

When hexane extracts made from the roots were stored in a cold room, there was a decline in activity after nine months while the same extract stored at room temperature exhibited a decrease in activity after three months. The methanol extract stored in a cold room resulted in a loss in activity after three months followed by an increase in activity after twelve months. The methanol extract stored at room temperature did not exhibit any change in activity (Figure 5.41).

TLC fingerprinting

At the time of collection, the hexane extract made from leaf material yielded seven spots when viewed under both 254 and 366 nm and after staining with anisaldehyde. Five of these spots fluorescenced red at UV light 366 nm. R_f values of the red spots are shown on Figure 5.42. The hexane extract made from roots resulted in four spots when viewed at the two UV wavelengths. More spots only appeared after the plate was stained. The methanol extract made from leaf material gave ten spots and five

of them fluorescenced red when viewed at 366 nm. R_f values of red spots are shown on Figure 5.42. The methanol extract made from roots resulted in seven spots when viewed at the two wavelengths (Figure 5.42).

Storage of plant material under all three sets of conditions for more than six months caused a decrease in the number of spots in the leaf hexane extract when viewed at 366 nm. The spots observed at 254 nm and after staining of the plate did not change (Figures 5.45 and 5.46). The hexane extracts made from root material stored under the three sets of storage conditions did not exhibit any change throughout the experiment (Figures 5.43-5.46). This was also noted from methanol extracts made from leaf and root material (Figures 5.47 to 5.50)

Hexane extracts made from leaves and roots and stored in a cold room and at room temperature showed no change in the number and colour of spots after six months of storage (Figure 5.52). Storage for longer than six months resulted in a decrease in the number and colour of the spots that could be detected (Figures 5.53-5.54). Methanol extracts made from leaf and root material and stored at the two storage conditions did not exhibit any change in number and colour of spots for up to nine months of storage. After twelve months, the spots disappeared from the extracts stored in a cold room (Figures 5.51 to 5.54).

5.5 Discussion

Storage of dried *M. parviflora*, *E. punctulatus* and *A. microraphis* plant material and organic extracts was carried out in order to determine the best storage condition to

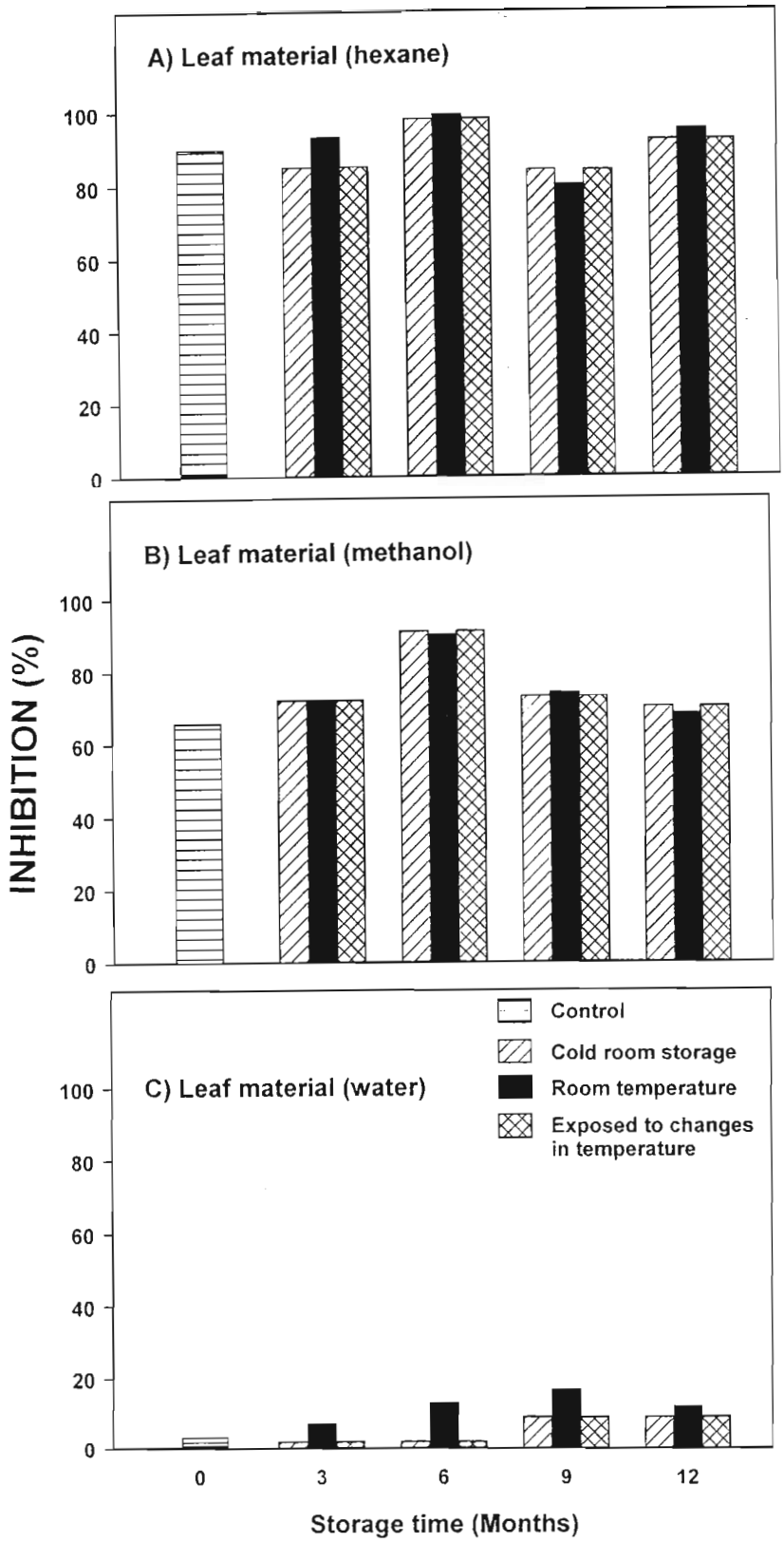


Figure 5.38: Effect of storage on anti-inflammatory activity of A) hexane, B) methanol and C) water extracts made from dried leaf material of *A. microraphis* stored in a cold room (10 ^{0}C), at room temperature and in the Botanical Garden. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 73 ± 7%

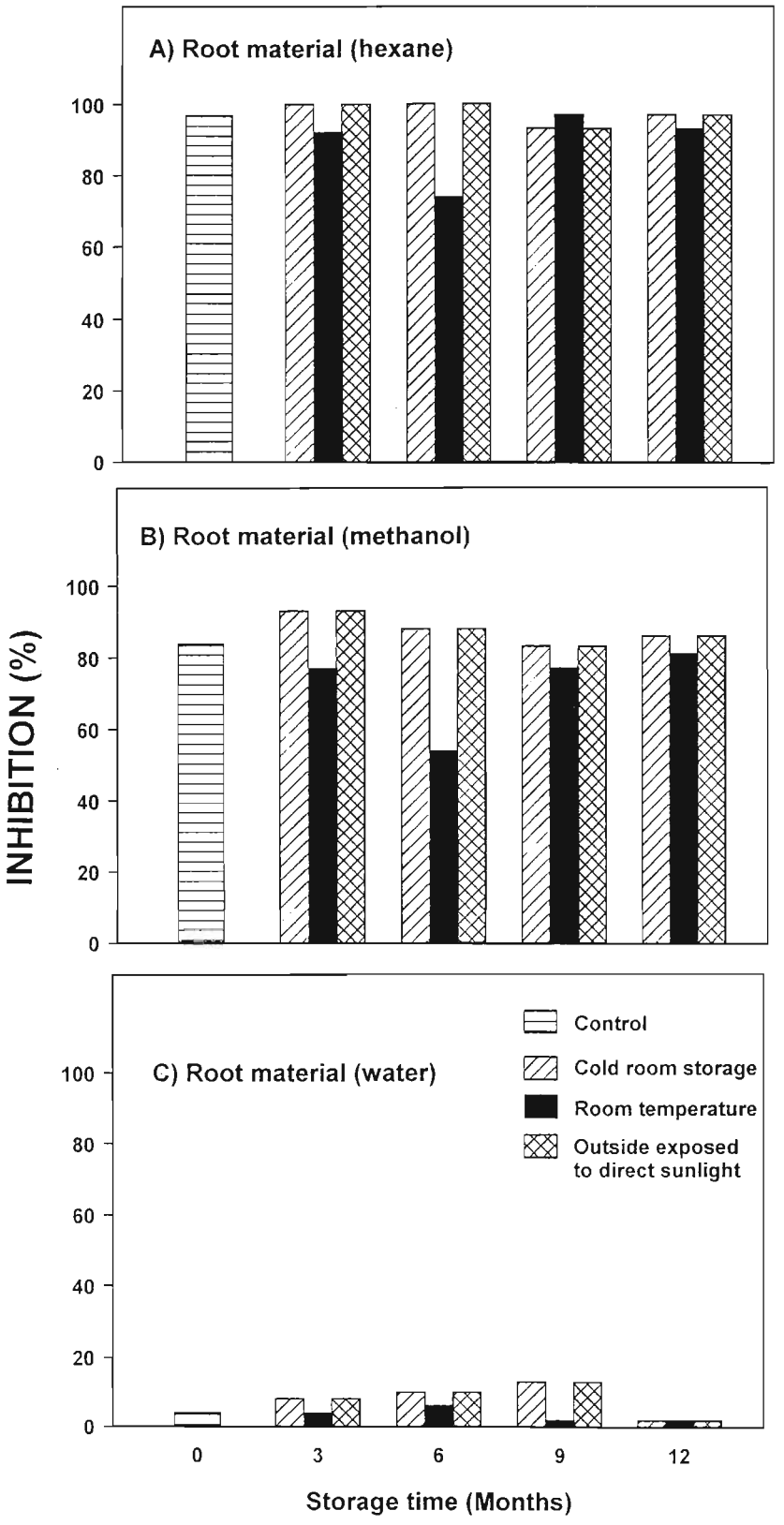


Figure 5.39: Effect of storage on anti-inflammatory activity of A) hexane, B) methanol and C) water extracts made from dried root material of *A. microraphis* stored in a cold room (10 ^{0}C), at room temperature and in the Botanical Garden. Indomethacin standard (50 µM ml^{-1}) inhibition (%) = 73 ± 7%.

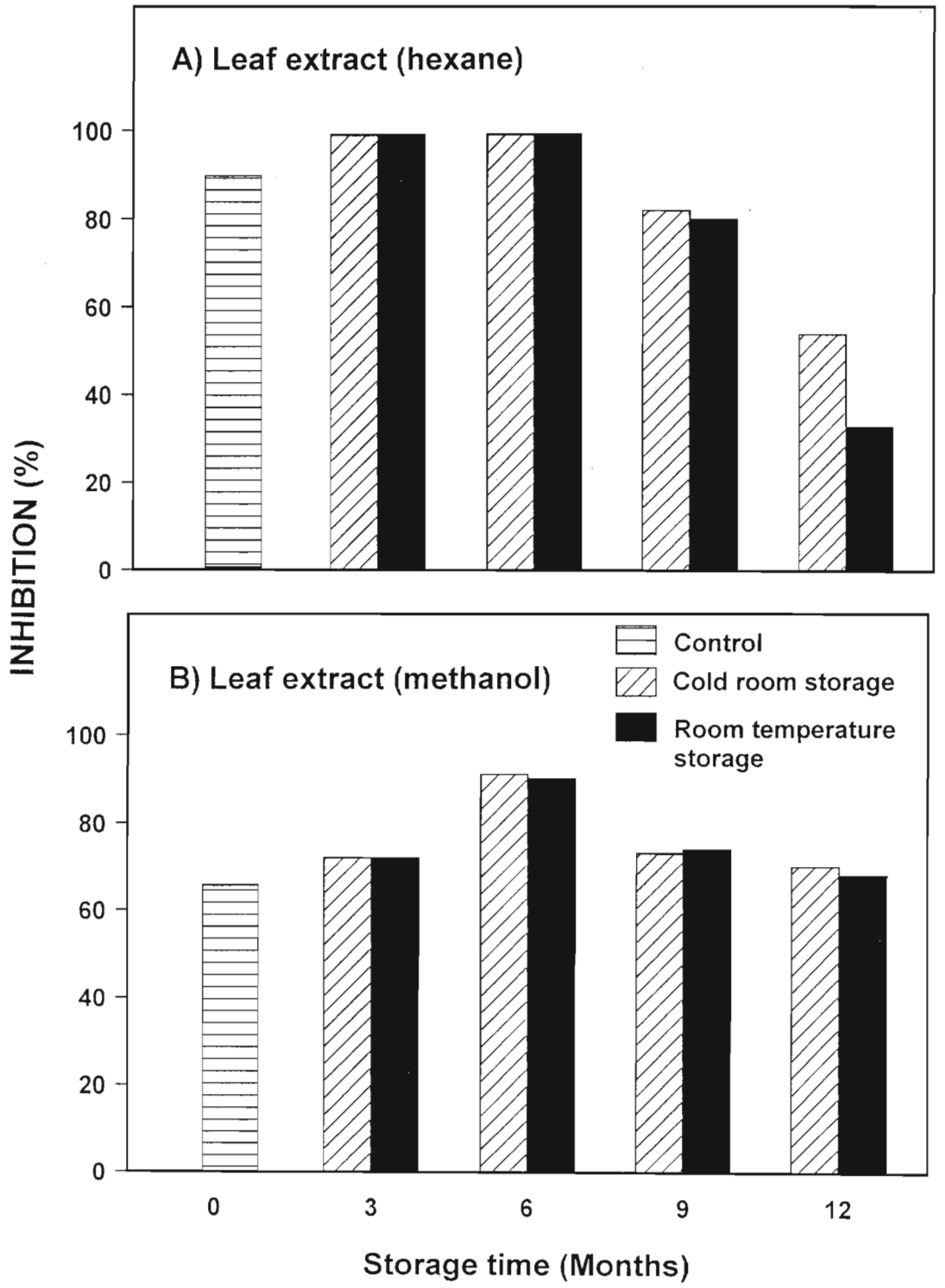


Figure 5.40: Effect of storage on anti-inflammatory activity of dried A) hexane and B) methanol extracts made from leaves of *A. microraphis* and stored in a cold room and at room temperature. Indomethacin standard (50 μM ml^{-1}) inhibition (%) = 73 ± 7%.

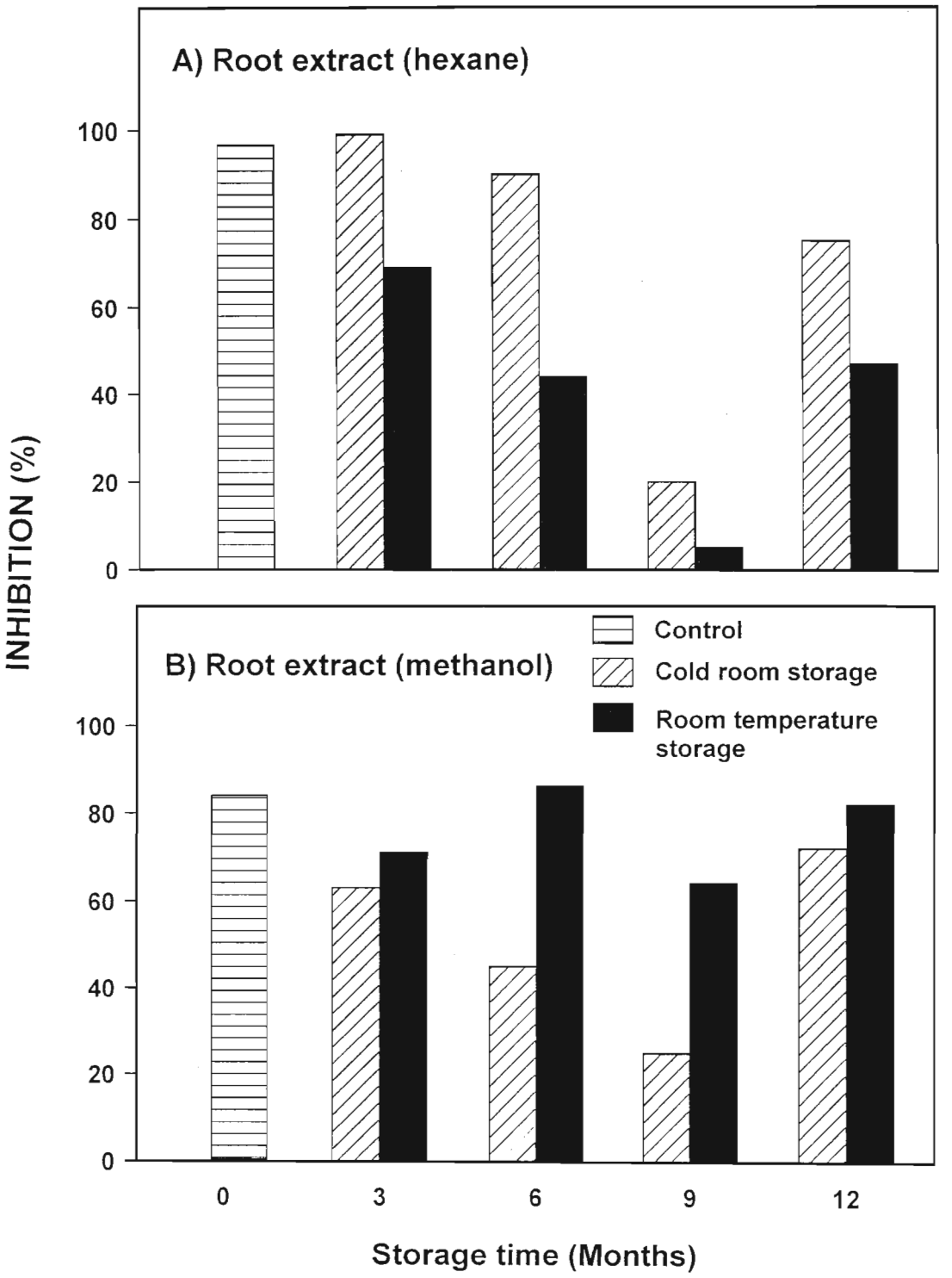


Figure 5.41: Effect of storage on anti-inflammatory activity of dried A) hexane and B) methanol extracts made from roots of *A. microraphis* and stored in a cold room and at room temperature. Indomethacin standard (50 μM ml^{-1}) inhibition (%) = 73 ± 7%.

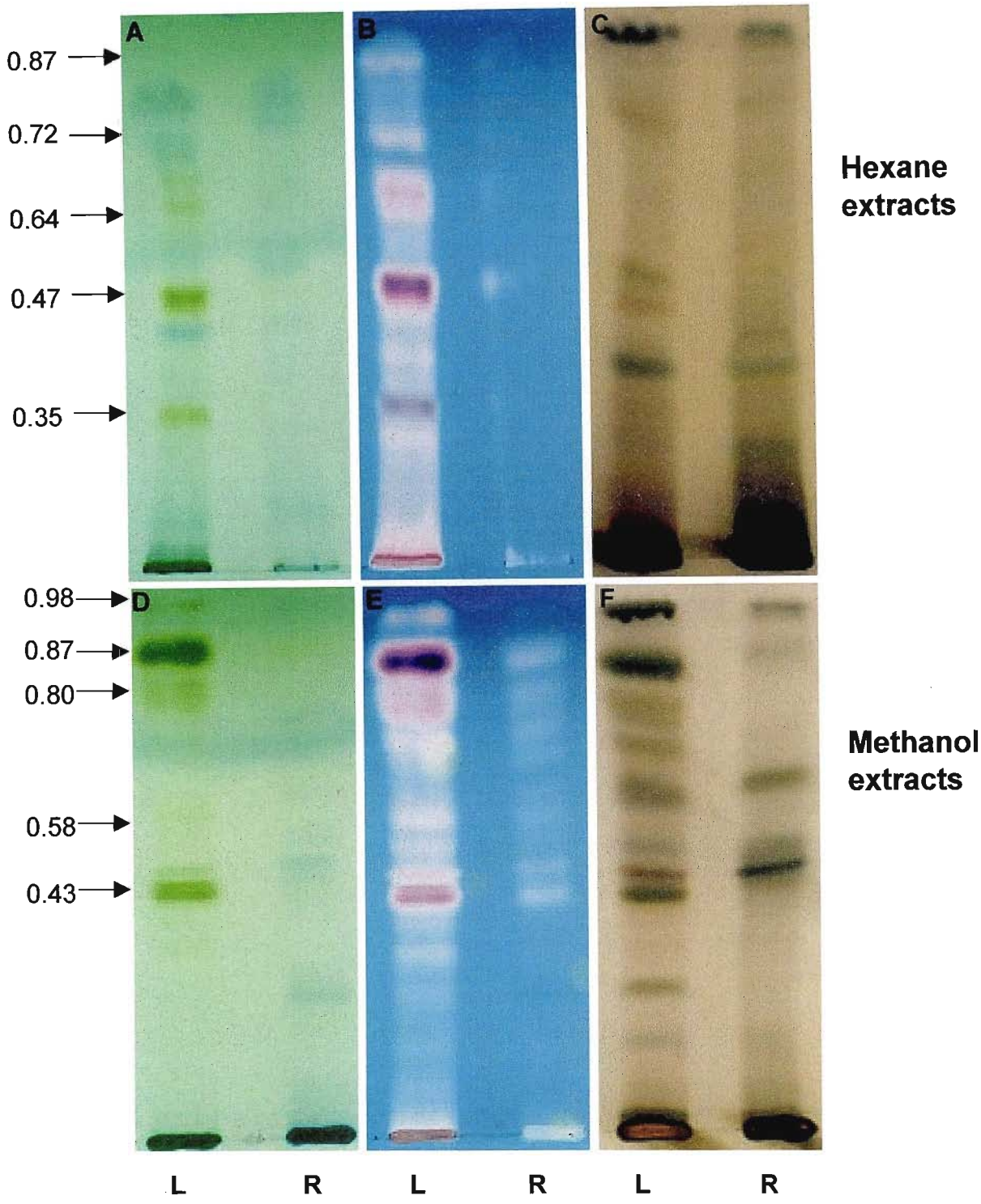


Figure 5.42: Fingerprint of hexane and methanol extracts made from leaf (L) and root (R) material of *A. microraphis*. TLC plates were run immediately after collection in January 2001 (0 months). These were viewed at 254 nm (A and D), 366 nm (B and E) and stained with anisaldehyde (C and F). The numbers refer to R_f values of the red spots

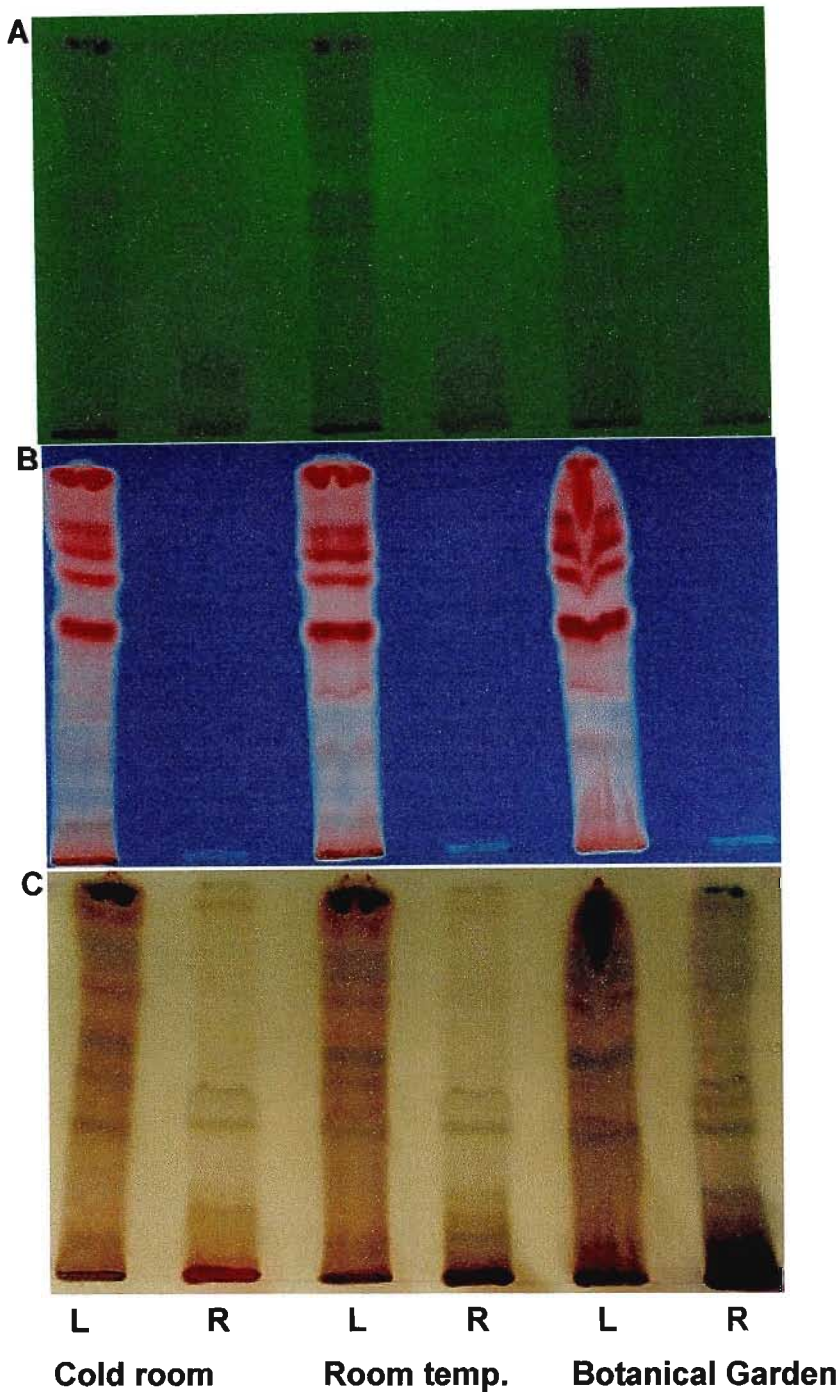


Figure 5.43: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for three months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

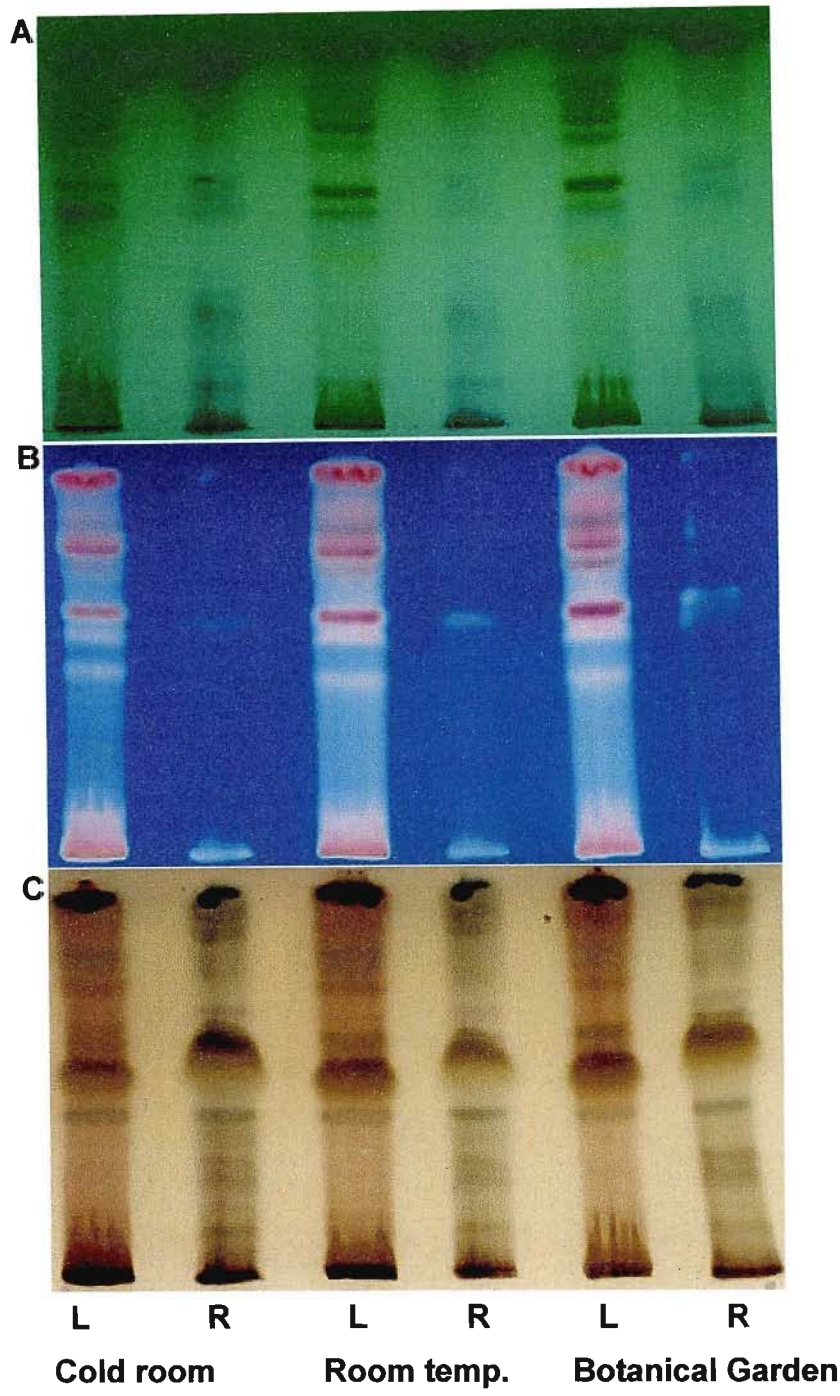


Figure 5.44: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for six months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

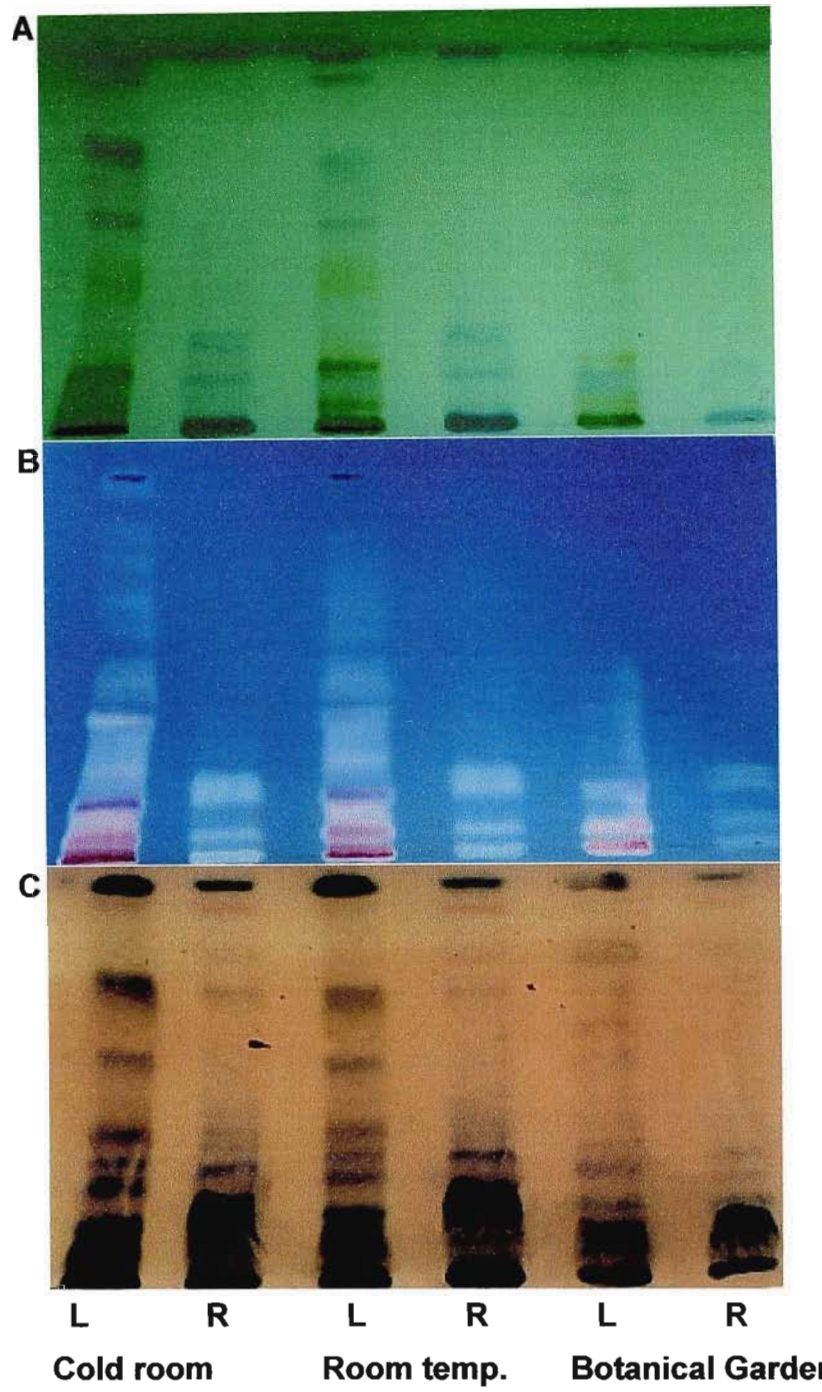


Figure 5.45: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for nine months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

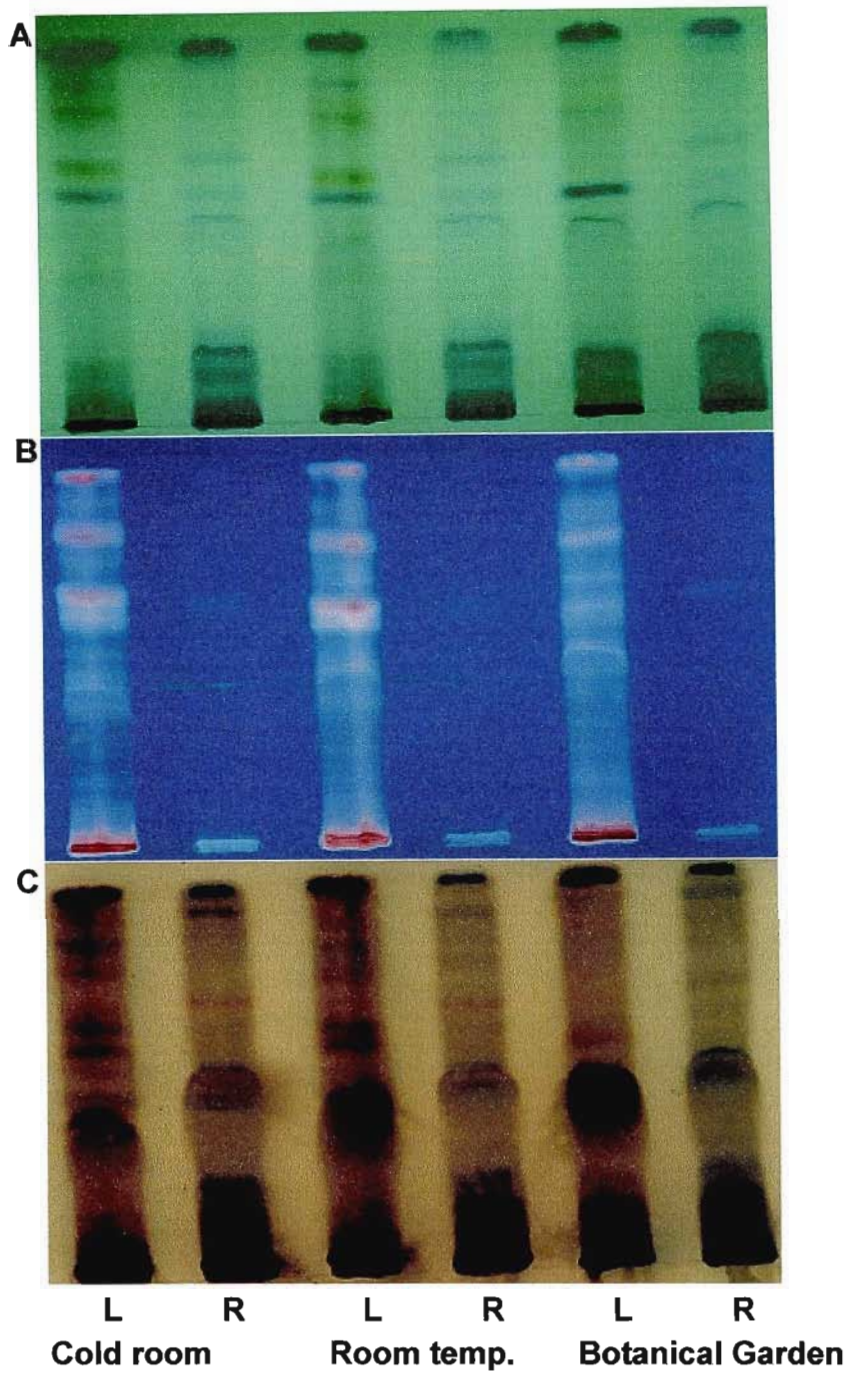


Figure 5.46: TLC fingerprints of hexane extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for twelve months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

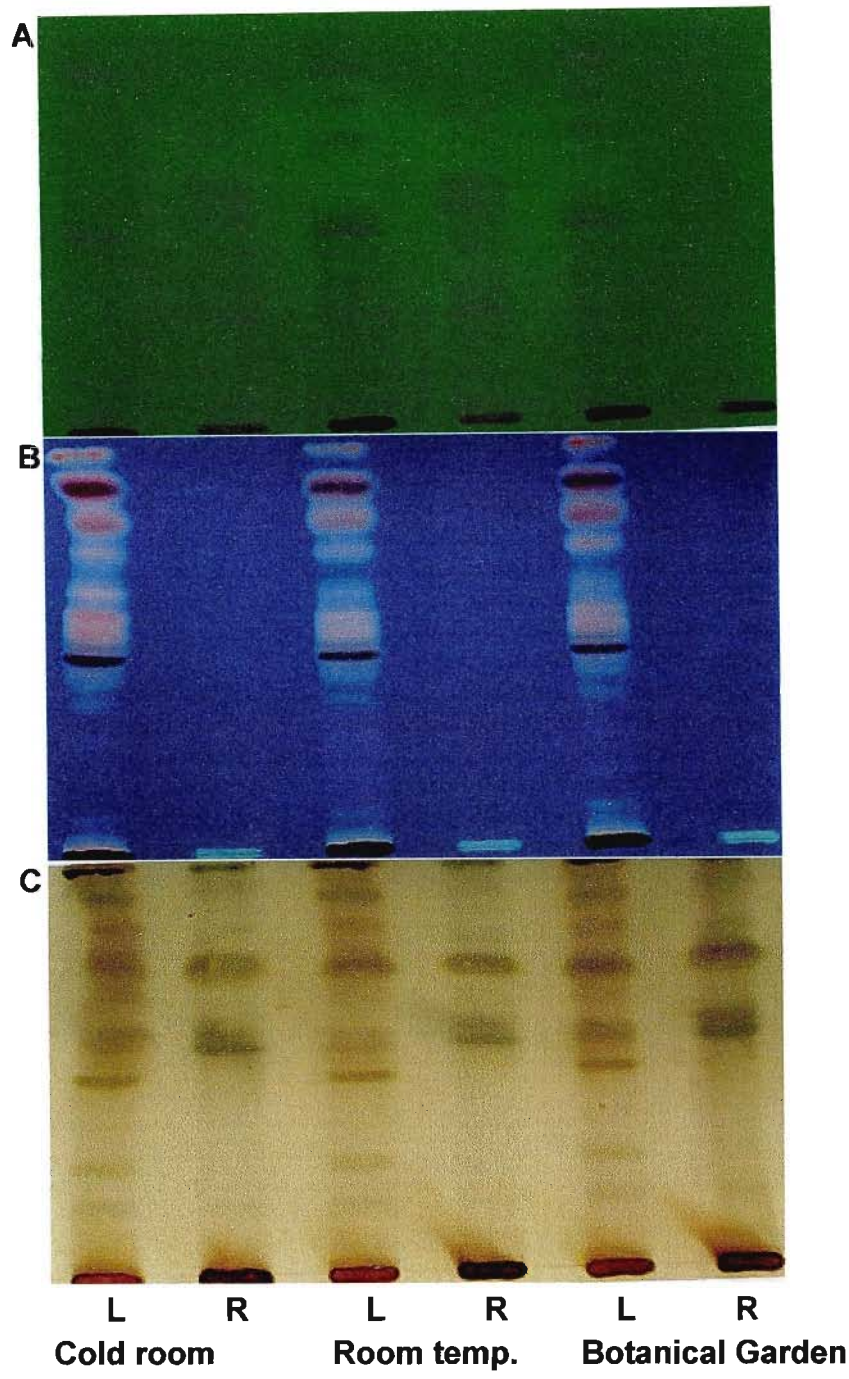


Figure 5.47: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for three months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

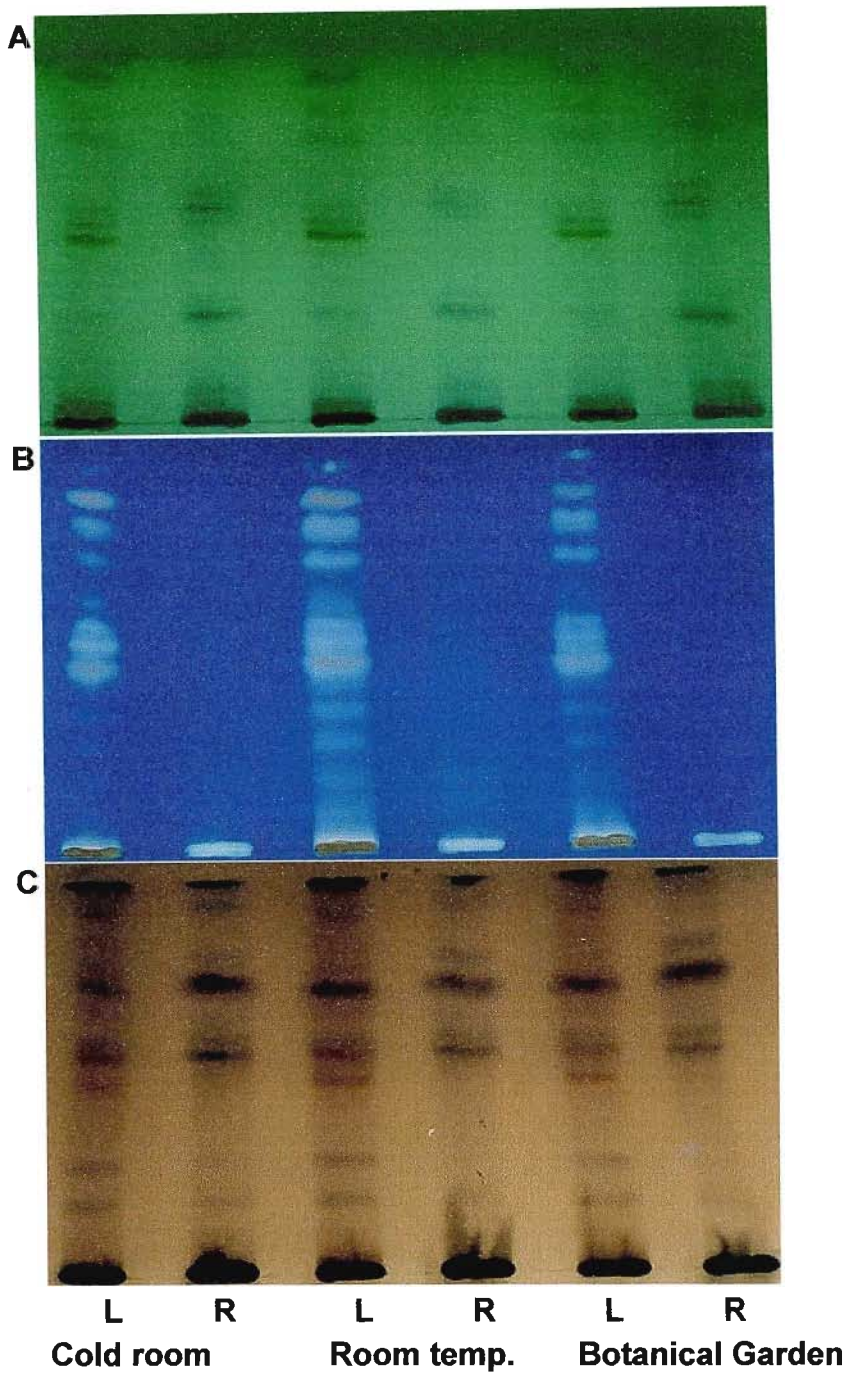


Figure 5.48: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for six months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

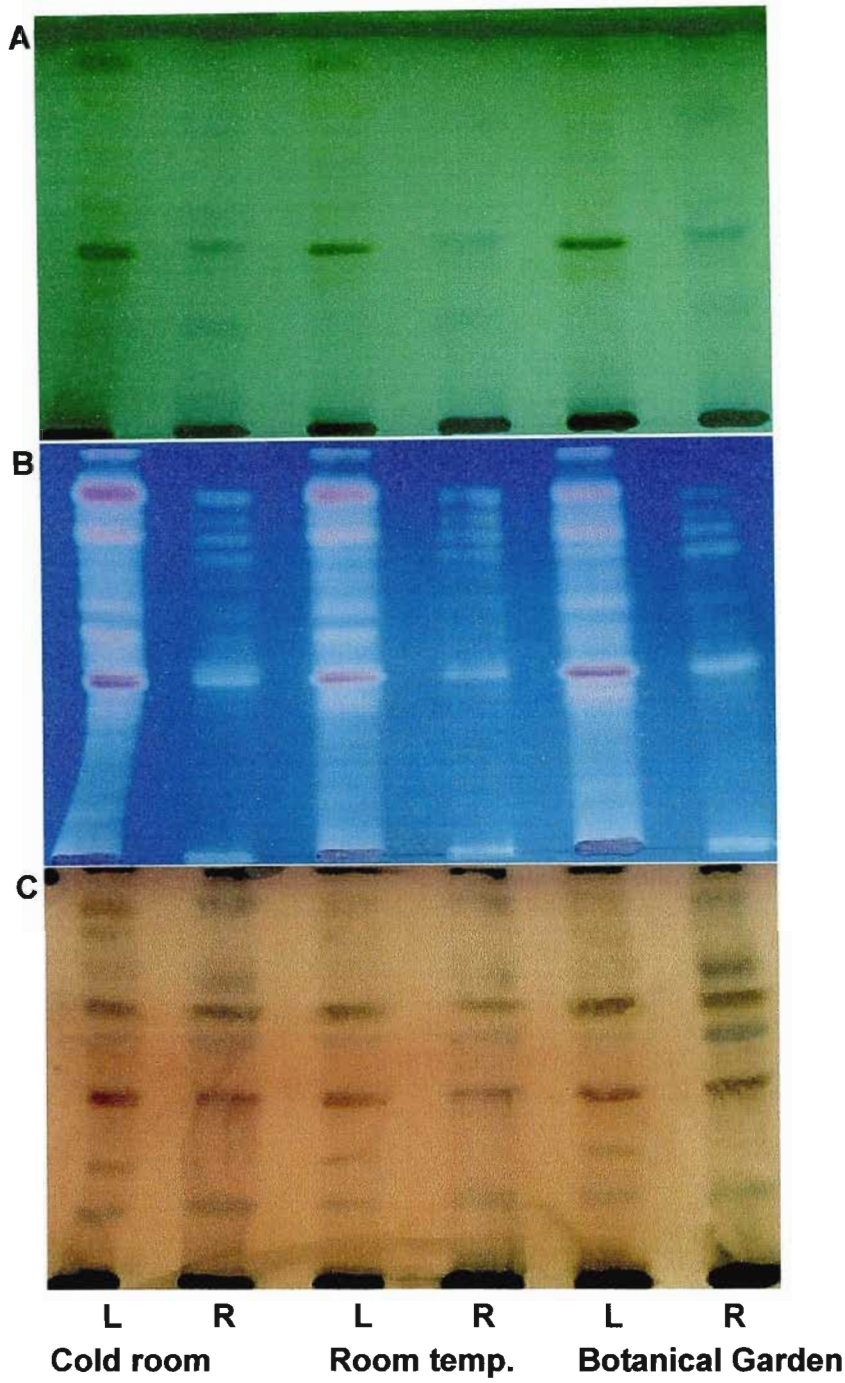


Figure 5.49: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for nine months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

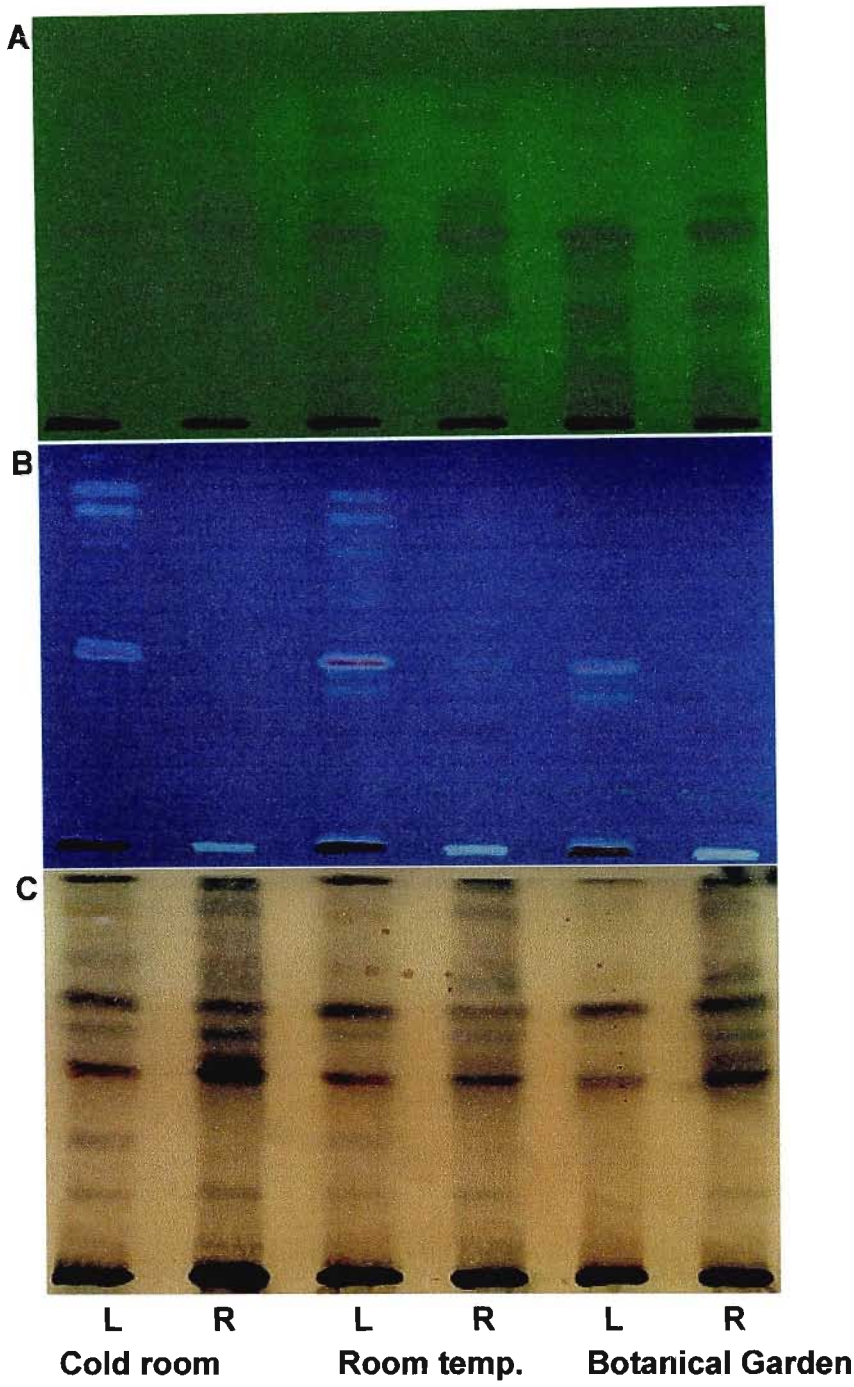


Figure 5.50: TLC fingerprints of methanol extract made from leaf (L) and root (R) material of *A. microraphis* stored in a cold room, at room temperature and in the Botanical Garden for twelve months. TLC plates were viewed at A) 254 nm; B) 366 nm and C) stained with anisaldehyde

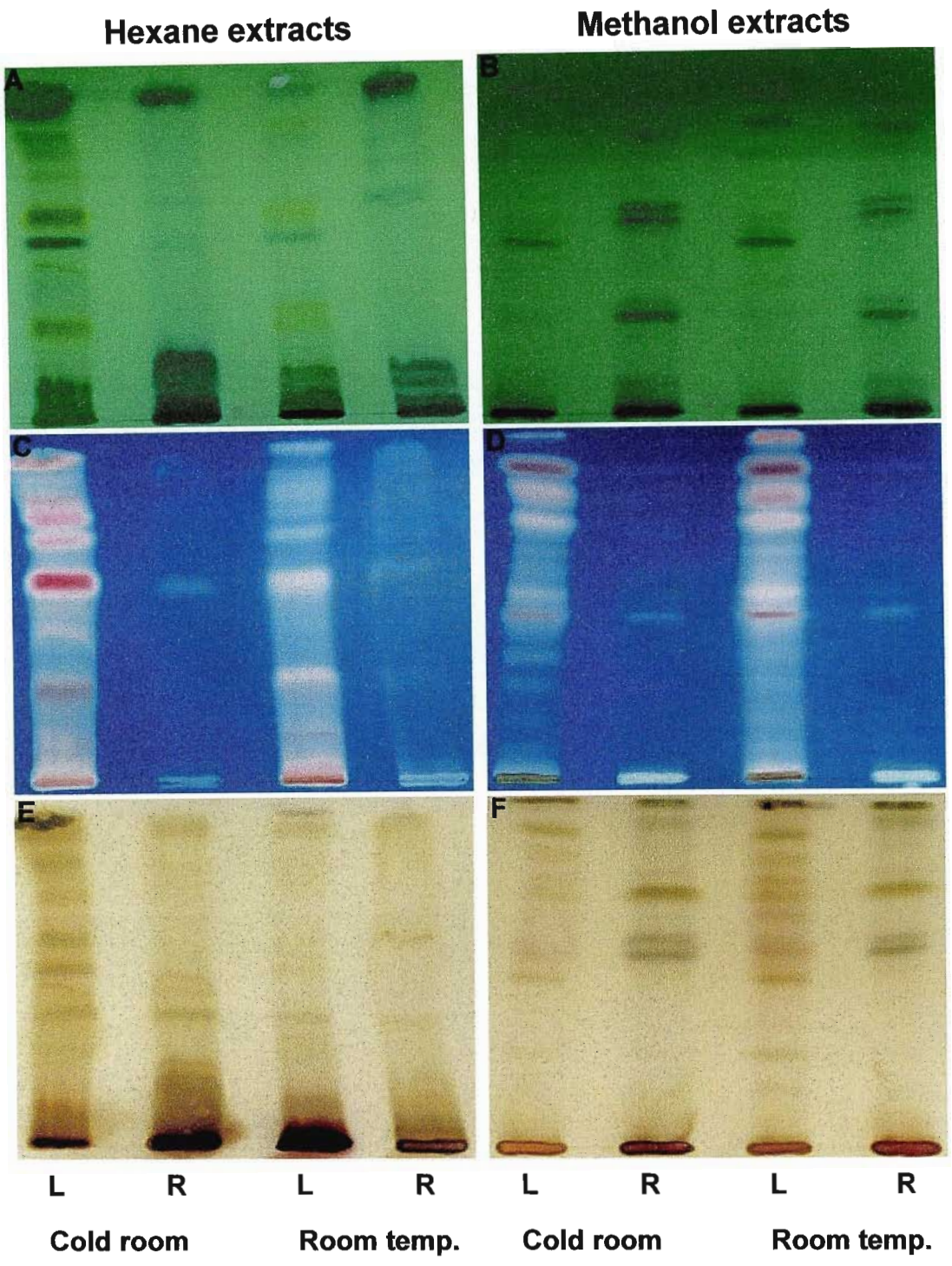


Figure 5.51: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of *A. microraphis* and stored in a cold room and at room temperature for three months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

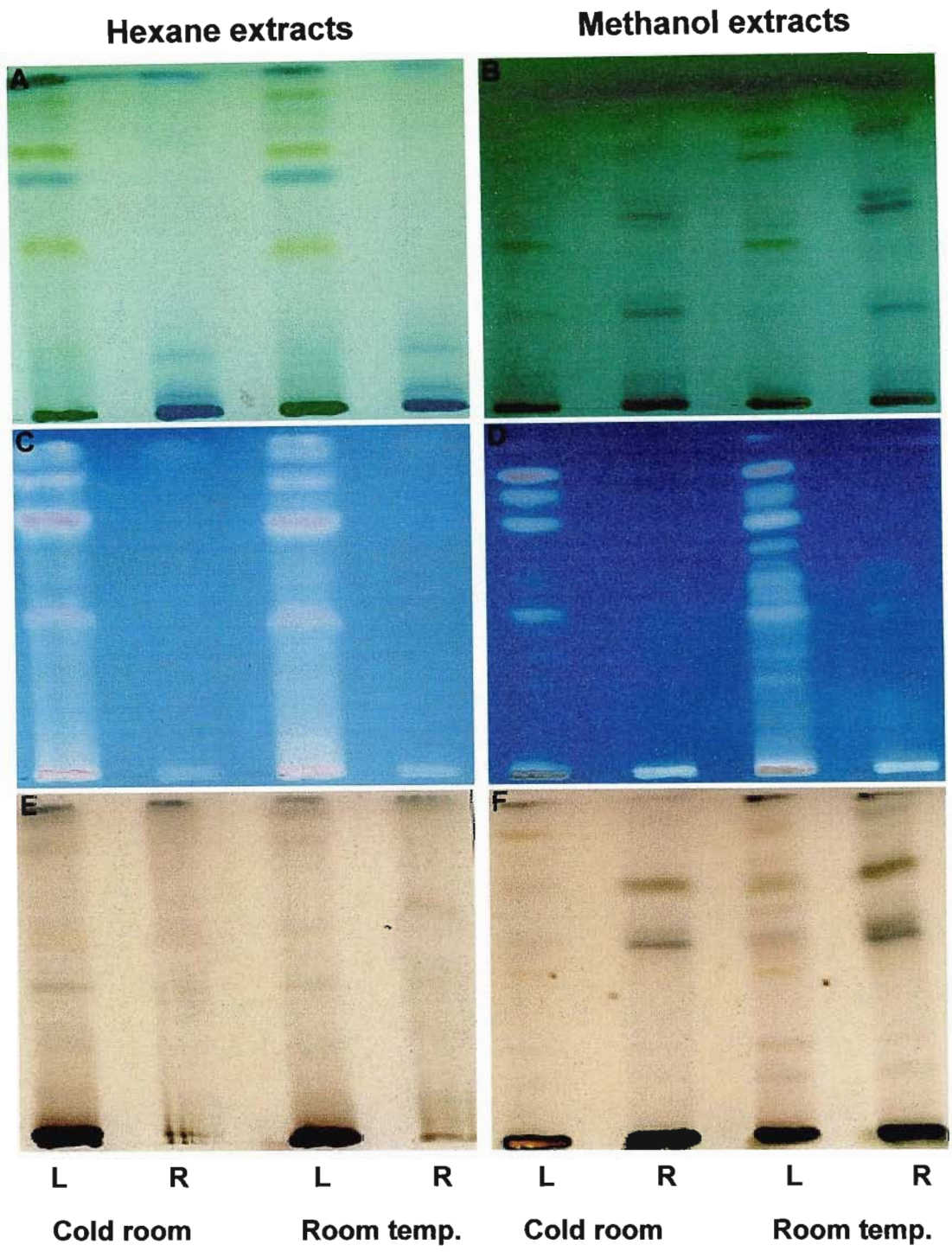


Figure 5.52: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of *A. microraphis* and stored in a cold room and at room temperature for six months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

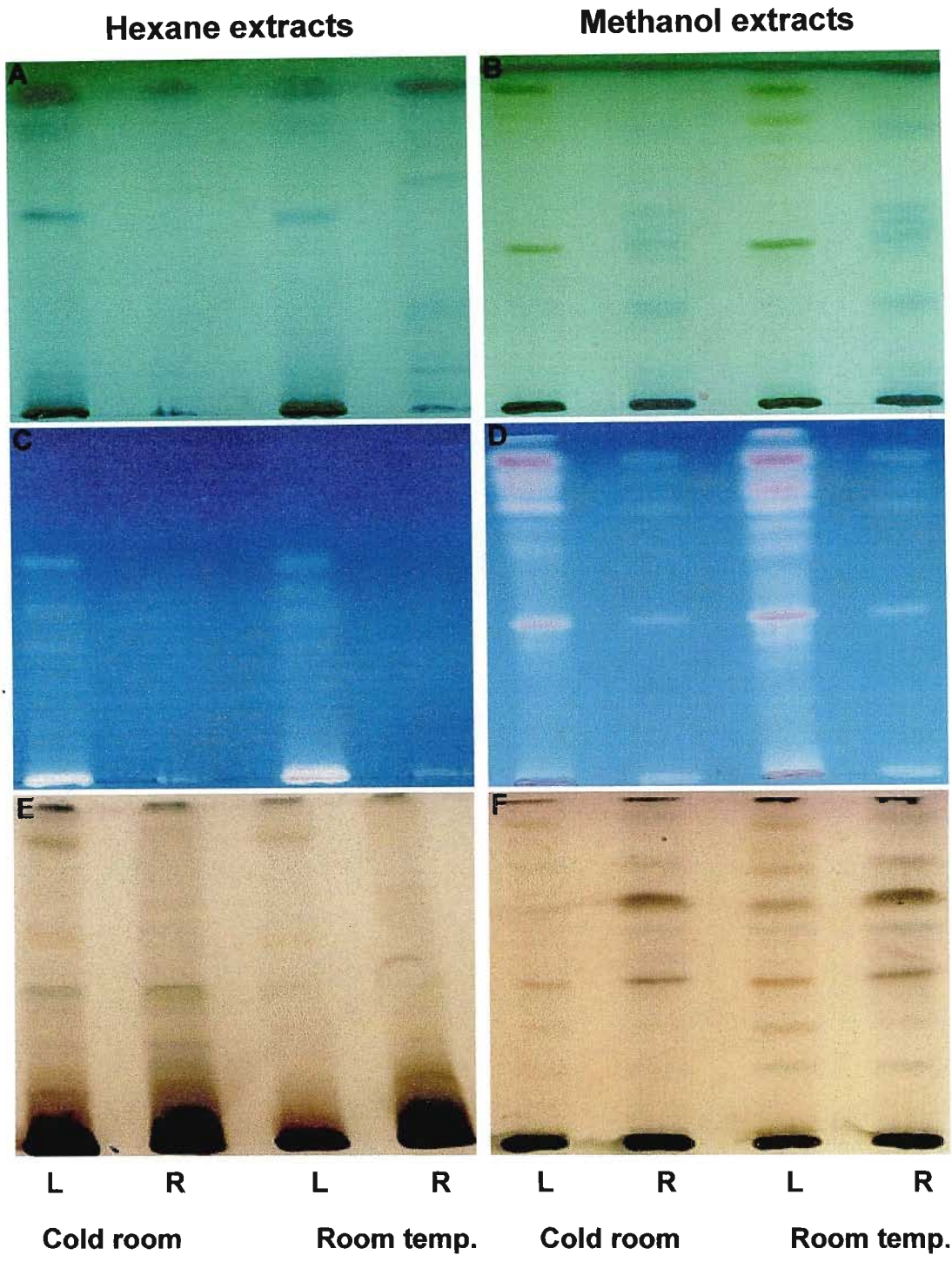


Figure 5.53: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of *A. microraphis* and stored in a cold room and at room temperature for nine months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

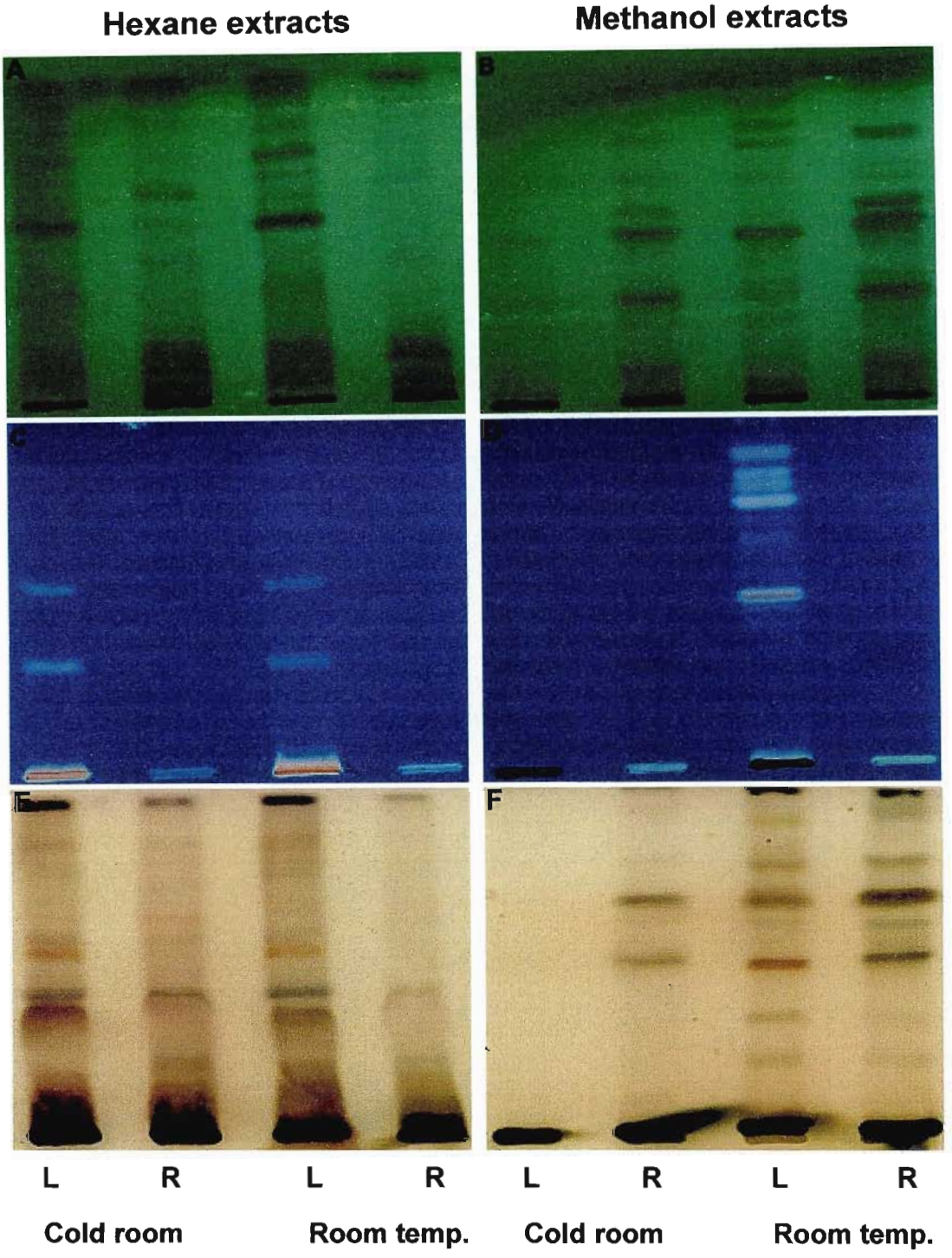


Figure 5.54: TLC fingerprints of hexane and methanol extracts made from leaves (L) and roots (R) of *A. microraphis* and stored in a cold room and at room temperature for twelve months. TLC plates were viewed at 254 nm (A and B), 366 nm (C and D) and stained with anisaldehyde (E and F).

retain viability of these medicinal plants. According to the interviewed healers and herbalists from Lesotho, storage of dried medicinal plants at room temperature, away from direct sunlight and large temperature changes enables plant material to retain activity for up to five years (CHAPTER 3). Other reports indicated that most marketed traditional plants which are spread out by vendors on pavements, known as an "open pharmacy", are subjected to loss of activity due to exposure to sunlight and large changes in temperature (BAGALE, NOAMESI and DAGNE, 1991). According to SILVA, LEE and KINGHORN (1998) plant material must be kept away from direct sunlight because ultraviolet radiation may produce chemical reactions giving rise to compound artifacts.

Anti-bacterial testing of *M. parviflora* and *E. punctulatus* showed that the inhibitory activity against Gram-positive and Gram-negative bacteria was high from hexane and methanol extracts made from leaf and root material. Water extracts, a form commonly utilised in traditional medicine, exhibited low or no anti-bacterial activity against the tested bacteria. The initial storage of plant material in a cold room, at room temperature and in the Botanical Garden caused an increase in anti-bacterial activity of the hexane, methanol and water extracts obtained from *M. parviflora* and *E. punctulatus*. Storage for a longer period resulted in a decrease in inhibitory activity (Figures 5.1-5.6 and 5.24-5.26). TLC fingerprint results obtained from hexane and methanol extracts made from *M. parviflora* and *E. punctulatus* material stored in the cold room and at room temperature showed a consistent number and colour of spots during the initial storage period. Prolonged storage resulted in a decline in number of spots and the disappearance of red fluorescence, probably chlorophyll, colour when plates were viewed at 366 nm (Figures 5.12-5.19 and 5.30-5.33). This is in line with

the decrease in activity of hexane and methanol extracts made from *M. parviflora* and *E. punctulatus* material stored for longer periods. The implication is that longer storage causes the degradation of compounds in the stored plant material, thus resulting in compounds with little or no anti-bacterial activity.

Testing for the effects of storage on anti-inflammatory activity of *M. parviflora, E. punctulatus* and *A. microraphis* extracts showed that the storage of plant material in a cold room, at room temperature and outside in the Botanical Garden did not cause any change in anti-inflammatory activity of hexane extracts obtained from leaves and roots of the three tested plants. Methanol and water extracts obtained from leaves and roots exhibited an increase in activity with prolonged storage of the plant material at the three storage conditions (Figures 5.7–5.10, 5.27–5.28 and 5.38-41). This increase in activity with time could be due to breakdown products caused by prolonged storage of plant material. The resulting products may potentiate anti-inflammatory activity, thus boosting the already existing activity from hexane, methanol and water extracts obtained from *M. parviflora, E. punctulatus* and *A. microraphis.*

Generally, exposure of the plant material to the three investigated storage conditions resulted in almost similar effects on anti-bacterial and anti-inflammatory activity of *M. parviflora, E. punctulatus* and *A. microraphis*. This disagrees with the reports from traditional healers and herbalists who emphasized that indoors and room temperature are the best storage method. The implication is that some medicinal plants used both by the healers who store plant material indoors and plants sold at open pharmacies by vendors retain anti-bacterial and anti-inflammatory activity for a

certain period depending on plant species. The work done by GRIGGS, MANANDHAR, TOWERS and TAYLOR (2001) showed that anti-bacterial and anti-fungal activity of medicinal plants varied among different plant species after six years storage. The activity of some plants was enhanced upon UV exposure while others which were initially noted to have activity enhanced by light, were found to have lost this activity upon re-testing as a result of storage. Prolonged storage often causes the constituents of the material to decompose or hydrolyse. This has been reported for *Antireha putminosa* which lost 50% of its alkaloids and flavonoid glycosides after two months storage (SILVA, LEE and KINGHORN, 1998).

Storage of hexane and methanol extracts of *M. parviflora, E. punctulatus* and *A. microraphis* still exhibited similar trends in changes in anti-bacterial and anti-inflammatory activity with time. However, the loss of anti-bacterial activity was faster from the stored extracts compared to the stored plant material (Figures 5.1–5.6; 5.9–5.10; 5.24-5.25 and 5.40–5.41). This shows that the stability of plant components depend to a great extend on the storage form of the plant with dried material generally having a longer shelf-life than plant extracts.

There is a concern when using stored plant material that could have inadequate medicinal activity. Better storage forms and ways of storing medicinal plants to maximise the "shelf life" needs attention. Knowledge of an efficient storage method could minimise loss of activity which usually occurs from most medicinal plants used in traditional remedies. One important consideration is how the harvested plant material is handled prior to extraction. For instance, plants that contain essential oils such as *E. punctulatus*, tend to loose some of its components during harvesting and

handling processes prior to storage. WEBBER, MAGWA and VAN STADEN (1999) observed that when vegetative material from *E. punctulatus* was transported over a long distance (further than 10 km) using an open truck, travelling at about 100 km/h, there was a reduction in the oil content of the plant material. A similar situation could have resulted in this study since the plant material was collected from Qacha's Nek district in Lesotho and transported over a long distance (350 km and 5 h drive) using public transport. The plant material was partially dried at room temperature prior to transportation to Pietermaritzburg where oven drying at 50 ^{0}C was used. Drying can give rise to a number of negative physical and chemical modifications which affect the quality of the marketed plants. There could be changes in appearance, smell and possible loss of compounds (BARITAUX, RICHARD, TOUCHE and DERBESY, 1992).

Compacted samples of fresh plant material with little air circulation may experience fungal infestation and elevated fermentation due to higher temperatures if the material is left to stand for several hours or days (SILVA, LEE and KINGHORN, 1998). These factors may then affect the initial results used as a reference point as well as contribute to the effects caused by storage. It is therefore advisable to fully extract a small portion of the fresh material and to keep a TLC chromatogram of the extract for future comparison purposes and for later detection of changes (SILVA, LEE and KINGHORN, 1998).

The testing of plants to determine the loss of activity over time will allow healers to make informed decisions with respect to prescribing stored plant material. The disposal of unstable plants will contribute to the efficacy of herbal medicines, while

plants that are known to have stable biological activity can safely be stored for longer periods of time. This will benefit both the healers and the environment, since fewer plants will be harvested (GRIGGS, MANANDHAR, TOWERS and TAYLOR, 2001).

Since both *M. parviflora* and *E. punctulatus* are used traditionally for diseases associated with bacterial infections and inflammation (CHAPTER 3), and the obtained results showed a decrease in anti-bacterial activity and an increase in anti-inflammatory activity with prolonged storage, one can recommend that the two plants could be stored for a certain time by the healers, herbalists and vendors prior to usage. The resulting stored material could still be effective for anti-inflammatory activity, provided that after long storage, the plants are prescribed for diseases associated with inflammation. *A. microraphis* can also be stored for longer periods since storage causes an increase in its anti-inflammatory activity. It is interesting to see that the stored plant material retains activity for a longer period with water extracts in particular exhibiting an increase in activity with time. This is beneficial both for the healers and patients since medicinal plants are usually stored in the form of plant material and not as extracts and water is commonly used as the extractant.

CHAPTER 6

ISOLATION OF ANTI-BACTERIAL AND ANTI-INFLAMMATORY COMPOUND(S) FROM *MALVA PARVIFLORA*

6.1 Introduction

Traditional healers and herbalists from Lesotho upon interview indicated that *M. parviflora* is used in traditional medicine for the treatment of wounds, sores and inflammation. An infusion made from both the leaves and roots is used to clean wounds and sores. Dried powdered plant material is then applied to the affected areas (CHAPTER 3). The Basotho people use a decoction made from *M. parviflora* as a remedy for tapeworm, profuse menstruation and as a lotion for bruised and broken limbs. Economically, *M. parviflora* is used to make glue or cement for mending broken or cracked clay pots (GUILLARMOD, 1971).

An infusion made from *M. parviflora* leaves is drunk by Europeans as a "nerve tonic". In the Western Cape, South Africa, *M. parviflora* is used as a remedy and douche for urinary tract problems including leucorrhoea. A hot poultice made from leaves is used for wounds and swelling. The Xhosa people use the leaves of this plant for drawing swollen, inflamed pulent wounds (WATT and BREYER-BRANDWIJK, 1962). Other species of *Malva* such as *Malva rotundifolia* L. are used medicinally by

Europeans as a decoction or poultice for sore throat, ophthalmia and maturing abscesses. Extracts made from the roots of this plant are reported to inhibit the growth of *Mycobacterium tuberculosis* (WATT and BREYER-BRANDWIJK, 1962). *Hibiscus trionum* L. is another member of the Malvaceae which is used by the Xhosa-speaking people from the Transkei region in the Eastern Cape. An infusion made from the shoot is used to wash wounds, thus killing pain and drying the wounds (TYISO and BHAT, 1998).

Other than for its medicinal uses in man, *M. parviflora* is reported to cause diseases in foraging livestock such as sheep, horses and cattle. This is due to the toxicity of nitrates present in the plant. The nitrates get converted to nitrites that combine with haemoglobin to form methaemoglobin, thus preventing adequate uptake of oxygen. Malvalic acid (an unsaturated fatty acid) previously referred to as halphen acid, also contributes to the toxic effects of *M. parviflora* (WATT and BREYER-BRANDWIJK, 1962; COOPER and JOHNSON, 1984).

Sheep are the most often affected and develop clinical signs including staggering, trembling arched back, laboured breathing and become uncoordinated. Severely affected animals may fall and die. The toxic principle is believed to be transmissible to the lamb through the mother's milk. A similar condition occurs, though much less frequently, in horses and cattle. In addition to the staggering characteristic due to poisoning, profuse and rapid breathing have been reported in horses. In southern Africa, the condition is common in riding and draught horses particularly after exertion. Cattle are also affected but have to eat a considerable quantity of *M. parviflora* before developing these symptoms (WATT and BREYER-BRANDWIJK,

1962; COOPER and JOHNSON, 1984). Ingestion of *M. parviflora* by chickens results in "pink-white" coloured eggs. This is due to malvic acid, which is a constituent of leaves and seeds of malvaceous plants. The acid causes iron to diffuse from the yolk and chelate with the albumin of the egg white (WATT and BREYER-BRANDWIJK, 1962; COOPER and JOHNSON, 1984). However, *Malva* species such as *Malva scabrosum* L. are not a threat to domesticated animals. Leaves of this plant are sticky and have stiff hairs as well as glands that secret aromatic oils. These result in emission of a tarry odour, which makes the plant unpalatable to grazing animals or damage by snails and caterpillars (WATT and BREYER-BRANDWIJK, 1962).

6.2 Aims

The aim of this part of the study was to extract, isolate and identify the active anti-bacterial and anti-inflammatory compounds from *M. parviflora*.

6.3 Materials, Methods and Results

6.3.1 Collection of *M. parviflora* for bulk extraction

The original collection site (Thaba Ts'oeu locality) where *M. parviflora* was collected for screening of anti-bacterial and anti-inflammatory activities did not have sufficient plant material for bulk collection. *M. parviflora* roots were thus collected at the Reserve locality from Qacha's Nek district in Lesotho in September 1998 for bulk extraction. The plant material was washed and dried at 50 ^{0}C, coarsely ground and stored at 10 ^{0}C until use.

6.3.2 Testing different extraction methods and solvents

Two different extraction methods were tested using different solvents to determine the most efficient method to maximise extraction.

Serial extraction of *M. parviflora* by Soxhlet

To determine the best solvent to use for extraction, 8 g of the ground plant material was serially extracted in a Soxhlet apparatus using 150 ml hexane, followed by the same volume of dichloromethane, methanol and water respectively. Each extraction was done for 90 minutes at boiling temperature and the resulting extracts were dried *in vacuo*. Dried extracts were redissolved at 100 mg ml^{-1} and tested in the disc diffusion anti-bacterial bioassay (Section 2.2.1). The same extracts were then dried in a stream of air and redissolved at 8 mg ml^{-1} (organic solvent extracts) and at 1 mg ml^{-1} (water extracts) and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

Serial extraction of *M. parviflora* by sonication

A further 2 g of ground plant material was serially extracted in 20 ml hexane followed by the same volumes of dichloromethane, methanol and water respectively for 30 minutes in an ultrasound bath. The extracts were left to stand at room temperature for a further 2 h and then filtered through Whatman No. 1 filter paper. Dried extracts were redissolved at 100 mg ml^{-1} and tested in the disc-diffusion bioassay (Section 2.2.1). The same dried extracts were redissolved at a concentration of 8 mg ml^{-1} (organic solvent extracts) and 1 mg ml^{-1} (water extracts) and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

Hexane, dichloromethane, methanol and water extracts obtained by Soxhlet extraction exhibited low anti-bacterial activity against *B. subtilis, S. aureus, S. epidermis* and *K. pneumoniae* (Table 6.1). Hexane and dichloromethane extracts obtained from the sonication extraction method did not yield any anti-bacterial activity against the tested bacteria. However, methanol extract from the sonication method resulted in high anti-bacterial activity against *E. coli.* The water extract inhibited the growth of *E. coli* and *S. aureus* (Table 6.1). The results indicated that the extraction of bacterial compounds was maximized using the sonication extraction method, possibly as lower temperatures are involved during the extractions. This suggests that anti-bacterial compounds have some degree of heat sensitivity so that Soxhlet extraction would not be ideal for bulk extraction.

Anti-inflammatory compound(s) did not show any change in activity between the extracts obtained from the Soxhlet and sonication extracting methods. Dichloromethane extracts from both extractions resulted in the detection of high anti-inflammatory activity (Table 6.1). Both Soxhlet and sonication extraction methods proved to be good for extracting anti-inflammatory compounds. Hexane, dichloromethane and methanol were used for the bulk extraction of *M. parviflora* roots.

6.3.3 Bulk extraction of *M. parviflora*

All solvents used in the isolation of the activity compounds were first distilled under vacuum to remove any impurities.

Table 6.1: Anti-bacterial and anti-inflammatory activity from serially extracted *M. parviflora* roots using different extraction methods and solvents. Anti-bacterial results of extracts with no activity against *M. luteus* and *P. aeruginosa* have been omitted from the table

Extraction Method	Solvents	Anti-bacterial activity					Anti-inflammatory activity
		Microorganisms tested					
		Gram-positive			Gram-negative		
		B.s	S.a	S.e	E.c	K.p	Inhibition %
Soxhlet	Hexane	0.3	-	0.2	-	-	57
	Dichloromethane	-	-	-	-	0.2	87
	Methanol	-	-	0.3	-	-	36
	Water	-	0.2	-	-	0.3	31
Sonication	Hexane	-	-	-	-	-	46
	Dichloromethane	-	-	-	-	-	81
	Methanol	0.4	0.3	-	0.6	-	12
	Water	-	0.3	-	0.4	-	10
	Indomethacin (20 µM)						65

Extraction of *M. parviflora* using a percolation method

Although the sonication extraction was shown to maximise the extraction of anti-bacterial compounds from *M. parviflora* roots (Table 6.1), this method only allowed

the extraction of a small amount of plant material, thus, a percolation method was used. As with sonication, this method also eliminated the use of heat during the extraction.

The ground *M. parviflora* roots (840 g) were soaked in 1.5 L of hexane for 90 minutes. The soaked plant material was loaded into a column (4 cm internal diameter). Two litres of hexane were added to the column and allowed to run slowly through the column. The collected extract was dried *in vacuo*. The extraction was repeated four times until the collected extract was almost clear of colour. This procedure was then repeated using 2 L X 4 extractions with dichloromethane, followed by 2 L X 4 extractions with methanol.

A small amount (1 g) from each of the dried extracts was redissolved at the concentration of 100 mg ml^{-1} and tested in the disc diffusion bioassay (Section 2.2.1) against *B. subtilis* and *E. coli.* as these were the bacteria most sensitive to the *M. parviflora* extract. The same dried extracts were redissolved at 8 mg ml^{-1} (organic solvent extracts) and 1 mg ml^{-1} (water extracts) and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

As before, the hexane and dichloromethane extracts obtained from serial extraction of *M. parviflora* root extracts obtained using the percolation method did not yield anti-bacterial activity against *B. subtilis* and *E. coli.* The methanol extract inhibited the growth of the tested bacteria (Table 6.2). Hexane and dichloromethane extracts yielded high anti-inflammatory activity while the methanol extract resulted in low activity (Table 6.2). The hexane and dichloromethane extracts were dried in a stream

of air and stored in a cold room (10 ^{0}C). The methanol extract was further analysed for the isolation of the active anti-bacterial compound(s).

Table 6.2: Anti-bacterial and anti-inflammatory activity of *M. parviflora* roots extracted serially with hexane, dichloromethane and methanol using a percolation method

Solvents	**Weight (Grams)**	**Anti-bacterial activity**		**Anti-inflammatory activity**
		Microorganisms		
		B. subtilis	***E. coli***	**Inhibition (%)**
Hexane	1	-	-	69
Dichloromethane	3	0.1	-	89
Methanol	86	0.4	0.4	10
Indomethacin (20 µM)				65

6.3.4 Isolation of anti-bacterial compound(s) from the methanol extract obtained from bulk extraction of *M. parviflora* roots

STEP 1: Vacuum Liquid Column Chromatography (VLC)

To determine the best solvent system for compound separation, a number of TLC plates were spotted with 2 µl of the methanol bulk extract (Section 6.3.3) redissolved at 50 mg ml^{-1}. The TLC plates were developed in different solvent systems and viewed at 254 and 366 nm wavelengths and after staining with

anisaldehyde as described in Section 2.4. Hexane:ethyl acetate (6:4) resulted in the best separation and was used for VLC chromatography.

Initially, a small VLC was run to test the separation ability of the system. Silica gel (12.5 g, particle size 0.04-0.063 mm) was poured into a column (2 cm internal diameter). The dried methanol extract (500 mg) was redissolved in 500 µl methanol. The extract was mixed with a small amount of silica and dried under nitrogen to form a powder. The powdered extract was spread over the top of the silica in the column. Glass wool was placed over the extract. A serial gradient of 50 ml hexane:ethyl acetate at different ratios (Table 6.3) was run through the column. The collected fractions were dried *in vacuo.* Dried extracts were redissolved in hexane:ethyl acetate (2:3) at 100 mg ml^{-1} and 50 mg ml^{-1} and tested in the disc diffusion and bioautographic bioassays respectively (Section 2.2.1 and 2.2.2) against *B. subtilis* and *E. coli.*

Anti-bacterial activity against *B. subtilis* and *E. coli* was detected from fractions collected at 60:40 and 50:50 hexane:ethyl acetate and 100% methanol during purification of *M. parviflora* root methanol extract by VLC chromatography (Table 6.3). Inhibition of the growth of the same bacteria was also observed from a bioautographic bioassay where clear zones formed on the TLC plate from the same fractions. However, the resulting anti-bacterial activity was much lower than for the initial screening results shown in Chapter 3 and Section 6.3.2, so no further isolation of the active compound(s) was attempted.

Table 6.3: Anti-bacterial activity of the fractions collected by VLC during the purification of methanol extract obtained from *M. parviflora* roots

Solvent system ratio			Anti-bacterial activity	
			Microorganisms tested	
Hexane	:	Ethyl acetate	*B. subtilis*	*E. coli*
100	:	0	-	-
80	:	20	-	-
60	:	40	0.3	0.3
50	:	50	0.3	0.2
40	:	60	-	-
30	:	70	-	-
20	:	80	-	-
Methanol 100%			0.3	-

6.3.5 Isolation of anti-inflammatory compound(s) from the dichloromethane extract obtained from bulk extraction of *M. parviflora* roots

STEP 1: Vacuum Liquid Column Chromatography (VLC)

The dichloromethane extract obtained from the serial extraction of bulk *M. parviflora* roots (Section 6.3.3) yielded high anti-inflammatory activity. This extract was used in the isolation of the active anti-inflammatory compound(s). A number of TLC plates were spotted with 2 µl of dichloromethane extract as described in Section 2.4. Hexane:ethyl acetate (8:2 and 7:3) resulted in the best separation and was used for VLC chromatography.

Silica gel (150 g, particle size 0.04-0.063 mm) was poured into a column (5 cm internal diameter). Dried plant extract (2.5 g) was resuspended in 5 ml methanol and mixed with 1 g of the silica. The resulting dried powdered extract was loaded onto the column as described in Section 6.3.4. A serial gradient of 100 ml hexane:ethyl acetate at different ratios (Table 6.4) was run through the column. The collected fractions were dried *in vacuo*, redissolved in ethanol at 4 and 1 mg ml $^{-1}$ and tested in the COX -1 anti-inflammatory bioassay (Section 2.3.1).

Hexane:ethyl acetate fractions of 80:20, 78:22 and 76:24 collected by VLC during the purification of the dichloromethane extract obtained from *M. parviflora* root, yielded high anti-inflammatory activity (Table 6.4). These fractions were combined and dried in a stream of air to give a combined dry weight of 1.043 g. The extract had a green colour indicating the presence of photosynthetic pigments.

STEP 2: Sephadex LH-20 Column chromatography

M. parviflora residues obtained from the combined hexane:ethyl acetate (80:20, 78:22 and 76:24) fractions (STEP1) were further purified using a Sephadex LH–20 column. Sephadex LH–20 was soaked in cyclo-hexane:dichloromethane:methanol (7:4:1) overnight. This was poured into the column (2.5 cm internal diameter and 50 cm long). Dried residue was redissolved in 500 µl cyclo-hexane:dichloromethane:methanol (7:4:1) and loaded onto the column. The same solvent system was used to elute the column. The extract separated into bands along the column and they were collected as individual fractions. Five µl of the collected fractions were spotted onto plastic TLC plates, developed in benzene:ethyl acetate (7:3), viewed under UV light and stained with anisaldehyde as described in

Table 6.4: COX-1 anti-inflammatory activity of the various fractions collected by VLC chromatography during the purification of the dichloromethane extract obtained from *M. parviflora* roots. The fractions highlighted in bold show the fractions which were combined for further purification

Fractions	**Solvent ratio**	**Weight (g)**	**Test concentration**	
	Hexane : ethyl acetate		**4 mg ml^{-1}**	**1 mg ml^{-1}**
A	100 : 0	22.9	36%	27%
B	**80 : 20**	**402.9**	**78%**	**71%**
C	**78 : 22**	**345.8**	**98%**	**86%**
D	**76 : 24**	**294.7**	**98%**	**83%**
E	74 : 26	65.8	88%	66%
F	72 : 28	17.6	96%	79%
G	70 : 30	16.9	93%	73%
H	60 : 40	32	92%	79%
I	50 : 50	94.2	89%	50%
J	40 : 60	118.1	89%	67%
K	100% methanol	1025	27%	24%

Section 2.4. Fractions showing similar spots were combined and dried *in vacuo*. The extracts were subsequently redissolved at 1, 0.5, 0.25 and 0.125 mg ml^{-1} and tested in the COX-1 and COX-2 anti-inflammatory bioassays (Sections 2.3.1 and 2.3.3).

Purification of the extract using a Sephadex LH-20 column chromatography resulted in compound(s) containing the green pigment being eluted first. These were collected as Fraction A (Table 6.5).

Fractions A to E yielded high anti-inflammatory activity at 1 and 0.5 mg ml^{-1} from the COX-1 bioassay. Lowering of the concentration to 0.25 mg ml^{-1} resulted in high anti-inflammatory activity from fractions B to E (Table 6.5). Testing of the same collected fractions for anti-inflammatory activity at 1 and 0.5 mg ml^{-1} using the COX-2 bioassay resulted in high anti-inflammatory activity being detected in fractions D and E (Table 6.5).

Fraction E yielded more dry weight (65.9 mg) and exhibited high anti-inflammatory activity in both COX-1 and COX-2 bioassays. This fraction was subsequently used for further purification of the active anti-inflammatory compound(s). The remaining Fractions were dried in a stream of air and stored in a cold room.

STEP 3: Thin Layer Column Chromatography (TLC)

Dried Fraction E collected by Sephadex LH-20 column chromatography (STEP 2) was resuspended in cyclo-hexane:dichloromethane:methanol (7:4:1) at 50 mg ml^{-1}, strip loaded onto one glass TLC plate (TLC plate 20 X 20 cm, silica gel 60 F_{254}) and developed in chloroform:benzene (7:3). The plate was viewed under UV light at 254 and 366 nm. A small piece (2 cm) of the TLC plate was cut off and stained with anisaldehyde as described in Section 2.4. Eight bands were visible. Their R_f values are given in Table 6.6. Each band was scrabed off, redissolved in chloroform:benzene (7:3), sonicated in an ultrasound bath for 5 minutes and filtered

Table 6.5: COX-1 and COX-2 anti-inflammatory activity of Fractions collected by Sephadex LH-20 column chromatography

Fractions	Weight (mg)	Inhibition (%)				
		COX-1			COX-2	
		1 mg ml^{-1}	0.5 mg ml^{-1}	0.25mg ml^{-1}	1 mg ml^{-1}	0.5 mg ml^{-1}
A	11.2	80	71	32	45	14
B	4.8	74	72	60	35	21
C	5.0	73	69	60	56	38
D	54.0	76	61	56	65	54
E	65.9	85	71	69	79	57
F	5.5	47	24	22	26	4
Indomethacin (20 µM)		75				
Nimesulide (200 µM)					49	
Indomethacin (200 µM)					34	

through a solvent Millipore filter (pore size 0.2 µM). Dried extracts were redissolved at 1 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

Very little anti-inflammatory activity was detected in the bioassay (Table 6.6). The filtered fractions were still contaminated with silica despite the filtering which affected the concentration of the tested fractions. Another attempt to remove the silica from the extracts was done by filtering through Celite Pasteur pipette columns.

STEP 4: Celite Pasteur pipette column purification

Pasteur pipettes were stoppered with glass wool and Celite (Celite analytical filter AID, BDH Chemicals Ltd Poole, England) packed to a height of 3 cm. Extracts obtained from STEP 3 were redissolved in 1 ml of methanol and run through Celite columns for the removal of the silica. Five ml of methanol (applied 1 ml at a time) was used to wash the extracts through the columns.

Table 6.6: COX-1 anti-inflammatory activity (1 mg ml^{-1}) of compound(s), which were separated by TLC

Bands	Rf values	Weight (mg)	Inhibition (%)
1	0.143	10.3	14
2	0.179	3.3	9
3	0.286	4.4	15
4	0.407	8.5	4
5	0.510	1.9	6
6	0.524	6.4	13
7	0.714	3.2	5
8	0.857	8.5	35
Indomethacin (20 µM)			63

Dried extracts were redissolved in ethanol at 1 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

The extracts run through the Celite Pasteur pipette column for the removal of silica resulted in low anti-inflammatory activity (Table 6.7). Further isolation of the active compounds could not be continued beyond this purification step due to the loss of the anti-inflammatory activity.

Table 6.7: COX-1 anti-inflammatory activity (1 mg ml^{-1}) of extracts run through Celite Pasteur pipette columns for the removal of silica from the extracts

Fractions	Dry weight (mg)	Inhibition (%)
1	3.8	17
2	4.1	7
3	2.0	1
4	1.0	7
5	1.6	10
6	1.8	11
7	1.9	6
8	1.3	20
Indomethacin (20 µM)		63

STEP 5: High Performance Liquid Chromatography (HPLC)

During STEP 2 (Sephadex LH-20 column chromatography) of the purification of the *M. parviflora* dichloromethane extract, Fraction D (dry weight 54 mg) also yielded high anti-inflammatory activity. The re-testing of this fraction at 1 mg ml^{-1} using the COX-1 anti-inflammatory bioassay (Section 2.3.1) confirmed this activity (72%

inhibition). This Fraction was then used in a further attempt to isolate the active anti-inflammatory compound(s) using HPLC.

Distilled water was filtered through a 0.22 µm aqueous filter and de-gassed by stirring with a magnetic rod under vacuum for 30 minutes. Water and HPLC grade methanol were used as solvents for the HPLC (Varian 5000 Liquid chromatograph) linked to a scanner (Spectrasystem UV 3000 HR). A semi-prep C_{18} column (Phenomenex Hypersil 5 C_{18} 25 cm X 10 mm diameter) was used.

Seven mg of the dried fraction D obtained from STEP 2 were redissolved in 1 ml of HPLC methanol and filtered through a Millipore solvent filter (GV type, pore size 0.2 µm). Five hundred µl of this extract were injected into the HPLC. Two programmes involving a water and methanol linear gradient shown in Table 6.8 were used to run the HPLC at a flow rate of 2.5 ml min^{-1}.

UV peaks were detected at 254 nm wavelengths and 0.1 absorbancy. Fractions were collected at 1 minute intervals, combined as shown in Table 6.9 and dried in a stream of air. Dried fractions were redissolved in 50 µl of ethanol and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

Anti-inflammatory activity was detected in fractions 17 to 30 collected by HPLC when programme 1 was used (Table 6.9). However, the detected anti-inflammatory activity was low compared to the activity of the extract (72%) before injection which was tested at lower concentrations.

Table 6.8: Programmes used to run the HPLC for the purification of Fraction D collected from the Sephadex LH-20 column during the purification of the dichloromethane extract obtained from *M. parviflora* roots

Time (min)	Solvent concentration			
	Programme 1		Programme 2	
	Water	Methanol	Water	Methanol
T_0	60	40	40	60
T_{40}	-	100	-	100
T_{50}	-	100	-	100

Separating by HPLC using Programme 2 (Table 6.8) resulted in better separation of the peaks but the collected fractions did not yield any anti-inflammatory activity. Combining and re-testing of the collected fractions for anti-inflammatory activity resulted in the re-appearance of the activity (Table 6.9)

6.3.6 Collection of *M. parviflora* from different localities

Bulk collection of *M. parviflora* with an upright growth form in September 1999 from a site (Reserve locality) different from the original (Thaba-tsoeu) site resulted in a decrease in anti-bacterial activity (Section 6.3.3). An investigation to determine variation in anti-bacterial and anti-inflammatory activity of *M. parviflora* collected at different localities was carried out. *M. parviflora* (leaves and roots) was collected at different localities from Qacha's Nek district in Lesotho in April 2001. The localities were Mpiti, TJ, Thaba-Tsoeu, Thifa and the Reserve (Appendix 5).

Table 6.9: COX-1 anti-inflammatory activity of Fractions collected from HPLC using programme 1 and 2

	Programme 1		Programme 2	
Sample	Fractions combined	Inhibition (%)	Fractions combined	Inhibition (%)
1	1-2	0	1-5	4
2	3-6	0	6-9	7
3	7-16	0	10-18	3
4	**17-21**	**69**	19-24	5
5	**22-25**	**65**	25-35	13
6	**26-30**	**53**	36-39	0
7	31-37	0	40-50	0
8	38-39	0	51-60	0
9	40-48	0	**Recombined 1-35**	**52%**
Indomethacin	20μM	65		61

Photographs of different growth forms of the collected *M. parviflora* were taken and are presented in Figure 6.1. The plant material was dried at 50 ^{0}C, coarsely ground and extracted with hexane, methanol or water as described in Section 3.3.2. Dried extracts were redissolved at 100 mg ml^{-1} and tested using the disc diffusion bioassay (Section 2.2.1). The same extracts were dried in a stream of air. Extracts made from organic solvents were redissolved in ethanol at 8 mg ml^{-1} and water extracts in water at 1 mg ml^{-1} and tested in the COX-1 bioassay (Section 2.3.1).

Collection of *M. parviflora* from different localities showed considerable variation in anti-bacterial and anti-inflammatory activity (Table 6.10 and Figure 6.2). *M. parviflora*

Figure 6.1: Upright (A and B) and creeping (C and D) growth forms of *M. parviflora* collected at Mpiti, TJ, Thifa and Reserve localities from Qacha ' s Nek district in Lesotho.

collected from TJ, Mpiti and Thaba-Tsoeu sites (Figure 6.1) had a creeping growth form. Hexane, methanol and water extracts obtained from these leaves and roots inhibited the growth of Gram-positive (*B. subtilis, S. aureus*) and Gram-negative (*E. coli*) bacteria (Table 6.10). *M. parviflora* collected from the Reserve and Thifa sites had an upright and erect growth form (Figure 6.1). Hexane, methanol and water extracts made from these leaves and roots inhibited the growth of Gram-positive (*B. subtilis, S. aureus*) bacteria only (Table 6.10).

Hexane and methanol extracts obtained from leaves and roots of *M. parviflora* collected from TJ, Thaba-Tsoeu, Mpiti, Reserve and Thifa localities did not exhibit any variation in anti-inflammatory activity. However, water extracts obtained from leaf and root material collected from TJ and Reserve localities yielded low anti-inflammatory activity (Figure. 6.2).

6.4 Discussion

6.4.1 Isolation of active compounds from *M. parviflora*

Purification of *M. parviflora* involved numerous techniques in an attempt to isolate the active compounds. Sephadex LH-20 column chromatography was among the techniques used. This technique is mostly used for separation of small hydrophobic natural products from their larger "contaminants" usually chlorophylls, which tend to be more lipophilic than many plant products (GIBBONS and GRAY, 1991). According to TAYLOR (1999), photosynthetic pigments are known to create false positives in the COX-1 anti-inflammatory bioassay. Purification of dichloromethane

Table 6.10: Anti-bacterial activity of hexane, methanol and water extracts obtained from leaves and roots of *M. parviflora* collected from different localities at Qacha's Nek district in Lesotho. Anti-bacterial activity results of extracts with no activity against *M.luteus, S. epidermis, P. aerugenosa,* and *K. pneumoniae* have been omitted from the table

Collection site	**Growth form**	**Plant parts**	**Extracting solvent**	**Microorganisms tested**		
				Gram-positive		**Gram-negative**
				B.s	**S.a**	**E.c**
TJ	Creeping	Leaves	Hexane	0.3	-	-
		Roots	Hexane	0.1	0.4	0.2
Mpiti		Roots	Hexane	-	0.3	0.3
			Methanol	0.1	0.4	0.4
			Water	0.1	-	-
Thaba-Tsoeu		Leaves	Hexane	0.2	0.1	-
		Roots	Hexane	0.1	0.4	0.4
			Methanol	0.2	-	-
Reserve	Upright	Leaves	Methanol	0.4	-	-
		Roots	Hexane	0.2	-	-
Thifa		Leaves	Hexane	0.3	-	-
			Methanol	0.3	-	-
			Water	0.3	0.3	-

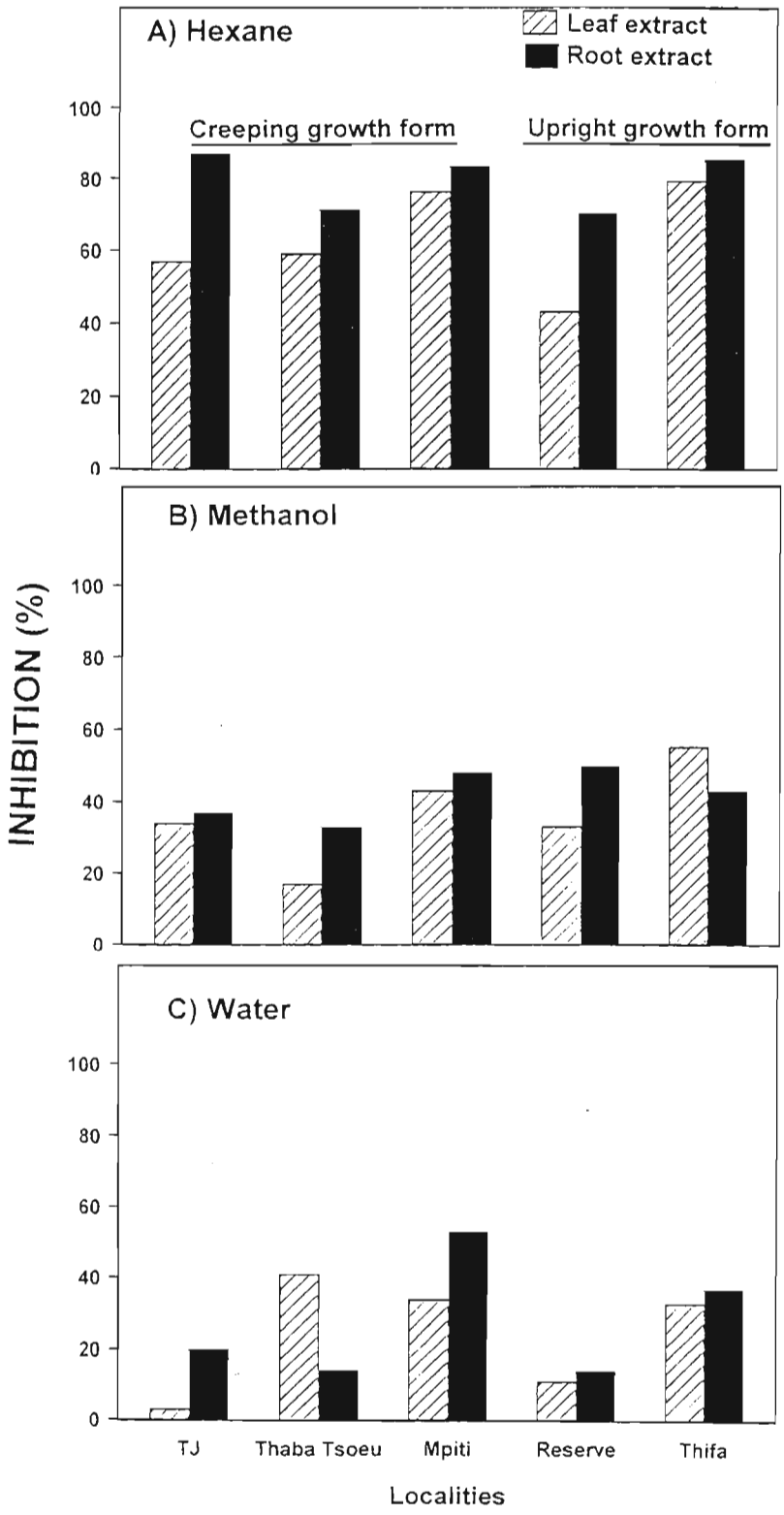


Figure 6.2: Anti-inflammatory activity of A) hexane, B) methanol and C) water extracts made from leaf and root material of *M. parviflora* collected from different localities. Plants collected from TJ, Thaba-Tsoeu and Mpiti showed a creeping growth form and the Reserve and Thifa collections showed upright growth form

extract obtained from the roots of *M. parviflora* using Sephadex LH-20 column chromatography, resulted in compounds containing a green pigment to elute first, followed by the fractions which yielded high anti-inflammatory activity using COX-1 and COX-2 bioassay (Table 6.5). Removal of the chlorophyll from the *Malva* extract still resulted in significant levels of COX-1 and COX-2 inhibitory activity showing that compounds other than the photosynthetic pigments are active in this anti-inflammatory assay.

Further purification of the extract using Thin Layer Chromatography (TLC) resulted in the extract being contaminated by silica. The removal of which proved difficult. Although chloroform:benzene (7:3) was the best solvent to redissolve the extract in, while attempting to remove the silica (STEP 3), the solvents may also have interfered with the active compounds in the extract. SILVA, LEE and KINGHORN (1998) indicated that chloroform and its impurities tend to react with some compounds (particularly alkaloids) and produce quaternary salts and other artifacts. Failure to remove the silica from the extract led to the consideration of another Fraction (Fraction D) which also had high anti-inflammatory activity in both the COX-1 and COX-2 bioassays (Table 6.5) after purification using Sephadex LH-20 column chromatography. Owing to the small amount of this Fraction (54 mg), HPLC was used for further purification. Although this technique is efficient in enabling a marked improvement in peak identification since it provides a simultaneous record of chromatograms obtained at different wavelengths, for compounds having closely related structures, the technique may not be sufficient for unambiguous identification (HOSTETTMANN, 1986).

M. parviflora fractions obtained from HPLC (Table 6.10) yielded some anti-inflammatory activity, but the separation of peaks was very poor. Further separation of the peaks resulted in loss of activity which then re-appeared when the fractions were combined. The results could imply that the active compounds in *M. parviflora* work collectively to exert the anti-inflammatory activity. FUGH-BERMAN (2000) and ELVIN-LEWIS (2001) stated that the interaction between herbs and drugs may affect the pharmacological and toxicological effects of the components through synergistic amplification or diminishment of the adverse effects.

There are always problems that serve to restrict the development of many plant-derived drugs. Such as limitation by low levels of secondary metabolites. In some cases, substances may be thermally or hydrolytically unstable or have unfavourable solubility properties (NIGG and SEIGLER, 1992).

6.4.2 Collection of *M. parviflora* from different localities

Collection of *M. parviflora* from different localities was done in order to investigate variation in levels of activity following a decline in anti-bacterial activity of the plant material collected for bulk extraction from a locality different from the original collection site.

Indigenous species, virtually all of which are gathered from the wild, vary greatly in their geographical distribution, population density, growth rate, growth form and population biology (CUNNINGHAM, 1991). Biological variation of the active plant constituents may result from recollection of the plant material from an area different

from the original locality. This may be due to the species of origin with promising natural product not being available in large quantity without disturbing the natural environment of the plant. Such recollection efforts may not result in the presence of the original biological activity and, hence re-isolation studies for the compound of interest would prove fruitless (NIGG and SEIGLER, 1992).

ELOFF (1999) indicated that the same taxon growing in different areas may have widely differing chemical components. He suggested that the plant material under study should be collected from the same area in order to minimize variation due to geographical factors. It is evident from Table 6.10 that *M. parviflora* collected from different localities exhibited variation in anti-bacterial activity as well as having different growth forms (Figure 6.1). The plant with a creeping growth form (collected from TJ, Mpiti and Thaba-Tsoeu localities, Table 6.10) inhibited the growth of Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative (*E. coli*) bacteria. In contrast, *M. parviflora* with an upright growth form (from Thifa and Reserve localities) inhibited only the Gram-positive (*B. subtilis* and *S. aureus*) bacteria. These results may explain low concentration levels of anti-bacterial compounds detected from the bulk extracted plant material (Table 6.3) collected from the Reserve locality compared to high anti-bacterial activity noted from the initially screened plant material collected from Thaba-Tsoeu (CHAPTER 3) as well as the material collected from other localities shown in Table 6.10. The results correspond with findings observed by other researchers on variation of components of the same taxon collected from different localities (CUNNINGHAM, 1991; ELOFF, 1999).

Variation in secondary metabolites have also been noted from the study on comparison of artemisinin content between *Artemisia annua* plants of different geographical origin. A considerable difference in the contents of artemisinin, artemisinic acid and dihydroartemisinic acid was noted. The highest levels of dihydroartemisinic acid and artemisinin were measured in *A. annua* plants of Vietnamese origin, while the lowest levels of these two compounds were found among the Chinese, European and New world plants (WALLAART, PRAS, BEEKMAN and QUAX, 2000).

6.4.3 Anti-bacterial and anti-inflammatory compounds isolated from other species in the family Malvaceae

Although the active anti-bacterial and anti-inflammatory compounds from *M. parviflora* have not been successfully isolated and identified during this study, a number of authors have reported on compounds isolated from other species of *Malva*. Their results may serve to highlight some possible compounds that may be found in *M. parviflora*.

Malvaceae plants such as *Gossypium hirsutum* L., *Malva sylvestris* L. and *Sida cordifolia* L. have been reported to potentiate some anti-bacterial, anti-inflammatory, anti-viral, insecticidal and fungicidal properties (BILLETER, MEIER and STICHER, 1990; DAVIS, EISENBRAUN and ESSENBERG, 1991; WILLIAMS, SATTLER, MOYNA, SCOTT, BELLS and VINSON, 1995; FRANZOTTI, SANTOS, RODRIGUES, MOURAU, ANDRADE and ANTONIOLLI, 2000). Compounds reported in species of Malvaceae include 2,7-dihydroxycadalene (DHC) and 2-

hydroxy-7-methoxycadalene (HMC). These are stress compounds produced in the leaves and cotyledons of upland cotton (*Gossypium hirsutum* L) during the hypersensitive response to incompatible races of bacterium pathogen. DHC has anti-bacterial activity against *Xanthomonas campestris* pv. *Malvacearum* (Smith), and is thus considered a phytoalexin. Sesquiterpenes are stored in subepidermal glands of leaves and buds of *Gossypium hirsutum* and the individual chemicals or mixtures from sesquiterpenes do possess insecticidal, fungicidal and bacteriocidal properties (DAVIS, EISENBRAUN and ESSENBERG, 1991; WILLIAMS, SATTLER, MOYNA, SCOTT, BELLS and VINSON, 1995).

Other compounds with chemotaxonomic significance for Malvaceae are the 8-hydroxy-flavonoids. Flavonoids are well known for their anti-bacterial, anti-fungal and anti-viral action. Several of them have been shown to be able to inhibit numerous diverse enzymes, including various hydrolases, cyclooxygenase, alkaline phosphatase, several ATPases, hydroxylases and kinases. The isolation of three 8-hydroxy-flavonoid sulphates and flavonoid glycoside has been reported from *Malva sylvestris* L. This plant is used in European folk medicine for the treatment of cough and inflammatory diseases of the mucous membrane (BILLETER, MEIER and STICHER, 1990; CORDELL, 1995; KUJUMGIEV, TSVETKOVA, SERKEDJIEVA, BANKOVA, CHRISTOV and POPOV, 1999). Phytochemical analysis of the leaves of *Sida cordifolia* L. demonstrated the presence of sympathomimetic amines, ephedrine and pseudoephedrine (a potent vasoconstrictor). This plant is used in folk medicine for treatment of stomatitis, blenorrhea, asthmatic bronchitis and nasal congestion (FRANZOTTI, SANTOS, RODRIGUES, MOURAU, ANDRADE and ANTONJOLLI, 2000).

CHAPTER 7

ISOLATION OF ANTI-BACTERIAL AND ANTI-INFLAMMATORY COMPOUND (S) FROM *ERIOCEPHALUS PUNCTULATUS*

7.1 Introduction

E. punctulatus contains highly priced essential oils used in the fragrance industry (WEBBER, MAGWA and VAN STADEN, 2000 A). Essential oils, also known as volatile oils, are usually complex mixtures of a wide variety of organic compounds such as hydrocarbons, alcohols, ketones, phenols, acids, ethers, aldehydes, esters, oxides and sulphur compounds (LEUNG and FOSTER, 1996). The oils are normally formed in special groups of cells or in glandular hairs found on organs of the plant including roots, stems, buds, leaves and flowers. However, oils are commonly concentrated in one particular region such as leaves, bark or fruits. When occurring in various organs in one plant, they may possess different individual chemical components (HILI, EVANS and VENESS, 1997).

Essential oils are generally isolated by distillation, the most common methods used being steam distillation, solvent extraction or expression (LEUNG and FOSTER, 1996). Essential oils of aromatic plant species are used in industries for the production of soaps, perfumes and toiletries. Many of them are also used in

traditional medicine for various purposes (CIMANGA, KAMBU, TONA, APERS, BRUYNE, HERMAN, TOTTE, PIETERS and VLIETINCK, 2002).

The essential oil obtained from *E. punctulatus* has a dark colour and a very attractive fresh fruity fragrance. The blue colour expressed in essential oil of *E. punctulatus* is believed to be due to the presence of azulenic compounds which are important perfumery constituents (WEBBER, MAGWA and VAN STADEN, 2000 A). Essential oils have been reported from other species of *Eriocephalus* such as *E. africanus* L. which yielded 0.3% of a green fairy dark attar when leaves and flowers were extracted with petroleum ether. Extraction of the fresh leaves of *E. umbellulatus* DC yielded 0.2% of a light yellow volatile oil with a sharp, unpleasant aroma and a burning taste (WATT and BREYER-BRANDWIJK, 1962).

E. punctulatus is used traditionally by the Southern Sotho to fumigate the hut of a person suffering from a cold or diarrhoea. The fumigation process is done during the illness or after death (WATT and BREYER-BRANDWIJK, 1962, GUILLARDMOND, 1971). When the traditional healers and herbalists in Lesotho were interviewed (CHAPTER 3) they indicated that *E. punctulatus* is also used to fumigate huts which had been invaded by lice. An infusion made from the leaves of *E. punctulatus* is used to clean wounds and sores and is drunk as a body cleanser and for the treatment of stomach pain.

Other species of *Eriocephalus* are reported to be used in the Cape (RSA) as diaphoretic and diuretic agents, e.g. *E. africanus* L. and *E. racemosus* L. An infusion made from *E. africanus* is used for the treatment of asthma and chest complaints, and the tea made from its leaves is drunk for coughs, colds, flatulence and colic

(WATT and BREYER-BRANDWIJK, 1962; SALIE, EAGLES and LENG, 1996). The Nama people use *E. umbellulatus* as a colic remedy. In the western Cape, *E. umbellulatus* is used as a household medicine for heart troubles, oedema and for swelling and pain arising from gynaecological conditions (WATT and BREYER-BRANDWIJK, 1962).

7.2 Aims

The aim of this part of the study was to extract, isolate and identify the active anti-bacterial and anti-inflammatory compounds from *E. punctulatus.*

7.3 Materials, Methods and Results

7.3.1 Plant material

E. punctulatus leaves were collected from Qacha's Nek district in Lesotho in May 2000. The plant material was dried at 50 ^{0}C, finely ground and stored in a cold room (10 ^{0}C) until needed.

7.3.2 Preliminary testing of bulk plant material for anti-bacterial and anti-inflammatory activities

Serial extraction of *E. punctulatus* by a Soxhlet apparatus

To determine the best solvent to use for bulk extraction, dried and ground *E. punctulatus* leaf material (8 g) was serially extracted in a Soxhlet apparatus using hexane, followed by the same amounts of dichloromethane, methanol and water as

described in Section 6.3.2. Dried extracts were redissolved at 100 mg ml^{-1} and tested in the disc-diffusion bioassay (Section 2.2.1) against *B. subtilis and S. aureus* bacteria. The same extracts were then dried in a stream of air and redissolved at 8 mg ml^{-1} (organic solvent extracts) and 1 mg ml^{-1} (water extracts) respectively to give a final test concentration of 200 µg ml^{-1}. The respective extracts were tested in the COX-1 anti-inflammatory bioassay as described in Section 2.3.1.

Hexane extracts exhibited the highest anti-bacterial activity against *S. aureus* and slightly lower inhibitory activity against *B. subtilis*. Dichloromethane and methanol extracts yielded lower anti-bacterial activity against the tested bacteria. The water extract showed no anti-bacterial activity. Hexane, dichloromethane and methanol extracts yielded high anti-inflammatory activity while low activity was noted with the water extract (Table 7.1). Hexane was thus used for the bulk extraction of *E. punctulatus* leaves.

7.3.3 Bulk extraction of *E. punctulatus* by Soxhlet

All solvents used in the isolation of the biologically active compounds were first distilled under vacuum to remove impurities.

Dried, ground plant material (490 g) was extracted with 3 X 2L of hexane using a Soxhlet apparatus. The extraction was run for 6 h and the extract was dried *in vacuo*. A dark thick oily extract was obtained (19.75 g dry weight). A small amount of the extract (1 g) was redissolved in hexane at 100 mg ml^{-1} and tested in the disc-diffusion bioassay (Section 2.2.1) against *B. subtilis and S. aureus*. The same extract

Table 7.1: Anti-bacterial and anti-inflammatory activity of the extracts obtained during the preliminary extraction of *E. punctulatus* using a Soxhlet apparatus

Extraction solvent	**Anti-bacterial activity**		**Anti-inflammatory activity**
	Microorganisms tested		**Inhibition (%)**
	B. subtilis	***S. aureus***	
Hexane	0.35	0.72	89
Dichloromethane	0.20	0.41	85
Methanol	0.33	0.30	80
Water	0.00	0.00	53
Indomethacin (20 μM)			63

was dried in a stream of air, redissolved at 8 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay as described in Section 2.3.1.

The crude hexane extract obtained from the extraction of *E. punctulatus* using a Soxhlet apparatus yielded high anti-bacterial activity against *S. aureus* and lower activity against *B. subtilis.* The extract also resulted in 85% inhibitory activity for the COX-1 anti-inflammatory bioassay. The indomethacin standard (20 μM) had 65% inhibitory activity.

7.3.4 Isolation of anti-bacterial and anti-inflammatory compounds from *E. punctulatus*

STEP 1: Vacuum liquid column chromatography (VLC)

The hexane extract obtained from the Soxhlet extraction (Section 7.3.3) was further purified for the isolation of the active anti-bacterial and anti-inflammatory compounds. The extract was spotted onto TLC plates (Section 2.4) and developed using various solvent systems to determine the best solvent system to use for separation on a VLC. Toluene:ethyl acetate (1:1) resulted in the best separation.

The maximum amount of extract that could be loaded onto the VLC was 5 g. Therefore, to prevent overloading, four separate columns were run simultaneously. The columns and the extract were prepared as described in Section 6.3.5. A serial gradient of 400 ml toluene:ethyl acetate (Table 7.2) was run through each column. Fractions collected from the four columns where the same ratio of solvent was used were combined and dried *in vacuo*. Dried extracts were redissolved in toluene:ethyl acetate (1:1) at 100 mg ml^{-1} and 50 mg ml^{-1} and tested in the disc-diffusion and bioautographic bioassays (Sections 2.2.1 and 2.2.2) against *B. subtilis and S. aureus*. The same extracts were dried in a stream of air, redissolved in ethanol at 1 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay as described in Section 2.3.1.

Fractions collected between toluene:ethyl acetate 60:40 to 40:60 all yielded high anti-bacterial activity against *B. subtilis* and medium activity against *S. aureus* (Table 7.2). Bioautographic results exhibited anti-bacterial activity against *B. subtilis* and *S. aureus* from fractions collected between toluene:ethyl acetate 60:40 to 40:60 (Figure 7.1). All of the fractions collected between toluene:ethyl acetate 80:20 to 30:70 gave anti-inflammatory activity above 60% (Table 7.2).

As the fractions collected between toluene:ethyl acetate 60:40 to 30:70 exhibited high anti-bacterial and anti-inflammatory activity, they were combined, dried in a stream of air. This residue was a dark thick oily extract (9.35 g dry weight).

Table 7.2: Anti-bacterial and anti-inflammatory activity of the fractions collected by VLC during the purification of the *E. punctulatus* hexane extract

Fraction	**Solvent system**	**Anti-bacterial activity**		**Anti-inflammatory activity**
	Toluene :Ethyl Acetate (%)	**Microorganisms**		**Inhibition (%)**
		B. subtilis	***S. aureus***	
1	100 : 0	-	-	69
2	80 : 20	-	-	89
3	60 : 40	0.42	0.30	90
4	50 : 50	0.52	0.32	76
5	40 : 60	0.40	0.33	85
6	30 : 70	0.23	0.30	73
7	20 : 80	-	-	64
8	100 methanol	-	-	72
	Indomethacin (20 μM)			65

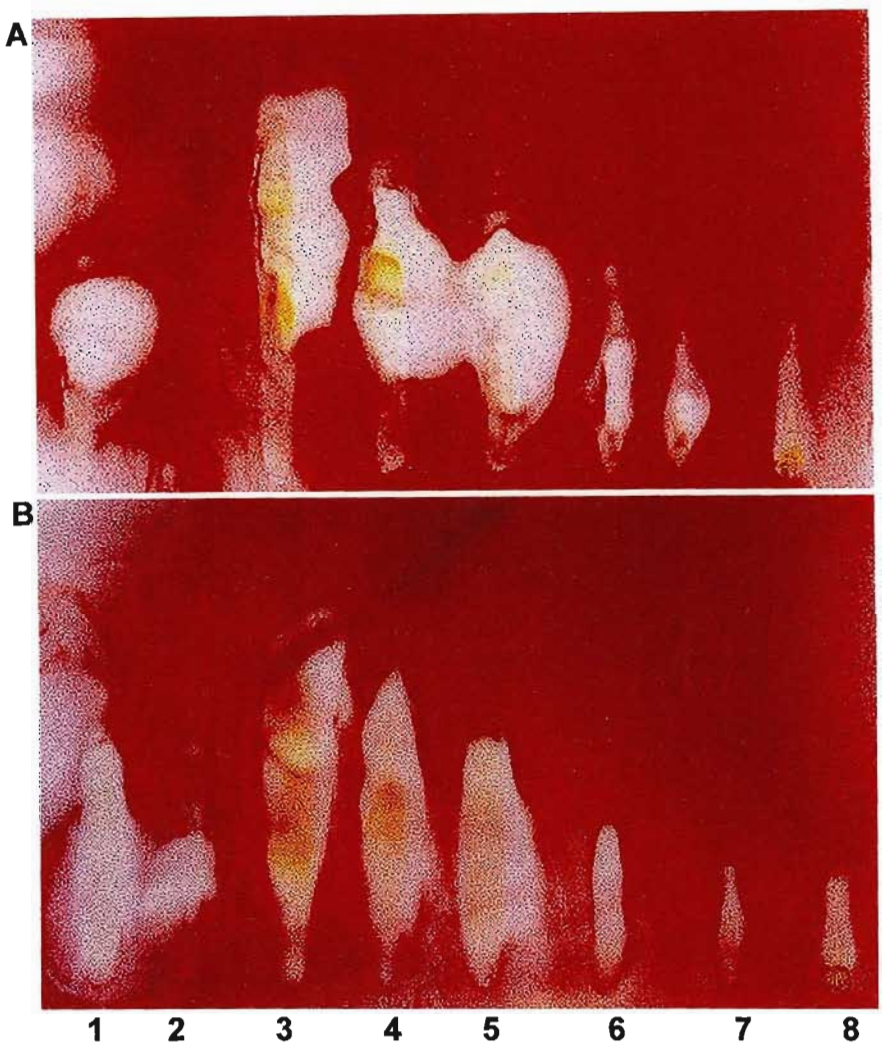


Figure 7.1: Results from the bioautographic bioassay showing anti-bacterial activity against A) *B. subtilis* and B) *S. aureus* from fractions collected during the purification of *E. punctulatus* hexane extract using VLC. The numbers refer to the fractions detailed in Table 7.2.

STEP 2: Second vacuum column chromatography

The extract obtained from STEP 1 was further purified using another VLC. This step was repeated for efficient separation of the extract which may have been overloading during the previous purification (STEP 1). Toluene:ethyl acetate (1:1) resulted in the best separation and was re-used for this VLC separation. Two columns were prepared as described in Section 6.3.5 and 4.5 g of the extract was run through each column using a serial gradient of 400 ml of toluene: ethyl acetate as shown in Table 7.3. Fractions collected from the columns were combined as described in STEP 1 and dried *in vauo*. Dried fractions were redissolved in toluene:ethyl acetate (1:1) at 100 mg ml^{-1} and 50 mg ml^{-1} respectively. These were tested in the disc-diffusion, bioautographic and minimum inhibitory concentration (MIC) microplate dilution bioassays (Sections 2.2.1, 2.2.2 and 2.2.3) against *B. subtilis* and *S. aureus*. The same fractions were dried in a stream of air, redissolved in ethanol at 1 mg ml^{-1} and tested in the COX-1 and COX-2 anti-inflammatory bioassays as described in Sections 2.3.1 and 2.3.3.

Anti-bacterial results obtained using the disc-diffusion bioassay showed activity against the two tested bacteria. Fractions collected at toluene:ethyl acetate 80:20 to 45:55 inhibited the growth of *B. subtilis* but showed lower anti-bacterial activity against *S. aureus*. However, the growth of *S. aureus* was inhibited by the fractions collected at toluene:ethyl acetate 40:60 and 30:70 with lower activity against *B. subtilis* (Table 7.3). Fractions that yielded anti-bacterial activity in the disc-diffusion bioassay also gave positive results in the bioautographic bioassay against the same bacteria, indicating the inhibition of their growth (Figure 7.2). The minimum inhibitory concentration (MIC) microplate dilution bioassay also gave positive results against *B.*

subtilis and *S. aureus* (Figure 7.3). Generally, *B. subtilis* seemed to be more sensitive than *S. aureus* since its growth was inhibited at lower concentrations of the plant extract (Table 7.3).

Table 7.3: Anti-bacterial activity from the disc-diffusion and MIC bioassays of the fractions collected by the second VLC during the purification of *E. punctulatus* hexane extract.

Fraction	Solvent system	Microorganisms tested			
	Toluene : ethyl acetate (%)	*B. subtilis*		*S. aureus*	
		Disc-diffusion (mm)	MIC (mg ml^{-1})	Disc-diffusion (mm)	MIC (mg ml^{-1})
1	100 : 0	-	-	-	-
2	80 : 20	0.58	0.78	0.20	3.13
3	70 : 30	0.45	0.78	0.11	6.25
4	60 : 40	0.40	1.56	0.20	3.13
5	50 : 50	0.38	1.56	0.11	6.25
6	45 : 55	0.38	3.13	0.11	6.25
7	40 : 60	0.23	3.13	1.50	6.25
8	30 : 70	0.33	3.13	1.13	12.5
9	25 : 75	-	-	-	-
10	20 : 80	-	-	-	-
11	100% methanol	-	-	-	-

Except for the fraction collected with 100% toluene, all of the collected fractions resulted in very high anti-inflammatory activity using the COX-1 bioassay. High anti-inflammatory activity in the COX-2 bioassay was detected from fractions collected at toluene:ethyl acetate at 70:30 to 30:70 (Table 7.4).

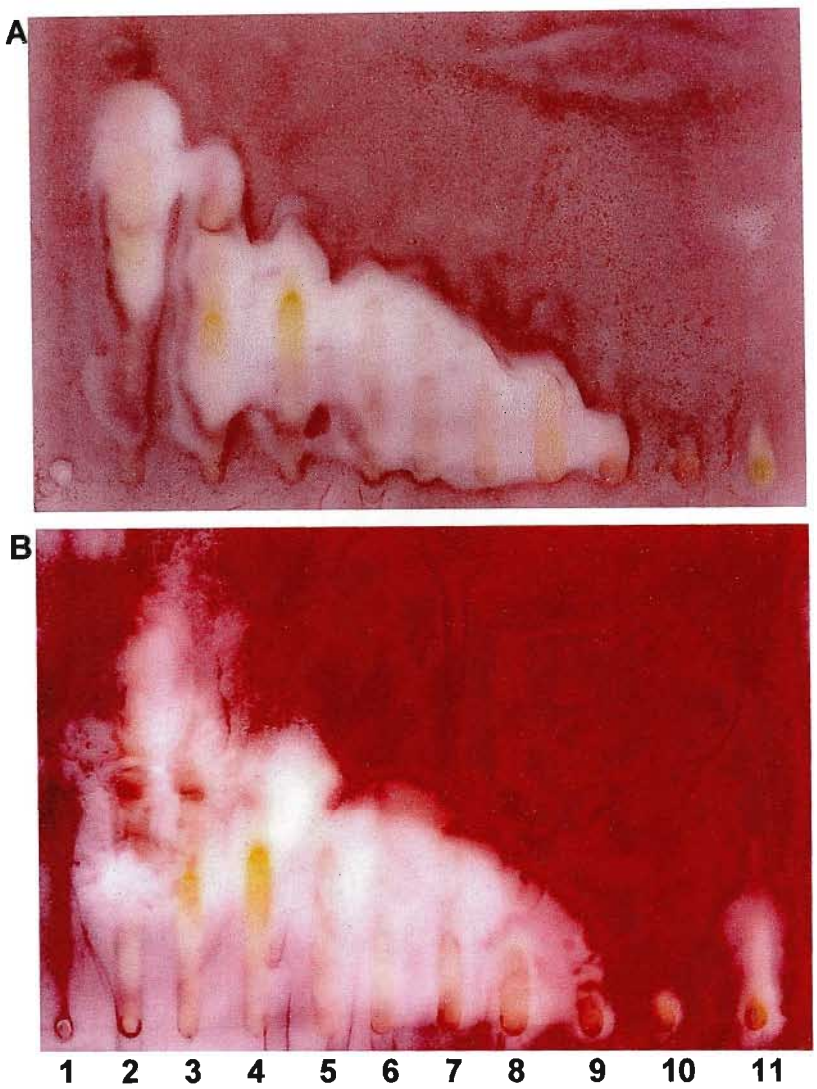


Figure 7.2: Results from the bioautographic bioassay showing anti-bacterial activity against A) *B. subtilis* and B) *S. aureus* from fractions collected during the second purification of *E. punctulatus* hexane extract using VLC. The numbers refer to the fractions detailed in Table 7.3.

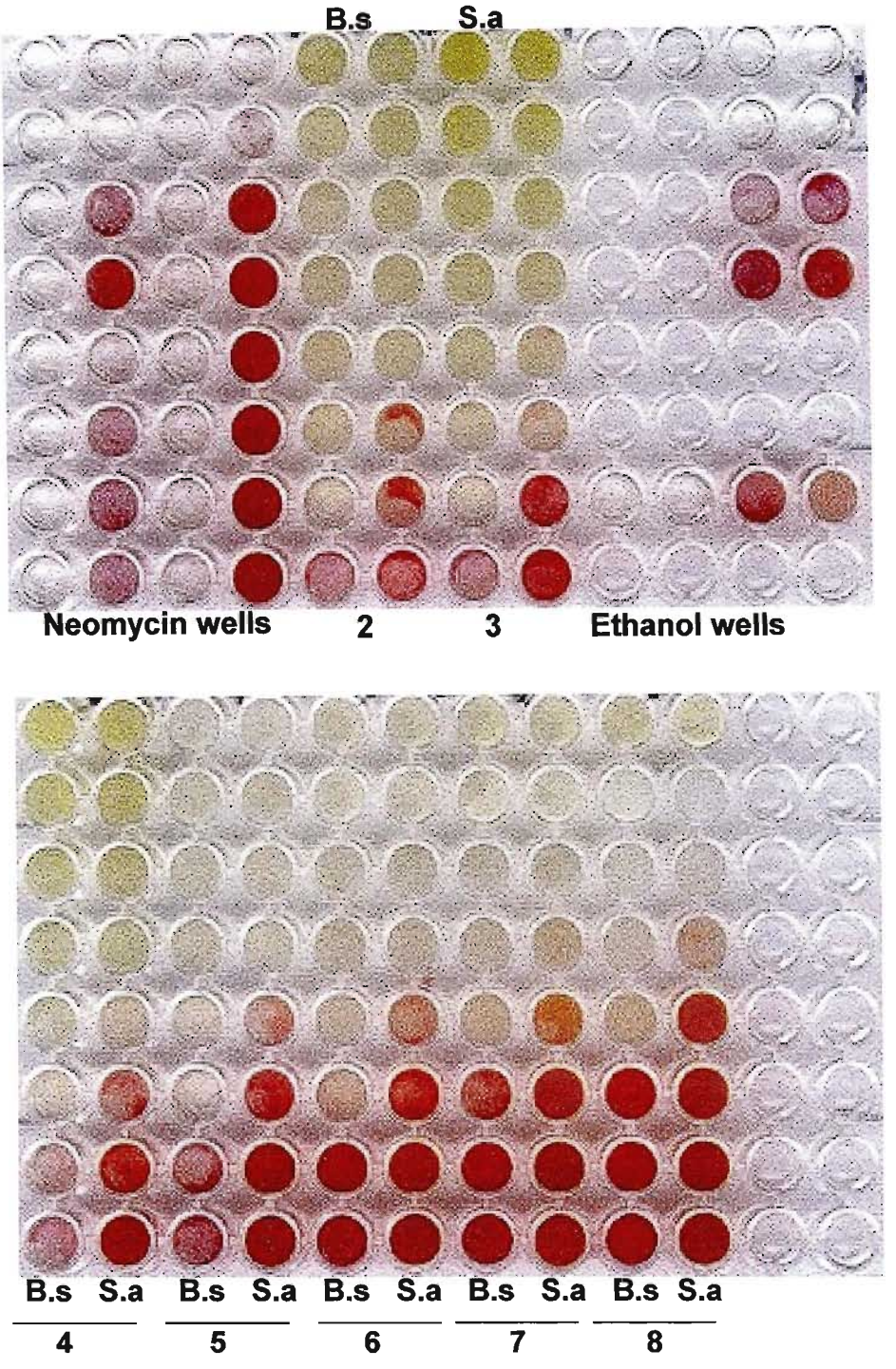


Figure 7.3: Results from the minimum inhibitory concentration (MIC) microplate dilution bioassay showing anti-bacterial activity against *B. subtilis* (B. s) and *S. aureus* (S. a) from fractions collected during the second purification of *E. punctulatus* using VLC. The numbers refer to the fractions detailed in Table 7.3.

Table 7.4: COX-1 and COX-2 anti-inflammatory activity of the Fractions collected from the second VLC chromatography column during the purification of the *E. punctulatus* hexane extract

Solvent system ratio	Inhibition (%)	
Toluene : ethyl acetate (%)	COX-1	COX-2
100 : 0	5	-
80 : 20	96	52
70 : 30	93	60
60 : 40	90	58
50 : 50	88	72
45 : 55	90	70
40 : 60	93	62
30 : 70	91	66
25 : 75	97	54
20 : 80	86	49
100% methanol	76	52
Indomethacin (20 μM)	65	
Indomethacin (200 μM)		38
Nimesulide (200 μM)		48

STEP 3: Gas chromatography (GC analysis)

Twenty μl of the fractions which showed high anti-bacterial and anti-inflammatory activity were pipetted into small brown bottles and dried in a stream of air (Table 7.5). Dried extracts were send to Dr. P. Boshoff at Cape Technikon, South Africa for

further analysis of essential oils. The essential oils of *E. punctulatus* were analysed by GC-Mass Spectroscopy.

Table 7.5: Fractions collected using VLC and analysed for essential oils

Sample	Solvent system ratio Toluene : ethyl acetate (%)	Extract dry weight (mg)
2	80 : 20	10
3	70 : 30	13
4	60 : 40	11
5	50 : 50	13
6	45 : 55	9
7	40 : 60	13
8	30 : 70	3
9	Crude extract	3

A number of compounds were detected from the fractions analysed for essential oils (Table 7.6). Further identification of the main peaks from each fraction was requested but the results were not obtained.

7.4 Discussion

7.4.1 Bioassays used in testing for essential oils found in *E. punctulatus* and their limitations

A large number of studies have been performed concerning the anti-microbial activity

Table 7.6: Compounds detected from Fractions collected by VLC during the purification of *E. punctulatus* and analysed using GC/MS

Fractions	Detection time (min)	Detected compounds
2 to 5	4.893	Azulene (CAS); Biocyl Cyclopentacycloheptene; Bicycl (5.3.0) decapentaene; Azunamic; Biocyclo 95.3.0)-1,3,5.7.9-decapentaene
	5.447	3-Hexyne –2,5-diol; 2,5-dimethyl; 2,-Dimethyl-3hexyn-2,5-diol; Kemitracin-50; Dimethylhexynediol; Tetramethy
	6.341	s-(+)-5(1-Hydroxyl-1-methylethyl)-2-methyl-2-cyclohexen-1-one; 2-Cyclohexen-1-one; 5-(1-hydroxy-1-methylethyl)-2-methyl
	6.747	1,2,4- Trihydroxy-p-menthane, 1,2,4-Cyclohexanetriol; 1-methyl-4-(methylethyl),
	6.876	Benzene; 1,1-(1,2-ethanediyl)bis-(CAS); Dibenzyl; Debenzil; Bibenzyl; Diphenylethylene; Sym-Diphenylethane,1,2-Diphen
	7.567	Benzenemethanol; 3,4,5-Trimethoxy-(CAS); Benzyl alcohol; 3,4,5-Trimethoxybenzy1 alcohol
6	7.886	Nordavanone; Davana oil
	7.988	Caryophyllene
	8.006	(-) Spathulenol (CAS) 1H-Cycloprop [e] azulen-7-ol; decahydro-1, 1,7-trimethyl-4-methylene
	8.218	2-Cyclopenten-1-one;2-(2-butenyl)-4-hydroxy-3-methyl, cinerolone, CIS-cinerolone, cinerolon
7	6.742	1,2,4-Trihydroxy-p-menthane; 1,2,4-cyclohexanetriol; 1-methyl-4-(1-methylethyl)
	7.968	4,4-Dimethyltricyclo [6.3.2.0 (2,5) trideca-8-ene-1-o-1
8	6.701	1,2,4-Trihydroxy-p-methane; 1,2,4-cyclohexanetriol; 1-methyl-4-(1-methyl)
	6.867	Benzene;1,1'-(1,2-ethanediyl)bi-(CAS);dibenzy1; diphenylethylene; sym-diphenylethane; 1,2-diphen
	14.442	Brucine

of essential oils. Testing and evaluation of the anti-bacterial activity of essential oils is difficult because of their volatility, water in solubility and complexity. Factors important when testing essential oils include the assay technique, the growth medium, the micro-organisms used and the oil composition itself. Usually, factors such as solubility and rate of vaporization of the oils are difficult to monitor and may lead to erroneous results (JANSSEN, SCHEFFER and SVENDSEN, 1986; HILI, EVANS and VENESS, 1997).

In this study, disc-diffusion, bioautographic and MIC bioassays were used to determine the anti-bacterial activity of extracts containing essential oils of *E. punctulatus* which is reported to be highly priced in the fragrance industry (WEBBER, MAGWA and VAN STADEN, 2000 A). The disc-diffusion involved the incorporation of essential oil into an agar media inoculated with the test bacteria. The method is highly dependent on water solubility and the ability of the test components to diffuse through agar. This implies that compounds of lower water solubility could show less activity in this assay than in other situations where water may not be involved. A TLC bioautographic technique was also used to track activity through the separation process. This assay is sensitive and gives accurate localization of active compounds. However, its limitation is that it does not distinguish between bacteriocidal and bacteriostatic metabolites. A liquid broth assay (e.g MIC assay) needs to be employed for efficient results. For the MIC bioassay used in this study, extracts tested for anti-bacterial activity were dissolved in ethanol. Addition of dispersing agents and solvents which maximise solubility of essential oil is needed to aid methods such as agar diffusion and serial dilution (MIC). However, emulsifiers and solvents such as ethanol are reported to have direct action on micro-organisms

by influencing the anti-microbial activity essential oils. These may cause changes in cell membrane permeability, thus leading to changes in bacterial sensitivity (GIBBONS and GRAY, 1991; REMMAL, BOUCHIKHI, RHAYOU and ETTAYEBI, 1993; HILI, EVANS and VENESS, 1997; GRIFFIN, WYLLIE, MARKHAM and LEACH, 1999).

7.4.2 Compounds found in *E. punctulatus*

The GC analysis of *E. punctulatus* essential oil resulted in the detection of a number of compounds (Table 7.6). It is interesting to note that azulene compounds were amongst the detected compounds. This confirmed findings by WEBBER, MAGWA and VAN STADEN (2000 A) who reported that the blue colour expressed by the essential oil of *E. punctulatus* is due to the presence of azulenic compounds which are important constituents in perfume manufacturing. Numerous compounds detected during the GC/MS analysis of *E. punctulatus* essential oil (Table 7.6) indicated the complexity of essential oils (LEUNG and FOSTER, 1996). The complexity is attributable to a variety of complex organic compounds comprising the oil. This makes it difficult to isolate the active individual compounds, which could also be synergistically combined/mixed for exerting medicinal properties detected from *E. punctulatus.*

Some of the *E. punctulatus* compounds detected during this study were the same as those detected by WEBBER, MAGWA and VAN STADEN (unpublished). e.g benzene and 2-methyl. However, a number of the detected compounds were different. This could be due to different extraction methods employed. Alternatively, different geographical locations of the analysed plant species may have contributed

to these differences. The plant analysed in this study grew wildly at Qacha's Nek district in Lesotho while the one studied by WEBBER, MAGWA and VAN STADEN (2000 A) was cultivated at the University of Fort Hare in South Africa. Genetic variation (genotype) can cause qualitative differences in different samples, while environmental factors can also affect qualitative results (TAYLOR, RABE, McGRAW, JÄGER and VAN STADEN, 2001).

Chromatography analysis of a methanol extract obtained from the extraction of aerial parts of *E. punctulatus* using a Soxhlet apparatus resulted in the isolation of the slightly bitter 8-isobutyl oxycumambrin-A and intensely bitter cummabrin-A and L-Z-O-methylchiroinositol (DAVIES-COLEMAN, ROBIN, ENGLISH and RIVETT, 1992). The bitter taste and chocking effects are common characteristics of *E. punctulatus.* According to the interviewed traditional healers and herbalists from Lesotho (CHAPTER 3), these properties contribute to medicinal activity of this plant when it is used to fumigate huts against lice.

7.4.3 Compounds found in other species of *Eriocephalus*

Phytochemical screening of other species of *Eriocephalus* such as *E. africanus* tested positive for tannins, flavonoids and triterpene steroids. Tannins are commonly found in high concentrations in plant extracts. They are reported to give false-positive results in many biological assays due to their tendency to precipitate proteins through multipoint hydrogen bonding and by reacting with flavonoids (SILVA, LEE and KINGHORN, 1998). Methanol and petroleum ether extracts made from stems and roots of this plant inhibited the growth of *S. aureus* while petroleum

ether and chloroform extracts made from the leaves showed anti-fungal activity against *Candida albicans* (SALIE, EAGLES and LENG, 1996).

7.4.4 Anti-microbial and anti-inflammatory activity of essential oils found in other plant species

The use of essential oils as anti-microbial agents have been reported by numerous researches. HILI, EVANS and VENESS (1997) reported that essential oils of the female and hermaphrodite *Thymus bacticus* Boiss (Thyme oil) showed marked activity against some Gram-positive and Gram-negative bacteria and yeasts. The activity was greater with essential oils which contained larger amounts of geraniol. Also, essential oils from *Melaleuca alternifolia* (tea tree), *Mentha piperita* (peppermint) and *Salvia officinalis* (sage) are reported to potentiate anti-microbial properties against facultative anaerobic oral bacteria. GALI-MUHTASIB, HILAN and KHATER (2000) further indicated that cineole components of the essential oils of *Salvia libanotica* possess anti-inflammatory properties. A decoction made from leaves of this plant is used for the relief of headaches, stomachaches and abdominal pain.

CHAPTER 8

ISOLATION OF ANTI-INFLAMMATORY COMPOUND(S) FROM *ASPARAGUS MICRORAPHIS*

8.1 Introduction

Traditional healers and herbalists from Lesotho reported that the decoction made from roots of *A. microraphis* is drunk as a cure for rheumatism and menstrual pains (CHAPTER 3). The plant is used by the southern Sotho and Xhosa people in treatment of venereal diseases. Extracts made from the whole plant can be taken orally or applied to patients with eruptions and gonorrhoea. The powdered plant material is placed in incisions on the bodies of young girls while at the initiation school (GUILLARMOND, 1971; POOLEY, 1998).

Other species of *Asparagus* are reported to be used in traditional remedies. Germans use root of *A. officinalis* L. in diuretic galenical preparations and fibers isolated from the leaves are reported to have mutagen-adsorbing (cancer-preventing) properties. Root extracts are also used for inflammatory diseases of the urinary tract and prevention of renal gravel. In South Africa, the diuretic root and fruits of this plant are used for "paralysis" of the bladder and heart disease. Indians use similar extracts for cardiac dropsy, rheumatism and chronic gout (WATT and BREYER-BRANDWIJK, 1962; LEUNG and FOSTER, 1996). Roots of *A. filicinus* are

considered as a vermifuge and taeniafuge in Vietnam. The plant is also given to cure cholera and acts as a powerful diuretic agent (SHARMA and THAKUR, 1994).

8.2 Aims

The aim of this part of the study was to extract, isolate and identify the active ant-inflammatory compound(s) from *A. microraphis*.

8.3 Materials, Methods and Results

8.3.1 Plant material

Leaves of *A. microraphis* were collected from Qacha's Nek district in Lesotho in September 1998. The plant material was dried at 50 °C, coarsely ground and stored at 10 °C. The total dry weight was 2.6 kg.

8.3.2 Preliminary testing of bulk plant material for anti-inflammatory activity

Serial extraction of *A. microraphis* using a Soxhlet apparatus

To determine the best solvent to use for bulk extraction, dried and ground *A. microraphis* leaf material (8 g) was serially extracted in a Soxhlet apparatus using hexane, followed by the same amount of dichloromethane, methanol and water as described in Section 6.3.2. Dried extracts were redissolved at 8 mg ml^{-1} (organic solvent extracts) and 1 mg ml^{-1} (water extracts) respectively. These were tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

Dichloromethane extract yielded high anti-inflammatory activity compared to hexane, methanol and water extracts which exhibited lower activity (Table 8.1). Dichloromethane was thus used for the bulk extraction of *A. microraphis* leaves.

Table 8.1: COX-1 anti-inflammatory activity of *A. microraphis* leaves extracted serially with hexane, dichloromethane, methanol and water using a Soxhlet apparatus

Extracting solvents	Inhibition (%)
Hexane	64
Dichloromethane	95
Methanol	65
Water	66
Indomethacin (20 μM)	67

8.3.3 Bulk extraction of *A. microraphis*

Dried, ground leaves of *A. microraphis* were extracted with dichloromethane using a Soxhlet apparatus. The amount of plant material that could be loaded into the Soxhlet vessel was 500 g. Therefore, four sets of extractions of the plant material were made. Three litres of dichloromethane was used for each extraction which ran for 6 h. The extracts obtained from each extraction were combined and dried *in vacuo*. The dry weight of the crude extract was 19.4 g. One gram of the extract was redissolved in ethanol at 8 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

The crude extract obtained from the extraction of *A. microraphis* using a Soxhlet apparatus still exhibited high anti-inflammatory activity (80%) as was noted from the preliminary tested bulk plant material. The indomethacin standard (20 µM) had 65% inhibitory activity. However, the omission of the extraction with hexane prior to dichloromethane resulted in a slight decrease in activity of the dichloromethane extract.

8.3.4 Isolation of anti-inflammatory compound(s) from *A. microraphis*

STEP 1: Vacuum liquid column chromatography (VLC)

The dichloromethane extract obtained from the Soxhlet extraction (Section 8.3.3) was purified further for the isolation of the active anti-inflammatory compound(s). A number of TLC plates were developed (Section 2.4) in order to determine the best solvent system to used to run VLC. Hexane:ethyl acetate (8:2) resulted in the best separation.

Four silica columns were prepared as described in Section 6.3.5 and 4.75 g of the extract was run through each column using a serial gradient of 400 ml of hexane:ethyl acetate as shown in Table 8.2. Fractions collected from the four columns where the same ratio of solvent was used were combined and dried *in vacuo*. Dried extracts were redissolved in ethanol at 8 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

Fractions collected between hexane:ethyl acetate 100:0 and 80:20 did not have any anti-inflammatory activity. High activity was noted for fractions collected between

hexane:ethyl acetate 60:40 to 30:70. These were combined, dried in a stream of air and resulted in a residue dry weight of 3.10 g.

Table 8.2: COX-1 anti-inflammatory activity of the fractions collected by VLC during the purification of dichloromethane extract obtained from *A. microraphis* leaves. The results highlighted in bold show fractions which were combined and further purified

Hexane : ethyl acetate	Weight (mg)	Inhibition (%)
100 : 0	3743	0
80 : 20	978.6	0
70 : 30	1495	75
60 : 40	**1063**	**93**
50 : 50	**779.4**	**94**
40 : 60	**632.4**	**90**
30 : 70	**628.2**	**86**
100% methanol	814.7	52
Indomethacin (20 µM)		67

STEP 2: Silica gel column chromatography

A number of TLC plates were developed (Section 2.4) to determine the best solvent system to run the silica gel column. Toluene:ethyl acetate (6:4) resulted in the best separation. The extract obtained from STEP 1 was further purified using silica gel column chromatography.

Silica gel (200 g, particle size 0.04-0.063 mm, Mesh ASTM) was suspended in minimal volume of toluene:ethyl acetate (6:4), stirred thoroughly to minimise air bubbles and quickly poured into the column (3.5 cm internal diameter). The extract was redissolved in 5 ml toluene:ethyl acetate (6:4) and slowly added to the column. The solvent was allowed to run out of the column until the extract was almost at the surface of the silica gel. Five ml of the solvent was gradually added and allowed to run through the silica column until the solvent above the silica was clear. Lots of the solvent was continuously added to the column. The extract formed bands along the column and fractions were collected at five minutes intervals. These fractions were spotted onto a TLC plate and developed in toluene:ethyl acetate (6:4) as described in Section 2.4. Fractions that showed similar spots were combined and dried *in vacuo*. The column was finally washed with 100% methanol. Dried extracts were redissolved in ethanol at 4 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

Fractions 1 - 22 showed low anti-inflammatory activity. High activity was recorded from fractions 23 - 245. However, this separation method did not prove to be appropriate for the isolation of the active compound(s) from *A. microraphis* since the activity was detected from a large range of the collected fractions showing inefficient separation of the extract (Table 8.3). The active fractions (23 - 245) were combined and dried *in vacuo*. The resulting dry extract was 2.003 g.

STEP 3: Chromatron

A. microraphis residue (2.003 g) obtained from the combined fractions 23 - 245 (STEP 2), was further purified using a chromatron. The maximum amount of extract

Table 8.3: COX-1 anti-inflammatory activity of the fractions collected by Silica column chromatography during the purification of dichloromethane extract obtained from *A. microraphis* leaves. The results highlighted in bold show the fractions which were combined and further purified

Fractions combined	Weight (mg)	Inhibition (%)
1-8	685	13
9-22	410	52
23-98	**1307**	**100**
99-162	**460**	**81**
163-245	**236**	**92**
100% methanol	8180	0
Indomethancin (20 μM)		64

that could be loaded onto the chromatron silica plate (2 mm thick) was 500 mg. Therefore, the extract was divided into four portions and each run on the chromatron. Each of the four extracts was redissolved in 5 ml hexane:ethyl acetate (1:1) and pipetted at the centre of the spinning chromatron silica plate. The plate was developed with 100 ml hexane:ethyl acetate at different ratio (Table 8.4). The extract separated into bands which were viewed under UV light at 254 and 366 nm. These were collected as fractions. The collected fractions were spotted on TLC plates and developed using toluene:ethyl acetate (6:4). Fractions that showed similar spots were combined and dried *in vacuo*.

Dried fractions were redissolved in ethanol at 1, 0.5 and 0.125 mg ml^{-1} and tested in COX-1 and COX-2 anti-inflammatory bioassays (Section 2.3.1 and 2.3.3).

The highest activity at 1 and 0.5 mg ml^{-1} for both COX-1 and COX-2 anti-inflammatory bioassays was recorded from the extract obtained from combining fractions 7-12. This was also the largest fraction (372.5 mg). Fractions 1 - 6 and 13 - 7 resulted in high activity at 1 mg ml^{-1} for COX-1 and slightly lower activity for COX-2 bioassay. However, these were small fractions (less than 200 mg) (Table 8.4).

STEP 4: High Performance Liquid Chromatography (HPLC)

Dried residue obtained from combined fractions 7-12 collected from chromatron (STEP 3) was further purified using HPLC as described in Section 6.3.5 (STEP 5).

A small amount of the extract (10 mg) was redissolved in 600 µl of HPLC grade methanol and filtered through a Millipore solvent filter (GV type, pore size 0.2 µM). Hundred µl of the extract was injected into the HPLC and run using the programmes shown in Table 8.5.

The initial fractions collected from the HPLC using programme 1 (Table 8.5) exhibited high anti-inflammatory activity from fractions collected between 18 – 55 minutes (Table 8.6). The programme could not be used for the final analysis of the extract because there was no consistency of the activity of the fractions collected from the three repeated runs. Also, fractions exhibiting activity were collected even where peaks were not detected at wavelengths 210, 305 and 400 nm by the scanner.

Table 8.4: COX-1 and COX-2 anti-inflammatory activity of the fractions collected by chromatron during the purification of dichloromethane extract obtained from *A. microraphis* leaves

Solvent ratio Hexane: ethyl acetate (%)	**Fractions combined**	**Weight (mg)**	**Inhibition (%)**			
			COX-1			**COX-2**
			1 mg ml^{-1}	**0.5 mg ml^{-1}**	**0.125 mg ml^{-1}**	**1 mg ml^{-1}**
6 : 4	1-6	191.8	80	58	21	69
	7-12	**372.5**	**83**	**73**	**36**	**77**
	13-20	193	81	62	24	72
	21-32	154.6	65	43	19	46
5.5 : 4.5	33-40	177.3	79	46	16	43
	41-48	148.8	74	49	10	61
	49-58	141.9	76	50	12	53
5 : 5	59-77	111.4	70	13	6	38
Methanol wash		8231.5	44	22	2	33
Indomethacin (20 µM)			67	60	62	
Indomethacin (200 µM)						40
Nimesulide (200 µM)						56

The extract was then run using programme 2 (Table 8.5) but very low activity was detected from the fractions collected at methanol wash between 49 – 53 minutes

(Table 8.6). These fractions were collected where the main peak was detected at 210 nm. However, the peak was not sharp and smooth indicating the impurity of the extract. The programme was run several times and fractions collected between 49 - 53 minutes were combined and dried in a stream of air (dry weight 10 mg). The extract was run through HPLC using programme 3 (Table 8.5). This programme was used in an attempt to achieve good separation of the extract. Low activity was noted from fractions collected between 23 – 27 minutes (Table 8.6). These were combined and dried in a stream of air (dry weight 3 mg). The extract was redissolved in ethanol at 1 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1). This resulted in 46% inhibitory activity.

Table 8.5: Programmes used to run the HPLC for the purification of the extract obtained from a combination of Fractions 7-12 collected from the chromatron during the purification of *A. microraphis* dichloromethane extract

Programme 1		**Programme 2**		**Programme 3**	
Time	**MeOH : H_2O**	**Time**	**MeOH : H_2O**	**Time**	**MeOH : H_2O**
T_0	40 : 60	T_0	20 : 80	T_0	45 : 55
T_{40}	100 : 0	T_{20}	45 : 55	T_{20}	95 : 5
T_{50}	100 : 0	T_{30}	45 : 55	T_{40}	100 : 0
T_{60}	40 : 60	T_{50}	100 : 0	T_{50}	100 : 0
		T_{60}	100 : 0	T_{60}	45 : 0
		T_{70}	20 : 0		

Table 8.6: COX-1 anti-inflammatory activity of fractions collected from HPLC using three different programmes during the purification of *A. microraphis* dichloromethane extract. Results of other collected fractions which did not exhibit any activity have been omitted.

Programme 1		**Programme 2**		**Programme 3**	
Fractions (min)	**Inhibition (%)**	**Fractions (min)**	**Inhibition (%)**	**Fractions (min)**	**Inhibition (%)**
18-33	96	49	4	21	0
45-47	95	50	3	22	0
50-55	90	51	5	23	7
		52	17	24	7
		53	5	25	4
				26	2
				27	3
				28	0
Indomethacin (20 µM)	67		63		63

STEP 5: Thin layer Chromatography

The extract (3 mg) obtained from a combination of the fractions collected between 23 – 27 minutes (STEP 4) was developed on a TLC plate. TLC plate was developed in order to determine the extend of the purity of the extract based on number of spots which separated from the extract. The extract was redissolved in methanol at 50

mg ml^{-1}, strip loaded on the TLC plate and developed in hexane:ethyl acetate (7:3). Photographs were taken under UV light at 254 and 366 nm. A strip of the plate was cut off and the cut piece was stained with anisaldehyde and photographed. Spots that separated on the unstained plate were scraped off, redissolved in hexane:ethyl acetate (7:3) and dried in a stream of air. Dried extracts were redissolved in ethanol at 4 mg ml^{-1} and tested in the COX-1 anti-inflammatory bioassay (Section 2.3.1).

The extract separated into five spots on the TLC plate showing the impurity of the extract (Table 8.7 and Figure 8.1). Four of the spots had high inhibitory activity. When considering that the spots were separated from 3 mg extract, the resulting spots each had a low dry weight and so could not be further isolated.

Table 8.7: COX-1 anti-inflammatory activity of spots scraped from TLC plate

Spot numbers	**R_{fs} values**	**Inhibition (%)**
1	0.01	61
2	0.07	91
3	0.18	72
4	0.26	99
5	0.61	80
Indomethacin		65

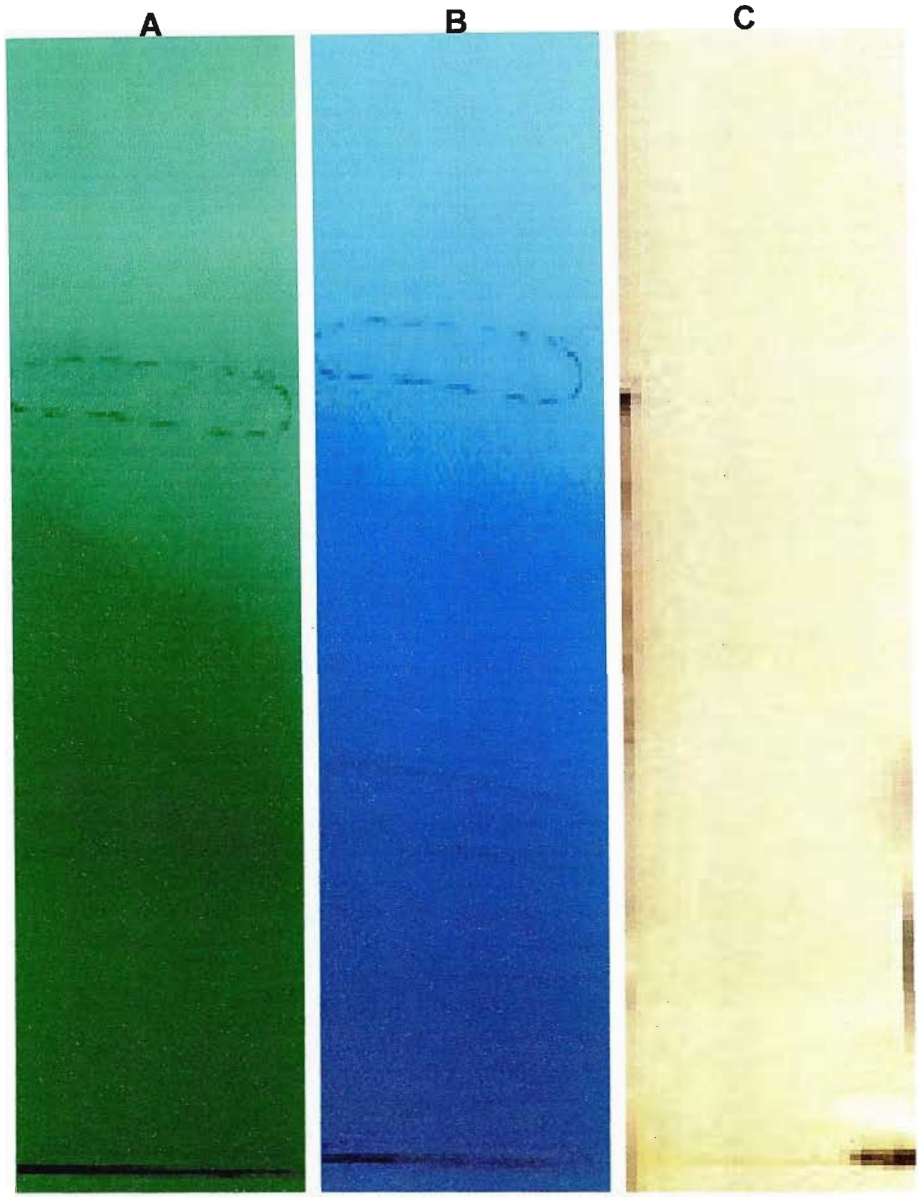


Figure 8.1: TLC fingerprints of the extract obtained from the combination of fractions 18-28 collected from HPLC during the purification of *A. microraphis* dichloromethane extract. TLC plates were viewed at A) 254 nm, B) 366 nm and C) stained with anisaldehyde.

8.4 Discussion

8.4.1 Isolation of the active compound(s) from *A. microraphis*

The isolation and purification of the active anti-inflammatory compound(s) from dichloromethane extract obtained from leaves of *A. microraphis* were not successful. Several techniques used in the isolation process did not prove to be appropriate for the extract obtained from the plant under study. The separation obtained from a silica gel column chromatography (STEP 2) resulted in the detection of anti-inflammatory activity from a wide range of the collected fractions, indicating poor separation (Table 8.3). Further purification of the extract using HPLC also exhibited poor separation regardless of different programmes used (STEP 4).

In the chemistry of natural product, the separation of large or small quantities of complex mixtures is seldom efficiently, rapidly and inexpensively achieved. In most cases, many naturally occurring compounds tend to interfere with the isolation and purification of a desired bioactive plant constituent. The long route from a crude plant extract, containing hundreds of constituents, to a pure compound is very tedious and often requires several separating steps involving various techniques (HOSTETTMANN, 1986; SILVA, LEE and KINGHORN, 1998).

8.4.2 Compounds found in other species of *Asparagus*

Although the isolation of the active anti-inflammatory compound(s) from *A. microraphis* have not been achieved, compounds isolated from other species of

Asparagus give some ideas on some possible compounds that may be found in *A. microraphis*.

Phytochemical investigation of roots of *A. africanus* led to the isolation of anti-protozoal compounds muzanzagenin and (-) -nysol. Isolation and structure elucidation of spirostanosides and oligofurostanoside have also been done from the root methanol extract of this plant (DEBELLA, HASLINGER, KUNERT, MICHL and ABEBE, 1999). Chemical analysis of ethanolic extract obtained from roots of *A. filicinus* showed the presence of two fuorostanosides, filicinoside A and B. Other compounds isolated and characterised from this plant are filicinin-A and B and oligospirostanosides isolated from a root ethanolic extract (SHARMA and THAKUR, 1996).

CHAPTER 9

GENERAL DISCUSSION

Knowledge of medicinal plants by traditional healers and herbalists is significant in primary health care system in many countries, particularly in developing countries where traditional medicine plays a major role in lives of people. Traditional healers and herbalists, together with other people (vendors and gatherers) knowledgeable about utilisation of medicinal plants need to be educated about certain Western diseases which are not likely to be diagnosed by indigenous people (e.g cancer and cardiovascular illness). Conservation of medicinal plants for sustainable use is another aspect which needs to be addressed. These could be achieved by encouraging the cooperation between traditional healers/herbalists and Western doctors through educational workshops, referral of patients between the two parties, acceptance and respect of each other 's skills.

9.1 Screening of medicinal plants for anti-bacterial and anti-inflammatory activity

Services provided by traditional healers and herbalists have been in existence for centuries and their effectiveness are highly respected among people utilising extracts prepared from plants. This stems from the fact that traditional medicine encourages and accepts local beliefs and behaviour related to health matters, a situation which is usually disregarded by Western medicine.

The continuity of utilisation of medicinal plants lead to efficiency of several traditional remedies prepared from plants. The results presented in CHAPTER 3 resulted in a large number of the screened plants giving positive activity with the disc diffusion anti-bacterial bioassay and COX-1 anti-inflammatory bioassay. The results substantiate the use of the tested plants in traditional remedies. Plants which exhibited less or no activity in this study may still have the active compounds but probably in too small amount to fully exert their effects. Alternatively, the active component may be less active due to the fact that most traditional preparations are a mixture of several plant extracts, which may lead to inter/intra synergistic effects of some active principles in plants. Testing of the individual plant extracts rather than mixtures could result in reduced or the disappearance of the activity. Nevertheless, traditional knowledge is a valuable guide in selection of plants from which compounds could be isolated and used in drug development.

9.2 Seasonal variation in medicinal activities and plant part substitution

Seasonal variation in medicinal activities of plants used in traditional remedies is an important factor to be investigated and the findings could enable scientists to encourage and educate traditional healers and herbalists on appropriate collection times and utilisation of aerial rather than underground parts of medicinal plants. This will benefit the healers and the environment since aerial parts are easily replaceable and sustainability of many endangered plant species will be achieved.

The results obtained from the investigation of seasonal variation in anti-bacterial and anti-inflammatory activity of *M. parviflora, E. punctulatus* and *A. microraphis* showed

variation in inhibitory activities of *M. parviflora* and *E. punctulatus* while the anti-inflammatory activity of *A. microraphis* was not affected by different seasons (CHAPTER 4). When considering the fact that most plants are collected from areas far from people utilising them, bulk plant material is usually collected for future use. Seasonal changes in activity of the collected plants may also be influenced by factors resulting from both seasonal and storage effects. Plant parts collected at the time when the activity is thriving may be lost or increased due to storage effects depending on plant species (CHAPTER 5). Cultivation of medicinal plants needs to be encouraged and emphasised. This will enable traditional healers and herbalists to collect plant parts at the appropriate time and perhaps at the time when there is a need to use them. This could also eliminate/minimise effect of storage on the activity of the plant material.

9.3 Effects of storage on inhibitory activity of medicinal plants

The investigation on the effect of storage on anti-bacterial and anti-inflammatory activity of *M. parviflora, E. punctulatus* and *A. microraphis* material and the stored extracts showed that different storage conditions (cold room, room temperature and exposure to high and large changes in temperature in the Botanical Garden) have minimum variation on the extend of effects on medicinal properties of the stored plants. However, the storage form of the plant does influence changes in activity, with the stored plant material retaining activity for a longer period than the stored extracts. TLC fingerprints also provided evidence on changes in composition of the tested extracts with the storage period (CHAPTER 5).

9.4 Isolation of active anti-bacterial and anti-inflammatory compound(s) from medicinal plants

Attempts to isolate the active compound(s) from *M. parviflora, E. punctulatus* and *A. microraphis* (CHAPTER 6, 7 and 8) have been made. Isolation and structure elucidation of the active compound(s) from *M. parviflora* were not successful. However, attempts highlighted the possible loss of activity due to the collection of the plant material from different geographic locations and the synergistic actions of plant components. A large number of compounds detected from the essential oils of *E. punctulatus* indicated the complexity of essential oils, thus making it difficult to isolate the active components but it indicated the presence of variety of compounds which possibly contribute to medicinal properties of plants. Studies on isolation processes require further investigation in order to determine compound(s) responsible for the activity. Failure to isolate the active compound(s) from *A. microraphis* showed constrains in methods employed in the isolation process. Cases like these may cause reluctance from companies involved in drug development to invest in natural product development as it is expensive and time consuming.

9.5 Conclusion

This thesis presents an extensive investigation on the involvement of traditional healers and herbalists in the health care system in Lesotho. The study also provides scientific verification of the efficiency of the tested medicinal plants for their use in traditional remedies. Furthermore, the insight of variation in inhibitory activities of medicinal plants due to seasonal and storage effects have been investigated. Future

research on seasonal and storage factors affecting other medicinal plants need to be investigated.

REFERENCES

AFIFI, F.U. and B. ABU-IRMAILEH (2000). Herbal medicine in Jordan with special emphasis on less commonly used medicinal herbs. *Journal of Ethnopharmacology* **72:** 101-110

AKERELE, O. (1991 A). Medicinal plants:policies and priorities. In: O. AKERELE, V. HEYWOOD and H. SYNGE (eds). **Conservation of Medicinal Plants**. Cambridge University Press, Britain pp 3-11. ISBN 0521392063

AKERELE, O. (1991 B). Registration and utilization of herbal remedies in some countries of east, central and southern Africa. In: K.E. MSHIGENI, M.H.H. NKUNYA, V. FUPI, R.L.A MAHUNNAH and E.N. MSHU (eds). **Proceedings of an International Conference of Expects from Developing Countries on Traditional Medicinal Plants.** Dar es Salaam University Press, Tanzania pp 3-8. ISBN 997660229

ANAND, N. and S. NIYANAND (1984). Integrated approach to development of new drugs from plants and indigenous remedies. In: P. KROGSGAARD-LARSEN, S. BROGGER-CHISTENSEN and P. KOFOD (eds). **Natural Products and Drug Development.** Munkggaard, Copehagen pp 28-91. ISBN 87-16-09563-4

ANDREWS, F.W. (1952). **The Flowering Plants of the Anglo-Egyptian Sudan.** Buncle and Co., Ltd, Arbroath, Scotland pp 234. ISBN 9.81

BAGALE, M., B.K. NOAMESI and E. DAGNE (1991). Chemical and pharmacological studies of marketed traditional drugs. In: K.E. MSHIGENI, M.H.H. NKUNYA, V. FUPI, R.L.A MAHUNNAH and E.N. MSHU (eds). **Proceedings of an International Conference of Expects from Developing Countries on Traditional Medicinal Plants.** Dar es Salaam University Press, Tanzania pp 21-27. ISBN 997660229

BALANDRIN, M.F, A.D. KINGHORN and N.R. FARNSWORTH (1993). Plant-derived natural products in drug discovery and development. In: A.D. KINGHORN and M.F. BALANDRIN (eds). **Human Medicinal Agents from Plants.** American Chemical Society, Washington DC pp 2-12. ISBN O-8412-2705-5

BANQUAR, S.R. (1993). The role of traditional medicine in rural medicine. In: I. SINDINGA, C. NYAIGATTI-CHACHA and M.P. KANUNAH (eds). **Traditional Medicine in Africa.** English Press Ltd, Nairobi pp 34-39. ISBN 9966-46548-0

BARITAUX, O., H. RICHARD, J. TOUCHE and M. DERBESY (1992). Effects of drying and storage of herbs and spices on the essential oil, part I basil, *Ocimum basilicum* L. *Flavour and Fragrance Journal* **7:** 267-271

BEER, L. and J. HOWIE (1985). **Growing Hibiscus**. Kangaroo Press, Hong Kong pp 88. ISBN 0-86417-045-9

BILLETER, M., B. MEIER and O. STICHER (1990). 8-Hydroxyflavonoid glucuronides from *Malva sylvestris*. *Journal Phytochemistry* **8:** 464 - 469

BRANTNER, A. and A. GREIN (1994). Antibacterial activity of plant extracts used externally in traditional medicine. *Journal of Ethnopharmacology* **44:** 35-40

BYE, S.N. and M.F. DUTTON (1991). The inappropriate use of traditional medicines in South Africa. *Journal of Ethnopharmacology* **34:** 253-259

CHALCHAT, J.; GARRY and MUHAYIMANA (1995). Essential oil of *Tagetes minuta* from Rwanda and France: chemical composition according to harvesting location, growth stage and part of plant extracted. *Journal of Essential oil Research* **7:** 375-386

CHRISTENSEN, L.P. and L.P. LAM (1990). Acetylenes and related compounds in Cynareae. *Journal of Phytochemistry* **29:** 2753-2785

CHU, D.T.W. and L.L. SHEN (1995). Quinolones:synthetic antibacterial agents. In: P.A. HUNTER, G.K. DARBY and N.J. RUSSELL (eds). **Fifty Years of Antimicrobial:Past Perspectives and Future Trends.** Society for General Microbiology, Cambridge pp 111-138. ISBN 0-521-48108-2

CIMANGA, K., K. KAMBU, L. TONA, S.ASPERS, T. DE BRUYNE, N. HERMANS, J. TOTTE, L. PIETERS and A.J. VLIETINCK (2002). Correlation between chemical composition and anti-bacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *Journal of Ethnopharmacology* **79:** 213-220

CLAPHAN, A.R., T.G. TUTIN and E.F. WARBURG (1962). **Flora of British Islesls**. Cambridge University Press, Great Britain pp 1269.

COOPER, M.R. and A.W. JOHNSON (1984). **Poisonous Plants in Britain and their Effects on Animals and Man**. Commonwealth Burea of Animal Health, Weybridge, London pp 305. ISBN 011 2425291

CORDELL, G. (1995). Changing strategies in natural products chemistry. *Journal of Phytochemistry* **40:** 1585-1612

COX, P.A. (1994). The ethnobotanical approach to drug discovery:strengths and limitations. In: D.J. CHADWICK and J. MARSH (eds). **Ethnobotany and Search for New Drugs:Cida Foundation Symposium 185.** John Wiley and Sons, New York pp 25-36. ISBN 0 471 95024 6

COX, P.A. (1995). Shaman as scientist:indigenous knowledge system in pharmacology research and conservation. In: K. HOSTETTMAANN, A. MARSTON, M. MAILALLARD and M. HUMBUGER (eds). **Phytochemistry of Plants used in Traditional Medicine.** Clarendon Press, Oxford pp 1-16. ISBN 0 19857775 3

CUNNINGHAM, A.B. (1991). Development of a conservation policy on commercially exploited medicinal plants:A case study from southern Africa. In: O. AKERELE, V. HEYWOOD and H.SYNGE (eds). **Conservation of Medicinal Plants**. Cambridge University Press, Britain pp 337-358. ISBN 0521392063

DAVIES-COLEMAN, M. T., B. ROBIN, R.B. ENGLISH and D.E.A. RIVETT (1992). Bitter guaianolides from *Eriocephalus punctulatus*. *Journal of Phytochemistry* **31:** 2165-2167

DAVIS, G.D., E.J. EISENBRAUN and M. ESSENBERG (1991). Tritium transfer during biosynthesis of cadalene stress compounds in cotton. *Journal Phytochemistry* **30:** 197-199

DEBELLA, A., E. HASLINGER, O. KUNERT, G. MICHL and D. ABEBE (1999). Steroidal saponins from *Asparagus africanus* **51:** 1069-1075

DILIKA, A.F., R.V. NIKOLOVA and T.V. JACOBS (1996). Plants used in circumcision rites of the Xhosa tribe in South Africa. *Acta Horticulture* **42:** 75-77

ELGORASHI, E.E., S.E. DREWES and J. VAN STADEN (2002). Organ-to-organ and seasonal variations of alkaloids from *Crinum moorei*. *South African Journal of Botany* **68:** 111-114

ELMI, A.S. (1991). Research into medicinal plants: The Somali experience. In: K.E. MSHIGENI, M.H.H. NKUNYA, V. FUPI, R.L.A. MAHUNNAH and E.N. MSHIU (eds). **Proceedings of an International Conference of Expects from Developing Countries on Traditional Medicinal Plants.** Dar es Salaam University Press, Tanzania pp27-32. ISBN 997660229

ELOFF, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* **64:** 711-713

ELOFF, J.N. (1999). It is possible to use herbarium specimens to screen for antibacterial components in some plants. *Journal of Ethnopharmacology* **67:** 355-360

ELVIN-LEWIS, M. (2001). Should we be concerned about herbal remedies. *Journal Ethnopharmacology* **75:** 141-164

FARBY, W., P.O. OKEMO and R. ANSORG (1998). Anti-bacterial activity of east African medicinal plants. *Journal of Ethnopharmacology* **60:** 79-84

FARNSWORTH, N.R. (1994). The role of medicinal plants in drug development. In: P. KROGSGAARD-LARSEN, S. BROGGER-CHRISTENSEN and H. KOFOD (eds). **Natural Products and Drug Development.** Munksgaard, Copenhagen pp 17-29. ISBN 87-16-0963-4

FRANZOTTI, E.M., C.V.F. SANTOS, H.M.S.L. RODRIGUES, R.H.V. MOURÃO, M.R. ANDRADE and A.R. ANTONIOLLI (2000). *Journal of Ethnopharmacology* **72:** 273-278

FUGH-BERMAN, A. (2000). Herb-drug interactions. *Lancet* **355:** 134-138

GALI-MUHTASIB, H., C. HILAN and C. KHATER (2000). Traditional uses of *Salvia libanotica* (East Mediterranean sage) and the effects of its essential oils. *Journal of Ethnopharmacology* **71:** 513-520

GIBBONS, S. and A. I. GRAY (1991). Isolation by planar chromatography. In: R.J.P. CANNELL (ed). **Natural Products Isolation**. Humana Press Inc., Totowa, NJ. ISBN 0-89603-362-7

GRIFFIN, S.G., S.G. WYLLIE, J.L. MARKHAM and D.N. LEACH (1999). The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. *Flavour and Fragrance Journal* **14:** 322-332

GRIGGS, B. (1981). **Green Pharmacy : A History of Herbal Medicine.** Jill Norman and Hobhouse, London pp 379. ISBN 0-96908-64-7

GRIGGS, J.K., N.P. MANANDHAR, G.H.N. TOWERS and R.S.L. TAYLOR (2001). The effects of storage on the biological activity of medicinal plants from Nepal. *Journal of Ethnopharmacology* **77:** 247-252

GUILLARMOD, A.J. (1971). **Flora of Lesotho.** Verlag von J. Cramer, Germany pp 474.

GUILLET, G., D. CHAURET and J.T ARNASON (1997). Phytotoxic polyacetylenes from *viguiera annua* and adaptations of a chrysomelid beetle, *Zygogramma continua* feeding on this plant. *Journal of Phytochemistry* **45:** 695-699

HALL, A.H. and H.R. BEHRMAN (1982). Prostaglandin:biosynthesis, metabolism and mechanism of cellular action. In: J.B. LEE (ed). **Prostaglandins**. Amsterman, New York pp 2-7. ISBN 0-444-006 45-1

HAMBURGER, M.O. and G.A. CORDELL (1987). A direct bioautographic TLC assay for compounds possessing anti-bacterial activity. *Journal of Natural Products* **50:** 19-22

HICKOCK, N.J., M. ALOSIO and R.S. BOCKMAN (1985). Prostaglandin endoperoxide synthase from human cell line. In: **Prostaglandin, Leukotrines and Lipoxins.** Plenum Press, New York

HILI, P., C.S. EVANS and R.G. VENESS (1997). Antimicrobial action of essential oils:the effect of dimethylsulphoxide on the activity of cinnamon oil. *Letters in Applied Microbiology* **24:** 269-275

HILLIARD, O.M. and B.L. BURTT (1987). **The Botany of the Southern Natal Drakensberg**. National Botanical Gardens, South Africa pp 253. ISBN 0620 10625 5

HOSTETTMANN, K. (1986). Current level of technology in the separation, isolation and structural analysis of natural products. In: D. BARTON and W.D. OLLIS (eds). **Advances in Medicinal Phytochemistry**. John Libbey and Company Ltd, Great Britain pp 67-76. ISBN 086196 092 0

HUTCHINGS, A. (1992). **Plants used for Some Stress-Related Ailments in Traditional Zulu, Xhosa and Sotho Medicine.** MSc. Thesis, University of Natal, Pietermaritzburg, South Africa

JÄGER, A.K., A. HUTCHINGS and J. VAN STADEN (1996). Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. *Journal of Ethnopharmacology* **52:** 95-100

JANSSEN, A.M., J.J.C. SCHEFFER and A.B. SVENDSEN (1986). Antimicrobial activity of essential oils:976-1986 literature review. Aspects of the test methods. *Plant Medica* **30:** 395-397

KALINOVIC, I. and V. ROZMAN (2000). Infestation of stored medicinal plants and herbal tea by insects and mites. *Plant Protection Science* **36:** 21-22

KERR, K.G. and R. W. LACEY (1995). Why do we still get epidemics?. In: P.A. HUNTER, G.K. DARBY and N.J. FUSSELL (eds). **Fifty years of Antimicrobial:Past Perspectives and Future Trends.** Society for General Microbiology, Cambridge pp 179-204. ISBN 0-521-48108-2

KOFI-TSEKPO, W.M. (1991). The role and use of ethnomedical data in the research on traditional medicines and medicinal plants. In: K.E. MSHIGENI, M.H.H. NKUNYA, V. FUPI, R.L.A. MAHUNNAH and E.N. MSHIU (eds). **Proceedings of an International Conference of Expects from Developing Countries on**

Traditional Medicinal Plants. Dar es Salaam University Press, Tanzania pp 202-208. ISBN 997660229

KUJUMGIEV, A., I. TSVETKOVA, Y. SERKEDJIEVA, V. BANKOVA, R. CHRISTOV and S. POPOV (1999). Anti-bacterial, antifungal and antiviral activity of propolis of different geographic origin. *Journal of Ethnopharmacology* **64:** 235-240

LESOTHO MEDICAL, DENTAL and PHARMACY COUNCIL (1998/99). **Register of Medical Practitioners, Dental Surgeons, Pharmacists and Para-medicals**. Maseru, Lesotho

LEUNG, A.Y. and S. FOSTER (1996). **Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics**. John Wiley & Sons, INC. New York pp 47. ISBN 0-471-04954-9

LI, T.S.C. and D.A. WARDLE (2001). Effects of root drying temperature and moisture content on the levels of active ingredients in *Echinacea* roots. *Journal of Herbs, Spices and Medicinal plants* **8:** 15-21

LIGHT, M.E., L.J. MCGAW, T. RABE, S.G. SPARG, M.B. TAYLOR, D.G. ERASMUS, A.K. JÄGER and J. VAN STADEN (2002). Investigation of the biological activities of *Siphonochilus aethiopicus* and the effect of seasonal senescence. *South African Journal of Botany* **68:** 55-61

LOPES, KATO, ANDRADE, MAIA and YOSHIDA (1997). Circadian and seasonal variation in the essential oil from *Virola surinamensis* leaves. *Phytochemistry* **46:** 689-693

MAHUNNAH, R.L.A. and E.N. MSHIU (1991). Ethnobotany and conservation of medicinal plants. In: K.E. MSHIGENI, M.H.H. NKUNYA, V. FUPI, R.L.A. MAHUNNAH and E.N. MSHIU (eds). **Proceedings of an International Conference of Expects from Developing Countries on Traditional Medicinal Plants.** Dar es Salaam University Press, Tanzania pp 83-86. ISBN 997660229

MAKINDE, AWE and SALAKO (1994). Seasonal variation in the antimalarial activity of *Morinda lucida* on *Plasmodium berghei berghei* in mice. *Fitoterapia* **LXV:** 124-130

MALLAVARAPU, G.R., R.N. KULKARNI, K. BASKARAN, L. RAO and S. RAMESH (1999). Influence of plant growth stage on the essential oil content and composition in davana (*Artemisia pallens* Wall.). *Journal of Agriculture and Food Chemistry* **47:** 254-257

MARSHALL, N.T. (1998). **Searching for a Cure:Conservation of Medicinal Wildlife Resources in East and Southern Africa**. TRAFFIC International Cambridge, UK pp 111 ISBN 185850 1512

MENKOVIC, N., K. ŠAVIKIN-FODULOVIC and K. SAVIN (2000). Chemical composition and seasonal variations in the amount of secondary compounds in *Gentiana lutea* leaves and flowers. *Plant Medica* **66:** 178-180

MITCHELL, J.P., P. AKARASEREENONT, C. THIEMERMANN and R.J. FLOWER (1984). Selectivity of nonsteroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proceeding of the National Academy of Science of the United States of America* **50:** 11691-11693

MSHIU, E.N. (1991). Biotechnology and medicinal plants. In: K.E. MSHIGENI, M.H.H. NKUNYA, V. FUPI, R.L.A. MAHUNNAH and E.N. MSHIU (eds). **Proceedings of an International Conference of Expects from Developing Countries on Traditional Medicinal Plants.** Dar es Salaam University Press, Tanzania pp 133-136. ISBN 997660229

NICK, A., T. RALI and O. STICHER (1995). Biological screening of traditional plants from Papua New Guinea. *Journal of Ethnopharmacology* **49:** 147-156

NIGG, H.N. and D. SEIGLER (1992). **Phytochemical Resources for Medicine and Agriculture.** Plenum Press, New York pp 445. ISBN 0-306-44245-0

NKUNYA, M.H.H., H. WEENEN and D.H. BRAY (1991). Chemical evaluation of Tanzanian medicinal plants for the active constituents as a basis for the medicinal usefulness of the plants. In : K.E. MSHIGENI, M.H.H. NKUNYA, V. FUPI, R.L.A. MAHUNNAH and E.N. MSHIU (eds). **Proceedings of an**

International Conference of Expects from Developing Countries on Traditional Medicinal Plants. Dar es Salaam University Press, Tanzania pp 101-11. ISBN 997660229

NOREEN, Y., T. RINGBOM, P. PERERA, H. DANIELSON and L. BOHLIN (1998). Development of radiochemical cyclooxygenase-1 and –2 *in vitro* assay for identification of natural products as inhibitors of prostaglandin biosynthesis. *Journal of Natural Products* **61:** 2-7

NYAMWAYA, D. (1992). **African Indigenous Medicine. An Anthropological Perspective for Policy Makers and Primary Health Care Managers.** African Medical and Research Foundation, Kenya pp 44. ISBN 9966-874-11-9

PLOTKIN, M.J. (1991). Traditional knowledge of medicinal plants – search for new jungle medicines. In: O. AKERELE, V. HEYWOOD and H. SYNGE (eds). **Conservation of Medicinal Plants**. Cambridge University Press, Britain ISBN 0521392063

POOLEY, E. (1998). **A Field Guide to Wild Flowers KwaZulu – Natal and the Eastern Region**. Natal Flora Publications Trust, South Africa pp 630. ISBN 0-620-21500-3

PRANCE, G.T. (1994). **Ethnobotany and the Search for New Drugs**. John Wiley and Sons, New York pp 1-3. ISBN 0 471 95024 6

PRIETO, J.M., M.C. RECIO, R.M. GINER, S. MANEZ and A. MASSMANIAN (1996). Tropical anti-inflammatory products from *Haplophyllum hispanicum*. *Verlag der Zeitschrift für Naturforschung* **51:** 618-622

PRINCIPE, P.P. (1991). Valuing the biodiversity of medicinal plants. In: O. AKERELE, V. HEYWOOD and H.SYNGE (eds). Conservation of Medicinal Plants. Cambridge University Press, Britain pp 79-124. ISBN 0521392063

RABE, T. and J. VAN STADEN (1997). Anti-bacterial activity of South African plants used for medicinal purposes..*Journal of Ethnopharmacology* **56:** 81-87

RAI, L.K., P. PRASAD and E. SHARMA (2000). Conservation threats to some important medicinal plants of the Sikkim Himalaya. *Biological Conservation* **93:** 27-33

RANG, H.P. and M.M. DALE (1987). **Pharmacology**. Churchhill Livingstone, UK pp 736.

RASOANAIVO, P. and S. RATSIMAMANGA-URVERG (1993). Biological evaluation of plants with reference to Malagasy flora. Napreca, Madagascar pp 9-43

REDL, K., W. BREW, B. DAVIS and R. BAVER (1994). Anti-inflammatory active polyacetylenes from *Biden campylotheca*. *Plant Medica* **60:** 58-62

REMMAL, A., T. BOUCHIKHI, K. RHAYOUR and M. ETTAYEBI (1993). Improved method for the determination of antimicrobial activity of essential oils in agar medium. *Journal of Essential Oil Research* **5:** 179-184

SALIE, F., P.F.K. EAGLES, H.M.J. LENG (1996). Preliminary antimicrobial screening of four South African Asteraceae species. *Journal of Ethnopharmacology* **52:** 27-33

SFORCIN, J.M., A. FERNANDES Jr, C.A.M. LOPES, V. BANKOVA and S.R.C. FUNARI (2000). Seasonal effect on Brazilian propolis anti-bacterial activity. *Jornal of Ethnopharmacology* **73:** 243-249

SHARMA, S.C. and N.K.THAKUR (1994). Furostanosides from *Asparagus filicinus* roots. *Phytochemistry* **36:** 469-471

SHARMA, S.C. and N.K.THAKUR (1996). Oligofurostanosides and oligospirostanosides from roots of *Asparagus filicinus. Phytochemistry* **41:** 599-603

SHELDON, J.W., M.J. BALICK and S.A. LAIRD (1997). **Medicinal Plants:Can Utilization and Conservation Coexist?**. The New York Botanical Garden, U.S.A pp 104. ISBN 0-89327-406-2

SILVA, G.L., I. LEE and A.D. KINGHORN (1998). Special problem with the extraction of plants. In: R.J.P. CANNELL. **Natural Products Isolation**. Humana Press Inc. Totowa, New Jersey pp 343-364 ISBN 0-89603-362

SINDINGA, I. (1995). Traditional medicine in Africa. In: I. SINDINGA, C. NYAIGOTTI-CHACHA and M.P. KANUNAH (eds). **Traditional Medicine in Africa**. East African Educational Publishers Ltd, Nairobi pp 1-11. ISBN 9966-46-548-0

SLUSARENKO, A.J., A.C. LONGLAND and I.M. WHITEHEAD (1989). A convenient, sensitive and rapid assay for anti-bacterial activity of phytoalexins. *Botanica Helvetica* **99/2:** 203-207

SOUTHWELL, I.A. and C.A. BOURKE (2001). Seasonal variation in hypericin content of *Hypericum perforatum* L. (St. John's Wort). *Phytochemistry* **56:** 437-441

SPARG, S.G, J. VAN STADEN and A.K. JÄGER (2000). Efficiency of traditionally used South African plants against schistosomiasis. *Journal Ethnopharmacology* **73:** 209-214

SRIVASTAVA, J., J. LAMBERT and N. VIETMEYER (1996). **Medicinal Plants:An Expanding Role in Development.** The World Bank, Washington D.C pp 18 ISBN 0-8213-3613-4

STEWART, M.J., V. STEENKAMP and ZUCKERMAN (1998). The toxicology of African herbal remedies. *Therapeutic Drug Monitoring* **20:** 510-516

TAKETO, M.M. (1998). Cyclooxygenase-2 inhibitors in tumorigenesis (part II). *Journal of the National Cancer Institute* **90:** 1609-1619

TAYLOR, J.L.S. (1999). **An investigation into the biological and medicinal properties of *Eucomis* species.** PhD. Thesis, University of Natal, Pietermaritzburg, South Africa

TAYLOR, J.L.S., T. RABE, L.J. MCGAW, A.K. JAGER and J. VAN STADEN (2001). Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation* **34:** 23-37

TYISO, S. and R.B. BHAT (1998). Medicinal plants used for child welfare in the Transkei region of the Eastern Cape (South Africa). *Vereinigung für Angewandandte Botanik, Gottingen* **72:** 92-98

VALSARAJ, R., P. PUSHPANGADAN, U.W. SMITT, A. ADSERSEN and U. NYMAN (1997). Antimicrobial screening of selected medicinal plants from India. *Journal of Ethnopharmacology* **58:** 75-83

VANE, J.R. and R.M. BOTTING (1996). Mechanism of action of anti-inflammatory drugs. *Scadanavian Journal of Rheumatology* **25:** 5-21

WAGNER, H. and K. JURIC (1991). Assays for immunomodulation and effects on mediators of inflammation. *Methods in Plant Biochemistry* **6:** 195-217

WALLAART, T.E., N. PRAS, A. BEEKMAN and W.J. QUAX (2000). Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin:proof for the existence of chemotypes. *Plant Medica* **66:** 57-62

WATT, J.M. and M.G. BREYER-BRANDWIJK (1962). **The Medicinal and Poisonous Plants of Southern and Eastern Africa:Being an Account of their Medicinal and Other uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animals.** E and S Livingstone (Ltd), London pp 1457.

WEBBER, L.N., M.L. MAGWA and J. VAN STADEN (1999). Field management practices that do not adversely affect plant vigour and longevity of *Eriocephalus punctulatus*. *South African Journal of Science* **95:** 351-356

WEBBER, L.N., M.L. MAGWA and J. VAN STADEN (2000 A). The effect of clone type and method of transportation on oil yield from vegetative material of *Eriocephalus punctulatus*. *South African Journal of Plant Soil* **17:** 10-14

WEBBER, L.N., M.L. MAGWA and J. VAN STADEN (2000 B). The development of harvesting schedules and post-harvesting management practices relative to oil yield of *Eriocephalus punctulatus*. *South African Journal of Plant Soil* **17:** 15-19

WHITTEN, G. (1997). **Herbal Harvest:Commercial Organic Production of Quality Dried Herbs (2ed).** Agmedia, Australia pp 556. ISBN 1876473045

WILLIAMS, V.L., K. BALKWILL and E.T.E. WITKOWSKI (2000). Unraveling the commercial market for medicinal plants and plant parts on the Witwaterstrand, South Africa. *Economic Botany* **54:** 310-327

WILLIAMS, H.J., I. SATTLER, G. MOYNA, A.I. SCOTT, A.A. BELLS and S. B. VINSON (1995). Diversity in cyclic sesquiterpene production by *Gossypium hirsutum*. *Phytochemistry* **40:** 1633-1636

ZSCHOCKE, S., T. RABE, J.L.S. TAYLOR, A.K. JÄGER and J. VAN STADEN (2000). Plant part substitution – a way to conserve endangered medicinal plants? *Journal of Ethnopharmacology* **71:** 281-292

ZURIER, R.B. (1982). Prostaglandins and inflammations. In: J.B. LEE (ed). **Prostaglandins**. Amsterham, New York pp 91-105. ISBN 0-444-00645-1

APPENDIX 1: Preparation of the cyclooxygenase-1 (COX-1) enzyme

The COX-1 enzyme used in testing of the anti-inflammatory activity of the plants studied in this research was prepared by TAYLOR (1999).

The enzyme was prepared from the fresh sheep seminal vesiscles obtained from 1-2 year old rams from the Cato Ridge Abattor. The vesiscles were frozen in an ice bath. In the laboratory, they were cut into small pieces (64.6 g) and transferred into a mixture of 150 ml 0.1 M potassium phosphate buffer (pH 7.4) and 1 mM ethylene diamine tetra-acetic acid disodium salt (EDTA). This was macerated using an Ultra Thurax at medium speed for 10 min followed by sonication in an ultrasound bath for 5 min.

The homogenate was centrifuged at 4000 g using Avanti J-251 centrifuge for 15 min. The supernatant was decanted and centrifuged at 17000 g for 10 min for the removal of mitochondria. Sixty ml of the supernatant was centrifuged for 1 h (Beckman L7-5S Centrifuge) for the isolation of the microsomes. The microsomal pellet was redissolved in 0.1 M potassium phosphate buffer (pH 7.4). Several bioassays (Section 2.2.2) were conducted for standardization of the optimal time and enzyme concentration for the COX-1 bioassay. Ten µl of the enzyme were pipetted into 1.5 ml eppendorfs and stored at –70 °C until use.

APPENDIX 2: Indomethacin standard curve

The inhibitory activity of indomethacin increased with the increase in concentration. Fifty percent inhibition (IC_{50}) resulted at 1.5 – 2 µM concentration (Figure A) This standard was included in each COX-1 assay at 20 µM and 200 µM for COX-2 assay.

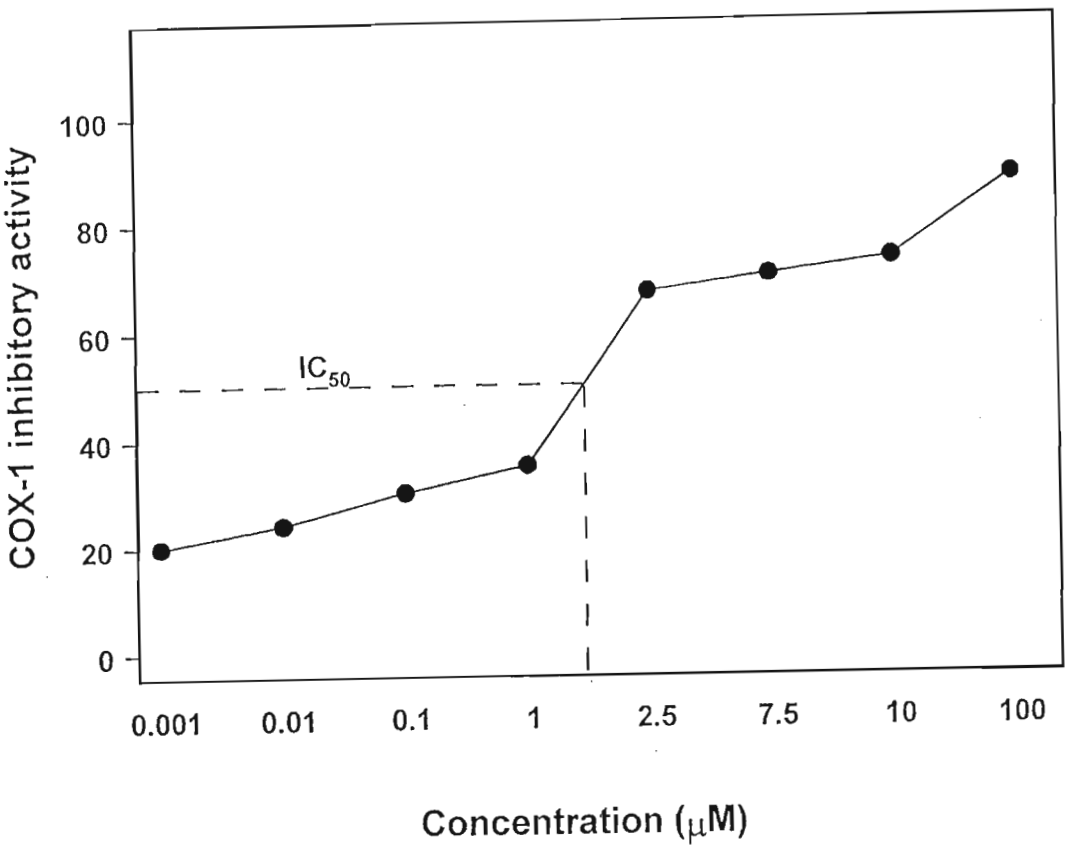


Figure A: Standard curve for inhibition of COX-1 by serially diluted indomethacin

APPENDIX 3: ANISALDEHYDE STAIN

Ingredients

465 ml ethanol

5 ml glacial acetic acid

13 ml concentrated sulphuric acid

13 ml para-anisaldehyde

Methods

Ethanol was cooled in a flask of ice. Other solvents were added to the ethanol following the order shown above. The mixture was kept cold all the time during mixing. The prepared anisaldehyde stain was stored in a shott bottle wrapped with aluminium foil and kept at 10 ^{0}C.

APPENDIX 4: QUESTIONNAIRE

The questionnaire was used as part of PhD research to investigate the role played by traditional healers and herbalists in health care system in Lesotho. Other aspects were to find out about ways in which medicinal plants are obtained, stored and administered in treatment of patients. The questions were directed to traditional healers and herbalists from Mohale's Hoek (lowland area) and Qacha's Nek (highland area) districts in Lesotho.

Structure of the Questionnaire

Name: Approximate age:

Religion: Sex:

1. When did you start healing people?
2. How did you acquire the healing knowledge?
    A. From the ancestors/gods
    B. Trained by other healers
    C. Through experience
    D. Others
3. How long did you train/study before you became a qualified traditional healer/herbalist?
4. Where do you obtain most of medicinal plants which you use to treat your patients?

5. Do you collect plants or have someone to do the collection for you?

6. How do you store your plants for future use?

7. What is the maximum period in which you can store plants before they are used?

8. In which form do you prescribe medication to your patients?

9. How do you prepare medicine from plants?

10. When preparing medicine from plants, which solvents do you use and why?

11. Do you prepare medicine from one plant or a mixture of extracts made from several plants?

12. Do you cultivate some of the plant which you use as source of medicine? Give reasons for your answer.

13. How many patients do you attend to per month?

14. Having prescribed certain medication, do you sometimes do the follow up on your patients' improvement?

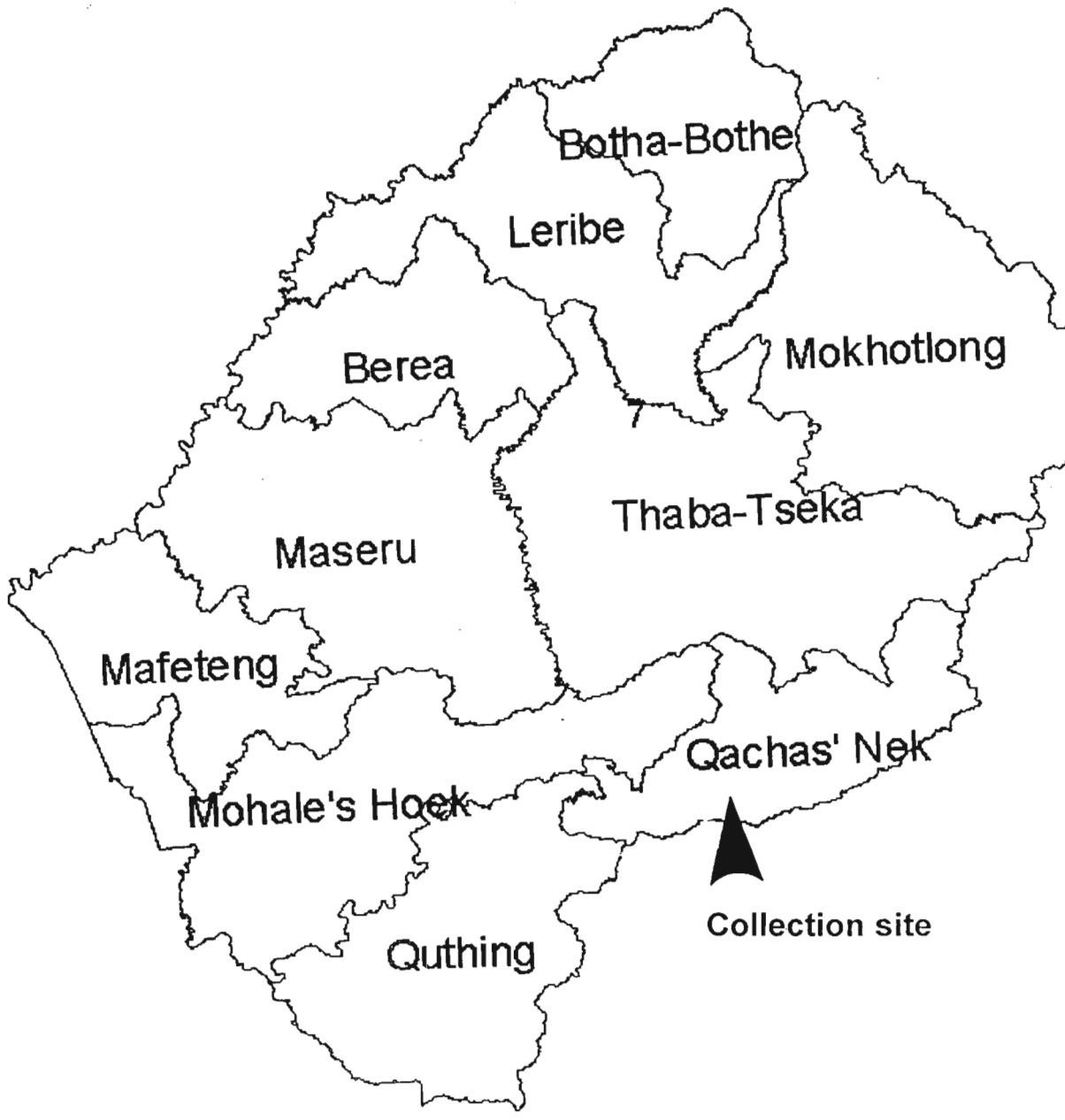
15. What are the most common medical dosage which you recommend for your patients?

16. Has it happened that some of your patients showed negative site effects to the given medicine? If so, what measures did you take?

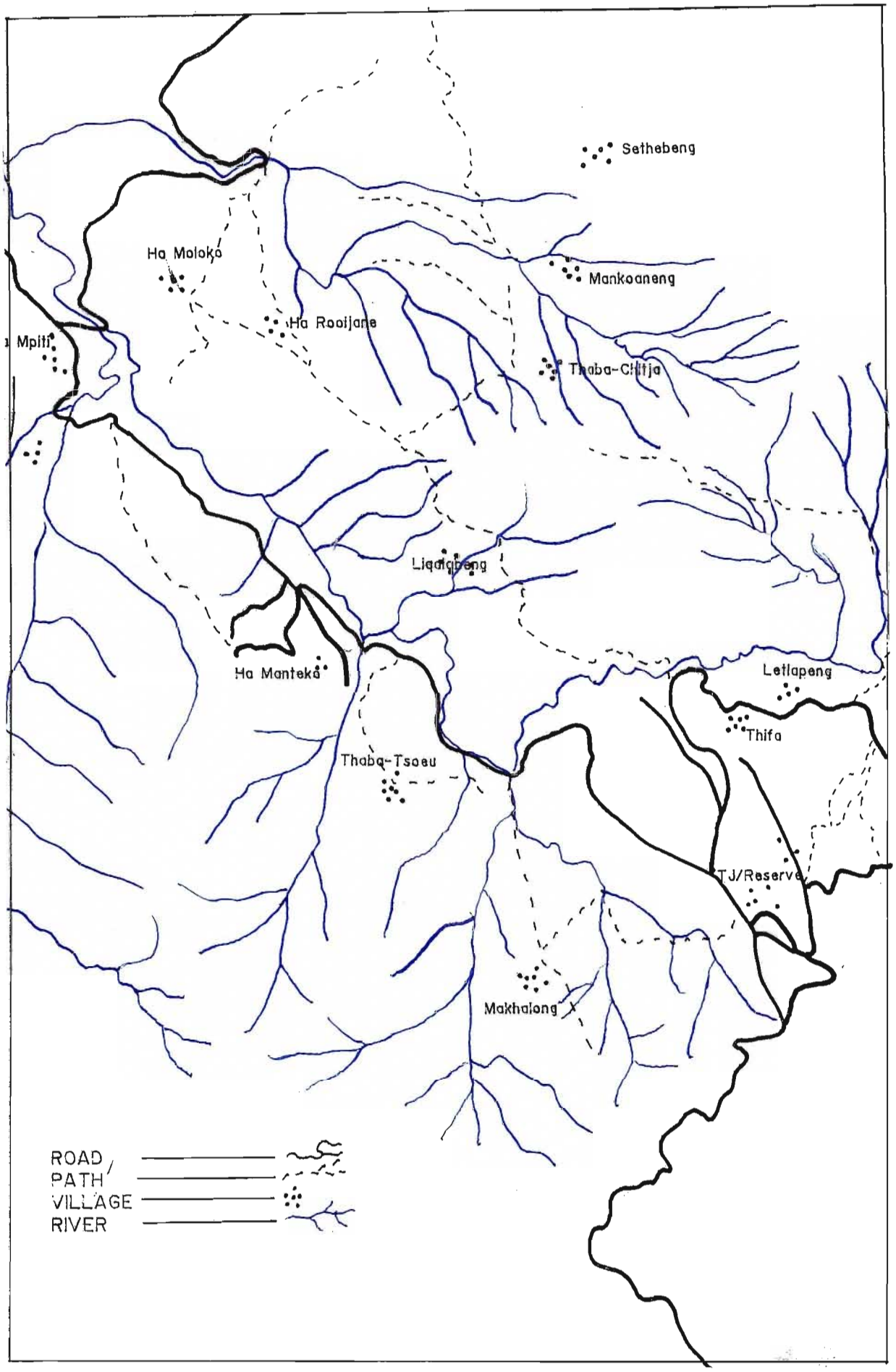
17. Do you sometimes refer your patients to Western Doctors (hospitals or clinics)? If so, during which situations? If not, why?

18. Do you sometimes meet and discuss with other traditional healers/herbalists in order to get help or advice? If so, how often? If not, why?

APPENDIX 5



Map of Lesotho showing area where *M. parviflora* was collected.



Map showing different localities in Qacha's Nek district, Lesotho where *M. parviflora* was collected