TESTING FOR MICROBIOLOGICALLY ACTIVE COMPOUNDS EXTRACTED FROM MEMBERS OF THE FAMILY LAMIACEAE AND OTHER INDIGENOUS PLANTS

by

Prenitha Gurlal

Submitted in partial fulfilment of the requirements for the degree of

Master of Science

In the
Discipline of Microbiology and Plant Pathology
School of Biochemistry, Genetics, Microbiology and Plant Pathology
Faculty of Science and Agriculture
University of KwaZulu-Natal
Pietermaritzburg
South Africa

2005

ABSTRACT

The Labiatae is a large family that occurs worldwide and have species that are adapted to almost all habitats and altitudes. *Plectranthus* is in this family. *Plectranthus* species are beautiful South African shrubs. The genus *Plectranthus* belongs to subfamily Nepetoideae of tribe Ocimeae. The test microorganisms were chosen carefully as each one belonged to a different taxonomic group of fungi and bacteria. Biologically active mono- and sesquiterpenoids are frequently found in many species of *Plectranthus* but there are little published data that directly link the presence of specific compounds in a species with the traditional uses of that species.

Various *Plectranthus spp.* were collected and dried, followed by chemical extraction using various solvents. Dichloromethane extracts of *P. fruticosus* and *P. ecklonii* were screened for antibacterial and antifungal activities using the agar well and trench diffusion methods. It was found that both methods produced inconsistent results. The trench method required a bigger volume of plant extract to be filled into the well, hence, better biological activity was observed with that method. The well method required a smaller volume therefore poor activity was noted with this assay. The size of inhibition zones are dosage dependent. Overall, both plant extracts exhibited antibacterial but no antifungal properties. The pure compound (**1**), 11-Hydroxy-2-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one, isolated from *P. ecklonii* was found to be the same as compound (**10**) which was isolated from *P. lucidus*.

P. hadiensis was extracted using dichloromethane and hexane. The dichloromethane extract proved to contain much higher biological activity than the hexane extract. Three pure compounds, identified as diterpenes, were isolated from the crude dichloromethane extract of *P. hadiensis*. 6,7-Dihydroxyroyleanone-6,7,12-trihydroxy-8,12-abietadiene-11,14-dione (**2**) and 7α-formoxy-6β-hydroxyroyleanone (**3**) exhibited good antibacterial and antifungal activity but not against all the test organisms. The remaining pure compound, 7α-acetoxy-6β-hydroxyroyleanone (**4**), exerted good antifungal activity. This was measured by the inhibition zone which measured up to 14mm when compound **4** was tested against *S. sclerotiorum*. When testing the hexane extract against the *Bacillus* formulations, the pellets that were suspended once in Ringer's solution produced bigger inhibition zones than the pellets that were suspended twice. This could be due to bacterial cells washing out of the suspension.

The dichloromethane extract of *P. praetermissus* proved to be very active against *X. campestris*, producing an inhibition zone of 8 – 20mm. Two pure compounds were isolated from the crude extract and identified as diterpenes. Compound **5,** 20(10→5)-abeo-1(10),6,8,11,13-abietapentaene-11,12,16-triol, and compound **6,** 11,12,15-trihydroxy-20-(10→5)-abeo-abieta-1-(10),6,8,11,13-pentaene are both known compounds which have previously been isolated from *Salvia apiana.*

P. cilatus was extracted with chloroform and tested against various microorganisms for antifungal and antibacterial activities. It showed poor biological activity overall, except against *S. sclerotiorum.*

The crude dichloromethane extract of *P. zuluensis* exhibited good antibacterial activity, which was limited to the Gram negative test organism. The extract produced an inhibition zone of 10-12mm when tested against *X. campestris.* Pure compound **7,** 2-hydroxy-4,6-dimethoxyacetophenone, exerted excellent inhibition against *B. subtilis* and *S. sclerotiorum.* Neither compound **8,** 1,2,4-trimethoxy-5-(2-propenyl)-benzene, nor compound **7,** inhibited *Candida* spp., *F. oxysporum* and *R. solani.*

Two diterpenes were isolated from the aerial plant parts of *P. lucidus* with dichloromethane and their structures elucidated by spectroscopic means. The pure compound **9,** 11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one, showed moderate antifungal activity whereas compound **10,** 11-hydroxy-2-(4-hroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one, showed high antifungal activity against *R. solani, S. sclerotiorum* and *F. oxysporum.* The crude and the pure compounds (formerly isolated from *P. parviflorus*) showed inhibition against *X. campestris.*

The dichloromethane extracts of *P. purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaensis* exhibit similar levels of biological activity when tested against the same test organisms. Poor antibacterial activity was noted with both extracts. However, excellent antifungal activity was depicted when both plant extracts were tested against *F. oxysporum, R. solani* and *S. sclerotiorum.* However, the highest biological activity was noted by *R. solani* which was totally inhibited by both dichloromethane extracts. The pure compound (**11**) isolated from *P. purpuratus* subsp. *purpuratus* was found to have the same chemical structure as compound (**9**) previously isolated from *P. lucidus.*

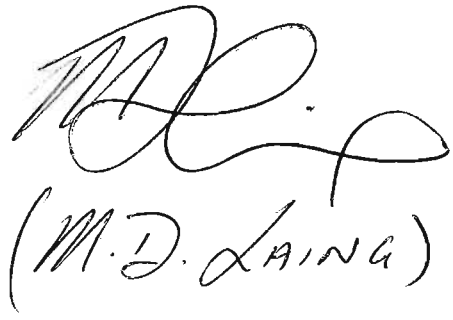
The bioautography assay was used to detect and activity–guide the fractionation of antimicrobial compounds from all the *Plectranthus* spp. tested. The TLC fingerprint showed a zone of clearing around the lower bands of *P. fruticosus* and *P. ecklonii* when the plate was sprayed with a suspension of *B. subtilis*. This result is consistent with the agar well diffusion method. Clear zones were also noted on some bands of the extracts of *P. zuluensis, P. ciliatus, P. hadiensis* and *P. praetermussis*. Clear zones indicate inhibition of growth.

Other plant extracts tested for biological activity were from the family Lamiaceae, however, not of the genus *Plectranthus*. *Persicaria senegalensis, Pycnostachys reticulata* and *Ficus sur* possessed moderate biological activity overall. It is interesting to note that *P. senegalensis* and *F. sur* exert high biological activity against *Candida* spp. This could be useful as herbal remedies for yeast infections.

DECLARATION

I, Prenitha Gurlal, declare that the research reported in this thesis, except where otherwise indicated, is my own original research. No-one else's data, pictures, graphs, writing or phrases lifted from the web, has been duplicated. This thesis has not been submitted for any degree or examination at any other university.

Prenitha Gurlal



ACKNOWLEDGEMENTS

I gratefully acknowledge:

Firstly I would like to thank God for giving me the strength to finish up my project.

My supervisor, Prof. M.D. Laing for his informative support and financial assistance. His guidance and enthusiasm has helped spur me on.

My co-supervisor, Professor S.E. Drewes, for his help and advice. He showed me that chemical structures are informative rather than daunting.

My parents, Satish and Monica Gurlal, for sacrifices made for my education.

My sister, Manisha, and brother Dinesh, for always believing in me.

A heartfelt gratitude to my dear husband Ravin, and my good friends for their understanding and support during this trying time of mine.

Finally, I should like to dedicate this thesis to the memory of my father, who had no tertiary education, but developed a passion for all things scientific, and ensured that I received the education that he had been denied.

In loving memory of my dad,
Mr Satish Gurlal,
because without him,
this would not have been possible.

CONTENTS

Part B: *Plectranthus* (Lamiaceae Family)

CHAPTER 2

Biological activity of *Plectranthus fruticosus* and *Plectranthus ecklonii* against selected bacteria and fungi

CHAPTER 3

Biological activity of the crude extract of *Plectranthus hadiensis* and the pure compounds isolated thereof

CHAPTER 4

Biological activity found in *Plectranthus praetermissus* and *Plectranthus ciliatus* sensu Jacot Guillarmod

CHAPTER 5

Biological activity of the crude extract and pure compounds isolated from *Plectranthus zuluensis* Codd.

CHAPTER 6

Crude extract and isolated pure compounds of *Plectranthus lucidus* Benth. tested for biological activity

CHAPTER 7

Biological activity of *Plectranthus purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaensis* against various microorganisms

CHAPTER 8

Chapter 8.1

A comparison of the biological activity between *Persicaria lapathifolia* and *Persicaria senegalensis*

Chapter 8.2

Examining the biological activity of *Pycnostachys reticulata*

Chapter 8.3

Screening of *Ficus sur* for biological activity

THE OBJECTIVES OF THE THESIS

The aim of this thesis was to examine the biological activity of *Plectranthus* species and, if possible, to isolate pure compounds thereof. From a plant pathology view, the study may provide the small-scale farmer with alternative methods of controlling bacterial and fungal diseases in the crops that they are growing. These natural control methods are both affordable and easy to apply. They also eliminate the dangers of toxic synthetic chemicals that could poison farmers and consumers.

At present, there is a growing movement in many countries to reduce the amount of chemicals being released into the environment. This action has been prompted in part by concern due to the misuse and overuse of pesticides. "*According to a WHO-UNEP report from 1985 the global annual pesticide consumption comprises of 46% herbicides, 31% insecticides and 18% fungicides. Third World countries account for 20-26% of the world's pesticide market which may increase in some of these countries*" (Berger and Mugoya, 1995). The most notable hazards of misuse and overuse of pesticides are the induction of resistance in pests, weeds and plant pathogens, occupational hazards, environmental pollution, destruction of natural enemies (ecological hazards) and residues in food that are poisonous and / or carcinogenic.

With regard to acute toxicity, fungicides have been reported to be less hazardous compared to insecticides and herbicides. The mercury compounds were an exception, but were banned in the 1970's. Most fungicides do not cause symptoms of acute poisoning in toxicological tests. Long-term exposure to chemicals for disease control and the chronic effect on humans thereof has not been determined directly. High doses of some plant extracts tested on animals have been shown to be mutagenic, antimitotic, teratogenic or carcinogenic. However, these doses are highly unlikely to be used under practical conditions (Berger and Mugoya, 1995).

Some fungicides have been withdrawn due to loss of efficacy. One example of this is the widespread failure of the benzimidazole fungicides (benomyl, thiabendazole, carbendazin, thiophanate methyl) due to the development of resistance in a number of pathogenic fungi" (Berger and Mugoya, 1995). Although fungicides apparently do not cause harmful environmental changes, concerted efforts should be made to keep the amounts released into the environment minimal. In Europe there is a growing pressure to reduce the amounts of

pesticides being used. During the next 10 years, Denmark intends to reduce the consumption of these pesticides by 50% (Warrel, 1990).

There is a need for safe and new biological fungicides due to environmental concerns. The biodegradability is another attribute that makes biological agents so attractive. They are safe to the environment as they do not pollute the air or water, and are safe to bees, ladybeetles and other beneficial insects. There is also a shorter re-entry period for fields sprayed with biopesticides as workers can return to the field in 4h after the use of a biopesticide. However, chemical biopesticides have a much longer re-entry period of 1 – 3d, during which nothing can be done in the field. The better environmental effects of organic farming are well known as there are no chemical pesticides to run off into the surface water or seep into the ground water. The use of organic pesticides rather than the conventional pesticides results in healthier soils with greater microbial diversity. The disadvantage for the farmers, however, is that fields need to sprayed more often when using biopesticides (http://www.ecoworld.org).

Resistance is a major concern when it comes to pesticides. The use of biopesticides is encouraged due to the loss of fungicides to resistance. When insects become resistant to a chemical, the pesticide is then rendered useless and farmers have to look elsewhere for a solution. Chemical pesticides have a single-site effect on a pest, which means, if a pest mutates just once it can become resistant. Natural pesticides are more complex and it is much harder to develop resistance to a biopesticide. There is an absence of effective fungicides versus many plant diseases such as *Fusarium* and *Anthracnose* (www.inchem.org).

The loss of some fungicides, for example, Rovilan, can be attributed to its carcinogenicity. Reports have indicated that certain dithiocarbamate fungicides may also be carcinogenic, affect reproductive physiology and disturb the thyroid function (www.inchem.org). There is an absence of new fungicides to control human mycoses, especially with AIDS, imitating susceptibility to fungi.

There is an imperative need for new antibacterials as there are relatively few classes of human antibiotics. Multidrug resistance is widespread, especially against antibiotics. There are no effective antibacterials in plant pathology.

The overall goal of this project is to discover, develop and foster commercialization of new bioactive natural products as new pharmaceuticals or agrichemicals, and to identify, characterize, and develop plants for production of pharmaceuticals or pesticides as potential alternative crops. There is no known literature published about the bioactivity of the pure compounds isolated.

A major problem in the discovery and development of natural products are the public concerns, which have increased the need for environmentally and toxicologically safer fungicides and bactericides. The solutions to this are natural products derived from plants that offer a broad array of molecules with great diversity in their structures and biological activity. Success for the future lies in discovery and development of these biologically useful natural products.

Literature cited

Berger, A. and Mugoya, C.F. (eds). 1995. Natural Plant Products as Pesticides. Proceedings from the First National Symposium in Zambia. Report 4. Swedish University of Agricultural Sciences, Department of Plant Protection Services, Sweden.

Warrel, E. 1990. Reducing pesticide use: The Danish experience. *Shell Agriculture* 8: 18-20.

Internet links

www.inchem.org
www.ecoworld.org

CHAPTER 1

Part A: LITERATURE REVIEW

1.1 Introduction

"*Traditional medicine is the sum total of all non-mainstream medical practices, usually excluding so-called 'Western' medicine. Ethnomedicine refers to the use of plants by humans as medicines. Ethnobotany is a broad term referring to the study of plants by humans. This includes plants used as foods, medicines, building materials and for any other economic application*" (Farnsworth, 1994).

Traditional medicine, which has always been practised in the indigenous cultures, is fast filling up the therapeutic gaps (Sindiga *et al.*, 1995). The inaccessibility of biomedicine to most of Africa's population because of increasing costs has necessitated a search for alternative ways of managing illnesses.

As sceptism against synthetic drugs and their side effects grows, the demand for plant-derived drugs by the population seems to increase. For acute ailments, there is no crude drug to offer an equivalent alternative to chemically defined drugs. Plant preparations are often considered useful for the treatment of less serious diseases, the supportive treatment of chronic diseases, for treatment over long periods of time and possibly for prophylactic medication, with no harmful side-effects. Thus, medicinal plants continue to receive attention of scientists for their chemical, pharmacological and clinical investigations worldwide (Govil *et al.*[a], 1993).

The ancient Ayurvedic and Siddha medical systems depend basically on the plant based material medica. Indigenous systems employ complex formulations with more than one plant ingredient and also prescribe a specific mode of application. These systems have stood the test of time for centuries. Similar experience has been recorded with Greek, Chinese and Arabian systems of medicine. The plant kingdom has supplied some excellent drugs like morphine, codeine, thebaine, digitoxin, quinine, ergotamine, etc. In addition, the natural plant drugs have served as useful prototypes for even better medicines (Govil *et al.*[b], 1993).

Plants are like chemical factories that produce a vast array of unusual chemical structures that display a variety of biological activities. Thus interest has risen among most major pharmaceutical firms in acquiring as many of the plants on this planet for their screening programmes as is possible. According to Farnsworth (1994), only small amounts of plant material are required for *in vitro* bioassays. If the extracts show activity, usually less than 500g are required to identify the active principle(s). The main aim from an industrial point of view, is to find biologically active molecules, and subsequently try to alter the structures in an attempt to enhance activity or decrease toxicity. Discovery of effective molecules are necessary to recover the costs of development. Usually, pharmaceutical firms do not intend to rely on the plants as a source of a profit-making drug because the final goal is complete synthesis. Due to this, there is little damage to plants being collected for screening programmes, because only small amounts are required. Hence, destruction of the tropical rain forests will not result from this type of small sampling (Farnsworth, 1994).

1.2 Phytochemical research in South Africa

South Africa is considered to be a "hotspot" for biodiversity. More than 22,000 plant species occur within its boundaries. This represents 10% of the world's species, although the land of South Africa is less than 1% of the earth. The country is divided into seven biomes and into 68 vegetation types (Low and Rebelo, 1996). It is especially the Flora Capensis that is unique. This, the Cape Floral Kingdom, is the smallest of the world's six Floral Kingdoms, and contains 8,700 species of which 68% are endemic.

Southern Africa has great cultural diversity, with many people still using a wide variety of plants in their daily lives for food, water, shelter, fuel, medicine and other necessities in life. Indigenous medicinal plants are used by more than 60% of South Africans in their health care needs or cultural practices (Van Wyk *et al.*, 1997). In the last few decades there have been great changes in access to modern health care and education. These changes have severely eroded the indigenous knowledge base. The study of the use of plants by local people, or ethnobotany, is still a relatively underdeveloped discipline in southern Africa. Collaborations are being forged between government departments, science councils, universities, local communities, traditional healers, farmers and entrepreneurs. Knowledge of indigenous plant

use in the region needs urgent significant documentation before it is irretrievably lost to future generations (Van Wyk and Gericke, 2000).

1.3 Phytochemical research world wide

During the last 20 years, there has been a resurgence of interest in herbals as remedies for self-medication in the Western world, which has been termed the 'green boom' (Mander *et al.*, 1997). Although southern Africa contains approximately 10% of the world's plant diversity, relatively little work has been done on the medicinal plants from this region (Eloff, 1998). Modern extraction and separation techniques have proved valuable in isolating pure compounds that can be applied against a myriad of biological activities. The reliance of the use of indigenous medicinal plants has a long history (Cunningham, 1998). Medicinal plants form a sizeable component of traditional medicine and are a mainstay for 80% of the people in developing nations. Estimates in 1996 showed that plant materials are present in, or have provided the models for 50% of Western drugs (Robbers *et al.*, 1996). In 1984/85, Farnsworth showed that 25% of the prescription drugs in the United States and Canada were modelled on plant-based products and 119 secondary plant metabolites were used globally as drugs, with hardly a dozen synthesized commercially or produced by simple chemical modification of active molecules (Farnsworth, 1984). At a national level, it is estimated that 20,000 tonnes of over 700 medicinal plant species are traded in South Africa every year, with a market value of some $60 million (approximately R450 million) (Mander *et al.*, 1997; Mander, 1998). Of approximately 3,000 species of higher plants used as medicine in South Africa, chemical investigations have mostly been on the 350 species commonly used and traded as medicines (Van Wyk *et al.*, 1997). This has led to renewed interest in biodiversity prospecting by both local and multinational companies. Phytochemical research since then has come a long way with several groups from both academia and industry being actively involved in screening programmes for wide-ranging plant activities.

1.4 International trade

International trade in phytomedicinal products is an estimated 500,000 tonnes a year, worth $1.5 billion. The herbal medicine trade is a booming business world-wide. In India, for

example, there are 46,000 licensed pharmacies manufacturing traditional remedies, 80% of which come from plants (Alok, 1991). Another example is Hong Kong, which is claimed to be the largest phytomedicinal market in the world, importing over US$190 million annually (Kong, 1982). In Durban (South Africa), in 1929 there were only two herbal traders; by 1987, there were over 70 herbal trader shops registered. The species-specific nature of the demand for medicinal plants is responsible for generating long distance trade across international boundaries. According to Malla (1982), 60-70% of the medicinal herbs collected in Nepal are exported to India, with 85-200 tons exported annually between 1972 and 1980. Similarly the Hong Kong market imports *Aquilaria* heart-wood for incense manufacture from rain forests in Thailand and Malaysia. This is devastating *Aquilaria* populations in core conservation areas such as Khao Yai National Park, Thailand (Cunningham, 1988). Africa is no exception to this pattern and an informal sector trade in medicinal plants spans long distances.

An average of 25% of prescription drugs sold in the USA during the period 1959-1973 contained active principles extracted from higher plants (Farnsworth and Soejarto, 1985). Many of these are derived from the same source as those used in traditional medicine. On a global scale, 74% of these chemicals have similar or related uses in traditional medicine (Farnsworth *et al.,* 1985). Similarly, many African plant species are the source of a number of active ingredients for the export market. Because of the low price demand by plant traders, even when technology for chemical synthesis is available, it can be cheaper for pharmaceutical companies to continue to extract the active ingredients from plants (http://www.biblicalgardens.org/spsMyWeb/Royal_Botanic_Gardens_Kew.htm).

1.5 Requirements for registering pesticides

Many people get poisoned every year by pesticides. It is illegal to use or sell a pesticide that is not registered. Based on the following information, a pesticide may be registered or denied registration due to:

a) **Toxicity:** A pesticide that is highly toxic to man and the environment in general may not be registered especially if alternative less toxic pesticides exist.
b) **Persistence:** It must not be stable in the environment over a period of years.
c) **Carcinogenic or teratogenic:** it may be rejected if it is implicated that the pesticide may cause cancer or reproductive problems.

d) **Shelf life:** It must have a reasonably long shelf life, preferably two years.

e) **Safety data:** this must include information in treating poisons.

While natural pesticides may seem to be safer, emphasis should not only be placed on efficacy tests but include mammalian toxicity and general environmental effects. Their use should be regulated to ensure minimal negative impacts on the environment (Berger and Mugoya, 1995).

1.6 Connections between agrochemicals and pharmaceuticals

Biological activity of a natural product involves several key characteristics that apply regardless of whether the activity is for an agrochemical or pharmaceutical application. One involves the classical dose-response relationship. Paracelsus recognized, in 1541, the need for proper experimentation to determine the toxic level of a chemical. He distinguished between therapeutic and toxic properties of a chemical and recognized that these may be indistinguishable except by dose. He stated: "*All substances (chemicals) are poisons; there is none which is not a poison. The only difference between a remedy and a poison is its dose*" (Berger and Mugoya, 1995). Antibiotics, antineoplastics, herbicides, and insecticides often originate from plant and microbial defense mechanisms. Secondary metabolites, once considered unimportant products, are now thought to mediate plant defense mechanisms by providing chemical barriers against animal and microbial predators. This chemical warfare between plants and their pathogens consistently provides new natural product leads. Discovery and development of new biologically derived and environmentally friendly chemicals are being aggressively pursued by leading chemical and pharmaceutical companies (Elwell and Maas, 1995).

1.7 Strategy in the search for new biologically active plant constituents

Many novel molecules are contained in the plant kingdom. Of the estimated 250,000 – 500,000 plant species on this earth, only a small percentage has been investigated phytochemically and the fraction subjected to biological or pharmacological screening is even lower (Hostettmann *et al.*, 1995). Rapidly disappearing tropical rainforests and other

important areas of vegetation have meant that it is essential to have access to methods which might lead to the rapid isolation and identification of bioactive natural products. To obtain an exploitable pure plant constituent involves interdisciplinary work in botany, pharmacognosy, pharmacology, chemistry and toxicology, and this approach can be formulated as follows (Fig. 1.1):

1. Selection, collection, botanical identification, and preparation of plant material;
2. Extraction with suitable solvents and primary analysis;
3. Biological and pharmacological screening of crude extracts;
4. Chromatographic separation of pure bioactive constituents, guided by bioassay (activity-guided fractionation);
5. Structure determination;
6. Analysis and pharmacological profile of pure compounds,
7. Toxicological testing;
8. Partial or total synthesis;
9. Preparation of derivatives for studying structure-activity relationships.

(Hostettmann *et al.*, 1995)

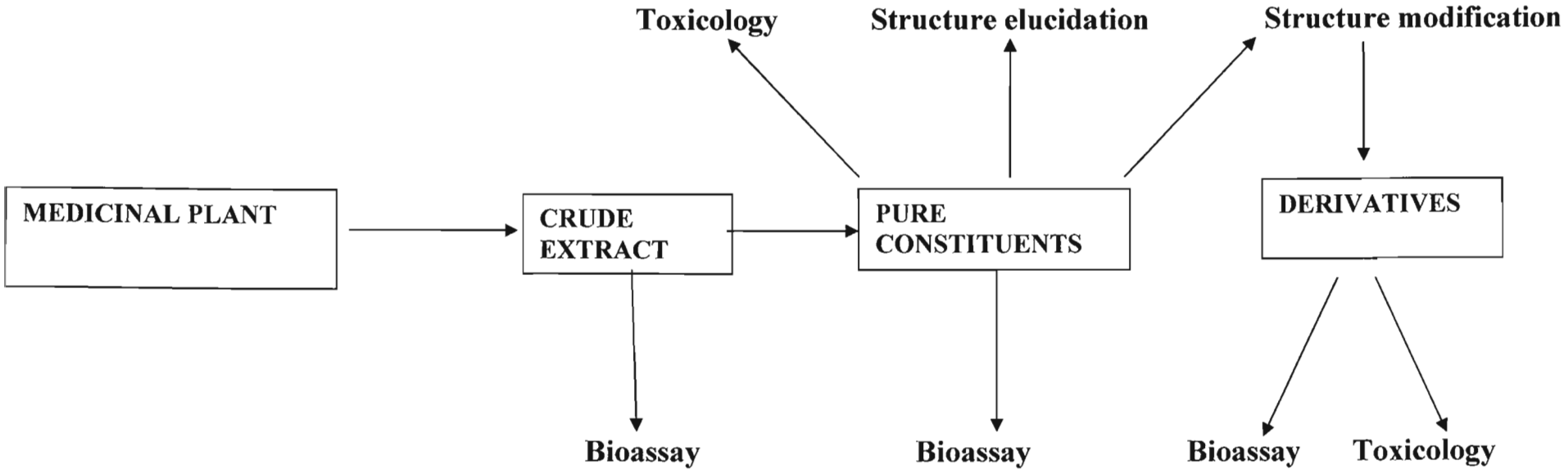


Fig. 1.1 A brief outline showing the steps involved in drug discovery from medicinal plants.

1.8 Selection of plant material

The ethnobotanical literature is rife with the varied bioactivity of plant parts, as well as their reported usage in traditional pharmacopoeia. The basis for all phytochemical research is raw material – various plant parts that are either underground (roots, bulbs, corms and rhizomes) or aerial parts (stem, stem-bark, leaves, inflorescence, fruit and seeds. It is estimated that three quarters of the pharmacologically active plant-derived components were discovered after following up on ethnomedical use of the plant by traditional healers (Farnsworth and Soejarto, 1991). Several groups have realized that drug exploration based on indigenous knowledge is cost-effective in terms of time and capital invested, rather than attempting to compete in expensive random receptor-based screening ventures. This is not to ignore the exceptional cases where leads were also obtained from serendipitous finds or observations (George *et al.*, 2001).

In order to arrive at useful compounds in the shortest possible time, careful selection of plant material is very important. Random collection is one method but it is more judicious to base the selection on certain criteria (Huxtable, 1992). Choice of plants according to chemotaxonomical considerations is another possibly. If, for example, a search for xanthones is being undertaken, it is advisable to start by investigating families that are known to contain this class of natural products – the Gentianaceae, the Polygalaceae, or the Guttiferae if prenylated xanthones are required. Field observations can be very important. If a bush or a tree shows no signs of being attacked by pests and has neither pieces eaten out of the leaves nor discolorations due to the presence of some foreign organism, there is a good chance that some metabolites are present which act as insecticides or antimicrobial agents. Aqueous plant extracts that foam copiously are an indication of the presence of saponins. Plant parts or extracts that are dark red in colour may show the presence of tannins (Hostettman *et al.*, 1995).

1.9 Collection of plant material

It is imperative to lodge preserved voucher specimens, after collection, at a nearby herbarium. Smith and Willis (1999) provide a comprehensive list of herbaria in South Africa.

SABONET (1999) can also be used to look up information on useful contact persons, postal and physical addresses, standard acronyms, expertise, etc.

Field guides are useful reference sources that illustrate habitat, habit and uses. Since the availability of several plants is seasonal, a good option is to start with those that are readily accessible in a quantity that can be procured during a particular time of the year. Local nurseries, private collectors, the National Botanical Institute and parks boards are ideal conduits for facilitating and sourcing plant material. When large quantities of material require screening, proper material transfer agreements for provisioning and adequate compensation or future benefit sharing to the trader is essential to secure uninterrupted supply (George *et al.*, 2001).

1.10 Processing of plant material

Fresh or dried material can be a source of secondary plant constituents. The quality of the material depends on when it is collected or harvested. The variety of compounds in plant material obtained from its wild native habitat may differ from that of cultivated plants. One of the examples is that of raponone, which was isolated in large quantities from wild plants of *Rapanae melanophloeos* Aubl. growing in the Karkloof forests of Kwa-Zulu Natal, but could not be detected from cultivated ones (George *et al.*, 2001). Dried material is preferred in cases where there is a delay between collection and processing as compared to fresh material in which the interfering water content can pose problems in subsequent processing by liquid-liquid extraction. Considering the methods of processing plant material by traditional healers, it is essential to standardize procedures, since most plants are used in dried form or in aqueous extracts (Eloff, 1998). Relative stability is a vital aspect to be considered, if a secondary plant component is to be used in sensitive bioassays. Activity may be observed after compound degradation has occurred. Although different drying methods may be employed prior to extraction, avoidance of harsh conditions retains valuable constituents. Drying in the shade or in a steady draft of warm air is recommended. For higher purity, the plant material can be freeze-dried to minimize loss of thermo-labile compounds. Powdering of the plant material increases the surface area accessible to the solvents used, resulting in better extraction (Eloff, 1998). Although the size of material particles obtained by milling or mechanical pounding varies, depending on individual requirements, filter sieves can be used to obtain uniformity of

particle size. In most cases, to prevent loss of heat-labile or oxidizable components of powdered plant material, it is advisable to subject the material to extraction. In cases where it is in bulk, the processed plant material must be stored. Whole or powdered plant material that is needed to be used later can be stored in clean, dry, air-tight, dark bottles or sealed brown paper bags and stored in a cool, dry place away from direct sunlight (George *et al.*, 2001).

1.11 Choosing a solvent system

Farnsworth (1994) stated that the biggest problem in drug development from plants is answering a very simple question, "*what kind of extract should we test*". Plant material consists of a complex mixture of cells and chemical substances, and although in herbal medicine it may be used in crude form, perhaps compressed into a tablet, for most purposes an extract will be needed: especially for biological screening (Williamson *et al.,* 1996). There has been a substantial increase in the number of papers where scientists screened plants for biological properties. From these papers, it is clear that these scientists used different extractants and extraction procedures for the final extracts (Taylor *et al.,* 1995; Vlietinck *et al.,* 1995; Salie *et al.,* 1996).

There are various extraction methods for screening plants for activity. The choice of the solvent is dependent of the end purpose of the extract. For example, if the plant is known to contain sugars, it would be pointless to extract with hexane. Ethyl acetate or ethanol, which is more polar, would be preferred. If all constituents are to be examined, however, a series of solvents with increasing dielectric constant should be used, e.g., hexane-chloroform-ether-ethyl acetate in turn. If the extraction is with a view solely to isolating chemical components (without any bioassay), toxicity of the solvent is not critical, since the extract can be made solvent-free, before subsequent isolation procedures (Eloff, 1998). If the extraction aims to screen for anti-microbial activity, it is essential that the solvent by itself should not inhibit the bioassay procedure. The biggest problem in drug development from plant extracts is the nature of the extract that requires testing. "*The extractants petroleum ether, chloroform, ethanol, methanol and water (Salie et al., 1996), 80% ethanol (Vlietinck et al., 1995), methanol (Taylor et al., 1995) acetone and methanol-chloroform-water mixtures (Eloff, 1998) have been used with varying success" (George et al., 2001). The widely employed Soxhlet extraction of wet and dried plant material using solvents with increasing polarity has*

limitations for thermo-labile compounds. Although the problem can be overcome by extracting under reduced pressure, it is a tedious process. Some solvents, like water, take longer and, if not properly evaporated, could result in microbial contamination.

Once the extract is obtained, employing bioassay-guided fractionation, pure constituents can be obtained by subjecting the crude extraction to separation techniques like open-column chromatography, High Pressure Liquid Chromatography (HPLC), Gas Chromatography (GC) and the structural elucidation of various constituents carried out by analytical methods including mass spectroscopy (MS) and nuclear magnetic resonance (NMR), circular dichroism(CD) and X-ray crystallography" (George *et al.*, 2001). If the compound is unstable and readily oxidized or photodegraded, it can be subjected to derivatization or encapsulation to obtain stable homologues, which are subjected to bioassays.

1.12 Choice of suitable separation method

The key to any successful programme involving the investigation of biologically active plant constituents is the availability and choice of chromatographic techniques for the separation of pure substances. The aim is to have maximum yield with a minimum of effort (to reduce the time and cost of the separation procedure). Preparative separation techniques can be tedious and time–consuming, especially when complex mixtures, such as crude plant extracts, have to be resolved. Over the past decade or so, several new techniques have been introduced, leading to the acceleration and simplification of difficult separation (Hostettmann *et al.*, 1986; Marston and Hostettmann, 1991). However, there is no universal technique capable of solving every isolation problem. All methods have limitations and advantages, so much so that the best results are often obtained by a combination of two or more of these (Hostettmann *et al.*, 1995).

The separation and purification of phytochemical constituents is mainly carried out using one or other, or a combination of chromatographic techniques (refer to Table 1). The choice of the technique depends largely on the physiochemical properties of the compounds in question. This list is divided into two halves, according to whether a solid stationary phase (or liquid fixed on a solid support) or an all-liquid partition procedure is used. Of the methods in the first category, column chromatography is very popular and used extensively. It can include

ion-exchange resins, polymeric columns, gel filtration, and chromatography over silica gel or chemically modified silica gel. Open-column chromatography has a high load capacity but the separation time is long and the resolution relatively low. Several new preparative systems that reduce separation times have been introduced, including flash chromatography (Still *et al.,* 1978) and vacuum liquid chromatography (Coll and Bowden, 1986). Preparative and semi-preparative HPLC are more efficient methods, with short separation times and high resolution. A high proportion of separations have been performed on chemically bonded silica gel supports and numerous applications of semi-preparative HPLC have been listed (Hostettmann *et al.,* 1986). Disadvantages of this method include the necessity to pre-purify the sample and the high cost of the columns involved. There has been continually mounting interest in chromatography systems involving liquid-liquid partition alone, without the need for a solid stationary phase. Their advantages lie in avoidance of irreversible adsorption of sample, the low cost of the phases and the minimization of denaturation (Hostettmann *et al.,* 1995).

Table 1. Preparative separation methods for plant constituents

SOLID PHASE CHROMATOGRAPHY
Paper chromatography
Preparative TLC, centrifugal TLC
Open-column chromatography
Vacuum liquid chromatography
Pressure column chromatography: flash chromatography, low pressure LC (LPLC), medium-pressure LC (MPLC), and high-pressure LC (HPLC)
LIQUID-LIQUID CHROMATOGRAPHY
Craig distribution
Droplet countercurrent chromatography (DCCC)
Rotation locular countercurrent chromatography (RLCC)
Centrifugal partition chromatography (CPC)

The advantages of TLC compared to high technology methods such as HPLC are:

- Sufficiently good sensitivity
- The technique can detect microgram quantities of material
- Good for qualitative investigation, because most compounds are not visible on the plate but may be visualised by spraying
- The technique is fast and efficient
- The equipment required is inexpensive and running costs are low
- It is adaptable to a wide range of conditions
- The technique is repeatable

(Harborne and Baxter, 1973)

1.12.1 Paper Chromatography

A useful technique for separating and identifying pigments and other molecules from cell extracts that contain a complex mixture of molecules is paper chromatography. By capillary action the solvent moves up the paper and this occurs as a result of the attraction of solvent molecules to the paper and the attraction of solvent molecules to one another. As the solvent moves up the paper, it carries along any substances dissolved in it. The pigments are carried along at different rates because they are not equally soluble in the solvent and because they are attracted, to different degrees, to the fibres in the paper through the formation of intermolecular bonds, such as hydrogen bonds (Martin, 1995).

Beta carotene, the most abundant carotene in plants, is carried along near the solvent front because it is very soluble in the solvent being used and because it forms no hydrogen bonds with cellulose. Another pigment, xanthophyll, differs from carotene in that it contains oxygen. Xanthophyll is found further from the solvent front because it is less soluble in the solvent and has been slowed down by hydrogen bonding to the cellulose. Chlorophylls contain oxygen and nitrogen and are bound more tightly to the paper than are the other pigments (Martin, 1995).

Chlorophyll A is the primary photosynthetic pigment in plants. A molecule of chlorophyll A is located at the reaction centre of photosystems. Other chlorophyll A molecules, chlorophyll B, and the carotenoids (that is, carotenes and xanthophylls) capture light energy and transfer it to the chlorophyll A at the reaction centre. Carotenoids also protect the photosynthetic system from the damaging effects of ultraviolet light (Schultes, 1978).

1.13 Bioassays

The availability of suitable bioassays is crucial to any investigation of plants with biological activities for monitoring the required effects. The capacity for high sample throughout is necessary in order to cope with the number of extracts and fractions from bioactivity-guided fractionation steps. The test systems should ideally be rapid, reproducible, inexpensive, and above all, simple. If active constituents are only present at low concentrations in the crude extract, the bioassay has to be of high enough sensitivity for their detection. At the same time, the number of false positives should also be minimized. Another factor of special importance to plant extracts is the solubility of the sample. Finding a suitable solvent can pose many problems (Hostettmann *et al.,* 1995).

When deciding which bioassays to employ in searching of plant constituents, the first step is to choose suitable target organisms. These can be any number of lower organisms (e.g. microorganisms, insects, crustaceans, or molluscs, isolated subcellular systems (enzymes, receptors or organelles), cultured cells of human or animal origin, isolated organs of vertebrates, or whole animals. However, the right target has to be found for the right disease. The complexity of the bioassay has to be designed according to the function of the facilities, resources and personnel available. In most phytochemical laboratories engaged in the investigation of bioactive medicinal plants, neither specialized animal facilities nor properly qualified technicians are available. Consequently, simple, inexpensive 'bench-top' bioassays for the rapid screening of plant extracts and fractions have been introduced. In the interpretation and predicative ability of these tests care must be taken as they provide very important preliminary information for the evaluation of vegetable (or other) material under study. Finding a suitable assay for antimicrobial agents occupy an important place in any screening programme. This is due to the problems of drug resistance and limited spectrum of activity; hence, new lead compounds are actively being sought (Mitscher and Rao, 1984).

Figure 1.2 shows the steps taken to perform the bioassay from preparation of the agar to disposal of the plates.

1.13.1 Agar well diffusion method

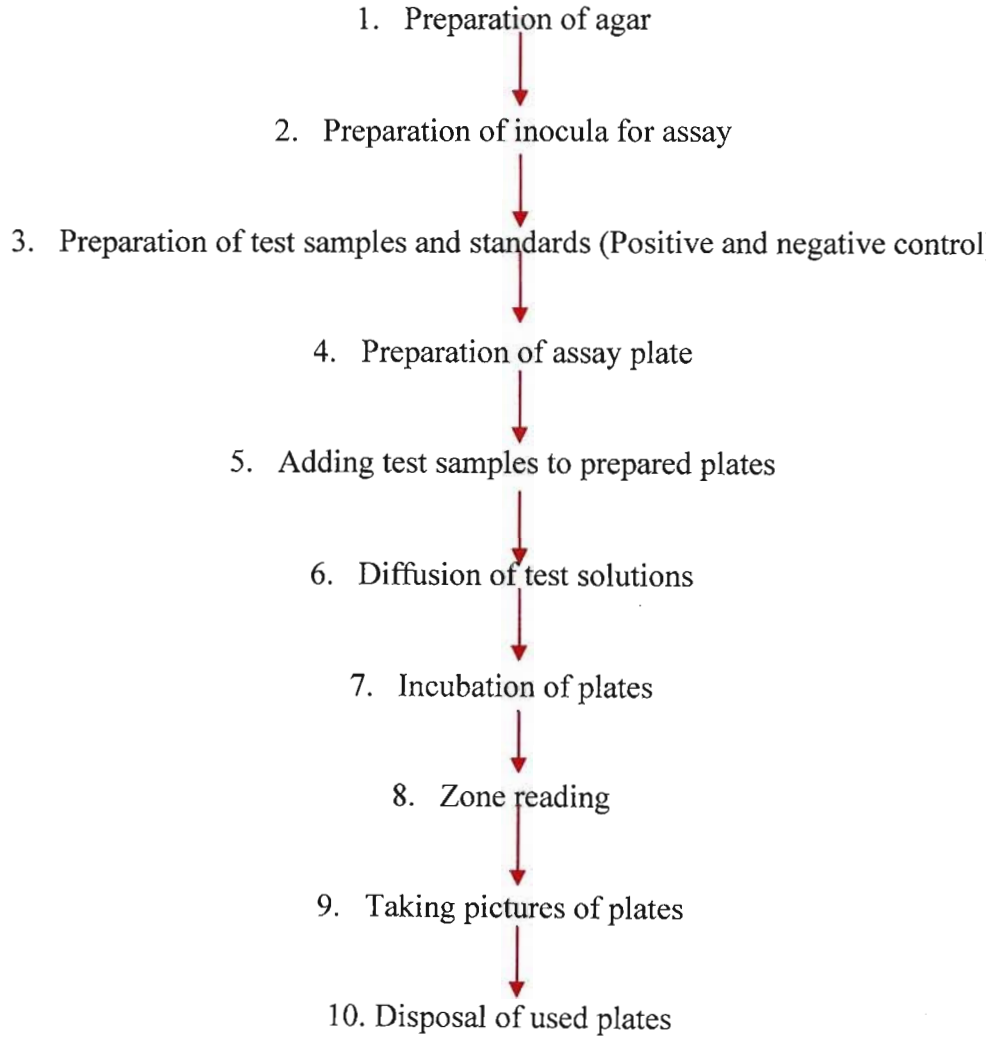


Fig. 1.2 Steps taken to perform the bioassay from preparation to disposal

1.14 Bioautography

A frequently used bioassay is the agar disc diffusion method (Shadomy *et al.*, 1985), which is suitable for screening a large number of samples for antifungal activity. This is the case, for example, with programmes involving microorganisms. However, bioautography is now the method of choice for the isolation of active compounds by bioactivity-guided fractionation. This new technique combines TLC with a bioassay *in situ* and shows the exact location of active constituents in a plant extract. In direct bioautographic procedures target organisms such as spore-producing fungi, like *Aspergillus, Penicillium* and *Cladosporium* spp. can all be employed (Homans and Fuchs, 1970; Van der Sluis *et al.*, 1981; Gottstein *et al.*, 1984). This method can also be applicable to the testing of antibacterial activity (Hamburger and Cordell, 1987). After migration and drying of the TLC solvent, the plates are sprayed with a mixture of the microorganisms in their nutritional medium. They are then incubated in a humid atmosphere like a humidifier. Zones of inhibition appear where spore growth is prevented by the active components of the plant extract. Since direct bioautography is not possible with yeasts such as *Candida albicans,* a simple and rapid agar overlay assay has been developed (Rahalison *et al.*, 1991). This contact bioautography technique relies on the transfer of active compounds from the stationary phase into the agar layer (which contains the microorganisms) by the process of diffusion. When the plate is sprayed with methyl thiazolytetrazolium chloride (MTT), a MTT formazan is produced and inhibition zones are observed against a purple background (Hamburger and Cordell, 1987).

1.15 Difficulties in determining antibacterial activity of plant extracts

Choice of test organism

There are major differences in susceptibility of different strains of the same species. In order to produce consistent results and to avoid mutations, strains recommended by the National Committee for Clinical Laboratory Standards USA, should be used (Eloff, 2001).

The test organisms chosen for this study were fungi and bacteria from different taxonomic groups, which represent plant and animal pathogens of these taxonomic groups. This is important because one often needs different fungicides and bactericides for fungi and bacteria from different taxonomic groups as they have different physiologies and morphologies.

Bacillus subtilis B69 (Gram positive), *Xanthomonas campestris* (Gram negative), *Candida* spp., *Fusarium oxysporum, Pythium ultimum, Rhizoctonia solani* and *Sclerotinia sclerotiorum* were chosen. The fungal phytopathogens belong to three classes – Ascomycetes (*F. oxysporum, S. sclerotiorum* and *Candida* spp.); Basidiomycetes (*R. solani*); and Oomycetes (*P. ultimum*).

Fusarium is one of the most drug-resistant fungi. Among the *Fusarium* spp., *Fusarium solani* in general tends to be most resistant of all (Arikan *et al.*, 1999). *Fusarium* strains yield quite high MICs for flucytosine, ketoconazole, miconazole, fluconazole, itraconazole, and posaconazole (Pujol *et al.*, 1997). The soilborne plant pathogen *F. oxysporum* causes vascular wilt disease on a wide variety of crops (Beckman, 1987). *Pythium ultimum* is an Oomycete. It has been shown to parasitize many fungi and other water moulds including *Botrytis, Fusarium and Phytophthora*, and has been licensed as a biocontrol agent in the Czech Republic (Brozova, 2002). *P. ultimum* causes damping-off which is a soil borne disease that occurs wherever plants are grown. The soil borne fungi causes' seed rot, pre-emergence damping-off, post emergence damping–off and stem rot. The symptoms of post emergence damping-off are a brown, watery, soft rot on the roots and stem, girdled stem and falling over of plants (Niere *et al.*, 1994). *R. solani* may cause damage at any time during the growing season, but it is more severe on young seedlings. It can cause seed rot, root rot, and lesions on hypocotyls. Damping-off occurs when germinating seedlings are infected prior to or just after emergence (Agrios, 1997). Common symptoms of *S. sclerotiorum* include water-soaked spots on fruits, stems, or leaves, petioles with altered morphology. The watery spots enlarge and develop into a cottony white mass that converts the plant to a slimy wet mass that produces abundant sclerotia (Agrios, 1997).

X. campestris is a gram-negative phytopathogenic bacterium (Weng *et al.*, 1999). It is the causal agent of black rot which is a serious disease that affects crucifers such as *Brassica* and *Arabidopsis* (Gottwald *et al.*, 2000). *X. campestris* is a vascular pathogen that invades the xylem and colonizes the mesophyll. Symptoms of black rot include marginal leaf chlorosis, necrosis and darkening of leaf veins and vascular tissue within the stem. Full leaf yellowing, wilting, and necrosis occur as the disease advances (Hayward, 1993). *X. campestris* is grown commercially to produce xanthan gum, an exopolysaccharide which is used as a viscosifying and stabilizing agent in the food and non-food industries (Becker *et al.*, 1998). In a previous

study, it was found that strains of *Xanthomonas* isolated in Taiwan are commonly resistant to ampicillin at a level of 50 μg/ml (Williams, 1980).

"*The most common fungal pathogens associated with invasive disease in humans are opportunistic yeasts (e.g., Candida albicans) or filamentous fungi (e.g., Aspergillus spp.). Fungi previously thought to be nonpathogenic for humans or only sporadically associated with human disease, such as Candida (except albicans), Fusarium, Trichosporon, and Malassezia spp., are emerging as important nosocomial fungal pathogens. These pathogens are associated with increasing morbidity and mortality. The emergence of these organisms and antifungal-resistant fungi, especially those that are azole-resistant, poses an important challenge to the clinician. This may be related to the increased use of antifungal agents, particularly as prophylactic or empiric agents. Another Candida species is Candida dubliniensis which presents a major problem in HIV positive patients receiving antifungal drugs* " (http://www.nfid.org/publications/clinicalupdates/fungal/noso.html).

One beneficial rhizobacterium is *Bacillus subtilis*, which is ubiquitous in soil. It promotes plant growth, protects against fungal pathogen attack (Utkhede and Smith, 1992; Asaka and Shoda, 1996; Emmert and Handelsman, 1999), and plays a role in the degradation of organic polymers in the soil (Emmert and Handelsman, 1999). Among the first successful biocontrol agents used against insects and pathogens were members of the genus *Bacillus* (Powell and Jutsum, 1993). Commercial strains of *B. subtilis* have been marketed as biocontrol agents for fungal diseases of crops (Emmert and Handelsman, 1999; Warrior *et al.,* 2002). The commercial biofungicide, Serenade, which contains a *B. subtilis* strain, is reported to be effective against a variety of pathogenic bacteria, including *Erwina, Pseudomonas*, and *Xanthomonas* strains (Warrior *et al.,* 2002). The mechanism of this antibacterial effect is uncertain, although it is known that *B. subtilis* can produce a variety of antibacterial agents, including a broad spectrum of lipopeptides, such as surfactin, that are potent biosurfactants (Zuber *et al.*, 1993; Peypoux *et al.*, 1999). *B. subtilis* has proven highly amenable to genetic manipulation, and has therefore become widely adopted as a model organism for laboratory studies, especially of sporulation, which is a simplified example of cellular differentiation.

1.15.1 Agar diffusion assay

An agar plate or narrow agar tube inoculated with bacteria is used. Diffusion of the active compound from a well / trench leads to growth or inhibition. Addition of a test solution by improvement paper disc or cup cut out of agar plate.

Factors influencing results of agar diffusion assay:

There are complications if the active compounds are not soluble in water because agar is in an aqueous matrix. The volume of the test solution, density of inoculum, incubation period, temperature, thickness and preparation of agar medium are very important. Length of diffusion phase should be at a low temperature to ensure diffusion is complete before growth starts, or else this may influence results. Quantification → a dose response logarithmic must be compared to a known standard. Calculating minimum inhibitory concentration [MIC] is more reproducible than calculating minimum lethal concentration [MLC]. When the assay is designed for a known compound with a standard available, it will work well. It can be misleading with unknown compounds or mixtures. Therefore a log dose curve is used. Many problems arise with volatile compounds if using the paper disc method (Eloff, 2001).

Presentation of results:

Many authors do not consider the width of the paper disc in presenting their results. If the disc is 6mm wide and no growth is observed, a value of 0 is presented, but if there is inhibition the whole diameter instead of the zone of inhibition is measured. A zone of 2 and 4mm would then be presented as 0.8 and 10 mm (Eloff, 2001).

Approaches to minimize difficulties:

The standard approach is to compare zones of inhibition to that of standard antibiotics, used as reference or internal standards. When plating with different concentrations of extract in agar for MIC tests, one should note the temperature, as most extracts are temperature sensitive. Plates must be sprayed with INT to differentiate bands more clearly (Eloff, 2001).

1.15.2 Liquid assays

A second general technique used for bioassay testing is by serial dilution of extract in a test tube followed by addition of a bacterial culture. MIC can be observed by viewing the turbidity due to growth after incubation. This assay requires large quantities of material and there may be problems due to precipitation. The problem with this assay is that when applied to plant extracts, one cannot detect growth by turbidity visually or with microplate reader due to the colour of extract and precipitation of compounds.

1.15.3 Bioautography gives insight on the quality of bioactives

All the other techniques discussed provide information on the antimicrobial activity, but do not give any qualitative information. By separating bioactive extracts by thin layer chromatography (TLC), one can obtain information on compounds present in the mixture. All traces of solvents must be removed when doing TLC. TLC plates must be sprayed with an actively growing suspension of test organism and incubated overnight at 100% relative humidity. Spray with tetrazolium violet (INT) or related compound.

Using INT as a redox indicator

Tetrazolium salts act as electron acceptor and change colour when reduced by living cells. However, tetrazolium chloride colours spontaneously in air. Tetrazolium violet [INT] on the other hand, stays clear in air and red in the presence of living cells.

1.15.4 Conclusions

Agar diffusion assays are frequently not suitable for investigating plant extracts. Earlier workers may have missed many important leads. The microplate technique works well and total activity is a useful measure to compare different plants or yields in bioassays guided fractionation. Bioautography however does give the most valuable information on composition of bioactives (Eloff, 2001).

1.16 Antifungal compounds from plants

Plants are an extremely rich source of diverse organic compounds that are antifungal (Nigg and Seigler, 1992). In general, the interest in such antifungal compounds depends on the concentration required for activity and the biological spectrum of activity. Some of the compounds are performed and are located in external plant tissues, i.e., bark, peel and cuticle. Others are located throughout the plant, often in vacuoles, and still others are produced by plants in response to physiological stress or infection. In the latter group are the phytoalexins, low molecular weight organic compounds produced by plants in response to infection or stress and localized at the site of infection or stress. This group of compounds is usually lipophilic and includes compounds as diverse as simple phenols, flavonoids, isoflavanoids, coumarins, isocoumarins, sesquiterpenoids, polyenes, stilbenes, furanoterpenoids and derivatives of these compounds. The levels of still another group of antifungal compounds are enhanced locally and systematically after infection or stress and a second infection often markedly enhances their levels. These are high molecular weight and include chitinases, β-1, 3-glucanases and thionins (Nigg and Seigler, 1992).

1.17 Insecticidal compounds from plants

Plants possess limited movement and cannot escape in space from their enemies. They are required to "*stand and fight*" to preserve their substance and progeny. Hence, they have developed superlative defensive mechanisms. Dense bark, hard wood, spines and thorns are classical morphological features of many plants. Less apparent, but probably more important in suppressing predation and disease, is the complex secondary chemistry of plants. Just as flowers elaborate a variety of alluring scents and tasty nectarines to attract birds, bees and other insects to assist in cross pollination, many plants produce repellents, poisons, antifeedants, growth regulators and antibiotics to reduce exploitation by herbivores and pathogens. Because insects compose more than 90% of all planetary species and are clearly the dominant herbivores, plants have evolved an extensive variety of defensive strategies targeted to limit their exploitation by pests (Menn *et al.*, 1989). The earliest insecticides used by man were simple powders or aqueous extracts of poisonous plants. Hundreds of toxic plants have been employed by primitive cultures for purposes of insect control and most of

these have been evaluated as prospective insecticides, but only a scant few have been found to possess useful commercial insecticidal properties (Nigg and Seigler, 1992).

While a few plant compounds like nicotine, rotenone and pyrethum continue to find use in specialized applications, most plant defensive chemicals are intrinsically labile, difficult or expensive to produce synthetically, and lack the activity spectrum required for commercial production, formulation and application. However, the discovery of biologically active natural products with insecticidal or growth regulant activity has opened simultaneously many new biochemical/physiological target sites for insect control. Botanical insecticides have been an important source of prototypic chemistry that has been vastly improved through biologically guided fractionation assays (Roark, 1947). The natural pyrethrins exemplify the opportunities essential within biological chemistry. Insects treated with pyrethrins are rapidly knocked down but often recover unless a synergist is included in the formulation. The synergists prevent oxidative decomposition of the pyrethrins at labile sites in the molecule, especially the allyl, carbomethoxy, isobutenyl and pentadienyl moieties (Yamamoto and Casida, 1966; Yamamoto *et al.*, 1969; Casida and Maddrell, 1971). Although natural synergists were used extensive in pyrethrum formulation, synthetic efforts soon capitalized on the chemistry and provided improved synergists such as piperonyl butoxide (Nigg and Seigler, 1992).

1.18 Future for Natural Products

Natural products are known as a host of plant, animal, fungal and bacterially derived chemicals that are involved in many aspects of human existence. These natural products may be used as pure compounds or as mixtures of complex components that serve as medicines, pesticides, flavourings, herbicides, dyestuffs, tanning agents, rubber, food-preservatives, detergents, perfumes, resins, gums, etc., as well as such esoteric uses as arrow poisons or piscicides (Breedt, 1999). Fungi produce many natural products that are frequent food contaminants.

To determine the future of man's usage of these compounds, one should examine the past as to why so many chemical entities have arisen. The answer to this lies in ecological and biological interactions. As evolution was taking place of all major kinds of organisms, new

pathways arose which differed from the basic steps involved in respiration and photosynthesis. As evolution progressed, the skies, water and ground were laced with many of these biologically active "messages" (Taylor *et al.*, 1995). Compounds with many types of activity are known; these may interact with most biochemical processes and affect many systems and receptors of organisms in the world around us (Nigg and Seigler, 1992).

Currently, the usage of these natural products are still being explored. In many aspects of modern civilization, biologically active compounds from a wide range of sources are still important. Many of the natural products have had compounds isolated and a large number are now available through synthesis; others have served as templates for the synthesis of derivative drugs with greater effectiveness, or special properties (Balandrin *et al.*, 1985).

There are many ways in which new biologically active and useful natural products can effectively be identified. These approaches range from random synthesis and screening (Menn, 1983; Menn and Henrick, 1981), basic biochemical, physiological and behavioural research (Menn and Pallos, 1975; Menn *et al.*, 1981, 1989; Menn, 1985), to the study of folklore and ethnobotany (Balick, 1990; Waterman, 1990; Fox, 1991). Many other useful natural products have been discovered by bioassay-guided serendipity, straight serendipity, chemotaxonomy, or selecting plants with few or no pest problems (Waterman, 1990).

By examining the biology and chemistry of the organisms involved in biological interactions (a field of study known as "chemical ecology"), the yield information about the mechanisms of biological communication can be evaluated. It can also provide many new leads to useful natural products. In an effort to discover new types of bioactive compounds, considerable attention has been given to ethnobotanical uses of plant drugs (Balick, 1990).

"A variety of natural sources yielding bioactive compounds have been proposed to be useful for treating a number of human maladies such as schistosomiasis (Hostettmann, 1984, Hamburger and Hostettmann, 1991), cancer (Svoboda, 1983; Kingston et al., 1990), viral diseases (Vanden Berghe et al., 1986; Che, 1991), and AIDS (Nonaka et al., 1990). Other medicinally important natural products include anti-inflammatory (Sertie et al., 1990), antihepatotoxic (Houghton and Hikino, 1989), antiphlogistic and antiallergic (Wagner, 1989), antimutagenic (Wall et al., 1988), antimicrobial (Metzner et al., 1979; Rehacek, 1990), and fungicidal drugs (Langcake, 1981; Lwande et al., 1986). There are also many reports of

bioactive compounds such as herbicides (Saito et al., 1989), insecticides, insect repellents and attractants (Jacobson, 1983), and molluscides (Lemma, 1983)" (Nigg and Seigler, 1992).

Several steps should be taken to take advantage of the host of natural products that have been produced in the evolution of living organisms. Studies of chemical ecology should be undertaken while many of the organisms still exist. There is poor support of this type of interdisciplinary research is at present. Timing is crucial; a large fraction of the world's organisms, especially those of rain forests, where some of the most pronounced and intricate biological interactions occur, are being destroyed at an alarming rate. Collection and testing of plants, animals, fungi, bacteria, etc. should be accelerated, but sensitivity and care should be given to the problems of dwindling populations of many organisms. Financing of studies for ethnobotanical, folkloric and traditional uses of natural products by various people should be established before the few remaining intact cultures disappear and the information is lost forever. New information, as well as exact literature, needs to be surveyed and stored in databases that are accessible to a wide range of investigators (Nigg and Seigler, 1992). Probably this would best be done though the oversight of major research foundations, one such being the South African National Research Foundation (NRF).

Finally, there is widespread perception and speculation that natural products research is not a good, solid, science, but rather a "fishing expedition" or "merely screening" biological materials. Projects are rarely sufficiently ecological, systematic or chemical to warrant support by specific programs representing these disciplines. Similar criticisms apply to other funding agencies as well. Changes in the prospective utilization of natural products are evident though. Attitudes towards interdisciplinary research and funding also seem to be changing. The realization that most natural products orientated problems require collaborative efforts of many scientific disciplines is being recognized. In the USA there has been renewed interest at the National Science Foundation, National Institutes of Health and USAID in funding natural product orientated projects from a broad perspective (Nigg and Seigler, 1992). Corporations have also expressed renewed interest in this area of research. Due to the heightened public interest in better quality food products, this will undoubtedly force new studies in this vital area. Although a few problems remain, the future payoff is too great not to attract many entrepreneurs and visionaries. A bright and exciting future for natural products seems to be promised as we systematically tap this vast resource for improvement of the quality of life (Nigg and Seigler, 1992).

1.19 Conclusion and Perspective

In order to exploit plant material fully, an endeavour should be made to have as many simple bioassays available as possible. The bioassays must be designed to have relevance to the disease state under consideration; they must also have the predicative capacity necessary for clinical efficacy. As these conditions are rarely easily satisfied, it is difficult to establish test systems that effectively represent complex pathological complications, because the number of effective screens is still rather low. Therefore, much remains to be done in the development of appropriate bioassays that fulfil the required criteria. "*A move in this direction is the use of mechanism-based assays with enzyme or receptor target systems. Care should, however, be exercised with this method of screening for active constituents of plants because compounds with unknown mechanisms of action are not detected. Furthermore, non-specific interactions, which are of little relevance to the disease under consideration, may be picked up*" (Nigg and Seigler, 1992).

Another condition for success in the discovery of new bioactive plant constituents is that effective collaborations between botanists, phytochemists and pharmacologists should be established in order to undertake all the steps involved, starting with plant material in the field and concluding with viable, pharmacologically pure active compounds. Only in this manner will it be possible to utilize effectively the riches still available to us in the plant kingdom.

An association has been made between antifungal compounds in plants with plant disease resistance. This, of course, excludes many compounds that may have antifungal activity and have not been tested for such activity and compounds of medicinal and industrial value that have not been investigated for a role in disease resistance. The thesis brings to the reader compounds of known antifungal activity and hopefully introduces, to those not in the plant field, compounds which might have otherwise escaped their attention. This is especially true for the phytoalexins, a broad spectrum of diverse compounds, which are not preformed but which accumulate at sites of infection or stress in plants and only relatively recently appeared in the literature. Though logic has its place in science, so does trial and error. There are likely to be many chemically characterized compounds in the literature that have interest for biological activity other than their antifungal properties. A general screening of such compounds is not out of place. Naturally occurring compounds may also serve as models for

metabolically active centres and derivatives of such compounds may find medicinal or industrial use.

Plant pathologists and breeders have realized for decades that phytochemical defense comes at an ecological cost; there are trade-offs between defense (resistance) and productivity. Elwell and Maas (1995), summarized 80 plant defense strategies into the optimal-defense theory by simply stating, "*you don't get something for nothing; there is a cost to everything*".

Humanity's future success in discovery and development of useful natural products will depend on knowledge and understanding of the diverse roles that phytochemicals play in the natural world and, of course, a healthy dose of serendipity.

1.20 Literature cited:

Agrios, G.N. 1997. Plant Pathology 4th edn. Academic Press, New York, Pp 20-68.

Alok, S.K. 1991. Medicinal plants in India. In: Conservation of Medicinal Plants. Approaches to exploitation and conservation, O. Akerele, V. Heywood, H. Synge (eds). Cambridge, Cambridge University Press, Pp. 295 - 304.

Arikan, S., Lozano-Chiu, M., Paetznick, V., Nangia, S. and Rex, J.H. 1999. Microdilution susceptibility testing of amphotericin B, itraconazole, and voriconazole against clinical isolates of *Aspergillus* and *Fusarium* species. *Journal of Clinical Microbiology* 37: 3946-3951.

Asaka, O. and Shoda, M. 1996. Biocontrol of *Rhizoctonia solani* damping off of Tomato with *Bacillus subtilis* RB14. *Applied Environmental Microbiology* 62: 4081-4085.

Balandrin, M.F., Klocke, J.A., Wurtele, E.S., and Bollinger, W.H. 1985. Natural plant chemicals: Sources of industrial and medicinal materials. *Science* 228: 1154-1160.

Balick, M.J. 1990. Ethnobotany and the identification of therapeutic agents from the rainforest. In: Bioactive Compounds from Plants, D.K. Chadwick and J. Marsh (eds). Ciba Foundation Symposium No. 54, John Wiley and Sons, Chichester, United Kingdom, Pp. 22-39.

Becker, A., Katzen, F., Puhler, A. and Ielpi, L. 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Applied Microbiology and Biotechnology* 50: 145-152.

Beckman, C.H. 1987. The Nature of Wilt Diseases of Plants. American Phytopathological Society, St. Paul, MN. pp94.

Berger, A. and Mugoya, C.F. (eds). 1995. Natural Plant Products as Pesticides. Proceedings from the First National Symposium in Zambia. Report 4. Swedish University of Agricultural Sciences, Department of Plant Protection Services, Sweden.

Breedt, P. 1999. Handbook on the Natural Control of Plant Diseases and Insects. Kwa-Zulu Natal Department of Agriculture, Cedara.

Brozova, J. 2002. Exploitation of the mycoparasitic fungus *Pythium oligandrum* in plant protection. *Plant Protection Science* 38: 29-35.

Casida, J.E. and Maddrell, S.H.P. 1971. Diuretic hormone release on poisoning *Rhodnius* with insecticide chemicals. *Pesticide Biochemistry and Physiology* 1: 71-83.

Che, C. 1991. Marine products as a source of antiviral drug leads. *Drug Development Research* 23: 201-218.

Coll, J.C. and Bowden, B.F. 1986. The application of vacuum liquid chromatography to the separation of terpene mixtures. *Journal of Natural Products* 49, 943-946.

Cunningham, A.B. 1988. An Investigation of the Herbal Medicine Trade in Natal / KwaZulu, Investigational Report No. 29, Institute of Natural Resources, University of Natal, Pietermaritzburg.

Eloff, J.N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Plant Medica* 64: 711–713.

Eloff, J.N. 2001. Indigenous Plants Use Forum Presentation (IPUF). Dept of Pharmacology, University of Pretoria.

Elwell, H. and Maas, A. 1995. Natural Pest and Disease Control. Natural Farming Network, Zimbabwe.

Emmert, E.A.B. and Handelsman, J. 1999. Biocontrol of plant disease: a Gram-positive perspective. *FEMS Microbiology Letters* 171: 1-9.

Farnsworth, N.R. 1984. The role of medicinal plants in drug development. In: Natural Products and Drug Development: Alfred Benzon Symposium 20, P Krogsgaard-Larsen, S Brogger-Christensen, and H Kofod, eds. Munksgaard, Copenhagen, Denmark. Pp 17-30.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D. and Guo, Z.-G. 1985. Medicinal plants in therapy. *Bulletin of the WHO* 63: 965-981.

Farnsworth, N.R. and Soejarto, D.D. 1985. Potential consequence of plant extinction in the United States on the current and future availability of prescription drugs. *Economic Botany* 39: 231-240.

Farnsworth, N.R. 1994. Ethnopharmacology and drug development. In: Ethnobotany and the Search for New Drugs, Ciba Foundation Symposium 185. John Wiley & Sons, Chichester.

Farnsworth, N.R. and Soejarto, D.D. 1991. Global Importance of Medicinal Plants. In: Conservation of medicinal plants. A Akarele, V Heywood and H Synge, Cambridge, UK, University Press. Pp 25-51.

Fox, B.W. 1991. Medicinal plants in tropical medicine. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85: 22-25.

George, J., Laing, M.D. and Drewes, S.E. 2001. Phytochemical Research in South Africa. *South African Journal of Sciences* 97: 93-103.

Gottstein, D., Gross, D. and Lehman, H. 1984. Mikrobiotest mit *Cladosporium cucumerinum* Ell. Et Arth. Zum Nachweis fungitoxischer Verbindungen auf Dunnschictplatten. *Archiv fur Phytopathologie und Pflanzenscutz* 20: 111-116.

Gottwald, T.R. and Graham, J.H. 2000. Compendium of citrus diseases. In: *Compendium of citrus diseases*. Timmer, L.W., Garnsey, S.M. and Graham, J.H. (eds.), American Phytopathological Society, St. Paul, pp. 34-35.

Govil, J.N., Singh, V.K. and Hashmi, S. (eds). 1993. Medicinal Plants - New Vistas of Researches (Pt. 1). Today and Tomorrow's Printers and Publishers, New Delhi, India.

Govil, J.N., Singh, V.K. and Hashmi, S. (eds). 1993. Medicinal Plants - New Vistas of Researches (Pt. 2). Today and Tomorrow's Printers and Publishers, New Delhi, India.

Hamburger, M.O. and Cordell, G.A. 1987. A direct bioautographic TLC assay for compounds possessing antibacterial activity. *Journal of Natural Products* 50: 19-22.

Hamburger, M. and Hostettmann, K. 1991. Bioactivity in plants: The link between phytochemistry and medicine. *Phytochemistry* 30: 3864-3874.

Harborne, J.B. and Baxter, H. 1973. Phytochemical Dictionary: Handbook of the bioactive compounds from plants. Taylor and Francis Ltd, London.

Hayward, A.C. 1993. The host of *Xanthomonas*. In: *Xanthomonas*. Swings, J.G. and Civerolo, E.L. (eds.), Chapman & Hall, London, pp. 51-54.

Homans, A.L. and Fuchs, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. *Journal of Chromatography* 52: 325-327.

Hostettmann, K. 1984. On the use of plants and plant-derived compounds for the control of schistosomiasis. *Natuurwissenschaften* 71: 247-251.

Hostettmann, K., Hostettmann, M., and Marston, A. 1986. Preparative chromatographic techniques; applications in natural product isolation. Springer-Verlag, Berlin, Germany.

Hostettmann, K., Marston, A., Maillard, M. and Hamburger, M. (eds) 1995. Phytochemistry of Plants used in Traditional Medicine. In: Proceedings of the Phytochemical Society of Europe. Claredon Press, Oxford.

Houghton, P.J. and Hikino, H. 1989. Anti-hepatotoxic activity of extracts and constituents of *Buddleja* species. *Planta Medica* 55: 123-126.

Huxtable, R.J. 1992. The pharmacology of extinction. *Journal of Ethnopharmacology* 37: 1-11.

Jacobson, M. 1983. Insecticides, repellents, and attractants from arid/semi-arid-land plants. In: Plants: The Potentials for Extracting Proteins, Medicines, and Other useful Chemicals - Workshop Proceedings. Washington D.C.: U.S Congress, Office of technology Assessment, September 1983), U.S. Government Printing Office, Washington, D.C., USA. , Pp. 138-146.

Kingston, D.G.I., Samaranayake, G. and Ivey, C.A. 1990. The chemistry of taxol, a clinically useful anticancer agent. *Journal of Natural Products* 53: 1-12.

Kong, Y.-C. 1982. The control of Chinese medicines – A scientific overview. *Yearly Pharmacology Society.* Hong Kong 1982:47-51.

Langcake, P. 1981. Alternative chemical agents for controlling plant disease. *Philosophical Transactions of the Royal Society of London Biological Sciences* 295: 83-101.

Lemma, A. 1983. Molluscicidal and other economic potentials of Endod. In: Plants: The Potentials for Extracting Protein, Medicines, and Other Useful Chemicals - Workshop Proceedings. Washington D.C: U.S. Congress, Office of Technology Assessment. U.S. Government Printing Office, Washington, D.C, USA. Pp. 111-125.

Low, A.B. and Rebelo, A.G. 1996. Vegetation of South Africa, Lesotho and Swaziland. Published by the Department of Environmental Affairs & Tourism, Pretoria, RSA.

Lwande W., Hassanali, A., Bentley, M.D., and Monache, F.D. 1986. 8-C-Prenylated flavones from the roots of *Tephrosia hildebrandtii*. *Journal of Natural Products* 49: 1157-1158.

Malla, S.B. 1982. Medicinal Plants of Nepal. FAO Regional Office for Asia and the Pacific. Report, No. 64, Bangkok, FAO.

Mander, M. 1998. Marketing of Indigenous Medicinal Plants in South Africa: A Case Study in Kwa-Zulu Natal. FAO, Rome.

Mander, M., Quinn, N.W. and Mander, M. 1997. Trade in Wildlife and Medicinals in South Africa. INR Investigational Report No. 157. Institute of Natural Resources, Pietermaritzburg.

Marston, A. and Hostettmann, K. 1991. Modern separation methods. *Natural Product Reports* 8: 391-423.

Martin, G.B. 1998. Gene discovery for crop improvement. *Current Opinions in Biotechnology* 9: 220-226.

Menn, J.J. 1983. Present insecticides and approaches to discovery of environmentally acceptable chemicals for pest management. In: Natural Products for Innovative Pest management, DL Whitehead (ed). Pergamon Press, Oxford and New York, New York, USA.

Menn, J.J. 1985. Prospects of exploitation of insect juvenile hormones for selective insect control. In: Approaches to New Leads for Insecticides, K von Keyserling, (ed). Springer-Verlag, Berlin and Heidelberg, Germany. Pp. 37-46.

Menn, J.J. and Henrick, C.A. 1981. Rational and Biorational Design of Pesticides. In: Philosophical Transactions of the Royal Society of London. *Series B-Biological Sciences* 295: 57-71.

Menn, J.J. and Pallos, F.M. 1975. Development of morphogenetic agents in insect control. *Environmental Letters* 8: 71-88.

Menn, J.J., Henrick, C.A. and Staal, G.B. 1981. Juvenoids: Bioactivity and prospects for insect management. In: Regulation of Insect development and Behaviour. Scientific Papers of the Institute of Organic and Physical Chemistry of Wroclaw Technical University, No.22, Conferences 7, Karpacz, Poland. Pp. 735-748.

Menn, J.J., King, E.G., and Coleman, R.J. (eds) 1989. Future control strategies for *Heliothis* in cotton. In: Pest Management in Cotton, Ellis Horwood Limited, Gweru.

Mitscher, L.A. and Rao, R.G.S. 1984. The search for new antimicrobial agents - unusual sources. In: Natural products and drug development. P. Krogsgaard-Larsen, L. Brogger-Christensen, and H. Kofod (eds), Munksgaard, Copenhagen. Pp.193-209.

Niere, J., DeAngelis, G. and Grant, B. 1994. The effect of phosphonate on the acid-soluble components in the genus *Phytophthora*. *Microbiology* 14: 1661-1670.

Nigg, H.N. and Seigler, D. (eds). 1992. Phytochemical Resources for Medicine and Agriculture. Plenum Press, New York. Pp 369-373.

Nonaka, G.-I., Nishioka, I., Nishizawa, M., Yamagishi, T., Kashiwada, Y., Dutschman, G.E., Bodner, A.J., Kilkuskie, R.E., Cheng, Y.-C., and Lee, K.-H., 1990. Anti-AIDS agents, 2: Inhibitory effects of tannins on HIV reverse transcriptase and HIV replication in H9 lymphocyte cells. *Journal of Natural Products* 53: 587-595.

Peypoux, F., Bonmatin, J.M. and Wallach, J. 1999. Recent trends in the biochemistry of surfactin. *Applied Microbiology and Biotechnology* 51: 553-563.

Powell, K.A. and Jutsum, A.R. 1993. Technical and commercial aspects of biological control products. *Pesticidal Science* 37: 315-321.

Pujol, I., Guarro, J., Gene, J. and Sala, J. 1997. *In vitro* antifungal susceptibility of clinical and environmental *Fusarium* spp. strains. *Journal of Antimicrobial Chemotherapy* 39: 163-167.

Rahalison, L., Hamburger, M., Hostettmann, K., Monod, M. and Frenk, E. 1991. A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. *Phytochemical Analysis* 2: 199-203.

Rehacek, Z. 1990. Research into biogenesis of biologically active microbial metabolites in Czechoslovakia: A review, *Process Biochemistry International* 25: 197-209.

Roark, R.C. 1947. Some promising insecticidal plants, *Economical Botany.* 1: 437–438.

Robbers, J., Speedie, M. and Tyler, V. 1996. Pharmacognosy and pharmacobiotechnology. Williams and Wilkins, Baltimore. Pp. 1–14.

Saito, K., Matsumoto, M., Sekine, T., Murakoshi, I., Morisaki, N. and Iwasaki, S. 1989. Inhibitory substances from *Myriophyllum brasiliense* on growth of blue-green algae. *Journal of Natural Products* 52: 1221-1226.

Salie, F., Eagle, P.F.K. and Leng, H.M.J. 1996. Preliminary antimicrobial screening of four South African asteraceae species. *Journal of Ethnopharmacology* 52: 27-33.

Schultes, R.E. 1978. The kingdom of plants. McGraw-Hill Book Co., New York. Pp 208.

Sertie, J.A.A., Basile, A.C., Panizza, S., Matida, A.K. and Zelnik, R. 1990. Anti-inflammatory activity and sub-acute toxicity of artemetin. *Planta Medica* 56: 36-40.

Shadomy, S., Espinel-Ingroff, A., and Cartwright, R.Y. 1985. Laboratory studies with antifungal agents: susceptibility tests and bioassays. In: *Manual of clinical microbiology*, 4th edn. E.H. Lennette, A. Balows, W.J. Hausler and H.J. Shadomy (eds). American Society for Microbiology, Washington D.C., USA. Pp. 991-999.

Sindiga, I., Nyaigotti-Chacha, C. and Kununah, M.P. (eds). 1995. Traditional Medicine in Africa. English Press Ltd., Nairobi.

Smith, G.F and Willis, C.K. 1999. Index Herbarium: southern African supplement, ed 2. *South African Botanical Diversity Network Report* 8: 1-81.

Still, W.C., Kahn, M., and Mitra, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *Journal of Organic Chemistry* 43: 2923-2925.

Svoboda, G.H. 1983. The role of the alkaloids of *Catharanthus roseus* (L.) G. Don (*Vinca rosea*) and their derivatives in cancer chemotherapy. In: Plants: The Potentials for Extracting protein, Medicines, and Other Useful Chemicals - Workshop Proceedings. Washington, D.C.: U.S. Congress, Office of Technology Assessment. U.S. Printing Office, Washington D.C., USA. Pp. 154-169.

Taylor, R.S., Mananhar, N.P., and Tower, G.H.N. 1995. Screening of selected medicinal plants of Nepal for antimicrobial properties. *Journal of Ethnopharmacology* 46: 153-159.

Utkhede, R.S. and Smith, E.M. 1992. Promotion of apple tree growth and fruit production by the EBW-4 strain of *Bacillus subtilis* in apple replant disease soil. *Canadian Journal of Microbiology* 38: 1270-1273.

Van den Berghe, D.A., Vlietinck, A.J. and Van hoof, L. 1986. Plant products as potential antiviral agents. *Bulletin de l'Institut Pasteur* 84: 101-147.

Van der Sluis, W.T., Van Arkel, J., Fischer, F.C., and Labadie, R.P. 1981. Thin-layer chromatographic assay of photoactive compounds (furocoumarins) using the fungus *Penicillium expansum* as a test organism. *Journal of Chromatography* 214: 349-359.

Van Wyk, B-E, Van Oudtshoorn, B. and Gericke, N. 1997. Medicinal Plants of South Africa. Briza Publications, South Africa.

Van Wyk, B.-E. and Gericke, N. 2000. People's plants: a guide to useful plants of southern Africa. Briza Publications, Arcadia, South Africa.

Vlietinck, A.J., Van Hoof, L., Totte, J., Lasure, A., Vanden Berghe, D., Rwangabo, P.C., and Mvukiyumwami, J. 1995. Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *Journal of Ethnopharmacology* 46: 31-47.

Wagner, H. 1989. Search for new plant constituents with potential antiphlogistic and antiallergic activity. *Planta Medica* 55: 235-241.

Wall, M.E., Wani, M.C., Hughes, T.J. and Taylor, H. 1988. Plant antimutagenic agents - General bioassay and isolation procedures. *Journal of Natural Products* 53: 13-22.

Warrior, P., Konduru, K. and Vasudevan, P. 2002. Formulation of biological control agents for pest and disease management. *In:* Biological Control of Crop Diseases. S.S. Gnanamanickam (ed), Marcel Dekker, New York, pp 421-442.

Waterman, P.G. 1990. Searching for bioactive compounds: Various strategies. *Journal of Natural Products* 53: 13-22.

Weng, S. F., Chen, C.Y., Lee, Y.S., Lin, J.W. and Tseng, Y.H. 1999. Identification of a novel β-lactamase produced by *Xanthomonas campestris*, a phytopathogenic bacterium. *Antimicrobial Agents in Chemotherapy 43*: 1792-1797.

Williams, P.H. 1980. Black rot: a continuing threat to world crucifers. *Plant Diseases* 64: 736-742.

Williamson, E.M., Okpako, D.T. and Evans, F.J. 1996. Selection, preparation and evaluation of plant material. In: Pharmacological methods in phytotherapy research, Vol. 1. John Wiley and Sons Ltd., England. Pp1-23.

Yamamoto, I. and Casida, J.E. 1966. O-Demethyl pyrethrin II analogues from oxidation of pyrethrin I, allethrin, dimethrin, and phthalthrin by a housefly enzyme system. *Journal of Economic Entomology* 59: 1542-1543.

Yamamoto, I., Kimmel, E.C. and Casida, J.E. 1969. Oxidative metabolism of pyrethroids in houseflies. *Journal of Agriculture and Food Chemistry* 17: 1227-1236.

Zuber, P., Nakano, M.M. and Marahiel, M.A. 1993. Peptide antibiotics. *In: Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics. AL Sonenshein, JA Hoch and R Losick (eds). American Society for Microbiology, Washington, DC, pp 897-916.

Internet references:

http://www.cbwinfo.com/Biological/PlantPath/SS.html

http://www.nfid.org/publications/clinicalupdates/fungal/noso.html

Part B: *Plectranthus* (Lamiaceae Family)

"Any aromatic plant is likely to have potential in pest control" (Elwell and Maas, 1995).

1.21 Introduction of *Plectranthus* species

Family = Lamiaceae

Genus = *Plectranthus*

***Plectranthus* - plek-tranth-us**

From Greek ***plectron*** (a spur) and ***anthos*** (a flower) referring to the spurred flowers of the type of species (http://davesgarden.com/botany/go/4503). The genus *Plectranthus* L'Herit belongs to the Sage family (Lamiaceae), known for their aromatic and herbal properties.

Plectranthus is a large genus of about 350 species distributed through Africa to Asia and Australia, with over 40 species indigenous to southern Africa (Codd, 1985). In South Africa it occurs in or along forest margins of woodlands, while some species occur in drier regions on rocky outcrops or in open grassland. There is a high concentration in Natal, east of the Drakensberg escarpment, where they are subjected to a moist and warm climate. *Plectranthus* are annual or perennial herbs or subshrubs with herbaceous, semi-succulent or succulent stems and leaves (Van Jaarsveld and Edwards, 1997).

"*Short-stalked epidermal glands bearing ethereal oils, often of the monoterpenoid, sesquiterpenoid type, are characteristic of the family Lamiaceae (Cronquist, 1981). Members of the family frequently produce triterpenoid substances, but generally not saponins. Acylated anthocyanins are common while alkaloids of the pyrorolidine or pyridine groups are seldom found. Iridoid compounds are sometimes found, usually in plants poor in essential oils, and plants are only rarely cyanogenic. Ordinary kinds of tannins are not generally present and both are ellagic acid and proanthocyanins are lacking. Potassium nitrate is often accumulated and carbohydrates, such as stachyose and/or oligogalactosides, are commonly stored. Diverse sorts of crystals of calcium oxalate are sometimes present in some cells of the parenchymatous tissues, but more often not*" (Hutchings *et al.*, 1996).

The Lamiaceae family has been well characterised with respect to their secondary metabolites, largely dominated by the flavonoids and terpenoids. They are a rich source of terpenoids possessing anti-insect, antibacterial and antifungal activity (Cole, 1992).

Plectranthus species, many which contain volatile oils in their leaves, have yielded a wide range of diterpenoids (Glasby, 1991), possessing antibacterial activity (Batista *et al.,* 1994; Batista *et al.,* 1995; Batista *et al.*, 1996; Dellar *et al.,* 1996; Teixeira *et al.,* 1997).

Plectranthus includes 45 southern African species, which are found in the subtropical forests and savannahs of the summer rainfall region (Van Jaarsveld and Edwards, 1997). Until recently, only a few species of this underrated genus were cultivated as ornamental foliage house-plants in Europe and North America. *P. verticillatus,* from the eastern region of southern Africa, has been grown for many years in hanging baskets for its trailing stems with bright green, glossy, rounded, slightly succulent leaves. It is commonly known as Swedish ivy (and is incorrectly known as *P. australis* and *P. parviflorus*). Also grown as Swedish ivy is *P. oertendahlii*, which has a similar habitat but is distinguished by the white veins on the upper side of the hairy, grey-green leaves (Shaw, 1999). In Kwa-Zulu Natal, its native home, plants have been found where white coloration extends over the whole leaf surface (http://darwin.bio.uci.edu/edu/aboretum/plectranthus.htm). This may prove to be an exciting new plant if introduced into Europe. Another old favourite is trailing *P. madagascariensis* 'Variegated Mintleaf', which has small, white-edged aromatic, leaves. In the right conditions this plant can produce stems hanging to 3m with clusters of small, white flowers in the leaf axils (Miller and Morgan, 2000).

The *Plectranthus* species are beautiful South African shrubs. Most of the species have fountain-like sprays of intensely coloured flowers borne above velvety, heart-shaped leaves. Taxonomically, the closest genus to *Plectranthus* is *Coleus* Lour., and some confusion can arise when distinguishing *Coleus* from *Plectranthus*. The unique feature of *Plectranthus* is the upper lip of the flower, which is unusually four-lobed, and the large shoe-shaped lower lip that is formed from a single lobe, whereas in *Coleus* the upper lip often consists of two lobes and the lower consisting of three (Abdel-Mogib *et al.*, 2002).

Many *Plectranthus* species are plants of economic and medicinal interest. In Polynesia, acute edematous otitis acuta of the ear is treated by applying the seed-oil of *P. amboinicus* (Lour.) Spreng. *P. esculentus* N.E. Brown, known as Livingstone potato tubers, is cultivated in tropical Africa for its edible tubers (Watt and Breyer-Brandwijk, 1962). *P. floribundus* N.E. Brown is cultivated in Nigeria, also for its edible tubers, and relished also in Kwa-Zulu Natal. In Africa, the leaves of *P. caninus* are chewed to relieve toothache and in East Africa the

leaves of *P. elegans* is used as a vermicide. The East African medicinal plant, *P. barbatus* Andr. is used as a remedy for stomach-ache and as a purgative (Hutchings, 1996). An aphid antifeedant diterpene has been isolated from this plant, which is resistant to insect attack. In Indian Ayurvedic medicine, *P. vettiverioides* (K.C. Jacob) H.I. Maas is prescribed as a remedy for vomiting and nausea. In Saudi Arabia, several species, such as *P. tenuiflorus*, are grown as ornamentals. In this country, the leaf extract of *P. tenuiflorus* is used to treat ear infections and the leaves of *P. asirensis* are used as an antiseptic dressing for wounds (Abdel-Mogib *et al.*, 2002).

The chemistry of *Plectranthus* has been extensively studied. The main phytochemical constituents of the genus *Plectranthus* are diterpenoids, essential oils and phenolics (Abdel-Mogib *et al.*, 2002). From the coloured leaf-glands of *Plectranthus* species, about 140 diterpenoids have been identified. *"The majority of them are highly modified abietanoids, in addition to some phyllocladanes, ent-kaurenes and a seco-kaurene structure. The abietanoids, in turn, could be classified, according to structure variation, into royleanones, spirocoleons, vinylogous quinones (also named extended quinones), quinone methides, acylhydroquinones, abeo-acylhydoquinones, phenolic abietanoids, phenanthraquinones, dimeric abietanoids and seco-abietanoids"* (Abdel-Mogib *et al.*, 2002).

Plectranthus is one of the oil-rich genera from the subfamily Nepetoideae. The main constituents of essential oils of *Plectranthus* are mono- and sequiterpenes. On a fused silica capillary column, the following were isolated from the essential oil of *P. fruticosus* L' Herit: α-humulene, aromadendrene, α-cubebene, β-bisabolene, γ-cadinene, α-elemene, *trans*-farnesol and *trans*-copaene (Abdel-Mogib *et al.*, 2002).

A group of long-chain alkylphenols has been isolated which are of possible taxonomic significance in the genus. Long-chain alkylphenols, which were isolated from *P. albidus*, showed significant *in vitro* antioxidant activity. Antioxidant activity guided fractionation of extracts of *P. sylvestris* Gurke and HPLC separation yielded the oxygenated long-chain alkylcatechols (Abdel-Mogib *et al.*, 2002).

From *P. hereroensis*, a single aristolane sesquiterpene was isolated. A new abietane diterpene has also been isolated from the acetone extract of the root of *P. hereroensis.* This compound shows antibacterial activity against *Staphylococcus aureus* and *Vibrio cholerae*, and antiviral

activity against *Herpes simplex* type II (Batista *et al.*, 1995). From *P. rugosus* Wall. ex Benth, five triterpenoids, namely, plectranthoic acid, acetylplectranthoic acid, plectranthadiol, plectranthoic acid A and plectranthoic acid B, in addition to β-sitosterol were isolated. Flavonoids seem to be rare in *Plectranthus*. Only two flavonoids have been identified, 4',7-dimethoxy-5,6-dihydroxyflavone from *P. ambiguous* Meyer and chrysosplenetin from *P. marruboides*. *P. zeylanicus* Benth, an Ayurvedic drug used for diarrhoea and grown in South India and Sri Lanka (Dassanayakae and Fosberg, 1981), has had diterpenoids isolated from it. It has been characterized as 7β-acetoxy-6β-hydroxyroyleanone and 7β, 6β-dihydroxyroyleanone (Mehrotra *et al.*, 1989).

1.22 Forskolin

Among the activators of adenylate cyclase, and apart from certain natural prostaglandins, there is at present only one specifically active substance available, forskolin (Seamon *et al.*, 1981). Forskolin is a novel labdane-type diterpene, isolated from the roots of *Coleus forskholi*, a member of the Labiatae, which originated in India. Biological activities of this diterpene have been demonstrated. Some authors think that forskolin might be able to prevent cancer mestastases (in animals, in 70% of cases, a single previous injection of the drug prevented the appearance and dissemination of lung tumours) (Barton and Ollis, 1986). Kilmer and Carlsen suggested that forskolin might stimulate nerve regeneration, since an increase in the level of neuronal cyclic AMP has been demonstrated in periphereal nerve lesions. Tests have been performed on frogs whose sciatic nerves had been damaged by cold and then treated with a daily injection of 500 μM forskolin into the dorsal lymphatic sac. Nerve generation was significantly faster in the treated animals than the controls. The work on forskolin suggests that other biological activities of the compound may be discovered Markstein *et al.*, 1984; Pfitzer and Ruegg, 1984). Oddly enough, it seems that this substance, just as it is, may already have the optimum chemical configuration, since of 50 semisynthetic derivatives tested, not one gave better results (Daly *et al.*, 1983).

"*Various biological activities (Table 1.22.1) have been reported for plant diterpenes, principally from members of the Ericaceae, Euphorbiaceae, Lamiaceae and Compositae*"

(Seaman *et al.*, 1990). The fact that this diterpene comes from the family Labiatae that contains no plant known for its major therapeutic activity, offers food for thought.

Table 2. Biological activities of some plant diterpenes of the Lamiaceae family (Seaman *et al.*, 1990)

Activity compound	**Source**
Antifeedant	
ajugarin I-III	*Ajuga remota*
teucjaponin A	*Teucrium japonicum*
Insecticidal	
ajugarin IV	*Ajuga remota*
Antibacterial	
longikaurins	*Rabdosia longituba*
Antibiotic	
przewaquinone	*Salvia przewalskii*
Antimicrobial	
7,18-dihydroxy-sandaracopimaradiene	*Iboza riparia*

1.23 *Plectranthus* spp. found in Port St Johns

The host substrate of the Pondoland Centre of Endemism (PCE) is Port St Johns (PSJ), which lies on an isolated outcrop of the Msikaba Formation. There are as many as thirty trees and at least as many grassland species that are endemic to the PC. Close to 1 000 species have been collected at PSJ, of which sixteen are PCE endemics. Seventeen species of *Plectranthus* are found in the immediate vicinity. Of these, one is endemic to the PC (*P. hilliardii*) and three are endemic to PSJ (*P. malvinus, P. praetermissus* and *P. reflexus*) (http://www.dwaf.gov.za/Dir_Forestry/IFM/Docs/CD1/Doc%2045%20-%20Cloete.doc).

"*Protection of these plants lies in the creation of large and small-scale protected areas, proper planning for urban and rural development, control of alien invasions, education of the local population and enforcement of existing regulations*" (http://www.dwaf.gov.za/Dir_Forestry/IFM/Docs/CD1/Doc%2045%20-%20Cloete.doc).

Table 3. Shows seventeen *Plectranthus* species that have been collected at PSJ:

Plectranthus ambiguus	*P. malvinus*
P. ciliatus	*P. petiolaris*
P. ecklonii	*P. praetermissus*
P. fruticosus	*P. reflexus*
P. hilliardiae	*P. saccatus* var. *saccatus*
P. laxiflorus	*P. strigosus*
P. lucidus	*P verticillatus*
P. madagascariensis var. *aliciae*	*P. zuluensis*
P. madagascariensis var. *madagascariensis*	

P. hilliardii is endemic to the Pondoland Centre and has been collected at Magwa, Fraser Falls and Umtamvuna. The three species that are endemic to PSJ are *P. malvinus, P. praetermissus* and *P. reflexus.*

The flowers of *P. praetermissus* Codd. are related to those of *P. oertendahlii*, but the vegetative characters differ significantly. Red compound gland dots occur on the undersurface of the leaves.

The taxon of *P. malvinus* E.J. van Jaarsveld and T.J Edwards is related to *P. strigosus* and *P. ciliatus* but is distinguished by its firm, succulent, ovate to obovate leaves which are serrate, have densely pilose purple veins and are densely punctate underneath.

The species, *P. reflexus* E.J. van Jaarsveld & T.J. Edwards, differs from related species in both floral and vegetative characters. The corolla is long (28 - 30mm) and narrow and is slightly saccate in the basal half. The upper lip is four-lobed and reflexed, often touching the corolla tube.

P. hilliardiae Codd has been recorded from Port St Johns for the first time only this year, indicating its rarity (http://www.dwaf.gov.za/Dir_Forestry/IFM/Docs/CD1/Doc%2045%20-%20Cloete.doc).

Conclusion

"*Several of the understorey taxa of Port St Johns have many small populations of plants that are clearly related, yet distinctly different (Begonia, Plectranthus). In addition there is an unusually large variety of certain groups such as Plectranthus and ferns. This high inter- and intra-species diversity is under threat and action should be taken without delay.*

A reserve created at PSJ would automatically conserve more than half of the ferns of Transkei and almost one-quarter of those found in South Africa, and more than one-third of the South African species of Plectranthus. Protection of these rare plants lies in creation of large and small-scale protected areas; proper planning for urban and rural development; control of alien invasions; education of the local population and enforcement of existing regulations"
(http://www.dwaf.gov.za/Dir_Forestry/IFM/Docs/CD1/Doc%2045%20%20Cloete.doc).

1.24 Evaluation of *Plectranthus esculentus* as a crop

It is well known that southern Africa has a harsh climate, and that the usual vegetable species are not very well-adapted to these conditions. Malnutrition is found in the areas of low agricultural potential as a result of a shortage of suitable vegetable crops. A great need to develop food plants that are well-adapted to these harsh climates conditions therefore exists. Fortunately there are a variety of plant species that have been utilized for many years by people in this country. One of these plant species is *P. esculentus.* It is an indigenous crop that can be used as a potato substitute in some areas. This plant is naturally distributed in the warmer eastern regions of South Africa, although it does occur in many African countries. Nutritionally it provides a large percentage of vitamin A in the form of β-carotene and calcium, while containing more than 100% of the RDA of iron. All three of these elements of a healthy diet, as well as protein, are lacking in rural diets in this country. The tubers contain between 7-10% protein, more than is found in both potatoes and sweet potatoes. The crop appears to be host to few pests and diseases and a specific niche market already exists which could be further exploited (Alleman *et al.,* 1998).

1.25 Hybridization of *Plectranthus*

Plectranthus are soft herbaceous plants, some with unusually beautiful tubular flowers in blue, violet, white and pink. In South Africa, *Plectranthus* spp. are widely used outdoors as indigenous bedding plants and groundcovers. They grow naturally in the subtropical south-eastern parts of South Africa as forest-floor plants and flowering times peak in autumn. Other salient qualities are easy cultivation and propagation, using stem cuttings; semi-drought tolerance and requirement for semi-shade.

A few species of this horticulturally neglected genus are used internationally as commercial foliage pot plants, with little or no breeding improvement. However, the first distinctive varieties of flowering pot plants have been developed in South Africa over the past 10 years. Species were found to hybridize with difficulty and the resulting interspecific hybrids are almost invariably seed sterile. In a breeding programme started in South Africa, a wide variety of compact plants with large and floriferous flower types and beautiful foliage were focussed upon. Improved foliage characters include texture (pleasant to touch and sight), shape (attractively rounded leaves), colour (silver-veined, variegated, underside red-tinted) and smell (pleasantly herbal typical of the mint family). A range of natural colours has been selected for and some selections now have the largest flowers in the genus. Product development focuses on flowering pot plants but also include hanging basket plants and floriferous outdoors plants for semi-shaded situations (http://www.actahort.org).

The new series of hybrid *Plectranthus* are introduced under the Trademark 'Blue Angels'. Research and development are done by a group of specialist nurseries in Australia, Europe, Japan, U.S.A and South Africa. This includes breeding, technical development, propagation/marketing and specialist administration, all by separate companies. Plant Breeders' Rights have been applied for the hybridization of *Plectranthus* (http://www.actahort.org).

1.26 Literature cited:

Abdel-Mogib, M., Albar, H.A. and Batterjee, S.M. 2002. Chemistry of the Genus *Plectranthus*. Molecules 7: 271-301.

Alleman, J., Viljoen, J., Venter, S.L. and van den Heever, E. 1998. Evaluation of *Plectranthus esculentus* as a crop. Mini-Symposium and Workshop, Agricultural Research Council Headquarters, Pretoria, RSA.

Barton, D. and Ollis, W.D. 1986. Advances in Medicinal Phytochemistry. John Libbey Eurotext Ltd.

Batista, O., Duarte, A., Nascimento, J., Simoes, M.F., De La Torre, M.C. and Rodriguez, B. 1994. Structure and antimicrobial activity of diterpenes from the roots of *Plectranthus hereroensis*. *Journal of Natural Products* 57: 858-861.

Batista, O., Simoes, M.F., Duarte, A., Valdeira, M.L., De La Torre, M.C. and Rodriguez, B. 1995. An antimicrobial diterpene from the root of *Plectranthus hereroensis*. *Phytochemistry* 38: 167-169.

Batista, O., Simoes, M.F., Nascimento, J., Riberio, S., Duarte, A., Rodriguez, B. and De La Torre, M.C. 1996. A re-arranged abietane diterpenoid from *Plectranthus hereroensis*. *Phytochemistry* 41: 571-573.

Codd, L.E. 1985. *Plectranthus.* In: Flora of Southern Africa, ed. O.A. Leistner, Vol. 28, Part 4 Lamiaceae. Pp. 137-172. Botanical Research Institute, Dept. Agriculture & Water Supply, Durban, RSA.

Cole, M.D. 1992. The significance of the terpenoids in the Labiatae. In: Advances in Labiate science. Eds. R.M. Harley and T.Reynolds. Royal Botanic Gardens, Kew, UK.

Daly, J.M., Metzger, H., De Souza, N.J. and Reden, J. 1983. Structure-activity relationship for activation of adenylate cyclase by the diterpene forskolin and its derivatives. *Journal of Medicinal Chemistry* 26: 436.

Dassanayakae, M.D. and Fosberg, F.R. 1981. A Revised Handbook of the Flora of Ceylon. Oxford and IBH, New Delhi. Pp 23-45.

Dellar, J.E., Cole, M.D. and Waterman, P.G. 1996. Antimicrobial abietane diterpenoids from *Plectranthus elegans*. *Phytochemistry* 41: 735–738.

Elwell, H. and Maas, A. 1995. Natural Pest and Disease Control. Natural Farming Network, Zimbabwe.

Glasby, J.S. 1991. Dictionary of Plants Containing Secondary Metabolites. Taylor and Francis, London.

Hutchings, A., Scott, A.H., Lewis, G. and Cunningham, A.B. 1996. Zulu Medicinal Plants. University of Natal Press, Pietermaritzburg. Pp 23-26.

Kilmer, S.L. and Carlsen, R.C. 1984. Forskolin activation of adenylate cyclase *in vivo* stimulates nerve regeneration. *Nature* 307: 455-457.

Markstein, R., Digges, K., Marshall, N.R. and Starke, K. 1984. Forskolin and the release of noradrenaline in cerebrocortical slices. *Naunyn-Schmiedeberg's Archae Pharmakologie* 325: 17-24.

Mehrotra, R., Vishwakarma, R.A. and Thakur, R.S. 1989. Abietane diterpenoids from *Coleus zeylanicus*. *Phytochemistry* 28: 3135-3137.

Miller, D. and Morgan, N. 2000. Focus on Plants: A New Leaf. *The Garden - Royal Horticultural Society* 125(11): 842-845.

Pfitzer, G. and Ruegg, J.C. 1984. Forskolin inhibits tension development in detergent-treated cardiac muscle fibres. *Experientia* 40: 65-67.

Seaman, F., Bohlmann, F., Zdero, C. and Mabry, T.J. 1990. Diterpenes of Flowering Plants. Springer-Verlag, New York.

Seamon, K.B., Padgett, W., Daly, J.W. 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proceedings of the National Academic Science, USA, Biochemistry* 78: 3363-3367.

Shaw, J.M.H. 1999. Notes on the Identity of Swedish Ivy and Other Cultivated *Plectranthus*. *The New Plantsman* 6(2): 71-74.

Teixeira, A.P., Batista, O., Simoes, M.F., Nascimento, J., Duarte, A., De La Torre, M.C. and Rodriguez, B. 1997. Abietane diterpenoids from *Plectranthus grandidentatus*. *Phytochemistry* 44: 325-327.

Watt, J.M. and Breyer-Brandwijk, M.G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa (2nd ed.). E. and S. Livingstone Ltd., London.

Van Jaarsveld, E.J. and Edwards, T.J. 1997. Notes on *Plectranthus* (Lamiaceae from southern Africa. *Bothalia* 27: 1-6.

Internet references:

(http://www.actahort.org)
(http://darwin.bio.uci.edu/edu/aboretum/plectranthus.htm)
(http://www.dwaf.gov.za/Dir_Forestry/IFM/Docs/CD1/Doc%2045%20-%20Cloete.doc)
(http://davesgarden.com/botany/go/4503)

Chapter 2

Biological activity of *Plectranthus fruticosus* L'Herit and *Plectranthus ecklonii* Benth against selected bacteria and fungi

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

Dichloromethane extracts of *P. fruticosus* and *P. ecklonii* were screened for antibacterial and antifungal activities using the agar well and trench diffusion methods. It was found that both methods produced inconsistent results. The trench method required a bigger volume of plant extract to be filled into the well, hence, better biological activity was observed with the above method. The well method required a smaller volume therefore less activity was noted with this assay. The size of inhibition zones are dosage dependent. Overall, both plant extracts exhibited antibacterial but no antifungal properties. 11-Hydroxy-2-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one (1) was isolated from the leaves of *P. ecklonii.*

2.1 Introduction

Botanical Name: ***Plectranthus ecklonii*** **Benth**

Common Name: Large Spur-Flower Bush / Mauve Plectranthus

Fig 2.1 ***P. ecklonii*** **in full bloom (www.plantzafrica.com)**

P. ecklonii is an upright shrub which grows up to 1.5m in height. The pink, white and blue forms are most attractive when grouped together. It can also be planted singly. This species is often grown for its attractive foliage. Spur flowers vary in their growth forms from low prostrate groundcovers to small, medium and large shrubs (www.plantzafrica.com). There are many attractive hybrids that have been developed locally in recent years. All *Plectranthus* have aromatic leaves when crushed or even brushed against. Although there are a few which are not very pleasant, many are spicy, sweet and mostly pleasant. This species, alone of the entire genus, is susceptible to giant whitefly. *P. ecklonii* is used in Zimbabwe to treat skin infections (Nyanyiwa and Gundidza, 1999). Both *P. ecklonii* and *P. montanus* have antibacterial and antifungal activity (Chadya and Gundidza, 1999 and Nyanyiwa and Gundidza, 1999).

Botanical Name: *Plectranthus fruticosus* L' Herit

Common Name: Forest Spur Flower / Pink Fly Bush

Fig 2.2 Foliage of *P. fruticosus* (www.plantzafrica.com)

P. fruticosus is an upright growing shrub. The inflorescence forms a striking plume 20-28cm long. The underside of the leaf is also an attractive mauve. It is well suited to mass plantings in light shade. Various colour forms exist (www.plantzafrica.com).

P. fruticosus is used in traditional Romanian medicine for its healing properties, especially for treating burns (Pages *et al.,* 1991). It is also antimicrobial and this activity may be attributed, in part, to the presence of kaurane diterpenoids (Gaspar-Marques *et al.*, 2003). *P. fruticosus*, *P. amboinicus* and *P. pseudomarrubioides* are also used as insect repellants (Roberts, 1990;

Prudent *et al.*, 1995 and Omolo *et al.*, 2004). The essential oils of *P. fruticosus* have teratogenic properties in mice, probably on account of the presence of saninal acetate (Pages *et al.*, 1988, 1991).

2.2 Materials and methods

Collection - The plants were collected from The Botanical Gardens, Pietermaritzburg. The taxonomic identification of the plants was confirmed by the Curator, Prof Trevor Edwards, Department of Botany, University of KwaZulu-Natal, Pietermaritzburg. Voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg.

Extraction - The leaves were air-dried and extracted after 1wk with dichloromethane followed by ethyl acetate. The plant extract was filtered from the solvent and the essential oils were removed from the solvent and concentrated using a rotary evaporator. Repeated flash column chromatography of the dichloromethane and ethyl acetate extracts of *P. fruticosus* and *P. ecklonii* allowed the isolation of pure compound **1** (EK4).

Paper chromatography was attempted as an alternative to column chromatography. However, this method was rejected because the technique is very slow relative to thin layer chromatography (TLC) which produces the same result in $1/10^{th}$ of the time. Hence, TLC was adopted as the standard chromatographic technique.

Microorganisms used were *Bacillus subtilis B69* (Gram positive), *Xanthomonas campestris* (Gram negative), *Candida* spp., *Fusarium oxysporum, Pythium ultimum, Rhizoctonia solani* and *Sclerotinia sclerotiorum.* The bacteria were subcultured onto Nutrient Agar (NA) plates and the fungi onto Potato Dextrose Agar (PDA) plates and used against the various extracts. All isolates were preserved and deposited in the Plant Protection Research Institute (PPRI – 600 Soutpansberg Road, Rietondale, Pretoria).

Maintenance of test organisms:

The bacterial cultures were transferred onto nutrient agar slopes and the fungal cultures onto PDA slopes. Fresh slopes were made from the original once a month. Inoculation of agar plates was done with the original cultures. The cultures on agar plates were kept air-sealed with parafilm to prevent contamination. Cultures were also stored in sterilized distilled water. This method of storage was most suitable for viability of *P. ultimum.*

Bioautography method:

This method takes advantage of TLC to separate chemically diverse mixtures found in crude plant extracts. TLC bioautography is best suited to resolving extracts that contain difficult-to-separate lipophilic natural products. Biologically active metabolites can be readily located on the plates by visually observing clear zones where active compounds inhibit fungal or bacterial growth. This method eliminates the need for the development of large numbers of plates in multiple solvent systems, reduces the amount of waste solvents for disposal, and substantially reduces the time needed to identify active compounds.

Procedure: TLC plates (10 X 10cm) were loaded with 100µg of each of the extracts preferably of the same final concentration (usually 10-20mg/ml), with a graduated micropipette. The prepared plates were each developed in separate mobile systems. The TLC plates were dried at room temperature under a stream of air overnight to remove the remaining solvent. Ten ml of dense bacterial culture were added to two test tubes and centrifuged at a high centrifugal force (3030 x g) for 20min to concentrate the bacteria. After discarding the supernatant, the pellet was visible at the bottom of the tube. The sedimented bacterial pellet was resuspended in fresh nutrient broth (started with small volume, mixed on the vortex shaker and then added more medium). For fungi, a spore suspension was made by adding 10ml sterile distilled water to the surface of a Petri dish covered with fungi. The plate was swirled around so that the spores were dislodged from the hyphae and dispersed into the water. The TLC plates were covered with a fine spray of the bacterial suspension. The same method was attempted with fungal suspensions. The plates were sprayed until they were just wet, and incubated overnight at 30°C in a clean chamber at 100% relative humidity. The following day plates were sprayed with a 2mg/ml solution of INT [p-iodonitrotetrazolium violet (3-{4-iodo-phenyl}-2-{4-nitrophenyl}-5-phenyl-2H-tetrazolium chloride)] (SIGMA). Clear zones on the chromatograms indicated inhibition of growth after 30min to 2h.

Agar well diffusion assay:

Bacteria: The bacteria were grown on nutrient agar and in nutrient broth. These microorganisms were incubated at 30°C overnight. Zero point one ml of bacterial suspension was spread plated over the surface of nutrient agar plates. A well was made by flaming the back of a glass Pasteur pipette and making a hole in the middle of the nutrient agar plate. The well was filled with 0.1g of extract dissolved in 1ml of the extracting solvent. The plates were done in duplicate and incubated at 30°C overnight. Dichloromethane, which was the

extracting solvent, was used as a control to determine if the solvent inhibited the growth of the microorganism in any way.

Fungi: A well was made by flaming the back of a glass Pasteur pipette and making a hole in the middle of a PDA plate. Small blocks of the fungal test cultures, measuring 5mm x 5mm, were cut and placed on opposite sides of the well at the far edge of the Petri dish. This was done so that the fungi would grow at the same rate, coming towards the well, and if there is any biological activity, a clear almost round zone would form by the fungi, while the rest of the plate would be covered with the fungus. The well was then filled with 0.1g of extract dissolved in 1ml of the extracting solvent. The plates were incubated at 30°C overnight. Dichloromethane, which was the extracting solvent, was used as a control to determine if the solvent inhibited the growth of the microorganism in any way.

Trench method:

This method was done in exactly the same manner as the agar well diffusion assay. The only difference was instead of making a well in the middle, a trench was made. This was done by cutting out a thin strip of agar, about 3mm wide, in the middle of the plate along the whole length. A solvent volume of 10ml was added to 1g of plant extract. The trench was then filled with 1ml of the extract dissolved in the extracting solvent. The plates were done in duplicate and incubated at 30°C overnight.

Scale:

The results were expressed as a scale in terms of the radius of the inhibition zone:
< 3mm, inactive; 3 - 6mm, partially active; 7 - 10mm, active; > 10mm, very active.

Antifungal activity with bioautography:

The extracts (1mg) were dissolved in a volatile solvent (100µl) and an aliquot (10µl) of each plant extract spotted onto TLC plates. Spore suspensions of the fungi were made by inoculating the different fungi into flasks containing nutrient media. However, this did not work as the fungi formed round pellets when incubated on a rotary shaker. A second attempt for a fungal suspension was made by adding 10ml of a 0.1% Tween solution to a fully grown PDA plate with the organism and scratching the surface of the plate lightly to obtain the spores. However, this did not work as the hyphae had blocked the nozzle of the spray and a

fine mist could not be evenly sprayed over the TLC plate. The bioautographic assay with fungi was changed for the agar well diffusion method.

Paper disc method:

Filter paper discs were sterilized by autoclaving at 121°C and saturated with the test plant extracts. It was observed that 0.1ml was enough to wet the discs. The discs were then transferred onto nutrient agar plates where the test organisms were already spread plated. The impregnated filter discs were placed on opposite sides of the plate. Two different plant extracts were tested in one Petri dish. The plates were left for 1h to allow diffusion to take place and thereafter incubated at 25°C for 48h.

Fractionation:

The fractionation process involved column and thin layer chromatographic techniques. Different sized columns, ranging from 1.5 – 6cm in diameter, were used depending on the amount of sample and the purification stage. Silica gel chromatography was conducted using Silica gel 60 (0.063 – 0.2mm) (Merck).

Thin layer chromatography was carried out on pre-coated silica aluminium-backed plates. The plates were first viewed under UV, developed using a vanillin:concentrate H_2SO_4 (1g:100ml) spray reagent and then heated.

Structure elucidation:

Pure compounds were identified and characterized by mass spectrometry and NMR experiments. The pure compounds are based on detailed studies of the high field ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) and the following two-dimensional (2D) NMR techniques (Croasmun and Carlson, 1987; Saunders and Hunter, 1987).

The proton-proton connectivity patterns were established by 2D (^{1}H, ^{1}H) correlation (COSY) experiments. The multiplicities of the different resonances in the 13C spectra were deduced from the proton-decoupled CH, CH_2 and CH_3 subspectra obtained using the DEPT (distortionless enhancement by polarization transfer) pulse sequence. The ^{13}C resonances were partly assigned by correlation of the proton-bearing carbon atoms with specific proton resonances in 2D (^{13}C, ^{1}H) heteronuclear single quantum correlation (HSQC) experiments.

The assignment of the quartenary carbon atoms and the deduction of the long-range (more than one bond) connectivity pattern was facilitated by 2D (^{13}C, ^{1}H) heteronuclear multiple bond correlation (HMBC) experiments. Correlations observed in nuclear Overhauser effect spectroscopy (NOESY) experiments provided information on the relative stereochemistry of the compounds.

2.3 Results

Paper disc method:

The paper disc method was unsuccessful because of the limited diffusion of the plant extract from the paper disc into the solid agar. There were no zones of inhibition as a result of this.

Trench method with dichloromethane extract of P. fruticosus:

The bioassay results show that while all of the other test microorganisms showed some form of inhibition, only *B. subtilis* and *R. solani* displayed significant biological ativity. The largest zone of inhibition observed was 12–15mm when *P. fruticosus* was tested against *R. solani*. No inhibition was noted when *P. fruticosus* was tested against *F. oxysporum* and *S. sclerotiorum.*

B. subtilis formed a zone of inhibition, termed as a 'halo' effect. A halo effect occurs when there is no complete inhibition of the organism against the test plant extract and growth of the organism is irregular. This was depicted by less growth for about 4mm around the trench, thereafter a clear zone for 4mm, sparse growth for 5mm and then dense growth towards the edge of the plate (Table 2.1).

Table 2.1 Biological activity of dichloromethane extract of *P. fruticosus* against various microorganisms using the trench method.

Microorganism	Zone of inhibition (mm)	Notes / Description
B. subtilis	4 – 13	Halo effect. Partially to very active.
X. campestris	4	Partially active.
F. oxysporum	No Inhibition	Inactive.
R. solani	12 – 15	Very active.
S. sclerotiorum	No Inhibition	Inactive.
Candida spp.	3 – 10	Partially active to active.

Agar well diffusion method with dichloromethane extract of P. fruticosus:

When *P. fruticosus* was tested against the test fungal pathogens using the well method, no inhibition was noted. However, this extract did possess some antibacterial activity but no antifungal activity (Fig 2.3 and Table 2.2).

Table 2.2 Biological activity of a dichloromethane extract of *P. fruticosus* against various microorganisms using the well method.

Microorganism	Zone of inhibition (mm)	Notes / Description
B. subtilis	2 – 5	Inactive to partially active.
X. campestris	5 – 10	Partially active to active.
F. oxysporum	No Inhibition	Inactive.
R. solani	No Inhibition	Inactive.
S. sclerotiorum	No Inhibition	Inactive.
Candida spp.	No Inhibition	Inactive.
P. ultimum	No Inhibition	Inactive.

Bioassay plates with *P. fruticosus*:

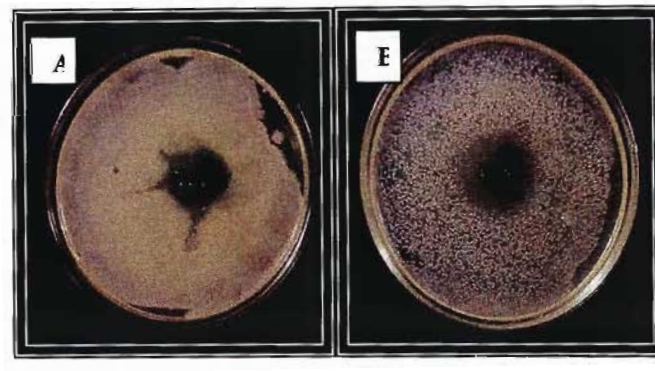


Fig 2.3 Bioassay plates showing biological activity of dichloromethane extract of *P. fruticosus* against *B. subtilis* (A) and *X. campestris* (B), using the agar well diffusion assay.

Trench method with dichloromethane extract of P. ecklonii:

There were no zones of inhibition when *P. ecklonii* was tested against *B. subtilis* and *F. oxysporum.* Growth was rampant all over the plate. The smallest inhibition zone was noted when *X. campestris* was tested, producing a zone of 1–6mm. Fairly large inhibition zones were observed for *R. solani, S. sclerotiorum* and *Candida* spp. The largest zone, which was 35mm, was formed when *P. ecklonii* inhibited *Candida* spp. (Table 2.3).

Table 2.3 Biological activity of a dichloromethane extract of *P. ecklonii* against various microorganisms using the trench method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	No Inhibition	Inactive.
X. campestris	1 – 6	Inactive to partially active.
F. oxysporum	No Inhibition	Inactive.
R. solani	5 – 15	Partially active to very active.
S. sclerotiorum	4 – 19	Partially active to very active.
Candida spp.	5 – 35	Partially active to very active.

Well-method with dichloromethane extract of P. ecklonii:

There was little or no inhibition produced by the crude extract of *P. ecklonii* when tested against the fungal pathogens *F. oxysporum, R. solani, S. sclerotiorum and P. ultimum.* When plated against *Candida* spp., a very slight inhibition zone of 1mm could be seen. *B. subtilis* also seemed to have about the same level of effect as *Candida* spp. and *S. sclerotiorum* on this extract, producing a zone of 3mm. The largest zone of inhibition produced was 20mm, when the plant extract was tested against *X. campestris* (Table 2.4).

Table 2.4 Biological activity of dichloromethane extract of *P. ecklonii* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	3	Partially active.
X. campestris	2 – 20	Very active.
F. oxysporum	No Inhibition	Inactive.
R. solani	No Inhibition	Inactive.
S. sclerotiorum	3	Slight inhibition - partially active.
Candida spp.	1	Inactive.
P. ultimum	No Inhibition	Inactive.

Bioassay plates with *P. ecklonii*:

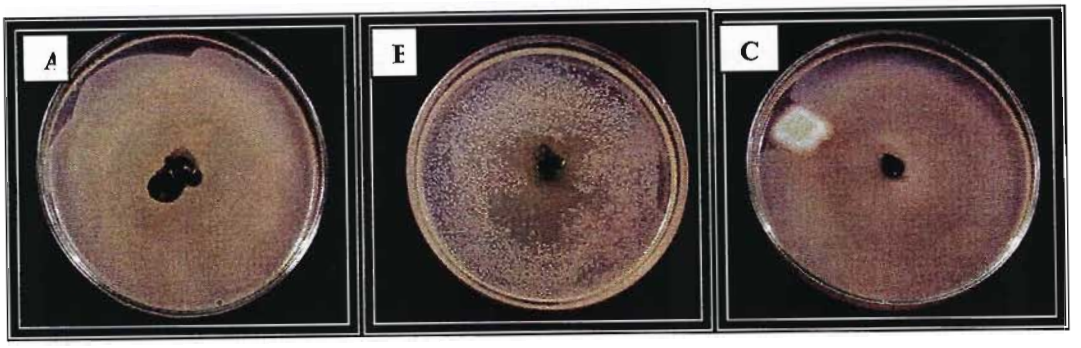
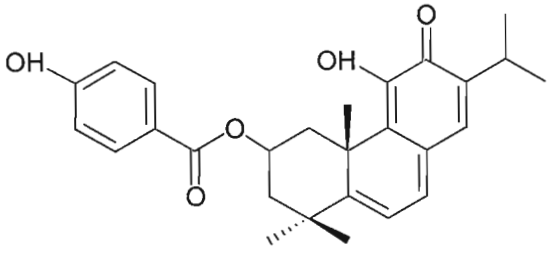


Fig 2.4 Bioassay plates showing biological activity of *P. ecklonii* by producing inhibition zones against *B. subtilis* (A) and *X. campestris* (B). There was a slight inhibition zone which can hardly be discerned, produced by *P. ecklonii* when tested against *S. sclerotiorum* (C).

Structure elucidated of pure compound of *P. ecklonii:*



1

Fig 2.5 Chemical structure of pure compound **1** (EK4) isolated from *P. ecklonii.*

Compound **1,** 11-Hydroxy-2-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one, known as Parviflorone D, is deep red in colour and has previously been isolated from *P. parviflorus* (Rüedi and Eugster, 1978).

Bioautography allowed for the detection of spots of growth inhibition after a broth culture of *B. subtilis* was sprayed directly onto the TLC plate. Spots of growth inhibition is indicated by zones of clearing around the individual bands (pure compounds) or around the whole extract. This procedure also allowed for the determination of antimicrobial activity which is due to compounds of probable terpenoid origin.

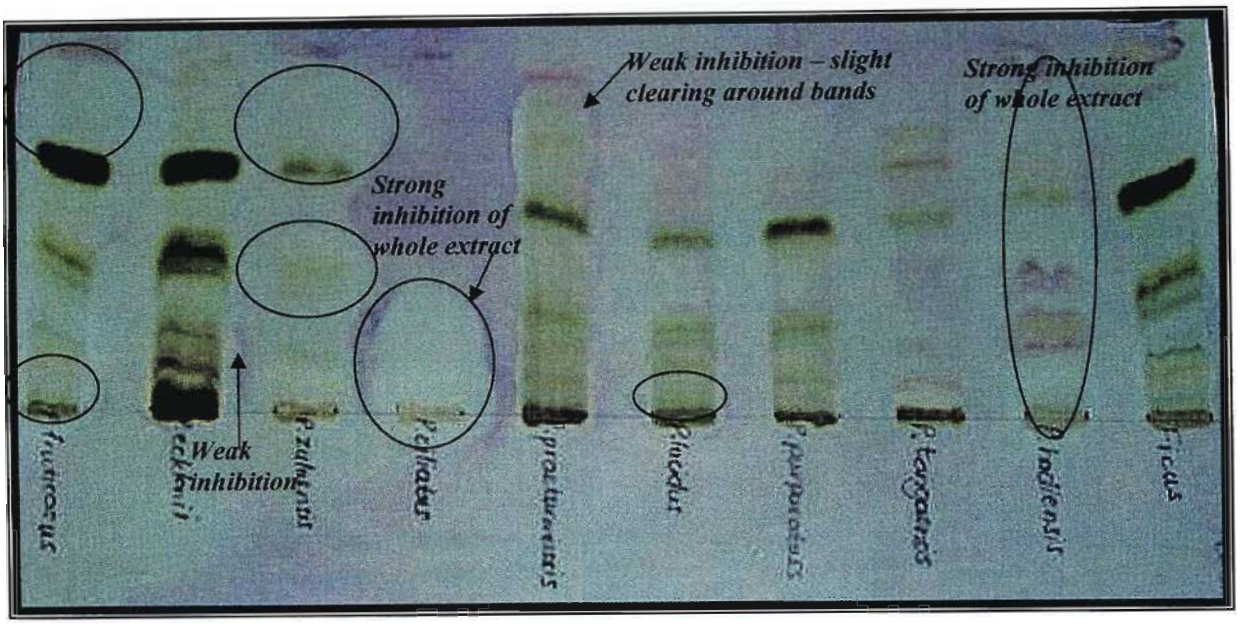


Fig 2.6 TLC fingerprint shows zones of inhibition produced by the crude extracts of all the *Plectranthus* species tested against *B. subtilis* using bioautography.

2.4 Discussion

In this chapter, *P. fruticosus* and *P. ecklonii* were evaluated for biological activity, and to determine if the trench and well method produced the same results. When *P. fruticosus* was tested against microorganisms using the trench method, it was found that this extract was very active against *B. subtilis* and *R. solani*. When the same plant extract was tested using the well method, moderate antibacterial and poor antifungal activity was exhibited. This information, testing the same plant extract but using two different methods showed that the results were inconsistent.

Good biological activity was observed when *P. ecklonii* was tested against the *Candida* spp. using the trench method. This extract also inhibited *R. solani* and *S. sclerotiorum*. Antibacterial activity ranged from inactive to partially active. By testing this same extract using the well method, the only zone of inhibition produced was with *X. campestris*. No antifungal activity was noted. This emphasises the above point which states that the two different agar diffusion methods produce different results.

It can be observed from Table 2.1 and 2.2 that when the trench method was used, higher biological activity was noted than when the well diffusion assay was used. This could be due

to 1ml of plant extract inserted into the trench and 0.1ml for the well. The same volume of extract to solvent was used for both bioassays, but different volumes were used. This was because it took a much smaller volume to fill the well than the trench. Hence, it can be said that the dosage plays an important role in evaluating how biologically active the extract is. For consistent results and deductions, only one bioassay method, the agar well diffusion assay was used.

The bioautography assay was used to detect and activity–guide the fractionation of antimicrobial compounds. This method only seemed to work when *B. subtilis* was sprayed on the plate (refer to Fig 2.6 for TLC fingerprint). When *X. campestris* was made as a bacterial suspension, the nozzle of the spray repeatedly blocked due to the slimy nature of the bacterium. The blocking of the nozzle of the spray also occurred when spore suspensions (including spores and hyphae) of the fungi were made. This caused large droplets to fall onto the TLC plate, instead of a fine mist, and the separated bands on the TLC plate ran into each other. Hence, no results were obtained when these plates were sprayed with *X. campestris, Candida* spp., *S. sclerotiorum, P. ultimum, R. solani* or *F. oxysporum.* The TLC fingerprint (Fig 2.6) shows a zone of clearing around the lower bands of *P. fruticosus* and *P. ecklonii* when the plate was sprayed with *B. subtilis* and stained. This result is consistent with the trench and well diffusion methods. Hence, it can be concluded that the plant extracts, *P. fruticosus* and *P. ecklonii* do possess antibacterial properties.

According to Rios *et al.* (1988), many factors can influence the results of bioassays. These include the extraction method, inocula volume, culture medium composition, pH and incubation temperature and blocking of the spray nozzles (Robert, 1999).

2.5 Literature cited

Chadya, M. and Gundidza, M. 1999. Extraction, quantification, structure elucidation and biological activities of selected essential oils from Zimbabwe. Honours Project Publications, Department of Pharmacy, University of Zimbabwe, Africa.

Croasmun, W.R. and Carlson, R.M.K. 1987. Two Dimensional NMR Spectroscopy: Applications for Chemists and Biochemists. VCH Publishers, New York.

Gaspar-Marques, C., Simões, M.F., Duarte, A. and Rodríguez, B. 2003. Labdane and kaurane derivatives from *Plectranthus fruticosus*. *Journal of Natural Products* 66: 491–496.

Nyanyiwa, M. and Gundidza, M. 1999. Antimicrobial activity of essential oils of *Ageratum conzoides*, *Plectranthus ecklonii*, *Conyza scabrida* and *Conyza bonariensis*. Student Publications, Pharmacy Department, University of Zimbabwe.

Pages, N., Fournier, G., Chamorro, G. and Salazar, M. 1991. Teratogenic effects of *Plectranthus fructicosus* essential oil in mice. *Phytotherapy Research* 5: 94–96.

Pages, N., Salazar, M., Chamorro, G., Fournier, G., Paris, M., Dumitresco, S.M. and Boudene, C. 1998. Teratogenic effects of *Plectranthus fructicosus* essential oil in mice. *Planta Medica* 54: 296–298.

Prudent, D., Perineau, F., Bessiere, J.M., Michel, G.M. and Baccou, J.C. 1995. Analysis of the essential oil of wild oregano from Martinique (*Coleus aromaticus* Benth.): evaluation of its bacteriostatic and fungistatic properties. *Journal of Essential Oil Research* 7: 165–173.

Rios, J.l., Recio, M.C. and Villar, A. 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. *Journal of Ethnopharmacology* 23: 127-149.

Robert, P.R. 1999. Antimicrobial resistance in Gram positive bacteria: The myth of the MIC. *Pharmacotherapy* 19: 112-119.

Roberts, M. 1990. Indigenous Healing Plants, Southern Book Publishers.

Rüedi, P. and Eugster, C.H. 1978. *Helvetica Chimica Acta,* 61: 709.

Omolo, M.O., Okinyo, D., Ndiege, I.O., Lwande, W. and Hassanali, A. 2004. Repellency of essential oils of some Kenyan plants against *Anopheles gambiae*. *Phytochemisty* 65: 2797–2802.

Saunders, J.M. and Hunter, B.K. 1987. Modern NMR Spectroscopy. A Guide for Chemists. Oxford University Press, Oxford.

Internet reference:

http://www.plantzafrica.com

Chapter 3

Biological activity of the crude extract of *Plectranthus hadiensis* Forssk. and the pure compounds isolated thereof

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

Compounds were extracted from *P. hadiensis* using dichloromethane and hexane. The dichloromethane extract contained much higher biological activity than the hexane extract. Three pure compounds, identified as diterpenes, were isolated from the crude dichloromethane extract of *P. hadiensis*. 6,7-Dihydroxyroyleanone-6,7,12-trihydroxy-8,12-abietadiene-11,14-dione (**2**) and 7α-formoxy-6β-hydroxyroyleanone (**3**) exhibited excellent antibacterial activity and antifungal activity on some of the test organisms. The remaining pure compound, 7α-acetoxy-6β-hydroxyroyleanone (**4**), possessed good antifungal activity. An inhibition zone measuring up to 14mm was noted when compound **4** was tested against *S. sclerotiorum*. The crude and all the pure compounds thereof, exerted great antifungal activity against *S. sclerotiorum*. When testing the hexane extract against the *Bacillus* formulations, the pellets that were suspended once in Ringer's solution produced bigger inhibition zones than the pellets that were suspended twice. This could be due to some of the bacterial cells washing out of the suspension.

3.1 Introduction

Research has been conducted worldwide on the use of botanical pesticides in plant disease control and extracts from many plant species have been found to have biological activity against phytopathogenic fungi (Berger and Mugoya, 1995).

In Zulu medicinal usage, infusions of *P. hadiensis* are used as sprinkling charms against evil spirits and are also administered as enemas but are not recommended for oral use as a cough medicine (Gerstner, 1938, 1941). Gerstner (1941) also reported it to have a strong smell and to be used as a fish poison.

P. hadiensis is easily distinguished from other *Plectranthus* species by the presence of coloured glands and its more erect habitat. In some places, particularly Angola, S. Malawi and E. Zimbabwe, *P. hadiensis* can have similar leaves to *P. porphyranthus*, with few dentations (Edwards *et al.*, 2000).

Miyase *et al.* (1977) stated, "*From leaf-glands of the South-African P. myrianthus (Labiatae) the following diterpenoids have been isolated and their structures established: coleon U, $C_{20}H_{26}O_5$ (6, 11, 12, 14-trihydroxy-abieta-8, 11, 13-tetraene-7-one; coleon V, $C_{20}H_{26}O_5$ (11, 12, 14-trihydroxy-abieta-8, 11, 13-triene-6, 7-dione; coleon W, $C_{22}H_{28}O_8$ (16(or 17)-acetoxy-6, 11, 12,14, 17 (or 16)-pentahydroxy-abieta-5,8,11,13-tetraene-7-one; 14-O-formyl-coleon-V, $C_{21}H_{26}O_6$ (14-formyloxy-11, 12-dihydroxy-abieta-8, 11, 13-triene-6, 7-dione; 7α-formyloxy-6β-hydroxyroyleanone. $C_{21}H_{26}O_6$ (7α-formyloxy-6β. 12-dihydroxy-abieta-8.12-diene-11.14-dione; the already known 6β.7α-dihydroxyroyleanone and a dimeric abietane derivative whose structure is not yet elucidated. This is the first record of a co-occurrence of coleons and royleanones in the same plant.*" *P. hadiensis* must be closely related to *P. myrianthus* because they contain the same compounds (pers. comm., Prof Drewes[2]).

Fig 3.1 Trailing species of *Plectranthus hadiensis var. tomentosus* (www.rhs.org.uk)

3.2 Materials and methods

The plant material was collected from the Botanical Gardens, Pietermaritzburg. Voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. All chemical extractions and structural identification for *P. hadiensis* was

done by F. Khan[2]. The aerial parts of *P. hadiensis* were air-dried and extracted successfully using dichloromethane and then hexane. The crude dichloromethane extract was subjected to silica gel column chromatography using 100% chloroform to yield a total of 5 pooled fractions. The third fraction was re-chromatographed on a silica gel column and eluted with 2:1 (hexane:ether). Two pure compounds were generated, with yellow needles from compound **2** and **3**, both showing crystalline properties. In addition, the yellow-orange needles of compound **4** were isolated and its structure established. This silica gel column was eluted with 85:15 (hexane:ether). These compounds were bioassayed against the test organisms. All compounds were subjected to NMR analysis.

Bioassay testing of *P. hadiensis*

The crude hexane and dichloromethane extract, including the pure compounds **2**, **3** and **4** of *P. hadiensis,* was screened for antifungal and antibacterial activity against one Gram-positive bacterium (*Bacillus subtilis*), one Gram-negative bacterium (*Xanthomonas campestris*), a yeast (*Candida* spp.) and four fungi, (*Fusarium oxysporum, Pythium ultimum, Rhizoctonia solani* and *Sclerotinia sclerotiorum).*

Refer to Chapter 2 for methodology on well agar diffusion assays.

***Bacillus* Formulations**

Bacillus isolates were grown in tryptone soy broth in the shaker incubator for 45–48h. Thereafter, the broth was centrifuged for 15min at 10,000 RPM. The suspension was referred to as the 'E' pellet. The pellets were then resuspended in Ringer's solution and centrifuged again. The second suspension was referred to as 'EE'. These isolates were spread plated onto nutrient agar plates and tested with the crude hexane extract of *P. hadiensis* using the agar well diffusion assay.

3.3 Results

The crude dichloromethane extract of *P. hadiensis* exerted the same level of activity against *S. sclerotiorum* and *Candida* spp., producing inhibition zones of 16mm and 15mm respectively. The biological activity of the extract on *B. subtilis* and *X. campestris* were also similar, producing inhibition zones of 8mm and 10mm, respectively. This extract had no antifungal activity against *F. oxysporum* and *R. solani*, as there were no zones of inhibition produced (Table 3.1).

Table 3.1 Biological activity of the crude dichloromethane extract of *P. hadiensis* against various microorganisms using the agar well diffusion assay.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	5 – 8	Active.
X. campestris	7 – 10	Active .
F. oxysporum	No Inhibition	Inactive.
R. solani	No Inhibition	Inactive.
S. sclerotiorum	3 – 16	Very active.
Candida spp.	7 – 15	Very active.
P. ultimum	0 – 5	Inactive.

- The biological activity of the extract against the specific organism is classified according to the scale (Chapter 2). The scale is based on the basis that the larger the zone of inhibition, the more biologically active the extract is against the specific organism.

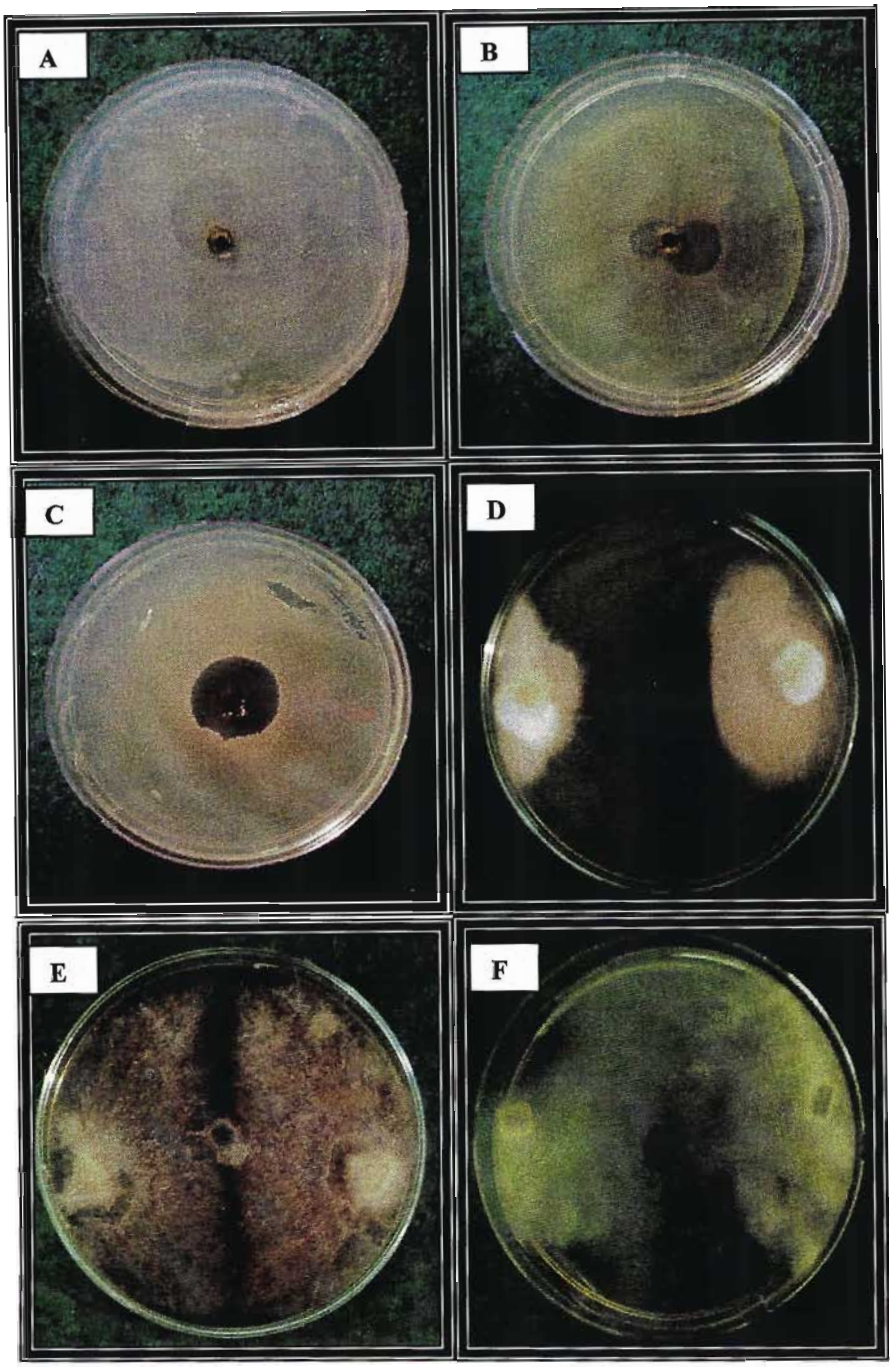


Fig 3.2 The bioassay plates show the biological activity of a crude dichloromethane extract of *P. hadiensis* against A – *Candida* spp., B – *X. campestris*, C – *B. subtilis*, D – *S. sclerotiorum*, E – *R. solani* and F – *P. ultimum*. On Plates A, B, C and D, clear zones of inhibition can be seen. On Plate E, *R. solani* has grown over the entire plate; hence, there is no inhibition.

The crude hexane extract of *P. hadiensis* showed no biological activity against most of the microorganisms, except *R. solani* and *S. sclerotiorum* (Table 3.2). This extract seems to exhibit specific antifungal activity but no antibacterial activity.

Table 3.2 Biological activity of crude hexane extract of *P. hadiensis* against various microorganisms using the agar well diffusion assay.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	No Inhibition	Inactive.
X. campestris	No Inhibition	Inactive.
F. oxysporum	No Inhibition	Inactive.
R. solani	12 – 22	Very active.
S. sclerotiorum	12 – 15	Very active.
Candida spp.	No Inhibition	Inactive.
P. ultimum	No Inhibition	Inactive.

Pure compound **2**, isolated from the crude dichloromethane extract of *P. hadiensis*, can be seen as a very active antibacterial and antifungal extract following the results (Table 3.3), against certain microorganisms. No inhibition was recorded against *F. oxysporum*. This pure compound is partially active against *Candida* spp.

Table 3.3 Biological activity of pure compound **2** against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	15 – 16	Very active.
X. campestris	10 – 11	Very active.
F. oxysporum	No Inhibition	Inactive.
R. solani	2 – 10	Very active.
S. sclerotiorum	10 – 11	Very active.
Candida spp.	3 – 5	Partially active.
P. ultimum	3 – 6	Partially active.

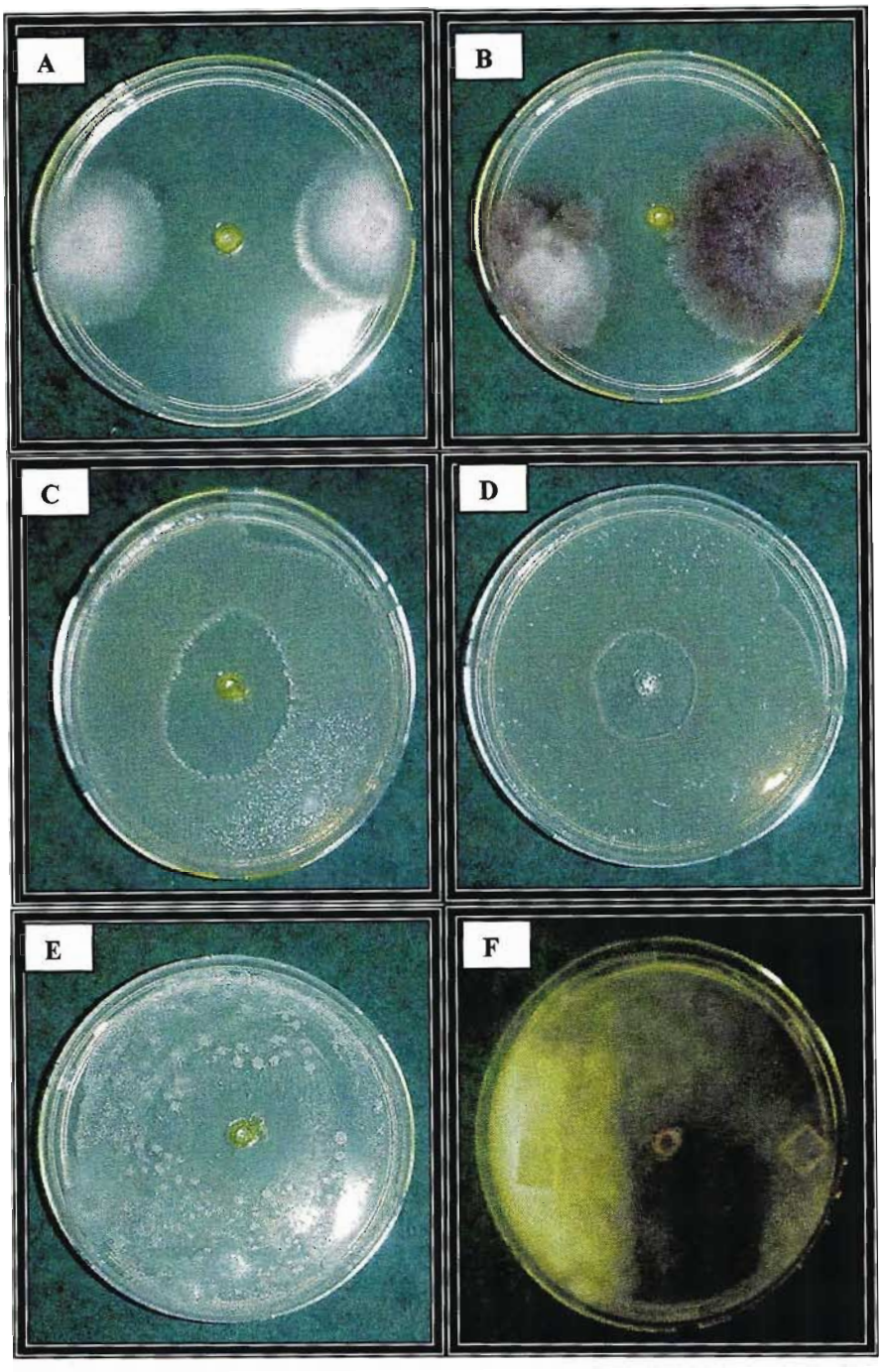


Fig 3.3 Plates showing the biological activity of pure compound **2** against: A – *S. sclerotiorum*, B – *R. solani*, C – *B. subtilis*, D – *X. campestris*, E – *Candida* spp. and F – *P. ultimum*. Plates A, B and F display good antifungal activity. Excellent antibacterial activity is seen on Plates C and D and these are depicted by the clear zones of inhibition.

This plant extract proved to be potent as there was total inhibition when pure compound **3** was tested against *X. campestris*. There was also very good activity against *B. subtilis*, *R. solani*, *Candida* spp., *P. ultimum* and *S. sclerotiorum*, with the latter producing the largest inhibition zone (Table 3.4).

Table 3.4 Biological activity of pure compound **3,** against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	10 – 11	Halo effect. Very active.
X. campestris	Total Inhibition	Very active.
F. oxysporum	No Inhibition	Inactive.
R. solani	1 – 5	Partially active.
S. sclerotiorum	10 – 17	Very active.
Candida spp.	5 – 9	Active.
P. ultimum	5 - 11	Active.

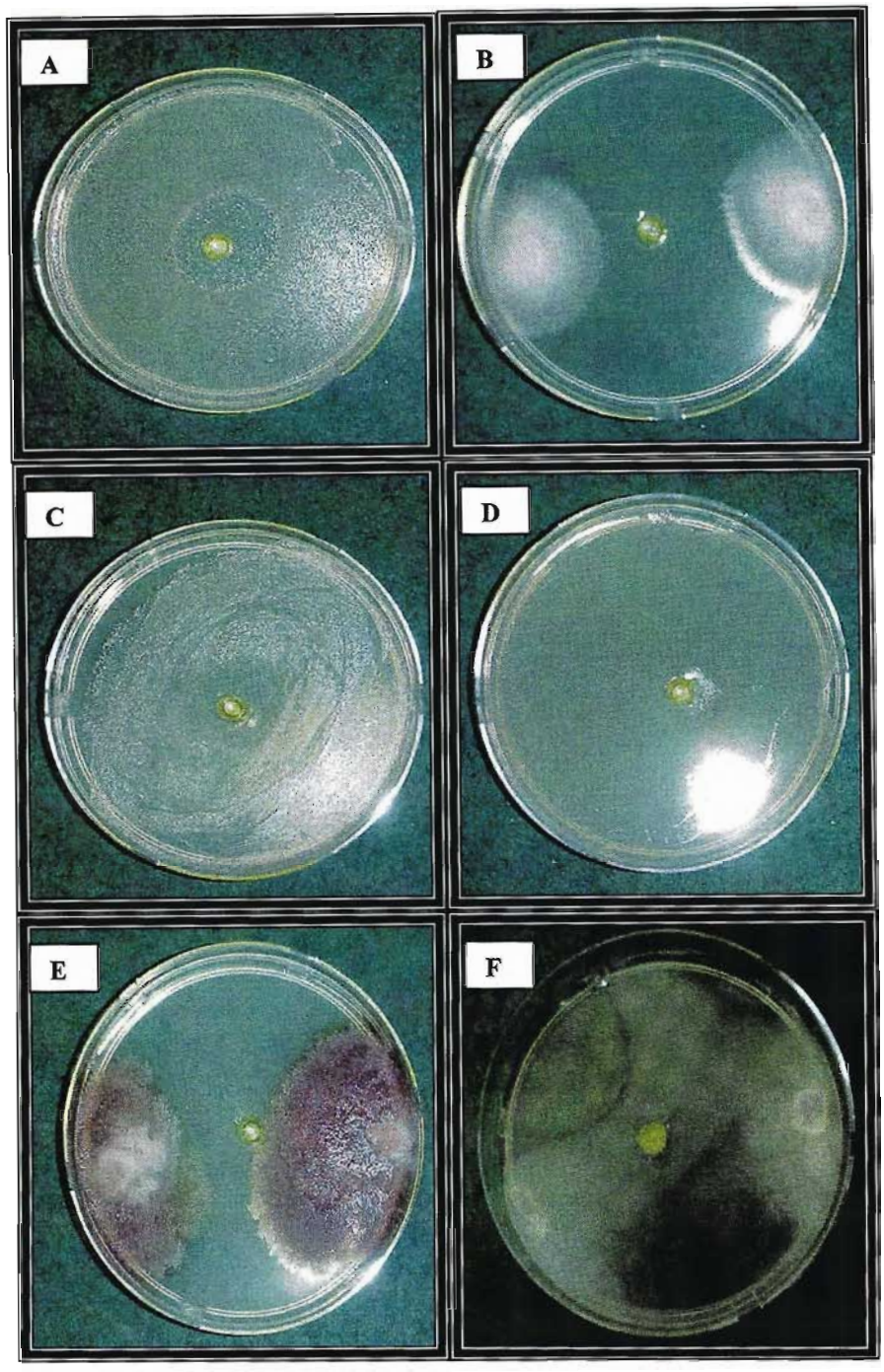


Fig 3.4 These plates show the bioassay results of the pure compound **3** against: A – *B. subtilis*, B – *S. sclerotiorum*, C – *Candida* spp., D – *X. campestris*, E – *R. solani* and F – *P. ultimum*. Plate A shows a distinct zone of inhibition, although there is still some growth of the organism within the zone. This effect is termed the halo effect. On Plate C a clear zone of inhibition is formed when this plant extract is tested against *Candida* spp. On Plate D there is total inhibition of *X. campestris* against this pure compound.

Pure compound **4** did not exhibit good biological activity overall. The only organism that was susceptible to this extract was *S. sclerotiorum*, producing a large inhibition zone of 9–14mm. *R. solani* covered two-thirds of the plate and the extract was rated as inactive. This compound produced a fairly small inhibition zone of 2–5mm when tested against *Candida* spp. No inhibition was recorded when tested against *B. subtilis, X. campestris, F. oxysporum* and *P. ultimum* (Table 3.5).

Table 3.5 Biological activity of pure compound **4**, against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	No Inhibition	Inactive.
X. campestris	No Inhibition	Inactive.
F. oxysporum	No Inhibition	Inactive.
R. solani	1 – 3	Inactive.
S. sclerotiorum	9 – 14	Very active.
Candida spp.	2 – 5	Partially active.
P. ultimum	No Inhibition	Inactive.

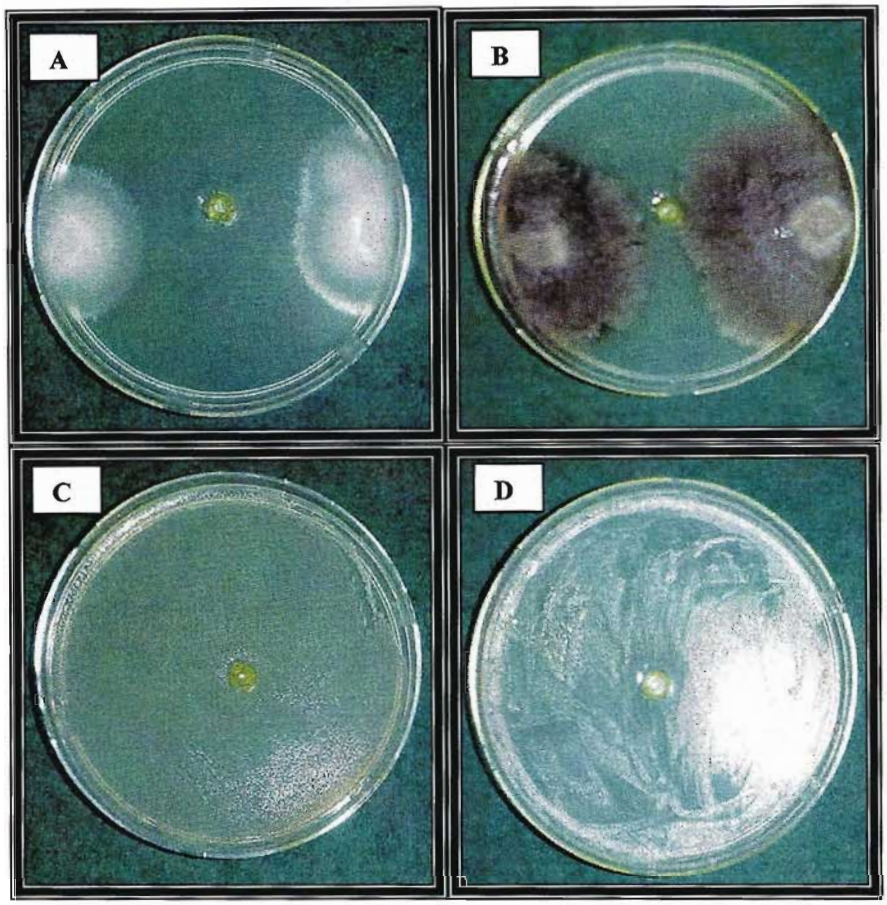


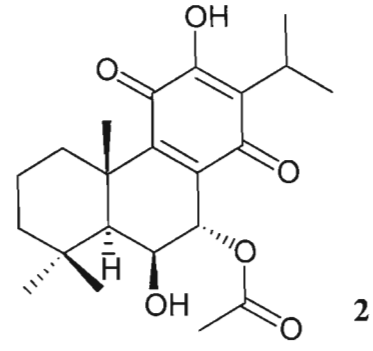
Fig 3.5 Bioassay plates showing biological activity of pure compound **4** against: A – *S. sclerotiorum*, B – *R. solani*, C – *B. subtilis* and D – *Candida* spp. Plate A depicts good antifungal activity of this compound against *S. sclerotiorum* whereas Plate B shows poor antifungal activity against *R. solani*. No biological activity was seen on Plate C against *B. subtilis*. There was slight inhibition by this compound against *Candida* spp. (Plate D).

Table 3.6 Shows biological activity of crude hexane extract of *P. hadiensis* against the different *Bacillus* formulations, *X. campestris* and *Candida* spp. using the agar well diffusion assay.

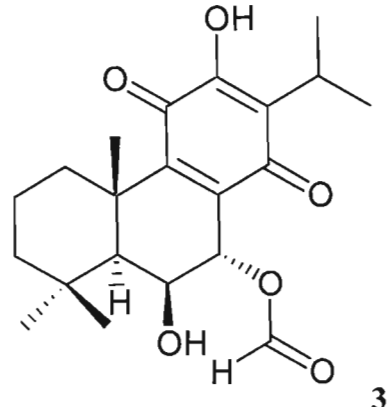
Bacillus formulation/ Microorganism	**Zone of inhibition (mm)**	***Biological activity**
B77 E	15 – 25	Very active.
B77 EE	2 – 5	Partially active.
B81 E	5 – 10	Active.
B81 EE	3 – 5	Partially active.
B69 E	5 – 15	Very active.
B69 EE	No Inhibition	Inactive.
B9 E	3 – 10	Active.
B9 EE	1 - 3	Inactive.
B11 E	10 - 15	Very active.
B11 EE	5 – 10	Active.
X. campestris	2 – 3	Inactive.
Candida spp.	6 – 7	Active.

*Biological activity was rated according to the scale described in Chapter 2.

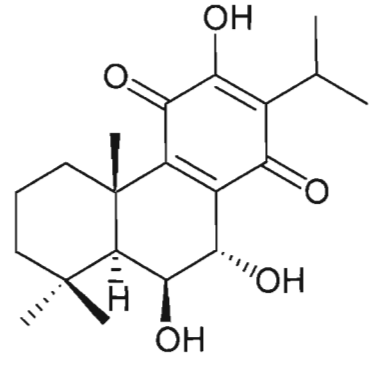
Structures elucidated of the pure compounds isolated from *P. hadiensis*:



2



3



4

Fig 3.6 Diterpene structures of pure compounds isolated from *P. hadiensis*

Compound **2**, 6,7-Dihydroxyroyleanone-6,7,12-trihydroxy-8,12-abietadiene-11,14-dione; compound **3**, 7α-formoxy-6β-hydroxyroyleanone and compound **4**, 7α-acetoxy-6β-hydroxyroyleanone, have been formerly isolated from *P. argentatus* and *P. myrianthus* (Miyase *et al.*, 1977 and Teixeira *et al.,* 1997).

Isolation of pure active constituents and elucidation of their structures is essential for the study of structure-activity relationship and the design of better synthetic drugs. The results in this section prove this clearly. The acyl group is important for antifungal activity, as its removal essentially eliminated toxicity to fungi (Oka *et al.,* 1988).

3.3 Discussion

The milled plant parts of *P. hadiensis* was extracted in dichloromethane and hexane and tested against various microorganisms. The crude dichloromethane extract exhibited stronger biological activity than the hexane extract. This could be due to hexane not being a suitable solvent for extraction of compounds from this plant. Heating of the plant extract should be discouraged, as it appears to reduce the activity of the plant extract against the test organisms. Compound **2** and compound **3** exhibited excellent antibacterial activity. The latter extract totally inhibited *X. campestris.* However, antifungal activity was limited to *S. sclerotiorum* and activity against the other fungi ranged from inactive to partially active. The results (Table 3.5) produced by compound **4** was the same as that of the crude hexane extract. Compound **4** is the main or one of the main diterpenes, and could be responsible for antifungal activity. No inhibition zones were recorded with the controls. This means that these organic solvents are not toxic to the pathogens and do not interfere with growth or inhibition.

In Fig 3.3, Plate A shows that although there was a distinct clear zone of inhibition, there was still some growth within the zone. This effect is termed the halo effect. This means that the extract does not totally inhibit the pathogen, but does decrease the rate of growth. This is reflected by the few colonies growing within the clearing compared to the dense growth of the bacteria outside the zone.

Bacillus Formulations

Plated out pellets (EE), which was resuspended in Ringer's, did not display as large inhibition zones as the initial suspensions (E) against the crude hexane extract of *P. hadiensis* (Table 3.6). This could be due to the bacterial cells washing out of the suspension when they were resuspended. The hexane extract was most active against B77E, producing a very large inhibition zone of 15–25mm.

3.5 Literature cited

Berger, A. and Mugoya, C.F. (edited by). 1995. Natural Plant Products as Pesticides. Proceedings from the First National Symposium in Zambia. Report 4. Swedish University of Agriculteral Sciences, Department of Plant Protection Services, Sweden.

Edwards, T.J., Paton, A. and Crouch, N.R. 2000. A new species of *Plectranthus* (*Lamiaceae*) from Zimbabwe. *Kew Bulletin* 55: 459-464.

Gerstner, J. 1938. A preliminary checklist of Zulu names of plants with short notes. *Bantu Studies* 12: 215-236.

Gerstner, J. 1941. A preliminary checklist of Zulu names of plants with short notes. *Bantu Studies* 15: 369-383.

Miyase, T., Ruedi, P. and Eugster, C.H. 1977. Leaf-gland Pigments: Coleons U, V, W, 14-0-formyl-coleon-V, and two royleanones from *Plectranthus myrianthus* BRIQ.; cis- and trans-A/B-6,7-dioxoroyleanones. *Helvetica Chimica Acta* 60: 2770-2771.

Oka, M., Numata, K., Nishiyama, Y., Kamei, H., Konishi, M., Oki, T. and Kawaguchi, H. 1988. Chemical modification of the antitumor antibiotic glidobactin. *Journal of Antibiotics* 41: 1812-1822.

Teixeira, A.P., Batista, O., Simoes, M.F., Nascimento, J., Duarte, A., dela Torre, M.C. and Rodriguez, B. 1997. *Phytochemistry* 44: 325.

Internet reference:

www.rhs.org.uk

Chapter 4

Biological activity found in *Plectranthus praetermissus* and *Plectranthus ciliatus* sensu Jacot Guillarmod

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

The dichloromethane extract of *P. praetermissus* proved to be very active against *X. campestris*, producing an inhibition zone of 8–20mm. Two pure compounds were isolated from the crude extract of *P. praetermissus*, 20(10→5)-abeo-1(10),6,8,11,13-abietapentaene-11,12,16-triol (**5**), and 11,12,15-trihydroxy-20-(10→5)-abeo-abieta-1-(10),6,8,11,13-pentaene (**6**), which were identified as diterpenes. *P. cilatus* was extracted with chloroform and tested against various microorganisms for antifungal and antibacterial activities. This extract showed poor antibacterial and antifungal activity, with the exception of *S. sclerotiorum.*

4.1 Introduction

Several *Plectranthus* species have been used in traditional medicine. However, the biological activity of compounds of *Plectranthus* remains relatively unknown. Diterpenoids are the more common secondary metabolites in found in this genus. The majority of them are highly modified abietanes (Cerqueira *et al.*, 2004). The diterpenes have been reported to have broad spectrum of biological activities (Chinou, 2005).

P. ciliatus is a low, spreading shrub, ranging from 0.5m – 1.0m wide. It has colourful leaves all year long. In autumn, this species blooms with large spikes of lilac flowers. This species should not be kept in too dark a shade, or else the purple reverse of the foliage will not develop fully. It can grow indoors, but will probably not develop full foliage colour. The roots of *P. ciliatus* are used in traditional medicine (Cunningham, 1988). It is used in personal hygiene and to wash clothes and animal skins (Watt and Breyer-Brandwijk, 1962).

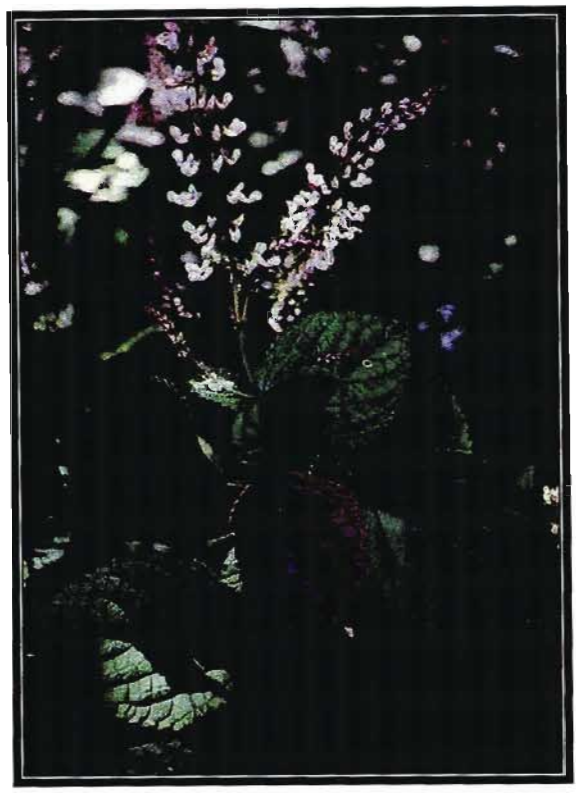


Fig 4.1 Foliage of *P. ciliatus* (www.plantsafari.com)

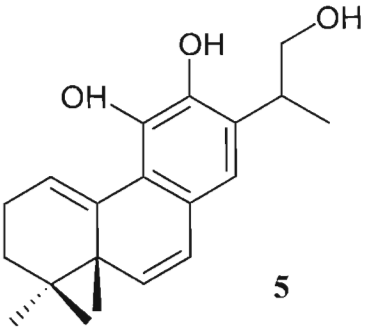
4.2 Materials and methods

The material was collected and voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg.

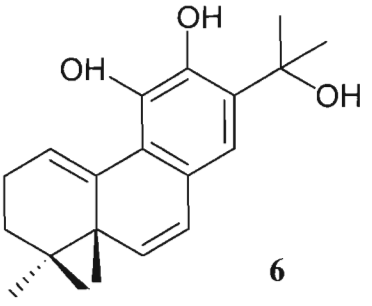
Extraction and isolation of the diterpenoids (done by F. Khan[2])

The whole plant of *P. praetermissus* was air dried and extracted with dichloromethane at room temperature. *P. ciliatus* was subjected to the same treatment but extracted with chloroform. Chloroform is a popular solvent, particularly for lipids of intermediate polarity (www.cyberlipid.org). The solvent was evaporated under vacuum and a thick oily extract resulted, which was subjected to column chromatography. A short chromatography column using silica gel was run using the crude extract of *P. praetermissus* and eluted with hexane:ethyl acetate (10:1). Five fractions were obtained, of which the fourth fraction was a deep reddish brown colour. When this fraction was spotted on a TLC plate, a single band appeared on the silica plate. It was purified further, identified and labelled as compound **5** (Fig 4.2). Repeated chromatography resulted in isolating another pure compound which was later identified by ^{1}H NMR as a diterpene (**6**).

Structure elucidated of pure compound from *P. praetermissus*:



5



6

Fig 4.2 Structures determined of the pure compounds of *P. praetermissus.*

Compound **5,** 20(10→5)-abeo-1(10),6,8,11,13-abietapentaene-11,12,16-triol, and compound **6,** 11,12,15-trihydroxy-20-(10→5)-abeo-abieta-1-(10),6,8,11,13-pentaene are both known compounds which have previously been isolated from *Salvia apiana* (González, 1992).

4.3 Results

P. praetermissus produced a very large zone of inhibition of 8–20mm when plated against *X. campestris*. With *B. subtilis* and *Candida* spp., although inhibition zones were observed, there was a halo effect. No inhibition zones were noted when this plant extract was tested against *F. oxysporum*, *R. solani* and *P. ultimum* (Table 4.1).

Table 4.1 Biological activity of crude dichloromethane extract of *P. praetermissus* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1 – 4	Inactive extract. Halo effect.
X. campestris	8 – 20	Very active extract.
F. oxysporum	No inhibition	Inactive extract.
R. solani	No inhibition	Inactive extract.
S. sclerotiorum	2 – 6	Partially active extract.
Candida spp.	2 – 15	Very active extract. Halo effect.
P. ultimum	No inhibition	Inactive extract.

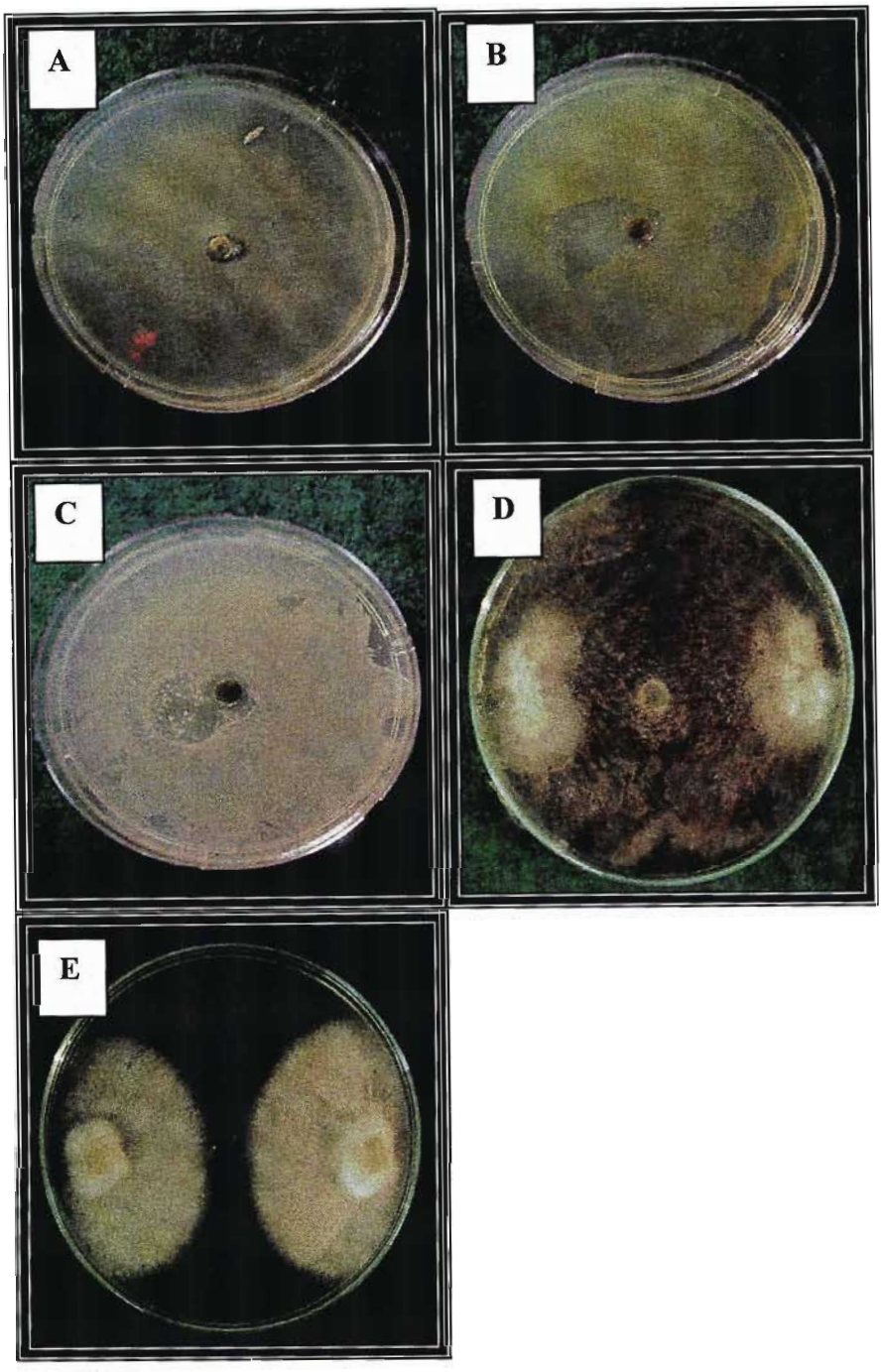


Fig 4.3 Bioassay plates showing biological activity of *P. praetermissus* against: *B. subtilis* (A), *X. campestris* (B) – zone of inhibition can be clearly seen, *Candida* spp. (C) – there is a halo effect, *R. solani* (D) – no inhibition, as fungus grows all over plate and *S. sclerotiorum* (E) – fungus covers 2/3 of plate.

F. oxysporum, R. solani, Candida spp., and *P. ultimum* were not inhibited by the crude chloroform extract of *P. ciliatus*. However, *S. sclerotiorum* showed a 6–12mm inhibition zone when tested against this plant extract. The agar well diffusion bioassay of the crude chloroform extract demonstrated a ring of inhibition against *B. subtilis* and *X. campestris* but inhibition proved to be only partially active.

Table 4.2 Biological activity of crude chloroform extract of *P. ciliatus* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	3 – 6	Partially active extract.
X. campestris	2 – 5	Partially active extract.
F. oxysporum	1	Inactive extract.
R. solani	No inhibition	Inactive extract.
S. sclerotiorum	6 – 12	Very active extract.
Candida spp.	No inhibition	Inactive extract.
P. ultimum	No inhibition	Inactive extract.

***P. ciliatus* bioassay pictures:**

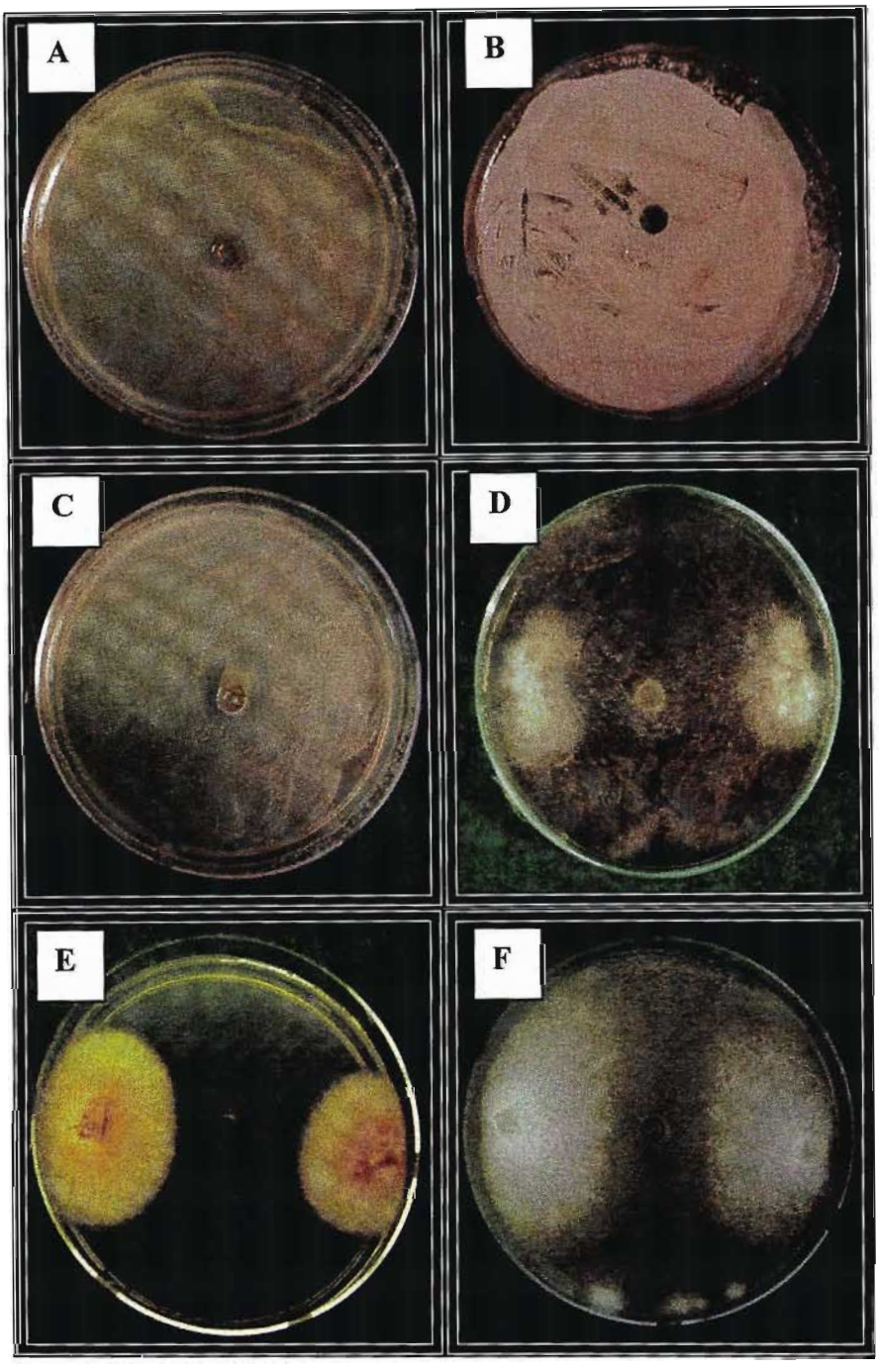


Fig 4.4 Bioassay plates showing biological activity of *P. ciliatus* extract against: *X. campestris* (A) – there is a zone of 2-5mm but not clearly visible, *Candida* spp. (B) – no inhibition, *B. subtilis* (C) – clear ring of inhibition around the well, *R. solani* (D) – no inhibition as fungus grows all over plate, *S. sclerotiorum* (E) – fungus covers 1/3 of plate and *F. oxysporum* (F) - fungus covers 2/3 of plate.

4.4 Discussion

The dichloromethane extract of *P. praetermissus* showed good antibacterial activity ranging from 8–20mm when tested against *X. campestris*. Excellent biological activity was also observed when tested against *Candida* spp. Two pure compounds, **5** and **6**, were isolated and identified as known diterpenes.

The chloroform extract of *P. ciliatus* was predominantly inactive, with the exception of *S. sclerotiorum,* producing an inhibition zone of up to 12mm. Chloroform was used for the extraction as we were interested in extracting terpenoids and flavonoids of this particular *Plectranthus* species. Several terpenoids, napthaquinones, flavonoids, alkaloids and glycosides are known to exert diverse biological activities (Ghisalberti and Lantana, 2000). Poor antibacterial activity was observed when this plant extract was tested against *B. subtilis* and *X. campestris*.

4.5 Literature cited

Cerqueira, F., Cordeiro-Da-Silva, A., Gaspar-Marques, C., Simoes, F., Pinto, M.M.M. and Nascimento, M.S.J. 2004. Effect of abietane diterpenes from *Plectranthus gradidentatus* on T- and B-lymphocyte proliferation. *Bioorganic and Medicinal Chemistry* 12: 217-223.

Chinou, I. 2005. Labdanes of natural origin-biological activities. *Current Medicinal Chemistry* 12: 1295-1317.

Cunningham, A.B. 1988. An Investigation of the Herbal Medicine Trade in Natal-KwaZulu. Investigational Report No. 29. Institute of Natural Resources, University of Natal, Pietermaritzburg.

González, A.G., Andrés, L.S., Aguiar, Z.E. and Luis, J.G. 1992. Diterpenes from *Salvia mellifera* and their biogenetic significance. *Phytochemistry* 31(4): 1297-1305.

Ghisalberti, E.L. and Lantana, C.L. 2000. Verbenaceae. *Fitoterapia* 71: 467-86.

Watt, J.M. and Breyer-Brandwijk, M.G. 1962. The medicinal and poisonous plants of Southern and Eastern Africa (2nd ed.). E. and S. Livingstone Ltd., London.

Internet references

www.cyberlipid.org
www.plantsafari.com

Chapter 5

Biological activity of the crude extract and pure compounds isolated from *Plectranthus zuluensis* Codd.

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

The crude dichloromethane extract of *P. zuluensis* exhibited good antibacterial activity. However, it was limited to the Gram negative organism. The extract produced an inhibition zone of 10-12mm when tested against *Xanthomonas campestris.* Two pure compounds, identified as terpenes, were isolated from this extract. 2-Hydroxy-4,6-dimethoxyacetophenone (**7**) and 1,2,4-trimethoxy-5-(2-propenyl)-benzene (**8**) exerted excellent inhibition against *B. subtilis* and *S. sclerotiorum.* However, both pure compounds did not inhibit *Candida* spp., *F. oxysporum* and *R. solani.*

5.1 Introduction

P. zuluensis is an upright medium shrub, growing up to 1m. The leaves have a sharp, pleasant odour. It bears baby blue flowers during warm weather (Fig. 5.1).

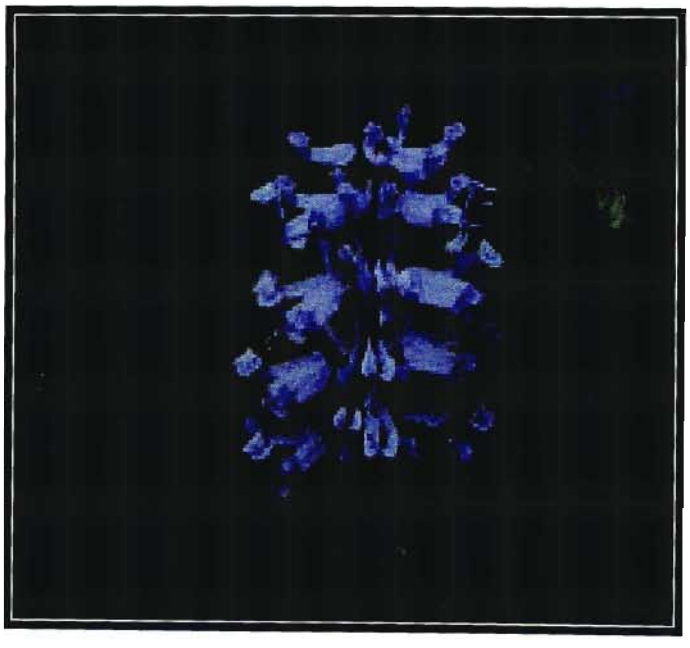


Fig 5.1 Flowers of *P. zuluensis* (www.oaklandnurseries.co.uk/)

5.2 Materials and methods

Extraction and isolation

The material was collected from the Botanical Gardens, Pietermaritzburg. Voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. All isolation and chemistry was done by F. Khan[2]. *P. zuluensis* was air-dried and extracted with dichloromethane at room temperature. The crude dichloromethane extract was subjected to silica gel column chromatography using the eluent chloroform to yield a total of 6 pooled fractions. The second fraction was chromatographed using a chromatatron and eluted with 20:1 (hexane:ether). Two pure compounds (**7** and **8**), were isolated from this fraction. Compound **7** was a yellow crystalline compound. Compound **8** was purified further by being subjected to silica gel column chromatography using a gradient eluent of increasing polarity 4:1 (hexane:ether).

Bioassay

Both pure compounds were tested on bacterial and fungal cultures. Refer to Chapter 2 for methodologies of the agar well diffusion method and autobiography.

5.3 Results

There were no inhibition zones produced by *R. solani* and *F. oxysporum* using the crude extract of *P. zuluensis*. This dichloromethane extract produced a large inhibition zone of 10-12mm when plated with *X. campestris,* but only a small zone of 1–2mm was noted when this plant extract was tested against *B. subtilis* (Table 5.1). This plant extract was active only against Gram negative but not Gram positive bacteria.

Table 5.1 Shows biological activity of crude dichloromethane extract of *P. zuluensis* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1 – 2	Inactive extract.
X. campestris	10 - 12	Very active extract.
F. oxysporum	No Inhibition	Inactive extract.
R. solani	No Inhibition	Inactive extract.
S. sclerotiorum	2 - 4	Inactive extract.
Candida spp.	8	Active extract.
P. ultimum	2 – 7	Partially active extract.

Plates showing bioassay results:

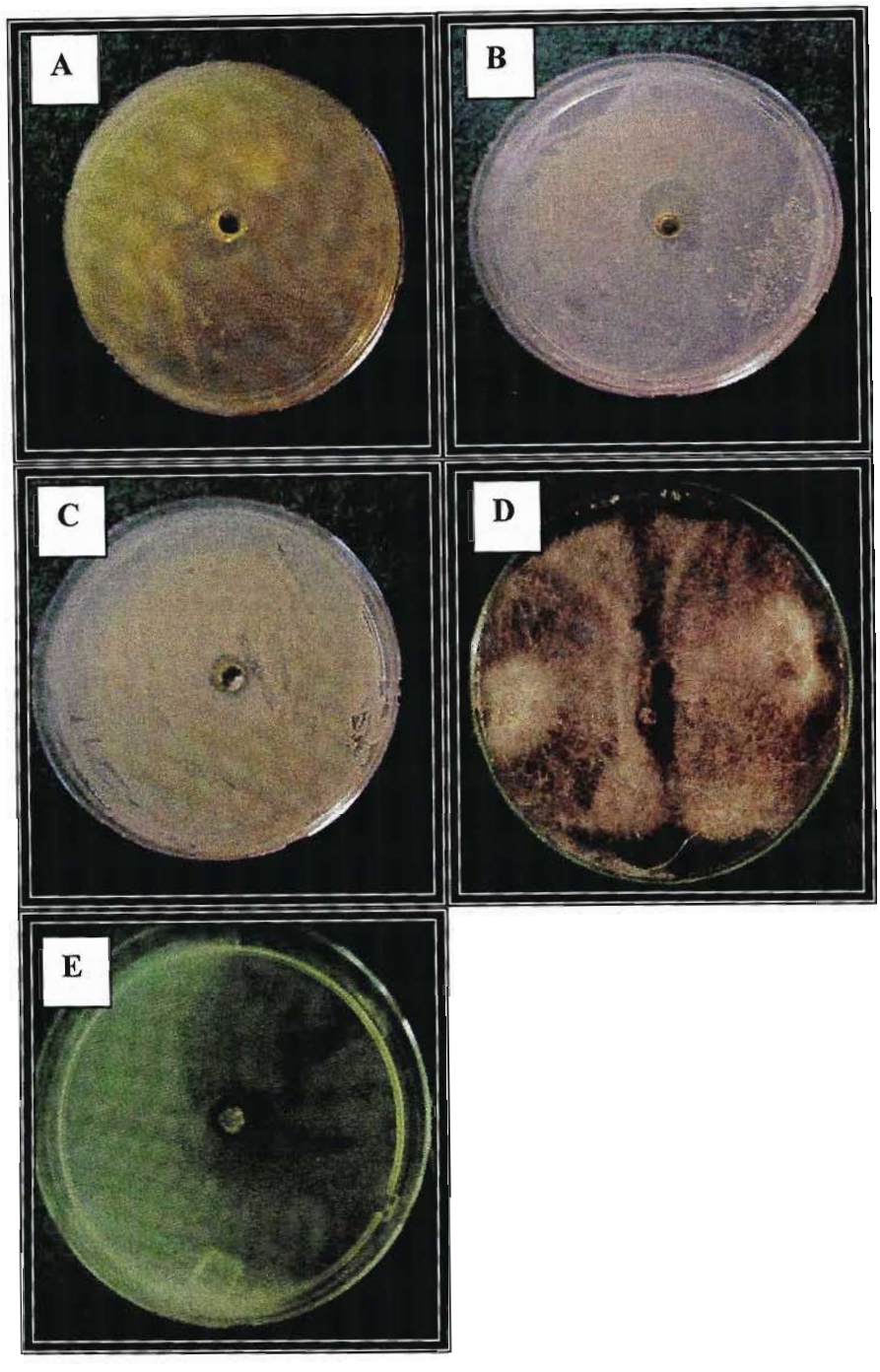


Figure 5.2 Bioassay plates showing the biological activity of *P. zuluensis* against: *X. campestris* (A) – clear zone of inhibition can be seen; *Candida* spp. (B) – irregular zone formed around well; *B. subtilis* (C) – slight ring of inhibition; *R. solani* (D) – no inhibition as the fungus grows from both sides and eventually covers the whole plate; and *P. ultimum* (E) – zone of inhibition cannot be seen clearly but ranges from 2 – 7mm.

Compound **7,** a yellow crystalline pure compound, was tested against *B. subtilis*, forming a large inhibition zone of 3 – 20mm. Poor antifungal activity was observed with the fungal test organisms.

Table 5.2 Shows biological activity of pure compound **7** against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	3 – 20	Very active extract.
X. campestris	4 – 5	Partially active extract.
F. oxysporum	2	Inactive extract.
R. solani	No Inhibition	Inactive extract.
S. sclerotiorum	4 – 11	Very active extract.
Candida spp.	No inhibition	Inactive extract.
P. ultimum	No Inhibition	Inactive extract.

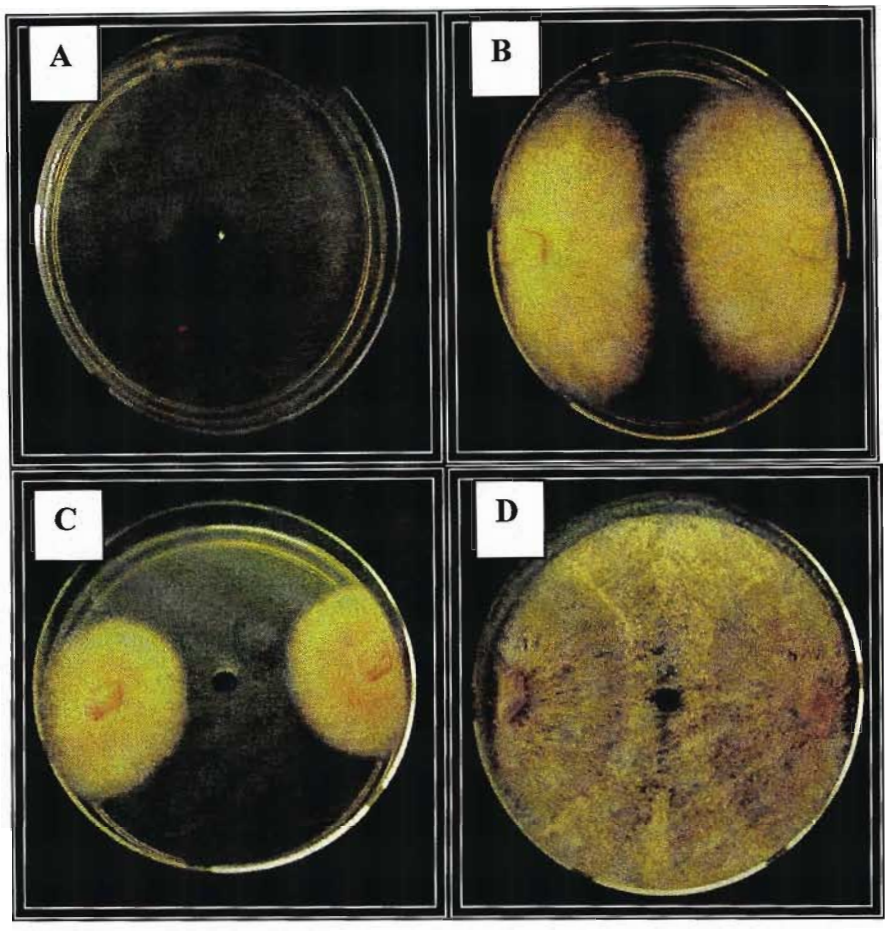


Fig 5.3 Bioassay plates showing the inhibitory activity of compound **7** against: *X. campestris* (A) – irregular zone of inhibition around well; *F. oxysporum* (B) – displaying poor antifungal activity; *S. sclerotiorum* (C) – shows good antifungal activity, only 1/3 of the plate is occupied; and *R. solani* (D) – no inhibition, pathogen grows over entire plate.

Compound **8** exerted poor biological activity against most of the microorganisms, except against *X. campestris* that produced a zone of 3-10mm. No inhibition was recorded for *R. solani* and *Candida* spp. (Table 5.3).

Table 5.3 Shows biological activity of pure compound **8** against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	4	Partially active extract.
X. campestris	3 – 10	Active extract.
F. oxysporum	2	Inactive extract.
R. solani	No Inhibition	Inactive extract.
S. sclerotiorum	4 – 8	Partially active extract.
Candida spp.	No Inhibition	Inactive extract.
P. ultimum	4 – 8	Partially active extract.

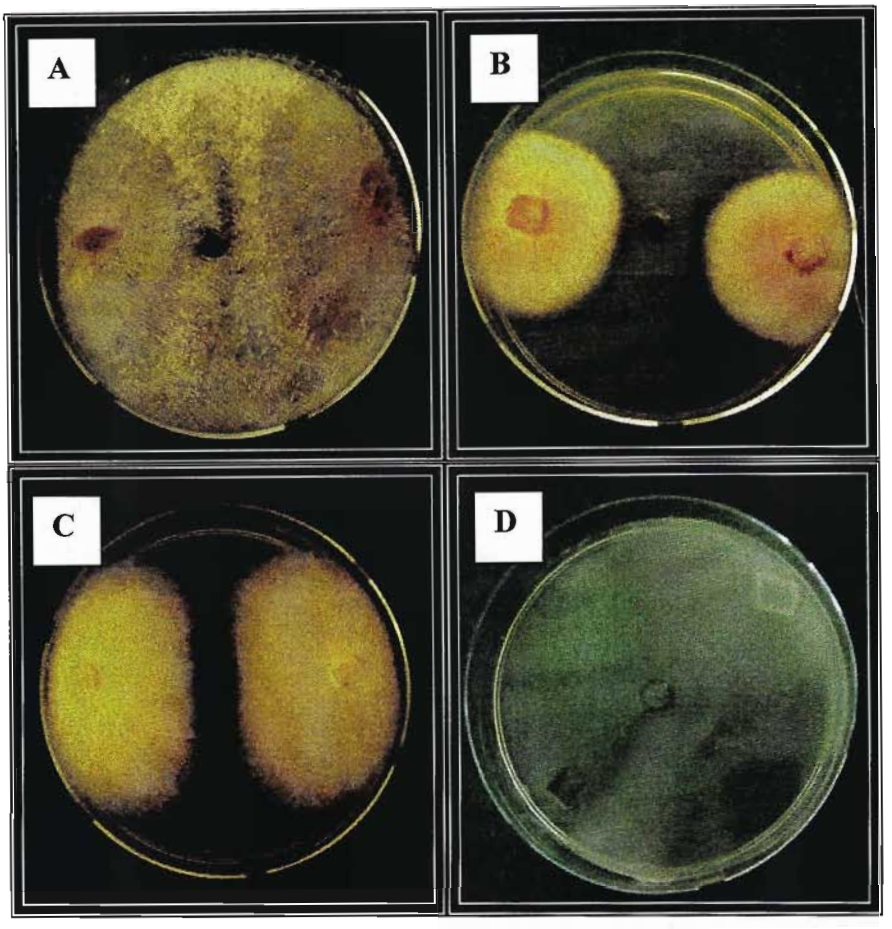
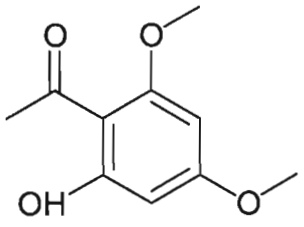
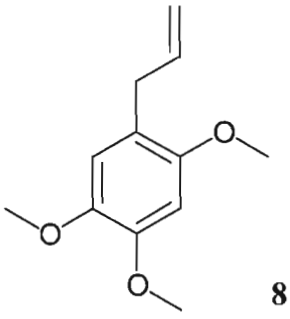


Fig 5.4 Bioassay plates showing the antifungal activity of compound **8** against: *R. solani* (A)- no inhibition; *S. sclerotiorum* (B) – clear area showing definite inhibition; *F. oxysporum* (C)- poor antifungal activity exhibited; *P. ultimum* (D) – inhibition zone is not clearly visible but measures up to 8mm.

Structures elucidated from the pure compounds:



7



8

Fig 5.5 Structures identified of the pure compounds isolated from *P. zuluensis.*

Compound **7,** 2-hydroxy-4,6-dimethoxyacetophenone, is commonly known as xanthoxylin. Xanthoxylin is obtained from *Zanthoxylum piperitum* (Japanese pepper tree), *Zanthoxylum alatum* (Rutaceae), *Artemisia brevifolia* (Compositae), *Hippomane mancinella* and *Sapium sebiferum* (Chinese tallowtree) (Euphorbiaceae) (Ascensao *et al.*, 1998).
Compound **8**, 1,2,4-trimethoxy-5-(2-propenyl)-benzene is also known as euasaron, isoasaron and sekishon. β-asarone is a natural constituent of some aromatic plants and their essential oil fractions, especially of species of the genus *Acorus* (Araceae) (http://www.emea.eu.int/pdfs/human/hmpc/13921505en.pdf).

5.4 Discussion

In Table 5.1, the largest inhibition zone was produced by the crude dichloromethane extract of *P. zuluensis* when plated against *X. campestris*. This large zone of 10 – 12mm can lead to the assumption that this plant extract may have good antibacterial properties against Gram negative bacteria. Poor antifungal activity was recorded when *P. zuluensis* was tested against various fungi. This can be visualized from the bioassay pictures (Fig 5.2).

When the crystals of compound **7** were re-suspended in dichloromethane and tested against *B. subtilis*, excellent biological activity was noted due to the production of a large inhibition zone of 3-20mm (Table 5.2). The crystals were examined via x-ray crystallography and NMR (nuclear magnetic resonance) and identified as 2-hydroxy-4,6-dimethoxyacetophenone (**7**) and 1,2,4-trimethoxy-5-(2-propenyl)-benzene (**8**). Terpenes are well known for their antibacterial activity (Abdel-Mogib *et al.*, 2002).

Little or no biological activity was observed with compound **7** against the fungal pathogens (Fig 5.3 C), with the exception of *S. sclerotiorum*. It can therefore be deduced that compound **7** would serve as a poor broad spectrum antifungal agent.

The results reflected in Table 5.2 and Table 5.3 for the two pure compounds are similar. Both pure compounds (**7** and **8**), were identified as terpenes (Fig 5.5). Diterpenes possess better antibacterial activity than antifungal activity (Berger and Mugoya, 1995). No inhibition was recorded when both pure compounds were tested against *Candida* spp., *F. oxysporum* and *R. solani.*

5.5 Literature cited

Abdel-Mogib, M., Albar, H.A. and Batterjee, S.M. 2002. Chemistry of the Genus *Plectranthus*. *Molecules* 7: 271-301.

Ascensao, L., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Schripsem, J., Deans, S.G. and Scheffer, J.C.J. 1998. *International Journal of Plant Science* 59: 31.

Berger, A. and Mugoya, C.F. (edited by). 1995. Natural Plant Products as Pesticides. Proceedings from the First National Symposium in Zambia. Report 4. Swedish University of Agriculteral Sciences, Department of Plant Protection Services, Sweden.

Interenet reference:

http://www.oaklandnurseries.co.uk

http://www.emea.eu.int/pdfs/human/hmpc/13921505en.pdf

Chapter 6

Crude extract and isolated pure compounds of *Plectranthus lucidus* Benth. tested for biological activity

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

Two pure compounds, 11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one (**9**) and 11-hydroxy-2-(4-hroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one (**10**), were isolated from the aerial plant parts of *P. lucidus* with dichloromethane and their structures elucidated by spectroscopic means. Compound **9** showed moderate antifungal activity whereas compound **10** showed high antifungal activity against *Rhizoctonia solani, Sclerotonia sclerotiorum* and *Fusarium oxysporum.* Both the crude extract and the pure compounds showed inhibition against *Xanthomonas campestris.*

6.1 Introduction

The distribution of *P. lucidus* is from Bathurst in the south eastern part of the Eastern Cape to Port St Johns in the north eastern part of the Eastern Cape. It forms mats on stabilized coastal sand dunes below trees and grows mainly with *Mimusops obovata* and *Allophyllus natalensis*. It shares its habitat with other shade-loving coastal plant species such as *P. madagascariensis* and *Scadoxus membranaceus* (Van Jaarsveld and Edwards, 1997).

"*This plant is a procumbent, succulent herb, rooting at nodes, glabrous or sparsely pubescent; roots fibrous. The stems are 4-angled, purplish to green. 2.5mm in diameter, strigose (white multicellular hairs), rubropunctate; internodes 5-20mm long. The leaves are ovate to broadly ovate, 12-15 X 10-15mm, serrate to crenate-dentate, with 3 or 4 pairs of teeth, green to purplish, abaxial surface sparsely strigose, veins densely strigose, rubropunctate, apex obtuse to acute; petioles are 5-15mm long, strigose and rubropunctate. The raceme is simple, 30-100mm long, rarely with a pair of side branches; cymes 1-flowered, verticillasters 5-10mm apart; bracts ovate-lanceolate, 2-3mm long; pedicel 4-5mm long. Calyxes at flowering time are 3-4mm long, enlarging to 7mm, consisting of a large, ovate*

upper lip 2 mm long (4mm after flowering) and 4 subulate lower lobes, 3.5mm long (5mm long after flowering), densely strigose, with longer purplish hairs. The corolla is 9-11mm long, white or mauve; tube straight, 5mm long, basally saccate, constricted distally; upper lip 5mm long, 2-lobed, upper lobes 3mm long, lateral lobes 2mm long, with long multicellular white hairs, lower lip boat-shaped, margins strongly involute. Nutlets are brown to black, ovoid, 1.5 X 1.0mm. Flowering time occurs from March to May.

P. lucidus is closely related to P. verticillatus (L.f.) Druce and P. strigosus Benth. It is distinguished by its 2-flowered verticillasters and short corolla (9-11mm) of which the lower lip margins are conspicuously inrolled. In both P. strigosus and P. lucidus the corolla is constricted at the throat, unlike P. verticillatus where the corolla is linear" (Van Jaarsveld and Edwards, 1997).

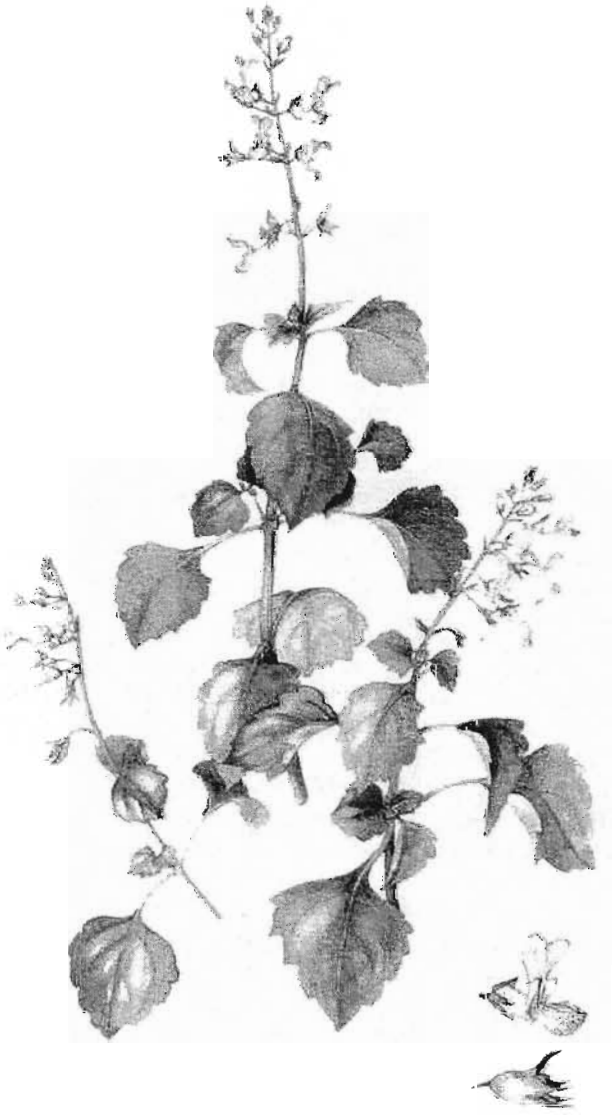


Figure 6.2 *P. lucidus* showing flowers and calyx (Van Jaarsveld and Edwards, 1997).

6.2 Materials and methods

Collection:

The material was collected from the Botanical Gardens, Pietermaritzburg. Voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg.

Extraction:

The aerial plant parts of *P. lucidus* were air dried and extracted with dichloromethane. The concentrated dichloromethane extract was chromatographed on a short silica gel column and eluted with chloroform to yield a total of four pooled fractions. The second fraction was separated via a chromatatron with an eluent 6:1 (hexane:ethyl acetate). It was from this second fraction that the orange-red compound **9** was isolated. The fourth fraction was separated using a chromatatron and the eluent used was 2:1 (hexane:ethyl acetate). Further separations using 5:1 (hexane:ethyl acetate) yielded the deep red coloured compound **10**.

Bioassay:

Compound **9** and compound **10** were tested on bacterial and fungal cultures. Refer to Chapter 2 for methodologies of the agar well diffusion method and autobiography.

6.3 Results

The bioassay results of the crude dichloromethane extract of *P. lucidus* showed inhibited growth of all the test microorganisms, with the exception of *P. ultimum*. Weak inhibition of growth was observed against *B. subtilis* and *Candida* spp., while significant inhibition occurred when tested against *X. campestris*.

Table 6.1 Shows biological activity of crude dichloromethane extract of *P. lucidus* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1 – 4	Inactive extract.
X. campestris	5 – 10	Active extract.
F. oxysporum	5 – 7	Partially active extract.
R. solani	5 – 7	Partially active extract.
S. sclerotiorum	5 – 7	Partially active extract.
Candida spp.	2 – 3	Inactive extract.
P. ultimum	No inhibition	Inactive extract.

Pure compound **9** was active only against the Gram negative bacterium, *X. campestris*. It exhibited partial or no activity against any of the other test microorganisms. It is a poor antifungal agent.

Table 6.2 Shows biological activity of dichloromethane extract of compound **9** against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1 - 2	Inactive extract.
X. campestris	2 – 10	Active extract.
Candida spp.	1 – 3	Inactive extract.
F. oxysporum	2 – 4	Partially active extract.
R. solani	3 – 4	Partially active extract.
S. sclerotiorum	3 – 4	Partially active extract.
P. ultimum	No inhibition	Inactive extract.

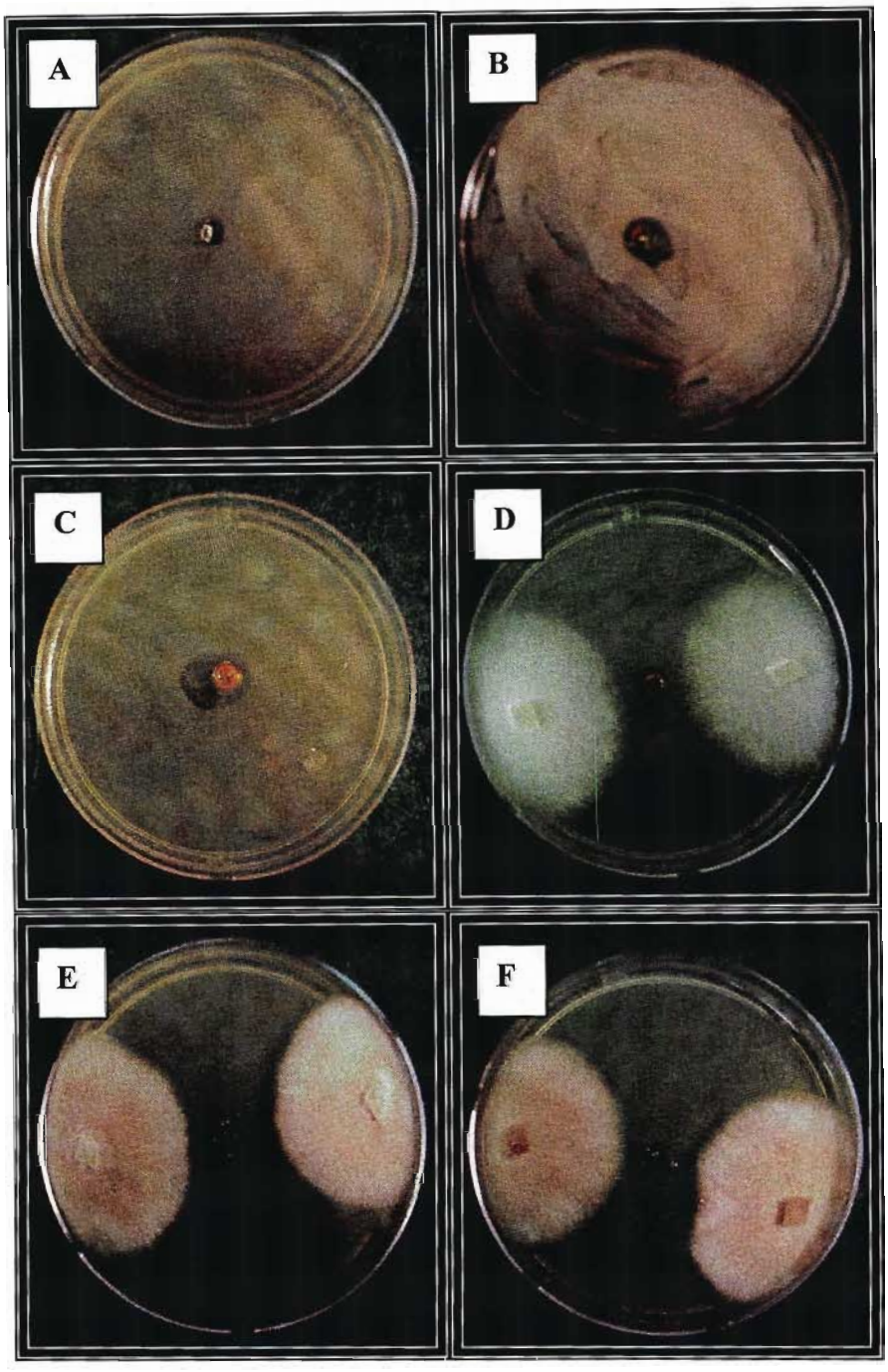


Fig 6.2 Bioassay plates showing the biological activity of pure compound **9** against: *B. subtilis* (A) – slight zone of inhibition seen; *Candida* spp. (B) – slight zone of inhibition; *X. campestris* (C) – clear zone can be seen; *S. sclerotiorum* (D); *R. solani* (E) and *F. oxysporum* (F). Plates D, E and F show similar levels of biological activity, i.e. very poor antifungal activity.

Pure compound **10** showed good antifungal activity against *F. oxysporum, R. solani* and *S. sclerotiorum*. Like compound **9**, it exhibited partial or no activity against the other pathogens. It is a weak antibacterial agent.

Table 6.3 Shows biological activity of dichloromethane extract of compound **10** against various microorganisms against the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1 – 2	Inactive extract.
X. campestris	1 – 5	Partially active extract.
F. oxysporum	2 – 7	Active extract.
R. solani	3 – 8	Active extract.
S. sclerotiorum	4 – 9	Active extract.
Candida spp.	1 – 4	Inactive extract.
P. ultimum	No inhibition	Inactive extract.

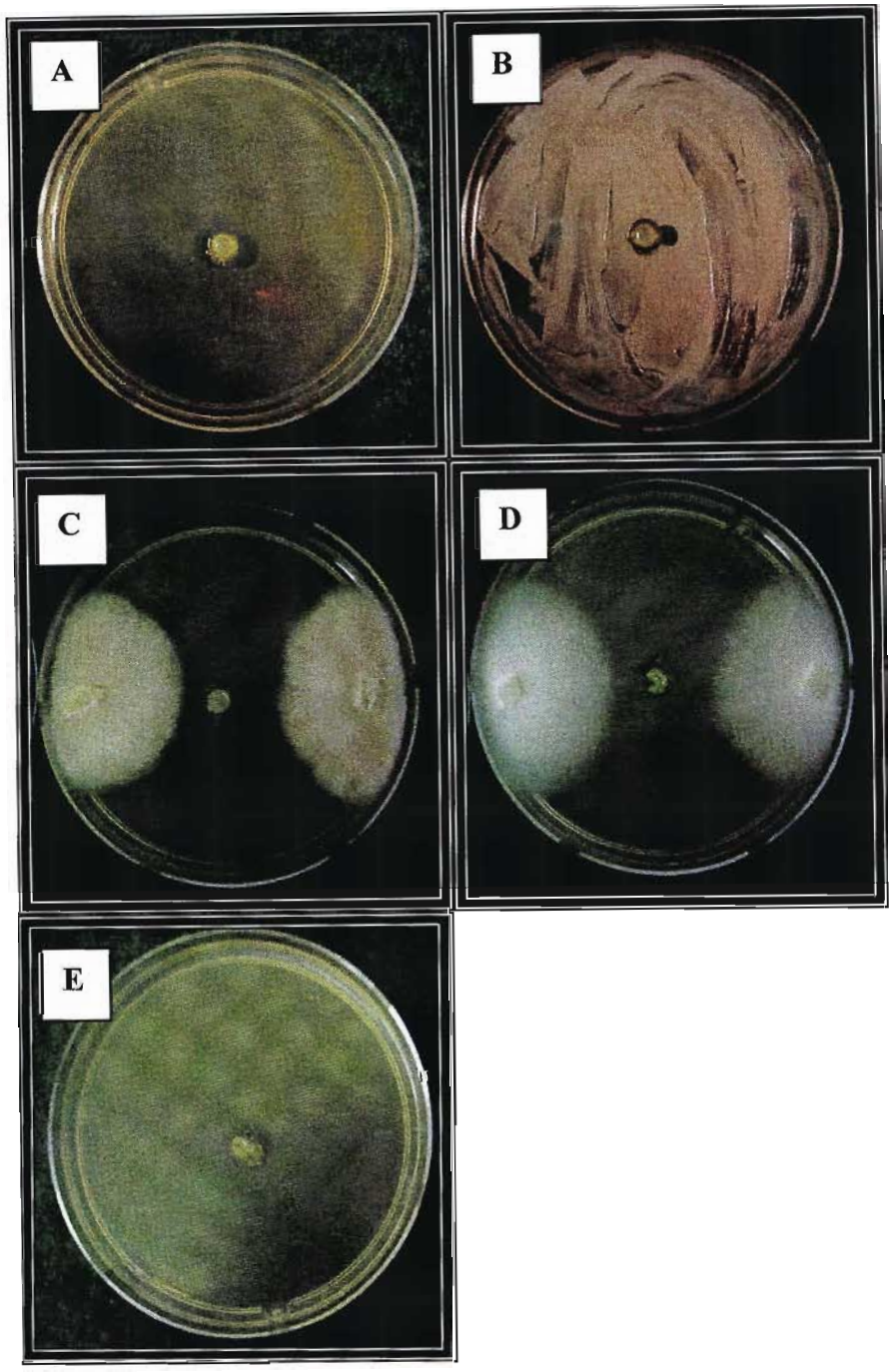
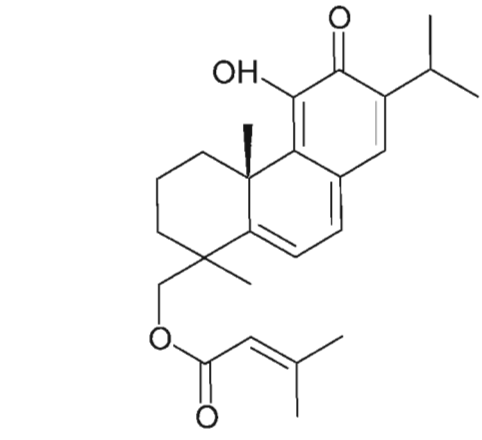
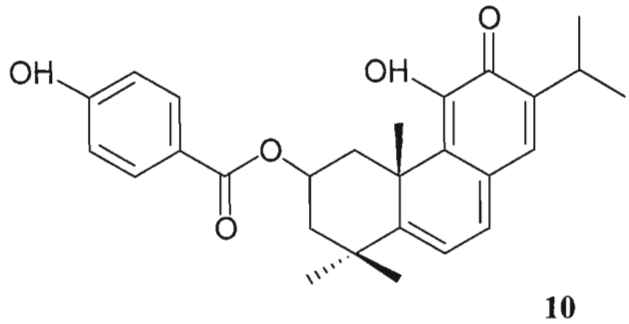


Fig 6.3 Bioassay plates showing biological activity of compound **10** against: *X. campestris* (A) – clear zone of inhibition exhibited; *Candida* spp.(B) – slight zone of inhibition; *F. oxysporum* (C) – partial biological activity depicted; *S. sclerotiorum* (D) – also partially active; and *B. subtilis* (E) – slight zone of inhibition (not clear in picture).

Structures elucidated from pure compounds of *P. lucidus*:



9



10

Fig 6.4 The pure compound structures determined from *P. lucidus*.

Compound **9**, 11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one, and compound **10**, 11-Hydroxy-2-(4-hroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one, were isolated formerly from *P. parviflorus* (Ruedi and Eugster, 1978). The former is known as Parviflorone A and the latter as Parviflorone D (Ruedi and Eugster, 1978).

6.4 Discussion

Antimicrobial activities of the two pure compounds **9** and **10**, and the crude dichloromethane extract was tested against several microorganisms and are summarized in Table 6.1, Table 6.2 and Table 6.3.

The crude dichloromethane extract of *P. lucidus* can be classified as having good biological activity overall, according to the scale used in Chapter 2 (Materials and methods).

Compound **9** showed significant biological activity against *X. campestris* only. Partial antifungal activity was observed when this plant extract was tested against the fungal pathogens.

Compound **10** showed to be in possession of potent antifungal properties as inhibition zones were formed when this pure compound was tested against *F. oxsporum, R. solani* and *S. sclerotiorum.*

Hence, from the above findings, it is evident that the crude extract of *P. lucidus* and pure compound **9** display similar levels of biological activity, especially with significant antimicrobial activity against *X. campestris* only. However, compound **10** exhibits more antifungal activity than the crude extract or pure compound **9**.

6.5 Literature cited

Ruedi, P. and Eugster, C.H. 1978. *Helvetica Chimica Acta* 61: 709-715.

Van Jaarsveld, E.J. and Edwards, T.J. 1997. Notes on *Plectranthus* (Lamiaceae from southern Africa). *Bothalia* 27: 1-6.

Chapter 7

Biological activity of *Plectranthus purpuratus* Harv. subsp. *purpuratus* and *P. purpuratus* subsp. *tongaensis* against various microorganisms

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

The dichloromethane extracts of *P. purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaensis* exhibited the same level of biological activity when tested against the same test organisms. Poor antibacterial activity was noted with both extracts. However, very high antifungal activity was discovered when both plant extracts were tested against *Fusarium oxysporum* and *Sclerotina sclerotiorum,* but the highest biological activity was noted against *Rhizoctonia solani* which was totally inhibited. Three pure compounds were isolated: 11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one (**11**) from *P. purpuratus* subsp. *purpuratus*, 11-hydroxy-19-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraene-12-one (**12**) and 11-hydroxy-19-(3,4-dihydroxybenzoyl)-abieta-5,7,9(11),13-tetraene-12-one (**13**) from *P. purpuratus* subsp. *tongaensis.*

7.1 Introduction

P. purpuratus* subsp. *purpuratus:

This species is confined to the Durban-Pietermaritzburg region of central KwaZulu-Natal (refer to Figure 7.1. for distribution). It occurs on rocky outcrops or south-facing cliffs in bushveld. It is commonly found in association with species such as *Aloe arborescens*, *Gasteria croucheri* and *Plectranthus hadiensis* var. *tomentosus* (Van Jaarsveld and Edwards, 1997).

"*The stems of P. purpuratus subsp. purpuratus are erect to decumbent, succulent. The leaves are subimbricate, subrotund to broadly ovate, succulent, 15-45 X 15-38mm, grey-green, entire, occasionally shallowly crenate, with 3 pairs of teeth, subglabrous, rubropunctate beneath, apex rounded, base truncate to cuneate. Racemes are 30-120mm long, often with a*

pair of side branches; cymes 3-flowered, 5-10mm apart; bracts ovate-lanceolate, 2mm long, persistent; pedicel 2-3mm long; fruiting calyx 5mm long. The corolla is 10-11mm long, white, tube +/- 4mm long, constricted in middle. The nutlets are brown and 1mm long.

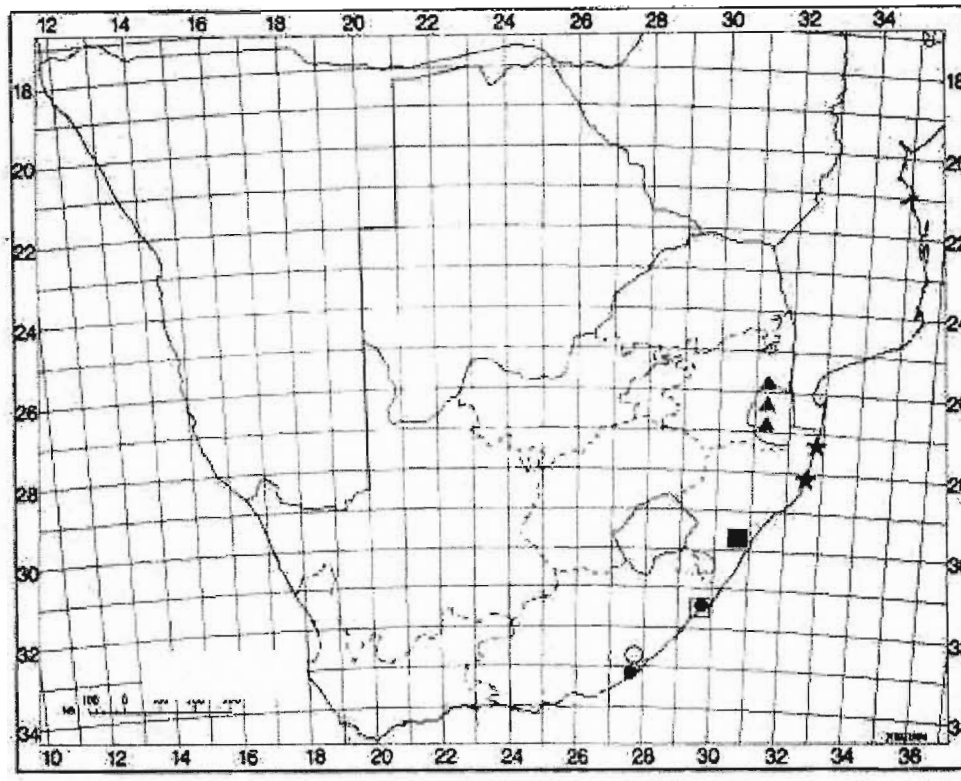


FIGURE 3.—Distribution of *P. lucidus*, ●; *P. purpuratus* subsp. *purpuratus*, ■; *P. purpuratus* subsp. *tongaensis*, ★; *P. purpuratus* subsp. *montanus*, ▲; *P. pentheri*, ○.

Fig 7.1 Map showing the distribution of *P. purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaensis* in southern Africa (Van Jaarsveld and Edwards, 1997).

Subsp. purpuratus is distinguished from subsp. montanus and subsp. tongaensis by its decumbent habit and succulent, glabrescent, subrotund leaves which are crowded and often subimbricate. Its leaves are entire or rarely obscurely crenate. The typical subspecies was named for its purple abaxial leaf surfaces. These plants have an erect to decumbent habit" (Van Jaarsveld and Edwards, 1997).

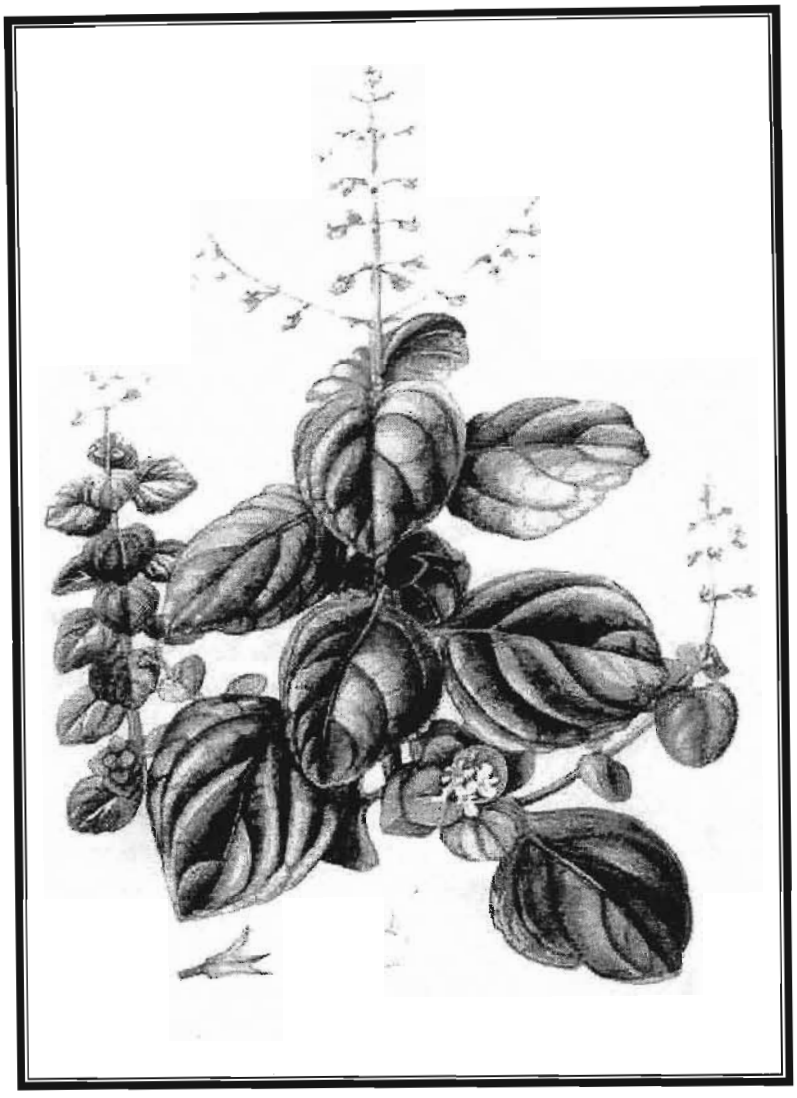


Fig 7.2 Leaves and flowers of *P. purpuratus subsp. purpuratus* (Van Jaarsveld and Edwards, 1997).

P. purpuratus* subsp. *tongaensis:

This species can be commonly found locally or sporadically in coastal sand forests from St Lucia to Kosi Bay in northern KwaZulu-Natal (see Fig. 7.1 for distribution). These plants are often found in association with *Crassula expansa* subsp. *fragilis, Plectranthus petiolaris* and *Cussonia arenicola.*

"*This plant is a procumbent, pubescent to glabrescent, succulent herb, rooting at nodes. The stems are 4-angled, purplish to green, 2-3mm in diameter, strigose (purplish to white hairs), rubropunctate; internodes 20-30 (-60)mm long. The leaves are ovate to broadly ovate, 30-34 X 15-18mm, green or purple tinged, coarsely serrate, with 3 or 4 pairs of shallow teeth, abaxial surface strigose to glabrescent, rubropunctate, veins densely strigose, apex acute,*

base broadly cuneate; petioles are 12-15mm long, strigose, rubropunctate. Racemes are 140-290mm long, occasionally with a pair of basal side branches; cymes 3-flowered, 8-20mm apart; bracts ovate-lanceolate, +/- 3mm long; pedicel +/- 4mm long. The calyx is +/- 3mm long, enlarging to 6mm, upper lip ovate, 2mm long (+/- 4mm after flowering), lower lobes 4, linear, +/- 1.5mm long (+/- 3.5mm long after flowering), densely strigose (flushed with blue). The corolla is 12-13mm long, white, bilabiate, tube basally saccate, 7mm long, upper lip 5-6mm long, 2-lobed, lateral lobes 2-3mm long, lower lip boat-shaped, 5-6 mm long. Nutlets are black, ovoid and 1.5 X 1.0mm long. Flowering time occurs from March to May" (Van Jaarsveld and Edwards, 1997).

Fig 7.3 Illustration showing *P. purpuratus*, subsp. *tongaensis* (A), and, subsp. *montanus* (B) (Van Jaarsveld and Edwards, 1997).

7.2 Materials and methods

P. purpuratus* subsp. *purpuratus

The material was collected and a voucher specimen deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. The leaves and stems were air-dried and extracted with dichloromethane at room temp. overnight. The dichloromethane extract was concentrated *in vacuo* to give a green semi-solid. The crude dichloromethane extract was subjected to silica gel column chromatography using the eluent chloroform to yield a total of 4 pooled fractions. The third fraction was further purified to yield the pure compound **11** (Fig 7.4) which was orange-red in colour.

P. purpuratus* subsp. *tongaensis

The material was collected and a voucher specimen deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. The aerial parts were air-dried and extracted with dichloromethane at room temp. overnight. The crude dichloromethane extract was subjected to silica gel chromatography using a gradient eluent of chloroform:ethyl acetate (9:1) to yield a total of 4 pooled fractions.

The third fraction was chromatographed on a chromatatron and eluted with a gradient eluent of increasing polarity [chloroform:ether (10:1) → chloroform:ether (25:1)]. A final separation of this fraction was chromatographed on a silica gel column and eluted with ethyl acetate:hexane (4:1) to yield compound **12**.

The fourth fraction was chromatographed further using a gradient eluent of increasing polarity [ethyl acetate:hexane (6:1) → ethyl acetate:hexane (10:1)]. A final separation of this fraction was chromatographed on a silica gel column and eluted with ethyl acetate:hexane (2:1) to yield compound **13**.

** All extractions and isolations of the diterpenoids was done by F. Khan*[2]

Bioassay

Refer to Chapter 2 for methodologies of the agar well diffusion method and autobiography.

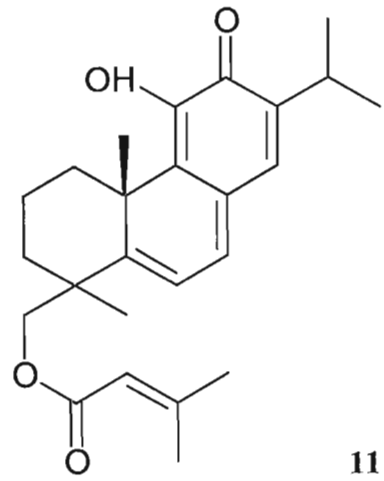
The pure compounds (**11**, **12**, **13**) were not tested as the sample volume was too less to perform the agar well diffusion assay, as agar plates were done in duplicate for a range of seven microorganisms.

7.3 Results

The dichloromethane extract of *P. purpuratus* subsp. *purpuratus* showed high antifungal but poor antibacterial activity when subjected to the agar well diffusion assay.

Table 7.1 Biological activity of dichloromethane extract of *P. purpuratus* subsp. *purpuratus* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1 - 2	Inactive extract.
X. campestris	No Inhibition	Inactive extract.
F. oxysporum	14 – 20	Very active extract.
R. solani	Total Inhibition	Totally active extract.
S. sclerotiorum	9 – 15	Very active extract.
Candida spp.	1 – 3	Inactive extract.
P. ultimum	No Inhibition	Inactive extract.

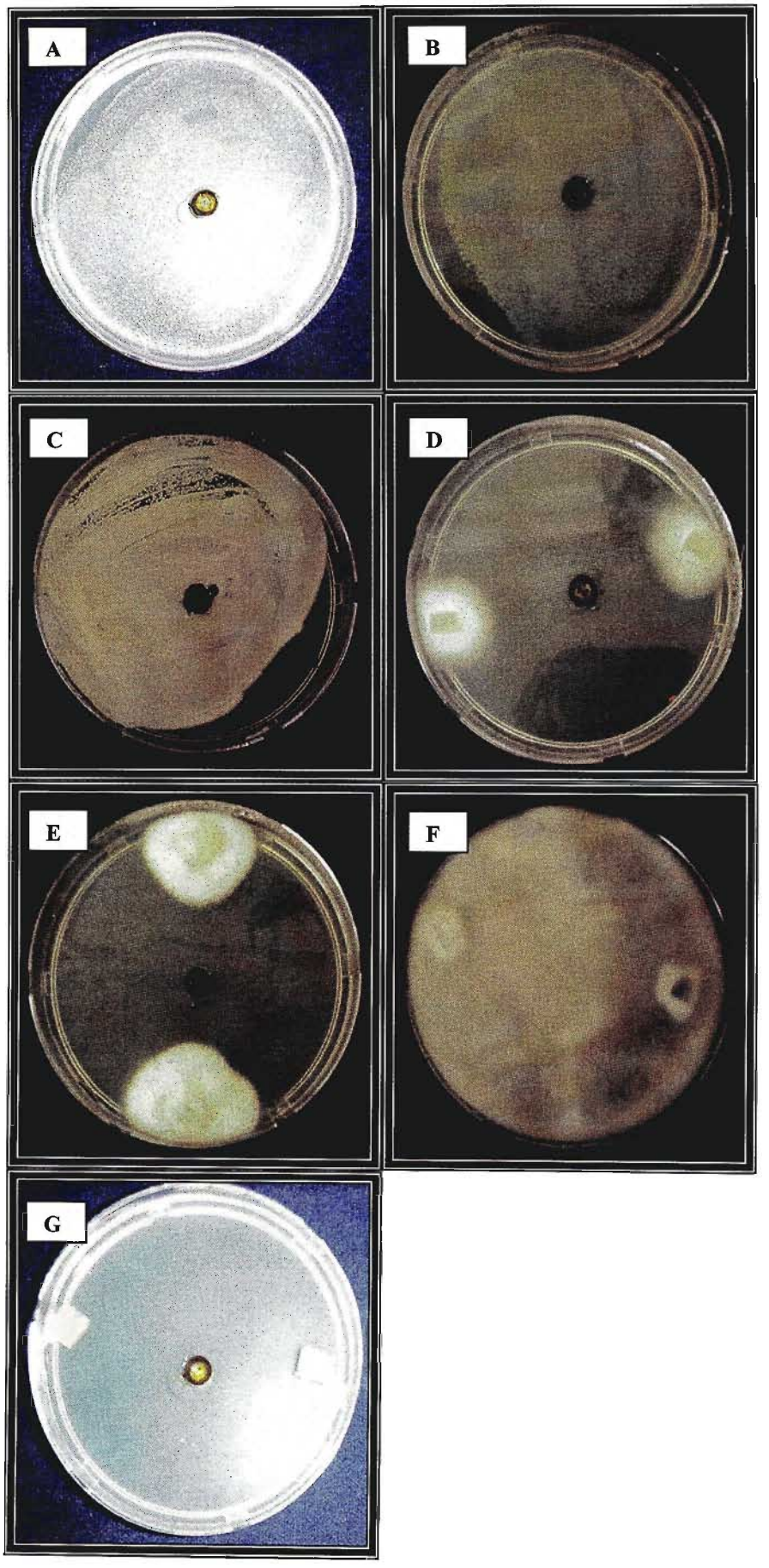


11

Fig 7.4 Octerpene (**11**) isolated from *P. purpuratus* subsp. *purpuratus*.

Compound **11,** 11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one, known as Parviflorone A, was previously isolated from *P. parviflorus* (Ruedi and Eugster, 1978).

Fig 7.5 Bioassay plates showing biological activity of *P. purpuratus* subsp. *purpuratus* against: *B. subtilis* (A) – a faint ring of inhibition occurs around the well; *X. campestris* (B) – no inhibition is noted; *Candida* spp. (C) – very slight ring of inhibition, *F. oxysporum* (D) – large inhibition zone noted, ranging from 14 - 20mm, *S. sclerotiorum* (E) – also displays very good antifungal activity; *P. ultimum* (F) – no inhibition and *R. solani* (G) - displayed the best result where there was total inhibition by the plant extract on this organism. The entire plate was clear.

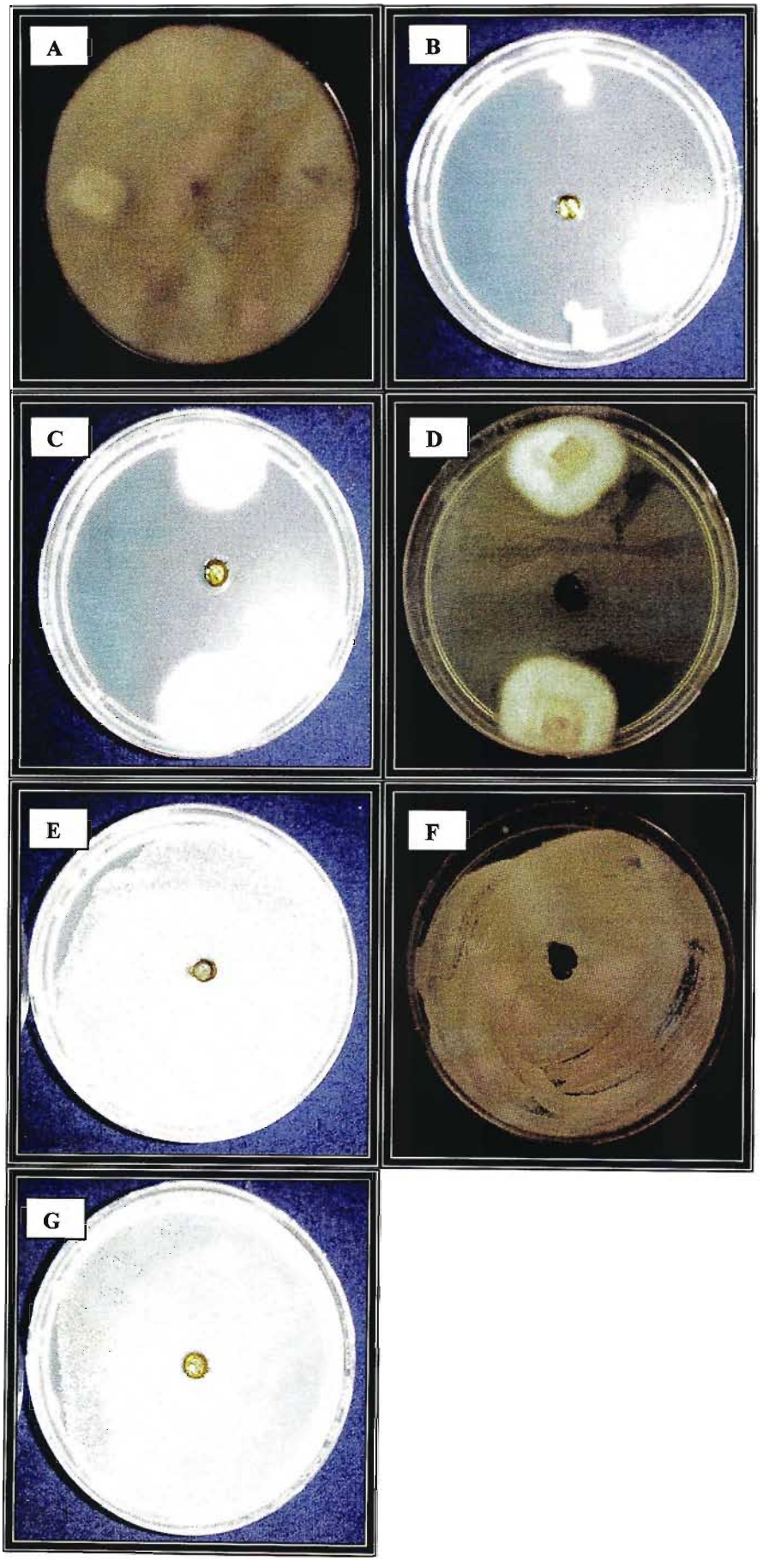

A
B
C
D
E
F
G

The dichloromethane extract of *P. purpuratus* subsp. *tongaensis* exhibited the same level of biological activity against all the test microorganisms as *P. purpuratus* subsp. *purpuratus.* There was high antifungal activity against *F. oxysporum* and *S. sclerotiorum* and total inhibition against *R. solani*. Poor antibacterial activity was observed.

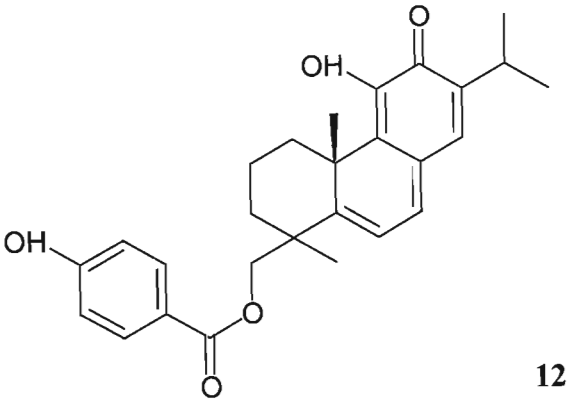
Table 7.2 Biological activity of dichloromethane extract of *P. purpuratus* subsp. *tongaensis* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1 – 2	Inactive extract.
X. campestris	No Inhibition	Inactive extract.
F. oxysporum	11 – 20	Very active extract.
R. solani	Total inhibition	Totally active extract.
S. sclerotiorum	10 – 15	Very active extract.
Candida spp.	2 – 3	Irregular zone. Inactive extract.
P. ultimum	No Inhibition	Inactive extract.

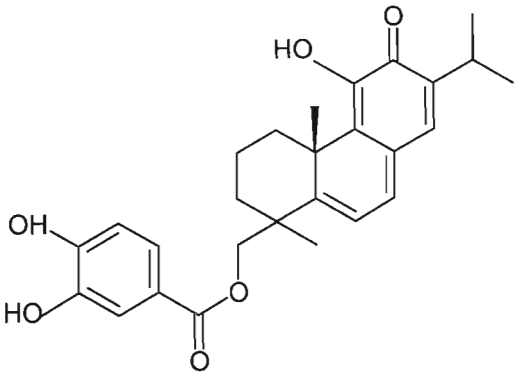
Fig 7.6 Bioassay plates showing biological activity of *P. purpuratus* subsp. *tongaensis* against: *P. ultimum* (A) – no inhibition, fungus grows over entire plate; *R. solani* (B) – the extract totally inhibits the pathogen; *F. oxysporum* (C) – displays good antifungal activity; *S. sclerotiorum* (D) – excellent antifungal activity is displayed by this extract; *B. subtilis* (E) – almost no zone of inhibition, thus not very good antibacterial activity displayed; *Candida* spp. (F) – zone of 2 – 3mm inhibition recorded; and *X. campestris* (G) – no zone of inhibition detected thus once again reconfirming the poor antibacterial activity of this extract.


A
B
C
D
E
F
G

Structures elucidated from pure compounds of *P. purpuratus* subsp. *tongaensis*:



12



13

Fig 7.7 The diterpenes *P. purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaensis*.

Compound **12**, 11-hydroxy-19-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraene-12-one, known as Parviflorone C, and compound **13**, 11-hydroxy-19-(3,4-dihydroxybenzoyl)-abieta-5,7,9(11),13-tetraene-12-one, known as Parviflorone E, have been isolated formerly from *P. parviflorus* (Ruedi and Eugster, 1978).

7.4 Discussion

Table 7.1, displays the biological activity of the dichloromethane extract of *P. purpuratus* subsp. *purpuratus*. It is evident that this plant extract do not possess very good antibacterial activity but has excellent antifungal properties. It exhibits total inhibition of *R. solani* and also shows very promising antifungal activity against *F. oxysporum* and *S. sclerotiorum*. However, *P. ultimum* is the exception from the list of fungal test organisms. No inhibition

was seen when it was tested against the crude extract. On the bacterial plates, slight zones of inhibition can be vaguely discerned. There was little or no inhibition observed when *P. purpuratus* subsp. *purpuratus* was plated against *B. subtilis* (Plate A), *X. campestris* (Plate B) and against *Candida* spp. (Plate C). The pure compound which was isolated via flash column chromatography was identified as 11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one (**11**). The more common secondary metabolites of *Plectranthus* are diterpenoids. The majority of them are highly modified abietanoids (Abdel-Mogib *et al.*, 2002).

Table 7.2 displays the biological activity of *P. purpuratus* subsp. *tongaensis*. The values obtained in Table 7.1 are similar to that from Table 7.2. *P. purpuratus* subsp. *tongaensis* also totally inhibits *R. solani* and displays very high antifungal activity against *F. oxysporum* and *S. sclerotiorum*. No inhibition was observed when it was plated against *P. ultimum*. This fungal pathogen covered the whole plate. This is not surprising as *Pythium* is an Oomycete, which has evolved from algae and hence has a totally different physiology to the higher fungi, which fall into a different Kingdom. The antibacterial activity was the same as seen for *P. purpuratus* subsp. *purpuratus*. The two pure compounds isolated from the crude extract of *P. purpuratus* subsp. *tongaensis*, were 11-hydroxy-19-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraene-12-one (**12**) and 11-hydroxy-19-(3,4-dihydroxybenzoyl)-abieta-5,7,9(11),13-tetraene-12-one (**13**).

The TLC fingerprint which was obtained by bioautography (Fig 2.6 – Chapter 2), showed no clearing around the bands for *P. purpuratus* subsp. *tongaensis* and *P. purpuratus* subsp. *purpuratus* when *B. subtilis* was sprayed onto the plate. This confirmed that these two plant extracts exhibited poor antibacterial activity.

Hence, by comparing Table 7.1 and Table 7.2, it can be deduced that *P. purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaenis* have similar plant compounds, and they display the same level of biological activity against the same pathogens.

7.5 Literature cited

Abdel-Mogib, M., Albar, H.A. and Batterjee, S.M. 2002. Chemistry of the Genus *Plectranthus*. *Molecules* 7: 271-301.

Ruedi, P. and Eugster, C.H. 1978. *Helvetica Chimica Acta* 61: 709-715.

Van Jaarsveld, E.J. and Edwards, T.J. 1997. Notes on *Plectranthus* (Lamiaceae from southern Africa). *Bothalia* 27: 1-6.

Chapter 8

Other plant extracts tested for biological activity

Several plant species of the genus Polygonum (Polygonaceae) are used in folk medicine in different parts of the world to treat many diseases including skin infections, dysentery, snake-bite, haemorrhoids, insomnia, heart diseases, and is also used for liver protection (Hutchings *et al.*, 1996).

Volatile oils of many plants are known to have antimicrobial activity (Deans *et al.*, 1992). This activity should act as a chemical defence against plant pathogenic diseases (Piccaglia *et al.*, 1993). Pathogens can readily penetrate at wound sites caused, for example, by herbivores. Wounding of leaves which are covered with volatile oil glands results in the rupture of glands causing oil to flow over the wound. The existence, therefore, of antimicrobial activity in the oil, would be of considerable benefit to the plant. Indeed, a good majority of aromatic and medicinal plants do not succumb to many of the commonest diseases. It is also suggested that a complex oil presents a greater barrier to pathogen adaptation than would a more simple mixture of monoterpenes (Svoboda and Deans, 1995). The complicated mixtures of monoterpenes and sesquiterpenes in the whole oil represents the strongest barrier to fungal infection.

Several active components might have a synergistic effect. To add to the complexity of volatile oils, there is evidence that the time of harvest influences the oil composition and consequently the potency of their biological activity (Deans and Svoboda, 1988).

Literature cited

Deans, S.G. and Svoboda, K.P. 1988. Anitibacterial activity of French tarragon (*Artemisia dracunculus* L.) essential oil and its constituents during ontogeny. *Journal of Horticulteral Science* 63: 503 – 508.

Deans, S.G., Svoboda, K.P., Gundidza, M. and Brechany, E.Y. 1992. Essential oil profiles of several temperate and tropical aromatic plants: their antimicrobial and antioxidant activities. *Acta Horticulturae* 306: 229 – 232.

Hutchings, A., Scott, A.H., Lewis, G. and Cunningham, A.B. 1996. Zulu Medicinal Plants. University of Natal Press, Pietermaritzburg.

Piccaglia, R., Marotti, M., Giovanelli, E., Deans, S.G. and Eaglesham, E. 1993. Antibacterial and antioxidant properties of Mediterranean aromatic plants. *Industrial Crops and Production* 2: 47 – 50.

Svoboda, K.P. and Deans, S.G. 1995. Biological activities of essential oils from selected aromatic plants. *Acta Horticulturae* 390: 203 - 209.

Chapter 8.1

A comparison of the biological activity between *Persicaria lapathifolia* (L.) S.F. Gray and *Persicaria senegalensis* (Meisn.) Soják

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

A comparative study on the antimicrobial properties of two plant extracts from the same family, Polygonaceae, was carried out. The screening for antimicrobial activity of the aqueous extract of *P. lapathifolia* and the dichloromethane extract from *P. senegalensis* was conducted by the agar well diffusion test against a Gram-positive, -negative bacterium, a yeast and four fungal organisms. The most active extract (inhibition diameter ≥20mm to total inhibition) was found to be *P. senegalensis* when this plant extract was assayed against *R. solani* and *Candida* spp. The results obtained indicate that the aqueous extract of *P. lapathifolia* was more inactive than the dichloromethane extract of *P. senegalensis*. However, the activity of the aqueous extract of *P. lapathifolia* was more pronounced against the Gram-positive bacterium than the Gram-negative bacterium or the fungal organisms.

Introduction

Persicaria lapathifolia

Synonyms: *Polygonum lapathifolium* L. subsp. *maculatum* (S.F. Gray) T. Dyer & Trim.; *Polygonum lapathifolium* L. var. *maculatum* T. Dyer & Trim.

Common name: hanekam

Zulu names: *idolo lenkonyane, umancibikela*

Fig 8.1.1 *P. lapathifolia* growing on a river bank (http://linnaeus.nrm.se/flora/di/polygona/persi)

This genus was considered by Mabberley (1990) to be synonymous with *Polygonum.* There are 6 *Polygonum* spp. and 10 *Persicaria* spp. found in South Africa. Of these, 3 *Polygonum* spp. and 7 *Persicaria* spp. occur in Kwa-Zulu Natal. Of the *Persicaria* spp., two are used for Zulu medicines (Mabberley, 1990).

Members of the genus *Persicaria* Mill. are erect, prostrate or scandent herbs or shrubs. Anthocyanin pigments, flavones and flavonols are common and quinones (anthraquinones, phenanthraquinones, anthrones and dianthrones) occur in many of the species (Trease and Evans, 1983). Potential toxins known in the genera include calcium oxalate, gallic acid, hydrocyanic acid, indican, quercitin, rutin and tannic acid (Duke, 1985). Plants accumulate free nitrates and may cause death or distress to cattle (Fuller and McClintock, 1986).

In Zulu medicinal usage, unspecified parts of *P. lapathifolia* are ingredients with substances known as 'white stone', 'blue stone' and *umesiswini* in medicines used to treat syphilis (Gerstner, 1941). One of the chemical constituents from the seeds is a flavonoid, 5,7-dihydroxychromone that inhibits germination of *Abutilon theophrasti* Medik. at molar concentrations from 1-6 μM (Spencer and Tjarks, 1985).

Persicaria senegalensis (Meisn.) Soják

Synonyms: *Persicaria senegalense*

Common name: German, Knoeterich

Fig 8.1.2 *P. senegalensis* (http://www.botanic.co.il)

P. senegalensis is widespread in tropical and subtropical Africa, including South Africa and Madagascar. The tender leaves of *P. senegalensis* are eaten cooked as a vegetable. Only in times of drought it is grazed by livestock (Thulin, 1993). In Senegal, a leaf decoction is taken in draught and by enema to treat syphilis in a mixture with other plants. In Nigeria, Tanzania and Ethiopia, the leaves are pounded and applied to swellings, syphylitic sores or skin affections (Kokwaro, 1993). The plant is also used as a veterinary medicine in Madagascar and elsewhere (Jansen, 1981).

Materials and methods

P. lapathifolia:

The material was collected from the Darville swamps, Pietermaritzburg. Voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. Plant material was dried and ground to a course powder using a hammer mill. Powdered plant material was sequentially extracted with purified water and ethyl acetate/water (50:50). For each extraction procedure the plant material was steeped in sufficient solvent overnight at room temperature, with occasional stirring. The solvent was subsequently drained and the

plant material was air-dried before extraction with the next solvent. The organic extract was concentrated by rotary vacuum evaporation below 45°C and then further dried *in vacuo* at ambient temperature for 24h. The same was done to the water fraction. The aqueous concentrate produced a viscous dark brownish-yellow oil mixture. The ethyl acetate concentrate was orange in colour. After a few days, the water extract had a layer of fungal growth on it. This was an indication that not much antifungal activity could be expected of this plant.

The crude ethyl acetate/water extract was fractionated on a silica gel column using a gradient eluent of increasing polarity (ethyl acetate:hexane 1:9 → ethyl acetate 3:2). A total of 8 pooled factions were generated. The column was stripped with methanol.

P. senegalensis:

The material was collected from the Darville swamps, Pietermaritzburg. Voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. Plant material was air-dried for 1wk and ground to a powder using a hammer mill. The leaves and the inflorescence were collected and milled together. The ground powdered plant material was sequentially extracted with dichloromethane and chloroform. For each extraction procedure the plant material was steeped in sufficient solvent overnight at room temperature, with occasional stirring. The solvent was subsequently filtered off and the plant material was air-dried before extraction with the next solvent. The organic extract was concentrated by rotary vacuum evaporation below 45°C and then further dried *in vacuo* at ambient temperature for 24h. The same was done to the chloroform fraction.

The crude dichloromethane extract was fractionated on a silica gel column using a gradient eluent of increasing polarity (dichloromethane:hexane 1:9 → dichloromethane 3:2). A total of 5 pooled factions were generated. The column was stripped with methanol.

Results

The table below reflects the biological activity of the aqueous extract of *P. lapathifolia* on the test microorganisms, using the trench method. No inhibition was recorded with this plant extract against any of the test microorganisms, hence, this extract was considered inactive.

Table 8.1.1 Biological activity of an aqueous extract of *P. lapathifolia* against various microorganisms using the trench method.

Microorganism	Zone of inhibition (mm)	Notes / Extract Description
B. subtilis	1	Inactive extract.
X. campestris	No Inhibition	Inactive extract.
F. oxysporum	No Inhibition	Growth is rampant over agar surface. Inactive extract.
R. solani	No Inhibition	Inactive extract.
S. sclerotiorum	No Inhibition	Inactive extract.
Candida spp.	No Inhibition	Inactive extract.

The aqueous extract of *P. lapathifolia* proved to be inactive as a biological control agent against most of the test microorganisms. However, this extract was very active against the Gram positive bacterium, *B. subtilis*, producing an irregular inhibition zone measuring up to 10mm in diameter.

Table 8.1.2 Biological activity of an aqueous extract of *P. lapathifolia* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Extract description**
B. subtilis	5 – 10	A white ring was formed. Active extract.
X. campestris	1	A slight ring was formed. Inactive extract.
F. oxysporum	No Inhibition	Inactive extract.
R. solani	No Inhibition	Inactive extract.
S. sclerotiorum	No Inhibition	Inactive extract.
Candida spp.	3	Inactive extract.
P. ultimum	0 – 4	Inactive extract.

Bioassay results for *P. senegalensis* showed no antimicrobial activity against *B. subtilis, S. sclerotiorum* and *F. oxysporum*, using the trench method. However, *R. solani* and *Candida* spp. produced significant results ranging from 10 – 20mm to total inhibition, respectively.

Table 8.1.3 Biological activity of a dichloromethane extract of *P. senegalensis* against various microorganisms against using the trench method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	No Inhibition	Inactive extract.
X. campestris	2	Inactive extract.
F. oxysporum	No Inhibition	Inactive extract.
R. solani	Total Inhibition	Very active extract.
S. sclerotiorum	No Inhibition	Inactive extract.
Candida spp.	10 - 20 to total inhibition	Clear zone on one plate while the other entire plate was clear. Very active extract.

No antimicrobial activity was observed against any of the test microbes, except *Candida* spp., that produced a significant result when the dichloromethane extract of *P. senegalensis* was tested. A ring of inhibition measuring 8 – 12mm was recorded for *Candida* spp.

Table 8.1.4 Biological activity of a dichloromethane extract of *P. senegalensis* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	1	Inactive extract.
X. campestris	1	Inactive extract.
F. oxysporum	2	Inactive extract. There was some clearing.
R. solani	No Inhibition	Inactive extract.
S. sclerotiorum	No Inhibition	Inactive extract.
Candida spp.	8 -12	Very active extract.
P. ultimum	No Inhibition	Inactive extract.

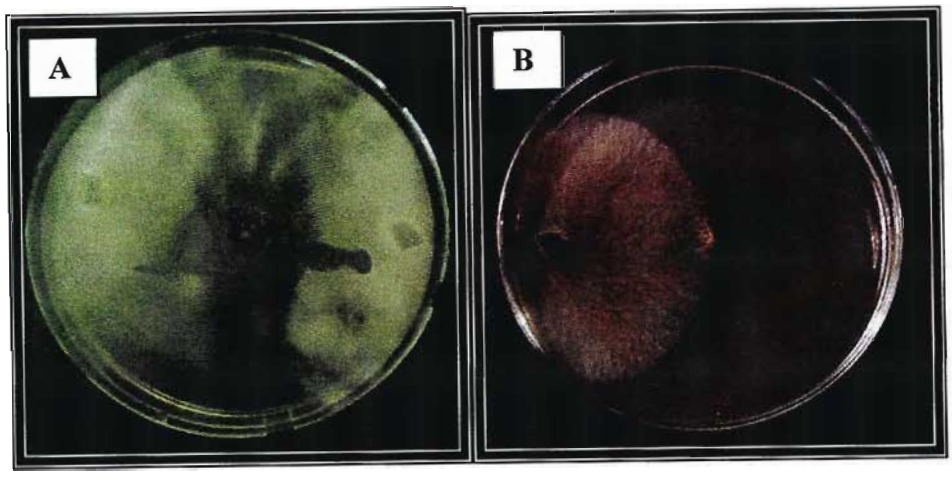


Fig 8.1.3 Bioassay plates showing poor biological activity of the aqueous extract of *P. lapathifolia* against *P. ultimum* (A) and *R. solani* (B).

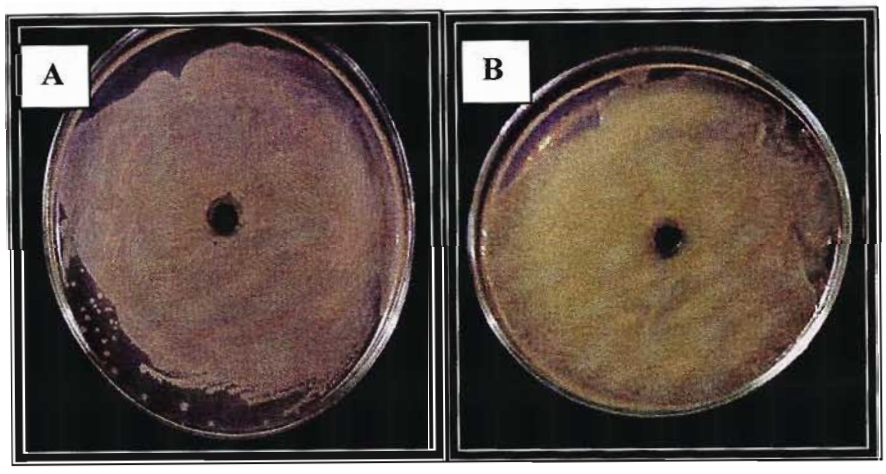


Fig 8.1.4 Bioassay plates showing poor antibacterial activity of *P. senegalensis* against *B. subtilis* (A) and *X. campestris* (B).

Discussion

The bioassay confirmed that the aqueous plant extract of *P. lapathifolia* exhibited low levels of inhibition when tested against the fungal microorganisms. This was evident from the consortium of fungi that formed a layer on the aqueous extract. However, there was moderate antibacterial activity of this aqueous extract against *B. subtilis* using the well method. Weak inhibition of growth was observed against *Candida* spp. and *P. ultimum*.

The bioassay testing, using the well method, showed the dichloromethane extract of *P. senegalensis* to exert weak inhibition of growth against all the test microorganisms, except *Candida* spp., where a significant amount of inhibition occurred. An inhibition zone measuring up to 12mm was recorded. This was observed again during the bioassay testing

using the trench method. An even larger inhibition zone to total inhibition was recorded when this extract was tested against *Candida* spp. and *R. solani*. The difference in the size of the inhibition zones can be attributed to volume of extract used for the different bioassay testing. The trench method requires 1ml of sample, whereas the well method requires 0.1ml. Therefore, it can be concluded that dosage plays a very important role with regards to how biologically active the plant extract appears to be.

Literature cited

Duke, J.A. 1985. Handbook of Medicinal Herbs. CRC, Florida.

Fuller, T.C. and McClintock, E. 1986. Poisonous plants in California. University of California Press, Berkeley.

Gerstner, J. 1941. A preliminary checklist of Zulu names of plants with short notes. *Bantu Studies* 15 : 369-383.

Jansen, P.C.M. 1981. Spices, condiments and medicinal plants in Ethiopia, their taxonomy and agricultural significance. Agricultural Research Reports 906. Centre for Agricultural Publishing and Documentation, Wageningen, Netherlands. Pp 327.

Kokwaro, J.O. 1993. Medicinal plants of East Africa. 2nd Edition. Kenya Literature Bureau, Nairobi, Kenya. Pp 401-410.

Mabberley, D.J.M. 1990. The plant-book. Cambridge University Press, London. Pp 312-323.

Spencer, G.F. and Tjarks, L.W. 1985. Germination inhibition by 5,7-dihydroxychromone, a flavanoid decomposition product. *Journal of Plant Growth Regulation* 4: 177-180.

Trease, G.E. and Evans, W.C. 1983. Pharmacognosy, 12th ed. Bailiere Tindall, London.

Thulin, M. 1993. Polygonaceae. In: Thulin, M. (Editor). Flora of Somalia. Volume 1. Pteridophyta; Gymnospermae; Angiospermae (Annonaceae-Fabaceae). Royal Botanic Gardens, Kew, Richmond, United Kingdom. Pp 175–179.

Internet references

http://linnaeus.nrm.se/flora/di/polygona/persi

http://www.botanic.co.il

Chapter 8.2

Examining the biological activity of *Pycnostachys reticulata* (E. Mey.) Benth.

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

In continuation of the research concerning the isolation and bioassay testing of crude and pure constituents of South African indigenous plants of the family Lamiaceae, *Pycnostachys reticulata*, of the genus *Pycnostachys* has been investigated. Extraction of the aerial parts of the plant with dichloromethane, followed by bioassay testing, proved it to contain extracts which are biologically active against *B. subtilis* (Gram positive) and *X. campestris* (Gram negative). No antifungal activity was observed with this extract when tested against *R. solani, P. ultimum, F. oxysporum* and *S. sclerotiorum.*

Introduction

Fig 8.2.1 *Pycnostachys reticulata* pale blue and mauve flowerheads (http://www.plantzafrica.com)

Synonyms: *Echinostachys reticulata* E. Mey.; *P. holophylla* Briq.; *P. purpurascens* Briq.; *P. reticulata* (E. Mey.) Benth. var. *angustifolia* Benth.; *P. schlecteri* Briq.

Common name: blue soldier salvia (Eng.); stekelsalie (Afr.)

Zulu name: *umvuthuza, uhlalawane*

Pycnostachys reticulata, also known as "Blue Boys", is a shrub of the Mint family. It grows to about 1.5m tall and bears curious white and purple flowers in dense clusters resembling a "witch's hat." It is a profuse bloomer in late summer & autumn. The flowers are held on long stems that are very nice for cutting (Watt and Breyer-Brandwijk, 1962).

P. reticulata is the most widespread of the southern African *Pycnostachys* species. It occurs in the Eastern Cape, KwaZulu Natal, Swaziland, Mpumalanga, Gauteng, North West, Limpopo and northwards as far as Tanzania. It grows in moist, swampy places and in grassland. There are 37 species of *Pycnostachys*, of which only three species occur in southern Africa: *P. reticulata, P. urticifolia* and *P. coerulea.* At Kirstenbosch, only the two former species are grown (Hutchings *et al.,* 1996).

P. reticulata roots are used in Zulu traditional medicine as a mouthwash for teeth suffering from neuralgia (nerve pain), not decay (Hulme, 1954). In the Democratic Republic of Congo, a species indigenous to that region, *P. erici-rosenii,* is used in traditional medicine in combination with other plants to treat rabies, madness, anaplasmosis, gastro-enteritis, diarrhoea, dysentery, coccidiosis and schistosomiasis (Chifundera, 1998). *P. reticulata* from Java is reported to be poisonous with emetic properties (Pammel, 1911).

Materials and methods

Seeds were obtained from Kirstenbosch and planted in Prof. Laing's[1] garden. A large sample weighing 2.75 kg of *P. reticulata* was collected from the garden. Voucher specimens were deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. The plant material was air-dried for 1wk and crushed to a powder using a hammer mill. The ground powdered plant material was extracted with dichloromethane. For the extraction procedure, the plant material was steeped in sufficient solvent overnight at room temperature, with occasional stirring. The solvent was subsequently filtered off and the organic extract was concentrated by rotary vacuum evaporation below 45°C resulting in a viscous and dark green extract.

The crude dichloromethane extract was fractionated on a silica gel column using a gradient eluent of increasing polarity (dichloromethane:hexane 1:9 → dichloromethane 3:2). A total of 6 pooled factions were generated. The column was stripped with methanol.

Bioassay

Refer to Chapter 2 for methodologies of the agar well diffusion method and autobiography.

Results

The dichloromethane extract of *P. reticulata* proved to be inactive in antifungal testing. However, this extract showed high antibacterial activity when tested against *B. subilis* and *X. campestris.*

Table 8.2.1 Biological activity of dichloromethane extract of *P. reticulata* against various mircroorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	4 – 10	Active extract. Red colouring from compound.
X. campestris	5 – 10	Active extract. Red colouring from compound.
F. oxysporum	No inhibition	Inactive extract.
R. solani	No inhibition	Inactive extract.
S. sclerotiorum	No inhibition	Inactive extract.
Candida spp.	1	Inactive extract.
P. ultimum	No inhibition	Inactive extract.

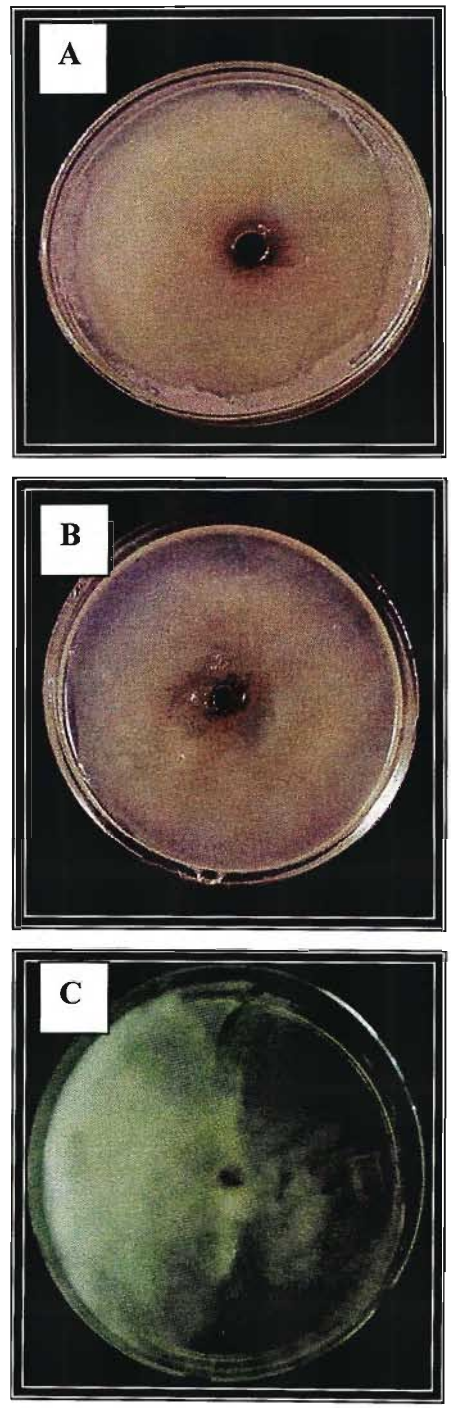


Fig 8.2.2 Bioassay plates showing the biological activity of *P. reticulata* against: *B. subtilis* (A), *X. campestris* (B) and *P. ultimum* (C).

Discussion

The dichloromethane extract of *P. reticulata* showed high antibacterial activity when tested against *B. subtilis* and *X. campestris.* This is depicted by Plate A and Plate B (Fig 8.2.2). No antifungal activity was noted with this plant extract. Due to the high antibacterial activity exhibited by this plant, it is no wonder that *P. reticulata* is used in Zulu traditional medicine as a mouthwash for teeth suffering from neuralgia (Hulme, 1954). No pure compounds from this plant extract were identified by chemical analysis.

Literature cited

Chifundera, K. 1998. Livestock diseases and the traditional medicine in the Bushi area, Kivu Province, Democratic Republic of Congo. *African Study of Monographs* 19: 13-33.

Hulme, M.M. 1954. Wild flowers of Natal. Shuter & Shooter, Pietermaritzburg.

Hutchings, A., Scott, A.H., Lewis, G. and Cunningham, A.B. 1996. Zulu Medicinal Plants. University of Natal Press, Pietermaritzburg.

Pammel, L.H. 1911. Poisonous Plants of the World. Torch Press, Iowa.

Watt, J.M. and Breyer-Brandwijk, M.G. 1962. The medicinal and poisonous plants of Southern and Eastern Africa. 2nd ed. Livingstone, London.

Internet reference

http://www.plantzafrica.com

Chapter 8.3

Screening of *Ficus sur* Forssk. for biological activity

P. Gurlal[1], M.D. Laing[1] and S.E. Drewes[2]

School of Biochemistry, Genetics, Microbiology and Plant Pathology[1], School of Chemistry[2], University of KwaZulu-Natal, Pietermaritzburg, P/Bag X01, Scottsville 3209, South Africa

Abstract

A halo effect occurred when the dichloromethane extract of *F. sur* was tested against *Candida* spp. This is a bacteriostatic effect whereby growth of the test organism is not totally inhibited but somewhat reduced by the plant extract. An inhibition zone of up to 15mm was recorded for this plant extract when tested against *Candida* spp. This plant extract also tested as an active biological control agent against the Gram negative bacterium, *Xanthomonas campestris,* forming an inhibition zone ranging from 2–9mm. Poor antifungal activity was observed with the test fungal microorganisms.

Introduction

Ficus sur

Family: Moraceae

Synonyms: *F. capensis* Thunb.; *F. mallotocarpa* Warb.

Common names: bosvy, bush/Cape fig, grootvy, komaan, koomaan, suurvy

Zulu names: *ingobozweni*, *intombi-kayibhinci*, *umkhiwane*

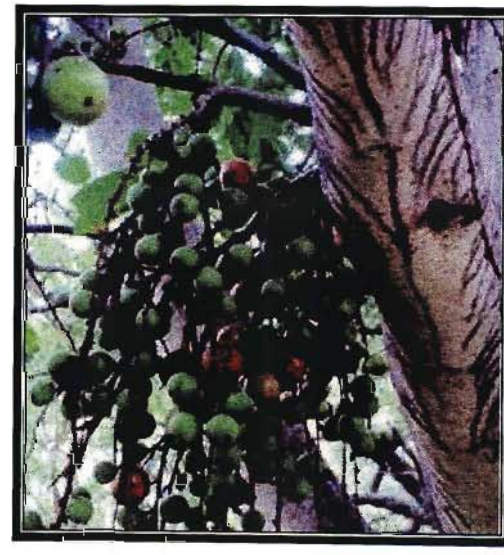


Fig 8.3.1 The fruit of *F. sur* (http://www.plantzafrica.com)

Ficus is a genus with over 750 species worldwide, of which 500 of them occurring in Asia and Australia. In Africa and Madagascar there are about 100 species, 26 of which are indigenous to southern Africa. It is used mainly for gastro-intestinal (GIT) disorders (Kunle *et al.*, 1999).

The species, *F. sur*, occurs in forests, swampy areas and on rocky outcrops in the southern and eastern coastal regions, as well as the bushveldt of Mpumalanga and Northern Province. A maximum height and spread of 10-15m is recorded for wild specimens. The fleshy figs (30mm and more in diameter) turn red when ripe and are borne in large bunches on fruiting branches which are retained after the figs have fallen. They are eaten by a variety of wild animals and birds. This species makes a good specimen plant for a large, frost-free garden. It is best planted in damp or boggy areas, where it will grow very quickly into an attractive shade and bird-attracting tree (Watt and Breyer-Brandwijk, 1962).

Zulu medicinal usage

Root and bark decoctions of *F. sur* are administered for suspected 'ulceration of the lung', possibly referring to pulmonary tuberculosis (Watt and Breyer-Brandwijk, 1962). Leaf and bark infusions are used as bovine galactagogues.

Other medicinal usage

The Vhavenda use the roots of *F. sur* for diarrhoea and the fruit for tuberculosis (Watt and Breyer-Brandwijk, 1962; Mabogo, 1990). Root infusions are used for sterility in men, infertility in women and for uterine pain and for swollen legs in Zimbabwe (Gelfand *et al.*, 1985). The latex is used for cataracts, sore eyes, general body pain and as an emetic. On the other hand, bark infusions are taken as galactagogues and administered to infants with oedemas. Bark is also applied as a powder to rashes (Watt and Breyer-Brandwijk, 1962). Fresh young aerial roots and inner bark are chewed with kola nuts for the alleviation of thirst and for sore throats in West Africa (Ayensu, 1978). Leaves are used as galactagogues while stems and twigs are used for dysentery, leprosy, epilepsy, rickets in infants and with other ingredients for oedema and poisoning. Unspecified parts of the plant are also reported to be used for lumbago, headaches, bronchial ailments, and stiffness from fever and as diuretics and aphrodisiacs (Watt and Breyer-Brandwijk, 1962). The Ila uses the plant as an anti-emetic,

and the tree is widely used as a protective charm and grown in sacred shrines in East Africa (Palmer and Pitman, 1972).

Chemical constituents and biological properties

A milky latex is produced and the plant is reported to yield 0,18% rubber (Watt and Breyer-Brandwijk, 1962). Traces of ascorbic acid are found in the fruit while traces of sterols are found in the leaves, stem and fruit. The bark is resinous and very bitter and reported to contain tannin (Hutchings, 1996).

Materials and methods

The leaves were collected and voucher specimens are deposited in the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg. The plant material was air-dried for 1wk and crushed to a powder using a hammer mill. The ground powdered plant material was extracted with dichloromethane. For the extraction procedure, the plant material was steeped in sufficient solvent overnight at room temperature. The solvent was subsequently filtered off and the organic extract was concentrated by rotary vacuum evaporation below 45°C resulting in a dark green extract.

The crude dichloromethane extract was fractionated on a silica gel column using a gradient eluent of increasing polarity (dichloromethane:hexane 1:9 → dichloromethane 3:2). A total of 8 pooled factions were generated. The column was stripped with methanol.

Bioassay

Refer to Chapter 2 for methodologies of the agar well diffusion method and autobiography.

Results

There was no biological activity by *F. sur* extracts against *F. oxysporum* and *R. solani*. There was a slight inhibition zone when *B. subtilis* and *P. ultimum* was plated out but the results were not very significant. This extract seems to work best on *Candida* spp. and *X. campestris*. Although *Candida* spp. yielded the largest inhibition zone of 15mm, it was a halo effect. This means that the extract is not an effective bacteriocide, but rather bacteriostatic in nature.

Table 8.3.1 Biological activity of dichloromethane extract of *F. sur* against various microorganisms using the well method.

Microorganism	**Zone of inhibition (mm)**	**Notes / Description**
B. subtilis	Not significant.	Inactive.
X. campestris	2 – 9	Active.
F. oxysporum	No inhibition	Inactive.
R. solani	No inhibition	Inactive.
S. sclerotiorum	2 – 4	Inactive to partially active.
Candida spp.	1 – 15	Halo effect. Very active.
P. ultimum	No inhibition	Inactive.

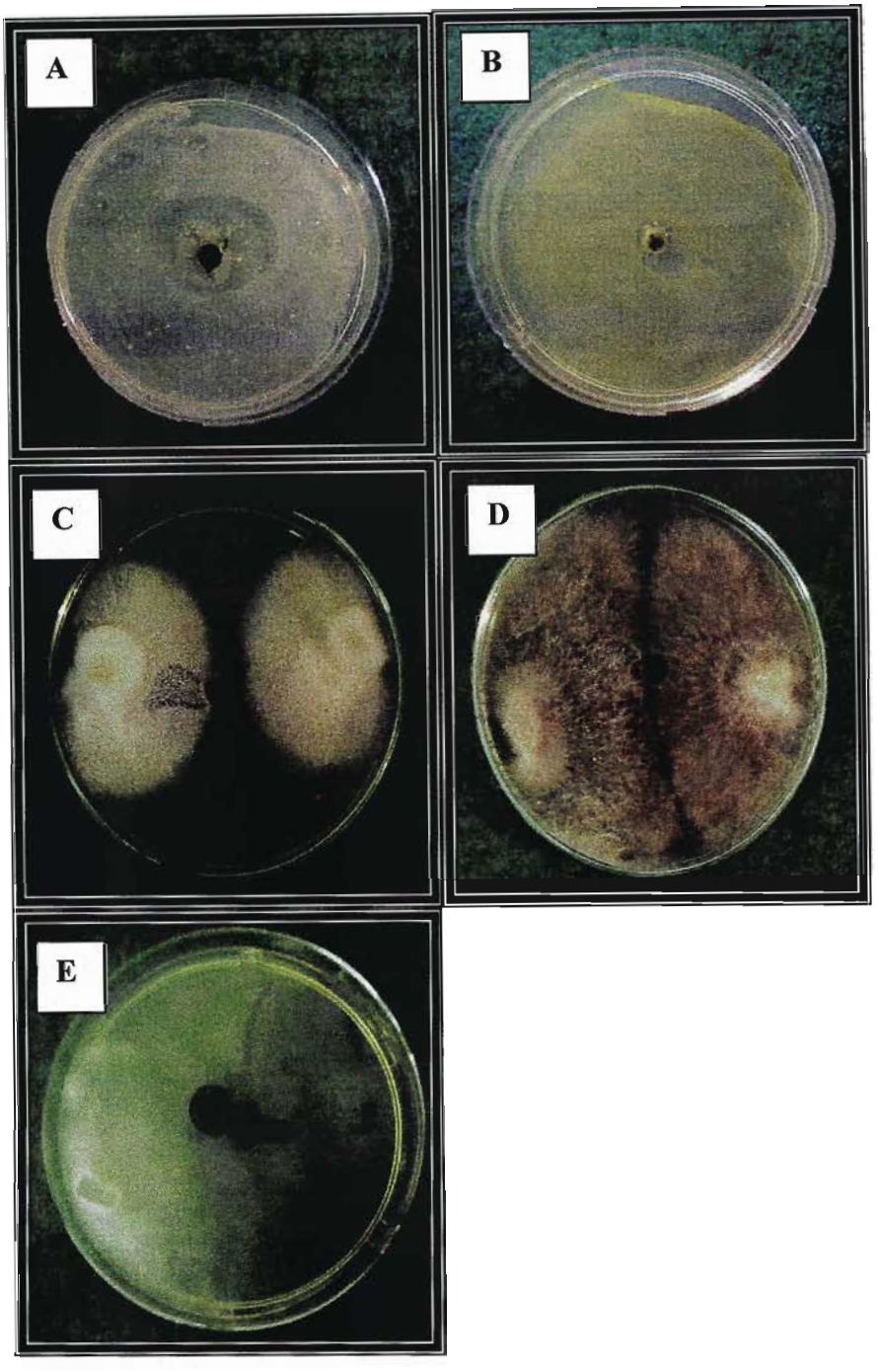


Fig 8.3.2 Bioassay plates showing the biological activity of *F. sur* against *Candida* spp. (A) – irregular inhibition zone around well; *X. campestris* (B) – small irregular zone can be seen; *S. sclerotiorum* (C) – average amount of inhibition is seen as fungus covers 2/3 of the plate; *R. solani* (D) – no inhibition zones as fungus grows over entire plate; and *P. ultimum* (E) – no inhibition can be seen.

Discussion

From the results presented in Table 8.3.1, it is evident that *F. oxysporum, R. solani, B. subtilis* and *P. ultimum* were not inhibited by the dichloromethane extract of *F. sur*. A large inhibition zone of up to 15mm was measured when this plant extract was tested against *Candida* spp. However, the inhibition zone was that of a halo effect, whereby growth of the organism is reduced but not totally inhibited. This can also be clearly viewed in Plate A, Fig 8.3.2. Poor antifungal activity was concluded after the antimicrobial testing. Besides *F. sur* exhibiting some level of biological control against *Candida* spp., this extract was also active against the Gram negative bacterium, *X. campestris,* with an inhibition zone ranging from 2 – 9mm.

Literature cited

Ayensu, E.S. 1978. Medicinal plants of West Africa. Reference Publications, Michigan.

Gelfand, M., Mavi, S., Drummond, R.B. and Ndemera, B. 1985. The traditional medical practitioner in Zimbabwe. Mambo Press, Gweru, Zimbabwe.

Hutchings, A. 1996. Zulu medicinal plants. University of Natal Press, Pietermaritzburg. Pp 75-76.

Kunle, O.O., Nasipuri, R.N., Oyewumi, M.O., Ojile, J.E. and Wambebe, C.N. 1999, Formulation studies on the hot water leaf extract of *Ficus sur* (Moraceae): Compaction characteristics and effect of magnesium stearate on granule and tablet properties. *Acta Pharm.* 49: 189-199.

Mabogo, D.E.N. 1990. The ethnobotany of the Vhavenda. Unpublished Master of Science Thesis, University of Pretoria.

Palmer, E. and Pitman, N. 1972. Trees of Southern Africa. Vol. 2. Balkema, Cape Town.

Watt, J.M. and Breyer-Brandwijk, M.G. 1962. The medicinal and poisonous plants of Southern and Eastern Africa. 2nd ed. Livingstone, London.

Internet reference

http://www.plantzafrica.com

Thesis Overview

Plectranthus is a large and widespread genus with a diversity of ethnobotanical uses. Monoterpenoids, sesquiterpenoids, diterpenoids and phenolics have been reported in species of *Plectranthus*. The abietane diterpenoids are the most diverse of the diterpenoids isolated from species of *Plectranthus* (Lukhoba *et al.*, 2005).

In this thesis a range of plant extracts were tested against representatives of the primary groups of fungi and bacteria. The effectiveness of some of these plant extracts, suggests that further research may develop these compounds into useful antifungal and antibacterial remedies, both for human and crop protection. However, to take the compounds further will take considerable funding and scientific commitment to test the compounds. Typically, efficacy, toxicology (chronic and acute) and eco-toxicology trials would need to be undertaken.

The results obtained from the bioassays, indicating biological activity of the plant extracts, are summarized in Table 9. A plant extract that is considered as having excellent biological activity was classified according to production of inhibition zones with a radius measuring greater than 10mm. The extracts that showed the most number of +++ for biological activity against the test organisms were considered the most potent. The most active extracts were the pure compounds, **2** and **3**, and the crude extracts *P. purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaenis*. From the seven microorganisms tested, these plant extracts showed excellent biological activity against three of the microorganisms. Pure compound **3** possesses good antifungal properties as *S. sclerotiorum* and *P. ultimum* were inhibited. It is also effective against *B. subtilis*, which is a gram positive bacterium. On the other hand, pure compounds **2** was active against the gram positive and gram negative bacteria, which could make it an effective antibacterial agent. *P. purpuratus* subsp. *purpuratus* and *P. purpuratus* subsp. *tongaenis* exert similar levels of biological activity. Although these two species differ morphologically, their chemical composition appears identical. They also appear to be excellent antifungal agents. The crude extract of *P. lucidus* appears slightly more potent than the pure compounds **9** and **10** isolated thereof. *P. senegalensis* and *F. sur* can be best used for yeast infections as they are most potent against the *Candida* spp. Good antibacterial activity is noted from *P. reticulata* as there was good inhibition against the gram positive and gram

negative bacteria. *P. fruticosus* and *P. ecklonii* do not possess any antifungal activity but some antibacterial activity. Table 10 summarizes the number of pure compounds isolated and the IUPAC nomenclature. Nothing is known or published about the bioactivity of the pure compounds isolated.

Scientists from divergent fields are investigating plants with a new eye to their antimicrobial usefulness. A sense of urgency accompanies the search as many plant species have become extinct already. Laboratories all over the world have found thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro.* More of these compounds should be subjected to microbial, animal and even human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic, faster and economical so that interpretation of results would be easily facilitated. Attention to these issues could usher in a badly needed new era of treatments of infection by using plant-derived principles.

Literature cited

Lukhoba, C.W., Simmonds, M.S.J. and Paton, A.J. 2005. *Plectranthus:* A review of ethnobotanical uses. *Journal of Ethnopharmacology.* Article in Press.

Table 9 Summary of all dichloromethane plant extracts tested against the various microorganisms

Plant extract	***B. subtilis***	***X. campestris***	***Candida* spp.**	***F. oxysporum***	***S. sclerotiorum***	***R. solani***	***P. ultimum***	***Control (dichloromethane)***
P. fruticosus crude	+	++	-	-	-	-	-	-
P. ecklonii crude	-	+++	-	-	-	-	-	-
P. hadiensis crude	++	++	+++	-	+++	-	+	-
Compound ***2***	+++	+++	+	-	+++	++	+	-
Compound ***3***	+++	+++	++	-	+++	+	+++	-
Compound ***4***	-	-	+	-	+++	-	-	-
P. praetermissus crude	+	+++	+++	-	+	-	-	-
P. ciliatus crude	+	+	-	-	+++	-	-	-
P. zuluensis crude	-	+++	++	-	+	-	++	-
Compound ***7***	+++	+	-	-	+++	-	-	-
Compound ***8***	+	++	-	-	++	-	++	-
P. lucidus crude	+	++	-	++	++	++	-	-
Compound ***9***	-	++	-	+	+	+	-	-
Compound ***10***	-	+	+	++	++	++	-	-
P. purpuratus subsp. *purpuratus* crude	-	-	-	+++	+++	+++	-	-
P. purpuratus subsp. *tongaenis* crude	-	-	-	+++	+++	+++	-	-
P. senegalensis crude	-	-	+++	-	-	-	-	-
P. reticulata crude	++	++	-	-	-	-	-	-
F. sur crude	-	++	+++	-	+	-	-	-

\- no activity, inhibition zone size < or = 3mm
\+ moderate activity, inhibition zone size > 3mm but < 7mm
++ good activity, inhibition zone size > 7mm to 10mm
+++ excellent activity, inhibition zone size > 10mm

Table 10 Summary of pure compounds isolated and IUPAC names

Compound No.	IUPAC Name	Synonym
1	11-Hydroxy-2-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one	Parviflorone D
2	6,7-Dihydroxyroyleanone-6,7,12-trihydroxy-8,12-abietadiene-11,14-dione	
3	7α-formoxy-6β-hydroxyroyleanone	
4	7α-acetoxy-6β-hydroxyroyleanone	
5	20(10→5)-abeo-1(10),6,8,11,13-abietapentaene-11,12,16-triol	
6	11,12,15-trihydroxy-20-(10→5)-abeo-abieta-1-(10),6,8,11,13-pentaene	
7	2'-hydroxy-4',6'-dimethoxyacetophenone	Xanthoxylin
8	1,2,4-trimethoxy-5-(2-propenyl)-benzene	Euasaron, isoasaron and sekishon
9	11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one	Parviflorone A
10	11-Hydroxy-2-(4-hroxybenzoyl)-5,7,9(11),13-abietatetraen-12-one,	Parviflorone D
11	11-hydroxy-19-(3-methyl-2-butenoyl)-5,7,9(11),13-abietatetraen-12-one	Parviflorone A
12	11-hydroxy-19-(4-hydroxybenzoyl)-5,7,9(11),13-abietatetraene-12-one	Parviflorone C
13	11-hydroxy-19-(4-3,4-dihydroxybenzoyl)-abieta-5,7,9(11),13-tetraene-12-one	Parviflorone E