ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUNDS FROM *GALENIA AFRICANA*

UNIVERSITY of the
WESTERN CAPE

Thesis submitted in the fulfilment of the requirements for the degree

MASTER OF SCIENCE

in the Department of Chemistry, University of the Western Cape

By

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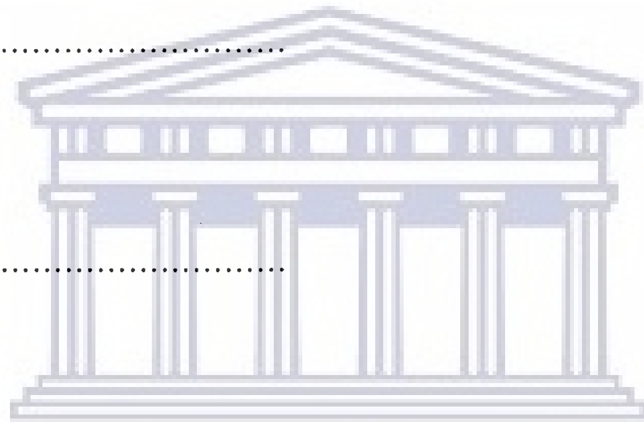
Declaration

I, Filicity Vries, hereby declare that the work on which this thesis is based is original work and that neither the whole nor any part of it has been, or is to be submitted for another degree in this or any other University.

Signed: ....



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SUMMARY

Galenia africana, commonly known as "kraalbos" or "geelbos" belonging to the family Aizoaceae, is a dominant plant throughout the Namaqualand area. It is an active invader and is especially abundant in disturbed areas around kraals, along roads, and water points which shows that it grows well on trampled soil. "Kraalbos" is not very palatable to livestock, but is grazed under pressure. The plant can cause liver damage and severe ascites, a condition referred to as "waterpens" in sheep and occasionally in goats. The Hottentots chew the plant to relieve toothache and it is said to blister the mucous membrane of the mouth if used too much. The plant was also used in the treatment of veneral diseases.

Dried samples of *G. africana* collected in Steinkopf and Concordia were sequentially extracted with various solvents starting with hexane, chloroform, ethanol and sterile distilled water. Each concentrated extract were tested for its inhibitory activity against fungi and bacteria by using the agar well diffusion method. The mycelial growth of *Fusarium graminearum* and *Fusarium verticillioides* cultured on PDA was inhibited by the $CHCl_3$ and EtOH extracts. The minimum inhibitory concentration (MIC) of these two extracts was calculated for both fungal and bacterial species. Overall results showed that the EtOH extract from *G. africana* has strong inhibitory activity *in vitro* against fungi and bacteria. The above findings advocate further investigation of the EtOH extract. It will be necessary to identify the active components and to evaluate their potential for use as antimicrobials.

The ethanol extract was purified using silica gel column chromatography and thin – layer chromatography. Four fractions showed significant antifungal activity as evidenced by a clear zone of inhibition. One fraction was tested against a range of fungi and a few bacterial isolates. This fraction inhibited the growth of most of the fungal isolates with *Alternaria* sp., *Fusarium equiseti*, *F. graminearum*, *F. verticillioides* and *Phaeomoniella clamydospora* being the most sensitive ones. Both *Bacillus* sp. and *Staphylococcus aureus* were inhibited in the presence of the fraction. The MIC`s for fungi showed values ranging from 10 to 2.5 % and bacteria from 2.5 to 1.25 %. The active fraction inoculated

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onto individual filter paper disks displayed potent antifungal activity on agar plates seeded with conidia of the various fungal isolates being tested. The filter paper disk method showed more sensitivity than the agar well diffusion method in the detection of the antifungal activity of the active fraction.

The purified plant extract fraction of *G. africana* was used to evaluate its antifungal activity on the germination of common plant pathogens. Twelve fungal species, i.e *Alternaria* sp., *Botrytis cinerea*, *Cylindrocarpon* sp., *Cylindrocladium* sp., *F. equiseti, Fusarium oxysporum, Fusarium pseudograminearum, Fusarium solani, Fusarium trinctinum, F. verticillioides, Penicillium expansum* and *P. clamydospora*, were used for the germination on water agar plates at different fraction concentrations, i. e 0.625, 1.25, 2.5, 5 and 10 %. Germ tube lengths were reduced at increasing concentrations of the active fraction for all the test fungi. Concentrations of the active fraction higher than 1.25 % prevented the spore germination of *Alternaria* sp., *B. cinerea, Cylindrocarpon* sp. and *F. verticillioides* and concentrations higher than 2.5 % prevented the spore germination of P. *expansum* and *P. clamydospora.*

The antifungal bioactive fraction which was isolated from *G. africana* was assayed for its efficacy to control gray mold (incited by *B. cinerea*) and blue mold (incited by *P. expansum*) *in vivo* on wounded apples. In the present study we demonstrated that the antifungal bioactive fraction of *G. africana* has both protective and curative effects against gray and blue mold of apple. The bioactive fraction of *G. africana* did not enhance the protective or curative activity of the yeast in this study. Although the bioactive fraction did not enhance the effect of the yeast, the results indicate that it will also not have a negative effect on decay control when applied in combination. In combinations therefore, the bioactive compound could control previously established infections while the antagonist confer a long term protective effect. Chemical studies of the bioactive fraction of *G. africana* identified an antimicrobial compound, 5,7 – dihyroxyflavanone **2** as a major component of this fraction.

The presentation of this thesis will follow the "authors instructions" of the "Phytochemistry" journal.

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CHAPTER 1

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GENERAL INTRODUCTION AND LITERATURE REVIEW

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Chapter 1

General Introduction and Literature Review

Plant diseases, caused primarily by fungal and bacterial pathogens, cause severe losses to agricultural and horticultural crops every year. These losses can result in reduced food supplies, poorer quality of agricultural products, economic hardship for growers and processors, and ultimately higher prices (website 1). Although these losses may be attenuated by the use of disease-tolerant cultivars, crop rotation, or sanitation practices, fungicides continue to be essential for the effective control of plant diseases (Knight *et al.*, 1997). These fungicides are not always economical or effective for many diseases and some may cause unwanted health, safety and environmental risks (website 1). This research project will seek to develop a biological pesticide. A biological pesticide is a product that uses naturally occurring ingredients, such as plant extracts to control pest populations. Biological control can offer an environmentally friendly approach to the management of plant disease and can be incorporated with cultural and physical controls and thereby limited chemical usage for an effective integrated disease management system (website 1).

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Many plants (eg. *Azadirachta indica*) and plant products have been reported to possess pest control properties (Grange and Ahmed, 1988; Grayer and Harborne, 1994). Although much of the literature on natural products in the agricultural field concerns insect control, a smaller but emerging body of papers report that plant extracts and plant essential oils are effective antimicrobials against food and grain storage fungi (Arras *et al.*, 1993; Bishop and Thorton, 1997; Chatterjee, 1989; Dixit *et al.*, 1995, Mishra and Dubey, 1994; Moleyar and Narasimham, 1986; Montes-Belmont and Carvajal, 1998; Paster *et al.*, 1995; Thompson, 1989; Wilson *et al.*, 1997), soilborne fungi (Awuah, 1994; Bianchi *et al.*, 1997; Dubey and Kishore, 1987; Kishore *et al.*, 1989; Kishore *et al.*, 1982; Muller-Riebau *et al.*, 1992; Pandey and Dubey, 1994; Shimoni *et al.*, 1993) and nematodes (Walker, 1998). This means that if natural plant products can reduce populations of plant pathogens and control disease development, then plant extracts have potential as environmentally sound alternatives to chemicals in integrated management

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programs (Bowers and Locke, 1999).

The alternative uses for some South African invader plants

Southern Africa is richly endowed with aromatic plants, most of which are unpalatable to livestock owing to terpenoidal oil located primarily in leaf glands. These plants (eg. *Agathosma betulina*, *Agathosma crenulata* and *Lippia javanica*) have no economic value to livestock farmers and consequently are regarded as invaders indicative of degraded veld. Some of these unpalatable plants are great survivors, as they are basically the only ones left standing during times of drought, and other severe environmental conditions. Before eradication of these plants from the veld their alternative use should be considered so that a farmer could benefit or receive an income, instead of incurring an expense (Webber *et al.*, 1999)

Agathosma betulina and *Agathosma crenulata*, generally known as buchu, are indigenous, aromatic, fynbos species found in the mountainous regions of the Western Cape. These plants have a medicinal value and are widely used by the local rural people and farmers for the treatment of stomach problems and a variety of other ailments (Webber *et al.*, 1999). *Aloe ferrox* is widely used for medicinal purposes by rural people for the treatment of constipation, eye infection and a number of other ailments, especially in the treatment of their livestock for internal parasites. The sap is used for either laxative or cosmetic reasons (Webber *et al.*, 1999). The oil *Artemisia afra* has value as a biological agent with greater antimycotic than antibacterial activity (Graven *et al.*, 1992; Mangena and Muyima, 1999). *Leonotis leonarus*, which is also known as wild dagga, klip dagga and mcafincanie, is one of the most widely used South African medicinal plants. Although it is used primarily for skin complaints, it has also been used against a host of other ailments. Its most promising healing property is for eczema (Webber *et al.*, 1999). Essential oil derived from *Lippia javanica* includes ipsdienol and ipsenon. Ipsdienol is used as an attractant for a beetle that causes great damage to pine trees in the forests of Canada and Europe (Webber *et al.*, 1999). Several compounds found in *Rosmarinus officinalis* oil have been reported to be inhibitory to micro-organisms (Guenther, 1974; Tyler *et al.*, 1976; Deans and Svoboda, 1993; Mangena and Muyima,

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1999).

The use of natural plant extracts to control fungi

In Nigeria, research has focused on the use of plant extracts and dried powders from neem (*Azadiracta indica*) (seed/kernel oil and cake, leaves bark, roots), dry chilli peppers (*Capsicum frutescens*), onion scale leaves (*Allium cepa*), ginger (*Zingiber officinale*), tobacco (*Nicotiana tabacum*), cocca pot husks (*Theobromo cacao*), vegetable oil, wood ash, smoke, cashew nut shell liquid (*Anacardium occidentale*), sand, organic manure, etc. for the control of pests and diseases in the field and in the store. Numerous studies have confirmed the importance of neem for the latter and the former have been shown to be comparable with some synthetic pyrethroids in control of cotton insect pest in Nigeria (Poswal and Akpa, 1991). Recently, neem-leaf powder, has been successfully used for controlling a nematode disease incited by *Meliodogyne* species (Mukerjee and Tiagi, 1978).

Aqueous extracts of 10 plant species collected in Israel were tested for their ability to inhibit *Trichophyton rubrum* and *Microsorium canis*, the aetiological agents of dermal fungal infections in humans, which are difficult to control effectively and in addition the pharmaceutical arsenal currently available against them is rather limited (Maoz and Neeman, 1998). Extracts were prepared from the indicated parts of the following 10 plant species: *Polygonum aviculareae* (roots), *Polygonum equistiforme* (upper parts), *Cynodon dactylon* (entire), *Inula viscosa* (leaves), *Prosopis farcta* (upper parts), *Ammi visnaga* (seeds), *Salvia fruticosa* (leaves), *Celltis australis* (bark), *Tamarix aphylla* (bark) and *Laurus nobilis* (leaves). According to indigenous medicinal knowledge these parts of the plants were known to have antifungal and /or other dermal qualities (Krispel, 1986). Aqueous extracts from the leaves of *Inula viscosa* produced detectable antifungal activity against these dermatophytes. Hence, plant products or botanicals that inhibit their growth without harming the host represent potential therapeutic agents (Maoz and Neeman, 1998).

The effect of aqueous extracts and the oil of neem (*Azadirachta indica*) on four soil-

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borne pathogens, *Fusarium oxysporum* f.sp *ciceri*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, which incite wilt and rot in gram seeds (*Cicer arietinum*) were studied (Singh *et al*., 1980). Growth of the four pathogens in liquid medium was inhibited by extracts of leaf, trunk, bark, fruit pulp, and oil. Of these four extracts, neem oil showed maximum inhibitory effects. The germination of gram seeds was inhibited at higher concentrations of oil. Oil- treated seeds sown in soil infested with the pathogens singly and intermixed produced disease-free seedlings whereas all seedlings from untreated seeds exhibited disease symptoms. The fruit pulp suppressed formation of *S. sclerotina* and *R. solani* at all the concentrations. A possible role of neem extracts and oil in controlling gram diseases in field conditions is thus suggested (Singh *et al*., 1980).

Orange peel is the primary waste fraction and is the source of orange essential-oil, which is widely used in the food, drug and cosmetic industries (Shaw, 1979; Shaw and Coleman, 1974). The oil exhibits important antifungal activity against *Fusarium culmorum*, *Aspergillus parasiticus* and *Penicillium italicum* and offers an interesting prospect as a food fungicide. Unsaturated C_{18} fatty acids isolated from rice leaves (*Oryzae sativa*) show fungicide activity against *Pyricularia oryzae* fungus (Kato *et al*., 1983) and, particularly, linoleil-monoglyceride has been previously reported as an inhibitor of the germination of spores of *Phytophora infestans* and *Cladosporium cucumerinum* (Oliva and Arditti, 1983). Recently, it has been reported that linoleil-monoglyceride also inhibits the germination of spores and the growth of the vegetative cells of *Bacillus cereus*, *Clostridium botulinum* and *C. sporogenes* (Chaibi *et al*., 1996 a, b). According to Vargas *et al*. (1999), the isolated hexa- and heptamethoxy flavones exhibited important antifungal activity against *Geotrichum candidum*, which is not inhibited by the broad-spectrum fungicide Benomyl. The use of natural plant products, which could possibly replace environmentally harmful synthetic herbicides (Rice, 1995), is fast becoming an important means of controlling noxious weeds. Among such products, essential oils have great significance in terms of their high phytotoxicity (Vicherkova and Patkova, 1982).

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The use of natural plant extracts to control insects

Integrated Pest Management (IPM) has become the predominant philosophy for insect pest control in the 1990's (Cousin, 1989; Xie and Isman, 1992 a, b). Major tenets of the IPM are the reduced use of pesticides and the use of those pesticides least disruptive to the environment. It is well known that oils (plant oils, animal oils and/ or mineral oils) can kill insects, both physically by interfering with insect respiration, and chemically (Martin and Woodcock, 1983). Mineral oils, especially petroleum oils, have been used as insecticides in agriculture for over two centuries (Miller, 1983). In the exploration of plant natural products as insecticides, tall oil, a by-product of the kraft process for pulping softwood, has attracted attention (Cousin, 1989; Xie and Isman, 1992 a, b).

Tall oil possesses properties somewhat similar to those of horticultural oils. However, major constituents of tall oil are resin acids (37 % by weight), which are known insect antifeedants and growth inhibitors (Ellinger *et al.*, 1976; Xie *et al.*, 1993; Wagner and Benjamin, 1984; Schuh and Benjamin, 1984). Tall oil could, therefore, be useful as a crop-protecting agent because of its toxic and/ or deterrent properties in addition to its physical properties. The comparative toxicity and deterrency of depitched tall oil (a by-product of the kraft process for pulping softwood) to the green peach aphid, *Myzus persicae* were investigated. The study provided evidence that depitched tall oil, an inexpensive and abundant by-product of the kraft pulping process of softwoods, has both deterrent effects and toxicity against *M. persicae*. The mechanisms of action of oils are thought to be mainly physical, i.e. the oils kill pests by suffocation. This study in addition demonstrated that depitched tall oil possesses significant deterrent activity on *M. persicae* nymphs in addition to contact toxicity (Xie *et al.*, 1993).

Cabbage is a staple vegetable in the rural areas of Southern Africa, with consumption increasing steadily (Van den Berg and Pretorius, 1995). The diamondback moth (DBM), *Plutella xylostella* is a serious pest of the cabbage family (*Brassicaceae*) in Africa. Developing farmers need safe, cost-effective and environmentally sound ways of controlling DBM. A possible solution to this problem would be the use of natural

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pesticides. These natural products are rich sources of biologically active compounds, which have evolved as components of the natural chemical defence mechanism used by plants. Natural pesticides have several active ingredients, making them more difficult for insects to develop resistance. Syringa, an invasive plant in South Africa, is closely related to neem (*Azadirachta indica*), which is renowned for its insecticidal properties and from which commercial formulations are available. Both plants are in the family *Meliaceae* (Van den Berg and Pretorius, 1995).

Extracts were prepared from dried syringa leaves and crushed into a fine powder (Van den Berg and Pretorius, 1995). The cabbage leaves were then dipped into the extract to test the effect on DBM and more than 90 % of the larvae died on the treated leaves. Diamondback moths were released into an insect cage containing both treated and untreated cabbage leaves, and left for 48 hours to lay eggs. It was found that up to four times as many eggs were laid on the untreated control leaves, an indication that syringa extracts deterred female moths from laying eggs on treated leaves. These results indicate that syringa extract shows great promise as a natural pesticide and its potential role resource-limited farming should be investigated further (Van den Berg and Pretorius, 1995).

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In Kerala, a southern state of India, fruit fly (*Dacus dorsalis* and *D. cucurditae*) incidence is rather severe in mango trees (Reghunath and Indira, 1993). A trial was undertaken in which fruit fly traps were installed in the mango trees grown in the garden land of the Rice Research Station, Kayamkulam and also in the homesteads of Kayamkulam. About 200 g of *Ocimum sanctum* (holy basil) leaves were crushed and the extract together with the crushed leaves was placed inside a coconut shell. The coconut shell was then filled by adding 100 ml water. For increasing the keeping quality of the extract, 0.5 g citric acid was added and the extract was then poisoned by mixing in 0.5 g carbofuran 3 G. The traps were then suspended in the tree branches at the rate of 4 traps per tree. The fruit flies fed on the *Ocimum* extract and died within a few minutes. In order to control insects successfully an integrated strategy is advisable (Reghunath and Indira, 1993).

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Ecology of *Galenia africana*

Namaqualand is a sparcely populated semi-desert comprising an area of 47700 km^2 located in the northwestern corner of South Africa (Boonzaaier *et al.*, 1990). Resource mining is being limited and thus farming activities are becoming more prevalent in Namaqualand. An increase in livestock populations has caused overgrazing of palatable and nutritious plants with a subsequent increase in non-palatable plant species. In this region unexplained problems exist regarding sheep and goat mortalities and their general well being. Some of these problems are related to the presence of toxic plant species (Boonzaaier *et al.*, 1990).

Galenia africana, commonly known as " kraalbos " or "geelbos" belonging to the family Aizoaceae is a dominant plant throughout Namaqualand and the Clanwilliam area. In recent years *G. africana* has become more widespread in the Western and Southern Karoo (Kellerman *et al.*, 1988).

G. africana is an active invader, and is especially abundant in disturbed areas around kraals, along roads and on trampled veld (Van der Lugt *et al.*, 1992). This plant is not only an indicator of disturbance, but is also a pioneer plant, being the first perennial to regrow after disturbance. Alternatively it can be the only remaining species after the veld has been heavily overgrazed. "Kraalbos" is an aromatic, woody perennial sub-shrub, growing 0.5 - 1 m high, having oppositely arranged green leaves (5 cm long and hairless) which turn yellow with age. Inflorescence is born at the ends of the twigs and is 30-100 mm long, with many small yellow flowers. The flowers are about 1.5 mm in diameter, yellowish green and born in large loose heads (Le Roux *et al.*, 1994).

Due to the severe drought and overgrazing, "kraalbos" is almost the only pasture plant on the farms in Namaqualand. Animals are forced to browse the plant and are more prone to develop clinical diseases (Van der Lugt *et al.*, 1992). The plant has been associated with liver damage and severe ascites, a condition referred to as "waterpens" in sheep and goats. "Waterpens" is characterised by the development of an extensive ascites

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in sheep and other small ruminants and seems to be associated with an atrophic or hypertonic arrhasis of the liver (Watt and Breyer-Brandwijk, 1962). The marked liver lesions in sheep and occasionally in goats with "kraalbos" have led farmers and researchers to believe that the plant is primarily heptotoxic to livestock. It was suggested that the plant contains an unidentified toxin responsible for severe hepatic damage and ascites (Van der Lugt *et al.*, 1992). "Kraalbos" can cause blood poisoning in human beings. Apart from weight loss, the habitus and appetite of sheep suffering from "waterpens" remain fair up to the terminal stages of the disease, after which the animals become apathetic and recumbent, and eventually die. At necropsy, the liver is always affected. Depending on the stage of the disease, the organ can either be smaller than normal or enlarged, the colour may range from a greyish-blue to a yellowish-brown and the morphology of the liver can be unaltered or distorted by nodular hyperplasia, atrophy and /or hypertrophy of certain parts (Kellerman *et al.*, 1988).

Local farmers claim that during the summer months the "kraalbos" is highly poisonous. This is the time when the plant is dry, woody with yellow leaves and it is these leaves, which are highly toxic. They claim that the leaves contain a high level of acidic material and when eaten by animals the acids "eat away" the stomach lining. As the acids eat away the stomach lining it becomes thinner and water permiates through this thinner lining. This is the time that the animal is unable to stand up again after lying down due to abdominal distension. According to the local farmers, if the plant is green it is palatable and not poisonous but if it is yellow and dry it is poisonous and non-palatable. The farmers further claimed that "kraalbos" has no side effects on donkeys and horses. These farmers are subsistence farmers and are not always financially able to provide medical attention for the affected animals, they have developed alternative methods to deal with the "waterpens" problem. The affected animal is stabbed with a knife in the groin and lifted up allowing the water to drain through the hole, and is only fed dry feed and is not given any water until all the water has drained from the abdomen. After the stab wound has healed, the animal is then allowed to drink water again.

During an outbreak of "waterpens", only a small percentage of the flock is affected,

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and some farmers are of the opinion that drainage of the ascitic fluid, coupled with deprivation of water for six to twelve days and an improved diet can lead to recovery of affected animals (Kellerman *et al.*, 1988).

The medicinal uses of *G. africana*

A decoction of *G. africana* is used as a lotion for wounds in man and animal (Watt and Breyer-Brandwijk, 1962). The Hottentots chew the plant to relieve toothache and it is said to blister the mucous membrane of the mouth if used too much. The plant was also used in the treatment of veneral diseases and a decoction as a lotion for skin diseases and for the relief of inflammation of the eyes. An ointment, made by frying the herb with *Cyanella lutea, Lobostemon fruticosus*, *Melianthus* major, *Melianthus comosus*, "*Tiendaegeneesblare*" and "*Jakkalsoorblare*" in butter, was used as a dressing for wounds especially wounds on the legs of women. In syphilis the external lesions were washed with a decoction of the plant and *Lobostemon fruticosus*, *Melianthus major* and *Melianthus comosus* and for lupus, a decoction of the plant with *Melianthus major*, *Melianthus comosus* and "*Berglelie*" (Watt and Breyer-Brandwijk, 1962).

The family Aizoaceae

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The family Aizoaceae is a largely southern hemisphere family of 100-150 genera and about 2500 species. It is placed in the suborder Caryophyllidae (order Caryophyllales) (Heywood, 1978). The family Aizoaceae is divided into five subfamilies (Table 1). Members of the family produce betalain pigments. Betalain pigments contain basic nitrogen and are responsible for the colouration of leaves (Brittrich and Hartmann, 1988).

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Table 1: Subfamilies in Aizoaceae with their different genera.

Subfamilies	Genera
Aizooideae	*Acrosanthes* Eckl. & Zeyh. *Aizoanthemum* Dinter ex Friedrich, *Aizoon* L., *Galenia* L., *Gunniopsis* Pax and *Plinthus* Fenzl.
Mesembryanthemoideae / Aptenioideae	*Aptenia* N.E.Br. , *Aspazoma* N.E.Br., *Brownanthis* Schwantes, *Dactylopsis* N.E.Br., *Pseudobrownanthus* Ihlenf. & Brittrich, *Psilocaulon* N.E.Br. *Synaptophyllum* N.E.Br and *Sceletium* N.E.Br.
Ruschioideae	About 120 genera comprising the subfamilies *Caryotophoroideae* Ihlenf., Schwantes & Straka, *Hymengynoideae* Schwantes and *Ruschioideae* Schwantes as in Herre (1971).
Sesuvioideae	*Cypselea* Turp., *Sesuvium* L., *Trianthema* L. and *Zaleya* N. L. Burman.
Tetragonioideae	*Tetragonia* L. and *Tribulocarpus* S. Moore

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Taxonomic confusion exists amongst certain authors regarding the classification of *Galenia* and *Sceletium*. *Sceletium* is classified by certain authors in the family Mesembryanthemaceae (Arnold and de Wet, 1993) and by others in the family Aizoaceae (Brittrich and Hartmann, 1988) while *Galenia* is always grouped in Aizoaceae. In the South African context Aizoaceae (non-succulent leaves) and Mesembryanthemaceae (succulent leaves) are recognised. As defined by Brittrich and Hartmann (1988), Chase *et al.* (1993) and Cronquist (1988), Mesembryanthemaceae is paraphyletic and should be included in Aizoaceae. Brittrich and Hartmann (1988) based his classification on morphology, anatomy and embryology, Chase *et al.* (1993) on the plastid rbc L gene and Cronquist (1988) on morphology and anatomy. *Sceletium* is classified in the subfamily Aptenioideae (succulent with petals present) in the family Aizoaceae and *Galenia* to the subfamily Aizooideae (petals absent and non-succulent) (Brittrich and Hartmann, 1988).

The results of general alkaloid tests have been recorded in an investigation of "kougoed", a preparation made from *Sceletium expansum* L. Bol. and *Sceletium tortuosum* N. E. Br. Popelak and Lettenbauer (1968) reported that levels of alkaloids in "kougoed" ranged from 1-1.5 %, while mesembrine and mesembrenine levels were 0.7 and 0.2 % respectively. According to Smith *et al.* (1996), *Sceletium* is a richer source of alkaloids than other Aizoaceae (Figure 1). The Hottentots chewed *Sceletium expansum* and S. *tortuosum* and later smoked the roots or leaves and then their animal spirits were awakened. Their eyes sparkled and thousands of delightful ideas appeared to them (website 1). If indulged in excess, it robbed them of their senses and they become intoxicated. In conclusion, there is a potential production of alkaloids or secondary compounds in the family Aizoaceae (Brittrich and Hartmann, 1988).

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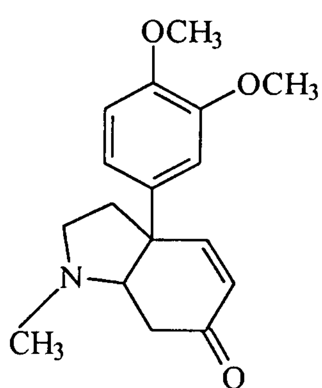
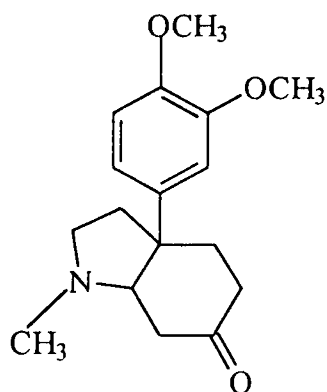
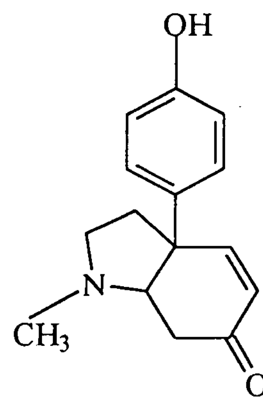


Mesembrenone



Mesembrine



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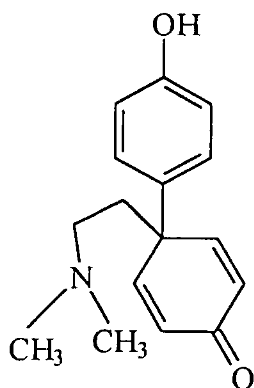
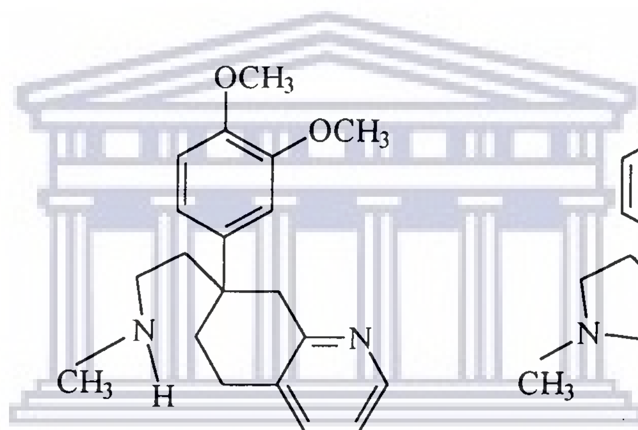
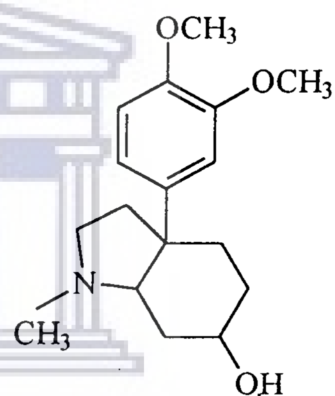


Dehydrojoubertamine



Tortuosamine



Mesembranol

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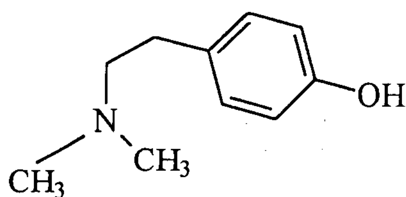

Hordenine

Figure 1: Alkaloids found in *Sceletium*.

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Secondary plant metabolite extraction, purification and characterization techniques

Secondary metabolites function as defence mechanisms for plants (Wink, 1999). Secondary metabolites can be utilized in agriculture to breed stronger crops and in the manufacture of biorational pesticides. They can also be exploited in the medical field as therapeutic agents. The secondary metabolites derived from cytoplasm, such as tannins, alkaloids, terpenes, flavonoids, cyanogenic glycosides and many others, have a negative influence on palatability and digestibility of forage, because of their irritant, toxic or unpalatable characteristics (Wink, 1999).

Alkaloids. The alkaloids comprise the largest single class of secondary plant products (Harborne and Turner, 1984). There are three main types of alkaloids a). true (have heterocyclic ring with nitrogen and derived from amino acids), b). proto alkaloids (do not have heterocyclic ring with nitrogen but derived from amino acids) and, c). pseudo alkaloids (have heterocyclic ring with nitrogen and are not derived from amino acids) (website 2). Alkaloids are highly reactive substances with biological activity in low doses. Alkaloids contain nitrogen and are usually derived from amino acids (website 2). A simple but by no means infallible test for alkaloids in fresh leaf or fruit material is the bitter taste they often impart (Harborne, 1984). They give a precipitate with heavy metal iodides. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer`s reagent (potassiomercuric iodide solution). Dragensdorff`s reagent (solution of potassium bismuth iodide) gives an orange coloured precipitate with alkaloids. Caffeine, a purine derivative, does not precipitate like most alkaloids. Alkaloids are usually colourless and basic. They form water-soluble salts and most alkaloids are well-defined crystalline substances, which combine with acids to form salts. In plants, they may exist in the free state, as salts or as N-oxides. They occur in a limited number of plants. Nucleic acids exist in all plants, whereas, morphine exists in only one plant species (Figure 2) (website 2).

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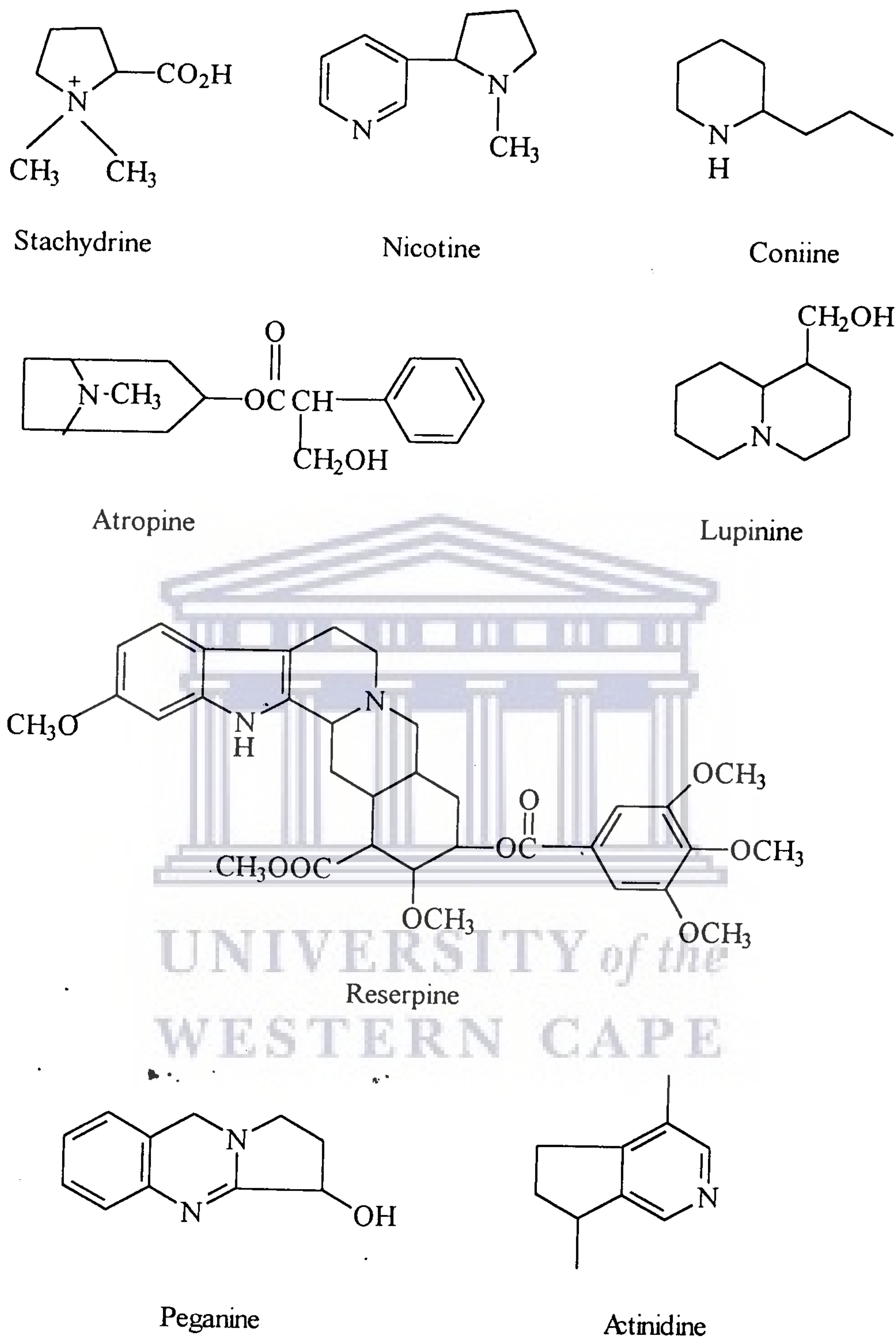

Figure 2: Different alkaloidal types.

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Functions of alkaloids. (I) alkaloids are nitrogen N-excretory products in the same way as urea and uric acid are in animals; (ii) they are a reserve of nitrogen; (iii) they act as growth regulators, possibly germination inhibitors; and (iv) they help to maintain ionic balance (Goodwin and Mercer, 1972).

Distribution. Alkaloids are poorly distributed in the Pteridophytes and the Gymnosperms, and unevenly and spasmodically in the Angiosperms. They are mainly found in the Centrospermae, Magnoliales, Ranunculaes, Papaveraceae, Leguminosae, Papulioaceae and Rutaceae. Monocotyledons are generally poor in alkaloids but exceptions are the Liliflora and Garminea. Alkaloids tend to accumulate mainly in four types of plant tissue: actively growing tissues, epidermal and hyperdermal cells, vascular sheaths and latex vessels (Goodwin and Mercer, 1972).

Detection

Preliminary detection. Alkaloids are normally extracted from plants into a weakly acid (1 M HCl / 10 % acetic acid) alcoholic solvent and are then precipitated with concentrated ammonia (Harborne, 1984). The alkaloid precipitate is recovered into chloroform and the crude extract is finally dissolved in aqueous acid and tested against a number of "alkaloid" reagents eg. Dragendorff (Harborne, 1984).

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Extraction of alkaloids. About 208 g of powdered stembark (*Erythroxylum lucidum*) was extracted following the method described by Christen *et al.* (1993). The petrol and Et_2O extracts afforded two residues of 20.4 mg and 32.2 mg, respectively. TLC was carried out on silica gel F_{254} with Me_2CO-conc. NH_3 (13:0.3) and on aluminium oxide 60 F_{254} with Et_2O-EtOH (90:1) (Brachet *et al.*, 1997).

Purification. Alkaloids were identified by gas chromatography - mass spectrometry (GC-MS) comparison of their R_fS and fragmentation patterns with those of authentic samples. GC-MS was performed in the EI mode at 70 εV. Helium (He) was used as carrier gas at a flow rate of 1 ml.min-1. Injection temperature was maintained at 260 °C and detection temperature at 280 °C. The injections were performed in the splitless mode and the

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injected volumes were 1µl. Different operating conditions were used: (i) a 30 m x 0.25 mm i.d. fused-silica capillary column coated with the phenyl-methyl silicone phase HP5 - MS (film thickness 0.25 µm). The temperature programme was isothermal 40 °C for 2 min, 40-100 °C at 30° min-1, 100-200 °C at 10° min^{-1}, 200-300 °C at 5° min^{-1}, isothermal 300 °C for 5 min^{-1} (ii) a 15 min x 0.25 mm i.d. fused-silica capillary column coated with the methyl silicone phase DBI (film thickness 0.25 µm). The temperature programme was, isothermal 45 °C for 2 min^{-1}, 45-100 °C at 30° min^{-1}, 100-300 °C at 5° min^{-1}, isothermal 300 °C for 5 min^{-1} (Brachet *et al.*, 1997).

Characterization. In *E. lucidum* 13 compounds were characterized hygrine, tropinone, tropine, 3-acetoxytropane, pseudopelletierine, nicotine, 2.1'- dehydrohygrine, 5-(2-oxopropyllhygrine), 5-(2-hydroxypropyl)-hygrine, N-methylpyrrolidinyl-hygrine A (or B pillary column coated with the methyl silicone phase DBI (film thickness 0.25µm). The temperature programme was, isothermal 45 °C for 2 min-1, 45-100 °C at 30 ° min^{-1}, 100-`300 °C at 5 ° min^{-1}, isothermal 300 °C for 5 min^{-1} (Brachet *et al.*, 1997).

Flavonoids. Flavonoids are plant pigments, water-soluble phenolic derivatives creating a rainbow of color (website 3). Chemically, flavonoids are phenolic glycosides, and their aglycones consist of two aromatic rings joined by a 3-C unit, in other words they are phenolylpropane derivatives (Goodwin and Mercer, 1972). The more well researched flavonoid classes are flavones, flavonols, isoflavones, quercetin, anthocyanidins and catechins (website 4).

Functions of the flavonoids. Flavonoids (water soluble) and carotenes (oil soluble) function as antioxidants and protect plants from damaging free radicals (website 5).

Distribution. Flavonoids are present in all vascular plants but some classes are more widely distributed than others. While flavones and flavonols are universal, isoflavones and biflavonyls are found in only a few plant families (Harborne, 1984). Some flavonoids are also found in chromoplasts and chloroplasts (Goodwin and Mercer, 1972).

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Detection

Extraction of flavonoids. The dried and ground roots (900 g) and the stems (900 g) of *Sophora tomentosa* (Figure 3) were respectively extracted with Me_2CO at room temperature, and each concentrated in vacuo to give a brownish syrup (61 g and 22 g respectively) (Tanaka *et al*., 1997).

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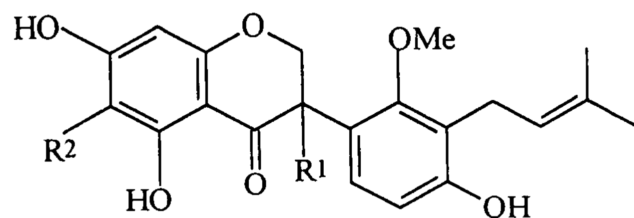
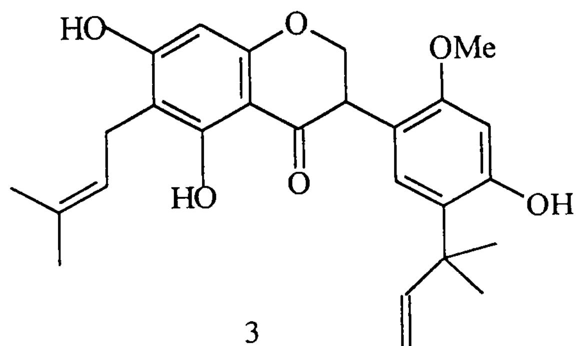


1: R1 = R2 = H
2: R1= H; R2 = isoprenyl
5: R1 = OH; R2 = H
6: R1 = OH; R2 = isoprenyl



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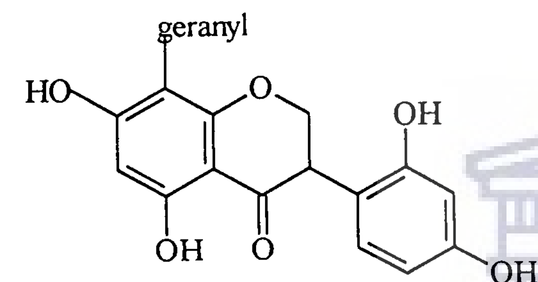

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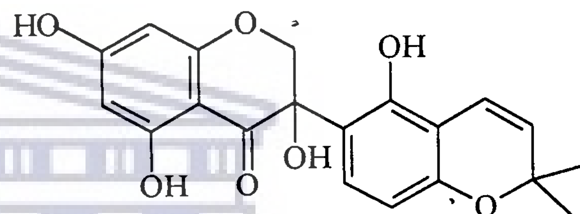

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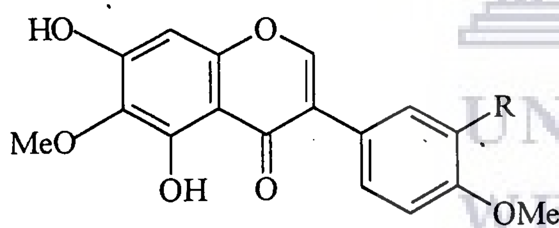
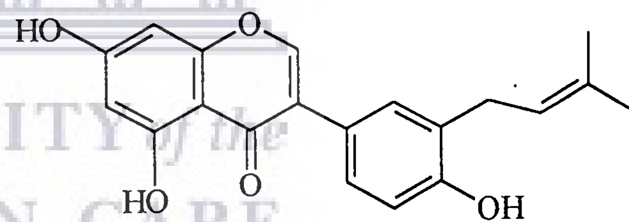


8: R = H
9: R = OH



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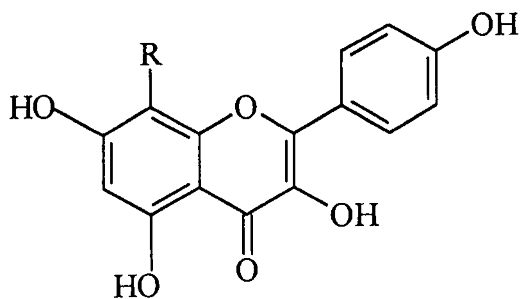
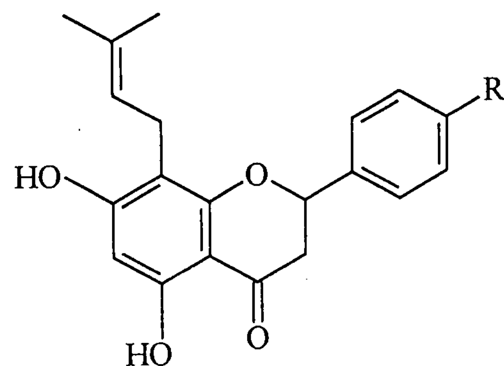


11: R = isoprenyl
12: R = geranyl



13: R = H
14: R = OH

gure 3: Flavanoids isolated from root and stem extracts of *Sophora tomentosa*.

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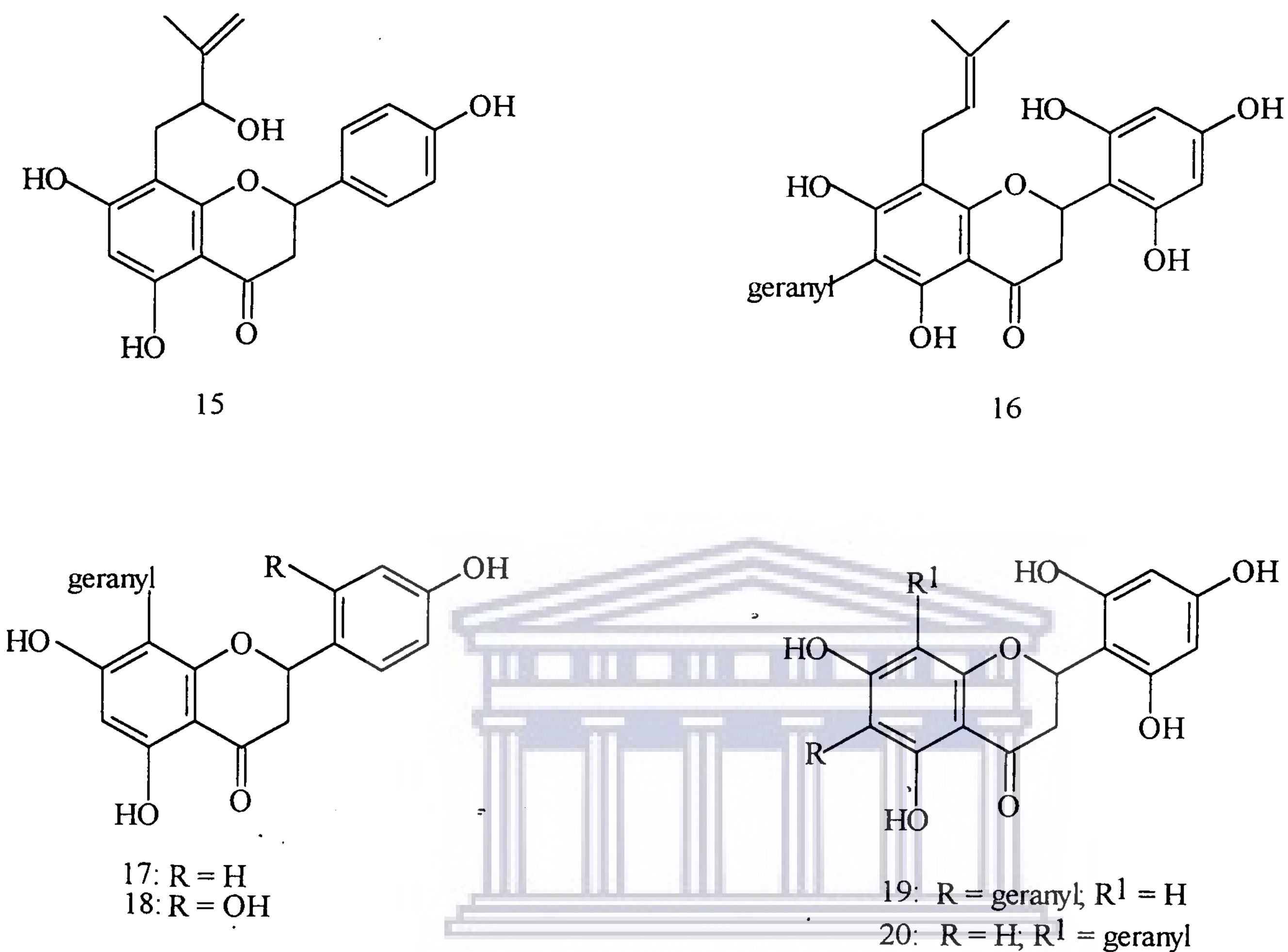

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Figure 3: Flavanoids isolated from root and stem extracts of *Sophora tomentosa*.

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Purification. A part of the root extract (54 g) was chromatographed on silica gel (2 kg) eluted with varying proportions of $CHCl_3$-MeOH. The $CHCl_3$-MeOH (15:1) fractions were repeatedly rechromatographed on silica gel (solvent system: $CHCl_3$-MeOH = 2:1, 10:1, n-hexane-Me_2CO = 3:1, C_6H_6 -EtoAc - MeOH =10:1:1), prepared TLC (same solvent systems as silica gel column chromatography), Sephadex LH 20 column chromatography with MeOH or Me_2CO and recrystallized to give 1 (12 mg), 2 (8 mg), 3 (4 mg), 4 (7 mg), 5 (4 mg), 6 (5 mg), 7 (1.2 mg), 9 (7 mg), 11 (3 mg), 12 (250 mg), 14 (12 mg), 15 (3 mg), 16 (10 mg), 17 (6 mg), 18 (6 mg), 19 (120 mg), 20 (100 mg), respectively. The stem extract were purified in the same manner to give 8 (12 mg), 10 (8 mg) and 13 (11 mg) in addition to the root constituents (Tanaka *et al*., 1997).

Characterization. By repeated chromatography, preparative TLC and recrystallization, 17 flavonoids (1-7, 9,11,12 and 14-20) were isolated from the root extract, and three (8, 10 and 13) were found in both root and stem extracts (Tanaka *et al*., 1997).

Isolation and extraction of flavanones. The isolation sequence was guided by a bioassay that measured repellency by monitoring ant choices among an array of treated and control food flakes (Roussis *et al*., 1987).

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Extraction. Approximately 10 kg *Lonchocarpus minimiflorus* leaves were collected from Santa Rosa National Park, Costa Rica, air-dried, and kept in sealed plastic bags until extracted (Figure 4). This plant material was successively extracted with hexane, $CHCl_3$, and MeOH in a soxhlet apparatus. The extracts were concentrated and each was bioassayed (Roussis *et al*., 1987).

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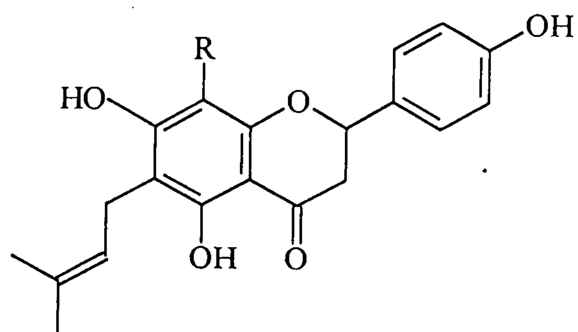

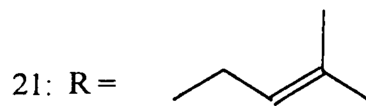

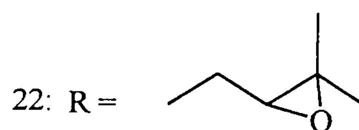

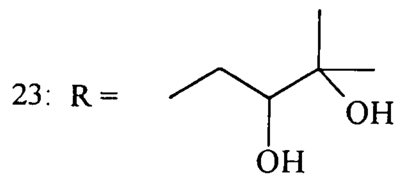

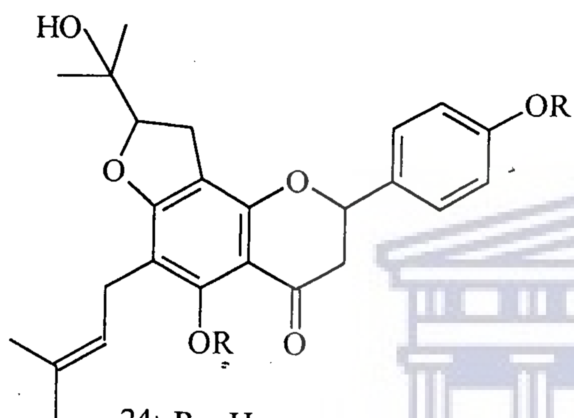

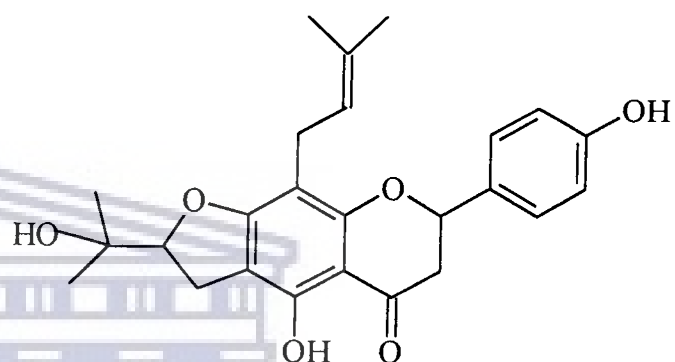

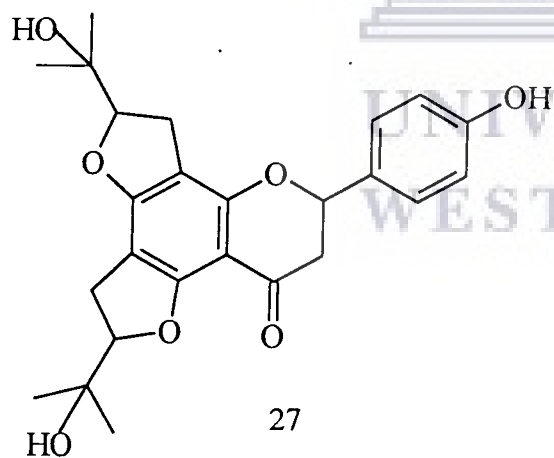

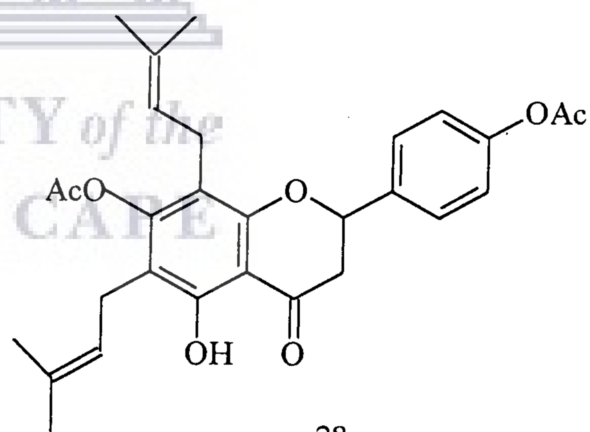

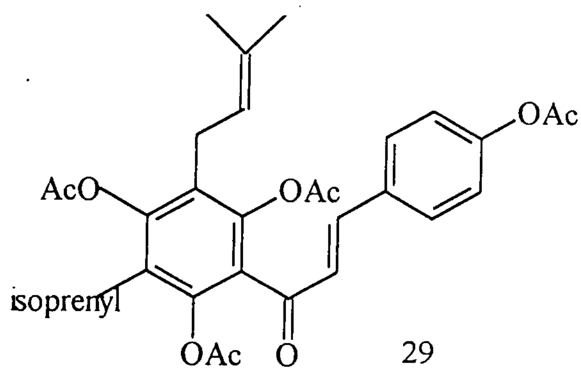

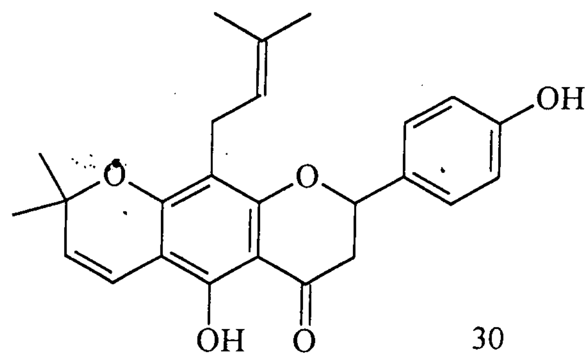

Figure 4: Compounds isolated from *Lonchocarpus minimiflorus*.

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Purification. The majority of the ant-repellency activity was found to reside in the hexane extract, which, after several chromatographic separations and purifications yielded only the common plant sterols, viz, stigmasterol and sitosterol. Column chromatography (EtOAc and hexane gradient, silica gel, 63-200 mesh) was used to fractionate the marginally active $CHCl_3$ extract. Between 40 % and 70 % EtOAc, compounds 21, 22, 23, 24 and 25 were eluted, in that order. Final purification was achieved by radial chromatography on silica gel using toluene-EtOAc-AcOH (80:19:1). Although not significantly active in the ant-repellency bioassay, compound 21 was found to be fungistatic when tested against the attine fungus, *Rhozites gongylophora* (Roussis *et al.*, 1987).

Tannins. Tannins are naturally occurring complex polyphenolics found in many plants. Animals are deterred from eating plants high in tannins because of their bitter astringent taste. Tannins are complex compounds with molecular weights of between 500 and 3000 bp. There are many different tannins, often divided into two groups: hydrolysable tannins and the condensed tannins. Tannins bind with proteins, starches, cellulose and minerals. Tannins have taken advantage of this strong protein binding ability to produce leather from animal hides for centuries (website 6).

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Functions. Nothing concrete is known of the function of tannins in plants (Goodwin and Mercer, 1972).

Distribution. Although tannin production is widespread, only a small number of plants produce sufficient for commercial exploitation. The highest concentrations of hydrolyzable tannins (64 %) are found in the galls produced on the leaves of *Rhus semialata* after infection with *Aphis chinensis*. In non-pathogenic tissues the highest concentration appears to be about 45 % found in the seed pods of Algarobilla (*Caesaepinia brevifolia*) and Divi-divi (*C. coriaria*). About 45 % is the highest concentration of condensed tannins recorded, in the bark of eg. eucalyptus and mangrove (*Rhizophora* species). Tannins have been found in almost every plant organ and tend to have their highest concentration in dead or dying cells (Goodwin and Mercer, 1972).

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Detection

Extraction of proanthocyanidins. Fresh plant tissues are extracted with hot 50-80 % aqueous methanol (Goodwin and Mercer, 1972).

Purification. The presence of mixtures of tannins are monitored by 2-D paper chromatography (Goodwin and Mercer, 1972). Tannins are separated on a preparative scale by chromatography on Sephadex and purification can be achieved on Sephadex G-50. The column is eluted with acetone-water (1:1) containing 0.1 % ascorbate to protect the tannin from aerial oxidation. The eluate is then applied to a Sephadex LH-20 column in aqueous methanol (1:1) (Goodwin and Mercer, 1972).

Characterization. The purified compounds are then characterized by chemical degradation and optical rotation measurements. The molecular weight and composition of the compounds are directly determined by ^{13}C-NMR spectral measurements (Harborne, 1984).

Terpenes and Terpenoids. The major components of the volatile steam-distillable "essential oil" fractions responsible in plants for characteristic odours are the terpenoids. Terpenoids are compounds with varying numbers of carbon atoms, which are clearly derived from C_5 Isoprene units (Goodwin and Mercer, 1972). They are largely water-insoluble acyclic or cyclic compounds (Ting, 1982). The essence of plant life, photosynthesis, depends on the existence of certain terpene derivatives and a number of plant hormones are terpenoids. Chemically, the terpene volatiles can be divided into two classes, the monoterpenoids and the sesquiterpenoids, C_{10} and C_{15} isoprenoids, which differ in volatility and boiling point range. Thus, monoterpenoids boil between 140° and 180 °C, whereas sesquiterpenoids tend to have boiling points of 200 °C or more (Harborne and Turner, 1984).

Functions. Monoterpenes with their penetrating odour function by attracting or repelling insects according to their intensions. The newly discovered growth inhibitory substance abscisic acid (I) which regulates dormancy in woody plants and leaf abscission in cotton

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plants is a sesquiterpene. The sweet potato responds to infection with the black rot fungus *Ceratocystis fimbriala* by synthesizing large amounts of the sesquiterpene ipomeamerone (xxiv), which has a potent anti-fungal action against the pathogen. Plant sterols are associated with cell organelles and they may be structural units of the membranes of cell particles as cholesterol is in animal cells. Terpenoids function as more complex molecules eg. as the chlorophylls, the plastoquinones, the ubiquinones and the tocopherylquinones (Goodwin and Mercer, 1972).

Distribution. Terpenoid eg. sterolins are widely distributed in plants in trace amounts. Simple monoterpenes are very widespread in nature and occur not only in higher plants, but also in bryophytes, algae and fungi (Harborne and Turner, 1984).

Detection of terpenoids.

Extraction. Aerial parts of *Pallenis spinosa* were collected near Orasei (NU) in June 1992. Powdered non-woody aerial parts (leaves and flowers) were extracted with Me_2CO (3x 1l) at room temperature (Figure 5). The extract was evaporated and the residue was dissolved in EtOH (500 ml), and an aqueous solution of $Pb(OAc)_2 .3H_2O$ (3 %, 500 ml) was added. After 2 h, the solution was filtered through Celite and the filtrate was concentrated to *ca* 20 ml, diluted with water (300 ml) and extracted with EtOAc (Giovanni *et al.*, 1997).

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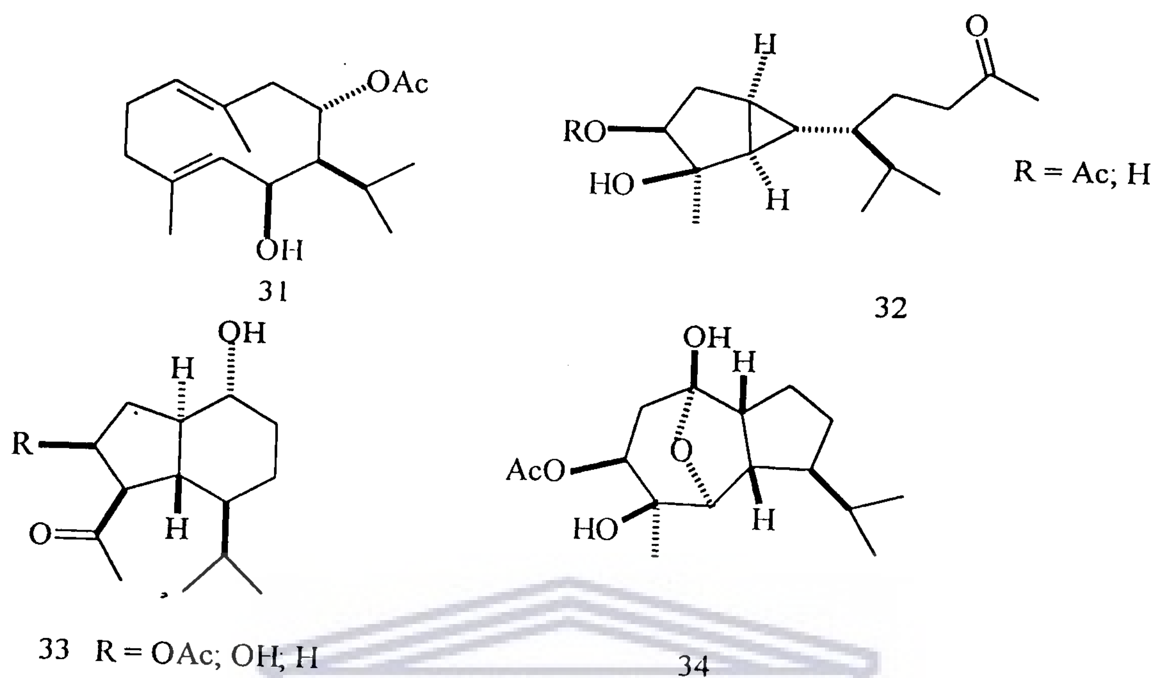

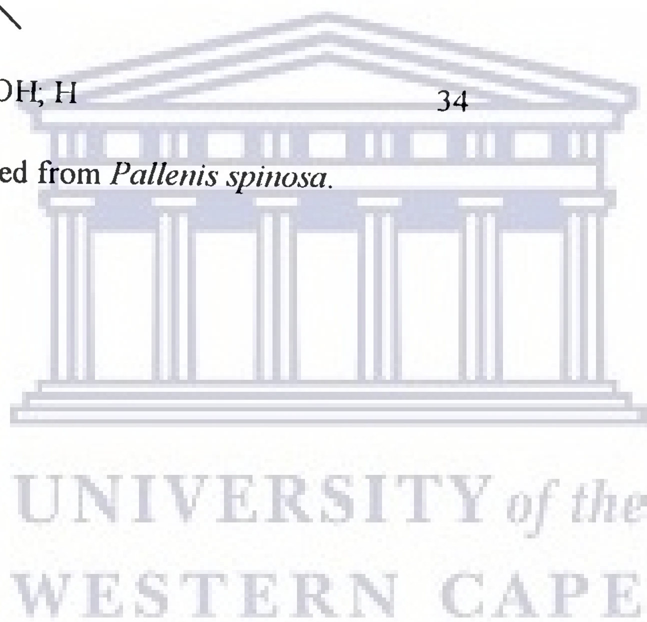

Figure 5: Compounds isolated from *Pallenis spinosa*.

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Purification. The removal of the solvent left a yellowish gum (4.0 g), which was separated by column chromatography [30 g silica, hexane-EtOAc. gradient (from hexane-EtOAc 3:7 to pure EtOAc)]. Four crude fractions (PS1-PS4) were obtained from TLC (705, 550, 615 and 200 mg, respectively). Fraction PS1 was further purified by column chromatography (silica gel, hexane-EtOAc 19:1 as eluent) to give 23 mg, 28, 37 mg 32 and 227 mg 30. Fractions PS-2 and PS-3 were further separated by column chromatography (hexane- EtOAc 9:1 and 7:3, respectively) to give 68 mg 26, 65 mg 30 (from PS-2), and 66 mg 31, 60 mg 28, and impure 27, 29 and 28 from PS-2. Further purification by HPLC (hexane-EtoAc 5:5) gave 33 mg, 27 mg and 35 mg pure 27, 29 and 28 respectively. Fractions PS-4 were further purified by column chromatography (hexane- EtOAc 7:3) to give 10 mg 28 and 13 mg 27 (Giovanni *et al*., 1997).

Characterization. Four new sesquiterpenoids were isolated using ^{1}H-and ^{13}C- NMR spectroscopy (Giovanni *et al*., 1997).

Phytoalexins. Phytoalexins are defined as antimicrobial compounds of low-molecular weight substances, that both are synthesized and accumulated in plants after the exposure of the plant to a microorganism and essentially absent from uninfected plant tissues (Ebel, 1986). In leaves undergoing a hypersensitive response (HR), phytoalexins accumulate more rapidly and reach higher concentrations than in leaves exhibiting a susceptible response (Sallaud *et al*., 1997). They are water-soluble and constitute a chemically heterogenous group of molecules typical of plant secondary metabolites, which belong predominantly to the families of the phenylpropanoids, isoprenoids and acetylenes (Bailey and Mansfield, 1982). These secondary metabolites are produced as a response to the localised death of plant cells (hypersensitivity). Phytoalexins do not move within host plants and accumulate at sites of pathogen invasion and are not detected at a distance from infection sites. This may occur because phytoalexins become bound to plant cell walls or are metabolised in nearby cells. Phytoalexin synthesis is rapidly induced by pathogen infection incompatible interactions and also by a number of biotic and abiotic molecules called elicitors (Bailey and Mansfield, 1982).

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Functions. Phytoalexins affect the growth of fungi, inhibiting germ tube elongation, colony growth and dry weight accumulation. There is also evidence that phytoalexins disrupt respiration pathways (Bailey and Mansfield, 1982).

Distribution. Resveratol is a phytoalexin found in greatest concentration in the skins of grapes (*Vitis vinifera*), which respond to invasion, by the fungus *Botrytis cinerea* (website 7). Red wine therefore contains a much higher resveratol concentration than white wine due to skin contact during fermentation (Armstrong *et al.*, unpublished).

Detection.

Danielone

Extraction. Papaya fruit (35 kg) was peeled, cut in 1.5 cm thick slices and soaked for 48 hr in $CuCl_2$ or $CuSO_4$ solution (0.1 M) with Tween 20 (0.5 %). Slices were then ground in 20 L of EtOH (95 %) in a blender, centrifuged and filtered through cheesecloth. The filtrate was concentrated *in vacuo* to give a dark-brown gum (1.4 g), which was re-extracted with n-hexane, CH_2Cl_2 and EtOAc. Sterile H_2O/Tween was used as a control treatment (Echerverri *et al.*, 1997).

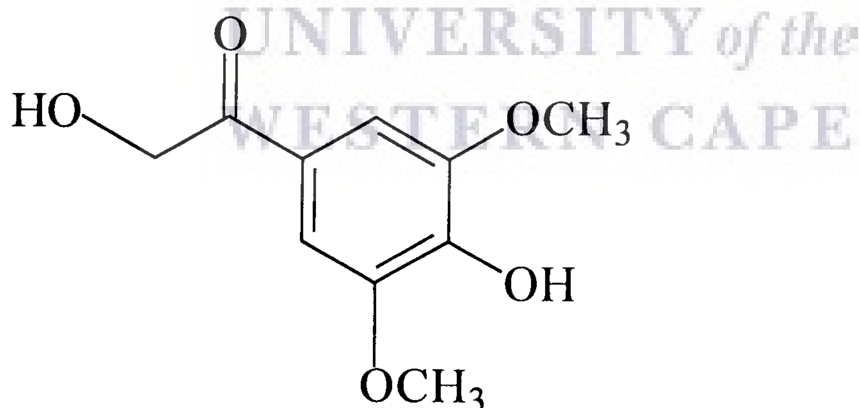

Figure 6: Danielone

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Purification. Danielone (50 mg) was purified from the dark gum by column chromatography of the fraction eluted with n-hexane-CH_2Cl_2 (9:1). Danielone: amorphous solid, mp 145 °C ; UV λ^{MeOH}_{max} 227, 298 nm. Ms: 70QV [M]+, 212.20, $C_{10}H_{12}O_5$ (22 %), 181 [M-OCH_3]+ (100 %), 153 (12), 123 (10), 95 (8), 67 (13). The structure was solved by direct methods using the SHELXS-86 program. The final discrepancy index was R = 0.013 (Echerverri *et al*., 1997).

Characterization. Danielone was isolated as a powder; the high-resolution EI-mass spectrum showed the [M]+ at m/z 212.20 with a molecular formula $C_{10}H_{12}O_5$. The presence of hydroxyl, cationic carbonyl and an aromatic ring was indicated by the IR absorptions at 3450, 1670, 820, 840 and 780 cm-1. The ^{1}H-NMR spectrum shows interalia two singlets at δ 3.95 (6 H) and δ 7.19 (2 H) ppm, that were attributed to methoxyl groups and aromatic protons, respectively (Echerverri *et al*., 1997).

Food spoilage caused by fungi and bacteria

The use of chemicals to enhance food safety of many foods is of great interest to the food industry. Effectiveness of chemical control depends on the types of microorganisms and the physical and chemical characteristics of foods. However, the presence of chemical residues in foods and labelling of preservatives on food packages are major concern to consumers these days. Therefore, the need for naturally derived compounds and other natural products with antimicrobial properties has been explored (Mau *et al*., 2001).

Fungal spoilage. Fungal spoilage falls into several categories depending on the strategy of the fungus. Some losses are caused by the ongoing activities of the pathogenic fungi already established in the substrate. Other fungi that caused storage problems, such as *Monilia fructigena* (soft rot of peaches and other stone fruits), are actually necrotrophs. Necrotrophs are fungi, which, although they grow on living hosts, are not really biotrophic (Frazer and Westhoff, 1978). They produce toxins that kill the plant cells

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before the fungal hyphae reach them, so the fungus is actually feeding saprobically. Other storage fungi are strictly saprobic, but have one or more of the following unusual abilities (a) coping with substrates of low moisture and / or high osmotic pressure (serotolerance), (b) surviving high temperatures (thermotolerance), (c) growing at low temperatures (psychrotolerance), (d) growing in extremely acid media and (e) growing at low levels of oxygen and / or high levels of carbon dioxide. Since fungal spoilage of food can happen only if a fungus is present and active, there are two possible avenues of prevention (Pitt, 1981). The first is to rigorously exclude fungi from the substrate. The second is to prevent them from growing even if they are present (website 9).

Alternaria. The various species of *Alternaria* cause decay on most, if not all, fresh fruits (eg. pears, apple) and vegetable (potato) either before or after harvest (Howard, 1983). The symptoms appear as brown or black, flat or sunken spots with definite margins, or as diffuse, large, decayed areas that are shallow or extend deep into the flesh of the fruit or vegetable. The fungus develops well at a wide range of temperatures, even in the refrigerator, although at a slower rate. The fungus may spread into and rot tissues internally with little or no mycelium appearing on the surface, but usually a mat of mycelium that is white at first but later turns brown to black forms on the surface of the rotted area (Agrios, 1997).

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Botrytis cinerea. Botrytis is the fungus which causes gray mold, Botrytis bunch rot (grapes), or stem-end rot (kiwifruit) of fruits and vegetables, both in the field and in storage. Almost all fresh fruits, vegetables, and bulbs are attacked by *Botrytis* in storage (Broome *et al.*, 1995). Some products, such as strawberries, lettuce, onion, and apple, are also attacked in the field near maturity or while green. The decay may start at the blossom or stem end of the fruit or any wound. The decay appears as a well-defined watersoaked, then brownish area that penetrates deeply and advances rapidly into the tissue. In most hosts and under humid conditions a grayish or brownish-gray, granular, velvety mold layer develops on the surface of decaying areas. Gray molds are most severe in cool, humid environments and continue to develop, although slowly, even at 0 °C (Agrios, 1997).

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Fusarium . The genus Fusarium is comprised of a large, complex group of fungi with ascomycete teleomorphs and contains numerous species that produce noxious secondary metabolites and / or cause serious plant diseases (Leslie *et al*., 1990). *Fusarium* species have been recognized as a great agricultural problem (Charmey *et al*., 1994). They occur worldwide on a variety of plant hosts and cereal grains. In a review covering the occurrence of *Fusarium* species in raw food and feed material and the mycotoxins produced by these moulds reported over the last 30 years, De Nijs *et al*. (1996) noted the occurrence of 61 different *Fusarium* spp. in a large variety of raw food products, such as cereals, vegetables and fruits. The six commonly reported species were: *Fusarium moniliforme*, *F. graminearum*, *F. sporotrichioides*, *F. oxysporum*, *F. equiseti*, and *F. culmorum* (Boeira *et al*., 2000). Contamination with *Fusarium* usually takes place in the field or in storage. Losses are particularly heavy with crops, such as potatoes, that are stored for long periods of time. Affected tissues appear fairly moist and light brown at first, but later they become darker brown and somewhat dry. As the decaying areas enlarge, they often become sunken, the skin is wrinkled, and small tufts of whitish, pink, or yellow mold appear. The infection of softer tissues such as tomatoes and cucurbits develops faster and is characterized by pink mycelium and pink, rotten tissues (Agrios, 1997).

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Penicillium. Blue mold is one of the most important diseases of pome fruit in storage, shipping, and in the market (Cappellini *et al*., 1987; Ceponis and Butterfield, 1974; Heald and Ruehle, 1931; Holmes, 1990; Rosenberger, 1990). Although a large number of *Penicillium* spp. have been implicated as causal agents of harvest decay of pome fruit, blue mold is usually attributed to infection by *P. expansum* (MacNab and Springer, 1983; Jones and Sutton, 1996). Species of *Penicillium* are ubiquitous soil inhabitants. Although some propagules of the various *Penicillium* spp. probably are transmitted to fruit surfaces by wind, it is likely that the majority of propagules are carried in soil and debris on field bins and equipment, and are transmitted to infection courts via water handling systems. Water systems are used to float pear and apple fruit from field bins to minimize mechanical damage to the fruit (Sanderson and Spotts, 1995). *Penicillium* infects fruit

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through wounds. However, it can spread from infected fruit in contact with healthy ones through the injured skin. Penicillium rots at first appear as soft, watery, slightly discolored spots of varying size and on any part of the fruit. The spots are rather shallow at first but quickly become deeper. At room temperature most or all of the fruit decays in a just a few days. Soon a white mold begins to grow on the surface of the fruit, near the centre of the spots, and starts producing spores. The sporulating areas has a blue, bluish green, or olive-green colour and is usually surrounded by white mycelium and a band of water-soaked tissue. The fungus develops on spots of any size as long as the air is moist and warm. Decaying fruit has a musty odor. Under dry conditions it may shrink and become mummified. Under moist conditions, secondary fungi and yeasts also enter the fruit, which is then reduced to a wet, soft mass (Agrios, 1997).

Other plant diseases caused by fungi

Cylindrocladium sp. Fungi belonging to the genus *Cylindrocladium* attack over 100 woody ornamentals including azaleas, rhododendrons, camellias, junipers, white pine, and holly. Many foliage plants including asparagus ferns, palms, and spathiphyllum are susceptible as well. Depending on the host plant and species of *Cylindrocladium* involved, damping-off, wilt, root rot, stem canker, crown rot or leaf spot may occur (Moorman, 1999).

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Eutypa lata. Eutypa dieback of grapevines, caused by *Eutypa lata*, is a lethal, perennial canker disease (Munkvold *et al*., 1994). The earliest symptoms of the disease are cankers formed around pruning wounds. The cankers are hard to detect as they are concealed by old, dead bark, which may become somewhat flattened. Symptoms of Eutypa dieback are apparent after the canker has become well established, perhaps 2 to 4 years after infection of the pruning wound (Munkvold *et al*., 1995). As new shoots develop on the trunk or arms above the cankered area, growth appears stunted and the internodes shortened. Symptoms are not readily seen until late spring because affected shoots are usually covered up by healthy shoots. Infected leaves are small, yellow, and crinkled. Symptoms on foliage of diseased arms become more extensive each year until eventually the diseased arms fails to produce shoots in the spring (Munkvold *et al*., 1994)

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Phaeomoniella clamydospora. One particular disease associated with young grapevines "black goo decline" or "*Phialophora*" grapevine decline. Ferreira *et al*. (1994) identified the causal organism as *Phialophora parasitica*. This fungus however differed from other species in the genus *Phialophora*, and consequently Crous *et al*. (1996) proposed a new hyphomycete genus, *Phaeocremonium*, including six species of which *P. clamydosporum*, *P. aleophilum*, *P. inflatipes* and *P. angustius* occurred on grapevines (Mugnai, 1998; Bertelli, Mugnai & Surico, 1998; Pascoe, 1998). *P. clamydosporum* is the most frequently isolated *Phaeocremonium* species from grapevines. In South Africa *P. clamydosporum* differed from the other species in the genus *Phaeocremonium* to such a degree (Dupont, Laloui & Roquebert, 1998, Groenewald, Bellstedt & Crous, 2000) that it had to be re-classified as a new genus, namely *Phaeomoniella clamydospora* (Crous & Gams, 1999). The biological name for the grapevine decline fungus identified by Ferreira *et al*. (1994) as *Phialophora parasitica* therefore changed to *Phaeoacremonium clamydosporum* and more recently to *Phaeomoniella clamydospora* (Fourie *et al*., 2000).

Although *Phaeomoniella clamydospora* appears to lack a sexual state, it has quite a complex life cycle and is able to form clamydospores (survival structures) as well as a synamorph (more than one asexual state). It produces conidia that infect the host tissue, invade the xylem, and the plant responds by producing tyloses (balloon-like structures that block the vessels) and phenolics (black discolouration). The tyloses block the xylem vessels resulting in vine decline, since the transport of water and minerals from the roots to the other metabolically active parts of the plant is impeded. When the plant dies or is cut, the fungus can produce conidia on the exposed surfaces, which could be dispersed to other potential infection sites. *P. clamydospora* also produces clamydospores, which in other pathosystems are able to survive for long periods in plant debris or soil. Based on extensive isolations made from apparently healthy rooted cuttings, authors found that *P. clamydospora* to be the one of most frequently isolated fungi from nursery material in Italy and France, occurring mostly from isolation sites near the base of the stem. Larignon (1998) concluded from aerial trapping experiments in France that *P. clamydospora* might be an airborne fungus that penetrates the plant via pruning wounds.

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Recent observations in the laboratories of ARC Infruitec-Nietvoorbij however indicate that *P. clamydospora* is present in apparently healthy propagation material in a latent or endophytic form. Typical symptoms include stunted growth, mildly chlorotic foliage and a general decline of young vines resulting in plant death. Cross-section of diseased vines reveal typical "black goo" symptoms. The blackened xylem tissue appears as black dots in the cross-section with a, shiny, tarry substance exuding from the severed vessels (Fourie *et al.*, 2000).

Bacterial Spoilage. Bacteria are the most troublesome and important biological foodborne hazard for the foodservice and food retail establishment. Bacteria are living microorganisms that are single cell. Bacterial cells can exist in two different states: the vegetative state and the spore state. All bacteria live in a vegetative state can grow and reproduce. Few bacteria are able to change into a special state called the spore state. Spores are reproduced when the bacterial cell is in an environment where it cannot grow (frozen foods, dried foods). Spores are not able to grow or reproduce. Instead spores are a means of protection when bacteria are in environment where they cannot grow. Bacteria are usually classified by their requirements needed for growth and as spoilage or pathogenic microorganisms (Harley *et al.*, 1990). Spoilage bacteria break down foods so that they look, taste, and smell bad. They affect quality. Pathogenic bacteria are disease – causing microorganisms and, if ingested in a food, can make people ill. Both spoilage and pathogenic bacteria are important to those preparing and serving foods. Bacteria have different required temperatures for growth. Psychrophiles (cold – loving) bacteria grow within a temperature range of 6 – 70 F. bacteria growing above 110 F are called thermophiles. All thermophiles are spoilage microorganisms. Bacteria also differ in their requirement of oxygen. Aerobic bacteria require oxygen level normally present in the air for growth. Anaerobic bacteria cannot tolerate any oxygen, it is toxic to them. Anaerobic bacteria grow well in vacuum packaged foods or canned foods where oxygen is not available. Facultative anaerobic bacteria can grow with or without oxygen. Microaerophilic bacteria require a specific amount of oxygen for growth. Important spore and non – spore forming pathogens in the food retail industry include *Bacillus*

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cereus, *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* (website 10).

Bacillus cereus. *B. cereus* is facultative anaerobic, spore – forming bacterium that has been associated with food poisoning. It is commonly found in soil, water, dust and air (Hashimoto *et al*., 1969b). A wide variety of foods including bread, meats, milk, vegetables and fish. (website 11). Illness due to *B. cereus* is most often attributed to foods that are improperly stored (cooled, hot – held) to permit the conversion of spores to vegetative cells (Jay, 2000). Vegetative cells then produce the toxin or grow to high enough numbers in the food to cause illness. To prevent sickness the food must be cooked and cooled rapidly (website 9).

Escherichia coli. The *E. coli* (non – spore forming) group of bacteria includes four strains of foodborne pathogens; enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enterohemorragic *E. coli* and enteroinvasive *E. coli* (Banwart, 1989; Jay, 2000; Frank, 1992). The most important of the group is a particular type of enterohemorragic E. *coli* called E. coli 0157: H7. this is a facultative anaerobic bacterium that we can found in the intestines of warm blooded animals (website 9). *E. coli* is found in soil, water, plants and various foods / products that are handled by people. Spoilage of the infected fruit concentrate or milk occur and result in slimy, unacceptable product by the customer. Vegetables and fruits can be infected through animal wastes and polluted irrigation water. Control is done through; personal hygiene, prepare foods in a sanitary manner, treat and protect water (by using chlorine), dispose of sewage in a sanitary manner, and cooking the meat at 60 ° C (website 11).

Salmonella. *Salmonella* are members of the family Enterobacteriaceae. They are Gram-negative bacilli growing aerobically and anaerobically at an optimum temperature of 37 °C, readily killed by temperatures above 55 °C. Clinical cases of salmonellosis caused by contaminated fruits and vegetables have been recently documented or reported with increasing frequency (Hedberg *et al*., 1994; Schwartz, 1995). Most of these cases involved, or were suspected of involving, ingestion of improperly stored or handled prepared foods that initially carried the bacteria as surface contamination (Geldreich and

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Bordner, 1970; Golden *et al.*, 1993; Gould, 1973; Lin and Wei, 1997). The level of *Salmonella* occurring epiphytically on fruits and vegetables retailed in the marketplace is a concern to epidemiologists and to the food industry. When foods are improperly handled, the level of this background contamination is suspected of being a contributing factor in development of public health problems. *Salmonella* bacteria have been isolated from fresh fruits and vegetables in marketplaces in the United States and in other countries. *Salmonella* that are epiphytic on fruits and vegetables can multiply if certain extrinsic factors are present, such as improper refrigeration during storage and preparation, poor product quality, or the presence of bacterial soft rot. The interaction of *Salmonella* with soft-rotting bacteria affecting a commodity raises the possibility of similar interactions with postharvest fungal decays or other disorders. Fungal rots are the leading cause of postharvest losses of fruits and many vegetables – among others, melons, tomatoes, strawberries, and sweet potatoes and have been associated with mycotoxin contamination. Their possible interaction with *Salmonella* on fruits and vegetables has not been previously investigated. Mechanical injury in the form of bruises, cuts, and punctures and physiological disorders are other important causes of postharvest losses. Injured tissues are open avenues for infection by bacterial soft rot and other pectolytic microorganisms, as well for colonization by human pathogens (Wells and Butterfield, 1999).

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Staphylococcus aureus. *S. aureus* is a facultative anaerobic, non - spore forming bacterium that produces a very heat stable toxin as it grows on foods (Jay, 2000; Murray, 1998). The microorganism is normally present on human skin, hands, nasal passages, and can be transferred to foods easily. It also survives in high salt conditions. Since humans are the primary source, cross – contamination from the worker`s hand is the most common way the microorganism is introduced into foods. Foods requiring large amounts of food preparation and handling are especially susceptible (website 11). People with Staphylococcal infection should avoid food preparation. Preparation should be done immediate to consumption. Always refrigerate food so the organism cannot grow and produce the enterotoxin. Thermal processing will kill vegetative cells (website 11).

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CHAPTER 2

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RELATIVE INHIBITION OF FUNGI AND BACTERIA BY FRACTIONS FROM DIFFERENT SOLVENT EXTRACTS OF *GALENIA AFRICANA*

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Chapter 2

Relative inhibition of fungi and bacteria by fractions from different solvent extracts of *Galenia africana*

Abstract

Dried samples of *Galenia africana,* a dominant plant throughout the Namaqualand area, were sequentially extracted with various solvents starting with hexane, chloroform, ethanol and sterile distilled water. Each extract were tested for its inhibitory activity against fungi and bacteria by using the agar well diffusion method. The mycelial growth of *Fusarium graminearum* and *Fusarium verticillioides* cultured on PDA was inhibited by the $CHCl_3$ and EtOH extracts. The growth of *Escherichia coli*, *Bacillus* sp. and *Salmonella typhi* were inhibited by $CHCl_3$ and EtOH extracts. The minimum inhibitory concentration (MIC) of these two extracts was calculated for both fungal and bacterial species mentioned above. The EtOH extract showed maximal inhibitory effect against *Alternaria* sp., *F. graminearum*, *Fusarium oxysporum* and *F. verticillioides* with MIC`s of 0.625 – 5 %. *F. graminearum* was most sensitive while the *Alternaria* sp., *F. oxysporum* and *F. verticillioides* were least sensitive. The EtOH extract showed more pronounced inhibitory effects against *Staphylococcus aureus* and *Bacillus* sp. with a MIC of 0.625 %. Therefore, the EtOH extracts of *G. africana* has potential in the management of diseases caused by these fungal isolates in several important crop plants or may have the potential to prevent the growth of foodborne bacteria in food products and can possibly extend the shelf – life of processed foods.

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INTRODUCTION

Southern Africa is richly endowed with aromatic plants, most of which are unpalatable to livestock, which have no economic value to livestock farmers and consequently are regarded as invaders indicative of degraded veld (Webber *et al.*, 1999). Some of these unpalatable plants are great survivors, as they are basically the only ones left standing during times of drought, and other severe environmental conditions. Webber *et al.* (1999) suggested therefore, that before eradication of these plants from the veld their alternative use should be considered so that a farmer could benefit or receive an income. Unpalatable plants may be useful as pesticide agents against different pests of agricultural importance. Plant-derived compounds are of interest in this context, because they comprise safer or more effective substitutes compared to synthetically produced antimicrobial agents (Dupuis *et al.*, 1972).

Galenia africana, commonly known as "kraalbos" or "geelbos" belonging to the family Aizoaceae, is a dominant plant throughout Namaqualand area (Kellerman *et al.*, 1988). It is an active invader and is especially abundant in disturbed areas around kraals, along roads, and water points which shows that it grows well on trampled soil (Van der Lugt *et al.*, 1992). "Kraalbos" is not very palatable to livestock, but is grazed under pressure. The plant can cause liver damage and severe ascites, a condition referred to as "waterpens" in sheep and occasionally in goats. A decoction of *G. africana* is used as a lotion for wounds in man and animal (Watt and Breyer-Brandwijk, 1962). The Hottentots chew the plant to relieve toothache and it is said to blister the mucous membrane of the mouth if used too much. The plant was also used in the treatment of veneral diseases. In Europe a decoction was used as a lotion for skin diseases and for the relief of inflammation of eyes. (Van der Lugt *et al.*, 1992).

The effect of aqueous extracts and the oil of neem (*Azadirachta indica*) on four soil-borne pathogens, *Fusarium oxysporum* f.sp. *ciceri*, *Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia sclerotiorum,* which incite wilt and rot in gram seeds (*Cicer arietinum*) were studied (Singh *et al.*, 1980). Growth of the four pathogens in liquid medium was inhibited by extracts and neem oil showed maximum inhibitory effects. Oil-treated seeds sown in

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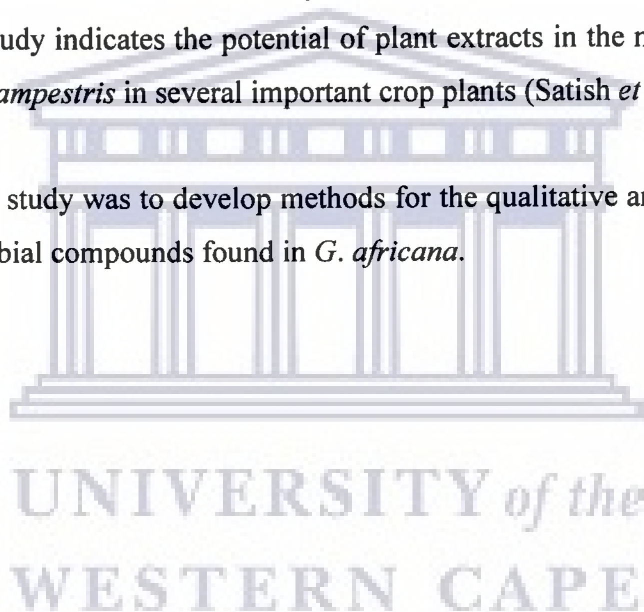
soil infested with the pathogens singly and intermixed produced disease-free seedlings whereas all seedlings from untreated seeds exhibited disease symptoms. A possible role of neem extracts and oil in controlling gram diseases in field conditions is thus suggested (Singh *et al.*, 1980). Aqueous extracts from leaves of 30 different plant species were screened *in vitro* for antibacterial activity against different pathovars of the phytopathogenic bacterium, *Xanthomonas campestris*. *X. campestris* causes bacterial blight, common blight, fuscous blight, cankers and leaf spots in French bean, cotton, paddy, tomato etc. Eight plant species (*Acacia arabica*, *Achras zapota*, *Enterolobium saman*, *Lawsonia inermis, Oxalis corniculata, Prosopis juliflora, Punica granatum* and *Viscum orientale*) showed antibacterial activity, based on the zone of inhibition in a diffusion assay. The study indicates the potential of plant extracts in the management of diseases caused by *X. campestris* in several important crop plants (Satish *et al.*, 1999).

The purpose of this study was to develop methods for the qualitative and quantitative evaluation of antimicrobial compounds found in *G. africana*.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RESULTS AND DISCUSSION

Screening solvent extracts for antifungal activity

G. africana was sequentially extracted with various solvents starting with hexane, which is non – polar and substances soluble in hexane were extracted. $CHCl_3$ is mildly polar and extracted particles not soluble in hexane but in $CHCl_3$. EtOH is a highly polar solvent and polar substances were removed. Although water is highly polar only a few substances were extracted due to previous solvents that were used. The extracts were reduced under vacuum using a rotary evaporator at 40°C and give 4.8 g (hexane extract), 11.4 g ($CHCl_3$), 11.0 g (EtOH extract) and 4.0 g (sterile distilled water) of the original plant material per ml. Each concentrated extract was screened for activity against the growth of fungal isolates. The hexane extract showed no inhibition against *F. graminearum* and *F. verticillioides* for Steinkopf and Concordia collected plant material (Table 1). The isolates grew up to the edge of the well showing no sensitivity to the hexane extract. Both the $CHCl_3$ and EtOH extracts produced clear zones of inhibition surrounding the wells on PDA plates seeded with spores of *F. graminearum* and *F. verticillioides*. The sterile distilled H_2O extract produced no antifungal activity against the two tested fungi. All the control wells were overgrown by the fungal isolate being tested. The $CHCl_3$ extract for Steinkopf showed larger inhibition (clear) zones at day 3 but a reduction in antifungal activity for day 5, whereas EtOH extract showed constant activity against the two tested fungi from day 3 until day 10 ranging from 3.1 – 6 mm inhibition (clear) zones. $CHCl_3$ and EtOH plant extracts of Concordia showed inhibition zones for *F. graminearum* only at day 3. For *F. verticillioides* the $CHCl_3$ extracts showed inhibition zones at days 3 to 5 and for the EtOH extract from days 3 to 10. The H_2O extract for Concordia was not analysed because no effects were obtained for the Steinkopf water extracts. The mycelial growth of both fungi in this test were found to be more sensitive to the EtOH extract than the $CHCl_3$ extract indicating that antifungal activity is inversely related to solvent polarity.

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Table 1. The relative inhibition[a] of *Fusarium graminearum* and *Fusarium verticillioides*

Area	Solvent extracts[b]	*Fusarium* isolates	Incubation time (days) 3	5	10	control
Steinkopf	Hexane	*F. graminearum*	-	-	-	-
		F. verticillioides	-	-	-	-
	$CHCl_3$	*F. graminearum*	++	+	-	-
		F. verticillioides	++	+	-	-
	EtOH	*F. graminearum*	++	++	++	-
		F. verticillioides	++	++	++	-
	dH_2O	*F. graminearum*	-	-	-	-
		F. verticillioides	-	-	-	-
Concordia	Hexane	*F. graminearum*	-	-	-	-
		F. verticillioides	-	-	-	-
	$CHCl_3$	*F. graminearum*	+	-	-	-
		F. verticillioides	+	+	-	-
	EtOH	*F. graminearum*	+	-	-	-
		F. verticillioides	+	+	+	-

[a] Relative inhibition of *Fusarium* isolates on agar well diffusion plates:

- = No inhibition zones present, + = Clear zones of inhibition from 1 – 3 mm,

++ = Clear zones of inhibition from 3.1 – 6 mm.

[b] $CHCl_3$ = Chloroform; EtOH = Ethanol; dH_2O = Sterile distilled water. The dH_2O extract for Concordia was not analyzed because no results were obtained for Steinkopf.

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Effect of solvent extracts on the growth of bacteria

The hexane extract did not show any inhibition against *E. coli, Bacillus* sp. and *S. typhi*, but only inhibited (clear zones) the growth of *S. aureus* (Table 2). Both the $CHCl_3$ and EtOH extracts produced clear zones of inhibition against *Bacillus sp*., *S. typhi* and *S. aureus.* No testing was done with dH_2O extract. All the control wells were overgrown by the bacterial isolates tested. Overall, S. *aureus* proved to be more sensitive to hexane, $CHCl_3$ and EtOH extracts while *Bacillus* sp. and *S. typhi* were less sensitive. *E. coli* proved to be the only foodborne pathogen that showed resistance to all the solvent extracts. This result could be explained due to the differences in the cell wall structure for the gram positive (*Bacillus*) and gram negative (*E. coli*) bacteria. The gram positive cell consists of a single 20-80nm thick homogenous peptidoglycan or murein layer lying outside the plasma membrane. The gram negative cell wall has a 1-3 nm peptidoglycan layer surrounded by a 7-8 nm thick outer membrane. The structures outside the plasma membrane is called the envelope and this includes the wall and structures like capsules when present. Frequently a space is seen between the plasma membrane and cell wall of gram negative bacteria and sometimes a similar but smaller gap is observed in gram positive bacteria. This space is called the periplasmic space or periplasm. The periplasmic space may be filled with a very loose network of peptidoglyacan. Possibly it is more a gel than a fluid-filled space. The periplasmic space of gram-negative bacteria contains many proteins that participate in nutrient acquisition – for example, hydrolytic enzymes attacking nucleic acids and phosphorylated molecules, and binding proteins involved in transport of materials into the cell. Gram positive bacteria do not appear to have as many periplasmic proteins; rather, they secrete several enzymes that would ordinarily be periplasmic in gram negative bacteria. Such sectrated enzymes are often referred to as exoenzymes (Harley *et al*., 1990). Gram negative bacteria could therefore be more resistant to the bioactive compound of *G. africana* due to its cell wall structure.

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Table 2. The relative inhibition of *Escherichia coli*, *Bacillus* sp., *Salmonella typhi* and *Staphylococcus aureus* by different solvent extracts of *Galenia africana* collected from Steinkopf after 24 h

Solvent extracts[b]	*E. coli*	*Bacillus* sp.	*S. typhi*	*S. aureus*
Hexane	-[a]	-	-	+++
$CHCl_3$	-	+	+	+++
EtOH	-	++	++	+++

[a] Relative inhibition:

- = No inhibition zones present, + = Clear zones of inhibition from 1-3 mm,

++ = Clear zones of inhibition from 3.1 – 6 mm, +++ = Clear zones of inhibition from 6.1 – 10 mm.

[b]$CHCl_3$ = Chloroform; EtOH = Ethanol.

Determination of minimum inhibitory concentration (MIC) for fungi and bacteria

Since most of the microorganisms tested were sensitive to the $CHCl_3$ and EtOH extracts it was necessary to identify the concentration of the extracts, which prevents the microorganisms from growing. The MIC is a quantitative indicator, defined as the minimum concentration of the plant extract capable of preventing the growth of a microorganism. Of the two extracts tested the EtOH extract showed a maximum inhibitory effect against the four fungal isolates with MIC`s of 0.625 – 5 % (g/vol) (Table 3). The $CHCl_3$ extract was also effective (MIC of 2.5 %) against *F. oxysporum* but the antifungal activity was much stronger for the EtOH extract (MIC of 1.25 %).

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Table 3. The minimum inhibitory concentration (MIC)[a] of different solvent extracts tested against different fungal isolates

Fungal isolates	Solvent extracts[b]	MIC (%)	Inhibition zones (mm)[c]
Alternaria sp.	$CHCl_3$	20	3.4
		10	-
		5	-
		2.5	-
		1.25	-
		0.625	-
	EtOH	20	3.1
		10	2.7
		5	2.3
		2.5	1.3
		1.25	0.6
		0.625	-
Fusarium graminearum	$CHCl_3$	20	3.0
		10	2.4
		5	-
		2.5	-
		1.25	-
		0.625	-
	EtOH	20	3.9
		10	3.0
		5	2.8
		2.5	2.1
		1.25	2.1
		0.625	1.9

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Table 3. (Cont.)

Fusarium oxysporum	$CHCl_3$	20	1.0
		10	0.9
		5	0.7
		2.5	0.7
		1.25	-
		0.625	-
	EtOH	20	3.9
		10	2.9
		5	2.3
		2.5	2.2
		1.25	2.0
		0.625	-
Fusarium verticillioides	$CHCl_3$	20	2.1
		10	1.9
		5	1.0
		2.5	-
		1.25	-
		0.625	-
	EtOH	20	2.8
		10	2.7
		5	2.0
		2.5	-
		1.25	-
		0.625	-

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[a] MIC`s were determined by the agar well diffusion method, reflecting the minimum concentration of the extracts preventing the growth of the fungal isolates, at least 3 d after start of growth of the control. Each test was performed in triplicate. The concentrations used were 0.625 %, 1.25 %, 2.5 %, 5 %, 10 % and 20 %. The concentrations given here are in g of the pure isolate per volume of $CHCl_3$ or g/vol of EtOH.

[b] $CHCl_3$ = Chloroform, EtOH = Ethanol.

[c] = Clear zones of inhibition or no inhibition zones present.

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The $CHCl_3$ extract showed maximal inhibitory effect only against one-tested bacteria with MIC of 0.625 % (*S. aureus*) and the EtOH extract against two tested bacteria with MIC of 0.625 % (*Bacillus* sp. and *S. aureus*) (Table 4).

Table 4. The minimum inhibitory concentration (MIC) [a] of different solvent extracts against *Bacillus* sp., *Salmonella typhi* and *Staphylococcus aureus*

Bacterial isolates	Solvent extracts[b]	MIC (%)	Inhibition zones (mm) [c]
Bacillus sp.	$CHCl_3$	20	4.3
		10	3.8
		5	3.1
		2.5	2.2
		1.25	2.0
		0.625	-
	EtOH	20	4.8
		10	4.4
		5	4.4
		2.5	3.7
		1.25	2.7
		0.625	1.2
Salmonella typhi	$CHCl_3$	20	4.1
		10	3.9
		5	2.1
		2.5	1.3
		1.25	1.1
		0.625	-
	EtOH	20	4.4
		10	4.2
		5	4.1

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Table 4. (Cont.)

		2.5	4.0
		1.25	3.9
		0.625	-
Staphylococcus aureus	$CHCl_3$	20	9.1
		10	8.0
		5	7.8
		2.5	6.0
		1.25	5.1
		0.625	4.4
	EtOH	20	10
		10	9.9
		5	8.0
		2.5	7.3
		1.25	6.4
		0.625	4.9

[a] MIC`s were determined by the agar well diffusion method, reflecting the minimum concentration of the extracts preventing the growth of the bacterial isolates, at least 24h after start of growth of the control. Each test was performed in triplicate. The concentrations used were 0.625 %, 1.25 %, 2.5 %, 5 %, 10 % and 20 %. The concentrations given here are in g of the pure isolate per volume of $CHCl_3$ or g/vol of EtOH.

[b]$CHCl_3$ = Chloroform, EtOH = Ethanol.

[c] = Clear zones of inhibition or no inhibition zones present.

At a concentration of 0.625 % (g/vol) the $CHCl_3$ extract only inhibited the growth of *S. aureus* while the EtOH extract inhibits both the growth of *Bacillus* sp. and *S. aureus* at this concentration. The inhibition zones for the $CHCl_3$ and EtOH extracts was similar (3.9 mm) at a MIC of 1.25 % and 10 %, respectively.

Overall results showed that the EtOH extract from *G. africana* has strong inhibitory activity *in vitro* against fungi and bacteria. The increased awareness of the antimicrobial problems associated with chemical pesticides has led to the search for non – conventional

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chemicals of biological origin for the management of these diseases (Bolkan and Reinert, 1994). Natural plant-derived compounds should provide a wide variety of compounds as alternatives to synthetic fungicides (Cuttler & Hill, 1994). This may also prove valuable as "lead structures" for the development of synthetic compounds. Reports are available on the use of active agents from higher plants, in place of chemical fungicides, that are non-phytotoxic, more systemic and easily biodegradable (Fawcett and Spencer, 1970). Reports are also available on the use of several plant by-products, which possess antimicrobial properties, on several pathogenic bacteria and fungi (Deans and Svoboda, 1990; Diker *et al.*, 1991; Hersey and Gorham, 1992; De Pooter *et al.*, 1995; Lis-Balchin and Deans, 1996; Hili *et al.*, 1997).

Laboratory screening of plant extracts has given encouraging results, indicating their potential use in the management of diseases caused by *Xanthomonas* species (Satish *et al.*, 1999). Singh *et al.* (1980) found that essential oils from *Cymbopogon martini*, *C. oliveri* and *Trachyspermum ammi* exhibited strong antifungal activity against *Helminthosporium oryzae* (Wilson *et al.*, 1997). The orange peel is the primary waste fraction and is the source of orange essential oil (Vargas *et al.*, 1999). Three permethoxylated flavones, dehydroabietic and linoleil monoglyceride were isolated from the nonvolatile residue of orange essential oil. All of them exhibited antifungal activity against phytopathogenic species (*Aspergillus parasiticus* and *Penicillium italicum*) and food contaminants. The isolated hexa – and heptamethoxy flavones exhibited important fungicidal activity against *Geotrichum candidum*, which is not inhibited by the commercial broadspectrum fungicide Benomyl (methyl – 1 – [butylcarbamoyl] - 2 – benzimidazolecarbamate) (Vargas *et al.*, 1999).

Some species are known to contain essential oils that possess antimicrobial activity, such as eugenol in cloves, allicin in garlic, and cinnamic aldehyde and eugenol in cinnamon (Bullerman *et al.*, 1977; Jay, 1986; Chang, 1995; Holt and Gamez – Almonte, 1995). Some vegetables and herbs also contain substances that inhibit microbial growth (Holt and Gamez – Almonte, 1995; Chiu and Chang, 1986; Zaika, 1988; Delaquis and Mazza, 1995). Fifteen species of common foodborne microorganisms (eg. *Bacillus*

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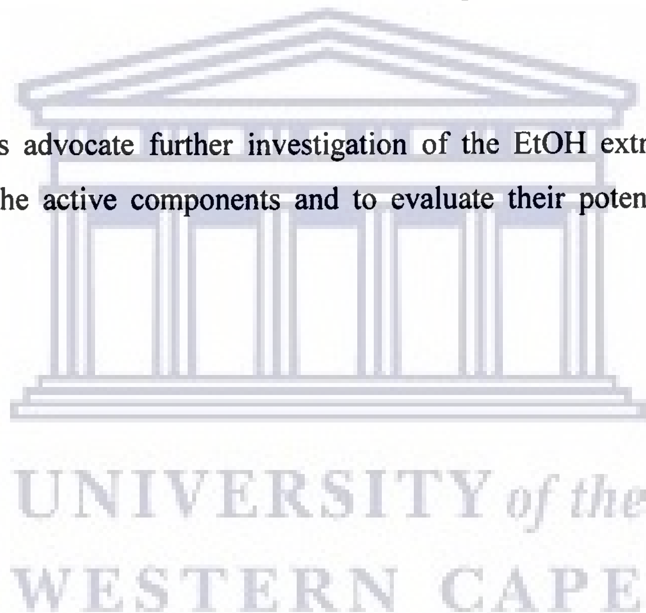
subtilis, *E. coli*, *Listeria monocytogenes*) were used to evaluate the antimicrobial effect of extracts and extract mixtures from Chinese chive, cinnamon, and corni fructus. Extracts from Chinese chive showed a broad antimicrobial spectrum (12 of 15 microorganisms). Extracts from cinnamon possessed extensive inhibitory effect on growth of test microorganisms. Extracts from corni fructus showed an inhibitory effect on growth of test bacteria, whereas no effect was observed on growth of yeast and molds (Jeng – Leun *et al.*, 2001). When two extracts were combined in equal volume, only the extract mixture of Chinese chive and corni fructus exhibited an extensive spectrum of antimicrobial effect. For the purpose of seasoning and antimicrobial effects, the mixed extract is of great interest for use as a natural additive in various food products (Jeng – Leun *et al.*, 2001).

The above findings advocate further investigation of the EtOH extract. It will be necessary to identify the active components and to evaluate their potential for use as antimicrobials.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MATERIALS AND METHODS

Microorganisms. Alternaria sp. and *Fusarium oxysporum* isolates were obtained from the culture collection of the Disease Management Division, Agriculture Research Council (ARC) Infruitec-Nietvoorbij, South Africa. *Fusarium graminearum* and *Fusarium verticillioides* isolates were obtained from the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council (MRC), South Africa. Cultures of the isolates were grown on carnation leaf agar (CLA) and incubated at 22 °C under white fluorescent light with a 12:12 h light: dark photoperiod for 7-14 days until well sporulated. The bacterial strains were *Escherichia coli*, *Bacillus* sp., *Salmonella typhi* and *Staphylococcus aureus*. These bacterial strains were obtained from the culture collection of the Department of Microbiology, University of the Western Cape, South Africa. The strains were maintained on their respective selective growth mediums (nutrient agar, xylose lysine deoxycholate agar, brain heart infusion agar and mannitol salt agar). A loopful of bacterial cells from the agar plates was inoculated into 200 ml brain heart infusion broth or lactose broth contained in a 250 ml Erleynmeyer flask. Incubation was done at 37 °C for 12 – 16 h. After incubation, the cultures were diluted into dilution series to give an optimal density ($O.D._{600}$) of 0.2.

Preparation of spore suspensions.

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Fungi. Standardised conidial suspensions were prepared. The spores were harvested by adding 5 ml of sterile distilled water to each CLA plate. Conidia were aseptically dislodged with a sterile inoculating loop into the water. Spore suspensions were aseptically filtered through two layers of sterile muslin cloth to remove mycelial debris. The spore suspensions were microscopically adjusted with the aid of a Neubauer haemocytometer to give a final concentration of approximately 1×10^6 spores/ml.

Bacteria. Standardised bacterial cell suspensions were prepared using the overnight cultures of the test organism. The overnight culture were inoculated into a dilution series and aseptically filtered through two layers of muslin cloth. It was microscopically

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adjusted with the aid of a Neubauer haemocytometer to give a cell density of about 1×10^6 viable organisms /ml.

Plant material. Two batches of *G. africana* were collected in April and December 1999 in Namaqualand (Northern Cape Province, South Africa). The first batch was collected in Concordia and the second one in Steinkopf. The plant material were separately ground in a mill into a fine powder and stored at 4°C until required. Both batches of plant materials were dried at room temperature (23 - 25 °C) before extraction.

Preparation of extractions. *G. africana* was sequentially extracted with various solvents starting with hexane (C_6H_{14}), chloroform ($CHCl_3$), ethanol (EtOH) and lastly sterile distilled water (dH_2O). Powdered, dried plant material (200 g) was extracted with hexane (500 ml) by agitation on a shaker (Innova 4000 incubator shaker, new Brunswick Scientific) for 1 h at room temperature. The extract was filtered through Whatman No. 4 qualitative filter paper. The residue was re-extracted with a further addition of hexane (500 ml) for 1 h at room temperature and filtered. After extraction with hexane the residue was individually extracted with $CHCl_3$ (2 x 500 ml), EtOH (2 x 500 ml) and lastly sterile dH_2O (2 x 500 ml) following the same procedure as described for hexane. The extracts were reduced under vacuum using a rotary evaporator at 40 °C. The extracts were stored at 4 °C until required. Each concentrated extract was bioassayed.

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Antifungal bioassay. The extract was filter sterilised by passing it through a Millipore filter (0.2 µm pore size) and was used to screen for activity against the growth of fungal species using the agar well diffusion method. Potato dextrose agar (PDA) plates were surface-inoculated with 200µl of the spore suspension and evenly spread with a L-shaped glass rod across the agar surface and allowed to dry. Four 5 mm diameter wells were aseptically made in each of the inoculated agar plates using a pre-sterilised cork borer. Each well was loaded with 50 µl of the respective extract. For the control the respective solvent was used. The extract was allowed to evaporate to dryness in a laminar flow hood. Plates were inverted and incubated at 27 °C for 5 days. The inhibition zones were

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measured in millimeters from the edge of the well and the means were determined. Triplicate determinations were made.

Antibacterial and antifungal bioassay. The activity of the extracts was tested against the growth of bacteria and fungi by the agar well diffusion method but 20 ml (in 90 mm petri dishes) agar plates were used. These agar plates (PDA, nutrient agar, xylose lysine deoxycholate agar, mannitol salt agar and brain heart infusion agar) were surface – inoculated with 200 µl of the prepared bacterial cell or fungal spore suspensions and evenly spread with a L-shaped glass rod across the agar surface and allowed to dry in a laminar flow hood. The same procedure were followed as described above except that the bacteria were incubated at 37 °C for 24 h.

Determination of Minimum Inhibitory Concentration (MIC). Once the sensitivity range for each microorganism (bacteria or fungi) was known, the MIC was determined by the agar well diffusion method. Two-fold serial dilutions of the extracts (0.156 %, 0.312 %, 0.625 %, 1.25 %, 2.5 %, 5 %, 10 %, and 20 %) were prepared within their respective solvent and used against the strain, which was sensitive. The MIC was defined as the lowest concentration of the extract preventing the growth of the microorganisms.

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CHAPTER 3

SEPARATION AND PURIFICATION OF THE ACTIVE AGENT IN THE SOLVENT EXTRACT

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Chapter 3

Separation and purification of the antimicrobial active agent in the solvent extract of Galenia africana

Abstract

The ethanol extract of *Galenia africana* was screened *in vitro* for antimicrobial activity against fungi and bacteria. The crude extract showed maximal inhibitory effect against four fungal isolates with MIC`s of 0.625 – 5 % (g/vol) and 2 tested bacteria with MIC`s of 0.625 % (g/vol). The ethanol extract was purified using silica gel column chromatography and thin – layer chromatography. Four fractions showed significant antifungal activity as evidenced by a clear zone of inhibition. One fraction was tested against a range of fungi and a few bacterial isolates. This fraction inhibited the growth of most of the fungal isolates with *Alternaria* sp., *Fusarium equiseti*, *Fusarium graminearum*, *Fusarium verticillioides* and *Phaeomoniella clamydospora* being the most sensitive ones. Both *Bacillus* sp. and *Staphylococcus aureus* were inhibited in the presence of the fraction. The MIC`s for fungi showed values ranging from 10 to 2.5 % and bacteria from 2.5 to 1.25 %. The Gram – positive bacteria were more sensitive to the inhibitory effects of the fraction.

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INTRODUCTION

Galenia africana, commonly known as kraalbos or geelbos, belonging to the family Aizoaceae is a dominant plant throughout the Namaqualand area (Kellerman *et al.*, 1988). *G. africana* is an active invader, and is especially abundant in disturbed areas around kraals, along roads and on trampled veld. Kraalbos is an aromatic woody perennial sub-shrub, growing 0.5 – 1 m high, and having small green leaves, which turn yellow with age. Inflorescences are born at the end of twigs and are 30 – 100 mm long, with many small yellow flowers. The plant has been associated with liver damage and severe ascites, referred to as "waterpens" in sheep and goats. Apart from severe abdominal distension, affected animals show mass loss, become apathetic and recumbent, and die (Van der Lugt *et al.*, 1981).

At present, quick and effective management of plant diseases and microbial contamination in several agricultural commodities is generally achieved by the use of synthetic pesticides (Agrios, 1997). However, the incessant and indiscriminate application of these chemical pesticides has caused health hazards in animals and humans due to their residual toxicity. In recent years, a large number of synthetic pesticides have been banned in the western world because of their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in the food chain and an extention of their power to destroy both useful and harmful pests. Many pathogenic microorganisms and insect pests have developed resistance against chemical pesticides. This seriously hinders the management of diseases of crops and agricultural products. Considering the deleterious effects of synthetic pesticides on life supporting systems, there is an urgent need for alternative agent's for the management of pathogenic microorganisms (Hili *et al.*, 1997).

Green plants represent a reservoir of effective chemotherapeutants and can provide valuable sources of natural pesticides (Satish *et al.*, 1999). Several compounds eg. essential oils, flavanones, etc. have antimicrobial activity. Essential oils are the odorous, volatile products of plant secondary metabolites, normally formed in special cells or groups of cells or as glandular hairs, found on many leaves and stems (Hili *et al.*, 1997).

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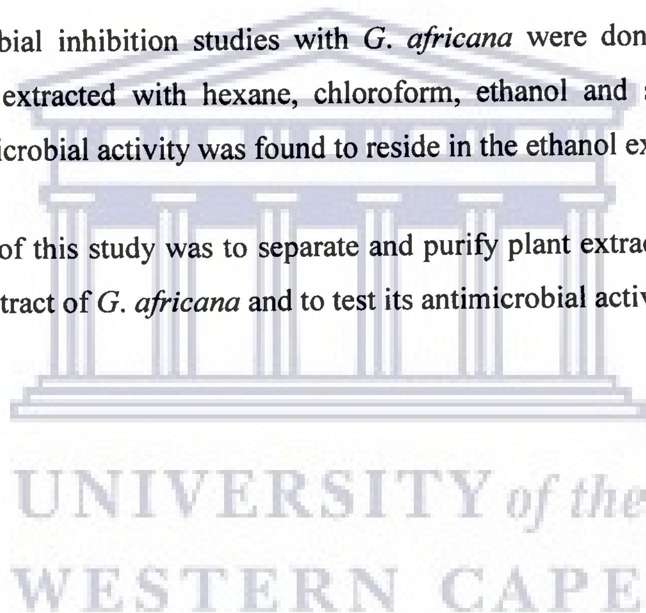
Essential oils are frequently used for flavour and fragnance in the perfume, pharmaceutical, cosmetic and food industries (Mangena and Muyima, 1999). Fifty-one essential oils extracted from plants (*Illicium verum*, *Ocimum basilicum*, *Piper nigrum*, *Salvia sclarea*, etc) were tested for their antimicrobial activity against three bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and four yeasts, *Torulopsis utilis*, *Schizosaccchararomyces pombe*, *Canidida albicans* and *Saccharomyces cerevisiae* using the drop diffusion method. All showed antimicrobial activity against at least one of the microorganisms. The concentration of most of the oils required for total inhibition of growth was >500μg ml^{-1} (Hili *et al.*, 1997).

Relative antimicrobial inhibition studies with *G. africana* were done with solvent fractions sequentially extracted with hexane, chloroform, ethanol and sterile distilled water. The main antimicrobial activity was found to reside in the ethanol extracts.

Thus, the purpose of this study was to separate and purify plant extracted substances present in the EtOH extract of *G. africana* and to test its antimicrobial activities.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RESULTS AND DISCUSSION

Screening combined and rechromatgraphed fractions for antifungal activity against F. verticillioides

The EtOH extract subjected to silica gel column chromatography eluted with CH_2Cl_2 – EtOAc – hex (75:10: 15) gave rise to 5 combined fractions for Steinkopf plant material and 4 combined fractions for Concordia plant material. The fractions were combined according the similarity they showed on a TLC plate. The fractions present in the extract are separated as they move down the column according to their relative strength of adsorption by the stationary phase and solubility in the mobile phase.

Steinkopf site collection

The Steinkopf combined fractions 1 – 29, 30-34 and 35 – 72 exhibited antifungal activity (Table 1). Combined fractions (1 – 29) gave rise to 3 subfractions (1-3; 7-9 and 11-13) in which fractions 11 – 13 (0.0110 g) retained the antifungal activity (Table 2). Combined fractions 35 – 72 were rechromatographed using CH_2Cl_2-EtOAc- hexane (50: 20: 30) as the solvent system and afforded major subfractions 9 – 29 (0.9616 g), which showed significant antifungal activity as evidenced by a clear zone of inhibition. The inhibition zones ranged between 3.1 – 6 mm for subfractions 9 – 29 and 1 – 3 mm for subfractions 11 – 13.

Concordia site collection

Of the Concordia site only the combined fraction 59-136 showed an inhibition zone (Table 1). The combined fractions 59-136 gave rise to 3 subfractions (7-16; 17-34 and 35-93) (Table 1). Subfractions 17-34 (0.4117 g) and 35-93 (0.6570 g) retained the antifungal activity. The zones of inhibition ranged between 2.0 - 3.3 mm for subfractions 17 – 34 and 4.1 – 7.1 mm for subfractions 35 – 93.

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Fractions were combined according the similarity they showed on a TLC plate. The combined fractions from Steinkopf (1-29 and 35-72) and Concordia (59-136) showed antifungal activity when tested against *F. verticillioides*.

Table 1. Antifungal activity of combined fractions against the growth of *F. verticillioides*

Area	Combined fraction[a]	Incubation time (day)		
		3	5	control
Steinkopf	1-29	++	++	-[b]
	30-34	++	+[c]	-
	35-72	++[d]	++	-
	79-105	-	-	-
	124-159	-	-	-
Concordia	1-11	-	-	-
	13-41	-	-	-
	43-57	-	-	-
	59-136	++	++	-

[a] Fractions combined according their similarity showed on TLC plates (solvent system CH_2Cl_2-EtOAc- hexane 75:10:15).

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Table 1 (Cont.)

[b] - = no inhibition zones present.

[c] + = clear zones of inhibition between 1- 3 mm.

[d] ++ = clear zones of inhibition between 3.1 – 7 mm.

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Table 2. Antifungal activity of rechromatographed fractions against the growth of *F. verticillioides*

Area	Combined[a] fractions	Rechrom.[b] fractions	Weight (g)	Incubation time (days) 3	5	control
Steinkopf	1-29	1-3	0.0449	-[c]	-	-
		7-9	0.0117	-	-	-
		11-13	0.0110	+[d]	+	-
	30-34	5-11	0.0078	-	-	-
	35-72	9-29	0.9616	++[e]	++	-
Concordia	59-136	7-16	0.0242	-	-	-
		17-34	0.4117	++	+	-
		35-93	0.6570	+++[f]	++	-

[a] Fractions were combined according their similarity on TLC (solvent system CH_2Cl_2-EtOAc-hexane 75:10:15).

[b] These fractions were rechromatographed by column chromatography (silica gel, CH_2Cl_2-EtOAc- hexane 50:20:30 were used as eluent).

[c] - = no inhibition zones present.

[d] + = clear zones of inhibition between 1- 3 mm.

[e] ++ = clear zones of inhibition between 3.1 – 5 mm.

[f] +++= clear zones of inhibition between 5.1 – 8 mm.

The effect of the rechromatographed fractions on the growth of different fungi

The rechromatographed fractions (9-29) were effective against most of the fungal isolates with *Alternaria* sp., *F.equiseti*, *F. graminearum*, *F. verticillioides* and *P. clamydospora* being the more sensitive ones (Table 3). All control wells were overgrown by the fungal isolate being tested. *P. expansum* proved to be the most resistant organism

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to the rechromatographed fractions although minimal growth was observed near the edges of the wells with the fraction compared to the growth of the control. In the case of *E. lata*, *F. solani*. *B. cinerea* and *Cylindrocarpon* the inhibitory activity disappeared quickly indicating that the fraction is not active for long periods against these isolates.

Table 3. Effect of the rechromatographed fractions (9-29) on the growth of different fungal species cultured on PDA (20 ml agar) with initial inoculum levels of 1 x 10^6 spores/ml at 27 °C for 2-10 days

Rechromatographed fractions[a]	Fungal isolates	Incubation time (days)						
		2	3	5	7	9	10	control
9-29	*Alternaria* sp.	+	+	+[b]				-[c]
	Eutypa lata	+	-	-				-
	Fusarium equiseti	++[d]	+	+				-
	Fusarium graminarum	+	-	-				-
	Fusarium oxysporum	+	+	-				-
	Fusarium solani	++	-	-				-
	Fusarium verticillioides	++	+	+				-
	Botrytis cinerea			++	-	-	-	-
	Cylindrocarpon sp.			++	-	-	-	-
	Penicillium expansum			-	-	-	-	-
	Phaeomoniella clamydospora			+	+	+	-	-

[a] Combined fractions 35 – 72 were rechromatographed using CH_2Cl_2-EtOAc- hexane 50: 20: 30 as the eluent and afforded a major subfractions 9 – 29 (0.9616 g).

[b]+ = clear zones of inhibition between 1- 3 mm.

[c]- = no inhibition zones present.

[d]++ = clear zones of inhibition ranging from 3.1 – 5 mm.

Effect of the rechromatographed fractions on the growth of different bacteria

The results of the antibacterial tests indicated that both *Bacillus* sp. and *S. aureus*

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were sensitive to the rechromatographed fraction (9-29) (Table 4). *S. aureus* showed the highest sensitivity to the rechromatographed fractions and *Bacillus* sp. had smaller zones of inhibition. During the incubation period of 5 days there was a reduction in the inhibitory activity caused by the fractions. All the control wells were overgrown by the bacterial isolate being tested. It appeared that the Gram - positive bacteria were more sensitive to the fractions than the Gram – negative bacteria. These bacteria (Gram - positive) are able to produce toxins responsible for food poisoning. Foods contaminated with pathogens pose a potential danger to the consumer's health. It is interesting to note that the fraction showed antibacterial activity towards organisms of importance to food poisoning. In view of the broad antibacterial activity, the fraction may have interesting preservative potential for industrial applications. The fraction can be suggested as a candidate for a natural preservation agent in the cosmetic and / or food industries. The use of the fraction could increase the shelf – life and decrease the possibilities of food poisoning in processed foods.

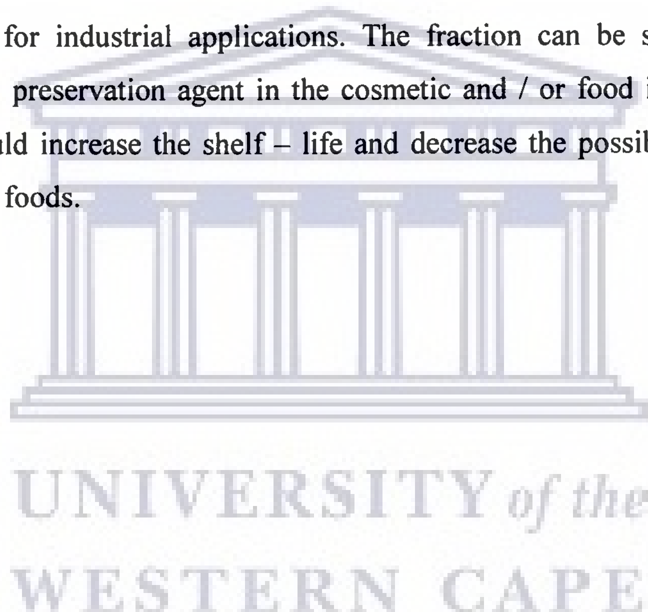
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Table 4. The effect of the rechromatographed fractions (9-29) on the growth of different bacterial species cultured on selective growth mediums with initial inoculum levels of 1 x 10^6 spores / ml at 37 °C for 1 – 5 days

Rechromatographed Fractions[a]	Bacterial isolates	Gram reaction	Incubation time (days) 1	3	5	control
9-29	*Bacillus* sp.	positive	++[c]	+	+[b]	-
	Escherichia coli	negative	-	-	-	-
	Salmonella typhi	negative	-	-	-	-
	Staphylococcus aureus	positive	+++[d]	++	++	-[e]

[a] Combined fractions 35 – 72 were rechromatographed using CH_2Cl_2-EtOAc- hexane 50: 20: 30 as the eluent and afforded a major subfraction 9 – 29 (0..9616 g).

[b] + = clear zones of inhibition between 1- 3 mm.

[c] ++ = clear zones of inhibition between 3.1 – 5 mm.

[d] (+++) = clear zones of inhibition between 5.1 – 8 mm.

[e] - = no inhibition zones present.

Determination of minimum inhibitory concentration (MIC) for fungi and bacteria

For *Alternaria* sp, *F. oxysporum* and *F. verticillioides* a concentration of 5 % is required to prevent the organisms from growing, and a concentration of 2.5 % of the active compound for *Cylindrocarpon*, *F. equiseti* and *P. clamydospora* (Table 5). *F. solani* requires a higher concentration (10 %) of the active fraction to prevent fungal growth. A concentration of 0.625 % and 1.25 %, respectively is required to inhibit the growth of *E. lata* and *B. cinerea*. Vigorous growth was observed at the control. *Bacillus* sp. and *S. aureus* were inhibited at low concentrations of 2.5 % and 1.25 %, respectively (Table 6).

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Table 5. The minimum inhibitory concentration (MIC)[a] of the rechromatographed fractions (9-29) tested against different fungal isolates

Fungal isolates	MIC (%)	Inhibition zones (mm)
Alternaria sp.	5	2.2
Botrytis cinerea	1.25	1.0
Cylindrocarpon sp.	2.5	2.4
Eutypa lata	0.625	0.7
Fusarium equiseti	2.5	3.2
Fusarium graminearum	2.5	1.9
Fusarium oxysporum	5	0.3
Fusarium solani	10	0.6
Fusarium verticillioides	5	0.4
Phaemoniella clamydospora	2.5	1.1

[a] MIC`s were determined by the agar well diffusion method, reflecting the lowest concentration of the fractions preventing the growth of the fungal isolates, at least 3 d after start of growth of the control. Each test was performed in triplicate. The concentrations used were 0.156 %, 0.312 %, 0.625 %, 1.25 %, 2.5 %, 5 % and 10 %. The concentrations given here are in g of the pure isolate per volume of CH_2Cl_2.

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Table 6. The minimum inhibitory concentration (MIC)[a] of the rechromatographed fractions (9-29) tested against *Bacillus* sp. and *Staphylococcus aureus*

Bacterial isolates	MIC (%)	Inhibition zones (mm)
Bacillus sp.	2.5	3.1
Staphylococcus aureus	1.25	4.1

[a] MIC`s were determined by the agar well diffusion method, reflecting the lowest concentration of the fractions preventing the growth of the bacterial isolates, at least 24h after start of growth of the control. Each test was performed in triplicate. The concentrations used were 0.156 %, 0.312 %, 0.625 %, 1.25 %, 2.5 %, 5 % and 10 %. The concentrations given here are in g of the pure isolate per volume of CH_2Cl_2.

The effect of the rechromatographed fractions on the growth of different fungi by the filter paper disk method

The active fraction inoculated onto individual filter paper disks displayed potent antifungal activity on agar plates seeded with conidia of the various fungal isolates being tested. The active fraction showed potent activity as measured by a zone of inhibition surrounding each disk against *B. cinerea*, *Alternaria* sp, *E. lata*, *F. equiseti*, *F. graminearum* and *F. pseudograminearum* at the lowest concentrations (0.312 and 0.625 % respectively). *F. solani* seems to be the only fungal isolate that can resist the activity of the bioactive fraction taking into account the small inhibition zone (0.3 mm) obtained surrounding the filter paper disks. The fraction was inhibitory to the growth of *Cylindrocladium*, *F. tricintum*, *Cylindrocarpon* sp., *F. oxysporum* and *P. expansum* at MIC`s of 5 and 2.5 %, respectively (Table 7). A concentration of 1.25 % is required to inhibit the growth of *F. verticillioides* and *P. clamydosporum*. The filter paper disk method was more sensitive than the agar well diffusion method in the detection of the antifungal activity of the active fraction. For the agar well diffusion method the active fraction was allowed to diffuse into the agar whereas for the filter paper disk method dried filter paper disks was placed equidistant on inoculated agar plates. Factors such as

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the rate of diffusion the active fraction are difficult to monitor and may lead to erroneous results. Thus the results of the filter paper disk method clearly show strong antifungal activity of the active fraction towards these fungal isolates. Several different methods exist to test the susceptibility of microorganisms against the rechromatographed fractions (9-29). These include broth dilution (Sandhu *et al.*, 1979), agar – diffusion (Keyser *et al.*, 1999), disk diffusion (Utz and Armstrong, 1977), and microtiter bioassays (Fisher and Armstrong, 1977). These bioassay methods were found to be useful in detecting proper minimum inhibitory concentration (MIC) zones. Results obtained with MIC`s are defined on the basis of visible mycelial growth and spore germination.

The increased awareness of the environmental problems associated with chemical pesticides has led to the search for non – conventional chemicals of biological origin for the management of these diseases. The products of plant origin are of great advantage to the user and the public. Laboratory screening of the fractions has given encouraging results, indicating the potential use in the management of diseases caused by certain fungal species.

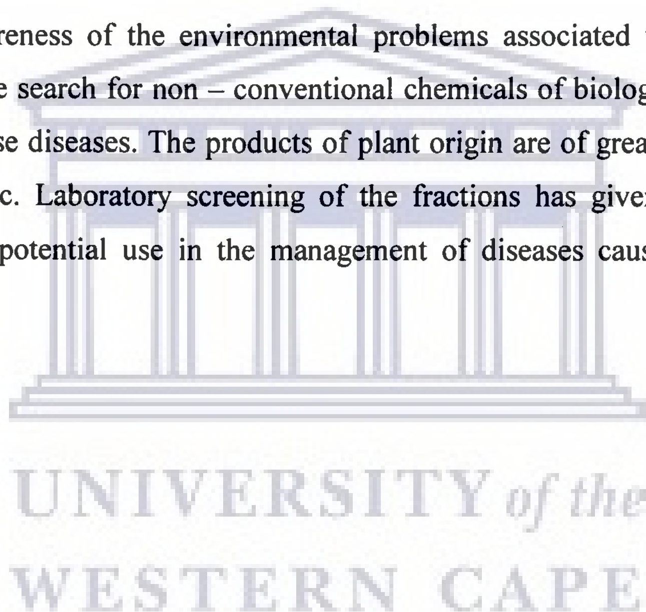
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Table 7. Effect of the rechromatographed fractions (9-29) on the growth of different fungal species by the filter paper method on PDA plates (20 ml agar) with initial inoculum levels of 1 x 10^6 spores/ml at 27 °C for 5 days

Rechromatographed fractions[a]	Fungal isolates	MIC[b]	Inhibition zones (mm
9-29	*Alternaria sp.*	0.625	0.5
	Botrytis cinerea	0.31	0.6
	Cylindrocarpon sp.	2.5	4.2
	Cylindrocladium sp.	5	0.4
	Eutypa lata	0.625	1.8
	Fusarium equiseti	0.625	3.2
	Fusarium graminearum	0.625	3.4
	Fusarium pseudograminearum	0.625	0.8
	Fusarium oxysporum	2.5	0.4
	Fusarium solani	10	0.3
	Fusarium trincintum	5	1.6
	Fusarium verticillioides	1.25	0.5
	Penicillium expansum	2.5	0.9
	Phaeomoniella clamydospora	1.25	2.2

[a] Combined fractions 35 – 72 were rechromatographed using CH_2Cl_2-EtOAc- hexane 50: 20: 30 as the eluent and afforded a major subfractions 9 – 29 (0.9616 g).

[b] MIC`s were determined by the filter paper method, reflecting the lowest concentration of the fractions preventing the growth of the fungal isolates, at least 5 d after start of growth of the control. Each test was performed in triplicate. The concentrations used were 0.156 %, 0.312 %, 0.625 %, 1.25 %, 2.5 %, 5 % and 10 %. The concentrations given here are in g of the pure isolate per volume of CH_2Cl_2.

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Plants are a source of naturally occurring pesticides. Plant extracts and essential oils show antifungal activity against a wide range of fungi. Garlic extracts contain a potent fungicide (Wilson *et al.*, 1997). Recently, it has been demonstrated that essential oils of female and hermaphrodite *Thymus bacticus* Boiss (thyme oil) showed marked activity against some Gram-positive and Gram-negative bacteria and yeast. The activity was greater in the essential oils containing larger amounts of geraniol (Cruz *et al.*, 1993). The antifungal activity of six essential oils was tested *in vitro* and 16 clinical isolates of the dermatophyte *Epidermophyton fluccosum*, *Microsporum canis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* isolated from patients with dermatophytosis (Lima *et al.*, 1992). The oils were obtained from the plants *Annona classifora*, *Cinnamomum zeylanicum*, *Cymbopogon citraus*, *Ocimum gratissimum, Protium heptaphyllum* and *Xylopin frutesens*. Oils (with the exception of that from *Protium heptaphyllum*) caused inhibition of 81 % of the studied strains of dermatophytes. The antimicrobial properties of oils from *Melaleuca alternifolia* (tea tree), *Mentha piperita* (peppermint) and *Salvia officinalis* (sage) were the most potent against facultatively anaerobic oral bacteria (Hili *et al.*, 1997).

The antimicrobial activities of the essential oils of *Artemisia afra*, *Pteronia incana* and *Rosmarinus officinalis* were tested against 41 microbial strains (Mangena and Muyima, 1999). The test organisms were selected on the basis of their significance as food spoilage and /or poisoning, common human and plant pathogens. The agar diffusion assay was performed using the nutrient agar and antibiotic medium. All the oils tested displayed some antimicrobial activities. However, the efficiency differed and depended both on the type and concentration of the oil, as well as the test microbial strain. *A. afra* and *R. officinalis* showed similar and higher antimicrobial activity than *P. incana*. *A. afra* is the one of the oldest known medicinal plants in Southern Africa. It is used to cure diseases such as the common cold, diabetes mellitus, bronchial complaints and stomach disorders. The volatile oil of *A. afra* has been reported to have several biological activities, notably antibacterial, antifungal and antioxidative properties. *P. incana* produces a yellowish oil with a strong fragrance. There is no further information eg. antimicrobial activity, available about this oil. *R. officinalis* produces a colourless or pale

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yellow oil with the characteristic of rosemary and a warm camphoraceous taste. It is used in the perfume industry and as a flavour agent (Mangena and Muyima, 1999).

In summary, current research offers possibilities for inhibition activity against fungi and bacteria. The level of antimicrobial activity exhibited by the active fraction could make an attractive option for the replacement of synthetic pesticides. As the fungal isolates are known to be transmitted through seeds, one important application of the active fraction is as a seed protectant. Fungicides or bactericides of plant origin can be one approach to disease management because of their eco-friendly nature. The products of plant origin will be of great advantage to the user (Bolkan and Reinert, 1994).

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MATERIALS AND METHOD

Test microorganisms. Compounds were tested for antifungal activity against 11 different fungal species: *Alternaria* sp., *Botrytis cinerea*, *Cylindrocarpon* sp., *Eutypa lata*, *Fusarium equiseti*, *F. oxysporum*, *F. graminearum*, *F. solani*, F. *verticillioides*, *Penicillium expansum* and *Phaemoniella clamydospora*. The cultures were obtained from the culture collection of the Disease Management Division, Agricultural Research Council Infruitec - Nietvoorbij, South Africa and from the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, South Africa. *Fusarium* spp. was maintained on carnation leaf agar (CLA) and the other fungi on potato dextrose agar (PDA). The PDA plates were incubated at 25 °C in the dark whereas the CLA plates were incubated at 22 °C under a white fluorescent light with a 12: 12 light: -dark photoperiod for 7-14 days. The compound were tested against bacteria and the bacterial isolates were, *Escherichia coli*, *Bacillus sp. Salmonella typhi* and *Staphylococcus aureus*. These isolates were obtained from the culture collection of the Department of Microbiology University of the Western Cape, South Africa. They were maintained on their respective selective growth mediums. All media were obtained from Difco Laboratories, Detroit, MI.

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Preparation of spore suspensions

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Fungi. Standardised conidial suspensions were prepared. The spores were harvested by adding 5 ml of sterile distilled water to each CLA plate and the conidia were aseptically dislodged with a sterile inoculating loop into the water. Spore suspensions were aseptically filtered through two layers of sterile muslin cloth to remove mycelial debris. The spore suspensions were microscopically adjusted with the aid of a Neubauer haemocytometer to give a final concentration of approximately $1x10^6$ spores/ml.

Bacteria. Standardised bacterial cell suspensions were prepared using the overnight cultures of the test organism. The overnight culture were inoculated into a dilution series and aseptically filtered through two layers of muslin cloth. It was microscopically

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adjusted with the aid of a Neubauer haemocytometer to give a cell density of about $1x10^6$ viable organisms /ml.

Plant material. G. africana was collected in Namaqualand (Steinkopf and Concordia), in April and December 1999, respectively and brought to the lab for analysis. The dried plant material was separately ground in a mill into a fine powder and stored at 4 °C until required. Although the plant material was collected in different areas it showed the same thin-layer chromatography (TLC) profile. No numerical differences were observed in the antimicrobial activity for the plant material collected in the two areas.

Chemicals. All the organic solvents were of analytical grade and distilled prior to use during chromatography.

Separative methods. Powdered, dried plant material (200 g) was sequentially extracted with various solvents (hexane, chloroform, ethanol and sterile distilled water) and filtered through Whatman No. 4 filter paper. The solvent was removed on a rotary evaporator and the EtOH (11 g) extract was subjected to column chromatography (particle size of silica gel 0.063-0.200 mm) with CH_2Cl_2-EtOAc-hexane 75:10:15 as eluent. The eluate was collected in tubes as fractions using a fraction collector. The fractions were spotted onto thin-layer chromatography (TLC) plates and were pooled into subfractions according to their similarity on TLC plates (same solvent system as for column chromatography, spots were visualized under UV light) and screened for antifungal activity. The active fractions were selected and repeatedly rechromatographed on silica gel (solvent system CH_2Cl_2-EtOAc-hexane 50:20:30). The antifungal activity was determined again. Three fractions of the Steinkopf plant material and one fraction of Concordia were fungistatic when tested against fungal species. Although the fractions showed activity only one fraction was selected and tested against a range of fungi and bacteria.

Bioassay . Antimicrobial activity was measured by the agar well diffusion method. Petri dishes (90 mm) containing 20 ml agar (nutrient agar, PDA, mannitol salt agar, brain heart infusion agar and xylose lysine deoxycholate agar) were surface–inoculated with 200µl

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of the prepared spore suspension (1 x 10^6 spores/ml) and evenly spread with a L-shaped glass rod across the agar surface and allowed to dry in a laminar flow hood. Four 5 mm diameter wells were aseptically made in each of the inoculated agar plates using a pre-sterilised cork-borer. The fraction (1 g) was dissolved in 10 ml CH_2Cl_2. Each well was loaded with 50µl of the fraction selected. The fraction was allowed to evaporate to dryness in a laminar flow hood. Plates were inverted and incubated at 27 °C for fungi and at 37 °C for bacteria over 5 days. The inhibition zones were measured in millimeters from the edge of the well and the means were determined. Triplicate determinations were made.

Determination of minimum inhibitory concentration (MIC). Once the sensitivity range for each organism was known, the MIC`s was determined by the agar well diffusion method. Two-fold serial dilutions of the active fraction (0.156 %, 0.315 %, 0.625 %, 1.25 %, 2.5 %, 5 % and 10 %) were prepared in 9 ml of CH_2Cl_2 and used against the test organism. The MIC is defined as the lowest concentration of the active fraction capable of preventing growth of an organism. Triplicate determinations were made.

Filter paper disks method. The active residue (1 g) was dissolved in 9 ml CH_2Cl_2 before the assay. Aliquots of the active residue (50 µl) were pipetted onto individual sterile analytical grade filter paper disks (Whatman No. 4, 10 mm diameter) in individual Petri dish lids and dried for 30 min in a laminar flow hood. Filter paper disks without the active residue represents the control disks. Four disks were placed equidistant from one another on the surface of inoculated PDA plates with standardised fungal spore suspensions (200 µl). The bioassay plates were incubated for 5 days at 27 °C and examined for the presence of a zone of inhibition surrounding the disk, which is evidence of the inhibition of germination and a measure of fungistatic activity. Triplicate determinations were made.

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CHAPTER 4

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EFFECT OF THE BIOACTIVE AGENT FOUND IN *GALENIA AFRICANA* ON GERMINATION OF INDIVIDUAL SPORES OF FUNGAL SPECIES

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Chapter 4

Comparative effects of the bioactive agent found in *Galenia africana* on spore germination, germ tube length and mycelial weight

Abstract

The purified plant extract fraction of *Galenia africana* was used to evaluate its antifungal activity on the spore germination of common plant pathogens. Twelve fungal species, i.e *Alternaria* sp., *Botrytis cinerea*, *Cylindrocarpon* sp., *Cylindrocladium*, *Fusarium equiseti*, *Fusarium oxysporum*, *Fusarium pseudograminearum*, *Fusarium solani*, *Fusarium trinctinum*, *Fusarium verticillioides*, *Penicllium expansum* and *Phamoniella clamydosporum*, were used for the germination on water agar plates at different fraction concentrations, i. e 0.625, 1.25, 2.5, 5 and 10 %. Germ tube lenghts were reduced at increasing concentrations of the active fraction for all the test fungi. Concentrations of the active fraction higher than 1.25 % prevented the germination of *Alternaria* sp., *B. cinerea*, *Cylindrocarpon* sp. and *F. verticillioides* and concentrations higher than 2.5% prevented the germination of *P. expansum* and *P. clamydospora*.

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INTRODUCTION

Galenia africana is an indigenous plant in South Africa and has been associated with liver damage and severe ascites a condition referred to as "waterpens" in sheep and goats (Watt & Breyer-Brandwijk, 1962). The plant has various medicinal properties which is used for various ailments. The Hottentots chew the plant to relieve toothache and it is said to blister the mucous membrane of the mouth if used too much. The plant was also used in the treatment of veneral diseases and a decoction is presently being used as a lotion for skin diseases and for the relief of inflammation of the eyes. A decoction of *G. africana* was used as a lotion for wounds in man and animal (Watt & Breyer-Brandwijk, 1962).

In a previous laboratory experiment the effect or activity of the bioactive fraction isolated from *G. africana* was tested against eleven fungi and a few bacterial isolates using the agar well diffusion method. This active fraction inhibited the growth of all fungal isolates with *Alternaria* sp., *Fusarium equiseti*, *F. graminearum*, *F. verticillioides* and *Phamoniella clamydospora* being the most sensitive ones. Both *Bacillus* sp. and *Staphylococcus aureus* were inhibited while *Eschericia coli* and *Salmonella typhi* were resistant in the presence of the bioactive fraction.

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Several different methods exist to test the susceptibility of fungi against plant extracts. These include broth dilution (Sandhu *et al.*, 1979), agar-diffusion (Keyser *et al.*, 1999), disk diffusion (Utz and Shacomy, 1976; Holt, 1978,) and microtiter bioassays (Fisher and Armstrong, 1977). These bioassay methods were found to be useful in detecting proper minimum inhibitory concentration (MIC) zones and also different deformations such as swelling and hyphal stunting caused by the specific antifungal agents. Results obtained with MIC`s are defined on the basis of visible mycelial growth. Conidia should germinate and produce germ tubes for monitoring the growth inhibition or stimulation and the fungicidal activity of the bioactive fraction, compared to the control. If a compound is capable of inhibiting the germination of conidia, while either affecting or not affecting the growth of the organism, the MIC is considered the concentration of the compound required for the inhibition of conidial germination, but not

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necessarily the concentration required for inhibition of the growth of the organism. The use of conidial germination has resulted in excellent reproducibility of germ tube inhibition results obtained in different laboratories (Manavathu *et al*., 1996: Denning *et al*., 1997, Pujol *et al*., 1997).

The present study was undertaken to demonstrate the effect of the bioactive agent on the germination of freshly harvested conidia of fungal species.

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RESULTS AND DISCUSSION

The effect of the active fraction of *G. africana* at different concentrations on the conidial germination of twelve test fungi was determined. The inhibitory effect of the active fraction was determined by means of germ tube length and percentage germination of conidia measurements. The germ tube lengths were reduced at increasing concentrations of the active compound for *Alternaria* sp., *B. cinerea*, *Cylindrocarpon* sp., *F. verticillioides*, *P. expansum* and *P. clamydospora* compared to the control (Table 1). At concentrations higher than 1.25 % (g/vol) the spore germination of *Alternaria* sp., *B. cinerea*, *Cylindrocarpon* sp. and *F. verticillioides* and at concentrations higher than 2.5 % (g/vol) the spore germination of *P. expansum* and *P. clamydospora* were completely prevented (Table 2 and Fig. 1-6). The spore germination of the *Cylindrocladium* sp., *F. equiseti, F. oxysporum*, *F. pseudograminearum*, *F. solani*, and *F. trinctinum* were completely inhibited by the active compound.

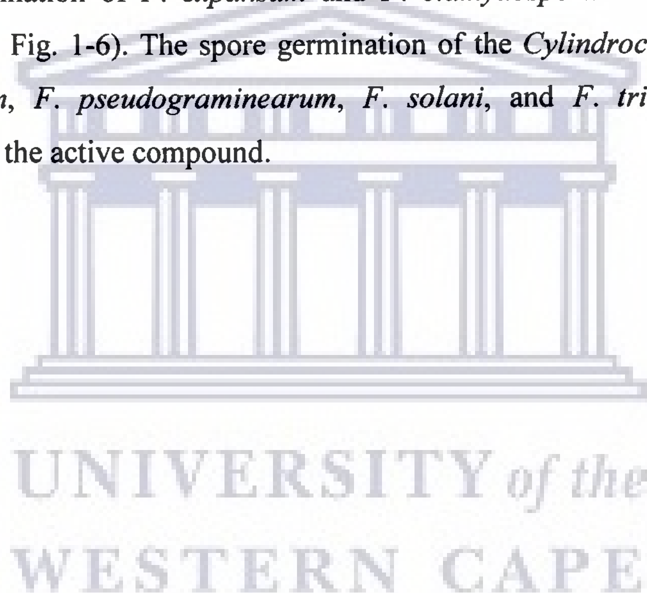
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Table 1. Effect of the bioactive fraction isolated from *Galenia africana* on germ tube length (μm) of twelve fungal isolates at different concentrations on water agar plates at 27 °C

	Active fraction concentration (g/vol)					
Fungi tested	Control[a]	0.625	1.25	2.5	5	10
	Germ tube length (μm)					
Alternaria sp.	3.0	0.4	ng[b]	ng	ng	ng
Botrytis cinerea	4.3	3.7	ng	ng	ng	ng
Cylindrocarpon sp.	9.1	2.5	ng	ng	ng	ng
Cylindrocladium sp.	6.2	ng	ng	ng	ng	ng
Fusarium equiseti	3.6	ng	ng	ng	ng	ng
Fusarium oxysporum	5.7	ng	ng	ng	ng	ng
Fusarium pseudograminearum	5.3	ng	ng	ng	ng	ng
Fusarium solani	11.5	ng	ng	ng	ng	ng
Fusarium trinctinum	3.3	ng	ng	ng	ng	ng
Fusarium verticillioides	8.0	6.0	ng	ng	ng	ng
Penicillium expansum	0.9	0.06	0.02	ng	ng	ng
Phamoniella clamydospora	5.5	1.7	0.9	ng	ng	ng

[a] control = EtOH and dH_2O (1:9) only without the active fraction.

[b] ng = no germ tube growth

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Table 2. The effect of the bioactive fraction isolated from *Galenia africana* at different concentrations on the spore germination (%) of various fungal isolates after incubation on water agar plates at 27 °C

	Active fraction concentration (g/vol)					
Fungi tested	Control[a]	0.625	1.25	2.5	5	10
	Spore germination (%)					
Alternaria sp.	48	8.8	ng[b]	ng	ng	ng
Botrytis cinerea	64	55.3	ng	ng	ng	ng
Cylindrocarpon sp.	48.7	38.7	ng	ng	ng	ng
Cylindrocladium sp.	67.3	ng	ng	ng	ng	ng
Fusarium equiseti	53.3	ng	ng	ng	ng	ng
Fusarium oxysporum	80	ng	ng	ng	ng	ng
Fusarium pseudograminearum	77.3	ng	ng	ng	ng	ng
Fusarium solani	98.7	ng	ng	ng	ng	ng
Fusarium trinctinum	66.7	ng	ng	ng	ng	ng
Fusarium verticillioides	76.7	30	ng	ng	ng	ng
Penicillium expansum	44	5	2	ng	ng	ng
Phaemoniella clamydospora	67.3	60.7	25.3	ng	ng	ng

[a] control = EtOH and dH_2O (1:9) only without the active fraction.

[b] ng = no germination.

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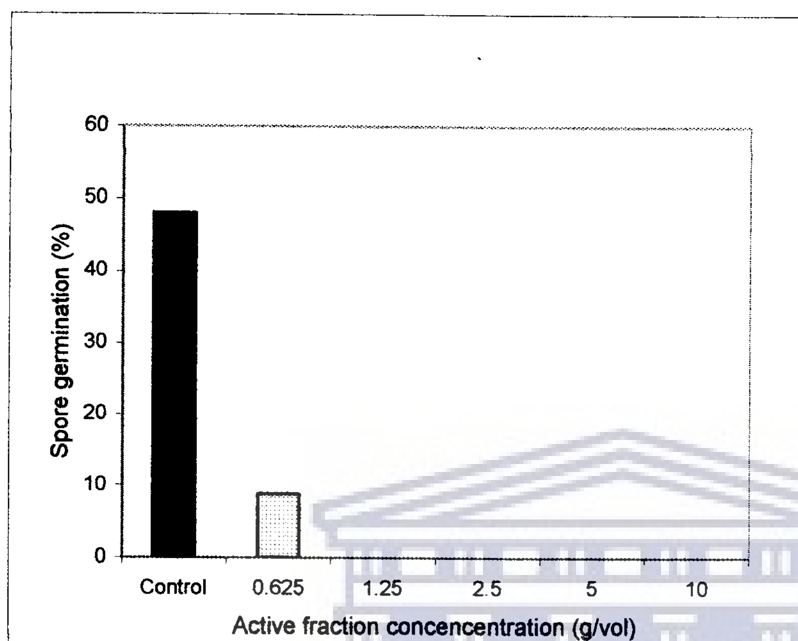

Figure 1. Mean percentage spore germination of *Alternaria* sp. at different concentrations of the active fraction after incubation in PDB at 27 °C.

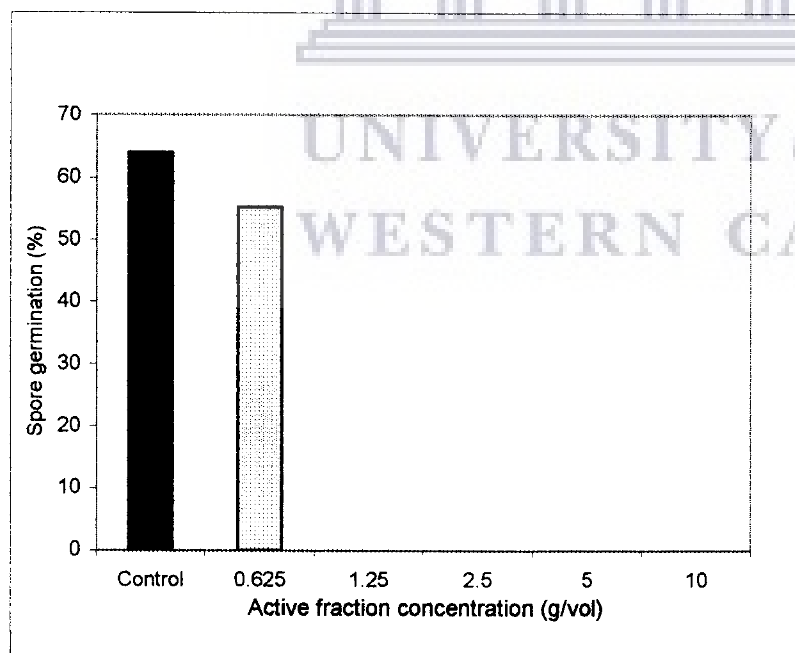

Figure 2. Mean percentage spore germination of *Botrytis cinerea* at different concentrations of the active fraction after incubation in PDB at 27 °C.

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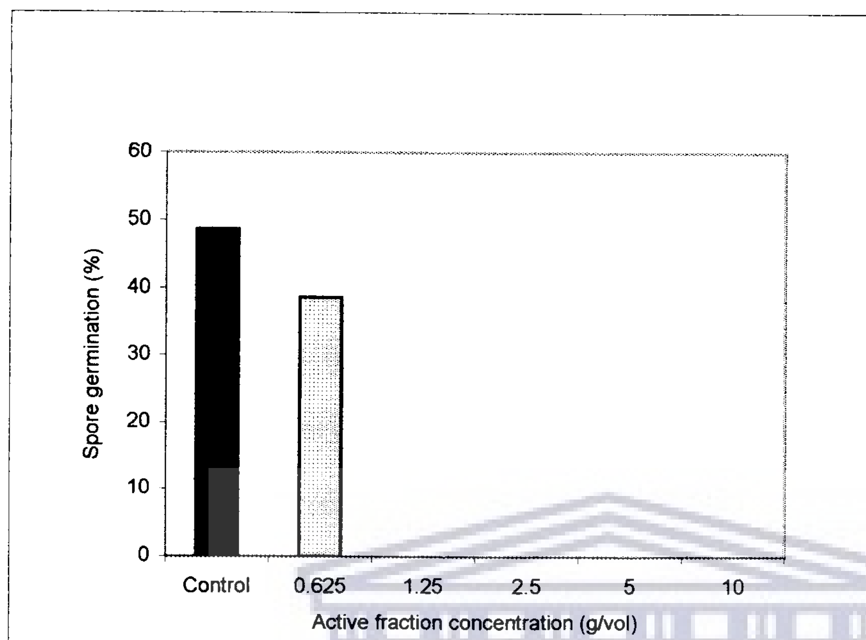

Figure 3. Mean percentage spore germination of *Cylindrocarpon* sp. at different concentrations of the active fraction after incubation in PDB at 27 °C.

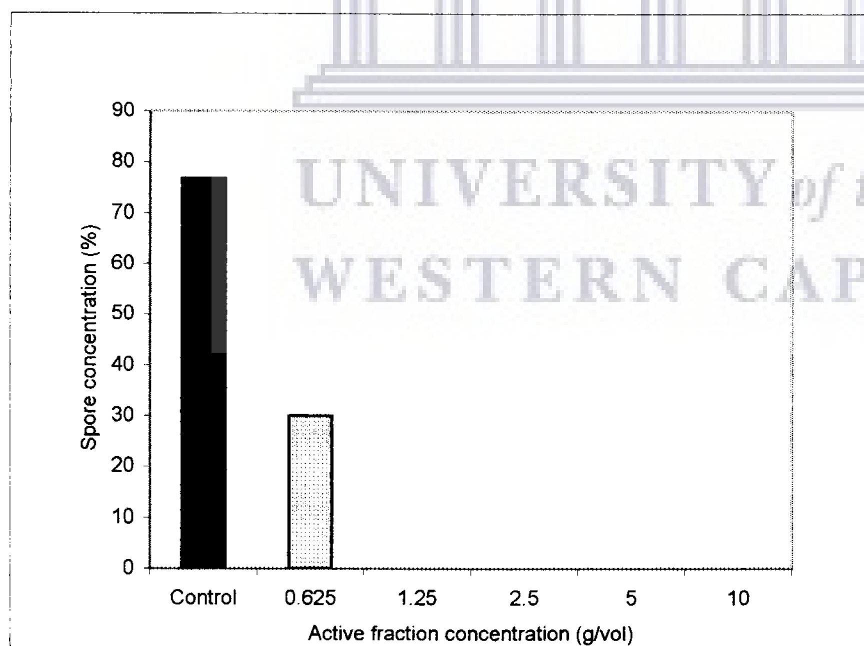

Figure 4. Mean percentage spore germination of *Fusarium verticillioides* at different concentrations of the active fraction after incubation in PDB at 27 °C.

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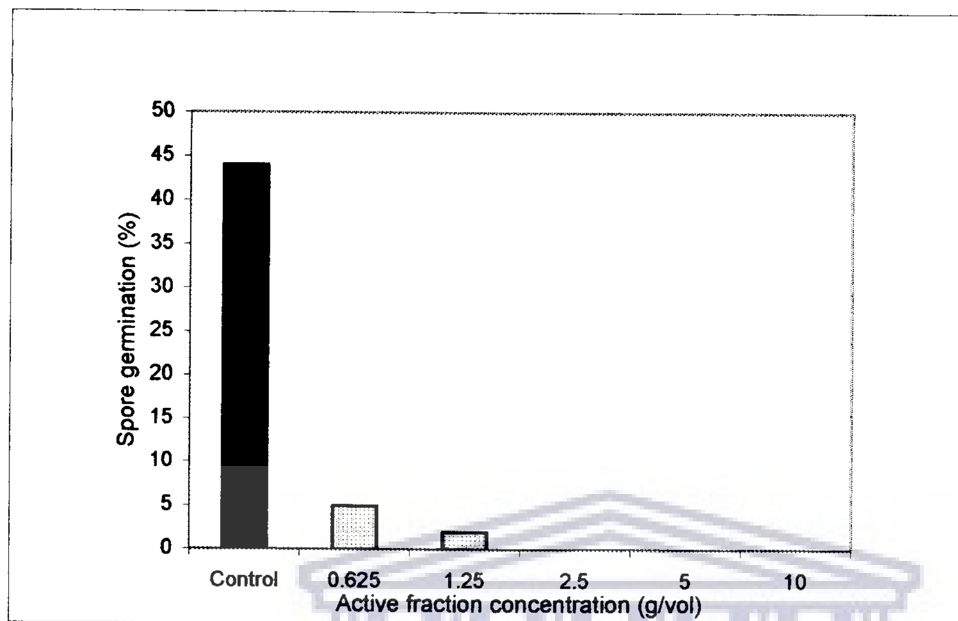

Figure 5. Mean percentage spore germination of *Penicillium expansum* at different concentrations of the active fraction after incubation in PDB at 27 °C.

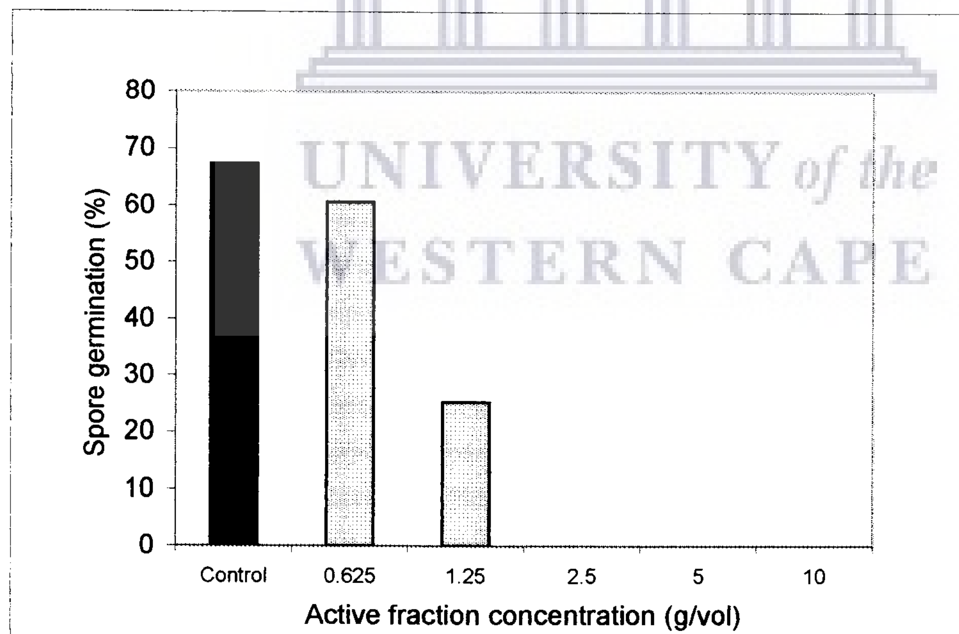

Figure 6. Mean percentage spore germination of *Phaeomoniella clamydospora* at different concentrations of the active fraction after incubation in PDB at 27 °C.

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Effect of the active compound on mycelial weight

The effect of the active compound on the mycelial weight of various fungal isolates grown in PDB for 14 days at 27 °C are presented in Table 3.

Table 3. Mycelial weight of various fungal isolates in potato dextrose broth (PDB) containing inoculum levels of 1 x 10^6 spores/ml and the active fraction of *Galenia africana* after 14 days of incubation at 27°C

	Active fraction concentration (g/vol)					
Fungi tested	Control[a]	0.625	1.25	2.5	5	10
	Mycelial weight (g dry wt)					
Alternaria sp.	0.3	0.2	0.2	0.1	NG[b]	NG
Botrytis cinerea	0.7	0.1	NG	NG	NG	NG
Cylindrocarpon sp.	0.3	0.2	0.2	NG	NG	NG
Eutypa lata	0.8	0.9	0.3	NG	NG	NG
Fusarium equiseti	0.2	0.2	0	0	NG	NG
Fusarium oxysporum	0.2	0.2	0.2	0.2	0.1	NG
Fusarium pseudograminearum	0.5	0.1	0.1	0	NG	NG
Fusarium solani	0.2	0.2	0.2	0.2	NG	NG
Fusarium verticillioides	0.3	0.3	0.2	0.2	0.2	NG
Penicillium expansum	0.2	0.2	0.1	0.1	0	NG
Phaemoniella clamydospora	0.1	0.1	0.1	0.1	NG	NG

[a] Control = without the active fraction.

[b]NG = No growth.

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The mycelial growth of all the fungal isolates tested were inhibited by the active compound at the highest concentration of 10 % of the active compound being used. *F. oxysporum*, *F. verticllioides* and *P. expansum* were the only fungal isolates of which the mycelial growth was not inhibited at a concentration of 5 %. At concentrations higher than 1.25 % and 2.5 % respectively, *B. cinerea* and *Cylindrocarpon* sp. were completely inhibited, indicating that these isolates are sensitive to higher concentrations of the active compound. The mycelial growth rate in control cultures for *F. equiseti, F. oxysporum*, *F. solani, F. verticillioides*, *P. expansum* and *P. clamydospora* were similar to the corresponding active concentration amended cultures. *E. lata* grows faster at a lower concentration (0.625 %) compared to the control but the growth rate decrease at a higher concentration (1.25 %) of the active compound. The mycelial growth of both *Alternaria* sp. and *F. pseudograminearum* were reduced at increasing concentrations of the active compound compared to the control, and at a concentration higher than 5 % both were completely inhibited.

The conidial germination bioassay was more sensitive in the detection of the antifungal activity of the active compound than the mycelial growth method. Since the MIC`s of the active compound to various fungi were defined on the basis of visible mycelial growth, this technique allowed the conidia to germinate and produce germ tubes for monitoring of the growth inhibition and fungicidal activity of the active compound. The MIC`s of the active compound for visible mycelial growth were comparable to those obtained from conidial germination. A concentration of 2.5 % of the active fraction is required to inhibit the mycelial growth of the *Alternaria* sp. compare to the germination bioassay where a concentration higher than 1.2 (g/vol) will completely prevent the germination of the *Alternaria* sp. The active compound completely inhibits the germination of *F. oxysporum* but a concentration of 10 % is required to inhibit the mycelial growth of the organism. The results of the germination studies clearly show strong antifungal activity of the active compound towards these fungal isolates. The antifungal assay allowed conidia to germinate almost synchronously, within short periods of time of incubation, to obtain reproducible results. Longer incubation could result in rapid growth of germ tubes, and hyphal or mycelial mass may increase rapidly.

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One of the concerns in the development of a standard method for the *in vitro* susceptibility testing of filamentous fungi is the nature of the inoculum. It has been shown that the MIC`s of antifungal agents for filamentous fungi are dependent on the nature and the size of the inoculum (Manavathu *et al.*, 1996; Guarro *et al.*, 1997; Keyser *et al.*, 1999). There are no universally accepted procedures for the determination of fungal susceptibilities. Variabilities in the test results are related to inoculum size, medium composition, medium pH, incubation temperature, incubation time and end point criteria (Natural Committee for Clinical Laboratory Standards, 1985). The use of conidia is therefore an attractive option in a study like this. This technique makes use of freshly harvested conidia which are allowed to germinate in the presence of the active compound. By allowing this it was demonstrated that the germination process was also affected by the active compound.

The results of our investigations strongly suggests that this extract possess remarkable antifungicidal activity, and is a good inhibitor of mycelial growth. In addition products of plant origin such as this one could be of great advantage to the user and public.

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MATERIALS AND METHODS

Fungal isolates. Alternaria sp., *Botrytis cinerea*, *Cylindrocarpon* sp., *Cylindrocladium*, *Fusarium equiseti*, *F. oxysporum*, *F. pseudograminearum*, *F. solani*, *F. verticillioides*, *F. trinctinum, Penicillium expansum* and *Phaemoniella clamydospora* were obtained from the culture collection of the Disease Management Division, Agriculture Research Council Infruitec-Nietvoorbij, South Africa. *Fusarium* sp. was maintained on carnation leaf agar (CLA) and the other fungi on potato dextrose agar (PDA). The plates were incubated at 25 °C in the dark whereas the CLA plates were incubated at 22 °C under a white fluorescent light with a 12:12 light:dark photoperiod for 7-14 days.

Preparation of spore suspension. The spores were harvested by adding 5 ml of sterile distilled water to each PDA and CLA plate and the conidia were aseptically dislodged with a sterile inoculating loop into the water. Spore suspension were aseptically filtered through two layers of sterile muslin cloth to remove mycelial debris. The spore suspensions were microscopically adjusted with the aid of a Neubauer haemocytometer to give a final concentration of approximately 1 x 10^6 spres/ml.

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Preparation of the bioactive compound. Powdered, dried plant material (200 g) was sequentially extracted with various solvents starting with hexane, chloroform, ethanol and sterile distilled water. Each concentrated extract was bioassayed. The major activity resided in the EtOH extract. The EtOH extract was purified using the silica gel column chromatography and thin-layer chromatography. Four fractions showed significant antifungal activity as evidenced by a clear zone of inhibition. One of the four fractions was used for further laboratory experiments. The active fraction which elutes as a single band from the silica gel (70-230 mesh) column using ethyl acetate-hexane (1:4) as eluent is a yellow-green semisolid soluble in ethanol, methanol, dichloromethane and tetrahydrofuran but insoluble in hexane and partially soluble in water. The active fraction (1 g) was dissolved in 1 ml of EtOH. The mixture was placed on a stirrer and 9 ml of distilled water was added slowly to it and left to stir until completely dissolved. The active fraction was filter-sterilized by using a Millipore filter (pore – size 0.22 µm,

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Corning Inc., Germany). A sterile dilution was made with dH_2O and EtOH to yield fraction concentrations of 0.625, 1.25, 2.5, 5, and 10 %, respectively.

Antifungal bioassay. Water agar plates were surface inoculated with 50 µl of the respective fraction concentration. The plates were allowed to dry in a laminar flow hood. The spore suspension (200 µl) was then evenly spread across the surface of the water agar plates and allowed to dry in a laminar flow. A control plate contains no active fraction but only dH_2O and EtOH (1:4). Conidia was allow to germinate in the presence of the active fraction and the plates were examined under the microscope. All experiments were stopped as soon as conidia on the control plate show signs of germination. Triplicate determinations were done. The percentage conidium germination was based on the random sampling of 50 conidia per fraction treatment. For each fraction treatment the germ tube length of the germinating conidia was measured. In the case of fungi with multi-celled conidia, a conidium was considered germinated if a germ tube was visible from at least one of the cells. Germ tube length of control and fraction treated conidia were measured under a microscope and an objective magnification of 10x was chosen. The germ tube length data given in the results represent the means for all tests done.

Culture conditions for inhibition tests of fungal growth. Tests for inhibition of fungal growth were assayed in flask cultures. Final spore suspensions (200 µl) containing 1 x 10^6 spores/ml were inoculated in 125-ml conical flasks containing 20 ml of potato dextrose broth (PDB). The active compound (50 µl) was added to each of the flasks containing mixtures of spores. Different concentrations (0.625, 1.25, 2.5, 5 and 10 %) of the active fraction were prepared and added to the flasks. The flasks not containing the active fraction served as the control. The flask cultures were then incubated for 14 days at 27 °C. Mold growth was observed visually throughout the incubation period, and mycelial mat weight was determined at the end of the incubation period. The mycelial mats were separated from culture broths by filtration and dried in a laminar flow hood for 16 h. The weight of the dried mycelial mats was then determined. Triplicate samples were taken for each assay.

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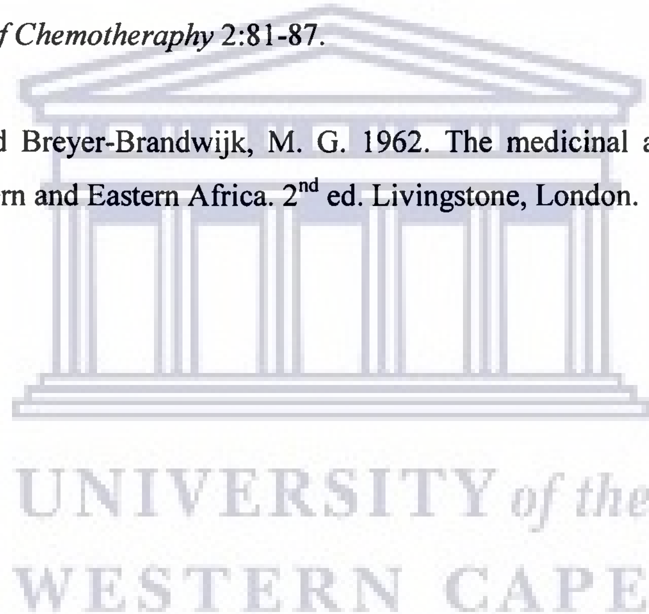
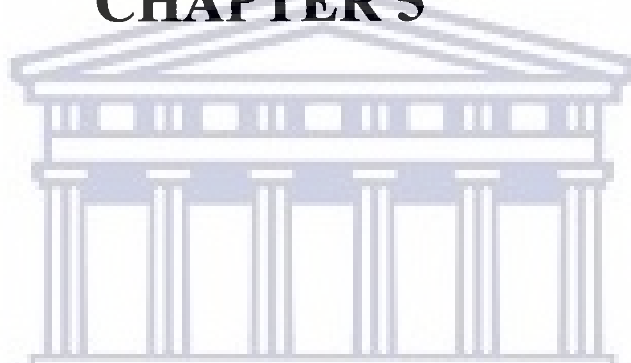


CHAPTER 5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THE CONTROL OF POSTHARVEST DECAY OF APPLES BY THE BIOACTIVE FRACTION FOUND IN *GALENIA AFRICANA*

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Chapter 5

The control of postharvest decay of apples by the bioactive fraction found in *Galenia africana*

Abstract

The antifungal bioactive fraction which was isolated from *Galenia africana* was assayed for its efficacy to control gray mold (incited by *Botrytis cinerea*) and blue mold (incited by *Penicillium expansum*) on wounded apples. Two types of treatments were used on Granny Smith apples. Firstly the bioactive fraction was applied to wounded fruits followed by spore inoculations. In the second treatment the spore suspension was inoculated first followed by the application of the bioactive fraction. This was done at different concentrations ranging from 0.5 to 2 %. The higher concentrations of the bioactive fraction provided control of both diseases on apples. *B. cinerea* gray mold was effectively controlled at the highest concentration of the bioactive fraction on apples than *P. expansum*.

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INTRODUCTION

Postharvest diseases are among the most important causes of losses and reduction of quality in fruits and vegetables. Most postharvest fungal decay on fruits is initiated at wounds that occur during harvest or packing. Subsequent infection occurs at the wound by one of several pathogens (Spotts *et al*., 1998 ; Sugar and Spotts, 1993). *Penicillium expansum* and *Botrytis cinerea* (causal agents of blue mold and gray mold, respectively) are relatively aggressive, fast-growing pathogens (Benbow and Sugar, 1999). Fungicides are routinely used to control these diseases after harvest (Bertrand and Saulie – Carter, 1979; Daines and Snee, 1969; Spalding, 1970; Spalding *et al*., 1969; Tepper and Yoder, 1982). However, the development of resistance by the pathogens and the recent association of health hazards with some fungicides used for postharvest treatment of fruit have necessitated the search for replacement fungicides and alternative non-chemical control methods. Postharvest treatment of fruit with biocontrol agents has emerged recently as a potential alternative disease management tool. Some essential oils components inhibit spore germination and mycelial growth of common postharvest pathogens (Vargas *et al*., 1999; Tsao and Zhou, 2000; Mishra and Dubey, 1994). Ark and Thompson showed that garlic extracts contains a potent fungicide. At a dilution of 10 % of a garlic extraction the spore germination of *B. cinerea* was completely inhibited after 24 h and 48 h (Wilson *et al*., 1997). Antagonistic yeasts and bacteria isolated from fruit surfaces were shown to have a broad spectrum of activity against a number of postharvest pathogens on a variety of fruit (Wilson and Chalutz, 1988). An antagonistic yeast (*Cryptococcus albidus*) is commercially available in South Africa under the trade name YIELDPLUS.

Galenia africana belonging to the family Aizoaceae is a dominant plant throughout Namaqualand (Kellerman *et al*., 1988). The plant is especially abundant in disturbed areas around kraals, along roads and on trampled veld. It is an aromatic woody perennial sub-shrub, growing 0.5-1 m high, and having small green leaves, which turn yellow with age. It can be the only remaining species after the veld has been heavily overgrazed (Van der Lugt *et al*., 1992). Webber *et al*. (1999) suggested that before eradication of these plants from the veld their alternative use should be considered so that a farmer could

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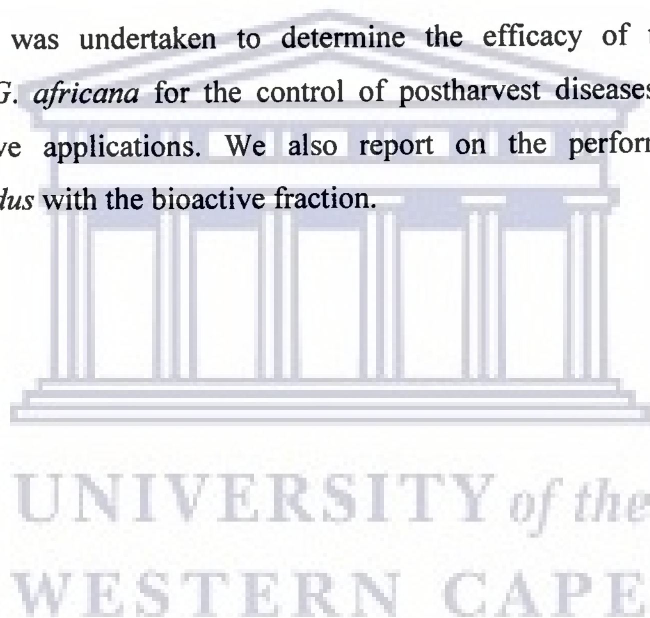
benefit or receive an income. Unpalatable plants may be useful as pesticide agents against different pests or diseases of agricultural importance (Dupuis *et al*., 1972).

Extracts from *G. africana* was screened for activity against microorganisms (especially fungi) causing economic important diseases to fruit trees in South Africa. The results indicate that the EtOH has strong inhibitory activity *in vitro* against fungi. The EtOH extract was purified using the silica gel column chromatography and thin layer chromatography. The active fractions showed significant antifungal activity as evidenced by a clear zone of inhibition.

This investigation was undertaken to determine the efficacy of the antifungal bioactive fraction of *G. africana* for the control of postharvest diseases of apples as protective and curative applications. We also report on the performance of the combination of *C. albidus* with the bioactive fraction.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RESULTS AND DICUSSION

Analysis of variance showed that the different types of treatment significantly ($P<0.0001$) affected lesion development for protective and curative treatments of fruit inoculated with *B. cinerea* and *P. expansum* (Table 1). The 1, 1.5 and 2 % bioactive fraction of *G. africana* reduced *Botrytis* significantly more than the control and *C. albidus* treatments when applied before inoculation (Table 2). Increasing the fraction concentration from 1 % to 2 % resulted in no significant difference in efficacy. Botrytis fruit decay of apples treated with the combination of 1 % fraction and *C. albidus* was not significantly different than decay in the fraction alone. The combination 0.5 % fraction and *C. albidus* give significantly better control than the 0.5 % fraction alone but this was significantly less than control provided by the yeast alone. The bioactive fraction of *G. africana* does therefore not enhance the efficacy of *C. albidus* against *Botrytis* decay on apples

For *Penicillium* decay the 2 % fraction alone and in combination with *C. albidus* provides significant protection against infection compared to the controls and the yeast alone (Table 2). Application of the 1.5 % and 2 % bioactive fractions after inoculation (curative) was significantly more effective in controlling *Botrytis* decay then the controls, yeast alone and 0.5 % and 1 % fraction and yeast combinations (Table 3). The 2 % fraction alone and *C. albidus* alone treatments give similar efficacy results against *Penicillium* decay, which was significantly less than the controls.

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In the present study we demonstrated that the antifungal bioactive fraction of *G. africana* has both protective and curative effects against gray and blue mold of apple. When applied prior to inoculation with a pathogen the 1 % fraction was effective against *B. cinerea* and the 2 % against *P. expansum*. When applied after inoculation the 1.5 % fraction was effective for *B. cinerea* and the 2 % fraction for *P. expansum*.

The bioactive fraction of *G. africana* did not enhance the protective or curative activity of the yeast in this study. Currently available microbial antagonists confer only protective effects that diminishes with ripening and provides no control of previously

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established infections (Roberts, 1990). Biological products, in order to be commercially acceptable, must protect wounds and control incipient infections in a manner similar to synthetic fungicides. Although the bioactive fraction did not enhance the effect of the yeast, the results indicate that it will also not have a negative effect on decay control when applied in combination. In combinations therefore, the bioactive compound could control previously established infections while the antagonist confer a long term protective effect.

Chemical studies of the bioactive fraction of *G. africana* identified 5,7 – dihyroxyflavanone **2** as a major component of this fraction. Numerous investigations have established the remarkable antifungal properties of essential oils; particularly flavonoids and isoflavonoids components (Tomás - Barberán *et al.*, 1988; Vargas *et al.*, 1999; Krämer *et al.*, 1984). The search for new and more bioactive natural flavonoids is warranted, these compounds are generally recognized as safe by the Food and Drug Administration (FDA), and thus will meet the new requirements for safe and effective control agents for use in postharvest treatment of fruits.

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Table 1. Summary analysis of variance for lesion development on apples cv Granny Smith, wounded and treated with *Galenia africana* antifungal fractions, yieldplus (*Crytococcus albidus*) or combinations of the two, inoculated with conidia of *Botrytis cinerea* or *Penicillium expansum*

	Botrytis cinerea			*Penicillium expansum*		
Source of variation	df	MS	*P>F*	df	MS	*P>F*
Protective treatments [b]						
Treatment concentrations	8	3309.7	0.0001	8	1032.3	0.0001
Error	261	68.6		261	14.2	
R^2		0.60				
Curative treatments [c]						
Treatment concentrations	8	819.0	0.0001	8	292.0	0.0001
Error	261	150.5		261	18.7	
R^2		0.14			0.32	

[a] df = Degrees of freedom, MS = mean square, $P > F$ = probabilities associated with individual F test. Mean squares were derived from the sum of squares for the general linear model and complete randomized design of treatments.

[b] Protective treatment = the bioactive fraction was applied to the wounds on the apple fruits and then the spore inoculation followed.

[c] Curative treatment = the spore suspension was inoculated first followed by the application of the bioactive fraction.

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Table 2. Protective effect of different concentrations of the *Galenia africana* bioactive fraction alone and with combinations of yieldplus (*Cryptococcus albidus*) on decay of apple cv. Granny Smith caused by *Botrytis cinerea* and *Penicillium expansum*

	Infected fruit lesion diamter (mm)[a]	
Treatment	*Botrytis cinerea*	*Penicillium expansum*
Control (dH_2O)	23.9 a	26.8 a
Control (ethanol 1 %)	22.2 a	21.2 a
0.5 % fraction	25.2 a	-[b]
1 % fraction	1.8 d	25.2 abc
1.5 % fraction	1.6 d	23.8 bcd
2 % fraction	1.2 d	9.2 g
0.5 % fraction + yieldplus	12.5 b	-
1 % fraction + yieldplus	1.1 d	23.4 cd
1.5 % fraction + yieldplus	-	21.9 e
2 % fraction + yieldplus	-	13.9 f
Yieldplus (0.75 g/100 ml)	6.4 c	25.7 ab

[a] Means are averaged values of an experiment with three replicates of 10 fruits each per treatment. Values followed by the same letter are not significantly different at P = 0.05, according to tests (LSD) for diameter.

[b] Treatment not included.

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Table 3. Curative effect of different concentrations of the *Galenia africana* bioactive fraction and with combinations of yieldplus (*Cryptococcus albidus*) on decay of apple cv. Granny Smith caused by *Botrytis cinerea* and *Penicillium expansum*

	Infected fruit lesion diamter (mm)[a]	
Treatment	*Botrytis cinerea*	*Penicillium expansum*
Control (dH_2O)	17.5 bcd	25.6 ab
Control (ethanol 1 %)	23.8 a	25.9 a
0.5 % fraction	23.1 ab	-
1 % fraction	13.9 de	23.6 bc
1.5 % fraction	9.2 e	21.9 dc
2 % fraction	9.7 e	17.8 e
0.5 % fraction + yieldplus	20.3 abc	-
1 % fraction + yieldplus	16.6 cd	21.7 cd
1.5 % fraction + yieldplus	-	21.2 d
2 % fraction + yieldplus	-	20.5 d
Yieldplus (0.75 g/100 ml)	1.64 cd	16.7 e

[a] Means are averaged values of an experiment with three replicates of 10 fruits each per treatment. Values followed by the same letter are not significantly different at P = 0.05, according to tests (LSD) for diameter.

[b] Treatment not included.

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MATERIALS AND METHODS

The pathogens: *P. expansum* and *B. cinerea* were obtained from the culture collection of Disease Management Division, Agricultural Research Council Infruitec-Nietvoorbij, South Africa. Both isolates are highly pathogenic to apples. The organisms were maintained on potato-dextrose agar (PDA) at 25 °C in the dark under a white fluorescent light with a 12:12 light:dark photoperiod for 7 – 14 days. Conidial suspensions were prepared by washing the colonies growing on PDA with 2 ml of sterile distilled water. Aliquots were then collected and diluted with water to the required concentration (1 x 10^4 spores/ml) as determined with a haemocytometer.

The fruit: Granny Smith apples were purchased from a local packing house. Prior to inoculation, fruit surfaces were lightly sprayed with 70 % ethanol and allowed to dry.

Treatments: Each fruit was then wounded in three locations with a sterile steel finishing nail. The resulting wounds were approximately 3 mm deep and 3 mm in diameter. EtOH (1 ml) was added to 0.2 g of the bioactive fraction and put on a stirrer until completely dissolved. Distilled water (9 ml) was added slowly to the mixture and left to stir until it was completely dissolved. Thereafter two-fold serial dilutions was prepared. The controls was distilled water and ethanol separately. Each of the three wounds on the Granny Smith apples was inoculated first with 15 µl of a spore suspension containing 1 x10^4 conidia of *P. expansum* or *B. cinerea* per milliliter. The spore suspension was allowed to dry for 2 hr. The bioactive fraction (15 µl) were then pipetted into wounds of apples at various concentrations (0.5 %, 1%, 1.5 % and 2 % g/vol). The bioactive formulation (15 µl) was applied to a set of wounds first which serve as a protective application. The fraction was allowed to dry for 2 hr and the spore suspensions (15 µl) was then pipetted into the wounded apple. For the second type of application, the curative treatment, the bioactive formulations was applied 2 hr after inoculating the wounds with a spore suspension. The control efficacy of different concentrations of the bioactive fraction in combination with yieldplus (*C. albidus*) was investigated. Apple fruit were wounded as described above. A 15 µl drop of a suspension (0.75 g/100ml dH_2O) of *C. albidus* was placed in each wound

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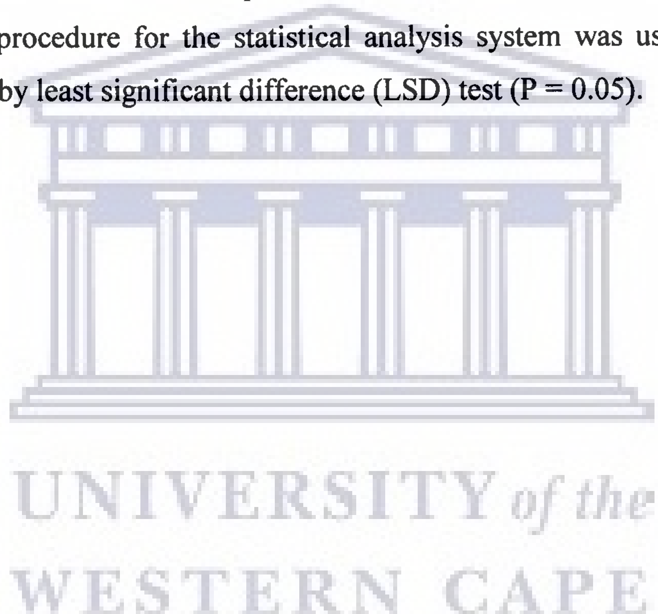
followed by a 15 µl drop of a formulation containing either 0.5, 1, 1.5 or 2 % (wt/vol) of the bioactive fraction and allowed to dry. After 2 hr of treatments wounds were inoculated with 15 µl of a conidial suspension of *B. cinerea* or *P. expansum*. Each treatment was applied to three replicates of 10 fruit. The fruits was placed on polystryrene or cardboard fruit tray-packs with the wounded face up in the fruit boxes. Fruit from each treatment were placed on separate trays in separate boxes. There was ten apples per treatment, and each treatment was repeated three times. The boxes was stored at 22°C for 7 days. At the end of the incubation period diameters of lesions were measured.

Data analysis. Analysis of variance was performed on the data on lesion diameter. The general linear model procedure for the statistical analysis system was used. Treatment means were separated by least significant difference (LSD) test ($P = 0.05$).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CHAPTER 6

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ASPECTS OF THE STRUCTURES COMPRISING THE ACTIVE FRACTION

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Chapter 6

Aspects of the structures comprising the active fraction

It must be clearly understood that at the outset of this work, the elucidation of the structure of the active agent isolated from *Galenia africana* was not be to be the focus of the study and would only form a very minor part of the investigation. Consequently the preliminary findings about some of the constituents present in the active fraction will be presented. The active fraction which elutes as a single band from the silica gel (70 – 230 mesh) column using ethyl acetate-hexane (1:4) as eluent is a yellow-green semisolid soluble in ethanol, methanol, dichloromethane and tetrahydrofuran but insoluble in hexane and partially soluble in water. Attempts to obtain crystalline material from a mixed solvent system viz., ethylacetate-hexane or dichloromethane-hexane failed and this suggested that the active fraction comprised more than one compound.

Rechromatography of the material on a long slow column using ethylacetate-hexane (1:5) eluent spread the band as it eluted. The yellow band that eluted was divided into two major fractions viz the first half to elute called fraction A and the latter half called fraction B. Both fractions have been re-crystallized from chloroform to give yellow crystals of impure material once again. Fraction A had a m.p.133-136 °C while fraction B had a m.p.143-148 °C. The infrared spectra run as Nujol mulls for both fraction A and B indicated broad bands at 3100-3500 cm^{-1} for hydroxyl groups as well as strong carbonyl stretching frequencies at 1644 cm^{-1} for conjugated systems. Before any sense could be made from the ^{1}H-n.m.r. spectra run in deutereochloroform at 25 °C on a Varian 200 MHz spectrometer due the fact that the samples where impure, it was considered appropriate to subject the samples to a Gas Chromotography-Mass Spectroscopy analysis. From the data produced by this latter analysis, one could look at the ^{1}H-n.m.r. spectra to see if the expected peaks are indeed observed for possible compounds identified from the GC-MS analysis.

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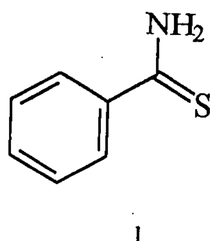
The GC-MS of fraction A showed that it comprised 3 major component. An initial minor component was identified from a library search in the QMD 1000 GC-MS as being benzenecarbothiamide **1**.



1

Since there is a large area of aromatic proton signals in the region between 7.0 and 8.0ppm in the ^{1}H-n.m.r. spectrum, the structure is acceptable. In addition, there are two exchangeable broadish peaks at 5.85and 5.95ppm. These could however be due to phenolic hydrogens as well. The second component, the major one has been identified as a 5,7-dihydroxyflavanone **2** from a search in the GC-MS library.

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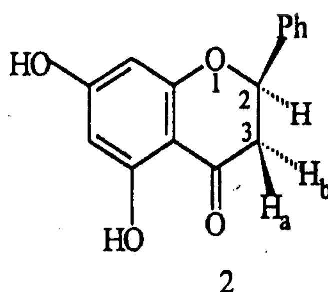

2

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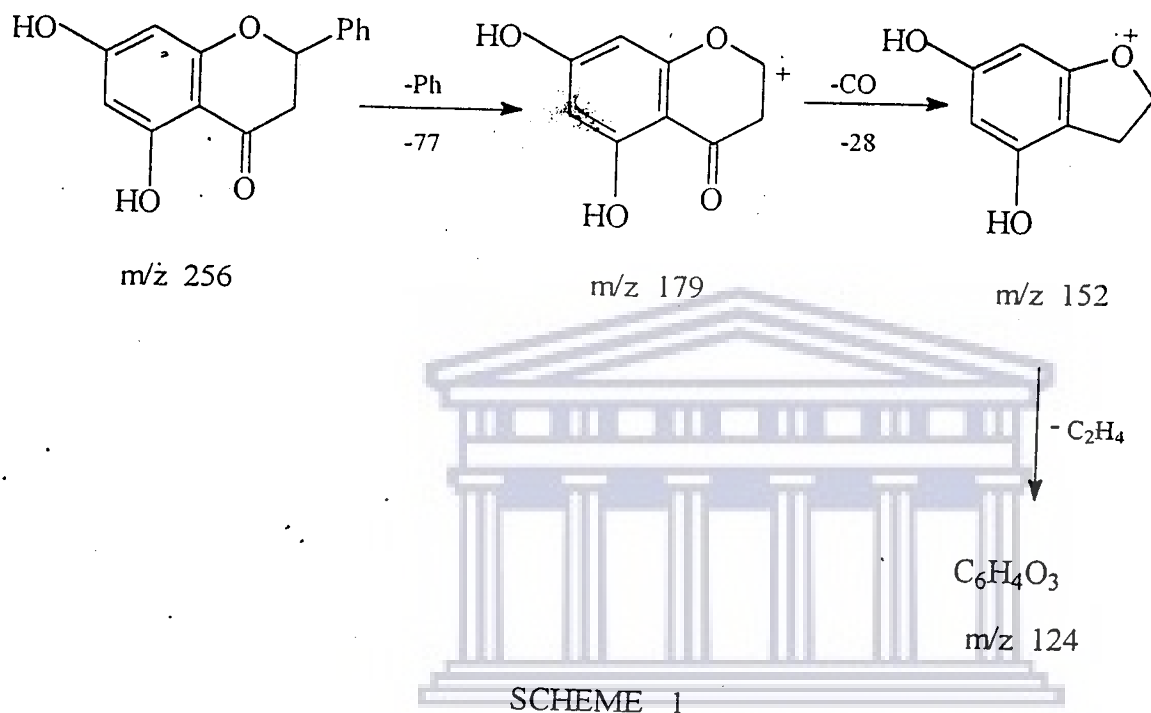
The molecular ion appears at m/z 256 with the first major fragmentation being the lost of the phenyl group to form the m/z 179 ion.. The fragmentation pattern is depicted below in Scheme 1 for the major fragments.



SCHEME 1

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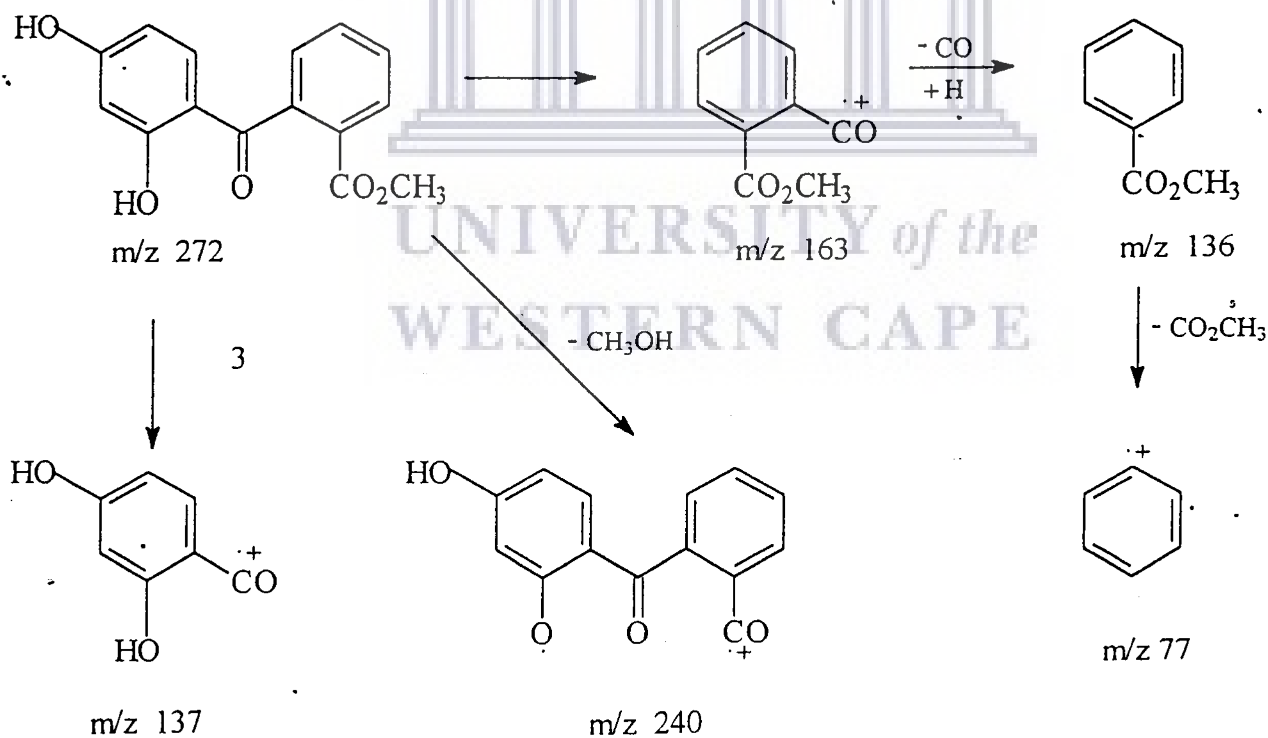
A 1-proton doublet of a doublet signal at 2.83ppm in the ^{1}H-n.m.r. spectrum is assigned to the 3-Hb proton with J 17.2 and 3.2Hz showing geminal or ^2J coupling to 3-Ha of 17.2Hz and syn-cis coupling of 3.2Hz to 2-H. Another 1-proton doublet of a doublet signal at 3.10ppm is assigned to the 3-Ha proton J 17.2 and 12.8Hz again demonstrating geminal coupling to the 3-Hb of 17.2Hz and with trans coupling to 2-H of 12.8Hz. The corresponding signal for 2-H appeared as a doublet of a doublet at 5.43ppm with the larger coupling of 12.8Hz to 3-Ha and the smaller coupling of 3.2Hz to 3-Hb. The 2-phenyl group appeared as a multiplet at 7.43ppm while 6-H and 8-H overlapped with peaks at 7.60 and 7.87ppm respectively. The 5- and 7-OH signals could not be assigned due to the complex nature of signals in the aromatic region between 7.0 and 8.0ppm and D_2O washing was not done since the fraction was not pure and ambiguous results could arise.

The third component which the GC-MS identified from its library search was the κeto ester **3**. A brief attempt at the major fragmentation patterns is presented below in Scheme 2.



SCHEME 2

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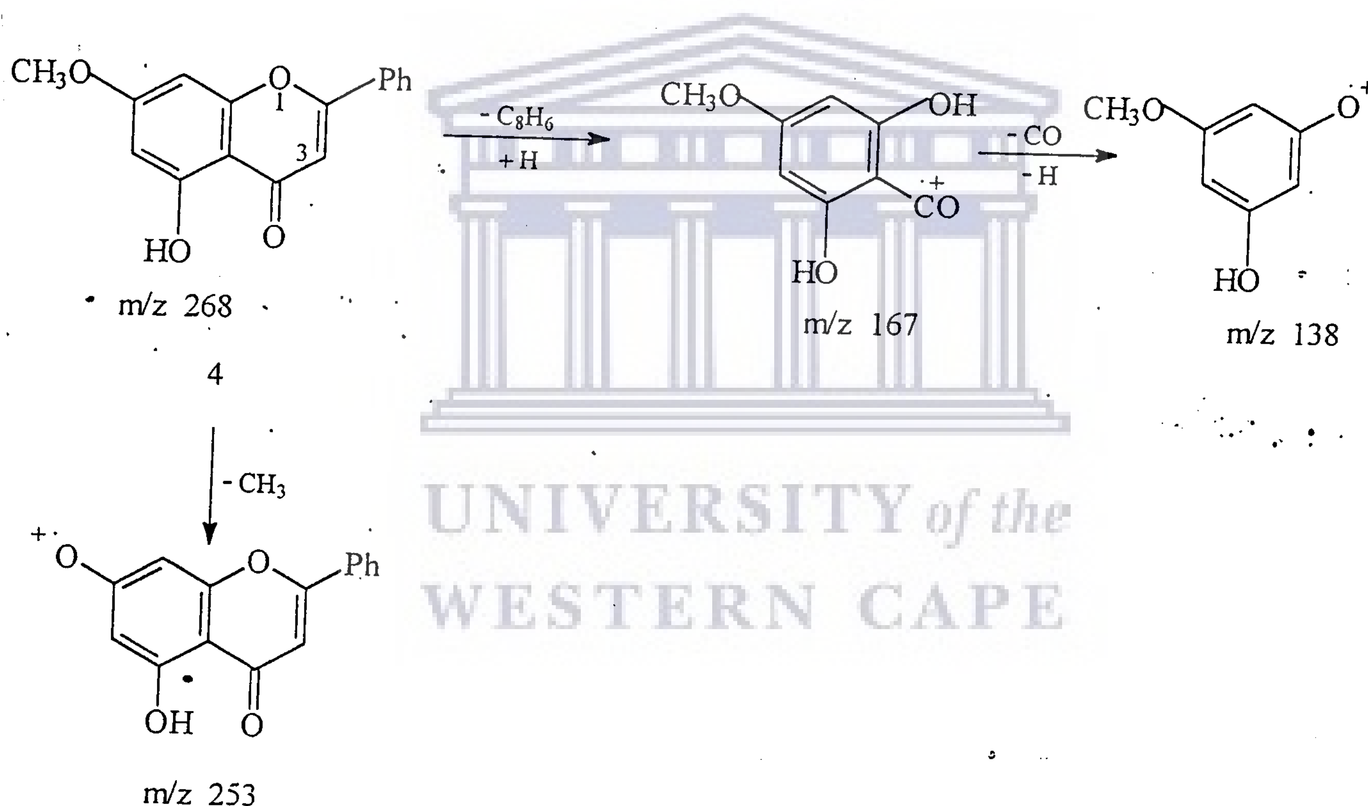
One of the diagnostic peaks one would expect to find for **3** in the ^{1}H-n.m.r. spectrum would be the methyl group in the benzoate ester. Indeed there is a strong signal at 3.81ppm to support the above assignment. For the rest there are abundant signals in the aromatic region.

GC-MS analysis of fraction B showed that it comprised of four major fractions. The first minor fraction is again assigned to benzenecarbothiamide **1** as found before and the second fraction has been assigned to ester **3** also found in fraction A. The third fraction was assigned to benzopyran-4-one **4** upon comparison of the fragmentation pattern of its mass spectrum in the library of the GC-MS. A brief fragmentation pattern of this component is presented in Scheme 3 below.



SCHEME 3

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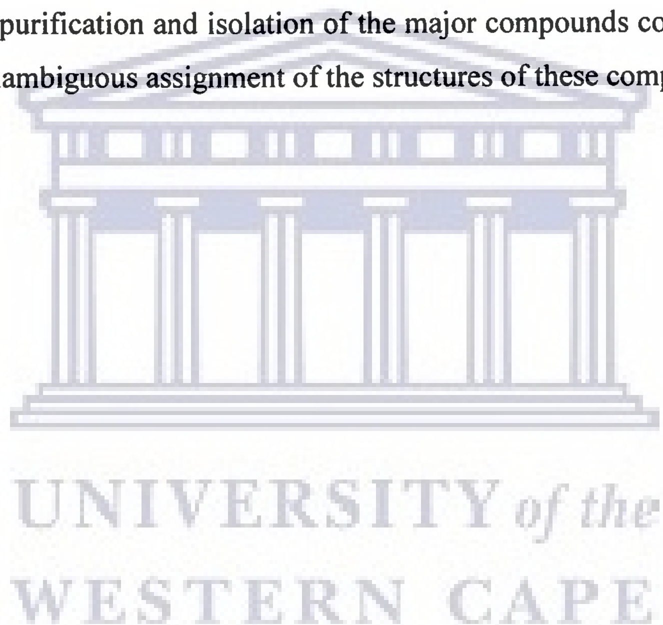
Again two diagnostic peaks one would expect to observe in the ^{1}H-n.m.r. spectrum would be the 7-CH_3O and the vinylic 3-H peaks. The peak at 3.81ppm which is a broadish signal assigned earlier to the ester CH_3O of compound **2** could also be due to the 7-CH_3O overlapping within this signal. Sharp signals at 6.47ppm which is where one might expect the 3-H to appear would support the structural assignment thus far. The fourth minor fragment could not be identified by a library search in the GC-MS and thus was not investigated further.

Thus from a very superficial analysis of the compounds contained in the active fraction, four have been tentatively identified. Clearly a great deal more work will have to be done in the proper purification and isolation of the major compounds contained in this fraction to make an unambiguous assignment of the structures of these compounds.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