CHAPTER 3

ANTIFUNGAL ACTIVITY OF *HELICHRYSUM* SPECIES

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3.1 Introduction

Fungi differ from bacteria in possessing a number of chromosomes within a well-defined nuclear membrane, mitochondria and an endoplasmic reticulum. Like plants they have definite cell walls, but these are usually composed of chitin rather than cellulose. They lack chlorophyll, so they live either on dead organic material as saprophytes, or on living organic matter as parasites. The cells may live separately (yeasts) or more commonly, they form long multicellular filaments or hyphae which may contain cross-walls or septa. A mass of hyphae is a mycelium. Many species have both yeast and mycelial forms which are dependent on the cultural conditions, a process known as dimorphism (Alexopoulos *et al.*, 1996).

The classification of fungi is based on the form of their sexual reproductive apparatus but there is a large group, containing most of the human parasites, which have never been known to undergo sexual reproduction. These are the fungi imperfecti or deuteromycetes. Where the perfect stages has subsequently been identified, members of this group have been found to be either Ascomycetes or Basidiomycetes (Alexepoulos *et al.*,1996).

3.1.1 Fungi and man

Parasitic fungi cause many different diseases, which may be superficial, subcutaneous or deep inside man and animals. In the superficial mycoses, the fungus is limited to the horny layer of the skin and to structures derived from it, while in the subcutaneous and deep mycoses there is a deeper invasion of the tissues (Laks and Pruner, 1989, Kwon-Chung and Bennett, 1992). Generally speaking, fungal infections of humans are most common in tropical regions of the world. However, in recent years the number of individuals infected with fungi have increased drastically in all regions of the world. This is due primarily to the fact that there are more individuals who are predisposed to fungal infection than ever

before. Individuals with compromised immune systems (including HIV/AIDS patients) are most at risk.

Some species possess enzymes, which digest keratin. Geophilic species normally inhabit the soil and occasionally colonize animal hair and infect man. Zoophilic species are primarily parasitic on particular animal host species but can also infect many others, including man. Anthrophilic species are adapted for living on man and only occasionally infect other species. A well-adapted species such as *Trichophyton rubrum* evokes very little inflammatory reaction from the host, and its presence is therefore tolerated for very long periods (Kwong-Chung andBennett, 1992). Poorly adapted species, geophilic or zoophilic, evoke a fierce inflammatory response and the infection is eventually terminated (Kwong-Chung and Bennett, 1992).

There are a wide variety of clinical diseases produced by the filamentous, septate fungi of the genus Aspergillus. *A. fumigatus* is most commonly involved in human infections, followed by *A. niger* and *A. flavus* (Kwon-Chung and Bennett, 1992). These organisms are widely distributed in nature, being found in soil, vegetation, grain and mouldy hay. Aspergillosis is also a common infection of birds. In human systemic infections there is lung invasion with tissue destruction and a purulent granulomatous reaction (Kwon-Chung and Bennett, 1992). Aspergillus strains are important as they are responsible for most human systemic infections.

3.1.2 Epidemiology

Some species are cosmopolitan, while others are strictly limited to certain parts of South Africa. In zoophilic species, this may reflect dependence on a particular animal host, but it is not clear why many human pathogenic fungal species have a restricted distribution (Tomas-Barberan *et al.*,1990, Alexopoulos *et al.*, 1996). Changes in distribution have taken place in the last century. Some species have a particular affinity for one site. *Tinea pedis* (toe clefts), *T. manuum* (hands), *T. cruri* (intertrinous parts of the groin), *T. barbae* (beard). *T. corporis* (glabrous skin), and *T. unguium* (nail) are typical examples.

3.1.3 Fungi and plants

Fungi are, tremendously important to humans because of the plant diseases they cause. Most species of plants are subjected to attack by a number of different types of fungal pathogens. The consequences of such infections are varied but range from widespread death of individuals of a particular species to the development of insignificant symptoms associated with little, if any, damage to the host. In addition to serious economic losses caused to agronomically important species, fungi have literally altered the course of history and have affected social customs on both a regional and global scale (Alexopoulos *et al.*, 1996). Many fungi such as *Penicillium digitatum*, *Phytophthora citophthora*, *Aspergillus niger* and *Cladosporium cladosporioides*, are well known as major pathogens causing decay in fruit, vegetable and other agricultural products, especially during storage (Adikaram *et al.*, 1992). *Phytophthora infestans* causes a severe disease of potatoes known as ‘ late blight’. It not only kills the foliage but also infects the tubers, causing them to rot rapidly. Examples of a number of classical fungal diseases include: corn smut, black stem rust of wheat, foolish seeding disease of rice, ergot of rye, club root of crucifers and Dutch elm disease and Chestnut blight (Alexopoulos *et al.*, 1996), to mention a few.

Plant pathogenic fungi are, of course, not limited to desirable or agronomically important hosts. In fact, one understudied aspect of ecology is the influence of fungal pathogens on natural plant populations. All types of plants are attacked by parasitic fungi including weedy species that can pose serious problems to farmers, golf course managers, individuals involved in commercial lawn and landscape businesses, and individual home owners who want weed free yards and gardens. In this regard, scientists are actively involved in the development of certain plant pathogenic fungi as biological control agents for weeds (Kwong-Chung and Bennett, 1992).

Not all fungi associated with higher plants are detrimental to the plants. The hyphae of some fungi form specialized organs in the roots of plants known as mycorrhizae (Alexopoulos *et al.*, 1996). These structures provide significant benefits to both the fungi

and the host plants involved. An astonishing diversity of fungi known as endophytes has also been shown to be present in the leaves and stems of healthy plants ranging from conifers to grasses (Alexopoulos *et al.*, 1996). Many of these fungi appear to protect their hosts from pathogenic bacteria and fungi as well as insects and grazing mammals. Unfortunately several popular forage grasses such as *Festuca arundinacea* and ryegrass (*Lolium perenne*) often contain endophytes that produce such high levels of physiologically active alkaloids that they are toxic to domestic mammals, causing alarming physical and behavioural disorders (Clay, 1989). Fungi are also known to colonize optical instruments resulting in extensive and costly damage.

3.1.4 Exploitation of *Helichrysum* species for new antifungal agents

Plants have been used to treat human, animal and plant diseases from time immemorial. In traditional medicine, this empirical knowledge belongs to societies in general where those plants are found, or to a limited group of people, such as a family. Generally, only a few individuals inherit such knowledge from traditional healers and pass it from one generation to another, using their knowledge to improve the well-being of their kin.

As a result of the increasing need for new and better drugs to heal diseases, researchers from different disciplines are jointly attempting to study rationally and scientifically the resources of medicinal plants. This process includes the use of plants in their crude form or as starting material for drugs. However, the research focus differs from researcher to researcher and from country to country, due to differences that prevail in technological development and scientific level between countries. Whichever type they belong to, the starting point for their investigation generally follows the same intellectual process based on ethnopharmacology, or, on data from the literature (Cragg *et al.*, 1994). We can control many human and animal pathogens by currently available antibiotics. However, the need for new antibiotics still exits. For example, systemic infections caused by fungi, especially in patients with impaired host defence mechanisms, have become increasingly serious. Various antifungal agents have been explored, but the control of many of the fungal diseases has not yet been achieved. This study examines the role of *Helichrysum* species as another source of antifungal agents.

3.2 Material and Methods

3.2.1 Plant material

Shoots of *Helichrysum* species were collected from the Drakensberg in the Mont-aux-Sources area in Qwaqwa. A taxonomist, Prof. R.O. Moffett verified their identity and voucher specimens were deposited in the herbarium of the Department of Botany, University of the North, QwaQwa Branch, South Africa and the National Botanical Institute herbarium, Pretoria.

3.2.2 Preparation of extracts

Shoots (excluding flowers) of the plants were air dried at room temperature. Each plant (80g) was shaken for five minutes in acetone and filtered through Whatman No 2 filter paper under suction to obtain the 'shaken extract'. The residue was then homogenized in acetone, and also filtered through Whatman No 2 filter paper under suction. Both extracts were concentrated to dryness under reduced pressure at 45^0C with a rotary evaporator. After determining the yields, extracts were stored at 4^0C until further use.

3.2.3 Fungal strains

Six fungal species (Table 3.1) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar (Biolab) and an inoculum was recovered for testing by growth on a potato dextrose nutrient agar (Biolab) for 24 hours.

3.2.4 Antifungal bioassay

The plant extracts (sterilized by filtering through a 0.22 mm filter) were added to 5 ml of nutrient agar medium in Petri dishes and swirled carefully before congealing. An aliquot of each extract was serially diluted (ten fold) to obtain a concentration range of 1.0 to 0.01 mg/ml in acetone. A negative blank containing only nutrient agar and a control containing nutrient agar and 2% acetone served as controls (Meyer and Afolayan, 1995). The prepared plates were inoculated with disks obtained from actively growing margins of the fungi plates (that is, before spore formation) and incubated at 25^0C in the

dark for two days. Plates were examined after 24 and 48 hours and complete suppression of growth was required for the extract to be declared bioactive. Three replications were used per treatment.

3.3 Results

Results of bioassays are summarised in Table 3.1. Of the 28 crude extracts tested, 27 (96.4%) showed varying degrees of antifungal activity. 21 (75%) extracts inhibited growth of all organisms tested, and in addition, six of these showed high activity in the bioassays, at 0.01 mg/ml, the highest dilution used in this investigation. The epicuticular extract of *H. pilosellum*, and 9 (32%) homogenized extracts did not show significant antifungal activity. Sixty-eight per cent of the homogenized extracts exhibited significant antifungal activity at the concentrations tested in this study. Results of the bioassays are summarized in Table 3.1. Of the 28 crude extracts tested, 27 (96.4%) showed varying degrees of antifungal activity. Twenty-one (75%) extracts inhibited growth of all organisms tested, and in addition, six of these showed high activity in the bioassays, at 0.01 mg/ml, the highest dilution used in this investigation. The been established that Helichrysum species can be divided into a number of chemical races, containing different compounds (Hilliard, 1983). However, some of the moderately active and least active plants were also reported to have similar and/or other active compounds but probably in smaller quantities. Different chemotypes of species could explain the observed variance in inhibitory activity.

3.4 Discussion

The present screening investigation has revealed a fairly high ‘hit’ rate for antifungal inhibition when selecting plants utilized in traditional medicines based upon the criteria given in Chapter 1. Some results obtained suggest the possible correlation between the folkloric uses of Helichrysum species and their activity.

For a biologically active compound like a fungicide to have activity it must first diffuse from its site of application, usually the exterior of the cell, to its site of action, often within the cell, and then partition itself onto the active site (Hansch, 1971). The rate of these

events will depend on the lipophilicity of the compound. Once at the active site, the compound has some chemical and physical effect that accounts for its activity. There is a growing consensus that, in most systems, antifungal agents exert their toxicity by some membrane-associated phenomenon (Laks and

Table 3.1 Antifungal activity (MIC) of the crude extracts of the aerial parts of *Helichrysum* species

Plant Species (Voucher specimen No.)	MIC (mg/ml)[a]						
		Fungi[b]					
		A. fla	*A. nig*	*C. cla*	*C. cuc*	*C. sph*	*P. cap*
H. appendiculatum	S[c]	1.0	1.0	na[e]	na	1.0	1.0
(M5135)	H[d]	na	na	na	na	na	na
H. argyrosphaerunm	S	1.0	1.0	na	na	1.0	1.0
(M5080)	H	na	na	na	na	na	na
H. aureonitens	S	1.0	1.0	na	na	1.0	1.0
(M5096)	H	na	na	na	na	na	na
H. bellum	S	1.0	1.0	0.10	0.10	1.0	1.0
(M5178)	H	na	na	na	na	1.0	na
H. caespititium	S	0.01	0.01	0.01	0.01	0.01	0.01
(M0011)	H	1.0	1.0	0.01	0.01	1.0	1.0
H. callicomum	S	0.01	0.01	1.0	0.01	0.01	0.01
(M5054)	H	0.01	na	na	na	na	na
H. candolleanum	S	1.0	na	1.0	1.0	1.0	na
(M3078)	H	na	na	1.0	1.0	1.0	na
H. chionosphaerum	S	1.0	0.10	0.10	1.0	0.10	1.0
(M5111)	H	na	1.0	1.0	na	1.0	na
H. decorum	S	1.0	1.0	1.0	1.0	1.0	1.0
(A0006)	H	1.0	0.10	0.10	0.10	0.10	na

Plant Species (Voucher specimen No.)		MIC (mg/ml)[a]					
		Fungi[b]					
		A. fla	*A. nig*	*C. cla*	*C. cuc*	*C. sph*	*P. cap*
H. glomeratum (M5055)	S	0.01	0.01	0.01	0.01	0.01	0.01
	H	na	na	1.0	1.0	na	na
H. herbaceum (M5272)	S	1.0	0.01	0.10	0.10	0.01	0.10
	H	1.0	1.0	0.10	1.0	1.0	0.10
H. hypoleucum (M5056)	S	1.0	0.01	0.01	0.01	0.01	0.01
	H	1.0	0.01	0.01	0.01	0.01	0.01
H. kraussii (M5173)	S	0.01	0.01	1.0	0.10	0.10	0.10
	H	1.0	1.0	1.0	1.0	1.0	na
H. longifolium (M5109)	S	0.10	0.10	0.10	0.10	0.10	0.10
	H	1.0	1.0	1.0	1.0	1.0	na
H. melanacme (M5110)	S	0.01	0.01	0.01	0.01	0.01	0.01
	H	0.01	0.01	0.01	0.01	0.01	0.01
H. microniifolium (5100)	S	1.0	1.0	na	na	1.0	1.0
	H	na	na	na	na	na	na
H. montanum (M3707)	S	1.0	1.0	1.0	1.0	1.0	1.0
	H	na	na	na	na	na	na
H. monticola (M5177)	S	1.0	1.0	1.0	1.0	1.0	na
	H	na	na	1.0	1.0	1.0	1.0
H. nudifolium (M3708)	S	0.10	0.10	0.10	0.10	0.10	0.10
	H	na	na	na	na	na	na
H. odoratissimum (M5061)	S	0.10	0.01	0.10	0.10	0.10	0.10
	H	na	1.0	na	na	na	na
H. oreophilum (M5097)	S	1.0	1.0	1.0	1.0	0.01	0.01
	H	na	na	na	1.0	0.01	0.01
H. pilosellum (M5059)	S	na	na	na	na	na	na
	H	na	na	na	na	1.0	na
H. psilolepis (M5081)	S	1.0	1.0	0.1	1.0	0.1	0.1
	H	na	na	1.0	1.0	1.0	1.0

Plant Species (Voucher specimen No.)	MIC (mg/ml)[a]						
		Fungi[b]					
		A. fla	*A. nig*	*C. cla*	*C. cuc*	*C. sph*	*P. cap*
H. rugulosum (M5060)	S	0.01	na	0.01	0.01	1.0	0.01
	H	0.01	na	na	0.01	na	0.01
H. simillimum (M0001)	S	1.0	na	na	1.0	1.0	na
	H	na	na	na	na	na	na
H. sutherlandii (M5179)	S	1.0	0.10	1.0	1.0	1.0	1.0
	H	1.0	1.0	na	na	na	na
H. trilineatum (M5172)	S	0.10	0.10	0.10	1.0	0.10	0.10
	H	na	na	na	na	0.10	na
H. umbraculigerum (M5174)	S	1.0	1.0	1.0	1.0	0.10	0.10
	H	1.0	1.0	1.0	1.0	0.10	0.10

[a] Minimum inhibition concentration

[b] A. fla (Aspergillus flavus), A. nig (Aspergillus niger), C. cla (Cladosporium cladosporioides), C. cuc (Cladosporium cucumericum), C. sph (Cladosporium sphaerospermum), and P. cap (Phytophthora capsici)

[c] Shaken extract

[d] Homogenized extract

[e] Not active

Pruner, 1989), again indicating the possible importance of lipophilicity for their activity.

The success of the ethnobotanical approach to drug discovery can no longer be questioned. Historical and current discoveries attest to its power (Cox, 1994). Medicinal plants are the 'backbone' of traditional medicine (Farnsworth, 1994). Focussing attention on those plants is the most effective way of identifying plants that contain bioactive compounds (Schultes, 1994). Internal uses predominate over external ones, but a decoction is the

primary form used. The types of diseases or complaints treated are ailments of the digestive tract, general pain, dermatological conditions, wound dressing and bronchiopulmonary disorders and inflammations (not necessarily in descending order of importance) but this natural antifungal activity can be rapidly lost because of seasonal changes, presumably due to chemical or enzymatic degradation of the active species (Prusky *et al*., 1983). The tested *Helichrysum* species represent a potential source of effective fungicides in food and medicine.

3.5 Conclusion

Helichrysum species have played an important role in the botanical pharmacopoeia of the indigenous people of South Africa. As described by Watt and Breyer-Brandwijk (1962), these plants have been used to treat a variety of ailments, many of which could have been caused or been complicated by fungal infection. In this investigation, the inhibitory effects produced by these Helichrysum species suggests that their agents may have played a medicinal role in the healing practice of the indigenous people of South Africa. All the fungal strains tested in this study were susceptible to most of the *Helichrysums* investigated.

It is not possible to make a direct correlation between the observed activity of the *Helichrysum* extracts *in vitro* and the actual effects when used *in vivo* for the diseases observed by the indigenous people and traditional healers. Therefore, it is important that the species which have demonstrated growth-inhibiting activity in this assay be further studied to evaluate the significance of these extracts' clinical role and, in the medical system of indigenous people. Additional research is also necessary to isolate and identify their active compounds for pharmacological testing.

Helichrysum species and the observations related to the use of these plants are open to extensive study. *Helichrysum* species not only function as important herbs, but also serve as nutritional and medicinal agents. It is certain, that, through observations made in this

study, *Helichrysum* species harbour many economically significant benefits awaiting 'discovery'.

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