SCREENING OF TRADITIONALLY USED SOUTH AFRICAN MEDICINAL PLANTS AGAINST *CANDIDA ALBICANS*

by

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DECLARATION

The experimental work describe in this thesis was carried out in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal (Pietermaritzburg), under the supervision of Doctor A.K. Jäger and Professor J. van Staden.

This work has been performed by the author and has not been submitted in any form to another University, except where the work of others is acknowledged in the text.

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We declare that the above statement is correct.

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ABSTRACT

Candida species were discovered more than a century ago as a causative organism of oral thrush. In HIV patients, the presence of oral candidiasis has been shown to be the earliest opportunistic infection. Candidiasis lesions associated with HIV infections are primarily a reflection of the specific change of the host's immune response caused by the virus. Studies of AIDS all over the world show that 58-81% of all patients contract a fungal infection at some time during the primordial stage or after developing AIDS and 10-20% have died as a direct consequence of fungal infections.

Twenty four South African medicinal plants were screened using a modification of the NCCSL broth microdilution antifungal test against *Candida albicans* standard strain ATCC 10231 and two clinical isolates from a 5-month-old baby and an adult. This assay was performed in order to find a traditional remedy to treat oral candidiasis. Of all the screened plants *Allium sativum* L., *Glycyrrhiza glabra* L., *Polygala myrtifolia* L. and *Tulbaghia violacea* L. aqueous extracts were found to have the best activity. *Allium sativum* and *Tulbaghia violacea* aqueous bulb extracts had MIC values of 0.56 $mgml^{-1}$ and 3.25 $mgml^{-1}$ respectively, whilst *Polygala myrtifolia* leaf extracts and *Glycyrrhiza glabra* rhizome extracts had MIC values of 1.56 $mgml^{-1}$ and 3.25 $mgml^{-1}$ respectively when tested against the isolate from a 5-month-old baby, which was the most susceptible of the isolates used. All the extracts had

higher MIC values against the standard strain (ATTC 10231), which was the least susceptible to the extracts used.

Stability testing was performed on fresh aqueous extracts of *A. sativum, G. glabra, T. violacea* and *P. myrtifolia* stored at 4°C, 23°C and 33°C over a period of one week, to determine the stability of the extracts in solution. All *A. sativum* extracts maintained stability for three days in solution, whilst *T. violacea* extracts remained stable for only two days in solution. TLC fingerprinting of *A. sativum* and *T. violacea* extracts indicated the presence of the known antibacterial and antifungal compound allicin. The activity of allicin and other active compounds was observed by using the bioautographic assay, which was performed on these extracts.

P. myrtifolia and *G. glabra* extracts lost stability 24 hours after preparation at all tested temperatures. However, it was clear with the four plant extracts tested that storage of solutions at higher temperatures reduced their activity and stability.

The unpleasant taste and smell of *A. sativum* and *G. glabra* could however not be masked, since the intake of these two extracts would result in HIV-patients being recognised. These two plants where therefore not considered for further investigation. *G. glabra* and *P. myrtifolia* are both saponin containing plants. These could be the active constituents responsible for the anticandidal action. *G. glabra* is known for its biological activity as an antibacterial agent, whilst other *Polygala* species have been reported to

possess antifungal saponins. Although *P. myrtifolia* and *G. glabra* are not stable for more than 24 hours, they do not have an unpleasant smell or taste. These plants are therefore further investigated for use as oral mouthwash in clinics and homes.

CHAPTER 1

ETHNOBOTANY AND DRUG DISCOVERY

1.1 Project Rationale

An approach by the Staff of Christ the King Hospital, located in Ixopo South Africa led to the initiation of this project. The hospital had severe cases of patients infected with oral candidiasis, especially babies. The number of people coming to the hospital is overwhelming, with most of the patients being admitted for a number of days. Most of the child patients have such severe infections of oral candidiasis that feeding is a problem. The feeding problem further compromises their condition as HIV positive people. Shortage of medication (antifungal drugs) aggravates the situation, and even when treatment is available most patients would return to hospital within two to three weeks with recurring symptoms.

After the initiation of this project Pfizer Pharmaceuticals made available the antifungal drug "Diflucan" (fluconazole), to state hospitals for HIV/AIDS patients in South Africa at no cost for a period of two years. However, the drug is only made available for severe or "life threatening" cases of candidiasis. As a result, the matter is serious, since many rural patients require readily available medication. Other available antifungal drugs, which could be prescribed to patients, are usually expensive. It is therefore evident that for

those patients where symptoms of oral candidiasis reoccur, and for cases where the microorganism has developed resistance to existing drugs, alternative medication is urgently required.

1.2 Ethnobotany and Traditional Medicine

Eighty five percent of traditional medicines are derived from plants and their extracts. Natural compounds, their derivatives and analogues still represent over 50% of all drugs in clinical use, with higher plant derived natural products representing 25% of the total (BALANDRIN *et al.*, 1993). Extracts of some plants are useful in a crude form, i.e. *Atropa belladonna* tincture as an antispasmodic, *Rauvolfia serpentina* roots for hypertension and as a tranquilizer, and *Papaver somniferum* extracts or tincutures as an analgesic (FARNSWORTH AND SOEJARTO, 1991).

For more than two decades the World Health Organisation (WHO) has encouraged the use of traditional medicine, especially in the developing countries by promoting the incorporation of useful elements into national healthcare systems (SINDIGA, 1995). It is estimated that 80% of the world population in less developed countries rely on traditional medicine as their sole primary health resource (FARNSWORTH, 1994). It is therefore essential that traditional medicine be promoted and plants be studied for safety and efficiency and to develop galenical products that are standardized and stable (FARNSWORTH, 1994). Promotion of herbal medicine at the national level

aims primarily at improving drug supply, lowering the cost for drugs and import constituents (GRAND AND WONDERGEM, 1989). At community levels promotional activities are focused on self-reliance, community participation and strengthening of local medical traditions as a whole (GRAND AND WONDERGEM, 1989).

The popularity of herbs and other alternative therapies is rising significantly worldwide. A survey conducted in the U.S. in 1997 showed that 42% of the people surveyed reported using some form of alternative medicine and herbal remedies showed the highest use increase (MORBIDONI, *et al.*, 2001). Despite the dramatic advances and advantages of conventional medicine, it is clear that herbal medicine has much to offer. Over the years, infectious organisms have developed resistance to synthesized drugs, and active constituents from herbs like *Artemisia annua* are being used to treat malaria in areas of the world where the protozoa causing the infection no longer respond to conventional treatment (CHEVALLIER, 1996). Herbal medicine often complements conventional treatments, providing safe, well-tolerated remedies for chronic illnesses (CHEVALLIER, 1996).

1.3 Traditional Medicine in South Africa

South Africa has a rich cultural diversity that is reflected in the formal and informal systems of medicine that is presently practised in different parts of the country. There are an estimated 200 000 indigenous traditional healers in

South Africa and up to 60% of the South Africans consult them (VAN WYK, *et al.*, 1997). These traditional healers are commonly divided into three types: 1. inyanga (herbalist); 2. isangoma (diviners) and 3. umthakathi (sorcerer) (PANTANOWITZ, 1994). These people are believed to be spiritually empowered and use these powers with plant extracts as remedies to heal patients (VAN WYK *et al.*, 1997).

Traditional healing provides for the primary health care needs of a large number of the black population in South Africa, and 80% of the Zulu population seen by medical practitioners also consult with traditional healers (JÄGER *et al.*, 1996; VAN WYK *et al.*, 1997). It is therefore estimated that there are 27 million consumers of traditional medicine in South Africa (MANDER, 1998). In KwaZulu-Natal alone over 400 plants are actively traded with an estimated trade volume of some 4 300 tonnes per year. Households are spending between 4% to 8% of their annual income on indigenous medicine and services (MANDER, 1998).

A large number of South African medicinal plants are widely used by traditional healers for their ability to cure a lot of the infectious diseases. Researchers in the ethnobotanical field have screened many of these plants for activity against bacteria, and fungi (COX AND BALICK, 1992). However, there has been little research performed on the activity of indigenous medicinal plants against the fungal species *Candida albicans*.

1.4 Infectious Diseases and Traditional Medicine

Infectious diseases account for a large proportion of the health problems in Africa (DESTA, 1993). A large number of traditional healers are using the diverse plant material at their disposal for the treatment of these diseases. With the knowledge these traditional healers have on medicinal plants, new and interesting natural products may well be discovered. Biologically active compounds could be isolated by bioassay-guided fractionation procedures in which various screening methods are employed to identify the biologically active compounds from crude extracts (MARSTON *et al.*, 1993).

1.5 The Threat and Conservation of Medicinal Plants

The importance of medicinal plants has been briefly outlined in the above paragraphs. The demand for indigenous medicine and medicinal plants is extensive, mostly by the black population and especially in KwaZulu-Natal (MANDER, 1998). Professional herb collectors and traditional healers have had a considerable impact on wild plant populations and the following result:

- Active trade and marketing of medicinal plant material;
- Fragmented distribution of medicinal plant species due to varied land use, topography and climate in the region;
- Large spatial concentrations of consumer demand;
- Local extinctions of plant populations and therefore the need to access more remote plant populations due to intensive harvesting; and

- The inadequate access to health care by rural communities, which when lacking maintains and stimulates demand (MANDER, 1998).

It is estimated that 340 tonnes of plants are traded through shops per year, with approximately 50% purchased directly from plant gathers (170 tones) and the remaining 50% purchased from street traders. These figures highlight the demand for indigenous medicine, which is estimated to increase with a population increase. The rapid increase of AIDS in South Africa also lead to an increased demand on traditional medicines since patients obtain little relief from western medicine prescriptions (MANDER, 1998). The demand is not only escalated by black patient use in rural communities but also pharmaceutical companies currently developing products for the African market in South Africa (MANDER, 1998).

The indigenous medicine market is based on indigenous plants, which are generally harvested from wild plant stocks throughout South Africa and neighbouring countries. Harvested plant stocks are not managed and little cultivation takes place. Wide ranges of plant species are showing indications of unsustainable use. This is visible through the decreased availability of plant products in the market, distances of collecting stocks increasing and availability of products becoming increasingly erratic in certain markets. The scarcity of popular plants and under-supply in some markets resulted considerable increases in product costs (MANDER, 1998).

Despite the fact that the fate of plants does not look bright, there are however methods which could be used to sustain the supply of plants to the markets or to ensure that the plants are not over exploited. These methods include effective management of grasslands, woodlands, thickets and private property that have not been intensively exploited. Proper storage facilities and trading infrastructure could lead to less wastage of already collected plant material, leading to less frequent harvesting (MANDER, 1998). Lastly, cultivation of medicinal plants could reduce the impact of poor management of wild medicinal plants.

CHAPTER 2

CANDIDA ALBICANS REVIEW

2.1 *Candida albicans*

Candida albicans is a yeast-like microorganism with fungal characteristics (SALTARELLI, 1989). The vegetative cells of *C. albicans* were first described in 1853 as spherical or broadly oval shape 2-8.5 µm to 3.0-14 µm in size and able to reproduces by multilateral budding. *C. albicans* can live on many other living organisms without inducing problems however; it may invade and cause infections and death in humans and animals (SALTARELLI, 1989). In humans it is mainly found in warm, dark, moist areas of the body. Antibiotics, steroid-hormones, birth control pills, excessive stress and too much sugar in the diet cause the yeast to overgrow. When *C. albicans* becomes active, it can affect every part of the body. Its toxins can even affect the brain and blood stream (BURTON, 1989). It is estimated that one out of three people in the Western world have *Candida* infections. This disease affects women, men, boys, girls and even babies, showing mild symptoms or even chronic conditions (BURTON, 1989).

Candida is a dimorphic fungus able to grow in the yeast and mycelium forms. This feature is associated with the morphological changes in the pathogenic fungus in that the morphology of the fungus in the infected tissue is different

from that of the propagule, which initiated the infection. This altering of morphology in the pathological state is also associated with the survival advantage of the fungus (SHEPHERD, 1991). The dimorphic nature of this yeast may therefore, be defined as an environmentally controlled reversible transition between yeast and mycelium forms (SALTARELLI, 1989).

The nutritional requirements of *C. albicans in vitro* are comparatively simple and as follows: A medium composed of glucose, $(NH_4)_2SO_4$, biotin and inorganic salts supports maximal growth of several different strains. The morphology of *Candida* is based on controllable modifications of well-defined media, with the organism growing at a wide range of pH, but flourishes best at pH 7 (SALTARELLI, 1989).

As mentioned *Candida* is a morphologically changing organism, therefore a number of external factors affect its existence and survival. Several environmental factors of which the most frequently cited are inoculum size, duration of incubation and medium composition are known to influence the outcome of susceptibility tests *in vitro* (ODDS, 1993).

Special attention should be taken with the strains to ensure that the correct morphological structure of the fungus is being used, ensuring that the inhibitory activity is not selective for only one of the yeast or mycelium stages. Temperature is another variable that influences the antifungal MIC's of yeasts in broth dilutions and should be well considered (ODDS, 1993).

2.2 *Candida albicans* in HIV/AIDS Patients

HIV/AIDS has become a devastating epidemic. Sub-Saharan Africa has 10% of the world's population and yet 70% of the global HIV burden and 90% of the deaths attributable to HIV/AIDS occur in this region. Of the estimated 13.2 million children orphaned due to AIDS, 12.1 million are Africans (MARSHALL, 2001). According to the UN, South Africa has the fastest HIV/AIDS growth in the world, with more than 4.5 million people diagnosed HIV positive, thousands of AIDS deaths, 2500 new infections daily and 100-150 000 AIDS orphans in the year 2000 alone (STEGMANN, 2001). Today AIDS accounts for nearly a third of all deaths nationally and more than 40% in KwaZulu-Natal i.e. some 500 deaths a day in total (MASLAN, 2001). A KwaZulu-Natal voluntary survey of University students demonstrated infection rates of 26% in women and 12% in men aged between 20 and 24, and 36% in women and 23% in men aged 25 to 29. This therefore emphasizes the need for top priority on prevention programmes for young people (Urban Health and Development, 2000).

Studies of AIDS all over the world show that 58-81% of all patients contract a fungal infection at some time during the primordial stage or after developing AIDS and 10-20 % die as a direct consequence of fungal infections (DROUHET AND DUPONT, 1989). The presence of oral candidiasis is considered the earliest of the opportunist infections (FAN-HARVARD *et al.*, 1991).

The natural history of HIV/AIDS is that it weakens the immune system, which renders the patients susceptible to a host of infections (DUBE AND MUTLOANE, 2001). Treatment of the opportunistic infections can make a large difference to the life quality of HIV-positive individuals. The best and most effective way to keep HIV-positive individuals healthy is to prevent and contain infection.

Candida was discovered more than a century ago as a causative organism of oral thrush (PRASAD, 1991). The yeast-like forms of the organism are non-invasive and sugar fermenting, but in immune deficient people the yeast overgrows. For the yeast to be invasive, it alters its form to the mycelium fungal stage, by producing rhizoids (long root-like structures) that are invasive and able to penetrate the intestine mucousal barriers, releasing metabolic toxins, and incompletely digested proteins into the blood stream (PRASAD, 1991; CHAITOW, 1996). This process initiates a series of adverse reactions that may cause tissue damage, and a wide range of problems, including food sensitivities or allergies (PRASAD, 1991).

Research indicates that many of the toxic effects observed with *Candida* infection results from its ability to manufacture acetaldehyde, under appropriate conditions, produced by both the clinical and laboratory characteristics of the candidal infection (CHAITOW, 1996).

Clinically the fungal infection is identified as "creamy-white, curd-like patches on the tongue or other oral mucousal surfaces which are removed by scrapings (DROUHET AND DUPONT, 1989). Progress to more severe oesophageal candidiasis is associated with difficulty in swallowing and hence causes dehydration, non-compliance with medication regimes and general malnutrition (CLARK AND HUFFORD, 1993), which is sometimes associated with severe diarrhoea (DROUHET AND DUPONT, 1989; DUBE AND MUTLOANE, 2001). Hence, those who suffer from oral candidiasis often lose a lot of weight due to a sore throat, which prevents them from eating (SANNE, 2001).

The presence of *C. albicans* is confirmed by positive KOH preparations or Gram strains (negative and positive), which shows masses of hyphae, pseudohyphae and yeast forms with positive culture (DROUHET AND DUPONT, 1989).

The infection causes lesions in a wide variety of morphological sites: mouth, bronchi, lungs, intestines, skin and vagina. It is most easily observed on the mouth and oesophagus. The distribution of candidiasis lesions associated with HIV infections is primarily a reflection of the specific alteration of host immune responses endangered by the virus (ODDS *et al.*, 1990). Immunosuppression and long-term treatment with antifungal agents associated with HIV infection create selective pressures (resistance) (ODDS *et al.*, 1990). Commencement of AIDS leads to increased use of antifungal

agents, with some of the infected patients requiring therapy for life. Most patients with symptomatic HIV infections or AIDS require long-term antifungal therapy either as treatment or as prophylaxis of oral and oesophageal thrush (FAN-HAVARD *et al.*, 1991).

The emergence of bacterial resistance in patients after prolonged use of antibiotics is well known and inevitable. The use of broad-spectrum antibiotics alters the normal bacterial flora and allows the more resistant bacteria to colonize (FAN-HAVARD *et al.*, 1991). *Candida* and other infections deplete the immune system. Treatment of these infections in an early stage can therefore help reduce the deterioration in the health of an individual (SANNE, 2001).

2.3 Existing Anticandidal Drugs

The selection of anticandidal drugs is based on their selective toxicity towards the fungal pathogen. The toxicity in turn depends on the cellular constituents and their biosynthetic pathways in the pathogen (BORLARD, 1991).

There has been extensive research on the development of antifungal drugs, but only six of these antifungal agents were licensed in 1995. These include polyene amphotericin B, the azoles, imiazoles, miconazole and ketoconazole, triazole and itraconazole and the pyrimidine synthesis inhibitor flucytosine (5-FC) (ESPINEL-INGROFF AND PFALLER, 1995).

Polyenes act by binding to ergosterol in the fungal cell membrane, causing osmotic instability and loss of membrane integrity. The azoles on the other hand inhibit fungal cytochrome P450-dependent enzymes, with resulting impairment of ergosterol synthesis and depletion of ergosterol in the fungal cell membrane (ESPINEL-INGROFF AND PFALLER, 1995). Fluconazole is a water-soluble, bifluorinated triazole, with low binding affinity for plasma protein. It distributes extensively throughout the body, and readily diffuses into saliva. This drug is highly successful in the treatment of AIDS patients whom had relapsed after amphotericin B and flucytosine (5-FC) treatment (DROUHET AND DUPONT, 1989).

The United States documented that nearly 10% of nosocomial bloodstream infections are due to *Candida albicans*. Despite the treatment with fluconazole and amphotericin B, the mortality associated with systemic *C. albicans* infections is ≥50% (LOUIE *et al.*, 1995).

However, it has been found that the treatment with these drugs, especially for extended periods of time, can lead to problems with toxicity to the patients (amphotericin B) or lead to the development of resistant fungal organisms during the course of therapy (5-fluorocystine) (BOONCHIRD AND FLEGEL, 1982). Since the incidence of these infections is on the increase, attempts are being made to develop new chemotherapeutic agents or a combination of agents to treat the fungus.

Amphotericin B induces several dose dependent effects on the growth of *C. albicans in vitro*:

- An increase in number of colony-forming units occurs at very low doses, although the exact dose may vary depending on the experimental conditions, this stimulatory effect has been seen in yeast treated with 0.01-0.04 $\mu g ml^{-1}$. It also shows that at sub-inhibitory doses, amphotericin induces chemical modification of the cell wall and plasmamembrane of *C. albicans*.
- Inhibition of growth and germ-tube formation occurs at intermediate concentrations (0.02-0.10 $\mu g ml^{-1}$).
- At higher concentrations (>0.30 $\mu g\ ml^{-1}$), amphotericin B is lethal to *C. albicans* cells. Here amphotericin-induced oxidative damage as the mechanism of cell killing had been suggested (BORLARD, 1991).

Due to the sterol binding action of amphotericin B in the fungal cell membrane, renal damage is found to occur in more than 80% of patients and can be permanent in patients receiving larger doses of the drug (CLARK AND HUFFORD, 1993). Flucytosine in combination with amphotericin B is designed to reduce the dosage of amphotericin (thereby reducing its dose-related toxicity) and to eliminate the development of resistance to flucytosine. However, it has been noted that flucytosine toxicity may increase when it is used in combination with amphotericin B (CLARK AND HUFFORD, 1993).

Fluconazole costs the South African consumer R57 per 200 mg capsule, while the public sector price is R36.33 for a 150 mg capsule. In pharmacies the retail price is R80 per 200 mg tablet, this information therefore emphasizes the non-affordability of the drug to the public sector at a cost of R20 000 per person a year. At these prices the private sector, medical aid and patients quickly exhaust their annual drug limits and become state patients (DUBE AND MUTLOANE, 2001). This drug is made available free of charge to South African patients for a period of two years and from then on will only be available to patients with severe oesophageal infection. This initiative was put into place in order to make the drug available to the poorest people in the developing world. However, this is the only form of medication available for free and none of the others are available at a cheap price. *Candida* is now resistant to nystatin, gliotoxin, comirin, candicidin, pimaricin, filipin (filimarisin) and triamicinolone acetonide (TA) in some form or another (SALTARELLI, 1989). Hence as most of the antibiotic medication on the market, the fungi develop resistance against fluconazole.

The most intensive efforts to develop new antifungal agents have centred on the synthesis of analogs of the existing azole antifungal agents. Features related to biological or therapeutic effects are due to skeletal structures, which tend to remain consistent throughout the class. This therefore means various azole analogs may exhibit varying degrees of affinity and selectivity for the molecular target, the fundamental mechanism remaining the same (CLARK AND HUFFORD, 1993).

Azoles' are fungistatic in their action requiring long-term (lifetime) use. Therefore modifying the structure of existing agents to produce new products is pointless as similar toxicity or resistance problems may recur (CLARK AND HUFFORD, 1993). It is therefore clear that novel antibiotics can serve two important functions:

a) As leads for production of novel agents; and

b) As probes for new molecular targets.

Searching for new prototypes of bioactive substances through natural products is more advantageous than chemical synthesis or modification of existing agents due to the likelihood of identifying novel prototype, drugs with unique and different chemical structures and hence less similar toxicities, cross resistance and mechanism of action (CLARK AND HUFFORD, 1993).

2.4 Plants with Activity against *Candida albicans*

The ongoing threat of synthetic agent resistance to yeast infections has led to researchers concentrating on naturally acquired agents. A variety of substances in food are known to act as antifungal or antimycotic agents. It was discovered long before the existence of refrigerators that antimicrobial properties of culinary herbs and spices could retard food spoilage (STILES *et al.*, 1995). Research has now focused on the ability of aromatic leaves, essential oils and their active principals to inhibit fungal infections.

Many researchers in the world have investigated antifungal properties, involving the screening of plants against *Candida albicans*. Numerous species from different plant families were tested and on the basis of their use in traditional medicine, arrays of plants have been screened. Plants are a logical choice chiefly because of their infinite variety of novel organic molecules. Compounds, which possess novel mechanisms of action regardless of whether they become clinically useful, and may serve as important biochemical tools to probe the critical biochemical pathways of the opportunistic pathogens (CLARK AND HUFFORD, 1993).

Many South African medicinal plants have been screened for antimicrobial and antibacterial activity, but there has been few researchers working on the anticandidal activity of the medicinal plants in South Africa *Eclipa prosta is* highly active against *Candida*, due to the presence of a polyacetylene isolated from the leaves. *Bidens pilosa* on the other hand contains an active compound phenylIheptatriyene in the leaf and root infusions (VAN WYK *et al.*, 1997).

Cáceres *et al.* (1991), Quiroga *et al.* (2001) and Agarwal *et al.* (2000) are some of the recent researchers who have associated the antifungal activity of most plants with secondary metabolites, which plants produce. These metabolites include flavonoids, phenols, phenolic glycosides, lactones and saponins. Agarwal *et al.* (2000) also reported on the antifungal and antibacterial activity of anthraquinone derivatives. These derivatives include

rhein, physcoin, aloe-emodin and chrysophanol, methanolic extracts from dried rhizomes containing the active constituents. Eugenol and vanillin are major constituents of clove oil and vanilla respectively, and both these compoundsare highly active against *Candida* (BOONCHIRD AND FLEGEL, 1982).

The fruit of *Solanum incanum*, a weed found in Tanzania and Kenya is extensively used for the treatment of cutaneous mycotic infections and other pathological conditions and are highly active against *Candida.* This is due to the presence of solanine, a glycoalkaloid (SAWHNEY *et al.*, 1978). In addition to the three known polyenes from Devil's club (*Oplopanax horridus*), Kobaisy *et al.*, (1997) isolated two more polyenes that exhibited anticandidal, antibacterial and antimycobacterial activity. Oregano, cloves, garlic, onions, thyme and savory are but a few of 44 herbs and spices found to inhibit the growth of *C. albicans* and other potentially pathogenic bacteria (STILES *et al.*, 1995). Table 2.4.1 lists different plants with anticandidal activity and activity against other microorganisms.

Table 2.4.1 Plants with anticandidal activity

Plant Species	Family	Plant Parts	Microorganisms	Country	Reference
Achyranthes aspera	AMARATHACEAE	Flower	*Sa, Pl*	Ethiopia	DESTA, 1993
Acokanhera schimperi	APOCYNACEAE	Flower	*Pa, Bs. Ms*	Ethiopia	DESTA, 1993
Asplenium trichomanes	ASPLENIACEAE	Root	*Sa; Ec; Pv; Pa*	Ethiopia	DESTA, 1993
Aspilia africana	ASTERACEAE	Leaf and root	*Bs; Ms*	Rwanda	BOILY and VAN PUYVELDE, 1986
Berberis holstii	BERBERIDACEAE	Root bark	*Ba, Ms*	Ethiopia	DESTA, 1993
Berchemia discolor	RHAMNACEAE	Leaf	*Sa; Ec; Pv; Pa*	Ethiopia	DESTA, 1993
Bidens borneana	COMPOSITAE	Flower	*Sa, Sg*	Ethiopia	DESTA, 1993
Bidens pilosa	ASTERACEAE	Leaf	*Bs; Ms; Sa; Sg*	Rwanda	BOILY and VAN PUYVELDE, 1986
Cajanus cajan	FABACEAE	Leaf	*Bs; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Coleus kilimandschari	LAMIACEAE	Stem and root	*Bs; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Dalbergia lactea	FABACEAE	Root	*Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Dryopteris inaequalis	ASPIDICEAE	Rhizomes	*Bs; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Gynandropsis gynadra	CAPPARIDACEAE	Leaf	*Ms*	Rwanda	BOILY and VAN PUYVELDE, 1986
Helicrysum hoschstetteri	ASTERACEAE	Flower	*Bs; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986

Hygrophila auriculata	ACANTHACEAE	Leaf	*Bs; Sa; Ms*	Rwanda	BOILY and VAN PUYVELDE, 1986
Iboza riparia	LAMIACEAE	Leaf	*Bs; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Macaranga kilimandscharika	EUPHOBIACEAE	Leaf	*Bs; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Mitragyna rubrostipulata	RUBIACEAE	Stem	*Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Momordic foetida	CUCURBITACEAE	Leaf	*Bs; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
		Stem	*Bs; Ms*		
Monechma subsessile	ACANTHACEAE	Leaf	*Bs; Sa*	Rwanda	BOILY and VAN PUYVELDE,1986
Rhus vulguris	ANACARDIACEAE	Bark of roots	*Bs; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Rumex abyssinicus	POLYGONACEAE	Root	*Ss; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Rumex usambarensis	POLYGONACEAE	Leaf	*Ss; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986
Schinus molle	ANACARDIACEAE	Whole plant	*Fo; Pn; An & T*	Argentina	QUIROGA, *et al.*, 2001
Schizozygia coffaeoides	APOCYNACEAE	Leaf	*Tm; Mg; Cc*	Kenya	KARIBA, *et al.*, 2001
Withania somnifera	SOLANACEAE	Leaf	*Ss; Ms; Sa*	Rwanda	BOILY and VAN PUYVELDE, 1986

Sa- Stapylococcus aerus, Sg- Salmonella gallinarum, Pa- Pseudomonas aeruginosa, Ms- Mycobacterium smegmatis, Bs- Bacillus subtilis, Mg- Microsporum gypseum, Cc- Cladosporium cucumerinum, Fo- Fusarium oxysporum, An- Aspergillus niger, T-Trichoderma spp, Pn- Penicilum notatum

2.5 Aims and Objectives of this Study

This project was aimed at:

- Identifying plants used by traditional healers to treat oral thrush;
- Establishment of an anticandidal bioassay;
- Screening of plant material for anticandidal activity, in order to identify the most active plants;
- Assess extract stability in solution; and
- Develop a fingerprinting system for quality control.

CHAPTER 3

SCREENING OF MEDICINAL PLANTS FOR ANTICANDIDAL ACTIVITY

3.1 INTRODUCTION

Plant derived natural products have long been, and will continue to be, extremely important as sources of medicinal agents and models for the design, synthesis and semisynthesis of novel substances for treating human diseases (BALANDRIN *et al.*, 1993). The goals of using plants as sources of therapeutic agents are; a) isolation of bioactive compounds for direct use as drugs; b) to produce bioactive compounds of novel and known structures as lead compounds for semisynthesis of entities with higher activity and/or lower toxicity; c) to use agents as pharmacological tools; and d) to use the whole plant or part of the plants as herbal remedy (FABRICANT AND FARNSWORTH, 2001).

Screening of South African medicinal plants for antifungal activity will be beneficial, in providing an alternative medication and availing cheap, easy to prepare medication. These benefits are aimed at the community level where medicinal plants are extensively used in primary health care. Plants with the best anticandidal activity will be identified using the MIC assay.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Plant material was selected according to their use by traditional healers and literature reports on their biological activity as anticandidal, antifungal and antibacterial agents. All plant parts were dried in an oven at 50°C for two days. The material was then ground to fine powders and stored in airtight containers at room temperature. The plants investigated are listed in Table 3.2.1.

Table 3.2.1 Plants screened for anticandidal activity

Species	Family	Part used	Site	Voucher No.	References
Artemisia afra Jacq.ex Willd	Asteraceae	leaves	1	MOTSEI 3 UN	BRUNETON, 1995
Bidens pilosa L.	Asteraceae	leaves	8	MOTSEI 24 UN	JÄGER *et al.*, 1996
Brachylaena huillensis L.	Asteraceae	leaves	1	MOTSEI 2 UN	WATT & BREYER- BRANDWIJK, 1962
Bulbine frutescens (L.) Willd	Asphodelaceae	leaves	1	MOTSEI 23 UN	WATT & BREYER- BRANDWIJK, 1962
Bulbine natalensis Wolf	Asphodelaceae	leaves	1	MOTSEI 5 UN	VAN WYK *et al.*, 1997
Carpobrotus edulis N.E.Br.	Mesembryanthe-maceae	leaves	6	MOTSEI 8 UN	VAN WYK *et al.*, 1997
Dodonaea angustifolia L.f.	Sapindaceae	leaves	4	MOTSEI 14 UN	VAN WYK *et al.*, 1997
Erythrina lysistemon Hutch.	Fabaceae	bark, leaves	1	MOTSEI 12 UN	HUTCHINGS *et al.*, 1996
Eucomis autumnalis L' Hér	Hyacinthaceae	bulbs	2	MOTSEI 14 UN	VAN WYK *et al., 1997*
Glycyrrhiza glabra L.	Fabaceae	rhizomes	7		VAN WYK *et al.*, 1997
Haemanthus albiflos Jacq.	Amaryllidaceae	bulbs, leaves	1	MOTSEI 9 UN	HUTCHINGS *et al.*, 1996
Hypoxis hemerocallidea (Fisch. & Mey.)	Hypoxidaceae	corms	2	MOTSEI 4 UN	VAN WYK *et al.*, 1997

Leonotis leonurus (Pers.) R.Br	Lamiaceae	leaves	2	MOTSEI 13 UN	
Plumbago auriculata Lam	Plumbaginaceae	leaves	2	MOTSEI 7 UN	HUTCHINGS *et al.*, 1996
Polygala myrtifolia L.	Polygalaceae	leaves	2	MOTSEI 17 UN	WATT & BREYER- BRANDWIJK, 1962
Rubus rigidus J.E. Sm.	Roseaceae	leaves	5	MOTSEI 11 UN	WATT & BREYER- BRANDWIJK, 1962
Siphonochilus aethiopicus Wood	Zingiberaceae	roots, rhizome	2	LIGHT 17 UN	VAN WYK *et al.*, 1997
Tagetes minuta L.	Asteraceae	leaves	8	MOTSEI 25 UN	BII *et al.*, 2000
Trichilia emetica Vahl.	Meliaceae	bark, leaves	2	MOTSEI 26 UN	BRUNETON, 1995
Tulbaghia violacea L.	Alliaceae	bulbs, leaves	1	MOTSEI 6 UN	WATT & BREYER- BRANDWIJK, 1962
Vetiveria zizaniodes (L.) Nash	Gramineae	leaves	3	MOTSEI 16 UN	HUTCHINGS *et al.*, 1996
Warburgia salutaris Engl.	Canellaceae	leaves	2,3	MOTSEI 15 UN	VAN WYK *et al.*, 1997
Zantedeschia aethiopica (L.)	Araceae	leaves	1	MOTSEI 6 UN	VAN WYK *et al.*, 1997
Zanthoxylum capense Thunb.Harvey	Rutaceae	leaves	1	MOTSEI 10 UN	HUTCHINGS *et al.*, 1996

1-Pietermaritzburg National Botanical Garden; 2- University of Natal Botanical Garden; 3- Silverglen Nature reserve; 4- Vallea-Vista Nursery; 5- Drakensberg; 6- KZN South Coast; 7- Health shop and 8- Grassland field University of Natal

3.2.2 Preparation of extracts

One gram of powdered plant material was weighed out and placed into a pill vial to which 10 ml of either ethanol, ethyl acetate, hexane or water was respectively added. The material was extracted in an ultrasound bath for 1 hour, and then allowed to stand for 30 minutes before being filtered using Whatman No. 1 filter paper in Büchner funnels. The extracts were dried in front of a desk fan.

3.2.3 Candida albicans strains and their maintenance

Three *Candida* strains were used. Two clinical isolates were prepared from hospital patients (a 5-month-old baby and an adult). A standard strain ATCC 10231 was obtained from SABS (South African Bureau of Standards). Scrapings of the clinical isolates were maintained on Sabouraud dextrose agar plates whilst the standard strain was maintained on YM (Yeast Mould) media agar plates. All the plates were kept in the fridge and subcultured every three to four weeks (ESPINELL-INGROFF AND PFALLER, 1995).

3.2.4 Screening of plant material

The water extract residues where dissolved in water and organic solvent extracts residues where dissolved in DMSO (Dimethyl sulfoxide). All extracts where dissolved to a concentration of 100 $mgml^{-1}$. All extracts were tested in triplicates.

3.2.5 Antifungal assay

A modification of the NCCLS proposed method (M27-P) broth microdilution test (ESPINELL-INGROFF AND PFALLER, 1995) was performed as follows: Four millilitres of sterile saline were added to 400 µl of 24 hr old *Candida* cultures. The absorbance was read at 530 nm and adjusted with sterile saline to match that of a 0.5 McFarland standard solution (Appendix 1). From the prepared stock yeast culture a 1:1000 dilution with broth (e.g. 10 µl stock yeast culture: 10 ml broth) was prepared.

One hundred µl of broth were added to each of a 96-well microplate. One hundred µl of the water extracts were added into well (A) and serially diluted from (A) by taking 100 µl into (B). This two-fold dilution was continued down the plate and a 100 µl from the last well (H) were discarded. In the case of organic solvents extracts 25 µl of the organic solvent extracts were added to 175 µl broth and serially diluted. Three replicates were prepared for each extract. All the wells were then filled with 100 µl of stock yeast culture. Amphotericin B was used as a standard for this experiment and the following controls were prepared: wells containing broth only; the different strains of fungi with no extract, and a serial dilution of the amphotericin B with the fungi at the recommended inhibitory concentrations. The plates were then read at 630 nm in an ELISA reader, covered with parafilm and incubated at 33°C overnight, whereafter their absorbance was reread.

3.3 RESULTS AND DISCUSSION

The results of anticandidal activity are presented in Table 3.3A and 3.3B. A number of plants exhibited activity against the *Candida albicans* strains used, with clear indications that the strain from the 5-month-old strain was the most susceptible, followed by the strain from an adult strain and lastly the standard strain, which was found to be resistant to most plant extracts screened.

Aqueous extracts of *A. sativum, G. glabra*, *P. myrtifolia* and *T. violacea* had the best activity amongst all the plants screened (Table 3.3A). *A. sativum* showed the best activity amongst the four extracts. It had an MIC value of 0.56 $mgml^{-1}$ with the strain from a 5-month-old baby and 6.25 $mgml^{-1}$ for both the strain from an adult and standard (ATCC 10231) strain. Garlic is one of the most popular used herbal alternative therapies, with over 300 varieties of garlic grown all over the world (MORBIDONI *et al.*, 2001). Garlic has for many years being used to treat arthritis, asthma, toothache, cancer, cardiovascular diseases and many other ailments (MORBIDONI *et al.*, 2001). Certain extracts of garlic are antibacterial, antifungal, antiviral and antithrombotic (YOSHIDA *et al.*, 1987; MORBIDINI *et al.*, 2001). Aqueous extracts from the bulb have been used in the past to control several human diseases, such as snakebites, haemorrhoids, rheumatism, abdominal pains and skin infections (SINGH *et al.*, 1990). In humans, antifungal activity was detected in serum after ingestion of garlic (YOSHIDA *et al.*, 1987).

The bulbs of *Tulbaghia violacea* showed activity with MIC values of 3.25 $mgml^{-1}$ for the clinical isolate from the 5-month-old baby, 6.25 mg ml^{-1} for the isolate from an adult and 12.5 $mgml^{-1}$ for the standard strain (ATCC 10231). It should be noted that the leaves of *T. violacea* were also screened, but no activity against any of the strains test strains was detected. *T. violacea* also known as wild garlic is a bulbous plant with long, narrow, hairless leaves arising from several white, fleshy bases. All parts of the plant have a strong smell of garlic when damaged, hence the common name. It has attractive mauve or pale purple flowers, which occur in groups at the tip of a slender stalk (VAN WYK *et al.*, 1997). This bulbous plant is traditionally used for fever and colds, but also for asthma and tuberculosis (WATT AND BREYER-BRANDWIJK, 1962). Decoctions are administered as enemas for stomach problems and the leaves are used to treat cancer of the oesophagus (WATT AND BREYER-BRANDWIJK, 1962; HUTCHINGS, 1996).

MIC values for *Glycyrrhiza glabra* rhizomes were 1.56 $mgml^{-1}$ for the isolate from a 5-month-old baby isolate and 12.5 $mgml^{-1}$ for both the isolate from an adult and the standard (ATCC 10231) strain. Liquorice, as *G. glabra* is commonly known belongs to the family Leguminosae. It is a robust perennial herb up to one metre in height, with a multi-branched, underground rhizome and an erect woody stem. The rhizomes, which are mostly referred to as roots, are harvested for medicinal and other uses such as flavouring of food products (VAN WYK *et al.*, 1997). Its main use is to sweeten decoctions, to mitigate the actions of drastic drugs and to relieve pain caused by muscle contraction (HIKINO, 1985). The dried rhizome or

extracts thereof are used to treat ulcers and coughs. In the Western Cape, South Africa it has been an early remedy for appendicitis and tuberculosis (WATT AND BREYER-BRANDWIJK, 1962; VAN WYK *et al.*, 1997). In European countries it is used as a corrective, an expectorant, an antitussive and an antiulcer agent (HIKINO, 1985).

Polygala myrtifolia also showed a strong inhibition against the isolate from the 5-month-old baby with an MIC value of 3.25 $mgml^{-1}$ and MIC values of 6.25 $mgml^{-1}$ for both the isolate from an adult and the standard (ATCC 10231) strain. *P. myrtifolia* is an evergreen small tree of up to 4 m in height, with a dense crown. The leaves are soft and light green with rounded tip endings. The flowers form in short clusters at the tips of the branches. It is distributed from Zululand in the north to the Western Cape in the south of South Africa. This plant occurs in forests, scrubveld and wooded grasslands (VENTER AND VENTER, 1996). The leaves are used in poultices for gout. Aqueous extracts of the green parts of this plant tested negative with *S. aureus* (WATT AND BREYER-BRANDWIJK, 1962).

Amongst the organic solvent extracts tested, *Bidens pilosa* (ethanol), *Dodonaea angustifolia* (ethanol and ethyl acetate), *G. glabra* (ethanol and ethyl acetate), *P. myrtifolia* (ethanol), *Leonotis leonurus*, *Siphonochilus aethiopicus* (ethanol, ethyl acetate and hexane), *Tagetes minuta* (ethanol) and *T. violacea* (ethanol and ethyl acetate) extracts were active against all *Candida* strains (Table 3.3B).

Table 3.3A Minimum inhibitory concentration of aqueous extracts against *Candida albicans* clinical isolates from a 5-month-old baby, an adult, and the standard strain (ATCC 10231)

		MIC ($mgml^{-1}$)		
Plant	**Part**	**5-month-old baby**	**Adult**	**ATCC 10231**
Allium sativum	bulbs	0.56	6.25	6.25
Bidens pilosa	leaves	25	25	25
Brachanellia hulleensis	leaves	>25	>25	>25
Bulbine frutescens	leaves	>25	>25	>25
Carpobrotus edulis	leaves	>25	>25	>25
Dodonaea angustifolia	leaves	>25	>25	>25
Erythrina lysistemon	bark	25	>25	25
	leaves	25	>25	>25
Eucomis autumnalis	bulbs	>25	>25	>25
Glycyrrhiza glabra	rhizomes	1.56	12.5	12.5
Haemanthus albiflos	bulbs	>25	>25	>25
	leaves	>25	>25	>25
Hypoxis hemerocallidea	corms	>25	>25	>25
Jatropha curcas	leaves	25	25	25
Leonotis leonurus	leaves	12.5	>25	>25
Polygala myrtifolia	leaves	1.56	6.25	6.25
Plumbago auriculata	leaves	>25	>25	>25

Rubus rigidus	leaves	>25	>25	>25
	stem	12.5	>25	25
Siphonochilus aethiopicus	bulbs	>25	>25	>25
Tagetes minuta	leaves	25	25	25
Trichilia emetica	leaves	>25	>25	>25
	bark	6.25	25	25
Tulbaghia violacea	bark	3.25	6.25	12.5
	leaves	>25	>25	>25
Vertiveria zizaniodes	leaves	25	25	12.5
Warburgia salutaris	bark	6.25	12.5	12.5
	leaves [1]	>25	>25	>25
	leaves [2]	12.5	12.5	25
Zantedeschia aethiopica	leaves	>25	>25	>25
Zanthoxylum capense	bark	>25	>25	>25

1-University of Natal Botanical garden collection, 2- Silverglen collection.

Table 3.3B Minimum inhibitory concentration of organic solvent extracts against *Candida albicans* clinical isolates from a 5-month-old baby and an adult, and standard strain (ATCC 10231)

			MIC ($mgml^{-1}$)		
Plant	**Part**	**Extract**	**5-month-old baby**	**Adult**	**ATCC 10231**
Bidens pilosa	leaves	EtOH	2.09	2.09	2.09
		EtOAc	8.35	8.35	8.35
		Hex	>8.35	>8.35	>8.35
Brachanellia hulleensis	leaves	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Bulbine frutescens	leaves	EtOH	>8.35	>8.35	>8.35
		EtOAc	2.09	8.35	8.35
		Hex	>8.35	>8.35	>8.35
Carpobrotus edulis	leaves	EtOH	>8.35	>8.35	>8.35
		EtOAc	2.09	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Dodonaea angustifolia	leaves & twigs	EtOH	2.09	2.09	2.09
		EtOAc	1.04	1.04	2.09
		Hex	>8.35	>8.35	>8.35
Erythrina lysistemon	bark	EtOAc	8.35	8.35	8.35
		EtOH	8.35	8.35	8.35
		Hex	>8.35	>8.35	>8.35
	leaves	EtOH	8.35	>8.35	>8.35

		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Eucomis autumnalis	bulbs	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Glycyrrhiza glabra	rhizomes	EtOH	0.52	2.09	2.09
		EtOAc	2.09	1.03	2.09
		Hex	>8.35	>8.35	>8.35
Haemanthus albiflos	bulbs	EtOH	8.35	>8.35	>8.35
		EtOAc	8.35	>8.35	>8.35
		Hex	8.35	>8.35	>8.35
	leaves	EtOH	2.09	8.35	8.35
		EtOAc	2.09	8.35	8.35
		Hex	>8.35	>8.35	>8.35
Hypoxis hemerocallidea	corms	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Jatropha curcas	leaves	EtOH	8.35	8.35	8.35
		EtOAc	8.35	8.35	8.35
		Hex	8.35	8.35	8.35
Leonotis leonurus	leaves	EtOH	1.03	1.03	2.09
		EtOAc	1.03	1.03	2.09
		Hex	>8.35	>8.35	>8.35
Polygala myrtifolia	leaves	EtOH	0.52	8.35	8.35

		EtOAc	8.35	8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Plumbago auriculata	leaves	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Rubus rigidus	stem	EtOH	>8.35	>8.35	>8.35
		EtOAc	8.35	8.35	8.35
		Hex	>8.35	>8.35	>8.35
	Leaves	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Siphonochilus aethiopicus	bulbs	EtOH	1.03	1.03	2.09
		EtOAc	1.03	1.03	2.09
		Hex	1.03	1.03	2.09
Tagetes minuta	leaves	EtOH	2.09	2.09	4.18
		EtOAc	8.35	8.35	8.35
		Hex	>8.35	>8.35	>8.35
Trichilia emetica	bark	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
	leaves	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Tulbaghia violacea	bulbs	EtOH	0.26	2.09	2.09
		EtOAc	0.13	2.09	2.09

		Hex	8.35	8.35	8.35
	leaves	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Vertiveria	leaves	EtOH	>8.35	>8.35	>8.35
zizaniodes		EtOAc	8.35	8.35	8.35
		Hex	>8.35	>8.35	>8.35
Warburgia	bark	EtOH	4.18	8.35	8.35
salutaris		EtOAc	8.35	8.35	8.35
		Hex	4.18	4.18	4.18
	leaves[1]	EtOH	>8.35	>8.35	>8.35
		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
	leaves[2]	EtOH	8.35	8.35	8.35
		EtOAc	8.35	8.35	8.35
		Hex	4.18	4.18	4.18
Zantedeschia	leaves	EtOH	>8.35	>8.35	>8.35
aethiopica		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Zanthoxylum	leaves	EtOH	>8.35	>8.35	>8.35
capense		EtOAc	>8.35	>8.35	>8.35
		Hex	>8.35	>8.35	>8.35
Amphotericin B (standard)			**1.56^{-3}**	**>8.35**	**>8.35**

EtOH- ethanol, EtOAc- ethyl acetate, Hex- Hexane

1-University of Natal Botanical garden collection, 2- Silverglen collection.

CHAPTER 4

FINGERPRINTING OF ACTIVE PLANT EXTRACTS

4.1 INTRODUCTION

Thin layer chromatography (TLC) is the most widely used of all the simple chromatographic methods for analysis of plant extracts (WAGNER *et al.*, 1984). This is due to its rapidity and ease of visual evaluation, and has become the most ideal analytical method for plant drugs and preparations that contain drug extracts or pure constituents. Advantages of this method include the short time required for the detection of characteristic constituents of a drug. TLC also provides semi-quantitative information on the chief active constituents of a drug, thus enabling an assessment of drug quality, in addition to its qualitative detection (WAGNER *et al.*, 1984). It is suitable for the monitoring of identity and purity of drugs against adulteration and substitution, as it provides a chromatographic drug fingerprint, and can also be used to analyse drug combinations and phytochemical preparations (WAGNER *et al.*, 1984).

4.2 MATERIAL AND METHODS

4.2.1 Plant material

All the plant material, which showed activity with the water, ethanol, ethyl acetate and hexane extracts were chosen for fingerprinting.

4.2.2 Extraction of plant material

Plant material was dried in an oven at 50°C and subsequently finely ground and stored at room temperature in glass bottles. One hundred grams of ground material were extracted with 10 ml of water or organic solvents in an ultrasound bath for one hour and left to stand for 30 minutes. The extracts were filtered using Whatman No. 1 filter paper and taken to dryness in front of a desk fan. The residues were diluted in water and organic solvents to a concentration of 50 $mgml^{-1}$.

Fresh bulb extracts of *Allium sativum* and *Tulbaghia violacea* were prepared using 10 g of garlic cloves and *T. violacea* bulbs; these were blended with 50 ml water, ethanol, ethyl acetate, hexane and dichloromethane respectively.

4.2.3 Fingerprinting

Five hundred µg of water, ethanol, ethyl acetate and hexane extracts of selected plants were applied to glass backed silica gel plates (10x10 cm, 0.25 mm) for fingerprinting. The extracts were loaded as one cm bands and the plates were developed in suitable solvent systems. Table 4.2.1 summarizes the solvent systems used with the different plant extracts. The TLC plates were removed from the tank and allowed to dry. The bands were viewed under UV-light (254 nm and 366 nm) and photographed for reference.

Table 4.2.1 Solvent systems used for the fingerprinting of screened plant extracts

Plant extract	Solvent system	Ratios (v/v)
Allium sativum	Toluene: ethyl acetate	100:30
Glycyrrhiza glabra	Toluene: ethyl acetate	100:30
Polygala myrtifolia	Toluene: ethyl acetate	100:30
Tulbaghia violacea	Toluene: ethyl acetate	100:30
Siphonochilus aethiopicus	Hexane: ethyl acetate	2:1
Leonotis leonurus	Hexane: ethyl acetate	2:1

4.2.4 Bioautography

All the above TLC plates were run in duplicate. The plates were developed and allowed to stand overnight, as this allowed the solvents to evaporate. The bioautographic assay was carried out using the fungal strain ATCC 10231. Ten ml of the overnight culture were prepared. The cultures were mixed with 50 ml YM broth and placed in a dip tank. The developed TLC plates were then dipped in the tank and placed on damp paper towels in a metal tray. The trays were covered with cling wrap, and incubated in an oven at 33°C overnight or until growth was observed. To detect the antifungal activity on the plates, they were sprayed with INT (*p*-iodonitrotetrazolium-violet, Sigma) prepared at two $mgml^{-1}$. Five ml of the INT was sprayed onto the plates. Positive results are observed when clear white areas appear on the red background due to the reduction of INT by the fungi.

4.3 RESULTS AND DISCUSSION

Figure 4.3.1 illustrates the TLC chromatogram of *Allium sativum* viewed under $UV_{254\ nm}$; the bands were a distinct blue-green for all prepared extracts, varying in number of bands depending on the extracting solvent. The TLC chromatogram of *Tulbaghia violacea* yielded two visible bands each for the ethanol and ethyl acetate extracts viewed under $UV_{254\ nm}$ (Figure 4.3.2). The dichloromethane and hexane extracts did not separate out into visible bands.

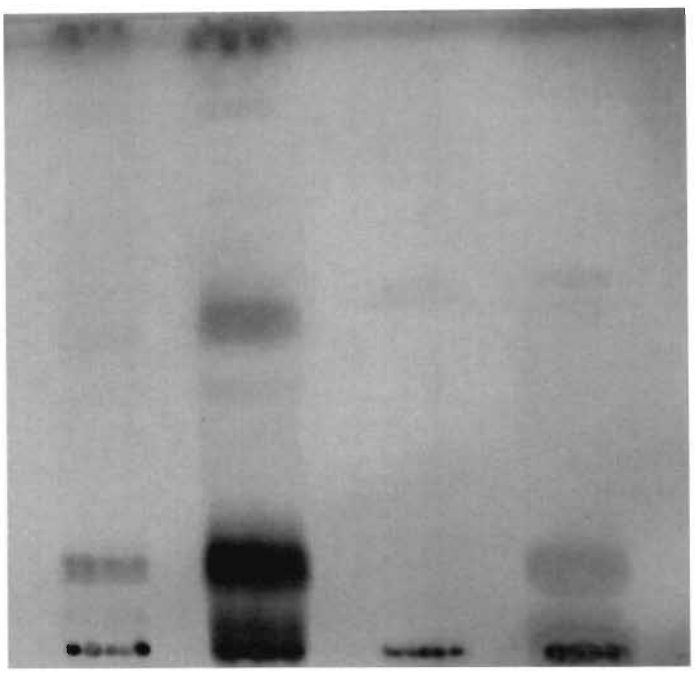

Figure 4.3.1 TLC chromatogram of *Allium sativum* extracts viewed under UV light (A) 254 nm. EtOH-ethanol; EtOAc-ethyl acetate; Hex-hexane; DiChl- dichloromethane.

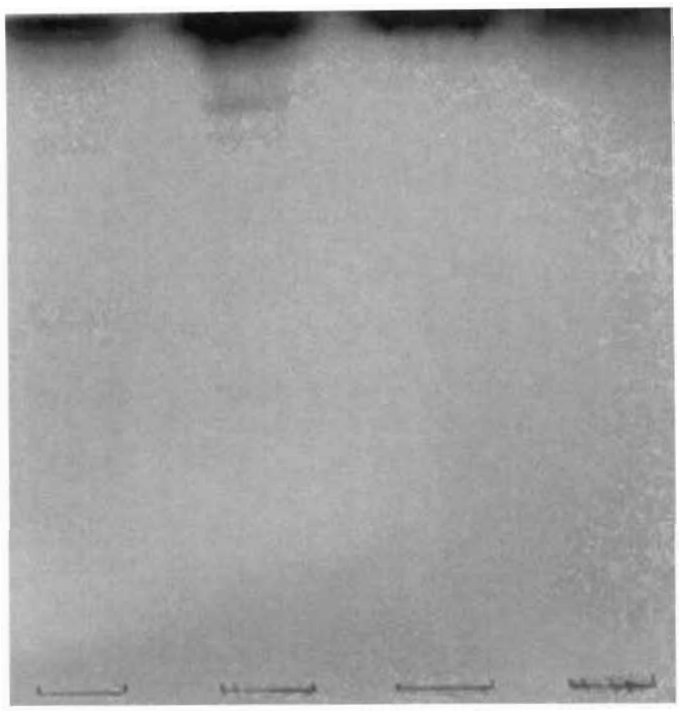

Figure 4.3.2 TLC chromatogram of *Tulbaghia violacea* extracts viewed under UV light 254 nm. EtOH-ethanol; EtOAc-ethyl acetate; Hex-hexane; DiChl- dichloromethane.

Palladium chloride is a stain specially used to detect allicin, which stains a brown-red colour (WAGNER, 1996). Allicin can be clearly identified on both the TLC chromatograms of *A. sativum* and *T. violacea* stained with this reagent (Figure 4.3.3A and Figure 4.3.4A) respectively. Allicin is one active principle of freshly crushed garlic homogenates. This main active constituent of garlic was isolated and identified in 1944. It is formed only when garlic gloves are minced or crushed as a result of enzyme action of its constituents (YAMADA AND AZUMA, 1977; HUGHES AND LAWSON, 1991). Allicin is an oxygenated sulphur compound, which is volatile and poorly miscible in aqueous solutions (ANKRI AND MIRELMAN, 1999). In pure form allicin exhibits antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria, including multi-drug-resistant entertoxicogenic strains of *Escherichial coli;* antifungal activity against *Candida albicans*; antiparisitic activity against some major human intestinal protozoans such as *Entamoeba histolytica* and *Giardia lamblia;* and also antiviral activity (ANKRI AND MIRELMAN, 1999).

Garlic extracts have strong antifungal effects; they inhibit the formation of mycotoxins like the aflatoxins of *Aspergillus parasiticus*. Allicin is the major component responsible for the inhibition of fungal growth, 34 % of concentrate garlic extracts is allicin and the remaining 44 % and 20 % comprising of thiosulfinates and vinyldinthiins respectively (ANKRI AND MIRELMAN, 1999). Garlic has also been used successfully in preventing and treating experimental infections with *Candida albicans* in chickens. Activity has also

been reported in human serum against seven species of *Candida* and two species of *Cryptococcus* (HUGHES AND LAWSON, 1991). Allicin is thought to disrupt cellular metabolism primarily by oxidation of essential thiols to the corresponding disulphide by the liable oxygen of allicin, thereby inactivating proteins. Another mechanism may be competitive inhibition of all sulphydryl compounds at low concentration. Non-competitive mechanisms of inhibiting enzymes of function, blockage of cellular lipid biosynthesis or decreasing fungi virulence through enhancement of yeast to mycelial conversion may also occur (RODE *et al.*, 1989).

E/Z-ajoene is the only water-soluble transformation compound of allicin and a minor component in oil-macerates of garlic and is known as a potent antithrombotic (SINGH *et al.*, 1990). This component also demonstrates anticandidal activity, although its activity is found to be less than that of thiosulphinates (YOSHIDA *et al.*, 1987; HUGHES AND LAWSON, 1991). E/Z-ajoene has also been found to inhibit spore germination of fungi, which cause diseases in crop plants (SINGH *et al.*, 1990). This compound however was found not to elicit antibacterial activity against Gram-positive and Gram - negative bacteria except for *Staphylococcus aureus* (YOSHIDA *et al.*, 1987)

Burton and Kaye (1992) isolated two sulphur compounds from *T. violacea*. As it is a general occurrence in the Alliaceae family and in *Allium* species, *T. violacea* was found to contain low molecular mass sulphur compounds. The two unidentified compounds in *T. violacea* therefore supported the modern

classification, which includes *Tulbaghia* species in the Alliaceae family (BURTON AND KAYE, 1992). The medical activity of sulphur compounds such as the activity of allicin in garlic was well established and observed in the bacteriostatic action of *T. violacea* extracts, and is attributed to the biologically active sulphur compounds (BURTON AND KAYE, 1992).

Duplicates of the palladium stained plates of *A. sativum* and *T. violacea* where prepared for bioautography, with *Candida albicans* (ATCC 10231) culture overlay. The inhibition of *C. albicans* on these plates is clearly seen by the contrast of white bands corresponding to separated bands of the extracts on the red background of the plates (Figure 4.3.3B and Figure 4.3.4B) respectively. The fungus uses INT as a terminal electron acceptor and is reduced to a white colour where it had grown, the red background therefore illustrating fungal growth. There are white bands on the INT stained plates, which correspond allicin bands on the palladium chloride stained chromatograms.

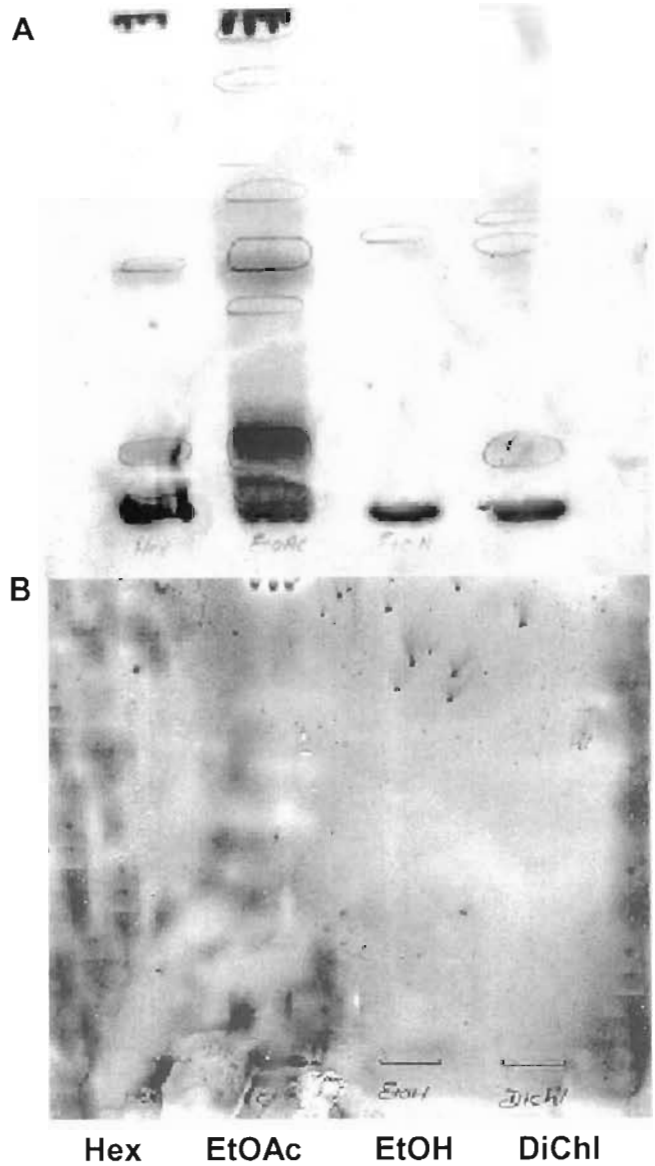

Figure 4.3.3 TLC fingerprinting of solvent extracts of *Allium sativum* (A) stained with palladium chloride. (B) Bioautographic plate with *Candida albicans* (ATCC 10231) after staining with INT, white spots indicate inhibition of fungal growth. Hex- hexane; EtOAc-ethyl acetate; EtOH-ethanol; DiChl-dichloromethane.

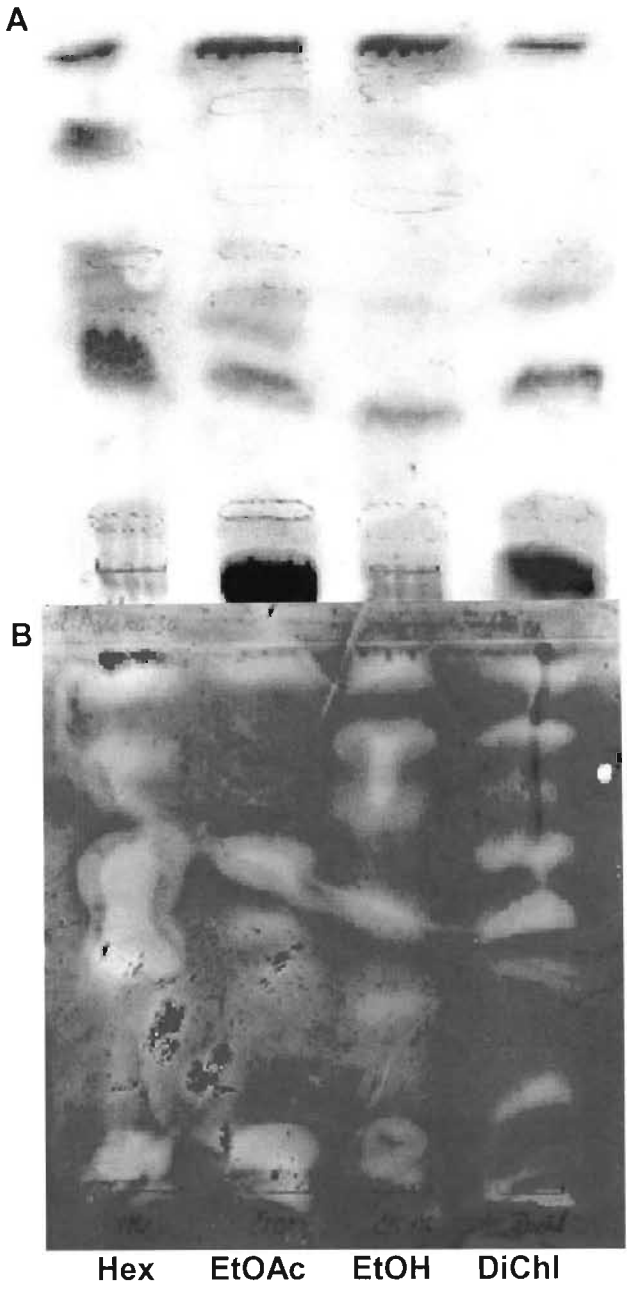

Figure 4.3.4 TLC fingerprinting of solvent extracts of *Tulbaghia violacea* (A) stained with palladium chloride. (B) Bioautographic plate with *Candida albicans* (ATCC 10231) after staining with INT, white spots indicate inhibition of fungal growth. Hex- hexane; EtOAc-ethyl acetate; EtOH-ethanol; DiChl-dichloromethane.

Glycyrrhiza glabra and *Polygala myrtifolia* are two other highly active plants, which were prepared for fingerprinting and bioautography. The water extracts of these two plants showed presence of saponins. Saponins were identified by formation of foam in the preparation of the extracts. These compounds constitute a vast group of glycosides, which are ubiquitous in plants. They are characterized by their surface-active properties and they dissolve in water to form foamy solutions. This surface activity is the reason why some drugs containing saponins have been used for ages as household detergents. Some saponins have haemolytic properties and are widely distributed in plants. Their industrial applications include starting materials for the semi-synthesis of steroidal drugs (BRUNETON, 1995). Other biological and pharmacological properties of saponins *in vivo* are known to be the defence mechanism, which plants have against fungal attack (BRUNETON, 1995). The activity against fungi has been established against pathogenic species, as well as towards various *Candida* species and dermatophytes. Their activity is attributed to monodesmosides, which are thought to result from the reaction of the saponins with the membrane sterols of the microorganism (BRUNETON, 1995). *Saponaris officinalis* is one plant, which has been widely used for its biologically active saponins (BRUNETON, 1995).

TLC fingerprints of *G. glabra* and *P. myrtifolia* were prepared from the aqueous extracts. Separation of the two water extracts is clearly seen in (Figure 4.3.5A) viewed under $UV_{254\ nm}$. Although both of the extracts showed characteristics of saponins the appearance to the bands showed that *P.*

myrtifolia separated into pink compounds whilst *G. glabra* separated into blue and white compounds. The same plate viewed at $UV_{366\ nm}$, also illustrates the bands in different colours, with those of *G. glabra* appearing a blue-green colour and those of *P. myrtifolia* appearing yellow-green colour and one major blue compound (Figure 4.3.5B).

G. glabra contains a variety of constituents, but its clinical effectiveness may be exerted mainly by three groups of substances: saponins, flavonoids and polysaccharides. Glycyrrhizin is one of the main components of liquorice root. It consists of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid. The liquorice saponins consist of one major component, glycyrrhizin (glycyrrhizin acid), and 13 minor components whose proportions vary depending on the species and collecting location. Glycyrrhizin is responsible for the liquorice-like sweetness and is 50 times sweeter than sucrose. This main saponin is present in concentrations of 2%- 6% (VAN WYK *et al.*, 1997). This property accounts for the widespread use of this crude drug in many composite prescriptions. It is thought that liquorice is effective for sore throat due to its sweetness and the acceleration of tracheal mucous secretion mediated by the saponin glycyrrhizin. This also explains its use as an expectorant and antitussive (HIKINO, 1985). Glycyrrhizin has anti-inflammatory activity and also weak antiviral, antibacterial, antihepatoxic, immunostimulating and healing activities (VAN WYK *et al.*, 1997).

Literature on the antibacterial and antifungal properties being identified in *P. myrtifolia* is not available, but other species of *Polygala* are known for the presence of antibacterial and antifungal compounds. A chromonocoumarin derivative was isolated from *Polygala fruticosa* and found to have antifungal activity (VAN WYK *et al.*, 1997). Chesne *et al.* (1983) conducted a study on the fungicidal activity of higher plants. Amongst the plants screened the aerial parts of *Polygala vulgaris* showed the presence of alkaloids, phenolic acids, flavonoids, tannins and saponins, but the fungicidal effect of the crude extracts was attributed to saponins.

Highlanders of Malawi and bordering countries use *Polygala nyikensis* to treat various skin diseases of fungal origin. Two antifungal xanthones were isolated from *Polygala nyikensis* and are active against the plant pathogen *Cladosporium cucumerinum* (MARSTON *et al.*, 1993). *Polygala senega* is one species, which has been highly studied. A drug has been developed form this plant and is known to contain lipids, methyl salicylate, phenolic acids and 5%-10 % saponins. The saponins present are glycoside of presenegin and the chief constituents are bidesmoides, however, the drug is mostly used as a treatment for coughs (BRUNETON, 1995).

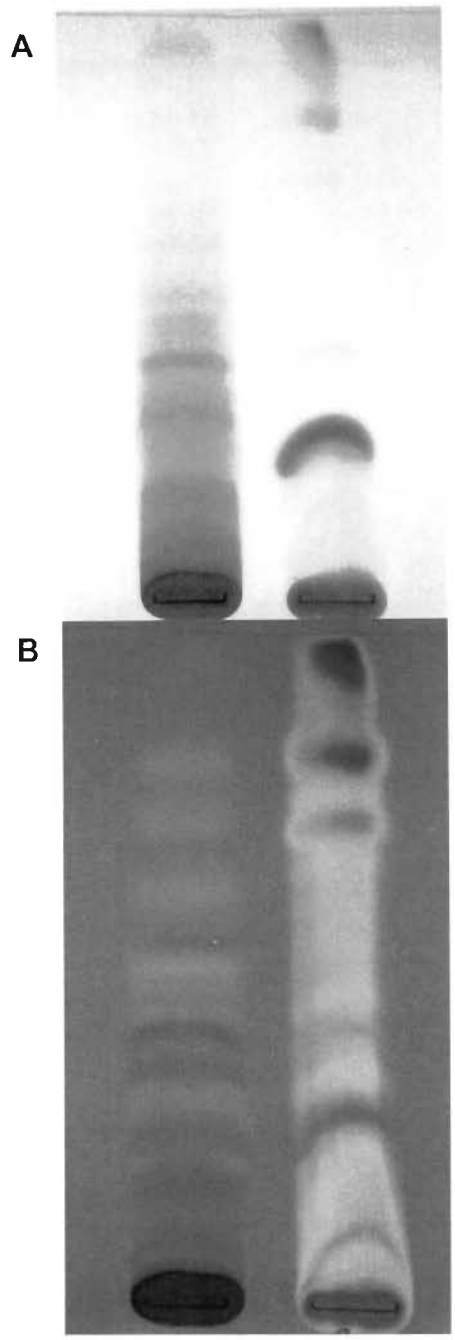

Figure 4.3.5 TLC chromatogram of *Glycyrrhiza glabra* and *Polygala myrtifolia* water extracts viewed under UV light (A) 254 nm; (B) 366 nm.

Extracts of *Leonotis leonurus, Siphonochilus aethiopicus* are a few of the organic solvent extracts, which showed activity. Figure 4.3.6A illustrates the TLC plate of *L. leonurus* viewed under UV_{254} nm, here the bands appear blue-green in colour with a distinct blue band which is similar in all three extracts. Figure 4.3.6B on the other hand is a view of the same plate at $UV_{366\ nm}$, the compounds separated into pink bands for the ethanolic extract and the ethyl acetate extract whilst the hexane extracts showed bands separating into white. From the TLC chromatograms of the above plant extracts it is clearly seen that hexane is a poor solvent to use for extracting compounds as fewer compounds were resolved. Antifungal activity has been established against ethanolic extracts of *Leonotis neptaefolia.*

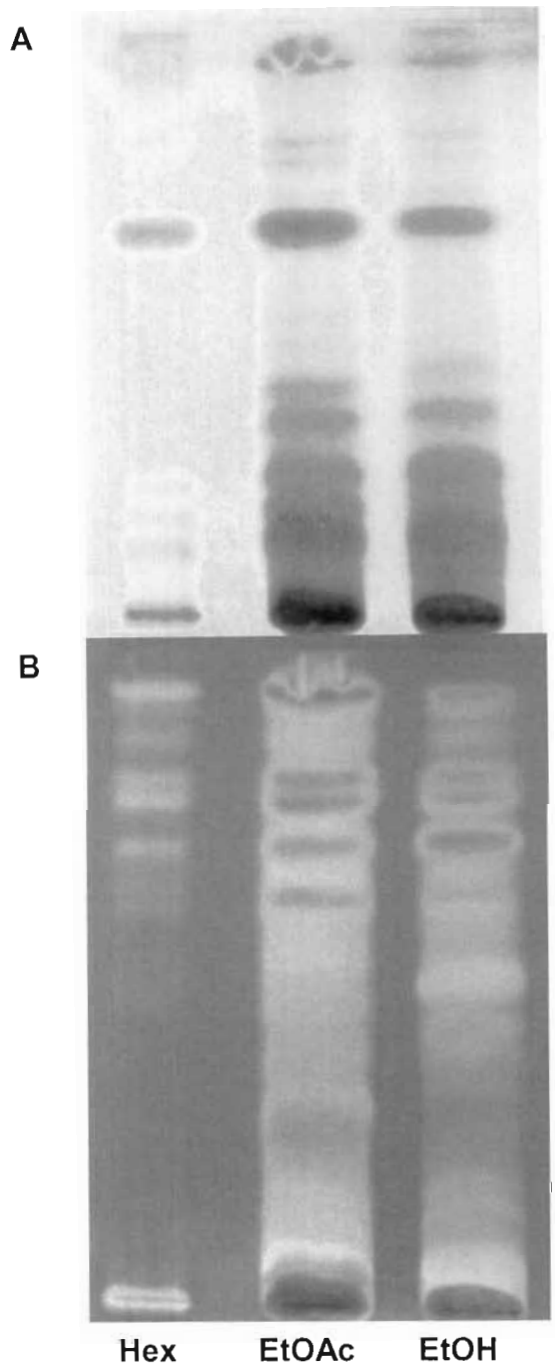

Figure 4.3.6 TLC chromatogram of *Leonotis leonurus* extracts viewed under UV light (A) 254 nm; (B) 366 nm. EtOH-ethanol; EtOAc-ethyl acetate; Hex-hexane.

The TLC chromatogram of *S. aethiopicus* (Figure 4.3.7) shows separation of compounds into blue and green bands viewed under $UV_{366\ nm}$. The ethanolic and the ethyl acetate extracts showed identical band separation, whilst the hexane extract showed separation of a few compounds, which also corresponded to those of the other two extracts. Amongst other recorded uses, *S. aethiopicus* underground parts are traditionally used for treatment of asthma and dysmennorhoea (WATT AND BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996). Rhizomes have also been found to be antibacterially active against numerous Gram-negative and Gram-positive bacteria (ZSCHOCKE *et al.*, 2000). Although the compounds responsible for the antifungal properties have not been identified, they are assumed to be the same ones responsible for the antibacterial activity.

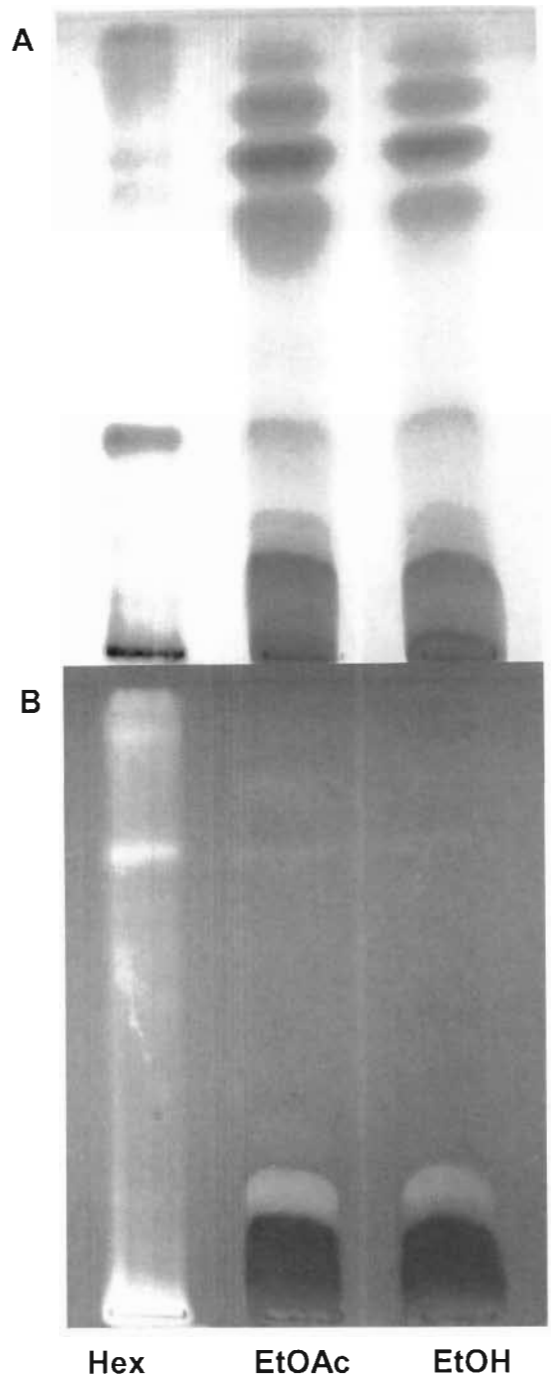

Figure 4.3.7 TLC chromatogram of *Siphonochilus aethiopicus* extracts viewed under UV light (A) 254 nm; (B) 366 nm. EtOH-ethanol; EtOAc-ethyl acetate; Hex-hexane.

CHAPTER 5

STABILITY TESTING OF ACTIVE PLANT EXTRACTS

5.1 INTRODUCTION

For the clinical use of plant extracts for candidiasis, either in hospitals or for home care, it is essential to know how long the extracts can be stored without losing of activity. Since chemical breakdown is temperature dependant, it would be necessary to take into consideration the storage condition in which home care products would be kept under, especially during hot summer days.

5.2 MATERIALS AND METHODS

5.2.1 Stability Testing

Stability tests were carried out on aqueous extracts of *Allium sativum*, *Glycyrrhiza glabra*, *Polygala myrtifolia* and *Tulbaghia violacea*. The aqueous extracts were prepared by diluting dried extracts to 100mg/ml. The diluted extracts were divided into three parts and stored at 4°C, 23 °C and 33°C (ambient temperature in Zululand) respectively. All the extracts were assayed for antifungal activity over a period of seven days.

5.2.2 Boiled extracts

Activity of boiled extracts was determined using the following procedure: One gram of fresh plant material was placed in a volumetric (250 ml), containing 100 ml distilled water. The volumetric flask was closed with a glass funnel to prevent over boiling. All prepared extracts were boiled for 10 minutes, then cooled and filtered using Whatman No. 1 filter paper.

5.2.3 Masking Tests

Allium sativum and *Tulbaghia violacea* extracts were prepared as in Chapter 3. One millilitre of each was added to a one ml sucrose solution and one ml of

flavouring (Robertsons' mint, strawberry and vanilla). These masked extracts where then tested for antifungal activity.

5.3 RESULTS AND DISCUSSION

Stability testing of extracts was performed in order to determine the activity of the extracts in solution over a period of time. Aqueous extracts were chosen for the stability testing given that a formulation would have to be prepared by patients in their homes and it would also need to be an easy and safe preparation. *Allium sativum* extracts remained active in solution for three days at all three temperatures. However, it was evident that the extract stored at 4°C remained stable for considerably longer periods than the extracts stored at 23°C and 33°C (Figure 5.3.1). When garlic is extracted at room temperature the major product is allicin (alkyl-2propene–thiosulphinate) which is formed by enzymatic action on alliin in the crushed tissue, and which has marked antibacterial, antiviral and antifungal activity. Allicin is very unstable and can undergo a variety of transformations, which results in products such as the ajoenes (BURTON AND KAYE, 1992). This therefore leads to stored extract solutions loosing their activity with time, as the allicin is transformed. The findings in this study confirms the work performed by Davis *et al.* (1994), where concentrated garlic extracts stored at low temperatures retained activity for a week and there after lost activity significantly. Extracts where biologically stable when frozen, but lost three-fourths of their biological potency when held at room temperature (DAVIS *et al.*, 1994).

Tulbaghia violacea remained stable in solution for an average of two days at the different temperatures (Figure 5.3.2). As with *A. sativum,* the extracts of *T. violacea* stored at 4°C remained stable for more days than those stored at

23°C and 33°C. *T. violacea* is also known to contain low molecular mass compounds which possess bacteriostatic activity. This activity is attributed to the sulphur compounds (BURTON AND KAYE, 1992). *T. violacea* has been reported to have presence of a carbon-sulphur lyase enzyme with similar action to that present in *Allium cepa* and *A. sativum,* which is hydrolysed to alkyl thiosulphinate and responsible for the biological activity (BURTON AND KAYE, 1992).

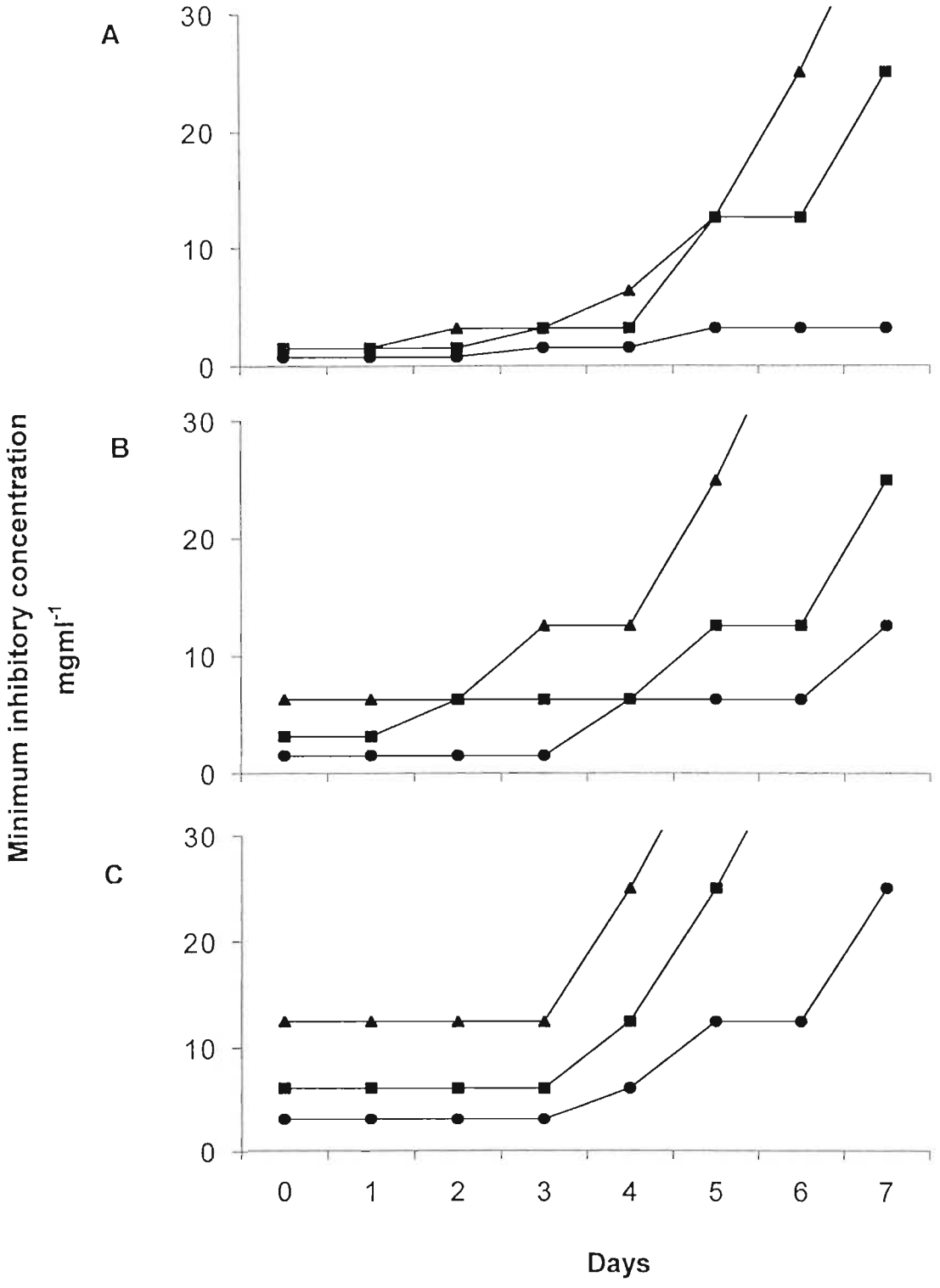

Figure 5.3.1 Stability of *Allium sativum* aqueous extracts over a one-week period against *Candida albicans* strains at different temperatures. (A) 5-month-old baby strain, (B) adult strain, (C) ATCC 10231. (circle) 4°C, (square) 23°C and (triangle) 33°C.

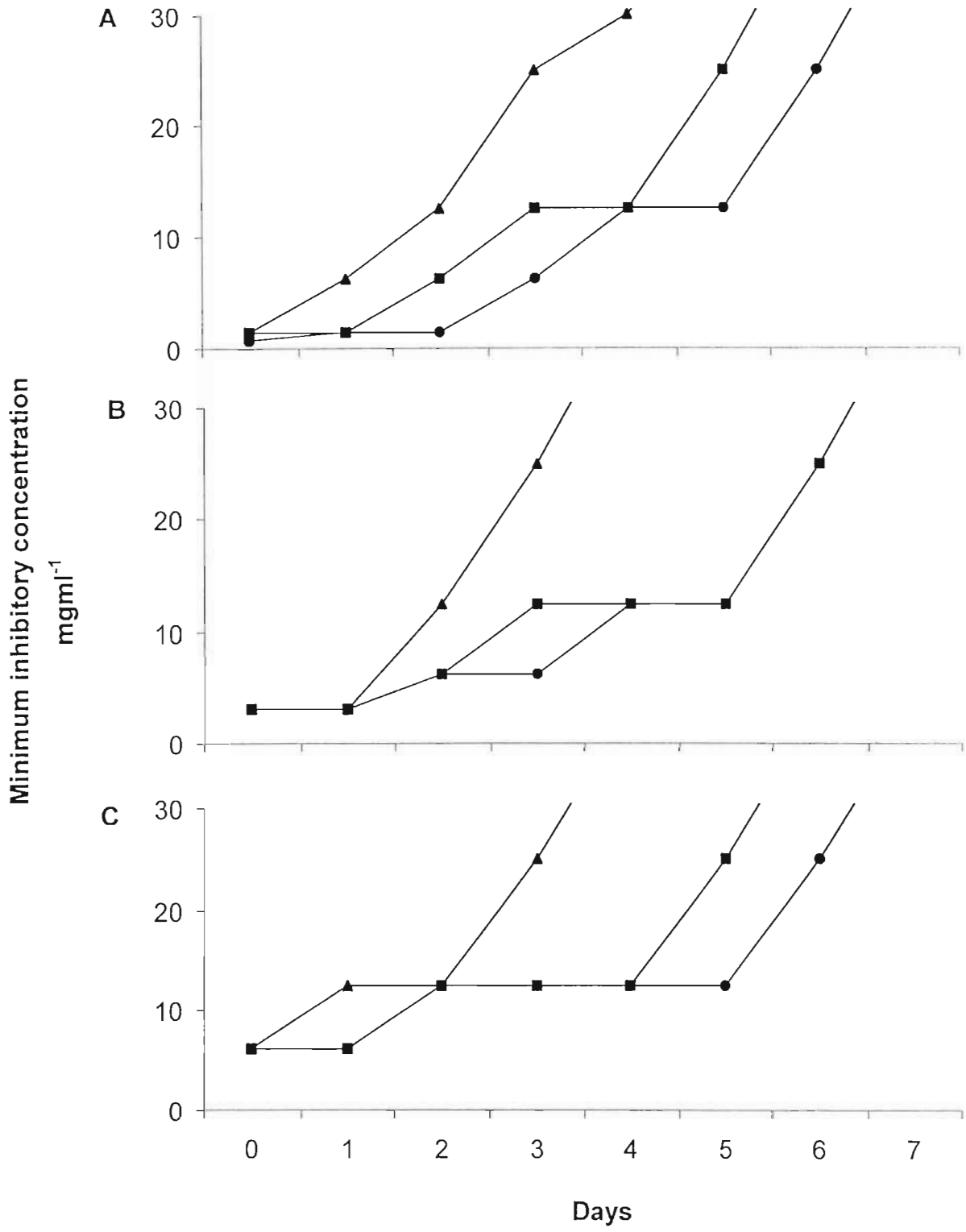

Figure 5.3.1 Stability of *Tulbaghia violacea* aqueous extracts over a one-week period against *Candida albicans* strains at different temperatures. (A) 5-month-old baby strain, (B) adult strain, (C) ATCC 10231. (circle) 4°C, (square) 23°C and (triangle) 33°C.

On the other hand *G. glabra* and *P. myrtifolia* were less stable. Both extracts lost activity within a 24 h period when stored non-refrigerated.

The results show that it would be necessary to prepare fresh extracts of *G. glabra* and *P. myrtifolia* on a daily basis, whereas extracts of *A. sativum* could be stored for two days and those of *T. violacea* for three to four days depending on the temperature of storage area. It would obviously be advantageous to be able to store the extracts for longer periods of time, and further the extracts of *T. violacea* would be preferred based on their stability in solution.

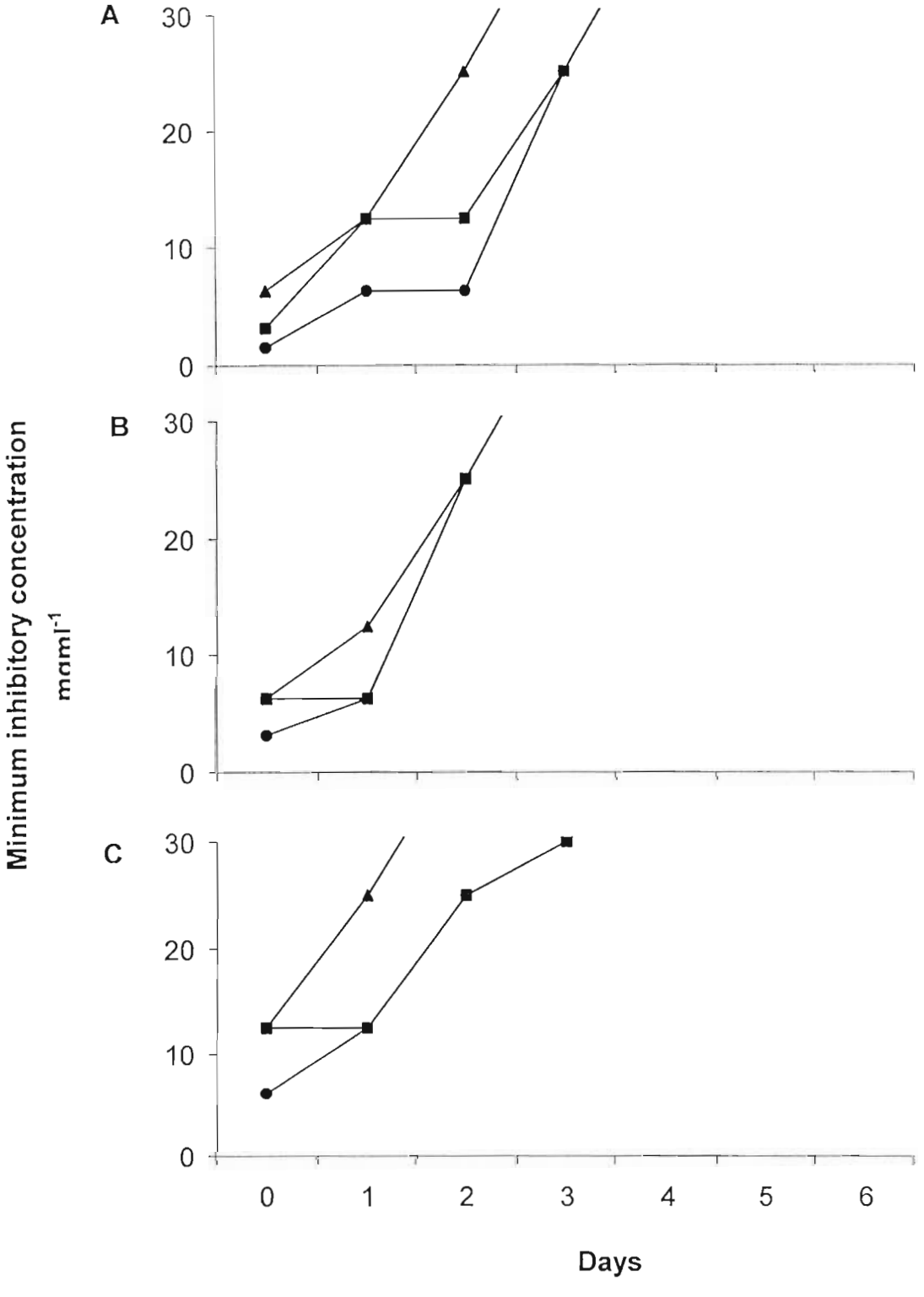

Figure 5.3.1 Stability of *Glycyrrhiza glabra* aqueous extracts over a one-week period against *Candida albicans* strains at different temperatures. (A) 5-month-old baby strain, (B) adult strain, (C) ATCC 10231. (circle) 4°C, (square) 23°C and (triangle) 33°C.

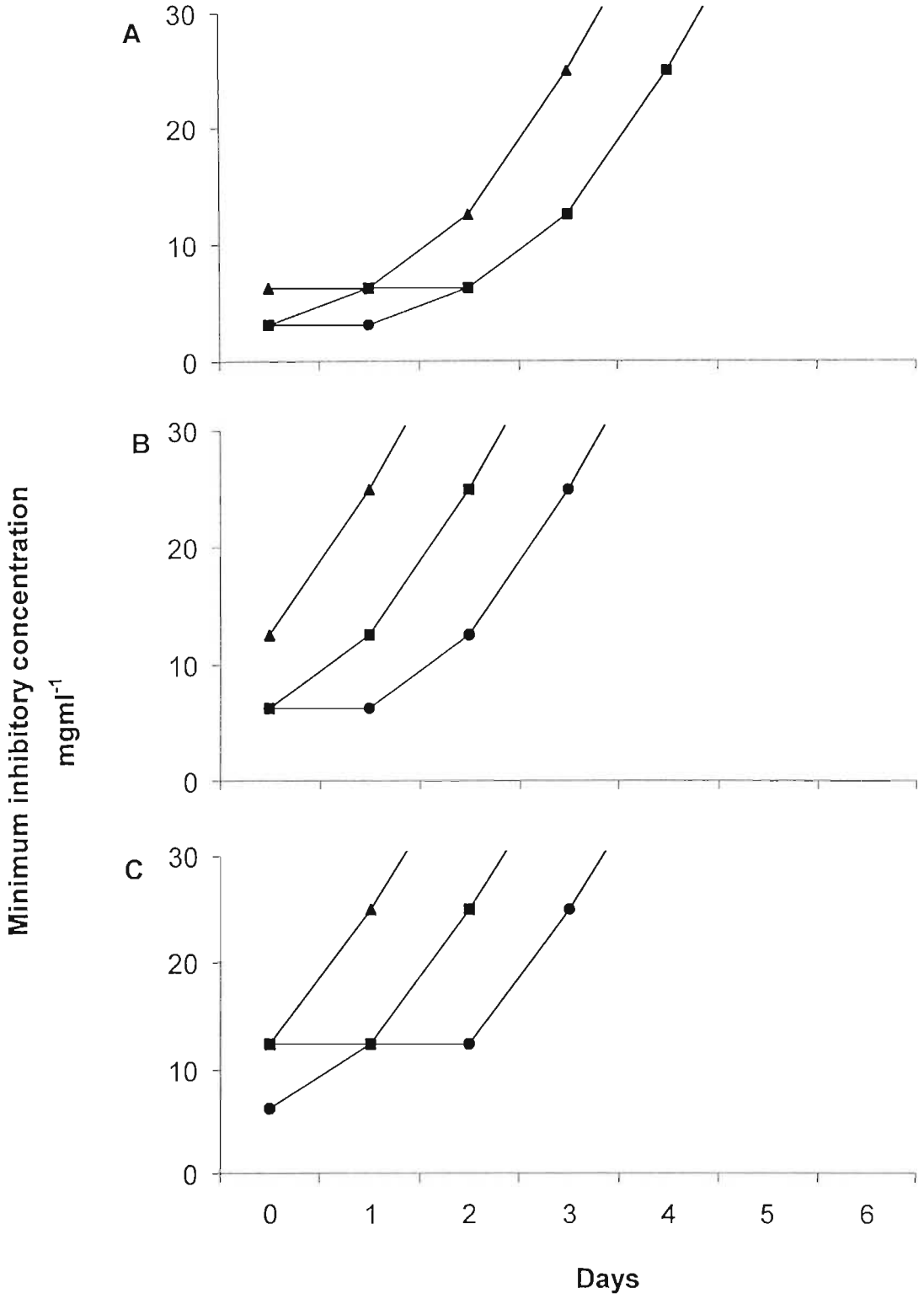

Figure 5.3.1 Stability of *Polygala myrtifolia* aqueous extracts over a one-week period against *Candida albicans* strains at different temperatures. (A) 5-month-old baby strain, (B) adult strain, (C) ATCC 10231. (circle) 4°C, (square) 23°C and (triangle) 33°C.

Boiled extracts

Difference in activity was evident with the boiled and fresh extracts of *A. sativum* (Figure 5.3.5). For all the three strains tested the activity of the boiled extracts was found to have MIC values of >25 mg/ml respectively, whilst fresh extracts had MIC values of 0.78 mg/ml, 3.25 mg/ml and 6.25 mg/ml for the strain from a 5-month-old, strain from an adult and standard (ATCC 10231) strain respectively.

Results obtained for *Polygala myrtifolia* boiled and fresh leaf extracts were similar to those observed in *A. sativum*, where the MIC values increased after boiling The MIC value increased after boiling (Figure 5.3.6). Boiling of the extracts could possibly inactivate the enzymes that breakdown saponins, the presumed active agents in *P. myrtifolia*, therefore leading to reduced activity in extracts.

Masking tests

Addition of sucrose and flavouring enhanced the extracts of *A. sativum* and *T. violacea* in sweetness, but this did not change the aftertaste and smell left by the active sulphur compound. Anticandidal activity was performed on the adulterated extracts. These extracts maintained activity without change in the MIC values when screened (Table 3.3.1). Extracts of *P. myrtifolia* did not have

an unpleasant taste or smell and extracts of *G. glabra* had a natural pleasant taste of liquorice, and therefore did not require masking.

The stigma attached to being infected with HIV/AIDS in the community lead to the attempt to masking the sulphur containing plant extracts. Those infected by the virus would have to remain unknown to the public and therefore, the failure to mask the two garlic extracts lead to the consideration of *G. glabra* and *P. myrtifolia* to be used in the clinical trials.

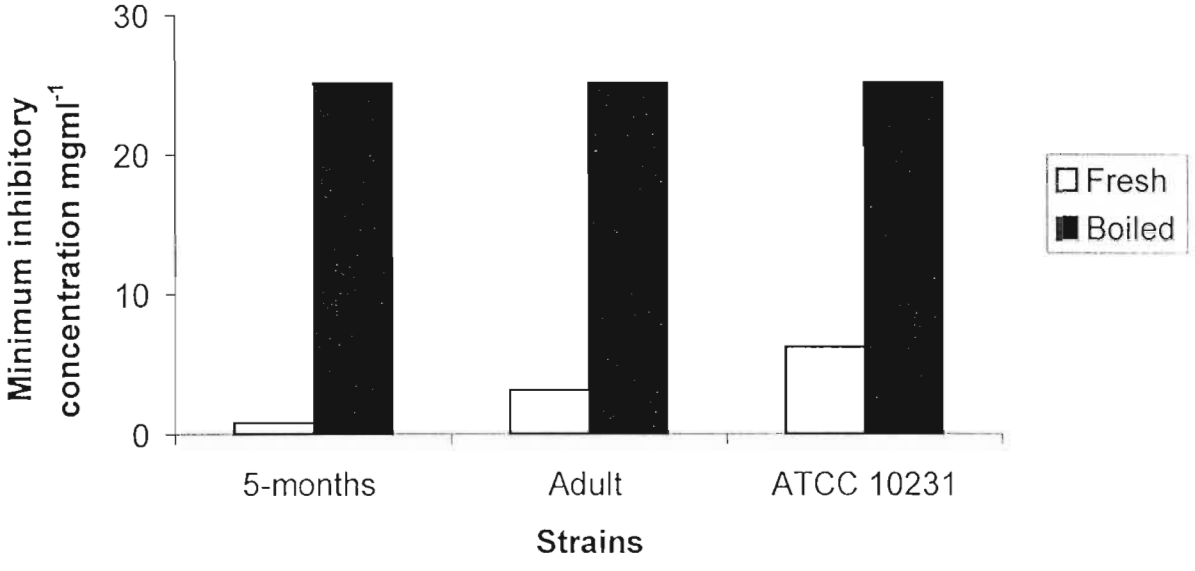

Figure 5.3.5 Antifungal activity of *Allium sativum* fresh aqueous and boiled extracts against *Candida albicans.*

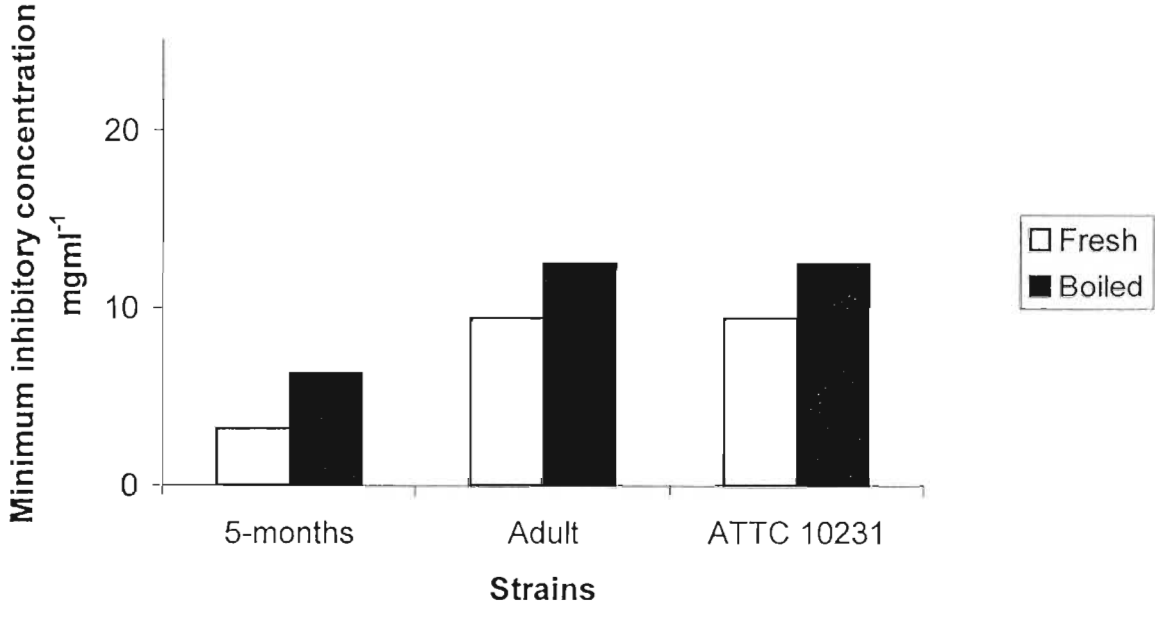

Figure 5.3.6 Antifungal activity of *Polygala myrtifolia* fresh aqueous extracts and boiled extracts against *Candida albicans*.

CHAPTER 6

CONCLUSION

An anticandidal assay was successfully set up and 24 traditionally used South African plants were screened against *Candida albicans*. *Allium sativum, Tulbaghia violacea, Glycyrrhiza glabra* and *Polygala myrtifolia* had the best activity. *A. sativum* and *T. violacea* were the most stable in solution when stored at a lower temperature of 4°C. However, the smell and bitter taste of garlic could however not be masked from both extracts. This odour leads to HIV/AIDS patients being identified. Due to the stigma attached to being infected by HIV/AIDS identification of patients by community members is not advised. These two plant extracts therefore had to be eliminated from use as mouthwash. *Glycyrrhiza glabra* and *Polygala myrtifolia* on the other hand did not need masking due to their pleasant smell and taste; *G. glabra* with the natural pleasant taste and smell of liquorice. Although these saponin containing extracts were not stable in solution for periods over 24 hours, they where however further investigated for use as mouthwash in homes and hospitals.

The presence of saponins and the assumption of these compounds as the active constituents in both *Glycyrrhiza glabra* and *Polygala myrtifolia* extracts, support the study of anticandidal compounds on other plant species known to contain saponins. The screening of *Glycyrrhiza glabra* for anticandidal activity is a further indication of its vast biological importance. Saponins in *P. myrtifolia* and its ability

to inhibit *Candida albicans* and its use as an anticandidal formulation could be justified by the use of *Polygala fruticosa* as an antifungal agent in traditional medicine.

Performance of stability tests on the extracts was of crucial importance to enable users to know the effects of temperature on the formulation and therefore know the precautions to take when preparations are made. After determining the stability status of the extracts, they were all found to maintain activity better when stored at lower temperatures. This was therefore an important step in determining the fact that daily preparations of extracts would have to be made.

Findings of this project could therefore lead to further studies on plants with anticandidal activity, paying special attention to species in the Alliaceae family and those which contain saponins. Secondly findings from this project could lead to the specific isolation of the active constituents from each active plant extract. Most importantly, findings from this project are a source of alternative antifungal remedies for HIV/AIDS patients, who cannot afford presently available antifungal agents and to those who find presently available antifungal agents ineffective.

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APPENDIX

McFarland Standard Solution

Solution A

1.75 g Barium chloride made up to 100 ml distilled water.

Solution B

1 ml sulphuric acid made up to 100 ml of distilled water

Add 0.5 ml Solution A with 99.5 ml Solution B, mix well and then dispense into a sealed container. Store in a dark place at room temperature. The solution should be replaced every 3 months.