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ANTIFUNGAL ACTIVITY OF EXTRACTS FROM SELECTED KENYAN MEDICINAL PLANTS

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A THESIS SUBMITTED IN FULFILMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE UNIVERSITY OF NAIROBI

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This thesis is my original work and has not been presented for any degree at this or any other University.

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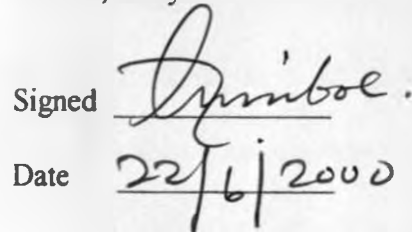

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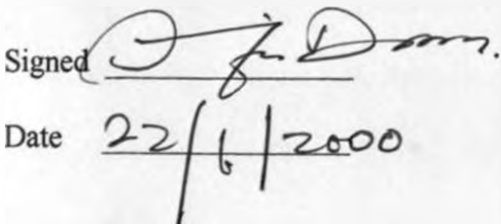

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DEDICATION

Dedicated to my parents, Mrs Selmith M'Ariba and the late Mr. M'Ariba M'Naituri who valued the search of knowledge above most things, and my husband Ndege who was always there. My daughter Lulu who always smiled even when I got home late from the laboratory takes the bigger share.

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ABSTRACT

The use of medicinal herbs in the treatment of diseases is an age-old practice. In Kenya several plant species are used by many ethnic groups for treatment of various diseases raging from minor infections to asthma, dysentery, skin diseases and a horde of others.

To establish the claimed therapeutic efficacy of some commonly used medicinal plants, *in vitro* antifungal activity tests of extracts from 28 Kenyan medicinal plant species was carried out. The activity of the extracts was studied on three human pathogens, *Trichophyton mentagrophytes* Robin, *Microsporum gypseum* Bodin and *Candida albicans* (Robin) Berkh. and one plant pathogenic fungus *Cladosporium cucumerinum* Ellis and Arth.. The disc assay method was used for the evaluation of fungitoxicity. Bioactivity guided fractionation was done for extracts that showed measurable activity.

Extracts from 17 plant species were variably active against *Trichophyton mentagrophytes, Microsporum gypseum*, *Candida albicans* and *Cladosporium cucumerinum*. The plant species included, *Acacia nilotica* (L.) Del, *Acacia senegal* (L.) Willd, *Adenia volkensii* Harms, *Ajuga remota* Benth., *Aporrhiza paniculata* Radlk., *Aspilia pluriseta* Schwieinf. Ex. Engl., *Azadirachta indica* A. Juss., *Senna didymobotrya* (Fresen.) Irwin and Barneby, *Commiphora*

africana (A. Rich.) Engl., *Hymenodictyon parvifolium* Oliv., *Plectranthus barbatus* Andr., *Plectranthus cylindraceus* Benth., *Premna resinosa* (Hochst Schauer, *Schizozygia coffaeoides* Baill, *Solanum arundo* Mattei, *Solanum nigrum* L. and *Tagetes minuta* L. Bioactivity guided fractionation led to the isolation of a potent antifungal compound from *Schizozygia coffaeoides* Baill. From spectroscopic and chemical analysis, the compound was determined as an indole alkaloid with a molecular formula $C_{21}H_{22}N_2O_4$ .The compound was fungistatic in action and affected the filamentous and yeast fungi by causing swelling and gloss morphological distortions.

From the results of this study, it is evident that some Kenyan medicinal plants have properties which could be exploited for the management of pathogenic fungi in humans and plants.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Some Problems Caused by Fungi

Since time immemorial humankind has had to struggle against several plant and animal diseases that affect his health and economic well-being. Quite a number of these diseases are caused by fungi, and there is substantive evidence that fungi have caused great suffering to humans both in the past and present. For example, some of the world's great famines can be blamed on pathogenic fungi. The great Irish famine of 1845-1847 was caused by the potato blight fungus *Phytophthora infestans* (Mont.) de Bary that completely destroyed the potato crop resulting in the death of more than one million people by starvation (Carefoot and Sprott 1967; Large 1940). The Brown spot of rice caused by *Helminthosporium oryzae* Breda de Haan caused loses of 40% to over 90% of rice crop in Bengal in 1942 and about 2 million people died of starvation (Strange 1993). Another fungus *Helminthosporium maydis* Nisikado caused loses of between 50% and 100% of corn crop in USA in 1971 (Ullstrup 1972).

Apart from damage to staple crops that has sometimes caused suffering and death by starvation, fungi have also caused loses in cash crops thereby affecting the economic well-being of mankind. A case in point is the effects on coffee of two fungal pathogens, *Colletotrichum coffeanum* Naock. the causal agent of Coffee Berry Disease, and the Coffee rust fungus *Hemilea vastatrix* Bark & Br. These two fungi

greatly reduce the value of coffee both in terms of yield and quality (Griffiths *et al* 1971; Schieber 1972).

Apart from the indirect effects, fungi also affect the human body directly. For example, *Aspergillus* species are a major cause of deterioration and spoilage amongst a wide range of stored materials particularly foodstuffs. Many species also produce mycotoxins harmful to the consumer, and exposure to their spores may result in respiratory allergies (Kozkiewicz 1989). Another effect of fungi directly on the human body includes dermatomycoses on the keratinized layers of the body caused by fungi belonging to the genera *Epidermophyton*, *Microsporum*, and *Trichophyton*. Dermatomycoses are a common affliction in the humid tropics and although they may not cause death, they cause serious discomfort and disfiguration (Campbel and Stewart 1980). Systemic fungal infections such as Cryptococcosis and Histoplasmosis caused by *Cryptococcus neoformans* Drechs. and *Histoplasma capsulatum* Darl. respectively are common opportunistic infections which have been reported to cause death in about 6-10% of HIV infected patients (Saag 1997). Further, oral lesions have been recognised as prominent features of Acquired Immunodeficiency Syndrome infection since the beginning of the epidemic and it is estimated that about 5.8% of people with AIDS have oral lesions caused by *Candida albicans* (Robin) Berkh. (Greenspan and Greenspan 1997).

To minimise all fungal problems antifungal agents are used. However, these antifungal agents are not without their disadvantages. Several have been found to cause considerable damage to both animals and plants. Some antifungal agents such as ketoconazole, useful in the treatment of cutaneous and systemic fungal diseases

has been known to have serious side effects such as liver damage (Fromtling *et al.* 1984). Another drug, amphotericin B, used in the treatment of systemic fungal diseases, is highly toxic (Colombo *et al.*1994). Many of the fungicides used in agriculture are also highly toxic, non biodegradable and therefore pollute the general environment (Menn 1983; McLaren 1986).

There is increasing occurrence of opportunistic systemic mycoses and a high rate of growing cases of microbial resistance to the time-honoured antibiotics. All these factors indicate that there is an urgent need to search for alternative, safe and biodegradable antifungal agents possibly of plant origin. This study was undertaken to contribute towards this goal.

1.2 The search for drugs and drug leads from higher plants

Plants synthesise a wide range of bioactive compounds which provide them with protection against external aggression from bacteria, fungi, viruses and insect pests (Mandava 1985). This principle suggests that plants offer a rich source of biologically active substances which can be harnessed to protect humans, livestock and crop plants against microbial diseases (McLaren 1986). Indeed medicinal herbs have been, and continue to be used for treatment of many diseases. The use of medicinal plants can be traced back many generations even in places where modern medicine has developed and completely overshadowed such practises (Hamburger *et al.* 1991). In a number of countries such as India, China and Australia, pharmaceutical companies are already marketing preparations of tablets, suspensions and capsules made directly from plant extracts for the treatment of specific diseases such as hepatitis, malaria,

cancer, allergy and opportunistic infections related to AIDS (Plotkin 1988; Decosterd *et al* 1991; Abrams 1997). In many industrialised countries, substances derived from higher plants constitute approximately 25% of the prescribed drugs (Principe 1989; Farnsworth and Bingel 1977). Natural products are also being used directly in their crude natural form in the "natural pharmaceutical industry" that is growing rapidly in the developed countries as well as in the traditional medical programs being incorporated into primary healthcare for example in China, Nigeria and Mexico (Cox 1990). Moreover, in the developing countries a large number of the population still depends on herbs for their healthcare. Some analysts have quoted a high incidence of this phenomenon, for example WHO estimates that, 60-90% of Africa's population partially or totally depends on plants to meet their healthcare needs (WHO 1978). Countries in Africa where herbal medicine is heavily relied on include West Africa; specifically, Ghana, Nigeria and Cote d' Ivoir; East Africa, especially, Ethiopia, Kenya and Tanzania; and South Eastern Africa including South Africa and Swaziland. It is estimated that over 70-80% of the rural population in these countries use herbal medicine (Cunningham 1993).

Although the traditional herbalists may not know the actual bioactive compounds in the medicinal plants they use, it is indeed the presence of the bioactive compounds that are the secret behind the effectiveness of the traditional herbal medicine against a wide range of human and animal ailments (Balick 1990; Farnsworth *et al.* 1985).

1.3 Selecting plants for analysis of bioactive compounds

Selecting a particular plant species for analysis is a very crucial stage in the process of investigating plants for any specific biological activity (Principe, 1989). Several criteria may be used in selecting plants to be studied.

1.3.1 Taxonomic criteria

Recently correlations have been established between plant taxonomy based on morphological characteristics and the occurrence of specific chemical constituents at different taxonomic levels. Most secondary metabolites are known to be family/genus/species specific (Harborne and Turner 1984; Gottlieb 1982). Thus selection could be limited to a particular family or genus known to contain a particular class of compounds. This approach however requires prior knowledge of the constituents of a group of plants and the particular compound being sought after.

1.3.2 Random sampling

The random sampling strategy involves picking plants for analysis from an area at random. It has been argued that this is not an effective approach often citing the case of National Cancer Institute (NCI) in U.S.A, where even after screening 35,000 species (114,000 plant extracts) no compound was found with clinical anticancer activity (Hostettman 1991). However the fact that a few of todays plant based commercial drugs including vincristine and vinbrastine, the anticancer alkaloids from

Catharanthus roseus were found in plants which had no previous use as medicines specific for any disease related to the activity for which the compounds are used, may imply that any wild plant may be a source of information that could be of great value to the pharmaceutical industry.

1.3.3 Screening plants used in traditional medicine

A correlation was found between plants used in folklore and pharmacological activity in the USA's National Cancer Institute (NCI) anticancer programme (Farnsworth 1990). Mortson and Hosttetman (1987) have also reported a correlation between plants used in Malawi, Tanzania and Panama to cure various diseases traditionally and their biological activity in biological screening systems including mulluscicidal , toxicological and fungitoxic activities. Selection of plants for analysis based on ethnopharmacological information increases the chances of success in finding bioactive compounds. The approach has been used successfuly in some West African countries under the auspices of the West African Pharmaceutical Federation (Sofowara 1982). This approach is probably one of the most successful in discovering bioactive compounds (Balick 1990; Farnsworth 1990; Hostettman 1991).

Further, scientific studies carried out on a few medicinal plants have proved the existence of bioactive compounds with antimicrobial activity. Several scientists have reported screenings of the crude extracts, but a few have isolated the antimicrobial compounds. Some examples include, screening of *Ficus septica* Burm. in the family Moraceae. *F. septica* is a small tree growing in Papua New Guinea and used by the local population for the treatment of colds, fever and skin diseases. A methanolic

extract of this plant displayed intense antifungal and antibacterial activity (Baumgartner *et al.* 1990). *Terminalia bellerica* Roxb. in the family Combretaceae is one of the most commonly used plants in Indian folk medicines. Bioactivity guided fractionation of the fruit extracts from this plant led to the isolation of compounds that have shown *in vitro* anti HIV-I, antimalarial and antifungal activity (Valsaraj *et al.* 1997). Barnabus and Nagarajan (1988) reported that aerial parts of ten south Indian medicinal plants yielded extracts showing antibacterial and antifungal activity. *Delphinium denudatum* L. in the family Ranunculaceae is used in the folk medicine of Pakistan for the treatment of itches and other skin eruptions. Compounds isolated from this plant demonstrated antifungal activities against human, animal and plant pathogenic fungi (Atta-ar- Rahman *et al.*, 1997). Anesini and Perez (1993) screened 132 species of plants used in Argentinian folk medicine and reported antifungal activity from several of them. *Dictamnus dasycarpus* L. in the family Rutaceae is used in traditional Chinese medicine for the treatment of jaundice, coughs, rheumatism and skin diseases. It was found out that water and dichloromethane extracts of the root bark of *D. dasycarpus* exhibited growth inhibitions against pathogenic fungi. Bioassay guided isolation led to the purification of six active components whose structures were elucidated on the basis of various spectroscopic methods (Wiemin Zhao *et al.*1998).

On plants from the African scene, Irobi and Daramola (1993) have reported that *Mitracapus villosus* (SW.) DC in the family Rubiaceae is one of the plants used for the treatment of skin diseases by the local people of Niger state in Nigeria. They reported that ethanolic extracts from this plant posses some antifungal activity against

Microsporum gypseum Bodin, *Candida albicans* (Robin) Berkh., *Fusarium solani* (Mart.) and Sacc. *and Aspergillus niger* Van tiegh. Irobi *et al* (1994) further reported antimicrobial activity of aqueous and ethanol extracts of *Bridelia ferruginea* Benth. Euphorbiaceae, a common shrub in many parts of Africa . The plant is used by medicine men in Cote D'Ivoir for the treatment of gonorrhoea, while in Nigeria portions of the bark are used for preparing medicines used as mouthwash and remedy for *Candida* oral thrush.

Premna oligotricha (hochst.) Schauer is used by the local Ethiopians as chewing sticks (twigs), while smoke formed from burning the plant is used to sterilise milk containers resulting in the milk remaining fresh for longer periods. These uses suggested the probable presence of antibacterial compounds. A study of the secondary metabolites from *P. oligotricha* lead to the isolation of two novel antibacterial compounds (Habtemariam *et al* 1992). Further, plants collected in East Africa on the basis of information gathered from native people, especially '*waganga*' (Kiswahili meaning medicine men), were evaluated for antimicrobial activities. Of the 79 species evaluated, 40 gave initial positive results indicative of antimicrobial activities against one or more pathogens. This observation led the researchers to conclude that the probability of finding antimicrobial compounds in *Dawa ya Miti* (Kiswahili meaning medicine from plants), is much higher than in common or garden plants (Taniguchi *et al.*1978). Similarly, Fabry *et al.* (1996) reported antifungal activities of extracts from six plant species collected from Kenya, that have been used in the traditional medicine of East Africa. Similarly, Lwande (1984) has reported antimicrobial activities of four Kenyan medicinal plants. On the same lines, Kariba

(1992) reported antifungal activities of extracts from *Solanum nigrum* L. a plant that is widely used in Kenya for treatment of ringworm.

Following such cues, recent screening of plants has led to the discovery and successful development of efficacious drugs such as artemisinin, originally isolated from *Artemisia annua* L. as an antimalarial drug, the anticancer drug etoposide derived from may apple *Podophyllum peltafum* L. (Berberidaceae) and vincristine and vinbrastine the anticancer alkaloids from *Catharanthus roseus* G. Don (Apocynaceae), (Farnsworth 1988; Trigg 1989; Bruhn 1989). More recently there has been successful trials of Taxol originally from *Taxus brevifolia* Nutt. as an anticancer drug (Kingston 1992). In addition, many natural compounds have shown encouraging activity against HIV virus and several tumour lines (Fleet *et al.* 1988; Suffness 1987).

However, data on actual efficacy and spectrum of activity exist only for a negligible number of medicinal plants. Out of the estimated 250,000 species of higher plants known to exist on earth, only a few have been studied for all aspects of their biological activity, and only about 1% have been acknowledged by scientists to have real therapeutic value (Farnsworth 1984, 1988). It is anticipated that on the basis of historical experience with many pharmacologically useful compounds that have been derived from plants, as well as recent experimental trials that there are probably innumerable potentially useful biotic compounds awaiting isolation and identification. However due to environmental pressure for human settlement, plants of medicinal value continue to be depleted from the ecosystem before adequate information on their efficacy is documented. In addition to this, the holders of the

valuable ethnomedical knowledge are getting phased out leaving no records for future reference of the plants that they used (Slikkerveer and Slikkerveer, 1995). In view of all these there is need to speed up scientific research on efficacy of traditionally used medicinal plants. Once the usefulness has been scientifically validated, and the active components identified, then the species can be conserved and probably domesticated for commercial exploitation. Moreover, when the active principles have been chemically identified, they can be synthesised economically and made into agricultural and/or pharmaceutical preparations, or modified to produce more active analogues. It is within this background that this study was conceptualised.

This study was designed to screen a selection of Kenyan medicinal plants for their antifungal compounds.

1.4 Objectives of the study:

1. To identify and collect medicinal plant species used for the treatment of various skin, mucous membrane and visceral organs ailments from different parts of Kenya;
2. To carry out fungitoxicity tests on the crude extracts from plant parts used traditionally;
3. To carry out bioactivity guided isolation and purification of antifungal compounds from extracts that showed measurable antifungal activity; and
4. To investigate some of the antifungal properties of the most potent isolated compound.

The study was guided by the hypothesis that :

Kenyan medicinal plants traditionally used for the treatment of skin mucous membrane, and visceral organs ailments could have antifungal compounds.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Identification and Selection of medicinal plants for antifungal activity

Ethnopharmacological information was used to identify potential plant candidates for the study. Because the aim of this study was to search for antifungal compounds, plants with traditional history of use in the treatment of skin diseases caused by fungi such as ringworm were given priority. The plants were collected from various parts of Kenya between March and May 1996. Voucher specimens of identified plants were deposited at the University of Nairobi Herbarium.

2.1.1 Preparation of plant materials

Upon collection from the field, a whole plant or portions of the plant were dried in open air at room temperature (25°C). The dried samples were crushed into powder using a warring blender. The material that was not being used immediately was kept dry at room temperature away from direct sunlight.

2.1.2 Test organisms

Three human pathogens and one phytopathogenic fungus were used. The human pathogens were *Trichophyton mentagrophytes* Robin and *Microsporum gypseum* Bodin (filamentous forms), the causal agents of dematomycoses, and *Candida albicans* (Robin) Berkh. (yeast form) which causes generalised cutaneous candidiasis and is the leading opportunistic pathogen in immuno-compromised patients. These were donated by Dr. G. Siboe of the Department of Botany, University of Nairobi. Stocks were maintained on slants and in sterile distilled water at 4°C and subcultured on Sabouraud's Dextrose Agar (SDA) at 26°C. The plant pathogen *Cladosporium cucumerinum* Ellis and Arth (1M1 No 299104) obtained from International Mycological Institute U. K.(now CABI Bioscience was maintained on Potato Dextrose Agar (PDA) slants at 4°C.

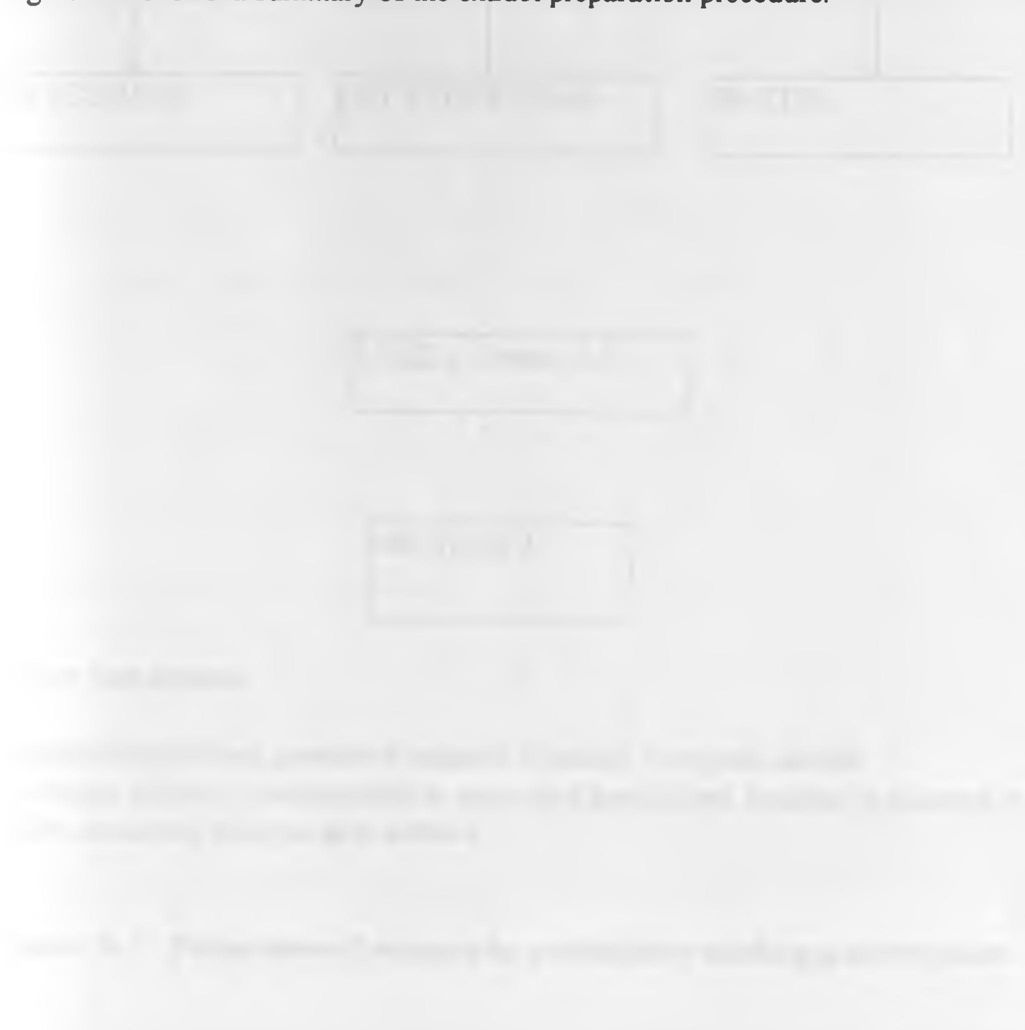
2.1.3 Preparation of crude plant extracts (fig. A.1)

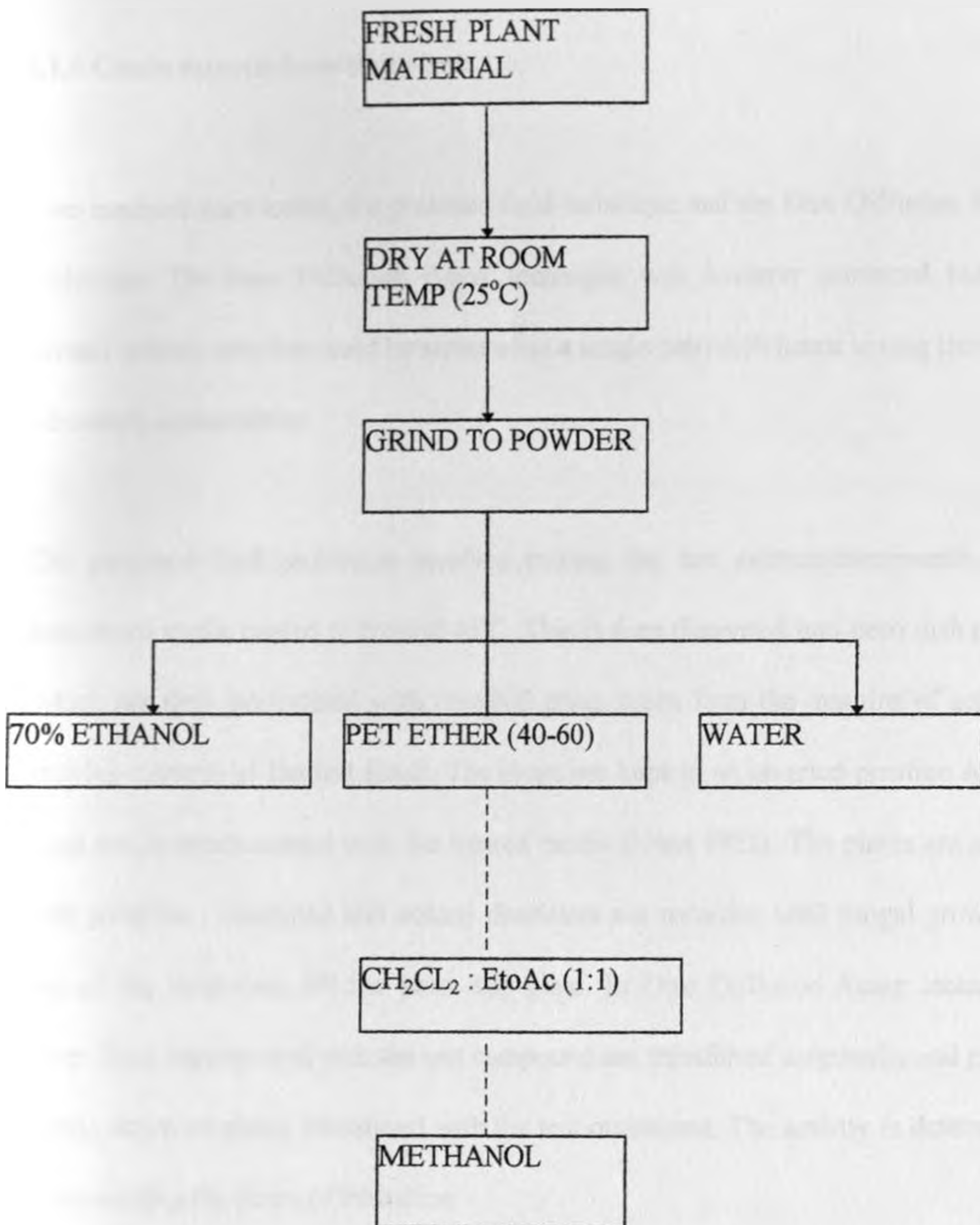
Crude plant extracts were obtained by a method described by Harborne (1984).The dry powdered material was divided into two portions. One portion was used to prepare organic extracts by cold percolation successively with petroleum ether 40-60°C, dichloromethane : ethylacetate (1:1) and methanol.

The powdered material was mixed separately with the solvent in the material to solvent ratio of 1:10. The mixture was kept in the fume cupboard at room temperature for 24 hours, after which the extract was passed through whatman filter

paper No.1 (whatman UK). The filtrates from each extraction were concentrated *in vacuo* using a rotary evaporator (Buchi laboratory techniques, Switzerland), at 40°C and freeze dried to remove all traces of water and solvents. After each extraction the material was spread out in the hood and the solvents allowed to evaporate before extraction with the next solvent. The other portion of the plant material was used to prepare aqueous extracts by soaking in water and 70% ethanol respectively for 24 hours. The filtrates were freeze dried to powder. The extracts were stored in scintillation vials and refrigerated at 4° C prior to use.

Figure A.1 shows a summary of the extract preparation procedure.





Broken line denotes;

Sequential extraction; powdered material is soaked in organic solvent for 24 hrs; filtrate is concentrated *in vacuo* and lyophilized. Residual is allowed to dry before extracting with the next solvent.

Figure A.1 Preparation of extracts for preliminary antifungal activity tests

2.1.4 Crude extracts bioactivity tests

Two methods were tested, the poisoned food technique and the Disc Diffusion Assay technique. The Disc Diffusion Assay technique was however preferred because several extract samples could be screened in a single petri dish hence saving time and laboratory consumables.

The poisoned food technique involves mixing the test extracts/compounds with autoclaved media cooled to around 40°C .This is then dispensed into petri dish plates which are then innoculated with mycelial plugs taken from the margins of actively growing cultures of the test fungi. The plugs are kept in an inverted position so that fungi are in direct contact with the treated media (Nene 1971). The plates are sealed with parafilm , incubated and colony diameters are recorded until fungal growth in one of the treatments fill the petri dish plate. In Disc Diffusion Assay technique, paper discs impregnated with the test compound are transferred aseptically and placed upside down on plates inoculated with the test organisms. The activity is determined by measuring the zones of inhibition.

Each of the extracts was separately reconstituted in the minimum amount of the extracting solvent or Dimethyl Sulfoxide (DMSO) (depending on the solubility of the test material) and tested at dilutions of 800, 400 and 200µg per disc. Sabourauds Dextrose Agar (SDA) was used as the medium for studies of antifungal activity for the human pathogens and Potato Dextrose Agar (PDA) was used for *Cladosporium cucumerinum*. Between 20 and 25 ml of the medium cooled to about 45-50°C was dispensed aseptically into sterile 9 mm diameter petri dish plates and left to set

uniformly under aseptic conditions.

Spore suspensions of four day old cultures of the test fungi were prepared using sterile distilled water. Two to three millilitres of each suspension was transferred into the petri dish plates and spread uniformly with a sterile glass rod.

Paper discs (5mm) were impregnated with 20μl of each of the extracts reconstituted at 40μg/μl, 20μg/μl and 10μg/μl to give concentrations of 800μg/disc, 400μg/disc and 200μg/disc respectively. The discs were aseptically transferred and placed upside down at the centre of the SDA or PDA plates previously inoculated with the test organisms. SDA and PDA Plates set with the same organisms with 1% Nystatin and griseofulvin (Sigma) respectively served as standards. Plates set with solvents in which the extracts were reconstituted and DMSO served as controls. All plates were set in quadruplicates. The plates were sealed with a laboratory parafilm to avoid contamination and any possible evaporation and incubated at 26°C. The activity of extracts were determined by measuring the zones of inhibition according to the methods described by Barry *et al* (1979) and Jacques *et al*.(1986).

2.2 Bioactivity guided fractionation.

2.2.1 Selection of plants for bioactivity guided fractination (fig A.2)

Four plant species were selected for further phytochemical analysis. Selection was done by eliminating those plants whose crude extracts showed very low antifungal

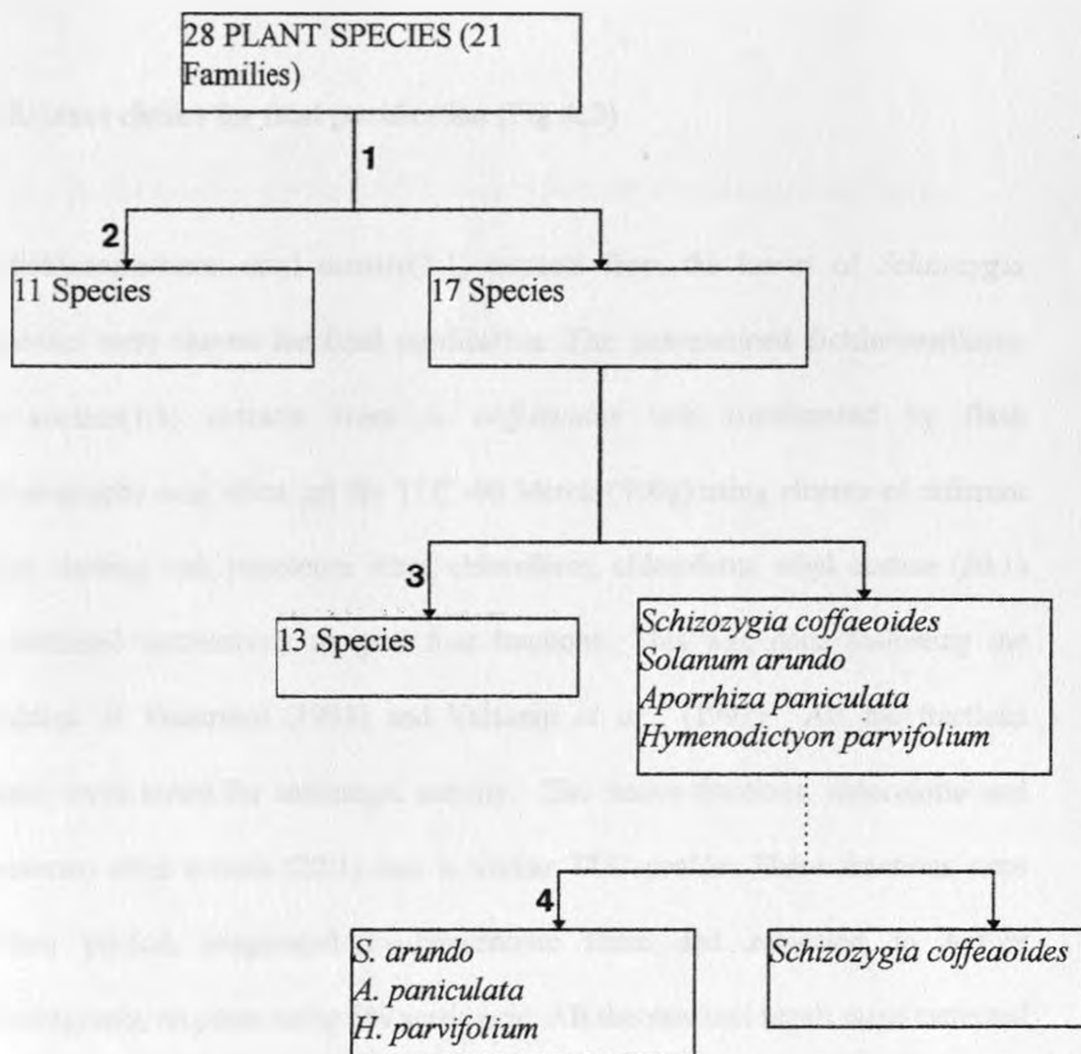
activity at 400µg and those that have been previously studied. Extracts from each of the four species were fractionated by column chromatography.

Two grams of each of petroleum ether, dichloromethane and methanol crude extracts from *Schizozygia coffaeoides* Bail., ethanol extracts from *Solanum arundo* Mattei., methanol extracts from *Aporrhiza paniculata* Radlk. and ethanol extracts from *Hymenodictyon parvifolium* Oliv., were separately adsorbed onto 2g silica gel and chromatographed over 80g silica gel column grade mesh 230-400 (Merck). Different elution solvents were used with increasing polarity. Finally the columns were washed with 100% methanol.

Fractions were collected and monitored on TLC according to the procedures of Stahl 1969. The plates were viewed under UV 254/366 and fractions of similar profile were pooled and subjected to *in vitro* antifungal tests.

The column fractions that showed antifungal activity were subjected to Preparative Thin Layer Chromatography (Stahl 1969; Harborne 1984). This involves spotting samples on silica gel precoated TLC plates (Schleicher and Schuell) with fluorescent indicator 254nm and running in an appropriate solvent system. All the resulting bands were extracted from the adsorbent and subjected to antifungal tests. The most active extract was chosen for final purification and further bioactivity tests.

Figure A.2 shows how the selection of plants for bioactivity guided fractionation was done



(1) Preliminary Screening
(2)Extracts with no inhibition zones at 800ug eliminated
(3) Extracts with inhibition zones < 10mm at 400ug eliminated
(4) Extracts whose column fractions had inhibition zones < 20mm at 400ug eliminated
······ Column fractionation and antifungal tests

Figure A.2 : Selection of plants for bioactivity guided fractionation

2.3.0 Extract chosen for final purification (Fig A.3)

The dichloromethane: ethyl acetate(1:1) extracts from the leaves of *Schizozygia coffaeoides* were chosen for final purification. The concentrated dichloromethane: ethyl acetate(1:1) extracts from *S. coffaeoides* was fractionated by flash chromatography over silica gel for TLC -60 Merck (100g) using eluents of different polarity starting with petroleum ether, chloroform, chloroform: ethyl acetate (20:1) and methanol successively to give four fractions. This was done following the procedures of Tsantrizos (1991) and Valsaraji *et al* . (1997) . All the fractions obtained were tested for antifungal activity. The active fractions, chloroform and chloroform: ethyl acetate (20:1) had a similar TLC profile. These fractions were therefore pooled, evaporated to concentrate them and subjected to further chromatography on paper using 5% acetic acid. All the resultant bands were extracted in 80% methanol overnight and subjected to antifungal tests. The active band was extracted, concentrated and re-chromatographed on the TLC silical gel with solvent system hexane: dichloromethane : ethyl acetate (2:2:1). All the resulting bands were extracted and subjected to antifungal tests. The presence of the active compound was also confirmed by the TLC bioassay method of Homans and Fuchs (1971).This is a bioactivity testing method which involves spoting the test sample on TLC plate and running it in an appropriate solvent system . The plate is then allowed to dry. The run TLC plate is sprayed with fungal spores in a nutrient broth and incubated in a moist chamber. Clear zones without any fungal growth are an indication of the presence of antifungal compounds.

2.3.1 Purification and identification by spectroscopic and chemical methods

Several methods were used to determine the purity of the compound isolated from *Schizozygia coffaeoides*. The compound was spotted on TLC plates and run using different solvent systems.

2.3.1.1 High Performance Liquid Chromatography (HPLC) analysis

The antifungal sample from *Schizozygia coffaeoides* was passed through High Performance Liquid Chromatography (HPLC) machine coupled to diode array detection. HPLC analysis and purification were carried out on reversed phase C^{18} columns, Bondapak 4 mm i.d x 30cm. Using Waters 600 multi-solvent system with Waters 994 programmable photodiode array detector connected to a Waters 5200 printer /plotter.

Helium gas 25 Psi was sparged 20% of the enabled time for degassing.

Solvent A was 2% acetic acid and solvent B was methanol: acetic acid: water (18:1:1). Gradient programme 75% A 25% B changing to 35% A and 65% B over 35 minutes in a linear mode at a flow rate of 1ml/minute and temperature of 25°C. UV detection was done at wavelength range of between 200 and 400nm.

This gave a single peak at retention time 23.95 minutes . The Peak was collected manually, vacuum evaporated and subjected to bioactivity tests.

2.3.1.2 Chemical tests

To get an idea of the group of compounds that the isolated compound belongs to, some chemical spray reagents were used. A pure sample collected after passing through HPLC, was spotted on TLC silica gel and ran on hexane : dichloromethane: Ethyl acetate (2:2:1). These were sprayed with Iodoplatinate, Dragendorff and Folin reagents to test for the presence of alkaloids and phenols respectively.

2.3.1.3 Identification by Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS)

Samples for NMR and mass spectrometry were prepared by filtration on sephadex LH- 20 with methanol as eluent. A small column was packed with sephadex LH-20 in methanol. Column was cleaned several times with HPLC water and the sample (10mg) was loaded. The loaded column was washed with HPLC water to clean the sample. Since the sample was known to dissolve in methanol, it was therefore added to wash the sample down the column. The sample was collected in a preweighied spectrophotometer pot and dried under liquid nitrogen. The pot was then put in a desiccator overnight to get rid of any water molecules. This sample was labelled SC-1 and given to Prof. Harborne of Phytochemistry Unit, University of Reading U.K. for analysis .

2.4 Further *in vitro* tests with the isolated compound

The aim of this study was to assess the efficacy of this compound against certain fungi that cause diseases to humans and cultivated plants, and to observe any morphological alteration caused by this product to the fungal mycelium of filamentous fungi and to the cells of the yeast fungi.

2.4.1 The pathogens used

In addition to the pathogens used for the preliminary screening (Section 2.1.2), five more pathogens were used. These included *Fusarium solani* , *Botrytis cinerea* Pers. Ex. Pers., *Cladosporium harbarum* (Pers.) Link ex. S. F. Gray, and *Alternaria tenuis* C. G Nees. The yeast fungi were *Saccharomyces cerevisae* Meyer ex. Hanson and *Candida albicans* (Robin) Berkh.

The fungitoxicity of the compound SC-1 was determined at concentrations levels of 6.25, 12.5, 25, 50, 100, 200 and 400 μg/disc using the methods outlined earlier (section 2.1.4).

2.4.2 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of SC-1 was determined by impregnating the paper discs, with various concentrations of the reconstituted sample. These were set as already mentioned. The MIC was regarded as the lowest concentration of SC-1 that

inhibited growth as compared to the drug free controls.

2.4.3 Inhibition of mycelial growth

The effect of SC-1 on mycelia growth was investigated in liquid culture using the methods of Papas and Fisher (1979) ; Fisher and Hayes (1982); and Weidenborner *et al.* (1990). Methanol served as the solvent for the addition of SC-1. Eight graded concentrations of the compound raging from 6.25 - 400 μg were used. 20μl methanolic solutions of the compound were added to 40ml potato dextrose broth in Erlenmeyer flasks and inoculated with a 5mm diameter mycelia plug got from the margins of seven day old cultures of *Cladosporium cucumerinum*, *Cladosporum harbarum*, *Botritis cinerea, Alternaria tenuis* and *Fusarium solani*. The flasks were incubated at 28°C on a shaker for seven days. At the end of this period mycelia were collected on filter papers, dried overnight at 60°C and the yield compared with control cultures grown in the absence of the fungicide. Three replicates were used for each concentration.

The data were evaluated by analysis of variance and the Duncan's multiple range test was applied to determine the significance of the difference between mean values where the test rejected the hypothesis that the treatment means are all equal (Zar 1984).

2.4.4 Studies on fungicidal and fungistatic properties

The fungistatic and fungicidal properties of the extracts were studied using the methods described by Scholar and Polak (1973), and Reiner (1982). A 5mm sterile paper disc was impregnated with the sample at concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400ug/disc. A mycelial plug measuring 5mm was then cut and removed from 4 day old cultures. The impregnated discs were placed on the surface of the plugs. Sterile water and reconstituting extracts were used for the controls. The mycelial plugs were removed at the time intervals of 2, 4, 10, 20 and 40 hours and transferred into fresh medium. These were then incubated at 26°C. The plates were examined for any mycelial growth and colony diameters were measured.

To ascertain whether the compound was fungicidal the method of Perrucci *et al.* (1993) came in handy. Plugs from zones of inhibition were re-incubated into fresh media and examined for growth.

2.4.5.0 Studies on morphological alteration of the fungal hyphae of filamentous fungi and cells of yeast fungi

For the studies of morphological alterations, Scanning Electron Microscopy was done according to the method of Zambonelli *et al.* (1996). The fungi used were , *Cladosporium cucumerinum* (filamentous) and *Saccharomyces cerevisiae* (yeast).

2.4.5.1 Preparation of material for Scanning Electron Microscopy

Mycelia plugs (1x1x0.3cm) were collected from different zones, (i) from the control plates, (ii) from the zones of inhibition at different concentrations

The material was prepared as follows:- The samples were fixed in phosphate buffered 25% EM grade glutaraldehyde. These were then dehydrated in graded aqueous series of Ethanol (30, 50, 70, 90, 95 ,100 and 100% V/V) .The samples were left in each step for 30 minutes. This was done according to Echlin (1971). The samples were then critical point dried using 100% ethanol as the intermediate fluid. After drying, the specimens were carefully fixed onto aluminium stubs 10mm diameter x10mm high (TAAB lab equip. Ltd). This was done using a special Adhesion silver glue. The specimens were kept under the fume cupboard for the glue to dry up. The pieces of agar were then sputter coated with gold palladium according to procedures of Cross (1972). The fungal hyphae and yeast cells were then examined under a Joel JSM -T20 Scanning Electron Microscope.

Figure (A.3) Shows steps leaing to the purification of the antifungal compound from *Schizozygia coffaeoides*.

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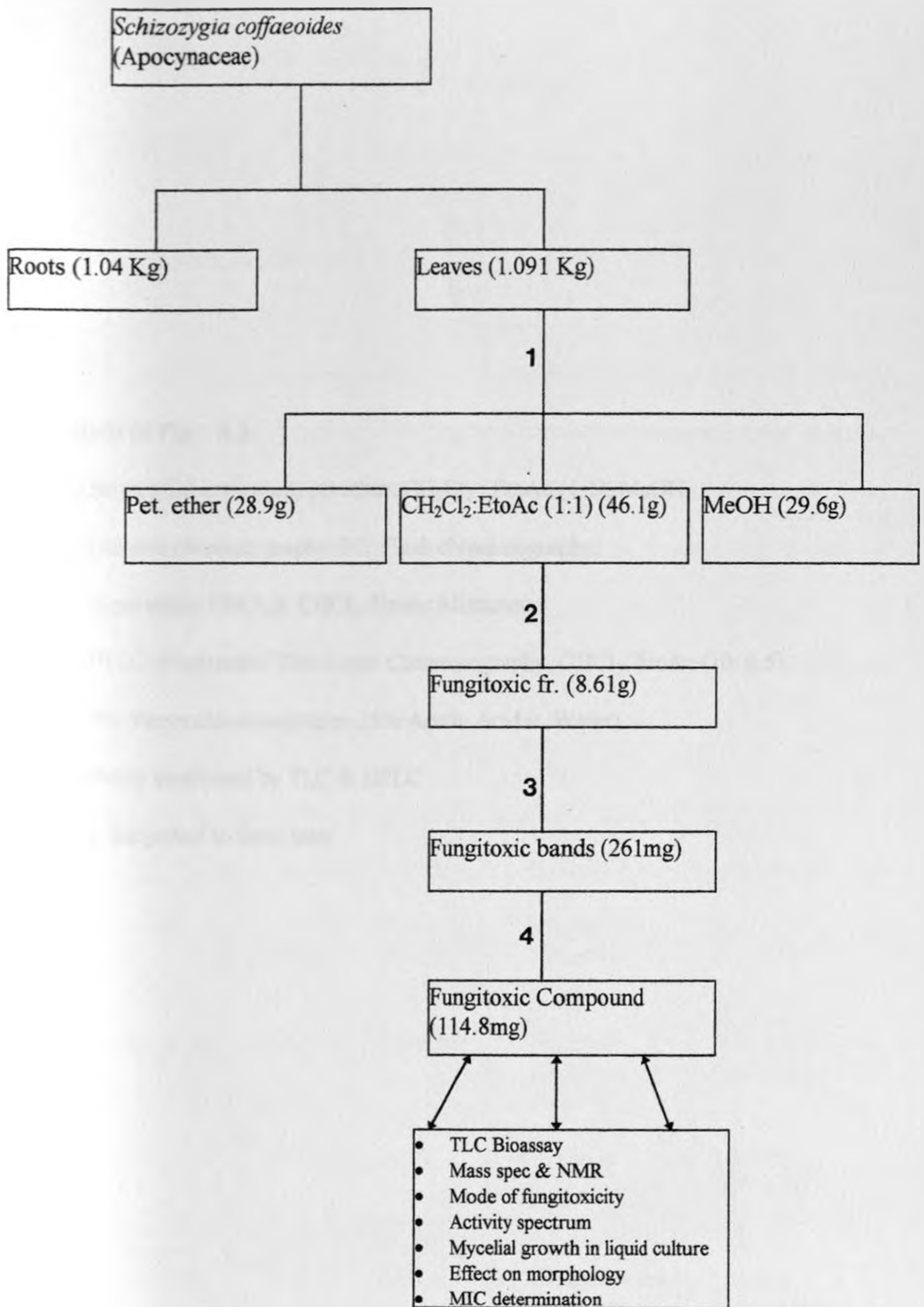


Fig A.3 : Purification of CH_2Cl_2 : EtoAc (1:1) extracts from *Schizozygia coffaeoides*

Details of Fig A.3

(1) Sequential extraction, pet ether; CH_2Cl_2 : EtoAc (1:1); MeOH

(2) Column chromatography: FC: Flash chromatography;

(pet ether- $CHCl_3$& $CHCl_3$: EtoAc Mixtures)

(3) PTLC: Preparative Thin Layer Chromatography, $CHCl_3$: EtoAc (10: 0.5)

PC: Paper chromatography ,(5% Acetic Acid in Water)

(4) Purity confirmed by TLC & HPLC

↔ Subjected to these tests

CHAPTER THREE

3.0 RESULTS

3.1 Plant species identified for study

Twenty eight plant species belonging to 21 families were selected for study. Table 1 shows the plant species, parts used, traditional methods of preparation, the ailments treated, localities from where they were collected and other possible localities in Kenya shown by botanical division numbers (K1-7) Fig. 1, according to Flora of Tropical East Africa (FTEA) Beentje, (1994).

Table 1. Species of medicinal plants identified as possible sources of antifungal compounds on the basis of their traditional ethnopharmacology.

Plant species	Parts used	Preparation method	Ailment treated	Collection locality (K*)
Acacia nilotica (L.) Del. (Mimosaceae)	Bark Fruit	Bark infusion, Fruit juice	wounds, eye infections	Athi River (K1234567)
Acacia senegal (L.) Willd. (Mimosaceae)	Bark	Infusion	Wounds, diarrhoea, malaria	Athi River (K123467)
Adenia volkensii Harms (Passifloraceae	Tuber	Juice	Wounds	Machakos (K134567)
Ajuga remota Benth. (Lamiaceae)	Leaves	Infusion	Skin diseases, toothache, headache, dysentery and high blood pressure	Langata (K123456)

Table 1 contd.

Aloe secundiflora Engl. (Aloeceae	Fleshy leaves	Sticky juice	Skin diseases and wounds, malaria	Athi River (K1234567)
Asparagus africana	leaves	Infusion	Wounds, gonorroea, malaria	Langata (K12346)
Aporrhiza paniculata Radlk. (Sapindaceae)	Bark	Infusion	Skin diseases, tuberculosis	Simba Hills (K7)
Aspilia pluriseta Schweinf. Ex Engl. (Compositae	Leaves	Infusion	Skin diseases, eye infections, malaria, headache	Meru (K3456)
Azadirachta indica Juss. (Meliaceae)	Bark	Infusion	Skin diseases, malaria, vomiting, intestinal worms, scabies and urinary diseases.	Mtitu Andei (K7)
Azanza garcheana (F. Hoffm.) Excell and Hillcoat (Malvaceae)	Bark	Infusion	Stomach problems, sore throat	Meru (K3456)
Commiphora africana (A. Rich.) Engl. (Burseraceae)	Fruits	Sap	Skin diseases, toothache	Athi River (K1267)
Datura stramonium L. (Solanaceae)	Leaves Seeds	Ground mixture	Ringworm Boils	Chiromo (K1234567)

Table 1 continued

Flueggea virosa (Willd.) Voigt. (Euphorbiaceae)	Twigs, Roots	Toothbrush Decoction	Dental hygiene, wounds and chest pain	Meru (K1234567)
Hymenodictyon parvifolium (Rubiaceae)	bark	Infusion	Skin diseases, venerael diseases, dysentery	Machakos (K124567)
Kigelia africana (Lam.) Benth. (Bignoniaceae)	Bark	Infusion	Sore throat, measles, sterility.	Machakos (K1234567)
Melia azedarach L. (Meliaceae)	Leaves Bark fruits	Infusion, fruit pulp	Skin diseases and wounds	Kabete (K347)
Oxalis corniculata L. (Oxalidaceae)	Leaves	Infusion	Ringworm	Ngong (K234567)
Pergularia daemia (Forsk.) Chiov. (Asclepiadaceae)	Leaves	Juice	Skin diseases	Chiromo (K23456)
Plectranthus barbatus Andr. (Labiatae)	Roots Leaves	Infusion	wounds, chest problems	Meru (K1234567)
Plectranthus cylindraceus Benth. (Labiatae)	Leaves	Infusion	Skin diseases and stomach-ache	Machakos (K1234567)
Pollichia campestris Aiton (Caryophyllaceae)	Leaves	Juice	wounds, bruises and swellings	Athi River (K23456)
Premna resinosa (Hochst.) Schauer Verbenaceae	Leaves	Juice	Wounds and unspecified skin problems	Machakos (K12347)

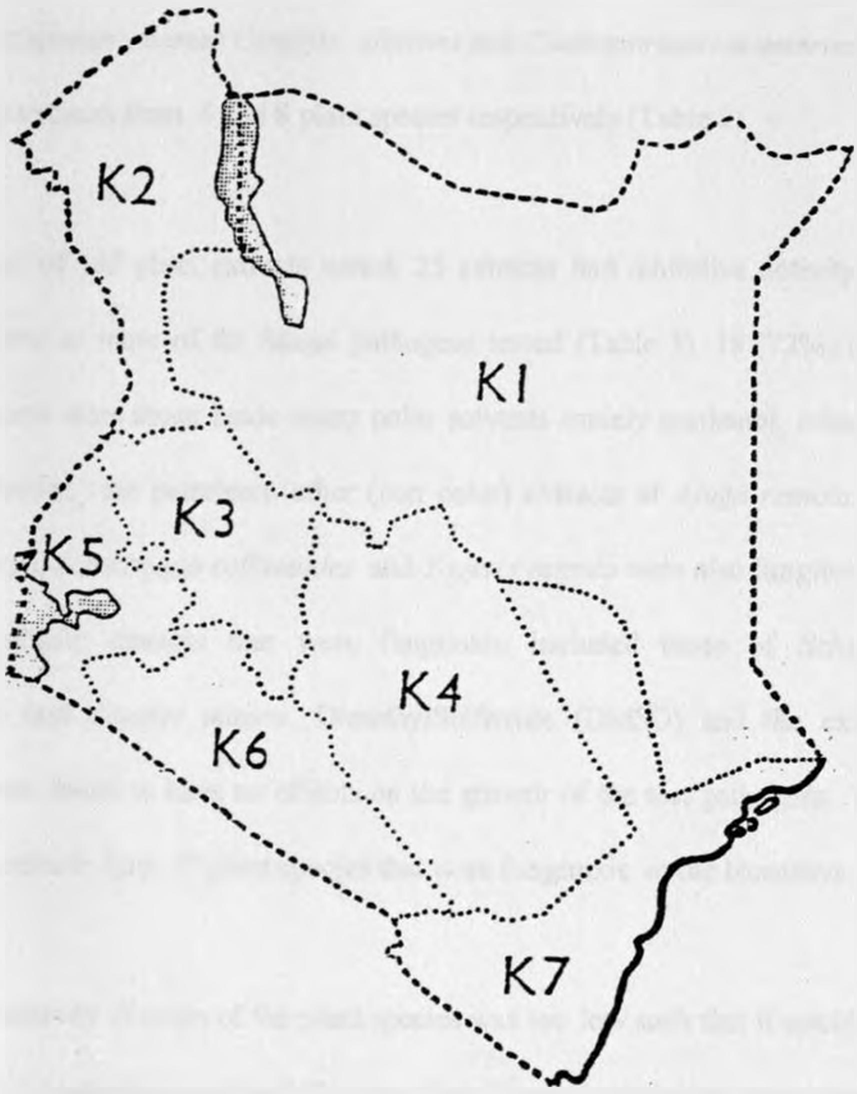
Table 1 contd

Senna didymobotrya (Fresen.) Irwin and Barneby. (Ceasalpinaceae)	Leaves Young stems	Infusion	Ringworm, gonorrhoea	Chiromo (K13456)
Sesbania sesban (L.) Merril (Papilinaceae)	Leaves	Infusion	Eye and ear infections, Afterbirth pain	Athi River (K134567)
Schizozygia coffaeoides Baill. (Apocynaceae)	Leaves Roots	Infusion	Skin diseases, wounds and eye infections	Simba Hills (K7)
Solanum arundo Mattei (Solanaceae)	Fruit	Juice	Itchy toes and feet	Athi River (K1467)
Solanum nigrum L. (Solanaceae)	leaves	Infusion	Ringworm, abdominal pains, urinary diseases	Kabete (K1234567)
Tagetes Minuta L. (Compositae)	Leaves & Flower	powder	wounds, nose breeding, constipation	Meru (K23467)

Sources: Watt and Breyerbrandwijk 1962; Jain 1976; Kokwaro 1976; Lindsay 1978, Bacquar 1989; Gachathi 1989; Beentje 1994 Sindiga *et al.* 1994; Olembo *et al* . 1995; Omino and Kokwaro1993; Personal communication.

(K*) The distribution information is according to the coded areas shown on the map of Kenya in Fig 1.

Fig. 1: Map of Kenya showing the botanical divisions according to Flora of Tropical East Africa (FTEA).



3.2 Bioactivity Tests

From the 28 plant species screened, extracts from 17 (60.7%) species gave initial positive results indicative of antifungal activity, whereas the remaining 11 (39.3%) had no fungitoxic activity (Table 2). The dermatophytic fungi *Trichophyton mentagrophytes* and *Microsporum gypseum* were sensitive to extracts from 13 out of the 17 plant species whereas *Candida albicans* and *Cladosporium cucumerinum* were sensitive to extracts from 6 and 8 plant species respectively (Table 2).

From a total of 155 plant extracts tested, 25 extracts had inhibitive activity on the growth of one or more of the fungal pathogens tested (Table 3). 18 (72%) of these active extracts were those made using polar solvents mainly methanol, ethanol and water. However, the petroleum ether (non polar) extracts of *Ajuga remota*, *Senna didymobotrya*, *Schizozygia coffaeoides* and *Tagetes minuta* were also fungitoxic. The medium polarity extracts that were fungitoxic included those of *Schizozygia coffaeoides* and *Tagetes minuta*. DimethylSulfoxide (DMSO) and the extracting solvents were found to have no effects on the growth of the test pathogens. Table 3 shows the extracts from 17 plant species that were fungitoxic in the bioassays.

Fungitoxic activity of some of the plant species was too low such that it could not be detected at concentrations below 800 μg per disc. These included the petroleum ether extracts of *Ajuga remota*, *Senna didymobotrya* and *Tagetes minuta*, the ethanol extracts of *Aspilia pluriseta* and *Plectranthus barbatus* and the methanol extracts of *Adenia volkensii* (Table 3).

Table 2. Preliminary screening of crude extracts from 28 plant species against *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Candida albicans* and *Cladosporium cucumerinum*.

Plant Species	Part	Test organisms			
		T.mentagrophytes	*M.gypseum*	*C. albicans*	*C. cucumerinum*
Acacia nilotica	bark	+	+	+	+
Acacia senegal	bark	+	+	+	+
Adenia volkensii	tuber	-	-	+	-
Ajuga remota	leaves	+	+	-	-
Aloe secundiflora	stem	-	-	-	-
Aporrhiza paniculata	bark	+	+	-	+
Asparagus africana	leaves	-	-	-	-
Aspilia pluriseta	leaves	-	-	+	-
Azanza garcheana	bark	-	-	-	-
Azadirachta indica	bark	+	+	-	+
Commiphora africana	fruits	-	-	+	-
Datura stramonium	leaves	-	-	-	-
Flueggea virosa	root	-	-	-	-

+= Active; -= Not active

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Table 2 continued

Plant Species	Part	Test organisms			
		T.mentagrophytes	*M.gypseum*	*C. albicans*	*C. cucumerinum*
Kigelia africana	bark	-	-	-	-
Melia azedarach	bark	-	-	-	-
	leaves	-	-	-	-
Oxalis corniculata	whole plant	-	-	-	-
Pergularia daemia	leaves	-	-	-	-
Plectranthus barbatus	leaves	-	+	-	-
Plectranthus cylindraceus	leaves	+	+	-	-
Pollichia campestris	leaves	-	-	-	-
Premna resinosa	leaves	+	+	-	-
Schizozygia coffaeoides	leaves	+	+	+	+
	roots	+	+	-	+
Senna didymobotrya	leaves	+	-	-	
Sesbania sesban	leaves	-	-	-	-
	bark	-	-	-	-
					+
Solanum arundo	fruits	+	+	-	
Solanum nigrum	leaves	+	+	-	-
Tagetes minuta	leaves & flowers	+	+	-	+

Key + = Active, -=Not active

Table 3: Bioactivity (% inhibition) of extracts from 17 plant species at concentrations raging from 200-800 μg/disc against species of *Trichophyton*, *Microsporum*, *Candida* and *Cladosporium* .

Plant species	Part/extracts	Test organisms/Concentrations in μg / disc											
		Trichophyton			*Microsporum*			*Candida*			*Cladosporium*		
		800	400	200	800	400	200	800	400	200	800	400	200
Acacia nilotica	Bark Methanol	31.1	20	7.8	26.7	14.4	7.8	11.1	-	-	15.6	6.7	-
Acacia senegal	Bark Methanol	24.4	15.6	-	24.4	13.3	-	10	-	-	18.9	10	-
Adenia volkensii	Tuber Methanol	-	-	-	-	-	-	10.6	-	-	-	-	-
Ajuga remota	leaves Pet ether	13.3	-	-	11.1	-	-	-	-	-	-	-	-
Aspilia pluriseta	Leaves 70% Ethanol	-	-	-	-	-	-	11.4	-	-	-	-	
Aporrhiza paniculata	Bark Methanol	20	16.7	10	18.9	13.1	-	7.8	-	-	11	-	-
Azadirachta indica	Bark Methanol	20	6.6	-	23.3	8.9	-	-	-	-	13.3	8.8	-
	70% Ethanol	11.1	-	-	-	-	-	-	-	-	8.8	-	-
Senna didymobotrya	Leaves Pet Ether	11.4	-	-	-	-	-	-	-	-	-	-	-
Commiphora africana	Fruits 70 %Ethanol	-	-	-	-	-	-	12.2	-	-	-	-	-
Hymenodictyon parvifolium	Bark 70% Ethanol	20.4	14.9	-	21.3	13.6	-	-	-	-	11.1	6.7	-
Plectranthus barbatus	Leaves 70% Ethanol	8.9	-	-	10	-	-	-	-	-	6.7	-	-
Plectranthus cylindraceus	Leaves 70% Ethanol	10	6.7	-	10.6	8.9	-	6.7	-	-	8.9	-	-
premna resinosa	Leaves 70% Ethanol	15.6	8.9	-	11.1	6.7	-	-	-	-	6.7	-	-
Schizozygia coffaeoides	Leaves Pet. Ether	32.2	22.2	8.9	28.9	21.1	17.8	12.3	8.9	6.7	21.1	12	-
	Dichl: EtoAc	42.3	35.3	25.6	35.6	30	24.8	18.9	12.2	10	28.9	14.8	11
	Methanol	26.7	21.1	15.6	22.2	20	14.4	10	7.7	-	13.6	7.8	-
	70% Ethanol	13.3	-	-	8.9	-	-	-	-	-	-	-	-
	Roots Dichl: EtoAc	16.7	8.9	-	14.4	8.3	-	-	-	-	8.9	-	-
	Methanol	14.2	6.7	-	13.8	7.1	-	-	-	-	6.7	-	-
Solanum arundo	Friuts 70% Ethanol	26.7	16.7	7.8	20	13.3	7.8	-	-	-	15.6	8.9	-
Solanum nigrum	Leaves Water	11.1	6.7	-	6.7	-	-	-	-	-	8.9	-	-
	70% Ethanol	11.1	-	-	10	-	-	-	-	-	-	-	-
Tagetes minuta	Leaves & Flowers Pet. Ether	11.1	-	-	11.1	-	-	-	-	-	-	-	-
	Dichl: EtoAc	20	13.3	8.9	17.8	13.3	6.7	8.9	-	-	13.3	-	-

3.3.0. Plant species selected for further bioactivity evaluation

After eliminating plants whose extracts had very low antifungal activity (less than 10% fungal growth inhibition at 400 µg per disc), together with those others that had been previously studied (*S. nigrum*, *A. nilotica. A. senegal* and *Premna resinosa*), four species were selected for further follow-up. These species were *Schizozygia coffaeoides*, *Solanum arundo*, *Aporrhiza paniculata* and *Hymenodictyon parvifolium).* Features of these plant species are shown in plates 1-4.

Plate 1: Features of *Schizozygia coffaeoides* ; grey- green stem bark (➡) and pale yellow flowers (⟶).

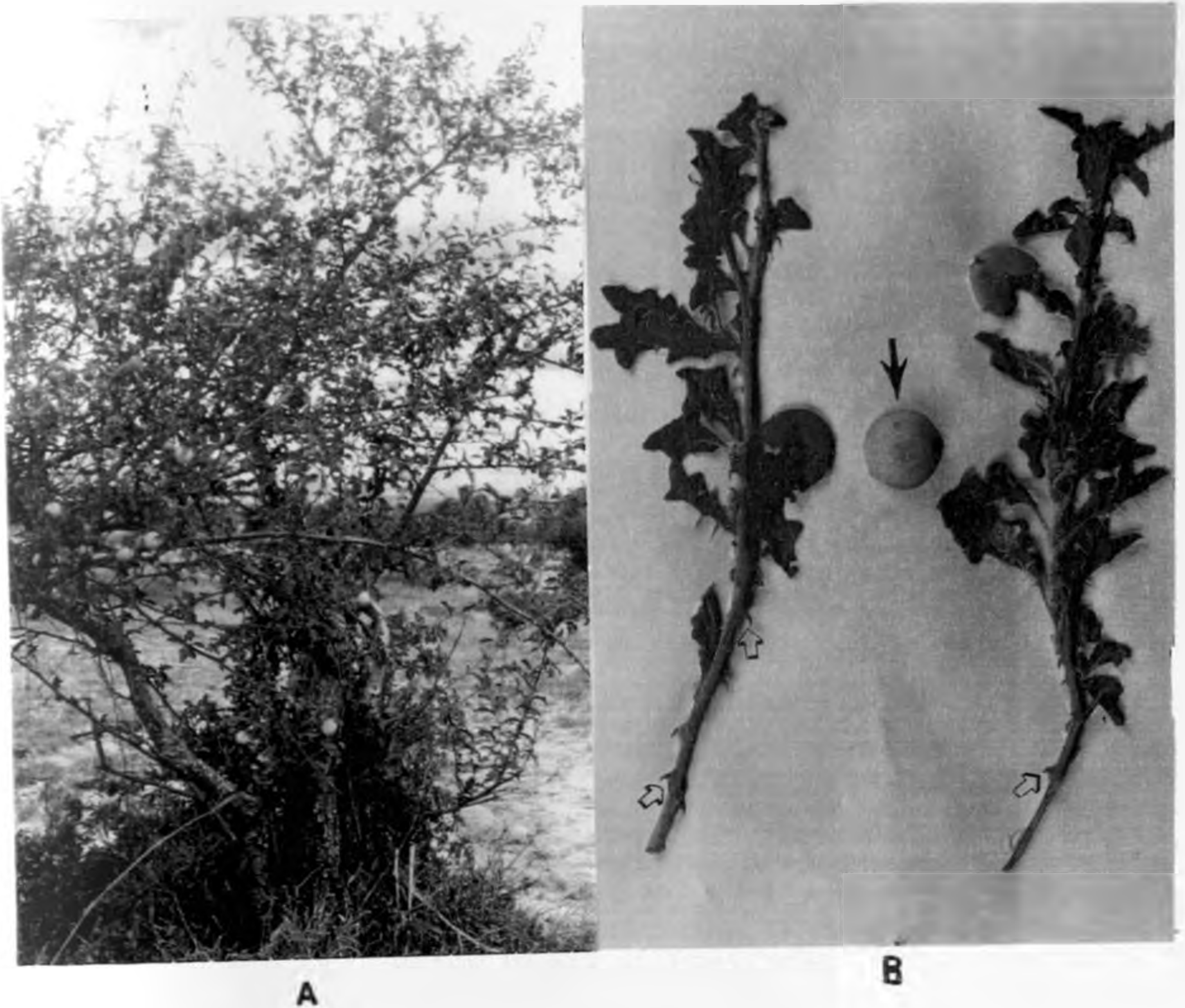


Plate 2: Features of *Solanum arundo* A: Specimen in the natural habitat; B- a closer view of a branch showing yellow globose fruits (➔) and stems with recurved thorns (⇨).

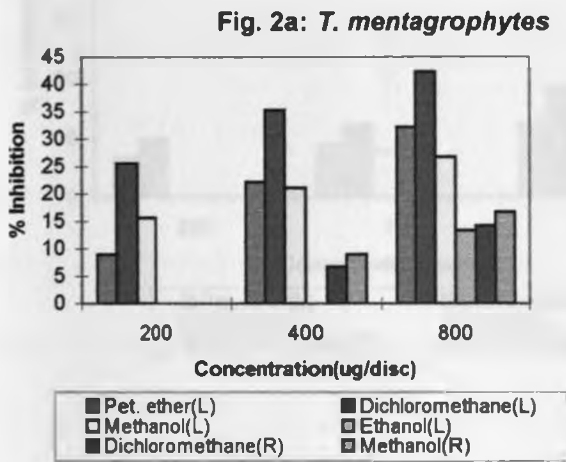
Plate 3: Features of *Aporrhiza paniculata*; leaves with cuneate base and obtuse apex, white flowers (⟶), and fruit with two flat almost round mericarps (➔).

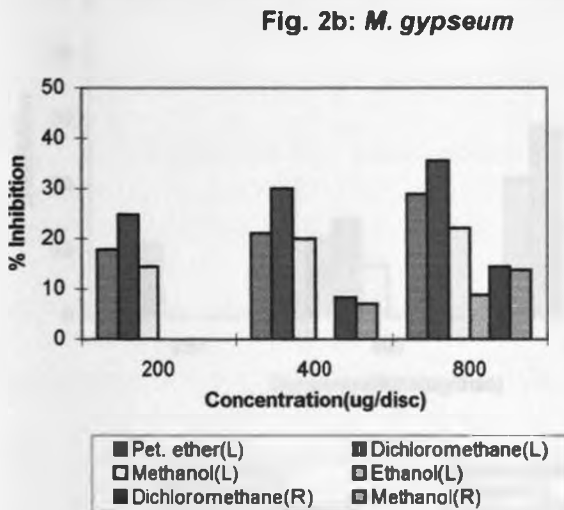
Plate 4: Features of *Hymenodictyon parvifolium* : elliptic leaves with a cuneate base and obtuse apex (⇨), dense cylindrical inflorescence (➛) and brown ellipsoid fruits (➞).

3.3.1 Bioactivity of extracts from *Schizozygia coffaeoides*

The petroleum ether, dichloromethane: ethyl acetate (1:1) and methanol extracts from the leaves of *Schizozygia coffaeoides* were fungitoxic to *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Candida albicans* and *Cladosporium cucumerinum* at concentrations of 400μg per disc. The ethanol extracts had growth inhibitory activity only against *T. mentagrophytes* and *M. gypseum* at a concentration of 800μg per disc (Fig. 2). Water extracts had no antifungal activity at concentrations of 800μg per disc. Methanol and dichloromethane extracts from the roots of *S. coffaeoides* had growth inhibition effects on *T. mentagrophytes*, *M. gypseum*, *C. albicans* and *C. cucumerinum* at concentration of 400 and 800μg per disc. However, the growth inhibitory effects of these extracts was less than 10% at a concentration of 400μg per disc. All other extracts from the roots of *S. coffaeoides* had no inhibitory effects on the growth of the test pathogens at concentrations below 800μg per disc. Data summarised in figure 2 shows that petroleum ether, dichloromethane and methanol extracts from the leaves of *S. coffaeoides* were the most fungitoxic and were therefore selected for further bioactivity tests.

Figure 2: Bioactivity (% inhibition) of extracts from *Schizozygia coffaeoides* at concentrations raging from 200-800μg/disc on *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum*





NB:

Six extracts tested at every concentration level

L= Extracts from leaves; R= Extracts from the roots

Fig. 2c: *C. albicans*

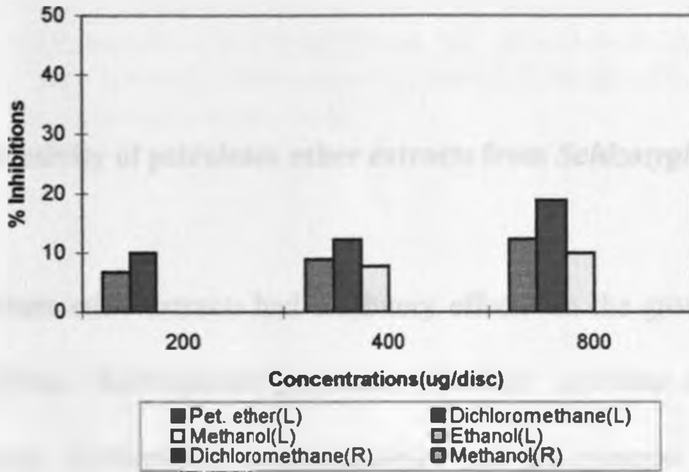
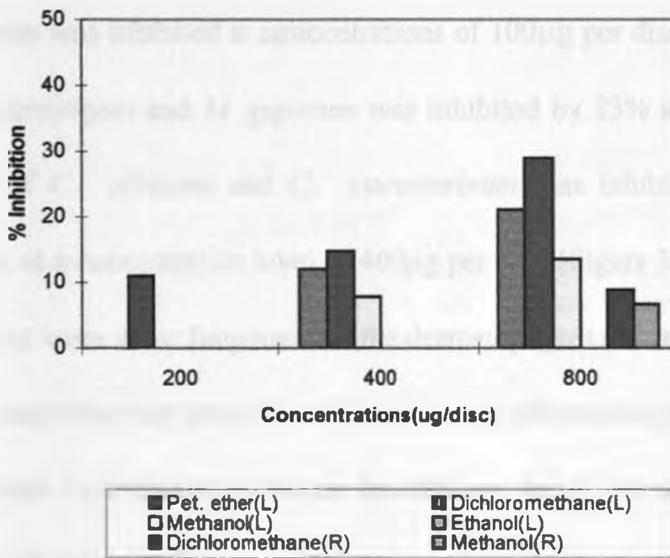


Fig. 2d: *C. cucumerinum*



NB:

Six extracts tested at every concentration level

L= Extracts from leaves; R= Extracts from the roots

3.3.1.1 Bioactivity of petroleum ether extracts from *Schizozygia coffaeoides*

The petroleum ether extracts had inhibitory effects on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum*. *Trichophyton mentagrophytes* and *M. gypseum* were sensitive at a concentration of 25μg per disc and the growth inhibitive property increased with concentrations. On the other hand the growth of *Candida albicans* and *Cladosporium cucumerinum* was inhibited at concentrations of 100μg per disc (fig. 3). The growth of *T. mentagrophytes* and *M. gypseum* was inhibited by 23% and 21% respectively, while that of *C. albicans* and *C. cucumerinum* was inhibited by 9% and 12% respectively at a concentration level of 400μg per disc (Figure 3). Thus the petroleum ether extracts were more fungitoxic to the dermatophytes (*T. mentagrophytes* and *M. gypseum*) than either the yeast (*C. albicans*) or the phytopathogen (*C. cucumerinum*). The difference in inhibitions between the different fungi was statistically significant at 99.9% level of probability.

When petroleum ether extracts were chromatographed over silica gel, the fractions eluted with petroleum ether : ethyl acetate (4:6) were found to be fungitoxic against the four test fungi. It was however found out that the antifungal activity of this fraction was lower than that of the unfractionated sample. The fungitoxicity of petroleum extracts decreased with fractionation by an average of between 2-10% for all the fungi tested (Figure 4).

Figure 3: Effects of Petroleum ether extracts from *Schizozygia coffaeoides* at concentrations of 0-400 µg/disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum.*

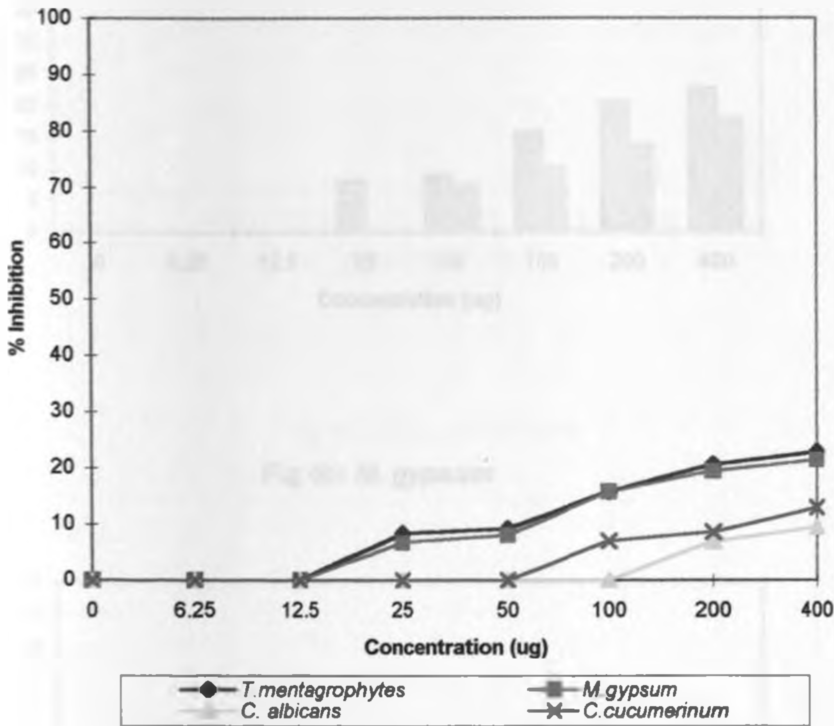


Figure 4: The effects of fractionated and unfractionated petroleum ether extracts at concentrations of 0-400 µg/disc on the on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum.*

Fig 4a : *T. mentagrophytes*

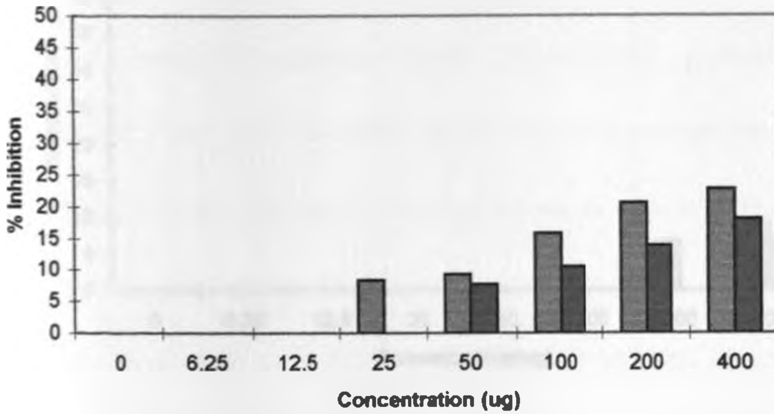


Fig 4b: *M. gypsum*

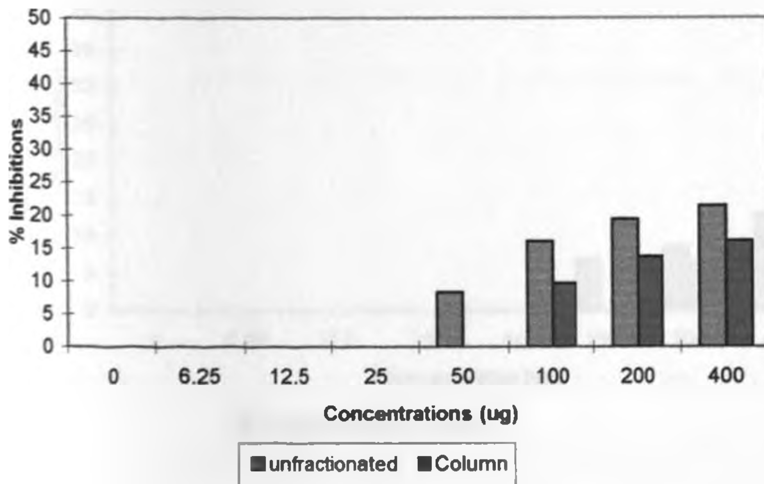


Fig 4c : *C. albicans*

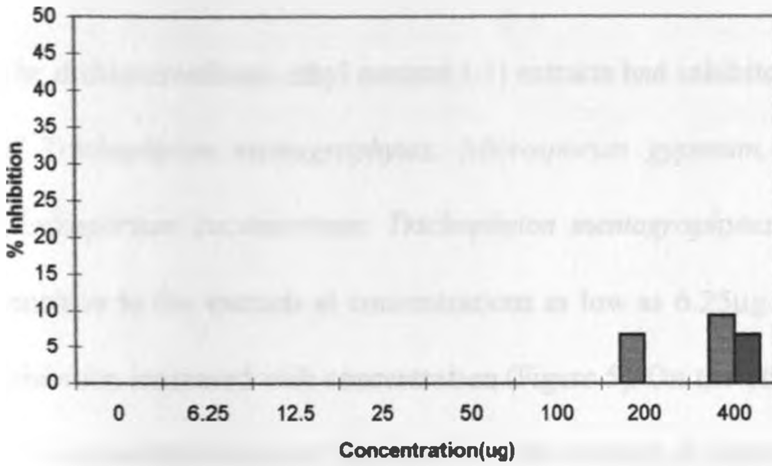
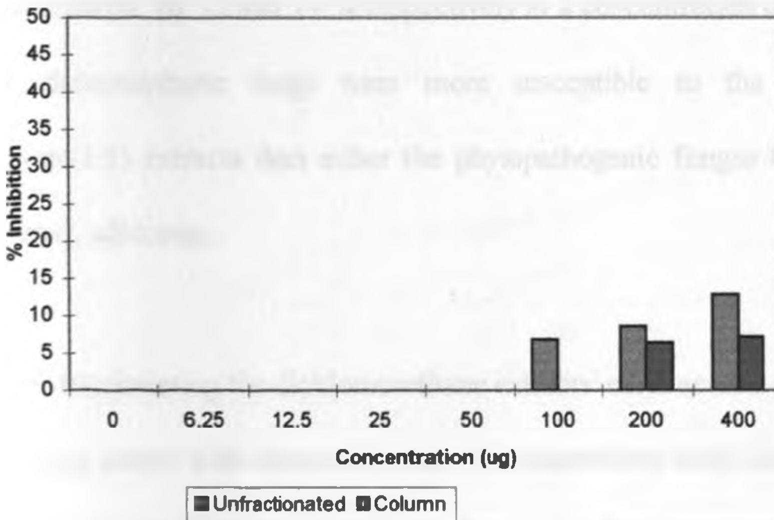


Fig 4d : *C.cucumerinum*



3.3.1.2 Bioactivity of dichloromethane: ethyl acetate (1:1) extracts from *Schizozygia coffaeoides*.

The dichloromethane: ethyl acetate(1:1) extracts had inhibitory effects on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum. Trichophyton mentagrophytes* and *M. gypseum* were sensitive to the extracts at concentrations as low as 6.25μg per disc and the growth inhibition increased with concentration (Figure 5). On the other hand *C. albicans* and *C. cucumerinum* were not susceptible to the extracts at concentrations below 100 and 50 μg per disc respectively. The growth of *T. mentagrophytes* and *M. gypseum* was reduced by 35% and 31% respectively while that of *C. albicans* and *C. cucumerinum* was reduced by 13 and 14 % respectively at a concentration of 400μg per disc. Thus the dermatophytic fungi were more susceptible to the dichloromethane:ethyl acetate(1:1) extracts than either the phytopathogenic fungus *C. cucumerinum* or the yeast *C. albicans*.

After fractionating the dichloromethane extracts: ethyl acetate extracts, column fractions eluted with chloroform and with chloroform: ethyl acetate (10:0.5) were fungitoxic against the test pathogens. Fractionated dichloromethane extracts were more fungitoxic than the unfractionated dichloromethane extracts by an average of 5% (Figure 6). Further purification by Preparative Thin Layer Chromatography (PTLC) gave a band that was about 12-16% more fungitoxic than the column fraction (Figure 6). The column fraction was therefore more active than the unfractionated

dichloromethane extracts but less active than the PTLC band (Figure 6). The differences in fungitoxicity between the different fractions was statistically significant at 99.9 level of probability.

Figure 5: Effects of dichloromethane: ethyl acetate (1:1) extracts from *Schizozygia coffaeoides* at concentrations of 0-400 μg/disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum*

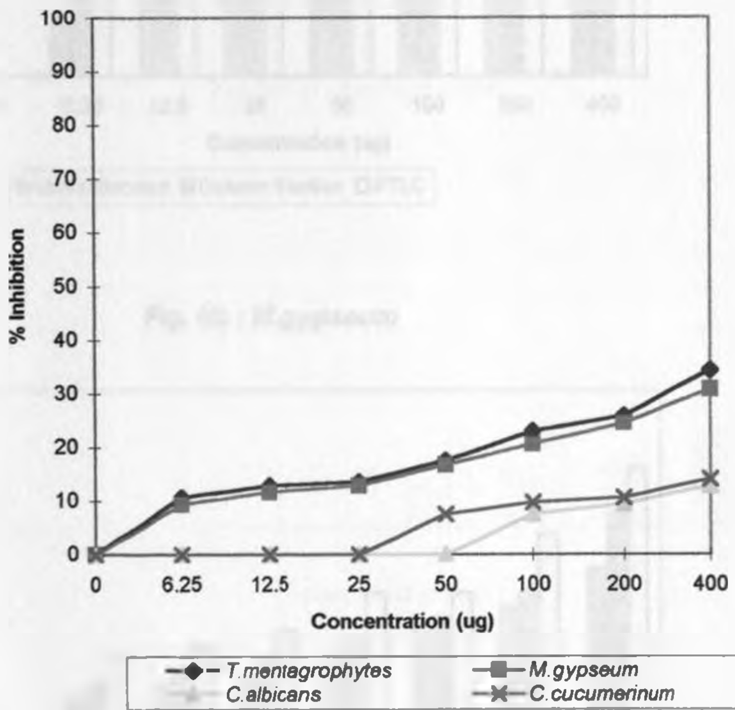
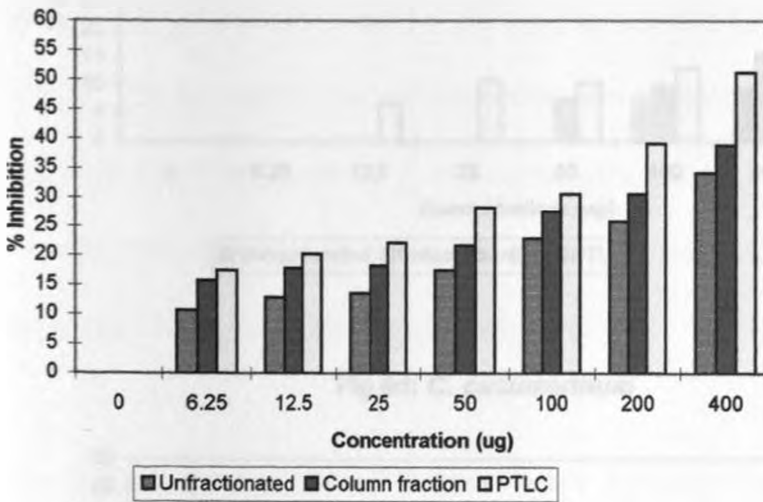


Figure 6. Effects of fractionated and unfractionated dichloromethane: ethyl acetate(1:1) extracts from *Schizozygia coffaeoides* at concentrations of 0-400 μg/disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum.*



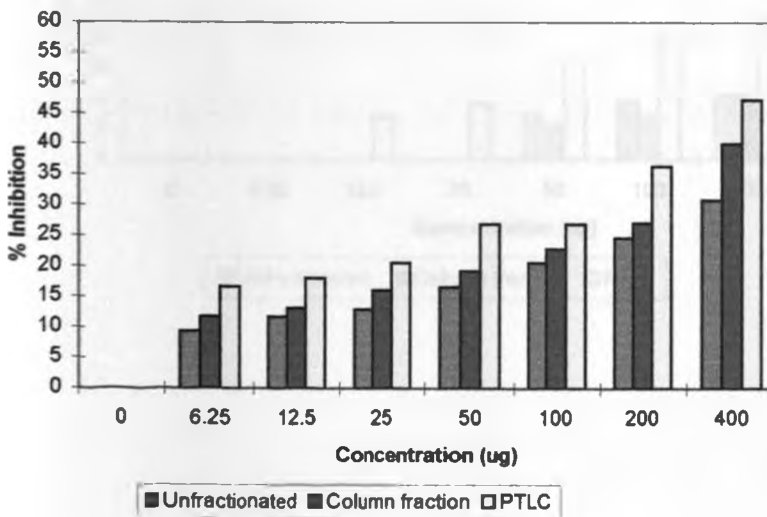


Fig 6c : *C. albicans*

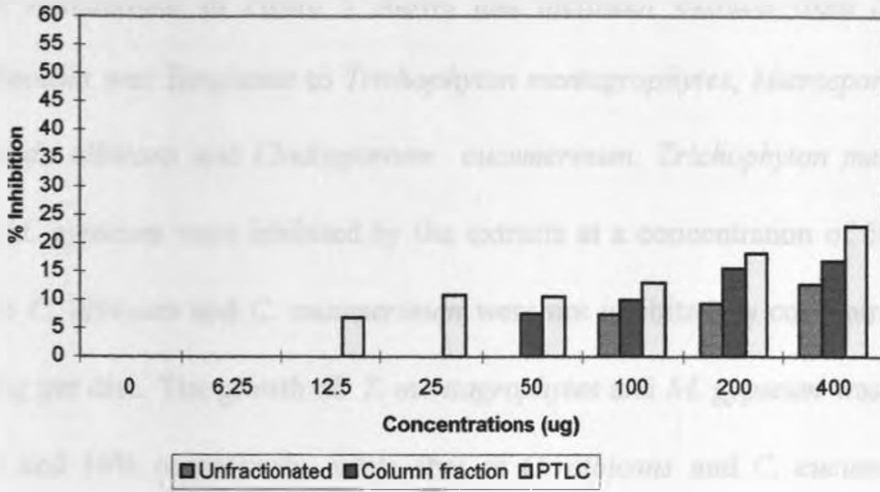
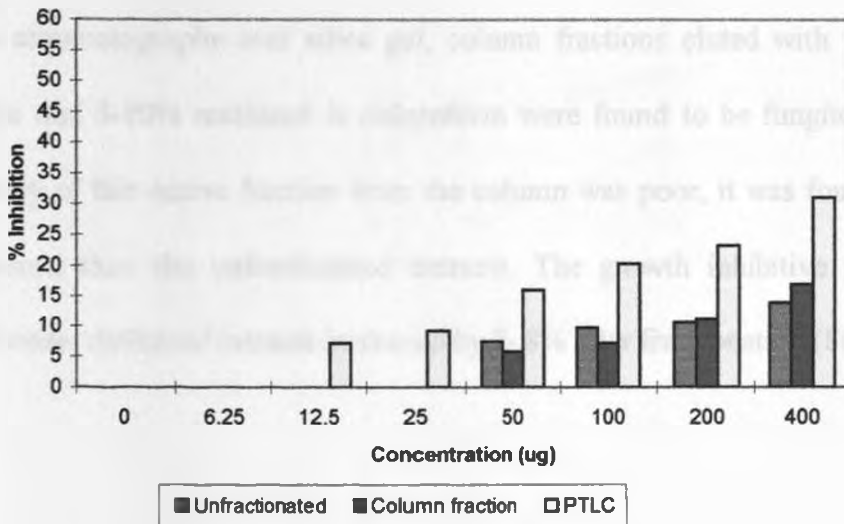


Fig 6d: *C. cucumerinum*



3.3.1.3 Bioactivity of methanol extracts from *Schizozygia coffaeoides*

Data summarized in Figure 7 shows that methanol extracts from *Schizogygia coffaeoides* was fungitoxic to *Trichophyton mentagrophytes*, *Microsporum gypseum Candida albicans* and *Cladosporium cucumerinum*. *Trichophyton mentagrophytes* and *M. gypseum* were inhibited by the extracts at a concentration of 50µg per disc while *C. albicans* and *C. cucumerinum* were not inhibited by concentrations below 200µg per disc. The growth of *T. mentagrophytes* and *M. gypseum* was inhibited by 21% and 19% respectively, while that of *C. albicans* and *C. cucumerinum* were inhibited by 8% at a concentration of 400µg per disc.

After chromatography over silica gel, column fractions eluted with 15-20% ethyl acetate and 5-10% methanol in chloroform were found to be fungitoxic. Although recovery of this active fraction from the column was poor, it was found to be more fungitoxic than the unfractionated extracts. The growth inhibitive property of *S. coffaeoides* methanol extracts increased by 2- 8% after fractionation (Figure 8)

Figure 7: Effects of methanol extracts from *Schizozygia coffaeoides* at concentrations of 0-400µg per disc on growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium. cucumerinum* .

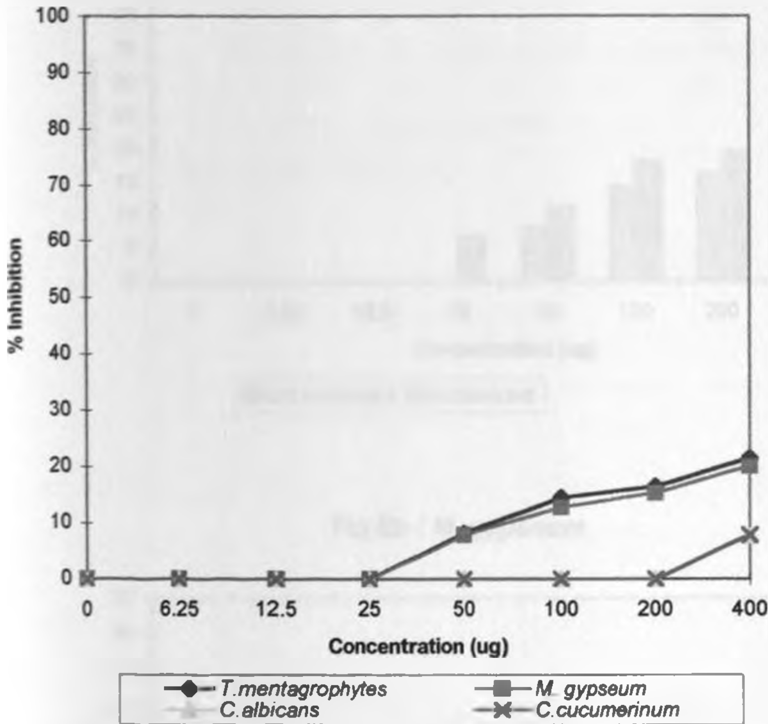
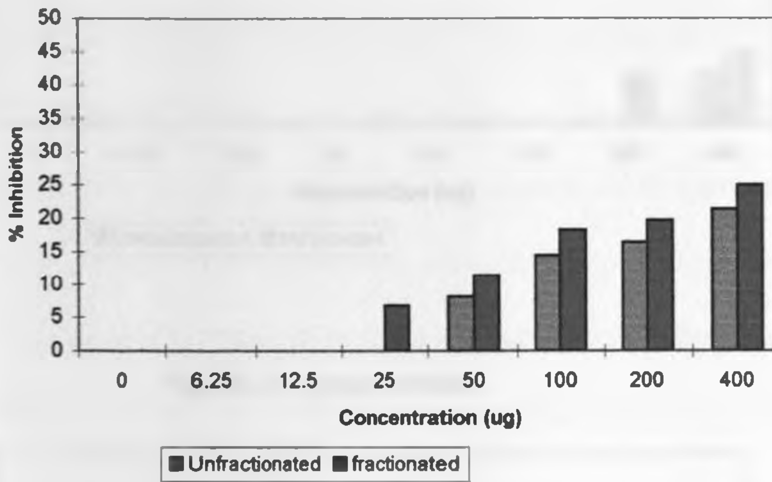


Figure 8. Effects of fractionated and unfractionated methanol extracts from *Schizozygia coffaeoides* at concentrations of 0-400µg per disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum*



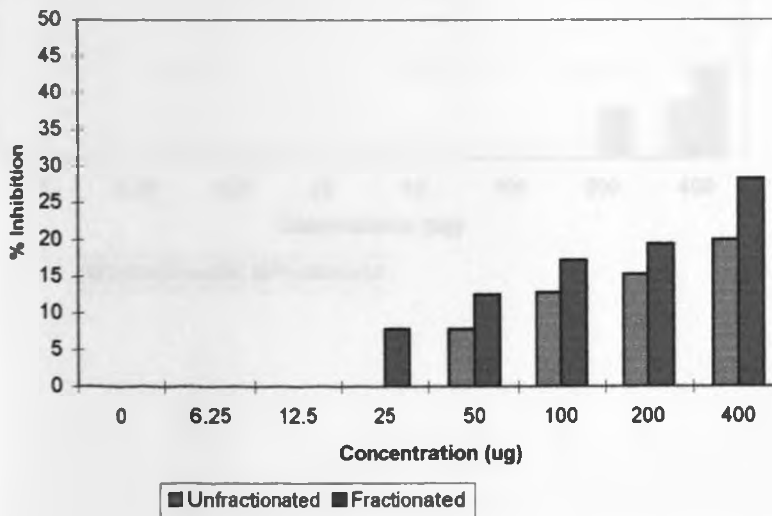


Fig 8c : *C. albicans*

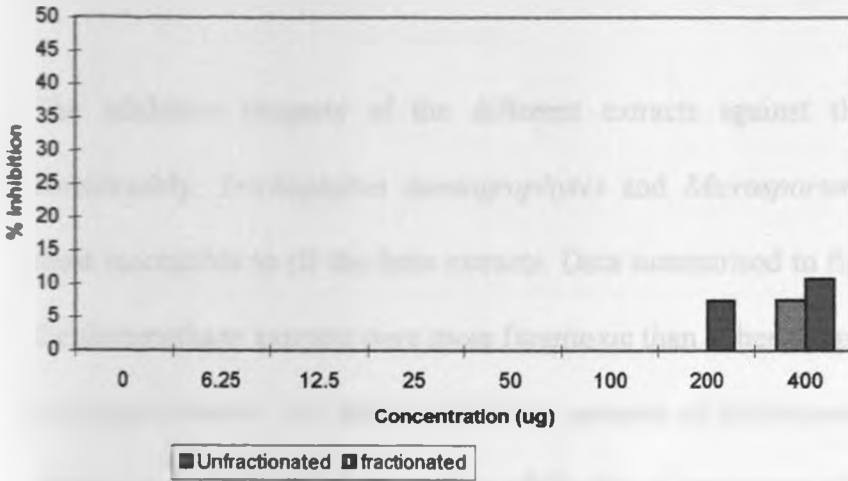
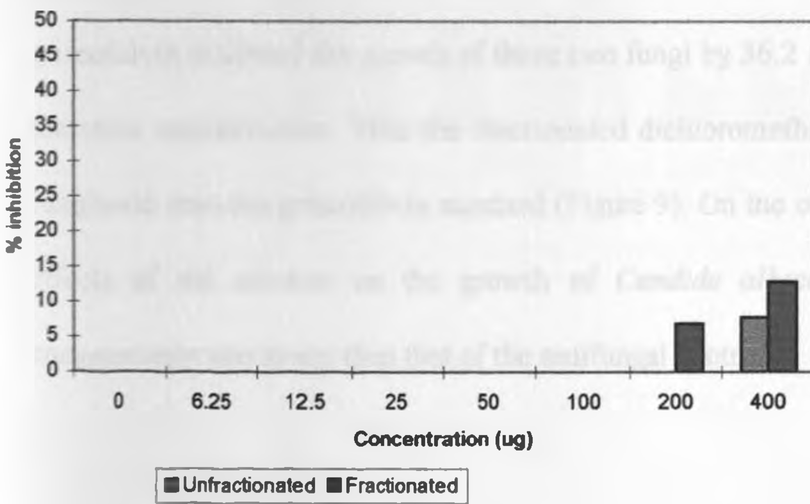


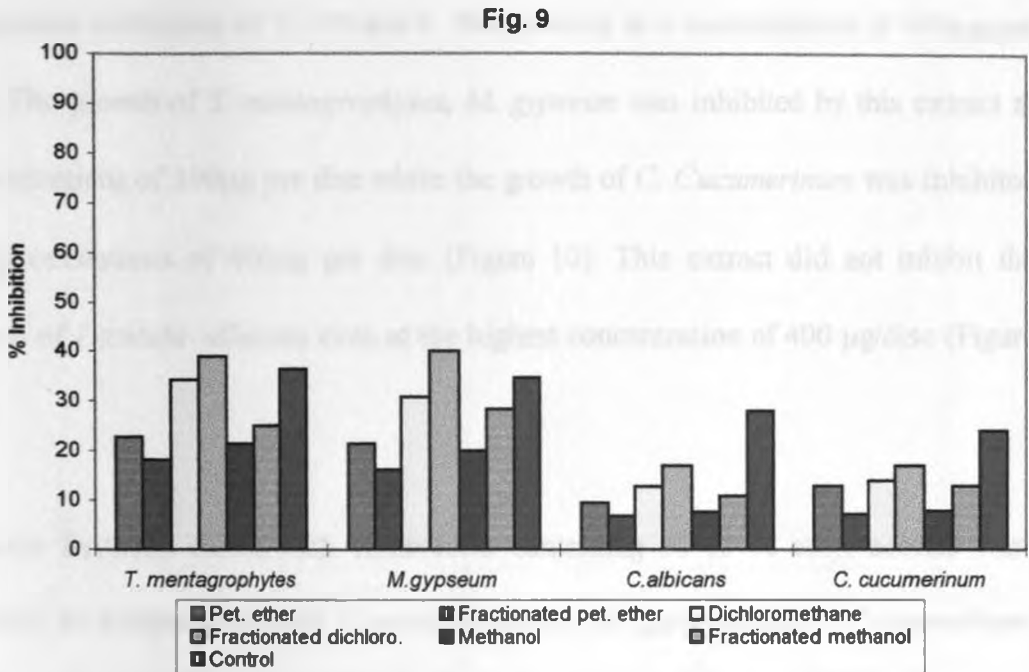
Fig 8d : *C. cucumerinum*



3.3.1.4 The growth inhibitory effects of petroleum ether, dichloromethane: EtoAc (1:1) and methanol extracts from *Schizozygia coffaeoides*

The inhibitive property of the different extracts against the test fungi varied considerably. *Trichophyton mentagrophytes* and *Microsporum gypseum* were the most susceptible to all the three extracts. Data summarised in figure 9 shows that the dichloromethane extracts were more fungitoxic than either the petroleum ether or the methanol extracts. The growth inhibitive property of dichloromethane and methanol extracts increased with fractionation while that of petroleum ether decreased (Figure 9). The fractionated dichloromethane extracts inhibited the growth of dermatophytic fungi *T. mentagrophytes* and *M. gypseum* by 38.9% and 40.1% respectively while griseofulvin inhibited the growth of these two fungi by 36.2 and 34.7 respectively at the same concentration. Thus the fractionated dichloromethane extracts were more fungitoxic than the griseofulvin standard (Figure 9). On the other hand the inhibitory effects of the extracts on the growth of *Candida albicans* and *Cladosporium cucumerinum* was lower than that of the antifungal controls.

Figure 9: Effects of different extracts from *Schizozygia coffaeoides* at a concentration of 400μg/disc on the growth of *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Candida albicans* and *Cladosporium cucumerinum*.



3.3.2 Bioactivity of extracts from *Solanum arundo*

The ethanol extracts from *Solanum arundo* were fungitoxic against *Trichophyton mentagrophytes*, *Microsporum gypseum* and *Cladosporium Cucumerinum* with percentage inhibitions of 17, 14 and 8 %resectively at a concentration of 400µg per disc .The growth of *T. mentagrophytes, M. gypseum* was inhibited by this extract at concentrations of 100µg per disc while the growth of *C. Cucumerinum* was inhibited by concentrations of 400µg per disc (Figure 10). This extract did not inhibit the growth of *Candida albicans* even at the highest concentration of 400 µg/disc (Figure 10).

Column fractions eluted with chloroform containing 30-65 % ethyl acetate were found to be fungitoxic against *T. mentagrophytes, M. gypseum* and *C. Cucumerinum*. The growth inhibitive property of *S. arundo* ethanol extracts on *T. mentagrophytes* and *M. gypseum* increased with fractionation by 2-10% and by 1.4% on *C. cucumerinum* at a concentration of 400 µg/disc (Figure 11). Just like the crude extracts, the column fraction had no inhibitory effects against *C. albicans*. Griseofulvin antifungal standard inhibited the growth of *T. mentagrophytes* and *M. gypseum* by an average of 36.1% and 34.2% respectively at a concentration of 400 µg/disc while the growth of *C. cucumerinum* was inhibited by 22.4% at the same concentration (Figure 11).

Figure 10: Effects of 70 % ethanol extracts from *Solanum arundo* at concentrations of 0-400 μg/disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum Cladosporium Cucumerinum* and *Candida albicans.*

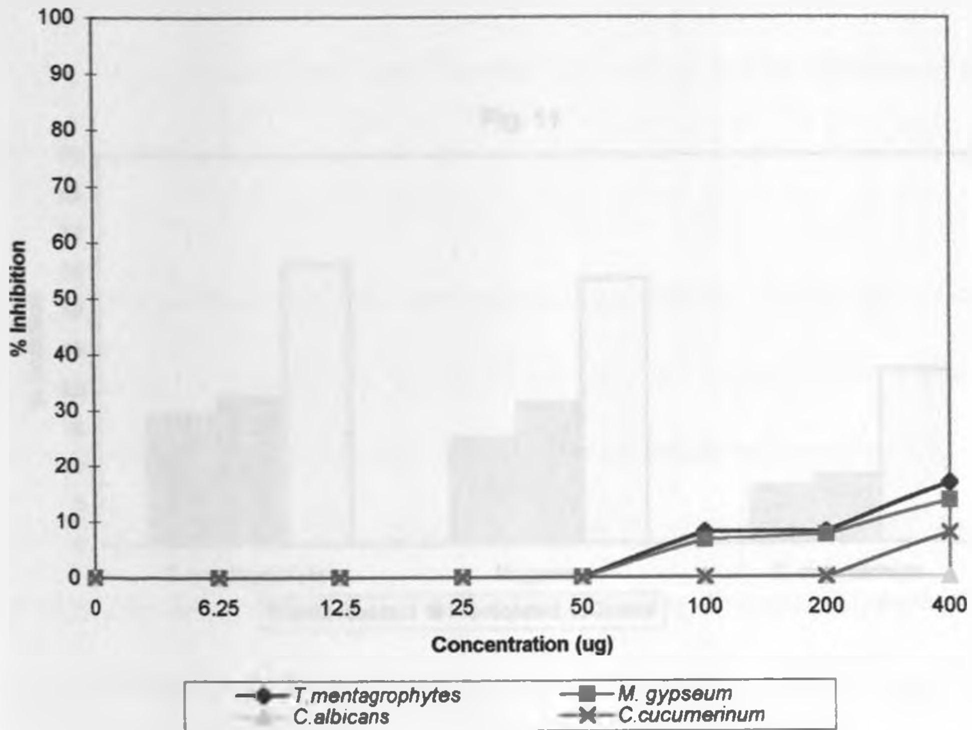
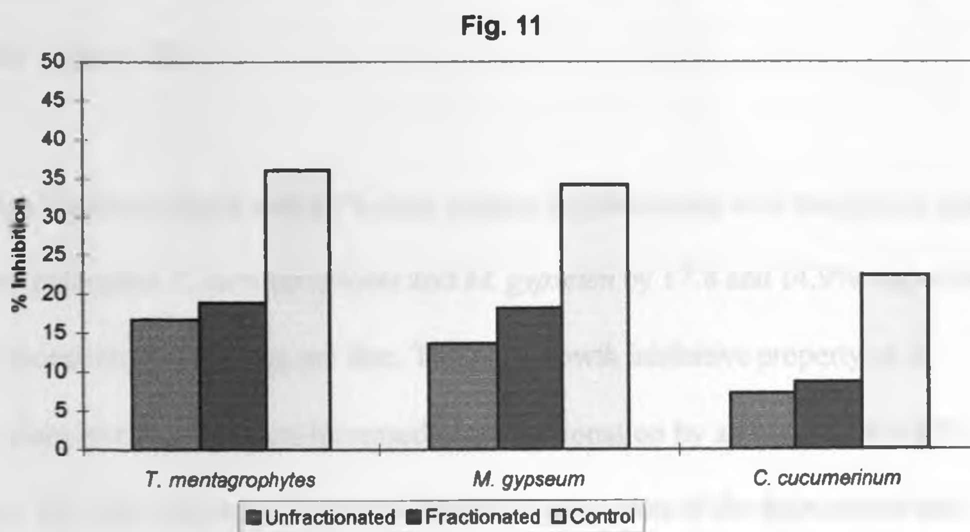


Figure 11: The effects of fractionated and unfractionated ethanol extracts from *Solanum arundo* at a concentration of 400 μg/disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum* , *Cladosporium cucumerinum* and *Candida albicans*



3.3.3 Bioactivity of extracts from *Aporrhiza paniculata*

Methanol extracts from *Aporrhiza paniculata* caused growth inhibitions of 17% and 14% against *Trichophyton mentagrophytes and Microsporum gypseum* respectively at a concentration of 400μg per disc. The methanol extract did not inhibit the growth of *Cladosporium cucumerinum* and *Candida albicans* at a concentration of 400μg per disc (Figure 12).

Column fractions eluted with 60% ethyl acetate in chloroform were fungitoxic against the test pathogens *T. mentagrophytes* and *M. gypseum* by 17.8 and 14.9% respectively at a concentration of 400 μg per disc. Thus the growth inhibitive property of *A. paniculata* methanol extracts increased after fractionation by an average of 0.85% (Figure 13). The difference in growth inhibitive properties of the fractionated and unfractionated methanol extracts from *A. paniculata* was not statistically significant at 99.9 level of probability. Griseofulvin antifungal control inhibited the growth of *T. mentagrophytes* and *M. gypseum* by 36% and 33.7% respectively at a concentration of 400μg per disc (Figure 13). The growth of *C. albicans* and *C. cucumerinum* was not inhibited by the fractionated *A. paniculata* extracts.

Figure 12: Effects of methanol extracts from *Aporrhiza paniculata* at concentrations of 0-400 μg/disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Cladosporium cucumerinum* and *Candida albicans.*

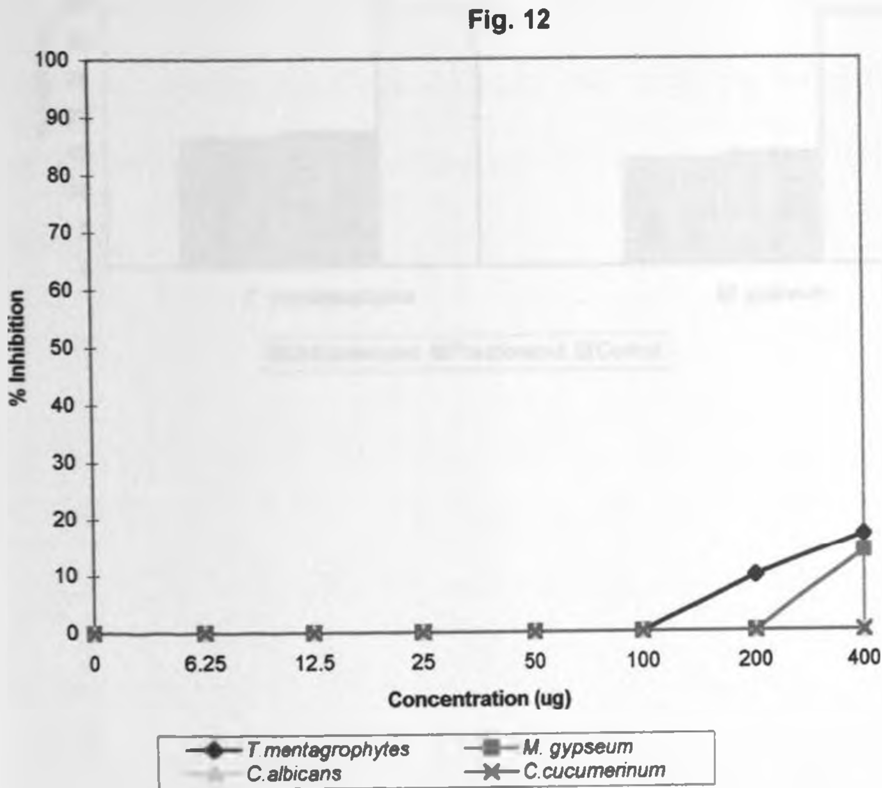
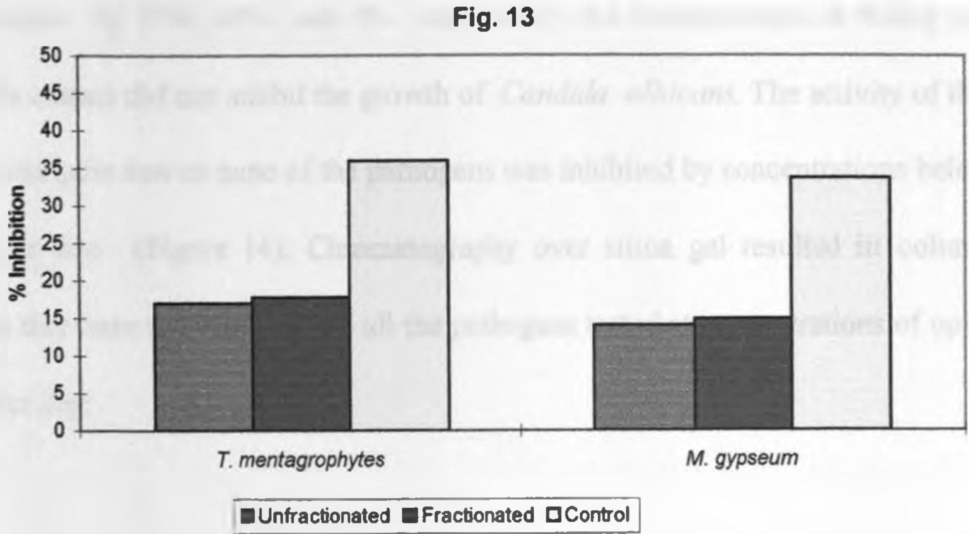


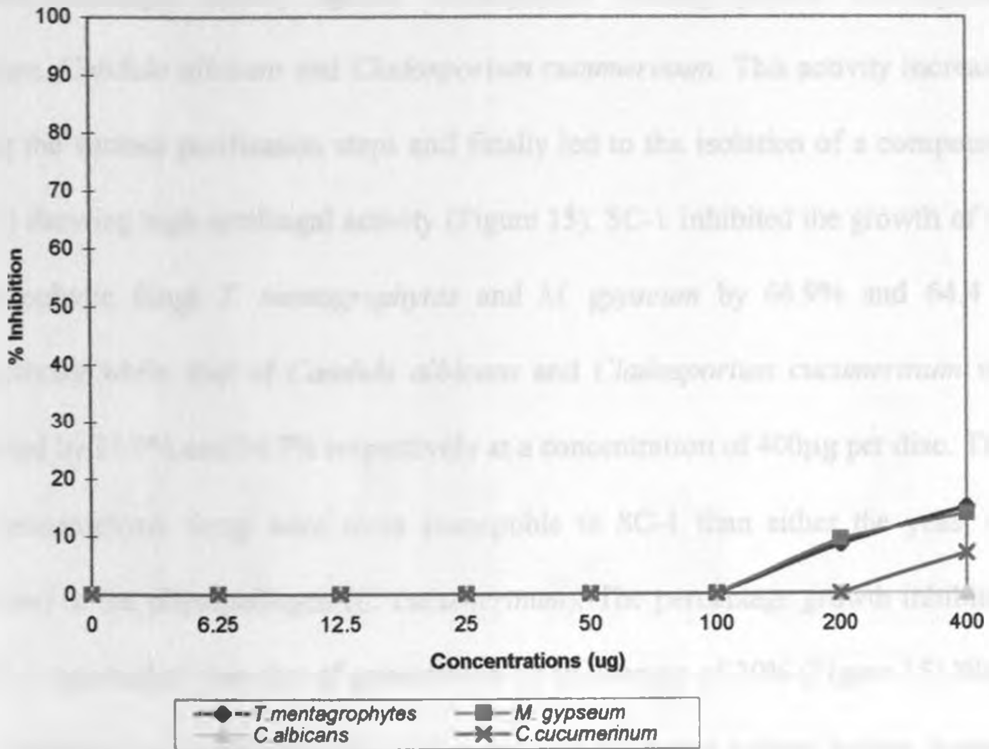
Figure 13: Effects of fractionated and unfractionated extracts from *Aporrhiza paniculata* at a concentration of 400 μg/disc on the growth of *Trichophyton mentagrophytes* and *Microsporum gypseum*.



3.3.4 Bioactivity of ethanol extracts from *Hymenodictyon parvifolium*

The ethanol extracts from *Hymenodictyon parvifolium* inhibited the growth of *Trichophyton mentagrophytes*, *Microsporum gypseum*, and *Cladosporium cucumerinum* by 15% ,14% and 7% respectively at a concentration of 400μg per disc. This extract did not inhibit the growth of *Candida albicans*. The activity of this extract was quite low as none of the pathogens was inhibited by concentrations below 200μg per disc (Figure 14). Chromatography over silica gel resulted in column fractions that were not fungitoxic to all the pathogens tested at concentrations of up to 400μg per disc.

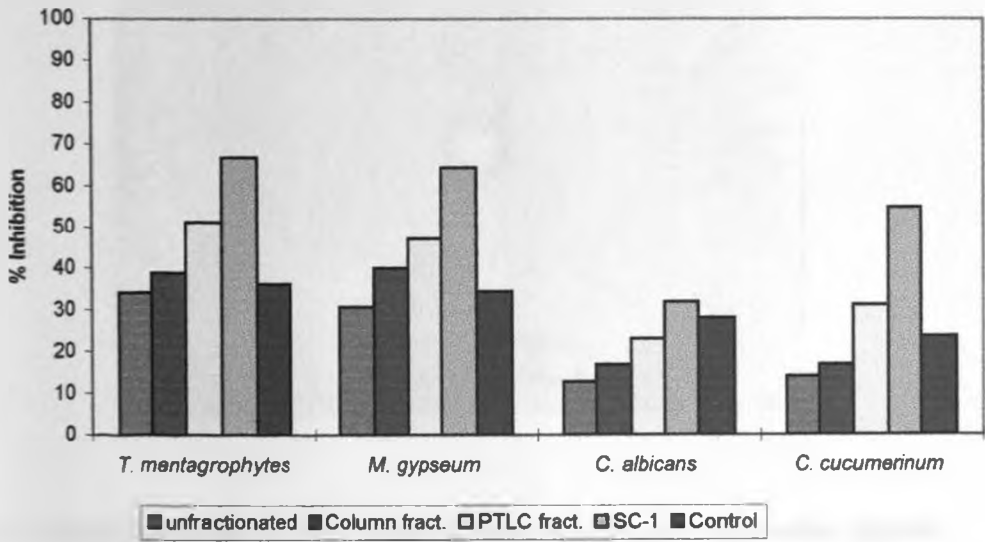
Figure 14 : Effects of 70% ethanol extracts from *Hymenodictyon parvifolium* at concentrations of 0-400 µg/disc on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Cladosporium cucumerinum* and *Candida albicans*.



3.4 Antifungal compounds from *Schizozygia coffaeoides*.

The dichloromethane: ethyl acetate (I:1) extracts from *Schizozygia coffaeoides* showed antifungal activity against *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum*. This activity increased during the various purification steps and finally led to the isolation of a compound (SC-1) showing high antifungal activity (Figure 15). SC-1 inhibited the growth of the dermatophytic fungi *T. mentagrophytes* and *M. gypseum* by 66.9% and 64.4 % respectively while that of *Candida albicans* and *Cladosporium cucumerinum* was inhibited by 31.9% and 54.7% respectively at a concentration of 400μg per disc. Thus the dermatophytic fungi were more susceptible to SC-1 than either the yeast (*C. albicans*) or the phytopathogen (*C. cucumerinum*). The percentage growth inhibition by SC-1 was higher than that of griseofulvin by an average of 30% (Figure 15).When the compound was applied to TLC silica gel and run using solvent system hexane: dichloromethane: ethyl acetate (2:2:1), it was seen to move as a single spot and it's presence was apparent at RF-0.42. This was confirmed by *Cladosporium* spray bioassay which showed a clear zone without any fungal growth (Plate 5).

Figure 15: Effects of dichloromethane: ethyl acetate(1:1) extracts from *Schizozygia coffaeoides* at different stages of purification on the growth of *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum* at a concentration of 400 μg/disc.



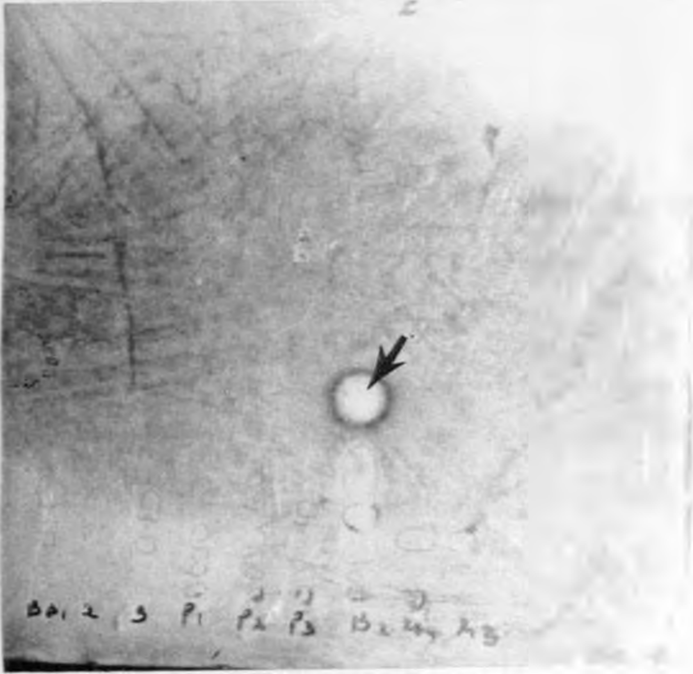


Plate 5: Inhibition zone of SC-1 isolated from *Schizozygia coffaeoides* against *Cladosporium cucumerinum* on TLC Silica gel. Note: clear zone without any fungal growth (→).

NB: The notations at the bottom of the plate denote other samples spotted alongside.

3.4.1 High Performance Liquid Chromatography (HPLC) for SC-1

A single absorption peak observed in the purity check based on analytical HPLC (Figure 16) and a single band shown on several TLC systems was a strong indication of purity and homogeneity of the sample.

The compound gave a single Peak on HPLC analysis at retention time 23.95 min. with a maximum of absorption bands at 237,274 (Sh -280) ,317 in its UV spectrum (Figure 17).

The compound was positive to Dragendorff, Folin and Iodoplatinate reagents indicating the presence of phenols and/or alkaloids.

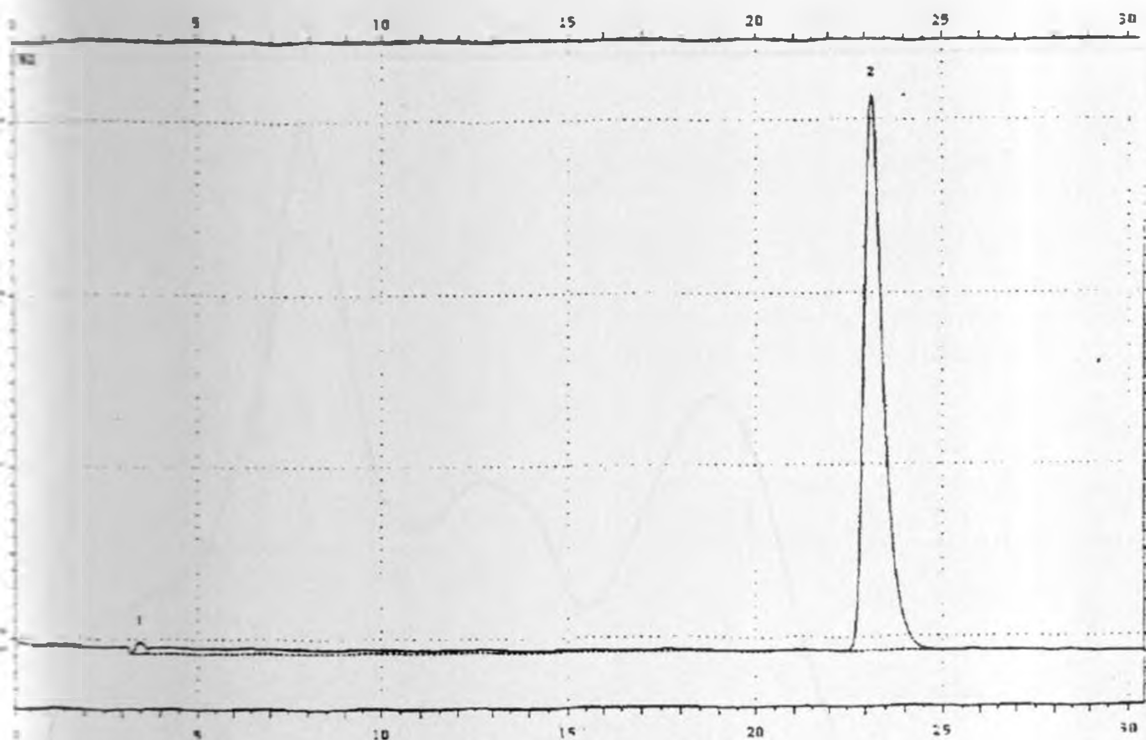


Figure 16: HPLC chromatogram of SC-1 isolated from *Schizozygia coffaeoides*.

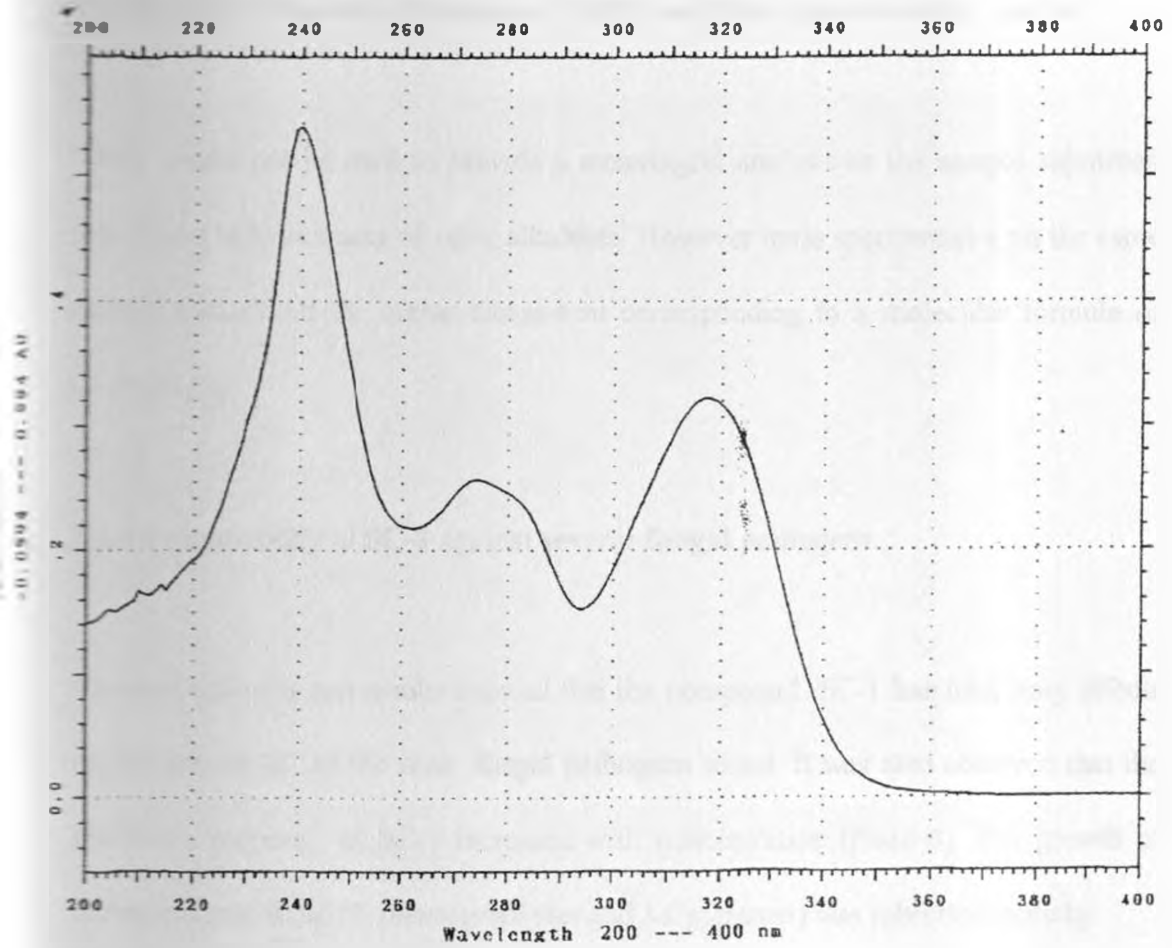


Figure 17: UV spectrum of SC-1 (Compound isolated from *Schizozygia coffaeoides*).

3.4.2 Nuclear Magnetic Resonance (NMR) and Mass Spectrometry results

NMR could not be used to provide a meaningful analysis as the sample submitted was found to have traces of other alkaloids. However mass spectrometry on the same sample picked out the major component corresponding to a molecular formula of $C_{21}H_{22}N_2O_4$.

3.5.0 Fungitoxicity of SC-1 against several fungal pathogens

The susceptibility test results showed that the compound SC-1 has inhibitory effects on the growth of all the nine fungal pathogens tested. It was also observed that the inhibitive property of SC-1 increased with concentration (Plate 6). The growth of dermatophytic fungi (*T. mentagrophytes* and *M. gypseum*) was inhibited more by SC-1 than by griseofulvin, the antifungal compound used as the control (Plate 7). The minimum inhibitory concentrations (MIC) values of SC-1 on the filamentous and yeast fungi varied considerably for the different pathogens. Among the pathogens tested, *T. mentagrophytes* had the lowest active concentration of 1.56 μg/disc and *Botrytis cinerea* had the highest at 200 μg/disc (Table 4).

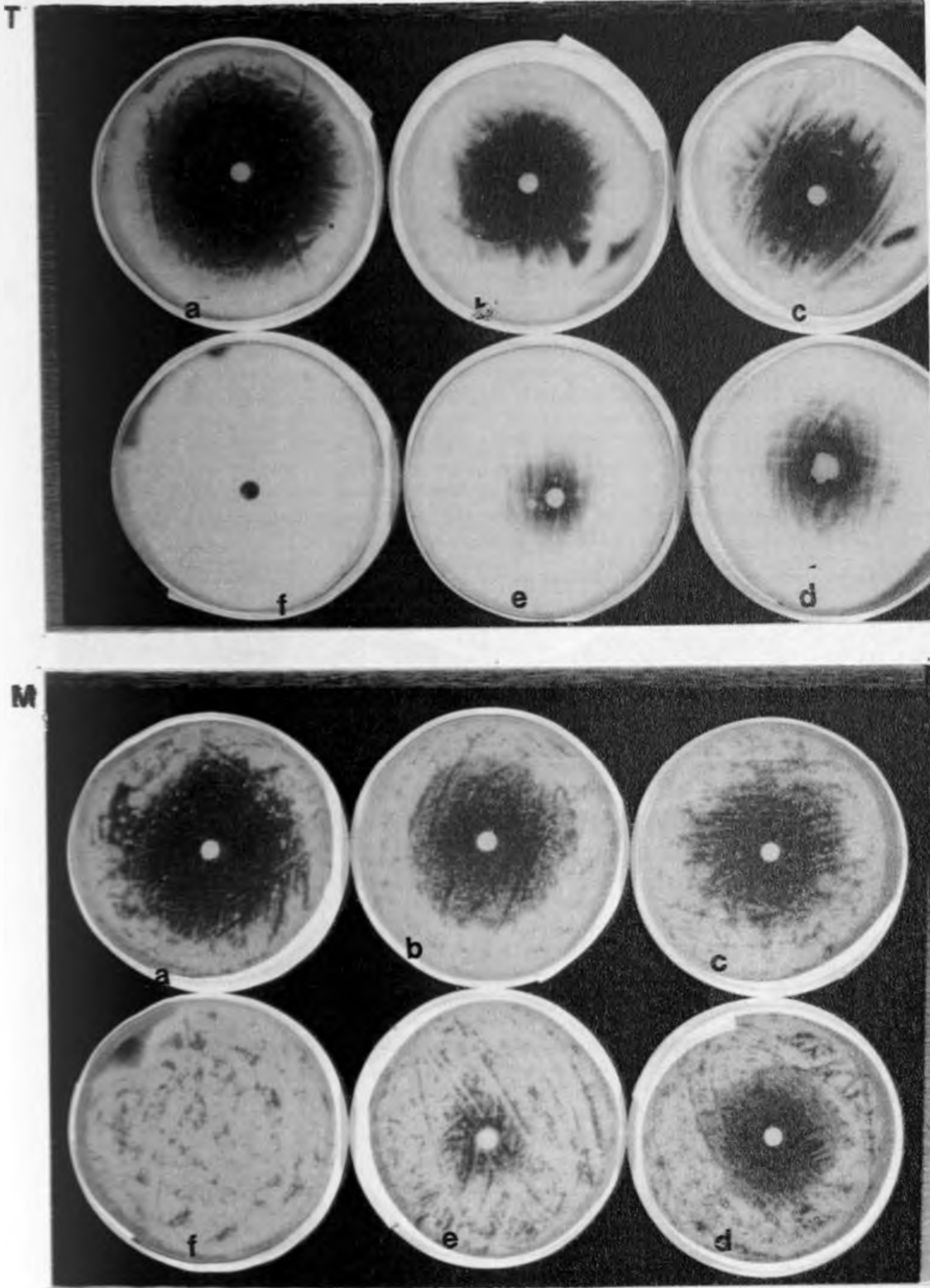


Plate 6: Effects of SC-1 isolated from *Schizozygia coffaeoides* on growth of *Trichophyton mentagrophytes* (T) and *Microsporum gypseum* (M) at concentrations of 0- 400 μg/disc

a- 400 μg/disc	d- 50μg/disc
b-200 μg/disc	e- 25μg/disc
c-100 μg/disc	f- 0μg/disc

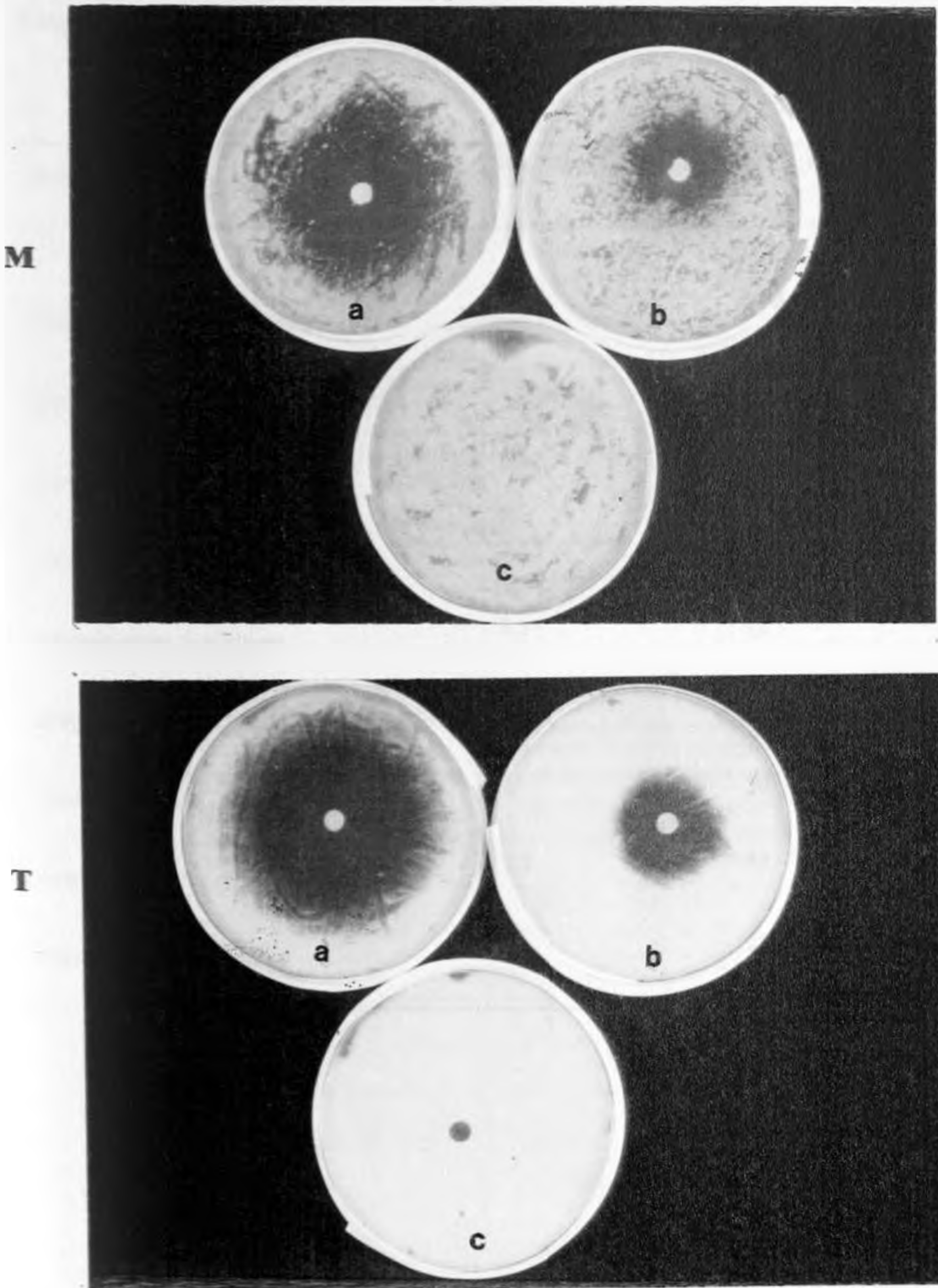


Plate 7: Effects of SC-1 (isolated from *Schizozygia coffaeoides* on the growth of *Microsporum gypseum* (M) and *Trichophyton mentagrophytes* (T) as compared to griseofulvin control at a concentration of 400μg/disc.
a- SC-1 b-Griseofulvin c-Control

Table 4. Minimum Inhibitory concentrations (MIC) of SC-1 on filamentous and yeast fungi.

Fungi	Inhibition zone (mm)	MIC (µg)
Trichophyton mentagrophytes	6.25	1.56
Microsporum gypseum	7.0	3.125
Candida albicans	7.75	6.25
Cladosporium cucumerinum	6.75	3.125
Cladosporium harbarum.	8.5	12.5
Botrytis cinerea	6.75	200
Alternaria tenuis	8	50
Fusarium solani	7.5	50
Saccharomyces cerevisae	6	25

3.5.1 Effects of SC-1 on mycelial dry weight

In liquid culture SC-1 reduced mycelia dry weight of the filamentous fungi in varying degrees. *Cladosporium cucumerinum* was the most inhibited with dry weight being reduced by up to 91% at 10μg/ml. Data summarised in Figure 18 show that percentage reduction in dry weight increased with concentration. The differences in dry weight reduction of *Cladosporium harbarum*, *Botrytis cinerea* , *Alternaria tenuis* and *Fusarium solani* by SC-1 was not statistically significant at 99.9% level of probability (Analysis of Variance Table 5).

Figure 18. Percentage reduction in dry weight of five fungal pathogens by SC-1 at concentrations raging from 0-10 μg/ml.

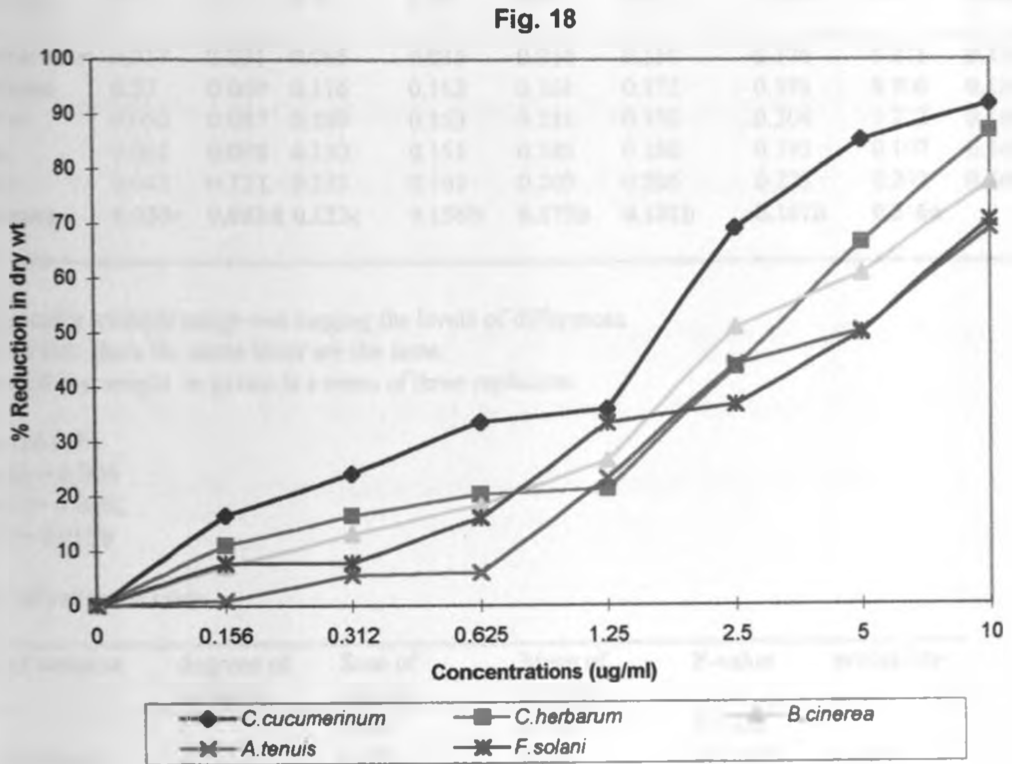


Table 5 : Effects of SC-1 on mycelial growth of five fungal pathogens (ANOVA)

Two way table of means

Test organisms/ /dry wt* (g)	Conc. (µg/ml) 10	5	2.5	1.25	0.63	0.31	0.156	0.0	**Fungi means**
C. cucumerinum	0.017	0.031	0.065	0.035	0.014	0.150	0.176	0.211	**0.119d**
C. harbarum	0.27	0.069	0.116	0.162	0.161	0.172	0.183	0.206	**0.133c**
B. cinerea	0.050	0.087	0.109	0.163	0.181	0.193	0.206	0.222	**0.160ab**
A. tenuis	0.061	0.098	0.110	0.151	0.185	0.186	0.195	0.197	**0.145bc**
F. solani	0.041	0.121	0.153	0.161	0.203	0.206	0.223	0.242	**0.169a**
conc. means	**0.035e**	**0.081d**	**0.123c**	**0.156b**	**0.175b**	**0.181b**	**0.181b**	**0.216a**	

a.b.c -Duncan's multiple range test ranging the levels of differences
Nb. Means that share the same letter are the same.
* = Mycelial dry weight in grams is a mean of three replicates.

CV(%)= 16.83%
SE(Fungi) = 0.049
SE(Conc.)= 0.0062
SE(F.C) = 0.0139

Analysis of variance table

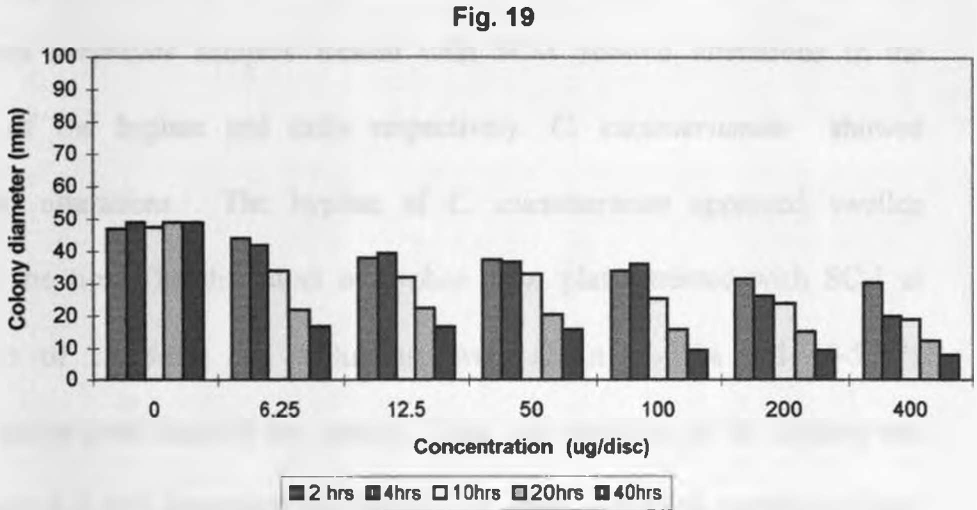
Source of variance	degrees of freedom	Sum of squares	Mean of squares	F-value	probability
Reps	2	0.001	0.000	0.7016	
Factor A(Fungi)	4	0.051	0.013	21.8472	0.0000*
Factor B(Conc.)	7	0.379	0.054	92.7284	0.0000*
Factor (AB)	28	0.021	0.001	1.2969	0.1852ns
Error	78	0.045	0.049		

* - very highly significant at 99.9% level of probability.

3.5.2 Effects of concentration and time of exposure on the inhibitive properties of SC-1

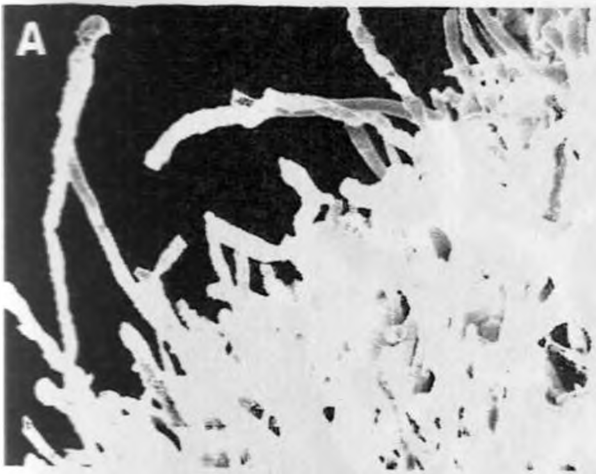
Results of an experiment in which mycelial plugs were exposed to SC-1 for varying periods of time at each concentration, showed that concentration and exposure time had a significant effect on the inhibitive property. Mycelial growth of *Cladosporium cucumerinum* exposed to SC-1 for 2 hours grew faster after incubation and therefore had larger colony diameters than those exposed for 40 hours at the same concentration. Thus colony diameters after incubation decreased with increasing hours of treatment(Fig. 19). Mycelia plugs of the test fungi taken from zones of inhibition were found to revive their growth on re-incubation into fresh media.

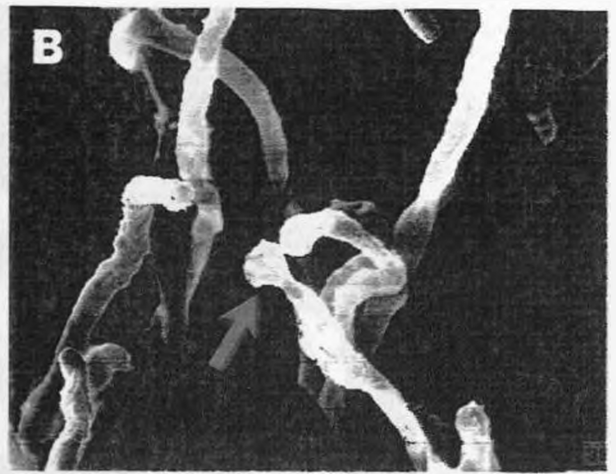
Figure 19: Effects of time of exposure to SC-1 at different concentrations on growth of *Cladosporium cucumerinum*.

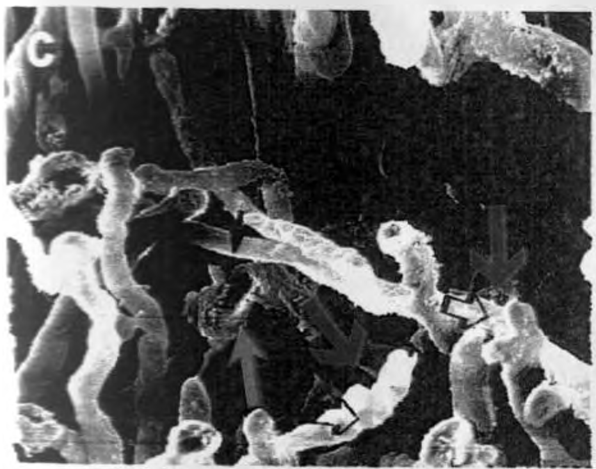


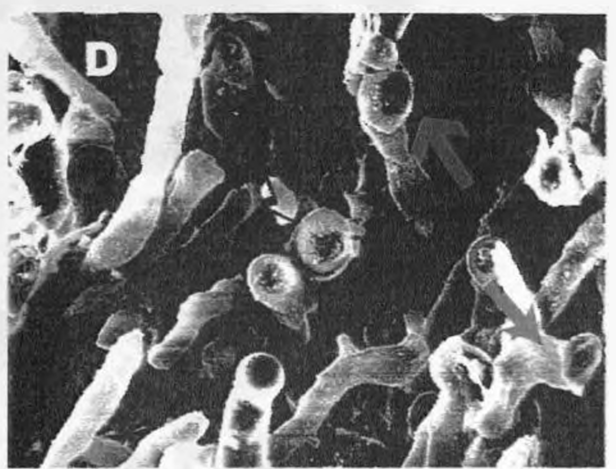
3.5.3 Effects of SC-1(compound isolated from *S. coffaeoides*) on the morphology of the fungal hyphae of filamentous fungi and the cells of yeast fungi.

From Scanning Electron Microscopy studies, *Cladosporium cucumerinum* and *Saccharomyces cerevisiae* samples treated with SC-1 showed alterations in the morphology of the hyphae and cells respectively. *C. cucumeriunum* showed morphological alterations. The hyphae of *C. cucumerinum* appeared swollen especially at the tips. The diameters of hyphae from plates treated with SC-1 at concentrations of 25μg/disc and 400μg/disc were about 25-33% and 40-52 % respectively larger than those of the control. Thus the swelling of the hyphae was more pronounced at high concentrations than at low ones. Lysed and morphologically distorted hyphae were common (Plate 8). The yeast cells were affected in a number of ways; some were deformed and still others produced pseudomycelium (Plate 9).









x 1500

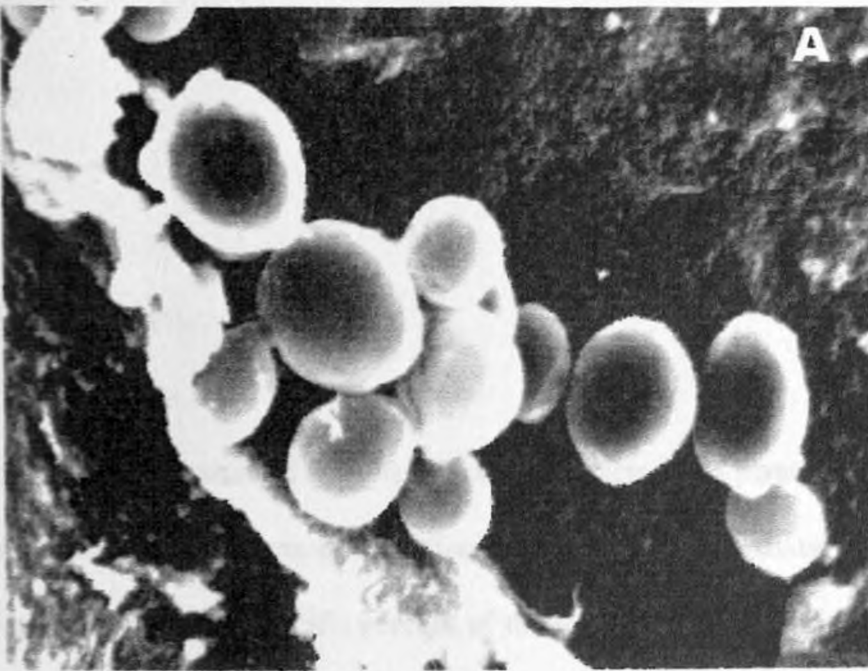
Plate 8: Effects of SC-1 on hyphae morphology of *Cladosporium cucumerinum*

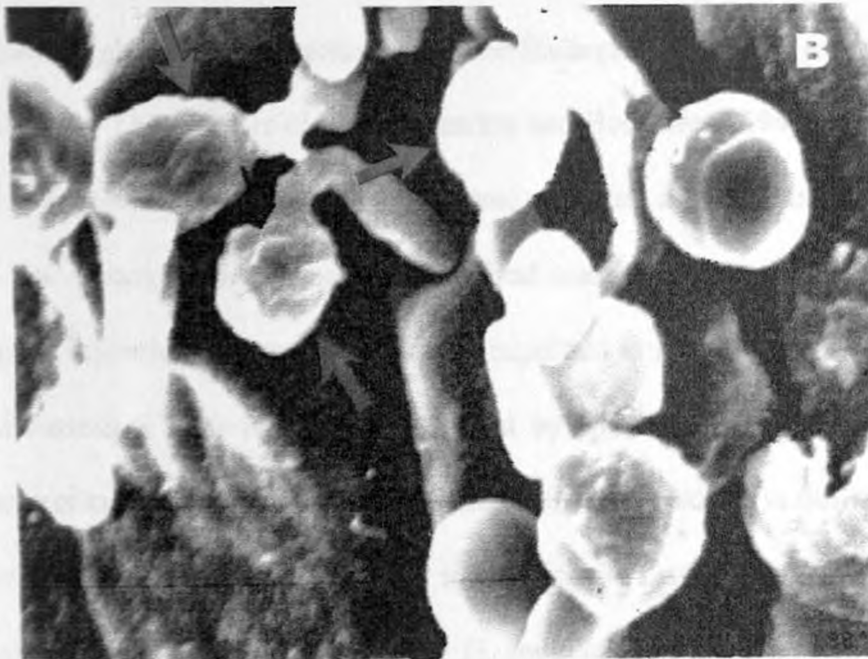
A: Control

B: Treated mycelium (25µg SC-1) Note the swollen hyphal tips →)

C: Treated: 200µg SC-1 (Note burst wall (Red→))
and the morphologically deformed hyphae (Blue→).

D: Treated: 400µg SC-1 (Note the swollen (Blue →)
and distorted hyphae (lower right Red →).





X3500

Plate 9: Effects of SC-1 on the cells of *Saccharomyces cerevisae* (yeast)
A: Control.
B: Treated (Note the distorted (Red→) and cell that have developed pseudomycelium (Green →)).

CHAPTER FOUR

4.0 DISCUSSION

The results obtained showed that 60.7% of the extracts from plants which are used traditionally for the treatment of skin diseases had antifungal activity against human and plant pathogenic fungi. Fifty percent of the screened plant extracts were active against dermatophytic fungi (*Trichophyton mentagrophytes* and *Microsporum gypseum*). This activity supports the folk therapy of infections of the skin whose symptoms might involve dermatophytes. The findings of this study are in agreement with those of Taniguchi *et al.* 1978; Marston and Hostettman 1987 and Fabry *et al.* 1996, who screened plants used in traditional medicine in Africa and found out that there was a correlation between ethnomedical uses of a plant and bioactivity in a biological screening system. However it is important to note that not all information on ethnomedical uses is reliable, as noted by Marston and Hostettman (1987); Slikkerveer and Slikkerveer (1995). A correlate to these findings in this study was the failure of 39.3 % of the plants screened to give positive results though reportedly used for the treatment of skin diseases (Table 2). Some of these plant species are probably used to treat skin diseases caused by non fungal pathogens. Among other possible reasons for this are the methods used in the preparation of the traditional medicine. For example the extracts from *Oxalis corniculata* were not fungitoxic, although traditionally a concoction of the pounded leaves of this plant is mixed with ghee and rubbed onto ringworm infected areas on the head. In this case the addition of ghee could have potentiated the fungitoxicity of the extracts. In other cases plants are used

in combined concoctions and therefore activity is synergistic. For example extracts from *Aspilia pluriseta* were not fungitoxic against dermatophytic fungi, although the plant has been reported to be a good remedy for fungal infections of the skin. According to Olembo *et al* 1995, *A. pluriseta* is used in combination with *Microgossa pyrifolia* and *Indigofera arrecta*. The reported antifungal activity may therefore be synergistic and hence the lack of activity when tested singly. Other times properties ascribed to a species are sometimes exaggerated. For example the traditional belief that *Azadirachta indica* can cure more than 40 ailments is most likely an exaggeration. Often properties may be ascribed to a plant due to improper identification by the traditional users. For example extracts from *Azadirachta indica* were fungitoxic but those of *Melia azedarach* were not. *M. azedarach* is in the same family with *A. indica* and the vegetative characteristics of the two plants closely resemble. Therefore the reported traditional use of extracts from *M. azedarach* may be due to being mistaken for *A. indica*. However when carefully sorted out, information from ethnomedical sources is a good pointer in the search of biologically active natural products as proved in this study.

The observation that several of the extracts that showed activity against the fungi were those made using polar solvents was of particular interest given that traditionally the preparation method is often with water (Table 1). The water extracts were however extremely difficult to dry in a rotary evaporator at 40°C. Further, even when lyophilised, most of the extracts were hygroscopic and hence difficult to handle. Therefore after the initial stage of preliminary screening, the water soluble materials were co- extracted with more lipophilic substances by use of 70% ethanol. However these extracts were less fungitoxic than those made using slightly more lipophilic

solvents like dichloromethane (Table 3). This finding seems to be in line with the observation by Harborne (1987), that a common feature of fungitoxic compounds from plants is that they appear to be mostly lipophilic but with a certain degree of water solubility. But we should note that these were not pure compounds and when purified may present characteristics which are different from Harborne's observation.

The reduction in activity after fractionation of petroleum ether extracts and the poor yield from the column may have been due to a reaction of the silica gel with hydrocarbon or oxygenated fractions or some compounds or their products adhering to or changing their properties in the presence of silica gel. It is also possible that some of the compounds could have been lost during the vacuo evaporation process. Such situations have also been reported by Banthorpe *et al.* (1990). The potentiation ability may also have been lost during the separation of the several constituents. Similarly the methanol extracts from *Aporrhiza paniculata* failed to increase in activity with fractionation (Figure 13). Just like in the case of petroleum ether, this failure may have resulted from the use of silica gel in the separation. In this respect, Fukuhara and Kubo (1991) have reported that the solid packaging material often causes irreversible absorption of large amounts of polar compounds. This might explain the poor yield from the column and the consequent decrease in activity to undetectable levels after fractionation of some of the extracts made in polar solvents. Other results however indicated a steady increase in fungitoxicity of dichloromethane extracts with fractionation (Figure 6).

The effectiveness of most extracts on filamentous fungi (*Trichophyton mentagrophytes* and *Microsporum gypseum*) only and not on the yeast (*Candida

albicans) (Figures 10, 12 and 14), may be due to differences in cell wall composition between yeast and filamentous fungi. Yeast forms of fungi contain glucans and mannan proteins in their cell walls compared to chitin and glycan in the cell walls of filamentous forms (Murrey *et al.*1988). Depending on composition the cell wall can act as a barrier preventing drugs from reaching the site of action (Mathison 1977). However, it was interesting to note the lack of susceptibility of a plant pathogen *Cladosporium cucumerinum* to extracts from *A. paniculata* (Figure 12) and the lower susceptibility to all the extracts compared to the animal pathogens (*T. mentagrophytes* and *M. gypsum*) despite the fact that they are all filamentous fungi (Figures 9,10 and 11). Similar results were obtained by Zacchino *et al.* (1997) who found that several antifungal compounds from Amazonian medicinal plants completely inhibited the growth of dermatophytes but had no effects on yeast and plant pathogenic fungi. Being a plant pathogen *C. cucumerinum* may have been pre-exposed to constitutive or induced antifungal compounds in host plants and therefore could have developed resistance thus rendering it less susceptible.

The results obtained also demonstrated that dichloromethane extracts from *Schizozygia coffaeoides* were the most fungitoxic, and activity increased with fractionation (Figure 9). The observation that these extracts were more fungitoxic than griseofulvin which is the commonly used antifungal drug against dermatophytes, suggests that extracts from S. *coffaeoides* could be used successfully for the treatment of dermatophytes. This is further supported by the fact that bioactivity guided separation led to the isolation of a compound that was highly fungitoxic against several fungal pathogens. Despite the lower susceptibility of the yeast and phytopathogenic fungi, there was enough evidence to suggest a broad spectrum of

activity since all tested fungi were susceptible to varying extents.

The results obtained showed that SC-1 (compound isolated from *S. coffaeoides*) had a strong fungistatic activity (Figure 19). When the inhibited mycelial plugs were transferred to fresh nutrient media, even those previously exposed to relatively high concentrations gave rise to colonies indicating that the inhibitory activity was reversible. Other researchers (Cabral and Cabral 1995) have reported reversible inhibitory activity of fungicides such as Vinclozolin. Further, it is also well established that most antifungal compounds including those in common clinical use such as griseofulvin are fungistatic in action (Finkelstein *et al.* 1996). Therefore the fact that SC-1 is fungistatic makes it a potential antifungal agent with the advantage of being a natural plant product.

Further analysis demonstrated that SC-I had antifungal activities at concentrations of below 10μg/ml. The potential of SC-1 is further demonstrated by the fact that compounds with activity only at 100μg/ml can be put into routine clinical use, provided they are non-toxic (Korolkovas *et al.* 1976). Toxicological work needs to be done on SC-1 to determine whether it will be toxic for general use, but in the meantime the employment of extracts of *Schizozygia coffaeoides* to treat skin diseases caused by fungi has been validated.

From Scanning Electron Microscopic studies it was obvious that SC-1 affected the morphology of the filamentous fungi by causing general morphological distortions and swelling of hyphae (Plate 8). Similar growth distortions caused by chemical

agents on filamentous fungi have been reported (Hippe and Grossman 1982; McGinnis and Rinaldi 1986; Zambonelli *et al.* 1996; Bianchi *et al.* 1997). According to McGinnis and Rinaldi 1986; Cabral and Cabral (1995), such observations point to membrane damage or inhibition of cell wall synthesis. When cell wall synthesis is inhibited and growth of protoplast continues, enlargement of the protoplasts stretches the cell wall and causes the cell to swell and sometimes twist. Therefore, although a comprehensive determination of mode of action of SC-1 was not achieved, the results obtained from the Scanning Electron Microscopic studies suggest that the compound may be a cell wall synthesis inhibitor. However more studies are required before the site and mode of action of SC-1 can be precisely defined. Although the benefits of this step are clear, it is generally not feasible to attain this goal for every compound generated. Constraints of time, effort and costs are usually prohibitive. As demonstrated by Kuhn (1989), the scale of inputs required for the determination of the site and mechanism of action of a compound may span more than fifteen years and involve high costs and collaboration of several research groups, both industrial and academic. It is due to such constraints that the determination of the exact mode of action of SC-1 could not be accomplished within the stipulated period, and resources available for this study.

The spectroscopic analysis and chemical data of the compound isolated from *S. coffaeoides* were indicative of alkaloids (Figure 17). This data is in agreement with that obtained by Renner and Kernweizs (1963), who isolated eleven alkaloids from *S. coffaeoides*. The UV absorption peaks of SC-1 were different from any of those isolated by these researchers. SC-1 had a molecular formula $C_{21}H_{22}N_2O_4$ which is within the range, though not exactly like that of any of those isolated by Renner and

Kernweizs (1963). This difference in UV absorption peaks and molecular mass points to a different compound from any of those already isolated and characterized . The structure of SC-1 was however not elucidated due to the presence of minute quantities of other alkaloids in the sample submitted for Nuclear Magnetic Resonance (NMR).

Although only one antifungal compound was successfully isolated from the leaves of *S. coffaeoides*, the presence of other antifungal compounds in this plant can not be ruled out . Petroleum ether and methanol fractions that were fungitoxic but could not be separated further, may still contain other compounds. Results obtained in this study also demonstrated that extracts from the roots were weakly fungitoxic which is an indication of the presence of antifungal compounds in the roots as well. However, leaves are a convenient renewable resource and therefore is a good material of choice for the extraction of natural products. The plant is evergreen and produces leaves abundantly (Beentje, 1994). Moreover it is a short shrub 1-4 meters tall and hence the leaves can be picked easily.

The yield of dried extract per gram of leaves for dichloromethane and methanol extracts were at 0.0146g and 0.1966g respectively. Therefore the cost of making this extracts may be much lower compared to the cost of commercial antifungal drugs. For example a 15g tube of Lamisil cream costs Ksh.795 (US$ 10.6) by July, 1999, and depending on the extent of infection this tube may not be enough. Further, it is recommended that treatment be continued for at least one more week after the symptoms have disappeared to prevent re-infection. Moreover, most of the antifungal drugs can only be bought with a prescription from a doctor which makes treatment even more expensive.

Schizozygia coffaeoides is a rare species in Kenya and is distributed along the coast (Figure 1). The results of this study indicate that there is need to promote this plant in other parts of the country, especially in communities where ethnomedicine is still widely used. A few species have been introduced at the Nairobi arboretum which is evidence that the plant can do well in other parts of the country.

5.0 RECOMMENDATIONS AND CONCLUSIONS

This study has demonstrated the potential of Kenyan medicinal plants as possible sources of new antifungal drugs. For example the extracts of *Acacia nilotica* (L.) Del, *Acacia senegal* (L.) Willd, *Adenia volkensii* Harms, *Ajuga remota* Benth., *Aporrhiza paniculata* Radlk., *Aspilia pluriseta* Schwieinf. Ex. Engl., *Azadirachta indica* A. Juss., *Cassia didymobotyra* (Fresen.) Irwin and Barneby, *Commiphora africana* (A. Rich.) Engl., *Hymenodictyon parvifolium* Oliv., *Plectranthus barbatus* Andr., *Plectranthus cylindraceus* Benth., *Premna resinosa* (Hochst.) Schauer, *Schizozygia coffaeoides* Baill, *Solanum arundo* Mattei, *Solanum nigrum* L. and *Tagetes minuta* L. are antifungal and can be used for the treatment of human mycotic diseases.

Only *in vitro* methods were used in assessing the activity of the extracts and the isolated compounds. In order to relate the use of these plants as herbal remedies, it would be necessary to perform clinical tests.

SC-1 (compound isolated from *S. coffaeoides*) is a potential antifungal drug. The results obtained in this study however warrant further investigation on this compound. Toxicological studies also need to be carried out before any clinical trials are initiated

and then the possibility of incorporating these extracts into creams and soaps for topical application should be explored.

Since *Schizozygia coffaeoides* is a rare species in Kenya, it should be conserved and probably domesticated for commercial exploitation. Possibilities of establishing plantations for large scale exploitation should also be explored.

The findings of this study indicate that it is worthwhile to scientifically follow up some of the leads suggested by ethnomedical uses of plants. This further confirms that possibilities still abound for developing many more natural plant-based compounds to help combat fungal pathogens harmful to humans, their plants and animals.

6.0 REFERENCES

Abrams, D. 1997. Alternative therapies for HIV (143-158). In: The Medical Management of AIDS. Eds. Merle, A.; Sande, M. D ; and Volberding, M. D. W.D saunders Company. London.

Anesini, C. and Perez, C. 1993. Screening of plants used in Argentina folk medicine for antimicrobial activity. J. Ethnopharm. 39: 119-128.

Atta-ur Rahman; Nasreeen, A.; Akhtar, F. Shekhani, M. S.; Clardy, J.; Parvez, M.; and Choudhary, M. I. 1997. Antifungal diterpene alkaloids from *Delphinium denudatum*. J. Nat. Prod. 60: 472-474.

Bacquar, S. R.1989. Medicinal and poisonous plants of Pakistan. Printas Karachi. Pakistan 506 pp.

Balick, M. J. (1990).Ethnobotany and identification of therapeutic agents from the rain forest. In: Bioactive compounds from plants. CIBA foundation symposium 154. John Wiley and sons. New York 23-39.

Banthorpe, D. V. and Charlwood, B. V. 1990. Secondary plant products (185-220): In Encyclopaedia of plant physiology 8 , Bell, E. A. and Charlwood, B. V. Eds. Springer- verlag. Berlin.

Barnabus ,G. G. and Nagarajan, S. 1988. Antimicrobial activity of flavanoids of some medicinal plants. Fitoterapia LIX 6

Barry, A. L., Coyle, M. B., Thornberry C, Gerlad, E. H. and Howkinson, R.W., 1979. Methods of measuring zones of inhibition with the Bauer-Kirby disk susceptibility test. J. clin. Microbiol 10:885-889.

Baumgatner, B.; Erdelmeier, A. J.; Wright, A. D; Rali, T. and Otto Sticher, 1990. Antimicrobial alkaloids from *Ficus septica*. Phytochem. 29 (10): 3327-3330.

Beentje, H. J. 1994. Kenya trees, Shrubs and Lianas. National museums of Kenya. Nairobi. 722pp

Bianchi, A.; Zambonelli, A.; Zechini D'Aulerio, A. and Bellesia F. 1997. Ultrastructural studies of the effects of *Allium sativum* on phytopathogenic fungi in vitro. Plant Dis. 81: 1241 - 1246.

Bruhn, S. 1989. Acta. Pharm. Nord. 1: 117-130

Cabral, S. M. J. and Cabral, J. P. S. 1995. The fungistatic and fungicidal activity of Vinclozolin against *Botrytis cinerea*. Mycological Research 99 (9): 1041- 1046.

Campbel, M. C. and Stewart, J. L. 1980. The Medical Mycology Handbook. John Wiley and Sons. Newyork. 410pp.

Carefoot, G. L. and Sprott , E. R. 1967. Famine on the wind; Plant disease and human history. Ebenezer Baylis and sons Ltd. London.

Colombo, L. A., Mcgough, D. A and Rinaldi M. G. 1994.Discrepancies between MIC and MLC values of amphotericin B against isolates of *Aspergillus* species. Mycopathologia (128) 129-133.

Cox, P. A. 1990. In Ethnopharmacology and the search for new drugs; Ethnobotany and the search for new drugs. (CIBA foundation symposium 154). Chadwick, D. J.' Derek, J. and Marsh, J eds. John Wiley and sons Ltd . Chichester.

Cunningham, A. B. 1993. African Medicinal Plants: People and plants working paper. Setting priorities at the interface between conservation and primary healthcare. UNESCO Paris.

Cross, P. M. 1972. Specimen Preparation (P75-86). In The use of Scanning Electron Microscope. Eds. Hearle,J. W. S. Sparrow, J. T. and Cross, P. M. Pergamon press. Oxford. New York.

Decosterd, D. L.; Hoffman E. Kyburz, R.; Bay, D. and Hostettman K. 1991. A new Phloroglunical derivatives from *Hypericum calycinum* with antifungal and *in vitro* antimalarial activity. Planta Med. 57: (6) 548

Echlin, P. 1971. Preparation of labile biological material for examination in Scanning Electron Microscope (P307-315). In Scanning Electron Microscopy Systematic and Evolutionary Applications. eds. V. H. Heywood. Academic press. London. New York. 325pp.

Fabry, W. ; Okemo, P. and Ansorg, R. 1996. Fungistatic and fungicidal activity of East African Medicinal plants. Mycoses 39, 67-70.

Farnsworth N. R. 1984. How can the well be dry when it is filled with water?. Econ. Bot. 38: 4-13.

Farnsworth N. R. 1988. Screening plants for new medicines. In: Biodiversity (Ed. Wilson, E. O.) National academy press. Washington D. C. (83-97)

Farnsworth N. R. 1990. Ethnopharmacology and the search for new drugs; Ethnobotany and the search for new drugs. (CIBA foundation symposium 154). Chadwick, D. J.' Derek, J. and Marsh, J eds. John Wiley and sons Ltd . Chichester.

Farnsworth, N. R. and Bingle, A. S. 1977. New natural products and plant drugs with pharmacological. Biological and therapeutic activity. In Wagner, H. and Wolf Eds. Springer verlag, New York pp 61-73.

Farnsworth, N. R. Akerele, O. and Bingle, A. S. 1985. Medicinal plants in therapy. Bull. World Health Organisation. 63: 965-981.

Fleet, G. W. J; Karpas, A; Dwek, R. A.; Fellows, L. E; Tyms, A. S; Peterson, S; Namgoog, S. K.; Ramsden, N. G. Smith, P. W. Son, J. C. 1988. Letters 237: 128-132.

Finkelstein, E. ; Amichai, B. ; Marcelo, H. G. 1996. Griseofulvin and it's uses. International Journal of antimicrobial agents. 6: 189-194.

Fisher, D. J. and Hayes, A. C. 1982. Mode of action of systemic fungicides Furalaxyl, Metalaxyl and Ofurace. Pesticide Science. 13: 330-339.

Fromtling, R. A. 1984. Imidazoles as medically important antifungal agents. An overview. Drugs Today (20) 325-349.

Fukuhara, K. and Kubo, I. 1991. Isolation of steroidal Glycoalkloids from Solanum incanum by two countercurrent chromatographic methods. Phytochem. (30) 2: 685-687.

Gachathi, F. N. 1989. Kikuyu Botanical Dictionary of Plant Names and Uses. Nairobi.

Gottlieb, O. R, 1982. Micromolecular Evolution. Systematics and Ecology. Springer-Verlag, Berlin, Heidelberg, Newyork.

Griffiths, S. E.; Gibbs, J. N. and Waller, I. M. 1971. Control of Coffee Berry Disease. Ann. Appl. Biol. 67: 43-74.

Greenspan, J. S. and Greenspan, D. 1997. Oral complications in HIV infections (169-180). In The Medical Management of AIDS. Eds Merle, A.; Sande, M. D ; Paul, A. ; Volberding, M. D. W.D saunders Company. London.

Habtemariam, S. Gray A. I. And Waterman P. G. 1992. Antibacterial diterpens from aerial parts of *Premna oligotricha*. Planta Medica 58 : 109 -111.

Harborne, J.B. 1984. Phytochemical Methods. A guide to modern techniques of plant analysis, 2nd edition, London, Chapman and Hall. 288pp.

Harborne, J.B. 1987. Natural fungitoxins. (195-211) In Biologically Active Natural products: Proceedings of the phytochemical society of Europe. Eds Hostettman, K. and Lea, P. J. Oxford.

Harborne, J.B and Turner, B. L. 1984. Plant Chemosystematics. Academic Press. London

Hippe, S. and Grossman, F. 1982. The ultrastructure of sporidia of *Ustilago avenae* after treatment with the fungicides Naurimal and Imazalil nitrate. Pesticide Sci. (13) 447 - 451

Homans, A. L. and Fuchs, A. 1971. Direct bioautography on thin-layer chromatograms as a method of detecting fungitoxic substances. J. Chromatog. 51: 327-329.

Hostettman, K. 1991. Assays for Bioactivity. Academic Press. London. Newyork,Tokyo.

Humberger, M. ; Mortson, A. ; Hostettman, K. 1991. Advances in drug research. Testa, B. ed; Academic press: London pp 167-216.

Irobi, O. N. and Daramola, S. O. 1993. Antifungal activity of crude extracts of *Mitracarpus villosus* (Rubiaceae). J Ethnopharm. 40: 137-140.

Irobi, O. N.; Moo-Young M.; Anderson W. A and Daramola, S. O 1994. Antimicrobial activity of bark extracts of *Bridelia ferruginea* (Euphorbiaceae) J. Ethnopharm. 43: 185-190.

Jain, S. K. 1979. Medicinal plants. National book trust. New Delhi. 178 pp

Jacques F. Acar, F.W. Goldstein. 1986. Disk susceptibility test in Antibiotics in laboratory medicine. 2nd Edition Ed. Victor Lorian, M.D. Williams and Wilkins. Boltimore. London Los Angeles Sydney. (27-63).

Kariba, R. M. 1992. Effects of Solanum nigrum extracts on four phytopathogenic species of colletotrichum. Msc. Thesis. University of Nairobi.

Kokwaro, J. O. 1976. Medicinal plants of East Africa. East Africa literature bureau Nairobi 384pp.

Korolkovas, A. and Burckhaller, J. H. 1976. Essentials of medicinal chemistry. John Wiley and sons, New York 541pp.

Kozkiewicz, Z 1989. *Aspergillus* species on stored products. Mycological Papers No. 161. C.A.B. International Mycological Institute.

Kingston, D. J. I. 1992. New drugs from natural sources: coombes, J.D. ed; IBC Technical services: London pp101-119

Kuhn, P. J. 1989. The discovery and development of fungicides. Does Biochemistry have a role?. Pesticide Sci. 25: 123-135.

Large, E. C. 1940. The Advance of Fungi. Pp 13-43. Henry Holt and Company. Jonathan Cape. London.

Lindsay, R. S. and Hepper, F. N. 1978. Medicinal plants of Marakwet- Kenya. 49pp.

Lwande, W. 1984. Phytochemical and bioactivity studiesof *Teclea trichocarpa, Tovomita mangle, Tephrosia hildebrandtii* and *Milletia thonongii*. Ph.D Thesis. University of Nairobi.

Mandava, B. N. 1985. Handbook of Natural Pesticides Methods. Vol 11. CRC press inc. Florida 545pp.

Mathison, E. 1977. Antifungal mechanisms in animal hosts. Bull. Brit. Mycol. Soc. ii Part 2 136-137.

McGinnis, M. R. and Rinaldi, M. G. 1986. Antifungal drugs: Mechanisms of action, Drug resistance, Susceptibility testing, and assays of activity in biological fluids (223-281). In Antibiotics in Laboratory Medicine .2nd edition. Eds Lorian V. Williams and Wilkins. Boltimore. London. 1259pp.

Menn, J. J. 1983.Natural products for innovative pest management. Current themes in tropical sciences. Eds. Odhiambo, T. R.; Whitehead L. and Bowers, W. S. Pergamon press. Oxford.

Mclaren,J. S. 1986. Biologically active substances from higher plants. Status and future potential. Pesticide Sci. 17: 559-578.

Morston, A. and Hostettman, K. 1987. Antifungal, Mulluscicidal and ctotoxic compounds from plants used in traditional medicine. 65-83 In: In Biologically Active Natural products; Proceedings of the phytochemical society of Europe. Eds Hostettman, K. and Lea, P. J. Oxford.

Murrey, R. K; Granner, D. K.; Mayers, P. A. and Rodwell, V. W, 1988. Membranes: Structures, Assembly and Function. Harpers Biochemistry. 21st Ed. 446-462.

Nene, Y. L. Fungicides in plant disease control. Gulab Oxford and IBH Publ. Co. New Dehli.

Olembo, K. N.; Fedha, S. S.; Ngaira, S. E. 1995. Medicinal and agricultural plants of Ikolomani division Kakamega districts. Signal press Ltd. Nairobi. 107pp.

Omino, E.A. and Kokwaro,.J.O. 1993. Ethnobotany of Apocynaceae species in Kenya. J. Ethnopharm. 40:167-180.

Pappas, A. C. and Fisher, D. J. 1979. A comparison of mechanism of action of vinclozolin, procymidone, iprodione and prochloraz against *Botrytis cinerea.* Pesticide Science. 10: 239-246.

Perrucci S; Mancianti F. ;Cioni, P. L.; Flamini, G.; Morelli, I. and Machioni G. 1993. *In vitro* antifungal activity of essential oils against some isolates of *Microsporum canis* and *Microsporum gypseum.* Planta Medica. 60: 184 - 187.

Plotkin, M. J. 1988 Traditional knowledge of medicinal plants. The search for new jungle medicines. In: Conservation of Medicinal plants. Proceedings of an international consultation held in Thailand March, 1988. Eds: Olayiwalo, A.; Heywood, V. and Synge, H. Cambridge Univ press, New York 362pp.

Principe, P. P. 1989. Economic and Medicinal Plants Research . Wagner H.; Hikino H. and Farnsworth N. R. eds. Academic press London (p 1-17).

Renner, U. and Kernwiesz, P. 1963. Alkaloids aus *Schizozygia coffaeoides* (Boj.) Baill. Experientia XIX/5

Reiner, R. 1982. Combination of antibiotics. Bactericidal and bacteriostatic antibiotics. Roche Scientific Services.8: 86-87.

Saag, M. S. 1977. Cryptococcosis and other fungal infections (Histoplasmosis, coccidioidomycosis) 327-342 In The Medical Management of AIDS. Eds Merle, A.; Sande, M. D ; Paul, A. ; Volberding, M. D. W.D saunders Company. London.

Schieber, C. 1972. Economic impact of coffee rust in Latin America. Annual Review of Phytopathology 10: 491-510

Scholar, H. J. and Polak, 1973. Fungicidal and fungistatic properties of 5-Flourocytosin: Methods of routine sensitivity tests (P. 162-163). The symposium international de mycologie medicale, Bukarest.

Slikkerveer, L. J. and Slikkerveer M. K. L. 1995. Indigenous Indonesian Medicine for self- reliance(13-34). In. The Cultural dimensions of Development. Indigenous knowledge systems. Bronkensha, D; Slikkerveer, L. J. Warren, D. M. eds; Intermediate Technology publications. London.

Sindiga I.; Chacha, N.C ; Kanunah, P. M. 1994. Traditional Medicine in Africa .East African Educational Publishers. Nairobi. 197pp

Sofowora, A. 1982. Medicinal plants and traditional medicine in Africa. John Wiley and Sons. Pitman press Ltd. Bath.

Stahl, E. J. 1968. Thin Layer Chromatography. A laboratory handbook. London. Academic press.

Strange, R. N. 1993. Plant Disease Control Towards Environmentally Acceptable Methods. Chapman and Hall. London. New York. Tokyo. 354pp.

Suffness, M. 1987. Biologically active natural products. Hostettman K. and Lea, P.J. eds; Claredon press: Oxford. (pp85-104).

Taniguchi, M., Chapya A, Kubo, I., and Nakanishi, K. 1978. Screening of East Africa plants for Antimicrobial Activity I. Chem. Pharm. Bull. 26:(9) 2910-2913.

Tsantrizos , Y.; Koppe, H. H.; Fortin, J. A. and Olgivie, K. E. 1991. Antifungal antibiotics from *Pisolithus tinctorius*. Phytochem. 30(4): 1113-1118.

Trigg, P. I. 1989. Economic and medicinal plant research. Wagner, H.; Hikini, H. and Farnsworth, N. R. Eds; Academic press. London (pp20-51).

Ullstrup, A. J. 1972. The impacts of southern corn leaf bright epidemics of 1970-1971. Annual Review of Phytopathology 10: 57-59.

Valsaraj, R.; Push, P.; Wagner,S.; Adersen A.;Christensen, S. B.; Sittie, A. Nyman, U.; Nielsen, C. and Olsen C. C. 1997. New anti HIV-1, antimalarial and antifungal compounds from *Terminalia bellerica*. J. Nat. Prod. 60: 739-742

Watt, J. M. and Breyer- Brandwijk, G.M. 1962. Medicinal and poisonous plants of Southern and Eastern Africa. Livingston Edinburg. 1198pp.

Weidenborner, M.; Hindorf, H.; Chandra,H.; Tsotsonos, P. and Egge H. 1990. Antifungal activities of Isoflavanoids in different reduced stages on *Rhizoctonia solani* . Phytochem. 29(3): 801-803

Weimin, Z; Wolfender, J; Hostettman, K. Xu R and Qin, G. 1998. Antifungal alkaloids and Limnoid derivatives fom *Dictamnus dasycarpus*. Phytochem. 47(1): 7-11.

WHO. 1978. World Health Organisation Technical report series (1-41).

Zacchino, S.; Rodriguez, G.; Pezzenati, G and Orellana, G. 1997. In vitro evaluation of antifungal properties of 8.o.4 neolignans. J. Nat. Prod. 60 (7): 659-662.

Zambonelli, A.; Zechini D' Aulerio; Bianchi, A. and Albasini, A. 1996. Effects of essential oils on phytopathogenic fungi in vitro. J. Phytopath. 144: 491-494.

Zar, J. H. Biostatistical Analysis. 2nd Edition. Prentice Hall International. London.

7.0 APPENDICES:

Appendix I:

Keys, Symbols and abbreviations used in the text.

AIDS	Acquired Immunodeficiency Syndrome
Cm	Centimeter
DMSO	Dimethylsulfoxide
EtoAc	Ethyl acetate
FTEA	Flora of Tropical East Africa
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
MIC	Minimum Inhibitory Concentration
ml	Millilitre
mg	Milligram
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
nm	Nanometer
Pet. ether	Petroleum ether
PTLC	Preparative Thin Layer Chromatography
TLC	Thin Layer Chromatography
UV	Ultra Violet
V/V	Volume to Volume
WHO	World Health Organisation
µl	Microliter
µg	Microgram
^0C	Degrees centigrade
%	Percent

Appendix II:

Yields per gram of plant material of extracts from *Schizozygia coffaeoides*, *Solanum arundo*, *Aporrhiza paniculata* and *Hymenodictyon parvifolium*.

Plant species	Part	Raw material	Solvent(1000ml)	Yield per gram
S. coffaeoides	Leaf	100g	Pet. ether	0.0289
		”	Dichloromethane	0.0146
		”	Methanol	0.1966
		”	70% Ethanol	0.1522
		”	Water	0.0972
	Root	100g	Pet. ether	0.0122
		”	Dichloromethane	0.0159
		”	Methanol	0.1430
		”	70% Ethanol	0.0961
		”	Water	0.1001
S. arundo	Fruit	100g	Pet. ether	0.0197
		”	Dichloromethane	0.0271
		”	Methanol	0.1618
		”	70% Ethanol	0.0115
		”	Water	0.0863
A. paniculata	Bark	100g	Pet. ether	0.0214
		”	Dichloromethane	0.0370
		”	Methanol	0.0638
		”	70% Ethanol	0.0702
		”	Water	0.0520
H. Parvifolium	Bark	100g	Pet. ether	0.0300
		”	Dichloromethane	0.0279
		”	Methanol	0.0226
		”	70% Ethanol	0.0303
		”	Water	0.0294

Appendix III

Average % inhibitions exhibited by leaf extracts from *Schizozygia coffaeoides.*

Test organisms	Test extracts	Concentrations(μg/disc) / Percentage inhibitions							
		0.0	6.25	12.5	25	50	100	200	400
T. mentagrophytes	i	0	0	0	8.33	9.17	15.8	20.6	22.8
	ii	0	10.7	12.9	13.6	17.5	22.9	25.8	34.3
	iii	0	0	0	0	8.15	14.4	16.6	21.4
M. gypseum	i	0	0	0	6.7	6.06	15.8	19.4	21.4
	ii	0	9.4	11.7	12.9	16.7	20.6	24.4	30.8
	iii	0	0	0	0	7.8	12.7	15.3	20
C. albicans	i	0	0	0	0	0	0	6.9	9.44
	ii	0	0	0	0	0	7.5	9.4	12.8
	iii	0	0	0	0	0	0	0	7.6
C.cucumerinum	i	0	0	0	0	0	6.94	8.6	12.9
	ii	0	0	0	0	7.8	9.7	10.6	14
	iii	0	0	0	0	0	0	0	7.8

Key: i- Petroleum ether
ii- Dichloromethane:EtoAc (1:1)
iii- Methanol

Appendix IV

Average % inhibitions exhibited by extracts from *Salanum arundo*, *Aporrhiza paniculata* and *Hymenodictyon parvifilium*

Test organisms	Extracts	Concentrations(µg/disc) / Percentage inhibitions							
		0.0	6.25	12.5	25	50	100	200	400
T. mentagrophytes	*S. arundo**	0	0	0	0	0	8.06	8.06	16.7
	A. paniculata #	0	0	0	0	0	0	9.7	16.9
	*H. parvifolium**	0	0	0	0	0	0	8.6	14.8
M. gypseum	*S. arundo**	0	0	0	0	0	6.7	7.5	13.6
	A. paniculata #	0	0	0	0	0	0	0	14.0
	*H. parvifolium**	0	0	0	0	0	0	9.4	13.9
C. albicans	*S. arundo**	0	0	0	0	0	0	0	0
	A. paniculata #	0	0	0	0	0	0	0	0
	*H. parvifolium**	0	0	0	0	0	0	0	0
C.cucumerinum	*S. arundo**	0	0	0	0	0	0	0	7.8
	A. paniculata #	0	0	0	0	0	0	0	0
	*H. parvifolium**	0	0	0	0	0	0	0	6.9

Key: * Ethanol extracts
Methanol extracts

Appendix V

The effects of SC-1 at different concentrations on the growth of nine pathogenic fungi.

Test organisms	Concentrations(μg/disc) of SC-1/ Percentage inhibitions										
	0.0	1.56	3.125	6.25	12.5	25	50	100	200	400	G	N
T. mentagrophytes	0.0	6.9	10	15.8	31.0	37.1	44.4	51.1	53.9	66.9	36.2	18.8
M. gypseum	0.0	0.0	7.8	13.6	26	31.1	43.1	45.8	47.2	64.4	35	34.4
C. cucumerinum	0.0	0.0	7.5	13.3	23.9	26.7	33.3	38.3	43.1	54.7	24	NT
C. albicans	0.0	0.0	0.0	8.6	12.2	14.3	18.3	22.1	25.6	31.9	13.3	30
C. harbarum	0.0	0.0	0.0	0.0	9.4	15.6	18.1	18.6	24.9	28.9	NT	NT
S. cerevisae	0.0	0.0	0.0	0.0	0.0	0.0	10.3	16.9	22.2	27.2	NT	NT
A. tenuis	0.0	0.0	0.0	0.0	0.0	0.0	8.9	15.3	17.8	25	NT	NT
F. solani	0.0	0.0	0.0	0.0	0.0	0.0	8.3	14.7	16.7	21.7	NT	NT
B. cinerea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.5	18.3	NT	NT

G= Griseofulvin(400μg), N= 1% Nystatin, NT= Not tested

Appendix VI

Minimum Inhibitory Concentrations (MIC) for extracts from *S.coffaeoides* against *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans* and *Cladosporium cucumerinum.*

Fungi	Extracts	MIC (mg/ml)
T. mentagrophytes	Petroleum ether	0.625
	Dichloromethane	0.3125
	Methanol	1.25
	Griseofulvin	0.3125
	SC-1	0.078
M. gypseum		
	Petroleum ether	0.625
	Dichloromethane	0.3125
	Methanol	0.625
	Griseofulvin	0.3125
	SC-1	0.156
C. albicans		
	Petroleum ether	2.5
	Dichloromethane	2.5
	Methanol	10.0
	Griseofulvin	5.0
	SC-1	0.3125
C. cucumerinum	Petroleum ether	5.0
	Dichloromethane	1.25
	Methanol	10.0
	Griseofulvin	0.625
	SC-1	0.3125

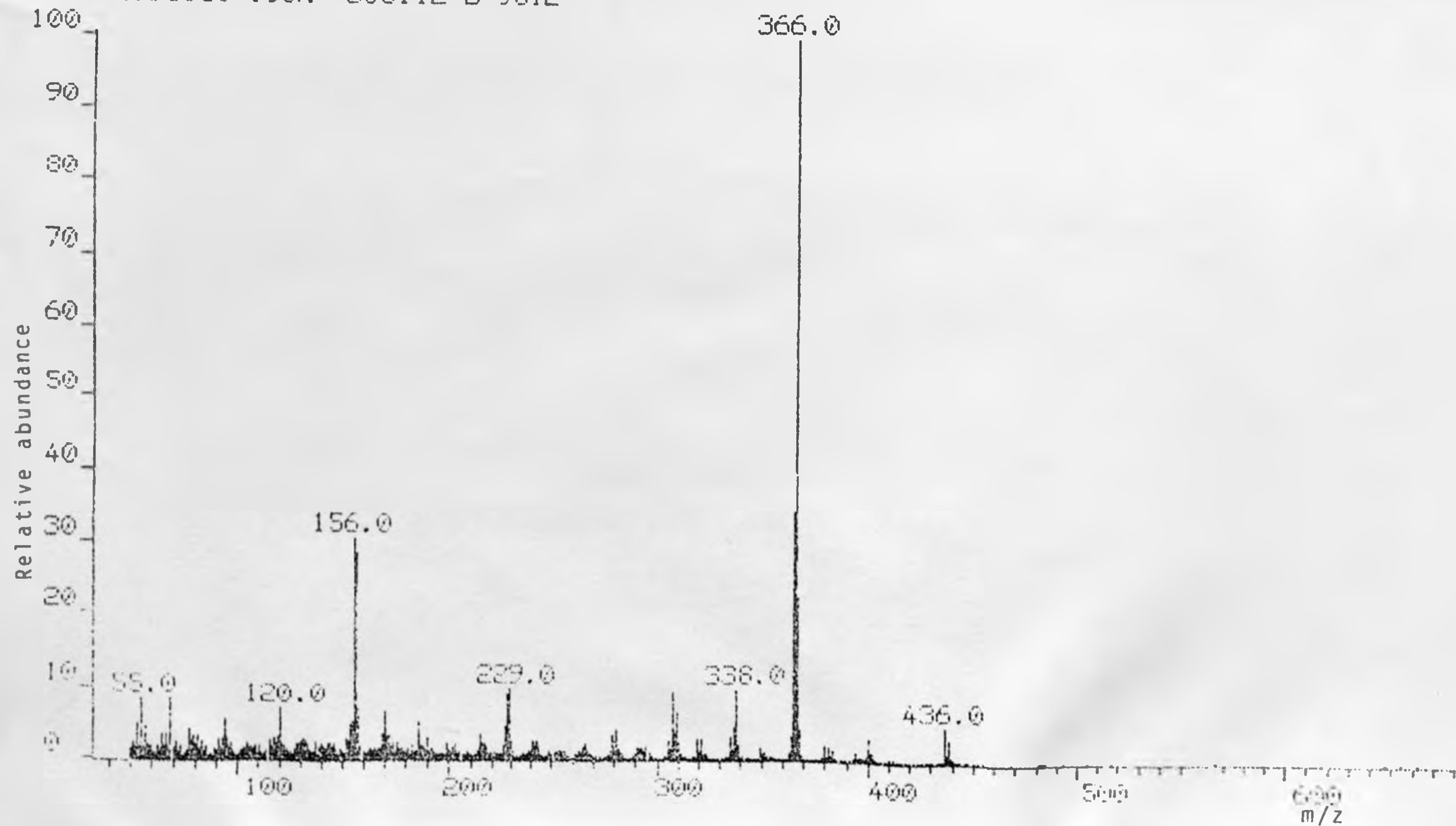


Fig. Mass Spectrum of SC-1