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ANTIFUNGAL METABOLITES FROM SUBMERGED CULTURE OF GANODERMA LUCIDUM (POLYPORE)

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ADANE BITEW

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TO YEWORKWUHA MENGISTU

AND

RAHELE ADANE

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ABSTRACT

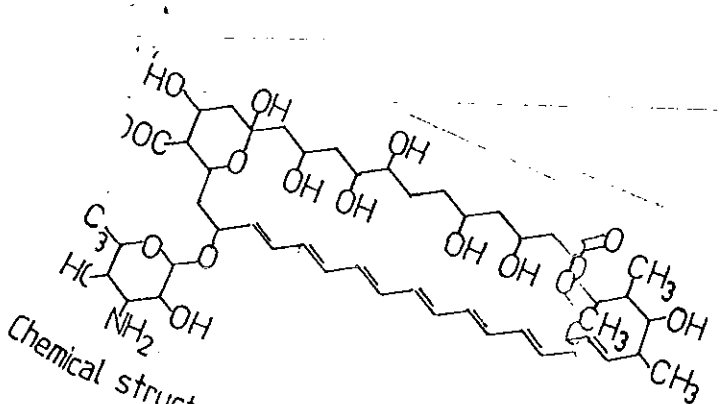
About 60 different basidiomycete cultures were screened for antimicrobial secondary metabolites in submerged culture grown in four different media. Ten (17%) of them produced antimicrobial secondary metabolites. Production medium, duration of growth, and the most susceptible test organisms for each producing strain were established. Among basidiomycetes screened for antimicrobial activity, the culture filtrate extract of the Ethiopian strain of the polypore, Ganoderma lucidum produced the most effective antifungal compounds. The cultural characteristics, growth in submerged culture of the polypore and isolation methods of the two antifungal antibiotics are described. Medium A (Yeast extract malt extract glucose medium) was a better medium for the production of the two antifungal agents. These compounds were released to the culture fluid and the maximum amount of antifungal compounds is obtained after 12 days of submerged growth at 120 revolution per minute (rpm). The two antifungal metabolites (201A and 201B) isolated from culture filtrated were biologically characterized. These metabolites had a wide spectrum of antifungal activity and affect the growth of several saprophytic as well as pathogenic fungi. The minimal inhibitory concentration (MIC) of 201A against Candida albicans and Candida pseudotropicalis was less than 1 mcg/ml and 1-5 mcg/ml respectively. Inhibition diameter zone of 36 mm was produced when 10 mcg/disc of 201A was applied on agar medium seeded with Aspergillus flavus. 201A was also a potent inhibitor of spore germination. No spores of Aspergillus niger were germinated at a concentration of 10 mcg/ml of the antibiotic. Bacteria were affected only at high concentration.

Antibiotic 201A was more active than antibiotic 201B. A comparison of antifungal activity against dermatophytes showed that the efficacy of 201A was comparable to griseofulvin. Treatment of sheep erythrocytes with the two antifungal antibiotics up to 100 mcg/disc did not show any lytic effect on sheep red blood cells. Application of 1 mg/10cm^2 of crude extract on a shaved rabbit skin showed no dermatotoxic reactions.

1. INTRODUCTION

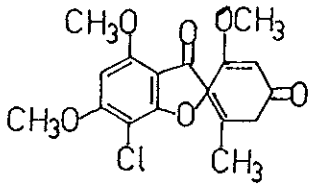
In the last several decades a large number of antimicrobial compounds have been discovered, of which only a small proportion of them are in clinical use. Major successes have been achieved particularly against bacterial infections. Though some important antifungal drugs of microbial and synthetic origin have also been discovered for the treatment of mycotic infections, they fail to achieve the desired results. Toxicity to the host, narrow spectrum of activity, difficulty of administration, drug resistance and recovery of the parasite after treatment are the major limitations of the existing antifungal drugs.

The systemically administrated antifungal drugs currently used have been shown to be toxic to the host. Amphotericin B, one of the polyene antifungal antibiotics isolated from Streptomyces nodosus is the most useful of the polyene antibiotics, for the treatment of systemic mycosis. However, it has been reported that this antifungal antibiotic has a serious toxic effect particularly on the kidney of the host and remains the treatment of choice in only life-threatening mycotic infections (Evans and Gentles, 1985).



Chemical structure of amphotericin B.

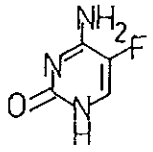
Spectrum of activity of antifungal antibiotics is an important factor to be considered, since a very large number of fungal species are capable of causing mycotic infections. However, griseofulvin an antifungal antibiotic isolated from Penicillium griseofulvum, has a narrow spectrum of activity and is primarily active only against dermatophytes (Russel, 1980). Flucytosine, a synthetic antifungal agent has also a narrow spectrum of antifungal activity and its high level of antifungal activity appears to be limited to yeast like fungi (Shadomy, 1969).



Chemical structure of griseofulvin.

Though administration by the oral rout is the simplest and most satisfactory, because of poor absorption or degradation in the gastrointestinal tract (Evans and Gentles, 1985) all antifungal agents for the treatment of deep-mycosis must be given intravenously, usually in a hospital settings (Graybill and Drutz, 1980).

Drug resistance is another factor of importance in the treatment of few mycotic infections. Development of drug resistance by Candida albicans and Cryptococcus species to flucytosine has been established (Graybill and Drutz, 1980). Development of resistance to flucytosine in 25% of clinically isolates have been reported by Block et al. (1973).

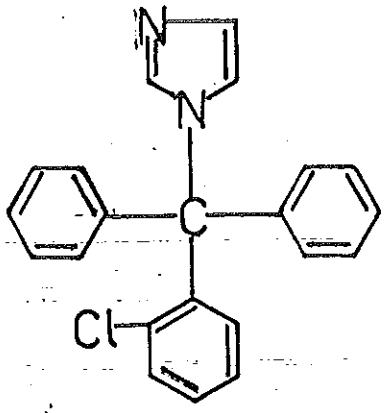


Chemical structure of flucytosine.

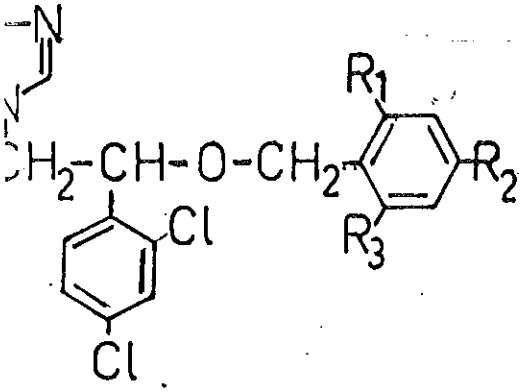
According to Evans and Gentles (1985) most clinically useful antifungal drugs are primarily fungistatic. Lack of fungicidal activity of griseofulvin against dermatophytes has been reported (Williams, 1960). This lack of fungicidal activity by most antifungal drugs has been known to contribute a narrow margin between therapeutic dose and occurrence of serious toxicity, a longer period of treatment of mycotic infections and recovery of the parasite after treatment.

The synthetic imidazole derivatives are a group of antifungal agents that have earned a place among the clinically useful antifungal agents. Many of the imidazole derivatives are widely used in the treatment of superficial mycosis (Utz, 1980). Ketoconazole is an imidazole derivative currently used as a therapeutic agent in a variety of systemic and local fungal infections. Preliminary data suggest that the drug is effective in superficial mycosis and may be beneficial in certain deep-seated mycotic infections (Symoens et al., 1980).

Mode of action of antifungal agents is also an essential factor to be considered in the development of antifungal agents. Polyene antifungal antibiotic and imidazole derivatives have cellular membranes as a common target organelle (Hamilton-miller, 1973; Borgers, 1980). They bind to the cell membrane, particularly to the sterol component, in an irreversible fashion, resulting in the destruction of membrane integrity, an event characterized by permeability change. Though reports concerning the primary site of action of griseofulvin is unclear, it has been suggested that the mode of action of griseofulvin is inhibition of synthesis

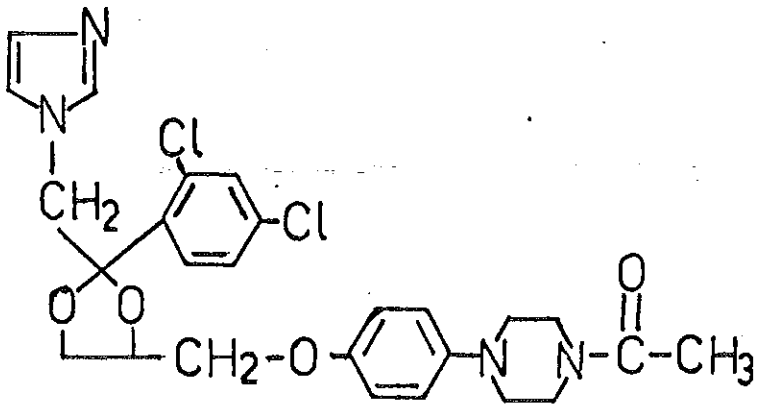


Chemical structure of clotrimazole



miconazole, $R_1=R_2=Cl$, $R_3=H$
econazole , $R_1=R_3=H$, $R_2=Cl$
isoconazole, $R_1=R_3=Cl$, $R_2=H$

Chemical structures of miconazole, econazole, and isoconazole.



Chemical structure of ketoconazole.

of nucleic acid and mitosis (Malawista et al., 1968) and hyphal cell wall material in chitinous fungi (Evans and White, 1967). Inhibition of protein synthesis by interfering with nucleic acid synthesis is the mode of action of flucytosine (Borgers, 1980). Therefore, absence of antifungal antibiotics, which selectively affect the fungal cell wall, the most obvious structural difference between the host and fungal cells is also one of the major hindrances in the development of antifungal agents (Evans and Gentles, 1985).

Therefore, like other infectious agents, mycotic infections, especially systemic mycosis still presents a significant morbidity and mortality.

Among the many superficial mycosis affecting the skin, hair, nail and mucosal membranes, ringworm, which is caused by a group of closely related fungi commonly referred to as dermatophytes is most common and is a major health problem. Because of its high morbidity it is a costly disease in terms of treatment and loss of working time (Evans and Gentles, 1985).

Subcutaneous mycosis which involves, the skin, the subcutaneous tissues, and bone is also an important fungal disease. Mycetoma the term used to denote a fungus tumour madurafoot (Abbot, 1956) is a major surgical problem and relapse rates after surgical treatment is very high (Delveloux et al., 1988) and responses to medical therapy is slow and inadequate (Bendle et al., 1987).

Among mycotic infections, systemic mycosis caused by a number of pathogenic fungi ranks first in morbidity and mortality.

Aspergillosis, particularly aspergillus granuloma is a serious systemic mycosis and is primarily a pulmonary disease, although in its most serious form it could invade a lung tissue from which dissemination to other organs occur (Evans and Gentles, 1985). Aspergillus granuloma which is caused by an opportunistic mold, Aspergillus fumigatus (Evans and Gentles, 1985) is associated with immunosupressed individuals (Veress et al., 1973; Dawately et al., 1988) and Aspergillus flavus as a causative agent of primary aspergillus granuloma (PPAG) affects healthy individuals (Dawately et al., 1988).

Coccidiodomycosis, primarily an infection of the lungs and systemic candidosis which is caused by Candida species particularly by Candida albicans are disease of major health importance. Coccidioidomycosis is generally the most difficult systemic mycosis to treat (Brass et al., 1980) and recurrence of infection after treatment is terminated and development of drug resistance by Candida species are the two major problems in the treatment of candidosis (Petersen et al., 1980).

The association of fungal infection with AIDS patients have attracted the interests of many workers in the field of medical mycology. According to Campbell and White (1989), AIDS patients are at special risk from Cryptococcus neoformans, Candida albicans, Histoplasma capsulatum, Coccidiodes immitis and

Aspergillus fumigatus. Among these, Cryptococcosis caused by Cryptococcus neoformans, is one of the most important manifestations of AIDS and of the infectious agents causing neurological disorders in patients with AIDS Cryptococcus neoformans ranks third in frequency (Dismukes, 1988). The clinical picture, the pathology, the geographical distribution of the disease, diagnosis, epidemiology and the therapy of cryptococcosis which is a serious systemic mycosis have been intensively discussed by Richardson et al., 1976; Dismukes, 1988; Evans and Gentles, 1985.

Before the advent of amphotericin B therapy, cryptococcal meningitis was invariably fatal (Geaney et al., 1956). Now, although most of these patients can be cured a mortality rate of 39 to 57% has been reported (Richardson et al., 1976, Edwards et al., 1970; Sarosi et al., 1969; Gould and Gould, 1985; Diamond and Bennett, 1974).

Clinical reports of mycotic infections from Ethiopia are relatively few. Two cases of rhinosporidiosis affecting the palpebral conjunctiva and the nasal membrane from Ethiopia have been reported by Beiske et al. (1982), and Roberts (1982). The clinical and pathological data and the diagnosis of the causative fungus (Rhinosporidium seeberi) are well documented. A case of Cryptococcosis from Ethiopia has also been reported by Lester et al. (1977).

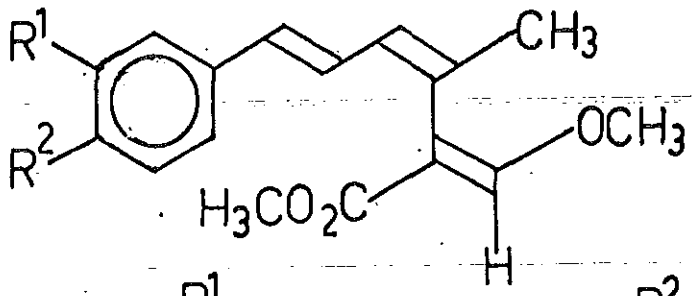
Drawbacks of the presently available antifungal agents and a clear understanding of the importance of disease of fungal origin particularly their

association with AIDS patients in the last few decades have initiated the interests of many academic institution and pharmaceutical industries to a search for new, better, less toxic and cheaper antifungal agents.

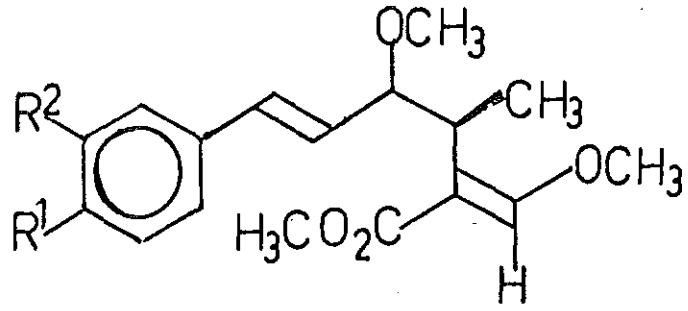
It has been pointed out that chemical synthesis and search for natural products from living organisms (higher plants and microorganisms) are the two sources for new biologically active compounds (Anke and Steglich, 1989).

Though many new biologically active metabolites have been isolated and characterized from basidiomycetes (Anke and Steglich, 1981, Anke, 1985) basidimycetes are still among the less extensively screened group of microorganisms (fungi). Among the biologically active metabolites isolated from basidiomycetes, such important antifungal antibiotics, as strobilurins and oudemasin, isolated from species belonging to the genera Oudemasiella, Strobilurus, Mycena, Hydropus, Cyphellopsis and Xerula (Anke et al., 1979, Anke et al., 1983) have attracted the interests of many workers. The same antifungal antibiotic by the name of mucidin has been isolated from fermentation culture of Oudemansiella mucida (Musilek et al., 1969).

These antifungal antibiotics have been shown to inhibit the growth of a variety of saprophytic and phytopathogenic fungi at a very low concentration. One of the strobilurins, mucidin, is commercially used for the treatment of dermatophytomycosis in animals in Czechoslovakia (Musielek et al., 1984).



	R^1	R^2
Strobilurin		
A	H	H
B	CH_3OH	Cl
C	$(CH_3)_2C=CHCH_2O$	H



	R^1	R^2
Oudemansin		
A	H	H
B	CH_3O	Cl

Chemical structures of Strobilurin and Oudemansin

Tiamulin a semisynthetic derivative of pleuromutilin isolated from basidiomycetes Pleurotus mutilus and Pleurotus passeckerianus (Kavanagh et al., 1951) was shown to be strongly active against mycoplasma and is used to treat mycoplasma infections in animals (Hogenauer, 1979).

Among the basidiomycetes the polypores also produce antifungal compounds. Oospolactone was isolated from fermentation culture of a polypore Gleophyllum sepiarium by Nakajima et al. (1976). The compound inhibits specifically the growth of fungi.

Ganoderma lucidum (Fr.) Karst is an important polypore that causes a white rot of wood of many species of plants (Adaskaveg and Gilbertson, 1987) and is a well known crude drug in China (Nishitoba et al., 1987; Arisawa et al., 1986; Tseng et al., 1984; Lin et al., 1988; Shiao et al., 1988; Shiao and Lin, 1987). It has been prescribed as a tonic and sedative drug and has been used to treat hepatopathy, hypertension, arthritis, neurasthenia and bronchitis (Arisawa et al., 1986). The infusion made by boiling the dried fruit body of the fungus with water was also used as a drink for medical and nutritive purposes in Singapore (Jones and Lim, 1989). Many Secondary metabolites with different chemical structures have been isolated from this fungus. Most of the metabolites isolated either from the fruiting body or mycelial extracts of the fungus are triterpenoids.

Chemical investigation of the fruiting body of Ganoderma lucidum led to the isolation of ganoderic acid A and B (Hirotani et al ., 1985), C and D (Kohda et al.,

1985), E and F (Hirotani and Furuya, 1986), H, M N, and O along with ganoderenic acid E and E_2 and Lucidenic acids H, I, J, K and M (Nishitoba et al., 1987), ganoderic acids Z, W, U, T, Y X (Toth et al., 1983) and G and I along with ganolucidic acid A and B (kikuchi et al., 1985).

Ganoderic acids C and D were shown to inhibit histamine release from rat mast cells (Kohda et al., 1985) and T and Z have shown in vitro cytotoxic activity against hepatoma cells (Toth et al., 1983).

Arisawa et al. (1986) have reported the isolation of three new lanostanoids, ganodermanonol ganodermadiol and ganodermatriol from the fruiting body of the fungus. In a continuing investigation of the methanolic extract of the fruit body of Ganoderma lucidum two more lanostanoids named ganodermanodiol and ganodermanontriol were isolated (Fujita et al., 1986).

Ganodermic acids D, E, F and H along with lucidenic acids D, E and F were isolated from the fruiting body of Ganoderma lucidum (Kikuchi et al., 1985) and ganodermic acids R, S, O, and Q from mycelial extracts (Shiao and Lin, 1987). Subsequent investigation of the mycelial extracts also resulted in the isolation of four more new triterpenes tentatively named ganodermic acids, Ja, Jb, P_1 and P_2 (Shiao et al., 1988). Three more new triterpenes tentatively named ganodermic acids T-N, T-O and T-Q from mycelial extracts were also reported (Lin et al., 1988). However, none of the metabolites isolated so far have been reported to have antifungal activity.

Basidiomycetes, particularly tropical basidiomycetes, have been little investigated from the view point of bioactive secondary metabolites and thus more intensive screening of tropical basidimycetes for new biologically active metabolites was suggested (Dawit, 1989).

In this, study different groups of basidiomycetes were collected and screened for antimicrobial compounds. Production medium, duration of growth, and the most susceptible test organisms for each producing cultures were determined. Cultural morphology, submerged culture of Ganoderma lucidum, isolation of two antifungal agents from the fungus, which produces the most effective antifungal principles than all basidiomycetes screened, biological and partial chemical characterization of the two antifungal agents tentatively named antibiotic 201A and 201B are described.

2 . MATERIALS AND METHODS

2.1 Collection and Culture of Basidiomycetes

Fruiting bodies of different groups of basidiomycetes (Agaricales, polypores and gastromycetes) were collected from Menagesha, Munesa, Wondogent forests, Bale national park and around Addis Ababa in July 1990.

Cultures of basidiomycetes were obtained from fresh fruit bodies. Pieces of fungal materials from the stipe and/or the pileus were transferred aseptically onto agar medium containing 4g yeast extract (Oxoid), 10g malt extract (Oxoid), 4g glucose (BDH), 100 mg penicillin (Epharm), 1g streptomycin (Epharm) and 20g agar (Oxoid) per liter of tap water. Culture plates were incubated at 25°C until young hyphae emerged, from which pieces of agar culture was transferred to the same medium without antibiotic to obtain pure culture.

Herbarium specimens were dried in an open air or in an oven. Small basidiomycetes were dried by leaving them in an open air in or on their paper bags in places with good ventilation. Larger basidiomycetes were dried in an oven at 50° C.

All Cultures are maintained on the same medium and transferred to fresh medium every three to six month

2.2. Screening of Basidiomycetes for Antimicrobial Metabolites.

Screening of basidiomycetes for biologically active compounds was done in submerged culture using culture media A, B, C and D.

Constituents of Media Used for Screening.

Medium A. (YMG- Medium)

Yeast extract	4g
Malt extract	10g
Glucose	4g
Tap water	1000 ml

Medium B. (Modified BAF Medium)

Maltose (BDH)	20g
Glucose	10g
Peptone (Oxoid)	2g
Yeast extract	1g
$MgSO_4.7H_2O$ (BDH)	1g
KH_2PO_4 (BDH)	500 mg
$CaCl_2$ (BDH)	50 mg
$FeCl_3$	10 mg
$Zn\ SO_4$ (Oxoid)	2 mg

About 60-80 ml of culture filtrate was extracted with equal volume of chloroform. The crude extract obtained after removing the solvent under reduced pressure (Rotavapour, Buchi, Model-140) was dissolved in 1 ml of methanol (MeOH). A maximum of 40 microliter (mcl) crude extract dissolved in methanol was adsorbed on filter paper discs (6 mm in diameter). The dried discs were placed on test plates seeded with the above test organisms. Presence of antimicrobial metabolites in a crude extract was determined by the appearance of zone of inhibition after incubating the plates for 24 hours at 37 °C.

2.3 Detection of Antimicrobial Principles in a Crude Extract.

Antimicrobial component (s) of crude extracts exhibiting biological activity in the above test system were detected by bioautography.

The crude extract was applied to silica gel plates (Merk 60) and left in an open air until the solvent evaporates. The plates were then developed in different solvent systems (Table 1) until the solvent system which best separates the spots was obtained. The solvent system which gave the best separation of spots of the crude extract was used to develop the chromatogram.

Table 1. Solvent systems

Toluene:	acetone:	acetic acid
70:	30:	1
Chloroform:	methanol	
90:	10	
Chloroform:	methanol	
95:	5	

To detect the active spot (s) the plates were dried prior to detection in order to remove the residues of the solvent from the plates. The dried plate was then carefully cut into pieces. Pieces of the plate containing each spot were placed upside down on test plates seeded with the most susceptible test organism.

Test plates were then incubated at an appropriate temperature for 24 hours and the active spot was detected from inhibition zone.

2.4 Identification of the Polypore and Cultural Characteristics.

The macroscopic and microscopic morphological characteristics of the fresh fruit body of Ganoderma lucidum was done according to Ryvarden and Johansen (1980). For microscopic examination, the specimen was sectioned with a razor blade. The section was then mounted in 5% KOH and squashed with a gentle tap on the cover glass. Then the preparation was stained with cotton blue in lactic acid. The hyphae,

cystidia and spores were examined under a light microscope and the results were recorded. The identity of the fungus was further confirmed by Dr. L. Ryvarden, University of Oslo, Norway.

The macroscopic cultural characteristics and its temperature relationship were also studied according to Advaskaveg and Gilbertson (1989). The organism was grown on 2 % malt extract agar at five different temperature, 20, 25, 30, 35 and 40°C. The macroscopic cultural characteristics and growth rate of the fungus with respect to different temperatures was also recorded.

2.5 Submerged Cultures and Isolation of Antifungal Metabolites from Ganoderma lucidum.

Equal number of about the same sized agar culture blocks of Ganoderma lucidum were transferred aseptically to 50 ml of medium A in 250 ml Erylenemer flasks. Culture flasks were then incubated at 20-22°C on a rotary shaker at 120 rpm. After 6 days of growth seed cultures (inoculum) were inoculated to 400 ml of the same medium (medium A). All flasks were then incubated at 20-22°C on orbital shaker at 120 rpm.

Growth (mycelial dry weight), pH-value and antibiotic production were determined using 100 ml of culture medium every other day. pH-values were determined by pH meter (Corning pH-meter mode 140), the dry weight was by oven dry method and antibiotic production by agar diffusion assay using Aspergillus niger

as test organism.

The culture was harvested when the antibiotic content of the culture filtrate reached its maximum. The culture filtrate was extracted with half volume of chloroform after it was dried over anhydrous Na_2SO_4. The chloroform extract was concentrated under reduced pressure.

The crude extract was applied on a column of silica gel Merk 60 (0.6 to 0.02 mm size), as thin layer on top of the column, after the material was dried with silica gel and eluted with increasing percentage of methanol in chloroform.

2.6 Physico-Chemical Properties of 201A and 201B.

The ultraviolet spectra were determined with Beckman spectrometer model Du-65 and the infrared spectra were recorded on IR- Spectrophotometer model 727B in KBr discs.

To detect color reactions after thin layer chromatography (TLC), reagents listed below were used.

Table 2. Colour reagents.

H_2SO_4- Vanillin (1% vanillin in Concentrated sulfuric acid)
KOH (30% solution)
$KMnO_4$ 0.05% solution)
Fast blue B salts 0.5% salt aqueous
+ 0.1N NaOH aqueous solution

Chromatographic behaviour of antibiotic 201A and 201B were also determined using the solvent systems listed in Table 1 above.

2.7 Biological Activity

2.7.1 Effects of 201A and 201B Against Yeasts and Bacteria.

The minimum inhibitory concentration (MIC), against bacteria and yeasts was determined by the conventional serial dilution assay method in 1-ml quantity of nutrient broth (bacteria) and yeast extract malt extract glucose broth medium (Medium A) for yeasts.

Test organisms (2-6 x 10^6/ml) were inoculated to test tubes containing different concentration of the test substances and incubated at appropriate temperature for 24 hours. The minimum inhibitory concentration (MIC), in the case of bacteria and yeasts was recorded as the lowest concentration of the test substance which completely prevents growth (visible turbidity) as determined by the naked eye after 24 hours of incubation.

The strain of bacteria and yeasts used to determine antimicrobial spectrum of the test substances are listed in Table 3.

2.7.2. Antifungal Activity

The activity of antibiotic 201A and antibiotic 201B against mycelial fungi was determined by the agar diffusion assay.

Heavy spores suspension (2-6 x 10^6 spores/ml) of each test fungus was suspended in Sabouruad dextrose agar (Oxoid) prior to pouring to sterile glass plates.

Different concentration of test substances were applied to antibiotic assay discs (6 mm in diameter) and placed to test plates to which spores of test fungi were seeded. Inhibition zone diameter were recorded after 24-48 hours of incubation at appropriate temperatures.

Strains of mycelial fungi used as test organisms are also listed in Table 3.

Table 3. List of Test Microorganisms.

Strain of test microorganisms	Incubation Temperature °C
Bacteria	
Staphylococcus aureus	37
Bacillus cereus	37
B. subtiles	37
Streptococcus faecalis	37
Aerobacter aerogeneus	37
Escherichia coli	37
Salmonella sp.	37
Proteus sp.	37
Yeasts	
Candida albicans	37
C. tropicalis	25
C. pseudotropicalis	25
Rhodotorula sp.	25
Saccharomyces cerviciae	25
Hansenia spora	25
Torulopsis cremoris	25
Mycelial fungi	
Aspergillus niger	25
A. ochraceus	25
A. flavus	25
A. fumigatus	25
Mucor sp.	25
Penicillium sp.	25
Fusarium sp.	25
Cladosprium sp.	25
Rhizopus sp.	25
Colletothricum coffeanum	25

2.7.3 Activity of Test Substances on Spore Germination.

A heavy spore suspension of Aspergillus niger was obtained by washing a slant with sterile distilled water and removal of hyphal material by filtration through sterile cotton. About 2-6 x 10^6 spores/ml were then inoculated into 10ml of minimal medium (Glucose 20g, NH_4SO_4 2g, KH_2PO_4 500 mg, $MgSO_4$ 100mg, $CaCl_2$ 10 mg and distilled water 1000ml) contained in 50 ml capacity Erylenemer flasks containing different concentration of the test substances. The flasks were then incubated on a rotary shaker set at 120 rpm at 25°C for 12-24 hours. About 300 spores were observed microscopically during the incubation period for the appearance of germ tubes as well as any morphological changes of the young germ tubes.

To obtain the minimum sporicidal concentration of test substances, spores which were completely inhibited in the above conditions were washed with sterile distilled water and transferred aseptically to same fresh medium with out the test substances. All flasks were incubated as described above and spore recovery was followed microscopically. The lowest concentration of the test substance that prevent any recovery of spores was recorded as the minimum sporicidal concentration of the test substance.

2.7.4 Antidermatophyte Activity

of antibiotic 201A and 201B.

Test dermatophytes, Trichophyton rubrum, Trichophyton interdigital and Trichophyton soudanese were obtained from National Research Institute of Health. Effects of antibiotic 201A and 201B against the test dermatophytes was determined by employing the modified paper-disc agar diffusion bioassay methods described by Gurusiddaiah et al. (1979).

Different concentration of antibiotic 201A, antibiotic 201B, crude extract obtained from submerged culture of Ganoderma lucidum and griseofulvin for comparative purpose were incorporated into Sabouruad dextrose agar medium just prior to pouring plates.

Equal sized agar culture blocks of test dermatophytes cut with a cork borer whose diameter is 8 mm were transferred aseptically on to agar plates (two replications of each concentration plus two controls). All plates were incubated at 27°C for 12 days.

The average growth rate was measured at 4 days interval and the minimum inhibitory concentrations were determined on the basis of percent control as follows: 100% of control equals no inhibition, and 0% of control is total inhibition of growth.

2.8 Hemolytic Effect of 201A and 201B.

Seven percent (7%) Sterile sheep blood was aseptically suspended in sterile basal blood agar medium (Oxoid). After solidification antibiotic assay discs containing different concentration of test substances and a detergent for comparative purpose were applied to plates. Diameters of lytic zone were recorded after 24 hours of incubation.

2.7.6 Dermatotoxicity Test.

Shaved rabbit skin was used to determine skin toxicity of the antifungal agent. Crude extract was dissolved in dimethylsulfoxide (DMSO) and applied to 10 sq cm of a shaved skin. The antifungal agent was applied topically every other day for a week. The applied area was compared with the control to which DMSO alone was applied. Observation on dermal reactions (dermatotoxicity) was followed every day for a week.

3.0 RESULTS

3.1 Growth and Screening of Basidiomycetes for Antimicrobial Metabolites.

About 120 different basidiomycete fruit bodies were collected for this study, from which 60 (50%) grew on laboratory cultures. Of the basidiomycetes collected 55 (45.8%) were species of Agaricales, 59 (49.2%) were polypores, and 6 (5%) were gastromycetes.

All the 60 different group of basidiomycetes which grew on medium A were tested against bacteria, yeasts and mycelial fungi. Among the different groups of basidiomycetes screened for antimicrobial activity 30 (50%) were different species of Agaricales, 27 (45%) were different species of polypores, and 3 (5%) were different species of gastromycetes. Ten (17%) of the different basidiomycetes produced antimicrobial secondary metabolites. The basidiomycetes which produced antimicrobial metabolites, the production medium, duration of growth and the most susceptible test organisms are shown in Table 4.

Table 4 list of basidiomycetes

Producing antimicrobial metabolites

Name and/or code of the fungus	Production medium	Duration of Growth in days	The most susceptible test organism
Ganoderma lucidum (ADA-201)	A	12	Aspergillus niger
Favolasihia sp. (DAB-17)	C	16-19	A. niger
Psathyrella sp. (ADA-218)	A	12-15	Bacillus cereus
Psathyrella sp. (ADA-3)	B	13	Staphylococcus aureus
Termetomyces microcarpus (ADA-1)	C	10	A. niger
Trametes pubescens (ADA-202)	B	10	A. niger
Agaricus sp. (ADA-2)	C	14	B. cereus
ADA-33 (Unidentified)	A	10-12	Staph. aureus
Cystidiodontia artoreas (ADA-310)	B	10	Staph. aureus
Coriolopsis strumosa (ADA-323)	A	10	B. cereus

Among the basidiomycetes screened, extract of culture filtrate of Ganoderma lucidum showed the strongest antifungal activity. Favolasihia sp. and Trametes pubescens (ADA-202) exhibited effective antifungal activity. Termetomyces microcarpus (ADA-1) has also produced a moderate activity against A.niger. Two species of Psathyrella (ADA-3 and ADA-218), Agaricus sp. (ADA-2), unidentified mushroom,(ADA-33), Cystidiodontia artocereas (ADA-310), a resupinate fungus and Coriolopsis strumosa (ADA-323), a polypore have displayed a moderate antibacterial activities.

As shown in Table 4, differences in antimicrobial activity was observed when certain fungus was grown in different media. Better antimicrobial activity was produced when G.lucidum (ADA-201) Psathyrella sp. (ADA-218), ADA-33 and Coriolopsis strumosa were grown in medium A. Psathyrella sp. (ADA-3), Trametes pubescens (ADA-202), Cystidiodontia artoreas (ADA-310) exhibited better antimicrobial activity when grown in medium B. Medium C was a better medium for the production of antimicrobial substances by Favolasihia sp.,Termtomyces microcarpus and Agaricus sp.

The antimicrobial activity of most fungi investigated increased with the age of the fungal culture. The age of each producing fungus at which better antimicrobial activity was produced is shown in Table 4.

3.2. Morphological and Cultural Characteristics of Ganoderma lucidum

The polypore, G.lucidum was collected in Munesa forest, Arsi. The fungus is wood inhabiting, mostly on angiosperms, more rarely on gymnosperms and is wide spread in Africa (Ryvarden and Johansen, 1980).

The fungus is characterized by its trimitic hyphal system. The fruiting body stipitate, is located either centrally, or often laterally. The fungus has glossy and shiny pileus and cylindrical to slightly flattened stipe which could extend up to 15 cm in length and up to 2.5 cm in diameter. The size of the spore is 7-12 x 6-8 micrometer, and the fungus produced clamp connection when grown in submerged culture.

Comparative data of macroscopic cultural characteristics of the polypore and its temperature relationships with other species of Ganodrma is given in Table 5. Cultural mat of the polypore had an even margin and was felty to floccose in texture. The fungus grew moderately on 2% malt extract agar plates covering the plate almost in 4 weeks time at 25°C. The optimum temperature was in the range of 20-25°C. As depicted in the Table, the Ethiopian strain of G.lucidum (ADA-201) is similar to G.tsugae and G.oregonese but different from the other four.

Table 5 Comparison of cultural characteristics of the polypore with other species of Ganoderma

Species*	Mat color	Growth habit+	Optimum temperature in °C
Ganoderma lucidum	White	even, felty	30-40
G. tsugae	White to pale yellow	even, felty to floccose	20-25
G. oregonense	White to pale yellow	even, felty to floccose	20-25
G. meridithiae	White to pale yellow	bayed, plumose	25-30
G. zonatum	White to reddish yellow	bayed to floccose	25-30
G. colossum	White, grey to tan	even wooly	35-40
Polypore under study (ADA-201)	White to pale yellow	even, felty to floccose	20-25

* Data of cultural characteristics of the first six species was obtained from Adaskaveg and Gilbertson (1989).

+ Growth habit refers to margin and texture of the cultural mat.

In the laboratory, the fungus was maintained on medium A, and transferred every 6 month to the same fresh medium. The fungus was maintained at 4°C on agar slants up to 6 months.

3.3 Submerged Culture and Isolation of Antifungal Metabolites from G. lucidum.

Production of antifungal metabolites was better in medium A. A culture grown in medium A for 6 days was used as an inoculum. The antifungal metabolites were found excreted into the culture filtrate.

The time course of submerged culture of the producing fungus was followed by measuring the dry weight of the mycelium, the pH-values and antifungal activity of the culture filtrate against Aspergillus niger and the results are shown in Fig. 1.

As shown in Fig.1, the pH-value of the culture broth decreased for the first six days of growth, followed by a continuous rise in pH. The optimum pH value corresponding to the highest production of antifungal agents was about 6.8.

Mycelial dry weight increased rapidly from the 6^{th} to the 10^{th} days of growth and showed no appreciable increase or decrease up to the 14^{th} day of growth.

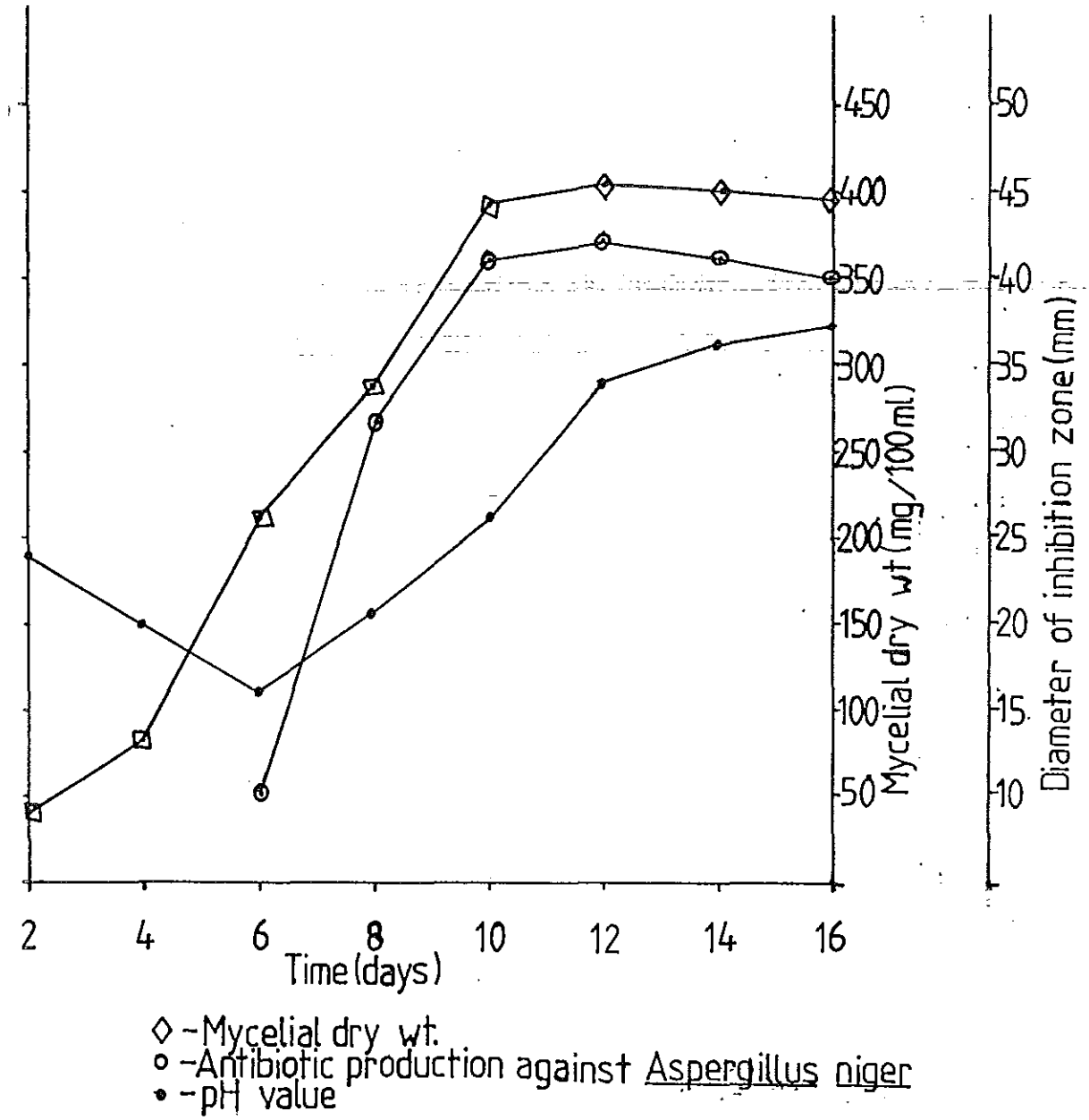


Fig. 1 Submerged culture of Ganoderma lucidum

The antibiotic production started from the 6th days of growth and reached its maximum in the 12th day of growth. After 14 days of growth a decrease in antibiotic production was observed.

Ten liters of culture filtrate obtained from growth of submerged culture was extracted with 5 liters of chloroform. The brown gummy crude extract (1.2 g) was then purified by a column of silica gel and preparative thin layer chromatography (PTLC) as shown in Fig. 2.

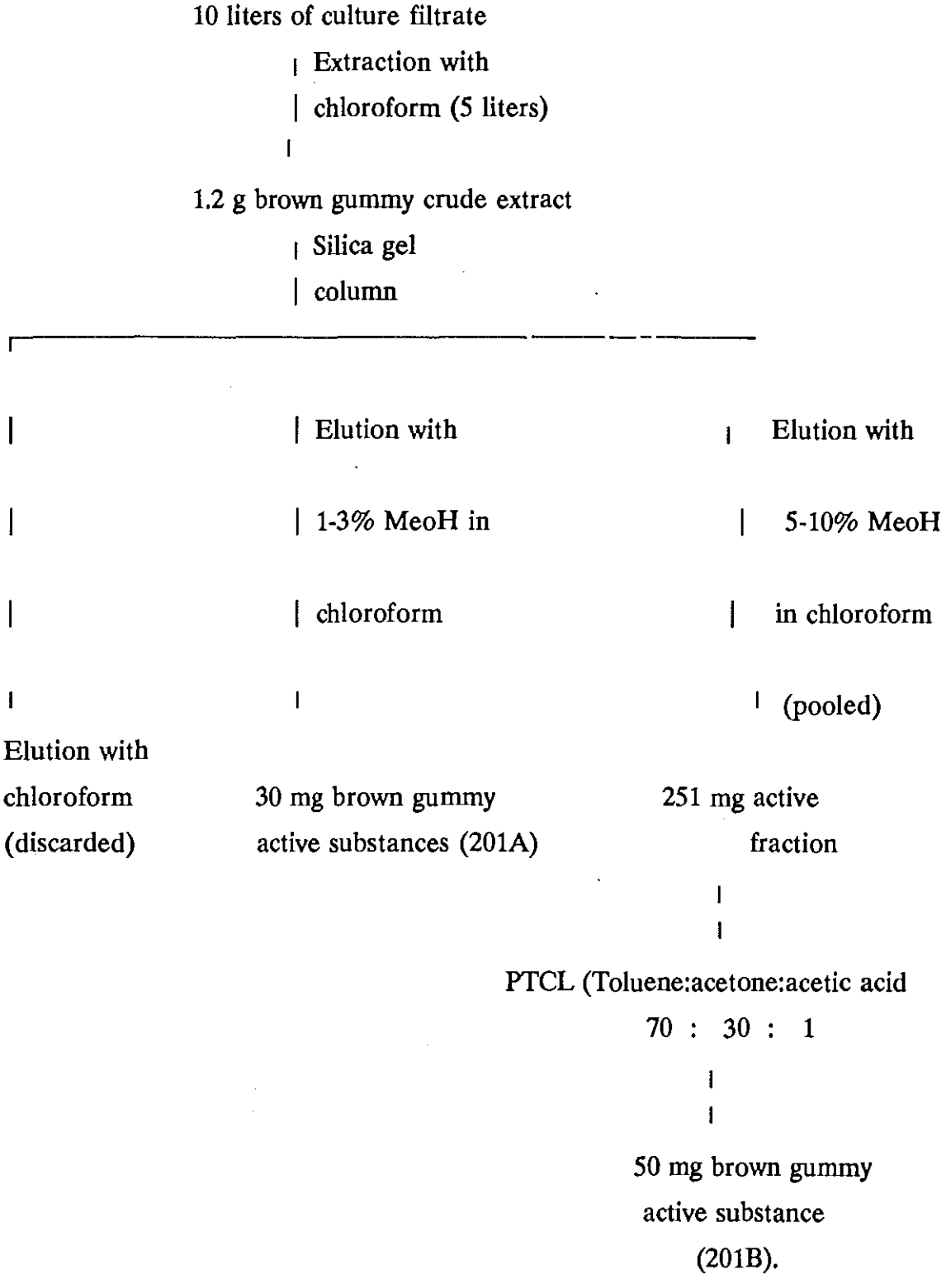


Fig 2 Purification of antibiotic
201A and 201B.

3.4. Physico-Chemical Properties of Antibiotic 201A and 201B.

Antibiotic 201A was brown gummy substance. It was soluble in chloroform, methanol, ethylacetate and dimethylsulfoxide (DMSO).

The antibiotic gave a light orange color reaction when sprayed with vanillin in sulfuric acid and no color reaction with 3% KOH, fast blue and $KMnO_4$.

The antibiotic had a UV absorption maximum at 247 nm in methanol (MeOH). The UV and IR- spectrum of 201A are given in Fig. 3 and 4, respectively.

Antibiotic 201B was also brown gummy substance. It was highly soluble in methanol, but slightly soluble in chloroform, dichloromethane and ethylacetate.

On thin layer chromatography plate (TLC), antibiotic 201B can be visualized by spraying with vanillin in sulfuric acid (brown).

The antibiotic had UV absorption maxima at 243 and 257 nm in MeoH. The IR-spectrum of 201B is depicted in Fig. 5.

The R_f-values (a term used to express the position of a substance on developed chromatogram) of the two substances in different solvent system are shown in Table 7.

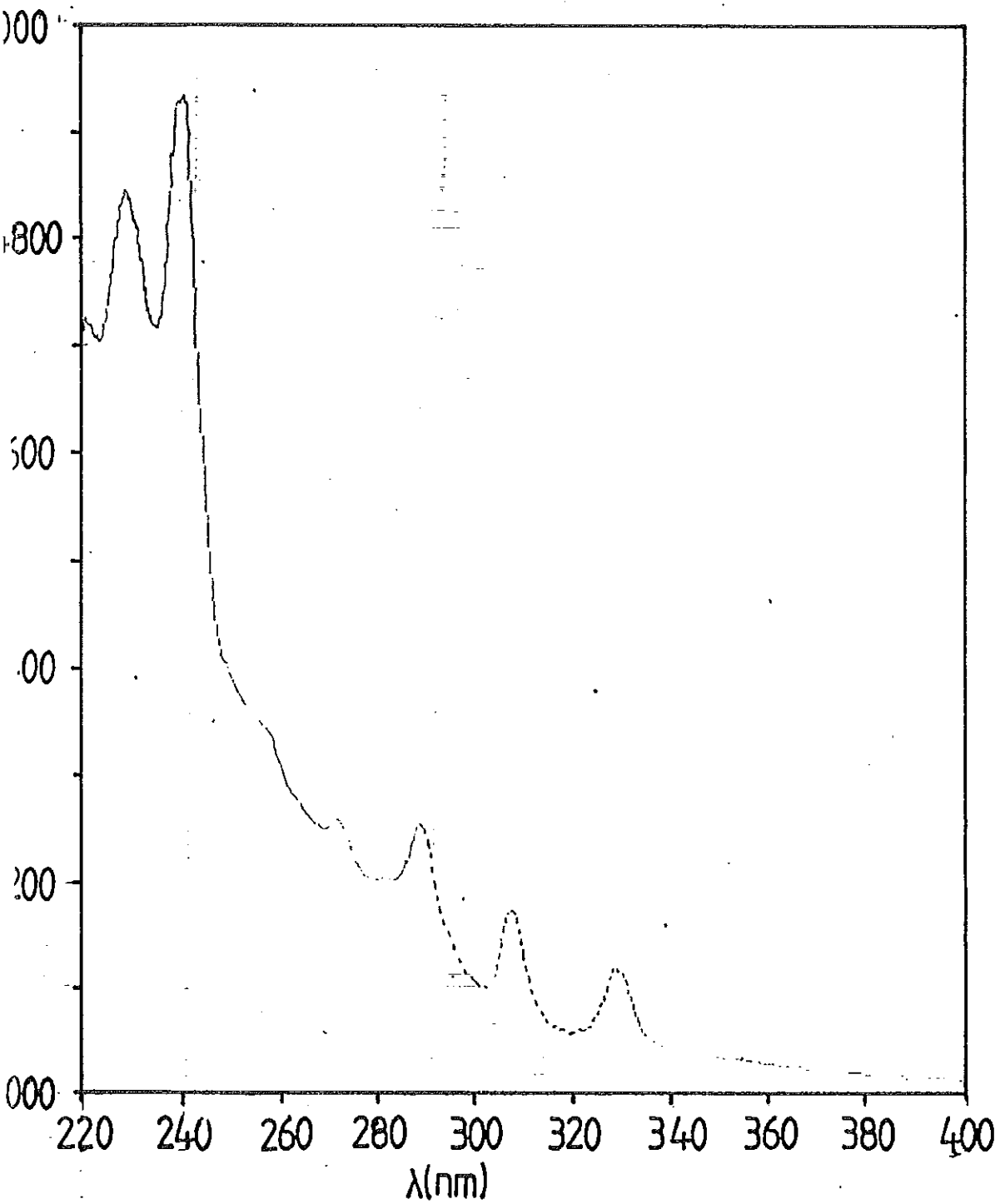


Fig. 3 UV-spectrum of 201A

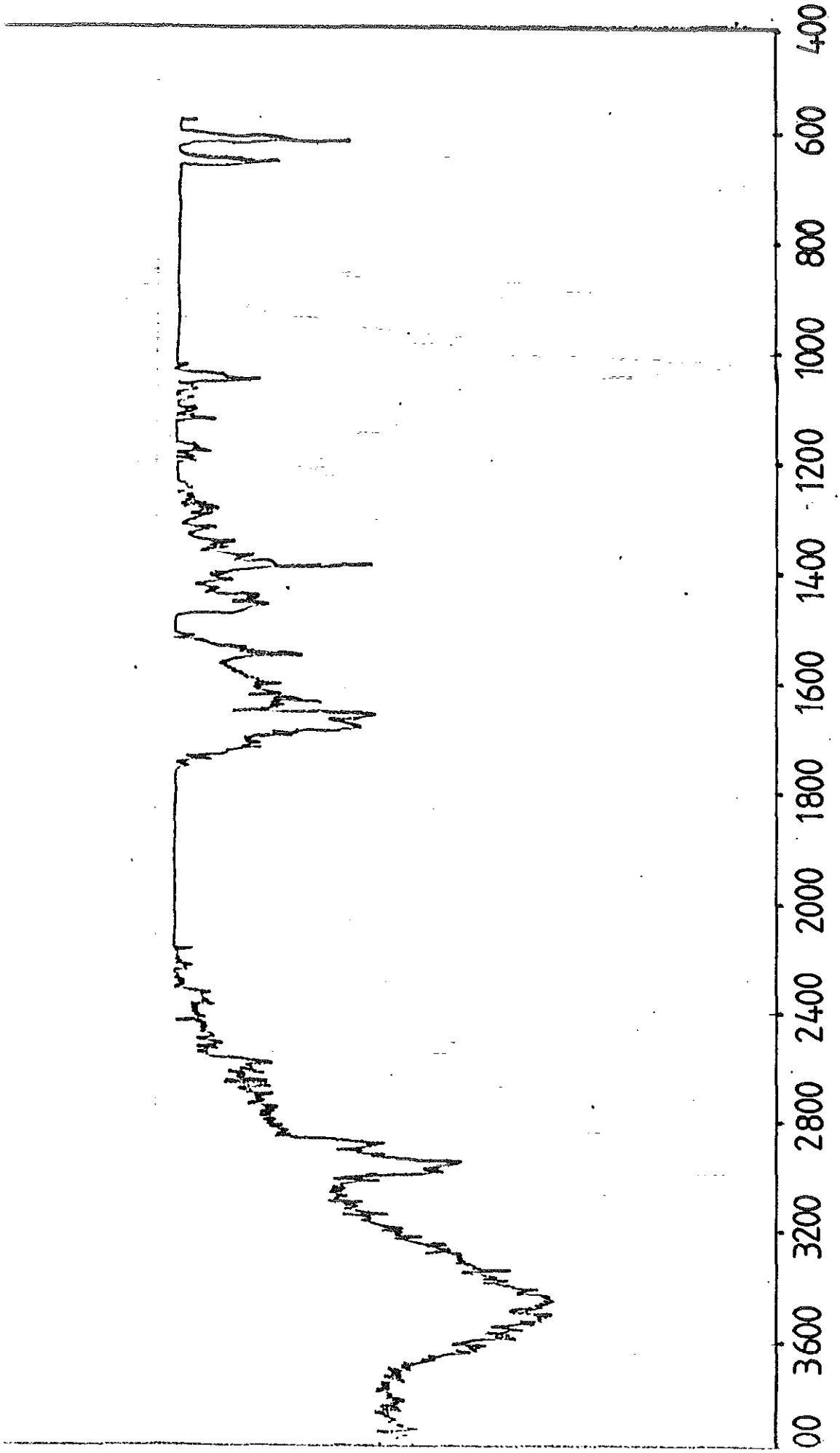


Fig. 4 IR-Spectrum of 201A in KBr discs

3.5 IR-spectrum of 201B in KBr discs

Table 6 Chromatographic behaviour

of antibiotic 201A and 201B.

Solvent system	R_f-value	
	201A	201B
Toluene : Acetone : acetic acid		
70 : 30 : 1	0.65	0.35
Chloroform : methanol		
90 : 10	0.85	0.51
Chloroform : methanol		
95 : 5	0.72	0.43

3.5. Biological Activity of Antibiotic 201A and 201B.

3.5.1. Effect Against Bacteria and Yeasts.

The minimum inhibitory concentrations (MIC) of 201A and 201B against an array of bacteria and yeasts are presented in Table 7. As is evident from this data, the antibiotics are broad spectrum and are effective against gram-positive bacteria and yeasts. The antibiotics are relatively ineffective against gram-negative bacteria.

Antibiotic 201A was particularly effective against the pathogenic yeast, Candida albicans. Antibiotic 201B was active at higher concentration when compared to 201A against yeasts and bacteria.

Table 7 Antimicrobial spectrum of antibiotic 201A and 201B (serial dilution assay)

Test organisms	MIC/mcg/ml	
	201A	201B
Bacteria		
Staphylococcus aureus	5-10	50-100
Bacillus cereus	10-20	50-100
B. subtiles	10-20	50-100
Streptococcus faecalis	10-20	50-100
Escherichia coli	>50	>100
Aerobacter aerogeneus	50	>100
Salmonell sp.	>50	>100
Proteus sp.	>50	>100
Yeasts		
Candida albicans	<1	10-20
C. pseudotropicalis	1-5	20-50
C. tropicalis	5-10	50-100
Saccharomyces cerevisiae	5-10	50-100
Rhodotrula sp.	1-5	20-50
Torulopsis cremoris	5-10	50-100
Hansenia spora	5-10	50-100

3.5.2 Activity against Mycelial Fungi.

The results of activity of antibiotic 201A and 201B against a variety of mycelial fungi is shown in Table 8. All the fungi tested were susceptible to the antibiotics. Species of Aspergillus were more senstive to both test substances than the other mycelial fungi. Aspergillus fumigatus, a pathogenic mycelial fungus was strongly affected by 201A. The effect of 201B against mycelial fungi was moderate.

Table 8 antifungal spectrum of antibiotic

201A and 201B (agar diffusion assay)

Test mycelial fungi	Diameter inhibition zone (mm) mcg/disc						
	201A			201B			
	10	20	50	10	20	50	100
Aspergillus niger	30	35	-	-	-	20	25
A. flavus	36	45	-	-	-	28	32
A. ochraceus	25	31	-	-	-	25	30
A. fumigatus	32	38	-	-	-	18	27
Colletothricum coffeanum	25	32	-	-	-	7	11
Penicillium sp.	17	32	38	-	-	0	10
Trichoderma sp.	10	22	31	-	-	0	7
Fusarium sp.	10	19	22	-	-	0	5
Rhizopus sp.	15	25	33	-	-	0	10
Mucor sp.	24	28	-	-	-	8	19

0 = no inhibition - = not tested.

3.5-3 Effect on Spore Germination.

The effect of antibiotic 201A and 201B on germination of spores of Aspergillus niger are given in Figures 6 and 7, respectively. Germination of spores of Aspergillus niger was completely inhibited in less than 10 mcg/ml when treated with antibiotic 201A. On the other hand, treatment of spores of A.niger with antibiotic 201B up to 100 mcg/ml did not show complete inhibition of spore germination.

No morphological deformation in the emerging germ tubes was also observed, when spores were treated with antibiotic 201A and 201B at concentration lower than those causing spore germination inhibition.

The antifungal compound 201A was sporicidal at higher concentration and sporostatic at lower concentrations (Table 9). Treatment of spores of Aspergillus niger with 201A at concentration higher than 15 mcg/ml for 24 hours irreversibly prevented germination of spores; no recovery of spores was seen after washing the spores with sterile distilled water.

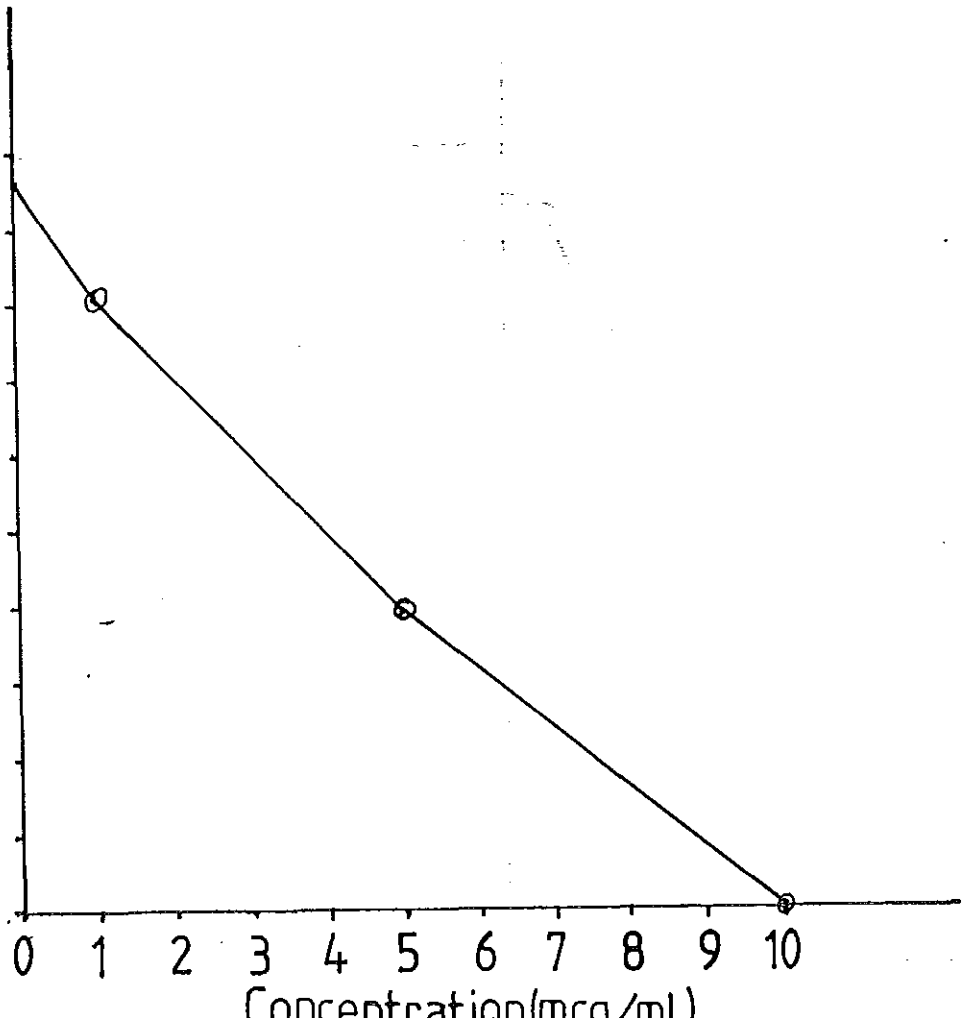


Fig. 6 Effect of 201A on germination of spores of Aspergillus niger

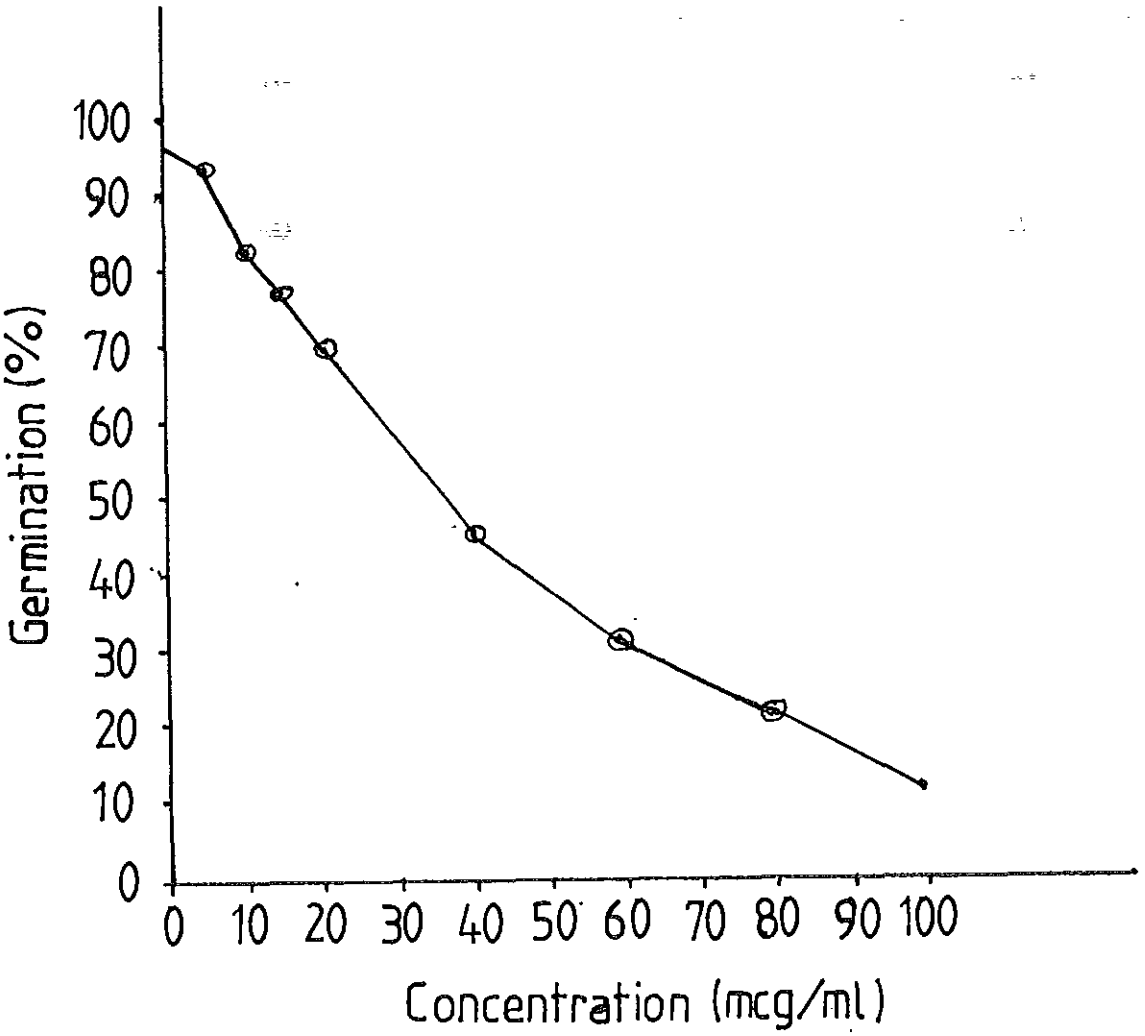


Fig.7 Effect of 201B on germination of A. niger spores

Table 9 Sporicidal activity of 201A.

Concentration (mcg/ml)	Recovery of spores (%)
10	31
15	7
20	0
40	0

3.5.4 Activity Against Dermatophytes.

The activities of 201A and 201B against dermatophytes were evaluated using the modified paper disc agar diffusion assay. The minimum inhibitory concentrations of the test substances against three anthropophilic dermatophytes are shown in Table 10.

Table 10 Actipity of 201A, 201B and Crude extract against dermatophytes(madified paper dipe a gar diffusion assay)

Test substances	dermatophyte	percent growth of control											
		20mcg/ml			40mcg/ml			50mcg/ml			100mcg/ml		
		4d	8d	12d	4d	8d	12d	4d	8d	12d	4d	8d	12d
201A	Trichophyton rubrum	50	42	28	0	0	0	-	-	-	-	-	-
	T interdigital	83	74	73	0	0	0	-	-	-	-	-	-
	T soudanense	40	28	25	0	0	0	-	-	-	-	-	-
201B	T rubrum	39	90	85	77	71	70	65	64	60	-	-	-
	T interdigital	100	98	95	92	92	88	87	85	82	-	-	-
	T soudanense	90	90	87	85	83	79	77	77	75	-	-	-
Griseofulvin	T rubrum	55	50	49	18	10	9	-	-	-	-	-	-
	T interdigital	35	30	13	0	0	0	-	-	-	-	-	-
	T soudanense	67	56	con	22	22	20	-	-	-	-	-	-
Crude extract	T rubrum	-	-	-	-	-	-	63	60	60	20	15	13
	T interdigital	-	-	-	-	-	-	100	96	95	50	46	38
	T soudanense	-	-	-	-	-	-	67	66	65	11	6	3

Con : Contaminated

\- : Not tested.

\= : griseofulvin was used for comparison

As is evident from Table 10 the growth of all of the three anthropophilic dermatophytes were completely inhibited by 201A. Since griseofulvin is an effective existing drug against dermatophytes (Evans and Gentles, 1985), it was compared with the two antibiotics. 201A showed a better inhibitory effect against T.rubrum and T.soudanense, while its effect against T.interdigital was comparable to griseofulvin. The result also indicate that 201B was less active against all test dermatophytes than both 201A and griseofulvin. The crude extract has been shown to have a strong effect against all the three human dermatophytes at concentration of 100 mcg/ml.

3.5.5 Hemolytic Effect on Sheep Erythrocytes

The lytic effect of antibiotic 201A and 201B was compared to a detergent. Treatment of sheep erythrocytes with the antibiotics did not cause lysis of red blood cells of a sheep (Table 11). Therefore both 201A and 201B do not seem to cause membrane damage of red blood cells.

Table 11 Lytic effect of 201A and 201B.

Concentration of test substances (mcg/disc)	Diameter of lytic zone (mm)		
	201A	201B	detergent
5	0	0	6
10	0	0	14
20	0	0	20
40	0	0	20
60	0	0	22
80	0	0	-
100	0	0	-

- = not tested

0 = no lysis

3.5.6 Effect of Crude Extract on Rabbit skin.

Dermatotoxicity tested by the rabbit skin toxicity test showed no detectable skin reaction (i.e, edema, necrosis or dermatitis) when compared to the control.

4.0 DISCUSSION

Not all basidiomycetes can be grown on conventional fungal media. About 50% of the 120 different species of basidiomycetes collected for this study grew on medium A. Contamination of fruit bodies with fast growing molds is one of the problem in obtaining pure cultures from basidiomycetes. This was responsible, in part, for the decrease in the percentage of basidiomycetes which grew on laboratory cultures.

The earliest screenings for antimicrobial activity from basidiomycetes (Robbins et al, 1945; Atkinson, 1946; Mathieson, 1946; Hervey, 1947) have shown that basidiomycetes are promising sources of antibiotics. Recent studies (Anke and Steglich, 1981, Anke, 1989) have also confirmed that basidiomycetes are good sources of bioactive secondary metabolites.

This study has also shown that from a relatively small number of basidiomycetes a fairly good number of them exhibited antimicrobial activity. Antimicrobial activity against bacteria, yeasts and/or mycelial fungi was produced by 17% of the different group of basidiomycetes investigated by the agar diffusion bioassay method (Table 4).

The main environmental factors to be considered in carrying out a successful fungal cultivation and antibiotic production are medium composition temperature, pH and aeration.

The influence of medium on growth and antibiotic production by various microorganisms has been described by various workers (Poster, 1976; Hockenhull, 1981; Turner, 1971; Demain, 1981; Martin and Demain, 1980; Drew and Demain, 1977; Aharozgwitz, 1980).

Production of antibiotics or yield differences from basidiomycetes could result from differences in the composition of fermentation medium (Dawit, 1989).

The effect of medium on antimicrobial activity was also observed in this study. In the preliminary screening of basidiomycetes for antimicrobial activity four different media were used and antimicrobial activity was variable (Table 4). Better antimicrobial activity was produced when Ganoderma lucidum, Psthyrella sp. (ADA-218) and Cystidiodontia artoreas (ADA- 310) were grown on medium A. Favolasihia sp. (DAB-17), Termtomyces microcarpus (ADA-1) and Agaricus sp.(ADA-2) exhibited better antimicrobial activity when cultivated in medium C. The remaining four species of basidiomycestes, Coriolopsis strumosa (ADA-232), Trametes pubsences (ADA- 202), Psathyrella sp (ADA-3) and ADA-33 (a non stipitate unidentified mushroom) showed better inhibitory activity when grown in medium B. Though wort medium was the best medium for the growth (biomass) of almost all different groups of basidiomycetes studied, no reproducible antibiotic production was evidenced by most basidiomycetes when grown in this medium. A good medium for the growth of a producing fungus is usually a poor medium for the production of antibiotics (Poster, 1976; Hervey, 1947).

The majority of fungi grow well at room temperature. An earlier study (Dawit, 1989) showed that no significant difference was observed in antibiotic production by basidiomycetes when grown at 22 or 27 °C. Thus in the preliminary screening of basidiomycetes for antimicrobial activity all submerged cultivation was performed in the range of 20-22°C.

Fungi are highly aerobic (Turner, 1971) and thus in this study, oxygen was provided to all submerged cultures by placing flasks on a rotary shaker at 120 rpm and by reducing the volume of the medium in each flask.

The effect of pH is an important factor for the production of antibiotics. Fungi grow better in acidic medium. Therefore, the unadjusted pH of the media used in this screening was in the range of 5.2 to 6.8.

The age (phase of growth) of the fungal culture is also an important factor in the investigation of basidiomycetes, for antimicrobial activity. In this study, duration of antibiotic production for each producing fungus was determined. The duration of antibiotic production was different for each strain of basidiomycete tested (Table 4). The antimicrobial activity of most of the fungi investigated may increase, decrease or disappear with the age of the strain under study.

Among all basidiomycetes screened for antifungal activity the polypore, Ganoderma lucidum produced the most effective antifungal compounds.

The need for revision of the genus Ganoderma especially G.lucidum has been recommended by Ryvarden and Johansen (1980) and Ganoderma lucidum complex is used here as described by the authors.

Currently, the use of cultural characters in developing the taxonomy of G.lucidum has been suggested (Adaskaveg and Gilbertson, 1989). Adaskaveg and Gilbertson (1989), using macroscopic and microscopic morphological characteristics and temperature relationships have separated the complex into six species (Table 6). Macroscopic cultural characteristics of the producing fungus, which include growth margin, mat texture and color of the mat on 2% malt extract agar and its growth with respect to different temperatures were studied. The results revealed that this strain of G.lucidum had an even margin, felty to floccose texture and white to pale yellow color of mat (Table 6). These cultural characteristics are similar to that of Ganoderma tsugae and Ganoderma oregonense than all the other species of Ganoderma.

Temperature relationships of the six species of the complex have been shown to correlate with geographical distribution of these fungi (Adaskaveg and Gilbertson, 1988). According to Adaskavegand Gilbertson (1988), G.colossum has a high optimum temperature range (35-40°C) and is found in tropical and subtropical regions. G.zonatum and G.meredithiae have an optimum temperature range of 25-30 °C and are found in warm temperate regions. G.lucidum has an optimum temperature range from 30-40 °C and is found through out the temperate regions. G.tsugae and G.oregonense are common in high elevation coniferous forests.

The Ethiopian strain of G.lucidum (the producing fungus) has an optimum temperature range of 20-25 °C. Its temperature relationships and our collection from Munesa forest an area which is highly dominated by gymnosperms may also further confirm the similarity of the producing fungus to G.tsugae and Ganoderma oregonense.

In submerged culture of Ganoderma lucidum, it has been shown that no significant antibiotic production was observed up to the first 8 days of growth. However, antibiotic production was found out to be maximum between 10 to 14 days of growth, when the growth of the producing fungus was completed (Fig. 1.). In batch cultures high levels of antibiotics are usually produced only after most of the cellular growth has already occurred (Weinberg, 1970). Depletion of one or more growth limiting substrates has been reported to arrest growth and initiate antibiotic synthesis (Martin and Demain, 1980; Bulock, 1961; Bulock et al; 1965).

It is also clear that the production of antibiotic was almost linear between 10 to 14 days of growth, followed by a decrease in antibiotic production. According to Martin and Demain (1980), depletion of the precursors of the antibiotic, irreversible decay of one or more antibiotic synthetase and feedback effect of antibiotic against its production are the most probable explanation for antibiotic synthesis cessation.

The pH of the broth medium decreased for the first few days of growth and such a decrease in pH in antibiotic fermentation with fermentation time could result in from production of organic acids. These are produced as a result of breakdown of

carbohydrates (Anke et al., 1987; Dawit, 1989; Poster, 1976). This was followed by a rise in pH to alkaline range and the maximum antibiotic production occurred during the earlier stage of this rise in pH. Exhaustion of carbohydrates as a cause of a rise in pH, and appearance of maximum antibiotic production in the earlier stage of a rise in pH in most antibiotic fermentation had been described (Poster, 1976). However, a shift of pH to a more alkaline range, due, to metabolic products is usually associated with a decrease in the yield or complete loss of antibiotic production (Dawit, 1989). This could also be a possible explanation for a decrease in antibiotic production in submerged cultures of Ganoderma lucidum.

Low yield of antibiotics from fermentation culture of basidiomycetes was pointed out as a major limitation in the investigation of these group of fungi for antimicrobial agents (Anke, 1985). Amounts of 3 mg of 201A and 5 mg of 201B were purified from a liter of culture filtrate (Fig. 2.). A number of factors that contribute to a reduction in yield of antibiotics have been discussed by many researchers working in the field of secondary metabolite production and regulation (Martin and Demain, 1980;Demain, 1981; Drew and Demain, 1977). According to Demain (1981) Penicillin, Cycloheximide, Cephalosporin and Chloramphenicol exert a feed back regulation on their biosynthesis. Inhibition of a common biosynthetic pathway of primary metabolism and secondary metabolism by primary end products has been reported to affect production of antibiotics (Martin and Demain, 1980).

In this work, the two antifungal antibiotics (antibiotic 201A and antibiotic 201B) isolated from submerged culture of Ganoderma lucidum have been biologically

characterized. Due to their effectiveness against disease causing fungi (dermatophytes, pathogenic yeasts and molds) the antifungal activity is described.

At present there are a number of antifungal agents of microbial or synthetic origin in therapeutic use. These antifungal agents, however differ in terms of spectrum of activity potency and mode of action and thus treatment of many mycotic infection is far from satisfactory.

Polyene antifungal antibiotics (nystatin, natamycin, amphotericin B) have a broad spectrum of activity against mycelial fungi and yeasts, but are inactive against gram-positive and gram-negative bacteria (Hamilton-miller, 1973). Narrow spectrum of activity is a major limitation of synthetic antifungal drug flucytosine and griseofulvin isolated from Penicillium griseofulvum. Activity of flucytosine appeared to be limited to yeast like fungi (Shadomy, 1969), while griseofulvin has been shown to posses fungistatic activity only against dermatophytes (Borgers, 1980).

With respect to a range of activity, both antibiotic 201A and 201B have a broad spectrum of activity against an array of bacteria, yeasts and mycelial fungi (Table 7 and 8).

The in vitro minimum inhibitory concentration (MIC) of antibiotic 201A and 201B against bacteria and yeasts show variability. Among the bacteria, gram-positive bacteria are more sensitive to both antibiotics than gram-negative bacteria. Of the

gram-positive bacteria Staphylococcus aureus has been shown to be the most susceptible. Gram-negative bacteria were almost completely resistant to both antibiotic 201A and 201B.

Both antibiotic 201A and 201B, were also active in vitro against a number of yeasts and mycelial fungi. Data on the in vitro antifungal properties of 201A and 201B showed that, the agents are active against the yeasts, C. albicans, C. tropicalis, C. pseudotropicalis, Saccharomyces cerevisiae, Rhodotrula sp. and molds belonging to the genera of Aspergillus, Rhizopus, Mucor, Penicillium, Fusarium, Cladosporium and Colletothricum.

The in vitro susceptibility data indicates that the potency of antibiotic 201A against bacteria and yeasts was much better than that of antibiotic 201B (Table 8). The MIC value of antibiotic 201A against C. albicans, the most frequent cause of superficial, mucocutaneous and systemic mycosis (Evans and Gentles, 1985) was less than 1 mcg/ml. The corresponding MIC value of antibiotic 201B was in the range of 10-20 mcg/ml. Comparing the MIC of antibiotic 201A against C. albicans with that of amphotericin B and ketoconazole from literature have revealed that the MIC of antibiotic 201A was similar to that of amphotericin B which is in the range of 0.01 to 0.5 mcg/ml (Hamilton-Miller, 1973) and that of ketoconzole which is in the range of 0.3 to 2.5 mcg/ml (Peterson et al 1980). These two antifungal drugs are known to have a wide spectrum of activity against a number of pathogenic fungi.

Results of antifungal activity testing with antibiotic 201A and 201B by agar diffusion assay against mycelial fungi (Table 8) showed that the potency of antibiotic 201A is strong against Aspergillus flavus and Aspergillus ochraceus. These molds are among the many fungi capable of producing aflatoxins and ochratoxin, respectively (Hesseltine et al, 1972). Aspergillus fumigatus, an opportunistic mold, implicated as a cause of systemic mycosis (Evans and gentles, 1985) was also sensitive to the two antifungal agents. Members of the genus Aspergillus are found out to be resistant organisms to both amphotericin B and flucytosine (Utz, 1980). Colletothricum coffeanum which is a known coffee pathogen was also sensitive to both antibiotics. The activity of antibiotic 201A against mycelial fungi by agar diffusion assay also compares favourably with that of strobilurins (Anke et al., 1979).

Not only the mycelial growth of various fungi but also the germination of fungal spores was affected by the two antifungal principles. The number of germinating spores of Aspergillus niger decreased with increasing concentration of both antifungal agents (Fig. 6 and 7). The percentages of spores showing some sort of germination response were 81% and 39% at concentrations of 1 mcg/ml and 5 mcg/ml of antibiotic 201A respectively. However, germination of conidiospores of A.niger was entirely absent at concentration of 10 mcg/ml and higher. This value (10 mcg/ml) is twice that of polyoxin D (Endo et al., 1970) and almost half of that of cycloheximide (Dawit, 1989), both of which are effective inhibitors of fungal spore germination. However, spores of A.niger were less sensitive to antibiotic 201B and no complete inhibition of spore germination was achieved by treating conidiospores up to 100 mcg/ml of the test substance.

Antifungal agents inhibit growth of fungi either by damaging structural components of the fungal cell or by interfering with fungal cell functions. For example, polyene antifungal antibiotics and imidazole derivatives affect the function of fungal cell membrane (Hamilton-Miller, 1973). Griseofulvin is known to prevent spindle and cytoplasmic microtubules, formation there by, influencing cell division (Malawista et al., 1968). The mechanism of action of other antifungal agents is inhibition of the sequence of reactions leading to the synthesis of nucleic acids or protein. Inhibition of protein synthesis is described as a primary site of action of flucytosine (Borgers, 1980) and cycloheximede (Van laere et al., 1976). Mucidin one of the antifungal antibiotic of the strobilurin series inhibits the mitochondrial electron transport between cytochromes b and C (Subic et al., 1974) thereby, affecting fungal cell respiration. A considerable interest has been also shown in chitin synthetase as a potential target for compounds with fungicidal and insecticidal activity, since chitin is found only in fungi and arthropods. Endo et al. (1970) have showed that chitin synthetase to be the site of action of an antifungal agent polyoxin D.

Cytochromes, synthesis of protein and/or nucleic acids are vital for fungal spore germination (Van Assche and Carlier, 1973, Keyhant et al.,1 972). Cycloheximide an effective inhibitor of spore germination, inhibits fungal spore germination by impairing protein synthesis (Van laere et al., 1972) and that of mucidin by affecting fungal cell respiration (Subic et al., 1974). Polypoxin D inhibits spore germination by affecting chitin synthesis (Endo, et al., 1970). Treatment of fungal spores with polyoxin D was shown to result in abnormal hyphal morphology in emerging germ tubes and such morphological deformation (formation of protoplast-like structures sensitive to osmotic

stress) at concentrations lower than those causing spore germination inhibition was reported to be due to the inhibitory effect of the antibiotic on chitin synthetase (Endo et al., 1974; Schmidt, 1987). Such morphological deformation either in the emerging germ tube or developing hyphae were not observed when spores of A.niger were treated with both antibiotic 201A and 201B at lower concentration. Therefore, it seems that both antibiotics do not affect chitin synthetase.

Treatment of spores of A.niger with antibiotic 201A at concentrations greater than 20 mcg/ml for 24 hours irreversibly prevented spore germination (Table 9). This sporicidal concentration of antibiotic 201A is found out to be twice that of the concentration of the test substance which results in complete inhibition of spore germination.

Dermatophytes are among the common infectious agents of humans and are cosmopolitan in distribution (Lagendre and Stetlz, 1980).

Since the introduction of griseofulvin for the treatment of dermatomycosis, there has not been many safe and effective drugs for the treatment of the disease (Welsh and Rodriguez, 1980). Thus in the present study, the efficacy of antibiotic 201A and 201B was compared with that of griseofulvin.

Antibiotic 201A and 201B are active in vitro against dermatophytes. The results in this comparative study demonstrated that antibiotic 201A appeared to be superior to 201B and griseofulvin (Table 10). The in vitro susceptibility data indicated that, the

MIC value for antibiotic 201A against dermatophytes tested was in the range of 20-40 mcg/ml. The comparative MIC value of griseofulvin against Trichophyton interdigital was similar to antibiotic 201A. However, griseofulvin was less active against Trichophyton rubrum and Trichophyton soudanense than antibiotic 201A. A possible reason for such variations in response could be due to the different level of susceptibility of the test dermatophytes to the test substances.

Inhibition of dermatophytes by antibiotic 201A at concentration of 20-40 mcg/ml is indicative of a definite levels of antidermatophyte activity and may reflect potential clinical usefulness of this agent in the treatment of dermatomycosis. Further more, due to the increasing number of patients that fail to respond to griseofulvin because either of resistance of dermatophytes to the drug or inadequate tissue levels of griseofulvin (Robertson et al., 1980) the in vitro and in vivo testings of the compound against many dermatophytes deserve further study.

In conclusion, the two antibiotics isolated from Ganoderma lucidum have been tested against only a few group of microorganisms. They could also be effective against many disease causing organisms (protozoa, helminths). Thus further study on this aspect is essential.

Systemic mycosis, currently requires amphotericin B, miconazole or flucytosine all of which have significant toxicity and have been a subject of recent reviews (Peterson et al, 1980). One of the major limitations of all polyene antifungal antibiotics

is their lytic effect on human red blood cell. As low as 5 mcg/ml of amphotericin B has been shown to cause lysis of human red blood cells (Kinsky et al., 1962). Our observation, however, indicated that no lytic effect was seen in sheep erythrocytes when treated with antibiotic 201A and 201B up to 100 mcg/ml (Table 11). This indicates that both antibiotics do not likely damage cell membrane of red blood cells.

Our observation also indicated that crude extract obtained from submerged culture of Ganoderma lucidum has not shown any marked dermal toxicity reactions when applied on shaved rabbit skin. Therefore, the two antifungal agents seem to be safe, though further work should reveal the potential uses of these antifungal agents for the treatment of fungal infectious of man and animals.

REFERENCES

Abbot P.H (1956). Mycetoma in the Sudan. Trans. Roy. Soc. Trop. Med. Hyg., 50: 11-24.

Adaskaveg J.E. and Gilbertson R.L. (1988). Basidiospores, Pilocystidia, and other basidiocarpcharacteristics of several species in Ganoderma lucidum complex. Mycologia, 80: 493-507.

Adaskaveg J.E. and Gilbertson R.L (1989). Cultural studies of four North American species of the Ganoderma lucidum complex with comparisons to Ganoderma lucidum and Ganoderma tsugae. Mycological Research, 92: 182-191.

Adaskaveg J.E. and Gilbertson R.L. (1987). Vegetative incompatibility between interspecific dikaryotic pairings of Ganoderma lucidum and Ganoderma tsugae. Mycologia, 79: 603-613.

Aharonowitz Y. (1980). Nitrogen metabolite regulation of antibiotic biosynthesis. Ann. Rev. Microbiol., 34: 209-233.

Anke H., Casser I., Steglich W., and Pommer E. H. (1987). Antibiotics from basidiomycetes 26. Phlebiakauranol aldehyde an antifungal and cytotoxic metabolite from Punctularia atropurpurascens. J. antibiotics, 40:443-449.

Anke T. (1985). Cytotoxic metabolites from basidiomycetes. In: Ebashi S. (ed.), Cellular Regulation and Malignant growth. Springer Verlag, Berlin, pp. 169-176.

Anke T., Hecht H. J., Schramm G., and Steglich W. (1979). Antibiotics from basidiomycetes IX. Oudemansin, an antifungal antibiotic from Oudemansiella mucida (Schader ex Fr.) Hoehnel (Agaricales). J. antibiotics, 32: 1112-1117.

Anke T. Besl H., Mocek U. and Steglich W. (1983). Antibiotics from basidiomycetes XVIII. Strobilurin C and oudemansin B, two new antifungal metabolites from Xerula species (Agaricales). J. Antibiotics, 36: 661-666.

Anke T. and Steglich W. (1981). Screening of basidiomycetes for new antibiotics. In M. Moo-Young, C. W. Robinson, C. Vezina (eds.), Advances in Biotechnology Vol 1. Pergamon Press, pp. 35-40.

Anke T. and Steglich W. (1989). Mtheoxyacrylate antibiotics: From biological activity to synthetic analogues. In: Biologically Active Molecules - Identification, characterization, and synthesis, U. P. Schunegger Ed., Springer Verlang.

Anke T. (1989). Basidiomycetes, a source for new bioactive metabolites. In: Progress in Industrial Microbiology, 27: 51-66.

Arisawa M., Fujita A., Saga M., Fukumura H., Hayashi T., Shimizu M. and Morita N. (1986). Three new lanostanoids from Ganoderma lucidum. J. Nat. Prod, 49: 621-625.

Atkinson N. (1946). Antibacterial activity in members of higher fungi. 1. Cortinarius rotundisporus and Psalliota xanthoderma. Aust. J. Exp. Biol. Med. Sci., 24: 169-173.

Beiske K., Lindtjorn B. and Olafsson J. (1982). Rhinosporidiosis affecting the palpebral conjunctiva : A case report. Ethiop. Med. J., 20: 135-139.

Bendle B.J., Mackey D., AL. Saati F., Sheth K.V., Ofola S.N. and Bailey T. M. (1987). Mycetoma in the Saudi Arabia. J. Trop. Med. Hyg., 90: 51-59.

Block E.R., Jennings A.E. and Bennett J.E. (1973). 5-fluorocytosine resistance in Cryptococcus neoformans. Antimicrob. Agents Chemother., 3: 649-656.

Borgers M. (1980). Mechanism of action of antifungal drugs, with special reference to the imidazole derivatives. Rev. Infec. Dis , , 2: 520-534.

Brass C., Galgiani J. N., Campbell S. C. and Stevens D. A. (1980). Therapy of disseminated or pulmonary coccidioidomycosis with ketoconazole. Rev. Infec. Dis., 2: 656-600.

Bu'lock. J. D (1961). Intermediary metabolism and antibiotic synthesis. Adv. appl. Microbiol., 3: 293-342.

B'ulock J.D., Hamilton D., Hulme M.A., Powell A.J., Smalley H.M., Shepherd D. and Smith G.N. (1965). Metabolic development and secondary biosynthesis in Penicillium urticae. Can. J. Microbiol., 11: 765-778.

Campbell C.K. and White G.C. (1989). Fungal infection in AIDS patients. The Mycologist, 3: 7-9.

Dawit A. (1989). Bioactive Metabolites from Fermentation Culture of Ethiopian Basidiomycetes. Ph. D. Thesis. University of Kaiserslautern, pp. 1-130.

Dawlately E.E., Anim J.T., Sowayan S. and EL-Hassan A-Y (1988). Primary paranasal aspergillus granuloma in Saudi Arabia. Tropical and Geographical Medicine, 40: 247-250.

Demain A.L. (1981). Applied Microbiology. A Personal view. In: Norris, J.R. and Richmond, M.H. (eds). Essays in Applied Microbiology. John Wiley and Sons Chichester. New York. Brisbane. Toronto, pp. 1-31.

Delveloux M., Audoin J., Treguer J., Vetter J.M., Warter A., and Cenac A. (1988). Mycetoma in the Republic of Niger. Clinical features and epidemiology. Am. J. Trop. Med. Hyg., 38: 386-390.

Diamond R. D. and Bennett J.E. (1974). Prognostic factors in Cryptococcal Meningitis. Annals. Internal. Medicine, 80: 176-181.

Dismukes W.E. (1988). Cryptococcal meningitis in patients with AIDS. J. Infect. Dis., 157: 624-625.

Drew S. W. and Demain A. L. (1977). Effect of primary metabolism on secondary metabolism. Ann. Rev. Microbiol., 31: 343-356.

Edwards V.E., Sutherland J.M. and Tyrer J.H (1970). Cryptoccosis of the central nervous system: Epidemiological, Clinical and Therapeutic features. J. Neurology, Neurosurgery, and Psychiatry, 33: 415-425.

Endo A., Kakiki. and Misato T. (1970). Mechanism of action of the antifungal agent polyoxin D. J. Bacteriol., 104: 189-196.

Evans E. G. V. and Gentles J.C. (1985). Essentials of Medical Mycology. Churchill Livingstone, Edenburgh, London, Melbowne and NewYork pp. 1-184.

Evans G., and White N. H. (1967). Effect of the antibiotics radicicolin and griseofulvin on the fine structure of fungi. J. Exp. Bot., 18: 465-470.

Fujita A., Arisawa M., Saga M., Hayashi J., and Morita T. (1986). Two new lanostanoids from Ganoderma lucidum. J. Nat. Prod., 49: 1122-1125.

Geaney B., Horsfall W. R., and Neilson G. (1956). Torulopsis in Queensland: report of fourteen cases. Med. J. Aust., 2: 378-382.

Gould P. R. J.S., and Gould I.M. (1985). Cryptococcosis in Zimbabwe. Trans. Roy. Soc. Trop. Med. Hyg., 79: 67-69.

Graybill J.P. and Drutz D.J. (1980). Ketoconazol: A major innovation for treatment of fungal disease. Annals. Internal. Medicine, 93: 921-923.

Gurusiddaiah S., Winward L.D. Burger D. and Graham S.D. (1979). Pantomycin: A new antimicrobial antibiotic. Mycologia, 71: 103-118.

Hamilton-Miller J. M. T. (1973). Chemistry and biology of the polyene macrolide antibiotics. Bacteriol. Rev., 37: 166-196.

Hervey A. H (1947). A survey of 500 basidiomycetes for antibacterial activity. Bull. Torrey Bot. Club, 74: 476-503.

Hesseltine G. W., Vandergaff E.E., Fennel D. I., Smith M.L. and Shotwell O.L. (1972). Aspergilli as ochratoxin Producer. Mycologia, 64: 538-549.

Hirotani M. and Furuya T. (1986). A ganoderic acid derivatives, highly oxygenated lanostane-type triterpenoids, from Ganoderma lucidum. Phytochemistry, 25: 1189-1193.

Hirotani M., Furuya T., and Shiro M. (1985). A ganodermic acid derivatives, a highly oxygenated lanostane-type triterpenoids from Ganoderma lucidum. Phytochemistry, 24: 2055-2061.

Hockenhull D. J. D. (1981). Production of antibiotics by fermentation. In: Norris, J. R. and Richmond M. H. (eds). Essays in Applied Microbiology. John Wiley and Sons, Chichester. New York. Brisbane. Toronto, pp. 4/1 - 4/31.

Hogenauer G. (1979). Tiamulin and Pleuromutilin. In: Hahn F. E. (ed.) Antibiotics, vol.v-1, Mechanism of Action of Antibacterial Agents. Springer Verlag, Berlin. Heidelberg. New York, pp. 344-360.

Jones E. B.G. and Lim. G. (1990). Edible mushrooms in Singapore and other Southeast Asian countries. The Mycologist, 4: 119-124.

Kavanagh F., Hervey A. and Robbins W.J. (1951). Antibiotic substances from basidiomycetes VIII.Pleurotus multilus (Fr.) Sacc. and Pleurotus passeckerianus pilat. Proc. Natl. Acad. Sci. U.S.A., 37: 570-574.

Keyhani J., Keyhani E. and Goodgal S.H. (1972). Studies on the cytochrome content of Phycomyces spores during germination. Eur. J. Biochem., 27: 527-534.

Kikuchi T., Matsuda S., Kadota S., Murai Y. and Ogita Z., (1985). Ganodermic acid D, E, F and H and lucidenic acid D, E and F, new triterpenes from Ganoderma lucidum. Chemical Abstract, 103: 175028.

Kikuchi T., Matsuda S., Murai Y. and Ogita Z. (1985). Ganoderic acid G and I and ganolucidic acid A and B, new triterpener from Ganoderma luccidum, Chemical Abstract, 103: 156942.

Kohda. H., Tokumota W., Sakamoto K. Fujii M., Hirani Y., Yamasakik., Komoda Y., Nakamura H., Ishihara S. and Uchida M. (1985). The biologically active constituents of Ganoderma lucidum. Histamine release-inhibitory triterpenes. Chemical Abstracts, 103: 47945.

Kinsky S.C., Avruch J., Permutt M., Rogers H.B. and Schonder A.A. (1962). The lytic effect of polyene antifungal antibiotics on mammalian erythrocytes. Biochem, Biophys. Res. Commun., 9: 503-507.

Legendre R. and Steltz M. (1980). A multicenter, doubleblind comparison of ketoconazole and griseofulvin in the treatment of infections due to dermatophytes. Rev. Infect. Dis., 2: 585-591.

Lester F. T., Gedebou M. and Akalu T. (1977). Crypotoccal meningitis in Ethiopia. Ethiop. Med. J., 20: 230.

Lin L.J., Shiao M.-S., and Yeh S. -F. (1988). Triterpenes from Ganoderma lucidum. Phytochemistry, 27: 2296-2271.

Malawista S.E., Sato H., Bensch, K.G. (1968). Vinblastine and griseofulvin reversibly disrupt the living mitotic spindle. Science, 160: 770-772.

Martin J. F. and Demain A. L. (1980). Control of antibiotic biosynthesis. Microbiol. Reviews, 44: 230-251.

Mathieson J. (1946). Antibiotics from Victorian basidiomycetes. Austral. J. Exp. Biol. Med. Sci., 24: 57-62.

Musilek V., Cerna J., Sasek V., Smerdzieva M. and Vondracek M. (1969). Antifungal antibiotic of the basidiomycete Oudemansiella mucida I. Isolation and cultivation of a producing strain. Folia Microbiol., 14: 377-387.

Musilek V., Volc J., Nerud F. and Sasek V. (1984). Present trends in the research of lignivorous basidiomycetes cultures. 3rd Symposium of Socialist Countries on Biotechnology, Bratislava pp. 603-609.

Nakajima S., Kawai K., Yamada S. and Sawai Y., (1976). Isolation of Oospolactone as an antifungal principle of Gloeophyllum sepiarium. Agr. Biol. Chem., 40: 811-812.

Nishitoba T., Sato H., and Sakamura S. (1987). Triterpenoids from the fungus Ganoderma lucidum. Phytochemistry, 26: 1777-1784.

Petersen E. A., Alling D.W., and Kirkpatrick C. M. (1980). Treatment of mucocutaneous candidosis with ketoconazole. Preliminary results of a controlled, double-blind clinical trial. Annals. Internal Medicine, 93: 791-795.

Poster J. N. (1976). Cultural condition for antibiotic-producing Microorganisms. In: Hash, J. H (ed.) Methods in Enzymology vol X1III (antibiotics). Academic press. New York San Francisco London.

Richardson P.M., Mohandas A., and Arumugasmy N. (1976). Cerebral Cryptococcosis in Malasia. J. Neurology, Neurosyrgery, and Psychiatry, 39: 330-337.

Robbins W. J., Hervey A., Davidson Ross W., Roberta M. and Robbins W.C. (1945). A survey of some wood destroying and other fungi for antibacterial activity. Bull. Torrey Club, 72: 165-190.

Robertson. M.H., Hanifin J. M. and Parker F. (1980). Oral therapy with ketoconazole for dermatophyte infections unresponsive to griseofulvin. Rev. Infect. Dis., 2: 578-811.

Roberts W.B.O. (1982). Rhinosporidiosis in the nasal membranes: A case report. Ethiop. Med J., 20: 141-143.

Russel A.D., (1980). Types of antibiotics and synthetic antimicrobial agents. In: Hung W B and Russel A.D (eds). Pharmaceutical Microbiology. Backwell Scientific Publications (2nd ed), Oxford London Edinburgh Melbourne.

Ryvarden L. and Johansen I. (1980). A preliminary polypore Flora of East Africa. Fungiflora, Oslo, pp. 84-92.

Sarosi G.A., Parker J.D. and Doto I.L. (1969). Amphotericin B in cryptococcal meningitis. Annals. Internal Medicine, 71: 1079-1087.

Schmidt E.L. (187). Influence of polyoxin D on basidiospore germination of two wood decaying fungi. Mycologia, 79: 627-630.

Shadomy T. (1969). In Vitro studies with 5-fluorocytosine. Appl. Microbiol., 17: 871-877.

Shiao M.-S. and Lin L. - J. (1987). Two new triterpenes of the fungus Ganoderma lucidum. J. Nat Prod., 50: 886-890.

Shiao M.-S., Lin L. -J and Yeh S. - F. (1988). Triterpenes from Ganoderma lucidum. Phytochemistry, 27: 873-875.

Subick J., Behun and Musilek (1974). Mode of action of a new antifungal antibiotic produced by the basidiomycetes Oudemansiella mucida. Biochem. Bioph. Acta., 343: 363-373.

Symoens J., Moens M., Dom J., Schejgrond H., Dony J., Schuermans V., Legendre R., and Finestine N. (1980). An evaluation of two years of clinical experience with ketoconazole. Rev. Infect Dis., 2: 674-687.

Toth J.O., Luu B., Beck J.P., and Ourisson G., (1983). Les acides Ganoderiques T a Z triterpenes cytotoxiquen DE. Ganoderma lucidum (polypore). Tetrahedron letters (abstract), 24:1081-1084.

Tseng T.-C., Shiao M.-S., Shieh Y.-S., and Hao Y.-Y. (1984). Studies on Ganoderma lucidum: Liquid culture and chemical composition. Bot. Bull. Academia. Simica, 25: 149-157.

Turner W.B. (1971). Fungal metabolites. Academic press, London, New York, pp. 11-23.

Utz J.P. (1980). Chemotherapy for systemic mycosis. The prelude to ketoconazole. Rev. Infect Dis., 2: 625-632.

Van Assche J.A., and Carlier A.R. (1973). The pattern of protein and nucleic acid synthesis in germinating spores of Phycomyces blakesleeanus. Arch. Mikrobiol., 93: 129-136.

Van Laere A.J., Carlier A.R and Van Asche J.A (1976). Effect of 5-fluorouracil and cycloheximide on the early development of Phycomyces blakesleeanus spores and the activity of N-glucoseamine synthesizing enzymes. Arch. Microbiol., 108: 113-116.

Veress B. Malik OA., EL. Tayeb AA., Daoud EL-S., and EL- Hassen AM. (1973). Further observations on the preliminary paranasal aspergillus granuloma in the Sudan, a morphological study on 46 Cases. Am. J. Trop Med. Hyg., 22: 765-772.

Weinberg E.C. (1970). Biosynthesis of secondary metabolites role of trace metals. Adv. Microbiol. Physiol., 4: 1-44.

Welsh O. and Rodriguez M. (1980). Treatment of dermatomycosis in ketoconazole. Rev. Infect Dis., 2: 582-585.

Williams D.I. (1960). Griseofulvin and Trichophyton rubrum infections. Arch. Dermatol., 81: 796-771.