ANTIFUNGAL METABOLITES FROM SUBMERGED CUL TORE OF GANODERMA LUCIDUM (POL YPORE)

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ABSTRACT: About 60 different basidiomycete cultures were screened for antimicrobial secondary metabolites. Among basidiomycetes screened for antimicrobial activity, the culture filtrate extract of the polypore, G. lucidum produced the most effective antifungal compounds. Growth in submerged culture of the polypore and isolation methods of the two antifungal antibiotics are described. These compounds were eleased to the culture fluid and the maximum amount of antifungal compounds was obtained after 12 days of
submerged growth at 120 revolution per minute (rpm). The culture filtrate were characterized biologically. These metabolites had a wide spectrum of antifungal activity and affected 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/m1 and 1-5 mcg/m1 respectively. Inhibition diameter zone of 36 mm was produced when 10 mcg/disc of 201A was applied on agar medium seeded with Aspergillus flavus .Bacteria were affected only at high concentration. Ethiop.J. Health Dev. 1994;8(1):63-70]

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 infection. Though some important antifungal drugs of microbial and synthetic origin have also been discovered for the treatment of mycotic infection, they fail to achieve the desired results. Toxicity to the host [1], narrow spectrum of activity [2, 3], difficulty of drug administration [1], development of drug resistance [4, 5] and recovery of fungi after treatment [1, 6] are the major limitations of the existing antifungal agents.

Drawbacks of the presently available antifungal agents and a clear understanding of the imponance of diseases of fungal origin panicularly their association with AIDS patients [7, 8] in the last few decades have initiated the interests of many academic institutions and pharmaceutical industries for a search for new, better, less toxic and cheaper antifungal agents.

It has been pointed out that chemical synthesis and a search for natural products from living organisms (higher plants and microorganisms) are the two sources for new biologically active compounds [9].

Basidiomycetes panicularly the tropical ones, have been little investigated from the view- point of bioactive secondary metabolites and thus more intensive screening of tropical basidiomycetes for new biologically active .metabolites was suggested [10]. In this paper the growth of G. lucidum in submerged culture, isolation and biological characterization of the two antifungal antibiotics tentatively named 201A and 201B are discussed.

METHODS

Submerged Cultures and Isolation of Antifungal Metabolites from G. lucidum Equal numbers of about the same sized agar culture blocks of G. lucidum were transferred aseptically to 50 mI of

4 9 yeast extract (Oxoid, Basingstoke, Hampshire, England) 10 9 malt extract (Oxoid) and 4 9 glucose (BDH, Poole Dorset, England) medium in 250 mI Erylenemer flasks. Culture flasks were then incubated at. 20-22°C on a rotary shaker set at 120 rpm. After 6 days of growth, seed cultures (inoculum) were inoculated to 400 mI of the _____

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same medium. All flasks were then incubated in the same condition as described above. Growth (mycelial dry weight), pH-value and antibiotic production were determined by taking aliquot of culture medium every other day. pH-values were determined by pH meter (Corning pH-meter mode 140), the dry weight was weighed 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 (Merk, Darmstadt) after it was dried over anhydrous Na2S04 (BDH). The chloroform extract was concentrated under reduced pressure and applied on a column of silica gel 60 (0.6 to 0.02 mm size; Merk) as a thin layer on top of the column, after the material was dried with silica gel and eluted with increasing percentage of methanol (Merk) in chloroform.

Biological Activity:

Effects of 20lA and 20lB Agaimt yeasts and Bacteria;

The minimum inhibitory concentration (MIC), against bacteria and yeasts was determined as described by Anke et. al [11j. In brief, test organisms (2-6 x 106/ml) were inoculated to test tubes containing different concentrations of the test substances and incubated at an appropriate temperature for 24 hours. The MIC was recorded as the lowest concentration of the test substances completely preventsing growth (visible turbidity) as determined by the naked eye after 24. hours of incubation.

Antifungal Activity Against Mycelial Fungi. The activity of antibiotic 201A and antibiotic 201B against mycelial fungi was determined by the agar diffusion assay as described by Dawit [10]. In brief, heavy spore
suspension (2-6 x 106 spores/ml) or macerated young mycelia (5 mg/ml) of each test fungus was suspended in Sabouruad dextrose agar (Oxoid) medium prior to pouring into sterile glass plates. Different concentration of test substances were applied to antibiotic assay discs (6 mm in diameter; Schleicher & Schuell, Dassel) and placed on test plates to which spores or macerated young mycelia of test fungi were seeded. Inhibition zone diameter was recorded after 24-48 hours of incubation at appropriate temperatures.

RESULTS

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

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. I, 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 61h to the IQ1h days of growth and showed no appreciable increase or decrease up to s the 141h day of growth. The antibiotic production started from e the 61h day of growth and reached its maximum It on the 121h day of growth. After 14 days of d growth, a decrease in antibiotic production was 4 observed.

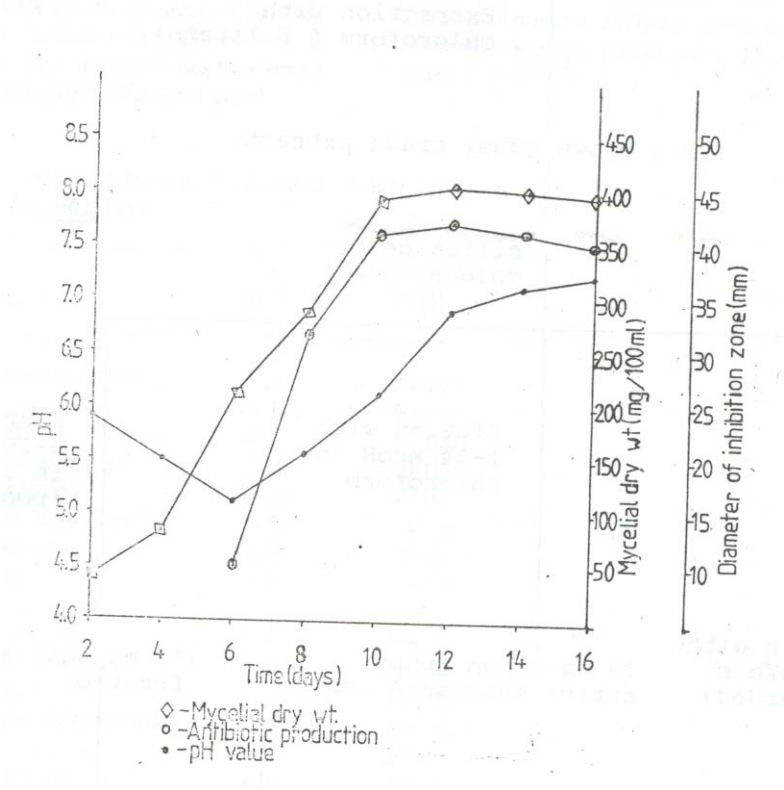

Figure 1 Submerged Culture of Granoderma lucidum

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. Three fig of 201A and 5 fig of 2018 were
obtained from a liter of culture filtrate.

Figure 2. Purification of antibiotic 201A and 201B

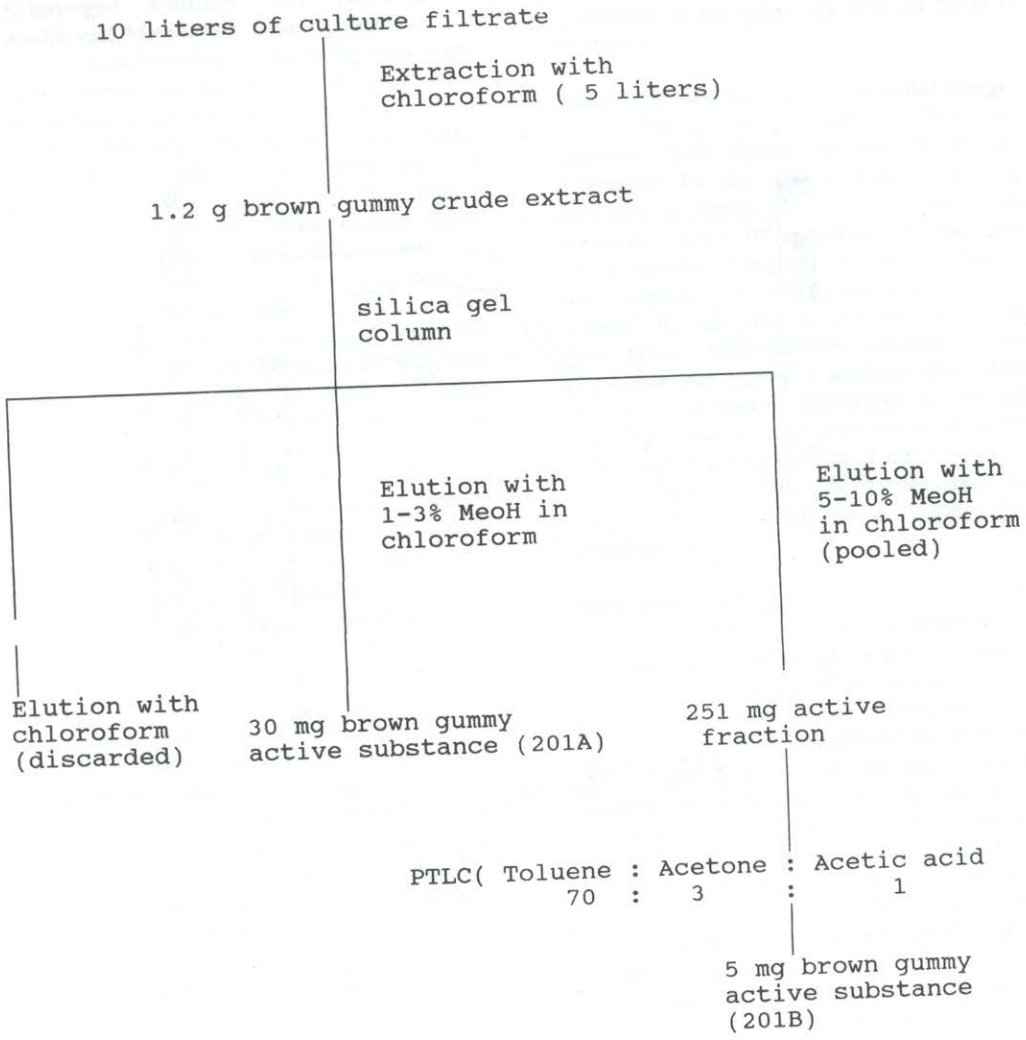

Fig. 2 Purification of antibiotic 201A and 201B

The approximate place of Fig 2 is immediately above biological activity of 201 A and 201B on page 6.

Biological Activity of Antibiotic 20lA and 20lB. Effect Against Bacteria and Yeasts.

The minimum inhibitory concentrations of 20lA and 2018 against an array of bacteria and yeasts are presented in Table 1. As is evident from this data, the antibiotics have broad spectrum and were effective against gram -positive bacteria and yeasts. The antibiotics were relatively ineffective against gram-negative
bacteria. Antibiotic 20lA was particularly effective against the pathogenic yeast, Candida lbicans .Antibiotic 20lB was active at a higher concentration when compared to 20lA against yeasts and bacteria.

Table 1. **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. subtilis	10-20	50-100
Streptococcus faecalis	10-20	50-100
Escherichia coli	10-20	50-100
Aerobacter aerogenes	>50	>100
Salmonella 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
Sacchromyces cerevisiae	5-10	50-100
Rhodotrula sP.	1-5	20-50
Torulopsis cremoris	5-10	50-100
Hansenia spora	5-10	50-100

The results of ;he activity of antibiotic 20lA and 20lB against a variety of mycelial fungi is shown in Table 2. All the fungi tested were susceptible to the antibiotics. Species of Aspergillus were more sensitive 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 2. **Antifungal Spectrum of Antibiotic 201A and 201B (agar diffusion assay)**

Test Mycelial Fungi	Diameter Inhibition Zone (mm) mcg/dsc						
	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
Trichodema 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 inhibitation - = not tested

DISCUSSION

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

In submerged culture of G. 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. I). In batch cultures high levels of antibiotics are usually produced only after most of the cellular growth has already occurred [12]. Depletion of one or more growth limiting substrates has been reported to arrest growth and initiate antibiotic synthesis [13]. It is also clear that the production of antibiotics was almost linear between 10 to 14 days of growth, followed by a steady decline in antibiiotic production. According to Martin and Demain[14], 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 time could result in from production of organic acids. These are produced as a result of breakdown of carbohydrates [14, 15]. 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 synthesis had been described [15]. 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 [10]. This could also be a possible explanation for a decrease in antibiotic production in submerged cultures of G. lucidum in the later stages of fermentation.

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 [16]. 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 [14, 17, 18]. According to Demain [18] penicillin, cycloheximide, cephalosporin and chloramphenicol exert a feedback 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 the production of antibiotics [14].

In this work, the two antifungal antibiotics (antibiotic 201A and antibiotic 201B), isolated from submerged culture of G. lucidum, have been biologically characterized. Due to their effectiveness against disease-causing fungi (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 infections is far from satisfactory .

Polyene antifungal aritibiotics (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 [19].
Narrow spectrum of activity is a. major limitation of the antifungal drugs, flucytosine and griseofulvin. Activity of flucytosine appeared to be limited to yeastlike fungi [2], while griseofulvin has been shown to posses fungistatic activity only against dermatophytes[20] .

With respect to a range of activities, both antibiotic 201A and 201B have a broad spectrum of activity against an array of bacteria, yeasts and mycelial fungi (Table 1 and 2). The in vitro MIC of antibio)ic 201A and 201B against bacteria and yeast show variability. Among the bacteria, gram-positive bacteria were 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, Rho4otrula sp. and molds belonging to the genera of Aspergillus, Rhizopus, Mucor, Penicillium, Fusarium, Cladosporium and Colletothricum. The in vitro susceptibility data indicated that the potency of antibiotic 201A against bacteria and yeasts was much better than that of antibiotic 201B (Table 1). The MIC value of antibiotic 201A against C. albicans, the most frequent cause of superficial, mucocutaneous and systemic mycosis [1] 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 [19] and that ofketoconazole which is in the range of 0.3 to 2.5 mcg/ml [21]. 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 2) showed that the potency of antibiotic 201A was strong against Aspergillus jlavus and Aspergillus ochraceus .These molds are among the many fungi capable of producing aflatoxins and ochratoxin, respectively [22]. Aspergillus umigatus. an opportunistic mold, implicated as a cause of systemic mycosis [1] was also sensitive to the two antifungal agents. Members of the genus Aspergillus are found to be resistant organisms to both amphotericin B and flucytosine [23]. Colletothricum coffeanum, which is a known coffee pathogen, was also sensitive to both antibiotics. The activity of the two antifungal antibiotics
against disease causing protozoa and helminths. their mode of action, toxicity and the elucidaion of their chemical structure are under investigation.

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