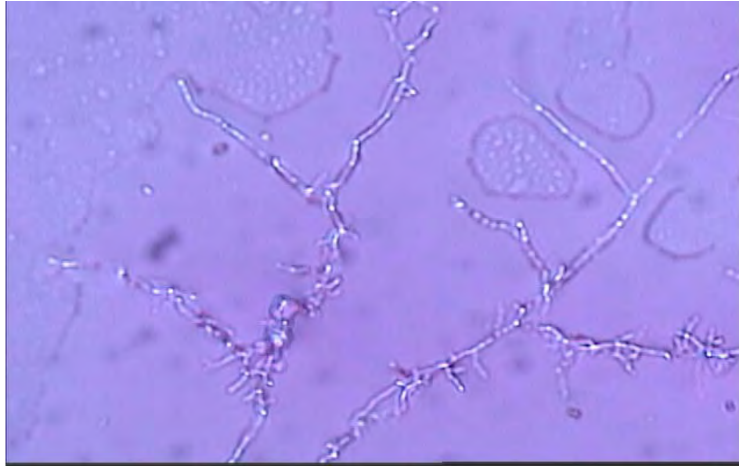
ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES



ISOLATION OF BIOACTIVE COMPOUNDS FROM A FOREST LITTER DECOMPOSING MICROFUNGUS

A Thesis submitted to the school of Graduate studies, Addis Ababa University in partial fulfillment for the requirement of the Degree of Masters OF Science in Biology

DAWIT TSEGAW

Aug, 2009

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Isolation of bioactive compounds from a forest litter decomposing microfungus

By

Dawit Tsegaw

A Thesis submitted to the school of Graduate studies, Addis Ababa University in partial fulfillment for the requirement of the Degree of Masters OF Science in Biology

Approved by Examining Board:

Dr. Amare Gessesse (Examiner) ____________________

Dr. Fassil Asefaw (Examiner) ____________________

Dr. Dawit Abate (Adivisor) ___________________.

ACKNOWLEGEMENT

Dr. Dawit was supervising the present work. 1 would like to thank him particularly for giving me the passion of mycology first as an excellent teacher and then by his own enthusiasm in applied mycology. I realize that even if I still have a long way to go in the domain of the research, he has already given me some good keys to arrive safely at the end, in addition , he supplied me with a relevant books, papers and materials.

Financial support from Addis Ababa University is gratefully acknowledged. The contribution of Zenbech Aytenew of Mycology Laboratory is highly appreciated. My thanks also go to all my colleagues and my friends with in the Mycology Laboratory.

I am highly grateful to my family especially my dear Mum and sisters.

I would deeply thank GOD who kept me doing all work according to his plan and will.

TABLE OF CONTENTS

ACKNOWLEGEMENT……………………………………………………………………i

TABLE OF CONTENTS……………………………………………………………...ii

LIST OF FIGURE……………………………………………………………………iv

LIST OF TABLE……………………………………………………………………..v

LIST OF ABBREVIATION…………………………………………………………vi

ABSTRACT…………………………………………………………………………..vii

1. INTRODUCTION………………………………………………………………… 1

2. LITRETUR REVIVEW ……………………………………………….............................4

2.1. Litter-decomposing microfungi ………………………………………………….4

2.2. Taxonomic and biotic status……………………………………………………5

2.3. Leaf litter diversity……………………………………………………………5

2.4. Isolation and Identification of Microfungi………………………………………..7

2.5. Solid state (or substrate) fermentation (SSF)…………………………………..7

2.6. Isolation of bioactive compounds…………………………………………………9

2..6.1. Anti-bacterial assay……………………………………………………10

2.6.2. Anti-fungal assay……………………………………………………..10

2.7. Minimum inhibitory concentration (MIC)…………………………………...10

3. OBJECTIVE OF THE STUDY………………………………………………………..12

4.MATERIALS AND METHOD……………………………………………………..13

4.1. Isolation and culture condition…………………………………………………13

4.1.1. Sample Collection…………………………………………………………13

4.1.2. Sample treatment…………………………………………………………13

4.1.3. Isolation and maintenance of pure culture………………………………...13

4.1.4. Identification of Fungus…………………………………………………..13

4.2. Solid-state cultivation……………………………………………………………..14

4.2.1. Condition for solid state fermentation…………………………………………14

4.2.2. Preparation of rice grain for solid state cultivation………………………………......15
4.3. Extraction and testing crude extracts……………………………………………………...15
4.3.1. Extraction of rice grain culture (SSF)...15
4.3.2. Antimicrobial activity assay…………………………………………………………16
4.3.2.1. Test Strains…………………………………………………………………………16
4.4. Determination and isolation of the biologically active compounds………………………..17
4.4.1. Preparative Thin Layer Chromatography (TLCP)……………………………………17
4.4.1.1. Preparation of Thin Layer Chromatography Plates (PTLCP)…………………17
4.4.2. Thin layer chromatography (TLC)……………………………………………………18
4.4.3. Column chromatography (CC)…………………………………………………………19
4.4.4. Antimicrobial Susceptibility Testing on bioactive compounds………………………19
5. Results...20
5.1. Isolated fungus……………………………………………………………………..20
5.2. Induction of bioactive metabolite production by Solid-state Fermentation on rice………21
5.3. Extraction……………………………………………………………………………………22
5.4. Antimicrobial activity……………………………………………………………………..23
5.5. TLC analysis……………………………………………………………………………..26
5.6. Preparative thin layer chromatography…………………………………………………26
5.7. Column chromatography………………………………………………………………..28
5.9. Antimicrobial Susceptibility Test on bioactive compound…………………………...29
6. Discussion…………………………………………………………………………………30
7. Conclusion……………………………………………………………………………32
8. Recommendation………………………………………………………………………..33
9. REFERENCES…………………………………………………………………………34
10.Appendices..39

LIST OF TABLES

Table 1. Solvent mixtures used in column chromatography……………………………19

Table 2. The mass (g) of crude extracts from Jp4 serially extracted with two extractants from 500g of fermented rice…………………..24

Table 3. Showing test organism (cfu/ml) used during the bioassay……………………..29

Table 4. Antimicrobial activity of fungal metabolite against various microorganisms……..31

Table 5. Showing data obtained from fractions of PTLC during antimicrobial testing...33

Table 6. Physical characteristics of the active compounds by LDFs, Jp4………………36

Table 7. Antimicrobial activity of Jp4 against different test organisms…………………….37

LIST OF FIGURES

Figure 1. Slide culture technique for the fungus Jp4……………………………………14

Figure 2. PTLC for the crude extract using SS Chloroform:Ethyl Acetate (1:1)………..18

Figure 3. Purification of the bioactive compound using CC……………………………..20

Figure 4. Morphological characteristics of culture Jp4 grown on PDA & MEA………...22

Figure 5. Fungus, Jp4 on phase contrast microscope 100x……………………………….23

Figure 6. Solid state cultivation of LDFs Jp4 on rice grain for secondary metabolite Production…………………………………………………………………….24

Figure 7. Growth curve of the test bacterial pathogens…………………………………28

Figure 8. Inhibition of the crude extract by disk diffusion assay………………………..30

Figure 9. TLC of the crude extracts of Jp4 at 254nm and 336nm……………………….32

Figure 10. Showing preparative thin layer chromatography under 366nm for the crude extract using solvent system Chloroform:Ethylacetate (1:1 v/v)………………..32

Figure 11. PTLCP &TLC on UV at 245nm…………………………………………………….34

Figure 12. Isolation of bioactive compound (yellow) using CC……………………………..34

Figure 13. Antimicrobial activity of the pure compound…………………………………….35

LIST OF ABBREVIATION

A_w	Water activity
CC	Column chromatography
CC	Colony count
CFU	Colony forming unit
DF	Dilution factor
EtOAc	Ethyl Acetate
GM+	Gram positive bacteria
GM-	Gram negative bacteria
LDMF's	Litter- decomposing microfungi
MEA	Malt extract agar
MIC	Minimum inhibitory concentration
MID	Minimum inhibitory dose
NB	Nutrient broth
OD	Optical density
PDA	Potato dextrose agar
PTLC	Preparative thin layer chromatography
PTLCP	Preparative thin layer chromatography plate
R_f	Retention Factor
SmF	Submerged fermentation
SSF	Solid-state fermentation
SS	Solvent system
T^o	Temperature
TDR	Transfer dilution rate
TLC	Thin layer chromatography

ABSTRACT

In a screening of fungi isolates associated with litter decomposition (litotrophic) for bioactive metabolite production, $LDMF_S$ JP_4 was isolated. It was identified to genus level using morphological (cultural) and microscopic characterization to belong to *Geotrichum* sp. A bioactive metabolite effective against bacteria and fungi, was isolated from this fungus at stationary phase of mycelium culture growing in solid substrate, rice. Through, bioassay guided fractionation of the crude extract using chromatographic methods, the active compound was isolated in pureform. The compound was found to exhibit activity. It was effective against gram positive (*S. pnumoneae, S. aureus and B. subtilis*) and gram negative bacteria (*P. aeruginosa, E. coli and S. boydii*) and the yeast, *Candidia albicans*. The active compound exhibited potent *in-vitro* antibacterial with a minimal inhibitory concentration (MIC) of 65-75µg/uL against Gram-positive bacteria and 75-80ug/uL against Gram-negative. It was less effective for the yeast, *Candidia albicans*. The pure compound is promising and further work on chemical characterization is recommended.

KEY WORD, LDFs, taxonomic studies, solid substrate, bioactive metabolite, fractionation, minimal inhibitory concentration

1. INTRODUCTION

Natural products from microbial or plant origin have played and still play an invaluable role in drug discovery, which accounts for more than 30% of worldwide human pharmaceutical sales (Rios *et al*., 1998), and constitute about 50% of the approved drugs over the period 1981-2002 (Anke, 1989). Fungi have proved to be capable of biosynthesizing secondary metabolites, out of the estimated 1.5 million species of fungi recorded worldwide (Hawksworth, 1991), approximately 4,000 secondary metabolites of fungal origin are known to possess biological activities (Dreyfuss and Chapela, 1994), the vast majority coming from the species of *Penicillium, Aspergillus, Acremonium* and *Fusarium.* Some of the important products of the pharmaceutical industries include Penicillin (*Penicillium notatum*), antibacterial agents Cephalosporin (*Cephalosporium cryptosporium*), cholesterol-lowering agents Mevastatin and Pravastatin (*Penicillium* species) (Gordon and David, 2005).

It is established that the wide spread use of antibiotics leads to the appeareance of resistant strains of pathogenic microorganism. Consequently, the existence of almost all known pathogens resistant to one or more of the commonly used antibiotics is well known. This necessitate the continuous search for new bioactive compounds.

Microfungi are defined as fungi with microscopic spore producing structure (Haeksworth *et al.,* 1995). They comprise the groups Ascomycota, Deutromycota, microscopic Basidiomycota and Zygomycota. The microfungi together with bacteria, other fungi and lower animal participate in the decomposition of leaf litter to CO_2 and humus (Bills and Polishook, 1994).

Microfungi grow on plant debris in every conceivable habitat, including those subjected to the extremes of climate that characterize the arctic and Antarctic and deserts and rain forests, as well as the milder subtropics and temperate environments.

The minute nature of microfungi makes their direct observation in the field difficult; consequently, collecting focuses on adequate sampling of entire substrates and niches rather than on the collection of individual species. Most microfungi are relatively long lived, but sporulate (and thus are directly identifiable) only for short periods. They may produce melanized fruiting and vegetative structures (chlamydospores, sclerotia, setae, etc) for dispersal or survival of adverse condition.

Over the past years, scientists have collected identified characterized fungi litter. Different study showed that a large variety of fungi exist on leaf litter, of which many new fungal taxa have been described (Hughes, 1989). It is also revealed that several genies of microfungi can produce bioactive compounds potentially useful in the pharmaceutical industry and agriculture (Manoch *et al.*, 2006).

Litter decomposing microfungus (LDMF's) are relatively easy to isolate and cultivate in the laboratory. Their spores can be isolated from substrate like decomposed forest litter and dead woods (Hughes, 1989). For isolation techniques the moist chamber, direct isolation and dilution plate methods were used. Fungi were cultivated on malt extract agar and potato dextrose agar and identification was based on morphological study examined under stereoscopic (Olympus SZ-PZ), light (Olympus BH) and scanning electron microscopes (Manoch *et al.*, 2006).

It is argued that extreme and peripheral ecosystem are of strategic importance in the discovery of new bioactive microbial secondary metabolites. Therefore, it is worthwhile isolating microorganism of a different ecosystem to find new compounds. In the present study, the source of the fungi screened for bioactive compounds was Menagesha forest, one of the oldest forest in Ethiopia dominated by the tree, *Juniperus procera* and other indeginous trees.

This work was also carried out to investigate secondary metabolite production by litter decomposing microfungus (LDMF's) cultivated on solid substrate rice, using SSF (Solid-state fermentation).

The use of SSF has been investigated for its production of higher yields. Similar results were also obtained in the screening of secondary metabolites by SSF (Wei pei-lian *et al.,*2006) when grown on solid substrates, numerous fungi have shown a markedly altered spectrum of secondary metabolites. Various secondary metabolites have even been shown to be produced solely during growth on solid substrates (Johns 1992; Nigam and Sing 1994). For this reason, SSF could play an interesting role in the screening of new metabolites and also in the production of these metabolites by wild-type fungi.

2. LITERATURE REVIEW

2.1. Litter-decomposing microfungi

Litter decomposing microfungi (LDMF's) belong to the Division Basidiomycota, Ascomycota, Zygomycota and Deuteromycota that participate in the decomposition of leaf litter to CO_2, and humus (Dix and Webster, 1995). The single criterion for inclusion in the group is the small size of the spore-producing structures (Hawksworth *et al*., 1995). Microfungi inhabit the ground litter layer of soil and the humus layer of forest and grasslands. A large variety of them are common on leaf litter in many studies, while many new fungal taxa have been described from decaying leaves and dead wood (Hughes, 1989).

Knowledge on the species diversity, biogeography and ecology is very limited, except the more well-known and widespread plant pathogens (Bills and Polishook, 1994). Furthermore, their small size and morphologically distinct features of their life cycle make their direct studies in the field difficult. But, recently many studies have been carried out, with regard to their ability to produce bioactive compounds that are very useful in pharmaceutical and agriculture (Bills and Polishook 1994; Saravanan, 2004; Tokumasu *et al*., 1997).

In Thailand, Rukachisirikul *et al*. (2005) found that *Beltrania rhombic* inhabiting on leaf litter produce secondary metabolites which inhibit *Staphylococcus aureus* and *Candida albicans*. Another study also showed that Arthrichitin from *Arthrinium phaeospermum* could decompose chitin, that was used to develop to fungicide and insecticide (Vijayakumar *et al*., 1996).

Microfungi overcome their environments constraints to colonize extreme habitats, by normally exploiting their defined microhabitats. This, together with their huge diversity makes them an exciting field of study in the search for new medicines against the back drop of the development of drug resistance by different microorganism causing diseases.

2.2. Taxonomic and biotic status

Although the knowledge of fungi, especially on the economically important groups (mainly plant pathogens) is extensive, the search and the number of collections of microfungal species is inadequate (Pirozynski and Weresub, 1979).

The number of species of microfungi isolated from dead plants is difficult to estimate, for lack of databases linked with correct synonyms (Cannon, 1991). However, different authors gave a conservative estimate of 72,000 described species, (Hawksworth *et al.,* 1995) and 100,000 species (Rossman, 1994). According to Hawksworth *et al.* (1995), from a total of 32,250 species of Ascomycota, 13,000 species (45%) are likely to be microfungi found on dead plant parts.

2.3. Leaf litter diversity of microfungi

The diversity of leaf litter fungi from various host plants is well known (Bills and Polishook, 1994; Saravanan, 2004; Tokumasu *et al*., 1997). Many new fungal taxa have been described from decaying leaves and dead wood (Hughes, 1989).

Considerable work has been carried out on a variety of herbaceous plants and also trees (Manoch *et al*., 2007). Analysis of any plant material result in the discovery of a range of different microfungi (the actual numbers can range up to many tens), and several of the fungi would likely be specific to that particular host (Tokumasu *et al*., 1997). Furthermore, environmental conditions such as soil, temperature and humidity etc, would also affect the nature and the population of microfungi (Dix and Webster 1995).

It is generally accepted that plants in unique environments that struggle to compete with other living organisms are likely to host or attract microfungi which can generate secondary metabolites (Anke *et al.,* 1977). Many scientists hope that plants growing in tropical rainforests, where competition for light and nutrients is severe, are most likely to host the greatest number of bio-active microfungi, and indeed a recent study noted that microfungi, from tropical regions produced significantly more bioactive secondary metabolites than those from temperate parts of

the world (Tokumasu *et al*., 1997). Consequently, most investigations in search of novel metabolites have tended to concentrate on organisms that inhabit biotypes that represent unique survival challenges to plants, such as tropical forests.

2.4. Isolation and Identification of Microfungi

The methods of analysis of litter microfungi in culture are similar to those described for soil fungi. Various factors can also influence the diversity of fungi isolated from litter samples such as, particle size, growth medium and growth inhibitors (Paopun *et al*., 2006). The most effective method described todate is that of Bills and Polishook (1994), in which plant material is mixed with sterile water and pulverized in a sterilized blender. The resulting suspension is pipetted onto agar plates on two selective media; a Malt Agar Medium amended with cyclosporine and Dichloran-rose Bengal Medium.

2.5. Solid state (or substrate) fermentation (SSF)

Solid state (or substrate) fermentation (SSF) is characterized by fermentation process on solid support, which has low moisture content (lower limit ~12%) and occurs in a non-septic and natural state (Nigam and Singh 1996; Holker and Hofer, 2004). In a broader definition, SSF can be seen as including processes during which microorganisms are cultivated in the presence of a liquid phase at maximal substrate concentrations or on inert carriers (Ashley *et al.,* 1999).

Several researchers have reviewed the different application of solid-state fermentation (Nigam and Singh 1996; Pandey *el al.,* 2000). SSF is mainly known for the production of traditional fermented foods such as “Kojl”, indonesian “tempeh” or Indian “ragi”. It has also been used for the production of high added value compounds (such as enzymes, organic acids, biopesticides, biofuel and new applications of SSF in the environmental control have been developed including bioremediation and biodergradation of hazardous compounds and the detoxification of agroindustrial residues (Pandey *et al.,* 2000).

Microorganism such as Bacteria, yeasts and fungi can grow on solid substrates, and find application in SSF processes. But filamentous fungi are the most important group of microorganisms used in SSF process owing to their physiological, enzymological and biochemical properties. The hyphal mode of fungal growth and their good tolerance to low water activity (AW) and tolerance to high osmotic pressure make them efficient and competitive in natural microflora for bioconversion of solid substrates.

The hitherto works were focused on the general applicability of SSF for the production of enzymes, secondary metabolites and spores, in that many different solid substrates (agricultural waste) have been combined with many different fungi and the productivity of each fermentation. SSF appears to possess several biotechnological advantages than submerged fermentation technology (SmF), such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi and lower demand on sterility due to the low water activity used in SSF (Holker and Hofer, 2004).

In laboratory, secondary metabolites are mostly produced under submerged (SmF) conditions, mainly because the processes associated with scale-up are much simplified, compared to those required for scale up in SSF. Liquid fermentation also allows greater control of parameters, such as pH, heat, nutrient conditions , etc; and it must also be taken into account that scientists in the west have less knowledge of the SSF process, compared to those in the East (Nigam and Singh, 1994).

The fact that liquid media have a detrimental effect on product formation by fungi, because highly viscous liquid media are required for successful metabolite production and this can interfere with oxygen transfer. The filamentous morphology of these micro-organisms and the secretion of these metabolites into the growth media can increase viscosity further. Therefore, SSF technology can be exploited as an alternative, allowing better oxygen circulation (Elibol and Mavituna 1997).

The use of SSF technology for the production of secondary metabolites from fungi is highly commended because the morphology is well suited to growth on a solid support (Barrios *et al.*, 1988). Similarly, many fungi have also shown diverse spectrum of secondary metabolites when

grown on solid substrates (Robinson *et al*., 2004). Therefore, the use of SSF should be considered by industry, especially when large quantities of secondary metabolites are required in short fermentation periods, with minimal expenditure on media and downstream processing.

As described in *Streptomycetes*, the production of secondary metabolites often seems to be associated with the formation of aerial hyphae and the onset of spore production in the stationary growth phase. Various secondary metabolites have even been shows to be produced solely during growth on solid substrates, although the fungi in question can be readily cultivated in submerged processes. For this reason, SSF could play an interesting role in the screening for new metabolites and also in the production of these metabolites by wild-type fungi.

2.6. Isolation of bioactive compounds

Once LDMFs are successfully cultured, bioassay-guided fractionation of growth media is used to isolate the most promising compounds. Here it must be stressed that having appropriate bio-assays must available in order to check the activity of the fungus because, it is crucial to the isolation and purification of the active components. The active microbial fungi are eventually transferred onto solid substrate in large flasks, and allowed to grow for several weeks. The compound synthesized in solid substrate is extracted with known amount of organic solvent before being tested for bio-active compounds (Abate, 1999). Isolation and purification of the bio-active compounds is attained through chromatographic techniques such as thin layer chromatography (TLC) and Column chromatography (CC). The type of bioassays used in this work depends largely on the interests of the investigators, but tests for anti-bacterial and anti-fungal are easy to prepare and conduct.

2.6.l. Anti-bacterial assay

Two simple methods can be utilized for the testing of crude filtered growth media for anti-bacterial properties; the first is a paper disc method and the second involves a liquid assay (Bauer *et al.,* 1996). For the former, a bacterial “lawn” of fast-growing *Pseudomonas aeruginosa* is generated in a petri dish containing Mueller Hinton agar using sterile glass beads.

Paper discs are then placed on this lawn and small aliquots of filter-sterilized LDFs growth media are pipetted onto the discs. Several different media can be used concurrently, to determine activity by a visual zone of inhibition surrounding the discs. The liquid anti-bacterial method involves adding the bacterium *Pseudomonas aeruginosa* in a broth to a 96-well plate and adding bacteria media at varying concentrations to the wells. Inhibition of growth in the wells is determined by optical transmittance through the wells.

2.6.2. Anti-fungal assay

There are two possible approaches to an anti-fungal assay. In a competitive assay, two fast-growing fungi (e.g. *Pythium* and *Geotricium*) are placed on opposite sides of a petri dish, and a plug containing a drop of the fungi liquid is placed at the center of the plate. If the microfungi has any anti-fungal activity, the center spot will have a strong zone of inhibition surrounding it. In a non-competitive assay, the liquid extracted from the fungi is placed in a 6-well plate containing M1D solution, and *Geotricium* or *Pythium* is added to the wells. The wells may be inspected at 24 hr intervals and the growth of the added fungus compared with that in a control medium.

2.7. Minimal Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the antibiotic that prevented visual turbidity after 18-36 hrs of incubation at 37 °C for bacterial cultures and longer for fungi (Hwang *et al.,* 2001). There are several established methods to determine MIC but, the most common one are Broth Microdilution Method (Polanco *et al*., 1995 and Microtitre broth dilution method (Singh, *et al*., 2000). These methods are standard methods for determining the levels of resistance to the bioactive compound. In both case serial dilutions of antibiotics are made in liquid medium which is inoculated with standardized number of organisms and incubated for prescribed time. For microtiter broth dilution method a sterile microtiter is used to inoculate the test organism rather than test tubes.

3. OBJECTIVE OF THE STUDY

This work was undertaken for the following objective

General objectives

To isolate, characterize and purify bioactive compounds from a forest litter decomposing fungi

Specific objectives

- To isolate, characterize and maintain a wide range of microfungi from Ethiopian forest
- To develop a SSF method for cultivation of fungi and production of 2° metabolite
- To extract, purify and evaluate the *in vitro* activity of the bioactive compound
- To determine the antimicrobial spectrum and MIC of the 2° metabolite

4. MATERIALS AND METHODS

4.1. Isolation and culture condition

4.1.1. Sample Collection

A few decomposed litter samples from the root surface of *Juniperus procera* in Menagesha Suba Forest, Ethiopia were collected on September 3,2008. The samples were stored in sterile plastic bags and sent to the Mycology Laboratory, Biology Department, Addis Ababa University where they were stored under refrigeration until LDMF isolation was performed.

4.1.2. Sample treatment

Isolation of fungal flora from the decomposed leaf suspensions were performed by dilution plate technique using Chloramphenicol-Malt Extract Agar (MEA), (consisting of g/l in distilled water: malt extract, 20.0; yeast extract, 2.0; agar, 20.0; chloramphenicol, 0.1) and Potato Dextrose Agar (PDA) (potatoes, 200.0; glucose, 20.0; agar, 20.0). 1gm of leaf suspensions was aseptically removed and suspended in 10ml of sterile distilled water in test tube. Then the sample was vigorously agitated using rotatory shaker for 1hour and left for 30 minute to settle. Later, the supernatant was diluted to 10^1 - 10^4 and from dilution 10^3 and 10^4 0.1mL was spread-plated in duplicates in pre-dried surfaces of Chloramphenicol-Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) and was incubated at 25^0C for five days.

4.1.3. Isolation and maintenance of pure culture

Five days after incubation, single colonies from the plates were picked and purified by inoculating in separate plates containing Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA). Then, the pure cultures were transferred in to slant cultures and preserved at 4^0C. Five isolates designated Ha4, Ha9, Eb1, Eb9, Jp4, LDMFs labeled Jp4 were selected for further investigation to test their ability to produce bioactive metabolites.

4.1.4. Identification of Fungus

For macroscopic morphology, the fungus Jp4 was grown in Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) at 22°C for five days. Colony characteristics and diameter were noted on the 2nd, 3rd and 4th days of incubation.

For microscopic analysis, Jp4 was grown on Potato Dextrose Agar (PDA) for 4 days in a slide culture technique (Boursnell, 1950) as follow. From matured fungus culture, a small pieces of the micellium was taken with inoculating niddle and placed on a sterile microscope slide. Then, the slide gently flattened with a cover slip and microscopic observations were performed with an Olympus B071 phase-contrast microscope at 10x, 40x and 100x magnification.

Figure 1. Showing slide culture technique for the fungus Jp4

4.2. Solid-state cultivation

4.2.1. Condition for solid state fermentation

Local rice called "Basmati" was used as solid substrate for the cultivation of Litter-decomposing fungus Jp4 (LDMF) for secondary metabolite production. The moisture content of the substrates (rice) that was suitable for the growth of the organism was adjusted prior to inoculation. Samples of 100-grams were taken from autoclaved substrate and were dried at 60^0C until constant weight was obtained. The moisture content of the rice was determined from the weight loss.

$$\text{Moisture level (\%) of substrate} = 100 \times \frac{\text{wet weight of the substrate} - \text{dry weight of the substrate}}{\text{Wet weight of the substrate}}$$

4.2.2. Preparation of rice grain for solid state cultivation

A method of preparation, described by Abate (1999) was employed. A weight of 200g of rice grains was boiled in 200ml of distilled water for 10 minutes in 1000ml Erlenmeyer flasks and transferred in 1000ml glass bottle and autoclaved at 121^{0}C for 25 minutes. A method of inoculating mycelial fungus by Anke *et al.,* (1977) was used, glass bottle containing sterile rice grains were kept for some hours to cool down and inoculated with 2mm x 2mm of mycelia culture (which was grown in PDA for five days) under aseptic conditions. The solid-state fermentation was lasted for twenty one days at 22^{0}C (Abate, 1999).

4.3. Extraction and testing crude extracts

Chemicals

Organic solvents used in this investigation were Ethyl Acetate (ALDRICH, Germany), Methanol ALDRICH, Germany), chloroform (Riedel-de Haen, Germany), and Petroleum ether (Riedel-de Haen Germany).

4.3.1. Extraction of rice grain culture (SSF)

Three weeks after inoculation, 500g of completely fermented rice (in five bottles) was ground with mortar and pestle. Then the biomass was kept at room T^{o} (22°C) for six hours to dry up. Later, it was extracted with 200mL Ethyl acetate and 200mL Methanol on rotary shaker at 120 rpm for twelve hours. Then the biomass was filtered with Whatman0.45um filter paper and the organic phase was separated from the aqueous phase using a Buchner funnel. Later, the extracts were concentrated using Rota-vapour set at 60^{0}C near dryness. Both solvents were recovered and reused for the next extraction before being decanted. The same quantity of solvent was added to the biomass and shaken once again for an hour for further extraction. The process was repeated three times with each solvent. The pooled extracts were allowed to dry in rotavapour once again and placed in pre-weighed beakers. The mass extract of each solvent for the fungus was calculated. Lastly, the dried crude extracts were assayed for antimicrobial activity and chemical profile was determined using TLC.

4.3.2. Antimicrobial activity assay

4.3.2.1. Test Strains

Altogether seven (six bacterial and one fungal) strains were used to evaluate the antimicrobial activity of crude extracts. *Streptococcus pneumonia* ATCC49619; *Pseudomonas aeruginosa* ATCC27853; *Staphylococcus aureus* ATCC25923; *Escherichia coli* ATCC25922 and *Shigella boydii* and *Basillus subtilis* (clinical isolate) were provided by Ethiopia Nutrition and Health Research Institute, Addis Ababa, and *Candida albicans*NCIM3471, provided by Black Lion Hospital, Addis Ababa, Ethiopia.

Agar diffusion assay by filter paper disc method (Bauer *et al.,* 1996) was carried out by impregnation of the crude extracts on filter paper discs of the same diameter (5mm) and agar plate dilution method was used to inoculate the bacterial pathogen in the plate (Singh *et al*., 2000).

First growth curve for the bacterial pathogens were determined by microbroth dilution method (Villanova, **1**991) and spectrometer reading at 660nm to measure their mass. Briefly, overnight cultures of the bacterial pathogens in Nutrient broth (100mL/500mL Erlenmeyer flask) at 37°C were diluted into a fresh prepared same media at two hour difference to a concentration ranging from 10^1 - 10^6cfu/ml. Later, from the appropriate dilution, 0.1mL of the right A_{660} cells from the exponential phase for each pathogen (six) was inoculated into sterilized flasks containing molten soft agar (20mL) at 50°C (Table.1). Then, the inoculated soft agar was immediately poured into sterilized plates (one for each pathogen).

After 15min, 80µg/uL of the crude extracts was spotted on the agar surface in the disk for each test organisms and disk impregnated with Tetracycline as positives and without as negative controls groups were taken. Later, the plate was incubated at 37°C for up to 24hrs and inhibition zones around each disc were measured in mm.

For fungal cultures, 24hrs yeast cells on Czapek broth (sucrose, 0.3 %; sodium chloride, 0.2 %; magnesium glycerophosphate, 0.05 %; potassium chloride, 0.05 %; dipotassium sulfate, 0.035

%; ferrous sulfate, 0.001%) were overlaid on the Czapek agar plate (one) and Positive control (10 µl of 10µg ketoconazole ml^{-1}) and Negative control (10µl of ethyl acetate) were taken. Then, incubation last for about 72 h at 30^0C. The diameter of the zone of inhibition [in mm] was measured excluding the disk impregnated with extracts.

.

Table.1 The population density of test organisms (cfu/ml) used during the bioassay

Test Organisms	Growth Curve/phase	Time (hrs)	OD	Dilution factor	Transfer dilution rate	Colony count	Formula CC / D.F X TDR	Cfu/mL
E. coli	Exponential	4	2.8	10^{-5}	0.1	117	117 / 10^{-5}x0.1	$1.17x10^8$
P. aeruginosa	Exponential	4	2.0	10^{-5}	0.1	119	119 / 10^{-5}x0.1	$1.19x10^8$
S. aureus	Exponential	4	0.18	10^{-5}	0.1	101	101 / 10^{-5}x0.1	$1.01x10^8$
S. pneumoneae	Exponential	4	0.14	10^{-5}	0.1	101	101 / 10^{-5}x0.1	$1.01x10^8$
B. subtilis	Exponential	6	0.54	10^{-5}	0.1	109	109 / 10^{-5}x0.1	$1.09x10^8$
S. boydii	Exponential	6	0.51	10^{-5}	0.1	111	111 / 10^{-5}x0.1	$1.11x10^8$
Candidia albicans		24	0.5	10^{-5}	0.1	107	107 / 10^{-5} x0.1	$1.07x10^8$

4.4. Determination and isolation of the biologically active compounds

4.4.1. Preparative Thin Layer Chromatography (TLCP)

For Preparative thin layer chromatography (TLCP) separations, glass plates coated (1mm) with silica gel GF254 Merck, Darmstadt, Germany were employed.

4.4.1.1. Preparation of Thin Layer Chromatography Plates (PTLCP)

25g of Merck silica gel was suspended in 50mL of deionized water and shaken vigorously for 45seconds in rubber-stopper Erlenmeyer flask (500mL). The thickened slurry was poured into the glass and pulled with a ruler in two sides at 1mm trailing edge to prepare similar plates. Then, the plates were allowed to air dry 30minutes in the oven at 50° C (until they turn white).

After preparation of the plates, a few drops of the ethyl acetate extracts were applied (using a capillary tube) to the bottom of each of the pre-coated and pre-heated (50°C for 30 minutes) glass plates (three glass plates).

After 5 minutes of drying, each of the plates was placed in the separate glass chamber with solvent system Chloroform: Ethyl acetate (1:1 v/v) as the mobile phase as shown in figure 2. After the movement of solvent at the top of the plates, each plate was removed from the glass chamber and separately air-dried. After air-drying, the produced spots were located by their fluorescence under long and short wave UV light (254 and 366 nm respectively).

The fluorescent spots visualized upon UV exposure were scraped off and eluded with ethyl acetate, filtered and allowed to dry. The dried residues were weighed and eluted with ethyl acetate to give a known concentration of the compounds. Lastly, each of the fractions was tested for antimicrobial activity against different bacterial and fungal strains as before to know the active fraction.

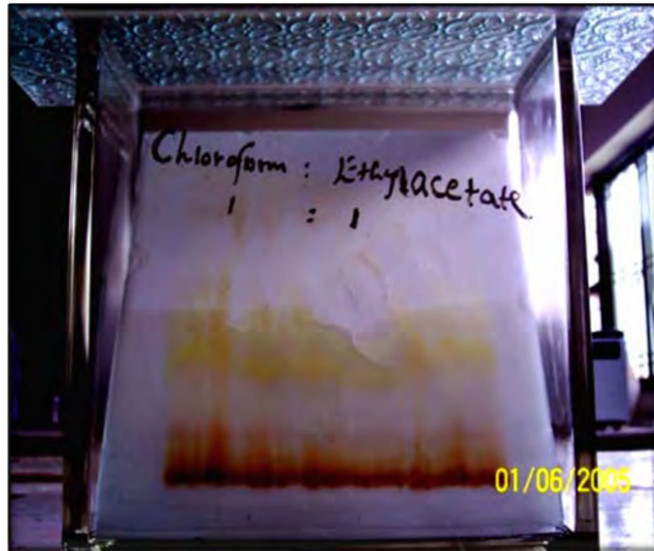


Figure 2. Preparative Thin Layer Chromatography (PTLC) for the crude extracts using Solvent system Chloroform: Ethyl acetate (1:1 v/v)

4.4.2. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) analyses were performed by silica gel 60-F254 on aluminum sheets (Merck, Darmstadt, Germany). Active compound was determined by thin layer chromatography (TLC). Crude extracts in ethyl acetate were spotted 2 cm from the bottom of a 20x20cm precoated aluminum sheet of silica gel 60 F254 (Merk). Glass jars were saturated overnight by the solvent system Chloroform: ethyl acetate (1:1, *v/v*). The silica gel sheet allowed to dry and then developed in an ascending order for few minutes until solvent front about 16cm length. Produced spots were located by their fluorescence under short and long wave UV light (254 and 366 nm respectively). The R_f values were determined.

$$R_f = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by solvent front}}$$

4.4.3. Column chromatography (CC)

Column chromatography was performed on silica gel 60, 230D400 mesh (Merck, Darmstadt, Germany) to isolate the active compound from the crude extracts. A column was prepared by adding a mixture of silica gel (30g) and 40mL petroleum ether to a column (3cm x 18cm). The silica gel was allowed to settle while petroleum ether was slowly passed through the column. The dried crude extracts (1gm) were dissolved in a minimal volume of petroleum ether and mixed with small amount of pure silica gel (5g) and applied in the column. Later, it was eluted with the solvent systems starting with petroleum ether followed by Petroleum ether: Chloroform (1:1 v/v) as in (table 1.) until the active compound was collected (figure 3). For each eluent mixture 20ml volumes were used and each fractions were collected in pill vials with 5ml volumes.

Table 2. Solvent mixtures used in column chromatography

Elution system	
Petroleum ether:	100%
Petroleum ether: Chloroform	90%
	80%
	70%
	60%
	50%
	30%
	10%
Chloroform:	100%
Chloroform: Petroleum ether	90%
	80%
	70%
	60%
	50%
	30%
	10%

Figure 3. Purification of the bioactive compound using solvent system Petroleum ether: Chloroform (1:1 v/v) in column chromatography

The collected fractions were concentrated using a Rotavapour. TLC was used to analyze the active compound, and those with similar chemical components were pooled together. The pure compound was dried in pre-weighed pill vial and its mass (g/ml) determined. Later, antimicrobial activity was evaluated.

4.4.4. Antimicrobial Susceptibility test on bioactive compounds.

To determine Minimum inhibitory concentrations (MIC) an antibiotic dilution assay technique was used (Markiewicz and Kwiatkowski, 2001), using nutrient broth (NB) for bacteria and Sabouroud's broth (Mycological peptone + Dextrose) media for the fungus. First, the bioactive compound was prepared in different concentrations (60; 65; 75; 80; 85; 90; 95; 100; 105; 110; 120 ug/mL) and dissolved in sterile capped test tubes (1-11) containing 1mL of suitable microbial growth medium. Then, serial dilutions of the compound were inoculated with 10ul of (predescribed) standard size (for MIC) bacterial inoculum from the exponential phase.

The bioactive compound was also compared with commercially available antibiotics, Tetracycline (positive control) for antibacterial activity against all bacterial pathogens and ketoconazole (positive control) for anticandidial activity using the above procedure.

The test microorganisms used in the assay are*: Pseudomonas aeruginosa* ATCC27853; *Escherichia coil* ATCC25922; *Staphylococcus aureus* ATCC25423; *Streptococcus pneumoneae* ATCC49619; *Shigella boydii*; *Bacillus subtilis* and *Candidia albicans* (clinical isolates).

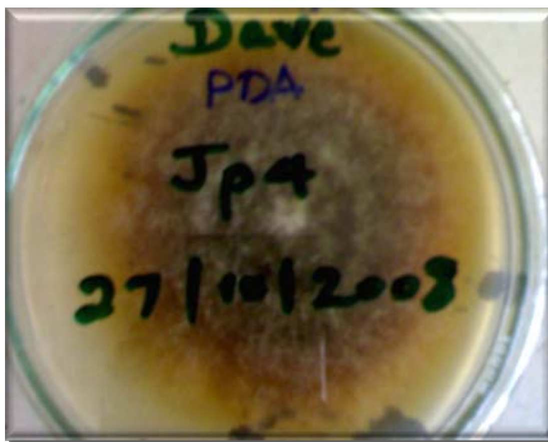
The antimicrobial activity was observed after 24-48h at 37°C for bacteria and 48-72h incubation time at 27°C for fungi. Lowest concentration of compound that showed antimicrobial activity against test organisms was recorded as MIC value (Hwang *et al.*, 2001).

5. Results

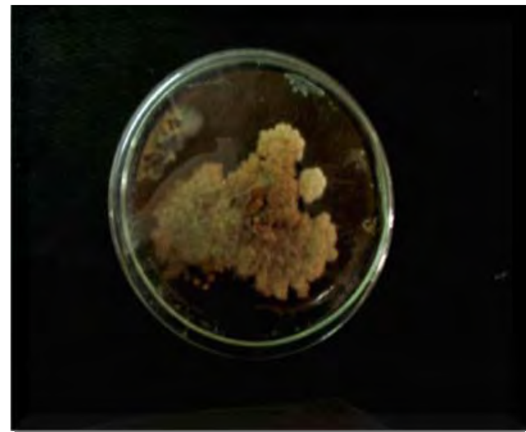
5.1. Isolated fungus

Culture Morphology

Growth of Jp4 on potato dextrose agar (PDA) and malt extract agar (MEA) showed that the organism grew well on the two media, but the most differentiation occurred on MEA (fig.4 B). Jp4 grew fast on PDA at 22°C reaching a diameter of 34mm after only 3rd days of growth and a maximum of 41mm by 4th days. Surface morphology was generally floccose to felty throughout the incubation period. The isolated fungus correspond in morphology to the genus *Geotrichum* described by Barnett and Barry (1972). Colony color progressed from white to yellow and with some dark-brown at the colony center on PDA and brown on MEA.



(A), On PDA for 4 days



(B), On MEA for 4days

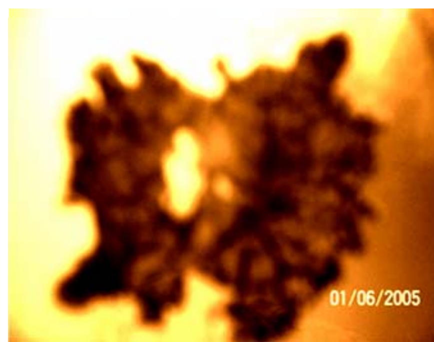


Figure 4. Morphological characteristics of culture Jp4 grown on PDA and MEA

Microscopic characterization

The fungus was characterized by highly branched and septated mycelium, without conidiophores and foot cell. Arthrospores were formed by segementation of hyphae. However for further analysis to species level the sample was sent to GERMANY.

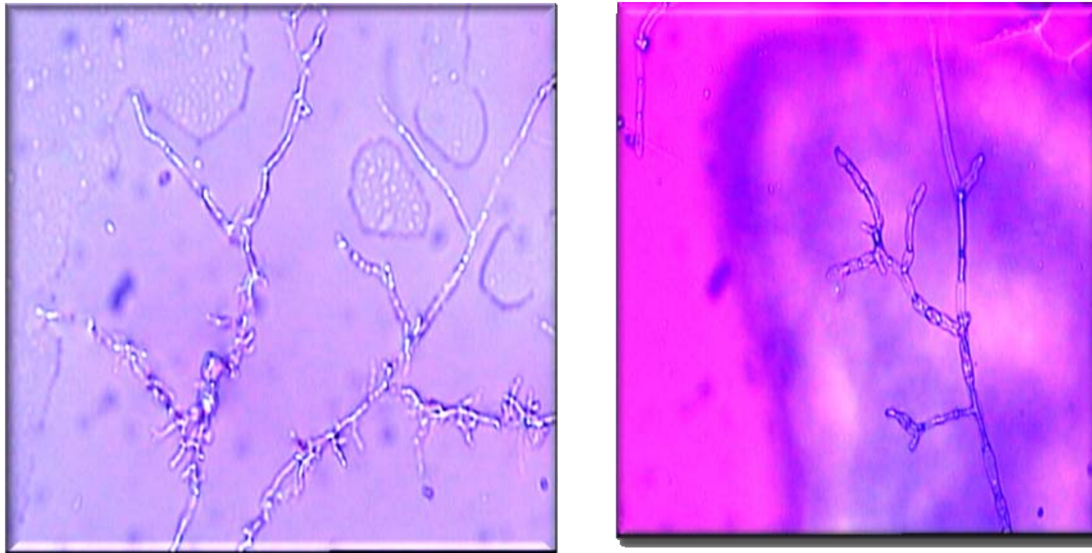


Figure 5. Showing Fungus, Jp4 on phase contrast microscope 100x.

5.2. Induction of bioactive production by Solid-state Fermentation on rice

Rice grain obtained from the local market, which is used as a substrate for bioactive metabolite production, supported a good mycelia invasion by Jp4. Complete colonization of the fungus on rice substrate lasted for about 21days. The moisture content of the sterile-moist rice (80%) was found to be suitable.

Figure 6. Solid state cultivation of Jp4 on rice grain for secondary metabolite production.

.

5.3. Extraction

Finally grounded 500g of dried fermented rice by fungus Jp4 were serially extracted with 200mL Ethyl acetate and 200mL Methanol as indicated above. The following masses in table 2.were obtained.

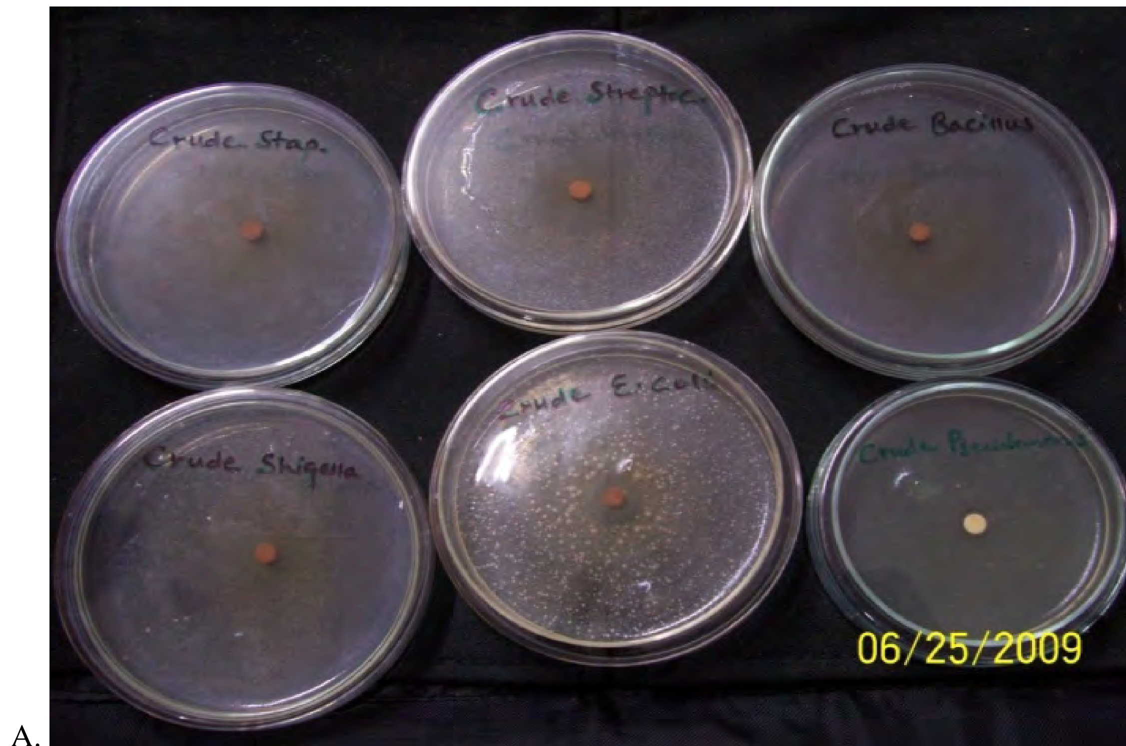
Table 3. The mass (g) of crude extracts from Jp4 serially extracted with two extractants from 500g of fermented rice.

Extractants		Mass residue extracted (g)	
		Mass	**Total**
Ethyl acetate	1	2.80	4.76
	2	1.76	
	3	0.2	
Methanol	1	3.1	6.6
	2	2.7	
	3	0.8	
Total			**11.36**

The total mass extract was 11.36 from 500g of fermented rice by the fungus Jp4. Methanol (6.6g) extracted the more mass than ethyl acetate (4.76).

5.4. Antimicrobial activity

Totally, seven pathogens were inhibited by the crude extract using disk diffusion assay (Bauer *et al.,* 1996) as shown in figure.8.

A. 

B.

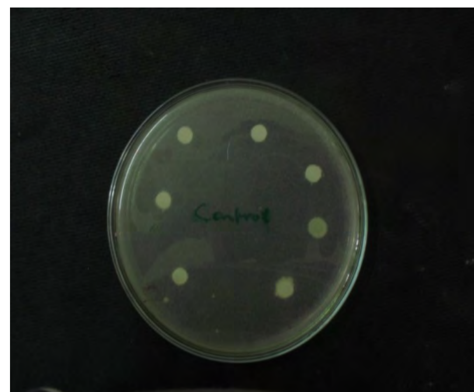
C. 

Figure 8. Inhibition of the crude extract by disk diffusion assay with different test organism

A. Bacterial strains (six)
B. Yeast, *Candidia albicans*
C. Control groups (Tetracycline)

Ethyl acetate and methanol extract of JP_4 from SSF cultures was tested for antimicrobial activity. The EtoAac extract inhibited the growth of all test microorganisms, but little or almost no activity was observed in the case of methanol extract (table 4.).

Table 4. **Antimicrobial activity of the crude fraction against various microorganisms**

Test microorganism	Amount of of inoculums used (cfu/ml)	Diameter of inhibition zone size (mm) methanol extract	EtoAac extract
Escherichia coli	**$1.17x10^8$**	**3**	**20**
Pseudomonas aeruginosa	**$1.19x10^8$**	**0**	**13**
Staphylococcus aureus	**$1.01x10^8$**	**2**	**15**
Shigella boydii	**$9.5x109^8$**	**3**	**18**
Bacillus subtilis	**$1.09x10^8$**	**2**	**15**
Streptococcus pneumoneae	**$1.01x10^8$**	**2**	**25**
Candida albicans	**$1.07x10^8$**	**3**	**11**

The crude metabolite exhibited antibacterial activity against three Gram-negative (*Eshershia coli, Pseudomonas aeroginosa, Shigella boydii*) and three Gram-positive (*Staphylococcus aureus, Bacillus subtallis and Streptococcus pneumonia*) bacterial strains and antifungal against *Candidia albicans*. However, higher activity towards *Streptococcus, Eshershia* and *Shigella* respectively.

5.5. TLC analysis

The results showed that the solvent system Chloroform: ethyl acetate (1:1 v/v) is the best solvent system. Since it separates a large number of compounds (ten) present in the base line. The compounds were separated from each other.

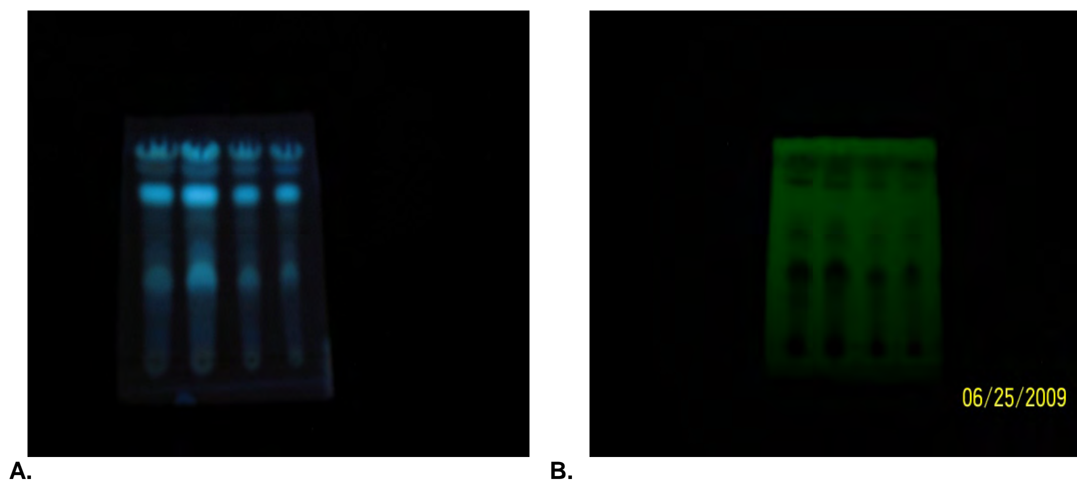


Figure 9. TLC of the crude extracts of Jp4 at A. 254nm (long) and B. 366nm (short)

5.6. Preparative thin layer chromatography

Active compound in the crude extracts of JP_4 was determined using PTLC separation and each of the fractions (figure 9.B) was further tested against six bacterial and one fungal pathogen (see Table 5.). TLCP on UV for the crude extract revealed ten fluorescent spots on UV (366nm) as shown in (fig 10A.) and they, along with the brown & yellow compounds.

A

Figure 10. Preparative thin layer chromatography under 366nm for the crude extracts using solvent system Chloroform: Ethyl acetate (1:1 v/v)

Table 5. Showing data obtained from fractions of PTLC during antimicrobial testing

		Fractions in ug/ul and Inhibition zone (mm)										
		F_1	**F_2**	**F_3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	Positive *controls* (Tetracycline) & Ketoconazole
N.0	**Test strain**	**75**	**75**	**75**	**75**	**75**	**75**	**75**	**75**	**75**	**75**	
1	*E. coli*	0	0	25mm	0	0	0	0	0	0	0	25
2	*S. aureus*	0	0	24mm	0	0	0	0	0	0	0	29
3	*P. aeruginosa*	0	0	14mm	0	0	0	0	0	0	0	17
4	*S. pneumoneae*	0	0	28mm	0	0	0	0	0	0	0	24
5	*Shigella boydii*	0	0	18mm	0	0	0	0	0	0	0	25
6	*Bacillus subtilis*	0	0	25mm	0	0	0	0	0	0	0	29
7	*Candidia albicans*	0	0	14mm	0	0	0	0	0	0	0	21
8	R_f			0.57								

Fraction three of the crude extract exhibited activity against bacteria (both GM+ and GM-) and *Candidia albicans*. Antimicrobial activity (six bacterial and yeast, *Candidia albicans* fungus) yielded inhibitory zones of 14mm against *Candidia albicans* and 28mm, 25mm, 24mm against GM+ bacteria (*S. pneumoneae*, *B. subtilis* and *S. aureus*) respectively. And 25mm, 18mm, 14mm against GM- bacteria (*E. coli, S. boydii and P. aeruginosa*) respectively at 75ug/ul of the active compound per/ disk and it was active against *S. pneumoneae* as compared to the positive control.

A TLC separation of the crude extracts showed almost similar fractions when compared with the PTLCP on UV absorption at 366nm (Short wave length) as shown in (figure 10). The active compound was easily detected with UV absorption at 245nm and the R_f value on silica get TLC was 0.57.

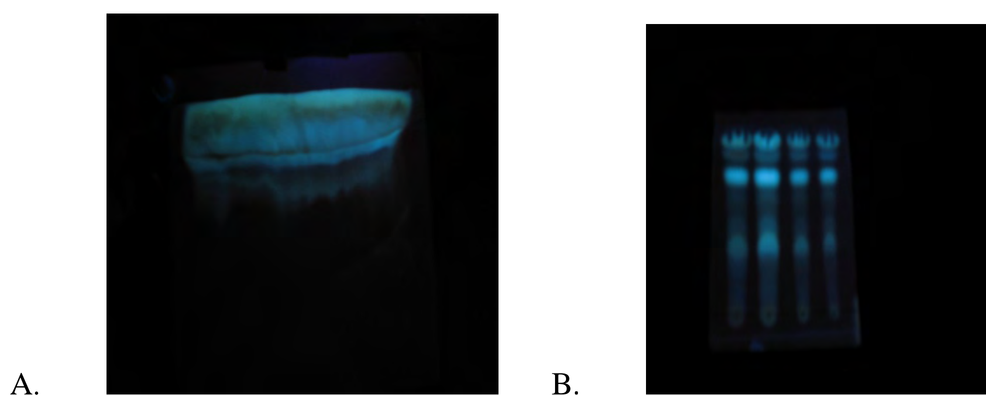


Figure 11. TLC on UV at 245nm (short wave length)

5.7. Column chromatography

The ethyl acetate extract (1g) was further partitioned by silica gel column chromatography (Petroleum ether: Chloroform /1:1) and six fractions were obtained. Fraction three (Rf=0.57, 264.8mg) of the crude extract was isolated and identified as active compound by its color (yellow) (figure 12) based on the preliminary results on PTLCP (Table 5.). Later, the pure

compound was investigated for antimicrobial activity against six bacterial and one fungal species by disk diffusion assay (Bauer *et al.,* 1996).

5.8. Antimicrobial Susceptibility test on bioactive compounds

The minimum inhibitory concentration (MIC) of the antimicrobial compound for the test organism ranged between 65 and 120ug/mL (Table 7.).

Table 7. Antimicrobial activity of f_3 against different test organisms

Test microorganism (OD)	MIC in µg/mL											Tetracycline250ug/ml
	60	65	75	80	85	90	95	100	105	110	120	
Staphylococcus aureus												
OD			**0**									**0**
Bacillus subtilis												
OD				**0**								**0**
Eshershia coli												
OD				**0**								**0**
Pseudomonas aeruginosa												
OD			**0**									**0**
Shigella boydii												
OD				**0**								**0**
Streptococcus pneumoneae												
OD		**0**										**0**
Candidia albicans												
OD											**0**	**0**

Amoung the tested GM+ bacteria, *Streptococcus pneumoneae* was highly susceptible with the decrease in cell number starting from the first concentration of the bioactive compound resulted in MIC value of 65ug/ml. Followed by *Staphylococcus aureus* at 75ug/ml and *Bacillus* sp. at 80ug/ul. From GM- bacteria, *Pseudomonas* was more susceptible with the decrease in cell number starting from 65ug/ul and resulted in MIC value of 75ug/ul continuing with *E.coli* with MIC value of 80ug/ul. Similar results were observed in the case of *Shigella* sp. In the case of *Candidia albicans* highest MIC value incountered. Susceptibility to the bioactive compound was observed at MIC value of 120ug/ul. The minimum inhibitory concentration (MIC) value of the sixbacterial pathogens with Tetracycline was 250ug/ml.

6. Discussion

In this study, four isolates of microfungi were collected from leaf-litter of Menagesha forest, which one isolate Jp4 was further characterized for the production of secondary metabolites (active compounds). Boiled pasteurized rice grain was used as a substrate for the cultivation of the isolate and induction of antimicrobial metabolite in a solid state fermentation (SSF). The fungus shows dense and heavy filaments mycelial growth on the entire surface of the substrate (rice grain) indicating that the grain provided good nutritional base for the isolate.

Mycelial colonization of the fungus was visually observed as it change the color of rice, from white in to brown. The fungus was found to show mycelia growth at moisture contents of 50% and 100 %. The ambitiant growth of the fungus on the rice substrate was at moisture content of 60%.

Recently, more attention has been focused for production of secondary metabolites (especially, pharmactical importance) in solid state fermentation (Jhons, 1992; Nigam and Singh, 1994; Vijaya Kumar *et al*., 1996).

During extraction, Methanol was found to be the best solvent to yield a large quantity of fungus extract (4.76g/500ml of rice substrate) than ethyl acetate (6.6g/500ml of rice substrate). Lin *et al.,* (1999) reported that success in isolating active compounds from biological material is largely dependent on the type of solvent used in the extraction procedure. However, the methanol extracts did not show any antimicrobial activity indicating that methanol was not a good organic solvent to extract active compounds from the isolate Jp4.

In this study, antimicrobial activity of the crude extracts was determined by disk diffusion assay (Bauer *et al.,* 1996) and the inoculums size used during the test for each organism were determined by micro broth dilution method (Villanova, **1**991) by subculturing of 24hour old culture. Although the assay disk diffusion is not a sensitive method to determine the number of test organisms inhibited by the active metabolites. It will indicate activity of the metabolite (Ely *et al.,* 2004). The different inocula taken for the bioassay was written in the range of 10^6-10^9cfu/ml (Table 3).

Inorder to identify the active ingredients from the crude fungal extracts, preparative thin layer chromatography (PTLC), Thin layer chromatography (TLC) and Column Chromatography methods were employed. The data showed that PTLC fractionated the crude extract into 10 components (Table 5). From the tested ten fractions, fraction three exhibited activity. High activity observed in *Streptococcus pneumoneae* followed by *P. aeruginosa* and *S. aureus* respectively. However, the active fraction isolated from TLCP was not significantly active in comparison to positive controls (Tetracycline). The active compound was detected as yellowish spots on a deep blue back ground, allowing the chromatographic retention factors (R_f) observation clear.

In this studies, MIC results of the bioactive compound reveals more active against GM+ than GM- bacteria, since the lowest MIC value recorded during the test is 65 and 75ug/ml. The reason for this may be due to the thin outer membrane which makes the GM+ bacteria susceptible to the active compound faster, but in the case of GM- bacteria they can tolerate at some concentration because of the presence of thick outer membrane (Villanova, **1**991)

In vitro antibacterial activity of the bioactive compound shows that it is effective than the commercially available antibacterial compound, Tetracycline (positive control). Therefore, this result suggest that the bioactive compound have the potential to be applied as antibacterial compound and used for further in vivo test as antibacterial metabolite.

7. Conclusion

The present paper investigated the cultivation (SSC), extraction, isolation and biological activities of antimicrobial compound from LDFs, Jp4. The bioactive compound from Jp4 is a promising compound as it exhibited antibacterial activity against gram+ as well as gram- bacteria and antifungal, against Candidia albicans. Finally, this paper will provide a scientific base especially in our country for the isolation of bioactive compounds from LDFs and could be the basis for novel bioactive compounds.

8. RECOMMENDATIONS

As time constraint did not allow to tell what the actual organism is, where the purified compound belongs, what it looks like and its *in vitro* potency, further research is needed. Further experiments with multiple strains of other organisms warranted to confirm the utility of the bioactive compound from *Geothrichum sp.* (Jp4) as an alternative medicine and also optimization of the SSF method for *Geothrichum sp.* (Jp4) would help in improving the scale-up process for industrial production of the active metabolite. Determination of the growth kinetics of the fungus would enable to estimate the exact period of extraction. Lastly, further investigation of this potential antimicrobial agent may help in developing new chemical classes of antibiotics.

9. REFERENCES:

Anke, T., Oberwinkler, F., Steglich, W. and Schramm, W. (1977). The strobilurins- new antifungal antibiotics from the basidiomycete *Strobilurus tenacellus* (Pers. ex Fr.) Sing. *J. Antibiotics.* **30:** 806-810.

Anke, T. (1989). Bioactive Metabolites from Microorganisms. *Prog. Ind. Microbiol*. pp 51-66.

Anke, T., Werle, A., Kappe, R. and Sterner, O. (2004). Laschiatrion, a new antifungal agent from a *Favolaschia sp*. (*Basidiomycetes*) active against human pathogens. *J. Antibiotics.* **57:** 496-501.

Aqueveque, P., Anke, T. and Sterner, T. (2002). The himanimides, new bioactive compounds from *Serpula himantoides* (Fr.) Karst. *Z. Naturforsch.* **57:** 257-262.

Barnett, H.L. and Barry, B. (1972). *Illustrated genera of imperfect fungi*. pp 165-177.

Bills, G.F. and Polishook, J.D. (1994). Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. *Mycologia.* **86:** 187-198.

Bills, G. F. and Polishook. J. D. (1999). Abundance and diversity of micro fungi in leaf litter of a low land rain forest in Costa Rica. *Exp. Mycologia.* **86:** 187-198.

Borders, D. B., Doyle, T. and Engel, W. (1993). Marcel Dekker, Inc New York, NY, pp. 17-27.

Boursnell, J. G. (1950). *Anal. of Bota*. **14**: 217-243.

Buswell, J. A. and Chang, S. T. (1993). Edible mushrooms: *Attributes and applications*. Chang, S.T., J.A. Buswell and Miles, P.G. (Eds.), Gordon and Breach, Philadelphia, pp 297-394.

Cappuccino, J.G. and Sherman, N. (1999). *Mycrobiology a laboratory manual.* p 263-264

Dawit, A. (1999). Secondary products by solid state cultivation. **In:**bChemistry, *Biological and Pharmatical properties of African Medical plants.* International Organization for chemical Science in Development.(IOCD)-2nd international symposium.BL10. Febrauary 28th – March3rd Addis Ababa, Ethiopia.

Davoli, P. Engel, G. Werle, A. Sterner, O. and Anke, T. (2002). Neosordarin and hydroxysordarin, two new antifungal agents from *Sordaria araneosa*. *J. Antibo*. **55:** 377-382.

Dix, N. J. and Webster, J. (1995). Fungal Ecology. Chapman & Hall, London, UK. p 497

Dreytuss, M. M. and Chapela, I. H. (1994). Potential of fungi in the discovery of novel, low molecular weight pharmaceuticals. **In:** *The Discovery of Natural Products with Therapeutic Potential* (V.P. Gullo, Ed), Butterworth-Heinemann, Boston, MA, pp: 49-80.

Durrant, A. J. Wood, D.A. and Cain, R.B. (1991). Lignocellulose biodegradation by *Agaricus bisporus* during solid substrate fermentation. *J. Gen. Microbiol.* **137:**751-755.

Ellis, M.B. (1971). Dematiaceous Hyphomycetes. *Commonwealth Mycological Institute*, Kew, Surrey, England. p 608.

Ely, R., Supriya, T. and Naik, C.G. (2004). Antimicrobial activity of marine organisms collected off the coast of South East India. *J. of Experimt. Marine Biol. and Ecol.* **304:** 121-127.

Farjon, A. (1993). The Taxonomy of Multi-seed Junipers (*Juniperus* sect. Sabina) in SW Asia and East Africa. *J. of Bota.* **49:** 251-283.

Furtado, N. M. Duarte, C. T. Albuquerque, A. J. Mello, S. C. and Bastos, J. K. (2005). Improvement of trypanocidal metabolites production by *Aspergillus fumigatus* using neutral networks. *Microb. Res.***160:** 141-148.

Gordon, M. C. and David, J. N. (2005). International collaboration in drug discovery and development from natural sources. *Pure Appl. Chem*. **77:** 1923-1942.

Greenstein, M. Wildey, M. J. and Maiese, W. M. (1989). The biochemical induction assay and its application in the detection of the calicheamicins. **In**: *Enediyne Antibiotics as Antitumor Agents*, Hughes, S.J. New Zealand fungi 33. Some new species and new record of *dematiaceous hyphomycetes*. *J. of Bota*. **27:** 449-459.

Hawhswarth, D.L.(1991). The fungal dimension of biodiversity: magnitude, significance and conservation. *Mycol. Res*. **95:** 641-655.

Houwink, A. L. (1987). Rect. Trav. Bot. Neert. p 32-51.

Hughes, S.J. 1989. New Zealand fungi 33. Some new species and new record of dematiaceous hyphomycetes. New Zealand *J. Bota.* **27:** 449-459.

Hwang, B. K. Lee, J. Y. and Kim, B. S. (2001). Isolation and in- vivo and in-vitro antifungal activity of phenolic acid and sodium phenolacetate from *Streptomycetes humidus. Appl. Environ. Microbiol.* **67:** 337-374.

Johns, M.R. (1992). Production of secondary metabolites. Solid Substrate Cultivation. *J. Microbiol*. **17:** 341–352.

Jonathan, S.C. and Fasidi, I. O. (2003). Antimicrobial activities of two Nigerian edible macro-fungi, *Lycoperdium pusilum* and *L. giganteum*. *Afr. J. Biomed. Res*. **6:** 85-90.

Kim, S. Shin, D. Lee, T. and Oh, K. (2004). Periconicins, two new fusicoccane diterpenes produced by an endophytioc fungus *Periconia* sp. with antibacterial activity. *J. Nat. Prod.* **37:** 448-450.

Lu, H., W.X.Z. Zou, Meng, J. C. Hu, J. and R. X. Tan, (2000). New bioactive metabolites produced by *Colletotrichum* sp. an endophytic fungus in *Artemisia annua. Plant Sci.,* **151:** 67-75.

Manoch, L. Kokaew, J. Jeamjitt, O. and Dethoup. T. (2006 a). Leaf litter fungi and studies on antagonistic effect against plant pathogenic fungi *in vitro*. pp. 771-780. **In**: *The Proceedings of 44th Kasetsart University Annual Conference*, Subject: plants. Kasetsart University, Bangkok. 30 January . 2 February 2006.

Manoch, L., O. Jeamjitt J. Kokaew and T. Dethoup. 2006 b. Morphological study of leaf litter fungi. *J. of Microbiol*. **20(1):** 231-232.

Manoch, L., Jeamjitt, T. Dethoup, O. Kokaew, J. Eamvijarn, A. Poochinya, P. and Paopun, Y. (2007). Morphological study of some noteworthy fungi from soil and plant. *J. of Microbiol.* **21:** 158-159.

Markiewicz, Z. and Kwiatkowski, Z. (2001). *Bacteria, antibiotics drug resistance* (polish).P.W.N Warsaw

Mierau, V., Anke, T. and Sterner, O. (2003). Dacrymenone and VM 3298-2- new antibiotics with antibacterial and antifungal activity. *Z. Naturforsch.* **58:** 541-546

Nigam, P. and Singh, D. (1994). Solid-state (substrate) fermentation systems and their applications in biotechnology. *J Basic Microbiol.* **34:** 405–423.

Paopun, Y., Poochinya, P., Umrung, P. and Kongpukdee, C. (2006). Electron microscopic techniques for pollen ornamentation of *Oryza sativa* L. *J. of Microscop. Soc. of Thailand.* **20:** 233-234.

Petit, K.E., Mondeguer, F., Roquebert, M.F., Biard, J.F. and Pouchus, Y.F. (2004). Detection of griseofulvin in a marine strain of *Penicillium waksmanii* by ion trab mass spectrometry. *J. of Microbiol. Met.* **58:** 59-65.

Rios, J. L., Recio, M. C. and Villar, A. (1998). Screening methods of natural products with antimicrobial activity: A review of the literature. *J. Ethnopharmacol*. **23:** 127-149.

Robinson, T., Singh, D. and Nigam P. (2004). Solid-state fermentation: a promising microbial technology for secondary metabolite production. *Appl. Microbiol. Biotechnol*. **26:**191-194.

Rodrigues, K. F., Hesse, M. and Werner, C. (2000). Antimicrobial activities of secondary metabolites produced by endophytic fungus of Spondia mombin. *J. Basic Microbil*. **40:** 261-267.

Rukachaisirikul, V., Kaewbumrung, C. Phongpaichi, S. and Hajiwangoh, Z. (2005). Eudesmane sesquiterpene from the aquatic fungua *Beltrania rhombica*. *Chem. Pharm. Bull.* **53:** 238-240.

Saparrat, M. C. N., Cabello, M. N. and Arambarri, A. M. (2002). Extracellular laccase activity in *Tetraploa aristata*. *Biotechnology Letters.* **24:** 1375-1377.

Saravanan, T. (2004). Studies on the Biodiversity of Micro-fungi in the Eastern Ghats of Tamilnadu, India. Ph.D. *Thesis.University of Madras*. India. p. 201

Shibaoka, T. (1988). Annu. Rev. Plant Physiology. p- 20-25.

Singh, M. P., Petersen, P. J. Weiss, W. J. Kong, F. Greenstein, and Saccharomicins, M. (2000**).** novel heptadecaglycoside antibiotics produced by *S. espanaensis*: Antibacterial and mechanistic activities. *Antimicrob. Agents Chemother. **44***: 2154-2159.

Spiteller, M. and Qazi, G. N. (2006). Bioactive molecules from endophytic microorganisms: http:www. Rrhajammu.org/people/GNQazi/Dr Qazi-homepage.htm.

Steffen, K. T., Hofrichter, M. and Hatakka, A. (2000). Mineralisation of 14C-labelled 52 synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidiomycetous fungi. *Appl. Microbiol. Biotechnol.* **54:**819-825.

Steffen, K. T., Hofrichter, M. and Hatakka, A. (2002). Purification and characterization of manganese peroxidases from the litter-decomposing basidiomycetes *Agrocybe praecox* and *Stropharia coronilla*. *Enzyme Microb. Technol*. **30:**550-555.

Suffness, M. and Douros, J. (1979). Drugs of plant origin. *Met. in canc. rese.* **16:** 73-126.

Tokumasu, S., Tubaki, K. and Manoch, L. (1997). Microfungal communities on decaying pine needles in Thailand. **In:** Tropical Mycology (eds. K.K. Janardhanan, K.R. Natarajan and D.L. Hawksworth). Science Publishers Inc, USA: 93-106.

Vijayakumar, E.K.S., K. Roy, S.Chatterjee, S.K. Deshmukh, B.N.Ganguli.,H.W. Fehlhaber and Kogler, H. (1996). Arthrichitin, a new cell wall active metabolite from *Arthrinium phaeospermum.* *J. Org. Chem*. **61:** 6591-6593.

Villanova, P.A. (**1991**). National Committee for Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard M7-A*; National Committee for Clinical Laboratory Standards. *J. Antibi*. **5:***432-439.*

Wei Pei-lian, Xu Zhi-nan and Cen, Pei-lin. (2006). Lovastatin production by *Aspergillus terreus* in solid-state fermentation, *Zhejiang University Press*. pp. 1521-1526,

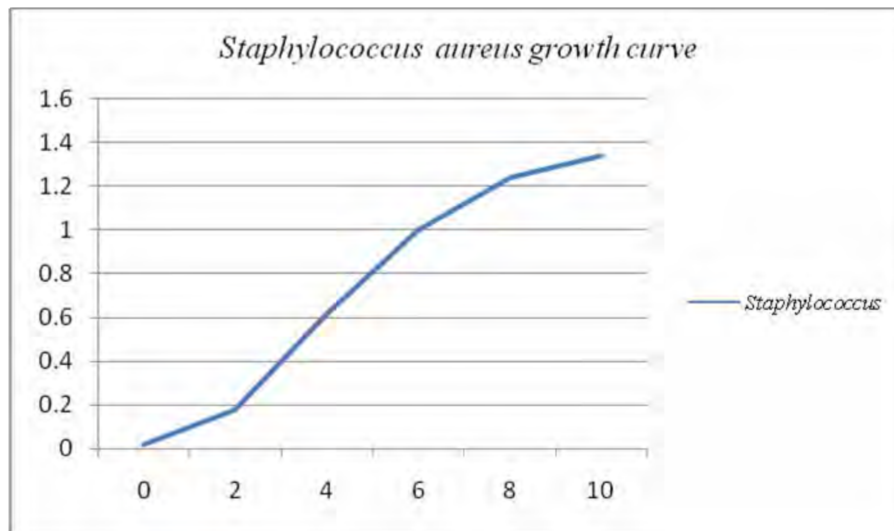
Zhang, B.S., Alituro, G. Szalkoqski, D. Zhang, Y. and Moller, D.E. (1999). Discovery of small molecules of insulin mimetic with antidiabetic activity in mice. *Science***. 284:** 974-977.

10. APPENDIES

Appendix.1

Time	0	2	4	6	8	10
OD	0.02	0.18	0.62	1.00	1.24	1.34

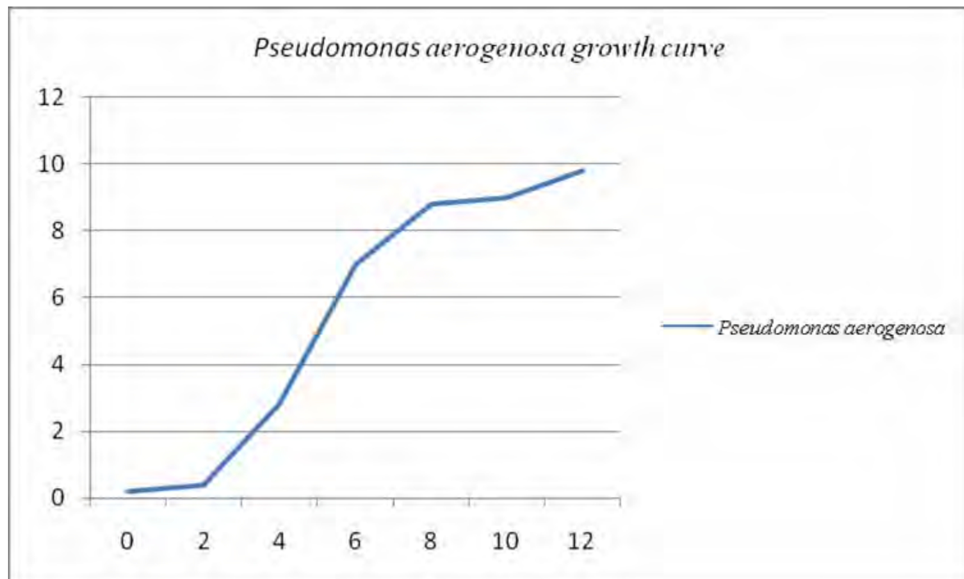
OD



Time

Time	0	2	4	6	8	10	12
OD	0.1	0.2	2.0	4.4	6.0	7.2	8.8

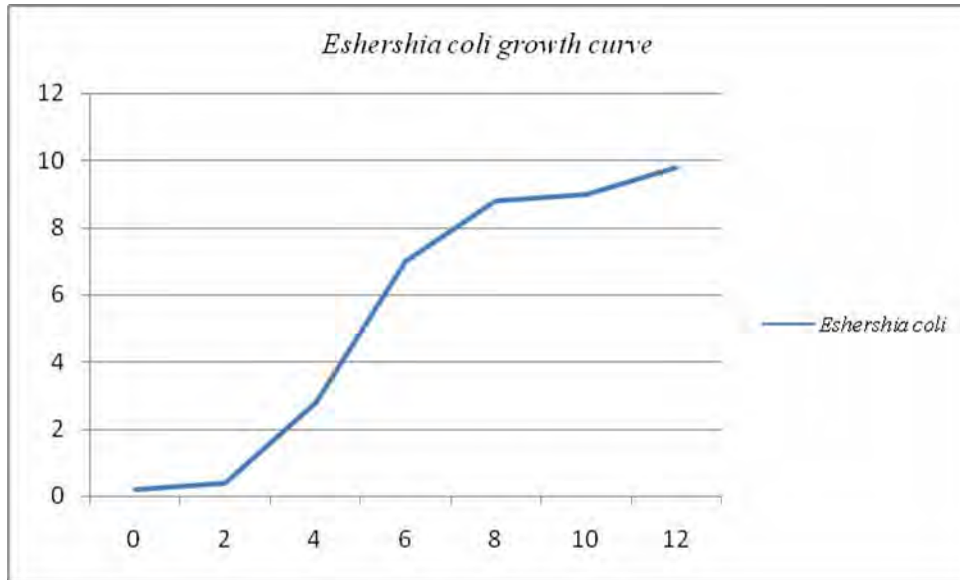
OD



Time

Time	0	2	4	6	8	10	12
OD	0.2	0.4	2.8	7.0	8.8	9.0	9.8

OD



Time

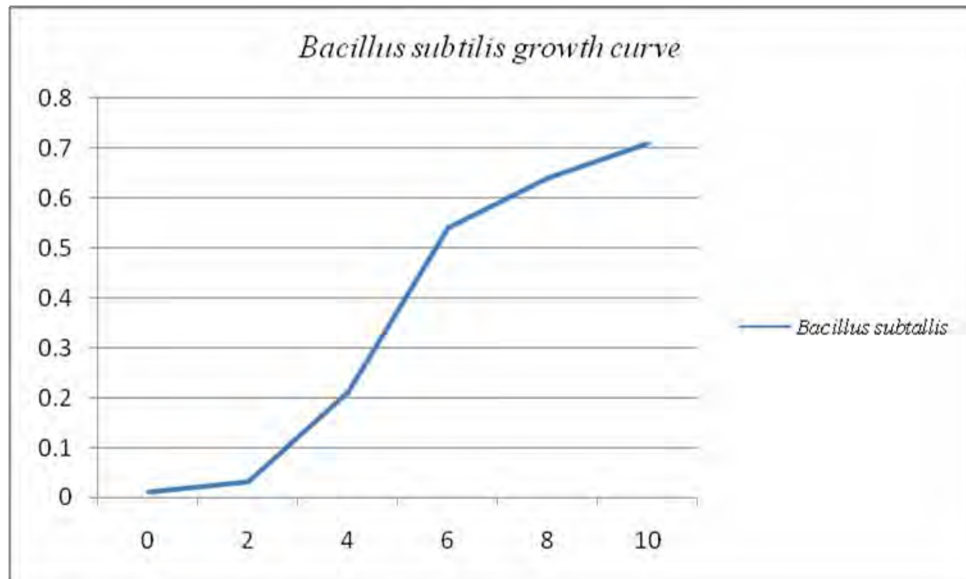
Time	0	2	4	6	8	10
OD	0.01	0.04	0.14	0.47	0.80	0.90

OD

Time

Time	0	2	4	6	8	10
OD	0.1	0.3	0.21	0.54	0.64	0.71

OD



Time

Time	0	2	4	6	8	10
OD	0.02	0.04	0.24	0.51	0.57	0.62

OD

Time

Figure 7. Showing growth curve of the test bacterial pathogens.

Appendix.2

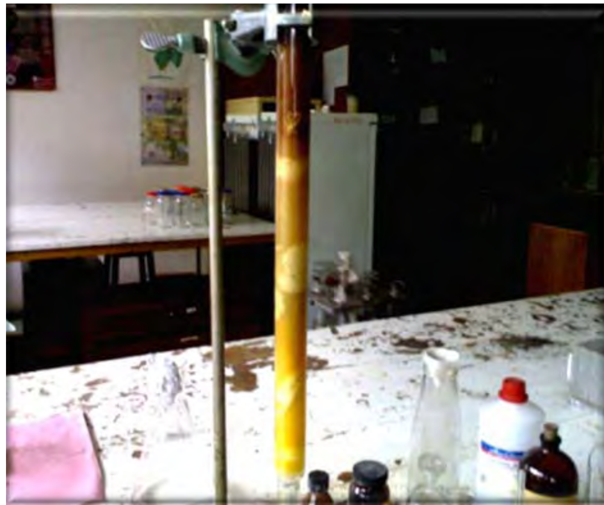
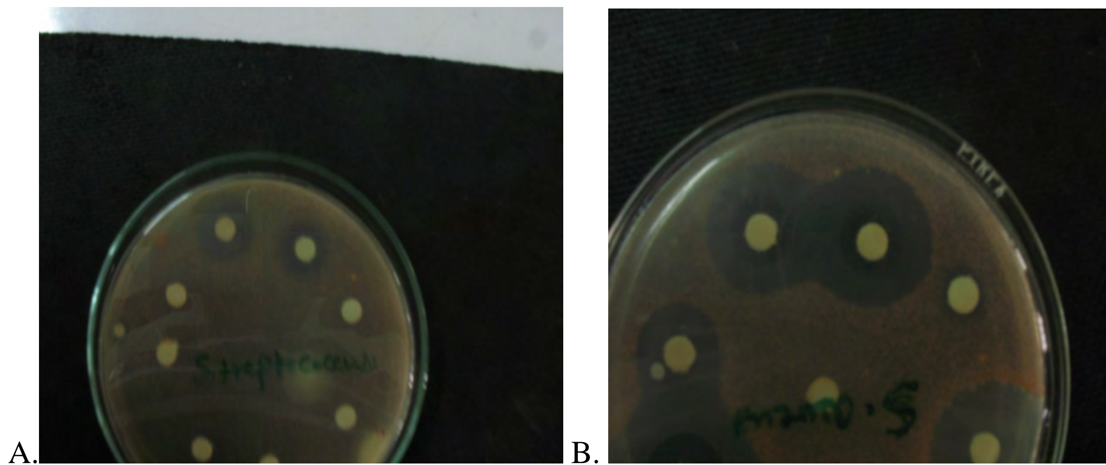


Figure 12. Isolation of bioactive compound (yellow) using CC

Appendix.3



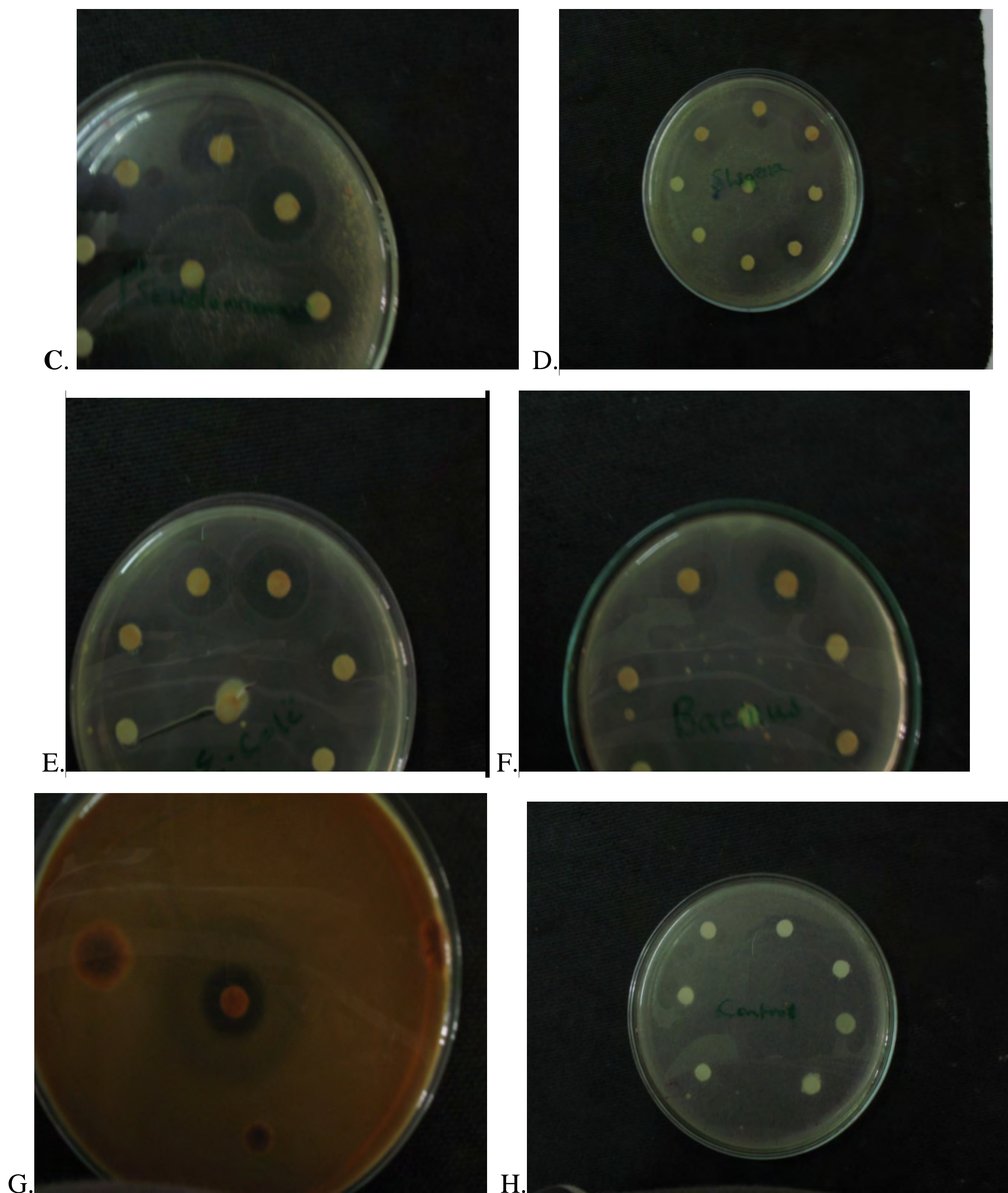


Figure 13. Antimicrobial activity of the pure compound

A. *Streptococcus pneumoneae*

B. *Staphylococcus aureus*

C. *Pseudomonas aerugenosa*

D. *Shigella obydii*

E. *Eshershia coli*

F. *Bacillus subtilis*

G. *Candidia albicans*

H. Control group (Chloramphenicol)

.