The Antibiotic Properties of Several Strains of Fungi

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Abstract: A fractions obtained from the culture fluids of a seven fungal species identified as *Verticillium albo-atrum*; *V. lecanii*; *V. bulbillosum*; *V. dahliae*; *Fusarium solani*; *F. oxysporum*, and *Aspergillus sclerociarum* were shown to contain a compounds with a biological activity against twenty two clinical bacterial strains including eleven isolates of gram positive and other were gram negative. Among tested fungal extracts *Verticillium albo-atrum* and *V. lecanii* exhibited significant antibacterial potency. Fractions were clearly more active on Gram-positive cocci than Gram-negative bacilli. Determination, isolation and purified of these fractions were carried out using column and one- and/or two-dimensional thin layer chromatography. IR, NMR, and mass spectrum analysis were used to identify the chemical structures of these active compounds. This study revealed that one isolate of fungal species could contain more than one compound acting as a broad spectrum antibacterial agents where two antibacterial compounds were isolated from *V. albo-atrum* extract and only one compound isolated from *V. lecanii*

Key words: Antibacterial, Verticillium, Fusarium, Fungi, Bioassay.

INTRODUCTION

Rapid emergence of antibacterial resistance is well documented as a serious problem worldwide. This situation shows that the potencies of prevalent antibiotics are decreasing steadily. This situation implies the need for searching new antimicrobial to replace with invalided ones or use in antibiotic rotation programs (Barbara, D.J. and E. Clewes, 2003 and Shahghasi, A., 2004). Discovery and development of new antibiotics is still very important because of the constant appearance of drug-resistant pathogenic bacteria. The broth micro-dilution test was applied for screening of antibacterial activity in extracts of fungi (Zrimec, M.B., 2004). Since the discovery of penicillin, a number of antibiotics have been discovered mostly from soil inhabiting microorganisms. During the last 20-30 years, the interest in the soil micro-flora increased due to the investigation of novel bioactive compounds, especially antibiotics and enzymes, active in severe environmental conditions (low temperature, low humidity, high radiation etc.) (Nedialkova, D. and M. Naidenova, 2004).

Plant roots surface (rhizoplane) and soil around the roots (rhizosphere) are the zones of intensified microbial activity due to secretion of root exudates (which contains sugars, amino acid vitamins, etc.) resulting in competition among the microbes for nutrition, a lot of sporulating and non-sporulating bacteria, some yeasts species, actinomycetes, and fungi were found (Jay, W., 2008; Townsend, A.M., 1990). The members of the last group were not less than other microorganisms but their number of species of these group were also large. Mainly, representatives of the genera *Verticillium, Fusarium, Paecilomyces, Chaetominum, Alternaria, Penicillium* and *Aspergillus*, etc were occurred. The competitive fungal species utilize their toxic metabolites enzymes, or antibiotics for their establishment in these regions, hence providing unexhausted source of antibiotic (Amey, R.C., 2003; Jay, W., 2008).

Parallel with the investigations of the microbial diversity of the permanently frozen continent, the biochemical characteristics of the microorganisms were examined too. It was found that most of the fungal isolates possessed high proteolytic, cellulase and chitinase activity and it was also mentioned about an antibacterial one. That points the possible directions in which the local microflora researches have to be developed (Khalidak, Z., 2003; Khiareddine, H.J., 2007).

*Verticillium; Fusarium* and *Aspergillus* are a large fungal species with representatives found all over the world. These fungi are often pathogenic, causing health problems for plants; animals and human they infect;
they also can be cause a serious problem for the agricultural industry (Miller, S.A., 2006; Yoshitaka, I., 2007). Certain Verticillium species can used in natural as pest control (Townsend, A.M., 1990; Goud, J.C., 2004). Farmers can spray fungus spores over a crop to protect it from attack of insects. Nematodes can also be managed with Verticillium applications (Veshkurova, O., 2006; Jeong, J.K., 2005).

A novel compounds derived from fungi of genus Verticillium, especially the fungus Verticillium balanoides useful for inhibiting activity of enzyme protein kinase C. Since the activation of protein kinase C has been implicated in several human disease processes, including cancer tumors, inflammation, and reperfusion injury, inhibition of protein kinase C should be of great therapeutic value in treating these conditions (Domenech, J., 2002; Goud, J.C., 2004; Damjan, J., 2007).

As well as being common plant pathogens, Fusarium spp. are causative agents of superficial and systemic infections in humans. Infections due to Fusarium spp. are collectively referred to as fusariosis. The most virulent Fusarium spp. are Fusarium solani (Mayayo, E., 1999). Trauma is the major predisposing factor for development of cutaneous infections due to Fusarium strains. Keratitis, endophthalmitis, otitis media, onychomycosis, cutaneous infections, particularly of burn wounds, mycetoma, sinusitis, pulmonary infections, endocarditis, peritonitis, central venous catheter infections, septic arthritis, disseminated infections, and fungemia due to Fusarium spp. have been reported (Arikan, S., 2001). Fusarium spp. produces mycotoxins. Ingestion of grains contaminated with these toxins may give rise to allergic symptoms or be carcinogenic in long-term consumption.

Fumonisins are the mycotoxins produced by Fusarium moniliforme and Fusarium proliferatum in maize. They may cause oesophageal cancer. Earlier papers described the production of an (Mandeel, Q. A., 1999; Kovacicova, G., 2001) antibiotic pigment, javanicin, from Fusarium javanimm, and the isolation of five antibiotic substances from strains of F. lateritium, F. fmtigenum, F. sambucinum and F. avenaceum.

MATERIALS AND METHODS

Source of Fungi Used:

Samples of fungal isolates used in this investigation were isolated from the soil of Vinnea and Egypt. Four Verticillium species (V. albo-atrum; V. lecanii; V. bulbillosum and V. dahliae) were isolated and investigated in Vienna soil. Prof. Dr. Gert Backman, University of Vienna, Austria generously provided the cultures. Two Fusarium species (F. solani and F. oxysporum) and Aspergillus sclerociarum isolated from soil of Egypt and identified in the identification unit of The Regional Center of Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt were kindly provided for this investigation.

Pathogenic Bacterial Isolates Used:

Twenty-two human pathogenic bacteria employed for this investigation including eleven-gram positive bacteria and the other gram-negative bacteria were listed in table (1). All strains were isolated, identified and kindly provided from culture collection of the El-Demertash Hospital.

<table>
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<th>Bacteria name</th>
<th>Serial code</th>
<th>Bacteria name</th>
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<td>Streptococcus pneumonia</td>
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<td>Salmonella typhi</td>
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Media and Cultivation Conditions:
Nutrient agar and Malt Extract Agar used for maintenance of stock bacterial and fungal cultures. Nutrient agar medium was used for bacterial growth [Beef Extract, 3.0g; Bacteriological Peptone, 5.0g; Agar, 20.0g,] the pH was adjusted at 6.2 ± 0.2 at 25 (±2)°C. while Malt Extract Agar (MEA) was used for fungal growth [Malt extract, 20.0g; Bacteriological Peptone, 1.0g; glucose, 20g; Agar, 20.0g]. The pH of the medium was adjusted to 5.4 ± 0.2 at 25 (±2)°C.

Yeast Extract Sucrose broth (YES) was used for preparation of fungal extract (their secondary metabolite profiles studies as antibacterial agents) [Yeast extract, 20; sucrose, 150]. All media used were prepared by dissolving the solid ingredients in 1 liter of cold distilled water and then heating to 60-70 °C with stirring. Media was sterilized by autoclaving at 121°C (1.5 atm.) for 15-20 minutes (Atlas, R.M., 1993).

Preparation of Crude Extracts from Fungal Isolates:
Inoculation of fungal isolates in flasks each contains 100 ml of YES medium incubate for 21 days at 28°C. The flasks were then aseptically filtered and the filtrate was concentrated using speed vacuum device (Maxi Dry Plus). Methanol/chloroform (1:2, v/v) was used for extraction of fungal secondary metabolites. Concentrated YES broth obtained from fungal cultures was mixed individually with chloroform/methanol. The mixture was shaken vigorously in a separating funnel and left to settle down forming a dense lower aqueous layer containing the secondary metabolites. Each extract was concentrated by evaporation of solvent under reduced pressure using an evaporator at a temperature not exceeding than 50 (±2)°C (Nedialkova, D. and M. Naidenova, 2004).

Antibacterial Assays:
Diffusion agar technique was carried out. Antibacterial potentiality of fungal extracts against several bacterial isolates was expressed as the measurement of diameter of their inhibition zone. 1 cm diameter of holes were made using sterile cork borer in Nutrient agar sterile plates (10x 10 cm), which had previously been seeded with tested bacterial isolates. Holes were filled with 100 μL of different fungal extracts. Control holes were filled with solvent used. Plates were left in a cooled incubator at 4 (±2)°C for one hour and then incubated at 37 (±2) °C. Inhibition zones developed was measured after 24 hours of incubation time (Nicola, S.I., 2005). Amoxicilli was used as a standard antibacterial agent.

Identification Assay for the Most Active Fungal Compounds:
For identifying the highly active compounds extracted from fungal isolates, purification techniques were carried out, the active crude extracts were subjected to fractionation as follows:

A- Column Chromatography:
For Column Packing and Equilibration: Column chromatography technique was carried out to separate the most pure and active compounds as antibacterial agents to determinate their chemical structures. Ten millilitre from each active extract were chromatographed on a column (1.5 cm diameter and 50 cm long) of silica gel (G 100) after activation at 80 (±2)°C for 30 min., the column was then eluted with chloroform and methanol (90: 10) v/v. The space above the silica gel was filled with the eluent to about 1 cm below the top of the column (Robbers, J.E., 1996).

Traces of air were removed through the air inlet in the top piece of the column. Elution was started immediately after filling the column in order to obtain an even sedimentation. In order to stabilize and equilibrate the bed, the gradient volume of crude extract was passed through the column; the fractions (each 1 ml) were collected separately. All fractions were stored at 4°C for repurified by TLC technique. Before starting any experiment, it was advisable to check the homogeneity of the bed by running through it a coloured substance. Before sample application, the upper surface of the gel bed was protected with a piece of suitable material for example Whatman No. 1 filter paper (Yousssry, A.A., 1998). In vitro, re-inoculation of all fractions with tested bacteria was carried out to determinate the most active fraction from each extract for further techniques.

B- Thin layer Chromatography (TLC):
Thin layer chromatography technique (TLC) was used for further separation and partial purification of the most active inhibiting components as antibacterial potency. TLC plates (20 x 20 cm Merk aluminum sheet,
silica gel 60, layer thickness 0.2 mm) were used. The diluted active (bacterial inhibitor) fractions were spotted at the start of the silica gel plates, and allowed to dry before applying other spots. Spot of terbinafine (Sigma) dissolved in chloroform / methanol (2:1, v/v) was used as a reference standard. Samples were chromatographed for 17 cm in toluene: ethyl acetate: 90% formic acid (5:4:1, v/v/v) (TEF) in a solvent saturated atmosphere, then allowed to air dry.

TLC plates were examined under white and UV light (365 nm) and the characteristics of the spots were recorded, also were examined under UV light (254 nm), and then back to 365 nm to visualize the intensity of spots and calculate their Rf values. TLC plates were sprayed with ceric sulphate in 3 M sulphuric acid, and examined under white and UV light (365 and 254 nm) (Bettelheim, F. and J. Landesberg, 1995; Evans, W.C., 1996). Scratching each band and re-examined there activity as antibacterial agent were done to determine the active and most pure compound for chemical identification.

C- Chemical Analyses:
For identify the chemical structure of the active compound the following spectra measurements were carried out:

1- Infra Red Spectra:
Infrared absorption spectrum was estimated using anicum infinity series FTIR, Perkin – Elmer 1650 Spectrophotometer, at Micro Analysis Center, Cairo University.

2- Nuclear Magnetic Resonance (NMR):
Proton (1H) NMR spectra were estimated using FT-NMR Braker Ac 200 spectrometer, at Micro Analysis Center, Cairo University.

3- Mass Spectroscopy:
Electron impact (MS) spectrometric spectrum was estimated using Shimadzu QP-5050 GC-MS at the Regional Center for Mycology and Biotechnology (RCMB) AL- Azhar University.

RESULTS AND DISCUSSION

Antibacterial Assay:
The antibacterial activities of extra-cellular secondary metabolites of local and international fungal species used were screened by an agar disc diffusion technique against selected groups of pathogenic Gram-positive and Gram-negative bacteria. A significant variation of inhibition of bacterial growth was recorded in table (1) and figures (1 &2).

Antibiotic potency is expressed as the highest dilution of fungal fluid tested which completely inhibited the growth of the test bacteria; thus generally, table (1) revealed that, gram positive bacteria were the most sensitive to several fungal extracts than gram negative. Extracts of Verticillium albo-atrum exhibited the most active antibacterial agent followed by Verticillium lecanii extract and then Fusarium solani while other fungal extracts varied in their potency as antibacterial agents.

However, Streptomyces clavuligerus was highly susceptible to Verticillium albo-atrum extract which exhibited 3.2 cm inhibition zones followed by Streptococcus pneumoniae (3.0 cm inhibition the growth), other bacterial strains exert varied sensitivity against Verticillium albo-atrum extract from 2.5 cm inhibition against Staphylococcus haemolyticus and Acinetobacter baumannii to 0.9 cm inhibition against Mycobacterium marinum growth (figure 1 &2).

As well as, Escherichia coli exhibited highest sensitive bacteria to Verticillium lecanii extract (3.5 cm inhibition) while Mycobacterium marinum was the least sensitive one to the same extract where (0.5 cm inhibition) was recorded in table (2). In addition, only Vibrio damsella organism showed high resist to same effect (0.0 cm inhibition).

In the present study revealed that Fusarium solani extract had differential antibacterial activity against most tested organisms, whereas 0.8 cm inhibition was the minimum effect that was obtained on Enterobacter aerogenes growth while 2.3 cm was the maximum effect inhibition that was observed against Bacillus cepatia. On contrast, Fusarium solani extract did not showed any ability to inhibit the growth of Streptomyces clavuligerus, Escherichia coli and Alcaligenes eutrophus (0.0 cm inhibition).

Previously, a total of 37 Fusarium isolates were recovered from Bahrain, using soil dilution and soil washing techniques, and plated onto Komada selective media. Of Among international Fusarium species, the highest inhibitory activity was reported against Gram-positive bacteria mor than Gram negative bacteria (Mandeel, Q., 1999) while F. oxysporium exhibited great reduction of S. aureus growth by another study (Marcato, P.D., 2005).
Fig. (1 A&B): *In vitro* antibacterial activity of fungal extra-metabolites on pathogenic gram positive bacterial

Fig. (2 A&B): *In vitro* antibacterial activity of fungal extra-metabolites on pathogenic gram negative bacterial
Table 2: *In vitro* antibacterial activity of fungal extra-metabolites on pathogenic bacterial strains

<table>
<thead>
<tr>
<th>Fungal Extracts</th>
<th>Verticillium lecanii</th>
<th>Verticillium dahliae</th>
<th>Verticillium bulbillose</th>
<th>Verticillium albo-atrum</th>
<th>Fusarium solani</th>
<th>Fusarium oxysporum</th>
<th>Aspergillus sclerociarum</th>
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<td>2.0</td>
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<td>2.3</td>
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<td>1.9</td>
<td>2.3</td>
<td>2.0</td>
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<td>1.5</td>
<td>2.1</td>
<td>2.0</td>
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<td>1.5</td>
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</table>

On the other hand, the present results obtained from figures (1 and 2) showed remarkable difference in the antibacterial effect of other extracts against tested bacterial strains. However, the bacterial inhibition ranged from 2.6 cm on *Nocardia asteroides* by *Fusarium oxysporum* extract to 0.5 cm inhibition against the growth of *Alcaligenes eutrophus* by *Verticillium dahliae* extract.

As well as, previous studies on antimicrobial properties of some fungi were carried out. A fraction obtained from the culture fluids of *Pycnoporus sanguineus* fungus was shown to contain a compound with biological activity against strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and members of the genus *Streptococcus*. The fraction was clearly more active on Gram-positive cocci than on Gram-negative bacilli (Smâniaa, A., 1995). Also, a total of 227 isolates of fungi were cultivated on different media and the secondary metabolite content of the extracts (ethyl acetate/chloroform/methanol 3:2:1) characterized by HPLC. The extracts were tested for antibacterial activity. A total of 7 were active towards *Vibrio parahaemolyticus* and 55 towards *Staphylococcus aureus*, representing 18 different fungal species from 8 ascomycetous genera. For 61 strains of *Penicillium citrinum* antibacterial activity correlated well with content of secondary metabolites as measured by HPLC. Thirteen isolates of *Penicillium steckii* produced very similar profiles of secondary metabolites and 6 of these had activity against either *V. parahaemolyticus* or *S. aureus* or both (Christophersen, C., 1999).

On the other hand, the antibacterial activity of fungi extracts was evaluated by a test of efficiency. The extracts of *Aspergillus ochraceus* and *Penicillium citrinum* present wide spectral antibacterial properties, inhibiting 100% and 80% of the developing germs, especially *Pseudomonas aeruginosa* and *Escherichia coli*. These fungi can be an important source of antibacterial secondary metabolites (Machalskis, I.C., 2000).
In a previous study, from 1,300 soil Actinomycete isolates collected from different localities *Streptomyces* sp. isolate showed widest antibacterial activity. The active principle named as Broadspectrim. It showed antibacterial activity against wide range of G+ and G- bacteria as *Bacillus anthracis*, *Bacillus subtilis*, *Citrobacter diversus*, *Citrobacter freundii*, *Corynebacterium diphtheriae*, *Enterobacter* sp., *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteum*, *Proteus vulgaris*, *Proteus rettgeri*, *Proteus mirabilis*, *Proteus morgani*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas syringae pv syringae*, *Salmonella Para typhi*, *Salmonella typhi*, *Sarcinia* sp., *S. marcescens*, *Shigella dysentery*, *S. flexneri*, *S. sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Vibrio cholera* and *Xanthomonas* sp. but *Shigella flexneri* and *Staphylococcus albus* were resistant to it (Shahghasi, A., 2004). And two fungi species, *Serpula lacrymans* and *Nectria vilior*, were found to be a potential new source of thermostable antibiotics. They were found to be a useful method for antibacterial activity screening from the samples of natural origin (Zrimec, M.B., 2004).

**B- Isolation & Identification of the Most Active Fungal Components Act as Antibacterial agent:**

The antibacterial properties of the most active two fungal extract (extra-cellular metabolites) have been recognized. In the present study, chloroform/methanol extract from *Verticillium albo-atrum* and *Verticillium lecanii* showed a great ability to inhibit most of pathogenic bacterial isolates, hence, this extracts was subjected to column and TLC techniques to determinate the most active purified compound. For determination the chemical structure of active fungal compounds GC/MS, IR and NMR analysis were occurred.

**B-I- Verticillium Albo-atrum Compound:**

Two fractions exhibited antibacterial activity from *Verticillium albo-atrum* extra-metabolite after fractionated by column chromatography technique (fraction number 5 and the other number 10).

**The First Fraction:**

IR spectroscopy analysis of the first fraction revealed the presence of OH group at 3776 cm⁻¹, (-CH) alkane at 2843 cm⁻¹. A band at 1713 appeared due to (C=O)group, while 1809 cm⁻¹ indicate the presence of alkene group (C=C) and a band at 1084 cm⁻¹ for C-O and confirmed of presence of OH group (figure 3 "A"). When the active pure compound was subjected to ¹H NMR analysis in DMSO (dimethylsulfoxide) it was found that; signals "δ" represented at 10.024 ppm for acid formation. While the signal at 7.267 indicated that an aromatic ring was present; Signal "δ" at 1.787 for (-OH) group; signal "δ" showed at 0.803 and 3.45 ppm indicated the presence of alkane (C-C) and alkene (C=C), respectively (figure 3 "B").

The mass in figure 3 (C) indicated the molecular formula of this compound is C₃₉ H₅₂ O₈ this compound could be identified as 3- [1-(1, methyl pent-1-enoi acid),4-[1,2-dimethyl propanoic acid] xylanlyl], 1-[1-(1,methyl-butanolic acid),4-pentanic acid) xylanlyl] prop-l-ene and its molecular structure was illustrated as:

![Molecular Structure](Attachment)

In previous study, polysaccharides compound were isolated from the cell-wall of three strains of *Verticillium fungicola* (Domenech, J., 2002).
The Second Fraction:

IR spectroscopy analysis showed the presence of NH amine group at 3410 cm⁻¹, aromatic ring at 1636 cm⁻¹. A band at 2920 appeared due to (CH) alkane group, (figure 4 "A"). ¹H NMR analysis found that; signals “δ” represented at 7.265 ppm for aromatic ring. While the signal at 1.668 indicated amine group (NH); Signal “δ” at 0.82 for (CH) alkane group (figure 4 "B"). The mass in figure 4 (C) indicated the molecular formula of this compound is C₄₀H₅₃N₈ identified as, [1',1′{(1-ethyl, 4-propyl) benzene diamine}, [1, ethyl, 4-methyl benzene diamine], 4 [(1- ethyl, 4- methyl benzene diamine, benzene diamine] ethane and molecular structure is illustrated as:

B-2- Verticillium Lecanii Compound:

One fraction act as antibacterial potency from Verticillium lecanii extract after purified by column and TLC chromatography techniques.

IR spectroscopy analysis of the first fraction revealed the presence of OH group at 3730 cm⁻¹, (CH) alkane at 2959 cm⁻¹. A band at 1736 appeared due to ketonic (C=O) group, while 1680 cm⁻¹ indicate the presence of alkene group (C=C) and a band at 1080 cm⁻¹ for C-O and a bant at 1462 to 1600 cm⁻¹ for aromatic ring (figure 5 “A”). ¹H NMR analysis showed that; signals “δ” represented at 10.024 ppm for acid formation. While the signal at 7.265 indicated that an aromatic ring was present; Signal “δ” at 1.529 for (–OH) group; signal “δ” showed at 0.845 for alkane (C-C) group and 3.485 ppm for alkene (C=C) group, signal at 3.41 ppm for CH₂-aromatic (figure 5 “B”). The mass in figure 5 (C) indicated the molecular formula of this compound is C₃₉H₅₁O₈. From the previous obtained data of compound it is closely similar to 1- [(2, methyl butanpicacid), 4-(pent-1-enoic acid) xylenyl], 3-(1-(1,methylbut-2- enoic),4-butanoic acid) xylenyl] prop-1-ene and its molecular structure is illustrated as:
Fig. 4: Spectra analysis of second compound extracted from extra-metabolites of *Verticillium albo-atrum*

- A - IR spectra
- B - NMR spectra
- C & D - Mass spectra

In previous studies, many compounds were isolated from the mycelial of *Ampelomyces* sp. fungus by antibacterial assay-guided fractionation. Their structures were elucidated on the basis of spectroscopic analysis (Zhang, H., 2008). Also twelve species of fungi were collected and identified as, *Ganoderma lucidum, Fomes lignosus, Schizophyllum commune, Pleurotus florida, Lentinus subnudus, Leptoporus sp, Panus fulvus, Coriolus versicolor, Trametes saepiara, Trametes betulina, Daedalea elegans* and *Auricularia auricula*. The best antibacterial activity was recorded from the ethanolic extracts of *Pl. florida* and *Pa. fulvus*. Both extracts were found to be active against *S. aureus, Streptococcus* sp., *Strept. pyogenes, E. coli, K. pneumoniae*, and *Flavobacterium* sp. at the concentration of 1,000mg/ml respectively. Ethanolic extracts of *S. commune, C. versicolor* showed no inhibition against any of the test bacteria (Fagade, O.E. and A. Oyelade, 2009).

**Conclusion:**

Resistance of pathogenic bacteria to antibiotics leads scientists to discover new antibacterial drugs especially from nature sources. Examination of pure antibiotic substances isolated from the culture fluids of seven of fungal strains showed them to be both bacteriostatic and bactericidal; they were very active against gram positive bacteria and effective to a lesser degree against other Gram negative bacteria.
Fig. 5: Spectra analysis of compound extracted from extrametabolites of *Verticillium lecanii*

A - IR spectra   B - NMR spectra   C&D - Mass spectra

REFERENCES


