

New Bioactive Compounds from *Verticillium alboatum* and *Verticillium leptobactrum*.

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Abstract: Three new fungal metabolites were isolated and purified from the broth culture of two entomopathogenic fungi *Verticillium alboatum* and *Verticillium leptobactrum*. The obtained compounds were screened for their antibacterial, antifungal, antiviral and antitumor activity. To test antibacterial, anti-yeast and antifungal activity, the secondary metabolites obtained by culturing fungi on YES broth for 21 days was screened by using agar well diffusion method. The active ingredients were purified and the chemical structures of the new metabolites were elucidated using the spectroscopic methods FTIR, ¹HNMR and EI-MS analyses. The cytotoxic and antitumor effects of the compounds on Vero and HEP2 cells, respectively, were assayed. The first compound was benzene diamine derivative produced from fraction 5 of *Verticillium alboatum* and showed activity against bacteria, fungi and polio virus. Compound 2 was benzylamine derivative produced from fraction 5 of *Verticillium alboatum* with broad spectrum antimicrobial as well as weak antitumor activity. Compound 3 was butylamideaniline derivative obtained from *Verticillium leptobactrum* with antibacterial activity. The compound 3 also showed antitumor activity with acceptable therapeutic indexes. In conclusion: The study illustrates the biological activities of new fungal metabolites from *Verticillium alboatum* and *Verticillium leptobactrum*, and is therefore, a potential drug and a good candidate for further studies and development.

Key words: Secondary metabolites, *Verticillium alboatum* and *Verticillium leptobactrum*, spectroscopic methods, antibacterial, antifungal, antiviral and antitumor.

INTRODUCTION

Verticillium belongs to fungi in the division Ascomycota. Within the genus, diverse groups are formed comprising saprotrophs and parasites of higher plants, insects, nematodes, mollusc eggs and other fungi thus it can be seen that the genus is a wide ranging group of taxa characterised by simple but ill-defined characters. The genus may be broadly divided into three ecologically based groups 1) mycopathogens 2) entomopathogens (Zare and Gams, 2001) and 3) plant pathogens and related saprotrophs (Barbara and Clewes, 2003).

Numerous reports have highlighted the remarkable potential of *Verticillium* as a powerful biocontrol agent against several arthropod pests and some plant pathogens. It has a very wide host range: it is especially parasitic of aphids and whiteflies – two of the most common greenhouse pests (Askary *et al.*, 1999; Drummonds *et al.*, 1987). This ability has been used for production of commercial preparations: Vertalec and Mycotol (Koike *et al.*, 2004).

Verticel is a biological insecticide manufactured from *Verticillium* sp. effective against all soft bodied sucking insect pests like aphids, thrips, mealy, bugs, whitefly, scale insects and all types of mites (Barbara and Clewes, 2003).

Verticillium was also reported as an effective mycoparasite of several rust fungi, green mold and fungi causing root rot diseases (*Pythium ultimum*), as well as of some powdery mildew pathogens (Askary *et al.*, 1997; Benhaumau & Brodeur, 2000; Koike *et al.*, 2004; Spencer *et al.*, 1981; Verhaap *et al.*, 1996).

Antimicrobial agents have been in widespread and largely effective therapeutic use since their discovery in the 20th century. However, the emergence of multi-drug resistant pathogens now presents an increasing global challenge to both human and veterinary medicine. It is now widely acknowledged that there is a need to develop novel antimicrobial agents to minimize the threat of further antimicrobial resistance (Rachel, *et al.*, 2009).

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Public awareness of the negative impact of synthetic fungicide and bactericide residues on human health and environment has prompted withdrawal of some chemical antibiotics from the market are the reasons why many research groups investigate potential strategies to develop alternatives to synthetic antibiotics (Gan-Mor and Matthews, 2003).

Currently, natural compounds are the focus of some biotechnological companies looking for new bioactive agents (Schachter and Slimy, 2003).

Secondary fungal metabolites represent a diverse group of bioactive compounds characterized by their origin and biosynthetic pathways. In fungi they serve as regulators, chemical messengers in developmental processes, or as defense system for the survival of the organism against their environment (Schneider *et al.*, 2008).

Given that there has been some evidence to date suggesting that *Verticillium* spp. may have some antimicrobial properties, this study is intended to evaluate whether *Verticillium alboatrum* and *Verticillium leptobactrum* have the ability to produce antibacterial, antifungal, antitumor and antiviral agents.

MATERIALS AND METHODS

Organisms:

The two entomopathogenic fungi *Verticillium alboatrum* and *Verticillium leptobactrum* were a kind gift from Dr. Gert Bachmann University of Vienna and subcultured in the culture collection of the Regional Center for Mycology and Biotechnology.

Tested microorganisms, including 9 bacteria and 10 fungi, were challenged in this study to ascertain the antimicrobial properties of the two *Verticillium* extracts and were obtained from the culture collection of the Regional Center for Mycology and Biotechnology. Of the bacterial isolates selected, included some Gram-negative organisms while the other Gram-positive organisms. Of the fungi examined, five were yeasts, with the remaining five being filamentous fungi.

Cell Lines:

HEp-2 cells (human epidermoid larynx carcinoma cells) were obtained from the American Type Culture Collection (ATCC). Vero cells (African green monkey kidney cell line) were obtained from European Collection of Cell Cultures (ECACC).

Polio Virus:

Polio type 1 virus was kindly provided by Prof. Dr. Rifky El-Karamany as monovalent poliomyelitis vaccine (HEATmaker™). Virus stocks were prepared by infecting cell lines (1 day after passage at a ratio of 1:4, infected to uninfected cells) in plastic flasks at 37°C. The cells were checked by microscopy for cytopathic effects (CPE). At 75% CPE, the medium was decanted and frozen at -70°C (Rovozzo, and Burke, 1973). The virus was propagated and quantified in terms of the 50% tissue culture infective dose (TCID₅₀) by endpoint dilution (Flint *et al.*, 2000).

Media Used:

Malt Extract Agar (MEA) (Smith and Onion, 1983):

Ingredients in g/l: malt extract, 20.0; peptone, 1.0; glucose 20.0; agar, 20.0 and distilled water 1L. The pH medium was adjusted at 5.5. This medium was used for cultivation of *Verticillium* species and the tested pathogenic fungi.

Nutrient Agar (NA):

The medium was used to cultivate tested pathogenic bacteria. It contains (g/l) Beef extract, 3; Peptone, 5 and distilled water 1L.

Biosynthesis of Fungal Secondary Metabolites:

For the biosynthesis of extracellular fungal secondary metabolites, a semi-synthetic medium of yeast-extract sucrose (YES) liquid medium was used. The YES medium contains (g/L); Yeast extract, 20.0; sucrose, 150.0; distilled water, 1.0 L (Paterson and Bridge, 1994). For enhancement of secondary metabolite production, 1 ml trace element solution was added to 1 L YES medium. The trace element solution is prepared by dissolving 0.5 g magnesium sulphate, 0.5 g cupric sulphate and 0.5 g zinc sulphate in 100 ml distilled water. The pH was adjusted to 6.5 ±0.2 and then autoclaved at 121°C for 15 min. The spores were scrapped from the

mycelium of two tested *Verticillium* spp. after 10 days of growth at 25°C on MEA medium and suspended in sterile distilled water. Aliquots of 2 ml of spore suspension were used to inoculate 250 ml Erlenmeyer flasks, each containing 100 ml sterile YES liquid medium. The inoculated flasks were incubated at 28 °C for 21 day. The mycelium was then harvested by filtration. The filtrate was then concentrated and used as concentrated crude extracellular secondary metabolites. Each concentrated crude fungal extract was separately sterilized by filtration and further dilutions were made from the stock.

Extraction of the Secondary Metabolites:

Yeast extract sucrose broths (200 ml) obtained from fungal cultures was mixed with (200 ml) chloroform / methanol (2: 1, v/v). The mixture was shaken vigorously in a separating funnel and left to settle down forming a dense lower organic layer containing the secondary metabolite. Extracted metabolites were then concentrated (Robbers *et al* 1996) by using a speed vacuum device (Maxi Dry Plus) to a volume of 5 ml.

Purification of the Secondary Metabolites:

Column packing and equilibration: Two millimeters of crude extract of previous method were subjected to separation using column chromatography (1.5 cm diameter and 50cm long) packed with silica gel (G100) after activated at 80 °C for 30min, then subjected to elution with chloroform:methanol (2:1, v/v). In order to stabilize and equilibrate the bed, the void volume of crude fungal extract was passed through the column, and then 1ml fractions were collected separately. All fractions were stored at 0-4 °C (Youssry, 1998).

Antimicrobial Activity:

Antibacterial and antifungal activities were expressed as the diameter of inhibition zones; well diffusion method was used. Holes (1 cm diameter) were digger in the agar using sterile cork borer in sterile malt agar plates for fungi and sterile nutrient agar plates for bacteria, which had previously been uniformly seeded with tested microorganisms. The holes were filled by fungal filtrates of two tested *Verticillium* spp. (100 µl). Plates were left in a cooled incubator at 4 °C for one hour for diffusion and then incubated at 37 °C for tested bacteria and 28°C for tested fungi (Abdel-Kader and Seddkey, 1995). Inhibition zones developed due to active antimicrobial metabolites were measured after 24 hour of incubation for bacteria and 48 hour of incubation for fungi. The antibiotic ciprofloxacin (5 mg disk) (MAST Diagnostics Ltd., Bootle, Merseyside, UK), was used as the positive control. The choice of ciprofloxacin was guided by the fact that it is a broad-spectrum antibiotic, thus having antibacterial properties for both Gram-positive and Gram-negative organisms.

Evaluation of the Antiviral Activity:

A plaque reduction assay was performed for the evaluation of the antiviral activity according to the standard method described (Hill *et al.*, 1991; Harper, 2000). Briefly, monolayers of Vero cells grown on 96-well culture plates were infected with polio type 1 virus. After incubation for 1h to allow viral adsorption, the inoculum was aspirated and the cultures were overlaid with maintaining medium [MEM with 2% fetal calf serum] and 1% methylcellulose containing dilutions of the fungal metabolites. After an incubation time of 48h at 37°C, the plates were fixed with formalin, stained with crystal violet, air-dried and the number of plaques in each well was counted under a light microscope. Six wells overlaid with methylcellulose medium without the fungal metabolite were used as controls. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control - mean number of plaques in sample)/(mean number of plaques in control)] x 100.

Evaluation of the Antitumor Activity:

HEp-2 cells were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The monolayers of 10,000 HEp-2 cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24h at 37°C in a humidified incubator with 5%CO₂. The monolayers of HEp-2 cells were then washed with sterile phosphate buffered saline (PBS; 0.01 M pH 7.2) and simultaneously the cells were treated with 100 µl from different dilutions of fungal metabolites in fresh maintenance medium and incubated at 37°C. A control of untreated HEp-2 cells was made in the absence of fungal metabolites. Six wells were used for each concentration of the test sample. Every 24 h the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and read the absorbance at 590nm using ELISA reader after well mixing. The absorbance values from untreated cells were considered as 100% proliferation.

Cytotoxicity Assay: Cell toxicity was monitored on vero cells. Vero cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. The cytotoxicity assay was carried out using 0.1ml of cell suspension, containing 10,000 cells seeded in each well of a 96-well microtitre plate (Falcon, NJ, USA). Fresh maintenance medium containing different dilutions of the test sample was added after 24 h of seeding. Control cells were incubated without test sample. The microtitre plates were incubated at 37°C in a humidified incubator with 5% CO₂ for a period of 48 h. Six wells were used for each concentration of the test sample. The morphology of the cells was inspected daily and observed for microscopically detectable alterations, *i.e.*, loss of monolayer, granulation and vacuolization in the cytoplasm. The cytopathic effect (CPE) was scored (Vijayan *et al.*, 2004). The number of viable cells was determined using ELISA reader as previously mentioned before and the percentage of viability was calculated as $[1-(ODt/ODc)] \times 100\%$ where ODt is the mean optical density of wells treated with the fungal metabolites and ODc is the mean optical density of untreated cells.

Characterization of the Purified Active Fraction:

In order to determine the chemical structure of the active compound, the following spectroscopic measurements were carried out:

1- Infra Red spectra: Infrared absorption spectrum was carried out using anicun infinity series FTIR, Perkin-Elmer 1650 Spectrophotometer, at Micro Analysis Center, Cairo University.

2- Nuclear Magnetic Resonance (NMR): Proton (¹H) NMR spectra was carried out using FT-NMR Bruker Ac 200 spectrometer, at Micro Analysis Center, Cairo University.

3- Mass spectroscopy: Electron impact mass spectrometric spectrum was carried out using direct inlet unit (DI-50) in the Shimadzu QP-5050 GC-MS at the Regional Center for Mycology and Biotechnology, Al-Azhar University.

RESULTS AND DISCUSSION

Given that the development of newer drugs continues to rely heavily on the isolation of natural sources rather than applications based on rational drug design and combinatorial chemistry (Cragg and Newman, 2000), we reasoned that fungal secondary metabolites are potential targets for the discovery of novel chemotherapeutic agents. To reiterate, little is known about the biological activity of *Verticillium* species. In an attempt to address this issue, the objectives of the present research were first to investigate the biological activity of *Verticillium alboatrum* and *Verticillium leptobactrum*.

Screening new organisms for antibacterial activity and searching for new antibacterial drugs is important due to the constant generation of new antibiotic-resistant strains of pathogenic bacteria (Janes *et al.*, 2006). This study highlighted, for the first time, the antimicrobial activity of these two *Verticillium* species.

The antimicrobial activity of extracellular secondary metabolites of *Verticillium alboatrum* and *Verticillium leptobactrum* metabolites against nineteen bacterial, yeast and fungal pathogens is shown in Table 1. *Verticillium leptobactrum* metabolites demonstrated antibacterial activity against 8/9 (88.8%) of tested bacteria (zone of inhibition range (13-20 mm) except for *Proteus* which was resistant. *Verticillium alboatrum* metabolites exhibited high antibacterial activity hence zone of inhibition ranged from 18 to 30 mm when tested against the positive control (ciprofloxacin 5 mg disk) and exerted their activities against all (9/9; 100 %) the bacteria tested. *Verticillium alboatrum* metabolites also exhibited an antifungal activity (9/10; 90 %) against four yeast species and five mould species except for the zygomycetous fungi *Syncephalastrum racemosum* and gave variable zone of inhibition (range: 12-26 mm zone of inhibition) with *Cryptococcus humicola* was the highly susceptible species followed by *Aspergillus niger*. None of *Verticillium leptobactrum* metabolites inhibited the growth of any of the ten yeast and mould species examined. Similarly, the ability of the entomopathogenic fungus *Verticillium lecanii* (Zimm.) to antagonize a number of pathogenic fungi including powdery mildew (Askary *et al.*, 1997&1998) and rusts (Spencer and Atkey, 1981) has been documented. *V. lecanii* has the ability to reduce green mold incidence caused by *Penicillium digitatum* and triggers the induction of host defense resistance thus would be of powerful value for the biological control of citrus green mold (Benhamou and Brodeur, 2000&2001). Also, the entomopathogenic *V. lecanii* HF238 strains produced clear antibiotic activity against *Bacillus* and *Saccharomyces*, but only in the presence of insect-derived materials, suggesting that the production of antibacterial/antifungal compounds by entomopathogenic fungi is triggered by the presence of insect-derived materials (Lee *et al.*, 2005). Similarly, *Verticillium lateritium* and *V. tenerum* isolated from water and sediment of the Kolubara River exerted inhibitory action on some species of pathogenic

bacteria (Ranković, 2005). Previous cytological investigation demonstrate that the beneficial effect of *V. lecanii* in repressing *Pythium* ingress in root tissues relies on a strong antifungal activity associated with an induction of structural (formation of elongated wall appositions, resembling papillae) and biochemical (occlusion of some intercellular spaces by an amorphous material or phenolic compound) barriers in host tissues (Benhamou *et al.*, 2001). Despite the amount of research devoted to elucidating the mechanisms by which *V. lecanii* exerts its antimicrobial activity (Bidochka *et al.*, 1999; Saksirirat, and Hoppe, 1990), the knowledge regarding the involvement of the host itself in plant–pathogen interactions is still developing, although a recent report demonstrated that *V. lecanii*-mediated induced resistance was a crucial event in the complex process of disease protection in cucumber (Benhamou and Brodeur, 2001; Benhamou, 2004). Many reports showed that treatment with the *Verticillium dahliae*, activated the expression of defense response in many plants (Benhamou, 1996; Hill *et al.*, 1999; McFadden *et al.*, 2001; Meyer *et al.*, 1994). The antimicrobial peptide poly(arginyl-histidine) is secreted by *Verticillium kibiense* and inhibits the growth of certain microorganisms more effectively than that chemically synthesized from the L-form of arginine and histidine. This peptide showed activity against a broad range of bacteria and fungi but lost its activity under conditions of high ionic strength (Nishikawa and Ogawa, 2004).

Table 1: Antimicrobial activities of *Verticillium alboatrum* and *Verticillium leptobactrum* metabolites against a range of pathogenic bacteria and fungi.

Microorganisms	<i>Verticillium alboatrum</i>	<i>Verticillium leptobactrum</i>	Reference control ⁽²⁾
<i>Streptococcus pyrogens</i>	21	16	19
<i>Bacillus thuringiensis</i>	25	19	24
<i>Shigella dysenteriae</i>	24	20	28
<i>Escherichia coli</i>	21	14	27
<i>Staphylococcus aureus</i>	18	17	25
<i>Pseudomonas auregenosa</i>	23	15	18
<i>Proteous vulgaris</i>	22	0	19
<i>Acinetobacter baumannii</i>	30	15	22
<i>Klebsiella pneumoniae</i>	28	13	24
<i>Aspergillus fumigatus</i>	12	0	ND ⁽²⁾
<i>Aspergillus niger</i>	24	0	ND
<i>Aspergillus flavus</i>	14	0	ND
<i>Penicillium expansum</i>	12	0	ND
<i>Syncephalastrum racemosum</i>	0	0	ND
<i>Candida albicans</i>	13	0	ND
<i>Geotrichum candidum</i>	14	0	ND
<i>Candida dubliniensis</i>	15	0	ND
<i>Cryptococcus humicola</i>	26	0	ND
<i>Trichophyton cutamueum</i>	13	0	ND

⁽¹⁾ Antibacterial and antifungal activities were expressed as the diameter of inhibition zones (mm) using well diffusion method.

⁽²⁾ Ciprofloxacin (5 mg disk) acted as the positive control against the tested bacteria.

⁽³⁾ ND = not determined.

The active ingredients from the two extracts were purified on G100 silica gel column using bioguided fractionation resulting in two active purified fractions (fraction numbers 5 & 10) were responsible for the antimicrobial activity in *Verticillium alboatrum* while one compound was the active principle obtained in fraction 5 in case of *Verticillium leptobactrum*.

To determine the effects of fungal metabolites upon viral pathology, Vero confluent cells in 96-well plates were incubated at 37°C in an atmosphere of 5% CO₂ for 1 h in the presence or absence of polio virus. The inoculum was aspirated and the cultures were overlaid with maintaining medium with 1% methylcellulose containing two-fold serial dilutions of the fungal metabolite. Two days post infection, cell monolayers were stained with crystal violet, and plaques were scored by inverted light microscopy. Plates overlaid with methylcellulose medium without the metabolite were used as controls.

The results shown in table 2 indicate that compound 1 inhibited the plaques formed from the virus infection in a concentration dependant manner. The inhibitory activity of compound 1 was ranged from 7-41%. Compounds 2 & 3 exhibited no reduction in plaques caused by polio virus infection in vero cells for all dilutions tested if compared with control wells under these experimental conditions.

Vertihemipterin A, ascochlorin analogs isolated from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 2370 have been reported to possess an antiviral activity against HSV-1 and cytotoxic activities (Seephonkai *et al.*, 2004).

Table 2: The inhibitory activity of the three compounds produced from *Verticillium albo-atrum* and *Verticillium leptobactrum* against polio type 1 virus⁽¹⁾.

Dilution	Antiviral activity ⁽²⁾				
	Neat	1/2	1/4	1/16	1/32
Treatment					
Compound 1	41 (±8)	36 (±7)	20 (±5)	7 (±5)	0
Compound 2	0	0	0	0	0
Compound 3	0	0	0	0	0

⁽¹⁾. A plaque reduction assay was performed for the evaluation of the antiviral activity

⁽²⁾. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control - mean number of plaques in sample)/(mean number of plaques in control)] x 100. Each value represents the mean ± SD of the three independent experiments.

To investigate whether fungal metabolites had an antitumor effect on HEp-2 cells, the cell lines were treated with various dilutions of fungal extracts. The normal HEp-2 cells monolayer was observed as short, polygonal-shaped epithelial cells with a number of rounded cells and numerous areas of cellular overgrowth. CPE caused in HEp-2 cells became apparent at 24 h post-treatment. Three independent experiments were assessed each containing six replicates per treatment. The morphology of the cells was inspected daily and observed for microscopically detectable alterations. The cytopathic effect (CPE) was scored for each treatment. CPE was apparent in the pattern of cell death observed by morphological changes. The rounded and refractile appearance of the cells was also observed in the treated cells that indicate dead or degenerating cells due to compound activity.

To investigate whether fungal metabolite extracts had an *in vitro* cytotoxic effects, vero cells were treated with various dilutions of fungal extracts. The morphology of the cells was inspected daily. Forty eight hours after incubation with fungal extracts, the cells were observed by inverted light microscopy and ELISA reader with comparing to control wells. Treatment of vero cells with the compound 1 of *Verticillium albo-atrum* resulted in no CPE in vero cells at almost all the tested dilutions under these experimental conditions. Compound 2 treated cells exhibited moderate toxicity when tested as crude or at 1:2 and 1:4 dilutions. In case of compound 3, weak inhibitory activity was observed against vero cell lines (Fig. 1).

Moderate inhibitory activity to HEp-2 cells was observed for the compound 3, while compounds 1 and 2 showed weak inhibitory activity against HEp-2 cells under these experimental conditions. The results of cell viability in figure (1) indicate that dilution 1:8 was the effective concentration of compound 3 that inhibited 50% from the tumor cells with almost no toxicity to the normal cells. In spite of the observed inhibitory activity of the two compounds produced from *Verticillium albo-atrum*, they were considered inactive due to the observed toxicity at the same dilutions.

Similarly, monorden that can be isolated from *Verticillium* species has been reported as an anticancer agent and also acting as promoters of nerve regeneration (Chanmugan, 1995; Pillay *et al.*, 1996; Arai *et al.*, 2003).

Characterization of the Bioactive Compounds:

Compound 1 (Fig. 2): Infra Red (IR) of this compound had three characteristic absorption bands at 3422cm⁻¹ due to the presence of NH group of amine and at 2920 cm⁻¹ for CH group of alkane as well as band at 1632 cm⁻¹ for the aromatic ring. The ¹H NMR spectrum (400 MHz, DMSO-d₆) showed only three characteristic signals 7.267 ppm integrating for aromatic ring, at 1.590 ppm for NH of amine and 0.840 ppm integrating for CH of alkane. The mass spectrum of this compound confirmed these findings with a characteristic fragmentation of m/z 43, 57, 71, 86, 118 and 316. Consequently, the expected molecular formula is C₄₀H₅₆N₈ and named as 1,1',1'[(1-ethyl, 4-propyl) benzene diamine], [(1-ethyl, 4-methyl) benzene diamine], 4 [(1-ethyl, 4-methyl) benzene diamine], benzene diamine] prop-3-ene.

Compound 2 (Fig. 2): The Infra Red (IR) spectrum of this compound had absorption band at 3707cm⁻¹ due to the presence of NH group of amine; band at 2920 cm⁻¹ for CH group of alkane; bands at 1450 and 1630 cm⁻¹ for the aromatic groups; band at 1810 cm⁻¹ for alkene group. The ¹H NMR spectrum (400 MHz, DMSO-d₆) showed only three characteristic signals 7.265 δ ppm integrating for aromatic ring, at 1.258 ppm for NH of amine with p-substitution and 0.852 ppm integrating for CH of alkane. It can be deduced that the molecule is symmetric but also that two carbons of the aromatic ring are substituted. A single sharp peak was obtained from mass spectroscopic analysis of the compound indicating its purity. The mass spectrum of this compound confirmed these findings with the molecular peak at m/z 450.1 [M-H] with a characteristic fragmentation of m/z 40, 55, 77, 149 and 230. Also, the mass spectrum revealed that the molecular weight of this peak is 450 Dalton. Consequently, the expected molecular formula is C₃₃H₂₆N₂ and named as Di-1, 3 [(4-(prop-1,3- dienyl) benzyl) benzylamine] prop-1,2-diene.

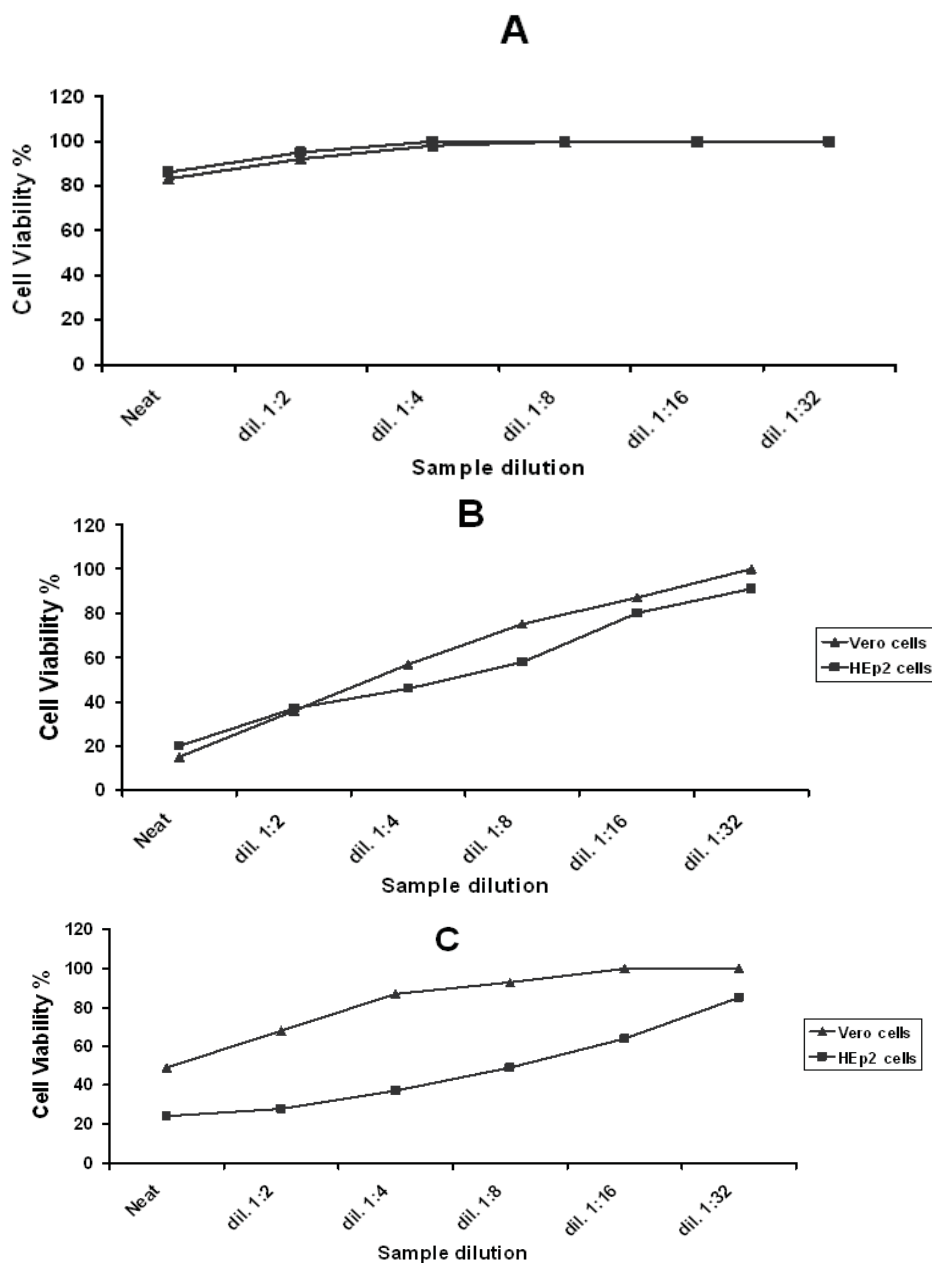


Fig. 1: Inhibitory activity of the metabolites on vero and HEp2 cells. (A) Compound 1 (B) Compound 2, (C) Compound 3.

Compound 3 (Fig. 2): Infra Red (IR) of this compound had absorption band at 2788cm^{-1} due to the presence of NH group of amine; band at 2920cm^{-1} for CH group of alkane; bands at 1450 and 1650cm^{-1} for the aromatic groups; band at 1597cm^{-1} for alkene group; band at 1817cm^{-1} for ketonic group. The ^1H NMR spectrum (400 MHz, DMSO-d_6) showed characteristic signals at 7.265 ppm integrating for aromatic ring, at 3.492 ppm for ketonic group; at 3.482 ppm for alkene group; at 1.436 ppm for NH of amine and 3.431 ppm integrating for CH_2 aromatic. The mass spectrum of this compound confirmed these findings with a characteristic fragmentation of m/z 41; 57; 135, 207 and 191. Consequently, the expected molecular formula is $\text{C}_{57}\text{H}_{76}\text{N}_7\text{O}_6$ and named as 1, 2, 3- [1-butylamideaniline, 4-dec-9-ene-3-one] amine.

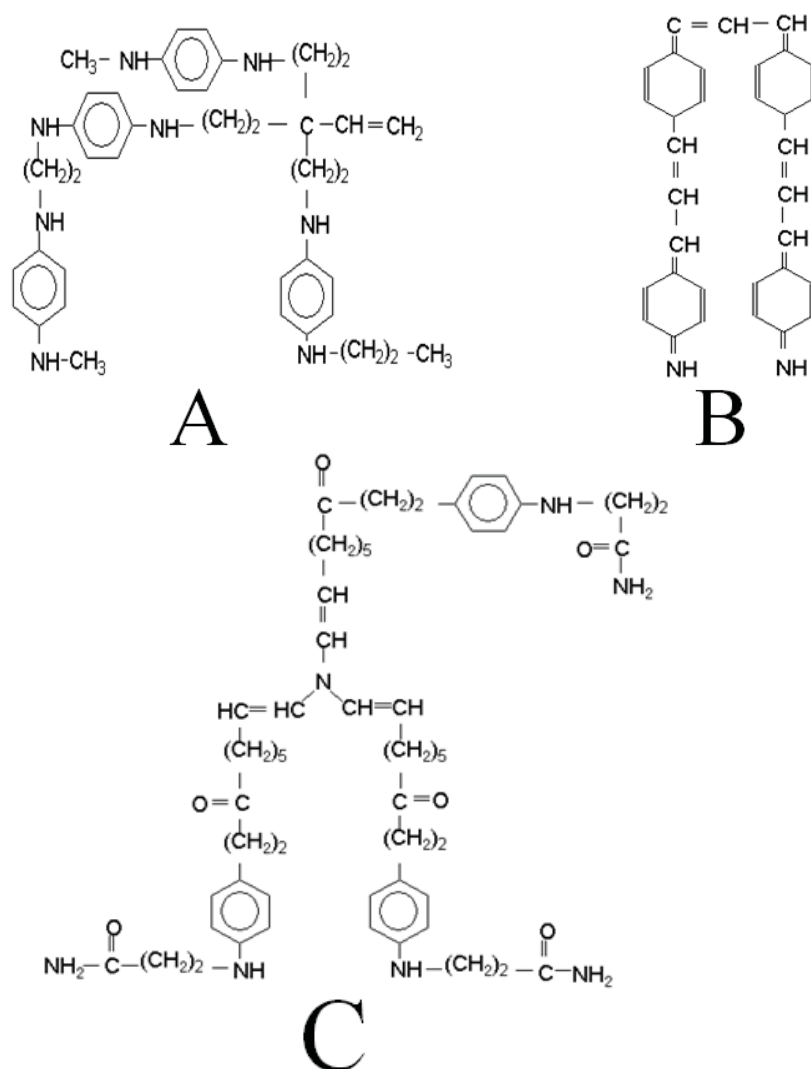


Fig. 2: The chemical structure of the active compounds. A, Compound 1: 1,1,1[(1-ethyl,4-propyl) benzene diamine], [(1, ethyl, 4-methyl) benzene diamine], 4[(1-ethyl, 4-methyl) benzene diamine], benzene diamine prop-3-ene; B, Compound 2: Di-1, 3 [(4-(prop 1, 3-dienyl) benzyl) benzylamine] prop-1, 2-diene; C, Compound 3: 1, 2, 3- [1-butylamideaniline, 4-dec-9-ene-3-one] amine.

Conclusion:

The study illustrates the biological activities of new fungal metabolites from *Verticillium alboatrum* and *Verticillium leptobactrum*, and is therefore, a potential drug and a good candidate for further studies and development. The first compound was benzene diamine derivative produced from fraction 5 of *Verticillium alboatrum* and showed activity against bacteria, fungi and polio virus. Compound 2 was benzylamine derivative produced from fraction 5 of *Verticillium alboatrum* with broad spectrum antimicrobial as well as weak antitumor activity. Compound 3 was butylamideaniline derivative obtained from *Verticillium leptobactrum* with antibacterial activity. The compound 3 also showed antitumor activity with acceptable therapeutic indexes.

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