



Cytotoxic and antibacterial activities of secondary metabolites from endophytic fungus *Pestalotiopsis virgatula* VN2

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Abstract

Development of new drugs, especially in area of oncology and infectious diseases, represents today one of the most important research. Fungal endophytes are receiving increasing attention by natural product chemists due to their diverse and structurally unprecedented compounds making them interesting candidates for drug discovery. In the present investigation, aims to study the cytotoxic and antibacterial activity of secondary metabolites from endophytic fungus *Pestalotiopsis virgatula* VN2. The ethyl acetate extract of partially purified fraction E had strong cytotoxic activity against MCF-7 and MDA MB-231 cell lines with concentration increase to 30 µg/mL the cell viability was decreased by 59.0% and 45.6% respectively. Further, fraction E had an effective antibacterial activity against multidrug resistant *S. aureus*. The fraction E produced a maximum inhibition zone of 19.8 mm against *S. aureus* strain 4. The minimal inhibitory concentration of the fraction E was found to be 31.2 to 500 µg/mL against *S. aureus* strains. In GC-MS analysis, one predominate peak with retention time of 20.9 and molecular ion peak at m/z 278.7 was observed.

Keywords – antibacterial activity – cytotoxic activity – *Pestalotiopsis virgatula* – *Staphylococcus aureus*

Introduction

Cancer is one of the leading causes of mortality worldwide, having significant impact on individuals and health care systems. Breast cancer, the most frequently diagnosed and second deadliest cancer among women is a global public health issue (Jemal et al. 2011). In 2008, approximately 1.38 million cases were diagnosed and 458,000 deaths occurred worldwide. The problems associated with hospital infections caused by drug resistant bacteria become increasingly evident and *Staphylococcus*

aureus is the most common pathogen associated with serious gram-positive bacterial infections (Corey 2009). The prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) has increased over the past several decades in most countries (Cornaglia & Rossolini 2009). Natural products still remain the most important resource for discovery of new and potential drug molecules (Strobel & Daisy 2003). Plant endophytic fungi are well-known as sources of bioactive secondary metabolites. The fungal species of the genus *Pestalotiopsis* have been demonstrated to be rich sources of bioactive secondary metabolites with diverse structural features (Harper et al. 2003). In the present study, the endophytic fungus *Pestalotiopsis virgatula* VN2 was isolated from the medicinal plant *Vitex negundo* L. and its partially purified extract was screened for their anticancer and antibacterial activities against human breast cancer cell lines and multidrug-resistant bacterial strains.

Materials and methods

Isolation of endophytic fungi

Healthy leaves of medicinal plant *Vitex negundo* L. were collected and washed thoroughly under running tap water and air dried. All the leaf samples were cut into about 5 mm² segments and surface sterilized successively with 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and rinsed with sterile water. After surface sterilization, the leaf samples were dried under sterile condition and aseptically transferred to Petri plates containing potato dextrose agar (PDA) with 50 µg/mL of streptomycin to suppress bacterial growth (Arnold et al. 2000). The identification of endophytic fungal strain *Pestalotiopsis virgatula* was confirmed by 18S rRNA sequence comparisons (Altschul et al. 1990). The 18S rRNA gene sequencing was done at Synergy Scientific Services, Chennai, India. The sequence alignment was done at BLAST server.

Extraction of bioactive metabolites

Endophytic fungal isolate was grown on potato carrot agar (PCA) at 30°C for 5 days. Three pieces (0.5 X 0.5 cm²) of an actively growing mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 200 mL of potato carrot broth and incubated at 30°C for three weeks under a static condition. After the incubation period, fungal mycelium was separated from the culture filtrate by cheesecloth. The filtrate and mycelia were extracted three times with ethyl acetate. The solvent was removed by rotatory vacuum evaporator. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) and study for anticancer and antibacterial activity. The DMSO solvent was used as control.

Partial purification of bioactive compounds

The dried fungal extracts was subjected to silica gel column chromatography (230-400 mesh) and eluted with n-hexane:ethyl acetate mixture by increasing polarity (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95, 0:100). About 50 mL per fraction were collected and made into six pooled fractions (A, B, C, D, E and F) on the basis of their thin layer chromatography (TLC) results. The pooled fraction such as fraction A (eluted with n-hexane:ethyl acetate from 100:0 to 80:20), fraction B (eluted with n-hexane:ethyl acetate from 75:25 to 60:40), fraction C (eluted with n-hexane:ethyl acetate from 55:45 to 45:55), fraction D (eluted with n-hexane:ethyl acetate from 40:60 to 30:70), fraction E (eluted with n-hexane:ethyl acetate from 25:75 to 15:85) and fraction F (eluted with n-hexane:ethyl acetate from 10:90 to 0:100) were used to determine the antimicrobial assay.

Cell culture

Human breast cancer cells MCF-7 and MDA MB-231 were obtained from ATCC (American

Type Culture Collection, Rockville, MD) and grown in MEM (minimum essential eagle medium) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) in 24-well tissue culture plates (Costar, Cambridge, MA).

MTT reduction cytotoxic assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), was taken up by cells and was reduced to a colored formazan product that could be detected by spectrophotometry ($k_{max} = 562$ nm). Reduction of MTT was dependent on the mitochondrial respiratory function, and thus, measures the relative number of viable cells in the culture. MCF-7 and MDA MB-231 cells were treated with various concentrations (0, 10, 20 and 30 $\mu\text{g/mL}$) of ethyl acetate extract of *P. virgatula* for 48 h. DMSO was used as a vehicle control. At the end of the treatments, media was aspirated, washed with DPBS and again incubated with 5 mg/mL of MTT for 1 h at 37°C. DMSO was used to dissolve the formazan. The absorbance was measured at 562 nm using automated TECAN multimode reader. The results represented are from three independent experiments.

Determination of antibiotics sensitivity of *S. aureus*

Clinical strains of *Staphylococcus aureus* were obtained from Bose Clinical Laboratory and X-ray (Madurai, Tamilnadu, India). The Kirby–Bauer disc diffusion test was used to determine the antibiotic resistance of *S. aureus* strains (1-10). The nutrient broth was prepared and well-isolated colonies of the same type from a culture agar plate were inoculated into it. The culture broth was incubated at 37°C until the culture equaled 0.5 McFarland standards. A McFarland 0.5 turbidity standard corresponded to an inoculum of 1×10^8 CFU/ml (Acar & Goldstein 1991). The Mueller-Hinton agar (MHA) medium supplemented with 2% NaCl was used for this study. The antibiotic discs such as penicillin (10 units/disc), methicillin (10 $\mu\text{g/disc}$) and vancomycin (30 $\mu\text{g/disc}$) were used. To ensure complete contact of the disks to the agar surface, the disks were pressed down with slight pressure. Inoculated plates were inverted and incubated at 37°C for 18 h. After the incubation period, the diameter of inhibition zone was measured and results were interpreted according to the standards of Clinical Laboratory Standards Institute (CLSI 2005).

Determination of minimum inhibitory concentration (MIC)

MIC of ethyl acetate extract was determined based on a broth micro dilution method in 96 well microplate (Al-bayati 2008). Briefly, *S. aureus* strains (1-10) were cultured overnight at 37°C on MH broth and adjusted to final density of 10^8 CFU/mL by 0.5 McFarland standards. The ethyl acetate extract (1mg/mL) was dissolved in DMSO, and twofold serial dilutions were made into concentration range from 7.8 $\mu\text{g/mL}$ to 1000 $\mu\text{g/mL}$. In 96 well plates, each well had 90 μL of MH broth supplemented with 2% NaCl, 10 μL of bacterial inoculum and 10 μL of different concentrations of fungal extract. The plate was incubated at 37°C for 18 h. Experiments were performed three independent times and each sample was assayed in duplicate.

Results and discussion

Identification of endophytic fungus

Totally 30 endophytic fungi were isolated from the medicinal plant *Vitex negundo* L. Among them the endophytic fungus VN2 was selected based on antibacterial and anticancer activity. The endophytic fungus VN2 growing on PDA was pale buff with sparse aerial mycelium and acervuli containing black, slimy spore masses (Fig. 1a). All isolates had 5-celled conidia, apical and basal cells were hyaline, while the three median cells were olivaceous; the upper two were slightly darker than the lower one (Fig. 1b). The endophytic fungus VN2 was identified as *Pestalotiopsis virgatula* by

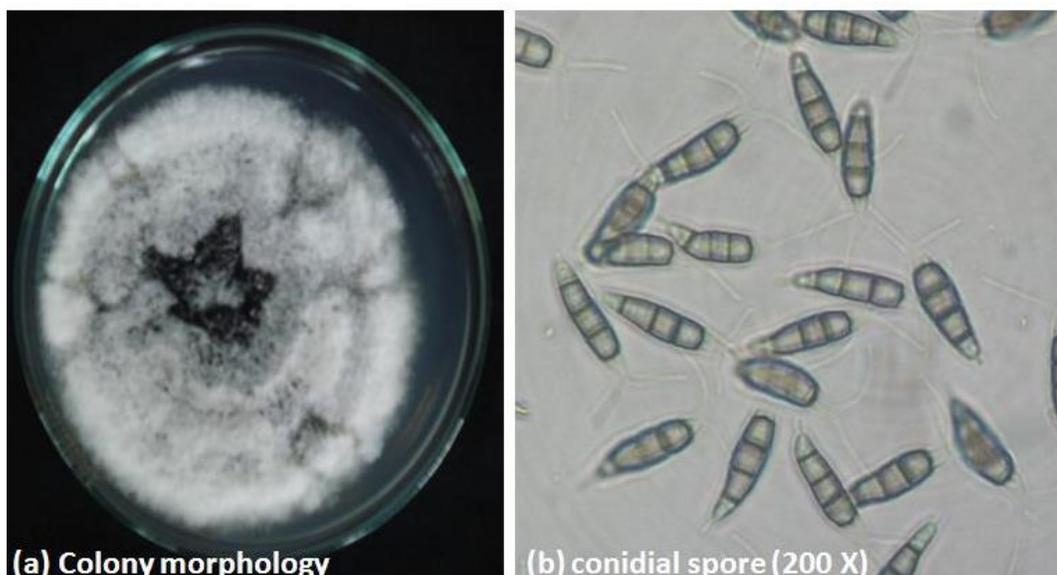


Fig. 1 – Colony morphology and conidial spore of endophytic fungus *Pestalotiopsis virgatula* VN2

comparing morphological and cultural characteristics (Size of the conidia, color and length of median cells, length and number of apical appendages and length of basal appendage) to those described in Guba's monograph of *Monochaetia* and *Pestalotia* (Guba 1961).

Morphological characteristics allowed the identification of the endophytic fungus as *Pestalotiopsis virgatula* which was reinforced by the sequence of its 18S rRNA gene that gave a 98% sequence similarity to those accessible at the BLAST of *Pestalotiopsis virgatula*. The endophytic fungus was designated as *Pestalotiopsis virgatula* VN2. The 18S ribosomal RNA gene (570 base pairs) of endophytic fungal isolate showed similarity with endophytic fungus *Pestalotiopsis virgatula* (GenBank No. AY 924283 and GenBank No. GU 595051) in nucleotide BLAST analysis against the GenBank database. The fungus was deposited in Genbank Accession No. JF 795287.

Cytotoxic activity of endophytic fungal extract

Cancer metastasis, which consists of uncontrolled growth and invasive behavior of cancer cells, is one of the major medical problems in breast cancer patients (Punglia et al. 2007). The cytotoxic activity of endophytic fungus *Pestalotiopsis virgatula* was investigated against the malignant MCF-7 and metastatic MDA MB-231 human breast cancer cell lines. Ethyl acetate extract of fraction E of *Pestalotiopsis virgatula* VN2 dose-dependently (0-30 $\mu\text{g}/\text{mL}$) increased cytotoxicity in both MCF-7 and MDA-MB-231 breast cancer cells. At 10 $\mu\text{g}/\text{mL}$, of fraction E of *P. virgatula*, the MCF-7 cell viability decreased to 72.1 %. When concentration of the fraction E increased at 30 $\mu\text{g}/\text{mL}$ the cell viability also decreased to 59.0% (Fig. 2). In the case of MDA MB-231 cells, the cell viability decreased to 65.0 % at 10 $\mu\text{g}/\text{mL}$, and further decreased to 45.6 % at 30 $\mu\text{g}/\text{mL}$ (Fig. 3). Nevertheless, both the cell lines exhibited significant dose-dependent cytotoxic effects against the fungal extract. Recently, Liu et al. (2013) isolated Pestalotficials Q-S from the plant endophytic fungus *Pestalotiopsis fici*. Two new isoprenylated chromone derivatives and one new benzofuran derivative of Pestaloficial. Along with three known metabolites, such as anofinic acid, siccayne and pyrenophorol. Siccayne showed cytotoxic activity against the human cancer cell lines, HeLa and HT29, with IC₅₀ values of 48.2 and 33.9 μM respectively.

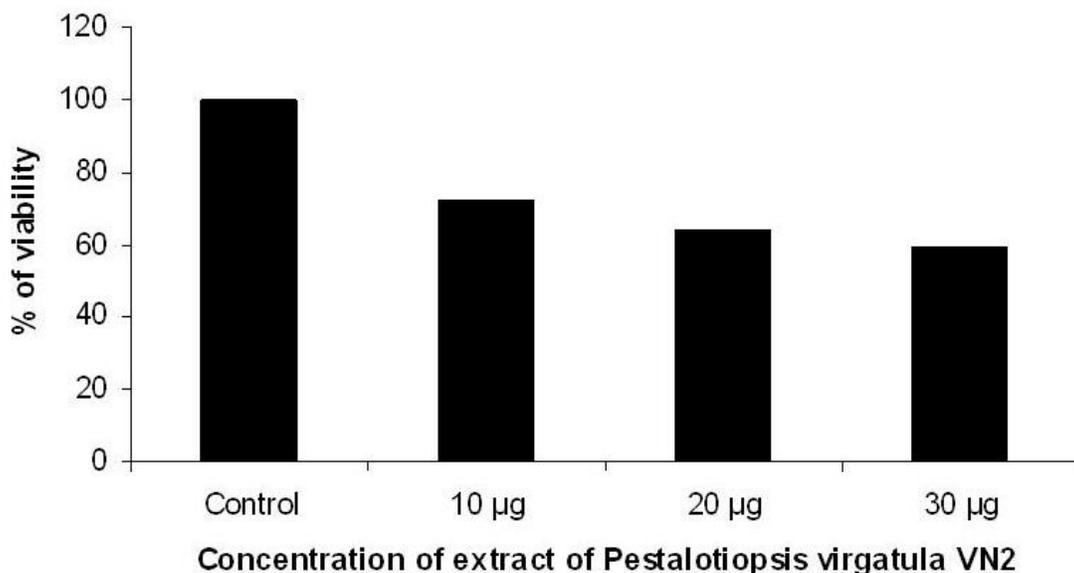


Fig. 2 – Cytotoxic activity of ethyl acetate extract of fraction E of *P. virgatula* VN2 against MCF-7

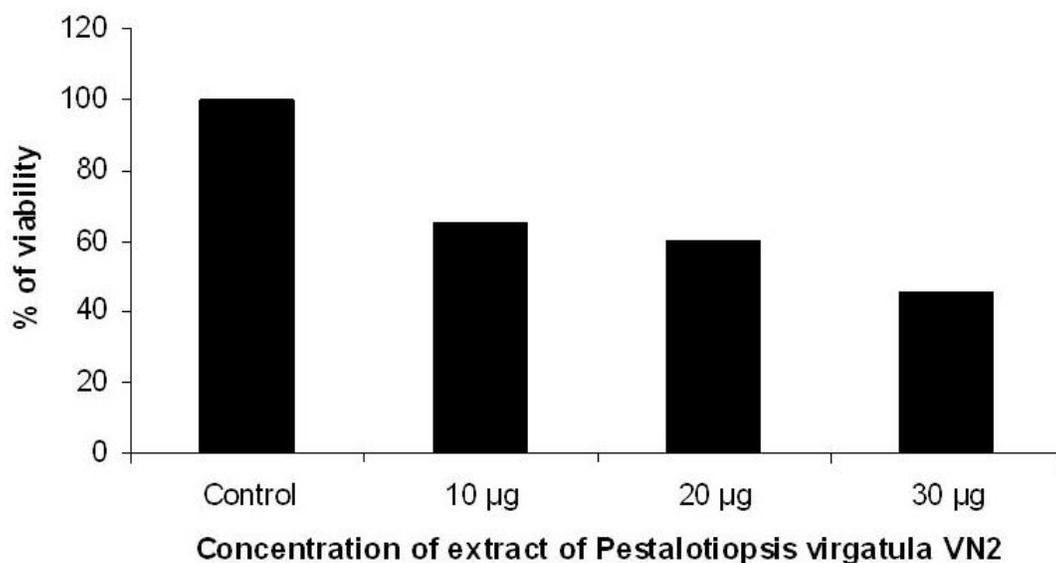


Fig. 3 – Cytotoxic activity of ethyl acetate extract of fraction E of *P. virgatula* VN2 against MDA MB-231

Antibacterial activity of endophytic fungal extract

The antibiotic resistant profile of *S. aureus* strains (1–10) was determined using commercial antibiotics such as penicillin, methicillin and vancomycin. All the *S. aureus* strains (1–10) were resistant to penicillin. The *S. aureus* strain 7 was sensitive to methicillin, all other strains (1–6 and 8–10) were resistant to methicillin. Strains 6, 8 and 9 were resistant to vancomycin whereas, the other strains (1–5, 7 and 10) were sensitive to vancomycin. The ethyl acetate extract of fraction E of *P.*

Table 1 Antibacterial activity of ethyl acetate extract of fraction E of *P. virgatula* VN2 against *S. aureus* strains

<i>S. aureus</i> strains	Zone of inhibition (mm)			
	Penicillin (10 unit/mL)	Methicillin (10 µg/mL)	Vancomycin (30 µg/mL)	Ethyl acetate extract fraction E (100 µg/mL)
Strain 1	12.8 (R)	10.8 (R)	16.5 (S)	18.1
Strain 2	12.8 (R)	11.2 (R)	15.4 (S)	16.7
Strain 3	14.2 (R)	12.1 (R)	16.0 (S)	15.4
Strain 4	11.5 (R)	10.2 (R)	17.2 (S)	19.8
Strain 5	10.8 (R)	8.9 (R)	18.5 (S)	18.9
Strain 6	10.3 (R)	9.5 (R)	12.4 (R)	14.4
Strain 7	13.8 (R)	15.5 (S)	16.9 (S)	19.4
Strain 8	10.5 (R)	8.8 (R)	13.5 (R)	18.2
Strain 9	14.0 (R)	12.2 (R)	14.8 (R)	17.3
Strain 10	14.9 (R)	8.9 (R)	17.4 (S)	18.6

R - Resistant, S – Sensitive

virgatula VN2 showed an effective antibacterial activity against all the *S. aureus* strains. A maximum inhibition zone of 19.8 mm was observed against *S. aureus* strain 4 (Table 1). The control (DMSO) had no inhibitory activity against *S. aureus* strains. Josiane et al. (2007) also reported that the metabolites of endophytic fungus *Pestalotiopsis* spp. inhibited the growth of the tested microorganisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus*. Xing et al. (2011) isolated two new compounds, 7-hydroxy-5-methoxy-4,6-dimethyl-7-O- α -L-rhamnosyl-phthalide and 7-hydroxy-5-methoxy-4,6-dimethyl-7-O- β -D-glucopyranosyl-phthalide were isolated from the ethyl acetate extract of endophytic fungus *Pestalotiopsis heterocomis*. Recently, Pinheiro et al. (2012) reported the antibacterial activity of alkaloids produced by endophytic fungus *Aspergillus* sp. EJC08 isolated from medical plant *Bauhinia guianensis*. Alkaloids known as fumigaclavine C and pseurotin A were broad spectrum antibacterial agents with good activity.

The lowest concentration of fungal extract at which no growth of microorganism observed upon visual observation after incubating at 37°C for 18 h was considered as the MIC value. Pellets formed on the bottom of wells were considered bacterial growth even if the wells were clear of turbidity. Lowest MIC value of 31.2 µg/mL was observed against vancomycin sensitive *S. aureus* strain 4 and 7, the highest MIC value of 250 µg/mL was observed against vancomycin resistant *S. aureus* strain 3, 6 and 8.

The partially purified fraction E of *P. virgatula* VN2 was subjected to GC-MS analysis to find out the components. The gas chromatogram of fraction E yielded one prominent peak with retention time of 20.9 min and other very small peaks with retention time of 14.8, 16.5 and 16.7 min (Fig. 4). The component with its retention time of 20.9 min was further analyzed by mass spectrometer. The mass spectrum gives the molecular ion peak at m/z 278.7 (Fig. 5).

Our results showed that the ethyl acetate extract of fraction E of *P. virgatula* VN2 had effective antibacterial activity against multidrug-resistant *S. aureus* strains. The fraction E had strong cytotoxic activity against human breast cancer cells MCF-7 and MDA MB-231. Further research on the purification of active compounds present in *P. virgatula* VN2 is needed in order to better understand the antimicrobial and anticancer properties.

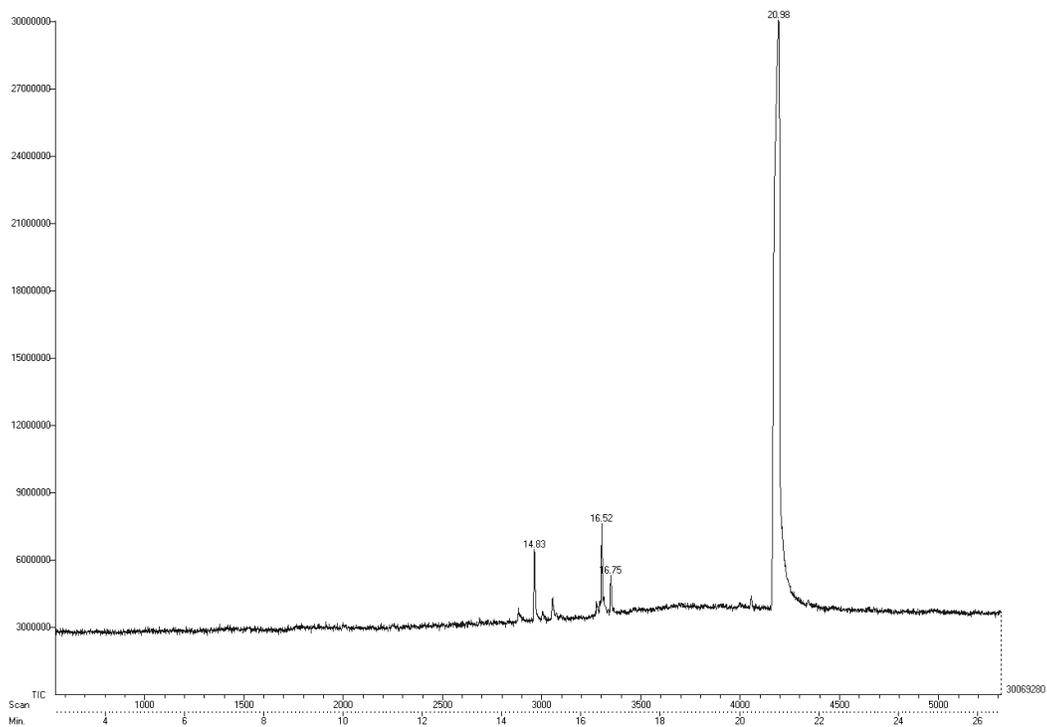


Fig. 4 – Gas chromatogram of ethyl acetate extract fraction E of *P. virgatula* VN2

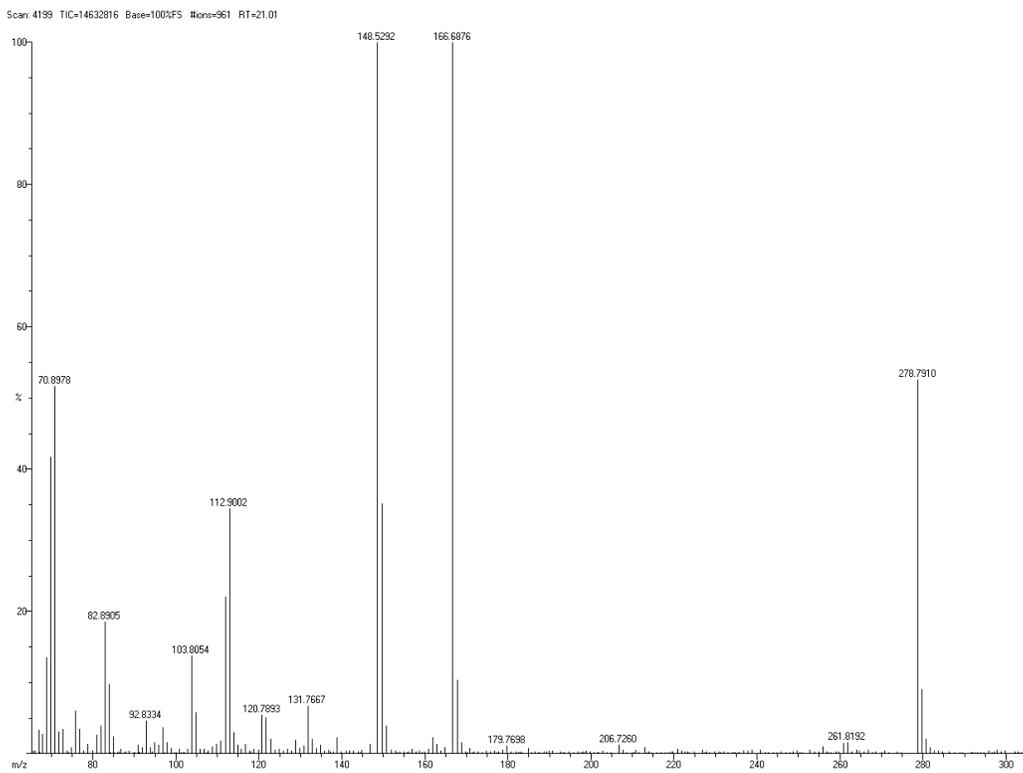


Fig. 5 – Mass Spectrum of ethyl acetate extract of fraction E of *P. virgatula* VN2

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