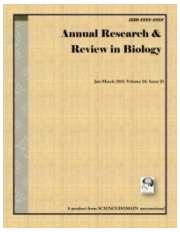

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Antimicrobial Activity of *Phomopsis sp*. ED2 Residing in Medicinal Plant *Orthosiphon stamineus* Benth

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Authors' contributions

All authors involved in the experimental design and manuscript writing. They also read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the antimicrobial activity of *Phomopsis sp*. ED2, endophytic fungus isolated from *Orthosiphon stamineus* Benth against various pathogenic microorganisms.
Study Design: Place and Duration of Study: Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia, between Dec 2011 to August 2013.
Methodology: The endophytic fungus *Phomopsis sp*. ED2 was cultivated in the culture medium supplemented with the host plant extract. Then the antimicrobial activities of the crude extracts were screened via disc diffusion assay. The minimal inhibitory concentration and minimal lethality concentration of the extract against selected test microorganisms by using broth microdilution assay. The bioactive constituents of the extract were analyzed by using gas chromatography-mass spectroscopy analysis.
Results: The ethyl acetate extract of this fungus showed broad spectrum inhibitory activity on both Gram positive and Gram negative bacteria with the minimal inhibitory concentrations ranged from 31.25 to 250 µg/mL. The gas chromatography-mass spectroscopy analysis of the extract showed that the bioactive constituents present in the extract were benzoic acid, lomustine and penicillic acid.
Conclusion: The endophytic fungus residing in *Orthosiphon stamineus* Benth, *Phomopsis*

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sp. ED2 exhibited significant antibacterial activity of the test microorganisms. It is a potential source of antibacterial compounds.

Keywords: Antimicrobial activity; endophyte; phomopsis; Orthosiphon stamineus Benth.

1. INTRODUCTION

Endophyte is generally defined as all living organisms residing plant organs that at some point of their life can colonize internal plant tissues without causing apparent disease to their host [1]. Endophytic fungi are a promising source of bioactive and chemically novel compounds with potential application in medical, agricultural and industrial areas [2]. The reported natural products from endophytes are highly diverse chemically and the biological activities exhibited include antibiotics, anticancer, immunosuppressant, antioxidant, anti-diabetic and anti-insecticidal [1].

Phomopsis is a genus that includes over 1000 species classified on the basis of their plant host and it is frequently isolated as an endophyte [3]. It is also well known for the production of bioactive compounds that exhibit antimicrotubule [4,5], antimalarial [6], antitubercular [7], antifungal [8,9], herbicidal [10], algicidal [11], anti-inflammatory [12], antimicrobial and plant growth regulatory activities [13,14,15,16]. This study is very important, as this is the first report of a *Phomopsis* isolated from *Orthosiphon stamineus*.

Basically, this study was initiated to investigate the antimicrobial activity of endophytic fungus *Phomopsis sp*. ED2 against various test microorganisms. Besides, it is also aimed to analyze the bioactive constituents of the extract by using gas chromatography-mass spectroscopy analysis.

2. MATERIALS AND METHODS

2.1 Storage of Endophytic Fungus

The endophytic fungus previously isolated from the leaf of medicinal herb, *O. stamineus* Benth. The fungal isolate was cultivated on Potato Dextrose Agar (AES) supplemented with powdered plant materials (10 g/L) and deposited at Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The culture was cultured on fresh medium every four weeks to ensure the viability of the isolate.

2.2 Culture Media

Yeast extract sucrose broth (sucrose 40 g/L, yeast extract (AES) 20 g/L and magnesium sulfate 0.5 g/L) supplemented with the aqueous extract of *O. stamineus* was used to cultivate the endophytic isolates in the shake-flask system. The plant extract was prepared by boiling 10 g of the powdered plant materials in 500 mL distilled water for 30 minutes. The extract was filtered and mixed with freshly prepared culture media and autoclaved at 121ºC for 15 min.

2.3 Identification of *Phomopsis sp.* ED2

To extract the genomic DNA, four pieces of sterile dialysis membrane were placed on the surface of PDA plate and inoculated with the fungal culture. The plate was incubated at 25ºC until the growth covered the whole membrane. An adequate amount of liquid nitrogen was added in a mortar and the mycelium was ground into fine powder. For DNA extraction, 20mg of powdered fungal materials were used and the DNA extraction was done with DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA was semi-quantified on a 1% (w/v) agarose-gel in 1 X Tris-borate-EDTA and visualized by staining with ethidium bromide. The internal transcribed spacer (ITS) regions of the extracted DNA sample was amplified in 50 µL reaction on a thermal cycler by using primer ITS 1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3'). The PCR was carried out with following reagents: 5 ng of DNA template, 10.0 µL of 10X PCR buffer, 1.5 mM magnesium chloride, 0.8 mM dNTP mix, 0.5 µM of both primers, 1 Unit *Taq* polymerase and 19.95 µL of deionized water. A negative control using water instead of DNA template was included. The reaction mixture was subjected to following temperature profile: 95ºC for 1min; followed by 34 cycles of 95ºC for 1 min, 52ºC for 30s and 72ºC for 1 min and finally 72ºC for 1 min. The PCR products were purified by using QIAquick PCR Purification Kit (Qiagen). For each PCR reaction, 2 µL of purified product was examined by electrophoresis at 90 V for 90 min in a 1% (w/v) agarose gel in 1X TBE buffer and visualized under UV transluminator (BioRad) after staining with ethidium bromide. DNA sequencing service was provided by 1st Base Laboratory. The fungal sequence was aligned using BioEdit and the alignment was adjusted manually where necessary to maximize alignment. A BLAST search was conducted to search for the closedly matched sequence in GenBank database. The multiple alignments of the sequence of the isolate with the sequences available in the data bank were carried out by Clustal W 1.83 version software. The phylogenetic tree was then constructed by MEGA software version 4.0 using the neighbor joining tree method with 100 replicates as bootstrap value.

2.4 Fermentation and Extraction of Fungal Cultures

The mycelial agar plug with 1 cm in diameter was excised from the periphery of 7-days-old fungal culture. Two mycelial plugs were then introduced into 250 mL Erlenmeyer flasks containing 100mL of the broth medium mentioned in section 2.2. After incubation at 30ºC with rotational speed of 120 rpm in a rotatory shaker, the fungal culture was harvested by centrifugation at 5311 g (Sigma, Model 4K15) to separate the fungal biomass and the fermented broth. The filtered broth was then extracted thrice with equal volume of hexane, dichloromethane, ethyl acetate and butanol (1:1, v/v). The organic phase was evaporated to dryness under reduced pressure by using a rotatory evaporator. The fungal mycelia were freeze-dried and soaked in methanol (1:50, w/v) overnight. The filter was then extracted sequentially with hexane, dichloromethane, ethyl acetate and butanol (1:1, v/v). The extracts were then concentrated by rotatory evaporator to obtain a crude extract paste. Besides, a control was included by extracting the sterile medium following exactly the same procedure as that for the fungal cultures.

2.5 Test Microorganisms

The test microorganisms used in the study included 5 gram-positive bacteria (*Proteus mirabilis*, *Streptococcus epidermidis, Streptococcus faecalis*, *Bacillus subtilis* and Methicilin-resistant *S. aureus* (MRSA)), 5 gram-negative bacteria (*Escherichia coli*, *Citrobacter freundii,*

Pseudomonas aeruginosa, *Klebsiella pneumoniae*, and *Shigella boydii)*, 2 yeasts (*Candida utilis* and *Cryptococcus neoformans)* and 6 fungi (*Fusarium solani*, *Rhizopus stolonifer, Trichoderma viridae, Aspergillus fumigatus, Microsporum gypseum* and *Trichophyton rubrum)*. The cultures were provided by Industrial Biotechnology Research Laboratory and Plant Pathology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The inoculum was prepared by adding 4 mL of sterile physiological saline to the agar slant and shake vigorously to get the cell or spore suspension.

2.6 Disc Diffusion Assay

The assay was conducted as per the procedure defined by Espinel-Ingroff et al. [17] and Jorgensen and Turnidge [18]. The crude extracts were prepared in 5% dimethyl sulfoxide (DMSO) and filter-sterilized with 0.2 µm Milipore filter. The Mueller Hinton agar medium (Hi-media) was used for cultivation of the test bacteria, the Mueller Hinton agar plate containing 2% dextrose and 0.5 µg/mL methylene blue for test yeasts or RPMI 1640 (Sigma) agar plates was used for test fungi. The RPMI 1640 agar plates were prepared by filter-sterilizing two times (2X) RPMI broth with 0.2 µm Milipore filter and followed with the addition of two times sterile agar after cooling at the time of experiment. The surfaces of the agar medium were inoculated with 100 µL of suspension containing 10^5 CFU/mL of bacteria, 10^6 CFU/mL of yeasts and 10^6 spores/mL of fungi. Twenty microliters of the extract with concentration 20mg/mL was added to a sterile Whatman antibiotic disc with 6mm in diameter. The disc was then placed on the surface of medium seeded with the test microorganisms. Five percent dimethyl sulfoxide (DMSO) was applied as a negative control to detect the solvent effects whereas 30µg/mL chloramphenicol was used as the positive controls for bacteria, 30µg/mL Amphotericin B for fungi and yeasts, respectively. The plates were incubated at 30ºC for 48 to 96 hours for fungi and at 37ºC for 24 hours for bacteria and yeasts. The diameters of the clear zones surrounding the disc were measured.

2.7 Determination of MIC and MLC

The minimal inhibitory concentration (MIC) was determined by using broth microdilution assay in sterile 96-well microtiter plate [17,18]. Only test microorganisms that showed significant inhibitory activity on disc diffusion assay were tested. Sterile Muller Hinton broth was used for test bacteria whereas RPMI 1640 medium containing 0.2% dextrose buffered with 0.165 M MOPS to a pH of 7.0 at 25ºC were used for test yeasts and test fungi. The prepared inoculum was diluted by sterile broth medium to the inoculum size required, 5 x 10^5 CFU/mL of bacteria, 1 x 10^6 CFU/mL of yeast and 1 x 10^6 spores/mL of fungi after the addition of the extract. The fungal extract was dissolved in 5% of DMSO to the concentration of 2 mg/mL, which then diluted to the highest concentration to be tested (1 mg/mL) after addition of 100 µL inoculum to achieve final volume of 200 µL. Then serial two fold dilution of the extract was carried out in a concentration range from 500 µg/mL to 15.63 µg/mL. The well containing only 5% DMSO and inoculum was used as the control. The plates were incubated at 30ºC for 48 to 96 hours for fungi, and at 37ºC for 24 hours for bacteria and yeasts. After the incubation period, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet salt (INT) (Sigma) dissolved in 99.5% ethanol was added to each well as a growth indicator for test bacteria and yeasts. The color of INT changed from yellow to purple where the microbial growth occurred. The MIC of test fungi was recorded as the lowest concentration of extract that prevents any discernible growth. To determine minimal lethality concentration (MLC) of the extract, 100 µL of the sample from each well was taken and suitably diluted before

streaking on agar plates to judge the viability. The MLC was recorded as the lowest concentration of extract that resulted in 99.9% growth reduction relative to the control.

2.8 GCMS Analysis

The ethyl acetate extract of fermentative broth of *Phomopsis sp*. ED2 was subjected to gas chromatography-mass spectroscopy (GCMS) to analyze the chemical constituents by using Hewlett-Packard 6890N Network GC system equipped with Hewlett-Packard 5973 inert mass selective detector mass spectrophotometer. The column used was HP-5MS (Agilent, USA). The oven temperature was set at 70ºC as initial temperature and held for 2 min, was then raised to 250ºC by 30ºC/min and held at this temperature for 20 min. The helium gas carrier was programmed to maintain a constant flow rate 1.2mL/min. The separated compounds were identified by comparison of their mass of the peaks with NIST02 library by computer-matching.

3. RESULTS AND DISCUSSION

3.1 Identification of *Phomopsis sp*. ED2

In 7 days old pure culture of isolate ED2 on potato dextrose agar, the isolate formed less abundant white mycelium, with a diameter of 10 cm. The reverse side of the culture was beige in color and had a light brown scattered spots at the central part of the colony at day 3, which turned into dark brown at day 7. Besides, the formation of simple dark stromata was observed on day 5. Yellowish spherical exudates were also observed at day 7. These characteristics showed isolate ED2 could be a member of genus *Phomopsis* [3].

After the PCR reaction, the size of amplified ITS product was estimated from gel electrophoresis to be about 700 base pairs for isolate *Phomopsis sp*. ED2. The isolate obtained in this study was found to have an identical ITS sequence in the GenBank database. The BLASTN result of the isolate showed high similarity to *Phomopsis sp*. Mfer5 (98%). This fungus has been previously isolated by Chomcheon et al. [19] and found to be an endophyte with anti-mycobacterial activity. The isolate was identified as *Phomopsis sp*. on the basis of the above mentioned characteristics. The use of the ITS gene for phylogenetic analysis in filamentous fungi is very common. The amount of the variation is suitable for studying phylogenetic relationships among closely related fungal species [20]. Fig. 1 shows the neighbor joining phylogenetic tree of isolate *Phomopsis sp*. ED2 based on the analysis of ITS sequence. In this study, the phylogenetic tree inferred from the sequence was well correlated with the species that were defined by cultural and morphological characteristics. The results were in agreed that isolate is a member of genus *Phomopsis* as most of the closely related isolates are belonged to genus *Phomopsis* or their sexual teleomorph known as *Diaporthe* [21]. Besides, it is also note that most of the closely related fungi were previously isolated as endophytes.

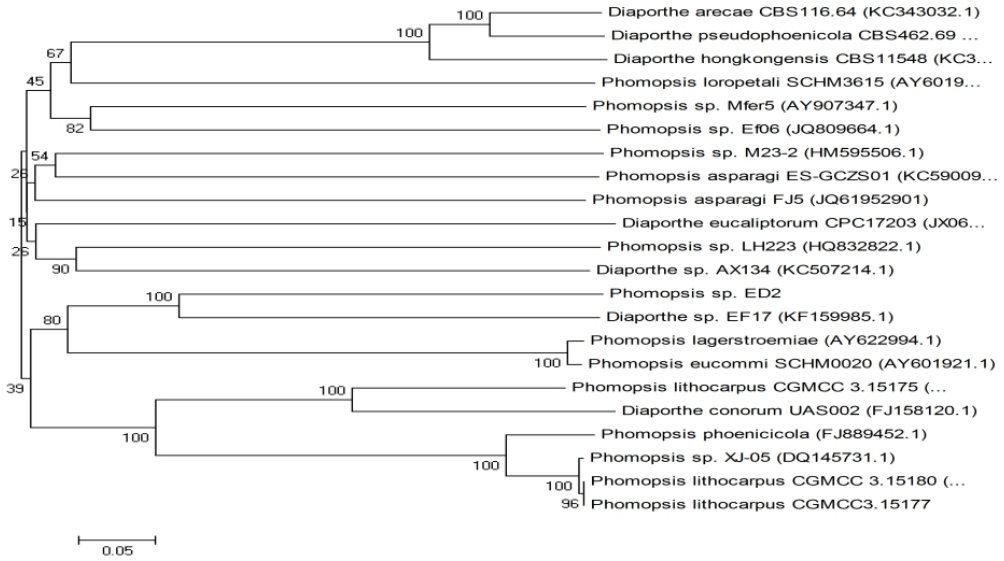

Fig. 1. Neighbor joining phylogenetic tree of *Phomopsis sp*. ED2. The isolate is closely related to the members of genus *Phomopsis* or their sexual teleomorph known as *Diaporthe.*

3.2 Antimicrobial Activity of *Phomopsis sp*. ED2

The antimicrobial activity of *Phomopsis sp*. ED2 in a disc-diffusion assay is shown in Table 1. In general, the extract prepared from the fungal biomass exhibited better antimicrobial activity. This indicates that the antimicrobial compounds were mainly associated with the fungal biomass. This has a benefit as the extraction yield is higher for fungal biomass. However, proper disruption of the fungal mycelia is necessary to release the antimicrobial compounds. The results indicated that all the Gram-positive test bacteria were inhibited by the ethyl acetate extract of the fungal biomass. Gram positive bacteria were more susceptible than the Gram-negative bacteria where only one Gram-negative bacteria (*K. pneumoniae*) was inhibited by the extract. The difference in sensitivity can be due to the morphological differences between these bacteria [22]. The cell wall of Gram-negative bacteria has an outer phospholipid membrane and lipopolysaccharide components, which hinder the access the bioactive metabolites through its outer covering [23]. The mutual interactions between the host and the endophytes are beneficial not only to the host but also to the endophytes by supplying nourishment to it [1]. Hence, the extract of the host was utilised as the basal medium for cultivating the endophytic fungi in this study.

Different organic solvent used in extraction was noticeably influenced the antimicrobial activity. In this study, most of the antimicrobial compounds were mainly present in the ethyl acetate fraction. The results are in agreement with those obtained from previous studies as the antimicrobial compounds from *Phomopsis* were mainly present in the ethyl acetate extract [14,16]. The finding also indicates that the antimicrobial compounds were mid-polar in nature and they can be extracted using ethyl acetate and dichloromethane. None of the antimicrobial compounds was present in the non-polar (hexane) fraction.

Table 1. Antimicrobial activity of *Phomopsis sp.* ED2 on disc diffusion assay. The culture medium was YES with host extract.

Test Microorganisms	Diameter of inhibition zone (mm)									
	Fungal Biomass					Fermentative Broth				Drug Control
	MeOH	Hexane	DCM	EtAc	Butanol	Hexane	DCM	EtAc	Butanol	
Gram Positive Bacteria										
P. mirabilis	15.7±1.2	-	16.7±0.6	17.0±4.4	-	-	-	15.0±1.0	-	22.7±1.2
S. epidermidis	-	-	-	15.0±1.0	-	-	22.7±1.1	19.7±0.6	-	19.7±0.6
S. faecalis	-	-	-	16.7±0.6	-	-	-	-	-	22.3±1.0
B. subtilis	19.7±2.1	-	17.3±1.2	23.3±1.2	-	-	19.7±0.6	15.3±0.5	-	19.7±2.1
MRSA	-	-	-	17.3±1.2	-	-	-	-	-	18.0±1.0
Gram Negative Bacteria										
E. coli	-	-	-	-	-	-	-	-	-	17.3±1.2
C. freundii	-	-	-	-	-	-	-	-	-	19.0±1.7
P. aeruginosa	-	-	-	-	-	-	-	-	-	22.3±1.6
K. pneumoniae	18.0±1.0	-	-	13.7±0.6	-	-	19.7±0.5	22.7±1.1	-	24.3±1.5
S. boydii	-	-	-	-	-	-	-	-	-	15.0±2.0
Yeasts										
C.utilis	-	-	-	-	-	-	-	17.7±0.6	-	22.7±1.5
C. neoformans	-	-	-	-	-	-	-	-	-	22.3±0.6
Filamentous Fungi										
F. solani	-	-	-	-	-	-	-	-	-	18.0±1.0
R. stolonifer	-	-	-	-	-	-	-	-	-	23.0±1.2
T. viridae	-	-	-	-	-	-	-	-	-	15.7±1.2
A. fumigatus	-	-	-	-	-	-	-	-	-	19.3±0.6
M. gypseum	-	-	-	-	-	-	-	-	-	19.6±1.8
T. rubrum	-	-	-		-	-	-	-	-	19.7±1.6

- = No inhibitory activity, MeOH = methanol, DCM = dichloromethane, EtAc = ethyl acetate.

It is also noteworthy that the ethyl acetate extract of the fungal biomass exhibited significant inhibitory activity on MRSA with an inhibition zone diameter comparable to that observed when 30 µg/mL of chloramphenicol was used (P=0.05). None of the test yeasts and filamentous fungi was inhibited by the extract of *Phomopsis sp*. ED2. This is probably due to the similarity of eukaryotic characteristics in the endophytic fungus and test yeasts [22]. The low inhibitory effect may caused by the exceptionally strong cell wall structure of the filamentous fungi which do not allow the diffusion of the bioactive compounds [24].

3.3 Determination of MIC and MLC

The MIC and MLC values of the fungal extracts on the selected test microorganisms are shown in Table 2. The antimicrobial activity of the extract was concentration-dependent. In general, the MIC values of the extract ranged from 31.25 to 250 µg/mL, thereby it is indicating the different susceptibility level of the test microorganisms towards the fungal extract. All of the extracts inhibited the growth of *B. subtilis* and it was found to be the most susceptible to the fungal extract. For most of the test microorganisms, the MLC was significantly higher than the MIC value, indicating that the concentration of the extract would have to be significantly increased to kill the microbial cells, instead of inhibiting their growth. Levison [25] reported that the MLC value for bactericidal drugs is usually not more than four-fold higher than their MIC. Hence, the activity of the extract can be considered as bactericidal because of the low MLC.

Table 2. MIC and MLC values of the crude extracts of *Phomopsis sp*. ED2 on selected test microorganisms

Test Microorganisms	Concentration of extract (µg/mL)									
	Fungal Biomass						Fermentative Broth			
	Methanol		DCM		EtAc		DCM		EtAc	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
Gram Positive Bacteria										
P. mirabilis	250	500	250	500	62.5	125	-	-	62.5	125
S. epidermidis	-	-	-	-	125	500	125	500	125	500
S. faecalis	-	-	-	-	250	500	-	-	-	-
B. subtilis	125	250	250	500	31.25	62.5	62.5	250	31.25	62.5
MRSA	-	-	-	-	125	125	-	-	-	-
Gram Negative Bacteria										
K. pneumoniae	125	500	-	-	62.5	250	125	500	62.5	125
Yeasts										
C. albicans	-	-	-	-	-	-	-	-	62.5	1000

- *Not tested*

Mirroring the result of disc diffusion assay, the ethyl acetate extract showed a very significant inhibitory on MRSA where the MIC and the MLC values are the same. Thus it indicates the bactericidal effect of the extract on MRSA. Besides, it is also showed that the extract that produces a bigger clear zone in disc diffusion assay did not showed a lower MIC values. This is because the diameter of the clear zone in disc diffusion assay allows only the diffusion of small or medium-sized polar compounds. Besides, the diameter of the clear zone was also affected by rate of diffusion and hydrophilicity of the bioactive metabolites [26]. Hence the broth microdilution assay is more sensitive and provides useful information in evaluating the relative degree of susceptibility of bacteria to the natural products [27].

3.4 GCMS Analysis of the Extract

GCMS analysis is important in identifying the non-volatile constituents of the active fraction. The chromatogram of the ethyl acetate extract was depicted in Fig. 2. The peaks in the chromatogram were numbered and the retention time, area and the matching factor of the compounds were characterized in Table 3. A total of 17 peaks were observed on the GCMS chromatogram, as most of the non-volatile constituents of the fraction was removed during the extraction process. Only 6 of them with the matching factor ≥90%. Generally, the type of compounds can be divided into 8 major groups, which are carboxylic acid, ketone, nitrosourea, amide, alkane, alcohol, phenol and arene based on the GCMS analysis. The major compound of this fraction is located at peak number 3, with an area of 38.03% and a matching factor of 92% with lomustine.

Based on the literature studies, three compounds that present in the extract exhibited antimicrobial activity, which are benzoic acid, lomustine and 3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid, or commonly known as penicillic acid. Benzoic acid and penicillic acid have been previously isolated from *Phomopsis*. Weber et al. [17] reported the isolation of benzoic acid from *Phomopsis sp*. E01105, an endophytic fungus from medicinal plant *Erythrine cristagalli*. Chilaka [28] reported the isolation of penicillic acid from *Phomopsis*, which exhibited antimicrobial activity. This is in agreement with the GCMS analysis of this extract that exhibited significant antibacterial activity.

Table 3. Characteristics of the compounds from GCMS analysis of the ethyl acetate extract of *Phomopsis sp*. ED2. A total of 17 volatile compounds were detected.

No	Compound	Retention time (min)	Area	Matching factor (%)
1	4,4,6-trimethyl-1,2-cyclohexen-1-one	5.15	0.21	33
2	Benzoic acid	5.51	0.61	93
3	Lomustine	5.65	38.03	92
4	Benzoic acid	6.88	6.84	94
5	N,N'-di-benzoyloxy-octanediamide	7.58	0.48	27
6	3-methoxy-benzoic acid	7.66	1.72	95
7	4-methoxy-benzoic acid	7.96	1.53	91
8	2,5-dimethyl-4-hexenoic acid	8.40	24.70	47
9	3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid	9.67	0.36	93
10	4,8,12-trimethyl-3,7,11-tridecatrienoic acid	11.98	0.45	42
11	6,6-dimethyl-2-2(2-methylpropyl)-bicyclo[3.1.1]heptan-3-one	12.32	0.20	30
12	3-hydroxy-5-methoxy-benzenemethanol	12.44	1.47	53
13	3-methyl-4-(methylthio)-phenol	12.53	2.84	47
14	1-chloro-2-propyl-benzene	13.45	1.50	41
15	1,2,3a,8b-tetrahydro-3a-(4-morpholyl)-3H-cyclopenta[b]-1H-benzofuran-7-ol	18.78	4.48	38
16	5,15-Dimethyl-2,4,7-trimethoxy-9H,10H-4b,9a-([1,2]benzenomethano)indeno[1,2-a]indene	25.83	14.41	50
17	5,15-Dimethyl-2,4,7-trimethoxy-9H,10H-4b,9a-([1,2]benzenomethano)indeno[1,2-a]indene	26.65	0.17	46

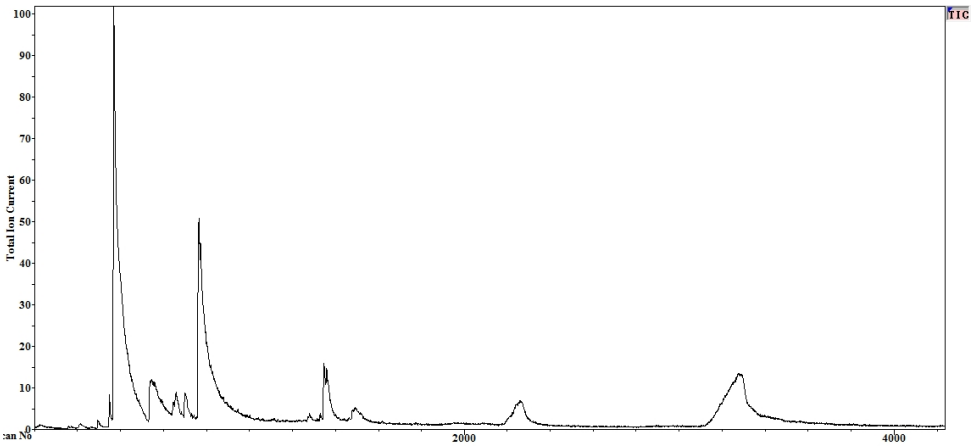

Fig. 2. The GCMS chromatogram of ethyl acetate extract of *Phomopsis sp*. ED2

4. CONCLUSION

The endophytic fungus *Phomopsis sp*. exhibited broad spectrum antibacterial activity against several test microorganisms. The GCMS analysis of the ethyl acetate extract showed the presence of 3 antimicrobial compounds. Further investigations should be carried out the isolate the antimicrobial compounds from this microorganism.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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