Insecticidal bioactive compounds derived from Cladosporium cladosporioides (Fresen.) G.A. de Vries and Acremonium zeylanicum (Petch) W. Gams & H.C. Evans

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Abstract

Entomopathogenic fungi (EPF) are the main microbiological control agents of insect pests. One of the key factors in the pathogenicity of EPF is the production of insecticidal bioactive compounds. Therefore, the metabolites of two isolates of EPF, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (AF98) and *Acremonium zeylanicum* (Petch) W. Gams & H.C. Evans (IR87), were analyzed. The presence of insecticidal toxic cyclic peptides such as Bassianolide in *C. cladosporioides* AF98 metabolites was detected by HPLC. Moreover, GC-MS analysis showed some toxic compounds, including 3,4-Dihydro-7,12-Dihydroxy-7,12-Imethylbenz[A]Anthracene, 1,2,3,4-Tetrahydro-1,1,4,4,6-Pentamethyl-5,7-Dinitronaphthalene, and 1,2,3,4-Tetrahydro-1,1,4,4,6-Pentamethyl-5,7-Dinitronaphthalene in the metabolites purified from *A. zeylanicum* IR87. Also, a few hazardous compounds, including 3,5-di-tert-butyl-4-trimethylsiloxytoluene, bis(2-Ethylhexyl) phthalate, di-n-octyl phthalate, 1-pentadecene, and 1-eicosene were found which might be toxic against insects. These results showed that multiple compounds are likely contributed to the insecticidal effects of the EPF.

Introduction

Entomopathogenic fungi (EPF) are the main microbiological control agents of insect pests, with 60%, in agricultural environments (Liu et al. 2017). The microbial agents play an important role in natural population regulations of insect pests in different ecosystems (Shah and Pell 2003). There is much public interest in the application of EPF as alternatives to chemical insecticides in integrated pest management (IPM) programs. The pathogens can directly penetrate in insect host cuticle despite other microbial control agents that must be ingested to infect their hosts (Lovett and Leger 2017). Furthermore, many EPF execrate some secondary metabolites considering as mycotoxins. The compounds may be toxic to the insect hosts and causing considerable mortalities when they are in contact with them. Furthermore, toxigenic isolates can kill the insect host faster than non-toxigenic fungi (Wang et al. 2018).

Based on biosynthetic pathways, two main classes of mycotoxins are nonribosomal peptides (NRPs) and synthetase mycotoxins and polyketide (PK) synthase mycotoxins (Hu et al. 2016). The class NRPs are composed of specific or modified amino acids and hydroxyl acids. The compounds are synthesized by using thiotemplate multienzyme mechanism. There are about twenty mycotoxins execrating from various isolates of EPF belonging to the genus *Beauveria*, *Metarhizium*, *Isaria*, *Paecilomyces*, *Akanthomyces*, etc. The NRP class is divided into groups of chain peptides, inclusing cicadapeptin and efrapeptin, and cyclic peptides, cyclopeptides, and cyclodepsipeptides. Cyclopeptides have cyclic structures which it is composed of amino acid residues through peptide bonding, such as cyclosporin. Cyclodepsipeptides as the main group of NRP class have lactone structure are composed of bonding amino acids with hydroxyl by using peptides (Hu and Dong 2015). The class PK synthase is the most abundant mycotoxins. The PK biosynthesis includes multiple enzymatic steps for the formation of the polyketides compose of different modules characteristic of each fungus, including keto synthases, acyl transferases, carboxylases, cyclases, dehydrases, aromatases, reductases, thioesterases, and laccases (Bräse et al. 2009).

Mycotoxins can be applied as bio-insecticides against some insect pests. There are some mycotoxins derived from EPF which currently commercially produced as biorational insecticides incuding beauvericin, cytochalasin C and cyclosporin H which are extracted from *Beauveria bassiana, Metarhizium anisopliae* and *Tolypocladium inflatum*, respectively (Boguś et al. 2021). Information about constituents of the mycotoxins produced by high potentials of EPF is crucial for developing the biorational insecticides.

Our previous study indicated that the isolate *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (AF98) and *Acremonium zeylanicum* (Petch) W. Gams & H.C. Evans (IR87) isolating from citrus hemipteran pests, *Pulvinaria aurantii* Cock. (Hemiptera: Coccidae) and *Aphis gossypii* Glover (Hemiptera: Aphididae), have appropriate potentials for controlling some aphids, including *Aphis fabae* Scopoli (Mousavi et al. 2022).

Determination of mycotoxin constituents can be serve as the first step for developing the compounds as biopesticides against target pests. There is no previous study on identifying the chemical composition of the EPF. Therefore, the study aimed to identify the chemical constituents of mycotoxins derived from the isolates.

Material And Methods

Fungal culture and maintenance

Two fungal isolates of *C. cladosporioides* and *A. zeylanicum* (AF98 and IR87, respectively) which had been previously isolated from *P. aurantii*and *A. gossypii* in citrus orchards of northern Iran, 36°54'24.2"N 50°39'26.7"E, and their pathogenicity had been confirmed against *A. fabae* Scopoli (Mousavi et al. 2022), were used in this study. The EPF were maintained on Sabouraud Dextrose Agar (SDA) Petri dish. The fungi were stored by preserving their conidia into 25% glycerol and kept at − 80°C, and sub-cultured for the following experiments (Zimmermann, 2007). The mycelia of the fungi were isolated from two-week-old plates and mixed with pre-autoclaved 0.05% liquid (w/v) Triton X-100. The fungi were then grown in 250 ml flasks containing 100 mL of Czapek Dox (CD) agar medium with 0.5% (w/v) bacto peptone, pH 7.0. The flasks were kept with 1 mL of conidial suspension containing 10^7 conidia/mL and incubated at 25 ± 1°C while shaking (150 rpm) for two weeks.

Preparation Of Crude Secondary Metabolites

Mycelium from the two-week-old culture of the fungi was harvested and isolated using centrifugation at 1000 *g*. The bioactive compounds were isolated from the fungi after the incubation period of two-weeks. Extracellular metabolites were isolated from the fungal cells using methanol: chloroform (1:2, v/v). Then, the mixture was centrifuged at 1000 *g* to remove fatty acids. The organic phase was separated and concentrated in a vacuum rotary evaporator. Relatively purified insecticidal toxic compounds were recovered and analyzed.

High-performance Liquid Chromatography (Hplc)

The crude extract of *C. cladosporioides* AF98 was dissolved in methanol-acetonitrile solution, the mobile phase was run in a K-60 silica gel column (230–400 mesh, 325 240 240 mm, Darmstadt, Germany) of chromatography machine (KNAUER, Germany), and started step by step in 50-mm methylene: First, methylene: methanol-dichloride (95: 5) was used and the slope of this solvent was used to achieve the desired polarities. The prepared sample was diluted with methanol and analyzed using a reverse phase HPLC column, and the peaks were detected by absorbing ultraviolet light at a wavelength of 240 nm. A typical slope of the thigh was used as follows: 0 minutes (0% acetonitrile), 30 minutes (40% acetonitrile), 40 minutes (50% acetonitrile), and 60 minutes (50% acetonitrile).

Gas Chromatography-mass Spectrometry (Gc-ms)

The resulted metabolites of *A. zeylanicum* IR87 were analyzed by using GC-MS with a quadrupole detector (GCMSQP2010 plus, Shimadzu, Japan). A Rtx®-5MS silica column (30 m × 25 mm, 25 µm film thickness, RESTEK) was used and the temperature was programmed from 80 to 320°C at 8°C/min and the holding temperature was for 5 min. The injection temperature was 280°C. The mass spectrum obtained by electron impact ionization was at 70 eV with a ratio of 25:1.

Results

Methanol/chloroform extracts of *C. cladosporioides* AF98 (990.0 mg) were examined following a fermentation process of two-week old liquid culture. The crude toxic matters were analyzed using HPLC and the materials were determined. The toxic cyclic peptides were detected within the metabolites. The bioactive compounds within the crude fungal metabolites were identified by using a mobile phase with a linear gradient of ddH_2O and acetonitrile at a flow rate of 1 mL/min throughout a 35 min time period. A major peak with high intensity was detected at the retention time of 3.3 min. Also, a minor peak was found at the retention time of 2.5 min (Fig. 1). The synthesis of fungal bioactive compounds was observed in fungal cultures submerged in CD broth. A few peaks related to impure materials were detected showing the rare impurity within the partially purified suspension.

Metabolites purified from *A. zeylanicum* IR87 were subjected to GC-MS analysis to identify their different components. Peaks were observed at different retention times 55 minutes after injection (Fig. 2). The identified compounds belonging to each of the observed peaks are listed in Table 1. The presence of cyclic peptide compounds with insecticidal properties (Sinha et al., 2016) was confirmed in the metabolites of the tested isolate.

Table 1
Details of the compounds identified in the metabolites of *A. zeylanicum* IR87 using GC-MS.

Number	Retention time (min)	Compound
1	8.933	Benzeneacetaldehyde
		Phenyl Acetaldehyde
		Styrene Oxide
2	10.297	Benzoic Acid
3	21.196	Phenol
4	30.097	Hexadecanoic Acid, Methyl Ester
		Pentadecanoic Acid
5	30.336	Methyl-3-(3,5-Ditertbutyl-4-Hydroxyphenyl) Propionate
		3,4-Dihydro-7,12-Dihydroxy-7,12-Dimethylbenz[A]Anthracene
		1,2,3,4-Tetrahydro-1,1,4,4,6-Pentamethyl-5,7-Dinitronaphthalene
		3,5-Di-Tert-Butyl-4-Trimethylsiloxytoluene
		1h-Indole-3-Acetic Acid, 5-[(Trimethylsilyl)Oxy]-, Methyl Ester
		(S)-2-Iodo-4-Methoxy-(1-Methoxyethyl)Benzene
6	33.349	9,12-Octadecadienoic Acid, Methyl Ester
		Ethyl Linoleate
		Ethylester
7	33.454	9-Octadecenoic Acid (Z)-, Ethyl Ester
		10-Octadecenoic Acid, Methyl Ester
8	33.565	Methyl Dihydromalvalate
		6-Octadecenoic Acid, Methyl Ester, (Z)-
		9-Octadecenoic Acid (Z)-, Methyl Ester
		E-1,9-Hexadecadiene
		15-Tetracosenoic Acid, Methyl Ester, (Z)-
		1-Propanesulfonic Acid, Methyl Ester
9	33.909	Octadecanoic Acid, Methyl Ester
		Heptadecanoic Acid
10	38.333	9-Octadecenamide

Number	Retention time (min)	Compound
		1-Cyclohexylnonene
		Butanal
		9,12-Octadecadienoyl Chloride
11	40.792	1,2-Benzenedicarboxylic Acid, Bis(2-Ethylhexyl) Ester
		Bis(2-Ethylhexyl) Phthalate
		Di-N-Octyl Phthalate
12	43.619	Heptadecene-(8)-Carbonic Acid-(1)
		9-Octadecenoic Acid, (E)-
		1-Pentadecene
		2-Octadecenal
		4-Cyclohexene-1,2-Dicarboximide, N-Butyl-, Cis-
13	44.639	2-Pentene, 3-(Chloroethylboryl)-2-(Chlorodimethylsilyl)-, (E)-
		Methyl 9,9-Dideutero-Octadecanoate
		12-Octadecenoic Acid, Methyl Ester
		E-2-Methyl-3-Tetradecen-1-Ol Acetate
		9-Octadecenoic Acid (Z)-
14	45.514	Propyleneglycol Monoleate
		Z-8-Pentadecen-1-Ol Acetate
		1-Hydroxy-1,7-Dimethyl-4-Isopropyl-2,7-Cyclodecadiene
		Linoleic Acid
		1h-Indene, 2-Butyl-5-Hexyloctahydro-
15	46.248	2-Methyl-Z,Z-3,13-Octadecadienol
		Heptadecene-(8)-Carbonic Acid-(1)
		1-Pentadecene
16	46.405	1-Eicosene
		2-Methyl-Z,Z-3,13-Octadecadienol
		9-Octadecenoic Acid
17	46.598	2h-1-Benzopyran

Number	Retention time (min)	Compound
		E-2-Methyl-3-Tetradecen-1-Ol Acetate
		Bis(Trimethylsiloxy)Methylsilane
		Z,Z-10,12-Hexadecadien-1-Ol Acetate
18	46.901	Z-8-Pentadecen-1-Ol Acetate
		2-Pentene, 3-(Chloroethylboryl)-2-(Chlorodimethylsilyl)-, (E)-
		2-Myristynoyl-Glycinamide
		Indole-2-One, 2,3-Dihydro-N-Hydroxy-4-Methoxy-3,3-Dimethyl-
		4-Cyclohexene-1,2-Dicarboximide, N-Butyl-, Cis-
		Z-4-Nonadecen-1-Ol Acetate
19	47.449	6-Nitro-Cylohexadecane-1,3-Dione
		6-Methyl-5-[1-Piperidinyl]-2,4-Pyrimidinediamine
		6,7-Dibromo-Z-11-Tetradecene-1-Ol Acetate
		Corydaldine
		2-Nitro-2-(3'-Hydroxybutyl)Cyclododecanone
		Pyrido[2,3-D]Pyrimidine, 4-Phenyl-
20	47.816	Octadec-9-Enoic Acid
		Heptadecene-(8)-Carbonic Acid-(1)

Discussion

EPF can produce a variety of secondary metabolites which have been considered as a key factor throughout their infection process (Vey et al. 2001). Although, it is documented that *A. zeylanicum* (isolate IR87) cause significant mortality against *A. fabae* (Mousavi et al. 2022), However, it is unknown whether the mortality is related to direct host infection, toxicity with mycotoxins execrating by the EPF, or both of them. Mousavi et al. (2022) indicated that the aphid mortality by the isolated was enhanced by increasing days after treatment. More mycotoxin production by the EPF may be a major reason for the mortality increase over the time.

Entomopathogenic effects of *C. cladosporioides* against some hemipteran pests, including *Brevicoryne brassicae* L. (Ibrahim, 2017), *Metopolophium dirhodum* Walker (Abdelaziz et al. 2018), and *Ceratovacuna lanigera* Zehntner (Hemiptera: Aphididae) have been reported previously (Kishore Varma et al. 2019). However, there are limited efforts to detect chemical constituents of EPF isolates of *C. cladosporioides*. Chemical analyses of secondary metabolites derived from Egyptian isolate of *C. cladosporioides*, isolated

from *Aphis craccivora* Koch (Hemiptera: Aphididae), implicated that 3-phenyl propanoic acid (6) and 3-(4β-hydroxy-6-pyranonyl)-5-isopropylpyrrolidin-2-one (7) are two major compounds in ethyl acetate extract of the EPF. Further bioassays showed that the extract led to high toxicity to *A. gossypii*, LC_{50} of 24.58 ppm and LC_{90} of 128.73 ppm (El-Sawy et al. 2019). However, the compounds were not detected from the extract of *C. cladosporioides* (isolate AF98). In EPF, histone alkylation (such as methylation or acetylation) status is related to the regulation of the secondary metabolite production. The manipulation of epigenetic regulators can induce or repress the production of multiple secondary metabolites by using chromatin remodeling that influences the biosynthetic gene clusters placed in heterochromatic zones (Zhang et al. 2020).

Akanthomyces lecanii Zimmermann is one of the EPF that has been well-studied as a microbial biocontrol agent and its compounds with toxic effects have been applied against various arthropodan pests (Butt et al. 1994; Isaka et al. 2005; Molnar et al. 2010). The cyclic peptides with toxic effects on insects have been found in *A. lecanii* (Ravindran et al. 2018). Similarly, the cyclic peptides were identified within *C. cladosporioides* AF98 metabolites analyzed in this study. Previous researches indicated that these compounds can be detected in different species of EPF such as *M. anisopliae* (Ravindranet al. 2016) and *B. bassiana* (Kanaoka et al. 1978). To the best of our knowledge, this is the first report on the detection of toxic peptides from *C. cladosporioides.*

Among the compounds found in *A. zeylanicum* IR87, 3,4-Dihydro-7,12-Dihydroxy-7,12-imethylbenz[A]Anthracene (synonym: 7,12-Dimethylbenz[A]Anthracene) has been identified as an active mutagenic compound in mouse cells (Schoepe et al. 1986). Moreover, 1,2,3,4-Tetrahydro-1,1,4,4,6-Pentamethyl-5,7-Dinitronaphthalene (synonym: methyl ionene [chemical family: Sesquiterpenes]) detected in the fungal metabolite was first isolated from *Sclerophoma pythiophila* Corda (Fraga 2013). Sesquiterpenes are natural terpenoids mainly distributed in plant and microbial communities (Kramer and Abraham 2012). Most of its oxygenated derivatives have a strong aroma and biological activity (Fraga 2013). It has been shown that these compounds have a satisfactory inhibition of *Plasmodium falciparum* Welch (Jaquet et al. 1994) and considerable antimalarial effects (Zhou et al., 1994). Also, 1-pentadecene was detected in *A. zeylanicum* IR87 used as aggregation pheromone of flour beetles, *Tribolium* spp. (Coleoptera: Tenebrionidae) to communicate between the beetles. Therefore, the compound can be applied in pheromone traps for developing IPM programs of the stored product pests (Arnaud et al. 2002). However, it is demonstrated that high concentrations of this compound have a significant repellent effect on the insect (Đukić et al. 2021). Some hazardous compounds, including 3,5-di-tert-butyl-4-trimethylsiloxytoluene, bis(2-Ethylhexyl) phthalate, di-n-octyl phthalate, 1-pentadecene, and 1-eicosene were found which might be toxic against insects. Our results show that several compounds probably contributed to the toxicity of *A. zeylanicum* metabolite against pests such as woolly aphid (*Ceratovacuna lanigera* Zehntner), spider mite (*Tetranychus truncatus* Ehara), cabbage aphid (*Brevicoryne brassicae* L.) and sorghum aphid (*Melanaphis sacchari* Zehntner).

Conclusion

The cyclic peptide, Bassianolide, may be the most important constituent with potential insecticidal property in the crude extract of the entomopathogen *C. cladosporioides* (AF98). In case of *A. zeylanicum* (IR87), a number of toxic compounds were detected in the fungal extract some of which, such as 4-Dihydro-7,12-Dihydroxy-7,12-Imethylbenz[A]Anthracene, 1,2,3,4-Tetrahydro-1,1,4,4,6-Pentamethyl-5,7-Dinitronaphthalene, and 1,2,3,4-Tetrahydro-1,1,4,4,6-Pentamethyl-5,7-Dinitronaphthalene, can be considered as potential bio insecticides for applying against some hemipteran pests including *A. fabae*. However, the experiment is preliminary and effect of each constituent on the target pest has to be evaluated before any commercial recommendations.

Declarations

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Competing interests: The authors declare that they have no competing interests.

Authors' contributions: AR, MHGP and FY designed the experiments. KM performed the

experiments. AR wrote the manuscript. The manuscript studied and certificated by all authors.

Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

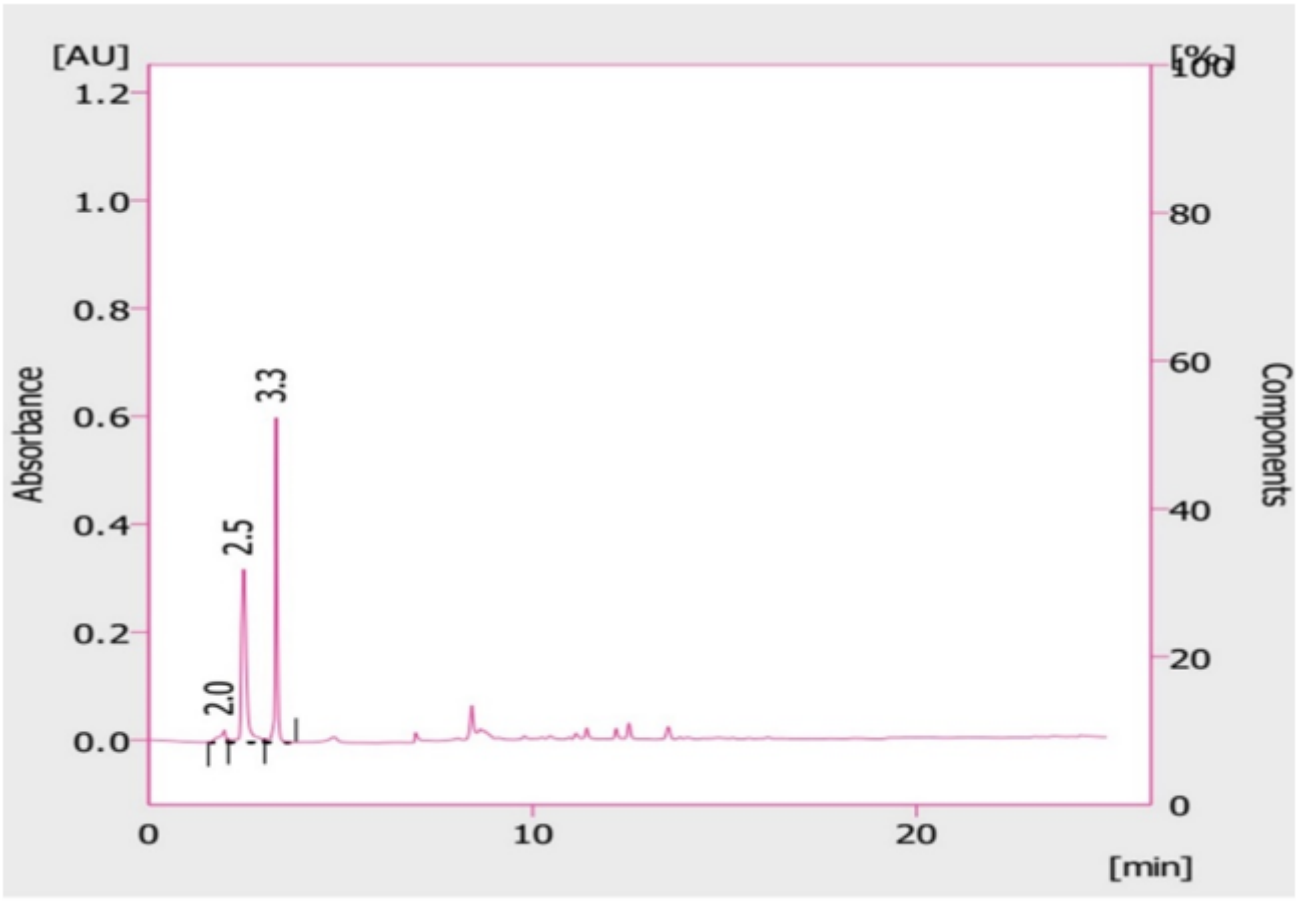

Figure 1

HPLC analysis of toxic compound produced by *C. cladosporioides* AF98.

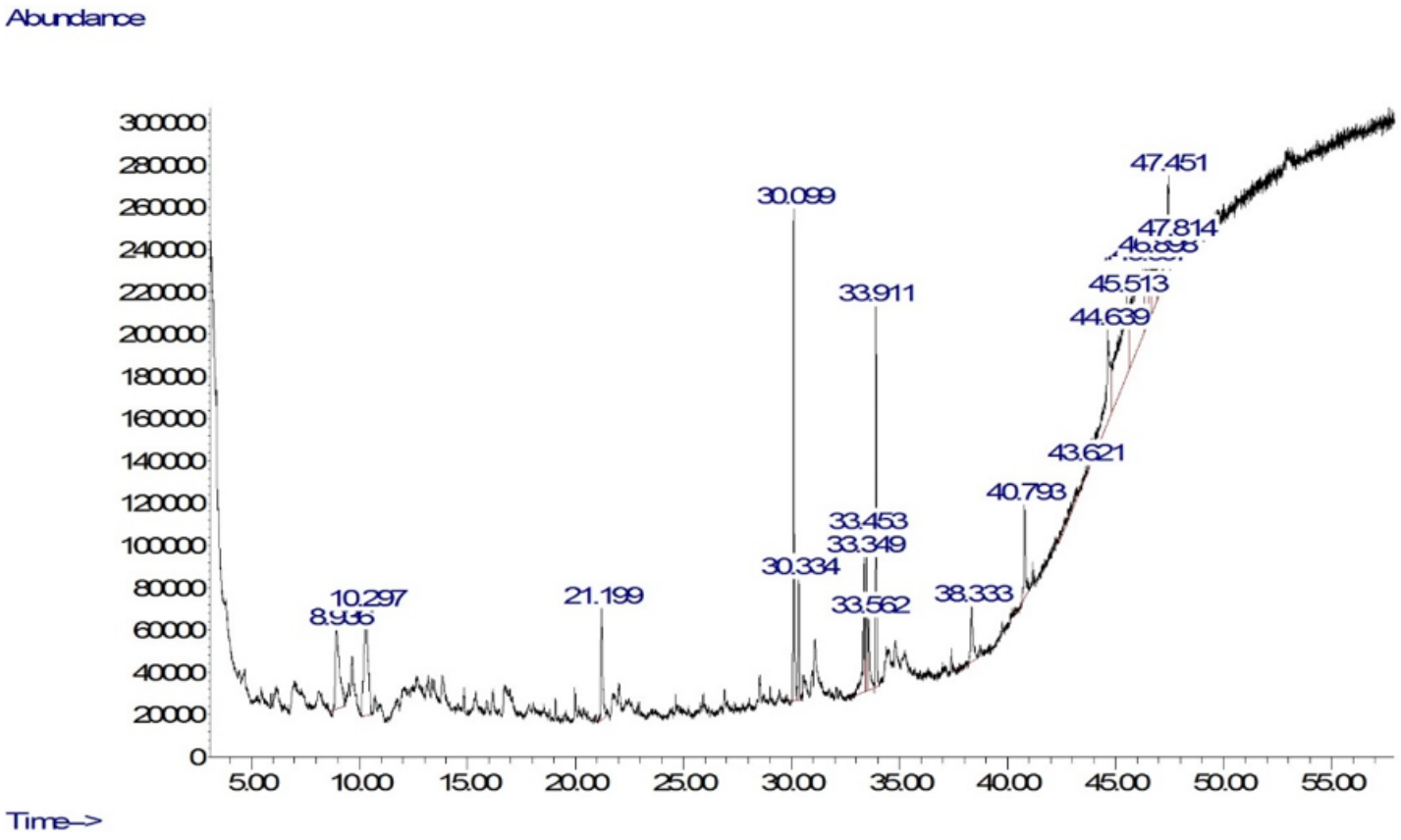

Figure 2

Chromatogram of *A. zeylanicum* IR87 analyzed by GC-MS.