



Bioactivity of *Alcaligenes faecalis* and *Lecanicillium* sp. isolated from crocker range of Sabah against *Erwinia psidii* of papaya dieback disease

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ABSTRACT

Aims: *Erwinia psidii* was first reported in 2017 to be the causal pathogen of papaya dieback disease (PDD) in Sabah, Malaysia. The present study aimed to isolate potential biocontrol agents against this pathogen.

Methodology and results: Out of the 20 samples collected from Crocker Range of Sabah, 154 bacteria and 55 fungi isolates were isolated and screened for their antagonistic activity against *E. psidii*. The fungi and bacteria which gave the highest inhibition to *E. psidii* were identified using molecular technique as *Alcaligenes faecalis* and *Lecanicillium* sp., respectively. Both isolates were selected for extraction of their secondary metabolites to determine their bioactivity against *E. psidii*. Micro-well dilution method was used to determine minimum inhibitory concentration (MIC) for each microbes' extract. GC-MS analysis was carried out to determine their secondary metabolites.

Conclusion, significance and impact of study: *Lecanicillium* sp. (Diethyl ether, chloroform and ethyl acetate) extracts and *A. faecalis* (Diethyl ether extract) showed positive inhibition against *E. psidii*. GC-MS analysis revealed that both *A. faecalis* and *Lecanicillium* sp. had secreted some secondary metabolites such as N-formylmaleamic acid, Oleamide and D-1-Piperidine-2-carboxylic acid that may relate to the growth inhibition. *Alcaligenes faecalis* and *Lecanicillium* sp. are potential to be further investigated as biocontrol against *E. psidii*.

Keywords: Papaya dieback disease, *Erwinia psidii*, *Alcaligenes faecalis*, *Lecanicillium* sp.

INTRODUCTION

Papaya (*Carica papaya*) is an economically important fruit crops grown in Malaysia with high export value. However, the production is hampered with the presence of papaya dieback disease (PDD). The disease causes great economic losses to the industry in Peninsular Malaysia and total yield losses were estimated at 200,000 metric tons, equivalent to US\$ 58 million (Maktar *et al.*, 2008). Although the losses from this disease are great in Peninsular Malaysia but it is rather new in Sabah and was first reported to cause by *Erwinia psidii* (Chai *et al.*, 2017). This pathogen threatens papaya plant and causes massive fruit yield loss. To date, no promising control has been reported in controlling this pathogen. A more drastic but sustainable approach such as using biological control may worth to be explored. Biological control has been proven significantly reduced disease severity such as basal stem rot of oil palm (Shamala *et al.*, 2009); potato scab (Han *et al.*, 2005); papaya antracnose (Rahman *et al.*, 2006). It has been noticed that soil microbes such as bacteria and fungi produce various secondary metabolites includes antibiotics, pigments and toxins which is

important in antimicrobial resistance (Demain, 1998; Bizuye *et al.*, 2013). Previous study of Sharifazizi *et al.* (2017) for instance has reported some of the potential bacteria strains such as *Pseudomonas* and *Pantoea* which were suitable as biocontrol agents against *E. amylovora*. Sabah is blessed with its rich biodiversity. However, the microbial biodiversity of Sabah is poorly understood and information on the soil microbial diversity is not well documented. Thus, this research was designed to study the potential of some biological control agents isolated from Crocker Range of Sabah and activity against *E. psidii* of PDD.

MATERIALS AND METHODS

The pathogen

The causal pathogen of PDD used in this experiment was obtained from the stock culture of Genetic Laboratory of Faculty of Science and Natural Resources, Universiti Malaysia Sabah (UMS). The pathogenicity of the isolate was confirmed previously using Koch Postulate and

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identified as *E. psidii* using molecular technique (Chai *et al.*, 2017).

Isolation of fungi and bacteria from soil samples

Twenty soil samples were collected from Crocker Range virgin forest of Sabah and transferred to UMS. The isolation of microbial of interest was conducted using the common dilution plate method (DPM) except different type of media were used for different target microorganisms, Potato Dextrose Agar (PDA)+ Streptomycin for fungi and Nutrient Agar (NA) for bacteria. Successful isolated microbes were differentiated based on their morphology. All isolates were maintained in their respective media for further investigation.

Soft agar overlay assays

Soil fungi were grown on PDA for five days and overlaid by a layer of soft agar (0.7%) containing *E. psidii*. Four fungi-free plates of PDA overlaid with test bacteria were used as control. The plates were incubated at room temperature for 48 h to observe any formation of inhibition zone (LiHan *et al.*, 2014). Bacteria were grown on LB plates and incubated for two days before overlaid with a layer of soft agar. Microorganisms that showed the formation of inhibition zone were selected for another cycle of soft agar overlay assay.

DNA extraction of microbes with the highest inhibition zone against *E. psidii*

DNA of isolates with the highest inhibition zone against the pathogen causal of PDD was extracted according to Yeates *et al.* (1998) and Kabir *et al.* (2003) with slight modification. Approximately 1.5 mL of bacteria culture in Nutrient Broth (NB) was centrifuged at 10,000 *g* for three min to collect the bacterial pellet. Meanwhile for fungi, 10 mg of dried pure culture-fungal mycelia was used. Extraction buffer (500 μ L of 100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 1.5 M NaCl containing proteinase K (0.5 mg) was mixed with both samples. The initial extraction procedure of fungi was aided with mechanical force from sterile glass rod to disrupt fungal thick cell wall. Both samples were then incubated in incubator shaker at 37 °C, 180 rpm for 30 min. Next, sodium dodecyl sulphate (SDS) was added (500 μ L; 20%) and the samples were incubated at 65 °C for 90 min. The supernatants were collected after centrifugation at 6000 *g* for 10 min at room temperature and were transferred to centrifuge tubes (1.5 mL) containing a half-volume of polyethylene glycol (30%)/ sodium chloride (1.6 M) and incubated at room temperature for 2 h. Samples were centrifuged (10,000 *g* for 20 min) and the partially purified nucleic acid pellet resuspended in 500 μ L of TE (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0). Potassium acetate (7.5 M) was added to a final concentration of 0.5 M. Samples were transferred to ice for 5 min then centrifuged (16,000 *g*, 30 min) at 4 °C to precipitate proteins and polysaccharides. The aqueous phase was

extracted with phenol/chloroform and chloroform/isoamyl alcohol and DNA was precipitated by adding 0.6 volume isopropanol. After 2 h at room temperature, DNA was pelleted by centrifugation (16,000 *g* for 30 min) and resuspended in TE (50 μ L). Quantity and quality of the resulting DNA was evaluated using Qubit 2.0, Invitrogen.

PCR and molecular identification

PCR protocol used was as described by Boyle *et al.* (2008). For bacteria, PCR amplification used the primers of Eub338_Reverse (5'-ACT CCT ACG GGA GGC AGC A-3') and Eub518_Forward (5'- ATT ACC GCG GCT GCT GG-3'). Meanwhile for fungi, PCR amplification used the primers of ITS1f_Reverse (5'-TCC GTA GGT GAA CCT GCG G-3') and 5.8s_Forward (5'-GCG TGC GTT CTT CAT CG-3'). Amplification is performed in a conventional thermocycler (Eppendorf Mastercycler). The following thermal cycle was performed; bacteria: 94 °C for 3 min (one cycle), 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min (35 cycles), 72 °C for 5 min (one cycle); fungi: 94 °C for 3 min (one cycle), 96 °C for 15 sec, 60 °C for 90 sec, 72 °C for 2 min (30 cycles), 72 °C for 5 min (one cycle). The resulting PCR products were kept at 4 °C for further usage. Molecular identification on the PCR product was performed at Q-myc, Gyeonggi-do, South Korea.

Determination of minimum inhibitory concentration (MIC) microbial extracts against *E. psidii*

Isolates with the highest percentage of inhibition zone were selected for their secondary metabolite extraction. Six different solvents with different polarity (Hexane, diethyl ether, chloroform, ethyl acetate, acetone and methanol) were used. The MIC value from each extract was evaluated according to micro-well dilution method with slight modification (Eloff, 1998). Extracts were first diluted with 99.9% (v/v) DMSO. Initial concentration of each extract was 80 mg/mL. Seven serials two-fold dilutions were conducted to obtain different concentrations of each extract (80 mg/mL, 40 mg/mL, 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL). Chloramphenicol and streptomycin were used as positive controls while DMSO was used as negative controls. Extracts (5 μ L) and 195 μ L of bacteria inoculum were filled in microplate and incubate at 28 °C for 20-22 h. An indicator of bacteria growth, INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, 95%) at initial concentration of 0.3 mg/mL was added into each well. The plate was incubated for another two hours at 28 °C. Any changes in solution to pinkish-red indicated bacteria growth, meanwhile no colour changes indicated bacteria inhibited by the extract. The test was performed in triplicate.

Identification of metabolites from potential antagonists against *E. psidii*

Bioactive compounds obtained from extracts were identified using Gas Chromatography-Mass Spectrometry

(GC-MS). Prior to GC-MS analysis, the crude extracts were diluted to 200 ppm and filtered using a 0.45 µm sterile PTFE filter. The GC-MS analysis of the extracts were carried out using Agilent 2000 Electron spray ionisation (ESI) GC-MS with fused silica 15 m × 0.2 mm ID × 1 µm of capillary column. Different extracts were using different GC-MS analysis procedures as followed:

Ethyl acetate extract

The instrument was set to an initial temperature of 50 °C for 5 min. At the end of this period the oven temperature was raised to 256 °C, at the rate of an increase of 4 °C/min and maintained for 10 min. Injection port temperature was maintained as 260 °C and helium flow rate as 1 mL/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 60-750 (m/z).

Chloroform extract

The instrument was set to an initial temperature of 110 °C, and maintained at this temperature for 5 min. At the end of this period the oven temperature was increased to 280 °C, at the rate of an increase of 3 °C/min and maintained for nine minutes. Injection port temperature was maintained as 250 °C and helium flow rate as 1 mL/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 60-750 (m/z).

Diethyl ether extract

The instrument was set to an initial temperature of 50 °C, and maintained at this temperature for 1 min. The oven temperature was increased to 145 °C, at the rate of an increase of 5 °C/min, increased to 175 °C at 7 °C/min, increased to 195 °C at 5 °C/min, and then ramped to 250 °C at 3 °C/min; 250 °C was maintained for 10 min. Injection port temperature was maintained as 250 °C and helium flow rate as 1 mL/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 60-750 (m/z).

Compounds identification

Chromatographic spectrum and m/z spectrum that obtained from GC-MS analysis were further processed using MZmine ver. 2.3 to facilitate the peaks and comparing the spectrum obtained through GC-MS analysis with computer searches on NIST Ver.2.1 MS data library. Other online database tools such as KEGG, Pubchem, LipidBank Database and Chemspider were also being utilized for the identification.

RESULTS AND DISCUSSION

A total of 209 pure isolates were obtained after three times of re-isolation from 20 soil samples (data not shown). Out of the total pure cultured, 154 bacteria and

55 were isolated. Isolated B93 (bacteria) and F8 (fungi) were selected for molecular identification due to their highest inhibition against *E. psidii*. Table 1 shows the BLAST analysis where query sequence for B93 showed high similarity (99.71%) to *A. faecalis* while the query sequence for F8 showed similarity of 100% to *Lecanicillium* sp. in Table 2. Figure 1 show the phylogenetic analysis where both B93 and F8 were identified as *A. faecalis* and *Lecanicillium* sp. respectively.

MIC was performed to determine the growth inhibition of *A. faecalis* and *Lecanicillium* sp. in six different solvents extracts against *E. psidii*. The results exhibited that all acetone and methanol extracts showed there was no inhibition against *E. psidii* (Table 3). *Lecanicillium* sp. extracted with diethyl ether, chloroform and ethyl acetate showed the same results where inhibition was only found at higher concentration of 2 mg/mL. Diethyl ether extracts of *A. faecalis* showed the best results when 1 mg/mL of extract was added into the well. The MIC for the best extracts was 2 mg/mL for *Lecanicillium* sp. and 1 mg/mL for *A. faecalis*. Both *A. faecalis* and *Lecanicillium* sp. appeared to have inhibitory effect against *E. psidii*.

GC-MS was carried out to study the potential metabolites that contribute to the inhibition against *E. psidii*. The best extracts for *Lecanicillium* sp. (diethyl ether, chloroform and ethyl acetate) and *A. faecalis* (diethyl ether) undergone GC-MS analysis. A total of nine metabolites were found in the diethyl ether extract of *A. faecalis* (Figure 2). The metabolites were (1) di[1-(3,4-methylenedioxyphenyl)-2-propyl]amine, (2) clindamycin, (3) N-formylmaleamic acid, (4) malonic acid, (5) sabinene hydrate, (6) methyl salicylate, (7) methylethylketone, (8) gamma-Crotonolactone and (9) valproic acid. Some metabolites detected in this extract were known to have antibacterial activities. Methyl salicylate is produced by microorganisms and has significant antioxidant and antimicrobial activities (Oloyede and Ayanbadejo, 2014). Similarly, gamma-Crotonolactone has been reported to have good antimicrobial properties (Aguedo *et al.*, 2003). N-formylmaleamic acid that was identified is a derivative of maleamic acid which is known to have antibacterial activities against *S. aureus* and *Pseudomonas aeruginosa* (Al-Ameri *et al.*, 2014). While, Sabinene hydrate found in the extract is active against *S. aureus*, *Enterococcus faecalis*, *E. coli* and *Klebsiella pneumonia* (Ramos *et al.*, 2011). Valproic acid has been reported to induce antimicrobial compound production in *Doratomyces microspores* (Zutz *et al.*, 2016).

Meanwhile for the GC-MS chromatogram of *Lecanicillium* sp., six metabolites were identified from diethyl ether extract (Figure 3). The metabolites were (1) dihydroliipoamide, (2) 16-methyl-6Z,9Z-heptadecadienoic acid, (3) linoleic acid, (4) 2-hydroxy-4-oxobutane-1,2,4-tricarboxylic acid, (5) 2-methyl-2Z-hexenoic acid and (6) dibenzylketone. Among the metabolites identified in diethyl ether extract, 2-hydroxy-4-oxobutane-1,2,4-tricarboxylic has been reported to possess antibacterial activity (Lee and Najjah, 2009). Linoleic acid is a polyunsaturated fatty acid that has antifungal activity against several plant pathogenic fungi (Liu *et al.*, 2008).

16-methyl-6Z,9Z-heptadecadienoic acid and 2-methyl-2Z-hexenoic acid are fatty acid constituents of plant which also commonly known to possess antimicrobial properties (McGaw *et al.*, 2002).

Table 1: BLAST analysis from NCBI gene bank, where the 16S rRNA sequence of sample B93 was sent as the query sequence. Note this isolate had 99.71% of similarity to *A. faecalis* strain NBRC 13111.

Description	Max Score	Total Score	Query Cover	E value	Identity	Accession
<i>A. faecalis</i> strain NBRC 13111 16S ribosomal RNA, partial sequence	2486	2486	100%	0.0	99.71%	NR 113606.1
<i>A. faecalis</i> strain IAM 12369 16S ribosomal RNA, partial sequence	2449	2449	100%	0.0	99.26%	NR 043445.1
<i>A. aquatilis</i> strain LMG 22996 16S ribosomal RNA, partial sequence	2442	2442	100%	0.0	99.04%	NR 104977.1
<i>A. faecalis</i> sub sp. <i>parafaecalis</i> strain 16S ribosomal RNA, partial sequence	2423	2423	100%	0.0	98.90%	NR 025357.1

Table 2: BLAST analysis from NCBI gene bank, where the nucleotide sequence of sample F8 was sent as the query sequence. Note this isolate had 100% of similarity to *Lecanicillium* sp. CCF 5201.

Description	Max Score	Total Score	Query Cover	E value	Identity	Accession
<i>Lecanicillium</i> sp. CCF 5201 genomic DNA sequence contains 28S rRNA gene, strain UBOCC-A-112180	1092	1092	100%	0.0	100%	LT 992876.1
<i>Lecanicillium</i> sp. CCF 5201 genomic DNA sequence contains 28S rRNA gene, strain UBOCC-A-116026	1092	1092	100%	0.0	100%	LT 992873.1
<i>Lecanicillium</i> sp. CCF 5201 genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, strain CCF 5201	1092	1092	100%	0.0	100%	LT 548278.1
<i>Lecanicillium</i> sp. CCF 5252 genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, strain CCF 5252	1075	1075	100%	0.0	99.49%	LT 548279.1

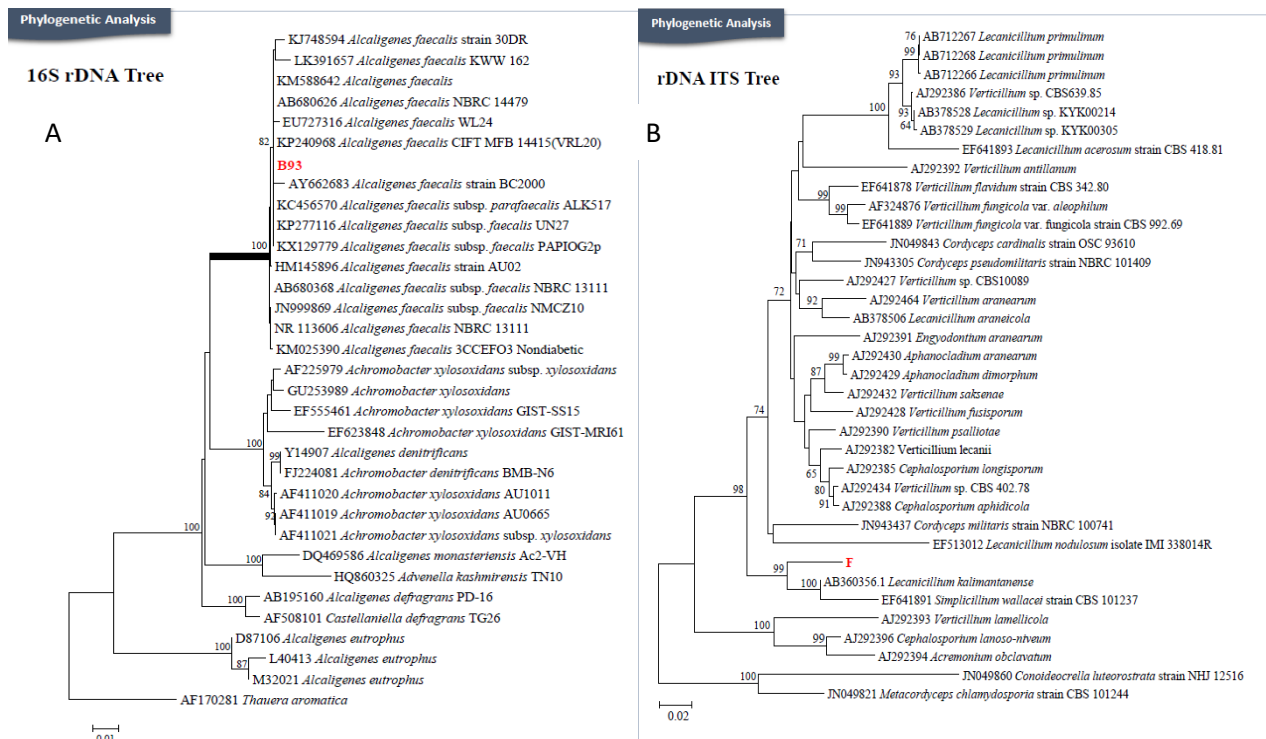


Figure 1: Phylogenetic analysis of potential antagonists against *E. psidii*: A, B93 identified as *A. faecalis*; B, F8 identified as *Lecanicillium* sp.

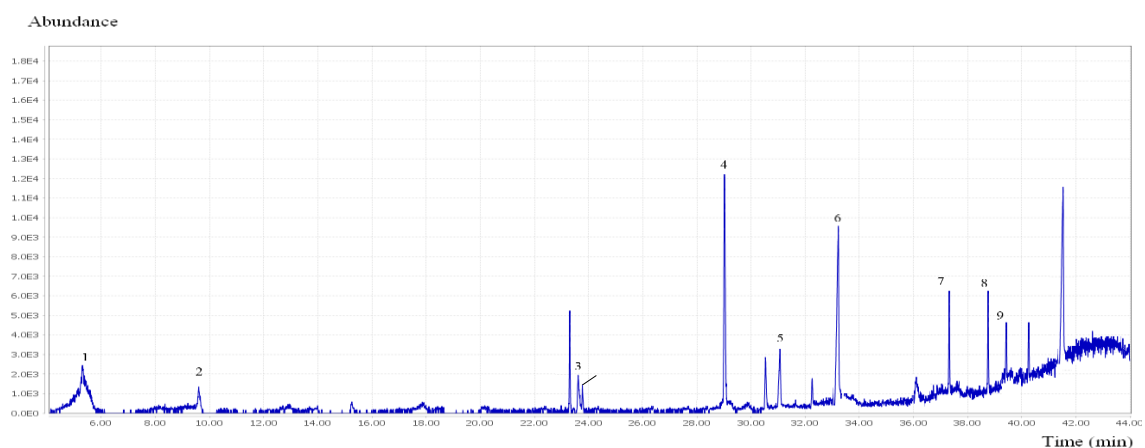


Figure 2: GC-MS profile of diethyl ether extract of *A. faecalis*: 1, di[1-(3,4-methylenedioxyphenyl)-2-propyl]amine; 2, Clindamycin; 3, N-formylmaleamic acid; 4, Malonic acid; 5, Sabinene hydrate; 6, Methyl salicylate; 7, Methyl ethyl ketone; 8, gamma-Crotonolactone; 9, Valproic acid.

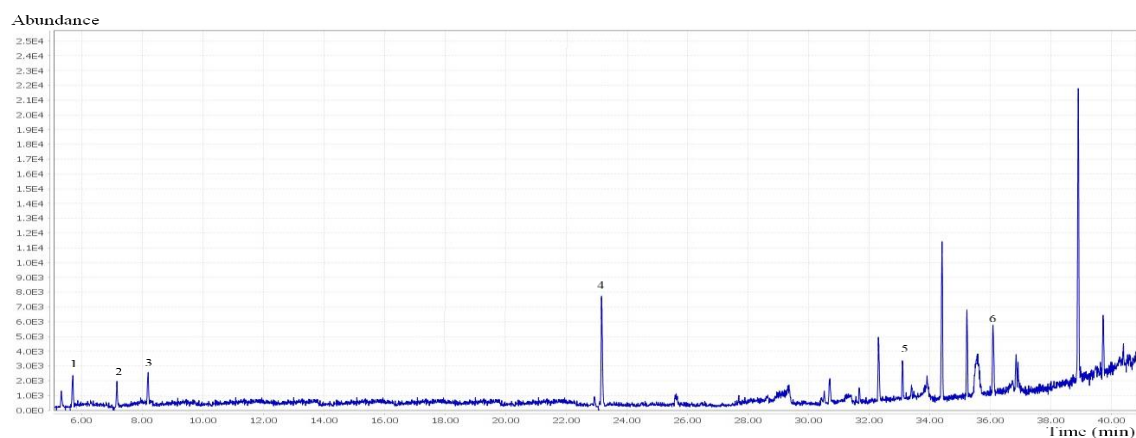


Figure 3: GC-MS profile of diethyl ether extract of *Lecanicillium* sp.: 1, Dihydrolipoamide; 2, 16-methyl-6Z,9Z-heptadecadienoic acid; 3, Linoleic acid; 4, 2-hydroxy-4-oxobutane-1,2,4-tricarboxylic acid; 5, 2-methyl-2Z-hexenoic acid; 6, Dibenzylketone.

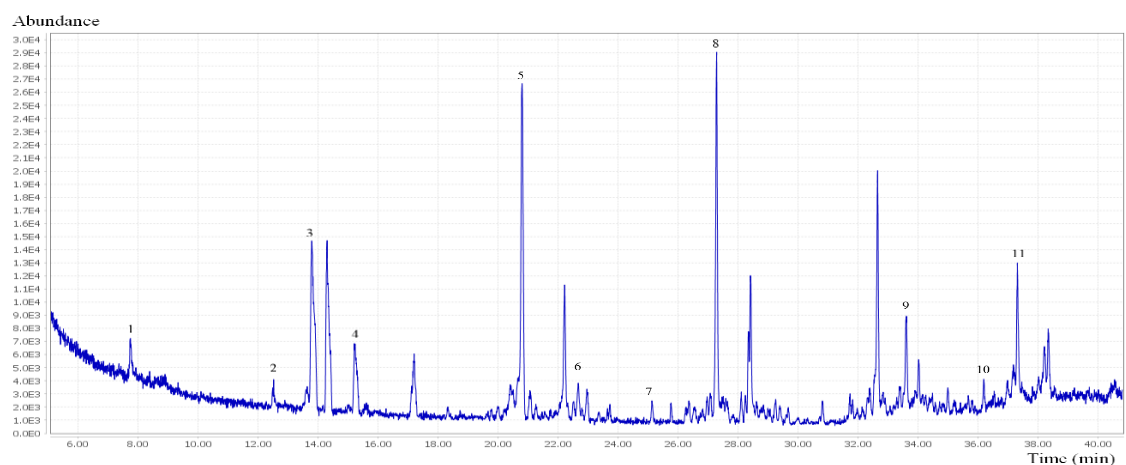


Figure 4: GC-MS profile of chloroform extract of *Lecanicillium* sp.: 1, Thiophene; 2, But-2-enoic acid; 3, Dihydrofuran-2(3H)-one; 4, 2-Pyrrolidinone; 5, Cyclopentanone; 6, 1-Pyrroline-5-carboxylic acid; 7, Piperidine; 8, D-1-Piperidine-2-carboxylic acid; 9, 2-methyl-16-heptadecenoic acid; 10, 1-Pyrroline; 11, 1-Pyrroline-5-carboxylic acid.

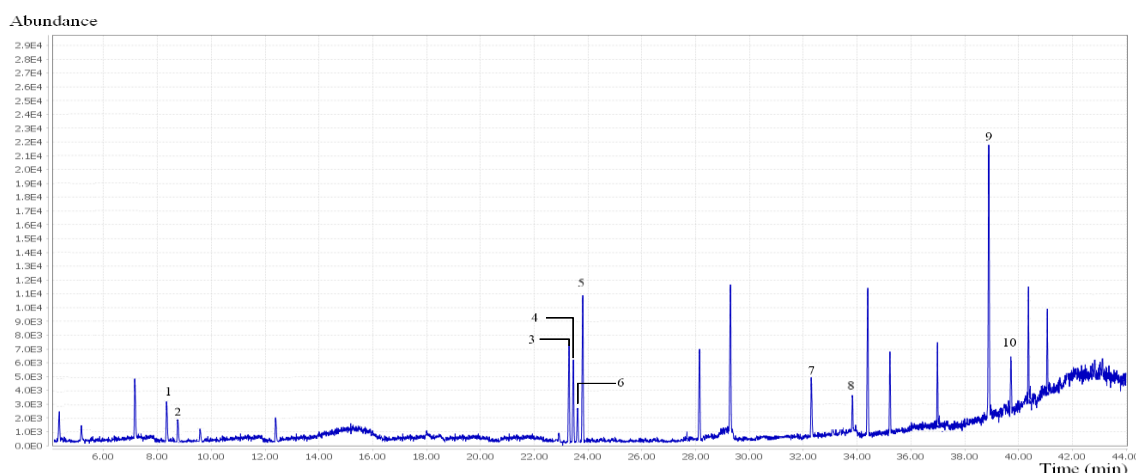


Figure 5: GC-MS profile of ethyl acetate extract of *Lecanicillium* sp.: 1, Oleamide; 2, 1,2-propanedione,1-(3,4-methylenedioxy) phenyl-; 3, 2-hydroxy-4-oxobutane-1,2,4-tricarboxylic acid; 4, 2E,4E,8E,10E-dodecatetraenedioic acid; 5, (Z,Z)-3-chloromuconic acid; 6, 2-hydroxy-2-butenedioic acid; 7, 1,4-butanediol; 8, Methyleneethylketone; 9, Cyclohexanone; 10, 2-phenylpropionaldehyde.

Table 3: MIC of *Lecanicillium* sp. and *A. faecalis* extracts in six different polarity solvents against *E. psidii* using micro-well dilution method.

Solvents	MIC of <i>Lecanicillium</i> sp. extracts (mg/mL)	MIC of <i>A. faecalis</i> extracts (mg/mL)
Hexane	>2	>2
Diethyl ether	2	1
Chloroform	2	>2
Ethyl acetate	2	>2
Acetone	>2	>2
Methanol	>2	>2

Eleven metabolites were identified from chloroform extract of *Lecanicillium* sp. (Figure 4). The metabolites are (1) thiophene, (2) But-2-enoic acid, (3) dihydrofuran-2(3H)-one, (4) 2-Pyrrolidinone, (5) cyclopentanone, (6) 1-Pyrroline-5-carboxylic acid, (7) piperidine, (8) D-1-Piperideine-2-carboxylic acid, (9) 2-methyl-16-heptadecenoic acid, (10) 1-Pyrroline and (11) 1-Pyrroline-5-carboxylic acid. The highest peak was noticed as D-1-Piperideine-2-carboxylic acid, a piperidine derivative with multiple pharmacological and physiological activities followed by cyclopentanone that exhibit antimicrobial activity (Trisuwan *et al.*, 2010). Dihydrofuran-2(3H)-one has been reported to be found in *Aspergillus terreus* and possess antifungal activity against another fungus, *Aspergillus fuigatus* (Awaad *et al.*, 2012).

A total of 10 metabolites were detected from ethyl acetate extract of *Lecanicillium* sp. (Figure 5). The metabolites were (1) oleamide, (2) 1,2-propanedione,1-(3,4 methylenedioxy)phenyl-, (3) 2-hydroxy-4-oxobutane-1,2,4-tricarboxylic acid, (4) 2E,4E,8E,10E-Dodecatetraenedioic acid, (5) (Z,Z)-3-chloromuconic acid, (6) 2-hydroxy-2-butenedioic acid, (7) 1,4 Butanediol, (8) methylethylketone, (9) cyclohexanone and (10) 2-

phenylpropionaldehyde. Some of the compounds have significant role in antimicrobial action. The major metabolites found in ethyl acetate extract were cyclohexanone that has been reported to exhibit antimicrobial action (Gein *et al.*, 2010). Oleamide which was identified in the same extract has a structural similarity to oleic acid that acts as antimicrobial agent. (Zaher *et al.*, 2015).

The results of GC-MS chromatography showed that several metabolites identified from different extracts of *A. faecalis* and *Lecanicillium* sp. have antimicrobial effects against different types of microorganisms. However, further studies to isolate and characterize the identified metabolites are necessary to confirm their activity and mechanism of action against *E. psidii*.

CONCLUSION

Lecanicillium sp. (F8) and *A. faecalis* (B93) from this study showed positive inhibition against *E. psidii*. Both GCMS analysis revealed *A. faecalis* and *Lecanicillium* sp. had secreted some secondary metabolites such as N-formylmaleamic acid, Oleamide and D-1-piperideine-2-carboxylic acid which may relate to the inhibition against *E. psidii*. *A. faecalis* (diethyl ether) extract and *Lecanicillium* sp. (diethyl ether, chloroform and ethyl acetate) extracts exhibit higher inhibition activity against *E. psidii* compared to other solvent extracts. *A. faecalis* and *Lecanicillium* spare potential to be further investigated as biocontrol against *E. psidii*.

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