Pertanika J. Trop. Agric. Sc. 41 (4): 1795-1809 (2018)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Characterization of Phenazine and Phenazine-1-carboxylic Acid Isolated from *Pseudomonas aeruginosa* UPMP3 and Their Antifungal Activities against *Ganoderma boninense*

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ABSTRACT

The structural characterization of two phenazines, produced simultaneously through biosynthesis by *Pseudomonas aeruginosa* UPMP3 were studied. The phenazine antibiotics play an important role in antimicrobial activities and potentially could be used in biopesticide formulation to control basal stem rot of oil palm. The antibiotics were identified through high-performance liquid chromatography (HPLC) and confirmed using Fourier Transform Infrared (FTIR) spectroscopy. The specific representations in the FTIR spectra of the purified compounds such as absorption peaks at 1353.68 (aromatic nitro compound), 1118.84 and 729.43 cm^{-1} (aromatic ring) were indications of phenazine (PHZ) whereas, absorption peaks at 1717.92 (carboxylic acid), 861.78 and 738.54 cm^{-1} (aromatic ring) were indicatives of phenazine-1-carboxylic acid (PCA). The structures of the compounds were further confirmed by ^{1}H NMR/^{13}C NMR spectroscopy as PHZ ($C_{12}H_8N_2$) and PCA ($C_{13}H_8N_2O_2$). *Ganoderma boninense* was sensitive to purified phenazine antibiotics especially phenazine and a concentration of 1000 ppm completely inhibited the mycelial growth. As far as we know, this is the first study where purified phenazine antibiotics isolated from *P. aeruginosa* UPMP3 were structurally characterised and tested to have positive antagonism against *G. boninense.*

ARTICLE INFO

Article history:
Received: 22 March 2018
Accepted: 26 June 2018
Published: 14 November 2018

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Keywords: Antibiotics, antifungal, bacteria, FTIR, NMR, phenazine, SEM

INTRODUCTION

Ganoderma boninense is the most noxious species of fungal plant pathogen in oil palm plantations. It causes basal stem rot (BSR) on different parts of the same host but lack

ISSN: 1511-3701
e-ISSN: 2231-8542

of information on the disease management causes severe economic losses due to this serious disease. The disease can cause losses up to 50% of palm trees and 45% yield from fresh fruit bunch (FFB) in oil palm plantations (Naher, Yusuf, Ismail, Tan, & Mondal, 2013). *Ganoderma* BSR disease currently has no cure because early disease symptoms are not detectable and the resistant stages of mycelium and basidiospores of *G. boninense* inhibit its control.

Previous studies using various control methods such as trench system and replanting techniques had been tried with great potential, while chemical control was the method which showed a significant reduction in BSR incidence when the oil palm trunk was injected with a combination of carboxin and quintozene fungicides or synthetic fungicides such as Anvil® with the active ingredient hexaconazole which was applied directly to the palm base. However, chemical fungicide applications might lead to fungicide resistance in fungus and inhibit the growth of good microbes. Since it is a synthetic formulation, growing concerns about the environment and high cost of chemicals have encouraged researchers to find an alternative way to control BSR. In this context, the development of a natural, effective and safe strategy for controlling *G. boninense* is necessary (Abraham et al., 2015). The use of pathogen-resistant cultivars and biological agents are the biological control measures.

Biological control through the use of antagonistic microbial can be an environmental-friendly and effective approach to control pathogenic fungi. Numerous studies have demonstrated that metabolites including antibiotics, enzymes and volatiles produced by antagonistic bacteria play key roles in the control of various plant pathogens. Natural antibiotics have been a subject of intense research for the past 70 years and natural antibiotics, together with their semi-synthetic derivatives and the formulation of modern antimicrobial therapy. The antibiotic mechanism is well documented and summarized in several review papers (Abraham et al., 2015).

Biological control of *Ganoderma* spp. has achieved notable attention. *In vitro* studies have shown that *Trichoderma* spp*., Aspergillus* spp. and *Penicillum* spp. are antagonistic agents of *G. boninense.* However, these biological agents can only protect the plants at the very early stage of infection, thus, are unable to cure highly infected palms (Abdullah, Ilias, Nelson, Izzati, & Yusuf, 2003). *Pseudomonas aeruginosa* UPMP3, a strain isolated from the rhizosphere of an oil palm plantation was found to have potential to become a biocontrol agent against *G. boninense* (Azadeh & Meon, 2009), being able to produce two types of phenazine antibiotics simultaneously in the culture broth. The antibiotics are phenazine (PHZ) and phenazine-1-carboxylic acid (PCA).

Phenazine is a nitrogen-containing heterocyclic antibiotic agent. More than 6000 phenazine derivatives have been identified and described during the last two centuries. Phenazine antibiotics are well-

known in demonstrating toxicity towards various pathogens by having the potent anti-fungal activity for control of economically important plant pathogens. A previous finding indicated that the isolated phenazine antibiotics exhibited antifungal activity on *G. boninense* (Parvin, Othman, Jaafar, & Wong, 2016).

Phenazines can be produced in two ways, namely biosynthesis and chemosynthesis (Cheluvappa, 2014). Biosynthesis, the natural way of antibiotics production, is achieved *via* phenazine-producing bacteria such as *P. aeruginosa* when the bacteria are incubated in a suitable medium and regulated by nutrient depletion. The high cell density converts the bacterium to biofilm form. Chemosynthesis of antibiotics is through serial oxidation of phenazine core (Nansathit, Phaosiri, Pongdontri, Chanthai, & Ruangviriyachai, 2011). In this present study, phenazine compounds were biosynthesized by an antagonistic bacterium, *P. aeruginosa* UPMP3. The objectives of this research were to characterize phenazine antibiotics produced by *P. aeruginosa* UPMP3 and to evaluate the inhibitory effect of the purified phenazines against *G. boninense in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Phenazine (PHZ) was purchased from Sigma-Aldrich (USA). Phenazine-1-carboxylic acid (PCA) was purchased from Toronto Research Chemicals Inc. (Canada). The purities of these compounds were more than 99%. Acetonitrile was of HPLC grade and obtained from Friedemann Schmidt Chemical (France). Methanol was of HPLC grade and obtained from RCI Labscan Ltd. (Thailand). Benzene was analytical grade and obtained from R & M Chemicals (UK). Water was triple distilled.

Bacterial and Fungal Cultures

The antagonistic rhizobacterium, *Pseudomonas aeruginosa* UPMP3 was obtained from the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia that was previously isolated from oil palm rhizosphere, and maintained on King's B agar at room temperature as working culture. *G. boninense* was obtained from Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia and maintained on Potato Dextrose Agar (PDA) at room temperature.

Antibiotics Biosynthesis

P. aeruginosa UPMP3 inoculum on agar was sub-cultivated in Luria Bertani (LB) broth at room temperature for three days in an orbital incubator maintained at 250 r.p.m. The inoculum then was centrifuged to remove all bacterial cells and the supernatant was lyophilized in a vacuum evaporator. The resultant powder form of the supernatant was dissolved in 100 ml distilled water followed by compounds extraction. The PCA and PHZ produced simultaneously in the supernatant were extracted using a previous method (Raio et al., 2011). The oily extract was dissolved in 2 ml methanol and purified by fractionation using analytical HPLC.

Antibiotics Fractionation by HPLC

An analytical reverse phase High Performance Liquid Chromatography (HPLC) column equipped with a fraction collector system (Agilent, USA) was used to fractionate the phenazine-compounds present in the extract. The antibiotics were identified according to a modified method previously described by Fernández and Pizarro (1997). A 150 × 4.6 mm Luna 5 µ C18 (2) 100A column and 30 × 4.6 mm Luna 5 µ 100A guard column (Phenomenex, USA) were used. The injection volume was 20 µL. The mobile phase A was water-trifluoroacetic acid (100:0.04, v/v) and mobile phase B was acetonitrile-water-trifluoroacetic acid (90:10:0.04, v/v/v). The flow rate was 1 mL/min. Chromatograms were developed using a Gold programmable solvent module 126 connected to a diode array detector module 168 (Water, USA). Elution was as follows: Solvent A was maintained for 15 min and then changed to 90% A and 10% B. This mixture was used for 10 min. A linear gradient of 70% A and 30% B in 15 min was then applied. These conditions were maintained for 5 min. Finally, the solvent composition was changed to 64% A and 36% B and maintained until the end of the run (65 min from the starting time). The antibiotics in the effluents of mobile phase were collected automatically by the fraction collection system.

Characterization of Purified Antibiotics

Fourier Transform Infrared (FTIR) spectroscopy was used to study the molecular structure of the antibiotics. One milligram dried antibiotics in amorphous powder was compacted with 0.1 g potassium bromide using a hydraulic pressure. For each spectrum, 21 scans between 400-4000 cm^{-1} were produced at a resolution of 4 cm^{-1}. The FTIR measurement was taken on a Perkin-Elmer spectrophotometer.

The purified antibiotics were characterized using spectroscopic techniques. ^{13}C and ^{1}H NMR spectra were obtained using a Varian Nuclear Magnetic Resonance System VNMRS-500 MHz instrument (Varian Inc., USA). Samples of 5 mg were dissolved in $CDCl_3$ and were analyzed in a liquid state NMR, ^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively. The same solvent was used as the internal standard.

The Scanning Electron Microscopy (SEM) analysis was carried out. For this purpose, the samples were fixed in a sample holder and covered with a gold layer for 3 min using a sputter coater (Cressington 108, JOEL Inc., Japan). Then, the samples were placed in an ETEC autoscan model JSM-6400 scanning electron microscope, SEM (JEOL Inc., Tokyo, Japan).

***In vitro* Antifungal Activity**

The inhibition activity of purified antibiotics was studied. Treatments of different concentrations (100, 200, 400, 800 and 1000 ppm) of PHZ and PCA were prepared. A dilution of aqueous methanol (80:20 v/v), was used as negative control. Poisonous agar method was applied, after incubation at 28°C for 10 days. The diameter of fungal

mycelia was measured to calculate the inhibition rate. This bioassay was performed with five replications. Inhibition rate was measured using the following formulae.

Percentage inhibition = (C-T)/C x 100

Where,

C = colony diameter (cm) of the control plate

T = colony diameter (cm) of the test plate

Statistical Analysis

The results were analyzed using statistical software JMP (9.3) (SAS Institute, Cary, NC). Data were expressed as means ± SD of replicated samples by one-way analysis of variance (ANOVA) using Tukey's honestly significant difference (HSD) test. Differences were considered significant at $p < 0.05$.

RESULTS

Fractionation of Antibiotics

PCA was collected from a single peak with retention time 51.17 min at 245 nm in the HPLC analysis. Similarly, PHZ was obtained from the peak with retention time 51.77 min at 252 nm. The quantification analysis indicated 685.0 mg of PHZ (Figure 1(a)) and 26.9 mg of PCA (Figure 1(b)) per litre of crude extract. The compounds were collected using a fraction collector and dried at 40°C. Approximately 0.05 mg/ml of yellow oily substance were obtained.

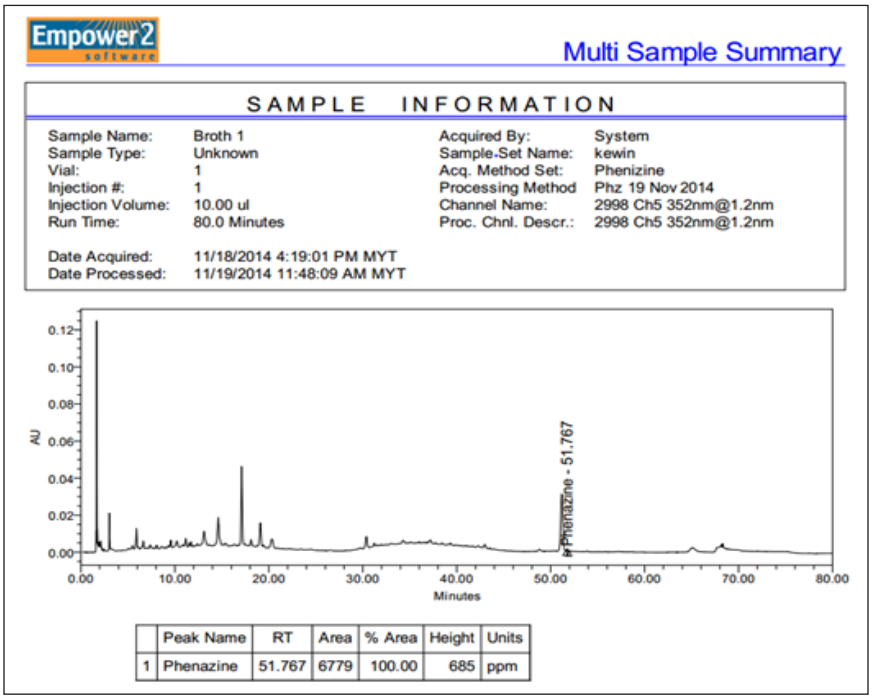


Figure 1(a). HPLC spectrum of PHZ at 252 nm

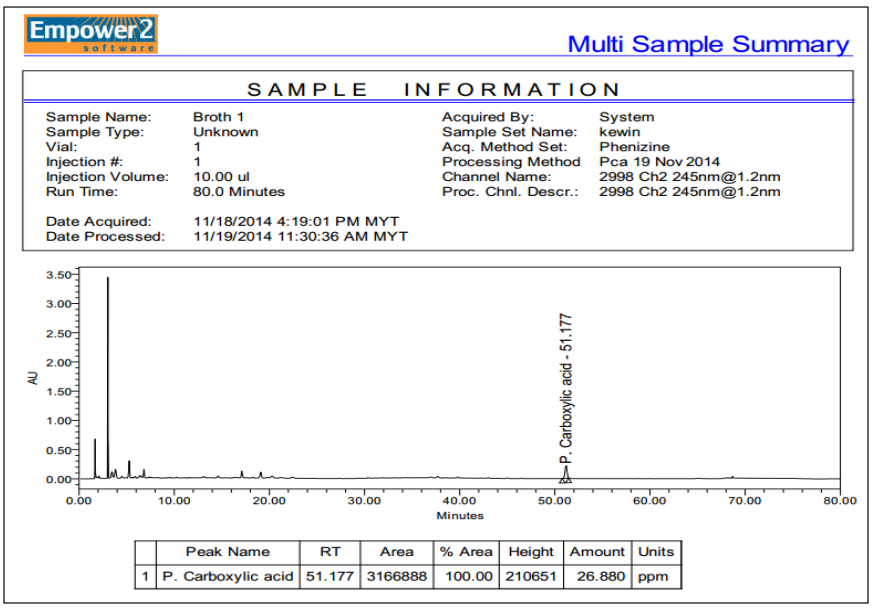


Figure 1(b). HPLC spectrum of PCA at 245 nm

Structure Elucidation of Phenazine

The IR spectrum of purified PHZ as shown in Figure 2(a) exhibited an absorption peak at 1499.23 cm^{-1} for aromatic stretch. Meanwhile, the absorption at 1353.68 cm^{-1} was indicative of an aromatic nitro compound. The absorption bands at 1118.84 cm^{-1} and 729.43 cm^{-1} were from aromatic C-H stretching vibrations.

The IR spectrum of purified PCA as shown in Figure 2(b) exhibited absorption peaks at 1717.92 cm^{-1} which were for the carboxylic acid vibration, 1461.21 cm^{-1} for the aromatic C=C stretch, while 861.78 cm^{-1} and 738.54 cm^{-1} were for aromatic CH stretching vibrations.

The structures of the purified compounds were further confirmed by ^{1}H and ^{13}C NMR spectroscopy. In the ^{1}H NMR spectrum of PHZ (Figure 3(a)) the two doublet peaks at δ7.71 and 8.13 were assigned to the 8 aromatic protons. PHZ being a symmetrical molecule, hence, the 8 aromatic protons only gave 2 overlapped signals in the ^{1}H NMR spectrum. H-2 and H-3 are equivalent to H-7 and H-6 respectively. H-2 and H-3 are coupled to their neighbouring protons H-1 and H-4 respectively resulting in 2 doublets and these signals are at the low field region. Thus, the doublet peak at δ8.13 was assigned to these four protons, H-2, H-3, H-6 and H-7 while the other doublet at δ7.71 was assigned to H-1, H-4, H-5 and H-8. Five well-resolved signals in the ^{13}C NMR spectrum of PHZ (Figure 3(b)) were also observed. The signals at δ143.3, 130.3, and 129.5 consisting of two overlapped peaks each indicated the presence of aromatic carbons in the structure. Looking at the symmetrical characteristic of the PHZ molecule, only 3

carbon signals could be seen in the ^{13}C NMR spectrum. C-5 and C-6 are equivalent to C-8 and C-7 respectively. The lower field carbon signal at 130.3 was hence assigned to C-5 and C-8 while δ129.5 was assigned to C-6 and C-7. Similarly, C-1 being equivalent to C-4 and C-2 to C-3 were assigned peaks at δ130.3 and 129.5 respectively. The

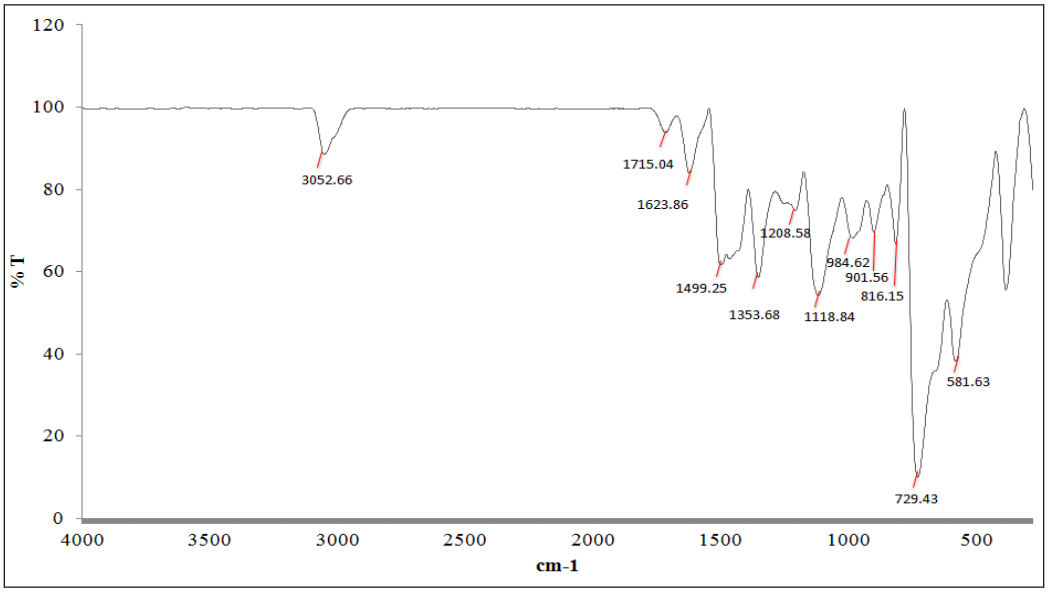


Figure 2(a). FTIR spectrum of PHZ

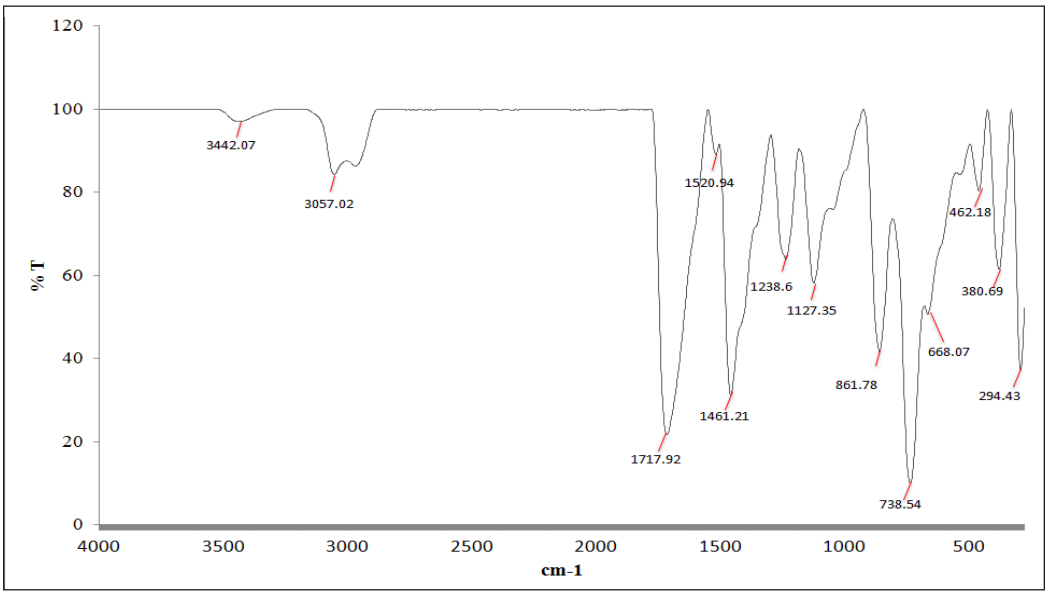


Figure 2(b). FTIR spectrum of PCA

quaternary carbons at position 4(a), and 5(a), 1(a) and 8(a) were duly assigned signals at δ 143.3 since they are all equivalent.

In the ^{1}H NMR spectrum of PCA (Figure 3(c)), seven signals between δ7.77-8.95 were duly assigned to the 8 aromatic protons. The carboxyl proton gave a very low field signal one-proton singlet at δ8.95. The presence of this carboxylic carbon was justified by the ^{13}C NMR signal at δ165.9. The ^{13}C NMR (Figure 3(d)) spectrum also gave 12 signals which agree with the structure of phenazine-1-carboxylic acid. The 4 low field signals at δ143.3, 139.9, 144.0 and 139.7 were assigned to the 4 quaternary carbons C-4a, C-5a, C-8a and C-1a. Seven doublet signals at δ8.50, 8.01, 8.02, 8.50, 8.25, 7.98 and 8.51 were assigned to their respective protons. In Tables 1 and 2, ^{1}H/^{13}C NMR spectral data of purified PHZ and PCA were compared to the previous finding in the literature review to correlate the signals allocation in molecules.

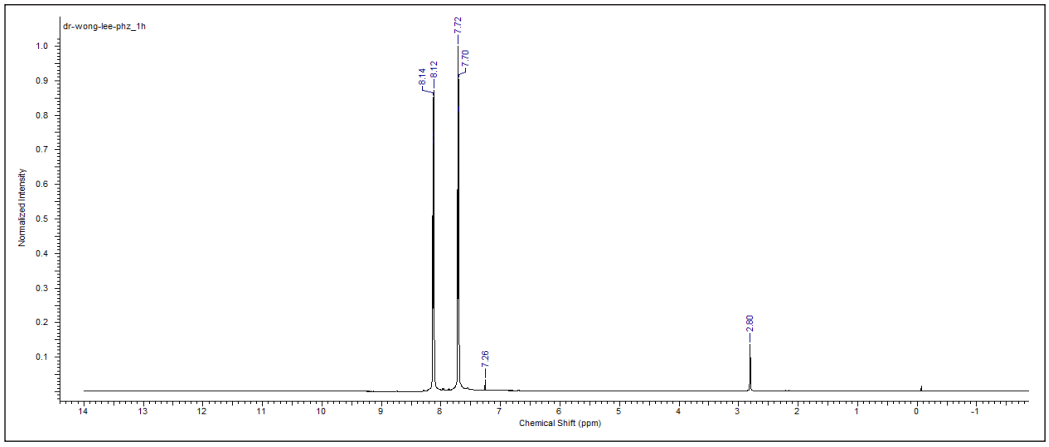


Figure 3(a). PHZ ^{1}H NMR

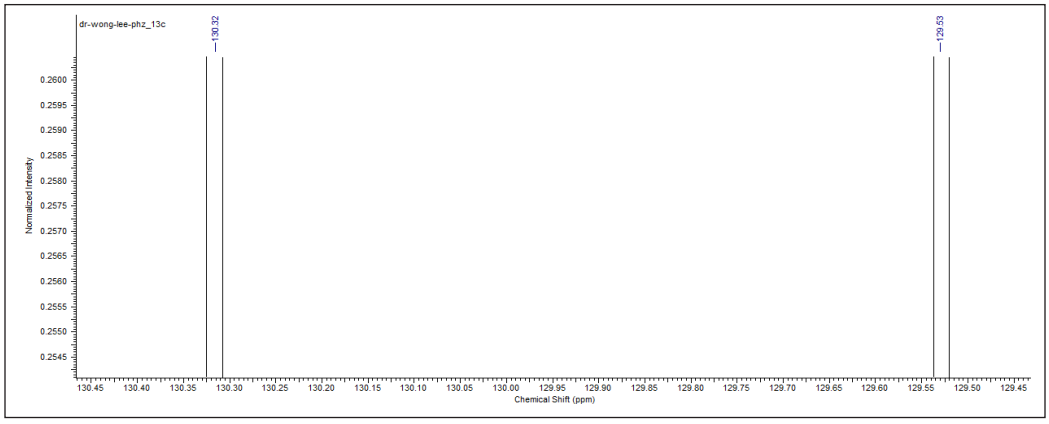


Figure 3(b). PHZ ^{13}C NMR

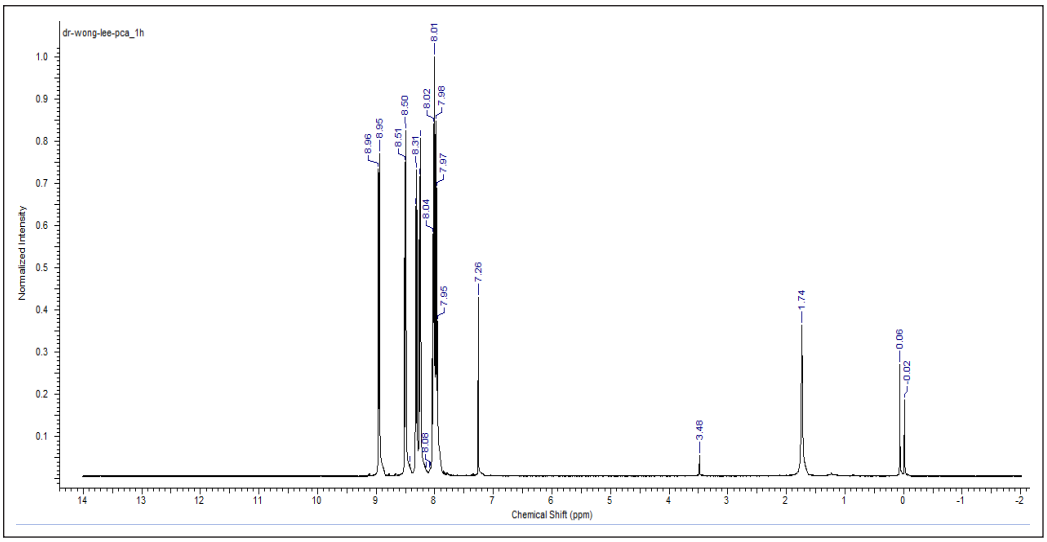


Figure 3(c). PCA ^{1}H NMR

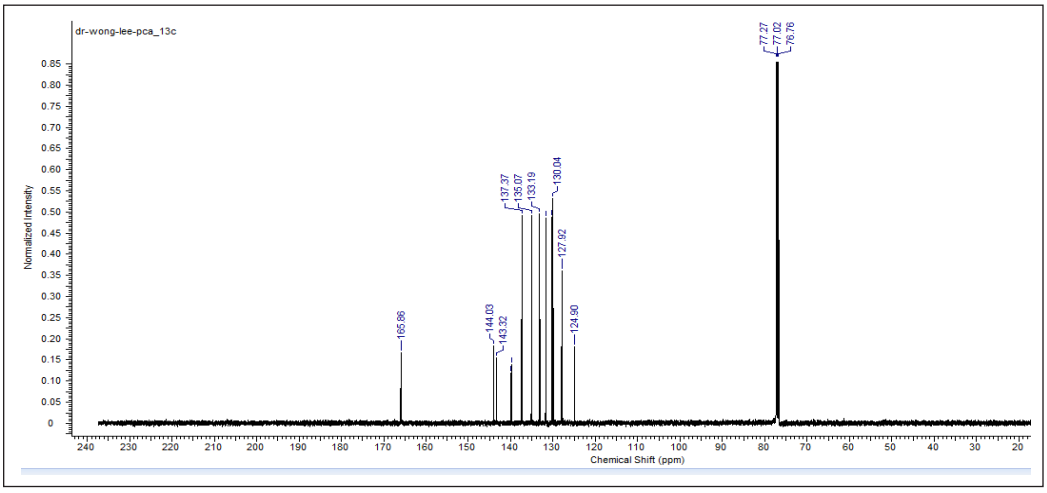


Figure 3(d). PCA ^{13}C NMR

The compounds formed were confirmed by SEM imaging (Figure 4(a)-(b)). The physical structures of purified compounds were demonstrated by electron beams. PHZ and PCA were detected to have crystalline needle-like structure. The PHZ crystalline structure is shown in Figure 4(a) at a magnification of ×7500 and the needle-crystal diameter is less than 1 µm. PCA crystalline structure is best figured at the magnification of ×43 and the size is less than 100 µm (Figure 4(b)).

Table 1
$^{1}H/^{13}C$ NMR spectral data of targeted PHZ purified from Pseudomonas aeruginosa *UPMP3*

Atom position	^{1}H NMR		^{13}C NMR	
	PHZ* (δ)	Purified PHZ (δ)	PHZ** (δ)	Purified PHZ (δ)
1	7.97	7.71, d	130.25	130.3
2	8.26	8.13, d	129.6	129.5
3	8.26	8.13, d	129.6	129.5
4	7.97	7.71, d	130.25	130.3
1a	-	-	143.45	143.3
4a	-	-	143.45	143.3
5a	-	-	143.45	143.3
8a	-	-	143.45	143.3
5	7.97	7.71,d	130.25	130.3
6	8.26	8.13,d	129.6	129.5
7	8.26	8.13,d	129.6	129.5
8	7.97	7.71,d	130.25	130.3

Note. δ in ppm, * (Griesbeck, 2014); ** (Breitmaier & Hollstein, 1976)

Table 2
$^{1}H/^{13}C$ NMR spectral data of targeted PCA purified from Pseudomonas aeruginosa *UPMP3*

Atom position	^{1}H NMR		^{13}C NMR	
	PCA* (δ)	Purified PCA (δ)	PCA* (δ)	Purified PCA (δ)
1	-	-	124,9	127.9
2	8.54	8.51, d	135.1	144.0
3	8.06	8.01, t	130.2	137.3
4	8.99	8.50, d	137.4	135.0
1a	-	-	143.4	144.0
4a	-	-	144.1	143.3
5a	-	-	140.1	139.9
8a	-	-	139.8	139.7
5	8.36	8.50, d	130.1	133.7
6	8.00	7.98, t	131.7	131.7
7	8.02	8.02, t	133.2	130.2
8	8.29	8.51, d	127.9	130.0
COOH	15.61	8.95, s	165.9	165.8

Note. δ in ppm, * (Abraham et al., 2015)

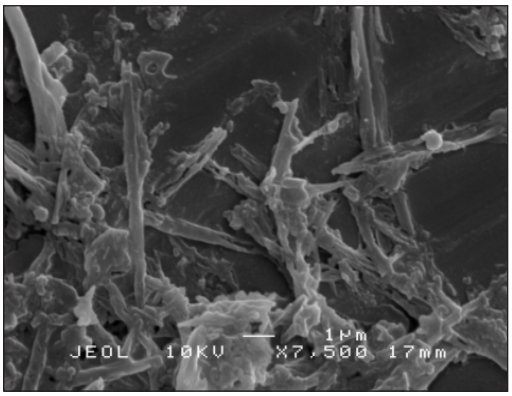


Figure 4(a). Purified PHZ viewed at magnification of × 7500

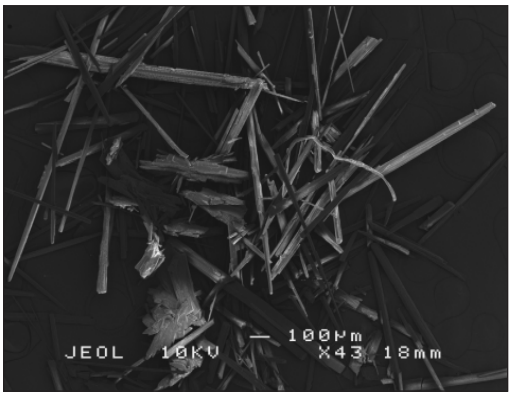


Figure 4(b). Purified PCA viewed at magnification of × 43

In vitro Antifungal Activity

Purified phenazine antibiotics showed antagonistic activity against *G. boninense*. The percentage inhibition of *G. boninense* mycelial growth increased with increasing concentration of purified compounds. Figure 5 indicates that PHZ has a better inhibition rate than PCA. PHZ inhibited 100% mycelial growth of *G. boninense* at 1000 ppm and at 800 ppm the inhibition rate was 92.31% which was more effective than PCA at 1000 ppm.

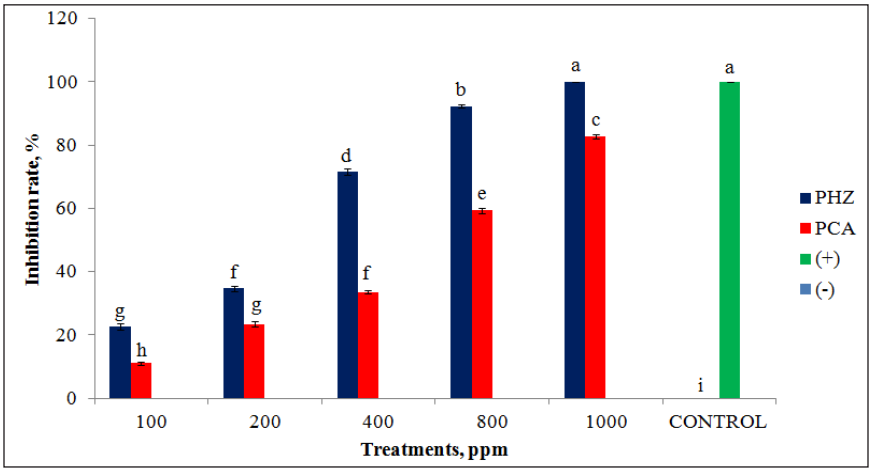


Figure 5. *In vitro* antifungal activities of PHZ and PCA at 100, 200, 400, 800 and 1000 ppm on *Ganoderma boninense* at Day 14

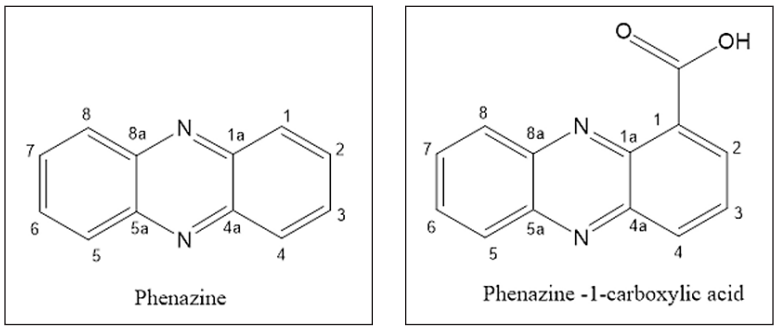
DISCUSSION

Disease caused by the pathogenic fungus *G. boninense* is the greatest threat to the oil palm industry. Increasing concerns over environmental problems due to chemical applications and labour safety supported the need for alternative pathogen control methods. Investigations on microbial

antibiotics are gaining greater momentum in the agrochemical industry as a source for the development of new products. Fungicides of microbial origin have been demonstrated to be not only specifically effective on the target organisms but also inherently biodegradable. Beneficial rhizobacteria have recently been a focus of interest as biocontrol agents and a source of bioactive metabolites (Strobel, Daisy, Castillo, & Harper, 2004). *P. aeruginosa* UPMP3 is indigenous to oil palm plantation rhizosphere, colonizing the tissue locally and systemically. In this study, *P. aeruginosa* UPMP3 was used to produce phenazine antibiotics and these compounds were proven to be the key antagonistic compounds that exhibited maximum inhibition to the growth of *G. boninense*. These compounds were extracted and purified through HPLC downstream processes (Abraham et al., 2015).

Phenazine compounds are structurally identical, which have 3 fused aromatic rings with conjugated dienes and two C-N bonds such as PHZ ($C_{12}H_8N_2$, Figure 6(a)) while PCA ($C_{13}H_8N_2O_2$, Figure 6(b)) has a molecular structure almost identical to PHZ but with a carboxylic side chain (Abraham et al., 2015). Diene is a hydrocarbon that contains two carbon double bonds. The counts of proton and carbon from ^{1}H to ^{13}C NMR spectra revealed the structure of protons and carbons (Figures 3(a)-(b)) which supported the molecular formula of phenazine antibiotics.

The phenazine antibiotics share similarities of having aromatic nitro compounds and several aromatic rings. From the FTIR spectrum of each compound, the presence of aromatic nitro compound was confirmed by the absorption peaks either between 1555 and 1484 cm^{-1} or 1355 and 1320 cm^{-1}. Aromatic C-H was confirmed by the absorption peaks between 1225 and 950 cm^{-1}. C=C-C aromatic ring stretch has peaks between 1510 and 1450 cm^{-1} while aromatic CH vibration have peaks of 900-670 cm^{-1}. The presence of a carboxylic acid moiety in PCA was confirmed by the absorption at 1717.92 cm^{-1}, a carbonyl compound group frequency for carboxylic acid should occur between 1725 and 1700 cm^{-1} (Coates, 2000).



(a) (b)

Figure 6. Structures of (a) phenazine and (b) phenazine-1-carboxylic acid

The indication of peaks in the compound elucidation in FTIR confirmed the presence of functional groups and structure of phenazine compounds.

Phenazines have been recognized for their antibiotic properties for centuries. They inhibit a wide range of plant pathogenic fungi and have a well-characterized mechanism of bacterial plant disease control (Bloemberg & Lugtenberg, 2003). In a medical aspect, pyocyanin caused ciliostasis with epithelial disruption. 1-hydroxyphenazine causes dyskinesia (Wilson et al., 1987) and lung infection of cystic fibrosis. The research showed that pyocyanin could react with glutathione to produce the toxin (Cheluvappa, Shimmon, Dawson, Hilmer, & Le Couteur, 2008). Despite the fact that phenazine antibiotics have the potential to cause infections in human, they are still studied for applications in plant protection.

In 1954, *Pseudomonas aeruginosa* was studied for its antibiotic production of streptomycin and dihydrostreptomycin, two compounds used against *Escherichia coli* (Lightbown, 1954). The pattern of phenazine pigment production by *P. aeruginosa* was studied and the result showed that PCA, pyocyanin, chloraraphin and oxychlororaphin were produced (Kanner, Gerber, & Bartha, 1978). PHZ and pyoluteorin were produced by *Pseudomonas* isolated from green pepper rhizosphere (Liu, Dong, Peng, Zhang, & Xu, 2006). *Pseudomonas aurantiaca* was able to produce HCN, siderophores and homoserine lactones that are antifungal to *Colletotrichum falcatum, Fusarium oxysporium* and *F. lateritium*, important pathogens of sugarcane (Mehnaz, Baig, Jamil, Weselowski, & Lazarovits, 2009). *P. chlororaphis* exhibited biocontrol of cypress canker, caused by the fungi *Lepteutypa cupressi* and *Seiridium unicorne* (Raio et al., 2011). *Pseudomonas* spp. such as *P. borealis, P. chlororaphis, P. fluorescens, P. mandelii, P. marginalis, P. poae, P. putida, P. syringae* and *P. vranovensis* were evaluated for their antifungal activities against root rot of wheat caused by *Rhizoctonia* sp. and *Pythium* sp. (Mavrodi, Walter, Elateek, Taylor, & Okubara, 2012). *P. chlororaphis* strain HT66 was able to inhibit various pathogens by producing antibiotics such as 2-OH-PHZ and PCA. These compounds also possessed potent insecticidal activity (Chen et al., 2015). PCA also showed antagonistic activity against oomycete pathogen *Phytophthora meadii* (Abraham et al., 2015). However, no phenazine derivatives have so far been reported as the active compounds demonstrating antibiosis against *G. boninense*.

The present study reported that PCA gave less inhibition compared to PHZ at the same concentration (Figure 5).

CONCLUSION

Based on the findings, it was concluded that *Pseudomonas aeruginosa* UPMP3 produces phenazine (PHZ) and phenazine-1-carboxylic acid (PCA) as secondary metabolites in the culture broth. Both antibiotics were detected simultaneously under the same HPLC conditions. Characterization of the structure of both

antibiotics by FTIR and NMR supported HPLC identification. Though both PHZ and PCA demonstrated effective inhibition (92-100%) of *G. boninense* mycelial growth *in vitro*, PCA showed lesser inhibition compared to PHZ at the same concentration (1000 ppm). As far as we know, this is the first study where purified phenazine antibiotics isolated from *P. aeruginosa* UPMP3 were structurally characterised and tested to have positive antagonism against *G. boninense*. The results of the present study suggest that phenazine antibiotics isolated from *P. aeruginosa* UPMP3 have the potential to be developed into antifungal formulations in future.

ACKNOWLEDGEMENTS

This study was partially supported by Fundamental Research Grant Scheme (FRGS) 2012 provided by the Ministry of Higher Education, Malaysia.

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