ISOLATION OF ANTIBACTERIAL COMPOUNDS FROM *PHOMOPSIS* SP. ISOLATED FROM LEAVES OF *PSIDIUM GUAJAVA* L.

Hnin Wit Mhon[1], Mon Mon Thu[2] , Yee Yee Thu[3]

Abstract

An endophytic fungal strain *Phomopsis* sp. was isolated from the leaves of *Psidium guajava* L. For the extraction of the bioactive compounds, 12L large fermentation of strain *Phomopsis* sp. and antimicrobial activity of fermented broth with *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus* were carried out at Microbiology Laboratory, Department of Botany, University of Yangon. Then, the filtrate was extracted with methanol on Ambilites XAD 2 resin column and the methanol extracts showed high against six test organisms. Isolation and purification of the bioactive compounds from the methanol extracts were done by utilizing silica gel columns and Sephadex LH 20 gel columns with various solvent systems at Department of Organic Chemistry, Ramkhamhaeng University, Bangkok, Thailand. The isolated compounds were characterized by FT-IR spectra, 1D-NMR (^{1}H-NMR, ^{13}C-NMR) and 2D-NMR (COSY, HSQC, and HMBC) spectra. The three isolated compounds were identified as phomopsolide B, adenosine and emerimidine C. Antimicrobial activity of the bioactive compounds was evaluated on six test organisms and showed highest activity against *Xanthomonas oryzae* and weak activity against *Candida albicans* and *Escherichia coli*.

Key words: *Adenosine, Emerimidine C, Phomopsis sp., Phomopsolide B*

Introduction

Bioactive metabolites produced by endophytic fungi are the major source of drugs, and the plant clearly provides the proper environment for its growth and survival (Kumaran *et al*., 2010). The bioactive compounds have

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been isolated from microorganisms originating from various terrestrial and marine environments (Strobel and Daisy, 2003). Endophytes have the ability to produce a range of secondary metabolites providing researchers with numerous leads for the compounds of pharmaceutical significance and possible developments as new drugs (Tan and Zou, 2001). Numerous antibiotics have been isolated from a variety of microorganisms, however, studies are still being conducted to identify novel antibiotics effective against pathogenic fungi and bacteria.

Endophytes are proved rich sources of the natural compounds, showing a variety of pharmacological and biological activities. The production and quality of the bioactive compounds from endophytic fungi depend on natural conditions of the association and the nature of the synthetic medium used (Strobe and Daisy, 2003). Several hundreds of the compounds with antibiotic activity have been isolated from microorganisms over the years, but only a few of them are clinically useful (Thomashow *et al.*, 2008).

Materials and Methods

Screening of Endophytic Fungus from *Psidium guajava* L.

The *Psiduum guajava* L. plant sample was collected from Nyaung-Hna-Pin area, Hmawbi Township. The isolation of endophytic fungus was carried out with the following scheme: (1) Plant parts were washed in running tap water for 15 min. (2) Plant parts were cut into about 1 cm pieces. (3) The surfaces of cut-plant pieces were sterilized by soaking it in 75% ethanol for 2 min. (4) Sterile surfaces were socked in 5.3% sodium hypocloride for 5 min. (5) Cut-plant pieces were washed out sodium hypocloride by socking in 75% ethanol for 0.5 min. (6) They were dried and cut into smaller pieces, and placed on agar plates and then incubated for 3 days to 3 weeks (Phay, 1997). Thus, the isolated microorganisms were transferred into a 10 ml test tube containing 5 ml of sucrose/yeast extract medium.

Antimicrobial Activity of 12L Fermentation of *Phomopsis* sp.

The small fungal piece (1 cm) from the plate culture of *Phomopsis* sp. was inoculated into 500 ml of conical flask containing 180 ml of sucrose, yeast extract seed medium. The flask was incubated at room temperature for two days for seed culture. Two days old seed culture (180 ml) was transferred into twelve flasks of 2L conical flask containing 1L of sucrose yeast fermentation medium. These flasks were incubated at 100 rpm for 3-7 days at room temperature. The fermentation flasks indicated antimicrobial activity on *Bacillus subtilis, Candida albicans, Escherichia coli, Malassezia furfur, Salmonella typhi and Staphylococcus aureus* (Strobel and Sullivan, 1999; Phay, 1997).

Extraction of Bioactive Compounds from Fermented Broth of *Phomopsis* sp.

After testing antimicrobial activity, 12L fermented broths were filtered with filter paper. The mycelia from fermented broth were filtered on the filter paper and then the filtrate was applied on an Amberlites XAD 2 resin column. The column was washed with water, followed by six liters of methanol. The methanol extract was evaporated on water bath at 55-60°C. The methanol extract was tested for antimicrobial activity against *Bacillus subtilis, Candida albicans, Escherichia coli, Malassezia furfur, Salmonella typhi* and *Staphylococcus aureus* (Strobel and Sullivan, 1999).

Isolation and Purification of Bioactive Compounds from *Phomopsis* sp.

According to TLC results, silica gel column chromatography was carried out. The silica 34 gel (100 g) column was eluted with hexane:ethyl acetate (100%, 10:1, 10:2, 10:3, 10:5, 1:1, 1:2, 1:3) and ethyl acetate : methanol (100%, 10:0.5, 10:1, 10:2, 10:3, 10:5, 1:1, 100% MeOH) and then fifteen fractions were collected. The column size was 4×17 cm and the flow rate was 2 ml per minute (Grabley *et al*., 1999).

Identification of Isolated Compounds from *Phomopsis* sp.

The identification of the isolated compounds was characterized by 1D-NMR (^{1}H-NMR and ^{13}C-NMR), 2D-NMR (^{1}H-^{1}H COSY, HMBC) 400 MHz at Nuclear Magnetic Resonance and FT-IR spectra at the Department of Chemistry, Ramkhamhaeng University, Bangkok, Thailand. The spectral data of the isolated compounds were compared by ACD (Advanced Chemistry Development) Labs (Robert and Francis, 2014).

Antimicrobial Activity of Isolated Compounds from *Phomopsis* sp.

All the isolated compounds were tested their antimicrobial activity with six test organisms. The volume of each compound was 10µg/disc (conc.1mg/ml).

Paper disc diffusion assay

Broth culture (50µl) of test organisms (*Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Salmonella enteric*, *Salmonella typhi*, *Staphylococcus aureus* and *Xanthomonas oryzae*) was added to 100 ml assay medium sucrose/yeast medium and then poured into the plates. After solidification paper discs infused with broth samples were applied on the test plates and incubated at 30°C for 24-48 hrs. When clear zones (inhibitory zones) showed around the paper discs, they were measured (Phay, 1997).

Results

Scientific Name - *Psidium guajara* L.
English Name - Guava
Myanmar Name - Malaka
Family - Myrtaceae

Figure 1. Habit of *Psidium guajava* L.

Antimicrobial Activity of 12L Fermented Broth of *Phomopsis* sp.

Twelve fermentation flasks of strain *Phomopsis* sp. showed very high activity against six test organisms. Fermentation flasks 2, 3, 4 and 10 of strain *Phomopsis* sp. indicated high activity against six test organisms. Fermentation flasks 5, 6, 7, 8, 9 and 12 of strain *Phomopsis* sp. exhibited weak activity against six test organisms.

Antimicrobial Activity of Methanol Extracts

The methanol extract showed higher activity than acetone extract against *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus* as shown in Table 1.

Table 1. Inhibitory zones (mm) of methanol extracts from *Phomopsis* sp.

Test Or. / Extracts	*Bacillus subtilis*	*Candida albicans*	*Escherichia coli*	*Malassezia furfur*	*Salmonella typhi*	*Staphylococcus aureus*
Methanol	45	40	45	45	40	35

10-12mm = weak activity, 13 - 18 mm = high activity, >18 mm = very high activity disc size = 6mm

Isolation and Purification of Bioactive Compounds from *Phomopsis* sp.

The two hundred and sixteenth small fractions were collected from silica gel 34 (100g) column. According to their R_f value and colors reaction by reagent on TLC plates under UV 254 nm, they were combined into the large fractions: F1 (1-5), F2 (16-25), F3 (26-40), F4 (41-50), F5 (51-65), F6 (66-80), F7 (81-96), F8 (97-120), F9 (121-146), F10 (148-162), F11 (152-162), F12 (163-180), F13 (181-200), F14 (201-216) and F15 (217-223). All of these fractions, the fraction 8 was crystal and after washing with methanol in three times, it was purified according to their spots on TLC plates under UV 254 nm.

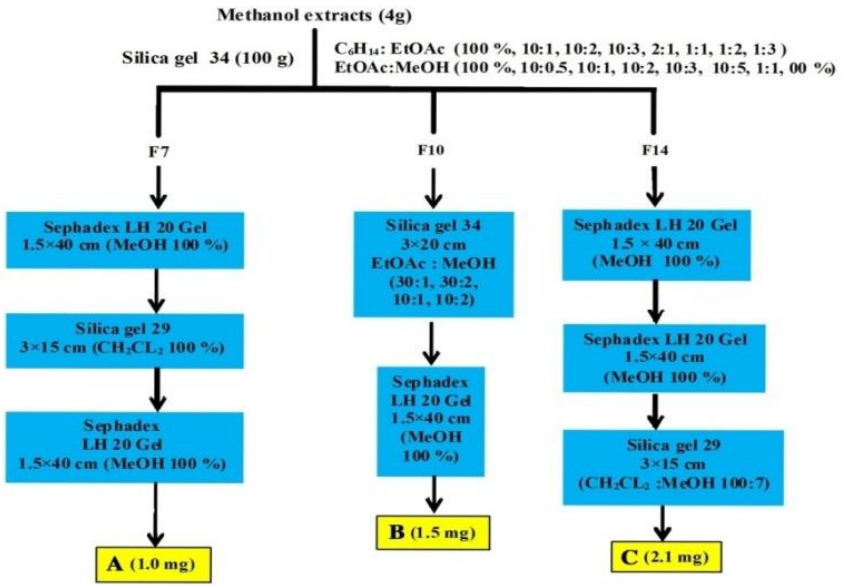
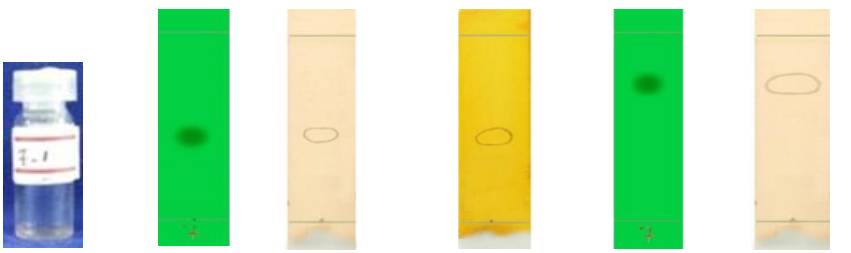


Figure 2. Flow chart for isolation procedure of the active compounds from *Phomopsis* sp.



Compound A | Under UV 254nm | Anisaldehyde | Dragendorff | Under UV 254 nm | Anisaldehyde

Solvent system - CH_2CL_2:MeOH, 5:0.3, 5:0.5,
R_f - 0.35 and 0.69

Figure 3. Identification of the isolated compound A by R_f value

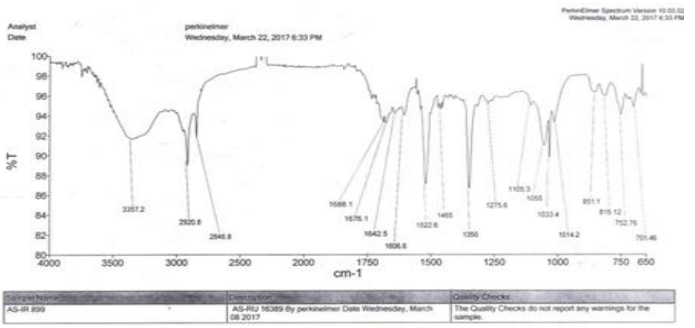


Figure 4. FT-IR spectrum of the isolated compound A

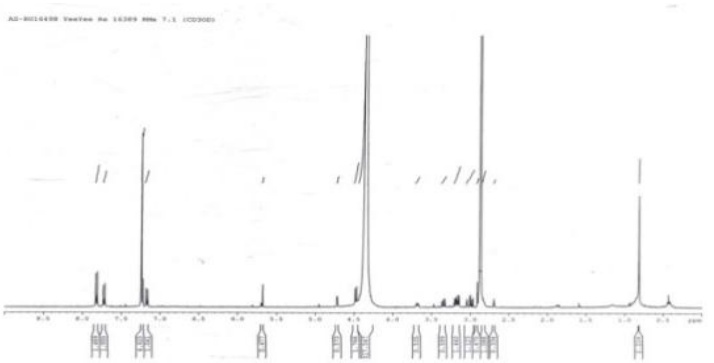


Figure 5. ^{1}H-NMR spectrum (400 MHz, CD_3OD) of the isolated compound A

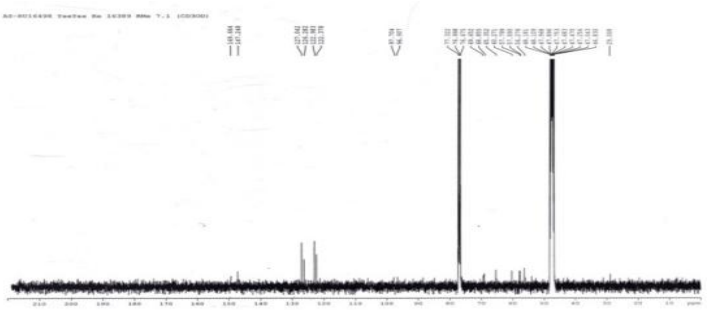


Figure 6. ^{13}C-NMR spectrums (400 MHz, CD_3OD) of the isolated compound A

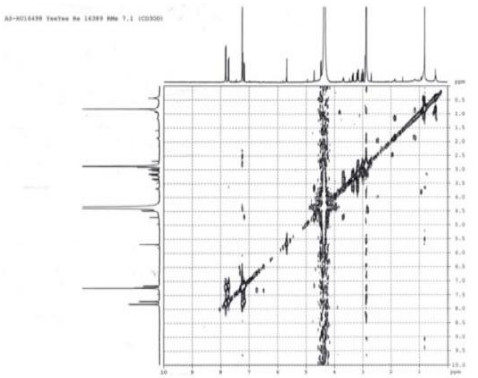


Figure 7. ^{1}H-^{1}H (COSY) spectrum (400 MHz, CD_3OD) of the isolated compound A

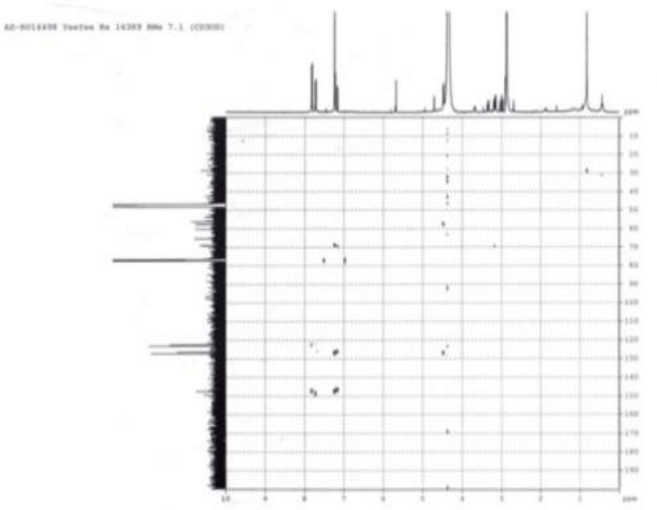


Figure 8. ^{1}H-^{13}C (HMBC) spectrum (400 MHz, CD_3OD) of the isolated compound A

Identification of Isolated Compound A

The isolated compound A was VU active at 254 nm and it does not give any color on TLC plate with anisaldehyde and dragendroff spray reagents. Its R_f value was 0.35 (CH_2CL_2: MeOH, 5:0.3) and 0.69 (CH_2CL_2:MeOH, 5:0.5). This substance is good soluble in chloroform or dichloromethane as shown in Figure 3.

In the FT-IR spectrum, the band for O-H stretching vibration (alcohol and phenol groups) was observed at 3357 cm^{-1}. The bands at 2920 and 2848 cm^{-1}were found for C-H stretching vibration of methyl, methylene and methine groups. C=O stretching vibration (ketone) was showed at 1688 cm^{-1} and C=C stretching vibration group was found at 1676, 1642, 1606, 1522 and 1465 cm^{-1}. The bands for 1350 and 1275 cm^{-1} were found due to the presence of C-H bending vibration of methyl and methylene groups. The bands at 1105, 1055, 1033 and 1014 cm^{-1} showed C-O-C stretching vibration as shown in Figure 4.

According to its ^{1}H-NMR spectrum, aromatic protons were as doublets (*d*) at 7.82 ppm, 7.72 ppm, 7.24 ppm and 7.18 ppm, olefinic protons (C=CH) as singlets (*s*) at 5.67 ppm and as doublets (*d*) at 4.73 ppm, CH_2 protons as doublets (*d*) at 4.47 ppm. CH_2 protons were observed as multiplets (*m*) at 3.69 ppm, as doublet of doublets (*dd*) at 3.35 ppm, as triplet of doublets (*td*) at 3.18 ppm, as doublet of doublets (*dd*) at 3.00 ppm, as singlets (*s*) at 2.93 ppm and as multiplets (*m*) at 2.70 ppm. Alkyl protons were as singlets (*s*) at 1.2 ppm in this compound as shown in Figure 5.

In this ^{13}C- NMR spectrum, aromatic or (C=C) olefinic carbons were found at 149.664 ppm, 147.248 ppm, 127.04 ppm, 126.28 ppm, 122.98 ppm and 122.378 ppm. Acohol (C-OH) groups were at 76.67 ppm, 69.65 ppm, 68.85 ppm, 65.35 ppm, 60.27 ppm, 57.799 ppm, 57.590 ppm and 56.27 ppm as shown in Figure 6.

According to its FT-IR spectral data, ^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectral data, it was identified as phomopsolide B and its molecular formula is $C_{15}H_{20}O_6$ as shown in Fig. 9.

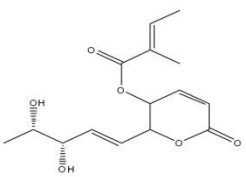
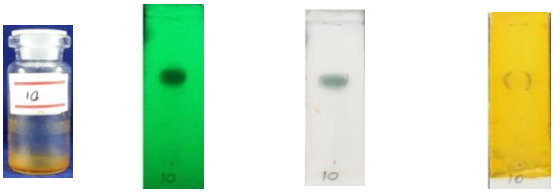


Figure 9. Phomopsolide B



Compound D	Under UV 254 nm	Anisaldehyde	Dragendorff
Solvent system	CH_2CL_2:MeOH, 5:0.3		
R_f	0.60		

Figure 10. Identification of the isolated compound B by R_f value

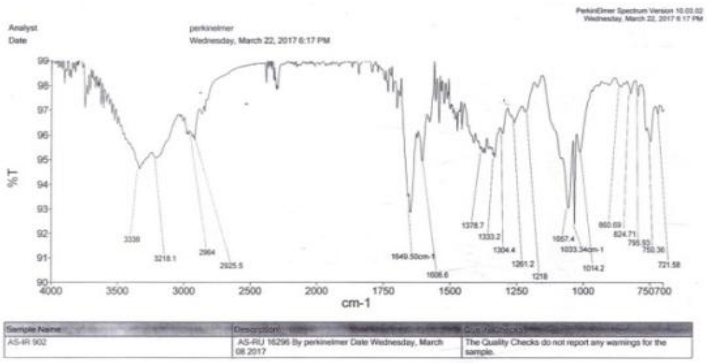


Figure 11. FT-IR spectrum of the isolated compound B

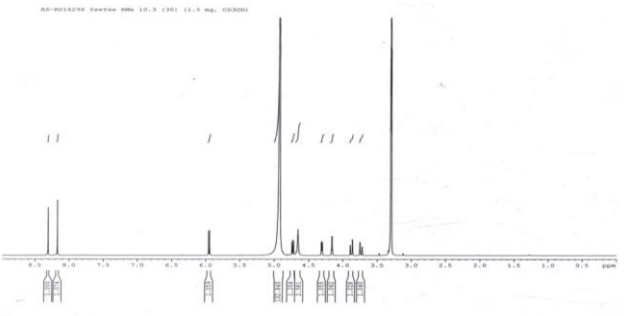


Figure 12. ^{1}H-NMR spectrum (400 MHz, CD_3OD) of the isolated compound B

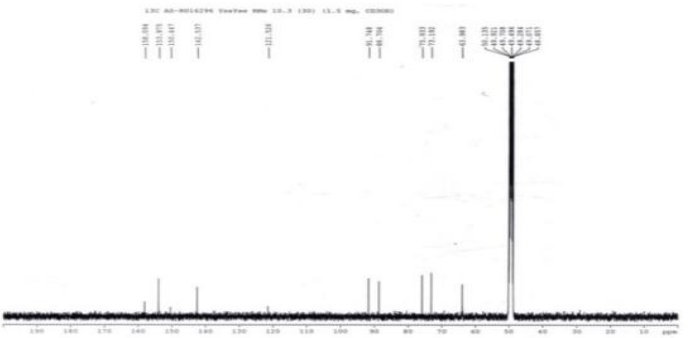


Figure 13. ^{13}C-NMR spectrum (400 MHz, CD_3OD) of the isolated compound B

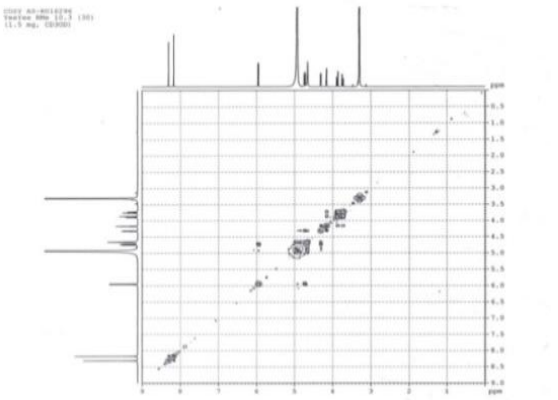


Figure 14. ^{1}H-^{1}H (COSY, 400 MHz, CD_3OD) of the isolated compound B

Identification of the isolated compound B

The isolated compound B was VU active under 254 nm and dark green spot on TLC plate with anisaldehyde spray reagent. Its R_f value was 0.60 (CH_2CL_2:MeOH, 5:0.3). This substance is good soluble in methanol as shown in Figure 10.

Its IR spectrum were showed N-H, O-H stretching vibration (amines, alcohol and phenol groups) at 3338 cm^{-1}. The bands at 2964 and 2925 cm^{-1} were for C-H stretching vibration of methyl and methylene groups. C=C stretching vibration were showed at 1649 and 1606 $cm^{-1}$and C=N stretching vibration was attributed at 1590 and 1690 cm^{-1}. CH_3 and CH_2 bending vibration were found at 1378, 1333, 1304, 1261 and 1218 cm^{-1} and C-N

stretching vibration at 1304 and 1261 cm^{-1}. The bands at 1057, 1033 and 1014 cm^{-1} were showed –C-O-C stretching vibration and the bands at 860, 795 and 750 cm^{1} were found =C-H bending vibration as shown in Figure 11.

According to ^{1}H-NMR spectrum, aromatic protons were found as singlets (*s*) at 8.3 ppm and 8.2 ppm and (CH_2) protons were as doublets (*d*) at 5.9 ppm, as doublet of doublets (*dd*) at 4.73 ppm and as singlets (*s*) 4.65 ppm. Olefinic protons (C=CH) were as quantets (*q*) at 4.31 ppm, as doublet of doublets (*dd*) at 4.16 ppm, at 3.86 ppm, 3.74 ppm in this compound as shown in Figure 12.

By means of ^{13}C NMR, aromatic carbons were at 158 ppm, 153 ppm, 150 ppm and the bands at 142 ppm, 121 ppm, 91 ppm, 88 ppm were found (C=C) olefinic carbons. The (C-OH) alcohol carbons groups were observed at 75 ppm, 73 ppm and 63 ppm as shown in Table 10 and Figure 13.

According to its FT-IR spectral data, ^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectral data, it was identified as adenosine and its molecular formula is $C_{10}H_{13}O_4N_5$ as shown in Figure 15.

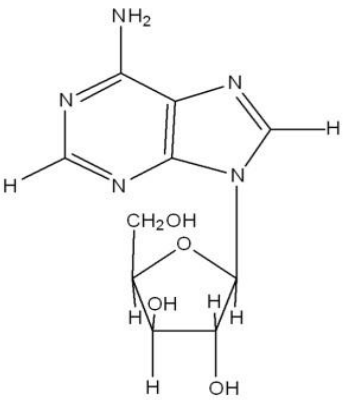
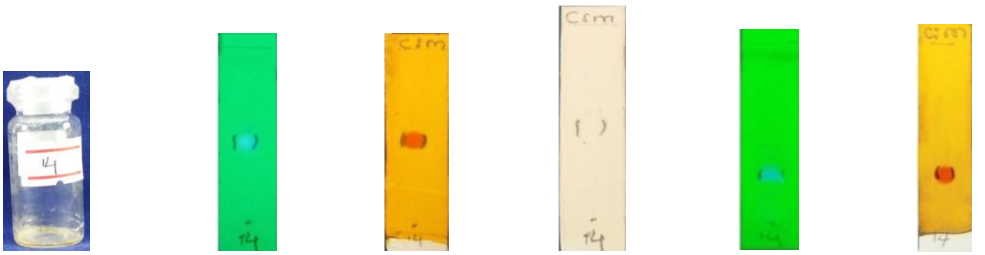


Figure 15. Adenosine



Compound H	Under UV 254 nm	Dragen dorff	Anisalde hyde	Under UV 254 nm	Dragen dorff
Solvent system	- CH_2CL_2:MeOH, 5:0.5, 5:0.3				
R_f	- 0.53, 0.25				

Figure 16. Identification of the isolated compound C by R_f value

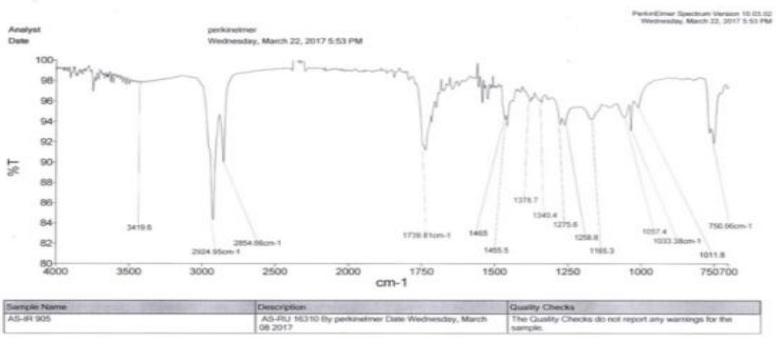


Figure 17. FT-IR spectrum of the isolated compound C

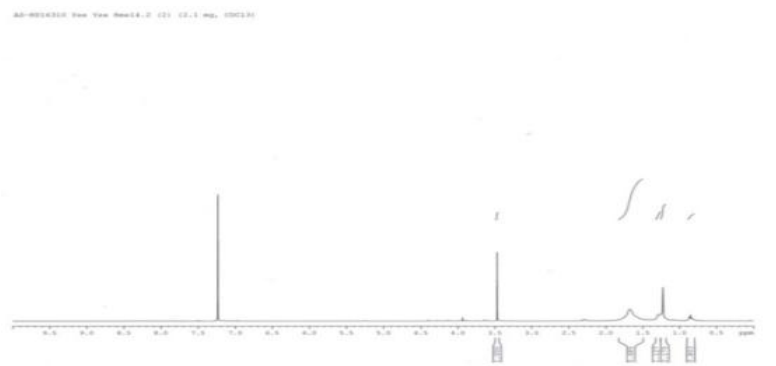


Figure 18. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the isolated compound C

Identification of the isolated compound C

The isolated compound H was VU active under 254 nm and it gave an orange colour

with dragendoff reagent. Its R_f value was 0.53 (CH_2CL_2:MeOH, 5:0.5) and 0.25 (CH_2CL_2:MeOH, 5:0.3). This substance is good soluble in chloroform or dichloromethane as shown in Figure16.

In the FT-IR spectrum, the band for N-H stretching vibration was observed at 3419 cm^{-1} and the bands at 2924 and 2854 cm^{-1} were found for C-H stretching vibration of methyl and methylene groups. C=O stretching vibration (ketone) was showed at 1739 cm^{-1} and aromatic carbons were found at 1465, 1455, 1378 and 1340 cm^{-1}. The bands at 1165, 1057, 1033 and 1011 cm^{-1} were found alkyl amines. The C-H bending vibration was found at 750 cm^{-1} as shown in Figure 17.

According to its ^{1}H-NMR spectrum, (CH_2) proton was as singlets (*s*) at 3.5 ppm and alkyl protons (CH_3) was as singlets (*s*) at 1.7 ppm, 1.3 ppm and 1.2 ppm in this compound as shown in Figure 18.

According to its FT-IR spectral data and ^{1}H-NMR spectral data, it was identified as emerimidine C and its molecular formula is $C_{10}H_{13}O_3$ as shown in Figure 19.

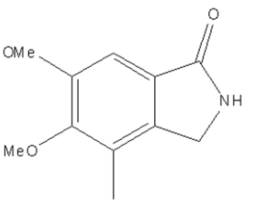


Figure 19. Emerimidine C

Antimicrobial Activity of Isolated Compounds from *Phomopsis* sp.

The compound B and C showed highest activity against *Xanthomonas oryzae* and indicated weak activity against *Candida albicans* and *Escherichia coli* as shown in Table 2.

Table 2. Inhibitory zones (mm) of the isolated compounds

T.O / Compounds	*Bacillus subtilis*	*Candida albicans*	*Escherichia coli*	*Salmonella enterica*	*Salmonella typhi*	*Xanthomonas oryzae*
A	-	-	-	-	-	-
B	-	10	12	-	-	25
C	-	12	10	-	-	32

10 -12 mm = weak activity, 13 - 18 mm = high activity, >18 mm = very high activity, disc size 6 mm

Discussion and Conclusion

In this research work, fungal strain *Phomopsis* sp. was isolated from leaves of *Psidium guajava* L. Twelve liters fermentation was undertaken at room temperature by sucrose/yeast medium and tested its antimicrobial activity with six test organisms. Then the fermentation, the filtrate was extracted with methanol on Ambilites XAD 2 resin column for crude extract. It is in agreement with the statement of Yee Yee Thu (2006). Bicas *et al.*, (2009) stated that the bioactive compounds could be isolated from microbial products through the fermentation.

The methanol extract were tested with six test organisms and it was observed that methanol extract gave good result. Kaczorowski *et al*., (2011) reported that methanol crude extracts of fungi inhibited the growth of all four human pathogens; *B. subtilis*, *S. aureus*, *E. coli* and *S. Typhimurium* by the agar diffusion method. Basha *et al*., (2012) stated that antimicrobial activity of methanol crude extract of fungi showed activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus faecalis*, *Escherichia coli*, *Salmonella typhimurium* by agar-well diffusion assay.

The compound A was identified as phomopsolide B. This compound was also isolated from endophytic fungus *Phomosis* sp. by Selim *et al.*, (2012). This compound was also isolated from *Aspergillus terreus* by Rizna *et al.*, (2015) and they also reported that it has antioxidant and antibacterial activities.

The compound B was identified as adenosine according to its spectroscopic methods. Yee Yee Thu (2006) also isolated adenosine from the fermented broth of endophytic fungus *Tricoderma* sp. Xin-guo Zhang *et al.*, (2017) reported that new uncompetitive inhibitor of adenosine deaminase identified from endophyte *Aspergillus niger* sp.

The compound C was identified emerimidine C. This compound was also isolated from endophytic fungi of medicinal plants and showed very high activity against *Xanthomonas oryzae* by Selim *et al.,* (2012).

In conclusion, the active compounds from *Phomopsis* sp. which was isolated from *Psidium guajava* L. exhibited higher activity on *Xanthomonas oryzae* than other test organisms. Therefore, it is essential to do further research concerning with leaf blight *Xanthomonas oryzae* on rice plants as biocontrol. After that, these compounds could be applied for the protection of leaf blight disease caused by bacterium *Xanthomonas oryzae* in the paddy fields. This finding would be helpful to our farmers who make rice, our staple food, as well as all of us in our nation.

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