BIOACTIVE COMPOUNDS OF AN ENDOPHYTIC FUNGUS *PHOMOPSIS* SP. ISOLATED FROM *PSIDIUM GUAJAVA* L.

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

Abstract

In this research work, an endophytic fungus *Phomopsis* sp. was isolated from the leaves of *Psidium guajava* L. In order to extract the bioactive compounds from this fungus, 12L fermentation was eluted on Amberlite XAD-2 resin column and extracted with methanol at microbiology laboratory, Department of Botany, University of Yangon. Isolation and purification of the bioactive compounds from the methanol extract were undertaken by utilizing silica gel columns and Sephardex LH20 gel columns with various solvent systems at Department of Organic Chemistry, Ramkhamhaeng University, Bangkok, Thailand. The isolated compounds were characterized by FT-IR spectra, 1-D NMR (^{1}H-NMR and ^{13}C-NMR), 2-D NMR (HSQC, HMBC and COSY) spectra. The three compounds I, II, III were identified as butyrolactone V, ligballinol and phomoxanthone A. Antimicrobial activity of the isolated compounds in 10 μg/disc (conc. 1 mg/ml) were evaluated on six test organisms at Department of Botany, University of Yangon. Among the isolated compounds, the compound I showed high activity against *Bacillus subtilis*, *Candida albicans* and *Escherichia coli* while the compound II and III indicated very high activity against *Bacillus subtilis* and *Xanthomonas oryzae* but weak activity against*Candida albicans* and *Escherichia coli*.

Key words: Butyrolactone, Endophytic *Phomopsis* sp., Ligballinol and Phomoxanthone

Introduction

Endophytes live in mutualistic association with plants for at least a part of their life cycle. Medicinal plants and their endophytic flora produce similar pharmaceutical products. The need for new anduseful bioactive compounds to provide assistance and relief in all aspects of the human condition is ever growing (Stone *et al*., 2000).

Many previous researchers reported endophytic fungi with the novel and bioactive natural products obtained from medicinal plants (Huang *et al.*, 2010). Natural products from fungal endophytes have a broad spectrum of biological activities, such as antimicrobial, immune suppressant, anticancer, and also may act as biocontrol agents (Borges *et al*., 2009). The bioactive metabolites produced by endophytic fungi are the major source of drugs. More than 20,000 bioactive metabolites of microbial origin are known (Kumaran *et al*., 2010).

The objectives of the present research work are to extract and isolate the bioactive compounds from an endophytic fungus *Phomopsis* sp. isolated from the leaves of *Psidium guajava*L. and to evaluate antimicrobial activity of the isolated compounds.

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Materials and Methods

Screening of the endophytic fungus from *Psidium guajava* L.

The *Psidium guajava* L. plant sample was collected from Nyaung-Hna-Pin area, Hmawbi Township. The screening 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 soaked in 5.3% sodium hypocloride for 5 min. (5) Cut-plant pieces were washed out sodium hypocloride by soaking in 75% ethanol for 0.5min. (6) They were dried and cut into smaller pieces, and placed on agar plates and then incubated for 3 days to 3 weeks. Isolated fungus was transferred into a 10 ml test tube containingsucrose/yeast extract medium and incubated for 2-5 days (Lee *et al*., 1996).

Antimicrobial activity of twelve liters fermentation of *Phomopsis* sp.

A piece of fungus from the slant culture of *Phomopsis* sp. was inoculated into 500 ml of conical flask containing 180 ml of sucrose/yeast seed medium and the flask was incubated for two days.Two days old seed culture (15 ml) was transferred into 2L conical flask containing 1L of sucrose/yeast fermentation medium. For 12L fermentation, twelve conical flasks were used and these flasks were incubated at 100 rpm for 3-7days at 30°C. The fermentation flasks were tested for antimicrobial activity on *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus* (Strobel & Sullivan, 1999; Phay, 1997).

Extraction of the bioactive compounds from fermented broth

After antimicrobial activity, 12L fermented broths were filtered into the mycelia and the filtrate. Then, the filtrate was extracted with Amberlites XAD-2 resin column. The column was washed with water followed by methanol. The methanol extract was evaporated on the water bath at 55˚C. The extract was tested for antimicrobial activity against *B. subtilis*, *C.albicans*, *E. coli*, *M. furfur*, *S. typhi*, *S. aureus* and *X. oryzae* (Strobel and Sullivan, 1999).

Isolation and purification of the bioactive compounds from *Phomopsis*sp.

Based on the TLC results of methanol extract, silica gel column chromatography was carried out. The silica 34 gel (200 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%) and then fifteen fractions were collected. Some fractions were combined according to their TLC behavior.The different sizes of silica gel columns and Sephadex LH20 columns were utilized for further purification (Grable*yetal*., 1999).

Identification of the isolated compounds from *Phomopsis*sp.

For the identification of the isolated compounds from *Phomopsis* sp., TLC profiles, the infrared spectra, the ^{1}H- and ^{13}C-NMR spectra and 2D-NMR spectra of the isolated compounds were recorded at the Department of Chemistry, Ramkhamhaeng University,Bangkok, Thailand. All data of the isolated compounds were compared to Advanced Chemistry Development ACD Labs(Robert and Francis, 2014).

Antimicrobial activity of the isolated compounds from *Phomopsis* sp.

All isolated compounds were tested their antimicrobial activity with six test organisms by paper disc diffusion assay. The volume of each compound was 10μg/disc(conc. 1.0mg/ml) (Phay, 1997).

Results

Antimicrobial activity of the methanol extract of *Phomopsis* sp.

The methanol extractof 12L fermentation also showed very high activity 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 the methanol extract from *Phomopsis*sp.

	B.subtilis	***C.albicans***	***E.coli***	***M.furfur***	***S.typhi***	***S.aureus***
Methanol extract	45	40	45	45	40	35

Isolation and purification of the bioactive compounds from *Phomopsis* sp.

The two hundred small fractions were collected from crude extract column.According to the same R_f value and color reactions by reagents on TLC plates, they were combined into the large fractions such as F1(30-50), F2 (80-120) and F3(152-180). The fraction F2 was crystaland after washing with methanol in four times, it was purified.

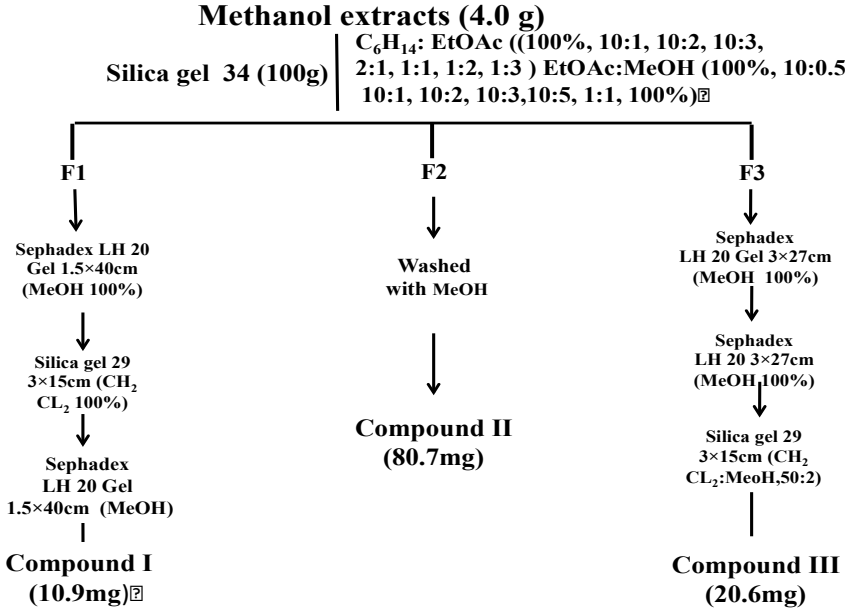


Figure 1. Isolation procedure of the active compounds from *Phomopsis* sp.

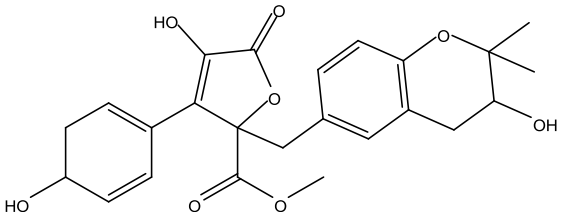
Identification of the isolated compound I

The isolated compound I was under UV absorbing band at 254 nm and light pink spot on TLC plate with anisaldehyde. Its R_f value was0.18 (CH_2CL_2 100%) and 0.66 (CH_2CL_2:MeOH, 5:0.3). This substance is well soluble in chloroform or dichloromethane.

In IR spectrum, O-H stretching vibration (alcohol and phenol groups) showed at 3500 cm^{-1}. C-H stretching vibrations of methyl and methylenegroups were found at 2978, 2935 and 2868 cm^{-1}. Its IR spectrum showed C=O stretching vibration (ketone)

at 1600 cm^{-1}. For olefiniccarbon groups, the bands for C=C were observed at 1275 and 1261 cm^{-1}. The bands at 1055, 1033, 1011 cm^{-1} were due to the presence of C-O-C stretching vibration of cyclic ether. The bands at 746 and 750 cm^{-1} were C-H bending vibration of methyl and methylenegroups as shown in Figure 2.

According to its ^{1}H-NMR spectrum, aromatic protons were found as multiplet (*m*) at 7.55 ppm and olefinic protons (C=CH) were as singlet(*s*) at 4.87ppm, 5.33ppm and3.5 ppm. Alkyl protons (CH_2) were observed as singlet(*s*) at 1.5 ppm in this compound as shown in Figure 3.According to its TLC profile, FT-IR and ^{1}H-NMR spectral data, the compound I was identified as butyrolactone V and its molecular formula is $C_{24}H_{25}O_8$.



Butyrolactone V

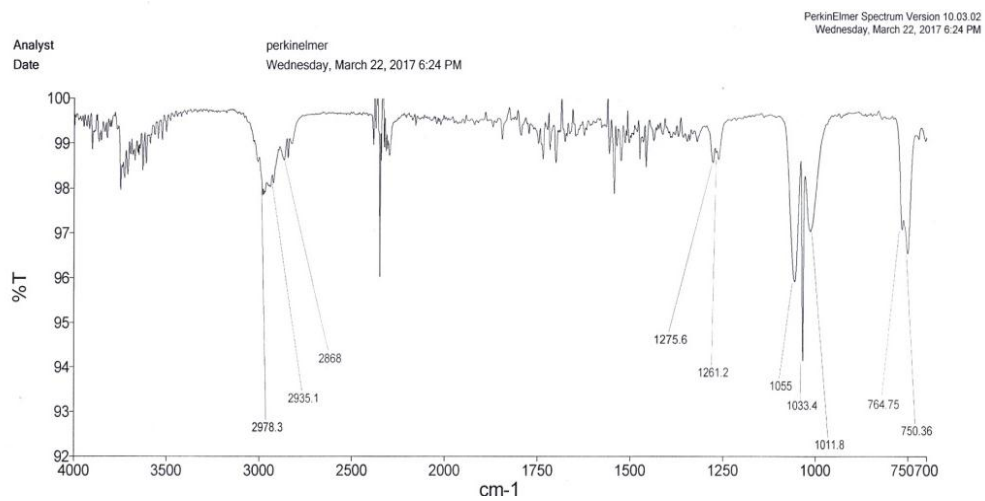


Figure 2. FT-IR spectrum of the isolated compound I

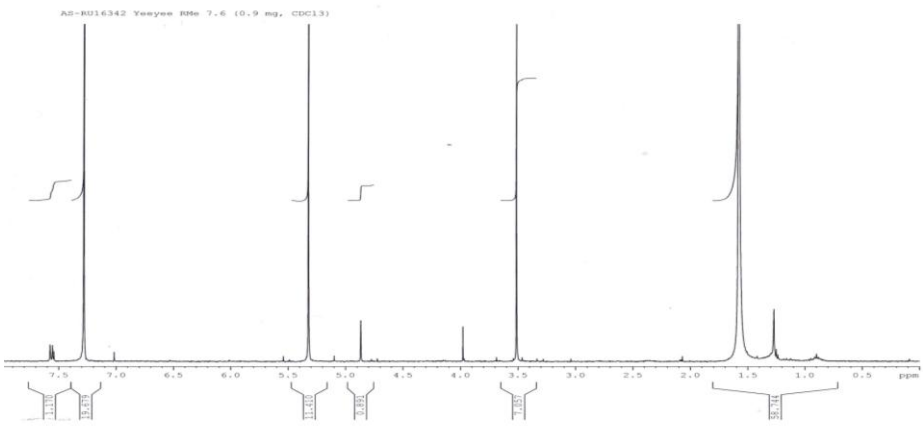


Figure 3.^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the isolated compound I

Identification of the isolated compound II

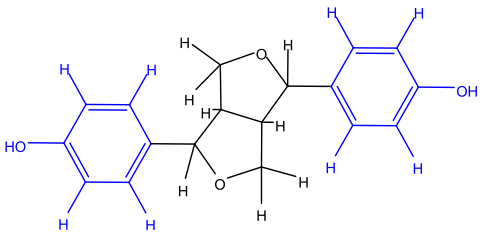
The isolated compound II was VU active at 254 nm and white color on TLC plate with anisaldehyde spray reagent. Its R_f value was 0.34 (CH_2CL_2:MeOH, 5:0.1) and 0.79 (EtOAc:MeOH, 3:0.1). This substance is well soluble in methanol.

In its FT-IR spectrum, NH and O-H in phenolic groups were observed at 3333, 3256, 3079cm^{-1}.The bands for 2964 and 2901cm^{-1} were due to the presence of C-H vibration of methyl and methylene groups. C=O stretching vibration (ketone) was shown at 1679 cm^{-1}. The bands at 1601, 1516 and 1446 cm^{-1} were for C=C aromatic group. The bandat 1563 cm^{-1} was found due to the presence of N-H bending. C-H bending vibrations of methyl and methylenegroups were shown at 1411, 1342 and 1244 cm^{-1}. C-C stretching was observed at 1107$cm^{-1}$and C-H out of plane bending was found at 972 cm^{-1}as seen inFigure 4.

According to its ^{1}H-NMR spectrum, aromatic protons were observed as doublets (*d*) at 8.2 ppm and 7.63 ppm. Olefinic protons (C=CH) were found as singlets(*s*)at 6.22ppm and5.15ppm.CH_2 protonswere as multiplets (*m*) at 4.13ppm, as quantets (*q*) at 3.8ppm and at 3.6ppmin this compound as shown in Figure 5.

In the ^{13}C-NMRspectrum, the group of carbon (C=O) ketone was found at 167 ppm and alcohol (C-OH) group was observed at 152ppm. Aromatic carbon C=C was at 149 ppm. The methine (CH) groups were observed at 128 ppm, 124 ppm, 71.8 ppm and 67.8 ppm. Methylene group was found at 62.7 ppm while the group of methyl at 59.0 ppm in this compound as shown in Figure 6.

According to its FT- IR spectral data, ^{1}H-NMR, ^{13}C-NMR and 2D-NMRspectral data, the compound II was identified asligballinol(or) 4,4'- (hexahydrofuro[3,4-c]furan-1,4-diyl) diphenol. Its molecular formula is $C_{18}H_{18}O_4$.



Ligballinol

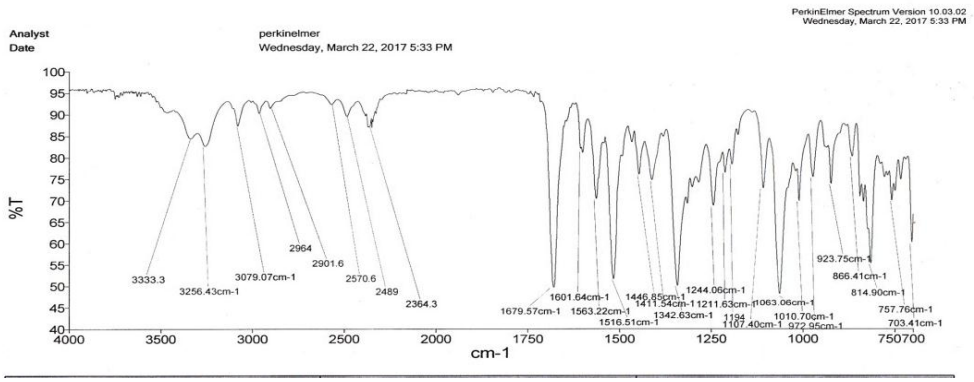


Figure 4. FT-IR spectrum of the compound II

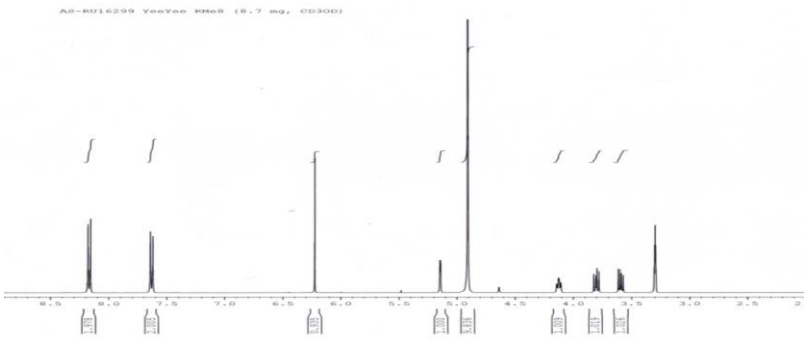


Figure 5.^{1}H-NMR spectrum (400 MHz, CD_3OD) of the isolated compound II

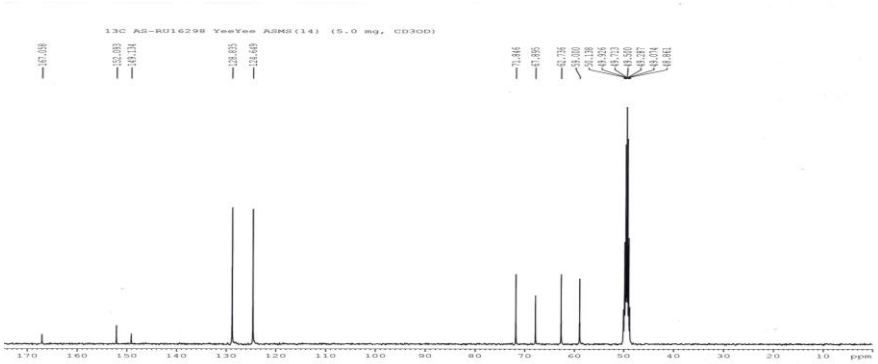


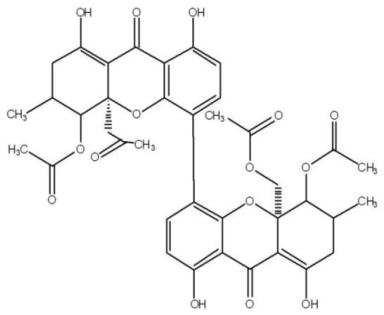
Figure 6. ^{13}C-NMR spectrum (400 MHz, CD_3OD)of the isolated compound II

Identification of the isolated compound III

The isolated compound III was VU active under 254 nm and its R_f value was 0.37 (CH_2CL_2:MeOH, 5:0.3). This substance is good soluble in chloroform or dichloromethane.

In the FT-IR spectrum, the band for O-H stretching vibration (alcohol and phenol groups) was observed at 3342 cm^{-1}. The bands at 2927 and 2858 cm^{-1} were for C-H stretching vibration of methyl and methylene groups. C=O stretching vibrations (ketone) were showed at 1700, 1697 cm^{-1} and C=C stretching vibration groups were found at 1690 and 1678 cm^{-1}. The bands at 1522, 1462, 1374 and 1275cm^{-1} were for C=C aromatic.The bands for 1107,1055 and 1016cm^{-1} were found due to the presence of C-O-C stretching vibration and showed C-H bending vibration of methyl and methylene groupswere showed at 851, 812 and 750 ppm as shown in Figure 7.

According to its ^{1}H-NMR spectrum, aromatic protons were observed as singlets (*s*)at 14.0ppm and as douplets(*d*) at 11.6 ppm, 7.40 ppm and 6.43ppm, as singlets (*s*) at 5.28 ppm and as doublets (*d*) at 5.25 ppm, 4.25ppm and 4.18 ppm. (CH_2) protons were found as multiplets(*m*) at 2.38 ppm and (CH_3) protons were found as doublets (*d*) at 0.98 ppmin this compound as shown in Figure 8.According to its TLC profile, FT-IR and ^{1}H-NMRspectral data, it was identified as phomoxanthone A and its molecular formula is $C_{38}H_{38}O_{16}$.



Phomoxanthone A

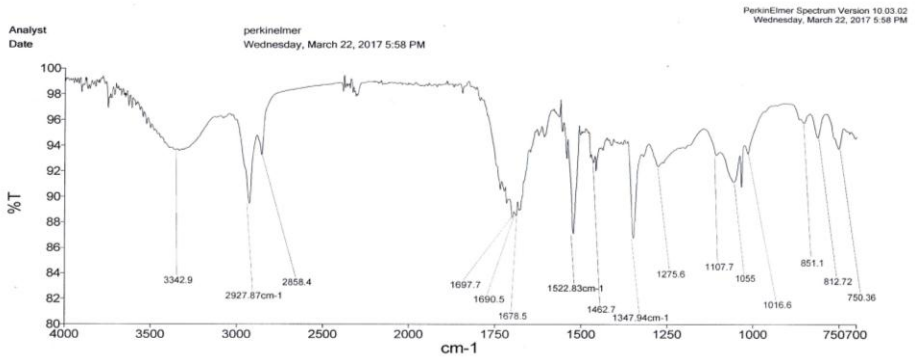


Figure 7. FT-IR spectrum of the isolated compound III

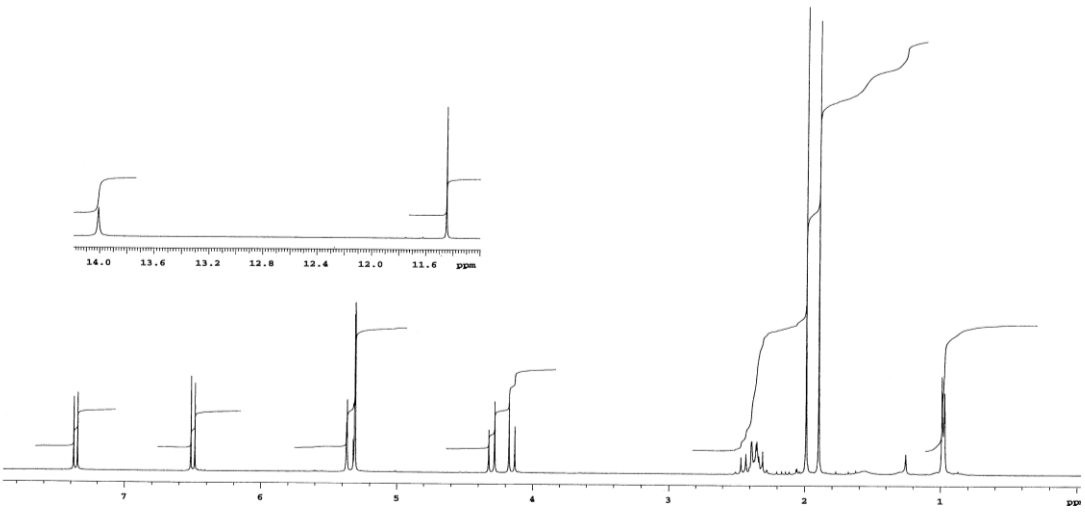


Figure 8. ^{1}H-NMR spectrum (400 MHz, DMSO) ofthe isolated

Antimicrobial activity of the isolated compounds from *Phomopsis* sp.

The compound I showed high activity against *Bacillus subtilis* (18mm) and *Candida albicans* (17mm) and *Escherichia coli* (19mm). The compound II indicated very high activity against *B. subtilis* (38mm) and *Xanthomonas oryzae* (25mm) but weak activity on *C. albicans* (13mm) and *E.coli* (14mm). The compound III exhibited very high activity against *B. subtilis* (27mm) and *X.oryzae* (18mm) but weak activity on *C.albicans* (13mm) and *E. coli* (15mm).

Discussion and Conclusion

In this research, a fungal strain *Phomopsis* sp. was isolated from the leaves of *Psidium guajava* L. Twelve liters fermentation of *Phomopsis* sp. was undertaken and tested antimicrobial activity on six test organisms. After the fermentation, the filtrate was extracted with methanol on Ambilites XAD-2 resin column for crude extracts. The methanol extract were tested with six test organisms and methanol extract showed good activity. Yee Yee Thu (2006) also isolated an endophytic fungus from the leaves of *Psidiumguajav*L.Kaczorowski*et al*. (2011) reported that the fungal methanol extract inhibited activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Salmonella typhimurium*. Basha*et al*. (2012) have also stated that the fungal crude extract indicated antimicrobial activity on *S. aureus, B. subtilis, Streptococcus faecalis, E. coli* and *S.typhimurium*. Bicas*et al.*(2009) stated that the bioactive compounds were isolated from microbial sources through the fermentation.

In this research work, the three bioactive compounds were isolated from the methanol extract of 12L fermentation. The compound I (butyrolactone V) showed high activity against *Bacillus subtilis* and *Candida albicans* and *Escherichia coli*. This compound was also isolated from an endophytic fungus *Phomopsis longicolla* by Selim*et al.,* (2012) and they reported that it has antibacterial activity.

The compound II (ligballinol) showed very high activity against *B. subtilis* and *X. oryzae* but weak activity on *C.albicans* and *E.coli*.This compound II was also isolated from *Aspergillusterreus*by Rizna*et al.,* (2015) and they also reported that it has antimicrobial, antioxidant and antibacterial activities.

The compound III (phomoxanthone A) and it has high activity against *B. subtilis*and *X. oryzae* but weak activity on *C. albicans* and *E. coli*. This is in agreement with the statement of Selim *et al.* 2012.This compound was also isolated from *Phmopsis longicolla* by Selim *et al.,* (2012) and they reported that it has antimicrobial activity.

In conclusion, the active compounds from *Phomopsis* sp. indicated high activity on *B.subtallis* which causes dysentery and diarrhea, *C. albicans* that causes vaginal and skin infections, *E. coli* that causes cholera, diarrhea and vomiting and *X. oryzae* that causes leaf blight disease on rice plants. Therefore, these compounds could be applied as antibiotics to treat some infectionsand diseases, and also to protect leaf blight disease caused by *Xanthomonas* sp.

It is essential to do further research concerning with leaf blight *Xanthomonas oryzae* on rice plants by applying the two active compounds, ligballinol and phomoxathone A. After that, these compounds could be applied as active agents for the protection of leaf blight disease caused by *Xanthomonas oryzae* in the paddy field be cause our nation is an agricultural one and rice is our staple food, then chemical pesticides are so dangerous to living things and their environments. The good findings in this research work are very beneficial to humans' health as well as farmers in our nation.

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