ISOLATION OF BIOACTIVE COMPOUNDS FROM *ASPERGILLUS* SP.PRODUCED FROM RHIZOME OF *ZINGIBERCASSUMUNAR* ROXB.

Soe Soe Yu Hnin[1], Mon Mon Thu[2], Yee Yee Thu[3] and Aye Pe[4]

Abstract

An Endophytic fungal strain*Aspergillus*sp. was isolated from the rhizome of *Zingibercassumunar*Roxb. For the extraction and isolation of the bioactive compounds, 10L fermentation of strain *Aspergillus*sp. and antimicrobial activity of fermented broth with six test organisms were carried out. After fermentation, the filtrate was extracted with methanol on Ambilite XAD 16 resin column at Microbiology Laboratory, Department of Botany, Universityof Yangon. The methanol extract showed high antimicrobial activity against *Bacillus subtilis*, *Candida albicans*,*Escherichia coli*,*Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus.* Isolation of the bioactive compounds from the methanol extract was carried out by using silica gel and Sephadex 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, 1D-NMR (^{1}H-NMR, ^{13}C-NMR) and 2D-NMR spectra.The compounds 1, 2 and 3 were identified as 4-amino-1-(1,3-dihydroxy-1- (4-nitrophenyl) propan-2-yl)-1H-1,2,3-triazol-5(4H)-one; 3,6-dibenzyl-3,6-dimethyl piperazine-2,5-dione and aspergillitine. Antimicrobial activity of all isolated compounds was evaluated on six test organisms and showed antibacterial activity on*Bacillus subtilis*,*Escherichia coli* and *Xanthomonasoryzae.*

Keywords: Antimicrobial activity, Aspergillitine,*Aspergillus*sp. And *Zingibercassumunar*Roxb.

Introduction

In the recent years, numerous metabolites possessing uncommon structures and potent bioactivity have been isolated from strains of bacteria and fungi collected from diverse environments, such as soils, animals, plants

1. Demonstrator, Department of Botany, University of Yangon
2. Associate Professor, Department of Chemistry, Kyaukse University
3. Associate Professor, Department of Botany, Sittway University
4. Professor and Head, Department of Botany, University of Yangon

and sediments (Faulkner *et al*., 2006 and Laatsch, 2010). Endophytic fungi are one of the most unexplored and diverse groups of organisms that make symbiotic associations with higher life forms and may produce beneficial substances for host (Weber, 1981 and Shiomi*et al.,* 2006). Fungi have been widely investigated as a source of the bioactive compounds (Zhang *et al*.,2011). Therefore, several research groups and pharmaceutical companies were motivated to start sampling and screening large collections of fungal strains for antibiotics, antimycotics, antivirals, anticancers and pharmacologically active agents (Song *et al*., 2004).

The objectives of this research work are extraction, isolation and structure elucidation of the bioactive compounds from fermented broth of *Aspergillus* sp., and evaluation of their antimicrobial activity.

Materials and Methods

Isolation of Endophytic Fungus from*Zingibercassumunar***Roxb.**

The *Zingibercassumunar*Roxb.plant sample was collected from Nyaung-Hna-Pin area, HmawbiTownship. 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 0.5min. **(**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 10ml test tube containing 5ml of sucrose/yeast extract medium.

Antimicrobial Activity of 10L Fermentation from *Aspergillus***sp.**

The small piece (1 cm) of fungus from the plate culture of *Aspergillus* 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 ten 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. Ten liters 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 *Aspergillus*sp.

After testing antimicrobial activity, 10L fermented broth was filtered with filter paper. The mycelia from fermented broth were filteredon the filter paper and then the filtrate was applied on an Amberlites XAD 16 resin column. The column was washed with water, followed by five 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 *Aspergillus*sp.

According to TLC result, silica gel column chromatography was carried out. The silica 34 gel (100 g) column was eluted with hexane : ethyl acetate (100%, 9:1, 8:2, 5:2, 2:1, 1:1, 1:2, 1:3, 1:5) and ethyl acetate : methanol (100% EA, 10:1, 10:2, 10:3, 10:5, 1:1, 100% MeOH) and then fourteen fractions were collected. The column size was 5 × 10 cm and flow rate was 2 ml per minute(Grableyet *al.*,1999).

Identification of Isolated Compounds from *Aspergillus*sp.

The identification of the isolated compounds 1, 2 and 3 were characterized by 1D-NMR (^{1}H-NMR and ^{13}C-NMR), 2D-NMR (^{1}H-^{1}H COSY) 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 *Aspergillus*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 (50 µl) of test organisms (*Bacillus subtilis*, *Candida albicans*,*Escherichia coli*,*Malassezia furfur*, *Salmonella enteric*, *Salmonella typhi*, *Staphylococcus aureus*and *Xanthomonasoryzae*) 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

Outstanding Characters of Plant Sample

Scientific name	-*Zingibercassumunar*Roxb.
English Name	- Bengal ginger
Myanmar name	- Meik-Tha-Lin
Family	- Zingiberaceae

Figure 1. Habit of *Zingibercassumunar*Roxb.

Outstanding characters

Herbs with aromatic rhizome, rhizomes bright yellow; Leaves opposite and distichous, simple; Inflorescence borne separately from the leaves, peduncle, ovate spike, bracteolate; Flowers pale yellow, complete, bisexual, irregular, zygomorphic, epigynous; Sepals (3), synpetalous; Petals (3),

synpetalous; Stamens 1+(2)st + 2st, epipetalous; Filaments exserted, anthers dithecous, dorsifixed, longitudinal dehiscence; Pistil 1, tricarpellary, syncarpous, axile placentation, style long and slender, stigma capitatesinferior; Fruits and seeds not seen.

Morphological and MicroscopicalCharacters of Isolated Fungus*Aspergillus*sp.

The surface and reverse colour of *Aspergillus* sp.was dark green and yellow. Conidiophores are upright, simple, terminating in a globose swelling bearing phialides at the apex, conidia 1 celled, globose, often variously colored in mass.Therefore, this strain was identified as *Aspergillus*sp.

Antimicrobial Activity of 10L Fermented Broth of*Aspergillus*sp.

In this study, allfermentation flasks showed antimicrobial activities against *Bacillus subtilis,Candida albicans, Escherichia coli, Malassezia furfur, Salmonella typhi* and *Staphylococcus*.

Antimicrobial Activity of Methanol Extract

Methanol extract showed highest activity against *Bacillus subtilis,Candida albicans, Escherichia coli, Malassezia furfur,Salmonella typhi*and *Staphylococcus aureus*in Table 1.

Table 1. Inhibitory zones (mm) of methanol extract of *Aspergillus*sp.

Extracts	*Bacillus subtilis*	*Candida albicans*	*Escherichia coli*	*Malassezia furfur*	*Salmonella typhi*	*Staphylococcus aureus*
Methanol	42	40	45	50	36	35

10 -12 mm = weak activity, 13 - 17 mm = high activity, >18 mm = very high activity, disc size: 6 mm

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

The two hundred and eight small fractions were collected from silica gel 34 column. According to theirR_f value and colouron TLC plates under UV 254 nm, they were combined into large fractions such as F1 (1-5), F2 (6-12), F3 (13-17), F4 (18-30), F5 (31-80), F6 (81-95), F7 (96-125), F8 (126-135), F9 (136-163), F10 (164-180), F11 (181-198), F12 (199-202), F13 (203-208).All of these fractions, the fraction 9 (the compound 1) was crystal after washing with dichloromethane as shown in Figure 2.

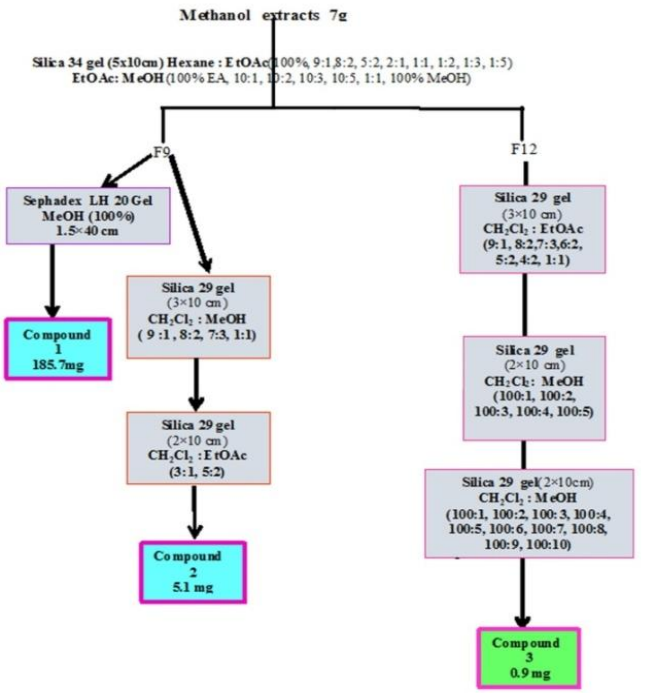
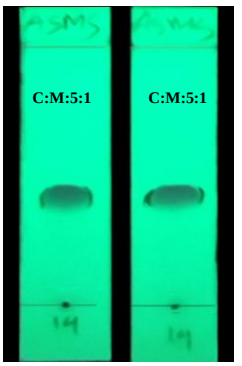


Figure 2. Isolation procedure of the bioactive compounds

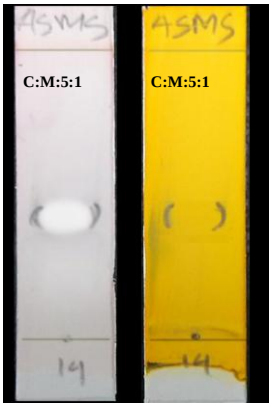
Identification of Isolated Compounds from *Aspergillus*sp.

Identification of the isolated compound 1

SephadexLH20 gel column 1.5×40 cm



UV 254nm



Staining colour (Anisaldehyde and Dragendroff)

Needle shaped crystal

Eluting solvent	= MeOH (100%)
Fraction size	= 1 ml
Flow rate	= 2ml / min
R_f	= 0.4 (CH_2Cl_2:MeOH; 5:1)

Figure 3. Identification of the isolated compound 1 by R_f value

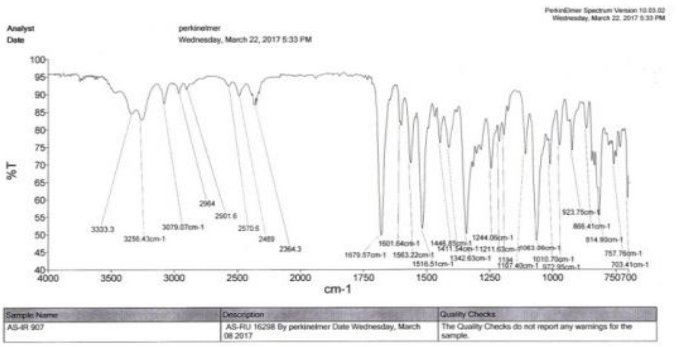


Figure 4. FT-IR spectrum of the isolated compound 1

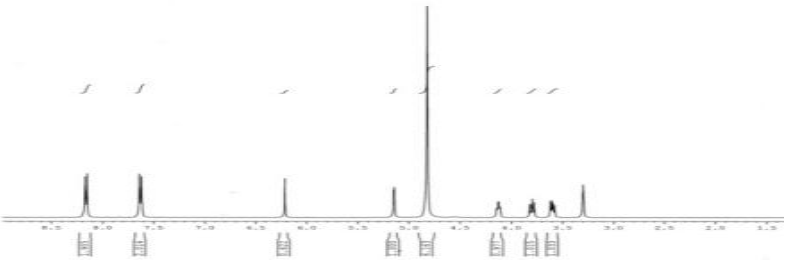


Figure 5. ^{1}H-NMR spectrum (400 MHz, CD_3OD) of the isolated compound 1

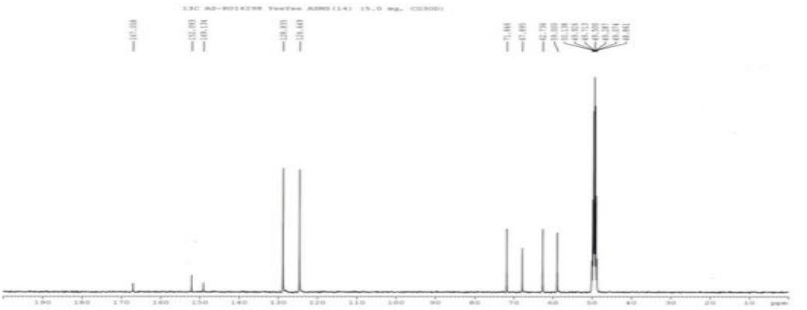


Figure 6. ^{13}C-NMR spectrum (400 MHz, CD_3OD) of the isolated compound 1

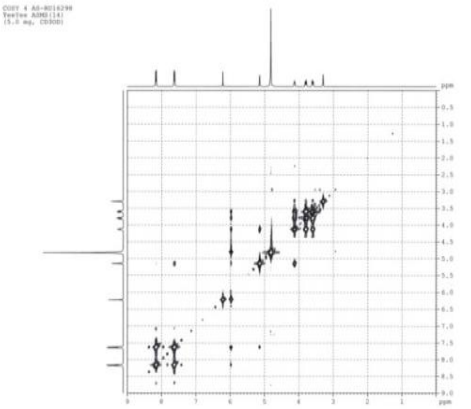


Figure 7. ^{1}H -^{1}H (COSY) spectrum (400 MHz, CD_3OD) of thecompound 1

The compound 1 was found in the fraction 9 as an UV absorbing band at 254 nm and has R_f0.4 (CH_2Cl_2 :MeOH,5:1).It showed white colour spot with anisaldehyde reagent but no active with dragendroff reagent(Figure 3). This substance is needle shaped crystal and good soluble in methanol. In FT-

IR spectrum, N-H and O-H in phenolic group were found at 3333, 3256, 3079 cm^{-1}. C-H stretching vibration of methyl and methylene groups was found at 2964, 2901 cm^{-1}. C=O stretching vibration of ketone was found at 1679 cm^{-1}. C=C aromatic group was observed at 1601, 1516, 1446 cm^{-1}. N-H bending was found at 1563 cm-1. C-H bending of methyl and methylene groups was found at 1411,1342, 1244 cm^{-1}. The bands at 1107 cm^{-1} were attributed C-C-stretching vibration. In addition, C-H out of plane bending vibration was found at 972 cm^{-1}as shown in Figure 4.

According to its ^{1}H-NMR spectrum, aromatic protons (Ar-H) between 8.2-6.22 ppm, as doublet at 8.2 and 7.63 ppm and as singlet at 6.22 ppm. Olefinic protons (C=CH) between 5.15-4.13 ppm, as singlet at 5.15-4.83 ppm,as multiplet at 4.13 ppmand methylene protons(CH_2) between 3.8-3.3 ppm, as quantet at 3.8-3.6 ppm and as singlet at 3.3 ppm are present in this compound as shown in Figure 5.

As a result of ^{13}C-NMR spectral data, C=Oketone carbon was found at 167 ppm, 152 and 149 ppm contained aromatic carbons, olefinic carbons was found at 128, 124 and 71.8 ppm, 67.8 ppm was C-OH carbon, 62.7 ppm contained methylene carbon and methine carbon was observed at 59.0 ppm are present in this compound as shown in Figure 6.

According to 1D-NMR (^{1}H-NMR and ^{13}C-NMR), 2D-NMR (HMBC, HSQC, ^{1}H-^{1}H COSY) and FT-IR spectral data, the compound 1 was identified as 4-amino-1-(1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)-1H-1,2,3-triazol-5(4H)-one. Its molecular formula is $C_{11}H_{13}O_4N_5$ as shown in Figure 8.

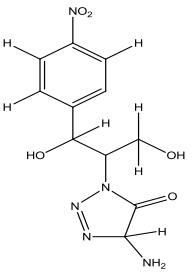
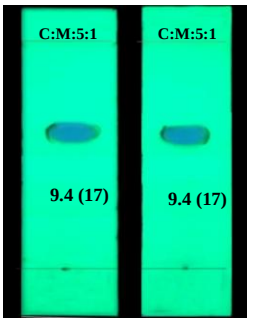


Figure 8. 4-amino-1-(1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)-1H-1,2,3-triazol-5(4H)-one

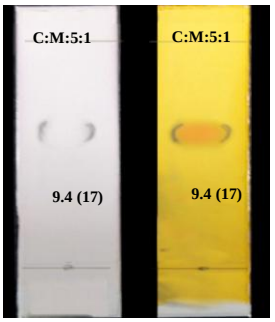
Identification of the isolated compound 2

Silica 29 gelcolumn (3 x10cm)

Silica 29 gelcolumn (2 x10 cm)



UV 254 nm



Staining colour(Anisaldehyde and Dragendroff)

Eluting solvent = CH_2Cl_2 :MeOH (9 :1 , 8 : 2, 7:3, 1:1)
= CH_2Cl_2 :EtOAc (3:1, 5:2)
Fraction size= 1.5 ml(3 x 10cm)
= 1 ml(2 x 10cm)
Flow rate = 2ml / min
R_f = 0.54 (CH_2Cl_2 :MeOH; 5:1)

Figure 9. Identification of the isolated compound 2 by R_f value

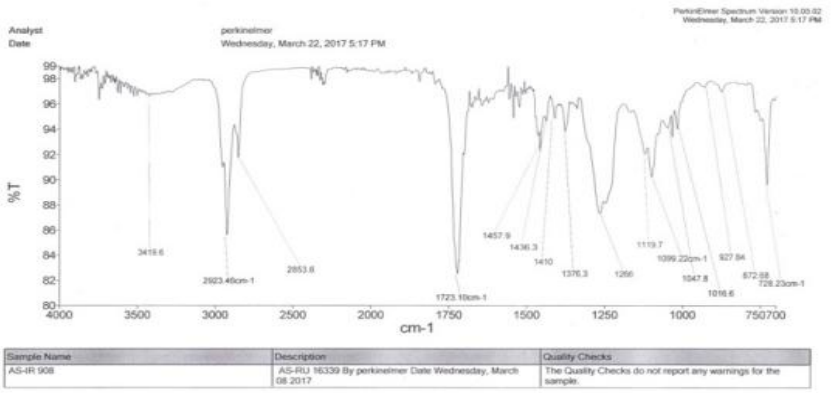


Figure 10. FT-IR spectrum of the isolated compound 2

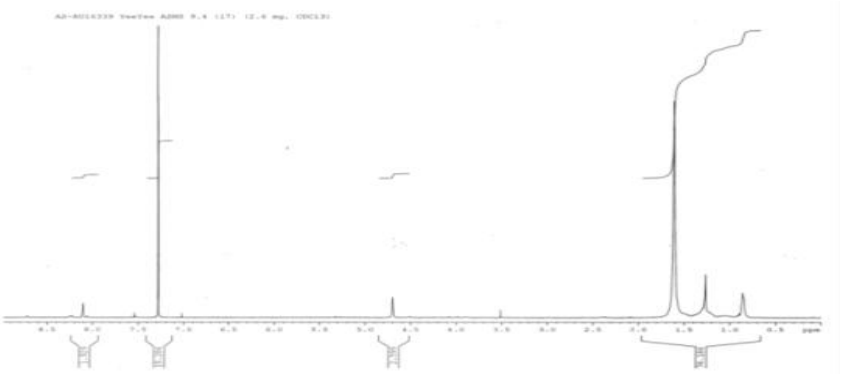


Figure 11. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the isolated compound 2

During the isolation of *Aspergillus* sp., an additional compound, the compound 2, was also isolated from the fraction 9 and has R_f0.54 (CH_2Cl_2 :MeOH,5:1). It gave an orange colour with dragendroffreagent (Figure 9). This substance is soluble in chloroform or dichloromethane.

According to the FT-IR spectral data, N-H stretching vibration group showed at 3419 cm^{-1}. C- H stretching of methyl and methylene groups was found at 2923 and 2853 cm^{-1}. C=O stretching vibration (Ketone) was observed at 1723 cm^{-1}. The bands at 1457, 1436 and 1410 cm^{-1} were attributed C-H bending of methyl and methylene groups. C-O-C- stretching

vibration of ether was found at 1376, 1266, 1099 and 1047 cm^{-1}. In addition, the bands for C-H out of plane bending vibration were observed at 927,872 and 728 cm^{-1}as shown in Figure 10.

According to its ^{1}H-NMR spectrum, aromatic protons (Ar-H) as singlet at 8.1 ppm, olefinic protons (C=CH) as singlet at 4.7 ppm and alkyl proton (CH_3) as singlet at 1.6-0.85 ppm are present in this compound as shown in Figure 11.

As a result of its ^{1}H-NMR spectrum and FT-IR spectral data, the compound 2 was identified as 3,6-dibenzyl-3,6-dimethylpiperazine-2,5-dione. Its molecular formula is $C_{20}H_{22}N_2O_2$ as shown in Figure 12.

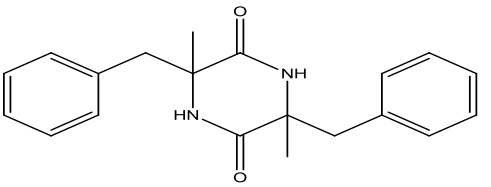
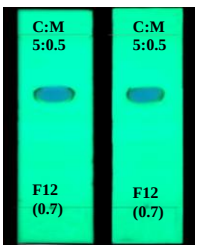


Figure 12. 3,6-dibenzyl-3,6-dimethylpiperazine-2,5-dione

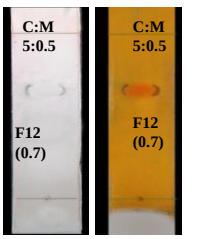
Identification of the isolated compound 3

Silica 29 gel Column(3x10 cm)

Silica 29 gelColumn (2 x10 cm)



UV 254 nm



Staining colour(Anisaldehyde and Dragendroff)

Eluting solvent	= CH_2Cl_2 :EtOAc (9:1, 8:2, 7:3, 6:2, 5:2, 4:2, 1:1) = CH_2Cl_2 :MeOH(100:1, 100:2, 100:3, 100:4, 100:5)
Fraction size	= 1.5 ml(3 x 10cm) = 1 ml(2 x 10cm)
Flow Rate	= 2ml / min
R_f	= 0.68 (CH_2Cl_2 :MeOH; 5:0.5)

Figure 13. Identification of the isolated compound 3 by R_f value

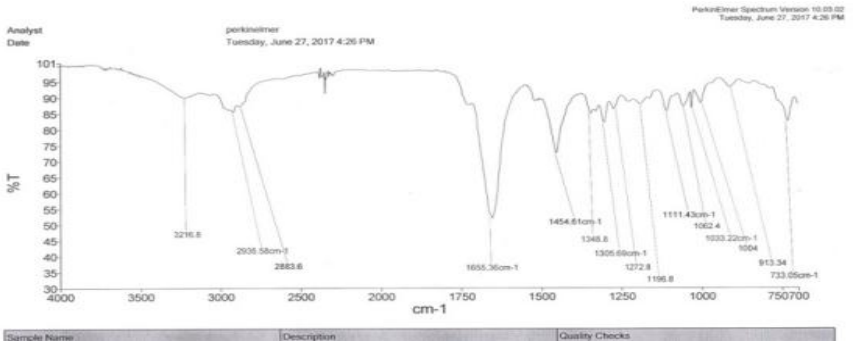


Figure 14. FT-IR spectrum of the isolated compound 3

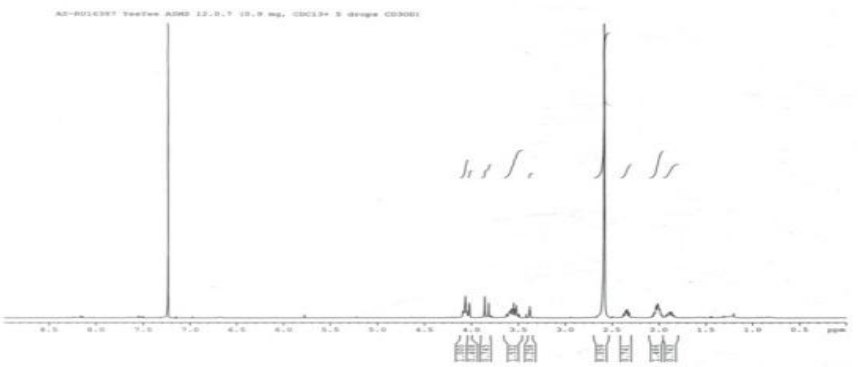


Figure 15. ^{1}H-NMR spectrum (400 MHz, CD_3OD) of the isolated compound 3

During the isolation of this strain, the compound 3 was isolated from the fraction 12 and has R_f0.68 (CH_2Cl_2 :MeOH,5:0.5). It showed orange colour with dragendroffreagent (Figure 13).In FT IR spectrum, N-H stretching vibration showed at 3216 cm^{-1}. C-H stretching of methyl andmethylene groups was found at 2935 and 2883 cm^{-1}. Its FT-IR spectrum showed C=O stretching vibration group at 1700 cm^{-1}. The bands at 1655 cm^{-1}were attributed C=N stretching vibration group. C=C aromatic stretching vibration was observed at 1454 cm^{-1}. Aromatic amines stretchingvibration showed at 1348 and 1305 cm^{-1}. C-O-C-stretching vibrationswere found at 1272 and 1196 cm^{-1}. In addition C-N bending vibration showed at 1111, 1062 and 1033 cm^{-1} as shown in Figure 14.

According to its ^{1}H-NMR spectrum, methine protons between 3.55-3.84 ppm, as multiplet at 3.55 ppm, as doublet at 3.84 ppm; allylic protons (C=C-CH2)as multiplet at 2.35 ppm and alkyl protons (CH3) 2.0-1.8 ppmas multiplet at 2.05 - 1.88 ppm are present in this compound as shown in Figure 15.

As a result of its ^{1}H-NMR spectrum and FT-IR spectral data, the compound 3 was identified as aspergillitine. Its molecular formula is $C_{15}H_{13}NO_2$as shown in Figure 16.

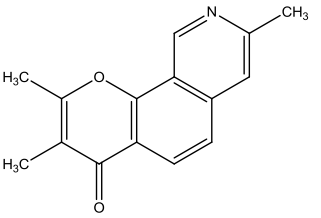


Figure 16. Aspergillitine

Antimicrobial Activity of the Isolated Compounds from*Aspergillus*sp.

Antimicrobial activity of the isolated compounds,the compounds 1;4-amino-1-(1,3-dihy-droxy-1-(4-nitrophenyl)propan-2-yl)-1H-1,2,3-triazol-5(4H)-oneshowed highest activity against *Bacillus subtilis*, *Escherichia coli* and*Xanthomonasoryzae*.The compounds 2;3,6-dibenzyl-3,6-dimethylpiperazine-2,5-dione; showed highest and high activities against *Escherichia coli* and s*Xanthomonasoryzae*. The compound 3 did not show antibacterial activity as shown in Table 2.

Table 2. Antibacterial activity of the isolated compounds

Test organisms	Compound		
	C-1	C-2	C-3
Bacillus subtilis	30	-	-
Escherichia coli	27	21	-
Xanthomonasoryzae	37	16	-

10 -12 mm = weak activity, 13 -17 mm = high activity, >18 mm = very highactivity, disc size = 6 mm

Discussion and Conclusion

Endophytic fungal strain *Aspergillus* sp. was isolated from the rhizome of *Zingibercassumunar*Roxb. in this research work. For the extraction of the bioactive compounds, 10L fermentation of*Aspergillus*sp. and antimicrobial activity of fermented broth with six test organisms were carried out. After testing the antimicrobial test, the active filtrate of fermented broth was applied on XAD 16 resin column followed bymethanol. Shaaban*et al*. (2013) also used XAD-16 resin toextract the filtrate with methanol.

The methanol extract were tested antimicrobial activity by six test organisms in this study.According to the result, methanol extract showed good result on six test organisms. In isolation and purification of the bioactive compounds from *Aspergillus*sp., the methanol extract was utilized on silica gel columns, 34 gel, 29 gel, SephadexLH 20 gel columnswith various solvent systems.

The compound 1 was identified as 4-amino-1-(1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)-1H-1,2,3-triazol-5(4H)-one and showed highest activity against *Batcillussubtilis*, *Escherichia coli* and *Xanthomonasoryzae*. This compound was also isolated from *Aspergillusterreus*by Riznа*etal*. (2015) and they also reported that it has antioxidant and antibacterial activities. The compound 2 was identified as 3,6-dibenzyl-3,6-dimethylpiperazine-2,5-dione compound. This compound 2 showed antibaterial activity on*Escherichia coli* and *Xanthomonasoryzae*. It was also isolated from *Aspergillusterreus* by Wen Gu and Chao (2012). They also reported that this compound has antibacterial activity on*Escherichia coli*.The compound 3 was identified as aspergillitine. This compoundwas also isolated from the *Aspergillusversicolor*by Vijaya (2017).

Manila*et al*. (2014) reported that alkaloids, phenolic and terpenoid compounds were the main phytochemicals presented in endophytes including *Aspergillus*sp. Furthermore they also stated that strains of various

*Aspergillus*sp. could exhibit the highest antioxidant activity ranging from 50% to 80%.In this study the isolated compound 1; 4-amino-1-(1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)-1H-1,2,3-triazol-5(4H)-one; and the compound2; 3,6-dibenzyl-3,6-dimethylpiperazine-2,5-dione showed antibacterial activity on *Bacillus subtilis*and*Xanthomonasoryzae.* This finding is in agreement with the statement of Hameed*et al*. (2015) who stated that *Aspergillusniger* produced many important secondary metabolites with high biological activities.

Moreover Hameed*et al*. (2015) also reported that drugs for the treatment of many diseases could produce based on the significance of employing bioactive compounds in pharmacy, the purification of compounds produced by *Aspergillusniger*can be useful. In this study the isolated compounds;4-amino-1-(1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)-1H-1,2, 3-triazol-5(4H)-one;showed antibacterial activity on *Bacillus subtilis*.This finding is in agreement with the statement of Olivia *et al.* (2015).

It could be concluded that the bioactive compounds were isolated from fermented broth of *Aspergillus* sp. produced from the rhizome of *Zingibercassumunar*Roxb. in this research. These active compounds indicated high activity on *Bacillus subtilis*, *Escherichia coli* and *Xanthomonasoryzae*. Therefore, these compounds could be applied to treat diseases caused by *Bacillus subtilis,Escherichia coli* and *Xanthomonasoryzae*.These findings could help the health of mankind and also help to suppress the *Xanthomonas* caused leaf blight disease in the paddy fields.

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