Antimicrobial Compounds of *aspergillus* sp. Isolated from *zingiber cassumunar* Roxb.

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Abstract

An Endophytic fungal strain *Aspergillus* sp. was isolated from the rhizome of *Zingiber cassumunar* Roxb. 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 at Department of Botany, University of Yangon. The filtrate was extracted with methanol on Ambilite XAD 16 resin column. The methanol extract showed high activity against *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*,*Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus.* Isolation and purification of the bioactive compounds from the methanol extract were carried out by utilizing 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, 1-D NMR (^{1}H-NMR, ^{13}C-NMR) and 2-D NMR (HSQC, HMBC and COSY) spectra. The three compounds A, B and C were identified as mephenamic acid or 2-(2,3-dimethylphenyl) amino) benzoic acid;eremophilanolide derivative and aspergillitine derivative. Antimicrobial activity of all isolated compounds was evaluated on six test organisms and showed high activity against*Bacillus subtilis*, *Escherichia coli* and *Xanthomonasoryzae.*

Keywords: *Aspergillus*sp., aspergillitine,eremophilanolide, mephenamic acid and*Z.cassumunar*Roxb.

Introduction

Endophytic fungi have been known as the source of secondary metabolites and some of which exhibited biological activities (Cui *et al*., 2011). Most of endophytes are capable of producing active metabolites and some of these compounds are proven to have medical values (Santiago *et al*., 2012). These are the latent sources of pharmaceutical leads and create novel bioactive metabolites such as antimicrobial, anticancer, and antiviral agents (Qin *et al*., 2009). Various types of the compounds produced by endophytes include terpenoids, alkaloids, phenylpropanoids, aliphatic compounds, and peptides that have been reported to have antimicrobial activities and many compounds showed bioactivities against microbial diseases and various types of cancer cell lines (Wu *et al*., 2012).

The objectives of this research work are to isolate and purify the isolated compounds from 10L fermentation of *Aspergillus* sp., to elucidate the molecular structures of the isolated compounds and to evaluate the antimicrobial activity of the isolated compounds.

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Materials and Methods

Isolation of endophytic fungus from *Zingiber cassumunar* Roxb.

The plant *Zingiber cassumunar* Roxb.sample was collected from Nyaung-Hna-Pin area, HmawbiTownship. The isolation of endophytic fungus was carried out with the following scheme: (1) The rhizomes were washed in running tap water for 15 min. **(**2) They were cut into about 1 cm pieces. **(**3) The surfaces of cut-rhizome 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-rhizome 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. Then, the isolated fungus was transferred into a 10ml test tube containing 5 ml of sucrose/yeast extract medium (Lee *et al*., 1996).

Antimicrobial activity of ten liters 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 160 ml of sucrose/yeast extract seed medium. The flask was incubated for two days for seed culture. Two days old seed culture (15 ml) was transferred into 2L conical flask containing 1L of sucrose/yeast fermentation medium. For ten liters fermentation, ten conical flasks were used andthese flasks were incubated at 100 rpm for 3-7 days. All the flasks were tested for antimicrobial activity on*Bacillus subtilis*, *Candida albicans,Escherichia coli*, *Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus* (Strobel and Sullivan,1999; Phay, 1997).

Extraction of the bioactive compounds from fermented broth of *Aspergillus*sp.

After testing antimicrobial activity, 10L fermented broth was filtered with filter paper. The mycelia were extracted with acetone by using filter paper while the filtrate was applied on an Amberlites XAD 16 resin column. The column was washed with water, followed bymethanol. The methanol extract was evaporated on water bath at 55°C. The extract was tested for antimicrobial activity against *B. subtilis,C. albicans*, *E. coli*, *M. furfur*, *S. typhi* and *S. aureus* (Strobel and Sullivan, 1999).

Isolation and purification of the 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). The column size was 5×10 cm and flow rate was 2 ml per minute(Grableyet *al.*,1999).

Identification of the isolated compounds from *Aspergillus*sp.

The identification of the isolated compounds was characterized by 1D-NMR (^{1}H-NMR and ^{13}C-NMR), 2D-NMR (HMBC, HSQC, ^{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 data of the compounds were compared to ACD Labs (Advanced Chemistry Development)(Robert and Francis, 2014).

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

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

Results

Antimicrobial activity of the methanol extract

The methanol extract showed highest activity against six test organisms as shown in Table 1.

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

Extract	*B.subtilis*	*C.albicans*	*E.coli*	*M.furfur*	*S.typhi*	*S.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 the bioactive compounds from *Aspergillus* sp.

The two hundred and eight small fractions were collected from silica gel 34 column. According to their R_f value and colour on 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 them, the fraction 2 was amorphous powder after washing with methanol. Then, this fraction was purified.

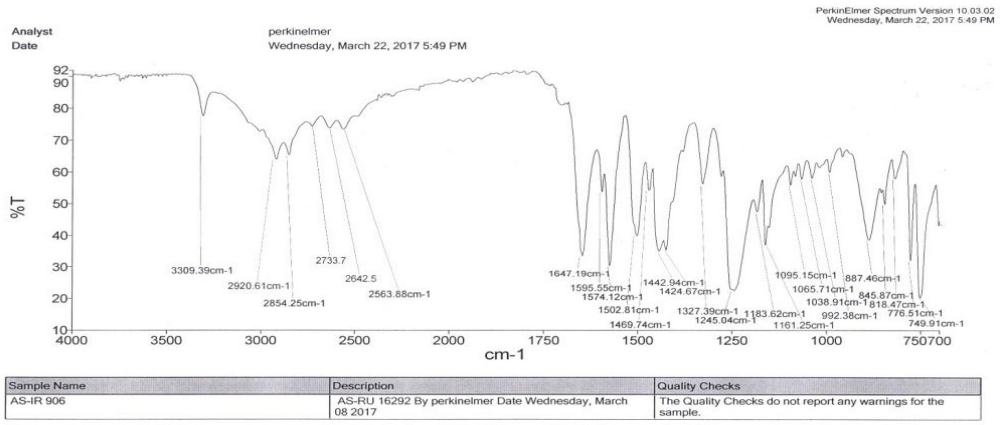

Figure1.FT-IR spectrum of the isolated compound A

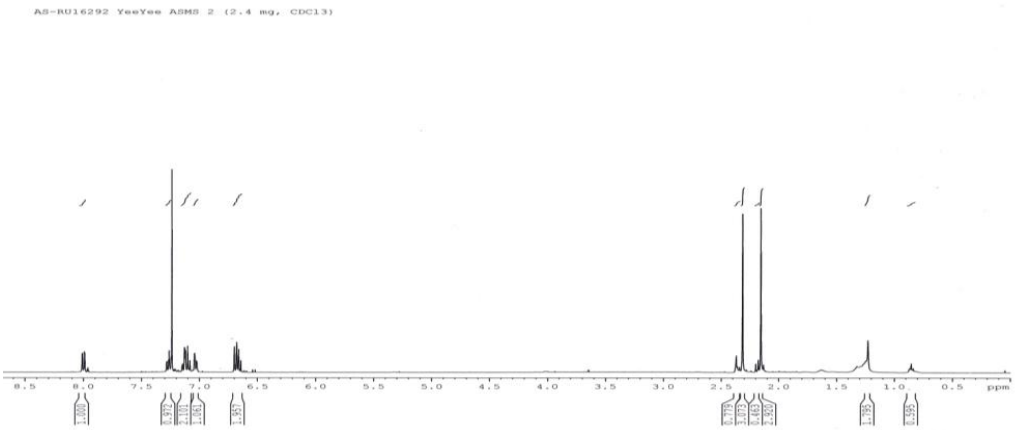

Figure 2.^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the isolated compound A

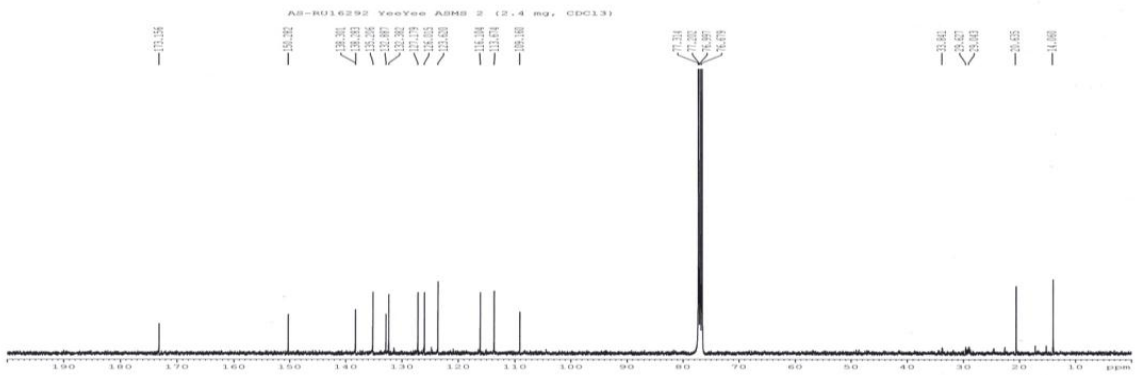

Figure 3.^{13}C-NMRspectrum (400 MHz, CD_3OD) of the isolated compound A

Identification of the isolated compound A

The compound A was isolated from the fraction 2 as a dark substance under UV absorbing band at 254 nm. It has R_f0.7 (CH_2Cl_2: MeOH; 5:0.2). It gave a dark pink colour with anisaldehyde reagent fast. Then, the colour changed into dark blue. This substance is amorphous powder and good soluble in chloroform or dichloromethane.

In the FT-IR spectrum of the isolated compound A,O-H in phenolic group showed at 3309 cm^{-1}. C-H stretching of methyl and methylene groups was found at 2920 and 2854 cm^{-1}. C-OOH (acid OH group) was observed at 2733, 2642 and 2563 cm^{-1} while C=C olefinic group was found at 1647 cm^{-1}. The bands at 1595, 1574, 1502 and 1469 cm^{-1} were showed C=C aromatic group. C-H bending of methyl and methylene groups showed at 1442 and 1327 cm^{-1}. In addition, the bands for aromatic ether were observed at 1245, 1161, 1095 and 1065 cm^{-1} while C-H bending of aromatic ring was found at 992 and 887 cm^{-1} as shown in Figure 1.

According to its ^{1}H-NMR spectrum, aromatic protons (Ar-H) between 8-6.7ppm, as doublet at 8.0, 7.13, 7.10 and 7.04 ppm, as doublet of doublets at 6.67 ppm and as triplets at 7.23 ppm. Allylic protons ($C=CH_2$) between 2-3 ppm, as singlet at 2.32 - 2.16 ppm and alkyl protons (CH_3) as singlet at 1.25 ppmare present in this compound as seen in Figure 2.

As a result of ^{13}C-NMR spectral data,173 ppm contained C=O carbons, 150 ppm was aromatic carbons, C=C oilefinic carbons were found at 138.30, 135.20, 132.88, 132.38, 127.17, 126.01, 123.62, 116.10, 113.67 and 109.16 ppm; 33.84 ppm was methane carbon and methyl carbons were observed at 20.63 and 14.06 ppm as seen in Figure 3.

According to its 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 A was identified as mephenamic acid or 2-(2-3 dimethylphenyl)amino benzoic acid. Its molecular formula is $C_{15}H_{15}NO_2$ as shown in Figure 4.

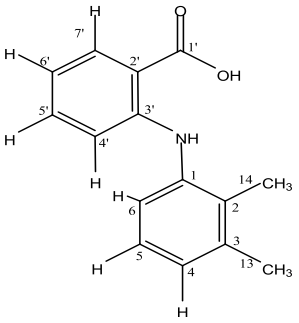

Figure 4. Mephenamic acid or 2-(2,3-dimethylphenyl)amino)benzoic acid

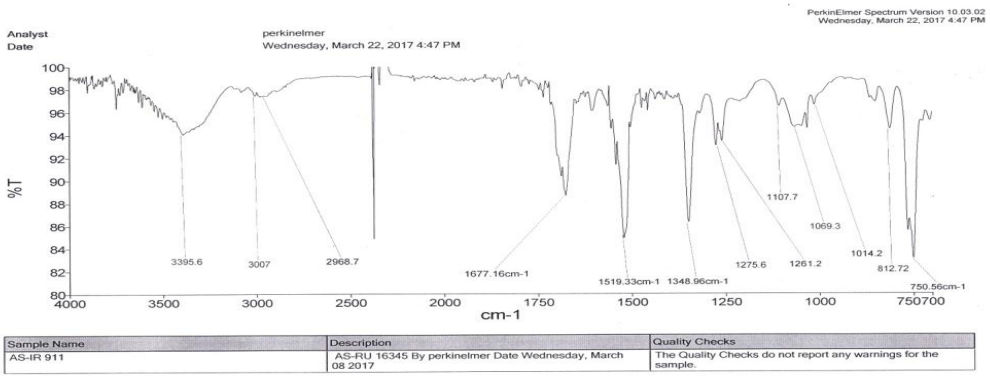

Figure 5.FT-IR spectrum of the isolated compound B

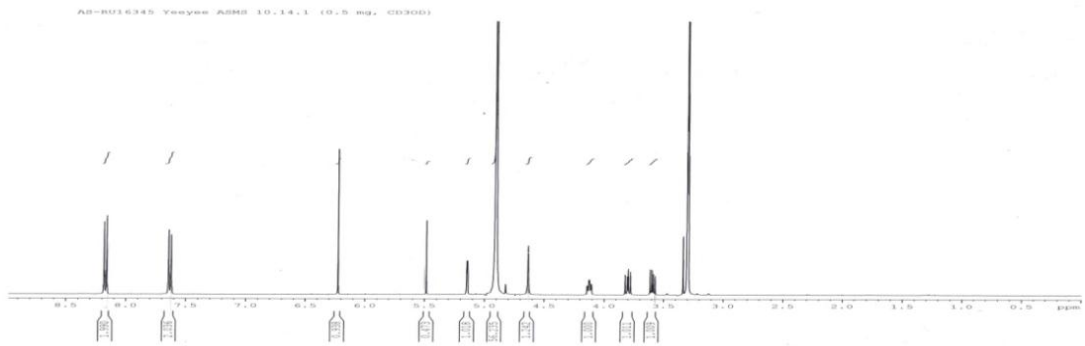

Figure 6.^{1}H-NMR spectrum (400 MHz, CD_3OD) of the isolated compound B

Identification of the isolated compound B

During the isolation of this strain, the compound B was isolated from the fraction 10 and has R_f 0.5 (CH_2Cl_2:MeOH; 5:1). It showed white colour with anisaldehyde reagent. This substance is good soluble in methanol.

According to FT-IR spectrum, O-H in phenolic group showed at 3395 cm^{-1}. The bands at 3007 cm^{-1} were attributed to C=C-H stretching vibration. C-H stretching of methyl and methylene groups was found at 2968 cm^{-1}. Its FT-IR spectrum showed C=O stretching vibration at 1667 cm^{-1}. C=C aromatic groups were found at 1519, 1348, 1275 and 1261 cm^{-1}. In addition, the bands at 1014, 1069 and 1107 cm^{-1} were attributed C-O-C- stretching vibration of ether as shown in Figure 5.

According to its ^{1}H-NMR spectrum, aromatic protons (Ar-H) between 8.15 - 7.63 ppm, as doublet at 8.15-7.63 ppm, as singlet at 6.23 ppm; olefinic protons (C=CH) between 5.5-4.6 ppm, as singlet at 5.15-4.62 ppm; methylene protons

between 4.1-3.6 ppm, as multipletat 3.6-3.8 ppm and as singlet at 4.12 ppm are present in this compound as shown in Figure 6.

As a result of its ^{1}H-NMR spectrum and FT-IR spectral data, the compound B was identified as an eremophilanolide derivative. Its molecular formula is $C_{12}H_{15}O_4$asshown in Figure 7.

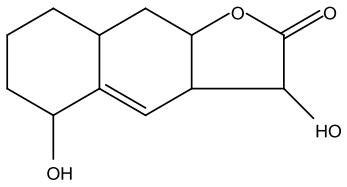

Figure 7.Eremophilanolide derivative

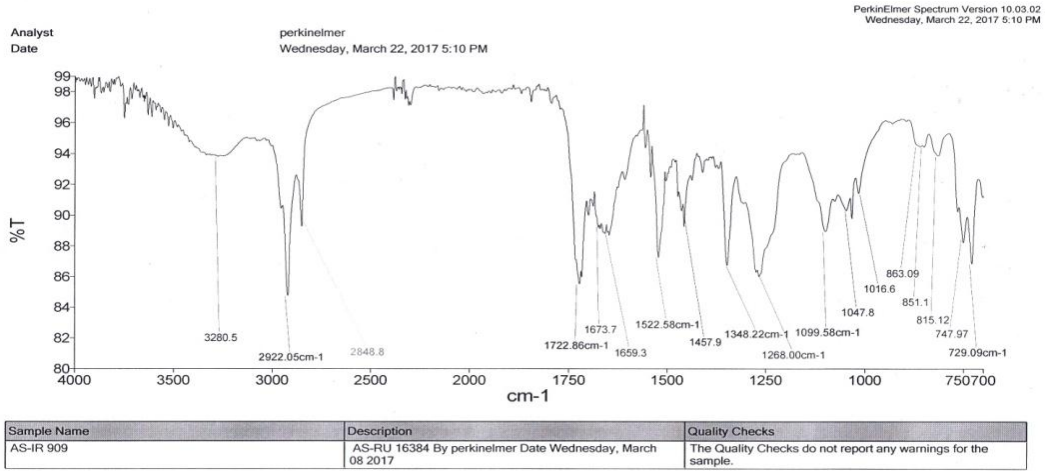

Figure 8.FT-IR spectrum of the isolated compound C

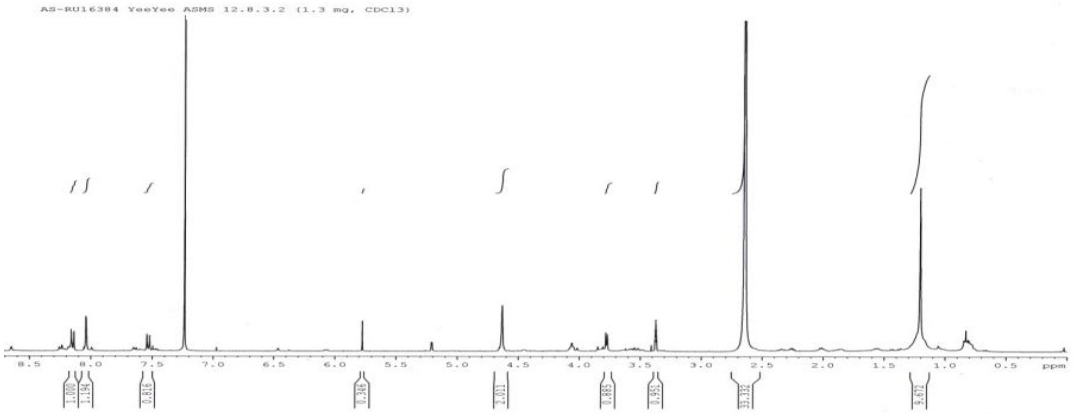

Figure 9. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the isolated compound C

Identification of the isolated compound C

During the isolation of this strain, the compound C was isolated from the fraction 12 and has R_f0.27 (CH_2Cl_2:MeOH; 5:0.3). It showed orange colour with dragendroff reagent. In FT-IR spectrum, O-H in phenolic group showed at 3280 cm^{-1}. C-H stretching of methyl and methylene groups was found at 2922 and 2848 cm^{-1}. The band at 1722 cm^{-1} was attributed at C=O stretching vibration. Aromatic vibrationwas found at 1673 and 1659 cm^{-1}. Its IR spectrum showed C=C aromatic vibration at 1522, 1457 and 1348 cm^{-1}. C-O-C-stretching vibration of ether was observed at 1268, 1099

and 1074 cm^{-1}. In addition, the band for C–N bending vibration was found at 1016 cm^{-1} shown in Figure 8.

According to its ^{1}H-NMR spectrum, aromatic protons (Ar-H) between 8.2 pm - 7.5 ppm as doublet at 7.53 - 8.05 ppm and as singlet at 8.15 ppm, olefinicprotons (C=CH) between 5.8 ppm - 4.6 ppmas singlet at 4.65 ppm, methylene protons between 3.8 ppm - 3.5 ppm as singlet at 3.35 ppm and as doublet at 3.8 ppm, alkyl proton (CH_3) as singlet at 1.2 ppm are present in this compound as shown in Figure 9. As a result of its ^{1}H-NMR spectrum and FT-IR spectral data, the compound C was identified as an aspergillitine derivative. Its molecular formula is $C_{15}H_{16}NO_5$ as shown in Figure 10.

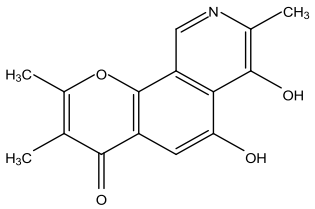

Figure 10.Aspergillitine derivative

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

Among all isolated compounds,the compound A,mephenamic acid, inhibited high activity against *Bacillus subtilis, Escherichia coli* but weak activity on *Xanthomonasoryzae*.The compounds B:eremophilanolide derivative showed very high activity against *Bacillus subtilis*, *Escherichia coli* and*Xanthomonasoryzae*while the compound C: aspergillitinederivative showed high activity against *Bacillus subtilis*, *Escherichia coli* and*Xanthomonasoryzae*as shown in Table 2.

Table 2.Antibacterial activity of the isolated compounds (mm)

Compound	*Bacillus subtilis*	*Escherichia coli*	*Xanthomonasoryzae*
A	17	16	13
B	25	24	31
C	17	15	18

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

DISCUSSION AND CONCLUSION

In this research work, an endophytic fungal strain *Aspergillus* sp. was isolated from the rhizome of *Zingiber cassumunar* Roxb.For extraction of the bioactive compounds, 10L fermentation of strain *Aspergillus* sp. on XAD-16 resin column was eluted with methanol. In isolation and purification of the bioactive compounds, the methanol extract was utilized on silica gel 34 gel and 29 gel columns andSephadex LH 20 gel columns with various solvent systems. Shaaban*et al*. (2013) also used XAD-16 resin to extract the active compounds from the filtrate and followed by methanol.

The compound A was a mephenamic acid and it showed high activity against on *Batcillus subtilis*, *Escherichia coli* but weak activity on *Xanthomonas oryzae*. This compound was also isolated from *Aspergillus*sp.of *Bauhinia guianensis* L.byFaculdade *et al.,* (2013). They also reported that this compound has antibacterial activity against *B.subtilis* and *E. coli.*

The compound B was an eremophilanolide derivative and it showed very high activity against *Bacillus subtilis*, *Escherichia coli* and *Xanthomonas oryzae*. This compound was also isolated from endophytic fungiof medicinal plants by Sanjana*et al.,* (2012). They also reported that this compound showed antibacterial activity.

The compound C was identified as an aspergillitinederivative and itshowed antibacterial activity on *Bacillus subtilis*, *Escherichia coli* and*Xanthomonas oryzae.* This compound was also isolated from the *Aspergillus versicolor* 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. exhibited the highest antioxidant activity.

In this study, the isolated compounds:mephenamic acid,eremophilanolide derivative and aspergillitinederivative showed antibacterial activity on *Bacillus subtilis Escherichia coli* and *Xanthomonas oryzae.*These findings are in agreement with the statements of Hameed *et al*. (2015) who stated that *Aspergillusniger*produced many important secondary metabolites with high biological activities. Moreover they also reported that the active compounds produced by *Aspergillus niger* can be useful in pharmacy. Antibacterial activity on *B. subtilis* and *E. coli* of all isolated compounds is in agreement with the statement of Olivia *et al.,* (2015).

It could be concluded that the bioactive compounds were produced from fermented broth of *Aspergillus* sp. isolated from the rhizome of *Zingiber cassumunar* Roxb. in this research. These active compounds indicated high activity on *Bacillus subtilis*, *Escherichia coli* and *Xanthomonas oryzae*.Therefore, these compounds could be applied to treat some diseases caused by *Bacillus subtilis*, *Escherichia coli* and *Xanthomonas oryzae*. These findings could help the health of mankind by producing antibacterial drugs to treat diseases causing *B.subtilis*and *E. coli*, moreover, it also help to control leaf blight bacteria caused by *Xanthomonas* in the paddy fields.

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