Bioactive Compounds Produced by Soil Bacterium *Bacillus* sp. against *Agrobacterium Tumefaciens*

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Abstract

In this research, the effective microorganism, selected soil bacterium *Bacillus* sp., was carried out by paper chromatography using the solvent 20% NH_4Cl, n-butanol saturated with water, n-butanol-acetic acid-water (3:1:1), ethyl acetate saturated with water for the extraction of antibacterial against *Agrobacterium tumefaciens.* And then, the bacterial culture filtrate acetate to fermented broth (1:1, 2:1, 3:1 v/v). The equal ratio(1:1v/v) n-butanol extract showed higher inhibitory effect (24.23 mm) than ethyl acetate extract (21.35 mm) and n-butanol layer was tested and adjusted at pH-7. Crude n-butanol extract 7.6 g was obtained from 15 liters of fermented broth and subjected to purification over silica gel column chromatography with various solvent systems. The major active ingredients from the culture filtrate were purified by silica gel column chromatography and identified to be linoleic acid (compound A) and α-tocopheryl quinine (compound B) by UV, FT-IR, NMR and mass spectral (MS) data. The minimum inhibitory concentration (MIC) value of compound A was 4.375 µg/L and compound B was 2.187 µg/mL on *Agrobacterium tumefaciens*. This research will provide the knowledge of antibacterial compounds derived from *Bacillus* sp. including their structure, classification, extraction and purification methods used to obtain these bioactive compounds.

Key words: Paper chromatography, silica gel column chromatography, minimum inhibitory concentration

INTRODUCTION

The biological diversity of microorganisms is mainly due to the diversity not only in their morphology but also in their role. There exists an antagonistic effect between each and every organisms, because of their tendency to produce biologically active compounds. Bioactive compounds are compounds that are produced by any living organisms and are known to exhibit as various biological activities both *in-vitro* and *in-vivo.* Bioactivity may be antimicrobial, antineoplastic, anticancerous, immunomodulation, antifertility and other (Loganathan *et al.,* 2014).

The recovery and purification of the product are one of the most critical aspects of industrial fermentation process. The type of extraction method, duration of extraction, temperature, and the polarity of solvent are used to influent the quality and the concentration of bioactive components isolated from the raw material (Annegowda *et al.,* 2013).

Based on the physical and chemical properties of compounds and their affinities for certain solid phase material (e.g., silica), a mixture can be separated into its individual compounds, or at least into mixtures containing fewer compounds with similar characteristics by selecting the appropriate elution solvent or solvent systems (Harris, 2003). The most common methods of detection for early stages are ultraviolet-visible spectroscopy (UV/Vis)

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that provides information on chromophores present in a compound and FT-IR provides information functional group present in a compound (Henke and Kelleher, 2016).

Early identification of activities is highly dependent upon paper chromatography or thin-layered chromatographic. Antibacterial compounds can be identified by Nuclear Magnetic Resonance (NMR) Spectroscopy and Mass Spectrometry (MS), the latter being after used for the determination of Molecular mass (Adeboye *et al.,* 2008).

Minimum inhibitory concentrations (MICs) are considered the "gold standard" for determining the susceptibility for organisms to antimicrobials and used to judge the performance of all other methods of suscepticlity testing (Andrews, 2001).

It is a well-known crude extract isolated from the soil bacteria metabolite contain complex chemical diversity which is difficult to identify and characterize. The present study was initiated to purify and characterize the antibacterial metabolite of selected bacterium *Bacillus* sp.

MATERIALS AND METHODS

Study on the paper chromatography (Tomita, 1988)

Paper chromatography was studied to know the suitable solvent system for the extraction of antibacterial compound from fermented broth. The fermented broth was filtered and dropped at the starting point of four filter paper strips (9 cm × 2 cm). After drying, each of four filter papers was chromatographed in each solvent such as ethyl acetate, n-butanol saturated with water, 20% ammonium chloride and n-butanol-acetic acid-water (3:1:1). When the solvent reached the end point, the filter papers were removed and allowed to dry. These papers were then placed on assay plate inoculated with test bacteria. After one hour, the papers were taken out and the plate was incubated at room temperature for 24-36 hours. Then, the distance from starting point to the centre of clear zone was measured and R_f values were calculated.

Preliminary extraction of antibacterial compounds (Saxena *et al.,* 2007)

The selected bacterium (72 hrs age and 30% size of inoculums) was inoculated in 500 mL conical flask containing 200 mL of fermentation medium. This flask was incubated at 25ºC for 3 days. Then the fermented broth was filtered. After that the filtrate was mixed with equal volume of n-butanol in a separating funnel. The organic layer was separated and collected.

Effect of pH on extraction with n-butanol (Vasconcelos *et al.,* 2015)

The collected organic layer (n-butanol layer) was tested and adjusted at pH 4, 5, 6, 7, 8, 9, 10 with desired 0.1 M NaOH or 0.1 M HCl. Then each adjusted pH sample was tested by using agar well diffusion assay.

Thin layer chromatography analysis (Verma *et al.,* 2014)

Thin layer chromatogramphy was developed by the methods of Touchstone, 1992. The obtained n-butanol extracted samples (20 µL) were applied on the TLC plate [60 F_{254}

silica gel precoated aluminium plate (Merck)] and chromatography was performed by employing solvent system chloroform-methanol (19:1, 9:1 and 4:1 v/v). Spots were visualized by spraying with sulphuric acid. The $R_f$values of the separated spots were determined subsequently.

Silica gel column chromatography (Simon and Gray, 1988)

According to the thin layer chromatography analysis, n-butanol extracted residue of isolated bacterium MMT-3 (*Bacillus* sp) metabolite was developed by silica gel column chromatography with chloroform to isolate the active compound: methanol as eluting solvent. The silica gel (ca-80 g) was dissolved in chloroform and the column was packed by the wet method. The n-butanol crude extract (4 g) was then passed through silica gel column and eluted with chloroform : methanol solvent (19:1, 9:1 and 4:1 v/v). Fractions of each equal to 2 mL were collected individually and the present compounds were checked with TLC.

Characterization of isolated antibacterial compounds

The isolated antibacterial compounds were characterized by the following tests.

Determination of R_f value

The isolated compounds were subjected to TLC analysis and their R_f values were determined. GF_{254}silic gel precoated aluminum plate (Merck) was employed and the chromatogram was developed in the appropriate solvent system. After the TLC plate was dried, the R_f values of isolated compounds were measured. Localization of spot was made by viewing directly under UV254 nm and 365 nm light or by treating with visualizing agent such as H_2SO_4.

Determination of solubility of isolated compounds

Each of isolated compounds (0.5 mg) was subjected to 0.5 mL of polar and non-polar solvents such as H_2O, MeOH, EtOAc, $CHCl_3$, PE and Hexane in order to know their solubilities.

Determination of some chemical properties of isolated compounds

Some coloured reagents such as 10% $KMnO_4$, I_2vapour, Anisaldehyde, 5% H_2SO_4, 5% $FeCl_3$, Lieberman Burchard, and 2, 4 Dinitrophenylhydrazine (DNP) were used to study their behaviour on TLC.

Study on UV-visible spectroscopy

For the identification of isolated compounds ultra violet absorption spectra were also recorded and examined by using a Shinmadzu UV-1800 UV-visible spectrophoto-meter at Chemistry Department, Pathein University.

Study under FT IR spectroscopy

The FT IR spectra of isolated compounds were recorded by using Perkin-Elmer Spectrum Two, Fourier TransformInfrard spectrophotometer at Chemistry Department, Pathein University.

NMR spectroscopy and GC-MS analysis

For the identification of isolated compounds, Nuclear Magnetic Resonance (NMR) spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS) analysis were recorded at Food Industry Research and Development Institute Bioresource Collection and Research Centre, Taiwan.

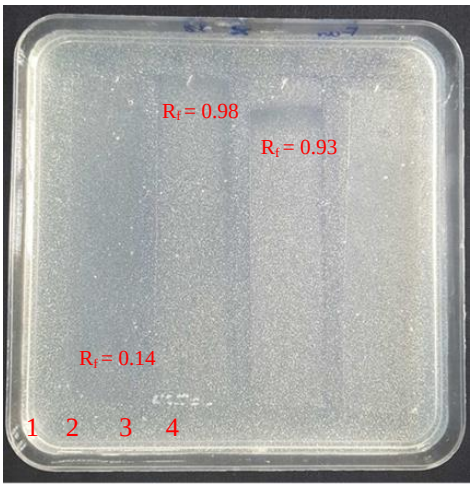
Minimum inhibitory concentration (MIC) of isolated compounds

The minimum inhibitory concentration (MIC) of metabolite was carried out by the method of two-fold serial dilution broth medium (Andrew, 2001). The concentration were 70 µg/mL, 35 µg/mL, 17.5 µg/mL, 8.75 µg/mL, 4.375 µg/mL, 2.187 µg/mL, 1.093 µg/mL, 0.546 µg/mL and 0.273 µg/mL respectively. The method of two-fold dilution on concentration 35 µg/mL to 0.273 µg/mL were prepared. Assay agar plate with antibacterial metabolite was incubated for 24 hrs at 27ºC. The MIC was determined by selecting the lowest concentration of metabolite which caused complete inhibition of test growth.

RESULTS

Paper chromatography

In this study, four kinds of 20% NH_4Cl, ethyl acetate saturated with water, n-butanol saturated with water, n-butanol-acetic acid-water (3:1:1) were used. According to the R_f value (0.98), n-butanol was more extractable antibacterial metabolite than other solvent, followed by n-butanol-acetic acid-water 3:1:1 (0.93) and 20% ammonium chloride NH_4Cl (0.14) but no activity of ethyl acetate(Figure 1).



1. 20% ammonium chloride
2. n-butanol saturated with water
3. n-butanol-acetic acid-water (3:1:1)
4. ethyl acetate saturated with water

Fig. 1. Paper chromatography bioautographic assay

Comparison of antibacterial activity of metabolite MMT-3 (*Bacillus* sp) extracted from different volume of EtOAc and n-BuOH extract against *Agrobacterium tumefaciens*

The n-butanol extract (1:1) of MMT-3 displayed higher inhibition zone (24.23 mm) than ethyl acetate extract (1:1) (21.35 mm). Therefore, antibacterial metabolite was extracted from equal volume of n-butanol extract (1:1) as solvent.

Extraction of antibacterial metabolite

Based on the results of paper chromatography (PPC), comparison with different ratio of EtOAc and n-BuOH, fermentation was studied. 15 liters of selected bacterium MMT-3 were fermented in suitable synthetic fermentation medium (temperature 25ºC, pH 7, 30% size of inoculums, 3 days fermentation period, under shaking culture) and extracted from equal ratio of n-BuOH for a period of four months to yield 7.6 g. Preparation of n-BuOH extract from fermented broth of selected bacterium MMT-3 was described in Figure 2.

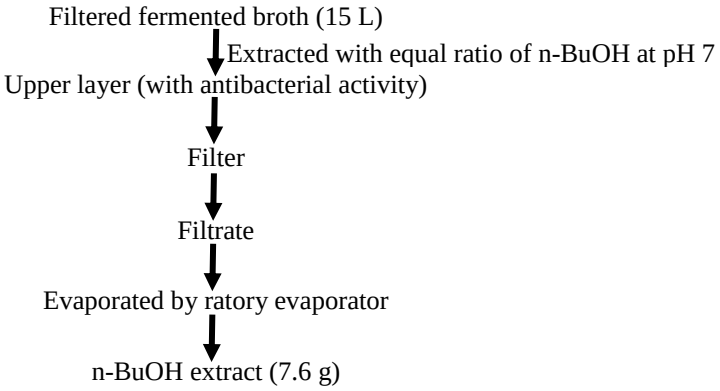


Fig. 2. Flow diagram for preparation of n-BuOH extract from fermented broth of isolated bacterium MMT-3 (*Bacillus* sp.)

Effect of pH on extraction of n-butanol

The collected upper organic layer (n-butanol layer) were tested and adjusted at pH 4, 5, 6, 7, 8, 9, 10. The minimum inhibitory zone was found at pH 5 (17.19 mm) while maximum inhibitory zone occurred in pH 7 (24.85 mm), followed by pH 6, 8 and 9 (21.31 mm, 22.16 mm, 17.46 mm) respectively. The negative results were found at pH 4 and 10. As shown in Table 1 and Figure 3.

Table 1.The bacterial activity on extracted from pH of MMT-3 (*Bacillus* sp)

pH values	Inhibitory zone (mm)
4	-
5	17.19
6	21.31
7	**24.85**
8	22.16
9	17.46
10	-

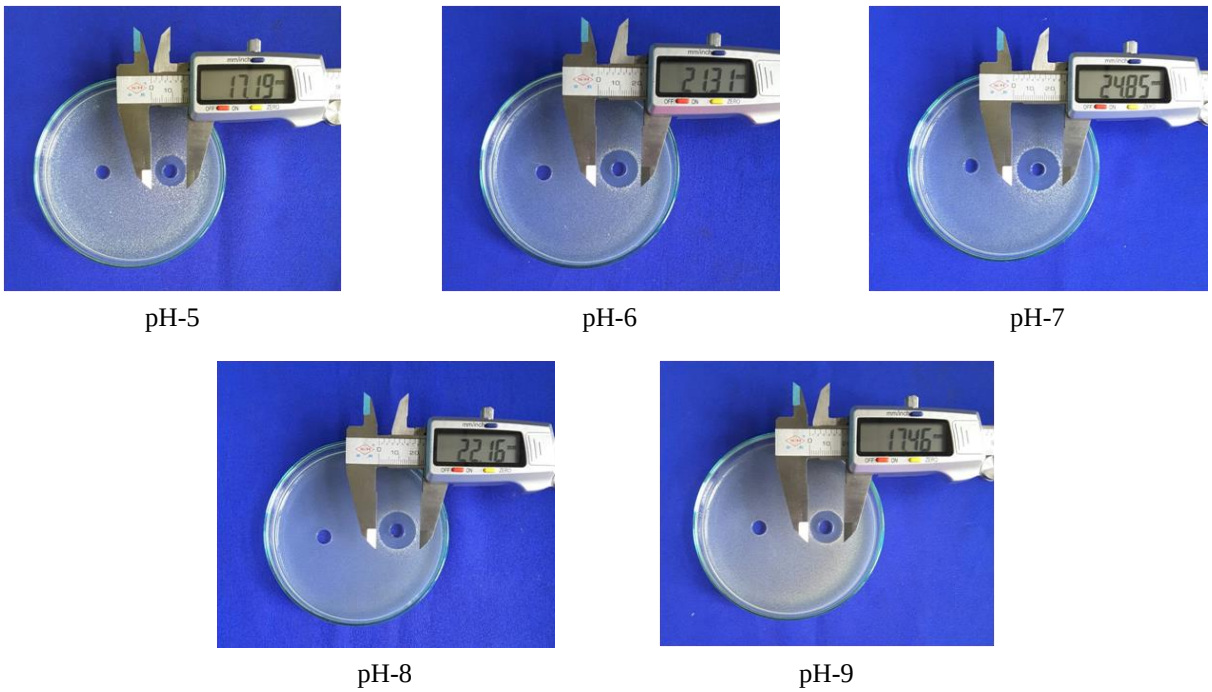


Fig. 3. The antibacterial activity on extracted pH of MMT-3 (*Bacillus* sp.)

Thin layer chromatography

Thin layer chromatography (TLC) was performed on n-butanol crude extract by employing chloroform: methanol solvent system (19:1 v/v, 9:1 v/v and 4:1 v/v). The extract showed well-separated spots on TLC by using chloroform: methanol solvent system under 365 nm UV and 254 nm UV. Therefore, these solvent systems were chosen to isolate pure compounds by silica gel column chromatography.

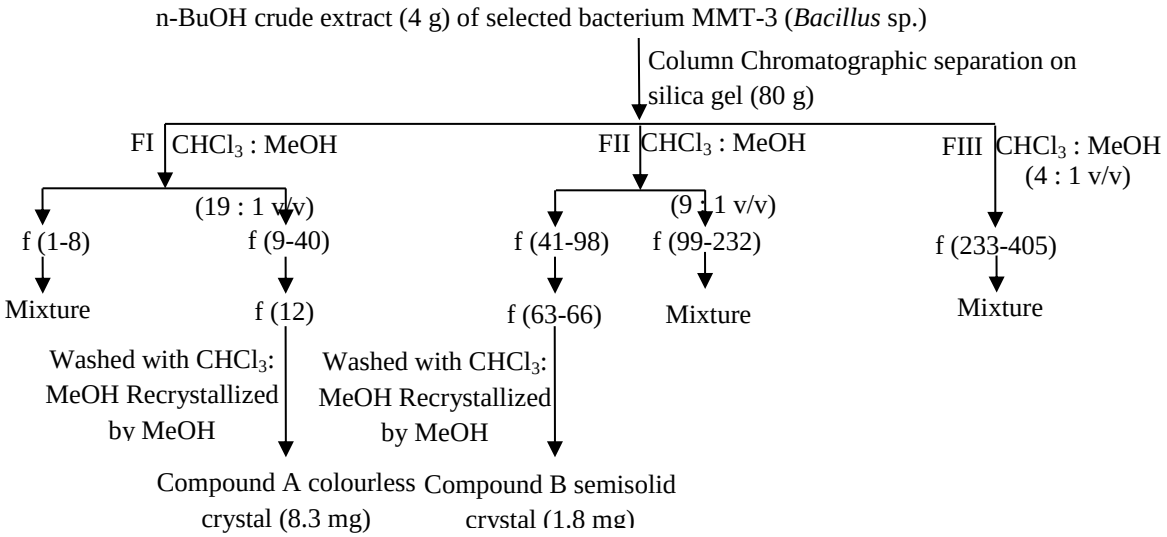
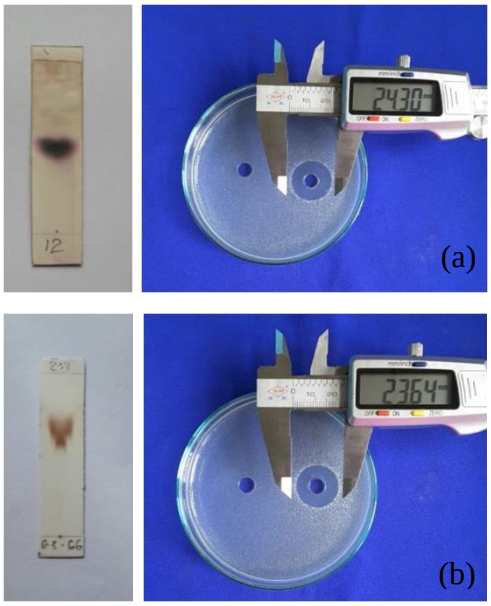


Fig. 4. Isolation of organic metabolites from n-butanol crude extract from culture broth of selected bacterium MMT-3 (*Bacillus* sp.) by column chromatography

Isolation of some organic metabolite from n-butanol extract of the fermented broth of MMT-3 (*Bacillus* sp)

Gradient elution was performed successively with increasing polarity (chloroform: methanol 19:1 and 9:1 v/v). According to the procedure in Figure4, compound A (gum, 6.3 mg) and compound B (yellowish oil, 1.8 mg) were obtained from the respective fractions F-I and F-II. The remainly fraction F-III was observed as mixture and no antibacterial activity was recorded. The isolated compounds A and B have significant activities on *Agrobacterium tumefaciens* with inhibitory zone 24.30 mm and 23.64 mm respectively. Thin layer chromatogram of compounds (A and B) and their antibacterial activities were presented in Figure 5.



Compound A

Solvent System = $CHCl_3$: MeOH (19:1 v/v)

R_f value = 0.45

Compound B

Solvent System = $CHCl_3$: MeOH (9:1 v/v)

R_f value = 0.66

Fig. 5. Thin layer chromatogram of isolated compounds and their antibacterial activities against *Agrobacterium tumefaciens* (a) compound A (b) compound B

Characterization of isolated antibacterial compounds

Some chemical properties of isolated compounds from MMT-3 (*Bacillus* sp.) metabolites with spraying agents on TLC chromatograms were described in Table 2. Moreover, the isolated compounds were characterized by solubility test, modern spectroscopic techniques such as UV and FTIR, ^{1}HNMR and GC-MS. These resultant data were given as follows:

Table 2. Some chemical properties of isolated compounds

Sr. No.	Spraying agent	Observation (compounds)	
		A	B
1	5% H_2SO_4	Deep violet	Pink
2	I_2vapour	Yellow	Yellow
3	5% $FeCl_3$	ND	ND
4	Libermann Burchard	Pink	Pink
5	Anisaldehyde	Violet	Violet
6	10% $KMnO_4$	ND	ND
7	2.4 DNP	ppt	ppt

ND = not detected
ppt = precipitate

Compound A

Compound A was isolated as semisolid (gum) from n-butanol extract of the fermented broth of selected bacterium MMT-3. It was soluble in EtOAc, MeOH, n-hexane and $CHCl_3$, but insoluble in PE and H_2O. The R_f value of compound A was found to be 0.45 in $CHCl_3$: MeOH (19:1 v/v) solvent system and it was UV active due to the presence of isolated double bond. According to the result obtained from chemical tests (Table 2), compound A was found as yellow spot on TLC chromatogram with iodine vapour, purple spot with anisaldehyde followed by heating, pale red colouration with libermann Burchard reagent, precipitate with 2, 4 DNP and the colour of compound A was deep violet spot on TLC plate while spraying with 5% H_2SO_4 followed by heating.

The UV absportion spectrum shows peak at 275.5 nm. This bond may be attributed to isolated double bond (Figure 6 and Table 3). The functional groups present in compound A was studied by FTIR spectroscopy. The FTIR spectrum was shown in Figure 7 and the interpreted spectral data are illustrated in Table 4. The FT IR spectrum of compound A showed the band at 3373cm^{-1} due to O-H stretching of acid. Absorption band at 3123$cm^{-1}$was due to =C-H stretching of alkene and -C-H stretching of $sp^3$was observed at 2920 cm^{-1} and 2848 cm^{-1}. Stretching band at 1717 cm^{-1} for C=O stretching of carbonyl and the band at 1666 cm^{-1} for C=C stretching of alkene were observed. Similarly, the band at 1377 cm^{-1} and 1346 cm^{-1} were due to C-H bending of alkyl group. And then, C-O stretching of acid group and C-H bending of sp^3 C-H were found at (1277 cm^{-1}, 1227 cm^{-1}) and (1130 cm^{-1}, 1101 cm^{-1}), respectively.

^{1}H NMR (200 MHz, $CDCl_3$): δ 0.88 (3H, t, *J* = 7.2 Hz, H-18), 1.25~1.31 (14H, br. s, H-4~7, H-15~17), 1.59 (2H, m, H-3), 2.04 (4H, m, H-8, 14), 2.30 (2H, t, *J* = 7.6 Hz, H-2), 2.78 (2H, t, *J* = 6.4 Hz, H-11), 5.35 (4H, m, H-9, 10, 12, 13). EI-MS *m/z* (rel. int): $C_{18}H_{32}O_2$; 264 $[M]^+$ (70).

According to the results from the physicochemical properties, R_f values, UV, FTIR, ^{1}HNMR and GC-MS spectral data, isolated compound A was identified as linoleic acid (O'Neil *et al.*, 2013).

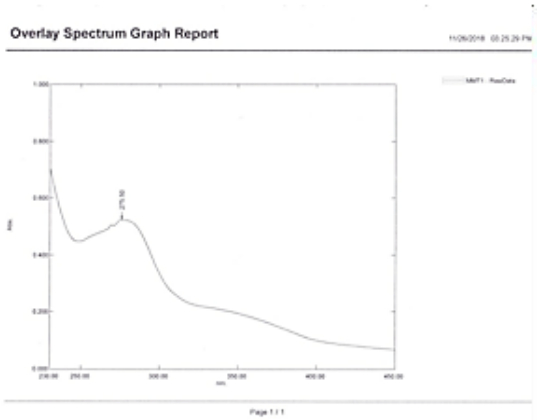


Fig. 6. UV spectrum of isolated compound A

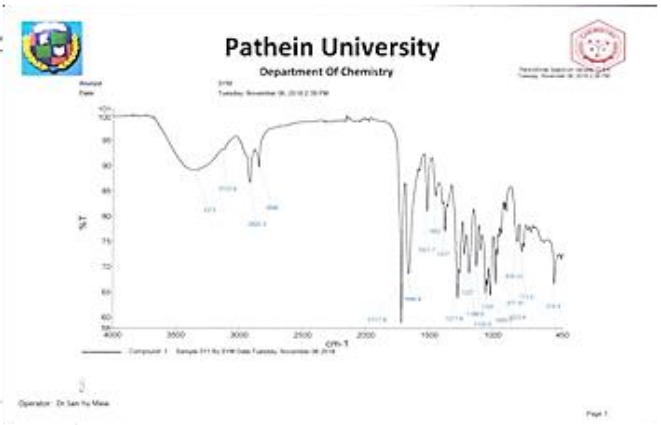


Fig. 7. FTIR spectrum of isolated compound A

Table 3. UV spectral data of isolated compound A

Solvent use	Observed λ_{max} (nm)	Remark
MeOH	275	Isolated double bond

In the study of UV spectrum of compound A, the maximum absorption band at max of λ_{275} nm indicates the presence of isolated double bond.

Table 4. FTIR spectral data of isolated compound A

Wave number (cm^{-1})	Literature Wave number (cm^{-1})	Band assignment
3373	3600~2500	O-H stretching of acid
3123	3050-3150	=C-H stretching of alkene
2920, 2848	2960, 2870	-C-H stretching of sp^3
1717	1700~1680	C=O stretching of carbonyl
1666	1680~1600	C=C stretching of alkene
1377, 1346	1390-1560	C-H bending of alkyl group
1277, 1227	1150-1350	C-O stretching of acid group
1130, 1101	1150-1000	C-H bending of sp^3 C-H

(Joseph *et al.*, 1987)

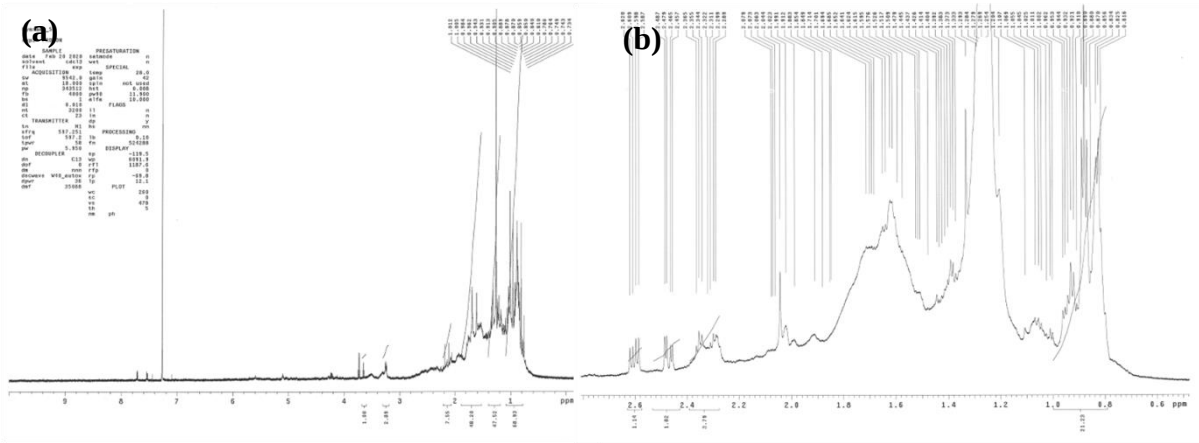


Fig. 8. (a, b) ^{1}HNMR spectrum of isolated compound A

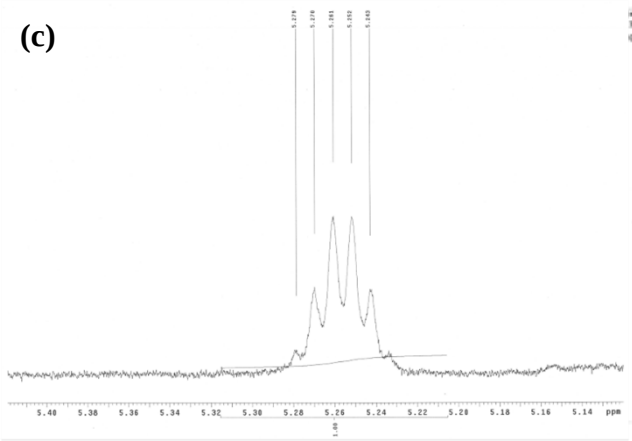


Fig. 8. (c) ^{1}HNMR spectrum of isolated compound A

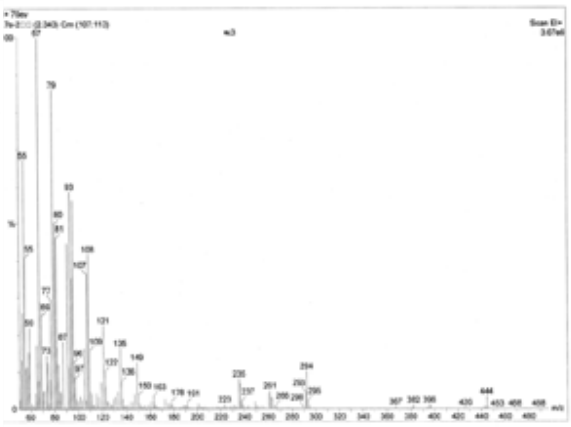


Fig. 9. GC-MS spectrum of isolated compound A

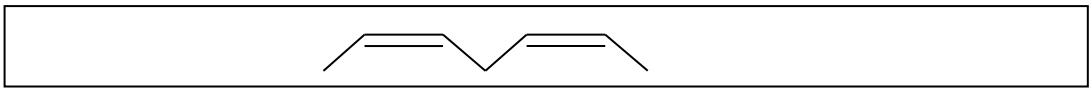


Fig. 10. Structure of isolated compound A (linoleic acid)

Compound B

Compound B was isolated as semisolid in yellowish oil from n-butanol extract of fermented broth of selected bacterium MMT-3. It was soluble in EtOAc, MeOH, n-hexane and $CHCl_3$ but insoluble in PE and H_2O. R_f value of compound B was found to be 0.66 in $CHCl_3$: MeOH (9:1 v/v) solvent system. In some chemical properties of isolated compound B, yellow spot was found with iodine vapour on TLC chromatogram. Purple spot with anisaldehyde followed by heating, pink colouration with Libermann burched reagent, precipitate with 2, 4 DNP and the colour of compound B was pink spot on TLC plate while spraying with 5% H_2SO_4 followed by heating. These results were described in (Table 2). Compound B was identified by using modern spectroscopic methods such as UV, FTIR^{1}HNMR and GC-MS spectral data. It was observed that there was absorption at ~290 nm in readily accessible UV region is due to double bond present in enone (Figure 11).

According to the FTIR spectrum (Figure 12) and its interpreted data (Table 5), absorption bond at 3327 cm^{-1} indicated that O-H stretching of alcohol, the band at 2924 cm^{-1}, 2855 cm^{-1} for -C-H stretching of sp^3 C-H, 1628 cm^{-1} showed C=O stretching of ketones. Stretching of carbonyl absorption band appeared at 1537 cm^{-1}, 1452 cm^{-1} indicated C-H bending of sp^3 C-H and C-O-H stretching of alcohol was observed at 1228 cm^{-1} and 1068 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.85 (3H, d, *J* = 6.4 Hz, CH_3-15'), 0.86 (9H, d, *J* = 6.4 Hz, CH_3-7', 11', 15'), 1.02-1.62 (20H, m, CH_2), 1.24 (3H, s, CH_3-3'), 2.01 (6H, s, CH_3-2, 3), 2.04 (3H, s, CH_3-5), 2.54 (2H, m, H-1').EI-MS *m/z* (rel. int): $C_{29}H_{50}O_3$; 446 $[M]^+$ (5), 237 (41), 235 (38), 221 (100), 194 (6), 167 (9), 151 (7), 137 (5), 71 (34), 69 (26), 57 (38), 55 (28).

According to the results from the physicochemical properties, R_f values, UV, FT IR, ^{1}HNMR and GC-MS and EI-MSspectral data isolated compound B was identified as *α*-tocopherylquinone (O'Neil *et al.*, 2013).

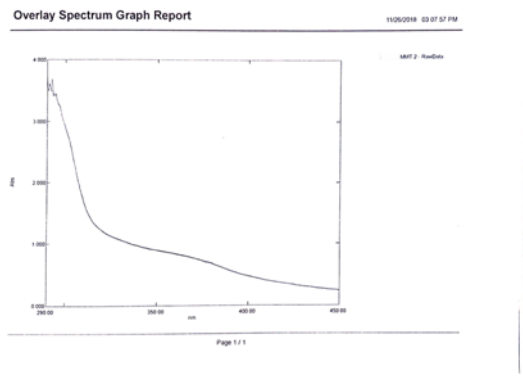


Fig. 11. UV spectrum of isolated compound B

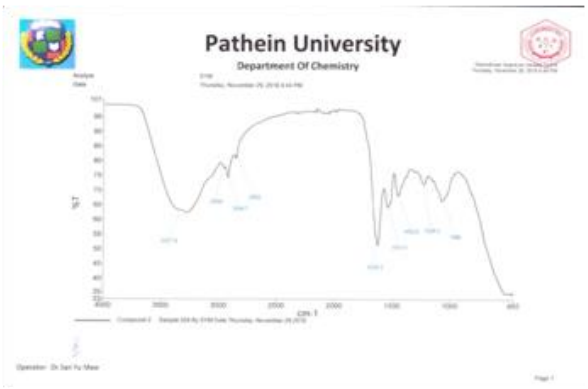


Fig. 12. FTIR spectrum of isolated compound B

Table 5. FTIR spectral data of isolated compound B

Wave number (cm^{-1})	Literature Wave number (cm^{-1})	Band assignment
3327	3600~2500	O-H stretching of alcohol
2924, 2855	2960, 2870	=C-H stretching of sp^3 C-H
1628	1680~1600	C=O stretching of carbonyl
1537, 1452	1480~1440	C-H bending of sp^3 C-H
1228, 1068	1200~1020	C-O-H stretching of alcohol

(Joseph *et al.*, 1987)

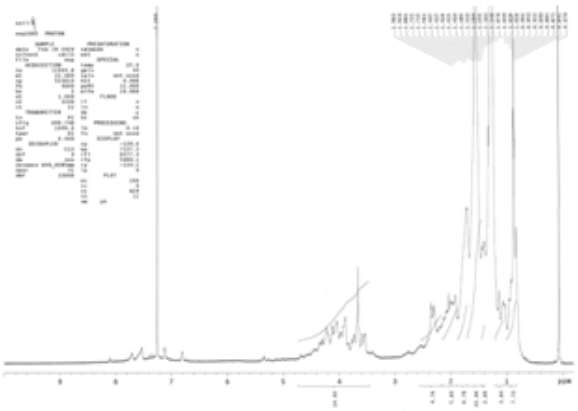


Fig. 13. ^{1}HNMR spectrum of isolated compound B

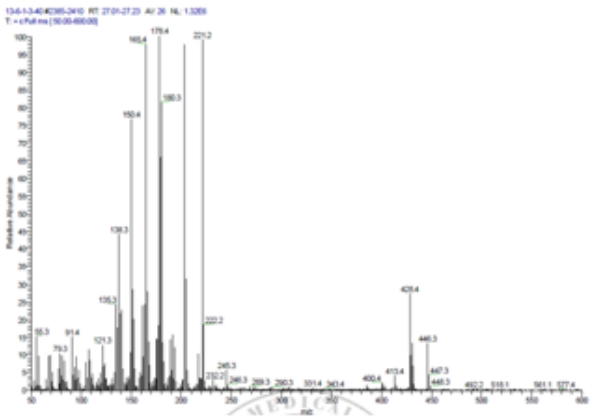


Fig. 14. GC-MS spectrum of isolated compound B

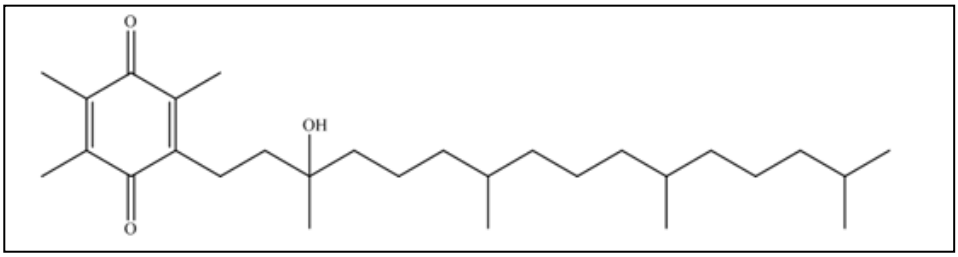
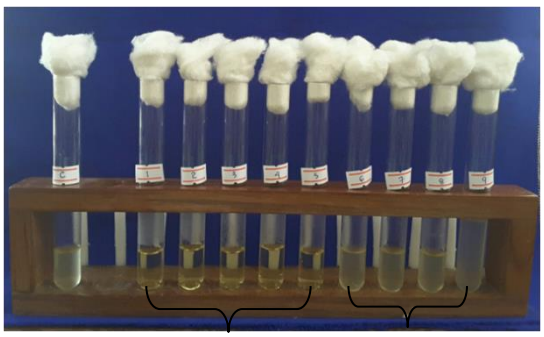


Fig. 15. Structure of isolated compound B (α-tocopherylquinone)

Minimum inhibitory concentrations (MIC) of isolated compounds

MIC values of compound A and B were determined by two fold serial dilution method ranging from 70 µg/mL to 0.273 µg/mL. MIC values were read in µg/mL after overnight incubation. It was observed that MIC value of compound A was 4.375 µg/mL when used against *Agrobacterium tumefaciens* and MIC value of compound B was 2.187 µg/mL.



C = Control
1 = 70 µg/mL
2 = 35 µg/mL
5 = 4.375 µg/mL
6 = 2.187 µg/mL
7 = 1.093 µg/mL

Fig. 16. MIC of antibacterial metabolite (compound A) with two-fold serial dilution

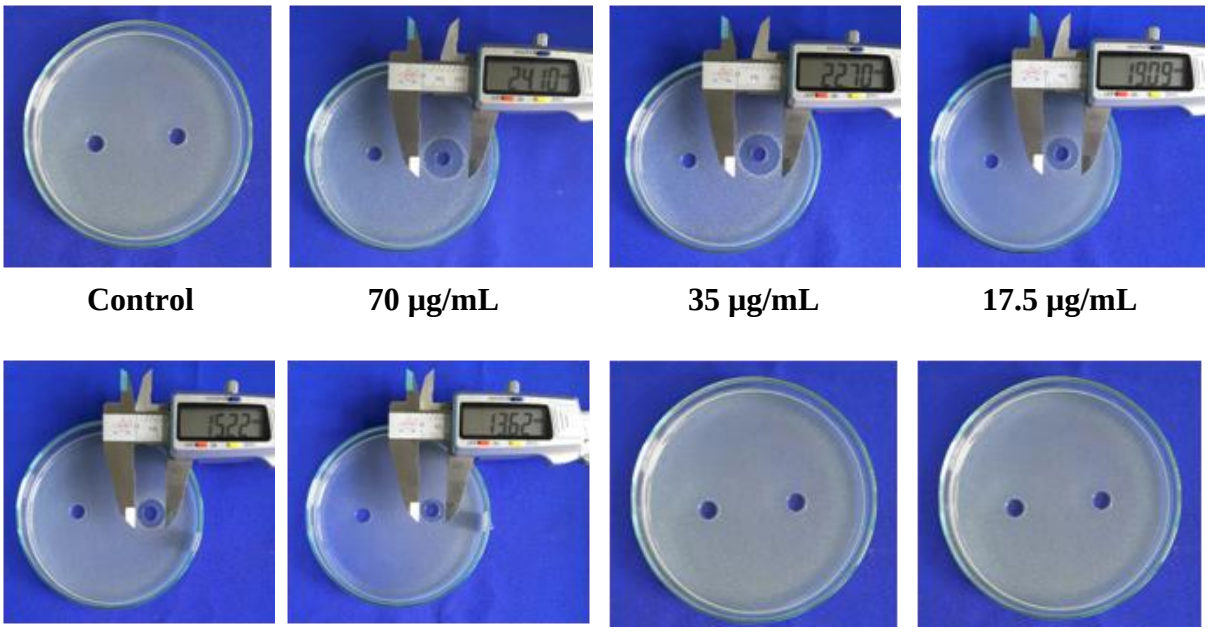
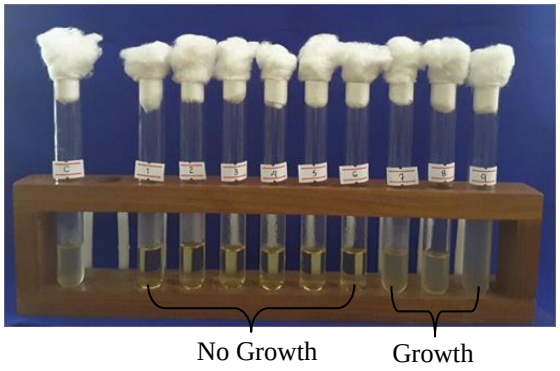


Fig. 17. MIC of secondary metabolite (compound A) on *Agrobacterium tumefaciens* (agar well diffusion method)



C = Control
1 = 70 µg/mL
2 = 35 µg/mL
5 = 4.375 µg/mL
6 = 2.187 µg/mL
7 = 1.093 µg/mL

Fig. 18. MIC of antibacterial metabolite (compound B) with two-fold serial dilution

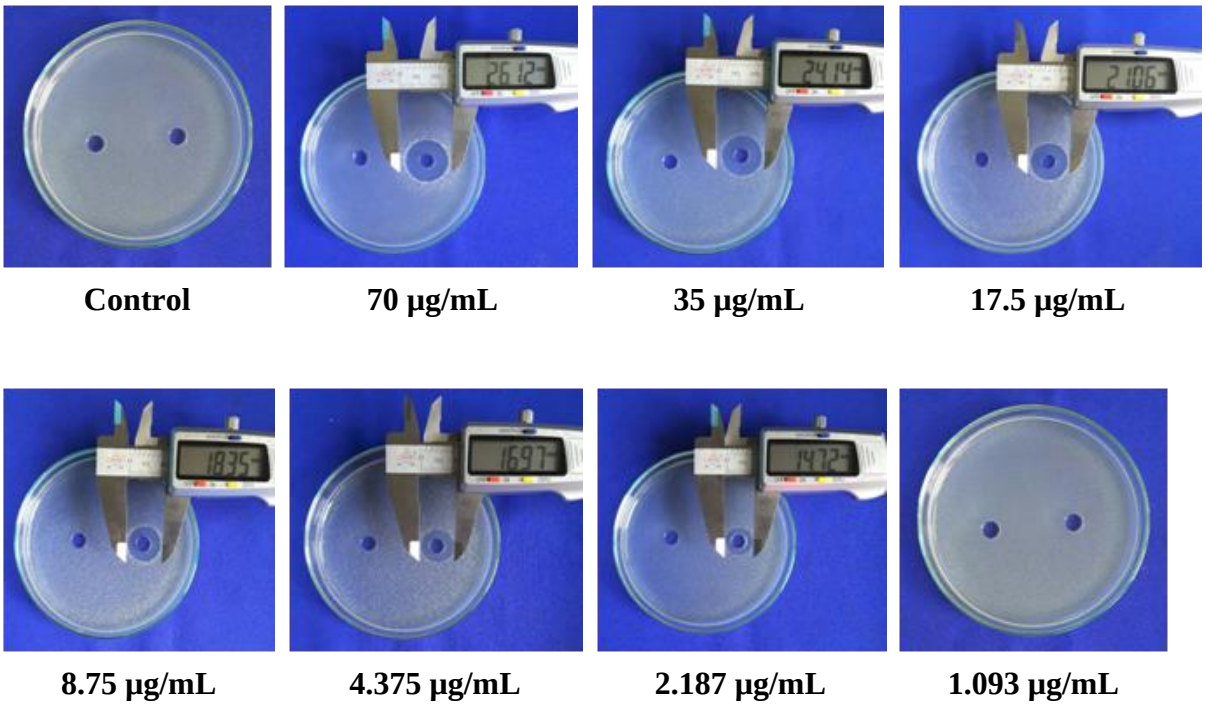


Fig. 19. MIC of secondary metabolite (compound B) on *Agrobacterium tumefaciens* (agar well diffusion method)

DISCUSSION AND CONCLUSION

Bacillus sp. was isolated from a soil sample, identified, cultivated and its crude extract and purified fractions were evaluated for their potent antibacterial activities. It was identified based on morphological, biochemical and physiological characteristics as well as on molecular level (Tarawni, *et al.,* 2015).

In the present study, isolation, improvement of yield and characterization of bioactive compounds from soil bacteria has been carried out. In the investigation of paper chromatography, four kinds of different solvents were applied to observe the optimum extraction ability of secondary metabolite.

TLC is the most common and efficient techniques used for detection and analysis and separation of phytoconstituents compound, and it is estimated that 60% of analyses are performed based on TLC over worldwide. Initially, TLC optimization of the solvent system for development and purification of secondary metabolites (Bipin *et al.,* 2014).

Successive fractions (F) obtained were combined on the basis of their behavior on TLC and tested their antibacterial against *Agrobacterium tumefaciens.* Identification of a number of new metabolites and evaluation of their biological activities has been studied. Several fractions were collected and analyzed on TLC plates. Similar fractions were combined into three major fractions (19:1, 9:1 and 4:1). Compound A from f (9-40) was observed a single spot which turned violet on TLC plate spray with 5% H_2SO_4 and the mass of the compound A (gum) was recorded as 6.3 mg. Compound B from f (41-98) observed a

single spot which gave pink on TLC plate sprayed with 5% H_2SO_4 and the mass of compound B (yellowish oil) was recorded as 1.8 mg.

Similarly, Bipin *et al.,* 2014 reported that, compound that is polar will be dissolved in polar solvent, compounds that are non-polar will dissolved in non-polar solvent system.

According to some chemical properties, UV, FT-IR and spectral data by compounds A (linoleic acid) and compound B (α- tocopherylquinone) were identified, respectively. All isolated compounds showed antibacterial activity, compound A (24.30 mm) and compound B (23.64 mm) against *Agrobacterium tumefaciens.*

In minimum inhibitory concentrations, the purified compound A showed minimum inhibitory zone (13.62 mm) against *Agrobacterium tumefaciens* with MIC 4.375 µg/mL. Similarly the active compounds B also showed the minimum inhibitory zone (14.72 mm) against *Agrobacterium tumefaciens* with MIC 2.187 µg/mL.

In pharmaceutical research, Minimum Inhibitory Concentration (MIC) is the least concentrated of an antimicrobial agent that inhibits the growth of a microorganism after a given period of incubation (Andrews, 2001). The present study indicated that *Bacillus* sp. had bioactive compounds with antibacterial potential. This may be due to the fact that soil microorganisms produced bioactive secondary metabolites.

It may be concluded that active antibacterial compounds are isolated from n-butanol extract of MMT-3 (*Bacillus* sp.) by column chromatography and identified by some chemical test and spectroscopic techniques. The MIC value of active compound A and B were screened by agar well method against *Agrobacterium tumefaciens.* Further work will be a study on compound, and investigation of molecular mechanisms can be a promising approach for further antibacterial drugs and biofertilizer development programs.

Acknowledgments

Firstly, we wish to express our gratitude to Dr Theingi Shwe (Rector, Hinthada University), Dr Yee Yee Than (Pro-Rector, Hinthada University) and Dr Cho Kyi Than (Pro-Rector, Hinthada University), for her valuable instruction and guidance. Then, we would like to convey our deep sense of gratitude to Dr Thida Oo, Professor and Head of Botany Department, Hinthada University, and Dr Wah Wah Myint, Professor, Department of Botany, Hinthada University. Finally, we would like to acknowledge our indebtedness to Dr. Min Jen Cheng and his group, for the purification of isolated compounds, Bioresource Collection and Research Centre (BCRC), Food Industry Research and Development Institute (FIRDI), Taiwan.

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