PURIFICATION, BIOLOGICAL PROPERTIES AND PARTIAL STRUCTURAL DETERMINATION OF ANTIBACTERIAL METABOLITES PRODUCED BY *ASPERGILLUS TAMARII*

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Abstract

The fungus *Aspergillus tamarii* was isolated from the soil sample of Pone Taung Pone Nyar Area, Magway Region. This fungus showed the antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA). The antibacterial metabolites against MRSA were isolated from the toluene extract of the fermented broth by column chromatography. Sephadex LH-20 resin columns were used finally to purify the metabolites. The active fractions eluted from theSephadex LH-20 column were separately combined and concentrated. These metabolites were checked for purity by thin layer chromatography (TLC), visualized under UV-254 nm and UV-365 nm light. Bioautography on TLC was also carried out to check the retardation factor (R_f) value and purity of the metabolites. From the chromatographic separations, 22.111 mg of metabolite-I and 21.026 mg of metabolite-II were obtained from 0.55 g of the toluene extract of fermented broth. From the physicochemical characterization, UV-vis, and FT IR spectra and comparison with FT IR library, metabolite-I may be a steroid derivative and metabolite-II may be an aromatic derivative compound containing alcohol and carbonyl groups.

Keywords: *Aspergillus tamarii*, metabolites, bioautography, spectroscopy**,** MRSA

Introduction

Pone Taung Pone Nyar region has been protected under the 1998 law. The fossils in Pone Taung Pone Nyar region are believed to be 4 million years older than the Egyptian counterparts which were thought to be the oldest before (Shwe, 2019). Production of the two antibacterial metabolites from *Aspergillus* was reported in the present research work. This research focuses on the study of the metabolites isolated from the soil sample of Pone Taung Pone Nyar Area. Modern and global healthcare today is facing the problem of multi-resistant bacteria. Many bacterial species have developed resistance mechanisms against several classes of antibiotics in a relatively short period of time after the clinical introduction of antibiotics (Stefan *et al.*, 2011).

From the history of drug discovery from microorganisms, fungal secondary metabolites have provided a number of important drugs (Alberts, 1980). In this study, the isolated soil fungus, *Aspergillus tamarii* of Pone Taung Pone Nyar Area produced antimicrobial substances against methicillin resistant *Staphylococcus aureus* (MRSA). Purifications of antibacterial metabolites were carried out through various chromatography. The functional groups present in the metabolites were studied by FT IR spectroscopic method. The absorption of maximum wavelength of the metabolites was investigated by ultraviolet-visible (UV-vis) spectrophotometry.

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Materials and Methods

Isolation of Antibacterial Metabolite by Silica Gel Column Chromatography

The silica gel was dissolved in *n*-hexane and column was packed. Concentrated toluene extract (0.55 g) of fermented broth was passed through silica gel column by elution with *n*-hexane and ethyl acetate solvent system in the ratio of (9:1, 4:1 and 7:3, v/v) (Simon and Gray, 1998; Phay, 1999). About 2.5 mL of each fraction was collected individually. One hundred and seven fractions were collected. All these fractions were tested the antibacterial activity against MRSA by paper disc diffusion assay. The active fractions were combined and concentrated separately and named as fraction A and fraction B. The active fractions were then rechromatographed on Sephadex LH-20 column.

Rechromatography of Active Fraction A on Sephadex LH-20 Column

Sephadex LH-20 was dissolved in chloroform and the column was packed. The residue of the active fractions (35-71) which was renamed as fraction A eluted from the silica gel column was transferred on to the Sephadex LH- 20 column and eluted with *n*-hexane. Eleven fractions (1 mL each) were collected. Then methanol was used as eluting solvent and another eleven fractions (1 mL each) were collected. The collected fractions were subjected to assessment of antibacterial activity against MRSA by paper disc diffusion assay.

Rechromatography of Active Fraction B on Sephadex LH-20 Column

The residue of the active fractions (82-94) which was named as fraction B from silica gel column chromatography eluting with *n*-hexane: ethyl acetate (7:3, v/v) solvent system was transferred on to a Sephadex LH- 20 column and eluted with *n*-hexane. Eleven fractions (1 mL each) were collected. Then ethyl acetate was used as another eluting solvent and eleven fractions (1 mL each) were collected. The collected fractions were tested the antibacterial activity by paper disc diffusion assay (Kumar, 2020).

Determination of R_f Value of the Isolated Antibacterial Metabolites

Each dissolved sample (100 µL) was applied on the TLC plates and allowed to dry. Each TLC plate was developed in the solvent system of benzene: ethyl acetate (4:1, v/v). The TLC plates developed in the solvent systems were allowed to dry and checked under UV (254 nm and 365 nm). Bioautography was also carried out to check the antibacterial activity against MRSA. Each TLC plate was placed on assay agar plates and allowed for 30 min. After 30 minutes, the TLC plates were carefully taken out from the assay plate. Then, the assay plates were incubated for 24 h at 30^{o}C. Clear zones of the active components indicated the presence of bioactive metabolites. The R_f value of active components were measured by localization of spots.

Physicochemical Characterization and Classification of Antibacterial Metabolites

The isolated antibacterial metabolites from the toluene extract of the fermented broth were characterized and classified by determination of their solubility, R_f value, some chemical tests and modern spectroscopic techniques such as UV-visible and FT IR spectrometry.

Determination of Some Chemical Properties of Isolated Metabolites

The isolated antibacterial metabolites were subjected to TLC analysis and then treated with some reagents such as 5% H_2SO_4, I_2 vapour, Liebermann-Burchard reagent, 5% $FeCl_3$

solution, 2,4-dinitrophenylhydrazine solution and hydroxamic acid test to study their behaviors on TLC and in test tube.

Study on UV-Visible Spectroscopy of Isolated Metabolites

The maximum wavelength of absorption of the metabolites was recorded and examined by UV 1800-UV spectrometer at the Department of Chemistry, Mandalay University and Department of Chemistry, Pathein University. UV-Visible spectroscopy of isolated metabolites was studied by the method of Diffey, 2023.

Determination of Functional Groups of Isolated Metabolites by FT IR Spectrometry

The function groups present in the isolated metabolites were studied by Fourier Transform Infrared Spectrophotometer (Perkin Elmer Spectrum Version 10.4.00) at the Department of Chemistry, Mandalay University. Determination of functional groups was studied by the methods of Silverstein *et al.*, 2005; Deng *et al.*, 2014.

Results and Discussion

Isolation of Antibacterial Metabolites by Silica Gel Column Chromatography

According to column chromatography, totally one hundred and seven fractions were collected. Each fraction was subjected to antibacterial activity against MRSA. It was found that fraction numbers (35-71) showed the activity on MRSA. These fractions were combined and renamed as fraction-A. It was also found that fraction numbers (82-94) showed the activity against MRSA.The fractions (82-94) were combined and renamed as fraction-B.The results are tabulated in Table 1 and the antimicrobial activities are shown in Figures 1 and 2. The resultant active fraction-A and B were needed to purify further to produce the bioactive metabolites.These active fractions- A and B were concentrated separately on rotary evaporator and were rechromatographed on Sephadex LH-20 resin column.

Table 1. Activity Against MRSA of the Fractions Eluted from Silica Gel Column Chromatography

Eluting solvent	Fraction No.	Activity
n-Hexane: EtOAc (9:1, v/v)	1-29	No activity
n-Hexane: EtOAc (4:1, v/v)	30-34	No activity
	35-71 (A)	Activity
	72-75	No activity
n-Hexane: EtOAc(7:3, v/v)	76-81	No activity
	82-94 (B)	Activity
	95-107	No activity

Figure 1. Antibacterial activity against MRSA of fractions1-32 eluted from silica gel column chromatography

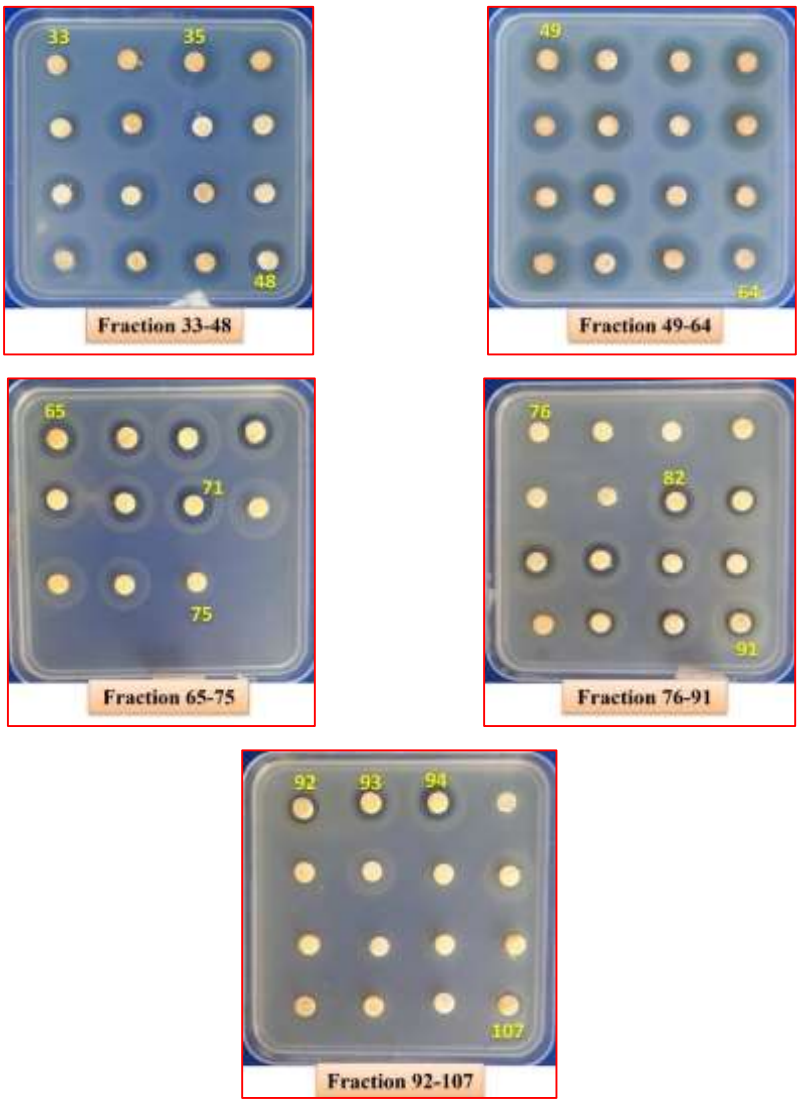


Figure 2. Antibacterial activity against MRSA of fractions 33-107 eluted from silica gel column chromatography

Rechromatography of Active Fraction A on Sephadex LH-20 Column

Active fraction-A was rechromatographed on Sephadex LH-20 using *n*-hexane and methanol as eluting solvent.Twenty two fractions were collected and tested for antibacterial activity against MRSA as shown in Table 2 and Figure 3. Active fractions were combined and concentrated. Then it was crystallized to yield 22.11 mg of metabolite-I.

Table 2. Activity Against MRSA of Fractions Eluted from Re-chromatography of Active Fraction AbySephadex LH-20 Resin Column

Eluting solvent	Fraction No.	Antibacterial Activity
n-Hexane	1-11	No activity
Methanol	12	No activity
	13-15	Activity
	16-22	No activity

Figure 3. Paper disc diffusion assay for antibacterial activity against MRSA

Rechromatography of Active Fraction B on Sephadex LH-20 Column

Active fraction-B was rechromatographed by Sephadex LH-20 using *n*-hexane and ethyl acetate. Active fractionated compound (13-14) from Sephadex LH-20 column chromatography developed by ethyl acetate was combined and concentrated. It was denoted as Metabolite-II. The results are shown in Table 3 and Figure 4.

Table 3. Activity Against MRSA of Fractions Eluted from Rechromatography of Active Fraction BbySephadex LH-20 Column

Eluting solvent	Fraction No.	Antibacterial Activity
n-Hexane	1-11	No activity
Ethyl Acetate	12	No activity
	13-14	Activity
	15-22	No activity

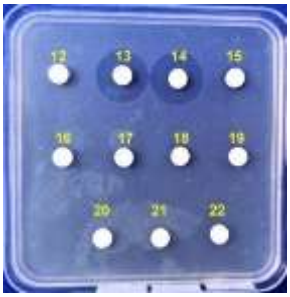
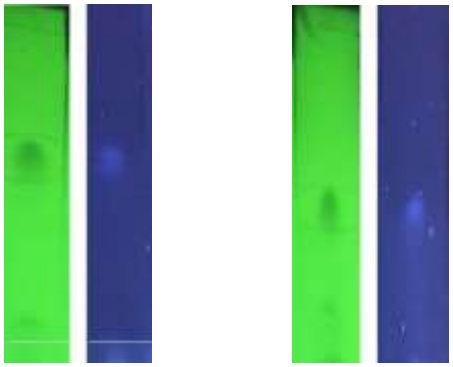


Figure 4. Paper disc diffusion assay for antibacterial activity against MRSA

Determination of R_f Values by Thin Layer Chromatography and Bioautography

In the TLC check of metabolite-I and metabolite-II by UV-254 nm and UV-365 nm, it was found that one active spot for each was found under the short wave and the long wave. The R_f value of metabolite-I was 0.65 and that of the metabolite-II was 0.47 when using the solvent system of benzene: ethyl acetate (4:1, v/v). Bioautography of TLC also showed the activity at the same R_f values with the same eluting solvent system as observed under UV-254 nm and UV-365 nm lights. TLC chromatograms of these purified metabolites are shown in Figures 5 and 6.



(a) (b) (c) (d)

Figure 5. TLC of metabolite-I and metabolite-II under UV light

(a)metabolite-I under UV 254 nm (b) metabolite-I under UV 365 nm

(c) metabolite-II under UV 254 nm (d) metabolite-II under UV 365 nm

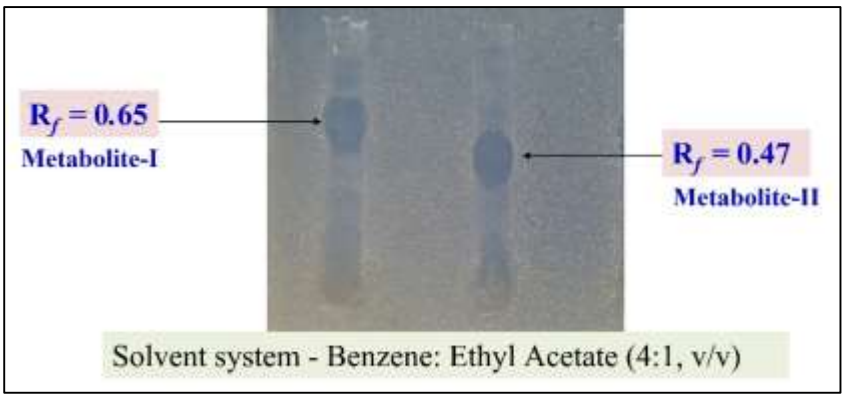


Figure 6. Bioautography of TLC

Physicochemical Characterization and Classification of the Isolated Metabolite-I

The metabolite-1 was firstly characterized by its physical properties such as solubility, R_f value and by some chemical reagent tests. The results for metabolite-I are summarized in Tables 4 and 5 and UV and FT IR spectral data are described in Tables 6 and 7 and Figures 7 and 8.

The solubility of metabolite-I was determined in various solvents. It was found that metabolite-I is soluble in methanol, ethanol, toluene and ethyl acetate; slightly soluble in benzene, dichloromethane and distilled water and insoluble in *n*-hexane and chloroform.

The R_f value of metabolite-I was found to be 0.65 ~~in~~ benzene: ethyl acetate (4:1, v/v) solvent system. According to the results obtained from chemical tests, metabolite-I gave yellow spot on the TLC chromatogram with iodine vapor while spraying with 5% H_2SO_4 followed by heating gave a pink spot. It gave green solution when treated with Liebermann-Burchard reagent, orange yellow precipitates when treated with 2,4-DNP reagent. Therefore, metabolite-I may be classified as a steroidal derivative containing carbonyl group.

In the UV absorption spectrum, maximum absorption bands at λ_{max} 234 nm and 248 nm indicating the $\pi \rightarrow \pi^*$ transition of electrons and at λ_{max} 273 nm was due to $n \rightarrow \pi^*$ transition of the nonbonding electron of carbonyl group.

The functional groups of metabolite-I were assigned by FT IR spectroscopy. According to the FT IR spectrum, physicochemical characterization, UV spectroscopic analysis and comparison with FT IR library, the isolated metabolite-I may be a derivative of steroid.

Table 4. Physical State, Color and R_f Value of Isolated Metabolite-I

Isolated Metabolite	Physical State	Color	R_f Value	Solvent System
Metabolite-I	Solid	Pale Yellow	0.65	Benzene: EtOAc (4:1, v/v)

Table 5. Some Chemical Reagent Tests of Isolated Metabolite-I

Reagent	Observation	Remark
5% H_2SO_4	pink (on TLC)	C=O present
I_2 vapour	yellow (on TLC)	C=C present
5% $FeCl_3$	no change in colour	Phenolic OH group is absent.
Liebermann-Burchard	green coloration	It may be a steroid.
Hydroxamic acid test	no change in colour	Ester group is absent.
2, 4-DNP	orange yellow precipitate	C=O group of aldehyde or ketone present
Mg, HCl (conc:)	no red coloration	flavonoid absent

Table 6. UV Spectral Data of Isolated Metabolite-I

Solvent Used	Observed λ_{max} (nm)	Remark
Methanol	234, 248	$\pi \longrightarrow \pi^*$ electron transition due to conjugated π bonds
Methanol	274	$n \longrightarrow \pi^*$ nonbonding electron transition due to conjugated π system

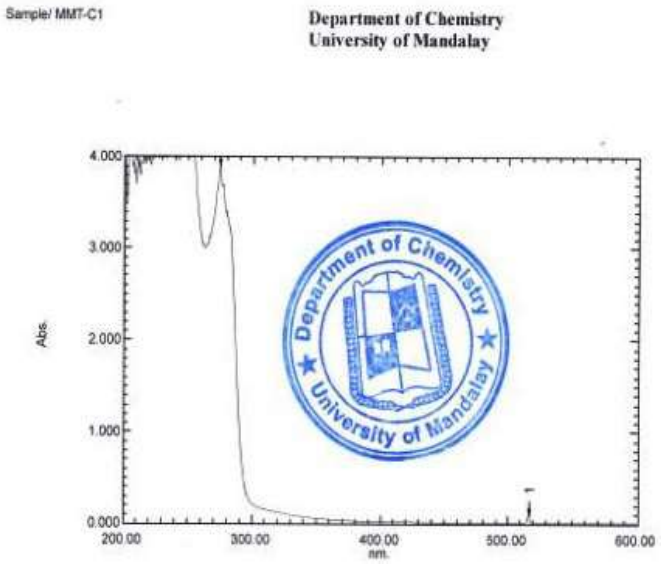


Figure 7. UV-visible spectrum of isolated metabolite-I

Table 7. Interpretation of FTIR Spectra (Metabolite-I)

No.	Metabolite-I (cm^{-1})	Literature Value*(cm^{-1})	Band Assignment
1	3433.29	3600-3200	-OH stretching vibration of alcohol group
2	3050.27	3100-3000	=C-H stretching vibration of olefinic group
3	2962.66, 2931.80, 2862.36	2962-2840	-C-H asym & symstretching vibration of -CH_2 and CH_3 groups
4	1728.22	1720-1710	-C=O stretching vibration of cyclic ketone
5	1597.06, 1581.63	1600-1450	-C=C stretching vibration of alkenic group
6	1465.90	1475-1445	-C-H asymmetric bending vibration of CH_3

No.	Metabolite-I (cm^{-1})	Literature Value*(cm^{-1})	Band Assignment
			group
7	1381.03	1390-1370	-C-H symmetric bending vibration of methylene and methyl group
8	1280.73	1260-1000	-C-C-O asymmetric stretching vibration of alcohol group
9	1126.43, 1072.42	1150-1085	C-F stretching frequency
10	740.67	900-675	-C-H out of plane bending of cis alkenic group

*****Silverstein *et al.*,2005

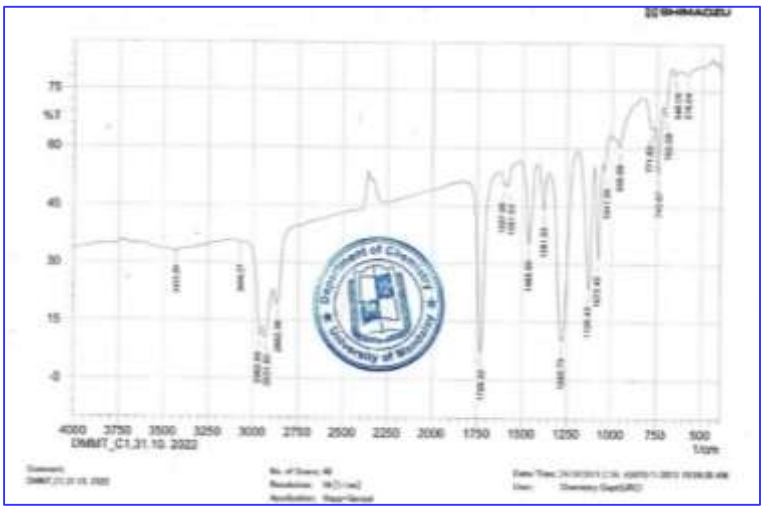


Figure 8. FT IR spectrum of metabolite-I

Physicochemical Characterization and Classification of the Isolated Metabolite-II

Physicochemical characterization and classification of the isolated metabolite-II were also undertaken. The isolated antibacterial metabolite-II from the toluene extract of the fermented broth of *A. tamarii* was firstly characterized by its physical properties such as solubility, R_f values and by some chemical reagent tests. The results for metabolite-II are summarized in Tables 8 and 9 and UV and FT IR spectral data are described in Tables 10 and 11 and Figures 9 and 10.

The solubility of metabolite-II was determined in various solvents. It was found that metabolite-II is soluble in methanol, ethanol, toluene and ethyl acetate; slightly soluble in benzene, dichloromethane and distilled water; and insoluble in *n*-hexane and chloroform.

The R_f value of metabolite-II was found to be 0.47 in benzene: ethyl acetate (4:1, v/v) solvent system. According to the results obtained from chemical tests, metabolite-II gave yellow spot on the TLC chromatogram with iodine vapor while spraying with 5% H_2SO_4 followed by heating gave a pale-yellow spot. There was a change in colour on TLC chromatogram by spraying with 5% $FeCl_3$. This metabolite did not give green solution when treated with

Liebermann-Burchard reagent. Orange yellow precipitates were found when treated with 2, 4-DNP reagent. Therefore, metabolite-II may be classified as aromatic derivative compound.

In the UV absorption spectrum, maximum absorption bands at λ_{max} 224nm, 232nm and 254 nm indicating the $\pi \rightarrow \pi^*$ transition of electrons due to conjugated π bond.

The functional groups of metabolite-II were assigned by FT IR spectroscopy. Based on the FT IR spectrum, UV spectroscopic analysis and chemical tests, it was suggested that metabolite-II may be classified as aromatic derivative compound containing alcohol group and carbonyl group.

Table 8. Physical State, Colour and R_f Value of Isolated Metabolite-II

Isolated Metabolite	Physical State	Colour	R_f Value	Solvent System
Metabolite-II	Solid	White	0.47	Benzene: Ethyl Acetate (4:1, v/v)

Table 9. Some Chemical Reagent Tests of Isolated Metabolite-II

Reagent	Observation	Remark
5% H_2SO_4	pale yellow (on TLC)	O-H or C=O present
I_2 vapour	yellow (on TLC)	C=C present
5% $FeCl_3$	change in colour	phenolic derivative
Liebermann-Burchard	not detected	It may not a steroid.
Hydroxamic acid test	no change in colour	Ester group is absent.
2, 4-DNP	orange yellow precipitate	C=O group of aldehyde or ketone present
Mg, HCl (conc:)	no red coloration	flavonoid absent

Table 10. UV Spectral Data of Isolated Metabolite-II

Solvent used	Observed λ_{max} (nm)	Remark
Methanol	224, 232, 254	$\pi \longrightarrow \pi^*$ electron transition due to conjugated π bonds

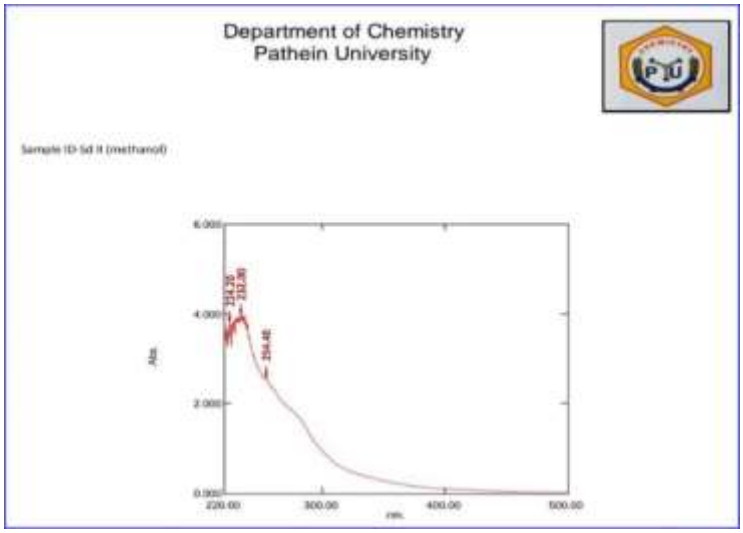


Figure 9. UV-visible spectrum of metabolite-II

Table 11. Interpretation of FTIR Spectra (Metabolite-II)

Sr.	Metabolite-II (cm^{-1})	Literature Value*(cm^{-1})	Band Assignment
1	3433.29	3600-3200	-OH stretching vibration of alcohol group
2	3020.27	3100-3000	=C-H stretching vibration of aromatic ring
3	2924.09, 2854.65	2962-2840	-C-H asym & symstretching vibration of -CH_2 and CH_3 groups
4	1743.65	1810-1715	-C=O stretching vibration of lactone and ester
5	1627.95, 1604.77,	1600-1400	-C=C stretching vibration of alkenic group
6	1465.90, 1411.89	1475-1400	-C-H asymmetric bending vibration of CH_3 group
7	1381	1390-1370	-C-H symmetric bending vibration of CH_3 and -CH_2 group
8	1103.28	1260-1000	-C-C-O asym stretching vibration of alcohol group
9	740.67	900-675	-C-H out of plane bending of aromatic group

*Silverstein *et al*.,2005

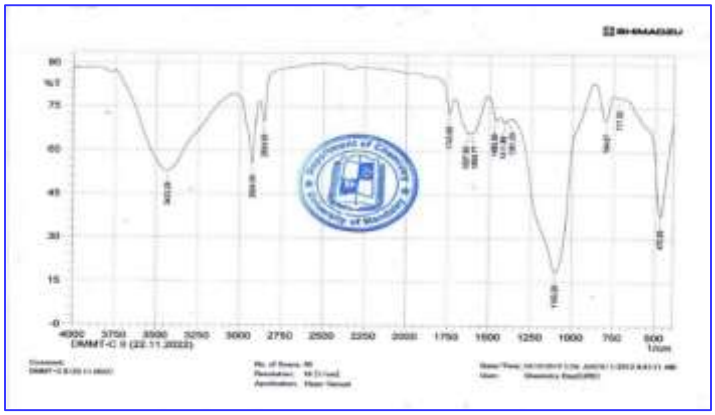


Figure 10. FT IR spectrum of metabolite-II

Conclusion

This study successfully isolated and partially characterized two antibacterial metabolites (metabolite-I and metabolite-II) produced by the soil fungus *Aspergillus tamarii* from Pone Taung Pone Nyar Area, Magway Region. Both metabolites exhibited promising activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA), a multidrug-resistant pathogen posing a significant threat to global health. These findings suggest that *A. tamarii* from this specific region could be a valuable source of novel antibiotics for combating drug-resistant bacteria. Further research on the complete structural elucidation and optimization of these metabolites' potency and selectivity is warranted to translate this discovery into potential therapeutic applications.

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