ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF ISOLATED ANTIFUNGAL COMPOUNDS FROM SOIL BACTERIUM *PSEUDOMONAS* SP.

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Abstract

The focus of the present research, the bacterium *Pseudomonas* sp, isolated from mangrove soil, Shwe thaung yan Township, Ayeyawady Region. It was carried out by paper chromatography with four solvents system. The bacterial culture filtrate was studied by the different ratio of ethyl acetate and fermented broth (1:1, 2:1, 3:1 v/v). The equal ratio (1:1 v/v) ethyl acetate extract showed higher inhibitory effect (19.79 mm) than the other ratio. Crude ethyl acetate extract 2.0 g was obtained from 10 liters of fermented broth with ethyl acetate (1:1 v/v) and subjected to purification was performed using both thin layer chromatography (TLC) method with various solvents system and silica gel column chromatography techniques. By silica gel column chromatographic separation, compound M-1 (colorless needle shape, 1.1 mg, may be sterol), compound M-2 (colorless amorphous powder, 5.6 mg, may be steroid) and compound M-3 (colorless amorphous powder, 5.8 mg, may be steroid) in chloroform: methanol solvent system were isolated. These isolated compounds were characterized by R_f value, physicochemical properties, modern spectroscopic methods such as UV and FT IR. In the further investigation of minimum inhibitory concentrations (MIC), it was observed that MIC value of antifungal compounds M-1 and M-2 were 1.25 µg/ mL and 0.625 µg/mL respectively but M-3 did not exhibit antifungal activity against *Candida albicans* in this experimental concentration range from 20 µg/mL to 0.078 µg/mL. In the present result, indicated that the selected bacterium *Pseudomonas* sp. may be utilized to treat the diseases caused by *Candida albicans.*

Keywords: paper chromatography, silica gel column chromatography, minimum inhibitory concentrations

Introduction

The recent decades are characterized by the novel discoveries of microorganisms capable of producing compounds as a potential source of new antibiotics. Antibiotics are antimicrobial agents produced by microorganisms that inhibit the growth or kill other microorganisms while being harmless to the host cells. Antibiotics are one of the most important commercially exploited secondary metabolites produced by the bacteria and employed in a wide range. Most of the antibiotic producers used today are the soil microbes (Arpigny and Jaeger, 1999). Thin layer chromatography (TLC) is one of the principal separation technique. It can be used in a search from optimum extraction solvents, for identification of known and unknown compounds (Fair *et al*., 2008).

Chromatography is a useful technique for the separation of compounds from a complex mixture, such as a bacterial extract. Base on the physical and chemical properties of compounds and their affinities for certain solid phase materials (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 system (Harris, 2003). The most common methods of detection for early stages are: ultraviolet-visible spectroscopy (UV/Vis) that provides information on chromophores present in a compound and FT IR provides information on functional group present in a compound (Henke and Kelleher, 2016).

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The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). It is well known crude extract isolated from these bacterial metabolites contains complex chemical diversity which is difficult to identify and characterize. Therefore, effort has been made to characterize a bioactive molecule synthesized by isolated bacterial in this study. The aim and objectives of this study were to isolate some organic compounds from the ethyl acetate extract of *Pseudomonas* sp. to characterize the isolated compounds by physicochemical tests and spectroscopic techniques such as UV, FT IR and to determine the Minimum Inhibitory Concentrations (MIC) of bacterial metabolites against *Candida albicans.*

Materials and Methods

Paper chromatography (Tomita, 1988)

The filter paper and four solvents such as 20% NH_4Cl, *n*-butanol saturated with water, *n*-butanol-acetic acid-water (3:1:1) and ethyl acetate saturated with water were used for preliminary characterization of antifungal metabolites. The obtained fermented broth sample was applied on the paper and allowed to dry. The papers were chromatogram in each solvent. Then, bioautography was done to check the antifungal activity of each. Each paper was placed on assay agar plate. After one hour the paper was taken out, and then the plates were incubated for 24-36 hours.

Extraction of antifungal metabolites (Natarajan *et al.*, 2010)

The bacterium was cultivated on nutrient agar medium by inoculating selected bacterium culture in 500 mL conical flask containing 250 mL of the medium. The flask was incubated at room temperature for 2 days. After incubation period, fermentation broth of the bacterium was filtered with filtered paper. The filtrate was extracted with equal ratio of ethyl acetate. Then the mixture was shaken in a separating funnel. The organic layer was separated and collected.

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

Thin layer chromatography (TLC) was performed to know the constituent of metabolites of ethyl acetate crude extract from the culture broth of *Pseudomonas* sp. Using pet-ether: ethyl acetate (10:1 and 1:1), chloroform: ethyl acetate, and chloroform: methanol (100:1-1:1) eluting solvent ratios were used. Active culture extract was applied on the TLC plate (1 cm × 6cm). The developing chromatogram was checked under UV lamp (254 nm and 365 nm) and noted down the fluorescence spots. TLC chromatogram was examined with some colour reaction tests such as 5% sulphuric acid, 5% ferric chloride, anisaldehyde/sulphuric acid, vanillin/sulphuric acid and iodine vapour.

Isolation of some organic metabolites by silica gel column chromatography (Simon and Gray, 1998)

According to thin layer chromatographic analysis, the ethyl acetate extract residue of isolated bacterium *Pseudomonas* sp. metabolite was developed to isolate the active compound by silica gel column chromatography with $CHCl_3$: MeOH as eluting solvent. Silica gel (60-200 mesh) (ca. 50g) was dissolved in chloroform and the column was packed by the wet method. EtOAc crude extract (0.58g) was then passed through silica gel column and eluted with chloroform: methanol 90:1-10:1v/v. Fractions of each equal to 2 mL, were collected individually, the compounds present were checked with TLC.

Characterization and identification of isolated antifungal compounds

In an attempt to characterize the isolated antifungal compounds, the following tests were performed:

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, $CHCl_3$ and PE in order to known their solubility.

Determination of some chemical properties of isolated compounds

Some coloured reagent such as aq. Potassium permanganate, Iodine vapour, Anisaldehyde/ sulphuric acid, 5% Sulphuric acid, 5% Ferric chloride, Vanillin/sulphuric acid and 2,4 Dinitrophenylhydrazine (DNP) were used to study their behavior on TLC.

Study under UV-visible spectroscopy

For the identification of isolated compounds, ultra violet absorption spectra were also recorded and examined. A Shimadzu UV-1800 UV- visible spectrophotometer at Department of Chemistry, Pathein University were used.

Study under FT IR spectroscopy

The FT IR spectra of isolated compounds were sample recorded by spectrum II spectrophotometer (Perkinelmer) FT IR Fourier Transform Infrared at Department of Chemistry, Pathein University.

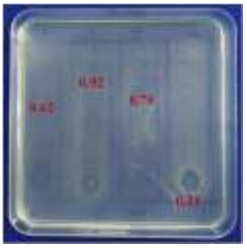
Minimum Inhibitory Concentration (MIC) of isolated compounds

Minimum Inhibitory Concentration (MIC) was carried out by two fold serial dilution method (Andrew, 2001). The using experimental concentrations were ranging from 20.0 µg/mL, 10.0 µg/mL, 5.0 µg/mL, 2. 5 µg/mL 1.25 µg/mL, 0.625 µg/mL, 0.312 µg/mL, 0.156 µg/mL and 0.078 µg/mL respectively. The test organism was *Candida albicans.* After incubation for 24 hours, the MIC were determined by selecting the lowest concentration of metabolite which caused complete inhibition of test growth.

Results

Paper chromatography

In this study, four kinds of solvents 20% NH_4Cl, *n*-butanol saturated with water, *n*-butanol acetic acid-water (3:1:1), ethyl acetate saturated with water were used. Ethyl acetate was more extractable the antifungal metabolites than other solvents. The chromatography bioautographic assay was shown in Figure 1.



1. 20% NH_4Cl
2. *n*-butanol saturated with water
3. *n*-butanol-acetic acid – water (3:1:1)
4. ethyl acetate saturated with water

Figure 1 Bioautographic assay of paper chromatography

Antifungal activity of metabolites in *Pseudomonas* sp. extracted with different volume of EtOAc

From the results of paper chromatography, using ethyl acetate extract (1:1 v/v) resulted in inhibition zone was 17.79 mm, followed by 16.83 mm and 16.60 mm in ethyl acetate extract with fermentation broth (2:1 v/v) and (3:1 v/v) respectively. These results were showed in Table 1 and Figure 2.

Table 1 Antifungal activity of *Pseudomonas* sp. extracted with different ratio of EtOAc to FB on *Candida albicans*

Different ratio of solvent: fermented broth(v/v)	Inhibition diameter Zone (mm)
1:1	19.79
2:1	16.83
3:1	16.60

Well size =8mm

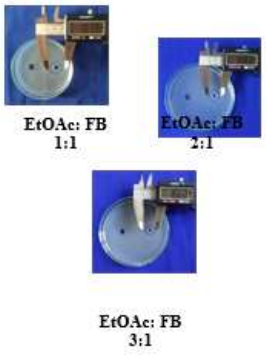


Figure 2 Antifungal activity of *Pseudomonas sp.* extracted with different ratio EtOAc to FB on *Candida albican*

Extraction of antifungal metabolites

10 liters of selected bacterium *Pseudomonas* sp. were fermented in suitable synthetic fermentation medium (48 hrs, 20% size of inoculum, temperature 35º C, pH 7, 2 days fermentation period, under shaking culture) and extracted with equal ratio of ethyl acetate to fermented broth (1:1 v/v) to yield 2.0 g was obtained from the culture filtrate.

Thin layer chromatographic analysis

Thin layer chromatography (TLC) was performed on ethyl acetate crude extracted by employing various solvent system. The extract showed well- separated spots on TLC by using $CHCl_3$:MeOH solvent systems under UV 254 nm and 365 nm and some color reagent tests. Therefore, the solvent system $CHCl_3$:MeOH was chosen to isolate pure compounds by silica gel column chromatography.

Isolation of some organic metabolites by silica gel column chromatography

Gradient elution was performed successively with increasing polarity. According to the procedure in Figure 4, compound M-1 (colorless needle shape 1.1 mg) and compound M-2 (colorless amorphous powder, 5.6 mg) and compound M-3 (colorless amorphous powder, 5.8 mg) were obtained from the respective fractions F-I, F III and F-IV. The remaining fractions F-II and F-V were observed as mixtures and no antifungal activity was recorded. These isolated compounds M-1, M-2 and M-3 have significant activity on *Candida albicans* with inhibitory zone 19.66 mm, 18.06 mm and 16.43 mm respectively. Thin layer chromatogram of compounds (M-1, M-2 and M-3) and their antifungal activity were presented in Figures 3 and 11.

(a)

Compound M-1
Solvent System : $CHCl_3$
R_f value : 0.61 ($CHCl_3$: MeOH, 120 : 1)
Spraying reagent : 5% H_2SO_4, heat
Amount : 1.1 mg

(b)

Compound M-2
Solvent System : $CHCl_3$:MeOH
R_f value : 0.43 ($CHCl_3$:MeOH 20:1)
Spraying reagent : 5% H_2SO_4, heat
Amount : 5.6 mg

(c)

Compound M-3
Solvent System : $CHCl_3$:MeOH
R_f value : 0.26 ($CHCl_3$:MeOH 20:1)
Spraying reagent : 5% H_2SO_4, heat
Amount : 5.8 mg

Figure 3 Thin layer chromatogram of isolated compounds (a) compound M-1, (b) compound M-2 and (c) compound M-3

Procedure for selection of stationary phase

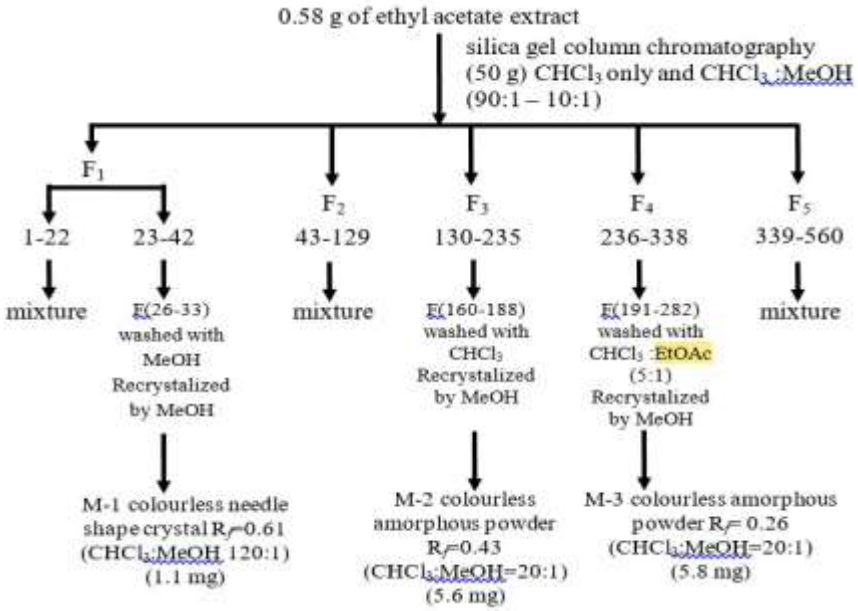


Figure 4 Flow diagram of separation of compounds from ethyl acetate extract of selected bacterium *Pseudomonas* sp. by column chromatography with $CHCl_3$:MeOH

Characterization of isolated antifungal compounds

The isolated compounds were characterized by physicochemical tests, solubility tests, modern spectroscopic techniques such as UV and FT IR. These resultant data were given as follow;

Table 2 Chemical Reagent Tests of Isolated Compounds M-1, M-2 and M-3

Reagent	Observation on Isolated Compounds			Remark		
	M-1	**M-2**	**M-3**	**M-1**	**M-2**	**M-3**
Anisaldehyde/ sulphuric acid, Δ	violet	purple	purple	steroid	steroid	steroid
Vanillin/ sulphuric acid, Δ	chared	chared	chared	steroid	steroid	steroid
5% $FeCl_3$	no brown colour	no brown colour	no brown colour	no	no	no
I_2vapour	yellow colour change purple to colourles	yellow colour immediately change purple to colourless	yellow colour immediately change purple to colourless	phenolic double bond double bond	phenolic double bond double bond	phenolic double bond double bond
aq.$KMnO_4$						
2, 4- Dinitrophenyl hydrazine	no ppt.	yellow ppt.	yellow ppt.	no carbonyl group	carbonyl group	carbonyl group

ppt. = precipitate (Δ) = heat

Identification of isolated compounds from ethyl acetate extract of bacterium *Pseudomonas* sp.

Compound M-1

It was soluble in EtOAc, MeOH, PE and $CHCl_3$ but insoluble in H_2O. The R_f value of compound M-1 was found to be 0.61 in $CHCl_3$ only solvent system, it gave yellow spot on TLC chromatogram with iodine vapour, violet spot with anisaldehyde/sulphuric acid followed by heating. According to the UV absorption spectral data of compound M-1, the maximum wavelength in methanol is 231 nm. This wavelength indicated to be the presence of π bond in compound M-1. The FT IR spectrum of compound M-1 is illustrated in Figure 5, 6 and the corresponding data assignments are interpreted in Table 3,4. According to the results of the physicochemical properties, R_f value, UV and FT IR spectral data, isolated compound M-1 may be sterol.

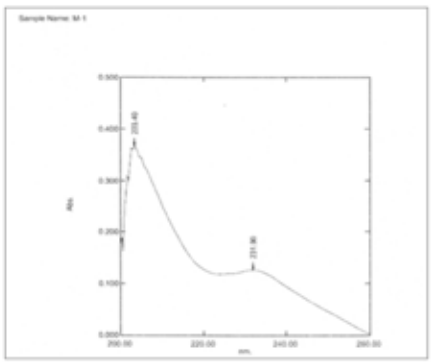


Figure 5 UV spectrum of isolated compound M-1

Table 3. UV Spectral Data of Isolated Compound M-1

Observed λ_{max} (nm) in methanol	* Remark
231	double bond

*(Kasal *et al.*, 2010)

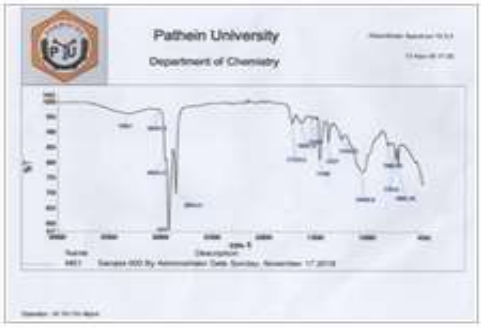


Figure 6 FT IR spectrum of isolated compound M-1

Table 4 FT IR Spectral Data of Isolated Compound M-1

Wave number (cm^{-1})		Band Assignment
Observed	*Literature	
3307	3625-3200	Streching O-H in alcohol
3024	3050-3000	Streching C-H for -HC=C-
2921	2970-2850	Streching C-H in CH_2 and CH_3
1632	1635-1605	Streching C=C for alkane
1459	1475-1448	Bending C-H in CH_3
1377	1390-1370	Bending C-H in CH_2
1048	1060-1000	Streching C-O in alcohol

(*Kasal *et al.*, 2010)

Compound M-2

It was soluble in EtOAc, MeOH and $CHCl_3$ but insoluble in PE, and H_2O. The R_f value of compound M-2 was found to be 0.43 in $CHCl_3$:MeOH (20:1 v/v) solvent system and it gave yellow spot on TLC chromatogram with iodine vapour, purple spot with anisaldehyde/sulphuric acid followed by heating, yellow color with 2, 4 DNP. According to the UV absorption spectral data of compound M-2, the maximum wavelength in methanol are 227 nm, 276 nm and 284 nm. This wavelength indicated to be the presence of π bond and atom with non bonded electrons. According to the results of the physicochemical properties, R_f value, UV and FT IR spectral data, isolated compound M-2 may be steroid Table 5, 6 and Figure 7, 8.

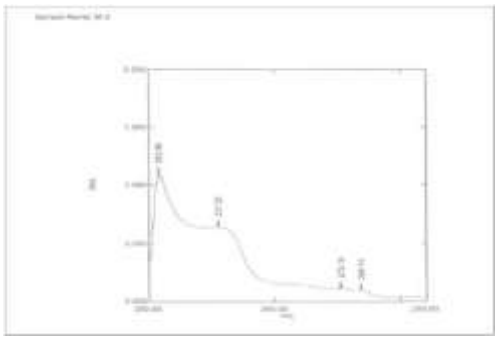


Table 5 UV Spectral Data of Isolated Compound M-2

λ_{max} in methanol (nm)	* Remark
227 276, 284	double bond atoms contained non bonded electrons

(*Kasal *et al.*, 2010)

Figure 7 UV spectrum of isolated compound M-2

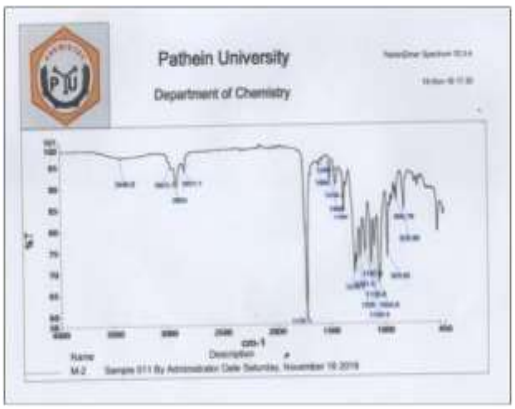


Figure 8 FT IR spectrum of isolated compound M-2

Table 6 FT IR Spectral Data of Isolated Compound M-2

Wave number (cm^{-1})		Band Assignment
Observed	*Literature	
2972-2851	2970-2850	Streching C-H in CH_2 and CH_3
1720	1800-1650	Streching C=O in carbonyl
1632-1605	1635-1605	Streching C=C in α, β unsaturated
1456	1475-1445	Bending C-H for methylene
1380	1390-1370	Bending C-H for methyl
1064	1060-1000	Streching C-O for alcohol
978, 896, 826	970-700	Streching of plane bending C-H in

(*Kasal *et al.*, 2010)

Compound M-3

It was soluble in EtOAc, MeOH, and $CHCl_3$ but insoluble in PE, and H_2O. The R_f value of compound M-3 was found to be 0.26 in $CHCl_3$:MeOH(20:1 v/v) solvent system and it gave yellow spot on TLC chromatogram with iodine vapour, purple spot with Anisaldehyde/sulphuric acid followed by heating, yellow color with 2, 4 DNP. According to the UV asorption spectral data of compound M-3, the maximum wavelength in methanol are 228 nm, 277 nm and 284 nm. This wavelength indicated to be the presence of π bond and atom with non bonded electrons. According to the results of the physicochemical properties, R_f value, UV and FT IR spectral data, isolated compound M-3 may be steroid.

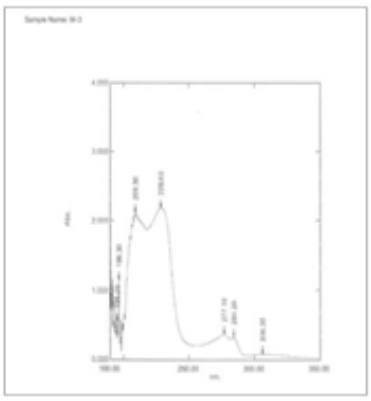


Figure 9 UV spectrum of isolated compound M-3

Table 7 UV Spectral Data of Isolated Compound M-3

λ_{max} in methanol (nm)	* Remark
228	double bond
277, 284	atom contained non bonded electrons

(*Kasal *et al.*, 2010)

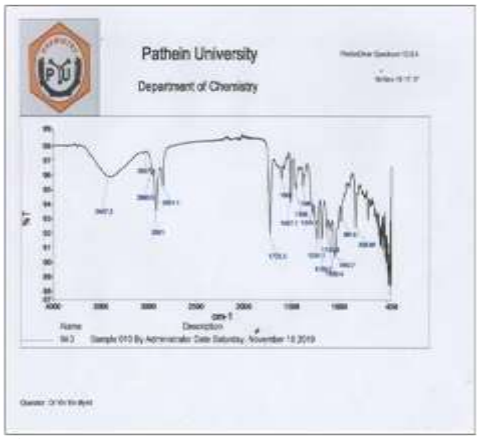


Figure 10 FT IR spectrum of isolated compound M-3

Table 8 FT IR Spectral Data of Isolated Compound M-3

Wave number (cm^{-1})		Band Assignment
Observe	***Literature**	
3407-2851	3550-3200	Streching O-H in alcohol
2921	2970-2850	Streching C-H in CH_2 and CH_3
2851	2970-2850	Streching C-H in CH_2 and CH_3
1723	1720-1700	Streching C=O in carbonyl
1608	1635-1605	Streching C=C in , β
1450	1457-1445	Bending C-H for methylene
1380	1390-1370	Bending C-H for methyl
1042	1260-1000	Streching C-O for OH
826	970-700	Bending C-H in CH_3

* (Kasal *et al.*, 2010)

Antifungal activity of isolated compounds

Bioassay for determination of isolated compounds were undertaken by agar well diffusion method. From the results, isolated compound M-1 showed antifungal activity (19.66 mm), compound M-2 exhibited (18.06 mm) and compound M-3 showed (16.43 mm) on *Candida albicans*

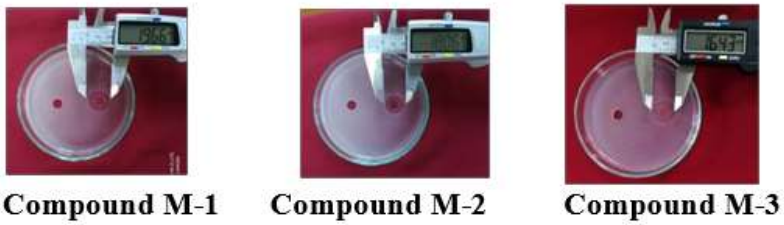


Figure 11 Antifungal activity of isolated compounds on *Candida albicans* by agar well diffusion method

Minimum Inhibitory Concentration (MIC) of isolated compounds

MICs were read in µg/mL after overnight incubation. It was observed that MIC value of compound M-1 was 1.25 µg/mL and for compound M-2 was 0.625 µg/mL but M-3 did not exhibit antifungal activity against *Candida albicans* in this experimental concentration range of 20 µg/mL to 0.078 µg/mL

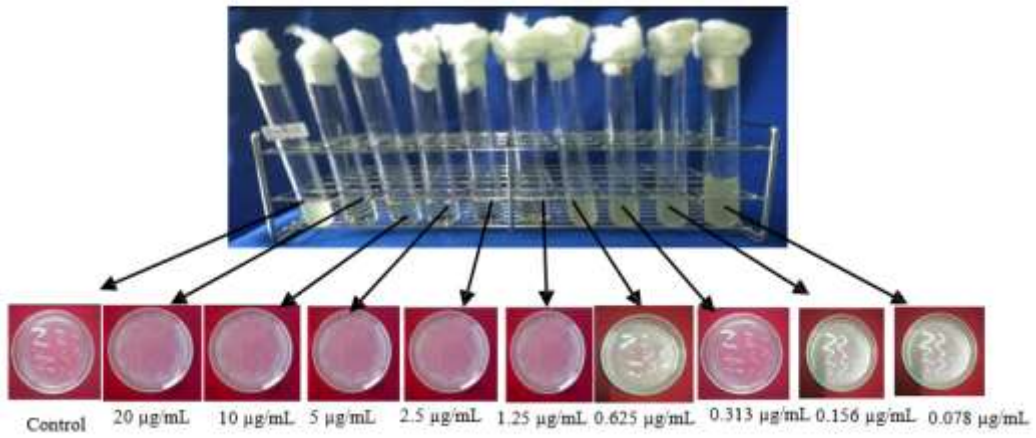


Figure 12 Minimum inhibitory concentration of secondary metabolites from compound M-1 on *Candida albicans*

Agar well size = 8 mm

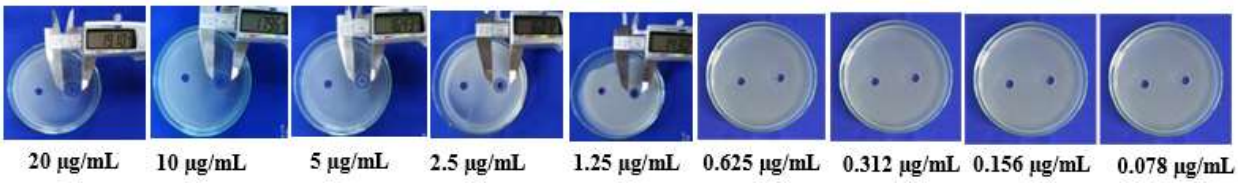


Figure 13 Minimum inhibitory concentration of secondary metabolites from compound M-1 on *Candida albicans* (agar well diffusion method

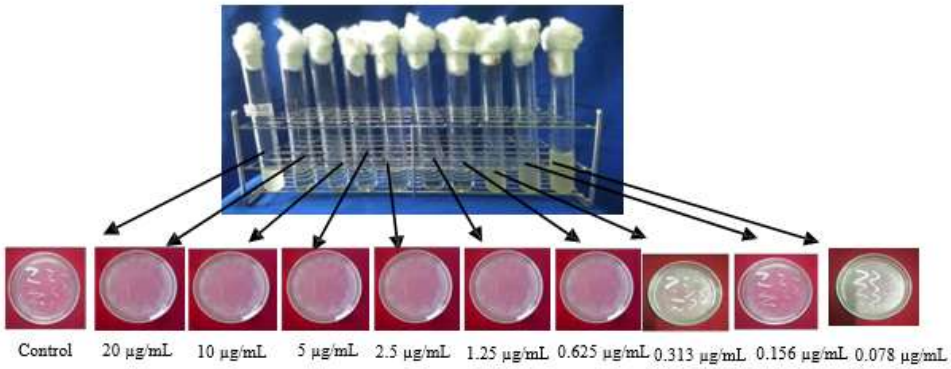


Figure 14 Minimum inhibitory concentration of secondary metabolites from compound M-2 on *Candida albicans*

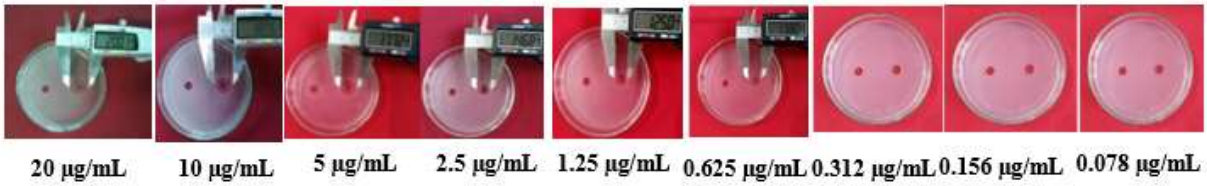


Figure 15 Minimum inhibitory concentrations of secondary metabolites from compound M-2 on *Candida albicans* (agar well diffusion method)

Discussion and Conclusion

The production of secondary metabolites from Pseudomonas species has been the most economical and biotechnological sources for the discovery of new bioactive compounds. In the investigation of paper chromatography, four kinds of different solvents were applied to observe the optimum extraction ability of secondary metabolites. Ethyl acetate was more extractable the antifungal metabolites than other solvents. On studying antifungal activity of bacterium extracted with different ratio (1:1, 2:1, 3:1 v/v) of EtOAc. The equal ratio of ethyl acetate extract to fermented broth showed the highest activity (19.79 mm). Jain and Pundir, 2011 reported that fermentation broth and ethyl acetate solvent (1:1 v/v) was applied and the maximum antimicrobial metabolite was obtained by using this ratio.

In this study, for containing the organic constituents in crude extract was examined by various solvent systems with TLC method. The crude extract showed well separated spots on TLC by using chloroform: methanol (1:1 v/v) solvent system. According to the TLC result, ethyl acetate crude extract (0.58 g) was subjected with silica gel column chromatography and eluted with starting from the optimized solvent systems used was chloroform: methanol at ratio 90:1, 70:1, 50:1, 30:1 and 10:1v/v. The isolated fractions were collected by same characteristic of TLC chromatogram which ultimately resulted in major five fractions after examined UV lamp (254 nm and 365 nm) as well as some color reaction tests. Compounds M-1, M-2 and M-3 were obtained from the respective fractions F-I, F-III, and F-IV and remaining fractions F-II and F-V were observed as mixture. The fraction (F-I) $f_{26\text{-}33}$; isolated compound M-1 with R_f value 0.61 showed antifungal activity 19.66 mm. Inhibitory zones 18.06 mm was observed in fraction (F-III) $f_{160\text{-}188}$; isolated compound M-2 with R_f value 0.43 and fraction (F-IV) $f_{191\text{-}282}$; isolated compound M-3 with R_f value 0.26 showed 16.43 mm inhibitory zone. The purified active compounds obtained were subjected to various examinations such as some chemical reagent tests, ultraviolet (UV) and FT IR (Fourier Transform Infrared). In order to these data, the isolated

compound, M1, M2 and M3 sterol and steroid respectively. Balandrin *et al*., 1998 described that steroids are a group of cholesterol derived lipophilic, low-molecular weight compounds found in derived from a variety of different marine, terrestrial, and synthetic sources. Steroid family includes the sterols, bile acids a number of hormones (both global and adrenal cortex hormones) and some hydrocarbons. In a study of Minimum Inhibitory Concentrations (MIC) of isolated compounds, the antifungal metabolites affected on the growth of *Candida albicans* at least MIC of 1.25 µg/mL for compound M-1 and 0.625 µg/mL for compound M-2 and compound M-3 did not exhibit antifungal activity against *Candida albicans* in this experimental range from 20 µg/mL to 0.078 µg/mL. A lower MIC is an indication of a better antimicrobial activity (Andrews, 2001).

The present study indicates that soil bacterium *Pseudomonas* sp. were isolated from mangrove soil for an antifungal compound against antibiotic resistant human pathogenic fungi *Candida albicans*. Purification and structure elucidation of active compound and investigation its molecular mechanisms can be a promising approach for further antimicrobial drug development programs.

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