# EXTRACTION OF ANTIFUNGAL COMPOUNDS FROM TR-2 OF MANGROVE WATER AND SLUDGE OF CHAUNG-THA AREA

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## Abstract

Extraction of antifungal metabolites from selected strain *Actinomycetes* was carried out by using paper chromatography and four solvent systems. Ethyl acetate was the most suitable solvent and $R_f$ value had 0.86. Crude ethyl acetate extract (2.0 g) was obtained from 10 L of fermented broth and purified over silica gel column chromatography. The ethyl acetate extract was carried out by employing various solvent systems on Thin Layer Chromatography (TLC). The results showed well separated spots on TLC by using the solvent system (pet ether: ethyl acetate). By silica gel column chromatographic separation, compound A (23 mg, white amorphous form) was isolated and subjected to various examinations such as chemical reagent tests, ultraviolet (UV) and FTIR (Fourier Transform Infrared). Based on the results of physicochemical properties and spectroscopic methods, compound A may be flavonoid. In the investigation of minimum inhibitory concentrations (MICs), the MIC value of compound A was 2.5 µg/mL on *Candida albicans*.

## Introduction

Chromatography is a separation method based on the partitioning of a solute between a mobile phase and a stationary phase. The mobile phase may be liquid, gas or a supercritical fluid. The stationary phase may be an immobilized liquid or a solid, in either a planar or column form. Based on the physicochemical characteristics of the analyte, and the availability of instrumentation, a chromatographic system is chosen to separate, identify and quantify the analyte (Tomita, 1988).

Marine sediment is an inexhaustible resource that has not been properly exploited. It is estimated that less than 1% of potentially useful chemicals from marine environment has been screened so far, with microbial products representing approximately 1% of the total number. Exploration of microbial secondary metabolites has led to the discovery of hundreds of biologically active compounds.

Members of the actinomycetes, which live in marine environment, are poorly understood and only few reports are available pertaining to microorganism from mangroves (Vikineswari 1997; Rathana and Chandrika, 1993).

Streptomycin was one of the first antibiotics found. It is produced by *Streptomyces griseus.* Today, various *Streptomyces* species are responsible for approximately 75% of both medical and commercial antibiotics and work very well in these areas. Due to the need for new antibiotics, studies have steered towards the isolation of streptomyces and the careful screening of different habitats in which they are used. It has also been found through research that different conditions such as nutrients, culturing, and other factors may affect how *Streptomyces* develop to form antibiotics (Schatz *et al.*, 2005).

Thin layer chromatography is the most familiar and efficient technique method used for the detection, analysis and separation of the bioactive compounds, so it is probably that 60% of the analyzed compounds are performed based on TLC over international.

Thus, it is important to know the basic operation and performance of the TLC protocol (Maitland *et al.*, 2010).

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Thin layer chromatography has been important for the separation of secondary metabolites, Polyphenol, saponin, alkaloids, flavonoid, aromatic amines, amino acids, alcohols, acids, glycols, proteins, amides, antibiotics, peptides, pesticides, bile acids, vitamins and porphyrins in soft drinks (Bhawani *et al.*, 2012).

The bioautography is one of the techniques useful in direct tracing out bioactive compounds from extracts on thin layer chromatogram. Antifungal bioautographic assays system have used classically one- dimensional thin layer chromatography (TLC) to separate the chemical constituent from the extract (Wedge and Nagle, 2000).

Antifungal metabolites can be readily located on the plates by visually observing clear zones where active compound inhibit fungal growth. 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). And MICs are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials (Mirbach & Ali, 2005).

The aim and objectives of this research were to study organic compound from the ethyl acetate extract of the isolate TR-2 by using column chromatographic separation techniques, to characterize the isolated compound by physiochemical tests and spectroscopic techniques such as UV, FT IR and to determine the Minimum Inhibitory Concentrations (MICs) of metabolite on *Candida albicans*.

## Materials and Methods

### Paper Chromatography Method (Tomita, 1988)

To study of extraction of antibacterial metabolites of selected TR-2 was work done by using paper chromatography. The filter paper and four solvents; 20% NH4Cl, n-butanol saturated water, n-butanol-acetic acid-water (3:1:1) and ethyl acetate saturated with water, were used for preliminary characterization of compound.

The obtained fermented broth samples (100 mL) were applied on the paper and allowed to dry. The papers were chromatographed 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, then the plate were incubated for 24-36 hours. In this case, the inhibitory zone was measured yielding a $R_f$ value for the corresponding bioactive compound.

$$R_f \text{ value} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

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

Thin layer chromatography (TLC) was performed on ethyl acetate crude extract from the culture broth. The crude fraction was spotted on TLC plate and performed by using various solvent systems. Spots were observed under UV light or by spraying spot with iodine vapour and $R_f$ values were recorded.

$$R_f \text{ value} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

### Silica gel column chromatography (Simon & Grey, 1998)

According to thin layer chromatographic analysis, the ethyl acetate extracted residue of TR-2 metabolite was developed to isolate the active compound by silica gel column chromatography with pet ether: ethyl acetate and ethyl acetate: methanol as eluting solvent. The

silica gel (ca. 50 g) was dissolved in chloroform and the column was packed by the wet method. EtOAc crude extract (2.0 g) was then passed through silica gel column and eluted with pet ether: ethyl acetate solvent (3:1, 2:1, 1:1 and ethyl acetate only). Fractions of each equal to 2 mL, were collected individually, the compound present were checked with TLC and examined the activity with *Candida albicans* by using agar well method.

Column size - (61 ×2 cm)
Flow rate - 1 mL/ 1 min
Eluted solvent - PE: EtOAc

**Figure 1** Silica gel column chromatography

**Determination of solubility of isolated compound**

Each of isolated compound (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 solubility.

**Characterization and Identification of Isolated Compounds**

- Determination of $R_f$ value
- Classification by using chemical reagents
- Identification of isolated compound by using modern spectroscopic methods such as: UV, FT IR spectroscopy as well as by comparing with reported data

**Determination of Minimum Inhibitory Concentrations (MICs) (Domain, 1999; Jennifer, 2006)**

Minimum inhibitory concentration (MICs) was determined by serial dilution method. The concenteations were 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL and 0.312 µg/mL respectively. The selected test organism was *Candida albicans*. After incubation for 24 hours, the MICs values were determined by selection the lowest concentration of antibacterial metabolites which caused complete inhibition of test growth.

## Results

**Extraction of antifungal compounds from TR-2**

In this study, TR-2 was carried out by using paper chromatography and four kinds of solvent 20% $NH_4Cl$, n-butanol saturated water, n-butanol-acetic acid-water (3:1:1) and ethyl acetate saturated with water, were used. According to $R_f$ value, ethyl acetate was the most suitable solvent and $R_f$ value was (0.86) in this solvent.

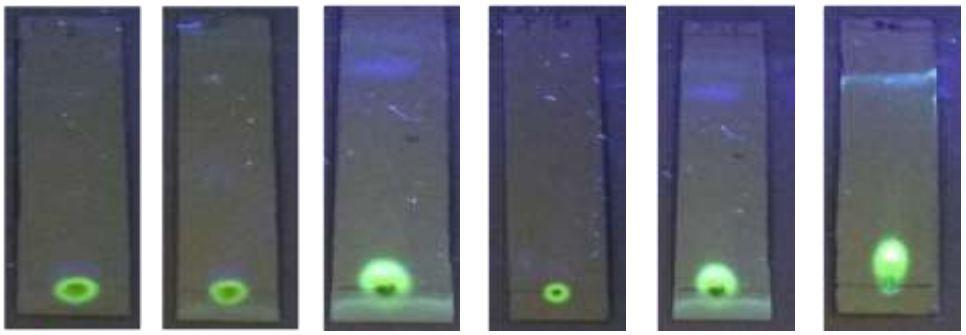
1. 20% $NH_4Cl$
2. n-butanol saturated with water
3. ethyl acetate-acetic acid water (3:1:1)
4. ethyl acetate saturated with water

**Figure 2** Paper Chromatography bioautographic assay

**Thin layer chromatographic analysis with various solvent systems**

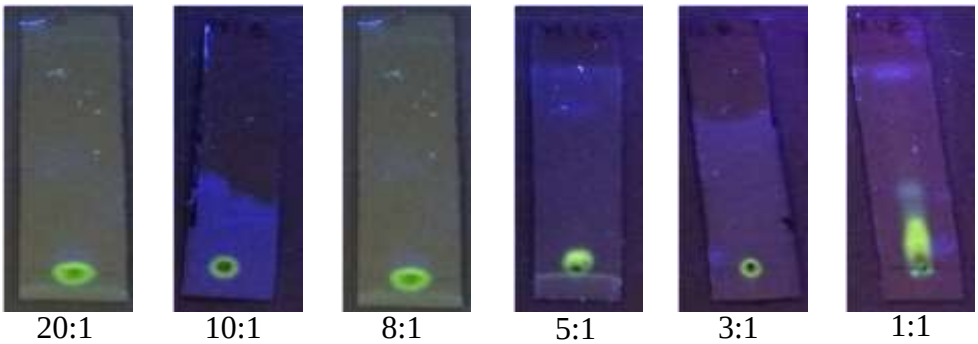
Thin Layer Chromatography was performed on ethyl acetate crude extracts by employing solvent systems: ethyl acetate only, chloroform only, pet ether only, methanol only, hexane only, pet ether: ethyl acetate (20:1, 10:1, 8:1, 5:1, 3:1 and 1:1 v/v), hexane: ethyl acetate (20:1, 10:1, 8:1, 5:1, 3:1 and 1:1 v/v), ethyl acetate: methanol(70:1, 30:1, 10:1, 8:1, 4:1 and 2:1), ethyl acetate: pet ether (20:1, 10:1, 5:1, 4:1, 2:1 and 1:1). The extract showed well- separated spots on TLC by using solvent systems. Therefore, (pet ether: ethyl acetate, ethyl acetate only and ethyl acetate: methanol) solvent systems were chosen to isolate pure compounds by silica gel column chromatography.


**Figure 3** Thin layer chromatographic analysis with various solvent systems



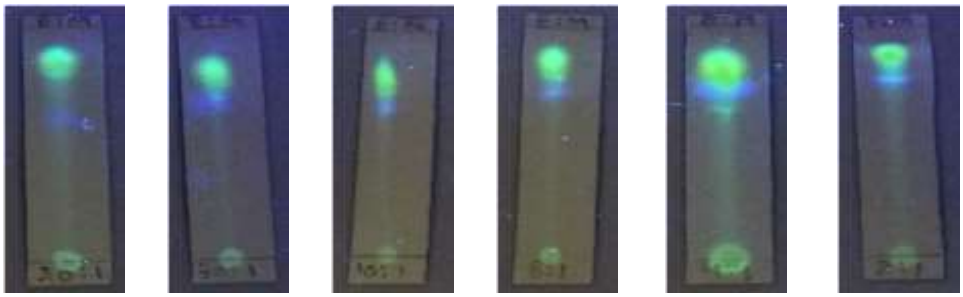

**PE: EtOAc**

**Figure 4** Thin layer chromatographic analysis with different ratios of pet ether: ethyl acetate




**Hexane: EtOAc**

**Figure 5** Thin layer chromatographic analysis with different ratios of Hexane: ethyl acetate




**EtOAc: ME**

**Figure 6** Thin layer chromatographic analysis with different ratios of ethyl acetae: methanol


**EtOAc: PE**

**Figure 7** Thin layer chromatographic analysis with different ratios of ethyl acetate: pet ether

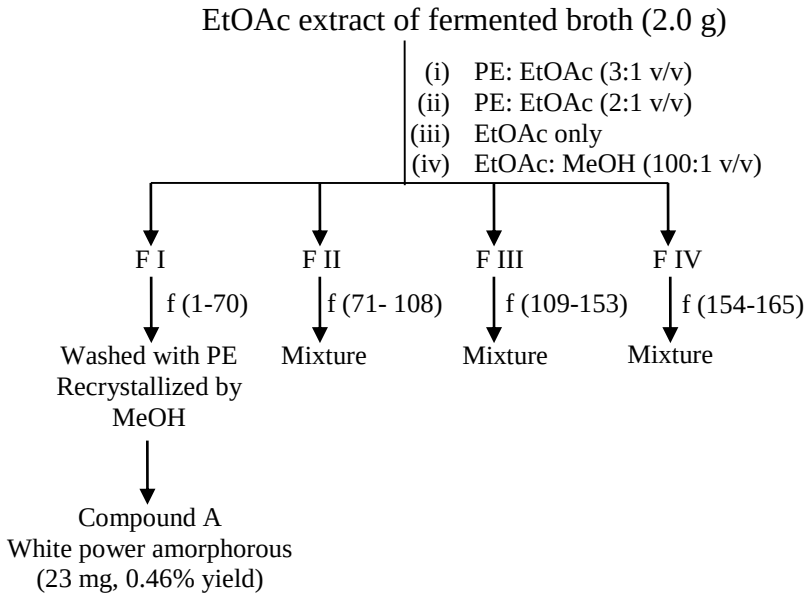


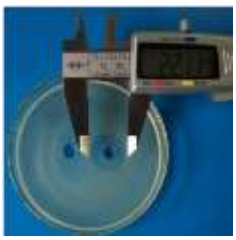

**Figure 8** Isolation of organic metabolites from ethyl acetate crude extract, culture broth of isolated Streptomyces TR-2 by column chromatography

### 3.3.3. Isolation of some organic compounds from ethyl acetate extract of the fermented broth TR-2

2.0 g of ethyl acetate crude extract from fermented broth TR-2 was separated by column chromatography using silica gel absorbed and eluted with pet ether : ethyl acetate (3:1, 2:1,

1:1 v/v), ethyl acetate only, ethyl acetate : methanol (100:1 v/v). Fraction were collected in accordance to their TLC profiles and tended for their antifungal activity with *Candida albicans.* The TLC plates revealed that the fractions were to be pooled into four main fraction (FI to FIV). The fraction FI (f 1-70) was evaporated and washed with pet ether and recrystallized with methanol, yielded 23 mg, white amorphous powder (Fig. 3.9). Its $R_f$ value was found to be 0.51 in (Hexane: EtOAc 15:1 v/v) solvent system and it gave brown colouration on TLC plate when spraying with 10% $FeCl_3$. The isolated compound A was active against on *Candida albicans* with inhibitory zone 22.11 mm. Thin layer chromatogram of compound A and its antifungal activity was presented in (Fig. 3.9). The remaining fractions (F II to F IV) were observed as mixture and no activity recorded against *Candida albicans.*

Compound A
Solvent system : Hexane: EtOAc (15:1)
$R_f$ value : 0.51
Spraying agent : $FeCl_3$



Inhibitory zone-22.11 mm

Compound A from ethyl acetate extract of TR-2

**Figure 9** Thin layer chromatogram of isolated compound A and its antifungal activity against *Candida albicans*

### 3.3.4. Characterization of isolated compound A

Some chemical properties of isolated compound from fermented broth TR-2 was characterized by spraying agents on TLC, solubility test, modern spectroscopic measurement such as UV and FT IR.

Compound A (23 mg, white amorphorous powder) was isolated from ethyl acetate extract of fermented broth TR-2. $R_f$ value of compound A was found to be 0.51 in Hexane: EtOAc (15:1 v/v) as shown in (Fig. 3.9.). Compound A was soluble in MeOH, EtOAc and $CH_2Cl_2$ but insoluble in PE, Hexane and EtOH as shown in (Table 3.4).

According to the result obtained from the chemical reagent tests, compound A gave yellow spot on TLC with iodine vapour, reddish brown spot with anisaldehyde followed by heating, brown spot with 10% $FeCl_3$ and 5% $H_2SO_4$ followed by heating and yellow ppt with 2, 4 DNP. These result was shown in Table 3.1.

Compound A was UV active because the presence of conjugated double bond. The UV absorption spectrum showed the absorption at 223, 267, 275 nm. These bands attributed to - * transistion and showed the presence of carbonyl compound (Fig. 3.11 and Table 3.2).

The functional groups present in compound A was studied by FT IR spectroscopy. FT IR spectrum was shown in (Fig. 3.12 and the interpreted spectral data was illustrated in Table 3.3. The FT IR spectrum of compound A showed the band at 3.383 $cm^{-1}$ due to O-H stretching vibration of

alcoholic group. Absorption band at 2924 $cm^{-1}$ and 2654 $cm^{-1}$ were due to C-H stretching vibration of $CH_2$ and $CH_3$ groups. Stretching band at 1729 $cm^{-1}$ indicated the presence of C=O stretching vibration of ketone group. The C=C stretching vibration of aromatic compound as observed at 1650 $cm^{-1}$. The band at 1462 $cm^{-1}$ was attributed to O-H bending vibration. The C=O stretching vibration of alcohol was shown as intense band at 1057 $cm^{-1}$. Absorption band at 796 $cm^{-1}$ was due to the C-H bending vibration of aromatic compound. From the results of physicochemical properties, $R_f$ value, UV and FT IR spectral data, the isolated compound A may be flavonoid.

**Table 1 Chemical properties of isolated compound from TR-2 metabolite**

| No | Separating agent | Compound A |
|---|---|---|
| 1 | 10% $KMnO_4$ | ND |
| 2 | Iodine | Yellow |
| 3 | Anisaldehyde | Reddish Brown |
| 4 | $FeCl_3$ | Brown |
| 5 | 5% $H_2SO_4$ | Brown |
| 6 | 2,4 DNP | Yellow ppt |

ND- not detected

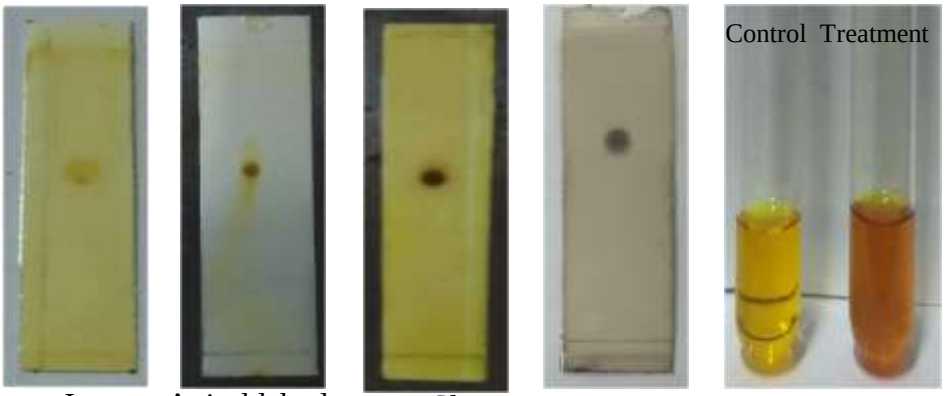



**Figure 10** Chemical reagent tests of isolated compound from TR-2 metabolite

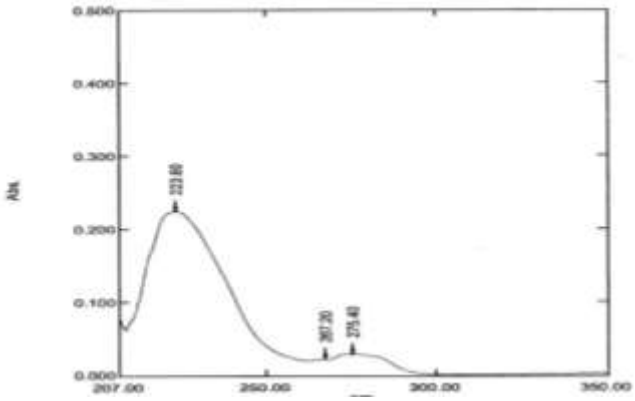



**Figure 11** UV spectrum of isolated compound A

**Table 2 UV spectrum of isolated compound A**

| Compound | $\lambda_{max}$ (nm) | Remark |
|---|---|---|
| A | 223, 267, 275 | $\pi \rightarrow \pi^*$ (carbonyl compound) |

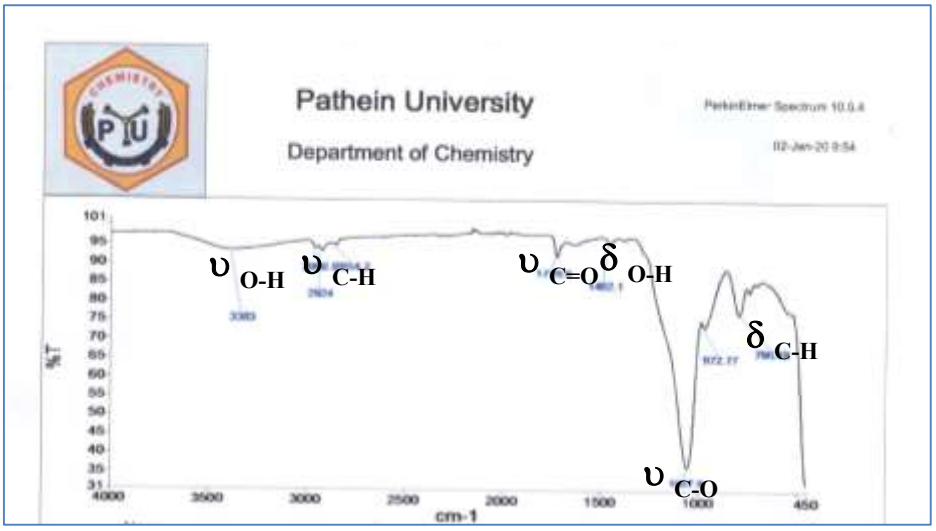



**Figure 12** FT IR spectrum of isolated compound A

**Table 3 FT IR spectral data of isolated compound A**

| Wave number ($cm^{-1}$) | Literature* Wave number ($cm^{-1}$) | Remark |
|---|---|---|
| 3383 | 3570-2900 | O-H stretching vibration |
| 2924, 2854 | 2935-2845 | C-H stretching vibration |
| 1729 | 1740-1675 | C=O stretching vibration |
| 1650 | 1655-1580 | C=C stretching vibration |
| 1462 | 1470-1280 | O-H bending vibration |
| 1057 | 1270-1025 | C=O stretching vibration |
| 796 | 900-670 | C-H bending vibration |

**Table 4 Solubility test for selected Streptomyces TR-2**

| No | Solvent | Result |
|---|---|---|
| 1. | MeOH | + |
| 2. | EtOAc | + |
| 3. | PE | - |
| 4. | $CH_3Cl_2$ | + |
| 5. | Hex | - |
| 6. | Ethanol | - |

\+ Soluble
\- Insoluble

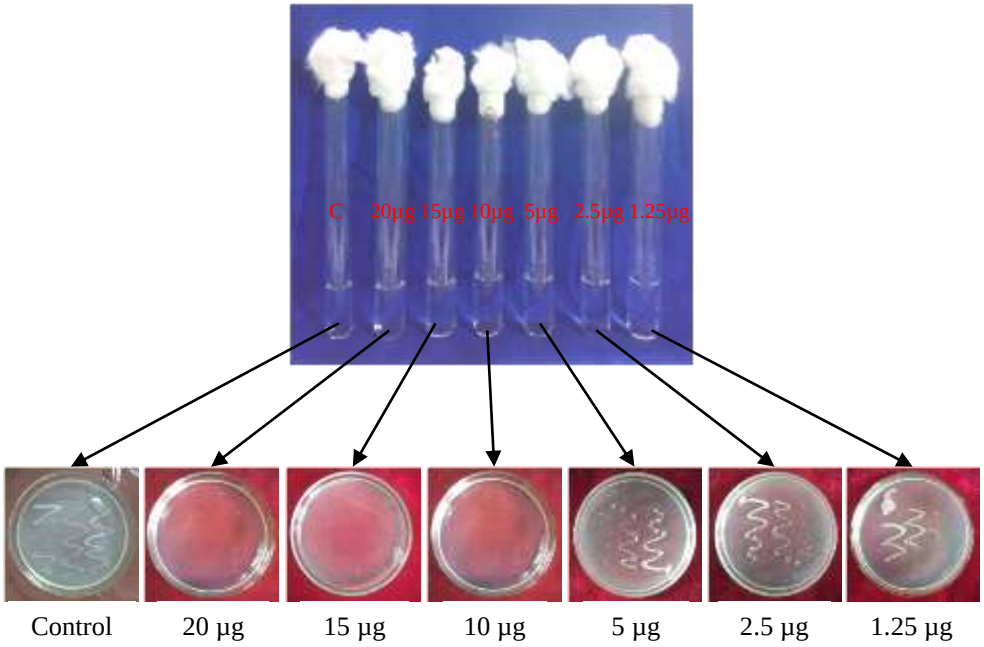



**Figure 13** Minimum inhibitory concentrations of secondary metabolites from compound A on *Candida albicans* (Streak culture method)

**Table 5 Minimum inhibitory concentrations of secondary metabolites from compound A on *Candida albicans***

| MIC values of compound A (µg/well) | Antifungal activity (mm) |
|---|---|
| Control | - |
| 10 | 10.78 |
| 5 | 8.91 |
| **2.5** | **8.87** |
| 1.25 | - |
| 0.625 | - |
| 0.312 | - |

- No activity

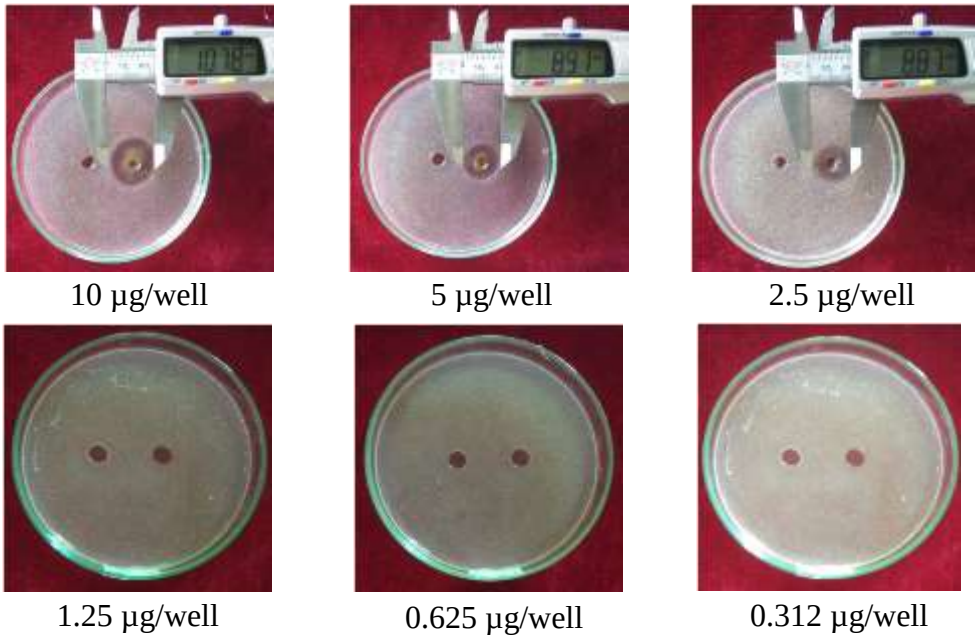



**Figure 14** Minimum inhibitory concentration of secondary metabolites from compound A on *Candida albicans* (Agar well diffusion method)

## Discussion and Conclusion

Abdul Wahab *et al.,* 2015, who documented that ethyl acetate provided the best solvent for the extraction of antimicrobial metabolites followed by chloroform.

Ripa *et al.,* 2010 noticed that ethyl acetate extract of *Streptomyces* sp. which showed antibacterial activity against a number of both Gram positive and Gram negative bacteria but did not have antifungal activity under in vitro conditions.

Differently, ethyl acetate extract of culture broth of selected *Streptomyces* TR-2 possessed antifungal activity on *Candida albicans*.

Thin layer chromatography (TLC) is very helpful in the identification and comparison of antibiotics, but very few results from the application of this technique have been published so far (Alfred and David, 1955).

Tirta *et al.,* 2017 reported that the metabolites from potent strain was produced by extraction of culture filtrate with ethyl acetate: methanol (4:1), it was tested for their antifungal activity by well diffusion method and it showed good antifungal activity.

In this study, thin layer chromatography (TLC) was performed on ethyl acetate crude extracted by employing solvent systems: 100% ethyl acetate, 100% chloroform, 100% pet ether, pet ether: ethyl acetate (20:1, 10:1, 8:1, 5:1, 3:1 and 1:1 v/v), 100% hexane only, hexane: ethyl acetate (20:1, 10:1, 8:1, 5:1, 3:1 and 1:1 v/v), 100% methanol only, ethyl acetate: methanol (70:1, 30:1, 10:1, 8:1, 4:1 and 2:1), ethyl acetate: pet ether (20:1, 10:1, 5:1, 4:1, 2:1 and 1:1). The extract showed well- separated spots on TLC by using pet ether: ethyl acetate solvent system (3:1 v/v).

Pandey *et al.,* 2011 suggested that the minimum inhibitory concentration is the minimum concentration of the antibacterial or antifungal agent in a given culture medium below which bacterial or fungal growth is not inhibited.

Likewise, on the studying the Minimum Inhibitory Concentrations (MICs), it was found that the MICs value of selected *Streptomyces* was 2.5 µg/ well on *Candida albicans* (8.87 mm).

Vineeta *et al.*, 2018 suggested that the compound extraction with ethyl acetate was found the maximum soluble in methanol, suggesting its polar nature.

In the study on the solubility test for compound A, it was observed that the compound was maximum soluble in methanol, ethyl acetate and ethanol and minimum soluble in petroleum ether, hexane and chloroform. Thus, compound A was the polar nature.

Oskay, 2011 also noticed that UV and Infrared spectrometry (IR) are being routinely used for the analytical estimation of various antibiotics. UV Scans of his strain dissolved in methanol were performed. Absorbance maxima were obtained at 275 and 286.5 nm, indicating the existence of a carbon- carbon double bond.

Similar to Oskay results, absorbance maximum of UV spectrum for the isolated compound A was obtained 275 nm.

According to the column chromatographic separation techniques, physiochemical tests and spectroscopic techniques such as UV and FT IR, it can be concluded that the isolate compound A may be flavonoid.

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