CHARACTERIZATION OF ANTIFUNGAL COMPOUNDS FROM ISOLATED ENDOPHYTIC FUNGUS *ASPERGILLUS DURICAULIS*

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Abstract

In the present research, the antifungal compounds isolated from endophytic fungus, *Aspergillus duricaulis* was carried out by paper chromatography using the solvent 20% NH_4Cl, ethyl acetate saturated with water, n-butanol saturated with water, n-butanol-acetic acid- water (3:1:1) for the extraction of antifungal metabolites against *Candida albicans*. And then, the fungal culture filtrate was studied by the ratio of two solvents, ethyl acetate and n-butanol to fermented broth (1:1, 2:1, 3:1v/v). The equal ratio (1:1 v/v) ethyl acetate extract showed higher inhibitory effect (26.75 mm) than n-butanol extract (20.49 mm). Crude ethyl acetate extract (5.0 g) was obtained from 17 liter of fermented broth and subjected to purification over silica gel column chromatography with various solvent systems. By silica gel column chromatographic separation, compound A (aromatic primary amide, 23 mg colourless crystal) and compound B (aliphatic ester, 24 mg yellow semisolid) in hexane: ethyl acetate 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 investigation of minimum inhibitory concentrations (MICs), it was observed that MICs value of antifungal compound A and B were 0.625 µg/mL and 1.25 µg/mL on *Candida albicans* respectively.

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

Introduction

Endophytic fungi have the capability to produce bioactive compounds such as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones (Lee *et al*., 2008). The recovery and purification of the product is one of the most critical aspects of industrial fermentation process. The type of extraction method, duration of extraction, temperature, and the polarity of solvent used influence the quality and the concentration of bioactive components isolated from the raw material (Annegowda *et al*., 2013). Chromatography is a useful technique for the separation of compounds from a complex mixture, such as a fungal extract.

Based 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).

The minimum inhibitory concentrations (MICs) 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 endophytic fungal 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 endophytic fungus *A. duricaulis* in this study. The aim and objectives of this study were to isolate some organic compounds from the ethyl acetate extract of *A. duricaulis*, to characterize the

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isolated compounds by physicochemical tests and spectroscopic techniques such as UV, FT IR and to determine the Minimum Inhibitory Concentrations (MICs) of fungal metabolites against *C.albicans.*

Materials and Methods

Paper chromatography (Tomita, 1988)

The filter paper and four solvents; 20% ammonium chloride (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 compound. The obtained fermented broth sample (100 µL) was 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, and then the plates were incubated for 24-36 hours. In this case, the inhibitory zone was measured yielding the R_f value for the corresponding bioactive compound.

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

The fungus was cultivated on PGA by inoculating selected endophyte culture in 500 mL conical flask containing 250 mL of the medium. The flask was incubated at 25˚C for 5 days. After incubation period, fermentation broth of the fungus was filtered with filter 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 on ethyl acetate crude extract from the culture broth of the endophyte. For this, the crude fraction was spotted (50 µL) on the TLC plate [GF_{254} silica gel precoated aluminium plate (Merck)] and chromatography was performed by employing solvent system chloroform: methanol (9:1, 8:2, 7:3 v/v), dichloromethane: methanol (9:1, 5:1, 2:1, 1:2 v/v) and hexane: ethyl acetate (9:1, 5:1, 2:1, 1:2 v/v). Spots were visualized by spraying with sulphuric acid.

Isolation of organic metabolites by silica gel column chromatography

(Simon and Gray, 1998)

According to thin layer chromatographic analysis, the ethyl acetate extract residue of isolated fungus *A. duricaulis* metabolite was developed to isolate the active compound by silica gel column chromatography with hexane: ethyl acetate as eluting solvent. Silica gel (60- 120 mesh) (ca.50g) was dissolved in Hexane: EtOAc 80:1 v/v and the column was packed by the wet method. EtOAc crude extract (3.0 g) was then passed through silica gel column and eluted with Hexane: EtOAc 80:1, 40:1, 20:1, 9:1, 5:1, 2:1, 1:1, 1:2 v/v. Fractions of each equal to 2 mL, were collected individually, the compounds presents were checked with TLC.

Characterization 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 water (H_2O), methanol (MeOH), ethyl acetate (EtOAc), chloroform ($CHCl_3$), pet-ether (PE) and Hexane (C_6H_{14}) in order to know their solubility.

Determination of some chemical properties of isolated compounds

Some coloured reagents such as 10% potassium permanganate ($KMnO_4$), iodine (I_2) vapour, Anisaldehyde, 5% sulphuric acid (H_2SO_4), 5% ferric chloride ($FeCl_3$), Lieberman Burchard, and 2,4 Dinitrophenylhydrazine (DNP) were used to study their behaviour on TLC.

Study under ultraviolet (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 Chemistry Department, Pathein University were used.

Study under Fourier transform (FT IR) spectroscopy

The FT IR spectra of isolated compounds were sampled with 1% KBr pellet and recorded by using Shimadzu FT IR- 8400 Fourier Transform Infrared spectrophotometer at Chemistry Department, Pathein University.

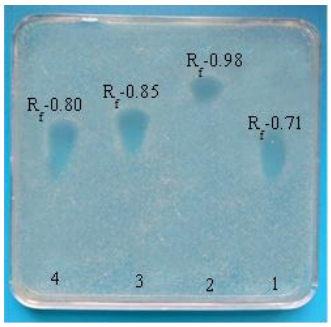
Minimum Inhibitory Concentration (MICs) of isolated compounds

Minimum Inhibitory Concentration (MICs) was carried out by two- fold serial dilution method (Domain, 1999 and Phay, 1997). The concentrations were 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL and 0.3.12 µg/mL respectively. The test organism was *Candida albicans*. After incubation for 24 hours, the MICs were determined by selecting the lowest concentration of metabolite.

Results

Paper chromatography

In this study, four kinds of solvent 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 R_f value (0.98), ethyl acetate was more extractable the antifungal metabolites than other solvent, followed by n- butanol solvent (0.85), ethyl acetate- acetic acid- water 3:1:1 (0.80) and the lowest R_f value at NH_4Cl (0.71) in Figure 1.



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

Figure 1 Paper chromatography bioautographic assay

Comparison of antifungal activity of metabolite in *A.duricaulis* extracted with different volume of EtOAc and n-BuOH

Using ethyl acetate extract (1:1v/v) resulted in inhibition zone was 26.75 mm, followed by 24.75 mm and 22.69 mm in ethyl acetate extract (2:1v/v) and (3:1v/v) respectively as well as inhibitory zone 20.49 mm was found in n- butanol extract (1:1v/v), 19.53 mm in n-butanol extract (2:1v/v) and 18.84 mm in n-butanol extract (3:1v/v). Therefore ethyl acetate extract (1:1v/v) of MZF-2 showed the higher inhibition zone than n- butanol extract (1:1v/v). There was no antifungal activity at all of lower layer. These results were shown in Table 1.

Table 1 Comparison of antifungal activity of isolated fungus *A. duricaulis* extracted with different ratio of EtOAc and n-BuOH against *C.albicans*

Different ratio of solvent: fermented broth (v/v)	Inhibition diameter zone (mm)	
	EtOAc extract	n-BuOH extract
1:1	26.75	20.49
2:1	24.75	19.53
3:1	22.69	18.84

Thin layer chromatographic analysis

Thin layer chromatography (TLC) was performed on ethyl acetate crude extracted by employing solvent system: chloroform ($CHCl_3$): methanol (MeOH) (9:1, 8:2, 7:3 v/v), hexane (C_6H_{14}): ethyl acetate (EtOAc) (9:1, 5:1, 2:1, 1:2 v/v) and dichloromethane (CH_2Cl_2): methanol (MeOH) (9:1, 5:1, 2:1, 1:2 v/v). The extract showed well- separated spots on TLC by using $CHCl_3$: MeOH and C_6H_{14}: EtOAc solvent systems. No spots were observed by using CH_2Cl_2: MeOH solvent system. These results were presented in Figure 2. Therefore the solvent system C_6H_{14}: EtOAc was chosen to isolate pure compounds by silica gel column chromatography.

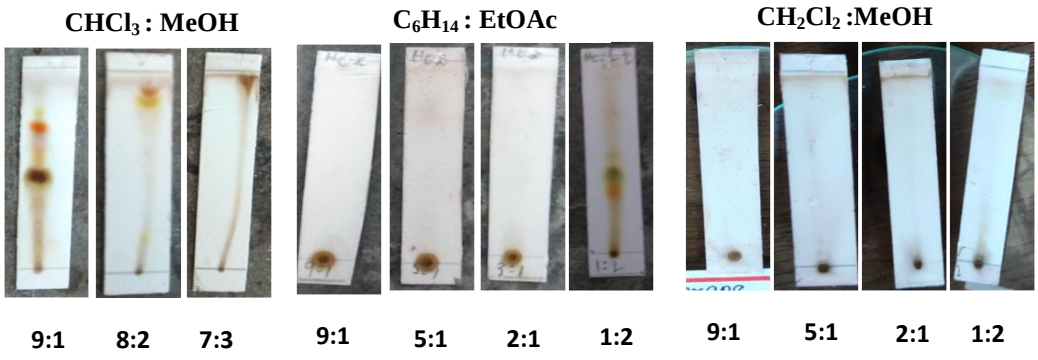
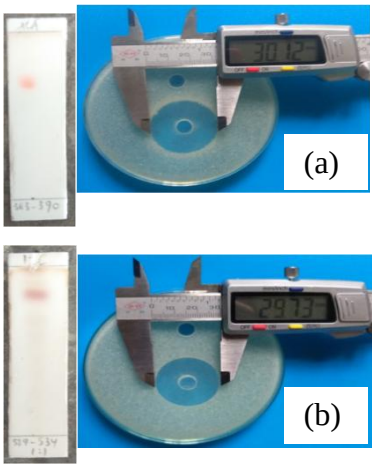


Figure 2 Thin layer chromatographic analysis with various solvent system

Isolation of some organic metabolites from ethyl acetate extract of the fermented broth of *A. duricaulis*

Gradient elution was performed successively with increasing polarity (Hexane: EtOAc, 80:1, 40:1, 20:1, 9:1, 5:1, 2:1, 1:1 and 1:2 v/v). According to the procedure in Figure 4, compound A (colourless crystal 23 mg) and compound B (semisolid in yellow 24 mg) were obtained from the respective fractions F-II and F-III. The remaining fractions FI, FIV and FV were observed as mixtures and no antifungal activity was recorded. This isolated compound A and B have significant activity on *C. albicans* with inhibitory zone 30.12 mm and 29.73mm respectively. Thin layer chromatogram of compounds (A and B) and their antifungal activity were presented in Figure 3.



Compound A

Solvent system : Hexane:EtOAc (1:1v/v)

R_f value : 0.6

Spraying agent : 5% H_2SO_4, heat

Compound B

Solvent system : Hexane: EtOAc (1:1v/v)

R_f value : 0.84

Spraying agent : 5% H_2SO_4, heat

Figure 3 Thin layer chromatogram of isolated compound and their antifungal activity against *Candida albicans* (a) compound A (b) compound B

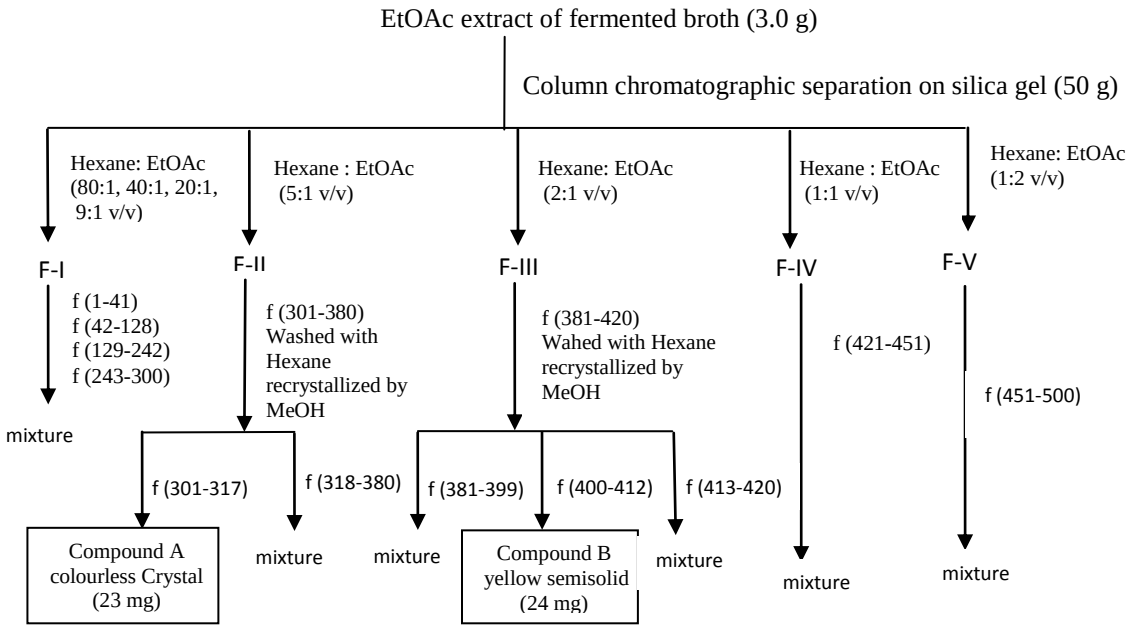


Figure 4 Isolation of organic metabolites from ethyl acetate crude extract,culture broth of isolated fungus *A.duricaulis* by column chromatography

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 Some chemical properties of isolated compounds

No.	Spraying agent	Observation (compounds)	
		A	**B**
1	10% $KMnO_4$	discharged	ND
2	I_2	Yellow	yellow
3	Anisaldehyde	Purple	ND
4	$FeCl_3$	ND	ND
5	Liebermann Burchard	Cherry red	pink
6	2,4 DNP	Yellow ppt	ND
7	5%H_2SO_4	pink	violet

ND- not detected

Compound A

It was soluble in EtOAc, EtOH, MeOH and $CHCl_3$ but insoluble in PE, Hexane and H_2O. R_f value of compound A was found to be 0.6 in Hexane: EtOAc (1:1 v/v) solvent system. According to the result obtained from chemical tests (Table 2), compound A gave yellow spot on TLC chromatogram with iodine vapour, purple spot with anisaldehyde followed by heating, cherry red colouration with Liebermann Burchard reagent, yellow colour with 2, 4 DNP and the colour of compound A was pink spot on TLC plate while spraying with 5% H_2SO_4 followed by heating.

The UV absorption spectrum shows peak at 296 nm. This band may be attributed to π-π^* transition (Figure 5 and Table 3). The functional groups present in compound A was studied by FT IR spectroscopy. The FT IR spectrum was shown in Figure 6 and the interpreted spectral data were illustrated in Table 4. The FT IR spectrum at compound A showed the bands at 3465 cm^{-1} and 3265 cm^{-1} due to N-H stretching of amide. Absorption band at 2960 cm^{-1} and 2853 cm^{-1} were due to -C-H stretching of alkyl group. C=O stretching of 1° amide was observed at 1685 cm^{-1} and 1603 cm^{-1}. Stretching band at 1532 cm^{-1} and 1436 cm^{-1} for C=C stretching of aromatic, and the band at 1407 cm^{-1} for C-C stretching of aromatic were observed. =C-H bending of aromatic group and C-H bending of aromatic compound were found at 1290 cm^{-1}, 1230 cm^{-1}, 1098 cm^{-1},

and 976 cm^{-1}, 909 cm^{-1} respectively. From the physicochemical properties, R_f value, UV and FT IR spectral data, isolated compound A may be aromatic primary amide.

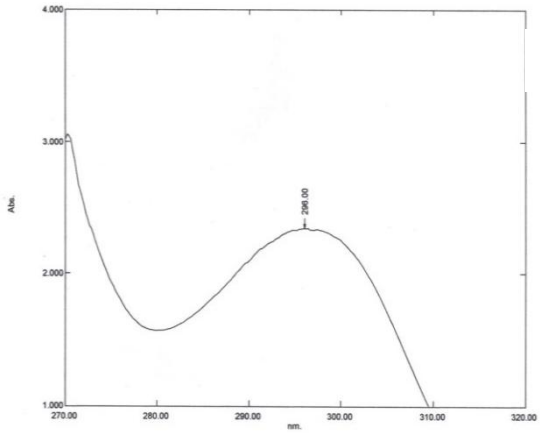


Table 3 UV spectral data of isolated compound A

Solvent used	Observed λ_{max} (nm)	Remark
MeOH	296	π-π* transition

Figure 5 UV spectrum of isolated compound A

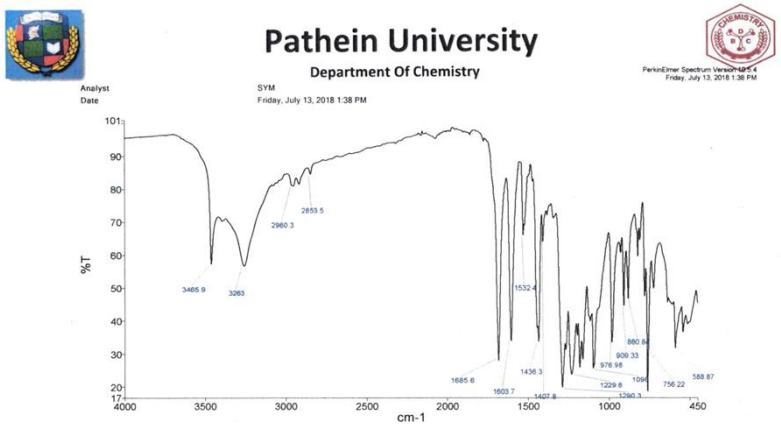


Figure 6 FT IR spectrum of isolated compound A

Table 4 FT IR spectral data of isolated compound A

Wave number (cm^{-1})	Literature* Wave number (cm^{-1})	Band assignment
3465, 3265	3500~3350	N-H stretching of amide
2960, 2853	2950~2840	-C-H stretching of alkyl group
1685, 1603	1700~15000	C=O stretching of 1° amide
1532, 1436	1600~1400	C=C stretching of aromatic
1407	~1430	C-C stretching of aromatic
1290, 1230, 1098	1250~1000	=C-H bending of aromatic group
976, 909	910~665	C-H bending of aromatic compound

*(Joseph *et al.*, 1987)

Compound B

It was soluble in EtOAc, EtOH, MeOH and $CHCl_3$ but insoluble in PE, Hexane and H_2O. Its R_f value was recorded 0.84 in Hexane: EtOAc (1:1 v/v) solvent system. Some chemical properties of isolated compound B was represented in Table 2. It showed a yellow spot on TLC plate with iodine vapour, pink colouration with Liebermann Burchard reagent and violet in colour when spraying with 5% H_2SO_4 followed by heating.

No absorption was observed in readily accessible UV region for isolated compound B. The IR results of the compound B shows the

presence of different bonds corresponding to the following functional group present in the molecular structure: stretching band at 2917 cm^{-1} and 2850 cm^{-1} for -C-H of alkyl group, stretching band at 1753 cm^{-1} for C=O of ester, 1476 cm^{-1} and 1461 cm^{-1} for C-H bending of an ester, 1372 cm^{-1} for -C-H bending of alkyl group. And the band at 1172 cm-1 was due to C-O-C stretching. These functional group of isolated compound and literature cited were shown in Table 5. All of physicochemical tested result, R_f value and spectroscopic data indicated that compound B may be aliphatic ester.

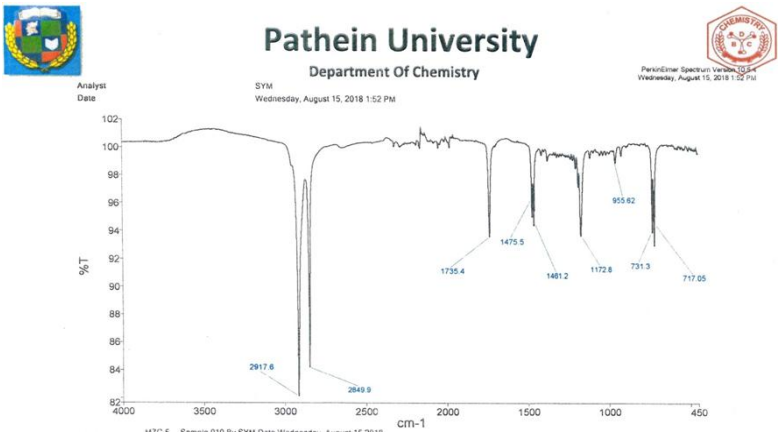


Figure 7 FT IR spectrum of isolated compound B

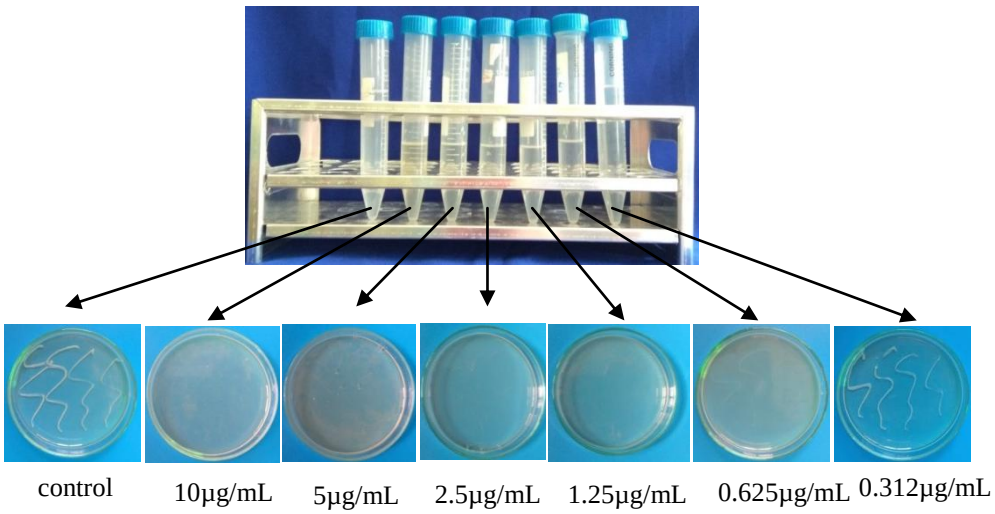
Table 5 FT IR spectral data of isolated compound B

Wave number	Literature* Wave number	Band assignment
2917, 2850	2950~2840	-C-H stretching of alkyl group
1753	1750~1720	C=O stretching of ester
1476, 1461	1480~1440	C-H bending of an ester
1372	1390~1365	-C-H bending of alkyl group
1172	1250~1050	C-O-C stretching

*(Joseph *et al.*, 1987)

Minimum Inhibitory Concentrations (MICs) of isolated compound

MICs compounds were determined by two fold serial dilution method ranging from 10 µg/ mL to 0.312 µg/ mL. MICs were read in µg/ mL after overnight incubation. It was observed that MICs value of compound A was 0.625 µg/ mL when used against *Candida albicans* and for compound B MICs value was 1.25 µg/ mL (Figure 8 to 11).



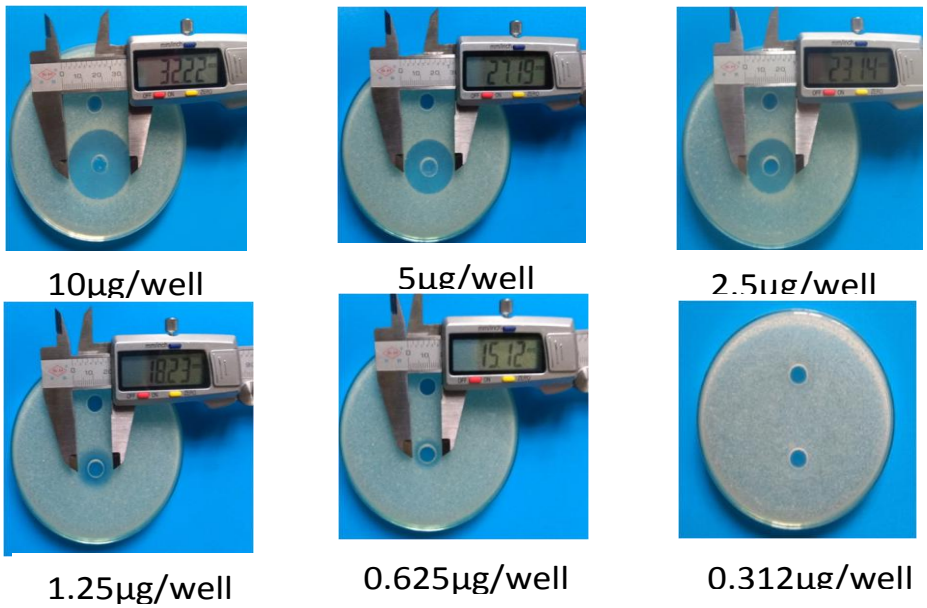


Figure 9 Minimum inhibitory concentrations of secondary metabolites from compound A on *Candida albicans* (agar well diffusion method)

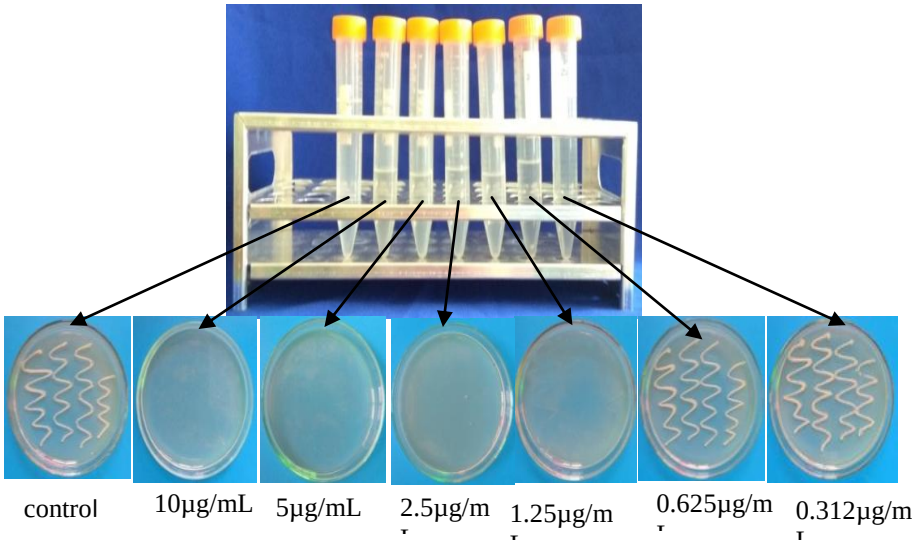


Figure 10 Minimum inhibitory concentrations of secondary metabolites from compound B on *Candida albicans* (streak method)

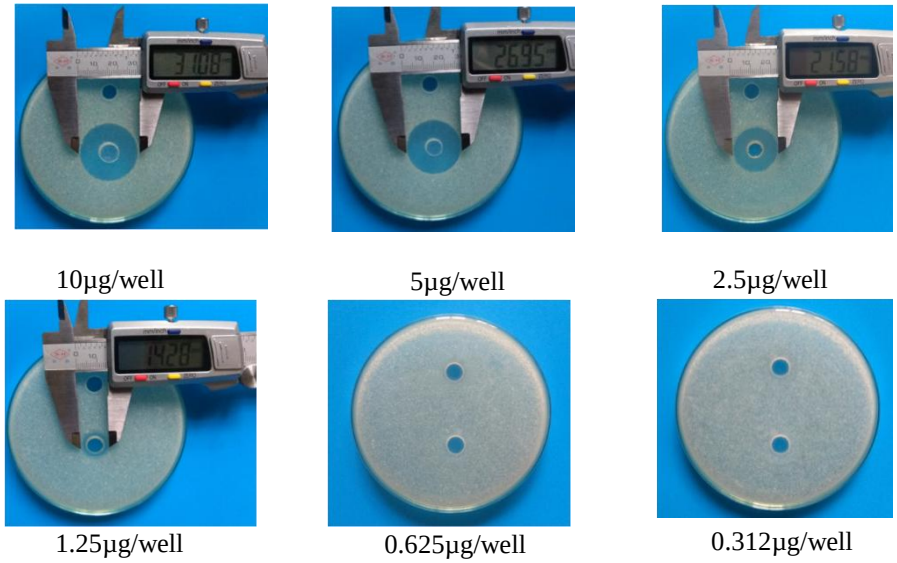


Figure 11 Minimum inhibitory concentrations of secondary metabolites from compound B on *Candida albicans* (agar well diffusion method)

Discussion and Conclusion

Fungal endophytes have been recognized as prolific producer of many chemical compounds having antibacterial, antifungal and other biological potential (Hoffman *et al*., 2008). In the investigation of paper chromatography, four kinds of different solvents were applied to observe the optimum extraction ability of secondary metabolites. According to R_f value, ethyl acetate was the excellent solvent for extracting secondary metabolites from *A. duricaulis*. There was in agreement with Garcia *et al*., 2012 who reported that ethyl acetate solvent system was

most efficient method to extract endophytic fungi principle compounds. On studying antifungal activity of *A.duricaulis* extracted with different ratio (1:1, 2:1, 3:1) of EtOAc and n-BuOH, equal ratio of ethyl acetate extract showed the highest activity with inhibition zone (26.75 mm). This result was in agreement with the description of Jain and Pundir 2011 the maximum antimicrobial metabolite was obtained by using the ratio of ethyl acetate to fermentation broth (1:1). Ayanbimpe *et al*., 2005 reported that the amount and nature of compounds produced depended on the strain of fungi and other conditions for extraction.

In this study, the optimized solvent system used was hexane: ethyl acetate at ratio 80:1, 40;1, 20:1, 9:1, 5:1, 2:1, 1:1 and 1:2. Similarly, Abdulwahid *et al*., 2013 used hexane: ethyl acetate solvent mixture in ratio of 8:2 and characterizied antibacterial compound from *Aspergillus niger*. The fractions (F) which gave the same R_f value on TLC were combined and tested their antifungal activity against *C.albicans.* The fraction (F-II) $f_{301-317}$; isolated compound A with R_f value 0.6] showed antifungal activity 30.12 mm. Inhibitory zones 29.73 mm was observed in fraction (F-III) $f_{400-412}$; isolated compound B with R_f value 0.84. 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 datas, the isolated compounds A and B may be aromatic primary amide and aliphatic ester respectively**.** In another study done by Yin *et al*., 2015, amine, aromatic, ketones, carboxylic acid ester, coumarin derivatives, dicarboxylic acid, heterocyclic compounds, hydrazide derivatives and imines identified were reported to exhibit antibacterial, antifungal and other biological activities. In a study of Minimum Inhibitory Concentrations (MICs) of isolated compounds, the antifungal metabolites affected on the growth of *C.albicans* at least MIC of 0.625 µg/mL for compound A and 1.25 µg/mL for compound B. A lower MIC is an indication of a better antimicrobial activity. In a study carried out by Suzuki *et al*., 1997, NF00659 A1, A2, A3, Bl and B2 novel metabolite produced by *Aspergillus* sp. showed antimicrobial activity at concentration of 1µg/ml against Gram-positive and Gram-negative bacteria and fungi.

The present study indicates that endophytic fungus *A.duricaulis* isolated from *Momordica charantia* L. had proved its capabilities of being a potential candidate in the search for an antifungal compound against antibiotic resistant human pathogenic fungi *C.albicans*. Further 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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