IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF SELECTED SOIL FUNGUS, HMF-33

Hlaing Myint Thu[1], Su Su Latt[2], San Yu Maw[3], Zar Zar Yin[4]

Abstract

The present research work was focused on the identification and extraction of antimicrobial active soil fungus HMF-33. This fungus was isolated by serial dilution method from the soil of Naung Taw village, Homalin Township. The macroscopic and microscopic characters of HMF-33 were observed on the Blaskeslee's Malt Extract Agar (BMEA Medium), Czapek-Dox Agar (CZA Medium), Malt Extract Agar (MEA Medium), Glucose Ammonium Nitrate Agar (GAN Medium), Potato Glucose Agar (PGA Medium) and incubated for 7 days at room temperature. According to the results, selected fungus HMF-33 was identified as the genus *Penicillium* sp. In the investigation of fermentation medium (FM), eight kinds of fermentation media was studied by using various carbon and nitrogen sources. Among them, FM 6 showed the higher antimicrobial activity than other fermentation media on seven test organisms. In the results of paper chromatography, n-butanol was the most suitable solvent for extraction of antimicrobial secondary metabolites from fermented broth of HMF-33 and bioautographic assay showed R_f values had 0.92 on *Bacillus subtilis* and 0.90 on *Candida albicans*. Furthermore, crude extract 33.0g yielded from 17.5L of fermented broth of *Penicillium sp.*, HMF-33 and showed highly antimicrobial activities (20.53-32.94mm) against (gram positive, gram negative bacteria and fungi) ten test organisms. Crude extract of *Penicillium sp*. (HMF 33) possessed broad spectrum bioactivity. Therefore, crude extract of *Penicillium sp*. may be used by the pharmaceutical industries for the production of antimicrobial compounds from local sources.

Keywords: bioautographic assay, secondary metabolites, antimicrobial activities, *Penicillium* sp., broad spectrum

Introduction

Penicillium derives its name from the latin word "Penicillius'' meaning "little brush'' (Pitt, 1979). The subgenus (verticillate nature) can be determined by the number of branch points (rami) between the phialide which bears the conidia on the tip and the stipe (hyphal stalk). Isolates with one such branch point are monoverticillate, two branches-biverticillate, three branches – terverticillate and four branches – quarterverticillate (pitt, 1991). Identification of *Penicillium* species should focus on the implementation of both morphological and molecular identification methods.

Solvent extraction provides the ease of liquid handing, the potential for high throughput operation, and the potential for adaptation to continuous operation (Schugerl, 1993). There are three main methods that are used to extract secondary metabolites namely; liquid-liquid extraction, reverse phase micelles and solid phase extraction. The most commonly used and favoured methods is the liquid- liquid extraction. [1]

Antibiotics play a very important role in controlling infectious diseases (Sarkar*et al.*, 2014). Antibiotics are produced by bacteria, fungi, sponges etc. Antimicrobial compounds are used to kill or retard the growth of the living organisms. Therefore, the aim of the research work was to identify genus level of HMF 33 and to produce antimicrobial metabolite from HMF 33, *Penicillium sp.*

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Materials and Methods

Method for Collection of Soil Sample

The soil sample was collected from Naung Taw village, Homalin Township, during July 2017. The soil sample was collected from Naung Taw village (up to 15 cm depth) into sterilized polythene bags after removing the surface soil for the isolation of fungi and brought to the laboratory of Biological Resources and Biotechnology Development Center at Pathein University.

Isolation of Fungi from the Soil Sample

Strain HMF-33 was isolated by the serial dilution method (Dubey and Maheshwari, 2002) from the soil of Naung Taw village (N 24º 49' 39.211"E 95˚ 07' 07.066"), Homalin Township.

Culture and identification of HMF-33

HMF-33 was identified in the level of genus on different media such as Blaskeslee's Malt Extract Agar (BMEA Medium), Czapek- Dox Agar (CZA Medium), Malt Extract Agar (MEA Medium), Dichloram Rose Bengal– Chloramphenicol Agar (DRBC Medium), Glucose Ammonium Nitrate Agar (GAN Medium), Potato Glucose Agar (PGA Medium). Morphological features of HMF-33 cultures were studied, the major and remarkable macroscopic features in species identification were the colony diameter and color (surface and reverse). Riddle's classic slide culture method (Riddle, 1950) was used for microscopic study of HMF-33. Microscopic characteristics for the identification were conidia length, conidia width, conidia shape, conidia ornamentation, stipe length, stipe width, stipe ornamentation, phialide shape and branching pattern. The identification of fungus HMF-33 was undertaken by the method of Ando, 2016, literature reviews and references key (Ando, 2004).

Agar Well Diffusion Method

Isolated strains were performed by agar well method (Collins, 1965) for the antimicrobial activities. Cork borer was used to make the wells (8 mm in diameter) in the autoclaved basal antimicrobial test-medium. Wells impregnated with 3-7 days old culture fermented broth (20µL/well) were incubated at room temperature for 24-28 h. After 24-28 h of incubation, the clear zones were measured. Therefore, the diameter of clear zones had been observed as potent activity as shown by respective strain. Clear zones surrounding the wells indicated the presence of antimicrobial activities which inhibit the growth of the test organisms selectively.

Test Organisms

The test organisms used for this experiment were *Agrobacterium tumefaciens* NITE 09678, *Bacillus pumilus*, *Bacillus subtilis* IFO 90571, *Candida albicans* NITE 09542, *Escherichia coli* AHU5436, *Pseudomonas fluorescens* IFO94307, *Malasseza furfur, Micrococcus luteus, Staphylococcus aureus* AHU8465, *Saccromyces cereviciae, Salmonella typhi*. The organisms were obtained from National Institute of Technology and Evaluation (NITE, Japan), and Pharmaceutical Research Department, Yangon, Myanmar.

Paper Chromatography

Paper chromatography was performed to know how to extract the bioactive compounds from fermentation broth by using which solvent system (Tomitta, 1988). The filter paper and four solvent systems; n-butanol-acetic acid –water (3:1:1), n-butanol saturated with water, ethyl acetate saturated with water and 20% NH_4Cl were used for preliminary characterization of antibiotics. The fermented broth (10µl) were spotted by capillary tube on the paper and allowed to dry. The papers

were chromatographed in each solvent. Then, bioautography was done to check the antimicrobial activity of each. Each paper was placed on assay agar plates, in the same method as paper disc assay, except that after one hour the paper was taken out, then the plates were incubated for 24-36 hours. The inhibitory zone place was measured yielding a R_f values were measured for the corresponding bioactive compounds.

Extraction of Bioactive Metabolite from Culture Filtrate of HMF-33

The fungal culture in fermentation medium was incubated with shaking for four days at room temperature. When the incubation period was complete, mycelia was filtered off, soaked on solvents (such as ethyl acetate and n- butanol) for 24 hrs and filtered, resulting in an organic layer. The antimicrobial metabolite was extracted by liquid-liquid extraction with solvents. Equal volume (1:1 v/v) of culture filtrate and solvents were taken in a separating funnel and agitated for about 30 minutes. Then the mixture was allowed to stand till complete separation to upper and lower layer (phase) was formed. The upper (solvent) layers were evaporated to get concentrated crude extract (Petit *et al*., 2009). The extracts were tested for antimicrobial activity by using agar well diffusion assay.

Results and Discussion

Colony Characters and Growth of HMF-33

In the study of morphological of HMF-33, six differential culture media were used for identification of HMF-33. A culture time of seven days was generally required for identification of HMF-33 strain. Using differential media like BMEA, CZA, MEA, GAN, DRBC, PGA media with colony and microscopic characters of HMF-33 growth on these culture media enable to discriminate HMF-33. Fungus HMF-33 has different growth, different surface and reverse color on different media. Isolates of single species may appear different if grown on various media (Raper and Thom, 1949). The surface color of HMF-33 were greenish yellow, gray, yellowish green, white on six different media and reverse color of HMF-33 were yellow, pink, white cream and yellow on BMEA, CZA, MEA, GAN, DRBC, PGA media. Mushimiyimana *et al.,* 2016 mentioned that colonies on CYA, MEA and YES with *Penicillium sp* and *Penicillium crustosum* are white to cream, yellow and dark green colour on observe whereas in reverse pale, yellow, orange, light brown, and dark brown. The reverse colony was usually pale to yellowish or brownish. Ando, 2004 stated that as the results of the observation of spore production, fungi are identified under genus level. For the identification of fungi under species level, media described and used in the descriptions or the monographs of each genus will be used. In generally, PDA, MEA, LCA, CZA and OA media are used for the identification media. Therefore, the media for identification varied with each fungus group. Ando, 2004 mentioned that many fungi grow robustly on BMEA medium. In the studying of colony growth of HMF-33, Colony growth of HMF-33 have excellent growth 39.00-45.89mm on BMEA medium, followed by 39.00-39.20mm on MEA medium, 37.13-37.48mm on PGA medium, 33.90-39.54mm on CZA, 24.55-24.65mm on DRBC, 19.88-21.00mm on GAN medium respectively (Table 1 and Figure 1) and colony texture of HMF-33 on six different media was almost valutinuous.

Table 1 Colony Morphology of HMF-33 on Different Media at Seven Days

Culture Media	Surface colour	Reverse colour	Size of Colonial Growth (mm)
BMEA	Greenish yellow	Yellow	39.00-45.89
CZA	Greenish yellow	Yellow	33.90-39.54
MEA	Gray in the center Yellow in the periphery	Yellow	39.00-39.20
GAN	Yellowish green	Pink	19.88-20.00
DRBC	White	White cream	24.55-24.65
PGA	Yellowish green	Yellow	37.13-37.48

10-20mm = Poor growth 20-30mm = Moderate growth
30-40mm = Good growth 40 to above = Excellent growth

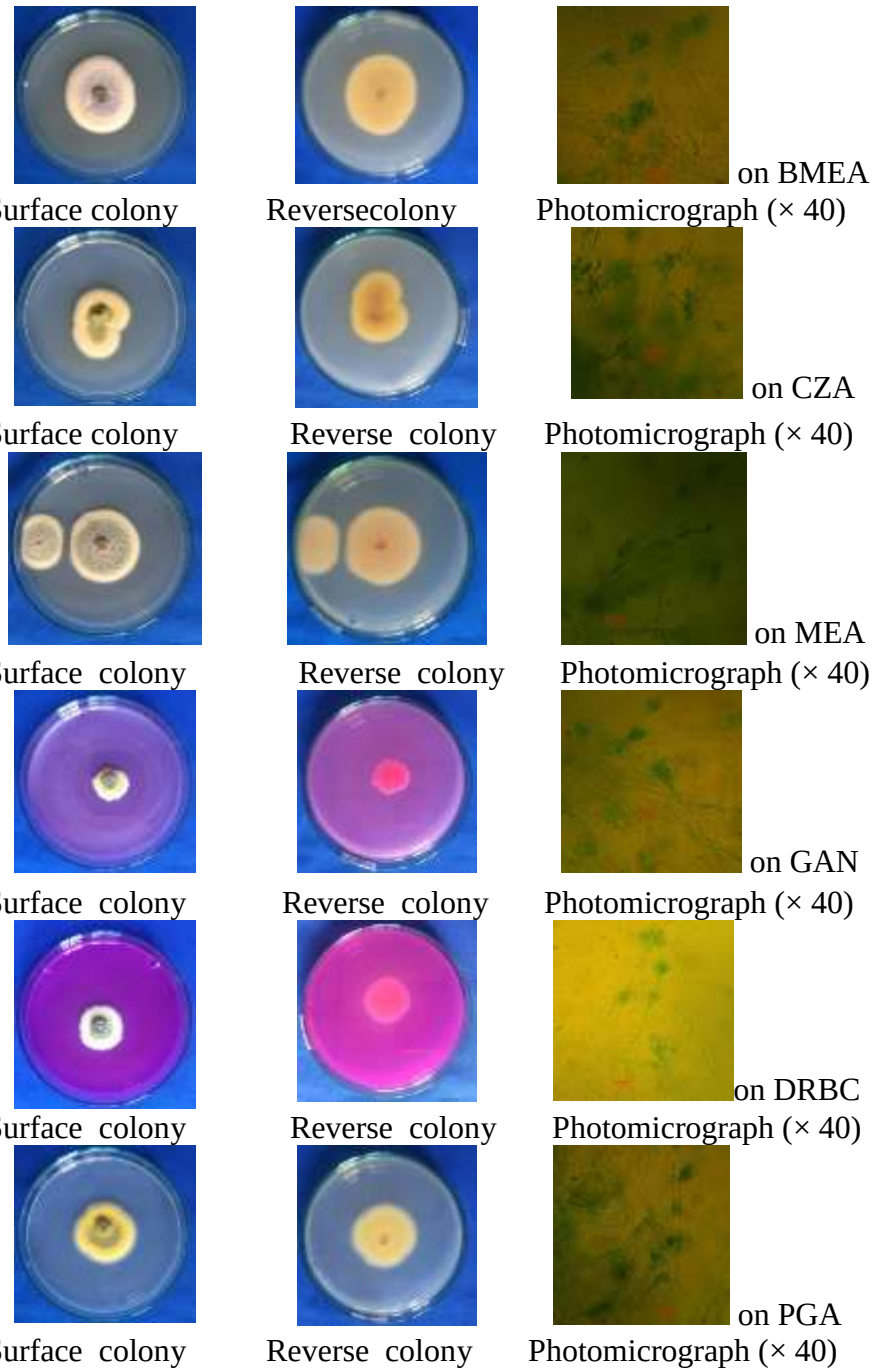


Figure 1 Colony and microscopic characters of isolated fungus HMF-33 in six (BMEA, CZA, MEA, GAN, DRBC, PGA) differential media at seven days

Microscopic Character of Fungus HMF-33

In the microscopic characters of HMF-33, conidiophores were biverticillate, appressed elements, born from surface hyphae, stipes were smooth walled, 100- 120µm × 2.5-3 µm and have a cluster of four metulae; cylindrical, 7.5- 8 µm × 2-2.5 µm. Phialids were cylindrical tapering to a distinct column, 7.5-8 µm × 2-2.5 µm. Conidia were small in size (2-2.5µm), globose in simple shape with smooth and chain in production (Figure 2). These microscopic characters were similar to the investigation of the *Penicillium* species of Ando (2004). Tiwari, 2011 also reported that *Penicillium rubrum* showed aerial mycelium; strips long smooth wall bearing biverticillate *penicillin* narrow and the conidia was smooth strongly. *Penicillium* variable showed smooth, much shorter hyphae, conidiophores typically biverticillate and *P. multicolor* showed septate hyphae and conidia were spheraidal. The regular production of biverticillate *Penicillin* with 4 to 6 terminal metulae, and phialides, which were flask shaped and distinctly shorter than metulae by *Penicillium* isolate are indicatives that it belongs to the subgenus Furcatum section (pitt, 2000). Barnett, 1969 also reported that conidiophores arising from the mycelium singly or less often in synnemata, branched near the apex to form a brush- like, conidia- bearing apparatus; ending in phialides which pinch off conidia in dry chain; conidia hyaline or brightly colored in mass, one celled, mostly globose or ovoid, produced basipetally. According to above evidence, HMF- 33 may be identified as the genus *Penicillium* sp.

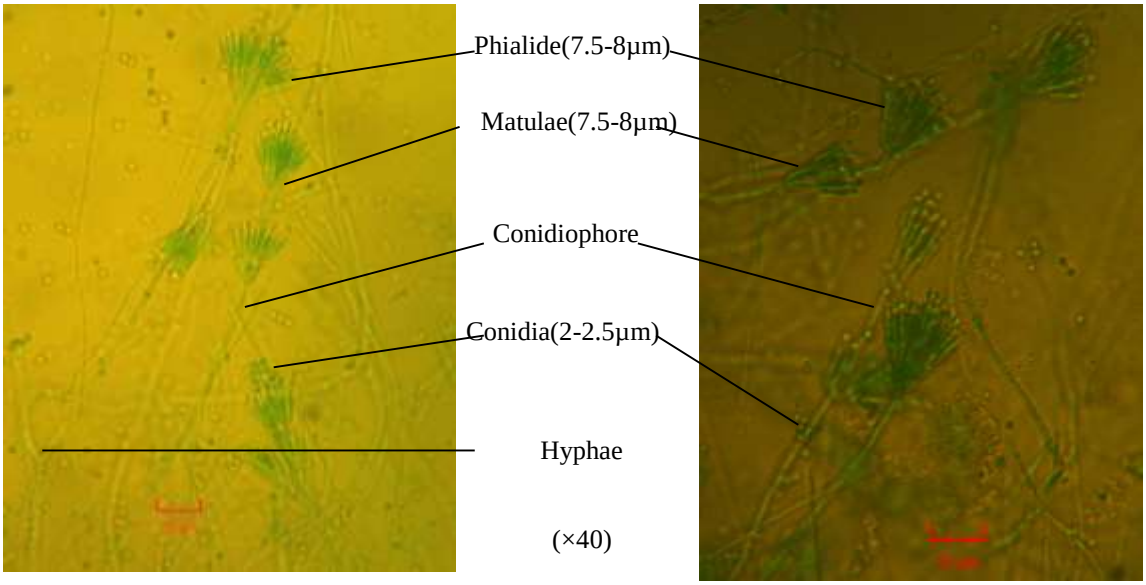


Figure 2 Microscopic Examination of Selected Fungus HMF-33

Effect of Different Concentration of Carbon and Nitrogen on Fermentation Media by HMF-33, *Penicillium* sp

Carbon and nitrogen sources from fermentation medium of Ando,2004 was substituted by dextrose3.2g, fructose 2.4g, casein 0.24g and yeast extract 2.4g in 100ml of fermentation medium. The eight fermentation media were performed for antimicrobial activities with test organisms such as *Bacillius subtilis* and *Candida albicans.* Among of all fermentation media, fermentation medium FM-6 showed maximum antimicrobial activity 30.88mm against *Bacillius subtilis* and 31.61mm against *Candida albicans,* followed by FM-3 (30.07mm and 31.08mm), FM-5 (28.71mm-29.11mm), FM-4 (28.28mm-29.47mm), FM-1 (27.50mm-29.68mm), FM-8 (25.68mm and 27.23mm), FM-2 (24.33mm and 24.66mm), FM-7 (22.68mm and 23.92mm) against *Bacillius subtilis* and *Candida albicans* (Table 2 and Figure 2). HMF-33, *Penicillium* sp produced red pigment in fermentation media including dextrose. Mendez *et al*., 2011 mentioned that *P.*

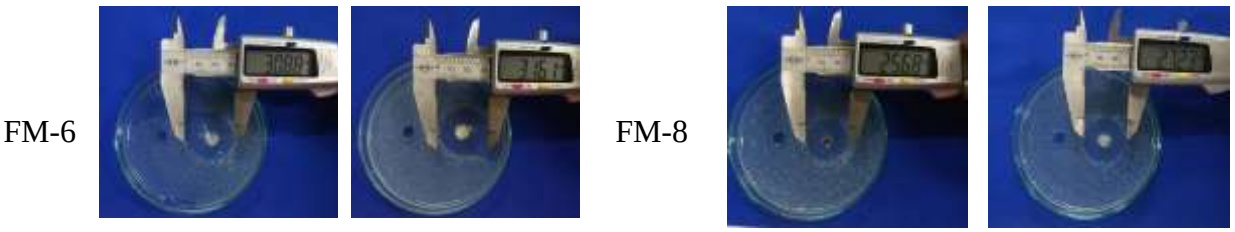
purpurogenum can produce colorants in both solid and liquid media. *P. purpurogenum* DPUA 1275 was studied to produce yellow, orange, and red extracellular colorants during culture on an orbital shaker (Santos-Ebinuma*et al*., 2013).

Table 2 Antimicrobial Activity of HMF 33, *Penicillium* sp on the Fermentation Medium with the Various Carbon and Nitrogen Concentrations

Fermentation media	Two test organisms and Inhibition Zone (mm)	
	Bacillus subtilis	***Candida albicans***
FM 1	27.50	29.68
FM 2	24.33	24.66
FM 3	30.07	31.08
FM 4	28.28	29.47
FM 5	28.71	29.11
FM 6	30.88	31.61
FM 7	22.68	23.92
FM 8	25.68	27.23

red pigment

Figure 2 (a) Production of red pigment by HMF-33 in Fermentation including dextrose



FM-6 FM-8

(A) (B) (C) (D)

Figure 2 (b) Antimicrobial Activity of HMF 33 on the Fermentation Medium with the Various Carbon and Nitrogen Concentrations against *Bacillus subtilis* (A, C) and *Candida albicans*(B, D), (FM-6, FM-8)

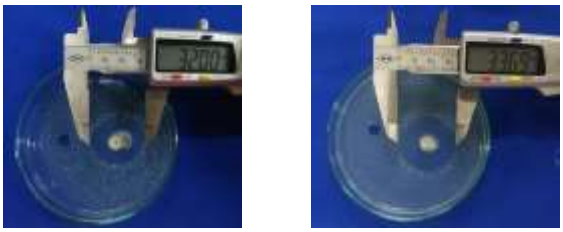
Antimicrobial Activity of HMF-33, *Penicillium* sp on the Suitable Synthetic Fermentation Medium

When synthetic fermentation medium was performed together with the optimal fermentation parameters such as 48h of seed culture,5% inoculums size, temperature 25 ˚C, pH 6.5, dextrose concentration 3.2 %, (w/v), yeast extract concentration 0.24 % (w/v), 250mL of fermentation vessel size with shaking culture, antimicrobial activity of HMF 33, *Penicillium spp.* exhibited inhibition zone 24.56mm against *Agrobacterium tumefaciens* at 4 days, 26.72mm against *Bacillus pumilus* at 4 days, 32.00mm against *Bacillus subtilis* at 4 days, 33.61mm against *Candida albicans* at 4 days, 26.42mm against *Escherichia coli* at 4 days, 25.08mm against *Pseudomonas fluorescens* at 5days, 25.56mm against *Staphylococcus aureus* at 5days (Table 3 and Figure 3) .

Table 3 Antimicrobial Activity of HMF-33, *Penicillium* sp on the Suitable Synthetic Fermentation Medium (6) against Seven Test Organisms

Fermentation Periods(Days)	Seven test organisms and Inhibition Zone (mm)						
	1	2	3	4	5	6	7
2	20.60	20.02	20.90	22.17	21.21	16.61	22.47
3	21.43	21.67	23.30	24.62	24.50	21.79	24.41
4	24.56	26.72	32.00	33.69	26.42	24.16	24.16
5	23.20	25.83	24.64	25.75	25.07	25.08	25.56
6	22.89	25.30	22.40	25.07	24.86	23.19	23.61
7	20.40	18.40	19.73	19.77	19.64	14.09	21.31

1. *Agrobacteriumtumefaciens*
2. *Bacillus pumilus*
3. *Bacillus subtilis*
4. *Candida albicans*
5. *Escherichia coli*
6. *Pseudomonasfluorescens*
7. *Staphylococcus aureus*

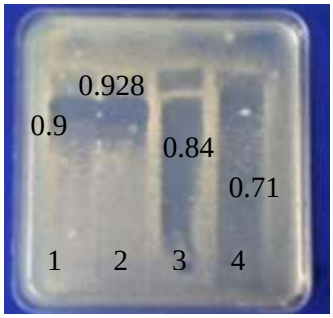


B. subtilis(4 days) *C. albicans*(4 days)

Figure 3 Antimicrobial Activity of HMF-33, *Penicillium* sp on the Suitable Synthetic Fermentation Medium (6) against Seven Test Organisms

Paper Chromatography of Fermented Broth of HMF-33, *Penicillium* sp

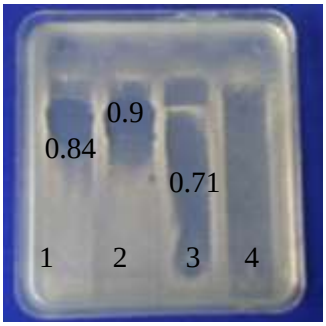
In this study, four solvents (20% NH_4CL, n-Butanol saturated with water, n-Butanol-Acetic acid-Water (3:1:1) and ethyl acetate saturated with water) were used. According to the R_f values, 0.92 and 0.90, n-Butanol was more extractable the antimicrobial metabolites than other solvents, followed by n-Butanol-Acetic acid-Water (3:1:1) 0.9 and 0.84 and the lower R_f value by ethyl acetate saturated with water (0.84 and 0.71), but 20% NH_4CL was not clearly showed inhibitory zones and R_f value (Fig 4 -a and b).



Solvent system

1. n-Butanol-Acetic acid-Water (3:1:1)
2. n-Butanol saturated with water
3. Ethyl acetate saturated with water
4. 20% NH_4CL

Figure 4 (a) Paper Chromatography bioautographic assay (against *Bacillus subtilis*)



Solvent system

1. n-Butanol-Acetic acid-Water (3:1:1)
2. n-Butanol saturated with water
3. Ethyl acetate saturated with water
4. 20% NH_4CL

(b) Paper Chromatography bioautographic assay (against *Candida albicans*)

Comparison of antimicrobial activity of metabolite in HMF-33, *Penicillium* sp extracted with different solvents of EtOAc and n-BuOH

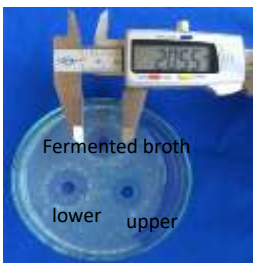
Candidaalbicans

When using EtoAc solvent, equal volume (1:1 v/v) of culture filtrate and ethyl acetate resulted in inhibition zone shown 19.15mm and 17.94mm against *Bacillius subtilis* and *Candida albicans* at upper layer. Antimicrobial activities showed22.30mm and 19.13mm against *Bacillius subtilis* and *Candida albicans* at lower layer. Fermented broth of HMF-33 showed 20.55mm, 17.10mm against *Bacillius subtilis* and *Candida albicans*. When using n-BuoH solvent, equal volume (1:1 v/v) of culture filtrate with n- butanol showed inhibition zone was 23.33mm against *Bacillius subtilis* and 21.79mm against *Candida albicans* at upper layer. Antimicrobial activities showed 15.60mm and 12.64mm against *Bacillius subtilis* and *Candida albicans* at lower layer. Fermented broth of HMF-33, *Penicillium* sp showed 20.60mm, 17.27mm against *Bacillius subtilis* and *Candida albicans*. These results were shown in (Table 5 and Figure 5).

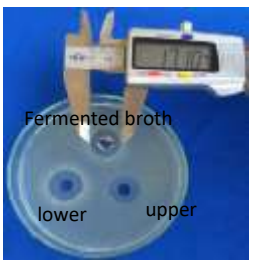
Table 5 Comparison of antimicrobial activity of metabolite in HMF-33, *Penicillium* sp extracted with different solvents

Extracted with different solvents (1:1 v/v)	Two test organisms and Inhibition Zone (mm)					
	Bacilliussubtilis			***Candida albicans***		
	1	**2**	**3**	**1**	**2**	**3**
EtoAc Extract	20.55	19.15	22.30	17.10	17.94	19.13
BuOH Extract	20.60	23.33	15.60	17.27	21.79	12.64

1. Fermented broth 2. Upper layer 3. Lower layer

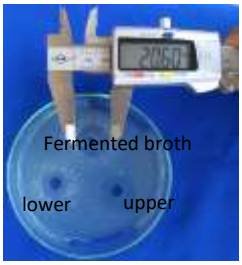


against Bacillius subtilis



against Candida albicans

Figure 5 (a) Antimicrobial activity of metabolite in HMF-33, *Penicillium* sp extracted with EtoAc solvent



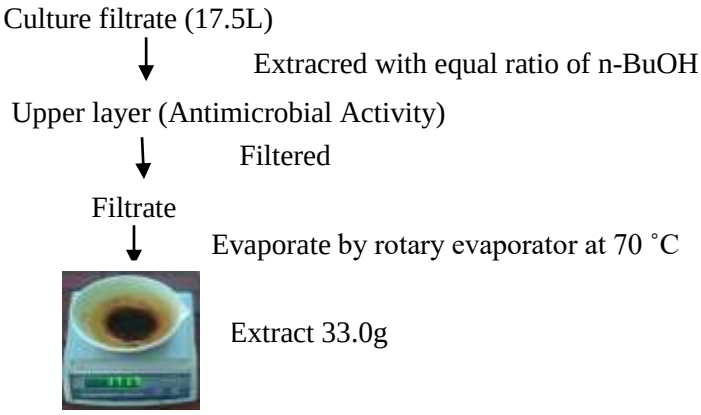
against Bacilliussubtilis

against Candida albicans

Figure 5 (b) Antimicrobial activity of metabolite in HMF-33, *Penicillium* sp extracted with BuOH solvent

Extraction Antimicrobial Metabolites from HMF-33, *Penicillium* sp

According to the results of paper chromatography (PPC), comparison with different solvents of EtOAc and n-BuOH, extracted with equal ratio (1:1 v/v) of culture fitrate and n-butanol to yield 33.0g of brown solid crude extract. Preparation of n-butanol extract from fermented broth of HMF-33, *Penicillium* sp was shown in Figure 6.



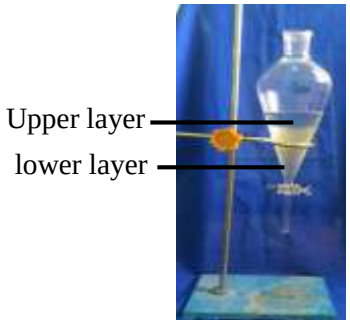


Figure 6 Flow diagram for preparation of n-BuOH extract from fermented broth of HMF-33, *Penicillium* sp

Antimicrobial Activity of Crude Extract of HMF-33

Antimicrobial Activity of Crude Extract of HMF-33 was found to be most effective against all test organisms. Crude Extract of HMF-33 showed antimicrobial activities 22.42mm against *Agrobacterium tumefaciens,* 26.86mmagainst *Bacillus subtilis,* 32.94mm against *Candidaalbicans,* 22.41 against *Escherichia coli,* 20.53mm against *Malasseza furfur*, 27.03mm against *Micrococcus luteus*, 27.56mm against *Pseudomonas fluorescens,* 26.00mm against *Staphylococcus aureus,* 22.58mm against *Saccromyces cereviciae*, 25.23mm against *Salmonella typhimurium* respectively (Table 7 and Figure 7).According to these results, crude extract of HMF-33, *Penicillium* sp shown broad spectrum in its mode of action as inhibited the growth of all test pathogens.

Table 7 Antimicrobial Activity of Crude Extract of HMF-33, *Penicillium* sp

Sample	Ten Test Organisms and Inhibition Zone (mm)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Crude extract (n-BuOH)	22.42	26.86	32.94	22.41	20.53	27.03	27.56	26.00	22.58	25.23

1. *Agrobacteriumtumefaciens*
2. *Bacillus subtilis*
3. *Candida albicans*
4. *Escherichia coli*
5. *Malasseza furfur*
6 .*Micrococcus luteus*
7. *Pseudomonas fluorescens*
8. *Staphylococcus aureus*
9. *Saccromycescereviciae*
10. *Salmonella typhimurium*

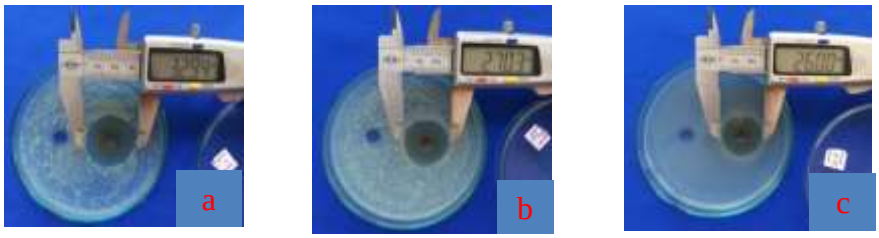


Figure 7 Antimicrobial Activity of Crude Extract of HMF-33, *Penicillium* sp on(a) *Candida albicans*(b) *Micrococcus luteus*(c) *Staphylococcus aureus*

Conclusion

This study revealed that selected fungus HMF-33 was identified as the genus level, *Penicillium sp* by observation of macroscopic and microscopic characters. According to the result of bioautographic assay, fermented broth of *Penicillium sp* extracted with equal ratio (1:1 v/v) of n-butanol yielded 33.0g of brown solid crude extract. Though it is not much about the chemical nature as identified the crude extract of *Penicillium sp*, HMF-33 showed broad spectrum in its mode of action as inhibited the growth of all test organisms (plant and animal pathogen). It may be suggested that *Penicillium sp* from soil of local sources may facilitate the new products (bioactive compounds) discovery process.

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