EXTRACTION OF ANTIBACTERIAL METABOLITES PRODUCED BY SLECTED SOIL FUNGUS (KM-16)

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Abstract

In the study, soil fungi were isolated from soil samples which were collected from the Pathein University Dhamma Yone campus and then air-dried at room temperature. After three days, ten different soil samples were isolated by feeding method and physical treatment dilution method. Thirty-one soil fungi were isolated from ten different soil samples. Among them isolated soil fungus KM-16 showed the highest antimicrobial activity against *Escherichia coli*. In this investigation, selected soil fungus (KM-16) was utilized by using paper chromatography for extracting of antibacterial metabolites. The extraction of antibacterial metabolites was carried out by Thin Layer Chromatography and antibacterial metabolites could be isolated from 20 Liters of fermented broth of KM-16.

Keyword: Soil Fungi, Feeding Method and Physical Treatment Dilution Method

Introduction

Soil is considered as one of the most suitable environments for microbial growth (Cavalcanti *et al.,* 2006). Fungi are fundamental for soil ecosystem functioning. Especially forest and agriculture soils play a key role in many essential processes such as organic matter decomposition and elemental release by mineralization (Haqeeqat and Nasreen, 2016). Fungi are one of the most common microbes living around us in the environment which could be pathogenic and sometimes life threatening. Lots of antifungal agents have been discovered so far and many are still the procedure to be defined as an effective fungicide.

The need for new, safe and more effective antifungals is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host (Thakur *et al*., 2007).

Materials and Methods

Sample collection, storage and transportation

Ten different soil samples were collected from ten different places around the University Campus (Pathein) during June 2019. The samples were taken near the tree by digging 6 cm depth under the soil. The soil sample were collected in sterile glass container, sealed and carefully placed in plasticbags and brought to the laboratory. The soil textures were measurement at Department of Agriculture (Land Use) Soil Interpretationin Yangon Township.

Isolation of soil fungi from different soil samples

The soil microorganisms were isolated by two methods, namely feeding method (Phay and Yamamura, 2005) and physical treatment dilution method (Hayakawa and Kobayashi, 2005) on different media such as low carbon agar (LCA)(Ando 2004), soil extract agar(SEA)(Ando 2004),potato glucose agar(PGA)(Ando 2004) and Czapek- dox agar medium.

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Isolation of Soil Fungi by Feeding Method

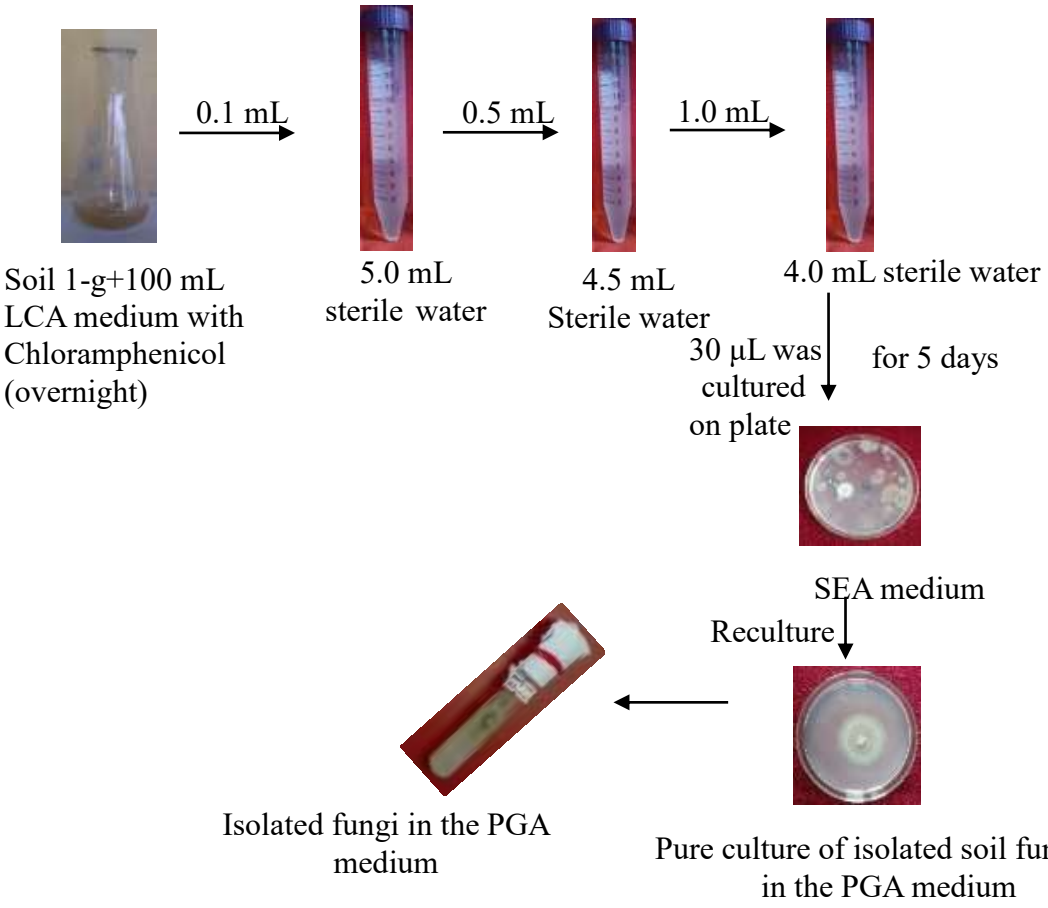


Figure 1 Procedure for isolation the fungi by using feeding method

The collected soil sample was air - dried at room temperature for 3 days. The soil sample was grounded and sieved in 2mm screen. One gram of soil sample was added LCA medium with chloramphenicol (overnight), and then prepared with 0.5 ml, 4.5 mL and 4 mL sterile water. The dilution series were cultured on SEA medium with chloramphenicol medium and incubated for 1 to 5 days at room temperature. After 6 days incubation, small piece colonies were appeared on the medium and transferred to a fresh PGA medium. Pure colonies were preserved into slant cultured containing in PGA medium (Figure.1).

Isolation of Soil Fungi by Physical Treatment Dilution Method

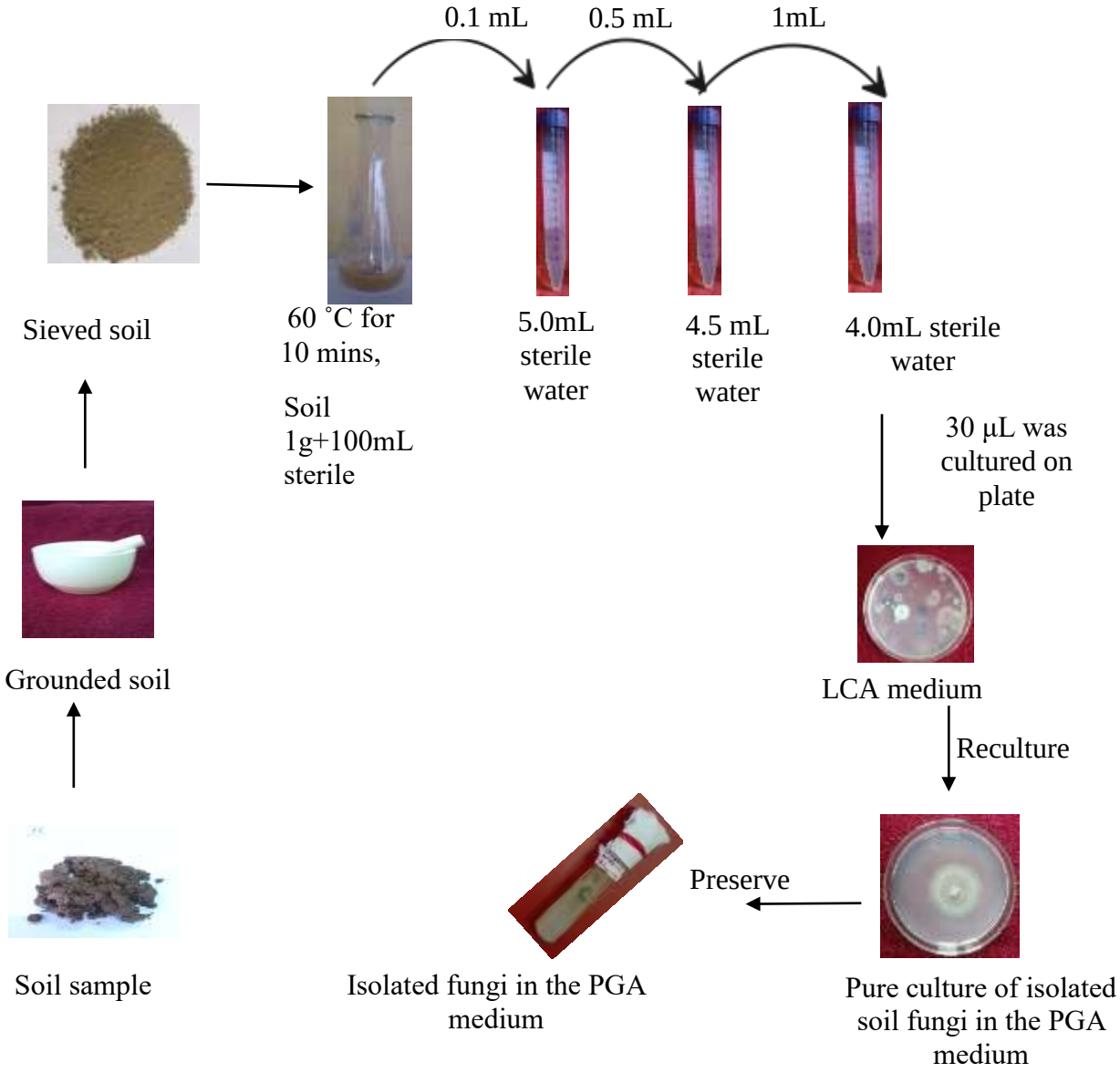


Figure 2 Procedure for isolation the fungi by using physical treatment dilution method

The collected soil sample was air - dried at room temperature for 3 days .The soil sample was grounded and sieved in 2mm screen. The soil sample was placed in the hot air oven at 60°C for 10 min. Soil sample was suspended series diluted with sterile water. The dilution were cultured on LCA medium and inoculated for 1 to 5 days at room temperature. Pure colonies were incubated into slant culture containing in PGA medium (Figure. 2)

Preliminary study of the activities of isolated microorganisms

The isolated soil microorganisms were inoculated into seed medium and incubated for 3 days at room temperature. Seed culture were transferred into the fermentation medium. It took 4 to 10 days for carrying out and it was tested with test organisms. After the end of fermentation, the fermented broth was used to check the antimicrobial activity against test organisms by paper disc diffusion assay. Paper disc having eight millimeter diameter were utilized for antimicrobial assays. The fermented broth (10-30 µL per disc) were dissolved and allowed to dry.

Assay Method

One percent of test organism was added to assay medium, then poured into plates. After solidification, paper discs impregnated with samples (fermented broth) were applied on the agar plates were incubated at room temperature for 24-36 hours.

Clear zones (inhibitory zones) surrounding the paper discs indicate the presence of bioactive metabolites which inhibit the growth of test organisms. The test organisms used in paper disc diffusion assay were supported by Department of Biotechnology of Pathein University for the cooperation research.

Paper Chromatography

Paper chromatography was carried out to extract the antibacterial compound from the fermented broth by the method of Tomita, (1988). The purpose of paper chromatography is how to extract the bioactive compound with which the suitable solvent systems.

The filter paper (Toyo Advantech Japan) and 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 for preliminary characterization of metabolites.

The obtained fermented broth samples were applied on the paper and allowed to dry. The paper were chromatographed in each solvent.

Then, bioautography was done to check the antibacterial activity of each. Each paper was placed on assay agar plates. 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 an R_f value for the corresponding metabolites.

Thin Layer Chromatography

The EtOAc extracted residue is necessary to be separated and purified more. Thus, TLC was developed by the methods of Touchstone, 1992 and Aszalos, 1987.

R_f values reported were acquired on Merck Kiesel gel GF_{254} silica gel precoated alumuinium plates (Merck), which were utilized for analytical preparative purpose.

The obtained EtOAc extracted samples (20µL) were applied on the TLC plates and allowed to dry. The TLC plates were developed in the solvent Hexane: EtOAc and Chloroform: EtOAc (100/1 v/v), (80:1 v/v), (60:1 v/v), (40:1 v/v), (20:1 v/v), (10:1 v/v), (5:1 v/v), (2:1 v/v) and (1:1 v/v).

The R_f values of isolated compounds were measured. Location of spot was made by viewed directly under UV 254 nm and 365 nm light or by treating with visualizing agents

Results and Discussion

In the course of the investigation of isolation, thirty one different fungi, KM-01 by feeding method and KM-02 to KM-31 by physical treatment dilution method) were isolated from the ten different soil samples of Pathein University (DhammaYone) campus. Among them, selected soil fungus (KM-16) showed antibacterial activities against *Escherichia coli* (Table 2 and Figure 3).

Table 2 Antibacterial activity of selected soil fungus(KM-16)

Sr.No	Isolated fungus	Antibacterial activity *Escherichia coli*
1	KM-16	20.59 mm clear zone

Figure 3 Antimicrobial activity of selected soil fungus (KM-16) against test organism (*E.coli*)

Morphology and Photomicrograph of selected soil fungus

Surface view of colony morphology

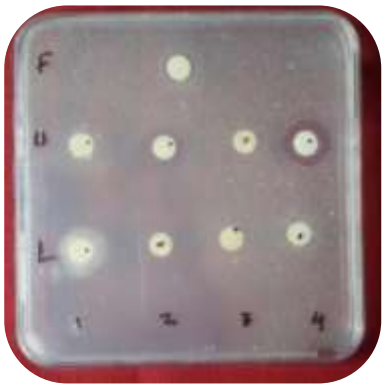
Reverse view of colony morphology

Photomicrograph (x 400)

Figure 4 Morphology and Photomicrograph characters of selected soil fungus KM-16

Paper Chromatography

According to the R_f value of paper chromatography bioassay (Figure. 5) and comparism of butanol extract and ethyl acetate extract, it was considered that the antibacterial compound of strain KM-16 could be extract from the fermented broth by ethyl acetate.



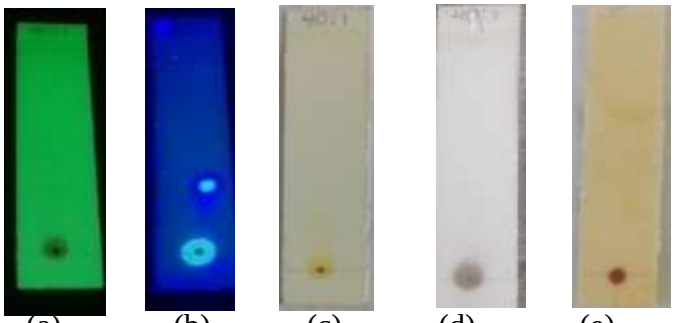
1 20% NH_4Cl 3 n-BuOH-aceticacid-water 3:1:1

2 n-BuOH satutated with water 4 EtOAc saturated with water

Figure 5 Paper chromatography bioautographic assay

Study on Thin Layer Chromatography of Ethyl Acetate Extract of *Aspergillus nidulans*

According to the results of TLC (Figure. 6-11), it may be considered that *Aspergillus nidulans* product was isolated to purify by silica gel column chromatography with n- hexane and ethyl acetate mixture as eluting solvents.



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

Figure 6 TLC chromatogram (a) under UV light 254 nm, (b) under 365 nm, (c) I_2 vapour, (d) 5% H_2SO_4 and (e) 1% $FeCl_3$ of n-hexane :ethyl acetate (40:1 v/v)

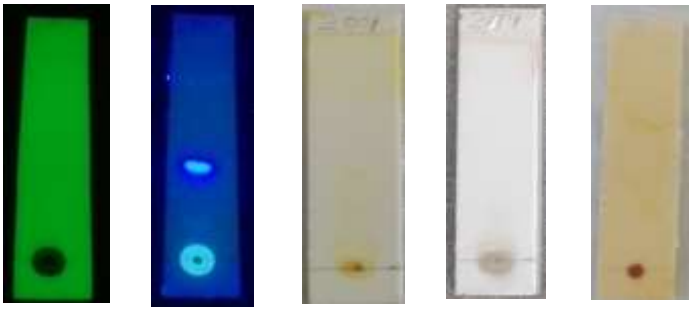


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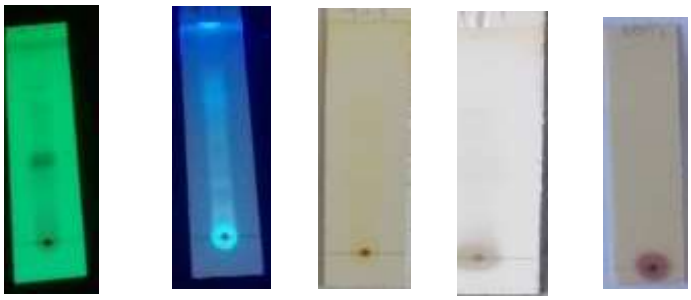
Figure 7 TLC chromatogram (a) under UV light 254 nm, (b) under 365 nm, (c) I_2 vapour, (d) 5% H_2SO_4 and (e) 1% $FeCl_3$ of n-hexane :ethyl acetate (20:1 v/v)

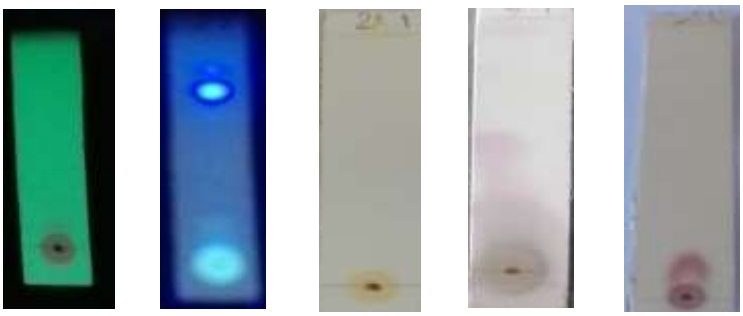


(a) (b) (c) (d) (e)

Figure 8 TLC chromatogram (a) under UV light 254 nm, (b) under 365 nm, (c) I_2 vapour, (d) 5% H_2SO_4 and (e) 1% $FeCl_3$ of n-hexane:ethyl acetate (10:1 v/v)

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

Figure 9 TLC chromatogram (a) under UV light 254 nm, (b) under 365 nm, (c) I_2 vapour, (d) 5% H_2SO_4 and (e) 1% $FeCl_3$ of n-hexane :ethyl acetate (5:1 v/v)



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

Figure 10 TLC chromatogram (a) under UV light 254 nm, (b) under 365 nm, (c) I_2 vapour, (d) 5% H_2SO_4 and (e) 1% $FeCl_3$ of n-hexane :ethyl acetate (2:1 v/v)

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

Figure 11 TLC chromatogram (a) under UV light 254 nm, (b) under 365 nm, (c) I_2 vapour, (d) 5% H_2SO_4 and (e) 1% $FeCl_3$ of n-hexane :ethyl acetate (1:1 v/v)

Conclusion

A number of antibiotics drugs have been discovered from soil inhabiting microorganisms. Antibiotics produced by fungi are widely used in current chemotherapy (Dobashi, 1998). Therefore, the isolation and screening of effective soils were investigated.

Thirty-one fungi were isolated from these 10 different soil samples. Most of soil are sandy loam. In these fungi, most of them are members of the *Aspergillus spp*. Optimal fermentation conditions are very important for maximal productivity of antibiotics (Cruegar, 1989). Moreover, the further investigation for extraction of antibacterial metabolite will be taken. According to the R_f value (Figure. 5), it was considered that antibacterial metabolite is suitable ethyl acetate solvent.

Therefore, solvent No. 4, ethyl acetate is suitable for the extraction of antibacterial metabolite from the fermented broth. The extraction of antibacterial compound was carried out by column chromatographic to get 9.5 g/ 20 liters.

Various chromatographic techniques have been used for successful fractionation and purification of biologically active compounds from variety of sample. Column-chromatography is one of the most popular and widely used separation techniques to characterize both organic and inorganic materials suggesting is potential usefulness in chemical analysis of complex extract material (Vivek *et al*., 2016).

In the screening of extracted substance, according to the TLC results (R_f value), gradient elution was performed successively with increasing polarity n-Hexane:EtOAc, (40:1 v/v), (20:1 v/v), (10:1 v/v), (5:1 v/v), (2:1 v/v) and (1:1 v/v) (Figure.6-11). Keeping in view the above justifications, the purification of antibacterial metabolites was carried out by using column chromatographic method. Identification of isolated compounds by modern spectroscopic techniques such as UV, FT IR and GC-MS would be carried out in further research.

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