# ANTIBACTERIAL COMPOUND PRODUCED BY *STACHYBOTRYS* SP. AGAINST *AGROBACTERIUM TUMEFACIENS*

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## Abstract

A total of 15 fungi were isolated from four different soil samples collected from Pathein Area. All 15 fungi showed no antimicrobial activity against eight test organisms except *Agrobacterium tumefaciens*. Among them, fungus TH-15 exhibited more activities than other fungi. After examining the biological activities, TH-15 was found to be similar with those of genus *Stachybotrys* sp. In the fermentation studies, the maximum activity reached at 72 hrs of fermentation for the production of antibacterial compound. After fermentation with optimal parameter, the broth was adjusted pH 7.0 and studied by paper chromatography. Thin layer chromatography (TLC) by using the crude extract to obtain the basal data. The purification of antibacterial compound was undertaken by silica gel column chromatographies with various elution solvents collected by Preparative Thin Layer Chromatography (PTLC).

## Introduction

Various microbial isolates may produce a wide variety of new or unusual compounds. Soil samples are good substrata for isolating microbes. Although microorganisms are harmful to human, they can be beneficial in medicines, industrials and various environment issues by means of secondary metabolite produced from these microorganisms (Hunter *et al*., 1999). *Agrobacterium tumefaciens* is the causal agent of crown gall disease (the formation of tumours) in over 140 species of eudicots (Young, *et al*., 2001).

The genus *Stachybotrys* is an asexually reproducing, belonging to the mitosporic Hypocreales group. *Stachybotrys chartarum* is a known producer of trichothecene mycotoxins and stachylysin (a hemolysin). The best

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characterized trichothecenes include satratoxins F, G, and H, roriden E, verrucarin J, and trichoverrols A and B (Dongyou and Paterson, 2011).

Verrucarin belongs in the class of tricothecenes, a group of sesquiterpene toxins produced by several fungi. Satratoxin-H, a trichothecene mycotoxin, is a naturally occurring mold byproduct of *Stachybotrys chartarum* which is toxic to humans and animals (Croft *et al.,* 1986). The aim and objectives are to collect soil samples in nature for the isolation of useful soil fungi and to identify soil fungi.

## Materials and Methods

### Collection and Preparation of soil samples

Four different soil samples were collected from Pathein Area, and were dried at room temperature in the laboratory.

### Isolation of microorganisms from different soil samples

The isolation of fungi was undertaken by the methods of chemical treatment dilution method (Phay and Yamamura, 2005) and feeding method (NITE, 2002) as shown in Figure 1.

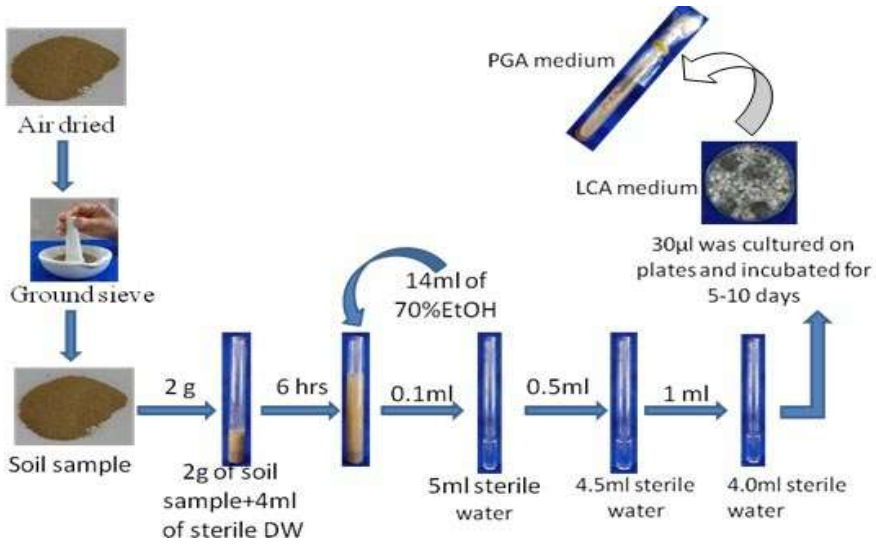
### Chemical treatment dilution method (Phay and Yamamura, 2005)

Soil sample was air dried at room temperature and was grounded and sieved. Two grams of the sieved soil was then put into 4 mL of sterilized distilled water and settle for 6 hours to germinate early-germinating soil fungi. Fourteen ml of 70% ethanol solution was then added into the tube containing soil suspension, and shaken for 1 minute and diluted with sterile water. After serial dilution, 30 µL of soil suspension was placed on low carbon agar medium (LCA medium) and then incubated for 5-7 days as shown in Figure 1.

### Feeding method (NITE, 2002)

Soil sample (1.0 g) was poured onto 100 mL LCA liquid culture medium and it was incubated overnight and 20 mL of 70% methanol was added. After shaking for 2 minutes, 30 µL sample was cultured on low carbon agar medium (LCA medium) plates and incubated for 5-10 days as shown in Figure 1.

**Method 1.** Chemical treatment dilution method (Phay and Yamamura , 2005)




**Method 2.** Feeding method (NITE, 2002)

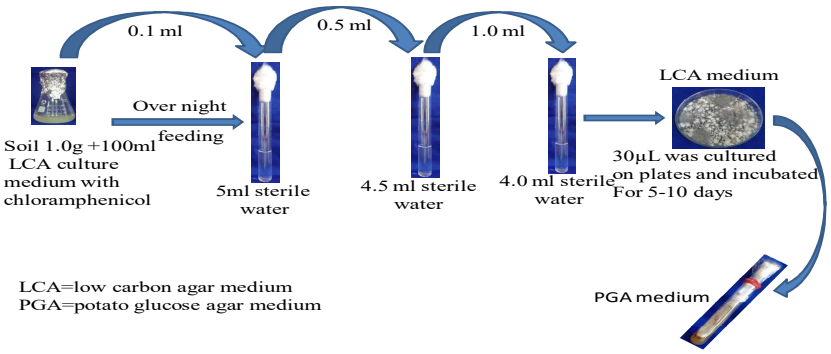



**Figure 1.** Procedure for chemical treatment dilution method and feeding method

**Preliminary study for antimicrobial activities by paper disc diffusion assay (Ando *et al*., 2004)**

The isolated fungi were inoculated on seed medium and incubated at room temperature for 3 days. Twenty mL of seed culture was transferred into the fermentation medium and incubated at room temperature for 7 days. Twenty µL of fermented broth was put on paper disc (size - 8mm) and placed

on assay plate containing test organisms and the plates were incubated for 24 hrs at 28˚C.

**Identification of the antibacterial metabolite producing fungus TH-15**

Morphological and microscopical characters of antibacterial metabolite producing fungus TH-15 was observed by the methods of identification of mitosporic fungi by Kasuhiko Ando (Biology Research Centre, 2015) and Barnett (1956).

**Studies on microbial growth kinetics on fungus TH-15 (Omura, 1985, Crueger, 1989)**

The strain TH-15 was inoculated into medium and cultured for 120 hrs at 100 rpm rotary shaker. The samples were taken out at 12 hrs intervals and PCV% (Packed cell volume) was calculated.

**Study on the effects of ages and sizes of seed culture on the fermentation**

The strain TH-15 was inoculated into the medium, 48 hrs, 60 hrs, 72 hrs, 84 hr, 96 hrs and 108 hrs were employed with 15 % seed culture in 12 hrs intervals for the growth.

In the investigation of sizes of inoculums, 5 %, 10 %, 15 %, 20 % and 25 % of 72 hrs seed culture were utilized for the fermentation. Fermentation was carried out 5 days and antibacterial activity was tested by paper disc diffusion assay reported by Ando, 2002.

**The effects of carbon and nitrogen sources on fermentation**

In the investigation of carbon sources, the 1.5 % of each carbon source used in this study such as glucose, sucrose, fructose, glycerol, glactose, tapioca powder and potato broth.

The 0.5 % 0f each nitrogen sources employed in this investigation were yeast extract, cornsteep liquor, $KNO_3$, peptone, fishcake and peanut cake. Antibacterial activity was examined at 12hrs intervals by paper disc diffusion assay of antibacterial activity against *Agrobacterium tumefaciens* respectively.

**Determination of solvents for the extraction of antibacterial metabolite by bio-assay of paper chromatography (Tomita, 1998)**

The antibacterial activity of each extract was measured and $R_f$ value for the corresponding metabolite was calculated.

**Extraction of antibacterial metabolites adjusted pH with organic solvents (Patrick, 1998; Simon and Gray, 1998)**

The *n-butanol* layer was tested the antibacterial activity compared with original fermented broth.

**Thin layer chromatography and bioautography overlay assay (Touchstone, 1992, and Aszalo, 1980)**

The TLC plates were developed in the solvents and these TLC plates were calculated by the following equation.

$$\text{This is defined as: } R_f \text{ value} = \frac{\text{Distance of compound from origin}}{\text{Solvent front from origin}}$$

**Investigation of silica gel column chromatography (Jarvis *et al*., 1986 and Marcelo *et al.*, 2006)**

Based on the TLC-bioautography's result, silica gel column chromatography was carried out and checked by TLC and tested with *Agrobacterium tumefaciens.* The column size and flow rate were the following.

Column size 14.5cm x50cm

Flow rate 0.75 mL/min

Eluting solvent $CHCl_3$, $CHCl_3$: MeOH (100:5 v/v) and $CHCl_3$: MeOH (100:10 v/v)

Fraction size 3mL / test tube

**Study on silica gel column re-chromatography**

Fraction two of silica gel column chromatography were combined and concentrated *in vacuo*. The UV spectrum and FTIR of isolated compound have been obtained. The UV spectrum of isolated compound ethanol is firstly dissolved in and subjected in the estimation of UV spectrum have been obtained. By using Amtt Company, Bago University and West Yangon

University of Department of Chemistry (UV - 1800 Spectrophotometer, Shimadzu, Japan). In FTIR were recorded by using FTIR-8400, Shimadzu, Japan at Universities′ Research Centre (URC), University of Yangon.

## Results

### Collection and Isolation of microorganisms from soil samples

In this study, 15 kinds of soil fungi were isolated from four different soil samples from Pathein Area, Ayeyarwady Region as shown in Table 1 and 2.

**Table 1.** Seven different soil samples collected at seven different places

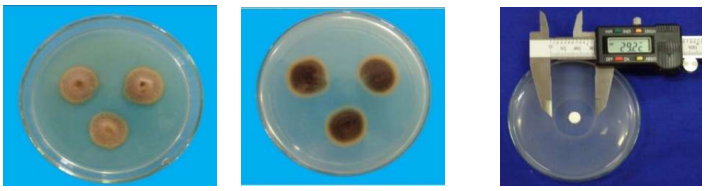
| Soil sample No. | pH | Soil type | Collect date | Collected place | Location |
|---|---|---|---|---|---|
| S-1 | 6.22 | Sandy Loam | 23.6.2013 | Shwe-wet-lue | N 16º 46.665″ E 094º 43.705″ |
| S-2 | 4.83 | Loam | 7.7.2013 | Thu-taw-gone | N 16º 48.978″ E 094º 42.540″ |
| S-3 | 5.28 | Sandy Clay Loam | 7.7.2013 | Tar-kaing | N 16º 45.392″ E 094º 42.757″ |
| S-4 | 4.86 | Silty Clay Loam | 28.7.2013 | Yae-oe-sin | N 16º 41.172″ E 094º 42.654″ |

**Table 2.**Numbers of fungi isolated from seven different soil samples by chemical dilution method and feeding method

| Soil No. | Collected places | Total isolated fungi | | | Fungi No. |
|---|---|---|---|---|---|
| | | Chemical treatment dilution method | Feeding method | Total fungi | |
| S-1 | Shwe-wet-lu | 2 | 1 | 3 | TH-01,02,03 |
| S-2 | Thu-taw-gone | 2 | 2 | 4 | TH-04,05,06,07 |
| S-3 | Tar-kaing | 2 | 2 | 4 | TH-08,09,10,11 |
| S-4 | Yae-o-sin | 2 | 2 | 5 | TH-12,13,14,15 |
| Total isolated fungi | | 8 | 7 | 15 | |

**Screening of effective fungi isolated from soil by paper disc diffusion assay**

The isolated 15 fungi were tested in nine kinds of test organism. Among them, TH-15 showed the best antibacterial activity (29.2mm of inhibitory zone) especially against *Agrobacterium tumefaciens.* According to the result, fungus TH-15 was selected for further investigation as shown in Figure 2.



Fungus TH-15 (Front and Reverse side) Antibacterial activity

**Figure 2.**Morphologies and activity of TH-15 agains *Agrobacterium tumefacie*ns

**Distinct characters of fungus TH-15**

In this study, the fungus TH-15 was cultured on PGA medium for seven days. It was found that, morphology of front color is brownish and reverse is black. The microscopical characters are conidial production - chain, type of conidial production – phialo, type of conidial ontogeny – enteroblastic, conidiophores - typical conidiophores but not branch, conidiophore elongates or not - conidiophores elongate along with conidial production, arrangement of conidiogenous cells - longitudinal (parallel), development of conidiogenous cells – stable, conidial production loci of conidiogenous cells – multi, conidia shape - simple spore, conidia septa – amerospore and hyphae - with septa regularly were observed.

According to the morphological and microscopical characters and based on the reference keys, fungus TH-15 was similar to genus *Stachybotrys* (Corda, 1837 & Jarvis *et al*.,1986) as shown in Figure 3 and morphology of TH-15 was mentioned above in Figure 2.

| | |
|---|---|
| Kingdom | Fungi |
| Division | Ascomycota |
| Class | Sordariomycetes |
| Order | Hypocreales |
| Family | Stachybotryaceae |
| Genus | *Stachybotrys* |

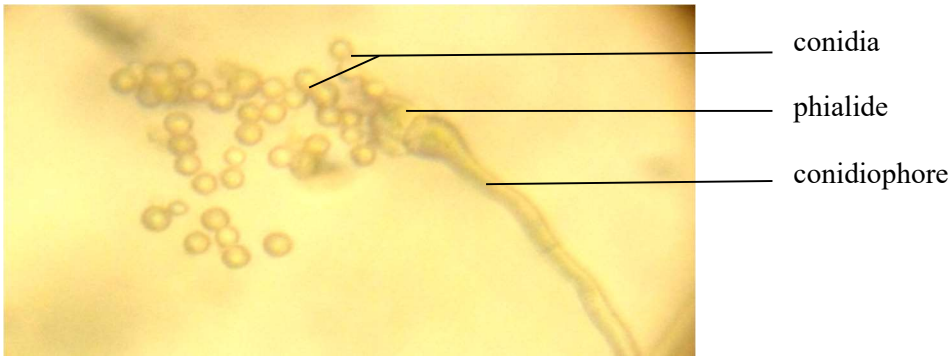



**Figure 3.** Photomicrograph of fungus TH-15 (x400)

**Studies on microbial growth kinetics of fungus TH-15**

In the studies of microbial growth kinetics, it was observed that growth phase of fungus TH-35 between 48hrs and 108hrs. According to Crueger and Crueger (1989), ages of inoculum 48hr, 60hr, 72hr, 84hr, 96hr and 108hr were utilized for the optimization of fermentation as shown in Figure 4.

**Studies on the ages and sizes of fungus TH-15 against on *Agrobacterium tumefaciens***

It was found that growth phase between 48hrs and 108hrs. According to Crueger and Crueger (1989), ages of inoculums (48, 60, 72, 84, 96 and 108hrs) with 20% sizes of inoculum were utilized for the optimization of fermentation. In this investigation, 72hrs seed culture was the best for fermentation in mention Table 3 and Figure 5. Based on the result of age of inoculum in Table 4 and Figure 5, 20% sizes of inoculums were optimized for the fermentation to produce the antibacterial metabolites.

**Studies on the effects of carbon and nitrogen sources of fungus TH-15 on the antimicroobial activity against on *Agrobacterium tumefaciens***

The best carbon source is glucose and yeast extract gave the best activity of nitrogen source were optimized in Figure 4.

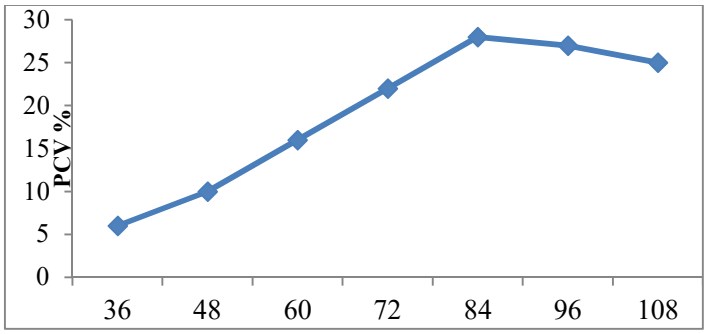


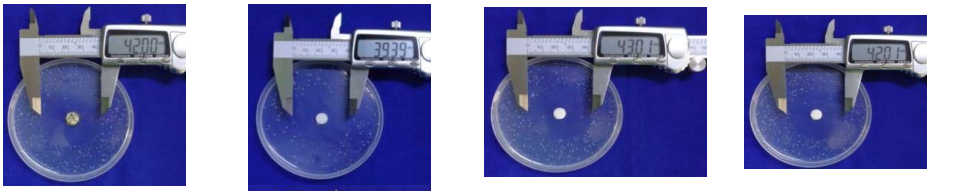

**Figure 4.** Microbial growth kinetics of fungus TH-15

**Table 3.**The effects of ages of culture on fermentation

| Culture time (Ages of culture, hrs) | Activity (clear zone, mm) |
|---|---|
| 48 | 38.45 |
| 60 | 39.67 |
| **72** | **42.00** |
| 84 | 41.17 |
| 96 | 40.54 |
| 108 | 39.04 |

Table 4.The effects of sizes of inoculum on the fermentation

| Sizes of inoculum at 72hrs (%) | Activity (clear zone, mm) |
|---|---|
| 5% | 42.65 |
| 10% | 40.54 |
| 15% | 39.39 |
| **20%** | **43.18** |
| 25% | 40.30 |

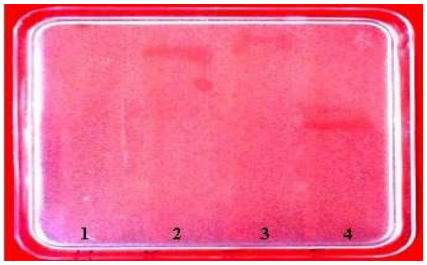


72hr (42.00mm) 20% (39.39mm) glucose (43.01 mm) Yeast extract (42.01 mm)

**Figure 5.**The best inhibitory zone of age, size, carbon source and nitrogen source against *Agrobacterium tumefaciens*

**Determination of solvent for the extraction of antibacterial metabolite by bio-assay of Paper chromatography**

According to the $R_f$ value, solvent 2 n-butanol more suitable for the extraction of antibacterial metabolite than solvent 4 ethyl acetate as shown in Figure 6.




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 6.**Paper chromatography Bioautographic overlay-assay

**Thin layer chromatography and bioautography assay**

Based on the TLC results ($R_f$ values), it was found that chloroform-acetone (9:1) solvent system was suitable for the separation of compound by silica gel column chromatography.

**Extraction of antibacterial metabolite adjusted pH with organic solvent**

It was found that antibacterial metabolite could be extracted with *n-butanol* at pH 7.0 and *n-butanol* was concentrated *in vacuo*.

**Silica gel column chromatography**

The presence of antibacterial compound produced by TH- 15 was detected by silica gel column chromatography, it was found that three main fractions (F-1, F-2 and F-3) were collected and checked by TLC and tested on *Agrobacterium tumefaciens*. In antibacterial activities, fraction two was showed the best inhibitory zone (33.58 mm) more than fraction one (30.02 mm of inhibitory zone) and fraction three (31.78 mm of inhibitory zone) respectively in Figure 7.

**Silica gel re-chromatography**

In the experiment of silica gel column re-chromatography, four fractions (F-A, F-B, F-C and F-D) were collected and then checked by TLC. By using PTLC method, compound 1and 2 were isolated from fraction B and

C, fraction A and D were showed tailing characters as shown in Figure 7 – 11and Table 5 and 6.

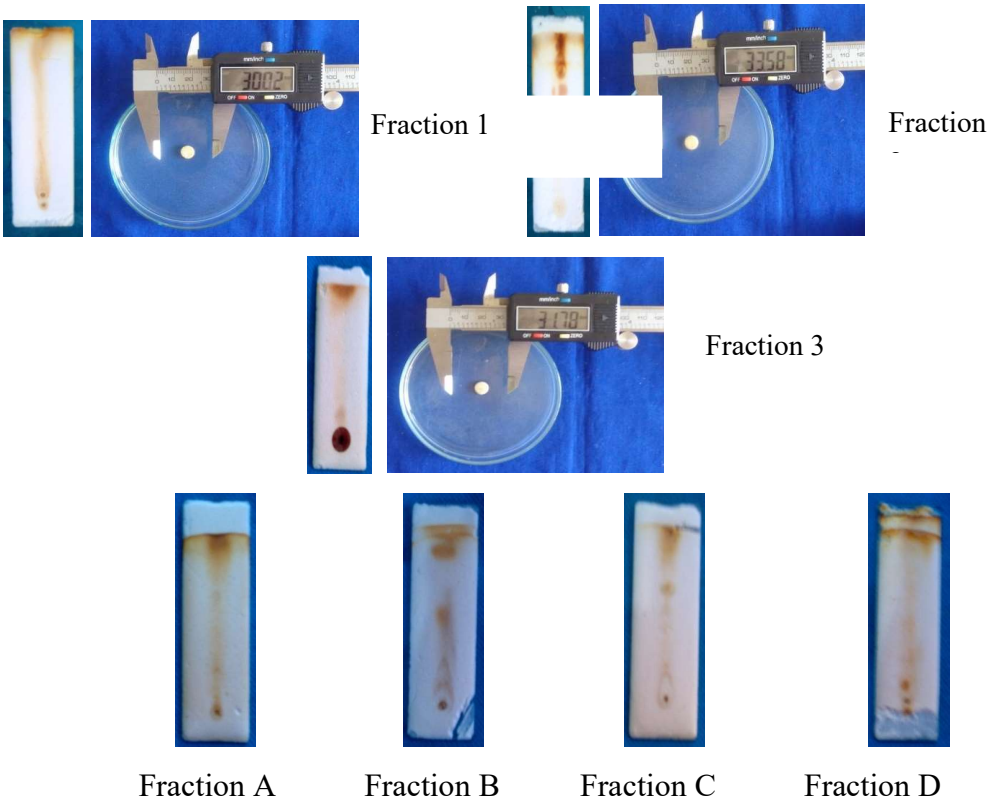
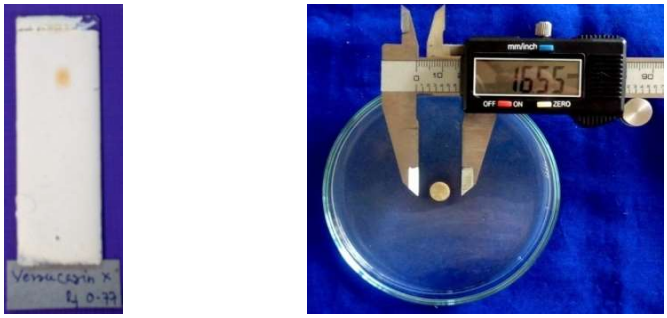


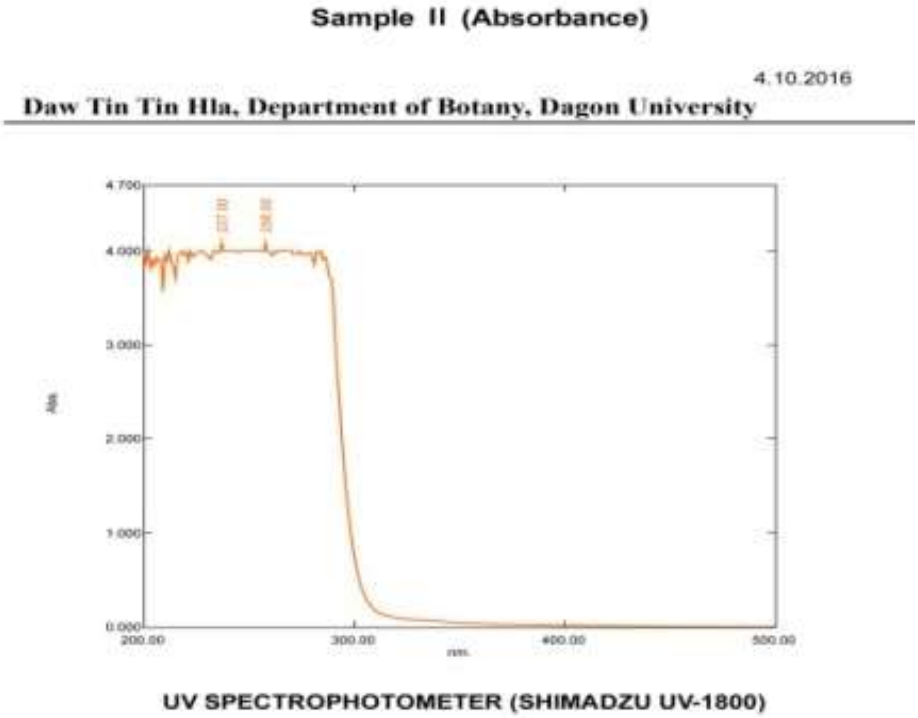

**Figure 7.**Checked by TLC and tested with *Agrobacterium tumefaciens*

| | |
|---|---|
| Solvent system | ethylacetate : hexane 7 : 3 v/v |
| Spray reagent | conc: $H_2SO_4$ (Δ) |
| $R_f$ value | 0.77 |
| UV | 258 nm observed (literature 259 nm) |
| Melting point | >300°C |




Inhibitory zone(16.55mm)

**Figure 8.**TLC plate and antibacterial activity against on *A tumefaciens*



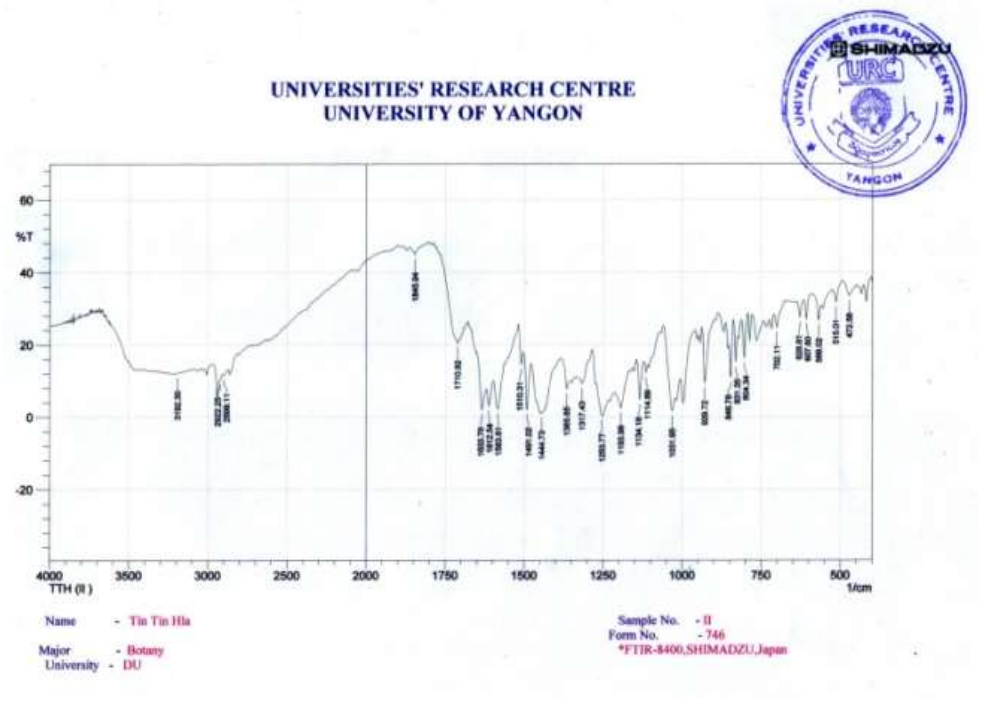


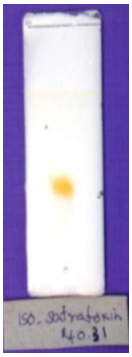

**Figure 9.**UV and FTIR spectrum of isolated compound 1 (in Amtt Company and URC, Yangon)

**Table 5.** FTIR spectrum of isolated compound 1 and comparison with literature value

| No. | Wave no. ($cm^{-1}$) | | Functional group |
|---|---|---|---|
| | isolated | literature | |
| 1 | 3445 | 3440 | OH stretching hydroxy group |
| 2 | 2922 | 2970 | CH stretching for CH =CH group |
| 3 | 1710 | 1715 | C=O stretching |
| 4 | 1630 | 1635 | C=C stretching for aromatic ring |
| 5 | 1444 | 1410 | CH banding |
| 6 | 1263, 1193 | 1270, 1190 | C-O- C stretching |
| 7 | 1031 | 1030 | C-O stretching in alcohol |
| 8 | 970, 625 | 973, 615 | CH out of plane banding |

S

| | |
|---|---|
| Solvent system | ethylacetate : hexane 7 : 3 v/v |
| Spray reagent | conc: $H_2SO_4$ (Δ) |
| $R_f$ value | 0.31 (literature 0.33) |
| UV | 225.10nm observed ( literature 226nm) |
| Melting point | 165-166°C ( literature 168-171) |



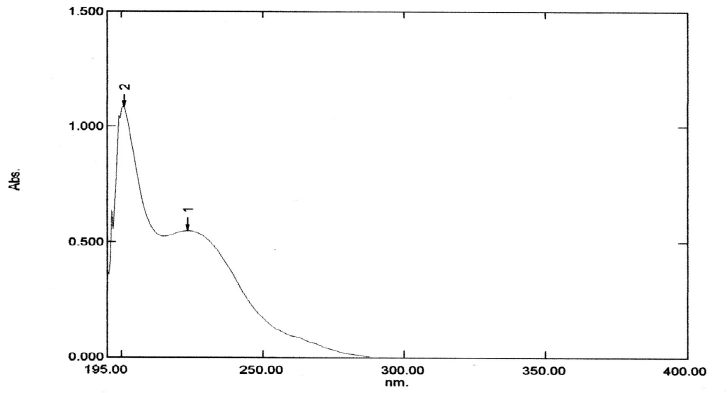


Inhibitory zone (18.88mm

**Figure 10.** TLC plate and antibacterial activity against o n *A. tumefaciens*

**WEST YANGON UNIVERSITY**
**DEPARTMENT OF CHEMISTRY**
**UV- 1800 SPECTROPHOTOMETER, SHIMADZU, JAPAN**

Sample Name : III
Operator : Kyaw Khaing



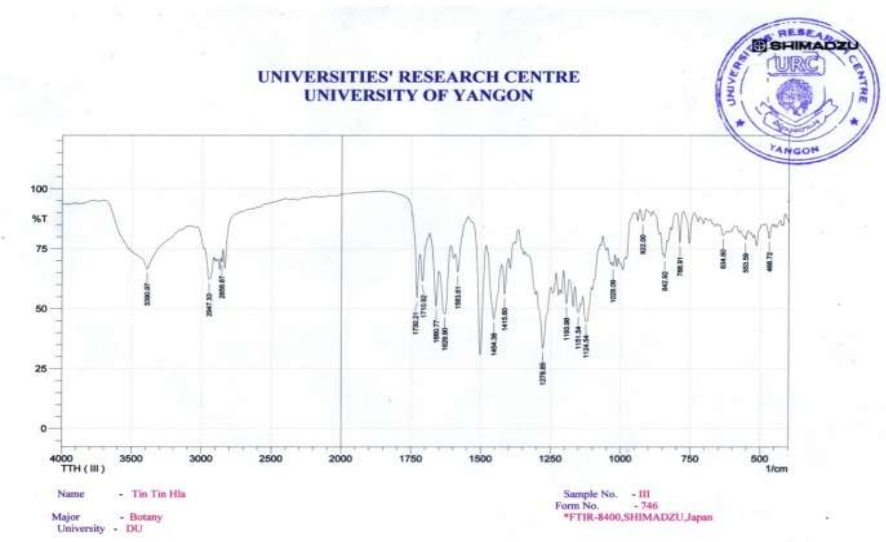



**Figure 11.**UV and FTIR spectrum of isolated compound 2

(in West Yangon University, Chemistry Department and URC, University of Yangon)

**Table 6**. FTIR spectrum of isolated compound 1 and comparison with literature value

| No. | Wave no. ($cm^{-1}$) | | Functional group |
|---|---|---|---|
| | isolated | literature | |
| 1 | 3550,3400 | 3560,3440 | OH stretching for hydroxy group |
| 2 | 2947, 2868 | 2975, 2855 | CH stretching for $CH_2$=$CH_3$ group |
| 3 | 1730, 1660 | 1730, 1660 | C=O stretching |
| 4 | 1583 | 1595 | C=C stretching for aromatic ring |
| 5 | 1278, 1193 | 1270, 1200 | C-O.C stretching (asymmetric) |
| 6 | 1151, 1124 | 1165, 1145 | C-O stretching (symmetric) |
| 7 | 1028 | 1080 | C-OH stretching |
| 8 | 970 | 975 | CH out of plane banding |

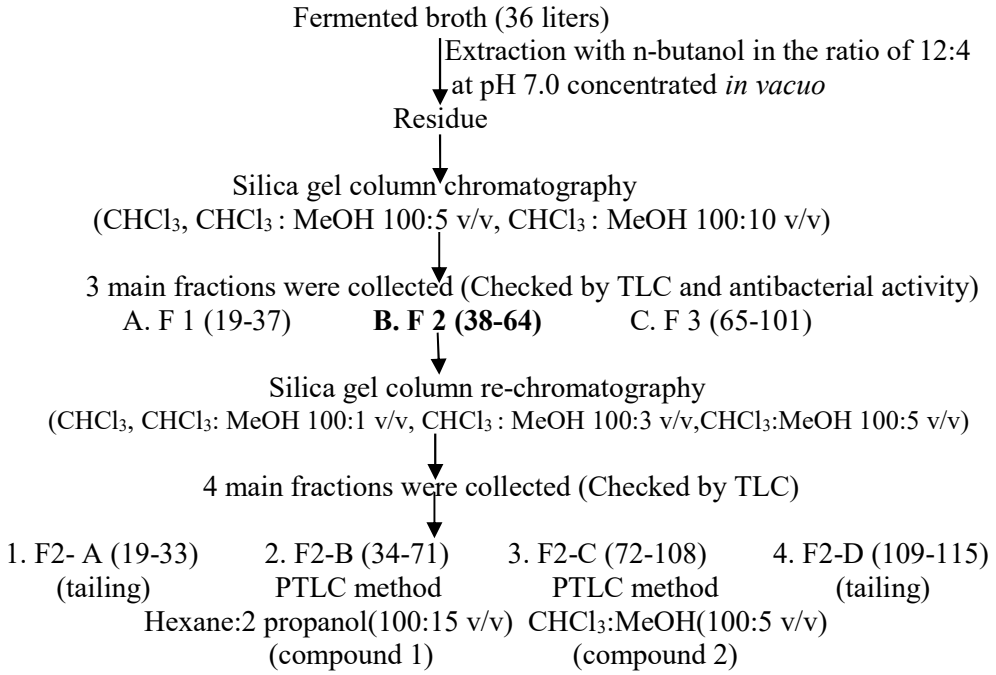



**Fig. 11.**Flow diagram of separation and isolation of antibacterial compound from extract TH-15

## Discussion and Conclusion

A total of 15 fungi were isolated from four different soil samples from Pathein Area. The isolated 15 fungi were tested in *Aspergillus paracitius, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas fluroscens, Saccharomyces cerevisiae* and *Staphylococcus aureus*, these isolated fungi showed no antimicrobial activity except for *Agrobacterium tumefaciens.* Among them, it was found that strain TH-15 distinctly showed the antimicrobial activity (29.2mm of inhibitory zone). Therefore, this fungus TH-15 was selected for the production of the antibacterial metabolite.

*Stachybotrys* is characterized by macronematous, mononematous, unbranched or branched conidiophores, with discrete terminal and phialidic conidiogenous cells, and aseptate, reniform, ellipsoidal to spherical, smooth or verrucose conidia (Brazilian Journal of Botany, 2010), in the investigation of fungus TH-15 is similar genus *Stachybotrys.*

The optimal parameter such as growth phase between 48 hrs and 108 hrs; 20% sizes and 72 hrs age of inoculum were observed. In the studies of

carbon and nitrogen sources utilization, glucose (43.01mm, inhibitory zone) and yeast extract (42.01mm, inhibitory zone) were the best for the production of the antibacterial metabolites. According to the result, the extraction of antibacterial metabolite was found in 5 days period.

Preliminary studies of paper chromatography are required to extract the compound from the fermented broth (Kyowa Hakko Co. Ltd., Japan, 1980). In the study of paper chromatography, it was found that n-butanol is suitable for the extraction of the antibacterial metabolite. In TLC, it was found that chloroform-methanol (9:1) solvent system was suitable. Base on the result, the active fractionated compounds, purified by various chromatographies and identification of pure compound had been studied.

In the study of isolation of the antibacterial compound from TH-15, two compounds were isolated from fraction two. Trichothecenes consists of verrucarin A, E and X, piperidine and satratoxins F, G, and H. Trichothecenes are mycotoxins, mycotoxins were used as antibiotics (Jarvis *et al*., 1986 and Marcelo *et al*., 2006). The metabolite extracted from fermented broth of the fungus TH-15, when the analysis of fraction two were isolated verrucarin X and satratoxin H. These isolated two compounds (compound 1 and compound 2) were re-checked by melting point, UV, FTIR, TLC and tested with *Agrobacterium tumefaciens*.

In compound 1, melting point is >300° C, in TLC of $R_f$ value is 0.77, UV 258 nm, tested with *Agrobacterium tumefaciens* (16.55 mm) inhibitory zone were observed compared as literature UV 259 nm (Marcelo *et al*., 2006). Therefore, isolated compound 1 suggested as verrucarin X. In compound 2, melting point is 165 - 166˚C, in TLC of $R_f$ value is 0.31, UV 225.10 nm and 18.88 mm inhibitory zone observed. In literature, melting point is 168 - 171°C, TLC of $R_f$ value 0.33, UV 226 nm (Rusell *et al.*, 1984). Therefore, the isolated compound 2 may be satratoxin H. These collected two compounds were mentioned that Figure flow chart.

The antibiotic trichothecene can be used for its antimicrobial in the form of pharmaceutical compositions containing verrucarin X and satratoxin H. The compositions may also contain other active antibacterial and/or antitumor agents and these made up in any pharmaceutical form appropriate (Rusell *et al.*, 1984). Thus, it is necessary to clarify how these isolated two compounds can be applied as novel pharmaceuticals will be conducted in the near future.

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