

ISOLATION OF ENDOPHYTIC FUNGI FROM *VITEX TRIFOLIA* L. AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS

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Abstract

Endophytic fungi (6 strains) were isolated from the leaf segments of *Vitex trifolia* L. collected from Kamayut Township in Yangon region. All the characters of vegetative and reproductive parts of the selected plants were checked with the help of available literatures. The leaves of *Vitex trifolia* L. were surface sterilized and the isolation of endophytic fungi was done on the sucrose yeast extract medium at 30°C for 3-7 days. All 6 different strains were sub-cultured into separate SY agar slants. The antimicrobial activity of 6 isolated endophytic fungi were investigated against 6 kinds of test organisms by paper disc diffusion method. Six types of endophytic fungi were observed to possess very high antibacterial activity. The selected strains T2, T3, T5 and T6 was extracted and isolation of bioactive compound from each selected broth culture by using TLC, UV and IR.

Keywords: *Vitex trifolia* L. leaves, endophytic fungi, TLC, UV and IR.

Introduction

Vitex trifolia L. is belong to the family Lamiaceae (Verbenaceae). It is also called chaste tree in English and jalanirgundi in Sanskrit, Nichinda in Hindi, and Manjingzi in Chinese. This plant is found in the tropical and subtropical regions around the world including India, Sri Lanka, China and Indonesia, Australia, and Singapore, East Africa and introduced to many islands in the Central Pacific and Hawaii. Meena Ajay Kumar, *et al*; (2011), Shashank Tiwari and Shreya Talreja, (2020). *Vitex trifolia* L. is a shrub or small tree, pungent smell. Hooker, (1897), Kirtiker and Basu. (1933), Backer,(1965), Julissa Rojas-Sandoval , (2022).

Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, fruits, roots, seeds etc. The most important bioactive compounds from medicinal plants include terpenes, alkaloids, phenolic compounds, steroidal compounds and flavonoids. Maroof Ahmed, *et al*; (2012).

This plant is known to possess various active constituents such as essential oils, diterpenes, Vitetrifolins with several pharmacological properties such as antipyretic, antibacterial, works against asthma and allergic diseases, anti-inflammatory and sedative for headache, rheumatism and for common cold and as anti-trypanosomal in Asian countries. Several oils were extracted from the leaves of the plant that showed considerable mosquito repellent activity. The plant is a Chinese folk medicine for the treatment of cancers, evaluated by sulforhodamine B, which is widely used in Chinese folk medicine. The fruit extracts of *V. trifolia* exhibited antipyretic, analgesic, and anti-inflammatory activity. Anita Rani and Anupam Sharma, (2013).

The leaves are traditionally made into decoction for oral inflammation, or externally applied as a poultice for rheumatic pain and sprains and exhibited moderate inhibition of both Gram-positive and Gram-negative bacteria. The flowers are administered orally as infusion for treating intermittent fever accompanied by vomiting and thirst, while the stems are used for

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dysentery. The roots are used as antiemetic, expectorant. Meena Ajay Kumar *et al*; (2011). Ning Wee Hai, *et al*; (2020).

Endophytes are microbes which colonize living, internal tissues of plants without causing any harm to their host. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. The endophytic fungi play important physiological and ecological roles in their host life. Khan Rezwana, *et al*; (2010). Today around 40% of modern medicines are plant-derived because of fewer side effects. Plants generate various secondary metabolites, which may be broken down into the chemical categories of phenolics, terpenes, and alkaloids. Sharma and Singh (2021).

In this study the morphological characters of plant sample and isolation of endophytic fungi from *Vitex trifolia* L. leaves, antimicrobial activity and then characterization of bioactive compound from selected strains. The main objectives of research work were to study morphological characters of *Vitex trifolia* L. to investigate the isolation of endophytic fungal strains from *Vitex trifolia* L. leaves and antimicrobial activity of endophytic fungi, to study the preparation for evaluation of bioactive compounds from isolated fungi.

Materials and Methods

Collection of Plant Samples

The plant sample of *Vitex trifolia* L. (Kyaung pan-galay) were collected from Kamayut Township, in Yangon region. All vegetative and reproductive parts of the collected sample were identified and studied with the help of available literatures Hooker, (1885), Kirtiker and Basu. (1933), Backer, (1965), Julissa Rojas-Sandoval, (2022) at Department of Botany, University of Yangon.

Isolation of endophytic fungi strains

The plants were washed in running water for fifteen minutes. These parts were sterilized by soaking in 75% ethanol for two minutes. Then they were sterilized by soaking in 5.3% sodium hypochlorite for one minute. After that, these parts were sterilized by soaking in 75% ethanol for fifteen seconds. These parts were dried on sterilized paper and then they were placed on agar plates containing nutrient sucrose yeast extract medium (Sucrose 10.0g, Yeast extract 3.0g, NaCl 0.5g, CaCO₃ 0.1g, Agar 18.0g, Distilled water 1L and pH 6.8±0.2) Strobel and Sullivan, (1999). Then, these plates were incubated at 30°C for 3-7 days and transferred to new plates. Then, isolated fungal strains were transferred into slant culture of test tubes Phay, (1997), Carbungco *et al.*, (2017). To suppress the growth of bacteria 0.001g of chloramphenicol was added to the above medium. Similarly, to deter the growth of fungi and yeasts about 0.001g of nystatin was added to isolation medium.



Figure 1. Isolation of Endophytic fungi from *Vitex trifolia* L.

Fermentation (broth culture)

The isolated fungal strains grown on slant culture were transferred into conical flasks containing 100 ml of SY broth (Sucrose 10.0g, Yeast extract 3.0g, NaCl 0.5g, CaCO₃ 0.1g, Distilled water 1L and pH 6.8±0.2). Then, isolated fungal strains were grown in broth media and these flasks were placed in chamber at room temperature for 3-7 days. Colony forms were showed on the surface of the broth media Strobel and Sullivan, (1999). Next step, experimentation of endophytic fungal activity against on test organisms on the culture media.

Antimicrobial activity test (Paper disc diffusion assay), Phay (1997)

After solidification, paper discs impregnated with broth samples were applied on the test plates and these plates were incubated at 30°C for 24-48 hrs. After 24-48 hrs clear zones (inhibitory zone) surrounding the test discs were measured. These zones indicate the presence of the bioactive compounds which inhibit the growth of test organisms.

Extraction of bioactive compound

The isolated individual endophytic fungi were inoculated in 150ml of conical flask containing sterilized SY broth medium. The flask was incubated at 28°C room temperature for 20 days with periodical shaking. After incubation period the fermented medium was filtered by using sterile qualitative filter paper ϕ 12.5cm and the filtrate was preserved and the mycelium part was removed. For the extraction of secondary metabolites, equal volume of filtrate and equal volume of ethyl acetate solvent was taken in the separating funnel and shaken continuously for 30 minutes and allowed to stand for 5 minutes until the formation of two immiscible layers. The upper portion of the solvent was separated and kept for evaporation. The culture filtrate was extracted thrice with ethyl acetate. Then the solvent phase was evaporated at 40°C. The residue was redissolved in methanol for subsequent separation and the crude extract was analyzed by thin layer chromatographic separation. Nithya and Muthumary, (2011), Sheeba *et al*, (2019).

Thin Layer Chromatography (TLC)

In TLC analysis, 6.5cm×1.5cm square pieces of TLC plates cut out from 20cm×20cm plate produced by Bezogen Von CAMAG (Germany) was used. The platting particle size of silica gel was 60 F254. The 20 days fermented broth of T2, T3, T5 and T6 was extracted with ethyl acetate

and about 0.5 µl was loaded onto TLC plate. After drying, the TLC plate was put into the chamber containing the solvent system of Petroleum ether : water: Ethyl acetate (9 : 3 : 2), Chloroform : Methanol (8 : 2), Hexane : Chloroform : Methanol (3 : 2 : 1), Methanol : ethyl acetate : Petroleum ether (9 : 3 : 1) in ascending condition. After 20-30 minutes the TLC plate was taken out and dried. Then the spot of metabolite was developed by using the spray containing vanillin: ethanol: sulphuric acid (5:2:0.5). The R_f value of the spot was calculated by using the following formula.

$$R_f = \frac{\text{Distance of compound from origin}}{\text{Distance of solvent from origin}}$$

Characterization Techniques

UV, FTIR and investigation of the ethyl acetate endophytic fungi extract and mixing with methanol solvent solution were carried out. The UV -visible spectrophotometer was recorded between 250-600 wavelength number by using (Evolution 220 UV-visible spectrophotometer). The mixing solution were subjected to FTIR (Nicolet summit PRO spectrophotometer in the range 450 - 4500 Spectrometry). The FTIR spectrum was recorded between 4500-500 cm^{-1} using the KBr pellet mode, at Department of Chemistry, University of Yangon.

Results

Scientific Name	- <i>Vitex trifolia</i> L.
Myanmar Name	- Kyaung Pan -kalay
English Name	- Indian Wild pepper
Family	- Lamiaceae /Verbenaceae

Outstanding characters

Habit perennial, shrub, stem long, erect, hairy present, aromatic smell present. Leaf simple, 3- trifoliate, opposite, short petioles, salivary hair present, exstipulate, ovate-oblong, serrate, acute. Inflorescence terminal panicle cyme. Flower pedicellate, bracteate, bracts small, ebracteolate, bisexual, zygomorphic complete, pale purple, hypogynous. Calyx (5), synsepalous, triangular acute, sepaloid, valvate. Corolla (5), syn petalous, bilabiate, corolla tube, petaloid (pale purple), upper lip 2-lobes, lower lip 3 lobes, imbricate. Androecium stamens 4, didynamous, epipetalous, anther ditheous, introrse, dorsifixed. Gynoecium 2, bicarpellary, syncarpous, ovary bilocular (early stage), tetra in mature stage, ovary superior, axile placentation one ovule in each locule, style long, stigma bifid. Fruit globose, salivary hair present.

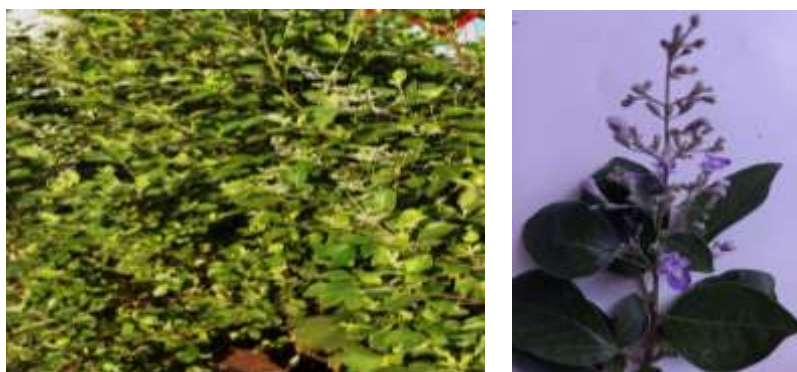


Figure 2. Habit and Inflorescence of *Vitex trifolia* L.

Six isolated endophytic fungi from *Vitex trifolia* L.

Table 1. Cultural Characters and Colony Morphology of Six isolated Endophytic Fungi

Strains	Cultural characters		Colony Morphology		
	Front view	Reverse view	Form	Elevation	Margin
T1	white	white, light yellow at the center	circular	umbonate	entire
T2	light grey	Pink, black at the center	filamentous	umbonate	filiform
T3	white	orange ,white at the margin	filamentous	flat	curled
T4	white	pink	circular	crateriform	undulate(wavy)
T5	white	white, brown at the center	circular	umbonate	filiform
T6	light green	white	circular	flat	entire

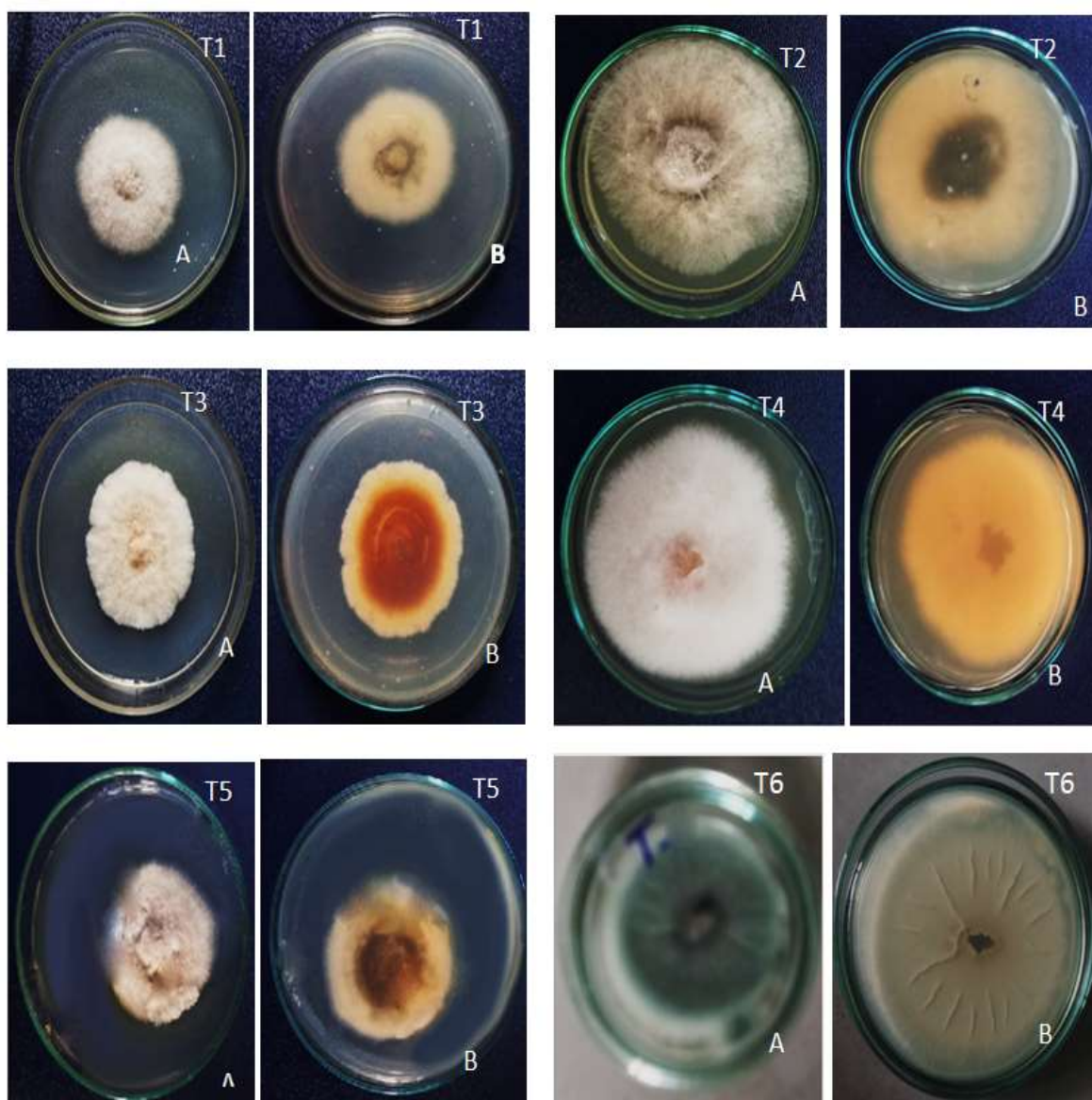


Figure 3. A. Front View, B. Reverse View of isolated Endophytic fungi T1,T2,T3,T4,T5 and T6.

Table 2. Test organisms and diseases

Test organisms	Code number	Diseases
<i>Bacillus subtilis</i>	JAP-0225025	Endocarditis, pneumonia, septicemia
<i>Bacillus pumilus</i>	IFO- 905571	Food poisoning the symptoms that resulted from infection included dizziness, headache, chill, back pain, stomach cramp, and diarrhea
<i>Candida albicans</i>	IFO-1060	Skin infection, vaginal candidiasis, alimentary tract infection, urogenital infection.
<i>Micrococcus luteus</i>	NITE-83297	Skin disease
<i>Pseudomonas</i> spp.	IFO-94307	Urinary tract infection, respiratory system infection, dermatitis, bone and joint infections, gastrointestinal infections. skin infection.
<i>Staphylococcus aureus</i>	ATCC-12877	Blood stream infection, burns, abscesses, skin disease, food poison wound infection, staphylococcal pneumonia.

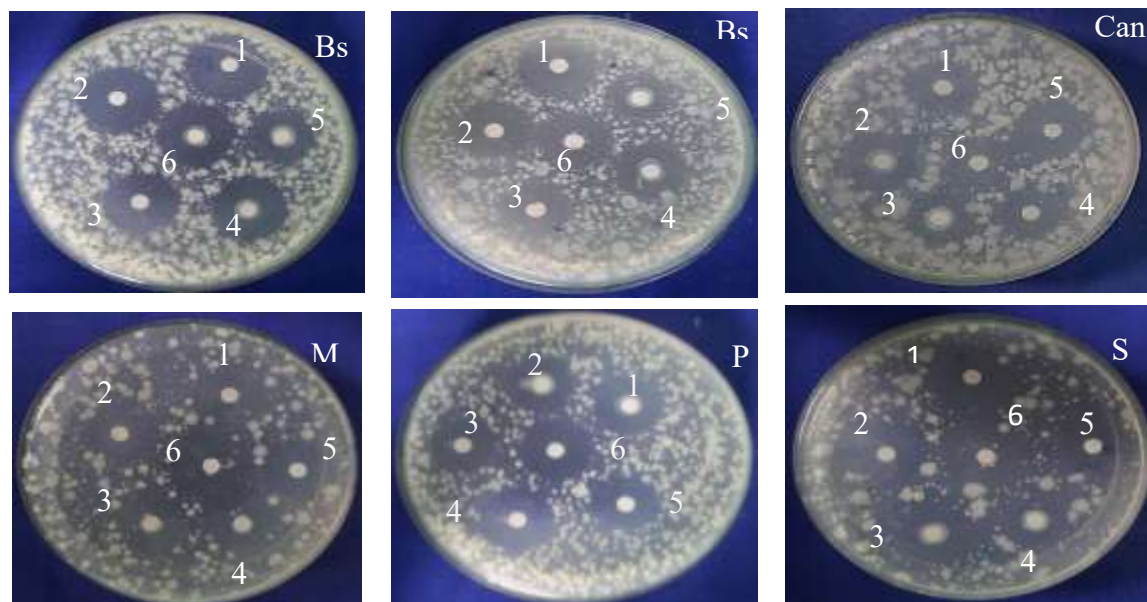
Screening for Antimicrobial Activity by paper disc diffusion method

In this screening it was observed that fungi T1, T2, T3, T4, T5 and T6 showed the antimicrobial activities on *Bacillus subtilis*, *Bacillus pumilus*, *Candida albicans*, *Micrococcus luteus*, *Staphylococcus aureus* and *Pseudomonas* spp. All strains were showed very high antimicrobial activity on six test organisms.

Table 3. Antimicrobial activities of isolated endophytic fungi from *Vitex trifolia* L. (Numbers shown in Tables are size of clear zones in mm)

Test organisms Strains	<i>Bacillus subtilis</i>	<i>Bacillus pumilus</i>	<i>Candida albicans</i>	<i>Micrococcus luteus</i>	<i>Pseudomonas</i> spp.	<i>Staphylococcus aureus</i>
T1	30	28	24	29	24	25
T2	30	30	26	27	26	26
T3	27	28	25	25	25	30
T4	28	26	22	27	23	30
T5	29	23	29	28	26	30
T6	30	25	26	30	27	30

Paper disc size = 6mm, 10mm-12mm weak activity, 13mm-17mm high activity, 18mm – above very high activity



BS= *Bacillus subtilis*, BP= *Bacillus pumilus*, Can= *Candida albicans*, M= *Micrococcus luteus*, P= *Pseudomonas* spp., S= *Staphylococcus aureus*

Figure 4. Antimicrobial activities of isolated endophytic fungi from *Vitex trifolia* L.

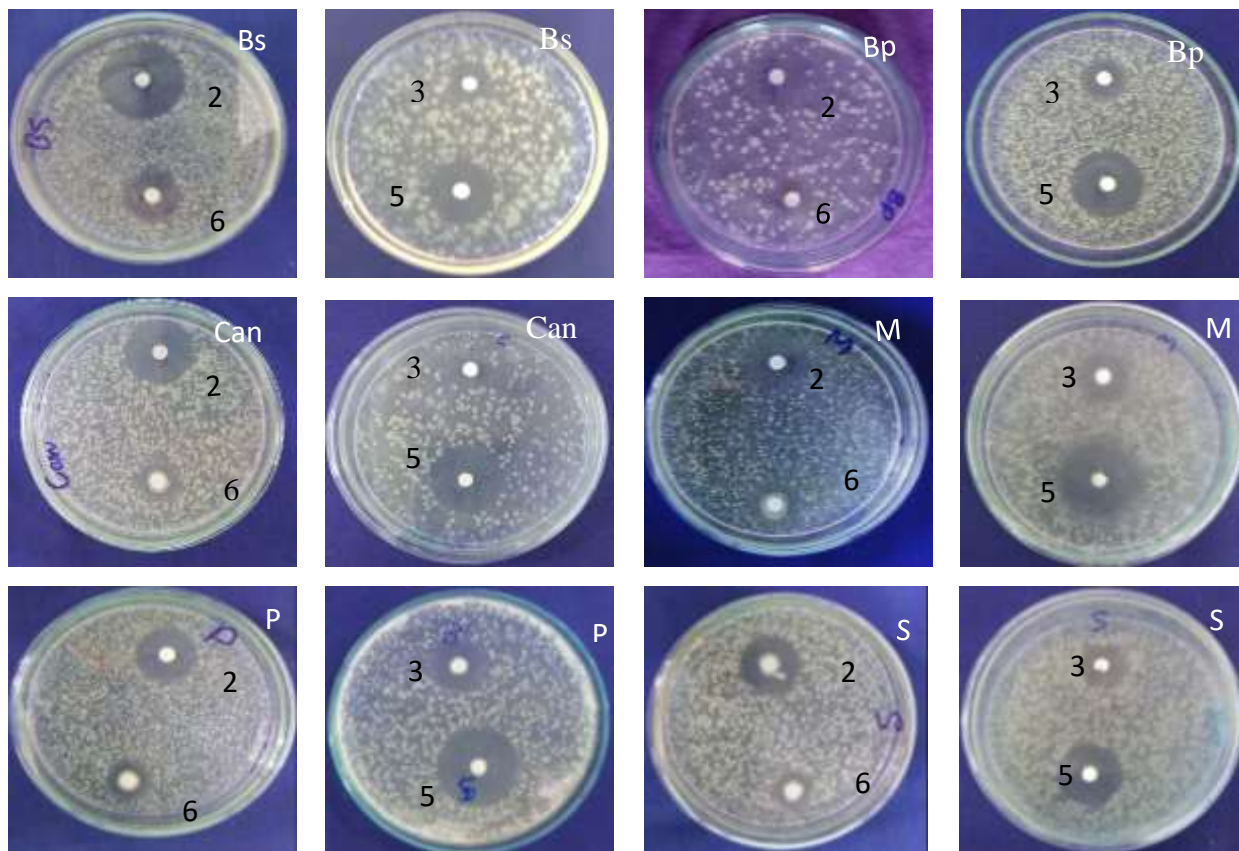
Screening for Antimicrobial activity of ethyl acetate extract from selected fungi T2,T3,T5 and T6 by paper disc diffusion method

In this experiment, only four isolates which revealed very high antimicrobial activity were selected for further investigation. They are T2, T3, T5 and T6. T2 extract was showed highest activity on *Bacillus subtilis*, *Bacillus pumilus* *Micrococcus luteus*, and *Pseudomonas* spp. T5 extract was found highest activity on all organisms and the rest of the extracts were showed high and very high activity on six test organisms.

Table 4. Screening for Antimicrobial activity of ethyl acetate extract from selected fungi T2,T3,T5 and T6 by paper disc diffusion method (Numbers shown in Tables are mm.)

Test of organisms \ Strains	T2	T3	T5	T6
<i>Bacillus subtilis</i>	33	17	26	23
<i>Bacillus pumilus</i>	31	17	29	23
<i>Candida albicans</i>	23	20	27	20
<i>Micrococcus luteus</i>	27	23	36	23
<i>Pseudomonas</i> spp.	27	22	30	20
<i>Staphylococcus aureus</i>	22	19	26	16

Paper disc size = 6mm, 10mm-12mm weak activity, 13mm-17mm high activity, 18mm – above very high activity.



BS= *Bacillus subtilis*, BP= *Bacillus pumilus*, Can= *Candida albicans*,

M= *Micrococcus luteus*, P= *Pseudomonas* spp., S= *Staphylococcus aureus*

Figure 5. Antimicrobial activity of ethyl acetate extract from isolated endophytic fungi T2,T3,T5 and T6
Preliminary detection of metabolite in the crude extract of endophytic fungi T2,T3,T5 and T6 by Thin Layer Chromatography

Solvent system T2= Petroleum ether : Water: Ethyl acetate (9 : 3 : 2)

T3 = Chloroform : Methanol (8 : 2)

T5 = Hexane : Chloroform :Methanol (3 :2 :1)

T6 = Methanol :Ethyl acetate : Petroleum ether (9 : 3 : 1)

Spraying = Vanillin : Ethanol :Sulphuric Acid (5:2:0.5)

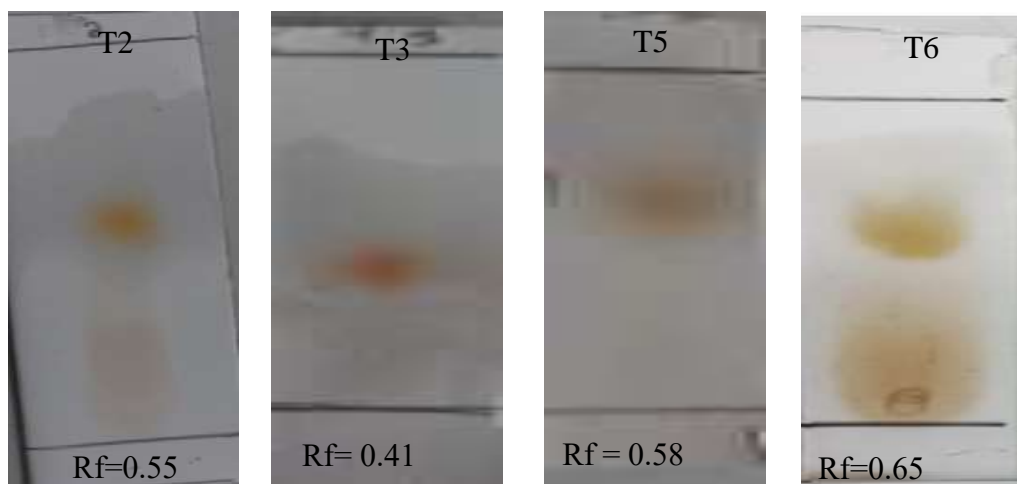


Figure 6. Crude bioactive compound from isolated endophytic fungi T2, T3, T5 and T6 by Thin Layer Chromatography (TLC)

Analysis of selected extract T2, T3, T5 and T6 by UV visible spectroscopy

The UV visible analysis data the wavelength number range 200 - 600 nm were recorded data of four selected extract sample. T2 was showed the wavelength number of (261, 283 and 303 nm), T3 showed the (290 and 403 nm), T5 was observed 297 and T6 was showed (294, 324 and 344 nm). This result was showed that the methanolic soluble compound on UV data.

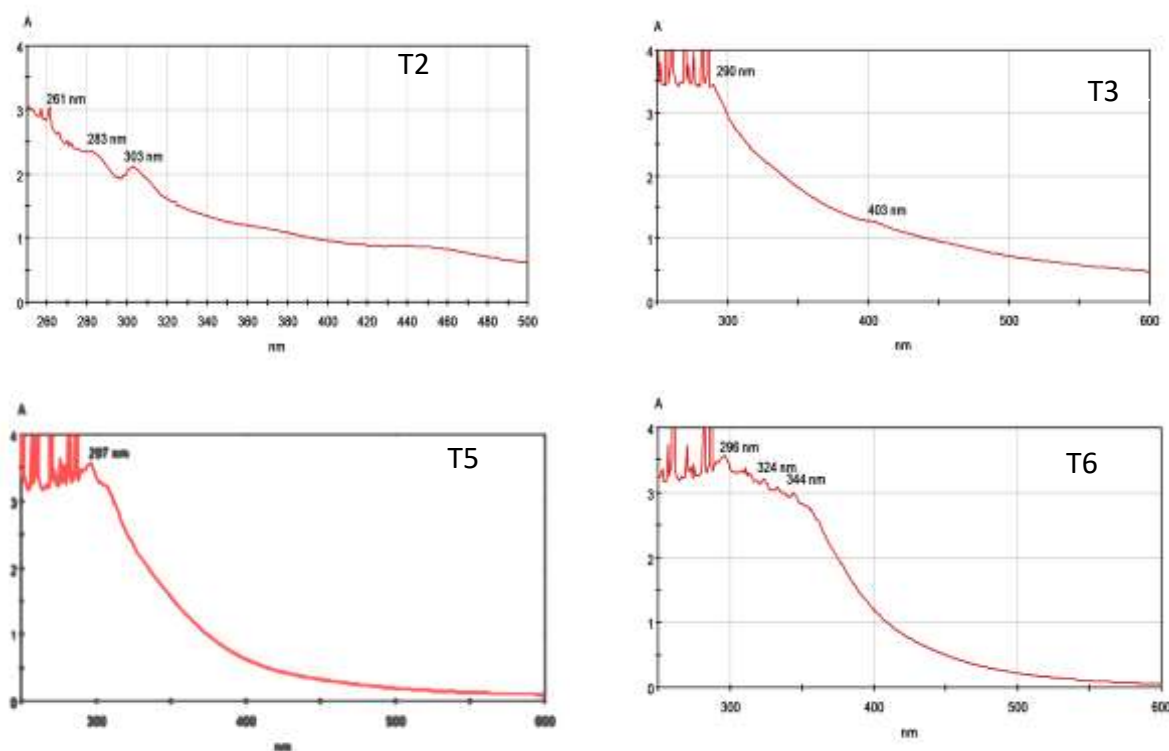


Figure 7. The UV data of four selected strains (T2,T3,T5,T6)

Infra-red spectral data

The FTIR spectra of the investigated T2, T3, T5 and T6 ethyl acetate sample extract and mixing with methanol solvent which solution analysis of the synthesized have showed in Fig 8 The IR spectrum data was recording by using Nicolet IS 50 FTIR spectrophotometer in the range 500 - 4500 cm^{-1} with the FTIR spectrophotometer. The determination of FTIR spectrum was obtained from the following data. The main peaks and their assignment to functional groups of methanol mixing sample. The results showed characteristic strong absorption bands at 3351, 3339., 3339., 3360, -OH stretching in alcohol, 2954, 2843 for -CH stretching for CH_3 group, 2120, 2115 cm^{-1} for $\text{C}\equiv\text{C}$ stretching for acetyl group, 1643, 1642, 1634 for $\text{C}=\text{O}$ stretching for keto-enol system and at 1457, 1453, 1450, 1411 cm^{-1} for $\text{C}=\text{C}$ stretching for aromatic ring 1015 for C-O stretching in alcohol, 610, 607, 559 (C-N banding out of plane) respectively.

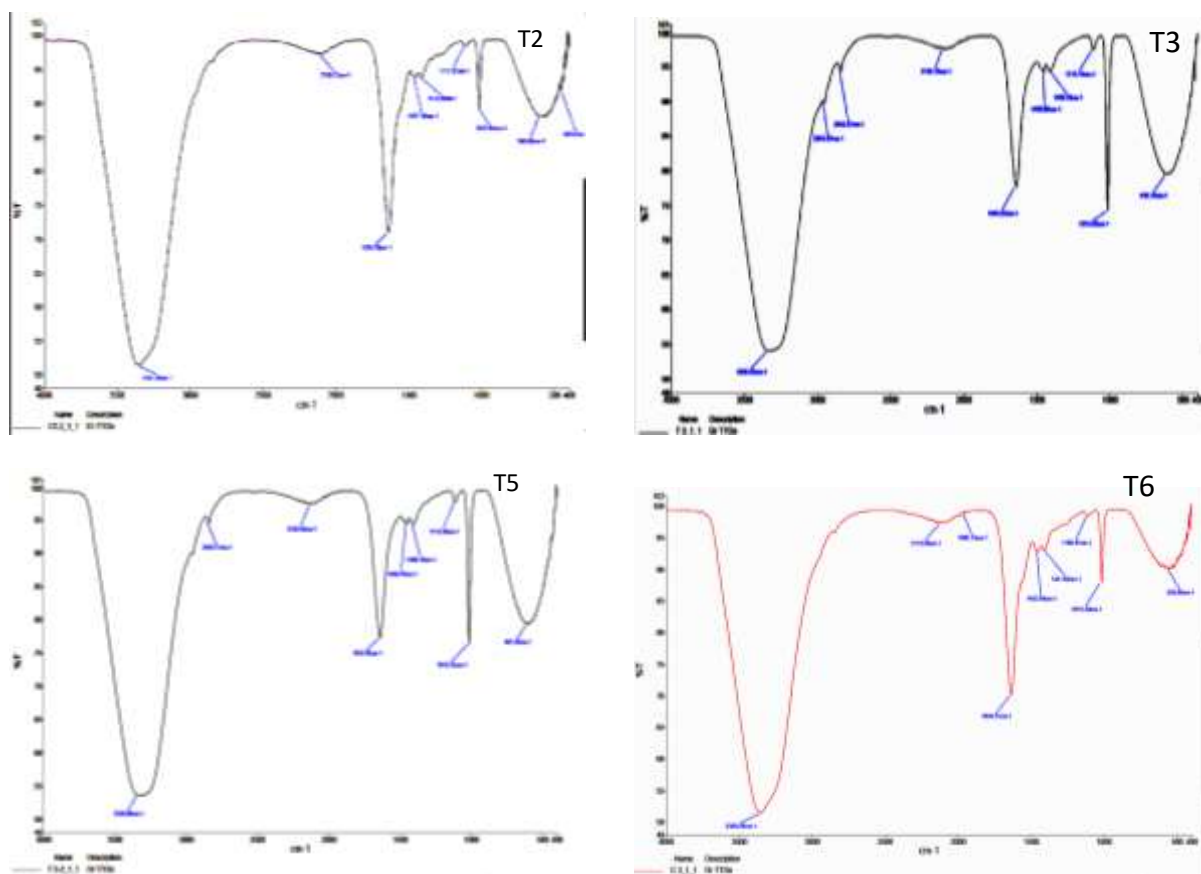


Figure. (8) FTIR spectral data of T2,T3,T5 and T6 sample extract solution

Discussion

Vitex trifolia L. plants were cultivated in tropical and subtropical area. Perennial sub-shrub, dioecious, salivary pubescent, pungent smell present, bilabiate tubular flower, purple. The fruit globose. According to this characters are in agreement with those mention by previous authors (Hooker (1885), Kirtiker and Basu. (1933). Backer and Bakhuizen, (1965), Julissa Rojas-Sandoval, (2022). Leaf , simple, 3- trifoliate, opposite, short petioles, leaflet sessile , bracts small , , pale purple, triangular acute are agreement with those mention by Julissa Rojas-Sandoval , (2022).

The six isolated endophytic fungi (T1, T2, T3, T4, T5 and T6,) from the leaves of *Vitex trifolia* L. Six strains were transferred to new plates (pure culture) and broth culture. And then fermentation process after one week, the fermented broth of endophytic fungi were tested with six

test organisms (*Bacillus subtilis*, *Bacillus pumilus*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas* spp., *Staphylococcus aureus*.). The first day, the isolated strains of T1, T5 and T6 were showed the highest antimicrobial activity on all test organisms. After second day, (T2,T3,) and T4 were observed the highest antimicrobial activity on (*Micrococcus luteus*) and *Candida albicans* . After three days, the antimicrobial activity indicated by size of clear zone was observed to be decreased in all isolates.

These crude bioactive compound was distinctly showed only one spot R_f value T2(0.55),T3 (0.41) ,T5(0.58) and T6(0.68) on thin layer chromatography (TLC) plate by using the solvent system of Petroleum ether : water: Ethyl acetate (9 : 3 : 2), Chloroform : Methanol (8 : 2), Hexane : Chloroform :Methanol (3 :2 :1),Methanol :ethyl acetate : Petroleum ether (9 : 3 : 1),the four extracted sample were showed the wavelength number of T2 (261,283,303), T3(290,403),T5(297), T6 (294,324,344) by checking with UV visible spectrophotometer and the functional group of C=O, C-H ,C-O ,CH₃ were observed in IR spectral data. Although there may be many points to clarify further, this research works shall to continue in the year 2024 to find out the actual nature of metabolites and genus and species of all isolates.

Conclusion

It can be concluded that ethyl acetate extract and methanol mixing solution of T5 provided the highest antimicrobial potential on six test organisms than the rest of T2, T3, and T6. All strains from *Vitex trifolia* L. leaves have showed good activity on six test organisms thus they have been abundantly bioactive compound and potentially wide range of medicinal effect. These fungi can be used to produce natural drugs, biopesticides, and biofertilizers that lead to decrease the dangers of synthetic chemicals. This can save the ecosystem and reduce the chemical residue in the environment (Laith, 2020). In the future, the bioactive compound from endophytic fungi of *Vitex trifolia* leaves can be used as natural drugs and endophytic fungi used for pesticides.

Acknowledgement

I am grateful to Professor and Head and all professors for the permission and supplying with the requirement of facilities for research work, I also thank to all member of the Department of Botany, University of Yangon for their help and encouragement.

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