Production of Biologically Active Compounds from Endophytic Strains Isolated from Different Plant Species

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Abstract

In screening of the bioactive compounds from microbial source, seven endophytic fungal strains were isolated from different plant species in this research. These fungal strains YT1 to YT7 indicated antimicrobial activity by paper disc diffusion assay. For extraction and isolation of the bioactive compounds, the methanol extracts of the most active strains were eluted on silica gel, flash silica gel, Sephadex LH20 gel columns with various solvent systems. The isolated compounds were identified and characterized by spectroscopic techniques. The two compounds: Butyrolactone I and Aspulvinone O from strain YT1 and Terreusinone from strain YT3 were isolated from six liter fermentation of each strain. All isolated compounds indicated antimicrobial activity on eight test organisms in 5 μg of 1.0 mg/mL *invitro*.

Key words:antimicrobial activity, aspulvinone O, butyrolactone I, endophytes, terreusinone

1. Introduction

The use of microorganisms to produce natural products and processes that benefit and improve our socioeconomic lifestyles has been a part of human history since the days of early civilization.

Since Alexander Fleming's outstanding discovery of a Penicillium colony, the production of the bioactive compounds by microorganisms and their antibiotic effect against pathogenic microbes are continuing to attract scientific and public interest (Demain, 1992).Since the discovery of penicillin in 1929, intensive studies of mainly soil derived bacteria and fungi have shown that the microorganisms are a rich source of structurally unique pharmaceutically important bioactive substances. Isolation of microorganisms from the environment is the microbiologist's first step in screening for natural products such as secondary metabolites. Microorganisms, in particular the bacteria, have had a profound effect on the development of medical science (Ankee 1989; Mann & Murder, 1994).

Over the past 60 years, about 28,000 natural products have been isolated from microorganisms. More than 10,000 of these compounds are biologically active and more than 8,000 are antibiotic and antitumor agents (Takahashi*et al.* 1990).Today over 1,000 microbial products continue to be used clinically as antibiotics, anti-tumor drugs and agrochemicals (Berdy, 1989).Antibiotics are defined as low-molecular weight (MW < 2000 Dalton) secondary metabolites from natural sources, and they show inhibition of the growth of higher organisms (e.g. tumour cells) or pathogens (e.g. bacteria, fungi, viruses) at low concentration (Fenical, 1993).

Nowadays, it is very important to find new antibiotics from microbial source because microbial agents continue to play a major role in drug discovery and development in the pharmaceutical industry.

The main goal of the present investigation was the isolation and identification of biologically active compounds produced by endophyticstrains in order to find new

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antibiotics. The objectives of this research work aretoisolate endophytic strains from different plant species,to investigate antimicrobial activity of isolated strains, to extract and isolate the bioactive compounds, to conduct structure elucidation of the bioactive compounds, and to evaluate antimicrobial activity of the isolated compounds.

2. Materials and Methods

2.1 Isolation of endophytic strains from plant parts

In the course of screening of endophytic strains, four different plant species were collected from the campus of University of Yangon to isolate endophytic strains as shown in Table 1.

Table 1. Plant species used for isolation of endophytes

No.	ScientificName	Family	MyanmarName
1	*Alternanthera dentata*Moench.	Amaranthaceae	Ywethla
2	*Laportea aestuans* L.	Urticaceae	Phyet ya
3	*Caessalpinia pulcherrima* L.	Caesalpiniaceae	Sein pan-gale
4	*Ervatamia divaricata* L.	Apocynaceae	Zalat-set kya

The isolation procedure was carried out with the following schemes: (1) Plant parts were washed in running tap water for 15 mins. (2) The leaves were cut into about 3 cm pieces. (3) The surfaces of cut-pieces were sterilized by soaking it in 75% ethanol for 2 min. (4) Next, sterile surfaces were socked in 5.3% sodium hypocloride for 5 min. (5) Cut-pieces were socked in 75% ethanol for 0.5 min to wash out sodium hypocloride. (6) They were dried and cut into smaller pieces, and placed on agar plates and then incubated for 3 days to 3 weeks.

Then, isolated fungal strain grown on SY medium (sucrose 1.0%, yeast extract 0.3%, agar 1.8% and distilled water 100 mL) agar plates were transferred into a 10 mL test tube containing 5 mL of SY medium and incubated for 2-5 days(Lee, *et al.*, 1996; Phay, 1997).

2.2 Antimicrobial activity of isolated fungal strains

Isolated strain grown on SY medium agar tubes was transferred into the SY medium plate and incubated for 3-5 days.Then, this strain was inoculated into seed medium (potato dextrose medium) and incubated for 3 days. After incubation, the seed culture 1% was transferred into fermentation flask and fermentation was carried out for 3-5 days. At the end of fermentation, the fermentation broth was used for the paper disc diffusion assay (Phay, 1997).

Paper disc diffusion assay

Test organisms:*Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Malassezia furfur*utilized in this research were incubated in fresh medium for 12 hrs. After 12 hrs, 50 µL of each test organism was added to 100 mL of assay medium (SM medium:sucrose 1.0%, malt extract 0.3%, agar 1.8% and distilled water 100 mL), then poured into test plates.

Paper discs (φ 5 mm, thickness 0.5 mm) were soaked with 20 μL of a fermented broth, dried under sterile flow box and put on agar plates inoculated with test organisms. Paper discs were impregnated with broth samples were allowed to dry at room temperature.

Dry paper discs impregnated with samples were applied on various test plates. Then, these plates were incubated for 24-36 hrs at 30°C. After 24-36 hours, clear zones (inhibitory zones) surrounding the test discs indicate the presence of the bioactive compounds which inhibit the growth of test organisms selectively. The diameter of clear zone including 6 mm disc were measured (Davis and Stout, 1971; Phay, 1997).

Table 2. Test organisms and diseases

Test organisms	Disease	Code number
Bacillus subtilis	Food spoilage and fever	JAP- 0225025
Candida albicans	Vaginal candidasis, alimentary tract infection, skin infection, sinus irritaion, intense itching and sores	IFO-1060
Escherichia coli	Diarrhoea and vomiting dysentery and urinary tract infection	ATCC-25922
Micrococus luteus	Skin diseases, sepsis	ATCC-23840
Pseudomonas aeruginosa	Chronic lung, urinary tract infection, inflammation in bone and joints, gastrointestinal and blood infections	-
Salmonella typhi	Typhid	ST.3/SEP 69
Staphylococcus aureus	Skin diseases, wound, burns, staphylococcal pneumonia, toxic shock syndrome and sepsis	ATCC-12877
Malassezia furfur	Dandruff and skin infections	AUW-0255

(Hudault, 2001; Hulse, 1993; Humphrey, 2004, Ogston, 1984; Reid *et al*, 2001; Ryan & Ray, 2004; Stewart, 1968)

2.3 Identification of active fungal strains

Morphological and microscopic characters of bioactive fungal strains were conducted to identify their taxonomic characters at Microbiology laboratory and Department of Botany and Universities' Research Centre, University of Yangon. The taxonomic characters of isolated fungal strains were identified according toBarnett and Hunter, 1998.

2.4 Fermentation of the most bioactive strains

In order to isolate the bioactive compounds from the most active strains,1 cm^2 pieces of mycelia ofstrainYT1were inoculated into 20 of 1L conical flasks containing 300 mL of SY fermentation medium. Then, these flasks were incubated for five days at 30°C.Fermentation medium was SY medium including sucrose 1.0%, yeast extract 0.3%, agar 1.8% and distilled water 100 mL(Strobel and Sullivan, 1999). For 6Lfermentation of strain YT3, the same procedure as YT1 was carried at Institute for Organic and Biomolecular Chemistry, Georg-August University, Goettingen, Germany.

2.5 Extraction, isolation and purification of the bioactive compounds

After the fermentation, the mycelia filtrate and the culture filtrate from each strain were separately extracted with various solvents such as acetone, ethyl acetate, methanol etc.The extracted samples were concentrated by using a 10L rotary evaporator and lyophilized. The bioactive compounds from strains YT1 and YT3 were isolated and purified by using various solvent systems on silica gel column, flash silica gel column, Sephadex

LH20 column, and preparative HPLC (High Performance Liquid Chromatography) column at Institute for Organic and Biomolecular Chemistry, Georg-August University, Goettingen, Germany (Grabley *et al.*, 1999).

2.6 Structural elucidation of the isolated compounds

The isolated compounds from strains YT1 and YT3 were characterized and identified by modern spectroscopic techniques such as ESI- or EI-MS (Electrospray Ionization/Electron Impact), IR (Infared Spectrum), UV (Ultra Violet), ^{1}H-NMR (Hydrogen-Nuclear Magnetic Resonance), ^{13}C-NMR (Carbon-Nuclear Magnetic Resonance) at Institute for Organic and Biomolecular Chemistry, Georg-August University, Goettingen, Germany(Laatsch, 2003).

2.7 Biological activity

It is essential to produce antibiotics that can fight serious microbial diseases. In this researchall the isolated compounds from strains YT1 and YT3 were evaluated their antimicrobial activity on *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Malassezia furfur* in 5 µL of 1.0 mg/mL (MIC) *in vitro*.

3. Results

3.1 Isolation of endophytic strains

Seven endophytic strains were isolated from four different plant species. The isolated strains were temporarily named as YT 1 to YT7 as shown in Table 3.

Table 3. Isolated strains and plant sources

Strain	Scientificname of plant source	Family
YT1 & YT2	*Alternanthera dentata* Moench.	Amaranthaceae
YT3 & YT4	*Laportea aestuans* L.	Urticaceae
YT5 & YT7	*Caessalpinia pulcherrima* L.	Caesalpiniaceae
YT6	*Ervatamia divaricata* L.	Apocynaceae

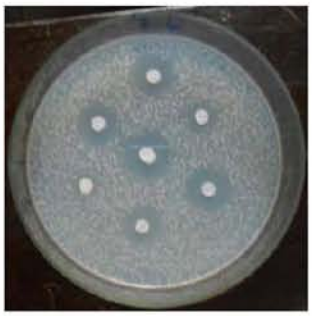
3.2 Antimicrobial activity of endophytic strains

All isolated strains (YT1 to YT7) showed good antimicrobial activity on six test organisms. Among seven strains, strainsYT1 and YT3 indicated very high activity against *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Malassezia furfur*. Therefore, these two strains YT1 and YT3 were chosen for isolation of the bioactive compounds (Table 4 and Figure 1).

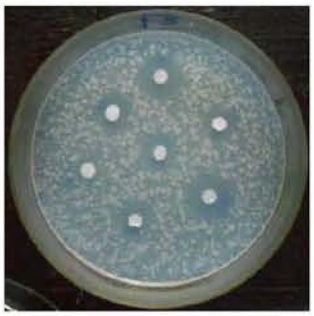
Table 4. Inhibitory zones (mm) of endophytic strains

Strain	*B. subtilis*	*C. albicans*	*E. coli*	*S. typhi*	*S. aureus*	*M. furfur*
YT1	20 ***	18 ***	22 ***	18 ***	22 ***	24 ***
YT2	12 *	13 *	18 ***	15 **	20 ***	20 ***
YT3	17 **	18 ***	22 ***	18 ***	22 ***	24 ***
YT4	15 **	14 **	15 **	13 **	20 ***	19 ***
YT5	10 *	10 *	12 *	12 *	16 **	16 **
YT6	15 **	13 **	13 **	12 *	17 **	18 ***
YT7	15 **	14 **	15 **	13 **	17 **	18 ***

(*) 10 -12 mm weak activity, (**) 13-17mm high activity, (***) >18 very high activity



B. subtilis



C. albicans

E. coli

S. aureus

S. typhi

M. furfur

Figure 1. Antimicrobial activity of seven endophytic strains

3.3 Identification of bioactive fungal strains

Surface color and reverse color of strain YT1 were white to gray (Figure 2). Mycelium of strain YT1 was typically appressed to substrate and fine. Its conidiophores were hyaline, simple, tapering upwards. Its conidia were globose, hyaline, single and apical. This fungus was identified as *Mortierella* sp. as shown in Figure 3.

Surface color of strain YT2 was white while its reverse color was gray (Figure 4). Its conidiophores were arising from mycelium singly,branched near the apex, penicillate and

ending in a group of phialides. Its conidia were hyaline, 1-celled and globose. This strain was identified as *Penicillium* sp. as shown in Figure 5.

Surface color of strain YT3 was white to brown whereas its reverse color was cream (Figure 6). Mycelium of strain YT3 was hyaline to brown while its conidiophores were sparsely branched and remaining in chain. Its conidia were 1-celled and hyaline (Figure 7). This fungus was identified as *Oidiodendron* sp.

Surface color and reverse color of strain YT4 were white to gray (Figure 8). Cells of its mycelium were long, septa of branches and set off from the main hyphae. Itsconidia were absent, sporodochium-like bodies and chlamydospore-like cells in chains (Figure 9). This fungus was identified as *Rhizoctonia* sp.

Surface color of strain YT5 was yellow whereas its reverse color was cream (Figure 10). Its conidiophores were upright, simple, terminating in a globose and bearing phialides at the apex.Its conidia (phialospores) were 1-celled and globose (Figure 11).This strain was identified as *Aspergillus* sp.

Surface color and reverse color of strain YT6 were white (Figure 12). Its mycelium was white and septate whereas conidiophores were absent. Its conidia were hyaline, 1-celled and formed by segmentation of hyphae (Figure 13). This fungus was identified as *Geotrichum* sp.

Surface color and reverse color of strain YT7 were white to gray (Figure 14). Cells of its mycelium were long, septa of branches and set off from the main hyphae. Itsconidia were absent, sporodochium-like bodies and chlamydospore-like cells in chains (Figure 15). This strain was identified as *Rhizoctonia* sp.

Figure 2. Morphological character of strain YT1

Figure 3.Microscopic character of strain YT1(X 40)

Surface color

Reverse color

Figure 4. Morphological character of strain YT2

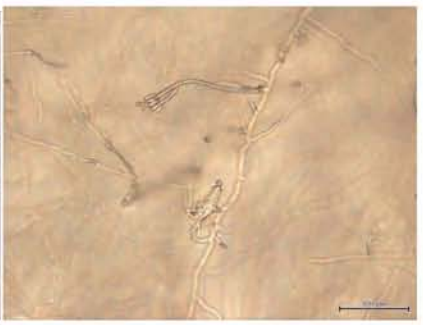
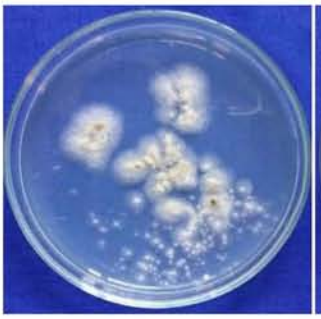
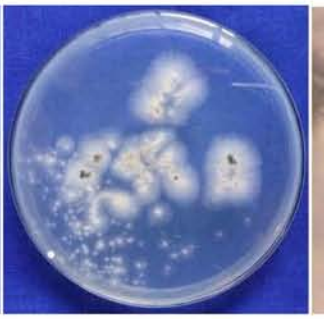


Figure 5.Microscopic character of strain YT2(X 40)

Surface color Reverse color
Figure 6. Morphological character of strain YT3

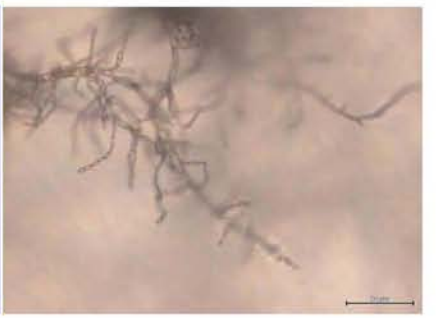
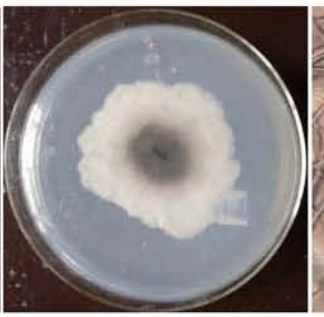


Figure 7.Microscopic character of strain YT3(X 40)

Surface color Reverse color
Figure 8. Morphological character of strain YT4

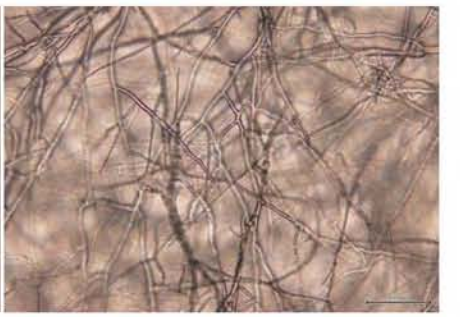
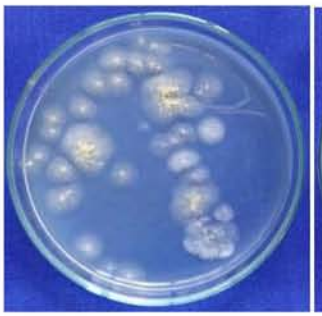


Figure 9.Microscopic character of strain YT4(X 40)




Surface color Reverse color
Figure 10. Morphological character of strain YT5

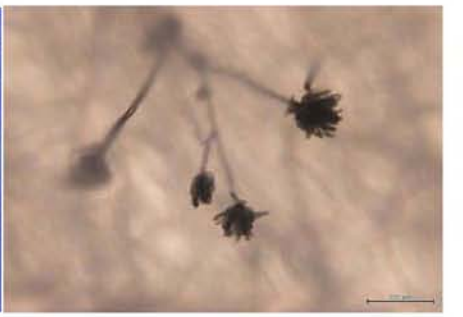
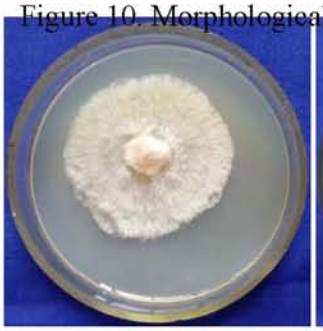


Figure 11.Microscopic character of strain YT5(X 40)




Surface color Reverse color
Figure 12. Morphological character of strain YT6

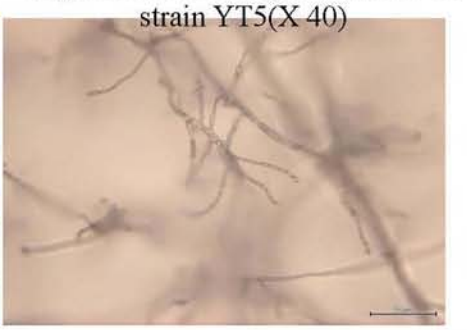


Figure 13.Microscopic character of strain YT6(X 40)

Surface color

Reverse color

Figure 14. Morphological character of strain YT7

Figure 15.Microscopic character of strain YT7(X 40)

3.4 Isolation of the bioactive compounds from the most active strains

The two compounds: "Butyrolactone I and Aspulvinone O" from strain YT1, and the compound "Terreusinone" from strain YT3 were isolated by using various solvent systems on silica gel, flash silica gel and Sephadex LH20 columns. The isolation procedures of these compounds are shown in Figures 16 and 17.

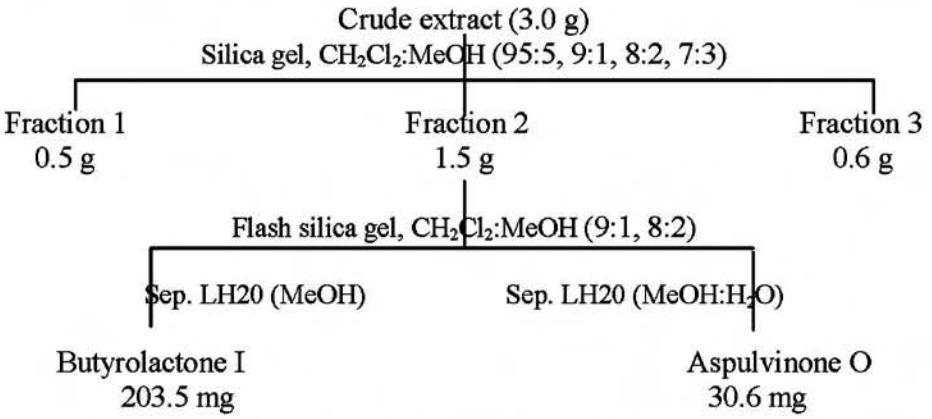


Fig. 16. Isolation procedure of the bioactive compounds from strain YT1

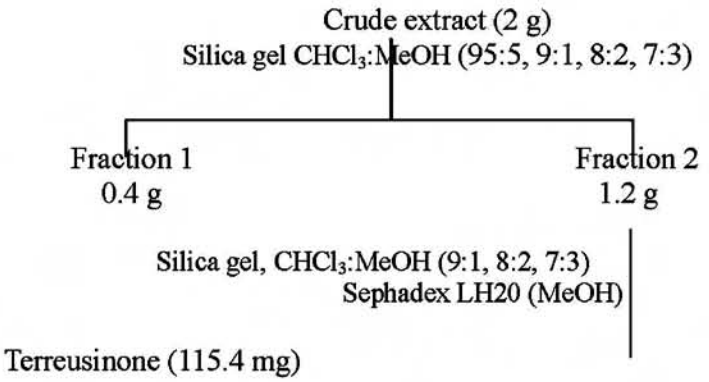
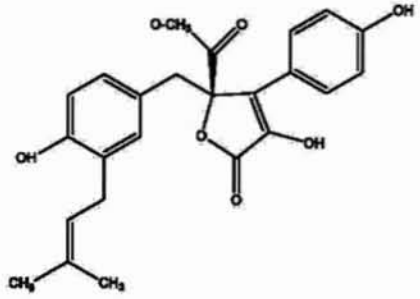


Fig17. Isolation procedure of the bioactive compounds from strain YT3

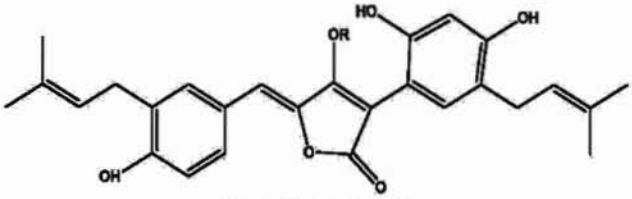
The compound Butyrolactone I was isolated from fraction 2 of crude extract of strain YT1 as a white solid. It showed an UV absorbing band at 254 nm. Its molecular weight was (424 g/mol) determined by ESI-MS spectrum (Fig. 20). Its molecular formula was $C_{24}H_{24}O_7$according to ^1H- and ^{13}C- NMR spectra (Fig. 21 & 22). It is good soluble in ethanol, methanol and DMSO.

Working up of fraction 2 of crude extract of strain YT1 led to an additional compound, Aspulvinone O was isolated as white crystals. It showed an UV absorbing band at 254 nm. The molecular weight of Aspulvinone O was (448 g/mol) determined by ESI-MS spectrum. Its molecular formula was $C_{27}H_{28}O_6$according to ^1H- and ^{13}C- NMR and ESI-MS spectra (Fig. 23, 24 & 25). This compound is good soluble in methanol and ethanol.

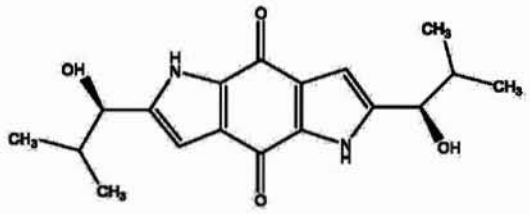
During the isolation of strain YT3, the compound "Terreusinone" was isolated as a white solid from fraction 2of crude extract. The molecular weight of Aspulvinone A was (330 g/mol) determined by ESI-MS spectrum. Its molecular formula was $C_{18}H_{22}N_2O_4$according to ^1H-NMR and ESI-MS spectra (Fig. 26 & 27). This substance is good soluble in DMSO and water.



Butyrolactone I



Aspulvinone O



Terreusinone

Figure 18. Molecular structure of the isolated compounds

3.5 Antimicrobial activity of the isolated compounds

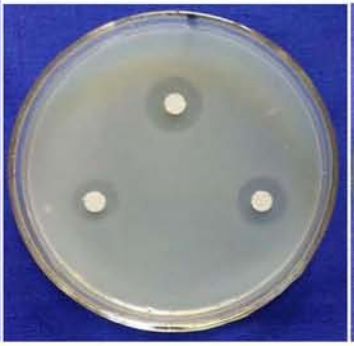
The compound Butyrolactone I showed high antimicrobial activity on *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Malassezia furfur*while it indicated very high activity on *Micrococcus luteus* and *Salmonella typhi*.Aspulvinone O indicated high antimicrobial activity on eight test organisms. Terreusinone indicated high antimicrobial activity on *Bacillus subtilis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Micococcus luteus*, *Salmonella typhi* and *Staphylococcus aureus* whereasit showed weak activity on *Escherichia coli* and *Malassezia furfur* (Table 5 and Figure 19).

Inhibitory zones against *B. subtilis*

Inhibitory zones against *C. albicans*

Inhibitory zones against *E. coli*

Inhibitory zones against *P. aeruginosa*



Inhibitory zones against *M. furfur*

Inhibitory zones against *M luteus*

Inhibitory zones against *S. typhi*

Inhibitory zones against *S. aureus*

Fig 19. Antimicrobial activity of the isolated compounds

Table5. Antimicrobial activity of the isolated compounds

Test organisms	Butyrolactone I 5 μg/disc (mm)	Aspulvinone O 5 μg/disc (mm)	Terreusinone 5 μg/disc (mm)
Bacillus subtilis	15	14	15
Cadida albicans	15	16	15
Escherichia coli	16	15	12
Malassezia furfur	16	14	12
Micrococcus luteus	18	17	14
P. aeroginosa	16	15	16
Salmonella typhi	15	15	14
Staphylococcusaureus	18	15	15

4. Discussion

In this research, seven endophytic strains (YT1 to YT7) were isolated from four different plant species. These strains showed antimicrobial activity on*Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Malassezia furfur*. Among these strains, strains YT1 and YT3 indicated very high activity on six test organisms.

Maria *etal.*, 2005 stated that endophytic fungi isolated from *Acanthus ilicifolius* and *Acrostichum aureum* in India showed anti-microbial property against *Bacillus subtilis*, *Candida* sp., *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. In 2006 Chareprasert *et al.*, reported an antimicrobial activity exhibited by endophytic fungi isolated from teak and rain trees.

Strain YT1 was identified as *Mortierella* sp. YT2 was *Penicillium* sp., YT3 was *Oidiodendron* sp., YT4 and YT7 were *Rhizoctonia* sp., YT5 was *Aspergillus* sp. and YT6 was *Geotrichum* sp. according to their morphological and microscopic characters that were agreed withthe statements of Barnett and Hunter, 1998.

Dobranic *etal.*, 1995 isolated endophytic fungi from Eastern Larch (*Larix larcina* L.) leaves from New Brunswick. In Myanmar, Aye Pe (2001), Yee Yee Thu (2006), Khin Thin Thin (2010), Kyawt Kyawt Aung (2014), Kyi Kyi Khine (2014) and others have isolated many endophytic fungal and bacterial strains from different plant species and they also isolated the bioactive compounds.

In this study, butyrolactone I and aspulvinone O from strain YT1 and terreusinone from strain YT3 were isolated from 6L fermentation of each strain.All isolated compounds showed antimicrobial activity oneight test organisms such as *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Malassezia furfur*in 5μg of 1.0 mg/mL (MIC) *in vitro*.

Suzuki*etal.*, 1999 reported that butyrolactone I induced cyclin B1 and caused G2/M arrest and skipping of mitosis in human prostate cell lines.Kitagawa *et al.*, 1994 stated that butyrolactone I was a selective inhibitor of the cyclin-dependent kinase (cdk) family and inhibited phosphorylation of RB protein and cell cycle progression.

Takahashi *etal.* 1978 isolated aspulvinones from *Aspergillusterreus*.Lee *etal.*, 2003 isolated terreusinone as a potent UV-A protecting agent from a marine algicolous fungus

Aspergillus terreus. Nurdiani *etal*., 2013 reported that *Penicillium* sp. isolated from *Rhizophoramucronata* indicated antibacterial activity on *E. coli* and *S. aureus*.

Gunatilaka, 2006 and Zhou *et al*., 2009 reported that endophytic fungi have produced many bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities. Dreyfuss and Chapela, 1994 stated that many endophytic fungi produced useful antibiotic compounds against human and plant pathogens.

5. Conclusion

In conclusion, it is very important to search for new antimicrobial drugs in the field of medicine. Since life-threatening fungal and bacterial diseases are strongly increasing nowadays, it is very essential to produce antibiotics that can fight serious microbial diseases. The bioactive compounds Butyrolactone I and Aspulvinone O isolated fromstrain YT1 (*Mortierella* sp.)and Terreusinone from strain YT3(*Oidiodendron* sp.) can effectively be used in the field of medicine as "antibiotics" to cure fungal and bacterial infections. It is very helpful and beneficial for human beings.

Acknowledgement

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APPENDIX

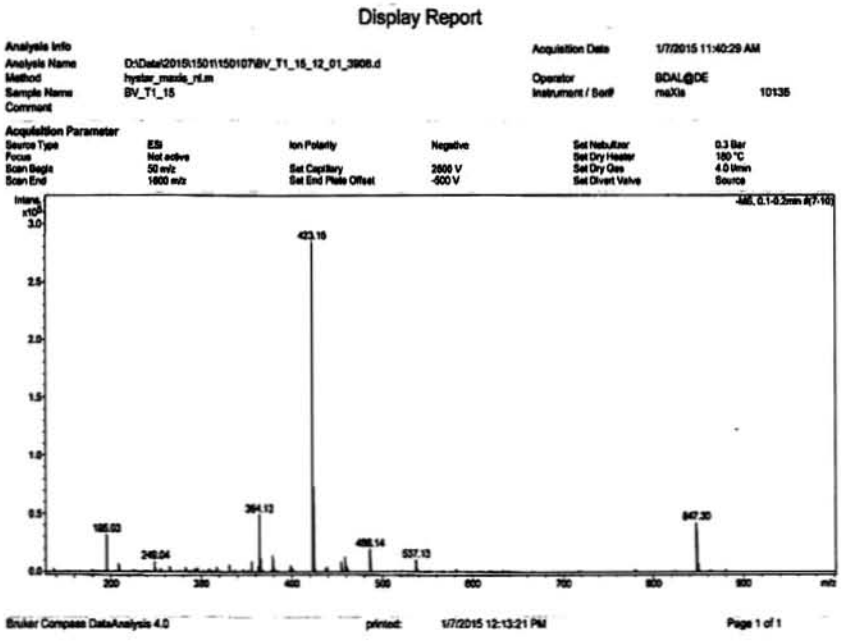

Figure 20. ESI-MS spectrum of Butyrolactone I

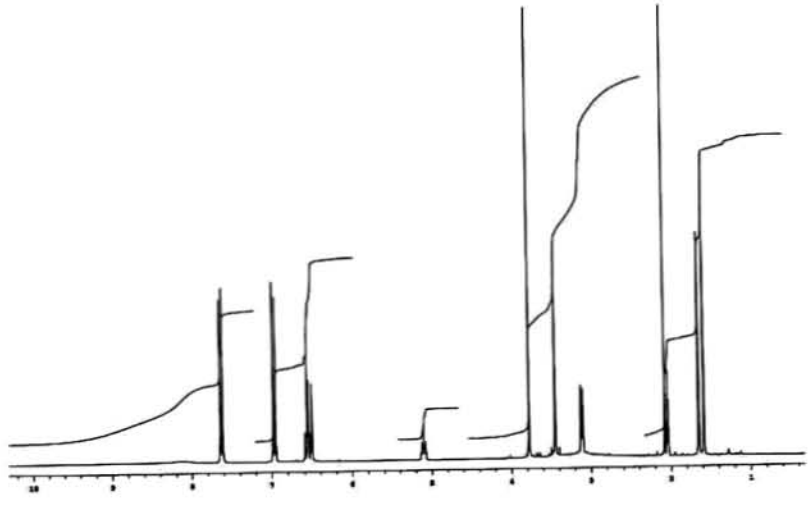

Figure 21. ^{1}H-NMR (300 MHz, acetone d_6) spectrum of Butyrolactone I

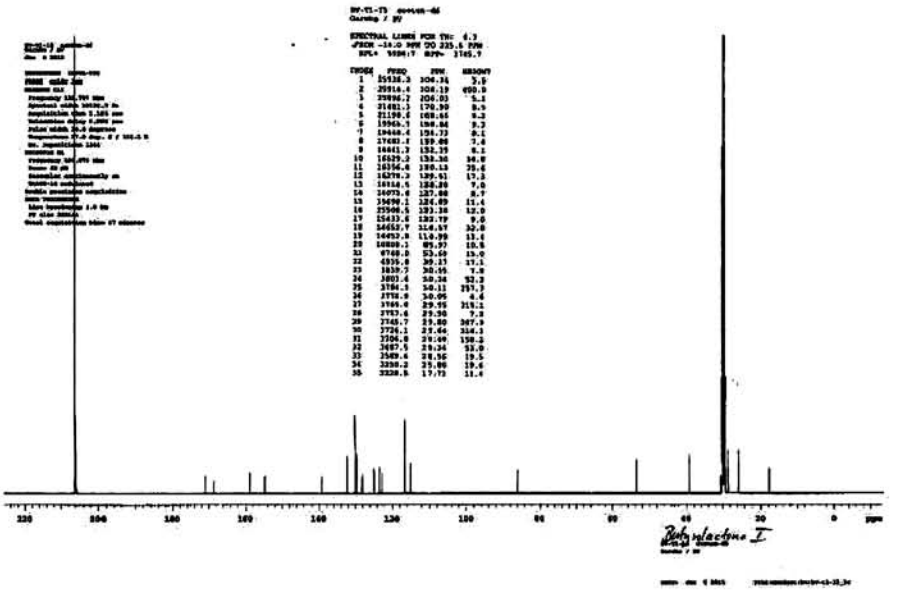

Figure 22. ^{13}C-NMR (150 MHz, $CDCl_3$) spectrum of Butyrolactone I

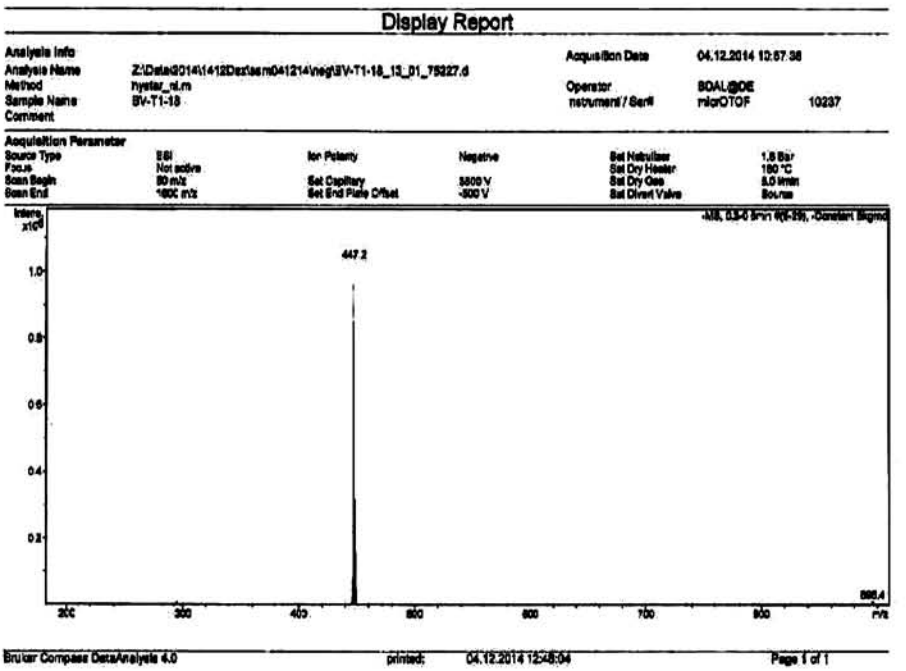

Figure 23. ESI-MS spectrum of Aspulvinone O

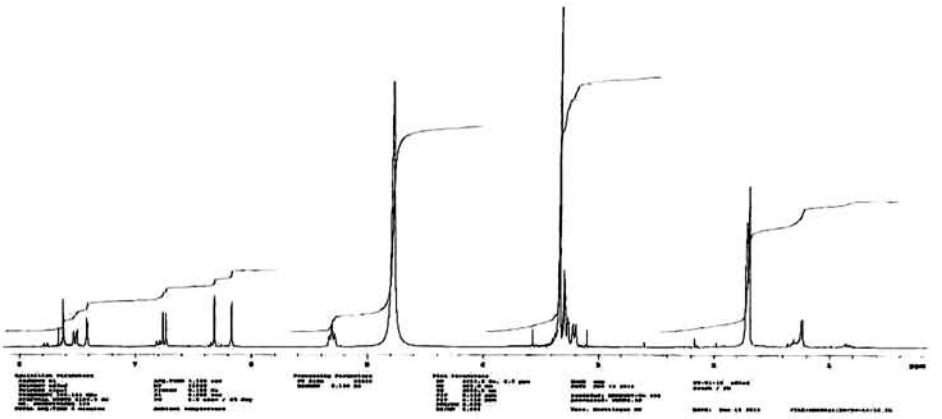

Figure 24. ^{1}H-NMR (300 MHz, CD_3OD) spectrum of Aspulvinone O

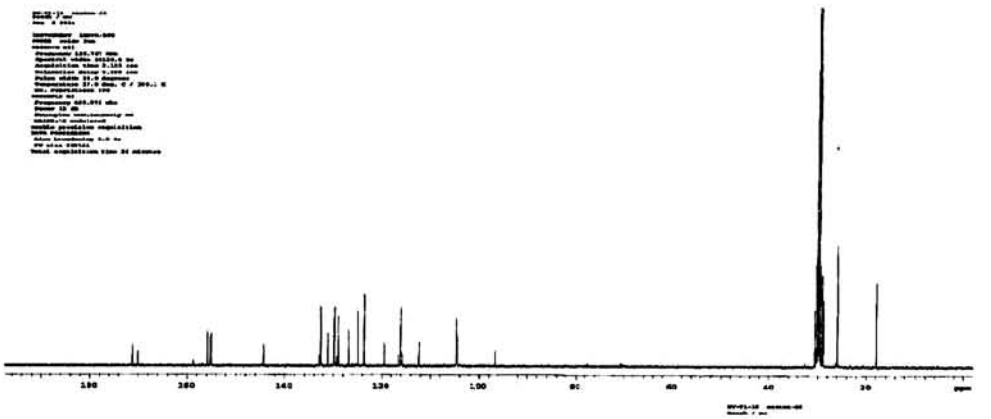

Figure 25. ^{13}C-NMR (150 MHz, acetone d_6) spectrum of Aspulvinone O

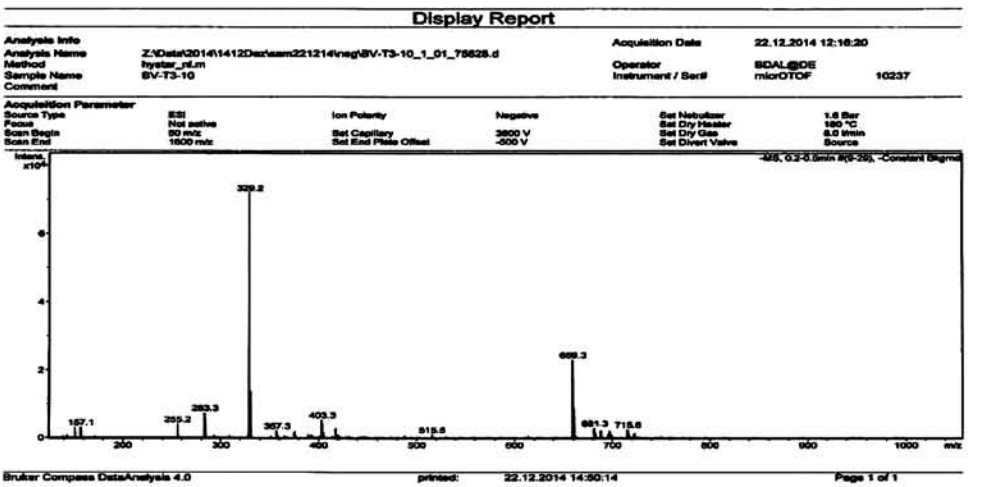

Figure 26. ESI-MS spectrum of Terreusinone

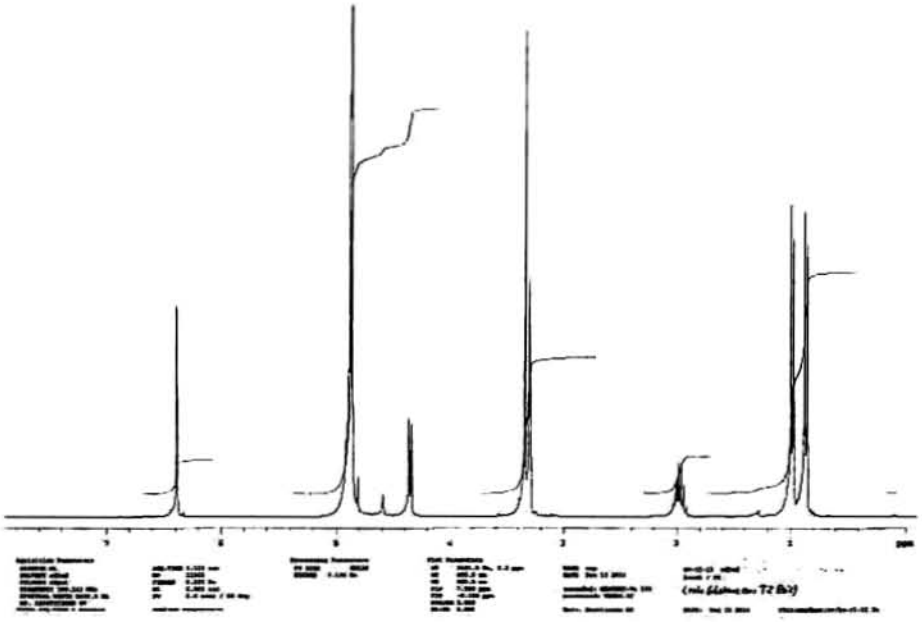

Figure 27. ^{1}H-NMR (300 MHz, CD_3OD) spectrum of Terreusinone