ISOLATION OF ENDOPHYTIC FUNGI FROM THE LEAVES OF *MUNTINGIA CALABURA* L. AND THEIR ANTIBACTERIAL ACTIVITIES

Phyo Phyo Soe San[1], Shoon Lae Yee Htwe[2], Nway Nway[3]

Abstract

In this study, the endophytic fungi were isolated from the leaves of *Muntingia calabura* L. in family Muntingiaceae. The plant sources were collected at Yadanabon University Campus, Amarapura Township of Mandalay Region. A total of 7 endophytic fungi were isolated by using surface sterilization method. Morphological characters of endophytic fungi were investigated and were coded MCF-01 to MCF-07. In the study of antibacterial activity of endophytic fungi, six fungi showed the act against test organisms. Among them, the fungus MCF-01 showed the highest activity against on *Salmonella typhi*. This fungus MCF-01 was isolated from the leaves of *M. calabura* L.. In the fermentation studies, maximum antibacterial activity of fungus MCF-01 on *Salmonella typhi* reached at 6 days fermentation with 84 hrs ages (22.25 mm clear zone) and 20% sizes of inoculum (23.49 mm clear zone). Furthermore, distinct characters of selected endophytic fungus MCF-01 was observed with the help of microscope. Endophytic fungus MCF-01 can fight back against typhoid fever disease by *S. typhi.* in humans. Therefore, fungus MCF-01 was selected for the further investigation such as paper chromatography and extraction of antibacterial metabolite.

Keywords: endophytic fungi, morphological character, antibacterial activities

Introduction

Microorganisms are present everywhere on Earth that will support life. These include habitats we are all familiar with-soil, water, animal, and plant-as well as virtually any structures made by humans. (Prescott *et al.,* 2002).

Endophytic fungi live in their host plants and due to this; they must develop certain chemical strategies that favor their existence. By producing metabolites, the endophytic fungi either protect the host from animal or herbivores attack or from other pathogenic microbes infection that will decrease the fungi's colonization (Tan and Zou, 2001).

In this study, the isolation of endophytic fungi was investigated from *Muntingia calabura* L. grown in the Yadanabon University Campus. This plant is belonging to the family Muntingia. It is known as Jamaica cherry in English. Its Myanmar name is Hnget-thagya (Hundley and Chit Ko Ko, 1987). This plant is grown in tropical climate area. It is said to help diabetic patients. A small reduction was recorded in patient's blood sugar levels after consumption. The aim and objectives of this study are to isolate the endophytic fungi of *Muntingia calabura* L., to study the morphology, to investigate the antibacterial activities and to optimize the fermentation conditions for the antibacterial activities.

Materials and Methods

Collection of plant samples

Muntingia calabura L. was collected at Yadanabon University Campus, Amarapura Township of Mandalay Region, during January in 2022. The healthy plant leaves were utilized for the isolation of endophytic fungi. Photograph of the source plant is shown in following (Figure 1).

[1] Department of Botany, Yadanabon University
[2] Department of Botany, Yadanabon University
[3] Department of Botany, Yadanabon University

The identification of the source plant was referred from the Flora of Java Vol.I (Backer, and Brink 1963).

Isolation of Endophytic Fungi

In the isolation of endophytic fungi from the leaves of *Muntingia calabura* L. was carried out by surface sterilization method (Ando, *et.al*, 2004). The leaves of plants samples were washed in running water and then the leaves were cut into pieces. The leaves pieces were sterilized by soaking it in 95% alcohol for 15 seconds and then rinsed several times in sterile distilled water. Then, the piece was dried on sterilized paper and cut into 2 pieces. The pieces were placed on agar plates. After isolation procedures of endophytes from the leaves were done, piece of the cut leaves were placed on petri dishes containing Low Carbon Agar (LCA) medium (Glucose 0.1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.02 g, Yeast extract 0.02 g, Agar 1.8 g, Distilled water 100 mL, pH 6.5) supplemented with chloramphenicol (250 µg) to suppress bacterial growth and incubated at 27ºC until the outgrowth of endophytic fungi was discerned. The selected endophytic fungi were transferred and cultured in Potato Glucose Agar (PGA) medium (Potato 20.0 g, Glucose 2.0 g, Agar 1.8 g, Distilled water 100 mL, pH 6.5) incubated for 3-7 days. Purified endophytic fungi were cultured in slant agar (Potato Glucose Agar) for keeping availability of microbes as long as possible.

Screening of Antibacterial Activities by Paper Disc Diffusion Assay (Omura, 1985 and Petrini *et.al,* 1986)

The isolated fungi were grown at 25 to 27 °C for 7 days on PGA medium. The isolated fungi were inoculated into seed medium and incubated at 25 to 27 °C for 3 days. The 10 ml of seed culture (Glucose 2.0 g, Sucrose 0.3 g, Yeast extract 0.3 g, KNO_3 0.1 g, K_2HPO_4 0.001g, Distilled water 100mL, pH 6.5) were transferred into the fermentation medium (Glucose 1.0 g, Soluble starch 0.5 g, Yeast extract 0.5 g, K_2HPO_4 0.001 g, $MgSO_4$ 0.001 g, $CaCO_3$ 0.01 g, Distilled water 100mL, pH 6.5). The fermentation was carried out for 7 days. After the end of fermentation, was alone the fermented broth (20 µl) was used to check the antimicrobial activity against test organisms by paper disc diffusion assay (Figure 2). Paper disc having eight millimeter diameter (Advantec, Toyo Roshi Kaisha Co., Ltd., Japan) were utilized for antimicrobial assays. The assay medium (Glucose 1 %, Polypepton- 0.3 %, KNO_3 0.1 %, Agar 1.8 %, Distilled water 100 ml, pH 6.5) was used for the antimicrobial activity test. One percent of test organism was added to assay medium, then poured into plates. After solidification, paper discs impregnated with samples were applied on the agar plates and the plates were incubated for 24 hours at 28 to 30 °C. clear zones surrounding the test discs indicate the presence of bioactive metabolites which inhibit the growth of test organisms.

The test organisms used in paper disc diffusion assay were *Bacillus pumalis* NITE 47239, *Escherichia coli* AHU 5436, *Micrococcus luteus* NITE 83279, *Salmonella typhi* AHU 9793 and *Staphylococcus aureus* AHU 8465. The test organisms were supported by NITE (National Institute of Technology and Evaluation, Japan) and Faculty of Agriculture, Hokkaido University, Japan (Table 1).

Table 1. Test organisms used in antimicrobial activities (NITE)

NO.	Test organisms	Infections/ Discusses
1.	*Bacillus pumalis* (NITE 47239)	Fever
2.	*Escherichia coli* (AHU 5436)	Diarrhoea
3.	*Micrococcus luteus* (NITE 83279)	Food Spoilage
4.	*Salmonella typhi* (AHU 9793)	Typhoid Fever
5.	*Staphylococcus aureus* (AHU 8465)	Food Poisoning, Skin disease

Fermentation Studies for the production of antibacterial metabolite against *Salmonella typhi* (Omura, 1985)

Study on the effects of ages of inoculums on the fermentation

The stain MCF-01 was inoculated into the medium (Glocuse 0.1g, Yeast extract 0.2g, NZ amine type A 0.3 g, Distilled water 100 mL) and incubated for 72 hours. The culture samples (10mL) were checked in 12 hours intervals for the growth.

Study on the effects of sizes of inoculums on the fermentation

In this study, 5%, 10%, 15%, 20% and 25% of 84 hours seed culture were utilized for the fermentation. The fermentation was carried out 7 days and antibacterial activity was tested by paper disc diffusion assay.

Distinctive characters of Fungus MCF-01

For the study of morphology and microscopical characters, fungus MCF-01was cultured at 25°C on Potato Glucose Agar medium for morphology and Water Agar medium for photomicrograph.

Results

Outstanding characters of *Muntingia calabura* L.

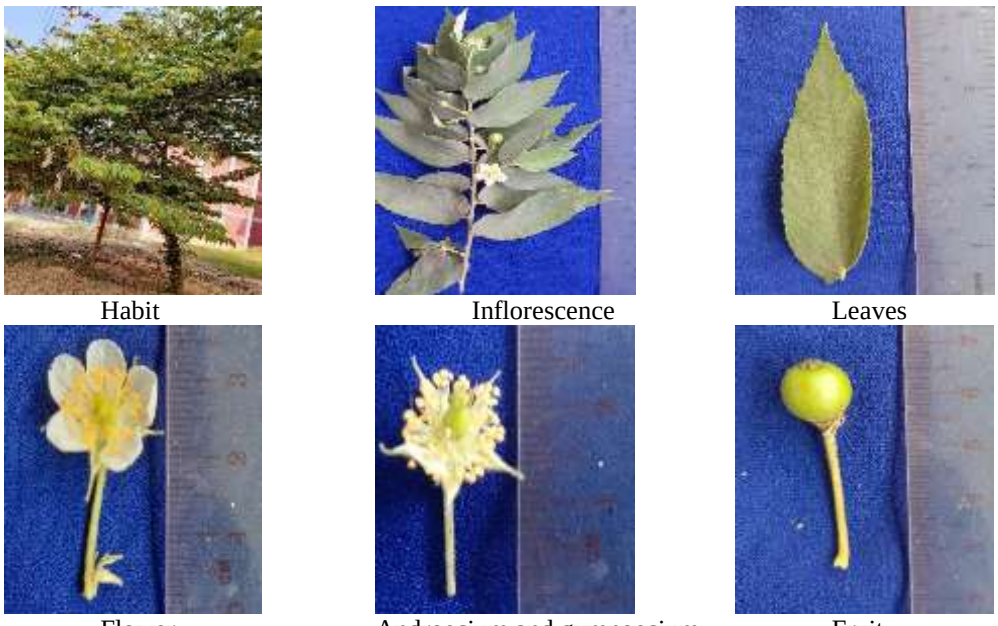


Figure.1. Morphological characters of *Muntingia calabura* L.

Family name : Muntingiaceae

Scientific names : *Muntingia calabura* L.

Myanmar name : Hnget-thagya

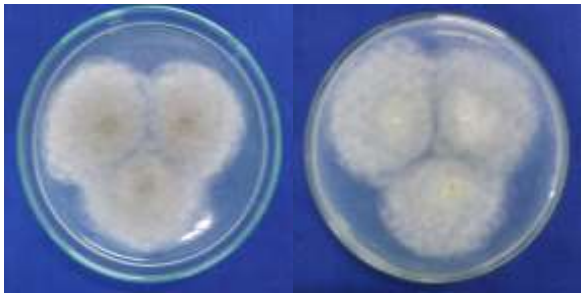
English name : Jamaica Cherry

Flowering period : throughout the year

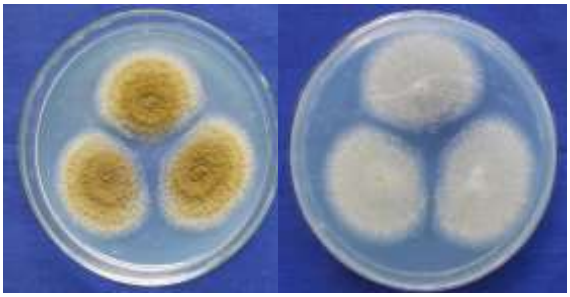
Perennial, shrubs or trees. Leaves simple, alternate; stipulate; petiolate; blades oblong-lanceolate, obliquely subcordate at the base, serrate along the margin**,** acuminate at the apex, softly pubescent beneath. Inflorescences supra-axillary, solitary cymes. Flowers bisexual, actinomorphic, pentamerous, hypogynous, white, pedicellate. Sepals 5, slightly connate at the base, lanceolate, green, densely pubescent. Petals 5, white, suborbicular, shortly clawed. Stamens numerous; filament filiform, connate at the base; anthers dithecous, ovoid, dorsifixed. Ovary superior, the axile placentae; style absent; stigma 5-lobed, glabrous. Fruit berry, sweet. Seeds numerous, minute.

Isolation of endophytic fungi

A total of 7 endophytic fungi were isolated from the leaves *Muntingia calabura* L. plant at Yadanabon University Campus. These endophytic fungi were coded MCF-01, MCF-02, MCF-03, MCF-04, MCF-05, MCF-06 and MCF-07 as shown in Figure 2.

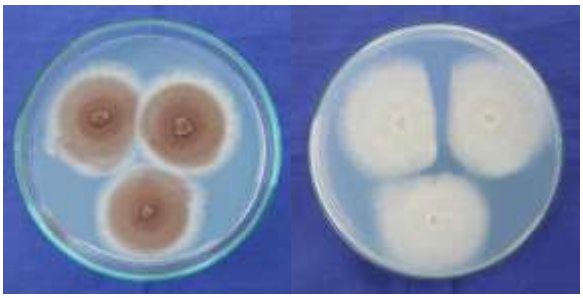


Front view and Reverse view of fungus MCF-01



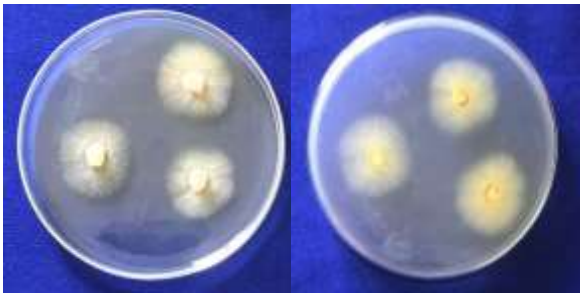
Front view and Reverse view of fungus MCF-02

Front view and Reverse view of fungus MCF-03

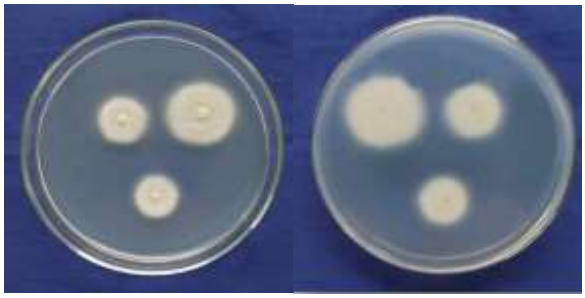


Front view and Reverse view of fungus MCF-04

Front view and Reverse view of fungus MCF-05



Front view and Reverse view of fungus MCF-06



Front view and Reverse view of fungus MCF-07

Figure.2. Morphologies of endophytic fungi on PGA medium

Table 2. The macroscopic characteristics of isolated endophytic fungi from the leaves of *Muntingia calabura* L.

No.	Isolated fungi	Color of Colony Front View	Color of Colony Reverse View	Size of the Colony
1.	MCF - 01	Smokey white	Smokey white	4.5 cm
2.	MCF - 02	Orange amber color	Pale yellow	4.0 cm
3.	MCF - 03	Milky white	Milky white	4.5 cm
4.	MCF - 04	Redish brown	Pale brown	4.0 cm
5.	MCF - 05	Dark slate grey	Greenish black	3.5 cm
6.	MCF - 06	Cream	Cream	2.5 cm
7.	MCF - 07	White	White	2.0 cm

Screening of Antibacterial Activities by Paper Disc Diffusion Assay

The study of 7 fungi were isolated from the leaves of *Muntingia calabura* L. collected at Yadanabon University Campus. During the study of biological properties of these fungi, six stains were exhibited antibacterial activities against *Salmonella typhi* (Figure 3).

Among them, fungus MCF-01 (22.07 mm clear zone) showed more hightly aelectibacterial activity against *Salmonella typhi.* than the other fungi. Therefore, this strain MCF-01 was selected for further investigations such as fermentation for the extraction of antibacterial metabolites.

Table 3. Antibacterial activity of isolated fungal strains

Stain No.	Inhibitory zone (mm) on *Salmonella typhi*
MCF-01	22. 07
MCF-02	17. 61
MCF-03	17. 78
MCF-04	18. 74
MCF-05	15. 02
MCF-06	12. 66
MCF-07	-

(-no activity)

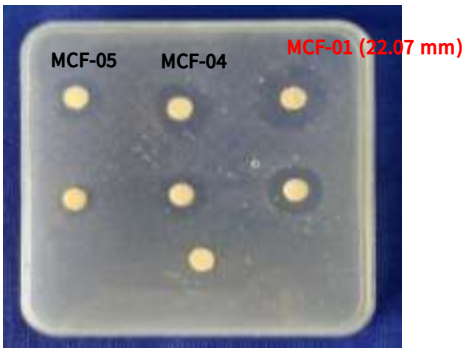


Figure. 3. Antibacterial activity of isolated fungal strains against *Salmonella typhi.*

Fermentation Studies for the Production of Antibacterial metaboliteagainst *Salmonella typhi*

It was observed that seed culture 84 hours age and 20% size were the best for the optimal fermentation as shown in Table 4 and 5, Figure 4.

Table 4. Effects of ages of inoculums on the fermentation

Culture time (hrs)	Inhibitory zone (mm) on *Salmonella typhi*
48	15.08
60	18.30
72	21.77
84	**22.25**
96	19.23

Table 5. Effects of sizes of inoculums on the fermentation

Size (%)	Inhibitory zone (mm) on *Salmonella typhi*
5%	16.54
10%	17.28
15%	20.48
20%	**23.49**
25%	20.76

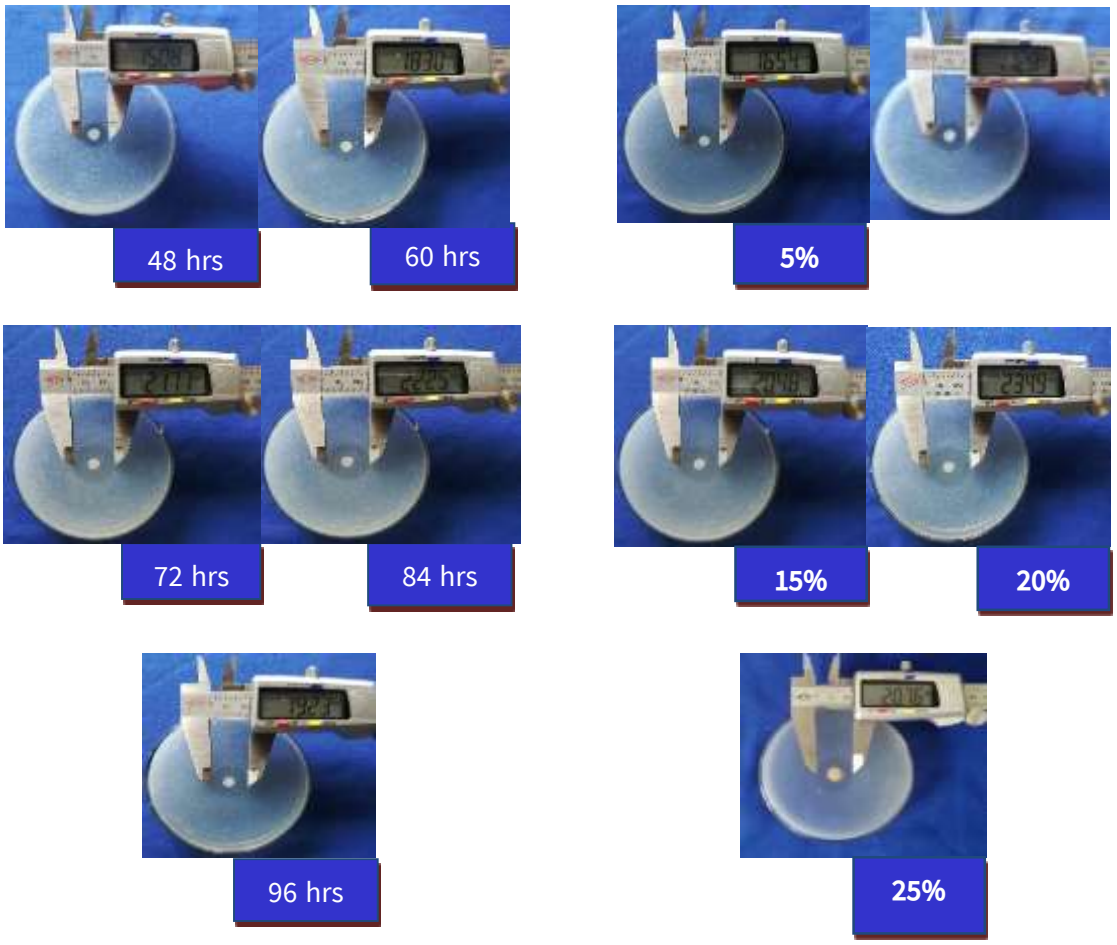


Figure. 4. The Effects of ages and sizes of inoculums on fermentation

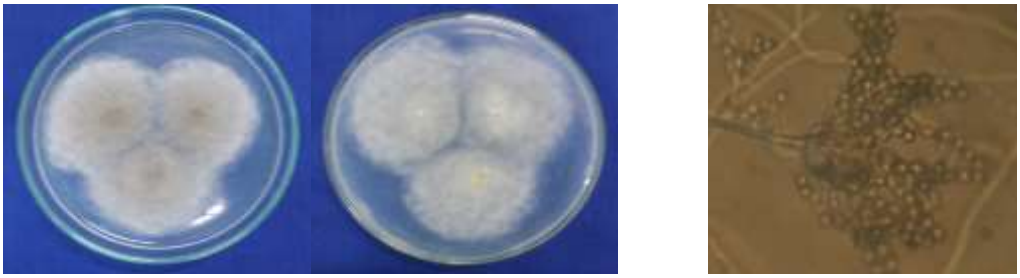
Distinct Microscopical characters of endophytic fungus MCF-01

In the investigation, microscopical study was done by planting the fungus on Water agar medium and incubating for 7 days. The growth appearance was then noted by observing both the reverse and front view of endophytic fungus MCF-01 was observed that the smokey white color.

The spore formation and mycelium of this endophytic fungus was studied with the help of microscope at ×40 magnifications. Distinct characters of fungus MCF-01 was observed and hyphae are long and abundantly branching; conidiophores, simple except at the apex where a cluster of thick, short phialides are produced, dark; conidia single, apical, globose or subglobose, brown, 1-celled (amerospore); some species also produce simple phialides and phialospores in chains, dark as shown in Table 4 and Figure 6. According to the reference of Ando, et al. (2004), this fungus MCF-01 was grouped as the fungi imperfecti.

Table 4. Morphology and Microscopical characters of Endophytic fungus MCF-01

Endophytic Fungus	Morphology	Hyphae Shape	Conidiophore Shape	Conidial Shape
MCF-01	surface and reverse colony color was smokey white color as a whole, the size of the colony reached about 4.0 cm in diameter for 5 days cultivation at 25±30℃	long and abundantly branching, septate	simple except at the apex where a cluster of thick, short phialides are produced, dark,	single, apical, globose or subglobose, brown, 1-celled (amerospore); some species also produce simple phialides and phialospores in chains, dark,



Front view Reverse view Photomicrograph (×40)

Figure. 5. Morphology and Photomicrograph of Endophytic fungus MCF-01

Discussion and Conclusion

A total of seven endophytic fungal stains were isolated from the leaves of the *Muntingia calabura* L. when cultured in the laboratory. These fungi are useful as a source of antibacterial metabolites.

In the investigation, these endophytic fungal stains were tested with five test organisms such as *Bacillus pumalis, Escherichia coli, Micrococcus luteus, Salmonella typhi* and *Staphylococcus aureus*. In this study, it was observed that six fungi (MCF-01, MCF-02, MCF-03, MCF-04, MCF-05 and MCF-06) were exhibited antibacterial against *Salmonella typhi* and remaining four test organisms did not show activity. Among them, fungus MCF-01 showed more activity against *Salmonella typhi* (22.07 mm clear zone) at 6 days fermentation period.

The fermentation studies for the antibacterial metabolite, it was observed that 84 hours ages of seed culture were optmized for fermentation. In the study of sizes of inoculum 20% contration,

it was the best for fermentation. The highest activity reached at 6 days fermentation with 84 hours ages and 20% sizes of inoculumns (23.49 mm clear zone) (Figure. 4).

The study of distinct morphological characters, the spore formation and mycelium of this endophytic fungus MCF-01 was observed with the help of microscope at ×40 magnifications.

Agung Bimantara *et.al* (2022), report that isolated fifteen endophytic fungi from *Muntingia calabura* L. and then investigated antifungal activity with *Candida parapsilosis*. These endophytic fungus (FDK-13) was found in inhibitory yeast growth of *Candida parapsilosis.*

The present studies isolated that endophytic fungus MCF-01 from the leaves of *Muntingia calabura* L. that was highest antibacterial activity against *Salmonella typhi.* Majority of fungi which produced secondary metabolites. That may be beneficial towards pharmaceutical effect and these metabolites are widely used in medicine. In conclusion, this selected endophytic fungus can be regarded as a source of antibiotic for human.

Acknowledgements

We wish to express our sincere gratitude to Dr Tint Moe Thuzar, Rector, Yadanabon University, for her permission to do this research. We are greatefully indebted to Dr Thwe Linn Ko, Dr U Khin Myot, Dr Myint Myint Oo and Dr Khin Maw Maw Soe Pro rectors, Yadanabon University, for their encouragement. Our special thanks go to Professor Dr Khin Pale`, Head of Botany Department, Yadanabon University, for her invaluable support and suggestions. We wish to thank, Dr Khin Myo Win and Dr Win Naing, Professors, Department of Botany, Yadanabon University for their kind help and advice.

References

Ando, K., M. Suto, and S. Inaba, (2004) *Sampling of isolation and identification methods of fungi*, Workshop at Univeristy of Pathein

Backer, C. A., and S.T. Brink, (1963) **Flora of Java, VoI**. I. N. V. P. Noordhoff

Bimantara, A., H. Widjajanti and E. Nurnawati, (2022) *Potency of Endophytic fungi Isolated from Muntingia calabura as Antifungal substances against Candida Parapsilosis***.** Biological Research Journal, V0lume 8, No. 2, Pg. 88-95.

Hundley, H. G., and Chit KoKo, (1987) *List of Trees, Shrubs, Herbs and Principal Climbers of Burma*. 4th end. Government printing and stationary, Rangoon.

Omura, S., (1985) *Microbial growth kinetics and secondary metabolites,* J. Fermentation Technology, 46: 134-140.

Petrini, O., T.N.Sieber, L. Toti, and O. Viret, (1986) *Ecology, metabolite production and substrate utilization in endophytic fungi.* Nat.Toh.1: 85-156

Prescott, L. M., J. P. Harley, and D. A. Klein., (2002) *Microbiology*. 5th ed. The McGraw-Hill Companies

Tan, R.X., and W.X. Zou, (2001) *Endophytes: a rich source of functional metabolites.* Natural Product Report 18:448-459

NITE (National Institute of Technology and Evaluation) (2004) *Amylase enzymestest activities methods.*