Isolation of Endophytic Fungi from Three Citrus Plants and their Antimicrobial Activities

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Abstract

In the course of isolation of endophytic fungi, essential oil bearing medicinal citrus plants were used at Taegyi-Kone village in Pathein Township. In this study on the isolation of endophytic fungi, the fungi were isolated from the *Citrus limon* (L.) Burm.f., *Citrus aurantifolia* (Christm.) Sw. and *Citrus maxima* (Burm. f.) Merr belonging to the family Rutaceae and the plant parts leaves and petioles were used. In the investigation, *Agrobacterium tumefaciens, Salmonella typhi, Bacillus substilis, Staphylococcus aureus, Escherichia coli, Candida albicans* and *Saccharomyces cerevisiae* were used for the test, throughout the research. Moreover, all strains were tested for preliminary studies of antimicrobial activities and these strains showed different levels of antimicrobial activities: MMT-04 showed the highest activity (25 mm) against the *Saccharomyces cerevisiae,* MMT-05 showed the highest activity (25 mm) against the *Salmonella typhi,* other strains showed the moderate against *Agrobacterium tumefaciens, Bacillus substilis, Staphylococcus aureus, Escherichia coli* and *Candida albicans.* So, in this study, isolated fungi MMT-04 and MMT-05 were selected for further investigation.

Keywords: Endophytic fungi, Colony Morphology, Antimicrobial Activities

Introduction

Citrus limon (L.) Burm.f., *Citrus aurantifolia* (Christm.) Sw. and *Citrus maxima* (Burm. f.) Merr are flowering plants belonging to the genus *Citrus,* family Rutaceae. These plants are found in warm templeate regions. They are also found in tropical regions. The fruits are edible and rich in vitamin C. It is also used in the preparation of cosmetic, perfumes in confectionery; they are also used in the traditional medicines (Nyo Maung, 2002). Endophytes are bacterial or fungal microorganisms that colonize healthy plant tissue intercellularly and or intracellularly without causing apparent symptoms of disease (Wilson, 1995).

Plants strictly limit the growth of endophytes, and these endophytes use many mechanisms to gradually adapt to their living environments (Dudeja, 2012). Endophyte microbes including fungi, bacteria and actinomycetes are uniquitous in most plant species growing in natural environment. They occupy a unique ecological niche and have major influence on a plant's good health, ecology, distribution and physiology. Communities of this group are known to contribute significantly to the biological diversity in the forest ecosystem (Bills, 1996). There is a need to search new ecological niches for potential sources of natural bioactive agents for different pharmaceutical, agricultural, and industrial applications, these should be renewable, eco-friendly and easily obtainable (Liu, 2001). The discovery of natural products involves isolation, structural elucidation and establishing the bio-synthetic pathway of the secondary metabolites. Citrus fruit intake is associated with a reduced risk of stomach cancer (Gonzalez, 2013). Lemons have the highest concentration of citrate of any citrus fruit, and daily consumption of lemonade has been shown to decrease the rate of kidney stone formation (Carr and Jackie, 2010).

According to the above references, endophytic fungi are industrial antibiotic producers. Antibiotics are special kind of chemotherapeutic agent usually obtained from living organisms. So, the isolation of endophytic fungi were studied in these research to get

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the secondary metabolite for human resources. The aim and objectives of this paper were to isolate the endophytic fungi, to study the antimicrobial activity of the fungal isolates and to investigate the effects of different fermentation periods.

Materials and Methods

The plant parts of *Citrus limon* (L.) Burm. f., *Citrus aurantifolia* (Christm.) Sw. and *Citrus maxima* (Burm. f.) Merr, potatoes, chloramphenicol, chemicals, and autoclave, balance, sterilized glass wares and other requisites were used for isolation of endophytic fungi and preliminary study on antimicrobial activity of fungal isolates.

Collection of plant samples

Figure (1) Map of plant samples collected area Tae-Gyi-Kone Village in Pathein Township

Source – Geography Department, Pathein University

SLNA medium
(Synthetic Low Nutrient Agar)

Glucose	2 g
Sucrose	2 g
KH_2PO_4	0.1 g
$MgSO_4.7H_2O$	0.5 g
KNO_3	0.1 g
KCl	0.5 g
Agar	1.8 g
D/W	100 ml
pH	± 6.5

WA medium
(Water Agar medium)

Agar	1.6 g
D/W	100 ml

Composition of PGA medium
(Potato Glucose Agar medium)

Potatoes	20 g
Glucose	2 g
Agar	2 g
D/W	100 ml

(After autoclaving chloramphenicol 0.8 µg was added to the medium).

Isolation procedure of endophytic fungi

The isolation of endophytic fungi was referred by the method of Ando and Inaba (2004) and the following procedures are shown in Figure (2).

The healthy leaves with petiole were collected from the plant. Leaves and petioles were rinsed under tap water for 10 minutes and dry in the air. Then leaves and petioles were cut into pieces and sterilize. The method of sterilization is firstly pieces of leaf and petiole sink in 70% EtOH for 1 minutes and rinse in the D/W for 1 minute. Then sink into 70% EtOH again for 30 seconds and rinse in D/W for 1 minute. After that they were making air dry by placing on the filter paper. When pieces of leaf and petiole were dried, margins were cut to ovoid contamination and culture on the culture medium by inverted position to attach inner surface and culture medium.

Isolation procedure for the endophytic fungi

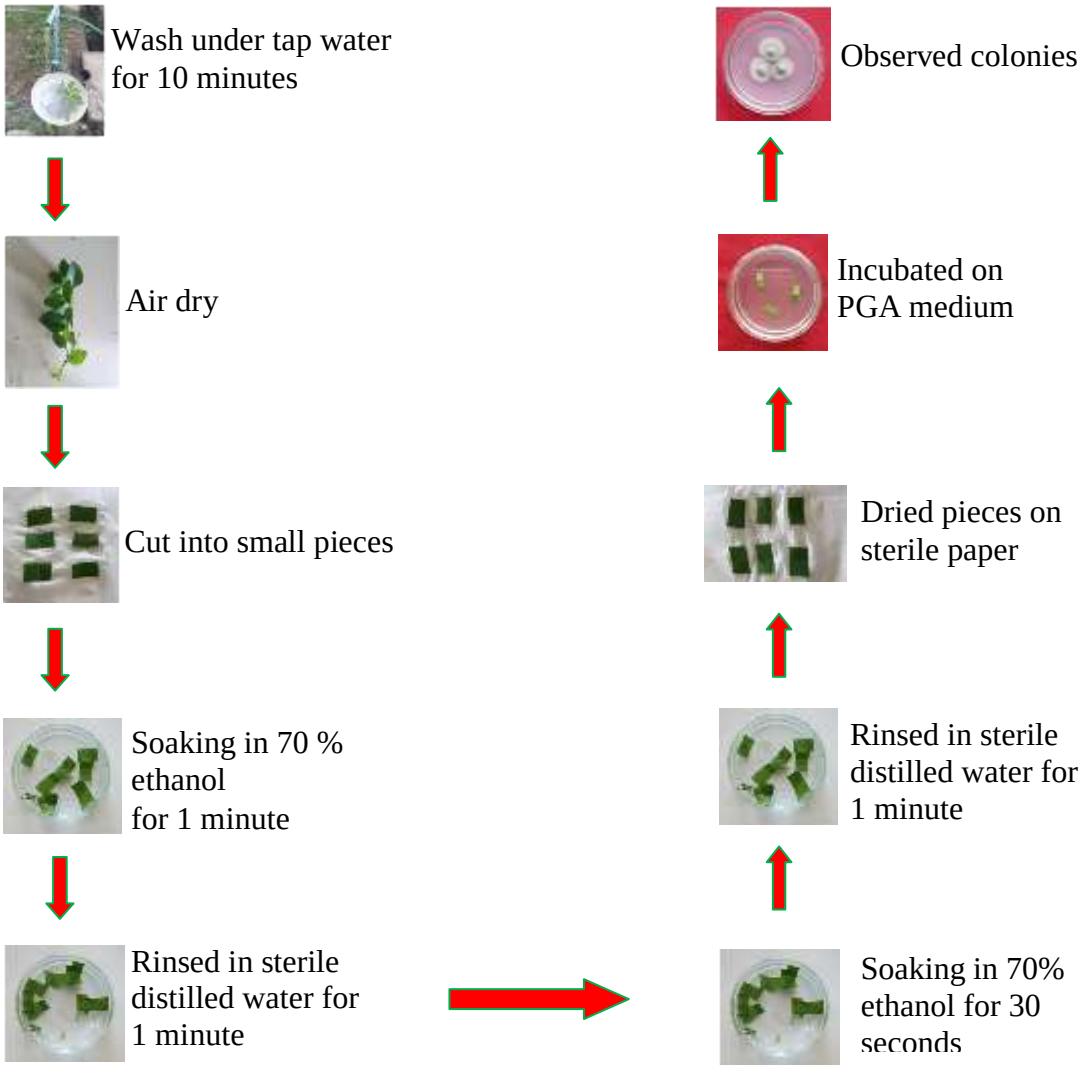


Figure (2) The isolation procedure of the endophytes

Screening of antimicrobial activities by paper disc diffusion assay (NITE, 2005)

1. The isolated fungi were grown at 25ºC for 5 days on PGA medium for sporulation.

5 days old culture

2. The isolated fungi were inoculated on seed medium and incubated at 25°C for 3 days.

100 ml conical flask contain 50 ml seed medium

Paper disc diffusion assay

4. 20 µl of fermented broth was put on paper disc and place on assay plate containing test organisms.

100 ml conical flask contain 50 ml seed medium

3. 10 ml of seed culture was transferred into the fermentation medium and incubate at 25ºC for 10 days.

Figure (3) Procedure for Screening of Effective Plant Isolated Microorganisms by Paper Disc Diffusion Assay (Antimicrobial Activity Test)

Preparation of seed culture and fermentation medium

Seed Medium (Ando, 2004)

Glucose	2.0 g
Sucrose	0.3 g
Yeast extract	0.3 g
KNO_3	0.1 g
K_2HPO_4	0.01 g
D/W	100 ml
pH	± 6.5

Fermentation Medium (Ando, 2004)

Glucose	3.0 g
Yeast extract	0.3 g
K_2HPO_4	0.001 g
$MgSO_4$	0.001 g
$CaCO_3$	0.1 g
D/W	100 ml
pH	± 6.5

Preparation of Assay medium

Nutrient Agar Medium (Atlas, 1993)

Beef extract	1.5 g
Yeast extract	1.5 g
Sodium chloride	5.0 g
Agar	1.5 g
D/W	1000 ml
pH	± 6.8

Nutrient Broth Medium (Dubey and Maheshwari, 2007)

Peptone	5.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
D/W	1000 ml
pH	6.8-7.02

Results

Scientific Name - *Citrus limon* (L.) Burm.f.

Vernacular Name - Shauk-chin

Family - Rutaceae

Distinguishing characters of *Citrus limon* (L.) Burm.f.

The plants are small trees. The leaves are compound, (8 to 12 cm) long and (3 to 5 cm) wide, alternate, exstipulate, obtuse, elliptic-lanceolate, crenate, petiole (winged petiole),

unifoliate, glabrous, dotted with gland, the leaves are reduced to spine, and reticulate vined. The inflorescences are axillary umbellate cyme. The flowers are bracteates, pedicellate, complete, actinomorphic, bisexual, hypogenous and cyclic. The sepals are 4-5, gamosepalous, valvate. The petals are 4-5, polypetalous, white, valvate. The stamens are numerous, dorsifixed, introrse, anther dithecous. The carpels are multilocular, style short, stigma capitate, axil placentation. The fruit may be hesperidium. The seeds are endospermic (Fig. 4).

Habit

Leaves

Figure (4) *Citrus Limon* (L.) Burm.f.

Scientific Name - *Citrus aurantifolia* (Christm.) Sw.

Vernacular Name - Thanbaya

Family - Rutaceae

Distinguishing characters of *Citrus aurantifolia* (Christm.) Sw.

The plants are small trees and slender branches. The leaves are compound, (4 to 7 cm) long and (2 to 4 cm) wide, alternate, exstipulate, ovate, bluntly pointed at the apex, margin short-serrate, base subcordate, petiolate (narrow winged petiole), unifoliate, dotted with gland, the leaves are reduced to spine, reticulate vined. The inflorescences are axillary umbellate cyme. The flowers are bracteates, pedicellate, complete, actinomorphic, bisexual, hypogeneous, white and they are bloom in cluster of 5-10 on the tip and several node bark. The sepals are 4-5 synsepalous, valvate. The petals are 4-5 apopetalous, white, imbricate. The stamens are numerous, basified, extrose, anther dithecous. The carpels are fused, the ovary is tri to multilocular, style short, stigma capitate, axil placentation. The fruit may be hesperidium. The seeds are endospermic (Fig. 5).

Habit

Leaves

Figure (5) *Citrus aurantifolia* (Christm.) Sw.

Scientific Name - *Citrus maxima* (Burm.f.) Merr.

Vernacular Name - Kywegaw

Family - Rutaceae

Distinguishing characters of *Citrus maxima* (Burm.f.) Merr.

The plants are medium size trees. The leaves are compound, (10 to 14 cm) long and (5 to 7 cm) wide, alternate, exstipulate, ovate, apex acuminate, margin short serrate, base subcordate, dotted with gland, petiolate (broad winged petiole), unifoliate, the leaves are reduced to spine, reticulate vined. Inflorescences umbellate cyme. The flowers are bracteates, pedicillate, complete, actinomorphic, bisexual, hypogenous and white. The sepals are 4-5 synsepalous, quincuncial. The petals are 4-5 apopetalous, white, united attaining, campantulate shape, valvate. The stamens are numerous, arranged in irregular bundles. The carpels are fused, multilocular, style short, capitate, the axil plancentation. The fruits may be hesperidium. The seeds are endospermic (Fig. 6).

Habit

Leaves

Figure (6) *Citrus maxima* (Burm.f.) Merr.

Table (1) Isolated endophytic fungi from *Citrus limon* (L.) Burm.f., *Citrus aurantifolia* (Christm.) Sw. and *Citrus maxima* (Burm.f.) Merr.

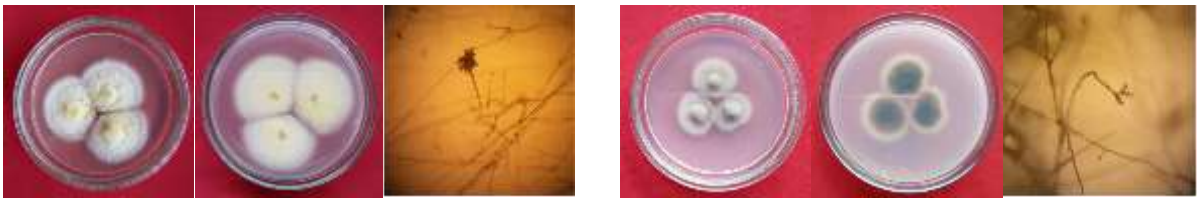
No	Part used	Isolated endophytic fungi	Plants	Colony colour	
				Front view	Reverse view
1	leaf	MMT-01	*Citrus limon*	white	center green, edge white
2	leaf	MMT-02		white	white
3	petiole	MMT-03		gray	center brown, edge cream
4	leaf	MMT-04	*Citrus aurantifolia*	white	center cream, edge brown
5	leaf	MMT-05		center yellow, edge white	White
6	leaf	MMT-06		cream	cream
7	leaf	MMT-07		white	white
8	petiole	MMT-08		yellow	yellow
9	leaf	MMT-09	*Citrus maxima*	white	white
10	petiole	MMT-10		white	yellow

Front view Reverse view Micrograph (X 40) Front view Reverse view Micrograph (X 40)

MMT-01 **MMT-02**

Front view Reverse view Micrograph (X 40) Front view Reverse view Micrograph (X 40)

MMT-03 **MMT-04**



Front view Reverse view Micrograph (X 40) Front view Reverse view Micrograph (X 40)

MMT-05 **MMT-06**

Front view Reverse view Micrograph (X 40) Front view Reverse view Micrograph (X 40)

MMT-07 **MMT-08**

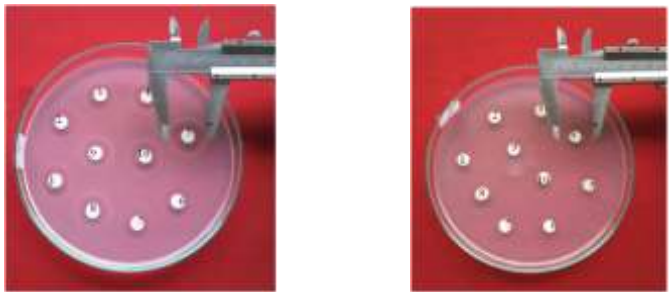
Front view Reverse view Micrograph (X 40) Front view Reverse view Micrograph (X 40)

MMT-09 **MMT-10**

Figure (7) Morphology and photomicrograph of endophytic fungi MMT-01 to MMT-10 from leaf and petiole (5 days old culture)

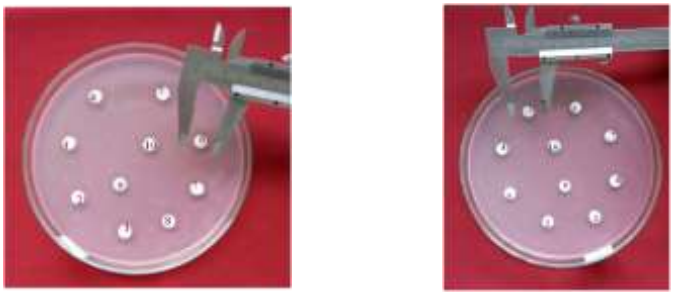
Table (2) Test organisms used in antimicrobial activities

No.	Test Organisms	Diseases
1	*Agrobacterium tumefaciens* NITE 09678	Plants diseases
2	*Bacillus subtilis* IFO 90571	Fever
3	*Candida albicans* NITE 09542	Candidiasis
4	*Salmonella typhi* AHU 7943	Typhoid
5	*Straphylococcus aureus* AHU 8465	Boil and food poisoning
6	*Escherichia coli* AHU 5436	Diarrhoea
7	*Saccharomyces serevisiae* NITE 52847	Food diseases



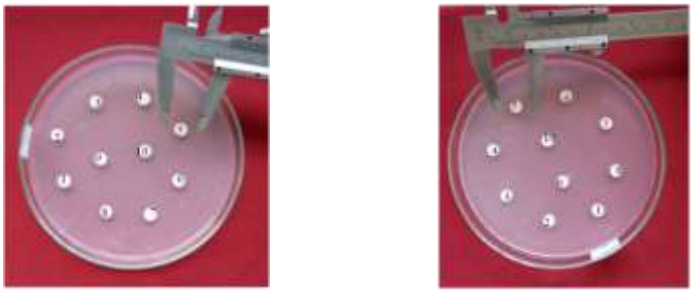
Salmonella typhi ***Saccharomyces cerevisiae***

Figure (8) Antimicrobial activity of isolated fungus against Salmonella typhi and Saccharomyces cerevisiae



Bacillus substilis ***Staphylococcus aureus***

Figure (9) Antimicrobial activity of isolated fungus against *Bacillus substilis* and *Staphylococcus aureus*



Escherichia coli ***Candida albicans***

Figure (10) Antimicrobial activity of isolated fungus against *Escherichia coli* and *Candida albicans*

Agrobacterium tumefaciens

Figure (11) Antimicrobial activity of isolated fungus against *Agrobacterium tumefaciens*

Discussion and Conclusion

In the present study, ten endophytic fungal strains were isolated from the leaves and petioles of three citrus plants. All the strains showed the antimicrobial activity on *Agrobacterium tumefaciens, Bacillus subtilis, Candida albicans, Salmonella typhi, Straphylococcus aureus, Escherichia coli* and *Saccharomyces serevisiae.* The isolated strains were designated as MMT-01 to MMT-10.

As the preliminary test, isolated fungal strains were tested by using seven kinds of test organisms. All of them, antimicrobial activity of fungal strains were evaluated by the paper disc diffusion assay with seven test organisms. Nearly all strains showed the antimicrobial activity. Among them, endophytic fungus MMT-04 showed the highest activity (25 mm) against *Saccharomyces cerevisiae* and MMT-05 showed the highest activity (25 mm) against *Salmonella typhi* in 5 day old culture. Other strains showed the moderate against *Agrobacterium tumefaciens, Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Candida albicans.* So, in the study, isolated fungi (MMT-04 and MMT-05) were selected for further investigation based on the results of the antimicrobial activity.

Many researchers have proved that endophyte is a new and potential source of novel natural products for exploitation in modern medicine, agriculture and industry (Bu'luck, 1974). Endophytic fungi have been recognized as a repository of novel secondly processing beneficial biological activities. One the most important properties of endophytic microorganisms especially fungi, is linked to their metabolic potential to produce a large variety of bioactive molecules that can protect the plant against pathogens (Strobel, 2003). So the most active strains, such as antimicrobial drug and higher crop yield need further investigation.

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References

Ando, K and S Inaba, (2004). *Workshop on Taxonomy and identification of Fungi***.** Pathein University Biotechnology Development Cetre.

Atlas, R.M., (1993). *Microbiology Fundamentals and Applications.* Mecmillan Publishing Co; advision of Macmillion, Inc.

Bills, (1996). *In Tropical Mycology:* Micromycetes; Watling, R., Frankland, J. C. Ainsworth, A.M., Issac; S., Robinson, C.H., Eds., CABI Publishing, New York.

Bu'luck, I.D., (1974). *Secondary metabolism in Fungi and its Relationship to growth and development.* Edward Arnold, London.

Carr, Jackie, (2010). *Five Ways to Prevent Kidney Stones UC San Diego***.** Retrieved April 22, 12-03

Dudeja, S.S., (2012). *Interaction of endophytic microbes with Legumes;* Journal of Basic Microbiology, Vol.53

Dubey R.C and D.K. Maheshwari, (2007). *Practical Microbiology*. S. Chang and Company Ltd. Ram Nagar, New Delhi 110 055 ELBS and E. and S. living stone ltd.

Gonzalez, CA, (2013). *Gastric cancer; epidermiologi aspects.* Helicobacter. 18 (Supplement. 1) 34-38

Hundley, H.G and Chit Ko Ko, (1987). *List of Trees, Shrubs, Herbs and Principal Climberes etc 4th ed.* Printing and stationary, Burma.

John Kress, W; *et.al.*, (2003). *A Checklist of the Trees, Shrubs, Herbs and Climbers of Myanmar.*

Liu, C.H., (2001). *Antifungal activity of Artemisia annua endophyte cultures against phytopathogenic fungi,* Journal of Biotechnology 88.

NITE (National Institute of Technology and Evaluation), (2005). *Medium for fermentation to produce the metabolites*

Nyo Maung, (2002). *Taxonomy of angiosperms,* Rutaceae

Owen NL, Hundley N. *Endophytes the chemical synthesizers inside plants.* Science Progress, 87; 79-99.

Strobel, G.A., (2003). *Endophytes as a sources of bioactive products.* Microbes Infect 5:535-544.

Wilson, D; (1995). *"Endophyte the evolution of a term, and clarification of its use and definition,"* Oikos, Vol.73.