ISOLATION OF ENDOPHYTES FROM SELECTED RHIZOMES OF GINGER FAMILY COLLECTED FROM KAMAYUT TOWNSHIP AND STUDY ON SOME BIOACTIVITIES

Than Than Soe[1], Nandar Wint[2]

Abstract

Endophytes which live on the surface of four underground rhizomes in the family Zingibraceae were isolated by using PDA, SM and SY media. The genus of isolated endophytic fungi were estimated based on their morphological structures of hyphae, mycelium and spores. After studying the preliminary strength of antimicrobial activity against six test organisms, they were used to test in optimizing age of culture, size of inoculum, fermentation time in glucose peptone medium. To understand the necessary experimental conditions in the estimation, extraction, purification of metabolites in the fermented medium of isolated fungal strains, the bioautographic assay with TLC was applied .The different ratios of Chloroform and Methnol (10:0, 9:1, and 8:2) were used in TLC. The secretion of hydrolytic enzymes (primary metabolite) were also measured in the media using different substrates. It was observed that *Penicillium* spp was isolated from *Zingiber officinales* Rose, *Mucor* spp. was from *Curcuma longa*, *Trichoderma* spp. was from *Alpinia galanga* (L.) Willd. and *Aspergillus* spp. from *Hedychium flavum* Roxb. In the estimation of antimicrobial potential, S_6 (possible genus *Trichoderma* spp.) isolated from *Alpinia galanga* (L.) Willd provided the highest clear zone of 22-25 mm and hence selected for further investigation. S_6 was found to gave high activity in 25% size of inoculum at 84 hours of age 4 days fermentation periods in Glucose Peptone medium. The highest size of clear zone (25 mm) was recorded against *Bacillus subtilis* in all antimicrobial tests. In bioautographic detection of suitable organic solvents ratio to be used in extraction and purification of metabolites from fermented broth, it was observed that 8:2 ratio of chloroform and methanol (v/v) resulted the best R_f value of 0.44 and was chosen for further tests. The final results also indicated that the isolated endophytic fungi can secrete the extracellular amylase, protease and cellulose enzymes into respective agar media.

Keywords : Endophyte (Zingibraceae), Antimicrobial activity

INTRODUCTION

Endophytes are the plant-associated microorganisms that live within the living tissues of their host plants without causing any harm to them. Almost all groups of microbes have been known to produce enormous variety of strange and wonderful metabolites. They have profound biological activities that can be exploited for human health and welfare. Some of the endophytic microorganisms can produce the same secondary metabolites as that of the plant thus making them a promising source of novel compounds. In the present investigation, endophytes were isolated for the first time from the underground rhizomes of the four selected plants in the Zingibraceae family. Family Zingiberaceae consists of the large number of medicinal plants and is well-known for its use in ethnomedicine and play a major role in Myanmar traditional Medicine. The ginger family of flowering plants, consists of 53 genera and more than 1,377 species. This family is rich in essential oil with terpenes such as borneol, camphor and cineole (all oxygen-containing monoterpenes), camphene, pinene (monoterpenes) and zingiberene (a sesquiterpene), as well as phenylpropanoids.

Typically, these compounds accumulate in oil cells, an important microscopical characteristic of the Zingiberaceae (Heinrich *et al*., 2003). Gingers are important natural resources, which provide many useful products for food, spices, medicines, dyes, perfume etc. The objective of this study is the documentation of antimicrobial activity shown by the endophytes isolated from rhizomes of

[1, 2] Lecturer, Dr., Department of Botany, Dagon University

Zingiberaceous plants widely used in Myanmar traditional medicine, adding information to the systematics and some bioactive potentials.

MATERIALS AND METHODS

Experimental Site

The plants were collected from different location in Kamayut Township, Yangon Region. Plants parts samples were collected from Yangon University Campus, Kamayut Township, Yangon Region.

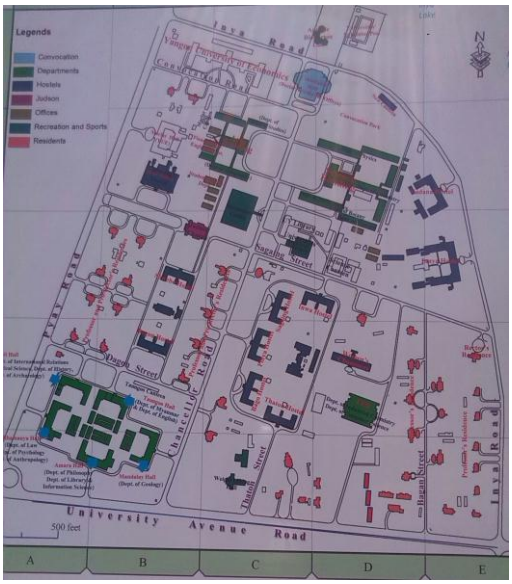


Figure 1. Plant samples collection site

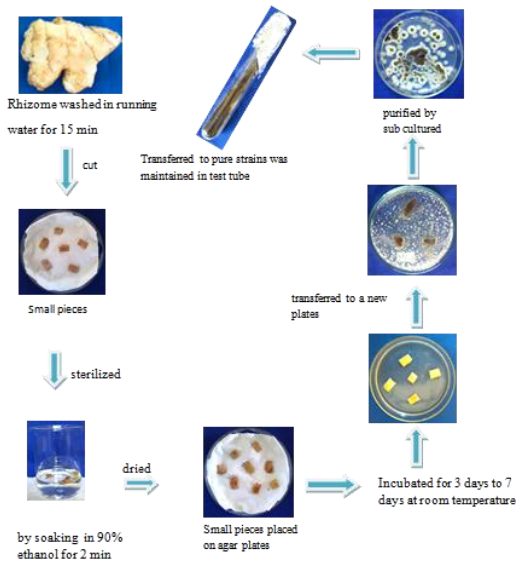


Figure 2. Isolation procedures of endophytes (Ando, 2004)

Table 1. Plants species used for isolation of endophytic fungal strains

No	Scientific Name	Myanmar name	Family
1	Zingiber officinalis Rosc	Gyin	Zingiberaceae
2	Curcuma longa L.	Na-nwin	Zingiberaceae
3	Alpinia galanga (L.) Wild	Pade gaw- gyi	Zingiberaceae
4	Hedychium falvum Roxb.	Shwe-pan	Zingiberaceae

Morphological and Microscopical Study for the Genus Identification

Botanical study of study plants

The preliminary identification of collected plant is studied in Botany Department of Yangon University using (Hooker, 1885), (Backer, 1965), (Kirtikar, 1973).

Identification of Isolated Fungi

For the identification of Genus of isolated endophyte the books of (Barnett, 1969), (Davie, 1987) was applied.

Study for Antimicrobial Activity by Paper Disc Diffusion Assay (Tomita, 1988)

The fungi used in this study were the endophytic fungi isolated from some rhizomes. Antimicrobial activities by paper disc diffusion assay of isolated fungi were investigated by the method of Tomita, (1988). The isolated fungi were grown at room temperature for 4 days on SY medium and were inoculated on seed broth medium and incubated at room temperature for 3 days. After the end of fermentation, the fermented broth (20µl) was used to check the antimicrobial activity against test organisms by paper disc diffusion assay. Each paper disc having six millimeter diameter was soaked with 20µl fermented broth for antimicrobial assays.

Fermentation Study

In the fermentation studies, ages of culture, size of inoculums and fermentation period were investigated by the method of Omura, 1985. In the study of ages of culture, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours, 96 hours and 108 hours were employed with 10 ml seed culture as shown in Figure.(3). In the investigation of sizes of inoculums, 5%, 10%, 15%, 20%, 25% and 30% were utilized with 84 hours ages of seed of culture as shown in Figure (4). Fermentation was carried out for 4 days with 84 hours ages of culture and 25% of sizes of inoculums at room temperature. Antimicrobial activity test was measured at 12 hours intervals by paper disc diffusion assay method (Tomita, 1988).

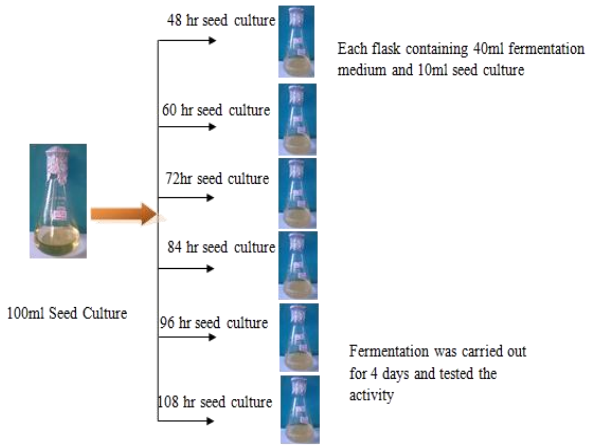


Figure 3. Effects of ages of inoculums for fermentation (Omura, 1985)

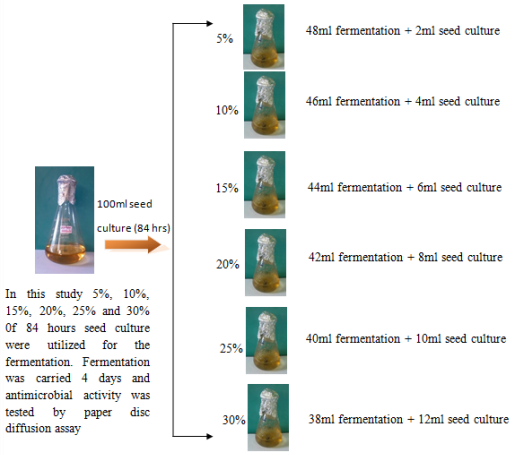


Figure 4. Effect of sizes of inoculum for fermentation

Bioautography Test (Choma and Grzelak, 2010)

Extraction by TLC plates (3 cm × 1 cm) saturated with ethyl acetate for the characterization of antimicrobial agent. One drop of extracted as loaded onto the TLC strip and dried. The loading was done 5 time and put into the TLC Tank (4.5 cm × 6.7 cm) as shown as figure (5) and chromatographed in chloroform and methanol in the ratio of (10:0, 9:1, 8:2). Each TLC strip, were placed on assay agar plates with test organisms. Each paper was placed on assay agar plates. The methods are as same as paper disc diffusion assay. After one hour the TLC strip was taken out and the plates were incubated for 24-36 hours. Then, the inhibitory zone appeared on the assay agar plate was recorded and the R_f value were measured.

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

RESULTS

Botanical Study

SN- *Zingiber officinal* Rosc
MN - Gin
F- Zingiberaceae

Figure 5. Habit and rhizome of ginger

SN- *Curcuma longa* L
MN- Nanwin
F- Zingiberaceae

Figure 6. Habit and rhizome of nanwin

SN - *Alpinia galanga* L.
MN - Padegaw-gyi
F- Zingiberaceae

Figure 7. Habit and rhizome of padegaw-gyi

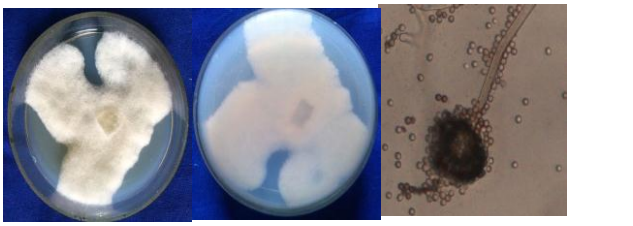
SN- *Hedychium flavum* Roxb.
MN- Shwe pan
F- Zingiberaceae

Figure 8. Habit and rhizome of shwe pan

Morphology and spore morphology of isolated fungi $S_1 – S_9$

Surface view Reverse view Hyphae spores (×100)

Figure 9. Colony morphology and Mycelium of isolated *Penicillium* sp

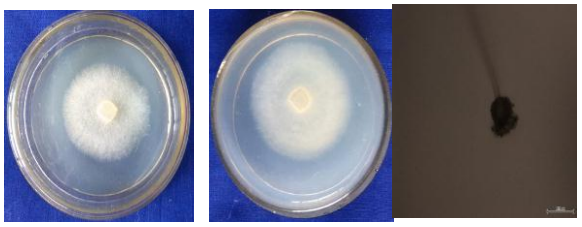


Surface view Reverse view Hyphae spores (×100)

Figure 10. Colony morphology and Mycelium of isolated *Mucor spp*

Surface view Reverse view Hyphae spores (×100)

Figure 11. Colony morphology and Mycelium of isolated *Trichoderma sp*



Surface view Reverse view Hyphae spores (×100)

Figure 12. Colony morphology and Mycelium of isolated *Aspergillus sp*

Preliminary Study for Antimicrobial Activity by Paper Disc Diffusion Assay

In the screening program, it was observed that 9 fungi endophytes (S_1 to S_9) were obtained (Table 3). When they are subjected in the antimicrobial test all the isolated endophytes showed the antimicrobial activity against 6 test organisms as shown in (Table 4). These results confirmed that S_6 provided the maximum antimicrobial activities among the 9 isolated endophytes. Therefore S_6 was used in fermentation studies.

Table 3. Possible Genus of isolated fungi

Designated Strain	Possible Genus
S_1	*Penicillium sp*
S_2	*Penicillium sp*
S_3	*Mucor sp*
S_4	*Mucor sp*
S_5	*Trichoderma sp*
S_6	*Trichoderma sp*
S_7	*Aspergillus sp*
S_8	*Aspergillus sp*
S_9	*Aspergillus sp*

Table 4. Antimicrobial Activities of Isolated Fungi S_6 Provided After 4 days of Fermentation Period

Test organisms	Size of clear zone(mm)								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9
Salmonella typhi	20	21	20	23	23	22	21	20	19
Malassaziza furfur	22	17	22	23	23	22	23	22	22
Staphylococcus aureus	15	12	16	15	16	17	17	15	13
Escherichia coli	20	14	22	19	22	23	22	21	19
Bacillus subtilis	20	11	16	17	18	25	15	21	10
Condida albicans	10	11	15	13	13	16	14	11	10

Table 5. The effects of ages of inoculum of S_6 on the antimicrobial activity Against *Bacillus subtilis* test organisms

Culture time (Ages of culture, hrs)	Antibacterial activity (mm)
48	10
60	11
72	24
84	25
96	17
108	14

Table 6. The effects of sizes of inoculum on antimicrobial activity against

Sizes %	**Clears zone (mm)**
5	15
10	17
15	19
20	20
25	**27**
30	20

Study on the fermentation periods for the fermentation

In the fermentation studies, fermentation periods was 1 to 5 days it was found that 84 hrs ages of culture was the best for the fermentation and 25 % sizes of inoculums was optimized to produce the antibacterial metabolite maximum activity reached at 4 days fermentation period with above age and sizes of inoculums.(Table 7, Figure. 17)

Table 7. Effects of Fermentation period Inoculum for Fermentation

Fermentation Periods (Days)	Inhibitory zone(mm)
1	10
2	17
3	21
4	**25**
5	22

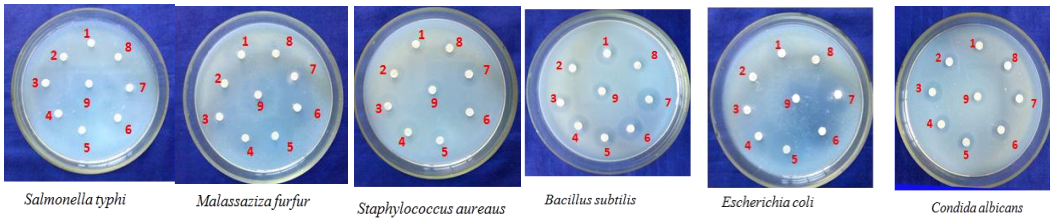


Figure 13. Antimicrobial activities shown by all isolated fungi from rhizomes

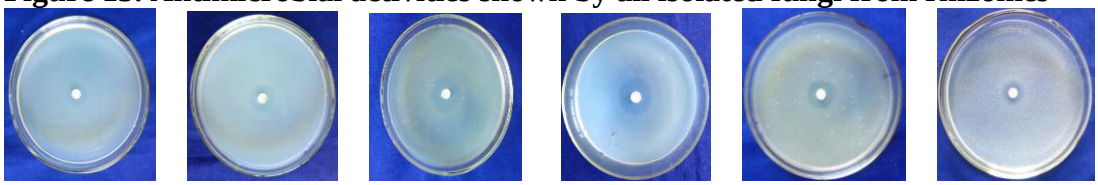


Figure 14. Effects of ages of inoculum of S_6 on for the Antimicrobial activity against *Bacillus subtilis*

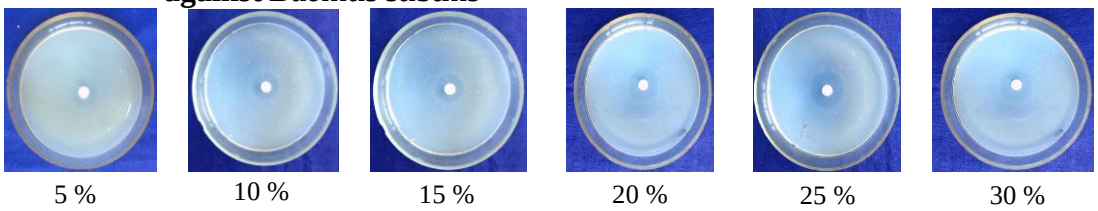


Figure 15. Effects of sizes of inoculum of S_6 for the antimicrobial activity against *Bacillus subtilis*

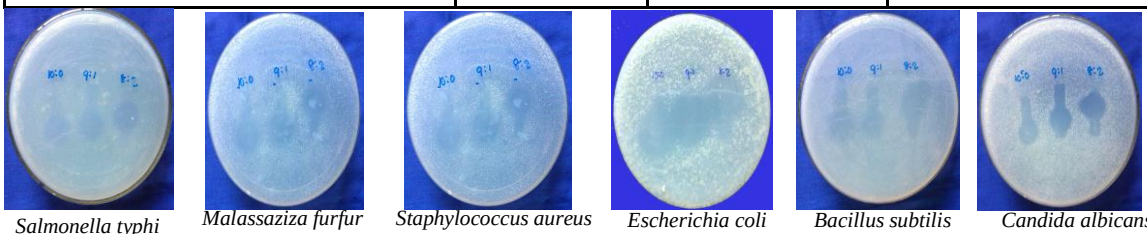
Figure 16. Antimicrobial activity of fermentation periods on *Bacillus subtilis*

Bioautography of Antimicrobial Metabolite

To determine the paper solvents in which antimicrobial metabolite secreted by S_6 can be extracted was done by Bioautography Technique (Choma, 2010). The R_f values of Antimicrobial Metabolite given by different ratio of Chloroform and methanol were shown in (Table VII and Figure 23). It was recorded that the metabolites produced by S_6 (*Trichoderma*) was the best (0.44 R_f value) in the solvent system 8:2 (Chloroform and methanol. It is meant that the get more better separation the methanol is needed in the solvent extraction in the cause of *Bacillus subtilis.*

Table 8. R_f values of Antimicrobial Metabolites produced S_6 (*Trichoderma*) extracted by using different ratio of chloroform and methanol on Bioautography

Ratio of Chloroform and Methanol			
Test Organism	**10 : 0**	**9 : 1**	**8 : 2**
Salmonella typhi	0.2	0.5	0.24
Malasseziza furfur	0.3	0.24	0.4
Staphylococcus aureus	0.24	0.18	0.42
Escherichia coli	0.3	0.4	**0.44**
Bacillus subtilis	0.24	0.26	**0.44**
Candida albicans	0.2	0.3	0.38



Salmonella typhi *Malassaziza furfur* *Staphylococcus aureus* *Escherichia coli* *Bacillus subtilis* *Candida albicans*

Figure 17. R_f value of antimicrobial activit

Estimation of Enzyme Activity

As described in materials and methods, three enzymes activities were estimated by using three basal substrates such as soluble starch, skimmed milk and cellulose. Two days after the inoculation of S_6 into individual medium, the clear zones of various sizes were detected in the all enzyme test plates, visually (Figure 19 to 21).

Figure 19. Amylase enzyme activity test

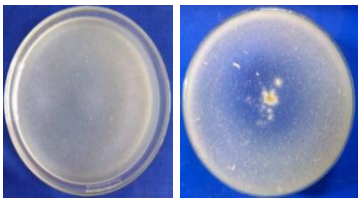


Figure 20. Protease enzyme activity test

Figure 21. Cellulose enzyme activity test

DISCUSSION AND CONCLUSION

Ginger and related plants have been used for centuries as remedies for human diseases. Treatments with the use of various plants have historically formed the basis of sophisticated Myanmar traditional medicine, preceding the established scientific literature by thousands of years. With the advancement of Endophytic Micro-biology, the source of the medicinal properties associated with microbes in the plant tissues been investigated. This quest for understanding has led to an explosion in the last few years in the areas of isolation of microbes, their identity, metabolites and biological activity, structural elucidation and the chemical synthesis of natural products. In the present work, 4 selected plants in Zingiberaceae family are important natural resources that provide many useful products for food, spices, medicines, dyes, perfume and aesthetics. These constitute a vital group of rhizomatous medicinal and aromatic plants characterized by the presence of volatile oils and oleoresins of export value and widely distributed in Myanmar, and in tropical and subtropical regions of

Asia. All 4 isolates showed considerable inhibitory activities towards 6 test organisms. All strains showed distinct inhibitory activities against bacterial pathogens.

The strongest activity was shown by S_6 against gram-positive, *Bacillus subtilis* (25 mm clear zone) followed by gram-negative, *Escherichia coli* (23 mm clear zone) while there was moderate evidence of inhibition against clinical yeast strain, *Candida albicans* (10 -16 mm). All strains were known as prominent antagonists of human pathogens against *Malassaziza furfur,* and *Candida albicans*. The four endophytic potential strains were identified as *Penicillium*, *Mucor*, *Trichoderma* and *Aspergillus* designated as S_1 to S_9. Anisa Lutfia *et al*., 2017, isolated the endophytic fungi from healthy rhizomes of the wild ginger *Amomum centrocephalum* A.D. Poulsen and evaluate the antagonistic properties of isolated endophytic fungi against selected pathogenic bacteria and phytopathogenic fungi assessed by dual culture plate assay. Methanolic extract of *Amomum centrocephalum* rhizome was also tested as a control. The results are in line with those found in present study. The present study suggests that the chloroform: methanol (8:2) extract of isolated S_6 (*Trichoderma* sp.) can be exploited as a natural antibiotic against bacterial diseases and as skin infections in line with its notable antimicrobial properties revealed in this study. Moreover, the results of enzyme activity tests indicated that S_6 may possess the hydrolyzing potentials on different raw materials of starch, protein and cellulose.

In 2019, Kazi Jannatul Ferdous *et al.* studied isolated endophytic fungi from the na nwine plant (*Curcuma longa)* and reported that endophytic fungi, had produced a wide array of secondary bioactive metabolites with the peculiar potential compounds, antibacterial, antifungal, anticancer, etc. These are the ideal targets for further drug discovery. These leads may play a major role in the recovery of infectious, inflammatory and also certain kinds of cancer diseases. So, extensive research is necessary in the area of endophytic fungi isolation and characterization of the compounds from the plants of Zingibraceae. Generally, the rhizomes of this family are aromatic, tonic and stimulant. They are rich sources of essential oils that consist of numerous complex terpenoid mixture. Many terpenoids compounds with varied physiological activities-antimicrobial, antiarthritic, antioxidant, anticancer, anti-inflammatory, antidiabetic, anti-HIV, neuroprotective and larvicidal etc. have been identified inthe essential oils of Zingiberaceous plants. The present study suggests that the chloroform: methanol (8:2) extract of isolated S_6 (*Trichoderma* sp.) can be exploited as a natural antibiotic against bacterial diseases and as skin infections in line with its notable antimicrobial properties revealed in this study. Moreover, the results of enzyme activity tests indicated that S_6 may possess the hydrolyzing potentials on different raw materials of starch, protein and cellulose.

REFERENCES

Ando, K. and S.Inba 2004. **Isolation and identification of fungi,** wprkshop, BDNRC- Pathein University.

Arnold, A. E., Z. Mayanard, G. S. Gilbert, P. D. Coley, 2000. **Are tropical fungal endophytes hyperdiverse**? Ecol. Left. 3, 267- 274

Barnett, H. I. 1969. **Illustrated Genera of Imperfect Fungi 2 Edition, Burgress Publishing Company.**

Bussaban, B., S. Lumyoung, P. Mekenzie, K.D Hyde. 2001. Endophytic **Fungi from Amomum Siamense, Canadian Journal of Microbiology,** 47(10) 943-948

Choma and Grzelak, 2010. **Bioautography detection in thin layer chromatography,** volume 1218, Issue 19, Pages 2684-2691

Daisy, B. and G. Strobel. 2003. **Bioprospecting for Microbial Endophytes and Their Nature Products**, Microbiol. Mol, Biol. Rev., 67(4); 491-502

Davie. H .Larone. 1987. **Medically Important Fungi,** Mycology Rescource Center, \Washing . DC, New York.

Hawksworth, D. L. 1991. **The fungal dimension of biodiversity; Magnitude, significance and conservation, Mycological Research** 95, 641- 655

Hooker, J. D. 1885. **The flora of British India (Vol.4) Englang**: L. Reeve and Co.ltd.

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

Policegoudra, R. S. and K.; Gowda, 2007. Journal of Chromatography B, 852, 40-48

Tomita, F. 1988. **Laboratory Method**, Hokkaiodo University, Japan