SCREENING OF EFFECTIVE ENDOPHYTIC FUNGI ISOLATED FROM LEAVES OF *ARISTOLOCHIA INDICA* L.

Nandar Wint[1], Than Than Soe[2], Thant Zin Nwe[3]

Abstract

In this study, the isolation of endophytic fungi from leaves of *Aristolochia indica* L.(Eik-thara-muli) was researched. The plants samples were collected from 31 ~~wart~~ ward, North Dagon Township, Yangon Region. The plant samples were identified and studied in accordance with taxonomic procedures. *Aristolochia indica* L. belong to the family Aristolochiaceae. Ten endophytic fungi were isolated from this plant. These strains were cultured on Potato Dextrose Agar medium and Czapex Dox Agar medium. In the biological properties of isolated fungi, AF-10 showed the high antibacterial activity of inhibitory zone (23.26 mm) against *Vibrio cholerae*. Therefore, this strain AF-10 was selected for further investigation. Based on the morphology and microscopical characteristic, this fungus AF-10 is preliminarily identified as *Aspergillus* sp. It was concluded that 72 hour culture time and 15% of inoculum size were suitable for the fermentation. The fermentation medium FM-3 was selected for the production of antimicrobial metabolite. Highly antibacterial activity reached ~~at~~ in 4days fermentation period with 72 hr of culture time and 15% of inoculum size.

Keywords: isolation, biological properties, identified, fermentation, inoculum, antimicrobial activity

INTRODUCTION

In Myanmar, medicinal plants had played an important role in traditional medicine for the treatment of diseases since ancient time. Apart from city dwellers, most of the people in rural and remote areas mainly depend on use of medicinal plants and their derivatives for healing their disease as a source of easily available and cheap therapeutic agents. About 80% of world's population is still using the herbal medicinal plants and their products. *Aristolochia indica* L. is one of the 500 species of the family Aristolochiaceae. It has wide spread distribution in tropical as well as temperate regions of the world including Myanmar. Aristolochia indica is called Eik-thara- muli in Myanmar. It is a popular medicinal plant.

The plant has a number of historical medicinal uses. Aristolochic acid, a major active constituent of the plant is reported to cause cancer. Most importantly, the plant exhibited significant antimicrobial activity.(Robert and Krupnick, 2018) Endophytic fungi have been discovered in many different types of plants. There are many ways in which plants can benefit from the presence of these fungi in their tissue. They get a relatively safe place to live within the tissues of a plant. Endophytic fungi usually live symptomatically within tissues of their host plants. The potential role of the endophyte and its biologically active metabolites in its association with its host has been investigated. (Mashra *et al*, 2014). The purpose of this research work was to investigate the various endophytic fungi, their antimicrobial activity and fermentation conditions of selected fungi.

MATERIAL AND METHOD

Collection and identification of plant samples

The plant samples were collected from 31 ~~wart~~ ward, North Dagon Township in Latitude 16˚86″N and 16˚89″N, Longitude 96˚19″E and 96˚22″E. The samples were then taken and the experiments were carried out at the microbiology laboratory of Botany Department, Dagon University from June, 2019. Identification of

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

specimens were accomplished in accordance with taxonomic procedures. The endophytic microorganisms were isolated from healthy leaves. According to the Hutchison (1967), Hooker (1882) and Dassanayake (1996), the plant samples were identified with taxonomic procedures. The local names were obtained from local inhabitants and records of Hundley and Chit Ko Ko (1987).

Isolation procedure of endophytes from plant parts (leaves).

Wash plant parts in running water for 15 mins. Cut the plant parts into about 1cm pieces. Sterilize the surface of plant part by soaking in 95% alcohol for 15 sec. Next sterilize the surface by soaking in 1% sodium hypochlorites for 5 min. Wash out sodium hypochlorite by soaking 95% alcohol for 15 sec. Dry the sample on sterilized paper. Then cut the sample into 2 pieces. The piece was placed on agar plates. The plates were incubated for 3 days to 1 week at room temperature (Tomita F, 1998).

Culture characteristics of isolated fungi strains

The distinct culture characteristic of isolated fungi on potato dextrose agar medium and czapex dox agar medium were studied according to illustrate genera of imperfect fungi by H.L Barnett (1960).

Screening of effective endophytic fungi by paper disc diffusion

The isolated fungi were grown on PDA medium for 5 days. The isolated fungi were inoculated into seed medium and incubated for 3 days at 27^{o}C. Seed culture (1%) was transferred to the fermentation medium. The fermentation was carried out for 7 days. The fermented broth was used to check the antimicrobial activity against test organisms by paper disc diffusion assay. In this study, nine test organisms were utilized for antimicrobial activity.

Screening of Antimicrobial Activity by Paper Disc Assay

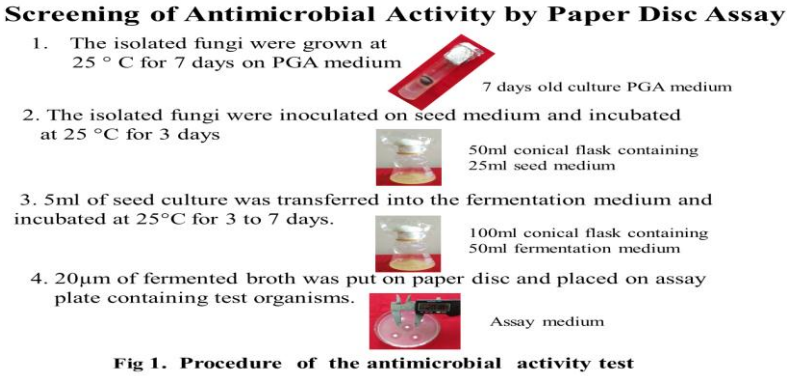

Fig 1. Procedure of the antimicrobial activity test

Table 1. Test organisms utilized in the antimicrobial activity of isolated fungi

No	Test organisms	Disease
1	*Vibrio cholerae*	Cholera, food infection
2	Pseudomonas aeruginosa	Infection of the respiratory tract , bone and joint infection, urinary tract infection
3	*Staphylococcus aureus*	Skin disease, food poison, wound infection, burns
4	*Klebsisella pneumonia*	Pneumonia, blood stream infection, meningitis
5	*Proteus mirabilis*	Urinary tract infection
6	*Candida albicans*	Alimentary tract, skin infection
7	*Escherichia coli*	Urinary tract infection, cholera, diarrhea and vomiting
8.	*Bacillus subtilis*	Ear, eye, respiratory tract infection, urinary tract and gastrointestinal tract infection
9.	*Bacillus pumalis*	Eye infection, soft tissue infection

Effects of ages of culture on the fermentation of endophytic fungi AF-10 (Comura, 1985 and Cruger, 1987)

The selected fungus AF-10 was inoculated into the seed medium and then transferred to the fermentation medium. The culture samples were checked in 12hrs intervals for the growth. Ages of culture with (48 hrs, 60 hrs, 72 hrs, 84 hrs, 96 hrs and 108 hrs) were utilized for the optimization of fermentation. The antimicrobial activity was carried out paper disc diffusion method by using with *Virbrio cholerae* test organisms.

Effects of size of inoculum on the fermentation of endophytic fungi AF-10 (Omura, 1985 and Cruger, 1987)

In this study, 5%, 10%, 15%, 20% and 25% of 72 hrs seed culture were utilized for the fermentation. Fermentation was carried out 7 days and antibacterial activity was tested by paper disc diffusion assay.

Results

Morphological Character of Plant

Scientific Name - *Aristolochia indica*
English Name - Indian Birthwort, Snake root
Myanmar Name - Eik- thara- muli
Family - Aristolochiaceae

Aristolochia indica L. is a twining perennial herb. Leaves alternate, petiolate, simple, entire and exstipulate. Flowers constitute of light purplish with inflorescence in axillary cymes hairy within limbs dilated. It is pedicellate, bract present, usually irregular, bisexual, perianth present, petaloid, fused. Stamens are six in number, adnate and filaments are not distinguishable from the style. Anthers are adnate to column and carpel is six locular with many ovules. Fruit is globose, oblong, septicidal, six valved, capsule containing many triangular seed.

Fig 2. Habit of the *Aristolochia indica* L.

Isolation of Endophytic Fungi

In the course of the investigation of endophytic fungi, 10 fungi strians were isolated from leaves of *Aristolochia indica* at 31 wart, North Dagon Township.

Table 2. Selection of site for leaves samples

Collection Sites	Latitude & Longitudes	Designated strains
North Dagon Township	16 ° 86’ N and 16 ° 89’ N 96 ° 19’ E and 96 ° 22’ E	AF -1 to AF-10

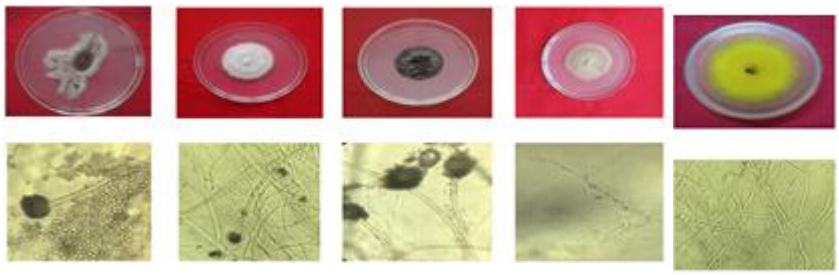

Fig 3. Morphology and micrograph of isolated fungi (AF-1 to AF-5)

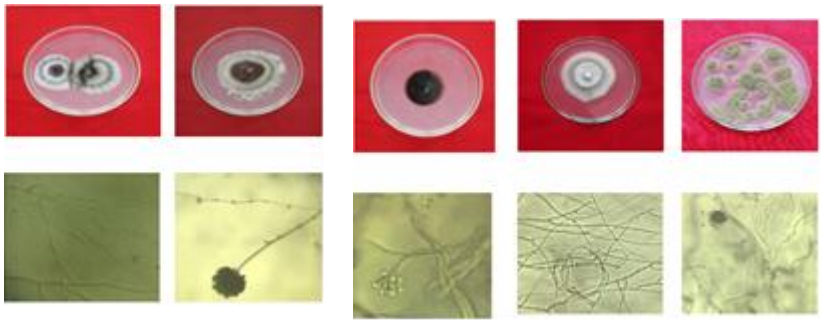

Fig 4. Morphology and micrograph of isolated fungi (AF-6 to AF -10)

Screening of effective isolated fungi by paper disc diffusion assay

The study of the biological properties of these endophytic fungi, two fungi exhibited antibacterial against *Vibrio cholerae*, two fungi against *Pseudomonas aeruginosa*, three fungi against *Staphylococcus aureus*, two strains exhibited antibacterial against *Klebsiella pneumonia*, three strains against *Proteus mirabilis*, three strains showed antifungal against *Candida albicans*, two strains against *E.coli*. Three strains against *Bacillus subtilis* and another three strains showed antibacterial against *Bacillus pumalis*. Among them AF-10 showed more highly antibacterial activity against *Vibrio cholerae*.

Table 3. Antimicrobial Activity of Isolated strains from *Aristilochia indica* L.

Table 3. Antimicrobial Activity of Isolated strains from *Aristilochia indica* L.

Test Organisms	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
Vibrio cholerae	-	-	-	-	-	-	15.14 mm	-	-	23.26 mm
Pseudomonas aeruginosa	-	-	-	-	-	-	15.33 mm	-	-	17.10 mm
Staphylococcus aureus	-	-	-	-	-	-	-	15.90 mm	15.82 mm	13.10 mm
Klebsiella pneumonia	-	-	-	-	-	-	19.09 mm	20.07 mm	-	-
Proteus mirabilis	-	-	13.58 mm	14.25 mm	12.72 mm	-	-	-	-	-
Candida albicans	-	-	-	-	-	-	-	15.23 mm	14.21 mm	14.33 mm
Escherichia coli	-	-	-	-	-	-	-	-	15.46 mm	1432 mm
Bacillus subtilis	-	-	-	-	-	17.65 mm	-	20.87 mm	19.18 mm	18.19 mm
Bacillus pumalis	-	13.15 mm	13.11 mm	-	12.81 mm	-	-	-	-	-

Paper disc size = 5mm

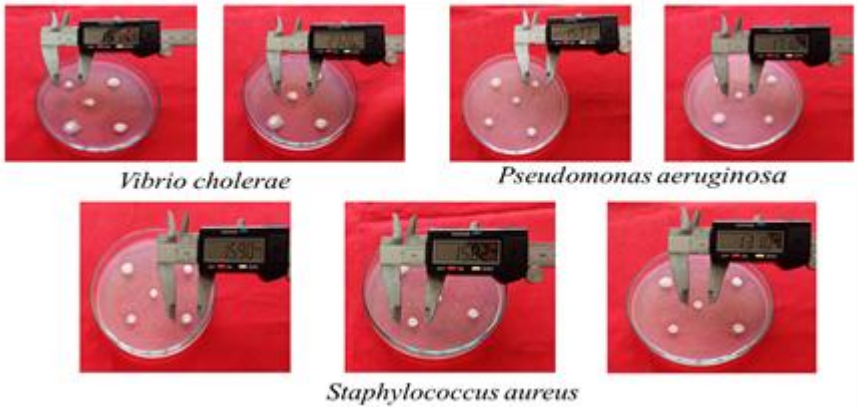

Fig 5 . Antimicrobial activities of endophytic fungi

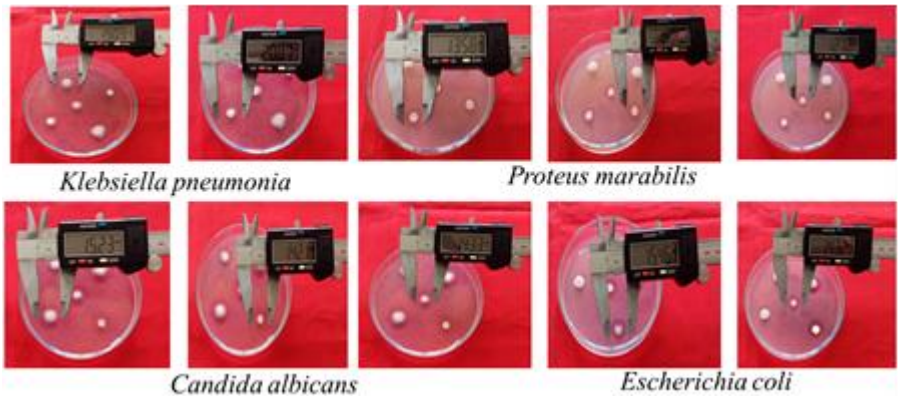

Fig 6. Antimicrobial activity od endophytic fungi

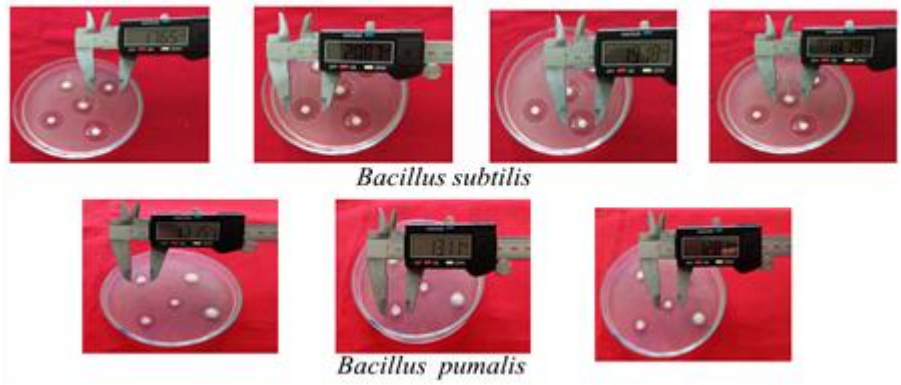

Fig 7. Antimicrobial activity of endophytic fungi

Identification of selected endophytic fungus AF-10

Macroscopic Characters

Fungi isolated are usually incubated on potato dextrose agar and czapex dox agar medium at 25˚C. Most species are sporulated within 7 days. The color of the extensive mycelium is green on medium.

Microscopic Characters

Conidiophores upright, simple, terminating in a globose, radiating from the entire surface, conidia 1- celled, globose, often variously colored in mass. According to the macroscopical and microscopical characteristic features and base on the references keys of Barnett 1972, Davise 1987, Colin *et al* 2013, it is assumed that AF-10 strain may be *Aspergillus* sp.

Kingdom - Mycota
Division - Eumycota
Sub-division - Deuteromycotina
Class - Eurotiomycetes
Order - Eurotiales
Family - Trichocomaceae
Genus - *Aspergillus*
Species - *Aspergillus* sp.

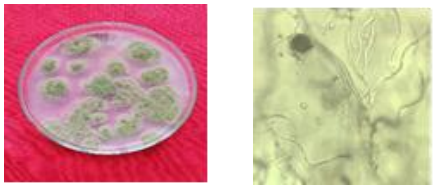

Fig 8 . Morphology and micrograph of isolated fungi AF-10

Study on the effects of ages of culture on the fermentation

According to the Omura (1985) and Cruger(1989), age of culture (48, 60, 72, 84, 96 and 108 hrs) were utilized for the fermentation. In the experiment, it was observed that 72 hrs age of seed culture were suitable for the fermentation (Table. 4, Fig, 9).

Table 4. Effects of ages of Culture on the fermentation by isolated fungi AF-10

Culture time (hrs)	Activity (inhibitory zone, mm)
48	12.55
60	14.33
72	25.74
84	24.00
96	16.78
108	16.02

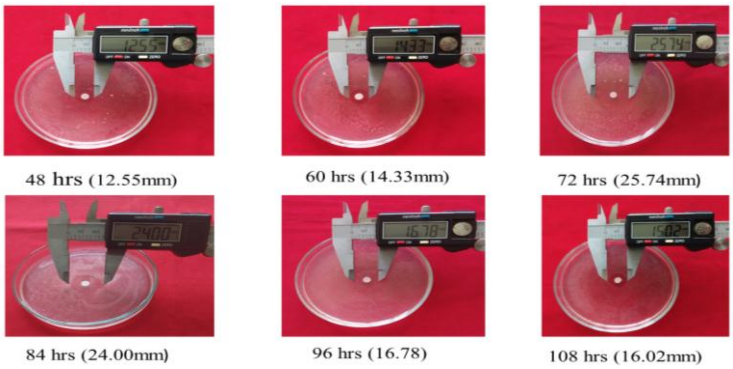

Fig 9 . Effects of ages of inoculum on fermentation (AF-10)

Study on the effects of size of inoculum on the fermentation

In this study, different sizes of inoculum (5%, 10%, 15%, 20% and 25 %) were tested. The size of 15 % inoculum was the best for the fermentation (Table -5, Fig -10).

Table 5. Effects of size of inoculum on the fermentation by isolated fungi AF-10

Size of inoculum (%)	Inhibitory zone (mm)
5 %	12.77
10 %	15.02
15 %	25.52
20 %	22.13
25 %	18.38

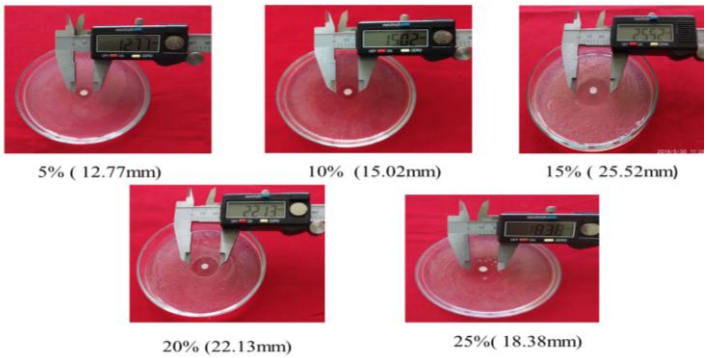

Fig 10. Effect of size of inoculum on fermentation (AF-10)

Table 6 .Three different fermentation condition of isolated fungi AF-10

Fermentation media	Inhibitory zone (mm)
FM - 1	-
FM - 2	-
FM - 3	21.42

Age of inoculum - 72 hrs
Size of inoculum - 15%
Medium - Assay medium
Fermentation time - 4 days
Test organisms - *Vibrio cholerae*

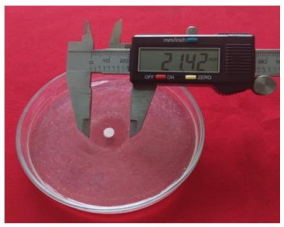

Fig 11 . Effect of medium on the fermentation of antimcrobial activity

Study on the Fermentation periods for the production of antibacterial metabolite against *Vibrio cholerae*

According to the results of antibacterial activity, fermentation medium FM-3 was selected for the production of antibacterial metabolite. In the fermentation study, highly antibacterial activity reached ~~at~~in 5 days fermentation. (Table 7, Fig-12).

Table 7. Effect of time course of fermentation by isolated fungi AF-10

Fermentation Times (Days)	Inhibitory zone (mm)
1	-
2	12.14
3	15.37
4	22.43
5	21. 42
6	13.96
7	12.14

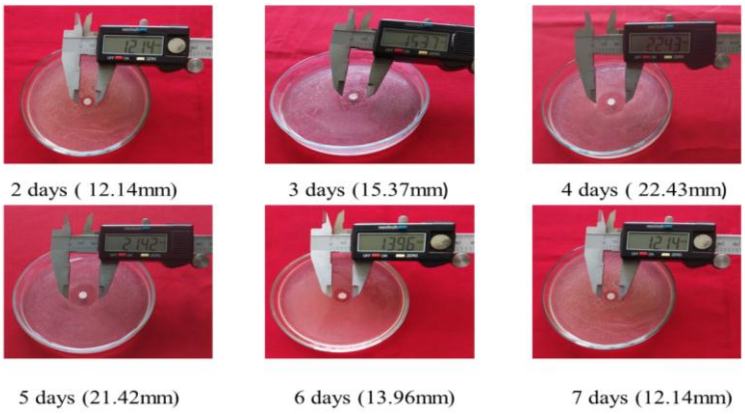

Fig 12 . Duration of fermentation for the production of antibacterial metabolite against *Vibrio cholera*

DISCUSSION AND CONCLUSION

The present investigation was under taken in order to isolated the endophytic fungi from leaves of *Aristolochia indica* L.Ten strains were isolated from this plants. The cultural characteristics and microscopical examination pointed to be fungi. During the study of biological properties of these fungi, two fungi exhibited antibacterial against *Vibrio cholerae*, two fungi against *Pseudomonas aeruginosa*, three fungi against *Staphylococcus aureus*, two fungi against *Klebsiella pneumonia*, three fungi against *Proteus mirabilis*, three strains against *Candida albicans*, two strains against *Escherichia coli*,three strains exhibited *Bacillus subtilis* and three strains against *Bacillus pumalis*. Among them AF-10 (23.26mm) showed more highly antibacterial activity on *Vibrio cholerae*. Therefore, this strain AF-10 was selected for further investigation such as ages of culture, size of inoculum and fermentation periods. The selected endophytic fungus AF-10 was observed that the vesical was in the shape of circular with filamentous extensions growing out from it. The hyphae allowed them to grow spread and continue reproducing across the surface of the substrate. Conidia are produced on conidiophores after completion of the spore

chains. Based on this morphology, microscopical characteristic and the references of Barnett (1972), Davise (1987) and Dube (1957), this endophytic fungusAF-10 may be identified as *Aspergillus* sp.

It was found that the most suitable parameter such as 72 hrs ofculture time and 15% of inoculum size were optimized to produce antibacterial activity during fermentation. Maximum activity reached on 4th day after fermentation. Strobel and Daisy (2003) and Huang *et al* (2008) reported that endophytic fungi are by now recognized as a potential source of anti- microbial secondary metabolites. It could be used for various medicinal purposes. Some of the isolates of *Aristolochia indica* also showed a very good antimicrobial activity against all test organisms. Maria *et al* (2005) and Madki *et al* (2010) evaluated that the species of *Aspergillus* genus are known by their production of substances having antibacterial effects. Above all these results were matched with Maria (2005) and Madki (2010). Fermentation was carried out with three media and antibacterial activity test paper disc diffusion assay method with test organisms used *Vibrio cholerae*. FM-3 medium was the best fermentation condition of isolated endophytic fungus AF-10 for the production of antibacterial activity. In conclusion, the endophytic fungus Aspergillus will offer further studies to clarify the identification of isolated fungus ~~up to~~ based on species level and to find out the nature of metabolites.

REFERENCES

Ando, K and S. Inaba (2004); Workshop on Isolation and Identification of Fungi, Pathein University, Biotechnology Development Center.

Atlas, R.M (1993); Handbook of Microbiological Media, CRC Press, printed in the United of America.

Backer, C.A (1963): Flora of Java Vol.1. Noordhoof and Groningen. The Netherlands.

Bal. S.N. and B. Gupta (1956); Morphology of *Aristolochia indica* Linn. Sci, Cult 22:276-277.

Barnett, H.L (1960); Illustrate Genera of Imperfect Fungi. Second Edition. Printed in the United State of America.

Bisen, P.S (1998): Handbook of Microbiology,CBS publishers and Distribulor Delhi.

Crueger, W and A. Crueger (1989); Methods of Fermentation, in Biotechnology, A Textbook of Industrial Microbiology, Internal Student Edition; 64-74.

Davise and H Larone (1987); Medically Important Fungi Mycology Resource Centre, Washinton DC, New York.

Dassanayake, M.D (1996); A revise handbook to the Flora of Ceylon, Vol 1,7 Washington. D.C., U.S.A

Dube, H.C (1997); An Introduction of Fungi. New Delhi.Vikas Publishing House Pvt. Ltd; 1983:616pp.

Hooker, J.DC (1882); Flora of British India, Vol-II.L Reeve and Co.,Ltd. Landon.

Huang, WY, Cai YZ, Hyde KD, Cork H and Sun M (2008); Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants, fungal diverse,33; 61-75

Hundley, H.G and Chit Ko Ko (1987); List of Trees, Shrubs, Herbs and Principle Climbers of Myanmar; Government Printing Press. Yangon Myanmar.

Hutchison, J.,(1967);Key to the Families of flowering plants of the World. Oxford University, At the Claredon Press Oxford, London.

Madki M.A, Manzoor A.S, Powar P.V, Patil K.S (2010); Isolation and Biological Activity of Endophytic Fungi from Withania Somnifera. Int.J.Pharm,Sci 2: 848-858.

Maria G.L, Sridhar K.R, Raviraja N.s (2005); Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. j. Agric, Technol.1;67-80.

Mishra, Y, Singh, A. Batra, A. and Sharma, M.M (2014); Understanding the biodiversity and biological application of endophytic fungi. A review.j.Microb.Biochem. Technol, 58-004.

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

Selim, K.A.,El. Beih AA, Abd El - Rahman TM and El – Diwany Ai(2012); Biology of Endophytic Fungi. Current Research in Environment & Applied Mycology 2(1), 31-82, Doi 10.5943/ cream/2/1/3.

Tomita F (1998); Isolation Procedure of Endophytes In Natural Products Isolation by R.J.P.Cannell 53-89