ISSN Online: 2349-7785

Bioactive Molecules (GC-MS) of Endophytic Fungi, *Xylaria* from *Nyctanthes arbor-trists* (Linn)

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Received 17 January 2017; accepted in revised form 115 February 2017

Abstract: This study deals with isolation and identification (GC-MS) of bioactive molecules from an endophytic fungus isolated from a traditional medicinal shrub *Nyctanthes arbor-trists* (Linn). Total 11 compounds were identified from the isolated fungal extract. On the basis of study of macroscopic and microscopic characters and ITS sequencing, isolated endophytic fungus was identified as *Xylaria*. Secondary metabolites isolated from *Xylaria* were screened for antimicrobial assay against three pathogenic bacteria. Crude extract of *Xylaria* was demonstrated strong inhibitor of three pathogenic bacteria *Micrococcus luteus* (clear zone 68.2 mm), *Citrobacter freundii* (clear zone of 59.16 mm) and *Chromobacterium violaceum (*clear zone 39.96 mm). Production of hydrolyzing enzymes like amylase and lipase from *Xylaria* was also detected.

Key words: Endophytic fungi, *Nyctanthes arbor-trists,* Secondary metabolites, *Xylaria.*

Introduction

Endophytes are considered as non-aggressive, often even postulating a mutualistic role within their host. Different groups of organisms such as fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plants [1]. Term "endophytes" refers to the organisms which throughout or part of their life cycle invade the tissue of living plant and cause asymptomatic infections. Most endophytes isolated to date have been ascomycetes and their amorphs, however, Rungjindamai *et al.*[2] reported several endophytes may also be basidiomycetes. However, the colonization rate and isolation rate of endophytic fungi from plants varied greatly. Some medicinal plants harbored more endophytic fungi than others [3]. Some of the common endophytes not only existed in most plant hosts but also have higher relative frequencies within each of the hosts. In contrast, some other endophytic fungi were detected in only plant host [3]. Endophytic fungi are of biotechnological interest due to their potential use as genetic vectors, metabolites and biological control agents.

Endophytes are ubiquitous and have been found in all the species of plants. Many economically important grasses carry fungal endophytes some of which may enhance host growth, may improve the plants ability to tolerate abiotic stress such as drought, as well as improve their resistance to insect and mammalian herbivores [4]. Some endophytes protect their host from insect by producing bioactive metabolites [5]. Recent studies suggest that endophytic fungi are not host specific [6].

Nyctanthes arbor-tristis is one of the most useful traditional medicinal plants, it is native to India. It is a night flowering sad tree of family Oleaceae is well known in India. It is a terrestrial woody perennial having life span of 5-20 yrs. It is

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usually a shrub or a small tree having brilliant, highly fragrant flower, which bloom at night and fall off before sunrise, giving the ground underneath a pleasing blend of white and red.

Nyctanthes arbor-tristis is a mythological plant and possesses high medicinal value in Ayurveda. The popular medicinal use of *N. arbor-tristis* include anthelminthic, antipyretic, skin ailments and as a sedative. Each part of the plant has some medicinal value and is thus commercially exploitable. It is now considered as a valuable source of several unique products for the medicines against various diseases and also for the development of some industrial products.

Objective

Objectives of the present study were: 1. Isolation of endophytic fungi, 2. Characterization and identification of isolated fungus. 3. Assessment of enzymatic activity of isolated endophytic fungi. 4. Isolation of secondary metabolites and assessment of its antimicrobial activity against three pathogenic bacterial strains. 5. Identification of compounds isolated in crude extract from isolated fungus through GC-MS.

Materials and methods

Isolation of endophytic fungi

Leaf of disease free plant *N. arbor-tristis* were surface sterilized and leaf pieces (1.2 cm) were inoculated on PDA media. Plates were then kept in BOD incubator (28±2°C) for 4-5 days, and monitored every day for growth of endophytic fungi.

Characterization of isolated fungus

The colonies of isolated fungus from leaf pieces were studied for their macroscopic features i.e. color, shape and growth of cultured colonies etc. Microscopic observations of endophytic fungal species were carried out by preparing slide of fungal mycelium, stained with lacto phenol cotton blue stain. The slide was observed under microscope for its microscopic characteristics i.e. structure of hyphae, type of septa in hypha, branching pattern of hypha, type and shape of spores, type of adherence of spores on hypha, no. of spores on each location, spore size, color of hypha and spores etc. Obtained data were then compared with the descriptions of endophytic fungus species in the literature and matches were recorded.

ITS and BLAST sequencing

ITS and BLAST [NCBI] sequence analysis have been carried out from the microbial culture collection, National Centre for Cell Science (NCCS), Pune by using following protocol:

DNA was extracted from the fungal culture using XcelGen Fungal gDNA Kit (Xcelris Labs), eluted in 200 µl of elution buffer and concentration of DNA was estimated using Nanodrop (ND-1000, Thermo scientific, USA). The genomic DNA was amplified using ITS1 and ITS4 primers[7] in PE 9700 thermo cyclers (PE, Applied Biosystems); initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, final extension at 72°C for 10 min. The PCR was carried out in 25 µl reaction mixture (10 U Taq polymerase buffer, New England Biolabs), 2 mMdNTPs, 10 pMprimers, 1 unit Taq polymerase (New England Biolabs), and 10 ng DNA). The positive amplicons were purified by PEG, and purified PCR products were sequenced for both strands on an ABI 3730 xl DNA analyzer using the Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA). A BLASTn sequence homology search was performed to compare it with available sequences of *Xylaria* in GenBank database [8]. Alignment [9] and phylogenetic analysis based on the neighbor joining (NJ), the maximum parsimony (MP) and maximum likelihood (ML) method was done with retrieved GenBank sequences of *Xylaria* in MEGA 5.0 [10].

Enzymatic activity of isolated endophytic fungi, *Xylaria sp.*

The endophytic fungal isolates were observed for the production of various hydrolyzing enzymes like amylase, lipase, protease and lactase.

Amylolytic activity

Amylase activity of isolated fungus was assessed by growing the fungi on Glucose Yeast Extract Peptone Agar (GYP) medium (glucose-1g, yeast extract 0.1 g, peptone 0.5 g, agar 16 g, dis-

tilled water 1L) with 0.2 % soluble starch (pH 6.0). After incubation on 28±2°C for 3-4 days the plates were flooded with 1 % iodine in 2 % potassium iodide

Lipolytic activity

For lipase activity, the fungi were grown on sterilized Peptone Agar medium (peptone-10 g, NaCl-5 g, $CaCl_2.H_2O$-0.1 g, agar 16 g, and distilled water 1L (pH 6.0). At the end of the incubation (28±2°C) period for 4-5 days a visible precipitate around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme indicated positive lipase activity.

Proteolytic activity

Glucose Yeast Extract Peptone Agar medium (containing 8 % gelatin) (pH 6.0) was used to analyze proteolytic activity of isolated endophytic fungus. After incubation (28±2°C, 4-5 days) degradation of the gelatin was seen as clear zone around the colonies. The plate was then flooded with 1 % mercuric chloride solution, which resulted in formation of a precipitate. This made the agar opaque and enhanced the clear zone around the fungal colony.

Biomass production

Autoclaved Potato Dextrose Broth (PDB) (150 mL) was inoculated with isolated endophytic fungus, kept for incubation at 28±2°C in a BOD incubator. After the incubation period, formation of the fungal mat was observed .Weight of a Whatman's No. 1 filter paper was taken. Filtration of fungal mat was performed using a funnel. Weight of fungal mat with filter paper was taken and weight of biomass production by the isolated endophytic fungi was calculated using following formula:-

Biomass production (g) = Weight of filter paper with fungal mat – weight of filter paper.

Secondary metabolites extractionfrom *Xylaria* sp.

After incubation mycelium containing flask was kept in an orbital shaker (Sonar) on 145 rpm for 4-5 days at 25±2. The broth after incubation was then filtered; filtrate containing the secreted secondary metabolite of endophytic fungus was used to observe antibacterial activities. Lyophilized extract send for GC-MS analysis to the laboratory of JNU, New Delhi.

Bacterial strains

Pure cultures of *Micrococcus luteus* [Causes sepsis, or endocarditis - an infection of the lining of the heart] (MTCC 7950), *Citrobacter freundii* [Leading pathogens of nosocomial infections] (MTCC 7029), *Chromobacterium violaceum* [Causes destruction of red blood cells and causes Listeriosis] (MTCC 7544), were procured from Microbial Type Culture Collection (MTCC), Chandigarh. The bacterial suspensions of the above pure cultures were prepared by inoculating the powdered form of the above strains into their respective nutritional broth.

Antimicrobial activity of secondary metabolites from *Xylaria* sp.

The isolated endophytic fungi were evaluated for their antibacterial activity against three species of human pathogenic bacteria. The antimicrobial activity of cell free extract (CFE) from isolated endophytic fungus was performed by agar well diffusion method. Nutrient agar plates were prepared and swabbed with pure culture of bacterial strains .The plates were kept for 20 minutes incubation. After the incubation period, wells were formed using well puncture and were inoculated with fungal metabolite, blank solvent and Abs disc (antibiotic disc) as a control. The plates were then kept for incubation at 37°C for 24 hours. Each experiment was set in three replicates.

Minimum inhibitory concentration (MIC) or zone of inhibition

After incubation, antibacterial activity of fungal metabolite was observed by measuring zone of inhibition surrounding each well. The zone of inhibition was measured by measuring the diameter of zone of inhibition (mm) against pathogenic bacteria by fungal metabolite. One set was kept as control.

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of crude fungal extracts was carried out at the advanced instrumentation research

facility (AIRF), JNU, New Delhi. GC-MS analysis of the crude was performed on a Shimadzu GC-MS-QP-2010 plus system. RTx-5 SilMS column (30 m × 0.25 mm id × 0.25 µm film thickness) was used with the following operating conditions: oven temperature program from 80°C-250°C at 5°C/ min with holding time of 4 min and from 250°C-310°C at 20°C/ min with holding time of 5 min, and the final temperature was kept for 24 min. The injector temperature was maintained at 270°C, pressure 81.7 kPa, total flow 16.3 ml/ min, column flow 1.21 ml/ min, linear velocity 40.5 cm/ sec, purge flow 3.0 ml/ min, split ratio 10.0, ion source temperature 230°C, scan mass range m/z 40-600, and interface line temperature 280°C.

The identification of compounds was performed by comparing the mass spectra with data from NIST (National Institute of Standards and Technology, US), Wiley, Pesticide Library 3rd edition, Drug Library, GC/MS Metabolite Mass Spectral Database and FFNSC (Flavor and Fragrance Natural and Synthetic Compounds) libraries.

Result and discussion

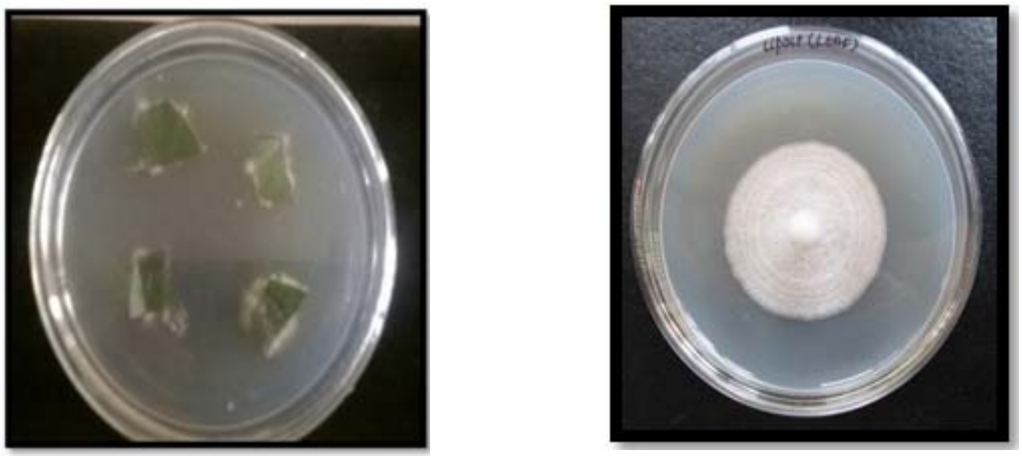
Macroscopic and microscopic characteristics of endophytic fungus isolated from leaf of N. arbor-tristis

The isolated fungal colony from leaf of *N. arbor-tristis* was round, velvety and regular. In the young stage, culture was white and turned gradually into pink background (Table 1) (Fig. 1a and 1b). Characteristic feature of isolated fungus was presence of mycelium with branched and septate hyphae (Fig. 2), with terminal 2-5 spores (conidia) in a cluster (Fig. 3).

The conidium is a specialized non motile asexual propagule usually not developing by cytoplasmic cleavage or free cell formation. It is a character-

Table 1. Macroscopic and microscopic characteristics of isolated endophytic fungi *Xylaria* of *N. arbor- tristis*

Characteristics	Features of isolated endophytic fungus
Growth rate	3-5 days
Growth media	Potato dextrose media (PDA)
Colonies	Round, velvety, regular
Color	Young culture is white
Background	White Pink
Mycelia pattern	Branched, septate
Types of spores	Conidia on conidiophores in the cluster of 2-5, apical in position



(a) **(b)**

Fig. 1(a), 1(b): Isolatedendophytic fungal colony of *Xylaria* from leaf of *N.arbor-tristis*

istic feature of Ascomycetes [11].

Fig. 2. Microscopic characteristics of isolated *Xylaria*from leaf *N.arbor-tristis*

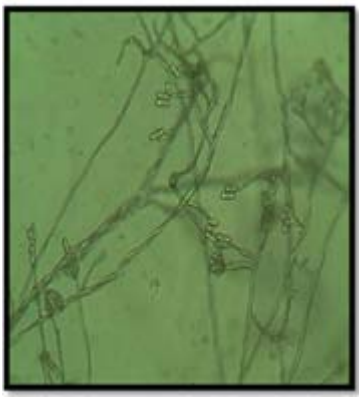


Fig. 3. Mycelium of *Xylaria* with terminal 2-5 spores (conidia) in a cluster

Identification of isolated fungus

On the basis of following gene sequence, result of ITS sequencing (carried out at the microbial culture collection, National Centre for Cell Science (NCCS), Pune), the isolated fungus was identified as *Xylaria* sp. The fungus was also isolated from an orchid (*Anoectochilus setaceus*) [12] and from *Coffea arabica* [13] as an endophyte .

>D_SEP_14_061(*Xylaria* sp.) TTATTGATA TGCTTAAGTTCAGCGGGTATTCCTACC TGATCCGAGGTCAACCTTGATAAATTAGG GGTTTTACGGCAGGGGACCGGTCCAACT AATAGGCGAGATAATATTTACTACGTCTAGA GTGTGAACCGA CTCCGCC ACTAATTTTAA GGGGCTACCGCCATACGGTAGGCCCCCAA CGCTAAGCAACAGAAGGCTTAAGG GTTGAAATGACGCTCGAACAGGCAT GCC CACTAGAATACTAATGGGCGCAATGTGC GTTCAAAGATTCG ATGATTCACTGAATTC TGCAATTCACATTACTTATCGCATTTCG CTGCGTTCTTCATCGATGCCAGAACCAA GAGATCCGTTGTTGAAAGTTTTAACTTAT TTAGTTATAATTCAGATATCCAGTAAT TAAACAGAGTTTAATGGGGCGCCGGCGG GCTTACCCGTGCCTACCGGGTAGGCACT TACAGGTAA GTG CA ATACAGG GTAGGTA CGACCCGCCGAGGCAACGTTAGGTATG TTCACATGGGGTTTGGGAGTTATAAACTCTT TAATGATCCCTCCGCAGGTTC.

Biomass production of *Xylaria* sp.

Fungal biomass production is a major of metabolite production [14], biomass of isolated fungus was quantified after 6 days of inoculation of in liquid broth of PDB (Potato dextrose broth). A thick fungal mycelia mat was produced. Fresh weight of this mycelia mat was 4.22 g. Biomass (with rate 0.703 g/day) utilized for secondary metabolite production (Fig. 4).

Fig. 4(a). Biomass production from leaf of *N.arbor-tristis*

GC-MS analysis

GC MS is an efficient method for identification of compounds present in the fungal extract. GC-MS chromatogram (Fig. 5) showing presence of 11 compounds identified on different Rt and with different % area (Table 2) from *Xylaria* extract. Percentage area in GC-MS chromatogram is representation of quantity of compound present in the extract. In the present study compound which

Table 2. Characteristics* of compounds from*Xylaria* extract identified through GC-MS

No.	Nameof compound	Chemical formula	RT	Area %	Applications
1	Cis-vaccenic acid	$CH_{34}O_2$	37.959	25.37	Trans-fatty acid in the fat of ruminants and in dairy products [15]
2	E,Z-1,3,12-Nonadecatriene	$C_{19}H_{34}$	46.316	16.50	-
3	Pentadecanoic acid (21)	$C_{15}H_{30}O_2$	34.469	4.62	Adhesives and sealantchemicals, agricultural chemicals [21]
4	(9E,12E)-9,12-octadecadienoyl chloride	C18H31ClO	43.607	4.05	
5	Octadecanoic acid 22	$C_{18}H_{36}O_2$	38.273	2.98	Ester of glycerol, animal and vegetable fats and oils, making candles, soaps, plastics, oil pastels, Lubricants [22]
6	Cyclododecyne	$C_{12}H_{20}$	43.546	1.84	-
7	9,12-Octadecadienoic acid, methyl ester (23)	$C_{19}H_{34}O_2$	37.013	1.33	Quick-drying oils, use in oil paints and varnishes antiinflammatory, moisture retentive [23]
8	9-Octadecenoicacid,methyl ester (24)	$C_{19}H_{36}O_2$	37.118	0.87	Agricultural products and colorant, Lubricants and adsorbents [24]
9	2-aminoethanethiol hydrogen-sulfate (est.)		44.776	0.34	-
10	Hexadec-anoic acid, methyl)	$CH_3(CH_2)_{14}COOH$	33.756	0.19	Boosts metastasis in mouse models of human oral cancer cells [16]
11	2-Hydroxycy-clopentadecanone	$C_{15}H_{28}O$	44.632	0.12	-

*As reported

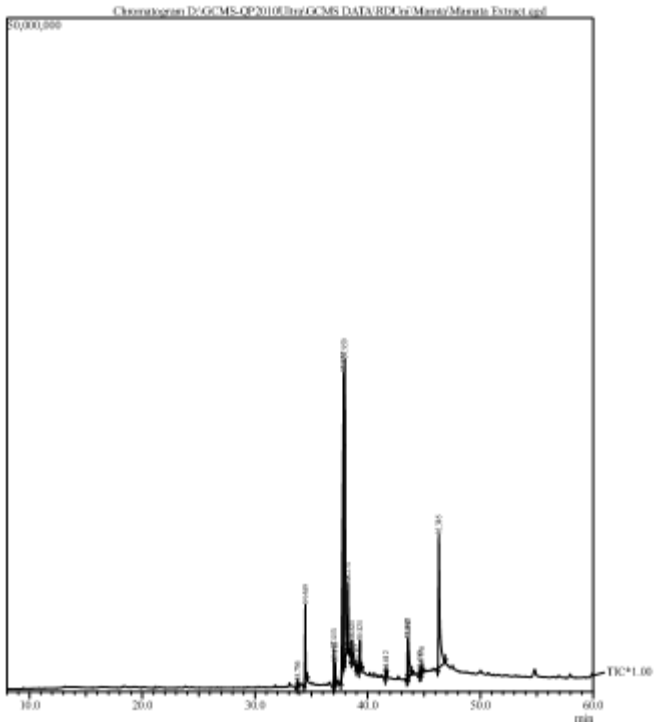


Fig. 5. GC-MS Chromatogram of *Xylaria* sp. extract

was isolated inmaximum quantity (25.37 %) is fatty acid [15]*cis*-Vaccenic acid (at Rt-37.95). Hexadec-anoic acid, which boosts metastasis in mouse models of human oral cancer cells [16], was the important compound isolated at Rt- 33.75 with 0.19 % area in the *Xylaria* extract.

Enzymatic activity of *Xylaria* sp.

The observations after enzymatic study indicated the capability of isolated fungi to produce high amount of lipase enzyme (Fig. 6). Formation of clear zone around the fungal colony was observed on Starch agar media (1.63 cm). This is an indication of high production of amylase enzyme (Fig. 7) (Table 3). *Xylariaceous* fungal taxa have also been known as producers of wood rotting enzymes. Production of xylarinase [17] and cellulose [18] was also reported in different *Xylaria* species. In the present study isolated *Xylaria* sp. is capable to produce amylase and lipase enzymes too.

Antimicrobial activity of secondary metabolite of *Xylaria* sp.

The present work reported the antimicrobial properties of fungal metabolite from leaf of *N.*

Fig. 6. Lipaseactivity of *Xylaria* from leaf of *N. arbor-tristis*

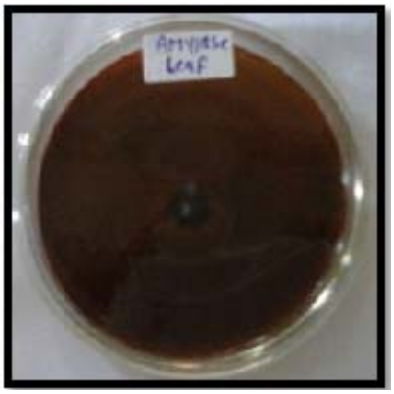


Fig. 7. Amylase activity of *Xylaria* from leaf of *N. arbor-tristis*

Table 3. Enzymatic activity of *Xylaria* of *N. arbor- tristis*

Replicate	Amylase (cm)	Protease (cm)	Lipase (cm)
R_1	1.72	-	2.5
R_2	1.52	-	2.8
R_3	1.77	-	2.2
Mean*	1.67±0.1	-	2.5±0.24

*results are mean of 3 replicates ±SD

arbor-trists. It was observed that the fungal metabolite were capable to inhibit the growth of pathogenic bacteria. The strongest antibacterial activity of fungal metabolite was observed against *Micrococcus luteus* (Fig. 8) (clear zone 68.2 mm). The isolated fungal metabolite forms a clear zone of 59.16 mm against *Citrobacter freundii* (Fig. 9) and of 39.96 mm against *Chromobacterium violaceum* (Fig. 10).

In the present study, it was observed that minimum antibacterial activity was found against *Chromobacterium violaceum*, whereas it was a strong inhibitor of *Micrococcus luteus* (Table 4) (Graph 1). Antimicrobial activities of endophytic *Xylaria* sp. were also reported previously [19, 20].

Acknowledgement

Authors are thankful to Principal, St. Aloysius (Autonomous) College, Jabalpur for providing lab facilities and Director, AIRF, JNU, New Delhi for GC-MS analysis. Corresponding author thanks, DST (WOS-A), New Delhi, for financial assistance. We are also thankful to Dr. Rohit Sharma, Microbial Culture Collection, NCCS, Pune for his help in sequencing and phylogenetic analysis.

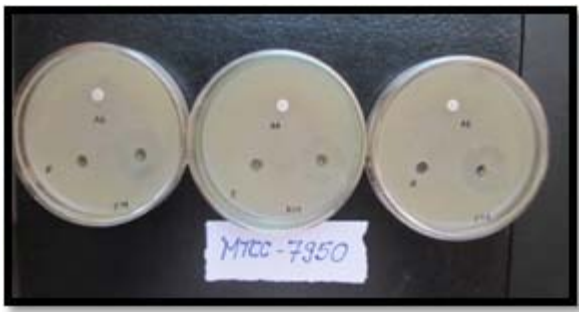


Fig. 8. Antimicrobial activity of fungal metabolite of *N. arbor- tristis* against *Micrococcus luteus*

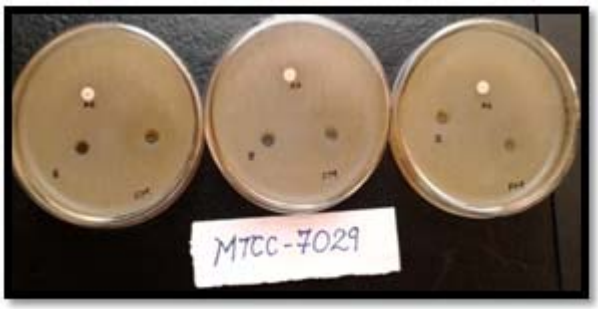


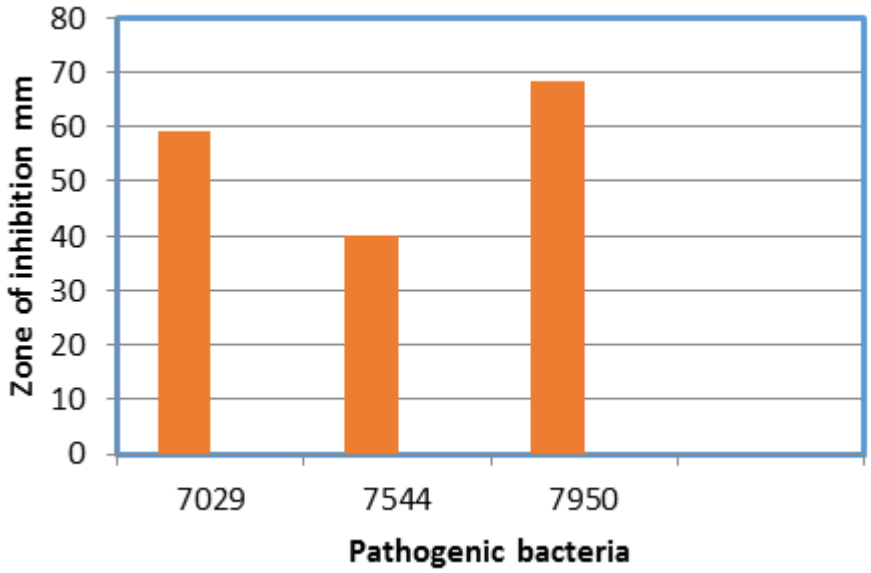
Fig. 9. Antimicrobial activity of fungal metabolite of *N. arbor- tristis* against *Citrobacter freundii*

Fig. 10. Antimicrobial activity of fungal metabolite of *N. arbor- tristis* against *Chromobacterium violaceum*

Table 4. Antimicrobial activity of fungal metabolite of *N. arbor-tristis* against human pathogens

Pathogenic bacterial strain	C	Zone of inhibition: Antibiotic disc mm 1	2	3	M	Isolated fungal metabolite (mm) 1	2	3	M
7029	-	13.3	9.5	3.2	8.6±4.17	65.3	59.5	52.7	59.16±5.15
7950	-	3.2	1.5	3.2	2.63±0.8	59.9	62.6	82.1	68.2±9.89
7544	-	5.1	1.5	1.5	2.7±1.7	37.1	37.1	45.7	39.96±4.02

7029: *Citrobacterfreundii*
7950: *Micrococcus luteus*
7544*: Chromobacteriumviolaceum*
*results are mean of 3 replicates±SD



Graph 1. Antimicrobial activity of secondary metabolite of *Xylaria* sp.

Reference

1. **Bandera, W.M.M.S., Seneviratne, Kulasooriya, S.A. (2006).** Interactions among endophytic bacteria and fungi: effects and potentials. Journal of Bio Science. 31(5): 645-650.

2. **Rungjindamai, N., Pinruan, U., Choeyklin, R., Hattori, T. and Jones, E.B.G. (2008).** Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeisguineensis*, in Thailand, Fungal Diversity. 33: 139-161.
3. **Huang, W.Y., Cai, Y.Z., Hyde, K.D., Corke, H. and Sun, M. (2008).** Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants, Fungal Diversity. 33: 61-75.
4. **Doty, S.L. (2011).** Growth-Promoting Endophytic Fungi of Forest Trees. Forestry Science. 80: 151-6.
5. **Jalgaonwala, R.E., Mohite, B.V., Mahajan, R.T. (2010).** Evaluation of endophytes for their antimicrobial activity from indigenous medicinal plants belonging to North Maharashtra region India. Int. J. Pharma Biomedical Resources. 1: 136-41.
6. **Budhiraja, A., Nepali, K., Sapra, S., Gupta, S., Kumar, S. and Dhâr, K.L. (2013).** Bioactive metabolites from an endophytic fungus of *Aspergillus* species from seed of *Gloriosasuperba* Linn, Med Chem Res. 22: 323-329.
7. **Zhang, Z., Schwartz, S., Wagner, L., Miller, W. (2000).** A greedy algorithm for aligning DNA sequences. J. Comput. Biology. 7: 203-14.
8. **Thompson, J.D., Higgins, D.G., Gibson, T.J., Clustal, W. (1994).** Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gappenalties and weight matrix choice. Nucleic Acids Research. 22: 4673-80.
9. **Tamura, K., Peterson, D., Peterson, N. (2011).** MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Molecular Biology Evolution. 28: 2731-39.
10. **Chen, L.J., David, E.G., Jones, J.N. (2002).** Isolation and identification off our flavonoid constituents from the seeds of *Oroxylumindicum*by high-speedcounter-current chromatography. J. Chromatography A. 988: 95-105.
11. **Ainsworth (1983).** Ainsworth and Bisby's Dictionary of the Fungi. 7th ed. Commonwealth Mycological Institute, Kew, Surrey, England.
12. **Pamoda, B.R., David, E.W., Dilip de Silva, E., Ravi, L.C.W., Doralyn, S.D., Raymond, J. A. (2014).** Helvolic acid, an antibacterial nortriterpenoid from a fungal endophyte, *Xylaria* sp. of orchid *Anoectochilussetaceus* endemic to Sri Lanka.Mycology. 5(1): 23-28.
13. **Saucedo-García, A., Anaya, A.L., Espinosa-García, F.J., González, M.C. (2014).** Diversity and Communities of Foliar Endophytic Fungi from Different Agro-ecosystems of *Coffeaarabica* L. in Two Regions of Veracruz, Mexico. PLoS ONE.9(6):e98454. doi:10.1371/journal. pone. 0098454.
14. **Sibtain Ahmed, Ghulam Mustafa, Muhammad Arshad and Muhammad Ibrahim Rajoka. (2017).** Fungal Biomass Protein Production from *Trichodermaharzianum* Using Rice Polishing BioMed Research International 9:BioMed Research International 2017:https://doi.org/10.1155/ 2017/6232793.
15. **ThiagoBruder-Nascimento, Dijon, H.S.C., Carlos, A., Samuel, T., Antônio, C.C., Sandra, C. (2013).** Effects of chronic stress and high-fat diet on metabolic and nutritional parameters in Wistar rats. Arq Bras EndocrinolMetab São Paulo 57(8): http://dx.doi.org/10.1590/S0004-2730 2013000800010.
16. **Kingsbury, K.J., Paul, S., Crossley, A., Morgan, D.M. (1961).** Composition of human adipose tissue from deep and subcutaneous sites. Biochemical J. 25: 377-380.
17. **Meshram, V., Saxena, S., Paul, K. (2016).** Xylarinase: a novel clot busting enzyme from an endophytic fungus *Xylaria*curta. J. Enzyme Inhib. Med. Chem. 31(6): 1502-11.
18. **Wei, D.L., Chang, S.C., Wei, Y.H., Lin, Y.W., Chuang, C.L. and Jong, S.C. (1992).** Production of cellulolytic enzymes from the *Xylaria* and *Hypoxylon* species of xylariaceae. World J. Microbiology and Biotechnology. 8(2): 141-146.

19. **Liu, X., Dong, M., Chen, X., Jiang, M., Lv, X., Zhou, J. (2008).** Antimicrobial activity of an endophytic Xylaria sp.YX-28 and identification of its antimicrobial compound 7-amino-4-methyl-coumarin. Appl. Microbiol. Biotechnol. 78(2): 241-7.
20. **Orachaipunlap, K., Suwannasai, N., WhalleyAnthony, J.S., Phosri, C., Sihanonth, P. (2016).** Biological activities of endophytic *Xylaria* sp. isolated from tropical forest in Chaiyapoom Province Thailand. Biological and Chemical Research. 3: 200-208.
21. **Hansen, R.P., Shorland, F.B., Cooke, N.J. (1954).** The branched-chain fatty acids of butterfat. IV. The isolation of (+)-12-methyltetradecanoic acid and of 13-methyltetradecanoic acid Biochem. J. 57-297.
22. **Akoh, C.C., Min, D.B. (2008).** In: Food lipids: chemistry, nutrition and biotechnology. CRC Press.
23. **Rogers, D.W., Siddiqui, N.A. (1975).** Heats of hydrogenation of large molecules. I. Esters of unsaturated fatty acids J. Phys. Chem. 79(6): 574-577
24. **Chow Ching, K. (2007).** Fatty acids in foods and their health implication, third edition CRC Press.