RESEARCH ARTICLE

Mycobiology 40(1) : 8-13 (2012)
http://dx.doi.org/10.5941/MYCO.2012.40.1.008

pISSN 1229-8093
eISSN 2092-9323

Diversity and Biological Activities of Endophytic Fungi of *Emblica officinalis*, an Ethnomedicinal Plant of India

Archana Nath[1], Prajwal Raghunatha[2] and S. R. Joshi[1]*

[1]*Microbiology Laboratory, Department of Biotechnology and Bioinformatics, North Eastern Hill University, Shillong 793 022, Meghalaya, India*
[2]*Yuvaraja College, University of Mysore, Mysore 570006, India*

(Received November 2, 2011. Revised December 14, 2011. Accepted March 2, 2012)

In the present study, an attempt to evaluate the antimicrobial and antioxidant activity of fungal endophytes inhabiting *Emblica officinalis* has been made keeping in view the medicinal importance of the selected host plant in Indian traditional practices. A total of four endophytic fungi belonging to Phylum Ascomycetes were isolated from different parts of the plant which were characterized morphologically and by using rDNA-internal transcribed spacer. The most frequently isolated endophyte was *Phomopsis* sp. The antioxidant activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power assay, and total phenol were evaluated using ethanolic extract of endophytic fungi. DPPH activities in all the ethanolic extract increased with the increase in concentrations. Endophytes, *Phomopsis* sp. and *Xylaria* sp. showed highest antioxidant activity and also had the higher levels of phenolics. Antimicrobial activity of fungal extract were tested against four bacteria namely, *Escherichia coli* MTCC730, *Enteroccocus faecalis* MTCC2729, *Salmonella enterica* ser. *paratyphi* MTCC735 and *Streptococcus pyogenes* MTCC1925, and the fungus *Candida albicans* MTCC183. In general, the fungal extracts inhibited the growth of test organisms except *E. coli.*

KEYWORDS : Antimicrobial activity, *Emblica officinalis*, Endophytic fungi, Reducing power assay, 2,2-Diphenyl-1-picrylhydrazyl

Introduction

A variety of relationship exists between the endophytic fungi and the host plants, ranging from mutuality or symbiotic to antagonistic or slightly pathogenic because of what appears to be their contribution to the host [1, 2]. Medicinal plants are reported to harbour endophytes [3, 4] which in turn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions. Metabolites produced by endophytes are being recognized as a versatile arsenal of antimicrobial agents. Endophytic fungi produce a number of substances such as antioxidants, novel antibiotics, antimycotics, immunosuppresants and anti-cancer compounds, and thus rich source of biologically active metabolites that find wide-ranging exploitation in medicine, agriculture, and industry [5, 6].

Investigating the secondary metabolites of micro-organisms from unusual or specialized niche may increase the chances of finding novel compounds, as plant endophytes are more subtle, rarely causing problems, coexisting with their hosts under most circumstances [7]. Several endophyte derived natural products have shown potential as antifungal agents against a variety of plant and human pathogens [8].

Emblica officinalis commonly known as "Amla" is an angiosperm of the order malpighiales and family Phyllanthaceae. The tree is small to medium in size, reaching 8 to 18 m in height, with a crooked trunk and spreading branches. The branchlets are glabrous or finely pubescent, 10~20 cm long, usually deciduous; the leaves are simple, subsessile and closely set along ranchlets, light green, resembling pinnate leaves [9]. The flowers are greenish-yellow. The fruit are nearly spherical, light greenish yellow, quite smooth and hard on appearance, with six vertical stripes or furrows.

Preliminary research of *Emblica officinalis* has demonstrated that it has anabolic, antibacterial, antidiarrhoeal, antidysenteric, expectorant, antispasmodic, antipyretic, antioxidative, antiviral, antiemetic, antihepatotoxic, immunomodulator and resistance building properties. Amla extract possesses anticancer, antisclerotic, lipid lowering, hepatoprotective and anti human immunodeficiency virus activities [10, 11].

*Corresponding author <E-mail : srjoshi2006@yahoo.co.in>

Reactive oxygen species and free radical mediated reactions can cause oxidative damage to biomolecules, eventually contributing to aging, cancer, atherosclerosis, coronary heart ailment, diabetes, Alzheimer's disease and other neurodegenerative disorders [12, 13]. *E. officinalis* has been reported to have many medicinal properties [14] and exploring the plant for endophytic fungi can be of immense value in screening for potential metabolites as was the case with *Taxus brevifoia* which had fungal endophyte that produced metabolites independently of the tree [15].

Materials and Methods

Sampling. Plant material was collected from various parts of Meghalaya, India, based on its ethno-medicinal usages by the ethnic tribals. Plants with no visible symptoms of disease were carefully selected, brought to the laboratory in sterile bags and processed within 24 hr after sampling.

Surface sterilization and Isolation of the endophytic fungi. Isolation of endophytic fungi was done according to the method described by Hallmann *et al.* [16] with minor modifications. The plant samples were rinsed gently in running water to remove adhered dust and debris. Samples were cut into 2 mm segments and were surface sterilized with 70% ethyl alcohol for 1 min, soaked in 4% sodium hypochlorite solution for 3 min, and then rinsed with 70% ethyl alcohol for 1 min. They were finally rinsed with sterile distilled water and blot dried on sterile filter paper. The excess water was dried under laminar airflow chamber. The surface sterilized explants were then inoculated into the Petri dishes containing potato dextrose agar (PDA) (Himedia, Mumbai, India) supplemented with 100 µg/mL of streptomycin and incubated at 25 ± 2°C for 15 days. The plates were periodically observed for fungal growth. The fungus growing out from the plant explants was then subcultured in PDA plates.

Morphological features. The endophytic fungal isolates were tentatively identified microscopically on the basis of their critical morphological structure such as hyphal features, arrangement of spores and reproductive structures.

Molecular characterization of endophytic fungus.

Genomic DNA extraction: Genomic DNA isolation for fungal endophytes in pure cultures was done using the HiPurA fungal DNA isolation kit (Himedia). Samples were stored at 4°C for immediate use and at −20°C for long-term storage.

PCR Amplification and sequencing of the internal transcribed spacer (ITS) region: Fungal rDNA-ITS region was amplified from the extracted genomic DNA by using the fungal domain specific ITS1 and ITS4 [17]. The PCR reaction mixture comprised of 10 µL fungal DNA, 5 µL 10 × PCR buffer, 1.5 µL of 50 mM $MgCl_2$, 1 µL of 10 mM dNTP, 0.25 µL Taq polymerase, 40 pM each of the forward and the reverse primers in a total reaction volume of 50 µL [18]. An initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 52°C for 30 sec, and 72°C for 1 min, with a final extension step of 72°C for 10 min. PCR was performed in a GeneAmp 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The amplification of ITS was confirmed by running the amplification product in 1.5% agarose gel electrophoresis in 1 × Tris-acetate-EDTA with a marker ladder of 100-bp and ethidium bromide staining.

Purification of ITS amplicon. The amplified ITS products were purified from the gel slice using QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction.

Sequencing of ITS genes. For sequencing reaction, Big Dye Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) was employed and sequencing was done in Applied Biosystems 3700 Genetic Analyser.

Sequence and phylogenetic analysis. Sequence analysis was performed with sequences in the NCBI database using BLAST and sequences were aligned by using the Clustal W program and phylogenetic tree was constructed using MEGA 4.1 software. The sequences were deposited to the NCBI database and accession numbers obtained.

Preparation of fungal extract. Each fungus was cultivated on potato dextrose broth by placing agar blocks of actively growing pure culture (3 mm in diameter) in 250 mL Erlenmeyer flask containing 100 mL of the medium. Each flask was incubated at 25 ± 2°C for 3 wk with periodical shaking at 150 rpm. After incubation, the culture filtrate was extracted and filtered through three layers of muslin cloth to remove mycelia. Then the culture filtrate was filtered thrice with equal volumes of solvent ethanol. The organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 45°C using rotary vacuum evaporator. The dry solid residue was re-dissolved in ethanol (2 mg per mL) and evaluated for their antimicrobial and antioxidant activities.

Test microorganisms. Test microorganisms used in the study included *Escherichia coli* MTCC730, *Enteroccocus faecalis* MTCC2729, *Salmonella enterica* ser *paratyphi* MTCC735, *Streptococcus pyogenes* MTCC1925 and *Candida albicans* MTCC183 procured from Institute of

Microbial technology (IMTECH, Chandigarh, India). Test bacteria were subcultured every two weeks on fresh nutrient agar slants and incubated at 37°C, whereas *C. albicans* was subcultured every four wk on fresh PDA slant and incubated at 37°C. All the cultures were kept at 4°C until further use.

Antimicrobial activity by well-diffusion method. The antimicrobial assay using the crude extract was carried out on Mueller Hinton agar medium for test bacteria and PDA for test fungus. One mL of inoculum was swapped on molten agar plates. Using the sterile cork borer 7 mm wells were made in the plates in which 20 µL of crude ethanol fraction was loaded. After incubation for 24 hr at 37°C for bacteria and at 25°C for fungus, the plates were observed for zone of inhibition.

Reducing power assay. The reducing power of the fungal extracts was determined according to Chang *et al.* [19]. An aliquot of 0.5 mL sample extract were taken in a test tube and 0.1 mL of 1% potassium ferricyanide solution was added. The mixture was incubated at 50°C for 30 min. After the incubation, 0.1 mL of 1% trichloroacetic acid and 0.1 mL of 1% ferric chloride solution was added to the mixture and left unattended for 20 min. The absorbance of the reaction mixture was read spectrophotometrically at 700 nm against water blank.

Radical scavenging activity using DPPH method. Different concentrations of the extracts were taken in separate test tubes and the volume was adjusted to 100 µL with ethanol. Five mL of 0.1 mM ethanolic solution of DPPH was added to these tubes. The mixture was shaken vigorously, left to stand for 30 min in the dark, and the absorbance was measured at 517 nm. The percentage inhibition of DPPH radical by the sample extract was expressed as the inhibition concentration (IC50) and was calculated using the following relation:

$$\text{DPPH scavenging effect (\%)} = [(A0 - A1/A0) \times 100],$$

where A0 is the absorbance of the control reaction and A1, the absorbance in presence of the extract.

Estimation of total phenolics content (TPC). The total polyphenolic content was determined calorimetrically following the Folin-Ciocalteau (FC) method according to Singleton *et al.* [20] with minor modifications. 0.5 mL of test sample was mixed with 0.2 mL of FC reagent and allowed to stand for 10 min to which 0.6 mL of 20% sodium carbonate was added and mixed. The reaction mixture was incubated at 40°C for 30 min and absorbance of the reaction mixture was measured at 765 nm with gallic acid taken as standard.

Statistical analysis. All the tests were performed in triplicates. The results are expressed as mean ± SE values and data were tested by one-way analysis of variance. Differences were considered significant at $p < 0.05$.

RESULTS

Isolation and identification. A total of eleven isolates were isolated from roots, stem and leaves of the host plant (Table 1). Based on colony and microscopic features, four isolates exhibiting different morphological features were considered for molecular characterization. The rDNA-ITS region was amplified, the sequence data was then assembled and submitted to the NCBI GenBank with accession Nos. JQ322820, JQ322821, JQ322823, JQ322824. Based on BLAST search of ribosomal RNA gene sequence, the endophytic fungi were found to be closest homolog of *Phomopsis* sp., *Epacris* sp., *Xylaria* sp. and *Diaporthe* sp. (Fig. 1).

Antimicrobial activity. The crude metabolite of the fungi displayed considerable antimicrobial activity against some clinically significant pathogens (Table 2). The crude extracts of two endophytic fungi, *Xylaria* sp. and *Diaporthe* sp. showed effective inhibition of three test strains. However, the endophyte *Epacris* sp. had no effect on all the five test strains. None of the fungal extracts showed inhibition of *E. coli* MTCC730. *C. albicans* MTCC183 was inhibited by the crude extracts of *Xylaria* sp. and *Phomopsis* sp. but the other fungal isolates had no effect on the test fungus.

Reducing power assay. The reducing power of ethanolic extract of the endophytic fungi showed significant activity but was lower as compared to ascorbic acid (Fig. 2). The reducing powers for the metabolites of different endophytes were in the order of *Phomopsis* sp. > *Xylaria* sp. > *Diaporthe* sp. > *Epacris* sp.

Table 1. Total fungal isolates and their presence in different parts of the host plant

Plant parts used	Total isolates observed	*Xylaria* sp. (PR6)	*Diporthe* sp. (PR8)	*Epacris* sp. (AS4)	*Phomopsis* sp. (AR3)
Stem	5	+	–	+	+
Roots	4	+	+	–	+
Leaves	2	–	–	–	+

+, presence of fungal sp. in the particular part of the plant; –, absence of fungal sp. in the particular part of the plant.

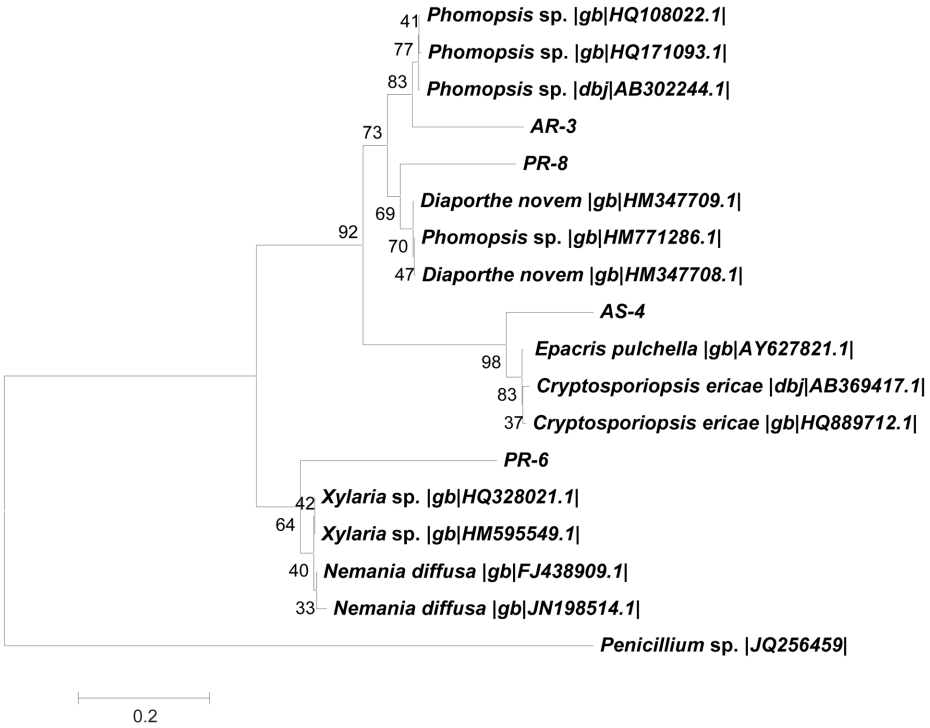

Fig. 1. Evolutionary positions of the four endophytic fungal isolates with other related fungal species based on internal transcribed spacer sequence similarity.

Table 2. Zone of inhibition (mm) of antimicrobial activity from endophytic extract by discdiffusion method

Plant parts used for isolation	Characterized isolates	*Candida albicans*	*Streptococcus pyogenes*	*Salmonella enterica* ser. *paratyphi*	*Escherichia coli*	*Enteroccocus faecalis*
Stem	*Phomopsis* sp. (AR3)	++	–	–	–	–
	Epacris sp. (AS4)	–	–	–	–	–
Root	*Xylaria* sp. (PR6)	+++		+++	–	++
	Diporthe sp. (PR8)	–	+	++	–	++

–, no zone of inhibition; +, inhibition zone between 10~12 mm; ++, inhibition zone between 12~14 mm; +++, inhibition zone between 14~16 mm.

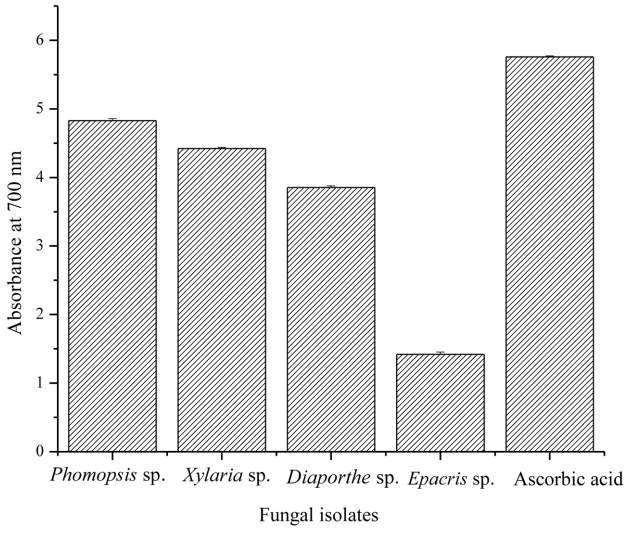

Fig. 2. Reducing power assay of ethanolic extracts of four different endophytic fungi.

DPPH radical scavenging activity. The highest scavenging activity was shown by *Phomopsis* sp., and minimum was by *Epacris* sp. The free radical scavenging activities of the ethanolic extracts were found to be in the order of *Phomopsis* sp. > *Diaporthe* sp. > *Xylaria* sp. > *Epacris* sp. Variation was observed in IC50 values (concentration of sample required to scavenge 50% of free radicals) of ethanolic extract of different endophytic fungi and ascorbic acid (Table 3).

Total phenolic content (TPC). The TPC of endophytic fungal ethanolic extracts have been expressed as gallic acid equivalent *i.e.*, mg gallic acid/g dry wt. A high phenolic content (12.5 ± 0.16) was found in *Xylaria* sp. and decreased in the order of *Xylaria* sp. > *Phomopsis* sp. > *Diaporthe* sp. > *Epacris* sp. (Table 4). Results showed that the levels of phenolic compounds in different endophytic fungi were significantly ($p < 0.05$) different from each other.

Table 3. Scavenging activity (%) on DPPH radicals (IC50) of ethanolic extract of different endophytic fungi at different concentration (μg/mL)

Concentration (μg/mL)	*Phomopsis* sp. (AR3)	*Diporthe* sp. (PR8)	*Xylaria* sp. (PR6)	*Epacris* sp. (AS4)	Ascorbic acid
10	17.36 ± 0.08	18.9 ± 0.06	20.11 ± 0.15	29.4 ± 0.03	15 ± 0.21
25	18.73 ± 0.11	19.53 ± 0.04	23.23 ± 0.26	33.23 ± 0.11	16.05 ± 0.30
50	21.2 ± 0.17	23.4 ± 0.11	26.76 ± 0.15	36.3 ± 0.01	17.75 ± 0.25
75	23 ± 0.11	23.93 ± 0.16	29.7 ± 0.11	39.3 ± 0.13	19.56 ± 0.19
100	23.76 ± 0.20	26.06 ± 0.21	34.1 ± 0.17	42.56 ± 0.09	21.36 ± 0.43

Table 4. Total phenolic content (TPC) of the ethanolic extract of endophytic fungi

Fungal isolates[a]	TPC[b]
Epacris sp. (AS4)	5.08 ± 0.31
Xylaria sp. (PR6)	12.5 ± 0.16
Diporthe sp. (PR8)	8.5 ± 0.18
Phomopsis sp. (AR3)	9.53 ± 0.17

Values are average of three replicates ± SE.

[a]Fungal sp. isolated from different parts of the plant *Emblica officinalis.*

[b]Total phenolic content of the different fungal sp. expressed as mg gallic acid equivalent/g dry wt.

DISCUSSION

Xylaria sp. and *Diaporthe* sp. showed the maximum inhibition of growth of the test microorganisms. *Diaporthe* sp. showed significant growth inhibition of both Gram positive and negative bacteria, but only one isolate, *Xylaria* sp. exhibited both antifungal and antibacterial activity. The crude extract of some fungal endophytes show greater antimicrobial activity compared to others which could be due to their higher efficiency in metabolite production. Vast diversity of microbes remain untapped for structurally diverse metabolites possessing highly valuable bioactivities including antioxidant activity [21]. It has been reported that many antioxidants compounds posses anti-inflammatory, anti-atherosclerotic, antitumor, anti-mutagenic, anticarcinogenic, antibacterial or antiviral properties [22]. In the present study, the crude extracts of endophytic fungi showed significant anti-oxidant activities.

The TPC of the ethanol extract in terms of GAE is indicative of high antioxidant potential of the extract, because the phenolic constituents can react with active oxygen radicals such as lipid peroxy radical [23], hydroxyl radical [24] and superoxide anion radical [25]. In our study, *Xylaria* sp. showed the highest phenolic content indicating good antioxidant potential. Liu *et al*. [26] have reported TPC in the range of 54.51 mg/g in intracellular extract of *Xylaria* sp. which in our case was 12.5 mg/g. The phenolic compounds in the endophytic fungi may have contributed significantly to their antioxidant activity. The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search the *in vitro* general antioxidant activity of pure compounds [27]. The lower the IC50 value obtained, the greater is the free radical-scavenging activity. A freshly prepared DPPH solution exhibited a deep purple color which disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals and convert them to a colorless product, resulting in a decrease in absorbance. The reducing capacity of compounds could serve as indicator of potential antioxidant property [28]. Considering the total phenolics, reducing power and the DPPH radical scavenging activity as indices of antioxidant activity of the extract, these findings reveal the potential of the extract as a source for natural antioxidants. It indicates that the metabolites of endophytic fungi isolated from *E. officinalis* could be potential agents in scavenging free radicals and treating diseases related to free radical reactions. In accordance with Ohwada *et al*. [29], a comparison of both antioxidant and antimicrobial activities in relation to the amount of total phenolics of different natural extracts certified that a moderate correlation exists between the amount of total phenolics and the biological activity. In this context, the crude extracts of the endophytes, *Xylaria* sp. and *Diaporthe* sp. presented a moderate antioxidant activity in the tested concentrations and, overall, a greater antimicrobial activity. The potent antioxidant activity of the ethanolic extract of endophyte supports its possible use as a natural antioxidant in food industries and other pharmaceutical preparations. This work provides an insight into understanding some basis of therapeutic properties of fungal endophytes of *E. officinalis* in traditional medicine. Furthermore, detailed studies on the isolation and characterization of the endophytic fungal metabolites as well as *in vivo* assays will be necessary in discovering new biological antioxidants.

Acknowledgements

AN and SRJ acknowledge the financial support received from DBT, Govt of India to carry out the present work.

References

1. Schulz B, Boyle C. The endophytic continuum. Mycol Res 2005;109(Pt 6):661-86.

2. Arnold AE. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol Rev 2007;21:55-66.
3. Strobel GA. Rainforest endophytes and bioactive products. Crit Rev Biotechnol 2002;22:315-33.
4. Bhagobaty RK, Joshi SR, Kumar R. *Penicillium verruculosum* RS7PF: a root fungal endophyte associated with an ethno-medicinal plant of the indigenous tribes of eastern India. Afr J Microbiol Res 2010;4:766-70.
5. Strobel GA, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003; 67:491-502.
6. Bhagobaty RK, Joshi SR. Metabolite profiling of endophytic fungal isolates of five ethno-pharmacologically important plants of Meghalaya, India. J Metabolomics Syst Biol 2011; 2:20-31.
7. Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D. Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat Prod Rep 2011;28: 1208-28.
8. Verma VC, Kharwar RN, Strobel GA. Chemical and functional diversity of natural products from plant associated endophytic fungi. Nat Prod Commun 2009;4:1511-32.
9. Pathak RK. Status report on genetic resources of Indian Gooseberry-*Aonla* (*Emblica officinalis* Gaertn.) in South and Southeast Asia. New Delhi: International Plant Genetic Resources Institute; 2003.
10. el-Mekkawy S, Meselhy MR, Kusumoto IT, Kadota S, Hattori M, Namba T. Inhibitory effects of Egyptian folk Medicines on human immunodeficiency virus (HIV) reverse transcriptase. Chem Pharm Bull (Tokyo) 1995;43:641-8.
11. Khan MT, Lampronti I, Martello D, Bianchi N, Jabbar S, Choudhuri MS, Datta BK, Gambari R. Identification of pyrogallol as an antiproliferative compound present in extracts from medicinal plant *Emblica officinalis*: effects on *in vitro* cell growth of human tumor cell lines. Int J Oncol 2002; 21:187-92.
12. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994;344:721-4.
13. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000;408:239-47.
14. Nadkarni KM, Nadkarni AK. Indian materia medica: with Ayurvedic, Unani-Tibbi, Siddha, allopathic, homeopathic, naturopathic and home remedies. Vol. 1. Bombay: Popular Prakashan Private Ltd.; 1999.
15. Stierle A, Strobel G, Stierle D. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 1993;260:214-6.
16. Hallmann J, Berg G, Schulz B. Isolation procedures for endophytic microorganisms. New York: Springer Brelin Heidelberg; 2007.
17. White TJ, Bruns T, Lee S, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. New York: Academic Press; 1990. p. 315-22.
18. Mirhendi H, Diba K, Kordbacheh P, Jalalizand N, Makimura K. Identification of pathogenic *Aspergillus* species by a PCR-restriction enzyme method. J Med Microbiol 2007;56(Pt 11): 1568-70.
19. Chang LW, Yen WJ, Huang SC, Duh PD. Antioxidant activity of sesame coat. Food Chem 2002;78:347-54.
20. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol 1999;299:152-78.
21. Motta AS, Cladera-Olivera F, Brandelli A. Screening for antimicrobial activity among bacteria isolated from the Amazon basin. Braz J Microbiol 2004;35:307-10.
22. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci 2004;74:2157-84.
23. Torel J, Cillard J, Cillard P. Antioxidant activity of flavonoids and reactivity with peroxy radical. Phytochemistry 1986;25: 383-5.
24. Husain SR, Cillard J, Cillard P. Hydroxyl radical scavenging activity of flavonoids. Phytochemistry 1987;26:2489-91.
25. Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochem Pharmacol 1989;38:1763-9.
26. Liu X, Dong M, Chen X, Jiang M, Lv X, Yan G. Antioxidant activity and phenolics of an endophytic *Xylaria* sp. from *Ginkgo biloba*. Food Chem 2007;105:548-54.
27. Koleva II, van Beek TA, Linssen JP, de Groot A, Evstatieva LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem Anal 2002;13:8-17.
28. Meir S, Kanner J, Akiri B, Philosoph-Hadas S. Determination and involvement of aqueous reducing compounds in oxidative defense system of various senescing leaves. J Agric Food Chem 1995;43:1813-9.
29. Ohwada J, Tsukazaki M, Hayase T, Oikawa N, Isshiki Y, Fukuda H, Mizyguchi E, Sakaitani M, Shiratori Y, Yamazaki T, et al. Design, synthesis and antifungal activity of a novel water soluble prodrug of antifungal triazole. Bioorg Med Chem Lett 2003;13:191-6.