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# Bioactive metabolites from an endophytic fungus *Penicillium* sp. isolated from *Centella asiatica*

**Devi NN and Prabakaran JJ**

*Department of Biotechnology, K.S.Rangasamy College of Arts and Science, Tiruchengode, Tamil Nadu, India.*email:nnirjan@gmail.com

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**Abstract**

Endophytes are important sources for the discovery of bioactive compounds. The potential role of the endophyte and its biologically active metabolites in its association with its host has been investigated. In the present study the antioxidant activity of the endophytic fungus *Penicillium* sp. isolated from medicinal plant *Centella asiatica* was evaluated by its ability to scavenge DPPH free radicals. Bioactive metabolites present in the ethyl acetate extract from the endophytic fungus *Penicillium* sp. were analysed by using GC-MS. The metabolites were investigated for cytotoxic activity. The extract of *Penicillium* sp. exhibited high antioxidant activity with $IC_{50}$ value, 54.72±2.19µg/ml. The ethyl acetate extract of *Penicillium* sp. has high antioxidant capacity of 325.76±0.14 mg equivalent to ascorbic acid. The ethyl acetate extract of *Penicillium* sp. demonstrated a promising cytotoxic activity against HeLa, A431 and human breast cancer (MCF7). These results indicate that endophytic fungi isolated from medicinal plants could be a potential source for bioactive compounds.

**Keywords** – *Centella asiatica* – DPPH – endophytes – GC-MS – *Penicillium* sp.

## Introduction

Natural products play a major role in the discovery of leads for the development of drugs in the treatment of human diseases. Natural products are an unsurpassed source of bioactive compounds and constitute a relevant economic resource for the pharmaceutical, cosmetic and food industry. Medicinal plants provide a unique environment for endophytes and have been recognized as a repository of endophytes with novel metabolites of pharmaceutical importance (Tan & Zou, 2001, Strobel *et al*. 2004). Medicinal plants and their endophytes are important resources for discovery of natural products. Endophytes are important components of microbial diversity. Endophytes are group of microorganism that resides asymptomatically inside the living plant tissues. Endophytic fungi represent an important and quantifiable component of fungal diversity, and are known to affect plant community diversity and structure (Krings *et al*. 2007). Endophytic fungi isolated from medicinal plants more likely exhibit pharmaceutical potentials. Plant endophytic fungi have been found in each plant species examined, and it is estimated that there are over one million fungal endophytes existed in the nature (Petrini 1991). Endophytes provide a wide

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Corresponding Author: Nameirakpam Nirjanta – e-mail – nnirjan@gmail.com

variety of structurally unique, bioactive natural products. These plentiful natural products represent a huge reservoir which offers an enormous potential for exploitation for medicinal, agricultural and industrial uses (Tan & Zou 2001, Zhang *et al*. 2006). There has been a great interest in endophytic fungi as potential producers of novel, biologically active products (Schulz *et al*. 2002, Strobel & Daisy 2003).The secondary metabolites produced by endophytes associated with medicinal plants can be exploited for curing diseases (Tejesvi *et al*. 2007).More exciting possibilities exist in the wild and unexplored part of the world for discovery of novel endophytes, their biology, and their potential usefulness. In this study, endophytic fungi were isolated and characterised from *Centella asiatica*. *Centella asiatica* is widely distributed in the Indian environment and has been used traditionally for treatment of various diseases. While much is known about the phytochemistry of *Centella asiatica* (L.)Urb, little information is available about its endophyte biology.

## Materials &Methods

### Isolation of endophytic fungi

Healthy and mature *Centella asiatica* were collected from different places in and around Namakkal District, Tamil Nadu and was used for the isolation of endophytic fungi. The plant was taxonomically identified and authenticated by Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore. The voucher specimen was deposited there with register number BSI/SRC/5/23/2011-12/Tech.-932.The fresh plant samples were taken to the laboratory and treated within few hours. Fresh materials were used for isolation of endophytes. A modified procedure was followed for surface sterilization and isolation of endophytic fungi as described by Schulz *et al.* 1993. Colonization Frequency (CF) was calculated as described by Suryanarayanan *et al.* 2003.

$$\text{Colonization frequency of endophyte} = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segment observed}} \times 100$$

### Identification of endophytic fungi

The endophytic fungi were identified based on the morphology of the fungal culture colony or hyphae, the characteristics of the spores (Barnett & Hunter 1998), and the identity of one major fungus was verified with molecular method. For inducing sporulation, each of the isolated fungal strains was separately inoculated on PDA in Petri dishes. Measurements of all fungal characters were made in water mounts, and the slides were subsequently mounted in lactophenol. All experiments and observations were repeated at least thrice.

### Cultivation of endophytic fungi

The fungi were cultured in appropriate media for the production of secondary metabolites. Primarily cultivation was done in small scale to perform bioassays for the detection of bioactive compounds. Both liquid and solid state fermentation was performed for the cultivation of endophytic fungi. Potato Dextrose Agar (PDA) was mainly used to culture the endophytic fungi. Liquid state fermentation was carried out for the production of metabolites. Medium was prepared for the cultivation of endophytic fungi. Two or three cut up of the agar medium (0.5 x 0.5 cm) with fungal cultures were inoculated in each 1000ml Erlenmeyer flask containing 400ml of potato dextrose broth medium, and incubated on a rotary shaker at 150 rpm and 28˚C for 21 to 30 days.

The culture broths were filtered, and the culture media and mycelia were separated. The mycelia were soaked in methanol. Metabolite was extracted by solvent extraction procedure using ethyl acetate as organic solvent. Equal volume of the filtrate and ethyl acetate was taken in a separating funnel and shaken vigorously for 10 min. The samples were extracted three times with ethyl acetate. Ethyl acetate collected after extraction was evaporated and the resultant compound was dried in vacuum evaporator using $MgSO_4$ to yield the crude metabolite. After evaporation, a brown coloured crude extract was obtained. The crude extract was then dissolved in Dimethyl sulphoxide (DMSO) until analysis. The methanolic extract of mycelia was collected after 7 days of

soaking. The organic extracts were blended and concentrated in a vacuum at 35°C. Crude extracts obtained were stored at −20°C until analysis (Choudhary *et al*. 2004).

**DPPH radical scavenging activity**

The free radical scavenging capacity of the plant and fungal extracts was determined using the DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay as described by (Braca *et al.* 2001) with a slight modification. Tested sample was mixed with 95 % methanol to prepare the stock solution (5mg/mL). One milliliter of DPPH (0.25mM) in methanol was taken in tubes and 2.0mL solution of various concentrations of plant extract was added. The reaction mixture was then allowed to stand at room temperature in a dark chamber for 30 min. The change in colour from deep violet to light yellow was then measured at 518 nm on a spectrophotometer. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (5mg/mL). Ninety-five percent methanol served as blank. Percentage scavenging of the DPPH free radical was measured by using the following equation:

$$\%\text{Scavenging Activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100$$

**Evaluation of total antioxidant activity by Phosphomolybdenum method**

The total antioxidant capacity of the fungal extracts was evaluated according to the method described by Prieto *et al*. 1999. An aliquot of 0.5mL of samples solution was combined with 4.5mL of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). Methanol (0.3mL) in the place of extract is used as the blank. The tubes were incubated in a boiling water bath at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each sample was measured at 695nm against blank in ELICO SL 159 UV-VIS Spectrophotometer. The total antioxidant activity is expressed as the number of gram equivalents of ascorbic acid. The higher absorbance value indicated higher antioxidant activity.

**Statistical analysis**

The experimental results were expressed as mean ± standard deviation of three replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA) and the differences between samples were determined by Duncan's Multiple Range test using the SPSS programme. P-value of $<0.05$ was regarded as highly significant.

**Cytotoxic Activity**

**Bioassay:** Anti cancer activity of endophytic fungi secondary metabolite was measured based on its cytotoxic effect. The cell lines HepG2 cells, MCF7 cells, A549 cells, A431 and HeLa cells obtained from the National Centre for Cell Science (NCCS), University of Pune Campus, Pune were used for the study. The tumour cells were maintained in the Minimum essential medium (Eagle's) supplemented with 2Mm L-glutamine and Earle's BSS adjusted to contain 1.5g/L Sodiumbicarbonate, 10% fetal calf serum and 100μg/mL streptomycin (Sigma). The cells were incubated at 37°C in an atmosphere of 5% $CO_2$ and 95% air with more than 95% humidity. 1000 μl of each cell suspension were added into each of the 24 multi-well plate followed by the addition of 10μL ethyl acetate extract of endophytic fungi metabolite and methanolic plant extract at three different concentrations 2.5, 5 and 10μg $mL^{-1}$ and incubated for 48 h. As a negative control, cells were treated with only Eagle's MEM with 10μL ethyl extract for endophytic fungi and methanol for plant sample. Each treatment was done in triplicates. The viable cells were counted in a haemocytometer using the trypan blue exclusion method (Kumala *et al*. 2006). Cytotoxic assay other than the MTT assay were used in this study, because direct cell counting by haemocytometer is one of the simplest method, inexpensive, accurate measure of cell number to assess the cytotoxic effect of secondary metabolites of endophytic fungi.

To calculate $IC_{50}$ values of secondary metabolites in ethyl acetate extract from endophytic fungi *Penicillium* sp. isolate, following formula was used:

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells per ml of aliquot}}{\text{Total number of cells per ml of aliquot}} \times 100$$

**Detection of bioactive compounds**

The extracts obtained were subjected to instrumental analysis such as, FTIR and GC-MS to identify the bioactive compounds present in the fungal extracts. The instrumental conditions and the compounds detected by various instruments were discussed below.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The crude extracts of endophytic fungus *Penicillium* sp. was analysed by FTIR (FTIR BRUKER ALPHA-E) to know the different functional groups present in the fungal extracts. The dried crude extract was subjected to analyze in FTIR in which the diffuse reflectance technique was followed. The crude dried sample was mixed with potassium bromide (KBr) to form a very fine powder. This powder was then compressed into a thin pellet which was analysed. KBr was also transparent in infra red light. The samples were irradiated by a broad spectrum of infra red light and the level of absorbance at a particular frequency was plotted after Fourier transformation of the data. The resulting spectrum was characteristic of the organic molecule present in the sample. The absorbance was measured at 400-600 nm for the identification and quantification of organic species. Compounds contained in the extracts were identified according to established criteria of Nyquist and Kagel (1997) and Socrates (2001), respectively.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The purified ethyl acetate extract of *Penicillium* sp. was subjected to GC- MS analysis to identify the bioactive compounds. The sample was analysed in Hewlett-Packard 5890 gas chromatograph equipped with HP 5972 MSD detector. The sample was introduced via an all-glass injector working in the split mode, with Helium as the carrier gas with a linear velocity of 32 cm/s. The HP-5 fused silica capillary column (Length – 30 m; Film thickness- 25 µm I.D - 0.2 mm) was used. The temperature program was as follows: 80- 240$^{o}$C at 8 deg/ min; 240-300$^{o}$C at 12 deg/ min and a 20 min hold at 300$^{o}$C. The identification of components was accomplished using computer searches in commercial libraries.

**Results**

**Isolation and identification**

The endophytic fungal diversity in medicinal plant *Centella asiatica* was studied to evaluate the production of bioactive compounds. The plant was taxonomically identified and authenticated by Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore. The voucher specimen was deposited there with register number BSI/SRC/5/23/2011-12/Tech.-932. In addition to the morphological characterization, molecular methods were carried out to confirm the identification of most promising endophytic fungal strain CA-01 isolated from the *C. asiatica* (L). Conidiophores erect, usually unbranched, septate, at the apex with a verticil of erect primary branches, each with a verticil of secondary and sometimes tertiary branch lets or with a vertical of conidia- bearing cells borne directly on the slightly inflated apex of the conidiophores, sometimes with secondary conidiophores borne on the apex of the main conidiophores. Conidia borne in chains which typically form a brush-like head. Conidia globose, ovate, or elliptical, smooth or rough. It was identified as *Penicillium* sp. The fungal sequence was submitted in National Centre for Biotechnology Information with accession number HM068965 with the name *Penicillium* sp. nirjan22.

**DPPH radical scavenging activity**

DPPH is a relatively stable free radical and had been widely used to evaluate the antioxidant activities of various biological samples. This method is based on the reduction of DPPH in the presence of a radical scavenger or hydrogen donors due to the formation of non radical form of DPPH-H. The antioxidant activity of the endophytic fungal culture *Penicillium* sp. was evaluated by its ability to scavenge DPPH free radicals. The radical scavenging activity of the compounds can be measured by the decolorizing effect following the trapping of the unpaired electrons of DPPH. The crude extract of *Penicillium* sp. exhibited high antioxidant activity with $IC_{50}$ value, 54.72±2.19µg/ml compared to the $IC_{50}$ value of ascorbic acid, 50.00±0.98µg/ml. BHT and ascorbic acid were used as positive controls. The inhibition % was found to be 70% for endophytic fungus *Penicillium* sp.

**Total antioxidant activity**

Total antioxidant capacity of the endophytic fungus, *Penicillium* sp. was expressed as the number of gram equivalent of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximum absorption at 695nm. The ethyl acetate extract of *Penicillium* sp. has high antioxidant capacity of 325.76±0.14mg equivalent to ascorbic acid than the plant extract with 298.98±0.14mg equivalent to ascorbic acid.

**Cytotoxic assay**

The cytotoxic activities ($IC_{50}$) of *Penicillium* sp. extract against five cancer cell lines were investigated. To assess the effect of ethyl acetate extract on cancer cell proliferation, a simple assay by cell counting method was used. The ethyl acetate extract of *Penicillium* sp. demonstrated a promising activity against HeLa, A431 and human breast cancer (MCF7) (95, 125 and 275µg/mL respectively), while that for HepG2 and A549 the $IC_{50}$ was not cytotoxic at the tested concentrations (up to 1,000µg/mL). Thus, the isolate may have potential as antitumour drugs and require further study.

**FTIR analysis**

The FTIR spectral analysis of the ethyl acetate crude extracts of *Penicillium* sp. nirjan22 showed the major peaks with intensity of 1658.84$cm^{-1}$ (Strong, stretch, C=O functional group ), 1257.63$cm^{-1}$ (strong, stretch, C-F group) respectively. The minor peaks in the FTIR spectrum were observed with the intensity of 3356.25$cm^{-1}$ (strong, broad, stretch, H-bonded, O-H group), 1033.88 $cm^{-1}$ (strong, stretch, C-F group), 918.85$cm^{-1}$ (Strong, bending, =C-H group), 825.56$cm^{-1}$ (Strong, bending, =C-H group), 763.83$cm^{-1}$(Strong, bending, =C-H group), 640.39$cm^{-1}$ (strong, stretch, C-Cl group), 555.52$cm^{-1}$ (strong, stretch, C-BR), 524.66$cm^{-1}$ (strong, stretch, C-BR) respectively as in Fig. 1.

**GC – MS analysis**

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The volatile compounds were tentatively identified on the basis of the NIST data base by virtue of comparisons made of the actual mass spectral data acquired on each compound to the data base. The chromatogram and the spectral analysis along with the name, molecular weight and structure of the components of the endophytic fungus *Penicillium* sp. were ascertained below in Fig. 2. Chemical structure of compounds present in ethyl acetate extract for *Penicillium* sp. are

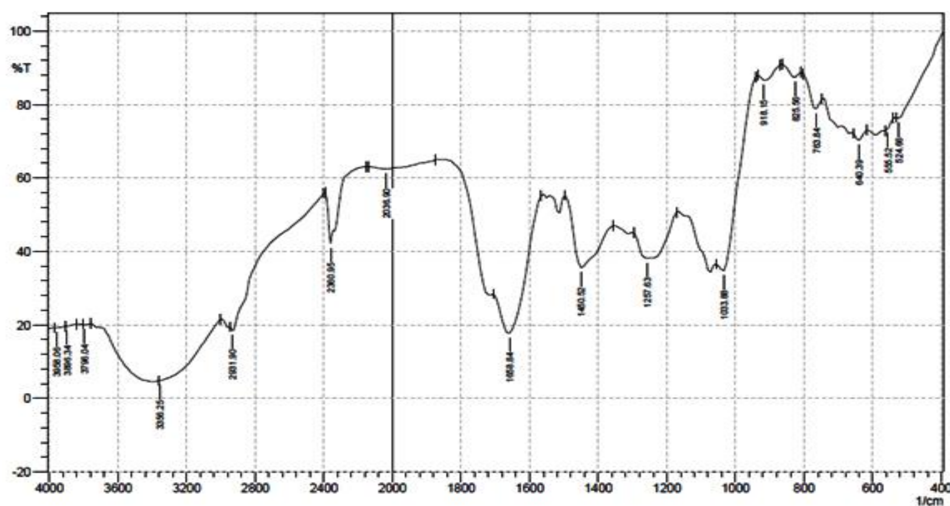


**Fig. 1**– FTIR results for ethyl acetate extract of *Penicillium*sp.

Benzeneethanol 4-hydroxy, 2-tert-Butyl-4-Isopropyl-1-5 methylphenol, Benzoic acid 4- hydroxyl-propyl ester, p-hydroxyphenylacetamide, N-[2-Methyl-1-prenylpropyl] formamide, Cyclo(L-Leucyl-L-Propyl), 3-(3-azidopropyl)-1H-indene and Dihydroergotamine (Fig.2).

## Discussion

Fungal endophytes have also been recognized as a warehouse of novel secondary metabolites, some of which have valuable biological activities (Bills & Polishook 1991). Endophytes are presumably ubiquitous in plants, with populations dependent on host species and location (Tan & Zou, 2001). As the endophytic fungi are a good source for bioactive compounds and a great demand arises for new drugs, there arises a need to exploit the endophytic fungi associated with medicinal plants. The present work analyzed fungal endophytes found in leaves. The leaves were selected for this study because, according to Arnold *et al*. (2000), they are especially rich and abundant. Most studies have focused on endophytes that colonize leaves.

*Penicillium* sp. is a major target of endophytic microorganism research, and many metabolites with cytotoxic and antibacterial effects have been isolated from this genus (Rukachaisirikul *et al*. 2007, Ge *et al*. 2008). In this study *Penicillium* sp. having cytotoxic activity was isolated from *C. asiatica.* Antioxidants are compounds that inhibit or delay the oxidation process by preventing the initiation or propagation of oxidizing chain reactions. DPPH radical scavenging assay is a swift and sensitive method for the antioxidant activity. A number of methods are available for the determination of free radical scavenging activity but the assay of using the stable 2, 2- diphenyl-1-picryl-hydrazyl radical (DPPH) has received the utmost attention owing to the ease of use and its convenience (Moreno *et al*. 1998).

In this study, DPPH radical scavenging and total antioxidant assay were respectively used to determine the radical scavenging and electron donating abilities of fungal extracts. The phosphomolybdate method has been used routinely to evaluate the total antioxidant capacity of plant extracts (Prieto *et al*. 1999). It is evident from the results that the fungal extract contains promising radical scavenging. Total antioxidant activities are found to be high in the endophytic fungus *Penicillium* sp. The crude extract of *Penicillium* sp. exhibited high antioxidant activity with IC50 value, 54.72±2.19µg/ml compared to the IC50 value of ascorbic acid, 50.00±0.98µg/ml. Even though the DPPH scavenging aptitude of the extracts was found to be lower than that of the commercial antioxidant, ascorbic acid, it still reached 70% inhibition at 200µg/ml concentration. Thus this study suggests that the *Penicillium* sp. has highlighted the potentiality of cytotoxic and also it is a persuasive resource of natural antioxidants.

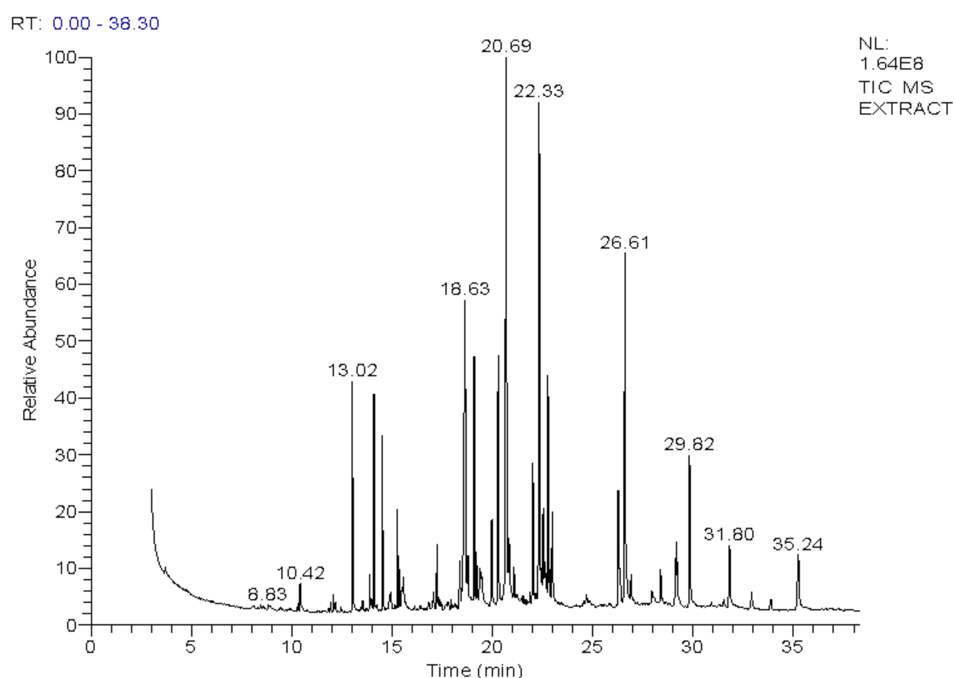


**Fig. 2** – GC/MS Chromatogram of volatile compounds from *Penicillium*sp. extract.

There is some previous research on the antioxidant activity of endophytic fungi from other medicinal plants. Strobel *et al*. (2002) & Harper *et al*. (2003) obtained two antioxidants, pestacin and isopestacin, from the endophytic fungus *Pestalotiopsis microspora*. The endophytic fungi residing in *Nerium oleander* were shown to have excellent antioxidant capacity and preliminary identification detected phenolics and volatile and aliphatic compounds by Huang *et al*. (2007). The results of this study are similar to those in previous reports and indicate that endophytic fungi may serve as a potential source of antioxidants.

In this study, *Penicillium* sp. exhibited cytotoxicity against HeLa, A431 and MCF7 cancer cell line. The activity was low against HepG2 and negligible against A549 cell line. Previous reports are available about the cytotoxic components isolated form endophytic *Penicillium* sp. (Ge *et al*., 2008). The metabolites of endophytic fungus *Penicillium* sp. from the leaf of *Hopea hainanensis* were reported to exhibit cytotoxic activity against HepG2 cell line (Wang *et al*., 2008). Cytotoxic activity of compounds against HeLa and HepG2 cell lines were evaluated by the strain *Penicillium* sp.P-1, an endophyte from the stems of *Huperzia serrata* (Ying *et al*. 2011). Mangrove endophytic fungus *Penicillium* sp. (ZH16) was found to exhibit cytotoxic activity against KB cell (Huang *et al*. 2012).

Many workers have demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicidal, bactericidal and cytotoxic metabolites (Wang *et al*. 2007). Cyclo (L-leucyl-L-prolyl) was detected from the endophytic fungal *Penicillium* sp. extract by GC-MS analysis. *Streptomyces* sp. KH-614 and *Aspergillus parasiticus* have been reported to produce Cyclo(L-leucyl-L-prolyl) that exhibit antimicrobial and anticancer activity. Cyclic dipeptides are known to have antiviral, antibiotic and antitumor properties (Rhee 2002, 2004, Yan *et al*. 2004). Two species of *Penicillium* were isolated from leaves of *Alibertia macrophylla* (Rubiaceae). *Penicillium* sp. 1 cultivated in potato-dextrose-broth furnished two different compounds, cyclo-(L-Pro-L-Val) and uracil (Oliveira *et al*. 2009).

The antibacterial constituents from the fermentation broth of endophytic fungus *Penicillium* sp. 0935030 from mangrove plant *Acrostichum aureurm* consist of cyclic dipeptide cyclo (Pro-Thr) and cyclo (Pro-Tyr). It showed inhibitory effect on *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (Cui *et al*. 2008). The extract of endophytes *Rahnella aquatilis* isolated from *Emilia sonchifolia*, Taiwanese herbal plants was found to produced two cyclic peptides: cyclo-Pro-Val (4) and cyclo-Pro-Phe (Hsieh *et al*. 2009).

Chemical investigations of the fermentation broth from the microfungus *Xylaria* sp. have afforded the new natural product 3-chloro-4- hydroxyphenylacetamide (Davis *et al*. 2005).Fungi in the genera *Epichloë* and *Neotyphodium*, which live as endophytic symbionts in grasses (Clay *et al*. 2002, Flieger *et al*.1997). Several *Penicillium* spp., also likely derived from ascomycete ancestors in the Eurotiales, also have been reported to produce ergot alkaloids (Coyle *et al.* 2005, Panaccione *et al*. 2005). In the result Ergotamine, an ergot alkaloid is one product obtained from *Penicillium* sp. which is confirmed by GC/MS analysis. The ergot alkaloids are the second group of amine and amide alkaloids discovered in cultures of *Neotyphodium* endophytes, all are being characterised previously from ergot sclerotia (Tan & Zou 2001).

Previous study has revealed that 2-tert-butyl-5-methylphenol and 4-Hydroxybenzoic acid exhibited high antimicrobial activity (Si *et al*., 2006). Highly substituted benzoic acid derivative were isolated from the ethyl acetate culture extract of a fungal endophyte, *Scytalidium* sp. (Krohn *et al*. 2004). Report from this study supports the growing evidence that bioactive compounds produced by fungal endophytes may not only be involved in the host-endophyte relationship, but may also ultimately have applicability in other industries also. Accordingly, because of their role in conferring plants the ability to adapt to stress conditions, and because they are proven or perceived sources of secondary metabolites with pharmaceutical importance, the study of fungal endophytes is expected to become an important component of fungal biology (Maheshwari, 2006). Endophytic fungus can be exploited for the bioactive compound. Endophytes are present in almost all plant species and have been recognized as a potential source of novel medicinal compounds. From this work we can conclude that the endophytic fungi have wide variety of bioactive compounds.

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