



ISSN No: 0975-7384
CODEN(USA): JCPRC5

J. Chem. Pharm. Res., 2011, 3(5):218-225

Antioxidant activity and phytochemical analysis of endophytic fungi isolated from *Lobelia nicotianifolia*

Nitya. K. Murthy, K. C. Pushpalatha* and Chandrashekhar G. Joshi

Department of Biochemistry, Mangalore University, Cauvery Campus, Madikeri, Karnataka, India

ABSTRACT

*The aim of the present study was to isolate endophytic fungi from the medicinal plant, *Lobelia nicotianifolia* (Lobeliaceae) and to evaluate the antioxidant potential of the methanolic extracts of the fungi. *Fusarium*, *Aspergillus*, *Penicillium* and *Mucor* species were isolated from *L. nicotianifolia* and subjected to antioxidant activity by DPPH method, total antioxidant potential by phosphomolybic acid method and total phenolic contents by Folin's Ciocaltue method. All the extracts showed significant antioxidant potential and the antioxidant nature of the extracts were dependent on the concentration. Phytochemical analysis showed the presence of various secondary metabolites including flavonoids. There was a positive correlation between the phenolic content and the antioxidant capacity of the endophyte extracts. These studies confirm the medicinal values of the endophytic fungi.*

Keywords: *Lobelia nicotianifolia*, antioxidant activity, methanol extract, phenolic compounds.

INTRODUCTION

Reactive oxygen /nitrogen species (ROS/RNS) produced during the cellular metabolism are essential for cell signaling, apoptosis, gene expression and ion transportation. However, ROS can cause oxidative stress if accumulated in the body in excess amount. The consequence of accumulation of ROS includes the damage of DNA, RNA, proteins and lipids resulting in the inhibition of their normal functions. The abnormal functioning of these biomolecules can enhance the risk for cardiovascular disease, cancer, autism and other diseases [1,2]. Therefore, minimizing oxidative stress will promote our physical condition and prevent some degenerative diseases in which free radicals are involved [3].

A myriad of both natural and synthetic antioxidants has been advised for use in the treatment of various human maladies [4]. Some synthetic antioxidant compounds like butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylhydroquinone commonly used in processed foods. However, synthetic antioxidants have shown potential health risks and toxicity, most notably possible carcinogenicity. Therefore; it is of great importance to find new sources of safe and inexpensive antioxidants of natural origin in order to use them in foods and pharmaceutical preparations to replace synthetic antioxidants [3, 5, 6].

Endophytes are symbiotically associated microorganisms of living plants and potential sources of biologically active natural products useful in medical, agricultural and industrial applications. They have been found in every plant species studied, and it is approximated to be around a million or more endophytic fungi in nature. There are hardly any studies have been carried out to on the plants and their relation to endophytic biology. Therefore, there is an ample opportunity to unearth a novel and interesting endophytic microorganisms with significant therapeutic efficacy [7]. Selection of appropriate higher plants, study and isolation of microfloral components can be achieved by superior understanding of the mechanism of endophyte's existence and their interactions with the surroundings. This procedure may help in unrevealing the new natural product. Although the attempts to capitalize on this immense wealth for therapeutic purpose have just begun, it has already proved to be of profound value for organism, product and utilitarian discovery. However, the extent of utilization of the endophytic microorganisms for food and health industries is still modest, compared to the ample number of useful microorganisms [8].

Lobelia nicotianifolia (Lobeliaceae) a tall, erect, much branched, somewhat hairy herb, which grows to 1.5-3 m in height. The leaves, resembling those of tobacco, are narrowly obovate-lanceolate, while the upper ones gradually become smaller. The flowers are large, white, and borne in terminal racemes 30-50 cm long. It is used in India to treat bronchitis, asthma, and insect and scorpion bites and to induce nausea and vomiting.

The present study was undertaken to isolate endophytic fungi from the leaves of *L. nicotianifolia* and to extract secondary metabolites in methanol. The methanol extract was subjected for antioxidant activity. This study showed the potential antioxidant nature of the methanol extracts of endophytic fungi and the positive correlation between phenolic content of the extracts to their antioxidant activity.

EXPERIMENTAL SECTION

Drugs and chemicals: 1,1-Diphenyl-2-picryl hydrazyl (DPPH) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Dimethylsulfoxide (DMSO) was from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Sodium carbonate (Na_2CO_3), tannic acid, ascorbic acid and Folin-Ciocalteu were from Merck (India) Ltd, India. All other chemicals used in the study were obtained commercially and were of analytical grade.

Location and study area: Mercara is located at 12.42° N and 75.73°E. It has an elevation of 1525 meters above sea level. It lies in western ghat regions of Karnataka. Temperature ranges

from 8.6° C in January to 35° C in May. Humidity ranges from 20%-97%, average rainfall of 2840.2mm and wind speed is 1m-60m/sec.

Isolation of endophytes: Healthy leaves of *Lobelia nicotianifolia* (Lobeliaceae) was collected in the month of April 2011 and the samples were processed within 24h following collection. The surface sterilization was carried out as explained by Petrini *et al* (1992)[8]. The collected sample is washed thoroughly with tap water. Surface sterilization was done by immersing the sample in 70% ethanol for 3min, 0.5% sodium hypochlorite for 30sec and 70% ethanol for 2min, rinsed with sterile water and dried on sterile filter paper. The outer tissue was removed and inner tissue of 0.5cm was placed on PDA plates supplemented with 100mg/L streptomycin.

Cultivation and extraction of fungal metabolites: Fungal endophytes were cultivated on 100ml potato dextrose broth and incubated at room temperature for 4-5 days with periodic shaking. After incubation, the culture was filtered through cheesecloth. The fungal metabolites were extracted by solvent extraction method. Equal volume of methanol and filtrate was taken in the separating funnel and shaken vigorously for 10min. The solution is allowed to stand and the cell mass separated and solvent was evaporated to obtain crude methanolic extract.

DPPH radical scavenging assay: To 2ml of DPPH (100 µM) solution, 100 µl of various concentrations of the extracts or the standard (ascorbic acid) solution were added separately. The reaction mixtures were incubated at 37 °C for 30 min. Absorbance of each solution was measured at 517 nm using methanol as blank reference [9]. DPPH scavenging activity (%) of the standard and extracts were determined using following formula,

$$\text{DPPH Scavenging effect (\%)} = \left(\frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100$$

Total Phenolic Contents: The total phenolic compounds in the extract were determined according to the method of Singleton and Rossi as cited in Çoruh *et al.* (2007)[10] with some modifications. To 1.0ml of methanolic extracts, 2ml of 2% (w/v) sodium carbonate solution was added and vortexed vigorously. After 5min, 0.1 ml of 1:1 diluted Folin Ciocalteu's phenol reagent was added and vortexed again. Same procedure was followed for the standard solution of tannic acid (0.05-0.3mg/ml). All the tubes were incubated at room temperature for 30min and the absorbance was measured at 750nm. The total phenolic content in the extracts were expressed as tannic acid equivalent in mg/g (TA mg/g).

Total antioxidant activity: Samples or standard (1ml) was mixed with 2ml reagent solution (ammonium molybdate{4mM}, sodium phosphate{28mM} and sulphuric acid({0.6M})). All the reaction mixtures were incubated at 30°C for 60min. The absorbance was measured at 665nm. Reducing capacity of the extract has been expressed as the ascorbic acid equivalents [11].

Phytochemical screening: Preliminary phytochemical screening of the crude extracts of the aerial parts was carried out with the methods with little modifications [12].

Statistical analysis: All experiments were performed in triplicate (n=3) and results were expressed as mean \pm SEM. Statistical analysis was carried out with (Prism package version 3.0) using ANOVA (P<0.05).

RESULTS AND DISCUSSION

Previous studies have reported about the various therapeutic efficacies of the crude extracts from culture broth of endophytic microorganisms [13]. However, there are no reports on the medicinal properties on the endophytes of *L. nicotianifolia*. In the present investigation, four strains of fungi isolated from the leaves of *L. nicotianifolia*. They are *Aspergillus*, *Mucor*, *Penicillium* and *Fusarium* species (Fig 1). Methanolic extracts of these strains were subjected to *in vitro* antioxidant studies by DPPH method. In this method, all the four extracts showed significant (p< 0.05) antioxidant activity and the activity was found to be concentration dependant.

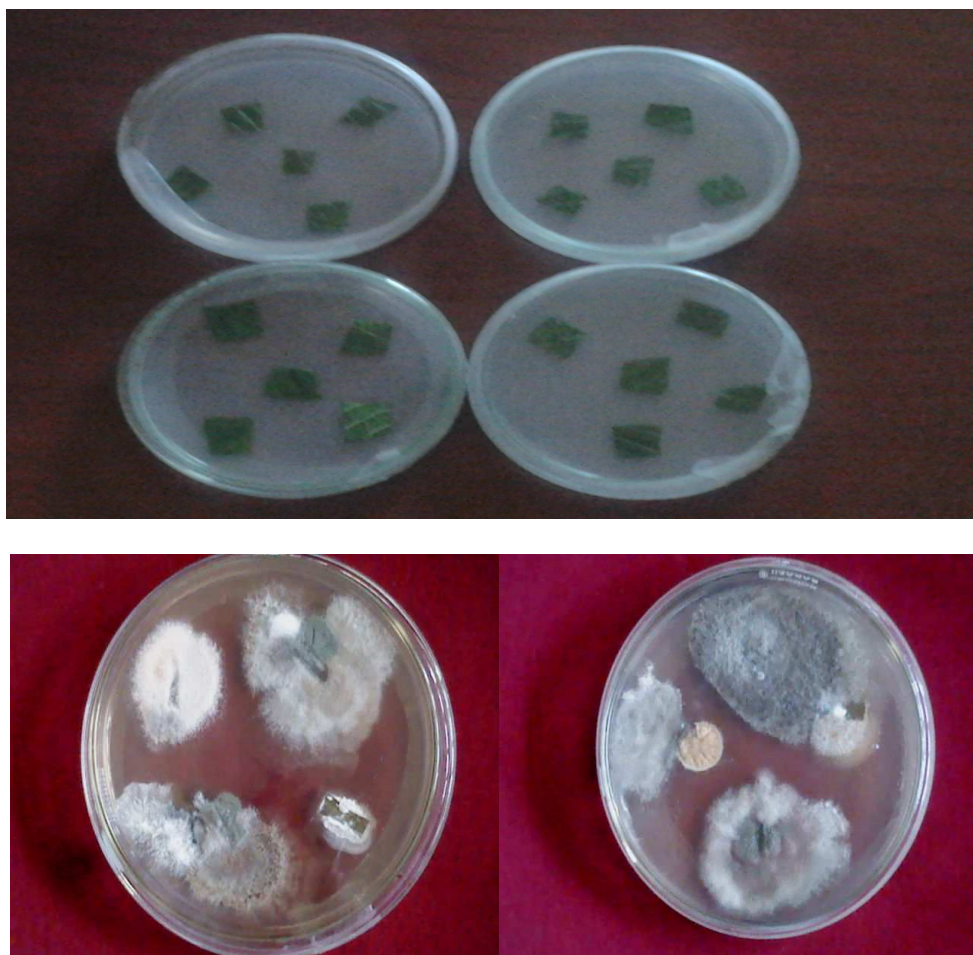


Fig 1. a) 1st day inoculation, b) emergence of endophytic fungi from the inoculated leaves of *Lobelia nicotianifolia*

Fusarium extract showed highest antioxidant potential ($160 \pm 1.21 \mu\text{g/ml}$) and the *penicillium* extract was least active ($320 \pm 3.72 \mu\text{g/ml}$) among the four endophytes. Though the antioxidant potential of these crude extracts (Fig.2 and Table.1) were lesser than standard ascorbic acid, the

studies have established the proton donating ability of the extracts as well as the capacity of these crude drugs to serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Free radicals generated during the course of metabolic process of an organism are known to play role in several disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases due to weak natural antioxidant defense mechanism. Antioxidant principles present in the plants have been shown to possess free radical scavenging activity. DPPH assay is one of the most widely used methods for screening antioxidant activity of natural products [14]. DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow colored product, diphenylpicryl hydrazine, with the addition of the fractions in a concentration-dependent manner. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. The significant DPPH scavenging potential of endopytic fungal extracts may be due to hydroxyl groups present in the extracts.

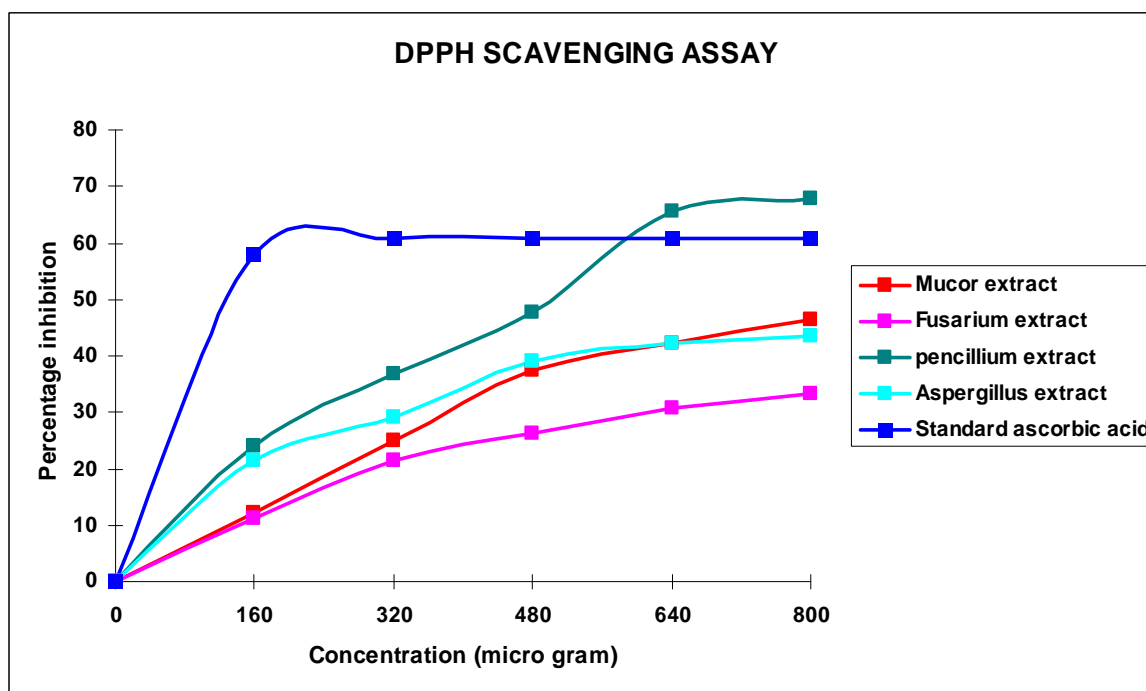


Fig.2 Antioxidant activity of methanol extract of endophtytic fungi.

Table No.1 –IC₅₀ values of Different fungal extracts to scavenge the DPPH radical.

Samples	IC ₅₀ values (µg/ml)
Ascorbic acid	6.5 ± 0.16
Fusarium extract	160 ± 1.21
Mucor extract	240 ± 2.33
Aspergillus extract	220 ± 1.07
Penicillium s extract	320 ± 3.72

Values are mean ± sem

Total antioxidant activities of the extracts were evaluated by phosphomolybdate method. The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of the extracts [13]. In the presence of the extracts, the Mo (VI) is reduced to Mo (V) and forms a green coloured phosphomolybdenum V complex which shows maximum absorbance at 695 nm. All the fractions showed significantly higher inhibition percentage {(stronger hydrogen – donating ability)(Fig.3)}. Aspergillus extract showed highest total antioxidant activity (25mg equivalents of ascorbic acid) followed by mucor(20mg AA equivalents), penicillium (15mg AA equivalents) and fusarium extract.

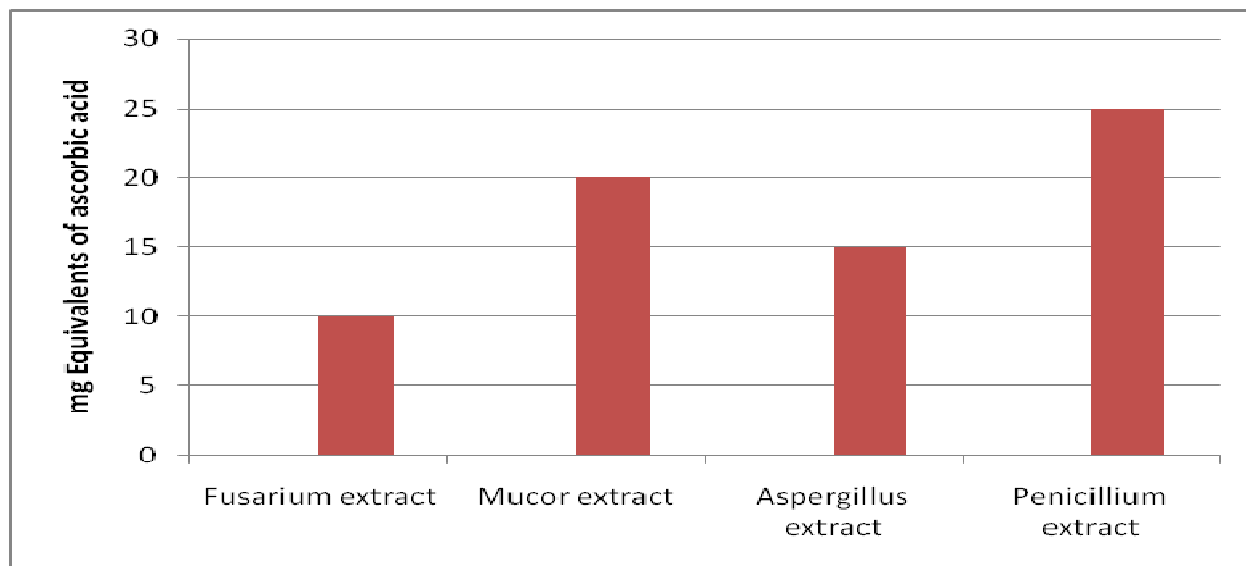


Fig.3 Total antioxidant activity of endophytic fungal extracts

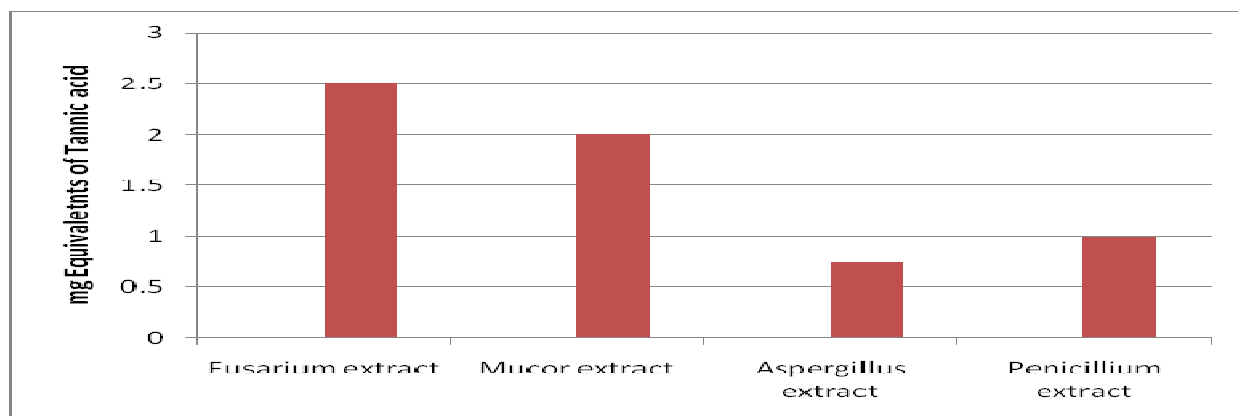


Fig.4 Total phenolic contents of endophytic fungal extracts

Phenolic content in the methanolic extracts showed a direct relationship between the antioxidant activities with phenolic content [15]. Fusarium extract showed highest amount of phenolic compounds(2.5mg equivalents of tannic acid) compared to other extracts(Fig.4) Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and

also decrease cardiovascular complications. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Total phenolic assay by using Folin-Ciocalteu reagent is a simple, convenient and reproducible method. It is employed routinely in studying phenolic antioxidants [16, 17]. The antioxidant capacities and total phenolic contents of endophytic fungi present in the leaves of *L. nicotianifolia* were evaluated for the first time and the study showed positive correlation between the phenol content of the extracts with their antioxidant. Many researchers have reported a positive relation between the phenolic contents to antioxidant activity [17, 18]. According to Huang and coworkers [19], phenolic content were the major antioxidant constituents of the endophytes. This is in agreement with the results reported by us in endophytic fungal extracts. Phytochemical analysis of the fungal extracts showed the presence of various secondary metabolites including flavonoids. These phytochemicals may be acting as antioxidants. Further studies are going on in our laboratory to find out the lead molecules involved in the antioxidant activity as well the mechanism of action.

Table .2 phytochemical analysis of Endophytic extracts.

Fungi	Saponin	Carbohydrates	Phenolics	Glycosides	Flavonoids
Aspergillus	-	+	-	+	-
Penicillium	-	-	+	+	-
Fusarium	-	-	-	+	+
Mucor	+	-	+	-	+

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