Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 2, 2012

Research Article

ANTIOXIDANT ACTIVITY OF FUNGAL ENDOPHYTES ISOLATED FROM SALVADORA OLEOIDES DECNE

[1]SEEMA DHANKHAR, [1]SANDEEP KUMAR, [2]SANDEEP DHANKHAR, [1*]JAYA PARKASH YADAV

[*1]Department of Genetics, Maharshi Dayanand University, Rohtak, Haryana, India. [2]Centre for Biotechnology, Maharshi Dayanand University, Rohtak, Haryana, India. Email: yadav1964@rediffmail.com.

Received: 23 Nov 2011, Revised and Accepted: 11 Feb 2012

ABSTRACT

A total of 3750 tissue segments (leaf, petiole and stem) of a medicinal plant, *Salvadora oleoides* from five different collection sites of Haryana, India were processed for isolation of fungal endophytes and 27 species belonging to 18 fungal genera were recovered. Seventeen culturable fungal endophytes were extracted with the three solvents in the order of polarity; (acetone, methanol and water). Crude extracts were screened for antioxidant activities by six potential assays, out of which four fungal endophyte (*Aspergillus sp.*JPY2*, Aspergillus sp.*JPY1, *Penicillium chrysogenum, Phoma sp.*) extracts have shown positive activity. The antioxidant potencies of acetonic extracts of all fungi were found to be superior to the methanolic and aqueous extract. The scavenging activities of the extracts were in a concentration dependent fashion. The *Phoma sp.* acetonic extract showed super oxide radical scavenging activity with IC_{50} value of 953µg/ml. The crude extracts here showed the moderate reducing power and ferrous ion chelating activity. Preliminary phytochemical screening of these four fungal endophytes acetonic, methanolic and water extracts revealed the presence of alkaloids, flavanoids, saponins, carbohydrates, tannins, sterols and terpenoids. Moreover, our results indicate that the acetonic extract of *Aspergillus sp.*JPY1 *and Phoma sp.* have good margin of safety and did not shown any lethal effects on the animals up to the doses of 1000mg/kg. This is the first report on antioxidant activities of endophytic fungi isolated from *S. oleoides*, an endangered species.

Keywords: *Salvadora oleoides*, Fungal endophytes, Antioxidant and Crude extracts.

INTRODUCTION

Antioxidants have become the topic of interest recently. Antioxidants act as radical scavengers, and inhibit lipid peroxidation and other free radical mediated processes; therefore, these are able to protect the human body from several diseases attributed to the reaction of radicals. Use of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects thus necessitating the search for natural antioxidants and free radical scavengers[1]. Clinical trials and epidemiological studies have established an inverse correlation between the intakes of fruits and vegetables and the occurrence of diseases such as inflammation, cardiovascular disease, cancer and age related disorders[2]. Dietary antioxidants, including polyphenolic compounds, vitamin E and C are believed to be the effective nutrients in the prevention of these oxidative stress related diseases[3].

It appears that all higher plants are hosts to one more endophytic microbes but one of the least studied biochemical systems in nature is the relationship between organisms and their plant hosts[4]. Endophytes are fungi or bacteria residing inside healthy plant tissues without any discernible infectious symptoms[5]. This group of microorganisms were poorly investigated group; they represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in a wide variety of medicinal, agriculture and industrial areas[6]. Globally, there are at least one million species of endophytic fungi in all plants[7], which can potentially provide a variety of structurally unique, bioactive natural products such as alkaloid, benzopyranones, chinones, flavanoids, phenols, steroids, tetralones, xanthones and others[5].

As part of ongoing efforts towards finding novel antioxidant from natural resources, we investigated the antioxidant potential of fungal endophytes associated with a traditional medicinal plant, *Salvadora oleoides*. This plant is an endangered (regional vulnerable) economic plant commonly known in India as meetha jaal[7]. It is oil yielding medicinal and multipurpose tree[8]. The leaves of *Salvadora oleoides* are used to relieve cough and for treatment of enlarged spleen and fever. The leaves of *S. Oleoides* are said to possess anti-inflammatory, analgesic and antiulcer activity[9]. However, the endophytes of this plant and their medicinal values have not been investigated.

We have isolated the fungal endophytes associated with *S. oleoides*. The leaves, petiole and stem samples were sampled thrice in the season from five different districts of Haryana. 27 different fungal species were recorded and out of these 17 culturable fungal endophytes were evaluated for antioxidant activities by different tests that have been important in assessing the antioxidant activity. Furthermore, we estimated the preliminary phytochemical screening of these extracts of fungal endophytes. This research will be helpful for searching new effective antioxidants from the fungal endophytes associated with this medicinal plant.

MATERIALS AND METHODS

Collection of *S. oleoides* Samples

Fresh and healthy leaves and stem samples of *S. oleoides* plants were collected from five different sites: site-1(Jhajjar district), site-2(Rohtak district), site-3 (Rewari district), site-4 (Bhiwani district) and site-5(Mahendergarh district) located in the southern belt of Haryana state (27°37' to 30°35' N latitude and between 74°28' and 77°36' E longitude), India. A total of 25 trees were examined, from each tree 10 leaves, 10 petioles and 10 stem samples were analyzed. Collections were made during summer (April to June), rainy (July to September) and winter season (December to February) from each site in 2008-2010. The collected samples were placed in sterile plastic bags and returned to the laboratory on the same day and kept at 4ºC until the next morning for the isolation of endophytes.

Isolation and Extract Preparation of Fungal Endophytes

Endophytes were isolated from various plant tissues following protocol[10] of Fisher et al. (1993) with some minor modifications. In brief: plant specimens were thoroughly washed in running tap water. All samples were surface sterilized by dipping in 75% ethanol for 1 minute, followed by 5% sodium hypochlorite for 3 minutes, and finally 75% ethanol for 30 seconds. The Surface sterilized samples were washed thrice with sterile distilled water. The surface sterilized samples leaf and stem were cut into small segments. The leaf was cut into three pieces apical, middle and basal parts of size 7 mm × 5mm and the stem was cut into 5mm pieces. The plant segments were then transferred to Potato Dextrose Agar (PDA) Petri plates amended with 150 mg/L streptomycin to inhibit bacterial growth. Plates were incubated at 26±1ºC for a week.

The endophytes were mass cultured on PDB and the mycelium were collected and dried. The dried powdered materials (100g) were then extracted with 1000ml of organic solvent (acetone, methanol and water) by using cold percolation for 24 hours. The obtained extract

was then filtered by using Whatman No. 1 filter paper and then concentrated under vacuum at 40^{0}C by using a rotary evaporator. The extract was then lyophilized (Allied Frost lyophilizer) to powdered form at -55^{0}C under vacuum conditions.

Identification of the Fungal Endophytes

Pure cultures were examined periodically. Fungal identification methods were based on the morphology of the fungal culture colony or hyphae and the characteristics of the spore. Temporary mounts of the fungi were made in lacto phenol cotton blue. The fungi were identified using relevant keys and taxonomic notes from various standard manuals. Moreover, the species of *Aspergillus* and *Penicillium* were identified by growing on Czapek's Dox agar for clear resolution. However, some of the fungal isolates were not distinctly identified by morphology so some of the fungal endophytes were identified molecularly using nuclear ribosomal DNA sequences including both (ITS1 and ITS4). DNA was extracted using Fungal DNA kit (Nucleospin). Purified DNA was subjected to PCR amplification using primers ITS1 and ITS4 which produce amplicons of approximately 500-600 bp of ITS region. The sequence data were analyzed using the BLAST software available at the National Centre of Biotechnology Information (NCBI) website to determine their identity. The phylogenetic trees were constructed using BLAST pair wise alignments in MEGA4.

Antioxidant Potential Assays

Reducing Power Assay

The reductive potential of the extract was determined according to the method of Oyaizu (1986)[11]. Different concentrations of extracts and standard (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1% w/v) was added to the mixture, which was then centrifuged for 10 minutes at 1000g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1% w/v) and the absorbance was measured at 700 nm in a spectrophotometer. High absorbance value of the reaction mixture indicates greater reductive potential.

Metal Chelating Activity

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis et al., (1994)[12]. To investigate 0.5 ml of extract, 1.6 ml of deionised water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30s, 0.1 ml ferrozine (5 mM) was added. Ferrozine react with the divalent iron to form stable magenta complex species that were soluble in water. Then after 10 min at room temperature, the absorbance of the Fe^{2+} – Ferrozine complex was measured at 562 nm. All test and analyses were done in triplicate and the percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below.

PI = $A_{(Control)} - A_{(Sample\ or\ Standard)} / A_{(Control)} \times 100$.

Where $A_{(Control)}$ = Absorbance of control reaction

$A_{(Sample\ or\ Standard)}$ = Absorbance of sample extract or standard

Superoxide-Radical Scavenging Assay

The scavenging activity of extracts towards superoxide anion radicals was measured by the method of Liu et al., (1997)[13]. The superoxide anions were generated in a non- enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 mL of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The super oxide anion scavenging activity was calculated according to the following equation:

Scavenging effect % = $A_{(Control)} - A_{(Sample\ or\ Standard)} / A_{(Control)} \times 100$.

Where $A_{(Control)}$ = Absorbance of control reaction

$A_{(Sample\ or\ Standard)}$ = Absorbance of sample extract or standard

Hydroxyl Radical Assay

The hydroxyl radical scavenging activity was measured by the deoxyribose method[14, 15]. The reaction mixture which contained extract in different concentrations (0.2 mg/ml to 1.0 mg/ml), deoxyribose (3.75 mM), H_2O_2 (1 mM), Potassium phosphate buffer (20 mM, pH 7.4), $FeCl_3$ (0.1 mM), EDTA (0.1 mM) and ascorbic acid (0.1 mM), was incubated in a water bath at 37±0.5^{0}C for 1 hour. The extent of deoxyribose degradation was measured by the TBA (Thio-barbituric acid) method[16]. Then, 1 ml of TBA (0.1% w/v) and 1 ml of TCA (2.8% w/v) were added to the mixture and heated in a water bath at 100^{0}C for 20 min. The absorbance of the resulting solution was measured at 532 nm. All the analysis was done in triplicates and the Percent Inhibition (PI) of deoxyribose degradation was calculated according to the equation.

PI = $A_{(Control)} - A_{(Sample\ or\ Standard)} / A_{(Control)} \times 100$.

Where $A_{(Control)}$ = Absorbance of control reaction

$A_{(Sample\ or\ Standard)}$ = Absorbance of sample extract or standard

Nitric Oxide Radical Scavenging Activity

Nitric oxide radical inhibition was estimated by the use of Griess Illosvoy reaction[17]. In this investigation, Griess Illosvoy reagent was generally modified by using naphthyl ethylene diamine dihydrochloride (0.1 % w/v) instead of the use of 1- naphthylamine (5 %). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and the extract (0.2-1.0 mg/ml) were incubated at 25^{o}C for 150 minutes. After incubation, 0.5 ml of the reaction mixture mixed with 1 ml sulfanilic acid reagent (0.33 % in 20 % glacial acetic acid) and allowed to stand for 5 min for completion of the reaction process of diazotization. Further, 1 ml of the naphthyl ethylene diamine dihydrochoride was added, mixed and was allowed to stand for 30 min at 25^{o}C. The absorbance was taken at 546 nm. Ascorbic acid was used as a standard. The percent inhibition (PI) was calculated using the formula.

PI = $A_{(Control)} - A_{(Sample\ or\ Standard)} / A_{(Control)} \times 100$.

Where $A_{(Control)}$ = Absorbance of control reaction

$A_{(Sample\ or\ Standard)}$ = Absorbance of sample extract or standard

β-Carotene -linoleic acid (linoleate) Assay

The antioxidative activity of extract was evaluated using a β-carotene-linoleic acid model system[18]. In briefly, 1 ml of β-carotene (0.2 mg/ml) dissolved in the chloroform was took in to a small round-bottom flask. After removing the chloroform by using a rotary evaporator, 20 mg of linoleic acid, 200 mg of Tween-40 and 50 ml of aerated distilled water were added to the flask with vigorous stirring. Then, 5 ml aliquot of the prepared emulsion were transferred to a series of tubes containing (0.2-1.0 mg/ml) the sample extract. Here, ascorbic acid was used as a positive control. The test systems were placed in the water bath at 50^{0}C for 2 hours. The reaction was performed in triplicates and absorbance was measured using the spectrophotometer at 470 nm, immediately after sample preparation (t=0 min) and then at the end of experiment (t=120 min). The antioxidant activity of the extracts under investigation was expressed as:

% AA = $100[1 - (A_{1(t=0)} - A_{1(t=120)}) / (A_{0(t=0)} - A_{0(t=120)})]$

Where, % AA = Antioxidant activity of the sample extract

$A_{1(t=0)}$ = Absorbance of the test sample/standard at zero time.

$A_{1(t=120)}$ = Absorbance of the test sample/standard after 120 min.

$A_{0(t=0)}$ = Absorbance of the aqueous control sample at zero time.

$A_{0(t=120)}$ = Absorbance of the aqueous control sample after 120 min

Preliminary Phytochemical Screening

Extracts obtained from fungal endophytes were subjected to various tests for the identification of various bioactive constituents present in these species[19, 20].

Acute Toxicity Studies

All the four fungal extracts at the range 100mg to 1000mg/kg were administered orally to the groups of rats comprised six rats in each group. Mortality and general behaviour was observed for 7 days.

Statistical Analysis

All the data are expressed as mean ± S.D. Data were analyzed by an analysis of variance ($P<0.05$) and the means separated by Duncan's multiple range test.

RESULTS

S. oleoides supports wide and diverse population of endophytic fungi. Out of 3750 samples (leaf segments, petiole and stem) from five different sites in three seasons (summer, rainy and winter) were examined, a total 27 species were recovered representing 18 fungal genera. 24 fungal isolates were identified up to species level morphologically; two fungal species were identified up to the genus level based on fungal sequences with the known identities in GenBank (accession number provided by the Genbank). Phylogenetic trees were constructed and analysis indicated that sequences of *Aspergillus* sp. JPY1 (JN900247) and *Aspergillus* sp. JPY2 (JN900248) are closely related to *Aspergillus* sp. (Figure-1, Figure-2).

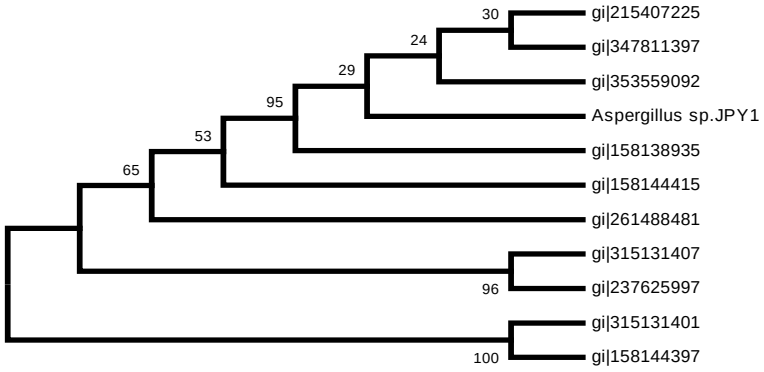

Fig. 1: The evolutionary history was inferred using neighbor - joining method.

The bootstrap consensus tree inferred from thousand replicates. Branches corresponding to the partitions reproduced in less than 50% bootstrap are collapsed. The percentage of replicates trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary tree was computed using the Kimura2- parameter method and are in the units of the number of base substitution per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). Phylogenetic analyses were conducted in MEGA4. *Aspergillus sp.*JPY1 is the fungal endophyte in the present study.

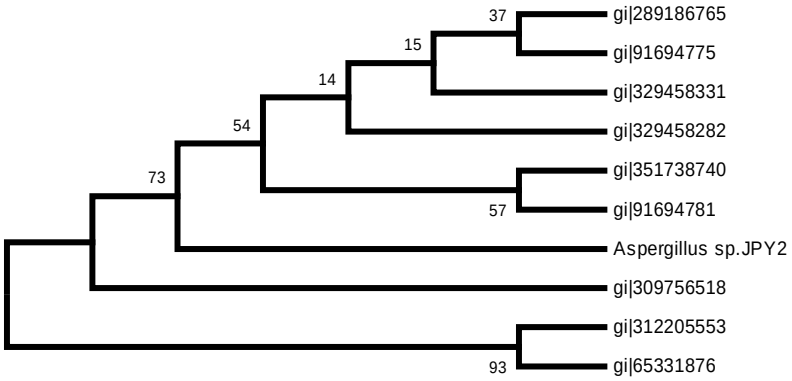

Fig. 2: The evolutionary history was inferred using neighbor - joining method.

The bootstrap consensus tree inferred from thousand replicates. Branches corresponding to the partitions reproduced in less than 50% bootstrap are collapsed. The percentage of replicates trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary tree was computed using the Kimura2- parameter method and are in the units of the number of base substitution per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). Phylogenetic analyses were conducted in MEGA4. *Aspergillus sp.*JPY2 is the fungal endophyte in the present study.

Scavenging Effect on Superoxide and Hydroxyl Radicals

The superoxide radical generated in a PMS/NADH and assayed by the reduction of NBT. As shown in Figure-3, the extracts scavenged superoxide radical in a concentration dependent fashion. At a concentration of 1.0 mg/ml, as much as 51.2% of superoxide radical were scavenged by the acetonic extract of *Phoma sp.*, the IC_{50} value of this extracts for inhibiting superoxide radical was close to be 954 µg/ml. The IC_{50} of BHT as positive control detected in the same experimental procedure was 300ug/ml. The hydroxyl radical scavenging activity of the methanolic, acetonic and aqueous extracts was assessed using fenton reaction (Smith et al 1992). All the extracts exhibited very low scavenging activity on hydroxyl radical. The minimum scavenging activity was found in *P. chrysogenum* as depicted in Figure-4.

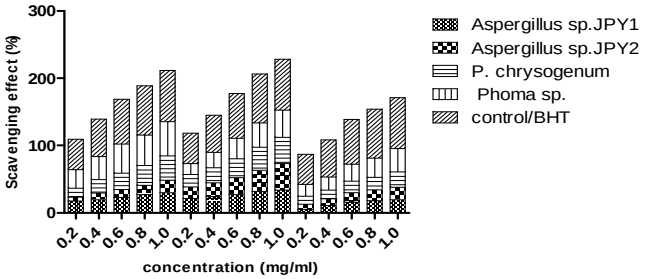

Fig. 3: **Superoxide Radical Scavenging Activity (in Percent) of different solvent extracts of different fungi**.

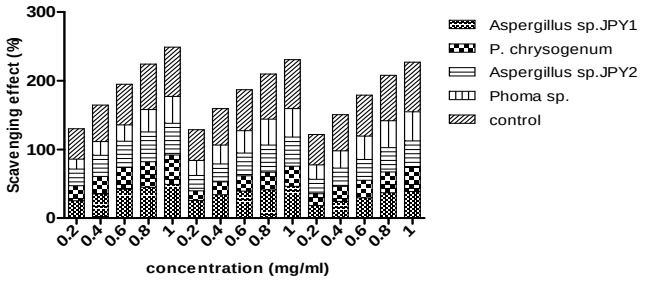

Fig. 4: Hydroxyl Radical Scavenging Activity (in Percent) of different solvent extracts of different fungi.

Reducing Power Assay

The reductive potential of the extracts were in a dose dependent manner. The percentage inhibition of the ferrozine-Fe^{2+} complex formation by the acetonic extract of *Phoma sp*. was found maximum at 1 mg/ml. Whereas that all other extracts of fungal endophyte have shown comparatively less activity (Figure-5).

Metal Chelating Activity

The extracts exhibited moderate chelating activity to Fe^{2+}. As shown in Figure-6, 1.0 mg/ml of EDTA chelated almost 71.8% Fe^{2+} whereas the *Aspergillus sp.* JPY1 extract such as acetonic, methanolic and aqueous chelated only 49%, 41.5% and 38.6% of Fe^{2+} at a concentration of 1mg/ml respectively.

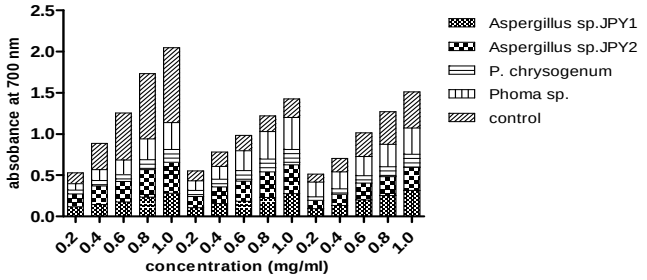

Fig. 5: **Reducing Power Activity of different solvent extracts of different fungi.**

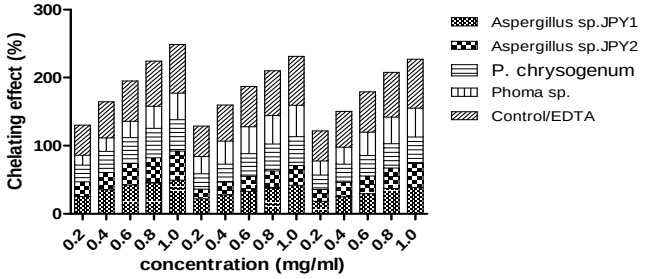

Fig. 6: Metal Chelating Activity (in Percent) of different solvent extracts of different fungi.s

Nitric Oxide Radical Scavenging Activity

The nitric oxide radical scavenging activity was observed maximum in the following order: *Aspergillus sp.* JPY1 (acetone) (49.1%) > *Aspergillus sp.* JPY2 (methanol) (46.4%)>. *Aspergillus sp.* JPY1 (methanol) (46.2%) (Figure-7).

β-Carotene- Linoleic Acid Assay

In this assay, the efficacies of the fungal extract possess inhibition in a range 8.49% to 35.8% from concentration range 0.2 mg/ml to 1.0 mg/ml as shown in Figure-8.

Preliminary Phytochemical Screening

Preliminary phytochemical screening of these four fungal endophytes acetonic, methanolic and water extract revealed the presence of alkaloids, flavanoids, saponins, carbohydrates, tannins, sterols and terpenoids (Table-1).

Acute toxicity studies

All the extracts treated rats showed no discernible behavioural changes up to 1000mg/kg by oral route. No mortality was observed at this dose during 7 days observation period.

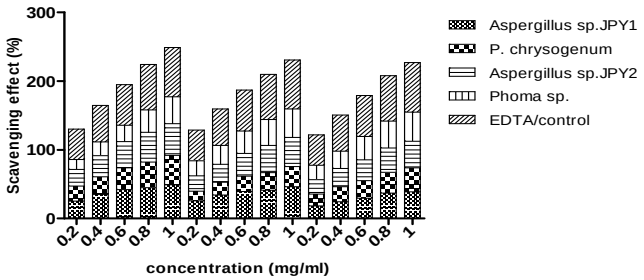

Fig. 7: Nitric Oxide Radical Scavenging Activity (in Percent) of different solvent extracts of different fungi.

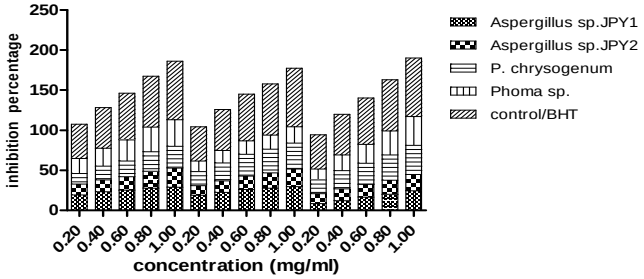

Fig. 8: Beta Carotene Linoleic Acid Activity (in Percent) of different solvent extracts of different fungi.

(In all the figures from 3 to 8 on concentration axis 0.2 to 1 mg/ml first is acetonic, second is methanolic and third is aqueous extract).

Table 1: Phytochemical present in the fungal endophyte extracts.

Fungal endophyte	Solvent	Phytochemical Present
*Aspergillus sp.*JPY1	water	Tannin, Terpenoid, Flavanoids, Carbohydrates
	methanol	Saponin, Terpenoid, Alkaloids, Carbohydrates
	acetone	Tannin, Terpenoid, Flavanoids, Phenols, Alkaloids, Carbohydrates
*Aspergillus sp.*JPY2	water	Tannin, Terpenoid, Carbohydrates
	methanol	Saponin, Terpenoid, Alkaloids, Carbohydrates
	acetone	Tannin, Terpenoid, Flavanoids, Phenols, Carbohydrates
Penicillium chrysogenum	water	Tannin, Terpenoid, Carbohydrates
	methanol	Alkaloids, Carbohydrates
	acetone	Tannin, Alkaloids, Carbohydrates
Phoma sp.	water	Tannin, Terpenoid, Carbohydrates
	methanol	Alkaloids, Carbohydrates
	acetone	Alkaloids, Phenols, Carbohydrates

DISCUSSION

All the tested extracts of the endophytes possessed some scavenging activity to a certain extent. The results obtained in the study indicate that *Aspergillus sp.* JPY1 (acetone) and *Phoma sp.* are potent antioxidant producer, having broad spectrum activity against various free radicals. It may also be possible that antioxidant activities of these crude extracts of the fungi is determined by the combination effects of the compounds present in a particular solvent extract. The high antioxidant activities of the acetonic extract of *Aspergillus sp.* JPY1, *P. chrysogenum* and *Phoma sp*. probably due to the extracted tannins, terpenes, sterols and flavanoids in the acetonic extract of these fungi (Table-1).

Previous studies have shown the linear correlation between total phenolic content and antioxidant activity, which is a consonance with earlier studies[21]. The phenolic compounds (e.g. phenolic acids and their derivatives, flavanoids and phenolic terpenoids) might be responsible to some extent for the antioxidant capacity of the host plant and the isolated endophytic fungi[22, 23]. The present study

reveals that methanolic and aqueous extract of these endophytic fungi exhibit comparatively low activity. Probably this may be due to partially solubilisation of the membranes of fungal cells and storage organs; moreover the phytochemical solubilized in these two extracts were also less than the acetonic extract.

The extracts exhibit lower antioxidant activity than the standard oxidant (BHT/EDTA/Ascorbic acid) or positive control taken the various assays used. It can be explained by the fact that we know that standard antioxidant used are in purified form whereas the crude extracts are the mixture of various compounds. Further, it has also been observed that certain other compounds may be of help in enhancing the potency of the active compounds resulting in an additive or synergistic positive effect[24] while others may neutralize or inhibit the same.

Out of an estimated 250000 higher plants, less than 1% has been screened pharmacologically[25] and very few in regard to fungal endophytes. Hence our study indicates that fungal endophyte *Aspergillus sp.* JPY1 (acetone) may serve as a good natural source of antioxidant compounds and offering a better scope for production, easier down streaming of such bioactive compounds as toxicity studies proved that the extract is safe. Therefore, in vivo antioxidant activities of these fungal extract; especially to assess the potential to attenuate or avoid the potential damages of the free radicals in pathophysiological conditions such as carcinogenic events is needed. In addition, further investigation is still needed to discover the unidentified antioxidant constituents in the extracts of endophytic fungal isolates.

ACKNOWLEDGMENT

The authors are very thankful to Department of Science and Technology, India for providing INSPIRE-DST-JRF to Mrs. Seema Dhankhar. We are also thankful to University Grant Commission, New Delhi, Govt of India, for financial support.

REFERENCES

1. Radulovic N, Stankov-Jovanovic V, Stojanovic G, Smelcerovic A, Spiteller M, Asakawa Y. Screening of in vitro antimicrobial and antioxidant activity of Nine Hypericum species from the Balkans. Food Chem 2007; 103: 15-21.
2. Willet WC. Eat, Drink, and be Healthy-The Harvard Medical School Guide to Healthy Eating; Simon and Schuster: New York; 2001.
3. Ames BN, Gold LS Willet WC. The causes and prevention of cancer. Proc. Natl. Acad. Sci. U.S.A. 1995; 92: 5258–5265
4. Sultan S, Shah SAA, Sun L, Ramasami K, Cole A, Blunt J, Munro HGM, Weber JFF. Bioactive fungal metabolites of 9PR2 isolated from roots of *Callophyllum ferrrugineum*. Int J Pharm Pharm Sci 2011; 3: 7-9.
5. Wilson D. Endophyte- the evolution of a term and clarification of its use and definition. Okios 1995; 73:274-276.
6. Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. Nat Prod Rep 2001; 18:448–459.
7. Ganley RJ, Brunsfeld SJ, Newcombe GA. Community of unknown, endophytic fungi in western white pine. Proc Nat Acad Sci USA. 2004; 101:10107–10112
8. Singh AK. Endangered economic species of Indian desert. Gen Res Crop Evo 2004; 51:371-380.
9. Anonymous. The Wealth of India-Raw Materials. IX, PID, Council of Scientific and Industrial Research, New Delhi 1972.
10. Fisher PJ, Petrini O, Sutton BC. A comparative study of fungal endophytes in leaves, xylem and bark of *Eucalyptus* in Australia and England. *Sydowia* 1993; 45:338-345.
11. Oyaizu M. Studies on the products of browning reaction prepared from glucose amine. Japan J Nutri 1986; 94: 307-31.
12. Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivates (acetoaminophen, salicilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxylradical scavengers. Arch of Biochem Biophys 1994; 315: 161-169.
13. Liu F, Ooi VEC, Chang ST. Free radical scavenging activity of mushroom polysaccharide extracts. Life Sci. 1997; 60: 763–771.
14. Mathew S, Abraham TE. Studies on the antioxidant activities of cinnamon *(Cinnamomum verum)* bark extracts through in vitro models. Food Chem 2006; 94: 520-28.
15. Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: a simple ''test tube'' assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem 1987; 165: 215–219.
16. Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.
17. Huong NT, Matsumoto K, Kasal R, Yamasaki K, Watanabe H. Invitro antioxidant activity of *Vietnamease ginsng* saponin and its components. Biopharm.Bull 1998; 21: 978-981.
18. Miller HE. A simplified method for evaluation of antioxidants. Journal of American oil Chemists Society 1971; 48: 91.
19. Harborne JB. Phytochemical methods: a guide to modern techniques of plant analysis. Chapman and Hall Limited, London 1980.
20. Kokote CK. Practical Pharmacognosy. Jain MK Vallabh Parkashan, Pitampura New Delhi 107.
21. Sultana B, Anwar F, Przybylski R. Antioxidant activity of phenolic components present in barks of *Azadirachta indica, Terminalia arjuna*, and *Eugenia jambolana* Lam. Trees. Food Chem 2007; 104: 1106-1114.
22. Yu J, Wang LM, Walzem RL, Miller EG, Pike LM, Patil BS. Antioxidant activity of citrus limonoids, flavonoids, and coumarins. J Agric Food Chem 2005; 53:2009–2014.
23. Dos Santos MD, Martins PR, dosSantos PA, Bortocan R, Iamamoto Y, Lopes NP. Oxidative metabolism of 5-O-caffeoylquinic acid (chlorogenic acid), a bioactive natural product, by metalloporphyrin and rat liver mitochondria. Eur J Pharm Sci 2005; 26:62–70.
24. Kulkarni RD. Principles of Pharmacology in Ayurveda. Ram Sangam Graphics, Mumbai 1997.
25. Kashikar VS, Kotkar T. Indigenous remedies for Diabetes Mellitus Int J Pharm Pharm Sci 2011; 3: 22-29.