Journal of Academia and Industrial Research (JAIR)
Volume 3, Issue 8 January 2015

ISSN: 2278-5213

RESEARCH ARTICLE

Biochemical Constituents and Antioxidant Potential of Endophytic Fungi isolated from the Leaves of *Azadirachta indica* A. Juss (Neem) from Chennai, India

S. Kumaresan*, V. Karthi, V. Senthilkumar, B.S. Balakumar and A. Stephen
Dept. of Botany, RKM Vivekananda College (Autonomous), Mylapore, Chennai-600004, India
kumareshrkm@yahoo.co.in*; +91 9445183736

Abstract

Four endophytic fungi identified from the leaves of *Azadirachta indica* A. Juss. (Meliaceae) were studied for their antioxidant potential. Biochemical constituents of endophytic fungal extracts were evaluated for their antioxidant activity by reducing power assay and nitric oxide radical inhibition by GriessIllosvoy reaction. The ascorbic acid content in *Chaetomium* sp., *Curvularia* sp., *Colletotrichum* sp. and *Trichoderma* sp. were 2.38, 1.21, 1.11, and 0.83 mg of quercetin equivalents/g respectively. The content of phenolic compounds followed the same pattern with values 28.5, 10.63, 9.82 and 7.51 mg of gallic acid equivalents/g, for *Chaetomium* sp., *Colletotrichum* sp., *Curvularia* sp. and *Trichoderma* sp. respectively. Apart from these, β-Carotene, tannin and total flavonoid content were also studied quantitatively. The antioxidant activities of fungal extracts increase with increasing amount of extracts (0.2-1 mg/mL). The variation in the content of phenolic components and ascorbic acid levels reflected the antioxidant activity of the organisms studied. The antioxidant activity of the extract of *Chaetomium* sp. is the highest followed by *Curvularia* sp., *Colletotrichum* sp. and *Trichoderma* sp. The methanolic extracts of these endophytic fungi showed promising antioxidant potential for bioprospecting.

Keywords: Endophytic fungi, *Azadirachta indica*, phenol, β-Carotene, tannin, total flavonoid, antioxidant.

Introduction

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. An antioxidant molecule can react with a free radical and is capable to neutralize by donating one of their own electron; ending the carbon-stealing reaction. Antioxidants prevent cell and tissue damage as they act as scavengers. Though the human body naturally produces antioxidants, the process is not effective in case of overwhelming production of free radicals besides antioxidant defense mechanism declines with aging (Sies, 1991; Goldfarb, 1993). Free radicals are an atom or a molecule of a chemical species which contains an unpaired electron in their outermost orbit that are capable of independent existence. These free radicals are highly unstable and can react with other molecules by giving out or accepting single electron (Haliwell, 1989). The reactive nature of the free radicals can damage all cellular macromolecules including proteins, carbohydrates, lipid and nucleic acids. It is found that the DNA of cells undergoes about 10,000 free radical attacks everyday (Frei *et al*., 1989). This involvement of free radical on cellular macromolecules results the development of pathological condition such as Down's syndrome, Alzheimer's disease, Atherosclerosis, Parkinson's disease, Hepatic damage, Hypertension, Diabetes and suppressed immune function. The most important free radicals in the body are reactive oxygen and reactive nitrogen species (ROS and RNS).

Some of the ROS and RNS have various important roles *in vivo*, such as energy production, phagocytosis, regulation of cell growth and intracellular signaling. Excess production of these free radicals causes damage to cells and tissues. Thus, a balance between ROS and RNS are maintained in the body by scavenging mechanisms called antioxidants (Halliwell and Gutteridge, 1999). Some of the exogenous factors like ionizing radiation, toxicity of lead, pesticide, cadmium, alcohol, cigarette smoke, UV light and drugs with reducing potential will also initiate the formation of free radicals (Langseth, 1996).

Recent years, endophytic fungi gained interest from taxonomists to chemists because of their bioactive metabolites (Aly *et al.,* 2011; Joseph and Priya, 2011). Endophytic fungi have been an important and potential resource of antioxidant (Huang *et al.,* 2007, 2008). The sodium hydroxide-extracted mycelial polysaccharide from the endophytic fungus *Fusarium oxysporum* was known to exhibit DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging activity and ferrous ions chelating ability (Li *et al.,* 2011). Endophytic fungi possess antioxidant components like flavonoids, carotenoids, phenolics, tannin and ascorbic acids that were also known to exhibit free radicals scavenging activity. The qualitative chemical analysis of the fungus *Penicillium* sp., isolated from *Centella asiatica,* a flowering plant showed positive results for alkaloids,

phenols, flavonoids, tannin and glycosides (Devi *et al.,* 2012). The natural antioxidant cajaninstilbene acid from endophytic fungi *Fusarium solani* and *F. proliferatum* isolated from pigeon pea by Zhao *et al.* (2012). Tejesvi *et al*. (2006) isolated endophytic fungal species *Fusarium, Pestalotiopsis, Myrothecium, Trichoswema, Erticillium and Chaetomium* from inner bark segments of ethno-pharmaceutically important tree species namely; *Terminalia arjuna, Crataeva magna, Azadirachta indica, Terminalia chebula* and *Butea monosperma* grown in different regions of Southern India that exhibited greater antifungal activity. The antioxidant, antibacterial and antihypertensive activities demonstrated the potential of extracts of *Pestalotiopsis* isolated from *Azadirachta indica* (Tejesvi *et al*., 2009). Products made from *Azadirachta indica* A. Juss. (Meliaceae), commonly known as Indian Neem or Indian lilac, have been used in India for over two millennia for their medicinal properties. Neem products are believed by Ayurvedic practitioners to be anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative (Biswas *et al.,* 2002). Sithisarn *et al.* (2005) reported that extracts from leaf, flower and stem bark of the Siamese neem tree have strong antioxidant potential. Flavanoids of *A. indica* have been reported to possess antioxidant activity (Bandyopadhyay *et al*., 2002) and anti-nociceptive and anti-inflammatory activities of carbon tetrachloride extract of fruit skin (Ilango *et al*., 2013). Endophytic fungi (*Eupenicillium parvum*) also synthesize azadirachtin which is produced by host neem plant. This warrant to explore biochemical nature of endophytic fungi isolated from medicinal plants (Kusari *et al.,* 2012). Hence, the bioactive nature of *Azadirachta indica* needs to be reexamined in the light of chemicals produced by endophytic fungal sp. Hence, the present study was attempted to investigate the antioxidant property of endophytic fungi isolated from *Azadirachta indica*.

Materials and methods

Sample collection: Healthy neem plants were selected for endophytic fungal isolation from Ramakrishna Mission Vivekananda College campus, Chennai, India. The leaves from each individual plant were collected and transported to the laboratory in separate sterile polythene bags and processed within 24 h of collection following the procedure of Fisher and Petrini (1987).

Isolation and identification of fungal endophytes: The collected leaves were thoroughly washed in running tap water, followed by surface sterilization by soaking in 4% sodium hypochloride for 3 min and finally in 75% ethanol for 30 sec. The surface sterilized samples were washed three times with sterile distilled water. The surface sterilized sample leaves were cut into (0.5 cm^2) pieces. They are then transferred to potato dextrose agar (PDA) medium petri plates amended with (150 mg/L) chloramphenicol to inhibit bacterial growth. Petri plates were incubated at 30°C for a week under regular monitoring.

The fungal colonies that grow from the leaf segments were sub-cultured repeatedly until a pure culture is obtained. The isolated fungus was identified based on the morphological characters and the same was sent to Dr. S. Muthumary, the Centre for Advance Studies in Botany, University of Madras, Chennai (India) for the confirmation.

Antioxidant constituents

Estimation of polyphenol: About 200 mg of dry fungal mycelia was homogenized with 80% ethanol and centrifuged at 10,000 rpm for 20 min and the supernatant was collected. The fungal extracts were analyzed for total polyphenol using the methods of Malick and Singh (1980). The content of total polyphenol was calculated based on the gallic acid standard calibration curve.

Estimation of ascorbic acid: About 1 g of the fungal mycelia was homogenized in 4% TCA. This was made up to 10 mL and centrifuged at 2000 rpm for 10 min. The supernatant was treated with a pinch of activated charcoal, shaken well and kept for 10 min. It was centrifuged once again to remove the charcoal residue and ascorbic acids of the fungal extracts were determined according to the method of Roe and Kuether (1943).

Determination of tannin: About 1 g of the fungal mycelia was grounded with a mortar and pestle by adding 50 mL methanol. The homogenate was centrifuged at 5,000 rpm for 5 min and the supernatant were saved. Tannin content in the sample is expressed as Catechin equivalents (Price *et al.*, 1978).

Estimation of β-Carotene: About 200 mg of fungal biomass was homogenized with mortar and pestle by adding 10x volume of ethanol: hexane (2:1). To the homogenate, 4 mL of double distilled water and 8 mL of hexane was added, mixed well and centrifuged for 5 min at 2,000 rpm. The hexane layer was aspirated and absorbance was measured at 450 nm in a spectrophotometer using hexane as a blank. The total content of β-Carotene in the sample was calculated as per Shaish *et al*. (1992).

Determination of total flavonoid: The dry 200 mg of fungal mycelia was homogenized with 90% ethanol and centrifuged at 3,000 rpm for 10 min and the supernatant was saved. Total flavonoid of the sample was determined according to Barros *et al*. (2007). The flavonoid content is expressed as milligram catechin equivalents/g sample.

Antioxidant activity

Reducing power assay: The reductive potential of the extracts were determined according to the method of Oyaizu (1986). Various concentrations of fungal methanolic extracts (2.5 mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of

1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After, 2.5 mL of 10% trichloroacetic acid (w/v) were added; the mixture was centrifuged at 1000 rpm for 10 min. The supernatant (5 mL) was mixed with 5 mL deionised water and 1 mL of 0.1% of ferric chloride and absorbance was measured at 700 nm using ascorbic acid as a standard. Higher absorbance value of the reaction mixture indicates greater reductive potential. The assay was carried out in triplicate and the results are expressed as mean ± standard deviation.

Nitric oxide radical scavenging activity: Nitric oxide radical inhibition was estimated by the use of GriessIllosvoy reaction (Garratt, 1964). The 3 mL reaction mixture contains 2 mL of 10 mM of sodium nitroprusside, 0.5 mL phosphate buffer saline and 0.5 mL of varied concentrations of the fungal extracts (0.2 to 1 mg/mL). They were incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture mixed with 1 mL sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was allowed to stand for 5 min for completion of the reaction of diazotization. Further, 1 mL of the naphthylethylene diamine di-hydrochloride (0.1% w/v) was added, mixed and was allowed to stand for 30 min at 25°C. The absorbance was taken at 546 nm using ascorbic acid as standard. The percent inhibition (PI) was calculated using the following formula:

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

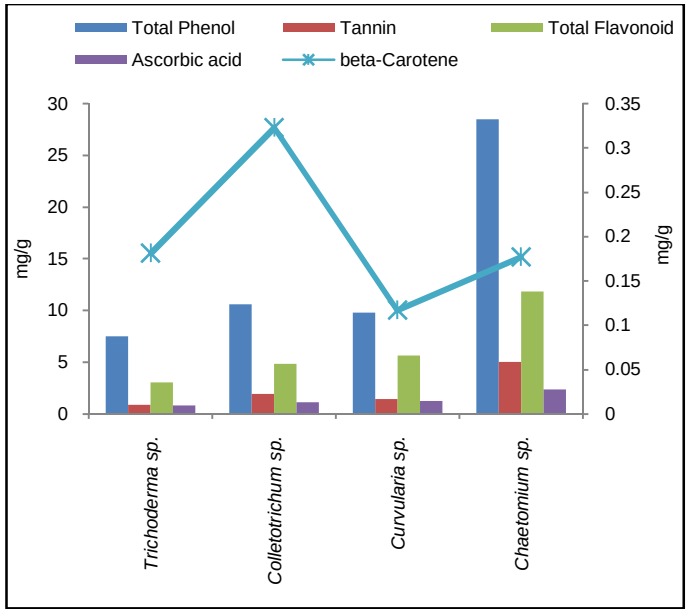
Where, $A_{(Control)}$ = Absorbance of control reaction
$A_{(Sample\ or\ Standard)}$ = Absorbance of sample extract or standard.

Results and discussion

Four endophytic fungi were identified based on morphological features from leaf tissues of *Azadirachta indica*. They are *Curvularia* sp., *Trichoderma* sp., *Chaetomium* sp. and *Colletotrichum* sp. Sterile cultures lacking reproductive structures were grouped under mycelia sterilia. Several endophytic fungal species were isolated and reported from inner bark (Mahesh *et al.,* 2005), bark, leaf and stem tissues of *A. indica* (Verma *et al.,* 2007).

Total phenolic contents: Phenolic content was found to be higher in *Chaetomium* sp. (28.5 mg/g) than the *Trichoderma* sp. (7.52 mg/g) (Fig. 1). Phenolic compounds are well-known antioxidant constituents; they are beneficial in terms of nutritional value (Ayala-Zavala *et al.,* 2012). In plants, they also serve as defense mechanism to counteract reactive oxygen species (ROS) in order to prevent the molecular damage in cells (Vaya *et al.,* 1997). Various fungal endophytes and mushrooms have been reported to produce antioxidant activity (Song and Yen, 2002). Murthy *et al.* (2011) reported that *Fusarium* sp. from *Lobelia nicotianifolia* produced phenolic compounds. Similarly, the ethanol extract of *Phyllostica* sp. isolated from *Guazuma tomentosa* also produced phenolic compounds (Srinivasan *et al.,* 2010).

Fig. 1. Antioxidant properties of endophytic fungi isolated from *Azadirachta indica.*



Whereas, *Chaetomium* sp. isolated from *Eugenia jambolana* possesses alkaloids, phenols, amino acids, carbohydrates and flavonoids (Yadav *et al.,* 2014).

Total flavonoid content: The most important and widespread polyphenolic secondary metabolite in the plant kingdom is the flavonoids. The quantitative estimation of the total flavonoid revealed that all the selected endophytic fungi were known to produce flavonoid in varied quantity ranging from 3.08–11.83 mg/g of Catechin equivalent (Fig. 1). *Chaetomium* sp. was found to have higher content of flavonoid (11.83 mg/g), whereas *Tricoderma* sp. synthesized low content of flavonoid (3.03 mg/g). Phenolics and flavonoids play an important role in stabilizing lipid peroxidation and by their antioxidant activity (Chandra and Arora, 2012). *Aspergillus nidulans* and *A. oryzae* isolated as an endophyte from *Ginkgo biloba* were found to produce flavonoids (Qiu *et al*., 2010). *Aspergillus niger* and *Fusarium oxysporum* isolated from *Crotalaria pallida* also produced flavonoids (Govindappa *et al*., 2011). The flavonoid compounds from Citrus have been shown to have anti-proliferative effects on squamous cell carcinoma in human (Kawaii *et al*., 1999). *Trichoderma longibrachiatum* isolated from *Eugenia jambolana* possess alkaloids, phenols, amino acids, carbohydrates, terpenes and flavonoids (Yadav *et al.,* 2014).

Ascorbic acid: The *Chaetomium* sp. exhibited high content of ascorbic acid (2.38 mg/g), whereas *Tricoderma* sp. exhibited a low content of ascorbic acid (0.83 mg/g) (Fig. 1). Ascorbic acid is naturally available form of vitamin C and is consequently the most important water soluble antioxidant vitamin in cells, effectively scavenging reactive oxygen species (ROS) (Gardner *et al.,* 2000).

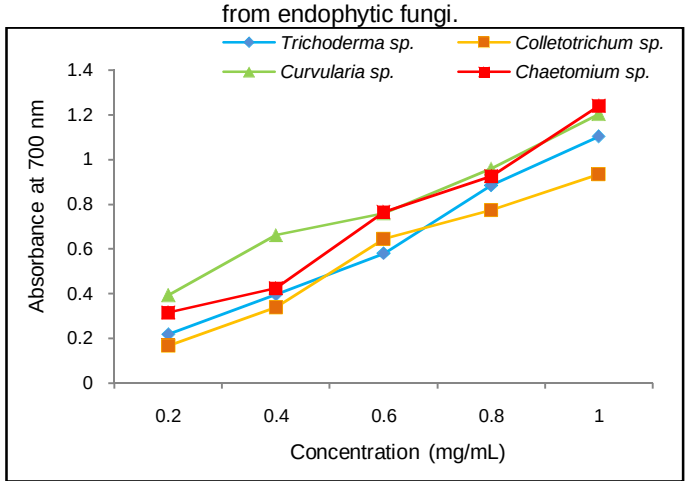
Vitamin C can act as an oxidant and pro-oxidant so as to protect DNA from free radical damage and mutagens (Hamid *et al.,* 2010; Sram *et al.,* 2012). Dietary supplementation of antioxidants such as vitamins C and E could prevent sodium arsenite induced toxicity and DNA damage in rats (Balakumar *et al.,* 2010a, b). Arsenic-exposed rats showed decreased level of lipid peroxidation when treated with α-tocopherol and ascorbic acid (Ramanathan *et al.,* 2002). Co-treatment of ascorbic acid and α-tocopherol modulated arsenic induced micronuclei formation in rats (Balakumar *et al.,* 2010c). Supplementation of antioxidants like vitamin C and E along with a chelating agent proves to be a better treatment regimen for skin lesions, peripheral vascular disease, hypertension, Blackfoot disease and cancer (Flora *et al.,* 2007).

Tannins: Chaetomium sp. was found to possess higher tannin content (5.0 mg/g) whereas *Tricoderma sp.* contains lower tannin content (0.86 mg/g). *Penicillium* sp. isolated from *Centella asiatica* was studied. The results suggested that the endophytic fungus found to produce phenols, alkaloids, tannins, flavonoids and glycosides (Devi *et al.,* 2012). Aqueous and acetone extract of *Aspergillus* sp., *Penicillium* sp. and *Phoma* sp. isolated as endophytes from *Salvadora oleoides* produced tannin (Dhankhar
et al., 2012).

β-Carotene: Colletotrichum sp. exhibited highest β-Carotene content (0.326 µg/g) and *Curvularia* sp. expressed low content (0.115 µg/g) (Fig. 1). Carotenoids have been found to enhance immune cell function in the body (Marie *et al*., 2012). B-Carotene functions as a provitamin A and also used as a food colorant (Bauernfeind, 1981). It possesses antioxidant properties and directly reacts with active oxygen species (Burton and Ingold, 1984; Krinsky, 1989; Girard *et al.,* 1994). High level production of β-Carotene was achieved from mutant strain of *Phaffia rhodozyma*, a red astaxanthin producing yeast (Girard *et al.,* 1994). *Phycomyces blakesleeanus* while grown on an aqueous medium synthesized considerable quantity of a single carotenoid, β-Carotene and traces of α-Carotene (Garton *et al*., 1951).

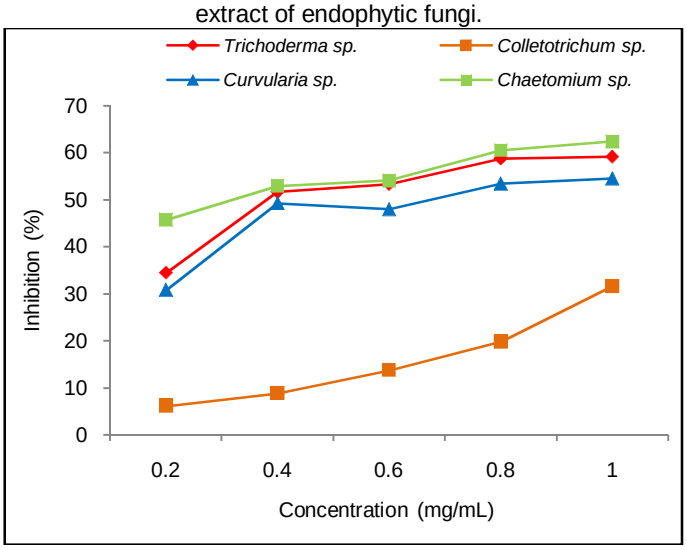
Reducing power assay: Figure 2 shows the reducing power of methanolic extracts of fungal spp. expressed against their concentration. The reducing power of the methanolic extracts of fungal spp. increased with concentration. The increase in absorbance of the reaction mixtures indicates increased reducing power (Kekuda *et al.,* 2010). Ethanolic extract of *Aspergillus* sp. isolated from *Potentilla fulgens* showed a potent reducing activity closer to ascorbic acid standard (Nath and Joshi, 2013). In the present analysis, *Chaetomium* sp. exhibited maximum reducing power (1.241) and *Colletotrichum* sp. showed a lowest reducing power (0.935) at 1 mg/mL.

Fig. 2. Reducing power of methanolic extract from endophytic fungi.



Zheng *et al*. (2011) reported that endophytic fungi *Chaetomium globossum* isolated from *Scapania versucosa* exhibited excellent reducing power potential, which is similar to the result obtained in the present study. The maximum reducing potential of *Chaetomium* sp. may be attributed to the potential of the extracts to act as reductants (Zhao *et al*., 2006). Various endophytic fungal species isolated from different plants showed greater antioxidant potential. *Phomopsis* sp. and *Xylaria* sp. isolated from *Emblica officinalis* exhibited higher level of reducing potential with increase in concentration (Nath *et al.,* 2012). *Aspergillus flavus* isolated from a mangrove plant *Avicennia officinalis* expressed a greater antioxidant potential (Ravindran *et al.,* 2012). *Aspergillus awamori* showed the highest antioxidant potential and also had the maximum phenolic content among different endophytes isolated from *Rauwolfia serpentina* (Nath *et al.,* 2013). Ethanolic extract of endophytic fungi *Aspergillus oryzae* CeR1 isolated from *Centella asiatica* showed higher radical scavenging activity than *Colletotrichum gloeosporioides* MKL1 from *Murraya koengii* (Nath *et al.,* 2014). Aqueous extract of *Tolypocladium* sp., an endophytic fungus isolated from wild *Cordyceps sinensis* exhibited moderate reducing power activity (Zheng *et al.,* 2008). Methonalic extract of wild edible mushroom species, *Lactarius deliciosus* and *Tricholoma portentosum* showed antioxidant potential; but absorbance value (1.2) was achieved at a concentration of 10 mg/mL (Ferreira *et al.,* 2007). A similarity was observed in our study of fungal species at 1 mg/mL concentration. This shows that these species possess better antioxidant potential than the wild edible mushrooms. It was reported that the reducing power of soybean oil might be due to their hydrogen-donating ability (Shimada *et al*., 1992). Similarly, endophytic fungi isolated from *Azadirachta indica* might contain higher amounts of reductone than wild edible mushrooms, which could react with free radicals to stabilize and block radical chain reactions effectively.

Fig. 3. Nitric oxide radical scavenging activity of methanolic extract of endophytic fungi.



Nitric oxide radical scavenging activity: Nitric oxide radical scavenging activity was found higher in *Chaetomium* sp. (62.39%), followed by *Tricoderma* sp. (59.20%) and the least of activity was observed in *Colletotrichum* sp. (31.66%) (Fig. 3). Nitric oxide can act both as a pro-oxidant and oxidant, the pro-oxidant effect of nitric oxide is by the formation of peroxynitrite, which is formed by its reaction with a superoxide free radical. *Armillaria mellea*, a medicinal edible mushroom of China, is a symbiotic fungus isolated from the underground tubers of wild orchid *Gastrodia elata,* showed notable free radical scavenging activity, inhibiting lipid peroxidation and DNA damage (Gao and Wang, 2012). Nitric oxide is implicated in inflammation, cancer and other pathological conditions like reactive oxygen species (Moncada *et al*., 1991). Endophytic fungi isolated from root of *Catharanthus roseus* exhibited nitric oxide scavenging capacity (Rosaline *et al.,* 2013). *Aspergillus fumigatus* exhibited 70.2% nitric oxide radical scavenging potential (Arora and Chandra, 2011).

Conclusion

In this study, the medicinal plant *Azadirachta indica* A. Juss. has been studied for its endophytic assemblage and its crude extract were screened for antioxidant constituents like phenols, tannin, flavonoid, ascorbic acid and β-carotene. The *in vitro* antioxidant potential is evaluated by reducing power assay and nitric oxide scavenging activity. The four fungal species namely *Tricoderma* sp., *Colletotrichum* sp., *Curvularia* sp. and *Chaetomium* sp. were isolated from the leaves of *Azadirachta indica*. Apart from these endophytic fungi, sterile culture lacking reproductive structure, which was grouped under mycelia sterilia was also isolated. It is evident that all the endophytic fungi studied here possess *in vitro* antioxidant activities. Biochemical screening of them revealed the presence of phenols, flavonoids, tannins, ascorbic acid and β-carotene but their quantities varied, this may be due to the biochemical, genetical and physiological conditions of the fungus. Bioactive compound found in endophytic fungi await a major break-through for a variety of medical applications. Further, feasible growth of endophytic fungi and easier downstream processing of the fungal compounds offer quantitative and qualitative development of chemotherapeutic agents especially antioxidants may be attempted.

Acknowledgements

Authors thank Management especially the Secretary, Revered Swami Shukhadevananda and the Principal Dr. K. Srinivasan of RKM Vivekananda College (Autonomous) for providing all the necessary facilities and encouragement.

References

1. Aly, A.H., Debbab, A. and Proksch, P. 2011. Fungal endophytes: Unique plant inhabitants with great promises. Appl. Microbiol. Biotechnol. 90(6): 1829-1845.
2. Arora, D.S. and Chandra, P. 2011. Antioxidants activity of *Aspergillus fumigatus*. *ISRN Pharmacol*10.5402/2011/619395.
3. Ayala-Zavala, J.F., Silva-Espinoza, B.A., Cruz-Valenzuela, M.R., Villegas-Ochoa, M. A., Esqueda, M., González-Aguilar, G.A. and Calderón-López, Y. 2012. Antioxidant and antifungal potential of methanol extracts of *Phellinus* spp. from Sonora, Mexico. *Revista Iberoamericana De Micología.* 29(3): 132-138.
4. Balakumar, B.S., Kumaresan, S., Venugopal, R. and Sivasubramanian, V. 2010a. Clastogenic effect of sodium arsenite in experimental rats and ameliorative effects of antioxidant vitamins C and E. *Ind. J. Sci. Technol.* 3(6): 642-647.
5. Balakumar, B.S., Ramanathan, K., Kumaresan, S. and Suresh, R. 2010b. DNA damage by sodium arsenite in experimental rats: Ameliorative effects of antioxidant vitamins C and E. *Ind. J. Sci. Technol.* 3(3): 322-327.
6. Balakumar, B.S., Suresh, R. and Venugopal, R. 2010c. Modulatory effects of Ascorbic acid and α–tochopherol on arsenic induced Micronuclei formation. *Int. J. Pharmacol.* 6(5): 676-680.
7. Bandyopadhyay, U., Biswas, K., Chatterjee, R., Bandyopadhyay, D., Chattopadhyay, I., Ganguly, C.K., Chakraborty, T., Bhattacharya, K. and Banerjee, R.K. 2002. Gastroprotective effect of neem (*Azadirachta indica*) bark extract: possible involvement of H^+-K^+-ATPase inhibition and scavenging of hydroxyl radical. *Life sci.* 71: 2845-2865.
8. Barros, L., Ferreira, M.J., Queiros, B., Ferreira, I.C.F.R. and Baptista, P. 2007. Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. *Food Chem.* 103: 413-419.
9. Bauerfeind, J.C. 1981. Carotenoids as colorants and vitamin A precursors. Academic Press, New York.
10. Biswas, K., Chattopadhyay, I., Banerjee, R.K. and Bandyopadhyay, U. 2002. Biological activities and medicinal properties of Neem (*Azadirachta indica*). *Curr. Sci.* 82(11): 1336-1345.
11. Burton, G.W. and Ingold, K.U. 1984. β-Carotene: An unusual type of lipid antioxidant. *Sci.* 224: 569-573.
12. Chandra, P. and Arora, D.S. 2012. Optimization of antioxidant potential of *Penicillium granulatum* Bainier by statistical approaches. *ISRN Microbiol.* 2012: 1-10.
13. Devi, N.N., Prabakaran, J.J. and Wahab, F. 2012. Phytochemical analysis and enzyme analysis of endophytic fungi from *Centella asiatica*. *Asian Pacific J. Trop. Biomed.* 2: S1280-S1284.

14. Dhankhar, S., Kumar, S., Dhankhar, S. and Yadav, J.P. 2012. Antioxidant activity of fungal endophytes isolated from *Salvadora oleoidesdecne*. *Int. J. Pharm. Sci.* 4: 0975-1491.
15. Ferreira, I.C.F.R., Baptista, P., Vilas-Boas, M. and Barros, L. 2007. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. *Food Chem.* 100: 1511-1516.
16. Fisher, P.J. and Petrini, O. 1987. Tissue specificity by fungi endophytic in *Ulex europaeus. Sydowia*. 40: 46-50.
17. Flora, S.J.S., Bhadauria, S., Kannan, G.M. and Singh, N. 2007. Arsenic induced oxidative stress and the role of antioxidant supplementation during chelation: A review. *J. Environ. Biol.* 28(2): 333-347.
18. Frei, B., England, L. and Ames, B.N. 1989. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. USA.* 86(16): 6377-6381.
19. Gao, L.W. and Wang, J.W. 2012. Antioxidant potential and DNA damage protecting activity of aqueous extract from *Armillaria mellea*. *J. Food Biochem.* 36(2): 139-148.
20. Gardner, P.T., White, T.A.C., McPhail, D.B. and Duthie, G.G. 2000. The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices-dietary flavonoids and phyto-estrogens. *Food Chem.* 68(4): 471-474.
21. Garratt, D.C. 1964. The quantitative analysis of drugs. Third Edition, Chapman and Hall Ltd., Tokyo, pp.456-458.
22. Garton, G.A., Goodwin, T.W. and Lijinsky, W. 1951. Studies in carotenogenisis. 1. General conditions governing β-carotene synthesis by the fungus *Phycomyces blakesleeanus* Burgeff. *Biochem. J.* 48: 154-163.
23. Girard, P., Falconnier, B., Bricout, J. and Vladescu, B. 1994. β-Carotene producing mutants of *phaffia rhodozyma. Appl. Micro. Biotech*. 41: 183-191.
24. Goldfarb, A.H. 1993. Antioxidants: Role of supplementation to prevent exercise-induced oxidative stress. *Med. Sci. Sports Exercise.* 25: 232-236.
25. Govindappa, M., Bharath, N., Shruthi, H.B. and Gustavo, S. 2011. *In vitro* antioxidant activity and phytochemical screening of endophytic extracts of *Crotalaria pallida*. *Free Rad. Antioxidants.* 1(3): 89-83.
26. Halliwell, B. and Gutteridge, J.M.C. 1989. In free radicals in biology and medicine (Second Edition) Clarendon Press, Oxford, U.K.
27. Halliwell, B. and Gutteridge, J.M.C. 1999. Free Radicals in Biology and Medicine, 2nd edition, *Clarendon Press, Oxford.*
28. Hamid, A.A., Aiyelaagbe, O.O., Usman, L.A., Ameen, O.M. and Lawal, A. 2010. Antioxidants: Its medicinal and pharmacological applications. *Afri. J. Pure Appl. Chem.* 4(8): 142-151.
29. Huang, W.Y., Cai, Y.Z., Xing, J., Corke, H. and Sun, M. 2007. A potential antioxidant resource: Endophytic fungi from medicinal plants. *Econ. Botany.* 61: 14-30.
30. Huang, W.Y., Cai, Y.Z., Hyde, K.D., Corke, H. and Sun, M. 2008. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Diversity.* 33: 61-75.
31. Ilango, K., Maharajan, G. and Narasimhan, S. 2013. Anti-nociceptive and anti-inflammatory activities of *Azadirachta indica* fruit skin extract and its isolated constituent azadiradione. *Nat. Prod. Res.* 27(16): 1463-1467.
32. Joseph, B. and Priya, R.M. 2011. Bioactive compounds from Endophytes and their potential in pharmaceutical effect: A review. *Amer. J. Biochem. Mol. Biol.* 1(3): 291-309.
33. Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. and Yano, M. 1999. Antiproliferative activity of flavonoids on several cancer cell lines. *Biosci. Biotechnol. Biochem.* 63(5): 896-899.
34. Kekuda, T.R.P., Shobha, K.S. and Onkarappa, R. 2010. Studies on antioxidant and anthelmintic activity of two *Streptomyces* species isolated from Western Ghat soils of Agumbe, Karnataka. *J. Pharm. Res.* 3(1): 26-29.
35. Krinsky, N.I. 1989. Antioxidant function of carotenoids. *Free Radical Biol. Med*. 7: 617-635.
36. Kusari, S., Verma, V.C., Lamshoeft, M. and Spiteller, M. 2012. An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. *World J. Microbiol. Biotechnol*. 28(3): 1287-1294.
37. Langesth, L. 1996. Oxidants, antioxidants and disease prevention. International Life Science Institute, ILSI Press, Brussels.
38. Li, P., Mou, Y., Shan, T., Xu, J., Li, Y., Lu, S. and Zhou, L. 2011. Effects of polysaccharide elicitors from Endophytic *Fusarium oxysporium* Dzf17 on growth and diosgenin production in cell suspension culture of *Dioscorea zingiberensis. Mol.* 16(11): 9003-9016.
39. Mahesh, B.T., Nalini, M.S., Prakash, H.S., Kini, K.R., Subbiah, V. and Shetty, H.S. 2005. Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss. *Curr. Sci.* 88(2): 218-219.
40. Malick, C.P. and Singh, M.B. 1980. Plant enzymology and histo-enzymology: A text manual. Kalyani Publishers, New Delhi.
41. Marie, C.F., Regine, M.Q., Stephane, W., Emilie, T., Joseph, R., Brigitte, W.R., Edmond, R. and Marie, P.V. 2012. Immune status is more affected by age than by carotenoid depletion-repletion in healthy human subjects. *Brit. J. Nutrit.* 108: 2054-2065.
42. Moncada, S., Palmer, R.M. and Hiiggs, E.A. 1991. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43: 109-142.
43. Murthy, N.K., Pushpalatha, K.C. and Joshi, C.G. 2011. Antioxidant activity and phytochemical analysis of endophytic fungi isolated from *Lobelia nicotianifolia*. *J. Chem. Pharm. Res*. 3: 218-225.
44. Nath, A. and Joshi, S.R. 2013. Bioactivity assessment of endophytic fungi associated with the ethnomedicinal plant *Potentilla fulgens*. *World J. Pharmaceut. Res.* 2(6): 2596-2607.
45. Nath, A., Chattopadhyay, A. and Joshi, S.R. 2013. Biological activity of endophytic fungi of *Rauwolfia serpentina* Benth: An ethnomedicinal plant used in folk medicines in Northeast India. *Proc. Natl. Acad. Sci. Ind. Sect. B Biol. Sci*. 85(1): 233-240.
46. Nath, A., Pathak, J. and Joshi, S.R. 2014. Bioactivity assessment of endophytic fungi associated with *Centella asiatica* and *Murraya koengii*. *J. Appl. Biol. Biotechnol.* 2(5): 6-11.
47. Nath, A., Raghunatha, P. and Joshi, S.R. 2012. Diversity and biological activities of endophytic fungi of *Emblica officinalis*, an ethnomedicinal plant of India. *Mycobiol.* 40(1): 8-13.
48. Oyaizu, M. 1986. Studies on the products of browning reaction prepared from glucose amine. *Japan J. Nutrit*. 94: 307-331.
49. Price, M.L., Scoyoc, S.V. and Butler, L.G. 1978. Critical evaluation of vanillin reaction as an assay for tannin in *Sorghum* grain. *J. Agric. Food Chem*. 26: 1214-1218.
50. Qiu, M., Xie, R.S., Shi, Y., Zhang, H.H. and Chen, H.M. 2010. Isolation and identification of two flavonoid producing endophytic fungi from *Ginkgo biloba* L. *Ann. Microbiol.* 60: 143-150.
51. Ramanathan, K., Balakumar, B.S. and Panneerselvam, C. 2002. Effects of ascorbic acid and alpha tocopherol on arsenic induced oxidative stress. *Human Exp. Toxicol.* 21: 675-680.
52. Ravindran, C., Naveenan, T., Govindaswamy, R., Varatharajan, R., Raju, R. and Meena, R.M. 2012. Antioxidants mangrove plants an endophytic fungal associations. *Bot. Mar.* 55: 269-279.
53. Roe, J.H. and Kuether, C.A. 1943. The determination of ascorbic acid in whole blood and urine through 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J. Biol. Chem*. 147: 399-407.
54. Rosaline, I. and Agastian, P. 2013. α-Glucosidase inhibitory activity of ethyl acetate extracts of endophytes isolated from the root of *Catharanthus roseus* L. *Int. J. Pharm. Bio. Sci*. 4: 1246-1258.

55. Shaish, A., Ben-Amotz, A. and Avron, M. 1992. Biosynthesis of β-Carotene in *Dunaliella*. *Meth. Enzymol.* 213: 439-444.
56. Shimada, K., Fujikawa, K., Yahara, K. and Nakamura, T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agri. Food Chem.* 40(6): 945-948.
57. Sies, H. 1991. Oxidative Stress Oxidants and Antioxidants. Academic Press, London.
58. Sithisarn, P., Supabphol, R. and Gritsanapan, W. 2005. Antioxidant activity of Siamese neem tree (VP1209). *J. Ethnopharmacol*. 99(1): 109-112.
59. Song, T.Y. and Yen, G.C. 2002. Antioxidant properties of *Antrodia camphorate* in submerged culture. *J. Agri. Food Chem.* 50: 3322-3327.
60. Sram, R.J., Binkova, B. and Rossner, Jr, P., 2012. Vitamin C for DNA damage prevention. *Mutat. Res./Fundamental Mol. Mech. Mutagenesis*. 733: 39-49.
61. Srinivasan, K., Jagadish, L.K., Shenbhagaraman, R. and Muthumary, J. 2010. Antioxidant activity of endophytic fungus *Phyllosticta* sp. Isolated from *Guazuma tomentosa. J. Phytol.* 2: 37-41.
62. Tejesvi, M.V., Mahesh, B., Nalini, M.S., Prakash, H.S., Kini, K.R., Subbiah, V. and Shetty, H.S. 2006. Fungal endophyte assemblages from ethnopharmaceutically important medicinal trees. *Can. J. Microbiol.* 52: 427-435.
63. Tejesvi, M.V., Tamhankar, S.A., Kini, K.R., Rao, V.S. and Prakash, H.S. 2009. Phylogenetic analysis of endophytic *Pestalotiopsis* species from ethnopharmaceutically important medicinal trees. *Fungal Diversity.* 38: 167-183.
64. Vaya, J., Belinky, P.A. and Aviram, M. 1997. Antioxidant constituents from licorice roots; Isolation, structure elucidation and antioxidative capacity toward LDL oxidation. *Free Rad. Biol. Med*. 23: 302-313.
65. Verma, V.C., Gond, S.K., Kumar, A., Kharwar, R.N. and Strobel, G. 2007. The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (Neem) from Varanasi (India). *Microbial Ecol.* 54: 119-125.
66. Yadav, M., Yadav, A. and Yadav, J.P. 2014. *In vitro* antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. *Asian Pac. J. Trop. Med.* 7(Suppl 1): S256-S261.
67. Zhao, G.R., Xiang, Z.J., Ye, T.X., Yuan, Y.J. and Guo, Z.X. 2006. Antioxidant activities of *Salvia miltiorrhiza* and *Panax notoginseng*. *Food Chem.* 99: 767-774.
68. Zhao, J., Fu, J., Luo, M., Zu, Y., Wang, W., Zhao, C. and Gu, C. 2012. Endophytic Fungi from Pigeon Pea [*Cajanus cajan* (L.) Millsp.] produce antioxidant cajaninstilbene acid. *J. Agric. Food Chem.* 60(17): 4314-4319.
69. Zhao, J., Shan, T., Mou, Y. and Zhou, L. 2011. Plant derived bioactive compounds produced by endophytic fungi. *Mini-Rev. Med. Chem.* 11: 159-168.
70. Zheng, L.P., Gao, L.W., Zhou, J.Q., Sima, Y.H. and Wang, J.W. 2008. Antioxidant activity of aqueous extract of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. *Afri. J. Biotech*. 7: 3004-3010.