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Research Article

A Comparative Study on the Antioxidant Activity of Four Different Fungal Endophytes

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Abstract

By definition, Endophytic fungi are the fungi that spend a part or whole of their life cycle. These fungi colonize the healthy tissues of host plants either inter or intracellularly typically without causing apparent symptoms of disease and without any immediate, negative overt results with the only exception that the fruit bearing structure of these endophytes might emerge out of the plant tissue for reproduction. A newly emerged leaf or any part of the plant may be colonized by a variety of fungal colonies in an asymptomatic or in some cases, symbiotic manner. The main objective of the current study is to scientifically validate the antioxidant activity of methanolic and aqueous extracts of endophytic fungi. Antioxidants are those that inhibit the oxidation by controlling the production of free radicals, thereby stopping the chain reaction that may damage the cells of the organism. The plant endophytes under investigation were *Phoma sp., Colletotrichum spiralis, Chaetomium sp.* (1) and *Chaetomium sp*. (2). Endophytes were isolated from plants and were cultured in Potato dextrose broth. The dry biomass yield of all the four endophytes was subjected for the study. The aqueous and methanolic extracts of the four endophytes were subjected to further investigation The total phenolic content of the four different fungal extracts were determined by using Folin-ciocalteau reagent method. DPPH (1,1- diphenyl-2-picryl-hydrazyl) radical assay was performed to determine the percentage of radical scavenging activity for all the four analytes. A general increase in the antioxidant activity was observed in methanolic extracts when compared with aqueous extracts.

Keywords: Endophytes; Antioxidants; Free Radicals; Folin-Ciocalteau; DPPH Radical Assay

Introduction

Endophytes are those microorganisms that inhabit interior of plants especially leaves, stems, roots and show no apparent harm to host [1]. Almost all classes of vascular plants and grasses examined to date are found to host endophytic organisms [2]. Different groups of organisms such as fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plants [3]. The endophytes existance has been known for over one hundred years. In literal translation, the word endophyte is derived from Greek, 'endo' or 'endon' meaning within, and 'phyte' or 'phyton' meaning plant. Research of endophytic fungi has a deep history and their diversity among plants has been found to be considerably huge. Each plant has been reported to harbor one or more endophytes [4,5]. Recently endophytes are regarded as an outstanding source of secondary metabolites that includes bioactive antimicrobial natural products.

Reactive oxygen species are molecules such as hypochlorite ions, superoxide anions, hydroxyl radical and hydrogen peroxide produced during the cellular metabolism are essential for cell signaling, apoptosis, gene expression and ion transportation. However, ROS can cause oxidative stress and cellular damage if accumulated in the body in excess. 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 [6]. Therefore,

minimizing the oxidation process will promote our physical condition and prevent some degenerative diseases in which free radicals are involved.

Free radicals are often produced as byproducts of biological reactions or from exogenous factors. The free radicals involvement in the pathogenesis of an ample diseases is well documented. Antioxidants have become the topic of interest recently due to their versatile roles in the human body such as radical scavengers, lipid peroxidation inhibitors, and felicitate other free radical mediated processes; therefore, these are able to protect the human body from several diseases caused to the reaction of radicals.

Antioxidants may protect the body against ROS toxicity either by averting the formation of ROS by bringing disruption in ROS attack, by converting them to less reactive molecules or by scavenging the reactive metabolites [7,8]. The fungal compounds are also said to exhibit natural antioxidants [9]. Therefore the uses of antioxidants, both natural and synthetic are gaining broad significance in prevention of diseases.

Usage of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects thus stressing the need for the search for natural antioxidants and free radical scavengers. Clinical trials in the past have confirmed through analysis that the probability of occurrence of diseases such as inflammation, cardiovascular disease, cancer and age related disorders are minimized on the intake of antioxidant rich fruits and vegetables comprising of 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 [10].

Generally, higher plants are hosts to one or more endophytic microbes but one of the least studied biochemical systems in nature is the relationship between organisms and their plant hosts [11]. Endophytes are fungi or bacteria residing inside healthy plant tissues without any discernible infectious symptoms. These groups 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 [12]. Globally, there are at least one million species of endophytic fungi in all plants [13], which can potentially provide a variety of structurally unique, bioactive natural products such as alkaloid, benzopyranones, chinones, flavanoids, phenols, steroids, tetralones, xanthones and others [14]. 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 on the plants and their relation to endophytic biology. Therefore, there is an ample opportunity to unearth novel and interesting endophytic microorganisms with significant therapeutic efficacy [15].

Materials and Methods

Preparation of fungal extracts [16]

Pure cultures of *Phoma sp., Colletotrichum spiralis, Chaetomium sp.* (1) and *Chaetomium sp*. (2) were inoculated in Potato Dextrose broth and were incubated for 6 days in orbital shaker at 100 rpm and at room temperature. After 6 days the broth containing fungal culture was aseptically filtered in laminar air flow chamber to obtain the fungal biomass and the broth as the filtrate. The fungal biomass thus obtained was carefully separated from the filter paper using a spatula and transferred on to a sterile petriplate. The petriplates containing the fungal samples were left in the Hot air oven to dry at 37 - 40 ˚C for 24 hours. The dried fungal samples after 24 hours were weighed and the dry weight of each sample was noted down. Each sample was then distributed into two parts of equal weight and which were homogenized into a thick paste using a mortar and pestle with water and methanol respectively. Caution was taken not to add excess of water or methanol during the process. The prepared fungal extracts were labeled and stored in sterile centrifuge tubes.

Determination of total phenolic content [17]

Total phenolic content of fungal extracts were determined by Folin-Ciocalteau (FC) method employing Gallic acid as standard (1mg/ml) as per the procedure of [17] with some modifications. Different concentrations of standard as well as the water and methanolic extracts were taken and one ml of FC reagent (1:1 dilution) was added, 3 - 5 min later 2.0 ml of sodium carbonate (20%, w/v) was added and the mixture was allowed to stand for 45 min under dark condition. After the specified incubation period, the absorbance of standard and samples were read at 765 nm using Spectrophotometer. The concentration of total phenolics was expressed in terms of mg/g GAE (Gallic acid equivalents)

DPPH free radical scavenging assay [16]

The DPPH radical assay is the most widely used and relatively quick method for determining radical scavenging activity of antioxidants. DPPH consists of stable free radicals and a decrease in the amount of DPPH molecules and consequently a decrease in the

absorbance value for which the visual reduction of color may serve as an external indicator with increasing concentration of fungal extracts indicates the free-radical scavenging potential of the sample under study. 0.1mM solution of DPPH in methanol was prepared. To 2ml of DPPH, different concentrations (100,200,300,400ul) of aqueous and methanolic extracts of the fungal samples were added. The test tubes containing the DPPH and the samples were incubated for 45 minutes in the dark. The absorbance was checked against the blank at 517 nm. Per cent free radical scavenging was calculated based on the extent of reduction in the color.

Results

Determination of total phenolic content

The fungal biomass was filtered from the broth and the after 24 hours of drying at 37˚C, the dry weight of the four different fungal samples were recorded. Maximum fungal growth was observed after 6 days of incubation (Figure 1).

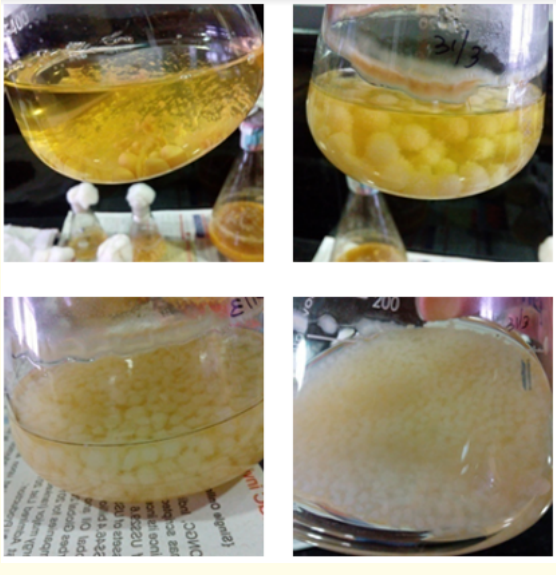


Figure 1: Broth containing fungal culture before filtration. From clockwise, a) *phoma sp*. b) *Colletotrichum spiralis*, c) *Chaetomium sp*. d) *Chaetomium sp*. (1).

Among the four endophytes under study, *Phoma sp.* projected the least growth in Potato dextrose agar, weighing 4.7 grams of dry weight while *Chaetomium sp. (1)* exhibited the maximum growth, weighing 31.47 grams of dry weight (Figure 2).

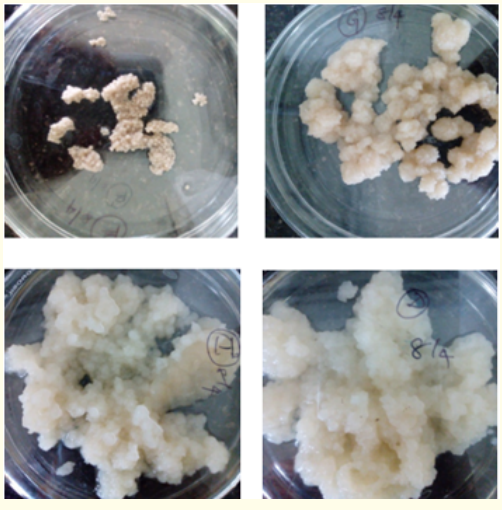


Figure 2: Petriplates containing fungal biomass after filtration. Clockwise, a) *phoma sp*. b) *Colletotrichum spiralis*, c) *Chaetomium sp*. (2), *Chaetomium sp*. (1).

Species	Dry Weight (in gms)
Phoma sp.	4.7
Colletotrichum spiralis	14.69
Chaetomium sp. (1)	31.47
Chaetomium sp. (2)	21.57

Table 1: Dry weight of fungal biomass (in gms).

Total phenolic content of fungal mycelia were determined by Folin-Ciocalteau (FC) method employing Gallic acid as standard (1mg/ml) as per the procedure of [16] with some modifications. Different concentrations of standard as well as the aqueous and methanolic extracts (200,400,600,800µg) were taken and one ml of FC reagent (1:1 dilution) was added, 3-5min later 2.0 ml of sodium carbonate (20%, w/v) was added and the mixture was allowed to stand for 45 min under dark condition. After the specified incubation period, the absorbance of standard and samples were read at 765 nm using Spectrophotometer. The concentration of total phenolic was expressed in terms of mg/g GAE (Gallic acid equivalents).

The Figure 3.1 represents the phenolic content of aqueous and methanolic extracts of *Phoma sp.* which was found out to be 3.2mg/ml and 3.4mg/ml respectively**.** The Figure 3.2 represents the phenolic content of aqueous and methanolic extracts of *Colletotrichum spiralis* which was found out to be 5.6mg/ml and 5.4mg/ml respectively**.**

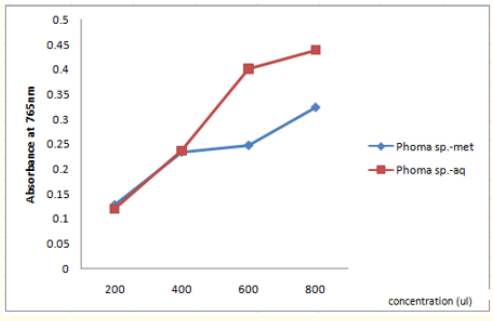


Figure 3.1: The phenolic content of aqueous and methanolic extracts of *phoma sp*. On performing folin-ciocalteau reagent assay for determination of total phenolic content.

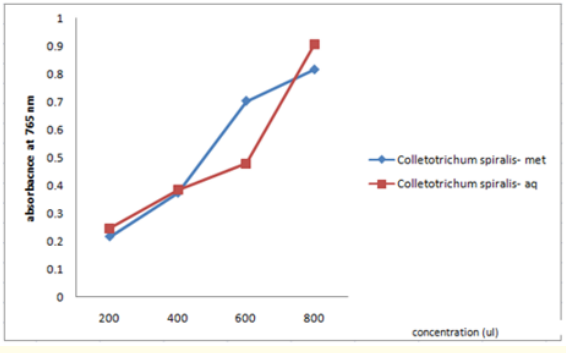


Figure 3.2: The phenolic content of aqueous and methanolic extracts of *Colletotrichum spiralis* on performing Folin-ciocalteau reagent assay for determination of total phenolic content.

The Figure 3.3 represents the phenolic content of aqueous and methanolic extracts of *Chaetomium sp.(2)* which was found out to be 3.81mg/ml and 3.89mg/ml respectively.

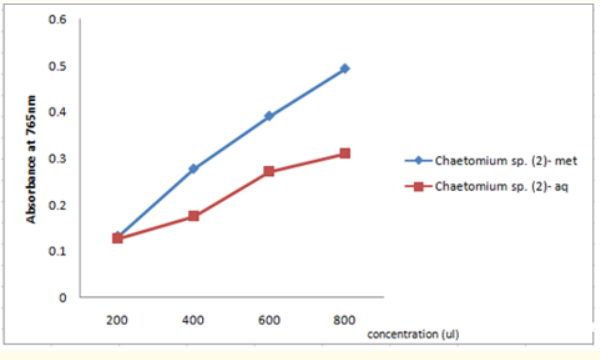


Figure 3.3: The phenolic content of aqueous and methanolic extracts of *Chaetomium* sp. (2) on performing Folin-ciocalteau reagent assay for determination of total phenolic.

The Figure 3.4 represents the phenolic content of aqueous and methanolic extracts of *Chaetomium sp.(1)* which was found out to be 2.93mg/ml and 2.0mg/ml respectively.

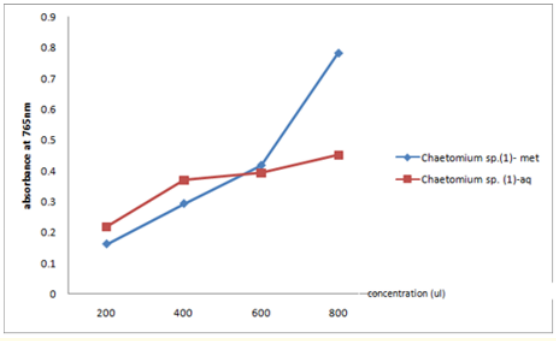
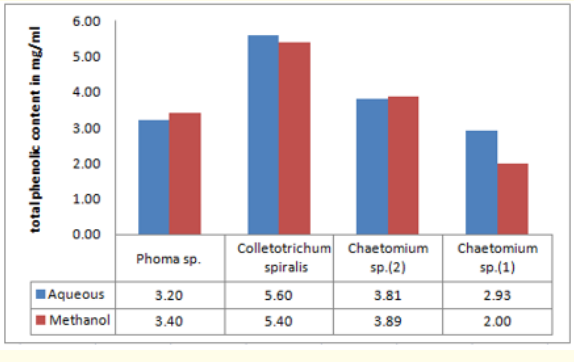


Figure 3.4: The phenolic content of aqueous and methanolic extracts of *Chaetomium* sp. (1) on performing folin-ciocalteau reagent assay for determination of total phenolic content.

The endophytic fungal samples were compared for their total phenolic content in polar solvent systems such as water and methanol. Folin-ciocalteau method for estimation of total phenolic content was performed with Gallic acid as standard. Absorbance values of the fungal extracts at different concentrations were taken at 765nm and the total phenolic content was estimated in GAE (Gallic acid equivalent). The phenolic content of *Phoma sp., Colletotrichum spiralis, Chaetomium sp.(2) and Chaeatomium sp.(1)* in aqueous extract was found to be 3.2 mg/ml, 5.6 mg/ml, 3.81 mg/ml and 2.93 mg/ml respectively and the phenolic content of *Phoma sp., Colletotrichum spiralis, Chaetomium sp.(2) and Chaeatomium sp.(1)* in methanolic extract was found to be 3.4 mg/ml, 5.4 mg/ml, 3.89 mg/ml and 2 mg/ml respectively.



	Phoma sp.	Colletotrichum spiralis	Chaetomium sp.(2)	Chaetomium sp.(1)
Aqueous	3.20	5.60	3.81	2.93
Methanol	3.40	5.40	3.89	2.00

Figure 3.4: The phenolic content of aqueous and methanolic extracts of *Chaetomium* sp. (1) on performing folin-ciocalteau reagent assay for determination of total phenolic content.

Determination of radical scavenging activity

To determine the effects of endophytic fungal extracts of *Phoma sp. Colletotrichum spiralis, Chaetomium sp. (2)* and *Chaetomium sp. (1)* on in vitro antioxidant activity, the DPPH scavenging rate was studied. The DPPH radical contains an old electron, which is accountable for the absorbance at 517 nm and also for a visible deep purple color. DPPH is decolorized when it accepts an electron donated by an antioxidant compound, which can be quantitatively measured from the changes in absorbance [18].

The decrease in the amount of DPPH free radicals is directly proportional to the amount of antioxidant activity.

The radical scavenging activity of aqueous and methanolic extracts of *Phoma sp.* revealed 32.66% activity in aqueous extract and 33% in methanolic extracts when absorbance values were taken at 517nm (Figure 5.1).

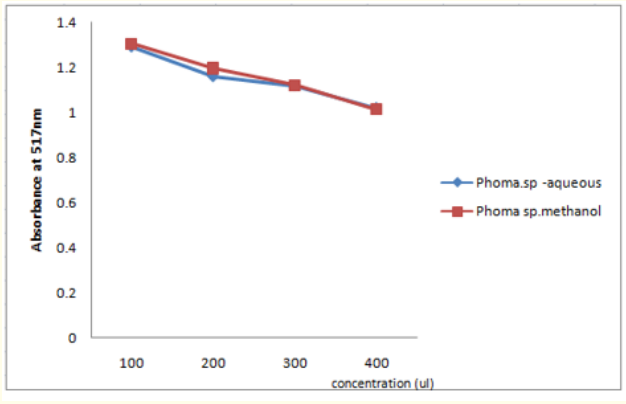


Figure 5.1: DPPH assay for determination of radical scavenging activity of aqueous and methanolic extracts of *phoma sp*.

The radical scavenging activity of aqueous and methanolic extracts of *Colletotrichum spiralis* revealed 64% activity in aqueous extract and 74% in methanolic extracts when absorbance values were taken at 517nm (Figure 5.2).

The radical scavenging activity of aqueous and methanolic extracts of *Chaetomium sp. (2)* revealed 65% activity in aqueous extract and 70% in methanolic extracts when absorbance values were taken at 517nm (Figure 5.3).

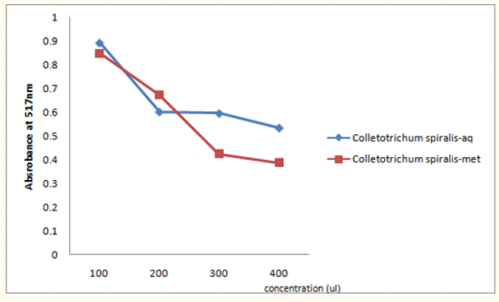


Figure 5.2: DPPH assay for determination of radical scavenging activity of aqueous and methanolic extracts of *Colletotrichum spiralis*.

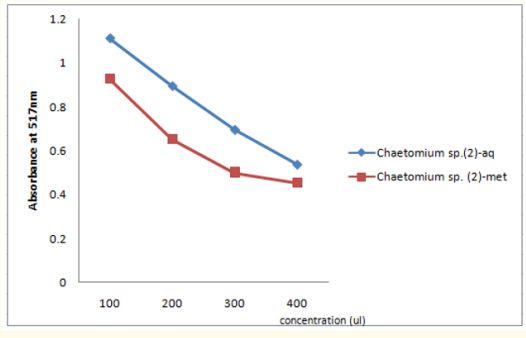


Figure 5.3: DPPH assay for determination of radical scavenging activity of aqueous and methanolic extracts of *Chaetomium sp*. (2).

The radical scavenging activity of aqueous and methanolic extracts of *Chaetomium sp. (1)* revealed 58% activity in aqueous extract and 63% in methanolic extracts when absorbance values were taken at 517nm (Figure 5.4)

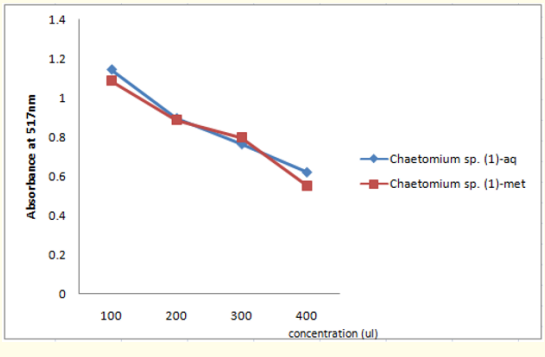


Figure 5.4: DPPH assay for determination of radical scavenging activity of aqueous and methanolic extracts of *chaetomium sp*. (1).

The percentage of radical scavenging activity can be calculated by the below formula,

$$\% \text{ of radical scavenging} = \frac{A_c - A_s}{A_c}$$

Where A_c= Absorbance value of Control and A_s = Absorbance value of sample [16].

The percentages of free radical scavenging of aqueous extracts of *Phoma s., Colletotrichum spiralis, Chaetomium sp. (2)* and *Chaetomium sp. (1)* were found out to be 34%, 64%,65% and 58% respectively. The percentages of free radical scavenging of methanolic extracts of *Phoma sp., Colletotrichum spiralis, Chaetomium sp. (2)* and *Chaetomium sp. (1)* were found out to be 33%, 74%,70% and 63% respectively (Figure 6). A decrease in the percentage of radical scavenging activity was observed in aqueous extracts of fungal samples (Table 2) when compared with the percentage of radical scavenging activity of methanolic extracts of fungal samples (Table3).

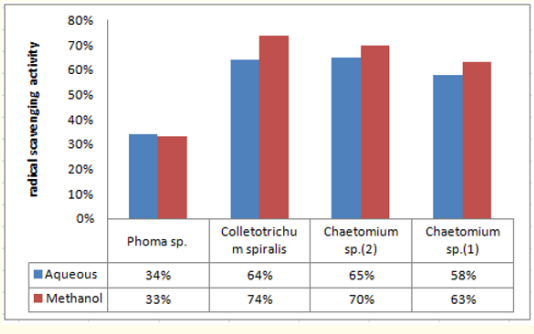


Figure 6: The percentage of radical scavenging activity of aqueous and methanolic extracts of the four species a) phoma sp. b) *Colletotrichum spiralis* c) *Chaetomium sp*. (2) and d) *Chaetomium sp*. (1).

Fungal extract/ Conc. ug/ml	100	200	300	400
Phoma sp.	19%	23.33%	26%	32.66%
Colletotrichum spiralis	40.6%	59%	60%	64%
Chaetomium sp.(2)	26%	40%	54%	64%
Chaetomium sp. (1)	24%	40%	49%	58%

Table 2: Antioxidant activity of aqueous extracts of fungal samples.

Fungal extract/ Conc. ug/ml	100	200	300	400
Phoma sp.	12.95%	20.2%	25.3%	33%
Colletotrichum spiralis	44%	55%	72%	74%
Chaetomium sp.(2)	38%	56%	66%	70%
Chaetomium sp. (1)	28%	41%	47%	63%

Table 3: Antioxidant activity of methanolic extracts of fungal samples.

Discussion

The primary objective of our study was to compare four different fungal endophytes namely *Phoma sp*., Colletotrichum spiralis, *Chaetomium sp*. (2) and *Chaetomium sp*. (1) based on their antioxidant activity which is directly related to the total phenolic content of the fungal extracts. The antioxidant activity was also quantitatively determined in terms of percentage of radical scavenging activity by subjecting the fungal extracts to DPPH radical assay. Methanolic extract of *Colletotrichum spiralis* displayed the highest percentage of radical scavenging activity of 74% and among the aqueous extracts *Chaetomium sp*. (2) recorded the highest percentage of radical scavenging activity which was 65% (Figure 6). In terms of total phenolic content which was estimated against the standard Gallic acid curve by Folin-ciocalteau method, *Colletotrichum spiralis* exhibited the highest phenolic content of 5.6mg/ml and 5.4mg/ml in aqueous and methanolic extracts respectively (Figure 6). Proper correlation could not be established in polyphenolic content and antioxidant activities which may be due to that FC reagent is not specific for just polyphenols as it can be reduced by many non phenolic compounds such as vitamins C, Cu (I) etc. Various others workers have also reported the poor specificity of the assay [19,20]. Further, variation in correlation coefficient among different antioxidant assays indicates that a single assay is not sufficient to evaluate the total antioxidant activity. Moreover, the polyphenolic contents of the fungi may not act as an index of their antioxidant activity as they possess many different enzymes, such as catalase and superoxide dismutase and many others components such as glutathione which may be responsible for antioxidant activity and the mechanism in fungi may be different from plants and it may become clearer in near future. Endophytic fungi are reported as sources of ample bioactive compounds and secondary metabolites. These bioactive compounds and secondary metabolites has application in biological control. Endophytes are thought to use chemical compounds to mediate interactions with other antagonists. In this study Colletot-

richum have been reported as the endophyte that has recorded the highest antioxidant activity among the four species under study, in comparison. Colletotrichum gloeosporioides is present as an endophytic fungus in Artemisia Mongolic and has been reported to produce a new antimicrobial metabolite, colletotric acid (Zou et al, 2000) [21]. Colletotrichum musae and *C. gloeosporioides* are found as an endophytes in banana plant (Photita *et al*., 2001) [22]. It is reported that the metabolites extracted from the endophytic fungus Colletotrichum sp. showed strong antimicrobial activity against various strain [23-57]. Recent reports have found that hundreds of natural products including alkaloids, flavonoids, and steroids, have been obtained from endophytes.

Conclusion

From the present study of four different endophytic fungi namely *Phoma sp., Colletotrichum spiralis, Chaetomium sp.(1)* and *Chaetomium sp.(2)* on the antioxidant activity of their aqueous and methanolic extracts by performing Folin-ciocalteau method for estimation of total phenolic content and DPPH radical assay to determine free radical scavenging activity in percentage, it can be concluded that among the four species, *Colletotrichum spiralis* exhibited the maximum phenolic content of 5.6 mg/ml and 5.4 mg/ml in GAE in aqueous and methanolic extracts respectively. Similarly, methanolic extract of *Colletotrichum spiralis* exhibited the maximum radical scavenging activity of 74% when compared with other species.

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