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ANTIOXIDANT POTENTIAL OF ENDOPHYTIC FUNGUS *COLLETOTRICHUM* SPECIES ISOLATED FROM *POLYGALA ELONGATA*

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ABSTRACT

Colletotrichum sp. was isolated as an endophyte of herbaceous plant *Polygala elongata* collected from Western Ghats, India. In vitro evaluation of antioxidant property of ethyl acetate extract of this fungus was done using ABTS and DPPH radicals. The ethyl acetate extract of *Colletotrichum* species showed potent antioxidant activity against both ABTS and DPPH radicals with the IC_{50} value of 9.312µg/ml and 21.22µg/ml, respectively. Total amount of phenol and flavonoid quantified were of 1614mg/g gallic acid equivalents and 160mg/g of quercetin equivalent, respectively. In addition, total antioxidant capacity of the crude extract was evaluated by the phosphomolybdenum method and quantified as 23.695mg/g of Gallic acid equivalent. This study suggests that, the crude extract of *Colletotrichum* sp. may have potential source of natural antioxidant.

KEYWORDS: **Antioxidant, DPPH, ABTS, phenol, *Colletotrichum* sp., *Polygala elongata***

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INTRODUCTION

Reactive oxygen species (ROS) produced during the cellular metabolism are essential for cell signalling, apoptosis, gene expression and ion transportation[1]. 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[2, 3]. This concept is supported by increasing evidence that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease[4]. Antioxidants are the substances that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance[5]. Apart from their role of health benefactors, antioxidants are added in foods to prevent or delay oxidation of food, initiated by free radicals formed during their exposure to environmental factors such as air, light and temperature[6]. A myriad of both natural and synthetic antioxidants has been advised for use in the treatment of various human maladies[7]. The main disadvantage with the synthetic antioxidants is the side effects when taken in vivo[8]. 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[9]. Endophytic fungi are microorganism hidden within healthy host plant were poorly investigated group among other microorganisms, they represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitations in a wide variety of medicinal, agricultural and industrial areas[10]. There are many reports and studies on the biological activities of endophytic species of genus *Colletotrichum* like anticancer and antimicrobial effects[11,12]. Apart from these biological properties, the reports published on the antioxidant properties of *Colletotrichum* were very few. Hence in present study *Collerotrichum* sp. was isolated from plant *Polygala elongata*, the isolated fungus cultivated under solid state fermentation was evaluated for its antioxidant activity.

MATERIALS AND METHODS

Isolation of Endophytic fungi

An endophytic *Colletotrichum* species was isolated from leaves of the medicinal plant *Polygala elongata* (Polygalacae) following previously described procedures[13]. Briefly, external tissues of plant parts were fully exposed to 70% ethanol (EtOH, v/v) prior to excision of internal tissues, which were cultured on standard water agar medium (WA) augmented with 50 µg/mL of streptomycin sulphate. Individual fungal colony growing out of plant tissues were then hyphal tipped and transferred on to potato dextrose agar (PDA). After proper growth and fruiting, on the basis of morphological and cultural characteristics, the isolated fungus was identified[14]. The axenic culture was maintained on potato dextrose agar.

Fermentation

Broken rice grain was used as substrate for cultivation of endophytic fungus for secondary metabolite production. A 100g of rice was washed with distilled water and transferred in 250ml Erlenmeyer flasks and autoclaved at 121^{0}C for 20 minutes, twice at alternate day. Erlenmeyer flasks containing sterile rice grains were kept for a few hours to cool down and inoculated with 2mm × 2mm of mycelia culture grown on PDA for 5-days in advance and under aseptic conditions. The inoculated flask was incubated at 28^{0}C. After twenty one days, fermented rice was extracted with ethyl acetate using Soxhlet apparatus. Obtained extract was then concentrated by evaporation of solvent.

Antioxidant assays

(i) DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging activity

The DPPH radical scavenging assay was performed as described by Miliauskas et al[15] with slight modifications. In brief, 2-ml of

DPPH solution (0.06mM) was added to 1-ml of test sample (dissolved in methanol) of concentrations (18.75, 37.5, 75, 150 and 300 µg/ml) leading to the final concentration of 6.25, 12.5, 25, 50, and 100 µg/ml. After 24 hours reaction, absorbance was read at 517 nm. Ascorbic acid was used as control. 1ml of methanol was used as negative control. The DPPH radical scavenging activity was calculated using the following equation:
% Radical scavenging activity = $[(A_0-A_1/A_0) \times 100]$, where A_0 was the absorbance of the control reaction and A1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (IC_{50}) was obtained by interpolation from linear regression analysis.

(ii) ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity

Two stock solutions included 7.4mM ABTS and 2.6mM potassium persulphate[16]. Working solution was then prepared by mixing two stock solutions in equal quantities and allowing them to react for 12-16 hrs at room temperature in dark. After 12-16 hrs, the solution was diluted using 50ml of methanol to obtain absorbance 1.1 ± 0.02 units at 734nm. 1ml samples (dissolved in methanol) of different concentrations (6.25-200µg/ml) were mixed with 2ml of ABTS solution and were left at room temperature for 2hr in the dark. The absorbance was then measured at 734nm. Ascorbic acid was used as a control. 1ml of methanol was used as negative control. The capability to scavenge the ABTS radical was calculated using the following equation: ABTS scavenging effect (%) = $[(A_0-A_1/A_0) \times 100]$ where A_0 was the absorbance of the control reaction and A_1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (IC_{50}) was obtained by interpolation from linear regression analysis.

Determination of Antioxidant Component

(i) Total Phenol

Total phenolic compounds were determined using Folin-Ciocalteu's method[17]. To 0.5ml of sample (100µg/ml) 2.5ml of 10% Folin-Ciocalteu's reagent and 2.5 ml of 7.5% sodium carbonate was added and incubated at 45^0C for 15mins and absorbance was measured at 765nm against blank. The content of total phenol was calculated on the basis of the calibration curve of gallic acid and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

(ii) Flavonoid

Total flavonoid was determined according to Barros et al[18]. The fungal extract (250µl) was mixed with distilled water (1.25 ml) and $NaNO_2$ solution (5%, 75µl). After 5 mins the $AlCl_3$ H_2O solution (10%, 150µl) was added. After 6 min, NaOH (1M, 500µl) and distilled water (275µl) were added to the mixture. The solution was mixed well and the intensity of the pink color was measured at 510 nm against the blank. The content of flavonoid was calculated on the basis of the calibration curve of quercetin and the results were expressed as mg of quercetin equivalents per g of extract.

Determination of antioxidant capacity by phosphomolybdenum assay

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method of Prieto, Pineda, & Aguilar[19]. An aliquot of 0.3 mL of sample solution (200µg/ml) was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). In case of the blank, 0.3 ml of the solvent was used in place of the sample. The tubes were capped and incubated in a water bath at 95^0 C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm. The antioxidant capacity was expressed as an equivalent of gallic acid (mg gallic acid/g dried extract).

RESULTS

Radical scavenging activity using DPPH

DPPH, a stable free radical with the characteristic absorption at 517nm, was used to the radical scavenging effects of ethyl acetate extract of *Colletotrichum* sp. As an antioxidant donate a proton to this radical, the absorption decreases. The sample was tested at different concentrations ranging from 6.25 to 100µg/ml and readings were observed by

decreasing the absorbance taken as a measure indicates the extent of radical scavenging property (Fig 1). The scavenging effect of the sample was evaluated along with standard Ascorbic acid. The extract showed maximum inhibition of 81.72% at maximum concentration 100 µg/ml. IC_{50} value against DPPH, obtained by interpolation from linear regression analysis was found to be 21.22µg/ml (Table 1) as compared to the IC_{50} value of ascorbic acid 1.74µg/ml.

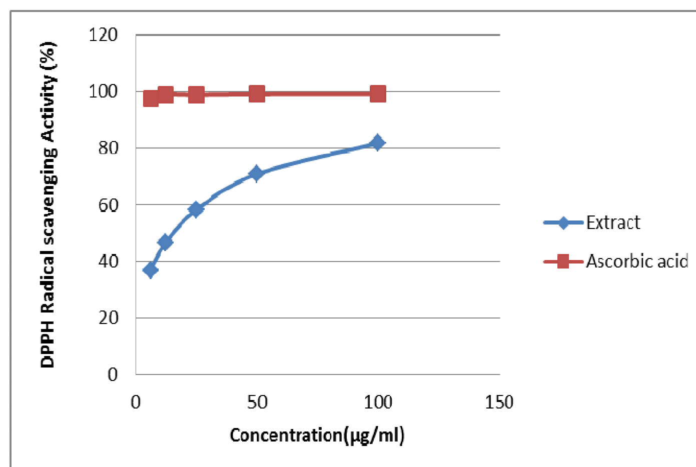

Figure 1
DPPH radical scavenging activity of Colletotrichum sp.

Radical scavenging activity against ABTS
ABTS a stable free radical with the characteristic absorption at 734 NM was used to study the radical scavenging effect of the extract. The results demonstrated that the extract reacted with ABTS at different concentration ranging from 6.25µg/ml to 100 µg/ml and the readings were observed by measuring the reduction of radical cation generated by $ABTS^{+}$ at 734 nm. The ethyl acetate extract of *Colletotrichum* sp. showed a maximum inhibition of 96.03% at a maximum concentration of 100µg/ml with the IC_{50} value 9.31µg/ml (Table 1) as compared to IC_{50} value of ascorbic acid 9.10µg/ml. The extent of decolourization is directly proportional to the increased concentration of the extract (Fig 2).

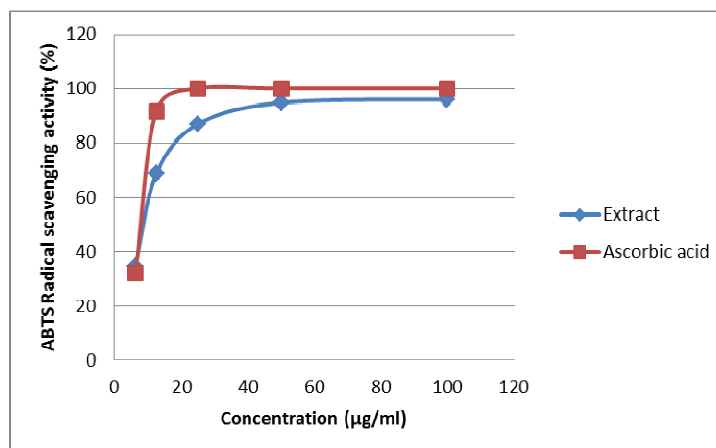

Figure 2
ABTS radical scavenging activity of Colletotrichum sp.

Determination of antioxidant compounds

The total phenolic content in ethyl acetate extract of *Colletotrichum* sp. was found out to be 1614mg/g of Gallic Acid Equivalent and flavonoid content was 160mg/g of quercetine equivalent (Table 1). Results revealed that ethyl acetate extract of *Colletotrichum* sp. contains significant amount of phenols and flavonoids.

Determination of antioxidant capacity by phosphomolybdenum assay

Total antioxidant capacity of the Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the ethyl acetate extract and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. Total antioxidant capacity was found to be 23.695mg/g of Gallic acid equivalent (Table 1).

Table 1

IC_{50} values and bioactive compounds from ethyl acetate extract of Colletotrichum sp.

Sample	IC_{50} (µg/ml)		Phenol (mg/g)	Flavonoid (mg/g)	Total antioxidant capacity (mg/g)
	DPPH	ABTS			
Colletotrichum sp.	21.22	9.31	1614	160	23.695

DISCUSSION

In present study, DPPH and ABTS radical scavenging activity of extract of *Colletotrichum* sp. have been evaluated. Even though the DPPH and ABTS scavenging aptitude of the extract were found to be lower at concentrations of 6.25-100µg/ml than that of the activity of commercial antioxidant, ascorbic acid at same concentrations, it still reached 81.72% inhibition against DPPH and 96.03% against ABTS at 100µg/ml concentration. The extract exhibited scavenging activity against ABTS radical with IC_{50} of 9.31µg/ml which is almost equivalent to IC_{50} value of ascorbic acid (9.10µg/ml). Though it exhibited activity against DPPH with IC_{50} 21.22 µg/ml higher than that of Ascorbic acid (1.74 µg/ml), studies have established the proton donating ability of the extract as well as the capacity of the crude extract to serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. Tianpanich et al[20] has evaluated radical scavenging activities of isocoumarins and a phthalide from the endophytic fungus *Colletotrichum* sp. Phenolic and flavonoid compound seem to have an important role in stabilizing lipid peroxidation, associated with antioxidant activity[21]. The results of the present study reveal that the ethyl acetate extract of *Colletotrichum* sp. contains significant amount of phenols and flavonoids. Srinivasan et al[22] have reported total phenolic content in range of 18.33 mg/g Gallic acid equivalent and flavonoid content of 6.44mg/g of quercetine equivalent in ethanolic extract of *Phyllosticta* sp. The antioxidant content range was less when compared with the current study. Total antioxidant activities of the extracts were evaluated by phosphomolybdate method that is routinely used to evaluate the total antioxidant capacity of the extracts[23]. The extract showed significant inhibition percentage. Pushpalatha et al[24] have reported total antioxidant activity of some endophytic fungi.

CONCLUSION

Present study concludes that the endophytic *Colletotrichum* sp. have phenolic and flavonoid content and showed excellent activity against DPPH and ABTS radicals. Hence it could be a source of natural antioxidants. Moreover, attempts should be made, using analytical chemistry procedures, in order to isolate and identify the bioactive compounds responsible for the antioxidant activity reported here.

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