In vitro Antioxidant Activity and Phytochemical Screening of Endophytic Extracts of *Crotalaria pallida*

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ABSTRACT

Introduction: The species *Crotalaria pallida*, which belongs to the Fabaceae family (Sub-family Faboideae), the members of which are herbs, shrubs and trees found in both temperate and tropical areas. All parts of the plants were incubated to know and isolate the endophytes. Antioxidants play an important role in protecting cellular damage by reactive oxygen species. Phenolic compounds from plants or endophytes have been reported to possess strong antioxidant properties. **Results:** Four different endophytic fungi isolated from *Crotalaria pallida* were tested for various phytochemicals. *Aspergillus niger* and *Fusarium oxysporum* yielded the tannin, flavonoids, tepenoids, phenol and saponins from ethanol extract. All four different endophytic extracts were used to evaluated *in vitro* antioxidant activity by ABTS, DPPH and FRAP method. Antioxidant compounds like total phenol and flavonoid were also determined. The ethanol extracts of *A. niger* and *F. oxysporum* showed potent antioxidant activity against ABTS, FRAP and DPPH radicals with EC_{50}. The total amount of phenol and flavonoid quantified were of 19.20, 19.23 gallic acid equivalent per gram of two endophytic fungi, *A. niger* and *F. oxysporum* and 7.25 and 6.41 µg/mg of quercetin equivalent respectively. **Conclusions:** The antioxidant potential may be directly linked to the phenolic compounds present in the endophytes, *A. niger* and *F. oxysporum* of *Crotalaria pallida*. The outcome of the present investigation clearly indicates that *A. niger* and *F. oxysporum* showed potential phytochemicals and they can used as antioxidants.

Keywords: Antioxidant, endophytic fungi, ABTS, FRAP, DPPH, Phenol

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INTRODUCTION

Oxygen is essential element for the survival of all living creatures on this earth. During the process of oxygen utilization in a normal physiological and catabolic process, approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals (ROS) like superoxide anions (O2 –), hydroxyl (.OH) nitric oxide (NO), which damage cellular components causing tissue injury through covalent binding.[1,2] Free radicals have been implicated in causation of diseases such as diabetes, inflammation, cancer, neurodegenerative disorders, atherosclerosis, liver cirrhosis, nephrotoxicity etc.[3] It has been suggested that fruits, vegetables, plants are the main source of antioxidant in the diet. Natural antioxidants may have free-radical scavengers, reducing agents, complexes of pro-oxidant metals, quenchers of singlet oxygen etc. Recently interest has been increased considerably in finding natural occurring antioxidants for use in foods or medicinal products to replace synthetic antioxidants, which are being restricted due to their adverse reaction such as carcinogenicity. Antioxidant constituents from natural resources possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance.[4]

Free radicals which have one or more unpraired electrons (superoxide, hydroxyl, peroxyl) are produced in normal or pathological cell metabolism and the compounds that can scavenge free radicals have great potential in ameliorating the diseases and pathological cells.[5,6,7] Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species. Free radicals or Reactive Oxygen Species (ROS) are produced *in vivo* from various biochemical reactions and also from the respiratory chain as a result occasional challenges. These free radicals are the main culprits in lipid peroxidation. Plants congaing bioactive compounds have been reported to possess strong

antioxidant properties. In many inflammatory disorders there is excessive activation of phagocytes, production of 0_2-, OH radicals as well as non free radicals species (H_20_2),[8] which can harm severely tissues either by powerful direct oxidizing action or indirect with hydrogen peroxide and –OH radical formed from O_2- which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors.[9] The reactive oxygen species are also known to activate matrix metello proteinase damage seen in various arthritic tissues.[10] The literature survey is giving the importance of plants and their antioxidant properties,[11] plant active compounds especially phenolic and flavonoid compounds have proved as potent antioxidant and free radical scavenger.[12] Endophytic fungi are microorganisms hidden within healthy host plant but they do not cause any harmful and they are able to produce the bioactive compounds what host plant is producing. These active molecules are exploiting from endophytes for variety of medicinal, agricultural and industrial purposes.[13] Many of the endophytes have showed few biological activities of antimicrobial, antioxidant, anticancer and anti-HIV.[14,15,16] Apart from the biological properties, the reports published on endophytic antioxidant properties were very few.

The literature survey indicates that no reports are available from India regarding *in vitro* antioxidant activity of endophytic extract of *C. pallida*. In present study was aimed to examine the total phenol and flavonoid content and phytochemical analysis. The findings from this work may add to the overall value of the medicinal potential of the plant.

MATERIALS AND METHODS

The plant was collected in November 2009 from our college campus (Shridevi Institute of Engineering & Technology, Sira Road, Tumkur, Karnataka, India). The plant was identified by their vernacular names and later it was compared with the herbarium of Department of Studies in Botany, Manasa Gangothri, University of Mysore, Mysore and Government Ayurvedic College, Mysore, India.

Isolation and identification of endophytic fungi

The protocol for isolation follow methods used in other endophyte study [17] but adjusted for the specific plant tissues used here following pilot experiments. The plant tissues were washed in running tap water for one hour. Fifty segments of leaves from each plant were cut into 5 mm 2 pieces, including a vein (25 samples) and intervein (25 samples). 25 segments of branches were then cut randomly to a length of 5 mm. Endophytic fungi were isolated from the bark of the plant (25 segments). Twenty five segments (5 mm long) were cut from the stems and the roots. The total 150 segments of plant material were treated by triple surface sterilization techniques.[18] Each piece was then placed on malt extract agar (malt extract (20g/l), rose Bengal (0.033 g/l), chloromphenicol (50 mg/l, agar (15g/l). All plates were incubated at 26 ± 2 °C until mycelium grew out hyphal tips were cut and transferred to Potato Dextrose Agar (PDA). Half strength PDA was used for subculture and stock culture. Identification was based on colony and hyphal morphology of the fungal cultures, characteristics of the spores.[19,20]

Fungal cultivation and extraction of metabolites

The fungal endophytes were cultivated on Potato Dextrose Broth (Himedia, Germany) by placing agar blocks of actively growing pure culture (3mm diameter) in 250 ml Erlenmeyer flasks containing 100ml of the medium. The flasks were incubated at 26 ± 2 °C for 1 week with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through sterile cheesecloth to remove the mycelia mats.

Solvents

Identification of the phytochemical active substances carried out using methanol solvent at 5 g/15ml (W/V).

Phytochemical analysis

Phytochemical analysis was carried out for saponins, flavonoids, cardiac glycosides, terpenoids, steroids, tannins, phenol, anthroquinone, alkaloids[21] and tannins[22] was performed as described by the authors. Wagner's and Heger's reagents was used for alkaloid foam test for saponins, Mg-HCl and Zn-HCl for flavonoids, Keller-Killani test for cardiac glycosides, Salkonoski test for terpenoids, acetic anhydride and sulphuric acid for steroids, chloride and gelatin for tannins, ferric chloride for phenol, hexane and diluted ammonia for anthraquinones test. All these experiments were carried out for ethanol extract of dry parts of stem, leaf and flower individually.

Determination of antioxidant activity

In order to investigate the antioxidant properties of the examined extracts, ferric ion reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS assays.

DPPH radical scavenging assay

The free radical scavenging activities of extracts were measured by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Briefly, extract concentration of (0.1-20 mg/ml) in methanol (4 ml) was mixed with 1 ml of methanol solution containing DPPH (Sigma) radicals of 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min in the dark and the absorbance was measured at 517 nm against a blank.[23] EC_{50} value (mg/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and the value was obtained by interpolation from linear regression analysis. BHT was used as standard for the comparison. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \left[\frac{\{A_0 - A_1\}}{A_0}\right] \times 100,$$

Whereas A_0 is the absorbance of the control reaction and A_1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (EC_{50}) was calculated was obtained by interpolation from linear regression analysis.

ABTS radical scavenging activity

The two stock solutions included 7.4 mM 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2.6 mM potassium persulphate was prepared.[24] The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was diluted by mixing with 1 ml ABTS solution prepared using 50 ml of methanol, in order to obtain absorbance 1.1 ± 0.02 units at 734 nm. Samples (1.5 ml) were mixed with 2.850 ml of ABTS solution and the mixture was left at room temperature for 2 h in dark. The capability to scavenge the ABTS radical was calculated using the following equation:

$$\text{ABTS scavenging effect (\%)} = \left[\frac{\{A_0 - A_1\}}{A_0}\right] \times 100,$$

Whereas A_0 is the absorbance of the control reaction and A_1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (EC_{50}) was calculated was obtained by interpolation from linear regression analysis.

FRAP assay

FRAP reagents was freshly prepared by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL 2,4,6-tris (2-pyridyl)-S-triazine (TPTZ) solution (10 mM TPTZ in 40 mM/L HCl) and 2.5 mL $FeCl_3$ (20 mM) water solution. Each sample (150 µL) (0.5 mg/mL) dissolved in methanol was added in 4.5 mL of freshly prepared FRAP reagent and stirred and after 5 min, absorbance was measured at 593nm, using FRAP working solution as blank.[25,26] A calibration curve of ferrous sulfate (100-1000 µmol/L) was used and results were expressed in µmol Fe^{2+}/mg dry weight extract. The relative activity of the samples was compared to L-ascorbic acid.

Determination of total phenolic content

Total Phenolic Content (TPC) in endophytic extracts was determined according to Taga et al.[27] using Folin-Ciocalteu's colorimetric method. To 5 ml of 0.3% HCl in methanol/deionised water (60:40, v/v), 100 mg of the ethanol extract was added. From the resulting mixture (100 µl) was added to 2 ml of 2% aqueous sodium carbonate. The mixture was incubated for 2 min. To that 100 µl of 50% Folin-Ciocalteu's reagent was added and incubated for 30 min, absorbance was measured at 750 nm 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.[28]

Flavonoid determination

Total flavonoid was determined according to Barros et al..[29] The fungal extract (250 µl) was mixed with distilled water (1.25 ml) and $NaNO_2$ solution (5%, 75 µl). After 5 min the $AlCl_3$ H_2O solution (10%, 150 µl) was added. After 6 min, NaOH (1M, 500 µl) and distilled water (275 µl) were added to the mixture. The solution was mixed well and the intensity of the pink color was measured at 510 nm against 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.

RESULTS AND DISCUSSION

Totally our different endophytic fungal species were identified in different parts of *C. Pallida* (Table 1). The four different fungal species are *Aspergillus flavus, Aspergillus niger, Fusarium oxysporum* and *Fusarium solani*.

The phytochemical screening showed that the *Aspergillus niger, A. flavus* and *Fusarium oxysporum* ethanol extracts yielded the tannins, flavonoids, terpenoids, phenols and saponins. The steroids, alkaloids, anthraquinones were absent in all the extracts except *F. oxysporum* extract. However, the *Fusarium solani* extract showed absence of all the phytochemicals tested, only one saponin was present (Table 2).

The antioxidant activity of the ethanol extract was measured by the ability to scavenge DPPH free radicals, was compared with the standards Butylated HydroxyToluene (BHT). It was observed that ethanol extract of the *Aspergillus niger, Fusarium oxysporum, Aspergillus flavus* had higher activity than that of *Fusarium solani*. At a concentration of 0.1 mg/ml, the scavenging activity of ethanol extract of the *A. niger* and *F. oxysporum* reached 88.61% and 86.72% respectively while at the concentration, that of *A. flavus* and *F. solani* was 51.66% and 49.13%. Though the DPPH radical scavenging abilities of the extract were less than those of (97.8%) at 0.1 mg/ml, the study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants (Figure 1). The performance of ethanol extract of endophytic fungi, *Phyllosticta* sp was higher than that the standard μ-tocopherol,[28] red alga *Polysiphonia urceolata* [30] and from different endophytes.[31]

ABTS a stable free radical with the characteristic absorption at 734 nm was used to study the radical scavenging effect of endophytic extract reacted with ABTS at different concentration ranging from 100, 200, 400, 800 and 1600 μg/ ml respectively and readings were observed by measuring the reduction of radical cation generated by $ABTS^+$ at 734 nm (Figure 2). The ethanol extract of *A. niger* and *F. oxysporum* showed maximum decolouration of 1600 μg/ml with the EC50 587.06 ± 0.74 and 566.71 ± 45 and the other two endophytes showed less decolouration (Table 3). ABTS assay is an excellent tool for determining the antioxidant activity of phytochemicals.[32] The edible basidiomycetes and endophytes assayed against ABTS radical and reported to have scavenging ability against these radicals.[32,33,28]

Table 1. List of endophytes from different parts of *Crotalaria pallida* on PDA media

Types of endophytes	leaves		bark	stem	root	petiole
	vein	Inter-vein				
Aspergillus niger	+	+	+	+	+	+
Aspergillus flavus	+	+	+	+	+	+
Fusarium oxysporum	+	+	+	+	+	+
Fusarium solani	+	+	+	+	+	+

Experiments were repeated for thrice for each sample, + = presence

Table 2. Phytochemical analysis of ethanol extract of different plant parts

Tests	*Aspergillus niger*	*Aspergillus flavus*	*Fusarium oxysporum*	*Fusarium solani*
Tannin	+ve	+ve	+ve	–ve
Steroids	–ve	–ve	–ve	–ve
Cardiac glycosides	–ve	–ve	+ve	–ve
Flavonoids	+ve	+ve	+ve	–ve
Terpenoids	+ve	+ve	+ve	–ve
Alkaloids	–ve	–ve	–ve	–ve
Phenol	+ve	+ve	+ve	–ve
Saponins	+ve	+ve	+ve	+ve
Anthraquinones	–ve	–ve	–ve	–ve

Experiments were repeated for thrice for each sample, +ve: positive, –ve: negative

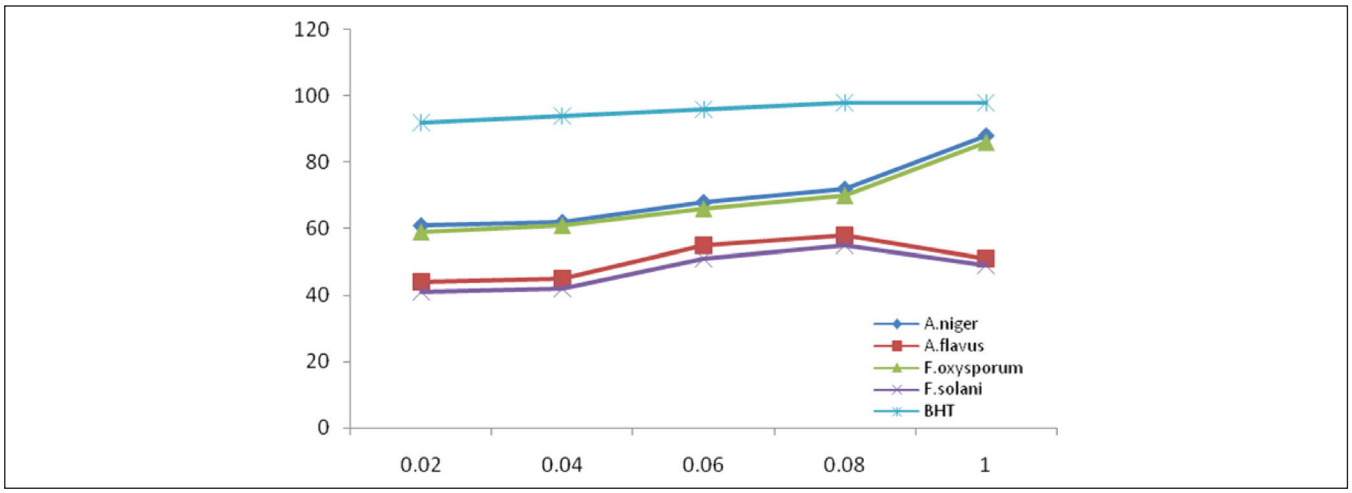

Figure 1. DPPH scavenging activities of the ethanol endophytic extracts

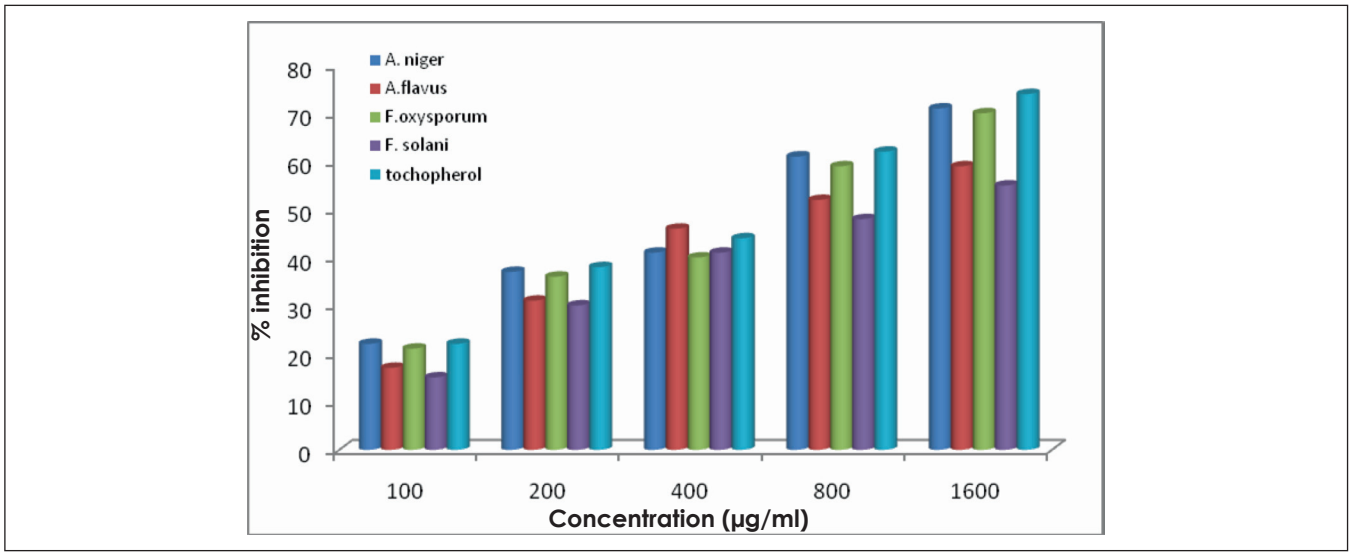

Figure 2. Free radical scavenging effect of different endophytic extracts against ABTS

Table 3. EC_{50} values of endyphytic ethanol extract

Sample	EC_{50} (µg/ml)	
	ABTS	DPPH
Aspergillus niger	587.06 ± 0.74^{b}	2054.63 ± 0.74^{a}
Aspergillus flavus	464.31 ± 0.74^{d}	1784.11 ± 0.74^{c}
Fusarium oxysporum	566.71 ± 0.74^{c}	2009.42 ± 0.74^{b}
Fusarium solani	448.22 ± 0.74^{e}	1544.63 ± 0.74^{d}
Plant stem extract	591.15 ± 0.74^{a}	2011.46 ± 0.74^{b}

Repeated the experiments three times for each replicates, According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at P ≤ 0.05, SE-standard error of the mean.

The reducing ability of the endophytic extracts was in the range of 448.26-1266.14 µm Fe (II)/mg (Table 4). The antioxidant potentials of the ethanol extracts of *A. niger* and *F. oxysporum* were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). The FRAP values for the ethanol endophytic extracts significantly lower that of ascorbic acid but higher that of BHT. Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenol and antioxidant activity appears to be the trend in many plant species.[34]

Phenolic and flavonoid compound seems to have an important role in stabilizing lipid oxidation, associated

with antioxidant activity.[35] Total phenol found to be in *Aspergillus niger* and *Fusarium oxysporum* of 19.20 and 18.23 mg/GAE/g dry weight and flavonoid content of *Aspergillus niger* and *Fusarium oxysporum* of 7.25 and 6.41 µg/mg equivalent respectively (Table 5). The present investigation results reveal that ethanol extract of *Aspergillus niger* and *Fusarium oxysporum* contains significant amount of phenols and flavonoids. Liu et al.[35] and Srinivasan et al.[28] have reported similar results from endophytic fungi.

In recent years, the search for phytochemicals possessing antioxidant properties have been on the rise due to their potential use in the therapy of various chronic and infectious diseases. Epidemiology and experimental studies have implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of cardiovascular, diseases, cancer, aging etc.[5] Due to risk of adverse effects encountered with the use of synthetic antibiotics, medicinal plants may offer an alternative source for antimicrobial agent with significant activity against pathogenic and infective microorganisms. In addition, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes.[37]

Results of our findings confirmed the use *Aspergillus niger* and *Fusarium oxysporum* extract can be as traditional medicine. We found strong antioxidants activities specifically in the ethanol *Aspergillus niger* and *Fusarium oxysporum* endophytic stem extracts. High phenolic and flavonoid values found in ethanol *Aspergillus niger* and *Fusarium oxysporum* extracts imply the role of phenolic compounds in contributing these activities. Plant phenolic compounds have been found to possess potent antioxidants.[38]

The flavonoids from endophytic extracts have been found to possess antioxidants properties in various studies.[39] The terpenoids act as antioxidant.[40] Strong presence of tannins in all extracts may explain its potent bioactivities are known to possess potent antioxidants.[41] The saponins have already shown as antioxidant activity.[42]

The present investigation has shown that the endophytic extracts have active phytochemicals and exhibited strong antioxidant properties. These activities may be due to strong occurrence of polyphenolic compounds such as flavonoids, tannins, terpenoids phenols and saponins. The antioxidant activity was comparable with standard ascorbic acid and BHT. These findings provide scientific evidence to support endophytic fungal medicinal uses and indicate a promising potential for the development of an antioxidant agent plant. These endophytic fungi by *in vitro* results appear as interesting and promising and may be effective as potential sources of novel antioxidant drugs.

Table 4. Total antioxidant (FRAP) activities of ethanol endophytic extracts

Samples	FRAP
Aspergillus niger	1266.14 ± 0.06^{b}
Aspergillus flavus	527.37 ± 0.06^{d}
Fusarium oxysporum	1151.53 ± 0.05^{c}
Fusarium solani	448.26 ± 0.06^{e}
Plant stem extract	1228.32 ± 0.06^{b}
Ascorbic Acid	1648.52 ± 0.06^{a}
BHT	64.84 ± 0.06^{f}

Repeated the experiments three times for each replicates,
According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at $P \leq 0.05$, SE-standard error of the mean.

Table 5. Determination of phenolic and flavonoid content from ethanol extract of endophytes of *C. pallida*

Sample	Phenol (mg/g)	Flavonoid (mg/g)
Aspergillus niger	19.20 ± 0.03^{a}	7.25 + 0.07^{a}
Aspergillus flavus	16.71 ± 0.07^{d}	5.16 + 0.07^{b}
Fusarium oxysporum	18.23 ± 0.07^{b}	6.41 + 0.07^{a}
Fusarium solani	14.88 + 0.07^{e}	4.56 + 0.07^{b}
Plant Stem	18.89 + 0.07^{b}	6.55 + 0.07^{a}

Repeated the experiments three times for each replicates,
According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at $P \leq 0.05$, SE-standard error of the mean.

CONCLUSION

Based on the above results, endophytes, *Aspergillus niger* and *Fusarium oxysporum* of *Crotalaria pallida* yielded medically important phytochemical compounds, may be due to these endophytes possess potent antioxidant potentials of ethanol and water extracts in all *in vitro* study. In addition, both endophytic extracts was found to possess significant amount of total phenolic content. Different ingredients of endophytes, *Aspergillus niger* and

Fusarium oxysporum of *Crotalaria pallida* have been claimed in different studies to possess biological properties related to antioxidant mechanisms. Hence, the significant antioxidant activity of ethanol and water extract in the present study may be attributed to these aforementioned potent antioxidant ingredients of endophytes, *Aspergillus niger* and *Fusarium oxysporum*.

ABBREVIATIONS

W/V: weight/volume
DPPH: 1,1-diphenyl-2-picryl-hydrazyl
EC50: required to induce a 50% effect
ABTS: 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
FRAP: The ferric reducing ability of plasma
TPTZ: 2,4,6-tris (2-pyridyl)-S-triazine
TPC: Total Phenolic Content
GAEs: gallic acid equivalents
AlCl3: Aluminium Chloride
BHT: Butylated HydroxyToluene
mg/g: milligram/gram

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