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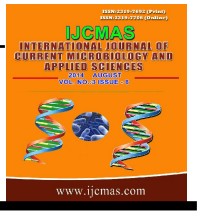

Original Research Article

In vitro antioxidant and antimicrobial activity of partially purified coumarins from fungal endophytes of *Crotalaria pallida*

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Crotalaria pallida, endophytes, MAE, coumarin(s), antioxidants, antimicrobials

A B S T R A C T

Two parts (leaf and stem) yielded five endophytic fungal species (leaf *Alternaria* sp and stem *Alternaria* sp, *Penicillium* sp1, *Penicillium* sp2 and *Aspergillus flavus*). All ethanol endophytic fungal extracts exhibited the presence of coumarin(s) (coumarin and O-coumaric acid) from MAE method at 2 cycles of 5 minutes each at 100^0C. The leaf *Alternaria* sp ethanol extract yielded more percentage of coumarins (3.772±0.03 and 1.701±0.082). All the five tests were positive for all endophytic fungal extracts in identification of coumarins. The leaf *Alternaria* sp extract showed highest scavenging DPPH free radical activity reached upto 97.2% followed by all stem endophytic fungal species. It is similar to standard BHT. High scavenging activities was observed in leaf *Alternaria* sp in ABTS and FRAP method. The flavonoid content was more in leaf *Alternaria* sp compared to other endophytes and plant part. The leaf *Alternaria* sp coumarin extract inhibited all the fungi and bacteria tested at maximum level. Further research work is needed to identify exact coumarin responsible for antioxidant and antimicrobial activity and their structure can be elucidated. The promised leaf *Alternaria* sp can be used for production of antioxidant and antimicrobial agent coumarin within a short period.

Introduction

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 (Yu, 1994; Katsube *et al*., 2006). Free radicals have been implicated in causation of diseases such as diabetes, inflammation, cancer, neurodegenerative disorders, atherosclerosis, liver cirrhosis, nephrotoxicity etc (Lachance *et al*., 2001). 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 (Kumaran and Karunakaran, 2007).

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 (Halliwell, 1995; Squadriato and Peyor, 1998; Gulcin *et al*., 2001). 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 02-, OH radicals as well as non free radicals species (H_20_2) (Gilham *et al*., 1997) which can harm severely tissues either by powerful direct oxidizing action or indirect with hydrogen peroxide and –OH radical formed from O2- which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors (Lewis, 1989). The reactive oxygen species are also known to activate matrix metello proteinase damage seen in various arthritic tissues (Cotran *et al*., 1994). The literature survey is giving the importance of plants and their antioxidant properties (Essawi and Srour, 2000) of plant active compounds especially phenolic and flavonoid compounds have proved as potent antioxidant and free radical scavenger (Silva *et al*., 2005).

Crotalaria pallida is an annual, erect herb, up to 150 cm tall, tap root, white or brown and system grooved, solid and glabrous. Novel antimicrobial peptides from *C. pallida* have shown strong antimicrobial activity (Pelegrin *et al*., 2009) and coumarin(s) was identified (Umashankar *et al*., 2012). Other species of *Crotalaria* have shown various folk and ayurvedic medicines for blood purifier, anemia, psoriasis (Chauhan and Singh, 2010), cancer activity (Kumar *et al*., 2008). And few species of *Crotalaria* have shown presence of coumarin (Bhakshu *et al*., 2008; Rao and Narukulla, 2007).

The *Crotalaria pallida* extracts have shown the presence of various bioactive phytochemicals and these compounds also showed significant inhibition of protease activity (Govindappa *et al*., 2011). Coumarin from different plants are possess anticancer (Devji *et al*., 2011), anti-HIV (Zhou *et al*., 2000) and antidiabetic (Gayathri *et al*., 2011). The aim of the work is to development of a rapid, reliable and reproducible method of extraction of coumarin and O-coumaric acid from different parts of *C.pallida* by using microwave assisted extraction method (MAE). Martino *et al*. (2006) have reported that for coumarin extraction is reliable and more reproducible from MAE from *Melilotus officinalis*. Waksmundzka-Hajnos

et al. (2004) have reported that the application of MAE to the extraction of secondary metabolites from the plants.

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 (Tan and Zhou, 2001) Many of the endophytes have showed few biological activities of antimicrobial, antioxidant, anticancer and anti-HIV (Guo, 2000; Strobel *et al*., 1996; Singh *et al*., 2004) 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 coumarin(s) and flavonoid content analysis. The findings from this work may add to the overall value of the medicinal potential of the plant.

Materials and Methods

Collection of plant material

Plant material *Crotalaria pallida* was collected from D.C. Bungalow, Sira Gate, Tumkur, Karnataka, India during January 2013. The collected plant was authenticated from the Department of Botany, Manasa Gangotri, University of Mysore and Government Ayurvedic College, Mysore. The fresh leaves and stem parts were used for isolation of fungal endophytes.

Isolation of endophytic fungi

Endophytic fungus isolation was carried out under aseptic condition (Theantana *et al*., 2009). The stem and leaves of the collected plant material were detached with a sterilized sharp blade, cleaned by washing with running tap water several times and soaked in 70% (v/v) ethanol for 10-20 min. It was then washed several times with sterilized water, dipped in 0.1% $HgCl_2$ for 1-2 min, again washed with sterilized water 3-5 times and then put into a beaker of sterilized distilled water.

The sterilized stem and leaves of collecting plant material were then cut into small pieces of 1 to 1.5cm, each piece put on a Petri plate containing Potato Dextrose Agar (PDA) medium and incubated at 30º C to promote fungal growth and sporulation. After 7-8 days Individual hyphal tips of the fungus were then picked up from each part and inoculated onto another PDA medium plate individually and incubated at 30º C for 1 week. The purified fungal isolates were numbered, transferred separately to PDA slants and kept at 4ºC for further use.

Identification of endophytic fungi

For the identification of endophytic fungal isolates, slides prepared from cultures and were stained with lactophenol cotton blue reagent and examined with a bright-field and phase contrast microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, the color of the colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and conidial characteristics using standard identification manuals (Ellis, 1971; Barnett and Hunter, 1972).

Mass production of identified fungi

Identified fungal species were cultured on PDB broth for large scale cultivation, which

was then incubated at room temperature (26±2ºC) for 8 days.

Identification of coumarin in the extracts

Test 1

3ml of ethanol extract was evaporated to dryness in a vessel and the residue was dissolved in hotdistilled water. It was then cooled and divided into two test portions, one was reference, second was the test. To the second test tube, 0.5 ml of 10 NH_4OH was added. The occurrence of intense/fluorescence under UV light is a positive test for the presence of coumarins and derivatives. The experiment was carried out for all the experiments in three replicates *(*Jagessar and Cox, 2010*)*.

Test 2

5ml of the extract was evaporated to dryness and the residue was dissolved in 2ml of distilled water. The aqueous solution was divided into two equal parts in test tubes. One part was the reference. To the other test tube, 0.5ml of 10%ammonia solution was added and the test tubes were observed under UV light indicated. The occurrence of a bluish green florescence under UV light indicated the presence of coumarin derivatives *(*Jagessar and Cox, 2010*)***.**

Test 3

To the concentrated alcoholic extract of drug few drops of alcoholic $FeCl_3$ solution was added. Formation of deep green colour, which turned yellow on addition of conc.HNO_3, indicates presence of coumarins.

Test 4

The alcoholic extract of drug was mixed with 1N NaOH solution (one ml each). Development of blue green fluorescence indicates presence of coumarins.

Test 5, for the Flavonoids

The ethanol extract (5 ml) was added to a concentrated sulphuric acid (1 ml) and 0.5g of Mg. A pink or red coloration that disappear on standing (3 min) indicates the presence of flavonoids.

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 *(*Zohra *et al*., 2012*)*.

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 (Shimada *et al*., 1992) 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:

DPPH scavenging effect (%) = $(\{A_0 - A_1\}A_0) \times 100$,

Whereas A_0 is the absorbance of the control reaction and A_1 is the absorbance of the presence of the sample.

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 *(*Arona *et al*., 2001*)*. 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:

ABTS scavenging effect (%) = *(*{A_0 – A_1}A_0*)* × 100,

Whereas A_0 is the absorbance of the control reaction and A_1 is the absorbance of the presence of the sample.

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 FeCl3 (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 (Szollosi and Szollosi Varga, 2002; Tomic *et al*., 2009). A calibration curve of ferrous sulfate (100-1000 μmol/L) was used and results were expressed in μmol Fe2+/mg dry weight extract. The relative activity of the samples was compared to L-ascorbic acid.

Flavonoid determination

Total flavonoid was determined according to Barros *et al*., (2007). The fungal extract (250 μl) was mixed with distilled water (1.25 ml) and $NaNO_2$ solution (5%, 75 μl). After 5 min the AlCl3 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.

Determination of antimicrobial activity antimicrobial assay

Bacterial species, *E. coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aueroginosa, Salmonella typhimurium, Stapholococcus aureus, Staphylococcus faecalis* and *Xanthomonas campestris* and fungal species *Alternaria brassicola, Alternaria geophila, Aspergillus flavus, A.fumigatus, A.tamari, C.tetramera, Fusarium equiseti, F. lateratium, F. moniliforme, F. oxysporum, F.udum, Penicillium chrysogenum* and *P. citrinum* were obtained from stock cultures presented at -80°C at Department of Studies in Microbiology and Biotechnology, Bangalore University, Jnana Bharathi, Bangalore, Karnataka, India. All bacteria were grown on nutrient agar media and all fungi were grown on potato dextrose agar medium.

Paper disc method

Diameter of zone of inhibition was determined using the paper disc diffusion method as described by Lai *et al*. (2009) and Adedapo *et al*. (2008). A swab of the

bacteria or fungi suspension containing $1x10^8$ CFU/ml was spread on to Petri plates containing nutrient agar and potato dextrose agar media separately. Ethanol endophytic fungal extracts were dissolved in each solvent to final concentration of 10mg/ml. Steriled filter paper discs (6mm in diameter) impregnated with 1mg of extracts were placed on culture plates separately for bacteria and fungi. The plates were incubated at 37°C for 24h for bacteria and 26 ± 2^0C for 7-8 days for fungi. Standard augmentin (10µg) for bacteria and blitox for fungi discs were used as a positive control. Antimicrobial activity was indicated by the presence of clear inhibition zone around the discs. The assay was repeated thrice and mean of three experiments was recorded.

Results and Discussion

From two parts (leaf and stem) parts totally four different fungal endophytes were isolated and identified. In leaf part, only one endophytic fungal species (*Alternaria* sp.) was observed and the stem part yielded *Alternaria* sp., *Penicillium* sp, (two species) and *Aspergillus flavus* (Table 1). Microwave assisted extraction method was used to isolate coumarin and o-coumaric acid from all the endophytic fungal species using the ratio sample weight/solvent constant with a value of 0.05 g/ml. Three replicates were maintained for all extracts. The extraction was performed employing as extraction solvent 50% aqueous ethanol at 100^0C in one and two cycle for 5 minutes each.

Table 2 depicts that, the 100^0C with 2 cycles have yielded high amount of coumarin(s) was noticed in *Alternaria* sp (3.772) isolated from leaf followed by stem fungal endophytes *Alternaria* sp (3.118), *Penicillium* sp1 (2.884), *Penicillium* sp2 (2.714) and *Aspergillus flavus* (2.233).

Employed all the MAE extracts of endophytic fungal species and plant were subjected to know the presence of coumarin using four different methods (Jagessar and Cox, 2010). All four different identification tests confirmed the presence of coumarin and coumarin related compounds (Table 3). This result confirmation gives strong evidence of coumarin presence.

The antioxidant activity of ethanol extract of all endophytic fungal extract were measured by the ability of scavenging DPPH free radicals, was compared with standard Butylated Hydroxy Toluene (BHT). It was observed that leaf *Alternaria* sp extract had higher activity followed by stem endophytes *Alternaria* sp, *Penicillium* sp1, *Penicillium* sp2 and *Aspergillus flavus*. At a concentration of 0.1 mg/ml, the scavenging activity of ethanol extract of leaf *Alternaria* sp and stem *Alternaria* sp reached to 97.2% and 95.4% respectively while at the same concentration, that of *Penicillium* sp1, *Penicillium* sp2 and *Aspergillus flavus* was 79.2%, 76.6% and 65.1%. Though the DPPH radical scavenging abilities of the extract were less than those of (99.1%) at 9.1 mg/ml, the study showed that the endophytic extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants (Fig 1). The performance of ethanol extract of endophytic fungi, *Phyllostica* sp was higher than that the standard µ-tocopherol *(*Srinivasan *et al*., 2010*)*, red alga *Polysiphonia urceolata (*Duan *et al*., 2006*)* and from different endophytes *(*Phongpaichit *et al*., 2007; Govindappa *et al*., 2011*)*.

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 (Fig 2). The ethanol extract of leaf endophyte *Alternaria* sp and stem *Alternaria* sp showed maximum decolouration. ABTS assay is an excellent tool for determining the antioxidant activity of phytochemicals *(*Jagadish *et al*., 2008*)*. The edible basidiomycetes and endophytes assayed against ABTS radical and reported to have scavenging ability against these radicals *(*Jagadish *et al*., 2008; Jagadish *et al*., 2009; Barros *et al*., 2007; Govindappa *et al*., 2011*)*.

The reducing ability of the endophytic fungal extracts was in the range of 491.96-1338.46 µm Fe (II)/mg (Fig 3). The antioxidant potentials of the ethanol extract of leaf *Alternaria* sp and stem *Alternaria* sp were estimated from their ability to reduce TPRZ-Fe (III) comply 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 flavonoids and antioxidant activity appears to be the trend in many plant species *(*Adedapo *et al*., 2008; Govindappa *et al*., 2011*)*.

Flavonoid compound seems to have an important role in stabilizing lipid oxidation, associated with antioxidant activity *(*Maslarova, 2001*)*. The flavonoid content of the leaf endophyte, *Alternaria* sp and stem *Alternaria* sp was 7.32 and 4.59 µg/mg equivalent respectively (Table 4). Results of our findings confirmed the use of leaf *Alternaria* sp and stem *Alternaria* sp extract having higher amount of coumarin(s) and flavonoids can be traditional medicine. We found strong antioxidant activities specifically in the ethanol extract of leaf *Alternaria* sp and stem *Alternaria* sp.

Higher flavonoid values found in same endophytic extracts imply the role of phenolic compounds in contributing these activities. Plant coumarin(s) and flavonoids have been found to possess potent antioxidants *(*Kostova *et al*., 2011; Brunetti *et al*., 2013*)*. The flavonoids and coumrins from endophytes have been found to possess antioxidants properties in various studies *(*Lin *et al*., 2008; Umashankar *et al*., 2012*)*. MAE endophytic fungal extracts have coumarin constituents showed inhibitory activity against some human pathogenic bacteria is presented in Table 5. The antifungal activity of all five different endophytic fungal extract of coumarin is presented in the Table 6.

Among five endophytic fungal extract, the leaf *Alternaria* sp extract showed higest antibacterial activity followed by stem *Alternaria* sp, *Penicillium* sp1, *Penicillium* sp2, and *Aspergillus flavus* (Table 5). But no significant activity was not found in *Penicillium* sp2 and *Aspergilus niger* extract. The control DMSO did not inhibit any of the bacteria tested. The leaf *Alternaria* sp has strongly inhibited the all bacteria tested. The maximum activity was noticed on *Proteus vulgaris* (18.4) followed by *Salmonella typhimurium* (17.8), *Stapholococcus aureus* (17.2), *Staphylococcus faecalis* (16.4), *Xanthomonas campestris* (15.8), *E. coli* (12.8) and *Klebsiella pneumonia* (11.4). The stem *Alternaria* sp also inhibited the all bacteria tested moderately when compared with leaf *Alternaria* sp. Other endophytic fungi stem *Penicillium* sp1, *Penicillium* sp2 and *Aspergillus flavus* are totally failed in inhibition of target organisms. Our results confirmation with the findings of Meng *et al*. (2012), Basile *et al*. (2009), Ojala *et al*. (2000), Widelski *et al*. (2009).

Table.1 List of endophytic fungal species from different parts of *Croatalaria pallida* on PDA media

Endophytes	Leaves	Twigs (Stem)
Alternaria sp.	+	+
Penicillium sp. 1	-	+
Penicillium sp. 2	-	+
Aspergillus flavus	-	+

+:presence, -: Not Present, data based repeated the three replicates of each experiment

Table.2 Effect of microwave assisted extracts on yield of coumarin & O-coumaric acid from different fungal endophytes of *Croatalaria pallida*

Extracts	Temperature (ºC)	Cycles x Minutes	Coumarin (mg/ g) ± SD	O-coumaric acid (mg/ g) ± SD
Alternaria sp. SIETBTCpL1	100	1x5	3.311±0.053	1.416±0.082
		2x5	3.772±0.053	1.701±0.082
Alternaria sp. SIETBTCpS2	100	1x5	2.966±0.053	1.229±0.082
		2x5	3.118±0.053	1.384±0.076
Penicillium sp. SIETBTCpS1	100	1x5	2.683±0.053	1.097±0.076
		2x5	2.844±0.053	1.188±0.076
Penicillium sp. SIETBTCpS2	100	1x5	2.598±0.053	1.068±0.076
		2x5	2.714±0.082	1.136±0.076
Aspergillus flavus SIETBTCpS1	100	1x5	2.116±0.082	1.008±0.076
		2x5	2.233±0.082	1.091±0.076
Flower	100	1x5	3.576±0.053	1.142±0.076
		2x5	3.881±0.082	1.224±0.058

Data based repeated the three replicates of each experiment

Table.3 Identification of coumarin and flavonoid from different endophytic fungal species of *C. pallid*

Samples	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 4**
Flower	+	+	+	+	+
Alternaria sp. SIETBTCpL1	+	+	+	+	+
Alternaria sp. SIETBTCpS2	+	+	+	+	+
Penicillium sp. SIETBTCpS1	+	+	+	+	+
Penicillium sp. SIETBTCpS2	+	+	+	+	+
Aspergillus flavus SIETBTCpS1	+	+	+	+	+

+: presence, data based repeated the three replicates of each experiment.

Table.4 Determination of flavonoid content in *Crotalaria pallida* endophytic fungal extract

Endophytic fungal Species	Flavonoid content
Alternaria sp. SIETBTCpL1	7.32±0.07
Alternaria sp. SIETBTCpS2	7.14±0.09
Penicillium sp. SIETBTCpS1	6.37±0.06
Penicillium sp. SIETBTCpS2	5.18±0.09
Aspergillus flavus SIETBTCpS1	4.59±0.07
Flower	6.71±0.07

data based repeated the three replicates of each experiment.

Table.5 Antibacterial activity of ethanol endophytic fungal coumarin extract

Bacteria tested	Endophytic fungal coumarin extract					
	1	2	3	4	5	Augmentin
E. coli	12.8±0.3	9.2±0.3	0.0±0.0	0.0±0.0	0.0±0.0	18.3±06
Klebsiella pneumonia	11.4±0.2	8.9±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Proteus vulgaris	18.4±0.3	8.1±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Pseudomonas aueroginosa	16.5±0.4	7.3±0.3	0.0±0.0	0.0±0.0	0.0±0.0	16.4+0.3
Salmonella typhimurium	17.8±0.3	6.8±0.3	0.0±0.0	0.0±0.0	0.0±0.0	16.9±0.3
Stapholococcus aureus	17.2±0.3	6.4±0.3	0.0±0.0	0.0±0.0	0.0±0.0	17.2±0.3
Staphylococcus faecalis	16.4±0.4	5.9±0.3	0.0±0.0	0.0±0.0	0.0±0.0	15.3±0.3
Xanthomonas campestris	15.8±0.4	6.1±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

1- *Alternaria* sp. SIETBTCpL1, 2- *Alternaria* sp. SIETBTCpS2, 3- *Penicillium* sp. SIETBTCpS1, 4- *Penicillium* sp. SIETBTCpS2, 5- *Aspergillus flavus* SIETBTCpS1

Table.6 Antifungal activity of ethanol endophytic fungal coumarin extract

Fungi tested	Endophytic fungal coumarin extract					
	1	2	3	4	5	Blitox
Alternaria brassicola	78.4±0.3	68.4±0.3	45.6±0.3	45.2±0.3	21.5±0.3	84.8±06
Alternaria geophila	88.3±0.2	81.9±0.3	48.9±0.3	46.2±0.3	22.6±0.3	91.2±0.3
Aspergillus flavus	87.6±0.3	82.7±0.3	45.1±0.3	41.5±0.3	28.7±0.3	92.8±0.3
A.fumigatus	91.6±0.4	84.5±0.3	39.2±0.3	37.4±0.3	26.2±0.3	96.7±0.3
A.tamari	90.8±0.3	82.1±0.3	38.4±0.3	35.6±0.3	20.4±0.3	96.2±0.3
C.tetramera	76.5±0.3	69.2±0.3	39.4±0.3	34.5±0.3	31.2±0.3	87.6±0.3
Fusarium equiseti	90.4±0.4	83.4±0.3	37.9±0.3	34.6±0.3	29.5±0.3	95.7±0.3
F. lateratium	76.4±0.4	69.4±0.3	41.1±0.3	39.7±0.3	28.2±0.3	84.4±0.3
F. moniliforme	88.2±0.3	81.7±0.3	42.4±0.3	39.8±0.3	24.1±0.3	93.2±0.3
F. oxysporum	82.5±0.3	78.4±0.3	43.2±0.3	41.3±0.3	21.2±0.3	90.7±0.3
F.udum	71.8±0.3	68.4±0.3	43.5±0.3	38.8±0.3	19.4±0.3	88.5±0.3
Penicillium chrysogenum	72.6±0.3	67.4±0.3	38.2±0.3	36.8±0.3	18.2±0.3	91.2±0.3
P. citrinum	85.6±0.4	77.9±0.3	38.6±0.3	39.7±0.3	18.6±0.3	94.6+0.3

1- *Alternaria* sp. SIETBTCpL1, 2- *Alternaria* sp. SIETBTCpS2, 3- *Penicillium* sp. SIETBTCpS1, 4- *Penicillium* sp. SIETBTCpS2, 5- *Aspergillus flavus* SIETBTCpS1

Fig.1 DPPH scavenging activities of *Crotalaria pallida* endophytic fungal ethanol extract

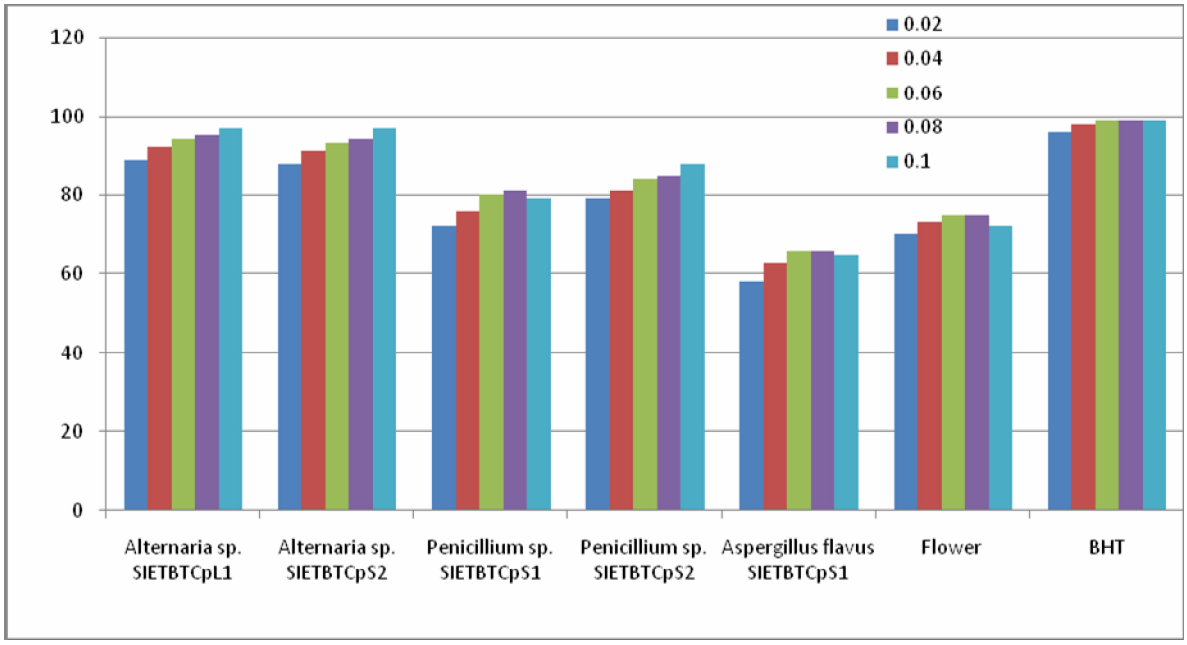


Fig.2 Free radical scavenging effect of diffeent endophytic fungal ethanol extract against ABTS

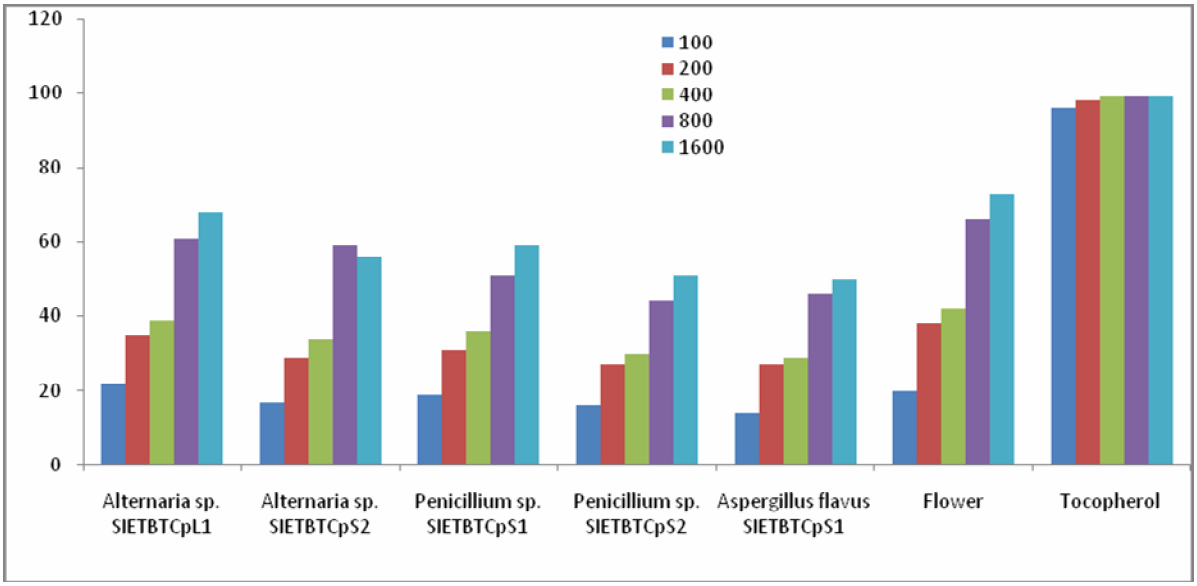


Fig.3 Total antioxidant (FRAP) activities of ethaol endophytic fungal extract

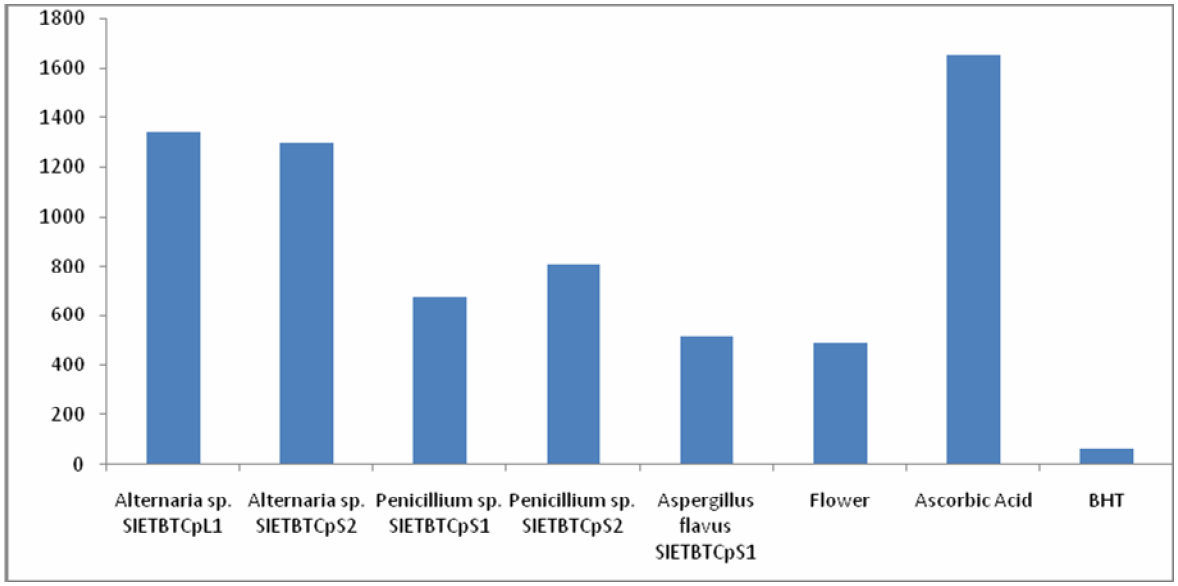
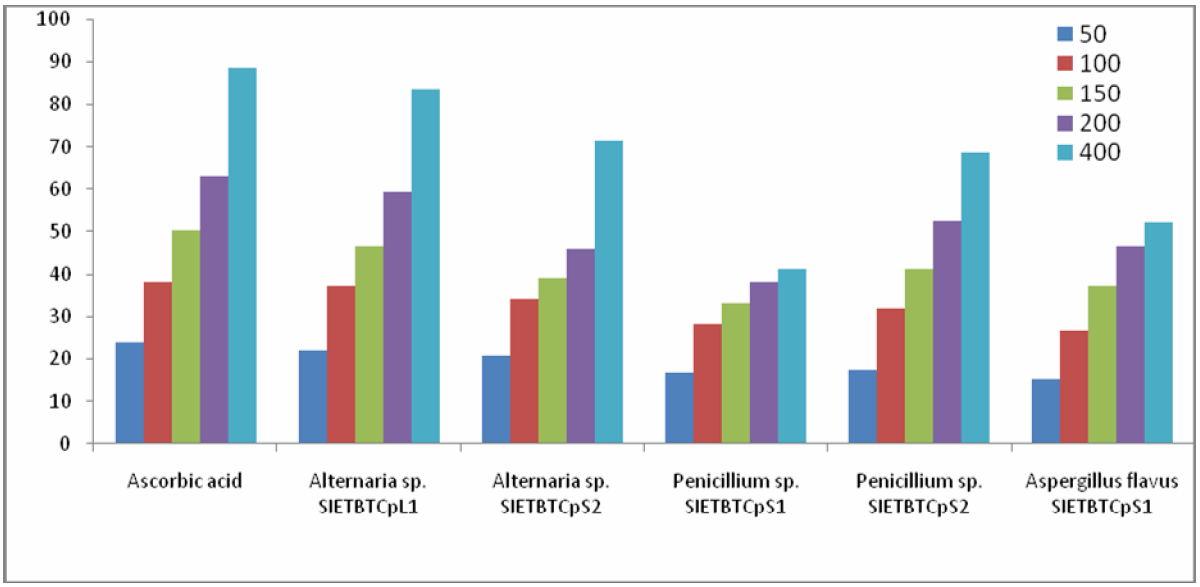


Fig.4 Effect of endophytic fungal extract on hydrogen peroxide radical scavenging activity



When compared with standard fungicide, blitox, the leaf *Alternaria* sp coumarin extract also dimished the growth of all fungi tested (Table 6). The laef endophytic coumarin extract may be has toxic material thus inhibited the grwoth of all plant pathogenic fungi, followed by stem *Alternaria* sp, *Penicillium* sp1, *Penicillium* sp2 and *Aspergillus flavus*. The leaf *Alternaria* sp dimished the growth of the plant pathogenic fungi greatly, the maximum inhibition was noticed against

Aspergillus fumigatus. The extract may having comarin compound may be toxic to the target fungi and similar reported were reported (Sardari *et al*., 1999; Montagner *et al*., 2008; Al-Amiery *et al*., 2012). The endophytic fungal flavonoids expressed as strong antioxidant compounds (Govindappa *et al*., 2011).

The present investigation has shown that the endophytic extracts have active phytochemical such as coumarin(s) or flavonoid and exhibited strong antioxidant properties. The antioxidant activity was comparable with standard ascorbic acid and BHT. The endophytic fungal species expressed as a stong antimicrobials. These findings provide evidence to produce promising antioxidant potetials from fungal endophytes of *Crotalaria pallida* and can be used as potential sources of novel antioxidant drugs.

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