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"Evaluation of *in vitro* antioxidant and free radical scavenging activity of leaf and twig endophytic fungal extract from *Mappia foetida*"

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Abstract:

The present study was aimed to investigate the *in vitro* free radical scavenging activity of leaf extracts. *In vitro* free radical scavenging activities of the endophytic extracts were assessed against DPPH and hydroxylradicals. The metal chelating activity and reducing power ability of the extracts of *M. foetida*. were also determined. The free radical scavenging activity was found to be high in methanolic extract for DPPH and hydroxyl radicals. The difference in scavenging potential of the endophytic extracts may be due to variation in the percentage of phytoconstituents extracted in

methanolic solvents. Thus, the result suggests that the methanolic leaf extract of ***M. foetida*** could serve as a potential source of antioxidants and can be explored as a therapeutic agent in free radical induced diseases.

Keywords:*M. foetida,* Endophyte, Anti-oxidant, DPPH.

1. Introduction

Medicinal plant used as a therapeutic agent for maintaining good health practices in the developing countries [1]. Many natural products including plants are in great demand for various factors like safety, dependability and lesser side effects [2]. Major side effects which are caused by cancer chemotherapeutic drugs may be used as an alternative medicine for curing cancer. Around 80% of the world's population mainly focus on traditional medicines for their primary healthcare which are responsible for curing diseases [3-5]. Bioprospecting of new drugs which are derived from plant source have more economical value [6].

Free radicals are said to be prominent and act as an essential part of aerobic life and metabolism [1]. They even cause oxidative damage to proteins, lipids and also DNA which leads to many diseases such as cancer, diabetes, aging etc. [2,3]. To protect the adverse effects of free radical, human cells generate enzymes such as superoxide dismutase (SOD) and catalase or compounds such as ascorbic acid, tocopherol, and glutathione [4]. Plants are very active in their biological activities especially in antioxidants, which produces many secondary metabolites such as alkaloids, flavonoids, tannins, cardiac glyceroids, sterols, amino acids and many more phytochemicals which are responsible for different pharmacological activities [5]. The present study proved that natural antioxidants can reduce the risk of cancer and many other diseases [6].

M. foetida now renamed as *Nothapodytes nimmoniana* is a moderate sized tree which grows up to 4-10 mts high. This plant has anti-cancer properties which has made it an endangered species. The present

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investigation was aimed to isolate different endophytic fungal species from different parts of *M. foetida*. All the isolated endophytes were screened for antioxidant activity. Total antioxidant activity of plant extract was evaluated by standard methods of DPPH and H_2O_2 assay.

2. Materials and Methods

2.1 Plant collection, sterilization and inoculation of implants.

The study area is situated at Western Ghats of Mookambika Wild life Sanctuary in Shivamogga district of Karnataka, India. Freshly collected plant material, *M. foetida* leaves and twigs is washed thoroughly under running tap water followed by sterile distilled water to remove the adhered debris. Twigs and leaves were surface sterilized under aseptic condition in sequential steps by immersing in mercuric chloride (1mg/1ml) for 10 min and 70 % ethanol for another min followed by washing finally with distilled water. Twigs and leaves of *M. foetida* were aseptically cut into small pieces (0.5-0.5cm^2) and placed 5-6 pieces on each of the solidified sterile Potato Dextrose Agar (PDA) media. The inoculated plant implants were incubated till the growth of distinguishable fungal endophytes.

2.2. Identification of fungal endophyte

For the identification of endophytic fungal isolates, slides were prepared from cultures and were stained with lacto phenol cotton blue and examined with a bright-field and phase contrast microscope. Identification was done based on morphological characteristics such as growth pattern, hyphae, the colour of the colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and conidia characteristics using standard identification manuals [7,8]. The identified fungi sub cultured in PDA slants for further use and stored in refrigerated conditions.

2.3. Estimation of antioxidant activity

2.3.1 DPPH assay.

The free radical scavenging activities of extract were measured by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) [9]. Extract concentration around 0.1 mg/ml in 4 mL of methanol was mixed with 1 mL of methanol solution containing DPPH radicals of 0.2 mM. The mixture was shaken vigorously and allowed to stand for 30 min in the dark chamber. The absorbance was measured at 517 nm against a blank. IC_{50} value was obtained by interpolation from linear regression analysis. Butylated hydroxy toluene (BHT) was used as standard for comparison studies. The capacity of radical scavenging activity 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.

2.3.2. Hydroxyl radical scavenging activity.

Hydrogen peroxide radical scavenging activity was determined by the method of Ruch *et al.,* (1989) [10]. The UV light absorption of hydrogen peroxide can be easily measured at 230 nm. On scavenging of hydrogen peroxide by the test samples, the absorption decreases at 230 nm wavelength and this property can be utilized to quantify their H_2O_2 scavenging activity.

Conduction.

The reducing power ability of the extracts was evaluated by the method described of Oyaizu. The reaction mixture contained 1.0 mL of various concentrations of extracts (2–10 mg/mL), 2.5 mL of 1% potassium ferricyanide and 2.5 mL of 0.2 mol/L sodium phosphate buffer. The mixture was incubated at 50 °C for 30 min and the reaction was terminated by the addition of 2.5 mL of 10% trichloroacetic acid, followed by centrifugation at 3000 r/min for 10 min. 2.5 mL of the upper layer was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm against

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blank that contained distilled water and phosphate buffer. Increase in absorbance indicates increased reducing power of the sample. Ascorbic acid was used as standard.

$$\% \text{ of } H_2O_2 \text{ radical scavenging activity} = \frac{AC - AT}{AC} \times 100$$

Where A_C is the absorbance of the control, A_T is the absorbance of the test sample.

Statistical Analysis:

Analysis of variance (ANOVA) was used to determine the significant difference between treatment groups ($p<0.05$). Means between treatment groups were compared for significance using Duncan's Multiple Range test.

3. Results and Discussion

3.1. Identification of an endophytic fungi

20 different endophytes were isolated and identified from *M. foetida.* Majority (70%) of endophytic isolates were repeated (Figure 1).

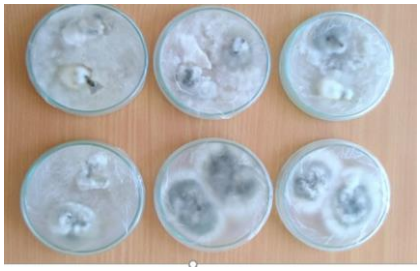

Fig 1: Fungal endophyte of different species

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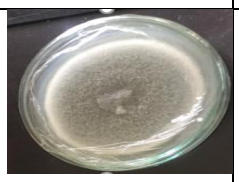
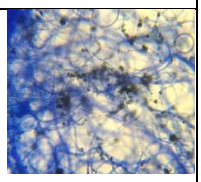
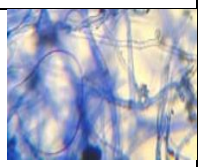
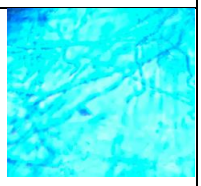
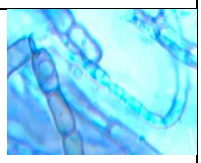
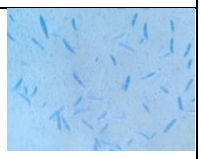
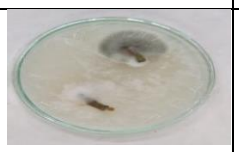
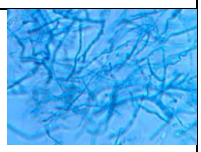
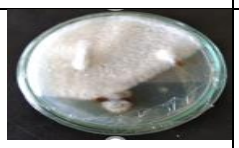
Fungal Endophytes	PDA Plates	Microscopic view (40X)
Alternaria alternata **(Fig. 1.1)**		
Bipolaris sp. **(Fig. 1.2)**		
Cladosporium cladosporioides **(Fig 1.3)**		
Cladosporium sp. **(Fig 1.3)**		
Colletotrichum sp. **(Fig. 1.4)**		
Drechslera dematioidea **(Fig. 1.5)**		
Fusarium verticilloides **(Fig. 1.6)**		

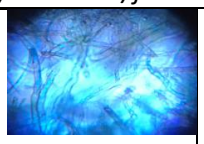
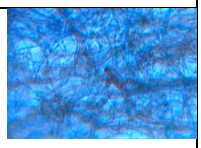
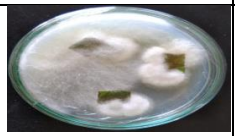
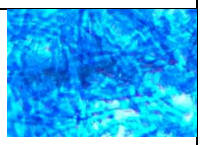
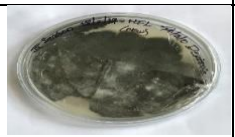
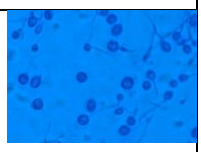
***Fusarium oxysporum* (Fig. 1.6)**		
***Fusarium sp* (Fig. 1.6)**		
***Fusarium moniliforme* (Fig. 1.6)**		
***Saprophytic fungi* (Fig. 1.7)**		

Fig. 1.1-1.7: Endophytic fungi on Potato dextrose agar media and their morphology under microscope (40X).

Antioxidant potential of endophytic fungi:

3.2. DPPH assay: The antioxidant activity of ethanol extract of all endophytic fungal extract measured by the ability of scavenging DPPH free radicals, was compared with standard Butylated Hydroxy Toluene (BHT). As antioxidants donate proton to DPPH radicals, the absorption decreases. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. The DPPH scavenging potential of endophytic extracts may be attributed to the hydroxyl groups present in the extracts (10). DPPH is a stable free radical with absorption at 570nm and the readings were observed by decreasing

the absorbance taken as a measure indicates the extent of radical scavenging property. The antioxidant activity of the twenty potential endophytic fungi was determined and the results are tabulated in (Table 1). The ethanol extracts (leaf and twig) of *Fusarium sp.*, *Saprophytic fungi*,*Alternaria alternata* and*Cladosporium sp*, showed higher DPPH activity. Hence 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 (Figure. 2).

Fig. 2.: DPPH activity of ethanol extracts of endophytic fungi.

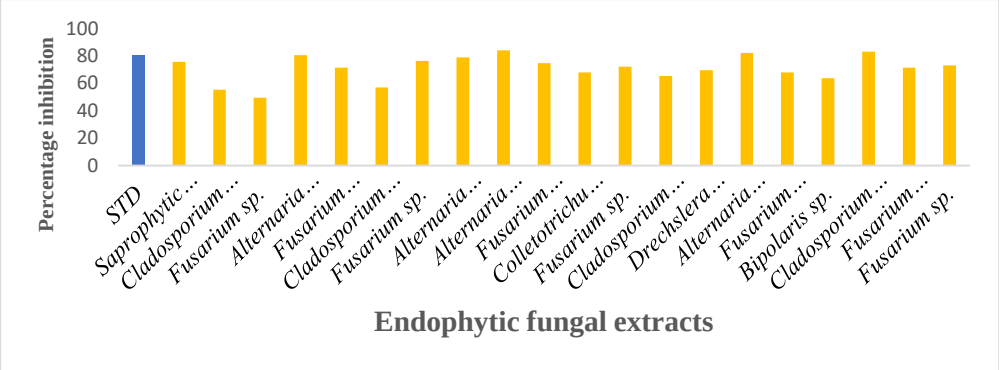


Table 1.: Antioxidant activity of endophytes isolated from endangered medicinal plant (Season I and Season II).

Endophytic fungi	**DPPH scavenging assay (%)**	**Radical scavenging assay (%)**
Saprophytic fungi	75.63±0.05	74.28±0.01
Cladosporium cladosporioides	55.46±0.02	67.14±0.03
Fusarium sp.	49.57±0.02	64.28±0.009

Alternaria alternata	80.67±0.005	81.42±0.006
Fusarium oxysporum	71.42±0.004	72.85±0.01
Cladosporium sp.	57.14±0.008	62.85±0.004
Fusarium sp.	76.47±0.02	77.14±0.004
Alternaria alternata	78.99±0.008	78.57±0.004
Standard Butylated Hydroxy Toluene for DPPH (BHT)*	80.67±0.02*	
Standard Ascorbic acid for Hydroxyl radical scavenging#		81.42±0.05#

Values represented in the results were mean ± SEM, shows significant difference ($p<0.05$).

3.3. **Hydroxyl radical scavenging activity:** The absorbance value of the reaction mixture was recorded at 230nm for H_2O_2 radical scavenging activity of the endophytic fungal extracts by comparing with standard ascorbic acid. The result showed that leaf endophytes had higher activity compared with twig endophytes. The hydroxyl radical scavenging activity of the twenty endophytic fungi was determined and the results are tabulated in (Table 2) (Figure. 3).

Fig. 3: Hydroxyl radical scavenging activity of ethanol extracts of endophytic fungi.

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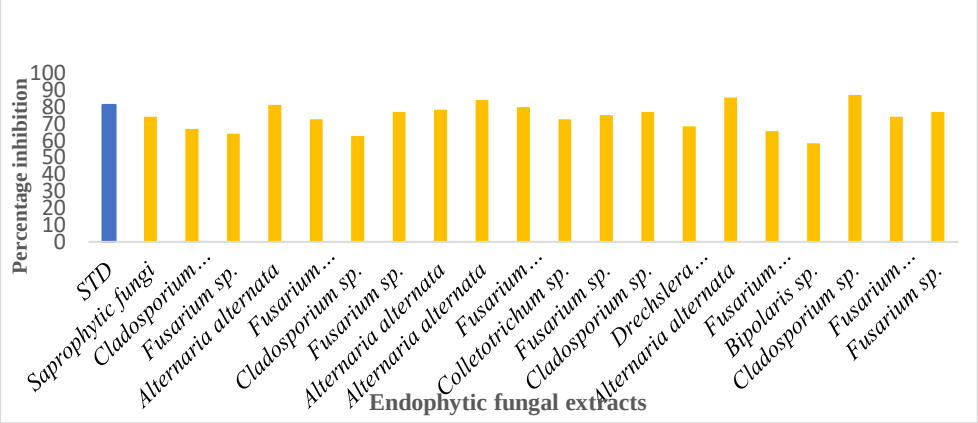

Table 2: Antioxidant activity of endophytes isolated from endangered medicinal plant (Season III and Season IV).

Endophytic fungi	DPPH scavenging assay (%)	Radical scavenging assay (%)
Alternaria alternata	84.03±0.008	84.28±0.004
Fusarium moniliforme	74.78±0.01	80±0.008
Colletotrichum sp.	68.06±0.01	72.85±0.008
Fusarium sp.	72.26±0.006	75.17±0.01
Cladosporium sp.	69.74±0.007	77.14±0.004
Drechslera dematioide a	65.54±0.01	68.57±0.004
Alternaria alternata	82.35±0.01	85.71±0.004
Fusarium moniliforme	*68.06*±0.008	*65.71*±0.004
Bipolaris sp.	63.86±0.01	58.57±0.004
Cladosporium sp.	83.19±0.006	87.14±0.01

Fusarium verticillioides	71.42±0.006	74.28±0.008
Fusarium sp.	73.1±0.006	77.14±0.004
Standard Butylated Hydroxy Toluene for DPPH (BHT)*	80.67±0.02*	
Standard Ascorbic acid for Hydroxyl radical scavenging #		81.42±0.05#

Values represented in the results were mean ± SEM, shows significant difference ($p<0.05$).

4. Conclusion

Plant based products are being used for medicinal and therapeutic purposes since the dawn of history. The use of plants as medicines has involved the isolation of active compounds. Isolation and characterization of pharmacologically active compounds from medicinal plants is continuing even today.

In this study endophytic fungal extracts have the potential activity against antioxidants. Endophytes from medicinal plants have a broad prospect in the future. Antioxidant activity of ethanol extract of the 20 potential endophytic fungi was determined. *Fusarium sp., Saprophytic fungi, A. alternata* and *Cladosporium* showed higher DPPH activity and free radical scavenging activity. Hence, we can conclude these endophytic extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants.

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