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Biomedical Potentials of *Talaromyces tratensis* – A New Endolichenic Fungi Associated with High Altitude Curstose Lichen *Lecanora sp*.

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ABSTRACT

Natural products remains a consistent source of drug leads with more than 40% of new chemical entities (NCEs). It has become imperative to explore microorganisms for NCEs and lead – drug - molecules for the drug discovery. Keeping this in view bioprospecting of microorganisms is carried out from every possible source, including extreme environments like ocean beds, geothermal vents, cold desserts etc., in search of novel strains with promising bioactivities. During the past two decades it has been observed that much wealth of microbial biodiversity with novel biochemistry and secondary metabolite production resides in endophytes. So far, numerous bioactive molecules have been isolated from endophytic fungi. An important step towards tapping their potentials for human welfare including drug discovery and sustainable agriculture, it is very essential to isolate endophytes from various ecological niches. Among the endophytes lichen associate fungi are unique organisms that have potential bioactive properties including, antibiotic, antioxidant, antiviral, anti-inflammatory, analgesic antipyretic, anti-proliferating and cytotoxic activities. In this study endolichenic fungi was isolated from crustose lichen *Lecanora sp.* collected from Horsley Hills, Andhra Pradesh. The isolated endolichenic fungi was identified as *Talaromyces tratensis* on the basis of ITS4and ITS5 ribosomal gene sequences. The fermented broth is potential source for anti-metabolites. The metabolites crude active against gram positive, gram negative bacteria and fungal pathogens. The most distinguished free radical scavenging activity was observed for Ethyl acetate extract of fungal mycelium. The EC_{50} values based on the DPPH (1, 1-Diphenyl-2- Picrylhydrazyl), Hydrogen peroxide and Nitric oxide were 45.50±0.01, 32.61±0.06 and 66.54±0.01 respectively.

Keywords: Antioxidant activity, Crustose Lichens, Endolichenic fungi and *Talaromyces tratensis*

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INTRODUCTION

The Name "endolichenic fungi" was introduced by Miadlikowsk in 2004 [1]. Endolichenic fungi signifies a vital ecological group of species that form close associations with lichens [2], which lives as endosymbiotic micro fungi in the thallus of lichens and resemble to endophytic fungi live in the intercellular spaces of the plant hosts [3-5]. To date about 100,000 fungal species are identified even if distant more than one million are expected. The diversity of species and the variety of their habitations, some of them unexplored, this lead to be fungi as a rich source of novel metabolites [6]. Besides that Endolichenic fungi are untapped and new treasured source for bioactive metabolite products [5, 7] Only a few investigations have been reported on the bioactive metabolites of endolichenic fungi, but they have shown great potential to be a new source for structurally diverse and biologically active natural products [5, 8-10]. Secondary metabolic products of endolichenic fungi shows distinct bioactivities like antimicrobial [5, 9, 11], antiviral [12], antioxidant [13-14] anticancer and cytotoxic [7, 9-10, 13-16]. These bioactive compounds have great prominence in development of pharmaceutical drugs, nutraceuticals and agrochemicals. The present study was carried out to investigate antimicrobial and antioxidant activities of endolichenic fungi *Talaromyces tratensis* inhabiting the lichen *Lecanora spp*. Collected from Horsley hills, Andhra Pradesh, India. This research was aimed determining the antimicrobial and antioxidant activity of secondary metabolites present in the ethyl acetate (EtOAc) extract of *Talaromyces tratensis* fermented in potato dextrose Broth (PDB) and their potential for the production of bioactive compounds.

MATERIALS AND METHODS

Sample Collection

The lichens were collected from Horsley hills (13.66°N 78.40°E), 147 km of a part of Sheshachalam Hills range, Andhra Pradesh. The lichens were located at an altitude of 1,290m above sea level. The lichen samples were collected from different substrates and transported into the laboratory in sterilized paper bags.

Isolation of Endolichenic Fungi

The fungi *Talaromyces tratensis* isolation was carried out by modified method of Guo et al.,2003 and Kannagara et al.,2009 [17-18]. Healthy lichen thalli were cleaned in running tap water to the remove dust particles, litter and then washed with milli-Q watter. The surface sterilized by consecutive immersion for 4min in 2% Sodium Hypochlorite, with Hydrogen peroxide for 2min followed by immersed in 30 s in 75 % ethanol. The thalli surface were dried with sterile filter papers and aseptically cut into small segments (0.5 × 1 cm) and were evenly placed in each 90mm Petri dishes containing Potato Dextrose agar (PDA) with Streptomycin Sulphate (50µg/ 100ml). The PDA plates were sealed with Paraffin film and incubated at 28ºC for 7days. Fungi grown from each lichen segment and make into pure cultures. Slides containing pure cultures were prepared using the slide culture method [19] and identified using identification keys [20]. The growing fungi *Talaromyces tratensis* were sub-cultured on PDA.

Molecular identification of the isolated endolichenic fungus

Genomic DNA isolated in the pure form from the fresh biomass of Endolichen fungus by CTAB (N-cetyl N,N, Ntrimethyl -ammonium bromide) method [21], the Identification of isolated pure strain of the endolichenic fungus was carried out using a molecular biological protocol by genomic DNA extraction, internal spacer transcribed (ITS) region amplification and sequencing.

The ITS region of rDNA was successfully amplified by PCR was set up with ABI BigDye® Terminatorv3.1 cycle sequencing kit and using fungal universal primers ITS4 (5' TCCTCCGCTTATTGATATGC 3') & ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') [22]. It was sequenced in both directions using the respective PCR primers. For this purpose, the Big Dye terminator sequencing kit (Version 3.1, Applied Biosystems) and an ABI 3100 automated DNA sequencer (Applied Biosystems) were used. Raw Gene sequence was manually edited for inconsistency and the predicted sequence data were aligned with public available sequences and analyzed to reach identity by using NCBI BLAST® (http://www.ncbi.nlm.nih.gov/blast/).

Fermentation and extraction:

The fermentation was carried out in Erlenmeyer flasks using a complex medium consisting of Potato Dextrose Broth (HIMEDIA Laboratories). The flasks containing 200 mL fermentation medium were inoculated with 5 days old actively growing *T. tratensis* mycelial agar discs (6mm), the Flask cultures allowed for inoculum development and fermentation at 28±2°C, pH 7.0 with orbital shaking at 120 rpm [23]. After 14days of Fermentation the fungal biomass was separated with Whatman No.1 filter paper from fermented broth and filtered broth was allowed to liquid-liquid separation with EtOAc (1:1 ratio) in a separatory funnel. After this procedure, the organic solvent was evaporated under reduced pressure to dryness to yield an EtOAc extract [24].

Antibacterial Activity:

To evaluate Antibacterial activity of *T. tratensis* EtOAc crude extract tested against gram positive (*Bacillus cereus* and *Staphylococcus aureus)* and gram negative bacterial pathogenic strains (*Escherecia coli, Pseudomonas fluorescence, Klebsiella pneumonia and Salmonella typhi)* by agar well diffusion method [25-26]. Antibacterial activity was expressed as the percent inhibition (%) of bacterial growth using the following formula C-T/C X 100.

Antifungal activity

The antibacterial activity in *in vitro* was dilution determined against the pathogenic fungi *Fusarium oxysporium, Colletotrichum capsisi and Aspergillus niger* by poison food technique [27]. 1 ml of tenfold of the EtOAc extracts were mixed with molten PDA separately and then poured into Petri dishes and control PDA plates supplemented with sterile distilled water. A mycelia disc of *tested pathogens* was transferred on the center of both test and control plates and incubated for 5days at 28°C. The mycelial radial was measured and the percentage of inhibition was expressed by using following formula $T_1 - T_2 / T_1 X 100$.

Screening for Antioxidant activity

DPPH Assay:

Free Radical-scavenging activity of *T. tratensis* extract against stable 2, 2 diphenyl 2 picrylhydrazyl hydrate (DPPH) was determined by the slightly modified method of Prior R.L. *et al.,* 2005 [28]. DPPH reacts with an antioxidant compound which can donate hydrogen and reduce DPPH. The change in colour (from deep violet to light yellow) was measured at 517 nm on a UV visible light spectrophotometer. The solution of DPPH in methanol 0.2mM was prepared fresh daily before UV measurements. One milliliter of this solution was individually mixed with ethyl acetate extracted crude sample of *T. tratensis* (25mg, 50mg, 100mg and 200mg). The samples were kept in the dark for 15 minutes at room temperature and the decrease in absorbance was measured. The experiment was carried out in triplicate. Radical-scavenging activity was calculated by the following formula

$$\text{Inhibition Percentage \%} = [(A_{\text{blank}} - A_{\text{sample}})]/A_{\text{blank}}] \times 100$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of purified molecules

Determination of Antioxidant Activity by Reducing Power Measurement

The reducing power of the extract was determined according to Oyaizu 1986 [29] with slight modifications. An amount of 25mg, 50mg, 100mg and 200mg of extracted sample was added to 2mL of 1% potassium ferricyanide. After incubating the mixture at 50°C for 30 min, during which ferricyanide was reduced to ferrocyanide, it was supplemented with 2mL of 1% trichloroacetic acid and 2% $FeCl_3$ and left for 20 min. Absorbance was read at 700 nm to determine the amount of ferric ferrocyanide (Prussian blue) formed. Higher absorbance of the reaction mixture indicates higher reducing power of the sample.

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Inhibition Percentage % = $[(A_{blank} - A_{sample})]/A_{blank}] \times 100$

Determination of Nitric Oxide (NO) Scavenging Activity

Nitric oxide production from sodium nitroprusside was measured according to Jagetia 2004 [30]. An equal amount (6 mL) of sodium nitroprusside (5mM) solution was mixed with extracted sample (25mg, 50mg, 100mg and 200mg) and incubated at 25°C for 180 min. After every 30 min, 0.5 mL of the reaction mixture was mixed with an equal amount of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% napthylethylene diamine dihydrochloride), and absorbance was taken at 546 nm and compared with absorbance of 1 mg/mL of standard solution (sodium nitrite) treated in the same way with Griess reagent.

Inhibition Percentage % = $[(A_{blank} - A_{sample})]/A_{blank}] \times 100$.

RESULTS AND DISCUSSION

Endolichenic fungi are residing in living thalli of lichens and that similar to endophytic fungi asymptomatically in internal tissues of all higher plants [3-5]. In Recently the biology of Endolichenic fungi are renowned to interesting novel sources of biologically active compounds. This study focuses on the biology of endolichenic fungi, their discovery, isolation, identification, and their biological activities in *invitro*.

In our present research, we isolated rare and interesting Endolichenic fungus from crustose type lichen *Lecanora spp.* (Fig.1) collected from Horsley Hills, Andhra Pradesh. The morphological characters of the isolate were slow-growing, yellow in colour, conidiophores having smooth, lateral branching, conidia aseptate, phialides and ascospores (Fig.3). The ITS sequence of endolichenic fungus 100% similarity with *Talaromyces tratensis* sequences from Gene-Bank and this endolichenic fungus was identified as *Talaromyces tratensis* (Fig.3). Previously several endolichenic fungi and their bioactive metabolites [7, 11-13] reported nevertheless *Talaromyces tratensis* newly reporting to produce and interesting bioactive metabolites with antimicrobial, and antioxidant properties. To our knowledge, this is the first report of this organism as an endolichenic fungi from Lichens.

Fig.1: *Lecanora spp* of Lichen

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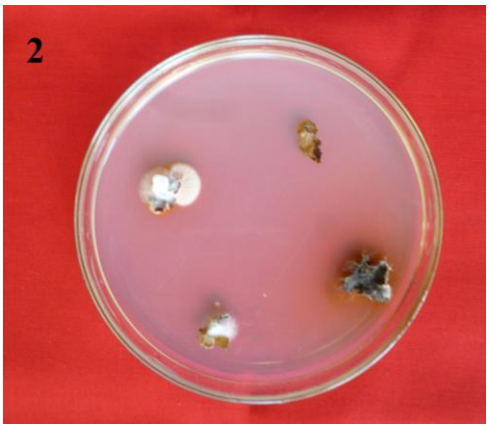

Fig. 2: Isolation of Endolichenic Fungi

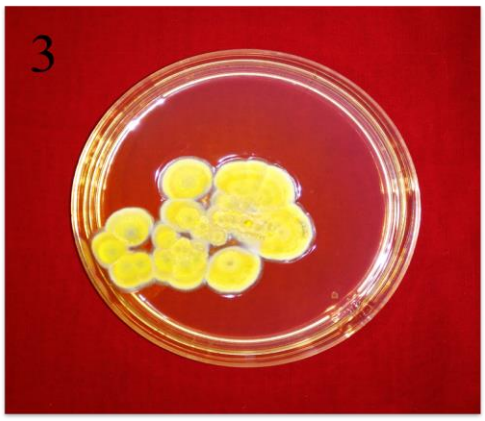

Fig. 3: Pure culture of Talaromyces tratensis

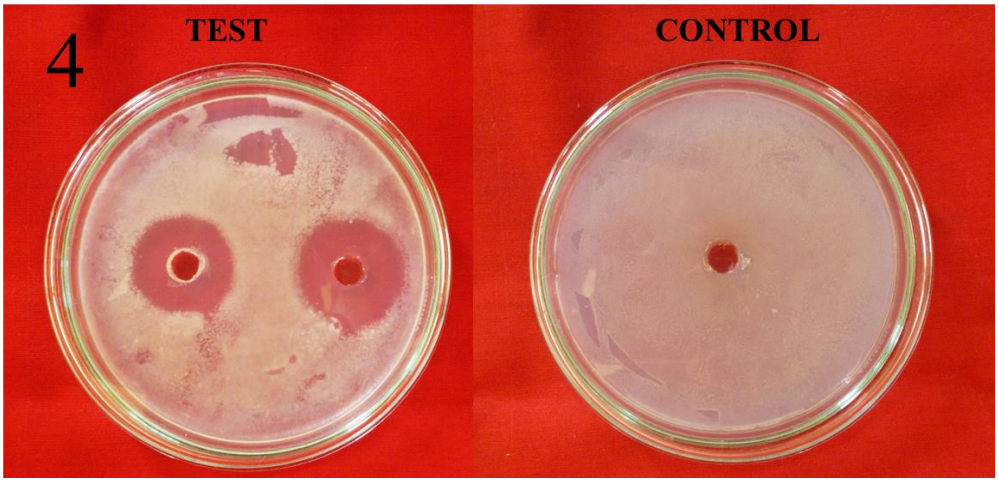

Fig.4: Antibacterial activity

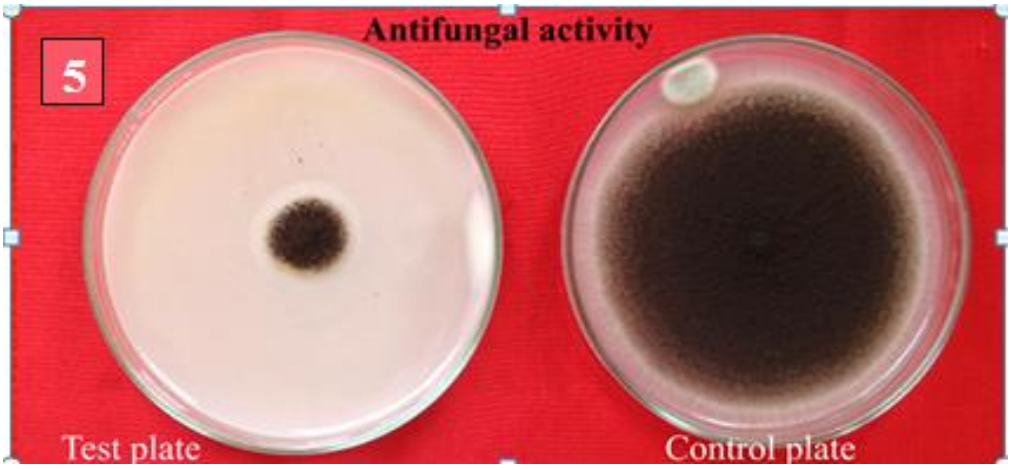

Fig.5: Antifungal activity

Crude metabolites of the *T. tratensis* were extracted with ethyl acetate as organic solvent by using solvent extraction procedure. The crude extract was evaluated for antibacterial and antifungal activity against some clinically significant microorganisms following agar well diffusion assay and poison food technique respectively. The metabolites displayed moderate to strong antibacterial activity (Fig. 4) against all the test pathogens. The metabolites showed highest in vitro activity against *Klebsiella pneumoniae* followed by *Escherichiacoli*, *Salmonella typhi, Proteus vulgaris*, *Bacillus substiles*, *Pseudomonas fluorescence* and *Staphylococcus aureus* (Table. 1). In food poison technique for antifungal activity (Fig. 5), it shows 82.59%

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highest growth inhibition on *Colletotrichum capsisi,* followed by *Aspergillus niger and Fusarium oxysporium* (Table. 2).

Table. 1: Antibacterial activity of *T. tratensis*

Name of Bacteria	% of growth inhibition at different concentration			
	25µl	50µl	75µl	100µl
Klebsiella pneumoniae	33.56	57.75	66.63	75.94
Escherichia coli	30.93	56.79	66.75	75.66
Salmonella typhi	30.98	56.32	66.52	74.39
Proteus vulgaris	31.70	55.28	66.00	69.83
Bacillus substiles	31.67	48.06	64.86	72.61
Pseudomonas fluorescence	29.38	49.47	64.95	72.61
Staphylococcus aureus	31.67	48.06	64.86	70.94

Table. 2: Antifungal activity of *T. tratensis*

Name of Fungal Pathogen	% of fungal growth inhibition				
	R_1	R_2	R_3	R_4	AVERAGE
Fusarium oxysporium	61.7	73.44	75.66	79	72.45 ± 7.52
Colletotrichum capsisi	86.86	81	79.69	82.8	82.59 ± 3.12
Aspergillus niger	71.89	72.9	75.87	70.93	72.90 ± 2.14

Table. 3 Antioxidant activity of *T. tratensis*

Sample concentration	DPPH	H2O2	NITRIC OXIDE
25mg	6.74±0.003	32.61±0.006	49.76±0.09
50mg	17.00±0.005	28.05±0.007	59.31±0.009
100mg	37.31±0.20	25.89±0.009	63.19±0.019
200mg	45.51±0.12	24.43±0.007	66.54±0.011
Control	0.00±0.00	0.00±0.00	0.00±0.00

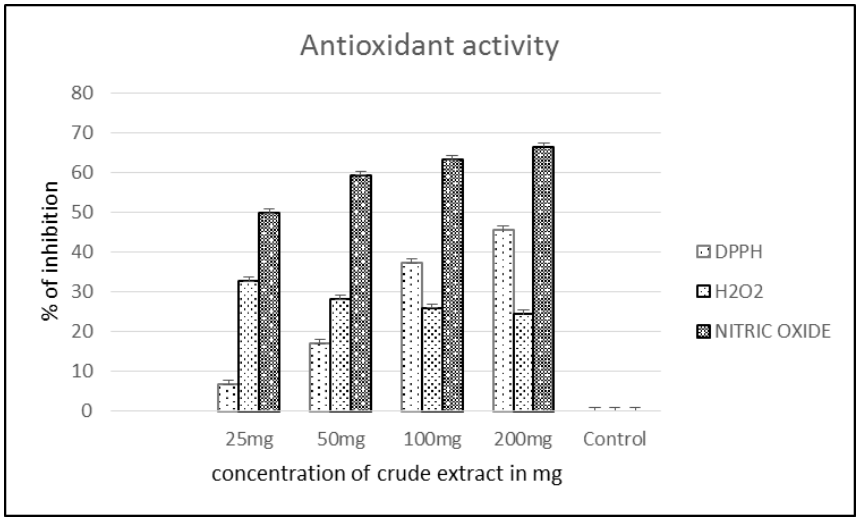

Fig.6: Antioxidant activity

The antioxidant potentiality of *T. tratensis* evaluated by using three different methods. The ethyl acetate crude extract showed significant antioxidant activity ranging from 49.76% to 66.54%. In DPPH radical scavenging activity, the reaction was visible as a colour change from Purple to Yellow and showed a high

antioxidant capacity value of 45.5% (Table. 3). Reduction potential of ethyl acetate crude extract in H_2O_2 assay showed 32% to 24% and In reducing power assay reducing ability was measured by change of Fe3+ to Fe2+absorbance values that indicated their greater reductive potential and electron donor ability for stabilizing free radicals (Table. 3). In the recent years of secondary metabolite of *Talaromyces* species research wasless prominence on biological testing, but increasingly there has been a focus on the biological properties of these compounds [31]. *Talaromyces sp.* are an important fungi [32], from many species of *Talaromyces* produced various kinds of biological activitie metabolites such as antibacterial, antifungal, antioxidant, cytotoxic and antivirals [31].

CONCLUSIONS

In this present investigation concludes that the presence of bioactive compounds in the extract which exhibited antimicrobial and antioxidant activity in *Talaromyces tratensis*. The evaluation of biological activity of Endolichenic fungi *T. tratensis*, especially those exclusive to their host lichen, is not only important from an ecological approach but also from a biochemical and molecular standpoint. Furthermore studies need to active crude extracts are being subjected to isolation and identification of active metabolites and different pharmacological evaluations for emerging new therapeutic drugs. To the best of our knowledge, this is the first report of the antimicrobial potency and antioxidant activity of *Talaromyces tratensis* isolated from Lichens. These results of the current study more strengthen our view that are potential treasure discovery of novel metabolites for bioactivities.

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