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Evaluating the *in vitro* antituberculosis, antibacterial and antioxidant potential of fungal endophytes isolated from *Glycyrrhiza glabra* L.

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Abstract

Endophytes, especially from medicinal plants and those from biodiversity rich ecoregions synthesize important bioactive molecules. The aim of the present study was to isolate and characterize the bioactive fungal endophytes from *Glycyrrhiza glabra* L. of Kashmir Himalayas-a biodiversity rich ecoregion in India. Plant material was collected from different location of Kashmir region for isolation of fungal endophytes. A total of thirty-three strains were isolated and their broth was screened for their antimicrobial activity by well diffusion assay. Potent endophytes were selected and identified by ITS-5.8S ribosomal gene sequence technique. The extracts of selected endophytes were evaluated for antibacterial as well as antimycobacterial and antioxidant activities by broth microdilution technique and DPPH assay, respectively. Thirteen isolates (40%) displayed antimicrobial activity against at least one pathogen. The extract of isolates identified as *Fusarium solani*strain (KT16646), *Fusarium oxysporum* strain (KT166447), *Colletotrichum gleosporoides* strain (KT166445) and *Alternaria alternate* strain (KT166448) displayed strong antimicrobial activity with MIC ranging from <2.34 to 125 µg/ml against various tested bacterial pathogens. *F. solani* and *C. gleosporoides* showed good activity against *Mycobacterium tuberculosis* (*M. tb*) strain H37Rv with MIC of 18.5 and 75 µg/ml, respectively. *F. oxysporum* exhibited good antioxidant activity with IC_{50} value of <100 µg/ml. To the best of our knowledge, this is the first study that reports antimycobacterial activity of any fungal endophyte isolated from *G. glabra* against the virulent strain of *M tb*. Thus, this study sets background towards the exploration of potential bioactive molecules that may have antituberculosis, antibacterial and antioxidant activity from the fungal endophytes of *G. glabra*.

Keywords: *Glycyrrhiza glabra* L., Kashmir Himalayas, endophytic fungi, ITS-5.8S ribosomal gene sequence, therapeutic activities

1. Introduction

Endophytes especially from medicinal plants of biodiversity rich ecoregions synthesize pharmacologically active molecules because of specialized habituation (Liu *et al*., 2004). Endophytic fungi are useful sources of biologically active substances having unique structures. There are around 300,000 plant species flourishing in uncharted quarter of the earth that harbor one or more endophytes (Schulz *et al*., 2002; Strobel 2003). Currently, every plant group ranging from large trees, palms, sea grasses, to even lichens serve as a source of endophytes. The number of plants studied for endophytic microbes is not much significant as compared to the 300,000 plant species expected to exist on the earth (Tan and Zou, 2001). Bioevaluation results of endophytic extracts and isolated chemical skeletons are a good rationale to explore new endophytes from untapped ecoregions for novel bioactive molecules (Strobel *et al*., 2004; Shah *et al.*, 2016). Identification and characterization of these compounds can facilitate the development of efficient therapeutic agents from this untapped natural source. Under the growing demand of current challenges like drug resistance among pathogens and the emergence of new diseases, their thorough exploration across all the highly diverse ecoregions of the earth is required. The Himalayan region of Jammu and Kashmir in India harbors a rich biodiversity of medicinal plants. Out of the 10,000 important medicinal plants, only fewer plants especially from Jammu and Kashmir region have been recognized for their endophytic micro flora (Deepu *et al.*, 2013). The aim of our study is to explore endophytes from '*G. glabra* (Liquorice)', which is ethno, pharmacologically a high value and an important medicinal plant grown in this high altitude Himalayan region of India. The plant is also found in the subtropical and temperate regions of the world. It has been used in medicine for morethan 4000 years (Mitscher *et al.*, 1980; Bombardelli *et al.*, 1989; Haraguchi *et al.*, 1998; Akao 2000; Seedi *et al.*, 2003; Cinatl *et al*., 2003; Hennell *et al.*, 2008). Screening of endophytic extracts is a preliminary step to check the biological efficacy rather than screening of pure compounds isolated from endophytes. Thus, the present study was designed to evaluate the *in vitro* antibacterial, antituberculosis and antioxidant activity of isolated fungal endophytes from the above said plant.

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2. Materials and Methods

2.1 Chemicals and reagents

Middle brook 7H9, Middle brook 7H11, ADC (albumin dextrose and catalase), OADC (Oleic-ADC), NaCl, Mueller Hinton broth/agar base, Potato Dextrose Broth/agar base, Ciprofloxacin, $Hgcl_2$ were obtained from HiMedia, Mumbai, India. Taq polymerase (3U/µl), Taq buffer (10 X) with 15 mM $MgCl_2$, dNTP Mix (2.5mM), 1kb ladder, Gel extraction kit were procured from Genei, Bangalore, India. ITS primers from IDT, Haryana, India. Isoniazid and Ethanol were purchased from Merck-Millipore. All other required analytical grade reagents were procured from standard companies and maintained under proper storage.

2.2 Collection of plant material

The plant material was collected from Aharbal, Yarikha and the farm of Indian Institute of Integrative Medicine, Srinagars, located between 34°5' N and 74°47'E at an elevation of around 1585 meters in Jammu and Kashmir State, India. The plant species was identified with the help of taxonomists IIIM Janaki Herbarium at Department of Biotechnology. A voucher specimen number was also deposited in the departmental herbarium. The accession is preserved as genetic source in the farm of IIIM, Srinagar. Following the selection of plant, its healthy leaves, roots and branches/stems were cut out with a sterile scalpel and were stored at 4°C until further use.

2.3 Isolation of endophytes

The endophytes were isolated from *G. glabra* by the slight modifications of the procedure as described earlier (Strobel and Daisy 2003). The freshly excised plant material (stem, branches and leaves) was cleaned for 10 min. using running tap water and subjected to sterilization by serially treating with 70% ethanol for 1 minute, 1.0% sodium hypochlorite (NaOCl) (v/v) for 1 min. and subsequently washed with sterile distilled water twice or thrice. Then, the stem leaves and branches were sliced into 1 cm small pieces following surface sterilization. The sterile samples were placed onto water agar and potato dextrose agar (PDA) media plates containing 250 µg/ml streptomycin to inhibit bacterial growth. These petridishes were covered by parafilm and incubated at 25 ± 2°C till the fungal mycelia began to grow from the samples. The well grown mycelia colonies were then transferred onto potato dextrose plates and plating of the microbial colonies was repeated continuously till pure cultures were obtained. The isolated fungal endophytes were stored at 4°C and samples were preserved in 30% glycerol at 70°C for later studies.

2.4 Screening for antimicrobial activity

The morphologically different isolated fungal endophytes were grown in 10 ml potato dextrose broth and incubated in shaker incubator (150 rpm) for about ten days at 28°C. The resulted broth was screened for antimicrobial activity by well diffusion assay against a series of human pathogens. A borer having a diameter of 8 mm formed wells on media containing petriplates spread over by pathogenic bacterial colonies. The wells were then treated with 30 µl of filtered broth and plates were kept overnight in an incubator at 37°C. Zones of inhibition around wells were measured in millimeters and recorded next day. The process was repeated thrice to strike out the average values.

2.5 Identification of potential endophytes

On the basis of preliminary screening, fungal strains showing strong antimicrobial activity were grown in PDA media for seven days at 28°C. The cell mass was separated by centrifugation and genomic DNA was isolated (Prabha *et al.*, 2013). The ITS region of the fungal DNA was amplified through PCR by using universal ITS primers, ITS1 (52-TCCGTAGGTGAACCTGCGG-32) and ITS4 (5'-TCCT CCGCTTATTGATATGC-3') (White *et al.*, 1990). PCR was performed as follows: denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 30s, primer-specific annealing temperature at 46°C for 30s, and 72°C for 1 min and a final extension for 10 min at 72°C. In the first step, PCR was carried out in a volume of 20 µl and then for preparative PCR, 50 - µL reaction mixture was used. The amplified product of around 500 bps was eluted by means of a Gel extraction Kit (Qiagen, USA) and 40 - 60 ng was employed in a 10 µl sequencing reaction using Big Dye Terminator sequencing kit (v. 3.1, Applied Biosystems). The resulted sequences of selected strains were deposited in NCBI database [www.ncbi.nlm.nih.gov/Blast.cgi]. Appropriate sequences were downloaded and aligned by employing the ClustalW program and a phylogenetic tree was constructed using MEGA6 software.

2.6 Fermentation and extraction

The selected strains that showed potent antimicrobial activity against test organisms in preliminary screening were subjected to small-scale fermentation. These fungal strains were grown in 500 ml potato dextrose broth and incubated in a shaking incubator (180 rpm) for a period of 10 d. at 28 ± 2°C. Culture broth was centrifuged and supernatant was extracted with equal volume of ethyl acetate. Ethyl acetate was concentrated using rotavapour and dried extracts were reconstituted in 100% dimethylsulfoxide and transferred for storage at -20°C until testing for their antimicrobial activities.

2.7 Antibacterial and antituberculosis activity

2.7.1 *Microbial strains*

A panel of lyophilized test bacteria and some fungal pathogens were acquired from Microbial Type Culture Collection (MTCC) and American Type Culture Collection (ATCC), *viz*., *Mycobacterium tuberculosis* H37R*v* strain (ATCC 25177), *Staphylococcus aureus* (MTCC 96), *Klebsiella pneumoniae* (MTCC 109), *Streptococcus pyogenes* (MTCC 442), *Bacillus subtilis* (MTCC 121), *Bacillus cereus* (IIIM 25), *Micrococcus luteus* (MTCC 2470) and *Escherichia coli* (MTCC 730).

2.7.2 *Preparation of stock and working solutions*

Stock antimicrobial solutions (4 mg/ml) of all the test extracts were prepared in dimethyl sulfoxide (DMSO). Stock solution (1mg/ml) of the reference antibiotics, isoniazid and ciprofloxacin purchased from HiMedia (India) was prepared in sterile distilled water. All stock antimicrobial solutions were stored in at -70°C till further use. The working solutions of extracts represented as E1, E2, E3, E4 of fungal endophytes coded as A1, A2, A4 and A7 having shown potent antimicrobial activity were prepared by diluting stock solutions in Mueller Hinton broth (MHB).

2.7.3 *Determination of minimum inhibitory concentration* (MIC)

The MIC was determined by broth micro-dilution method as done earlier (Ahmad *et al.*, 2005) and as per the guidelines of Clinical and

Laboratory Standards Institute (Wayne, 2006), with some modifications. Briefly, test extracts were mixed with MHB and Middle brook 7H9 (supplemented with 10% ADC: albumin, dextrose and catalase) for non-mycobacteria and mycobacteria, respectively. 200 µl of the above media was added to the first well of each row. All the remaining wells initially received 100 µl of sample free media. This was followed by performing serial two-fold dilutions by transferring 100 µl from column 1 to column 2 and then continued through column 10. 100 µl of excess medium was discarded from the wells in column 10. Bacterial inocula were prepared from mid log phase grown culture. The turbidity of each bacterial suspension was adjusted to 0.5 McFarland standards. The size of the inocula was adjusted to (1.5×10^8 CFU/ml) by appropriate dilutions with respective media and 50 µl of the same was added to each well of micro-titre plate to obtain a required inoculums of 5×10^5 CFU/ml in each well. The concentration of extract samples ranged from 2.34 to 1000 µg/ml Columns 11 and 12 served as growth control and media controls respectively. The plates were then incubated at 37°C for 24 h and read with an unaided eye for the absence or presence of visible growth. The MIC was taken as the lowest concentration of the sample showing no visible gsrowth. All the assays were done thrice in triplicate.

2.8 Evaluation of antioxidant activity

Measurement of radical scavenging properties of extracts of selected strains was tested for antioxidant activity. The antioxidant activity was carried out according to the method described by Baba *et al.* (2015) with slight modifications. Ascorbic acid was employed as positive control and % inhibition was determined according to the following equation:

$$\% \text{ Inhibition} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Three experimental replicates were taken for the assay. The IC_{50} values were calculated as the concentration of extracts causing 50% inhibition of DPPH radical, a lower IC_{50} value corresponds to a higher antioxidant activity of sample.

2.9 Statistical analysis

All experiments were carried out in three biological replicates and expressed as Mean ± Standard deviation (SD).

3. Results and Discussion

3.1 Isolation and characterization of endophytes

A total of thirty-three fungal isolates were isolated from the different tissue parts of *G. glabra*. Among these isolates, thirteen showed antibacterial activities against at least one of the tested pathogen in preliminary screening (Table 1). The activity was measured as zone of inhibition of filtered broth against various pathogens in three independent experiments. The mean value of the zone of inhibition in millimeters (mm) ± standard deviation (SD) as given in table 1.

Table 1: Antimicrobial Activity (zone of inhibition in mm) of ten days old culture broth of some of the isolated endophytes

Isolate code	*E. coli*	*S. aureus*	*S. pyogenes*	*K. pneumonia*	*B. subtilis*	*B. cereus*	*M. luteus*	*C. albicans*	*S. cerevisiae*
A1	12+1.00	15+1.00	10+0.5	16+1.00	18+1.00	14+1.00	20+2.00	_	
A2	10+1.00	20+1.00	18+1.5	12+1.00	20+1.00	11+0.5	30+2.00	_	11+1.00
A3	_	_	_	_	_	_	_	12+1.00	
A4	12+1.00	30+1.00	15+1.00	18+2.00	13+1.00	19+1.00	24+2.00	_	10+1.00
A5	_	_	_	_	_	_	16+1.00	_	
A6	_	_	_	_	_	_	_	_	14+1.00
A7	10+1.00	20+0.5	_	15+1.00	20+1.00		18+1.00	_	_
A8	_	_	_	_	_	_	_	_	12+1.00
A9	_	_	_	_	_	_	_	_	16+2.00
A10	_	_	_	10+1.00	_	_	_	_	_
A11	_	_	_	_	_	_	12+1.00	_	11+1.00
A12	_	_	_	_	_	_	11+1.00	_	_
A13	_	_	_	_	_	_	11+1.00	_	_

Among thirteen isolates, four potential isolates were characterized on the basis of ITS-5.8S ribosomal gene sequence technique. The identified endophytes were: *Fusarium solani* strain (KT16646), *Fusarium oxysporum* strain (KT166447), *Colletotrichum gleosporoides* strain (KT166445) and *Alternaria alternata* strain (KT166448). Further, ITS-5.8S ribosomal gene sequences of *Fusarium solani* strain (KT16646), *Fusarium oxysporum* strain (KT166447) showed 99% and 100% homology with *Fusarium solani* and *Fusarium oxysporum* respectively, while *Colletotrichum gleosporoides* strain (KT166445) and *Alternaria alternata* strain (KT166448) showed 96% homology with *Colletotrichum gleosporoides* and *Alternaria alternata*. Results based on ITS-5.8S ribosomal gene sequences of these strains were correlated well with morphological findings and their phylogenetic position (Figure 1). The four selected isolates with their crude extracts were evaluated for their activity.

3.2 Antibacterial activity

The crude ethylacetate extract these potent strains exhibited antibacterial activity with MIC values ranging from 2.34 to 125 µg/ml (Table 2). However *F solani* was found more potent against *S aureus* with MIC of 18.75 µg as compared to ciprofloxacin whose MIC against this strain is 50 µg. It also showed good activity against *M. tb*. Currently, there is immense need of newer and effective antimicrobials to defeat the growing challenge of antimicrobial drug resistance.

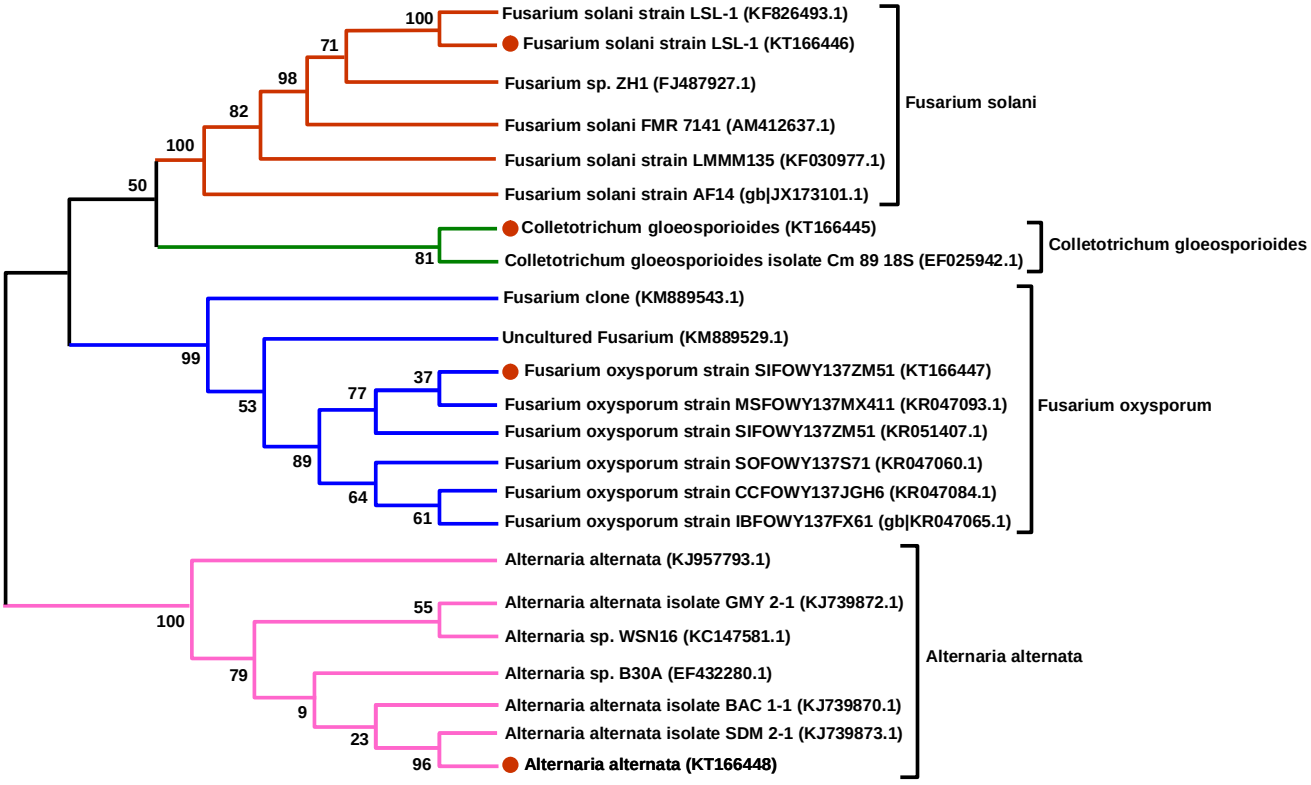

Figure 1: Phylogenetic tree of the identified strains of endophytes

Table 2: MIC values (µg/ml) of the extracts of selected endophytes

Bacterial strain	MIC of endophytic extracts (µg/ml)				Std
	E1	E2	E3	E4	Cipro
M. tuberculosis (ATCC25177)	18.5	75	>500	>500	H
E. coli (MTCC 730)	37.5	75	37.5	125	25
S. pyogenes (MTCC 442)	125	18.75	18.5	125	10
B cereus(IIIM 25)	<2.34	125	9.37	125	0.15
S. aureus (MTCC 96)	18.75	125	37.5	125	50
B. subtilis (MTCC 12)	9.37	4.68	4.68	<2.34	0.15
M. luteus (MTCC 2470)	9.37	37.5	9.37	125	0.78
K. pneumonia(ATCC 75388)	N.D	N.D	N.D	N.D	25

where E1, E2, E3 and E4 are ethyl acetate extracts of *Fusarium solani* strain (KT16646), *Colletotrichum gleosporoides* strain (KT166445), *Fusarium oxysporum* strain (KT166447), and *Alternaria alternate* strain (KT166448), respectively.

H= Isoniazid with MIC of 0.156 µg/ml against *M. tb*

From the endophytes reported in this study, some previous studies have identified various important metabolites after isolating them from different plant hosts of different ecoregions. To the best of our knowledge, this is the first study that reports antimycobacterial activity of any fungal endophyte isolated from *G. glabra* against the virulent strain of *M tb*. The variation in metabolite profile and biological activity of endophytes even between the isolates of same species can be due to the chemical diversity of host plants (Paulus *et al.*, 2006). An aggressive environment may lead to the evolution of increased synthetic ability of the endophytes. This perhaps explains the observation that why a species of endophyte isolated from a plant host is able to produce a bioactive compound but fails to do so when isolated from another plant species (Li *et al.*, 1996). The endophytes reported in our study are of significant value because some previous studies have reported different important molecules from the same endophytic species while isolating them from different plant hosts of different ecoregions. The metabolites of *Fusarium solani* from *Taxus baccata* bark in Dibang Valley of Arunachal Pradesh were characterized as 1-tetradecene, 8-octadecanone, 8-pentadecanone, octylcyclohexane and 10-nonadecanone (Tayung *et al.*, 2011). Camptothecin and 10-hydroxycamptothecin are two important metabolites obtained from the same endophyte when isolated from *Apodytes dimidiata* (Icacinaceae), a tree in the Western Ghats of India (Shweta *et al.*, 2010). Fumitremorgin B, Fumitremorgin C, Helvolic acid, Bisdethiobis (methylthio) gliotoxin, Bis-N-norgliovietin and Gliotoxin were isolated from the endophyte *Fusarium solani* of *Ficus carica* (Zhang *et al.*, 2012). *Fusarium oxysporum* when isolated as an endophyte from the Indian *Catharanthus roseus* plant produced the anticancer drugs vinblastine and vincristine but reported to produce taxol after its isolation from the mangrove leaves of *Rhizophora annamalayan* (Elavarasi *et al.*, 2012; Kumar *et al.*, 2013). *Colletotrichum gloeosporioides* isolated from *Piper nigrum* produced piperine, which possess a wide range of bioactivity including antimicrobial, antidepressant, anti-inflammatory, antioxidative and anticancer activities. Interestingly, it also increases the bioavailability of many drugs (Chithra *et al.*, 2013). This indicates that chemoprofile of same endophyte species varies from one ecoregion to another ecoregion because biodiversity can have considerable role in ecosystems (Naeem, 2002). Therefore, there is possibility of isolating some different important bioactive molecules from the endophytes of *G. glabra*.

3.3 Antioxidant activity

Among the four extracts, only the extract of *Fusarium oxysporum* has shown promising antioxidant ability (Figure 2). Its antioxidant potential was found close to that of standard antioxidant, ascorbic acid. This indicates that the chemoprofile of its extract can be somewhat different from the extract of other endophytes and may contain compounds with such activity. There is dire need of effective antioxidants to combat oxidative stress, which is involved in the progression of many diseases like cancer and neurodegenerative diseases (Shah *et al.*, 2016).

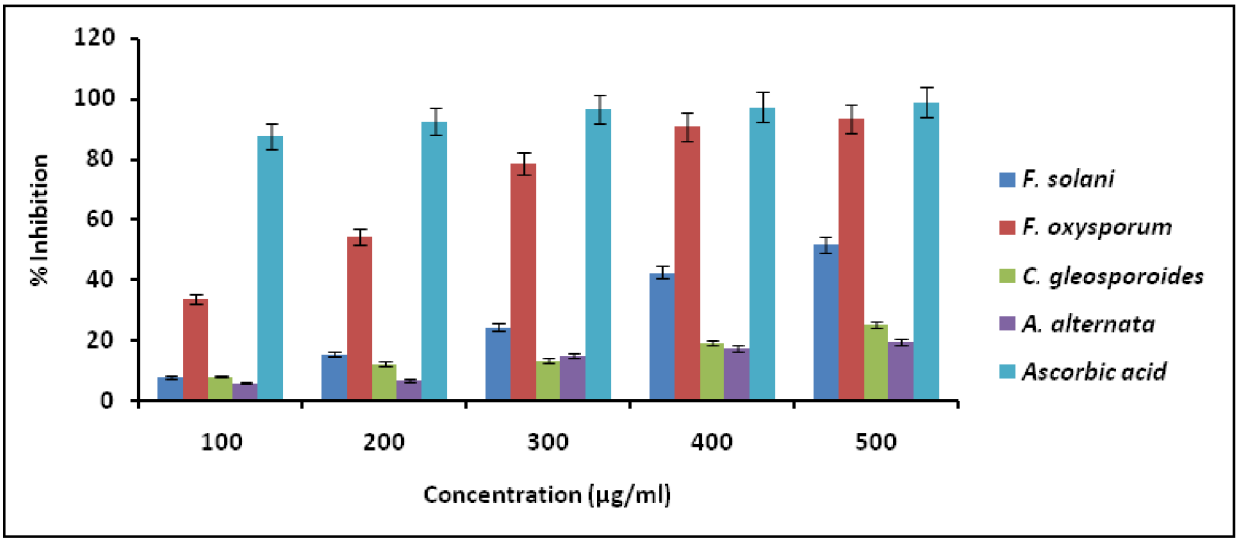

Figure 2: Antioxidant activity of extracts of selected endophytes

This study was successful in the isolation and investigation of antagonistic fungal isolates from *G. glabra*. The ethylacetate extract of four isolates demonstrated potent antibacterial activity and the extract of *Fusarium oxysporum* showed antioxidant property. The metabolites of same endophyte vary with respect to source plant and ecoregions. Therefore, the exploration for new secondary metabolites must focus on endophytes existing in biodiversity rich ecoregions. There exist a number of accessible biodiversity rich ecoregions (around 238) throughout all the continents, whose endophytic biodiversity is still unexplored. These consist of African, Australian, American and Himalayan regions of Asia (David and Eric, 2002). Since the Himalayan region of Jammu and Kashmir in India provide specialized habituation for exploration of endophytes due to its different climatic zones with temperate, tropical and arid

regions. It is known as the Botanical Garden of Globe due to its rich biodiversity of medicinal plants. It is one among the 26 hotspots in India with around 4439 species of Angiosperms, 19 species of Gymnosperms and 168 species of ferns (Deepu *et al.*, 2013). As a part of the research mandate of our Institute to explore medicinal plants from high altitude regions of Kashmir Himalayas, our research team is actively engaged in the exploration of such medicinal plants (Mushtaq *et al.*, 2016). However, they and the bioactive endophytes associated with them need to be explored fully. Some of the endophytes isolated from the medicinal plants of this ecoregion have shown diverse biological activities like antimicrobial, anticancer and cytotoxic activities (Maroof *et al.*, 2012; Masroor *et al.*, 2013; Meenu *et al.*, 2014). From a few native medicinal plants, our research team has previously reported some bioactive endophytes synthesizing chief metabolites (Amardeep *et al.*, 2008; Rehman *et al.*, 2011; Refaz *et al.*, 2013). Thus, this study sets background towards the exploration of potential bioactive molecules that may have antituberculosis, antibacterial and antioxidant activity from the fungal endophytes of *G. glabra*. With this notion, we isolated and characterized potent bioactive fungal endophytes from *G. glabra*.

4. Conclusion

These studies show that endophytes especially from medicinal plants of biodiversity rich ecoregions and unusual habitats are able to produce important and or novel bioactive molecules. Therefore, further studies are required to explore endophytes of medicinal plants of Kashmir Himalayas and other unusual biodiversity rich ecoregions for important and or novel bioactive molecules. Our results suggest that these potent endophytes may be a source of natural antimicrobial, antituberculosis and antioxidant agents for drug discovery programme. The presence of bioactive endophytes can also be a reason of improved disease resistance in the plant as it is least susceptible to most common phytopathogens. They may have helped the plant to resist and adapt to the changing harsh environmental conditions since its age-old survival in the history of ethno medicinal utility. Thus, the role of these endophytes in the plant itself needs to be elucidated.

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Conflict of interest

We declare that we have no conflict of interest.

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