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Phytochemical distribution, antimicrobial activity, enzyme production of phylogenetically differentiated endophytes from *Solanum violaceum* Ortega fruits.

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Abstract

The aim of the present work is to isolate, identify endophytic fungi from edible fruits and to test the efficacy on nosocomial microbes. Healthy and asymptomatic unripe and ripe fruits of *Solanum violaceum* were studied for the presence of endophytes. Twelve endophytic fungi were isolated and identified on the basis of Internal Transcribed Spacer sequences. Screened the fungi for presence and absence of phytochemicals and enzymes; and antimicrobial activity of endophytes was studied by testing against human nosocomial pathogens. Diversity of fungal endophytes was more in ripe fruits as evidenced by diversity indices *viz*., Simpson's diversity (0.834) and Shannon–Weiner indices (H'- 0.474). Phytochemical analysis revealed the presence of alkaloids, saponins, glycoside, gum and mucilage. Isolates produced amylases (33.33%), lipases (58.32) and asparaginase (80%) but not laccase and protease enzymes. *Alternaria* sp, *Cladosporium tenuissimum*, *Mycosphaerellaceae* sp., *Curvularia beasleyi* and *Alternaria alternata* showed activity against *S. aureus*, *E. coli* and *K. pneumoniae*. *Curvularia beasleyi* exhibited similar antifungal activity against *C. albicans* and *C. tropicalis* (20±2mm). Morphological changes of mycelia were noted after 96 h of treatment. To conclude, fungal endophytes might be potential agents for production of useful enzymes and to control microbes.

Keywords – Agar plug – Cell free supernatant – Cross streak assay – Extracellular enzymes – Fungal endophytes – Internal transcribed spacer ribosomal-deoxyribonucleic acid – Nosocomial pathogens

Introduction

Solanum violaceum Ortega., synonym, *Solanum indicum* var. *recurvatum* C.Y. Wu & S.C. Huang commonly called as poison berry belongs to the family *Solanaceae*. It is a widespread, spiny, highly branched shrub, and grows up to 5 m height. The younger branches are covered heavily with minute star-shaped hairs. *Solanum violaceum* grows in tropical and sub-tropical areas of India and Nepal. Plants found usually on wastelands, roadsides and in open scrublands. Whole

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plant is rich with fatty acids, wax and solanine (alkaloid). The fruits are edible, rich in phosphorous content along with carotene, carpesterol, solanocarpone, lanostrerol, solasonine, solanidine and β-sitosterol etc. The different parts (fruits, leaves, roots) of this plant are used to treat loss of appetite, anorexia, asthma, colic, digestive disorder, heart diseases, nasal ulcers, body pains, toothache, vomiting and worm infestation caused by roundworms, whipworms, hookworms, tapeworms and flukes (Jain & Borthakur 1986, Raju et al. 2013).

Plants are fundamental sources of biologically active compounds that treat innumerable diseases worldwide. The wide range of naturally occurring pharmaceuticals exhibit and play an important role in drug discovery and development (Alvin et al. 2014). Plants harbour a few microbes in them and both benefit mutually. Endophytes are non-pathogenic microorganisms, often fungi or bacteria, residing in extracellular spaces of tissues with no apparent symptoms in the host. It is known that plants and their endophytes produce similar therapeutic products (Kusari et al. 2013). Endophytic fungi are capable of helping their host plants during biotic and abiotic stress conditions.

Medicinal plants are the rich source of endophytic fungi that are known to produce bioactive compounds (Arnold et al. 2003, Waqas et al. 2012).

Secondary metabolites of endophytic fungi are significant agriculturally, industrially, pharmaceutically and ecologically and these organisms are recognized as valuable medicinal sources (Deshmukh et al. 2014). According to chemical structures and biosynthetic pathways, secondary metabolites (phytochemicals) are mainly divided into flavonoids, glycosides, polyketides, peptides, alkaloids, terpenes and many more (Aly et al. 2010, Hari et al. 2014). As part of metabolic processes in cells, unique enzyme production is the capability of an endophyte to carry out a specific biochemical reaction (Carrim et al. 2006). Enzymes, in general, are specific in their action on substrates. The principal function of fungal enzymes is to hydrolyse food substances or to control pathogens (Pavithra et al. 2012). Additionally, these microbes are easy to grow at large scale in fermentation process for the enzyme production.

The key health threat globally is microbial resistance. Pathogens turn into resistance to available antibiotics by limiting the drug target access and altering the drug. Therefore, a methodical study about endophytic fungi may prove to be a new tool for finding novel bioactive molecules against pathogenic microbes (Sahu et al. 2014).

Since there is no data on the endophytic fungi from *Solanum violaceum* Ortega and the active compounds produced by endophytes, we made an attempt to isolate and identify cultivable endophytic fungi from *S. violaceum* fruits and uncover their bio-active potential against microorganisms.

Materials & Methods

Sample collection

Plant, *S. violaceum*, growing in Dhanvanthari herbal garden, was identified and authenticated. The voucher specimen number (USAB-4589) and collection number (MAP1) were given by Taxonomist, Dr. K. T. Prasanna, Professor and Head, Department of Forestry and Environmental Science, Gandhi Krishi Vignana Kendra (G.K.V.K.), University of Agricultural Sciences (U.A.S), Bengaluru. Healthy (showing no visible disease), uniform, matured (ripe/yellow) and unripe (green) fruits of *S. violaceum* were collected during spring season, 2019 and transported carefully in paper bags with wet cotton to avoid moisture loss.

Sterilization and endophyte isolation

The endophytes were isolated from the above-mentioned parts as per the protocol (Jinu & Jayabaskaran 2015) with slight modifications. Fruits were selected for isolation of endophytes because they are edible and used as vegetable. They are rich in phosphorous content along with carotene, carpesterol, solanocarpone, lanostrerol, solasonine, solanidine and β-sitosterol etc. The sterilization process is as follows: water wash to remove the dust and dirt from the surface,

followed by 4% sodium hypochlorite for 30 seconds, followed by sterile distilled water wash for four times to remove sodium hypochlorite.

The last water wash was tested for microbial growth on appropriate medium; no growth gave us information of proper surface sterilization. The surface sterilized ripe and unripe whole fruits were aseptically crushed in an autoclaved mortar and pestle inside laminar airflow to separate pulp and seeds. Small pieces of the pulp, broken seeds and the whole fruits were used for isolation and carefully positioned the same on Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDB) and Czapek dox agar media supplemented with 50 mg/L of streptomycin and 35mg/L ampicillin antibiotics to suppress bacterial growth (Waller et al. 2001). All the plates were incubated at 27°C for 2 weeks until visible mycelium emerges. The actively growing hyphal tips were subcultured for another week on their corresponding media. The fungal mycelia were preserved in 50% (v/v) autoclaved glycerol at 4°C.

Identification by staining and molecular methods

Genus identification of isolated fungal endophytes was performed by comparable morphological features, shape, colony colour, and texture of the mycelium. Lactophenol cotton blue stained hyphae and spores were viewed and captured and under microscope (Petrini 1986).

Genomic DNA was isolated from all the isolated fungi by Cetyl Trimethyl Ammonium Bromide (CTAB) method (Camacho et al. 1997, Chiang et al. 2001). DNA purity and concentrations were checked in Nanodrop. Polymerase Chain Reaction (PCR) was performed by using Internal transcribed spacer 1 (forward 5'- TCC GTA GGT GAA CCT GCG G- 3') and ITS 4 (reverse 5'- TCC TCC GCT TAT TGA TAT GC–3'). PCR reaction mixture contained 0.1 µg genomic DNA (3 µL), 10 pmol/µL forward and reverse primers (1.5 µL), *Taq* polymerase master mix containing 400 µM dNTPs and 3mM $MgCl_2$ (pH8.5) (15 µL), and 9 µL molecular biology grade distilled water. The PCR conditions adopted were: initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 8 min. The PCR product was loaded in 1% agarose gel along with 1kb DNA ladder (Kuriakose et al. 2014) followed by unidirectional sequence with ITS 1 at Biokart sequencing services (Bengaluru). The obtained sequences were analysed, dropped in NCBI GenBank (http://www.ncbi.nlm.nih.gov) for comparing with the existing genera and species sequences using BLASTN algorithm.

Phylogenetic analysis and diversity indices calculation

By using the above obtained sequences and from the sequences available in GenBank, a dendrogram was made for the phylogenetic analysis. Clustal X aligned sequences were used to make dendrogram in MEGA5 (Tamura et al. 2013) by neighbour joining. The divergence was evaluated using indices such as Simpson's dominance (D), Simpson's diversity index, Sorensen's index of similarity (QS) (Dhayanithy et al. 2019, Katoch et al. 2017, Uzma et al. 2016).

Extraction of extrolites

To maintain uniformity, all the fungi were cultured on Potato Dextrose Broth (PDB). Actively growing hyphal tips of all the fungi were inoculated in Erlenmeyer flasks containing PDB and cultivated for three weeks at 27°C (Atalla et al. 2008). The broth was filtered through Whatman filter paper 1 to separate the mycelial mat from fungal filtrate. The mats were placed in an oven at 40°C for drying and fine powder was made. The filtrates were further clarified by spinning at 4°C at 12,000 rpm for 10 min. Cell Free Supernatants (CFSs) and the dry mat powders were then stored at 4°C untill further use.

Screening for extracellular enzyme from endophytic fungi

All the isolated fungi were screened for their capacity to produce amylases, lipases, proteases, laccases and asparaginases (Mani et al. 2018, Moharram et al. 2016, Sunitha et al. 2013). For all the enzymes, inoculated actively growing hyphae tips on the respective media. Every experiment was

carried out three times and further evaluation was done after week long incubation at 27°C. By using the formula mentioned below, the relative enzyme activity index was estimated (Hankin & Anagnostakis 1975) and results were tabulated.

$$\text{Clear zone ration} = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

Phytochemical screening

The CFSs and powdered mats were screened for the qualitative presence of phytochemicals *viz.*, alkaloids, flavonoids, phenols, tannins, saponins, steroids, reducing sugars, cardiac glycosides, gums and mucilage, fats and oils by standard protocols (Devi et al. 2012, Harborne 1998). The following tests were performed for the above-mentioned phytochemicals:

Wagner's for alkaloids, foam and froth for saponins; Killer-Killiani for glycosides, ferric chloride for phenolics, gelatin for tannins, alkaline reagent for flavonoids, Benedict's for carbohydrates, filter paper test for fats and fixed oils, production of slimy mass test for gums and mucilage.

Test organisms, media and standard antibiotics used in this study

Human nosocomial pathogens such as *Candida albicans* (ATCC 10231) and *Candida tropicalis* (ATTC 201380); *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 35657) were collected from Department of Microbiology, Sri Devaraj Urs Academy of Higher Education and Research. In this study, for antimicrobial assay, Mueller Hinton Agar medium (MHA) (for bacteria) and PDA (for fungi) were used (Marcellano et al. 2017, Pai & Chandra 2018, Yasser et al. 2019); Fluconazole for fungi; ceftazidime and chloramphenicol for bacteria were used as positive controls. The overnight pathogen suspensions were adjusted to 0.5 McFarland standard for the test analysis.

Preliminary antimicrobial activity by different diffusion methods

Agar well-diffusion method

All the filtrates were filter sterilized before performing the experiments. Test bacteria were spread on MHA plates with sterile cotton swabs and test fungi were mixed with PDA. Wells were made with sterilized cork borer (5mm) and 30 µL of crude CFSs were added. To facilitate proper diffusion, plates were refrigerated for half an hour, later incubated at 37°C for 24 h (bacteria) and 28°C for 48-72 h (fungi) and measured the inhibition zone. The experiment was carried out for three times.

Agar plug diffusion

All isolated fungi were grown on PDA for three weeks at 27°C. Fungal agar plugs (8 mm) were cut from them, pressed against the test bacteria/ fungi which were already spread on the respective plates, and the plates were sealed. The plates were refrigerated for 12 h to facilitate metabolite diffusion and left undisturbed at 37°C to enable microbial growth. After incubation, the zone of inhibition was measured (Marcellano et al. 2017).

Cross-streak plate methods

Method employed was based on the protocol of Aljuraifani et al. (2019) with slight modifications. Briefly, the actively growing endophytic fungal strains were seeded in centre of the blood agar plate as a single streak. Blood agar medium was used because; this medium is suitable for both bacteria and fungi. After an incubation period, depending upon growth of the strain, the plates were streaked with the pathogenic organisms on either side of fungus. After incubation, the antimicrobial interactions were analysed by observing the growth.

Stability test for cell free supernatants

To find out the thermal stability, based on the antimicrobial potential, the selected CFSs were kept in a boiling water bath (98-100°C) for 10 min. In order to check its protease stability, the CFSs were treated with Proteinase-K (1 mg/mL) for 2 h at 37°C. These treated CFSs and positive control (antibiotic discs) were tested for antimicrobial activity (Chatterjee et al. 2019).

Effect of CFSs on fungal colony forming units and morphology

The extracts that showed activity against pathogenic fungi (*Candida albicans* and *Candida tropicalis*) were selected for this assay. Method employed is based on the protocol of Chatterjee et al. (2019) with slight modifications. The pathogenic fungi (*C. albicans* and *C. tropicalis*), PDB medium (supplemented with 50 mg/L of Streptomycin to avoid bacteria contamination) and CFSs were mixed in 1:1:1 proportions. Pathogenic fungi without CFSs were considered as controls. Treated and untreated cultures were kept undisturbed for 24 h, 48 h, 72 h and 96 h at 37°C. Colony forming units were counted by spreading on PDA. Microscopic observations were recorded for morphological changes under 40X.

Statistical Analysis

All the experiments were performed in triplicate and data are represented in mean. For calculating mean and standard deviation, Microsoft Excel program version 2019 was used.

Results

Isolation and identification

From healthy unripe and ripe fruits (fruit pulp, whole fruit and seed) of *S. violaceum*, twelve fungi were isolated using different media. The DNAs from the isolated organisms were amplified (approximate size was between 500 and 600 bp) by using universal ITS 1 and ITS 4 primers (Fig. 1) and all the fragments were sequenced. The nucleotide BLAST search provided the 96% to 100% homology with the organisms from GenBank. The rate of fungal growth was higher in ripe fruit (7) followed by unripe (5) (Fig. 2).

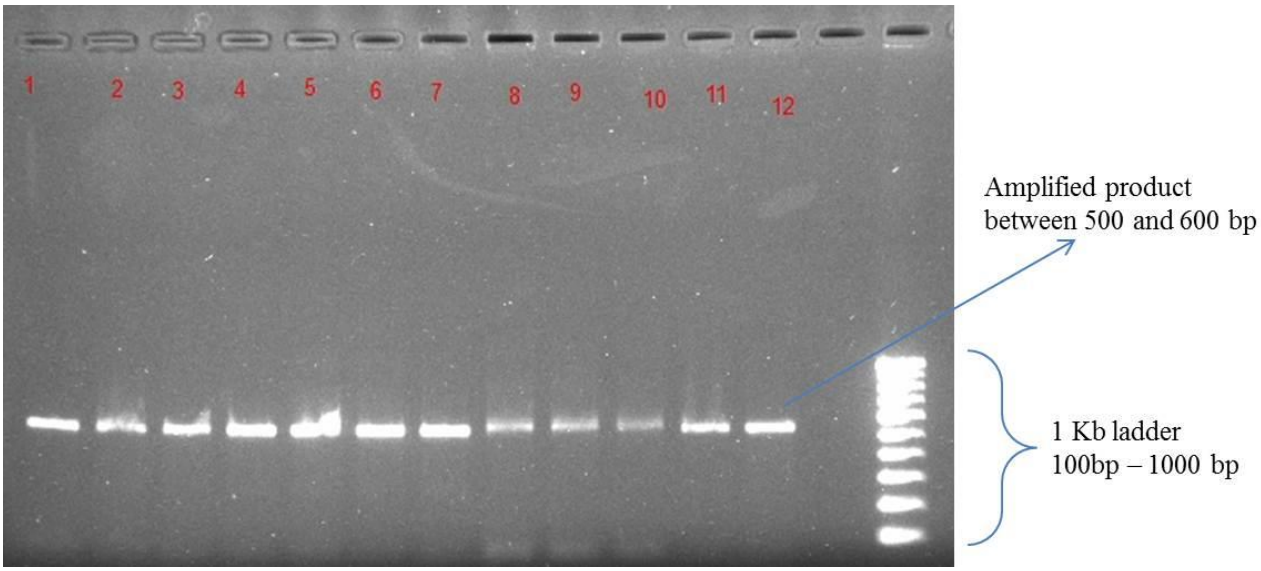


Fig. 1 – The Internal Transcribed Sequence 1 and Internal Transcribed Sequence 4 PCR amplified product from 1 to 12 endophytic fungi isolated from *S. violaceum* in 1.5% agarose gel. The amplified product was between 500 and 600 bp.

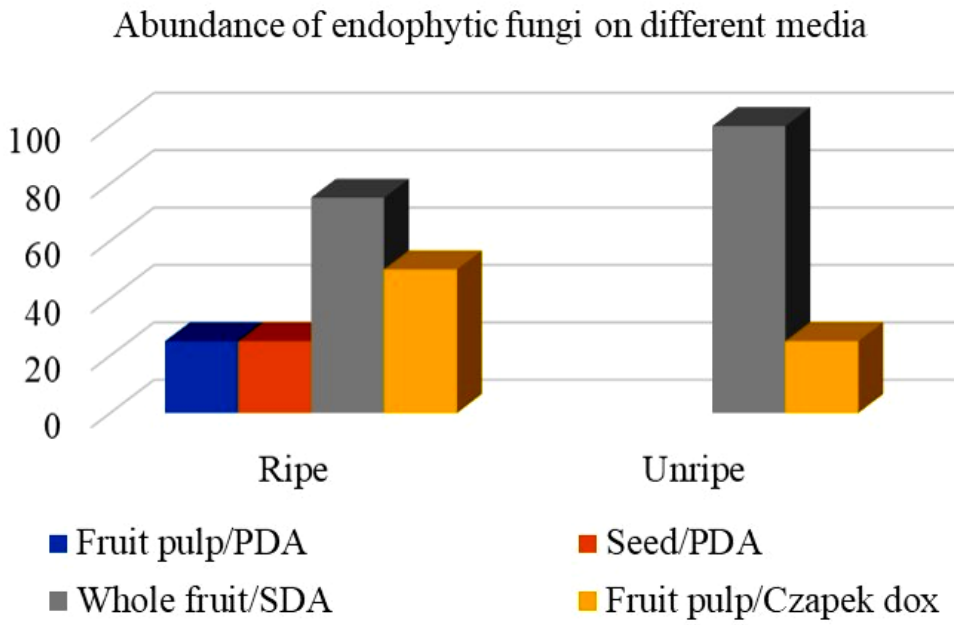


Fig. 2 – Percent abundance of fungal endophytes in both ripe and unripe fruits. X-axis indicates the part of the plant used; Y-axis indicates the percentage of fungi.

All the organisms were inoculated on PDA to maintain uniformity, and the isolated organisms belonged to Ascomycota phylum. *Cladosporium* sp., had the highest colonization frequency in ripe fruit, whereas *Alternaria* sp., had the highest colonization frequency in unripe fruit (Fig. 3). Molecular identification was carried out based on the obtained sequences. The obtained ITS sequence of each endophyte was blasted against the nucleotide database of NCBI for the most homologous sequence. Taking into consideration the blast results, the species to which each endophyte belonged were determined accordingly. The identified fungal endophytes data is given in Table 1. The neighbour joining phylogenetic tree also showed its close relationship with the strains (Fig. 4, Table 1).

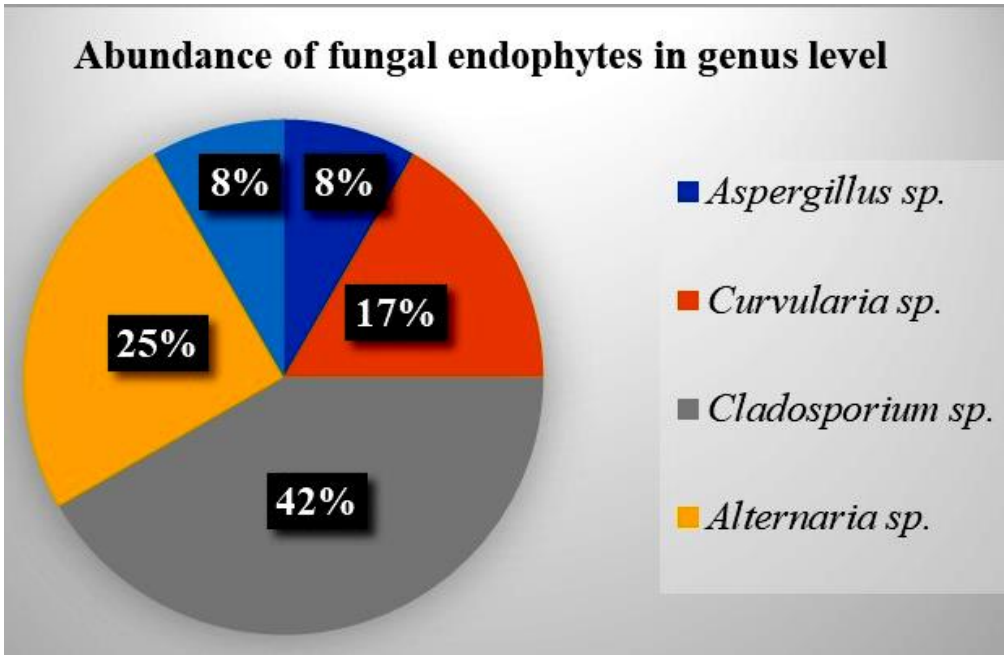


Fig. 3 – Per cent abundance of endophytic fungi isolated from ripe and unripe fruits of *S. violaceum*.

Table 2 summarizes the indices related to the diversity of fungal endophytes in *S. violaceum.* In this experiment, species diversity was evaluated based on Simpson's diversity index (1-D) and Margalef and Menhinick index can reflect the richness of endophytic fungal species. Similarity among the species was calculated based on Sorensen's index of similarity.

Qualitative analysis of phytochemicals

Phytochemical analysis was carried out on the isolated fungal endophytes to determine the presence of chemical constituent as a potential source for medicinal and industrial use. Phytochemicals were qualitatively analysed from the fungi and are listed in Table 3.

Dry mats did not show the presence of phytochemicals except alkaloids (SRF-1,2,5,7; SUF-1, 2 and 4) and carbohydrates (SRF-5, SUF-1 and 5). Alkaloids, carbohydrates, glycosides were noted as the major phytochemical constituents of the isolated fungal CFS.

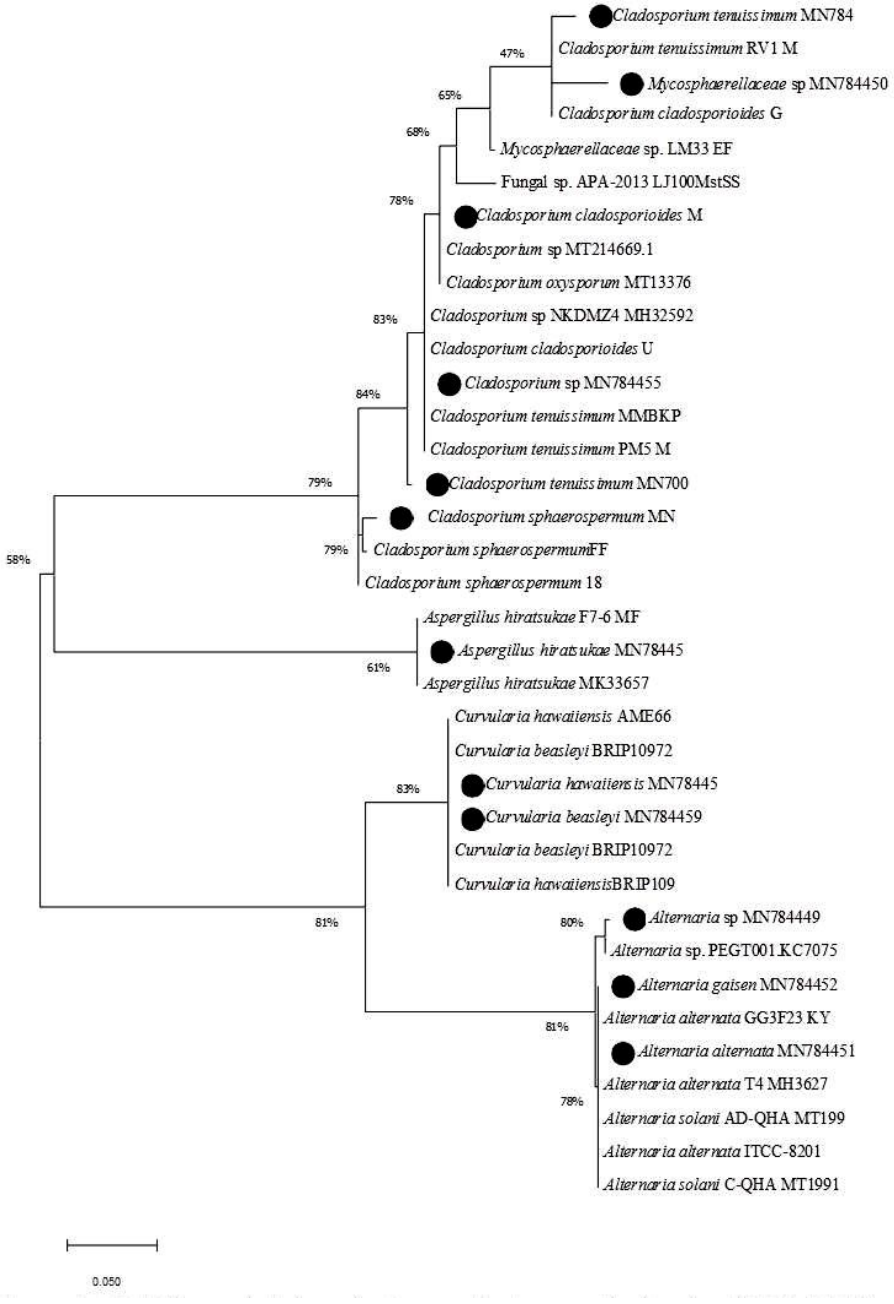
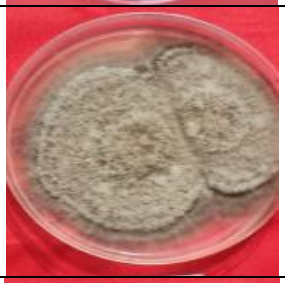
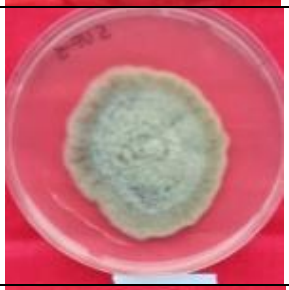


Fig. 4 – Fungal diversity analysis by neighbour joining phylogenetic tree on the basis of ITS rDNA sequence.

Table 1 Macroscopic observation of *S. violaceum* endophytic fungal isolates with GenBank accession numbers, percentage homology and amplicon size

GenBank accession number, name and division of the isolates from *S. violaceum*	**Plate Photo**	**Homologous organisms from NCBI GenBank**	**% Query coverage**	**Size (bp) from ITS 1**	**ID given to the organism**
MN784457 ***Aspergillus hiratsukae*** **(Ascomycota)**		*Aspergillus hiratsukae* strain MK336575.1	100%	386	SRF-1
MN784459 ***Curvularia beasleyi*** **(Ascomycota)**		*Curvularia beasleyi* NR158442.1	100%	434	SRF-2
MN784458 ***Curvularia hawaiiensis*** **(Ascomycota)**		*Curvularia hawaiiensis* MG571422.1	100%	502	SRF-3
MN784448 ***Cladosporium sphaerospermum*** **(Ascomycota)**		*Cladosporium sphaerospermum* KP794157.1	100%	498	SRF-4
MN784453 ***Cladosporium tenuissimum*** **(Ascomycota)**		*Cladosporium tenuissimum strain RV-1* MK957179.1	100%	296	SRF-5
MN784454 ***Cladosporium tenuissimum*** **(Ascomycota)**		*Cladosporium tenuissimum* isolate KIK MN700643.1	100%	440	SRF-6

Table 1 Continued.

GenBank accession number, name and division of the isolates from *S. violaceum*	**Plate Photo**	**Homologous organisms from NCBI GenBank**	**% Query coverage**	**Size (bp) from ITS 1**	**ID given to the organism**
MN784455 *Cladosporium* sp. (Ascomycota)		*Cladosporium* sp. isolate NKDMZ-4 MH325926.1	100%	444	SRF-7
MN784456 *Cladosporium Cladosporiodes* (Ascomycota)		*Cladosporium cladosporiodes* MG228421.1	100%	477	SUF-1
MN784449 *Alternaria* sp. (Ascomycota)		*Alternaria* sp. PEGT001 KC707558.1	100%	510	SUF-2
MN784450 *Mycosphaerellaceae* sp. (Ascomycota)		*Mycosphaerellaceae* sp. EF060417.1	100%	408	SUF-3
MN784451 *Alternaria alternata* (Ascomycota)		*Alternaria longipes strain* MN853398.1	100%	511	SUF-4
MN784452 *Alternaria gaisen* (Ascomycota)		*Alternaria alternata* MT077170.1	100%	492	SUF-5

Isolates were named after the source.
Abbreviations: SRF = *Solanum* Ripened Fruit, SUF = *Solanum* Unripened Fruit

Table 2 Common ways to measure the diversity of isolated fungi from *S. violaceum*

Indices	Ripe			Unripe		
	FP	S	WF	FP	S	WF
Species richness	3	1	2	1	0	2
Simpson's diversity	0	0	0.834	0	0	0.667
Menhenik (R1 for WF)	1.229			1		
Margalef (R2 for WF)	2.567			1.661		
Sorensen's index	0.538			0.666		

Abbreviations: FP = Fruit pulp, S = Seed, WF = Whole fruit

Extracellular enzyme production by the isolates

The results of presence and absence of extracellular enzymes were shown in Fig. 5, Table 4. Each strain was able to produce one or the other enzymes. None was capable to synthesize all five enzymes. Out of 12, only four isolates produced clear zone around the colony by digesting the starch, a positive result for amylase. Among these, *Cladosporium* sp., was the potential amylase producer with a maximum halo of 13mm. White precipitate around the colony was an indicative of lipase production in seven isolated endophytes. Among the positive isolates, *A. gaisen* was found to be potent lipase producer based on the clear zone around the colony. Asparaginase enzyme was produced by 80% of the isolates, *C. sphaerospermum* has been identified as potential producer. None of the isolates produced laccase and protease. The enzyme production by the plant's counterpart is partially or completely dependent on host's habitat (Uzma et al. 2016).

Amylase positive isolates – *Cladosporium tenuissimum* (MN784453), *Cladosporium tenuissimum* (MN784454), *Cladosporium* sp., *Cladosporium cladosporiodes*

Lipase positive isolates – *Cladosporium sphaerospermum, Cladosporium tenuissimum, Cladosporium* sp., *Cladosporium cladosporioides, Alternaria* sp., *Alternaria alternata, Alternaria gaisen*

Asparaginase positive isolates – *Aspergillus hiratsukae, Curvularia beasleyi, Curvularia hawaiiensis, Cladosporium sphaerospermum, Cladosporium tenuissimum, Cladosporium tenuissimum, Cladosporium* sp., *Cladosporium cladosporioides, Mycosphaerellaceae* sp. and *Alternaria gaisen*

Antimicrobial activity

All the obtained CFSs and agar plugs were assessed for the initial antimicrobial activity on human pathogenic nosocomial organisms. *Alternaria* sp, *C. tenuissimum, Mycosphaerellaceae* sp., and *A. alternata* CFSs tested against *S. aureus, E. coli,* and *K. pneumoniae*. *C. beasleyi* alone showed antimicrobial activity against all pathogens, except on *P. aeruginosa*. A range of 10 to 22mm in diameter inhibition was noted by agar well diffusion method. *C. beasleyi* showed strong inhibition toward *C. albicans* and *C. tropicalis* (20mm) which was nearly equivalent to the standard antibiotics tested (Fig. 6). In agar plug diffusion method, the fungal agar plugs showed activity against only with *E. coli* and *S. aureus* (Fig. 7). In cross streak method *Alternaria gaisen* showed good activity against the test bacteria and the results are similar to the agar well diffusion assay (Fig. 8) while none of the other isolated endophytic fungi showed activity on pathogenic fungi. Fungal positive control, Fluconozole, showed 22mm diameter, whereas bacterial positive control Chloramphenicol showed 20mm diameter as inhibition zone.

Nature of antimicrobial principle of stable cell free supernatants:

Like untreated CFS (no heat inactivation or Proteinase-K treatment), clean zones of inhibitions were noted for all the pathogens tested. No halos were observed in control (uninoculated

broth and 1 mg/ml of Proteinase-K). Halos produced by both heat inactivated and Proteinase-K treated CFS indicated heat and enzyme stability nature of the antimicrobial principle(s) (Table 5).

Outcome of *C. beasleyi* CFS on fungal colony forming units and morphology:

Of the identified organisms, CFS of *C. beasleyi* demonstrated antifungal activity. When treated with CFS of *C. beasleyi*, the number of colonies of *C. albicans* and *C. tropicalis* was reduced in a time dependent manner. Incubation for 96 h with CFS showed no growing cell of *Candida* species. Growth was noted in 24 h and 48 h, then further decline in growth was observed in terms of number of colonies (Figs 9, 10) and the morphological changes of *Candida* species after 96 h treatment are shown in Fig. 11. No such changes were noted in controls.

The lactophenol cotton blue stained mycelia of *Candida* species were observed at 40X magnification after 96 h to find out the changes in mycelial and spore morphology (Fig. 11). Controls of both the species were not affected (Fig. 11 1a, 2a), whereas treated mycelia of both the species were noted as shrunken and mycelial debris (Fig. 11 1b, 2b). Also, reduction in spore formation was noted in both the *Candia* species after treatment with *C. beasleyi* extract.

Table 3 Qualitative analysis of phytochemicals from cell free supernatants (CFSs) and dry fungal mat

Phytochemicals	Alkaloids		Carbohydrates		Glycosides		Saponins		Tannins		Phenols		Flavonoids		Fats and oils		Gum and mucilages	
	CFS	mat	CFS	Mat	CFS	mat	CFS	Mat	CFS	mat	CFS	mat	CFS	mat	CFS	Mat	CFS	mat
Isolates																		
SRF-1	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SRF-2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SRF-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
SRF-4	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SRF-5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SRF-6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SRF-7	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SUF-1	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
SUF-2	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
SUF-3	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
SUF-4	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
SUF-5	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-

Abbreviations: SRF = *Solanum* ripe fruit, SUF = *Solanum* unripe fruit

Table 4 List of extracellular enzymes and enzyme index from isolated endophytic fungi

Sl. No	Endophytic fungi	Amylase	Lipase	Laccase	Protease	Asparaginase
		Enzyme activity in mm				
1.	*Aspergillus hiratsukae* (MN784457)	0	0	0	0	20
2.	*Curvularia beasleyi* (MN784459)	0	0	0	0	09
3.	*Curvularia hawaiiensis* (MN784458)	0	0	0	0	10
4.	*Cladosporium sphaerospermum* (MN784448)	0	12	0	0	11
5.	*Cladosporium tenuissimum* (MN784453)	11	0	0	0	21
6.	*Cladosporium tenuissimum* **(**MN784454)	07	15	0	0	12
7.	*Cladosporium* sp. (MN784455)	13	16	0	0	20
8.	*Cladosporium cladosporiodes* (MN784456)	10	20	0	0	23
9.	*Alternaria* sp. (MN784449)	0	13	0	0	0
10.	*Mycosphaerellaceae* sp. (MN784450)	0	0	0	0	11
11.	*Alternaria alternata* (MN784451)	0	20	0	0	0
12.	*Alternaria gaisen* (MN784452)	0	15	0	0	05

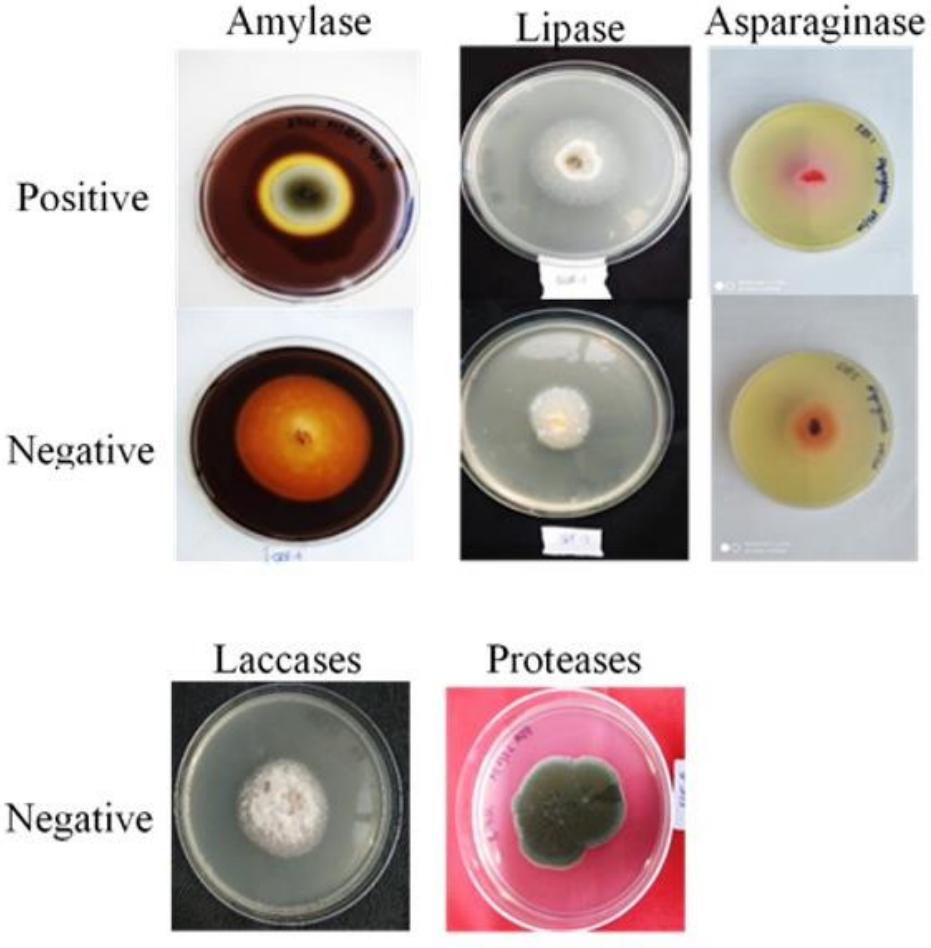


Fig. 5 – Screening of endophytic fungi for extracellular enzymes on solid medium.

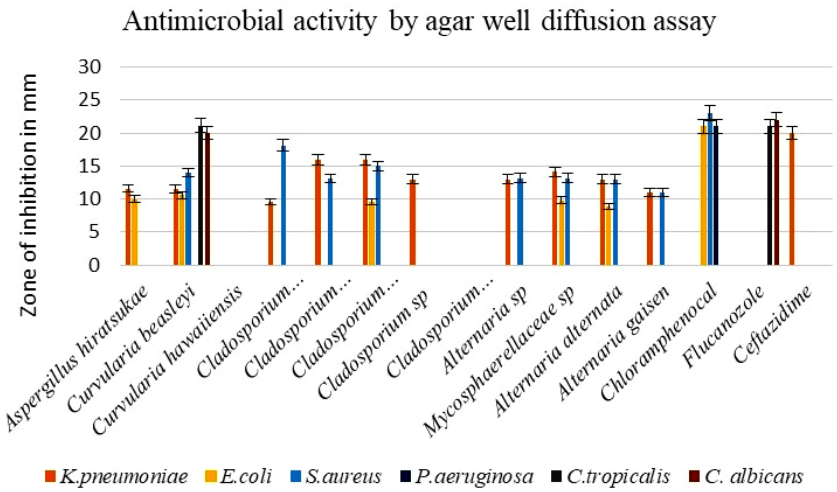


Fig. 6 – Antimicrobial activity of cell free supernatants of endophytic fungi isolated from *S. violaceum* on pathogenic bacteria and fungi.

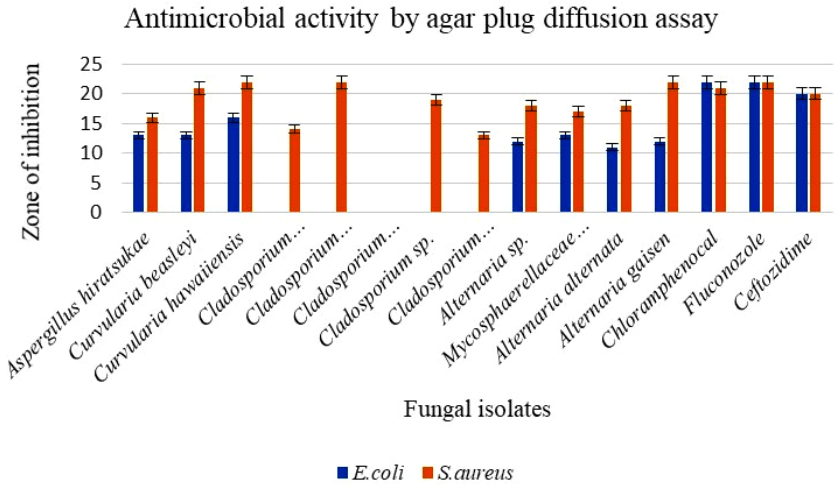


Fig. 7 – Antimicrobial activity of agar plug of endophytic fungi isolated from *S. violaceum* on pathogenic bacteria and fungi.

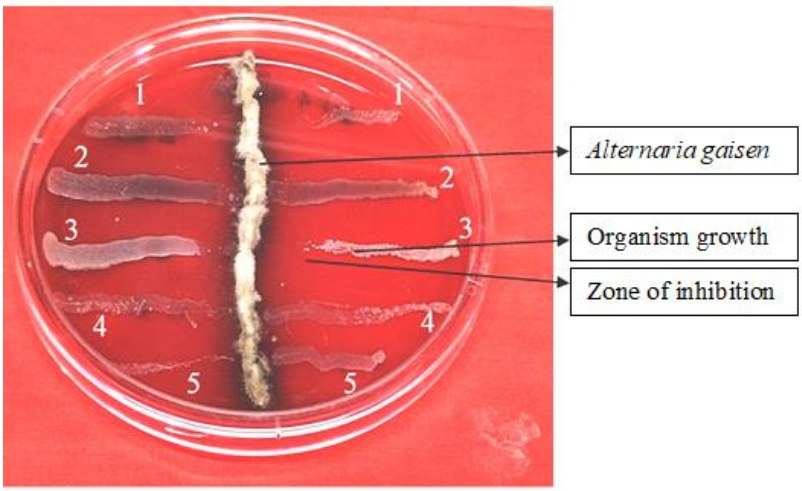


Fig. 8 – Cross streak method of testing antimicrobial activity of *Alternaria gaisen* on pathogenic organisms tested on blood agar medium. 1 *E. coli.* 2 *K. pneumonia*. 3 *S. aureus.* 4 *C. albicans*. 5 *C. tropicalis.*

Table 5 Temperature and Proteinase-K induced antimicrobial effect of the cell free supernatants (Zone of inhibition in mm)

Sl. No.	Isolates	*E. coli*		*S. aureus*		*K. pneumonia*		*C. albicans*		*C. tropicalis*	
		T	P-K	T	P-K	T	P-K	T	P-K	T	P-K
1.	SRF-1	10.0±0.2	-	10±0.2	8±0.2	12±0.2	-	-	-	-	-
2.	SRF-2	11±0.4	-	12±0.2	-	6±0.2	-	-	-	-	10±0.2
3.	SRF-3	7±0.1	-	12±0.2	10±0.3	10±0.1	-	-	-	-	-
4.	SRF-4	9±0.2	12±0.2	15±0.2	16±0.2	11±0.1	10±0.2	-	-	-	-
5.	SRF-5	15±0.1	14±0.2	13±0.2	13±0.1	9±0.2	12±0.3	-	-	-	-
6.	SRF-6	9±0.2	-		9±0.2	14±0.2		-	-	-	-
7.	SRF-7	14±0.2	13±0.2	10±0.2	13±0.1	12±0.2	13±0.2	-	-	-	-
8.	SUF-1	11±0.1	11±0.3	14±0.2	14±0.2	13±0.2	13±0.1	-	-	-	-
9.	SUF-4	8±0.1	--	13±0.1	14±0.2	14±0.1	13±0.1	-	-	-	-
10.	SUF-5	7±0.2	-	11±0.1	10±0.1	10±0.2	10±0.2	-	-	-	-
Sa	Chloramphenicol	21±0.2	21±0.2	22±0.1	22±0.1	-	-	-	-	-	-
	Ceftazidime	-	-	-	-	20±0.1	20±0.2	-	-	-	-
	Fluconozole	-	-	-	-	-	-	21±0.2	21±0.2	21±0.2	21±0.2

Abbreviations: T = Temperature (98-100°C), P-K = Proteinase-K (1 mg/mL), Sa = Standard antibiotics, SRF = *Solanum* ripe fruit, SUF = *Solanum* unripe fruit

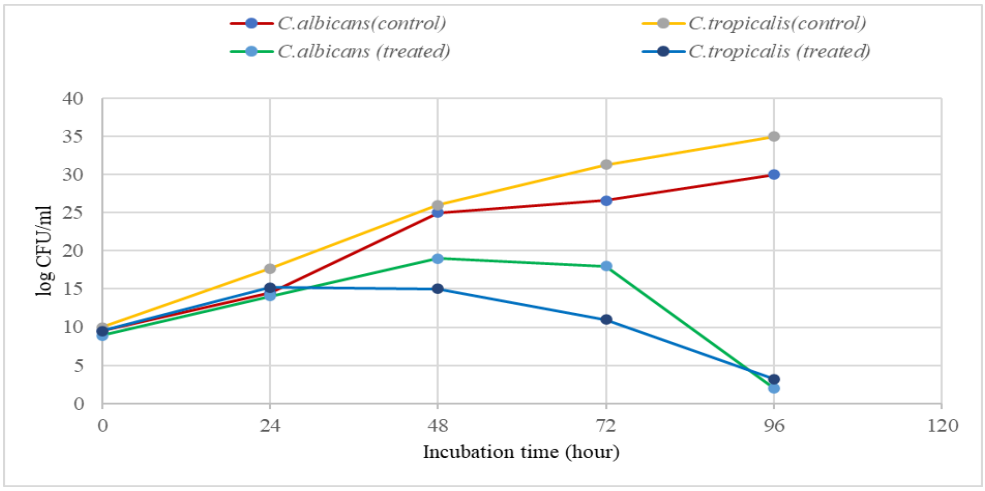
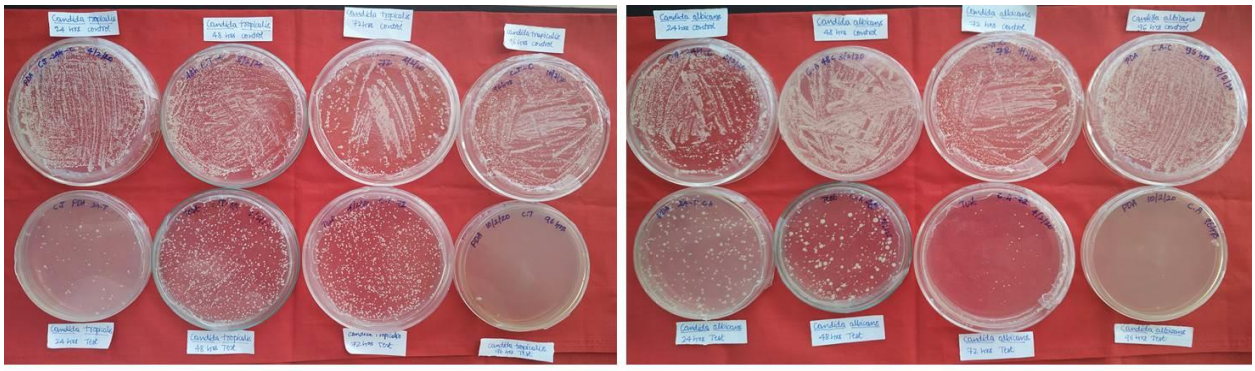


Fig. 9 – Effect of *C. beasleyi* CSF on colony frequency unit of pathogenic fungi at different time intervals.



Candida albicans *Candida tropicalis*

Fig. 10 – Antifungal activity of *C. beasleyi* cell free supernatants on *C. albicans* and *C. tropicalis* by plate count method.

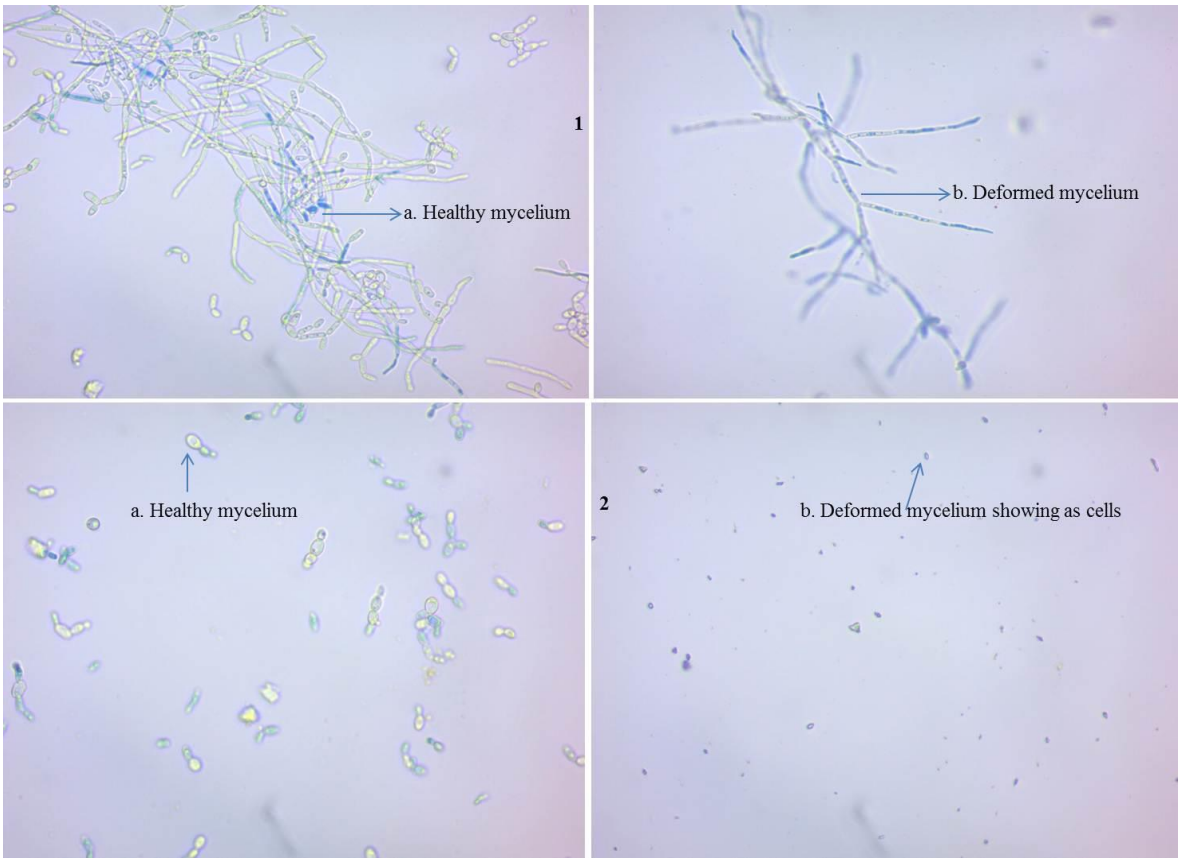


Fig. 11 – Images of fungal mycelium at 40 X after 96 hours of incubation with *C. beasleyi* extract. 1 *C. albicans* (upper panel) 2 *C. tropicalis* (lower panel) (left side: control; right side: treated). Arrows indicate the mycelia nature.

Discussion

In this study, the diversity, characterization and bioactivities of cultured fungi obtained from fresh unripe and ripe fruits of *S. violaceum* were investigated. A total of 12 cultivable fungal colonies were isolated and identified. The number of colony forming rate of endophytic fungi was higher in ripe fruit (seven) followed by unripe (five). The isolates belonged to the Ascomycota phylum. Porter (1991) in his findings demonstrated that the average size of ITS region in fungal kingdom belongs to Ascomycota, which is in agreement with our results.

This result supports the finding of Rakotoniriana et al. (2008) wherein fungal endophytes mainly belong to the Ascomycetes. *Cladosporium* sp., had the highest colonization frequency in ripe fruit, whereas *Alternaria* sp., had the highest colonization frequency in unripe fruit. *Cladosporium, Curvularia* were dominant in our work and it might be due to their cosmopolitan nature, which increases their chance to get accepted as endophytes (Raviraja 2005). The ways to measure fungal diversity are richness (number of species), relative abundance (proportion of a species in a community) and evenness (well representation of a species). Diversity at different levels were analysed by Simpson indices which indicated differences in endophytic fungal isolates and species richness. These results are in line with the work done on endophytic fungi isolated from *Piper nigrum* by Uzma et al. (2016), wherein high Simpson's diversity indices were noted in ripe fruit pulp.

In the present study, phytochemical analysis of CFSs from all fungal isolates revealed the presence of alkaloids, carbohydrates, saponins, and glucosides and the absence of flavonoids, tannins and phenols. On contrary, dry mat powder showed only alkaloids (Selim et al. 2012). Ramesha & Srinivas (2014) isolated endophytic fungi from *Plumeria acuminata* L. and *Plumeria obtusifolia* L. and analysed for phytochemicals which revealed the presence of alkaloids, flavonoids, steroid and phenolic compounds.

Choi et al. (2005) described the endophytes as potential enzyme synthesizers. The hypothesis is that the extracellular enzymes vary from fungi to fungi; also influenced by the hosts and their growing conditions. Out of 12 endophytic fungi isolated, four of them were able to degrade starch by amylase, an enzyme that has huge industrial applications. Based on enzyme production capacities, a set of fungi showed a very clear zone, whereas, another set showed no or less significant zone. Our results specify that some of these might produce large amounts (high activity) of amylase enzyme on large scale fermentation, if performed. Literature review emphasized that 72% of the isolated endophytes were able to produce amylase from *Adhatoda vasica, Costus igneus, Coleus aromaticus* and *Lawsonia inerims* (Amirita et al. 2012). This result is in conformity with those of Sunitha et al. (2013) wherein the endophytic isolates of *Bixa orellana* and *Catharanthus roseus* were not active producers of the amylase enzyme. In our study seven isolates showed lipase activity, out of them, *A. gaisen* was able to produce visibly more lipase enzyme when compared to others. Panuthai et al. (2012) reported ten isolates were potential lipase producers among 65 fungal isolates from uninfected leaves of *Croton oblongifolium* in Thailand, among them *F. oxysporum* was the potential isolate for lipase production. Studies of Chow & Ting (2015) revealed that endophytes are good alternative sources for L-asparaginase. Out of 12, ten isolated were able to produce asparaginase. Isolates of *C. cladosporioides* and *A. hiratsukae* exhibited maximum asparaginase enzyme activity in present study. In one of the studies, out of 17, only eight isolates were positive for L-asparaginase enzyme (Kalyanasundaram et al. 2015). *Aspergillus* sp., showed maximum enzyme activity as that of our study. An interesting finding was that, none were able to produce laccase and protease enzymes. Uzma et al. (2016) also stated absence of laccase enzyme producers from the endophytic isolates.

We conclude that enzyme production by endophytic microbes depends on the type of host and its habitats. Endophytes are group of microorganisms that represent an abundantly free or reliant source of bioactive and chemically novel materials with potential for pharmaceutical and industrial importance (Tiwari 2015). There is a need to explore more for such kind of microbes from various plant parts. Antibiotic resistance is greatly increased and extended beyond the clinical application of drugs (Malik & Bhattacharyya 2019). There is always a ray of hope to bring new drugs from microbes which fight against the other microbes. In the present study *Alternaria* sp., *C. tenuissimum*, *Mycosphaerellaceae* sp., and *A. alternata* endophytic fungal isolates demonstrated bacterial controlling capacity (against *S. aureus*, *E. coli* and *K. pneumoniae). P. aeruginosa* is known for its antibiotic drug resistant potential and we also noted that no endophytic fungi controlled *P. aeruginosa*. *P. aeruginosa (*ATCC 27853) contains virulence genes, *lecA*, *lecB* and quorum sensing regulators, LasI/R. This might be the possible reason for resistance to the all the endophytic fungi. (Cao et al. 2017). Yadav et al. (2014) stated that out of 22 endophytic fungi from the *E. jambolana*, 68% fungal isolates controlled the tested microbes. According to Pai & Chandra (2018) *Cladosporium cladosporioides* was the most effective endophytic fungus against *P. aeruginosa*. On the contrary, in our study, *Cladosporium cladosporioides* did not show any activity on test organisms. Rani et al. (2017) studied the endophytic fungal extracts of *Aspergillus nidulans*, *Curvularia hawaiiensis*, *Chaetomium arcuatum* and *Chaetomium atrobrunneum* and demonstrated significant microbial controlling capacity. The broad spectrum antibacterial and antimycotic property of *C. beasley* is significantly linked to tested microorganisms and it is nearly equivalent to the standard antibiotics tested. The results are on par with the results Pavithra et al. (2012). As we noted, all the isolates inhibited *S. aureus* and a few isolates inhibited *E. coli*.

A good number of methods are in practice to estimate or monitor the antimicrobial activity of extracts. The most common ones are well and disc diffusion methods for bacteria and poisoned food methods for fungi. Every method has its own merits and demerits. Bacterial growth inhibition will not describe whether the compound has bactericidal or bacteriostatic property. In widely used well method, the chosen concentration of the test sample will be introduced in a known size hole and as the organisms are thoroughly mixed with the agar (pour plate), organisms are in close proximity of the test sample. The suitable method to get to know the antagonistic activity is the agar plug diffusion method, though it is similar to disk diffusion method. In this method, the

secreted molecules of the fungus will diffuse and inhibition zone around the test organism will be visualized (Mounyr et al. 2016). For cross steak method, in the present study, commercially available blood agar has been used and is considered as a standard media for the analysis of food samples (Coman et al. 1997).

C. cladosporioides did not show any activity in both agar well and agar plug diffusion method on all test organisms. Varying growth inhibitory percentages were observed suggesting the chemo-diversity of endophytic fungi under three different assay conditions. These results obliged the concept of OSMAC (one strain many compounds), wherein, production of different microbial molecules depends on the culture conditions (Bode et al. 2002). Observations showed that isolates are less powerful on Gram -ve bacteria as compared to Gram +ve bacteria. This might be due to the cell wall components of bacteria (Scherrer & Gerhardt 1971).

Conclusion

Our results in the study specified that these cultivable endophytic fungi from the fruits of *S. violaceum* have potential pharmaceutical properties as they produced antimicrobial compounds. The medicinal properties might be a consequence of the capacity of inherently growing microbes that synthesize bio active compounds. Further in-depth research is required to find out the active ingredient with antimicrobial activity.

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