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ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF ENDOPHYTIC FUNGI ASSOCIATED WITH *CASSIA FISTULA*

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Abstract: Various medicinal plant species are moving from fringe to mainstream and being used by a large numbers of people who are seeking remedies and health approaches from natural products. *Cassia fistula* is well known for its wealth of antimicrobial metabolites and medicinal property. Therefore, the endophytic fungi that inhabiting such environment are expected to mimic the host for the antimicrobial and medicinal potential. In present study, five fungal endophytes were isolated from different parts of *C. fistula* and screened *in-vitro* for antimicrobial activity and presence of important phytochemicals. The results revealed that the isolated fungal endophytes have possible presence of alkaloids, phenolics and tannins, flavonoids, carbohydrates and glycosides, terpenoids, amino acids and proteins.

Keywords: *Cassia fistula,* fungal endophyte, antimicrobial activity, phytochemicals

Introduction

Cassia fistula, commonly known as golden shower**,** Indian laburnum or amaltas, is widely distributed in different parts of world including Asia, South Africa, China, West Indies and Brazil (Kumar *et al.,* 2006). Each part of this tree has its own traditional and medicinal importance. Tribal people are using this plant to treat ringworm and other fungal skin infections (Rajan *et al.*, 2001). The bark and leaves of *C. fistula* are useful for the treatment of skin diseases, flowers for fever, root as a diuretic, febrifuge, for gout and rheumatism. It is one of the ingredient in Constivac (Lupin Herbal) a bowel regulator which relieves constipation and Pilex, Purian (Himalaya Drug Company) for curing piles and as detoxifier respectively (Agarwal and Paridhavi, 2005).

Earlier work on phytochemicals of *C. fistula* revealed that it comprises of some antimicrobial compounds like anthroquinone, alkaloids, catachols, flavonoids, phenolic compounds, saponins, steroids, tannins and triterpenoids (Shah *et al.*, 1981; Rabe and Van Stadin, 1997; Santhi *et al.*, 2006). However, work on its fungal endophytes and their potential as antimicrobial agent is lacking. Endophytes are known to be associated with all plant species but their relationships with host, the importance and existence is not well defined (Faeth, 2002, 2009). Endophytic fungi are able to produce antimicrobial, anticancer compounds like Taxol (Walker and Croteau, 2001) and antimalarial activities (Wiyakrutta *et al.*, 2004). Study of Woropong *et al.* (2001) revealed that the endophytic fungi are able to produce a range of volatile organic compounds that are lethal to pathogenic fungi and bacteria. Endophytes may also produce chemicals, which inhibit the growth of competitors, including pathogenic organisms. In addition to production of

bioactive compounds, the endophytes are also known to produces antibiotic substances which enable them to act as biocontrol agents (BCA) for management of various plant diseases. Fungal based BCAs have gained wide acceptance next to bacteria, primarily because of their broad spectrum action on disease control and yield. *Trichoderma* spp. are the centre of attraction for most of researchers working on the biological control of plant diseases (Verma *et al.*, 2007). Although, *Trichoderma* spp. are managing successfully the variety phytopathogens, but the present investigation aims on the isolation of fungal endophytes associated with *C. fistula* and their screening for different phytochemicals and antimicrobial activity.

Materials and Methods:

Isolation of fungal endophytes:

Different parts of *C. fistula* (bark, wood, leaves and twigs) were collected and surface sterilized before inoculation. Four media [potato dextrose agar (PDA), czapek dox agar (CDA), yeast mannitol agar (YMA), and water agar (WA)] were tried. Pure cultures were raised by subsequent subculturing on PDA.

Screening of fungal endophytes against *Alternaria alternata* in dual culture*:*

Alternaria alternata, the common pathogen of *C. fistula* was procured from Forest Pathology Division, Forest Research Institute, Dehradun. Fungal endophytes were screened against *C. fistula* by dual cultures technique on PDA (Dennis and Webster, 1971). Percentage of growth inhibition was determined by the formula:

Percent inhibition = $(A_1-A_2)/A_1x100$

Where A_1 is area covered by pathogen in control and A_2 is area covered by pathogen in dual culture. Whole of experiment was carried out in triplicates.

Effect of non-volatile compounds released by fungal endophytes on the growth of *A. alternata*:

Poisoned food technique (Nene and Thapliyal, 1993) was followed to study the effect of non-volatile compounds release by the fungal endophyte on the growth of pathogen. The endophytes were grown on potato dextrose broth and incubated at 25±1°C. After 15 days of incubation, the media containing fungal endophyte were filtered through Whatman-I filter paper and finally passed through syringe filter (Ran Disc, PVD 0.45 µm) under aseptic conditions. The PDA was amended with culture filtrate (20%) just before pouring. Five mm discs of the pathogen were inoculated in the Petri plates amended with culture filtrate and incubated at 25±1°C. The colony diameter of the pathogen was measured and compared with control. PDA without culture filtrate served as control.

Extract preparation for phytochemical screening:

Fungal endophytes were cultivated in potato dextrose broth for 15 days at 25±1°C in shaking conditions. Mycelial biomass was harvested by filtering through Whatman No. 1 filter paper. The filter papers containing mycelial mat were oven dried to get constant weight. The mycelial mat was crushed and mixed with distilled water and ethanol. Finally filtered and the filtrate was utilized for phytochemical screening.

Phytochemical screening:

Preliminary phytochemical screening of the fungal endophytes for the presence or absence of important constitutes was carried out by adapting following methodologies:

1. **Test for Alkaloids: Dragendroff's reagent test–** Few ml of filtrate was taken in a test tube and 1-2 ml of Dragendroff"s reagent was added. Appearance of yellow colored precipitate confirmed the presence of alkaloids.
2. **Test for Phenolics and Tannins: Ferric Chloride test-** Extract was dissolved in the distilled water and few drops of 5% ferric chloride solution were added. A dark green color indicated the presence of Phenolics and Tannins.
3. **Test for Flavonoids:** 5 ml of dilute ammonia solution was added to the portion of extract, followed by the addition of few drop of concentrated sulphuric acid. Appearance of yellow color confirmed the presence of flavonoids.
4. **Test for carbohydrates and glycosides: Molisch test-** 2 ml of filtrate was taken in the test tube followed by addition of 1-2 drops of alcoholic of α-napthol, mixture shaken well and concentrated sulphuric acid was added along sides of the test tube. Formation of a violet color ring confirmed the presence of carbohydrates.
5. **Fehling's solution test-** 1 ml of filtrate was boiled in water followed by addition of 1 ml of Fehling solution. Red coloured precipitate indicated the presence of reducing sugars.
6. **Test for Terpenoids**: **Salkowski test-** 5 ml of extract was mixed with 2 ml of chloroform and 3 ml of sulphuric acid was added from the sides of the test tube. Formation of reddish brown coloration at interface indicated the presence of terpenoids.
7. **Test for steroids: Libermann burchard's test-** Extract was dissolved in 2 ml of acetic anhydride and 1-2 drops of sulphuric acid was added along sides of the test tube. Blue green ring appears or the array of color changes indicate the presence of steroids.
8. **Test for amino acids:** 1-2 drops of phenolphthalein was added to the extract and of dilute sodium hydroxide solution was added drop by drop. Appearance of pink color confirmed the presence of amino acids.
9. **Test for protein: Biuret test-** 2 ml of filtrate was treated with one drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) and excess of potassium hydroxide pellets were added. A pink color appears in the ethanolic layer confirmed the presence of proteins.

Results and Discussions

Fungal endophytes:

Five fungal endophytes associated with *C. fistula* isolated and identified as *Thelioviopsis* strain-1&2, *Periconia* sp., *Papulospora* sp. and *Mycelia sterilia* (table-1). All fungal endophytes were recovered on four selected media except *Thelioviopsis* (strain 2) and *Mycelia sterilia*, which were not encountered on YMA and WA respectively. *Thelioviopsis* strain 1&2 were isolated from leaves, *Periconia* sp., *Papulospora* sp. were isolated from bark and *mycelia sterilia* was isolated from the fruit.

Table 1-Fungal Endophytes isolated on different media from *Cassia fistula*

S. No.	Fungal Endophytes	Media			
		PDA	CDA	YMA	WA
1.	*Thelioviopsis* (strain1)	+	+	+	+
2.	*Thelioviopsis* (strain 2)	+	+	-	+
3.	*Periconia* sp.	+	+	+	+
4.	*Papulospora* sp.	+	+	+	+

5.	*Mycelia sterilia*	+	+	+

In vitro antimicrobial activity of fungal endophytes against *A. alternata*

A. alternata is one of destructive plant pathogen that affecting a wide range of host plants, causing leaf spots, blights, blossom rots, and fruit rots (Mmbaga *et al.*, 2011). The results of dual culture trials revealed that the fungal endophytes have restricted the growth of *A. alternata* (table-2) and a zone of inhibition was observed at the point of contact. The results revealed that on fifth day of incubation *Periconia* sp. recorded maximum growth inhibition (65.6%) followed by *Papulospora* sp. (45.6%), *Thelioviopsis* sp. (strain 1) (29.6%), *Thelioviopsis* sp. (strain 2) (22.0%) and minimum by mycelia sterilia (27.2%).

In comparison, low growth inhibition (2.85-16.88%) of *A. alternata* was recorded by the non-volatile metabolites of the fungal endophytes (table-2). Maximum growth inhibition (16.88%) of *A. alternata* was recorded by *Thelioviopsis* (strain 1) followed by *Thelioviopsis* (strain 2) (8.77%), *Periconia* sp. (5.04%), *Papulospora* sp. (3.51%) and minimum by mycelia sterilia (2.85%).

Table 2 – *In vitro* antimicrobial activity of fungal endophytes against *A. alternata*

S. No.	Fungal endophytes	Growth inhibition (%)	
		Dual culture	Volatile compounds
1.	*Thelioviopsis* (strain 1)	29.6	8.77
2.	*Thelioviopsis* (strain 2)	22.0	16.88
3.	*Periconia* sp.	65.6	5.04
4.	*Papulospora* sp.	45.6	3.51
5.	*Mycelia sterilia*	27.2	2.85

Phytochemical analysis of fungal endophyte

Preliminary phytochemical analysis of fungal endophytes revealed that phenolic and tannins, carbohydrates and glycosides, steroids, amino acids and proteins were present in all endophytes (table-3). While flavonoids were present in all endophytes except *Thelioviopsis* (strain 2) and alkaloids and teprpenoids were confirmed only for *Thelioviopsis* (strain 1).

Table 3 Preliminary phytochemical screening of fungal endophytes

S. No.	Phytochemical	Phytochemical secretion by fungal endophytes				
		Thelioviopsis (Strain 1)	*Thelioviopsis* (Strain 2)	*Periconia* sp.	*Papulospora* sp.	*Mycelia sterilia*
1.	Alkaloids	+	-	-	-	-
2.	Phenolics and tannins	+	+	+	+	+
3.	Flavonoids	+	-	+	+	+
4.	Carbohydrates and glycosides	+	+	+	+	-
5.	Terpenoids	+	-	-	-	-
6.	Steroids	+	+	+	+	+
7.	Amino acids	+	+	+	+	+
8.	Proteins	+	+	+	+	+

Discussion

Cassia fistula has the great medicinal uses for the treatment of

variety of human and veterinary diseases. The plant is widely used by tribal peoples to treat various ailments including ringworm, jock itch, irritation, swelling and other fungal skin infection. Phongpaichit *et al.* (2004) recorded antifungal activity of crude methanol extracts of *C. alata, C. fistula* and *C. tora* leaves on *Microsporum gypseum, Trichophyton rubrum* and *Penicillium marneffei.* The hyphae and macroconidia of pathogenic fungi treated with leaf extracts were shrunken and collapsed, which might be due to cell fluid leakage. Nartey *et al.* (2012) observed significant ferric reducing antioxidant power and DPPH scavenging activity of the roots bark extract of *C. sieberiana*. Mali *et al.* (2013) investigated the effect of methanolic extracts of *C. auriculata* leaves in various experimental models of pain and inflammation in rats and observed that the ethyl acetate fraction were more effective. Phytochemical analysis revealed the presence of steroids, flavonoids and tannins. Ahmed and Baig (2014) recorded antimicrobial activity by ethanolic and aqueous extracts of leaves and bark of *C. fistula* against number of bacteria and fungi. Most of studies highlight the medicinal importance of the *Cassia* species. Although most of earlier studies deals with the phytochemical screening and antimicrobial potential of *Cassia* species, but almost no information is available on fungal endophytes of *C. fistula* and their utility for the production of useful phytochemicals and antimicrobial activity.

Fungal endophytes are endosymbiotic fungi, which live within a plant for least part of his life without causing apparent harm. Endophytes are also having an ability to produce novel secondary metabolites for medical, agricultural and industrial use. They may secrete the similar metabolite of their host plant. By culturing such endophyte on artificial media, important compounds of interest can be produced on commercial scale without putting pressure on harvesting of host plant. Nigg *et al.* (2014) isolated *Nodulisporium* sp. (Ti-13) as an endophyte from *Cassia fistula* and observed that it produces a variety of volatile organic compounds like ethanol, acetaldehyde, and 1,8-cineole as major components.

The fungal endophytes *Thelioviopsis, Periconia*, *Papulospora* spp. and mycelia sterilia isolated from different parts of *C. fistula* have exhibited considerable antimicrobial potential against *A. alternata* in dual culture. The endophytes were grown in liquid media with a hypothesis that actively growing fungi will secrete its secondary metabolite (non-volatile compounds) into media, which may be toxic the pathogen. In present study, the growth inhibition by non-volatile is fewer in comparison to dual culture trial, this may due to lesser period of incubation of fungal endophytes in liquid media and require further investigation. All of the endophytic fungi contains the important phytochemical which can be harvested. Dhankhar *et al.* (2012) have isolated 27 fungal endophytes from different parts of *Salvadora oleoides* out of which the species of *Aspergillus, Penicillium* and *Phoma* have exhibited considerable antioxidant activity and presence of alkaloids, flavanoids, saponins, carbohydrates, tannins, sterols and terpenoids. Sadrati *et al.* (2013) isolated 20 endophytic fungi and 23 endophytic actinomycetes from wheat (*Triticum durum*) and found considerable antimicrobial activity against twelve pathogenic bacteria, yeast, and two phytopathogenic fungi.

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