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Research Article

Study of Endophytic Fungi and their Metabolites from the Medicinal Plant of *Withania somnifera*

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ABSTRACT

The study of fungal endophytes and their metabolites isolated from medicinal plants of *Withania somnifera* reported dominant fungal species *Gliocladium deliquescens, Mycelia sterilia, Phoma glomerata, Phoma humicola* and *Fusarium xylarioides*. The fungi *Fusarium xylarioides* and *Phoma humicola* were reported to inhibit both gram positive and negative bacteria. The fungus *Gliocladium deliquescens* showed an inhibition for gram positive bacteria *S. aureus*. They show an efficacy of antibacterial property on *E. coli, K. pneumoniae* and *S. aureus*. These fungal isolates were found to produce metabolites rich in aldehydes, esters and ketones.

Keywords: Fungal endophytes, *Withania somnifera*, Antibacterial property, *E.coli*

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INTRODUCTION

Soil is a media with macro and micro nutrients influencing the growth of microorganisms which is supported by the root exudates of plants. It is a complex biotransformation reaction influenced by humifications and cycling of elements. The study of endophytes in the rhizosphere and rhizoplane of plant roots plays an important role in understanding the symbiotic associations. The endophytic fungi can influence the plant at different stages of growth and production of metabolites. A study of endophytes in medicinal plants has played an important role in exploring the metabolites produced by the endophytes (Khan R, *et al.*, 2010). The study of fungal endophytes and their metabolites isolated from medicinal plants of *Withania somnifera* reported dominant fungal species *Gliocladium deliquescens, Mycelia sterilia, Phoma glomerata, Phoma humicola* and *Fusarium xylarioides*. The fungi *Fusarium xylarioides* and *Phoma humicola* were reported to inhibit both gram negative bacteria and gram positive bacteria. These fungal isolates are found to produce metabolites rich in aldehydes, esters and ketones. They show an efficacy of antibacterial property on *E. coli*, and *S. aureus*.

MATERIALS AND METHODS

Sampling site

The endophytic fungal samples were collected from the roots of medicinal plants of *Withania somnifera* from Dhanvantri Vana is located at Jnana Bharathi, Department of forestry, Government of Karnataka, Bengaluru, Karnataka, India.

Medicinal plant

Withania somnifera: It is commonly known as Ashwagandha-an evergreen shrub-flowering in November to February. It is also known as Indian ginseng or winter cherry. It belongs to Solanaceae family (night shade family), which is found in North Africa, West and South Asia, Southern Europe and Mediterranean region. Leaves and roots are used for infections like carbuncles, ulcers, cough and nerve disorders.

Isolation and screening of endophytic fungi

The collected root samples from *Withania somnifera* was washed and cut into small bits and subjected to surface sterilization. It was disinfected with 75% alcohol for 1 minute followed by immersion in 5% of sodium hypochlorite for 8 minutes. The sterilized root bits were later immersed in 75% alcohol for 30 seconds and then rinsed with sterilized distilled water to remove the traces of sterilant left on the root bits. Finally the root bits were blot dried on a sterile blotting paper a modified method (Huang WY, *et al.*, 2008).

The processed root bits were placed on sterilized Potato Dextrose Agar (PDA) medium containing streptomycin and incubated at 28°C for 21 days and observed for the growth of fungus. The isolated fungal colonies were observed for the colony characteristics and sub cultured in PDA slants and identified for their sporulation characters and the morphological characters by mounting with lactophenol cotton blue.

RESULTS AND DISCUSSION

Study of endophytic fungi for anti-bacterial activity

Agar-plate technique: A plate of nutrient agar medium was inoculated with the test organism by spread plate technique for uniform growth of bacterial lawn. Circular sterile paper discs soaked in fungal extract is placed on the agar medium. The fungal broth extracted with ethyl acetate and dissolved in Dimethyl Sulfoxide (DMSO) was used for the study of antibacterial property. The plates were then incubated at 37°C for 36-48 hours and observed for the zone of inhibition. The control used was an antibiotic ciprofloxacin was used to compare the zone of inhibition of test results. The diameter of the inhibition zone around the discs is measured in millimeter (mm). Agar disc diffusion method was followed by the method given by (Li H, *et al.*, 2005; Azevedo JL, *et al.*, 2000).

Organic analysis to detect organic compounds present in the fungal broth

It is categorized into 4 groups. Group 1 includes water soluble compounds namely acetic acid, and acetone.

- **Acetic acid:** It is available in physical state which has odour and is colourless.

Experiment, observation and inference: A little of the fungal

crude broth is ignited in a spatula. It is observed that it burns with a non-smoky flame. So, it is an aliphatic compound. Further, to 3 drops of the crude broth and little sodium bicarbonate solution is added and shaken. Effervescence takes place and carbon dioxide gas is liberated, thus the compound is an acid.

- **Acetone:** It is available in physical state, having alcohol odour and is colourless liquid.

Experiment, observation and inference: 4 drops of fungal crude broth+sodium bicarbonate solution gave no effervescence, confirming that the compound is not an acid.

Group 2 includes aniline which is available in physical state. It is brown liquid with fishy smell.

Experiment, observation and inference: 2 drops of the crude broth is shaken well with 2 ml of dilute Hydrocholric Acid (HCL) and allowed to stand. As per the observation, the compound is dissolved. Thus, it belongs to III solubility group and is an amine.

Further, diazotization test was carried out. 3 drops of the broth was dissolved in 1 ml of dilute HCL and the solution is cooled in ice. To the ice cold solution, 0.2 gm of sodium nitrite was added and solution of beta naphthol was added. Red dye is produced and thus can be confirmed that it is a primary aromatic amine.

Group 3 includes benzoic acid, salicylic acid and phenol.

- **Benzoic acid:** It is a colourless, crystalline solid which is available in physical state.

Experiment, observation and inference: 0.1 ml of the crude broth is shaken with 2 ml of cold dilute HCL and the compound does not dissolve. Thus, the compound does not belong to III solubility group. Further, 0.1 ml of the broth is shaken well with 2 ml of 5% NaOH solution. The compound dissolves to give a clear solution. This confirms that the compound belongs to IV solubility group. So, it may be phenol or acid. The next test used 0.1 ml of the broth which is shaken with 2 ml of sodium bicarbonate solution. Effervescence takes place and carbon dioxide gas is liberated, denoting that the compound is an acid. Similarly, in a test 0.2 ml of the broth is treated with neutral ferric chloride solution. No violet colour is produced, indicating that it is not salicylic acid, hence it is benzoic acid.

- **Salicylic acid:** It is a colourless crystalline acid, available in physical state.

Experiment, observation and inference: 0.2 ml of the broth is shaken with neutral ferric chloride solution. Violet colour is produced, indicating that the compound is a phenolic acid (salicylic acid).

- **Phenol:** It is a colourless crystalline solid with phenolic odour and is available in physical state.

Experiment, observation and inference: 0.2 ml of the broth+2 ml of sodium bicarbonate were added. No effervescence and no carbon dioxide gas was liberated, denoting that it is not an acid. Further, 2 drops of the broth taken in a dry test tube and little sodium nitrite is added (Liebermann's reaction). The mixture was warmed, shaken and cooled. To the mixture, 4 drops of conc. sulphuric acid is added. The mixture was added to plenty of cold water. A red solution was formed, and thus it is phenol.

Group 4 includes benzaldehyde, acetophenone and ethyl benzoate.

- **Benzaldehyde:** It has available in physical state. It is a colourless liquid having the smell of bitter almonds.

Experiment, observation and inference: 3 drops of the crude broth is shaken with 2 ml of conc. sulphuric acid. The compound dissolves, and thus the compound belongs to IV solubility group. It may be an aldehyde, ketone, ester or alcohol. 2 drops of the broth is shaken with 1 ml of Schiff's reagent (Schiff's test). An orange yellow crystalline solid is formed. So, the compound may be an aldehyde or ketone. 2 drops of the broth, 2ml of Tollen's reagent is added and the mixture is shaken well and gently heated (Tollen's test). A bright silver mirror is formed on the side of the test tube and the compound is an aldehyde.

- **Acetophenone:** It is available in physical state, which is colourless liquid with pleasant smell.

Experiment, observation and inference: 2 drops of the broth and 1 ml of Schiff's reagent is added and the mixture is shaken. No pink colour is produced. It is not an aldehyde. So it is ketone. 2 drops of the broth with 2 ml of Tollen's reagent is added and shaken well and gently heated. No silver mirror is formed, thus indicating that it is not an aldehyde. So it is a ketone.

- **Ethyl benzoate:** It is a colourless liquid with a fruity smell, in physical state.

Experiment, observation and inference: 5 drops of broth and 5 ml of NaOH were added and the mixture is boiled for 5 minutes (Saponification). Then it is added and acidified with dilute sulphuric acid. A white precipitate is formed and the compound is an ester.

Isolation of endophytic fungi from the roots of Withania somnifera

The plant *Withania somnifera* was isolated with five fungi *Gliocladium deliquescens, Fusarium xylarioides, Mycelia sterilia, Phoma humicola* and *Phoma glomerata* from rhizosphere while three fungi *Gliocladium deliquescens, Mycelia sterilia* and *Phoma glomerata* were isolated as endophytes from the plant *Withania somnifera* (Bacon CW and White J, 2000).

Two types of rhizosphere fungi *Fusarium xylarioides* and *Phoma humicola* were found to be 40% of the population of total fungi isolated while there were no isolates of specific endophytes. The fungi common both as rhizospheric and endophytic include three isolates *Gliocladium deliquescens, Mycelia sterilia* and *Phoma glomerata* being 60% of the total population of the isolates (Cannon PF and Simmons CM, 2002; Girivasan KP and Suryanarayanan TS, 2004). The results are presented in *Tables 1* and *2* (*Figures 1 and 2*).

Table 1: Rhizospheric and endophytic fungi isolated from *Withania somnifera*

S.no	Fungal isolates	Rhizosphere fungi	Endophytic fungi
1	*Gliocladium deliquescens*	+	+
2	*Fusarium xylarioides*	+	-
3	*Mycelia sterilia*	+	+
4	*Phoma humicola*	+	-
5	*Phoma glomerata*	+	+

Table 2: Effect of endophytic fungi as an antibacterial property against *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*

S.no	Name of the fungi	Zone of inhibition (mm)		
		E. coli	*S. aureus*	*K. pneumoniae*
1	*Phoma glomerata*	0	0	0
2	*Gliocladium deliquescens*	0	7	0
3	*Phoma humicola*	7	0	7
4	*Mycelia sterilia*	0	0	0
5	*Fusarium xylarioides*	5	0	0
6	Standard ciproflaxacin (5µl) 1mg/ml)	20	21	13

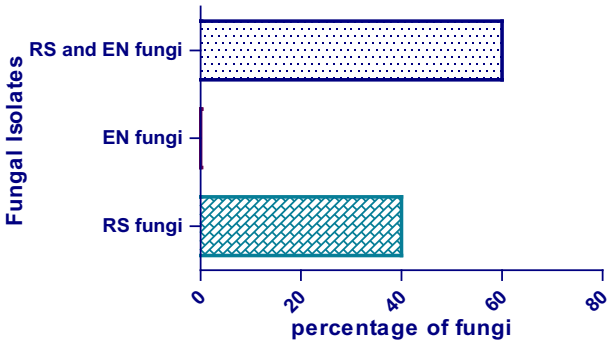
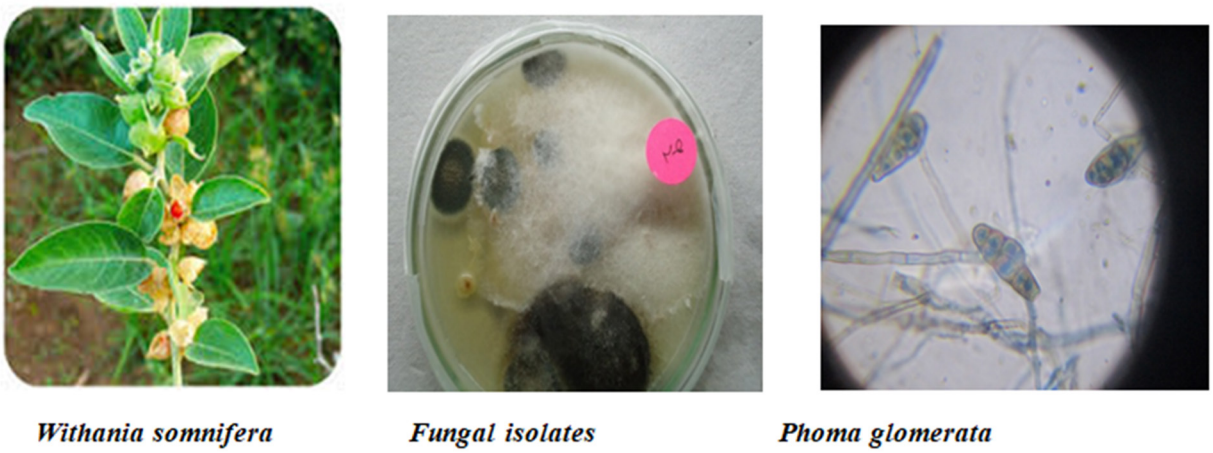


Figure 1: Percentage of rhizospheric, endophytic fungi and total fungal isolates from *Withania somnifera*



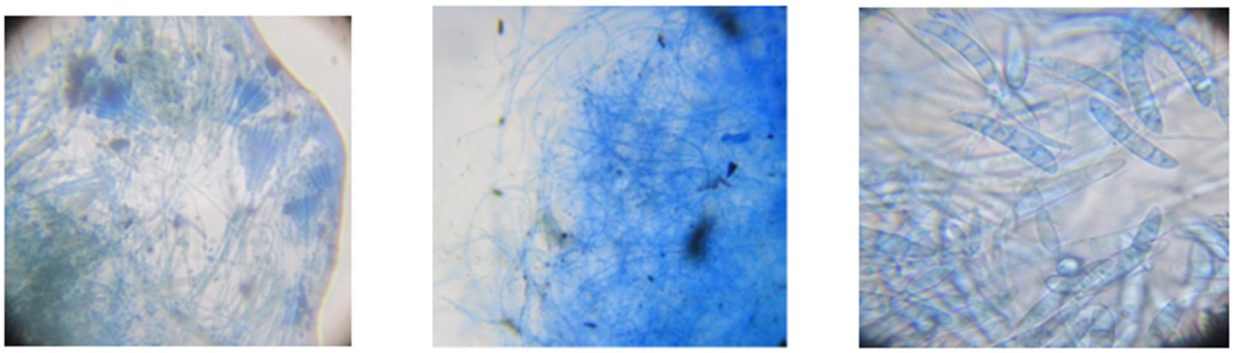


Figure 2: *Withania somnifera* plant, fungal isolates and microscopic observations of the fungal isolates

Study of endophytic fungi for antibacterial property

The plant *Withania somnifera* showed predominant fungus *Gliocladium deliquescens* which showed antibacterial property against gram positive bacteria *S. aureus*. The fungus *Phoma humicola* showed inhibition of *E.coli* and *K.pneumoniae*. The fungus *Fusarium xylarioides* showed inhibition of only *E.coli*. The fungi *Mycelia sterilia* and *Phoma glomerata* did not show inhibition of all the three test bacteria (Karthikeyan B, *et al.*, 2012; Kharwar RN, *et al.*, 2009).

The fungal isolate *Fusarium xylarioides* showed the presence of aliphatic compounds and acidic compounds, whereas the fungus *Phoma humicola* showed the presence of aromatic compounds and aldehydes (Manoharachary C, *et al.*, 2005). The fungus *Gliocladium deliquescens* showed the presence of aliphatic and aldehydes.

CONCLUSION

The medicinal plant, *Withania somnifera* even though used for antibacterial activities, the endophytic fungi can also be used, since it showed a similar anti-bacterial activity. In the present study the fungal isolates included *Gliocladium deliquescens, Mycelia sterilia, Phoma glomerata, Phoma humicola* and *Fusarium xylarioides*. These fungal isolates showed varied levels of efficacy as an antibacterial property on *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus.*

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