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Original Research Article

Antimicrobial Activity of Endophytic Fungi Isolated From *Vitex negundo* Linn.

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A B S T R A C T

Endophytes are the microorganisms present in living tissues of various plants, establishing mutual relationship without apparently any symptoms of diseases (Strobel and Daisy, 2003). Endophytes have received attention of the scientific community due to their capacity to produce novel bioactive compounds. In the present study, bioprospecting of fungal endophytes from *Vitex negundo* L. was studied and screened for their antimicrobial potential. Seventeen endophytic fungi were isolated from different parts of the plant. *Phomopsis sp*. isolated from the leaves showed significant antimicrobial potential. The crude extract of this fungal isolate with Hexane, Ethyl acetate and Methanol were screened for their antimicrobial potential. The extract by ethyl acetate showed significant antimicrobial activity against *E. coli, S. typhimurium, B. cereus, B. subtilis, K. pneumoniae and S. aureus*. The antimicrobial activity was highest against *E. coli, followed by S. typhimurium* and *B. cereus* . The present study helped to justify the traditional use of *Vitex negundo* L. against human pathogenic bacteria. Further, it is confirmed that the antimicrobial activity is attributable to the presence of endophytic fungi. It also justifies that the studies on isolation and identification of these bioactive compounds can be a crucial approach to search of novel natural products.

Introduction

In view of the increased global health concern over the failure of currently used antibiotics to many super resistant strains, indiscriminate exploitation of medicinal plants for extraction of antimicrobial agents of plant origin and limitations of plant resources due to various factors like requirement of land for cultivation, environmental competence of plants, seasonal specificity etc., the search for new and effective antimicrobial agents is becoming a necessity. Therefore,

worldwide, there is an increased interest in searching novel bioactive compounds having high effectiveness, low toxicity and negligible environmental impacts. Microbes have been an abundant source of novel chemo-types and pharmacophores from thousands of years. In recent past, Endophytes have received attention of the scientific community due to their capacity to produce novel bioactive compounds (Aly *et al*, 2010; Strobel, 2003; Schulz *et al*, 2002; Tan and Zou ,2001)Endophytes are the microorganisms which are present in living tissues of various plants, establishing mutual relationship without apparently any symptoms of diseases (Petrini *et al*, 1991 ; Bacon *et al*, 2000) Some of the endophytes are known to produce bioactive metabolites with important applications. Endophytes have proved to be the promising sources of biologically active products which are of interest for specific health care applications. (Strobel *et al*, 2001; Suthep *et al*, 2004; Strobel, 2002.) Endophytic fungal strains are also found to be potentially useful in the production of pigments, bioactive metabolites, immuno-suppressants, anticancer compounds and bio-control agents (Wang *et al*, 2002; Stinson *et al*, 2003; Gangadevi and Muthumary, 2007).

Therefore, various traditionally used medicinal plants are being studied world-wide for their ability to host endophytic fungi having antimicrobial potential. However, being recent development, the endophytes although relatively less studied, are potential sources for novel natural products. The practitioners of traditional systems of medicine have been using *Vitex negundo* L. for curing various ailments and conditions due to its substantial therapeutic potential and widespread occurrence in India. (Perry, 1980; Jayaweera, 1980; Anonymous., 1992).

Every part of *Vitex negundo* L. has medicinal value for various skin diseases. Leaves of this plant are useful in rheumatism.(Nadkarni, 1976) It is widely used in Chinese herbal medicines. It is very useful in the treatment of chronic bronchitis and cold. Despite the therapeutic potential of medicinal plants in general and that of *Vitex negundo* L. in particular as reported, a scientific and rational approach to the traditional medical practice with modern system of medicine is found lacking. Identification of endophytic fungi has potential to establish a scientific basis for the traditional therapeutic uses of *Vitex negundo*. Hence, the work for the search for potential antibacterial compounds from indigenous endophytic fungi from *Vitex negundo* was carried out with the view of human health care and drug discovery.

Materials and Methods

Isolation and Identification of Endophytic Fungus

For fungal isolation, twelve different sterilization protocols were analyzed and the protocol of Petrini *et al*,(1986) was followed with minor modifications. The leaves of *Vitex negundo* were first rinsed under running tap water, immersed in 75% ethanol (1 min) followed by NaOCl (1 to 13% depending upon type of tissue) (3 to 5 min.), and then with 75% ethanol (30 sec). All the segments were then washed three times with sterile distilled water and allowed to surface-dry on sterilized filter paper. The efficiency of surface sterilization procedure was ascertained for every segment of tissue

following the imprint method described by Schulz *et al*, (1993). All the segments were placed in Petri dishes containing potato dextrose agar (PDA) medium supplemented with chloramphenicol (50 µg/mL) to inhibit bacterial growth. The hypal tips were transferred to fresh PDA to obtain pure culture.

Sporulating structures were considered as diagnostic features for the morphological identification of endophytic fungi. Among several endophytic isolates, *Phomopsis* sp. showed significant antimicrobial activity against selected microorganisms and hence was selected for further study. The fungus was induced to sporulate by inoculating in potato carrot agar medium and identified by morphological as well as by ITS rDNA sequence analysis.

Isolation of Secondary Metabolites

Isolation of secondary metabolites from liquid media was carried out by the method described by Choudhary *et al*, (2004). The culture media and the mycelia were separated from each other by filtration. The mycelia were soaked in methanol and the methanolic extract was collected after 7 to 10 days of soaking. Organic solvents, hexane and ethyl acetate were used to extract the filter. The filtrate was extracted three times with equal volume of Hexane, Ethyl acetate. Each solvent was subjected to liquid - liquid extraction for 3 to 4 times. Solid residues were obtained by evaporating organic extracts under reduced pressure.

Test Microorganisms

Antibacterial activity of metabolites isolated from endophytic fungi was screened against pathogenic and non pathogenic bacteria using agar well diffusion method. Six bacteria, gram positive *B. subtilis* (NCIM No. 2063), *S. aureus* (NCIM No. 2079) , *B. cereus* (NCIM No. 2155) and gram negative *E. coli* (NCIM No. 2345), *K. pneumoniae* (NCIM No. 2706) and *S. typhimurium* (NCIM No.2501) were grown on nutrient agar media and used for antimicrobial activity. 0.5 McFarland standard suspension was used for this assay.

Antimicrobial Activity

The antimicrobial activity was carried out by agar well diffusion technique. The respective wells were poured with 30µL/mL (1mg/mL Concentration) of the sample. In other wells, supplements of DMSO and reference antimicrobial drug (Cloramphenicol) were used as negative and positive controls, respectively. The experiment was carried out in triplicate. The plates were incubated at 37ºC for overnight and results were recorded as zone of inhibition in mm.

Process Optimization for Production of Active Metabolites

For process optimization, different parameters like temperature, pH, incubation period were optimized with respect to fungal biomass and crude metabolite production in shake culture condition. The mycelial mats were filtered and dried at 50^0C until constant weight was obtained. The final fungal biomass was recorded in mg/100mL. Temperature (25^0C, 27^0C, 30^0C), pH (3,5,7) and incubation period (15 and 21 day) were studied by inoculating the fungus in potato dextrose agar and the effect on crude metabolite production was observed.

Isolation of genomic DNA, PCR amplification and sequencing

Fungal genomic DNA was isolated using gene O-spin Microbial DNA isolation kit (GeneOmbio technologies, Pune, India). The ITS1, ITS2 and inverting 5.8S coding rDNA were amplified using Universal ITS rDNA typing primers ITS1 and ITS4 in standard PCR reaction. After amplification, products were purified by using a gene0-spin PCR product purification kit and were directly sequenced using an ABI PRISM Big Dye Terminator V3.1 kit. (Applied Biosystem, USA). The sequences were analysed using sequencing analysis 5.2 software. BLAST analysis was performed at BlastN site at NCBI server (http://www.ncbi.nlm. nih.gov/BLAST).

Results and Discussion

Total of 150 segments from *Vitex negundo* L. were processed to investigate the presence of endophytic fungi. A total of 17 endophytic fungi were isolated from all the segments of different parts of *Vitex negundo* L. as summarised in Table 1.

Table.1 Endophytic Fungi Isolated from Different Parts of *Vitex negundo* L..

Site of Isolation	Number of samples	Number of Fungi Isolated
Leaves	50	6
Stems	50	7
Roots	50	4
Total number of isolates	150	17

Table.2 Endophytic Fungi Isolated from Different Parts of *Vitex negundo* L.

Source	Identification Remark
Leaf	*Colletotrichum gloeosporioides* Penz.
	Phomopsis archeri B. sutton
	Aspergillus flavus gr.
	Nigrospora sphaerica(Sacc.)Mason
	Nonsporulating dematiaceous form
	Colletotrichum gloeosporioides Penz.
Stem	*Phomopsis* sp. aff. *P. archeri* B. sutton
	Colletotrichum gloeosporioides Penz.
	Nonsporulating dematiaceous form
	Alternaria raphani JW Groves &skolko
	Penicillum sp.
	Mucor hiemalis Wehmer
	Nonsporulating dematiaceous form
Root	*Monodictys paradoxa* (Corda) Hughes
	Nonsporulating dematiaceous form
	Mucor hiemalis Wehmer
	Nigrospora state of *Khuskia oryzae* H.J. Hudson

Table.3 Antibacterial Activity of Various Extracts of Phomopsis sp. Zone of Inhibition (Diameter In mm) (300µg/well)

S.No	Name of Organisms	Extracts (DMSO –ve control)			
		Hexane	Ethyl acetate	Methanol	Cloramphenicol (+ve control)
1.	*E. coli* (NCIM No.2345)	16	24	12	22
2.	*S. typhimurium* (NCIM No.2501)	13	22	12	24
3.	*B. cereus* (NCIM No.2155)	10	16	10	20
4.	*S. aureus* (NCIM No.2079)	12	18	-	18
5.	*K. pneumoniae* (NCIM No.2706)	-	10	12	18
6.	*B. subtilis* (NCIM No.2063)	10	8	6	22

Among the endophytic flora, *Colletotrichum* was found to be the most prominent genus.

Among all endophytic fungi, 13 exhibited prominent antibacterial activity whereas 4 did not exhibit any antibacterial activity. Among the species showing antibacterial properties, the fungus showing the highest inhibitory activity was identified as *Phomopsis* sp. by molecular analysis. The least antibacterial activity was shown by species *Aspergillus.*

The crude extract of these fungal isolates with Hexane, Ethyl acetate and Methanol were screened for their antimicrobial potential. The extract of phomopsis by ethyl acetate showed significant antimicrobial activity against *E.coli, S. typhimurium, B. cereus, B. subtilis, K. pneumoniae* and *S. aureus*. The antimicrobial activity was highest against *E. coli* (24mm), followed by *S. typhimurium* (22mm), and *B. cereus* (16mm). The hexane and methanol extracts showed moderate activity against all the pathogenic organisms.

Figure.1 Antimicrobial activity of *Phomopsis* against bacterial pathogens

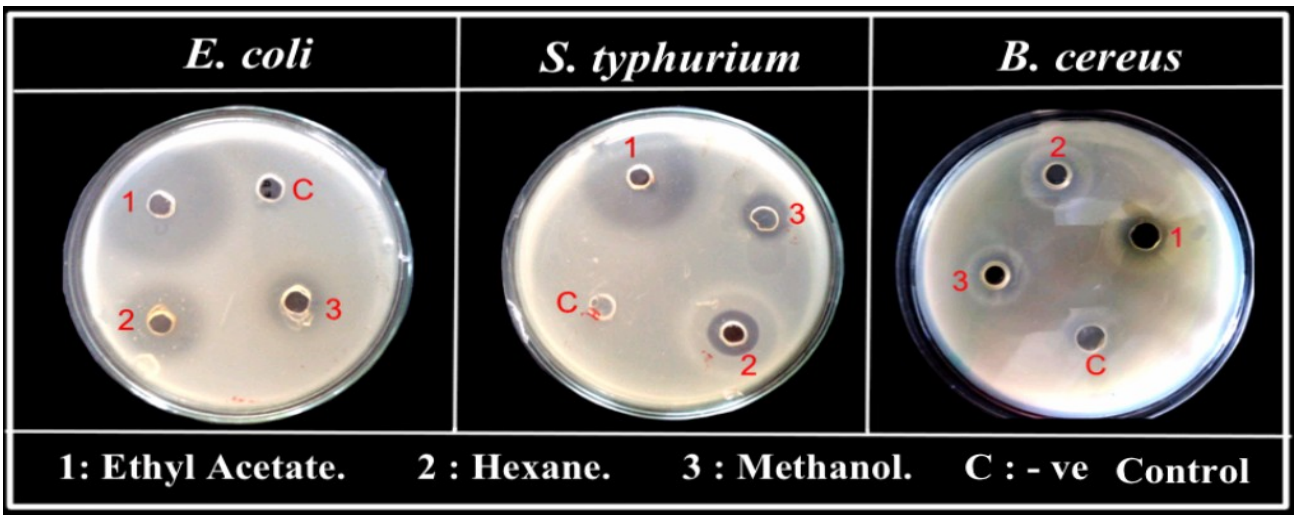
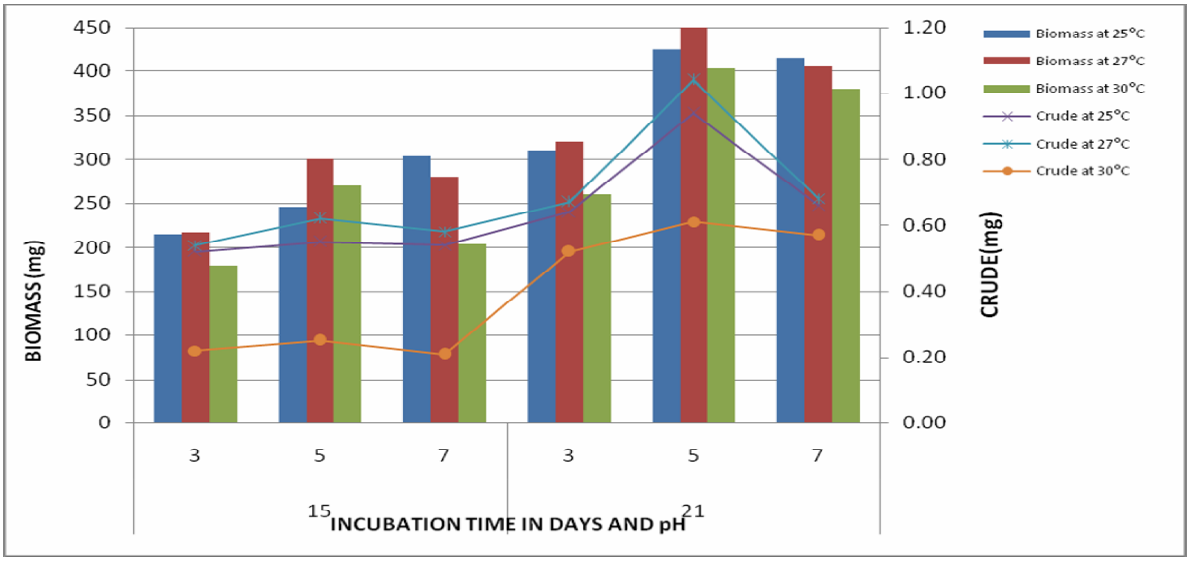


Figure.2 Effect of pH, Incubation Time and Temperature on growth and production of crude metabolite (extract) by *phomopsis* sp. in PD broth



The growth of fungal isolate was studied on potato dextrose broth aerobically at stationary phase. The isolated fungus was estimated for biomass and crude metabolite at different pH and temperature on 15th and 21st day. Maximum growth was recorded at 27^{0}C at pH 5 on 21st day of incubation.

Conclusion

The medicinal plant *Vitex negundo* harbors diverse species of endophytic fungi (Banerjee *et al.*, 2006). The stem of *Vitex negundo* L. harbor largest number of endophytic fungi as compared to roots and leaves. *Colletotrichum sp.* is the most prominent genus among the mycoflora. Some of the isolates of endophytic fungi exhibited significant inhibitory activity on selected test organisms. *Phomopsis sp.* exhibited the most significant inhibitory activity against all the test human pathogens. Further studies on isolation of these antimicrobial compounds and identification of bioactive compounds can be a crucial approach to search of novel natural products.

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