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SCREENING OF SECONDARY METABOLITES, ENDOPHYTIC DIVERSITY AND ANTIMICROBIAL ACTIVITY OF ENDOPHYTES ISOLATED FROM AN ENDANGERED PLANT *VATERIA INDICA* (LINN) SARJA.

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Abstract:
The current study was aimed to evaluate the endophytic diversity and its biological activity of V. indica, an endangered plant. It is also known as white dammar, is a species of plant in the Dipterocarpaceae family. It is a slow- growing species, endemic and found predominantly in the south west coast evergreen forests. The majority of endophtyes are isolated from stem and leaves. The ethanolic extract of all endophytes shows antibacterial activity with maximum 20mm and a minimum of 4mm zone of inhibition against different bacteria in disc diffusion assay. From the present study we conclude that the compounds synthesized by bio endophytes are used for the treatment of many diseases. Screening of secondary metabolites which include alkaloids, flavonoids, saponins, terpenoids, steroids, glycosides, tannins, volatile eoils etc., are used for curing many diseases. Few isolates showed the maximum antimicrobial activity exhibiting the clearzone of inhibition against few human pathogens. Pharmacological studies have mainly focused on potential efficacy on tumors, bacteria, Alzheimer's disease, cardiovascular diseases, and others.

Keywords: V. indica, Secondary metabolites, Antimicrobial activity, Endophytic diversity.

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INTRODUCTION:

The term endophytes was proposed by[1], described as any organism that develops within plant tissues, but now they are more accurately illustrated in terms of their types (fungal and bacterial) and relationships[2]. This well distinct group of fungi can have intense impressions on plant communities through enhancing fitness by conferring abiotic and biotic stress tolerance[3]. Its shows several applications in the field of medicine, agriculture and industries.

Endophytes are rich source of secondary metabolites with multifold significance[4] in contrast to other endophytic microorganisms, fungal endophytes yield large number of secondary activities. The fungi belonged to eight groups, i.e six dematiaceous genera (*Alternaria*, *Cladosporium*, *Chaetomium*, *Curvularia*, *Drechslera* and *Scopulariopsis*) and the non-dematiaceous genera (*Acremonium*, *Aspergillus*, *Colletotrichum*, *Fusarium*, *Paecilomyces* and *penicillium* along with some Mycelia sterilia[5]. The current work comprises the evaluation of endophytic fungi diversity and to determine more effective endophtyic fungi bioactive compounds based on the activity and screening of secondary metabolites from the endophytes.

MATERIALS AND METHODS:

Plant material collection

Plant material, *V. indica* was collected from Mookambika Wildlife Sanctuary, Western Ghats of India Agumbe, Shivamogga district, Karnataka, India.

Figure 1 : *V. indica* morphology.

Study site:

The study site is situated at Western Ghats of India, Agumbe, in Shivamogga district of Karnataka. It is part of the Bio-diversity hot-spot of the Western Ghats and boasts of a large number of endemic flora and fauna. These forests have been classified as tropical wet evergreen forest with various medicinal plants. Agumbe region falls in 13°30' 36''N and 75° 6' 9''E with elevation ranges 650 meters above mean sea level (MSL) with a mean typical annual rainfall of 5000 – 8000mm.

Surface sterilization of plant material:

Freshly collected plant materials are washed carefully under running tap water followed by sterile distilled water to eliminate the adhered debris. Stem and leaves were surface sterilized under aseptic condition in consecutive step by immersing in mercuric chloride (1mg/1mL) for 10 min and 70% ethanol for another min followed by washing finally with distilled water to remove the traces of mercuric chloride.

Inoculation of implants:

After sequential sterilization of stem and leaves of *V. indica* parts were cut into small pieces aseptically and placed 2-3 pieces on each of the solidified Potato Dextrose Agar (PDA) media. The inoculated plant implants were incubated till the growth of distinguishable fungal endophytes.

Figure 2: *V. indica* leaves inoculated on PDA media

Figure 3: *V. indica* stem inoculated on PDA media

Identification of endophytic fungi:
For the identification of endophytic fungal isolates, slides were prepared from the culture and were stained with lactophenol cotton blue and examined with a bright field and phase contrast microscopes. Identification was based on morphological characteristics such as growth pattern, hyphae, the color of the colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and conidia characteristic using standard identification manual[6,7]. The identified fungus was sub cultured in PDA slants for further use and stored in refrigerated conditions.

Mass production of identified fungi:
Identified fungal species were cultured on PDB broth for large scale production. The inoculated flasks were incubated at room temperature (26±2°C) for 8-15 days and allowed to grow the fungal mats. Further these mats were used as a fungal extract for the further analysis.

Antibacterial screening by disc diffusion method:
Disc diffusion method is done to test antibacterial activity for all the endophytic fungal species. All fungal cultures were inoculated into MHA (Muller Hinton Agar) plates by using sterile cotton swabs. Around 5mm diameter well was made by using cork borer which is sterilized and approximately 200µl of culture supernatants were added. Later all plates were placed in bacterial incubator for 48 hours around 37°C for the development of zone of inhibition. Streptomycin is used as a positive control.

Screening of secondary metabolites of ethanolic extract of *V. indica*
Terpenoids: 2mL of fungal extract and 2mL of chloroform is added. 3mL of conc. sulphuric acid is added to form a layer, if reddish brown color appears indicates the presence of terpenoids.

Steroids: 1mg of extract was taken in a test tube and dissolved with chloroform (10mL), and then added equal volume of conc. Sulphuric acid to the test tube by sides. The upper layer in the test tube was turns into red color indicate the presence of steroids.

Tannins: Ferric chloride test: plant extract is mixed with 1% $FeCl_3$ solution which produces green or brownish green, blue color.

Saponin: Fungal extract of 1mL is mixed using 20mL of distilled water and kept on stirring for 15 min if foam forms, indicates the presence of saponins.

Alkaloids: Extraction is made with ethanol. Then the extract can be used for qualitative detection test, crude extract can be used for chemical test.

Mayer's test: For 3mL of extract, a few drops of Mayer's reagent are added. Brownish precipitate indicates the presence of alkaloids.

Flavonoids: Fungal extract of 2mL is added to the test tube containing 1 mL of dilute ammonia and mixed well with 1mL of conc. sulphuric acid. Formation of yellow color indicates the presence of flavonoids.

Triterpenoids: Fungal extract of 2mL is mixed with 5 drops of conc. Sulphuric acid. Appearance of greenish blue color indicates the presence of triterpenoids.

Cardiac glycosides: To 0.5g of extract diluted to 5 mL in water was added 2mL of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1mL of conc. sulphuric acid. A brown ring at the interface indicates the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above

the brown ring and gradually spread throughout this layer.

RESULTS:

Table 1 shows the endophytic fungus associated with the plant *V. indica* were studied to evaluate the production of bioactive compounds. The plant was taxonomically identified and authenticated by the taxonomist Prof. K. Gopalakrishna Bhat, Taxonomy Research Centre, Department of Botany, Poornaprajna College, Udupi. A total of sixteen different endophytic fungi associated with leaves and twig of *V. indica* were isolated, and morphotypically identified as *Phyllosticta, Cladosporium sp. Fusarium sp. Tritirachum, Coniothyrium sp. Chaetomium, Colletotrichum.*

Table 2 shows the fungal isolates which were obtained from the twig and leaves of *V. indica*. All isolated endophytes hindered at least one pathogenic bacterium in the disc diffusion assay and created zones of inhibition ranging from 4 to 20mm (Table 2). Among the six tested bacteria, *S. aureus* and *E. coli* was inhibited by most endophytic fungal extract. Highest antibacterial activity was shown by *Cladosporium sp.* From the leaf part which inhibited all test bacteria. Lowest antibacterial activity was shown for *Cladosporium sp.* From the twig part which shows the minimum zone of inhibition.

Table 3 shows the presence of secondary metabolites from different kind of endophytic fungi. The active metabolites contain chemical groups such as flavonoids, terpenoids, alkaloids, tannins, saponins etc. The analyses of the crude extracts of *Cladosporium sp.* have shown the presence of secondary metabolites. These different fungal isolates have an ability to produce various secondary metabolites which may be used in the area of pharmacology and also as a prospective source of valuable novel drugs.

Table 1: Different types of identified endophytes

Sl. No.	Plant parts	Endophytic fungi	Class
01.	Twig	*Phyllosticta*	Dothideomycetes
02.	Leaf	*Cladosporium sp.*	Dothideomycetes
03.	Twig	*Fusarium sp.*	Sordariomycetes
04.	Leaf	*Fusarium sp.(crescent shape)*	Hyphomycetes
05.	Twig	*Tritirachum*	Tritirachiomycetes
06.	Leaf	*Fusarium sp.*	Sordariomycetes
07.	Twig	*Fusarium sp.*	Hyphomycetes
08.	Leaf	*Fusarium sp.*	Hyphomycetes
09.	Twig	*Cladosporium sp.*	Dothideomycetes
10.	Leaf	*Fusarium sp.*	Sordariomycetes
11.	Twig	*Coniothyrium sp.*	Dothideomycetes
12.	Leaf	*Cladosporium sp.*	Dothideomycetes
13.	Twig	*Chaetomium*	Sordariomycetes
14.	Twig	*Cladosporium sp.*	Dothideomycetes
15.	Twig	*Colletotrichum*	Sordariomycetes
16.	Leaf	*Cladosporium sp.*	Dothideomycetes

Table 2: Evaluation of anti-bacterial activities based on fungal diversity:

Endophytes isolated From *V. indica* Leaves / Twigs	Zone of Inhibition in mm						+ve Control (Strep)
	E. coli	*P. syrin*	*P. aero*	*B. sub*	*S. aure*	*K. sine*	
Twig- *Phyllosticta*	12	04	14	11	14	14	26
Twig- *Fusarium sp.*	10	16	16	17	19	17	22
Leaf- *Cladosporium sp.*	16	14	10	12	12	20	24
Twig- *Cladosporium sp..*	10	09	08	08	09	10	22
Twig- *Coniothyrium*	15	12	12	16	15	10	26
Leaf- *Fusarium sp.*	14	12	15	10	13	11	24
Twig-*Tritirachum*	12	08	10	10	11	08	22
Twig- *Fusarium sp.*	10	14	16	11	18	12	20
Twig- *Cheatomella*	19	11	14	13	14	13	20
Twig- *Cladosporium sp.*	14	11	12	20	12	15	22

Whereas;
E. coli - Escherichia coli
P. syringes-Pseudomonas syringae
P. aero – Pseudomonas aeruginosa
B. sub -Bacillus subtilis
S. aureus- Staphylococcus aureus
K. sine – Knoellia sinensis

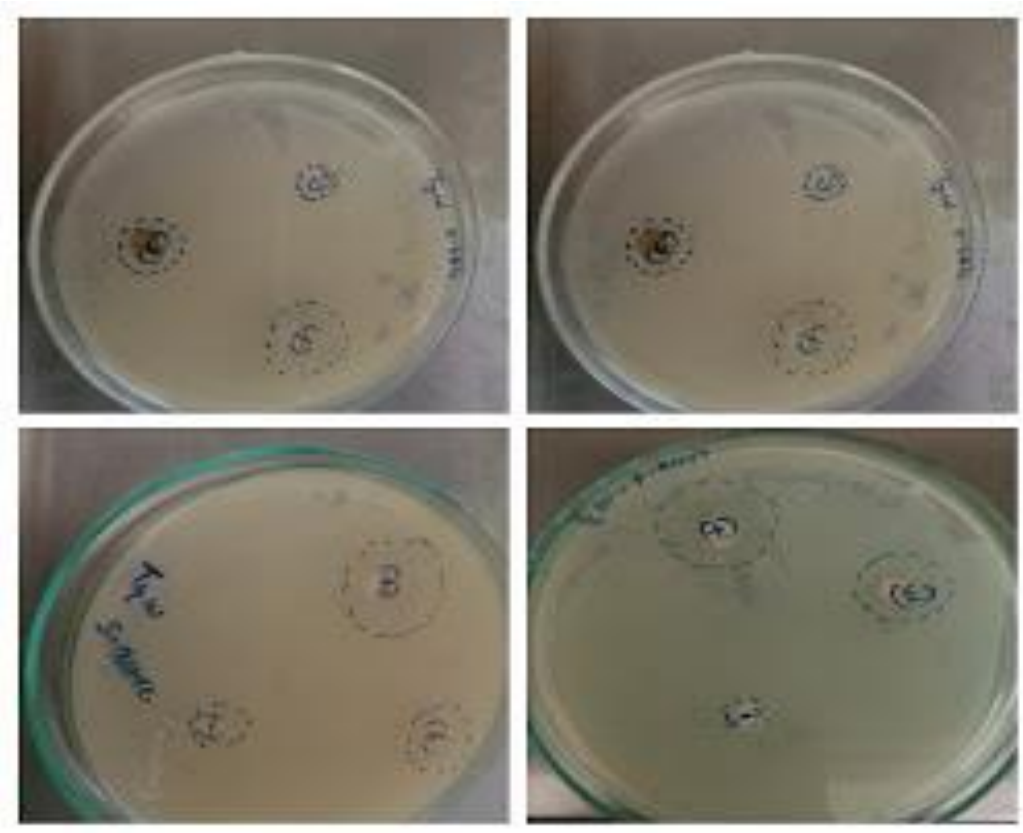

Figure 4 illustrates the antimicrobial of isolates against bacterial pathogens.

Table 3: Screening of secondary metabolites of ethanolic extract of *V. indica*

Secondary Metabolites	VIT-P	VIT- F	VIT- T	VIT-F	VIT- F	VIT-C	VIT Con	VIT-Ch
Alkaloids	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	-	+	+	+	+
Saponins	+	+	-	+	+	+	+	+
Tanins	+	+	-	-	+	+	+	+
Terpenoids	+	+	-	-	-	+	+	+
Cardiac glycoside	+	+	-	+	-	+	+	+
Sterols	-	-	-	-	-	-	-	-
Triterpenoids	+	+	-	-	-	+	-	-

Secondary metabolites	VIT-Cl	VIT-Cl	VIT-Co	VIL-Cl	VIL-F	VIL-F	VIL-F	VIL-Cl
Alkaloids	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	-	+	+	+
Saponins	-	-	+	+	+	+	-	+
Tanins	+	-	+	+	-	+	+	+
Terpenoids	+	+	-	+	-	+	-	+
Cardiac glycoside	+	+	+	+	+	+	+	+
Sterols	-	-	-	-	-	-	-	-
Triterpenoids	-	-	-	+	-	+	+	-

Where;
VIT-P =*V. indica Phyllosticta*
VIT-F=*V. indica Fusarium*
VIT-T= *V. indica Tritirachum*
VIT-C= *V. indica Cladosporium*
VIT-Con = *V. indica Coniothyrium*
VIT-Ch= *V. indica Cheatomium*
VIT-Cl= *V. indica Cladosporium*
VIT- Co= *V. indica Colletotrichum*
VIL-F = *V. indica Fusarium sp.*
VIL-Cl = *V. indica Cladosporium*

DISCUSSION:

This study showed the promising antimicrobial activity against different human pathogens. Endophytic fungi have broad application in diverse fields. The diverse array of endophytic bacterial which forms a non pathogenic relationship with their hosts also confers benefit to plants [8]. It has the ability to produce numerous bioactive compounds. The secondary metabolites produced by the endophytic fungi have the capability to act as biocontrol agent. Endophytic fungi isolated from the medicinal plants would be a encouraging source for many pharmaceutical constituents and industries. Around more than 15 types of endophytic fungal species have been isolated from the plant *V. indica.* This study is mainly conducted on the diversity of the endophytes present in *V. indica* from the biodiversity hotspot in the Western Ghats of India. A single leaf of a plant can harbor many species of endophytes, both bacteria and fungi [9]. The diversity of endophytes from the leaves of traditional medicinal plants that has anti phyto pathogenic properties were reported [10]. Some of these fungal species are subjected to antibacterial activity against pathogenic Gram positive and Gram negative bacteria. Zone of inhibition was observed in the plates. All endophytic extracts showed growth inhibitory activity against at least one of the test pathogens [11]. Many of them are capable of synthesizing active compounds that can be used by plants for defense against human pathogens. Compounds of medicinal value derivative from various endophytic fungi have made massive impact towards the betterment of human health and act as a source of revelation for novel drug compounds [12]. We can conclude that the endophytic fungi can used for the various purposes by investigation to prove its

potential produce of novel bioactive compounds and it will lead for the discovery of new drugs.

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