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Studies on Endophytes and Antibacterial activity of *Trillium govanianum* Wall. ex D. Don.

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Abstract
Trillium govanianum commonly called as "Nag chhatri" is an endangered plant species being traditionally over exploited by the tribal people to curve diseases like diarrhoea, dysentery, ulcerous wounds and menustrual and sexual disorders. Studies conducted on endophytes and antibacterial activity of this plant revealed that its leaves, stem and rhizome harbour nine genera of fungi (*Alternaria, Aspergillus, Fusarium, Mucor, Phoma, Pythium, Rhizopus, Stachybotrys* and *Trichoderma* as endophytes. The antibacterial activity of methanol, ethanol, acetone and distilled water rhizome extracts of *T. govanianum* was determined *in vitro* against three human pathogenic bacteria (i.e. *Escherichia coli*, *Yersinia pestis* and *Staphylococcus aureus*) following agar well diffusion method using different concentrations of plant parts extracts (25%, 50%, 75% and 100%). The screening revealed that methanol rhizome extract of *T. govanianum* was most effective in inhibiting the growth of *S. aureus*. There is a need to analyze the bio active molecules and secretions of endophytes present in this medicinal plant.

Keywords: *Trillium govanianum*, endophytes, antibacterial activity, rhizome extract, *in vitro*, agar-well diffusion

1. Introduction
There is a vast microbial flora inhabiting in the earth and micro-organisms are found in all types of soil which are cultivated, sands, deserts, thermal soils, snow covered soils, sediments, semi aquatic ecosystem and on rocks etc. The dominating groups of micro-organisms are bacterial, actinomycetes, fungi, nematodes and protozoans [1]. Fungi comprises a large groups of organisms which differ in behaviour and life patterns, they produce a large number of sexual and asexual spores which are liberated and dispersed with the great efficiency, hence they found in every niche. Fungi shows symbiotic, saprophytic or parasitic mode of nutrition. The fungi colonizing the root surface enhance the absorption of water and nutrient from the soil was first observed by Frank [2]. The fungal endophytes are micro-fungi that colonize living tissues of plants without producing any symptoms or negative effects [3]. Some endophytes have high metabolic versatility and produce secondary metabolite of industrial importance [4]. Endophyte plays a significant role in plant growth, development, stress tolerance and adaptation. Fungal endophytes have been found in thousands of plants, including many agricultural plants such as wheat [5], bananas [6, 7] and tomatoes [8]. Plants are affected by endophytes in various ways, but in the most cases the presences of endophytic microorganisms in the host plants are beneficial to their host plants. Endophytes can actively or passively promote the plant growth through a variety of mechanisms, as endophytic metabolites provide a variety of nutrition to host plants as well as enhance plant resistance to biotic and abiotic stresses and enhance plant growth.
Endophytic fungi protect their host plants from pathogens and pests [9, 10]. Endophytes usually produce the enzymes necessary for the colonization of plant tissues. Many endophytic fungi have been reported to produce novel antibacterial, antifungal, antiviral and anti-inflammatory agents belonging to the alkaloids, steroids, flavonoids and terpenoids derivatives [11, 12].
Medicinal plants contain the active constituents which can be used in the treatment of many human diseases [13]. The use of medicinal plants is a universal phenomenon. Medicinal plants have ability to cure both infectious and non-infectious diseases. The use of plants and plant products as medicines could be traced as back as the beginning of the human civilization. Plants used in traditional medicine contain a vast array of substances that can be used in the treatment of chronic and infectious diseases. Medicinal plants are sources of verification of pharmacological products and as a natural composite that acts as a new anti-infectious agent [14]. Higher plants have the capacity to produce a large number of organic phytochemicals that is known as secondary metabolites. Sometime secondary metabolites are produced by the plants for self-defence [15]. In the last 20 years, a large number of secondary metabolites have been reported from different plants for their antimicrobial activity. The demands of plants based products have increased in both developed and developing countries due to growing recognition that they are natural products, non-narcotic, easily biodegradable and pose minimum environmental hazards. They have no side effects and are easily available at affordable prices [16].
In 2011, due to unscrupulous extraction of *Trillium* from wild in Kullu district of Himachal Pradesh its population has decreased to the level of an endangered species. Amongst the threatened medicinal plants *T. govanianum* (Wall. ex D. Don) is a native species of the Himalayas usually preferring shady areas in forest for its profuse growth [17, 18] and it is mainly distributed in the Himalaya, Pakistan, Bhutan, Nepal and China between the attitudinal ranges of 2500 to 4,000 m above the sea level. Commonly called as Nag chhatri, it is an endangered plant which belong the family Lilliaceae or Melanthiaceae. Its rhizome is used to cure infectious diseases (healing of wounds, antiseptic, bacterial diarrhoea and dysentery) and menstrual and sexual disorders [19, 20, 21]. It also serves as a source for preparing steroidal and sex

hormones. This study gives good information on antibacterial activity and isolation of endophytes of this plant. At present, there is an urgent need of exploration and development of cheaper and effective plant based drugs with better bioactive potential and least side effect.

2. Materials and Methods

Banjar Valley of District Kullu in Himachal Pradesh was selected for the collection of study material (leaves, stem and rhizomes) of plant *Trillium govanianum* during summer and rainy seasons.

Processing of Plant Material

The collected samples were first washed thoroughly in running tap water. The plant materials from different tissues (i.e. leaves, stem and rhizomes) of *Trillium govanianum* were cut into small pieces (5 mm approx.) and screened for the presence of fungal endophytes.

2.1 Methodology for Isolation of Endophytes

(a) Hot water treatment

The Endophytes were isolated from small pieces of leaves, bark and roots. These were washed with hot water (60°C) for 15 min in a test tube. Then three pieces of each sample were inoculated on Petriplates containing PDA medium supplemented with Penicillin or Streptomycin (150 mgl^{-1}). These Petriplates were incubated at 25°C±2 in an incubator for one week. After the fungal growth, sub-culturing was done on PDA slants and slants were preserved in refrigerator.

(b) Three step method

The samples were washed with sterilized distilled water. Then these were surface sterilized with 25% methanol for 5 minutes, followed by 50% methanol for 3 min, again followed by 75% methanol for 2 min. Finally these samples were washed in sterilized water for 5 minutes. Then three pieces of each sample were inoculated on Petriplates containing PDA medium supplemented with Penicillin or Streptomycin (150 mgL^{-1}). The Petriplates were incubated at 25±2 °C for few days. The growing fungal colonies were then transferred on PDA slants.

2.2 Slide preparation

For identification, temporary mounts of fungi were made in 0.1% cotton blue and lactophenol. Adequate high power microscopes were used for observing the slides. Fungi were identified following Nagmani *et al.* [22].

2.3 Maintenance and Preservation of cultures

Pure cultures of different fungal genera were maintained on PDA which was preserved in refrigerator. Sub-culturing was done at regular intervals in order to maintain cultures. Each fungal species was transferred from parent source to a fresh slant in order to maintain and preserve the cultures.

2.4 Methodology for antibacterial activity

Procurement of bacteria

Different strains of bacteria (*Staphylococcus aureus, Escherichia coli* and *Yersinia pestis*) have been procured from Indira Gandhi Medical Collage (IGMC) Shimla and Department of Microbiology, HPU Shimla for screening antibacterial properties of plant extracts.

Revival of pathogen

The collected pathogens were revived in nutrient broth and stored in nutrient agar slants at 4°C.

Maintenance and preservation of pure culture

Pure cultures of all the bacteria were maintained on nutrient medium broth and preserved in refrigerator. Sub culturing was done at regular interval in order to maintain the cultures. Each bacterial species was transferred from parent source to maintain and preserve the cultures.

Extract preparation

Extracts (acetone, ethanol, methanol and distilled water) of plant have been prepared to check antimicrobial activity. 5 gm dried plant material was taken in separate Erlenmeyer flasks to which 50 mL of required solvents i.e. methanol, acetone, ethanol and distilled water were added. The flasks were covered with aluminium foil and allowed to stand for 3-5 days for extraction. These extracts were filtered through Whatman filter paper no. 1 and evaporated at 40°C using rotary evaporator. The extracts were collected and weighed. Finally, stock solution of conc. 50 mg/mL was prepared.

2.5 Screening of Plant Extracts for Antibacterial Activity

Agar-well Diffusion Method

Screening of plant extract rhizome (methanol, acetone, ethanol and distilled water) of *Trillium govanianum* was done using agar-well diffusion method. Nutrient agar medium (Beef extract 1 g, Yeast extract 2 g, Sodium Chloride 1 g, Peptone 5 g, agar 20 g, Distilled Water 1000 mL) was used throughout the investigation. The medium was autoclaved at 121.6°C for 30 minutes and poured into Petri plates. Bacteria were grown in nutrient broth for 24 hours. A 100 µl of bacterial suspension was spread on each nutrient agar plate. Agar wells of 8 mm diameter were prepared with the help of sterilized stainless steel cork borer in each Petri plate. The wells in each plate were loaded with 25%, 50%, 75% and 100% concentration of prepared plant extracts. The Petri plate kept as a control contained pure solvent only. The plates were incubated at 37 ± 2^0C for 24 hours in the incubation chamber. The zone of growth inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in perpendicular direction in all the three replicates and the average values were tabulated. Percentage inhibition of bacterial species was calculated after subtracting control from the values of inhibition diameter using control as standard [23].

Percentage of growth inhibition = (Control - Test/Control) x100

Control = average diameter of bacterial colony in control

Test = average diameter of bacterial colony in treatment sets [16]

3. Observation and Results

3.1. Qualitative assessment of endophytic fungi isolated from various plant parts of *Trillium govanianum.*

In the present investigation, thirteen species of fungi were isolated from different plant parts (i.e. leave, stem and

rhizome) in two seasons which fall into 9 genera (Table-1). These genera were *Alternaria, Aspergillus, Fusarium, Mucor, Phoma, Pythium, Rhizopus, Stachybotrys* and *Trichoderma*. The genus *Aspergillus* was represented by three species (*A. niger, A. nidulans* and *A. wentii*). The genus *Fusarium* was represented by two species (*F. oxysporum* and *F. solani*) and genus *Rhizopus* was represented by two species (*R. nigricans* and *R. oryzae*). The genus *Alternaria, Mucor, Phoma, Pythium, Stachybotrys* and *Trichoderma* were all represented by one species each (i.e. *Alternaria* sp., *Mucor plumbeus, Phoma* sp., *Pythium* sp., *Stachybotrys atra* and *Trichoderma viride*).

A comparision of seasonal distribution of these isolates revealed that maximum number of fungi were recorded in rainy season (10 spp.) followed by summer season (8 spp.). These fungal isolates from different plant parts (viz. Leaves, stem and rhizomes) of *Trillium govanianum* were further grouped into Zygomycota (i.e. *Mucor* and *Rhizopus*), Ascomycota (i.e. *Alternaria, Aspergillus, Stachybotrys, Fusarium, Phoma* and *Trichoderma*) and Eumycota (i.e. *Pythium*). Maximum representatives belong to Ascomycota.

Table 1: List of Isolated Endophytes from various plant parts (viz. Stem, leaves and rhizomes) of *Trillium govanianum* Wall. ex D. Don.

S. No.	Isolated Endophytes
1.	*Alternaria sp.*
2.	*Aspergillus nidulans*
3.	*Aspergillus niger*
4.	*Aspergillus wentii*
5.	*Fusarium oxisporum*
6.	*Fusarium solani*
7.	*Mucor plumbeus*
8.	*Phoma sp.*
9.	*Pythium sp.*
10.	*Rhizopus nigricans*
11.	*Rhizopus oryzae*
12.	*Stachybotrys atra*
13.	*Trichoderma viride*

Table 2: Comparison of occurrence of isolated endophytes from various plant parts (viz. leaves, stem and rhizomes) of *Trillium govanianum* during summer and rainy seasons

S. No.	Isolated Endophytes	Summer	Rainy
1.	*Alternaria sp.*	-	+
2.	*Aspergillus nidulans*	+	-
3.	*Aspergillus niger*	+	+
4.	*Aspergillus wentii*	-	+
5.	*Fusarium oxysporum*	-	+
6.	*Fusarium solani*	+	+
7.	*Mucor plumbeus*	+	-
8.	*Phoma sp.*	+	-
9.	*Pythium sp.*	-	+
10.	*Rhizopus nigricans*	+	+
11.	*Rhizopus oryzae*	+	+
12.	*Stachybotrys atra*	-	+
13.	*Trichoderma viride*	+	+

(+) present
(-)Absent

Table 3: Categorization of isolated endophytes from various plant parts viz. leaves, stem and rhizomes of *Trillium govanianum* into different fungal divisions

S. No.	Division	Genus
1.	Ascomycota	*Alternaria, Aspergillus, Stachybotrys, Fusarium, Phoma, Trichoderma*
2.	Zygomycota	*Mucor, Rhizopus*
3.	Eumycota	*Pythium*

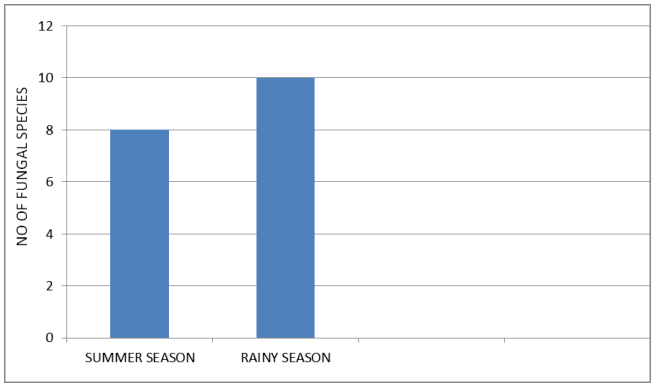

Fig 1: Histogram showing the distribution of endophytic fungal species (in summer and rainy seasons) isolated from various plant parts (viz. leaves, stem and rhizomes) of *Trillium govanianum.*

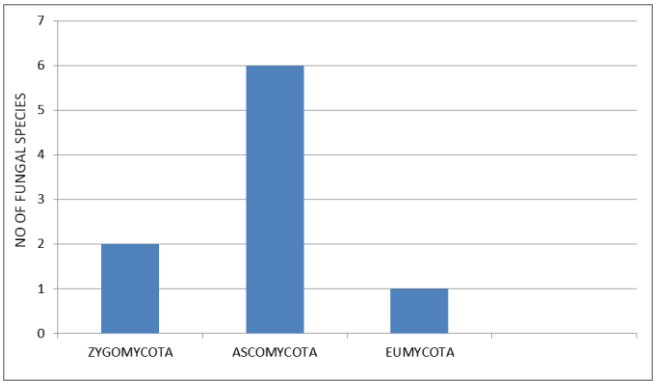

Fig 2: Histogram showing the distribution of endophytic fungal species (in different fungal divisions) isolated from various plant parts (viz. leaves, stem and rhizomes) of *Trillium govanianum.*

3.2. Antibacterial screening of rhizome extracts (methanol, ethanol, acetone and distilled water) of *Trillium govanianum* Wall. ex D. Don. against *Staphylococcus aureus, Escherichia coli* and *Yersinia pestis* bacteria.

The rhizomes of *T. govanianum* were tested for their antibacterial properties against selected human pathogens. Results obtained revealed that the tested plant extracts possess considerable potential antibacterial activity against *Staphylococcus aureus, Escherchia coli* and *Yersinia pestis* bacteria.

Table 4: antibacterial screning of methanol, ethanol, acetone and distilled water rhizome extract of *Trillium govanianum* against *S. aureus*, *E. coli* and *Y. Pestis*.

	Concentrations (In %)	Inhibition zone diameter (In mm±S.E)		
		S. aureus	***E. coli***	***Y. pestis***
Methanol Extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	8.66 ± 0.87	9.66 ± 0.87	0.00 ± 0.00
	50	11.66 ± 0.87	11.00 ± 0.57	0.00 ± 0.00
	75	14.33 ± 1.45	13.00 ± 1.45	0.00 ± 0.00
	100	18.00 ± 1.15	17.00 ± 1.15	8.33 ± 1.48
Ethanol extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	5.66 ± 1.76	8.33 ± 1.76	6.66 ± 0.87
	50	9.00 ± 0.57	11.33 ± 0.65	7.33 ± 0.90
	75	12.66 ± 0.87	12.00 ± 1.15	9.66 ± 0.87
	100	15.33 ± 0.87	13.33 ± 0.87	12.66 ± 0.87
Acetone extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	10.66 ± 0.85	8.66 ± 0.87	2.66 ± 1.15
	50	12.66 ± 0.87	11.00 ± 0.57	10.00 ± 0.57
	75	14.00 ± 1.52	13.00 ± 1.15	12.00 ± 0.57
	100	16.33 ± 1.19	14.33 ± 1.45	15.00 ± 1.15
Distilled Water extract	Control	0.00 ±0.00	0.00±0.00	0.00 ±0.00
	25	10.66 ± 0.87	0.00 ± 0.00	10.00 ± 0.57
	50	14.00 ± 1.15	0.00 ± 0.00	13.00 ± 1.15
	75	15.00 ± 0.57	0.00 ±0.00	17.00 ± 1.20
	100	16.33 ± 1.19	3.00 ± 1.15	19.00 ± 0.57

Each data represents mean of three replicates ± S.E.

It is evident from Table no.4 that the *T. govanianum* rhizome extract showed maximum zone of inhibition against the growth of *S. aureus* in methanol extracts of around 15.33 mm at 100% concentration.

4. Discussion

In the last few decades, extensive work has been done pertaining to endophytes and antibacterial activity of medicinal pants. In the present investigation, thirteen fungal species belonging to nine genera were isolated as endophytes from leaves, stem and rhizomes of *Trillium govanianum* during summer and rainy seasons. These different genera were *Alternaria, Aspergillus, Fusarium, Mucor, Phoma, Pythium, Rhizopus, Stachybotrys* and *Trichoderma*. Various workers have reported similar fungal endophytes from different plants. Gulati [24] isolated *Alternaria alternata, Aspergillus niger* and *Phoma* sp. as endophytes of *Taxus baccata*. Sagar and Chauhan [25] observed five species of fungal endophytes belonging to four genera viz. *Penicillium, Rhizopus, Gliocladium* and *Trichoderma* from leaves, bark and roots of *Quercus leucotrichophora.* Shilpa *et al.* [26] have also reported similar records of fungi from a medicinal plant *Rhododendron compannlatum*.

Rhizome extract of *T. govanianum* was screened *in vitro* for antibacterial activity against three pathogenic bacteria (*E. coli, S. aureus* and *Y. pestis*). Table no. 4 shows result of antibacterial screening of methanol, ethanol, acetone and distilled water rhizome extract of *T. govanianum* against three pathogenic bacterial spp. The screening revealed that ethanol rhizome extract of *T. govanianum* was most effective in inhibiting the growth of *S. aureus* (15.33 mm at 100% conc.) followed by (12.66 mm at 75% conc., 11 mm at 50% conc. and 10.33 mm at 25% conc.) where as in case of *E. coli* inhibition was (13.33 mm at 100% conc.) followed by 12 mm at 75% conc., 11.33 mm at 50% conc. and 8.33 mm at 25% conc.), and in case of *Y. pestis* inhibition was (12.66 mm at 100% conc.) followed by (9.66 mm at 75% conc., 7.33 mm at 50% conc. and 6.66 mm at 25% conc.) respectively.

The antibacterial screening of methanol rhizome extract was found to be most effective against *S. aureus* followed by 14.33 mm at 75%, 11.66 mm at 50% and 8.66 mm at 25%), where as in case of *E. coli* inhibition was (17 mm at 100%) followed by (14.33 mm at 75%, 11 mm at 50% and 9.66 mm at 25%) and in case of *Y. pestis* inhibition was (8.33 mm at 100% conc.) and (0.00 mm at 75%, 50%, 25%).

The acetone extract was most effective against *S. aureus* was (16.33 mm at 100%) followed by (14 mm at 75%, 12.66 mm at 50%, 10.66 mm at 25%) where as in case of *Y. pestis* inhibition was (15 mm at 100%) followed by (12 mm at 75%, 10 mm at 50% and 2.66 mm at 25%) and in case of *E. coli* inhibition was (14.33 mm at 100%) which is followed by (13 mm at 75%, 11 mm at 50% and 8.66 mm at 25%).

The antibacterial screening of distilled water extract against these bacteria was most effective against *Y. pestis* (inhibition was 19 mm at 100%) which is followed by (17 mm at 75%, 13 mm at 50% and 10 mm at 25%) where as in case of *S. aureus* inhibition was (16.33 mm at 100%) followed by (15 mm at 75%, 14 mm at 50% and 10.66 mm at 25%) and in case of *E. coli* inhibition was (3 mm at 100%) and showed (0.00 mm at 75%, 50% and 25%).

Hufford *et al.* [27] reported that extracts of the rhizome and aboveground portion of *Trillium grandiflourm* showed significant *in vitro* activity against *Candida albirans.* The compounds were found to exhibit some *in vitro* activity against four other genera of fungi.

It is evident from the results that methanol, ethanol, acetone as well as distilled water rhizome extract of *Trillium govanianum* were found to be effective in inhibiting the growth of bacteria. Possible reasons for this antibacterial activity are presence of alkaloids, phenolics and flavanoids

in the rhizomes of *T. Govanianum* which need futher investigations.

5. Conclusions

It was concluded from the above experimental observations that *Trillium govanianum* was more effective against *S. aureus* at all concentration as compared to *E. coli* and *Y. pestis* in three solvents such as ethanol, methanol and acetone but in case of distilled water rhizome extract the *Y. pestis* was found to be more effective as compared to *S. aureus* and *E. coli.*

In the present investigations showed thirteen fungal endophytes were isolated and identified from *T. govanianum* (viz. Stem, leaves and rhizomes). Comparing of the results of our study with the existing work of previous researchers revealed that majority of our results are in agreement with the existing reports. Some variation in the results can be attributed to adverse or favourable climatic conditions prevalent in this specific zone.

6. Acknowledgements

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7. References

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