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Studies on endophytes and antibacterial activity of *Plumbago zeylanica* L

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Abstract
Plumbago zeylanica L. is a medicinal plant commonly known as chitrak, belonging to family Plumbaginaceae. In the present investigation, plant parts of *Plumbago zeylanica* were collected to isolate the endophytes and to analyse the antibacterial activity. Thirteen species of endophytic fungi belonging to eight genera (*Aspergillus, Curvularia, Fusarium, Penicillium, Pythium, Rhizopus, Sporotrichum* and *Trichoderma*) were isolated from leaves, stem and roots of *P. zeylanica* during summer, rainy and winter seasons. The antibacterial activity of methanol, acetone and ethanol stem and root extracts of *P. zeylanica* was determined *in vitro* against medically important pathogens (i.e. *Escherichia coli, Staphylococcus aureus* and *Yersinia pestis*) following Agar-well diffusion method using different concentrations (25%, 50%, 75% and 100%). Results showed low to significant antibacterial activity against the mentioned bacterial strains. Methanol leaf extract was found to be more effective against selected pathogenic bacteria as compared to acetone and aqueous leaf extracts.

Keywords: *plumbago zeylanica,* plant extracts, pathogenic bacteria, endophytic fungi, antibacterial, agar-well diffusion

1. Introduction
Fungi includes all those organisms which are having a thalloid body, build-up of single cell or cells having a definite cell wall and nucleus but lack chlorophyll pigment necessary for the process of photosynthesis. They occur in almost every kind of habitat where organic matter is available. In most of the cases, fungal thallus consists of branched, long, tubular multinucleate hyphae which together form mycelial structure. Fungal hyphae have cell wall composed of fungal cellulose, pectose, callose, chitin or any other related compounds [1]. Fungi generally reproduce by both asexual and sexual means via the formation of spores.

Since fungi depend on organic material for their nutrition, they cause a great harm to food, household and commercial goods, our vegetation particularly forest trees causing diseases to them [2]. But fungi are not harmful to plants every time. They also occur as endophytes in mutual symbiotic association with plants. Endophytes are fungi or bacteria that colonize and reside in living and internal tissues of plants without causing any disease to them [3]. Fungal endophytes are known from plants which grow in tropical, temperate, arctic, alpine and xeric environments. These microfungi from healthy aerial plant tissues of stem and leaves are mostly documented from conifers. Fungal surveys of various hosts during the past twenty years have demonstrated that colonization of land plants by fungi is ubiquitous.

Fungi described as endophytic characteristically exhibit a prolonged, inconspicuous period in which growth and colonization cease temporarily, resuming after a physical or maturational change in the host. Most of the endophytic fungi are reported mainly from classes Ascomycetes, Basidiomycetes and Deuteromycetes [4]. The class and species of fungi depend upon the host plant. Fungal endophytes are known to produce antibacterial substances and have been shown to improve the tolerance of host plants to a variety of biotic and abiotic stresses. The presence of fungal endophytes has been shown to increase the survival and persistence of their host plants in a diverse range of environments and may also protect them from insects, pathogens, and herbivores [5].

In ancient times, plants were used traditionally as medicines due to their better cultural acceptability and fewer side effects. Even today, in developing countries like India, people of low income groups, farmers and native communities use folk medicines obtained from plant resources. People all over the world are facing serious health related problems particularly of skin and mucosal surface infections. Methicillin-resistant *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Candida albicans* and *Yersinia pestis* were found to be the most frequent skin pathogens. For the control of these kind of disease causing organisms, synthetic pesticides are highly effective [6], but due to appearance of side effects of certain antibiotics and development of drug resistance in these kind of microorganisms [7], research has been carried out in the discovery of new antibacterial agents particularly from medicinal plants.

A diverse range of bioactive molecules are produced by plants, thus making them rich sources of different types of medicines and foods. Because of non-violent and non-toxic nature of herbal medicines, people all around the globe are dependent on them since time immortal. Traditionally, for the treatment of human infectious diseases crude plant extracts are used as herbal medicine. These can be derived from any part of the plant like bark, leaves, flowers, seeds i.e., any part of the plant may contain active components like, alkaloids, flavonoids, glucosides, tannins, gums, resins, essential oils, fatty oils, nitrogen salts of some chemicals [8].

Plumbago zeylanica Linn. is one such plant of great medicinal importance. This plant is commonly known as chitrak, ceylon or lead wort is innate to South Asia. It is dispersed in tropical and sub-tropical countries of the world. In India, it is sprinkled in central India to West Bengal, Maharashtra, Uttar Pradesh and some parts of South India.

Plumbago from Plumbaginaceae family comprises of 10 genera and 280 species. Genus *Plumbago* takes account of

three species *i.e. Plumbago zeylanica*, *P. indica* and *P. capensis*. Of all these 3 species *P. zeylanica* is most cultivated because of its therapeutic uses. It grows wild in India and also refined commercially. It is a perennial bushy shrub. Roots are light yellow in colour when plant is garden fresh, but change to reddish brown in colour on drying. The texture of the roots are in the form of hard, straight, long, unbranched or slightly branched unbroken form. Roots are usually strong having a distinctive odour with bitter taste. Researchers reported anticancerous [9], antimicrobial and antibiotic [10] antifungal and antibacterial activities [11] from *P. zeylanica*. It is also believed that malaria, rheumatism, intestinal parasites, trauma and toxic swellings can be treated with this plant. This study gives good information on endophytic isolation and antibacterial properties of *P. zeylanica.* 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

2.1 Collection of plant material

Fresh leaves, stem and roots of *P. zeylanica* were collected from Jarot village, District Kangra Himachal Pradesh, India. Plant was properly identified and authenticated in Ethno-botany Laboratory, Department of Bio sciences, Shimla Himachal Pradesh. The collection of plant material was made during summer, rainy and winter seasons for endophyte isolation from its different parts.

2.2 Processing of Plant Material

For endophytic isolation, Leaves stem and roots of *P. zeylanica* were washed thoroughly under tap water and then with 2% Mercuric chloride. After that these all were cut into smaller pieces for quick drying. Cleaned leaves, stem and roots were shade dried for 15-20 days. The dried plant material was crushed into fine powder with the help of pestle mortar. Finally the fine powder was stored in an air tight container at room temperature. In order to determine the antibacterial activity of the plant, the powdered plant materials were extracted using solvents of different polarity i.e., methanol, ethanol and acetone. These extracts were used for the *in vitro* antibacterial assays.

2.3 Methodology for Endophytic fungal isolation

(a) Hot water treatment

The samples from the stem and roots were taken and were washed in water at 60ºC for fifteen minutes. Each sample was taken in three pieces and was inoculated on separate petriplate each containing PDA medium which was supplemented with streptomycin (150 mgL^{-1}). These petriplates were incubated at 25±2 ºC in incubator for one week. After the fungal growth in these plates, sub-culturing was done on PDA slants and the slants were preserved in refrigerator.

(b) Three step method

Firstly, samples were washed with sterilized distilled water. Then these were surface sterilized with 25% methanol for 5 min, followed by 50% methanol for 3 min and after that 75% methanol was used for 2 min. At last, these samples were washed in sterilized water for five minutes. Inoculation of three pieces of each sample was then done on petriplates containing PDA medium supplemented with streptomycin (150 mgL^{-1}). Petriplates were incubated at 25±2ºC for few days. The fungal colony growing in the petriplates was then transferred on PDA slants for sub-culturing.

2.4 Methodology for antibacterial activity of *P. zeylanica*

2.4.1 Preparation of plant extracts

5 g each dried roots and stem of *P. zeylanica* were taken in separate Erlenmeyer flasks to which 50 mL of required solvents i.e. methanol, acetone and ethanol 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 concentration 50 mg/mL was prepared.

2.4.2 Procurement of Bacteria

Bacterial spp. used for antibacterial studies were procured from Department of Biotechnology, Himachal Pradesh University, Summer Hill Shimla, India. Pathogens used in the study were *Escherichia coli*, *Yersinia pestis* and *Staphylococcus aureus.*

2.4.3 Revival of Pathogen

For antibacterial testing, fresh inoculum was prepared for each bacterium and they were incubated at 37 °C for 24 h.

2.4.4 Screening the antibacterial activity of *P. zeylanica*

The antibacterial screening was carried out using the 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 and distilled water 1 lt) was used throughout the investigation for the growth of microorganisms. The medium was autoclaved at 121.6ºC for 30 min. The plates were left over night at room temperature to check for any contamination to appear. Bacteria were grown in nutrient broth for 24 h. A 100 µl nutrient broth culture of each bacterial species was used to prepare bacterial lawns. Nutrient agar plates were spread with 100 µL of bacterial suspension. With the help of sterilized stainless steel cork bore, agar wells of 8mm diameter were prepared. Five wells were prepared in agar plates. The wells in each plate were loaded with 25%, 50%, 75%, 100% concentrations of root, stem extract of solvent with central well for control were prepared separately by dissolving extract in requisite amount of solvent. The plates containing bacterial colonies were incubated at 37ºC for 24 h (*S. aureus, E. coli* and *Y. pestis*) in incubation chamber. All the tests were repeated in triplicates. Diameter of bacterial colonies of treatment and control sets were measured in mutually perpendicular direction on second day. Percentage inhibition of each bacterial species was calculated after subtracting the value of control from the value of extracts using control as standard [12].

Percentage of growth inhibition = Control – Test/Control ×100 [13]

3. Results and Discussion

Total thirteen species of endophytic fungi belonging to eight genera (*Aspergillus, Curvularia, Fusarium, Penicillium, Pythium*, *Rhizopus, Sporotrichum* and *Trichoderma*) were isolated from leaves, stem and roots of *P. zeylanica* plant during summer, rainy and winter seasons. The genera

Curvularia, *Fusarium*, *Pythium*, *Sporotrichum* and *Trichoderma* were represented by one species each. The spp. of *Curvularia* and *Fusarium* were *Curvularia prasadii* and *Fusarium oxysporum*. The genus *Aspergillus* was represented by two species namely *A. niger* and *A. ustus*. The genus *Penicillium* was found to be dominant with four species, which were *Penicillium citrinum*, *Penicillium chrysogenum*, *Penicillium funiculosum* and *Penicillium restrictum*. The genus *Rhizopus* was represented by two species i.e. *R. nigricans* and *R. oryzae* (Table-1). Out of the identified genera, one genus each belonged to division Eumycota, Zygomycota, Basidiomycota and five genera belonged to Ascomycota (Table-2). Further, maximum number of endophytic fungi were observed during rainy season followed by winter and least during summer season. These findings are in accordance with the work carried out by many research workers. However, some variations in results can be attributed to host specificity and climatic conditions.

Table 1: List of endophytic fungi isolated from root, stem and leaves of *Plumbago zeylanica* Linn.

Sr. No.	Endophytic fungus isolated
1.	*Aspergillus niger*
2.	*Aspergillus ustus*
3.	*Curvularia prasadii*
4.	*Fusarium oxysporum*
5.	*Penicillium citrinum*
6.	*Penicillium chrysogenum*
7.	*Penicillium funiculosum*
8.	*Penicillium restrictum*
9.	*Pythium* sp.
10.	*Rhizopus nigricans*
11.	*Rhizopus oryzae*
12.	*Sporotrichum* sp.
13.	*Trichoderma viride*

Table 2: Categorization of endophytic fungi of *P. zeylanica* into different Divisions

Sr. No.	Division	Genus
1.	Eumycota	*Pythium*
2.	Zygomycota	*Rhizopus*
3.	Ascomycota	*Aspergillus*, *Curvularia*, *Fusarium*, *Penicillium* and *Trichoderma*
4.	Basidiomycota	*Sporotrichum*

Table 3: Seasonal distribution of endophytic fungi isolated from *P. zeylanica* Linn.

Sr. No.	Endophytic fungi isolated	Summer	Rainy	Winter
1.	*Aspergillus niger*	_	+	+
2.	*Aspergillus ustus*	_	_	+
3.	*Curvularia prasadii*	_	+	_
4.	*Fusarium oxysporum*	_	+	_
5.	*Penicillium citrinum*	_	_	+
6.	*Penicillium chrysogenum*	_	+	+
7.	*Penicillium funiculosum*	_	+	_
8.	*Penicillium restrictum*	_	+	+
9.	*Pythium* sp.	+	+	_
10.	*Rhizopus nigricans*	+	+	+
11.	*Rhizopus oryzae*	+	+	+
12.	*Sporotrichum* sp.	+	_	_
13.	*Trichoderma viride*	_	+	_

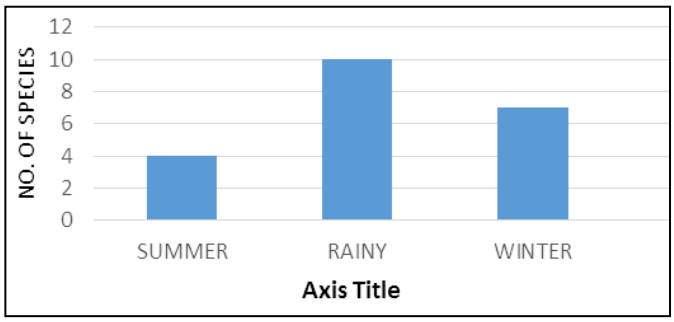

Fig 1: Histogram showing the seasonal distribution of different fungal endophytes isolated from different parts of *Plumbago zeylanica* Linn.

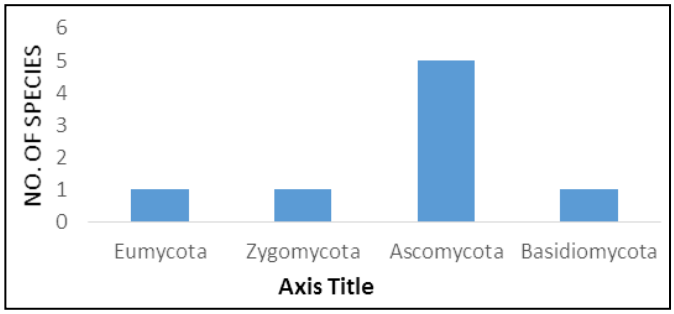

Fig 2: Histogram showing the distribution of different fungal endophytes isolated from different parts of *P. zeylanica* Linn.

Antibacterial screening of root and stem extracts of *P. zeylanica* against *Escherichia coli*, *Staphylococcus aureus* and *Yersinia pestis*

It is evident from Table-4 that three different root extracts (methanol, ethanol and acetone) of *P. zeylanica* showed gradual increase in inhibition of radial growth of three test bacteria at different concentrations. The present study revealed that methanol, acetone and ethanol root extracts of *P. zeylanica* proved themselves as good antibacterial agents. The methanol extract of *P. zeylanica* showed considerable growth inhibition of test bacteria at different concentrations (25%, 50%, 75%, 100%) as compared to acetone and ethanol root extract of the plant. The methanol extract was found to be most effective against *Y. pestis* at (30 mm at 100%) followed by (26.00 mm at 75%), (20.60 mm at 50%), (19.30 mm at 25%).Minimum inhibition of root extract against test bacteria was given against *S. aureus* in ethanol solvent at (15.60 mm at 100%) followed by (13.60 mm at 75%), 13.30 mm at 50%) and (11.00 mm at 25%) as shown in Table-4. Likewise, stem extract in methanol as solvent showed maximum zone of inhibition *Y. pestis* at (25.00 mm at 100%) followed by (19.00 mm at 75%), (17.00 mm at 50%), (14.00 mm at 25%) as shown in Table-4. Ethanol stem extract showed minimum inhibition against *E. coli* at (13.30 mm at 100%) followed by (12.60 mm at 75%), (10.60 mm at 50%) and (8.60 mm at 25%) as shown in Table-4.

It can be viewed from Table-4 that both root and stem extracts of *Plumbago zeylanica* Linn. plant show inhibition against all the three bacteria namely *Yersinia pestis*, *Staphylococcus aureus* and *Escherichia coli* respectively. But root extracts in different solvents show more inhibition against the three bacteria as compared to stem extracts. Zones of inhibition for the three bacteria differ for the stem and root extracts. In case of root, extracts show maximum inhibition against *Yersinia pestis* followed by *Staphylococcus aureus* and least inhibition

against *Escherichia coli.* In contrast, stem extracts show maximum inhibition against *Yersinia pestis* followed by *Escherichia coli* and least inhibition against *Staphylococcus aureus.* It is evident from the results that methanol as well as acetone root and stem extracts of *P. zeylanica* were quite effective in inhibiting the growth of bacteria.

Table 4: Antibacterial screening of different root and stem extracts (methanol, ethanol and acetone) of *Plumbago zeylanica* Linn. against *E. coli*, *S. aureus* and *Y. pestis*

Concentration of extract (in %)	Percent inhibition of growth of test bacteria (mm ± S. E.)					
	Root			Stem		
	E. coli	***S. aureus***	***Y. pestis***	***E. coli***	***S. aureus***	***Y. pestis***
Control	0.000.00	0.000.00	0.000.00	0.000.00	0.000.00	0.000.00
25	12.30±0.18	15.60±0.21	19.30±0.23	13.30±0.13	12.60±0.54	14.00±0.22
50	20.60±0.22	22.00±0.21	20.60±0.06	15.60±0.34	15.50±0.30	17.00±0.43
75	26.30±0.32	26.00±0.19	26.00±0.24	20.00±0.50	18.00±0.28	19.00±0.17
100	28.60±0.08	27.60±0.33	30.00±0.31	22.60±0.18	23.30±0.06	25.00±0.15
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
25	11.30±0.08	11.00±0.40	10.30±0.37	08.60±0.46	09.30±0.50	11.60±0.09
50	12.60±0.27	13.30±0.29	15.60±0.18	10.60±0.23	11.60±0.06	14.00±0.39
75	14.60±0.23	13.60±0.52	19.00±0.06	12.60±0.55	13.60±0.23	16.30±0.53
100	15.60±0.21	15.60±0.30	23.00±0.15	13.30±0.08	15.30±0.27	19.00±0.25
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
25	09.60±0.08	15.30±0.29	13.30±0.32	11.60±0.43	10.00±0.50	14.30±0.06
50	13.30±0.25	19.00±0.08	17.60±0.16	12.60±0.26	11.00±0.18	16.00±0.08
75	14.30±0.06	20.30±0.50	23.00±0.23	15.00±0.15	12.00±0.21	21.30±0.50
100	16.00±0.22	21.60±0.33	26.00±0.08	16.60±0.33	14.00±0.33	22.60±0.13

4. Conclusions

The results from this particular study provide relevant information on the diversity and seasonal influence on the colonization frequencies of endophytic fungi from *Plumbago zeylanica*. A number of fungal endophytic species were found in different parts of this plant. Maximum number of endophytic species were recorded in rainy season. It was also concluded from the above experimental observations that the plant *P. zeylanica* showed satisfactory antibacterial activity at different concentrations in three different solvents. The best solvent for extraction was methanol and maximum antibacterial activity was shown by methanol root extract against *Y. pestis* followed by *S. aureus* and *E. coli* respectively. Least antibacterial activity was shown by ethanol stem extract of *Plumbago zeylanica* Linn. against the test bacteria.

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6. References

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