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STUDIES ON ENDOPHYTES AND ANTIBACTERIAL ACTIVITY OF SAUSSUREA COSTUS (FALC.) LIPSCH

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ABSTRACT

Different plant parts (root, stem, leaf) of *Saussurea costus* were used to isolate and investigate endophytic fungal species in summer, rainy and winter seasons. Total ten species of endophytic fungi belonging to seven genera (*Aspergillus*, *Cunninghamella*, *Myrothecium*, *Penicillium*, *Pythium*, *Rhizopus* and *Trichoderma*) were isolated from the root, stem and leaf in different seasons. The genus *Aspergillus* was found to be dominant with three species (*A. nustus*, *A. wentii* and *A. niger*). The genus *Rhizopus* was represented by two species (*R. oryzae* and *R. nigricans*). The genera *Cunninghamella*, *Myrothecium*, *Penicillium*, *Trichoderma* and *Pythium* were represented by one species each i.e. *Cunninghamella elegans*, *Myrothecium roridum*, *Penicillium chrysogenum*, *Trichoderma viride* and *Pythium* sp. respectively. Antibacterial activity of root of *S. costus* has been investigated using different solvents (methanol, ethanol and acetone) at different concentrations (25%, 50%, 75% and 100%) against three test bacteria namely *S. aureus*, *E. coli* and *Y. pestis*. Root extract of *S. costus* showed greater antibacterial activity using methanol as solvent followed by acetone and ethanol solvent. In case of methanol extract, maximum inhibition activity was shown against *S. aureus* and minimum against *E. coli*. Maximum inhibition was shown against *Y. pestis* and minimum against *E. coli* in case of acetone extract whereas ethanol extract exhibited highest inhibitory activity against *E. coli* and lowest against *Y. pestis*. Therefore, it is evident that *S. costus* exhibited antibacterial activity against all the test bacteria in all the solvents used in this study thereby conforming it as a good antibacterial agent for future study.

Keywords: *Saussurea costus*, endophyte isolation, antibacterial activity, agar well diffusion

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INTRODUCTION

Fungi are achlorophylous eukaryotic organisms which grow as single cell (yeasts) or as multicellular filaments (moulds/fungi), obtaining nutrition by absorption from their surroundings. Fungi play important role in the daily life of human beings such as in agriculture, food industry, medicine, textiles, bioremediation, natural cycling, as bio-fertilizer and in many other ways. Fungi are omnipresent on Earth, and represent essential components of many ecosystems where they are involved in many vital processes[1].

Endophytic fungi are those which spend whole or part of their life-cycle colonizing inter-cellular or intra-cellular inside the healthy tissues of the host plants, typically causing no visible symptoms of disease. They play significant role and constitute a significant component of plant micro-ecosystems[2]. Endophytes occur in a wide variety of plant parts such as roots, stems, leaves, tubers, buds, ovules, seeds, fruits, xylem and bark. Among the host plants, medicinal herbs are one of the important groups of hosts for endophytic fungi[3,4]. Endophytes normally found in above ground plant parts, but also occasionally found in roots.

Endophytes benefit the plant by increasing nutrient absorption, tolerance against stress, help in seed germination and also phyto-remediation of environmental pollutants[5].

Plants contain many biologically active compounds, many of which show antimicrobial properties[6]. Plants are used as traditional healthcare in most parts of the world for thousands of years and there is increasing interest in plants as source of medicinal agents to fight microbial and fungal diseases[7]. Use of plant and its products has a long history. Plants produce a wide range of bioactive molecules, making them rich source of different types of medicine[8]. Secondary metabolites in plants include alkaloids, flavonoids, steroids, resins, fatty acids, tannins and phenolic compounds etc. Compounds extracted from different parts of the plants can be used in treatment of diarrhoea, dysentery, cough, cold, fever, bronchitis, cholera etc[9].

Herbal medicines are more accessible to most of the population. About 60 to 85% of the population of every country of the developing world is dependent on herbal or native forms of medicine. The Himalayas have a great wealth of medicinal plants and traditional medicinal knowledge. Medicinal plants have played very important role in primary health care system among the local people of Himalayan region. *Saussurea costus* (Falc.) Lipsch. (Syn: *Saussurea lappa* C.B. Clarke family Asteraceae; Vern. Kuth) is commercially produced and well known medicinal plant of Indian Himalayan region which grows at an altitude of 2600 to 4000 m[10].

This plant is endemic in India in the sub-alpine regions of Jammu and Kashmir, Himachal Pradesh and Uttaranchal, from an altitude of 3200-3800 m. The wild availability of this important plant is decreasing day by day due to over-exploitation for different medicinal and commercial purposes. This critically endangered medicinal plant species is enlisted in Convention on International Trade in Endangered Species of Fauna and Flora (CITES) and is one of the 37 Himalayan endangered medicinal plants that have been listed for *in situ* and *ex situ* conservation[11].

Saussurea costus (roots and root oil) has become an important drug in the international market. In India, *S. lappa* grows naturally in Jammu and Kashmir while in Uttarakhand and Himachal Pradesh, the species is being cultivated since 1920. Traditionally, *S. lappa* roots are used in the treatment of asthma, arthritis, chronic gastritis, dysentery, diarrhea, chronic skin diseases, fever, headache, abdominal pain and bronchitis[12]. Hence present study was undertaken on endophytes and anti bacterial activity of *S. costus*.

MATERIALS AND METHODS

Plant material

Keylong Headquarter (Lahaul and Spiti) of Himachal Pradesh was selected for the collection of study material named *Saussurea costus* (Falc.). The collection was made during summer, rainy and winter seasons. The material used for present study was root, stem, and leaf of *Saussurea costus.*

Methodology for endophytic fungal isolation

(a) Hot water treatment

The samples from the root, stem and leaf were taken and were washed in water at 60^{o}C for fifteen minutes. Each sample was cut in three pieces and these were inoculated in separate Petri plates each containing PDA (Potato Dextrose Agar) medium which was supplemented with streptomycin (150 mg/l). These Petri plates were incubated at 25±2^{o}C 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 minutes, followed by 50% methanol for 3 minutes and after that 75% methanol was used for 2 minutes. 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. Petri plates were incubated at 25±2^{o}C for few days. The fungal colony growing in the petriplates were then transferred on PDA slants for sub-culturing.

Maintenance and Preservation of Culture

Maintenance of pure culture of different fungal genera was then done on PDA which was preserved in refrigerator. Sub-culturing was done at regular intervals in order to maintain cultures. Transfer of each fungal species from parent source to a fresh slant was done to maintain the pure cultures.

Methodology for antibacterial screening

Maintenance and preservation of pure culture of bacteria

Preservation of pure culture of all bacteria was done on nutrient broth and was kept in refrigerator. Sub-culturing was done at regular intervals.

Preparation of root extract of *Saussurea costus*

Fresh roots of *S. costus* were washed thoroughly for 4-5 times with tap water to remove dust and other foreign material from surface and then with 2% mercuric chloride. Then these were washed in distilled water for 2-3 times. Decantation of water from the material was then done. In order to dry out the material, it was kept in between the folds of filter paper. Plant material was weighed 50 g, chopped into small pieces and with known volume of distilled water (1:1 w/v); fine slurry of plant was prepared using sterile mortar and pestle at room temperature. To get the clear solution, it was then filtered with Whatman No. 1 filter paper. It was considered as 100% concentration.

Methodology for antibacterial screening with root extracts of *Saussurea costus*

Antibacterial screening was done using Agar well diffusion method. Nutrient Agar Medium (Beef extract

1g, Yeast extract 2g, Sodium chloride 1g, Peptone 5g, Agar 20g and distilled water 1 Lt.) was used throughout the investigation for the growth of microorganisms. The medium was autoclaved at 121.6°C for 30 minutes. 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 8 mm diameter were prepared. Five wells were prepared in agar plates. The wells in each plate were loaded with 25%, 50%, 75%, 100% concentration with central well for control. The plates containing bacterial colonies were incubated at 37°C for 24 hours (*S. aureus*, *E. coli* and *Y. pestis*) in an incubation chamber. All the tests were repeated in triplicates. Diameter of bacterial colonies of treatment and control sets was 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[13].

Percentage (%) of growth inhibition = Control – Test/Control ×100[14]

RESULTS

Ten species of endophytic fungi belonging to seven genera (*Aspergillus*, *Cunninghamella*, *Myrothecium*, *Penicillium*, *Pythium*, *Rhizopus* and *Trichoderma*) were isolated from root, stem and leaf of *Saussurea costus* during summer, rainy and winter seasons. The genus *Aspergillus* was represented by three species (*A. nustus*, *A. wentii* and *A. niger*). The genus *Rhizopus* was represented by two species i.e. (*R. oryzae* and *R. nigricans*). The genera *Cunninghamella Myrothecium*, *Penicillium*, *Trichoderma* and *Pythium* were represented by one species each i.e. *Cunninghamella elegans*, *Myrothecium roridum*, *Penicillium chrysogenum*, *Trichoderma viride* and *Pythium* sp. respectively (Table 1). Out of identified genera, four belonged to division Ascomycota, two belonged to Zygomycota and one belonged to Oomycota (Table 2). Further, maximum numbers of endophytic fungi were observed during rainy season followed by winter and summer (Table 3).

Table 1: List of endophytic fungi isolated from leaves, stem and root of *Saussurea costus*

Sr. No.	Endophytic fungi isolated
1.	*Aspergillus ustus*
2.	*Aspergillus niger*
3.	*Aspergillus wentii*
4.	*Cunninghamella elegans*
5.	*Myrothecium roridum*
6.	*Pythium* sp.
7.	*Penicillium chrysogenum*
8.	*Rhizopus oryzae*
9.	*Rhizopus nigricans*
10.	*Trichoderma viride*

Table 2: Categorization of endophytic fungi from *Saussurea costus* into different divisions

S. N.	Division	Genus
1.	Ascomycota	*Aspergillus, Penicillum, Myrothecium, Trichoderma*
2.	Zygomycota	*Rhizopus, Cunninghamella*
3.	Oomycota	*Pythium*

Table 3: Seasonal distribution of endophytic fungi isolated from *Saussurea costus*

Sr.No.	Endophytic fungi isolated	Summer	Rainy	Winter
1.	*Aspergillus ustus*	_	+	_
2.	*Aspergillus niger*	+	+	+
3.	*Aspergillus wentii*	-	+	+
4.	*Cunninghamella elegans*	_	_	+
5.	*Myrothecium roridum*	+	_	_
6.	*Pythium* sp.	_	+	+
7.	*Penicillium chrysogenum*	+	_	_
8.	*Rhizopus oryzae*	_	+	_
9.	*Rhizopus nigricans*	+	+	+
10.	*Trichoderma viride*	_	+	_

+ Present; - Absent

Table 4: Antibacterial screening of methanol root extract of *Saussurea costus* against *S. aureus, E. coli* and *Y. pestis*

Concentration (In %)	Percent inhibition of growth of test bacteria (mm±S.E.)		
	S.aureus	***E.coli***	***Y.pestis***
25	15.30± 0.088	14.33±0.088	14.66±0.021
50	18.00±0.057	17.00±0.057	16.33±0.088
75	21.33±0.046	20.33±0.021	19.00±0.033
100	25.33±0.033	22.00±0.033	23.66±0.046

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

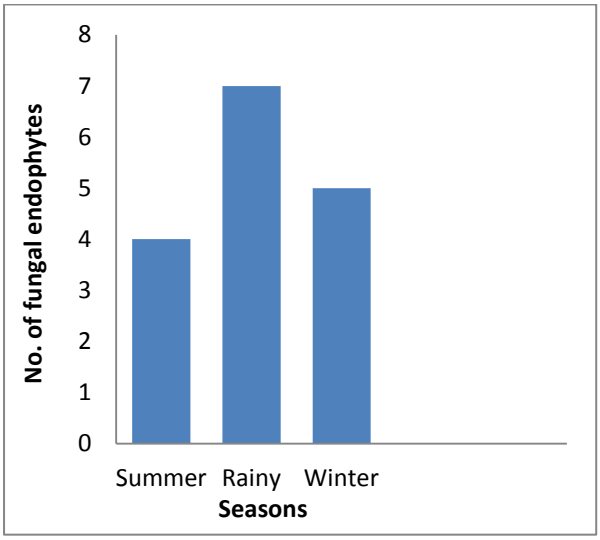

Figure 1: Histogram showing the seasonal distribution of different fungal endophytes isolated from different parts of *Saussurea costus*

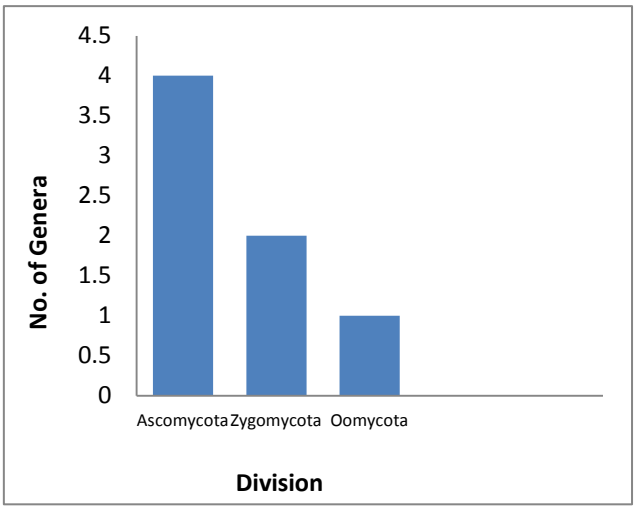

Figure 2: Histogram showing the distribution of different fungal endophytes isolated from different parts of *S. costus* into divisions

Table 4 showing data pertaining to antimicrobial activity of methanol root extract of *Saussurea costus* at different concentrations. At 25% concentration, zone of inhibition recorded was 15.30 mm, 14.33 mm and 14.66 mm for *S. aureus*, *E. coli* and *Y. pestis* respectively. At 50% concentration, zone of inhibition recorded was 18.00 mm, 17.00 mm and 16.33 mm. At 75% concentration, zone of inhibition recorded was 21.33 mm, 20.33 mm and 19.00 mm. Maximum zone of inhibition was recorded at 100% concentration that was 25.33 mm, 22.00 mm and 23.66 mm respectively.

Table 5 showing data pertaining to antibacterial activity of ethanol root extract of *Saussurea costus* for *S.aureus*, *E.coli* and *Y.pestis* at different concentrations. At 25% concentration, zone of inhibition recorded were 13.66 mm, 12.33 mm and 11.00 mm for *S. aureus*, *E. coli* and *Y. pestis* respectively. At 50% concentration, 15.66 mm, 14.66 mm and 12.33 mm zone of inhibition were recorded. At 75% concentration, zone of inhibition recorded was17.00 mm, 17.66 mm and 15. 33 mm. Maximum zone of inhibition was recorded at 100% concentration that was 19.00 mm, 19.66 mm and 17.66 mm respectively.

Table 5: Antibacterial screening of ethanol root extract of *S. costus* against *S. aureus, E. coli* and *Y. pestis*

Concentration (In %)	Percent inhibition of zone of test bacteria (mm±S.E)		
	S.aureus	***E.coli***	***Y.pestis***
25	13.66± 0.088	12.33± 0.033	11.00± 0.021
50	15.66± 0.057	14.66± 0.021	12.33± 0.088
75	17.00± 0.033	17.66± 0.088	15.33± 0.057
100	19.00± 0.046	19.66± 0.057	17.66± 0.033

Each data point represent mean of three replicates ± S.E.

Table 6: Antibacterial screening of acetone root extract of *S. costus* against *S. aureus, E. coli* and *Y. pestis*

Concentration (In %)	Percent inhibition of growth of test bacteria (mm±S.E.)		
	S.aureus	***E.coli***	***Y.pestis***
25	13.66± 0.033	12.33±0.021	13.33± 0.088
50	15.33± 0.057	14.33± 0.088	16.66± 0.057
75	18.66± 0.088	17.66± 0.033	18.66± 0.021
100	22.33± 0.046	21.00± 0.046	20.33± 0.046

Each data point represent mean of three replicates ± S.E.

Table 6 showing data pertaining to antibacterial activity of acetone root extract of *Saussurea costus* for *S. aureus*, *E. coli* and *Y. pestis* at different concentrations. At 25% concentration, zone of inhibition recorded was 13.66 mm, 12.33 mm and 13.33 mm for *S. aureus*, *E. coli* and *Y. pestis* respectively. At 50% concentration, 15.33 mm, 14. 33 mm and 16.66 mm zone of inhibition was recorded. At 75% concentration, 18.66 mm, 17.66 mm and 18.66 mm zone of inhibition was recorded. Maximum zone of inhibition was recorded at 100% concentration that was 22.33 mm, 21.00 mm and 20.33 mm respectively.

DISCUSSION

Saussurea costus is an important medicinal plant of Himalayan region which helps in treating various diseases. Present investigation was carried out on *S. costus* with the objective of isolating and identifying its fungal endophytic species and antibacterial activity of root extract of this plant against three pathogenic bacteria (*E. coli, S. aureus* and *Y. pestis*)

1) Endophytic fungi isolated from *Saussurea costus*

In the present investigation, ten species of endophytic fungi belonging to seven genera (*Aspergillus*, *Cunninghamella*, *Myrothecium*, *Penicillium*, *Pythium*, *Rhizopus* and *Trichoderma*) were isolated from root, stem and leaf of *Saussurea costus* during summer, rainy and winter seasons. This could be attributed to the reason that environmental conditions greatly influence the occurrence of fungi in the plant[15]. Isolation of endophytes from various plant parts showed greater numbers of endophytes during rainy than winter and summer seasons.

Various workers have reported similar fungal endophytes from different plants. Sagar and Chauhan[16] reported five fungal endophytic species belonging to four genera viz. *Penicillium, Rhizopus, Gliocladium* and *Trichoderma* from leaves, bark and root of *Quercus leucotrichophora.* Sagar and Kaur[17] isolated four species of endophytic fungi (*Aspergillus flavus, A.niger, Cephalosporium Acremonium, Gliocladium fimbriatum and Myrothecium sp.*) from roots, bark and leaves samples of *Aesculus indicia.* Tejesvi *et al*[18] investigated fungal endophytes from inner bark segments of some medicinal tree species viz *Holarrhena antidysenterica*, *Butea monosperm* and *Crataeva magna, Azadirachta indica*, and *Terminalia chebula*. *Fusarium, Verticillium, Trichoderma, Chaetomium, Myrothecium* and *Pestalotiopsis* species were identified from these tree species. Bzerra *et al*[19] worked on Cactus (*Cereus jamacaru*) and identified 59 fungal species, of which 30.3% were sterile mycelia. Fungal species isolated were *Cladosporium cladopsporioides, Aspergillus flavus, Trichoderma viride* and *Fusarium oxisporum.* There is no previous report variable regarding endophyte isolation of *S. costus*.

2) Antibacterial screening of root extract of *Saussurea costus*

Result of antibacterial activity revealed that methanol extract showed greater zone of inhibition followed by acetone than ethanol. It has been found that with increase in concentration of root extract, zone of inhibition also increases. In case of methanol root extract maximum zone of inhibition recorded was 25.33 mm against *S.aureus* and minimum zone of inhibition was 22.00 mm against *E. coli.* In case of acetone root extract maximum zone of inhibition recorded was 22.33 mm against *S. aureus* and minimum zone of inhibition was 20.33 mm against *Y. pestis*. In case of ethanol root extract maximum zone of inhibition recorded was 19.66 mm against *E. coli* and minimum zone of inhibition was 17.66 mm against *Y. pestis*.

Alaagib and Ayoub[20] investigated antibacterial activity of root of *Saussurea lappa* using petroleum ether, chloroform, methanol and water extracts as solvents. It was revealed that chloroform extract showed the highest antibacterial activity. Chang *et al*[21] also investigated ethanol extracts of *S. lappa* with various solvents (*n*-hexane, chloroform, and *n*-butanol). The antimicrobial activity of *S. lappa* was examined against six food-borne pathogens (*L. monocytogenes*, *B. cereus*, *B. subtilis*, *S. aureus*, *S. choleraesuis* and *V. parahaemolyticus*) and also compared to that of the synthetic antibiotics. It is found that the *S. lappa* ethanol extract and *n*-hexane fraction have strong activity against *B. cereus* and *V. parahaemolyticus* strains compared to ampicillin against food-borne pathogens. Even though, present investigation are of preliminary type, yet they have established a base for further extension of this work towards isolation and purification of bioactive compounds present in this medicinal plant.

CONCLUSIONS

The results obtained in the present investigation indicated that fungal endophytic species were found in different plant parts of *Saussurea costus.* Number of endophytic species varies with different seasons and maximum number was investigated in rainy Seasons. Ten species of endophytic fungi belonging to seven genera (*Aspergillus, Rhizopus, Myrothecium, Penicillium, Pythium* and *Trichoderma*) were isolated. The genus *Aspergillus* was dominant with three species. Antibacterial activity of root extract of *S. costus* showed greater activity using methanol as solvent followed by acetone and ethanol.

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CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest regarding the manuscript and experimentation done.

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