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PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTES OF EMBELIA TSJERIAM COTTAM LINN

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ABSTRACT

Four different fungal endophytes (Cladosporium cladosporiodes, Penicillium sp., Aspergillus niger and Alternaria sp.) were isolated from buds and leaf of Embelia tsjeriam cottam. Three different solvents were used to analyze the phytochemicals in all the endophytes. The Cladosporium cladosporiodes yielded flavonoids (in ethyl acetate), phenols, tannins and cardiac glycosides (in petroleum ether and hexane). Saponins and tannins (in ethyl acetate), phenols (in hexane) and cardiac glycosides present in Penicillium sp. Aspergillus niger showed the presence of saponins (in ethyl acetate), phenols and cardiac glycosides (in all solvents). Alternaria sp yielded the flavonoids (in petroleum ether), phenols (in hexane) and cardiac glycosides (in hexane and ethyl acetate). In antibacterial activity, the Aspergillus niger(ethyl acetae extract) inhibited the Pseudomonas aeuroginosa, Bacillus subtilis and Shigella flexneri strongly whereas hexane also inhibited the growth of Klebseilla pneumoniae and Bacillus subtilis. Ethyl acetate extract of Alternaria sp controlled the growth of Klebseilla pneumoniae and Bacillus subtilis and ethyl acetate extract of Penicillium sp inhibited the growth of Klebseilla pneumoniae and Bacillus subtilis. The activity may be due to the presence of phytochemicals (phenols, flavonoids, tannins or cardiac glycosides). Cladosporium cladosporiodes have not evaluated antibacterial activity against tested bacteria.

KEY WORDS

Embelia tsjeriam cottam,endophytes , phytochemicals, antibacterial activity

Bioactive natural compounds produced by endophytes have been promising potential usefulness in safety and human health concerns, although there is still a significant demand of drug industry for synthetic products due to economic and time-consuming reasons (Strobel *et al*., 2004). Medicinal plants are reported to harbour endophytes, which inturn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions. It is therefore important to determine the entophyte diversity of medicinal plants (Strobel *et al*., 2002). *Embelia tsjeriam cottam* Linn. (Myrsinaceae) commonly known as *Vidanga, Ambati* (common name) is a climbing shrub distributed in the mountains of the Western Ghats of Karnataka, Kerala and Malabar. It has considerable reputation as a potent medicament in the treatment of various ailments such as antifertility, antioestrogenic, and anthelminitic. One of its components, embelin, is reported to possess anti-inflammatory activity (Vite *et al.,* 2011).

In the present investigation was aimed to identify and isolate different fungal endophytes from different parts of *Embelia tsjeriam cottam,* their

phytochemical analysis and antibacterial activity. The literature survey indicates that, no reports are available on identification, isolation, phytochemical and antibacterial activity of *Embelia tsjeriam cottam* fungal endophytes.

MATERIALS AND METHODS

Collection of plant material

The Plant material was collected from the surroundings of Alur, near Shakaleshapura, Hassan district of Karnataka, India during the month of January 2012. The plant was identified by their vernacular names and later it was compared with the herbarium of the Department of Studies in Botany, Manasa Gangothri, University of Mysore, Mysore and Government Ayurvedic College, Mysore, India.

Isolation of endophytes

The method used for isolation of other endophytes was followed (Rungjindamai *et al.,* 2008; Oses *et al.,* 2008; Theantana *et al.,* 2009) for isolation of endophytes from *Embelia tsjeriam cottam* with slight modifications. The plant tissues were washed in running tap water for one hour. Twenty five segments of leaves from plant were cut into 5 mm pieces. Endophytic fungi were isolated from the leaf of the plant (25 segments) and 10 segments (2mm diameter) of the buds. The total 35 segments of plant material were treated by triple surface sterilization techniques (Bussaban *et al.,* 2001). Each piece was then placed on malt extract agar (malt extract 20 gm. L^{-}), Rose Bengal (0.033 gm. L^{-}), chloramphenicol (50 mg. L^{-}; agar 15 gm. L^{-}). All plates were incubated at 26±2°C until mycelium grew out; hyphal tips were cut and transferred to Potato Dextrose Agar (PDA). Half strength PDA was used for subculture and stock culture. Identification was based on colony, hyphal morphology of the fungal cultures and characteristics of the spores (Sadananda *et al.,* 2011).

Mass culturing of endophytes

The isolated endophytes were mass cultured on a Potato Dextrose Broth (PDB) and incubated at 26± 2°C for 1 week for the development of fungal mycelia mats and then the cultures were taken out and filtered through sterile cheesecloth to remove the mycelia mats and grinded. The fungal metabolites from different endophytic mycelia mats were extracted by using solvents such as hexane, ethyl acetate and petroleum ether and the sample was filtered using Whatman filter paper No 2. Equal volume of the filtrate and solvents were taken in a separating funnel and was shaken vigorously for 10 min. The solution was then allowed to stand, the cell mass got separated and the solvent so obtained, was collected. All solvents were evaporated and the resultant was dried in vacuum evaporator using $MgSO_4$ to yield the crude extract (Sadananda *et al.*, 2011).

Phytochemical screening

Phytochemical analysis is intended to serve as a major resource for information on analytical and instrumental methodology in the plant sciences. This determines the presence of bioactive compounds in extracts of endophytes. Phytochemical analysis was carried out according to Bandoni *et al* (Sadananda *et al.*, 2011) with slight modifications.

Test for saponins

1 ml aliquots of the various endophytic extracts were combined with 5 ml water which is at 60°C, then, shaken for 2 min, as saponins are known to possess frothing activity, the volume of froth produced in this experiment was observed and recorded every 10 min for a period of 30 min (Sadananda *et al.*, 2011).

Test for phenolic compounds

1 ml of test solution was treated with 10% ethanolic ferric chloride. Phenolic compounds were considered present when a colour changes to blue green (Sadananda *et al.*, 2011).

Test for anthraquinones

The Borntrager test was used for the detection of anthraquinones. 2 ml of the test sample was shaken with 4 ml of hexane. The upper lypophilic layer was separated and treated with 4 ml of dilute ammonia. If the lower layer changed to violet pink, it indicates the presence of anthraquinones (Sadananda *et al.*, 2011).

Test for steroids

1 ml of the respective endophytic extract was treated with three drops of acetic anhydride and one drop of concentrated sulphuric acid. A colour change from deep green, turning to brown indicated the presence of sterols (Sadananda *et al.*, 2011).

Cardiac glycosides

1 ml of sample solution was mixed with 1 ml of glacial acetic acid then treated with one drop of 5% ethanolic ferric chloride solution. 1ml of concentrated sulphuric acid was carefully poured down the sides of test tube. The appearance of a brownish ring between the two layers with lower acidic layer turning blue green upon standing indicates the presence of cardiac glycosides (Sadananda *et al.*, 2011)

Antibacterial activity

The antibacterial activity of fungal (endophytic) extracts was done by using agar well diffusion method (Ganjewala *et al.,* 2010).

Growth and maintenance of test microorganism for antibacterial activity

Bacterial cultures of *Pseudomonos aeuroginosa (*MTCC1034*)*, *Klebseilla pneumoniae (*MTCC39*),* *Shigella flexneri (*MTCC1457*)* and *Bacillus subtilis (*MTCC441*)*were obtained authenticated cultures from Institute of Microbial Technology (IMTECH) Chandigarh. The bacteria were maintained on nutrient broth (NB) at 37^{0}C (Mahesh *et al.,* 2008).

Agar well diffusion method

The strains that had been incubated for 24 h were used for the assay. A sterile cotton swab was dipped into the bacterial suspension and then evenly streaked over the entire surface of a sterile Mueller Hinton agar plate to obtain a uniform inoculum. Wells were made on the seeded plates using a 1000 µl sterile micro tip (8 mm) and the plates and allowed to dry for 5 min. The endophytes extracts (20 µl) were dispensed into each well using a sterile micropipette. Dimethyl sulfoxide was used as a negative control and ampicillin (10 µl) was used as a positive control. The plates were incubated overnight at 37°C and the antibacterial activity was determined by measuring the diameter of zone of inhibition (mm) with slight modification (Mahesh *et al.,* 2008).

RESULTS

Isolation of endophytes

The buds and leaf of the *Embelia tsjeriam cottam* were subjected to isolate the endophytes and the results are presented in **Table 1**.

Phytochemical analysis

We have used three different solvents to analyze the phytochemicals from different fungal endophytes. The entophytes were subjected to phytochemical analysis to check the presence of phytochemicals and the results are presented in **Table 2**.

Antibacterial activity

Size of zone of inhibition against bacterial strains *Klebsiella pneumonia, Pseudomonas aeuroginosa, Bacillus subtilis* and *Shigella flexneri* was determined by measuring the clear zone surrounding the well made on the nutrient agar and results are presented in **Table 3 and Table 4**.

Table 1. Incidence of endophytes in *Embelia tsjeriam cottam*

Endophytes	Parts	
	Bud	Leaf
Cladosporium cladosporioides	+	-

Penicillium sp	+	-
Aspergillus niger	-	+
Alternaria sp	-	+

*+ = Present *- = Absent, Repeated the each experiment thrice.

Table 2. Phytochemical analysis of *Embelia tsjerium cottam*

Phytochemicals	**Endophytes**											
	Cladosporium cladosporioides			***Penicillium sp***			***Aspergillus niger***			***Alternaria sp***		
	PE	HE	EA	PE	HE	EA	PE	HE	EA	PE	HE	EA
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	-	-	++	-	-	-	-	-	+	+	-	-
Saponins	-	-	-	-	-	+	-	-	-	-	-	-
Phenols	+	+	++	-	++	-	+	+	+	-	+	-
Tannins	+	+	+	-	-	-	-	-	+	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	+	+	-	++	++	+	+	++	++	-	+	++
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids	-	-	-	-	-	-	-	-	-	-	-	-
Cardenolides	-	-	-	-	-	-	-	-	-	-	-	-

*PE= Petroleum Ether, HE= Hexane, EA= Ethyl Acetate

*++ = More presence, + = Moderate presence, - = Not present, Repeated the each experiment thrice.

Table 3. Antibacterial activity of *ETC* leaf (*Aspergillus niger*)

Bacterial strains	**Inhibition zone (mm)**			
	Klebsiella pneumoniae	***Pseudomonas aeuroginosa***	***Bacillus subtilis***	***Shigella flexneri***
Dimethyl sulfoxide (DMSO)	-	-	-	-
Ampicillin	16	-	20	24
Ethyl acetate extract	-	11	10	13
Hexane extract	10	-	13	-
Petroleum ether extract	-	-	-	-

Repeated the each experiment thrice.

Table 4. Antibacterial activity of *ETC* leaf (*Alternaria sp*)

Bacterial strains →	**Inhibition zone (mm)**			
	Klebsiella pneumoniae	***Pseudomonas aeuroginosa***	***Bacillus subtilis***	***Shigella flexneri***
Dimethyl sulfoxide (DMSO)	-	-	-	-
Ampicillin	-	-	12	-
Ethyl acetate extract	8	-	8	-
Hexane extract	-	-	-	-
Petroleum ether extract	-	-	-	-

Repeated the each experiment thrice.

Table 5: Antibacterial activity of *ETC* bud (*Pencillium sp*)

Bacterial strains →	**Inhibition zone (mm)**			
	Klebsilla pneumoniae	***Pseudomonas aeuroginosa***	***Bacillus subtilis***	***Shigella flexneri***

Dimethyl sulfoxide (DMSO)	-	-	-	-
Ampicillin	-	-	12	-
Ethyl acetate extract	8	-	8	-
Hexane extract	-	-	-	-
Petroleum ether extract	-	-	-	-

DISCUSSION

Totally four different fungal endophytes were isolated from buds and leaf of *Embelia tsjeriam cottam*. The buds have shown the presence of *Cladosporium cladosporoides* and *Penicillium* sp. whereas the leaf part has yielded *Aspergillus niger* and *Alternaria* sp.

The presence of phytochemicals in endophytes is an indicator that they can be potential source of precursors in the development of synthetic drugs (Sadananda *et al.*, 2011).

Cardiac glycoside, flavonoids and phenols are present in high concentrations, tannins, terpenoids, cardenolids and saponins are present whereas alkaloids, anthraquinones and phlobatannins are totally absent. Petroleum ether, hexane and ethyl acetate extracts of *Cladosporium cladosporioides* isolated from buds of *Embelia tsjeriam cottam* have revealed the presence of phenols and tanins. Petroleum ether and hexane extracts of *Cladosporium cladosporioides* isolated from buds also have shown the presence of cardiac glycosides and presence of flavonoids in ethyl acetate extract. Alkaloids, saponins, anthroquinones, phlobatannins, terpenoids and cardenolides were not present in petroleum ether, hexane and ethyl acetate extracts of *Cladosporium cladosporioides*. Cardiac glycosides were present in petroleum ether, hexane and ethyl acetate extracts of *Penicillium sp* isolated from buds of *Embelia tsjeriam cottam*. Ethyl acetate extract of *Penicillium sp* have shown the presence of saponins. Phenols were also present in the hexane extract of *Penicillium sp* and petroleum ether, hexane and ethyl acetate have not revealed the presence of alkaloids, flavonoids, tannins, anthraquinones, phlobatannins, terpenoids and cardenolides in *Penicillium sp* isolated from buds of *Embelia tsjeriam cottam.*

Petroleum ether, hexane and ethyl acetate extracts of *Aspergillus niger* isolated from leaf of *Embelia tsjeriam cottam* has been revealed the presence of cardiac glycosides and phenols. Flavonoids and tanins were also present in the extracts of ethyl acetate. Extracts of petroleum ether, hexane and ethyl acetate have not shown the presence of alkaloids, saponins, anthraquinones, phlobatannins, Terpenoids and cardenolides in *Aspergillus niger.* Cardiac glycosides were present in hexane and ethyl acetate extract and also present flavonoids, cardenolides and phenols in the extracts of petroleum ether and hexane respectively of *Alternaria sp*. Alkaloids, saponins, tannins, anthraquinones, phlobatannins and terpenoids were not present in petroleum ether, hexane and ethyl acetate extracts of *Alternaria sp* isolated from *Embelia tsjeriam cottam*.

Ethyl acetate extract of *Aspergillus niger* have shown maximum inhibition zone against *Shigella flexneri* followed by *Pseudomonas aeuroginosa* and *Bacillus subtilis* whereas no inhibition zone has been showed against *Klebseilla pneumonia.* Hexane extract of *Aspergillus niger* isolated from leaf of *Embelia tsjeriam cottam* have shown maximum inhibition zone against *Bacillus subtilis* followed by *Klebseilla pneumonia* whereas no activity against *Pseudomonas aeuroginosa* and *Shigella flexneri*. Petroleum extracts of *Aspergillus niger* have not shown inhibition zone against all the bacterial starins. It was found that the ethyl acetate extract of *Alternaria* sp isolated from leaf of *Embelia tsjeriam cottam* have shown

maximum inhibition zone against *Klebsiella pneumonia* and *Bacillus subtilis* whereas other extracts of *Alternaria* sp have not shown any inhibition zone against *Pseudomonas aeuroginosa* and *Shigella flexneri.*

The size of zone of inhibition of extracts of *Pencillium sp* against bacterial strains *Klebsiella pneumonia, Pseudomonas aeuroginosa, Bacillus subtilis* and *Shigella flexneri* are presented in table 5. The ethyl acetate extract of *Pencillium* sp have shown least inhibition zone against *Klebsiella pneumonia* and *Bacillus subtilis* whereas petroleum ether, hexane extracts have not any inhibition zone against all the bacterial strains mentioned. Endophytic microorganisms are excellent sources of bioactive natural products that can be used to satisfy demand of Pharmaceutical and Medical Industries (Baby *et al.,* 2011). A large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes like alkaloids, peptides, sterols, terpenoids, phenols, quinines and flavonoids (Cushnie *et al.,* 2005). The four different fungal endophytes have exhibited the presence of flavonoids, phenols, tannins and cardiac glycosides at different concentration. The antibacterial activity is due to potent activity present phytochemicals in endophytes. Our results are confirmed with the finding of Cushine and Lamb (2005), Saravanakumar et al. (2009) (Saravanakumar *et al.,* 2009, Doss *et al.,* 2009 and Maneemegalai *et al.,* 2010). This is the preliminary and first attempt was taken up to add more work in plant. Further work needs to fullfil the gaps in the plants and endophytes and these endophytes can be exploiting to use phytochemicals for treatment of various diseases.

CONCLUSION

The antibacterial activity of the endophytes isolated from *Embelia tsjeriam cottam* can be due to the presence of various phytochemicals. The endophytes exhibited antibacterial activity against four bacteria and therefore these can be considered as a source of antibacterial agents and for further analysis in future.

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