

Antimicrobial Evaluation of Some Fungal Endophytes Isolated from the Bark of Himalayan Yew

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Abstract: Endophytic fungi are potent source of important bioactive compounds like taxol, cryptocin, cryptocandin, munumbicin and ambuic acid. *Taxus baccata* (Himalayan yew), an evergreen tree of temperate forests of Arunachal Pradesh, is an important medicinal plant because its leaves and bark yield Taxol- a proven anticancer compound. During the present investigation we have isolated 33 fungal endophytes from the bark of *Taxus baccata* collected from different areas of Arunachal Pradesh. Twenty-three isolates were evaluated for antimicrobial activities. Crude extracts of these endophytic fungal strains were tested against *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoniae* and *C. albican* using agar cup diffusion techniques. Crude extracts of five endophytic fungal isolates effectively inhibited the growth of all the test organisms. Highest Antimicrobial activity was observed in the fungal isolate no. DEF3 which inhibited the growth of all the tested organisms followed by DEF9 > DEF6 > DEF1 > TEF7. The Strain DEF3 was most effective against *S. aureus* (zone of inhibition 24 mm) and least against *E. coli* (zone of inhibition 17 mm). The results indicated great potential of these strains in pharmaceutical and/or agricultural industries.

Key words: Himalayan yew • Arunachal Pradesh • fungal endophytes • antimicrobial evaluation

INTRODUCTION

Fungal endophytes live internally and asymptotically within the plant tissues. They are the untapped microbial resources that are likely to have several economically important applications in the future. In recent years these fungi have gained importance because of their ability to produce good number of bioactive molecules [1-5]. Perhaps the greatest discovery was the isolation of Taxol producing endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia* [6]. Taxol, a diterpenoid, is an important medicine for treatment of variety of cancer cells [1]. The discovery of Taxol producing fungus from *Taxus brevifolia* has enthused workers all over the world to isolate and study other endophytes associated with other *Taxus* spp. and screen them for their bioactivity. *Taxus wallichinia*, commonly known as Nepalese yew, remain associated with many endophytic fungi, one of these is identified as *Phoma* sp., that produced two antibiotics i.e. altersolanol A and α -hydroxy-6-methyl benzoic acid [7]. Similarly other workers from same *Taxus* sp. have isolated three different endophytic fungi namely *Sporomium minima*, *Trichothecium* sp. and an unidentified dimorphic fungi. Each of these fungi has been shown to produce Paclitaxel

in culture medium [8]. Other species of *Taxus* like *Taxus mairei* have also yielded an endophytic fungus identified as *Tubercularia* sp. which have been reported to produce Taxol when grown in potato dextrose liquid medium [9]. It is assumed that other *Taxus* sp. around the world may also harbor other endophytes that may produce important bioactive molecules [1].

Endophytes that are relatively unstudied produce novel natural products for exploitation in the field of medicine, agriculture and industry. It is noteworthy that both the host and the associated endophytes produce the same natural product, as evidenced by the discovery of Taxol from *Taxomyces andreanae* an endophytes on *Taxus brevifolia* and other endophytes of *Taxus* sp. [6, 8]. *Taxus* spp. worldwide has been extensively studied for fungal endophytes and for the production of Taxol [8, 9]. The fungal endophytes isolated from medicinal and non-medicinal plants have been investigated for their Antimicrobial activity [10-12]. Works, however, on endophytic fungi of *Taxus baccata* growing in Arunachal Pradesh and evaluation of their antimicrobial activity and production of Paclitaxel have not been reported so far. This investigation, therefore, intends to report the isolation of fungal endophytes from *Taxus baccata* and their evaluation for their antimicrobial properties.

MATERIALS AND METHODS

Sampling sites: Arunachal Pradesh is located between 28° North latitudes and 95° East longitudes. The state by virtue of its geographical position, climatic conditions and altitudinal variations, is a biodiversity rich regions in the northeast India, with large tracts of tropical wet evergreen, subtropical and alpine forests. The state (83,743 sq. km) occupies a major portion of the Indian Eastern Himalayas and has 82% forest cover [13]. It is located in the Eastern Himalayan- global biodiversity hotspot [14]. Bark of *Taxus baccata* was collected from two different locations of Arunachal Pradesh namely Tawang and Dibang valley situated at an altitude of 3200 m and 2600 m, respectively. Geographically and climatically these two locations differed from each other.

Field and laboratory procedure: Fresh *Taxus* barks were cut from the selected plants and placed in plastic bags in order to avoid moisture loss. The materials were transported to the laboratory within 48 h and stored at 4°C until isolation procedures were completed. For isolation of endophytes, *Taxus* barks were washed thoroughly in distilled sterile water. The washed bark materials were then, surface sterilized with 70% ethanol for 3 min and 0.5% NaOCl for 1 min under a laminar hood until dried [15, 16]. Then, with a sterile scalpel, outer tissues were removed from the bark samples and the inner tissues were carefully dissected and placed on Petri-Plates in media (Hi-media) and also media amended with the bark extract (Table 1). The plates were incubated in BOD incubator at 24±1°C for specified time period. The hyphal tips of the fungal cultures growing out of the plated barks were removed with a fine sterile needle and transferred onto the freshly prepared potato dextrose agar slants after the incubation period was over (Table 1). The slants were incubated for 1-2 weeks and were periodically checked for purity. The fungal isolates were observed under microscope for pure culture and their morphological characters were noted. The pure fungal isolates were then preserved in refrigerator for further work.

Antimicrobial evaluation: The selected fungal endophytes were evaluated for their antimicrobial activity. The endophytes were grown in 250 ml Erlenmeyer flasks containing 500 ml of potato dextrose broth (PDB). The flasks were incubated in BOD shaking incubator for 3-4 weeks at 24±1°C with periodic shaking at 150 rpm. The flasks were taken out of the incubator and filtered through sterile muslin cloth to remove the mycelial mats. The liquid

Table 1: Recovery of endophytes from *Taxus baccata* bark on various media combinations

Sample	Media*	Incubation Time (in weeks)	No. of colonies isolated
Bark	PDA	2	4
	MEA	2	6
	WA	2-3	3
	PDA+BE	2	6
	MEA+BE	2	8
	WA+BE	1-2	6

*Media: PDA-Potato dextrose agar; MEA-Malt extract agar; WA-Water agar; BE-Bark extract

broth were collected and extracted with equal volume of ethyl acetate in a separating funnel by vigorous shaking for 10 min. The cell mass were separated and the solvent obtained were collected and dried with MgSO₄ and concentrated to yield the crude extract [11]. The crude extracts were then dissolved in Dimethyl sulphoxide (DMSO) for antimicrobial bioassay.

Test Organisms: *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumoniae*, which were obtained from MTCC, Chandigarh, India (No. ATCC-29676) and *Candida albicans* that was obtained from microbiology division of Defense Research Laboratory, Tezpur, India were used as the test organisms. All the test organisms were maintained on freshly prepared nutrient agar slant. *C. albicans* was cultured in Sabouraud's agar slant. All media component were procured from Hi Media, Mumbai, India.

Bioassay: Agar cup diffusion method was used for antimicrobial screening [17]. Nutrient agar plates were inoculated with 0.2 ml of overnight culture of each bacterial suspension containing 1.0x10⁹ cells/ml. Similarly Sabouraud's agar plate was inoculated with 0.2 ml of *C. albicans* containing 1.0x10⁹ cells/ml. The plates were evenly spread out. Then agar cup were prepared in the plates with a cork borer. Each cup was then loaded with 200 µl of the crude extracts. The plates were incubated at 37°C for 24 h and the zone of inhibition was measured (Table 2).

RESULTS

A total of 33 fungal endophytes were isolated from the barks of *Taxus baccata*. Twenty-three morphologically different isolates were selected for

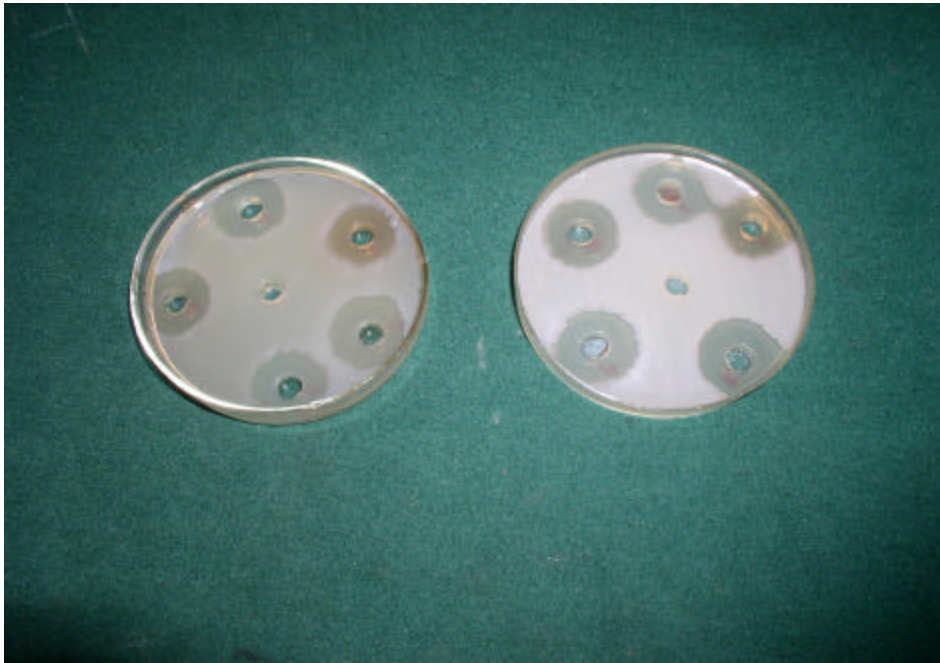


Figure 1: Antimicrobial activity of the crude extracts of endophytic fungal isolates against *E. coli* and *B. subtilis*

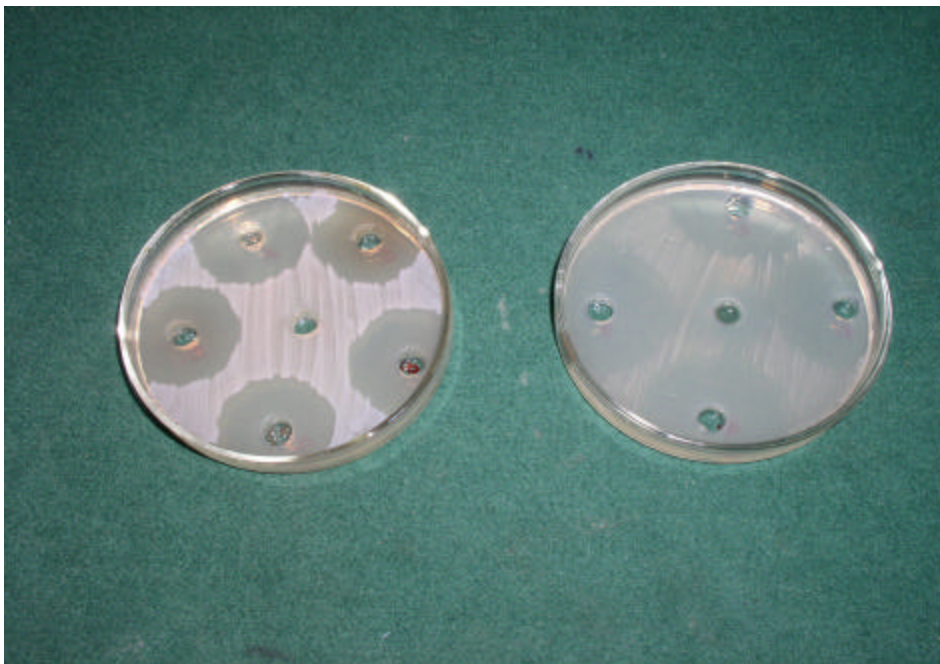


Figure 2: Antimicrobial activity of the crude extracts of endophytic fungal isolates against *C. albicans* and *S. aureus*

Table 2: Antimicrobial activity of fungal endophytes isolated from *Taxus baccata* bark

Isolate(s)	Zone of inhibition (mm)				
	<i>S. aureus</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>K. pneumoniae</i>	<i>C. albicans</i>
Tawang valley					
TEF1	-	-	-	-	-
TEF2	8	-	-	-	-
TEF3	-	-	-	-	-
TEF4	-	-	-	-	-
TEF5	6	-	-	-	-
TEF6	10	-	12*	-	8
TEF7	18	12	15	10	16
TEF8	-	-	-	-	-
TEF9	8	-	7	8	12
TEF10	-	10	-	-	-
TEF11	11	-	14	-	-
TEF12	-	-	-	-	-
Dibang valley					
DEF1	19	12	17	11	18
DEF2	10	-	8	-	-
DEF3	24	17	22	18	22
DEF4	8	6	5	-	-
DEF5	-	-	-	-	-
DEF6	20	11	17	12	18
DEF7	-	-	-	-	-
DEF8	4	-	-	-	-
DEF9	23	15	21	18	22
DEF11	7	-	5	-	9

*Zone diameter > 10 is indicated in bold; - indicates no activity

testing the antimicrobial activities. Twenty-two percent isolates showed good antimicrobial activity against all the test organisms (Figs. 1 & 2). The endophytes were isolated using different normal and modified media (Table 1). More endophytes were isolated on media amended with bark extracts of the host plant than those not amended. Maximum fungal endophytes were isolated from *Taxus* barks on Malt Extract Agar media amended with bark extracts (MEA+BE) and minimum on Potato Dextrose Agar media (PDA). We, however, observed that almost identical incubation period was required in all the cases irrespective of whether the media were amended with bark extract or not. Endophytes, however, took just 1-2 weeks on bark extract amended Water Agar medium than other media indicating lesser incubation time.

The crude extracts obtained from the culture broth of endophytic fungi, grown aerobically on potato dextrose broth medium, showed considerable antimicrobial activity against two gram-positive bacteria (*B. subtilis* and *S. aureus*), two gram-negative bacteria (*E. coli* and *K.*

pneumoniae) and one pathogenic fungus (*C. albicans*). Among the five potent endophytes highest antimicrobial activity was recorded for isolate No. DEF3, followed by DEF 9 > DEF 6 > DEF 1 and lowest for isolate No. TEF7 (Table 2). All the potent isolates effectively inhibited *S. aureus* with zone of inhibition (diameter) ranging from 24 mm to 18 mm. The zone of inhibition in case of *C. albicans*, however, ranged from from 22 mm to 16 mm. This indicated that both these organisms were more sensitive to the broth extracts. Good antimicrobial activity was also observed against *B. subtilis*. The gram-negative organisms namely *E. coli* and *K. pneumoniae* were less sensitive to the broth extracts compared to the gram-positive organisms.

DISCUSSION

The endophytes were isolated using different normal and modified media (Table 1). More endophytes were isolated on media amended with bark extracts of the host

plant than those not amended. This indicated the presence of some molecules in the bark extracts that might have favored the growth of the endophytes. It has been reported that some fungal endophytes sporulate only when autoclaved host tissues are added to the culture media [18, 19]. This suggests that compounds found in host plants might be necessary for the fungus to complete its life cycle. Crude extracts obtained from the broth cultures of fungal endophytes have been reported to show antimicrobial activity against *E. coli*, *S. aureus*, *Saccharomyces cerevisiae* and *Penicillium cendadensis* [10]. It has also been reported to be active against *Mycobacterium tuberculosis*, *Plasmodium falciparum* and *Herpes simplex* virus type 1 [20]. This suggests the great potentiality of fungal endophytes as a source of new bioactive molecules considering the present health problem of the world owing to cancer, multi drugs resistance, parasitic protozoans and fungi, besides the new emerging diseases. Fungal endophyte isolated from European yew (*Taxus baccata*) has reportedly produced an antifungal-anticancer agent known as Leucinostatins that is active against human cancer cell lines [4]. But studies with regards to antimicrobial activities of fungal isolates of Himalayan yew has not been reported so far. The results of the present investigation revealed that Himalayan Yew (*Taxus baccata*) of Arunachal Pradesh harbored fungal endophytes, which produced bioactive molecules. The evaluation of the capability of these fungal endophytes to produce compounds having antibacterial and antifungal activities confirmed the potentiality of these groups for screening programs of bioactive natural products. The observations can also be taken as lead for production of Paclitaxel in these endophytes. There are certain limitations in natural product screening programs as the "referm" problem (rare culture that produces an activity of interest the first time they are grown cannot be made to produce that activity again when they are re-fermented) that need further investigation [12]. Therefore, any information or research on endophytes-plant symbiosis, such as in this study is of value, especially when taken into account of the positive biological activities. Thus isolation of endophytes associated with Himalayan yew of Arunachal Pradesh and their screening for Antimicrobial activity is an important direction in drugs discovery program considering the present health problems and importance of endophytes in this regards. The fungal endophytes could not be identified because majority of them did not produce spores. Efforts are on for identification of the

potent isolates. The results of the present investigation suggested either good antimicrobial potentiality of the extract or of a high concentration of an active principle in the crude extracts of the potent strains. The crude extracts obtained from other endophytic fungal isolates that showed low antibacterial activity in the bioassay might have active compound but probably in smaller amount. In the present study, out of five potent endophytic fungal isolates one was isolated from Tawang valley and four from Dibang valley. Dibang valley of Arunachal Pradesh falls within the Indo-Burma mega biodiversity hot spots of the world [14]. This region has higher species diversity and annual rainfall with moist environment than Tawang valley, which is comparatively drier. The isolation of more number of potent fungal endophytes from Dibang valley might be due to altitudinal variations and favourable climatic conditions. Productions of bioactive molecules might be understood as the survival strategy by the microbial endophytes arising due to the plant-microbe interactions. Such kind of interaction are observed in plants growing in moist environments or in rain forests, which are prone to attack by certain group of extremely pathogenic fungi and defense strategy is necessary for survival [1].

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