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RESEARCH ARTICLE

Antimicrobial properties of endophytic actinomycetes isolated from *Combretum latifolium* Blume, a medicinal shrub from Western Ghats of India

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Abstract **Endophytic actinomycetes were isolated from *Combretum latifolium* Blume (Combretaceae), Western Ghats of Southern India and identified by its characteristic culture morphology and molecular analysis of 16S rRNA gene sequences. In this survey of endophytic actinomycetes, a total of 117 isolates representing 9 different genera of endophytic actinomycetes were obtained using four different isolation media and several of them seemed to be novel taxa. *Streptomyces* genera (35%) was the most frequently isolated strains, followed by *Nocordiopsis* (17%) and *Micromonospora* (13%). ISP-4 medium recovered more isolates (47%) when compared to rest of the media used. Preliminary antibacterial activity of the isolates was carried out by confrontation test. Ethyl acetate fraction of selected isolates in disc diffusion assay exhibited broad spectrum antimicrobial activity against test human pathogens. All *Streptomyces* spp. strains displayed significant antimicrobial activity against test pathogens. Strain CLA-66 and CLA-68 which are *Nocordipsis* spp. inhibited both bacterial and fungal pathogens where as other isolates inhibited atleast three test human pathogens in disc diffusion assay. Antimicrobial screening of endophytic actinomycetes from this host may represent a unique potential niche for antimicrobial compounds of industrial and pharmaceutical applications. This work is the first comprehensive report on incidence of potential endophytic actinomycetes inhabiting *C. latifolium* Blume.**

Keywords endophytic actinomycetes, *Combretum latifolium* Blume, *Streptomyces*, antimicrobial activity

Introduction

The hunt for new biologically active secondary metabolites synthesized by microorganisms continues to be an important area in order to cope with the increasing demand for the treatment of human diseases (Liu et al., 2013). Over the last several decades, natural products have contributed a highly significant role in the area of drug discovery (Ji et al., 2009; Rao and Satish, 2015). Secondary metabolites produced by actinomycetes are considered to be an excellent starting scaffold for the development of antibiotics, anticancer agents, enzymes, anti-infection agents, immunomodulators, anthelminthic agents, insect control agents and plant growth hormones (Demain, 1995; Fiedler et al., 2008; Schulz et al., 2009; Strobel et al., 2004; Baltz, 2010; Qin et al., 2011). Interestingly, about 70%–80% of all isolated compounds are from actinomycetes (El-Tarabily et al., 2000; Bérdy, 2005; Nagpure et al., 2014). Meantime, promising approaches of mining unexplored microbial ecological niches for use as antibiotics have also been emerging (Fischbach and Walsh, 2009). These microbial endophytes may represent an underexplored reservoir for novel species of potential interest in the discovery of novel lead compounds and for exploitation in pharmaceutical, agriculture and industry (Qin et al., 2011; Rao et al., 2015).

Microbial endophytes of medicinal plants participate in the metabolic pathways of medicinal plants and produce

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analogous or novel bioactive secondary metabolites (Yu et al., 2010). Endophytic actinomycetes as one of the substantial residents in plant tissues, which are rarely studied have attracted more attention in recent years since there is an ever increasing need for novel organisms and their bioactive products (Li et al., 2012). Antimicrobial agents produced by endophytic actinomycetes may provide new alternatives to combat multidrug-resistant human pathogens, the prevalence of which is increasing in the recent days (Verma et al., 2013). They are considered as a potential source towards the discovery of novel bioactive metabolites (Lam, 2006).

Species distribution, species richness and biologic diversity are significantly influenced by ecological environs (Sheil, 1999; Hou et al., 2009). The diversity and antimicrobial activity of endophytic actinomycetes isolated from medicinal plants of tropical regions have been reported in previous studies (Li et al., 2008; Bascom-Slack et al., 2009; Qin et al., 2009), while the medicinal plants from Western Ghats of India have not gained much research attention. Therefore, screening for endophytic actinomycetes able to produce bioactive compounds, the exploration of new habitats has been recommended (Nolan and Cross, 1988; Takahashi and Omura, 2003).

Combretum latifolium Blume (Combretaceae) is a large climbing shrub which has great ethnomedicinal values (Shrisha et al., 2011). Stem and bark of this shrub is used as insecticides (Suthari et al., 2014), Leaves juice is used in the treatment for dysentery (Debnath et al., 2014). No reports are available in search of endophytic actinomycetes in *C. latifolium* Blume, in spite of the fact that it has great medicinal value and has huge importance to the mankind. Thus, the present study was designed to explore the hidden endophytic actinomycetes of *C. latifolium* Blume using different isolation media for greater diversity.

Materials and methods

Study site characteristics and selection of plant

Western Ghats are a mountain range that runs almost parallel to the Western coast of the Indian peninsula, located entirely in India. It is one of the eight 'hottest hotspots' of biological diversity in the world (Myres et al., 2000). Pushpagiri Sanctuary (12°35′N 75°40′E, elevation 1748 m) is located in the Western Ghats of Kodagu (Coorg), Karnataka which is the part of Southern India covered with thick evergreen and semi-evergreen forests and shoal grassland habitat. The imposing Kumaraparvata Peak forms the core of the Sanctuary, with dense sholas on the upper reaches and evergreen forests lower down. *C. latifolium* Blume was selected for the study from this region. The selection of plant for the isolation endophytes was based on its local ethnobotanical properties, including its antibacterial, insecticidal, antitumor, and wound-healing properties (Debnath et al., 2014; Suthari et al., 2014).

Collection of samples

Healthy and asymptomatic bark, root and leaf samples of *C. latifolium* Blume were collected. To secure the endophytic nature of the isolates, the cut ends of root and bark were sealed with wax. All the samples were immediately brought to the laboratory in an icebox and stored at 4°C. Each sample tissues were used for the isolation of endophytic actinomycetes within 24 h from collection.

Isolation media

Four isolation media (HiMedia, Mumbai, India): Yeast extract-malt extract agar (ISP-2), Inorganic Salt Starch Agar (ISP-4), Chitin agar (CA) and Humic-vitamin agar (HV) which is supplemented with 1.8% agar were used in the study to isolate endophytic actinomycetes. To inhibit the growth of nonactinomycetes, the isolation media were supplemented with nalidixic acid and $K_2Cr_2O_7$ (You et al., 2005) both to a final concentration of 50 μg/ml and 75 μg/ml of filter-sterilized cycloheximide as an antifungal agent to reduce fungal contamination (Jensen et al., 1991).

Isolation of endophytic actinomycetes

Isolation of endophytic actinomycetes was carried out according to method described by Johannes et al. (2006) with some modifications. Each sample tissues were washed under running water for 15 min and dried at room temperature prior to surface-sterilization. All the slight visibly damaged material was excluded. Samples were washed by sonification for 10 min at 150 w to dislodge soil and organic matter. Surface sterilization: the tissue segments were rinsed in 0.1% Tween 20 for 30 s, sequentially immersed in 75% ethanol for 5 min, 2% sodium hypochlorite for 5 min and rinsed with 10% $NaHCO_3$ for 10 min to inhibit the growth of fungi. All samples were rinsed three times in sterile distilled water after each treatment. Finally, the tissue samples were dried thoroughly in the sterile conditions. To confirm the success of surface disinfection process, aliquots of the sterile distilled water from the final rinse were inoculated on the isolation media plates. The surface-sterilized tissue samples were aseptically cut into small pieces, placed on different isolation media (100 each from leaf, root and bark) and incubated at 28ºC for 1 month. The plates were regularly checked for the growth of endophytic actinomycetes isolates. Pure cultures were obtained by repeated streaking on ISP-4 agar medium cultivated at 28ºC.

Growth characteristics of endophytic actinomycetes

All the isolates were sub cultured on ISP-4 medium at 28± 2ºC and the growth rate was monitored every two days up to 25 days. The isolates, which showed good growth in 4 days were considered as fast growers and those that showed good growth between 4 to 8 days were classified as moderate growers and the slow growers took more than 8 days for their growth. In addition to growth, color of mycelia was monitored in all the isolates of endophytic actinomycetes and documented.

Preliminary identification of endophytic actinomycetes

Actinomycetes were recognized on basis of morphological features following directions of International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). The appearance and growth of endophytic actinomycetes were observed regularly. They were dereplicated by cultural and morphological characteristics, including chalky to leathery appearance and color of diffusible pigment and growth. Further, the isolates were observed using light microscope for their filamentous nature, spores, spiral sporospores, hyphe and Gram-straining were performed for further identification (Yan, 1992). Individual colonies were picked up, maintained on ISP-4 slope medium and stored at 4ºC after profuse growth of isolates.

Confrontation test

Diversity of active endophytic actinomycetes isolates was determined by the confrontation test. Isolates were suspended in sterile distilled water and inoculated on the surface of modified nutrient agar (50% NA + 50% ISP-4). After 72 h of incubation at 28ºC, test human pathogens were inoculated in perpendicular directions of the endophytic isolates and incubated at 28ºC for 48 h (Highley and Ricard, 1988; Bensultana et al., 2010). The diversity in producing antibiotics is expressed by the inhibition zones between the test isolates. The inhibition zone against human pathogens was measured after 48 h of incubation. Plates with the same medium without inoculation of endophytic actinomycetes but with simultaneous streaking of test human pathogens were maintained as control.

Fermentation and extraction

Selected active isolates were cultured in 1 L Erlenmeyer flask containing 300 mL of ISP-4 broth. The cultures were incubated for 30 days at 28ºC under static conditions. The culture broth was then filtered to separate the filtrate and mycelium. Filtrate was blended thoroughly and centrifuged at 4000 r/min for 10 min. Liquid supernatant was extracted with ethyl acetate (v/v) thrice separately and this was evaporated to dryness under reduced pressure at 45ºC using rotary flash evaporator (Anibou et al., 2008).

Antimicrobial susceptibility testing

Antimicrobial susceptibility test was carried out by disc diffusion assay. The sterile discs (6 mm) were impregnated with 20 μL (100 μg/disc) of ethyl acetate extract. Discs impregnated with ethyl acetate extract were dried in laminar air flow and placed on the surface of the media seeded with test human pathogen in Petri plates. A disc impregnated with only 20 μL of ethyl acetate was also placed for each test human pathogen with a positive control. The plates were incubated at 37±2°C and room temperature (for test bacteria and fungi respectively) and the diameter of the zone of inhibition was recorded (Ramesh and Mathivanan, 2009).

Genomic DNA extraction and 16S rRNA gene sequence analysis

The selected potential endophytic actinomycetes isolates were grown for 7 days at 28ºC with agitation in 250 mL flasks containing 100 mL of ISP-4 broth medium. Biomass was harvested by centrifugation (8000 r/min for 5 min). 200 mg of mycelium was used for genomic DNA extraction as described by Kieser et al. (2000). The PCR amplification of 16S rDNA was carried out with universal primers: PA 5′ AGAGTTT-GATCCTGGCTCAG 3′ and PH 5′ AAGGAGGTGATC-CAGCCGCA 3′ (Loqman et al., 2009) in a thermal cycler (Bio-Rad, USA). PCR reaction was performed using the extracted genomic DNA as template under following conditions: DNA was denatured at 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 55ºC for 30 s, and 72ºC for 1 min 30 s, with a final 10 min extension at 72ºC. The sequence was compared with similar 16S rDNA gene sequences retrieved from the DNA databases using the BLAST search program in the National Center for Biotechnology Information (NCBI).

Results and discussion

Taxonomic diversity of endophytic actinomycetes

Actinomycetes constantly hold a special significance in the research arena for the past few decades as the members of this group, especially *Streptomyces* spp. are known to produce a vast array of biological compounds. The searching of new natural products derived from microorganisms, especially from actinomycetes, has been focused on the isolation of species from unexplored niches. In this backdrop, the present work has been initiated to exploit hidden endophytic actinomycetes from Western Ghats of India because of its rich microbial diversity which has been studied only to a limited extent. Endophytic actinomycetes were isolated from internal part leaf, bark and root tissues of *C. latifolium* Blume. Cultures that exhibited growth morphology indicative of actinomycetes were selected for further study. A total of 117 isolates belonging to nine well identified distinct taxa (Fig. 3) were recovered from 200 segments of three different tissue samples of *Combretum latifolium* Blume after 1–1.5-month incubation using four different isolation media. No colonies emerged from the final rinses of the sterilization procedure showing that the surface sterilization was effective and the subsequent isolates were endophytes.

All three tissues showed variation in the recovery of endophytic actinomycetes (Fig. 1). Maximum recovery of isolates was found in root (54.7%), secondly from stem (26.5%) and least in leaf (18.8%). The higher recovery of endophytic actinomycetes from root samples compared to stem and leaf is supported by previous literature of endophytes diversity in leaves, stem and roots of *Citrus reticulate* L. (Shutsrirung et al., 2013), *Azadirachta indica* A. Juss. (Verma et al., 2009) and three other medicinal plants (Gangwar et al., 2014). The prominent recovery of endophytic actinomycetes from roots compared to stem and leaf suggests that, property of root tissue which has soil adhesion around its surface enriches nutrient supplements for the growth of endophytes. Interestingly, among 117 isolates, 68 isolates (72.59%) exhibited fast growth, 29 isolates (4.8%) showed moderate growth and the remaining of 10 isolates (4.8%) showed slow growth (Fig. 2). The result clearly implies that the endophytic actinomycetes are not slow growing microorganisms and most of their growth is comparable with filamentous fungi (Ramesh and Mathivanan, 2009).

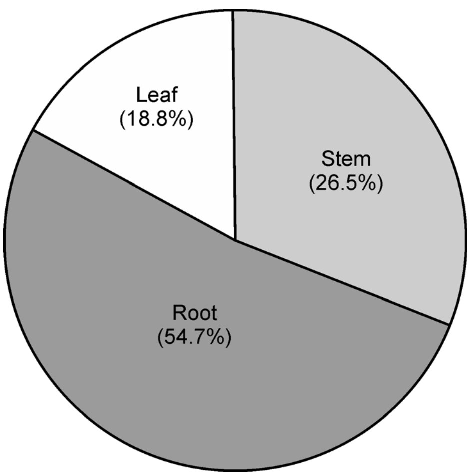

Figure 1 Recovery of endophytic actinomycetes from different tissues of *C. latifolium* Blume.

Effect of isolation media on recovery of endophytic bioactive isolates

Four media were utilized to retrieve the isolates from the host plant. These isolation media were helpful in acquiring to the diverse isolates obtained in this study. These isolation media seem to be specific and sensitive for endophytic actinomycetes (Barakate et al., 2002). According to the results (Table 1), ISP-4 medium retrieved more isolates followed by ISP-2 medium, whereas least recovery of isolates was observed in CA medium. This suggests that, ISP-4 medium seems to be the most favorable medium for the recovery of endophytic actinomycetes from the host and best suitable for the isolation of antibiotic producing strains. It can be considered that, different isolation media employed in this study were productive and resulted in the successful isolation of numerous endophytic actinomycetes.

Preliminary antibacterial activity: Confrontation test

The antimicrobial activities of endophytic strains could involve in the protection of host plant against invading pathogens which are a combination of physical, chemical and biological factors. In this study, a total of 117 isolates were subjected to primary screening (Fig. 4) and the distribution of active isolates is shown in Table 2. Despite the observed abundance of other strains, the highest percentage of bioactive strains found was genus *Streptomyces* (Fig. 5) which is consistent with other reports (Qin et al., 2009; Li et al., 2012). Some strains of *Streptomyces* spp. and *Nocordiopsis* spp. were active against all the tested Gram positive

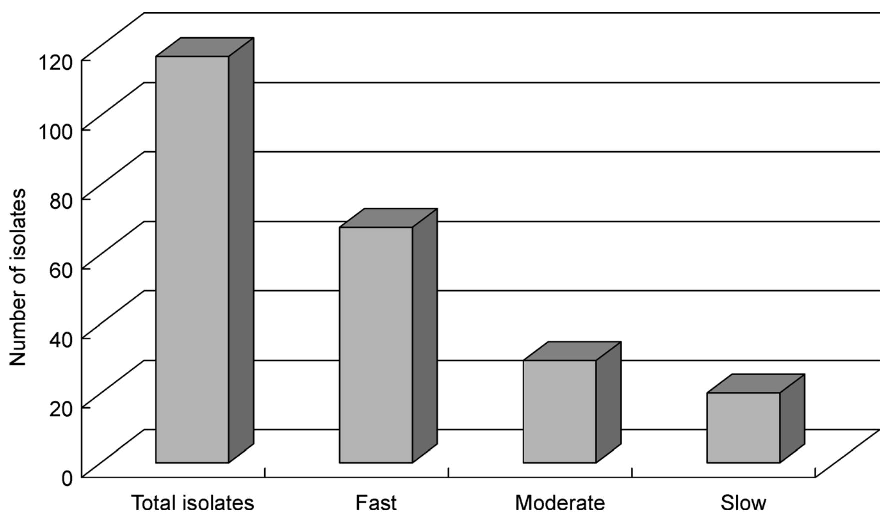

Figure 2 Growth rate of endophytic actinomycetes isolates from *C. latifolium* Blume.

Table 1 Effect of isolation media on recovery of endophytic bioactive isolates

Isolation media*	Number of isolated isolates	Number of potential isolates	% of bioactive isolates
ISP-2	27	16	59.25
ISP-4	56	41	73.21
CA	13	5	38.46
HV	21	7	33.33
Total	117	69	58.97

*Note: ISP-2 = Yeast extract-malt extract agar, ISP-4 = Inorganic Salt Starch Agar, CA = Chitin agar, HV = Humic vitamin agar

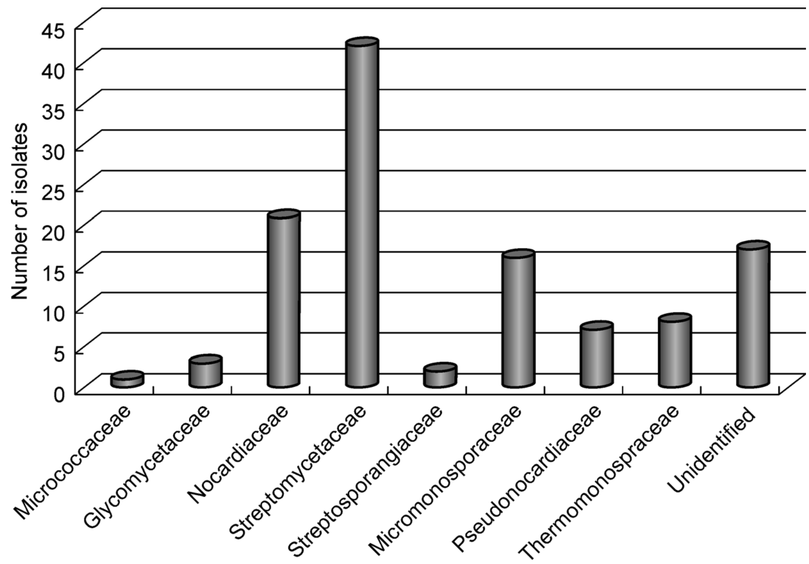

Figure 3 Distribution of endophytic actinomycetes to different actinomycetes group sampled from *C. latifolium* Blume.

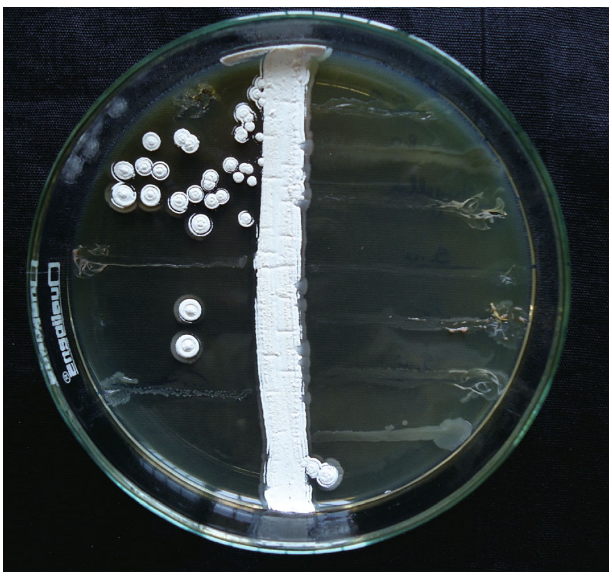

Figure 4 Preliminary screening for antibacterial activity of endophytic actinomycetes against human pathogens by confrontation test.

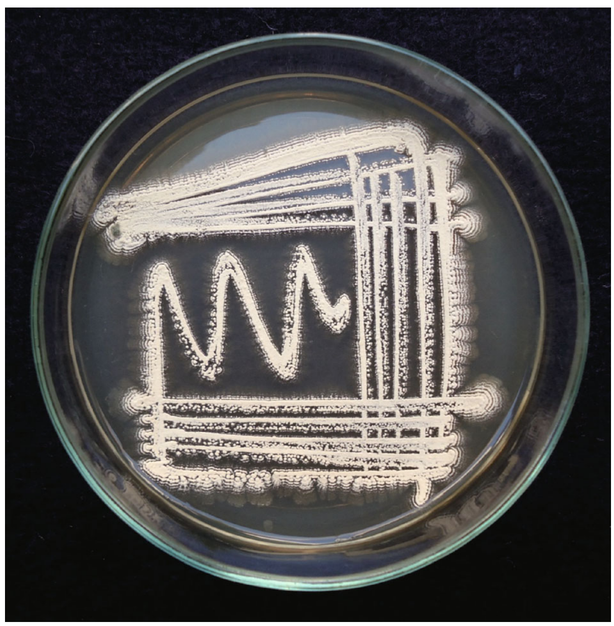

Figure 5 Colony morphology of endophytic *Streptomyces* sp. on ISP-4 agar after 14 days.

and Gram negative bacteria which confirm their broad spectrum of activity. *Micromonaspora* isolates effectively inhibited *S. aureus* and *B. subtilis*. It is especially interesting that isolates belonging to genera not often or not all reported to exhibit antimicrobial activity were found to inhibit the growth of at least one test pathogen. Even though our isolation media were not designed to focus on the rare actinomycetes, we recovered few unique sporing structure isolates. Since similar isolation media have been used in the isolation of endophytic actinomycetes from *Ocimum sanctum* (Singh and Padmavathy, 2015), findings of Li et al., (2012) and the results of our study indicates that medicinal plants in general and the medicinal plants of Western Ghats in particular host has a great diversity of endophytic actinomycetes resources. However, to catch more rare endophytic actinomycetes in Western Ghats habitat, a wider range of isolation media should be employed in further studies.

Morphological and 16S rRNA gene sequence analysis for identification

Several rare genera were obtained by using different isolation media (Table 2). The colonies of the endophytic actinomycetes were elevated, convex and powdery in nature. Many of such morphological characteristics are common in most of the *Streptomycetes* spp. (Miyadoh et al., 1997; Sujatha et al., 2005; Fguira et al., 2005). Analysis of the actinomycete isolates using their colony appearance, spore chain morphology helped for the preliminary identification. To confirm the reliability of morphological identification, selected bioactive endophytic actinomycetes strains were subjected to 16S rRNA gene sequence analysis. The consensus DNA sequences in BLASTN search suggested that the isolates belonged to nine genera: *Kocuria, Glycomyces, Nocordiopsis, Streptomyces, Microbispora, Micromonospora, Pseudonocardia, Spirillospora, Actinomadura*. The results showed that the endophytic community is diverse and that the most abundant isolates recovered were *Streptomyces* genus (35%) which was consistent with various reports about other hosts (Miyadoh et al., 1997; Qin et al., 2009; Li et al., 2012; Shutsrirung et al., 2013). This finding implies that *Streptomyces* spp. can dominate over the rest of the taxa in endophytic colonization. However, *Kocuria, Glycomyces* and *Microbispora* were the least frequent in the host. Notably, some uncommon genera from the endophytic environment, such as *Glycomyces, Micromonospora, Pseudonocardia* and *Spirillospora* were recovered. It is worth mentioning that such a large number of rare actinomycetes genera isolated from a single type of host. Many of them could be considered unique species or novel strains with potential to produce new secondary metabolites. This work is the first comprehensive report concerning the endophytic actinomycetes of *C. latifolium* Blume.

Antimicrobial susceptibility test: Disc diffusion assay

Based on primary screening for antibacterial activity by confrontation test, genus specificity and sporing structure we selected 12 isolates for antimicrobial susceptibility testing of their ethyl acetate extract cultured in ISP-4 broth. All strains demonstrated robust antimicrobial activity against the test pathogens tested human pathogens (Table 3). A prominent difference in inhibitory activities and target test organism specificity was associated with each endophytic actinomycete. *Streptomyces* spp. harbored a significant higher proportion of isolates with antimicrobial activities similar to that of rare actinomycetes. Strains CLA-1, CLA-12 and CLA-72 which are *Streptomyces* spp. exhibited strong broad spectrum antimicrobial activity compared to rest of the isolates. However, *Nocordiopsis* sp. (strain CLA-68), *Micromonospora* (strain CLA-56), strain CLA-98 and CLA-104 also exhibited significant activity against all the test human

Table 2 Genus distribution of endophytic actinomycetes isolates from *C. latifolium* Blume and their primary screening for antibacterial activity by confrontation test

Family	Genus	Total number of isolates	Number of bioactive isolates against test pathogens				
			SA	BC	EC	ST	PA
Micrococcaceae	*Kocuria*	1	0	1	0	0	0
Glycomycetaceae	*Glycomyces*	3	0	1	0	0	0
Nocardiaceae	*Nocordiopsis*	21	13	15	09	12	06
Streptomycetaceae	*Streptomyces*	42	31	38	27	33	28
Streptosporangiaceae	*Microbispora*	2	1	1	0	0	0
Micromonosporaceae	*Micromonospora*	16	3	4	2	2	1
Pseudonocardiaceae	*Pseudonocardia*	04	1	2	0	0	0
	Spirillospora	03	0	1	0	0	0
Thermomonosporaceae	*Actinomadura*	08	0	1	2	0	2
	Unidentified	17	4	8	4	5	4
	Total	117	53	72	44	52	41

SA- *Staphylococcus aureus (*MTCC 7443), BC- *Bacillus subtilis* (MTCC 121), EC- *Escherichia coli* (MTCC 7410), ST- *Salmonella typhi* (MTCC 733), PA- *Pseudomonas aeruginosa* (MTCC 7903). Antagonism was estimated by confrontation test between actinomycetes and test human pathogens.

Table 3 Determination of activity of ethyl acetate extract of selected endophytic actinomycetes by disc diffusion assay

Strain code	Genus	Inhibition zones in diameter (mm)					
		Test human pathogens					
		S. aureus (MTCC 7443)	*L. monocyto-genes* (MTCC 839)	*P. aeruginosa* (MTCC 7903)	*S. typhi* (MTCC 733)	*C. albicans* (MTCC 183)	*M. canis* (MTCC 2820)
CLA01	*Streptomyces* sp.	+++	+++	+++	+++	++	+++
CLA34	*Actinomadura* sp.	-	++	+++	+	-	-
CLA56	*Micromonospora* sp.	++	++	++	+	++	++
CLA66	*Nocordiopsis* sp.	++	++	+++	++	++	-
CLA68	Nocordiopsis sp.	++	+++	++	+++	+++	++
CLA12	*Streptomyces* sp.	+++	+++	+++	+++	+++	+++
CLA14	*Glycomyces* sp.	++	++	+++	++	-	++
CLA34	*Streptomyces* sp.	++	+++	+++	+++	+++	++
CLA56	*Microbispora* sp.	+	++	+	++	-	++
CLA72	*Streptomyces* sp.	+++	++	+++	+++	+++	+++
CLA98	Unidentified	+++	++	+++	+++	++	+++
CLA-104	Unidentified	+++	+++	+++	++	+++	++

(-) no inhibition, (+) inhibition zone > 5mm, (++) inhibition zone > 10mm, (+++) inhibition zone > 15 mm

pathogens. Moreover, our findings indicates that the bioactivity against test Gram negative bacteria was significantly higher than the Gram positive bacteria. In addition, the rare actinomycetes obtained from the host are metabolically active and produce interesting bioactive molecules. Endophytic *Streptomyces longisporoflavus* was isolated from *L. ciliata* Benth. (Lamiaceae*)* of Western Ghats, India which exhibited anti-diabetic activity (Akshatha et al., 2014). Search for novel secondary metabolites with diverse biological activity in assorted environment has gained greater attention in recent years due to the increased incidence of multiple resistances to the available drugs (Dharmaraj, 2011). In view of this, these potential endophytic strains were promising source of producing new antibiotics.

Conclusion

C. latifolium Blume provides a beneficial source for potential endophytic actinomycetes and these strains might represent a promising source of new biologically active compounds. ISP-4 medium serves as the most favorable medium for the recovery of endophytic actinomycetes from the host. The study implies that targeting medicinal plants from unique niche to explore endophytic actinomycetes will facilitates for the discovery of new antimicrobial agents with substantial biologic activity. This work is the first comprehensive report concerning the endophytic actinomycetes of *C. latifolium* Blume. from Western Ghats of Southern India.

Compliance with ethics guidelines

H. C. Yashavantha Rao, Devarju Rakshith and Sreedharamurthy Satish declare that they have no conflict of interest

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