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Antimicrobial Activity of Endophytic Fungi from Leaves and Barks of *Litsea cubeba* Pers., a Traditionally Important Medicinal Plant of North East India

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Abstract

The present research work was carried out to study the endophytic fungal flora associated with the leaves and barks of *Litsea cubeba* and antibacterial activity of the crude metabolites produced by the endophytes. *L. cubeba* is an endemic plant to Southeast Asia and is commonly known as Mezankari in Assam. A total of 12 morphologically different endophytic fungi were isolated from *L. cubeba*. *Acremonium falciforme* was the most dominant fungi that inhabited both the leaves and barks of *L. cubeba* and, respectively, had 42.41% and 31.42% relative frequency of dominance. The ethyl acetate extracts of the crude metabolites of all the isolates, showed antagonistic activity against at least one of the tested bacteria. *Acremonium falciforme* showed the highest zone of inhibition (12.3±0.50 mm) against *Staphylococcus epidermidis* (MTCC 435). The results of the present study indicated that the isolated endophytes produced bioactive compounds which might have potential application in pharmaceutical industry.

Keywords: *Acremonium falcifome*, Antimicrobial activity, Endophytic fungi, Inhibition zone, *Litsea cubeba*.

1. Introduction

Endophytes are microorganisms colonizing healthy plant tissues without causing overt symptoms or apparent injuries to the host (Bills, 1996). Since the discovery of endophytes in Darnel, Germany, in 1904, various investigators have defined endophytes differently depending on the perspective from which the endophytes were being isolated and subsequently examined (Strobel and Daisy, 2003). The most common endophytes in plants were fungi (Tayung, 2008). According to Petrini (1991) endophytes, include all those fungi that during quite a prolonged period of their life remain present in the living internal tissues of their host without producing any symptoms. Mostly Ascomycetes, Deuteromycetes and Basidiomycetes class of fungi are reported as endophytic fungi (Petrini, 1986; Dayle *et al.*, 2001). Many genera and species of fungi belonging to first two classes could live endophytically in plants (Khan, 2007; Dissanayake *et al.*, 2016). Fungi are a rich source of many therapeutic substances. Metabolites of endophytic *Fusarium* sp. isolated from *Selaginella pallescens*, collected from Guanacaste Conservation Area of Costa Rica, showed antifungal activity (Brady and Clardy, 2000). The secondary metabolites produced by *Guignardia sp.* was active against *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Geotrichum* sp. and *Penicillium hennebertii*. Phomopsilactone, an antifungal compound, was isolated from *Phomopsis cassia*, an endophyte of *Cassia spectabilis* (Silva *et al.*, 2005). Nineteen out of 73 endophytic fungi produced antimicrobial compounds that inhibited several plant and human pathogens (Tuppad and Shishupala, 2014). Katoch *et al.* (2014) observed that twenty-six endophytic fungi isolated from *Bacopa monnieri* possessed antimicrobial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Candida albicans.*

Litsea cubeba is an important medicinal plant. The fruit and leaf of *L. cubeba* produce an essential oil that primarily contains Citral and 1,8- Cineol, respectively (Ho *et al.*, 2010). This oil exhibited cytotoxic activity against human lung, liver and oral cancer cells besides antimicrobial activity (Ho *et al.*, 2010). It is also used as a raw material for the synthesis of Vitamin-A. In Assam, it is economically important and is widely used as a secondary food plant for the Muga silkworms (*Antheraea assamensis*), which yields valuable golden yellow muga silk fiber ("the golden fiber"). The medicinal as well as the economic importance of *Litsea cubeba* enthused us to carry out the present investigation on endophytic fungi, which has been properly explored so far as a source of noble compounds. Till now, meagre work has been done related to isolation and bioactivities of endophytic fungi

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associated with *L. cubeba*. The objectives of the present work, therefore, were to isolate the endophytic fungi associated with *L. cubeba* and to investigate the antibacterial properties of their secondary crude metabolites against some important bacterial pathogens.

2. Materials and Methods

2.1. Collection of the Plant Materials

The present study was conducted in the Botanical Garden, Department of Botany, Gauhati University, Guwahati, Assam, which is located between 25°45' N to 26°25' N latitude and 91°10' E to 92°E longitudes, at an altitude of 62.0 masl. The present study was conducted between March 2012 and February 2013. Healthy leaves and barks of *L. cubeba* were collected aseptically. Three samples each from the bark and leaf totaling to six samples were collected for isolation of endophytic fungi. The samples were immediately brought to the laboratory in sterilized bags and were kept in a refrigerator at 4°C until they were processed. The materials were used for the analysis within 24 hours.

2.2. Isolation of Endophytes

Samples were washed thoroughly with distilled water, air-dried and were cut aseptically into about 2 cm long and 0.5 cm broad segments with a sterile knife and were surface-sterilized. A total of 144 segments were made from the plant, 72 each from barks and leaves for isolation of the endophytic fungi. For surface-sterilization, segments were immersed in 70% ethanol for 3 minutes and 4% aqueous solution of sodium hypochlorite for 5 minutes there after again with 70% ethanol 1 minute and 0.1% mercury chloride ($HgCl_2$) for 3 minutes (Bills and Polishook, 1993; Strobel, 2002). Finally, the segments were rinsed with sterile distilled water until the traces of $HgCl_2$ were washed off. The efficiency of surface sterilization was ascertained for every segment following the imprint method of Schulz *et al*. (1993). After surface drying under sterile conditions (Arnold *et al.*, 2000) in laminar air flow chamber to remove the excess water, segments were inoculated in plates containing Czapeck-Dox-Agar (CDA), Potato-Dextrose-Agar (PDA) media (Hi-Media, India) and media amended with bark and leaf extracts separately. Bark and leaf extracts were prepared by boiling 500 g of the plant's bark and leaf in 250 ml of distilled water separately for 10-15 minutes (Tayung, 2008). The preparation was cooled and filtered through sterile Whatman No.1 filter paper to get the bark and leaf extracts. The medium was supplemented with streptomycin (50 µg/ml) to prevent bacterial contamination. The plates were sealed with parafilm and then incubated at 25±1°C until the mycelium appeared surrounding the segments. The plates were checked every other day continuously for 30 days. The individual fungal colonies were transferred onto other plates with PDA for pure culture and pure culture was maintained on PDA slants.

2.3. Identification of Isolates

The fungal endophytes were identified based on their morphological and reproductive characters using identification manuals of Nagamani *et al.* (2006) and Gilman (1950). Sporulation was induced in non-sporulating isolates by inoculating them in different media and incubating them at different temperatures for different period of time. Those without distinct morphological and reproductive characters were recorded as mycelia sterilia.

2.4. Production of Crude Metabolites

All the isolates were cultivated to produce crude metabolites according to the protocols of Phongpaichit *et al.* (2007). Endophytic fungal isolates were grown in 1000 ml Erlenmeyer flask containing 500 ml potato dextrose broth media and incubated at 25±1ºC for 3-4 weeks under a stationary condition. The crude fermentation broth was filtered using Whatman filter paper No. 1 and the supernatant was blended thoroughly and centrifuged at 3600 rpm for 10 minutes. Finally, the crude metabolite was extracted three times with ethyl acetate and then it was concentrated to dryness by using rotary vacuum evaporator (Model: EYELA/NVC-2100) at 40°C. The resulting extracts from each isolate was diluted with Dimethyl Sulfoxide (DMSO) at a concentration of 10 mg/ml. The solution was sterilized by filtration through 0.4 µm Cellulose Acetate (hydrophilic) filter and was examined for antimicrobial activity against some bacteria.

2.5. Antibacterial Activity Assay

It was assayed by Kirby-Bauer disc diffusion method (Bauer *et al.*, 1996). The antimicrobial activity of the crude extract was determined against two-gram negative, viz. *Escherichia coli* (MTCC 443) and *Klebsiella pneumoniae* (MTCC 619), and two gram-positive, viz. *Bacillus subtilis* (MTCC 441) and *Staphylococcus epidermidis* (MTCC 435) bacteria. The test organisms, except for *S. epidermidis*, were collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India. *S. epidermidis* was collected from Regional Institute of Medical Sciences (RIMS), Imphal, India. Prior to testing, the pathogens were cultured in Nutrient broth at 28±1°C until their growth was observed. Then, with sterile cotton-buds swabbing was done on the Nutrient Agar (NA) medium in Petri dishes using the four test bacteria, after solidification. The sterile paper disc (0.6 cm in diameter) soaked in crude extract was placed on the NA media to evaluate of antimicrobial activity. Tetracycline antibiotic discs (10 µg/disc) was used as positive control and discs immersed with DMSO were used as negative control in the experiment. The plates were incubated at 28±1°C for 4-7 days and diameter of the inhibition zone was measured. Three replicates were maintained in each case.

2.6. Data Analysis

The Colonization Frequency (CF %) of endophytic fungi was calculated using the following formula, given by Fisher and Petrini (1987):

$$CF = (N_{COL} / N_{t)} \times 100$$

where, N_{COL} = Number of bark/leaf segments colonized by specific fungus; N_t = Total number of bark/leaf segments plated.

Frequency of dominant endophytes was calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes x 100 (Kumaresan and Suryanarayanan, 2002).

Similarity co-efficient ($SC = 2w/a+b+c$) was calculated to compare the endophytic colonization in different organs of the plants, by using Carroll and Carroll (1978) formula

and was expressed as a percentage, where: a = the sum of colonization frequency for all fungal species in a tissue; b,c = the similar sum for another tissue; w = the sum of lower colonization frequencies for fungal endophytes in common between the tissues.

2.7. Statistical Analysis

Standard error was calculated for the antimicrobial activity assay using Microsoft office excel 2016. One-way analysis of variance (ANOVA) was used to analyze the differences between the number of isolates of the endophytic fungi in the media amended with plant extract and un-amended medium followed by Least Significant Difference (LSD) test. P value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Endophytic Fungi Isolated from L. cubeba:

A total of sixty-nine isolates were obtained from healthy barks and leaves of *L. cubeba*. Thirty-six isolates were obtained from bark and thirty-three from leaf samples. Out of sixty-nine isolates, sixty-three isolates belonged to eight different genera. Four isolates did not show any reproductive structures, i.e., they did not sporulate due to which they could not be identified. These four isolates were termed as mycelia sterilia (Table 1). The occurrence of Ascomycota was the highest both in leaves and bark as compared to Chytridiomycota and mycelia sterilia (Figure 1). Species of *Acremonium* and *Nigrospora* were the most frequently isolated endophytes during the present investigation. Along with *Acremonium* and *Nigrospora*, genera, like *Allomyces*, *Penicillium*, *Aureobasidium*, *Periconia*, *Chaetomium* and *Acrophialophora*, were also isolated in the present work (Table 1). The colony morphology and microphotograph of *Nigrospora sphaerica* and *Acremonium falciforme* have been shown, respectively, in Figure 2 (a & b) and Figure 3 (a & b). Colonizing Frequency (%) and the frequency of dominance (%) were highest for *Acremonium falciforme* (Table 1).

Table 1. Occurrence, Colonizing Frequency (%) and Frequency of dominance (%) of endophytic fungi isolated from different parts of *L. cubeba*

Plant part	Endophytic fungi	Total no. of isolates	Colonizing Frequency (%)	Frequency of dominant Endophytes (%)
Bark	1.*Nigrospora sphaerica*	10	13.89	28.56
	2.*Acremonium falciforme*	11	15.28	31.42
	3. *Periconia hispidula*	2	2.78	5.72
	4.*Allomyces arbuscula*	4	4.17	8.57
	5. *Aureobasidium sp.*	1	1.39	2.86
	6. *Chaetomium sp.*	1	2.78	5.72
	7.*Penicillium chrysogenum*	2	1.39	2.86
	8. Mycelia sterilia (1)	1	1.39	2.86
	9. Mycelia sterilia (2)	1	1.39	2.86
	10. Mycelia sterilia (3)	1	1.39	2.86
	11. Mycelia sterilia (4)	2	2.78	5.72
Leaf	1.*Nigrospora sphaerica*	12	16.67	36.37
	2.*Acremonium falciforme*	14	19.44	42.41
	3.*Allomyces arbuscula*	3	1.39	3.03
	4.*Penicillium chrysogenum*	2	4.17	9.10
	5. *Acrophialophora* sp.	1	2.78	6.06
	6. Mycelia sterilia (3)	1	1.39	3.03

The Colony frequency was calculated based on 72 segments of plant parts plated

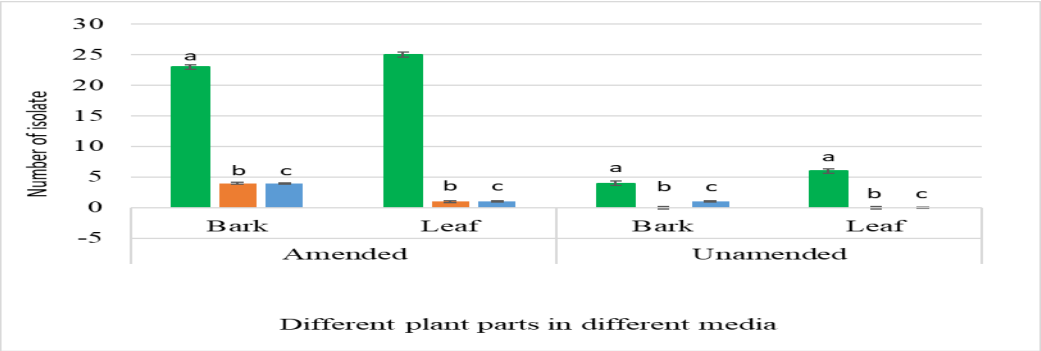

Figure 1. Occurance of (a) Ascomycota, (b) Chytridiomycota and (c) unidentified (sterile) fungal endophytes isolated from bark and leaf of *L. cubeba* using amended and unamended media. Results are expressed as Mean±SE. Isolation of the fungal endophytes (a, b and c) from bark and leaf are significantly different in media amended with plant extract from unamended media (p<0.05).

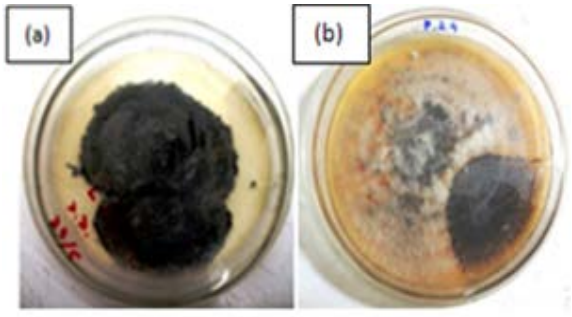

Figure 2. Colony morphology of (a) *Nigrospora sphaerica* and (b) *Acremonium falciforme*

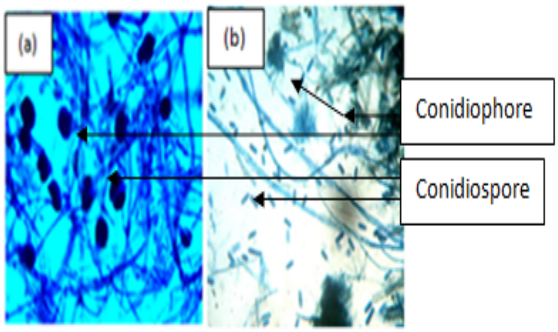

Figure 3. Photo micrograph of (a) *Nigrospora sphaerica* and (b) *Acremonium falciforme*

3.2. Effect of Different Media on the Growth of Endophytic Fungi

The endophytic fungi grew optimally and produced spores on Potato Dextrose Agar media than other media used. A significant difference in the number of isolates of the endophytic fungi was observed when the medium was amended with bark and leaf extracts than un-amended medium (Figure 1) (P<0.05). Out of sixty-nine fungal isolates of *L. cubeba*, 58 isolates were obtained from sample segments placed in media amended either with bark or leaf extracts.

3.3. Organ Specificity of Endophytic Fungi in the Host

The recovery of endophytes from the bark of *L. cubeba* (52.17%) was more than that of leaf (47.83%). The colonization frequency, in case of bark, was 48.63%, while the same for leaf was 45.84%. The fungi, viz. *Periconia hispidula*, *Aureobasidium sp.*, *Chaetomium sp.*, mycelia sterilia (1), mycelia sterilia (2) and mycelia sterilia (4) were isolated only from bark showing their organ specificity. Moreover, the fungus *Acrophialophora sp.* colonized only leaf segments showing its organ specificity for leaves (Table 1). The similarity coefficient between leaf and bark was 35.29%.

3.4. Antimicrobial Activity of Ethyl Acetate Extracts of Crude Metabolites against Some Bacteria

Ethyl acetate extracts of crude metabolites of all the isolates were tested for antimicrobial activity against four test bacteria. Amongst all, the crude extract of *Nigrospora sphaerica* showed activity against all the four test microbes and it inhibited *B. subtilis* mostly (Table 2, Figure 4a). *Acremonium falciforme* showed the highest zone of inhibition of 12.3±0.50 mm diameter, against *S. epidermidis* (Table 2, Figure 4b).

Table 2. Zone of inhibition of crude metabolites obtained from different endophytic fungi isolated from *L. cubeba* against different gram-positive and gram-negative bacteria

	Zone of inhibition (mm)			
	Gram- positive bacteria		Gram- negative bacteria	
Endophytic fungi	Se	Bs	Kp	Ec
Nigrospora sphaerica	4 ±0.75	8±0.25	6±0.29	3±0.36
Acremonium falciforme	12.3±0.50	5±0.9	2±0.25	-
Periconia hispidula	-	5± 0.55	5±0.29	-
Allomyces arbuscula	-	7±0.32	9±0.19	-
Aureobasidium sp.	-	3±0.50	2.15±0.75	-
Chaetomium sp.	-	7 ±0.45	-	3±0.35
Penicillium chrysogenum	-	3±0.61	2±0.16	-
Acrophialophora sp	-	2 ±0.19	-	-
Mycelia sterilia (1)	-	2±0.23	-	-
Mycelia sterilia (2)	-	3±0.32	-	-
Mycelia sterilia (3)	-	-	3±0.70	-
Mycelia sterilia (4)	2±0.12	3±0.29	-	-
Tetracycline	18.8±0.21	15.05±0.19	4±0.6	13±0.15
Negative control	0	0	0	0

Positive control: Co-assayed antibiotics (Tetracycline-30mcg/disc). Negative control: Sterile disc (5 mm diameter) immersed in Dimethyl sulphoxide (DMSO)

Se=*Staphylococcus epidermidis*, *Bs=Bacillus subtilis*, *Kp= Klebsiella pneumoniae*, *Ec=Escherichia coli*. Data mean of three replicates ± SE.

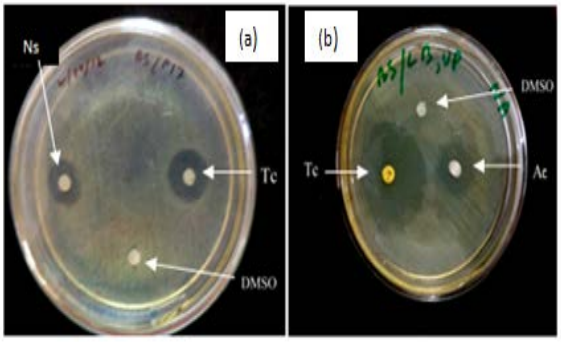

Figure 4. Antibacterial activity of the metabolites produced by (a) *Nigrospora sphaerica* (Ns) against *Bacillus subtilis* and (b) Antibacterial activity of the metabolites produced by *Acremonium falciforme* against *Staphylococcus epidermidis.* Co-assayed antibiotic-tetracycline (Tc-30mcg/disc) and Negative control-Dimethyl Sulfoxide (DMSO).

4. Discussion

Litsea cubeba is of great economic importance due to its high medicinal properties. Meager work has been done on endophytes associated with *L. cubeba.* Over exploitation of these plants for medicinal and commercial purposes has threatened the existence of this plant. Therefore, the present work was carried out with an aim to study the endophytic fungi associated with *L. cubeba* so

that edophytes may be used for antimicrobial metabolites instead of plants, thus conserving the plants. The major objectives of the present work include studying the occurrence of endophytic fungi and to screen and evaluate these microorganisms for the presence of antimicrobial bioactive metabolites. The endophytic fungi were isolated from bark and leaf samples of the plant to screen organ specific endophytes regarding the host plant. Endophytes were generally not considered as organ-specific microbes and it is likely that many of the species isolated from bark may also occur in leaves (Dix *et al.*, 1995). A similar type of study was carried out to evaluate the organ specificity of the endophytes in the host plant. The endophytic fungi viz., *Nigrospora sphaerica*, *Acremonium falciforme*, *P. chrysogenum*, *Allomyces arbuscula* were recovered both from bark and leaf samples of *Litsea cubeba* while some were restricted to a particular organ of the plant. The fungus *Aureobasidium* sp., *Chaetomium* sp., *Periconia hispidula*, and the three mycelia sterilia were isolated only from the bark throughout the study period. The fungus *Acrophialophora* sp. was isolated only from the leaf samples. Tejesvi *et al.* (2005) while working on the endophytic fungi of *Terminalia arjuna* found that the distribution of some taxa and their density was more in inner bark segments compared to the twigs. Chareprasert *et al*. (2006) also recovered more endophytic fungi from leaves of *Tectona grandis L.* and *Samanea saman* Merr. Tejesvi *et al.* (2005) isolated *Chaetomium* and *Penicillium* from *T. arjuna*, a medicinal plant. Some species of endophytes, like *Nigrospora* sp., *Penicillium* sp., *Chaetomium* sp., *Aspergillus* sp. Etc., were isolated from *Rauvolfia serpentina*, a medicinal plant (Daleyi, 2002). *Nigrospora* sp., *Penicillium* sp., *Chaetomium* sp. were also isolated during the present work. Endophytic fungi, *Nigrospora sphaerica*, was also isolated from medicinal plants, viz. *Adhatoda vasica*, *Costus igneus*, *Coleus aromaticus* and *Lawsonia inermis* by Amirita *et al*. (2012). The genera *Penicillium* was amongst the most commonly isolated genera (Santos *et al.*, 2003). *Aureobasidium pullulans*, endophytic fungi isolated from grapevine **(***Vitis vinifera*), play a potential role as biological control agents against grapevine pathogens (Martini *et al.*, 2009). *Aureobasidium pullulans* were also isolated from *L. cubeba* during the present work. *Acremonium sp.*, the dominant endophytic fungi of *L. cubeba* of the present work, was also isolated from grass and found to be antagonistic towards several grass pathogens (White and Cole, 1985).

The culture media can affect the endophytic fungi that produce secondary metabolites. In the present experiment recovery of endophytic fungi differed in different media. The PDA media appeared as the suitable media for isolation of a large number of isolates. It might be due to the nature of carbon and nitrogen constituents of the media (Tayung, 2008). A large number of species were isolated from *L. cubeba* on media amended with bark and leaf extracts due to the addition of some extra nutrients through the host plant part extracts which had a positive effect on the growth of endophytes. This indicates the presence of some substances in the host plant which encourage the growth of the endophytes.

Endophytic fungi are by now recognized as a potential source of anti-microbial secondary metabolites (Strobel and Daisy, 2003; Li *et al.*, 2005; Huang *et al.*, 2008) that could be used for various medicinal purposes. The crude extracts of some endophytic fungi, namely *Acremonium* sp., *Aspergillus terreus*, *A. flavus*, *Alternaria* sp., showed an antimicrobial activity against pathogenic *E. coli*, *Proteus mirabilis*, *S. typhi*, *K. pneumoniae* (Kalyanasundaram *et al.*, 2015). Nwakanma *et al.* (2016) studied antagonistic activity of the crude secondary metabolites of 16 different endophytic fungi isolated from leaves of Bush mango against *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, *P. chrysogenum* and *A. fumigatus.* Pinheiro *et al.* (2017) also found that, among seventeen endophytic fungi isolated from *Bauhinia guianensis*, the fungus *Exserohilum rostratum* showed the highest activity against *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923), *B. subtilis* (ATCC6633) and *S. typhimurium* (ATCC14028). During the present investigation, some of the isolates of *L. cubeba* also showed a very good antimicrobial activity against some microbes, which are of pharmaceutical importance. Endophytic fungi isolated from *L. cubeba*, produced antimicrobial secondary metabolites as most of the crude extracts showed an inhibitory activity against all the test organisms. Gram-positive test bacteria (*B. subtilis*) was more sensitive to the crude extracts of the isolated endophytes than that of gram-negative bacteria which supported the findings of Rakshith and Sreedharamurthy (2011) and Dzoyem *et al.* (2017).

The results of the present work, thus, suggest that *L. cubeba* harbor some endophytic fungi producing antimicrobial secondary metabolite which may have noble compounds. These endophytes may be used as source of therapeutic agents in pharmaceutical industries. However, further investigation is needed for the characterization of these endophytes within the host plant, proper establishment of their role and chemical characterization of secondary metabolites produced by them for their future applications as bio-control and pharmaceutical agents (Dissanayake *et al.*, 2016).

5. Conclusion

The present study reveals that a total of sixty-nine isolates, thirty-six isolates from bark and thirty-three from leaf samples, sheltered *L. cubeba*. These sixty-nine isolates, excluding four mycelia sterilia isolates, belonged to 8 different genera. *Acremonium falciforme* was the most dominant and potent endophyte showing highest antimicrobial activity against *Staphylococcus epidermidis* (MTCC 435). All the isolates showed antimicrobial activity against the test organisms. Thus, it can be concluded from the present investigation that endophytic fungi, isolated from *L. cubeba*, can be used for pharmaceutical purposes. More aggressive investigation is required to better understanding of the metabolomics and endophyte biology of *L. cubeba*.

Conflict of Interest

No conflicts of interest have been declared by the authors.

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