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Antimicrobial Activity of Endophytic Fungi Isolated from a Traditionally Important Plant of NE India–*Litsea chinensis* Lam

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ABSTRACT

Finding new and effective antimicrobial metabolites has become more important as bacterial pathogens have developed a major resistance to antibiotics. Several active metabolites and their antimicrobial properties have been reported from endophytes isolated from medicinal plants. The goal of the current study was to isolate endophytes from the leaves of *Litsea chinensis* and assess the antibacterial activity of the crude metabolites produced by these endophytes. The endophytes were isolated from surface-sterilized healthy leaves inoculated on different mycological media – Potato Dextrose Agar (PDA), Host extract+Agar (HEA) and Water Agar (WA). A total of 27 endophytes were isolated from the three distinct media with the highest colonization frequency in WA (50%) media followed by HEA (20%) and PDA (20%) media. The highest fungal colonies were recovered from WA media, whereas more yeast colonies were isolated from PDA and HEA media. The pure cultures of the isolated endophytes were cultured on Potato Dextrose Broth (PDB) and five different isolates were obtained. The crude metabolites of only one of the isolates (LCEA1) showed promising antimicrobial activity against all the test organisms. The isolate LCEA1 was again tested for antimicrobial activity at 14 and 21 days of incubation, with the maximum antibacterial activity reported at 21 days. However, the highest zone of inhibition (18 mm) was observed against MTCC-737 (*Staphylococcus aureus*) followed by MTCC-736 (*Bacillus subtilis*) (12 mm) and MTCC-424 (*Pseudomonas aeruginosa*) (11 mm). The crude metabolites had a λ_{max} value of 0.53, indicating the presence of some bioactive metabolites responsible for antimicrobial activity.

Key words: Endophytic fungi, *Litsea chinensis*, antimicrobial activity, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*

INTRODUCTION

A pertinent issue for health services is the emergence of pathogenic bacteria and fungi that are resistant to commercial medicines, which has grown to be a major global concern (Smani *et al.*, 2019). Now-a-days, this scenario has been promoted due to several factors, including the excessive and frequently improper use of antibiotics, unsanitary conditions, constant travel, an increase in patients with impaired immune systems and a delay in the detection of infections (dos Santos *et al.*, 2015). As a result, researchers are looking for new and effective antimicrobial agents that are also safe for humans (Savage, 2020).

Endophytes, a particular class of microorganisms can colonize the plants without appearing to harm them (Kandel *et al.*, 2017). Among all endophytic microorganisms, fungi are the most notable ones that shield plants from various diseases (Fontana *et al.*, 2021). Endophytic fungi are well known for producing bioactive secondary metabolites, including alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and lactones (Manganyi and Ateba, 2020). As a result, they are an important source of research for the creation of novel pharmaceuticals for application in industry, agriculture and medicine (Burragoni and Jeon, 2021).

According to several studies, the discovery of new bioactive strains of endophytic fungi has been facilitated by plant species utilized in traditional medicine (Talukdar and Tayung, 2019). Additionally, some studies claim that the therapeutic qualities of medicinal plants may be somewhat influenced by the

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metabolites produced by the endophytic fungi (Caruso *et al.*, 2020). Considering this potential, research into the endophytic composition of a variety of therapeutic plants needs to be done (Liu-Xu *et al.*, 2022). *Litsea* is a significant genus of plants that has been used for centuries for treating a variety of diseases, including stomach-aches, gastroenteritis, diabetes, edema, colds, arthritis, asthma, dyspepsia, traumatic injuries, diarrhea, etc. (Kong *et al.*, 2015). Among the different species under the genus *Litsea*, *L. chinensis* is an important species which possesses significant beneficial activity against cough, swelling, fracture, diarrhea and dryness of skin (Prashanth, 2017). Due to the therapeutic qualities of *L. chinensis*, this species was the focus of the current study to investigate the leaf's antimicrobial capacity of endophytic fungi present in this plant leaves.

MATERIALS AND METHODS

For the investigation of the antimicrobial activity of yeast endophytes, leaves of *L. chinensis* Lam were selected as the source plant material. The plant material was collected from Raha town of Nagaon district of Assam (26°2′N latitude, 92°4′E longitude and 64 m altitude above the sea level; Fig. 1A). The general climate of this area is characterized by hot and wet summers and dry and cool winters which makes it suitable for the excellent growth of several sub-tropical plants. Fresh and healthy leaf tissues were selected and collected for the study (Fig. 1B, C). The leaves were cut off from the selected healthy plants with an ethanol-disinfected sickle and placed immediately in separate zip-lock polythene bags to avoid moisture loss. The samples were then transported to the laboratory within 24 h and stored at 4°C until the isolation procedure was completed.

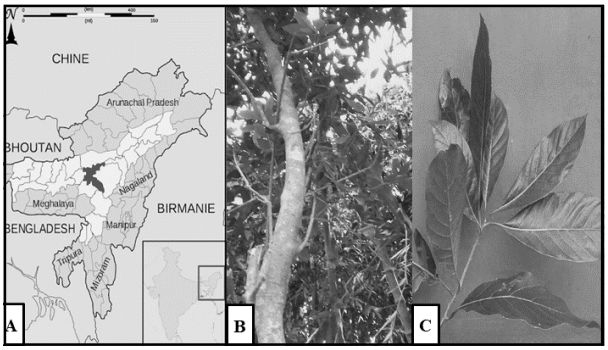

Fig. 1. *L. chinensis*: (A) Map of the study area, (B) Habit of *L. chinensis* and (C) Leaves of *L. chinensis*.

For the isolation of endophytes, the plant material was washed under running tap water to remove any residue and then rinsed with distilled water before surface sterilization. It was air-dried and then the leaf was cut into fragments of medium size to facilitate the further sterilizing process. The fragments of leaves were then subjected to surface sterilization by immersing sequentially in 70% alcohol for 3min, 0.5% NaOCl for 1 min and again in 90% alcohol for 30 sec and were transferred to a laminar airflow hood where they were rinsed thoroughly with sterile distilled water and excess was dried out using sterilized blotting paper. The fragments of leaves were cut into small pieces with the help of a sterilized puncher. A total of 90 leaf pieces were then inoculated on Petri plates using three different media, namely, Potato Dextrose Agar (PDA), Host Extract+Agar (HEA) and Water Agar (WA). The Petri plates were sealed with parafilm and incubated in a BOD incubator at 24±1°C for several days until fungal growth on the plate was visible. The filamentous fungi growing out of the plated samples were removed with a fine sterile needle and transferred to freshly prepared PDA slants, incubated for 1-2 weeks and periodically checked for purity. The fungal isolates were observed under the microscope and their characters were noted. Cultures that failed to sporulate were recorded as sterile mycelia. The pure endophytic fungal isolates obtained were preserved in a refrigerator for further work.

The pure fungal endophytes obtained were transferred to slants and from the slants, they were cultivated in Potato Dextrose Broth (PDB) for determination of antimicrobial activity. Agar blocks of actively developing culture were used to culture the fungal isolates in conical flasks containing 100 ml of the PDB medium. The flasks were then incubated at 30°C in a BOD incubator for two weeks while being shaken three times per day. After the incubation period, the flasks were then taken out and filtered through sterile Whatman filter paper to remove the mycelia mats. The liquid broth was collected and extracted with an equal volume of ethyl acetate in a separating funnel by vigorous shaking for 10 min. The cell mass was separated and the solvent so obtained was collected. The solvent was then evaporated and

the crude metabolite thus obtained was used to test for antimicrobial activities against some common human pathogens using the Agar cup Diffusion method.

The crude metabolites obtained from the endophytic isolates were dissolved in ethyl acetate solvent and the metabolites were screened for the presence of bioactives using a spectrophotometer for the λ_{max} in scanning mode at wavelength 230-440 nm. The peak obtained was noted.

Both gram-positive and gram-negative bacteria were used as test organisms for the evaluation of the antimicrobial activity of the endophytic isolates. The gram-positive organisms include MTCC-736 (*Bacillus subtilis*), MTCC-737 (*Staphylococcus aureus*) and the gram-negative organism include MTCC-424 (*Pseudomonas aeruginosa*). The strains were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The bacterial strains were maintained on freshly prepared nutrient agar slants and stored at 4°C for further use.

The crude metabolites obtained were determined for antimicrobial activity following the agar cup diffusion method. Petriplates containing nutrient broth and agar medium were prepared according to the manufacturer's instructions. The test organisms stored at 4°C were taken out and cultured in test tubes containing nutrient broth and keeping them for overnight in the incubator. The test organisms were then swabbed over the media with a cotton swab on the petriplates and agar cups were prepared at an equal distance using a cork borer (5 agar cups in each petriplate). Each agar cup was loaded with 100 µl of the crude metabolites. Antimicrobial activity was then determined at 14 and 21 days of incubation.

The colonization frequency (CF%) of endophytic fungi was calculated using the formula:

$$\text{Colonization frequency} = \frac{\text{Total number of leaf segments colonized by endophytes}}{\text{Total number of leaf segments inoculated}} \times 100$$

RESULTS AND DISCUSSION

Fresh and young healthy leaves of *L. chinensis* were selected for endophytic study. By culturing on different mediums, the leaves were found to be colonized with endophytic fungi (Fig. 2A-C). It was observed that the recovery of endophytes was more in WA media as compared with HEA and PDA media. A similar result was also reported where WA media showed higher efficiency during the recovery of endophytes from the leaves of *Eyngium foetidum* as compared with the other media (Talukdar and Tayung, 2019). The potential cause for this might be due to the fact that fungal endophytes work well on WA medium and might not be required to a greater extent of nutrients like PDA media. In total, 27 endophytes were isolated from the three distinct media (Table 1). The growth of endophytes (incubation period) was found to be fairly constant in all the nutrient media. The incubation periods for the growth of endophytes vary according to their host tissue but the effects of different media on incubation periods for the emergence of endophytes are still under research (Murphy *et al.*, 2015; Zheng *et al.*, 2021). In WA media, other fungal colonies were more than yeast colonies. This might be due to the yeast having a higher nutrient requirement, which may be provided by the PDA medium, compared to other endophytic fungi, which have a lower nutrient requirement and can therefore flourish in WA

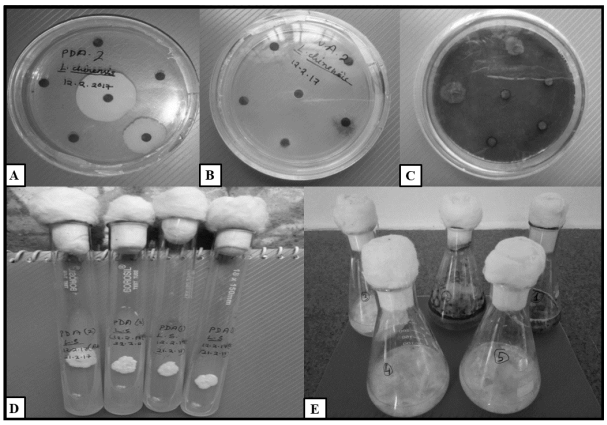

Fig. 2. Culture of endophytes on different media: (A) PDA medium, (B) WA medium, (C) HEA medium, (D) Pure culture of some endophytic isolates and (E) Endophytic isolates cultured in PDB.

Table 1. Isolation of endophytes in different media from surface sterilized leaf tissue of *L. chinensis*

Media	No. of colonies
Potato dextrose agar (PDA)	06
Water agar (WA)	15
Host extract+agar (HEA)	06

media (Sia *et al.*, 2013). It was observed that the highest numbers of fungal colonies were isolated from the WA medium, whereas the highest numbers of yeast colonies were recovered from HEA and PDA media. This might be due to the high concentration of dextrose present in the PDA media which may fulfil the nutrient requirements for the growth of endophytic yeast (Sia *et al.*, 2013). Similar studies were also reported in *Olea europaea* (Sia *et al.*, 2013), *Paullinia cupana* (Sia *et al.*, 2013), *Aegle marmelos* (Mani *et al.*, 2015), *Triticum* spp. (Ripa *et al.*, 2019), etc. Also, the colonization frequency of fungal endophytes was analyzed in their colonies recovered in different media, which showed that the colonization frequency was highest in WA (50%) than in the HEA (20%) and PDA (20%) media (Table 2). A similar result was also reported from the leaves of *Eyngium foetidum* (Talukdar and Tayung, 2019). The endophytes were also studied for their colonial morphology and microscopic characters for identification. It was observed that most of the endophytes did not produce spores and the cultures that failed to sporulate were recorded as sterile mycelia. The isolated fungi were grown on a PDA medium to obtain pure cultures (Fig. 2D). The isolated, pure fungi were then stored in the refrigerator for further study.

Table 2. Colonization frequency of endophytes in different media

Media	Colonization frequency (%)
Potato dextrose agar (PDA)	20
Water agar (WA)	50
Host extract+agar (HEA)	20

Pure cultures of the isolated yeast and other fungal endophytes were selected and cultured on a PDB medium for testing antimicrobial activities. From the PDB medium, the culture filtrates of five different fungal isolates were obtained (Fig. 2E). Among all, only one of the endophytic fungal isolates (LCEA1) showed promising antimicrobial activity inhibiting all the test organisms (Table 3). The highest antimicrobial activity was observed against *Staphylococcus aureus* followed by *Bacillus subtilis* and *Pseudomonas aeruginosa*. This is consistent with the result obtained by Deka and Jha (2017). The endophytic isolate LCEA1 was chosen for further investigation based on the outcome of the preliminary antimicrobial activity.

Table 3. Antimicrobial activity of crude metabolites of fungal endophytic isolates against test pathogens

Isolate No.	*Pa*	*Bs*	*Sa*
LCEA1	+	+	++
LCWA2	--	--	--
LCWA3	--	--	--
LCWA4	--	--	--
LCWA5	--	--	--

Pa: *P. aeruginosa*, *Bs*: *B. subtilis*, *Sa*: *S. aureus*, indicating no inhibition zone, + indicates zone of inhibition > 10 mm < 12 mm and ++ indicates zone of inhibition > 12 mm < 15 mm.

The fungal isolate LCEA1 was again cultivated in PDB media and the antimicrobial activity of the crude metabolite was determined after 14 and 21 days of incubation. The results indicated that higher antimicrobial activity was observed in 21 days of incubation against the entire test organism (Fig. 3A). Similar observations were also reported in crude metabolite from *Vitex negundo* where higher antimicrobial activity was observed after 21 days of incubation (Desale, 2016). However, it was found that the highest zone of inhibition was observed against *Staphylococcus aureus* (Fig. 3B-C). The crude metabolite was effective against MTCC-737 (*S. aureus*) with a zone of inhibition of 18 mm, followed by MTCC-736 (*B. subtilis*) and MTCC-424 (*P. aeruginosa*) with a zone of inhibition of 12 and 11 mm,

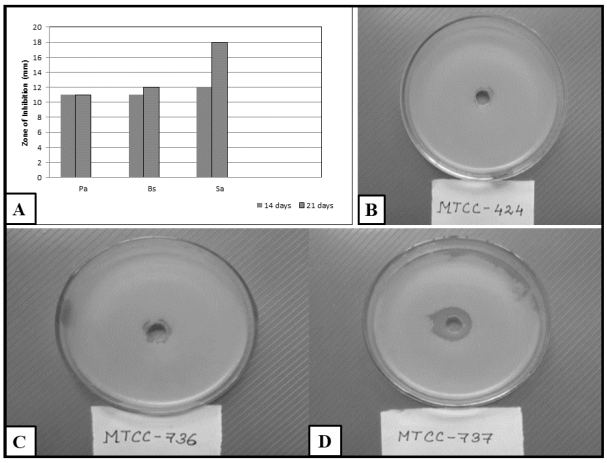

Fig. 3. (A) Antimicrobial activity of endophytic isolate LCEA1 at 14 and 21 days of incubation period (Pa–*P. aeruginosa*; Bs–*B. subtilis*; Sa–*S. aureus*); (B) Antimicrobial activity of the crude metabolite of isolate LCEA1 against (B) MTCC-424 (*P. aeruginosa*); (C) MTCC-736 (*B. subtilis*) and (D) MTCC-737 (*S. aureus*).

respectively, at 21 days from the incubation period. Also, the crude metabolite was subjected to UV-spectrophotometer for detection of the presence of bioactive compound. The crude metabolite showed a λ_{max} value of 0.53 with an absorbance of 260 indicating the presence of some bioactive metabolites responsible for antimicrobial activity (Fig. 4). Similar observations were also reported in *Terminalia mantaly*, *Terminalia catappa*, *Cananga odorata* (Mbekou *et al.*, 2021) and *Annona muricata* (Yimgang *et al.*, 2022), etc.

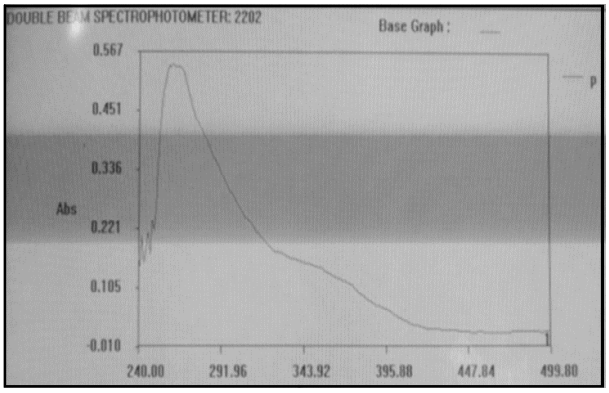

Fig. 4. λ_{max} value (0.53) of the crude metabolite of endophytic isolate LCEA1 in spectrophotometer.

CONCLUSION

The ubiquity of endophytes in the plant kingdom has been well established as they have been found to be distributed in all species investigated including both higher plants and algae. The current study reported antimicrobial activity of endophytic isolates from *L. chinensis*, in which the isolate LCEA1 demonstrated the ability to produce bioactive agents against all the test pathogens, and may provide a new lead in the pursuit of new biological sources of drug candidates. Furthermore, our spectrophotometric findings suggest that the ability of this isolate to produce bioactive compounds may be contributing factor to its antimicrobial activity and hence the plant's recognition for its medicinal properties. The results, therefore, indicate its potentiality in pharmaceutical and/or agricultural industries and could be an alternative source of antimicrobial agents. However, more research is needed to identify the active compounds produced to discover new antimicrobial drugs.

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