EXPLORING ANTIBACTERIAL AND CYTOTOXIC POTENTIAL OF ENDOPHYTIC Streptomyces ISOLATED FROM THE MEDICINAL PLANT Litsea cubeba (Lour.) Pers

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Abstract. Over the past decades, researchers and scientists have paid more and more attention to the discovery of novel antibiotics and anticancer agents in fighting infection and cancer diseases. It is believed that endophytic actinomycetes from medicinal plants provide abundant resources for new compounds with significant bioactive properties. In the present study, we assessed antibacterial and cytotoxic activities of endophytic actinomycetes associated with Litsea cubeba (Lour.) Pers. A total of 45 endophytic actinomycetes were isolated from Litsea cubeba (Lour.) Pers were collected at Lai Chau province, Viet Nam, with the maximum number of isolates obtained from roots (44.4 %), followed by stems (35.5 %), and leaves (20.1 %). Among them, 3 isolates LCL08, LCL25, LCL28 showed a broad spectrum of antibacterial activity against six tested bacteria, which were subsequently identified as Streptomyces albogriseolus LCL08, S. olivaceus LCL25, and S. cavourens LCL28 by using 16S rRNA sequence analysis. This is the first report of S. albogriseolus isolated as endophyte. Ethyl acetate extracted from these 3 strains exhibited strong antibacterial activity against 6 tested pathogens with a minimum inhibitory concentration ranging from 2 to 64 μg/mL. Moreover, these extracts showed remarkable cytotoxic effects against Hep3B human hepatoma, MCF-7 breast cancer, and A549 lung cancer cell lines with the recorded viability of tested cancer cells ranging from 13.87 to 65.69 %. These findings suggest that Streptomyces spp. recovered from Litsea cubeba (Lour.) Pers are an excellent source of natural antibacterial and cancer compounds that could be promising for further research.

Keywords: antibacterial activity, cytotoxic activity, endophytic actinomycetes, Lai Chau province, Litsea cubeba (Lour.) Pers, Streptomyces.

Classification numbers: 1.2.1, 1.2.3

1. INTRODUCTION

The misuse and overuse of antibiotic have lead to the emerging threat of multidrug resistant pathogens in recent decades, causing immense medical and environmental problems worldwide.
Additionally, cancer, being a major cause of death globally, has yet been completely cured without a competent cancer drug [3]. The rapid rise of antimicrobial resistance along with cancer drug resistance has reduced the efficacy of current therapeutic agents, pressing an urgent need for novel natural compounds with remarkable antimicrobial and cytotoxic potential [4].

Endophytic actinomycetes associated with medicinal plants are known to be a producer of novel bioactive compounds, which have been used in industry and pharmacy [5]. Among endophytic actinomycetes found in medicinal plants, *Streptomyces* was the most frequently detected genera followed by *Micromonospora*, *Actinopolyspora*, *Nocardia*, *Saccharopolyspora*, and *Streptosporangium* [6]. To date, the genus *Streptomyces* is considered a prolific producer of about 70% of natural antibiotics in clinical practices [7]. Numerous antimicrobial antibiotics including cedarmycins, munumbicins, kakadumycins, xiamycins, and profilic producer *Saccharopolyspora* spp. have been used as chemotherapeutic agents in the fight against multidrug-resistant bacteria including several Gram-positive bacteria such as *Bacillus anthracis*, *Enterococcus faecalis*, and *Staphylococcus aureus* and multidrug-resistant bacteria including methicillin-resistant strain of *S. aureus* (MRSA), vancomycin-resistant *E. faecalis*, and multiple-drug-resistant *Mycobacterium tuberculosis* [13]. In addition, *Streptomyces* spp. bear a fascinating ability to synthesize anticancer agents. Under laboratory conditions, two novel compounds including azalomycin F4a 2-ethylpentyl ester and azalomycin F5a 2-ethylpentyl ester from endophytic mangrove *Streptomyces* sp. 211726 were proved to inhibit HCT-116 colon cancer cell line with IC50 values as low as 5.64 µg/mL and 2.58 µg/mL, respectively [14]. Halichobelides isolated from mangrove-derived *Streptomyces* sp. 219807 exhibited strong cytotoxic activity against the human cancer cells including HeLa and breast cancer MCF-7 cells [15]. Doxorubicin and its precursor daunorubicin from *Streptomyces peucetius* were among the first of its kind as chemotherapy drugs, playing an essential role in cancer treatment [16].

The medicinal plant *Litsea cubeba* (Lour) Pers. is an evergreen tree distributed mainly in tropical and subtropical regions such as China, India and Viet Nam, which has been used as a therapeutic agent for inflammation, headache and intoxication [17]. *L. cubeba* essential oils combined with *Streptomyces griseorubens* MT42 extract exhibited synergistic antimicrobial activity against multidrug resistant bacteria including several Gram-positive and negative pathogens [18]. Although the distribution of endophytic actinomycetes isolated from *Litsea cubeba* (Lour.) Pers was revealed previously [19], therapeutic potential of actinomycetes has not been systematically explored. In this study, we explored antibacterial and anticancer potential of endophytic actinomycetes from *Litsea cubeba* (Lour.) Pers. collected in Lai Chau province, Viet Nam. The findings will contribute to the wealth of knowledge of endophytic actinomycetes from medicinal plants and their potential as alternative antimicrobial and anticancer agents in the fight against diseases.

### 2. MATERIAL AND METHODS

#### 2.1. Materials

Six pathogenic bacteria including *Escherichia coli* ATCC 11,105, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027, methicillin-resistant *Staphylococcus epidermidis* (MRSE) ATCC 35984, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 25923, and *Bacillus cereus* ATCC 11778 were obtained from the Institute of Biotechnology, Vietnam Academy of Science and Technology.
Human hepatoma Hep3B, MCF-7 breast cancer and A549 lung cancer cell lines were kindly provided by Prof. Jeong-Hyung Lee, Department of Biochemistry, College of Natural Sciences, Kangwon National University, Korea.

2.2. Methods

2.2.1. Isolation of endophytic actinomycetes associated with Litsea Cubeba

*Litsea cubeba* samples divided into 3 organs including roots, stems, and leaves were collected from Lai Chau province, a Northwest high-mountainous area of Viet Nam (22°21’41”N, 103°16’4’’E). The samples were separately placed in plastic bags, transported to the laboratory of the Institute of Biotechnology, Vietnam Academy of Science and Technology, and then kept at 4 °C. The plant voucher specimens were then identified as *Litsea cubeba* (Lour.) Per by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology as described previously [18, 19]. All plant samples collected were surface-sterilized, homogenized and then inoculated on nine selective media commonly used for the isolation of actinomycetes as described previously [20]. Apparent actinomycete colonies were rapidly picked up and streaked onto ISP2 agar medium. Pure isolates were recovered and stored in 15% glycerol at –80 °C.

2.2.2. Identification using 16S rRNA gene and phylogenetic analysis

Genomic DNA was extracted using a G-spin™ Total DNA Extraction Mini Kit (Intron Bio, Korea) following the manufacturer’s instructions. The universal primers of 27F (5′-TAACACATGCAAGTCGAACG-3′) and 1492R (5′-GGTGTGACGGGCGGTGTGA-3′) were utilized for PCR amplification [21]. The 16S rRNA was sequenced by First BASE Laboratories Sdn. Bhd. (Malaysia). The resulting sequences were analyzed using BioEdit software (version 7.2) and aligned with reference sequences retrieved from GenBank based on BLAST searches. The phylogenetic tree was computed using the maximum-likelihood method based on the Tamura-Nei model, Gamma distributed with a bootstrap of 1000 replications in MEGA7. *Bacillus aryabhattai* B8W22 was used as an outgroup branch.

2.2.3 Evaluation of antibacterial activities

Antimicrobial activity against pathogenic bacteria was assessed using agar-well dilution method [22]. Briefly, all endophytic isolates were cultivated in YIM38 medium at 30 °C for 5 days under shaking conditions (150 rpm). The obtained cell-free supernatant of each strain was used to evaluate antimicrobial activity against 6 pathogenic bacteria including *E. coli* ATCC 11105, *S. typhimurium* ATCC 14028, *P. aeruginosa* ATCC 9027, MRSAATCC 25923, MRSE ATCC 35984, and *B. cereus* ATCC 11778. Wells were cut using a sterile cork borer, and filled with 0.1 mL of cell-free supernatant. The experiment was performed in triplicate and the diameter of the inhibition zone was determined after 12-16 h of incubation at 37 °C.

The cell-free supernatant of bioactive strains was extracted with ethyl acetate (1:1, v/v). The mixture was vigorously shaken for 30 min and kept stationary for 60 min to separate the aqeous and organic phases. The organic phase was then evaporated on a rotary evaporator (Scilogex RE100-Pro, USA) to determine the weight of the extract. The antibacterial activity of crude extracts was assessed using the minimum inhibitory concentration (MIC) [23]. All tested bacteria were grown in LB medium and transferred to a 96-well microtitre plate. Serially
diluted fraction of the extract dissolved in DMSO was added to the well to give final concentrations (0.5–128 μg/mL), followed by incubation at 37 °C. MIC values were considered as the lowest concentration with no visible growth of microbes after 24 h of incubation.

2.2.4 Cytotoxic assay

The cytotoxic effect of ethyl acetate extract obtained from bioactive actinomycetes was tested against human hepatoma Hep3B, MCF-7 breast cancer and A549 lung cancer cell lines using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [24]. Briefly, the cells were incubated at 37 °C with 5 % CO₂ in RMPI medium supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin to avoid contaminations. The cells were then seeded and grown on 96-well plates at 37 °C for 48 h. The cells were then treated with 30 μg/mL and 100 μg/mL extracts for 24 h. After the incubation, 20 μL of MTT (5 mg/mL in PBS) was added to each well and the plates were incubated at 37 °C with 5% CO₂ for 4 h. After removing the supernatant, formazan crystals were dissolved in isopropanol and the optical density was measured at 570 nm. Camptothecin was used as a positive control (concentrations of 1 μM and 10 μM), and 0.1 % DMSO served as a negative control. A decrease in cell viability to less than 50 % was noted as a positive outcome.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of bioactive endophytic actinomycetes

A total of 45 endophytic actinomycetes were recovered from healthy medicinal plants Litsea cubeba based on morphological characteristics. In the control samples, no colonies were observed on the isolation medium after the last wash of the sterilization process. Of the 45 isolates, those from roots accounted for the highest number of isolates (n = 20; 44.4 %), followed by stems (n = 16; 35.5 %), and leaves (n = 9; 20.1 %). Most colonies grew slowly on the isolation media and formed different colors such as white, grey, blackish white, and brownish white (Figure 1). The distribution of endophytic actinomycetes was in agreement with previous studies in which actinomycetes isolated from roots were predominant [25, 26]. In Cinnamomum cassia Presl, actinomycetes were mainly isolated from stems [20]. This indicated that the distribution of endophytes might vary depending on plant species, specific plant tissues and isolation approaches.

![Figure 1. Colony characteristics of some endophytic actinomycetes isolated from Litsea cubeba.](image)

All isolates were evaluated for their antibacterial activities against six tested bacteria (E. coli ATCC 11105, S. typhimurium ATCC 14028, P. aeruginosa ATCC 9027, MRSA, MRSE, and B. cereus ATCC 11778). Of the 45 isolates, 13 isolates (28.9 %) exhibited antibacterial
activity against at least one pathogen (Figure 2). Various studies proved that the proportion of endophytic actinomycetes with remarkable antimicrobial activity found in medicinal plants is always higher than that of crop plants [13]. The percentage of bioactive isolates obtained in this study was lower than that of C. cassia Presl (34.2 %) and Rhynchotoechum ellipticum (47.9 %) [20, 27]. Gram-positive bacteria including MRSAATCC 25923, MRSEATCC 35984, and B. cereus ATCC 11778 were much more sensitive to the obtained isolates. Surprisingly, three isolates displaying significant antibacterial potential against six pathogenic bacteria formed a group as shown in Figure 2. Isolate LCL28 showed strong inhibitory effects against E. coli ATCC 11105 (16.0 ± 0.4 mm), S. typhimurium ATCC 14028 (17.9 ± 0.1 mm), P. aeruginosa ATCC 9027 (7.9 ± 0.1 mm), MRSAATCC 25923 (32.5 ± 0.1 mm), MRSEATCC 35984 (23.6 ± 0.6 mm), and B. cereus ATCC 11778 (12.3 ± 0.1 mm). Similar to isolate LCL28, LCL08 and LCL25 also exhibited significant broad-spectrum antibacterial activity against all tested pathogens. These results suggested that these three actinomycetes were promising candidates to exploit new antibacterial compounds.

Figure 2. Antibacterial activities of endophytic actinomycetes. Pathogenic bacteria: 1, E. coli ATCC 11105; 2, S. typhimurium ATCC 14028; 3, P. aeruginosa ATCC 9027; 4, MRSAATCC 25923; 5, MRSEATCC 35984; 6, B. cereus ATCC 11778.

3.2. Molecular identification of the bioactive strains

By analyzing 16S rRNA gene sequence, three isolated strains LCL08, LCL25, and LCL28 possessing potent antibacterial activity belonged to the genus Streptomyces. LCL08 and LCL25
showed 100% similarity to strains of \textit{S. alborgriseolus} DSM 40003\textsuperscript{T} and \textit{S. olivaceus} NBRC 12805\textsuperscript{T}, respectively. Meanwhile, LCL 28 showed a close phylogenetic relationship with \textit{S. cavourensis} NRRL 2740\textsuperscript{T} and \textit{S. cavourensis} NRBC 13026\textsuperscript{T} with similarity of about 99%. The phylogenetic tree showed three clusters in which LCL08, LCL25, and LCL28, each formed its distinct cluster with closely related \textit{Streptomyces} species (Figure 3). Taken together, LCL08, LCL25, and LCL28 were identified as \textit{S. alborgriseolus}, \textit{S. olivaceus}, and \textit{S. cavourensis}, respectively. The 16S rRNA gene sequences of LCL08, LCL25, and LCL28 were deposited in GenBank under accession numbers OK614088, OK614089, and OK614090, respectively.

In recent years, researchers have focused on exploring the possibility of synthesizing bioactive metabolites from medicinal plants involving actinobacteria, with an emphasis on \textit{Streptomyces}, a prolific producer of novel antimicrobial compounds [1, 2, 5, 18, 21, 28]. \textit{Streptomyces} spp. isolated from mangrove ecosystem showed strong antibacterial activity against clinical isolates such as MRSA, MRSE, and \textit{S. Typhimurium} [29]. Secondary metabolites from \textit{Streptomyces} sp. HUST012 showed significant inhibition of MRSA, MRSE, \textit{E. coli}, and \textit{Klebsiella pneumoniae} with MIC values ranging from 0.04 to 62.5 μg/mL [21]. In this study, while \textit{S. olivaceus} and \textit{S. cavourensis} were previously isolated from several plants such as \textit{R. ellipticum}, \textit{C.cassia} Presl, and \textit{Triticum aestivum} [20, 27, 30], this is the first report of \textit{S. alborgriseolus} isolated as endophyte.

\section*{3.3. Evaluation of antibacterial activity}

To assess precisely antibacterial activities, the antibacterial potential of crude extracts of three bioactive actinomycetes was determined using MIC assay. It was shown that all three strains exhibited effective antibacterial activity with MIC values from 2 to 64 μg/mL (Table 1). Regarding the Gram-negative bacteria, MIC ranges from 16 to 32 μg/mL for \textit{E. coli} ATCC 11105, 2-8 μg/mL for both \textit{S. typhimurium} ATCC 14028 and \textit{P. aeruginosa} ATCC 9027.
Gram-positive bacteria were resistant to the extracts with MIC value ranging from 2 to 64 µg/mL. The S. olivaceus LCL25 extract showed significant broad-spectrum antibacterial activity against 6 pathogenic bacteria followed by S. Albogriseolus LCL08 and S. Cavourensis LCL28. These results proved that lower concentrations of the extract succeeded in antibacterial activity, indicating an excellent antibacterial property.

As described previously, S. Albogriseolus has been mainly isolated from soils and marine environments, which was reported to produce a bioactive fatty acid methyl ester - methyl-4,8-dimethylnodecanate and silver nanoparticles (AgNPs) against pathogens [31, 32]. Since a few antimicrobial compounds have been revealed in S. albogriseolus, S. Albogriseolus LCL08 is a promising candidate in the search for a novel compound. Different to S. albogriseolus, marine S. olivaceus FXJ8.012 produced mycemycins A-D that inhibited E.coli and P.aeruginosa [33]. A recent study proved that S. olivaceus LEP7 recovered from lichens yielded cyclopentene inhibiting wound pathogens [28]. It would be interesting to discover antibacterial compounds extracted from endophytic S. olivaceus LCL25.

### Table 1. Antibacterial activity of the ethyl acetate extracts from 3 Streptomyces strains against six pathogens.

<table>
<thead>
<tr>
<th>Bacterial Pathogens</th>
<th>MIC (µg/mL)</th>
<th>S. albogriseolus LCL08</th>
<th>S. olivaceolus LCL25</th>
<th>S. cavourensis LCL28</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli ATCC 11105</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>S. typhimurium ATCC 14028</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa ATCC 9027</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MRSA ATCC 25923</td>
<td>16</td>
<td>2</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>MRSE ATCC 35984</td>
<td>32</td>
<td>2</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>B. cereus ATCC 11778</td>
<td>2</td>
<td>8</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

On the other hand, we observed that S. cavourensis LCL28 extract displayed the strongest antimicrobial activity against P. aeruginosa ATCC 9027 and S. typhimurium ATCC 14028 with MIC of 2 µg/mL and 8 µg/mL, respectively (Table 1). Hoyos et al. reported that the extract from S. cavourensis 1AS2a exhibited strong antimicrobial activities against plant pathogens [34]. Our previous study proved that 5 bioactive compounds including 1-monolinolein, bafilomycin D, non-actic acid, daidzein, and 3'-hydroxydaidzein from S. cavourensis YBQ59 associated with C. cassia Presl displayed high potential effects against MRSA and MRSE, but not with E. coli ATCC 11105, S. typhimurium ATCC 14028, and P. aeruginosa ATCC 9027 [35]. These evidences signify the diverse antimicrobial properties of S. cavourensis, which might significantly vary depending on the host plants.

### 3.4. Evaluation of the cytotoxic activity

The crude extracts from strains LCL08, LCL25, and LCL28 were evaluated for their cytotoxic activity against several human cancer cell lines such as A549, Hep3B and MCF-7. At the highest concentration (100 µg/mL), the extracts exhibited significant growth inhibitory activity against tested cell lines indicated by the recorded viability of the cancer cells tested ranging from 13.87 to 65.69 % (Table 2). Reducing the extract concentration to 30 µg/mL resulted in a lower cytotoxic effect. When treated with 30 µg/mL extract, the cytotoxic potential of strain LCL28 on A549, Hep3B and MCF-7 turned out to be negligible, while significant growth inhibitory efficacy was observed in strains LCL08 and LCL25. A549 was much more
susceptible to three extracts, while Hep3B was tolerant to all strains with higher rates of cell viability observed for both concentrations.

The anticancer or antitumor effects may be driven by the response of various cellular homeostasis components such as the p53 tumor suppressor or cyclophilin A upon the presence of bioactive metabolites [16, 36, 37]. In fact, migrastatin from *Streptomyces platensis* was shown to increase the expression of p53 leading to apoptosis of human liver cancer cells [36]. Moreover, sanglifehrin A isolated from *Streptomyces* sp. A92-308110 was a CypA-binding compound [37] which was reported to synergistically enhance cisplatin-induced apoptosis in liver cancer cell line by abrogating chemotherapy resistance [38]. In this study, the remarkable anticancer activity against all cell lines obtained at 100 μg/mL may be resulted from the inhibitory effects on p53 tumor suppressor protein [30]. Further studies should explore secondary metabolites secreted by strains LCL08, LCL25, and LCL28 revealed in this study to elucidate their cytotoxic activities on specific cellular targets.

Table 2. The cytotoxic activity of 3 bioactive strains against human carcinoma cell lines.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration</th>
<th>A549</th>
<th>Hep3B</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. albogriseolus</em></td>
<td>30 μg/mL</td>
<td>29.77 ± 2.97</td>
<td>65.21 ± 2.78</td>
<td>53.79 ± 2.49</td>
</tr>
<tr>
<td></td>
<td>100 μg/mL</td>
<td>13.87 ± 2.17</td>
<td>38.11 ± 2.03</td>
<td>29.95 ± 3.08</td>
</tr>
<tr>
<td><em>S. olivaceus</em></td>
<td>30 μg/mL</td>
<td>31.53 ± 1.74</td>
<td>48.83 ± 1.45</td>
<td>51.16 ± 1.71</td>
</tr>
<tr>
<td></td>
<td>100 μg/mL</td>
<td>24.16 ± 1.92</td>
<td>31.56 ± 1.79</td>
<td>21.80 ± 3.04</td>
</tr>
<tr>
<td><em>S. cavourensis</em></td>
<td>30 μg/mL</td>
<td>52.16 ± 2.13</td>
<td>94.94 ± 1.26</td>
<td>62.36 ± 1.54</td>
</tr>
<tr>
<td></td>
<td>100 μg/mL</td>
<td>35.56 ± 1.51</td>
<td>65.69 ± 1.13</td>
<td>41.11 ± 2.74</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>0.1 μM</td>
<td>66.12 ± 3.10</td>
<td>52.76 ± 1.89</td>
<td>62.56 ± 3.22</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>39.23 ± 2.52</td>
<td>26.87 ± 1.54</td>
<td>43.25 ± 3.68</td>
</tr>
</tbody>
</table>

Camptothecin: anticancer drug used as the standard.

4. CONCLUSION

The current investigation sheds light on the endophytic *Streptomyces* spp. associated with *Litsea cubeba* (Lour.) Pers that are a prolific source to exploit new antibacterial and anticancer agents. Endophytic actinomycetes inhabited mainly in roots, followed by stems and leaves. Among them, *S. albogriseolus* LCL08, *S. olivaceus* LCL25, and *S. cavourensis* LCL28 could serve as a producer of bioactive compounds in combating cancer and infectious diseases. To our knowledge, this is the first study showing systematically endophytic actinomycetes derived from *Litsea cubeba* (Lour.) Pers collected from Lai Chau province, Viet Nam and their antibacterial and anticancer potential. Further studies are under progress to identify bioactive metabolites from these three strains, which can be used for pharmaceutical applications.

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Declaration of competing interest. We have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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