

Antibacterial and antibiofilm activity of *Lagotis brachystachya* extract against extended-spectrum β -lactamases-producing *Escherichia coli* from broiler chickens

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ABSTRACT *Lagotis brachystachya* Maxim (*L. brachystachya*) is an herb widely used in traditional Tibetan medicine. In the present study, the antibacterial activity of *L. brachystachya* extract to extended-spectrum-lactamases (ESBLs)-producing *E. coli* was determined by Kirby-Bauer disc diffusion, minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) methods as well as time-kill curve assay. Meanwhile, the biofilm inhibition and eradication effects of *L. brachystachya* extract on the ESBLs-producing *E. coli* were evaluated by crystal violet staining, and further confirmed by confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM). The results indicated that *L. brachystachya* extract exhibited moderate antibacterial activity, with diameter of inhibition zones varying from 15.4 to 20.3 mm, and the MIC and MBC values were 6.25 to 25 mg/mL and

12.5 to 100 mg/mL, respectively. Time-kill curve showed that $4 \times$ MIC level of *L. brachystachya* extract concentration of was able to kill 99.9% of ESBLs-producing *E. coli* after 16 h treatment. The biofilm inhibition rate and eradication rate for the ESBLs-producing *E. coli* were 35.66 to 79.91% and 22.18 to 56.21% at MIC level of extract concentration, respectively. CLSM images showed that the biofilm became thinner as the ESBLs-producing *E. coli* isolate exposed to *L. brachystachya* extract with a concentration-dependent manner from $1/4 \times$ MIC to MIC compared with the control isolate. SEM images indicated that *L. brachystachya* extract at $1/2 \times$ MIC and MIC levels could evidently inhibit the biofilm formation or eradicate the mature biofilms. The effect of *L. brachystachya* highlights its potential of antibacterial and antibiofilm activities against the ESBLs-producing *E. coli*.

Key words: *Lagotis brachystachya*, *Escherichia coli*, ESBLs, biofilm, broiler

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INTRODUCTION

The excessive use and misuse of antibiotics led to the escalation of the extended-spectrum β -lactamases (ESBLs)-producing bacteria that is becoming increasingly difficult to treat (Yigin, 2021). The ESBLs-producing bacteria confer resistance to β -lactams antibiotics, especially the third and fourth-generation extended-spectrum β -lactam antibiotics. Moreover, ESBLs-producing bacteria also typically exhibit resistance to other classes of antibiotics, and express the multidrug resistance (MDR) phenotype (Jo et al., 2021). MDR isolates have escalated and become a significant cause of morbidity and mortality worldwide (Lay et al., 2021). The prevalence of MDR ESBLs-producing *E. coli* may pose a

serious challenge to the clinically sick and healthy broiler chickens because of the transmission of ESBLs producers as well as horizontal plasmid and gene transfer between broiler and humans (Li et al., 2016).

Biofilms were defined by Donlan and Costerton (Donlan and Costerton, 2002), and the bacteria survive by forming biofilm, which is a complex matrix of microorganisms, and the biofilm formation can make most of the bacterial species resistance to antibiotics, even the conventional disinfectants (Charlebois et al., 2017; Lu et al., 2021). Biofilm is an important resistant mechanism of diverse bacteria as it could assist the bacteria withstands the antimicrobial agents, and immunological defense systems (Lee et al., 2019). Moreover, there is a positive correlation between the biofilm formation and ESBLs-producing bacteria that confer resistance to antibiotics as biofilm may create a suitable environment for horizontal gene transfer (Shrestha et al., 2019). Therefore, it is urgent to explore more effective biofilm inhibitors, involving the use of plant origin (Xie et al., 2017). As one of the China's traditional medical systems, Tibetan medicine is

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receiving greater attention from the public, scholars and the media due to their special geographical environment on the Qinghai-Tibet Plateau and particular biological activities, such as anti-inflammatory, antioxidant, and immunotropic activity (Zhang et al., 2008). A number of Tibetan herbal medicines have been well-documented due to their excellent antimicrobial activities and good safety profile (Zhang et al., 2008; Radomska-Lesniewska et al., 2013; Shang et al., 2014).

The purpose of this study was to evaluate the antibacterial activity of *Lagotis brachystachya* Maxim (*L. brachystachya*), an herb widely used in traditional Tibetan medicine, against ESBLs-producing *E. coli* from broiler chicken, and to further investigate the biofilm formation inhibition and biofilm eradication capabilities of *L. brachystachya* against ESBLs-producing *E. coli*.

MATERIALS AND METHODS

Bacterial Isolates

Twenty-one ESBLs-producing *E. coli* isolates were obtained from a primary surveillance study population consisting of 180 *E. coli* isolates acquired from broiler chickens. Isolates were firstly identified based on cultural, morphological analysis, Gram staining, biochemical tests, 16S rRNA gene sequencing, and the ESBL-producing phenotype was further confirmed by the double-disk synergy test using both cefotaxime and ceftazidime in the presence or absence of clavulanic acid as recommended by the CLSI (CLSI, 2011). In addition, the ESBLs-producing *E. coli* isolates were tested for the antimicrobial resistant phenotype and biofilm formation. Finally, the ESBLs-producing *E. coli* isolates with moderate or strong biofilm-forming abilities were used to evaluate the anti-biofilm activity of *L. brachystachya* extract in this study.

Preparation of the *L. brachystachya* Extract

L. brachystachya was obtained from a pharmacy located in Lhasa City of Tibet, it was naturally collected at an elevation of 3,700 m, in August 2020 from Shannan City, in the southern part of Tibet, and it has been authenticated by the pharmacy and Dr. Jin of Tibet Vocational Technical College. The herb extract was made in an Electrical Herb Pot (Cangnan Dongqi electric apparatus Co. Ltd., Zhejiang, China). First, the oven-dried *L. brachystachya* was ground into coarse powder. Second, 50 g of herb powder was soaked in 500 mL of the distilled water for 24 h, boiled for 1 h, and filtered through the four-layer sterile gauze. The decoction and filtration were performed in triplicate, and the filtrates were combined, concentrated to a volume of about 100 mL. The collected decoction was centrifuged at 4,000 rpm for 5 min and filtered through a 0.22- μ m microporous membrane. Finally, the filtrate was further concentrated to a final volume of 50 mL, with a concentration of 1 g/mL. The prepared decoction was stored in the refrigerator at 4°C for use.

In Vitro Antibacterial Activity of *L. brachystachya* Extract Against ESBLs-Producing *E. coli*

The Kirby-Bauer disc diffusion method was applied to evaluate the in vitro antibacterial activity of *L. brachystachya* against ESBLs-producing *E. coli* according to CLSI protocols. All tests were performed on Mueller-Hinton (MH) agar. Briefly, a sterile cotton swab was soaked in a bacterial suspension with a turbidity of 0.5 McFarland, and squeezing the extra suspension, and then lightly and uniformly inoculated on the surface of MH agar. Moreover, the 6 millimeter in diameter sterile paper discs were immersed in prepared decoctions, and dried at room temperature. The impregnated with prepared decoctions were placed at equal distances on the MH agar. Finally, the plates were incubated at 37°C for 18 to 24 h to measure the diameter of inhibition zones.

Moreover, the MICs and MBCs of *L. brachystachya* against ESBLs-producing *E. coli* were performed using microdilution method. MIC was recorded as *L. brachystachya* extract with the lowest concentration and has shown absolute inhibition of observable growth (Sharifzadeh and Shokri, 2016). MBC was determined following the MIC assay (Mohammadi et al., 2019). The wells that exhibited no evident growth were streaked on MH agar and followed by incubation at 37°C for 18 to 24 h. The MBC was regarded as the lowest concentration at which no colonies were observed.

Time-Kill Assay of *L. brachystachya* Extract

Time-kill kinetic experiment of *L. brachystachya* extract was evaluated in MH broth according to previous literatures (Saising et al., 2008; Neta et al., 2017) with modifications. Briefly, bacterial cultures were adjusted with fresh MH broth to approximately 1×10^6 CFU/mL, and incubated at 37°C for 24 h. Then, 0.5 mL of adjusted inoculum was added to the tubes containing the *L. brachystachya* extract to the final concentrations of $1/2 \times \text{MIC}$, MIC, $2 \times \text{MIC}$ and $4 \times \text{MIC}$, and incubated at 37°C. Extract-free inoculated medium was used as growth control. Aliquots of 10 μ L of the sample were taken at 0, 4, 8, 12, 16, 20, and 24 h of incubation, and spread on MH agar plates after serial 10-fold dilutions with normal saline, and then the numbers of viable cells were determined after incubation for 24 h at 37°C. The lower limit of detection was 10^2 CFU/mL. The experiments were carried out in duplicate.

Anti-Biofilm Activity of *L. brachystachya* Extract Against ESBLs-Producing *E. coli*

Eight of 21 ESBLs-producing *E. coli* showed moderate or strong biofilm-forming abilities according to the biofilm formation capacity. The potency of different concentrations of *L. brachystachya* extract (MIC, $1/2 \times \text{MIC}$, $1/4 \times \text{MIC}$) to inhibit the ESBLs-producing *E. coli* biofilm formation as well as to eradicate the

established biofilm were assessed. In the biofilm inhibition tests, 100 μL extract and 100 μL bacterial suspension was added to the 96-well polystyrene plates, and the mature biofilm was obtained by growing the isolates at 37°C for 48 h. The plates were washed 3 times with the sterile PBS to remove the planktonic bacteria, and 200 μL of methanol was added each well for 15 min to fix the biofilms, and finally dried naturally. Each well was stained with 200 μL 1% crystal violet and incubated at room temperature for 5 min, and excess dye was removed by washing the plates 3 times with sterile PBS. The bound crystal violet was eluted by adding 200 μL of 95% ethanol followed by incubation for 10 min at room temperature. The OD_{570 nm} was measured to quantify biofilm formation. MH broth was used as the negative control and all determinations were performed in triplicate. For the biofilm eradication test, whereas, the difference was that the *L. brachystachya* extract was added to 96-well plate after the mature biofilm was obtained, and kept for further incubation at 37°C for 24 h (Lemos et al., 2018), and then the biofilm was quantified by crystal violet method as aforementioned. The percentage of biofilm inhibition and eradication was determined by the formula: inhibition/eradication % = (OD control - OD sample) / OD control \times 100% as described in previous study (Maselli et al., 2020).

Confocal Laser Scanning Microscopy Analysis of ESBLs-Producing *E. coli* Biofilm

Confocal laser scanning microscopy (CLSM; Nikon A1+/A1R+, Nikon, Tokyo, Japan) was used to evaluate the biofilm inhibition and eradication activities against the ESBLs-producing *E. coli* at different concentrations of *L. brachystachya* extract (MIC, 1/2 \times MIC, 1/4 \times MIC). The biofilm culturing and extract adding methods were the same as the protocol used in the aforementioned antibiofilm test with slight modification. The supernatants obtained during the bacterial growth were discarded, and the material that remained fixed to the 24-well plate was washed with 400 μL of 0.9% saline solution to discard the planktonic cells. Biofilms were stained with fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA) and propidium iodide (PI) in the dark condition for 30 min at 4°C, and then visualized by CLSM.

Scanning Electron Microscopy Analysis of ESBLs-Producing *E. coli* Biofilm

The morphological effects on ESBLs-producing *E. coli* biofilm at different concentrations of *L. brachystachya* extract (MIC, 1/2 \times MIC, 1/4 \times MIC) were assessed by a scanning electron microscopy (SEM; Nano SEM-450, FEI Company, OR) according to previous studies (Melo et al., 2019; Adnan et al., 2020) with modification. Briefly, the biofilm culturing and extracts adding methods were the same as the protocol used in the aforementioned antibiofilm test with a slight adjustment. The

biofilm formed in 24-well plates containing microscope slides. After 24-h incubation at 37°C, the slides were washed 3 times with 0.1 M PBS, and fixed with 2% glutaraldehyde, dehydrated with an increasing concentrations of ethanol at 30, 50, 70, 80, 90, and 100% for 15 min each, and finally dried in the critical point drying apparatus using liquid carbon dioxide. The slides were mounted on the aluminum stubs, coated with gold by an ion sputter, and visualized under SEM at 10 kV.

Statistical Analysis

All experiments were carried out in triplicates. Mean and standard deviation, Student's *t* test was used to compare the inhibition and eradication rate with the control isolates. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA). A *P* < 0.05 was interpreted as statistically significant.

RESULTS

Antimicrobial Activity of *L. brachystachya* Extract

The results of antimicrobial susceptibility indicated that the diameter of inhibition zones of *L. brachystachya* extract against ESBLs-producing *E. coli* isolates ranged from 15.4 to 20.3 mm. The MIC values of *L. brachystachya* extract were 6.25 to 25 mg/mL, and the corresponding MBC values were 12.5 to 100 mg/mL.

Time-Kill Analysis

The antibacterial activity of the *L. brachystachya* extract against ESBLs-producing *E. coli* was evaluated by time-kill assay at 1/2 \times MIC, MIC, 2 \times MIC and 4 \times MIC level of extract concentration. The results were illustrated in Figure 1. Time-kill curve showed that the difference in bacterial counts in the control and test concentrations was not significant (*P* > 0.05) at 0 h. The control isolate has a typical bacterial growth curve with slow growth at the lag phase and the rapid growth at the exponential phase. The growth of ESBLs-producing *E. coli* isolate slowly increased within 12 h, and then kept a stagnant growth after a transitory decline after treated with 1/2 \times MIC level of extract. However, a decline throughout the incubation period was observed at MIC, 2 \times MIC and 4 \times MIC groups, and the bactericidal activity occurred after 16 h with concentrations equal to or above 2 \times MIC level of *L. brachystachya* extract. 4 \times MIC and 2 \times MIC levels of extract were sufficient to kill more than 99.9% of the bacteria cells after 16 h and 24 h, respectively. The time-kill revealed that *L. brachystachya* extract displayed concentration- and exposure time-dependent activity over a range of 1/2 \times MIC to 4 \times MIC.

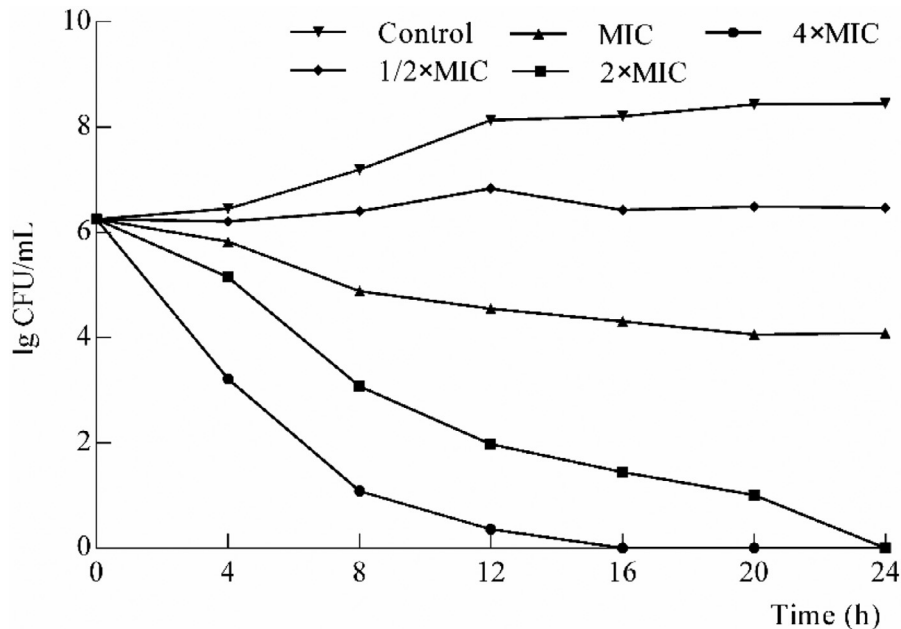


Figure 1. Time-kill curve of *L. brachystachya* extract against ESBLs-producing *E. coli* isolate.

Biofilm Inhibition and Eradication Analysis

The antibiofilm activity of *L. brachystachya* extract against ESBLs-producing *E. coli* was showed in Figure 2. As shown in Figures 2A and 2B, *L. brachystachya* extract exhibited different levels of antibiofilm activities to 6 ESBLs-producing *E. coli* isolates with a concentration-dependent manner, and it showed significant biofilm inhibition and biofilm eradication activities at MIC and $1/2 \times$ MIC levels of extract compared with $1/4 \times$ MIC ($P < 0.01$), and the biofilm eradication activities were weaker than biofilm inhibition abilities at different concentrations except for Ec07 isolate (Figures 2A and 2B). It is worth noting that *L. brachystachya* extract showed stronger biofilm eradication activity than biofilm inhibition activity to Ec07 isolate. Moreover, the biofilm inhibition and eradication activities were weak for a few isolates at $1/4 \times$ MIC (Figures 2A and 2B). The biofilm inhibition rates for ESBLs-producing *E. coli* biofilm at $1/4 \times$ MIC, $1/2 \times$ MIC and MIC levels of extract were 0 to 51.43%, 20.01 to 71.27%, and 35.66 to 79.91%, respectively (Figure 2A), and the corresponding biofilm eradication rates were 0 to 33.47%, 7.23 to 36.53% and 22.18 to 56.21%, respectively (Figure 2B).

CLSM and SEM Analysis of ESBLs-Producing *E. coli* Biofilm

CLSM was used to visualize the effect of *L. brachystachya* extract on the biofilms using PI (red) for staining dead cells and FITC-ConA (green) for staining live cells. As shown in Figure 3, red fluorescence sporadically appeared in the control isolates, whereas the intensity of red fluorescence significantly enhanced as the concentrations of *L. brachystachya* extract increased from $1/4 \times$ MIC to MIC. The ESBLs-producing *E. coli* isolates

exposed to MIC level of *L. brachystachya* extract exhibited the most conspicuous red fluorescence intensity, followed by those exposed to $1/2 \times$ MIC and $1/4 \times$ MIC levels of extract concentration (Figures 3B–3D and Figures 4B1–4D1) with a concentration-dependent manner. It is indicated that some isolates died under the *L. brachystachya* pressure. CLSM images showed that *L. brachystachya* extract could decrease the amount of live cells by the presence of a large number of uniformly distributed red fluorescent bacterial colonies. Meanwhile, the isolates of control group formed a compact and uniform biofilm as evidenced by the presence of green fluorescence, whereas the intensity of green fluorescence decreased as the isolate exposed to *L. brachystachya* extract with a concentration-dependent manner from $1/4 \times$ MIC to MIC. It is indicated that *L. brachystachya* extract could reduce the thickness of biofilm. The above results suggest that *L. brachystachya* extract can not only inhibit but also disperses established biofilm of ESBL-producing *E. coli* isolates.

The effect of different concentrations of *L. brachystachya* extract on the biofilm structures of ESBLs-producing *E. coli* was also visualized by SEM images at 12,000 \times magnifications. The SEM images of control isolate and *L. brachystachya*-treated isolates were showed in Figures 5 and 6. As shown in Figures 5A and a, the control isolate showed an intact compact biofilm structure with a particulate surface. While the *L. brachystachya*-treated isolates exhibited the abnormal morphology (Figures 5B–5D and Figures 6B–6D), the SEM images showed an evident reduction in adherent cells, the isolate colonies had not clear cell edges, and collapsed with loss of turgidity and few surface particles after contact with *L. brachystachya* extract (Figures 5B and 5C). Moreover, the biofilm mass was significantly reduced, and the biofilm became thinner and looser under *L. brachystachya* pressure with the concentration

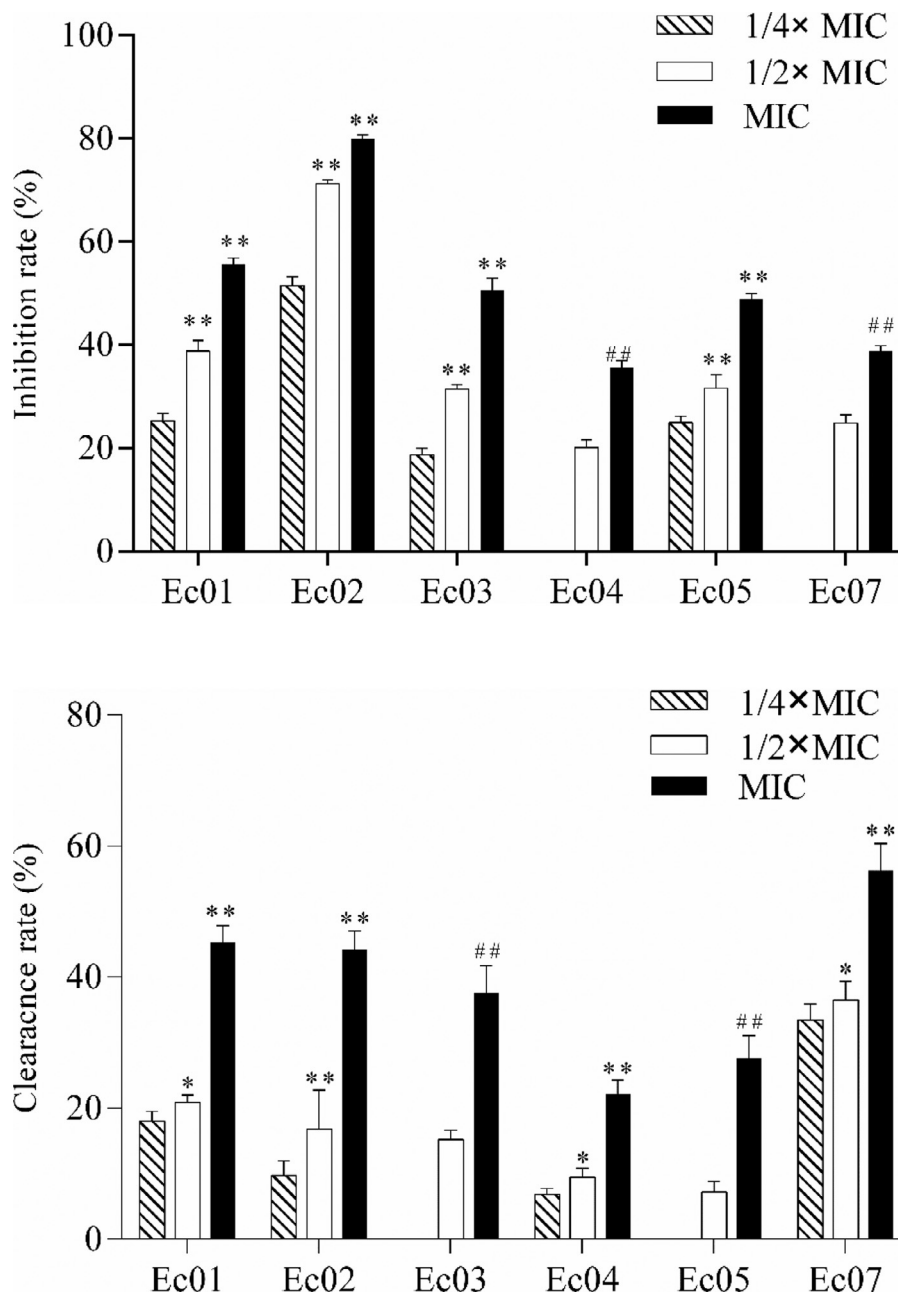


Figure 2. (A) The biofilm inhibition activity against ESBLs-producing *E. coli* after cultured with different concentrations of *L. brachystachya* extract ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC). (B) The biofilm eradication activity against ESBLs-producing *E. coli* after cultured with different concentrations of *L. brachystachya* extract ($1/4 \times \text{MIC}$, $1/2 \times \text{MIC}$, MIC). * and # on the column indicate a significant difference, * indicates $P < 0.05$ compared with $1/4 \text{ MIC}$ group; ** indicates $P < 0.01$ compared with $1/4 \text{ MIC}$ group, and # # indicates $P < 0.01$ compared with $1/2 \text{ MIC}$ group. Abbreviations: ESBLs, extended-spectrum-lactamases; MICs, minimum inhibitory concentrations.

increasing compared with the control, and the biofilm formation ability of ESBLs-producing *E. coli* decreased with the concentration increasing of *L. brachystachya* extract. In the inhibition group, it was observed that the integrity of the biofilm structure was disrupted, the isolates treated with MIC and $1/2 \times \text{MIC}$ levels of extract concentration exhibited well-defined rod-shaped contours with smooth surfaces, and were uniform in size after exposure to *L. brachystachya* extract for 48 h (Figures 6B-6D). However, *L. brachystachya*-treated isolates of the eradication group showed the relatively normal morphology in comparison to the isolates of inhibition group, and the surface of cells was regular

although the aggregation and adherence properties of the isolates were poor.

DISCUSSION

Traditional Chinese medicine is commonly used as disease treatment or the supplement to the health care. In the recent years, more and more plant-derived products, especially some plant essential oils have gained interest as potential antimicrobial agents for combating bacterial biofilms (Orhan-Yanikan et al., 2019; Hassanshahian et al., 2020; Qian et al., 2020b). Tibetan

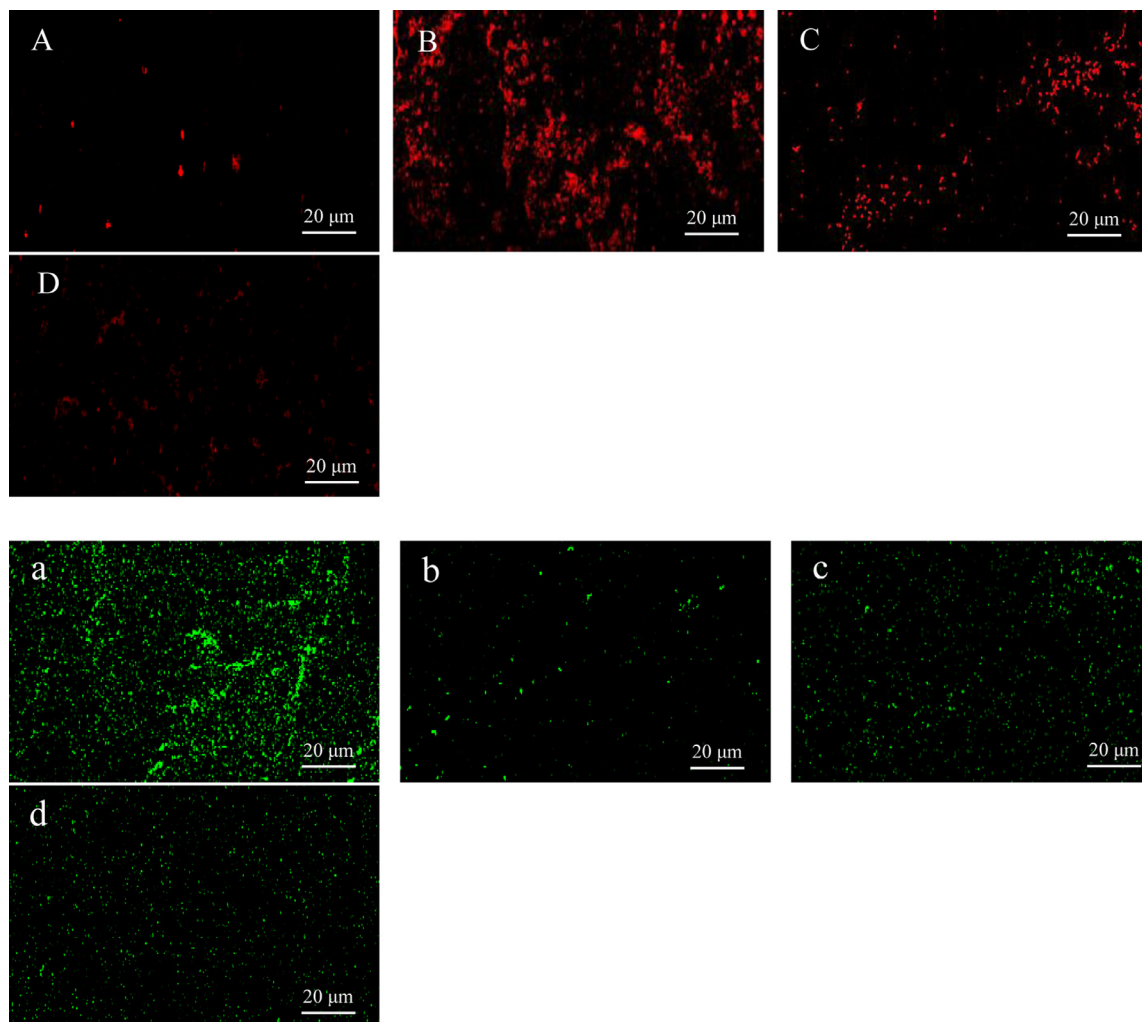


Figure 3. CLSM images of biofilm inhibition activity against ESBLs-producing *E. coli* at different concentrations of *L. brachystachya* extract. Scale bar is 20 μm . Note: A–D were stained with PI. (A) Negative control; B–D were treated with MIC, $1/2 \times \text{MIC}$ and $1/4 \times \text{MIC}$ levels of *L. brachystachya* extract, respectively. A–D were stained with FITC-ConA. (A) Negative control; B–D were treated with MIC, $1/2 \times \text{MIC}$ and $1/4 \times \text{MIC}$ levels of *L. brachystachya* extract, respectively. Abbreviations: CLSM, confocal laser scanning microscope; ESBLs, extended-spectrum-lactamases; MICs, minimum inhibitory concentrations.

medicine has a long history as one of the world's oldest known medical systems, and it plays an important role in the health care system in Qinghai-Tibet Plateau of China. Moreover, Tibetan medicine has gradually developed into a unique medical system by incorporating the theories of early traditional Chinese medicine, Indian medicine, and Arabian medicine (van der Valk, 2021). However, no study focused on the antibiofilm potential of the Tibetan medicinal plants with antibacterial activity.

L. brachystachya is an herb widely used in traditional Tibetan medicine. Modern pharmacological researches showed that *L. brachystachya* was extensively used for antioxidant, antibacterial, antitumor, liver damage, anti-inflammatory, antigout, and other pharmacological activities (Zhu et al., 2019; Zhu et al., 2021), and no adverse reaction was reported since *L. brachystachya* was used in clinical practice for a long history. Hence, we conclude that it should be safe if *L. brachystachya* was used in appropriate patients, correct dose levels and administration. In this study, *L. brachystachya* was

investigated for the first time for its antibacterial and antibiofilm activities against the ESBLs-producing *E. coli*, and it exhibited moderate antibacterial activity according to the MICs, while the average diameter of inhibition zones were more than 18 mm according to the results of the Kirby-Bauer disc diffusion test. One possible reason is that the latter method is more sensitive than microdilution method in determination of in vitro antibacterial activity of the crude extract of *L. brachystachya*. The antibacterial activity of was similar with cinnamon extract, and weaker than some pure compounds of medical plants (Hassanshahian et al., 2020; Lu et al., 2021). Additionally, we compared the water extract and ethanol extract of *L. brachystachya* in the preliminary experiment, and the water extract displayed a better antibacterial activity against the ESBLs-producing *E. coli* isolates. Usually, time-kill assay was used to evaluate the pharmacodynamics of an antibacterial agent as it is more precisely to define the profile of bactericidal activity of antibacterial agents over time, and provide more information than the MIC values

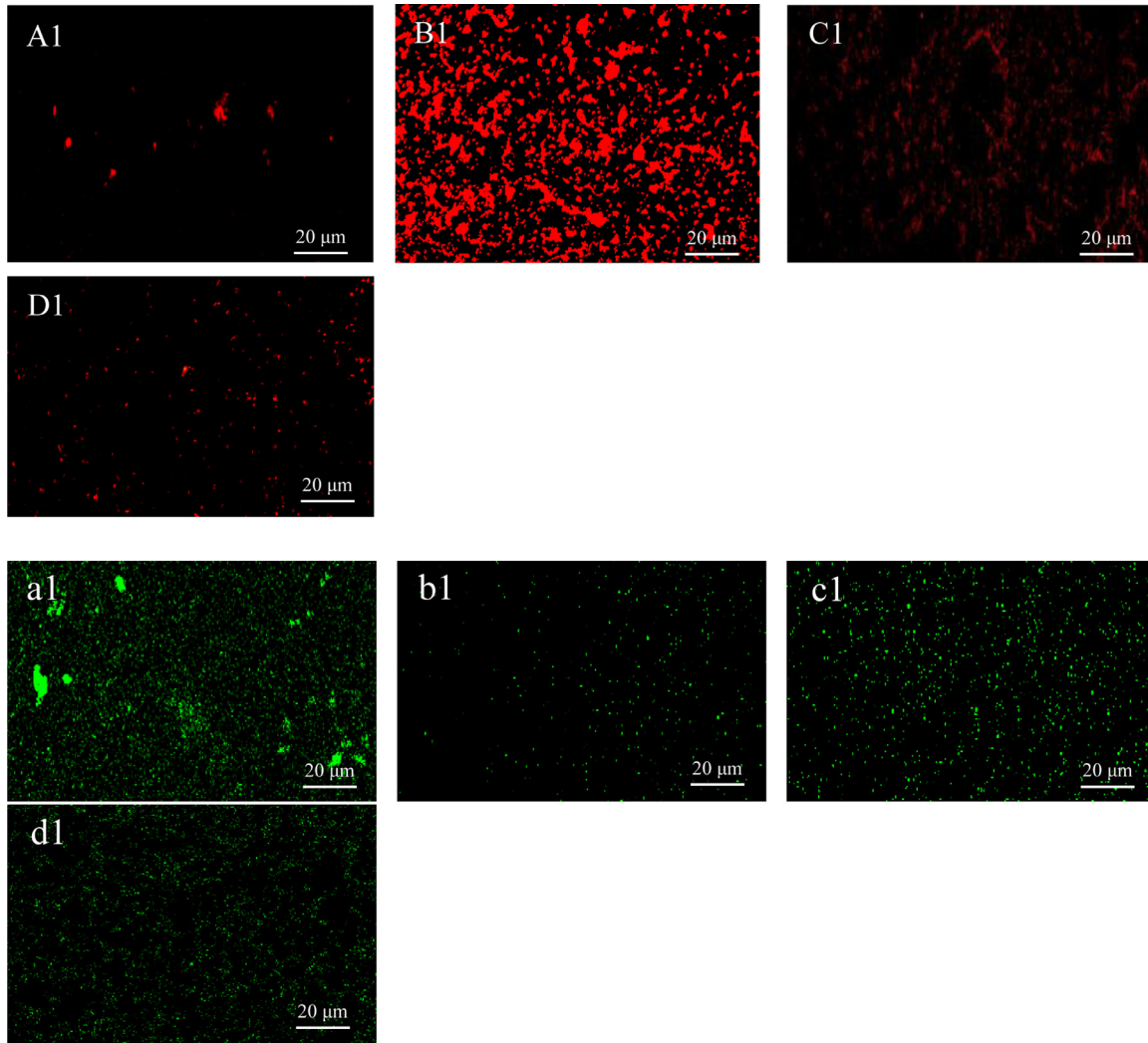


Figure 4. CLSM images of biofilm eradication activity against ESBLs-producing *E. coli* at different concentrations of *L. brachystachya* extract. Scale bar is 20 μm . Note: A1–D1 were stained with PI. (A1) Negative control; B1–D1 were treated with MIC, $1/2 \times \text{MIC}$ and $1/4 \times \text{MIC}$ levels of *L. brachystachya* extract, respectively. a1–d1 were stained with FITC-ConA. a1. Negative control; b1–d1 were treated with MIC, $1/2 \times \text{MIC}$ and $1/4 \times \text{MIC}$ levels of *L. brachystachya* extract, respectively. Abbreviations: CLSM, confocal laser scanning microscope; ESBLs, extended-spectrum-lactamases.

(Ferro et al., 2015). Our study revealed that the concentrations equal to or above $2 \times \text{MIC}$ level of *L. brachystachya* extract had obvious bactericidal activity toward ESBLs-producing *E. coli*. The time-kill assay is useful for assessing the bactericidal effect of the *L. brachystachya* extract by providing dynamic information between the bacteria and drug. Moreover, antibacterial activity of *L. brachystachya* extract was concentration- and exposure time-dependent manner. Combining our previous study that *L. brachystachya* extract had the antibacterial activity against the *S. aureus*. We can infer that *L. brachystachya* had broad-spectrum antibacterial activity, and might be used in the treatment of infectious diseases caused by Gram-negative and Gram-positive bacteria, including the resistant isolates.

Biofilm is a complex matrix of microorganisms in which cells bind together with the extracellular polysaccharides (EPS), which can insulate cells from antibacterial substances and reduced the antibacterial activity (Lu et al., 2021). Adhesion is the first and crucial step to the formation of biofilm because it determines the

subsequent development and maturity of biofilm (Liu et al., 2017). The current study indicated that $1/2 \times \text{MIC}$ and MIC levels of *L. brachystachya* extract could inhibit the biofilm formation or eradicate the established biofilms, and obstruct the adhesion of ESBLs-producing *E. coli* isolates. The ESBLs-producing *E. coli* were treated with different concentrations of *L. brachystachya* extract (MIC, $1/2 \times \text{MIC}$ and $1/4 \times \text{MIC}$) for 24 to 48 h to analyze the biofilm inhibition and biofilm eradication activities, and significant reduction ($P < 0.01$) in bacterial count was observed in treated isolates as compared to the control isolate with a concentration-dependent manner. Crystal violet staining also confirmed that the isolates treated with MIC and $1/2 \times \text{MIC}$ levels of extract concentrations showed significant reduction of viable cell count. Our results indicated that the *L. brachystachya* extract exhibited prominent effects in controlling the biofilms of ESBL-producing *E. coli*, and it could inhibit the formation of biofilms, and even eradicate the established biofilms based on CLSM and SEM analysis. Previous studies

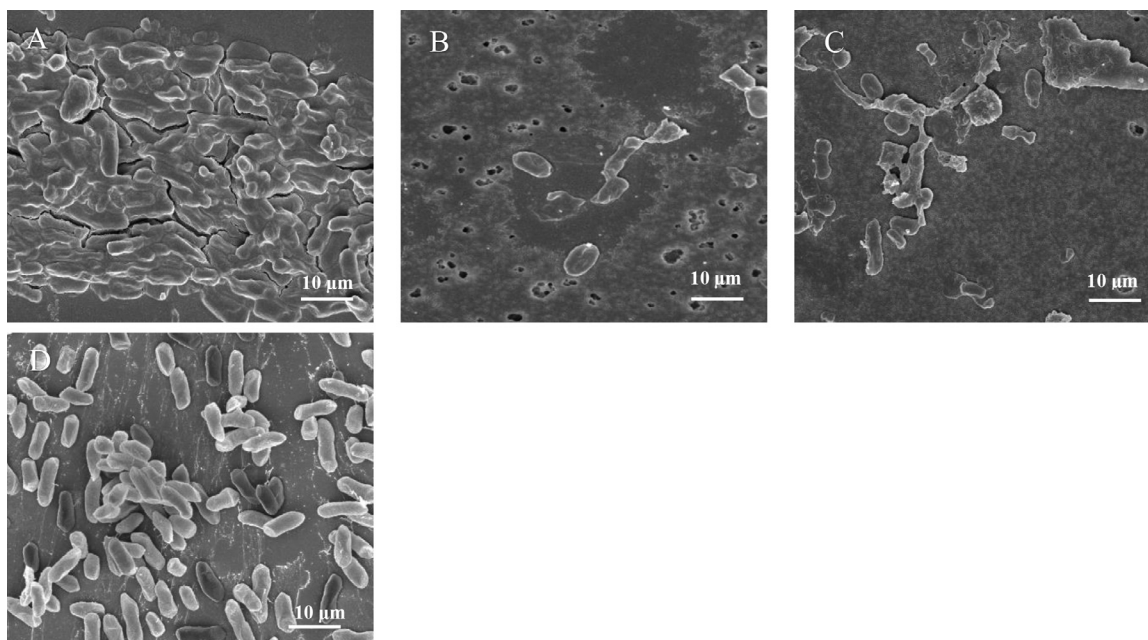


Figure 5. SEM images of biofilm inhibition activity against ESBLs-producing *E. coli* at different concentrations of *L. brachystachya* extract. Scale bar is 10 μm . Abbreviations: ESBLs, extended-spectrum-lactamases; SEM, scanning electron microscopy.

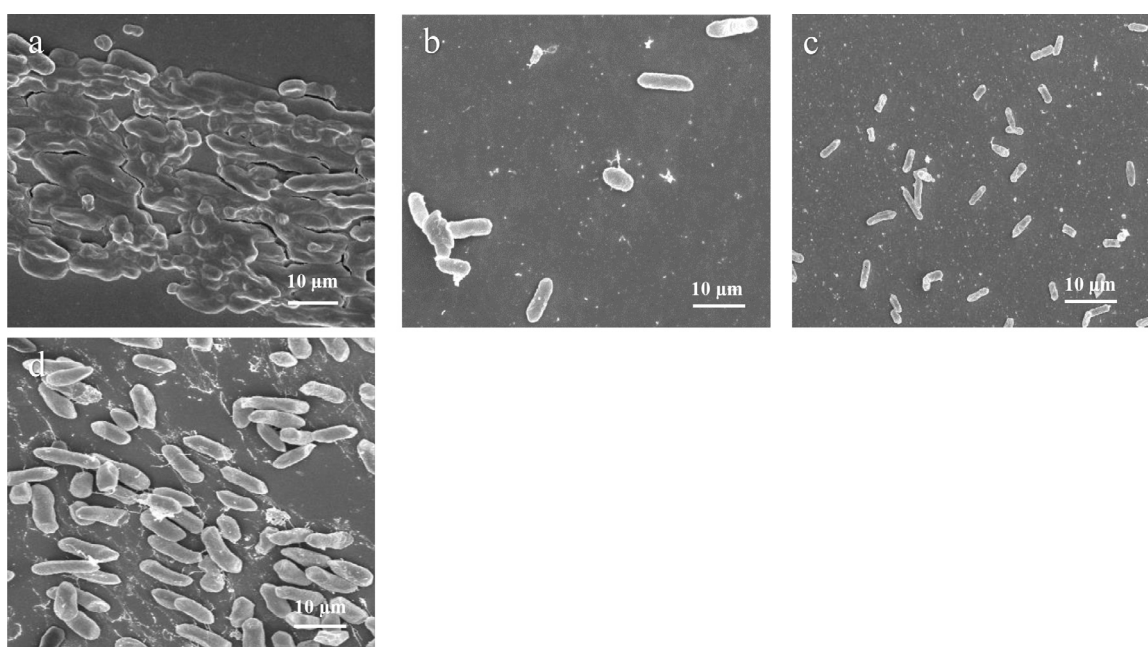


Figure 6. SEM images of biofilm eradication activity against ESBLs-producing *E. coli* at different concentrations of *L. brachystachya* extract. Abbreviations: ESBLs, extended-spectrum-lactamases; SEM, scanning electron microscopy.

showed that the crude extracts or pure compounds from the plant sources exhibited the antibacterial and anti-biofilm activities, and the crude extracts usually exhibit weaker activity than pure component (Bhandari et al., 2021). The different phytochemicals of the medicinal plants, such as flavonoid, glycosides, tannic acid, phenolics, and chlorogenic acid, can inhibit or eradicate the biofilm by inhibiting the growth of biofilm-producing bacteria, breaking the polysaccharides in EPS, destroying the bacteria membrane integrity, inhibiting the activity of related enzymes, disrupting fimbriae for adhesion of bacteria, inhibiting the expressions of biofilm-

related genes, or inhibiting the quorum-sensing system (Niu and Gilbert, 2004; Antunes et al., 2010; Vikram et al., 2010). *L. brachystachya* contain more than a dozen chemical constituents, including flavonoids (luteolin, luteoloside and apigenin), vanillic acid, iridoid glycosides, phenylpropanoid glycosides, steroids, terpenoids, and other chemical constituents. We speculate that the synergistic effect of phytoconstituents in *L. brachystachya* might be responsible for antibacterial as well as biofilm formation inhibition of ESBL-producing *E. coli*, especially the rich flavonoids and vanillic acid, which had shown the antibacterial activities and biofilm

inhibition (Christena et al., 2015; Sharma et al., 2015; Qian et al., 2020a). The exact mechanisms involved still need to be fully elucidated.

In conclusion, this study revealed the potential antibacterial and antibiofilm activities of *L. brachystachya* extract against ESBLs-producing *E. coli*, and it is worthwhile noting that the *L. brachystachya* extract was able to eradicate the mature biofilm of the ESBLs-producing *E. coli*. Therefore, *L. brachystachya* could be a candidate for combating the bacterial infections, including the infections caused by resistant and biofilm-producing bacteria.

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DISCLOSURES

We declare that we have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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