The investigation of inhibiting quorum sensing and methicillin-resistant *Staphylococcus aureus* biofilm formation from *Liriodendron hybrid*

Xiaojuan Tan[1], Dongting Yang[2], Guoxu Yang[1], Jinhui Chen[3], Wei Dong[2], Jisen Shi[3] and Aiqun Jia[1]*

[1]Center for Molecular Metabolism, School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing, China
[2]School of Chemical Engineering, Nanjing University of Science and Technology, Nanjing, China
[3]Key Laboratory of Forest Genetics and Biotechnology of the Ministry of Education of China, Nanjing Forestry University, Nanjing, China

Abstract: The quorum sensing (QS) of pathogens has been found to affect their biofilm forming ability, making it a potential target for anti-microbial therapy. The present research aimed to evaluate the anti-QS activities of different extracts and isolated phytochemicals from *Liriodendron hybrid* barks and their roles in the inhibition of the growth and biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA). The assays on the inhibition of QS by the five extracts (*n*-hexane, dichloromethane, ethyl acetate, acetone, and methanol) and eight isolated compounds were carried out by using both the indicator strains *Chromobacrerium violaceum* CV026 and *C. violaceum* ATCC12472. The *in vitro* effects of the five extracts and eight isolated compounds on MRSA biofilm were also preliminarily evaluated using crystal violet micro titer plate assays. The results suggested that the dichloromethane extract showed anti-QS and MRSA biofilm inhibitory activities and the *n*-hexane extract possessed only MRSA biofilm inhibitory effect. The dichloromethane extract could serve as a source for developing bacterial intervention strategies targeting microbial QS system. All eight isolated compounds showed no anti-QS and biofilm formation inhibiting activities. So further researches are still being required to purify and identify the compounds possessing anti-QS and biofilm inhibitory effects from the dichloromethane and *n*-hexane extracts.

Keywords: *Liriodendron hybrid*, quorum sensing inhibitors, biofilm formation inhibitors, phytochemicals.

INTRODUCTION

Quorum sensing (QS) is considered to play a major role in regulating the bacterial gene expression related to human infections and food spoilage (Antunes *et al*., 2010; Fuqua *et al*., 1994; Truchado *et al*., 2009). QS is a population density-dependent phenomenon and was first found in luminescent marine species of *Vibrio* (Nealson *et al*., 1970; Hastings and Greenberg, 1999). Moreover, QS systems are widespread in bacteria and have been found to regulate various cellular functions including luminescence, biofilm formation, virulence factors expression, etc (Whitehead *et al*., 2001; Fuqua and Greenberg, 2002). Small signaling molecules termed as autoinducers (AIs) enable the bacterial cells to regulate some genes expression (Dotsch *et al*., 2012). Several signaling molecules have been identified, and N-acylhomoserine lactones (AHLs) have been characterized in Gram-negative bacteria (Dong and Zhang, 2005). AHLs-mediated QS systems have been detected in bacteria such as *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Clostridium difficile*, *Burkholderia cepacia*, and *Escherichia coli* (Adonizio *et al*., 2006). Prevent bacterial infection by inhibiting bacterial growth has been a primary and traditional anti-microbial therapy strategy (Vattem *et al*., 2007). Several types of antibiotics such as penicillins and sulphonamides can increase antibiotic resistance. Inhibiting bacterial QS by reducing AIs becomes an alternative strategy to hinder the spread of bacterial virulence (Raffa *et al*., 2005; Lyon and Muir, 2003; Schauder *et al*., 2001). Although halogenated furanones (Manefield *et al*., 2002; Manefield *et al*., 1999; Manefield *et al*., 2000) have been shown to directly combine with the AHLs-binding site in LuxR (Stauff and Bassler, 2011), such halogenated compounds have been found to exert *in vitro* toxicity to human cells (Anguige *et al*., 2004; Gao *et al*., 2003; Hentzer and Givskov, 2003). Therefore, the current searching new anti-microbials aimed at exploring non-toxic QS inhibitors from the natural products. The advantage of this alternative approach is that these new anti-microbials could inhibit the virulence rather than growth. Consequently, the problems of resistance associated with many bactericidal or bacteriostatic drugs could be avoided (Singh *et al*., 2009). The ideal QS inhibitors should be a low-molecular mass molecule causing a remarkable reduction in the expression of QS-regulated genes (Rasmussen and Givskov, 2006).

Biofilm formation is closely related to pathogenicity.

**Corresponding author:* e-mail: jiaaiqun@gmail.com

Reports have suggested that AIs play a vital role in biofilm formation (Wang *et al*., 2009). Natural products could represent an interesting strategy to inhibit the emergence and diffusion of bacteria forming biofilm (Nostro *et al*., 2007). The barks of *Liriodendron tulipofera* have been served by the Native Americans as a tonic, stimulant and febrifuge. They are used to remedy the intermittent fever caused by malaria. The species *L. chinese* are used as traditional Chinese medicines to remedy rheumatism and cough due to wind-cold evil. Zeng *et al*. reported antibiotic activity of *n*-butanol portion against *Staphylococcus aureus*, *Bacillus subtilis*, and *E. coli* in *L. Chinese* (Zeng *et al*., 2009). In addition, Ye *et al* successfully cultivated a new hybrid strain, *L. chinese* x *L. tulipifera* (*L. hybrid*) in 1963 (Chen *et al*., 2013). However, the medicinal values of the hybrid tree, especially its antibacterial activities, have been little investigated (Chen *et al*., 2013).

In the present research, five extracts and eight isolated compounds were obtained from *L. hybrid* barks and were tested for their ability to inhibit QS-controlled behaviors by employing the biosensor strains *Chromobacrerium violaceum* CV026 and *C. violaceum* ATCC12472. In addition, we also preliminarily evaluate their inhibiting effect on methicillin-resistant *S. aureus* (MRSA) biofilm formation *in vitro*.

MATERIALS AND METHODS

Collection of plant materials and preparation of extracts and isolation of eight compounds

L. hybrid species was identified by Prof. Jisen Shi of Nanjing Forestry University, China, A voucher specimen (LX-001-B) was deposited with Nanjing University of Science and Technology, Nanjing, China, and the barks were harvested in the university in September 2011. The dried and powdered barks were refluxed thrice with 95% ethanol for 1h each and evaporated to dryness to yield ethanol extract. This extract was then suspended in water and partitioned successively with *n*-hexane, dichloromethane, and ethyl acetate; and the residue was evaporated again *in vacuo* and extracted with acetone and methanol, respectively to yield five fractions after the evaporation of five organic solvents (*n*-hexane, dichloromethane, ethyl acetate, acetone, and methanol). Eight phytochemicals, four (1, 2, 5 and 8) from the *n*-hexane extract and four (3, 4, 6 and 7) from the dichloromethane extract, were isolated under bioassay-guided screening, The stock concentrations of these extracts in dimethyl sulfoxide (DMSO) were 21.2mg/mL, 22.2mg/mL, 22.8mg/mL, 24.4mg/mL and 21.6mg/mL, respectively and they were used to study the inhibitory effect of MRSA biofilm formation. The stock concentrations of 0.1mg/mL, 1.0mg/mL, 2.0mg/mL, 3.0mg/mL, and 4.0mg/mL of these extracts in DMSO, respectively were prepared for QS inhibitory tests. All stock solutions were sterile-filtered (0.22μm) and then stored at 4^{o}C.

Bacterial strains and media

C. violaceum CV026 and *C. violaceum* ATCC12472 were used to detect the QS inhibitory activity. The signaling molecule C6-HSL was purchased from Sigma Chemical Co. (USA), and was dissolved in acidified ethyl acetate (0.1mL acetic acid per liter). The stock concentration of 1×10^{-3} mol/L was supplemented in *C. violaceum* CV026 cultures to induce violacein production. Prior to the experiments, these two strains were routinely cultured aerobically in Luria-Bertani (LB) broth at 30^{o}C with an agitation of 140 rpm in a shaking incubator. The medium was supplemented with appropriate antibiotics (*C. violaceum* CV026, kanamycin 20μg/mL) when required. The solid medium containing 1.2% agar was used to measure the QS inhibition by the extracts and eight isolated compounds of *L. hybrid* barks.

The clinical isolate of MRSA was used in this study. The strain was isolated from the human blood sample at the Clinical Laboratory, Jiangsu Province People's Hospital, China. The blood agar medium was used for culturing of colonies, nutrient broth (NB) medium was used for routine culturing of the strain, and tryptic soy broth (TSB) medium was used to study the biofilm formation in flat bottom 96-well polystyrene culture plates (Costar 3599, USA).

Disc diffusion method for QS inhibition assays

The QS inhibition assay was performed as previously described methods with few modifications (Zhu *et al*., 2011; Chong *et al*., 2011; Vattem *et al*., 2007). *C. violaceum* was incubated for 24h and 100μL of the cultures were grown overnight in LB broth. After adjusting the OD_{600nm} to 0.1 (c. 1×10^{8} CFU mL^{-1}), it was spread on agar plates. The paper discs (8mm diameter) were placed on the plates, and 5μL of extracts and compounds in DMSO with different concentrations was loaded on the discs. Violacein was examined after the plates were incubated at 30^{o}C for 24 h or 36h. The QS inhibition was determined by a colorless, opaque, viable and halo around the discs. DMSO was used as a negative control.

Effect of the plant extracts on the growth of MRSA and biofilm formation

The quantification of biofilm was performed according to the reported methods with few modifications (Thein *et al*., 2007; Wang *et al*., 2011). The overnight cultures were inoculated (1: 100) in TSB medium. To assess the impact of the plant extracts on the growth of MRSA and biofilm formation, *n*-hexane, dichloromethane, ethyl acetate, acetone, and methanol extracts were added to these culture suspensions to make the final concentrations of 212μg/mL, 222μg/mL, 228μg/mL, 244μg/mL and

216μg/mL, respectively. These culture suspensions (200 μL) were added to each well of the sterile flat bottom 96-well polystyrene plates. A negative control (1.0% DMSO in culture) was included in each experiment. The plates were incubated at 37°C for 24h with shaking (150 rpm). After incubation, the OD was measured at 600_{nm} to obtain the impact of the plant extracts on the growth of MRSA. The suspension cultures were removed, and the plates were washed thrice with deionized water. The remaining biofilms were fixed in 200μL methanol for 15 min and then dried at 60°C. The biofilms were stained with 50μL of 0.1% crystal violet (w/v) for 15 min. The wells were washed thrice with water to remove the unbound crystal violet dye and were dried for 2h at 60°C. After adding 200μL of 95% ethanol (v/v) to each well, the plates were shaken for 1h to release the stain from the biofilms and OD_{570nm} was measured with a microplate reader (BioTek, USA). All assays were performed in triplicate and repeated thrice with the new cultures.

STATISTICAL ANALYSIS

At least three independent replicates of each 96-well plate experiment were performed. The results of biofilm inhibition and growth inhibition were statistically analyzed using SPSS software version 18.0 (SPSS, Chicago, IL, USA). P values ≤ 0.05 were considered significant.

RESULTS

Five extracts (*n*-hexane, dichloromethane, ethyl acetate, acetone, and methanol) were obtained from dry sliced *L. hybrid* species (10 kg), the yields of five extracts were 2.14%, 1.12%, 0.37%, 1.00% and 1.49% respectively. Four (1, 2, 5 and 8) phytochemicals from the *n*-hexane extract and four (3, 4, 6 and 7) from the dichloromethane extract were isolated under bioassay-guided screening, they were elucidated as 5,6,7-trimethoxycoumarin (1) (97 mg, purity on HPLC: 98.1%), 6,7-dimethoxycoumarin (2) (61 mg, purity on HPLC: 96.5%), 5-hydroxyl-6,7-dimethoxycoumarin (3) (19 mg, purity on HPLC: 98.4%), 7-hydroxyl-6-methoxycourmarin (4) (182mg, purity on HPLC: 96.8%), N-phenethylbenzamide (5) (228mg, purity on HPLC: 97.3%), N-(1-hydroxy-2-phenylethyl)-benzamide (6) (210mg, purity on HPLC: 96.7%), N-acetylnornuciferine (7) (10 mg, purity on HPLC: 97.6%), Tulipinolide (8) (84mg, purity on HPLC: 97.7%) by NMR and MS data (data not shown). The purity of compounds was measured on an Agilent HP1100 equipped with a quaternary solvent delivery system, an auto sampler and a DAD detector. The chemical structures of eight phytochemicals were illustrated in fig. 1.

The assay on the extracts inhibiting QS was carried out by using both the indicator strains *C. violaceum* CV026 and *C. violaceum* ATCC12472. Lack of purple pigment surrounding the test extracts demonstrated that the extracts have inhibitory effect on QS. The opaque, not-transparent, white zone observed around the discs indicated that the halo was caused by inhibiting violacein secretion instead of inhibiting cell growth. Among the different extracts tested, only the dichloromethane extract was found to be effective for anti-QS activity (fig. 2). As shown in fig. 2E-F, C6-HSL-induced violacein production was inhibited by the dichloromethane extract at different concentrations with different effects. On further analysis, the biomonitor *C. violaceum* CV026 was found to produce bigger halos than ATCC12472 in the dichloromethane extract in the screening QS inhibition assays (fig. 2E-F, fig. 3).

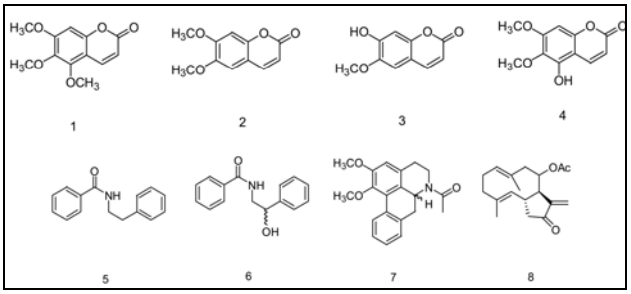

Fig. 1: Chemical structures of compounds isolated from *Liriodendron hybrid*, four compounds (1, 2, 5, and 8) were isolated from the *n*-hexane extract; the others (3, 4, 6 and 7) were isolated from the dichloromethane extract

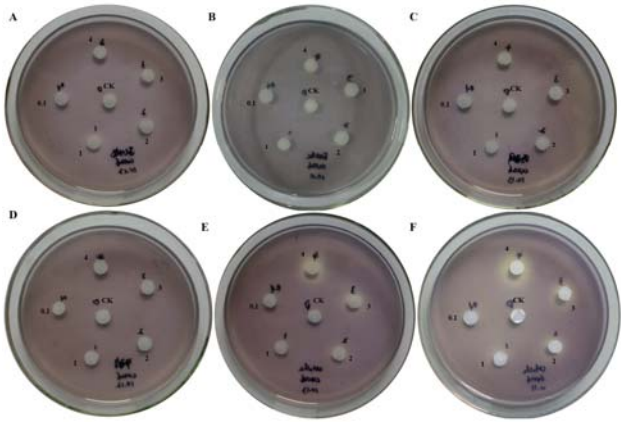

Fig. 2: Inhibition of violacein production in *C. violaceum* CV026 by the extracts of *Liriodendron hybrid* barks at concentrations of 0.1mg/mL, 1.0mg/mL, 2.0mg/mL, 3.0mg/mL and 4.0mg/mL, respectively, CK, dimethyl sulfoxide as negative control; (A) the *n*-hexane extract; (B) the ethyl acetate extract; (C) the acetone extract; (D) the methanol extract; (E) the dichloromethane extract for 24h; (F) the dichloromethane extract for 36h. The experiments were carried out on an LB agar plate overlain with a lawn of C6-HSL- induced CV026, which were incubated for 24h, except for (F): 36h

The *in vitro* effects of the five extracts on MRSA biofilm were preliminarily evaluated by crystal violet micro titer

plate assays. MRSA can form biofilm on the microtiter plate after incubating for 24h. The amount of cells was quantified by OD_{600nm}, and it was found that the effect of the dichloromethane extract inhibiting the growth of MRSA was obvious (48.9%) (fig. 4). When OD_{570nm} was used for quantifying the amount of crystal violet stained biofilm, the results showed that the dichloromethane and the *n*-hexane extracts inhibited MRSA biofilm formation with the inhibitory rates of 25.5%±0.066 and 29.0%±0.217, respectively (fig. 4).

Fig. 3: Inhibition of violacein production in *C. violaceum* ATCC12472 by the dichloromethane extract of *Liriodendron hybrid* barks at concentrations of 0.1mg/mL, 1.0mg/mL, 2.0mg/mL, 3.0mg/mL and 4.0 mg/mL, respectively. The experiment was carried out on an LB agar plate overlain with *C. violaceum* ATCC12472, which was incubated for 24 h. CK, dimethyl sulfoxide as negative control

DISCUSSION

To date, only a few bioactive studies have been conducted on the tumor cytotoxic effects of extracts from *L. hybrid* barks (Chen *et al*., 2013). For the first time, the present study attempted to discuss the anti-QS effects of *L. hybrid* bark extracts and their inhibitory effects on the biofilm formation of MRSA. Using multiple biomonitors to screen QS inhibitors could eliminate the artifact effects and perhaps explains a pathway. *C. violaceum* ATCC12472 produces and responds to C6-HSL and C4-HSL (Bosgelmez-Tinaz *et al*., 2007; Brackman *et al*., 2009). The second biomonitor strain, *C. violaceum* CV026, could not produce its own AHL signal, but could respond to exogenous AIs such as C6-HSL (Chen *et al*., 2011; Musthafa *et al*., 2010). In this study, the results showed that *C. violaceum* CV026 produced bigger halos around the paper discs. Therefore, it was presumed that the dichloromethane extract might disturb the combining of C6-HSL with receptor protein CviR and might not affect the interaction of C4-HSL with receptor protein or might affect at least only to a minor degree.

When we screened five extracts for their anti-QS activity, we also screened four different compounds (3, 4, 6, and **7**) (fig. 1) isolated from the dichloromethane extract. The results showed that these four compounds did not inhibit QS-controlled violacein production in *C. violaceum* and did not interfere with its growth (data not shown). However, the dichloromethane extract might be evaluated as QS inhibitor, so further studies involve in the isolation of anti-QS compounds from the dichloromethane extract.

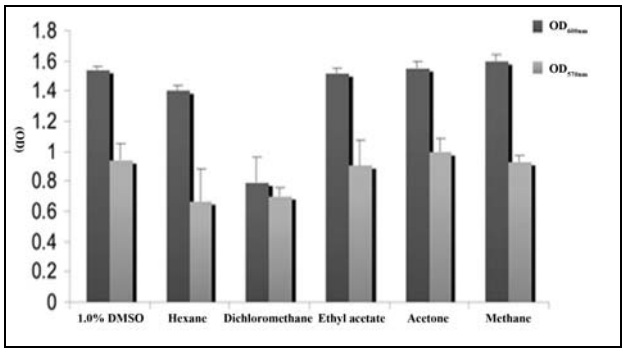

Fig. 4: Inhibition of growth and biofilm formation of MRSA by the addition of five extracts. At least three independent experiments were conducted, and the error bars indicate one standard deviation. As for OD_{600nm}, Value treated with dichloromethane is significantly different from the untreated control (P value<0.05 assigned using Student's t-test). Values treated with others are not significantly different from the untreated control (P value>0.05 assigned using Student's t-test). As for OD_{570nm}, Values treated with hexane and dichloromethane are significantly different from the untreated control (P value<0.05 assigned using Student's t-test). Values treated with others are not significantly different from the untreated control (P value>0.05 assigned using Student's t-test)

Several publications suggested that targeting QS could be a new strategy in controlling biofilm infections (Kjelleberg and Molin, 2002; Parsek and Greenberg, 2005). The role of QS inhibitors against biofilm formation has been evaluated in Gram-negative bacteria (Rudrappa and Bais, 2008; Chong *et al*., 2011; Truchado *et al*., 2009). However, such inhibitors have not been evaluated with *C. violaceum* as biomonitor strain against Gram-positive bacteria biofilms. The present study not only investigated the effect of *L. hybrid* bark extracts on the QS systems, but also preliminarily screened their *in vitro* inhibiting effect on MRSA biofilm formation. Among the five extracts tested, only the dichloromethane and *n*-hexane extracts partially inhibit MRSA biofilm formation.

Moreover, the dichloromethane extract obviously inhibited the growth of MRSA (48.9%±0.178), while the *n*-hexane extract had less inhibitory activity (8.6%± 0.028). Based on the ability to inhibit MRSA biofilm formation by the dichloromethane and *n*-hexane extracts, we also evaluated the four compounds isolated from the *n*-hexane extracts (1, 2, 5 and 8) (fig. 1) for their ability to inhibit MRSA biofilm formation. The results showed that all four compounds also did not have biofilm inhibitory activity (data was not shown). Interestingly, the dichloromethane extract not only possessed QS inhibitory activity but also showed the inhibitory effect on MRSA biofilm formation. However, the *n*-hexane extract could possess biofilm-inhibiting activity without anti-QS activity. These findings could reveal some relationships between the QS inhibitors screened using *C. violaceum* and biofilm inhibitors of Gram-positive strains. But such relationship remains to be clarified. As many phytochemicals present in the dichloromethane extract bearing anti-QS and biofilm inhibitory effects, further investigations are required to 'catch' one or some compounds showing anti-QS and biofilm inhibitory activities.

CONCLUSION

Overall, the anti-QS and biofilm inhibiting effects of *L. hybrid* may be important to the antibacterial effect. In this study, the dichloromethane extract showed anti-QS and MRSA biofilm inhibitory activities, and the *n*-hexane extract possessed only MRSA biofilm inhibitory effect. Further researches are required to isolate and identify the compounds from the dichloromethane and *n*-hexane extracts and evaluates their further roles on QS and biofilm inhibition.

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DISCLOSERS

The authors report no conflicts of interest in this manuscript.

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