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An Aqueous Extract of Yunnan Baiyao Inhibits the Quorum-Sensing-Related Virulence of *Pseudomonas aeruginosa*

Zu-Guo Zhao[1†], Shuang-Shuang Yan[3†], Yun-Mei Yu[2], Na Mi[1], La-Xi Zhang[2], Jun Liu[1], Xiao-Ling Li[1], Fang Liu[1], Jun-Fa Xu[3], Wei-Qing Yang[1], and Guo-Ming Li[1*]

[1]*Department of Microbiology and Immunology, Guangdong Medical College, Zhanjiang, 524023, P. R. China*
[2]*The 422nd Central Hospital, the People's Liberation Army, Zhanjiang, 524023, P. R. China*
[3]*Institute of Medical Laboratory, Guangdong Medical College, Dongguan, 523808, P. R. China*

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Yunnan Baiyao is a famous Chinese medicine that has long been directly applied to wounds to reduce bleeding, pain, and swelling without causing infection. However, little is known about its ability to prevent infection. The present study aimed to assess *in vitro* the anti-virulence activity of an aqueous extract of Yunnan Baiyao (YBX) using *Pseudomonas aeruginosa* as a pathogenic model. We found that a sub-MIC (2.5 mg/ml) of YBX can efficiently interfere with the quorum-sensing (QS) signaling circuit. Real-time polymerase chain reaction analysis showed that a sub-MIC of YBX down-regulated the transcriptions of *lasR*, *lasI*, *rhlR*, and *rhlI*, which resulted in global attenuation of QS-regulated virulence activities, such as biofilm formation, and secretion of LasA protease, LasB elastase and pyocyanin. Further, YBX reduced the motility of *P. aeruginosa* related to QS, and impaired the formation of biofilms. These results suggest that YBX may possess global inhibitory activity against the virulence of *P. aeruginosa* and that YBX may also exhibit antimicrobial activity *in vivo*. The present study suggests that Yunnan Baiyao represents a potential source for isolating novel, safe, and efficacious antimicrobial agents.

Keywords: *P. aeruginosa*, quorum sensing, virulence, motility, Yunnan Baiyao

Introduction

The treatment of bacterial infections with antibiotics has become increasingly compromised by the development of microbial antibiotic resistance. The isolation of multidrug-resistant bacterial strains, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, is becoming more frequent (Bereket *et al*., 2012). Therefore, there is an urgent need for novel antibiotics that can evade microbial resistance.

An important question is how the mechanisms of drug resistance can be circumvented. Bacterial virulence makes an attractive and ideal target. In most cases, virulence is a prerequisite for infection, although it is not required for bacterial survival as illustrated by the development of attenuated live vaccines (Cardona *et al*., 2009; Wu *et al*., 2011). Therefore, anti-virulence strategies that disarm pathogens rather than killing them may apply milder evolutionary pressure that does not favor the development of resistance, as is the case for currently available antibiotics (Veesenmeyer *et al*., 2009; Rasko and Sperandio, 2010).

To develop novel anti-virulence agents, researchers focus on plants, because they serve as a robust reservoir for drug discovery. Recently, many medical and edible plants that have long been used worldwide have been demonstrated to possess anti-virulence activity (Song *et al*., 2010; Koh and Tham, 2011; Jakobsen *et al*., 2012) and low or moderate toxicity. Yunnan Baiyao (YB), one of the most famous Chinese herbal medicines, consists mainly of components derived from medicinal plants (U.S. Food and Drug Administration, 2002). It has long been widely used to reduce bleeding, pain, and swelling. Interestingly, although it is not necessary to sterilize YB, over the past nearly 100 years there is no documented case of it causing an infection itself when applied to a lesion. We speculate, therefore, that YB may represent the "perfect" antimicrobial agent. However, since the complete formula of YB is kept as a "national secret" by the Chinese government, little is known about its exact components and their antimicrobial activity. *Panax pseudoginseng* is the only component of YB that we were able to find in a search of the PubMed database. *P. pseudoginseng* exhibits a quorum-sensing inhibitory (QSI) effect on *P. aeruginosa* (Koh and Tham, 2011).

P. aeruginosa virulence is mainly regulated by an intercellular signaling process called quorum sensing (QS). There are two well-studied *P. aeruginosa* QS systems, *las* and *rhl*. N-3-oxo-dodecanoyl homoserine lactone (OdDHL) and N-butanoyl homoserine lactone (BHL) act as autoinducers (AI), and LasR and RhlR function as transcriptional regulators (Antunes *et al*., 2010). The *P. aeruginosa* QS system is responsible for many virulent behaviors, such as biofilm formation, and secretion of LasA protease, LasB elastase, exotoxin A, alkaline protease, and pyocyanin (Antunes *et al*., 2010). In addition to QS, type IV pili (TFP) and flagella as well as type II and type III secretion systems are *P. aeruginosa* virulence factors (Veesenmeyer *et al*., 2009). Here, we report for the first time that an extract prepared from

† These authors contributed equally to this work.
*For correspondence. E-mail: gdmcgml@yahoo.cn; Tel.: +867592388270; Fax: +867692388364

YB exerts anti-virulence effects and significantly attenuates virulence factor of *P. aeruginosa*.

Materials and Methods

Bacteria strains and culture conditions

P. aeruginosa strain PAO1 carrying the chromosomal *PlasB*-encoded labile green fluorescent protein (GFP, $T_{1/2} \approx 40$ min) reporter and a *dsred* expression cassette (Hentzer *et al.*, 2002) was a kind gift of M. Givskov, University of Copenhagen. The media used for motility testing were as follows: swimming plates contained 10 g/L tryptone (Difco, USA), 5 g/L NaCl and 0.3% agarose; swarming plates contained 0.5% Difco Bacto Agar, 8 g/L Difco nutrient broth, and 5 g/L glucose; and twitching plates contained 10 g/of tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 1% Difco Granulated Agar. Gentamicin (15 μg/ml) and carbenicillin (300 μg/ml) were added to cultures of *P. aeruginosa*. Azithromycin (AZM), a well-studied QS inhibitor (Tateda *et al.*, 2001; Bala *et al.*, 2011), was used (2 μg/ml) as a positive control.

Preparation of drug extract

Eighty grams of YB powder (the National Pharmacy, China) was extracted with 800 ml ultrapure water prepared with a Milli-Q® Integral system (EMD Millipore Corporation, USA) on a shaker at room temperature for 24 h. The extract was centrifuged twice at 25,000×*g* for 60 min to remove particles and was then lyophilized (Heto PowerDry LL1500, Thermo Fisher Scientific Inc., USA). Approximately 650 mg of lyophilized powder was obtained from 80 g of YB powder. Before use, the drug powder was dissolved in media and sterilized by passing it through a 0.22-μm syringe filter (EMD Millipore Corporation, USA).

Determination of MIC

The MIC of YBX for *P. aeruginosa* was determined by two-old macrodilutions in Mueller-Hinton broth with an inoculum of 10^5 CFU/ml (Bala *et al.*, 2011). The MIC was defined as the lowest concentration of YBX allowing no visible growth, and the sub-MIC was defined as the highest concentration of YBX that did not inhibit growth by measuring cell density (Bala *et al.*, 2011). For other experiments, *P. aeruginosa* was cultured in a 20 ml conical flask with shaking at 37°C in LB broth containing appropriate concentrations of YBX. Bacterial cultures were sampled at intervals of 1 h. Cell density was determined by measuring absorbance at 600 nm. Total and extracellular bacterial protein levels of 16-h *P. aeruginosa* cultures were determined using the Bradford assay (Bradford, 1976).

Quantitative assay of QS inhibitory activity using a GFP reporter

Micrographs of *P. aeruginosa* were taken as described by Hentzer *et al.* (2002) using a fluorescence microscope (TE 2000-U, Nikon). For testing the influence of YBX on the establishment of the QS circuit, *P. aeruginosa* was cultured in the presence of appropriate concentration of YBX or AZM and sampled at 1-h intervals for fluorescence emitted by GFP (GFV) using a Synergy HT Multi-Mode Microplate Reader (Hentzer *et al.*, 2002). For testing the influence of YBX after the QS circuit was established, cells were grown to OD_{600}=1.0, and then either YBX or AZM was added to the culture to the desired concentration. Cells were recultured at 37°C, and GFV was determined at 2-h intervals.

RNA extraction and quantitation of QS gene expression

The primers used for QS gene quantification were as follows (sense and antisense): *lasI*, 5′-GCCCCTACATGCTG AAGAACA-3′ and 5′-CGAGCAAGGCGCTTCCT-3′; *rhlI* 5′-GCAGCTGGCGATGAAGATATTC-3′ and 5′-CGAAC GAAATAGCGCTCCAT-3′ (Takaya *et al.*, 2008); *lasR*, 5′-AAGGAAGTGTTGCAGTGGTG-3′ and 5′-GAGCAGTT GCAGATAACCGA-3′; and *rhlR*, 5′-GACCAGGAGTTCG ACCAGTT-3′ and 5′-GGTAGGCGAAGACTTCCTTG-3′ (Bratu *et al.*, 2006). The relative levels of transcription were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) and were normalized to that of 16S rRNA. Total bacterial RNA from 16 h cultures was isolated using an RNA isolation Kit $RNA_{fast200}$ (Feijie Biotechnology Co. LTD., China). PrimeScript II 1st Strand cDNA Synthesis and SYBR® *Premix Ex Taq*™ Kits (TaKaRa Biotechnology, China) were used for cDNA synthesis and real-time PCR, respectively. Control reactions without reverse transcriptase mixture were performed to assess contamination with genomic DNA. All reactions were performed using an ABI 7500 Fast Real-Time PCR System (ABI, USA).

Quantification of secreted of virulence factors and static biofilms

Pyocyanin was sequentially extracted with chloroform and 0.2 N HCl and was quantified by its absorption at OD_{520}. LasA protease activity in culture supernatants was determined by measuring the rate of lysis of a *S. aureus* cell suspension. LasB elastase activity was determined using an Elastin-Congo Red (ECR; Sigma-Aldrich, USA) assay described by Kong *et al.* (2005). To determine if the inhibitory effects were reversed by addition of exogenous acyl-homoserine lactones (HSLs), either synthetic OdDHL or BHL (Sigma-Aldrich) was added to cultures in the early logarithmic growth phase to 0.13 μmol/L (for OdDHL) or 5.2 μmol/L (for BHL), which are the maximal autoinducer (AI) concentrations for *P. aeruginosa* PAO1 (Cataldi *et al.*, 2009). Twenty-four static *P. aeruginosa* biofilms were quantified using crystal violet (Adonizio *et al.*, 2008).

Motility assays

Swimming, swarming, and twitching motility tests were performed as described by Rashid and Kornberg (2000). Briefly, for swimming and swarming tests, overnight cultures initiated with a single colony were inoculated using a toothpick onto swimming and swarming plates containing YBX or AZM, respectively, and incubated at 30°C for 24 h. For the twitching test, a bacterial colony was stab-inoculated to the bottom of the Petri dish using a toothpick. After incubation at 37°C for 24 h, the twitching zone at the agar-Petri dish interface was measured.

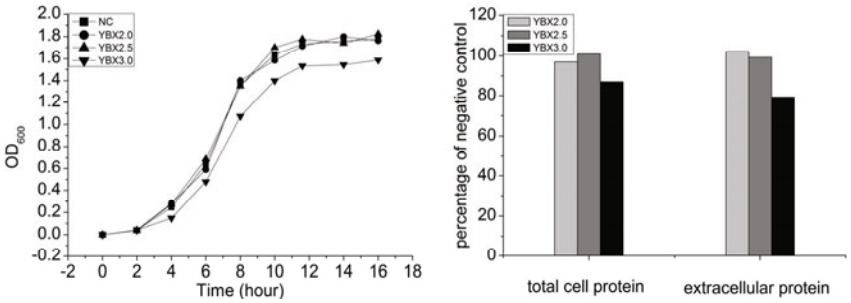

Fig. 1. Growth curve and protein quantification of *P. aeruginosa* PAO1. (A) Growth curve in the presence of YBX; (B) expression level of total and extracellular protein of *P. aeruginosa* in the presence of YBX. Total cellular or extracellular protein at different YB extract concentrations is designated Vt, and the value of the corresponding negative control (NC) without YB is designated Vc. The result of 100%×Vt/Vc is used to evaluate the inhibitory effect of the YBX on *P. aeruginosa* growth and protein synthesis. The numbers associated with YBX indicate its concentration (mg/ml).

Statistical analysis

All quantitative experiments were performed independently in triplicate. Data was analyzed by one-way analysis of variance using the SPSS 11.0 statistical software package. P=0.05 was considered significant.

Results

Antibacterial effect of YBX

The MIC and sub-MIC values of YBX were 55 mg/ml and 2.5 mg/ml, respectively (data not shown). At 3.0 mg/ml, cell density and total bacterial and extracellular protein were reduced by 13, 13, and 21%, respectively (Figs. 1A and 1B). However, 2.0 or 2.5 mg/ml of YBX had no significant impact on growth or protein synthesis (Figs. 1A and 1B). Therefore 2.5 mg/ml was designated as the sub-MIC of YBX.

Effects of YBX on QS signaling

The QSI effect of YBX was determined using labile GFP expressed under the control of the *lasB* promoter (Hentzer *et al.*, 2002). When YBX was applied at the beginning of culture, a sub-MIC concentration of YBX delayed the expression of GFP by 4 h and reduced the maximum GFV by 63% (Fig. 2A), indicating that the establishment of the QS signaling circuit was impeded. When YBX was added to a *P. aeruginosa* culture of 1.0 OD, it reduced *P. aeruginosa* GFV by 35.2% during the first 2 h (Fig. 2B), which suggested that an established QS signaling circuit was also disrupted by YBX. YBX added to 2 mg/ml exhibited a weaker QSI effect, and 3.0 mg/ml exhibited a stronger QSI effect compared with 2.5 mg/ml YBX (Figs. 2B and 2C), indicating that YBX exerted a dose-dependent QSI effect. Similar to the results of Tateda *et al.* (2001), 2.0 μg/ml of AZM also exerted a QSI effect (Figs. 2A and 2B).

Quantification of QS gene expression

The *lasI-lasR* and *rhlI-rhlR* QS systems of *P. aeruginosa* regulate the expression of numerous virulence factor genes and play important roles in the development of biofilms. The *las* and *rhl* systems are directly or indirectly regulated by environmental conditions (Duan and Surette, 2007). Our data presented here show that transcriptional levels of *lasR*, *lasI*, *rhlR*, and *rhlI* were down-regulated by at least 64% (Fig. 3) by 2.5 mg/ml of YBX, indicating that YBX may globally inhibit the QS system. The inhibitory effect of 2.0 mg/ml of YBX was weaker than that of 2.5 mg/ml.

Suppression of QS-controlled virulence factors

Global down-regulation of quorum-sensing gene transcription implies the reduction of *P. aeruginosa* virulence. Although the expression of GFP was used to represent the expression of LasB elastase, we quantified LasB elastase directly together with two other representative virulence factors, LasA protease and pyocyanin. Our data show that the expression of the LasA protease (68.85% decrease), LasB elastase (65.64% decrease), and pyocyanin (76.47% decrease) were significantly suppressed by 2.5 mg/ml of YBX (Table 1). Similar to its QSI effect, the inhibitory effect of YBX on QS-controlled virulent factors was also dose-dependent. Interestingly, addition of OdDHL, an AI of the *las* system, significantly relieved the inhibitory effect of YBX on the three toxins. In contrast, BHL, an AI of the *rhl* system, significantly antagonized the inhibitory effect of YBX on the secretion of pyocyanin (Table 1).

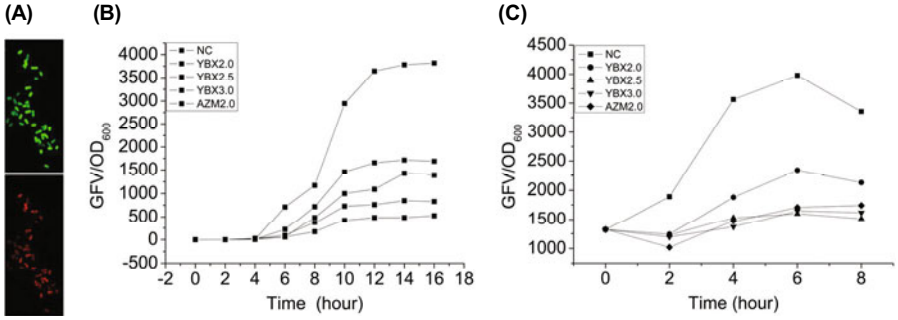

Fig. 2. YBX interferes with QS signaling. (A) Fluorescent micrograph of *P. aeruginosa* strain PAO1 carrying the chromosomal *PlasB*-encoded labile GFP reporter and a *dsred* expression cassette. Green fluorescence indicates transcription of *lasB*, namely activation of the *las* system, and red fluorescence indicates bacterial cells. (B) YBX was applied at the beginning of culture to study its interference with the establishment of *P. aeruginosa* QS signaling circuit. (C) YBX was added to a *P. aeruginosa* culture of 1.0 OD to study its interference with the already established *P. aeruginosa* QS signaling circuit. NC indicates negative control. The numbers associated with YBX and AZM indicate concentration (mg/ml for YBX, μg/ml for AZM).

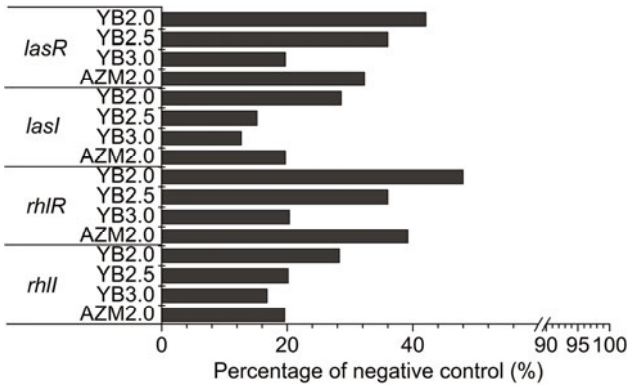

Fig. 3. Inhibitory effect of YBX on transcription of QS gene. Transcriptional level of each gene in the presence of YBX is expressed as percentage of that of negative control. The numbers associated with YBX and AZM indicate concentration (mg/ml for YBX, μg/ml for AZM).

Biofilm is another important virulence factor, and it was reduced by 58.7% in the presence of 2.5 mg/ml of YBX (Table 1). This suggests that YBX may also be used for the treatment of chronic infections.

The effects of YBX on motility

P. aeruginosa displays three major forms of motility (Rashid and Kornberg, 2000) as follows: (i) TFP-mediated twitching on solid surfaces, (ii) flagellum-mediated swimming in an aqueous environment, and (iii) swarming on semisolid (viscous) surfaces (Rashid and Kornberg, 2000). The mean twitching diameters in the presence of a sub-MIC of YBX or on AZM-treated cultures were significantly less than that of controls (Fig. 4A). YBX significantly reduced *P. aeruginosa* to swarming ablility in a manner similar to that of AZM (Fig. 4B). This indicates that YBX damaged flagella and TFP of *P. aeruginosa*. Unexpectedly, no decrease in swimming was observed in the presence of a sub-MIC of YBX (Fig. 4C).

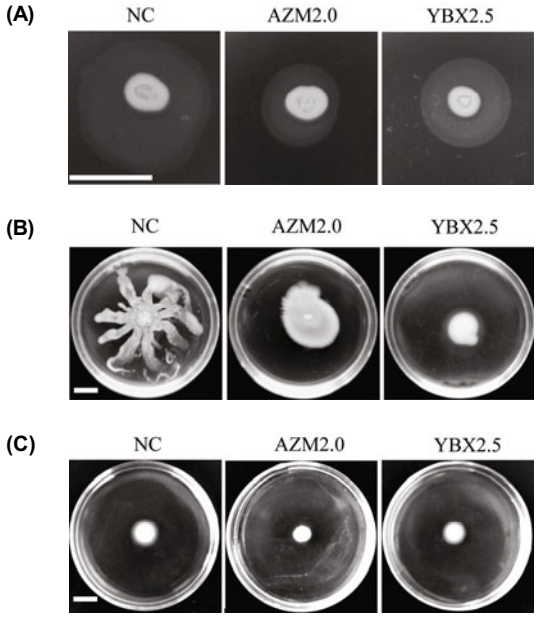

Fig. 4. Impairment of motility by a sub-MIC level of YBX. (A) twitching motility; mean twitching diameter at sub-MIC of YBX and AZM-treated *P. aeruginosa* cultures are 1.9 (±0.23) cm and 2.21 (±0.27) cm, respectively, and both are significantly less than that [3.31 (±0.29) cm] of the NC ($P<0.05$); white bar indicates 1 cm. (B) swarming motility; YBX and AZM-treated *P. aeruginosa* cultures exhibit significantly impaired swarming motility. (C) swimming motility; mean diameter of YBX-treated *P. aeruginosa* cultures is 1.10 (±0.14) cm and is not significantly shorter than that [(1.15 (±0.17) cm] of NC ($P>0.05$). The mean diameter of AZM-treated *P. aeruginosa* cultures is 0.7 (±0.11) cm and is significantly shorter than that of NC ($P<0.05$). NC, negative control. The numbers associated with YBX and AZM indicate concentration (mg/ml for YBX, μg/ml for AZM).

Discussion

The PubMed database includes studies on YB that address its procoagulant, anti-inflammatory, and wound healing-promoting activities (Li *et al.*, 2011; He *et al.*, 2012; Jia *et al.*, 2012); however, there are no reports regarding its antimicrobial activity. Therefore, the goal of the present study was to determine whether an aqueous extract (YBX) prepared from YB could inhibit the growth of the model pathogen, *P. aeruginosa.* We report here that the MIC of YBX was 55 mg/ml and its sub-MIC was 2.5 mg/ml. However, 55 mg/ml is not a feasible concentration for use as an antibiotic. Therefore, we focused our experiments on the effects of YBX on virulence using a near-sub-MIC.

YB is not a prescription drug in China and is only allowed

Table 1. Inhibitory effect of the YB extract on QS-regulated virulence

LasA activity is expressed as the change in OD_{600}/h/μg protein; elastase activity is expressed as the change in OD_{495} per microgram protein; pyocyanin concentration is expressed as micrograms of pyocyanin produced per microgram total protein. In all assays, the concentrations of OdDHL and BHL are 0.13 μmol/L and 5.2 μmol/L, respectively. NC, negative control; ND, not detected. The numbers associated with YBX and AZM indicate concentrations (mg/ml for YBX, μg/ml for AZM). [a] P value<0.05 vs NC; [b] P value<0.05 vs. the same concentration of YBX or AZM alone.

Drug	LasA protease	LasB elastase	Pyocyanin	Biofilm
NC	0.61 (±0.037)	35.8 (±5.15)	0.68 (±0.07)	1.530 (±0.12)
YBX 2.0	0.26 (±0.03)[a]	17.0 (±2.10)[a]	0.20 (±0.03)[a]	0.785 (±0.09)[a]
YBX 2.5	0.19 (±0.03)[a]	12.3 (±2.42)[a]	0.16 (±0.02)[a]	0.632 (±0.05)[a]
YBX3.0	0.13 (±0.04)[a]	8.2 (±1.36)[a]	0.12 (±0.02)[a]	0.512 (±0.05)[a]
AZM2.0	0.15 (±0.02)[a]	13.3 (±1.92)[a]	0.18 (±0.02)[a]	0.561 (±0.06)[a]
YBX 2.5+OdDHL	0.50 (±0.08)[a, b]	27.2 (±3.56)[a, b]	0.46 (±0.07)[a, b]	ND
YBX 2.5+BHL	0.21 (±0.03)[a, b]	13.5 (±1.43)[a, b]	0.43 (±0.04)[a, b]	ND
AZM2.0+OdDHL	0.52 (±0.07)[a, b]	24.2 (±3.19)[a, b]	0.42 (±0.06)[a, c]	ND
AZM2.0+BHL	0.22 (±0.04)[a, b]	13.0 (±2.01)[a, b]	0.51 (±0.03)[a, b]	ND

to be used as a dietary supplement in the United States (U.S. Food and Drug Administration, 2002). Consequently, the dose and frequency of administration are at the complete discretion of users, making evaluation of the drug's efficacy impossible. To address this issue, we used a genetically engineered *P. aeruginosa* strain that expresses GFP under control of the *lasB* promoter (Hentzer *et al.*, 2002) to allow rapid evaluation of the QSI effect of YB. As we expected, YBX interfered with the establishment of the QS signaling circuit and disrupted an established QS circuit at or below the sub-MIC.

Sub-MIC doses of AZM inhibit QS by suppressing the *las* and *rhl* systems (Tateda *et al.*, 2001); however, many plants reduce the virulence of *P. aeruginosa* by unknown QS-inhibiting (Adonizio *et al.*, 2008). We investigated here the QS target of YBX by quantifying the transcriptional levels of QS genes, and found that all four QS genes (*lasR*, *lasI*, *rhlR*, and *lasI*) were significantly down-regulated. These findings indicate that YBX may have a global inhibitory effect on *P. aeruginosa* QS signaling and QS-related virulence. Because YB comprises a complex mixture of components derived from medicinal plants, it is reasonable to conclude that its mechanism or mechanisms of action are complex. For example, multiple chemicals present in YB may cause direct effects on different aspects of the *las* and *rhl* system or act on a global regulator high in the QS hierarchy, such as *Vfr* (Liang *et al.*, 2012) or *gacA* (De Souza *et al.*, 2003).

Secreted virulence factors are key mediators of acute infection. The LasA protease and LasB elastase are *las*-controlled virulence factors (Bala *et al.*, 2011), and pyocyanin (Adonizio *et al.*, 2008) is controlled by *rhl* system. The three representative secreted virulence factors are often used as indicators of QS-regulated virulence factors (Dekimpe and Deziel, 2009). The results of our present study show that the effects of toxins controlled by *las* and *rhl* can be significantly inhibited by YBX at or below sub-MIC, indicating that it can be used to treat acute infections caused by *P. aeruginosa*. Because sub-MICs of YB did not inhibit the growth or protein synthesis of *P. aeruginosa*, the suppression of secreted virulence factors appears to be a specific behavior. Other plants also have specific inhibitory effects on the virulence of *P. aeruginosa* (Hentzer *et al.*, 2002; Adonizio *et al.*, 2008; Jakobsen, 2012). Interestingly, exogenous AIs can relieve the inhibitory effect on virulence factors, which indicates that the attenuation of *P. aeruginosa* virulence by YBX may partially due to the decrease in synthesis of HSLs, which are critical signal molecules that activate the QS circuit and then the production of many virulence factors (Antunes *et al.*, 2010).

Biofilms can be thought of as highly organized bacterial cities encased in a polysaccharide matrix and attached to a surface. They are highly resistant to adverse conditions and are key factors in chronic infection (Davies *et al.*, 1998). Similar to the effects of other plant extracts (Hentzer *et al.*, 2002, 2003; Rasmussen *et al.*, 2005; Rice *et al.*, 2005; Jagani, *et al.*, 2009), we show here that YBX can also significantly reduce static biofilm produced by *P. aeruginosa*, which means that YBX may be used to treat chronic infection. Because the *P. aeruginosa las* system contributes greatly to biofilm formation (Davies *et al.*, 1998), substantial down-regulated transcription of *lasR* and *lasI* may be directly responsible for the reduction of *P. aeruginosa* biofilms. As we expected, AZM at a sub-MIC can also significantly reduce the formation of static *P. aeruginosa* static biofilms. However, much remains to be learned about YBX 's inhibitory effect on the formation of continuous biofilms.

Motility plays an important role in the pathogenesis of *P. aeruginosa* infections. It is crucial for colonization, biofilm formation, and expression of full virulence (Beatson *et al.*, 2002; Balloy *et al.*, 2007). Although YBX did not reduce the ability of *P. aeruginosa* to swim, it significantly affected swarming and twitching motility, which suggests that YBX can interfere with the functions of flagella and TFP. *P. aeruginosa* motility is regulated by the QS system (Glessner *et al.*, 1999; Patriquin *et al.*, 2008), and YBX may inhibit motility by interfering with QS or by acting directly on TFP and flagella, thus contributing partially to the inhibition of biofilm formation and the expression of other virulence factors.

The results of the present study demonstrate that an aqueous extract of YB exerts excellent anti-virulence activity by interfering with *las* and *rhl* QS signaling and by partially reducing motility. The effect of YB on other bacteria that are associated with infected wounds, such as *S. aureus*, *E. coli* and *Enterococci*, requires further study. Although our results do not reveal the major mechanism responsible for the anti-virulence activity of YBX, the successful history of the application of YB as a drug, together with our results, strongly indicates that YB may provide a source for discovering natural compounds with significant antimicrobial activity.

Acknowledgements

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