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ฤทธิ์ต้านแบคทีเรีย และฤทธิ์ต้านระบบ Quorum sensing ของแบคทีเรีย จากพืชสมุนไพรบางชนิดในจังหวัดนครราชสีมา ประเทศไทย

Anti-bacterial and Anti-quorum Sensing Properties of Selected Medicinal Plants from Nakhon Ratchasima Province, Thailand

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Abstract

Quorum sensing (QS) is an intercellular signaling system and gene regulatory mechanism used by bacteria to respond to their cell population. Several physiological processes in pathogenesis of medically important bacteria are associated with this system. As QS controls bacterial virulence factors, the inhibition of the system is being considered as a novel approach for antibacterial substance. Hence, the present study was determined the effect of 48 Thai medicinal plant species on bacterial QS, *Chromobacterium violaceum*. Screening test using disc diffusion assay showed that five plant extracts exhibited anti-QS activity including *Alpinia galanga* Sw., *Feronia limonia* (L.) Swing, *Millingtonia hortensis* Linn., *Oroxylum indicum* (L.) Kurz., and *Sesbania grandiflora* (L.) Poiret. All extracts, except *A. galanga*, reduced strong violacein production. The sixteen extracts from 60 plant extracts which exhibited an antibacterial activity, were than determined minimal inhibitory concentration (MIC) and was observed the violacein production. The extract of *A. galanga* and *P. betle* gave MIC values at 5 and 1.25 mg/ml, respectively. In addition, the two extracts also possessed an anti-QS activity at sub-MIC. The results reveal the mode of action of botanical plants including anti-QS property and this activity should be further examinations.

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1. Introduction

Quorum sensing (QS) is an intercellular signaling system in bacteria. This system lets bacteria to communicate their cell population and to regulate a variety of physiological processes [1]. The most pathogenic bacteria have QS systems to control their phenotypes, including pigmentation, swarming motility, secretion of virulence factors and biofilm formation [2]. In different environments, many bacteria are attached to various surface materials to form biofilms. The bacterial biofilms are more resistant to antibiotic treatment, host immune, and contribute to bacterial persistence in chronic infections than cells in suspension [3]. Thus, method of controlling biofilms refers to inhibit the QS system is considered as an alternative therapeutic method for treating microbial infections [4].

Chromobacterium violaceum is Gram-negative bacteria has been found in a QS system by synthesis of violacein, the purple pigment with antibacterial activity [6]. This bacterium has been used as a model to investigate natural products for anti-QS activity [6, 7, 8]. Any alteration in the pigment-producing ability of *C. violaceum* under the influence of test extract(s) can easily be observed.

Traditional treatment of diseases generally relies on natural sources for remedies. Many plant species have been used for treating microbial infections or as antiseptics. They have been developed to contribute significantly in pharmaceutical products [9]. The emergence of antibiotic resistance microorganisms requires for novel alternative therapeutics. Conventional researches to control pathogenic bacteria are studied on the antimicrobial agents that aim to kill or inhibit bacterial growth [10]. This approach is concerned the observed development of resistance to anti-microbial compounds. The discovery of bacterial communication systems (QS systems) is an opportunity to challenge bacterial infection [6]. In many reports, medicinal plant extracts have been reported to kill or inhibit bacteria. Therefore, the present study aimed to investigate the anti-QS and anti-bacterial potentials of Thai medicinal plant extracts against *C. violaceum* using paper disc agar diffusion and broth microdilution methods.

2. Materials and Experiment

2.1 Medicinal plants

Forty eight medicinal plants were used in this study as showed in Table 1. They were collected in Nakhon Ratchasima Province, Thailand. All of the plant materials were dried at 50°C for 3-4 days or until dry. They were crushed and soaked with 95% ethanol for 7 days. The solvent was then distilled under reduced pressure in a rotary evaporator until it became completely

dry. The extracts were dissolved in dimethyl sulfoxide (DMSO, Himedia, India) before use.

2.2 Bacterial strain

Chromobacterium violaceum DMST 21761, obtained from Department of Microbiology and Natural Products Research Center, Faculty of Science, Prince of Songkla University, was used in this study. The bacterium was cultured in trypticase soy agar (TSA, Himedia) at 30°C for 18 h. The bacterial strains were maintained in TSB containing 20% glycerol at -80°C.

2.3 Anti-bacterial and anti-quorum sensing activities

The paper disc agar diffusion method was used to detect anti-QS activity of the extracts [11]. The extracts were dissolved in DMSO, 10 µl (250 mg/ml) of the crude extracts was applied to sterile filter paper discs (Whatman no. 1; 6 mm in diameter) therefore each disc was saturated with 2.5 mg of the extract. Than the discs were dried at 30°C for overnight and were applied to the surface of TSA seeded with 3–5 h tripticase soy broth (TSB) culture of *C. violaceum*. The plates were incubated at 30°C for 24 h and examined for diameter of violacein production and inhibition zone. Quorum-sensing inhibition in *C. violaceum* has been presented as the inhibition of purple pigmentation around the disc containing the extracts also demonstrates viable cell growth. A clear halo indicated an anti-bacterial activity. DMSO was used as control. All the experiments were performed in triplicate, and measurements were reported as mean ± SD.

2.4 Determination of minimal inhibitory concentration (MIC) of active plant extracts and violacein inhibitory assay

The MICs of the active ethanolic extracts were examined using a broth microdilution method according to CLSI guideline [12]. The extracts were serially diluted twofold in TSB in 96-well microtiter plates to obtain final concentrations ranged from 0.15-10 mg/ml. Then, an equal volume of 100 µl of log phase culture of *C. violaceum*, approximately 10^6 CFU/ml, was then added to each well. After incubation at 30°C for 16-18 h, MIC was defined as the lowest concentration of the extracts at which the microorganism did not exhibit visible growth, as indicated by the turbidity of the medium compared with 1% DMSO as a negative control.

For violacein inhibition assay, the microtiter plates were further incubated at 30°C for 48 h and observed for the reduction or increase in purple pigment production compared with the untreated condition. An inhibited pigment production at sub-MIC was showed as a minus (—). At sub-MIC condition, the treatment as same as the control presented in a plus (+) and if the pigmentation was more intensive than the control, the result was read as stimulated pigment production which presented in two-plus (++). All experiments were done in triplicate.

3. Results and Discussion

3.1 Screening of anti-bacterial and anti-quorum sensing activities in plants using *Chromobacterium violaceum* assays

The paper disc agar diffusion method was initially carried out to determine an antibacterial activity of the 48 medicinal plants against *C. violaceum*. Out of the 60 medicinal plant extracts, the 16 ethanolic extracts of *Alpinia galanga*, *Andrographis paniculata*, *Careya arborea*, *Feronia limonia*, *Limnophila aromatica*, *Melastoma malabathricum*, *Millingtonia hortensis*, *Oroxylum indicum*, bark and leaf extracts of *Peltophorum pterocarpum*, *Persicaria odorata*, *Piper betle*, *Rhodomyrtus tomentosa*, *Syzygium cumini*, *Xanthostemon chrysanthus* and *Ziziphus mauritiana* demonstrated inhibition of bacterial growth against the test bacterium (Table 1). The plant extracts exhibited clear zones ranging from 6.5 mm to 18.8 mm diameter, with the most remarkable results presented by leaf extract of *P. betle*.

The expression of violet pigment, violacein, in *C. violaceum* was suggested that the bacterium was regulated via QS system. Loss of violacein in *C. violaceum* was demonstrated QS was inhibited by the plant extracts. The yellowish zone of inhibition was observed opaque rather than transparent, indicating that the halo around the disc was caused by inhibition of quorum sensing, not inhibition of cell growth. Out of 60 ethanolic extracts (Table 1) screened for anti-QS activity, five extracts proved to be effective activity. Strong quorum-sensing inhibition was observed in the extract of *A. galanga*. Four extracts such as *F. limonia*, *M. hortensis*, *O. indicum*, and *S. grandiflora* showed partial inhibition with a slight incomplete zone.

As a large number of scientific studies, various plant species produce secondary metabolites to control the growth of microorganisms and have traditionally been used to treat human diseases, particularly bacterial infections [10]. *C. violaceum* is a Gram-negative bacillus, facultative anaerobe and motile. The bacterium is widely distributed in natural aquatic environments. It typically can produce an anti-oxidant pigment, violacein, with purple color [5]. Pathogenic infection of *C. violaceum* is infrequent. However, increasing human infections have been reported [13]. Also, it is noteworthy to emphasize the multidrug-resistant bacteria [13]. This study introduces 16 medicinal plant species possessing anti-Chromobacterium activity. In addition, five of 60 plant species contained anti-QS activity.

3.2 Determination of MIC and violacein production of the active extracts

The antibacterial activities of 16 active extracts against *C. violaceum* were determined MICs using doubling dilution method with the concentrations varying from 0.15-10 mg/ml (as showed in Table 2). Along with the plants investigated, *P. betle* showed a good MIC at 1.25 mg/ml. Whereas, extract of *A. galanga*, *C. arborea*, *A. paniculata*, *R. tomentosa*, and *X. chrysanthus* exhibited MIC values ranging 2.5-5

mg/ml. On the other hand, MIC values of 10 extracts ranged from 10 to >10 mg/ml.

After verify MIC values, anti-QS potential of 16 extracts was observed at 48 h (as showed in Table 2 and Fig. 2). The extracts of *P. betle* and *A. galanga* at sub-MIC concentrations caused a decrease in violacein production compared with the control. In contrast, sub-MICs of *C. arborea*, *L. aromatica*, *M. malabathricum*, bark of *P. pterocarpum*, *R. tomentosa*, *S. cumini*, and *X. chrysanthus* was observed which stimulated over production of violacein in *C. violaceum*.

Ethanolic extracts of *P. betle* and *A. galangal* possessed an anti-QS activity in broth microdilution method. In contract, the *P. betle* extract did not give the result using paper disc diffusion assay, whereas, the extract of *A. galanga* was showed an inhibit violacein production. This appearance also has been reported in determination of anti-bacterial activity by disc diffusion technique [16]. Therefore, using the diameter of inhibition zone to indicate relative antibacterial or anti-QS activities was not typically quantitative and sufficient. Many factors such as the evaporation, solubility, and diffusion rate of the active compounds throughout the medium may involved the inhibition zone or purple pigment reduction zone. Consequently, in this study, liquid culture by determination of MIC was carried out to further assess the effects of the active plant extracts on violacein production.

Piper betle or Betel is a glabrous climbing vine belonging to the family Piperaceae. It is plentifully dispersed in many Asian countries. The Betel leaves have been used in traditional medicine also as antibacterial agent. The volatile oil known as Betel oil is the chief constituent of the leaves [14]. *Piper betle* can be of great benefit in folk treating diseases caused by bacteria and fungi. Previous studies on the Betel leaves, roots and whole extract (mixture of volatile and non-volatile) of the green variety showed a very strong antimicrobial activity. The extract (400 µg/disc) showed highest zone of inhibition against the Gram positive *S. aureus* (6.77±0.25 mm) [15]. On *Streptococcus pyogenes*, the extract gave high inhibition zone with 29 mm however, MIC/MBC values demonstrated moderate activity (500-1000 µg/ml) [16]. In this study, sub-MIC of *P. betle* reduced violacein production which indicated this extract may process an anti-biofilm activity. According to recent study, the hexane, chloroform and methanol extracts from *P. betle* showed the most potent anti-QS activity against *C. violaceum* CV026 [17]. In addition, leaves of *P. betle* extract showed a potent anti-biofilm agent on *C. albicans* that contains dual actions of preventing biofilm formation and removing existing biofilm [18].

Alpinia galanga commonly called greater galangal, belonging to the family Zingiberaceae is a rhizomatous herb distributed in various parts of India and throughout Southeast Asia [19]. It has been used as food additive in Thailand and other countries in Asia for a long time. Recent study, plant parts of *A. galanga* like the root, rhizome and leaf extracted with methanol,

acetone and diethyl ether have showed good activity against Gram-positive and Gram-negative pathogens [20].

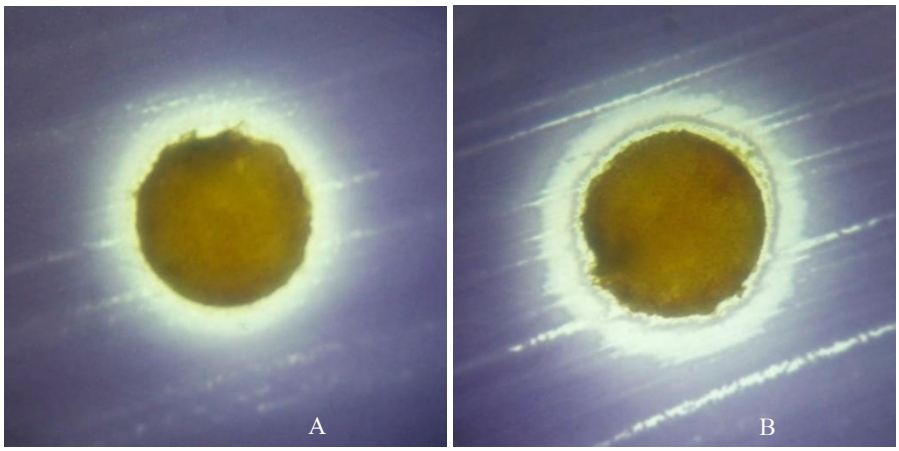

Fig. 1 Result of anti-quorum sensing screening of *Alpinia galanga against Chromobacterium violaceum* DMST 21761. Magnification of the areas surrounding a disc containing ethanolic extract of *A. galanga* at 2.5 mg/ml under the same magnification was observed inhibition of violacein pigmentation (8.92 ± 0.70 mm, in Table 1) and inhibition of the bacterial growth (6.5 ± 0.00 mm, in Table 1) after incubated for 48 h (A) and 4 days (B).

Table 1 Anti-bacterial and anti-quorum sensing activity of selected Thai medicinal plants against *Chromobacterium violaceum* DMST 21761

Medicinal plants	**Plant part**	**Inhibition zone (mm)**	
		Antibacterial activity	**Anti-quorum sensing activity**
Abutilon indicum (L.) Sweet	leaf	ND	ND
	fruit	ND	ND
Aegle marmelos (L.) Correa ex Roxb.	fruit	ND	ND
Alpinia galanga Sw.	rhizome	6.5 ± 0.00	8.92 ± 0.70
Andrographis paniculata (Burm.f.) Wall ex Nees.	whole plant	7.0 ± 0.87	ND
Artocarpus lakoocha Roxb.	leaf	ND	ND
Asparagus racemosus Willd.	leaf	ND	ND
	root	ND	ND
	root bark	ND	ND
Averrhoa carambola Linn.	leaf	ND	ND
Azadirachta indica A. Juss	leaf	ND	ND
Bauhinia purpurea L.	leaf	ND	ND
Boesenbergia rotunda (Roxb.) Schltr.	rhizome	ND	ND
Cardiospermum Halicacabum Linn.	leaf	ND	ND
	fruit	ND	ND
Cerbera odollam Gaertn.	flower	ND	ND
Careya arborea Roxb	leaf	7.73 ± 1.66	ND
Caseria grewiifolia Vent.	leaf	ND	ND
	bark	ND	ND
	fruit	ND	ND
Centella asiatica (L.) Urban.	whole plant	ND	ND
Chromolaena odorata (L.) R.M.King & H. Rob.	leaf	ND	ND
Cleome gynandra L.	leaf	ND	ND
Crateva adansonii DC. subsp. trifoliata (Roxb.) Jacobs	leaf	ND	ND

ND: not detected the activity. The results were obtained from 3 separated experiments (±SD values).

+: from observing, the bacterial growth around the disc has a slight loss of purple pigmentation.

Table 1 Anti-bacterial and anti-quorum sensing activity of selected Thai medicinal plants against *Chromobacterium violaceum* DMST 21761 (continued)

Medicinal plants	**Plant part**	**Inhibition zone (mm)**	
		Antibacterial activity	**Anti-quorum sensing activity**
Diospyros rhodocalyx Kurz.	leaf	ND	ND
Dolichandrone serrulata (DC.) Seem.	leaf	ND	ND
Feronia limonia (L.) Swing	leaf	8.75 ± 2.60	+
Limnophila aromatica (Lam.) Merr	whole plant	7.4 ± 1.15	ND
Maerua siamensis (Kurz.) Pax	leaf	ND	ND
Melastoma malabathricum Linn	leaf	8.33 ± 0.76	ND
Metha cordifolia Opiz.	leaf	ND	ND
Millingtonia hortensis Linn.	leaf	8.3 ± 0.77	+
Momordica cochinchinensis (Lour.) Spreng.	leaf	ND	ND
Moringa oleifera Lam.	leaf	ND	ND
Oroxylum indicum (L.) Kurz.	leaf	8.38 ± 0.63	+
Ocimumbasilicum L.	leaf	ND	ND
Passiflora foetida Linn.	leaf	ND	ND
Peltophorum pterocarpum (DC.) Backer ex K.Heyne	leaf	6.57 ± 0.98	ND
	bark	7.25 ± 1.06	ND
	fruit	ND	ND
Persicaria odorata	whole plant	6.5 ± 0.00	ND
Phyllanthus acidus (L.) Skeels	leaf	ND	ND
Piper betle Linn.	leaf	18.83 ± 3.24	ND
Rhodomyrtus tomentosa Wight	leaf	9.75 ± 1.64	ND
Ricinus communis Linn.	leaf	ND	ND
Schleichera oleosa (Lour.) Oken.	leaf	ND	ND
	seed	ND	ND
	peel	ND	ND
	bark	ND	ND

ND: not detected the activity. The results were obtained from 3 separated experiments (±SD values).

+: from observing, the bacterial growth around the disc has a slight loss of purple pigmentation.

Table 1 Anti-bacterial and anti-quorum sensing activity of selected Thai medicinal plants against *Chromobacterium violaceum* DMST 21761 (continued)

Medicinal plants	**Plant part**	**Inhibition zone (mm)**	
		Antibacterial activity	**Anti-quorum sensing activity**
Sesbania grandiflora (L.) Poiret	leaf	ND	+
Streblus asper Lour.	leaf	ND	ND
	fruit	ND	ND
Syzygium cumini Linn.	leaf	9.25 ± 0.64	ND
Tamarindus indica L	bark	ND	ND
Thunbergia laurifolia Lindl.	leaf	ND	ND
Tiliacora triandra (Colebr.) Diels	leaf	ND	ND
Xanthostemon chrysanthus	leaf	10.13 ± 1.22	ND
Zingiber officinale Roscoe.	rhizome	ND	ND
Ziziphus mauritiana Lam.	leaf	9.05 ± 0.42	ND
Zizyphus oenoplia (L.) Mill.	leaf	ND	ND

ND: not detected the activity. The results were obtained from 3 separated experiments (±SD values).

+: from observing, the bacterial growth around the disc has a slight loss of purple pigmentation.

Table 2 Minimum inhibitory concentration (MIC) of 16 effective plant extracts and anti-quorum sensing activity against *Chromobacterium violaceum* DMST 21761

Medicinal plants	**Plant part**	**MIC (mg/ml)**	**Activity of extract against violacein production***
Alpinia galanga Sw.	rhizome	5.00	—
Andrographis paniculata (Burm.f.) Wall ex Nees.	whole plant	10.00	+
Careya arborea Roxb	leaf	5.00	++
Feronia limonia (L.) Swing	leaf	10.00	+
Limnophila aromatica (Lam.) Merr	whole plant	>10.00	++
Melastoma malabathricum Linn	leaf	10.00	++
Millingtonia hortensis Linn.	leaf	>10.00	+
Oroxylum indicum (L.) Kurz.	leaf	10.00	+
Peltophorum pterocarpum (DC.) Backer ex K.Heyne	leaf	10.00	+
	bark	10.00	++
Persicaria odorata	whole plant	10.00	+
Piper betle Linn.	leaf	1.25	—
Rhodomyrtus tomentosa Wight	leaf	5.00	++
Syzygium cumini Linn.	leaf	10.00	++
Xanthostemon chrysanthus	leaf	2.50	++
Ziziphus mauritiana Lam.	leaf	>10.00	+

* Violacein inhibition assay was observed for the reduction or increase in purple pigmentation compared with the untreated condition. A minus (—) presented an inhibited pigment production at sub-MIC, one plus (+) presented the pigment production in a test condition as same as the control, two plus (++) displayed the extract stimulated pigment production at sub-MIC

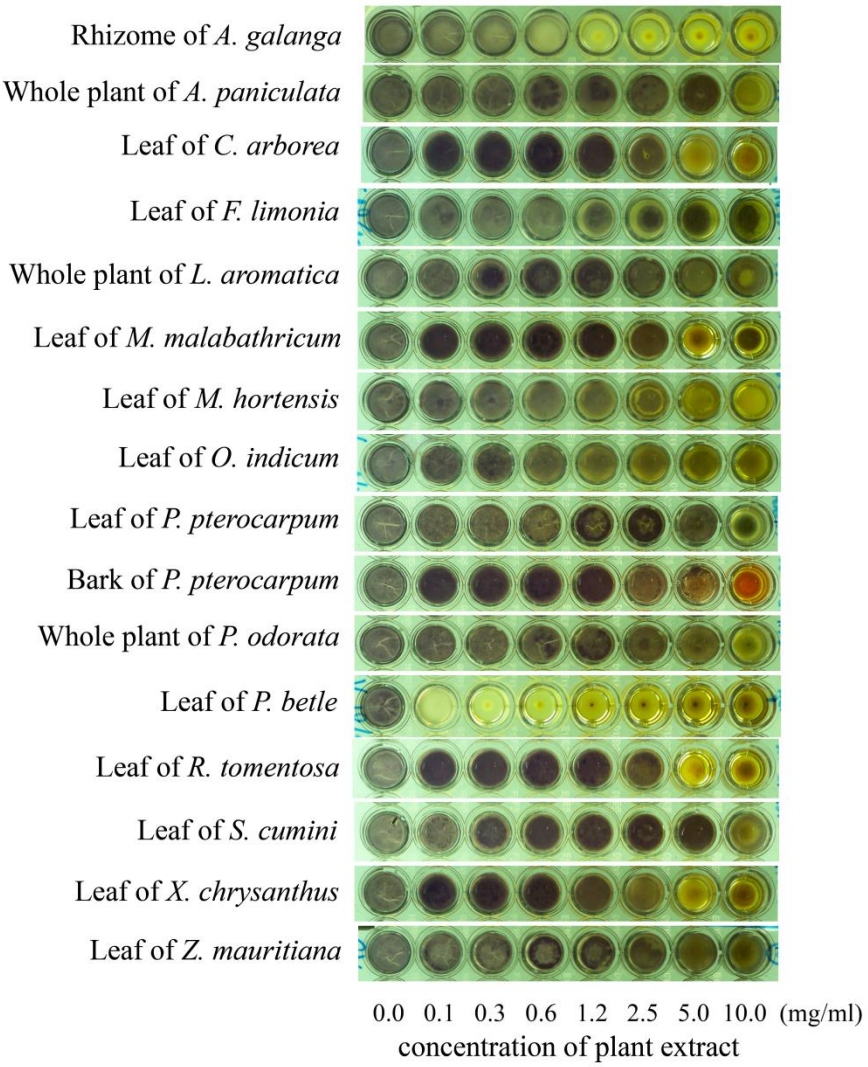

Fig. 2 Effects of plant extracts on violacein production of *C. violaceum* DMST 21761. Using broth microdilution assay, the inhibition of violacein production was observed at 48 h. Extracts of *A. galanga* and *P. bettle* showed an anti-quorum sensing activity against *C. violaceum* by an inhibiting violacein production at sub-MIC of the extracts.

4. Conclusions

In conclusion, the anti-QS potential of medicinal plants was proposed significantly as an antibacterial effect. This study was determined the effect of 48 Thai medicinal plant species on bacterial QS, *Chromobacterium violaceum*. The results were introduced two ethanolic extract of *A. galanga* and *P. betle* produced the anti-bacterial and anti-QS activities. This research revealed the mode of action of the medicinal plants including anti-QS property and this activity should be further examinations such as quantitative assay of

violacein production, biofilm formation, bacterial motility and virulence factor production.

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