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Anti-quorum Sensing Activity of Medicinal Plants Used in Thai Traditional Medicine for Infections

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Quorum sensing is a control of bacterial gene expression in response to cell density. Several processes in pathogenesis of many medically-important bacteria are associated with quorum sensing. As quorum sensing controls bacterial virulence, the inhibition of quorum sensing is being considered as a novel strategy for antibacterial drug. This study investigated the effect of 44 Thai medicinal plant species on bacterial quorum sensing. Anti-quorum sensing activity was indicated by the inhibition of violacein pigment producing of biomonitor strain, *Chromobacterium violaceum* DMST 21761. Screening test by disc diffusion assay revealed that 9 plant extracts exhibited anti-quorum sensing activity including *Cinnamomum bejolghota*, *Cinnamomum porrectum*, *Holarrhena antidysenterica*, *Punica granatum*, *Quercus infectoria*, *Quisqualis indica*, *Terminalia bellirica*, *Terminalia chebula*, and *Terminalia* sp. Flask incubation assay was used for quantifying the inhibitory activity. All extracts, except *Q. infectoria*, reduced violacein production but did not suppress the cell growth. *Holarrhena antidysenterica*, *T. bellirica*, and *Terminalia* sp. produced pronounced inhibitory activity. This finding may uncover the potential mode of action of these plants. Anti-quorum sensing property of these medicinal plants may play an important role as the antimicrobial activity for their efficacy in traditional use as medicines and this should not be overlooked.

Key words: Quorum sensing, Thai medicinal plants, *Holarrhena antidysenterica*, *Terminalia bellirica, Terminalia* sp., *Chromobacterium violaceum*.

The widespread use and misuse of antibiotics are playing a significant role in the emergence and spread of resistant bacteria[1,2]. Use of antibiotics may result in a selective pressure by killing susceptible bacteria, allowing antibiotic-resistant bacteria to survive and grow. Increase in antibiotic resistance in many pathogenic bacteria leads to an urgent requirement for new antibacterial drugs with novel modes of actions in order to fight against their resistance[3,4]. Inhibition of bacterial virulence factors is an attractive target for antibacterial drug and could represent an innovative therapeutic strategy[5,6]. Antivirulence approaches do not stress bacteria by killing activity but they just lower their virulence factors and increase the odds that antibiotics or immune system can clear bacterial infections, hence, selective pressure will not occur. Moreover, the specificity of antivirulence drugs could preserve the bacteria constitutive of the normal microbiota[6,7].

Bacterial communication, or quorum sensing, is a cell-population dependent expression of species in bacteria mediated by signaling molecules called autoinducers. Gram-negative bacteria use derivatives of homoserine lactones as autoinducers while gram-positive bacteria use

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secreted peptides[8,9]. Quorum sensing system controls several processes in pathogenesis and regulates the expression of virulence factors of many medically-important bacteria. Many bacterial virulence factors are controlled by quorum sensing system, including biofilm formation, enzyme and toxin productions[9-11]. As quorum sensing system controls bacterial virulence, inhibition of quorum sensing is being considered as an alternative approach to antibacterial therapy. Several bacterial models such as *Agrobacterium tumefaciens*, *Chromobacterium violaceum*, and *Pseudomonas aeruginosa* have been used to study the inhibition of quorum sensing. Among these bacterial models, *C. violaceum* is the most popular bacterium that used for anti-quorum sensing screening. This bacterium synthesizes violet pigment named violacein due to the regulation of quorum sensing system mediated by its autoinducer *N*-hexanoyl homoserine lactone[12].

Many natural products such as fruit, herb, and spice have been reported to demonstrate anti-quorum sensing activity[13-21]. A wide variety of plants in traditional Thai medicine have long been used as remedy for many infectious diseases. Most recent researches have reported only the antibacterial property of plants used to treat infection[22-24]. Nonetheless, anti-quorum sensing activity of plants, a new issue which can contribute useful information has not been investigated. The objective of this study was to investigate the effect of Thai medicinal plants on bacterial quorum sensing using *C. violaceum* as a biomonitor strain.

MATERIALS AND METHODS

Plant collection and extract preparation

Forty-four selected medicinal plant species used in traditional Thai medicine were used in this study. Parts of plants were collected on the basis of traditional practices by Dr. Oratai Neamsuvan, an ethnobotanist. Reference voucher specimens were made and deposited at Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The plant materials were cleaned and dried at 60°C overnight. They were crushed and soaked with 95% ethanol for 7 days. The solvent was then filtrated and distilled under reduced pressure in a rotary evaporator until it became completely dry. The plant extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) before use. All extracts were stored at -4°C. The aliquots were check for sterility using a sterile loop streaking on Tryptic soy agar (TSA, Difco) and incubated at 37°C for overnight.

Screening for anti-quorum sensing activity

Paper disc agar diffusion method was used to detect anti-quorum sensing activity of the extracts[14]. The extracts were dissolved in DMSO, 10 µL of the extracts (100 and 200 mg/mL) was applied to sterile filter paper discs (Whatman no. 1; 6 mm in diameter) so that each disc was saturated with 1 or 2 mg of the extract. Dry discs (dried at 37°C overnight) were applied to the surface of TSA plates seeded with 3 to 5 h Tryptic soy broth (TSB, Difco) culture of *C. violaceum* DMST 21761 (1.5×10^8 CFU/mL). The plates were incubated overnight at 30°C and examined for violacein production. Inhibition of quorum sensing system in the bacterial population was detected by a colourless, opaque halo around the disc. The paper disc with DMSO was used as control. The experiment was replicated twice.

Quantification of violacein production

A modified flask incubation assay described by Choo *et al*[15] was used to quantify quorum sensing inhibitory activity of the extracts. *C. violaceum* DMST 21761 was cultured in TSB and incubated for 18 to 24 h. One mL of the culture ($OD_{600} = 0.1$) was inoculated to Erlenmeyer flasks containing 9 mL of TSB supplemented with the plant extracts at concentration of 2.0, 1.0 and 0.5 mg/mL. The flasks were incubated at 30°C with 150 rpm agitation for 24 h in a shaking incubator. 1% DMSO was used as control. After incubation, 1 mL culture from each flask was centrifuged at 5,000 rpm for 5 min to precipitate the insoluble violacein. The culture supernatant was discarded and 1 mL of DMSO was added to the pellet. The solution was vortexed vigorously for 30 s and leaved for 24 h to completely solubilize violacein and centrifuged at 5,000 rpm for 5 min to remove the cells. Two hundred µL of the violacein-containing supernatants were added to 96-well flat bottomed microtiter plate (Corning Life Sciences, USA) and the absorbance was read with a microtiter plate reader (Perkin-Elmer, Finland) at a wavelength of 595 nm. The experiment was replicated three times.

In parallel experiments, each culture was spread on TSA plates to confirm any antibacterial activity of the plant extracts. Each culture was serially diluted and spread on TSA plates. The plates were incubated at 30°C for 24 h, and bacterial counts were compared with control (1% DMSO). The experiment was replicated twice.

RESULTS

Quorum sensing screening test

The paper disc diffusion assay was performed to test anti-quorum sensing activity of 48 extracts from 44 Thai medicinal plant species used in traditional Thai medicine. We found that 9 (20%) out of 44 Thai medicinal plant species demonstrated anti-quorum sensing activity with *C. violaceum* as a bacterial reporter strain. These include *Cinnamomum bejolghota*, *Cinnamomum porrectum*, *Holarrhena antidysenterica*, *Punica granatum*, *Quercus infectoria*, *Quisqualis indica*, *Terminalia bellirica*, *Terminalia chebula*, and *Terminalia* sp. (Table 1). Strong quorum-sensing inhibition was observed in the extract of *H. antidysenterica* (Fig. 1), followed by *Terminalia* sp., *T. bellirica*. *Quercus infectoria* extract produced both growth and quorum sensing inhibition zones. The quorum sensing inhibition zone occurred after the margin of the inhibition zone. Meanwhile, *Piper betle* extract exhibited the inhibition of cell growth without quorum sensing inhibition zone.

Quantification of violacein production

The flask incubation assay was used to quantify quorum sensing inhibitory activity of the extracts that produced quorum-sensing inhibition zones. All the test extracts, except *C. porrectum*, demonstrated concentration-dependent violacein inhibitory activity. A decrease level in violacein content was demonstrated in the extracts at all tested concentrations (Fig. 2). At concentration of 0.5, 1.0, and 2.0 mg/mL, *H. antidysenterica*, *T. bellirica*, and *Terminalia* sp. extracts did not inhibit cell growth, but significantly reduced violacein production with percentage of inhibition ranging from 26 to 71, 42 to 59, and 32 to 61, respectively. The bacterial cell count performed on TSA plates at 24 h showed no significant difference in the numbers of CFU in all the tested extracts (Fig. 3), except for *Q. infectoria* (the number of CFU at concentration of 0.5, 1.0, and 2.0 mg/mL were decreased 1.7, 1.8, and 3.4 log, respectively). This correlated to the results of the paper disc diffusion assay and further confirmed that all extracts, except *Q. infectoria*, did not possess antibacterial activity against *C. violaceum*.

DISCUSSION

Chromobacterium violaceum synthesizes violet pigment violacein as a result of quorum sensing[12]. Loss of the purple pigment violacein in *C. violaceum* is an indicative of quorum sensing inhibition by the plant extracts. In this study, we found that 20% of Thai medicinal plant species demonstrated anti-quorum sensing activity with *C. violaceum* as a bacterial reporter strain. Anti-quorum sensing activity screening can be improved by using several bacterial biomonitors because the ability to detect this activity depends on each system used[14,21]. Koh and Tham[21] reported 70 and 40% out of 10 Chinese medicinal plant species with anti-quorum sensing activity when *C. violaceum* and *P. aeruginosa* were used as reporter strains, respectively.

Strong quorum sensing inhibition was observed in the extract of *H. antidysenterica*, followed by *Terminalia* sp., and *T. bellirica*. At 2 mg/ml, these extracts inhibited violacein production more than 50%. Our previous study with the same plant extracts found that these plant extracts did not show strong antibacterial activity, especially the activity against gram-negative bacteria[22]. However, these plants have had a long history of use in traditional Thai medicine as therapies for ailment caused by bacterial infections particularly for diarrhoea. Due to their weak antibacterial activity, the anti-quorum sensing activity or other mechanisms may be responsible for their therapeutic efficacies.

Quercus infectoria exhibited antibacterial activity as well as anti-quorum sensing activity. Similarly, Adonizio *et al*[14] found some medicinal plants from southern Florida, *Conocarpus erectus* and *Bucida buceras*, demonstrated these two activities. From this previous study and our result, the same question is arisen whether reduced concentration of antibacterial compounds in the extract can improve an anti-quorum sensing activity by reducing the cell population to a number below

Table 1. Anti-quorum sensing activity of ethanolic extracts of Thai medicinal plants using agar disc diffusion method and *Chromobacterium violaceum* DMST 21761 as a biomonitor strain.

Botanical name (Family name)	Plant part (% yield)	Herbal remedies	[a]Quorum sensing inhibition zone (mm±SD)	
			1 mg/disc	2 mg/disc
Acacia catechu (L.f.) Willd. (Fabaceae)	core (5.6)	asthma, body pain, cancer, cough, eczema, diarrhoea, pile, leprosy, sore mouth, sore throat, ulceration	-	-
Aegle marmelos (L.) Corrêa (Rutaceae)	fruit (5.4)	diarrhoea, dysentery, stomachache	-	-
Ardisia colorata Roxb. (Myrsinaceae)	fruit (4.4)	diarrhoea, fever, haematinic	-	-
Asclepias curassavica L. (Asclepiadaceae)	wood (1.0)	asthma, cough, pain, typhus fever	-	-
Centella asiatica (L.) Urb. (Apiaceae)	leaf (6.0)	fever	-	-
Cinnamomum bejolghota (Buch.-Ham.)	bark (14.7)	diarrhoea, biliousness, flatulence, carminative	7.0±0.0	7.5±0.0
Sweet (Lauraceae)	wood (2.3)		-	-
Cinnamomum porrectum (Roxb.) Kosterm.	bark (7.1)	tonic, flatulence	8.2±1.4	9.5±0.7
(Lauraceae)	wood (11.2)		-	-
Curcuma zedoaria (Christm.) Roscoe (Zingiberaceae)	rhizome (9.6)	asthma, cough, leueorrhoea, tonsillitis	-	-
Curcuma longa L. (Zingiberaceae)	rhizome (3.3)	arthritis, inflammatory, skin disease	-	-
Derris scandens Roxb. Benth. (Leguminosae)	stem (3.2)	inflammatory, analgesic	-	-
Dryopteris syrmatica O. Kze. (Polypodiaceae)	wood (4.5)	diarrhoea	-	-
Eleutherine americana Merr. (Iridaceae)	bulb (4.8)	cold, diuretic, gastralgia	-	-
Euphorbia thymifolia L. (Euphorbiaceae)	whole plant (1.3)	diarrhoea, dysentery, enteritis	-	-
Gymnopetalum cochinchinensis (Lour.) Kurz (Cucurbitaceae)	fruit (7.7)	tonic, fever	-	-
Holarrhena antidysenterica (L.) Wall. ex A. DC. (Apocynaceae)	bark (2.1)	amoebic dysentery, diarrhoea, fever, stomachache, tonic	19.5±0.7	23.0±0.0
Impatiens balsamina L. (Balsaminaceae)	leaf (5.2)	wart, wound, abscess, chronic ulcer	-	-
Manilkara achras (Mill.) Fosberg (Sapotaceae)	fruit (26.8)	diarrhoea, pulmonary complaint	-	-
Millingtonia hortensis L. f. (Bignoniaceae)	flower (25.4)	asthma, sinusitis, tonic	-	-
Mimosa pudica L. (Fabaceae)	whole plant(4.9)	asthma, biliousness, blood disease, burning sensation, dysentery, fatigue, inflammatory, leprosy, vaginal and uterine complaint	-	-
Mitragyna speciosa (Korth.) Havil(Rubiaceae)	leaf (6.0)	diarrhoea	-	-
Momordica charantia L. (Cucurbitaceae)	vine (3.0)	aid in childbirth, cough, diabetes, fever, headache, hepatitis, hypertension, infection, malaria, measles, menstrual disorder, skin disease, stomachache	-	-

Morinda citrifolia L. (Rubiaceae)	fruit (7.4)	asthma, dysentery, fever, wound, stomachache, tonic	-	-
Murdannia loriformis (Hassk.) R.S. Rao & Kammathy (Commelinaceae)	whole plant (7.7)	cough, diabetes	-	-
Oroxylum indicum (L.) Kurz (Bignoniaceae)	bark (3.7)	astringent, diarrhoea, dysentery, rheumatism, tonic	-	-
Phyllanthus niruri L. (Euphorbiaceae)	whole plant (7.8)	anemia, asthma, biliousness, bronchitis, diuretic, leprosy, urinary discharge	-	-
Piper betle L. (Piperaceae)	leaf (9.2)	antiseptic, astringent, carminative, cough, dental care, nose bleeding, mouth odour, stomachache, tonic	[b]19.5±0.7	[b]22.0±0.0
Piper chaba Hunter (Piperaceae)	fruit (9.0)	carminative, diaphoretic, diuretic, skin liniment	-	-
Piper nigrum L. (Piperaceae)	fruit (6.3)	cough, diarrhoea, dyspepsia, headache, pharyngitis	-	-
Piper sarmentosum Roxb. (Piperaceae)	leaf (4.7)	asthma, cough, diabetes, fever, pleurisy, toothache	-	-
Pluchea indica (L.) Less. (Asteraceae)	leaf (17.8)	diabetes, diuretic, haemorrhoid	-	-
Punica granatum L. (Punicaceae)	pericarp (9.6)	anthelmintic, diarrhoea, dyspepsia	8.5±0.7	11.2±1.1
Quercus infectoria G. Olivier (Fagaceae)	nut gall (57.2)	biliousness, chronic dysentery, diarrhoea, fever, skin infection	[c]16.0±0.0	[c]18.0±0.0
Quisqualis indica L. (Combretaceae)	flower (11.1)	biliousness, chronic dysentery, diarrhoea, fever	7.5±0.7	9.5±0.7
Rhizophora mucronata Lam. (Rhizophoraceae)	bark (11.7)	angina, astringent, diabetes, diarrhoea, dysentery	-	-
	fruit (10.8)		-	-
Rhodomyrtus tomentosa (Aiton) Hassk. (Myrtaceae)	stem (7.2)	diarrhoea, stomachache	-	-
Sandoricum indicum Cav. (Meliaceae)	root (4.0)	astringent, diarrhoea, dysentery, fever, skin disease, stomachache	-	-
Tamarindus indica L. (Fabaceae)	leaf (4.8)	fever, anthelmintic, dysentery	-	-
Terminalia bellirica (Gaertn.) Roxb. (Combretaceae)	fruit (14.9)	diarrhoea	10.8±0.4	11.8±1.1
Terminalia chebula Retz. (Combretaceae)	fruit (17.2)	bacterial infection, skin disease	7.5±0.0	8.0±0.0
Terminalia sp. (Combretaceae)	fruit (23.9)	diarrhoea, fever	12.5±0.7	14±0.0
Theobroma cacao L. (Sterculiaceae)	pericarp (3.7)	antiseptic, diuretic, emmenagogue, parasiticide, vulnerary	-	-
Uncaria gambir (Rubiaceae)	leaf, branch (65.4)	inflammatory, enhancement immune,	-	-
Wrightia tomentosa (Roxb.) Roem. & Schult. (Apocynaceae)	stem (3.9)	bitter tonic, dysentery	-	-
Xylocarpus granatum J. König (Meliaceae)	pericarp (2.7)	diarrhoea, dysentery	-	-
	seed (6.8)		-	-

[a]Loss of purple pigment violacein in *C. violaceum* is an indicative of quorum-sensing inhibition by the plant extracts, not inhibition of cell growth.
[b]Inhibition of cell growth without quorum sensing inhibition zone.
[c]Quorum sensing inhibition zone with some inhibition of cell growth.
[-] No quorum sensing inhibition zone.

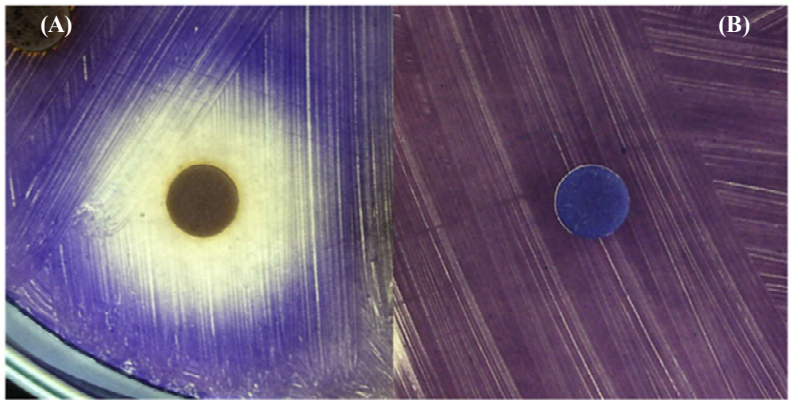

Fig. 1. Anti-quorum sensing activity using agar disc diffusion method and *Chromobacterium violaceum* DMST 21761 as a biomonitor strain. Loss of purple pigment violacein in *C. violaceum* is an indicative of quorum-sensing inhibition by the plant extracts. (A) *Holarrhena antidysenterica* extract at 2 mg/disc. (B) Dimethyl sulfoxide (DMSO) was used as control.

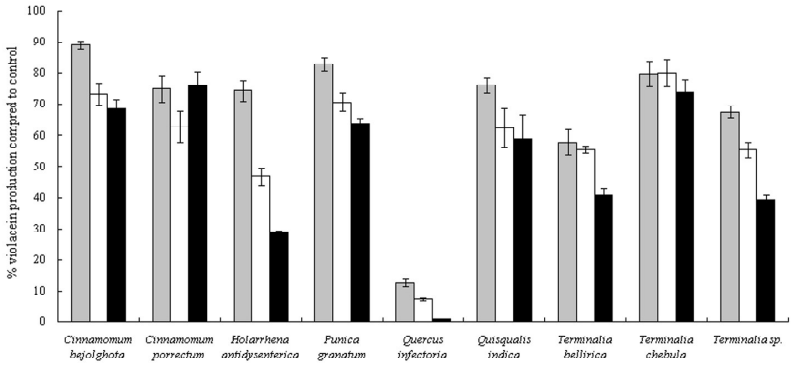

Fig. 2. Inhibition of violacein production by the plant extracts at 0.5 (▭), 1.0 (▭), and 2.0 (▬) mg/mL. Violacein production by *Chromobacterium violaceum* DMST 21761 was measured spectrophotometrically at 24 h. Data are presented as mean ± SD of absorbance at 595 nm.

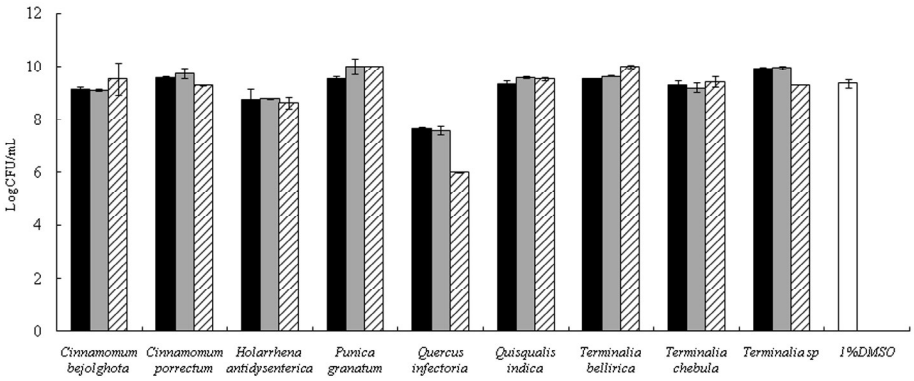

Fig. 3. Effect of the plant extracts at 0.5 (▬), 1.0 (▭), and 2.0 (▭) mg/mL on the growth of *Chromobacterium violaceum* DMST 21761. Bacterial growth was quantified using spread plate method at 24 h. 1% dimethyl sulfoxide (DMSO) was used as control (▭). The mean values of duplicate independent experiments and SDs are shown.

the quorum. Nevertheless, the study by disc diffusion assay with some antibiotics and our result evidenced this untrue. Previous work reported that at difference concentrations of tetracycline and gentamycin[14] as well as *P. betle* extract from this study possessed a distinct change of inhibition zone, but there was no loss of violacein pigment surrounding the zone. Our recent study found that *Q. infectoria* demonstrated very strong and broad spectrum of antibacterial activity against both gram-negative bacteria and gram-positive bacteria[22]. However, it is still unclear whether the antibacterial and anti-quorum sensing activities of this extract are from the same or different compounds.

Promising anti-quorum sensing compounds have been demonstrated to disrupt bacterial biofilms and make the bacteria more susceptible to antibiotics, and these compounds also provide the ability to reduce bacterial virulence factors as well as promote clearance of bacteria in infectious animal models[25-28]. Many mechanism of actions have been proposed to interfere the quorum sensing system such as (1) inhibition of autoinducer molecule biosynthesis, (2) inactivation or degradation of autoinducer, (3) interference with the signal receptor[25], and (4) inhibition of genetic regulation system[29]. Our research is ongoing to isolate the anti-quorum sensing compounds together with the investigation of their mechanism of actions and their effects on bacterial virulence factors.

In summary, the inhibition of bacterial quorum sensing introduces a new strategy for antibacterial chemotherapy and provides the prevention of antibiotic resistant bacteria. Our study indicated 9 Thai medicinal plants with anti-quorum sensing activity. This finding may uncover the potential mode of action of these plants and may lead to a new therapeutic direction for the treatment of bacterial infections. Anti-quorum sensing property of these medicinal plants may play an important role as the antibacterial activity for their efficacy in traditional use as medicines and this should not be overlooked.

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