

Anti-quorum sensing activity of medicinal plants in southern Florida

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Abstract

Bacterial intercellular communication, or quorum sensing (QS), controls the pathogenesis of many medically important organisms. Anti-QS compounds are known to exist in marine algae and have the ability to attenuate bacterial pathogenicity. We hypothesized that terrestrial plants traditionally used as medicines may also produce anti-QS compounds. To test this hypothesis, 50 medicinal plants from southern Florida were screened for anti-QS activity using two biomonitor strains, *Chromobacterium violaceum* and *Agrobacterium tumefaciens*. Of these plants, six showed QS inhibition: *Conocarpus erectus* L. (Combretaceae), *Chamaecybe hypericifolia* (L.) Millsp. (Euphorbiaceae), *Callistemon viminalis* (Sol. ex Gaertn.) G. Don (Myrtaceae), *Bucida burceras* L. (Combretaceae), *Tetrazygia bicolor* (Mill.) Cogn. (Melastomataceae), and *Quercus virginiana* Mill. (Fagaceae). This study introduces not only a new mode of action and possible validation for traditional plant use, but also a potentially new therapeutic direction for the treatment of bacterial infections.

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1. Introduction

Whether for traditional use-validation or drug discovery purposes, previous studies have focused on the antibacterial potential of medicinal plants (e.g., Cowan, 1999; Wallace, 2004). Investigations were restricted to whether or not a plant could kill or inhibit the growth of bacteria. However, this is only one facet of a plant's anti-infective potential. The antipathogenic properties of plants have received much less attention, but may be just as important in combating disease as their antibacterial counterparts. The interruption of quorum sensing (QS), or bacterial cell-to-cell communication, is one example of an antipathogenic effect. Since a large number of systems affecting pathogenicity are controlled by QS, interrupting this communication system can render pathogenic bacteria non-virulent (Zhang and Dong, 2004).

Quorum sensing (QS) is a population-dependent phenomenon first characterized in the 1970s in luminescent marine

species of *Vibrio* (Nealson et al., 1970; Hastings and Greenberg, 1999). QS systems are ubiquitous in bacteria, and have since been found to regulate diverse cellular functions including luminescence, biofilm formation, antibiotic production, virulence factor expression, pigment production, plant-microbe interactions, and motility (Whitehead et al., 2001; Fuqua and Greenberg, 2002). In QS, small signaling molecules called autoinducers mediate the ability to sense the size of a bacterial population (Eberhard et al., 1981). Autoinducers are constantly produced and received at a basal level by bacterial cells. With high population density, there is a surplus of such molecules in the environment (Hastings and Greenberg, 1999). These molecules then interact with a transcriptional regulator to activate the expression of genes involved in light production in luminescent *Vibrio* species (Fuqua et al., 1994). In other bacteria, these chemical signals are important for the establishment of infection and can serve as a switch to a pathogenic state (De Kievit and Iglewski, 2000; Wu et al., 2001; Sircili et al., 2004).

Several signaling molecules have been identified (Dong and Zhang, 2005); the best-characterized being the acyl-homoserine lactones (AHLs) in Gram-negative bacteria (Eberhard et al., 1981). The AHLs are highly conserved, having the same homoserine lactone moiety but different acyl side chains and substitution (carbonyl or hydroxyl) at the C3 carbon (Fuqua

Abbreviations: AHL, acyl-homoserine lactone; QS, quorum sensing; wt, wild type; X-gal, 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

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and Greenberg, 2002). AHL-mediated quorum sensing systems have been characterized in bacteria associated with human disease, e.g., *Pseudomonas aeruginosa* (Smith and Iglewski, 2003), *Yersinia pseudotuberculosis* (Atkinson et al., 1999), *Clostridium difficile* (Carter et al., 2005), *Burkholderia cepacia* (Lutter et al., 2001), and *Escherichia coli* (Surette and Bassler, 1998); as well as plant associated bacteria, e.g., *Rhizobium leguminosarum* (Rodelas et al., 1999), *Ralstonia solanacearum*, and *Erwinia carotovora* (Von Bodman et al., 2003). The discovery of the QS system and its critical role in bacterial virulence and survival has revealed a new target—a novel way to attack and attenuate bacterial pathogenicity.

There are a number of ways to interrupt the QS system. Thus, anti-quorum sensing (anti-QS) compounds can be of great interest in the treatment of bacterial infections (Fast, 2003; Rice et al., 2005). A number of quorum-quenching enzymes that hydrolyze AHLs have been identified in bacteria (Dong and Zhang, 2005). To date, the only known anti-QS compounds of non-bacterial origin are halogenated furanones from the red alga *Delisea pulchra* (Manefield et al., 1999). Anti-QS activity has also been shown in a number of southern Florida seaweeds (Cumberbatch, 2002) and a few terrestrial plants (Teplitski et al., 2000; Gao et al., 2003). However, so far, only a handful of higher plants have been studied, and nothing has been published with regard to anti-QS activity in medicinal plants.

The plant kingdom has long been a source of medicines and continues to contribute significantly to the development of today's pharmaceuticals (Cragg et al., 1997). The emergence of antibiotic resistance begs the need for novel therapeutics. It has been suggested that targeting the QS system, instead of killing bacteria, may provide a solution to antibiotic resistance (Hentzer and Givskov, 2003). With the promise of anti-QS compounds, one should be compelled to search for these agents by the most efficient method possible. There have been many ethnobotanically directed searches for agents to treat infection, demonstrating not only the need for these drugs, but also the large number of plants utilized for bacterial conditions (e.g., Cowan, 1999; Camporese et al., 2003; Gnanamani et al., 2003; Hernandez et al., 2003). Although this antibacterial effect is important, it is not the only source of a plant's medicinal properties. Shifting the focus from the strictly antibacterial to anti-QS properties of plants may reveal new quorum quenching compounds and provide use-validation for traditional medicinals.

Using two different biomonitor strains selected for their QS genetics, we were able to screen southern Florida plants for anti-QS activity. We have found anti-QS activity in 6 out of 50 plants, and in most cases this activity is retained upon extraction into common solvents.

2. Materials and methods

2.1. Collection of plant material and extract preparation

Fifty plant species (Table 1) were collected in the Florida International University Environmental Preserve and Miami-

Dade County, FL from February–May 2004. Plant selection was based on availability and ethnobotanical use categories relating to infection (Duke, 1972, 1985, 2000; Morton, 1981; Burkhill, 1985; Liogier, 1990). These categories include plants used to treat wounds, burns, and dermatitis; conditions that favor microbial colonization. Plants were collected and processed in triplicate. All plants were identified by the authors and vouchers were deposited in the herbarium at Fairchild Tropical Botanic Garden (FTG), Miami, FL.

As a preliminary screen, unwashed leaves from 10 species were compared with leaves washed in ethanol to determine the effect of surface microbes and epiphylls. Plants were then processed and tested in four stages using two biomonitor strains (described in the following section). Testing of fresh material composed the primary screen, as it was faster and ethnomedicinally analogous to poultices. Plants that showed anti-QS activity as fresh material were tested in dried form. Those that still retained activity after drying were tested as water and ethanol extractions. Screening was performed in this manner, rather than testing extracts only, since certain chemicals may have been lost or altered during processing.

For ethanolic extracts, plants were separated into component parts and dried in a plant drier for approximately 24 h. Dried plant matter was ground and added to 95% ethanol (100 g dry wt/L), and allowed to stand for 24 h before vacuum filtration with filter paper (Whatman, 1001-270, Florham Park, NJ) to remove particulate matter. An aliquot was removed to test for anti-QS activity at the 100 g/L concentration. The remainder was evaporated to dryness using a rotoevaporator (Buechi R-114, Uster, Switzerland) and stored at -20°C .

In addition, water extracts were prepared from the six species that proved active as fresh material. Plants were processed as above, dried material was added to sterile water at 100 g dry wt/L, and boiled for 5 min. An aliquot was removed for testing and the remainder of the decoction was frozen at -80°C for 24 h and then freeze-dried using a lyophilizer. The lyophilized extracts remained at -20°C to be reconstituted in sterile water as needed. Filtration of the water extracts using a 0.2 μm (pore size) filter into autoclaved vials ensured sterility of the samples. Extracts were tested for microbial contamination at every step of processing, by streaking to LB agar plates, to minimize the potential for introduction of exogenous anti-QS compounds.

2.2. Biomonitor organisms

2.2.1. *Chromobacterium violaceum*

In *Chromobacterium violaceum* (ATCC 12472), production of a purple pigment, violacein, is under QS-control (Lichstein and Van De Sand, 1945; Throup et al., 1995). This wild type strain produces and responds to the cognate autoinducer molecules C6-AHL and C4-AHL. The second biomonitor strain, CVO26, is a mutant of the wild type strain that is unable to produce its own AHL signal, but responds to exogenous active signal molecules (Cha et al., 1998). Anti-QS compounds inhibit production of violacein in both cases making these strains excellent for screening (McClellan et al., 2004).

Inhibition of Quorum Sensing-Controlled Virulence Factor Production in *Pseudomonas aeruginosa* by South Florida Plant Extracts[∇]

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Quorum sensing (QS) is a key regulator of virulence and biofilm formation in *Pseudomonas aeruginosa* and other medically relevant bacteria. Aqueous extracts of six plants, *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras*, *Tetrazygia bicolor*, and *Quercus virginiana*, were examined in this study for their effects on *P. aeruginosa* virulence factors and the QS system. *C. erectus*, *B. buceras*, and *C. viminalis* caused a significant inhibition of LasA protease, LasB elastase, pyoverdinin production, and biofilm formation. Additionally, each plant presented a distinct effect profile on the *las* and *rhl* QS genes and their respective signaling molecules, suggesting that different mechanisms are responsible for efficacy. Extracts of all plants caused the inhibition of QS genes and QS-controlled factors, with marginal effects on bacterial growth, suggesting that the quorum-quenching mechanisms are unrelated to static or cidal effects.

Pneumonia due to microbial infections is a major cause of morbidity and mortality in immunocompromised patients. *Pseudomonas aeruginosa* hails as the leading pathogen among patients with cystic fibrosis, diffused panbronchitis, and chronic obstructive pulmonary disease (16, 29, 37). In addition, *P. aeruginosa* remains one of the major causes of nosocomial infections (10). The success of this organism is largely due to the production of a myriad of virulence factors (including LasA protease, LasB elastase, pyoverdinin, pyocyanin, and alginate) and its ability to form intractable biofilms (38).

Expression of many of the virulence factors in *P. aeruginosa* is controlled by a quorum-sensing (QS) system (59), an intercellular communication scheme in which bacteria are able to detect the population density (via signaling molecules and receptors) and control gene expression accordingly (55). *P. aeruginosa* elaborates two main sets of QS systems: *lasI-lasR* and *rhlI-rhlR* (55). LasI and RhlI are synthetases that manufacture the autoinducer signaling molecules *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL), respectively. These molecules diffuse out into the environment, and when they reach a putative threshold concentration, they activate the receptors *lasR* and *rhlR*. These receptors, in turn, coordinate the regulation of pathogenicity. A third signal, the *Pseudomonas* quinolone signal, also plays an integral role in the QS system (50). This secondary metabolite of *P. aeruginosa* is incorporated into the QS hierarchy in times of cell stress (43). This intricate communication system of *P. aeruginosa* is mirrored in many gram-negative pathogenic bacteria, where it coordinates the regula-

tion of virulence, including motility, biofilm formation, and toxin production (18, 19, 21, 48, 54).

The misuse and abuse of antibiotics in pharmacotherapy have led to the development of widespread resistance in the target organism. The failure of existing antibiotics to control infection makes it crucial to find alternatives to currently available drugs. Since pathogenicity in many bacteria is regulated by QS, inhibition of this system may cause the attenuation of virulence and protect against infection (25, 32, 56). In fact, an anti-QS approach has already shown promise in the battle against *P. aeruginosa* infections (27, 62).

Anti-QS agents were first characterized in the red marine alga (*Delisea pulchra*) (40, 41) and, more recently, in a south Florida alga (15) and a few higher plants (6, 23, 57). It has been shown that terrestrial plants not only produce autoinducer mimics to confound the bacterial QS system but also receive and respond to microbial signals (1, 3). Given the promise of anti-QS compounds, efficient screening for these agents becomes imperative. In a previous study we utilized an ethnobotanically directed search for anti-QS activity (1). We confirmed that six south Florida medicinal plants, *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras*, *Tetrazygia bicolor*, and *Quercus virginiana*, have anti-QS properties using *Chromobacterium violaceum* and *Agrobacterium tumefaciens* NTL4 strains as biomonitors (1). These plants were chosen on the basis of their traditional use against respiratory and skin infections, conditions potentially caused or complicated by bacteria such as *P. aeruginosa*.

In the study described here we have taken this work a step further by exploring the effects of these six plants on the production of virulence factors and biofilms, acylated homoserine lactone (AHL) levels, and QS gene transcription in this organism. We demonstrate a significant decrease in the production of LasA protease, LasB elastase, pyoverdinin, and biofilms in the presence of the extracts. Furthermore, each plant has a unique pattern of effect on the QS genes *lasI-lasR* and *rhlI-rhlR* and their respective signaling molecules, OdDHL and BHL.

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Attenuation of *Pseudomonas aeruginosa* virulence by medicinal plants in a *Caenorhabditis elegans* model system

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Expression of a myriad of virulence factors and innate antibiotic resistance enables the opportunistic human pathogen *Pseudomonas aeruginosa* to create intractable infections. Using a nematode model, we screened for novel inhibitors of this pathogen. Aqueous extracts of three plants, *Conocarpus erectus*, *Callistemon viminalis* and *Bucida buceras*, were examined for their effects on *P. aeruginosa* killing of the nematode *Caenorhabditis elegans*. The results were evaluated in toxin-based and infection-based assays using *P. aeruginosa* strains PAO1 and PA14. The tested plant extracts prevented mortality via gut infection in approximately 60% of the worms and caused a 50–90% reduction in death from toxin production. All extracts inhibited nematode death by *P. aeruginosa* without host toxicity, indicating their potential for further development as anti-infectives.

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INTRODUCTION

Pseudomonas aeruginosa is one of the leading pathogens among patients suffering from cystic fibrosis, diffused pan-bronchitis and chronic obstructive pulmonary disease (Hoiby, 1994; Lieberman, 2003; Registry, 2005). In addition, it remains one of the major causes of nosocomial infections (National Nosocomial Infections Surveillance System, 2004). The success of this organism is attributed to numerous virulence factors (Smith & Iglewski, 2003b; Tang *et al.*, 1996), its ability to form biofilms (Costerton *et al.*, 1995) and innate antibiotic resistance (De Kievit *et al.*, 2001; Fisher *et al.*, 2005).

Conventional anti-pseudomonal treatment includes elevated doses of β -lactam, fluoroquinolone or aminoglycoside antibiotics (Hauser & Sriram, 2005). However, these drugs possess a high degree of toxicity, and mucoid strains of *P. aeruginosa* are rarely eradicated by these treatments (Hauser & Sriram, 2005; Pedersen, 1992). **The failure of existing antibiotics to control infection makes it crucial to find alternatives to currently available drugs. Since pathogenicity in many bacteria is regulated by quorum sensing (QS), or cell-to-cell communication, inhibition of this system can cause attenuation of virulence and protect**

against infection (Hentzer & Givskov, 2003; Juhas *et al.*, 2005; Smith & Iglewski, 2003a).

Plants have evolved numerous chemical strategies for deterring pathogen attack, including the production of bactericidal and anti-infective compounds, leading to their use as medicines (reviewed by Lewis & Ausubel, 2006). In our previous work, we demonstrated that a number of medicinal plants exhibit anti-QS activity (Adonizio *et al.*, 2006). Extracts of these plants were later shown to have an effect on virulence factor production, biofilm formation, QS gene expression and autoinducer production in *P. aeruginosa* (Adonizio *et al.*, 2008). In this study, we assessed the ability of three plant extracts to attenuate *P. aeruginosa* killing of the nematode *Caenorhabditis elegans*.

Caenorhabditis elegans is well established as a pertinent and practical model for studying bacterial virulence (Darby *et al.*, 1999; Tan & Ausubel, 2000), as a number of *P. aeruginosa* factors important in the killing of *Caenorhabditis elegans* are also relevant to mammalian systems (Rahme *et al.*, 1995; Tan *et al.*, 1999a). ‘Fast killing’ of *Caenorhabditis elegans* by *P. aeruginosa* strain PA14 (on rich media) is mediated through the production of virulence factors such as phenazines, whereas ‘slow killing’ (on minimal media) occurs via ingestion of the bacteria and subsequent infection (Mahajan-Miklos *et al.*, 1999;

Abbreviation: QS, quorum sensing.

(Mahajan-Miklos *et al.*, 1999). A $\Delta phnAphnB$ deletion mutant has been shown to abolish nematode death completely, whilst a $TnphoA$ mutation of the related gene *phzB* has been shown to greatly reduce mortality in mice and *Arabidopsis* (Mahajan-Miklos *et al.*, 1999). Like many virulence factors, phenazines are partially under the control of the QS gene *rhlR* (Brint & Ohman, 1995; Latifi *et al.*, 1995). The results from the PA14 fast-killing assay suggested that the addition of extracts affected phenazine production, either directly through the *phz* and *phn* genes or indirectly through the QS system via *rhlR*. All three extracts were shown previously to significantly affect the *rhlI/R* system; however, *Conocarpus erectus* has less of an effect on N-acylhomoserine lactone production and biofilm formation (strain PAO1; Adonizio *et al.*, 2008) than either *B. buceras* or *Callistemon viminalis*. Although still successful, *Conocarpus erectus* was less efficient in preventing nematode death in the fast-killing assay than the other extracts.

Plant extracts reduce the mortality of *Caenorhabditis elegans* due to slow killing by *P. aeruginosa*

The slow-killing assay left 50% of nematodes dead on PA14 between 48 and 50 h, with all worms dead by 58 h (Fig. 1c). The control worms on *E. coli* OP50 remained alive throughout the assay (not shown). The QS mutant (PA14 $\Delta lasR$) reduced nematode death, with 75% alive between 48 and 50 h and 53% alive at 58 h. At this time, approximately 60, 59 and 57% of worms were alive on PA14 plates with added *Conocarpus erectus*, *Callistemon viminalis* and *B. buceras* extract, respectively (Fig. 1c). All three of the plant extracts, when added to plates containing wild-type PA14, suppressed killing to the level of the QS mutant. There was no significant difference between PA14 $\Delta lasR$ and the extract plates or between individual extracts at 58 h ($P > 0.05$ in all cases); however, all extracts were significantly different from PA14 without treatment, suggesting a marked effect of the plant extracts on *P. aeruginosa* infection of *Caenorhabditis elegans*.

Slow killing of *Caenorhabditis elegans* occurs over approximately 60 h due to ingestion of and subsequent infection by *P. aeruginosa* (Tan *et al.*, 1999a). Nematode mortality is attenuated by $TnphoA$ mutations of *lasR* and *gacA* (Tan *et al.*, 1999b), suggesting that QS is required for the infection process. The addition of plant extracts in this assay drastically reduced nematode death, suggesting an effect on *lasR* or *gacA*. Previous work on these extracts corroborated the inhibitory effect on *lasR*; however, the effect on *gacA* was not tested directly (Adonizio *et al.*, 2008). An effect on either of these factors remains a plausible hypothesis.

Conclusions

The three plant extracts from *Conocarpus erectus* (Combretaceae), *Callistemon viminalis* (Myrtaceae) and *B. buceras* (Combretaceae), in all three assays, showed a highly

significant reduction in virulence when compared with wild-type PAO1 and PA14 without treatment. Overall, the tested plant extracts reduced nematode death by approximately 60–90% on wild-type *P. aeruginosa*. In each case, this reduction was equal to or greater than that of the corresponding QS mutant strain. The fact that the plant extracts reduced virulence across the board suggests that they are possibly affecting an upstream QS gene such as *las* or *rhl*, or perhaps a global regulator such as GacA. This further corroborates our previous data on the anti-QS effect of these plant extracts (Adonizio *et al.*, 2008). All extracts inhibited nematode death without any significant bactericidal effect, leaving QS inhibition as a plausible hypothesis. In addition, none of the tested plants showed any toxicity in the nematode model, making them reasonable candidates for purification and drug development.

Conocarpus erectus, *B. buceras* and *Callistemon viminalis* (and other closely related species) have been used medicinally to treat bacterial infections either as teas or as poultices (Burkhill, 1985; Irvine, 1961; Melendez, 1982; Morton, 1981; Stewart & Percival, 1997). Thus the plants were extracted with hot water to provide greater congruity with traditional preparation methods. Although teas and poultices are many steps removed from modern formulae, traditional use suggests the potential success of topical or enteral routes of administration.

With the increase in bacterial resistance to antibiotics, we should look to the past in the hope of finding solutions for the future. Plants have been used medicinally for thousands of years and, even without marked antibiotic activity, these three plants are still efficacious in ameliorating disease. We have previously shown the activity of these plants on *P. aeruginosa* alone and, although the exact mechanism of action is not yet known, the nematode experiments described in this paper are consistent with their previous and potential further use as anti-infectives.

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