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, diffuse panbronchiolitis, 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, pyoverdin, 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-oxodecanoyl)-L-homoserine lactone (OdhDHL) 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 intricate communication system of *P. aeruginosa* is mirrored in many gram-negative pathogenic bacteria, where it coordinates the regulation 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 ethno-botanically directed search for anti-QS activity (1). We confirmed that six south Florida medicinal plants, *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras*, *Tetragynia 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. hypericifolia* caused a significant inhibition of LasA protease, LasB elastase, pyoverdin 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.

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, pyoverdin, 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, OdhDHL and BHL.
MATERIALS AND METHODS

Plant extraction. Conocarpus erectus (Combretaceae), Chamaesyce hypericifolia (Euphorbiaceae), Callistemon viminalis (Myrtaceae) (inflorescence), Bacuta buceras (Combretaceae), Tetrazygia bicolor (Melastomataceae), and Quercus virginiana (Fagaceae) were collected and processed by the methods described previously (1). Briefly, pulverized plant material was extracted into boiling water, freeze-dried with a lyophilizer, and stored at −20°C until it was needed. Leaf extracts were tested unless otherwise noted. To complement the six active plants from the previous study, an additional plant with no anti-QS activity (Schefflera actinophylla, Apocynaceae) was chosen as a negative control.

Strains and media. Prototypic P. aeruginosa strain PAO1 (30) and its derivatives (35) were used throughout this study. In addition, Staphylococcus aureus (ATCC 12600) was used in the LasA assay. Cells were maintained on Luria-Bertani (LB) plates and in LB liquid for overnight cultures. For quantitative assays, either LB (LasA assay only) or Agrobacterium (AB) minimum medium (to which glucose and casein amino acids [20%, wt/vol] was added) was used (11).

Culture conditions. For all assays except those for biofilm formation, the culture conditions were as follows. Overnight cultures of strain PAO1 were grown in LB medium at 37°C with shaking. The cultures were then diluted 100-fold in AB or LB medium and allowed to grow to an optical density at 600 nm (OD600) of 1.7 (early stationary phase). At this point, the culture was divided into 10-ml aliquots and an additional 1 ml of fresh medium containing crude plant extract (or media control) was added to a final concentration of 1 mg/ml. Cultures were recovered at late stationary phase (approximately 12 h after addition). The cells were separated from the growth medium by centrifugation at 10,000 × g for 10 min.

LasA staphyloptic assay. LasA protease activity was determined by measuring the ability of culture supernatants to lyse boiled S. aureus cells (35). A 100-μl aliquot of P. aeruginosa LB medium culture supernatant with or without plant extracts was added to 900 μl of a boiled S. aureus suspension. The OD600 was determined after 0, 5, 10, 20, 30, 45, and 60 min. Activity was expressed as the change in the OD600 (OD600/hour per μg protein).

LasB elastolytic assay. The elastolytic activity of the AB medium culture supernatants was determined by using elastin Congo red (ECMR, Sigma, St. Louis, MO) (46). A 100-μl aliquot was added to 900 μl of ECMR buffer (100 mM Tris, 1 mM CaCl2, pH 7.5) containing 20 mg ECMR. This mixture was then incubated with shaking for 3 h at 37°C. Insoluble ECMR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. Cell-free AB medium alone and AB medium with plant extracts were used as negative controls. Activity was expressed as the change in the OD600 per μg protein.

Pyoverdin assay. The pyoverdin assay was adapted from the methods of Cox and Adams (14). The AB medium culture supernatant was diluted 10-fold in Tris-HCl buffer (pH 7.4), and 100-μl aliquots were added to 96-well microtiter plates on ice. The relative concentration of pyoverdin was based on the fluorescence of the supernatant at an excitation wavelength of 405 nm and an emission wavelength of 450 nm (Tecan Genios FluorSpec instrument). Activity was expressed in relative fluorescence units. Although we consider pyoverdin to be a marker of QS, a drop in production may be due to an indirect effect via pH or iron concentration changes (31). To eliminate the chance of false-positive results, the iron concentration was tested by using 1,10-phenanthroline and desferal (63), and their sodium adducts (pLP170) (35). The cultures were grown in AB medium and monitored under the same conditions used for strain PAO1, with the extract added once the cultures reached an OD600 of 1.7. Assays for β-galactosidase activity in P. aeruginosa were performed with o-nitrophenyl-β-galactopyranoside, as described previously (42).

Bradford assay. In addition to growth curve monitoring, the Bradford assay (7) was performed to confirm that the reduction in virulence factors was not due to a decrease in cell density. Raw data from all assays were normalized to the total protein concentration; however, there was no significant difference between sample sets.

Statistical analysis. All experiments were performed independently in triplicate with pooled samples of biological replicates, as described by Adonizio et al. (1). Data were analyzed by one-way analysis of variance, with a P value of 0.05 being significant, by using the SPSS (Chicago, IL) statistical software package.

RESULTS

LasA protease activity in the presence of plant extracts. LasA staphyloptic protease is a 20-kDa zinc metalloendopeptidase belonging to the β-lytic endopeptidase family of proteases (33). There was a significant decrease in LasA activity compared to that of the control when strain PAO1 was grown in the presence of B. buceras (96% decrease), C. erectus (94% decrease), T. bicolor (89% decrease), C. viminalis (71% decrease), or C. hypericifolia (49% decrease) (Table 1). Addition of Q. virginiana had no significant effect on LasA protease production. As expected, the negative control, S. actinophylla, also showed no significant change in LasA activity (Table 1).

LasB elastase activity in the presence of plant extracts. LasB elastase is a zinc metalloprotease capable of destroying or inactivating a wide range of biological tissues and immunological agents (5). There was a significant decrease in LasB activity compared to that of the control when strain PAO1 was grown in the presence of C. erectus (65% decrease) or C. viminalis (63% decrease). The growth of PAO1 with B. buceras, T. bicolor, or S. actinophylla (negative control) caused no significant effect, whereas the growth of PAO1 with C. hypericifolia and Q. virginiana caused an increase in elastase activity.

Extracts alter pyoverdin production. Pyoverdins are virulence factors, in that they compete with mammalian transferrin for iron, the successful sequestration of which essentially starves the host tissues (44). They also promote pathogenicity by stimulating bacterial growth (14). One of the pyoverdins is suggested to be a QS-like molecule, regulating both itself and the production of other toxins (4, 36). All plant extracts with the exception of those of Q. virginiana and S. actinophylla (negative control) showed a significant reduction of pyoverdin production; however, the results for C. hypericifolia were only marginally significant (Table 1). The most active extracts were C. erectus and B. buceras, with a substantial decrease in pyoverdin activity (91% and 84% decreases, respectively) compared to that of the control (Table 1). All of the culture supernatants were visualized by staining with a crystal violet solution. The plates were rinsed with AB medium, and the OD600 was monitored at 45-min intervals until an OD600 of ~1.7 was obtained (approximately 8 h). The culture was then diluted into 28-ml aliquots, to which 2 ml of AB or LB medium (controls) or 2 ml concentrated extract was added. The final extract concentration was 1 mg/ml. The OD600 of cultures with added extracts was normalized to the control OD600 of 1.7 at this time to account for plant pigmentation. The OD was monitored at 1.5-h intervals until a final time point of 24 h. All OD600 measurements were verified at a 1/10 dilution for greater accuracy.

β-Galactosidase assay. The transcriptional activity of the QS gene promoters was assayed by using strain PA01-derived strains harboring the promoter-lacZ fusions Puvr/lacZ (pPC223), Puvr/lacZ (pPSC1001), Puvr/lacZ (pLP1R), and Puvr/lacZ (pPSC1002) and, as a control, a promoterless-lacZ fusion strain (pLP710) (35). The cultures were grown in AB medium and monitored under the same conditions used for strain PAO1, with the extract added once the cultures reached an OD600 of 1.7. Assays for β-galactosidase activity in P. aeruginosa were performed with o-nitrophenyl-β-galactopyranoside, as described previously (42).
retained a pH of ~7.0, regardless of the amount or the type of extract added. Although statistically significant differences between the iron concentrations in the extracts were found, there was no trend which could be correlated with activity against pyoverdin (data not shown).

Plant extracts have an inhibitory effect on biofilm formation. *P. aeruginosa* has the ability to form biofilms, a partially QS-controlled phenomenon (17) in which cells are organized into layers and enmeshed in a matrix of mucoid polysaccharides (12). A switch to the biofilm mode of growth confers more severe infection in the lungs of patients with cystic fibrosis (38). There was a significant decrease in biofilm formation in the presence of *C. viminalis* (89% decrease), *Q. virginiana* (88% decrease), *T. bicolor* (80% decrease), or *B. buceras* (78% decrease) (Table 1). *C. erectus* caused a 41% decrease in biofilm formation; however, this result was marginally significant.

Plant extracts affect QS gene expression. With the exception of *Q. virginiana*, *C. hypericifolia*, and the negative plant control (*S. actinophylla*), most extracts had a significant effect on QS gene expression (Table 2). The most significant decreases in LasA expression were found with *C. viminalis* (80% decrease), *B. buceras* (49% decrease), *T. bicolor* (41% decrease), and *C. erectus* (38% decrease). *C. erectus* and *C. viminalis* showed the greatest reduction of lasR expression (56% and 48% decreases, respectively), whereas *T. bicolor* (42% decrease), *B. buceras* (55% decrease), and *C. erectus* (40% decrease) showed the greatest reductions in *rhl* expression. The same three plants also effected the greatest reductions in *rhlR* expression (66%, 62%, and 66% decreases, respectively).

Plant extracts affect the production of AHL molecules. Notable decreases in OdDHL levels were seen with *C. viminalis* (46% reduction from that for the control) and *B. buceras* (38% reduction). Significant decreases in BHL levels corresponded to the addition of *B. buceras*, *T. bicolor*, and *C. hypericifolia* extracts, with reductions of 41%, 39%, and 35%, respectively.

Extracts have a minimal effect on PAO1 growth after log phase. Addition of extracts at early stationary phase was chosen to limit any confounding effects on growth. When the extracts were added at the beginning of the cell cycle (time zero), there were changes (a slight delay or acceleration) in the logarithmic phase (data not shown). This did not, however, affect the endpoint cell density in most cases. To confirm an anti-QS mode of action rather than logarithmic changes, a growth curve was taken by controlling for the latter (Fig. 1). Cultures of strain PAO1 were grown to early stationary phase before addition of the compound (Fig. 1, arrow). This ensures that all samples would have the same opportunity (length of time and cell density) to reach the point of QS-controlled production of virulence factors. Stationary phase was reached in all samples (including the control) approximately 8 h after extract addition. Addition of the extracts did not significantly affect the cell density or the total protein concentration.

The majority of the samples had little effect on the growth curve of strain PAO1 after log phase (Fig. 1). The growth curve...
Plants are not due to cell death. However, it verifies that the anti-QS effects of these plant extracts. However, the total protein concentration (measured by the Bradford assay) of these samples did not differ significantly from that of the control.

For cultures to which T. bicolor was added closely followed that of the medium-only control, whereas the growth curves for Q. virginiana, B. buceras, C. hypericifolia, C. erectus, and S. actinophylla all exhibited slight increases in cell density (Fig. 1). This increase was not significant and was possibly due to the added nutrients. However, it verifies that the anti-QS effects of these plants are not due to cell death.

Incidentally, addition of T. bicolor, and Q. virginiana at time zero resulted in cell densities below the cell density for the control (data not shown). This suggests an additional antibacterial effect of these plant extracts. However, the total protein concentration (measured by the Bradford assay) of these samples did not differ significantly from that of the control.

**DISCUSSION**

Six south Florida medicinal plants, C. erectus (Combretaceae), C. hypericifolia (Euphorbiaceae), C. viminalis (Myrtaceae), B. buceras (Combretaceae), T. bicolor (Melastomataceae), and Q. virginiana (Fagaceae), were examined for their anti-QS activities against P. aeruginosa PAO1. The virulence of P. aeruginosa is owed to its capacity to degrade host tissue with proteases and toxins and to evade antibiotic attack by the formation of biofilms. Biofilm formation and the virulence factors examined in this study are under QS control (17, 34, 44, 45). Thus, the plant extracts were examined for their ability to interfere with the QS-dependent production of the P. aeruginosa virulence factors LasA, LasB, and pyoverdin. In addition, we examined the ability of the extracts to inhibit biofilm formation, QS gene expression, and AHL synthesis.

**Plant extracts differentially affect biofilm formation.** Since QS is involved in biofilm formation (17), we expected the plants possessing anti-QS activity to have a significant effect. Indeed, all extracts tested, with the exception of the extract of C. hypericifolia, effected a decrease in biofilm formation. Interestingly, Q. virginiana exhibited an effect on biofilm production, but not on any other virulence factors, suggesting either a physical inhibition of biofilm growth or the repression of biofilm genes and components outside the QS system. Disruption of the QS system with furanones has also been shown to inhibit biofilm growth (26). Previous work with garlic and D. pulchra furanones showed a qualitative change in biofilm morphology and a reduction in thickness; however, these analyses were not quantified (26, 27, 51).

**Plant extracts differentially affect production of virulence factors.** P. aeruginosa proteases LasA and LasB are believed to play a major role in pathogenesis via host tissue degradation (34, 45). With the exception of growth with Q. virginiana extracts, growth with the extracts of all other plant species tested resulted in a significant decrease in the LasA activity of P. aeruginosa, with the most drastic reductions seen in B. buceras, C. erectus, and T. bicolor extract cultures. No prior studies have considered LasA activity in the presence of anti-QS compounds.

The LasB elastase activity was significantly reduced in the presence of C. erectus and C. viminalis extracts. Extracts from B. buceras and T. bicolor had no significant effect, whereas those from C. hypericifolia and Q. virginiana caused an increase in elastase activity. It is likely that the compounds in the last two plants may upregulate the production of LasB and/or enhance the elastase activity. The compounds are not likely to be elastase-like proteins in the plants, since the extraction process would have denatured most proteins. In comparison, recent studies with garlic (at 2%, vol/vol) showed a 50% decrease in LasB activity (51), whereas studies with purified halogenated furanone from D. pulchra (10 μM) resulted in an approximately 90% decrease (27).

All plant extracts, with the exception of the Q. virginiana extract, caused a significant reduction in pyoverdin production. Mixed results on pyoverdin production have previously been observed with furanones. A naturally occurring furanone from D. pulchra [(5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone] actually increased the production of pyoverdin (53), whereas furanone C-30, a synthetic derivative of a compound from D. pulchra, conferred a 90% reduction in pyoverdin levels (27). Neither iron limitation nor pH is sufficient to explain the effect on virulence factor production. There were also no significant changes in cell growth corresponding to pyoverdin production, leaving an anti-QS effect as the most plausible hypothesis.

**Mechanistic musings: multiple targets or global effect.** QS inhibition occurs via a number of different mechanisms, with the most well known being signal mimicry, such as in the case of the furanones (40, 41). Other methods include signal degradation by proteins such as lactonase and acylase (20), signal binding, the inhibition of genetic regulation systems, or the interruption of downstream virulence and biofilm genes (for a review, see reference 60).
The current view of the *P. aeruginosa* QS hierarchy suggests that las controls *rhl* with virulence proteins at the bottom of the ladder (55). The virulence factors LasA (staphylolytic protease) and LasB (elastase) are generally thought to be under the control of the *lasI-lasR* system (22, 55); however, *rhlI-rhlR* also controls activity to a lesser extent (8, 49). Pyoverdin is believed to be under the control of *rhlI-rhlR* (8, 61), whereas biofilm production is only partially under the control of the QS system (17). The redundant and autoregulatory nature of the QS system is quite convoluted (58). This fact, compounded with the complexity of the QS system with each of the plant extracts tested (with the exception of *S. actinophylla* extract), suggests that the effect is not directly on the QS system. The QS system is quite convoluted (58). The second explanation is that the effect is not directly on the QS system but, rather, on a more global QS regulator, such as Vfr (2) or GacA (52).

There is also a trend in which the plant species that have less of an effect on AHL production and *las-rhl* expression also have less activity against *P. aeruginosa* virulence factors. This was most clearly seen with *Q. virginiana*, *C. hypericifolia*, and the control, *S. actinophylla* (Tables 1 and 2). Although it is not absolute, the converse is generally true. Overall, the most significant effects on the QS system were found with *C. viminalis*, *B. buceras*, and *C. erectus*.

The distinct patterns of thin-layer chromatography migration of these three extracts (data not shown) suggest that they contain multiple active compounds and that they perhaps function with separate mechanisms. At this point we do not have sufficient data to pinpoint the mechanism of quorum quenching. We will therefore withhold further speculation on the mechanism of action until these compounds are purified from the plants.

In summary, this work describes six plant species, representing five distinct families, which have a differential but significant effects on virulence factors, biofilms, QS gene expression, and signal production at a concentration of 1 mg/ml. This concentration, although high, is relevant to traditional medicinal use in teas and poultices. More significantly, this concentration represents that of a crude aqueous extract, and therefore, the concentration should be much lower when the purified extract is used as a putative anti-QS compound. Concentrations down to 0.25 mg/ml were tested; and some extracts, notably, that of *C. erectus*, still had an effect on virulence factor production.

None of the plant extracts tested had a significant effect on the growth of strain PAO1 when they were added at early stationary phase (Fig. 1, arrow). However, addition of *T. bi-color* and *Q. virginiana* at time zero resulted in cell densities below the density for the control (data not shown), which suggests that these plant extracts have additional antibacterial effects. All other plant extracts significantly reduced one to four QS-controlled virulence factors, genes, and AHL levels without a reduction in growth. This would strongly suggest an anti-QS effect rather than an antibacterial effect.

The top candidates for further anti-QS investigation are *C. erectus*, *B. buceras*, and *C. viminalis*. *C. erectus* significantly decreases LasA, LasB, and pyoverdin production but not biofilm formation, whereas *B. buceras* affects all but elastase activity. *B. buceras* also has a significant effect on the expression of *lasI*, *lasR*, *rhlI*, and *rhlR* and the concentrations of OdDHL and BHL. *C. erectus* exhibits the same pattern of effect on QS genes and signaling molecules, although to a lesser extent than *B. buceras*. *C. viminalis* decreases all three virulence factors and biofilm formation, all QS genes tested, and OdDHL concentrations. Research is under way in our laboratory to isolate the active chemicals from these species.

The plant kingdom has long been a source of medicines, and as such, there have been many ethnomedicinally directed searches for agents that can be used to treat infections (e.g., (9, 13, 24, 28). However, most studies focus solely on bactericidal effects. Since the plants in this study showed little, if any, cidal activity (1), quorum inhibition remains a potential mode of action. A shift of our focus to anti-QS and antivirulence properties may reveal new quorum-quenching compounds from medicinal plants and provide a novel method for the treatment of infections.

In conclusion, the effects of the plant extracts studied on *P. aeruginosa* are quite complicated and perhaps extend beyond the domain of the QS control hypothesis. However, the reduction of QS gene expression and signaling molecule levels and the end effect on virulence factor production provide some insight into why these plants were used in the past and how they can be used in the future to combat *P. aeruginosa* and other bacterial infections.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the support of the National Institutes of Health National Center for Alternative and Complementary Medicine (grant NRSRA 1-T32-AT01006-01 to A.A. and R15-AT00262-01 to K.M.), Cystic Fibrosis Foundation Traineeship Grant ADONIZ06H0 (to A.A.), NIGMS Research Initiative Award R25 GM61347 (to A.A.), Biomedical Research Initiative Award R25 GM61347, and Florida International University Teaching Assistantship (to K.-F.K.). We thank John Makemson, members of the K. MaTeec lab for assistance and support (especially Melissa Doud, Deepak Balasubramanian, and Lance Umansky), and members of the D. Roy lab for gracious use of the fluorescence spectrophotometer. We also thank the reviewers for their critical reading of the manuscript and their invaluable suggestions and comments.

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