

Identification of Catechin as One of the Flavonoids from *Combretum albiflorum* Bark Extract That Reduces the Production of Quorum-Sensing-Controlled Virulence Factors in *Pseudomonas aeruginosa* PAO1[∇]

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Quorum-sensing (QS) regulates the production of key virulence factors in *Pseudomonas aeruginosa* and other important pathogenic bacteria. In this report, extracts of leaves and bark of *Combretum albiflorum* (Tul.) Jongkind (*Combretaceae*) were found to quench the production of QS-dependent factors in *P. aeruginosa* PAO1. Chromatographic fractionation of the crude active extract generated several active fractions containing flavonoids, as shown by their typical spectral features. Purification and structural characterization of one of the active compounds led to the identification of the flavan-3-ol catechin [(2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol]. The identity of catechin as one of the active molecules was confirmed by comparing the high-pressure liquid chromatography profiles and the mass spectrometry spectra obtained for a catechin standard and for the active *C. albiflorum* fraction. Moreover, standard catechin had a significant negative effect on pyocyanin and elastase productions and biofilm formation, as well as on the expression of the QS-regulated genes *lasB* and *rhlA* and of the key QS regulatory genes *lasI*, *lasR*, *rhlI*, and *rhlR*. The use of RhlR- and LasR-based biosensors indicated that catechin might interfere with the perception of the QS signal *N*-butanoyl-L-homoserine lactone by RhlR, thereby leading to a reduction of the production of QS factors. Hence, catechin, along with other flavonoids produced by higher plants, might constitute a first line of defense against pathogenic attacks by affecting QS mechanisms and thereby virulence factor production.

Pseudomonas aeruginosa is a gram-negative bacterium infecting insects, plants, animals, and humans (65). As an opportunistic pathogen, *P. aeruginosa* is a major cause of nosocomial diseases and mortality in immunocompromised patients and particularly in patients with cystic fibrosis, diffused bronchitis, and pulmonary deficiencies (21, 54). Successful infection of diverse hosts is due to the profusion and diversity of virulence factors secreted by *P. aeruginosa* such as proteases, exopolysaccharides and redox-active compounds, as well as to its capacity to form biofilms (9, 60, 62).

Many pathogenic bacteria trigger the production of their virulence factors in a population density-dependent manner, a cell-to-cell communication mechanism known as quorum sensing (QS) (24). This mechanism enables bacteria to detect their population density through the production, release, and perception of small diffusible molecules called autoinducers and to coordinate gene expression accordingly (7, 9, 13, 24, 84). In *P. aeruginosa*, two QS systems (*las* and *rhl*) drive the production (by the synthetases LasI and RhlI) and the perception (by the transcription factors LasR and RhlR) of the acyl-homo-

serine lactones (AHL) *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), respectively (9, 62). Once LasR interacts with 3-oxo-C12-HSL, it induces the *las* system (by increasing *lasI* expression) and triggers the production of LasB elastase, LasA protease, Apr alkaline protease, and exotoxin A (9, 25, 26, 73, 79, 80). The *las* system is also required for the development of *P. aeruginosa* biofilms (16). RhlR interacts with C4-HSL, resulting in an enhancement of the production of rhamnolipids, pyocyanin, LasB elastase, hydrogen cyanide, and cytotoxic lectins (9, 12, 42, 57, 85). The *rhl* system is also regulated, at the transcriptional and posttranscriptional levels, by the *las* system in a hierarchical manner (62). In *P. aeruginosa*, a third QS system, based on quinolone signals, interacts with the AHL systems in an intricate way (61, 81). Since QS systems regulate fundamental virulence processes in many pathogenic bacteria, interfering with this cell-to-cell communication mechanism is a rational strategy to attenuate their virulence with the hope of developing a new drug arsenal to counterbalance the emergence of antibiotic-resistant pathogens (8, 29, 52). Anti-QS compounds have been identified from chemical synthesis programs (34, 55) and from natural sources (28, 30, 44, 75). Moreover, many higher plants have been shown to contain QS mimics and/or anti-QS activities (1, 2, 10, 27, 40, 77, 78).

Ethnobotanical searches have evidenced that members of the *Combretaceae* family (*Viridiplantae*) are commonly used in

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics ^a | Reference |
|------------------------------|--|-----------|
| Strains | | |
| <i>C. violaceum</i> CV026 | Violacein-negative, <i>cviI</i> mini-Tn5 mutant of <i>C. violaceum</i> | 51 |
| <i>P. aeruginosa</i> PAO1 | Wild type (strain PAO0001; http://www.pseudomonas.med.ecu.edu/) | |
| <i>P. aeruginosa</i> ΔPA1430 | <i>P. aeruginosa</i> transposon mutant ID 17281 | 35 |
| <i>P. aeruginosa</i> ΔPA1432 | <i>P. aeruginosa</i> transposon mutant ID 11174 | 35 |
| <i>P. aeruginosa</i> ΔPA3476 | <i>P. aeruginosa</i> transposon mutant ID 32454 | 35 |
| <i>P. aeruginosa</i> ΔPA3477 | <i>P. aeruginosa</i> transposon mutant ID 3452 | 35 |
| <i>E. coli</i> JLD271 | <i>E. coli</i> K-12 Δ <i>lacX74 sdiA271::Cam</i> | 43 |
| Plasmids | | |
| pQF50 | Broad-host-range promoterless <i>lacZ</i> transcriptional fusion vector; Cb ^r | 34 |
| pβ01 | pQF50-derivative containing P _{lasB} - <i>lacZ</i> transcriptional fusion | 34 |
| pβ02 | pQF50-derivative containing P _{rhlA} - <i>lacZ</i> transcriptional fusion | 34 |
| pβ03 | pQF50-derivative containing P _{lasR} - <i>lacZ</i> transcriptional fusion | 34 |
| pLP170 | Broad-host-range <i>lacZ</i> transcriptional fusion vector that contains an RNase III splice sequence positioned between the multiple cloning site and <i>lacZ</i> ; Cb ^r | 62 |
| pPCS223 | pLP170-derivative containing P _{lasR} - <i>lacZ</i> transcriptional fusion | 80a |
| pPCS1001 | pLP170-derivative containing P _{lasR} - <i>lacZ</i> transcriptional fusion | 62 |
| pLPR1 | pLP170-derivative containing P _{rhlI} - <i>lacZ</i> transcriptional fusion | 80a |
| pPCS1002 | pLP170-derivative containing P _{rhlI} - <i>lacZ</i> transcriptional fusion | 62 |
| pAL101 | pSB401-derivative containing <i>rhlR</i> ⁺ <i>rhlI::luxCDABE</i> ; Tet ^r | 43 |
| pAL102 | pSB401-derivative containing <i>rhlI::luxCDABE</i> ; Tet ^r | 43 |
| pAL105 | pSB401-derivative containing <i>lasR</i> ⁺ <i>lasI::luxCDABE</i> ; Tet ^r | 43 |
| pAL106 | pSB401-derivative containing <i>lasI::luxCDABE</i> ; Tet ^r | 43 |
| pTB4124 | pQF50-derivative containing P _{aceA} - <i>lacZ</i> transcriptional fusion | 41 |

^a Cb^r, carbenicillin resistance; Tet^r, tetracycline resistance.

traditional medicine, representing close to 90 medical indications including bacterial infections, fever, leprosy, and pneumonia (23, 53). In this plant family, species of the genus *Combretum*, which is the largest genus (with about 370 species), are known to contain a wide variety of secondary metabolites (23) and have been screened for their antimicrobial, antifungal, or antiviral activities (23, 46–48, 53). To the best of our knowledge, *Combretum* species have not been screened for their capacity to inhibit QS mechanisms in bacteria. However, two other *Combretaceae* species (*Conocarpus erectus* and *Bucida buceras*) have been identified as having anti-QS activities (1, 2), but the active compounds have still to be identified. *C. albiflorum* (Tul.) Jongkind, which is endemic in Madagascar and formerly known as *C. phaneropetalum* (Baker) (36), was screened for the presence of compounds reducing the production of extracellular virulence factors that are regulated by QS mechanisms. The screening of samples from *C. albiflorum* for their capacity to inhibit the production of violacein in *Chromobacterium violaceum* CV026 and pyocyanin in *P. aeruginosa* PAO1 generated several active fractions containing flavonoid-like compounds, among which the flavan-3-ol catechin. Catechin was found to have a negative impact on the transcription of several QS related genes (i.e., *lasI*, *lasR*, *rhlI*, *rhlR*, *lasB*, and *rhlA*), confirming the inhibitory effect of catechin on QS mechanisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *C. violaceum* CV026 was grown in liquid LB medium at 28°C (51). *P. aeruginosa* PAO1 was obtained from the *Pseudomonas* Genetic Stock Center (strain PAO0001 [<http://www.pseudomonas.med.ecu.edu/>]) and was grown in liquid LB cultures (5 ml) supplemented with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7.0) at 37°C as described elsewhere (55). Plasmids (listed in Table 1) were introduced in *P. aeruginosa* PAO1 as described previously (76), and positive clones were selected on solid LB medium containing carbenicillin (300 μg/ml) and X-Gal

(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Mutant strains ΔPA1430 (ID 17281), ΔPA1432 (ID 11174), ΔPA3476 (ID 32454) and ΔPA3477 (ID 3452) were obtained from the Transposon Mutant Collection (Department of Genome Sciences, University of Washington-<http://www.gs.washington.edu/labs/manoil/libraryindex.htm>) and grown in LB medium supplemented with tetracycline according to (35). *Escherichia coli* biosensor strains harboring LasR- and RhlR-based plasmids pAL105 and pAL101 and control plasmids pAL106 (LasR⁻) and pAL102 (RhlR⁻) (see Table 1) were grown in LB medium supplemented with tetracycline and chloramphenicol as described elsewhere (43). When required, the medium was supplemented with 10 μM (final concentration) of 3-oxo-C12-HSL or C4-HSL from Sigma.

β-Galactosidase and luminescence measurements. Transcription of the QS genes was assayed by using PAO1-derived strains harboring promoter-*lacZ* fusions (Table 1). PAO1 reporter strains were prepared as described for pyocyanin and elastase quantification (see below). PAO1 strains (50 μl) were grown in 1 ml of LB medium at 37°C with agitation (175 rpm) supplemented with 10 μl of catechin (4 mM final) or dimethyl sulfoxide (DMSO; 1% [vol/vol], final concentration) and incubated for 8 and 18 h. After incubation, cell growth was assessed by spectrophotometry (i.e., the optical density at 600 nm [OD₆₀₀]) using a SpectraMax M2 device (Molecular Devices), and the absorbance of the medium (OD₆₀₀) after centrifugation of the bacteria (16,000 × g) was used as a blank. The sample used for cell growth assessment was used to perform the β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside as previously described (92). Promoterless *lacZ* fusion strains were used as controls. *E. coli* biosensor strains were grown in LB medium for 24 h, and 50-μl portions were subcultured for 8 h at 37°C in 1 ml of LB medium (the starting OD₆₀₀ ranged between 0.02 and 0.025 corresponding to ~5 × 10⁶ CFU) supplemented with 10 μl of DMSO (1% [vol/vol] final), 10 μl of catechin dissolved in DMSO (4 mM, final concentration), 10 μl of the appropriate homoserine lactone (10 μM, final concentration), or 10 μl of catechin (4 mM, final concentration) and the appropriate homoserine lactone (10 μM, final concentration). C4-HSL was added to pAL101 and pAL102, while 3-oxo-C12-HSL was added to pAL105 and pAL106. After incubation for 8 h at 37°C with agitation (175 rpm), 200 μl of culture was transferred to 96-well OptiPlate-96 F plates from Perkin-Elmer, and the luminescence of each sample was measured by using a TopCount NXT device from Perkin-Elmer. The LasR⁻ (pAL106) and RhlR⁻ (pAL102) biosensors were used for background subtraction, and the OD₆₀₀ values were measured to account for the differences in cell density. All experiments were performed in six replicates (*n* = 6). The statistical significance of each test was evaluated by conducting

Student *t* tests using GraphPad Prism software, and *P* values of ≤ 0.05 were considered significant.

***C. albiflorum* sample preparation, chromatography, and electrospray ionization mass spectrometry (ESI-MS) analysis.** Dried powdered samples (5 g) of leaf and stem bark of *C. albiflorum* (Tul.) Jongkind (*Combretaceae*) were macerated overnight at room temperature in MilliQ water (25 ml). After centrifugation and filtration on Whatman paper, followed by lyophilization, the resulting residue was dissolved in 1 ml of MilliQ water and loaded onto a Sephadex LH-20 (GE Healthcare Life Sciences) column (2.5 by 7.0 cm) that was eluted successively with 100 ml of water (fractions 1 to 10), water-ethanol (9:1) (fractions 11 to 14), water-ethanol (8:2) (fractions 15 to 18), water-ethanol (1:1) (fractions 19 to 22), water-ethanol-acetone (1:0.5:0.5) (fractions 23 to 24), and water-acetone (1:1) (fractions 25 to 27). Fractions were evaporated and stored at -20°C until required for further analysis.

One active fraction (fraction 21) was further fractionated by HPLC using a reverse-phase C18 column (Symmetry 300, 2.4 by 150 mm) that was eluted with a gradient of water-methanol containing 1% acetic acid (from 0 to 100% methanol in 30 min). Analytical high-pressure liquid chromatography (HPLC) was performed by using a Waters apparatus equipped with a 626 pump, a 626 controller, and a 996 photodiode array detector. Samples were analyzed on an HPLC apparatus (Waters 600 system) coupled to both UV (Waters 2487 detector) and mass (Micromass Waters VG Quattro II mass spectrometer) detectors. A BIO Wide Pore C-18 column (250 by 4.6 mm; 5 μm) was used, and the solvent elution consisted of a linear gradient of water and acetonitrile (from 5 to 100% acetonitrile in 30 min) at a flow rate of 1 ml/min. After UV detection at 215 and 254 nm, the column eluate was split (LC Packings splitter), and 0.1 ml/min was directed to the mass spectrometer fitted with an ESI interface. For mass detection, analyte ionization was achieved by using the positive electrospray mode. The ESI parameters were as follows: nebulizing gas (N_2 , 20 liters/h), drying gas (N_2 , 250 liters/h), source temperature (80°C), cone voltage (35 V), and capillary voltage (35 kV). The total ion current scanning was from *m/z* 115 to 1,000 with 1 s/scan.

Commercially available catechin [(2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol] and epicatechin standards used for HPLC and ESI-MS analysis or for QS inhibition tests in *C. violaceum* CV026 or *P. aeruginosa* PAO1 were purchased from Sigma and dissolved in DMSO. The addition of catechin, epicatechin, or DMSO did not result in an increase of the LB broth pH (i.e., the anti-QS effect observed did not result from an opening of the lactone ring of the QS molecules through alkaline hydrolysis) (1).

Quantitative analysis of violacein production in *C. violaceum* CV026. Inhibition of violacein production in *C. violaceum* CV026 by plant extracts (10 μl), chromatographic fractions (10 μl), or the authentic catechin and epicatechin standards (both at 4 mM final concentrations) was tested by using a liquid assay according to previously reported protocols (11, 51). Violacein production was induced in *C. violaceum* CV026 by adding *N*-hexanoyl-L-homoserine lactone (HHL; Sigma) at a final concentration of 3 μM . For violacein quantification, an overnight culture of *C. violaceum* CV026 was diluted 100 times in 250 μl of LB medium that were subsequently incubated for 18 h at 28°C with agitation (150 rpm) and supplemented with the appropriate sample. After assessment of bacterial growth by measuring the OD_{600} by using a SpectraMax M2 device (Molecular Devices), violacein contents were quantified as described previously (11). Briefly, cells were centrifuged ($16,000 \times g$) and washed twice with fresh LB medium. After centrifugation, cells were suspended in 200 μl of ethanol and sonicated. Cell debris were discarded by centrifugation ($16,000 \times g$), and the absorbance (A_{575}) of the solution was measured. The statistical significance of each test ($n = 4$) was evaluated by conducting Student *t* tests using the GraphPad Prism software, and a *P* value of ≤ 0.01 was considered significant.

Quantitative analysis of pyocyanin and elastase production in *P. aeruginosa* wild-type and mutant strains. Inhibition of pyocyanin and elastase production was assessed according to previously described procedures (34, 55). *P. aeruginosa* PAO1 or mutant cells (single colony from LB agar plates) were grown for 18 h in 5 ml of LB-MOPS medium (37°C and agitation at 175 rpm). The cells were washed twice in fresh LB-MOPS medium to eliminate the homoserine lactones, and the pellets were suspended in LB-MOPS medium. Then, 50- μl portions of the cell suspension were added to 1 ml of LB-MOPS (in order to obtain a starting OD_{600} of the LB-MOPS medium ranging between 0.020 and 0.025 and corresponding to $\sim 10^7$ CFU/ml) supplemented with 10 μl of plant extract or authentic catechin standard (4 mM [final concentration] or as stated in the text) dissolved in DMSO and, when appropriate, with 3-oxo-C12-HSL or C4-HSL. After 8 h of growth, samples were taken to assess the growth (OD_{600}). After centrifugation ($16,000 \times g$), the absorbance at 600 nm of the medium was used as blank, and 900 μl was used for pyocyanin determination (A_{380}). Elastase production was assessed through the measurement of elastase activity using elastin-Congo red (A_{495}). The statistical significance of each test ($n = 4$) was evaluated by conducting Student *t* tests using GraphPad Prism software, and a *P* value of ≤ 0.01 was considered significant.

Biofilm formation and quantification. *P. aeruginosa* PAO1 was grown overnight in LB medium at 37°C with agitation. After growth, the culture was diluted with TB (tryptone broth) medium (OD_{600} of ~ 0.02), and 50 μl of the diluted culture was added to 940 μl of TB medium supplemented with 10 μl of DMSO or catechin (4 mM, final concentration). PAO1 cells were incubated statically for 18 h at 37°C in 24-well polystyrene plates. After incubation, planktonic bacteria were discarded, and the biofilms were washed three times with phosphate-buffered saline buffer. Washed biofilms were fixed with 1 ml of methanol (99%). After 15 min, the methanol was discarded, and the plates were dried at room temperature. Crystal violet (0.1% in water) was then added to each well (1 ml/well), and the plates were incubated for 15 min at room temperature. Crystal violet was then discarded, and stained biofilms were washed three times with 1 ml of water. Acetic acid (33% in water) was added to the stained biofilms (2 ml) in order to solubilize the crystal violet, and the absorbance of the solution was read at 590 nm with a SpectraMax M2 device (Molecular Devices). The statistical significance of each test ($n = 6$) was evaluated by conducting Student *t* tests and using the GraphPad Prism software; a *P* value of ≤ 0.01 was considered significant.

Assessment of *P. aeruginosa* PAO1 viability. *P. aeruginosa* PAO1 cells incubated with DMSO or catechin (4 mM) for 8 and 18 h were stained with SYTO-9 (3.34 mM) (Molecular Probes/Invitrogen) and propidium iodide (PI) (20 mM) (Molecular Probes/Invitrogen). *P. aeruginosa* PAO1 cultures were adjusted to an OD_{600} of 0.10 to 0.15, and a 100- μl mix of SYTO-9 and PI was added to 100 μl of diluted bacteria according to the Molecular Probes protocol. The cells were transferred to a 96-well OptiPlate-96 F (Perkin-Elmer), and the fluorescence intensities were determined (excitation, 485 nm; emission, 530 nm [SYTO-9] and 630 nm [PI]) by using a SpectraMax M2 device (Molecular Devices). Ratios between the green and red fluorescences (530 nm/630 nm) were compared to assess the cytotoxicity of catechin and epicatechin. DMSO-supplied cultures were used as controls. The statistical significance of each test ($n = 6$) was evaluated by conducting Student *t* tests using the GraphPad Prism software, and a *P* value of ≤ 0.01 was considered significant.

RESULTS

***C. albiflorum* extracts reduce the production of QS-regulated factors.** The leaves and bark of *Combretum* spp. are predominantly used in traditional medicine, while their fruits do not feature in traditional uses due to their toxicity to humans (23). Therefore, leaf and bark aqueous extracts of *C. albiflorum* (Tul.) Jongkind (*Combretaceae*) were screened for the occurrence of QS inhibitors. Assays were carried out using the *C. violaceum* CV026 reporter strain mutated in the AHL synthase gene *cvlI*. This strain is able to produce violacein when the natural inducer HHL is supplied to the growth medium (51). Filter-sterilized extracts were added to noninduced (data not shown) or HHL-induced (Fig. 1A) *C. violaceum* CV026 cultures. When *C. albiflorum* extracts were added, violacein was not produced in either noninduced or HHL-induced *C. violaceum* CV026 cultures compared to the control cultures, indicating that *C. albiflorum* extracts do not contain HHL mimic compounds (data not shown) but contain QS inhibitory compounds (Fig. 1A). To evaluate the degree of inhibition of violacein production, violacein was extracted and quantified. As shown in Fig. 1A, both extracts inhibited violacein production by more than 80%. However, the growth of *C. violaceum* CV026 was inhibited by ca. 50% in the presence of leaf extract, whereas bark extract had no growth-inhibiting effect (Fig. 1A). These extracts were also tested on *P. aeruginosa* PAO1, where the extracellular virulence factor pyocyanin can be easily detected (62). As shown in Fig. 1B, both extracts abolished the QS-dependent production of pyocyanin. As observed with *C. violaceum* CV026, the bark extract had no effect on *P. aeruginosa* PAO1 growth, while the leaf extract did but to a lesser extent than with CV026 (Fig. 1B).

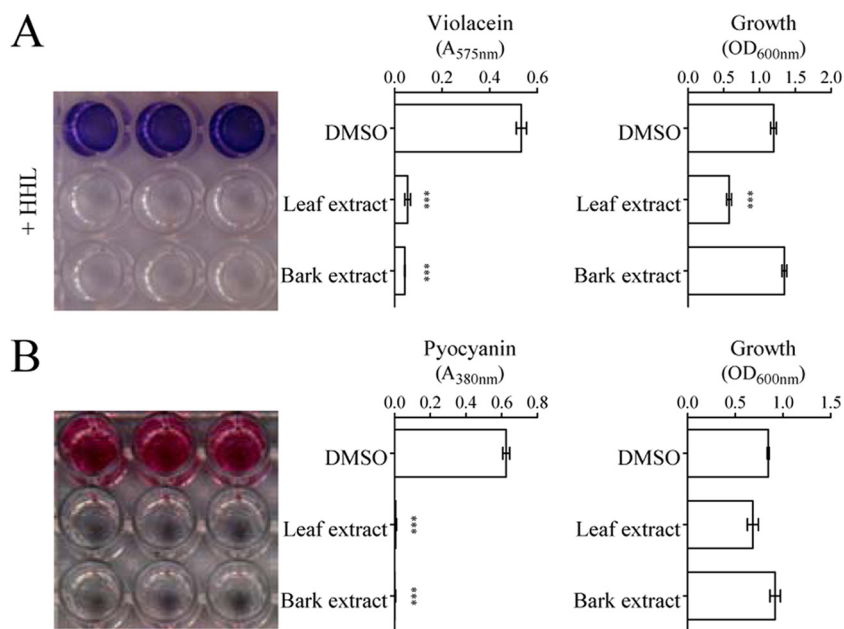


FIG. 1. Screening of *C. albiflorum* leaf and bark extracts for anti-QS activity using *C. violaceum* CV026 (A) and *P. aeruginosa* PAO1 (B). (A) Growth and violacein production by *C. violaceum* CV026 when HHL is supplied at a final concentration of 3 μ M and in the presence of *C. albiflorum* extracts. (B) Growth and pyocyanin production by *P. aeruginosa* PAO1 when *C. albiflorum* extracts are added to the growth medium. DMSO-supplied cultures were used as controls. Violacein and pyocyanin were extracted as described in Materials and Methods and quantified by spectrophotometry (575 nm for violacein and 380 nm for pyocyanin). Bacteria growth was assessed at 600 nm. All graphs show the OD values associated with each parameter measured, and each test was performed four times. ***, Data that are statistically different ($P \leq 0.01$).

Chromatographic fractionation of the *C. albiflorum* bark extract and identification of flavonoid-containing active fractions. In order to identify the inhibitory compound(s) responsible for the QS inhibition activity, the *C. albiflorum* aqueous bark extract was subjected to gel filtration chromatography (Fig. 2A). Twenty-seven fractions were collected, and the last seven eluted fractions (fractions 21 to 27) were found to inhibit violacein production (Fig. 2A). The first active fraction (fraction 21) was further fractionated, using reversed-phase HPLC (RP-HPLC), into 19 subfractions, and subfractions F4 to F16 inhibited violacein production (Fig. 2B). Fraction 21 was chosen because (i) preliminary thin-layer chromatography showed that this fraction contained few different compounds compared to the following fractions, (ii) growth of the bacteria was not affected, and (iii) the amount of residue obtained allowed the further chemical characterization of the active compound(s) (data not shown). The UV spectra of these subfractions showed that most of the peaks presented typical flavonoid-type UV spectra profiles, i.e., two main absorption bands comprised between 240 to 270 nm and 320 to 380 nm, respectively (31; data not shown). These data suggest that the active fractions of the *C. albiflorum* bark extract contain flavonoid-like structures interfering with violacein production in *C. violaceum* CV026 without any bactericidal or bacteriostatic activity.

Catechin is one of the *C. albiflorum* flavonoid inhibiting the production of violacein in *C. violaceum* CV026 and pyocyanin in *P. aeruginosa* PAO1. The peak purity of the HPLC subfractions F4 to F16 was assessed using the Waters Millennium chromatography manager. The presence of a single peak in subfractions F7 (with a retention time of 11 min) and F9 (with a retention time of 13 min) prompted us to further investigate

the structural characterization of the QS inhibitory compound present in these subfractions. First, as shown in Fig. 2C and D, the UV spectrum of the major peaks in subfractions F7 is similar to that of catechin (of which the structure is given in Fig. 2E). Second, analysis of a standard of catechin in the same HPLC conditions showed that catechin has the same retention time as the compound present in subfraction F7 (data not shown). Third, coinjection of the catechin standard with subfraction F7 showed no difference in the retention times, which further supported the presence of catechin in this subfraction (data not shown). Fourth, the ESI-MS analysis indicated that the MS spectrum of subfraction F7 was similar to that of authentic catechin standard showing an identical quasimolecular ion at m/z (Da/electron [e]) 291 with ion at 139 and 123 (Fig. 2F and G, respectively) in agreement with data reported previously (22). Epicatechin, which is the epimer of catechin, has the same UV and mass spectra but is found in fraction F9, as demonstrated by HPLC separation of an epicatechin standard (which had a retention time of 13 min, as did fraction F9) and coinjection of epicatechin and fraction F9 in the same HPLC conditions (data not shown). Finally, the catechin standard (Fig. 2E) inhibited violacein production when added to HHL-induced liquid cultures of *C. violaceum* CV026, as did subfraction F7, further confirming that catechin is the QS inhibitory compound. Indeed, as shown in Fig. 3, serial dilutions of the catechin standard were tested on HHL-induced cultures of *C. violaceum* CV026 in order to determine the range of concentrations inhibiting violacein production with a limited effect on bacterial growth (Fig. 3A and C). When concentrations of catechin higher than 4 mM were used, *C. violaceum* CV026 growth was strongly reduced and, as a consequence, no viola-

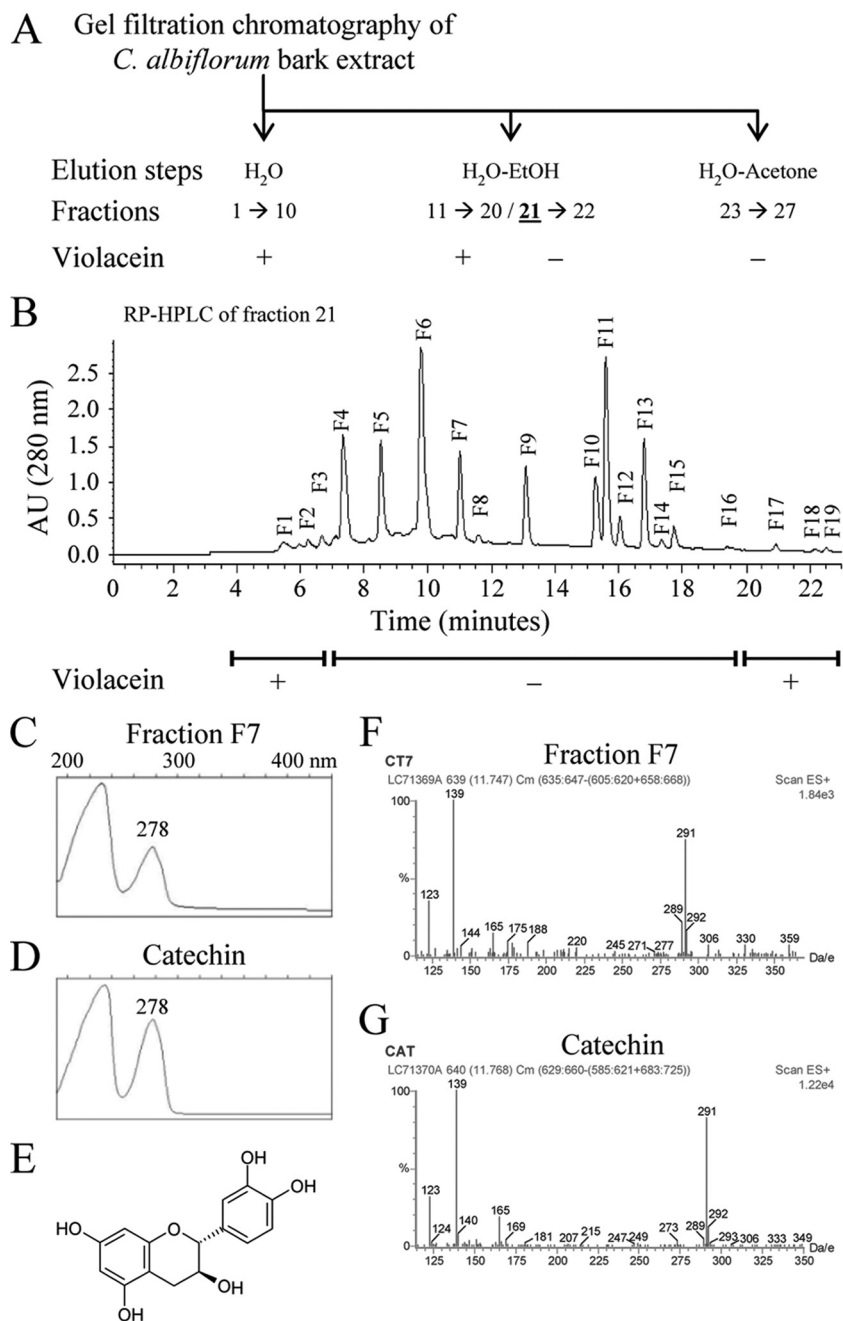


FIG. 2. Chromatographic scheme of *C. albiflorum* bark extract fractionation steps used to isolate and identify the anti-QS compounds and identification of catechin as the main compound present in the active fraction F7. (A) Gel filtration fractionation of the crude *C. albiflorum* bark extract. The column was successively eluted with water, water-ethanol, and water-acetone, and 27 fractions were collected. These fractions were added to HHL-induced *C. violaceum* CV026, and inhibition of violacein production was observed starting from fraction 21. (B) RP-HPLC profile of fraction 21 (280 nm). Nineteen subfractions were collected and tested on HHL-induced *C. violaceum* CV026, and inhibition of violacein production was observed with fractions F4 to F16. AU, arbitrary unit. (C) UV spectrum of fraction F7. (D) UV spectrum of an authentic standard of catechin showing the characteristic absorption maximum of catechin at 278 nm. (E) Chemical structure of catechin with the two benzene cycles connected by the heterocycle typical of flavonoids. (F and G) ESI-MS of fraction F7 (F) and of the authentic standard of catechin (G) with the main peaks (Da/e) at 123, 139, and 291.

cein production occurred (Fig. 3A and C). From 0.25 to 4 mM, violacein production was significantly affected by catechin (Fig. 3A), an effect that was not associated with a decrease in growth of *C. violaceum* CV026 (Fig. 3C). Serial dilutions of catechin were also tested on *P. aeruginosa* PAO1. As shown in Fig. 3B, concentrations between 0.125 and 16 mM had a significant

negative effect on pyocyanin production without affecting *P. aeruginosa* PAO1 growth (Fig. 3D). The epicatechin standard, tested at 4 mM, had an inhibitory effect on pyocyanin production in *P. aeruginosa* PAO1, as did subfraction F9, while this compound had a bactericidal effect on *C. violaceum* CV026 (data not shown). The effect of catechin and epicatechin (at 4

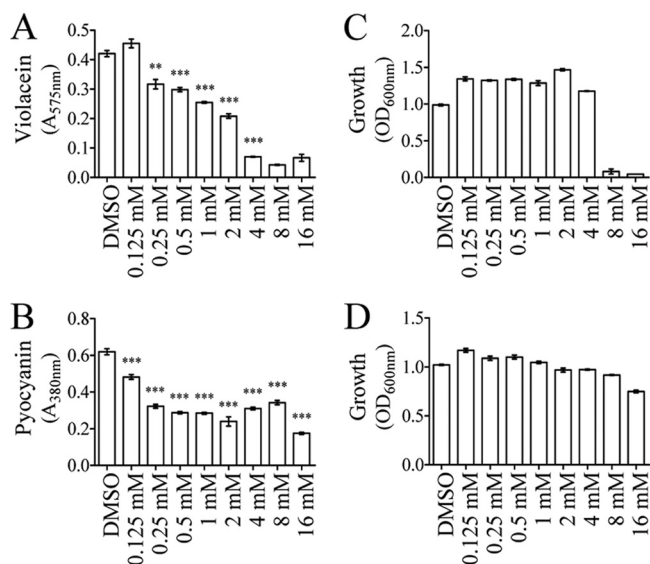


FIG. 3. Effect of serial dilutions of an authentic standard of catechin on violacein (A) and pyocyanin (B) production in *C. violaceum* CV026 and *P. aeruginosa* PAO1, respectively. (C and D) Growth of both bacteria assessed at 600 nm. Violacein and pyocyanin were extracted as described in Materials and Methods and quantified by reading the OD values of the solution at 575 nm (A) and 380 nm (B), respectively. DMSO-supplied cultures were used as controls. The statistical significance of each test ($n = 4$) was evaluated by conducting Student *t* tests, and a *P* value of ≤ 0.01 was considered significant. ***, Data that are statistically different ($P \leq 0.01$).

mM) was also tested on elastase production in *P. aeruginosa* PAO1 by performing an elastolysis assay as described in Material and Methods. As shown in Fig. 4A, after 8 h, the addition of catechin (as well as epicatechin) reduced the production of elastase by ca. 30%. For comparison, the production of pyocyanin by the Δ PA3476 (Δ *rhII*) and Δ PA3477 (Δ *rhIR*) mutants was lower than in the wild-type strain PAO1 by $69\% \pm 6\%$ and $73\% \pm 4\%$, respectively, whereas the production of elastase (as measured by the elastolysis assay) by the Δ PA1432 (Δ *lasI*) and Δ PA1430 (Δ *lasR*) mutants was lower than in the wild-type strain PAO1 by $53\% \pm 5\%$ and $76\% \pm 6\%$, respectively.

Effect of catechin on biofilm formation in *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 can switch to a biofilm mode of growth. Biofilms are matrixes of polysaccharides in which bacteria are enmeshed and protected from the environment, increasing their resistance to antibiotics and the immune system, thereby increasing their capacity to remain in the infected host (15, 56). Since biofilm formation is partially controlled by QS mechanisms (16), the effect of catechin and epicatechin on *P. aeruginosa* PAO1 biofilm formation was assessed after 18 h of growth. As shown in Fig. 4B, catechin reduced biofilm formation by ca. 30%, while epicatechin had no effect. To determine whether the effect of both compounds on pyocyanin, elastase, and biofilm was due to a drop in cell viability, *P. aeruginosa* PAO1 viability was assessed after 8 and 18 h. As shown in Fig. 4C and D, none of the compounds had an effect after 8 h of incubation (Fig. 4C) but, after 18 h, epicatechin-treated *P. aeruginosa* PAO1 cells had a viability of 66%, whereas catechin-treated cells were not affected in their viability compared to DMSO-treated cells (Fig. 4D). Consistently, catechin had

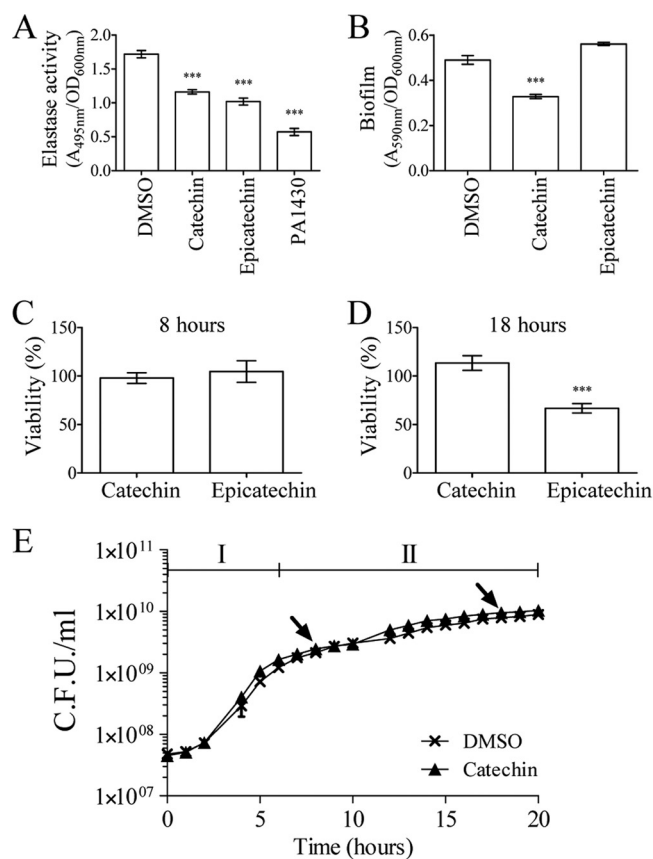


FIG. 4. Effect of authentic standards of catechin (4 mM) and epicatechin (4 mM) on elastase production (A), biofilm formation (B), *P. aeruginosa* PAO1 viability after 8 h (C) and 18 h (D) of culture, and *P. aeruginosa* PAO1 growth kinetics (E). DMSO-supplied cultures were used as controls. Elastase production by *P. aeruginosa* PAO1 (A) was quantified through an elastolysis assay as described in Materials and Methods. No difference in cell densities (OD_{600}) was observed between the different treatments (data not shown). Elastase production by the Δ *lasR* mutant strain PA1430 was used as a comparison to assess the potency of catechin. (B) Biofilm formation by *P. aeruginosa* PAO1 after 18 h of incubation was assessed by using crystal violet. (C and D) The viability of *P. aeruginosa* PAO1 cells was assessed by using SYTO-9 and PI as described in Materials and Methods after 8 h (C) and 18 h (D) of culture. DMSO-supplied cultures were used as controls, and their viability was used to plot the viability of catechin- and epicatechin-treated cultures. The statistical significance of each test ($n = 6$) was evaluated by conducting Student *t* tests, and a *P* value of ≤ 0.01 was considered significant. ***, Data that are statistically different ($P \leq 0.01$). (E) Growth kinetics of DMSO- and catechin-treated *P. aeruginosa* PAO1. The growth kinetics were divided into two phases: phase I was between 0 and 6 h (corresponding to the exponential growth phase), and phase II was between 6 and 20 h (corresponding to the stationary phase). Doubling times were calculated for both phases by using GraphPad Prism software ($n = 6$). Arrows indicate the time points at which samples were taken for pyocyanin, elastase, biofilm, or gene expression analysis.

no significant effect on *P. aeruginosa* PAO1 growth parameters, as shown in Fig. 4E. Indeed, the doubling times for control and catechin-treated *P. aeruginosa* PAO1 cells were, respectively, 0.94 ± 0.13 and 0.92 ± 0.03 h during the first phase of the kinetics (phase I) and 8.02 ± 0.24 and 7.46 ± 0.30 h during the second phase of the kinetics (phase II).

TABLE 2. Effect of catechin and salicylic acid on *P. aeruginosa* PAO1 QS genes expression after 8 and 18 h of incubation

| Time (h) | Culture condition | Gene expression (mean Miller units \pm SD) ^a | | | | | | |
|----------|-----------------------|---|------------------|------------------|------------------|-------------------|------------------|-----------------|
| | | <i>lasI</i> ^b | <i>lasR</i> | <i>lasB</i> | <i>rhlI</i> | <i>rhlR</i> | <i>rhlA</i> | <i>aceA</i> |
| 8 | DMSO | 521 \pm 31 | 6,055 \pm 220 | 3,286 \pm 526 | 9,403 \pm 634 | 7,520 \pm 415 | 2,782 \pm 145 | 610 \pm 107 |
| | Salicylic acid (4 mM) | 455 \pm 71 | 4,659 \pm 369* | 2,022 \pm 282* | 7,804 \pm 905† | 4,944 \pm 673* | 2,208 \pm 230* | ND |
| | Catechin (4 mM) | 314 \pm 42* | 4,892 \pm 321* | 2,116 \pm 181† | 5,298 \pm 654* | 4,629 \pm 729* | 2,150 \pm 118* | 525 \pm 116 |
| 18 | DMSO | 1,505 \pm 222 | 4,199 \pm 945 | 453 \pm 34 | 2,718 \pm 575 | 5,096 \pm 1,323 | 2,754 \pm 73 | 2,029 \pm 143 |
| | Salicylic acid (4 mM) | 1,338 \pm 152 | 1,719 \pm 472* | 459 \pm 13 | 1,114 \pm 134* | 2,582 \pm 741* | 2,233 \pm 187* | ND |
| | Catechin (4 mM) | 1,107 \pm 371* | 2,002 \pm 371* | 332 \pm 23* | 1,132 \pm 214* | 2,276 \pm 663* | 2,525 \pm 163* | 2,221 \pm 46 |

^a Gene expression was measured as the β -galactosidase activity of the *lacZ* gene fusions expressed in Miller units. *, Significance at $P < 0.01$; †, significance at $P < 0.05$. ND, not determined.

^b Similar results were obtained with plasmids p β 03 and pPCS223. The results shown were obtained with plasmid pPCS223.

Catechin affects *lasI*, *lasR*, *rhlI*, and *rhlR* and downstream genes expression. If the diminution of pyocyanin and elastase production and biofilm formation was due to an interference of catechin with QS mechanisms, this should be reflected in the expression of the main regulatory genes involved in QS mechanisms in *P. aeruginosa* PAO1. The effect of catechin on QS systems was therefore further characterized by evaluating the expression of the AHL synthetase genes *lasI* and *rhlI* and the QS regulator genes *lasR* and *rhlR*. Salicylic acid, which is known to affect QS signaling in *P. aeruginosa* and *Agrobacterium tumefaciens* (6, 64, 87, 89, 90), was used as a positive control. As shown in Table 2, after 8 h, the addition of catechin resulted in a significantly reduced expression of the regulatory genes of the *las* system by 40% (*lasI*) and 20% (*lasR*). The regulatory genes of the *rhl* system were also significantly affected. The expression of *rhlI* and *rhlR* was reduced by 44 and 38%, respectively. After 18 h of incubation, these reductions were of 26% for *lasI* and 52% for *lasR*, while the expression of *rhlI* and *rhlR* was reduced by 58 and 55%, respectively (Table 2). Compared to salicylic acid, catechin had an equivalent or a more pronounced effect on the expression of these genes after 8 and 18 h of incubation (Table 2). Elastolysis measurements showed that catechin likely reduced elastase production by ca. 30% (Fig. 4A). To strengthen this observation, the expression of the *lasB* gene, encoding LasB elastase (58, 79, 86), was quantified. As shown in Table 2, the addition of catechin reduced the expression of *lasB* by ca. 36% after 8 h and by 27% after 18 h, whereas salicylic acid was more effective after 8 h (ca. 40% of repression) than after 18 h. This reduction of *lasB* expression is in accordance with the concomitant reduction in expression of the *las* system. The effect of catechin on the expression of the *rhlA* gene, contributing to the production of rhamnolipids (58), was also investigated. As shown in Table 2, after 8 h of incubation, the addition of catechin or salicylic acid reduced *rhlA* expression by 23 and 21%, respectively. Salicylic acid had a greater impact on *rhlA* expression (20% reduction) than catechin after 18 h, which seemed not effective anymore (only 10% reduction [Table 2]). These results highlight that the expression of QS genes is affected by catechin (as well as salicylic acid) to various extents depending on the QS system, likely as a result of the complex, redundant, and hierarchical mechanisms underlying this cell-to-cell communication process (18). This reduction in QS gene expression was not linked to a drop in cell viability (data not shown), cell growth retardation (data not shown), or nonspecific reduction of the transcription

machinery of the bacteria. Indeed, *P. aeruginosa* PAO1 grown in the presence of catechin (4 mM, final concentration) had growth characteristics similar to those of DMSO-treated cells. Finally, the activity of the *aceA* promoter (regulating the expression of the isocitrate lyase gene PA2634) from *P. aeruginosa* (41) was studied in strain PAO1 grown in the presence or absence of catechin. As shown in Table 2, the addition of catechin had no effect on the transcription of the *aceA* gene, indicating that catechin affects the expression of QS-related genes without affecting the transcription machinery of *P. aeruginosa* PAO1. To determine whether catechin could interfere with the perception of C4-HSL or 3-oxo-C12-HSL by their cognate receptors (i.e., RhlR or LasR, respectively), *E. coli* biosensor strains were used. As shown in Fig. 5A, the addition of catechin to the C4-HSL-treated pAL101 *E. coli* biosensor strain reduced the expression of the *lux* operon, indicating that catechin interferes with the capacity of RhlR to interact with the promoter of the *rhlI* gene (driving *lux* expression). The effect of catechin on the induction of the pAL105 biosensor strain was minor (data not shown), suggesting that catechin might mainly affect the *rhlRI* system, the effect on the *las* system being a side effect of the reduction of the *rhl* system. Besides, the effect of catechin could not be compensated by the addition of exogenous homoserine lactones. Indeed, as shown in Fig. 5B, the production of pyocyanin in the wild-type strain PAO1 could not be restored by the addition of homoserine lactones. In addition, as shown in Fig. 5C, the addition of HSL to catechin-treated wild-type strain PAO1 did not enhance the production of elastase as evaluated by the elastolysis assay. Consistent with the effect of catechin on the pAL101 biosensor (Fig. 5A), pyocyanin production was significantly reduced after 8 h of growth in the Δ PA3476 mutant supplemented with catechin (4 mM, final concentration) and C4-HSL compared to the C4-HSL-treated mutant cells, where pyocyanin production was optimal (Fig. 5D). A slight induction of pyocyanin production was observed when 3-oxo-C12-HSL was added to this mutant, but the addition of catechin reduced this production to basal levels (Fig. 5D).

DISCUSSION

Plants used by traditional healers for the treatment of various diseases constitute an important source of chemical diversity for the potential identification and development of new drugs useful as antimicrobials. Here, it has been shown that

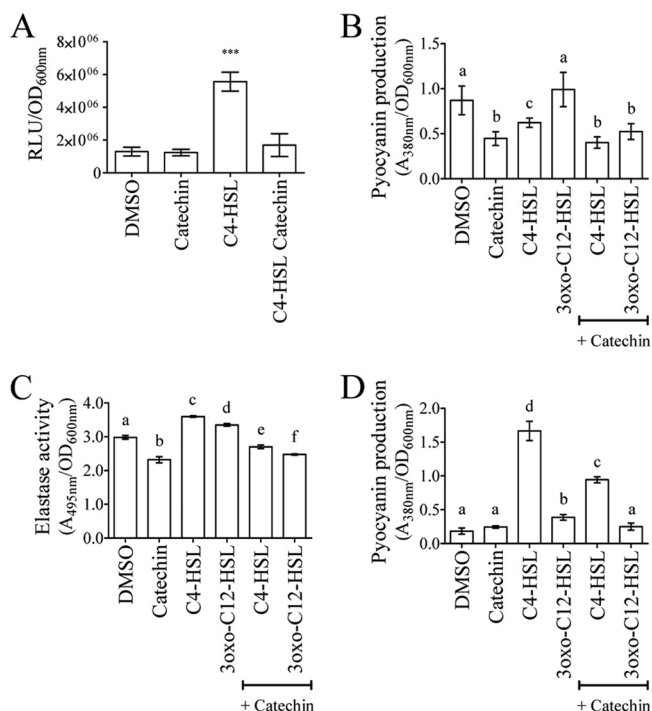


FIG. 5. Effect of catechin on exogenous addition of homoserine lactones. (A) Effect of catechin on the perception of C4-HSL by RhlR. The pAL101 and pAL102 *E. coli* biosensor strains were incubated for 8 h with DMSO (1% [vol/vol]), catechin (4 mM), C4-HSL only (10 μ M), or C4-HSL (10 μ M) plus catechin (4 mM). Samples were taken to record the luminescence of the culture in relative light units (RLU) and to measure the OD_{600} . The pAL102 strain was used as background. The statistical significance of each test ($n = 6$) was evaluated by conducting Student *t* tests, and a *P* value of ≤ 0.01 was considered significant. (B and C) Production of pyocyanin (B) and elastase (C) by the wild-type strain PAO1 after the addition of C4-HSL or 3-oxo-C12-HSL (10 μ M) in the presence or absence of catechin after 8 h of incubation. Pyocyanin (A_{380}) and elastase (A_{495}) were quantified as described in Materials and Methods, and values were corrected for the difference in cell density (OD_{600}). (D) Pyocyanin production in the $\Delta rhlI$ mutant (PA3476) after the addition of C4-HSL or 3-oxo-C12-HSL (10 μ M) in the presence or absence of catechin after 8 h of incubation. ***, Data that are statistically different ($P \leq 0.01$). The different letters above the histograms indicate that the data are statistically different from each other ($P \leq 0.01$).

extracts from the leaves and bark of the Malagasy plant *C. albiflorum* reduce the production of two QS-controlled factors in two different types of bacteria, namely, violacein in *C. violaceum* CV026 and pyocyanin in *P. aeruginosa* PAO1 (Fig. 1). Nevertheless, the leaf extract affected significantly the growth of both bacterial species and was not further investigated. *Combretum* species are indeed known to contain many secondary metabolites such as tannins, flavonoids, terpenoids, and stilbenoids that have been associated to their antimicrobial potential (5, 23, 46–49, 53). Further analytical investigations of the bark extract (that had no effect on bacteria growth) showed that flavonoids (possibly acting synergistically) are likely responsible for these activities and that catechin is one of the active molecules among other bark components with an effect on QS (Fig. 2). The decrease in production of violacein and pyocyanin could not be attributed to any bactericidal or bacteriostatic effect of catechin on *P. aeruginosa* PAO1 or *C.*

violaceum CV026 (Fig. 3). This observation is consistent with other reports showing that catechin has no effect on different gram-positive and gram-negative bacteria, including *P. aeruginosa* (3, 39), or increases growth of *Lactobacillus hilgardii* (3).

Compounds from other natural sources having an anti-QS activity include also L-canavanine from *Medicago sativa* which interferes with QS in *Sinorhizobium meliloti* likely through hindering the folding of the QS regulator protein ExpR at concentrations comprised between 56 μ M and 5.6 mM (40); patulin (8 μ M) from *Penicillium coprobium* and penicillic acid (147 μ M) from *P. radicicola*, which both decreased QS-controlled gene expression in *P. aeruginosa* PAO1 (with patulin accelerating LuxR turnover and increasing sensitivity of *P. aeruginosa* biofilms to tobramycin when used at 3.5 and 8 μ M, respectively) (68); garlic extract (used at 2%) and isolated components and some of their derivatives (10, 59, 67); various antibiotics or drugs (including salicylic acid used at concentrations ranging from 1 to 30 mM), which decrease the expression of QS-related genes and corresponding virulence factors (6, 64, 74, 87, 89, 90); the well-known and characterized furanones synthesized by the Australian red alga *Delisea pulchra* (28, 44, 45); manoalide, manoalide monoacetate, and secmanoalide used at micromolar concentrations from the marine sponge *Luffariella variabilis* (75); and the polyphenol curcumin (used at 1.5 and 3 μ g/ml), which attenuates *P. aeruginosa* PAO1 pathogenicity by reducing the production of QS regulated virulence factors and biofilm formation (70). Quenchers of QS mechanisms produced by eukaryote organisms is likely not limited to these compounds since QS inhibitory activities have also been found in numerous plant extracts including *Chlamydomonas reinhardtii*, *Pisum sativum*, *M. sativa*, *M. truncatula*, *Allium sativum*, *Callistemon viminalis*, bean sprout, chamomile, *Daucus carota*, *Capsicum chinense*, *B. buceras*, and *C. erectus* (1, 2, 10, 27, 38, 40, 50, 67, 77, 78). Interestingly, the two last plant species belong to the *Combretaceae* plant family and have been shown to reduce the expression of the *las* and *rhl* genes, as well as the production of various virulence factors in *P. aeruginosa* PAO1 (1). Preliminary thin-layer chromatography characterization of the crude extracts of these plants revealed that they contain multiple active compounds (1). In the present study, *C. albiflorum* fractionation by HPLC and UV spectrum analysis revealed that several compounds (flavonoids in particular) are probably responsible for the activity of the crude extract and that the synergistic action of these compounds might explain the strong inhibitory activity observed in *C. violaceum* CV026 and *P. aeruginosa* PAO1. Whether the compounds present in *B. buceras* and *C. erectus* are the same or belong to the flavonoids remains an open question.

QS mechanisms can be inhibited in various ways: through signal mimicry (such as with furanones or synthetic signal analogs) resulting in a decrease in QS gene expression, through enzymatic degradation of homoserine lactones, through anti-activator proteins or negative transcriptional regulator homologs (such as the LasR and RhlR homolog QscR in *P. aeruginosa*) or through small regulatory RNAs (29, 82). In *P. aeruginosa*, the QS hierarchy is a complex process (18, 71, 72) with the *las* system regulating the *rhl* system through LasR, and recent evidence suggest that the *rhl* system is only delayed in a *las* negative background and that RhlR controls *lasI* expression and quinolone synthesis (a third signal in *P. aeruginosa*) (18,

72). Elastase (LasB) is mainly under the control of the *lasI-lasR* system (25), while pyocyanin production (12, 83), as well as rhamnolipid production (*rhlA*) (58), is under the control of the *rhlI-rhlR* system, which contributes to a lesser extent to the control of *lasB* expression (12, 58). Biofilm formation involves different stages consisting in attachment, proliferation and differentiation, and QS is thought to be involved mainly in this last stage, the *las* system being the main QS regulator of biofilm (16). The addition of catechin affected all of these processes to different extents (Table 2), and the use of *E. coli* biosensors suggests that catechin acts by interfering with the perception of C4-HSL by RhlR (Fig. 5A), which likely explains the reduction in *rhlR*, *rhlI*, and *rhlA* and, to some extent, *lasB* expression and the reduction in pyocyanin production. Nevertheless, it might be that, as suggested for the extracts of *B. buceras* and *C. erectus* (1), catechin affects also an upstream QS regulator such as GacA, Vfr, VqsR, RsaL, or QscR (4, 14, 17, 19, 32, 37, 63, 69).

Catechin derives from the phenylpropanoid pathway and is classified among the flavan-3-ols of the flavonoid family of polyphenols (20, 88). (–)-Epigallocatechin gallate (resulting from the esterification of epigallocatechin and gallic acid) has been demonstrated (at 100 μ M) to repress the expression of *lasB* from *P. aeruginosa* in *P. putida* cells hosting the pKR-C12 plasmid (carrying a translational *lasB-GFP* fusion and the *lasR* gene under the control of a *lac*-type promoter) and the expression of *luxI* from *Photobacterium fischeri* in *E. coli* cells hosting the pSB403 plasmid (carrying the *luxR* gene and the *luxI* promoter fused to the *luxCDABE* operon) (33). More recently, using an in silico approach, it has been evidenced that baicalein, which is a flavone reported in *Scutellaria baicalensis* (a plant used in traditional Chinese medicine), inhibits the formation of biofilm by *P. aeruginosa* PAO1 when used at 200 μ M and promotes the proteolysis of the signal reporter TraR protein from *A. tumefaciens* expressed in *E. coli* at concentrations ranging from 4 to 40 mM (91). Moreover, baicalein, whose chemical structure differs from catechin by its hydroxylation status and the carbonyl in the heterocycle, has been shown to have a high in silico TraR docking score (91), a characteristic catechin might also have. Both studies used reporter bacteria with a *E. coli* or *P. putida* genomic background differing from those of the target genes (i.e., *luxI/luxR* from *P. fischeri*, *lasB* from *P. aeruginosa*, and TraR from *A. tumefaciens*), while it seems that QS inhibitors should be used and screened in the specific organism targeted (66). Here, we have shown that the expression of *P. aeruginosa* QS genes is downregulated in the *P. aeruginosa* strain PAO1 genomic background, possibly as a result of the disruption of the perception of C4-HSL by RhlR, when catechin is added to the growth medium, which further supports the possible activity of some flavonoids, natural products widely found in plants, as QS inhibitors.

Consistent with a possible role of flavonoids in messing with QS communication among bacteria, a proteomic analysis of *M. trunculata* roots treated with nanomolar to micromolar concentrations of *N*-acyl homoserine lactones from the symbiont *Sinorhizobium meliloti* and from *P. aeruginosa* revealed that, among the 150 proteins whose accumulation was modified, the expression of proteins related to flavonoid metabolism is affected (50). In addition, by using transgenic *Trifolium repens* plants with a relevant GUS reporter fusion, these authors also

showed that the exposure to 3-oxo-C12-HSL (produced by *P. aeruginosa*) substantially increased the expression of three chalcone synthase promoters involved in the flavonoid pathway. Although the exact consequence of these increased expressions is not known, these results and our data suggest a link between the flavonoid pathway in plant and bacterial QS signaling, playing important roles in the beneficial (symbiosis) or pathogenic outcomes of plant-prokaryote interactions. Hence, flavonoids might also constitute a first line of defense against pathogenic attacks by affecting QS mechanisms and thereby virulence factor production.

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