**Endemic Malagasy *Dalbergia* species inhibit quorum sensing in *Pseudomonas aeruginosa PAO1***

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Various species of the plant genus *Dalbergia* are traditionally used as medicine for sundry ailments and some of them have been shown recently to quench the virulence of Gram-positive and Gram-negative bacteria. Cell-to-cell communication mechanisms, quorum sensing (QS) in particular, are key regulators of virulence in many pathogenic bacteria. Screening n-hexane extracts of leaves, roots and bark of endemic Malagasy *Dalbergia* species for their capacity to antagonize QS mechanisms in *Pseudomonas aeruginosa PAO1* showed that many reduced the expression of the QS-regulated genes *lasB* and *rhlA*. However, only the extract of *Dalbergia trichocarpa* bark (DTB) showed a significant reduction of QS gene expression without any effect on the *aceA* gene encoding a QS-independent isocitrate lyase. Further characterization of DTB impact on QS revealed that the QS systems *las* and *rhl* are inhibited and that swarming, twitching, biofilm formation and the production of pyocyanin, elastase and proteases are also hampered in the presence of the DTB extract. Importantly, compared with the known QS inhibitor naringenin, the DTB extract showed a stronger negative effect on twitching, biofilm formation and tobramycin resistance. Preliminary structural characterization of these potent biofilm disrupters suggests that they belong to the phytosterols. The strong inhibition of motility and biofilm formation suggests that the DTB extract contains agents disrupting biofilm architecture, which is an important observation in the context of the design of new drugs targeting biofilm-encapsulated pathogens.

**INTRODUCTION**

*Dalbergia* is a genus of trees and shrubs of the family Fabaceae widely distributed in tropical and subtropical regions and is best known for the high quality and value of its woods (Barrett *et al.*, 2010). Beyond their commercial value, various species are traditionally used as medicine for sundry ailments and some of them have shown significant
antibacterial activity against Gram-positive and Gram-negative bacteria (Okwute et al., 2009; Vasudeva et al., 2009). Forty-seven of the 48 Dalbergia species found in Madagascar are endemic (Bosser & Rabevohitra, 2002, 2005), and although Dalbergia species, such as Dalbergia trichocarpa, are widely used in traditional pharmacopoeia (Lemmens, 2008; Rajaonson et al., 2011), their use and mode of action is poorly documented and only a few species have been screened for their antibacterial activity and their capacity to inhibit virulence factors of bacteria.

Recently, a new type of antibacterial activity was associated with the Madagascar endemic Dalbergia pervillei (Rajaonson et al., 2011). Indeed, this Dalbergia species contains a prenylated isoflavonane, perbergin, quenching the virulence of the phytopathogen Rhodococcus fascians by inhibiting the genetic network driving the production of its virulence factors (Rajaonson et al., 2011; Stes et al., 2011).

Moreover, Brijesh et al. (2006) showed that decoction of Dalbergia sissou leaves negatively impacted the host-cell colonization capacity and enterotoxin production of diarrhoeic Escherichia coli without any antimicrobial effect, providing evidence for antivirulence compounds in the genus Dalbergia. We therefore reasoned that the genus Dalbergia could be a source of compounds inhibiting the expression of virulence factors mediated by quorum sensing (QS).

QS is a cell-to-cell communication mechanism controlling the expression of bacterial virulence factors (Ng & Bassler, 2009; Smith & Iglewski, 2003; Williams, 2007). Through QS, bacteria are able to detect their population density by producing, releasing and perceiving small diffusible molecules called autoinducers and allowing them to coordinate a common action such as expression of virulence factors (Girard & Bloemberg, 2008; Jimenez et al., 2012; Smith & Iglewski, 2003; Williams, 2007). Autoinducer-regulated processes such as virulence factor expression, motility, biofilm formation and toxin production by pathogenic bacteria directly contribute to their capacity to colonize and disseminate through their hosts, which determines the course and outcome of infectious diseases (Bjarnsholt & Givskov, 2007; Deep et al., 2011; Gupta et al., 2011). In the ongoing struggle against bacterial infection, antibiotics are commonly used to kill pathogenic bacteria. However, bacteria are increasingly exhibiting resistance against available antimicrobial drugs due to their misuse and/or abuse (Epps & Walker, 2006; English & Gaur, 2010). Moreover, antibiotics kill indigenous bacteria that are beneficial to their host. To cope with these limitations, the alternative approach of attenuating bacteria virulence factor expression without affecting their viability by using anti-QS agents has become a rational preventive strategy (Bjarnsholt & Givskov, 2008; Hentzer & Givskov, 2003).

In the present study, four endemic Malagasy Dalbergia species (i.e. D. chlorocarpa, D. pervillei, D. trichocarpa and D. lemurica) were selected based on their ethnopharmacological uses (Du Puy et al., 2002; Lemmens, 2008; Rajaonson et al., 2011) to assess their capacity to interfere with QS systems in Pseudomonas aeruginosa PA01. Herein, we show that n-hexane extracts of some Dalbergia species interfere with the expression of QS-related genes. Furthermore, we demonstrate that the extract of D. trichocarpa bark (DTB) shows an inhibitory effect on the production of QS-controlled virulence factors, including swarming, twitching, biofilm formation, and the production of pyocyanin, elastase and proteases, validating its traditional uses in the Malagasy pharmacopoeia.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** P. aeruginosa wild-type and reporter strains were grown (37°C, agitation at 175 r.p.m.) in LB-MOPS broth (50 mM, pH 7) supplemented with carbenicillin (300 μg ml⁻¹) when appropriate as described previously (Vandeputte et al., 2010, 2011). Plasmids listed in Table 1 were used and introduced into PA01 as previously described (Vandeputte et al., 2010). P. aeruginosa PA01 mutant strains were obtained from the Transposon Mutant Collection (Department of Genome Sciences, University of Washington; http://www.gs.washington.edu/labs/manoil/libraryindex.htm) and include mutants 11174 (ΔPA41432, Δlas) and 32454 (ΔPA34476, Δhil) (Jacobs et al., 2003). Mutant strains were grown at 37°C with agitation (175 r.p.m.) for 18 h in liquid Luria–Bertani (LB) medium (5 ml) supplemented with 50 mM MOPS at pH 7.0 and with 60 μg tetracycline ml⁻¹. The N-acylhomoserine lactones (AHLs) N-butanol–t–homoserine lactone (C4-HSL) and N-(3-oxodecanoyl)–t–homoserine lactone (3xooC12-HSL) were purchased from Sigma-Aldrich and dissolved in 100% DMSO. AHLs were added to wild-type and mutant strains at a final concentration of 10 μM as described elsewhere (Vandeputte et al., 2010, 2011).

**Plant material and extracts.** Leaf, bark and root samples from four endemic Malagasy Dalbergia species (D. chlorocarpa R.Vig., D. pervillei Vatke, D. trichocarpa Baker and D. lemurica Bosser & Rabevohitra) were collected from trees growing close to the city of Morondava. Bark samples were collected from six different specimens of D. trichocarpa trees growing in three areas of the Menabe region (DTB1, Kirindy forest; DTB2, around Miandrivazo village; and DTB3, around Antsidika village) and in three areas of the Boeny forest (DTB4, around Ambalanjanakomy village; DTB5, Ambalanjanakomy forest; and DTB6, around Anrandosafia village). The six harvested trees have the following GPS coordinates: 20° 04.120’S 44° 39.250’E, elevation 88 m (DTB1); 19° 43.502’S 45° 28.433’E, elevation 108 m (DTB2); 19° 46.451’S 45° 31.147’E, elevation 168 m (DTB3); 16° 56.356’S 46° 56.242’E, elevation 224 m (DTB4); 16° 20.402’S 46° 50.381’E, elevation 106 m (DTB5); and 16° 21.461’S 46° 49.175’E, elevation 106 m (DTB6). Voucher specimens of these Madagascar endemic species were identified and deposited at both FOIFA (Centre National de la Recherche Appliquée du Développement Rural, Antananarivo, Madagascar) and PBZT herbarium (Botanical and Zoological Park of Tsimbazaza, Antananarivo, Madagascar). Dried plant powders (5 g) were soaked overnight (25°C, agitation at 50 r.p.m.) in n-hexane (50 ml), filtered and concentrated to dryness using a speedvac device. Extracts were dissolved in DMSO to get appropriate concentrations of the plant extracts, which were stored at −20°C until use.

**Gene expression and β-galactosidase measurements.** β-Galactosidase measurements were performed as previously described (Vandeputte et al., 2010) to monitor gene expression. Briefly, after growth in liquid LB-MOPS-carbenicillin at 37°C and 175 r.p.m. for 18 h, PA01 reporter strains were washed twice in fresh LB medium and resuspended in liquid LB-MOPS-carbenicillin. PA01 reporter
strain inocula (50 μl) were incubated at 37 °C with agitation at 175 r.p.m. for 18 h in 1 ml LB-MOPS-carbenicillin (initial OD600 of the culture was between 0.020 and 0.025) supplemented with 10 μl of Dalbergia extracts dissolved in DMSO (300 μg ml⁻¹ final concentration) or 10 μl DMSO. Additionally, the commercially available flavanone naringenin (Sigma-Aldrich) known as a QS inhibitor in P. aeruginosa PAO1 and its glycoside form, naringin (Sigma-Aldrich), were used as a positive and negative control, respectively (Vandeputte et al., 2011). After incubation, bacterial density was assessed by spectrophotometry (OD600) and the sample was precipitated with 1.2 ml of trichloroacetic acid (10 %, w/v) for 10 min. Five hundred microlitres of the supernatant was neutralized with 500 μl of water. Acetic acid (33 % in water) was added to the stained biofilms (2 ml) to solubilize the crystal violet and the absorbance of the solution was read at 590 nm with a SpectraMax M2 device (Molecular Devices).

Quantitative analysis of pyocyanin, LasB elastase production and proteolytic activity. Pyocyanin and elastase production were assessed according to previously described procedures (Ishida et al., 2007; Müh et al., 2006; Vandeputte et al., 2010, 2011). P. aeruginosa PAO1 or mutant strain cell suspension (50 μl) was added to 1 ml of LB-MOPS (starting OD600 ranged between 0.02 and 0.025) supplemented with 10 μl of plant extract dissolved in DMSO (300 μg ml⁻¹ final concentration), DMSO (1 %, v/v), naringenin (4 mM final concentration) or naringin (4 mM final concentration) or fractions (F1–F9 and F4-1) at 150 gm l⁻¹. After 18 h growth, samples were taken to assess growth (OD600) and the remaining volume was used for pyocyanin determination. LasB elastase production was assessed through the measurement of elastase activity using elastin-Congo red. Estimation of exogenous proteolytic activity was carried out as described by Hentzer et al. (2002), with some modifications. Briefly, 200 μl of culture supernatant, centrifuged at 15000 g and 4 °C for 15 min, was mixed with 300 μl of an azocasein solution [2 % (w/v) in 2 mM CaCl₂ and 50 mM Tris/HCl (pH 7.8); Sigma-Aldrich] and allowed to react for 45 min at 37 °C. Undigested substrate was precipitated with 1.2 ml of trichloroacetic acid (33 %, w/v) for 30 min at room temperature and subsequently centrifuged at 15000 g for 10 min. Five hundred microlitres of the supernatant was neutralized with 500 μl of 1 M NaOH. Proteolytic activity was measured as the A₅₇₀ against a blank sample run in parallel but precipitated at time zero and divided by the OD₆₀₀ of the culture to estimate the relative protease production. Moreover, extract samples diluted in LB medium free of bacteria were run in parallel with the same procedure to account for the occurrence of elastase-like or protease-like activities.

Biofilm formation, quantification and antibiotic tolerance. P. aeruginosa PAO1 was grown overnight in LB medium at 37 °C with agitation. After growth, PAO1 strains were washed twice and diluted in fresh biofilm broth (BB) medium (Na₂HPO₄, 1.25 g l⁻¹; FeSO₄.7H₂O, 0.0005 g l⁻¹; glucose, 0.05 g l⁻¹; (NH₄)₂SO₄, 0.1 g l⁻¹; MgSO₄.7H₂O, 0.2 g l⁻¹; KH₂PO₄, 0.5 g l⁻¹) as described by Khalilzadeh et al. (2010) and 50 μl of the diluted culture was added to 940 μl of BB medium (initial OD600 of the culture between 0.14 and 0.16) supplemented with 10 μl of DMSO (1 %, v/v), plant extract (300 μg ml⁻¹ final concentration), naringenin (4 mM final concentration) or naringin (4 mM final concentration). PAO1 cells were incubated statically for 24 h at 37 °C in 24-well polystyrene plates. After incubation, planktonic bacteria were discarded and the biofilms were washed three times with water. Washed biofilms were fixed with 2 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 (2 ml per well), and the plates were incubated for 30 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) to solubilize the crystal violet and the absorbance of the solution was read at 590 nm with a SpectraMax M2 device (Molecular Devices).

The tolerance of biofilm-encapsulated PAO1 cells to antibiotics was assessed by growing PAO1 cells under the same conditions as those described above and tobramycin (350 μg ml⁻¹) was added to 1-day-old biofilms. Tobramycin was chosen because it has been shown that QS inhibition greatly enhances the sensitivity of P. aeruginosa PAO1 to this antibiotic (Jakobsen et al., 2012 and references therein). After a further 24 h incubation, planktonic bacteria were discarded and biofilms were washed three times with water. Biofilm development and bacterial viability in biofilms were assessed using the LIVE/DEAD BacLight bacterial viability staining kit (Invitrogen). The growth medium was removed and replaced by 500 μl of a solution of SYTO 9 and propidium iodide diluted 400× in BB medium. Biofilms were incubated for 15 min and PAO1 cells were examined using a Leica DM IRE2 inverted fluorescence microscope coupled to a CCD camera (Leica DC350 FX) and equipped with FITC and Texas red filters.

Motility assays. Swarming motility was examined using LB agar plates as described by Kohler et al. (2000) with slight modification. Briefly, LB agar (0.6 %) medium supplemented with glutamate (0.05 %) and glucose (0.2 %) was autoclaved for sterilization. Sterilized medium was cooled at 45–50 °C and then supplemented with the desired compound (DMSO 1 %, plant extract at 300 μg ml⁻¹, naringenin at 1 mM or naringin at 1 mM final concentration) before being poured into compartmented Petri dishes. Dried plates with each supplement were inoculated at their centre with 5 μl of PAO1 culture diluted to an OD₆₀₀ of 1 and placed at 37 °C when inoculum spots were dry. The diameter of the spreading zone of PAO1 from the
inoculation spot was measured after 24 h. Twitching motility was assayed as described by Glessner et al. (1999) with slight modification. Briefly, plates with LB agar (1%) supplemented as above poured to an average depth of 3 mm were prepared and dried briefly. A hole (about 1 mm in diameter) was dug in the centre of each compartment of the Petri dish through the agar and 5 μl of P. aeruginosa PA01 cells diluted to an OD600 of 1 was spotted inside the hole and then plates were air-dried briefly before incubation at 37 °C for 48 h. After the incubation period, the twitching zone (occurring at the agar/plate interface) was measured. For diameter measurement, the agar was removed and twitching motility zones were easily visualized by staining for 5 min with 0.1% (w/v) of crystal violet as proposed by Darzins (1993).

Assessment of bacterial growth. Relative growth of P. aeruginosa PA01 after 18 h in the different experimental conditions was evaluated by measuring the cell density at an OD600 with a SpectraMax M2 device (Molecular Devices). The growth kinetics of P. aeruginosa PA01 was evaluated by measuring the OD600 of PA01 cells grown over a 24 h period with bacterial cell counts (c.f.u.) for two points (8 and 18 h).

D. trichocarpa sample preparation, chromatography, ESI-MS analysis and 1H-NMR spectroscopy. Dried powdered samples of D. trichocarpa stem bark (10 kg) were macerated overnight at room temperature in n-hexane (500 g l⁻¹) and extracted with n-hexane by percolation (2 litres for 1 h). Collected n-hexane extracts were filtered on Whatman paper, evaporated with a Buchi Rotavapor and the resulting residue (20 g) was stored at −20 °C. For fractionation, the residue was dissolved in 30 ml of n-hexane and loaded onto silica gel 60 F254 (63–200 μm/70–230 mesh; Merck) poured in a chromatography column (35 by 4 cm). The sample was eluted with n-hexane and a step gradient of ethyl acetate (100: 10 to 10: 100) (fractions F1–F6) and then with ethyl acetate and a step gradient of methanol (100: 10 to 10: 100) (fractions F7–F9). Consecutive fractions showing similar chromatographic profiles under UV illumination and after developing by spraying with 10% (v/v) H2SO4 followed by heating at 110 °C for 10 min were pooled together. TLC was conducted on precoated Kieselgel 60 F254 plates (Merck). The fractions were evaporated and stored at −20 °C until required for further analysis. One active fraction (F4) was further fractionated by prepHPLC using a reversed-phase C18 column (Altima HP, 13 by 250 mm) that was eluted with a gradient of water and acetonitrile (10: 90 for 3 min, 10: 90 to 0: 100 in 4 min, 0: 100 for 2 min, 0: 100 to 10: 90 in 1 min, 10: 90 for 5 min) and a step gradient of ethyl acetate (100: 10 to 10: 100) (fractions F1–F6). The enzyme activity and, consequently, they were not considered for further analyses.

Statistics. All experiments were performed in quintuplicate and repeated in three independent assays. The data were statistically analysed by conducting Student’s t tests (i.e. each test was compared with the DMSO-treated cells) and a P-value ≤ 0.01 was considered as being significant.

RESULTS

Dalbergia extracts inhibit lasB and rhlA gene expression in P. aeruginosa

Bark, root and leaf samples collected from standing plants in Madagascar were extracted with n-hexane and their effect on the expression of lasB (encoding LasB elastase) and rhlA (required for production of rhamnolipids), two QS-regulated genes, was investigated. n-Hexane extracts were chosen according to a preliminary screening that demonstrated the negative impact of D. pervillei and D. trichocarpa n-hexane extracts while dichloromethane and methanol extracts were not active (Rajaonson et al., 2011; data not shown). With a final concentration of 300 μg ml⁻¹, no negative impact on end-point cell density of PA01 was noted and a significant reduction of lasB as well as rhlA gene expression was found for eight extracts when compared with the DMSO treatment [Fig. 1(a, b) for D. trichocarpa and Fig. S1 (available with the online version of this paper) for D. chlorocarpa, D. pervillei and D. lemurica]. As we were specifically interested in compounds directly interfering with QS gene expression without any antimicrobial activity, we also looked at the impact of these extracts on the expression of the QS-independent gene aceA (encoding an isocitrate lyase) to clarify if the drop in β-galactosidase activity recorded was not due to a putative effect on the transcription/translation mechanisms as described earlier (Vandeputte et al., 2010, 2011). As shown in Figs 1(c) and S1, seven of the eight interesting extracts had a negative effect on the expression of the aceA gene. Hence, we concluded that these seven extracts may affect the expression of QS-related genes by affecting housekeeping functions of P. aeruginosa PA01 or may disrupt the β-galactosidase enzyme activity and, consequently, they were not considered for further analyses. Thus, only the DTB extract had no effect on the transcription of the aceA gene, demonstrating its putative specific effect on the expression of QS-related genes without affecting the global transcription machinery of P. aeruginosa PA01, and this extract was therefore selected for further investigations.

DTB extracts from different regions of Madagascar exhibit the same inhibitory effect on QS-regulated lasB and rhlA genes

To clarify if the inhibitory effect on lasB and rhlA gene expression does not represent an exception or an isolated case from the selected group of D. trichocarpa, we collected bark of D. trichocarpa trees from different locations in western Madagascar. Six specimens were collected from trees growing in three areas of the Menabe region (DTB1–3) and three areas of the Boeny region (DTB4–6). As shown in Fig. S2, all bark extracts of D. trichocarpa exhibited the same inhibitory effect (with marginal difference) on rhlA and lasB
The variation among the different DTB extracts is probably linked to a difference in the quantitative production of the active compounds linked to ecological factors such as climate and soil conditions. The DTB extract collected in the Menabe region Kirindy (DTB1) was used for all further experiments.

**DTB extract reduces the expression of QS-regulated lasB and rhlA genes in a dose-dependent manner**

To show a progressive inhibitory effect on the expression of lasB and rhlA genes, different concentrations of DTB (100–400 µg ml⁻¹) were assessed. The data summarized in Fig. 2 show that the inhibitory effects on lasB and rhlA expression were greatly enhanced by increasing the DTB extract concentration, suggesting that the inhibitory effect is dose-dependent. DTB showed significantly less inhibitory activity on lasB expression at 100 µg ml⁻¹ final concentration (14 ± 4 %) than on rhlA expression (27 ± 3 %). At 400 µg ml⁻¹, the DTB extract exhibited a similar inhibitory effect on lasB (44 ± 3 %) and rhlA (45 ± 5 %) expression to naringenin (Fig. 2). Additionally, the DTB effect on aceA expression was also assessed in a dose-dependent manner and no significant inhibitory effect at any of the concentrations tested was observed and, consequently, 300 µg ml⁻¹ of DTB extract was used in all further experiments.

**DTB extract alters QS gene expression**

Since the DTB extract reduced the expression of QS-regulated lasB and rhlA genes, we were interested in assessing its effect on P. aeruginosa PA01 QS systems by measuring the expression of the AHL synthetase genes lasI and rhlI and the QS regulator genes lasR and rhlR. As shown in Fig. 3, DTB reduced significantly (P≤0.01) the expression of the AHL synthetase rhlI gene (33 ± 2 % inhibition) and slightly expression of the lasI gene (18 ± 5 % inhibition). Similarly, DTB reduced significantly the expression of the QS regulator rhlR gene (29 ± 3 % inhibition) but slightly the lasR gene (10 ± 3 % inhibition). As previously reported, naringenin and naringin had a strong inhibitory effect and no effect, respectively, on the expression of the las and rhl genes (Vandeputte et al., 2011). The DTB extract was less potent than naringenin, as would be expected from a crude extract containing a mixture of molecules with an opposite impact on QS gene expression.

**DTB reduces LasB elastase and pyocyanin production but marginally reduces exogenous protease production by P. aeruginosa PA01**

The major role of proteases in P. aeruginosa virulence in various infection models is thought to involve tissue penetration (Tang et al., 1996; Twining et al., 1993). Proteases synthesized by P. aeruginosa include mainly LasB, LasA (encoded by the lasA gene), alkaline protease
grown in the presence of DTB compared with growth with DMSO. However, the effect of DTB (25 ± 6 % inhibition) was not similar to naringenin (38 ± 5 % inhibition). The production of exogenous proteases was assessed indirectly by measuring the ability of culture supernatants to degrade the coloured protein substrate azocasein. DTB showed only a marginally significant effect (15 ± 4 % inhibition) on the production of exogenous proteases (Fig. 4b) compared with naringenin (36 ± 3 % inhibition). Additionally, no elastase or protease-like activities (which could interfere with the tests) were observed when the DTB extract was used in bacteria-free control tests (data not shown). The DTB extract was further tested for its impact on pyocyanin production (encoded by the \( \text{phz} \) operon). As shown in Fig. 4c, the DTB extract decreased significantly \((P \leq 0.01)\) the amount of pyocyanin in bacterial culture supernatant compared with that of the control (DMSO).

**P. aeruginosa PAO1 swarming and twitching are affected by the DTB extract**

Swarming movement is a form of organized surface translocation that depends on extensive flagellation and cell-to-cell contact induced on semisolid surfaces (Daniels et al., 2004; Fraser & Hughes, 1999; Harshey, 1994). Twitching motility is a flagella-independent form of bacterial translocation occurring by successive extension and retraction of polar type IV pili that enables bacteria to move across moist solid surfaces (Mattick, 2002). Because addition of the control compounds naringenin and naringin affected the consistency of the LB agar used for the swarming and twitching assays, their concentration was lowered to 1 mM (final concentration). The swarming ability of PAO1 was significantly affected when grown on LB agar supplemented with DTB or naringenin (1 mM) compared with PAO1 cells inoculated into DMSO- and naringin-supplemented medium (Fig. 5a). Besides, PAO1 wild-type was not able to twitch when grown on LB agar supplemented with the DTB extract, in contrast to the PAO1 wild-type grown on LB agar supplemented with DMSO, naringenin or naringin (Fig. 5b). In both cases, the DTB extract was more potent than naringenin with a 50 % reduction of the swarming and twitching capacities of *P. aeruginosa* PAO1.

**DTB reduces biofilm formation and increases the sensitivity of *P. aeruginosa* PAO1 to tobramycin**

Since biofilm formation is partially controlled by QS mechanisms (Davies et al., 1998; Favre-Bonté et al., 2003; Sauer et al., 2002), the effect of the DTB extract on *P. aeruginosa* PAO1 biofilm formation was assessed. Noticeably, there was a significant decrease in biofilm biomass when PAO1 was grown in the presence of DTB compared with the DMSO treatment. As shown in Fig. 5c, the inhibitory effect of DTB was markedly significant \((49 ± 4 \%)\) after 24 h of incubation and similar results were observed after 48 h of incubation (data not shown).

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**(a)** Effect of DTB on QS-regulated *lasB* gene expression. The amount of pyocyanin was calculated as the β-galactosidase activity of the lacZ-gene fusions and expressed in Miller units. Naringenin (Nar) was used as a QS inhibitor control and naringin (Nin) as a negative control. Error bars represent SEM and all experiments were performed in quintuplicate with three independent assays; asterisks indicate samples that were significantly different from the DMSO (Student’s *t*-tests; \(P \leq 0.01\)).


**(c)** Effect of DTB on *aceA* gene expression. The amount of pyocyanin was calculated as the β-galactosidase activity of the lacZ-gene fusions and expressed in Miller units. Naringenin (Nar) was used as a QS inhibitor control and naringin (Nin) as a negative control. Error bars represent SEM and all experiments were performed in quintuplicate with three independent assays; asterisks indicate samples that were significantly different from the DMSO (Student’s *t*-tests; \(P \leq 0.01\)).

**Fig. 2.** Effect of *Dalbergia trichocarpa* bark (DTB) extract at different concentrations (100–400 \(\mu\text{g ml}^{-1}\)) on the expression of PAO1 QS-regulated genes *lasB* and *rhlA* and the QS-independent gene *aceA* after 18 h of incubation. (a) Effect of DTB on QS-regulated *lasB* gene expression. (b) Effect of DTB on QS-regulated *rhlA* gene expression. (c) Effect of DTB on *aceA* gene expression. Gene expression was measured as the β-galactosidase activity of the lacZ-gene fusions and expressed in Miller units. Naringenin (Nar) was used as a QS inhibitor control and naringin (Nin) as a negative control. Error bars represent SEM and all experiments were performed in quintuplicate with three independent assays; asterisks indicate samples that were significantly different from the DMSO (Student’s *t*-tests; \(P \leq 0.01\)).

(encoded by the *aprA* gene) and protease IV (Caballero et al., 2001). As shown in Fig. 4a, a significant reduction in elastase activity was observed when strain PAO1 was grown in the presence of DTB compared with growth with DMSO. However, the effect of DTB (25 ± 6 % inhibition) was not similar to naringenin (38 ± 5 % inhibition). The production of exogenous proteases was assessed indirectly by measuring the ability of culture supernatants to degrade the coloured protein substrate azocasein. DTB showed only a marginally significant effect (15 ± 4 % inhibition) on the production of exogenous proteases (Fig. 4b) compared with naringenin (36 ± 3 % inhibition). Additionally, no elastase or protease-like activities (which could interfere with the tests) were observed when the DTB extract was used in bacteria-free control tests (data not shown). The DTB extract was further tested for its impact on pyocyanin production (encoded by the *phz* operon). As shown in Fig. 4c, the DTB extract decreased significantly \((P \leq 0.01)\) the amount of pyocyanin in bacterial culture supernatant compared with that of the control (DMSO).

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**DTB reduces biofilm formation and increases the sensitivity of *P. aeruginosa* PAO1 to tobramycin**

Since biofilm formation is partially controlled by QS mechanisms (Davies et al., 1998; Favre-Bonté et al., 2003; Sauer et al., 2002), the effect of the DTB extract on *P. aeruginosa* PAO1 biofilm formation was assessed. Noticeably, there was a significant decrease in biofilm biomass when PAO1 was grown in the presence of DTB compared with the DMSO treatment. As shown in Fig. 5c, the inhibitory effect of DTB was markedly significant \((49 ± 4 \%)\) after 24 h of incubation and similar results were observed after 48 h of incubation (data not shown).
Interestingly, the DTB extract had a stronger impact on biofilm formation than naringenin. Besides, for DTB and naringenin treatments, the formed biofilm showed an irregular texture as compared with DMSO and negative control naringin (Fig. 5c, right), suggesting that DTB and naringenin may affect biofilm architecture and/or composition.

Since biofilm cells are highly resistant to antibiotics (Rasmussen et al., 2005) and biofilm formation was disrupted by naringenin and DTB, the tolerance of PAO1 cells to antibiotics was assessed by adding tobramycin to their growth medium. Biofilms were allowed to form for 24 h under the various treatments (i.e. DMSO, naringin, naringenin and DTB). Formed biofilms were incubated for an extra 24 h with or without tobramycin (350 \(\mu\text{g ml}^{-1}\) final concentration) and the effect of the treatments on PAO1 viability was assessed by LIVE/DEAD staining. As shown in Fig. 6, compared with DMSO and naringin (Fig. 6a, b), naringenin and DTB (Fig. 6c, d) disrupted biofilm architecture, confirming the observation made using the crystal violet staining (Fig. 5c). Assessing the viability of \(P.\ aeruginosa\) showed that the capacity of the formed biofilm to protect PAO1 cells was decreased. Indeed, as shown in Fig. 6(e–h), naringenin and DTB considerably increased the sensitivity of PAO1 to tobramycin, as shown by the increased proportion of dead cells compared with DMSO and naringin, which both showed a moderate proportion of dead cells. DTB seemed very potent in inhibiting the protective nature of biofilm given that almost all PAO1 cells died.

DTB has no influence on PAO1 growth

Addition of the DTB extract at the beginning of the lag phase (time zero) was chosen to assess the effect of DTB on PAO1 wild-type growth kinetics. Compared with the DMSO condition, no significant difference was found in any growth phase according to culture turbidity (\(\text{OD}_{600}\)) measurement (Fig. S3a) and c.f.u. quantifications at 8 h (Fig. S3b) and 18 h (Fig. S3c). Noticeably, no negative impact was observed on bacterial growth, suggesting that the decrease in QS gene expression, pyocyanin, LasB elastase, exogenous protease production and biofilm formation, as well as the inability of swarming and twitching, were not due to a drop in cell viability.
Structural and QS inhibitory features of the active compounds occurring in DTB extract

To identify the QS inhibitory compound(s) occurring in the DTB extract, the n-hexane extract was fractionated using chromatography. Nine fractions were extracted (see Methods) and four of them (F3–F6) showed a significant inhibitory effect on the expression of both lasB and rhlA genes while none of them had an effect on the expression of the QS-independent gene aceA (Fig. S4). Fraction F4 was selected for further fractionation through prepHPLC and subfraction F4-1, eluted after 21.5 min (Fig. S5), was isolated as a white powder. Subfraction F4-1 showed the strongest QS inhibitory activity (data not shown). After spotting and elution on a TLC plate, compounds occurring in F4-1 were visualized by spraying with 1 % (w/v) vanillin in 1 % (v/v) ethanolic H2SO4 followed by heating at 110 °C for 5 min. Subfraction F4-1 displayed pinkish to dark purple spots, suggesting the presence of terpenes (Jermain & Evans, 2009). The UV spectrum profile ($\lambda_{max}$: 203, 226, 308.8 nm; Fig. S6a) suggested the presence of a 4-oxygenated cinnamic acid derivative (Collins et al., 1991; Stalikas 2007; Määtä et al., 2003). ESI-MS analysis of F4-1 revealed a pseudomolecular peak ion at m/z (relative intensity percentage) 585.54 ([M–H]+, 100) and five peaks at m/z 145.04 (80), 621.14 (72), 623.15 (25), 630.60 (10) and 1171, 17 (2[M–H]+, 10) (Fig. S6b). 1H-NMR data indicated the presence of two groups of signals, one group corresponding to sterol derivatives and the other group to cinnamic acid derivatives (Fig. S6c). First, the presence of two proton multiplets at $\delta$ 3.73 and $\delta$ 5.34 was typical for hydrogens of a steroidal nucleus. Secondly, the presence of two 1H doublets at $\delta$ 6.30 and $\delta$ 7.61 indicated a double bond with trans-hydrogens and the two 2H doublets at $\delta$ 7.45 and $\delta$ 6.84 were suggestive of aromatic hydrogens, precisely in a para-substituted aromatic ring. Finally, the presence of one proton multiplet at $\delta$ 4.62 suggested the presence of an ester carbonyl group. Based on these preliminary data and as compared with the spectroscopic data reported in the literature, the above-described characteristics of active subfraction F4-1 show similarities with those of the sitosterol-coumarates isolated from Glycine soja (Zhou et al., 2011) and Vigna vexillata (Leu et al., 2012). The impact of F4-1 on the expression of the las and rhl systems was further detailed by analysing the expression of the lasI, lasR, rhlI, and rhlR genes as well as the production of pyocyanin and elastase. As shown in Fig. S7, the expression of the lasIR (Fig. S7a, b) and rhlIR (Fig. S7c, d) genes was downregulated when F4-1 was added to the growth medium (150 μg ml$^{-1}$) while the expression of the aceA gene was not affected (Fig. S7e), showing that the impact of F4-1 is probably limited to QS-regulated genes. Besides, the effect of F4-1 could not be compensated for by the addition of exogenous homoserine lactones. Indeed, as shown in Fig. 7(a), the production of pyocyanin by wild-type strain PAO1 was strongly inhibited by F4-1 and could not be restored by the addition of C4-HSL or 3oxoC12-HSL, suggesting that F4-1 interferes with the perception of...
the HSLs by LasR and/or RhlR as does naringenin (Vandeputte et al., 2011). The inhibition of pyocyanin production by F4-1 was stronger than that with naringenin (50% inhibition with naringenin versus 65% with F4-1) (Fig. 7a). In addition, as shown in Fig. 7(b), the production of elastase as evaluated by the elastolysis assay could not be

**Fig. 5.** Effect of the DTB extract on mobility of and biofilm formation by *P. aeruginosa* PAO1. (a) Swarming motility of *P. aeruginosa* PAO1 onto LB agar (0.6%) supplemented with glutamate (0.05%) and glucose (0.2%) and DMSO (1%) or DTB (300 μg ml⁻¹ final concentration), naringenin (Nar; 1 mM final concentration) or naringin (Nin; 1 mM final concentration). After incubation at 37 °C for 24 h, the zones of migration from the point of inoculation were measured for each condition (left). (b) Twitching motility of *P. aeruginosa* PAO1 onto LB agar (1%) supplemented with DMSO (1%), DTB (300 μg ml⁻¹ final concentration), Nar (1 mM final concentration) or Nin (1 mM final concentration). The twitching zones were stained (right) and their diameters measured (left) after incubation at 37 °C for 48 h. (c) Quantification of biofilm formation by PAO1 after static incubation at 37 °C for 24 h. Biofilm formation was visualized on the bottom of the wells of polystyrene plates by crystal violet staining (right) and indirect measurement of biofilm biomass (left). DMSO (1%), bark extract of *D. trichocarpa* (DTB; 300 μg ml⁻¹ final concentration), naringenin (Nar; 4 mM final concentration) and naringin (Nin; 4 mM final concentration) were tested. Error bars represent SEM and all experiments were performed in quintuplicate with three independent assays; asterisks indicate samples that were significantly different from the DMSO (Student’s t-tests; *P* < 0.01).
enhanced by the addition of HSL to F4-1-treated strain PAO1. Indeed, while adding C4-HSL or 3oxoC12-HSL to PAO1 increased the production of elastase, adding F4-1 or naringenin limited this production to DMSO-treated PAO1 cultures. Consistent with the effect of F4-1 on wild-type strain PAO1 supplemented with HSLs, HSL-supplemented mutant strains ΔlasI and ΔrhlI were impaired in their capacity to produce elastase and pyocyanin when naringenin or F4-1 was added to the growth medium. Indeed, as shown in Fig. 8, while production of elastase and pyocyanin was restored when the appropriate HSL was added to the growth medium of mutant strains ΔlasI (Fig. 8a) and ΔrhlI (Fig. 8b), adding naringenin or F4-1 reduced these values by 20 or 30 % for elastase and 40 or 60 % for pyocyanin, respectively. In each case, F4-1 had a stronger effect than naringenin, suggesting that the phytosterols occurring in F4-1 are more effective in affecting QS mechanisms in P. aeruginosa PAO1.

**DISCUSSION**

African pharmacopeias are invaluably important as sources of new antibacterial compounds and for drug discovery and design. The genus *Dalbergia* is used in traditional medicine and is known for its antimicrobial activities, validating its traditional uses (e.g. Lemmens, 2008; Yadav et al., 2008; Okwute et al., 2009; Vasudeva et al., 2009; Rajaonson et al., 2011). Besides, recent evidence points towards the occurrence of antivirulence...
compounds in this plant genus (Brijesh et al., 2006; Rajaonson et al., 2011), suggesting that it is a potential source to discover new drugs targeting the production of virulence factors by pathogenic bacteria.

Many of the extracts tested here were found to affect the expression of two QS-regulated genes, i.e. lasB and rhlA, but a careful analysis of their impact on a QS-independent gene, i.e. aceA encoding an isocitrate lyase (Kretzschmar et al., 2008), led to the identification of the bark extract of D. trichocarpa (DTB extract) as a potent inhibitor of QS-related genes. Although we focused on the DTB extract, we cannot exclude that other Dalbergia species contain QS inhibitors (QSI). None of the extracts showed an antibacterial activity while Dalbergia species (e.g. D. melanoxylon and D. saxatilis) are indeed known to contain antimicrobial compounds active against Gram-positive and Gram-negative bacteria, including P. aeruginosa (Gundidza & Gaza, 1993; Okwute et al., 2009). Since these activities were associated with ethanolic extracts, the antimicrobial compounds occurring in Dalbergia species are probably polar while the molecules extracted with n-hexane are mainly apolar, which could explain why no antimicrobial activity was observed.

As with D. pervillei and D. sissou, which affect the virulence of the phytopathogen Rhodococcus fascians and of diar-rhoeic Escherichia coli, respectively (Brijesh et al., 2006; Rajaonson et al., 2011), D. trichocarpa contains antivirulence compounds targeting QS mechanisms in P. aeruginosa. In this bacterium, QS regulates the expression of the lasIR and rhlIR genes as well as the production of elastase (lasB), LasA protease (lasA), alkaline protease (aprA), rhamnolipids (through the rhlAB operon) and pyocyanin (phz operon) among other virulence factors (Brint & Ohman, 1995; Gambello & Iglewski, 1991; Pearson et al., 1997; Van Delden & Iglewski, 1998; Whiteley et al., 1999).

Taken together, our results suggest that the DTB extract slightly affects the las system but mainly interferes with the rhl system as all the dramatic effects concern rhl-dependent factors. Indeed, rhlA, lasB, twitching, swarming and pyocyanin are under the strict or partial control of the rhl system (Brint & Ohman, 1995; Daniels et al., 2004; Gambello & Iglewski, 1991; Glessner et al., 1999; Köhler et al., 2000; Pearson et al., 1997; Van Delden & Iglewski, 1998). The lower impact of the DTB extract on LasB elastase production and the slight inhibitory effect on proteolytic activity could be explained by the fact that, although these virulence factors are mainly under las regulation, they are under partial rhl control (Brint & Ohman, 1995; Gambello et al., 1993; Pearson et al., 1997; Van Delden & Iglewski, 1998). Indeed, even if the LasR transcriptional regulator is generally considered to sit at the top of the QS hierarchy in P. aeruginosa (Pesci et al., 1997), the QS hierarchy is more complex than the model presenting the las system above the rhl system, given that Dekimpe & Déziel (2009) demonstrated that RhlR is able to induce LasR-regulated genes when LasR is non-functional.

The virulence of P. aeruginosa is multifactorial but relies also on its ability to rapidly colonise its hosts and to form biofilms (Davies et al., 1998). The effect of the DTB extract on the formation of biofilm and on swarming and twitching (both of which contribute to biofilm formation and architecture; Klausen et al., 2003a, b; O’Toole & Kolter, 1998; Davey et al., 2003; Soberón-Chávez et al., 2005) was therefore tested. Consistent with the reduced expression of rhlA, swarming, twitching and biofilm formation were significantly reduced. Indeed, it has been shown that the rhl system, C4-HSL production, rhamnolipids and their precursor [3-(3-hydroxyalkanoyloxy)-alkanoic acid (HAA)] represent putative regulators of P. aeruginosa swarming behaviour (Daniels et al., 2004; Déziel et al., 2003). Déziel et al. (2003) demonstrated that the rhlA gene, required for HAA production, promotes swarming motility in P. aeruginosa. Besides, rhamnolipids, the production of which depends on rhlA (Van Gennip et al., 2009), are required to maintain tendril organization, and their involvement in modulating swarming motility has been demonstrated (Caiazza et al., 2005). Other studies demonstrated the role of rhamnolipids in the architecture and maintenance of biofilms produced by P. aeruginosa (Chrzanowski et al., 2012; Davey et al., 2003; Soberón-Chávez et al., 2005). As a consequence, disruption of rhamnolipid production when P. aeruginosa PA01 is grown in the presence of the DTB extract could explain the observed disruption of biofilm architecture (Fig. 5, right; Fig. 6). If biofilm differentiation and maturation are indeed mainly regulated by the las system (Daniels et al., 1998; Sauer et al., 2002), Favre-Bonté et al. (2003) demonstrated that C4-HSL is also required for optimal biofilm formation.

Besides, swarming and twitching motilities, which are regulated by the rhl system (Patriquin et al., 2008; Shrout et al., 2006), also contribute to the initial stages of biofilm formation and architecture (Klausen et al., 2003a, b; Chrzanowski et al., 2012; Davey et al., 2003; Soberón-Chávez et al., 2005). In addition, DTB and naringenin drastically increased the sensitivity of P. aeruginosa to tobramycin, confirming that biofilm formation is impaired by these treatments. The preliminary structural characterization of the responsible compounds could have medical applications as biofilm formation/structure greatly contributes to chronic infection by increasing antimicrobial resistance properties of pathogenic bacteria (Hentzer & Givskov, 2003; Ito et al., 2009; Landry et al., 2006; Tré-Hardy et al., 2009). Naringenin and the responsible compounds in DTB are therefore important lead compounds for the development of new therapeutic compounds against biofilm-encapsulated pathogens, acting by disrupting biofilm structure and so increasing pathogen exposure to antibiotics.

QS systems are new targets for the development of new antimicrobial strategies aiming at inhibiting the production of virulence factors by pathogenic bacteria and thereby their deleterious effects (Bjarnsholt et al., 2010). Higher plants have been shown to contain QS mimics and/or anti-QS activities (Teplitski et al., 2011) and are reservoirs of...
countless QSIs, including flavonoids, essential oils, limonoids, salicylic acid and $\gamma$-aminobutyric acid (GABA) (Chevrot et al., 2006; Szabó et al., 2010; Truchado et al., 2012; Vandeputte et al., 2010, 2011; Vikram et al., 2010, 2011; Yuan et al., 2007, 2008; Zeng et al., 2008). Based on the preliminary structural characterization of the compound(s) present in F4-1, $p$-coumarate derivatives might be responsible for the QS inhibitory activity of $D. trichocarpa$. Although $p$-coumaroyl-AHL, a $p$-coumarate derivative, has been shown to regulate QS in the photosynthetic bacterium Rhodopseudomonas palustris (Schaefer et al., 2008), cinnamaldehyde analogues and derivatives as well as $p$-coumaric acid have also been shown to antagonize QS in different models (Bodini et al., 2009; Brackman et al., 2011). Subfraction F4-1 contains sitosterol $p$-coumarate analogues and further studies are required to determine the precise structure of the QSI compounds and to identify which part of the molecule (sterol versus $p$-coumarate) is responsible for the QSI activity.

Fig. 7. Effect of fraction F4-1 on the production of (a) pyocyanin and (b) elastase after exogenous supply of homoserine lactones to wild-type strain PAO1. Production of pyocyanin was quantified as described in Methods and is expressed as the ratio between $A_{380}$ and $OD_{600}$. Elastase production was evaluated via an elastolysis assay as described in Methods. In each case, bacteria were incubated with DMSO, naringenin (Nar), F4-1, $N$-butanoyl-$L$-homoserine lactone (C4-HSL) or $N$-(3-oxodecanoyl)-$L$-homoserine lactone (3oxoC12-HSL). Bacteria were also induced with the appropriate AHL and simultaneously treated with naringenin (+Nar) or F4-1 (+F4-1). C4-HSL and 3oxoC12-HSL were added at 10 $\mu$M final concentration. DMSO-treated cultures were used as controls, the statistical significance of each test ($n=4$) was evaluated by conducting one-way ANOVA with Tukey’s multiple comparison tests and a $P$-value of $\leq 0.01$ was considered significant.

Fig. 8. Effect of fraction F4-1 on the production of (a) elastase and (b) pyocyanin after exogenous supply of the appropriate AHLS to, respectively, $\Delta$lasI (APA1432, mutant ID 11174) and $\Delta$rhII (APA3476, mutant ID 32454) mutant strains. See legend to Fig. 7 for further details. The different letters above the histograms indicate that the data are statistically different from each other ($P \leq 0.01$).
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