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Glycosylflavonoids from *Cecropia pachystachya* Trécul are quorum sensing inhibitors

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ABSTRACT

The *Cecropia* genus is widely distributed in Latin America including at least 60 species, and some of them are commonly used in traditional medicine for the treatment of several diseases. We used *Cecropia pachystachya* Trécul to search for quorum sensing (QS) inhibitors compounds and found that the aqueous extract of *C. pachystachya* leaves is a promising source of substances with this activity. Using as biosensor *Chromobacterium violaceum* ATCC 31532 and *Escherichia coli* pSB403, the compounds chlorogenic acid (**2**), isoorientin (**3**), orientin (**4**), isovitexin (**6**), vitexin (**7**), and rutin (**9**) were identified as QS inhibitors. None of these compounds inhibited the growth of neither the used biosensors nor the microorganisms *Staphylococcus aureus* ATCC 23591, *Escherichia coli* ATCC 25922 and *Saccharomyces cerevisiae*, used here as growth inhibition controls. Along with the rutin, here we presented for the first time the QS-inhibition potential of the *C*-glycosyl flavonoids. The prospective of this evidence lead to the use of these compounds as antipathogenic drugs or antifoulants.

Introduction

QS inhibitors compounds are emerging as a potentially approach for the design of antipathogenic compounds with new pharmaceutical targets, useful in controlling microbial chronic infections (Rasmussen and Givskov 2006), and bacterial biofilms (Romero et al. 2012). Compounds with this activity have been search in natural and synthetic sources (Rasmussen and Givskov 2006; Osorno et al. 2012).

In preliminary studies we screened for quorum sensing inhibition more than 40 extracts including marine organisms, algae and plants (Brango 2012). Considering that the methanolic extract of *C. pachystachya* was the most active when the biosensor *Chromobacterium violaceum* ATCC 31532 was used, the aim of the present work was to identify the compounds responsible for the quorum sensing inhibition activity observed in polar extract, using *C. violaceum* ATCC 31532 and *Escherichia coli* pSB403 as biosensors for QS inhibition tests. In order to evaluate the effect of crude extract and the isolated compounds over microorganism growth, we investigated their antibacterial activity against three Gram positive strains and two Gram negative strains. Additionally, the antifungal activity was evaluated against *Saccharomyces cerevisiae*.

Material and methods

General

UV spectra were recorded on an UV-Vis Cary 50-Agilent spectrometer; the compounds were dissolved in methanol. IR spectra were recorded on an IR Prestige-21 FTIR-Shimadzu. Optical rotations were measured in a Polartronic ADP440+, Bellinghan + Stanley polarimeter. ^{1}H and ^{13}C NMR (1D and 2D) spectra were recorded on a Bruker Avance 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) using CD_3OD or DMSO-d_6 as solvents and the residual solvent signals as internal standards. The HRMS analyses were performed on Shimadzu LC-IT-TOF by direct injection. Electrospray ion source ESI-MS spectra were acquired in both positive and negative ion modes, and the interface and MS detector parameters were as follows: detector voltage 1.5 kV; curved desolvation line (CDL) temperature 300 °C and 150 V. Full scan MS analysis were done in the range of m/z 200–2000, using nitrogen as the nebulizing gas

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(1 l/min). Digital images of the plates were captured using an imaging system ChemiDoc™ XRS+. Model Universal Hood II-BIO-RAD.

Preparative HPLC was conducted on a Merck-Hitachi 6000 instrument with a UV/Vis L-4250 detector at 340 nm using a LichroCART RP-18 (250 × 10 mm i.d., 10 μm) column, with isocratic elution of solvent A (THF/ACN/IPA, 8:3:2) and solvent B (formic acid 0.5%) in a 85:15 (v/v) ratio using a flow rate of 2.5 ml/min. The analytical HPLC were carried out in a Merck-Hitachi 6000 HPLC equipped with L-6200A pump and DAD L-4500 detector, using a Luna® C18 Phenomenex column (250 × 4.6 mm; i.d., 5 μm) and the same solvents used for the preparative isolation as the mobile phase with a flow rate of 1.0 ml/min.

Methanol, butanol, dichloromethane and formic acid (A.R. grade); acetonitrile, THF, isopropyl alcohol and formic acid (HPLC grade) were purchased from Merck® (Darmstadt – Germany). Water was purified with a Milli-Q system (Millipore®, Bedford, USA). All the solutions prepared for HPLC analysis were filtered through a 0.45 μm membrane before use. Chlorogenic acid (3-*O*-caffeoylquinic acid, ≥98.0%), vitexin (4′,5,7-tetrahydroxyflavone-8-glucoside, ≥96.0%), isovitexin (4′,5,7-tetrahydroxyflavone-6-glucoside, ≥98.0%) and rutin (3′,4′,5,7,-tetrahydroxyflavonol-3-*O*-rutinoside) were purchased from Sigma-Aldrich® Co. (St. Louis, USA). Isoorientin (3′,4′,5,7-tetrahydroxyflavone-6-glucoside, ≥98.0%) and orientin (3′,4′,5,7-tetrahydroxyflavone-8-glucoside, ≥98.0%) were purchased from Extrasynthèse (France). Culture media were purchased as follows: Mueller Hinton (Scharlau®) and Sabouraud dextrose (Merck®), and PDA (Scharlau®), Triptic Soy Broth (TSB) – Oxoid®. LB agar was prepared by components.

Plant material

Aerial parts of *Cecropia pachystachya* Trécul were collected in Viamão, in the State of Rio Grande do Sul, Brazil, in March 2007 and a voucher specimen (ICN 150025) was deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. The collection and identification of plant material was done by Prof. Dr. Lilian A. Mentz.

Extraction

The collected leaves were air-dried (35–40 °C) and crushed; afterwards, the powdered material (100 g) was extracted by infusion with boiling distilled water (1 l) for 30 min and then filtered. This crude extract was stirred with 50 g of Amberlite® XAD-16 for 1 h, the mixture was filtered and the resin stirred again with 500 ml of methanol for 1 h. This MeOH solution was further filtered and the solvent removed under reduced pressure. This procedure was repeated three times to yield 5 g of MeOH fraction (FM). A portion of the FM (1 g) was partitioned between CH_2Cl_2 (FD) and water. The aqueous layer was subsequently extracted with butanol, the organic layer was evaporated under vacuum to obtain 400 mg of butanolic fraction (FB), and the residual aqueous layer was freeze-dried (FW, 598 mg).

Isolation and compounds identification

The FM fraction and its sub-fractions (FB and FW) were analyzed by HPLC and evaluated in the QSI assay, finding that FB and FW had similar composition and activity. Because of that we decided to study just the parental fraction FM (100 mg) that was separated by preparative reversed-phase HPLC to yield nine pure compounds (**1–9**). Each compound was evaluated in the QS inhibition test, and only the active compounds were identified by the analysis of their spectral data and HPLC co-injection with pure and certified standards. In this way, we identified one derivate of cinnamic acid (**2**), four *C*-glycosylated flavonoids (**3**, **4**, **6** and **7**) and one *C*-diglycosylated flavonoid (**9**).

Bacterial strains and culture conditions

Two different biosensors were used to evaluate QS inhibition activity: *Chromobacterium violaceum* (ATCC 31532); and *Escherichia coli* (pSB403) coupled with *Pseudomonas putida* (isoF WT) as the exogenous source of AHLs to induce bioluminescence production. All those strains were kindly provided by Prof. Dr. Katharina Riedel (Institute for Microbiology, Ernst-Moritz-Arndt-University in Greifswald, Germany).

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 23591) were kindly donated by Professor Edelberto Silva (Departamento de Farmacia, Universidad Nacional de Colombia – Bogotá). Additionally, a strain of *Saccharomyces cerevisiae* was provided by Professor Angélica Kundson (Facultad de Medicina, Universidad Nacional de Colombia). *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) were taken from our own collection.

Quorum sensing inhibition assays

Violacein production

The bioassay was done using the same procedure described by Tello et al. (2013). The crude extract were evaluated at 100, 200 and 300 μg/disc; and for pure compounds the discs were loaded with 7.5, 15, 30, 40, 50, 75 and 100 μg.

Bacterial bioluminescence

The assay was performed according to methodology described by Tello et al. (2009), using *E. coli* (pSB403) and *P. putida* (isoF WT), employing 3 mm diameter wells instead of paper discs. The pure compounds were applied at 15, 30 and 35 μg/well; while the fractions or extracts were applied at 100, 200 and 300 μg/well. Assays were performed by triplicate.

Antimicrobial assays

Sensi-disc Growth inhibition test

E. coli (ATCC 25922) and *S. aureus* (ATCC 23591), inoculated in Mueller Hilton agar (1.2%), and *S. cerevisiae* inoculated in Sabourau dextrose agar 1.2%, were used in order to evaluate the antimicrobial activity of the crude extracts by the growth inhibition test using sterile sensi-disc. The discs were loaded with 100, 200 and 300 μg/discs. As positive control tetracycline (1.5 μg/well) for bacteria, and cycloheximide (2.1 μg/well) for the yeast were used. As a negative control 10 ml of methanol was used. The Petri dishes were incubated at 37 °C and the growth inhibition halo was measured after 24 h for bacteria and 48 h for yeast.

Microdilution test

The assays were conducted in 96 well plates (MICROTEST™). *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923) were grown in TSB at 37 °C under shaking until an optical density of between 0.2 and 0.3 Abs at 600 nm. In order to obtain the solutions of known concentration of the compounds, these were dissolved medium-DMSO (Final concentration of DMSO, 0.5%). To each well was added the suspension of the bacterium (20 μl), an appropriate aliquot of the solution of the compound, and completed to 200 μl with sterile medium. Final concentrations of compounds in the wells were 2.5, 12.5 and 100 ppm. Experiments were performed by triplicate. The plates were incubated for 48 h at 37 °C. Later, absorbance measurements were taken of the culture at 621 nm for each treatment. The percent inhibition of bacterial growth (%IBG) was calculated with the Formula $\%IBG = 100 - 100(P^{\circ}/P)$, where P° is the average absorbance obtained for each treatment;

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Table 1
Quorum sensing inhibition test against *Chromobacterium violaceum* ATCC 31532 and *Escherichia coli* pSB403 of fraction and pure compounds isolated from *Cecropia pachystachya*.

Fractions/compounds	Biosensor *C. violaceum*[a] (quantity in μg/disc)[b]	Biosensor *E. coli*[c] (quantity in μg/disc)[d]
FM	30 μg {+}/100 μg (+++)	100 μg (++)
FW	100 μg (++)	ND
FB	100 μg (+++)	ND
FD	–	–
1	–	ND
2	50 μg {+}/100 (++)	–
3	40 μg (+++)	45 μg (++)
4	50 μg (+++)	15 μg (+)
5	50 μg {+}*	–
6	50 μg {+}*	45 μg (+++)
7	7.5 μg (+)	–
8	–	ND
9	15 μg (+++)	30 μg (++)

–, no zone of inhibition was observed, even at 300 μg/disc for extracts and 50 μg/disc for pure compounds. The inhibitory quorum sensing activity is expressed as the diameter of the inhibition zone (–, no inhibition; ND, no determined; +, 5–7 mm; ++, 8–9 mm; +++, 10–12 mm; ++++, >12). For diffuse halos with decreased amount of violacein: {+/++}*, the meaning of the number of + is the same as before.

[a] Activity was measured by the inhibition of violacein pigment.
[b] Minimum quantity in μg per disc of compound required to inhibit violacein pigment.
[c] Activity was measured by bioluminescent inhibition.
[d] Minimum quantity in μg per disc of compound required to inhibit bioluminescent.

P is the absorbance of the blank of cell viability (200 μl of fresh medium).

Results

Two different biosensors were used to evaluate the quorum sensing inhibition properties of compounds and extracts. For *C. violaceum*, the absence of violet pigment, and for *E. coli* the presence of a dark shadow surrounding the disc were interpreted as QS inhibition positive result only when there was not antibacterial activity against these biosensors. The results of QS inhibition of the extract and fractions against *C. violaceum* showed that the FM, and the fractions FB and FW were active (Table 1, Fig. 1A–C), while the fraction FD did not show activity. Additionally, when the biosensor *E. coli* was used the same results of QS inhibition activity of FM, FB and FW fractions were obtained (Table 1, Fig. 1D–F).

The FB, FW and FM were analyzed by HPLC. The fractions FB and FW were less complex than the original fraction FM (data not show); however, FB and FW still shared five compounds, the FB fraction was enriched in low polar compounds while the FW was enriched in more polar compounds, and their QSI activity was quite similar among them, showing that the partition procedure was not good enough to lead the active compounds isolation. Because of that, we decided to purify the compounds from FM fraction by HPLC preparative. The HPLC chromatogram showed nine major compounds (Fig. 2A). Each isolated compound was tested in the QS inhibition assay using both biosensors. The compounds **2**, **3**, **4**, **6**, **7** and **9** showed to be active as QS inhibitor compounds (Fig. 2B, Table 1), while the compounds **1**, **5**, and **8** did not show QSI activity.

On the basis of the UV spectra analysis for identification of the flavonoid nucleus, and the high resolution mass spectra for

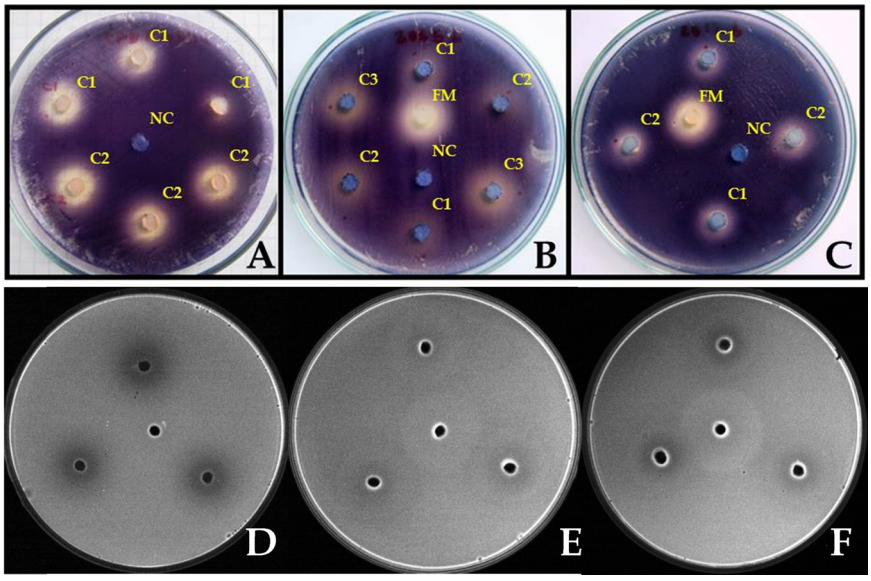

Fig. 1. Quorum sensing inhibition assays using *Chromobacterium violaceum* ATCC 31532 and *Escherichia coli* pSB403 as biosensors. For assays with *C. violaceum* ATCC 31532 the absence of violet pigment indicate a QSI activity (A) FM: C1 = 100 μg/disc, C2 = 200 μg/disc; (B) Compound **4**: C1 = 50 μg/disc, C2 = 75 μg/disc, C3 = 100 μg/disc; and (C) Compound **9**: C1 = 40 μg/disc, C2 = 50 μg/disc; NC = negative control (methanol). In the assays B and C the FM was used at 100 μg/disc as control. For assays using *Escherichia coli* pSB403 as biosensor, a QS inhibition positive result was the inhibition of the expression of bioluminescence (dark shadow zone). (D) Results using FM fraction at 300 μg/well; (E) Compound **3** 30 μg/well; and (F) Compound **4** 30 μg/well. The central wells in all cases are the negative control (10 μl of a solution DMSO 5% in water).

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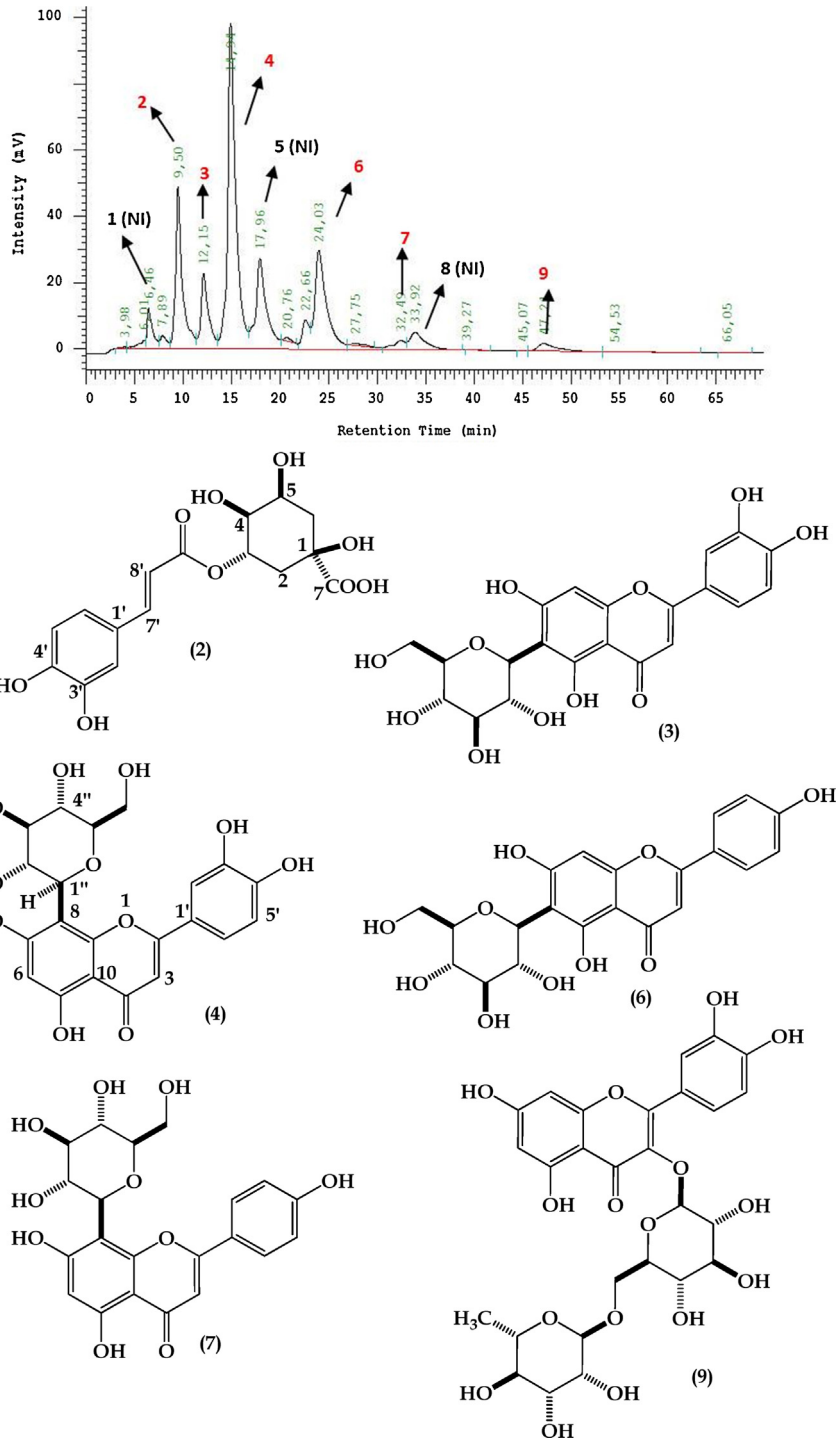

Fig. 2. (A) Methanolic fraction HPLC chromatogram (340 nm). The red highlighted compounds showed QS inhibition activity against *C. violaceum* ATCC 31532 biosensor and/or against *Escherichia coli* pSB403. NI, not identified. (B) Structure of quorum sensing inhibitors compounds isolated from the polar extract of *Cecropia pachystachya*. Chlorogenic acid (**2**), isoorientin (**3**), orientin (**4**), isovitexin (**6**), vitexin (**7**), and rutin (**9**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proposing the molecular formula of each compound it was possible to identify of both *C*-glycosyl and *O*-glycosyl flavonoids (Zucolotto et al. 2012; Marston and Hostettmann 2006). Their identity was confirmed by co-injection of the isolated compound with standards. For the differentiation between the C-6 and C-8 *C*-glycosyl flavonoids, MS/MS experiments were carried out, due the striking differences in the product ions spectra of their $^{0,2}X^{+}$ fragments ($[M+H-120]^{+}$) (Waridel et al. 2001; Marston and Hostettmann 2006). Additionally, the NMR spectra were recorded to confirm their identity (see Experimental section). The identified bioactive compounds are presented in Fig. 2B.

The results of QS inhibition assay with pure compounds using *C. violaceum* as biosensor are showed in Table 1. The compounds **7** and **9** were the most active ones, showing inhibition of violacein production at 7.5 μg/disc. On the other hand, the compounds **3** and **4** showed mild activity (40 and 50 μg/disc, respectively), while the compound **2** was the less active of the tested compounds (80 μg/disc). All the compounds, and extracts, showed a

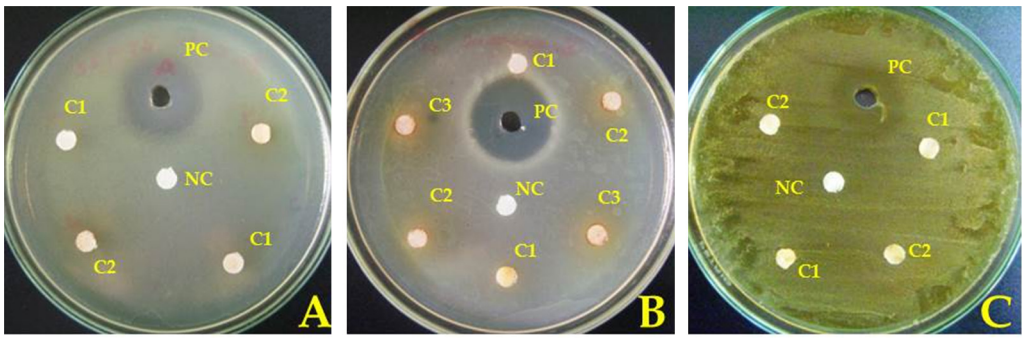

Fig. 3. Growth inhibition assay of FM fraction against: (A) *Escherichia coli* ATCC 25922, (B) *Staphylococcus aureus* ATCC 23591, and (C) *Saccharomyces cerevisiae*. C1 = 100 μg/disc, C2 = 200 μg/disc, C3 = 300 μg/disc; PC = positive control (for assays with bacteria: tetracycline 1.5 μg/well; for yeast assay: cycloheximide 2.1 μg/well); NC = negative control (10 μl/disc of solvent).

dose dependent response. None of the tested compounds, whether they were active or not active, showed bactericidal effects against this biosensor at 80 μg/disc.

In addition, compounds **3**, **4**, **6** and **9** were active as QS inhibitors when tested on the *E. coli* biosensor model (Table 1). The compounds **4** and **9** were the most active ones (15 and 30 μg/disc, respectively) while **3** and **6** presented moderate activity. The compounds **2** and **7** were not active as inhibitors of QS on the *E. coli* model, since they showed activity as QS inhibitors on the *C. violaceum* model.

In order to evaluate the effect of the FM and their related fractions on the growth of microorganisms, these samples were tested in a growth inhibition test using *S. aureus* (23591), *E. coli* (25922) and *S. cerevisiae*. The fraction FM was tested at 100, 200 and 300 μg/disc against the three microorganisms, and did not show any growth inhibition activity (Fig. 3). The used amounts were the same and even higher than the amount used for QS inhibition test (100 μg/disc). Additionally, for isolated compounds the grown inhibition was quantitative evaluated in a different model, at three different concentrations (2.5, 12.5 and 100 ppm) and employing other bacterial strains: *S. aureus* (25923) and *P. aeruginosa* (27853). All compounds showed a grown inhibition concentration dependent. However, none of them presented a high activity even at concentrations as high as 100 ppm, with the growth inhibition smaller that 32% in all cases (Figs. S1 and S2 in the supporting information), confirming the results obtained in de sensi-disc assay.

To summarize, we isolated nine pure compounds from the FM original fraction, which were tested in the QS inhibition assay. The compounds **2**, **3**, **4**, **6**, **7** and **9** showed QS inhibition against *C. violaceum*, while compounds **3**, **4**, **6** and **9** were active as QS disruptors against *E coli*.

Discussion

In our continuous research on quorum sensing inhibitors (Brango 2012; Tello et al. 2013) we focused on the *Cecropia pachystachya* polar extract, since it showed high activity as quorum sensing inhibitor using the biosensors *C. violaceum* (ATCC 31532) and *E. coli* (pSB403). Additionally, this extract does not present growth inhibition properties against the same biosensors.

The six isolated compounds were identified by their chromatographic, MS and NMR data as the known compounds chlorogenic acid (**2**) (Lacaille-Dubois et al. 2001; Dürüst et al. 2001), isoorientin (**3**), orientin (**4**), isovitexin (**6**), vitexin (**7**) (Aragão et al. 2010; Cheng-Ning et al. 2010) and rutin (**9**) (Zhang et al. 2010). The first one is a cinnamic acid derivative, while compounds **3**, **4**, **6** and **7** are *C*-glycosyl flavonoids previously reported from *C. pachystachya* (Costa et al. 2011a,b), and the last one of the active compounds is an *O*-diglycosylated flavonoid. The *C*-glycosylated compounds have a broad distribution in the *Cecropia* genus (Costa et al. 2011a). Other *O*-glycosylflavonoids, e.g. naringin, neohesperidin and hesperidin, have been reported as quorum sensing disruptors, reducing the AHLs production of *Yersinia enterocolitica*, inhibiting biofilm formation, swimming and swarming motility (Truchado et al. 2012).

The most active compound tested with both biosensors was rutin (**9**), the only *O*-diglycosylated flavonoid isolated. Among the *C*-glycosylated flavonoids, vitexin (**7**) was most active against *C. violaceum* followed by chlorogenic acid (**2**), but these compounds were not effective to inhibit bioluminescence of *E. coli*. In contrast, orientin (**4**) presented strong activity tested with *E. coli* biosensor, but when *C. violaceum* was used, only a mild activity could be detected for this compound. Moreover, chlorogenic acid (**2**) that was expected to have a strong activity, because the derivatives of cinnamic acid have been recognized as potent QS inhibitor compounds, was the less active among all the tested compounds. The structural analogs of cinnamic acid have the capability of acting as Michael acceptors in the α,β-insaturated system, and it was proposed as a possible mechanism for inhibitors in *Vibrio* spp. quorum sensing systems (Brackman et al. 2011).

In order to evaluate the antimicrobial activity, the FM fraction was evaluated against the strains *S. aureus* ATCC 53591 and *E. coli* ATCC 25922, and the yeast *S. cerevisiae*. The crude extract did not show antibiotic activity even at concentrations highest as 300 μg/disc.

Additionally, the active compounds were evaluated in a growth inhibition test using *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. The IC_{50} value for all compounds is higher than 100 ppm, suggesting a low growth inhibition activity. The most active compounds as growth inhibitors were **2**, **7** and **9**. Interestingly, when the compounds were assayed at 100 ppm against *P. aeruginosa* a decreasing of pyocyanine production was observed. This green pigment, which is reported as a quorum sensing regulated virulence factor (Morkunas et al. 2012), helps bacteria to scavenge iron from the media (Lu et al. 2012).

In summary, the methanolic extract of *Cecropia pachystachya* Trécul and its glycosylflavonoids showed a potent activity as quorum sensing inhibitors, using *C. violaceum* (ATCC 31532) and *Escherichia coli* (pSB403) as biosensors. Additionally, they did not show growth inhibition properties against to the gram positive, the gram negative bacteria tested, as well as against the yeast *Saccharomyces cerevisiae*. Thus, the extract and the compounds have a potential to be used in the development of antipathogenic drugs or for antifouling coatings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2014.01.001.

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