Revista Brasileira de Farmacognosia 25 (2015) 199–207

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Marine organisms as source of extracts to disrupt bacterial communication: bioguided isolation and identification of quorum sensing inhibitors from *Ircinia felix*

Jairo Quintana[a], José Brango-Vanegas[a], Geison M. Costa[a], Leonardo Castellanos[a,*], Catalina Arévalo[a,b], Carmenza Duque[a]

[a] *Grupo de Estudio y Aprovechamiento de Productos Naturales Marinos y Frutas de Colombia, Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Bogotá, Colombia*
[b] *Grupo de Comunicación y Comunidades Bacterianas, Departamento de Biología, Facultad de Ciencias, Universidad Nacional de Colombia, Bogotá, Colombia*

ARTICLE INFO

Article history:
Received 14 November 2014
Accepted 26 March 2015
Available online 29 April 2015

Keywords:
Quorum sensing
Marine products
Ircinia felix
Furanosesterterpenes
Biofilm

ABSTRACT

In this study, 39 extracts from marine organisms were evaluated as quorum sensing inhibitors, collected in the Colombian Caribbean Sea and the Brazilian Coast including 26 sponges, seven soft corals, five algae and one zooanthid. The results showed that crude extracts from the soft coral *Eunicea laciniata*, and the sponges *Svenzea tubulosa*, *Ircinia felix* and *Neopetrosia carbonaria* were the most promising source of quorum sensing inhibitors compounds without affecting bacterial growth, unlike the raw extracts of *Agelas citrina*, *Agelas tubulata*, *Iotrochota arenosa*, *Topsentia ophiraphidites*, *Niphates caycedoi*, *Cliona tenuis*, *Ptilocaulis walpersi*, *Petrosia pellasarca*, and the algae *Laurencia catarinensis* and *Laurencia obtusa*, which displayed potent antibacterial activity against the biosensors employed. The crude extract from the sponge *I. felix* was fractionated, obtaining furanosesterterpenes which were identified and evaluated as quorum sensing inhibitors, showing a moderate activity without affecting the biosensor's growth.

Introduction

Due to the great chemical variability provided by biological diversity existing in the marine environment, research in marine natural products has received great interest (Blunt et al., 2010). Many of these molecules are responsible for stability and adaptability of marine organisms to a changing environment; besides they are involved in the symbiotic relationship between marine macro-organisms and associated microorganisms that has been thoroughly documented. This entire chemical signaling makes this multiorganismal consortium behave as a biochemical and physiological unit fundamental to the equilibrium of the whole ecosystem (Harvell et al., 2007).

Structure and relative density of microbial populations are associated with macro-organisms' change in time and location. This alteration directly influences the organism health as well as the production of bioactive substances. Therefore, multiorganism consortium could regulate settlement of exogenous bacteria using compounds to avoid a biofilm with an undesirable composition (Teplitski and Ritchie, 2009). Coordination between macro-organisms and associated bacteria is done, as well as between bacteria, by means of communication systems regulated by chemical molecules. In bacteria, this phenomenon is known as quorum sensing (QS), and signals [*N*-acyl homoserine lactones (AHL) for Gram negative bacteria] involved in this mechanism are also responsible for inter-kingdom communication (Lowery et al., 2008).

Due to QS systems, bacteria behave in a very different way when they are organized as a complex community because in such a way, they coordinate different phenotypes that provide the community with more resistance and adaptability to the environment, like antibiotic production, sporulation, DNA exchange, expression and secretion of virulence factors, bioluminescence, pigment production, and remarkable biofilm maturation (Ng and Bassler, 2009; Greenberg, 2003; Boyer and Wisniewski-Dyé, 2009).

Bacterial biofilms are sessile communities of differentiated cells associated with a surface, submerged in an extracellular matrix of excreted polysaccharides (also termed exopolysaccharides, EPS), proteins and DNA. Specific structure and composition of biofilms vary with the resident species and environmental conditions (Dickschat, 2010). Biofilm maturation is also controlled by QS that regulates many phenotypes as EPS production, adhesines

* Corresponding author.
E-mail: lcastellanosh@unal.edu.co (L. Castellanos).

http://dx.doi.org/10.1016/j.bjp.2015.03.013

and other fundamental requirements for the consolidation of a strong matrix (Dickschat, 2010). Since biofilms are known to be important cues for settlement of larvae of many marine invertebrates (Dobretsov et al., 2009), the control of biofilm formation on the surface becomes a target for biofouling control. Furthermore, biofilm has been disrupted over unanimated surfaces using quorum sensing inhibitors (QSI), leading to the reduction of macrofouling (Dobretsov et al., 2009).

One of the most promising alternatives for biofilm control is the use of marine natural products, which are extracted from some invertebrate organisms with a macro-fouling free surface that could be an indicator of the production of compounds with settlement deterrence properties (Qian et al., 2007; Meseguer et al., 2004). Following the necessity of antifouling compounds without toxic effects on colonizing species, quorum sensing disruption has become a promising alternative. QSI may act in four different ways: first, inhibiting the signal molecules biosynthesis (*i.e.* AHL); second, inducing degradation of the signal molecules; third, blocking specific bind sites of AHL to LuxR type proteins; and finally, by inhibition of DNA transcription (Dobretsov et al., 2009). A quorum sensing inhibitor suppresses specific genetic expression of bacteria without causing death. Since survivable mechanisms of bacteria are not induced by QSI compound, bacterial resistance is not developed; thus it has also been considered for years by the pharmaceutical industry as a promising strategy for the design and development of antipathogenic compounds, useful in controlling microbial chronic infections, due to the observed microbial development of resistance to antibiotics (Rasmussen and Givskov, 2006). These increasing efforts focused on new alternatives intended to prevent and treat severe infections in order to minimize the total antimicrobial exposure, and includes several QS-modulating therapies such as macrolide antibiotics, QS vaccines, and competitive QS inhibitors that have been proved to be helpful in diminishing the translation of QS-directed toxins or by prematurely activating the QS response to alert the immune system to bacteria hiding in a low cell density (Martin et al., 2008).

This study is aimed to extend knowledge about the Colombian and Brazilian marine biodiversity and to evaluate the QSI activity of marine organisms' extracts, which have not been widely studied around the world and, as far as we are aware, never done before at the South-Eastern Caribbean Sea and South Brazilian Coast. We present evaluations of the QSI activity of 39 marine organisms' extracts from the Colombian Caribbean Sea and the Brazilian Coast (Santa Catarina State), using *Chromobacterium violaceum* ATCC 31532 and *Escherichia coli* pSB401 as biosensors coupled with *Pseudomonas putida* IsoF by disc diffusion assays. Besides, we evaluated a furanosesterterpene enriched-fraction from the active sponge *Ircinia felix*, as an approach to identify some of the possible active compounds.

Materials and methods

Organisms and extract preparation

Organisms were collected by scuba diving in Santa Marta bay and Islas del Rosario at the Colombian Caribbean Sea, and in Santa Catarina State on the Brazilian Atlantic Coast (Table 1; authorization code: DTC-C-33 11/7 of UAESPNN; permission No. 4 of 10/02/2010). Organisms were dried and kept frozen until extraction. Dried organisms were then cut into small pieces and extracted three times with dichloromethane:methanol (1:1); solvent was then evaporated until the crude extracts were obtained, and stored at 4 °C before using. The extracts of *Svenzea tubulosa* and *Neopetrosia carbonaria* were partitioned using Kupchan methodology (Anta et al., 2002) and the fractions soluble in dichloromethane (DCM), butanol (WB) and water (WW) were tested.

Extraction and isolation of furanosesterterpenes from I. felix

The crude extract (35.3 g) of *I. felix*, obtained as described above from 562.2 g of fresh material, was partitioned with a mixture of DCM:H_2O (1:1, v/v), yielding the dichloromethane fraction that was concentrated under reduced pressure. This fraction (4.5 g) was submitted to vacuum column chromatography over silica gel (Merck 60) using a gradient increasing in polarity, starting with 100% benzene to ethyl acetate:methanol (1:1, v/v). Seven fractions (IFF1-IFF7) were yielded and evaluated in the QSI assay. Fraction IFF3 (1.7 g), eluted with benzene:ethyl acetate (7:3, v/v), showed QSI activity and was further chromatographed again in vacuum column chromatography over silica gel (Merck 60); and eluted with hexane:ethyl acetate (7:3, v/v), hexane:ethyl acetate (1:1, v/v) and 100% ethyl acetate, led to nine fractions (Fractions IFF3-1 to IFF3-9). Fraction IFF3-3 (600 mg), eluted with hexane:ethyl acetate (7:3, v/v), containing a mixture of sesterterpenes compounds, showing QSI activity, was acetylated. Briefly, an aliquot (300 mg) of fraction IFF3-3 was submitted to acetylation with 6 ml of a mixture of acetic anhydride:pyridine (1:2, v/v) under agitation for 24 h. The reactions' products were monitored by thin-layer chromatography. The solvent mixture was poured over 10 ml of hydrochloric acid (5%) cold solution and extracted with ethyl acetate. The organic phase was dried with anhydrous sodium sulfate, filtrated and dried under reduced pressure, yielding 332.7 mg of an acetylated furanosesterterpene fraction. This fraction was submitted to high-performance liquid chromatography (HPLC) (for chromatographic conditions, see 'Preparative HPLC separations' section) to yield compounds **1a** and **1b** (2.0 mg), **2a** and **2b** (2.5 mg) and **3** (6.0 mg), identified by ^{1}H and ^{13}C NMR, and by comparison with the literature data (Martínez et al., 1995, 1997).

Fraction I (mixture of the two compounds)

Colorless oil. $[\alpha]_D^{25}$ 23.0 (*c* 0.193, CH_3OH). **1a**. (7*Z*,13*Z*,18*R*,20*Z*)-felixinin acetate. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.35 (1H, m, H-1), 7.21 (1H, s, H-4), 6.27 (1H, s, H-2), 5.15 (1H, t, *J* = 6.7 Hz, H-7), 5.08 (1H, t, *J* = 6.8 Hz, H-15), 5.04 (1H, d, *J* = 10.2 Hz, H-20), 2.82 (1H, m, H-18), 2.43 (2H, t, H-5a and H-5b), 2.35 (3H, s, H-27), 2.22 (2H, c, *J* = 7.5 Hz, H-6a and H-6b), 1.96–2.06 (m, H-16, H-12 and H-10), 1.82 (3H, s, H-25), 1.65 (3H, s, H-14 and H-9), 1.43–1.33 (m, H-17 and H-11), 1.05 (3H, d, *J* = 6.8 Hz, H-19). **1b**. (8*Z*,13*Z*,18*R*,20*Z*)-strobilinin acetate. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.34 (1H, m, H-1), 7.21 (1H, s, H-4), 6.27 (1H, s, H-2), 5.12 (1H, t, *J* = 6.7 Hz, H-10), 5.08 (1H, t, *J* = 6.8 Hz, H-15), 5.04 (1H, d, *J* = 10.2 Hz, H-20), 2.82 (1H, m, H-18), 2.39 (2H, t, H-5a and H-5b), 2.34 (3H, s, H-27), 2.03 (2H, m, H-7), 1.96–2.06 (m, H-16 and H-12), 1.82 (3H, s, H-25), 1.67 (3H, s, H-14 and H-9), 1.62 (m, H-6), 1.43–1.33 (m, H-17 and H-11), 1.05 (3H, d, *J* = 6.8 Hz, H-19).

Fraction II (mixture of the two compounds)

Colorless oil. $[\alpha]_D^{25}$ 23.5 (*c* 0.20, CH_3OH). **2a**. (7*E*,13*Z*,18*R*,20*Z*)-felixinin acetate. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.34 (1H, m, H-1), 7.20 (1H, s, H-4), 6.27 (1H, s, H-2), 5.16 (1H, m, H-7), 5.08 (m, H-15), 5.05 (1H, d, *J* = 10.3 Hz, H-20), 2.82 (1H, m, H-18), 2.44 (2H, t, *J* = 6.5 Hz, H-5a and H-5b), 2.35 (3H, s, H-27), 2.25 (2H, t, *J* = 6.5 Hz, H-6a and H-6b), 1.95–2.09 (m, H-11 and H-10), 1.88–1.95 (m, H-16, H-12), 1.81 (3H, s, H-25), 1.65 (3H, s, H-14), 1.57 (3H, s, H-9), 1.30–1.50 (m, H-17), 1.06 (3H, d, *J* = 6.7 Hz, H-19). **2b**. (8*E*,13*Z*,18*R*,20*Z*)-strobilinin acetate. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.34 (1H, m, H-1), 7.20 (1H, s, H-4), 6.26 (1H, s, H-2), 5.08 (m, H-10), 5.08 (1H, m, H-15 and

Table 1
QSI and antibacterial results of marine extracts from Colombian Caribbean Sea and Brazilian South Coast.

Type of collected sample	Marine organism extract source	Collection number	Collection date	Geographic origin	*Escherichia coli* pSB401				*Chromobacterium violaceum* ATCC 31532					
					Growing inhibition[a]		QS inhibition[a]		Growing inhibition[a]			QS inhibition[a]		
					200 μg	400 μg	200 μg	400 μg	100 μg	200 μg	400 μg	100 μg	200 μg	400 μg
Sponges	*Agelas citrina*	INV-POR 969	05/04/2008	Islas del Rosario (Col)	7–9	7–9	5–7	>12	–	–	–	–	–	7–9
	Agelas tubulata	ICN-MHN(Po) 154	07/12/2005	Santa Marta (Col)	7–9	7–9	–	>12	–	–	*NT*	–	–	*NT*
	Agelas marron	ICN-MHN(Po) 0149	07/12/2005	Islas del Rosario (Col)	–	–	–	–	–	–	–	–	–	–
	Aka cachacrouense	INV-POR-412	07/12/2005	Santa Marta (Col)	–	5–7	–	–	–	–	*NT*	9–12	9–12	*NT*
	Biemna cribaria	INV-POR-890	07/12/2005	Santa Marta (Col)	7–9	5–7	–	7–9	*NT*	–	–	*NT*	–	–
	Cinachyrella kuekenthalli	INV-POR-878	18/11/2009	Santa Marta (Col)	–	–	5–7	5–7	*NT*	*NT*	*NT*	*NT*	*NT*	*NT*
	Cliona delitrix	INV-MHN(Po)-189	18/09/2005	San Andrés (Col)	–	–	–	7–9	*NT*	–	–	*NT*	–	–
	Cliona tenuis	INV-POR-669	06/04/2008	Islas del Rosario (Col)	–	7–9	–	–	9–12	9–12	*NT*	–	–	*NT*
	Cliona varians	INV-POR-339	07/09/2005	Santa Marta (Col)	–	–	–	–	*NT*	–	–	*NT*	–	–
	Iotrochota arenosa (*Iotrochota inminuta*)[b]	INV-POR-883	15/11/2006	Santa Marta (Col)	>12	–	–	7–9	–	–	–	–	–	–
	Ircinia felix	INV-POR-014	18/11/2009	Santa Marta (Col)	*NT*	*NT*	*NT*	*NT*	–	–	–	7–9	7–9	7–9
	Lissodendoryx colombiensis	ICN-MHN-PO 0246	06/04/2008	Islas del Rosario (Col)	–	–	–	–	*NT*	–	–	*NT*	–	–
	Muriceopsis sp.	ICN-MHN-PO 0254	13/02/2006	Santa Marta (Col)	–	5–7	–	–	–	–	*NT*	-	–	*NT*
	Neopetrosia carbonaria (DCM) (*Xestospongia carbonaria*)[b]	ICN-MHN-PO 0244	05/04/2008	Islas del Rosario (Col)	–	–	–	7–9	–	–	–	>12	–	9–12
	Neopetrosia carbonaria (WB)	ICN-MHN-PO 0244	05/04/2008	Islas del Rosario (Col)	–	–	7–9	7–9	–	–	–	–	–	>12
	Neopetrosia carbonaria (WW)	ICN-MHN-PO 0244	05/04/2008	Islas del Rosario (Col)	–	–	–	–	*NT*	–	–	*NT*	–	–
	Neopetrosia muta (*Xestospongia muta*)[b]	ICN-MHN-PO 0247	06/04/2008	Islas del Rosario (Col)	–	–	–	–	*NT*	–	–	*NT*	–	–
	Neopetrosia proxima (*Xestospongia proxima*)[b]	ICN-MHN-PO 0239	04/04/2008	Islas del Rosario (Col)	–	–	–	–	*NT*	–	–	*NT*	–	–
	Neopetrosia rosariensis (*Xestospongia rosariensis*)[b]	ICN-MHN-PO 0245	05/04/2008	Islas del Rosario (Col)	–	–	–	-	*NT*	–	–	*NT*	–	–
	Neopetrosia subtriangularis (*Xestospongia subtriangularis*)[b]	ICN-MHN-PO 0242	05/04/2008	Islas del Rosario (Col)	–	–	–	–	–	–	–	>12	7–9	7–9
	Ptilocaulis walpersi	ICN-MHN-PO 0248	06/04/2008	Islas del Rosario (Col)	–	–	–	5–7	*NT*	9–12	9–12	*NT*	–	–
	Svenzea tubulosa (crude extract)	ICN-MHN-PO 0249	06/04/2008	Islas del Rosario (Col)	–	–	9–12	>12	–	–	–	–	>12	9–12
	Svenzea tubulosa (DCM)	ICN-MHN-PO 0249	06/04/2008	Islas del Rosario (Col)	*NT*	*NT*	*NT*	*NT*	–	–	–	7–9	9–12	9–12
	Topsentia ophiraphidites	ICN-MHN-PO 0243	05/04/2008	Islas del Rosario (Col)	–	9–12	–	5–7	–	–	–	–	–	–

Table 1 (*Continued*)

Type of collected sample	Marine organism extract source	Collection number	Collection date	Geographic origin	*Escherichia coli* pSB401				*Chromobacterium violaceum* ATCC 31532					
					Growing inhibition[a]		QS inhibition[a]		Growing inhibition[a]			QS inhibition[a]		
					200 μg	400 μg	200 μg	400 μg	100 μg	200 μg	400 μg	100 μg	200 μg	400 μg
Soft corals	*Eunicea laciniata*	ICN-MHN-CR-106	13/02/2006	Santa Marta (Col)	–	–	7–9	7–9	*NT*	*NT*	*NT*	*NT*	*NT*	*NT*
	Eunicea succinea	ICN-MHN-PO 0251	13/02/2006	Santa Marta (Col)	–	–	–	5–7	*NT*	–	–	*NT*	–	–
	Eunicea fusca	ICN-MHN-PO 0252	13/02/2006	Santa Marta (Col)	–	–	–	5–7	*NT*	–	–	*NT*	–	–
	Eunicea sp1	–	13/02/2006	Santa Marta (Col)	5–7	5–7	–	–	*NT*	–	–	*NT*	–	–
	Erythropodium caribaeorum	INV-CNI-1193	07/09/2005	Santa Marta (Col)	–	–	–	–	–	–	–	–	–	9–12
	Pseudopterogorgia elisabethae (Providencia)	INV-CNI-1612–1614	25/05/2008	Providencia and San Andrés (Col)	5–7	–	–	–	–	–	*NT*	–	–	*NT*
	Pseudopterogorgia elisabethae (San Andrés)	INV-CNI-1615-1616	18/09/2005	Providencia and San Andrés (Col)	–	–	–	9–12	*NT*	–	–	*NT*	–	–
Zoanthid	*Palythoa caribaeorum*	ICN-MHN-PO 0255	07/09/2005	Santa Marta (Col)	–	–	–	–	–	–	–	–	9–12	–
Algae	*Laurencia catarinensis*	FLOR 14516	02/2008	Ilha do Arvoredo, Florianópolis (Bra)	–	–	–	–	7–9	9–12	9–12	–	–	–
	Laurencia flagellifera	FLOR 14521	03/2006	Praia da Sepultura, Bombinhas (Bra)	–	–	–	–	–	–	–	–	–	–
	Laurencia majuscule	FLOR 14524	11/2006	Ilha do Francês, Florianópolis (Bra)	–	–	–	–	–	7–9	–	–	–	–
	Laurencia microcladia	FLOR 14520	03/2008	Praia da Sepultura, Bombinhas (Bra)	–	–	–	–	–	–	–	–	–	–
	Laurencia obtusa	FLOR 14512	08/2009	Praia de Canasvieiras, Florianópolis (Bra)	–	–	–	–	7–9	9–12	>12	–	–	–

[a] halo, in mm.

[b] Formerly known as: –, no activity halo, <5 mm; *NT*, no tested; DCM, dichloromethane fraction; WB, butanol fraction; WW, aqueous fraction; Col, Colombia; Bra, Brasil.

H-10), 5.04 (1H, d, J = 10.3 Hz, H-20), 2.82 (1H, m, H-18), 2.37 (2H, t, H-5a and H-5b), 2.34 (3H, s, H-27), 1.95–2.09 (m, H-16, H-12, H-7), 1.81 (3H, s, H-25), 1.67 (3H, s, H-14), 1.58 (3H, s, H-9), 1.64 (m, H-6), 1.30–1.50 (m, H-17 and H-11), 1.05 (3H, d, J = 6.7 Hz, H-19).

*Fraction III (**3**)*

(7*E*,12*E*,18*R*,20*Z*)-variabilin acetate, colorless oil. $[\alpha]_D^{25}$ 26.1 (*c* 0.167, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.33 (1H, s, H-1), 7.20 (1H, s, H-4), 6.28 (1H, s, H-2), 5.16 (1H, t, J = 6.9 Hz, H-7), 5.07 (1H, t, J = 6.9 Hz, H-12), 5.04 (1H, d, J = 10.2 Hz, H-20), 2.81 (1H, m, H-18), 2.44 (2H, t, J = 7.4 Hz, H-5a and H-5b), 2.35 (3H, s, H-27), 2.23 (2H, c, J = 14.7 and 7.4 Hz, H-6a and H-6b), 2.05 (2H, m, J = 8.0 and 7.6 Hz, H-11a and H-11b), 1.98 (2H, t, J = 7.8 Hz, H-10a and H-10b), 1.94 (2H, t, J = 7.0 Hz, H-15a and H-15b), 1.82 (3H, s, H-25), 1.58 (3H, s, H-9), 1.55 (3H, s, H-14), 1.34(4H, m, H-16), 1.34 (4H, m, H-17), 1.05 (3H, d, J = 6.7 Hz, H-19). RMN ^{13}C (100 MHz, $CDCl_3$) δ (ppm): 142.5 (CH, C1), 138.8 (CH, C4), 124.4 (CH, C12), 123.7 (CH, C7), 111.1 (CH, C2), 39.7 (CH_2, C10), 39.5 (CH_2, C15), 36.6 (CH_2, C17), 31.1 (CH, C18), 28.4 (CH_2, C6), 26.6 (CH_2, C11), 25.7 (CH_2, C16), 25.0 (CH_2, C5), 20.6 (CH_3, C27), 20.5 (CH_3, C19), 16.1 (CH_3, C9), 15.8 (CH_3, C14), 116.7 (CH, C20), 8.4 (CH_3, C26).

Preparative HPLC separations

HPLC separations were carried out on a Merck-Hitachi 6000 system equipped with a L-6000A binary pump, Rheodyne injector and a L-4250 UV–Vis detector. The stationary phase employed was Scharlau Science Nucleosil 120 C-18 column, (300 mm × 8 mm; i.d.; 10 μm), using isocratic MeOH:H_2O (95:5) as mobile phase, with a flow rate at 2 ml/min, and detection at 310 nm.

Bacterial strains and culture conditions

Two different biosensors were used to evaluate QSI activity: *C. violaceum* ATCC 31532 and *E. coli* pSB401 coupled with *P. putida* isoF WT as the exogenous source of AHL to induce bioluminescence production. *E. coli* and *P. putida* isoF WT were cultured in Luria–Bertani broth at 37 °C, and those strains were kindly provided by Prof. Dr. Kathrin Riedel (Department of Microbiology, Institute of Plant Biology, University of Zürich). *C. violaceum* was cultured in CASO Agar® (Merck, Germany) at room temperature.

QSI assays

C. violaceum ATCC 31532 was inoculated overnight at room temperature in CASO broth and 100 μl of bacteria were spread on agar plates. In contrast, *E. coli* pSB401 and *P. putida* isoF WT were inoculated separately at 37 °C overnight to get a cellular density equal to 0.5 MacFarland standard. An equal amount of LB medium with agar at 1.6% was prepared and mixed with bacteria cultures with the intention of preparing 0.8% agar plates, already inoculated with both, the biosensor and the AHL producer strain. Sterile paper discs (5 mm diameter) were loaded with the appropriate volume of crude extracts solutions, dissolved in methanol, in order to obtain 100, 200 and 400 μg per disc. The solvent was left to evaporate. Methanol was used as blank control and methanolic fraction from *Cecropia pachystachya* was used as positive control. Discs were placed on plates previously inoculated with the appropriate biosensor and were incubated for 24 h at 37 °C for the assay with *E. coli* pSB401 and *P. putida* isoF WT; and at 28 °C for *C. violaceum* ATCC 31532 assay. A QSI positive result was the inhibition of phenotype expression (bioluminescence or violacein, respectively) without affecting the growing process of bacteria. The presence of a growth inhibition zone around the discs was taken as antibacterial activity. This procedure was made by a triplicate. The inhibition of quorum sensing in *C. violaceum* was measured by visual analysis of violacein production, and for the bioassay with *E. coli* pSB401 and *P. putida* isoF WT the bioluminescence inhibition was registered by autoradiography.

Results and discussion

Many different studies have demonstrated the ability of natural compounds to block QS systems. Ornamental, medicinal and edible plants have been the most studied sources of QSI compounds (Dickschat, 2010; Dobretsov et al., 2009); however, only a few examples of systematic screening studies have been documented using raw extracts from marine sources. An investigation based on 78 extracts of 25 species of marine organisms collected from Florida waters showed that cyanobacteria have high QS inhibitory potential (Dobretsov et al., 2011). Another study used marine crude extracts of organisms from the Great Barrier Reef in Australia. The authors identified in the sponge *Luffariella variabilis* the manoalide as responsible for the QSI activity of the raw extract. This compound is known as an anti-inflammatory agent, and its structure has a lactonic ring which resembles the structure of AHL, and has been found to be an active QSI compound in the fusion system *lasB::gfp* (ASV) of *Pseudomonas aeruginosa* (Skindersoe et al., 2008). Some other compounds have been identified as QSI from marine sources, and the most representative QSI compounds are the halogenated furanones, isolated from the red alga *Delisea pulchra*, which have been demonstrated to be one of the most promising QSI compounds and potent antifouling agents (Boyer and Wisniewski-Dyé, 2009). On the other hand, three compounds (floridoside, betonicine and isethionic acid) isolated from the red alga *Ahnfeltiopsis flabelliformis* showed promising QSI activity with the *Agrobacterium tumefaciens* NTL4 QS system (Kim et al., 2007). Another example is the brominated triptamine-like alkaloids that were found in bryozoan *Flustra foliacea* and were tested using the *P. putida* (pKR-C12), *P. putida* (pAS-C8) and *E. coli* (pSB403) QS systems as biosensors (Peters et al., 2003).

Extracts tested in the present study were obtained from marine organisms selected specially because of their clean surface; such organisms have microorganism communities in the surfaces which may have an ecological control that avoid settlement of macroorganisms. This control can be done by chemical compounds produced by the marine organism (the host) or by associated microorganisms as bacteria, or by both, the holobiont. As previously mentioned, in this ecological control, QS plays a major role due to regulation of biofilm maturation, causing a selective settlement of bacteria, larvae or other eukaryotic microorganisms (Boyer and Wisniewski-Dyé, 2009).

Disc diffusion assays were used to test QSI and antibacterial activity of 39 marine organisms extracts. Table 1 shows the results using different concentrations of extracts with both biosensors. A promising extract as source of QSI compounds must show a QS inhibition without affecting the bacterial growing process to avoid later development of bacterial resistance.

Using *E. coli* pSB401 coupled with *P. putida* as a biosensor, fifteen of the marine extracts (40.5%) gave a positive result inhibiting the quorum sensing system regulated by *luxI*/*luxR*, at one or both concentrations. Soft corals extracts were the most active, which proportionally contributed to the highest number of active extracts (4 out of 7 were QSI active), 11 of 25 sponges extracts resulted active as QSI, and *Laurencia* marine algal extracts did not present QSI activity. Fig. 1 shows the results of two of the most active raw extracts in this assay, the raw extracts of octocoral *Eunicea laciniata*, and the sponge *S. tubulosa* additionally to the raw extracts of octocoral *Pseudopterogorgia elisabethae* chemotype 2 (San Andres Island – Colombian Caribbean Sea), and the butanol fraction (WB)

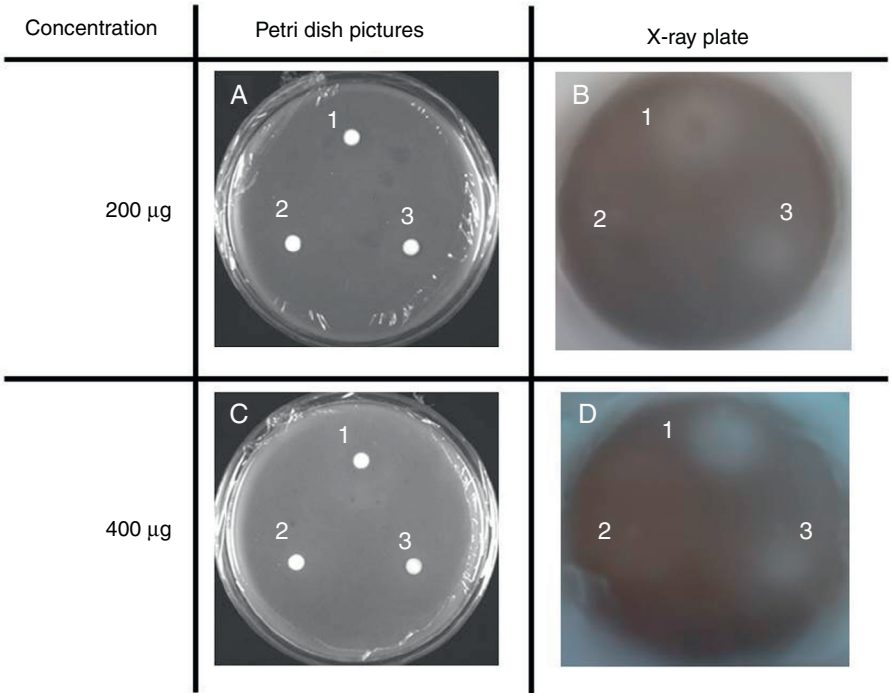


Fig. 1. Disc diffusion assay showing inhibition of bioluminescence of *Escherichia coli* pSB401 coupled with *P. putida* IsoF by marine extracts. A and C are pictures of Petri dishes and sensi-discs. B and D are the X-ray plaques of these petri dishes. 1: *Svenzea tubulosa* (crude extract), 2: *Petrosia pellasarca* (crude extract) and 3: *Eunicea laciniata* (crude extract). The clear zones in autoradiography (B and D) and the absence of growth inhibition zones (A and C) show QS inhibitory activity of the extracts.

of sponge *N. carbonaria*. A mild QSI activity was shown by crude extracts from the sponges *Cliona delitrix*, dichloromethane fraction of *N. carbonaria* and a low activity was shown by crude extracts from the octocorals *Eunicea succinea*, *Eunicea fusca* and the sponge *Ptilocaulis walpersi*.

When *C. violaceum* ATCC 31532 was used as a biosensor, nine (23%) of the 39 extracts resulted active. Of these, seven corresponded to sponges, one to an octocoral, and the other to a zoanthid. These results are in contrast with the ones obtained by using the *E. coli* biosensor because with *C. violaceum* the most active extract belonged to sponges, 27% of the extracts were active (7 of 26) and so were just 14.3% of the octocoral extracts (1 of 7). Likewise, algae extracts showed no activity with this biosensor. Fig. 2 shows the results for the raw extracts of the sponges *S. tubulosa* and *I. felix* in the assay with *C. violaceum* which, in conjunction with the crude extracts from sponges *Neopetrosia subtriangularis* and *Aka cachacrouense*, and as well as extracts from octocoral *Erythropodium caribaeorum*, dichloromethane extracts of *S. tubulosa* and *N. carbonaria*, and butanol fraction of *N. carbonaria*, showed high bacterial communication disruption (QSI) activity. Extracts from the sponge *E. caribaeorum* and the zoanthid *Palythoa caribaeorum* showed moderate activity.

With these tests we could also see some extracts with antibacterial activities against three Gram negative bacteria: *E. coli*, *P. putida* and *C. violaceum*. Extracts from the sponges *Agelas citrina*, *Agelas tubulata*, *Iotrochota arenosa* and *Topsentia ophiraphidites* were the most potent antibacterial against *E. coli* and *P. putida* IsoF, while extracts from the sponges *Niphates caycedoi*, *Cliona tenuis*, *P. walpersi*, *Petrosia pellasarca* and the algae *Laurencia catarinensis* and *Laurencia obtusa* showed high antibacterial activity against *C. violaceum* ATCC 31532. These results could be explained due to the fact that the sponges belonging to the genus *Agelas* are recognized as a source of bromopyrrolidinic alkaloids (Braekman et al., 1992) with an important ecological key role as fish feeding deterrents (Assmann et al., 2000). It has also been found that such compounds possess interesting antiviral (Keifer et al., 1991), antihistaminic (Cafieri et al., 1997) and antimicrobial activities (Shen et al., 1998). The sponges of the genus *Iotrochota* are known for producing brominated alkaloids (Carletti et al., 2000) with mild antibacterial activity, and sulfated pyrrole alkaloids with promising anti-HIV-1 activity (Fan et al., 2010). The sponges of the genus *Topsentia* are recognized as source of sulfated steroids with a widespread range of bioactivities, for example, ophirapstanol trisulfate – a sulfate steroid isolated from *T. ophiraphidites* which has shown activity as an inhibitor in the guanosine diphosphate/G-protein RAS exchange assay (GDPX), which is directly related with anticancer activity (Gunasekera et al., 1994). The sponges of the genus *Ptilocaulis* have been reported by producing guanidine type alkaloids with antimicrobial activity, among others. In 2003, a cyclic guanidine, with antibacterial activity against *Staphylococcus aureus*, was identified from the sponge *Ptilocaulis spiculifer* (Yang et al., 2003). Algae of the genus *Laurencia* have shown sesquiterpenes, diterpenes, triterpenes and acetogenins that usually have one or more halogen atoms. These types of compounds were recognized for various activities, such as cytotoxic and antibacterial (Lhullie et al., 2010).

Those results are in agreement with a previous work done in our laboratory, where many of the extracts used in this study were tested against a collection of twelve marine bacteria strains, isolated from colonized surfaces (Mora-Cristancho et al., 2011). Additionally, for many of these marine strains, presence of QS systems by identification of its AHL production was demonstrated (Cuadrado, 2010). Therefore, it could be a challenge to evaluate the properties of these QSI active extracts in these marine bacteria involved in the biofouling process of QS systems.

The results of the QSI test with both biosensors employed showed that crude extracts of the sponges *S. tubulosa*, *I. felix*, and the butanol fraction of *N. carbonaria* are the most promising as a source of QSI active compounds. Indeed, these extracts did not show antibacterial activity but they inhibit QS system in both biosensors. These results could be an indicator of a safe use as antifoulants. These organisms could also represent a promising source of molecules that could act as antipathogenic compounds

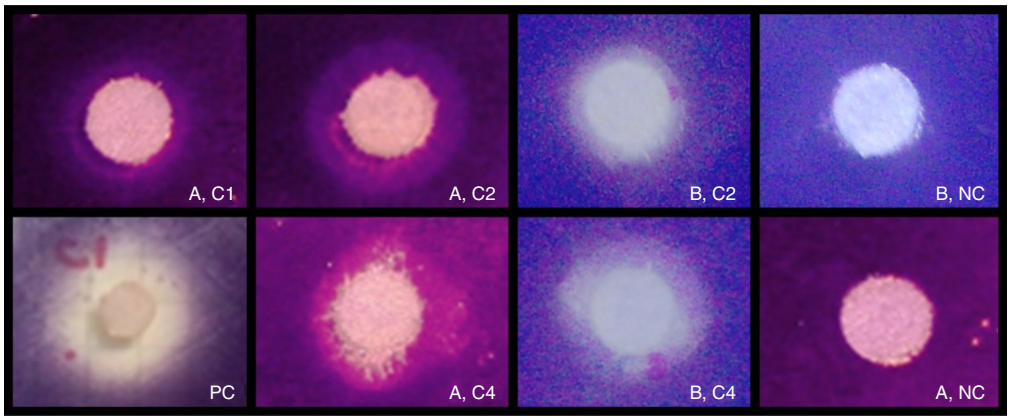
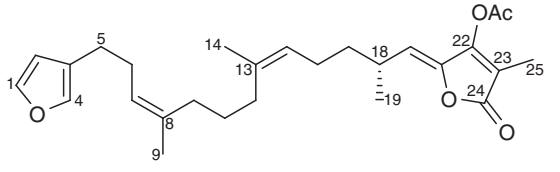


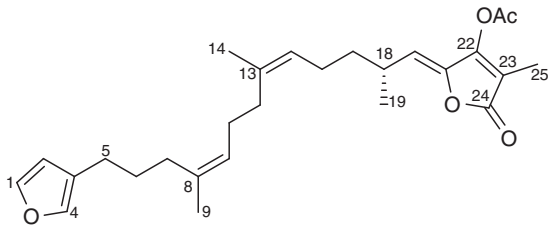
Fig. 2. Disc diffusion assay showing inhibition of violaceina production in *C. violaceum* ATCC 31532. The picture (A) is dichloromethane fraction from *Svenzea tubulosa* and (B) is crude extract from *Ircinia felix*. C1 = 100 μg, C2 = 200 μg, C3 = 300 μg, and C4 = 400 μg per disco. Positive control, PC = 100 μg of methanol fraction from *Cecropia pachystachya*; negative control, NC = 20 μl of methanol with prior evaporation before to placing. The clear zone surrounding the paper disc shows QS inhibitory activity of the extracts.

able to combat against antibiotic resistant bacterial strains that represent a serious threat to human health.

In this sense, bioguided isolation was conducted with the crude extract of *I. felix*. Throughout different chromatographic steps, we obtained a furanosesterterpenes-enriched fraction (IFF3-3) that presented a moderate QSI activity. Due to the known instability of these compounds, this fraction was acetylated and submitted to a preparative HPLC to yield three sub-fractions (Fraction I–III). Fraction I showed a mixture of (7*Z*,13*Z*,18*R*,20*Z*)-felixinin acetate (**1a**) and (8*Z*,13*Z*,18*R*,20*Z*)-strobilinin acetate (**1b**). Very similarly, fraction II also presented a mixture of these furanosesterterpenes, but with a distinct configuration: (7*E*,13*Z*,18*R*,20*Z*)-felixinin acetate (**2a**) and (8*E*,13*Z*,18*R*,20*Z*)-strobilinin acetate (**2b**). In fraction III, the compound (7*E*,12*E*,18*R*,20*Z*)-variabilin acetate (**3**) was isolated. All these five compounds, which present similar structure to AHL (Dobretsov et al., 2009), have already been reported for *I. felix* (Martínez et al., 1995, 1997). Besides, properties such as analgesic, antimicrobial, cytotoxic and anti-tumor of this furanosesterterpenes are well documented (Martínez et al., 1997).



1a



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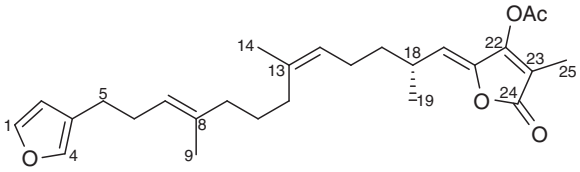
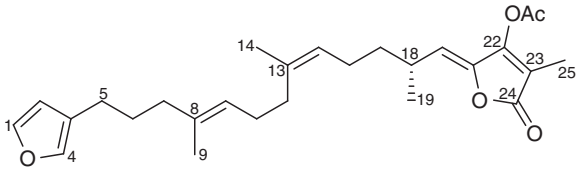


2a



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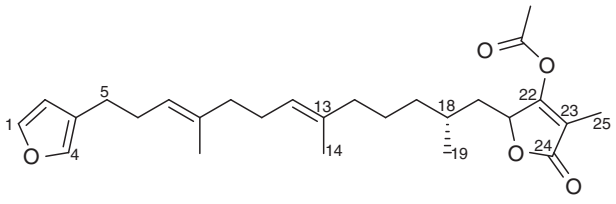

3

The other two promising QSI sponges, *S. tubulosa*, formerly known as *Pseudoaxinella tubulosa*, have no reports about its chemistry, but the sponges of this genus are known as source of long chain fatty acids, steroidal, and peptide compounds, some of them with antibacterial activities (Kong et al., 1992; Sjöstrand et al., 1981). In our preliminary QSI bioassay-guided fractionation of this sponge, we found that organic fractions are responsible for the activity, and these fractions are rich in epidioxysterols and lipids, which could be the QS inhibitors in the crude extract (unpublished results) considering that, for some fatty acids, QSI activity is reported (Osorno et al., 2012). The genus *Neopetrosia* is known as a source of quinolinic type alkaloids and other nitrogen compounds that exhibit cytotoxic, antimicrobial activity, among others (Nakao et al., 2004; De Almeida et al., 2008). Particularly from *N. carbonaria*, previously known as *Xestospongia carbonaria*, it was possible to isolate acridine type polar alkaloids (Thale et al., 2002) with recognized anticancer activity. In a previous study, these alkaloids were present in the butanolic fraction which, in the present study, is also active disrupting QS biosensor systems. Consequently, it could be assumed that these alkaloids are responsible for the QSI activity in our extract, but it has not been demonstrated yet.

In conclusion, we were able to identify the extracts of the sponges *N. carbonaria*, *S. tubulosa* and *I. felix* as the most promising Quorum sensing inhibitors disrupting the expression of both biosensors' phenotypes without affecting their growing process. Different from these extracts, the extracts of the sponges *A. citrina*, *A. tubulata*, *I. arenosa*, and *T. ophiraphidites* showed antibacterial activity against *E. coli* and *P. putida*, and the extracts of the sponges *N. caycedoi*, *C. tenuis*, *Ptilocaulis wallpersi*, *P. pellasarca* and the algae *L. catarinensis* and *Laurencia obtuse* were highly antibacterial against *C. violaceum*. From the sponge *I. felix*, a furanosesterterpenes enriched-fraction showed a moderate QSI activity without affecting the biosensor's growth. In general, these furanosesterterpenes are structurally related with AHL and other QSI, which could explain the observed activity against *C. violaceum*, additionally possessing a similar mechanism of action. Notwithstanding, other studies are required to improve the real potential of these compounds as QSI exploitable by man.

Authors' contributions

JQ and JBV (PhD students) contributed by running the laboratory work, biological studies, analysis of the data and drafting the paper. GMC (Postdoctoral researcher) contributed to chromatographic procedures and to critical reading of the manuscript. CA contributed by collecting samples, identification, and biological studies. LC and CD designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was conducted as part of a joint project financially supported by CNPq/MCT/Brazil (grant number 490151/2007-8) and COLCIENCIAS/Colombia (grant number 358-2007). The work was also financed by other grants from Colciencias and "Fundación para la Promoción de la Investigación y la Tecnología del Banco de la República". The Colombian authors greatly acknowledge Prof. Dr. Kathrin Riedel (Department of Microbiology, Institute of Plant Biology, University of Zürich) and Prof. Dr. Sven Zea from the Universidad Nacional de Colombia for their interest and assistance in this work, and Prof. Dr. Paulo Horta, Dr. Cintia Llhulier and Prof. Dr. Eloir P. Schenkel for the collection and identification of the algae.

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