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Original Article

Biofilm inhibition activity of compounds isolated from two *Eunicea* species collected at the Caribbean Sea

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ABSTRACT

Biofilm has a primary role in the pathogenesis of diseases and in the attachment of multicellular organisms to a fouled surface. Because of that, the control of bacterial biofilms has been identified as an important target. In the present study, five lipid compounds isolated from soft coral *Eunicea* sp. and three terpenoids together with a mixture of sterols from *Eunicea fusca* collected at the Colombian Caribbean Sea showed different effectiveness against biofilm formation by three marine bacteria associated with immersed fouled surfaces, *Ochrobactrum pseudogringnonense*, *Alteromona macleodii* and *Vibrio harveyi*, and against two known biofilm forming bacteria, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. The pure compounds were characterized by NMR, HRESI-MS, HRGC-MS and optical rotation. The most effective compounds were batyl alcohol (**1**) and fuscoside E peracetate (**6**), acting against four strains without affecting their microbial growth. Compound **1** showed biofilm inhibition greater than 30% against *A. macleodii*, and up to 60% against *O. pseudogringnonense*, *V. harveyi* and *S. aureus*. Compound **6** inhibited *O. pseudogringnonense* and *V. harveyi* between 25 and 50%, and *P. aeruginosa* or *S. aureus* up to 60% at 0.5 mg/ml. The results suggest that these compounds exhibit specific biofilm inhibition with lower antimicrobial effect against the bacterial species assayed.

Introduction

All immersed surfaces are subject to colonization by microorganisms as bacteria, microalgae and then macroorganisms such as invertebrates, in a complex layer called biofouling (Viano et al., 2009). Bacteria are the first colonizers and their biofilm formation takes place in multiple stages (Jadhav et al., 2013). Biofilms are abundant in nature and are developed when planktonic bacteria adhere to a surface and initiate the formation of a microcolony that exists as a community encased in an extracellular matrix that confers a high degree of protection (O'toole et al., 2000; Huigens et al., 2007).

In marine environments, biofilms are formed by bacteria, diatoms and protozoa (Viano et al., 2009; Dobretsov et al., 2013). Bacterial biofilms are known for being implicated in the regulation of a subsequent settlement by invertebrate larvae, mediate larval metamorphosis, provide suitable food source to newly metamorphosed juveniles, and serve as biofilters to absorb and biodegrade excessive nutrients (Yang et al., 2014). On the other hand, biofilms are prevalent in medical, dental, industrial and marine environmental settings, where they are undesirable due to their pathogenicity, and resistance toward antimicrobial agents or anti-biofouling technologies (Dusane et al., 2011; Raut et al., 2013). In this sense, inhibition of biofilms may play an important role in biofouling prevention (Quinn et al., 2012; Dobretsov et al., 2013).

Although a diversity of strategies has been developed to control biofouling, the search for novel and effective technologies continues (Dusane et al., 2011). One of the most ecologically relevant antifouling strategies is to apply the chemical defenses of sessile

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marine organisms that keep their body surfaces free of fouling (Jadhav et al., 2013; Majik and Parvatkar, 2014). Those antifouling compounds could be isolated from sponges, algae, bryozoans, and corals, which produce such compounds presumably as a means of protection from predation or fouling or to reduce competition for space (Mora-Cristancho et al., 2011). The use of these marine natural products is more favored than commonly used biocides because of their biocompatibility, stability, biodegradability and low toxicity under different environmental conditions (Qi et al., 2008; Dusane et al., 2011; Tello et al., 2011). Therefore, continuous searches for antifouling compounds from marine invertebrates of the Colombian Caribbean Sea have been reported (Mayorga et al., 2011; Tello et al., 2011, 2012; Cuadrado et al., 2013). However, an anti-biofilm possibility of the compounds assayed in the present investigation has not been studied before.

The aim of the present study was to evaluate the anti-biofilm activity of lipids and terpenoid compounds isolated from *Eunicea* sp. and *Eunicea fusca* Duchassaing and Michelotti (phylum Cnidaria, class Anthozoa, subclass Octocorallia, order Alcyonacea, family Plexauridae), collected at Santa Marta Bay (Colombia), against three marine bacteria and two known biofilm forming bacteria by the microtiter plate method.

Materials and methods

General experimental procedures

For high-resolution electrospray mass spectrometry (HRESIMS), a LC-MS-IT-TOF was used on positive mode, and for ESIMS a LCMS-2010-ESI (Shimadzu, Tokyo, Japan) was likewise used. Optical rotation was measured on a Polax-2L (Atago, Japan) and an ADP440 (Bellingham + Stanley, USA). The NMR spectra at 400 MHz for ^{1}H and 100 MHz for ^{13}C and DEPT in $CDCl_3$ were recorded on a Bruker Avance 400 spectrometer, with TMS. High performance liquid chromatography HPLC was performed on a Merck-Hitachi (L-4250 UV/Vis detector, USA) at 210 nm, and on a Luna C18 column 250 mm × 4.60 mm i.d. × 5 μm (Phenomenex). For gas chromatography mass spectrometry HRGCMS, Agilent equipment model 7890A-5975C with a DB-1 (30 m × 250 μm × 0.25 μm) capillary column was employed. All solvents used were analytical grade.

Coral material

Eunicea sp. and *Eunicea fusca* Duchassaing and Michelotti, samples were collected by hand at Santa Marta Bay (Latitude 11.25 N, Longitude 74.2 W) in 2007, through scuba diving at 6–14 m deep by Prof. Dr. S. Zea, and voucher specimens were deposited at the Instituto de Ciencias Naturales de la Universidad Nacional de Colombia coded as ICN-MHN-PO No. 250 and No. 252 respectively. The collection of fresh coral colonies was superficially air-dried in the shade, immediately frozen and was then transported by airplane to laboratory in Bogota, where was kept in freezer until extraction.

Extraction and isolation of compounds

The soft coral *Eunicea* sp. was cut into small pieces and was partially air-dried (wet weight 850 g, 600 g dry weight), extracted three times with dichloromethane:methanol (1:1) at room temperature. The combined extracts were concentrated by rotatory evaporation, and the crude extract (25 g) was partitioned between dichloromethane:water (1:1). The organic fraction (15 g) was subjected to silica gel, column chromatography (CC) with solvents of increasing polarity hexane, benzene, ethyl acetate and methanol, to obtain fifteen fractions (F1–F15) in a bioguided manner using an antimicrobial test with several marine bacteria isolated from heavy colonized surfaces by Mora-Cristancho et al. (2011). The bioactive fractions were further fractionated over SiO_2 CC as follows. F4 (0.3 g), eluted with hexane-benzene (1:1), was chromatographed using an elution gradient with hexane:benzene (10:0–0:10) to give compound **3** (60 mg). Fraction F7 (0.8 g), eluted with benzene:ethyl acetate (9:1), was subjected to CC with benzene-ethyl acetate (10:0–2:8) and subfraction F7.1 was purified by HPLC using methanol to obtain compounds **4** (5 mg) and **5** (7 mg). Fraction F10 (1.1 g), eluted with benzene:ethyl acetate (2:8), was separated on CC using benzene-ethyl acetate (5:5–0:10). Subsequently, subfraction F10.6, eluted with benzene:ethyl acetate (4:6), contains compound **1** (150 mg) and subfraction F10.9, was purified by reverse-phase CC (Lichroprep RP-18, Merck) and finally by HPLC to afford compound **2** (9 mg).

From *E. fusca*, the following products were previously isolated: fuscoside E, fuscoside B (**7**), (+)-germacrene D (**8**), the mixture of six sterols (**9**–**14**), and the compound fuscoside E peracetate (**6**) was semi-synthesized (Reina et al., 2011).

Compounds identification and fatty acid analysis

To determine the structure of compounds from *Eunicea* sp., optical rotation, NMR, ESIMS and HRESIMS data were recorded. Compounds **2**, **4** and **5** were subjected to methanolysis as previously reported (Ramos et al., 2006), and the mixture of fatty acid methyl esters was analyzed by HRGCMS as described earlier (Castellanos et al., 2010).

Bacterial strains

Two reference gram-positive strains, *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 51400310) and two gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (206), commonly reported as biofilms forming strains, were used (Quinn et al., 2012). They were supplied by the molecular epidemiology laboratory of the Biotechnology Institute (Universidad Nacional de Colombia). Wild type marine bacteria associated with fouled surfaces were also used; these gram-positive strains: *Kokuria* sp. (10-4DEP), *Bacillus megaterium* (16-Ains5) and *Oceanobacillus iheyensis* (31-C), and these gram-negative strains: *Vibrio campbellii* (6-8PIN), *Ochrobactrum pseudogringnonense* (4-4DEP), *Alteromona macleodii* (29-C) and *Vibrio harveyi* (Phy-2A), as was reported for Mora-Cristancho et al. (2011).

Microtiter plate biofilm production assay

In order to select the most suitable reference bacteria as control for the assay with marine strains, the biofilm formation was tested by their ability to adhere to wells of polystyrene (353072-BD Falcon™ Clear 96-well) using a modification of protocol by O'toole et al. (2000). Pre-inoculums were grown in trypticase soy broth (TSB) to an optical density at 600 nm (OD_{600}) of 0.2–0.3. From each cultured broth, 20 μl was inoculated to the wells and incubated for 24 h and 48 h at 37 °C. After removal of the planktonic cells by inversion, the wells were rinsed repeatedly with distilled water. Crystal violet (CV) solution at 1% (w/v) was added to each well for 5 min to stain the adherent bacteria, and discarded by inversion. The wells were rinsed twice and destined with ethanol-acetone 8:2 (v/v). Ten minutes later, the differential staining absorbance was quantified at 621 nm using a plate reader, Sensident Scan (Merck, Darmstadt, Germany). The absorbance of CV bound to uninoculated wells was used as negative control, and microbial growth was monitored. Additionally, due to established growth conditions of the marine strains, inoculation with an OD_{600} of 0.1–0.2 and incubation at 25 °C (Mora-Cristancho et al., 2011), the reference strains had to be tested

in order to establish whether the change from 37 °C to 25 °C had affected their biofilm formation, for the subsequent assay under those conditions with marine strains.

Biofilm inhibition activity assay

Three marine strains: *O. pseudogringnonense*, *A. macleodii* and *V. harveyi*, and two reference strains: *P. aeruginosa* and *S. aureus*, selected because of their strong biofilm-forming ability confirmed in this study, were used in the assay of compounds (**1**–**14**), as previously reported (Tello et al., 2011), and following the biofilm production assay described above. Pre-inoculums were grown to an OD_{600} of 0.1–0.2 in marine broth (MB) and TSB respectively. Twenty μl of inoculated culture were mixed with two different aliquot, 100 μg/well or 5 μg/well of pure compound completed with medium to concentrations at 0.5 mg/ml or 0.025 mg/ml, and incubated for 48 h at 25 °C. Planktonic cells were discarded, and finally the differential staining was quantified using a CV assay. The values are expressed in terms of the percentage of biofilm inhibited in comparison to the untreated control biofilms (positive control). The percentage of growth inhibition was calculated for each assay as a control. Additionally, to evaluate the compounds' effect on the remaining viable bacteria, after the assay, 10 μl of the suspension from each well was inoculated into Luria Bertani (LB) agar plate solid medium, and bacterial growth was tested.

Antimicrobial activity assay

Bioguided separation of compounds from *Eunicea* sp. was performed against the seven marine strains set up as described by Mora-Cristancho et al. (2011). Moreover, the concentration at which compounds (**1**–**14**) could be bactericidal was established through a modification of the Kirby-Bauer disk diffusion susceptibility testing (Wilkins et al., 1972), using two reference strains, *K. pneumoniae* and *S. aureus*, and two marine strains, *A. macleodii* and *V. harveyi*. LB solid agar plates were inoculated with the tested microorganisms. Then filter paper disks of 5 mm diameter impregnated with different amounts of compounds (3 μg/disk, 7.5 μg/disk, 15 μg/disk and 30 μg/disk) were placed on the inoculated plates and incubated at 37 °C for reference strains or at 25 °C for marine strains. The growth inhibition halos were measured for 24 and 48 h of incubation.

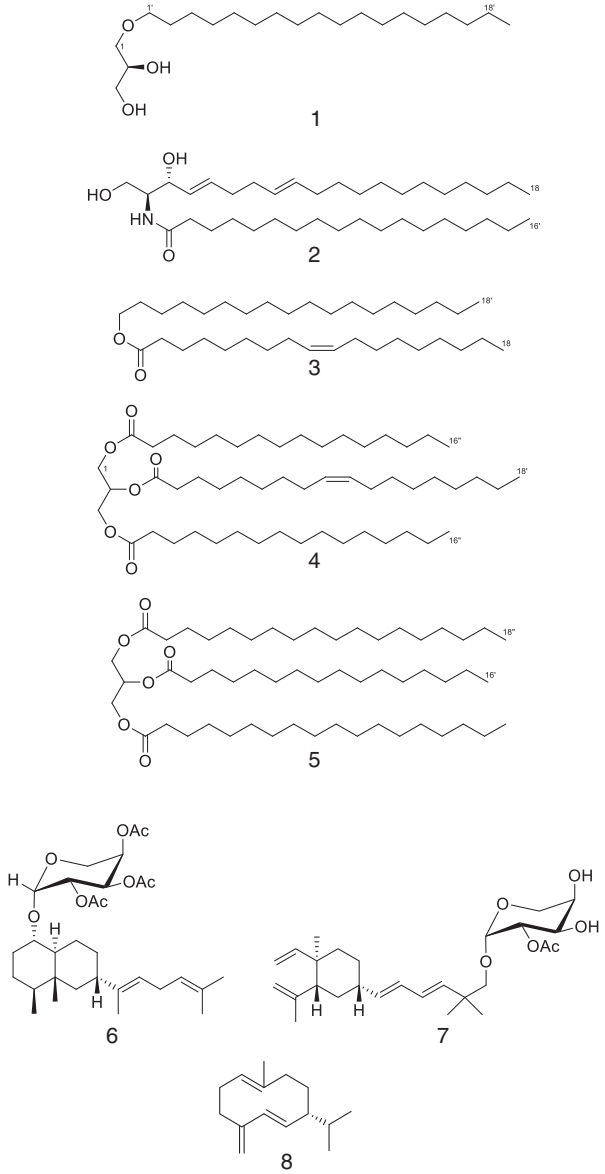
Statistical analyses

Biofilm inhibition and growth inhibition data were statistically analyzed by ANOVA, and all experiments were conducted in triplicate for each strain. To detect differences between and among the groups of data or when significant differences were detected, pairwise comparisons were made among all the groups using the Turkey's method to adjust for multiple comparisons (Montgomery, 2007).

Results

Identification of compounds isolated from Eunicea sp

Compounds from *Eunicea* sp. were identified by comparing the spectroscopic data with those previously published. Compound **1** (176 mg/kg yield from the coral) had the molecular formula $C_{21}H_{44}O_3$, as deduced from HRESIMS with a $[M+Na]^+$ peak at *m*/*z* 367.3194 (calcd. for $C_{21}H_{44}O_3Na^+$, 367.3183). Optical rotation $[\alpha]_D^{25}$ +3.2 (*c* 1.5, $CHCl_3$) and its ^1H and ^{13}C NMR spectra showed a close resemblance to those published by Quijano et al. (1994) and Sun et al. (2005) for (*S*)-(+)-3-octadecyloxy-l,2-propane diol, an alkylglycerol also known as batyl alcohol. Compound **2** (10.6 mg/kg) had the molecular formula $C_{34}H_{65}NO_3$ as established by HRESIMS. Its methanolysis and analysis by HRGCMS displayed a single volatile compound identified as methyl hexadecanoate. The negative optical rotation $[\alpha]_D^{25}$ −9.7 (*c* 0.3, $CHCl_3$) and NMR spectra suggest that **2**, (2*S*,3*R*,4*E*,8*E*)-*N*-hexadecanoyl-2-amino-4,8-octadecadiene-1,3-diol, was a ceramide as reported by Shin and Seo (1995) and Han et al. (2005). Compound **3** (70 mg/kg) was recognized as octadecyl (9*Z*)-9-octadecenoate (stearyl oleate), on the basis of HRESIMS, and NMR data were coincident to those of the wax previously synthetized (Vieville et al., 1995). Compound **4** (5.9 mg/kg) was optically inactive and its methanolysis gave a mixture of methyl hexadecanoate and methyl (9*Z*)-9-octadecenoate in proportion 2:1 by HRGCMS. According to ESIMS, the NMR data were identical to those of the synthesized glycerolipid 1,3-dihexadecanoyl-2-(9*Z*-octadecenoyl)-glycerol as reported by Stamatov and Stawinski (2007). NMR spectroscopic features of **5** (8.2 mg/kg yield from the coral) were similar to those of **4** except for the lack of double bond signals and it was also optically inactive. It revealed the presence of methyl hexadecanoate and methyl octadecanoate in a 1:2 ratio as per HRGCMS. In agreement with ESIMS analysis, compound **5** was postulated as 1,3-dioctadecanoyl-2-hexadecanoyl-glycerol.



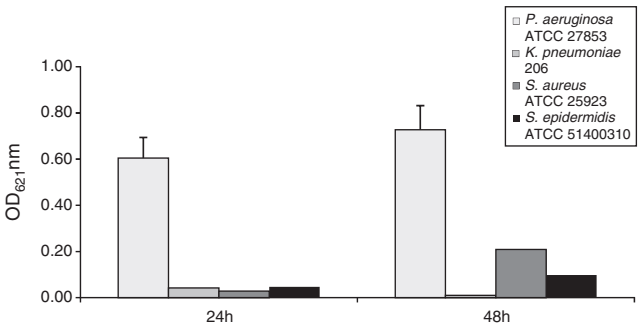


Fig. 1. Biofilm forming ability of reference strains at 37 °C. Microtiter plate method was used for quantifying biofilm formation, dyed with crystal violet. The bars on the graph represent mean ± SD of the biofilm formation from three independent experiments. The *X*-axis gives the incubation time (h).

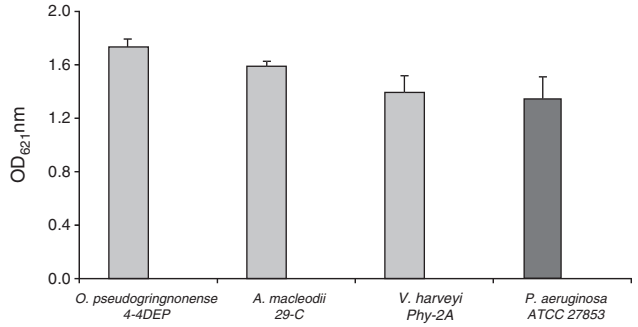


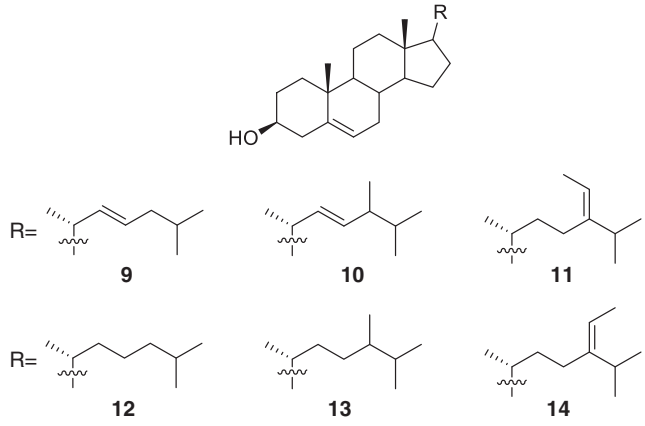
Fig. 2. Biofilm forming ability of marine strains during 48 h at 25 °C. Microtiter plate method and CV staining to quantify were used. Between the reference strains, *P. aeruginosa* was used as positive control in the present study due to its higher biofilm formation level. The bars on the graph represent mean ± SD of biofilm formation from three independent experiments. The ANOVA test showed a statically significant difference relative to control ($p < 0.05$).

Biofilm production assay by microtiter plate test

The results showed that the biofilm was higher after 48 h of incubation for three reference strains. From these, *P. aeruginosa* exhibited the highest biofilm formation level, significantly more than other strains (Fig. 1), followed by *S. aureus* being the selected reference strains. On the other hand, the assay with those two strains at 25 °C determined that the *S. aureus*' biofilm decreased while *P. aeruginosa* showed higher levels in its biofilm formation. Thus, *P. aeruginosa* was used as positive control in biofilms formation assay of marine strains. Then, those results showed that the marine strains *O. pseudogringnonese*, *A. macleodii* and *V. harveyi* had higher biofilm formation, such as shown by the control strain *P. aeruginosa* (Fig. 2), while the other marine bacteria practically did not show any biofilm production.

Biofilm inhibition activity assay of compounds

The anti-biofilm activity of compounds **1**–**8** and sterols **9**–**14** was determined toward three marine strains, and two reference strains, all selected in the present study due to their strong biofilm-forming ability. As a result, *O. pseudogringnonense* was inhibited over 50% only by compound **1**, up to 61% at 0.5 mg/ml, while at 0.025 mg/ml, compounds **1**, **7**, **8** had an inhibition close to 30% (Fig. 3). The strain exhibited low response to other compounds. Only compound **1** was up to 33% and the mixture **9**–**14** decreased the biofilm formation of *A. macleodii* at 0.5 mg/ml (Fig. 3). Other compounds slightly reduced microbial growth without effect on biofilms. In *V. harveyi*, biofilm inhibition was shown by **1** up to 64% at 0.5 mg/ml; and at 0.025 mg/ml compounds **7** and **8** were the most active. Results with the reference strain *P. aeruginosa*, shown as compounds **6**, (**9**–**14**) and **2** at 0.5 mg/ml in their order, had the highest level of effectiveness reaching **6** up to 66%, whereas at 0.025 mg/ml, **2** and (**9**–**14**) showed effectiveness as well. Additionally, *S. aureus* as the only gram-positive strain included was the most susceptible and compounds **1**, **3**, **6** and **7** showed high percentages of inhibition up to 100%, at 0.5 mg/ml (Table 1) and at 0.025 mg/ml these exhibited low activity. In all cases, the compounds were less active against bacterial growth and the assessment of bacterial viability by plating on agar showed that each strain grew again.



Biofilms formation was significantly reduced as compared to untreated biofilms of each of these bacteria at the tested concentrations of compounds. This was validated by ANOVA, with a significance level of 95% and a mean comparison Tukey test, with an α error of 0.05.

Antimicrobial activity in extracts and compounds from Eunicea *soft corals*

In this study, the crude extract of *Eunicea* sp. evidenced moderate growth inhibition against six of the seven marine bacteria. The organic fraction and isolated compounds **1** to **5** showed mild antimicrobial activity toward *O. pseudogringnonense*, *Kokuria* sp., and *V. harveyi*. The deacetylated of **6**, the terpene **8** and the sterols (**9**–**14**) from *Eunicea fusca* also had low activity against *Bacillus* sp., *O. pseudogringnonense* and *V. harbeyi* as detailed by Reina et al. (2011). Finally, the disk diffusion susceptibility test did not evidence that compounds **1**–**14** could be bactericide, even at the highest assayed concentration (30 μg/disk), confirming their low antibacterial activity.

Discussion

Bacterial biofilms has a primary role in the pathogenesis of diseases (Al-Sohaibani and Murugan, 2012) and are important in biofouling processes as fundamental settlement cues, for many invertebrate larvae that colonize hard substrata (Fusetani, 2011). In the present study, evaluation of crude extract and fractions of *Eunicea* sp. against several bacteria previously isolated from heavily fouled marine surfaces (Mora-Cristancho et al., 2011) showed low antibacterial activity. From the organic fraction by bioguided chromatography, five lipid compounds were isolated and identified through spectroscopic analysis and compared with literature data known as alkylglycerol, batyl alcohol (**1**); ceramide: (2*S*,3*R*,4*E*,8*E*)-*N*-hexadecanoyl-2-amino-4,8-octadecadiene-1,3-diol (**2**); wax ester: stearyl oleate (**3**);

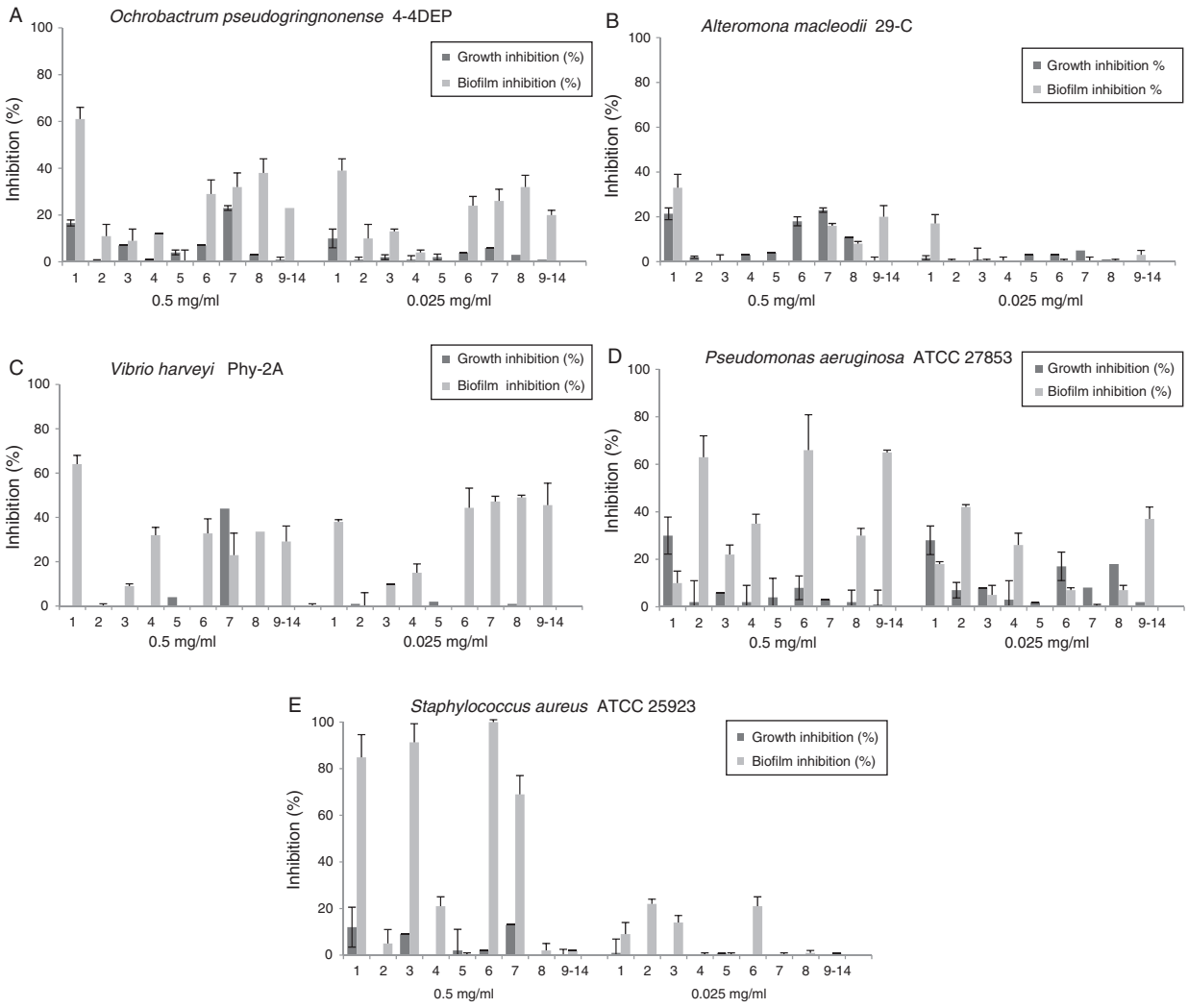


Fig. 3. Biofilm inhibition and growth inhibition effect of isolated compounds **1** to mixture **9**–**14** from *Eunicea* sp. and *Eunicea fusca* against marine and reference bacteria. Biofilm was quantified using the microtiter plate test and crystal violet assay. The *X*-axis gives the number of the isolated compound and its concentration at 0.5 mg/ml or 0.025 mg/ml. The bars on the graph represent mean ± SD as a percentage of biofilm inhibition of triplicate experiments. Biofilms formation was significantly reduced as compared to untreated biofilms (control) of each of these bacteria at the tested concentrations of the compounds (ANOVA $p < 0.05$) with a significance level of 95% and a mean comparison Tukey test with an α error of 0.05.

acylglycerols: 1,3-dihexadecanoyl-2-(9Z-octadecenoyl)-glycerol (**4**), and 1,3-dioctadecanoyl-2-hexadecanoyl-glycerol (**5**). For decades, the family of bioactive lipids has grown tremendously (McAnoy et al., 2005; Deniau et al., 2010; Blunt et al., 2014). Therefore, the possibilities of biofilm inhibition of lipids from *Eunicea* sp., together with terpenoids, fuscoside E peracetate (**6**), fuscoside B (**7**), (+)-germacrene D (**8**), and sterols (**9**–**14**), from *Eunicea fusca* (Reina et al., 2011), were essayed by microtiter

Table 1
Biofilm inhibition (values from 25%) of compounds isolated from *Eunicea* sp. and *Eunicea fusca* at 0.5 or 0.025 mg/ml, against three marine strains and two reference bacteria.

Compound	Mean percentage inhibition of biofilm*									
	O. pseudogringnonense 4-4DEP		*A. macleodii* 29-C		*V. harveyi* Phy-2A		*P. aeruginosa* ATCC 27853		*S. aureus* ATCC 25923	
	0.5	0.025	0.5	0.025	0.5 mg/ml	0.025	0.5	0.025	0.5	0.025
1	61 ± 6.2	39 ± 5.8	33 ± 6.0		64 ± 4.2	38 ± 1.3			85 ± 10.3	
2							63 ± 9.2	42 ± 1.8		
3									91 ± 9.0	
4					32 ± 3.7		35 ± 4.6	26 ± 4.8		
5										
6	29 ± 6.5				33 ± 6.4	44 ± 9.0	66 ± 14.3		100 ± 0.0	
7	32 ± 6.1	26 ± 5.3				47 ± 2.9			69 ± 8.4	
8	38 ± 6.8	32 ± 5.0			34 ± 0.0	49 ± 1.6	30 ± 3.4			
9–14					29 ± 7.0	45 ± 10.0	65 ± 1.4	37 ± 5.4		

Biofilm was quantified by microtiter plate test and crystal violet. In this table, the effectiveness of more than 25% of inhibition was considered in comparison to the untreated control biofilms (positive control).

* Data represent mean ± SD as a percentage of biofilm inhibition of the compound from three independent experiments. Biofilms formation was significantly reduced as compared to control (ANOVA $p < 0.05$), with a significance level of 95% and a mean comparison Tukey test with an α error of 0.05.

plate tests, a frequently used technique for quantifying biofilm formation (O'toole et al., 2000; Al-Sohaibani and Murugan, 2012).

According to the results obtained in this study, out of the four reference strains, *P. aeruginosa* evidenced the best biofilm formation, followed by *S. aureus* and they were used for further essays (Fig. 1). *P. aeruginosa* and *S. aureus* are model organisms, known for their capacity of biofilm formation and their inherent resistance to antimicrobial agents (O'toole et al., 2000; Quinn et al., 2012). On the other hand, the results about marine strains showed that *O. pseudogringnonense*, *A. macleodii* and *V. harveyi* had the highest biofilm formation (Fig. 2), as the control strain *P. aeruginosa* at the same culture condition. As published by Vikram et al. (2011), biofilm formation and bioluminescence production in *V. harveyi* are a known quorum sensing-controlled process, and it was recently studied also in *O. pseudogringnonense* (Cuadrado et al., 2013), while *A. macleodii* has been commonly found in biofilms from different depths of oceans (Ivars-Martinez et al., 2008). These marine bacteria together with reference strains are more efficient, and were selected as positive control for the further biofilm inhibition tests. Consequently, compounds **1**–**8** and sterols **9**–**14** were tested for assessing the anti-biofilm ability, and had different effectiveness depending on the bacteria, and some were active against various microorganisms (Fig. 3). Compounds **1** and **6** were the most effective as they acted against four strains. Compound **1** inhibited *A. macleodii*, *O. pseudogringnonense*, *V. harveyi* and *S. aureus* to a percentage greater than 30% against the first, and above 60% for the last three. Compound **6** affected *O. pseudogringnonense* and *V. harveyi* with effectiveness between 25 and 50%, and *P. aeruginosa* and *S. aureus* above 60% of biofilm inhibition (Table 1). Compounds **7** and **8** had an effect greater than 25% against three microorganisms. Compound **4** and mixture of sterols **9**–**14** were effective against two strains, with a percentage higher than 25%. Compounds **2** and **3** showed effectiveness only to one strain but above 50%. In contrast, compound **5** was inactive to biofilm inhibition against all strains.

Additionally, compounds **1**–**5**, **8** and sterols **9**–**14** had low antibacterial activity by the disk-diffusion test against marine bacteria. Results, consistent with those of the disk diffusion susceptibility test, showed that none of the compounds **1**–**8** and (**9**–**14**) displayed a bactericidal effect even at the highest assayed concentration and that they only evidenced their low antibacterial activity. Interestingly, the results confirmed that the compounds exhibited growth inhibition much lower than the anti-biofilm activity, and that a proportion of the biofilm bacteria still remained viable, suggesting that there is biofilm inhibition without affecting their growth, and that these compounds are little toxic.

In the present study, the alkylglycerol – batyl alcohol **1** – is described for the first time as an anti-biofilm compound. Alkylglycerols are found in marine invertebrates in higher levels than in land vertebrates (Watschinger and Werner, 2013). Their biological activities comprise stimulation of hematopoiesis, immunological defenses, antitumor and antimetastasis (Deniau et al., 2010; Iannitti and Palmieri, 2010). Batyl alcohol **1** was previously isolated from shark liver oil (Myers and Crews, 1983), from the invertebrate sponge *Desmapsamna anchorata* (Quijano et al., 1994), from the soft coral *Plexaura flexuosa* (Kind and Bergmann, 1942), from *Cladiella* species (Radhika, 2006), and from the gorgonia *Scirpearia gracilis*, showing moderate growth inhibition for marine bacterial species as *Pseudoalteromonas piscida*, *Ruegeria* sp., *Vibrio alginolyticus*, *V. furnissii* and *V. harveyi* (Qi et al., 2008). Only dodecylglycerol was found as an effective antibacterial against *Streptococcus faecium* (Ved et al., 1984). In previous studies, other structurally related natural lipids showed potential anti-biofilm, such as a glycolipid composed of glucose and palmitic acid, which disrupted biofilms of *Candida albicans*, *P. aeruginosa* and *Bacillus pumilus* (Dusane et al., 2011), and the linoleic acid that reduced biofilm accumulation of *Streptococcus mutans* (Jung et al., 2014).

In earlier works, ceramide **2** was obtained from anemone *Paracondylactis indicus*, gorgonian *Acabaria unhlata* (Shin and Seo, 1995), soft coral *Dendronephthya gigantean* (Han et al., 2005), and zoanthids, *Palythoa caribaeorum* and *Protopalyhtoa variabilis*. Other ceramides have been recognized for creating an epidermal permeability barrier, and showed cytotoxic or antitumor activities (Almeida et al., 2012; Blunt et al., 2014). Waxes and glycerolipids have been commonly detected by analytical techniques (Vieville et al., 1995; McAnoy et al., 2005). Wax **3** was previously biosynthetized by *Corynebacterium* sp. (Seo et al., 1982) and has been detected in the eyelids of humans and mammalians (Butovich et al., 2007). The purification of glycerolipids **4** and **5** has not been reported from natural sources. Nevertheless, another regioisomer of **4**, called 1,2-dihexadecanoyl-3-(9*Z*-octadecenoyl)-glycerol, which is quiral, was detected in bovine heart (Shinzawa-Itoh et al., 2007). In the same way, the regioisomer of **5**, 1,2-dioctadecenoyl-3-hexadecanoyl-glycerol, was detected in rapeseed oil (Beermann et al., 2007).

Eunicea fusca is the source of fuscol and fuscosides A–E having anti-inflammatory activity, as well as (+)-germacrene D **8** and the sterols mixture (**9**–**14**) (Shin and Fenical, 1991; Reina et al., 2011). The (−)-germacrene D is a common compound of plants (Reina et al., 2011), and the essential oil that contains germacrene from *Achillea millefolium* inhibits biofilms of *Listeria monocytogenes* and *Listeria innocua* (Jadhav et al., 2013). Raut et al. (2013) demonstrated the biofilm inhibition of other terpenoids against *Candida albicans*. Moreover, bioactive sterols mixture or pure compounds exhibit antibacterial or antifouling activities (Al-Lihaibi et al., 2010; Castellanos et al., 2010; Blunt et al., 2014). In the meanwhile, other diterpenoids such as xenicanes, dolabelanes and cembranoids from marine sources possess anti-biofilm activity against marine bacterial isolates (Viano et al., 2009; Tello et al., 2011, 2012). Notwithstanding, previous works have not yet reported the possibility of lipids and terpenoids here assayed as anti-biofilm compounds.

In the present study, batyl alcohol **1** and fuscoside E peracetate **6** were the most effective compounds against the best biofilm formation by the bacteria tested here, and due to their low antibacterial effect they could be considered as less toxic anti-biofilm compounds to the environment. The results are stimulating for further research and better understanding the structure-activity relationship or the action mode of the most active compounds found in this study. In this sense, further studies to determine the minimal inhibitory concentration (MIC) with some of these synthesized compounds are under development in our laboratory.

Conclusion

The present study showed the anti-biofilm capacity of five lipid compounds isolated from soft coral *Eunicea* sp., together with three terpenoids and a mixture of sterols from *E. fusca* collected at Caribbean Sea, against three marine bacteria, *O. pseudogringnonense*, *A. macleodii* and *V. harveyi*, and two known biofilm forming bacteria, *P. aeruginosa* and *S. aureus*. They showed different effectiveness and the most active compounds were batyl alcohol and fuscoside E peracetate. These compounds exhibit specific biofilm inhibition and could be considered less toxic to the environment by having lower antimicrobial activity.

Author's contributions

YRMD (MSc student) performed inhibitions assays and biofilm formation. GPVL and LERG were responsible for running the experimental work. HMW supervised the laboratory work and contributed to preparation of the paper. CAF supervised the laboratory

work and contributed to the biological studies. FARR supervised the laboratory work and contributed to critical reading of the manuscript. CDB contributed with study design and supervised the research project. LCH supervised the research project as head of the research team, 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.

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