INHIBITION OF QUORUM SENSING BY COMPOUNDS FROM TWO EUNICEA SPECIES AND SYNTHETIC SATURATED ALKYLGLYCEROLS

INHIBICIÓN DEL QUORUM SENSING POR COMPUESTOS DE DOS ESPECIES DE EUNICEA Y ALQUILGLICEROLES SATURADOS SINTÉTICOS

Clara BARRAGÁN A., M.Sc.¹, Edelberto SILVA G., M.Sc.², Bárbara MORENO M. Esp. P.N.¹, Humberto MAYORGA W., PhD.¹*

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ABSTRACT

Background: the emergence of bacterial resistance has led to a search for new natural products as alternatives starting points to prevent and control diseases caused by microorganisms. Among the potential candidates were bioactive compounds from octocorals of the genus Eunicea due to their chemical structures and their wide range of biological activities. Objective: the purpose of this study was to evaluate the quorum sensing inhibition (QSI) and antibacterial activity of compounds previously isolated from two Eunicea species and synthesized alkylglycerols (AKG).

Methods: the QSI of three nonpolar compounds and a mixture of AKGs from Eunicea were evaluated by a microtiter plate assay using Chromobacterium violaceum (ATCC 31532). Four naturally occurring, saturated, and enantiomerically pure AKGs, all of which were derived from the chiral precursor (R)-solketal, were synthesized from alkyl chains of 12, 14, 16 and 18 carbons, and their structures were spectroscopically verified by NMR, ESI-MS and optical rotation data. Their QSI by the disc diffusion assay and minimum inhibitory concentrations (MIC) against 14 clinical bacterial isolates in microtiter plates were determined. Results: cembradiene 1, the AKG mixture and AKG (2S)-3-O-dodecyl-1,2-propanediol 4 inhibit QS at the same concentration as kojic acid (10 µg/well or 20 µg/disc, respectively). In this study, the bioactive compounds 1, stearyl oleate 2, acylglycerol 3 and AKGs 4 and (2S)-3-O-tetradecyl-1,2-propanediol 5 showed in vitro IQS activity for the first time. Additionally, 4 and 5 displayed in vitro antibacterial activity against Listeria innocua and Staphylococcus aureus (MIC = 32 µg/mL for both 4 and 5), Enterococcus faecalis (128 µg/mL and 64 µg/mL respectively), Micrococcus luteus (128 µg/mL for both) and Brevibacillus brevis (Bacillus brevis) (512 µg/mL and 64 µg/mL respectively). Conclusion: results suggest that natural compounds 1, 2, 3, 4 and 5 showed QSI, also 4 and 5 have antibacterial activity and they are an interesting alternative to continue researching their effect against pathogenic microorganisms.

Keywords: Natural products, alkylglycerols, synthesis, quorum sensing inhibition, antibacterial.
RESUMEN

**Antecedentes:** la aparición de resistencia bacteriana ha llevado a la búsqueda de nuevos productos naturales como puntos de partida alternativos para prevenir y controlar las enfermedades causadas por microorganismos. Entre los posibles candidatos se encontraban los compuestos bioactivos de octocorales del género *Eunicea* debido a sus estructuras químicas y su gama amplia de actividades biológicas. **Objetivos:** el propósito de este estudio fue evaluar la inhibición del quorum sensing (IQS) y la actividad antibacteriana de compuestos aislados previamente de dos especies de *Eunicea* y de alquilgliceroles (AQG) sintetizados. **Métodos:** la IQS de tres compuestos no polares y de una mezcla de AQGs de *Eunicea* se evaluaron mediante un ensayo de placa de microtitulación usando *Chromobacterium violaceum* (ATCC 31532). Cuatro AQG naturales, saturados y enantioméricamente puros, todos los cuales se derivaron del precursor quiral (R)-solketal, se sintetizaron a partir de cadenas alquímicas de 12, 14, 16 y 18 carbonos, y sus estructuras se verificaron espectroscópicamente mediante RMN, ESI-MS y datos de rotación óptica. Se determinó su IQS mediante el ensayo de difusión de disco y las concentraciones inhibitorias mínimas (CIM) en placas de microtitulación frente a 14 aislamientos bacterianos clínicos. **Resultados:** cembradieno 1, la mezcla de AQGs y AQG (2S)-3-O-dodecild-1,2-propanodiol 4 inhiben el QS a la misma concentración que el ácido kójico (10 µg/pocillo o 20 µg/disco, respectivamente). En este estudio, los compuestos bioactivos 1, estearil oleato 2, acilglicerol 3 y AQGs 4 y (2S)-3-O-tetradecild-1,2-propanodiol 5 mostraron actividad de IQS in vitro por primera vez. Además, 4 y 5 presentaron actividad antibacteriana in vitro contra *Listeria innocua* y *Staphylococcus aureus* (CIM = 32 µg/mL para 4 y 5), *Enterococcus faecalis* (128 µg/mL y 64 µg/mL respectivamente), *Micrococcus luteus* (128 µg/mL para ambos) y *Brevibacillus brevis* (*Bacillus brevis*) (512 µg/mL y 64 µg/mL respectivamente). **Conclusions:** los resultados sugieren que los compuestos naturales 1, 2, 3, 4 y 5 mostraron IQS, también 4 y 5 tenían actividad antibacteriana y son una alternativa interesante para continuar investigando su efecto contra microorganismos patógenos.

**Palabras clave:** Productos naturales, alquilgliceroles, síntesis, inhibición del *quorum sensing*, antibacterial.

INTRODUCTION

The emergence and rapid spread of resistant bacterial strains and the overuse of antibiotics is a significant public health problem, and the development of other strategies requires searching for new natural products that are active against pathogenic microorganisms. In recent years, several known or newly discovered bioactive molecules have been evaluated against microorganisms (1-3). In the same way, quorum sensing inhibitors are potential leads for the design of new anti-pathogenic drugs (4). A process called quorum sensing (QS) is a form of population density-dependent cell-cell communication and gene regulation that allows bacteria to coordinate settlement, virulence, luminescence, metabolite production and biofilm maturation (5, 6). The interruption of QS may prevent the development of bacterial biofilms, and in this sense, it constitutes an opportunity to attenuate the pathogenicity of bacteria resistant to available antibiotics (7). In addition, promising QSI compounds have been shown to make biofilms more susceptible to antimicrobial treatments, and this indicates that a combination treatment of both QSI and antibiotics may prove useful against resistant bacteria (8).

In previous research with sponges and octocorals from the Colombian Caribbean Sea, some biologically active compounds were identified (9-11). Two cembradiene diterpenoids and other nonpolar compounds were isolated from two octocoral species of the genus *Eunicea* collected in Santa Marta Bay (*Eunicea* sp.), and they afforded only modest activity against a variety of isolated marine bacteria from immersed fouled surfaces (10,12). However, an isolated alkylglycerol (AKG), the 7 (Figure 1), showed remarkable effectiveness against biofilm formation by three of these marine bacteria, namely, *Ochrobactrum pseudogringonense*, *Aleromonas macleodii* and *Vibrio harveyi*, as well as against a known biofilm-forming bacterium, *Staphylococcus aureus* ATCC 25923 (12), but other AKGs could not be purified from these *Eunicea* species due to their low natural abundances.

Alkylglycerols are ether lipids naturally found in a variety of organisms such as microorganisms, fish, and invertebrates such as corals and sponges, milk, bone marrow, mammalian blood cells...
including human blood cells and to a lesser extent in higher plants. AKGs have multiple biological and therapeutic properties, such as the stimulation of hematopoiesis, immunological defenses, anti-tumor activity, and anti-metastasis activity as well as improving sperm quality and vaccination efficiency (13, 14). Natural AKGs were found as complex mixtures of compounds that vary by length and the degree of unsaturation of the alkyl chain; however, the absolute configuration at their asymmetric carbon is always $S$ (15). Although biological testing has been done on AKGs isolated from natural sources, the separation of individual components is tedious (16), and therefore, some of the biological assays have been performed with synthesized AKGs (14). As an example, in the antibacterial activity studies, the synthetic and racemic AKG dodecylglycerol was the most potent against *Enterococcus faecium*, *Streptococcus mutans* and *S. aureus* (17, 18).

Continuing our studies on bioactive compounds against microorganisms, the aim of this work was to assess the QSI using *C. violaceum* of three compounds, a mixture of AKGs previously isolated from two *Eunicea* species, of four synthesized natural enantiomers of saturated AKGs (Figure 1) and evaluate the antibacterial activity of the AKGs against clinical isolates of bacteria, some of them known for their pathogenic capacity. The results exhibited that some compounds from those two octocorals may have potential for the control of bacterial infections.

**Figure 1.** Chemical structures of the natural compounds and synthetic alkylglycerols (AKG) included in this study: a cembradiene diterpenoid, (+)-(15S, 2R, 11S, 12R, 15S, 3E, 7E)-11-acetoxy-2,12-oxa-15,17-epoxy-cembra-3,7-diene 1; a wax ester, stearyl oleate 2; an acylglycerol, 1,3-dihexadecanoyl-2-(9Z-octadecenoyl)-glycerol 3; the alkylglycerols (AKGs), (2S)-3-O-dodecyl-1,2-propanediol 4; (2S)-3-O-tetradecyl-1,2-propanediol 5; (2S)-3-O-hexadecyl-1,2-propanediol 6; and (2S)-3-O-octadecyl-1,2-propanediol 7.
MATERIALS AND METHODS

Reagents and equipment

The reagents (R)-(-)-2,3-O-isopropylideneglycerol ((R)-solketal), tetra-n-butyl ammonium bromide (n-Bu₄NBr), sodium bis-(2-methoxyethoxy)aluminum hydride (Red-Al), kojic acid (KA) and alkyl bromides were purchased from Alfa Aesar (USA); methanesulfonyl chloride (MsCl), p-toluenesulfonic acid (p-TsOH), p-hydroxybenzaldehyde (p-HB) and methyl octadecanoate were from Merck (Germany). All reagents were of analytical grade, and solvents were dried and distilled prior to use. TLC was performed on silica gel 60 F 254 (Merck) plates and visualized by UV light and 0.5 % (p/v) ceric ammonium sulfate in a 10 % sulfuric acid solution. ¹H and ¹³C NMR spectra were recorded on an Avance 400 spectrometer (Bruker, USA) or an Avance 300, using CDCl₃ and TMS as internal standards. For liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS), an LCMS-2010-ESI system (Shimadzu, Japan) was used in the positive ion mode with a RP-18 column (50 mm x 4.6 mm id x 5 µm, Waters, USA). Ten microliters of the fraction dissolved at 1mg/mL in chloroform:methanol:water:ammonium acetate 0.5:9:0.5:0.004 (v/v/v/v) was injected and eluted with a gradient of 9:1:0.004 methanol:water:ammonium acetate-10:0.004 methanol:ammonium acetate for 10 min at a flux of 1.5 mL/min. Additionally, 5 µL samples of pure compounds were eluted with 9:1 methanol:water-methanol. Optical rotations were measured on an ADP440 (Bellingham + Stanley, USA), and a Spectronic 20 Genesys spectrophotometer (Thermo Scientific, USA) for measuring the optical density at 600 nm was used for the evaluation of the bacterial growth.

Analysis of compounds and a fraction from two octocorals of the genus Eunicea

In our earlier works, organic compounds 1, 2, and 3 (Figure 1) and a nonpolar fraction of two octocoral species of the genus Eunicea from Santa Marta Bay, Colombia, were obtained by bio-guided isolation using an antibacterial test against several marine bacteria and they were previously assigned (10, 12). In this work, the natural samples were used for QSI assays, and the nonpolar fraction was subjected to further analysis by ¹H-NMR, ¹³C-NMR and LC-ESI-MS.

Synthesis of saturated alkylglycerols 4-7

Natural saturated AKGs 4-6 were synthesized to obtain enough material for their bioassays, following the procedures described in the literature (15, 16, 19). To the chiral precursor (R)-solketal (0.60 g, 4.54 mmol), the corresponding alkyl bromide (1.13 g, 4.54 mmol) and n-Bu₄NBr (0.29 g, 0.91 mmol), KOH (0.32 g, 5.68 mmol) was added, and the resulting mixture was stirred for 15 hours at 40°C. The reaction was quenched by the addition of distilled water (3 mL), extracted with ethyl ether (3 x 3 mL) and washed with H₂O until the pH was neutral. The organic phase was concentrated under vacuum using a rotary evaporator, and a semisolid enriched in the alkyl isopropylidene-glycerol was obtained (Scheme 1). This intermediate was deprotected in 7 mL of THF:water (15:5) with p-TsOH (55.4 mg, 0.30 mmol) and refluxed for 15 h. After extraction with chloroform (3x3 mL) and washing with H₂O, the organic phase was dried with anhydrous MgSO₄ and concentrated under vacuum. The obtained solid was recrystallized from hexane at 0°C to afford pure AKGs 4, 5 and 6. Each step in the synthesis was monitored by TLC.

Scheme 1: Synthesis of alkylglycerols 4-6.
The synthesis of \( \text{7} \) was performed according to methods published by some authors \((16, 19, 20)\). Red-Al \((0.83 \text{ g}, 4.15 \text{ mmol})\) was added to methyl octadecanoate \((0.62 \text{ g}, 2.08 \text{ mmol})\) dissolved in anhydrous THF \((5 \text{ mL})\) at \(0^\circ\text{C}\) and stirred for \(5\) h at \(24^\circ\text{C}\). NaOH \((10\% \text{ in water})\) was then added at \(10^\circ\text{C}\), and the organic layer was washed with water, dried, and concentrated. Octadecanol was subsequently mesylated in dichloromethane with anhydrous pyridine \((0.3 \text{ mL}, 4.53 \text{ mmol})\) at \(0^\circ\text{C}\) by the addition of MsCl \((0.3 \text{ mL}, 4.53 \text{ mmol})\) and stirring for \(24\) h at \(25^\circ\text{C}\) (Scheme 2). After quenching the reaction with distilled water, the organic phase was extracted with \(1\% \text{ HCl}\), washed with water, dried, and concentrated. Finally, the newly formed mesylate was converted to AKG \( \text{7} \) as described above, using \((R)-\text{solketal (0.4 g, 2.92 mmol)}, n-\text{Bu}_4\text{NBr (0.2 g, 0.58 mmol)}, \) and KOH \((0.3 \text{ g}, 5.31 \text{ mmol})\).

**Scheme 2: Synthesis of alkylglycerol 7.**

**Quorum sensing inhibition assays**

The QSI abilities of compounds \( \text{1, 2, and 3} \) as well as the fraction from *Eunicea* sp. were evaluated *in vitro* in 96-well microtiter plates (TPP, Switzerland) \((6)\), using *C. violaceum* (ATCC 31532) as the biosensor \((4)\). Pre-inoculum was cultured overnight in tryptic soy broth (TSB, Merck), and concentration was adjusted to an optical density of \(0.2-0.3\) at \(600\) nm. Then, \(100 \mu\text{L}\) was added to each well, followed by \(100 \mu\text{L}\) of natural samples dissolved in dimethylsulphoxide (DMSO) at final concentrations of \(5, 10, 20\), and \(40 \mu\text{g}\), respectively, and the plate was incubated for \(24\) h at \(28^\circ\text{C}\). Thus, the QSI activity was established by the appearance of a colorless and opaque well but without affecting bacterial growth, and it was evaluated as the minimum quantity in \(\mu\text{g}\) per well of sample required to inhibit violacin pigment. The sample solvent (DMSO), as solvent without antimicrobial activity it served as the negative control and KA was used as the positive control. The QSI potential of synthesized AKGs was assessed using the standard disk diffusion method \((21, 22)\), with *C. violaceum* (ATCC 31532) and Luria-Bertani broth (LB, Merck) as growth medium. Sterile filter paper discs \(5\) mm in diameter (Whatman, USA) were impregnated with \(10, 20, 30\), and \(50 \mu\text{g}\) of each AKG dissolved in dichloromethane (DCM) and dried at room temperature for \(20\) minutes. The inoculum was adjusted to \(10^6\) CFU/mL \((0.5\%\text{ McFarland})\) in LB agar in Petri dishes. The discs were placed on the agar, the plates were incubated for \(24-48\) h at \(27^\circ\text{C}\) and the inhibition halo was measured. This assay also measures the minimum amount of each compound that inhibits pigment production (violacein) around the disc and indicates that the QS of *C. violaceum* was disrupted but does not interfere with bacterial growth \((22)\). As positive controls, KA \((5)\), and \(p\)-HB \((28)\), were used at the same concentrations and treated as described above per disc, and DCM as inactive solvent to antimicrobial activity was used as a negative control.

**Evaluation of the antibacterial activity**

To further investigate the antibacterial properties, the synthesized AKGs were selected since all natural compounds were isolated from these octocorals in low quantities. Their MICs were determined
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in vitro in microtiter plates against 14 clinical bacterial isolates (23-25). Each AKG was dissolved in dimethylformamide (DMF) (1 mg/mL) to final concentrations of 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/mL in wells. Bacterial strains were grown with Mueller-Hinton agar (MHA, Merck). The inoculum (100 µL, 10⁴ CFU/mL) in each well was mixed with the AKG in MH broth to 200 µL, and the plates were incubated at 37°C for 18-24 h. An inoculated well with DMF in culture medium and blank well containing only assay medium were used as growth controls. The MIC of 64 µg/mL of racemic dodecylglycerol against S. aureus (18) was used as a standard reference data point. Among the clinical isolates, the gram-positive bacteria Listeria innocua, Staphylococcus aureus, Enterococcus faecalis, Brevibacillus brevis, and Micrococcus luteus as well as the gram-negative bacteria Escherichia coli, Salmonella enteritidis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Enterobacter agglomerans, Klebsiella oxytoca, Acinetobacter baumannii, and Proteus mirabilis were provided by the Hospital of Neiva (Huila), Hospital of Tunal, Hospital of Engativá and Universidad del Bosque (Bogotá).

Statistical analysis

Bioassay determinations were replicated three times and reported as the mean values using the standard error and analysis of variance ANOVA’s Tukey test. A difference was considered statistically significant when p<0.05 (26).

RESULTS

Analysis of compounds and a fraction from two octocorals of the genus Eunicea

In this work, natural compounds and a nonpolar fraction were reserved for QSI assays. The nonpolar fraction was found by NMR to be a mixture of AKGs, and by LC-MS-ESI, it was found to have the abundant ions [M + Na]⁺, [M + H]⁺, and [M + NH₄]⁺ (Figure 2).

Figure 2. TIC (total ion chromatogram) obtained by LC-MS-ESI of a fraction from Eunicea sp., showing the separation and spectra of the major identified AKGs 6 and 7.
Synthesis of saturated alkylglycerols 4-7

Chemical structures of the synthesized AKGs were confirmed by NMR and LC-ESI-MS spectra along with the specific optical rotations as follows.

**Compound 4.** Yield 50% as a bright white solid, (560 mg, 2.15 mmol). 1H NMR (400 MHz, CDCl3/TMS): δ 3.89-3.83 (m, 1H, H-2), 3.71 (dd, J = 11.4, 3.8 Hz, 1H, H-1a), 3.63 (dd, J = 11.4, 5.4 Hz, 1H, H-1b), 3.52 (dd, J = 9.7, 4.0 Hz, 1H, H-1), 3.48 (dd, J = 9.8, 5.9 Hz, 1H, H-3b), 3.45 (2x dt, J = 9.1, 6.7 Hz, 2H, H-1'), 2.56 (s, 2H, CH2OHx2), 1.56 (br. quint, J = 6.8 Hz, 2H, H-2'), 1.35-1.16 (m, 18H, CαH12-14'), 1.25 (br. s, 26H, CαH1-15), 0.87 (t, J = 6.8 Hz, 3H, H-16'). 13C NMR (100 MHz, CDCl3): δ 72.60 (C-3'), 70.64 (C-2), 64.40 (C-1), 32.05 (C-16'), 29.83, 29.81, 29.80, 29.74, 29.72, 29.59, 29.49 (7C, C-2'- and C-4'-C-9'), 26.21 (C-3'), 22.82 (C-11') ppm. 13C NMR and 13C NMR APT (75 MHz, CDCl3) δ 72.57 (C-3), 71.98 (C-1'), 70.66 (C-2'), 64.38 (C-1'), 32.05 (C-14'), 29.82, 29.79, 29.74, 29.72, 29.60, 29.49 (11C, C-2' and C-4'-C13'), 26.21 (C-3'), 22.81 (C-15'), 14.23 (C-16'). LC-MS-ESI m/z 317.20 [M + H]+ (34%), 383.05 [M + Na]+ (100%). [α]20D +2.2 (c 4.3, CHCl3).

**Compound 5.** Yield 52% as a white solid (385 mg, 1.33 mmol). 1H NMR (400 MHz, CDCl3/TMS): δ 3.89-3.83 (m, 1H, H-2), 3.71 (dd, J = 11.4, 3.8 Hz, 1H, H-1a), 3.63 (dd, J = 11.4, 5.3 Hz, 1H, H-1b), 3.52 (dd, J = 9.7, 4.0 Hz, 1H, H-1), 3.48 (dd, J = 9.7, 6.1 Hz, 1H, H-3b), 3.45 (2x dt, J = 9.3, 6.7 Hz, 2H, H-1'), 2.80 (br. s, 1H, CHO), 2.40 (br. s, 1H, CH2OH), 1.57 (br. quint, J = 6.8 Hz, 2H, H-2'), 1.25 (br. s, 22H, CH2x11), 0.87 (t, J = 6.8 Hz, 3H, H-16'). 13C NMR (100 MHz, CDCl3): δ 72.62 (C-3), 72.00 (C-1'), 70.60 (C-2), 64.40 (C-1), 32.06 (C-12'), 29.83, 29.81, 29.80, 29.75, 29.72, 29.71, 29.59, 29.50 (9C, C-2' and C-4'-C11'), 26.21 (C-3'), 22.83 (C-13'), 14.26 (C-14'). LC-MS-ESI m/z 289.10 [M + H]+ (40%), 311.10 [M + Na]+ (100%). [α]20D +2.1 (c 4.1, CHCl3).

**Compound 6.** Yield 27% as a white greasy solid (307 mg, 0.97 mmol). 1H NMR (300 MHz, CDCl3/TMS): δ 3.89-3.81 (m, 1H, H-2), 3.70 (dd, J = 11.4, 3.8 Hz, 1H, H-1a), 3.64 (dd, J = 11.4, 5.4 Hz, 1H, H-1b), 3.55-3.49 (m, 1H, H-3a), 3.51-3.46 (m, 1H, H-3b), 3.47-3.40 (m, 2H, H-1'), 2.20 (br. s, 2H, CHOHx2), 1.59-1.52 (m, 2H, H-2'), 1.25 (br. s, 26H, CH2x13), 0.87 (t, J = 6.7 Hz, 3H, H-16'). 13C NMR and 13C NMR APT (75 MHz, CDCl3) δ 72.57 (C-3), 71.98 (C-1'), 70.66 (C-2), 64.38 (C-1'), 32.05 (C-14'), 29.82, 29.79, 29.74, 29.72, 29.60, 29.49 (11C, C-2' and C-4'-C13'), 26.21 (C-3'), 22.81 (C-15'), 14.23 (C-16'). LC-MS-ESI m/z 345.25 [M + H]+ (31%), 367.25 [M + Na]+ (100%). [α]20D +2.7 (c 4.0, CHCl3).

Quorum sensing inhibition assays

Cembradiene 1 and the mixture of AKGs presented inhibition at the same concentration as KA, the control, while 2 and 3 were less active (Table 1). It was observed that neither AKGs affected the growth of *C. violaceum*.

### Table 1. Quorum sensing inhibition of an AKG mixture and compounds 1, 2, and 3 isolated from *Eunicea* sp. in *C. violaceum* (ATCC 31532) by the microtiter plate assay.

| Compounds | 1* | 2* | 3* | AKG mixture* | KA* | DMSO* |
|-----------|----|----|----|-------------|-----|-------|
| values in µg/well* | 10 | 20 | 40 | 10 | 10 | -- |

*Minimum quantity in µg of compound per well required to inhibit violacein pigment. KA was used as a positive control. DMSO was used as a negative control. --, No inhibition was observed in the assay conditions. Data represent the mean from three independent experiments. The ANOVA showed a statistically significant difference between the controls and treated groups (p < 0.05).

Moreover, the results of QSI showed that AKG 4 exhibited the same minimum inhibition halo of 14 ± 1 mm at 20 µg/disc as KA, while AKG 5 and the other control, p-HB gave a halo of 12 ± 3 mm at 50 µg/disc (Figure 3, Table 2). The *C. violaceum* growth confirmed that only they inhibited violacein production.
Inhibition of quorum sensing by compounds from two *Eunicea* species and synthetic saturated alkylglycerols

**Figure 3.** Inhibition halo (in mm) of QSI by the disc diffusion assay of synthesized alkylglycerols 4-7 in *C. violaceum* (ATCC 31532). KA and p-HB were positive controls. DCM was a negative control. Bars indicate the standard deviation of three replicates. Statistically significant differences (p < 0.05, ANOVA) between the treatment and the negative control (without AKG) are marked by asterisks.

**Table 2.** Quorum sensing inhibitory activity of synthesized alkylglycerols 4-7 against the biosensor strain *C. violaceum* (ATCC 31532) by the disc diffusion assay.

| Compounds | Quorum sensing inhibition of AKGs | 4* | 5* | 6 | 7 | KA | p-HB | DCM |
|-----------|----------------------------------|----|----|---|---|----|------|------|
| values in µg/disc |                                  | 20 | 50 | -- | -- | 20 | 50 | --  |

*Minimum quantity in µg of compound per disc required to inhibit violacein pigment. KA and p-HB were used as a positive control. Dichloromethane (DCM) was used as a negative control. --, No inhibition zone observed in the assay conditions. Only the synthesized AKGs 4-7 were selected for this assay since all natural 1, 2, 3 compounds were isolated from these two octocorals in insufficient amount. Data are the mean of three separate experiments. *The ANOVA showed a statistically significant difference relative to controls (p < 0.05).*

**Evaluation of the antibacterial activity**

The results show that AKGs 4 and 5 were the most active against five of the gram-positive bacterial strains (Table 3), while against the gram-negative strains *S. enteritidis, K. pneumonia, E. agglomerans, K. oxytoca,* and *A. baumannii,* the AKGs were inactive. However, AKG 4 was active against *P. aeruginosa* and *P. mirabilis* at the highest concentration assayed.

**Table 3.** Growth inhibitory activities of synthesized alkylglycerols (AKGs) 4-7 against clinical bacterial isolates by the microtiter plate assay.

| Clinical isolates | Minimum inhibitory concentrations (MICs) of AKGs (µg/mL) |
|-------------------|--------------------------------------------------------|
|                   | 4* | 5* | 6* | 7* |
| Gram-positives    |    |    |    |    |
| *L. innocua*      | 32 | 32 | 512| 512|
| *S. aureus*       | 32 | 32 | 512| 512|
| *E. faecalis*     | 128| 64 | 512| 512|
| *B. brevis*       | 512| 64 | 512| 512|
| *M. luteus*       | 128| 128| 64 | 512|
| Gram-negatives    |    |    |    |    |
| *E. coli*         | -- | -- | 512| b |
| *P. aeruginosa*   | 512| -- | -- | 512|
| *S. marcescens*   | -- | -- | 32 | -- |
| *P. mirabilis*    | 512| 512| -- | b |

*Racemic dodecylglycerol has an MIC of 64 µg/mL against *S. aureus* (17), and this was used as a reference value. A treatment with DMF in culture medium without compound was used as bacterial growth control, while the treatment containing only assay medium served as blank control. --, Not detected under the assay conditions. Data represent the mean of three different experiments. *The ANOVA revealed significant differences (p < 0.05) between the tests and the growth control of each of these bacteria. *No significant difference was observed between treated and the bacterial growth control.*
**DISCUSSION**

In this work, natural compounds 1, 2, and 3 as well as the AKG mixture were subjected to QSI assays. The LC-MS-ESI of the AKG mixture had ions corresponding with molecular masses of 316 g/mol and 344 g/mol, respectively, and thus, 6 and 7 were identified in a 1:2 ratio as the major AKGs, along with other probable saturated AKGs with lower masses (Figure 2). Recently, only 7 had been purified (12), and unfortunately, we were unable to investigate the effects of all compounds from the two octocorals in the QSI and antibacterial assays because some of them were available in low quantities. Particularly, alkylglycerols (AKGs) are found in low concentrations in marine sponges and corals and at trace levels in mammal, including humans (16). Previous studies were developed with the most abundant AKGs or their mixtures, and recent advances in AKG synthesis have been crucial for the exploration of their biological activities (13, 14). In this work, the most common natural enantiomers of saturated AKGs 4-7 (13), were synthesized (Figure 1). One of the most suitable and well-established routes for their preparation is from a chiral precursor with an alkyl halide or sulfonate (15, 16, 19, 20). Thus, yields of the AKGs were satisfactory, ranging from 25% to 52%, as no further chromatographic purifications were performed, and sufficient material was obtained for the biological tests. Their chemical structures were confirmed by spectroscopic data with those previously published. Mass spectrometry analysis of compound 4 by LC-MS-ESI showed the followed two peaks: m/z 261.05 (34%) corresponding to the ion [M + H]+ and m/z 283.05 (100%) corresponding to the ion [M + Na]+, which are consistent with a molecular mass of 260.05 g/mol and formula of C_{15}H_{33}O_{3}. The positive optical rotation, [α]_{D}^{20} +2.2 (c 4.3, CHCl_3), is typical of the S configuration of the natural AKGs (16), confirming its optically purity, and the NMR data were similar to those obtained for AKGs 6 and 7, allowing 4 to be assigned as (2S)-3-O-dodecyl-1,2-propanediol, a natural AKG previously synthesized (13). Compound 5 had the formula C_{14}H_{30}O_{3}, by LC-MS-ESI. Its optical rotation and NMR spectra were consistent with those of 4, and it was confirmed to be (2S)-3-O-tetradecyl-1,2-propanediol, a natural and previously synthesized AKG (13). Compound 6 was C_{19}H_{40}O_{3}, its optical rotation and NMR data are similar to those of the AKG synthesized (15), and thus, it was named (2S)-3-O-hexadecyl-1,2-propanediol, a natural compound known as chimyl alcohol. Compound 7 had the formula C_{21}H_{44}O_{3}, and its optical rotation and NMR data are in agreement with those of the natural batyl alcohol (12) previously synthesized (15); therefore, it was assigned as (2S)-3-O-octadecyl-1,2-propanediol.

In relation to the natural samples tested for QSI by the microtiter plate assay, compounds 1, 2, and 3 as well as the mixture of AKGs display activity. 1 and the AKG mixture presented the largest inhibition at the same concentration as the positive control, KA (10 µg/well), while 2 and 3 were less active. Compound 1 was first isolated from a specimen of *Eunicea* collected near Providencia Island and displayed antiparasoidal activity against *Pseudoplexaura* (27). In our recent research, we isolated 1 again from *Eunicea* sp. collected in Santa Marta Bay, and its absolute configuration was determined, but it showed weak antimicrobial activity against marine bacteria (10). In this work, 1 displayed significant QSI and was as active as KA, the positive control used and a known inhibitor of QS systems (5). Other *Eunicea* and *Pseudoplexaura* cembranol diterpenes have shown prominent QSI activity (11). Surprisingly, the nonpolar fraction was as active as KA or as 1, and since this fraction is a mixture of alkylglycerols, it indicated that natural AKGs could be interesting QS inhibitors, which was another reason that motivated us to synthesize some of its most likely constituents, four of the major natural saturated alkylglycerols (13). Compound 2 was previously biosynthesized and it has been detected in the eyelids of humans and mammalians. It was isolated from *Eunicea* sp. and showed biofilm inhibition up to 91% against *S. aureus* ATCC 25923 (12). 3 only has been isolated from *Eunicea* sp., and it was effective against biofilm formation by *V. harveyi* (Phy-2A) and *P. aeruginosa* (ATCC 27853), with a percentage higher than 25% (12). In this work, compounds 2 and 3 showed QSI at 20 and 40 µg/well, respectively, but they were less active than KA.

When synthetic AKGs were evaluated by the disc diffusion assay, 4 was the most active, exhibiting the same QSI as KA (20 µg/disc), followed by 5, which had the same QSI as p-HB at 50 µg/disc, all of them without affecting the microbial growth of *C. violaceum* (ATCC 31532). Meanwhile, 6 and
7 were practically inactive to the concentrations assayed. AKGs 4 and 5 could be responsible for the QSI activity determined in the AKG mixture from *Eunicea* sp. In addition to KA, p-HB was selected as a control because it demonstrated QSI against *C. violaceum* (ATCC 31532) (28), and it is a natural component of vanilla extract, which is also a QS inhibitor (29). Other nonpolar and natural compounds that can interfere with QS include, furanones, chlorinated metabolites (called homauccins), unsaturated lactones (nocapyrones), furanosesquiterpenoid (felinixin), sesquiterpene alcohol (farnesol), unsaturated fatty acids (pitinoic acid and cis-9-octadecenoic acid), and a cycloprenyl fatty acid (lyngbyoic acid) (30, 31). In the current work, the bioactive natural and known compounds, 1, 2, 3, 4 and 5 displayed in vitro QSI for the first time.

The antibacterial potential of the synthesized AKGs was evaluated against clinical isolates of bacteria, and the MICs were determined in a microtiter plate assay. In this way, 4 and 5 showed in vitro antibacterial activity against five of the gram-positive strains: *L. innocua* and *S. aureus* (MICs of 32 µg/mL), with less effectiveness against *E. faecalis, B. brevis* and *M. luteus*. The AKGs, 4 and 5, were much less active against gram-negative clinical isolates, as shown in Table 3. These AKGs showed bacteriostatic effects on the clinical isolates because evaluation of the viability by seeding in fresh culture medium led to the recovery of each bacterial species (data not shown). The results from this study are consistent with previous observations for synthesized and racemic AKGs with chains of 8, 10, 12, 14 and 16 carbons, and among them, racemic dodecylglycerol was an effective antibacterial against the gram-positive strains of *S. aureus, E. faecium*, and *S. mutans*. It also showed low activity against the gram-negative bacteria *P. aeruginosa* and *K. pneumonia* (17, 18). However, in the present work, a MIC value of 32 µg/mL was found for the pure enantiomer 4 against *S. aureus*, which was better than that of racemic dodecylglycerol with the published value of 64 µg/mL (17). Nevertheless, previously 7 showed low activity against the gram-negative marine bacteria *Pseudoalteromonas piscida, Ruegeria* sp., *Vibrio alginolyticus, V. furnissii* and *V. harveyi* (32). We have reported that 7 isolated from *Eunicea* sp. had moderate activity against the gram-negative marine bacteria *O. pseudogringnonense* (4-4DEP) and *A. macleodii* (29-C), the reference strain *P. aeruginosa* ATCC 27853, and even the gram-positive *S. aureus* ATCC 25923 (12). Many other natural lipids show antimicrobial activity (14, 17, 30), but the considerable resistance of gram-negative clinical isolates to AKGs could be attributed to their outer membrane, which creates a protective barrier against hydrophobic compounds such as lipids (33). Among the strains that were susceptible to AKGs, *L. innocua*, which is widely distributed in nature, is able to overcome extreme conditions of pH, temperature, and salinity, and although it is not considered pathogenic, it has caused some infections in humans requiring treatments with prolonged doses of antibiotics (34). Its powerful ability to grow in certain foods has been suggested to be an expression of QS (35). *S. aureus* is a widespread pathogen and known food contaminant that has developed antibiotic resistance and can cause a variety of QS-regulated infectious diseases (36, 37). *E. faecalis* is a multiantibiotic resistant pathogen that resides in the human gastrointestinal tract, but studies have proven the potential for developing QS antagonists that could help control this pathogen (38). *M. luteus* has been identified from several sources, such as human tissues, and although it is not considered pathogenic, is it characterized by withstanding various environmental conditions (39). *B. brevis* is found in soils, air, and water, and it exhibits moderate resistance (40).

Present research provides additional information. It constitutes the first report of the QSI activity of natural compounds 1, 2, 3, 4 and 5. Additionally, the optically pure AKGs 4 and 5 showed more potent antimicrobial activity against *L. innocua* and *S. aureus* with a MIC of 32 µg/mL, and they were active against *E. faecalis, B. brevis*, and *M. luteus*.

The main limitation of this research was that the natural compounds were available in low quantities; however, these compounds may be an alternative for new studies against pathogenic microorganisms, and the results motivated us to produce other natural AKGs because of their relatively simple structures and short chemical synthesis. Also, these advances open the possibilities to obtain by synthesis that class of compounds, specifically the alkylglycerols, since they have already found some applications in several industrial and pharmaceutical domains and could be considered as promising compounds either from an economic or an ecological point of view (13, 14).
CONCLUSIONS

This study illustrated, for the first time, the importance of bioactive natural compounds 1, 2, 3, and AKGs 4 and 5 as QS inhibitors in C. violaceum (ATCC 31532). The naturally occurring enantiomers of four AKGs (4-7) were synthesized. Cembradiene 1 together with the AKG mixture from Eunicea and AKG 4 inhibit QS at the same concentration as KA. In addition, 4 and 5 displayed specific antibacterial activity against gram-positive clinical isolates in the following order: L. innocua and S. aureus (MIC = 32 µg/mL for both 4 and 5), E. faecalis (128 µg/mL and 64 µg/mL respectively), M. luteus (128 µg/mL for both) and B. brevis (512 µg/mL and 64 µg/mL, respectively). The interesting results show that additional studies are necessary to explore the prospects of controlling pathogenic microorganisms with these natural compounds or other natural AKGs obtained by synthesis.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS’ CONTRIBUTIONS

CBA performed the experimental work. ESG supervised the biological assays and contributed to the critical reading of the manuscript. BMM made contributions to the preparation of the paper as a leader of the research team. HMW supervised the research project and the drafting of the article. All authors approved the final version of the manuscript.

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