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REPORT

Natural products from Caribbean octocorals demonstrate bioactivity against *Vibrio coralliilyticus* strains

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Abstract Caribbean coral reefs are currently facing a rapid decline caused by a plethora of threats including disease outbreaks. Octocorals appear to be unaffected by the majority of diseases impacting scleractinian corals, including stony coral tissue loss disease (SCTLD) that emerged in 2014 and resulted in a mass mortality of scleractinian coral populations inhabiting Florida, the USA, and Caribbean reefs. Although the Caribbean Sea is considered a disease hot spot, few investigations into the mechanism(s) responsible for the resistance of octocorals have been conducted. In response, the capacity for octocoral-derived extracts and natural products to inhibit strains of *Vibrio coralliilyticus*, pathogenic bacteria that can cause bleaching and disease in stony corals and can co-occur in SCTLD infections, was explored. Extracts obtained from each of the four octocoral species studied demonstrated antimicrobial activity against *V. coralliilyticus*. Bioassay-guided fractionations of crude extracts from *Antillogorgia americana* were employed to identify the antimicrobial compounds, revealing the presence of secosterols in the most bioactive fractions. These results suggest that octocoral species may utilize chemical defenses to protect themselves against infection by strains of a known coral pathogen and contribute to the body of knowledge regarding the success of octocorals on Caribbean reefs.

Keywords Octocorals · Natural products · Bioactivity · *Vibrio coralliilyticus* · Coral diseases · Stony coral tissue loss

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Introduction

Diseases have been shown to play a major role in the decline of coral reef ecosystems around the world (Webster 2007; Francini-Filho et al. 2008; Rogers and Miller 2013; Montano et al. 2015; Estrada-Saldívar et al. 2020). The prevalence of these diseases has increased in recent years, leading to the devastation of some foundational reef species (e.g., the reef-building elkhorn and staghorn corals, *Acropora palmata* and *A. cervicornis,* respectively; Aronson and Precht 2001; Sutherland et al. 2011). Although our understanding of putative etiological agents, disease vectors, and transmission mechanisms remains limited, members of the Vibrionaceae, a ubiquitous marine bacterial family, have been implicated in several coral diseases (reviewed by Munn 2015). In particular, exposure to some strains of *Vibrio coralliilyticus* resulted in the onset of disease symptoms in several scleractinian coral species, suggesting that this bacterium may be a pathogen of notable concern (Ben-Haim et al. 2003; Sussman et al. 2008; Ushijima et al. 2014a, 2016). However, other strains of *V. coralliilyticus* have been isolated from both healthy and diseased corals worldwide, suggesting that the presence of this species does not always result in disease (Arboleda and Reichardt 2009; Arotsker et al. 2009; Kvennefors et al. 2010). Although *V. coralliilyticus* can be present at low density in the bacterial consortium of healthy corals, its abundance and virulence increase when temperatures are

raised (Kimes et al. 2012; Garren et al. 2014, 2016; van de Water et al. 2018). As a result, it is considered one of the most important coral pathogens under the current global climate change scenario.

Although a wide array of coral diseases has been reported across the globe, the Caribbean Sea is considered a disease hot spot due to the rapid emergence of novel diseases that have wide geographical and host ranges and the frequency of epizootic events (Bruckner 2016). One of the most devastating coral diseases to date emerged in 2014 in Broward and Miami-Dade counties of Florida and rapidly spread through the entire Florida Reef Tract and eventually to Caribbean reefs (Precht et al. 2016; NOAA Stony Coral Tissue Loss Disease Case Definition 2018; Alvarez-Filip et al. 2019; Estrada-Saldívar et al. 2020; Muller et al 2020; Brandt et al. 2021; Heres et al. 2021). Recent efforts to characterize the microbial communities associated with this novel disease, referred to as stony coral tissue loss disease (SCTLD; NOAA Stony Coral Tissue Loss Disease Case Definition, 2018), identified several potentially pathogenic and/or opportunistic prokaryotic taxa including various *V. coralliilyticus* strains that may contribute to disease initiation, cause secondary infections, and/or exacerbate tissue loss in infected corals (Meyer et al. 2019; Ushijima et al. 2020). Although *V. coralliilyticus* strains have been implicated in diseases affecting a wide range of scleractinian corals, resulting in altered coral populations and abundances on the reefs of the Caribbean Sea (Hayes et al. 2022; Álvarez-Filip et al. 2022), *Caribbean octocorals* do not appear to be affected by these diseases, including SCTLD, and their populations are thriving (Tsounis and Edmunds 2017).

Overall, despite a previous report of *V. coralliilyticus* in six *A. americana* colonies (previously classified as *Pseudopterogorgia americana*) (Vizcaino et al. 2010) and the isolation of this bacteria from diseased tissues of the temperate octocoral *Paramuricea clavata* in the Mediterranean (Bally and Garrabou 2007), no investigations into why Caribbean octocorals appear to not be susceptible to *V. coralliilyticus* have been conducted to date, and little is known about its role in octocoral disease.

Octocorals are known to produce a plethora of natural products (e.g., Look et al. 1984; Standing et al. 1984; Bandurraga and Fenical 1985; Harvell et al. 1988), including a wide variety of terpenes that have been extensively studied for pharmacological properties (i.e., antibacterial, antifungal, anticancer, and antiviral) of interest for human health and for their potential to augment the blue economy (e.g., Fenical et al. 1991; Jensen et al. 1996; Berrue and Kerr 2009; Rocha et al. 2011; Blunt et al. 2016; Raimundo et al. 2018). Despite these efforts, the bioactivity of octocoral-derived chemical compounds against marine pathogens remains largely unknown. To address this knowledge gap and potentially explain why octocorals may not be impacted by some coral diseases, this study investigated whether common Caribbean octocoral species could produce bioactive natural products that inhibit the growth of *V. coralliilyticus* strains.

Material and methods

Field collection

Five to ten small samples (3–4 cm) from multiple branches of four visually healthy colonies from each of the four common octocoral species studied (*Antillogorgia americana*, *Eunicea flexuosa*, *Gorgonia ventalina,* and *Plexaura homomalla*) were collected while SCUBA diving in May 2022 from American Shoal reef, Florida Keys, Florida, USA (24.55293° N–81.51861° W), at a depth of ~5 m using sea snips which were changed between coral species to avoid contamination. Corals were visually identified in the field before their taxonomy was confirmed in the laboratory through stereomicroscopic observations of anatomic and phenotypic features. The five to ten samples collected from each individual octocoral were placed into a separate prelabeled, resealable plastic bag (16 bags total) and transported on ice to the Mote Marine Laboratory's Elizabeth Moore International Center for Coral Reef Research and Restoration (Summerland Key, FL) and stored overnight at −20 °C. Frozen samples were transported on dry ice to the Smithsonian Marine Station (Fort Pierce, FL) and stored at −20 °C until processing. Samples were collected under Florida Keys National Marine Sanctuary Research Permit FKNMS-2019–078 and Florida Fish and Wildlife Conservation Commission Division of Marine Fisheries Management Special Activity Licenses SAL-21-2138-SRP.

Chemical extraction of octocorals samples for natural products

The four bags containing samples for each of the targeted octocoral species were thawed, combined, excess seawater removed, and weighed. To obtain the widest breadth of compounds, the pooled samples for each species were extracted three times with organic solvents at room temperature. The first two extractions used a mixture of ethyl acetate and methanol (EtOAc–MeOH, 1:1). The extractions were first sonicated for 5 min using an Edmund Scientific sonicator (Barrington, NJ, USA) and allowed to soak for about 5 h. A third extraction was carried out similarly but used 30% aqueous ethanol (EtOH). The ethanol was removed from the polar extract under vacuum at 35 °C by rotary evaporator (Buchi R300 rotavapor, New Castle, DE, USA), and the remaining water was then partitioned in a separatory funnel with n-butanol (n-BuOH-H_2O, 2:1) and allowed to stand

overnight for complete separation (Fig. 1A). This n-BuOH extract was eventually combined with the n-BuOH partition derived from the EtOAc–MeOH extract (see below). The H_2O extract was discarded as it contained mostly salts (Fig. 1A, B). The nonpolar EtOAc–MeOH extract was first partitioned in a separatory funnel using EtOAc-H_2O, 1:1 (Fig. 1B). After separating the EtOAc partitioned fraction, the water-soluble fraction was re-partitioned with n-butanol (n-BuOH-H_2O, 1:6). The resulting n-BuOH partition fraction was combined with the n-BuOH extract obtained from the polar extract (Fig. 1A, B). The extracts and partitions were filtered through grade 1 Whatman® filter papers to remove suspended particles before the solvents were evaporated under vacuum at 35 °C using a Buchi rotavapor. The dried material was resuspended in MeOH and transferred to pre-weighed 20 ml scintillation vials. A Savant Speed-Vac Vacuum Concentrator (SPD121P; Thermo Scientific) at 35 °C was used to remove the MeOH. The dried EtOAc, n-BuOH, and water partitions were weighed and stored at -20 °C until use.

Antimicrobial assays

All three partitions (EtOAc, n-BuOH, and H_2O) of the organic extracts obtained for the four octocoral species were tested for antimicrobial activity using a modified disk diffusion method (Bauer 1966; Monti et al. 2022) against five strains of *V. coralliilyticus* (Cn26H-1; Cn52H-1; OfT6-17; OfT6-21; and OfT7-21) isolated from both apparently healthy and SCTLD-affected scleractinian corals in the Florida Keys and Broward County (FL) and from SCTLD transmission experiments (Ushijima et al. 2020). Three additional *V. coralliilyticus* strains (ATCC BAA-450^T; and OCN-008 and OCN-014, both generously provided by Dr. Blake Ushijima, University of North Carolina Wilmington, NC, USA) known to elicit disease symptoms in scleractinian corals from the Indian and Pacific oceans were also tested (Ben-Haim et al. 2003; Ushijima 2014a, 2014b) as was *Pseudoalteromonas* sp. McH1-7, a putative probiotic strain active against SCTLD (Ushijima et al. 2023). Octocoral partitions resuspended in methanol at a standard

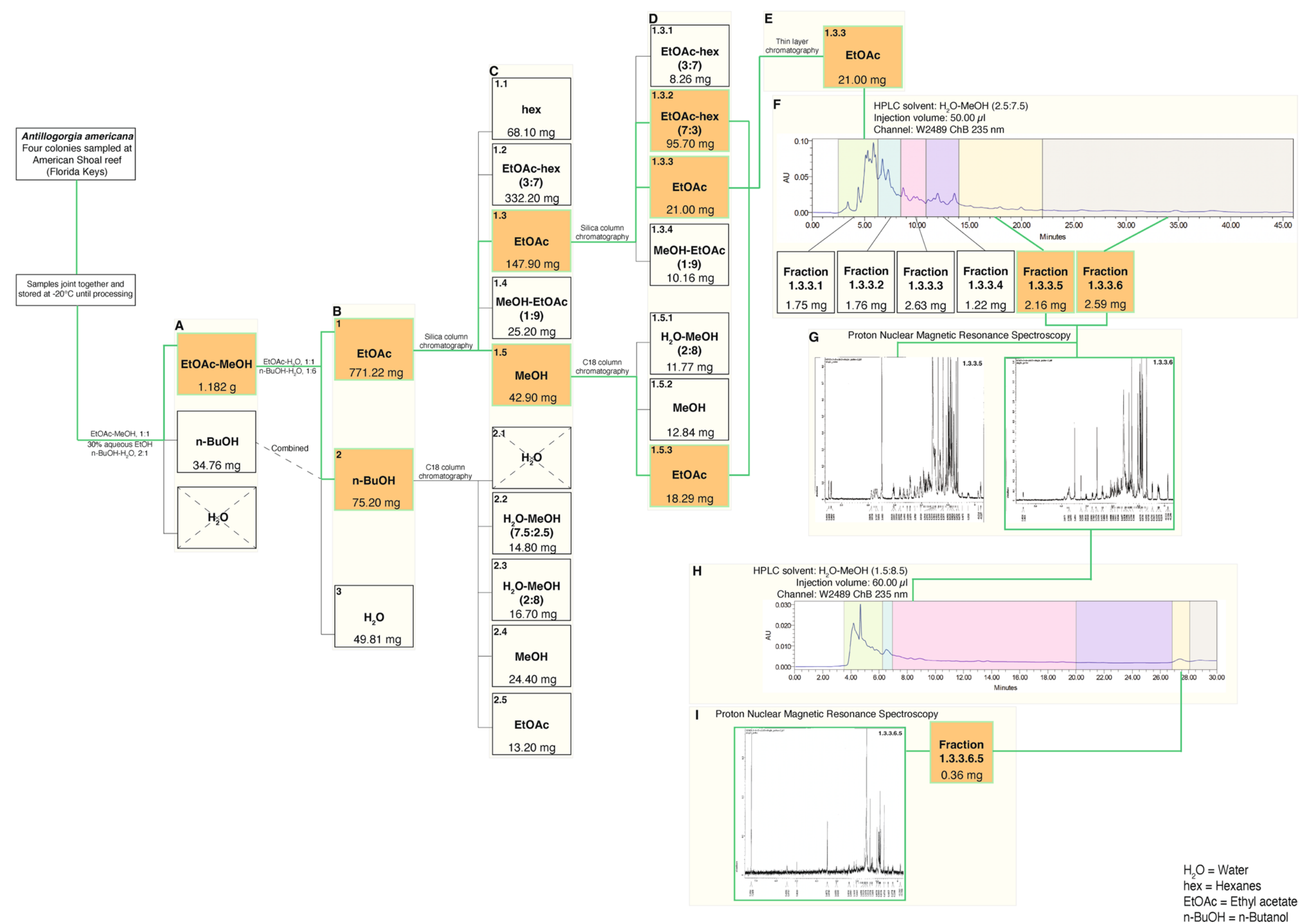


Fig. 1 Workflow for the identification and characterization of compound(s) obtained from *A. americana* active against *V. coralliilyticus* strains Cn52H-1 and OfT6-21. Extract/Partitions/Fractions highlighted in orange were selected for further analyses based on their antimicrobial activity. Solvents employed: MeOH = Methanol; EtOAc = Ethyl acetate; n-BuOH = n-Butanol; and hex = Hexanes

concentration of 31.25 mg l^{-1} were tested in triplicate using sterile paper disks (7 mm diameter) (Fisher, Whatman, cat. no. 1001–125) impregnated with 4.0 µL (125 µg) of material following Deutsch et al. (2022). The *V. coralliilyticus* strains and *Pseudoalteromonas* sp. McH1-7 were grown overnight in seawater broth (SWB: 4 g tryptone, 2 g yeast extract, 1000 ml 0.22 µm filtered seawater) at 28 °C and 220 rpm and diluted with sterile SWB until an OD_{600} between 0.5 and 0.6 was obtained. Seawater agar plates (150 × 150 mm; SWA: 4 g tryptone, 2 g yeast extract, 15 g agar, 1000 ml 0.22 µm filtered seawater) were seeded with the test organisms by spreading 200 µl of culture with sterile glass beads and dried for approximately 10 min before adding the test disks. An OD_{600} between 0.5 and 0.6 yielded a confluent yet thin lawn for each of the test organisms. The partition impregnated disks were dried before being placed onto the seeded plates along with disks impregnated with 100% MeOH (solvent control) or nalidixic acid (positive control) at 15.62 mg l^{-1} (62.50 µg) (after Deutsch et al. 2022). SWA plates were incubated for 24 h at 28 °C before bioactivity, if any, of the octocoral partitions against the *V. coralliilyticus* and *Pseudoalteromonas* strains was determined by measuring the zones of inhibition [ZOI (mm) = diameter of the inhibition zone—diameter of the paper disk] with a digital caliper to the nearest 0.01 mm (as in Monti et al. 2022).

Chemical analysis of *Antillogorgia americana* for bioactive compounds

Because species within the genus *Antillogorgia* are some of the most widespread and abundant octocorals in the Caribbean (Jordán-Dahlgren 2002; Lenz et al. 2015; Lasker and Porto-Hannes 2021), the EtOAc and n-BuOH extracts from *A. americana* were further analyzed against *V. coralliilyticus* strains to determine the active compound(s). The dried EtOAc partition was dissolved in the smallest possible volume of EtOAc–MeOH (1:1) and mixed with 4.0 g of column chromatography silica gel. The solvent was evaporated before the residue was placed on a packed silica gel column (20.0 g) and fractionated using five solvent mixtures applied to the column based on polarity (hexanes, most nonpolar, [150.0 ml]; EtOAc-hexanes, 3:7, [45.0 ml:105.0 ml]; EtOAc [150.0 ml]; MeOH-EtOAc, 1:9, [15.0 ml:135.0 ml]; MeOH, most polar, [50.0 ml]; Fig. 1C). The n-BuOH fraction was solubilized in MeOH, mixed with 1.0 g of chromatography C-18, and the dried C-18 powder was placed onto a packed C-18 reversed-phase chromatography column (5.4 g). This n-BuOH partition was subjected to column chromatography using a five-step gradient solvent system (H_2O, most polar, [20.0 ml]; H_2O-MeOH, 7.5:2.5, [30.0 ml: 10.0 ml]; H_2O-MeOH, 2:8, [6.0 ml: 24 ml], MeOH, [30.0 ml]; and EtOAc, most nonpolar, [30.0 ml]). The initial water fraction was used to remove salts from the n-BuOH partition and was not retained (Fig. 1C).

All fractions were subjected to bioassay-guided screening using disk diffusion assays to identify the most bioactive fractions. Two strains of *V. coralliilyticus,* Cn52H-1 and OfT6-21, were selected as target pathogens in accordance with Deutsch et al. (2022). Strain Cn52H-1 was found to possess the largest number of unique metabolites from a known pathogenic genus (Deutsch et al. 2022), making it an ideal candidate for bioactivity assays. Disk diffusion assays were performed as above, and fractions were tested at proportional concentrations calculated as Fraction 'i' concentration (ml/mg) = [Fraction 'i' dry weight / (Sum of *n* fraction dry weights)] * sum of *n* fraction concentrations.

Bioactive fractions were chosen for further investigation based on significant differences in size and clarity of the inhibition zones produced on the lawns of pathogens compared to the solvent control (see 'Statistical analyses'). The selected bioactive fractions were subjected to an additional round of fractionation using either normal phase column chromatography (solvent system: EtOAc-hexanes, 3:7, [15.0 ml:35.0 ml]; EtOAc-hexanes, 7:3, [35.0 ml:15.0 ml]; EtOAc [50.0 ml]; MeOH-EtOAc, 1:9, [5.0 ml:45.0 ml]) or reversed-phase chromatography (solvent system: H_2O-MeOH, 2:8, [4.0 ml:16.0 ml]; MeOH [20.0 ml], EtOAc [20.0 ml]). The newly obtained fractions were tested for bioactivity using disk diffusion assays (Fig. 1D). Three fractions exhibited substantial antimicrobial activity against the *V. coralliilyticus* strains. These active fractions were analyzed by thin layer chromatography (TLC) using different mobile phases (EtOAc; EtOAc-hexanes, 1:1; H_2O-MeOH, 1:9; MeOH [Fig. 1E]) to evaluate their complexity and possible similarity. Among these fractions, one (selected based on the clarity of the inhibition zones produced on the *Vibrio* lawns and TLC compound separation spectrum) was further purified through repeated reversed-phase (RP) high-performance liquid chromatography (HPLC). RP-HPLC was performed at room temperature of 23 °C using a Waters (Milford, MA, USA) 1525 binary HPLC pump connected to a YMC (Devens, MA, USA) HPLC semi-prep column of 250 × 10 mm RP-C-18 and using a solvent mixture of H_2O-MeOH (2.5:7.5) at a flow rate of 3.0 ml/min. The outflow was monitored using a Waters 2489 UV/visible detector with the Breeze 2 program at UV 220 and 235 nm. This method was repeated using a solvent mixture of 1.5:8.5 H_2O-MeOH to give pure compounds (Fig. 1F, H). These compounds were subjected to proton nuclear magnetic resonance (H-NMR) spectroscopy and high-resolution mass spectroscopy (HRMS) analysis. The H-NMR spectra were obtained in $CDCl_3$ on a JEOL (JEOL USA, Peabody, MA, USA) 600 MHz spectrometer running Delta software (version 4.3.6). The electrospray ionization (ESI) HRMS data were obtained using a JEOL AccuTOF-DART 4G

equipped with an ESI source operating in positive mode. The DART-HRMS data were obtained using the same instrument equipped with a Direct Analysis in Real Time (DART) ionization source (IonSense, Saugua, MA, USA) operating at 250 °C and ion guide RF voltage of 1000 V. The H-NMR, ESI-HRMS, and DART-HRMS were performed at Florida Atlantic University's Harbor Branch Oceanographic Institute (Fort Pierce, FL, USA).

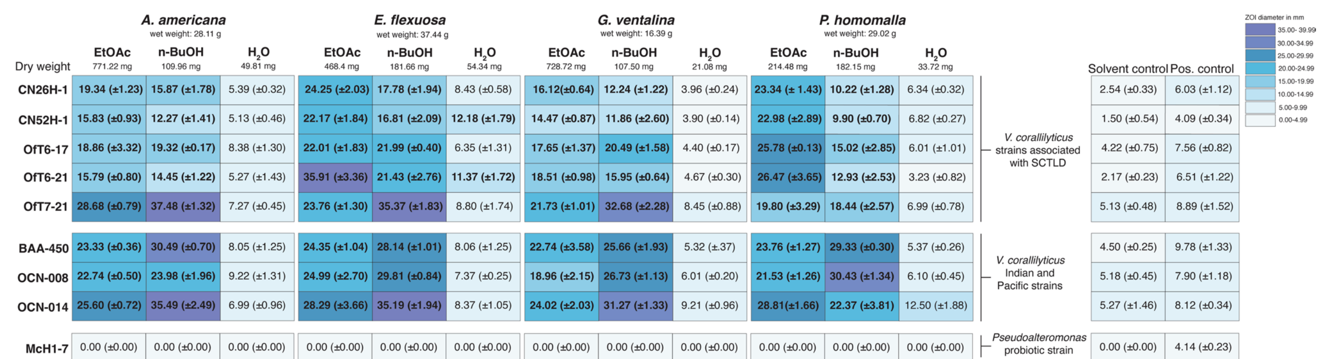
Statistical analyses

All analyses were performed using R version 3.4.3 (R Core Team 2017). Measures of zones of inhibition are expressed as the mean value of the three replicates ± standard error. To verify non-significant differences in homogeneity of variances of the data, the function *leveneTest* in the *car* package (Fox and Weisberg 2012) was performed followed by a visual inspection of the residuals. Generalized linear models (GLMs) were employed to test for possible significant differences between the sizes of the ZOIs produced by the solvent controls and those of the octocoral partitions and fractions (codes are provided in Suppl. 1). Because multiple comparisons were calculated in the GLMs, Bonferroni corrections were applied for each model.

Results

Antimicrobial activity of octocoral natural products

A total of 28.11 g, 16.39 g, 29.02 g, and 37.44 g (wet weight) of coral material was obtained from the pooled samples from four individuals of *A. americana*, *G. ventalina*, *P. homomalla*, and *E. flexuosa*,, respectively. After three rounds of extraction with different solvents and separation of the EtOAc–MeOH crude extract, three partitions for each octocoral species were obtained (Fig. 2), which were then tested for bioactivity against eight *V. coralliilyticus* strains and the putative probiotic strain *Pseudoalteromonas* sp. McH1-7. When tested at a standard concentration against *V. coralliilyticus*, the EtOAc partitions from the EtOAc–MeOH extracts from the four octocoral species produced significantly larger ZOIs compared to the solvent controls (GLMs Bonferroni adjusted $p < 0.0125$, Fig. 2, Suppl. 1), ranging from a minimum of 14.47 (± 0.87) mm obtained from *G. ventalina* on a lawn of strain Cn52H-1 to a maximum of 35.91 (± 3.36) mm from *E. flexuosa* on a lawn of strain OfT6-21. The n-BuOH partitions combined with the initial n-BuOH extract from all octocorals yielded similar significant results, with ZOIs ranging from a minimum of 9.90 (± 0.70) mm from *P. homomalla* against strain CN52H-1 to 37.48 (± 1.32) mm from *A. americana* against strain OfT7-21 (GLMs Bonferroni adjusted $p < 0.0125$, Fig. 2, Suppl. 1). *V. coralliilyticus* strains OfT7-21 and OCN-014 appeared to be most susceptible (Fig. 2). Water partitions from all octocoral species generated non-significant inhibition against all pathogen strains with the exception of those from *E. flexuosa* against strains CN52H-1 and OfT6-21 (Fig. 2, Suppl. 1). Overall, the *V. coralliilyticus* isolates from the Indian and Pacific Oceans appeared to be more susceptible to the octocoral compounds than the Florida *V. coralliilyticus* isolates, as larger ZOIs were recorded on the plates seeded with the Pacific strains. Finally, the MeOH solvent control disks produced small ZOIs on all pathogen lawns, ranging from a minimum of 1.50 (± 0.54) mm to a maximum of 5.27 (± 1.46) mm on the lawns of CN52H-1 and OfT7-21 strains respectively.



	A. americana wet weight: 28.11 g			*E. flexuosa* wet weight: 37.44 g			*G. ventalina* wet weight: 16.39 g			*P. homomalla* wet weight: 29.02 g					
Dry weight	EtOAc 771.22 mg	n-BuOH 109.96 mg	H_2O 49.81 mg	EtOAc 468.4 mg	n-BuOH 181.66 mg	H_2O 54.34 mg	EtOAc 728.72 mg	n-BuOH 107.50 mg	H_2O 21.08 mg	EtOAc 214.48 mg	n-BuOH 182.15 mg	H_2O 33.72 mg		Solvent control	Pos. control
CN26H-1	19.34 (±1.23)	15.87 (±1.78)	5.39 (±0.32)	24.25 (±2.03)	17.78 (±1.94)	8.43 (±0.58)	16.12(±0.64)	12.24 (±1.22)	3.96 (±0.24)	23.34 (± 1.43)	10.22 (±1.28)	6.34 (±0.32)	*V. coralliilyticus* strains associated with SCTLD	2.54 (±0.33)	6.03 (±1.12)
CN52H-1	15.83 (±0.93)	12.27 (±1.41)	5.13 (±0.46)	22.17 (±1.84)	16.81 (±2.09)	12.18 (±1.79)	14.47 (±0.87)	11.86 (±2.60)	3.90 (±0.14)	22.98 (±2.89)	9.90 (±0.70)	6.82 (±0.27)		1.50 (±0.54)	4.09 (±0.34)
OfT6-17	18.86 (±3.32)	19.32 (±0.17)	8.38 (±1.30)	22.01 (±1.83)	21.99 (±0.40)	6.35 (±1.31)	17.65 (±1.37)	20.49 (±1.58)	4.40 (±0.17)	25.78 (±0.13)	15.02 (±2.85)	6.01 (±1.01)		4.22 (±0.75)	7.56 (±0.82)
OfT6-21	15.79 (±0.80)	14.45 (±1.22)	5.27 (±1.43)	35.91 (±3.36)	21.43 (±2.76)	11.37 (±1.72)	18.51 (±0.98)	15.95 (±0.64)	4.67 (±0.30)	26.47 (±3.65)	12.93 (±2.53)	3.23 (±0.82)		2.17 (±0.23)	6.51 (±1.22)
OfT7-21	28.68 (±0.79)	37.48 (±1.32)	7.27 (±0.45)	23.76 (±1.30)	35.37 (±1.83)	8.80 (±1.74)	21.73 (±1.01)	32.68 (±2.28)	8.45 (±0.88)	19.80 (±3.29)	18.44 (±2.57)	6.99 (±0.78)		5.13 (±0.48)	8.89 (±1.52)
BAA-450	23.33 (±0.36)	30.49 (±0.70)	8.05 (±1.25)	24.35 (±1.04)	28.14 (±1.01)	8.06 (±1.25)	22.74 (±3.58)	25.66 (±1.93)	5.32 (±.37)	23.76 (±1.27)	29.33 (±0.30)	5.37 (±0.26)	*V. coralliilyticus* Indian and Pacific strains	4.50 (±0.25)	9.78 (±1.33)
OCN-008	22.74 (±0.50)	23.98 (±1.96)	9.22 (±1.31)	24.99 (±2.70)	29.81 (±0.84)	7.37 (±0.25)	18.96 (±2.15)	26.73 (±1.13)	6.01 (±0.20)	21.53 (±1.26)	30.43 (±1.34)	6.10 (±0.45)		5.18 (±0.45)	7.90 (±1.18)
OCN-014	25.60 (±0.72)	35.49 (±2.49)	6.99 (±0.96)	28.29 (±3.66)	35.19 (±1.94)	8.37 (±1.05)	24.02 (±2.03)	31.27 (±1.33)	9.21 (±0.96)	28.81(±1.66)	22.37 (±3.81)	12.50 (±1.88)		5.27 (±1.46)	8.12 (±0.34)
McH1-7	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	*Pseudoalteromonas* probiotic strain	0.00 (±0.00)	4.14 (±0.23)

Fig. 2 Zones of inhibition (average ± standard error in mm) produced by the partitions (EtOAc = Ethyl acetate; n-BuOH = n-Butanol; H_2O = Water) of the organic crude extracts obtained from *A. americana*, *E. flexuosa*, *G. ventalina* and *P. homomalla* when tested against *V. coralliilyticus* strains and *Pseudoalteromonas* strain McH1-7. Solvent control = 100% methanol; positive control = nalidixic acid at 15.62 mg l^{-1}. Darker color = larger zone of inhibition

Bioactive compounds from *Antillogorgia americana* active against *Vibrio coralliilyticus*

A series of bioassay-guided fractionations employing normal- and reversed-phase chromatographic separations, TLC, and HPLC of the organic extracts obtained from *A. americana* allowed for the investigation of bioactive chemical compounds produced by this octocoral against two strains of *V. coralliilyticus.* Among the three partitions obtained from separation of the EtOAc–MeOH crude extract (Fig. 1A), the EtOAc partition (Fig. 1B) demonstrated bioactivity with clear and significantly larger zones of inhibition when tested at 125 µg compared to the MeOH control (ZOI on Cn52H-1 = 15.83 (± 0.97) mm GLM Estimate = 14.327, Bonferroni adjusted $p < 0.0125$; ZOI on OfT6-21 = 15.79 (± 0.82) mm, GLM Estimate = 13.623, Bonferroni adjusted $p < 0.0125$; Table 1, Suppl. 1).

Similarly, the n-BuOH partition (Fig. 1B) tested at the same concentration was able to significantly inhibit the growth of *V. coralliilyticus* compared to the MeOH control (ZOI on Cn52H-1 = 12.27 (± 1.40) mm, GLM Estimate = 10.767, Bonferroni adjusted $p < 0.0125$; ZOI on OfT6-21 = 11.45 (± 1.22) mm, GLM Estimate = 12.277, Bonferroni adjusted $p < 0.0125$; Table 1, Suppl. 1). Conversely, the water partition did not show significant bioactivity compared to the solvent control. The EtOAc and n-BuOH partitions (Fig. 1B) were each further separated into five fractions through column chromatography (Fig. 1C). When tested against the two strains of *V. coralliilyticus*, only fraction 1.1 (hexanes) from the original EtOAc partition did not show significant differences in antimicrobial activity when tested at 13.80 µg compared to the MeOH control (Cn52H-1 GLM Estimate = 4.820, Bonferroni adjusted $p > 0.008$; OfT6-21 GLM Estimate = 6.593, Bonferroni adjusted $p > 0.008$) (Table 1, Suppl. 1). For the five fractions obtained from the original n-BuOH partition, only fraction 2.5 (EtOAc) produced significantly larger ZOIs than the MeOH control (Cn52H-1 GLM Estimate = 33.720, Bonferroni adjusted $p < 0.008$; OfT6-21 GLM Estimate = 65.880, Bonferroni adjusted $p < 0.008$; Table 1, Suppl. 1) showing that the activity was in the nonpolar portion of the n-BuOH partition. Because there were many significantly active fractions, only those that produced very clear (e.g., no haze) ZOIs, indicating complete growth inhibition, were selected for further analyses. Fraction 1.3 (EtOAc) was subjected to additional column chromatography to generate four new fractions, fractions 1.3.1 through 1.3.4 (Fig. 1D; Table 1). Fraction 1.5 (MeOH) was also selected for additional C-18 chromatography, resulting in three new fractions, fractions 1.5.1 through 1.5.3 (Fig. 1D; Table 1). Among these fractions, 1.3.2, 1.3.3, and 1.5.3 demonstrated significant bioactivity against both *V. coralliilyticus* strains Cn52H-1 and OfT6-21 (Table 1, Suppl. 1).

The three fractions with significant bioactivity were subjected to TLC (Fig. 1E), and based on the results fraction 1.3.3 was selected for HPLC separation using the solvent mixture H_2O-MeOH (2.5:7.5). HPLC guided by UV trace (235 nm) separated the fraction 1.3.3 into six new fractions (1.3.3.1–1.3.3.6), which were again tested for bioactivity (Fig. 1F; Table 1; Suppl. 1).

The two less-polar fractions (1.3.3.5 and 1.3.3.6) tested at 4.47 µg demonstrated the greatest antimicrobial inhibition against both *Vibrio* strains (Table 1; Suppl. 1). Fraction 1.3.3.6 produced clearer, although smaller, ZOIs than 1.3.3.5 (Table 1) on lawns of both pathogens. Proton NMR spectroscopy analysis of both fractions (Fig. 1G; Suppl. Figure 1, 2) indicated the presence of at least seven methyl singlets and one or two methyl doublets in the methyl region of the spectra. In addition, there were signals for the presence of hydroxy groups, unsaturation, and a cyclopropyl ring system in these molecules. The H-NMR spectrum of fraction 1.3.3.5 indicated the presence of a mixture of three or four sterols, while the proton spectrum of fraction 1.3.3.6 showed the presence of two sterols with cyclopropyl rings. This fraction was further separated through HPLC using the solvent mixture of H_2O-MeOH (1.5:8.5) (Fig. 1H). From this separation, the second most nonpolar fraction (1.3.3.6.5) eluted as a single peak at a retention time between 26.9 and 28.0 min. This HPLC peak demonstrated significant bioactivity against both *Vibrio* strains (Cn52H-1 GLM Estimate = 5.157, Bonferroni adjusted $p < 0.017$; OfT6-21 GLM Estimate = 4.883, Bonferroni adjusted $p < 0.017$; Table 1 Suppl. 1), and its proton NMR spectrum appeared to be a 9:1 mixture of two sterols. The H-NMR spectrum of the major compound showed close similarities to cyclopropyl ring-containing secosterols reported in the literature (Enwall et al. 1972; Bonini et al. 1983; Capon and Faulkner 1985; Pika et al. 1992; Migliuolo et al. 1992; Pika and Andersen 1993; Lopp et al. 1994). The proton NMR showed the presence of four methyl singlets at δ1.37, 1.02, 0.87 and 0.67 and three methyl doublets at δ0.94 ($J = 6.9$ Hz), 0.92 ($J = 6.8$ Hz) and 0.85 ($J = 6.2$ Hz). Three characteristic high field multiplets at δ0.48 (1H, m), 0.23 (1H, m), −0.13 (1H, m) indicated the presence of a tri substituted cyclopropyl ring system. The spectrum also indicated the presence of a C-3 hydroxymethine at δ3.48 (1H, m), hydroxymethylene group at δ3.88 (1H, m), 3.73 (1H, m) at C-11 position of the secosterol skeleton and an olefinic proton at δ5.48 (1H, m) at C-6 position.

The presence of a carbonyl at the C-9 position was evident from the presence of C-10 methyl at δ1.33 and C-8 proton at δ3.04. The presence of the primary hydroxyl group at C-11, a keto group at C-9, seven methyl groups, and trisubstituted cyclopropyl ring system suggested that the major compound is a cyclopropyl group-containing 9-11 secosterol. These proton NMR data closely match those reported for secogorgosterol ($C_{30}H_{50}O_3$) in the literature (Enwall et al.

Table 1 Partitions and fractions of the chemical extracts obtained from *A. americana* with data regarding their dry weights (mg), concentrations (mg/ml) used for bioassays, average diameter (± standard error) of the zones of inhibition (ZOI) produced on lawns of *Vibrio coralliilyticus* strains OfT6-21 and Cn52H-1 (mm), and average diameter (± standard error) of the ZOIs produced by the methanol solvent control and nalidixic acid positive control on lawns of strains OfT6-21 and Cn52H-1 (mm). Significant differences between ZOIs produced by partitions/fractions and the solvent control are bolded. Fractions and partitions highlighted in blue were chosen for further analyses. Significance levels were adjusted with Bonferroni corrections

Partition/fraction	Solvents	Dry weight (mg)	Test concentration (mg/ml)	ZOI Oft6-21 (mm)	ZOI neg. control (mm)	GLM p-value	ZOI pos. control (mm)	ZOI Cn52H-1 (mm)	ZOI neg. control (mm)	GLM p-value	ZOI pos. control (mm)	Significance
1	EtOAc	771.22	31.25	15.79 (±0.82)	2.17 (±0.27)	**<0.0125**	6.51 (±1.22)	15.83 (±0.97)	1.50 (±0.23)	**<0.0125**	4.09 (±0.34)	Bonferroni $p < 0.0125$
2	n-BuOH	75.20	31.25	14.45 (±1.22)	2.17 (±0.27)	**<0.0125**	6.51 (±1.22)	12.27 (±1.40)	1.50 (±0.23)	**<0.0125**	4.09 (±0.34)	
3	H_2O	49.81	31.25	5.27 (±1.44)	2.17 (±0.27)	0.038	6.51 (±1.22)	5.13 (±0.46)	1.50 (±0.23)	0.024	4.09 (±0.34)	
1.1	hex	68.10	3.45	9.33 (±0.73)	3.74 (±0.31)	0.081	8.50 (±1.19)	6.70 (±0.93)	1.86 (±0.08)	0.013	7.44 (±0.56)	Bonferroni $p < 0.008$
1.2	EtOAc-hex, 3:7	332.20	16.84	17.33 (±1.65)	3.74 (±0.31)	**<0.008**	8.50 (±1.19)	12.13 (±0.36)	1.86 (±0.08)	**<0.008**	7.44 (±0.56)	
1.3	EtOAc	147.90	7.49	16.94 (±4.97)	3.74 (±0.31)	**<0.008**	8.50 (±1.19)	9.71 (±0.72)	1.86 (±0.08)	**<0.008**	7.44 (±0.56)	
1.4	MeOH-EtOAc, 1:9	25.20	1.28	18.12 (±4.97)	3.74 (±0.31)	**<0.008**	8.50 (±1.19)	24.11 (±2.33)	1.86 (±0.08)	**<0.008**	7.44 (±0.56)	
1.5	MeOH	42.90	2.17	32.03 (±1.09)	3.74 (±0.31)	**<0.008**	8.50 (±1.19)	29.04 (±0.81)	1.86 (±0.08)	**<0.008**	7.44 (±0.56)	
2.1	H_2O	Discarded	Discarded	/	/	/	/	/	/	/	/	Bonferroni $p < 0.008$
2.2	H_2O-MeOH, 7.5:2.5	14.80	6.69	7.79 (±0.75)	3.74 (±0.31)	0.181	8.50 (±1.19)	5.12 (±2.62)	1.86 (±0.08)	0.145	7.44 (±0.56)	
2.3	H_2O-MeOH, 2:8	16.70	7.55	8.86 (±0.67)	3.74 (±0.31)	0.475	8.50 (±1.19)	4.26 (±0.26)	1.86 (±0.08)	0.274	7.44 (±0.56)	
2.4	MeOH	24.40	11.04	10.21 (±0.33)	3.74 (±0.31)	0.386	8.50 (±1.19)	9.27 (±2.45)	1.86 (±0.08)	0.039	7.44 (±0.56)	
2.5	EtOAc	13.20	5.97	68.62 (±14.34)	3.74 (±0.31)	**<0.008**	8.50 (±1.19)	36.60 (±6.43)	1.86 (±0.08)	**<0.008**	7.44 (±0.56)	
1.3.1	EtOAc-hex, 3:7	8.26	0.46	9.59 (±0.42)	2.78 (±0.78)	0.002	7.89 (±0.87)	5.29 (±0.59)	2.12 (±1.08)	0.036	7.57 (±0.23)	Bonferroni $p < 0.006$
1.3.2	EtOAc-hex, 7:3	95.70	5.30	18.28 (±1.72)	2.78 (±0.78)	**<0.006**	7.89 (±0.87)	11.62 (±1.89)	2.12 (±1.08)	**<0.006**	7.57 (±0.23)	
1.3.3	EtOAc	21.00	1.17	11.08 (±0.24)	2.78 (±0.78)	**<0.006**	7.89 (±0.87)	8.68 (±0.98)	2.12 (±1.08)	**<0.006**	7.57 (±0.23)	
1.3.4	MeOH-EtOAc, 1:9	10.16	0.56	7.67 (±0.24)	2.78 (±0.78)	0.003	7.89 (±0.87)	4.00 (±0.73)	2.12 (±1.08)	0.195	7.57 (±0.23)	

Table 1 (continued)

Partition/fraction	Solvents	Dry weight (mg)	Test concentration (mg/ml)	ZOI Oft6-21 (mm)	ZOI neg. control (mm)	GLM p-value	ZOI pos. control (mm)	ZOI Cn52H-1 (mm)	ZOI neg. control (mm)	GLM p-value	ZOI pos. control (mm)	Significance
1.5.1	H_2O-MeOH, 2:8	11.77	0.60	4.72 (±0.38)	2.78 (±0.78)	0.122	7.89 (±0.87)	3.74 (±0.07)	2.12 (±1.08)	0.262	7.57 (±0.23)	Bonferroni $p < 0.006$
1.5.2	MeOH	12.84	0.65	11.43 (±0.93)	2.78 (±0.78)	**< 0.006**	7.89 (±0.87)	6.29 (±1.13)	2.12 (±1.08)	0.008	7.57 (±0.23)	
1.5.3	EtOAc	18.29	0.92	17.50 (±1.16)	2.78 (±0.78)	**< 0.006**	7.89 (±0.87)	12.50 (±1.42)	2.12 (±1.08)	**< 0.006**	7.57 (±0.23)	
1.3.3.1	H_2O-MeOH, 2.5:7.5	1.75	1.17	6.77 (±0.12)	2.86 (±0.08)	0.008	6.94 (±0.46)	5.52 (±0.15)	1.95 (±0.22)	**< 0.007**	7.14 (±0.12)	Bonferroni $p < 0.007$
1.3.3.2	H_2O-MeOH, 2.5:7.6	1.76	1.17	6.27 (±0.11)	2.86 (±0.08)	0.008	6.94 (±0.46)	3.17 (±0.62)	1.95 (±0.22)	0.101	7.14 (±0.12)	
1.3.3.3	H_2O-MeOH, 2.5:7.7	2.63	1.17	8.04 (±1.53)	2.86 (±0.08)	**< 0.007**	6.94 (±0.46)	4.01 (±0.31)	1.95 (±0.22)	0.010	7.14 (±0.12)	
1.3.3.4	H_2O-MeOH, 2.5:7.8	1.22	1.17	5.49 (±0.71)	2.86 (±0.08)	0.032	6.94 (±0.46)	4.66 (±0.37)	1.95 (±0.22)	**< 0.007**	7.14 (±0.12)	
1.3.3.5	H_2O-MeOH, 2.5:7.9	2.16	1.17	12.25 (±1.14)	2.86 (±0.08)	**< 0.007**	6.94 (±0.46)	9.55 (±1.01)	1.95 (±0.22)	**< 0.007**	7.14 (±0.12)	
1.3.3.6	H_2O-MeOH, 2.5:7.5	2.59	1.17	7.21 (±0.89)	2.86 (±0.08)	**< 0.007**	6.94 (±0.46)	5.54 (±0.52)	1.95 (±0.22)	**< 0.007**	7.14 (±0.12)	
1.3.3.5.6	H_2O-MeOH, 1.5:8.5	0.36	1.17	7.33 (±0.85)	2.43 (±0.24)	**< 0.017**	8.40 (±0.22)	7.29 (±0.47)	2.14 (±0.42)	**< 0.017**	7.94 (±0.18)	Bonferroni $p < 0.017$

Significant differences between ZOIs produced by partitions/fractions and the solvent control are bolded

1972; Bonini et al. 1983; Capon and Faulkner 1985; Pika et al. 1992; Migliuolo et al. 1992; Pika and Andersen 1993; Lopp et al. 1994; He et al. 1995). The compound appeared to be unstable in storage at − 20 °C. High-resolution mass spectral analysis using ESI and DART methods of the stored compound 1.3.3.6.5 gave two strong peaks for molecular ion plus sodium at *m/z* 509.3637 and 513.3159 in addition to several minor peaks. ESI-HRMS gave the strongest peak at *m/z* 509.3637 for $(M+Na)^+$ (calc'd for $C_{31}H_{50}O_4Na$, 509.3606) suggesting a molecular formula of $C_{31}H_{50}O_4$ for the major component in the mixture.

Due to the small quantity of the compound, our analyses were limited to mass spectrometry and proton NMR. Therefore, the tentative structure was determined using the high-resolution mass data and H-NMR data in $CDCl_3$. The H-NMR spectrum indicated the presence of four methyl singlets: a high field singlet at δ0.67 assigned to C-18, a second methyl singlet at δ0.87 assigned to C-30, a third methyl singlet at δ1.02 assigned to C-19 methyl, and its low field shift indicated the presence of a carbonyl group at adjacent C-9 position. The presence of a carbonyl group at position-9 is characteristic for secosterols. The fourth methyl singlet at δ1.37 was assigned to the C-29 methyl. Its downfield shift suggested the presence of a hydroxy group attached to the same carbon atom C-25. The three methyl doublets at δ0.85 ($J=6.2$ Hz), 0.92 ($J=6.8$ Hz), 0.94 ($J=6.9$ Hz) were assigned to positions C-21, C-27, and C-28. A multiplet at δ3.03 indicted the presence of the C-3 hydroxymethine proton. Similarly, the signals at δ3.73 (1H) and 3.88 (1H) showed the presence of the secosterol primary hydroxyl group at C-12. A single down field signal at δ5.47 (1H) was assigned to the C-6 olefinic proton. Further, the characteristic three down field coupled multiplets at δ0.23 (1H), 0.48 (1H), and − 0.13 (1H) indicated the presence of one cyclopropyl group likely attached to the C-22 and C-23 positions. These data suggested that the bioactive compound isolated was a 31-carbon 3, 12 dihydroxy 9-oxo 5-6-ene 22-23-cyclopropyl 9-11 secosterol (Supplementary Material 2). Additional carbon-13 and several 2D NMR data are required to confirm the complete stereochemical structure of this compound. Instability of the compound and insufficient material prevented acquisition of these additional NMR data.

Discussion

Despite the increasing number of coral diseases recorded worldwide, only a few microorganisms have been identified as etiological agents with many more proposed as putative pathogens involved in the onset and/or progress of different diseases (Pollock et al. 2011; Sweet et al. 2012). Among the bacterial pathogens, *V. coralliilyticus,* which has been implicated in several diseases and syndromes affecting a wide range of scleractinian species as well as other marine invertebrates in the Indian, Atlantic, and Pacific oceans (Ben-Haim et al. 2003; Sussman et al. 2008; Ushijima et al. 2014a), is one of the best characterized. Recent studies identified strains of *V. coralliilyticus* associated with virulent SCTLD lesions, suggesting that this organism may play an important role in this unprecedented threat to Caribbean scleractinians (Ushijima et al 2020; Huntley et al. 2022). As coral diseases, including SCTLD, continue to decimate scleractinian populations on Florida and Caribbean coral reefs, the benthic assemblages of some reefs in these locations have shifted towards dominance of octocorals (Ruzicka et al. 2013; Lenz et al. 2015), as these organisms do not appear to be affected by the majority of scleractinian coral diseases (Weil et al. 2016; Rioja-Nieto and Alvarez-Filip 2019).

Here, we demonstrated the presence of antimicrobial compounds in the organic extracts of four common Caribbean octocoral species, *A. americana*, *E. flexuosa*, *G. ventalina*, and *P. homomalla*, that inhibited the growth of eight strains of *V. coralliilyticus* isolated during previous studies of scleractinian coral diseases. These diseases included tissue lysis in *Pocillopora damicornis* (Ben-Haim et al. 2003), white syndromes in the genera *Acropora* and *Montipora* (Ushijima et al. 2014a, b), and SCTLD (Ushijima et al. 2020). Our results support the body of research on octocoral bioactive compounds (e.g., Puglisi et al. 2014; Cerri et al. 2022) although few prior studies evaluated their activity against marine bacteria (Kim 1994; Jensen et al. 1996). Interestingly, these earlier studies tested organic extracts from a variety of *Caribbean octocorals*, reporting that octocorals generally did not possess potent broad-spectrum bioactivity against opportunistic marine pathogens, although extracts from individual species, including *A. americana,* were able to inhibit the growth of several bacterial strains (Jensen et al. 1996). There are a number of possible explanations for the discrepancies between our results and those of previous studies, including the type of bioassay procedures employed, the identity of the test organisms used, or changes to the composition of the octocoral-associated microbial communities that may have occurred over time. Nevertheless, the antimicrobial activity of the octocoral natural products extracted from the four Caribbean octocoral species against the suite of *V. coralliilyticus* strains tested in this study may represent a mechanism to allow octocorals to avoid colonization by or control the proliferation of this opportunistic pathogen and may in part explain their success on Caribbean reefs.

Equally intriguing is the lack of bioactivity of the organic extracts from the four species of octocorals against the putative coral probiotic *Pseudoalteromonas* sp. McH1-7. Studies showed that the application of this organism to SCTLD-affected scleractinian corals stopped or slowed the progression of disease both ex situ (Ushijima et al.

2023) and in early field tests (Meyer et al. 2019; Paul et al. 2021). This lack of bioactivity against *Pseudoalteromonas* sp. McH1-7 may permit the expansion of current efforts to treat SCTLD, as the active components in the octocoral-derived organic extracts could be used as probiotic adjuvants targeting opportunistic pathogens, such as *V. coralliilyticus*. *V. coralliilyticus* has been shown to co-infect diseased scleractinian corals and increase the rate of tissue loss (Ushijima et al. 2020). Additional studies are warranted to assess the specificity of the bioactivity of the octocoral chemical extracts and/or purified active compounds on the coral-associated microbial community, as negative impacts on beneficial community members may result in further damage to host health.

Although both polar and nonpolar fractions of the chemical extracts derived from *A. americana* demonstrated bioactivity against the *V. coralliilyticus* strains tested, the compounds obtained from nonpolar fractions created clearer zones of inhibition on pathogen lawns. Similarly, Kim (1994) reported that polar fractions obtained from eight octocorals were less effective than nonpolar fractions against six bacterial species. Interestingly, all fractions analyzed showed some level of inhibition against *V. coralliilyticus* strains OfT6-21 and Cn52H-1, suggesting the presence of multiple bioactive compounds across a wide range of polarity. Our results support other studies that found the genus *Antillogorgia* to be one of the most highly chemically defended *Caribbean octocorals* (Pawlik et al. 1987; Fenical et al. 1987; Harvell et al. 1988; O'Neal and Pawlik 2002; Epifanio et al. 2007; Berrue and Kerr 2009).

The bioactivity of nonpolar EtOAc fractions of *A. americana* that consistently demonstrated bioactivity against *V. coralliilyticus* strains became the focus for compound structure elucidation. HPLC separations and NMR analyses revealed the presence of secosterols, natural products that have been previously found in octocorals (e.g., Ciereszko et al. 1989; Epifanio et al. 2007; Sarma et al. 2009; Marrero et al. 2010; Rocha et al. 2011). Our proton NMR spectra were similar to the proton NMR spectra reported for secogorgosterol and suggested that the major bioactive compound is a cyclopropyl group-containing 9-11 secosterol. Although *A. americana* is known to produce several 9-11 secosterols (Enwall et al. 1972; Musmar and Weinheimern 1990; He et al. 1995, 2017; Naz et al. 2000; Sica and Musumeci 2004; Epifanio et al. 2007), the study on the potential ecological functions of these compounds remains still relatively unexplored. One study conducted by Epifanio et al. (2007) identified two secosterols (9-11 secogorgosterol and 9-11 secodinosterol) from Bahamian *A. americana* colonies that deterred fish feeding activity both in aquaria and in situ, suggesting that these molecules provide chemical defense against predation.

Because octocoral holobionts were used for chemical extractions, the origin of the natural products encountered could not be elucidated (e.g., produced by the host octocoral, by members of its associated microbial community including Symbiodiniaceae, or by an interaction between holobiont members). Culture experiments have shown that the dinoflagellates living in octocoral tissues have the ability to produce gorgosterol and dinosterol (Withers et al. 1982; Ciereszko 1989), while Kerr et al. (1996) experimentally showed that radiolabeled gorgosterol was transformed into 9-11 secogorgosterol by an enzyme extract of *A. americana*. Accordingly, Epifanio et al. (2007) suggested that antipredatory secosterols in *A. americana* were dinoflagellate-produced prior to subsequent oxidation by the octocoral host to form C-ring-seco-sterols. Further studies are warranted to determine the origin and biosynthetic pathway of the bioactive compounds in our study.

Despite the extensive body of the literature regarding octocoral-derived chemical compounds and their pharmaceutical properties, their potential bioactivity against marine pathogens remains surprisingly unexplored. To the best of our knowledge, this study is the first to investigate the activity of octocoral-derived natural products against a suite of pathogenic *V. coralliilyticus* strains and provides a baseline for additional research. The results generated information that might explain the apparent resistance of octocorals to many scleractinian coral diseases and provide insights into the success of these organisms within the benthic communities of Caribbean and Mesoamerican reefs.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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