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CHEMICAL SCIENCES

The Brazilian octocoral *Phyllogorgia dilatata* as a source of cytotoxic compounds

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Abstract: The extensive marine biodiversity has proved to be a promising source of substances with biomedical potential. In this study, the cytotoxicity of the Brazilian octocoral *Phyllogorgia dilatata* (Gorgoniidae) was evaluated against two tumor cell lines and three bacterial strains. The methanol/dichloromethane crude extract presented no antibacterial activity up to the highest concentration tested (512 µg/mL), however it revealed a noteworthy antiproliferative effect against HCT-116 (80%) and MCF-7 (54%) cell lines at 50 µg/mL. Therefore, guided by the cytotoxic activity, a multistep chemical fractionation of the extract provided the subfraction 5 (PDPH2-5) with IC_{50} values of 3.18 and 17.80 µg/mL against HCT-116 and MCF-7, respectively. The LC-HRMS/MS analysis of PDPH2-5 showed ions of *m/z* 219.1742 and 219.1743, characterized as (*E,E*) and (*Z,E*) germacrone, after a LC-DAD-SPE/NMR analysis of the hexanic fraction and comparisons of NMR data with the literature. Previously reported assessments to the cytotoxic activity of the (*E,E*)-diastereoisomer disclosed higher IC_{50} values than that obtained for the PDPH2-5 fraction, suggesting, herein, a potentiated effect of the diastereoisomeric mixture. Such remark encourage further bioactivity studies with stereoisomer mixtures and reduce the urge for compound isolation.

Key words: Anticancer, cytotoxic, germacrone, gorgonian, marine natural products, octocoral.

INTRODUCTION

Marine organisms have been presented as an alternative source of compounds with potential use in cancer chemotherapy. Substances such as cytosine arabinoside (Cytarabine®) and trabectedin (Yondelis®) are anticancer drugs in clinical use that are related to compounds from marine invertebrates (Jimenez et al. 2018).

Octocorals from the Gorgoniidae family have been reported for their vast biological properties, such as antiplasmodial, antituberculosis, anti-inflammatory, antiviral, antimalarial, and anticancer, which, in turn, have been attributed to secondary metabolites, mostly diterpene structures (Berrue & Kerr 2009, Almeida et al. 2014). This family is very abundant and diverse in the Atlantic Ocean, however, *Phyllogorgia dilatata* Esper, an endemic species from the Brazilian coast, has only few compounds described, including a sterol, nardosinane and germacrane sesquiterpenes, an epoxipukalide diterpene, carotenoids and long-chain polyenal pigments (Oigman et al. 2015). This species is known to possess antimicrobial activity (de Lima et al. 2013), as well as feeding deterrent (Epifanio et al. 1999) and antifouling properties (Epifanio et al. 2006), but no evaluation of the anticancer

activity has been carried out, so far. Therefore, the present study aimed to assess the cytotoxicity against tumor cell lines and antibacterial activity of methanol/dichloromethane crude extract and fractions obtained from *P. dilatata* and further identify the compounds present in the active fractions.

MATERIALS AND METHODS

Collection and identification of octocoral material

The *Phyllogorgia dilatata* Esper (Gorgoniidae) collection at Tartaruga beach, Armação dos Búzios, Rio de Janeiro State, Brazil (22°45´S; 41°51´W), as well as the taxonomic identification and voucher specimen deposit (PD1295) was described in previous studies (Martins & Epifanio 1998, Epifanio et al. 2006). The study is registered in National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) under the number AB724BB.

Preparation and fractionation of crude extract

Phyllogorgia dilatata tissues (1,364 g of dry weight) were cut into small pieces and extracted at room temperature by maceration with a mixture of methanol/dichloromethane (1:1) (once - during 6 hours), and pure dichloromethane (twice - during 20 hours each). The extracts were combined and evaporated under reduced pressure yielding 151 g of a viscous dark brown crude extract (PDEB - 11% of dry weight). A portion of the crude extract (35.14 g) was stirred with a mixture of distilled water and methanol (1:4) then partitioned twice with hexane, affording two low polarity fractions (PDPH1, 30.7 g, and PDPH2, 1.0 g) and one high polarity fraction (PDPMe, 2.4 g).

PDPH2 (971 mg) was subjected to a flash column chromatography (Still et al. 1978) using silica gel F60 (230-400 mesh) Silicycle®. Initially, the column was eluted with dichloromethane/ethyl acetate (95:5) (19 fractions), then with dichloromethane/ethyl acetate (1:1) (3 fractions) and, finally, with pure methanol (3 fractions). The fractions were analyzed by thin layer chromatography (TLC) on silica gel and comparable fractions were combined, yielding 17 fractions (PDPH2 1-17).

LC-HRMS/MS analysis

LC-HRMS/MS analysis were carried out on an ultra-high-resolution liquid chromatography system (Nexera®, Shimadzu) equipped with two LC-30AD quaternary pumps, DGU-20A5R degasser, SIL-20AC autoinjector, SPD-30MA diode array detector, CTO-20AC oven, 6-port column switching selector valve, and a CBM-20A interface, coupled to a high-resolution mass spectrometer (HRMS/MS) equipped by an electrospray interface (ESI) and a quadrupole time-of-flight (QqTOF) hybrid system Impact HD (Bruker Daltonics, Bremen, Germany). Data acquisition and processing was performed using Data Analysis® 4.0 Software (Bruker Daltonics GmbH, Bremen, Germany).

The chromatographic analysis of sample PDPH2-5 (1 mg/mL) was performed on an Acquity UPLC BEH C-18 column (Waters®, 50 x 2.1 mm, 1.7 µm), with 5 µL of injection volume and flow 0.4 mL/min. The mobile phase was ultrapure water added with 0.1% of formic acid (v/v) (solvent A) and acetonitrile (solvent B) and the linear gradient elution profile was 30 to 80% of B in 20 min and kept at this concentration for 1 min, then the initial condition was reestablished (30% B) and kept for 4 min at this concentration (total run time of 25 min). The temperature of the auto-sampler was set at 4 °C. The LC-HRMS/MS analyses were carried out at 30 °C.

The analysis was done with mass spectrometer operating in positive ion modes

(*m/z* 100–1000). The capillary voltage applied was 4500 V, end plate offset 500 V, nitrogen was employed as the nebulizer gas (1 bar), dry heater temperature 200 °C, drying gas (8 L/min) and collision cell energy 8 eV. The mass spectrometer was programmed to perform acquisition in auto MS/MS mode (number of precursors 4) in experiments with different collision energy of 18-45 eV for all *m/z* range analyzed.

LC-DAD-SPE/NMR analysis

Liquid chromatography with diode array detector-solid phase extraction/nuclear magnetic resonance (LC-DAD-SPE/NMR) analyses were carried out using an Agilent liquid chromatographic system (1200 series, Agilent GmbH) equipped with a quaternary pump (G1311A) and a degasser (G1322A), a variable wavelength diode array detector (G1315D), an autosampler (G1329A), and an automatic cartridge exchanger (ACE™ Spark Holland, Germany). The LC system was controlled by the HyStar® 2.3 software (Bruker). A Knauer (K120 Knauer Smartline Pump Control 100, Bruker Daltonik GmbH©, V01.11) makeup pump diluted the post column flow with water before the peaks were trapped using a Prospekt II SPE® unit.

Fraction PDPH2 was prepared in THF (10 mg/mL) and submitted to thirty consecutive chromatographic runs, using an analytical C-18 Luna® column (150 × 4.6 mm, 10 µm) with 10 µL injection volumes and flow 1 mL/min. The mobile phase was ultrapure water (solvent A) and acetonitrile (solvent B) and the linear gradient elution profile was 30 to 80% (v/v) of B in 60 min. The elution was monitored based on UV absorption at 200 nm. Two fractions were collected by time-slice. The substances were pumped into a HySfere Resin GP cartridge (10 mm × 2 mm × 10 mm) of spherical phase of polyvinylbenzene. After the adsorption process, cartridges were dried with nitrogen gas for 30 min to remove residual solvents and eluted with acetone-d_6 directly into NMR tubes (Deutero 3 mm o.d).

The 1D (^{1}H and ^{13}C) and 2D (HSQC, HMBC, ^{1}H-^{1}H COSY) NMR spectra of fractions were acquired using a Bruker Avance III instrument (14.1 Tesla, 600 MHz, Ultrashield Plus®) equipped with an automatic sample changer and a triple resonance inverse cryoprobe of 5 mm (^{1}H/^{13}C/^{15}N) with *z*-field gradient. Data collection and processing were carried out using the Bruker software Topspin 3.2 version. Chemical shifts (δ) were reported in ppm and coupling constant (*J*) reported in Hertz.

(*E*,*E*)-Germacra-1(10),4,7(11)-trien-8-one (**1**): ^{1}H NMR (600.00 MHz, acetone-d_6) δ: 1.42 (3H, s, H-15); 1.62 (3H, s, H-14); 1.73 (3H, s, H-12); 1.79 (3H, s, H-13); 2.06 (1H, s, H-2a); 2.16 (2H, m, H-3); 2.42 (1H, m, H-2b); 2.81 (1H, m, H-6a); 2.90 (m, H-9a); 2.93 (m, H-6b); 3.40 (1H, d, *J*=10.3 Hz, H-9b); 4.75 (1H, dd, *J*=11.55, 2.57 Hz, H-5); 4.99 (1H, m, H-1). ^{13}C NMR (150.0 MHz, acetone-d_6) δ: 15.1 (C-15); 16.4 (C-14); 19.3 (C-12); 21.8 (C-13); 24.1 (C-2); 29.2 (C-6); 38.1 (C-3); 55.7 (C-9); 125.7 (C-5); 126.9 (C-10); 129.3 (C-7); 132.5 (C-1); 134.9 (C-4); 137.5 (C-11); 205.9 (C-8). LC-ESI-HRMS/MS - *m/z* (relative abundance in 23.4/20.2 eV): 219.1742 $[M+H]^+$ (219.1743 calcd. for $C_{15}H_{23}O$, Δ = 0.6 ppm – 30.0/74.7), 204.1513 $[M+H-CH_3]^{\cdot+}$ (17.8/16.3), 201.1651 $[M+H-H_2O]^+$ (0.9/5.7), 189.1279 $[M+H-C_2H_6]^+$ (11.3/5.5), 177.1265 $[M+H-C_3H_6]^+$ (1.6/5.6), 161.1331 $[M+H-C_3H_6O]^+$ (8.9/21.6), 149.1327 $[M+H-C_4H_6O]^+$ (0.7/1.79), 149.0963 $[M+H-C_5H_{10}]^+$ (100/100), 123.1170 $[M+H-C_6H_8O]^+$ (24.9/32.2), 111.0809 $[M+H-C_8H_{12}]^+$ (7.2/7.9).

(*Z*,*E*)-Germacra-1(10),4,7(11)-trien-8-one (**2**): ^{1}H NMR (600.00 MHz, acetone *d*-$_6$) δ: 1.57 (3H, s, H-15); 1.65 (3H, s, H-12); 1.74 (3H, s, H-13); 1.89 (3H, m, H-14); 1.95 (s, H-3); 2.11 (m, H-2); 2.91 (H-6); 3.10 (2H, s, H-9); 5.11 (1H, t, *J*=7.52, H-5); 5.30 (1H, td, *J*=8.34, 1.28, H-1). ^{13}C NMR (150.0 MHz, acetone-d_6) δ: 17.7 (C-15); 18.8 (C-13); 21.5 (C-12); 25.3 (C-14); 28.4 (C-6); 28.6 (C-2); 37.4 (C-3); 47.5 (C-9); 121.7

(C-5); 125.8 (C-1); 132.8 (C-7); 136.7 (C-4); 136.6 (C-11); 209.9 (C-8). LC-ESI-HRMS/MS - *m/z* (relative abundance in 23.4/20.2 eV): 219.1743 $[M+H]^+$ (219.1743 calcd. for $C_{15}H_{23}O$, Δ = 0 ppm – 20.9/61.6), 204.1497 $[M+H-CH_3]^{\cdot+}$ (1.7/-), 201.1651 $[M+H-H_2O]^+$ (4.1/29.0), 189.1227 $[M+H-C_2H_6]^+$ (0.7/-), 177.1622 $[M+H-C_2H_2O]^+$ (1.5/-), 177.1277 $[M+H-C_3H_6]^+$ (1.3/7.6), 163.1125 $[M+H-C_4H_8]^+$ (23.9/68.2), 149.0975 $[M+H-C_5H_{10}]^+$ (12.4/25.1), 135.0809 $[M+H-C_6H_{12}]^+$ (4.8/8.7), 121.1017 $[M+H-C_6H_{10}O]^+$ (4.9/19.3), 109.1016 $[M+H-C_7H_{10}O]^+$ (100/100).

Cytotoxicity assay

Human tumor cell lines HCT-116 (colorectal carcinoma; CCL-247) and MCF-7 (breast adenocarcinoma; HTB-22) were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 (HCT-116) or DMEM/F-12 (MCF-7) media supplemented with 10% fetal bovine serum, supplemented with 1% antibiotics (penicillin + streptomycin) at 37 °C with 5% CO_2 and 95% relative humidity. To maintain cultures in exponential growth, cells were split periodically.

Cytotoxicity of PDEB and fractions was evaluated by the MTT assay (Mosmann 1983) against both cell lines, initially, in a qualitative approach. In such cases, cells were plated in 96-well plates (10^4 cells/well in 200 µL/well) and left to adhere for 24 h. Each sample, dissolved in DMSO, was added to their respective wells, making up to final concentrations of 5 and 50 µg/mL, then incubated during 72 h. Selected cytotoxic samples were tested for a quantitative approach, in a 7-concentration curve, between 0.003 and 50 µg/mL, and cells were also exposed for 72 h. Doxorubicin was used as a positive control, while negative control groups received the sample dilution vehicle, DMSO. At the end of the incubation period, the supernatant in each well was substituted with fresh media added with 0.5 mg/mL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide). After 3 h, the supernatant was removed, the precipitate product was dissolved in 150 µL DMSO and the absorbance was measured using a multiplate reader at 570 nm.

Absorbance values for each treatment were transformed to percentage of cell growth inhibition based on positive (100% inhibition) and negative (0% inhibition) controls parameters, for analysis of qualitative data. Nevertheless, for the quantitative analysis, IC_{50} and respective 95% CI (confidence interval) and R^2 were obtained by non-linear regression of transformed absorbance values using GraphPad Prism 7.0 software.

Antimicrobial assay

Minimal Inhibitory Concentration (MIC) against *Staphylococcus aureus* HU25, *Staphylococcus epidermidis* ATCC12228 and *Escherichia coli* ATCC 11775 were determined by the broth microdilution according the Clinical & Laboratory Standard Institute (2009). Colonies were collected from TSA plates, added into 5 mL of BHI medium. The bacteria were grown in aerobic conditions at 37.5 °C for 4–5 hours. The suspension was prepared based on the density of 0.5 McFarland standard and 100 µL of the suspension of the respective strain was added to each well of a 96-well plate containing 100 µL of BHI media with the crude extract diluted in DMSO to make up the final test concentrations, ranging from 4 µg/mL to 512 µg/mL. After 24 h incubation at 37 °C, 20 µL of resazurin at 1 mg/mL was added to each well, further incubated for 4 h and visually analyzed for color change from pink to blue. MIC was defined as the lowest concentration of the extract that inhibited the growth of 99.9% of the initial inoculum.

RESULTS

The *Phyllogorgia dilatata* cytotoxic methanol/dichloromethane crude extract (PDEB) was subjected to a cytotoxicity-guided fractionation. Initially, PDEB was fractionated by liquid-liquid partition, giving two fractions with hexane (PDPH1, PDPH2) and one with methanol/water (PDPMe). TLC analysis showed distinct profiles for the hexane fractions, therefore those were not combined. The cytotoxic fraction PDPH2 was thus subjected to another fractionation step yielding 17 subfractions. Fraction 5 (PDPH2-5) displayed the strongest cytotoxicity and its chemical profile was investigated by LC-HRMS. After optimization of the chromatographic conditions, the sample was analyzed in positive ionization mode. The chromatographic profile revealed two $[M+H]^+$ ions at *m/z* 219.1742 and 219.1743 (Figure 1), further suggesting isomers of $C_{15}H_{22}O$ with errors below 1.0 ppm.

LC-DAD-SPE/NMR were performed to isolate the two stereoisomers from PDPH2 fraction. The compounds were characterized as (*E*,*E*)-germacra-1(10),4,7(11)-trien-8-one (**1**, t_R = 38.29–40.30 min) and (*Z*,*E*)-germacra-1(10),4,7(11)-trien-8-one (**2**, t_R = 41.74–43.95 min) by 1D (^{1}H and ^{13}C) and 2D NMR (COSY, HSQC and HMBC) and the data were according to the literature (Simova et al. 1984, Oigman et al. 2015). These compounds have already been reported for *P. dilatata* (Oigman et al. 2015).

The MS/MS spectra of **1** and **2** also confirmed their *E*,*E* and *Z*,*E* configurations, respectively. The major differences rely on the base peak and in the presence of the fragment *m/z* 201, relative to the 1,4-dehydration of **2.** For compound **1** the methyl group in C-10 is less accessible for interaction with carbonyl group in C-8. On the other hand, after the loss of the isopropyl moiety from C-7 (*m/z* 177 $[M+H-C_3H_6]^+$), the electronic repulsion caused by the position of the methyl groups in **2** restrict the hydride rearrangement for the formation of the more stable tertiary/allylic carbocation. Therefore, *m/z* 149 $[M+H-C_5H_{10}]^+$ is unstable in **2**, presenting very low abundance, while in **1** it is the base peak. Proposed fragmentation pathways are showed in Figures 2 and 3.

The crude extract (PDEB) and its fractions were evaluated for qualitative cytotoxicity against two human cancer cell lines (HCT-116: colorectal carcinoma; MCF-7: breast adenocarcinoma). PDEB presented a relevant inhibition of cell proliferation at the highest concentration tested, reducing over 80% growth of HCT-116 cells and nearly 60% of MCF-7 cells (Figure 4).

Among all samples obtained from the bioguided fractionation protocol carried out herein, faction 5 (PDPH2-5) displayed the strongest activity, inhibiting over 90% proliferation of HCT-116 and MCF-7 cells at 50 µg/mL and, remarkably, above 70% at 5 µg/mL (Figure 5). Quantitative assessment was also performed to establish the IC_{50} values for PDEB and for the most active fractions PDPH2 and PDPH2-5 (Table I). MCF-7 cells were consistently less sensitive to tested samples, while PDPH2-5 showed an IC_{50} value as low as 3.18 µg/mL for HCT-116 cell line.

Antimicrobial potential against Gram-positive (*Staphylococcus aureus, Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli*) bacterial strains was also investigated. No antimicrobial activity of crude extract was observed until the highest concentration tested (512 µg/mL).

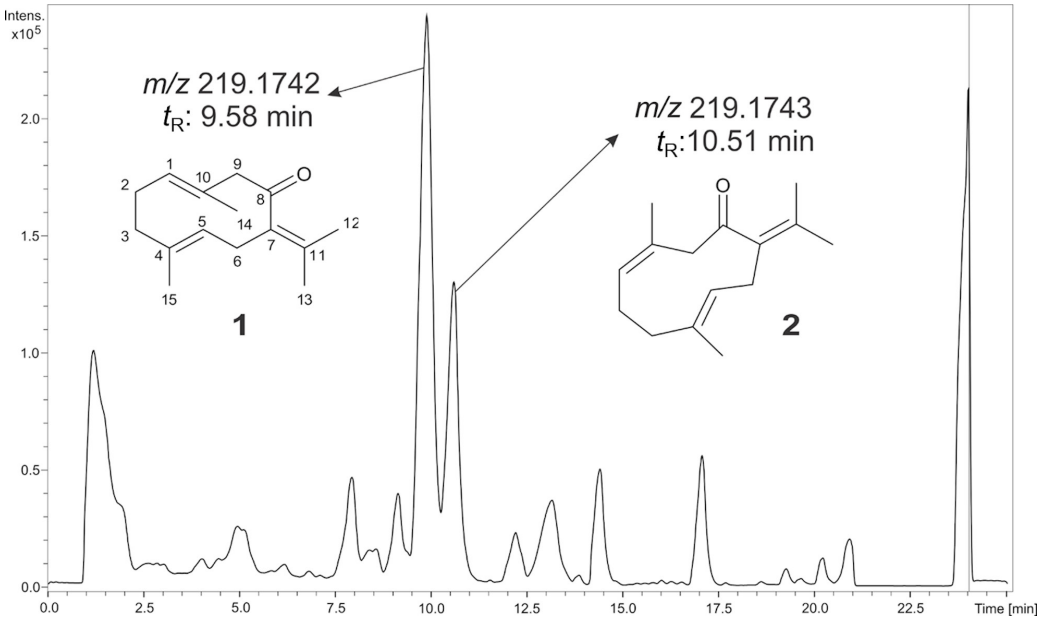

Figure 1. **Base peak chromatogram of fraction PDPH2-5 using Acquity UPLC BEH C-18 column, 5 µL injection volume, flow 0.4 mL/min, mobile phase ultrapure water with 0.1% of formic acid (v/v) (solvent A) and acetonitrile (solvent B) in gradient elution 30 to 95% of B in 21 min.**

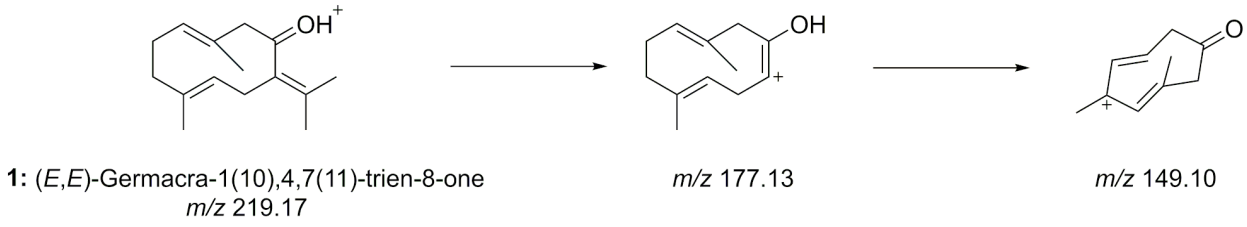

Figure 2. **Proposed fragmentation pathways of (*E*,*E*)-germacra-1(10),4,7(11)-trien-8-one (1).**

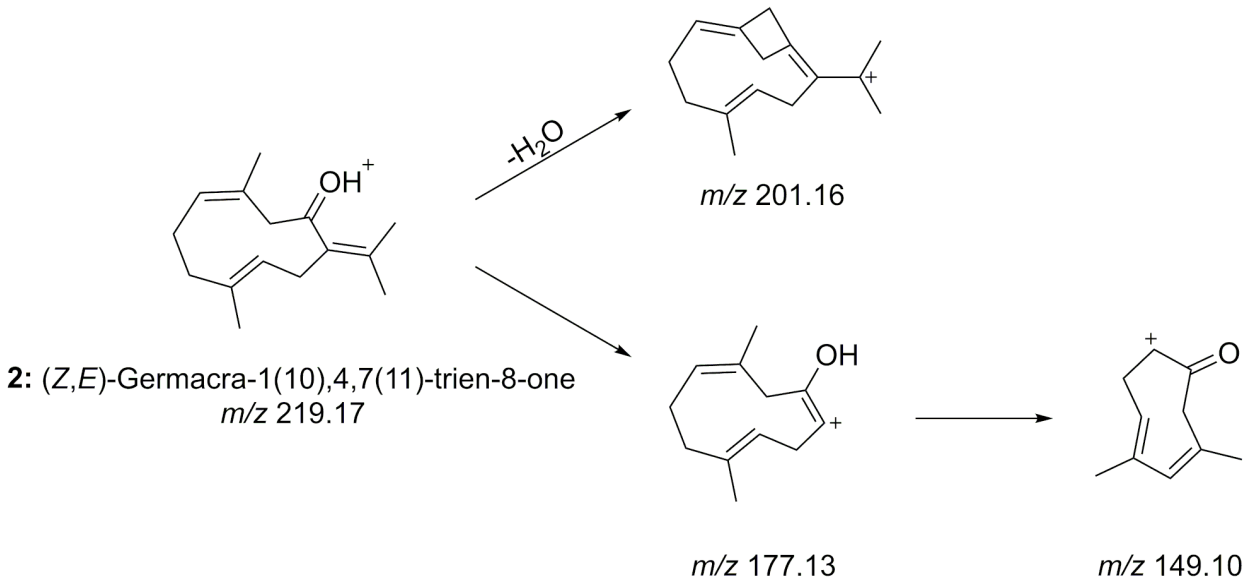

Figure 3. **Proposed fragmentation pathways of (*Z*,*E*)-germacra-1(10),4,7(11)-trien-8-one (2).**

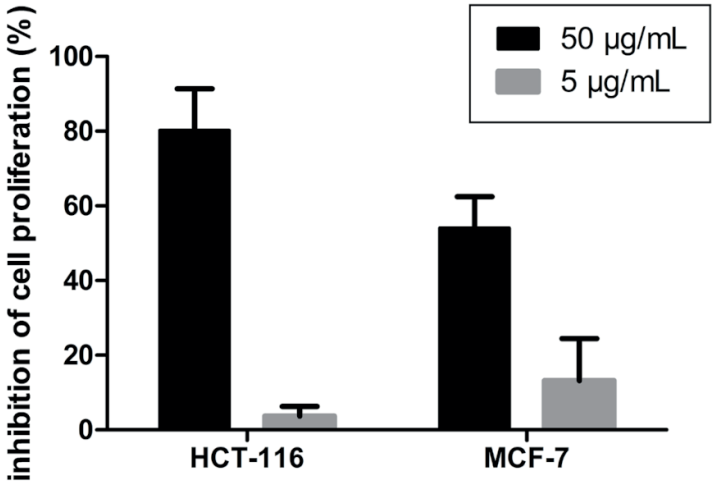

Figure 4. Inhibition of cell proliferation by the crude extract of *Phyllogorgia dilatata*. Two human tumor cell lines, HCT-116 and MCF-7, were exposed to 50 µg/mL and 5 µg/mL of *Phyllogorgia dilatata* crude extract for 72 h. Cell proliferation was assessed by the MTT assay. Data are presented as mean ± SEM from 2 independent experiments performed in duplicate.

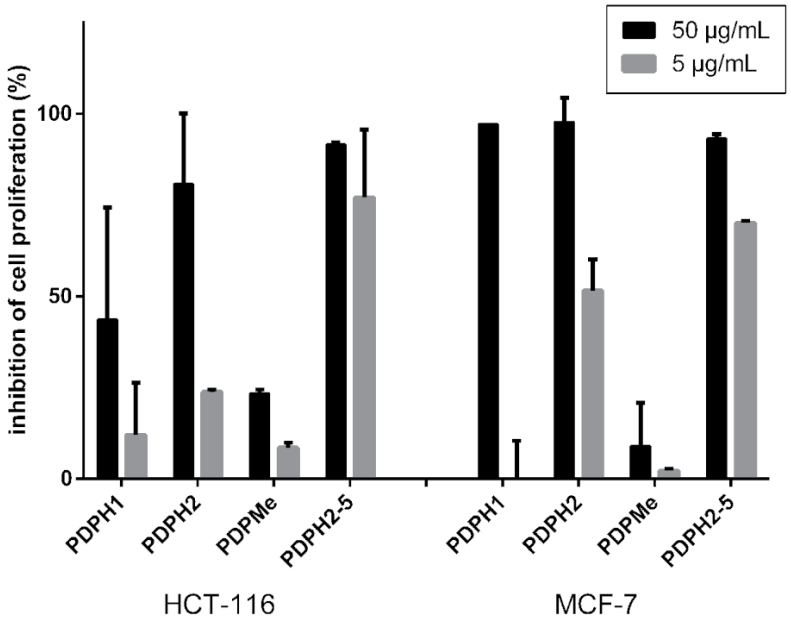

Figure 5. Inhibition of cell proliferation by fractions of the crude extract of *Phyllogorgia dilatata*. The cytotoxicity of the samples against HCT-116 and MCF-7 cells was assessed by MTT assay after treatment with 50 µg/mL and 5 µg/mL for 72 h. Data are presented as mean ± SEM from 2 independent experiments performed in duplicate.

Table I. Cytotoxic activity of *Phyllogorgia dilatata* crude extract (PDEB) and fractions (PDPH2 and PDPH2-5) in HCT-116 ($IC_{50\ HCT-116}$) and MCF-7 ($IC_{50\ MCF-7}$) cells.

Sample	$IC_{50\ HCT-116}$ (µg/mL)[a]	CI95%	R^2	$IC_{50\ MCF-7}$ (µg/mL)[a]	CI95%	R^2
PDEB	29.31	10.78 – 181.80	0.7797	> 50.00	–	–
PDPH2	10.82	4.19 – 21.63	0.7907	25.39	11.11 – 90.27	0.8523
PDPH2-5	3.18	2.06 – 4.79	0.9456	17.80	12.24 – 27.07	0.8694
Doxorubicin[b]	0.03	0.02 – 0.05	0.9809	0.15	0.04 – 0.63	0.9262

[a] the cytotoxic activity was evaluated by the MTT assay after 72 h incubation. IC_{50}, CI 95% and R^2 values were calculated through a non-linear regression curve. Results are expressed in µg/mL.
[b] Doxorubicin was used as a positive control.

DISCUSSION

The results obtained through a bioassay guided fractionation approach of the PDEB extract revealed a mixture of known diastereoisomers **1** and **2** (fraction PDPH2-5) with promising cytotoxic activity against HCT-116 and MCF-7 cell lines. Previously, compound **1** isolated from the plant *Curcuma zedoaria*, revealed antiproliferative activity against MCF-7 cells, with IC_{50} = 59.1 µg/mL (Hamdi et al. 2014). In the present study, the fraction enriched with the mixture of stereoisomers **1** and **2** displayed IC_{50} = 17.80 µg/mL, a value more than 3 times below that of substance **1** alone in the same cell line. It is known that isomerism can lead to different therapeutic uses, once stereoisomers differ in pharmacokinetic and pharmacodynamic properties (Chhabra et al. 2013).

Some studies have explored the dissimilar cytotoxicity or anticancer activity of *Z*/*E* isomers. Iwata et al. (1997) illustrate this contrast in their work with (*Z*)- and (*E*)-3-hydroxy-3'-methylchalcone, where the (*Z*)-isomer inhibited proliferation of HGC-27 cells nearly twice as much than the (*E*)-isomer. Recently, Ubel et al. (2019) showed different IC_{50} values for *α* and *β*-asarone in HepG2 cells. The authors also demonstrated the synergistic effect reached by different proportioned mixtures of these isomers.

Therefore, our finds suggest two hypotheses: **2** is more active than **1** and these compounds present additive effect, or **1** is more potent than **2** and they present synergistic effects. Nevertheless, a proper study of these particular propositions would require an extensive analysis with the isolated compounds, which are beyond the scope of this work.

The identification of synergistic compound combinations is essential and particularly attractive for clinical use once it allows lower doses of future drugs to be used, reducing the side-effects (Gregan et al. 2014). The observations pointed here stimulate new studies of bioactivity with mixtures of stereoisomers and reduce the need for isolation of the compounds, which is often a laborious step in the search for bioactive substances of natural origin.

Herein, PDEB did not retrieve a potential antimicrobial activity, as the outcome reported by de Lima et al. (2013) in their assessment of such bioactivity with an aqueous crude extract and peptides obtained from *P. dilatata*. This indicates that the antimicrobial compounds of this organism must be restricted to primary metabolites instead of secondary metabolites, as explored in the present study.

CONCLUSION

In conclusion, crude extract obtained from Brazilian octocoral *P. dilatata* and its fractions showed *in vitro* cytotoxic effects against human breast and colorectal tumor cell lines. The LC-HRMS was able to identify the two stereoisomers which were isolated by LC-DAD-SPE and further characterized by NMR as (*E*,*E*) and (*Z*,*E*)-germacra-1(10),4,7(11)-trien-8-one, which may be responsible for the observed activity.

Acknowledgements

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