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Research Paper

Anti-inflammatory potential of invasive sun corals (Scleractinia: Tubastraea spp.) from Brazil: alternative use for management?

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Abstract

Aim The objective was to analyse the anti-inflammatory potential of the invasive coral species *Tubastraea coccinea* and *Tubastraea tagusensis*.

Methods Methanolic extracts, fractions and synthesized compounds were evaluated for their anti-inflammatory ability, and their composition was elucidated through chemical analysis.

Key findings The genus *Tubastraea* (Order Scleractinia, Family Dendrophylliidae) (known as sun corals) presents compounds with pharmacological value. The introduction of these azooxanthellate hard corals into Brazil, initially in Rio de Janeiro state, occurred through their fouling of oil and gas platforms from the Campos oil Basin. The two invasive species have successfully expanded along the Brazilian coast and threaten endemic species and biodiversity. The HPLC-MS and GC-MS data suggest the presence of aplysinopsin analogues (alkaloids). Anti-inflammatory activity was observed in all samples tested in *in-vivo* assays, especially in *T. coccinea*. The ethyl acetate fraction from this sample was more effective in *in-vitro* assays for anti-inflammatory activity. Depending on the concentration, this fraction showed cytotoxic responses.

Conclusions These species have potential pharmacological use, and considering their invasive nature, this study presents a potential alternative use, which may enhance the management of this biological invasion.

Introduction

Natural products research has as one of its final objectives the discovery of new compounds for the treatment of human diseases. In recent years, the sources of these substances have shifted from the terrestrial to the marine environment, as the marine molecules generally demonstrate unique structural patterns due to the greater marine biodiversity.[1,2] The marine invertebrates, among them corals, are responsible for most of these substances, whose associated activity comprises the anti-inflammatory effect.[3,4] Recently (2012–2013), marine compounds in the preclinical stage with several related pharmacological activities, including the anti-inflammatory effect, were reviewed.[5] In that study, 257 marine compounds were described of which 19, mostly terpenoids, are from corals and have anti-inflammatory activity. In a previous study (2009–2011) by the same group using the same parameters, 226 marine compounds were described, of which 17 substances with anti-inflammatory activity were identified from cnidarians, and most of them also terpenoids.[6]

Anti-inflammatories can be divided into three groups: biological drugs, steroidal and non-steroidal anti-inflammatory drugs. While the use of biological drugs predisposes a high cost to the treatment, the other two groups present adverse reactions due to their continuous use. Steroidal anti-inflammatory drugs, also called corticosteroids, are a set of hormones that interact with a glucocorticoid receptor

and regulate protein expression. The anti-inflammatory effect generated by this group occurs by blocking the release of prostaglandins by inhibiting the activity of the enzyme phospholipase A2, thus interfering in the cascade of arachidonic acid reactions. Non-steroidal anti-inflammatory drugs act by inhibiting cyclooxygenase (COX) enzymes, which may be non-selective (COX-1 and COX-2) or selective (COX-2 isoform only).[7–9] However, both corticosteroids and non-steroidal, selective and non-selective anti-inflammatory drugs have side effects as a result of prolonged use. Steroidal anti-inflammatory compounds increase susceptibility to infections because of their immunosuppressive effect, alter blood pressure and are related to gastric ulcers. Non-selective COX inhibitors generate gastrointestinal complications, and COX-2-selective drugs induce or aggravate arterial hypertension.[10–12]

Corals of the Order Scleractinia secrete a calcium carbonate skeleton, which provides some physical defence against their predators and competitors.[13–15] The production of secondary metabolites also contributes to defence. These substances are of particular interest since they have considerable pharmacological potential. There are records in the literature that have demonstrated the isolation of secondary metabolites with biological activity originating from scleractinian corals (see Table S1).[16–21]

The genus *Tubastraea* was first recorded on the Brazilian coast in the late 1990s.[22] The introduction of these invasive azooxantellate corals (Dendrophyllydae), also known as sun corals, was through fouling on oil and gas platforms brought to the Campos Basin in the northern region of Rio de Janeiro state, in the 1980s. Over the years, these invasive species, identified as *T. coccinea* Lesson, 1829 and *T. tagusensis* Wells, 1982, have successfully expanded along the Brazilian coast.[23,24] The biological invasion has reached the coast of Santa Catarina, São Paulo, Espírito Santo and Bahia states,[25,26] thus increasing national awareness of this phenomenon, where the introduction of these exotic species into local ecosystems has had significant impacts upon the biodiversity of the invaded ecosystems.[23,24,27,28]

Tubastraea spp. are a threat to endemic organisms of the invaded regions, which characterizes them as invasive species. Among the threatened organisms, the endemic coral *Mussismilia hispida* (Scleractinia, Mussidae) can be highlighted. Studies have shown that interactions between these species generated adverse effects, with tissue necrosis of *M. hispida*.[29] Although there is evidence that these invading corals can produce allelochemicals against potential competitors for space,[26,28,30] a physical response using the mesenteric filaments is also quite effective in corals.[31] The production of secondary metabolites may also be responsible for the low rates of predation by generalist fish and for preventing the fouling of other marine organisms in competition for space in the substrate. Both factors, together with their rapid growth and active sexual reproduction, have contributed to the expansion of the *Tubastraea* genus in Brazilian waters.[30,32]

In response to the bioinvasion process, the Sun-Coral Project was launched in 2006 aiming to contribute to the formulation of public policies on issues relating to the sun coral in Brazil. Part of this initiative involves investigating potential uses for these species, which may enhance control efforts and offset the environmental costs. Further studies of the potential of biological and chemical activities of these species are thus further justified by the value attributed to these corals, which are discarded in the management process.[33,34] This study aims to investigate the anti-inflammatory potential of extracts and fractions of the invasive corals *T. coccinea* and *T. tagusensis* collected in Ilha Grande Bay (RJ), to establish their potential pharmacological use. An underlying rationale for this study was that human use of wild invasive species can provide beneficial consequences for conservation of the environment by providing an alternative pathway to manage biological invasions such as the sun corals on the Brazilian coast.

Materials and Methods

Collection

The colonies of the invasive scleractinian corals *T. coccinea* and *T. tagusensis* were collected by SCUBA diving at 8 m depth, in September 2014, at Ilha dos Macacos, Ilha Grande Bay (S 23°04′58″ – W 44°14′13″), Rio de Janeiro, Brazil. The material was collected by the Sun-Coral Project technical team. Samples were sorted, weighed and frozen, until the extraction process was started. The total wet weight of colonies of *T. coccinea* was 19.5 kg and *T. tagusensis* was 19.4 kg. For this study, 10.4 kg of *T. coccinea* and 10.9 kg of *T. tagusensis* were used.

Extraction

The colonies were dried by lyophilizer K 105, Liotop. The lyophilized colonies were screened a second time for the removal of fouling organisms. The samples were macerated and extracted on ultrasound Ultra Cleaner 1600, Unique, three times consecutively with MeOH. The extracted material was filtered on Qualy filter paper and stored at room temperature in amber bottles, protected from light. This material was concentrated under reduced pressure to remove the solvent. The crude methanolic extracts of *T. coccinea* and *T. tagusensis*, respectively, EbMTc and EbMTt, were obtained at the end of this process.

Due to the EbMTc results obtained in carrageenan-induced paw oedema assay, fractions were prepared from

10 g in 500 mL ethanol and water (1:1 v/v). After solubilization, the partition was performed sequentially from low to high polarity solvents. The materials generated after the fractionation were stored in amber bottles. These samples were also rotary dried under reduced pressure to remove organic solvents. The solvents used were hexane, dichloromethane, ethyl acetate and n-butanol, yielding the respective fractions: f-Hex, f-DCM, f-AcOEt and f-BuOH. The residual material was named f-Residual.

Preparation of 5-(indol-3-ylmethylene)-hydantoin (3′-deimino-2′,4′-bis(demethyl)-3′-oxoaplysinopsin) – APL01

Indolecarbaldehyde (0.500 g, 3.4 mmol) and hydantoin (1.00 g, 10.0 mmol) were combined with morpholine (2.00 g) and heated at 105°C for 3 h. The reaction readily formed a thick yellow paste. The mixture was allowed to cool and was then diluted with aqueous acetone. The solid was isolated by filtration and washed with aqueous acetone. The solid was dried in a warm oven (50°C) to constant mass. The dried yellow coloured solid weighed 0.720 g, 92% yield. Spectroscopic data were consistent with previously published data.[35] ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.77 (bs, NH), 10.86 (bs, NH), 10.10 (bs, NH), 8.10 (s, 1H), 7.72 (d, J = 8.5 Hz, 1H), 7.39 (d, J = 8.5 Hz, 1H), 7.12 (m, 2H), 6.73 (s, 1H). ^{13}C NMR (75 MHz, DMSO) δ 166.06, 155.96, 136.50, 127.61, 127.45, 124.31, 123.01, 120.85, 118.70, 112.57, 109.07, 102.47.

Gas Chromatography–Mass Spectrometry (GC-MS)

The filtered fractions from EbMTc were analysed using a gas chromatograph (Agilent 6890N model) coupled to a high-resolution mass spectrometer (model 5973N) (CG-MS) with an automated injector (model 7683) and a DB-5MS column (30 m, 0.250 mm and 0.250 μm). Helium was used as carrier gas with a flow rate of 1.5 mL/min, and the samples were injected in split mode (with flow division), at 1:20 rate. The method employed an initial temperature of 60 °C/min, heating rate from 10 °C/min to 220 °C, another ramp heated up to 290 °C, with a heating rate of 5 °C/min with final isotherm of 30 min. Three replicate injections of each sample were performed. The mass spectra obtained were compared to those contained in the Wiley 275 library for identification.[36]

Chromatograms were analysed using Agilent software, MSD ChemStation version E.02.02.1431. All peaks that represented up to 10% of the major peak area of each fraction were selected to be identified by the libraries and sampled their abundances.

High Performance Liquid Chromatography–Mass Spectrometry (HPLC-MS)

EbMTc, EbMTt and f-AcOEt were used in this methodology. The samples were solubilized in 95:5 (v/v) proportion with mobile phases A (water with formic acid 0.1% (v/v)) and B (MeOH with formic acid 0.1% (v/v)), at 0.5 mg/mL concentration, filtered and injected into the system. The equipment used was Shimadzu's Prominence Liquid Chromatography System, with detector module with diode/UV array, scanning range of 190 to 800 nm and Rheodyne manual injector with 20 μL loop. The column used was Thermo's Hypersyl C18, dimensions 150 x 2.1 mm and 3 μm particle diameter and 10 x 2.1 mm precolumn, from Thermo. The mobile phase flow occurred at 0.2 mL/min, oven temperature at 40 °C with a gradient elution of 5% B over 1 min, 5%–95% B over 30 min, 95% B over 13 min, 95–5% B over 2 min and 5% B over 15 min.

The mass spectrometer used was the Bruker Maxis Impact model, ESI-Q-TOF configuration, with a scanning range of 20 to 1200 m/z, internal calibration with 100 μM sodium formate in water and isopropanol (1:1 v/v). Data-dependent acquisition (DDA/AutoMS), with isolation/fragmentation of 1 precursor per cycle, electrospray ionization source at positive polarity, nebulizer pressure at 1,5 bar, gas flow at 8 L/min and temperature of 210 °C. The UV light absorption readings for all substances analysed were 194 nm due to the use of formic acid in the mobile phase. This reagent has absorption in 193 to 250 nm range.[37]

The software Compass Data Analysis Ink and Compass Isotope Pattern (Bruker Daltonics) were used for the result analysis as the followed comparison parameters: (1) arbitrary intensity and overlap; (2) isotope pattern; and (3) experimental mass accuracy at 5 ppm,[38] by equation:

$$\text{ppm} = \left(\frac{\text{accurate mass} - \text{measured mass}}{\text{accurate mass}}\right) \times 10^6, \quad (1)$$

The base peak chromatogram (BPC; black), where the base peak is the ion with the maximum abundance, represents the samples, and the extracted ion chromatogram (EIC; grey) expresses the masses concerning the suggested molecules described in the literature.

Animals

Male mice aged 3–4 months, weighing 25–35 g of the Swiss Webster (SW) lineage raised at the Biotherm of UERJ Biochemistry Department, were used. The experiments were carried out according to ethical standards for experimental animal use and approved by the Ethics Committee of IBRAG-UERJ (CEUA-IBRAG) according to protocol 05/2009 and 007/2013.

Carrageenan-induced paw oedema model

The samples used in this experiment were EbMTc, EbMTt and APL01 according to Ref. [39] with adaptations. The animals were separated in different groups ($n = 5$), pretreated with intraperitoneal (i.p.) injection and had water and food removed. Paw volumes were measured in Ugo Basile plethysmometer (t0). The negative control group animals were treated with the vehicle (15% ethanol, 1.25% Tween-20 in distilled water), and the treatment with the standard drug (indomethacin 10 mg/kg) was used for the positive control, while the other groups received the treatment in determined doses (0.2; 20; 200 mg/kg) by body weight.

After 1 h of pretreatment, the groups were injected with 50 µL (subplantar) of carrageenan (6 mg/mL) into the right hind paw. Paw volumes were measured at t1 (1 h), t2 (2 h), t3 (3 h) and t4 (4 h) after carrageenan injection.

The following formulas were used to calculate the oedema index:

$$\text{Edema index } (\%) = \left(\frac{\text{edema value } t_x \times 100}{\text{edema value } t_0}\right) - 100, \quad (2)$$

and the inhibition of oedema:

$$\text{Edema inhibition } (\%) = 100 - \left[\frac{(\text{edema value } t_x \times 100)}{\text{mean of control edema}}\right], \quad (3)$$

where:

t_x = paw volume at different times evaluated;

t_0 = paw volume before injection of the carrageenan phlogistic agent.

The experiments were performed in duplicate, and at the end, the animals were euthanized in a CO_2 chamber. The literature describes that the peak of oedema in the negative control group is observed after 3 h of carrageenan injection and from the second check of the inflamed paw. Due to the previous treatment, it is expected that there will be no significant increase in the paws, but rather that values similar to those measured in t0. The results are expressed as a percentage of the oedema index as a function of time (h).[39–41]

NO releasing by LPS-stimulated RAW 264.7 macrophages

The RAW 264.7 cell line was added in 96-well plates at 5.0 x 10^4 cells/well concentration in DMEM supplemented with 10% FBS and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h for cell adhesion. The cells were then incubated in the presence and absence of lipopolysaccharide (LPS) at 1.0 µg/mL final concentration and treated for 24 h. The EbMTc, EbMTt, APL01, f-Hex, f-DCM, f-AcOEt, f-BuOH and f-Residual samples were used in treatment at 0.25; 2.5; 5.0; 50; 100 µg/mL concentrations. DMEM was used to dilute the samples. 50 µL of supernatant from each well was transferred to another plate, and the cells had their volume completed at 100 µL for the cytotoxic test.[41] In supernatant, sulfanilic acid at 10 mg/mL concentration was added, and the plate was incubated for 10 min. Then, *N*-naphthyl ethylenediamine at 1 mg/mL concentration was added, and the plate was again incubated for 10 min, protected from light and at room temperature. Finally, the absorbance was obtained at 550 nm wavelength in a microplate reader. The NO concentration in µM was established with sodium nitrite ($NaNO_2$) as reference.[42,43]

Cell culture

The HepG2 cell line (human hepatocellular carcinoma) was provided by Dr. Danielle Palma de Oliveira, Faculty of Pharmaceutical Sciences of Ribeirão Preto, São Paulo, Brazil. The RAW 264.7 cell line was provided by Dr. Marsen Garcia Pinto Coelho, Department of Biochemistry of the University of the State of Rio de Janeiro, Rio de Janeiro, Brazil. HepG2 was maintained in Eagle's minimum essential medium (MEM) (9.5 g/L MEM; 1.76 g/L $NaHCO_3$; 0.88 g/L sodium pyruvate; 21.6 mg/L aspartic acid; 16.8 mg/L L-serine; streptomycin 100 mg/L; penicillin 70 mg/L; pH 7.4; Sigma-Aldrich), and RAW 264.7 was maintained in Dulbecco's modified Eagle's medium (DMEM) (17.3 g/L DMEM; 3.7 g/L $NaHCO_3$; streptomycin 100 mg/mL; penicillin 70 mg/mL; pH 7.4; Life Technologies, São Paulo, SP, Brazil). Both supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), incubating at 37 °C in a humidified atmosphere containing 5% CO_2.

Mitochondrial enzymatic activity through WST-1 assay

Fresh cells were added in 96-well culture plates at 1.0 x 10^4 cells/ml (HepG2 in MEM) and 5.0 x 10^4 cells/ml (RAW 264.7 in DMEM) concentrations, supplemented with 10% FBS. After plating, the culture plates were placed in a 5% CO_2 atmosphere at 37 °C for 24 h for cell adhesion. After the 24-, 48- and 72-h treatment with f-AcOEt concentrations (0.25; 2.5; 5.0; 50 and 100 µg/mL) or 2% Triton X-100 (positive control), culture medium was replaced by 90 µL fresh medium and 10 µL WST-1 reagent (Cell Proliferation Kit WST-1; Cat. No. 05015944001; Roche, South San Francisco, CA, USA) and incubated at 37°C with 5% CO_2 for 3 h. This assay consists of a tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate) in formazan crystal reduction, both

water-soluble. This reduction occurs through the mitochondrial metabolism by dehydrogenase enzyme action, which allows evaluating the presence of metabolically active cells in culture.[44] The absorbances were obtained by ELISA microplate reader (CELER, Polaris model), with 450 nm wavelength. Treatment with the respective culture media was used as negative control. The mitochondrial enzymatic activity was established as a percentage of negative control absorbances. All experiments were performed in triplicate and according to the manufacturer's instructions.

Lactate dehydrogenase release assay

This assay is based on the relationship between cells in lysis or with damage to cytoplasmic membrane and the release and activity of LDH enzyme in the supernatant. With membrane rupture, this enzyme is released into extracellular medium and acts on lactate to oxidative pyruvate reaction, leading to nicotinamide adenine dinucleotide molecule reduction (NAD^+ for $NADH^+H^+$). In a second step, the catalytic reagent diaphorase transfers the H/H^+ to tetrazolium salt (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) which it has reduced in formazan salt.[45] Fresh cells were added in 96-well culture plates at 1.0 x 10^4 cells/ml (HepG2 in MEM) and 5.0 x 10^4 cells/ml (RAW 264.7 in DMEM) concentrations, supplemented with 10% FBS. After plating, the culture plates were placed in a 5% CO_2 atmosphere at 37 °C for 24 h for cell adhesion. After the 24-, 48- and 72-h treatment with f-AcOEt concentrations (0.25; 2.5; 5.0; 50 and 100 μg/mL) or 2% Triton X-100 (positive control), the supernatant (100 μL) was replaced in a new 96-well culture dish. Then, 100 μL of LDH reagent (Cytotoxicity Detection Kit (LDH), Cat. No. 11644793001; Roche) was added to the supernatant, and the reaction occurred protected from light for 30 min. Lastly, the absorbances were obtained by ELISA microplate reader at 492 nm wavelength. Treatment with the respective culture media was used as negative control. Interaction analysis between f-AcOEt concentrations and LDH reagent was performed due to the staining present in samples (background). LDH release in supernatant was quantitated relative to positive control used (Triton X-100) absorbances with background being discounted for each sample concentration. All experiments were performed in triplicate and according to the manufacturer's instructions.

MTT survival assay

The assay was performed in 96-well plates with 80-90% confluent and adherent cell by 100 μL volume culture medium/well. The samples were solubilized in DMEM and added to the cells, and then maintained for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. After 21 h, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium) at 5 mg/mL was added. After 3 h, the plate was centrifuged at 3000 rpm/5 min for MTT crystals formed deposition. The supernatant was discarded and the crystals diluted by 100 μL of DMSO (100%) addition per well. The reading was performed on a microplate reader at 492 nm. Cell cultures in treatment absence were used as a negative control, and as a positive control, the cells were cultured in 100% MeOH presence.[46]

Statistical analysis

The data are expressed as the mean and standard deviation using one-way ANOVA test ($P < 0.05$) followed by Dunnett or Tukey ($P < 0.01$ and $P < 0.001$) according to the applied methodology.[47] The Microsoft® Office Excel and GraphPad Prism® 5 programs (Targetware Informática Ltd, São Paulo, SP, Brazil) for Windows were used. All the experiments were repeated at least twice.

Results

GC-MS analysis

The analysis of EbMTc fractions by GC-MS showed a total of 47 compounds. The percentage values of these compounds were 38.30% for fatty acid esters, 27.66% for sterols, 12.77% for fatty acids, 8.51% for hydrocarbonates, 4.26% for alkaloids, 2.13% for aldehydes, 2.13% for alcohols, 2.13% for ether and 2.13% for fatty acid amides. The observed compounds are shown in Table 1.

HPLC-MS analysis

The HPLC-MS results of EbMTc, EbMTt and f-AcOEt (Table 2) were compared with the substances found in *Tubastraea* described in the literature. According to the parameters used, the protonated masses of 3'-deimino-3'-oxoaplysinopsin as m/z of 256.1081 $[M + H]^+$ and its 6-bromo halogenated analogues as m/z of 334.0186 $[M + H]^+$ and 336.0168 $[(M + 2)+H]^+$ were identified in all analysed samples by extracted ion chromatogram (EIC). In 3'-deimino-3'-oxoaplysinopsin chromatogram, it is noted that there is a change in peak signal intensity in EbMTc and EbMTt base peak chromatograms (BPC), which may indicate the presence of its E and Z isomers. The tubastrine protonated mass corresponded to m/z of 194.0924 $[M + H]^+$ was observed in EbMTc and f-AcOEt. The 3'-deimino-2',4'-bis(demethyl)-3'-oxoaplysinopsin (APL01) protonated mass corresponded to m/z of 228.0768 $[M + H]^+$ was identified in EbMTc and f-AcOEt as well as its 6-bromo halogenated derivatives m/z of 305.9873

Table 1 Composition of fractions from EbMTc by GC-MS analysis

MM[a]	MF[b]	Compound
164	$C_{12}H_{20}$	(E, E, E)-1,4,8-dodecatriene
224	$C_{16}H_{32}$	Cyclohexadecane
228	$C_{14}H_{28}O_2$	Myristic acid
252	$C_{18}H_{36}$	1-octadecene
254	$C_{16}H_{30}O_2$	Palmitoleic acid
255	$C_{14}H_{13}N_3O_2$	3′-deimino-3′-oxoaplysinopsin
255	$C_{16}H_{33}NO$	Palmitamide
256	$C_{16}H_{32}O_2$	Palmitic acid
264	$C_{17}H_{28}O_2$	Methyl-(7E,10E,13E)-7,10,13-hexadecatrienoate
264	$C_{18}H_{32}O$	(9Z)-9,17-octadecadienal
268	$C_{17}H_{32}O_2$	Methyl palmitoleate
270	$C_{17}H_{34}O_2$	Methyl palmitate
270	$C_{18}H_{38}O$	Stearyl alcohol
281	$C_{20}H_{40}$	Cycloeicosane
282	$C_{18}H_{34}O_2$	Oleic acid
283	$C_{19}H_{38}O$	1-methoxy-1-octadecene
284	$C_{18}H_{36}O_2$	Heptadecanoic acid
284	$C_{18}H_{36}O_2$	Stearic acid
294	$C_{19}H_{34}O_2$	Methyl linoleate
296	$C_{19}H_{36}O_2$	Methyl oleate
296	$C_{19}H_{36}O_2$	Methyl trans-8-octadecenoate
299	$C_{19}H_{38}O_2$	Methyl stearate
308	$C_{20}H_{36}O_2$	Ethyl linoleate
318	$C_{21}H_{34}O_2$	Methyl arachidonate
333	$C_{22}H_{36}O_2$	Ethyl arachidonate
334	$C_{14}H_{12}BrN_3O_2$	6-bromo-3′-deimino-3′-oxoaplysinopsin
371	$C_{26}H_{42}O$	(22Z)-26,27-dinorergosta-5,22-dien-3β-ol
385	$C_{27}H_{44}O$	(22Z)-27-norergosta-5,22-dien-3β-ol
385	$C_{27}H_{44}O$	22-dehydrocholesterol
387	$C_{27}H_{46}O$	Cholesterol
397	$C_{28}H_{44}O$	Ergosterol
399	$C_{28}H_{46}O$	(22E, 24S)-crinosterol
399	$C_{28}H_{46}O$	24-methyldesmosterol
401	$C_{28}H_{48}O$	23R-methylcholesterol
411	$C_{29}H_{46}O$	(3β)-stigmasta-5,7,22-trien-3-ol
413	$C_{29}H_{48}O$	Stigmasta-5,22E-dien-3β-ol
413	$C_{29}H_{48}O$	(24E)-Stigmasta-5,24(28)-dien-3β-ol
413	$C_{29}H_{48}O$	Fucosterol
431	$C_{29}H_{50}O_2$	Tocopherol
453	$C_{30}H_{60}O_2$	Cetyl myristate
479	$C_{32}H_{62}O_2$	Myristyl oleate
481	$C_{32}H_{64}O_2$	Cetyl palmitate
507	$C_{34}H_{66}O_2$	Cetyl oleate
509	$C_{34}H_{68}O_2$	Stearyl palmitate
535	$C_{36}H_{70}O_2$	Arachidonic palmitoleate
535	$C_{36}H_{70}O_2$	Stearyl oleate
563	$C_{38}H_{74}O_2$	Eicosyl oleate

[a]Molecular mass. [b]Molecular formula.

$[M + H]^+$ and 307.9852 $[(M + 2)+H]^+$ in the same samples. The signal intensities of these two substances were observed to increase in f-AcOEt, which is derived from EbMTc. This may have occurred because of refinement generated by EbMTc fractionation process. The protonated mass of 2'-N-demethylaplysinopsin as m/z of 241.1084 $[M + H]^+$ was also identified in f-AcOEt fraction. The values of retention time (RT) and arbitrary intensity (I; $x10^5$) of the peaks are found in Table 2. All the chromatograms are presented in supporting information (Figures S1–S6).

Anti-inflammatory potential of EbMTc, EbMTt and APL01 in carrageenan-induced paw oedema model

Figure 1a and Figure 1b represents the samples of EbMTc and EbMTt, respectively. In both figures, the peak of oedema induced by carrageenan paw injection was at the third hour, plotted on the negative control curve (vehicle). The treatment with EbMTc (Figure 1a) induced anti-inflammatory activity from the first hour, which was maintained until the end of the experiment (4 h). All doses were able to decrease induction of oedema similarly to the indomethacin-positive control and presented statistically significant differences throughout the experiment.

All doses of EbMTt (Figure 1b) showed statistically significant differences concerning the negative control (vehicle) at the peak of inflammation generated by carrageenan (3 h) but did not produce an equal or more significant effect than indomethacin. The treatment with 20 mg/kg EbMTt showed the best performance, maintaining a statistically significant lower oedema index in relation to the vehicle at 3 and 4 h. No dose achieved better results than indomethacin throughout the experiment, except that of 20 mg/kg at 4 h where results were similar.

A dose–response relationship was observed for APL01 injection with three doses used (Figure 1c). All concentrations were able to reduce oedema generated by carrageenan at peak oedema (3 h). Treatment with 20 mg/kg dose presented the lowest oedema reduction performance in relation to 0.02 and 200 mg/kg doses, the larger dose being the most efficient in reducing the oedema after 1 h relative to the positive control. Similar behaviour to indomethacin was exhibited by treatment with both 0.02 and 200 mg/kg APL01 doses at 3 h and 4 h.

Inhibitory effect on LPS-Induced nitrite production in murine RAW 264.7 macrophages

NO is a mediator of the inflammatory process, and its release is one of the functions performed by macrophages. Among its various actions in the body, nitric oxide acts as a stimulus for vasodilation, an essential step for locomotion of defence cells, and presents antimicrobial activity through active nitrogen species action. This molecule is highly unstable and converts to nitrite and nitrate within seconds. It can be detected by the use of the Griess reaction, where

Table 2 Compounds suggested in samples EbMTc, EbMTt and f-AcOEt by HPLC analysis, with respective *m/z*, retention time (RT) and intensity (I)

Compound	*m/z*	EbMTc		EbMTt		f-AcOEt	
		RT(min)	I	RT(min)	I	RT(min)	I
3′-deimino-3′-oxoaplysinopsin	256.1081	22.73025.295	1.122.61	22.73823.603	2.300.80	22.73025.297	1.212.73
6-bromo-3′-deimino-3′-oxoaplysinopsin	334.0186336.0168	26.912	0.37	26.263	0.44	26.906	0.41
Tubastrine	194.0924	15.575	1.34			15.602	1.52
3′-deimino-2′,4′-bis(demethyl)-3′-oxoaplysinopsin (APL01)	228.0768	19.998	0.33			19.995	2.93
6-bromo-3′-deimino-2′,4′-bis (demethyl)-3′-oxoaplysinopsin	305.9873307.9852	24.259	0.41			24.251	1.76
2′-N-demethylaplysinopsin	241.1084					25.909	1.30

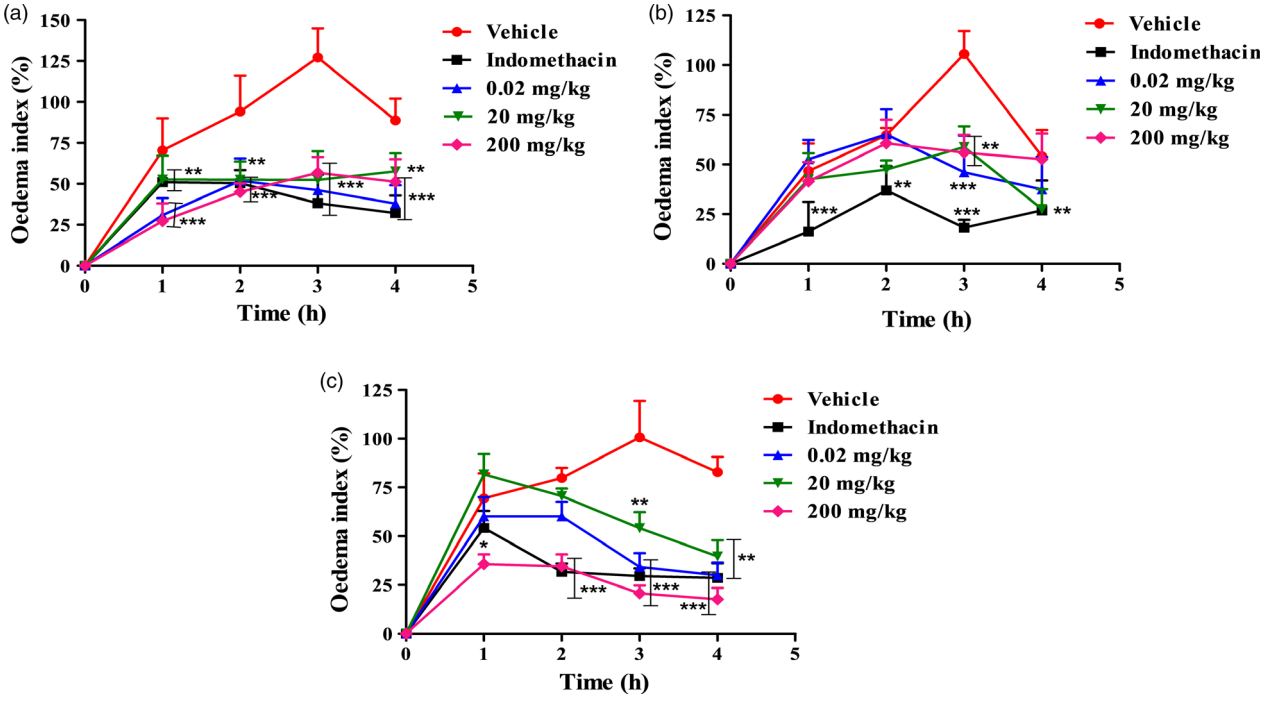

Figure 1 Effect of (a) EbMTc, (b) EbMTt and (c) APL01 in carrageenan-induced paw oedema model. Groups of male SW mice (5/group) were treated with the vehicle (15% ethanol, 1.25% Tween-20 in distilled water) as a negative control, or extract (0.02, 20 or 200 mg/kg), or indomethacin (10 mg/kg) by intraperitoneal injection. The results express mean ± standard deviation (**$P < 0.01$; *** $P < 0.001$) in relation to the vehicle (ANOVA followed by Dunnett's test).

nitrite reacts with sulfanilamide in acidic medium, generating diazo compound, which will react with N-naphthyl ethylenediamine, producing a red coloured compound. Figure 2 shows the results found for the analysed samples.

EbMTc and EbMTt reduced the NO release in the two highest concentrations (50 and 100 µg/mL), with EbMTc performing better at the lower concentration (50 µg/mL). However, the action of APL01 was more effective than the crude extracts at 0.25, 2.5, 5.0 and 50 µg/mL concentrations. Statistical analysis corroborates these observations. At 100 µg/mL, the three samples showed similar responses to one another. There was no evidence of macrophage killing at any concentration tested for these samples in the MTT survival assay.

The analysis of the anti-inflammatory response generated by the EbMTc fractions showed stronger effects in relation to the origin (EbMTc) and the synthesized alkaloid. The f-Hex, f-DCM, f-AcOEt and f-BuOH fractions were able to decrease the NO release at the two highest concentrations, especially f-DCM and f-AcOEt, which reached NO levels close to zero.

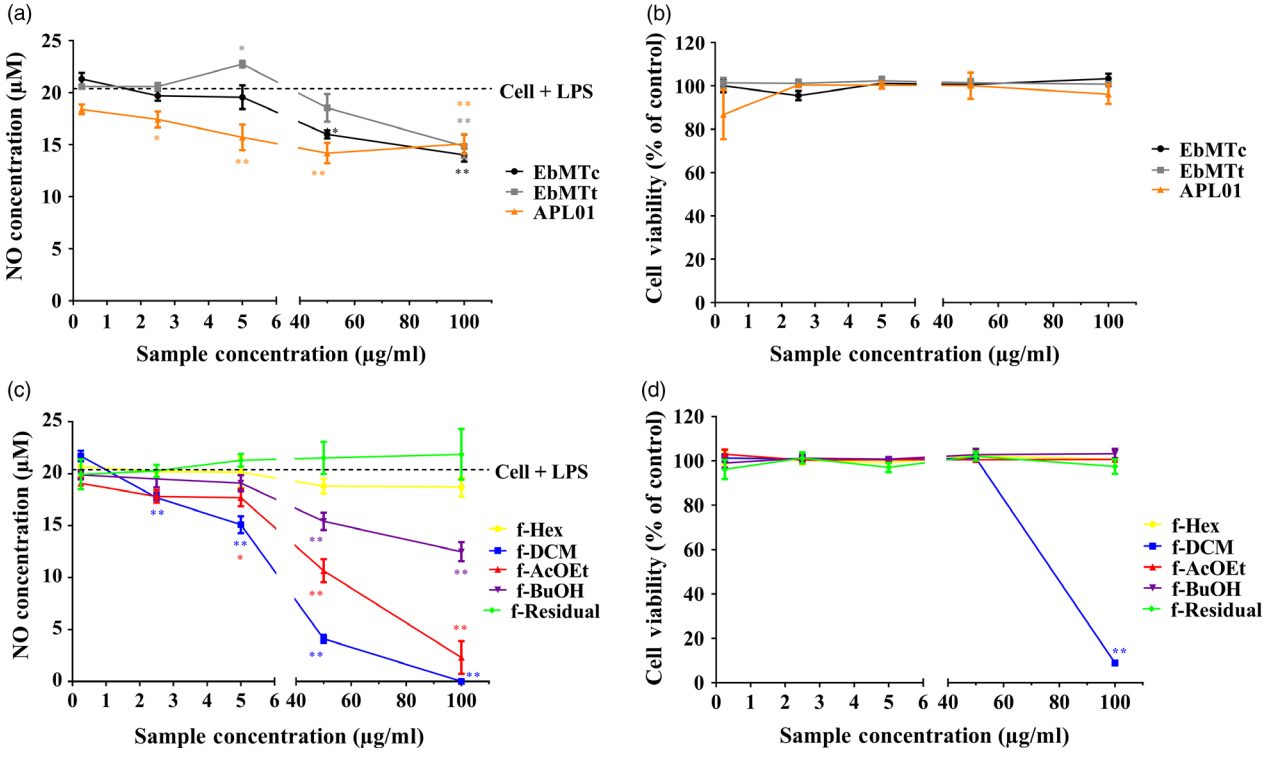

Figure 2 Survival and concentration of nitric oxide generated by LPS-stimulated macrophages in the presence of crude extracts and synthesized alkaloids (a) and (b) and fractions derived from EbMTc (c) and (d). The results express mean ± standard deviation (**$P < 0.01$) in relation to the Cell + LPS (ANOVA followed by Tukey's test).

However, in survival analysis for MTT assay, f-DCM revealed indications of cytotoxicity at 100 μg/mL concentration, suggesting that the decrease in NO release was probably due to cell death. Therefore, f-AcOEt was the sample that presented better performance in this assay as it reduced NO production without interfering with cell viability.

Cytotoxic assays of the f-AcOEt fraction

WST-1 Colorimetric Test

The f-AcOEt sample was able to alter the cellular proliferation in relation to the control group of both cell lines used, according to the applied methodology. The presence of mitochondrial metabolism observed through the reduction of crystal salt tetrazolium formazan by dehydrogenase enzymes action demonstrated that the HepG2 and RAW 264.7 cultures were able to multiply in the presence of f-AcOEt, depending on the concentration used (Figure 3).

The f-AcOEt sample, from the crude extract EbMTc, demonstrated a cytotoxic capacity for the HepG2 cell line. However, the highest concentration tested (100 μg/mL) was not responsible for this effect. Treatment periods of 48 and 72 h were not influential in generating a cytotoxic response, as cell proliferation remained between 95% and 105%, except for the 24-h treatment period, whose mean percentage was slightly below 80 % and statistical analysis indicated a difference in relation to the negative control. The 2.5 and 5.0 μg/mL concentrations showed cytotoxicity with proliferation below 50% at 48 and 72 h times.

A decline in proliferation is observed along concentrations where the most toxic level is 5.0 μg/mL concentration in 48 h. At 72 h, this concentration has its proliferation increased, but not similar to the negative control. In this case, the association between prolonged exposure time and increased cytotoxicity had not been observed. At 24 h, a concentration-dependent relationship is found throughout the concentrations.

For RAW 264.7 cell line, f-AcOEt showed cytotoxic potential at the two highest concentrations (50 and 100 μg/mL), where cell proliferation percentages at 48 and 72 h were below 30% in relation to the control group, with values similar to those found by the action of 2% Triton X. In 24 h, these concentrations presented results between 75 and 80%, but statistically different from the negative control. The lowest concentrations (0.25, 2.5 and 5.0 μg/mL) did not induce positive responses to cytotoxicity in any treatment period used.

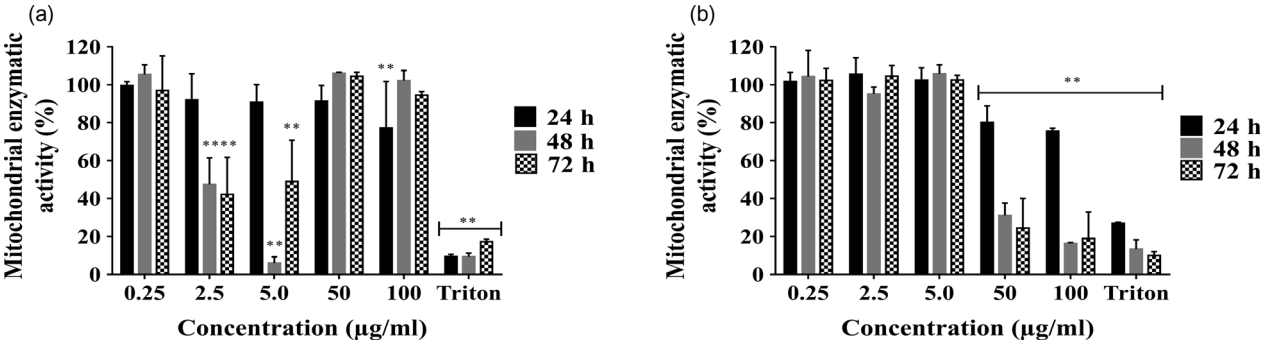

Figure 3 Cell proliferation by WST-1 colorimetric assay of HepG2 (a) and RAW 264.7 (b) cell lines in f-AcOEt presence. Percentage values express mean and standard deviation of replicates (n = 6). statistically significant difference compared to the negative control MEM or DMEM (100%): ANOVA test followed by Dunnett (**P < 0.01).

Lactate Dehydrogenase (LDH) Colorimetric Test

The assay to detect the cytotoxic activity by reaction with the LDH enzyme demonstrated positive results. The LDH presence in the extracellular environment is an indication that the cytoplasmic membrane has suffered some lesion, leading to extravasation of intracellular content, or cellular lysis has occurred. Both cases lead to cell death, which allows associating the observed cytotoxicity with sample action. Figure 4 illustrates the results found.

The f-AcOEt showed the capacity to generate damage in the cytoplasmic membrane of the HepG2 cell line. In the 48-h treatment, f-AcOEt demonstrated positive responses to this effect at 2.5 and 5.0 µg/mL concentrations, reaching 90% cytotoxicity. The 24- and 72-h periods showed no cytotoxic activity at any concentration tested.

The f-AcOEt effect on RAW 264.7 cell line showed negative responses for cytotoxicity. No concentration in any treatment was able to induce damage to cytoplasmic membrane in macrophages and therefore did not intensify the reaction with the LDH enzyme present in the intracellular environment.

Discussion

Forty-seven compounds were identified, comparing the mass spectra contained in the Wiley 275 library and with probabilities above 90%. The presence of fractions of 3'-deimino-3'-oxoaplysinopsin and its 6-bromo halogenated analogue is emphasized because of the class and the substance itself being present in *Tubastraea* genus. In this study, we used the same methodology as applied [with few variations in fractions originated from EbMTc (f-Hex, f-DCM, f-AcOEt, f-BuOH and f-Residual)] previously by the research group of UERJ Benthic Marine Ecology Laboratory. They also elucidated the chemical composition of the invading corals *T. coccinea* and *T. tagusensis* through GC/MS in Hex, Hex and DCM (1:1 v/v), DCM and DCM and MeOH fractions (1:1 v/v).[28,48]

In comparison with Ref. [48], the occurrence of methyl palmitoleate, heptadecanoic acid (methyl margarate), (22Z)-26,27-dinorergosta-5,22-dien-3β-ol (isomer), (22E,24S)-crinosterol, 23R-methylcholesterol, stigmasta-5,22E-dien-3β-ol, cetyl oleate and stearyl palmitate is observed. The presence of methyl

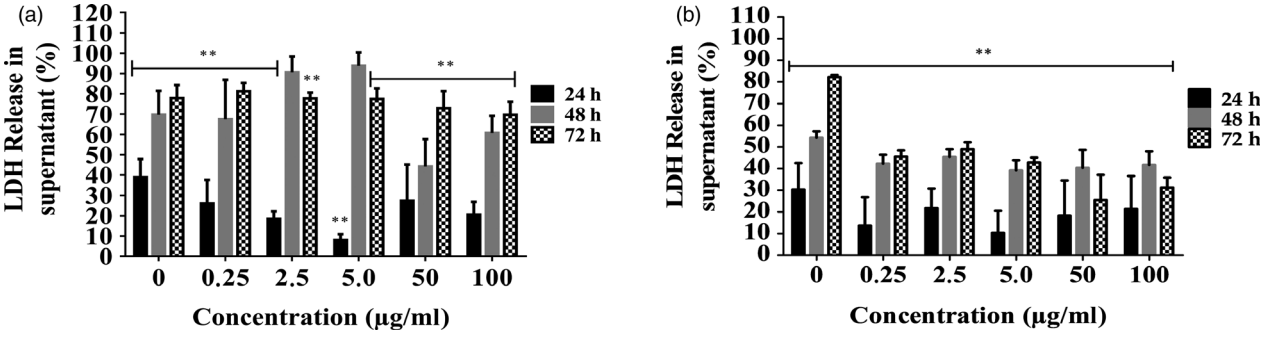

Figure 4 Cellular cytotoxicity assessed by LDH colorimetric assay of HepG2 (a) and RAW 264.7 (b) cell lines in the presence of f-AcOEt. Percentage values expressed as mean and standard deviation of replicates (n = 6). statistically significant difference in relation to the positive control 2% Triton X-100 (100%): ANOVA test followed by Dunnett (**P < 0.01).

(7E, 10E, 13E)-7,10,13-hexadecatrienoate, methyl linoleate (methyl 9,12-octadecadienoate), methyl arachidonate, ergosterol (ergosta-5,7,22-trien-3β-ol), 24-methyldesmosterol (ergosta-5,24-dien-3β-ol) and cetyl palmitate (hexadecanoic acid) is also noted in the literature.[28] Taking the three studies yields 1-octadecene, methyl palmitate, oleic acid, methyl oleate, methyl stearate, (22Z)-27-norergosta-5,22-dien-3β-ol, cholesterol (Cholest-5-en-3β-ol) and stearyl oleate (9-octadecenoic acid). The (24E)-stigmasta-5,24(28)-dien-3β-ol compound is a fucosterol isomer, which is described in Lages' works, but unspecified as to its isomerism. The substance myristyl oleate is described in Ref.[48], but only in the Hex and DCM fraction (1:1 v/v) of *T. tagusensis*. Fatty acids, sterols, lipids and hydrocarbons play several roles in cnidarians. In the alcyonacean coral *Chromonephthea braziliensis*, the 23-keto-cladielin-A steroid was isolated from hexane extract and showed antipredatory activity against generalist fish. In scleractinian corals, steroids may play a role as sex hormones since the presence of enzymes involved in the metabolism of these hormones has been confirmed. Fatty acids and sterols are structural components of the cell membrane, and they can act as allelopathic compounds in marine organisms.[26,49–52]

The HPLC and mass spectrometry results of EbMTc, EbMTt and f-AcOEt were compared with the substances described in *Tubastraea* genus. According to the adopted parameters, 3'-deimino-3'-oxoaplysinopsin and 6-bromo-3'-deimino-3'-oxoaplysinopsin were present in all three samples. The chromatogram for the supposed presence of 3'-deimino-3'-oxoaplysinopsin presents two alternating peaks in intensity, which suggests the presence of isomers. The occurrence of 8E and 8Z isomer mixture of 3'-deimino-3'-oxoaplysinopsin in the *Tubastraea* genus is described in the literature.[20,53,54] This mixture demonstrated weak antagonistic activity for alpha 3 glycine-controlled chloride channel receptors (α3 GlyR), whereas tubastrindol B was shown to be a potent antagonist for α1 GlyR subunit. Substances that can modulate different GlyR isoforms are essential in drugs development for the treatment of diseases caused by respiratory and movement disorders such as spasticity and hyperekplexia.[55,56]

The alkaloid tubastrine was present in the EbMTc and f-AcOET samples. This molecule was first identified in *T. coccinea* and showed antiviral activity. Subsequent studies have demonstrated that tubastrine and derivatives isolated from the sponge *Spongosorites* spp. were able to moderately inhibit the bacterial growth of *S. aureus*, *Serratia* sp. and *E. coli*. Tubastrine isolated from ascidian *Aplidium orthium* has been shown to inhibit the production of superoxide in human neutrophils stimulated by PMA (phorbol-12-myristate-13-acetate), an NADPH oxidase enzyme complex stimulating agent in *in-vitro* assays. In *in-vivo* assays, tubastrine demonstrated anti-inflammatory capacity in acute models for gouty arthritis and inhibition of neutrophil infiltration.[57–60]

The APL01 synthesized alkaloid and its 6-bromo derivative masses were observed in EbMTc and f-AcOEt samples. Note that peak intensities increase in this fraction, which may indicate a purification process. The sequential fractionation process adopted in EbMTc fractions preparation promoted this purification, which is supported by GC/MS analyses, where the f-Hex fraction held most of the low polarity compounds. APL01 is identified as 3'-deimino-2',4'-bis(demethyl)-3'-oxoaplysinopsin. Both compounds have been found in the scleractinian coral *Leptopsammia pruvoti*, collected in the Marseilles region.[16,53]

The 2'-N-demethylaplysinopsin was only observed in EbMTc. It is a metabolite that was first found in the sponge *Dercitus* sp. and later in *T. coccinea* and the mollusc *Phestilla melanobrachia*; this mollusc almost exclusively predates *Tubastraea* so it was hypothesized that the mollusc accumulated aplysinopsins in its mantle through feeding, where they act as a defence against predators.[61–63] The scleractinian coral *Dendrophyllia* sp. also has this metabolite in its tissues and was found, along with its 6-bromo halogenated analogue among other substances in the sponge *Smenospongia aurea*.[19,62] However, this 6-bromo derivative has interesting pharmacological activity. The 6-bromo-2'-N-demethylaplysinopsin found in *S. aurea* showed moderate antimalarial activity for *Plasmodium falciparum* D6 clones and inhibited the plasmepsin enzyme action *in-vitro* assays, presenting low cytotoxicity. This substance demonstrated high affinity as an antagonistic ligand for serotonin 5-HT2 receptors (5-HT2A and 5-HT2B). From the sponge *Hyrtios* sp., it acts as a potent antioxidant agent.[62,64]

The aplysinopsin protonated mass was not observed in the samples although according to the literature, it is present in *Tubastraea* genus.[20,65–67] It should be noted that aplysinopsin has been identified in both methanol extracts of *T. coccinea* and *T. tagusensis* through the Raman spectroscopic technique. This method has been used successfully to characterize conjugated polyenes, without the need to isolate specific compounds, in different organisms such as microorganisms, plants, corals, fishes and birds.[67]

The anti-inflammatory activity through the carrageenan-induced paw oedema model revealed that the EbMTc, EbMTt and APL01 samples have anti-inflammatory potential. Between the two extracts, EbMTc showed a better performance at 0.02 mg/kg concentration throughout the experiment, demonstrating a better or similar effect to the indomethacin control drug. The concentration of 200 mg/kg showed a behaviour similar to 0.02 mg/kg in EbMTc throughout the experiment, but at the peak of oedema induction (3 h), where carrageenan showed its most potent

effect, 0.02 mg/kg maintains the oedema index below 50%, similar to indomethacin.

APL01 also presented interesting results, whose concentration of 200 mg/kg showed better activity when compared to positive control. However, it is a pure synthesized substance and a concentration twenty times greater than positive control (10 mg/kg) was required to obtain the same intensity of the anti-inflammatory response. It is noteworthy that its 0.02 mg/kg concentration demonstrated similar results to indomethacin at 1 h, 3 h and 4 h and better than 20 mg/kg concentration. When the lower concentration of a molecule generates a more potent response than larger concentrations, the hormesis effect can be considered.[68]

The effect of these three samples on NO release by LPS-stimulated RAW 264.7 macrophages was compared with those from EbMTc-derived fractions. The EbMTc, EbMTt and APL01 samples were able to decrease the NO release when compared to the control group with statistically significant difference associated with no signs of cytotoxicity. The NO decreasing was more evident in the two higher concentrations (50 and 100 μg/mL) with emphasis on EbMTc and APL01 samples. However, the best results of this experiment came from EbMTc fractions, specifically the f-AcOEt sample at 50 and 100 μg/mL concentrations, whose inhibitory activity reached values close to zero referring to NO concentration. The f-DCM sample generated more intense responses but could not be associated with anti-inflammatory activity since it induced cytotoxicity in macrophages in MTT survival assay.

According to the chemical analyses, it is possible to infer some points about the origin of the observed anti-inflammatory effect when considering the presence of terpenes, alkaloids and aplysinopsin derivatives in the samples. As described previously, extensive reviews of marine substances originating from corals in the preclinical stage of drug development are found in the literature, from which the probable mechanisms of action responsible for various pharmacological activities are found, among them anti-inflammatory activity. In general, the inhibition of the enzymes nitric oxide synthase (iNOS isoform) and COX-2 expressions and the NO release inhibition are the primary mechanisms by which compounds from corals act as anti-inflammatory agents. However, most of these molecules are terpenes and terpenoid derivatives from soft corals. In contrast to marine alkaloids, and including organisms from other phyla, the pathways that induce the anti-inflammatory effect may occur by inhibiting the release of NO in macrophages, iNOS, COX-2, TNF-α, interleukin 6, NF-κB, prostaglandin E2, among others.[5,6,69,70]

One of the activities associated with aplysinopsin is neuromodulation through the nitric oxide synthase (NOS) selective inhibition. There are three isoforms of NOS; two are constitutively expressed and one induced. Isoform I or nNOS (NO neuron synthase) is present in brain and isoform III or eNOS (NO endothelial NO synthase) in endothelial cells. Both are Ca^{+2} binding dependent through calmodulin protein for activation, whereas iNOS (NO synthase induced) is Ca^{+2}/calmodulin complex formation independent for maintenance, being induced in macrophages and other cells by bacterial LPS or cytokines. Aplysinopsin derivatives such as 6-bromo-2'-N-demethylaplysinopsin that is found in *T. coccinea* can inhibit the action of both nNOS and iNOS. NO is an important signalling molecule and has functions relating to neurotransmission, blood pressure regulation, platelet adhesion, control of placental circulation, regulation of uterine contractions in labour, endocrine actions, autoimmunity mechanisms and inflammatory process such as in the fight against invading microorganisms. Therefore, substances that can regulate NOS enzymes are essential therapeutic targets.[16,63,71,72] Another factor that is directly related to the anti-inflammatory action of aplysinopsins is the presence of the imidazolidine ring or hydantoin, which confers this ability, as well as other potential actions, such as antimicrobial, antifungal, herbicide and cytotoxic effects. Adolf von Baeyer discovered this structure in 1861, and since then, hydantoins and their derivatives have been investigated due to their pharmacological properties.[73–77]

The EbMTc, EbMTt, APL01 and AcOEt samples were also evaluated for their toxicological profile,[78] and a safe concentration of 50 μg/mL has been established. The f-AcOEt fraction, from the crude extract EbMTc, was tested at lower concentrations, but corresponding to those tested for anti-inflammatory effect *in-vitro* test. In WST-1 assay, f-AcOEt demonstrated cytotoxic responses for HepG2 cell line at the two highest exposure times associated with 2.5 and 5.0 μg/mL concentrations, whereas for RAW 264.7, the two largest concentrations were cytotoxic with a higher intensity of this effect at 48- and 72-h period. In LDH reagent assay, f-AcOEt did not promote cytotoxic effect for RAW 264.7 in any test condition, and for HepG2, the RAW 264.7 cytotoxicity was only observed at 2.5 and 5.0 μg/mL concentrations in 48 h of treatment considering the statistical analysis. This sample is less complex in its composition than the extracts. However, it may present other molecules with different characteristics such as the fatty acid ester methyl oleate and the indole alkaloid 3'-deimino-3'-oxoaplysinopsin making synergy possible. The presence of hydantoin nucleus may be related to the cytotoxic effect observed in both assays for tumoral cell line used.[56,77,79]

In summary, these invasive corals possess anti-inflammatory activity, which was observed in all samples tested for *in-vivo* assays, especially in *T. coccinea*. The ethyl acetate

fraction demonstrated greater efficacy in *in-vitro* assays for anti-inflammatory activity. The amount of necessary material for the preclinical and clinical trials is a limiting factor for pharmacological development and research from marine natural products.[56] However, the sun coral species management can provide biological material enough to supply this necessity.[33,34] Therefore, the continued expansion of these species along the Brazilian coast implies that there will be plenty of biological material for further studies of pharmacological application in the future in Brazil.

Conclusions

We conclude that EbMTc and EbMTt have low polarity compounds identified in the literature with occurrence in *T. coccinea* and *T. tagusensis* coral species. The presence of alkaloids in EbMTc, EbMTt and f-AcOEt was observed. The extracts and the synthetic alkaloid APL01 were able to decrease mouse paw oedema generated by carrageenan, whose EbMTc results were the most promising. The f-AcOET sample, from EbMTc, showed anti-inflammatory activity as it inhibited the *in-vitro* NO production by macrophages. This sample showed cytotoxic potential for permanent cell culture generating cytotoxicity through the interference of mitochondrial dehydrogenase enzyme activity, independent of metabolism of substances present in its composition. The cytotoxicity caused by cytoplasmic membrane damage depended on metabolism.

Declarations

Conflicts of interest

The Authors declare that they have no conflicts of interest to disclose.

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Author's contribution

RMC designed the experiments, wrote the proposal, participated in data collection, and analysed and draft the manuscript. DCF and MGPC participated in carrageenan-induced paw oedema model and NO releasing by LPS-stimulated RAW 264.7 macrophages. SJG participated in the preparation of 5-(indol-3-ylmethylene)-hydantoin (3'-deimino-2',4'-bis(demethyl)-3'-oxoaplysinopsin) – APL01. JCC, BGF and IF participated in the proposal, result analysis, review supervision and interpretation. All authors reviewed literature and participated in the paper final version.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Suggested compound (a) 3'-deimino-3'-oxoaplysinopsin. BPC (black), EIC (gray) of *m/z* of 256.1081 and overlapped chromatograms of: (b) EbMTc (RT: 22.730 min and 25.295 min / I: 1.12 and 2.61); (c) EbMTt (RT: 22.738 min and 23.603 min / I: 2.30 and 0.80); (d) f-AcOEt (RT: 22.730 min and 25.297 min / I: 1.21 and 2.73).

Figure S2. Suggested compound (a) 6-bromo-3'-deimino-3'-oxoaplysinopsin. BPC (black), EIC (gray) of *m/z* of 334.0186 $[M + H]^+$ and 336.0168 $[(M+2) + H]^+$ and overlapped chromatograms of: (b) EbMTc (RT: 26.912 min / I: 0.37); (c) EbMTt (RT: 26.263 min / I: 0.44); (d) f-AcOEt (RT: 26.906 min / I: 0.41).

Figure S3. Suggested compound (a) tubastrine. BPC (black), EIC (gray) of *m/z* of 194.0924 and overlapped chromatograms of: (b) EbMTc (RT: 15.575 min / I: 1.34); (c) f-AcOEt (RT: 15.602 min / I: 1.52).

Figure S4. Suggested compound (a) APL01. BPC (black), EIC (gray) of *m/z* of 228.0768 and overlapped chromatograms of: (b) EbMTc (RT: 19.998 min / I: 0.33); (c) f-AcOEt (RT: 19.995 min / I: 2.93).

Figure S5. Suggested compound (a) 6-bromo-3'-deimino-2',4'-bis (demethyl)-3'- oxoaplysinopsin. BPC (black), EIC (gray) of *m/z* of 305.9873 $[M + H]^+$ and 307.9852 $[(M+2) + H]^+$ and overlapped chromatograms of: (b) EbMTc (RT: 24.259 min / I: 0.41); (c) f-AcOEt (RT: 24.251 min / I: 1.76).

Figure S6. Suggested compound (a) 2'-N-demethylaplysinopsin. BPC (black), EIC (gray) of *m/z* of 241.1084 and overlapped chromatograms of (b) f-AcOEt (RT: 25.909 min / I: 1.30).

Table S1. Molecular structures of substances isolated from the genus *Tubastraea*.