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Mutagenicity, genotoxicity, and scavenging activities of extracts from the soft coral *Chromonephthea braziliensis*: a possibility of new bioactive compounds

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ABSTRACT. Coral reefs are diverse ecosystems that have a high density of biodiversity leading to intense competition among species. These species may produce unknown substances, many with pharmacological value. *Chromonephthea braziliensis* is an invasive soft coral from the Indo-Pacific Ocean that is possibly transported by oil platforms and whose presence can be a threat to a region's biodiversity. This species produces secondary metabolites that are responsible for inducing damage to the local ecosystem. In the present study, extracts

were prepared from dried colonies of *C. braziliensis* (solvents: hexane, dichloromethane, ethyl acetate, and methanol). We evaluated their mutagenicity using the *Salmonella* reverse mutation assay (TA97, TA98, TA100, and TA102 strains), their genotoxicity using the DNA breakage analysis and micronucleus assay, and scavenging activity using the 1,1-diphenyl-2-picrylhydrazyl-free radical assay. Cytotoxicity and mutagenicity were not observed for any of the extracts. Genotoxicity was observed for the dichloromethane, ethyl acetate, and methanol extracts at high concentrations, but no DNA damage was observed in the micronucleus assay. Scavenging activity was not detected.

Key words: *Chromonephthea braziliensis*; Mutagenicity; Toxicity; Secondary metabolites

INTRODUCTION

The marine environment contains an as yet untapped wealth of useful products for the treatment of infectious diseases (Donia and Hamann, 2003). It can be argued that pharmacological research involving marine organisms is intrinsically slower and has disadvantages compared to programs based on synthesis, but the number and quality of leads generated more than justify research in marine pharmacology (Faulkner, 2000).

A growing number of discoveries have been made of marine compounds with biological activity or properties of great interest. In the marine environment, sponges are responsible for the highest proportion of these findings (Sipkema et al., 2005; Stankevicins et al., 2008). However, coral reefs are the most diverse ecosystems in the sea and have the highest density of biodiversity globally. High species diversity gives rise to intense competition amongst species, with the surviving organisms having the capability to construct exotic defensive and offensive chemicals, many with pharmacological value (Adey, 2000).

Soft corals are very common in the Indo-Pacific reefs and in certain areas of the Great Barrier Reef. Although common species of corals are potentially rich in proteins, carbohydrates, and lipids, they are subject to relatively low levels of predation since they produce large amounts of protective secondary metabolites (Coll, 1992; Sammarco and Coll, 1992). Studies of the chemical defenses of corals have been limited to species of the Anthozoa class, comprising the Gorgonacea and Alcyonacea orders (Octocorallia subclass) (Pawlik, 1993).

Terpenes and their derivatives (terpenoids) comprise the prevailing class of substances in Octocorallia, and therefore in Alcyonacea. Studies of these substances have identified functional properties of great interest. For example, compounds like limonene, perillyl alcohol, and carvone that can act in the prevention of degenerative diseases, among others that are being studied as chemotherapeutic agents (Maróstica Júnior, 2008). Research in other properties, such as natural insecticides and antimicrobials, are examples of the importance that these chemicals have received (Maróstica Júnior, 2008).

The soft coral *Chromonephthea braziliensis* (Alcyonacea, Nephtheidae; Ofwegen, 2005) was first recorded in Arraial do Cabo, southeastern Brazil (Ferreira, 2003). Studies have shown that the presence of *C. braziliensis* in Brazilian waters can be a real threat to the region's biodiversity, especially endemic species of corals, such as the gorgonian *Phyllogorgia*

dilatata (Lages et al., 2006). This exotic coral produces secondary metabolites that confer it with beneficial action against native species, contributing to its defense against fish predators and increasing its success in competition for space (Lages et al., 2006; Fleury et al., 2008; Oliveira and Medeiros, 2008).

This study aimed to carry out qualitative and quantitative cytotoxic, mutagenic, genotoxic, and scavenging evaluations of hexane (n-Hex), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH) extracts from *C. braziliensis*. The motivation of this analysis is the development of future drugs for general chemotherapy based on the structure of bioactive compounds.

MATERIAL AND METHODS

Collection and extraction

The *C. braziliensis* colonies were collected by scuba diving to 8 m depth in May 2004 in a marine reserve in the Arraial do Cabo region of Rio de Janeiro State (23°44'S-42°02'W), southeastern Brazil. The samples generated a total mass of 286 g freeze-dried coral and were extracted in 3 consecutive times, then sonicated with each solvent sequentially. The results are shown in Table 1.

Table 1. Yield from the extracts.

Chromonephthea braziliensis	
Freeze-dried coral	286 g
Hexane extract (n-Hex)	9.20 g
Dichloromethane extract (DCM)	3.00 g
Ethyl acetate extract (EtOAc)	0.387 g
Methanol extract (MeOH)	2.93 g

Bacterial strains

The features of *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102 (from our stock) are shown in Table 2.

Table 2. Genotypic and phenotypic characteristics of standard strains derived from *Salmonella typhimurium* LT2.

Strain	*His* mutation	Plasmids	Other mutations	Type of mutation detected	
TA97	*hisD6610; hisO1242*	pKM101	*rfa Δ* (*uvrB chl bio*)	*Frameshift*	G:C pair addition
TA98	*hisD3052*	pKM101	*rfa Δ* (*uvrB chl bio*)	*Frameshift*	G:C pair deletion
TA100	*his G46*	pKM101	*rfa Δ* (*uvrB chl bio*)	*Substitution*	G:C to A:T
TA102	pAQ1(*hisG428*)	pKM101,pAQ1	*rfa*	*Substitution*	A:T to G:C

Bacterial reverse mutation test

The test tube contained a mixture of 100 µL of 1 of the 4 extracts of *C. braziliensis* concentrations (0.5, 5, 10, and 20 µg/plate), 500 µL sodium phosphate buffer (27.6 g/L $NaH_2PO_4 \cdot H_2O$ and 28.4 g/L Na_2HPO_4; 0.2 M, pH 7.4) and 100 µL bacterial suspension (2 x

10^9 cells/mL). Then, 2 mL top agar (7 g/L agar; 5 g/L NaCl; 0.0105 g/L L-histidine; 0.0122 g/L biotin, pH 7.4, 45°C) was added to the test tube and the final mixture was poured onto a Petri dish with minimal agar [15 g/L agar, Vogel-Bonner E medium 10X (10 g/L $MgSO_4 \cdot 7H_2O$; 100 g/L $C_6H_8O_7 \cdot H_2O$; 500 g/L K_2HPO_4; 175 g/L $Na(NH_4)HPO_4 \cdot 4H_2O$)] containing 20 g/L glucose. This final mixture was incubated at 37°C for 72 h, and the *His*$^+$ revertant colonies were counted.

The positive controls for assays in the absence of S9 mix were as follows: 4-nitroquinoline 1-oxide (CAS: 56-57-5) at 1.0 µg/plate for TA97; 4-NQO at 0.5 µg/plate for TA98; sodium azide (CAS: 26628-22-8) at 0.5 µg/plate for TA100; and mitomycin C (CAS: 50-07-7) at 0.5 µg/plate for TA102. All the chemicals used were from Sigma (USA).

The substance or sample was considered positive for mutagenicity when: a) the number of revertant colonies in the test assay was at least twice the number of spontaneous revertants (mutagenicity induction ≥2), calculated as the number of *His*$^+$ induced in the sample divided by the number of spontaneous *His*$^+$ in the negative control; b) a significant response for analysis of variance (ANOVA, $P \leq 0.05$) and the Student *t*-test was found; c) a reproducible positive dose-response curve ($P \leq 0.01$) was present. All experiments were done in triplicate (Maron and Ames, 1983).

Survival experiments

Quantitative evaluations were made to determine the cytotoxic effects of the concentrations of the 4 extracts of *C. braziliensis*. In the assay, 10 µL bacterial suspension treated as described for the Ames test was diluted with a saline solution (9 g/L NaCl). Then, 100 µL mix solution was put on a Petri dish with solid LB medium. The total dilution was 10^{-7}-fold. These dishes were incubated at 37°C for 24 h. The colonies were counted and a percentage calculation was made relative to the negative control.

DNA breakage analysis

The plasmid pUC18 was extracted from *Escherichia coli* DH5α strain using a kit for plasmid DNA extraction (Miniprep Kit for plasmid) from Axygen Biosciences (USA). Electrophoresis was performed on 0.8% agarose gel in order to separate different structural conformations of pUC18: form I, supercoiled native conformation; form II, open circle resulting from single-strand breaks; and form III, linear resulting from double-strand breaks. The electrophoresis assay was also used to verify if there was a delay in DNA migration. Aliquots (5 µL) of each extract at different concentrations (28.6, 50, 100, and 250 µg/mL) with the plasmid (0.4 µg) were incubated for 10 min at 37°C and submitted to electrophoretic migration for 15 min (E-Gel® iBase™ Power System) and visualized by ultraviolet transilluminator (E-Gel® Safe Imager™ Real-Time Transilluminator) both from Invitrogen (USA). The images were digitalized in a photo documentation system using the ImageJ 1.36b software to perform the quantification of the bands. The quantitative analysis was done only once.

The assay included a positive control using stannous chloride ($SnCl_2$, CAS: 7772-99-8) at 100 and 200 µg/mL concentrations (Sigma) (Gomes et al., 1996; Felzenszwalb et al., 1998).

Micronuclei in macrophages

The RAW264.7 macrophage strain was used from a confluence culture. Eagle's minimum essential medium (950 µL MEM; 1.8 mM Ca^{2+}, pH 7.6; Gibco, USA) was supplemented with 1.76 g/L $NaHCO_3$, 0.88 g/L pyruvate, 21.6 mg/L aspartic acid, and 16.8 mg/L L-serine, with 10% fetal bovine serum (FBS), both at 37°C. Next, 50 µL 2 x 10^5 cells/mL suspended cells was added to 24 wells of a microtiter plate containing a coverslip that had been pre-treated with 0.1 M nitric acid for 15 min. This suspension was maintained in Eagle's MEM Ca^{2+} 1.8 mM, containing 10% FBS, 100 mg/L streptomycin, and 70 mg/L penicillin. The plates were placed in an incubator with an atmosphere of 5% CO_2 for 24 h.

For cell treatment, 100 µL of the 4 concentrations (20, 200, 350, and 500 µg/mL) of the 4 extracts of *C. braziliensis* (n-Hex, DCM, EtOAc, and MeOH) was added, equivalent to 10% of total volume, and the plates were incubated for 24 h. After the incubation period, the medium was removed and the cells were rinsed with 1 mL Eagle's MEM. One milliliter medium supplemented with FBS (10%) was added and the cells were re-incubated for an additional 24 h in an incubator with an atmosphere of 5% CO_2. The positive control was N-methyl-N-nitro-N-nitrosoguanidine at a concentration of 0.5 mM.

The Eagle's MEM was replaced with cold fixative solution methanol-glacial acetic acid (3:1) for 15 min. The fixed cells were rinsed with McIlvaine buffer (MI buffer: 21.01 g/L citric acid and 35.60 g/L Na_2HPO_4, pH 7.5) for 2 min and dried at room temperature. The fixed cells were stained with 0.2 µg/mL 4'-6-diamidino-2-phenylindole dissolved in MI buffer for 40 min. Cells were washed with MI buffer for 2 min followed by distilled water and dried again at room temperature. To determine the mitotic index, the number of cells with micronuclei and the percentages of necrosis and apoptosis (1000 cells per concentration, triplicate) were analyzed in a fluorescence microscope (Reichert Univar) with an excitation wavelength of 350 nm (Eckl et al., 1987).

Protein quantification

We evaluated the protein composition using Qubit™ Protein Assay Kit (Q33211) as recommended by Invitrogen. Three different considerations can be made regarding the protein concentration: a) the increased protein concentration was related to the decreased bioavailability of secondary metabolites in the form of free secondary metabolites; b) the decrease in the protein concentration could be due to sequential extraction; and c) the proteins present in the extracts could act as scavengers.

DPPH-free radical scavenging activity assay

An aliquot of 1.0 mL 0.25 mM DPPH solution in ethanol and 1.0 mL of 1 of the 4 concentrations (0.001; 0.01; 0.1; 1.0 mg/mL) for each extract were mixed. The mixture was vigorously shaken and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring spectrophotometric absorbance at 517 nm. The capability to scavenge DPPH radicals was calculated by the following equation: scavenging rate = [1 - (absorbance of the sample at 517 nm / absorbance of the control at

517 nm)] x 100%. The same procedure was done using proteinase K in order to isolate substances from proteins present in the extracts. The samples were incubated with 250 µg/mL proteinase K for 60 min at 50°C. One milliliter 0.25 mM DPPH was added to the final mix and the decolorization of DPPH was measured at 517 nm after 30 min at room temperature (Amarowicz, 2000).

Statistical analysis

For the *S. typhimurium* reverse mutation assay, mutagenicity was identified when the mutagenic index was at least twice the spontaneous rate (in the negative control). Survival rates of 70% less than the negative control indicated cytotoxicity. Significant statistical differences between negative and tested concentrations under the same experimental conditions were verified using the Student *t*-test ($P < 0.05$) (Stankevicins et al., 2008).

The data for micronuclei were analyzed using a one-way ANOVA and the Tukey-Kramer multiple comparison test using the GraphPad Instat® software, version 3.01 (GraphPad Software, Inc., USA). Results were considered to be statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Quantitative survival and bacterial reverse mutation assays

We used the 4 bacterial strains in the mutagenic and cytotoxic assays due to their high sensitivity to various compounds. TA97 presents a mutation which, when altered by base pair deletion (frameshift), generates resistance to mutagens. This mutation allows growth on minimal medium. TA98 has a spontaneous mutation that decreases its level of nitroredutase activity. TA100 detects agents that induce methylation and cause the replacement of base pairs. The replacement of A:T by G:C triggers the start of the biosynthesis of histidine, restoring the wild phenotype. TA98 and TA100 are used to study the metabolism and mutagenicity of carcinogens, even though they are nitroredutase deficient. TA102 has an auxotrophic nature; it contains an A:T base pair at the critical site for reversion (the other strains have G:C base pairs). It detects a variety of oxidative mutagens and crosslink agents that preferentially attack A:T base pairs (Levin et al., 1982; Maron and Ames, 1983). However, no positive response was observed for mutagenic and cytotoxic assays.

Tables 3 and 4 present the analysis of the mutagenic and/or cytotoxic response for n-Hex, DCM, EtOAc, and MeOH extracts of *C. braziliensis*.

The n-Hex extract did not show a mutagenic response, but a toxicity signal was observed for TA102 at the highest concentration by a decrease in the percentage of survival. In the DCM extract, the mutagenic and cytotoxic effects were negative, but the survival rate decreased in a dose-dependent fashion when the extract was in contact with the TA97 strain.

The EtOAc extract did not induce cytotoxic or mutagenic effects, although the survival rate did decrease when the extract was in contact with the TA97 and TA100 strains. The MeOH extract followed the patterns of the DCM and EtOAc extracts, without mutagenic or cytotoxic effects.

Table 3. Mutagenicity and cytotoxicity for hexane and dichloromethane extracts of *Chromonephthea braziliensis*.

Strain	*C. braziliensis* (µg/plate)	Hexane extract			Dichloromethane extract		
		M.I.[a]	*His*$^+$ ± SD[b]	% Survival[c]	M.I.[a]	*His*$^+$ ± SD[b]	% Survival[c]
TA97	DMSO	1.0	132 ± 5	100	1.0	176 ± 49	100
	0.5	0.7	94 ± 9*	100	0.9	162 ± 52	95
	5	0.8	100 ± 11*	100	0.9	162 ± 34	90
	10	0.8	110 ± 16	100	1.0	170 ± 38	80
	20	1.0	131 ± 13	100	0.9	165 ± 12	80
	4-NQO (1.0 µg/plate)	2.8	368 ± 27		5.3	934 ± 361	
TA98	DMSO	1.0	18 ± 3	100	1.0	28 ± 4	100
	0.5	1.4	26 ± 7	100	1.1	29 ± 8	100
	5	1.1	21 ± 2	100	1.0	28 ± 6	100
	10	0.7	12 ± 3	87	1.1	30 ± 2	100
	20	0.8	14 ± 7	100	1.0	28 ± 7	100
	4-NQO (0.5 µg/plate)	10	183 ± 0		35.9	993 ± 272	
TA100	DMSO	1.0	146 ± 12	100	1.0	246 ± 16	100
	0.5	0.7	100 ± 8*	89	1.0	248 ± 25	100
	5	0.9	131 ± 12	86	1.1	262 ± 21	100
	10	0.9	133 ± 20	96	1.1	263 ± 34	100
	20	0.7	96 ± 18*	88	0.9	227 ± 18	100
	SA (0.5 µg/plate)	5.1	751 ± 61		11	2673 ± 805	
TA102	DMSO	1.0	229 ± 43	100	1.0	346 ± 65	100
	0.5	0.7	170 ± 36	90	1.1	370 ± 95	100
	5	1.0	220 ± 19	90	1.1	390 ± 72	100
	10	0.9	216 ± 99	90	1.1	372 ± 90	100
	20	1.0	230 ± 162	84	0.8	277 ± 78	100
	MitC (0.5 µg/plate)	4.3	989 ± 95		3.0	1036 ± 464	

[a]Mutagenic index: No. of *His*$^+$ induced in the sample/number of spontaneous *His*$^+$ in the negative control (DMSO). [b]*His*$^+$/plate: mean values of at least 3 replicate plates. [c]Percent survival relative to the negative control: toxicity is considered when percent survival <70% related to the control group. Positive controls are described in the Experimental section. Significant statistical differences between negative control and tested concentrations under the same experimental conditions are identified by an asterisk ($P < 0.05$). 4-NQO = DMSO = dimethyl sulfoxide; 4-nitroquinoline 1-oxide; SA = sodium azide; MitC = mitomycin C.

DNA breakage analysis

The results shown in Figure 1 are for the highest concentrations of the extracts of *C. braziliensis* (250 µg/mL). No positive response was observed for the other concentrations (28.6, 50, and 100 µg/mL; data not shown). These concentrations were used to compare with the mutagenic and cytotoxic assays at the highest concentration (20 µg/plate) and the genotoxic assay with eukaryotic cells (WHO, 2002).

Melatonin has a potent-free radical scavenger and antioxidant effect (Reiter et al., 1995). It can detoxify highly toxic hydroxyl radicals and peroxyl radicals *in vitro* (Reiter et al., 2001). In this way, melatonin exerts a protective effect under certain pathological conditions such as damage caused by drug toxicity in which a common feature is the existence of mitochondrial damage (Reiter et al., 1995; Karbownik and Reiter, 2000). One millimolar melatonin (N-acetyl-5-methoxytryptamine) was dissolved in 50 mM sodium phosphate buffer, pH 7.4, with 10% methanol (Onuki et al., 2005). The reaction mixtures were incubated at 37°C for 2 h. Melatonin was able to detoxify reactive oxygen species generated by DCM, EtOAc, and MeOH extracts avoiding the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo) (Burkhardt et al., 2001) (Figure 2).

Cytotoxic effects of terpenes and terpenoids on different organelles, such as mitochondria, were already known. They generate chain reactions from the cell wall or the outer cell membrane and invade the whole cell, which affects activities such as membrane potential, ionic channels, and regulation of pH gradients, ultimately leading to cell death by apoptosis and necrosis (Bakkali et al., 2008).

Table 4. Mutagenicity and cytotoxicity for ethyl acetate and methanol extracts of *Chromonephthea braziliensis*.

Strain	*C. braziliensis* (µg/plate)	Ethyl acetate extract			Methanol extract		
		M.I.[a]	*His*$^+$ ± SD[b]	% Survival[c]	M.I.[a]	*His*$^+$ ± SD[b]	% Survival[c]
TA97	DMSO	1.0	128 ± 25	100	1.0	112 ± 30	100
	0.5	1.4	174 ± 33	95	1.1	128 ± 8	100
	5	1.3	164 ± 31	95	0.8	91 ± 5	100
	10	1.2	151 ± 20	90	0.9	99 ± 6	100
	20	1.0	135 ± 10	80	1.1	126 ± 40	100
	4-NQO (1.0 µg/plate)	6.6	853 ± 188		8.7	974 ± 108	
TA98	DMSO	1.0	37 ± 2	100	1.0	24 ± 1	100
	0.5	1.1	39 ± 7	100	1.0	23 ± 5	100
	5	0.8	30 ± 4	100	1.1	26 ± 4	100
	10	0.9	31 ± 9	100	0.7	17 ± 3	100
	20	1.1	39 ± 12	100	0.9	20 ± 2	100
	4-NQO (0.5 µg/plate)	9.9	362 ± 42		20	479 ± 97	
TA100	DMSO	1.0	201 ± 35	100	1.0	195 ± 7	100
	0.5	1.0	208 ± 4	100	0.8	160 ± 3*	100
	5	0.9	184 ± 22	95	0.9	182 ± 12	100
	10	0.9	184 ± 27	90	1.0	193 ± 5	100
	20	1.1	218 ± 25	90	1.0	186 ± 18	95
	SA (0.5 µg/plate)	15	2929 ± 478		14	2680 ± 103	
TA102	DMSO	1.0	329 ± 27	100	1.0	334 ± 9	100
	0.5	1.1	361 ± 19	100	1.0	350 ± 29	100
	5	0.7	229 ± 93	100	0.9	310 ± 34	100
	10	0.8	265 ± 6	100	0.9	293 ± 48	100
	20	0.9	295 ± 6	100	0.9	297 ± 25	100
	MitC (0.5 µg/plate)	5.2	1703 ± 120		3.2	1067 ± 219	

[a]Mutagenic index: No. of *His*$^+$ induced in the sample/number of spontaneous *His*$^+$ in the negative control (DMSO). [b]*His*$^+$/plate: mean values of at least 3 replicate plates. [c]Percent survival relative to the negative control: toxicity is considered when percent survival <70% related to the control group. Positive controls are described in the Experimental section. Significant statistical differences between negative control and tested concentrations under the same experimental conditions are identified by an asterisk ($P < 0.05$). For abbreviations, see legend to Table 3.

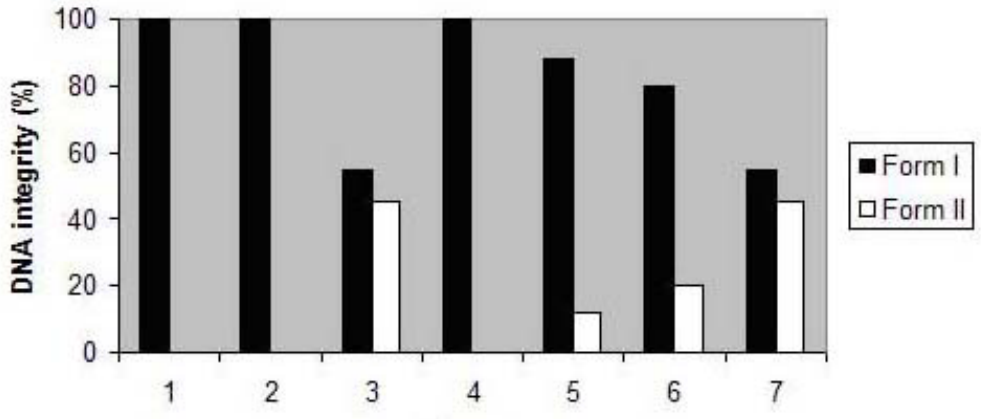

Figure 1. DNA breakage analysis using 0.8% agarose gel incubating 0.4 µg pUC18 plasmid with each of the 4 extracts of *Chromonephthea braziliensis*. 1 = H_2O; 2 = DMSO; 3 = 200 µg/mL $SnCl_2$; 4 = 250 µg/mL hexanic extract; 5 = 250 µg/mL dichloromethane extract; 6 = 250 µg/mL ethyl acetate extract; 7 = 250 µg/mL methanol extract. Form I = supercoiled native conformation; form II = open circle resulting from single-strand breaks.

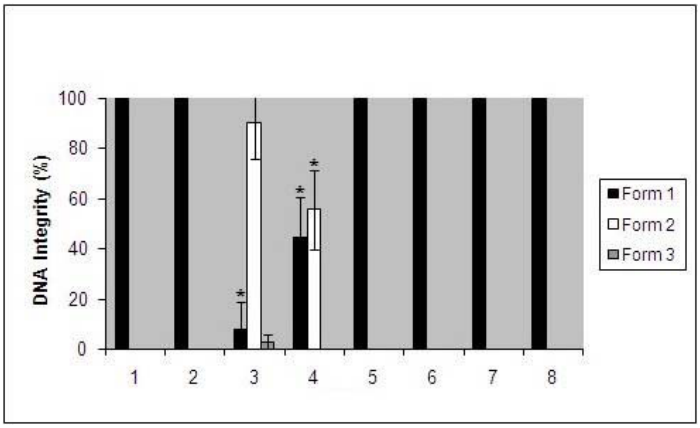

Figure 2. DNA breakage analysis using 0.8% agarose gel incubating 0.4 µg pUC18 plasmid with each of the 4 extracts of *Chromonephthea braziliensis* with melatonin (500 µM). 1 = dimethyl sulfoxide (DMSO); 2 = DMSO with melatonin; 3 = 200 µg/mL $SnCl_2$; 4 = 200 µg/mL $SnCl_2$ with melatonin; 5 = 250 µg/mL hexanic extract; 6 = 250 µg/mL dichloromethane extract; 7 = 250 µg/mL ethyl acetate extract; 8 = 250 µg/mL methanol extract. Significant statistical differences between negative control and tested concentrations under the same experimental conditions are identified by an asterisk ($P < 0.05$). Form I = supercoiled native conformation; form II = open circle resulting from single-strand breaks; form III = linear resulting from double-strand breaks.

Micronuclei in macrophages

Table 5 shows the results obtained using the 4 extracts of *C. braziliensis* at final concentrations of 20, 200, 350, and 500 µg/mL. Although there is a dose-dependent increase there is no evidence of cytotoxicity being induced, as no significant differences were found between each group and its control.

Table 5. Micronucleus induction in macrophage assay using the 4 extracts of *Chromonephthea braziliensis*.

Extract	*C. braziliensis* (µg/mL)	‰M.I. ± SD[a]	% Apoptosis	% Necrosis	% Micronucleus ± SD
	DMSO	3.7 ± 0.7	0.2	0.1	1.4 ± 0.3
n-Hex	20	2.8 ± 0.4	0.1	0.1	2.1 ± 0.3
	200	3.1 ± 0.7	0.2	0.0	1.5 ± 0.1
	350	2.2 ± 1.3	0.1	0.1	2.3 ± 0.3
	500	2.4 ± 0.8	0.1	0.1	2.0 ± 1.0
DCM	20	1.7 ± 0.7	0.3	0.1	2.1 ± 0.4
	200	2.2 ± 0.1	0.3	0.1	1.8 ± 0.1
	350	4.8 ± 1.3	0.0	0.1	2.4 ± 0.8
	500	2.3 ± 0.3	0.2	0.1	2.3 ± 0.7
EtOAc	20	3.9 ± 0.3	0.1	0.0	2.5 ± 1.0
	200	3.3 ± 1.1	0.1	0.0	1.3 ± 0.1
	350	4.7 ± 0.4	0.3	0.1	1.7 ± 0.8
	500	3.5 ± 0.1	0.2	0.1	1.8 ± 1.0
MeOH	20	2.4 ± 0.8	0.2	0.2	1.9 ± 0.8
	200	2.6 ± 0.4	0.1	0.1	1.3 ± 0.3
	350	3.9 ± 0.6	0.3	0.1	1.6 ± 0.1
	500	3.1 ± 0.1	0.2	0.1	1.8 ± 0.3
	MNNG	2.5 ± 0.6	0.4	0.2	2.9 ± 0.1*

[a]Mitotic index per thousand. DMSO = dimethyl sulfoxide (negative control); MNNG = 0.5 µM N-methyl-N-nitro-N-nitrosoguanidine (positive control). Significant statistical difference between the negative control and concentrations tested under the same experimental conditions is identified by an asterisk ($P < 0.05$). For abbreviations, see Table 1.

Protein concentration and DPPH activity

The protein concentration measured in each extract was 309 µg/mL for n-Hex; 492.5 µg/mL for DCM; 373 µg/mL for EtOAc; and only 69.1 µg/mL for MeOH. The decrease in the protein concentration observed in the extracts could be due to the sequential extraction.

The incubation of the extracts at different concentrations could suggest the ability of proteins to act as scavengers or form complexes with secondary metabolites, which would lead to decreases in antioxidant activity of the extracts. No significant differences ($P > 0.05$) were detected in the presence or absence of proteinase K (250 µg/mL), suggesting that the proteins present in the extracts did not interfere with the antioxidant ability of the secondary metabolites (Figure 3).

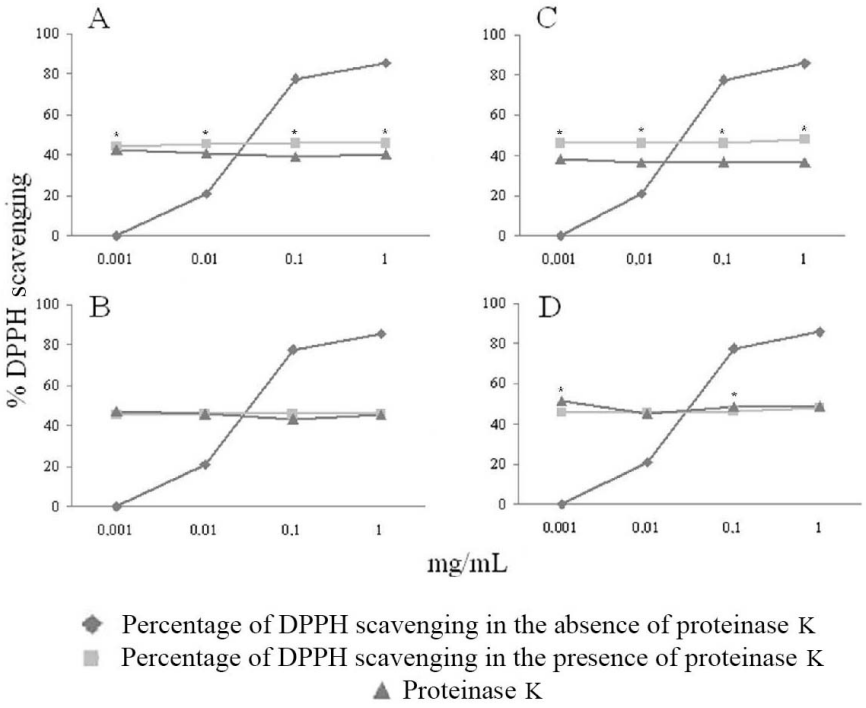

Figure 3. Percentage of DPPH scavenging in the absence or in the presence of proteinase K (250 µg/mL) at diferent concentrations (0.001, 0.01, 0.1, and 1 mg/mL). **A.** Hexane extract; **B.** dichloromethane extract; **C.** ethyl acetate extract; **D.** methanol extract. Significant statistical differences between absence and presence of proteinase K under the same concentrations are identified by an asterisk ($P < 0.05$).

In the Nephtheidae family, some species can be distinguished by their terpene distribution. *Nephthea* spp produce sesquiterpenes and/or diterpenes (70% of cembranoid skeletons), *Litophyton* spp produce up to 100% cembranoid diterpenes, and *Lemnalia*, *Paralemnalia*, and *Capnella* spp produce 100% sesquiterpenes (Coll, 1992). These secondary metabolites play various ecological roles for octocorals, namely in antipredation activity, and in the production of allelopathic agents, antifouling agents, and sex pheromones (Lages, 2003).

Terpene activity in *Sinularia* spp (Alcyonacea), soft corals, has been described as having antipredation activity when in its colonial form, and dependent on inorganic compounds. Allelopathy is also a feature of the secondary metabolites and has been studied in parallel with antipredation activity. Soft corals have proved to be aggressive competitors in the race for space, leading to neighboring scleractinian coral mortality (Lages, 2003).

Terpenes can react with atmospheric ozone inducing hydrogen peroxide, a highly reactive oxygen species (Becker et al., 1990). In the same way, α-pinene, a monoterpene, can cause oxidative damage (Singh et al., 2006). Assays for the antimutagenic and mutagenic activity of linalool, linalyl acetate, and β-caryophyllene - all terpenes - showed positive responses to mutagenicity for linalyl acetate and antimutagenic activity for linalool and β-caryophyllene (Di Sotto et al., 2008).

Different end points were evaluated in the present study. The negative mutagenicity and cytotoxicity responses were evaluated by *Salmonella* reverse assays, while genotoxicity was evaluated by micronucleus formation and DNA breakage. For the first assay, cells were incubated with the extracts. In the second one, there was direct interaction with the electrophilic compounds (plasmid DNA).

We only observed induced DNA breakage for DCM, EtOAc, and MeOH extracts. As these solvents have different polarities, the products extracted by them should in general be different. Quantitatively, MeOH extract presented greater DNA breakage than the others. This means that more polar substances in the extract present this potential. Recently, new composition of the monohydroxylated sterol fraction has been described: [22(E)-cholesta-5,22-dien-3 β-ol; cholesta-5-en-3 β-ol; 22(E), 24(S)-Ergosta-5,22-3β-ol; 22(E)-24(S)-24-methylcholesta-5,22-dien-3β-ol; 24(R)-24-methylcholesta-5,24-dien-3β-ol; 24(Z)-24-ethylcholesta-5-24(28)-dien-3β-ol; 28(E)-ethylcholesta-5,22-dien-3β-ol; 24-ethylcholesterol(24ξ)-ethylcholesta-5-3β-ol; 24(E)-propylcholesta-5,24-dien-3β-ol or 24(E)-isopropylcholesta-5,24-dien-3β-ol]. A new hemiketal steroid, 23-keto-cladiellin-A, has also been isolated from the unpalatable hexane extract of *C. braziliensis* (Fleury et al., 2008). We now believe that the above mentioned products are not involved in DNA breakage.

Although macrophage micronucleus formation was not observed, the presence of DNA damage in free cell extracts (plasmid incubation) with the DCM, EtOAc, and MeOH extracts suggests that simple DNA breaks were induced. We suggest that among the secondary metabolites produced by the coral *C. braziliensis*, there is some substance, possibly a terpene or terpenoid, capable of generating oxidative stress that may be linked to some of its ecological interactions, enabling its continuation elsewhere.

CONCLUSIONS

The n-Hex, DCM, EtOAc, and MeOH extracts of *C. braziliensis* showed no cytotoxicity or mutagenicity, but genotoxicity was found for the DCM, EtOAc, and MeOH extracts. However, factors like the process of fractioning or test conditions to which these samples were subjected could have prevented their activation. Therefore, a methodology, which includes these questions, is needed for a more complete toxicological evaluation.

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