Arquivos de Ciências do Mar

BIOLOGICAL ACTIVITY OF AQUEOUS AND ORGANIC EXTRACTS OF SEAWEEDS FROM CEARÁ STATE, BRAZIL

Atividade biológica de extratos orgânicos e aquosos de macroalgas marinhas do Estado do Ceará, Brasil

Márcia Rocha Torres[1], Alessandra de Paula Alves Sousa[1], Eduardo Augusto Torres da Silva Filho[1], Cláudia Pessoa[1], Maria Elisabete Amaral de Moraes[1], Manoel Odorico de Moraes[1], Letícia Veras Costa-Lotufo[1,2]

RESUMO

O presente estudo avaliou a atividade biológica de 12 macroalgas marinhas da costa do estado do Ceará, Brasil. Das espécies coletadas foram obtidos 48 extratos (extratos aquosos, diclorometânicos, acetônicos e metanólicos) de cada espécie. A atividade dos extratos foi avaliada quanto à letalidade em náuplios de artêmia, ao potencial hemolítico, à inibição do desenvolvimento de embriões de ouriço-do-mar e ao efeito antiproliferativo sobre células tumorais através do método do MTT. Este estudo revelou que 9 das 12 espécies testadas apresentaram alguma atividade nos ensaios aplicados, sendo que Botryocladia occidentalis *foi a mais potente. O extrato acetônico obtido de* B. occidentalis *inibiu o crescimento de quatro das cinco linhagens de células tumorais usadas com uma CI_{50} variando de 5,0 a 24,5 μg/mL, demonstrou atividade antimitótica em ovos de ouriço em concentrações até 100 μg/mL, não apresentou atividade hemolítica, mas mostrou uma moderada toxicidade para* Artemia salina*. Estudos futuros são necessários para caracterização química dos princípios ativos, além de avaliações biológicas mais extensas.*

Palavras-chaves: *algas marinhas, citotoxicidade, hemólise, ovos de ouriço-do-mar, células tumorais.*

ABSTRACT

The present study evaluated the biological activity of 12 marine macroalgae from Ceará State, Brazil. From the collected species 48 extracts (aqueous, dichloromethane, acetone and methanol extracts for each species) were obtained. The activity of extracts were rated at the following bioactivities: lethality in brine shrimp nauplii, inhibition of the development of sea urchin eggs, hemolytic activity on mice erythrocytes and inhibition of in vitro cellular proliferation on tumor cell lines using MTT assay. This study revealed that nine among the 12 tested species presented some activity in the applied assays, being that of the red algae Botryocladia occidentalis *the most potent one The acetone extract obtained from* B. occidentalis *inhibited the growth of four out of five used tumor cell lines with an IC_{50} in the range of 5.0 to 24.5 μg/ml, possessed antimitotic activity on sea urchin eggs at concentrations up to 100 μg/ml, with no hemolylic activity and moderate toxicity to brine shrimp nauplii. Further studies are necessary for chemical characterization of the active principles and more extensive biological evaluation*

Key words: *marine macroalgae, cytotoxicity, hemolysis, sea urchin embryos, tumor cell lines*

[1] Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, CE, Brasil.
[2] Instituto de Ciências do Mar, Universidade Federal do Ceará, Fortaleza, CE, Brasil, e-mail: lvcosta@secrel.com.br

INTRODUCTION

Historically, seaweeds have been consumed as healthy food in many Asiatic countries like China, Korea and Japan. They have also been used by cosmetic and chemical industries (Round, 1983) and at last they have caused an emerging interest in the biomedical area due to their content on pharmacologically bioactive substances with great chances to be employed against bacteria, virus, other pathogens and tumors (Blunden, 1993; Ireland *et al*., 1993; Smit, 2004).

New anticancer agents have been found in marine sources like peptides, polyesters, alkaloids, prostanoids, lactones, terpenoids, hidroquinones and so on. These compounds have been isolated from sponges, corals, seaweeds, tunicates, bryozoans and others (Kitagawa & Kobayashi, 1991; Newman & Cragg, 2004). Despite the ascending number of new findings about seaweed metabolites possessing biological activity on the last three decades few products having actual potential have been identified or developed (Smit, 2004). Among those substances that received most attention from pharmaceutical companies for development of new drugs are the sulfated polysaccharides (antivirals), the halogenated furanones (antifouling compounds) and the kahalide F (anti-cancer and anti-HIV compounds) (reviewed by Smit, 2004).

Kahalide F was first isolated from the sacoglossan mollusk *Elysia rufescens*, however later it was elucidated that the producer is a green alga from the genus *Bryopsis*, being concentrated in the mollusk through its diet (Hamann and Scheuer, 1993; Hamann *et al*., 1996). This depsipeptide now undergo phase II clinical studies for liver carcinoma treatment (Newman & Cragg, 2004). This compound has a unique target, acting on the lysosomal membrane, leading to a necrotic cell death, called oncosis (Newman & Cragg, 2004; Smit, 2004).

The significant number of compounds from marine sources that have been entered into antitumor preclinical and clinical trials stimulates continuous efforts on this research area. Previous data on the screening of ascidians and sponges collected in the northeastern Brazilian coast showed two interesting findings: first, the high degree of endemism of this region and second, that approximately half of the studied species presented promising results (Jimenez *et al*., 2003 and 2004).

The aim of the present study was to screen for cytotoxic and antimitotic activities seaweed extracts collected from the coastal waters of Ceará in the Northeast, Brazil. The intention of this screening program is to expand the knowledge of the flora of this region and find new substances with possible pharmaceutical applications.

MATERIALS AND METHODS

Seaweed harvesting

The seaweeds were collected from Pacheco Beach (Fortaleza-Ceará-Brazil) on the months of January and February, 2004. Once in the lab they were rinsed with filtered ocean water, packed in plastic bags and frozen. Their classification was made in accordance to the checklist of Wynne (1986). Table I shows the studied species separated by divisions (Chlorophyta, Rhodophyta, Phaeophyta) and some of the previous reported pharmacological activities.

Preparation of the crude seaweed extracts

From the 12 collected species it has been obtained 48 extracts (aqueous, dichloromethane, acetone and methanol extracts for each species). The frozen seaweeds were dried out at 60°C. Thereafter, they were grounded to a fine powder, weighed and subjected to successive extractions with a sample mass to solvent volume proportion of 1:10 at room temperature. The solvent sequence showed an increasing polarity starting with dichloromethane, followed by methanol and saline solution. Each extraction procedure was made under constant agitation for 24 hours. After this time, the extracts were filtered through a nylon cloth and the resulting solutions (just the organic ones) were concentrated in a rotary vacuum evaporator. The remaining concentrates were dried out at 40°C. Each methanol fraction was applied into a cellulose column and eluted with acetone followed by methanol. Once dried all the extracts were weighed. The aqueous extracts were concentrated, dialisated, and then, freeze-dried. The aqueous residues were reconstituted in water, while organic ones were reconstituted in DMSO before testing.

MTT assay

The cytotoxicity of the extracts was tested against B-16 (murine melanoma), HCT-8 (human colon carcinoma), MCF-7 (human breast carcinoma) CEM and HL-60 (leukemia) tumor cell lines (National Cancer Institute, MD, USA). Cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C with 5% CO_2. For experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.3×10^6 cells/well for suspended cells in 100 µl of medium). In a first set of

Table I – List of algal species used in this study.

Classification	Dry Weight (g)	Extraction solvent and yield (%)	Some reported pharmacological activies	Ref.
CHLOROPHYTA				
Caulerpa racemosa (Forsskal) J.Agardh (Caulerpaceae)	5.0	Dichloromethane (3.5) Acetone (0.3) Methanol (0.2) Aqueous (0.5)	Antiherpetic Antitumor Brine shrimp toxicity	Ghosh *et al*. (2004) Harada *et al*. (1997) Ara et al. (1999)
Caulerpa sertularioides (S. G. Gmelin) Howe (Caulerpaceae)	1.2	Dichloromethane (9.5) Acetone (0.5) Methanol (4.3) Aqueous (0.03)	Inhibit telomerase activity Antitumor	Kanegawa *et al*. 2000 Harada *et al*. (1997)
Codium decorticatum (Woodward) Howe (Codiaceae)	32.0	Dichloromethane (1.5) Acetone (0.9) Methanol (10.8) Aqueous (2.5)	Antiherpetic	Santos *et al*. (1999)
Enteromorpha intestinalis (Linnaeus) Link (Ulvaceae)	6.0	Dichloromethane (2.1) Acetone (1.1) Methanol (0.5) Aqueous (0.8)	Antiviral Antitumor	Kamat *et al*. (1992) Harada *et al*. (1997)
Ulva fasciata Delile (Ulvaceae)	20.0	Dichloromethane (1.5) Acetone (0.1) Methanol (0.2) Aqueous (7.0)	Antibacterial Antiherpetic Antiviral Metal accumulation Brine shrimp toxicity	Pinheiro- Vieira and Caland-Noronha (1971), Selvin and Lipton (2004) Santos *et al*. (1999) Tringali (1997), Garg *et al*. (1992) Lee and Wang (2001) Selvin and Lipton (2004)
RHODOPHYTA				
Botryocladia occidentalis (B_{ϕ}rgesen) Kylin (Rhodymeniaceae)	6.0	Dichloromethane (0.9) Acetone (0.3) Methanol (2.4) Aqueous (0.5)	Anticoagulant	Farias *et al*. (2000; 2001), Matsubara (2004), Pereira *et al*. (2002)
Gracilaria domingensis Sonder ex Kützing (Gracilariaceae)	20.0	Dichloromethane (0.3) Acetone (0.1) Methanol (0.3) Aqueous (3.5)	Antibacterial Antitumor	Pinheiro-Vieira and Caland-Noronha (1971) Fernández *et al*. (1989)
Gracilaria lemaneiformis (Bory) Weber-van Bosse (Gracilariaceae)	20.0	Dichloromethane (0.3) Acetone (0.3) Methanol (0.4) Aqueous (1.2)	Hemagglutinating activity	Freitas *et al*. (1992)
Hypnea musciformis (Wulfen in Jacquin) Lamouroux (Hypneaceae)	8.0	Dichloromethane (0.5) Acetone (0.2) Methanol (0.5) Aqueous (0.2)	Antiherpetic Antiviral Antifungal Antispasmodic Anti-inflammatory Hemagglutinating activity Brine shrimp toxicity	Santos *et al*. (1999) Kamat *et al*. (1992) Melo *et al*. (1997) Salimabi (1980) Salimabi (1980) Nagano *et al*. (2002) Selvin and Lipton (2004)
Laurencia papillosa (C. Agardh) Grreville (Rhodomelaceae)	19.0	Dichloromethane (1.0) Acetone (0.2) Methanol (0.5) Aqueous (1.2)	Antibacterial Inhibit telomerase activity	Pinheiro- Vieira and Caland-Noronha (1971) Kanegawa *et al*. (2000)
Pterocladia capillacea (S. G. Gmelin) Bornet & Thuret (Gelidiaceae)	10.0	Dichloromethane (0.3) Acetone (0.3) Methanol (0.3) Aqueous (0.2)	Anticoagulant Antiherpetic	Shanmugam and Mody (2000) Santos *et al*. (1999), Pujol *et al*. (1996)
PHAEOPHYTA				
Lobophora variegata (Lamouroux) Womersley (Dictyotaceae)	11.3	Dichloromethane (1.2) Acetone (1.7) Methanol (0.6) Aqueous (0.9)	Antifungal	Ballesteros *et al*. (1992)

experiments, the extracts (125 µg/ml) dissolved in DMSO (1%) were added to each well after 24 hours and, then, incubated for 3 days (72 h). Control groups received the same amount of DMSO. Doxorubicin (0.058 - 0.58 µg/ml) was employed as positive control. Growth of tumor cells was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a blue formazan product (Mosmann, 1983). At the end of a 72 h incubation period, the medium in each well was replaced by fresh medium (200 µl) containing 0.5 mg/ml of MTT. Three hours later, the

formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a multi-plate reader (Spectra Count, Packard, Ontario, Canada). Drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm. The extracts that exhibited a growth inhibitory effect greater than 90% in this pre-screen were selected for a second experiment in order to determine the IC_{50} values. In these experiments, the extract concentration ranged from 2 to 125 µg/ml.

Assay on sea urchins

The test was performed in 24-well plates following the method described by Jimenez *et al.* (2003). Adult sea urchins (*Lytechinus variegatus*) were collected at Lagoinha Beach (Trairí, Ceará State, Brazil). The gamete elimination was induced by injecting 3.0 ml of 0.5M KCl into the urchins coelomic cavity via the periostomial membrane. The eggs were washed twice using filtered sea water to remove the jelly coat surrounding the cells. Concentrated sperm was collected with a Pasteur pipette and maintained under low temperatures until the moment of fertilization. For fertilization, 1 ml of a sperm suspension (0.05 ml of concentrated sperm in 2.45 ml of filtered sea water) was added to every 50 ml of egg solution. Each well received 1 ml of fertilized egg suspension. The algal extracts were added immediately after fertilization (within 2 min) and in order to get concentrations of 100 and 1000 µg/ml in a final volume of 2 ml. Only the extracts considered active in the pre-screen were tested in this assay. Doxorubicin (58.0 µg/ml) was used as positive control. The plates were then shaken on a constant temperature water bath at 26 ± 2°C. At appropriate intervals, aliquots of 200 µl were fixed in the same volume of 10% formaldehyde to obtain first and third cleavages, and blastulae. One hundred eggs or embryos were counted for each concentration of extracts to obtain the percentage of normal cells.

Brine shrimp assay

Brine shrimp (*Artemia salina* Leach) eggs were hatched in a beaker filled with sea water under constant aeration. After 48 hours the phototrophic nauplii were collected by pipette. The nauplii were macroscopically counted in the stem of the pipette against a lighted background. Ten shrimps were transferred to each well of 24-multiwell plates containing the samples. The extract concentrations were 100 and 1000 µg/ml. Only the extracts considered active in the pre-screen were tested in this assay. The plates were maintained under illumination. Survivors were counted after 24 hours of incubation and the percentage of deaths at each dose and control (sea water plus vehicle) were determined (Meyer *et al*., 1982).

Hemolytic assay

The test was performed in 96-well plates following the method described by Jimenez *et al*. (2003). Each well received 100 µl of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well was the negative control that contained only the vehicle (distilled water or DMSO 10 %), and, in the second well, 100 µl of test substance that was diluted in half was added. The extracts were tested at concentrations ranging from 0.39 to 200 µg/ml. Only the extracts considered active in the pre-screen were tested in this assay. The serial dilution continued until the 11th well. The last well received 20 µl of 0.1% triton X-100 (in 0.85% saline) to obtain 100% hemolysis (positive control). Then, each well received 100 µl of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm.

Statistical analysis

Data are presented as mean ± S.E.M from three independent experiments. The IC_{50} or EC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA).

RESULTS

MTT assay

Among forty-eight tested extracts in the pre-screen, sixteen were considered active, since they caused a cell growth inhibition greater than 90%. All tested species presented some kind of activity in the acetone extract, while only the dichloromethane extracts obtained from *Gracilaria domingensis* and *Gracilaria lemaneiformis* were active and also the aqueous extracts of *Codium decorticatum* and *Lobophora variegata*. In a second set of experiments, the IC_{50} of these extracts were determined (Table II). The acetone extract obtained from *Botryocladia occidentalis* and the aqueous extract obtained from *Codium decorticatum* were the most active in this assay, exhibiting lower IC_{50} values against all tested cell lines, 5.0 and 28.2 µg/ml for HL-60, 43.1 and 16.0 µg/ml for CEM, 24.5 and 14.6 for HCT-8, 10.3 and 45.6 µg/ml for B-16 and

Table II - *In vitro* cytotoxicity of the alga l extracts on five tumor cell lines measured by the MTT assay.

Alga species	Extract	Cell line IC_{50} (µg/ml)				
		HL-60	CEM	HCT-8	B-16	MCF-7
Positive Control (Doxorubicin)	-	0.02 0.01 – 0.02	0.02 0.01 – 0.02	0.04 0.03 – 0.05	0.03 0.02 – 0.04	0.20 0.17 – 0.24
Caulerpa racemosa	Acetone	34.5 32.5 – 36.6	35.8 32.5 – 39.5	29.7 23.4 – 37.6	29.2 22.9 – 37.1	42.3 36.8 – 48.6
Caulerpa sertularioides	Acetone	58.7 56.1 – 61.4	59.5 54.6 – 64.7	55.8 49.8 – 62.6	37.7 34.0 – 41.8	58.1 51.6 – 65.4
Codium decorticatum	Acetone	35.9 32.9 – 39.2	29.7 27.5 – 32.1	35.8 31.9 – 40.2	37.8 31.3 – 45.5	62.4 16.6 - 23.3
	Aqueous	28.2 23.8 – 34.9	16.0 11.7 – 21.8	14.6 13.4 – 15.9	45.6 41.6 – 50.1	10.6 7.9 – 14.1
Enteromorpha intestinalis	Acetone	18.3 14.9 – 22.4	34.6 21.1 – 56.8	42.3 35.7 – 50.0	13.5 12.4 – 14.7	37.7 17.9 – 79.6
Ulva fasciata	Acetone	31.5 26.1 – 38.0	46.6 43.1 – 50.3	77.2 71.7 – 83.1	34.5 28.4 – 41.8	61.1 49.1 – 76.0
Botryocladia occidentalis	Acetone	5.0 3.6 – 7.21	43.1 33.1 – 56.1	24.5 20.7 – 28.9	10.3 9.1 – 11.6	20.3 14.6 – 28.2
Gracilaria domingensis	Acetone	17.2 11.6 – 25.4	42.8 36.5 – 50.2	57.1 48.5 – 67.1	36.8 30.2 – 45.0	52.3 44.5 – 61.5
	Dichloromethane	26.7 21.3 – 33.5	59.5 37.0 -95.7	49.8 43.9 – 56.5	58.7 47.3 – 72.9	148.8 118.2 – 187.3
Gracilaria lemaneiformis	Acetone	10.9 7.4 – 16.2	30.7 23.1 – 40.9	33.7 25.5 – 44.5	14.6 13.6 – 15.7	18.9 7.2 – 49.2
	Dichloromethane	18.8 7.0 – 50.3	57.0 40.6 – 80.0	16.3 11.1 – 23.9	42.2 34.1 – 52.3	25.6 9.9 – 66.3
Hypnea musciformis	Acetone	30.5 26.5 – 35.1	34.8 24.8 – 48.9	58.0 30.0 – 112.3	30.5 21.6 – 43.1	38.2 31.2 – 46.6
Laurencia papillosa	Acetone	9.9 7.4 – 13.0	49.0 22.3 – 107.8	15.6 12.5 – 19.4	13.9 10.7 – 18.2	31.6 6.0 – 146.1
Pterocladia capillacea	Acetone	45.5 35.9 – 57.5	ND	64.9 50.1 – 84.1	48.5 19.9 – 118.0	83.9 15.4 – 455.8
Lobophora variegata	Acetone	96.4 64.4 – 144.2	55.5 49.5 – 62.2	97.7 65.6 – 145.6	32.4 23.3 – 44.9	48.2 26.9 – 86.3
	Aqueous	ND	57.0 39.3 – 82.8	73.0 57.0 – 93.6	59.4 44.9 – 78.4	62.5 54.7 – 71.3

The IC_{50} and its 95 % confidence interval (CI 95%) were obtained by non-linear regression. ND (Not determined)

20.3 and 10.6 µg/ml for MCF-7, respectively. The acetone extracts obtained from *Laurencia papillosa* (3 cell lines), *Gracilaria lemaneiformis* (3 cell lines), *Gracilaria domingensis* (1 cell line), *Caulerpa racemosa* (2 cell lines), *Enteromorpha intestinalis* (1 cell line), *Codium decorticatum* (1 cell line) and the dichloromethane extracts of *Gracilaria lemaneiformis* (2 cell lines) and *Gracilaria domingensis* (1 cell line) also showed cytotoxic activity.

Assay of sea urchins

Table III shows the results for the sea urchin embryo assay using the algal extracts. The acetone extracts from *Botryocladia occidentalis*, *Ulva fasciata*, *Gracilaria lemaneiformis* and *Hypnea musciformis* were the most active ones, inhibiting more than 50% of normal development in all analyzed phases at the smallest tested concentration (100 µg/ml). The aqueous extract of *Lobophora variegata* (100 µg/ml) seemed to lose activity with an increasing time of incubation, as observed by the low inhibition observed in the blastulae stage, 12.6 ± 3.9 %, when compared to the ones observed in the first and third cleavages, 95.0 ± 0.6 and 95.4 ± 0.6 %, respectively. On the other hand, the acetone extract of *Gracilaria domingensis*, and the extracts obtained with dichloromethane of both *Gracilaria* species showed enhanced activity with an increasing time of incubation, being more active after the first cleavage at the concentration of 100 µg/ml. All extracts presented significant activity at the concentration of 1000 µg/ml.

Table III – Cytotoxic activity on the sea urchin, *Lytechinus variegatus*.

Algal species	Extract	Concentration (µg/mL)/	1st Cleavage mean ± SEM	3rd Cleavage mean ± SEM	Blastulae mean ± SEM
Positive Control Doxorubicin	-	88	100.0 ±0.0	100 ±0.0	100.0 ±0.0
Caulerpa racemosa	Acetone	100	0.0 ± 0.6	14.6 ± 3.4	8.3 ± 2.0
		1000	100.0 ±0.0	99.6 ± 0.4	98.6 ±0.9
Caulerpa sertularioides	Acetone	100	0.0 ± 0.0	38.2 ± 4.2	32.3 ± 10.8
		1000	100.0 ±0.0	100.0 ± 0.0	100.0 ±0.0
Codium decorticatum	Acetone	100	0.0 ± 0.0	25.8 ± 4.0	6.5 ± 0.9
		1000	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
	Aqueous	100	10.3 ± 2.4	98.1 ± 0.7	45.5 ±6.6
		1000	28.6 ± 1.6	99.6 ± 0.3	31.8 ±9.0
Enteromorpha intestinalis	Acetone	100	0.0 ± 0.0	0.0 ± 0.0	22.8 ±0.9
		1000	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
Ulva fasciata	Acetone	100	83.6 ± 3.1	98.4 ± 0.7	86.5 ±1.8
		1000	100.0 ±0.0	100.0 ±0.0	86.2 ± 2.9
Botryocladia occidentalis	Acetone	100	89.68 ±0.3	99.25 ±0.7	99.65 ±0.3
		1000	100.0 ±0.0	100.0 ±/ 0.0	100.0 ±0.0
Gracilaria domingensis	Acetone	100	28.8 ± 0.9	81.4 ± 1.3	93.1 ±2.4
		1000	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
	Dichloromethane	100	0.0 ± 0.0	61.7 ± 2.9	100.0 ±0.0
		1000	100.0 ±0.0	100.0 ±0.0	61.7 ±0.0
Gracilaria lemaneiformis	Acetone	100	65.8 ± 15.6	80.3 ± 1.4	52.3 ±1.4
		1000	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
	Dichloromethane	100	23.9 ± 6.2	100.0 ±0.0	100.0 ±0.0
		1000	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
Hypnea musciformis	Acetone	100	56.4 ± 0.2	100.0 ±0.0	100.0 ±0.0
		1000	99.6 ± 0.1	100.0 ±0.0	100.0 ±0.0
Laurencia papillosa	Acetone	100	4.29 ± 1.4	41.3 ± 4.8	4.82 ±2.0
		1000	100.0 ±0.0	99.6 ± 0.4	100.0 ±0.0
Pterocladia capillacea	Acetone	100	2.5 ± 1.4	12.7 ± 3.0	5.5 ± 2.1
		1000	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
Lobophora variegata	Acetone	100	18.3 ± 1.0	89.5 ± 1.3	16.3 ±3.9
		1000	100.0 ±0.4	100.0 ±0.0	100.0 ±0.0
	Aqueous	100	95.0 ± 0.6	95.4 ± 0.6	12.6 ±3.9
		1000	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0

Brine shrimp assay

Among the sixteen tested extracts, six killed all nauplii at the concentration of 100 µg/ml: *Caulerpa racemosa* (acetone), *Codium decorticatum* (aqueous), *Lobophora variegata* (acetone), *Gracilaria domingensis* (acetone and dichloromethane) and *Gracilaria lemaneiformis* (dichloromethane), being the most active extracts in this assay (Table IV). The acetone extracts of *Caulerpa sertularioides*, *Ulva fasciata* and *Botryocladia occidentalis* were moderately active, presenting lethality higher than 50% at the concentration of 100 µg/ml. The other tested extracts presented low toxicity in this assay, being active only at the highest concentration (1000 µg/ml).

Hemolytic assay

In order to verify whether the observed cytotoxicity is related to membrane disruption, the selected algal extracts were tested for their ability to induce lysis of mouse erythrocytes. The results obtained from the hemolytic assay are presented in table V.

Table IV – Acute toxicity of the alga extracts on *Artemia salina nauplii*.

Algal species	Extract	Concentration (µg/ml)	Lethality mean ± SEM (%)
Caulerpa racemosa	Acetone	100	100.0 ± 0.0
		1000	100.0 ± 0.0
Caulerpa serrulatoides	Acetone	100	73.3 ± 14.5
		1000	90.0 ± 0.0
Codium decorticatum	Acetone	100	26.6 ± 0.0
		1000	100.0 ± 3.3
	Aqueous	100	100.0 ± 0.0
		1000	100.0 ± 0.0
Enteromorpha intestinalis	Acetone	100	30.0 ± 0.0
		1000	100.0 ± 0.0
Ulva fasciata	Acetone	100	80.0 ± 5.8
		1000	100.0 ± 0
Botryocladia occidentalis	Acetone	100	63.3 ± 3.3
		1000	100.0 ± 0.0
Gracilaria domingensis	Acetone	100	96.6 ± 3.3
		1000	100.0 ± 0.0
	Dichloromethane	100	100.0 ± 0.0
		1000	100.0 ± 0.0
Gracilaria lemaneiformis	Acetone	100	0.0 ± 0.0
		1000	100.0 ± 0.0
	Dichloromethane	100	100.0 ± 0.0
		1000	100.0 ± 0.0
Hypnea musciformis	Acetone	100	0.0 ± 0.0
		1000	100.0 ± 0.0
Laurencia papillosa	Acetone	100	36.6 ± 0
		1000	100.0 ± 0
Pterocladia capillacea	Acetone	100	0.0 ± 0.0
		1000	100.0 ± 0.0
Lobophora variegata	Acetone	100	100.0 ± 0.0
		1000	100.0 ± 0.0
	Aqueous	100	23.3 ± 13.3
		1000	100.0 ± 0.0

Table V – Hemolytic activity of the alga extracts on 2% mouse erythrocytes.

Algal species	Extract	EC_{50} (CI 95%) µg/ml
Caulerpa racemosa	Acetone	> 200.0
Caulerpa serrulatoides	Acetone	> 200.0
Codium decorticatum	Acetone	>200.0
	Aqueous	16.1 (6.9 – 37.1)
Enteromorpha intestinalis	Acetone	> 200.0
Ulva fasciata	Acetone	> 200.0
Botryocladia occidentales	Acetone	> 200.0
Gracilaria domingensis	Dichloromethane	> 200.0
	Acetone	> 200.0
Gracilaria lemaneiformis	Dichloromethane	129.8 (123.0 – 137.0)
	Acetone	> 200.0
Hypnea musciformis	Acetone	> 200.0
Laurencia papillosa	Acetone	> 200.0
Pterocladia capillacea	Acetone	108.6 (35.6 - 331.7)
Lobophora variegata	Acetone	105.1 (104.3 – 106.0)
	Aqueous	78.2 (64.6 – 90.2)

The EC_{50} and 95 % confidence interval (CI 95%) were obtained by non-linear regression

The aqueous extract obtained from the green algae *Codium decorticatum* was the most active in this assay (EC_{50} = 16.1 µg/ml), followed by the extracts from *Lobophora variegata* (aqueous and acetone), *Pterocladia capillacea* (acetone), and *Gracilaria lemaneiformis* (dichloromethane), which presented EC_{50} values of 78.2, 105.1, 108.6 and 129.8 µg/ml, respectively. The other tested extracts were inactive in this assay.

DISCUSSION AND CONCLUSIONS

In this study, the antimitotic potential of 12 macroalgae collected from the northeastern Brazilian coast was investigated. The *in vitro* antimitotic potential was estimated as the ability of these extracts to inhibit tumor cell line growth and sea urchin egg development. The toxicity to *A. salina* nauplii and the hemolytic activity on mouse erythrocytes were also evaluated.

According to the criteria of the American National Cancer Institute, the IC_{50} limit to consider a crude extract promising for further purification is lower than 30 µg/mL (Suffness & Pezzuto, 1990). Considering this criteria, seven of twelve species of marine algae (four red and three green algae species) studied presented promising results, given a particularly high incidence (58.3%) of cytotoxic activity. Harada *et al*. (1997) and Xu *et al*. (2004) emphasized the antitumor potential of brown and red algae. Generally, their antitumor potential is related to the presence of polysaccharides. The extraction protocol used in the present work led to a differential extraction according to the polarity of the compounds, being the presence of polysaccharides expected to the aqueous extracts, which were, indeed, not very active. In the screening performed by Harada *et al*. (1997), cytotoxic activity was frequently found in methanol extracts, while Xu *et al*. (2004) showed that ethanol was the best solvent for extracting purposes, in the present work the acetone extracts were the most active.

The most interesting result was obtained with acetone extract of *Botryocladia occidentalis*, that inhibited the growth of four out of five tumor cell lines used in this study with an IC_{50} lower than 25 µg/ml. The extract also showed antimitotic activity on sea urchin eggs at 100 µg/ml. These activities seemed not to be related with membrane disruption once this extract presented no hemolytic activity. Moreover, it presented only a moderate lethality potential on the brine shrimp assay. This is the first report on the antitumor potential of this species. Previous studies with this alga are restricted to the isolation of a unique sulfated galactan, that in fact has a very interesting

antithrombotic action, simultaneously inducing platelet aggregation (Farias *et al*., 2000 e 2001).

The aqueous extract of the green algae *Codium decorticatum* also presented strong cytotoxicity on four out of five tested tumor cell lines. However, it was extremely potent on hemolytic assay (EC_{50} = 16.1 µg/ml), suggesting that the presence of lytic substances could be responsible for the observed cytotoxic activity. It was also observed a high lethality potential for this extract in the brine shrimp assay. Previous work with *Codium decorticatum* described the presence of an alcohol with strong toxicity in the brine shrimp assay (Ahmad *et al*., 1994), while the antitumor potential was already described for other species of the genus *Codium* (El-Marsy *et al*., 1995; Harada *et al*., 1997; Xu *et al*., 2004)

The red algae *Gracilaria lemaneiformis* also presented interesting results. The acetone extract inhibited cell proliferation in three out of five tested tumor cell lines, and also inhibited the urchin development, while the dichloromethane extract induced lyses of mice erythrocytes and led to nauplii death. These data suggest the presence of different bioactive substances in these extracts.

The extracts of *Laurencia papillosa*, *Gracilaria domingensis*, *Caulerpa racemosa*, *Enteromorpha intestinalis*, *Ulva fasciata* and *Hypnea musciformis* also presented moderate cytotoxic activity. Previous studies have already demonstrated the occurrence of cytotoxic activity in some of these species, like *Caulerpa racemosa*, *Enteromorpha intestinalis* and *Gracilaria domingensis* (Fernández *et al*., 1989; Harada *et al*., 1997). On the other hand, no interesting cytotoxic activity was observed in *Caulerpa sertularioides*, *Lobophora variegata* and *Pterocladia capillacea* extracts.

Finally, this study revealed that 9 among 12 tested species of marine macroalgae presented some activity in the applied assays, being that of *Botryocladia occidentalis* the most potent one. Further studies are necessary for chemical characterization of the active principles and more extensive biological evaluation.

Acknowledgements - We wish to thank the Brazilian National Research Council (CNPq), Claude Bernard Institute and the Research Support Foundation of Ceará (FUNCAP) for financial support in the form of grants and fellowship awards. The technical assistance of Silvana França and Fátima Teixeira are gratefully acknowledged.

REFERENCES

Ara, J.; Sultana, V.; Ehteshamul-Haque, S.; Qasim, R. & Ahmad, V. U. Cytotoxic activity of marine macroalgae on *Artemia salina* (brine shrimp). *Phytother. Res*., v. 13, p. 304 – 307, 1999.

Ahmad, V. U; Ahmad, W. U; Aliya, R.; Baqai, F. T.; Ghazala, I. S.; Khatoon, R., Mohammad, F.V.; Noorwala, M.; Perveen, S.; Pervez, A.; Saba, N.; Shah, M.G; & Siddiqui, S. New natural products from terrestrial medicinal plants and marine algae. *Pure & Appl. Chem*., v. 66, p. 2311 – 2314, 1994.

Ballesteros, E.; Martin, D. & Uris, M.J. Biological activity of extracts from some Mediterranean macrophytes. *Bot. Mar*., v. 35, p. 481-485,1992.

Blunden, G. Marine algae as sources of biologically active compounds. *Interdiscipl. Sci. Rev*., v. 18, p. 73-80, 1993.

El-Masry, M. H.; Mostafa, M. H.; Ibrahim, M. A. & El-Naggar, M. M. A. Marine algae that display anti-tumorigenic activity against *Agrobacterium tumefaciens*. *FEMS Microbiol. Lett*., v. 128, p. 151–156,1995

Farias, W. R.; Valente, A.P.; Pereira, S. M. & Mourão, P. A. Structure and anticoagulant activity of sulfated galactans. Isolation of a unique sulfated galactan from the red algae *Botryocladia occidentalis* and comparison of its anticoagulant action with that of a sulfated galactans from invertebrates. *J. Biol. Chem*., v. 275, p. 29299-29307, 2000.

Farias, W. R.; Nazareth, R. A. & Mourão, P. A. Dual effects of sulfated D-galactans from the red algae *Botryocladia occidentalis* preventing thrombosis and inducing platelet aggregation. *J. Thromb.Haemost*., v. 86, p. 1540-1546, 2001.

Fernandez, L. E.; Valiente, G.; Mainardi, V.; Bello, J. L.; Vélez, H. & Rosado, A. Isolation and characterization of an antitumor active agar-type polysaccharide of *Gracilaria dominguensis*. *Carbohydrate Res*., v. 190, p.77-83, 1989.

Freitas, A. L. P.; Ainouz, I. L. & Sampaio, A. H. Agglutination of enzyme treated erythrocytes by brazilian marine algae. *Bot. Mar*., v. 35, p. 475-479, 1992.

Garg, H. S.; Sharma, T.; Bhakuni, D. S.; Pramanik, B. N. & Bose, A. K. An antiviral sphingosine derivative from green alga *Ulva fasciata*. *Tetrahedron Lett*., v. 33, p. 1641-1644, 1992.

Ghosh, P.; Adhikari, U.; Ghosal, P. K.; Pujol, C. A.; Carlucci, M. J.; Damonte, E. B. & Ray, B. *In vitro* anti-herpetic activity of sulfated polysaccharide fractions from *Caulerpa racemosa*. *Phytochem*., p. 3151-3157, 2004.

Hamann, M. T.; Scheuer, P. J. & Kahalide F. A bioactive depsipeptide from the sacoglossan mollusk, *Elysia rufescens* and the green algae *Bryopsis* sp. *J. Am. Chem. Soc*., v. 115, p. 5825-5826, 1993.

Hamann, M.T.; Otto, C.S.; Scheuer, P.J. & Dunbar, D.C. Kahalides: Bioactive peptides from a marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. *J. Org. Chem*., v. 61, p. 6594-6600, 1996.

Harada, H.; Naxo, T. & Kamel, Y. Selective antitumor activity *in vitro* from marine algae from Japan coasts. *Biol. Pharm. Bull.*, v. 20, p. 541-546, 1997.

Ireland, C. M.; Copp, B.R.; Foster, M. P.; McDonald, L.A.; Radisky, D.C. & Swersey, C. Biomedical potential of marine natural products, p. 1-37, *in* Attaway D. H. & Zaborsky, O. R. (eds.), *Marine biotechnology: pharmaceutical and bioactive natural products.* Plenum Publishing Corporation, 1993.

Jimenez, P. C.; Fortier, S. C.; Lotufo, T. M. C.; Pessoa, C.; Moraes, M. E. A.; Moraes, M. O. & Costa-Lotufo, L.V. Biological activity in extracts of ascidians (Tunicata, Ascidiacea) from the northeastern Brazilian coast. *J. Exp. Mar.Biol. Ecol.*, v. 287, p. 93 – 101, 2003.

Jimenez, P.C.; Teixeira, G. L. S.; Wilke, D. V.; Nogueira, N. A. P.; Hajdu, E.; Pessoa, C.; Moraes, M. O. & Costa-Lotufo, L. V. Cytotoxic and antimicrobial activities of hydromethanolic extracts of sponges (Porifera) from Ceará State, Brazil. *Arq. Ciên. Mar*, Fortaleza, v. 37, p. 85-92, 2004.

Kamat, S. Y.; Wahidullah, S.; Sousa, L.; Naik, C. G.; Ambiye, V.; Bhakuni, D. S.; Goel, A. K.; Garg, H. S. & Shimal, R. C. Bioactivity of marine organisms. 6. Antiviral evaluation of marine algae extracts from the Indian coast. *Bot. Mar.*, v. 35, p. 161-164, 1992.

Kanegawa, K.; Harada, H.; Myouga, H.; Katakura, Y.; Shirahata, S. & Kamei, Y. Telomerase inhibiting activity *in vitro* from natural resources, marine algae extracts. *Cytotechnol.,* v. 33, p. 221-227, 2000.

Kitagawa, I. & Kobayashi, M. Antitumor marine natural-products. *J. Synt.Org. Chem. Japan*, v. 49, p. 1053-1061, 1991.

Lee, W. Y. & Wang ,W. X. Metal accumulation in the green macroalga *Ulva fasciata*: effects of nitrate, ammonium and phosphate. *Sci. Total Environ.*, v. 278, p. 11-22, 2001.

Matsubara, K. Recents advances in marine algae anticoagulants. *Current Med. Chem. Cardiovasc. Hematol Agents*, v. 2, p. 13-19, 2004.

Melo, V. M. M.; Medeiros, D. A. & Rios, F. J. B. Antifungal properties of proteins (agglutinins) from the red alga *Hypnea musciformis* (wulfen) Lamouroux. *Bot.Mar.*, v. 40, p. 281-284, 1997.

Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E. & McLaughlin, J. L. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med.*, v. 45, p. 31-34, 1982.

Mosmann, T. Rapid colorimetric assay for cellular growth anf survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.*, v. 65, p. 55-63, 1983.

Nagano, C. S.; Moreno, F. B.; Bloch, C.; Prates, M. V.; Calvete, J. J.; Saker-Sampaio, S.; Farias, W. R.; Tavares, T. D.; Nascimento, K. S.; Grangeiro, T. B. & Sampaio, A.H. Purification and characterization of a new lectin from the red marine algae *Hypnea musciformes. Protein Pept. Lett.* , v. 9, p. 159-166, 2002.

Newman, D. J. & Cragg, G. M. Marine natural products and related compounds in clinical and advanced preclinical trials. *J. Nat. Prod.*, v. 67, p. 1216 – 1238, 2004.

Pereira, S. M.; Melo, R. F. & Mourão, P. A. Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans?. *Glycobiology,* v. 12, p. 573-580, 2002.

Pinheiro-Vieira, F. & Caland-Noronha, M. Atividade antibiótica de algumas algas marinhas do estado do Ceará. *Arq. Ciên. Mar*, v. 11, p. 91-93, 1971.

Pujol, C. A.; Errea, M. I.; Matulewicz, M. C. & Damonte, E.B. Antiherpetic activity of s1, an algae derived sulfated galactan. *Phytother. Res.*, v. 10, p. 410-413, 1996.

Round, E. F. *Biologia das algas*. Guanabara Dois, 2ª edição, 263p., Rio de Janeiro, 1983.

Salimabi, D. B. Antispasmodic and anti-inflammatory activity of carragenan from *Hypnea musciformis* Wulfen. *Indian J. Pharmacol.,* v. 12, p. 259-261, 1980.

Santos, M. G. M.; Lagrota, M. H. C.; Miranda, M. M. F.; Yoneshigue-Valentin, Y. & Wigg, M. D. A screening for the antiviral effect of extracts from Brazilian marine algae against acyclovir resistant herpes simplex virus type 1. *Bot. Mar.*, v. 42, p. iii–v, 1999.

Selvin, J. & Lipton, A. P. Biopotentials of *Ulva fasciata* and *Hypnea musciformis* collected from the peninsular coast of India. *J. Mar. Sci. Technol.*, v. 12, p. 1-6, 2004.

Shanmugam, M. & Mody, K.H. Heparinoid-active sulphated polysaccharides from marine algae as potential blood anticoagulant agentes. *Curr. Sci.*, v. 79, p. 1672-1683, 2000.

Smit, A. J. Medicinal and pharmaceutical uses of seaweed natural products: A review. *J. Appl. Phycol* ,v. 16, p. 245-262, 2004.

Suffness, M. & Pezzuto, J. M. Assays related to cancer drug discovery, p. 71-133, *in* Hostettmann, K. (ed.), *Methods in plant biochemistry: assays for bioactivity*. Academic Press, London, 1990.

Tringali, C. Bioactive metabolites from marine algae: Recent Results. *Curr. Org. Chem.*, v. 1, p. 375- 394, 1997.

Wynne, M. J. A checklist of benthic marine algae of the tropical and subtropical Western Atlantic. *Canadian J. Bot.*, v. 64, p. 2239-2281. 1986.

Xu, N.; Fan, X.; Yan, X. & Tseng, C. K. Screening marine algae from China for their antitumor activities. *J. Appl. Phycol.*, in press, 2004.