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Glycolipids from macroalgae: potential biomolecules for marine biotechnology?

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Abstract: Brown, red and green algae from the Southeastern coast of Brazil were successively extracted with chloroform/methanol 2:1 and 1:2 (v/v). The crude lipid extract was partitioned according to Folch and the lower phase enriched in glycolipids was fractionated on a silica gel column chromatography eluted with chloroform, acetone and methanol. Three major orcinol-reactive bands present in the acetone and methanol fractions were detected by thin-layer chromatography with chromatographic mobilities corresponding to sulfoglycolipids and glycosyldiacylglycerols. These fractions exhibited potent antiviral activity against HSV-1-ACVs and HSV-1-ACVr and present low toxicity for cell cultures. Purification and identification of these bioactive glycolipids will be necessary in order to elucidate their primary structures and mechanism of action.

Introduction

Glycolipids constitute an important class of membrane lipids that are synthesized by both prokaryotic and eukaryotic organisms. Many reports have been published about isolated compounds from algae with biological activity, demonstrating their ability to produce metabolites unlike those in terrestrial species, with high complexity and unlimited diversity of pharmacological and/or biological properties (Mayer & Hamman, 2004).

Monogalactosyl diacyl glycerols have been isolated from *Sargassum thunbergii* (Kim et al., 2007), *Exophlyllum wentii* (Illijas et al., 2009) and *Chondria armata* (Al-Fadhli et al., 2006). Sulfoglycolipids such as sulfoquinavosyldiacylglycerols presenting antivirus activity were identified in *Porphyridium purpureum* and other microalgae (Naumann et al., 2007).

In search for potentially useful bioactive molecules of marine origin, we have investigated glycolipids of red, green and brown algae from Southeastern Brazilian coast.

Material and Methods

Biological material

Specimens of *Ulva fasciata, Caulerpa racemosa, Dictyota cervicomis, Dictyota menstrualis, Hypnea musciformis, Osmundaria obtusiloba, Porphyra acanthophora, Pterocladiella capillacea* were collected at Praia Rasa, located at the city of Búzios, Rio de Janeiro, Brazil, in September 2007. The seaweeds were washed with local sea water and separated from sediments, epiphytes and other associated organisms.

Extraction and fractionation of lipids

Green, red and brown algae were successively extracted at room temperature with chloroform/methanol 2:1 and 1:2 (v/v). Extracts were combined, dried and the crude lipid extract was partionated according to Folch and coworkers (1957). The lipids recovered from Folch lower phase were fractionated on a silica gel column eluted with chloroform, acetone and then methanol. Fractions were collected and analyzed by HPTLC developed with chloroform/methanol/water 65:25:4 (v/v). The spots were visualized with iodine and by spraying with orcinol/H_2SO_4.The enriched-glycosphingolipid fractions were further purified by a second silica gel chromatography.

Cells and viruses

Vero cells (African Green monkey kidney cells) were grown in Eagle`s minimum essential medium

(Eagle-MEM) supplemented with 10% (v/v) fetal bovine serum, glutamine (0.03 mg/mL), garamycin (50 µg/mL), fungizone (amphothericin B) (2.5 mg/mL), $NaHCO_3$ (0.25%), HEPES (10 mM) and maintained at 37 °C in a 5% CO_2 atmosphere.

Samples of HSV-1 (herpes virus simplex type 1) acyclovir-sensible (HSV-1-ACVs) and acyclovir-resistant (HSV-1-ACVr) were isolated in the Laboratório Experimental de Drogas Antivirais e Citotóxicas, Departamento de Virologia, Instituto de Microbiologia, UFRJ, Brasil.

Citotoxicity assay

To determine the maximum non-toxic concentrations (MNTC) of the acetone and methanol fractions, different concentrations (200, 100, 50, 25, 12.5, 6.25 and 3.15 µg/mL) were placed in contact with confluent Vero cell monolayers and incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. After incubation, the morphological alterations of the treated cells were observed in an inverted optical microscope (Leitz) and the maximum non-toxic concentrations (MNTC) were determined (Walker et al., 1971). Cellular viability was further evaluated by the neutral red dye-uptake method (Neyndorff et al., 1990). Briefly, cells were incubated in the presence of 0.01% neutral red solution for 3 h at 37 °C in a 5% CO_2 atmosphere. Then, the medium was removed and the cells were fixed with 4% formalin in phosphate-buffered saline, pH 7.2. The dye incorporated by the viable cells was eluted using a mixture of methanol:acetic acid:water (50:1:49), and the dye uptake was determined by measuring the optical density (OD) of the eluate at 490 nm in an automatic spectrophotometer (ELx800TM-Bio-TeK Instruments, Inc.). The 50% cytotoxic concentration (CC50) was defined as the concentration that caused a 50% reduction in dye uptake.

Antiviral activity assay

The antiviral activity of the acetone and methanol fractions was evaluated by the titer reductions. The virus titers were calculated using the Reed & Muench (1938) statistical method and expressed as 50% tissue culture infective dose (TCID50) per mL.

Vero cell monolayers were treated with the acetone and methanol fractions at the MNTC and viral suspension (100 TCID50/mL) was added to treated and untreated cell cultures. After 48 h incubation at 37 °C in a 5% CO_2 atmosphere the supernatants were collected and the virus titers in treated and untreated cells were determined. The results were expressed as Viral Inhibition Index (VII) and Percentage of Inhibition (PI) (Nishimura et al., 1977)

Results and Discussion

Total lipids from brown, red and green algae from the Southeastern coast of Brazil were extracted using previously established methods. After Folch´s partition, the lower phase enriched in glycolipids, was fractionated by silica gel chromatography, and the separation monitored by thin-layer chromatography. Figure 1 shows all the steps of purification. Thin-layer chromatography of the partially purified acetone fractions revealed a single orcinol reactive band (Figure 2, lanes 2, 4 and 6). Two major glycolipid bands were also isolated from the methanol fractions of brown, red and green algae analyzed (Figure 2, lanes 3, 5 and 7). These bands presented TLC mobilities similar to sulfonoglycolipids and glycosyldiacylglycerols isolated from different organisms and this result could suggest that these compounds are conserved molecules in algae. Distinct sulfonoglycolipid fractions were isolated from the basidiolichen *Dyctionema glabratum* (Sassaki et al., 2001) and from the red algae *Chondria armata* (Al-Fadhli et al., 2006). Glycoglycerol lipids occur widely in green seaweeds (Mancini et al., 1998), cyanobacteria (Reshef et al., 1997), marine dinoflagellates (Oshima, et al., 1994) and the freshwater alga *Chlorella vulgaris* (Morimoto et al., 1995).

Experiments to evaluate the cytotoxicity of the acetone and methanol fractions were performed and shown in Table 1. Our results have demonstrated that both fractions had potent antiviral activity against HSV-1- ACVs and HSV-1-ACVr and present low toxicity for cell cultures.

Purification and identification of these bioactive glycolipids will be necessary in order to elucidate their primary structures and mechanism of action.

Table 1. Cytotoxicity and antiviral activity of methanol and acetone fractions from *Osmundaria obtusiloba.*

Material	MNTC µg mL^{-1}	CC50 µg mL^{-1}	ACVs-HSV-1 PI%	ACVs-HSV-1 VII	ACVr-HSV-1 PI%	ACVr-HSV-1 VIII
MeOH	100	171.53	99.5	2.35	99.9	4.5
Acetone	50	>200	82.2	0.75	99.7	2.5

ACVs-HSV-1: Acyclovir-sensible Herpes Simplex Virus Type 1; ACVr-HSV-1: Acyclovir-resistant Herpes Simplex Virus Type 1; MNTC: Maximum Non-toxic Concentration; CC50: 50% Cytotoxic Concentration; PI: Percentage of Inhibition; VII Viral Inhidition Index.

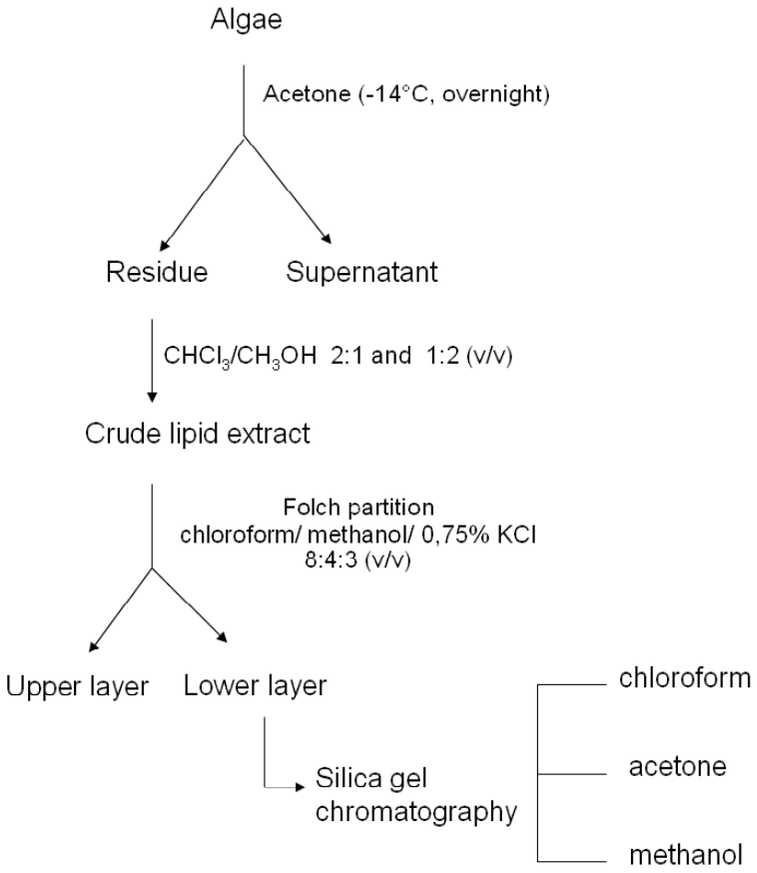

Figure 1. Isolation and partial purification of glycolipids from algae.

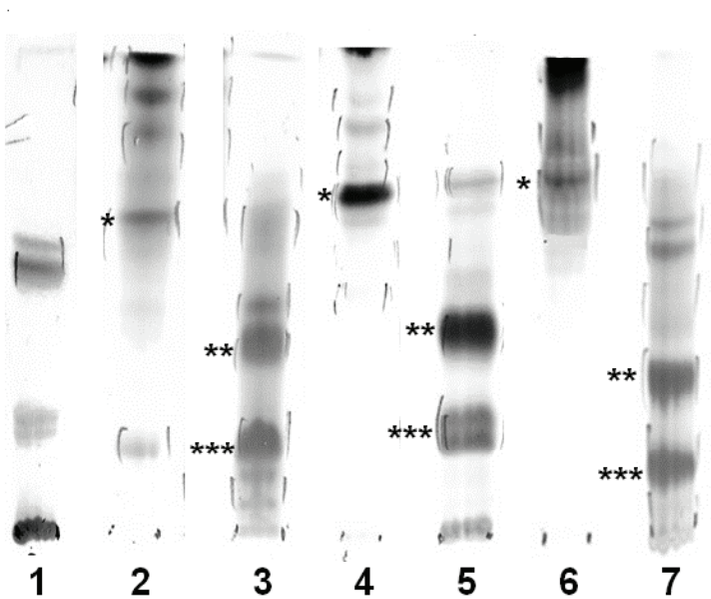

Figure 2. Thin-layer chromatography (TLC) of partially purified glycolipids eluted from a silica gel column chromatography with acetone and methanol and visualized by reaction with orcinol- sulfuric acid. Lane 1: crude lipid extract from bovine brain. Lanes 2, 4 and 6: acetone fractions from *Dictyota menstrualis* (brown alga), *Osmundaria obtusiloba* (red alga) and *Caulerpa racemosa* (green alga). Lanes 3, 5 and 7: methanol fractions from *Dictyota menstrualis* (brown alga), *Osmundaria obtusiloba* (red alga) and *Caulerpa racemosa* (green alga).Asteristics show the presence of three different glycolipid species present in algae.

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