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Cytotoxicity in the marine dinoflagellate *Prorocentrum mexicanum* from Brazil

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Abstract

The microscopic algae in the oceans are crucial food for filter feeding bivalve shellfish (oysters, mussels, scallops, clams, etc.) as well as for the larvae of commercially important crustaceans. Some species of microalgae have the capacity to produce potent toxins, such as saxitoxins and ciguatoxins, which may intoxicate humans. Among the marine phytoplankton, the dinoflagellates are the main toxin producers. Studies on the marine phytoplankton from the São Sebastião Channel, southeastern coast of Brazil, showed a great diversity of dinoflagellates. Some species were collected and cultured at the Marine Biology Center of the São Paulo University (USP). The polar (PEs) (aqueous) and apolar (AEs) (methylene chloride) extracts of the cultivated dinoflagellate species were tested on different stages of the sea urchin development, on mouse erythrocytes and on microfilaments organization in a neuroblastoma cell line. *Prorocentrum mexicanum* PE and AE induced cells anomalies and cell division inhibition of sea urchin eggs at EC_{50} of 78.75 μg/mL (95% CI from 32.56 to 190.50) and 22.50 μg/mL (95% CI from 2.96 to 170.80) respectively ($n=3$). Both AE and PE of *P. mexicanum* induced hemolysis with EC_{50} of 65.07 μg/mL (95% CI from 27.40 to 154.60) and 84.29 μg/mL (95% CI from 53.26 to 133.40 μg/mL), respectively. *P. mexicanum* PE was tested in immunofluorescence for actin filaments organization in neuroblastoma cultured cell.

Keywords: *Prorocentrum mexicanum*; Dinoflagellate; Marine toxins; Microalgae; Hemolytic assay; Neuroblastoma; Antimitotic assay; Cytotoxicity

1. Introduction

Microalgae play an important role in marine biological ecosystems, because of their photosynthetic activity. They are the major producers of biomass and organic compounds in the oceans (Madigans et al., 1999; Morel and Price, 2003). Sometimes, a variety of species may proliferate and contaminate seafood, such as clams and fishes, with toxins that cause intoxications when eaten by humans. The class Dinophyceae, which comprises the dinoflagellates, contains the majority of toxic species (Steidinger, 1983; Shumway, 1990; Sournia, 1995).

Toxins from dinoflagellates are often classified according to their effects on humans, e.g.: DSPs (diarrheic shellfish poisoning), which causes gastrointestinal problems, and PSPs (paralytic shellfish poisoning), that have neurological paralytic effects (Daranas et al., 2001). Bioassays constitute a first step approach to detect and quantify toxin effects in crude extracts from marine microalgae, shellfish and fish. The development of cellular models has allowed more sensitivity for a large number of samples in a short period of time (Diogène et al., 1995). The sea urchin eggs development assay and hemolytic assay have been extensively used to identify toxins from marine algae (Bignami, 1993; Miralto et al., 1999, Buttino et al., 1999; Shimojo and Iwaoka, 2000; Sato et al., 2002; Hansen et al., 2003). Some bioactive compounds produced by microalgae may have minimal effects on mammalian systems, but they can more seriously affect aquatic organisms because of their mode of activity in an aqueous medium (Landsberg, 2002), hence we choose mammalian and invertebrate cells for comparative studies.

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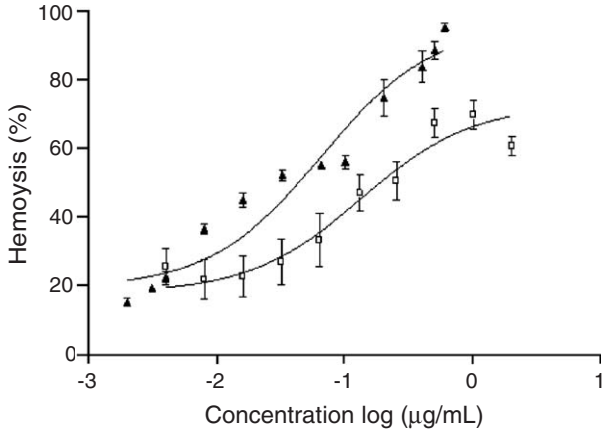

Fig. 1. Hemolytic effect induced by different concentrations of PE and AE of *P. mexicanum* in mouse erythrocytes. Data are reported as means±SEM of percent of hemolysis (n=4). ▲ AE □ PE.

In the present work we investigated the toxicity of extracts obtained from *Prorocentrum mexicanum*, a dinoflagellate from the São Sebastião Channel, Southeastern Brazilian coast. This specie is associate with ciguatera studies (Tindall et al., 1984; Bomber and Aikman, 1989; Steidinger, 1993) that include lipid-soluble toxins, ciguatoxins and gambierol and water-soluble toxins, maitotoxin and palytoxin (Lehane and Lewis, 2000; Daranas et al., 2001). Shimojo and Iwaoka (2000) reported hemolytic activity to *P. mexicanum* extract suggesting the presence of a sodium channel activator in this extract.

We evaluated the effects of the *P. mexicanum* extracts on different stages of the sea urchin development, on mouse erythrocytes and on microfilaments organization in neuroblastoma cell line.

2. Materials and methods

2.1. Culture and extraction of toxins

A benthic (epiphytic) dinoflagellate identified as *P. mexicanum* was collected at the São Sebastião Channel, Southeastern of São Paulo State, Brazil. The isolation and purification of specimens were made with a micropipette followed by a serial-dilution technique, using 24-multiwell plates (Corning™, USA), containing modified f/2 medium (without silicate, to suppress the diatoms), obtaining an unialgal culture. The microplates containing f/2 culture broth were maintained in a chamber under constant temperature (24 °C±2) and 12/12 h photoperiod. At the exponential phase of growth, the cells were inoculated into a 1 L culture flask. For growth measurement the cell counts were made for each sample with Sedgwick–Rafter counting slides.

Cultures were harvested during the log phase of growth using glass microfibre filters (Whatman GF/C, USA). The filters were homogenized with MeOH/H_2O (80:20 v/v) and the insoluble materials were removed by centrifugation and filtration with 0.45 µm filter. The insoluble materials were then re-extracted with methanol. The combined methanol extracts were dried on a rotary vacuum-evaporator (50 °C). The residue was submitted to a partition using water and methylene chloride (3:1 v/v). The water-soluble fraction was lyophilized and non-polar fraction was dried by solvent evaporation.

2.2. Hemolytic assay

Blood was collected from decapitated Balb-C white mice (*Mus musculus*) and a 0.5% erythrocyte suspension (ES) was obtained according to Malpezzi and Freitas (1991).

The test was performed using polyethylene tubes (eppendorf) and the dose response curves were determined for the apolar and polar fractions. Each tube received 100 µl of Krebs–Henseleit mammalian physiological solution (PS). Test tubes contained serial dilutions of the fractions (apolar and polar) in 100 µl volume and were challenged against 100 µl of 0.5% ES. Negative control contained 100 µl of PS and positive control (100% of hemolysis) was obtained with 100 µl Triton X-100 (1%). After 2 h incubation, at room temperature (25±2 °C) under constant shaking, this suspension was centrifuged and percent of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm. The experiments were performed in triplicates. The EC_{50} and their 95% confidence interval were obtained by nonlinear regression using the Graphpad software (Intuitive Software for Science, San Diego, CA).

2.3. Antimitotic assay

Antimitotic activity was evaluated as the ability of the extract to inhibit the cleavage of eggs of the sea urchin *Lytechinus variegatus*, collected at the same location as the dinoflagellate. Gamete elimination was induced by injecting 0,5 M KCl into the sea urchin's perivisceral cavity. The eggs were washed twice in filtered seawater to remove the jelly coat surrounding the cells. The sperm was maintained in a refrigerator without dilution until use. The assays were carried out in 24-multiwell plates (Corning, USA). The eggs were fertilized in a beaker mixing 50 mL of eggs with 50 µL sperm suspension a couple minutes before

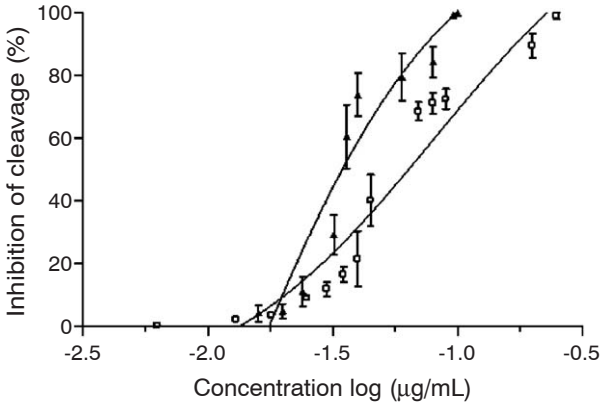

Fig. 2. Antimitotic effect induced by different concentrations of extracts of *P. mexicanum* on sea urchin (*Lytechinus variegatus*) egg cleavage. Data are reported as percent inhibition±SEM of the first cleavage (n=4). ▲ AE □ PE.