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Cytotoxic activity of a dichloromethane extract and fractions obtained from *Eudistoma vannamei* (Tunicata: Ascidiacea)☆

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Abstract

This study consists of the bioassay-guided fractionation of the dichloromethane extract from *Eudistoma vannamei* and the pharmacological characterization of the active fractions. The dried hydromethanolic extract dissolved in aqueous methanol was partitioned with dichloromethane and chromatographed on a silica gel flash column. The anti-proliferative effect was monitored by the MTT assay. Four of the latest fractions, numbered **14** to **17**, which held many chemical similarities amongst each other, were found to be the most active. The selected fractions were tested for viability, proliferation and death induction on cultures of HL-60 promyeloblastic leukemia cells. The results suggested that the observed cytotoxicity is related to apoptosis induction.

Keywords: Ascidians; Apoptosis; Cyotoxicity; *Eudistoma vannamei*; MTT assay; tumor cell lines

1. Introduction

Ascidians are marine invertebrates present in almost every marine community and particularly abundant in coastal regions (Seed and O'Connor, 1981; Schmidt and Warner, 1986; Todd and Turner, 1988; Teo and Ryland, 1994). One of the reasons for this ecological success is the ability of these animals to synthesize secondary metabolites with important defensive roles, including antimicrobial peptides (Lee et al., 1997), cytokine-like compounds (Raftos and Nair, 2004), lectins (Green et al., 2006) and antileukemic compounds (Takeara et al., 2008).

Regarding the marine invertebrates, ascidians from the families Didemnidae and Polycitoridae are among the most prolific sources of biologically active compounds (Waters and van den Brenk, 1993). The genus *Eudistoma* is the most diverse of the family Polycitoridae, with most of its species living in tropical regions (Kott, 1990). Several cytotoxic alkaloids have been isolated from *Eudistoma* ascidians, including eudistomins and eudistomidins (Kobayashi et al., 1984; Kinzer and Cardelina, 1987; Rinehart et al., 1987; Murata et al., 1991; Adesanya et al., 1992; Davis et al., 2003; Makarieva et al., 2001; Rashid et al., 2001; Schupp, et al., 2003). On the Brazilian coast, only one species of *Eudistoma* was found in temperate waters, while the warmer Northeast region has a record of seven species of this genus. The species *Eudistoma vannamei* Millar, 1977 is endemic from the Northeast coast of

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Brazil, and its occurrence has been recorded across the states of Ceará down to Bahia (Lotufo, 2002).

Information concerning the biomedical properties of the marine fauna from the northeast coast of Brazil remains scanty. Initial screening for cytotoxicity of the most abundant ascidian species found on the intertidal waters of Ceará state has already been reported (Jimenez et al., 2003) and showed them to be a rich source of compounds with bioactive proprieties. *E. vannamei* provided many interesting results on the various cell models on which it was assayed.

Engaging a wide range of bioactivities and a highly potent crude extract, *E. vannamei* was decidedly suitable for a more in-depth chemical and biological characterization. This is what the present study intends to accomplish.

2. Materials and methods

2.1. Collection and identification

Samples of *E. vannamei* were collected in crevices or on the underside of beach-rocks in the intertidal zone of Taíba Beach (03°34,931′S; 038°54,469′W), on the west coast of Ceará state, Brazil. The material was immediately immersed in methanol and stored refrigerated at −4 °C. Part of the material was fixed in 70% ethanol and sent for identification. A voucher specimen #198 is deposited at the ascidian collection of the Departamento de Engenharia de Pesca, Universidade Federal do Ceará.

2.2. Bioassay-guided fractionation

The crude extract was prepared in methanol 1:5 (w/v, 1.34 kg/6.7 L), evaporated under pressure to dryness (66 g), and partitioned with dichloromethane (DCM). The dried DCM phase (200 mg) was chromatographed on a flash silica gel column affording 102 fractions. These fractions were organized by similarity and amount of samples in thin layer chromatography (TLC) on silica gel plates using *n*-hexane/ethyl acetate (60:40) as developing solvent and UV light and cerium sulfate in H_2SO_4 as detection system, yielding 20 fractions. The activity of the crude methanolic extract, the DCM extract and fractions were monitored by the MTT assay using concentrations of 100 μg/mL and 50 μg/mL, respectively. The active fractions were analyzed by ^{1}H NMR and the data compared with the previous publish data of chemical constituent from ascidians.

2.3. MTT assay

The fractionation of the crude extract was bioguided by the evaluation of its cytotoxicity against two tumor cell lines (National Cancer Institute, Bethesda, MD, USA), HCT-8 (human colon) and HL-60 (human leukemia), using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay as described by Mosmann (1983), for 72 h of incubation. For those samples with a positive result, three more cell lines were also tested: B-16 (murine skin), MCF-7 (human breast) and CEM (human leukaemia). DCM fractions **14**, **15**, **16** and **17** showed a very similar profile in TLC with an intensive UV absorption. Based on previous information of the presence of aromatic cytotoxic compounds they were analysed separated by ^{1}H NMR and also tested at 24 h incubation period on HL-60 cell line. Doxorubicin (0.3 μg/mL) was used as positive control.

The fractions **14** through **17** were evaluated in order to elucidate the mechanism of their detected cytotoxicity by using HL-60 cells as a model.

2.4. Cell viability assay

Cell viability was determined by the Trypan blue dye exclusion test on HL-60 cells over a 24 h incubation period. The cells (3×10^5 cells/mL) were incubated with **14**, **15**, **16** and **17** at concentrations of 0.1 and 1.0 μg/mL. After the incubation period, they were counted by using a haemocytometer. Untreated cells were used as a negative control and doxorubicin (0.3 μg/mL) was used as a positive control.

2.5. Cell proliferation

HL-60 cells were plated onto 24-well tissue culture plates and incubated with **14**, **15**, **16** and **17** at 0.1 and 1.0 μg/mL. Every 6 h, over a 72 h period, cell aliquots were stained with Trypan blue dye and counted in a haemocytometer for viability. The growth curve was obtained by the values plotted on a cell count x time graph. Untreated cells were used as a negative control and doxorubicin (0.3 μg/mL) was used as a positive control.

2.6. Effect on cell DNA synthesis

HL-60 cells (3×10^5 cells/mL) were plated onto 24-well tissue culture plates and treated with **14**, **15**, **16** and **17** for 24 h at 0.1 and 1.0 μg/mL. Ten microliters of 5-bromo-2′-deoxyuridine (BrdU, final concentration of 10 mM) were added to each well and incubated for 3 h at 37 °C before the completion of the incubation period. To access the BrdU incorporation on cell DNA, cells were harvested, placed on a glass slide using cytospin, and left to dry for 2 h at room temperature. Cells that incorporated BrdU were labelled by direct peroxidase immunocytochemistry using the chromogen diaminobenzidine. Slides were counterstained with hematoxylin, mounted, and coverslipped. Untreated cells were used as a negative control and doxorubicin (0.3 μg/mL) was used as a positive control (Pera et al., 1977). Evaluation of BrdU uptake was accomplished by light microscopy. Two hundred cells were counted per sample to calculate the percentage of positive cells.

2.7. Morphological changes analysis

HL-60 cells were treated for 24 h with 0.1 and 1.0 μg/mL of **14**, **15**, **16** and **17** to be examined for morphological changes. To access the nuclear and membrane morphology, cells were harvested, placed on a glass slide using cytospin, fixed with 96% ethanol for 1 h and stained with eosin–hematoxylin differential dye. Visual analysis was accomplished using light microscopy.