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Antitumor effect of laticifer proteins of *Himatanthus drasticus* (Mart.) Plumel – Apocynaceae

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ABSTRACT

Ethnopharmacological relevance: Himatanthus drasticus (Mart.) Plumel – Apocynaceae is a medicinal plant popularly known as Janaguba. Its bark and latex have been used by the public for cancer treatment, among other medicinal uses. However, there is almost no scientific research report on its medicinal properties.
Aim of the study: The aim of this study was to investigate the antitumor effects of *Himatanthus drasticus* latex proteins (HdLP) in experimental models.
Materials and methods: The *in vitro* cytotoxic activity of the HdLP was determined on cultured tumor cells. HdLP was also tested for its ability to induce lysis of mouse erythrocytes. *In vivo* antitumor activity was assessed in two experimental models, Sarcoma 180 and Walker 256 carcinosarcoma. Additionally, its effects on the immunological system were also investigated.
Results: HdLP did not show any significant *in vitro* cytotoxic effect at experimental exposure levels. When intraperitoneally administered, HdLP was active against both *in vivo* experimental tumors. However, it was inactive by oral administration. The histopathological analysis indicates that the liver and kidney were only weakly affected by HdLP treatment. It was also demonstrated that HdLP acts as an immunomodulatory agent, increasing the production of OVA-specific antibodies. Additionally, it increased relative spleen weight and the incidence of megakaryocyte colonies.
Conclusion: In summary, HdLP has some interesting anticancer activity that could be associated with its immunostimulating properties.

1. Introduction

Himatanthus is a small Apocynaceae genus composed of 14 species (Plumel, 1991). The importance of the genus *Himatanthus* in traditional medicine is supported by reports where preparations were evaluated for mainly anti-tumor (Bolzani et al., 1999), anti-inflammatory (Miranda et al., 2000), anti-ulcerous (Baggio et al., 2005), anti-spasmodic (Rattmann et al., 2005), antimicrobial (Souza et al., 2004), and antileishmanial (Castillo et al., 2007) activities.

Himatanthus drasticus (Mart.) Plumel – Apocynaceae is a medium-sized tree growing on firm ground in South America. It is a medicinal plant popularly known as janaguba, tiboma, jasmim-manga, raivosa, pau-de-leite, joanaguba, and sucuúba (Plumel, 1991). Its bark and latex have been used by the public mainly for cancer treatment, as an anti-inflammatory medication, and to stimulate the immune system (Amaro et al., 2006). Usually, latex plus water (janaguba milk) or decoctions of the bark or latex are taken at a dose of one cup about three times a day. Although several medicinal uses have been described, there are almost no scientific research reports to this regard.

In a preliminary study of the phytochemical and biological effects of *Himatanthus drasticus*, the ethanolic extract of stem bark was evaluated biologically. This extract was found to be toxic against brine shrimp, but showed no antimicrobial effect

Abbreviations: 5-FU, 5-fluorouracil; SBCAL, Sociedade Brasileira de Ciência em Animais de Laboratório; HDLP, Himatanthus drasticus latex protein; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; OVA, ovabulmin; PBMC, peripheral blood mononuclear cells; TGF-β, transforming growth factor-β; TNF-α, tumor nuclear factor-α.

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against the pathogens tested *in vitro* (*Enterobacter*, *Streptococcus* and *Escherichia coli*). Additionally, it showed antinociceptive (writhing test in rats) affect (Colares et al., 2008). Leite et al. (2009) demonstrated the latex from *Himatanthus drasticus* to be a cytoprotective agent against ethanol-induced ulcer formation in mice.

The aim of this study was to investigate the antitumor effects of *Himatanthus drasticus* latex proteins (HdLP) in experimental models. In order to evaluate the toxicological aspects related to HdLP treatment, histopathological and morphological analyses of treated animals were also performed.

2. Material and methods

2.1. Reagents

5-Fluorouracil (5-FU), *O*-phenylenidiamine dihydrochloride, Ficoll-Hypaque, phytohemagglutinin, resazurin, and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA); rabbit antimouse total Ig (IgG, A, M) was from Serotec (Kidlington, Oxford, UK). All other reagents were of analytical grade.

2.2. Plant material, latex collection and extraction of *Himatanthus drasticus* latex proteins (HdLP)

Latex was obtained in May 2010 from uncultivated plants located in the vicinity of Fortaleza, State of Ceará, Brazil. Botanical material was identified at the Herbarium Prisco Bezerra (Departamento de Biologia, Universidade Federal do Ceará). A voucher was registered under the code 40408.

The latex was obtained after cutting stem and allowing it to flow into tap water in order to give an equal mixture of volumes. The samples were initially centrifuged ($5000 \times g$) at 10 °C for 25 min. The pellet was discarded and the soluble phase was dialyzed against distilled water for 60 h at 25 °C with water being renewed three times daily. Finally, the dialyzed material was centrifuged as previously done, and clean supernatant was recovered, freeze dried and used in all further determinations. This fraction, comprising almost all soluble latex protein was called HdLP.

2.3. Animals

A total of 56 Swiss mice (female, 25–30 g) and 40 Wistar rats (female, 180–220 g), obtained from the central animal house of Universidade Federal do Ceará – Brazil, were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12:12 h light–dark cycle (lights on at 6:00 a.m.). Animals were treated according to the ethical principles for animal experimentation of SBCAL (Sociedade Brasileira de Ciência em Animais de Laboratório), Brazil. The Animal Studies Committee of Universidade Federal do Ceará approved the experimental protocol (number 08/08).

2.4. Cells

The cytotoxicity of HdLP was tested against HL-60 (leukemia), MDA-MB-435 (melanoma), SF-295 (brain), and HCT-8 (colon) human cancer cell lines, all obtained from the National Cancer Institute, Bethesda, MD, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin, and incubated at 37 °C with a 5% CO_2 atmosphere.

In order to get healthy human peripheral blood mononuclear cells (PBMC), heparinized blood (from healthy, non-smoker donors who had not taken any drug at least 15 days prior to sampling) was collected, and PBMC were isolated by a standard method of density-gradient centrifugation over Ficoll-Hypaque. PBMC were washed and resuspended. Cells were grown under the same conditions as above plus the addition of phytohemagglutinin (4%).

Sarcoma 180 tumor cells had been maintained in the peritoneal cavity of Swiss mice and Walker 256 carcinosarcoma tumor cells had been maintained by intramuscular inoculation of the medial side of the thigh of Wistar rats in the Laboratory of Experimental Oncology from the Universidade Federal do Ceará since the mid-1980s.

2.5. In vitro cytotoxic evaluation of HdLP

2.5.1. Determination of the effect of HdLP on cultured tumor cells

Tumor cell growth 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 purple formazan product (Mossman, 1983). For all experiments, cells were seeded in 96-well plates (10^5 cells/well for adherent cells or 0.5×10^5 cells/well for suspended cells in 100 μl of medium). After 24 h, the test substance (0.39–50 μg/ml), dissolved in saline, was added to each well (using the HTS—high-throughput screening—Biomek 3000; Beckman Coulter Inc., Fullerton, CA, USA) and incubated for 72 h. 5-FU was used as the positive control. At the end of incubation, the plates were centrifuged and the medium was replaced by fresh medium (150 μl) containing 0.5 mg/ml MTT. Three hours later, the formazan product was dissolved in 150 μl DMSO and the absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter Inc., Fullerton, CA, USA). The drug effect was quantified as the percentage of control absorbance of reduced dye at 595 nm.

PBMC cell growth was determined by the Alamar blue assay (Ahmed et al., 1994). For all experiments, cells were seeded in 96-well plates (0.3×10^6 cells/well for suspended cells in 100 μl of medium). After 24 h, the test substance (0.39–50 μg/ml), dissolved in saline, was added to each well (using the HTS – high-throughput screening – Biomek 3000 – Beckman Coulter, Inc., Fullerton, CA, USA) and incubated for 72 h. 5-FU was used as the positive control. Twenty-four hours before the end of incubation, 10 μl of stock solution (0.312 mg/ml) of Alamar Blue (Resazurin) was added to each well. The absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter®) and the drug effect was quantified as the percentage of control absorbance at 570 nm and 595 nm. The absorbance of Alamar Blue in culture medium is measured at a higher wavelength and lower wavelength. The absorbance of the medium is also measured at the higher and lower wavelengths. The absorbance of the medium alone is subtracted from the absorbance of medium plus Alamar Blue at the higher wavelength. This value is called AO_{HW}. The absorbance of the medium alone is subtracted from the absorbance of medium plus Alamar Blue at the lower wavelength. This value is called AO_{LW}. A correction factor R_0 can be calculated from AO_{HW} and AO_{LW}, where $R_0 = AO_{LW}/AO_{HW}$. The percent Alamar Blue reduced is then expressed as follows: % reduced $= A_{LW} - (A_{HW} \times R_0) \times 100$.

2.5.2. Determination of the effect of HdLP on mouse erythrocytes

The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mm $CaCl_2$, following the method described by Jimenez et al. (2003). HdLP was tested at concentrations ranging from 8 to 200 μg/ml. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the hemoglobin released was measured spectrophotometrically as the absorbance at 540 nm.