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Aqueous extract of *Plinia edulis* leaves: Antioxidant activity and cytotoxicity to human breast cancer MCF-7 cell line

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Abstract

The aqueous extract of *Plinia edulis* leaves (AEP) was evaluated for its *in vitro* antioxidant potential and cytotoxicity to MCF-7 cells, a human breast adenocarcinoma cell-line that express the estrogen receptor α (ER+). AEP exhibited total antioxidant capacity (324.20±3.14 mg/g) and strong scavenging activity on DPPH free radical, with maximum effect at 20 μg/mL. AEP and cyclophosphamide (cytotoxic agent) treatment for 24 and 48 h decreased the cell protein content, as compared to control cells. Morphological analysis, after hematoxylin–eosin staining of cells, showed changes such as cell rounding-up, shrinkage, nuclear condensation and reduction of colony and cell diameter ($p<0.01$), thereby indicating that AEP is cytotoxic to MCF-7 cells. AEP treatment also induced cell death, with a maximum effect at 5 μg/mL, and its mechanism of action seems to include the induction of apoptosis, as a DNA ladder-pattern was obtained in the DNA analysis. Oxidative stress is supposed to play an important role in cancer initiation and progression. Considering that AEP showed to be antioxidant and cytotoxic, the extract seems to be a chemopreventive agent and a good candidate for antineoplastic drug development.

Keywords: Anticancer; Cambucá; Cell death; Cell morphology; DNA ladder; Plant extract

1. Introduction

Breast cancer is a large problem of public health all around the world, and is the second most commonly diagnosed cancer type (Anderson, 2010; Jemal et al., 2010). Breast cancer-related mortality rates continue to increase in Brazil and in other less developed countries (Bines and Eniu, 2008). Increasing breast cancer incidence is associated with risk factor diversity, genetic characteristics involved in its etiology, later stage disease diagnosis, and lack of adequate adjuvant, systemic treatment (Bines and Eniu, 2008; Anderson, 2010; Brasil, 2011). Tissue invasiveness and metastatic spread of breast cancer cells are responsible for most of the morbidity and mortality associated with the disease (Nukumi et al., 2007).

Breast cancers exhibit remarkable heterogeneity not only with respect to estrogen (ER), progesterone (PR), and human epidermal growth factor-2 (HER-2) receptor expression but also with respect to tumor size, grade, and nodal status. Thus, breast carcinoma is a mixture of diverse phenotypes, which raises different treatment needs (Jatoi et al., 2008) and at the present moment there is no cure for metastatic breast cancer (Pagani et al., 2010), whereby the necessary search for new drugs to treat and control this disease is much needed.

Cancer initiation and progression and a number of human diseases including cardiovascular, metabolic, inflammatory, and

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neurodegenerative diseases are related to reactive oxygen species (ROS) and reactive nitrogen species (RNS), which once accumulated inside the cell, can attack proteins, lipids, and DNA, causing a state of oxidative stress (Halliwell, 2007; Vurusaner et al., 2012). Living organisms are equipped with an antioxidant defense system that regulates the toxic impact of ROS and RNS. However, a disturbance in the balance between the production of ROS or RNS and antioxidant defenses may lead to cell molecule or tissue injury (Gouvêa, 2004; Halliwell, 2007). There is strong evidence that the antioxidants prevent carcinogenesis, and natural products have proven to be an important source of new and effective antioxidant and anticancer agents (Milaeva, 2011). Secondary plant metabolites, such as phenolic compounds, have been found to be strong antioxidants, which can scavenge or suppress ROS and RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production, and up-regulate or protect antioxidant defense, thereby preventing carcinogenesis (Gouvêa, 2004; Halliwell, 2007; Huang et al., 2010).

Plinia edulis (Vell.) Sobral, Myrtaceae, popularly known as "cambucá", is an arboreous species with edible fruits, which grows naturally in Brazilian Atlantic Rain Forest (Lorenzi et al., 2006). In folk medicine, *P. edulis* is used to treat stomach problems, sore throat, and diabetes and as tonic (Nascente, 2008). This plant is a source of flavonoids, tannins, saponins and terpenoids (Ishikawa et al., 2008b), and the aqueous ethanol extract of *P. edulis* leaves is antiulcerogenic (Ishikawa et al., 2008a).

The aim of the present work was to evaluate the aqueous extract of *P. edulis* (AEP) *in vitro* antioxidant activity and its cytotoxicity to MCF-7 cells that express the estrogen receptor α (ER+), a model for the human *in situ* breast carcinoma.

2. Materials and methods

2.1. Plant material

Leaves of *P. edulis* (Vell.) Sobral, Myrtaceae were collected in Trindade (Paraty, Rio de Janeiro, Brazil) in the morning during flowering, identified by Dr. Lúcia Rossi, and voucher specimens (SP 356.472) were deposited at the Herbarium of the Instituto de Botânica de São Paulo, Brazil.

2.2. Extract preparation

Leaves were dried at 40–45 °C and pulverized and the aqueous extract (AEP) was obtained by decoction of 10 g leaves in 100 mL deionized water, at 90 °C for 30 min (Farmacopéia, 1959), next lyophilized and stored in a desiccator until use. For use, AEP was dissolved in deionized water.

2.3. Determination of total phenolic and flavonoid content

The total phenolic compounds, present in AEP, were determined using Folin–Ciocalteau's method (Rai et al., 2006) and gallic acid as standard. Total flavonoids were estimated by the aluminum chelating method (Ebrahimzadeh et al., 2008), using quercetin as standard.

2.4. Antioxidant assays

The total antioxidant capacity was determined (Prieto et al., 1999), and the results were expressed as mg equivalents of ascorbic acid/g of extract. The hydrogen atom or electron donation ability of AEP was measured from the bleaching of purple colored methanol solution of DPPH according to Yen and Wu (1999). L-Ascorbic acid was used as the reference standard.

2.5. Cell line and culture

Human breast cancer MCF-7 cell line (ER+) was purchased from Rio de Janeiro Cell Bank (BCRJ 0162) and cultured in RPMI 1640 medium, supplemented with 20% (v/v) inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was replaced every 2 days and cells were sub-cultured every 5 days, after 0.25% trypsin–EDTA solution treatment. Cell viability was assessed, before beginning each experiment, by the Trypan blue-dye exclusion method and 2×10^4 viable cells/mL were used in all experiments. Before treatment, viable cells were cultivated for 24 h to reach exponential growth.

2.6. Sulforhodamine B (SRB) uptake assay

The effect of different concentrations of AEP on cell protein content was determined by the sulforhodamine B (SRB) colorimetric assay (Vichai and Kirtikara, 2006). Briefly, cells were seeded onto 96-well plates and treated with different concentrations of AEP (0.1, 0.2, 0.5, 1.0, 1.5, 2.5, 5.0, 10, 25, 50 and 100.0 μg/mL) for 24 and 48 h and fixed with 10% trichloroacetic acid (w/v) for 30 min at 4 °C. The plate contents were carefully removed and each well was washed with distilled water. The plate was then dried for 24 h and stained with 0.4% SRB (w/v) in 1% acetic acid (v/v) for 30 min, after which excess SRB was removed, and the wells were washed 4 times with 1% acetic acid (v/v). The bound SRB was dissolved by adding 100 μL of 10 μM Tris, pH 10.5 for 10 min, and absorbance read at 510 nm. Treated-cells with culture medium were the negative control, and with 550 μg/mL cyclophosphamide, the positive.

2.7. Cell morphological analysis

Cells cultured on coverslips were treated with various concentrations of AEP (0.1, 0.2, 0.5, 1.0, 1.5 5.0 μg/mL) for 24 and 48 h. After treatment, cells were fixed with 70% acetone for 15 min, washed with PBS, and stained with hematoxylin–eosin. Slides were mounted in Entellan, and observed by light microscopy. Cell digital images were acquired using an Olympus BX52 microscope and Motic Images Plus 2.0 software. Fifteen random fields were analyzed per treatment, to describe