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.

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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...
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 (Mialeva, 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).

2. Materials and methods

2.1. Plant material

Leaves of *Plinia edulis* (Vell.) Sobral, Myrtaceae, popularly known as “cambuca”, 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 as well 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.

2.2. 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% CO₂ 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 × 10⁵ viable cells/mL were used in all experiments. Before treatment, viable cells were cultivated for 24 h to reach exponential growth.

2.6. Sulfurhodamine 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 BXX2 microscope and Motic Images Plus 2.0 software. Fifteen random fields were analyzed per treatment, to describe
cell morphology. Treated-cells with culture medium were the negative control, and with 550 μg/mL cyclophosphamide, the positive. Cell and colony diameters were determined by measuring 150 cells and 20 colonies per treatment (Campanella et al., 2012).

2.8. Cell death assay

Cell death was estimated using the Fast green-dye exclusion method, according to Weisenthal et al. (1983), with modifications. The rationale of this method is based on the principle that viable cells are able to exclude the dye while dead cells lose this ability. Cells were treated with several concentrations of AEP (0.1, 0.2, 0.5, 1.0 and 5.0 μg/mL) for 24 h, whereupon cells were stained with 2% Fast green, followed by hematoxylin–eosin, and slides were mounted in Entellan. With this procedure, viable cells (i.e., cells excluding Fast green) were stained reddish-pink, while dead cells (cells unable to exclude Fast green) were stained green. To achieve the percentage of dead cells, a total of 600 cells/treatment was analyzed using an Olympus BX52 microscope and Motic Images Plus 2.0 software. Treated-cells with culture medium were the negative control, and with 550 μg/mL cyclophosphamide, the positive.

2.9. DNA isolation and electrophoresis

MCF-7 cell genomic DNA was isolated using the SDS/Proteinase K/RNase A extraction method (De Siervi et al., 2002). Samples were electrophoresed in 0.8% (w/v) agarose gel and DNA was visualized by ethidium bromide staining. The presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments on the agarose gel.

2.10. Statistical analysis

Obtained data were compared by one-way analysis of variance (ANOVA) followed by the Tukey test, when p < 0.05. Data are shown as the mean±SEM of three independent experiments.

3. Results and discussion

3.1. Phenolic compound determination

AEP presented 54.34± 0.02 mg/g total phenols and 35.16± 0.01 mg/g flavonoids, a higher content of flavonoids than the hydroalcoholic extract from Plinia cauliflora leaves (1 mg/g; Souza-Moreira et al., 2011), and also that reported by other previously examined sources of antioxidants (Kim et al., 2011; Santos Filho et al., 2011).

Phenolic compounds from medicinal and dietary plants are bioactive and play an important role in prevention of cancer. They have a complementary and overlapping mode of action, including antioxidant activity, scavenging free radicals, and modulation of carcinogen metabolism that alter important cellular and molecular mechanisms related to carcinogenesis, a multistep process involving the transformation, survival, proliferation, invasion, angiogenesis, and metastasis of the tumor cells (Huang et al., 2010). Epidemiological studies confirmed that phenolics, namely flavonoids, appear to be beneficial compounds in various stages of carcinogenesis (Clere et al., 2011; Fresco et al., 2010). In fact, little is known about the chemical composition of the genus Plinia. Research is currently in progress in our laboratory to better identify the AEP constituents.

3.2. Antioxidant activity

The total antioxidant capacity of AEP was 324.20 ±3.14 mg/g. The extract also showed strong DPPH scavenging activity (Fig. 1), which was concentration-dependent, showing the maximum effect with 20 μg/mL AEP, an activity similar to ascorbic acid. The results of the present work indicate that AEP acts as an antioxidant and it could be attributed to the extract constituents. Phenolic compounds are regarded to be the most important antioxidant constituents of plants. In general, the phenolic OH is considered a scavenger of free radicals with consequently antioxidant activity. Flavonoids may also contribute to AEP antioxidant activity, due to its hydrogen-donating ability (Rice-
Different letters in the same column indicate significant differences (p < 0.01) by the Tukey test. Note cell-diameter reduction after treatment, in comparison to control cells, thereby indicating AEP cytotoxicity.

Table 1
MCF-7 cell colony diameter (μm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (h)</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>12.72±1.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.48±1.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>8.65±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.53±1.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>AEP0.1</td>
<td>12.05±1.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.30±1.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

NC — negative control cells, CP — 550 μg/mL cyclophosphamide-treated cells, and AEP0.1 — 0.1 μg/mL aqueous extract of Plinia edulis leaves-treated cells. Results are expressed as mean±SEM of three independent experiments. Different letters in the same column indicate significant differences (p < 0.01) by the Tukey test.

Evans et al., 1996). The interplay between free radicals and antioxidants is important in maintaining health and preventing the onset and progression of several pathologies, including cancer (Gouvêa, 2004; Halliwell, 2007; Huang et al., 2010).

3.3. Determination of cytotoxicity

We evaluated the cytotoxicity of AEP using the SRB assay (Fig. 2). SRB, in moderated acid conditions, binds stoichiometrically to basic protein amino acids so that the decrease in OD observed with AEP treatment could be correlated to cell number, thus being an indicator of cell growth (Vichai and Kirtikara, 2006) or the extract could affect the protein turnover, affecting cell viability. The results depicted in Fig. 2 show that the extract reduced cell protein content in a concentration-dependent manner. Cell treatment with 0.1 μg/mL AEP for 48 h or with 0.2 to 2.5 μg/mL for 24 and 48 h reduced protein content by 80% and the maximum effect was obtained with 5 μg/mL AEP. No further significant (p > 0.05) hindrance was achieved by increasing AEP concentration or incubation time. Therefore, it is possible to sustain the positive cytotoxic role of AEP in the inhibition of MCF-7 cell growth and spread, thus affecting cell viability.

The most readily observed effect, following cell exposure to toxicants, is morphological alteration in the cell-layer and/or cell-shape in monolayer cultures (Ekwall et al., 1990). AEP and CP treatment induced alterations in cell morphology. Untreated control cells grow as colonies, presented irregular morphology, and were homogeneously stained, with the cytoplasm less stained than the nucleus and the nucleolus plainly visible. On the other hand, when exposed to AEP cells shrank, became rounded and individually separated, with wrinkled cytoplasmic membrane and nuclear condensation, indicating cytoskeleton disruption (Fig. 3). Cell shrinkage was further confirmed by the determination of cell and colony diameter that were significantly (p < 0.01) decreased in comparison to control cells (Fig. 4 and Table 1). AEP seemed to be more effective than CP, even at lower concentrations (Fig. 4). Cell colonies were visible only with 0.1 μg/mL AEP treatment; upon higher AEP concentrations only individually separated cells were seen.

A cytotoxic compound induces short-term loss of cell viability either by triggering cell-death or decreasing cell survival (Sumantran, 2011), so we next investigated the AEP effect on cell death. AEP and CP treatment induced significant (p < 0.01) cell death, in a concentration-dependent manner with maximum effect at 5 μg/mL (Fig. 5).

DNA fragmentation analysis (Fig. 6) indicates that CP and AEP induced apoptosis, as a DNA ladder pattern was obtained. Laddering-DNA is one of the best-studied biochemical
already been established (Fresco et al., 2010). Therefore AEP prevent the further promotion or progression of lesions that have agents are effective as blocking agents that prevent the mutagenic chemopreventive role of the extract. Cancer chemopreventive oxidant and free radical scavenging properties, which indicate a tumer cells and this activity may originate from the AEP anti-

ticity. This is the first report on the AEP cytotoxicity to breast death and DNA fragmentation induction indicated AEP cytotox-
ing characteristics of apoptosis that demonstrates the internucleosomal DNA cleavage (Yan and Shi, 2005). Altogether, protein decreasing, morphological alterations, cell death and DNA fragmentation induction indicated AEP cytotoxicity. This is the first report on the AEP cytotoxicity to breast tumor cells and this activity may originate from the AEP anti-

oxidant and free radical scavenging properties, which indicate a chemopreventive role of the extract. Cancer chemopreventive agents are effective as blocking agents that prevent the mutagenic initiation of the carcinogenic process and suppressing agents that prevent the further promotion or progression of lesions that have already been established (Fresco et al., 2010). Therefore AEP could act as a blocking agent by scavenging the reactive forms of carcinogens or even as a suppressor, considering the results on the direct action of AEP against the MCF-7 tumor cells.

As there are no satisfactory drugs to treat and cure most breast cancers, research to develop new treatment, is needed. In this context P. edulis seems to be a chemopreventive agent and a rich source of potential compounds for antineoplastic drug development.

The potential toxic effects of a compound are strongly dependent on its concentration and chemical environment, which must be thoroughly understood before any preventive or therapeutic use is considered. Research on the safety of this plant for medicinal use is currently in progress in our laboratory, including in vivo experiments.

4. Conclusion

In conclusion, the aqueous extract of P. edulis leaves exhibits good antioxidant and cytotoxic activities. The obtained results in human breast cancer MCF-7 cell line may provide support for P. edulis’ potentiality as a chemopreventive agent and as a promising candidate for antineoplastic drug development.

Acknowledgments

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