Mitochondria and redox homoeostasis as chemotherapeutic targets of *Araucaria angustifolia* (Bert.) O. Kuntze in human larynx HEp-2 cancer cells

Cátia dos Santos Branco, Émilin Dreher de Lima, Tiago Selau Rodrigues, Thamiris Becker Scheffel, Gustavo Scola, Claudia Cilene Fernandes Correia Laurino, Sidnei Moura, Mirian Salvador

*Laboratório de Estresse Oxidativo e Antioxidantes, Instituto de Biotecnologia, Universidade de Caxias do Sul, RS 95070560, Brazil*  
*University of Toronto, Medical Science Building, Room 4204, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada*  
*Laboratório de Biotecnologia de Produtos Naturais e Sintéticos, Instituto de Biotecnologia, Universidade de Caxias do Sul, RS 95070560, Brazil*

**ARTICLE INFO**

Article history:
Received 9 October 2014  
Received in revised form 24 February 2015  
Accepted 3 March 2015  
Available online 11 March 2015  

Keywords:  
Mitochondria  
Apoptosis  
HEp-2  
*Araucaria angustifolia*  
HRMS

**ABSTRACT**

Natural products are among one of the most promising fields in finding new molecular targets in cancer therapy. Laryngeal carcinoma is one of the most common cancers affecting the head and neck regions, and is associated with high morbidity rate if left untreated. The aim of this study was to examine the antiproliferative effect of *Araucaria angustifolia* on laryngeal carcinoma HEp-2 cells. The results showed that *A. angustifolia* extract (AAE) induced a significant cytotoxicity in HEp-2 cells compared to the non-tumor human epithelial (HEK-293) cells, indicating a selective activity of AAE for the cancer cells. *A. angustifolia* extract was able to increase oxidative damage to lipids and proteins, and the production of nitric oxide, along with the depletion of enzymatic antioxidant defenses (superoxide dismutase and catalase) in the tumor cell line. Moreover, AAE was able to induce DNA damage, nuclear fragmentation and chromatin condensation. A significant increase in the Apoptosis Inducing Factor (AIF), Bax, poly-(ADP-ribose) polymerase (PARP) and caspase-3 cleavage expression were also found. These effects could be related to the ability of AAE to increase the production of reactive oxygen species through inhibition of the mitochondrial electron transport chain complex I activity and ATP production by the tumor cells. The phytochemical analysis of *A. angustifolia*, performed using High Resolution Mass Spectrometry (HRMS) in MS and MS/MS mode, showed the presence of dodecanoic and hexadecanoic acids, and phenolic compounds, which may be associated with the chemotherapeutic effect observed in this study.

© 2015 Elsevier Ireland Ltd. All rights reserved.

**1. Introduction**

Cancer represents the second leading cause of death in occidental countries. Laryngeal carcinoma is one of the most common form of tumor cells that reach the head and neck, and it represents about 30% of all malignancies in these body areas [1]. Although there are effective drugs for treating several types of cancer, laryngeal cancer is one of the many cancers that still lack appropriate treatment, thus giving rise to considerable morbidity and mortality rates in patients. The resistance of tumor cells depend mainly on their capacity to resist to apoptosis and growth-inhibitory signals, thereby leading to tissue invasion and metastasis [2]. Some of these resistance characteristics are dependent on redox homoeostasis alterations in cancer cells [3]. Therefore, it is important to investigate new therapeutic agents, which may present selective properties to induce cytotoxicity through the activation of cell death signaling pathways.

Plant-derived products possess a wide range of pharmacological actions, including chemopreventive, anticancer and apoptotic properties [4–6]. *Araucaria angustifolia* (Bertolini) Otto Kuntze belongs to the Araucariaceae family and is one of the main pine species found in South America. In Brazil, native populations of this species occur essentially in the southern highlands, constituting the Araucaria forests. *A. angustifolia* is a dioecious species, meaning male and female specimens have their own distinct strobili. Female strobilus consists of seeds (the edible part named “pinhão”) and bracts, which are undeveloped seeds commonly discarded into the environment. Moreover, *A. angustifolia* presents great relevance in the Brazilian folk medicine. Different parts of the tree (bark,
needles and resin) are used to treat various illnesses, particularly diseases of the respiratory tract. Recently, our group has characterized an *A. angustifolia* extract obtained from the bracts, which is rich in phenolic compounds such as flavonoids and tannins [7,8].

Phenolic compounds are multifaceted molecules, which have powerful antioxidant abilities. Nevertheless, some of these compounds may promote the formation of reactive oxygen species (ROS) [6,9] mainly through the mitochondria. The effect resulting from ROS production occurs through additional mechanisms of action in which the polyphenols are able to generate o-semiquinones and to oxidize NADH, resulting in inhibition of mitochondrial respiration [10]. Mitochondrial dysfunction is often associated with oxidative damage, defective ATP synthesis, and cell death, showing that mitochondria might be an important therapeutic target for cancer treatment [11,12]. Inhibition of mitochondrial respiration has been associated with the ability of polyphenols to modulate the dynamics of cancer cells in order to exert selective anticancer effects [6]. However, the mechanism of action of polyphenols in implicating this effect still remains unclear.

In this context, the objective of this study was to (1) evaluate the antiproliferative activity of an extract obtained from *A. angustifolia* bracts on human laryngeal carcinoma (HEp-2) and non-tumor (HEK-293) cells; (2) explore the cytotoxic mechanism of action of the extract in tumor cells and to; (3) evaluate the chemical composition of AAE to better examine the biological properties of this extract.

2. Materials and methods

2.1. Chemicals

Complete Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), trypsin–EDTA and penicillin–streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Apoptosis antibody sampler kits (CS9915, CS9942 and CS4670) were acquired from Cell Signaling Technology (Danvers, MA, USA). Complex I Enzyme Activity Microplate Assay Kit was purchased from Cell Signaling Technology (Danvers, MA, USA). Apoptosis antibody sampler kits (CS9915, CS9942 and CS4670) were purchased from Gibco BRL (Grand Island, NY, USA). Complex I Enzyme Activity Microplate Assay Kit was purchased from Cell Signaling Technology (Danvers, MA, USA). The Cell Titer-Glo® Luminescent Cell Viability Assay Kit from Promega (Madison, WI, USA). Low-melting point agarose and normal agarose were purchased from Invitrogen (Carlsbad, CA, USA). Acrylamide kit was acquired from Sigma–Aldrich (St. Louis, MO, USA). Other reagents and solvents were obtained from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade.

2.2. *A. angustifolia* extract

Female strobili of *A. angustifolia* were collected in Caxias do Sul, Rio Grande do Sul (29°9'34.90"S, 51°8'45.34"W); Ibania n° 02001.001127/2013–94, Brazil. Voucher specimens were identified by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (HUCS 40710/40711). Bracts were manually separated by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil. 

2.3. Mass Spectrometry

The lyophilized extract of *A. angustifolia* was dissolved in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid or 0.1% ammonia hydroxide for ESI(+) or ESI(−) respectively. The solutions were individually infused directly or with HPLC (Shymadzu) assistance into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μL min⁻¹. ESI(+)-MS, tandem ESI(+)-MS/MS and ESI(−)-MS were acquired using a hybrid high-resolution and high accuracy (5 μL/L) microTOF-QII mass spectrometer (Bruker® Daltonics) under the following conditions: capillary and cone voltages were set to +3500 and +40 V, respectively, with a de-solvation temperature of 100 °C. Diagnostic ions in different fractions were identified by the comparison of exact m/z with compounds identified in previous studies. For data acquisition and processing, Hystar software (Bruker® Daltonics) was used. The data were collected in the m/z range of 70–800 at the speed of two scans per second, providing the resolution of 50,000 (FWHM) at m/z 200. No important ions were observed below m/z 150 or above m/z 500, therefore data is shown in the m/z 180–500 range.

2.4. Cell culture

Human laryngeal cancer cells (HEp-2 line) and human non-tumor cells (HEK-293 line) were obtained from American Type Culture Collection (ATCC) bank. Both epithelial cell lines were cultivated under standardized conditions in DMEM medium, supplemented with 10% heat-inactivated FBS and penicillin–streptomycin (10,000 U/mL). Cells were seeded in culture flasks and maintained in a humidified atmosphere at 37 °C with 5% CO₂. Studies were conducted when the cells reached 80–90% confluency.

2.5. Cell viability assays

In order to determine the antiproliferative activity of the *A. angustifolia* extract (AAE) on HEp-2 cells, 3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyl tetrazolium bromide (MTT) [14], and the trypan blue exclusion assays were performed [15]. For the MTT assay, seeded cells (1 × 10⁶ cells/well in 96-well plate) were treated with different concentrations (100, 250 and 500 μg/mL) of AAE, and incubated at 37 °C in 5% CO₂ for 24, 48 and 72 h. After this time, the medium was removed and 1 mg/mL MTT dye in serum-free medium was added to the wells. Plates were incubated at 37 °C for 3 h. Subsequently, MTT solution was removed and the resulting formazan violet product was dissolved in 100 μL dimethylsulfoxide (DMSO), stirred for 15 min, and the absorbance was measured using a microplate reader (Victor-X3, multilabel counter, Perkin Elmer, Finland) at 517 nm. The cell viability in each well was expressed as percentage compared to non-AAE treated control cells. In addition to the MTT assay, trypan blue stain (0.4%) dissolved in Phosphate Buffered Saline (PBS) was used to assess cell viability. Cells (2.5 × 10⁵ cells/well in a six-well plate) were treated with increasing concentrations of AAE (100, 250 and 500 μg/mL) for 24 h. The number of viable and dead cells was counted using an optical microscope. The percentage of viability was calculated by number of unstained cells/total number of cells × 100. Normal human embryonic kidney-293 (HEK-293) epithelial cell line was used as non-tumor control.

2.6. Cell and nuclear morphology analysis

Changes in cellular morphology were observed in culture flasks after 24 h of different AAE treatments (100, 250 and 500 μg/mL). Nuclear morphology evaluation was performed according to Jaganathan et al. [16]. The cells (2 × 10⁵ per well) were grown on 12 mm cover slips in 24-well culture plates and exposed to different concentrations of AAE (100, 250 and 500 μg/mL) for 24 h. The monolayer of cells were washed with PBS and fixed with 3% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and...
incubated with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were analyzed. Images were obtained using an inverted fluorescent microscope (Optiphase-403F, USA).

2.7. Oxidative damage to lipids and proteins, and nitric oxide levels

With the aim to study the cytotoxic mechanisms of the AAE, oxidative damage to lipids and proteins, and nitric oxide (NO) production were assessed in HEp-2 cells (1 \times 10^7) after 24 h treatment with different AAE concentrations (100, 250 and 500 \mu g/mL). Oxidative parameters were assessed after the incubation of the cells with RIPA lysis buffer for 30 min, and centrifugation at 1500g at 4°C for 5 min. The supernatants were used for the assays.

Lipid peroxidation was monitored by the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction, according to Wills [17] with modifications. Specifically, 400 \mu l of supernatant from each sample was combined with 600 \mu l of 15% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA). The mixture was heated at 100°C for 20 min. After cooling to room temperature, the samples were centrifuged at 1300g for 10 min. The supernatants were isolated, and their absorbance were measured at 530 nm. Hydrolyzed 1,1,3,3-tetramethoxypropane (TMP) was used as standard, and the results were expressed as nmol TMP/mg of protein. Oxidative damage to proteins were measured based on the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) [18]. For the control, 200 \mu l of DNPH (10 mM) or 200 \mu l of HCl (2 M) were added to 50 \mu l of cell supernatants. The reaction mixture was incubated in the dark for 30 min, and vortexed every 10 min. After, 250 \mu l of 20% TCA was added to each reaction mixture and centrifuged at 1400g for 8 min. The supernatants from each sample were discarded, and the pellets were washed 3 times with ethanol–ethyl acetate (1:1) to remove free reagent. Samples were centrifuged and the pellets were resuspended in 1000 \mu l of urea solution (8 M) at 37°C for 15 min. Absorbance was read at 365 nm, and results were expressed as nmol DNPH/mg protein. Nitric oxide production was determined based on the Griess reaction [19]. Taking into account that accurate NO measurements are very difficult to assess in biological specimens, nitrite concentration was estimated as an index of NO production. A standard curve was performed into account that accurate NO measurements are very difficult to assess in biological specimens, nitrite concentration was estimated as an index of NO production. A standard curve was performed using sodium nitroprusside (SNP) for calibration. Optical density

was quantified at 535 nm and the results were expressed as nmol SNP/mg protein. Protein concentration was determined by the Lowry et al. [20] method using bovine serum albumin as standard.

2.8. Superoxide dismutase and catalase activities

After 24 h of treatment with different concentrations of AAE (100, 250 and 500 \mu g/mL), HEp-2 cells (1 \times 10^7) were incubated with RIPA lysis buffer for 30 min, and centrifuged at 8000g at 4°C for 10 min. The supernatants were used for both enzymatic assays. Superoxide dismutase (Sod) activity was measured by the inhibition of self-catalytic adrenochrome formation rate at 480 nm, in a reaction medium containing 1 mmol/L adrenaline (pH 2.0) and 50 mmol/L glycine (pH 10.2) at 30°C for 3 min [21]. Results were expressed as USod (units of enzyme activity)/mg protein. One unit is defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50%. Catalase (Cat) activity was measured according to the method described by Aebi [22]. The assay determines the rate of H2O2 decomposition at 30°C for 1 min in 240 nm. Results were expressed as UCat/mg of protein. One unit is defined as the amount of enzyme that decomposes 1 mmol of H2O2 in 1 min at pH 7.4. Protein concentration was determined by the Lowry method [20] using bovine serum albumin as standard. All absorbance were measured in spectrophotometer model UV-1700.

2.9. DNA damage

Single cell gel electrophoresis (Comet assay) was performed to assess the genotoxic effects of AAE on 24 h treated HEp-2 cells, as described by Singh et al. [23]. Cells (2.5 \times 10^5) were washed with ice-cold PBS, trypsinized, and resuspended in complete medium. Slides were prepared by mixing 20 \mu l of suspended cells and 80 \mu l of low melting point agarose (0.75%). The mixture was poured onto a frosted microscope slide coated with normal melting point agarose (1%). After solidification, the coverslip was removed and the slides were placed in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and 10% DMSO) for 24 h. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH = 12.6) for 20 min. The DNA was electrophoresed for 20 min at 25 V (0.9 V/cm) and 300 mA, and the buffer was neutralized with 0.4 M Tris (pH 7.5). Finally, DNA was

Fig. 1. HRMS full mass spectra of the Araucaria angustifolia extract in positive (A) ESI (+) and negative (B) ESI (−) mode.
stained with silver nitrate. Images of 100 randomly selected cells (of four replicated slides) were analyzed from each sample. DNA damage was visually scored according to tail size into five classes, from no tail (0) to maximal (4) long tail. Therefore, a group damage index (DI) could range from 0 (all cells with no tail, 100 cells/C2) to 400 (all cells with maximally long tails, 100 cells/C2). The frequency (%) of the different classes of DNA damage was also evaluated.

2.10. Immunoblotting analysis

HEp-2 cells (6 \times 10^5) treated with AAE during 24 h were harvested and mixed with Laemmli sample buffer, denatured by boiling (100 °C) for 5 min. Briefly, 3000 cells/µL (20 µL) were loaded and the proteins were separated on 7.5–15% SDS–PAGE gels. After, the proteins were electro-transferred onto nitrocellulose membranes (Amersham Hybond™-C Extra). The membranes were blocked with 5% non-fat dry milk and were stained overnight at 4 °C with primary antibodies. Specifically, cleaved and total Poly-(ADP-ribose) polymerase (PARP), Apoptosis Inducing Factor (AIF), p53, cleaved caspase-3, Bax, Bad, and Bim were labeled with primary antibodies, and β-actin was used as a loading control. The following dilutions were used: PARP and AIF (1:2000), p53 (1:500), cleaved caspase3, Bax, Bad, Bim and β-actin (1:1000). After incubation with primary antibodies, the membranes were washed with PBS-T (PBS-buffered saline containing 0.1% Tween-20) to remove unbound primary antibodies. The membranes were then stained with anti-Rabbit IgG conjugated–peroxidase or Mouse IgG secondary antibodies (1:2000) for 1 h at room temperature. The membranes were washed repeatedly. Protein detection was performed by using a chemiluminescence protocol (Amersham Bioscience). Protein band images were captured using Image Scanner™ III (GE Healthcare) and pairwise comparisons of the protein bands on the immunoblot were performed using Image-J 1.45 software.

2.11. Mitochondrial complex I activity

HEp-2 cells (1 \times 10^7) were grown in culture flasks and treated with 100 µg/mL of AAE for 24 h. After AAE-treatment, cells were washed with PBS, scraped and homogenized with ice-cold PBS. Mitochondrial complex I activity was assayed in the cell extracts using the Complex I Enzyme Activity Microplate Assay Kit, by following the manufacturer’s instructions. Mitochondrial complex I activity was measured based on the oxidation of NADH to NAD^+ at 450 nm in the microplate reader (Victor-X3, Perkin Elmer, Finland). Positive control was obtained with cells exposed to rotenone (20 µM, a complex I inhibitor) for 30 min. Mitochondrial complex I activity was also evaluated in HEK-293 cells treated with AAE (100 µg/mL) for 24 h, using rotenone (20 µM; 30 min.) as positive control. Data were presented as percentage of control (%).

2.12. ATP quantification

HEp-2 cells (5 \times 10^4 cells/well in opaque-walled 96-well plate) were incubated with 100 µg/mL of AAE for 24 h. ATP levels were measured by using the Cell-Titer-Glo® Assay Kit, according to the manufacturer’s instructions. Luminescence was recorded using a multi-mode microplate reader (Victor-X3, Perkin Elmer, Finland). Positive control was obtained with cells exposed to rotenone (20 µM, a complex I inhibitor) for 30 min. ATP levels were also

---

Table 1

<table>
<thead>
<tr>
<th>Entry</th>
<th>Precursor ion m/z (%)</th>
<th>Identification</th>
<th>Elem. comp.</th>
<th>Diff. ppm</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESI(+) [M+H]^+ 193.0703</td>
<td>1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid</td>
<td>C7H10O6</td>
<td>4.73</td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td>195.0868</td>
<td>3-O-methyl-α-chiro-inositol</td>
<td>C7H10O5</td>
<td>0.32</td>
<td>[27]</td>
<td></td>
</tr>
<tr>
<td>302.0870</td>
<td>4-Nitrophenyl-β-D-glucopyranoside</td>
<td>C12H14NO4</td>
<td>1.95</td>
<td>[28]</td>
<td></td>
</tr>
<tr>
<td>315.0862</td>
<td>4'-Methoxytectorigenin</td>
<td>C17H14O6</td>
<td>2.11</td>
<td>[29]</td>
<td></td>
</tr>
<tr>
<td>467.1177</td>
<td>3-Glucoside-dihydroquercetin</td>
<td>C21H22O12</td>
<td>1.18</td>
<td>[30]</td>
<td></td>
</tr>
<tr>
<td>595.1598</td>
<td>Amentoflavone 4,4&quot;,7,7&quot;-tetramethyl ether</td>
<td>C34H27O10</td>
<td>1.04</td>
<td>[31]</td>
<td></td>
</tr>
<tr>
<td>ESI(-) [M-H]^- 191.0560</td>
<td>1,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid</td>
<td>C7H10O6</td>
<td>2.28</td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td>199.1703</td>
<td>Dodecanoic acid</td>
<td>C12H22O2</td>
<td>2.48</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td>255.2334</td>
<td>Hexadecanoic acid</td>
<td>C16H30O2</td>
<td>3.90</td>
<td>[33]</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Chemical structures of the compounds identified in Araucaria angustifolia extract.
Both lines present epithelial morphology. The analysis of complex mixtures such as plant extracts and have been used by our group to rapidly analyze natural products [24]. HRMS-ESI has been elected as one of the most powerful tools for the parametric distribution of data. Statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. Results are deemed significant if p-value was less than 0.05.

2.13. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 21.0) for Windows (Illinois, USA). Kolmogorov–Smirnov test was used to assess for normality of distribution of data. Statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. Results are deemed significant if p-value was less than 0.05.

3. Results

3.1. Phytochemical characterization

To investigate the chemical composition of AAE, High Resolution Mass Spectrometry (HRMS) was used with Q-TOF-II (Bruker® Daltons). The electrospray ionization (ESI) technique allows for the analysis of a wide range of compounds. Thus, HRMS-ESI has been elected as one of the most powerful tools for the analysis of complex mixtures such as plant extracts and have been used by our group to rapidly analyze natural products [24]. In accordance, the positive (A) and negative mode (B) were tested quantified in HEK-293 cells treated with AAE (100 µg/mL) for 24 h, using rotenone (20 µM; 30 min.) as positive control. Results were expressed as percentage of control (%).

3.2. AAE exhibits antiproliferative activity

The sensitivity of HEp-2 cell line to AAE treatment was evaluated by the MIT assay (Fig. 3) and trypan blue exclusion method (Fig. 4). Non-tumor (HEK-293) cell line was used as a control. It was observed that AAE treatments induced a significant time-dependent antiproliferative activity in HEp-2 cells. The concentrations of 250 and 500 µg/mL were able to inhibit tumor cell growth by approximately 50% in 24 h of treatment. In 48 h, tumor reduction reached 65.0 4.9% and 70.0 ± 2.8% for 250 and 500 µg/mL, respectively. In 72 h, higher cytotoxicity rate was observed as tumor reduction reached approximately 80% in the treatment with 250 and 500 µg/mL, indicating a dose-dependent effect only in treatments below 250 µg/mL. It is important to observe the selective effect of AAE, which presented no significant cytotoxicity for non-tumor HEK-293 cells treated with 250 µg/mL of AAE. Trypan blue assay (Fig. 4) confirmed the results found in MIT assay, reinforcing the selective antiproliferative activity of AAE to tumor cells.

3.3. AAE induces morphological alterations in HEp-2 cells

Microscopic analysis of HEp-2 cells treated with different concentrations of AAE (100, 250 and 500 µg/mL) for 24 h of treatment demonstrated a decrease in cell number, along with characteristic structural changes, including vacuolization of the cytoplasm and permeabilization of the plasma membrane. In addition, AAE changed the symmetry of the cells, reducing their growth and cell adherence (Fig. 5A). To assess the nuclear morphology, the cells were stained with DAPI, a DNA-specific fluorescent dye, and were then examined by fluorescent microscopy. HEp-2 cells treated with 250 and 500 µg/mL of AAE exhibited condensed and fragmented nuclei when compared to control cells, which showed clear and interphase nuclei (Fig. 5B).

3.4. AAE pro-oxidants effects

To evaluate the possible mechanisms by which AAE leads to cell death, oxidative damage markers to biomolecules, production of NO and enzymatic antioxidant activities were assayed. As shown in Table 2, AAE was able to induce oxidative damage to lipids and proteins, and to increase the production of NO. Antioxidant activities of Sod and Cat enzymes were significantly decreased under all treatments, indicating that AAE could be generating high levels of superoxide anion radical (O2⁻), thus increasing redox imbalance in tumor cells.
3.5. AAE induces DNA damage

The genotoxic effect of AAE on HEp-2 cells was determined through Single cell gel electrophoresis assay (Fig. 6). AAE treatment was able to increase DNA damage levels in all tested concentrations when compared to non-treated control group. DNA damage indexes were 158.0 ± 11.31, 176.0 ± 16.18, and 280.0 ± 14.43 for 100, 250 and 500 μg/mL of AAE, respectively (Fig. 6A). AAE treatments increased the frequency of classes 2, 3, and 4 of DNA damage in a dose-dependent manner, with high levels of the maximal DNA damage (class 4) (Fig. 6B).

3.6. AAE modulates apoptotic proteins expression

To assess the involvement of apoptotic signaling molecules following AAE treatment, the expression levels of cleaved and total PARP, AIF, p53, cleaved caspase-3, Bax, Bad, and Bim were examined. In AAE-treated HEp-2 cells (100, 250 and 500 μg/mL), the expression levels of total PARP were found to be 3, 5, and 9 times higher than control, respectively (Fig. 7A). In addition, our results showed that AAE treatment with 500 μg/mL was able to induce PARP cleavage, which is an important substrate for apoptosis. Expression levels of AIF were also upregulated by AAE treatment, as shown in Fig. 7B. Similar results were observed for cleaved caspase-3 expression levels, which showed upregulation after AAE treatment (Fig. 7D). AAE treatment did not influence the expression of p53 (Fig. 7C), suggesting an induction of p53-independent apoptosis. Proteins of the mitochondrial intrinsic pathway (Bax, Bad and Bim) were also analyzed. Bax expression was significantly increased following treatment with AAE, only at higher concentrations (Fig. 7E). On the other hand, the expression levels of Bad and Bim were not modulated by AAE treatment (Fig. 7F and G).

3.7. AAE induces mitochondrial dysfunction

In order to evaluate the possible effects of AAE on mitochondrial electron transport chain complex I, the lowest cytotoxic concentration (100 μg/mL) of the extract was used. In HEp-2 cells AAE treatment was able to reduce mitochondrial complex I activity by 55.05% (Fig. 8A) when compared to control group. Rotenone, a specific inhibitor of complex I, caused a significant reduction in complex I activity (76.36 ± 5.14%; Fig. 8A) following 30 min of exposure. In addition, solvent control (DMSO) did not inhibit complex I activity (data not shown). Furthermore, HEp-2 cells exposed to AAE treatment showed decreased levels of ATP production when compared to untreated cells (20.86 ± 3.37%), and this decrease was more accentuated than those observed for rotenone exposure (57.26 ± 1.03%; Fig. 8B). In order to assess the effect of AAE on mitochondrial function in normal cells, mitochondrial complex I activity and ATP levels were evaluated in HEK-293 cell line. It was observed that rotenone presented higher inhibition on complex I activity (around 75% of inhibition related to control group) and consequently lower production of ATP (around 60% in comparison to control group). Moreover, HEK-293 cells treated with AAE presented less inhibition on mitochondrial complex I activity (around 35% of inhibition in relation to control group) along with no significant reduction on ATP levels (around 92% of production compared to control) (Fig. 8A and B).
4. Discussion

Some polyphenols [6,34] and fatty acids [35,36] found in plants have already been described as chemotherapeutic agents; however, the mechanisms implicated in this effect have not been clearly defined. Therefore, the study of chemical composition and biological effects of plants with chemotherapeutic potential is useful to the search of new compounds for cancer treatment. In this study, we investigated the chemical composition, antiproliferative activity and cytotoxic mechanism of action of \textit{A. angustifolia}, an important native species from South America, on HEp-2 cells.

From the full mass spectrum and in accordance with a set of characteristics for each ion, some compounds were identified by ESI-MS (+) and ESI-MS (−). In this work, for example, the presence of 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid (determined by \( m/z \) 193.0703 and 191.0560 in positive and negative mode respectively) is a fragment indicative of 5-p-cis-coumaroylquinic acid presence, which had its chemical structure recently determined by Chen et al. [26]. Among the compounds identified by HRMS analysis, it could be highlighted the presence of 4′-methoxy-tectorigenin, 3-glucoside-dihydroquercetin and biflavonoid amentofoavone 4′,4″,7,7″-tetramethyl ether, important derivatives of polyphenols; phenolic compounds that were previously reported to exhibit anticancer and apoptosis-inducing properties in cell lines and animal models [6,37]. Moreover, two important fatty acids, dodecanoic acid (lauric acid) and hexadecanoic acid (palmitic acid) were identified, both of which present antiproliferative actions, capacity to modulate apoptosis, and ability to regulate the cell cycle of human colon cancer cells [35,36,38].

In the present study, AAE showed a selective capacity to inhibit the proliferation of HEp-2 cells, with no cytotoxicity to normal epithelial cells. After 72 h of treatment, the cytotoxicity rate induced by 250 µg/mL AAE reached around 80% of the tumor cells, however it was not cytotoxic to non-tumor cells. This selectivity is an important factor in cancer therapy and natural products that present this particular selectivity are promising candidates to be developed into new chemotherapeutic agents. Cancer cells utilize high amounts of glucose to produce lactate even in the presence of oxygen ("Warburg effect"), differently from normal cells [39]. This metabolic choice reduces concentration of pyruvate destined to mitochondrial oxidative phosphorylation, and is probably related to the overexpression of pyruvate kinase isoenzyme M2 (PKM2), which is able to inactivate the pyruvate dehydrogenase complex (PDHC) [40–42]. PDHC is a key enzyme that catalyzes the oxidative decarboxylation of pyruvate in order to produce acetyl-CoA, linking glycolysis to the tricarboxylic acid cycle [43], and thereby promoting mitochondrial respiration. Amongst the possible mechanisms associated with selectivity elicited by AAE, we hypothesized that AAE is able to inhibit PKM2, therefore activating PDHC. Similar mechanisms have been already described to dichloroacetate, which is able to induce a metabolic switch from glycolysis to mitochondrial respiration in tumor cells (for review, see [44]).

Besides the assumption that AAE could interfere with cytosolic metabolism, our experimental data demonstrated that AAE may act as a mitochondrial complex I inhibitor, with consequent ATP depletion in HEp-2 tumor cells. In this sense, the AAE exerts a biphasic effect, first reversing the "Warburg effect" on HEp-2 cells and posteriorly inhibiting the mitochondrial activity in these cells. Tumor cells present a high proliferative index and require more ATP generation [45,46], being more sensitive to inhibition of oxidative phosphorylation than non-tumor cells.

It has already been shown that some phenolic compounds are able to inhibit mitochondrial respiration, resulting in the overproduction of ROS (for review, see [10]). Furthermore, it has been shown previously that polyphenols diffuse easily through the biological membranes and enter into the mitochondria [47–49]. Flavonoids, an important group of polyphenols, exhibit chemical
Fig. 7. Effect of AAE on protein expression of apoptotic cascade in HEp-2 cells. Protein expression evaluation of PARP (Poly-(ADP-ribose) polymerase) (A); AIF (Apoptosis Inducing Factor) (B); p53 (C); cleaved caspase-3 (D); Bax (E); Bad (F); Bim (G). The cells were treated with AAE at different concentrations of 100, 250 and 500 μg/mL for 24 h. Western blot data are quantified using β-actin as an internal control. Expression levels of proteins are expressed as the relative intensity of the bands. *p < 0.05 compared to the respective control.
homology with the quinone moiety of coenzyme Q, suggesting a competitive inhibition through binding to the quinone sites in the mitochondrial electron chain [50]. Similarly, three phenolic compounds found in AAE (4′-methoxytectorigenin, 3′-glucosidedihydroquercetin, and amentoflavone 4′, 4″, 7″-tetramethyl ether) present chemical homology with the quinone moiety of coenzyme Q. This may explain the inhibition of mitochondrial complex I observed in treated HEp-2 cells.

Complex I of the mitochondrial electron transport chain is one of the major sites of superoxide radical (O2•−) production [51]. Superoxide anion radical can be dismutated by the enzyme Sod, producing H2O2. Increase in ROS levels along with reduced activities of the antioxidant enzymes observed in this study could lead to tumor cell death. Along with the depletion of enzymatic antioxidant defenses (Sod and Cat), we found high levels of lipid peroxidation, protein carbonyl contents, and NO in AAE-treated HEp-2 cells. This redox imbalance may lead to a loss of cell integrity and consequent cell death. Nitric oxide has been implicated in the modulation of different cancer-related events [52] by being able to induce cytoplastic and/or cytotoxic effect on tumor cells through caspase signaling [53]. NO can freely diffuse from the cytosol to the mitochondria, facilitating cytochrome c release. Released cytochrome c is associated with up-regulation of AIF (AIF mediator of cell death) and Bim (Bcl-2-interacting mediator of cell death) and other Bcl-2 family members with conserved homology with the mitochondrial death pathway.

Another important target in cancer therapeutics is PARP. Our study demonstrated that AAE was able to increase total PARP expression and induce PARP cleavage. When the levels of DNA damage are irreparable, PARP plays a decisive role in the apoptotic process, becoming a key substrate for the degradation of damaged DNA by caspases [62,63]. In fact, our results showed upregulation in the levels of cleaved caspase-3 and PARP, indicating that AAE induces apoptosis also via substrate cleavage. Additionally, expression of protein p53, related to maintaining genomic stability had no significative change in HEp-2 cells. Similar results were found in previous reports [16,64] that showed induction of apoptosis independent of p53 function.

**Scheme 1.** Hypothetical model of *Araucaria angustifolia* extract (AAE) mechanism of action on HEp-2 cells. AAE inhibit mitochondrial complex I activity leading to ATP depletion and redox stress by ROS generation. Superoxide radical anion (O2•−) can be dismutated by action of the mitochondrial superoxide dismutase (Sod) enzyme and/or may react with NO generating ONOO−, a potent oxidant that can diffuse into the cytosol, cleaving DNA. H2O2 is easily diffusible through biological membranes, reacting with the catalase (Cat) enzyme or form OH via Fenton reaction. Cellular stress and genotoxic insults increase Bax expression levels, which translocates from the cytosol to the mitochondria, facilitating cytochrome c release. Released cytochrome c is associated with up-regulation of cleaved caspase-3 expression, which in turn cleaves nuclear proteins, such as PARP, leading to apoptosis by mitochondrial death pathway.

**Fig. 8.** Effects of AAE treatment (100 μg/mL) on complex I mitochondrial activity (A) and ATP production (B) in HEp-2 and HEK-293 cells. Results are expressed as mean ± SD. Superscript letters indicate significant differences among groups in each cell line according to ANOVA and Tukey’s post hoc test (p < 0.05).
The study of cell death signaling in different families of proteins is very important in the discovery of new potential anticancer agents. In fact, AAE displays a diverse range of molecular targets (Scheme 1), mainly through mitochondrial dysfunction and redox stress generation, demonstrating an important antitumor potential. However, it is important to evaluate the same molecular parameters in non-tumor cells. Moreover, the theoretical explanation on how AAE may be modeling the PKM2 and PDHC activities needs to be further investigated, along with other regulatory enzymes of cytosolic and mitochondrial carbohydrate metabolism. Although future studies are needed, our results brings forward opportunities for future investigations that will lead to the development of new therapeutic or adjuvant agents in the cancer research field.

5. Conclusions

The findings of our study provide evidence that the chemical composition of AAE demonstrates selective cytotoxicity and pro-apoptotic activity in HEp-2 tumor cells. The cytotoxic mechanisms of AAE include inhibition of the mitochondrial complex I activity, induction of redox stress and DNA breakage. The apoptosis pathway activation occurs via Bax-triggered, along with AIF and cytochrome c release, and it is independent of p53 incement. Lastly, this study showed that mitochondria and redox homeostasis are chemotherapeutic targets of AAE in human larynx HEp-2 cancer cells.

Conflict of Interest

The authors declare no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and from Coordenação de Apoio de Pessoal de Nível Superior (CAPES). Cátia Branco is the recipient of a CNPq Research Fellowship and Mirian Salvador is recipient of a CNPq Research Fellowship.

References


