Brazilian green propolis induced apoptosis in human lung cancer A549 cells through mitochondrial-mediated pathway

Yahima Frión-Herrera, Alexis Díaz-García, Jenny Ruiz-Fuentes, Hermis Rodríguez-Sánchez and José Maurício Sforcina

Keywords
apoptosis; lung cancer; mitochondria; pathway; propolis

Abstract
Objectives Propolis effect on the growth and apoptosis of human lung adenocarcinoma (A549 cells) was investigated as well as its mechanisms.

Methods Cells were incubated with propolis for 72 h, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assays were employed to assess cell viability and the inhibitory concentration (IC). Apoptosis was detected by Acridine Orange/Ethidium Bromide and 4′,6-diamidino-2-phenylindole staining after 24 and 48 h of incubation with ¼ IC_{50} of propolis by testing the mitochondrial membrane potential (ΔΨm) and the expression of apoptosis-related genes (p53, Caspase-3, Bax, Bcl-2, Bcl-XL, Noxa, Puma and p21) by reverse transcription polymerase chain reaction.

Key findings Propolis displayed antiproliferative and cytotoxic effects on A549 cells in a dose- and time-dependent manner, but it did not suppress the growth of normal Vero cells. An enhanced apoptosis was seen in A549 propolis-treated cells after 48 h compared with the control cells. Propolis decreased mitochondrial membrane potential by overexpression of pro-apoptotic genes (Bax and Noxa) and reduction of the antiapoptotic gene Bcl-XL. The expression level of other genes remained unchanged (p53, Caspase-3 and Bax), whereas p21 expression was increased. Propolis induced caspase-independent apoptosis through a p53-independent mitochondrial pathway, and cell cycle arrest by upregulation of p21.

Conclusions Although propolis induces apoptosis mainly by p53-independent manner, it may be induced by another pathway, and new insights may arise for preventing or treating lung cancer.

Introduction
Lung cancer has high morbidity and mortality rates and has drawn significant attention in prevention, diagnosis and therapy.[1] Despite improvements in surgical treatment and radiotherapy, the 5-year survival rate for all patients diagnosed with lung cancer remains between 15% and 20%.[2] It is therefore necessary to develop more effective and less harmful therapies to reduce lung cancer mortality.

In recent years, several groups have reported the efficacies of different natural products for the treatment of lung cancer with few side effects.[3] The therapeutic potential of propolis to treat several diseases, such as cancer, is well recognized.[4,5] The chemical composition of this resinous material collected by bees defines its pharmacological properties and varies in accordance with the phytogeographical diversity of the area from which it is collected. In the apiary of our university (UNESP, Campus of Botucatu, Brazil), the main vegetal sources of propolis produced by Africanized honeybees are Baccharis dracunculifolia, Eucalyptus citriodora and Araucaria angustifolia.[7]

Brazil has great diversity and rich flora, and due to this, propolis collected from diverse regions of this country show distinct colours and chemical compositions depending on the local flora at the site of collection.[8] According to different reports, the Brazilian propolis characterized as ‘green’

Propolis from southeastern Brazil has previously been found to exert a cytotoxic action against human laryngeal epidermoid carcinoma, leukaemia and prostate cancer cells.[16,18,19] Lung cancer is one of the most common cancers in the world, and the objective of this study was to investigate the mechanisms by which propolis may induce the death of human lung carcinoma cells. This paper assays for apoptosis, mitochondrial transmembrane potential disruption and gene expression of pro- and anti-apoptotic proteins (p53, Caspase-3, Bax, Bcl-2, Bcl-XL, Puma, Noxa and p21).

Materials and Methods

Cell culture

Human lung carcinoma cell line A549 (ATCC CCL-185) and Vero cells (normal African green monkey kidney ATCC CRL-1586) were grown in Dulbecco’s Modified Eagle Medium (Gibco Laboratories, Grand Island, NY, USA) (90% v/v) and heat inactivated fetal bovine serum (10% v/v) containing penicillin (100 U/ml), and streptomycin (100 μg/ml). Culture cells were maintained at 37°C under an atmosphere of 5% CO₂ for a maximum of 12 passages.

Propolis sample

Propolis was produced by Africanized honeybees and provided by UNESP, Campus of Botucatu (Brazil) and obtained as described previously.[16] Propolis was ground and 30% ethanolic extracts of propolis were prepared in the absence of bright light, at room temperature and with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated. Concentrations of 6.25, 12.5, 25, 50 and 100 μg/ml of propolis crude hydroethanolic extract were made by diluting the stock solution (130 mg/ml) with the medium prepared as described above.

Cell viability by MTT assay

A549 and Vero cells (2 × 10⁵ cells/ml) were seeded into 96-well plates and treated with different concentrations of propolis (6.25, 12.5, 25, 50 and 100 μg/ml). Untreated cells were used as a negative control. The cells were then cultured for 72 h before the addition of 10 μl of 5 mg/ml solution of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) into each well. The incubation was continued for another 3 h before the medium was removed. Dimethyl sulfoxide (150 μl) was added to each well and mixed to ensure cell lysis and dissolution of the formazan crystals.

Absorbance (A) was read in a ultraviolet-visible spectrophotometer multiplate reader (MRX Revelation Dynex Technologies; Denkendorf, Germany) at 570 nm. Absorbance from untreated cells was considered as 100% of growth and used for viability calculation. The effect of propolis extract on the viability for cell lines panel was expressed as the % viability, using the formula: % viability = A570 of treated cells / A570 of control cells × 100%. The median inhibitory concentration (IC₅₀) values from line cells were determined.

Cytotoxicity assay

The cytotoxic effects of propolis on cells were measured by lactate dehydrogenase (LDH) assay after incubation with various concentrations of propolis (6.25, 12.5, 25, 50 and 100 μg/ml) for 72 h. The assays were carried out in triplicate, and untreated cells were used as a negative control. Briefly, 100 μl/well of the supernatant was transferred to the corresponding well of an optically clear 96-well flat-bottom microlitre plate and analyzed using a Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, St Louis, MO, USA). The absorbance of each sample was measured at 490 nm in a microplate reader (MRX Revelation Dynex Technologies). LDH release was expressed according to the formula: % LDH release = (LDH activity in the medium / total LDH activity) × 100.

Morphological changes and apoptosis assessment

Apoptosis was analyzed by Acridine Orange/Ethidium Bromide (AO/EB) and 4′,6-diamidino-2-phenylindole (DAPI) staining. A549 cells (2 × 10⁴ cell/ml) were grown in 24-well culture plates and treated with propolis for 24 and 48 h using ¼ IC₅₀ of propolis, because cells died using the IC₅₀ or ½ IC₅₀. After these periods, cells were incubated with a mixture of AO/EB in phosphate-buffered saline (PBS) (100 μg/ml AO/100 μg/ml EB). The nuclei of viable cells were stained by the membrane-permeable dye AO, whereas necrotic cells were stained by the highly fluorescent EB. To evaluate the changes in the chromatin morphology of cells by DAPI staining, cells were fixed with 50 μl of acetic acid fixing solution (acetic acid (Quimipur, Madrid, Spain)/ methanol (Merck, Darmstadt, Germany) – 1:2 v/v). After 5 min, cells were washed with PBS and stained with DAPI (1 μg/ml) for 10 min. Apoptotic nuclei were identified by the condensed chromatin gathered at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. In both stains, untreated cells were used as a negative control. A total of 200 cells were analyzed and counted in triplicate in three independent experiments.
using an Olympus IX-71 fluorescent microscope equipped with a Color View camera Olympus DP72, filters with emission shift from red (480 nm) to green (520 nm) and a magnification of 400× (Melville, NY, USA).

**Measurement of mitochondrial transmembrane potential (ΔΨm) disruption**

The changes in the mitochondrial potential were detected using a JC-1 mitochondrial membrane potential assay (Mitochondria Staining Kit, Sigma-Aldrich). A549 cells were cultured in a 24-well plate at a density of 2×10⁵ cells/ml and treated with ¼ IC₅₀ of propolis for 24 and 48 h (triplicate wells per condition). Untreated cells were used as a negative control and valinomycin-treated cells were considered as positive control. All cells were imaged with Olympus IX-71 fluorescent microscope equipped with a Color View camera Olympus DP72 using filters with emission shift from red (590 nm) to green (525 nm) and a magnification of 400×.

**Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR) for pro- and anti-apoptotic genes (p53, Caspase-3, Bax, Bcl-2, Bcl-Xₐ, Puma, Noxa and p21) evaluation**

A549 cells (2×10⁵ cell/ml) were seeded on 24-well plate and treated with ¼ IC₅₀ of propolis for 24 and 48 h (triplicate wells per condition). Total RNA was isolated from untreated or treated A549 cells with TRIzol reagent (LS Reagent, Sigma-Aldrich, St Louis, MO, USA). The RNA final concentration was determined using a Biophotometer plus (Eppendorf, Hauppauge, NY, USA). Each sample of isolated RNA (1 μg) was reverse transcribed in a thermal cycler (AUXILAB) by Moloney Murine Leukemia Virus reverse transcriptase system (Promega Inc., Madison, WI, USA) in a 50-μl volume reaction.

Each PCR was carried out in a master mix containing 1X Green Go Taq Flexi Buffer, MgCl₂, (2 mM), dNTPs (10 mM) and 1.25 U GoTaq DNA polymerase (Promega Inc.) containing 0.2 mM of respective forward and reverse primers, and 5 μl of complementary DNA. The reaction mix was made up to 25 μl with water molecular biology reagent (Sigma-Aldrich, EUA).

The PCR amplification was carried out in a Thermal cycler (AUXILAB) using specific primers (Table 1). Each reverse-transcribed mRNA product was internally controlled by β-actin gene.

PCR conditions for every one of genes were 35 cycles at 94°C for 1.5 min, at 59°C for 1 min, at 72°C for 1 min and at 72°C for 7 min. Amplified PCR products were subjected to electrophoresis at 70 V in 1.5% (w/v) agarose gel for 1.5 h. A 50-bp DNA ladder marker was used as a molecular marker. Gels were visualized with EB in 1×TBE (Tris-borate-EDTA) buffer. The gels were examined and the intensity of each band was measured by using Gel Doc imaging system UViSave D-55/20M version 15.08 (UVitec, Cambridge, England). Size of the amplified PCR products were 191 pb for β-actin, 100 pb for p53, 100 pb for Caspase-3, 283 pb for Bax, 100 pb for Bcl-2, 492 pb for Bcl-Xₐ, 209 pb for Puma, 295 pb for Noxa and 257 pb for p21.

**Statistical analysis**

The IC₅₀ values were determined by interpolation of the graph of propolis concentration vs cell viability. Band intensity of each gene from propolis-treated and non-treated cells was compared using the Kruskal–Wallis test for multiple comparisons and Dunn’s post-hoc test. For all analysis, we used the GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences were considered for P < 0.05.

**Results**

**MTT assay**

The A549 cell viability after 72-h treatment with propolis was determined by MTT reduction assay. A dose–response curve for the percentage of viable cells was obtained against the concentration displayed a significant reduction of cell viability.

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**Table 1: Primers used in polymerase chain reaction reactions**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>CCTTCTGGGATT GGAGTCTCCT</td>
<td>GGAGAATGTGATC CTCTTC</td>
</tr>
<tr>
<td>p53</td>
<td>GGGTGTATTCAATCAGCCACT</td>
<td>GGCTTGAGATGAGAAAATC</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>AGAACATTGGGATCCCTG GGGC</td>
<td>ATCCAGGGGCACTTG ACGAC</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>ATGGGCGGTACATTGGGACT</td>
<td>GGAGGTAGCGAGAAGAT</td>
</tr>
<tr>
<td>Bax</td>
<td>ATGGGCGGTACATTGGGACT</td>
<td>CCATAAAGACGTGTCGGGCA</td>
</tr>
<tr>
<td>Bcl-Xₐ</td>
<td>ACCCCCTAGGCTCTCCTGAA</td>
<td>CCCCAAACGCTGTCGGGCA</td>
</tr>
<tr>
<td>Puma</td>
<td>GCTCGGAGGCCGCTGCTTAC</td>
<td>GTCTGTCGGCCTGCTG TCC</td>
</tr>
<tr>
<td>Noxa</td>
<td>TTCCTTCATGCGGCTCCTGGGC</td>
<td>GTGCAAGGGGAGATGTG</td>
</tr>
<tr>
<td>p21</td>
<td>CAGGGGACAGCAGAGGAAGA</td>
<td>GGCAGAATGAGATGAGC</td>
</tr>
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viability (Figure 1). The IC50 value obtained for the mean of the three independent experiments was 69.17 ± 11.28 μg/ml (P < 0.05).

We have also determined the effects of propolis on the growth of Vero cells (Figure 2). The IC50 obtained from three independent experiments was >100 μg/ml, showing that propolis exhibited a high selectivity for tumour cells over normal Vero cells.

**Lactate dehydrogenase leakage assay**

To determine the cytotoxic effects of propolis on A549 using LDH assay, cells were incubated with increasing concentrations of propolis for 72 h. LDH release increased slightly with increasing propolis concentrations to a maximum of 21.35% for the highest concentration of propolis assayed (100 μg/ml) (Figure 1), suggesting the loss of membrane integrity.

**Analysis of apoptosis induction by fluorescence staining**

Analysis of changes in cell morphology was evaluated using AO/EB and DAPI fluorescence staining. AO/EB staining allows separate enumeration of populations of viable non-apoptotic, necrotic and non-viable apoptotic cells. DAPI is known to form blue fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for A-T rich regions of DNA. When DAPI binds to DNA, the blue fluorescence is enhanced greatly. Direct observation of the morphological changes of A549 cells revealed induction of cell death after treatment with propolis. Apoptotic cells were clearly observed with the AO/EB staining (Figure 3a). Control cells were seen to be uniform green with normal nuclear morphology and cells treated with propolis at 48 h showed an increase of non-viable apoptotic cells with a green fluorescent coloration relative to the cells treated with propolis at 24 h and negative control. DAPI staining also exhibited significant changes (Figure 3b). Cells incubated with propolis at 48 h showed nuclear fragmentation, chromatin condensation and apoptotic bodies compared with the control group and the cells treated during 24 h.

**JC-1 staining to measure mitochondrial membrane potential**

The effect of propolis on mitochondrial membrane potential (ΔΨm) was evaluated by JC-1 mitochondrial membrane potential assay. Fluorescent microscopic observation revealed that incubation of A564 cells with 1/4 IC50 of propolis during 24 and 48 h markedly increased the green fluorescence, similar to the positive control (valinomycin), indicating a significant disruption of mitochondrial potential and onset of cell death (Figure 4).

**Alteration in gene expression profile by propolis**

In order to identify the pathways responsible in induced apoptosis by propolis, we have analyzed the altered gene expression profile in A549 cells treated with this extract (1/4 IC50 of propolis for 24 and 48 h). The genes p53, Caspase-3, Bax, Bcl-2, Bcl-Xl, Noxa, Puma and p21 were detected by RT-PCR. As shown in Figure 5a and 5b, propolis treatment of A549 cells resulted in downregulation of the expression of Bcl-Xl and upregulation of the expression of Bax, Noxa and p21 in a time-dependent manner. Propolis did not affect the level of p53, Caspase-3, Bcl-2, Puma and β-actin when it was employed as housekeeping gene.
Discussion

Globally, lung cancer is the second most common cancer in both men and women and is by far the leading cause of cancer death. Propolis has long been used in the treatment of different diseases including cancer and has the advantages of being inexpensive and possessing low toxicity. This study demonstrated that Brazilian propolis decreased cell viability by exerting a cytotoxic effect on cells of human lung adenocarcinoma (A549 cells) associated with the induction of apoptosis.

The study of antiproliferative and cytotoxic activities of Brazilian green propolis against A549 cells showed that it could inhibit the growth of such cells in a dose-dependent manner, showing a selectivity toward tumour cells compared with normal Vero cells. To further confirm the cytotoxic effect of propolis on A549 cells, LDH leakage assay was also performed as another indicator of A549 cytotoxicity. LDH leakage of A549 cells was slightly increased with the presence of propolis. Orsolic et al. showed that several water-soluble compounds of propolis could be extremely useful for controlling tumour growth in experimental models and could inhibit cell growth, depending on the geographical source and composition of propolis. The main constituents of the propolis used in this study were phenolic compounds (flavonoids, aromatic acids) including artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) and triterpenes. Artepillin C has demonstrated a cytotoxic activity against other tumour cells and one may speculate that artepillin C may be one of propolis constituents related to its antiproliferative and cytotoxic against A549 cells.

The inhibitory effect of propolis on A549 proliferation is of great value for exploring the related signalling pathways. Using AO/EB and DAPI fluorescence staining, our results
indicated the appearance of conventional morphological signs of apoptosis. The number of apoptotic cells increased after incubation for 48 h at a propolis concentration of $\frac{1}{4}$ IC$_{50}$. These results indicated that the decrease in A549 cell viability by propolis was due to the induction of apoptosis. Several authors have reported that apoptosis can be the primary mechanism for the antitumour activity described of propolis.\[22,30\] Considering that artepillin C is known to inhibit the growth of different tumour cell lines via the induction of apoptosis,\[25,29\] our observations may be attributable to the presence of this chemical constituent.

During apoptosis, the mitochondrial membrane potential is disrupted by the formation of permeability transition pores due to the effect of different stimuli, which produce intracellular signals that cause the loss of $\Delta \Psi_m$. JC-1 staining indicated that the structure of the mitochondria had changed, suggesting that disruption of the mitochondrial membrane is indeed an important mechanism in the induction of apoptosis by this propolis sample.

Sawicka \textit{et al.} reported that the mechanism of apoptosis induced by different types of propolis depends on the chemical composition and the concentration of the propolis extract.\[31\] Additionally, several studies have demonstrated that propolis and its isolated compounds can induce the intrinsic or extrinsic pathways of apoptosis.\[28–31\] The intrinsic apoptotic pathway is mediated by mitochondria and pro-apoptotic proteins,\[32,33\] and our results clearly indicate for the first time that Brazilian propolis induces apoptosis through the mitochondrial-mediated pathway in A549 cells.

Mitochondrial membrane potential, and subsequently apoptosis, is regulated by the Bcl-2 protein family. The family is divided into three classes based on their Bcl-2 homology (BH) domains: the multidomain antiapoptotic proteins (Bcl-2, Bcl-X$_L$, Mcl-1, Bcl-w and A1), multidomain...
pro-apoptotic proteins (Bak and Bax) and BH3-only pro-apoptotic proteins (Bid, Bad, Bim, Puma and Noxa).

The expression of genes, which encode many of these proteins, is regulated by p53, a tumour suppressor gene, which, together with p21 (an inhibitor of cyclin-dependent kinases), induces cell cycle arrest and apoptosis.

In this study, the treatment of A549 cells with our extract resulted in the upregulation of Bax, Noxa and p21 levels and the downregulation of bcl-xL. Expression levels of p53, caspase-3, bcl-2 and puma were not affected. The Bcl-2 protein family is an important endogenous regulator of cellular activity that depends on the protein encoded by Bax and its translocation to the mitochondrial membrane.

Propolis can induce apoptosis by interfering with the Bcl-2/Bax-mediated regulation of mitochondrial membrane potential. In our case, Brazilian propolis upregulated the expression levels of Bax and did not alter the levels of Bcl-2 in A564. The overexpression of Bcl-2 is generally associated with many cancers as well as resistance to chemotherapy. However, it is reportedly expressed at low levels in alveolar epithelial cells, unlike Bcl-XL, which shows a high basal expression in these cells. This observation could explain the unchanged expression of Bcl-2 in the A549 cell line and suggests that altering Bcl-2 expression alone is insufficient to affect a cell’s susceptibility to apoptosis.

In contrast, the expression of the anti-apoptotic gene Bcl-XL was also downregulated by propolis. The protein encoded by this gene is found in the outer mitochondrial membrane and plays a role in maintaining membrane integrity. Anti-apoptotic effect of Bcl-XL protein could be suppressed by forming a heterodimer with Bax, which activates Bcl-XL and compromises the integrity of the mitochondrial membrane.

Furthermore, it has been also shown that there is an association between Noxa and Bcl-XL protein, suggesting that the upregulation of Noxa could affect the anti-apoptotic effect of Bcl-XL. Our results, in which Bcl-XL expression was decreased but Noxa expression was increased (thereby increasing the relative expression of Noxa to Bcl-XL), could be consistent with these findings. As such, we conclude that apoptosis induced by Brazilian green propolis is associated with increased Bax and Noxa expression.

Hassan et al. reported that expression of Noxa not only triggered apoptosis via the classical mitochondrial pathway, but also resulted in the activation of apoptosis signal-regulating kinase 1 and its downstream effectors c-Jun N-terminal kinase and p38, leading to cell death via a mechanism independent of caspase.

Interestingly, we observed that the expression of gene Caspase-3 not was changed; however, the ΔΨm was disrupted by our sample in A549 cells. Several studies revealed that mitochondria contain different apoptogenic factors, including apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which have crucial roles in caspase-independent apoptosis. In fact, it has been reported that Bax-induced cell death may not require caspase activity. Our results suggest that Brazilian propolis or some of its bioactive constituents could induce Caspase-independent apoptosis by two different routes.

Our results also demonstrated that p53 deficiency does not alter apoptosis rates in A549 cells treated with Brazilian green propolis. It has previously been demonstrated that this gene plays a major role in p53-mediated apoptosis, which explains why Puma expression similarly remained unchanged by Brazilian green propolis.

Interestingly, elevated expression of p21 was observed in cells treated with green propolis. Expression of this gene can be regulated by several pathways, inclusive by p53. The gene p21 is a critical inhibitor of the G1 kinase of the cell cycle pathway. The mechanism by which this occurs is unclear, but several studies indicate that the modulation of pro-apoptotic or anti-apoptotic genes is responsible for p21-induced apoptosis. Thus, p21 overexpression induced by Brazilian green propolis or some of its constituents could trigger two distinct pathways: cell cycle arrest or mitochondrial apoptosis in a manner independent of p53.

Conclusions

Brazilian propolis suppressed the proliferation of A459 cells by inducing apoptosis via the intrinsic pathway. The activation of this pathway was associated with decreased Bcl-XL expression and increased Bax and Noxa expression, resulting in a loss of mitochondrial membrane potential. Furthermore, cell death was found to be independent of Caspase-3, and the upregulation of p21 without p53 in response to treatment of A549 cells with propolis suggested a novel mechanism underlying propolis-induced apoptosis in lung cancer cells. We believe that Brazilian propolis (or its bioactive constituents) could be a promising candidate for the treatment of lung cancer.

Declaration

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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