The cytotoxic effects of propolis on breast cancer cells involve PI3K/Akt and ERK1/2 pathways, mitochondrial membrane potential, and reactive oxygen species generation

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Abstract

Propolis has been extensively used to improve health and prevent inflammatory diseases. Different types of Cuban propolis (red, brown and yellow) have been documented. The purpose of this research was to investigate the cytotoxic effects of Cuban red propolis (CP) on MDA MB-231 cell line, since breast cancer is considered one of the most common causes of mortality among women. Antiproliferative and cytotoxic activity of CP against MDA MB-231 cells were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) and lactate dehydrogenase (LDH) assays. Apoptosis/necrosis, involvement of PI3K/Akt and ERK1/2 pathways, mitochondrial membrane potential and expression of genes were investigated. CP extract exhibited antiproliferative and cytotoxic effects on MDA MB-231 cells, what may be probably related to PI3K/Akt and ERK1/2 pathways. A decreased expression of apoptosis-related genes (TP53, CASP3, BAX and P21) was seen, whereas the expressions of BCL-2, BCL-XL, NOXA and PUMA were unaffected. CP extract induced mitochondrial dysfunction and LDH release, what indicated cell necrosis associated with reactive oxygen species production and decreased cell migration. Our findings provide a basis for future investigation of chemopreventive and/or therapeutic studies against apoptosis-resistant breast cancer, in animals and humans.

Keywords Breast cancer · Propolis · Cytotoxicity · Necrosis

Introduction

Propolis is a sticky product made by bees (Apis mellifera) from resinous materials of plants and trees and has been used empirically in folk medicine as a traditional remedy (Silva-Carvalho et al. 2015). Propolis has been extensively used to improve health and prevent inflammatory diseases. There are various types of propolis depending on the zone where it was collected, such as Brazil, Cuba, the Canarian Islands, Europe, India, China, New Zealand and others (Sforcin 2016), and all of them contain several polyphenol compounds. Propolis samples from the tropics show unusual compositions including prenylated derivatives of p-coumaric acid and of acetophenone, terpenes, lignans, flavonoids and many others (Acikelli et al. 2013; Szliszka and Krol 2013; Bankova et al. 2014). The proportion of these compounds in propolis is related to its anti-inflammatory, immunomodulatory, antimicrobial, regenerative, antioxidant, hepatoprotective and antitumor activity (Orsolic et al. 2006; Wang et al. 2015).

In Cuba, propolis ethanolic extract is popular as a domestic medicine (Andreu et al. 2015). Chemical studies have classified different types of Cuban propolis (red, brown and yellow) with significant differences in their chemical composition (Cuesta-Rubio et al. 2007). Currently, Cuban red propolis (CP) has been the focus due to its pharmacological properties, such as antiparasite, antimicrobial, hepatoprotective and antitumor (Monzote et al. 2011; Frión-Herrera et al. 2013; Conti et al. 2015; Frión-Herrera et al. 2015). CP is derived from the resin of Dalbergia ecastaphyllum (Leguminosae), and isoflavonoids are its main chemical constituents (Cuesta-Rubio et al. 2007).
Propolis has been reported to interfere with the proliferation of several neoplastic cells (Patel 2016; Sforcin 2016). Breast cancer is considered one of the most common causes of mortality among women. Although different and more effective treatments have improved breast cancer survival rates, there is still a need for new research due to several factors, such as survival by stage of diagnosis, patients with de novo metastatic breast cancer, among others. Estrogen receptor (ER)-negative breast cancers do not respond to anti-estrogen therapies and it is the most aggressive type of breast cancer. Therapies for ER-negative breast cancer patients are usually correlated with a strong toxicity and side effects (Eckhardt et al. 2012). Therefore, the development of more effective and less toxic therapies are necessary to treat breast cancer.

In search of anticarcinogenic therapies from natural sources, we have attempted to determine the intracellular mechanisms involved in CP effects on MDA MB-231 cells in vitro, assessing apoptosis/necrosis, inhibition of PI3K/Akt and ERK1/2 pathways, mitochondrial membrane potential dysfunction and gene expression.

Materials and methods

Reagents

Dulbecco’s Modified Eagle Medium (DMEM), trypsin–EDTA, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco/BRL (Grand Island, NY, USA). Normal agarose and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, USA). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium-bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), acridine orange (AO), ethidium bromide (EB), N-acetyl-L-cysteine (NAC), dimethyl sulfoxide (DMSO), lactate dehydrogenase (LDH) activity assay kit, mitochondria staining kit (JC-1-5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′ tetraethylbenzimidazolcarbocyanine iodide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). One Step RT-PCR Kit was obtained from Qiagen (Hilden, Germany). Acetic acid was purchased from Quimipur (Madrid, Spain) and methanol from Merck (Darmstadt, Germany). PI3K/Akt inhibitor LY-294002 and ERK1/2 inhibitor PD-98059 were purchased from Selleckchem (Houston, TX, USA).

Cuban propolis (CP) was provided by “La Estación Experimental Apícola”, Havana, Cuba. The extract was obtained and characterized as red propolis by spectroscopic techniques as previously described (Cuesta-Rubio et al. 2007). CP final concentrations used in all assays (6.25, 12.5, 25, 50, and 100 μg/ml) were prepared immediately from the stock solution containing 100 mg (dry weight) of CP adding 1 ml of 70% ethanol. Propolis solvent (70% ethanol) was assayed in all experiments.

Breast cancer cells

The human breast cancer line MDA MB-231 (adenocarcinoma) derived from metastatic site pleural effusion (ATCC HTB-26™) was grown in DMEM (90% v/v) and FBS (10% v/v) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) according to the manufacturer’s recommendations. Culture cells were maintained at 37 °C under an atmosphere of 5% CO₂ for a maximum of 18 passages.

This work was approved by the Ethics Committee of the Medical School, UNESP (1.356.549).

Cell proliferation (MTT assay)

MTT assay was used to investigate the effect of CP extract on cell proliferation. Cells (2 × 10⁵/ml) were seeded into 96-well plates. After 24 h, different concentrations of CP dissolved in the culture medium were added and cells were incubated for 72 h. Untreated cells were used as a negative control. MTT (10 μl) stock solution (5 mg/ml) was added to each well and incubated for 3 h at 37 °C. MTT-formazan product in viable cells was dissolved by adding 150 μl of DMSO. Plates were incubated for 30 min at 37 °C and the absorbance (A) was measured at 570 nm using an Elisa reader (MRX Revelation Dynex Technologies, Denkendorf, Germany). Absorbance from untreated cells was considered as 100% and cell viability was calculated using the formula: % cell viability = [A₅₇₀ treated cells/A₅₇₀ control cells] × 100.

Lactate dehydrogenase release assay

To assess CP cytotoxic effects, the release of the cytosolic enzyme LDH to the culture medium was determined as a marker of cell membrane integrity. Briefly, cells (2 × 10⁵ cells/ml) were seeded in a 96-well plate and incubated overnight. The medium was replaced by a serum-free medium containing different concentrations of CP and incubated for 72 h. LDH activity was analyzed in the supernatants using the in vitro toxicity assay kit according to manufacturer’s instructions and reading in a spectrophotometer multiplate reader at 490 nm.

Effects of CP extract on PI3K-Akt and ERK1/2 signal transduction pathway

In breast cancer cells, the activation of PI3K/Akt and ERK1/2 signal transduction pathways play an essential role
in the regulation of multiple cellular processes to promote cell growth, survival, and metastasis. The mechanisms underlying the inhibitory effects of CP extract on MDA MB-231 cells were investigated using cellular-signaling pathway-specific inhibitors and MTT assay after 72 h. MDA MB-231 cells were pre-incubated in a 96-well plate (2 × 10^5 cells/ml) for 1 h with inhibitors of ERK1/2 and PI3 K/Akt pathway (PD-98059—80 µM and LY-294002—80 µM, respectively) prior to CP (IC_{50}) extract exposure. Cell viability was calculated as the percentage of viable cells in each treatment (CP, CP/PD-98059, CP/LY-294002, PD-98059 and LY-294002) vs untreated control.

**Measurement of mitochondrial membrane potential (ΔΨ_m) and reactive oxygen species (ROS) production**

Changes in the mitochondrial structure were detected using a mitochondria staining kit (JC-1). MDA MB-231 cells (2 × 10^5 cells/ml) were cultured in a 24-well plate and treated with ¼ IC_{50} of CP extract for 24 and 48 h in triplicate. Untreated cells were used as a negative control. Cells were stained with JC-1 according to the manufacturer’s protocol and analyzed using a Olympus IX-71 (Melville, NY, USA) inverted fluorescent microscope.

To examine a possible influence of ROS on the survival of treated cells, MDA MB-231 cells were co-treated with 5 µM of N-acetyl-l-cysteine (NAC) simultaneously with CP extract (IC_{50}) for 72 h. The survival percentage of MDA MB-231 cells was assessed by MTT assay in comparison to NAC-treated cells, considered as 100%.

**Fluorescence microscopic analysis of cell death**

Nuclear morphological changes were determined using a differential acridine orange/ethidium bromide (AO/EB) staining, DAPI staining and the fluorescence microscopy Olympus IX-71. MDA MB 231 cells (2 × 10^5 cell/ml) were grown in 24-well culture plates and treated with ¼ IC_{50} of CP extract. After 24 and 48 h, cells were incubated with AO/EB solution in a final concentration of 100 µg/ml. Both viable and dead cells were stained with AO. AO emits a green fluorescence by binding into a RNA or a single stranded DNA; on the other hand, cells with ruptured membranes (necrotic cells) stain with AO and their DNA emits a red fluorescence. To evaluate morphological characteristics of chromatin condensation and fragmented nuclei by DAPI staining, cells were fixed with 500 µl of fixing solution (acetic acid/methanol—1:2 v/v) and stained with DAPI (1 µg/ml). Untreated cells were used as a negative control. A total of 200 cells were analyzed, photographed and counted in triplicate in three independent experiments.

**Total RNA isolation and real-time polymerase chain reaction (RT-PCR)**

The expression levels of genes were evaluated by RT-PCR: TP53 (regulates the cell cycle and acts as a tumor suppressor), anti-apoptotic (BCL2, BCL-XL) and pro-apoptotic genes (CASp3, BAX, NOXA and PUMA) and P21 (promotes cell cycle arrest).

Total RNA was isolated from cultured MDA MB-231 cells using Trizol according to manufacturer’s protocol. RNA concentration was determined using a Biophotometer plus (Eppendorf, Hauppauge, NY, USA). RT-PCRs for the genes TP53, CASP3, BAX, BCL2, BCL-XL, NOXA, PUMA and P21 were run on the thermocycler (AUXILAB, Navarra, Spain) according to One step RT-PCR kit and using specific primers (Table 1). The thermal cycling condition for all genes were: initial denaturation at 95 °C for 1.5 min followed by 35 cycles at 94 °C for 1.5 min, 59 °C for 1 min and 72 °C for 1 min.

Amplified PCR products were subjected to electrophoresis at 70 V in 1.5% (w/v) agarose gel for 90 min. A 50-base pairs (bp) DNA ladder marker was used as a molecular marker. Gels were visualized with EB in 1 x TBE (Tris-borate-EDTA) buffer. Gels were analyzed and the intensity of each band was measured by the Gel Doc imaging system UVIsave D-55/20 M version 15.08 (UVItel, Cambridge, England).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>β-ACTIN</td>
<td>CTCCTCCGAGCAGGAGTCTCTG</td>
<td>GGAGCATTGATCTGATCTCT</td>
</tr>
<tr>
<td>TP53</td>
<td>GGGTTAGTATACACACACCAATT</td>
<td>GCCCTGTAGTTAGAAATTCAT</td>
</tr>
<tr>
<td>CASP3</td>
<td>AGAACCTAGCATCTGTTG</td>
<td>ATCCAGGGGCTATTAGCAC</td>
</tr>
<tr>
<td>BCL2</td>
<td>ATGGCGCTGAACTGTG</td>
<td>GGGATTGTCAGAGGAGTAGAT</td>
</tr>
<tr>
<td>BAX</td>
<td>ATGGGGCTGGCAATTG</td>
<td>CCATAAACGTCCTGAGGGCA</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>ACCCTTCTGACCTTCTG</td>
<td>CCATAAACGCTCTGGGGCA</td>
</tr>
<tr>
<td>NOXA</td>
<td>TTCAATGTGTCTGGTGGGC</td>
<td>GTGACAAAGGAGCATTCTC</td>
</tr>
<tr>
<td>PUMA</td>
<td>GTCCCTACCCCTGTCCTC</td>
<td>CTGTCGCTCTCTGTCCTC</td>
</tr>
<tr>
<td>P21</td>
<td>CAGGGGACAGGAGGAGGAAGA</td>
<td>GGCAGAAGATGAGAGCGG</td>
</tr>
</tbody>
</table>

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**Cell migration assay**

MDA MB-231 cells (2 × 10^5 cells/ml) were grown on 24 well plates to form confluent monolayer. Gaps were created in confluent cell layers using micropipette tips, followed by a rinse with PBS. Cells were treated with ¼ IC₅₀ of CP extract and cells growing in complete media were considered as control. Wound closure was monitored by phase-contrast microscopy and photographed at 0, 24 and 48 h. The wound-healed distance was measured from each picture and the migrated cells were counted from five random fields using the Image J software (NIH, USA). Each experiment was repeated at least three times independently and the percentage of migration was calculated by the formula:

\[
\% \text{ Migration} = \frac{\text{Width of the wound at 0 h} - \text{Width of the wound at (24 h or 48 h)}}{\text{Width of the wound at 0 h}} \times 100.
\]

**Statistical analysis**

The experiments were repeated at least three times. The values are reported as mean ± standard deviation (SD). The IC₅₀ values were determined by interpolation of the graph of propolis concentration vs cell viability. Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Results were analyzed using Kruskal–Wallis test for multiple comparisons and Dunn’s post hoc test (\(P < 0.05\)).

**Results**

**CP extract-induced morphological changes and decreased MDA MB-231 cells viability in a concentration-dependent manner**

The morphological changes in MDA MB-231 cells after treatment for 72 h with CP extract are represented in Fig. 1a. Untreated cells showed an elongated shape while treated cells showed cellular shrinkage, oval or irregular shape and condensed cytoplasm. The highest concentrations of CP extract (50 and 100 µg/ml) led to detached and round-shaped dead cells.

Cell membrane integrity and cytotoxic effects caused by CP extract on MDA MB-231 cells were detected by LDH release. The release of LDH increased in a dose-dependent manner by MDA MB-231 cells, and the percentage of cytotoxic cells reached 71.0% using 100 µg/ml of CP extract (Fig. 1b). These data are in agreement with the cell viability assay, which suggested a specific cytotoxic effect of CP extract against MDA MB-231 cells.

**CP extract decreased mitochondrial membrane potential (ΔΨₘ)**

The cationic fluorescent indicator JC-1 was used to evaluate changes in mitochondrial transmembrane potential. In intact mitochondria, J-aggregates fluoresce with emission at 583 nm (red), and in the cytoplasm J-monomers fluoresce with emission at 525 nm (green) at 488 nm. Treatment of MDA MB-231 cells with CP (¼ IC₅₀) for 24 and 48 h revealed a notable increased green fluorescence, indicating disruption of mitochondrial potential and onset of cell death (Fig. 3).

**A ROS scavenger (NAC) decreased CP cytotoxic effects on MDA MB-231 cells**

The co-treatment of NAC with CP extract increased cell viability from 48.3 to 69.8%, as shown in Fig. 4. These results suggested that CP cytotoxic effects on MDA MB-231 cells might be associated with the intracellular ROS production.

**CP extract induced necrosis in MDA MB-231 cells**

Identification of apoptosis and necrosis was analyzed by AO/EB and DAPI staining. AO/EB staining distinguishes viable, apoptotic and necrotic cells, whereas DAPI forms a blue fluorescent complex with DNA, showing a specific fluorescence for A-T-rich regions of DNA broken chains. The percentage of viable, apoptotic and necrotic cells were calculated with AO/EB staining (Fig. 5a). The number of viable cells reduced by increasing the exposure time to CP extract (¼ IC₅₀). CP-treated cells for 24 h exhibited a similar percentage of viable, apoptotic and necrotic cells, indicating that CP extract induced both apoptosis and necrosis.
The cytotoxic effects of propolis on breast cancer cells involve PI3K/Akt and ERK1/2 pathways,…

However, after 48 h of treatment with CP extract, the number of orange-colored nuclei increased compared to control and apoptotic cells, showing a severe membrane disruption and a higher prevalence of necrotic cells (Fig. 5b). Additionally, morphological changes of chromatin condensation and fragmented nuclei evaluated by DAPI staining were undetectable (Fig. 5c). Data revealed that CP caused necrosis in MDA MB-231 cells in a time-dependent manner.

Fig. 1 Viability and cytotoxicity of MDA MB-231 cells after incubation with various concentrations of Cuban propolis (CP 6.25–100 μg/ml) for 72 h. a A decreased viability and morphological changes according to the concentration (magnification ×20). b The percentage (%) of cell viability was measured by MTT assay and the cytotoxicity by lactate dehydrogenase (LDH) release. The results are expressed as mean ± SD of three independent experiments in triplicate.

CP extract inhibited the expression of apoptosis-related genes

Figure 6 shows that the expression of apoptosis-related genes TP53, CASP3, BAX and P21 decreased in CP-treated MDA MB-231 cells at 24 and 48 h. Moreover, the expression of BCL2, BCL-XL, NOXA and PUMA in propolis-treated cells was unchanged compared to untreated cells, indicating that CP extract induced non-apoptotic cell death.
Breast cancer is associated with a higher dissemination rate; therefore, we also performed wound-healing experiments to determine the effects of CP on MDA MB-231 cells migration. Cells were treated with 1/4 IC_{50} of CP for 24 and 48 h and the percentage of cell migration in CP-treated cells was 33.2 ± 11.0% at 24 h and 51.4 ± 9.1% at 48 h vs 45.7 ± 4.5% at 24 h and 67.7 ± 15.0% at 48 h in the control group. Wound-healing assay revealed that CP decreased cell migration in comparison to control (Fig. 7).

**Discussion**

In this work, we demonstrated the sensitivity of MDA MB-231 cells to Cuban propolis. MTT assay showed the inhibition of cell proliferation in a concentration-dependent manner. LDH release by treated cells suggested that CP-induced growth inhibition occurred due to its influence on membrane integrity. It has been reported that propolis cytotoxic effects on animal and human tumor cells depend on its components (Watanabe et al. 2011; Sawicka et al. 2012; Patel 2016). The chemical composition of our propolis sample was characterized by spectroscopic techniques (Cuesta-Rubio et al. 2007) and the main components found in our propolis, such as vestitol, neovestitol, liquiritigenin, isoliquiritigenin, isosativan, and medicarpin may be related to the cytotoxic potential of CP extract.
The cytotoxic effects of propolis on breast cancer cells involve PI3K/Akt and ERK1/2 pathways...

The activation of PI3K/Akt and ERK1/2 signal transduction pathways was evaluated using the inhibitors LY-294002 and PD-98059. CP may kill MDA-MB-231 cells through these pathways because CP and LY or PD worked additively.

Several researches have shown a close relationship between PI3K/Akt, ERK1/2 pathways and apoptosis (Osaki et al. 2004; Kolch 2005; Franke 2008; Mebratu and Tesfaigzi 2009). PI3 K/Akt signaling pathway can promote the phosphorylation or inactivation of Bcl-2 family proteins and block the cytochrome C release from mitochondria and caspase function, which play an essential role in apoptosis of tumor cells (Datta et al. 1999). On the other hand, several molecules can be targeted by ERK1/2 to promote proliferation and inhibit apoptosis. They include Bcl-2 family proteins, caspase-3, cyclin D1, and death associated to protein kinase (DAPK) (Chen et al. 2005). This data is in agreement with the results of AO/EB and DAPI staining and indicate that CP extract induces cell death by necrosis. In addition, CP extract decreased the expression of apoptosis-related genes (TP53, CASP3, and BAX). No alterations were seen in the expression levels of anti-apoptotic (BCL2, BCL-XL) and pro-apoptotic (PUMA, NOXA) genes. The downregulation of CASP3, BAX, P21 and unchanged regulation of PUMA and NOXA indicate the involvement of a transcription-p53-dependent apoptosis (Findley et al. 1997; Hong et al. 2014). It is well known that Bcl-2 family members can influence apoptosis, and alterations in the levels of anti- and
pro-apoptotic genes can regulate the type of cell death program (Ouyang et al. 2012; Su et al. 2015). Furthermore, the downregulation of p21 indicated that other mechanism(s) may be triggered by CP extract to replace p21-dependent checkpoint and to induce inhibition of cell proliferation. These findings suggest that a death pathway triggered by CP in MDA MB-231 cells is clearly related to necrosis. CP-induced ROS generation was reduced after cotreatment with the antioxidant NAC, which increased the percentage of viable cells, suggesting that CP-induced necrotic-related cell death could be associated with ROS production.

Necrosis death is associated with mitochondrial dysfunction and our propolis sample reduced the ΔΨm and increased LDH levels. During cellular stress, mitochondria exert its pro-death function (Ott et al. 2007). The consequence of mitochondrial dysfunction includes a rapid impairment of ΔΨm, rupture of mitochondrial membrane, loss of adenosine triphosphate (ATP), excessive ROS production, intracellular [Ca^{2+}] elevation, osmotic shock, loss of ion homeostasis and cell integrity, LDH release, what finally results in necrosis. Our findings are in accordance with other reports demonstrating that ROS production, LDH release, and ΔΨm impairment in cancer cells are signals related to mitochondrial-regulated necrosis (Lu et al. 2014; Shahsavari et al. 2015).

In contrast, it has been reported that propolis may display inhibitory effects towards a variety of tumor cells through apoptosis (Frión-Herrera et al. 2015). This data, which seems initially contradictory, may be explained by differences in the chemical composition of propolis samples obtained in different geographic zones from different botanical sources and/or by the behavior inherent to each cellular model.

Our data indicated that CP extract not only induced necrosis but also inhibited cell migration. MDA MB-231 cancer cells are widely used as a model to study breast cancer cell metastasis (Jiang et al. 2016; Zhang et al. 2016). Here, we found that CP extract exerted a remarkable effect on the aggressive behavior of cancer cells that was evident by the decreased potential of migration. Propolis composition is heterogeneous; however, this natural product and its main constituents have shown efficiency in the prevention of metastasis (Orsolic et al. 2006). Different components from various propolis samples with antimetastatic activity have been identified such as isoflavonoids, caffeic acid phenethyl ester, artepillin C, nemorosone, galangin, cardanol (Peng et al. 2012; Chien et al. 2015; Guan et al. 2016; Patel 2016). These constituents target various genetic and biochemical pathways involved in cancer progression, and the chemical composition of our propolis sample demonstrated the ability of inhibiting breast cancer cells metastasis.

CP may serve as an alternative therapy due to necrosis induction and could offer an effective approach to eliminate apoptosis-resistant cancer cells. Further studies are still needed to evaluate the CP-induced necrotic pathways and the compounds involved in the antitumor and antimetastatic activity of our propolis sample.

In conclusion, this study has indicated that CP suppressed the growth and migration of MDA-MB 231 breast cancer cells, and necrosis was the main mechanism induced by CP extract in such cells. Our findings provide a basis for future investigation of chemopreventive and/or therapeutic studies, in animals and humans.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. This work was approved by the Ethics Committee, UNESP, Campus of Botucatu (CEP 1.356.549-2015).
The cytotoxic effects of propolis on breast cancer cells involve PI3K/Akt and ERK1/2 pathways,

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