



## Antiproliferative effects of Tubi-bee propolis in glioblastoma cell lines

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### Abstract

Propolis is a resin formed by a complex chemical composition of substances that bees collect from plants. Since ancient times, propolis has been used in folk medicine, due to its biological properties, that include antimicrobial, anti-inflammatory, antitumoral and immunomodulatory activities. Glioblastoma is the most common human brain tumor. Despite the improvements in GBM standard treatment, patients' prognosis is still very poor. The aim of this work was to evaluate *in vitro* the Tubi-bee propolis effects on human glioblastoma (U251 and U343) and fibroblast (MRC-5) cell lines. Proliferation, clonogenic capacity and apoptosis were analyzed after treatment with 1 mg/mL and 2 mg/mL propolis concentrations for different time periods. Additionally, glioblastoma cell lines were submitted to treatment with propolis combined with temozolomide (TMZ). Data showed an antiproliferative effect of tubi-bee propolis against glioblastoma and fibroblast cell lines. Combination of propolis with TMZ had a synergic antiproliferative effect. Moreover, propolis caused decrease in colony formation in glioblastoma cell lines. Propolis treatment had no effects on apoptosis, demonstrating a cytostatic action. Further investigations are needed to elucidate the molecular mechanism of the antitumor effect of propolis, and the study of its individual components may reveal specific molecules with antiproliferative capacity.

*Key words:* glioblastoma, propolis, temozolomide, U251, U343.

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Propolis is a resinous product composed of various botanical exudates, collected from plants and used by bees as a protective hive barrier against different pathogens. Its chemical composition includes flavonoids, aromatic acids, esters, aldehydes, ketones, fatty acids, terpenes, steroids, amino acids, polysaccharides, hydrocarbons, alcohols, hydroxybenzene, and several other compounds in trace amounts (Bankova *et al.*, 1983; Marcucci, 1995). Its composition varies according to the resin sources in the hive specific region. Propolis has been widely used in folk medicine, with therapeutic or preventive effects against inflammation, heart disease, diabetes mellitus, microbial hepatotoxicity and cancer (Bankova *et al.*, 1998; Burdock, 1998).

Glioblastoma is the most frequent and aggressive primary brain tumor in adults. Despite standard treatments, consisting of surgery and postoperative radiotherapy, patient survival remains poor, mainly attributed to tumor-

inherent radio- and chemoresistance (Esteller *et al.*, 2000; Scrideli *et al.*, 2008). In recent years, the combination of the alkylating agent temozolomide (TMZ) with standard daily fractionated irradiation therapy followed by adjuvant TMZ have shown to improve prognosis, therefore becoming a part of the standard therapy for patients with newly diagnosed glioblastoma (van Niftrik *et al.* 2007; Brandes *et al.*, 2010). This chemotherapeutic pro-drug is transformed under physiological conditions into its active unstable methylating metabolite, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC). Methylation of the DNA by MTIC results in O<sup>6</sup>-methylguanine adducts, which are considered to be responsible for the cytotoxic effect of TMZ (Esteller *et al.*, 2000; Brandes *et al.*, 2010). O<sup>6</sup>-methylguanine adducts can result in futile attempts of the mismatch repair system, leading to DNA double-strand breakage and eventually cell death (van Niftrik *et al.*, 2007)

Several reports have shown the antiproliferative effects of propolis from different origins and their fractions in several cancer cell lines (Grunberger *et al.*, 1988; Khalil, 2006). In the present study, we describe the anti-cancer effects of ethanolic propolis extract produced by *Scaptotrigona sp* (Tubi-bee propolis) in glioblastoma cell

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lines, associated or not with TMZ, and in one non-neoplastic fibroblast cell line.

Propolis samples obtained from the stingless “Tubi” beehives (*Scaptotrigona sp*) were collected in the Serra do Corda region (Maranhão State, Brazil). Tubi-bee propolis extracts were obtained as previously described (Farnesi *et al.*, 2009). Briefly, propolis was ground and an ethanol extract was prepared, as follows: 30 g of propolis/100 mL ethanol (70%). The solution was kept at room temperature for 20 days and shaken once a day. After filtration, the solvent was totally evaporated in a water bath, at temperatures not exceeding 50 °C. For cell assays the crude extract was diluted in dimethylsulphoxide (DMSO, Sigma-Aldrich).

TMZ, known commercially as Temodal®, was acquired from Schering-Plough Brazil and diluted according to the manufacturer's instructions. The capsule content was diluted in water at a ratio of 22 mg drug/100 mL water. This solution was placed in a shaker for 30 min at 37 °C and then filtered through a Millipore® filter (0.5 µm). By this procedure, 85% of dissolved active principle was obtained. Aliquots of the drug were stored at -20 °C. TMZ concentrations of 20, 50 and 100 µM were used in the experiments.

Human adult glioblastoma cell lines U251 and U343 and the human fibroblast cell line MRC5 were purchased from the American Type Culture Collection. Cells were cultured in HAM F10 medium (Gibco BRL, Life Technologies®, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL), at 37 °C in a humidified 5% CO<sub>2</sub> incubator.

The effects of propolis on clonogenic capacity were evaluated by a clonogenic assay (Franken *et al.*, 2006). After trypsinization, single cell suspensions of 300 cells were seeded into 6-well plates and treated with propolis extract at the concentrations of 1 and 2 mg/mL for 48 h. After this treatment, the culture medium was removed and replaced with extract-free medium. The cell cultures were then incubated for 7-10 days and thereafter the colonies were rinsed with PBS, fixed with methanol and stained with Giemsa. All colonies with > 50 cells were counted. Assays were performed in triplicate.

For the proliferation assay, cells were seeded on 96-well plates (1x10<sup>3</sup> cells/well). After 24 h, the medium was replaced with fresh media containing the treatment (propolis, TMZ or DMSO at 0.5%) and then cultured for 24, 48 and 72 h. After the treatment, the culture medium was removed and replaced with medium containing 10 µL of XTT dye (3 mg/mL) (XTT II; Roche Molecular Biochemicals, Indianapolis, USA) in each well. The plates were incubated for 2 h at 37 °C, and the formazan product was measured at 450 nm in an iMark microplate reader (Bio-Rad Laboratories). All experiments were performed in triplicate. Values are shown as mean ± SD.

For apoptosis assessment, a total of 3x10<sup>5</sup> cells were seeded in 25 cm<sup>2</sup> tissue culture flasks containing 5 mL of culture medium. After 24 h, the medium was replaced,

propolis and DMSO were added, and then the cells were cultured for additional 48 h. Apoptotic cells were recognized by nuclear condensation and fragmentation, according to Lee and Shacter (1999). Treated cells were centrifuged and incubated for 5 min at 37 °C with bisbenzimidazole (Hoechst 33342), propidium iodide and fluorescein diacetate (Sigma Chemical Co., St. Louis, USA). Then, samples were mounted on slides, coverslipped and analyzed by fluorescence microscopy with a triple filter. Cells were scored and categorized according to differential staining: (1) normal: blue nucleus and green cytoplasm, (2) apoptotic: fragmented blue nucleus and green cytoplasm, and (3) necrotic: spherical red nucleus. 500 nuclei were analyzed per treatment.

One-way or two-way ANOVA followed by the appropriate *post-hoc* test (Bonferroni) were used to check for significant differences between groups (differences between doses or times). Differences were considered significant at  $p < 0.05$ .

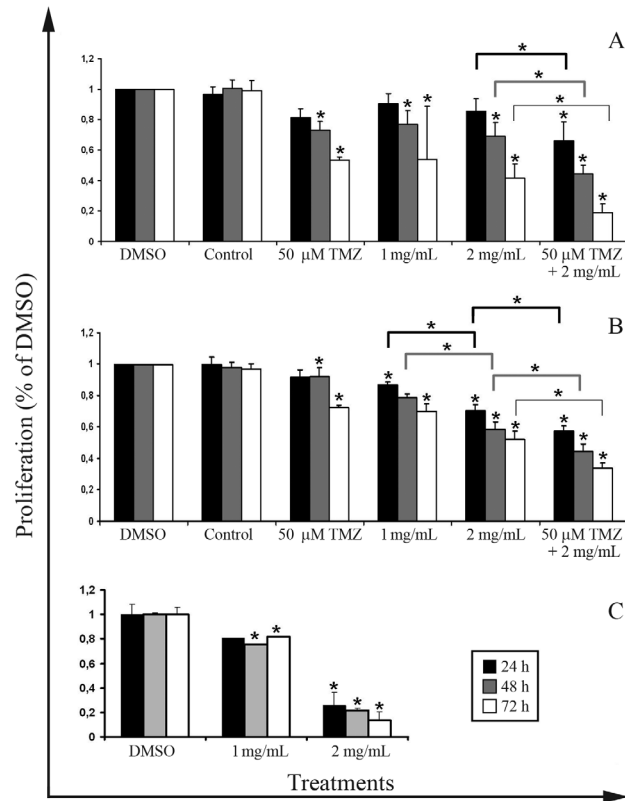
To determine the TMZ concentration for combination with propolis, U343 and U251 cells were submitted to different concentrations of TMZ for 24, 48 and 72 h. The TMZ concentration chosen for combined treatment was 50 µM, which reduced proliferation at 48 h for U251 and U343 cell lines (Figure 2).

U251, U343 and MCR-5 cell lines were treated with propolis extract at the concentrations of 1 and 2 mg/mL for 24, 48 and 72 h. The glioblastoma cell lines were also treated with a propolis concentration of 2 mg/mL associated with 50 µM of temozolomide. Cell viability was determined by an XTT assay, as described above.

Propolis extract concentrations inhibited growth of the three cell lines when compared with DMSO (0.5%) ( $p < 0.05$ ) (Figure 1). For cell line U251, proliferation inhibition was observed at 24, 48 and 72 h corresponding to a 10, 24 and 46% decrease with the 1 mg/mL dose, and a 15, 32 and 59% decrease with the 2 mg/mL dose, respectively. However, there was no statistically significant difference between the concentrations for this cell line at the times studied (Figure 1A).

In cell line U343, a decrease in proliferation was observed at 24, 48 and 72 h for both dosages, corresponding to a 14, 21 and 30% decrease for the 1 mg/mL, and a 30, 42 and 48% decrease for the 2 mg/mL dose. For this cell line, all treatments except the 72 h treatment showed statistically significant differences between the concentrations (Figure 1B).

Statistically significant effects of dose and time dependence were observed only for the U343 cell line ( $p < 0.05$ ). The association of propolis and TMZ showed a synergistic effect on both glioblastoma cell lines at all times analyzed, except for the U343 cell line at 72 h (Figure 1A and B). In the fibroblast MRC-5 cell line a decrease in proliferation was also observed, although this effect was neither dose- nor time-dependent (Figure 1C).



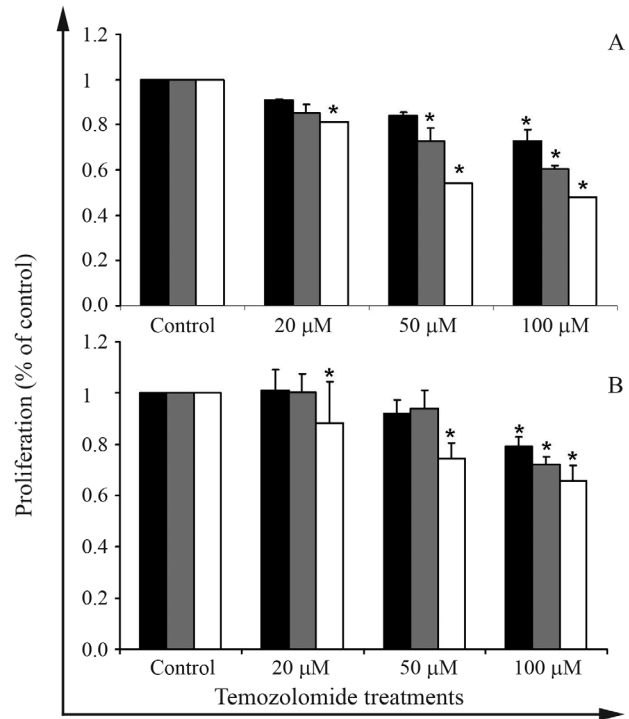
**Figure 1** - Proliferation assay of cell lines treated with Tubi-bee propolis at the concentrations of 1 and 2 mg/mL for 24, 48 and 72 h. Glioblastoma cells were also treated with temozolomide (TMZ) combined with propolis. Asterisks indicate a statistically significant difference between groups. A. U251, B. U343, C. MRC5.

In both the U251 and U343 cell lines treated with 1 and 2 mg/mL propolis extract for 48 h, a decrease in colony formation capacity was observed; however, there was no difference between the two treatments (data not shown).

To determine the occurrence of apoptosis in GBM cells treated with the propolis extract, the cells were differentially stained. Apoptosis was not observed after the treatment with propolis at neither of the concentrations tested. The methodology applied also allowed the detection of necrotic cells, observed at a low number and without differences between treatments (data not shown).

Propolis, a complex mixture of plant metabolites, shows a broad spectrum of biological activities including antibiotic, antioxidative, anti-inflammatory and anticancer effects (Bankova *et al.*, 1983; Marcucci, 1995; Banskota *et al.*, 2001). Its cytotoxicity in cultures of human and animal tumor cells, including breast carcinoma, melanoma, colon and renal carcinoma cell lines, has been frequently reported in the literature (Khalil, 2006).

The present study showed that propolis extract inhibited proliferation in glioblastoma and fibroblast cell lines, as already demonstrated by previous studies. Propolis extract from the Netherlands showed an interesting antiproliferative activity against highly metastatic liver murine



**Figure 2** - Proliferation assay of cell lines treated with TMZ at the concentrations of 20, 50 and 100 µM, for 24, 48 and 72 h. Asterisks indicates a statistically significant difference between the TMZ-treated and the control group.

colon 26-L5 carcinoma cells (Banskota *et al.*, 2000). A butanolic Greek propolis extract was also found to be cytotoxic in two malignant human cell lines (HT-1080 fibrosarcoma and HT-29 colon adenocarcinoma), whereas it was not equally toxic when tested in normal human skin fibroblasts (Pratsinis *et al.*, 2010).

In cell line U343, the antiproliferative effect of propolis was dose- and time-dependent, suggesting that this cell line is more sensitive than U251 that did not present the same effect. These differences could be associated with *p53* status (mutant or wild type). Cell line U343, but not U251, carries the wild type gene (Ishii *et al.*, 1999) and the antiproliferative effects of propolis may be *p53*-dependent. Other studies have shown increased expression of *p53* after treatment with different propolis extracts (Weng *et al.*, 2007; Ishihara *et al.*, 2009; Xuan *et al.*, 2010). Several functions and activities are attributed to *p53*, and it also acts in different cellular metabolism processes, such as cell cycle, apoptosis, senescence and DNA repair (Joerger and Fersht, 2008).

The combination of propolis with temozolomide, a chemotherapeutic drug used in the treatment of glioblastoma which produces DNA alkylation (Esteller *et al.*, 2000), evidenced synergistic antiproliferative effects, demonstrating the ability of propolis to predispose cells to the action of chemotherapy (Figures 1A and B). However, this effect should be further investigated.

The effects observed in this work can be related with the chemical composition of propolis, which is highly dependent on the flora of the region where it is collected. Sawaya *et al.* (2009) studied the same Tubi-bee propolis used in this work and showed that its composition varied seasonally. The mass spectra ions found in this extract were  $m/z$  371, 373, 401 and 471. This latter was the more important one; its formula  $C_{30}H_{47}O_4$  suggests that it has at least one acid function. Electrospray Ionization – Mass Spectrometry (ESI-MS) of these ions showed to be compatible with terpenes and with acid groups. All are marker ions of *Schinus terebenthifolius*, also known in Brazil as “aroeira mansa”, a preferred source of resins in stingless bee propolis in many regions of Brazil.

Propolis from temperate zones predominantly contains phenolic compounds, including flavonoids and cinnamic acid derivatives (Marcucci, 1995). On the other hand, diterpenes and prenylated compounds, which are virtually absent in propolis from temperate zones, have been reported to be the major constituents of propolis from tropical South America, along with lignans, flavonoids and other classes of compounds (Sawaya *et al.*, 2009).

Several reports have shown apoptosis induction caused by propolis extracts (Weng *et al.*, 2007; Szliszka *et al.*, 2009; Xuan *et al.*, 2010). However, in the present study, this effect was not observed, suggesting that in the concentrations used this type of propolis presents only cytostatic effects.

In summary, this investigation of the potential antiproliferative effects of propolis in human glioblastoma and normal fibroblast cell lines showed a strong inhibitory effect on the proliferation of all cell lines tested. Dose and time dependence were only observed for cell line U343. Moreover, the association of propolis with temozolomide produced synergistic antiproliferative effects. Propolis treatment also inhibited the clonogenic capacity in GBM cell lines, but the antitumor effects observed here were not caused by apoptosis. Further investigations are needed to elucidate the molecular mechanism of the antitumor effect of propolis, and the study of its individual constituents may reveal specific molecules with antiproliferative capacity.

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