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 anti-proliferative 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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lines, associated or not with TMZ, and in one non-neo-
plastic 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 ex-
tact was prepared, as follows: 30 g of propolis/100 mL eth-
anol (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 di-
luted in dimethylsulphoxide (DMSO, Sigma-Aldrich).

TMZ, known commercially as Temodal®, was ac-
quired 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
diluted in water at a ratio of 22 mg drug/100 mL water. This
quired from Schering-Plough Brazil and diluted ac-
luted in dimethylsulphoxide (DMSO, Sigma-Aldrich).

Human adult glioblastoma cell lines U251 and U343
and the human fibroblast cell line MRC-5 were purchased
from the American Type Culture Collection. Cells were
 cultured in HAM F10 medium (Gibco BRL, Life Technol-
gies®, 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% CO2 incubator.

The effects of propolis on clonogenic capacity were
evaluated by a clonogenic assay (Franken et al., 2006). Af-
ter 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 incub-
bated 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 per-
formed in triplicate.

For the proliferation assay, cells were seeded on 96-
well plates (1x10^3 cells/well). After 24 h, the medium was
replaced with fresh media containing the treatment (pro-
opolis, TMZ or DMSO at 0.5%) and then cultured for 24, 48
and 72 h. After the treatment, the culture medium was re-
moved 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 formazon product was measured at
450 nm in an iMark microplate reader (Bio-Rad Labora-
tories). All experiments were performed in triplicate. Va-
ues are shown as mean ± SD.

For apoptosis assessment, a total of 3x10^5 cells were
seeded in 25 cm^2 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 cul-
tured for additional 48 h. Apoptotic cells were recog-
nized by nuclear condensation and fragmentation, accord-
ing to Lee and Shaeter (1999). Treated cells were cen-
trifuged and incubated for 5 min at 37 °C with bisbenzimide
(Hoechst 33342), propidium iodide and fluorescein dia-
cetate (Sigma Chemical Co., St. Louis, USA). Then, sam-
ples 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) apo-
ptotic: fragmented blue nucleus and green cytoplasm, and
(3) necrotic: spherical red nucleus. 500 nuclei were ana-
lyzed per treatment.

One-way or two-way ANOVA followed by the ap-
propriate post-hoc test (Bonferroni) were used to check for
significant differences between groups (differences be-
tween doses or times). Differences were considered signifi-
cant at p < 0.05.

To determine the TMZ concentration for combination
with propolis, U343 and U251 cells were submitted to dif-
ferent 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 associ-
ated with 50 µM of temozolomide. Cell viability was deter-
mined by an XTT assay, as described above.

Propolis extract concentrations inhibited growth of the
tree cell lines when compared with DMSO (0.5%)
(p < 0.05) (Figure 1). For cell line U251, proliferation inhib-
tion was observed at 24, 48 and 72 h supporting 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 ob-
erved 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 (Fig-
ure 1B).

Statistically significant effects of dose and time de-
pendence 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 pro-
liferation was also observed, although this effect was nei-
ther dose- nor time-dependent (Figure 1C).
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 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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