Oncology and Translational Medicine
DOI 10.1007/s10330-014-0047-7

February 2016, Vol. 2, No. 1, P34–P38

ORIGINAL ARTICLE

Ethanolic extract of propolis induces apoptosis of HL-60 cells in vitro*

Yan Shi[1], Yana Li[1] (Co-first author), Naie Li[2], Min Yu[1], Dong Wang[1], Lijun Kong[3] (✉)

[1] Department of Histology and Embryology, Binzhou Medical College, Yantai 264003, China
[2] Department of Diagnostics, Binzhou Medical College, Yantai 264003, China
[3] Department of Biochemistry, Binzhou Medical College, Yantai 264003, China

Abstract

Objective The aim of the study was to investigate whether ethanolic extract of propolis inhibits the growth and induces apoptosis of HL-60 cells.

Methods HL-60 cells were treated for 24, 48, 72 h with various concentrations ethanolic extracts of propolis (0, 50, 100, and 200 μg/mL). The proliferation of HL-60 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Subsequently, Hochest 33258 staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) were used to test the apoptosis of HL-60 cells. We observed the expression levels of Bax and Bcl-2 in HL-60 cells by immunohistochemistry.

Results MTT assay showed that various concentrations of ethanolic extract of propolis had significant inhibitory effect on HL-60 cell proliferation ($P < 0.05$). Typical morphologic changes could be observed by fluorescence microscope and TUNEL. By immunohistochemistry, we found the expression level of Bax was up-regulated, whereas that of Bc1-2 was down-regulated ($P < 0.05$).

Conclusion Ethanolic extract of propolis inhibits leukemia cell proliferation and induces apoptosis *in vitro*. Its mechanism may be related to the regulation of Bax and Bcl-2 expression and up-regulation of Bcl-2/Bax ratio.

Received: 29 December 2014
Revised: 22 March 2015
Accepted: 25 December 2015

Key words: propolis; leukemia; apoptosis; Bcl-2/Bax

Leukemia is one of the most common human malignant tumors of the hemopoietic system and poses a serious threat to human health. Currently, there is no effective therapy for leukemia because its true cause is unknown. Combination chemotherapy is widely used to treat leukemia, but chemotherapy is also toxic to normal cells.

Propolis is a resinoid produced by the mixing of bee palate gland secretion with resins from plants. The main active compounds of propolis include flavonoids, terpenoids, and phenols [1]. Propolis has many kinds of medical and health care effects, such as antibacterial activities [2], immunomodulatory [3], and antiviral activity [4]. Leukemia has significant higher morbidity and mortality in malignant hematopoietic diseases. The treatment of traditional Chinese medicine for leukemia shows unique effects compared with combined treatment with chemotherapy and bone marrow transplantation. Recent studies on propolis indicate that some of its components can kill and inhibit the proliferation of tumors. As a natural antitumor drug, propolis has gained significant research attention. Here, we studied the effect of ethanol extract of propolis on HL-60 cells to explore its anticancer mechanism.

Materials and methods

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Company (USA). Hochest 33258 dying kit was obtained from Nanjing Keygen Biotech Company (China). TUNEL apoptosis detection kit (In Situ Cell Death Detection kit, POD kit) was bought from Roche Molecular Bioch (USA). RPMI 1640 was purchased from Gibco Company (USA). Immunohistochemical SP Kit, rabbit anti-human Bcl-2

✉ Correspondence to: Lijun Kong. Email: kong_lijun@163.com
* Supported by grants from the Shandong Province Natural Science Fund Project (No. ZR2013HL004), Colleges and Universities in Shandong Province Science and Technology Plan (No. J10LF57) and Binzhou Medical College Students of Science and Technology Innovation Fund Project (No. BY2014DKCX003).

and anti-human Bax McAb were purchased from Beijing Zhongshan Biotechnology Company (China).

Cell lines and cell culture

Human acute myeloid leukemia HL-60 cells were obtained from Blood Research Institute of Shanghai Second Medical University, China. The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FBS) and antibiotics [penicillin (100 U/mL) and streptomycin (100 U/mL)] at 5% CO_2 in a humidified incubator at 37 ℃. Cells were passaged every 2–3 days. Drug was added when the cultures were in the logarithmic growth phase.

Ethanolic extract of propolis

Propolis was comminuted under low temperature. Next, 50 mL of alcohol was added and the mixture was stirred for 24 h at room temperature. After centrifugation of the solution, the supernatant was freeze-dried. The dried extract was dissolved in alcohol and mixed with RPMI 1640 medium (1 mg/mL).

Drug experiment

HL-60 cells were seeded into a 24-well plate at a density of 2×10^5 cells per well. The cells were treated with ethanolic extract of propolis at various concentrations (0, 50, 100, and 200 μg/mL) for different durations (0, 12, 24, and 48 h, respectively). Ethanol group was set as the control, and cells were collected at 24, 48, and 72 h.

MTT assay

HL-60 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and treated with propolis ethanol extract at indicated concentrations (0, 50, 100, and 200 μg/mL) at 37 ℃ for 24, 48, and 72 h, respectively. The blank control group was treated with normal saline and ethanol, each concentration being set 6 parallel wells. Next, the cells were washed twice with PBS and incubated with 200 μL of MTT (5 mg/mL) solution for 4 h at 37 ℃. After carefully discarding the supernatant, 180 μL of DMSO was used to dissolve the precipitate. The optical density was measured at 570 nm. The cell growth inhibition rate (inhibition ratio, IR) was calculated as follows: IR = (1 − experimental group mean OD value / control group mean OD value) × 100%.

Hochest 33258 staining test

Cells collected after 72 h were smeared on slides coated with polylysine. After staining with Hochest 33258 for 15 min, the cells were examined for morphological changes under a fluorescent microscope.

Immunohistochemistry

The expression of Bax and Bcl-2 was measured using immunohistochemical analysis (SP). Endogenous peroxidases were inactivated in 3% H_2O_2, and then the rabbit anti-human Bax (1:100) and Bcl-2 (1:100) antibodies were processed according to reagents instructions. We used chromogenic reagent diaminobenzidine (DAB) for the staining and observed the sections under microscope. The optical density of detected proteins was calculated using ImageProPlus software.

We used ImageProPlus software to interpret the results. Under the light microscope, we randomly chose 100 cells to calculate optical density.

TdT-mediated dUTP nick end labeling (TUNEL) test to evaluate cell apoptosis

The slides were processed as above, and the experimental procedure was also carried out according to the manufacture's instruction. At least 200 cells were counted to calculate apoptosis rate.

Statistical analysis

Experimental results were analyzed using SPSS 17.0 statistical package. Data obtained were expressed as means ± SD. Data analysis among groups was performed using analysis of variance. The data were considered statistically significant at $P < 0.05$.

Results

Ethanolic extract of propolis induced apoptosis in HL-60 cells

To determine the inhibitory effect of ethanolic extract of propolis, we evaluated apoptosis induction in treatment group. The morphologic changes were first examined by Hoechst 33258 staining (Fig. 1). After HL-60 cells were treated with different concentrations of the extract for 24 h, the apoptosis-related morphologic changes were observed in comparison with the vehicle control. In the vehicle control group, the nuclei of HL-60 cells were round and homogeneously stained (Fig. 1a), while the extract-treated cells exhibited evident late apoptotic characteristics, including cell shrinkage and membrane integrity loss or deformation, nuclear fragmentation, and chromatin compaction (Fig. 1b).

Ethanolic extract of propolis suppressed the proliferation of HL-60 cells

Compared with the control group, the group treated with various concentrations (50, 100, and 200 μg/mL) of ethanolic extract of propolis showed significantly lower

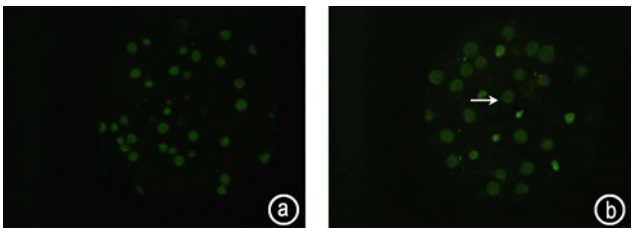

Fig. 1 Morphological changes in HL-60 cells (Hoechst 33258, ×400). (a) Control group; (b) Propolis extract group

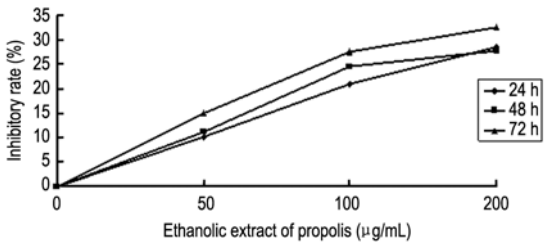

Fig. 2 Inhibition of HL-60 cell proliferation by ethanolic extract of propolis in a time- and dose-dependent manner, detected by MTT assay

Table 2 Effect of different concentration of ethanolic extract of propolis on leukemia cells with TUNEL (mean ± SD, n = 5)

Groups	Drug concentration (μg/mL)	Apoptosis index (%)
Control	0	7.06 ± 0.80
Ethanolic extract of propolis	50	8.10 ± 0.75
	100	11.14 ± 0.50*
	200	15.60 ± 1.40*

* P < 0.05, vs control group

cell proliferation at 24, 48, and 72 h (P < 0.05). The inhibitory effect of the extract gradually increased with the increase in time and drug concentration (P < 0.05; Table 1 and Fig. 2). This result suggests that ethanolic extract of propolis inhibited HL-60 cell proliferation *in vitro* (Table 1 and Fig. 2).

Apoptosis analysis by TUNEL

Brown particles observed in the nucleus under microscopy were apoptotic cells (Fig. 3). The control group showed a small number of apoptotic cells. The number of

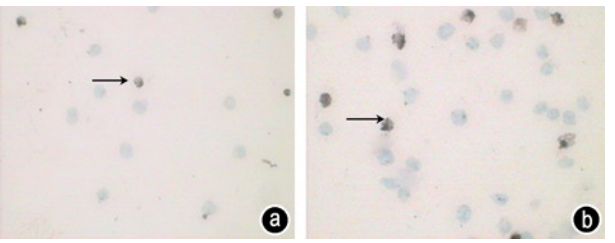

Fig. 3 TUNEL-positive HL-60 cells (×400). (a) Control group; (b) Propolis extract group

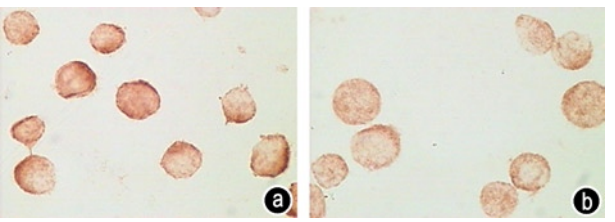

Fig. 4 Bcl-2 expression in HL-60 cells (SP stain, × 400). (a) Intensely expressed Bcl-2 in control group; (b) Weakly expressed Bcl-2 in group treated with ethanolic extract of propolis

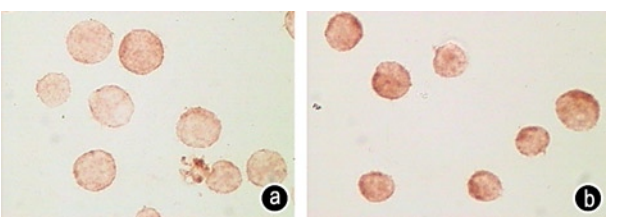

Fig. 5 Bax expression in HL-60 cells (SP stain, ×400). (a) Weakly expressed Bax in control group; (b) Strongly expressed Bax in group treated with ethanolic extract of propolis

apoptotic cells in the group treated with ethanolic extract of propolis (100 and 200 μg/mL) was significantly more than that in the control group. The number of apoptotic cells in each group was compared with that of the control group, and the difference was statistically significant (P < 0.05, Table 2). When we used PBS instead of marker solution, no positive cells were observed in the negative slides.

Table 1 Inhibitory effects of ethanolic extract of propolis on proliferation of HL-60 cells (mean ± SD, n = 6)

Drug concentration (μg/mL)	24 h		48 h		72 h	
	OD value	Inhibitory rate (%)	OD value	Inhibitory rate (%)	OD value	Inhibitory rate (%)
0	0.570 ± 0.062	–	0.758 ± 0.012	–	0.939 ± 0.011	–
50	0.551 ± 0.021	10.30*	0.796 ± 0.033	11.20*	0.870 ± 0.097	11.50*
100	0.489 ± 0.015	20.91*	0.601 ± 0.029	24.59*	0.770 ± 0.052	27.59*
200	0.310 ± 0.028	28.56*	0.541 ± 0.014	27.72*	0.570 ± 0.038	32.56*

* Compared with control group (drug concentration = 0 μg/mL), P < 0.05

Table 3 Effect of ethanolic extract of propolis on the expression of Bcl-2 and Bax in HL-60 cells (mean ± SD, n = 6)

Groups	Drug concentration (μg/mL)	Optical density value	
		Bcl-2	Bax
Control	0	45.23 ± 2.41	26.51 ± 0.73
Ethanolic extract of propolis	50	43.08 ± 1.24	28.84 ± 0.21
	100	25.14 ± 1.74*	35.23 ± 0.36*
	200	20.60 ± 0.45*	37.23 ± 0.28*

* P < 0.05 vs control group

Effect of ethanolic extract of propolis on expression of Bcl-2 and Bax

Bcl-2- and Bax-positive products were brown, granular and mainly distributed in the cytoplasm of HL-60 cells. Bcl-2 and Bax showed a weak positive reaction in normal HL-60 cells. Compared with control group, the group treated with ethanolic extract of propolis (100 and 200 μg/mL for 72 h showed significantly decreased expression of Bcl-2 and increased expression of Bax (P < 0.05, Fig. 4, Fig. 5 and Table 3).

Discussion

Natural products continue to be an invaluable resource for anticancer drug discovery. The idea of using natural products to develop more selective and effective cancer treatment has become a reality. Propolis possesses strong anti-tumor potential. In the present study, we evaluated the effects of propolis ethanolic extract on human leukemia cells. In our *in vitro* experiments, treatment with propolis ethanolic extracts at concentrations above 100 μg/mL resulted in morphological changes, significant anti-proliferative effect, and cytotoxic effect on leukemia cell line. Interestingly, we also found that propolis ethanolic extract (100 and 200 μg/mL) significantly induced HL-60 cell apoptosis *in vitro*. These data show the effectiveness of propolis extract on leukemia cells.

Propolis is a traditional animal medicine in China. It is one of the richest sources of phenolic acids and flavonoids [5]. In the past few years, several studies have been conducted worldwide on the anti-tumor effects of propolis. Its anti-tumor activity is related to improvement in immunity, and induction of tumor cell apoptosis. Ethanolic extract of propolis and its phenolic compounds have been known to possess various biological activities, including immunopotentiation and anti-tumor effects [6–7]. As an active component of propolis, caffeic acid phenethyl ester (CAPE) has anti-inflammatory effects [8], can inhibit the growth of breast cancer stem cells [9], and induces apoptosis of cancer cells [10–11]. It can obviously induce apoptosis of solid tumor and leukemia cells in humans and murids [12].

Apoptosis induction is one of the mechanisms proposed for the anticancer effects of propolis. Apoptosis is a well-characterized type of programmed cell death and is considered a highly regulated process that allows unwanted or dysfunctional cells to self-degrade. Conventional anticancer treatments, such as chemotherapy and radiotherapy, kill tumor cells primarily by the induction of apoptosis or apoptosis-like PCD. In this study, we demonstrated by Hoechst and TUNEL staining that propolis ethanolic extract can induce apoptosis in HL-60 cells. Moreover, the mechanism of apoptosis induced by Bax/Bcl-2 seems to be dependent on the concentration of the propolis extract.

Bcl-2 and Bax are two important genes of the Bcl-2 gene family. The Bcl-2 subgroup inhibits apoptosis and the Bax subgroup promotes apoptosis [13]. Studies have shown that Bax/Bcl-2 ratio is a key factor in determining the strength of inhibition of cell apoptosis. Therefore, down-regulating the expression of Bcl-2 in tumor cells and up-regulating the expression of Bax protein are effective means to induce apoptosis. Our immunohistochemical studies revealed that the expression of Bcl-2 decreased significantly and that of Bax increased significantly in a concentration-dependent manner following treatment with propolis ethanolic extract. Our findings suggest that propolis ethanolic extract may promote apoptosis of leukemia cells through the dual action of Bcl-2 down-regulation and Bax up-regulation.

Conclusion

Propolis ethanolic extract can inhibit the proliferation and induces apoptosis of HL-60 cells. It likely exerts its effects through regulation of Bax and Bcl-2 expression. Our findings suggest that ethanolic extract of propolis is a potential therapeutic agent for leukemia.

Conflicts of interest

The authors declare no potential conflicts of interest.

References

1. Mishima S, Ono Y, Araki Y, *et al*. Two related cinnamic Acid derivatives from Brazilian honey bee propolis, baccharin and drupanin, induce growth inhibition in allografted sarcoma S-180 in mice. Biol Pharm Bull, 2005, 28:1025–1030.
2. Cardoso RL, Maboni F, Machado G, *et al*. Antimicrobial activity of propolis extract against Staphylococcus coagulase positive and Malassezia pachydermatis of canine otitis. Vet Microbiol, 2010, 142: 432–434.
3. Girgin G, Baydar T, Ledochowski M, *et al*. Immunomodulatory effects of Turkish propolis: changes in neopterin release and tryptophan degradation. Immunobiology, 2009, 214: 129–134.
4. Schnitzler P, Neuner A, Nolkemper S, *et al*. Antiviral activity and mode of action of propolis extracts and selected compounds. Phytother Res, 2010, 24 Suppl 1: S20–28.
5. Volpi N, Bergonzini G. Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry. J Pharm Biomed Anal, 2006,

42: 354–361.
6. Szliszka E, Czuba ZP, Domino M, *et al*. Ethanolic extract of propolis (EEP) enhances the apoptosis- inducing potential of TRAIL in cancer cells. Molecules, 2009, 14: 738–754.
7. Díaz-Carballo D, Malak S, Bardenheuer W, *et al.* The contribution of plukenetione A to the anti-tumoral activity of Cuban propolis. Bioorg Med Chem, 2008, 16: 9635–9643.
8. Toyoda T, Tsukamoto T, Takasu S, *et al*. Anti-inflammatory effects of caffeic acid phenethyl ester (CAPE), a nuclear factor-kappaB inhibitor, on Helicobacter pylori-induced gastritis in Mongolian gerbils. Int J Cancer, 2009, 125: 1786–1795.
9. Omene CO, Wu J, Frenkel K. Caffeic Acid Phenethyl Ester (CAPE) derived from propolis, a honeybee product, inhibits growth of breast cancer stem cells. Invest New Drugs, 2012, 30: 1279–1288.
10. Kamiya T, Nishihara H, Hara H, *et al*. Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. J Agric Food Chem, 2012, 60: 11065–11070.
11. Kim EY, Ryu JH, Kim AK. CAPE promotes TRAIL-induced apoptosis through the upregulation of TRAIL receptors via activation of p38 and suppression of JNK in SK-Hep1 hepatocellular carcinoma cells. Int J Oncol, 2013, 43: 1291–1300.
12. Tomizawa A, Kanno S, Osanai Y, *et al*. Induction of apoptosis by a potent caffeic acid derivative, caffeic acid undecyl ester, is mediated by mitochondrial damage in NALM-6 human B cell leukemia cells. Oncol Rep, 2013, 29: 425–429.
13. Stefanaki C, Antoniou C, Stefanaki K, *et al.* Bcl-2 and Bax in congenital naevi. Br J Dermatol, 2006, 154: 1175–1179.

DOI 10.1007/s10330-014-0047-7
Cite this article as: Shi Y, Li YN, Li NE, *et al*. Ethanolic extract of propolis induces apoptosis of HL-60 cells *in vitro*. Oncol Transl Med, 2016, 2: 34–38.