BULGARIAN PROPOLIS INDUCES APOPTOSIS IN LYMPHOBLAST CELL LINE L5178Y

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ABSTRACT

Propolis is a natural product resulting from the vital activity of the honey bee. It has many biological effects, including antitumoral, immunomodulatory, anti-inflammatory, antioxidant, antimicrobial, etc. Current investigations focus on its ability to modulate the immune response and to protect the organism from tumor formation.

The aim of our study is to investigate the influence of propolis on cell proliferation and death on lymphoblast cell line L5178Y**.**

96% ethanolic extract of Bulgarian propolis was used in concentrations 0.1; 1.0 and 10 mg/L. A cell line was cultivated in an incubator of 37^{o}C, 5% CO_2 and high humidity. The cells were treated with increasing concentration of propolis for 24 hours. The inhibitory effect on cell vitality was detected by cytotoxcisity MTT-test and Trypan blue. IC50 was counted. Immunocytochemical detection of proliferative (PCNA) and proapoptotic (p53) markers was used.

The results from Trypan blue и MTT-test were statistically significant. The concentration-dependent cytotoxic effect on tumor cells was proved. IC50 for L5178Y cell line was 3,1mg/L propolis. The expression of PCNA decreased with increasing of propolis concentrations in contrast to p53 expression. These results determine the potential of the natural product to induce apoptosis in tumor cells.

Our results confirm the ability of propolis to reduce tumor growth *in vitro* and it is a prerequisite for further investigation of the use of propolis in therapy.

Key words: *propolis, apoptosis, tumor cell line*

Introduction

Propolis is a natural product resulting from the vital activity of the honey bee. It has many biological effects, including antitumoral, immunomodulatory, antiinflammatory, antioxidant, antimicrobial, etc (1,2). Current investigations focus on its ability to modulate the immune response and to protect the organism from tumor formation.

Establishing a scientific basis of its effects at a cellular level explains its potential influence on the human body. The main problem in therapy is the discovery of selective products that have a protective effect on normal cells and at the same time a cytotoxic one on the tumor cells. Propolis has the ability to affect the process of cell division and death at a cellular level (3). Its antiproliferative and proapoptic effect on tumor cells has been proven *in vitro* (4).

Induction of apoptosis and/or inhibition of cell proliferation are related to activation of various intracellular signals. As a result of DNA-damage the arrest of cell cycle can be achieved at G1 or G2 phase. In the first case replication is obstructed and in the second- mitosis of the damaged cell (5). The mechanisms that control the cell cycle arrest are based on the activation and interaction of specific signaling molecules, transcription factors, cyclins and cyclin-dependent kinases (6). As a tumor suppressor gene p53 regulates positively or negatively the expression of a large group of genes. Some of them mediate the cell cycle arrest, cellular senescence and apoptosis (7). The change in the expression of the proliferative antigen PCNA and the proapoptic marker p53 is evidence of changes in the control and regulatory mechanisms of cell division (8).

P53-dependent control mechanism is activated in case of stress signals and activation of mediators of signaling interactions (8). The protein, synthesized under the control of p53 gene, is a basic transcription factor for the PCNA-gene. The regulation of PCNA expression is accomplished at a transcriptional and post-translational level (9). The change in the expression of cell markers under the influence of the tested substances, allows analysis of the cell cycle progression.

Materials and methods

96% ethanolic extract of propolis from the Eastern Rodope mountains at concentrations 0.1, 1.0 and 10 mg/L is used.

Cytotoxicity tests

Mouse lymphoma cell line L5178Y is grown in a culture medium RPMI-1640 in the presence of 10% FCS and the addition of 100 U/ml Penicillin and 100 mg/ml Streptomycin. The cells are grown in an incubator Heareus at 37^{o}C and 5% CO_2 for 24 hours and are treated with increasing propolis concentrations. To demonstrate the inhibitory effect of the natural product on viability, a cytotoxic MTT test and Trypan blue staining are used. The MTT-assay is carried out according to protocol, adapted according to (10). After being treated for 24-hour, the cells are centrifuged and MTT is added at a final concentration of 500 μg/ml. The cells are incubated for two hours and then the dye is extracted with DMSO. The adsorption of propolis treated cells and a control cells is read on Ranscan spirit spectrophometer at wavelength of 540 and 690 nm. Viability is calculated in %, using a formula: absorption of treated cells/absorption of control cells x 100. Based on the results of the MTT assay, IC50 is calculated. When the dye Trypan blue is applied, the stained cells are counted out of every 100 and the percentage of cell viability is calculated. The average results are given after a minimum of 6 repetitions.

Immunocytochemical technique

Cells are cultivated on adhesive slides and a biotin-streptavidin peroxidase method is carried out for examination of expression of proliferative and proapoptic markers. A universal *DAKO* kit is used for immunocytochemical detection of the expression of the proliferating cell nuclear antigen (PCNA) and the tumour-suppressor protein p53.

For negative control PBS is used. The antigene-antibody complex is observed as red-brown granular staining. Hematoxylin is used for contrasting of nuclei. The expression of the markers is evaluated in terms of their localization and intensity. The intensity of reaction is defined by means of the semi-quantity scale, reflecting positivation of a given number of cells out of every 100 ("+" – slight staining in single cells, "2+" – presence of expression in 50% of the 100 counted cells "3+" – intensive homogenous expression in 100% of cells).

Statistics

Processing of statistical data is carried out by means of the statistical product Statistica 4.5 (StatSoft, Inc. Microsoft) and SPSS 11.5.0. The parametric t-test is used to define the statistically significant difference between the relative shares and average values in the groups at a level of significance $p < 0.05$.

Results

In propolis treated tumor cells L5178Y the following pattern is observed – the percentage of living cells decreases with increasing of propolis concentrations. The results of MMT assay show that concentration 0.1 mg/L leads to 73% cell survival, 1.0 mg/L to 69%, and the highest concentration – 10 mg/L causes a decrease in the percentage of surviving cells to 40% (figure 1). The Trypan blue staining shows similar results. When treated with 0.1 mg/L viability of cells is 92%, 1 mg/L – 62%, 10 mg/L – 38% (figure1). Results from both tests are statistically similar. The calculated value of IC50 is 3.1 mg/L propolis.

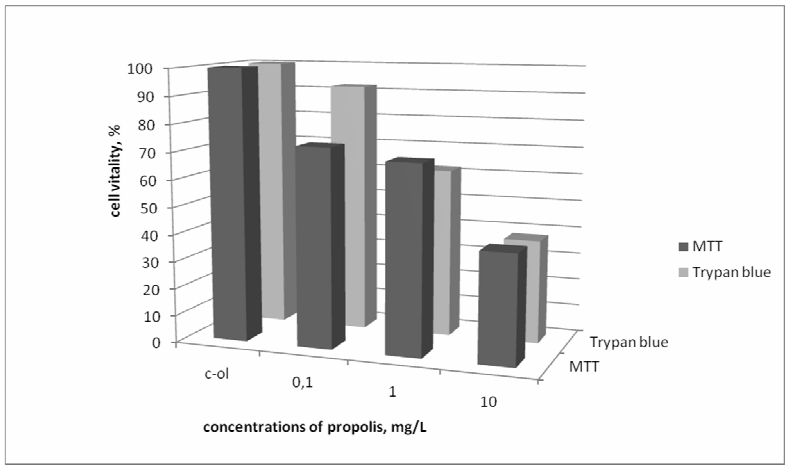
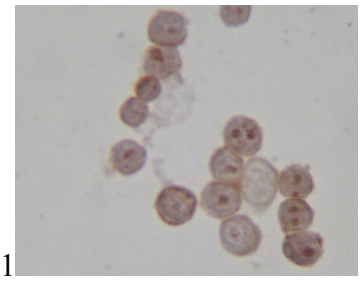
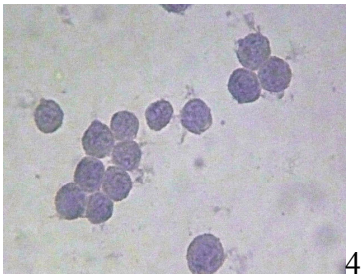


Figure1. Vitality of L5178Y cell line after propolis treatment. Results from MTT-test and Trypan blue. Decreasing of cell vitality depending of propolis concentration is observed. Between two tests no significant differences.

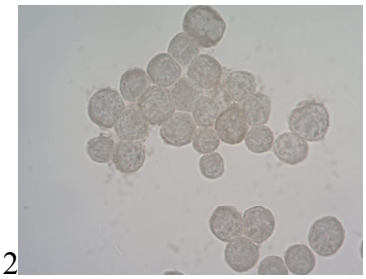
PCNA is expressed with high intensity in L5178Y control cells. In propolis treated cells the marker expression decreases depending on the concentration. In control cells and those, treated with 0.1 mg/L propolis, the intensive positive reaction is observed in the cytoplasm and in the nucleus. In cells treated with 1 and 10 mg/L propolis the expression decreases. In the highest concentration in parallel of the lack of positive response, morphological characteristic of apoptosis are observed (figure 2.1 – 2.3). p53 also changes its intensity. It is missing in untreated cells. When treated with 0.1 mg/L positivation is not observed, but with 1 and 10 mg/L, the marker is visualized with intensity „++" and „+++" in the cytoplasm (figure 2.4 – 2.6).



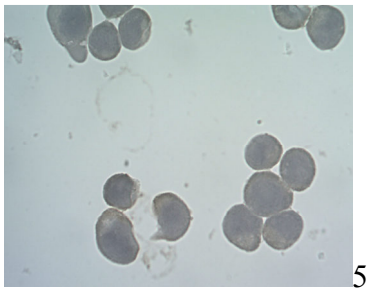
1. PCNA in cells treated with 0,1 mg/L propolis for 24h. High intensity of cytoplasmic expression "+++". Magnification x 1000.



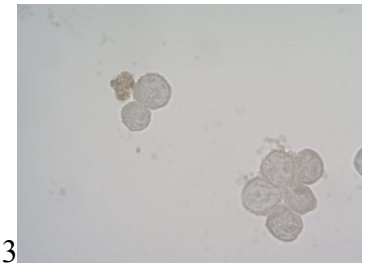
4. p53 in cells treated with 0,1 mg/L propolis for 24h. There is no expression. Magnification x 1000.



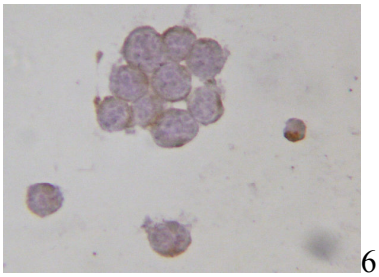
2. PCNA in cells treated with 1,0 mg/L propolis for 24h. Cytoplasmic expression with intensity "++". Magnification x 1000.



5. p53 in cells treated with 1,0 mg/L propolis for 24h. Cytoplasmic expression with intensity "++". Magnification x 1000.



3. PCNA in cells treated with 10 mg/L propolis for 24h. Sporadic cytoplasmic expression "+". Cells with apoptosis tipical morphology are observed. Magnification x 1000.



6. p53 in cells treated with 10 mg/L propolis for 24h. High intensity of cytoplasmic expression "+++". Apoptotic cells are observed. Magnification x 1000.

Figure2. Immunocytochemical detection of expression of proliferative (PCNA) and proapoptotic (p53) markers in L5178Y cell line.

Discussion

Propolis can interact with membrane-associated molecules and unlock transmembrane pathways for cell proliferation and death. Ethanol extract of propolis passes through cell membranes and affects cell activity. Propolis can affect tumor formations, which is due to its ability to activate cell metabolism, activating different signal molecules (11). In case of treatment with increasing concentrations of propolis, morphologic and functional changes in the lymphoblast cell line L5178Y occur. Cell viability is decreasing with increasing of concentrations. These data support the results established by other researchers on other cell lines (12, 13).

The tumor suppressor protein p53 plays an important role in apoptosis disregulation of numerous cancer diseases. In clinic its decreasing expression or missing activity is associated with bad prognosis. Its reactivation induces cell arrest, underlying therapeutic strategies. Propolis causes p53 expression in L5178Y cells, which is evidence of DNA damage. Expression of p53 in treated cells supposes its participation in induction of G1 arrest and as a result cell death. There are two possible pathways of cell suicide – membrane-associated and mitochondrial and they are interrelated. p53 takes part in both, but it is predominant in the internal one. In eukaryotic cells the DNA damage induces p21 expression, which blocks the cell cycle progression from G1 to S phase. The link between p21 and PCNA *in vitro* suppresses replication, but not the reparation of DNA (13). In our study we have observed that the increase in propolis concentration leads to a decrease in PCNA level and increase in p53 expression. The complex interactions between these regulatory proteins allow a fine regulation and modulation of the cellular response to various external signals.

The ability of the natural bee product to reduce the proliferative activity of tumor cells *in vitro* is a prerequisite for a new study of its possible therapeutic use.

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