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The Ethanol Extract of Polish Propolis Exhibits Anti-Proliferative and/or Pro-Apoptotic Effect on HCT 116 Colon Cancer and Me45 Malignant Melanoma Cells *In Vitro* Conditions

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Propolis is a natural product widely consumed in folk medicine. Different biological activities, such as anticancer, antioxidant, anti-inflammatory, antibiotic and antifungal effects have been reported for propolis and its constituents.
Objectives. An *in vitro* study focused on an evaluation of the biological activity of EEPP, including its anti-proliferative influence on selected neoplastic cells, considering qualitative-quantitative chemical characterization of Polish propolis.
Material and Methods. Cytotoxicity was evaluated by means of the MTT and LDH assays. The apoptosis was determined using fluorescence microscopy with annexin V-FITC. Additional EEPP composition was analyzed by a High Performance Liquid Chromatography (HPLC) method. The antimicrobial activity was evaluated by minimal inhibitory concentrations (MIC) against *Streptococcus aureus*, *Enetecoccus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*.
Results. The total content of flavonoids per quercetin in the examined propolis extract amounted to 0.442 ± 0.091 mg/mL. The flavonoid compounds identified in Polish propolis included flavones, flavonones, flavonolols, flavonols and phenolic acids. The multi-directional interactions among the various chemical compounds in propolis seem to be the essential biological activities when considering its anticancer effects. The results showed that in case of Me45 and HCT 116 cell lines, the ethanol extract of propolis could inhibit cell growth as well as cell size reduction. Regarding antimicrobial activity, EEPP showed MICs ranging from 0.39 to 6.25 mg/mL.
Conclusions. Ethanol extract of propolis from Poland obtained in the study exhibits anti-proliferative activity in different carcinoma cells (**Adv Clin Exp Med 2015, 24, 2, 203–212**).

Key words: antiproliferative, cancer cell line, proapoptotic, chemoprevention, ethanol extract of Polish propolis.

Growing interest in unconventional treatment methods validated by a tradition with many centuries of results is the reason that propolis has become increasingly popular in Poland and around the world. Propolis is a resin substance collected by bees from various plant species. This substance is used by bees to seal frames and walls of a hive as well as to build a barrier surrounding its entrance hole. Bees use this product also for maintaining a proper homeostasis inside the hive. The vast majority of studies demonstrated that propolis reveals substantial properties, including the promotion of soft tissues healing and regeneration/reparation. Apart from its antibacterial [1–6],

anti-inflammatory [7, 8], antioxidant [9–11], immunoprotective [12], hepatoprotective activities [13, 14], propolis has also anti-proliferative [15–19] and proapoptotic ones [20–23]. Composition of propolis is diverse and depends on both place of origin and time in which it has been collected. It is known that the composition of propolis includes over 300 biologically active substances, guaranteeing curative effects of propolis. Among these substances, flavonoids, phenolic acid and phenolic acid esters draw the most attention. Epidemiologic data suggested that flavonoids and phenolic acid consumption may protect against cancer induction in several human tissues. Flavonoids, such as quercetin, apigenin, galangin, pinocembrin, pinostrobin are antioxidants – they scavenge damaging particles in the body known as free radicals, which damage cell membranes, tamper with DNA, and even cause cell death. Substances exhibiting potent anti-proliferative and proapoptotic properties are: caffeic acid phenethyl ester (CAPE), quercetin, apigenin, galangin which enjoys attention of researchers from all over the world. The usefulness of propolis in therapy of neoplastic diseases is connected predominantly with its ability to induce apoptosis and anti-proliferative activities. In numerous *in vitro* research, propolis exhibited proapoptotic activities on various types of neoplastic cells such as in the cases of: laryngeal cancer, lung carcinoma, pancreatic cancer, thyroid neoplasm, colorectal cancer, breast cancer, prostate cancer and malignant glioma [24, 25].

Nowadays, standard chemiotherapeutic agents are used as a supportive or main treatment of malignant melanoma and colon cancer. However, chemotherapy applied systemically may result in severe adverse effects and affects blood cells production leading to the impairment of homeostasis: anaemia, neutropenia, and/or thrombopenia. For that reason, it is important to find additional measures as supplemental adjuvants to overcome these drawbacks and reduce these cancer therapy problems [26]. According to our knowledge, Polish propolis has not yet been investigated in the context of on its anti-tumor potential towards malignant melanoma and colorectal carcinoma.

Our study was aimed to assess the cytotoxic effect on malignant melanoma Me45 and colorectal cancer HCT 116 cells in the aspect of potential propolis chemoprevention protocols for clinical use in hospital setting.

Experimental Section

Propolis obtained from bees from an apiary in Kamienna in Nowy Sącz Voivodeship (Poland) constituted the material for the research. The region of Poland mentioned above is the main area enriched in alder (*Alnus glutinosa*), horsechestnut (*Aesculus hippocastanum*), black poplar (*Populus nigra*), beech (*Fagus sylvatica*) and birch (*Betula alba*).

Ethanol Extract of Propolis

Propolis was extracted during a two-week period in order to obtain ethanol extract of Polish propolis (EEPP). EEPP was then dissolved in dimethyl sulfoxide to produce 100 mg/mL of working concentration. To reach this aim, 10 g propolis was ground mechanically and then 100 g of 75% (v/v) ethanol was added. The flask was inserted into laboratory shaker in a dark container for the time period of 14 days in a standard temperature of 21°C. The ethanol extract of propolis was cooled in 4°C for 24 h to allow the precipitation of insoluble to sediment. Then, it was filtered using Watman no. 4 filter. Filtrate obtained in this way was evaporated using rotary vacuum evaporator in 40°C. This way, a viscous substance of brown colour was obtained, which was dissolved in dimethyl sulfoxide in order to receive a working concentration. Final concentration of dimethyl sulfoxide in cell culture did not exceed 0.1% [27].

Marking the Content of Flavonoids in EEPP

Concentration of flavonoids was marked using colorimetry method based on the possibility to form flavonoid complexes with aluminium chloride. In order to perform the marking, 5 μL of EEPP was taken, then it was added to 2 cm^3 of 2% aluminium chloride ethanol solution (POCh, Poland) and incubated for 1 h in room temperature. After the lapse of this period, absorption with wavelength of $\lambda = 420$ nm in cuvette with light-way 10 mm using spectrometer was surveyed. Concentration of flavonoids was determined on the basis of analytical curve made for quercetin (Sigma-Aldrich, St. Louis, MO, USA).

HPLC-DAD Analysis of EEPP

To assess the chemical characterization of EEPP we applied a HPLC (high performance liquid chromatography) method. This chemical assessment was carried out with the use of Varian 920-LC HPLC (Harbor City, CA, USA). The autosampler (900-LC model), gradient pump, 330 model DAD, and software (Galaxie) for data

validating and processing were used. Chromatographic separation was obtained on a Pursuit C18 (5 μm particle size) column (250 × 4.6 mm i.d.) with gradient mode consisting of solvents: water and formic acid (95 : 5, v/v) and acetonitrile. The elution was achieved at a flow rate of 0.6 mL/ /min. The separations carried out using a gradient elution: 20–30% solvent B (for 15 min), 30% solvent B (15–28 min), 30–80% solvent B (28–50 min), 80% solvent B (50–54 min), 80–40% solvent B (54–60 min) and 40–20% solvent B (60–65 min). Data acquisition was performed at 254 and 340 nm and the ingredients were specified after having been compared with commercial standards isolated previously. Ethanol extract of Polish propolis were purified with a 0.22 μm filter prior to injection of 20 μL into the HPLC autosampler. The unified mixtures in ethyl alcohol containing pinostrombin, apigenin, chrysin, ferulic acid, quercetin, galangin, kaempferol, coumaric acid, gallic acid and caffeic acid were obtained from stock solutions (Carl Roth GmbH and Sigma Chemical Company).

Microbiological EEPP Standardization

In order to assess the biological activity of Polish propolis from Kamianna, we carried out a microbiological investigation including assessment of Minimal Inhibitory Concentration of bacteria. Antimicrobial activity was estimated by means of serial dilution methods using the following reference strains: *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29123, *Enetecoccus faecalis* ATCC 29212, *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 60193.

In order to do so, to 2 mL of Müller-Hinton liquid growth material (MH Broth) 2 mL of DMSO solution of Polish propolis were added and a series of 6 dilutions of propolis from 12.5 mg/ /mL to 0.39 mg/mL were performed. In the next stage, to each test tube from the series of dilutions 50 μL of bacterial suspension was added. In the 15^{th} min, the whole was incubated 18 to 24 h in 35°C and in the 12^{th} and the 24^{th} h the minimal inhibitory concentration (MIC) growth of microorganisms and the lowest concentration of EEPP visually inhibiting the growth of bacteria were determined. Using the same method, a control study with pure DMSO (Sigma-Aldrich, St. Louis, MO, USA) without addition of propolis was performed.

Conditions of *In Vitro* Cell Culture

Cell lines of human malignant melanoma Me45 (cell line was purchased from the Silesian University of Technology, as a kind gift from dr M. Wideł [28] from the Marie Curie Memorial Cancer Centre and Institute of Oncology, Gliwice, Poland. The cells were derived from metastatic lesions of a 35-year-old patient of the Institute of Oncology) and colorectal cancer HCT 116 (purchased from ATCC catalog no. CCL-247) were cultivated with the use of modified McConkey culture medium (PAA) with an addition of fetal bovine serum (FBS; PAA), whose final content amounted to 10%. Antibiotic-Antimycotic Solution (PAA) was added to cell culture. Cells were cultivated in CO_2 incubator, produced by Heraeus Instruments, in 37°C, in air atmosphere containing 5% of carbon dioxide. Subsequent passages were made by treating confluent cell culture with trypsin solution (PAA) and then, cells were plated into a new cell culture receptacle (PAA) in the ratio of 1 to 4.

Cell viability was determined by light microscope method using 0.4% of trypan blue solution in physiological saline (Sigma-Aldrich, St. Louis, MO, USA) in disposable hemocytometer (C-Chip, PAA, USA) with the use of Zeiss Axiostar Plus microscope (Carl Zeiss, Jena, Germany)

Evaluation of Anti-Proliferative Properties

As an indicator of cytotoxic activity of ethanol extract of propolis an MTT assay was used. It is a colorimetric method consisting in the evaluation of neoplastic cells' metabolic activity (using conversion of tetrazolium salts in mitochondria into insoluble formazan product; the amount of created formazan is proportional to the amount of living cells).

In order to evaluate EEPP cytotoxic properties, cells of examined lines were plated on 96-well plates in the amount of 10,000 cells per well and then a culture medium in the amount of 0.1 mL was added. Cells were left for 24 h in order to attach to the culture medium. After the lapse of this period, culture medium was decanted and to each well a culture medium containing EEPP with concentration from 100 μg/mL to 5 μg/mL was added and left for 48 h. After the lapse of this period, the culture medium was decanted and to each well 10 μL of MTT reagent (Biotium, USA) was added. Cells were left for 4 h. After the lapse of this period, to each well 200 μL of DMSO (POCh, Polska) was added in order to dissolve formed

formazan crystals. The measurement of absorbance was performed by using a EL × 800 microplate reader (BioTek Instruments, USA) with 570 nm wavelength. In order to deduce the number of dead cells, an appropriate formula was used: (dead cells) = (1-[absorbance of examined sample/control absorbance]) ×100%.

Determination of Percentage of Apoptotic and Necrotic Cells

As a result of apoptosis, content of phospholipids in external cytoplasmic membrane changes. Phosphatidylserine moves from cytoplasmic side towards external layer. Detection of phosphatidylserine is possible thanks to the use of annexin V. It exhibits a relation with phosphatidylserine. Coupling of this protein with fluorescent dye (fluorescein isothiocyanate – FITC) enables the detection in fluorescence microscope thanks to green fluorescent signal ($\lambda_{abs}/\lambda_{em}$= 492/514 nm). In order to evaluate the type of cell death, the examined cell lines were plated onto 96-well plates Imaging Plates FC (PAA, USA) in the amount of 10,000 cells per well, then a nutrient medium was added and it was left for 24 h so that cells attach to the culture medium. After the lapse of this time, a nutrient medium containing EEPP with concentration analogous to MTT assay (200 μg/mL to 5 μg/mL) was added. Then, cells were rinsed with buffer attached to Apoptotic & Necrotic & Healthy Cells Quantification Kit (Biotium, USA). To each well, appropriate dyes were added and left for 15 min in a dark room. Cells were counted using an Axiovert (Carl Zeiss, Germany) fluorescence microscope with suitable fluorochromes. Proper control tests (cell cultures not treated with EEPP) were taken into account. For examined samples and control, an apoptotic cell indicator that corresponded to the percentage of cells with apoptotic cell morphology, as regards 100 evaluated cells in analyzed fields of vision of preparations, was determined.

Statistical Analysis

Statistical analysis was performed using statistical software package STATISTICA (version 8.0, Statsoft Poland). The results were shown in the form of average and standard deviation obtained from 4 independent tests (n = 96 for each examined concentration in the case of MTT assay) and from 3 independent tests (n = 12 for each examined concentration in the case of Apoptotic & Necrotic & Healthy Cells Quantification Kit test). Normality of distribution was determined with Shapiro-Wilk test. In order to determine statistical significance, a Student's *t*-test as well as one-way ANOVA were used. Statistical significance was accepted on the level of $p < 0.05$.

Results

Evaluation of Flavonoid Content in EEPP

Process of marking flavonoids' concentration was made in 12 repetitions. The obtained results were shown as an average and standard deviation. The conducted analysis showed that in the examined sample of EEPP, total content of flavonoids per quercetin amounted to 0.442 ± 0.091 mg/mL.

High Performance Liquid Chromatography Results

The identification of chromatographic peaks was accomplished by combining the information obtained from different sources. When a reference standard was available (p-coumaric acid, benzoic acid, ferulic acid, gallic acid, caffeic acid, apigenin, pinostrobin, kaempferol, galangin, chrysin, quercetin), the identification was confirmed by direct comparison of the retention times and spectra acquired in the same analytical conditions. Table 1 shows the concentrations of the compounds identified in propolis samples. In general, phenolic acids and their esters were the predominant class of substances in propolis extracts, followed by flavones and flavonols.

Biological Activity of EEPP Confirmed by Antimicrobial MIC Tests

Analyzing ethanolic extract of Polish propolis obtained from Kamianna on 5 strains of Gram-positive bacteria, Gram-negative bacteria and yeast-like fungi we demonstrated that all of them exhibited antimicrobial activity, and MIC value was within the range from 0.39 to 6.25 mg/mL. In the cases of examined strains *S. aureus* ATTC 25923 and *S. aureus* ATCC 29123, the MIC value range was 390 μg/mL. In the case of Gram-negative bacteria MIC values were considerably higher and amounted to 6.25 mg/mL analyzing strains *Enetecoccus faecalis* ATCC 29212, *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 60193. Ethanol extract of propolis demonstrates diverse

Table 1. The content of the test flavonoids and phenolic acid in Polish propolis sample. Data gathered by HPLC-DAD analysis. The main detected ingredients belonging to phenolic compounds and flavonoids are respectively: caffeic acid and quercetin. The increasing sequence of quantitative EEPP content for selected compounds is presenting as follow – flavones < aromatic carboxylic acid

Name the compound identified	Chemical group	Chemical name	The content of the test compound [mg/g EEEP]
Pinostrobin	flavones	(*RS*)-2,3-dihydro-5-hydroxy-7-methoxy-2-phenyl-4*H*-1-benzopyran-4-one	1.62
Kaempferol	flavonols	3,4',5,7-tetrahydroxyflavone	1.17
Galangin	flavonols	3,5,7-trihydroxyflavone	3.75
Chrysin	flavones	5,7-dihydroxyflavone	2.01
Apigenin	flavones	4',5,7-trihydroxyflavone	4.02
Quercetin	flavonols	3,3',4',5,6-pentahydroxyflavone	4.42
Gallic acid	phenolic acid	3,4,5-trihydroksybenzoic acid	3.75
Ferulic acid	phenolic acid	*trans*-4-hydroxy-3-methoxycinnamic acid	5.26
Caffeic acid	phenolic acid	3,4-dihydroxycinnamic acid	16.60
Coumaric acid	phenolic acid	trans-4-hydroxycinnamic acid	1.20
Benzoic acid	aromatic carboxylic acid	benzenecarboxylic acid	8.53

antimicrobial properties depending on species of the examined strain and activity time. Results of MIC value have been demonstrated in Table 2.

Evaluation of Anti-Proliferative Properties

Using MTT assay it has been showed that antiproliferative activity of EEPP is dependent on the applied concentration. For the analysis, EEPP with a from 5 to 100 μg/mL was used, in which DMSO final concentration did not exceed 0.1%. When EEPP in 3.125 μg/mL concentration was used, after 48 h of incubation cytotoxicity amounted to 7.36 ± 3.36% ($p > 0.05$ vs C) and 9.40 ± 1.38% ($p < 0.05$ vs C) respectively for Me45 cell line of malignant melanoma and for HCT 116 cell line of colorectal cancer. In 25 μg/mL concentration the obtained values were 12.2 ± 1.77% ($p < 0.05$ vs C) and 20.52 ± 2.46% ($p < 0.05$ vs C), in 50 μg/mL the values for Me45 amounted to 16.94 ± 5.27% ($p < 0.05$ vs C) and for HCT 116 23.09 ± 6.84% ($p < 0.05$ vs C). When 100 μg/mL concentrations were applied, then the obtained results were as follows: 25.76 ± 5.13% ($p < 0.05$ vs C) xand 20.84 ± 2.93% ($p < 0.05$ vs C) respectively for HCT 116 and Me45 cell line. It was also noticed that EEPP in all examined concentrations showed more powerful cytotoxic properties as regards HCT 116 cell line of colorectal cancer. Results of cytotoxicity have been demonstrated in Fig. 1–3. The viability of melanoma cells line Me45 and colorectal cancer cell line have been demonstrated in Fig. 4.

Table 2. Minimal inhibitory concentration of Polish EEPP sample. Minimum inhibitory concentration (MIC) is the lowest concentration of antimicrobial drug that will inhibit the visible growth of a microorganism after 24 h incubation

Reference strains	MIC [mg/mL]
S. aureus ATTC 25923	0.39
S. aureus ATCC 29123	0.39
Enetecoccus faecalis ATCC 29212	6.25
Escherichia coli ATCC 11775	6.25
Pseudomonas aeruginosa ATCC 27853	6.25
Candida albicans ATCC 60193	6.25

Evaluation of Proapoptotic Properties

The apoptosis process plays a significant role in pathogenesis of neoplasmic diseases. Controls constituted Me45 and HCT 116 lines cultivated on the DMEM culture medium without addition of ethanol extract of propolis. For Me45 line the percentage of apoptotic cells amounted to 0.45 ± 0.51%, while for HCT 116 line it amounted to 1.0 ± 1.12%. In order to perform an assessment, we have calculated apoptotic index. In order to better illustrate this, the percentage of apoptotic

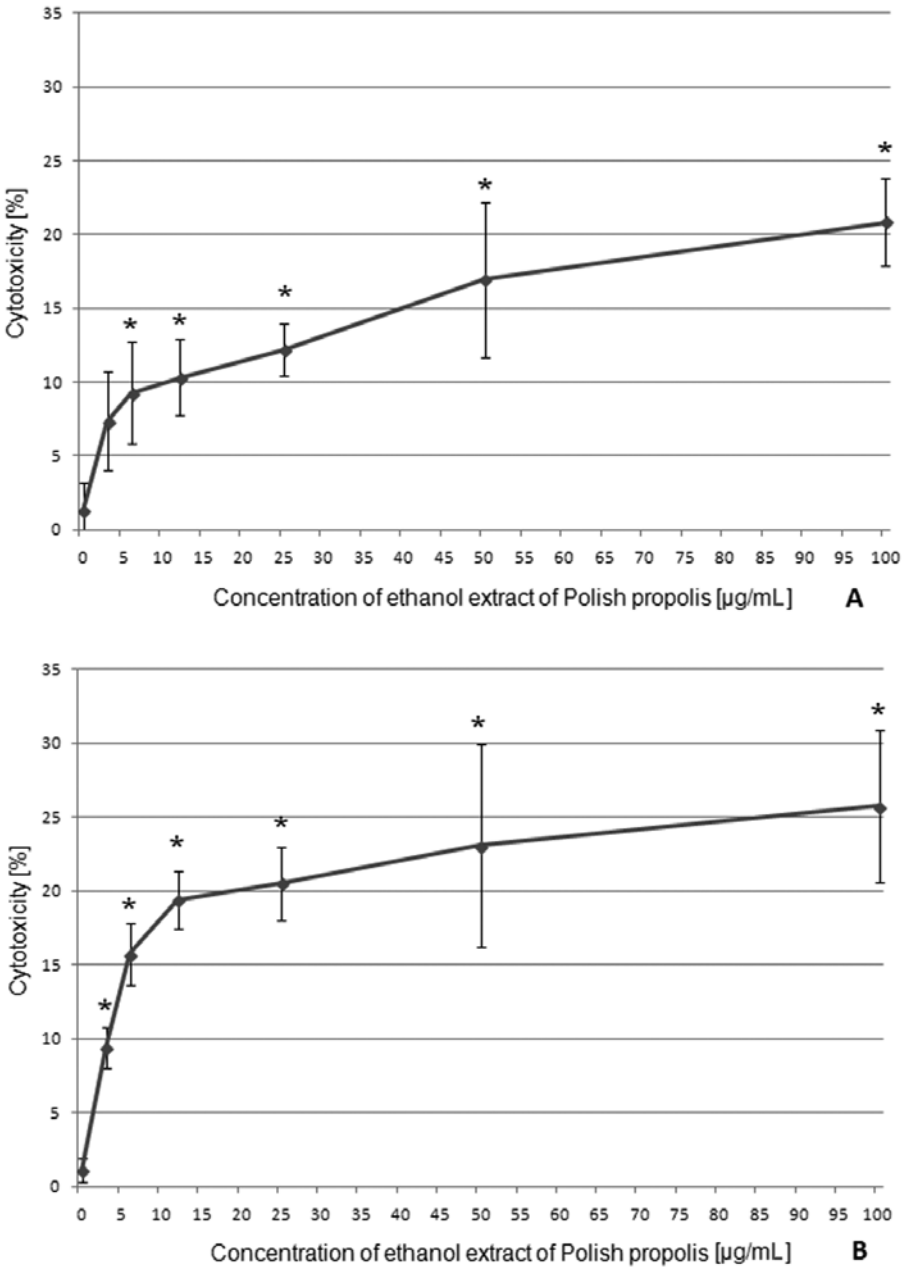

Fig. 1. Results of cytotoxicity of ethanol extract of propolis as regards Me45 (Fig. 1A) cell line of malignant melanoma and for HCT 116 (Fig. 1B) cell line of colorectal cancer

* $p < 0.05$ vs C.

cells for Me45 and HCT 116 lines are demonstrated in Table 3 and Fig. 5.

Discussion

Ethanol extract of propolis shows antineoplastic effects thanks to the stimulation of the apoptosis process. It also has cytotoxic and anti-proliferative effects on neoplasmic cells. In the research conducted by Szliszka et al. [27], it was shown that ethanol extract of propolis demonstrates proapoptotic effects on HeLa cervical cancer line. In their research, the authors showed that ethanol extract of propolis attenuated growth and induced apoptosis in HeLa carcinoma cells in relation to dose of ethanol extract of propolis. They showed that after incubation for 48 h, ethanol extract of propolis at the concentrations of 5, 25 and 50 µg/mL induced 1.45 ± 0.54%, 4.21 ± 0.5%, 19.62 ± 0.96% cell death, respectively. They have demonstrated that treatment of cancer cells with propolis inhibited cell proliferation by induced cytotoxicity and apoptosis.

Research conducted by Syamsudin et al. [29] showed that ethyl acetate extract of propolis demonstrates stronger proapoptotic effects as regards MCF-7 line originating in breast cancer. In this research propolis extract with 47.45 µg/mL was used, which caused a 13.21% increase in the percentage of apoptotic cells. In their research, the authors in order to compare apoptotic properties of apitherapeutic used chemotherapeutic – doxorubicin. The obtained results allowed us to assume that the

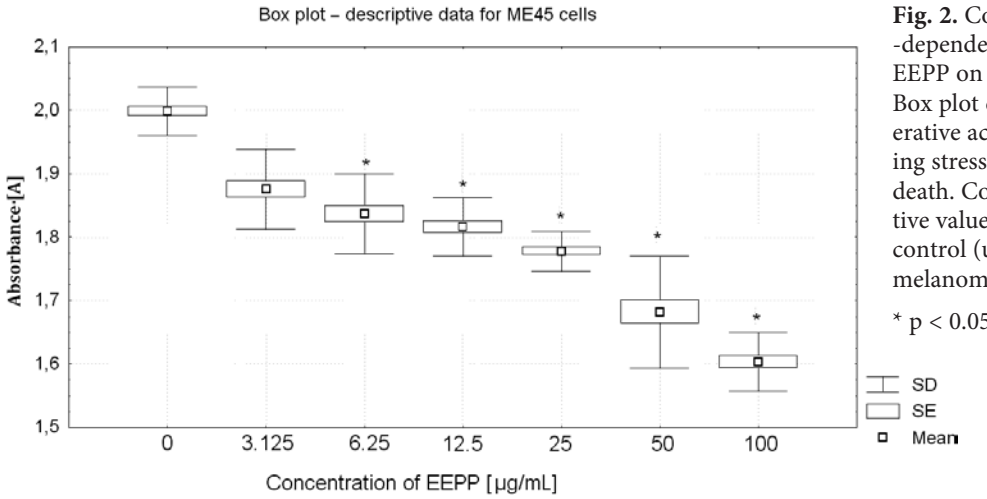

Fig. 2. Concentration--depended cytotoxic effect of EEPP on Me45 cancer cells. Box plot displaying antiproliferative activity of EEPP causing stress-induced Me45 cells death. Comparison of descriptive values for Me45 cells and control (untreated) malignant melanoma cancer cells

* $p < 0.05$ vs C.

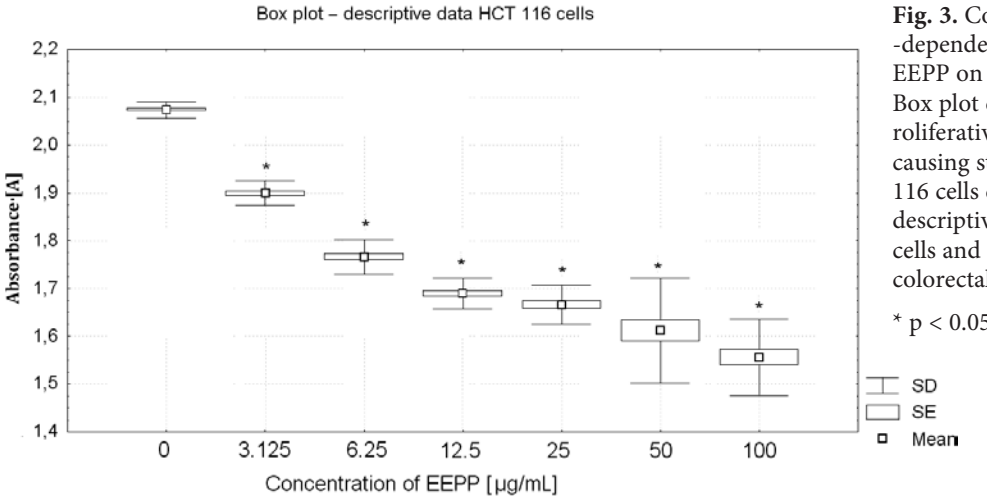

Fig. 3. Concentration--depended cytotoxic effect of EEPP on Me45 cancer cells. Box plot displaying antiproliferative activity of EEPP causing stress-induced HCT 116 cells death. Comparison of descriptive values for HCT 116 cells and control (untreated) colorectal cancer cells

* $p < 0.05$ vs C.

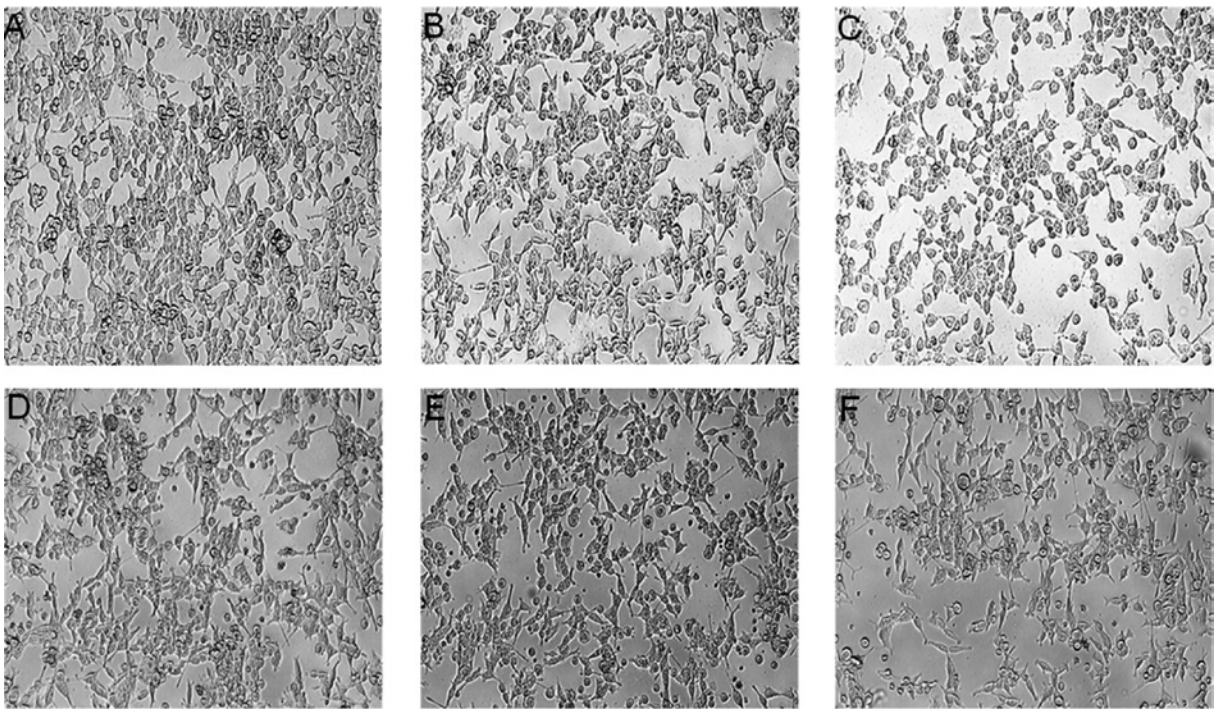

Fig. 4. The viability of melanoma cells line Me45 (A-controla without EEPP addition; B-12 h incubation with EEPP addition in concentration 100 µg/mL; C-24 h of incubation with EEPP in concentration 100 µg/mL) and the viability of colorectal cancer (D-control without EEPP addition; E-12 h of incubation with EEPP addition in concentration 100 µg/mL; F-24 h of incubation with EEPP addition in concentration 100 µg/mL). The optical microscope observation (JuLI, Digital-Bio ×200 magnification)

Table 3. Percentage of apoptotic cells for Me45 and HCT 116 cell line induced by different concentrations of EEPP. After treating with EEPP, cells were stained with apoptotic, necrotic, and healthy cells quantification kit and analyzed by fluorescence microscopy

EEPP concentration	Percentage of apoptotic cells [%]	
	name of cell line	
	malignant melanoma Me45	colorectal cancer HCT 116
0 μg/mL	0.45 ± 0.51	0.85 ± 0.49
3.125 μg/mL	5.55 ± 1.27	2.84 ± 1.55
6.25 μg/mL	7.19 ± 2.03*	2.81 ± 1.64
12.5 μg/mL	8.61 ± 3.39*	2.99 ± 0.87
25 μg/mL	13.45 ± 4.14*	2.50 ± 1.88
50 μg/mL	15.45 ± 4.57*	2.95 ± 1.70
100 μg/mL	18.25 ± 2.88*	2.20 ± 1.94

* $p < 0.05$ vs C.

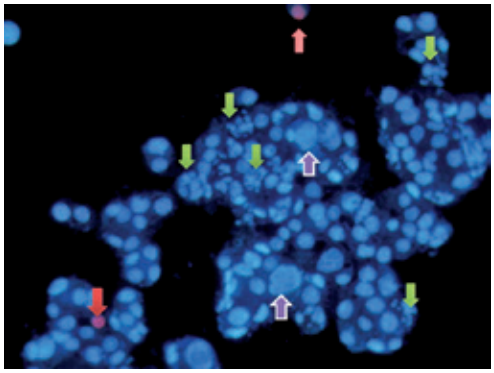

Fig. 5. The microscopic evaluation of the morphological changes of melanoma cells Me45 treated by ethanol extract of Polish propolis in concentration 100 μg/mL (fluorescence microscope, Zeiss, Germany, ×400 magnification). Green arrow indicates apoptotic cells during formation of apoptotic elements. Red arrow indicates necrotic or late-apoptotic cells. Purple arrow indicates a giant-poliploidal cell. Representative microphotographs were selected

chemotherapeutic administered with 145.57 nM concentration caused an 18.89% increase in apoptotic cells. The authors draw attention to the fact that even if cytotoxic activity of the propolis extract is lower than in the case of conventional chemotherapeutics, such as doxorubicin, the IC_{50} value (the half maximal inhibitory concentration) of the extract is promising with regard to designing new chemopreventive methods.

Scientists from Kyonggi University [30] in their research confirmed the proapoptotic effects of ethanol extract of propolis. They carried out an experiment whose purpose was to define DNA fragmentation that is considered to be one of the morphological symptoms of physiological cell death process. HL-60 acute myeloid leukemia cells were incubated with different ethanol extract of propolis condensations and then, quantitative indicator of DNA fragmentation was determined with the use of DPA reaction method. They showed that propolis triggers DNA fragmentation of HL-60 cells to the extent dependent on condensation and time of incubation. When propolis with condensation higher than 10 μg/ml was used, indicator of DNA fragmentation was considerably higher. In order to confirm the proapoptotic effects of ethanol extract of propolis, DNA of neoplasmic cells was also isolated. An analysis by means of agarose gel was performed and the result of it was a typical model of DNA ladder in cells treated with propolis with condensation higher than 10 μg/mL. These results confirm that propolis induces apoptosis in HL-60 cells.

In the recent years, scientists have focused on the isolation of substances present in propolis and on the evaluation of their biological properties. Thanks to potent antineoplastic properties, caffeic acid phenethyl ester is of great interest. Avci et al. [31] analyzed in their research proapoptotic properties of CAPE. They proved that this compound causes an increase in apoptotic index of human lymphoblastic leukemia cells treated with 10 μM CAPE. They also observed with the use of fluorescence microscopy a considerable accumulation of JC-1 dye. It suggests a loss of potential of mitochondrial membrane in CCRF-CEM cell lines exposed to CAPE. Using flow cytometry technique, they confirmed 45% growth of necrotic cells.

Research of Ishihara et al. [32] provide detailed information about anti-proliferative activity of ethanol extract of propolis coming from China and Brazil as regards cell line of colorectal cancer

($Caco_2$, HcCT 116, HT29 and SW480). In the research they demonstrated that EEP inhibits growth of human cell lines of colorectal cancer. Obtained results suggest that propolis includes a biologically active substance with anti-proliferative properties. Among 4 examined neoplasmic lines, HCT116, HT29 and SW480 lines were more susceptible to ethanol extract of propolis effects than CaCo2 line, which required considerably higher EEP concentrations (> 50 μg/mL) to inhibit the growth.

Interesting observations were found by Lee et al. [33], who investigated the influence of phenyloethyl ester of caffeic acid (CAPE) on the activity of protein p53 and protein p38 in glioblastoma cells C6. These results confirmed that propolis exibits cytotoxic activity and CAPE induces the release of cytochrome c from mitochondrium to cytosole and caspase-3 acvivation. Moreover, the expression of p53 protein, Bax and Bak were found to be increased after 3 h of incubation with CAPE causing simultaneously the decrease of expression of anti-apoptotic protein Bcl-2 following 36 h of incubation. The qualitative investigation carried out by our team with the use of fluorescent microscope revealed that melanoma malignant cells death was mainly through apoptocic pathway as opposed to colorectal cancer cells which after being exposed to ethanol extract of propolis showed typical changes for cell necrosis.

Similar results were obtained by Jin et al. [34], who performed a similar experiment on human leucoma cells line U937 and stated that CAPE has a citotoxic activity non-related to concentration and time exposure on cancer cells. Using the DAPI dye application, they observed the changes characteristic for apoptosis in cells nuclei. However, the did not report protein Fas expression on the surface of tested cells but confirmed a release of cytochrme c, inhibition of antiapoptotic Bcl-2 protein expression and the increase of expression of pro-apoptotic Bax protein.

Due to the severe impact of colon cancer and malignant melanoma worldwide, this aspect could be of significant clinical relevance. Since the present data was gathered within a *in vitro* setting and a selected cancer cells line, further investigations should take into account different types of malignant neo-cells and synergistic effect of EEPP with various anti-cancer drugs to support this potential intriguing aspect of cancer and its chemiotherapy. These studies, including model of anti-neoplastic regime, may deliver novel findings, which may provide insights on development or suppression of human cancers.

Ethanol extract of propolis from South-East region of Poland exhibits anti-proliferative activity in tested malignant melanoma Me45 and colorectal cancer HCT 116 cells. The results showed that in case of Me45 and HCT 116 cell lines, the ethanol extract of propolis could inhibit cell growth as well as cell size reduction. It is cytotoxic as regards cells of both examined lines and indicates the apoptosis stimulation for malignant melanoma cells.

However, their complex endocytotic pathways require thorough investigations. Further *ex vivo* studies on these malignant neoplasms should consider the combination of certain propolis compounds and ingredients with specific chemiotherapeutic drugs, representing anti-tumor drugs regime.

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