Chemical composition, cytotoxic and antioxidative activities of ethanolic extracts of propolis on HCT-116 cell line

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Abstract

BACKGROUND: Propolis is a complex resinous sticky substance that honeybees collect from buds and exudates of various plants. Due to propolis versatile biological and pharmacological activities, it is widely used in medicine, cosmetics and food industry. The aim of this study was to evaluate cytotoxic and antioxidative effects of various ethanolic extracts of propolis (EEP) on human colon cancer cell line (HCT-116) and compare it with their composition. HPLC-DAD method was used to determine the chemical composition of propolis samples.

RESULTS: The most abundant flavonoids in all samples were chrysin, pinocembrin and galangin (12.697- 40.811 μg mg^{-1}). On the other hand, main phenolic acids were caffeic, ferulic and isoferulic acid. Dose and time-dependent inhibition of cell growth of HCT-116 cells was observed in all propolis samples, with IC_{50} values ranging from 26.33 to 143.09 μg mL^{-1}. Differences in cytotoxic activity of propolis samples were associated with differences in their composition. Our results showed that all EEP samples reduced both superoxide anion radical and nitrite levels and also had strong DPPH scavenging activity.

CONCLUSION: All tested propolis samples had pronounced cytotoxic and antioxidative activities.

Key words: Propolis; Polyphenols; Cytotoxicity; Antioxidants; HCT-116 cell line

INTRODUCTION

Propolis (bee glue) is a complex resinous sticky substance that honeybees collect from buds and exudates of various plants and mix it with their own salivary secretions and waxes. It is thought to be used as a protective barrier and sterilant in beehives. Due to its numerous pharmacological properties, it has been used in folk medicine since ancient times.[1] The precise composition of raw propolis varies with the source. In general, it is composed of 50 % resin and vegetable balsam, 30 % wax, 10 % essential and aromatic oils, 5 % pollen and 5 % other various substances, including organic debris.[2] More than 300 constituents have been identified in different propolis samples.[3] Propolis contains a variety of chemical compounds such as polyphenols (flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones), sesquiterpene quinones, coumarins, steroids, amino acids, and inorganic compounds.[4]

Due to propolis versatile biological and pharmacological activities, it is widely used in medicine, cosmetics and food industry. Propolis and its derivatives possess numerous biological properties such as antibacterial, antioxidant, antiviral, anti-inflammatory, antitumoral, immunomodulatory, anti-HIV-1, antineurodegenerative and antituberculosis.[5-10] Furthermore, most of its components are natural constituents of food and recognized as safe substances.[11]

In literature, no data can be found about the composition and biological activities of Serbian propolis extracts. Therefore, this paper aims to characterize the phenolic composition of propolis samples by HPLC-DAD, as well as to characterize their cytotoxic and antioxidant activities.

EXPERIMENTAL

Chemicals

The phenolic standards apigenin, tectochrysin, galangin, hesperetin, kaempferol, luteolin, myricetin, naringenin, pinocembrin, chrysin, isorhamnetin, quercetin, *p*-hydroxybenzoic acid, caffeic acid, ferulic acid, and *p*-coumaric acid were purchased from Extrasynthese (Genay-Sedex, France). The analytical grade reagents - formic acid and ethanol, were obtained from Sigma (USA), and methanol and acetonitrile with HPLC purity were purchased from J.T. Baker (Holland). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA). Sodium nitrite ($NaNO_2$) and phosphoric acid were purchased from "Zorka pharma", Serbia. Dublecco's Modified Eagle Medium (DMEM) was obtained from GIBCO, Invitrogen, USA. Fetal bovine serum (FBS) and trypsin-EDTA were from PAA (The cell culture company), Austria. Dimethyl sulfoxide (DMSO), nitro blue tetrazolium (NBT), ethidium bromide and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from SERVA (Germany) and sulfanilic acid from MP Hemija, Serbia. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical was obtained from Acros organic, New Jersey, USA. N-(1-Naphthyl)ethylenediamine was purchased from Fluka Chemie AG, Buchs, Switzerland. 5-Fluorouracil was obtained from Sigma, USA.

Sample collection and propolis extract preparation

On the basis of literature data that seasonal variations in propolis composition are not significant (only minor quantitative changes) and the fact that composition of the plant source determines the chemical composition of propolis, we chose seven different locations that cover a relatively large area for our experiment.[12, 13]

Seven distinct propolis samples (S1–S7) were collected in the summer of 2011 from *Apis mellifera* hives located in different apiaries in the southwest of Serbia (locations of samples: S1, Mrckovina; S2, Babine; S3: Miljevici, S4: Jabuka, S5: Velika Zupa, S6: Zalug, S7: Kacevo). Raw propolis samples were obtained by scraping the frames of beehives, and stored at 4 °C until analysis. Prior to the extraction, the samples of propolis (10 g) (S1–S7) were grounded and homogenized. The samples were extracted in dark with 96 % ethanol (1:20 w/v), and mixed with magnetic stirrer at room temperature for 24 h. The resulting mixtures were filtered and stored overnight at 4 °C to induce the crystallization of dissolved waxes. The resultant solutions were filtered, concentrated on a rotary evaporator under reduced pressure at 40 °C, giving resinous red to brown products (EEP). The extracts were stored at 4 °C, protected from light, until use.

HPLC-DAD analysis of ethanolic extracts of propolis (EEP)

Experiments were performed on an Agilent 1200 Series HPLC system (Agilent Technologies, USA), equipped with degasser, autosampler and DAD detector. For chromatographic analysis, a Zorbax Eclipse Plus C18 column (150×4.6 mm i.d.; 1.8 μm) was used. The mobile phase consisted of 0.2 % (v/v) solution of formic acid in water (A) and acetonitrile (B). The gradient program was used as follows, with a total analysis time of 120 min: (0-3 min) 10 % B, (3-8 min) 10-25 % B, (8-11 min) 25 % B, (11-18 min) 25-30 % B, (18-48 min) 30-40 % B, (48-68 min) 40-60 % B, (68-88 min) 60-90 % B, (88-100 min) 90 % B, (100-101 min) 90-100 % B, and (101-120 min) 10 % B. The flow rate was 1.20 mL min^{-1}, the injection volume was 5 μL, while the temperature of the column oven was set at 40 °C. The chromatographic data were processed using Agilent Technologies ChemStation software, equipped with a spectral identification module of the compounds separated on the column.

By using DAD detector, absorption was detected in a range of wavelengths from 190 nm to 450 nm, while chromatograms were recorded at following wavelengths: 260 nm for p-hydroxybenzoic acid, myricetin, quercetin, kaempferol, isorhamnetin, chrysin, galangin and tectochrysin; 280 nm for naringenin, hesperetin and pinocembrin; 320 nm for caffeic acid, caffeic acid phenylethyl ester (CAPE), p-coumaric acid and luteolin; and 340 nm for ferulic acid, isoferulic acid and apigenin.

For quantitative analysis of the seven samples of ethanolic extracts of propolis, each analyte was dissolved in methanol at a concentration of 1000 μg mL^{-1}, and then diluted with methanol to appropriate concentrations (0.2-500 μg mL^{-1}) for the establishment of calibration curves. All of the standard solutions were kept at 4 °C.

Amounts of 10 mg of each extract (S1-S7) were dissolved in 1 mL of methanol and filtered through 0.45 μm filter (Millipore) prior to HPLC injection. An aliquot of 5 μL of solution was injected in triplicate for HPLC analysis.

Range of linearity

An aliquot of 5 μL of solution for each calibration standard solution was injected in triplicate for HPLC analysis. The calibration curve was constructed by plotting the peak areas versus the concentration for each analyte (Appendix 1).

Limits of detection (LOD) and limits of quantification (LOQ)

The stock solutions were diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions was injected for HPLC analysis. The limits of detection (LOD) and limits of quantification (LOQ) for each analyte were determined under the present chromatographic conditions at a signal-to-noise ratio (S/N) of about 3 and 10, respectively (Appendix 1).

Determination of cytotoxic and antioxidative activities of ethanolic extracts of propolis (EEP)

Cell preparation and culturing

Human colon cancer, HCT-116 cell line was obtained from American Type Culture Collection. Cells were maintained in DMEM medium, supplemented with 100 g L^{-1} heat-inactivated FBS, 100 IU mL^{-1} of penicillin and 100 µg mL^{-1} of streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were grown in 75 cm^2 culture bottles supplied with 15 mL of DMEM.

Treatment of cell line

EEP samples (S1–S7) were prepared as stock solutions (1000 µg mL^{-1}) in 10 g L^{-1} DMSO. Working solutions in the concentration range of 1–500 µg mL^{-1} were prepared prior to testing. HCT-116 cells (10000 cells per well) were seeded in a 96-well microtitre plates (exponentially growing viable cells were used throughout the assay) and 24 h later, after cell adherence, culturing medium was replaced with 100 µL of medium containing various doses of ethanolic propolis extracts at different concentrations (1, 10, 50, 100, 250 and 500 µg mL^{-1}) for cell viability assay and concentrations (10, 50, 100 and 500 µg mL^{-1}) for NBT and Griess assay, except in control wells, where only the nutrient medium was added to the cells. Cells were incubated with EEP samples for 24 h and 72 h prior to testing.

Cell Viability Assay (MTT Assay)

Cell viability was determined by MTT assay.[14] At the end of the treatment period, 25 µL of MTT solution (final concentration 5 mg mL^{-1} PBS) was added to each well and incubated at 37 °C in 5% CO_2 for 3 h. The colored crystals of produced formazan were dissolved in 150

μL DMSO. The absorbance was measured at 570 nm on microplate reader (ELISA 2100C). To determine cell viability (%), the absorbance (A) of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided by the control (the A of control cells grown only in culturing medium) and multiplied by 100. It was implied that the A of the blank was always subtracted from the A of the corresponding sample with target cells. We have also calculated the half maximal inhibitory concentration (IC_{50}), delineated as concentration of substance eliciting inhibition of cell growth by 50 % compared with a vehicle-treated control. As a positive control, 5-Fluorouracil (5-FU) was used. A DMSO solution was used as a negative control. All experiments were done in triplicate.

Determination of superoxide anion radical (NBT assay)

The concentration of superoxide anion radical ($O_2^{\cdot-}$) in the sample was determined by spectrophotometric method and is based on the reduction of nitroblue tetrazolium (NBT) to nitroblue-formazan in the presence of $O_2^{\cdot-}$.[15] Assay was performed by adding 10 μL of 5 mg mL^{-1} NBT to each well and then the cells were incubated for 45 min at 37 ^{o}C in 5% CO_2. To quantify the formazan product, formazan was solubilized in 10 μL DMSO and the resulting colour reaction was measured spectrophotometrically on microplate reader at 570 nm (ELISA 2100C). The amount of NBT reduced was determined by the change in absorbance at 560 nm, based on molar extinction coefficient for monoformazan that is 15,000 M^{-1} cm^{-1} and the results were expressed as nmol NBT mL^{-1}.

Nitric oxide (NO) measurement (Griess Assay)

The spectrophotometric determination of nitrites – NO_2^- (indicator of the nitric oxide – NO level) was performed by using the Griess method.[16] Experiments were performed at room temperature or at 37 °C in a warm room. Nitrite standard solution (100 mM) was serially

diluted from 100–1.6 μM in triplicate in a 96-well plate. Equal volumes 1 mg mL^{-1} of N-(1-naphthyl)ethylenediamine and 10 mg mL^{-1} of sulfanilic acid (solution in 50 g L^{-1} phosphoric acid) were mixed to form the Griess reagent immediately prior to application to cells. The absorbance at 550 nm was measured by using microplate reader (ELISA 2100C) following incubation (usually 5–10 min). The results were expressed in nmol nitrite mL^{-1} from a standard curve established in each test, constituted of known molar concentrations of nitrite.

DPPH assay

The method used by Takao et al.[17], was adopted with suitable modifications. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (8 mg) was dissolved in methanol (100 mL) to obtain a concentration of 80 μg mL^{-1}. Serial dilutions were carried out with stock solutions (1000 μg mL^{-1}) of the EEP to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.9 and 1.95 μg mL^{-1}. Diluted solutions (2 mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 and 60 min for any reaction to occur. The absorbance was recorded at 517 nm by using a Jenway 6105 UV/VIS spectrophotometer. The experiment was performed in triplicate and the average absorbance was noted for each concentration. The IC_{50} value, which is the concentration of the test extract that reduces 50 % of the initial free radical concentration, was calculated as l μg mL^{-1}. Ascorbic acid and BHT were used as reference standards, at the same concentrations in methanol as were used for the tested extracts. The control sample was prepared containing the same volume without test extracts and reference compounds. The radical-scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula:

$IC\ (\%) = [(A_0 - A_t) / A_0] \times 100;$

where A_t is the absorbance value of the tested sample and A_0 is the absorbance value of blank sample, at a particular time. Percentage inhibition after 30 and 60 min was plotted against

concentration, and the equation for the line was used to obtain the IC_{50} value. A lower IC_{50} value indicates greater antioxidant activity.

Statistical Analysis

The data is expressed as mean values ± standard errors (SE). Biological activity was performed in triplicate for each dose. Data were expressed as mean ± standard error and one-way analysis of variance (ANOVA) was performed to test for significant differences and between means and Fisher's Least Significant Difference (LSD) post hoc analysis using SPSS statistical software package (SPSS for Windows, version 17, 2008). Differences at $p \leq 0.05$ were considered significant. The magnitude of correlation between variables and the IC_{50} values were calculated from the dose curves by a computer program (CalcuSyn).

RESULTS AND DISCUSSION

Analysis by HPLC-DAD

Qualitative analysis

Analyzing the ethanolic extracts of propolis by the HPLC-DAD on the basis of the retention times, as well as UV spectra of standard compounds, five phenolic acids (*p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid and ferulic acid), one phenolic acid ester (caffeic acid phenylethyl ester, CAPE) and twelve flavonoids (myricetin, luteolin, quercetin, naringenin, apigenin, kaempferol, hesperetin, isorhamnetin, chrysin, pinocembrin, galangin and tectochrysin) were unequivocally identified **(**Figure 1**).**

Quantitative analysis

Equations for regression lines obtained from calibration curves, correlation coefficients, limits of detection, and limits of quantification and range of linearity of the target compounds were presented in Appendix 1**.**

Linear regressions of these standard compounds showed good linearity in the range of 0.098–500.000 μg mL^{-1} with correlation coefficient (r^2) ranging from 0.9986 to 0.9999. This allows the determination of these compounds over a wide range of concentrations. On the other hand, limits of detection were found within the range of 0.058–1.271 μg mL^{-1}, while the limits of quantification were within 0.098–2.119 μg mL^{-1} **(**Appendix 1**).**

The content of phenolic acids and flavonoids of the seven ethanolic propolis samples (S1-S7) is reported in Table 1. In analyzed samples, with the total concentration of 25.415 μg mg^{-1}, S5 contains the highest level of phenolic acids. The most common compound from this group was caffeic acid found in the ranges of 5.989 μg mg^{-1} (in S7) to 8.931 μg mg^{-1} (in S1) (except in S3; 3.372 μg mg^{-1}). The content of caffeic acid phenylethyl ester (CAPE) was found in the range 6.053 μg mg^{-1} (in sample S3) to 8.209 μg mg^{-1} (in sample S5). Also, ferulic acid was found in high amount in S3 (7.584 μg mg^{-1}), as well as isoferulic acid in samples S4, S5 and S6 (5.490 μg mg^{-1}, 6.624 μg $mg^{-1}$and 5.263 μg mg^{-1}, respectively). The lowest concentration was observed in the case of *p*-coumaric acid (0.180 μg/mg-0.281 μg mg^{-1}). These results are quite similar to those obtained in investigations performed on propolis samples from Croatia, Bosnia and Hercegovina and Macedonia.[18]

Similarly to the above mentioned, the sample labeled as S5 contains the highest level of analyzed flavonoids and CAPE (139.875 μg mg^{-1} and 8.209 μg mg^{-1}, respectively). On the other hand, with the total concentration of 59.058 μg mg^{-1}, S3 showed the lowest level of these compounds (Table 1). The most abundant compounds in all samples were chrysin,

pinocembrin and galangin (12.697 μg mg^{-1}-40.811 μg mg^{-1}), while other flavonoids and CAPE were detected in small amounts. Samples S5 and S1 were especially characteristic, showing a high level of chrysin, pinocembrin and galangin (S5: 40.811 μg mg^{-1}, 40.324 μg mg^{-1} and 35.586 μg mg^{-1}, respectively; S1: 34.124 μg mg^{-1}, 35.546 μg $mg^{-1}$and 30.432 μg mg^{-1}, respectively). From this aspect, our propolis samples showed similarity to analyzed samples from Bulgaria, Italy and Switzerland.[19] In all analyzed extracts, myricetin, luteolin and hesperetin were observed in a concentration lower than 1.000 μg mg^{-1}.

Cytotoxic effects of ethanolic extracts of propolis

Cytotoxic effects of the seven EEP samples were assessed on HCT-116 cell line. Results are presented as a percentage of cell viability compared to untreated, control cells (Figure 2). Dose and time-dependent inhibition of cell growth was observed in all cells treated with all seven propolis samples at tested concentration range. All propolis samples had pronounced cytotoxic effects at highest applied concentration ranging from 19 to 47 % for 24 h treatment and 15 to 23 % for 72 h treatment.

Results of *in vitro* cytotoxic activity of seven investigated EEP were also expressed by IC_{50} values, presented in Table 2. The most pronounced cytotoxic effect 24 h after treatment exhibited samples EEP S1 and S4 (184.08 and 191.13 μg mL^{-1}, respectively) and 72 h after treatment propolis samples S4, S5 and S6 (26.33, 39.81 and 38.54 μg mL^{-1}, respectively). Other propolis extracts exhibited only weak cytotoxic effect. According to the American National Cancer Institute (NCI), the criteria of cytotoxic activity for the crude extracts is IC_{50} < 30 μg/mL.[20] In consideration to this criteria and observed IC_{50} values, we can conclude that propolis sample S4 has a strong antiproliferative effect on the HCT-116 cell line 72 h after treatment and that sample S5 and S6 also have significant antiproliferative effects, their IC_{50} values being on the upper limit.

Several research groups showed that phenolic compounds, flavonoids and CAPE from propolis elicited protective effect both in *in vitro* and *in vivo* colon cancer models.[21-25] Differences in cytotoxic activity of propolis samples are associated with differences in composition of phenolic acid, flavonoids and CAPE, which was observed by HPLC-DAD analysis. This corresponds with very pronounced antiproliferative effect of samples S4 and S5, having the highest level of phenolic acids, flavonoids and CAPE.

Cytotoxic effect of 5-fluorouracil was used as positive control to evaluate cytotoxic effects of propolis samples. Our data showed that IC_{50} value for 5-fluorouracil (0.81 μg mL^{-1}) was around 32 times higher than IC_{50} value of most cytotoxic propolis sample S4 (26.33 μg mL^{-1}). Conversely, 5-fluorouracil used as a standard chemotherapeutic drug has around 62 times higher acute oral toxicity (LD_{50} = 115 mg kg^{-1}) than propolis (LD_{50} = 2000–7300 mg kg^{-1}) in mouse model.[26] Due to its antiproliferative effects and low oral toxicity, propolis could be used over a prolonged period of time for chemoprevention and supportive therapy. The negative control DMSO had no inhibitory effect on the tested cell lines.

Antioxidative properties of EEP on HCT-116 cells production of superoxide anion radical and nitrites

In order to explain the mechanism of cytotoxic activities of EEP on the HCT-116 cell line, we followed antioxidative properties of investigated extracts on HCT-116 cells. The data presented in Table 3 present the release of $O_2^{\cdot -}$ as nmol NBT mL^{-1}, 24 h and 72 h after incubation with seven ethanolic propolis extracts. Samples S1 and S2, at all concentrations (10, 50, 100 and 500 μg mL^{-1}), induced significantly lower level of superoxide anion radical production in HCT-116 cells compared to control cells 24 h after treatment. All other samples did not have any significant impact on superoxide anion radical production at 500 μg mL^{-1} concentration. All propolis samples induced significantly lower level of superoxide anion radical in HCT-116 cells compared to control cells, except samples S5 and S7 at highest

concentration applied that caused higher level of superoxide anion radical compared to control cells, 72 h after treatment.

Determination of the nitrite (NO_2^-) concentration demonstrated that all ethanolic extracts of propolis at all concentrations applied were able to reduce the release of NO (Table 4) in comparison with control cells. All propolis extracts, except S7 at lowest and highest concentration, significantly reduced the level of nitrites after 72 h of exposure compared to 24 h exposure.

Some studies suggested that the antiproliferative effects of some polyphenol antioxidants on cancer cells are partially due to their prooxidant actions.[27] In our experiment, propolis samples S5 and S7 that have highest phenolic acids content induced the highest levels of superoxide anion radical 72 h after treatment at highest concentration applied and maybe, at least in part, that could be the source of their good antiproliferative potential. On the other hand, due to their ability to scavenge and reduce the production of free radicals, and because they act as transition metal chelators, natural phenolic compounds may exert a major chemopreventive activity.[28] Our data showed that all propolis samples had stronger antioxidant properties 72 h after treatment compared to 24 h treatment, since they reduce both levels of superoxide anion radical and nitrites and may have protective role against free radical production, that together with other factors are responsible for cellular aging and many conditions such as cancer, cardiovascular diseases, diabetes, arthritis, Parkinson disease and Alzheimer.[29]

Free radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl radical by ethanolic propolis extracts

This assay is based on the measurements of the scavenging ability of extracts towards the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The antioxidant activity was expressed

as the 50 % inhibitory concentration (IC_{50}) based on the amount of extract required for a 50 % decrease of the initial DPPH radical concentration. IC_{50} values of EEP samples S1–S7 are presented in Table 5 and are ranging from 55.45 to 118.46 μg mL^{-1}. All samples showed lower radical-scavenging activities in comparison to butylated hydroxytoluene (BHT) and ascorbic acid standards. Samples S1, S4 and S7 had most effective scavenging activity (IC_{50} values were 70.42, 55.45 and 56.68 μg mL^{-1}, respectively).

Propolis samples with strong DPPH scavenging activity contained higher concentrations of antioxidative phenolic compounds compared to other samples, such as caffeic acid (S7 and S1), isoferulic acid (S4) and especially high levels of flavonoids with most abundant being chrysin, pinocembrin and galangin (S1). This positive corelation, however is not observed in all samples, since we should expect that sample S5 with highest amount of phenolic acids, flavonoids and CAPE has more pronounced scavenging activity because flavonoids and CAPE are found to be most effective antioxidant in propolis.[30, 31] Studies on volatiles, especially aroma-active components are reported to help improve the medicinal effect of propolis.[32]

Polyphenolic compounds identified in our propolis samples, such as flavonols, are the most abundant flavonoids and are common compounds in the human diet. They are found in onions, apples and tea, with quercetin, kaempferol and myricetin being the three most common flavonols. Flavanones are mainly found in citrus fruit and flavones in celery.

Many research groups have done *in vivo* and *in vitro* studies of colon cancer and showed that tea, apples and onions extracts exhibited pronounced antioxidative and cytotoxic effects comparable to our results with propolis.[33-35]

CONCLUSIONS

Today propolis is widely used as a dietary supplement and is one of the richest sources of plant phenolics and polyphenolics and is claimed to improve health and prevent diseases such

as inflammation, heart disease, diabetes and even cancer. We have determined chemical composition, on the basis of five standards of phenolic acids and twelve standards of flavonoids, by HPLC-DAD analysis in order to characterize phenolic acid and flavonoid content in propolis samples from Serbia.

We have evaluated antiproliferative effects of ethanolic extracts of propolis on human colon cancer cell line, HCT-116 and we observed that differences in cytotoxic activity of propolis samples are correlated with their differences in composition of phenolic acids and flavonoids, since samples rich in phenolic acids, flavonoids and CAPE also exhibited stronger cytotoxic effects.

Propolis is also reported to exert antioxidative activity due to its phenolic constituents. Our results showed that ethanolic extracts of propolis reduced both superoxide anion radical and nitrite levels and also had strong DPPH scavenging activity, but also that not only phenolic compounds are involved in this process, since there is no obvious correlation with their content of phenolic compounds and their antioxidative activity. For that reason additional qualitative and quantitative analyses of the propolis compounds are needed to explain exactly antioxidant activity of propolis.

Tested propolis samples had pronounced antiproliferative and antioxidative activities and may be considered as safe and healthy food supplements with chemopreventive properties, since relatively large amounts may be ingested without side effects, because of propolis low oral toxicity compared to standard chemotherapeutic drugs.

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Table 1. Concentration of analyzed phenolic compounds in ethanolic extracts of propolis

Compound	*Retention time* (min)	*Concentration* (μg mg^{-1} of EEP)						
		S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**
p-hydroxybenzoic acid	4.64	0.256±0.003	0.251±0.003	0.180±0.002	0.235±0.003	0.281±0.003	0.232±0.003	0.215±0.002
caffeic acid	5.91	8.931±0.098	7.919±0.087	3.372±0.040	7.271±0.079	8.872±0.095	6.968±0.077	5.989±0.072
p-coumaric acid	7.75	3.553±0.043	3.515±0.047	4.488±0.049	3.582±0.047	4.649±0.047	3.663±0.045	3.726±0.049
ferulic acid	8.61	2.601±0.033	2.209±0.029	7.584±0.086	3.482±0.053	4.989±0.059	3.382±0.047	4.546±0.058
isoferulic acid*	8.99	0.336±0.041	0.289±0.034	2.448±0.036	5.490±0.061	6.624±0.063	5.263±0.059	4.460±0.039
myricetin	10.51	0.613±0.007	0.647±0.009	0.500±0.006	0.614±0.008	0.703±0.008	0.635±0.007	0.572±0.006
luteolin	13.81	0.853±0.010	0.922±0.011	0.667±0.007	0.823±0.010	0.898±0.011	0.789±0.009	0.750±0.008
quercetin	14.27	2.849±0.034	2.893±0.035	1.696±0.023	2.746±0.029	3.457±0.045	2.664±0.031	2.427±0.027
naringenin	17.87	0.928±0.011	0.960±0.012	1.328±0.018	0.961±0.012	1.230±0.015	0.981±0.014	1.668±0.019
apigenin	18.33	3.860±0.047	3.841±0.045	2.469±0.021	3.929±0.049	4.658±0.056	3.653±0.035	3.345±0.029
kaempferol	19.20	4.625±0.051	3.834±0.041	2.445±0.031	3.588±0.037	4.636±0.052	3.520±0.028	3.197±0.024
hesperetin	19.79	0.415±0.035	0.389±0.032	0.661±0.039	0.347±0.028	0.448±0.036	0.372±0.039	0.453±0.036
isorhamnetin	20.05	0.950±0.048	0.840±0.041	1.855±0.019	0.804±0.039	0.869±0.042	0.779±0.038	0.763±0.037
chrysin	33.03	34.124±0.375	29.687±0.327	16.665±0.183	35.555±0.381	40.811±0.449	28.490±0.311	26.729±0.294
pinocembrin	34.87	35.546±0.379	35.322±0.368	15.244±0.175	32.672±0.325	40.324±0.401	31.934±0.311	27.150±0.302
galangin	36.21	30.432±0.287	29.771±0.276	12.697±0.128	27.389±0.295	35.586±0.374	27.122±0.288	23.119±0.254
caffeic acid phenylethyl ester**	38.71	6.552±0.072	7.075±0.079	6.053±0.067	6.869±0.076	8.209±0.090	7.153±0.079	6.637±0.073
tectochrysin	57.31	6.247±0.074	5.571±0.068	2.831±0.029	4.336±0.049	6.255±0.075	5.239±0.067	4.477±0.051

Results are mean values ± SD from at least three experiments
* Semiquantitatively determined from the calibration curve of ferulic acid
**Semiquantitatively determined from the calibration curve of caffeic acid

Table 2. Growth inhibitory effects-IC_{50} values (μg mL^{-1}) of ethanolic extracts from different propolis samples on HCT-116 cell line after 24 h and 72 h of exposure. IC_{50} values were determined by linear regression analysis.

Sample	IC_{50} (μg mL^{-1})	
	24 h	72 h
S1	184.08±14.83	65.58±3.24
S2	466.72±0.53	143.09±2.66
S3	464.21±19.2	65.13±1.13
S4	191.13±5.62	26.33±2.97
S5	451.04±7.16	39.81±1.01
S6	388.55±8.17	38.54±1.56
S7	294.62±10.67	93.64±8.19
5-Fluorouracil	0.018±0.004	0.81±0.49

Results are mean values ± SE from at least three experiments. 5-Fluorouracil is positive control for propolis cytotoxic evaluation.

Table 3. Effect of EEP samples, on HCT-116 cell line after 24 and 72 h of exposure, on superoxide anion radical ($O_2^{\cdot-}$) production expressed as nmol mL^{-1}.

	Concetration of EEP				
Sample	**0 μg mL^{-1}**	**10 μg mL^{-1}**	**50 μg mL^{-1}**	**100 μg mL^{-1}**	**500 μg mL^{-1}**
	after 24 h				
S1	33.02±2.05	29.32±0.60*	30.03±0.84*	29.53±0.57*	30.96±0.42*
S2	33.02±2.05	29.40±0.28*#	29.87±0.63*#	30.48±0.44*#	30.18±0.11*#
S3	33.02±2.05	31.69±1#	30.04±0.11*	30.48±0.88*	32.36±0.68#
S4	33.02±2.05	32.17±0.50#	30.04±0.69*	30.70±0.10*#	32.94±1.75
S5	33.02±2.05	29.10±0.61*#	31.60±0.43#	30.70±0.33#	31.90±0.47#
S6	33.02±2.05	28.99±1.12*#	29.98±1.16*#	33.12±0.50#	31.26±0.42
S7	33.02±2.05	30.70±0.37*#	29.23±0.52*#	30.36±0.60*	32.74±0.24#
Sample	**0 μg mL^{-1}**	**10 μg mL^{-1}**	**50 μg mL^{-1}**	**100 μg mL^{-1}**	**500 μg mL^{-1}**
	after 72 h				
S1	32.16±0.40	29.48±1.24*	29.66±0.64*	28.22±0.53*	28.25±1.74*
S2	32.16±0.40	27.57±1.10*	27.76±0.44*	28.70±0.85*	28.49±0.68*
S3	32.16±0.40	28.66±0.48*	29.30±0.42*	29.09±0.41*	30.54±0.35*
S4	32.16±0.40	28.76±0.47*	29.30±0.70*	28.86±0.33*	32.01±0.47
S5	32.16±0.40	32.68±1.86	22.76±0.72*	28.86±0.69*	33.32±0.99*
S6	32.16±0.40	18.81±0.90*	23.04±0.32*	29.80±0.50*	32.54±0.52
S7	32.16±0.40	23.50±0.71*	23.48±0.59*	28.59±0.37*	34.42±0.25*

All values are mean values ± SE from at least three experiments, *$p < 0.05$ compared with control and # $p < 0.05$ comparision after 24 h and 72 h of treatment.

Table 4. Effect of ethanolic extracts from different propolis samples, on HCT-116 cell line after 24 and 72 h of exposure, on the nitrite (NO_2^-) production expressed as nmol mL^{-1}.

Sample	Concetration of EEP				
	0 µg mL^{-1}	**10 µg mL^{-1}**	**50 µg mL^{-1}**	**100 µg mL^{-1}**	**500 µg mL^{-1}**
	after 24 h				
S1	19.30±0.14	16.07±0.15*#	14.73±0.41*#	14.63±0.70*#	14.70±0.15*#
S2	19.30±0.14	16.45±0.05*#	15.75±0.27*#	15.78±0.79*#	14.29±0.40*#
S3	19.30±0.14	16.11±0.30*#	14.75±0.73*#	14.61±0.60*#	16.77±0.24*#
S4	19.30±0.14	17.90± 1.03#	16.33±0.82*#	16.17±0.70*#	17.06±0.30*#
S5	19.30±0.14	16.84±0.20*#	16.69±0.16*#	16.63±0.26*#	16.08±0.13*#
S6	19.30±0.14	17.47±0.43*#	16.22±0.31*#	16.01±0.19*#	15.72±0.30*#
S7	19.30±0.14	17.78±0.20*#	17.04±0.04*#	17.90±0.26*#	18.13±0.18*#
Sample	**0 µg mL^{-1}**	**10 µg mL^{-1}**	**50 µg mL^{-1}**	**100 µg mL^{-1}**	**500 µg mL^{-1}**
	after 72 h				
S1	17.49±0.42	12.85±0.22*	9.90±0.27*	9.62±0.45*	10.54±0.37*
S2	17.49±0.42	14.33±0.55*	7.38±0.44*	0.70±0*	1.48±0.01*
S3	17.49±0.42	7.02±0.75*	2.60±0.21*	2.67±0.40*	13.49±0.72*
S4	17.49±0.42	5.76± 0.07*	7.87±0.23*	2.04±0.18*	19.81±0.60
S5	17.49±0.42	1.48±0.11*	3.79±0.18*	1.69±0.10*	6.18±0.19*
S6	17.49±0.42	13.77±0.65*	3.79±0.23*	1.69±0.10*	9.48±0.40*
S7	17.49±0.42	23.81±0.38*	9.27±0.27*	8.08±0.31*	29.15±0.43*

All values are mean values ± SE from at least three experiments, *$p < 0.05$ compared with control and #$p < 0.05$ comparision after 24 h and 72 h of treatment.

Table 5. Free radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl radical by ethanolic propolis extracts. IC_{50} values were determined by linear regression analysis.

Sample	**IC_{50} (µg mL^{-1})**	
	30 min	60 min
S1	70.42±20.32	57.75±17.34
S2	103.88±27.66	95.76±25.97
S3	87.13±27.42	81.81±26
S4	55.45±20.61	51.28±18.57
S5	118.46±36.44	74.69±30.56
S6	75.75±23.21	69.15±20.59
S7	56.68±19.62	53.97±18.46
BHT	25.4 ± 22.70	12.78 ± 7.60
Asc	42.4 ± 22.70	27.22 ±14.30

Results are mean values ± SE from at least three experiments

Figure 1. HPLC-DAD chromatogram (λ=260 nm) for EEP of sample S2

HPLC DAD chromatogram at 260 nm of EEP of sample S2. Peaks: 1, *p*-hydroxybenzoic acid; 2, caffeic acid; 3, *p*-coumaric acid; 4, ferulic acid; 5, isoferulic acid; 6, myricetin; 7, luteolin; 8, quercetin; 9, naringenin; 10, apigenin; 11, kaempferol; 12, hesperetin; 13, isorhamnetin; 14, chrysin; 15, pinocembrin; 16, galangin; 17, caffeic acid phenylethyl ester; 18, tectochrysin.

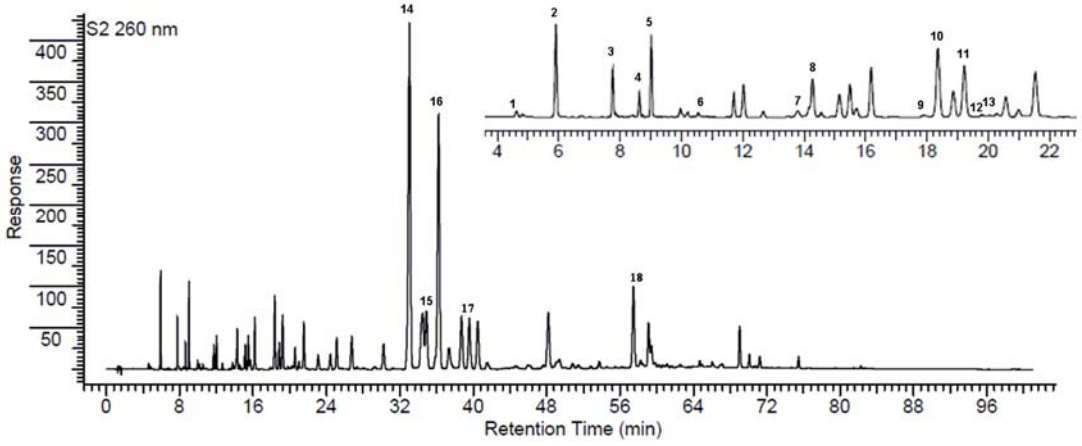

Figure 2. The dose-response curves of the effects of ethanolic propolis extracts (S1-S7) on cell growth of HCT-116 cells. The cells were treated with various concentrations of drugs for 24 h and 72 h. The cytotoxic effects were measured by MTT assay.

Results were expressed as mean values ± SE for three independent determinations.