JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Oxidant/Antioxidant Properties of Croatian Native Propolis

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Native propolis was defined as propolis powder collected from the continental part of Croatia and prepared according to a patented process that preserves all the propolis natural nutritional and organoleptic qualities. Nine phenolic compounds (out of thirteen tested) in propolis sample were detected by high performance liquid chromatography (HPLC) analysis. Among them chrysin was the most abundant (2478.5 μg/g propolis). Contrary to moderate antioxidant activity of propolis examined in vitro (ferric reduction antioxidant power; FRAP-assay), propolis as a food supplement modulated antioxidant enzymes (AOE) and significantly decreased lipid peroxidation processes (LPO) in plasma, liver, lungs, and brain of mice. The effect was dose- and tissue-dependent. The lower dose (100 mg/kg bw) protected plasma from oxidation, whereas the higher dose (300 mg/kg bw) was pro-oxidative. Hyperoxia (long-term normobaric 100% oxygen) increased LPO in all three organs tested. The highest vulnerability to oxidative stress was observed in lungs where hyperoxia was not associated with augmentation of AOE. Propolis protected lungs from hyperoxia by increased catalase (CAT) activity. This is of special importance for lungs since lungs of adult animals are highly vulnerable to oxidative stress because of their inability to augment AOE activity. Because of its strong antioxidant and scavenging abilities, native propolis might be used as a strong plant-based antioxidant effective not only in physiological conditions but also in cases that require prolonged high concentration of oxygen.

KEYWORDS: Propolis; antioxidant enzymes; lipid peroxidation; flavonoids.

INTRODUCTION

Honey-bee products have been used since ancient times in folk medicine. However, the study of bee products is no longer relegated to the status of folk medicine. Propolis, as one of the most investigated bee products, is considered to have antiviral (*1*), antibacterial (*2*), antifungal (*3*), immunomodulatory, anti-metastatic (*4*), and antioxidant properties (*5*) due to its scavenging capacity of free radicals (*6*).

Diverse smell, color, constitution, and chemical composition of propolis are due to different sources from which it is collected. In general, it is composed of 50% balsams (including phenolics) and resins, 30% wax, 5% pollen, and 10% essential oils. Flavonoids are potent antioxidants that can prevent cell damage caused by free radicals (*7*, *8*). Even neuroprotection by flavonoids has been demonstrated (*9*). In contrast to their beneficial effects, some flavonoids have been found to be mutagenic in vitro (*10*). These harmful effects were suspected to result from pro-oxidant rather than antioxidant action of the related flavonoids (*11*, *12*). Identification of the active functional ingredient(s) and the question about dose−effect responses for beneficial versus harmful effects are still of interest. Besides, to the best of our knowledge most of the studies of antioxidative properties of propolis were done by measuring antioxidative effects in vitro mostly by 2,2-diphenyl-1-picrylhydrazyl (DPPH) or ferric-reducing antioxidant potential (FRAP) assay (*13*, *14*) on different cell lines (*15*) or isolated hepatocytes (*16*). Among the few reports of in vivo treatment is the study by Sforcin et al. (*17*) of superoxide dismutase (SOD) in plasma of rats treated with Brazilian propolis. No such in vivo study of the effect of Croatian propolis on LPO and AOE in different tissues of mice has been done so far.

Supplemental oxygen used in the treatment of several diseases (*18*) and bronchopulmonary dysplasia after premature birth (*19*) or by military divers induces tissue damage due to free radical formation which might be prevented by propolis because of its potential antioxidant properties.

In this study we aimed to assess the possible modulation of oxidant (LPO)/ antioxidant (total-tSOD, MnSOD, CuZnSOD, CAT, glutathione peroxidase-Gpx) status in mice liver, brain,

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10.1021/jf0612023 CCC: $33.50 © 2006 American Chemical Society
Published on Web 09/27/2006

lungs, and blood by food supplemented with two doses of propolis harvested in naturally preserved, continental regions of Croatia. Also, the possible contribution to oxidant/antioxidant status of such propolis treatment was evaluated in mice subjected to oxidative stress induced by long-term, normobaric oxygen (100% oxygen for 18 h).

MATERIALS AND METHODS

Chemicals. Horse heart cytochrome C (Type VI) and human blood CuZnSOD (Type I, lyophilized powder, 2400 U/mg protein), bovine serum albumin, hydrogen peroxide (30%), bovine liver catalase and xantine, xantine oxidase, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane, dodecyl sulfate sodium salt, and flavonoids (naringenin, quercetin, taxifolin, pinocembrin, genistein) as well as Folin Ciocalteu reagent, buthylated hydroxytoluene (BHT), Fe^{2+}, vitamin C, (+)-catechin, and trolox, were purchased from Sigma, St. Louis, MO. Other flavonoids and phenolics (galangin, isorhamnetin, myricetin, chrysin, kaempherol, luteolin, daidzein, phenylpropanoid caffeic acid) were purchased from Fluka, Switzerland.

Gpx (RANSEL) and SOD (RANSOD) assay kit was obtained from RANDOX, San Diego, CA. HPLC-grade solvents were purchased from Merck, Germany. Nylon filters Schleicher & Schnell (0.2 μm pore) were used. All other chemicals were of analytical grade.

Propolis. In our study, we used a representative mixture of propolis obtained from apiaries located in naturally preserved areas of continental Croatia. Propolis was collected from spring to winter by special ecological nets introduced into hives. Bees filled holes in the plastic nets with propolis. Nets were taken and frozen to promote propolis removal. Samples of propolis were pooled by seasons. In this way, a clean origin propolis without any mechanical impurities and parts of dead bees was obtained. To pulverize the viscous, crude propolis into a powder, an original treatment by firm HEDERA d.o.o.(Strobreé Croatia) was performed with no chemical refinement. Chemical analysis of this native propolis, using standard methods, showed that it contained 4.0% water, 25.6% fat (*20*), 1.6% protein (*21*), 0.7% ashes and minerals (*22*) as follows: lead (Pb) 2.15 mg/kg, iron (Fe) 344.5 mg/ kg, copper (Cu) 1.85 mg/kg, mercury (Hg) 0.0078 mg/kg, zinc (Zn) 131 mg/kg, manganese (Mn) 7.61 mg/kg, chromium (Cr) 0.707 mg/kg, calcium (Ca) 770 mg/kg, magnesium (Mg) 271 mg/kg. Analyses of vitamin (*23*) content in this native propolis were as follows: vitamin A < 10 μg/100 g, vitamin C < 1 mg/100 g, vitamin B_1 1.45 mg/100 g, B_2 0.062 mg/100 g, vitamin B_6 2.25 mg/100 g. This native propolis sample was used in our study for determination of LPO and AOE activity.

Propolis Extract. Propolis (1 g) was homogenized in a chilled mortar and mixed vigorously with 10.45 mL of 80% (V/V) ethanol during 72 h at the room temperature. The extract was filtered through Whatman No.1 paper and the residue was washed with 0.5 mL of 80% ethanol. Such prepared extract was kept at −20 °C, at least for 24 h. This extract was filtered through nylon filter Schleicher&Schnell (0.2 μm pore) and submitted to HPLC analysis and FRAP assay.

HPLC Analyses. Qualitative and quantitative chromatographic analysis of phenolics was performed on a HPLC system (Agilent 1100 Series) equipped with a quaternary pump, multiwave UV/vis detector, autosampler, and fraction collector. The column used was a 5 μm Zorbax RX-C18 (250 × 4.6 mm, Agilent Technologies). Injection volume was 200 μL, flow rate 1.0 mL/min, and temperature was 45 °C. The propolis extract was fractionated. Fractions 1 (t_R 9.8−10.6 min), 2 (t_R 12.8−13.6 min), 3 (t_R 18.9−20.3 min), 4 (t_R 22.7−23.7 min), 5 (t_R 24.3−25.3 min), 6 (t_R 26.5−27.8 min), 7 (t_R 28−29 min), 8 (t_R 30−31 min), 9 (t_R 32.6−34.1 min) were obtained using elution profile consisting of solvent A (5% formic acid) and solvent B (methanol). Linear gradient from 10% to 90% B within 45 min was used.

Phenolic compounds of collected fractions were identified by UV−vis spectroscopy at 268 nm 280 nm, 374 nm, 310 nm, 350 nm (to cover possible two maximal absorption values from 240−280 nm and 340−380 nm). In addition, chromatography (HPLC) with authentic standards was performed. Fractions 1, 2, 3, 4, 5, 6, 7, 8 were analyzed using solvents A (5% formic acid) and B (acetonitrile) on linear gradients from 5% to 53% solvent B within 30 min. Fraction 9 was analyzed using isocratic separation on 33.5% solvent B in 30 min.

Total Phenol Analysis. The procedure used is based on the colorimetric method of Singleton and Rossi (*24*) outlined by Folin-Ciocalteau. Catechin was used as a standard, and the results were expressed as catechin equivalents. The total phenolic content in methanolic extract of propolis was 10160 ± 360 mg CE/L or 101.6 ± 3.6 mg CE/g of propolis. Data presented mean ± SEM are average of three measurements.

Phenolic Standards. The following compounds, representative of various subclasses of phenolics, were used in our study: flavonol (quercetin, isorhamnetin, kaempherol, galangin, myricetin, taxifolin), flavone (luteolin), flavanone (pinocembrin, naringenin), isoflavone (chrysin, genistein, daidzein), and phenolic acid (phenylpropanoid caffeic acid). All of the standards were dissolved in ethanol (96%, V/V) to give 0.01 mg/mL solutions.

Determination of Antioxidant Activity of Propolis in Vitro. The total antioxidant potential of each sample was determined using a FRAP assay (*25*). Standard curves were prepared using different concentrations (100−1000 μmol/L) of $FeSO_4 \times 7H_2O$. All solutions were used on the day of preparation. In the FRAP assay, the antioxidant efficiency of the antioxidant under the test was calculated by reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. Ascorbate (vitamin C), (+)-catechin, Fe^{2+}, trolox, and BHT were measured within 1 h after preparation. All determinations were performed in triplicate. The propolis extract to be analyzed was first adequately diluted to fit within the linearity range. The same vehicle was used in preparation of propolis and the controls.

Animals and Experimental Design. Female CBA/Hr mice aged 4 months from a breeding colony at the Ruđer Bošković Institute (Zagreb, Croatia) were used. The animals were maintained under the following laboratory conditions: four to a cage; light on from 06:00 to 18:00; 22 ± 2 °C room-temperature; access to food pellets, and tap water ad lib. Experimental groups consisted of 8 mice each. Mice were fed 14 days before testing either with commercial food pellet (control group) or with commercial food mixed with propolis powder (100 mg/kg bw or 300 mg/kg bw, respectively). Doses of 100 mg/kg or 300 mg/kg of native propolis were used to correspond the doses usually used in humans with correction for mouse metabolism.

Other groups of mice were subjected to normobaric oxygen (100% O_2 for 18 h) in a hyperbaric chamber after feeding mice for 14 days with commercial food pellet (control normobaric group) or by commercial food mixed with propolis powder (100 mg/kg bw or 300 mg/kg bw, respectively). Normobaric oxygen conditions were carried out by flushing the chamber (Đuro Đaković, Slavonski Brod, Croatia) by pure oxygen (25 L/min for 10 min) to replace room air. To test the best way of introducing propolis to mice, we studied the possibility of gauge feeding too. In several experiments, to test this possibility, mice received buffer saline once a day for 2 weeks by gauge feeding.

This study was in compliance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ministry of Agriculture Forestry and Water Management, Republic of Croatia.

Acquisition of Samples. Mice were anesthetized with diethyl ether and bled from the jugular vein into heparinized tubes. Whole blood was centrifuged (2000*g* for 10 min), and plasma was used for LPO determination by following the formation of malondialdehyde according to the presence of thiobarbituric acid reactive substances (TBARS). Erythrocytes in the remaining pellet were lysed in cold deionized water, and the AOE activities were determined in the lysate. Hemoglobin concentration was measured by Cell-Dyn 1600 (Abbot, USA). Liver, brain, and lung were removed immediately by manual dissection, blotted on filter paper, and weighed. A portion of the tissue was placed into 1.15% KCl on ice for determination of TBARS production and homogenized (1300 rpm; 10% w/v for liver and lungs and 20% w/v for brain tissue) using an ice-packed Potter-Elvehjem homogenizer (Braun, Biotech. Int., Germany). To determine the AOE a section of tissue was placed in 50 mM phosphate buffer (pH = 7.8), homogenized (10% w/v for liver and lungs and 20% w/v for brain tissue), sonicated on ice for 30 s in three 10 s intervals, and centrifuged at 4 °C (20000*g*

for 15 min). LPO was determined on the same day while aliquots of the resulting supernatant for determination of AOE were stored in plastic tubes at −70 °C until assayed. The absorbance of LPO product and AOE activity was monitored using a Camspec M330 UV−vis spectrophotometer equipped with M330 Camspec software package (Camspec Ltd., Cambridge, UK).

Assay for LPO. The lipid peroxides in the liver, lungs, and brain were estimated by the measurement of TBARS according to the method by Ohkawa et al. (*26*). In the plasma, TBARS concentration was determined as described by Schlorff et al. (*27*). Briefly, tissue homogenate or plasma was mixed with sodium dodecyl sulfate, acetate buffer (pH 3.5), and an aqueous solution of thiobarbituric acid. After being heated at 95 °C for 60 min, the red pigment produced was extracted with *n*-butanol−pyridine mixture and estimated by the absorbance at 532 nm. The results were expressed as nmol/mg of protein in liver, lung, and brain tissue and as nmol/ mL plasma in blood according to a standard curve which was prepared with serial dilutions of standard 1,1,3,3-tetramethoxypropane.

Assay for tSOD, MnSOD, and CuZnSOD. tSOD in the lysate of erythrocytes was determined by RANSOD assay kit. In the liver, lungs, and brain, tSOD was assayed by the method of Flohé and Ötting (*28*). MnSOD activity was determined under the same conditions with addition of 3 mM KCN for 30 min in the assay buffer to inhibit CuZnSOD (*29*). CuZnSOD was obtained by subtracting the MnSOD from the tSOD activity. One unit of tSOD, CuZnSOD, and MnSOD activity was defined as the amount of enzyme required to give 50% inhibition in the typical calibration curve obtained with standard SOD and was expressed as IU/mg protein. In the lysate of erythrocyte, SOD activity was expressed as U/mg Hb.

Determination of CAT Activity. CAT activity in lysate of erythrocytes and tissues was determined according to Aebi (*30*) by measuring absorbance changes in the reaction mixture using the final concentrations of 10 mM H_2O_2 and 50 mM phosphate buffer (pH=7.0) at 240 nm during the time interval of 30 s after sample addition. The CAT activity was expressed as IU/mg Hb in lysate of erythrocytes and as IU/mg protein for activity in tissues.

Determination of Gpx Activity. The Gpx activity in lysate of erythrocytes and tissues was measured by Gpx assay kit (RANSEL) based on the method of Paglia and Valentine (*31*). Gpx activity was assayed spectrophotometrically (340 nm) using Camspec M330 equipped with M330 Camspec software package. GPX activity is determined indirectly with RANSEL kit by measuring the rate of NADPH oxidation to $NADP^+$, which is accompanied by a decrease in absorbance at 340 nm per minute. One GPX unit is proportional to the amount of NADPH consumed in nmol per minute at 37 °C and pH 7.2. To obtain the linearity of the assay, if the absorbency change per minute exceeded 0.1, the sample was diluted with the diluting agent. Gpx activity was expressed as IU/mg Hb in lysate of erythrocytes and IU/ mg protein in tissues.

Determination of Protein Concentration. Protein concentration in the tissue samples (mg/g) was estimated by the method of Lowry et al. (*32*) using bovine serum albumin as the standard.

Statistical Analysis. The data were analyzed using the statistical package SPSS for windows (v.11.00) and were presented as mean ± SEM. The significance of the differences between control and propolis-treated groups were compared with one way analysis of variance (ANOVA) followed by Scheff's post hoc test. A mean difference was significant at the 0.05 level.

RESULTS

Concentration of Phenolics in the Ethanolic Extract of Propolis. Concentration of detected phenolics in ethanolic extract of propolis is presented in **Figure 1**. Out of 13 phenolics determined, 4 were not detectable (genistein, daidzain, myricetin, taxifolin), and chrysin was the most abundant (2478.5 μg/g propolis), followed by pinocembrin (2069.1 μg/g propolis) and galangin (1441.6 μg/g propolis) while isorhamnetin was detected in the least amount (32.2 μg/g propolis). Some compounds

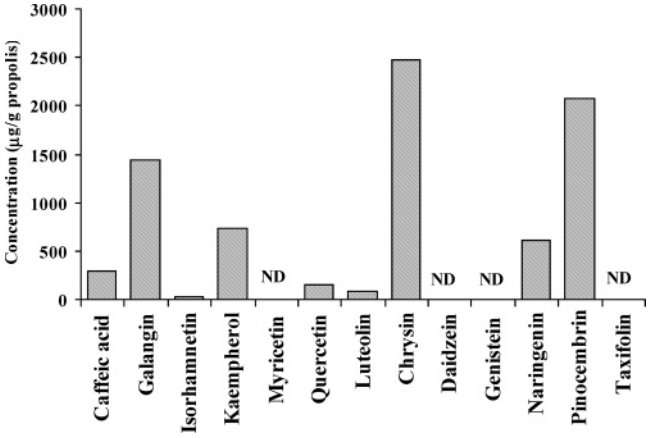

Figure 1. Phenol contents of propolis from the continental region of Croatia. ND = not detectable.

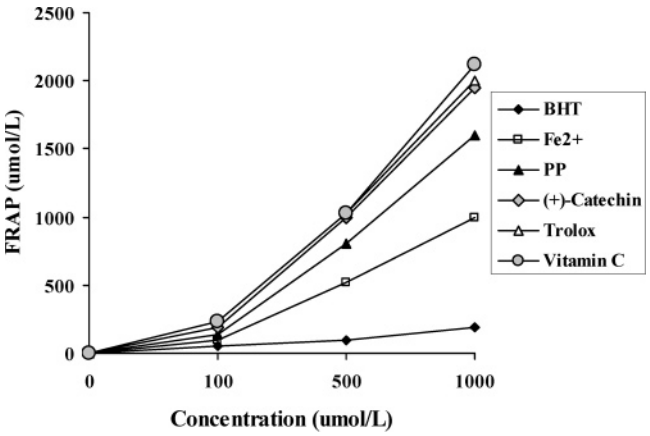

Figure 2. Dose–response line of vitamin C, trolox, (+)-catechin, Fe^{2+}, BHT, and native propolis extract (PP), over the concentration range of 100–1000 μM, in the ferric reduction/antioxidant power assay (FRAP).

remained unidentified because of the lack of authentic samples and library spectra of the corresponding compounds.

Antioxidant Activity of Propolis in Vitro. To compare the antioxidant capacity of propolis with known antioxidants, FRAP assay of vitamin C, trolox, (+)-catechin, BHT, Fe^{2+}, and propolis were carried out (**Figure 2**). The antioxidant efficiency of the antioxidant under test was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration. Fe^{2+} was representing a one-electron exchange reaction in the FRAP assay. Over the concentration range of 100−1000 μM, vitamin C, trolox, and (+)-catechin had the highest relative antioxidant efficiency. The relative antioxidant efficiency of propolis was 1.6 of trolox equivalent.

Effect of Gauge Feeding on TBARS Level in the Liver, Brain, and Lungs. As shown in **Table 1**, gauge feeding itself caused statistically significant increase of TBARS level in tissues examined. We therefore chose to feed the animals in our study by supplementing commercial food with chosen dosage of propolis.

Effect of Propolis on TBARS Level in Plasma and AOE Activity in Lysate of Erythrocytes. Effect of propolis on TBARS level in plasma was dose dependent and decreased with 100 mg/kg bw of propolis ($p = 0.005$), and it increased with 300 mg/ kg bw of propolis ($p = 0.01$) (**Figure 3**). In case of oxidative stress (normobaric 100% oxygen for 18 h), propolis in a dose of 300 mg/kg bw increased TBARS level ($p = 0.0001$) while oxidative stress per se was not affected. tSOD in the lysate

Table 1. TBARS Concentration in Liver, Brain, Lungs and Plasma of Control and Gauge Groups of Mice[a]

	liver (nmol/mg protein)	brain (nmol/mg protein)	lungs (nmol/mg protein)	plasma (nmol/mL plasma)
control	0.81 ± 0.14	0.37 ± 0.31	1.36 ± 0.17	11.69 ± 3.85
gauge	1.12 ± 0.19**	0.70 ± 0.16*	2.10 ± 0.78*	6.48 ± 2.20**

[a] Data are expressed as mean ± SD of 10 animals. *p < 0.05 control vs gauge. **p < 0.001 control vs gauge

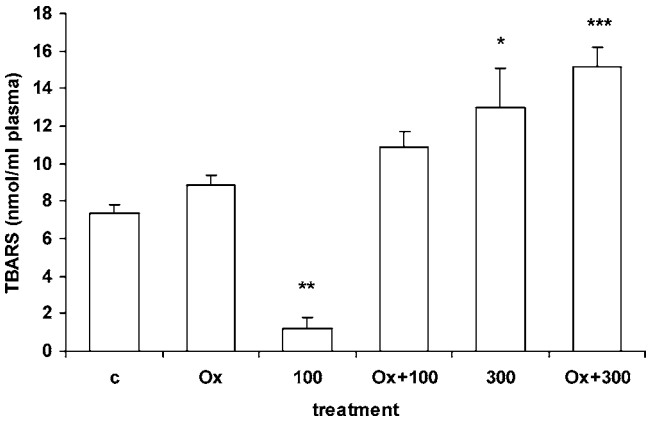

Figure 3. TBARS concentration in plasma of mice fed with commercial diet (c), commercial diet and propolis in a dose of 100 mg/kg bw (100) or 300 mg/kg bw (300), normobaric 100% oxygen after commercial diet (Ox), normobaric 100% oxygen after commercial diet and propolis in a dose of 100 mg/kg bw (Ox+100) or 300 mg/kg bw (Ox+300). *p = 0.01 c versus 300; **p = 0.005 c versus 100; ***p = 0.0001 c versus Ox+300.

of erythrocytes was significantly increased (p = 0.0001) with 300 mg of propolis (**Figure 4**A). The same amount of tSOD increase was observed with oxidative stress (p = 0.0001), which was not abolished by feeding mice with propolis prior to oxidative stress. The activity of CAT remained unchanged either by propolis or oxidative stress or the treatment of mice with propolis prior to oxidative stress (**Figure 4B)**. Pro-oxidative effect of 300 mg/bw propolis on Gpx activity was also observed (p = 0.016) (**Figure 4C**). Oxidative stress per se had no effect on Gpx activity, while pro-oxidative effect of 300 mg/kg bw of propolis under normobaric oxygen condition (100% O_2 for 18 h) was abolished.

Effect of Propolis on TBARS Level in Liver, Lungs, and Brain. Propolis in a dose of 100 mg/kg bw significantly decreased TBARS concentration in liver (p = 0.001), lungs (p = 0.001), and brain (p = 0.042) (**Figure 5**A). Propolis in a dose of 300 mg/kg bw affected none of the organs tested. Oxidative stress significantly increased (p = 0.0001 and p = 0.043, respectively) TBARS level in liver and lungs but was without effect in the brain. Both concentrations of propolis in the normobaric 100% oxygen condition returned the elevated TBARS level to control values. (**Figure 5B**).

Effect of Propolis on CuZnSOD and MnSOD Activity in the Liver, Brain, and Lungs. In lungs and brain, propolis had no effect on CuZnSOD activity. Contrary to that, dose of 100 mg/kg bw propolis increased significantly CuZnSOD activity in the liver (p = 0.001) (**Figure 6**A). Neither oxidative stress nor oxidative stress combined with propolis affected CuZnSOD activity in the organs tested (**Figure 6B**). MnSOD activity was unaffected by propolis in the brain and liver but significantly increased in lungs with 100 mg/kg bw of propolis (p = 0.002) (**Figure 6C**). Oxidative stress increased MnSOD activity only in the liver of treated animals (p = 0.0001) (**Figure 6D**). This was abolished with 300 mg/kg bw of propolis. The increased

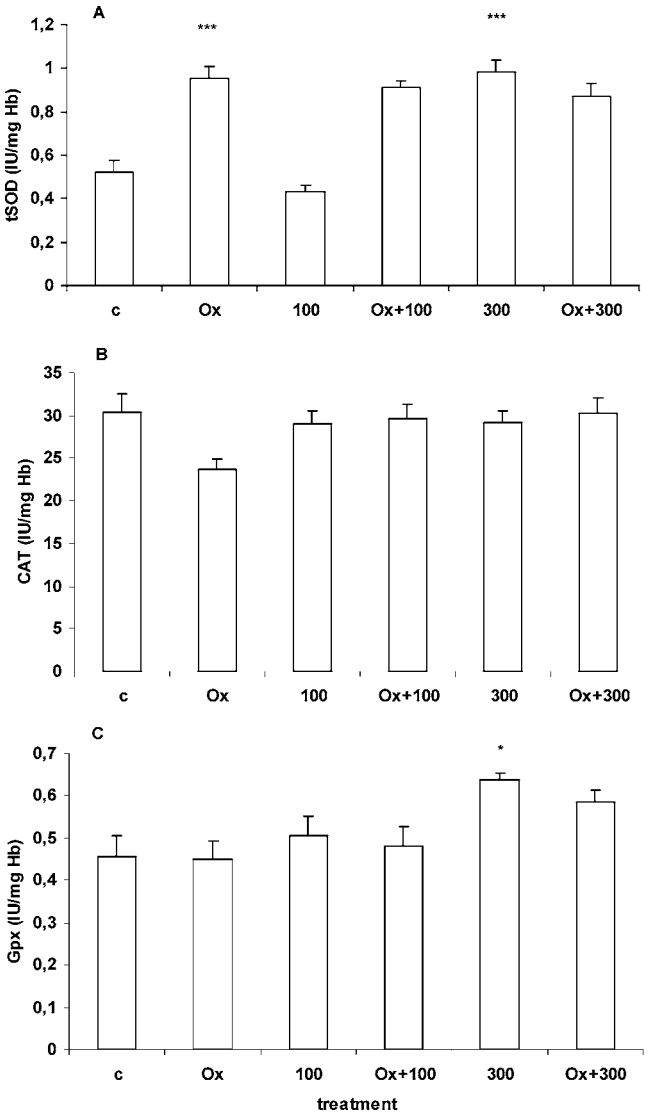

Figure 4. tSOD (A), CAT (B), and Gpx (C) activity in the lysate of erythrocytes of mice fed with commercial diet (c), commercial diet and propolis in a dose of 100 mg/kg bw (100) or 300 mg/kg bw (300), normobaric 100% oxygen after commercial diet (Ox), normobaric 100% oxygen after commercial diet and propolis in a dose of 100 mg/kg bw (Ox+100) or 300 mg/kg bw (Ox+300). (A) ***p = 0.0001 c versus 300 and c versus Ox; (C) *p = 0.016 c versus 300.

MnSOD activity (p = 0.032) in the lungs after oxidative stress and 100 mg/kg bw of propolis was an effect of propolis per se.

Effect of Propolis on CAT and Gpx Activity in the Liver, Brain, and Lungs. The tendency of increased CAT activity induced by propolis in the liver (**Figure 7**A) was not statistically significant. Both doses of propolis increased CAT activity in lungs (100 mg/kg bw p = 0.01; 300 mg/kg bw p = 0.0001) (**Figure 7C**). The increased CAT activity in brain of animals treated with 300 mg/kg bw of propolis was on the margin of statistical significance (p = 0.057) (**Figure 7C**). Neither

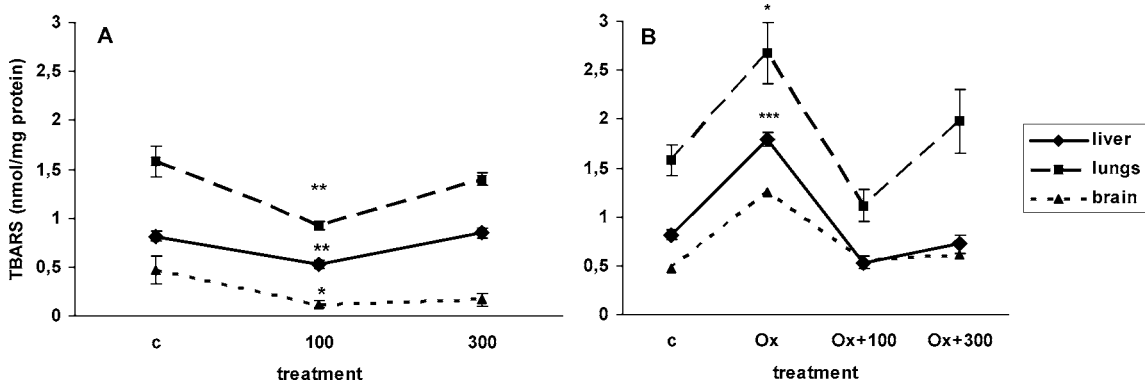

Figure 5. TBARS concentration (A) in liver, lungs and brain of mice fed with commercial diet (c), commercial diet and propolis in a dose of 100 mg/kg bw (100) or 300 mg/kg bw (300). (A) $*p = 0.042$ c versus 100 in brain; $**p = 0.001$ c versus 100 in liver and lungs. TBARS concentration (B) in liver, lungs, and brain of mice fed with commercial diet (c), normobaric 100% oxygen after commercial diet (Ox), normobaric 100% oxygen after commercial diet and propolis in a dose of 100 mg/kg bw (Ox+100) or 300 mg/kg bw (Ox+300). (B) $*p = 0.043$ c versus Ox in lungs; $***p = 0.0001$ c versus Ox in liver.

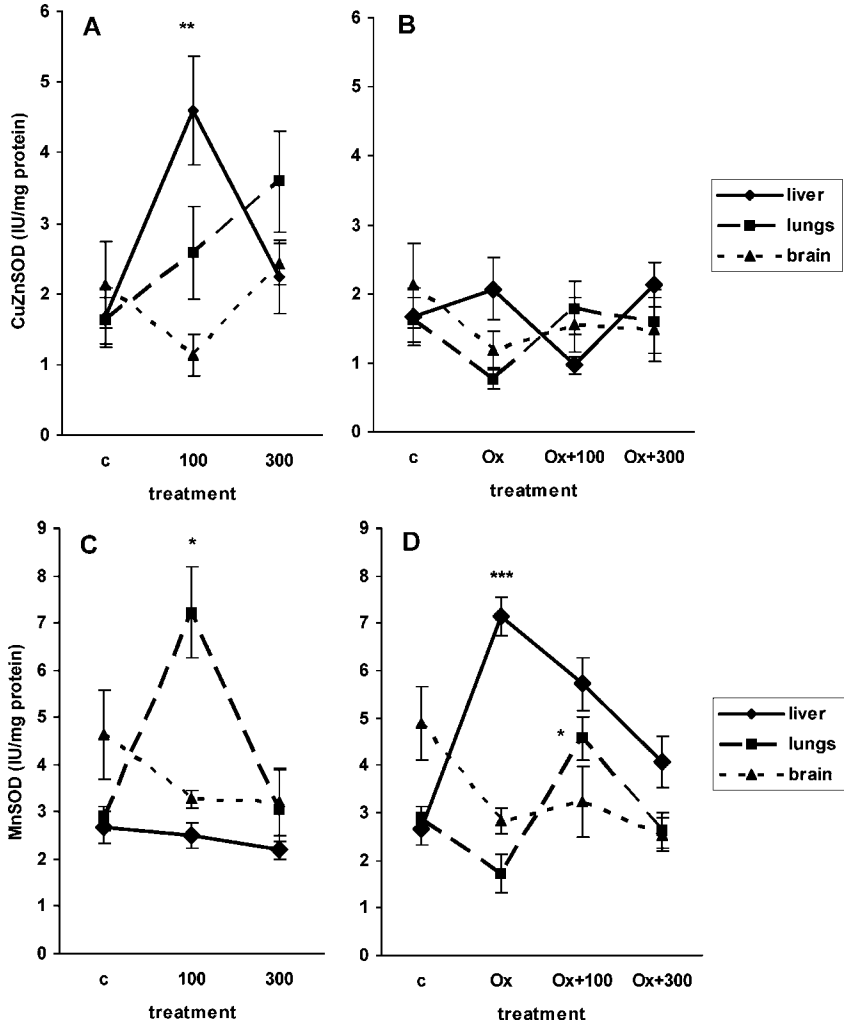

Figure 6. CuZnSOD (A) and MnSOD (C) activity in liver, lungs, and brain of mice fed with commercial diet (c), commercial diet and propolis in a dose of 100 mg/kg bw (100) or 300 mg/kg bw (300). (A) $**p = 0.001$ c versus 100 in the liver; (C) $*p = 0.002$ c versus 100 in the lungs CuZnSOD (B) and MnSOD (D) activity in liver, lungs, and brain of mice fed with commercial diet (c), normobaric 100% oxygen after commercial diet (Ox), normobaric 100% oxygen after commercial diet and propolis in a dose of 100 mg/kg bw (Ox+100) or 300 mg/kg bw (Ox+300). (D) $*p = 0.032$ Ox versus Ox+100 in the lung; $***p = 0.0001$ c versus Ox in the liver.

oxidative stress nor oxidative stress combined with propolis affected CAT activity in the liver (**Figure 7B**). However, in the brain a significant increase of CAT activity induced by oxidative stress ($p = 0.0001$) returned to control values in mice fed with both doses of propolis (**Figure 7D**). While in lungs, oxidative stress by itself had no effect on CAT activity; the increase with both doses of propolis in case of hyperoxygenation was due to propolis ($p = 0.0001$) (**Figure 7D**). Propolis had no effect on Gpx activity in the liver, brain, and lungs (**Figure 7E**). The same was established for liver, brain, and lung from

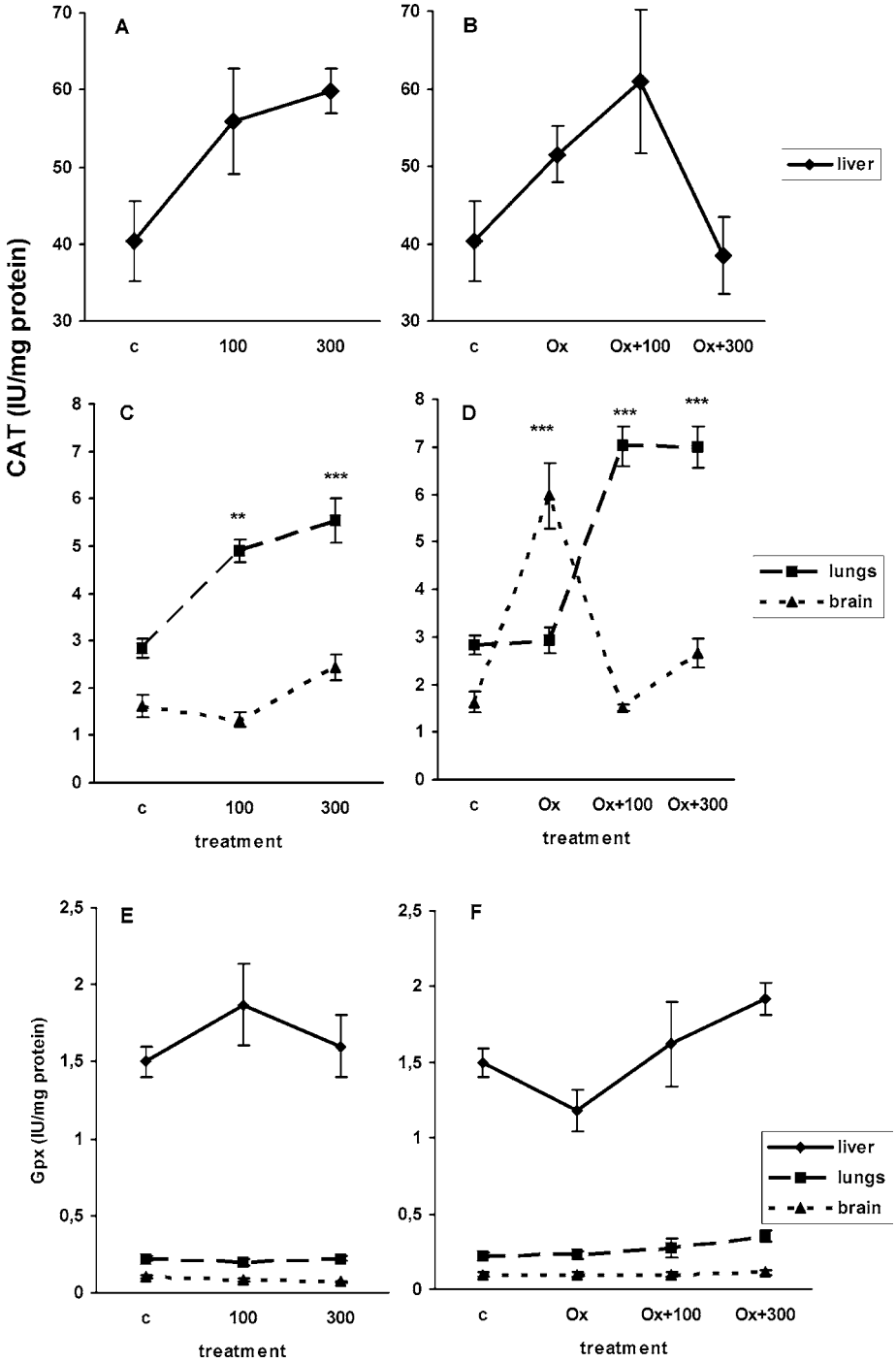

Figure 7. CAT activity in the liver (A), lungs and brain (C) and Gpx activity (E) in liver, lungs, and brain of mice fed with commercial diet (c), commercial diet and propolis in a dose of 100 mg/kg bw (100) or 300 mg/kg bw (300). (C) $^{**}p = 0.01$ c versus 100 in lungs; $^{***}p = 0.0001$ c versus 300 in lungs CAT activity in liver (B), lung, and brain (D) and Gpx activity (F) in liver, lung, and brain of mice fed with commercial diet (c), normobaric 100% oxygen after commercial diet (Ox), normobaric 100% oxygen after commercial diet and propolis in a dose of 100 mg/ kg bw (Ox+100) or 300 mg/kg bw (Ox+300). (D) $^{***}p = 0.0001$ c versus Ox in brain; $^{***}p = 0.0001$ Ox versus Ox+100 and Ox+300 in lungs.

mice subjected to oxidative stress or oxidative stress combined with propolis (**Figure 7F**).

DISCUSSION

The qualitative and quantitative chromatographic analysis showed that flavon chrysin (followed by pinocembrin and galangin) was the main phenolic compound detected in the sample of propolis investigated in our study. Differences in chemical composition of our sample of propolis versus samples from the continental region of Croatia studied by Kosalec et al. (*33*) were predominantly of quantitative nature. Namely, in the study of Kosalec, pinocembrin was reported as the dominant flavonoid followed by chrysin. Different flavonoid composition and/or quantities in propolis samples are not surprising since besides having the place of origin, variation may arise from local flora influences, seasonal differences, and environmental conditions (*34*). Besides, propolis used in our study was prepared with no chemical refinement, the result of which is minimal

loss of active ingredients. The content of chrysin in our study (9.8 μmol/g of propolis) was similar to the content in Brazilian propolis (8.0 μmol/g of propolis) (*15*). Besides literature data that chrysin expresses phytoestrogenic, antioxidant, antiinflammatory (35), anxiolytic activities (*36*), and inhibitory activities of aromatase (*37*) and as such may modulate gonadal function in both sexes (*38*) or can be used clinically in cases of estrogen-dependent carcinoma (*39*), its oral bioavailability is poorly understood. Indeed, Walle et al. (*40*) suggested that the oral bioavailability of chrysin in humans may be low depending on extensive oxidative metabolism and efflux of metabolites back into intestine for hydrolysis. Although oxidative metabolism of chrysin may facilitate its elimination, the oxidation of other flavonoids, specifically galangin to kaempherol which can proceed further to quercetin (*41*, *42*) yields increasingly active molecules with regard to antioxidant properties (*43*). Our propolis sample although of relatively low antioxidant capacity in vitro as demonstrated in the FRAP assay (1.6 μmol/L for propolis versus 2.0 μmol/L for trolox) proved to be a strong antioxidant in vivo. Moreover, content of antioxidative relevant vitamins in our native propolis was very low so its biological effects may be mostly due to flavonoids. This may be of importance because literature data showed contrasting results of in vitro and in vivo oxidant/antioxidant capacity of flavonoid-rich foods (*44*). The effect in our study was, however, tissue- and dose-related. In plasma, the TBARS level was affected by propolis; 100 mg/kg bw decreased while 300 mg/kg bw increased the TBARS level. This is in accordance with the paradoxical effect of higher versus lower doses of antioxidants (*45*, *46*). Some other literature data support the fact that propolis components may have both pro-oxidant and antioxidant properties (*47*). Quercetin pro-oxidative activity in erythrocytes was attributed by Galati et al. (*48*). However, the pro-oxidative effect of the high dose of propolis in plasma in our study was probably not due to quercetin since in our propolis sample the very low quantities were insufficient for inducing oxidative effect. Many flavonoids (luteolin, chrysin, apigenin, naringenin) show beside antioxidative also pro-oxidative characteristics in concentrations where other flavonoids were still antioxidants mainly depending on enzymatic and/or chemical (auto)oxidation or metal ion concentration *(49*, *50*). Increase in both tSOD and Gpx in the lysate of erythrocytes of animals fed with 300 mg/kg bw of propolis is likely due to pro-oxidant effect of this dose of propolis.

Indeed as pointed by Scapagnini et al. (51) caffeic acid phenethyl ester (CAPE), a phenolic originating from plants and active component of propolis, and curcumin are potent inducers of heme-oxygenase-1 protein (HO-1) which is a redox sensitive inducible protein which provides protection from various forms of stress. These two phytochemicals can efficiently inhibit LPO as shown by Balogun et al., (*52*) in that the induction of heme-oxygenase-1 by curcumin and CAPE requires the activation of the transcription factor Nrf2/antioxidant-responsive element complex pathway (*53*). Thus, beside the activation of "classic" detoxifying enzymes, the induction of heme-oxygenase-1 protein by phenolic natural substances has to be considered. Heme-oxygenase-1 protein is a novel aspect of the mode of action of polyphenolics like CAPE since so far they have been considered as chemicals reactive toward free radicals.

Hyperoxia by itself did not affect the TBARS level in plasma probably because of the concomitant increment of tSOD. Contrary to physiological conditions, in hyperoxia, propolis at a dose of 100 mg/kg bw was without effect. Beneficial effect of propolis (100 mg/kg) in physiological conditions was observed in liver, lungs, and brain, while the dose of 300 mg/kg was without effect. Decreased TBARS levels in investigated tissues of treated animals in physiological conditions were probably the result of increased CuZnSOD activity in the liver and MnSOD and CAT activity in lungs. In addition, CAPE may be effective in protecting tissue damage caused by oxidative stress *(54*). Gpx level appeared to be unaffected by propolis in investigated tissues in physiological conditions.

Oxidative stress significantly increased TBARS level in liver and lungs of mice, and the same pattern, although insignificant, was observed in the brain. The brain was protected from damage by the large increment of CAT activity (over 350%) while the liver, with only slightly increased CAT activity, remained vulnerable to hyperoxia in spite of increased MnSOD activity. Since hyperoxia in lungs was not associated with augmentation of the oxidant defenses, lungs demonstrated the highest TBARS increase among all the tissues tested. Hyperoxia induced increase of the TBARS level in all three tissues tested was abrogated by both doses of propolis. Propolis in a dose of 300 mg/kg bw abolished the increment of MnSOD and CAT activity in liver. In the brain, CAT activity increased by hyperoxia was abolished by propolis, too. In lungs of mice treated with propolis before hyperoxia, MnSOD and CAT activity were significantly augmented. This is of great importance since lungs of adult animals are, contrary to neonatal animals, vulnerable to hyperoxia because they are not able to mount protective lung biochemical responses rapidly (*55*, *56*). It seems that this important feature can be given back to adult animals by administration of propolis before hyperoxia.

ABBREVIATIONS USED:

AOE, antioxidant enzymes; BHT, butylated hydroxytoluene; CAT, catalase; CuZnSOD, CuZn superoxide dismutase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant potential; Gpx, glutathione peroxidase; HPLC, high performance liquid chromatography; LPO, lipid peroxidation; MnSOD, Mn superoxide dismutase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; tSOD, total superoxide dismutase.

ACKNOWLEDGMENT

The authors thank Prof. W.L. Ragland for his helpful comments and discussion. We thank V. Matešić for her most helpful technical support and preparation of manuscript.

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Received for review April 28, 2006. Revised manuscript received August 23, 2006. Accepted August 30, 2006.

JF0612023