Revista Brasileira de Farmacognosia 29 (2019) 301–308

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

The role of seasonality on the chemical composition, antioxidant activity and cytotoxicity of Polish propolis in human erythrocytes

Magdalena Woźniak [a], Lucyna Mrówczyńska [b,*], Agnieszka Waśkiewicz [a], Tomasz Rogoziński [c], Izabela Ratajczak [a,*]

[a] Department of Chemistry, Faculty of Wood Technology, Poznań University of Life Sciences, Poznań, Poland
[b] Department of Cell Biology, Faculty of Biology, Adam Mickiewicz University, Poznań, Poland
[c] Department of Furniture Design, Faculty of Wood Technology, Poznań University of Life Sciences, Poznań, Poland

A R T I C L E I N F O

Article history:
Received 31 October 2018
Accepted 4 February 2019
Available online 7 March 2019

Keywords:
Propolis
Phenolic acids
Flavonoids
Antioxidant potential
Human erythrocytes

A B S T R A C T

Propolis extracts possess beneficial biological effects, such as antioxidant activity. However, the composition of propolis and biological properties of its extracts depend on many factor, including time of harvesting. The main purpose of this study was to evaluate the seasonal effect on the phenolic profile of Polish propolis extracts and their antioxidant activity. Propolis samples were collected from the same apiary during three seasons of the year. The chemical composition (contents of phenolic acids and flavonoids) of ethanolic propolis extracts was determined by ultra-performance liquid chromatography equipped with a photodiode detector and a triple quadrupole mass spectrometer. The antioxidant potential of propolis extracts was evaluated. Additionally, *in vitro* effects of propolis extracts on the morphology of human red blood cells and the selective permeability of their membrane were determined. The capacity of propolis extracts to protect human red blood cells against free radical-induced hemolysis was also studied. The analyze of the chemical composition of propolis extracts collected in three season of the year indicated that the sum of determined flavonoids and phenolic acids was the highest in the sample harvested in the spring (125.14 mg/g) and it was the lowest in the extract of material collected in the fall (110.09 mg/g), but the differences were slightly. The concentration of examined phenols in propolis samples collected in different seasons was similar and only content of seven among fifteen determined compound was significantly different in extracts according to statistical analysis. The propolis extracts possess high antioxidant potential and significantly protect human red blood cells from oxidative damage. There was no significant differences with regard to the seasonal effect on the chemical profile and antioxidant potential of Polish propolis extracts. These results indicate that the time of Polish propolis harvesting have no influence on phenolic profile and antioxidant activity of its extract.

© 2019 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Reactive oxygen species (ROS) play a role in various physiological conditions including the synthesis of thyroid hormones or in cellular signaling as second messengers (Di Dalmazi et al., 2016). The free radicals are generated by human organism under the influence of both endogenous sources (*e.g.* mitochondria or endoplasmic reticulum) and exogenous sources (*e.g.* pesticides, heavy metals and drugs like paracetamol) (Phaniendra et al., 2015). Overproduction of ROS can adversely affect various biomolecules, such as lipids, proteins or nucleic acids, leading to increased oxidative stress (Lobo et al., 2010; Phaniendra et al., 2015). The oxidative stress has been associated with different type of chronic diseases, such as diabetes mellitus, cardiovascular diseases, respiratory diseases or in various types of cancer (Cai et al., 2004; Lobo et al., 2010; Phaniendra et al., 2015). Therefore, a balance between free radicals and antioxidants, which possess ability in scavenging radicals, is necessary for proper function of human organisms (Lobo et al., 2010). The literature reports extensive date on the antioxidant activity of phenolic compounds, mainly flavonoids and phenolic acids (Havsteen, 2002; Leopoldini et al., 2004).

* Corresponding authors.
E-mails: lumro@amu.edu.pl (L. Mrówczyńska), iza.ratajczak@up.poznan.pl (I. Ratajczak).

https://doi.org/10.1016/j.bjp.2019.02.002
0102-695X/© 2019 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

The rich source of phenols is propolis, which is a resinous material collected by honeybees from the leaf buds of numerous trees, shrubs and other plant species, to be modified and stored inside their hives. Propolis has a long history in traditional medicine in many regions of world dating back at least to 300 BC (Ghisalberti, 1979). Egyptians used the anti-putrefactive properties of propolis to embalm cadavers. In the Middle Ages and among Arab physicians propolis was used as an antiseptic and cicatrizant in wound treatment and as a mouth disinfectant (Castaldo and Capasso, 2002; Toreti et al., 2013). Modern herbalists recommend this bee product for its beneficial properties to increase the natural resistance of human organisms (Castaldo and Capasso, 2002). Numerous scientific reports describe its biological activities, including antibacterial (Kalogeropoulos et al., 2009), antifungal (Salas et al., 2016), antitumoral (Ahn et al., 2007b), antioxidant (Kumazawa et al., 2004; Ahn et al., 2007a) and anticancer (Li et al., 2009) properties. For this reason, propolis is currently used in many applications, including over-the-counter preparations for cold syndrome, dermatological preparations useful in wound, acne, boils and dermatitis treatment (Banskota et al., 2001; Castaldo and Capasso, 2002; Kuropatnicki et al., 2013). Moreover, propolis is constituents of nutritional supplements, health foods or alternative medicine products, such as candy or syrup (Valencia et al., 2012). Propolis, mainly in form of extracts, is also used in home remedies, both in internally and externally applications (Valencia et al., 2012; Wagh, 2013). It is commercially available in the form of extracts, capsules, a mouthwash solutions, powder or creams (Castaldo and Capasso, 2002; Wagh, 2013).

Propolis contains over 500 constituents, including phenolic compounds (flavonoids, phenolic acids and their esters), fatty acids, sugars, mineral elements and terpenoids (Melliou et al., 2007; Gong et al., 2012; Huang et al., 2014; Kurek-Gorecka et al., 2014; Kasote et al., 2017). The composition of propolis varies quantitatively and qualitatively depending on many factors, such as geographical and botanical origin, time and method of harvest or solvent used in extraction (Kumazawa et al., 2004; Ahn et al., 2007a; Simoes-Ambrosio et al., 2010; Teixeira et al., 2010; Papotti et al., 2012). There are several studies involving the effect of time of propolis harvest to its chemical composition (Bankova et al., 1998; Isla et al., 2012; Souza et al., 2016). Extracts of Argentinian propolis collected in the spring and summer contained greater amounts of phenols than samples of propolis collected in the other seasons of the year (Isla et al., 2012). The HPLC analysis of Brazilian propolis indicated relatively similar qualitative profiles, but a huge variation in the levels of aromadendrin-4′-methyl ether, baccharin and artepillin C in propolis samples harvested in different parts of year (Simoes-Ambrosio et al., 2010). The seasonal effect on the chemical composition of Brazilian propolis was also confirmed by research described by Neto et al. (2017) and Teixeira et al. (2010). On the other hand, the total content of flavonoids and phenolic compounds in Mexican propolis collected during the four seasons of year was similar in all samples (Valencia et al., 2012).

The literature also described the effect of seasonality on biological activities of propolis extracts. Isla et al. (2012) reported that Argentinian propolis collected during the summer exhibited greater antibacterial and antifungal activity than propolis samples collected in the other seasons. In turn, results described by Salas et al. (2016) indicated that extracts obtained from propolis collected in the same location in Argentine, but in two different months (December and March), exhibited similar antifungal, antioxidant and nematicidal effects. Simoes-Ambrosio et al. (2010) found that seasonality plays an important role in the inhibitory effect of green propolis extract from Brazil on the oxidative metabolism of neutrophils. Valencia et al. (2012) examined the seasonal effect on the antioxidant properties and antiproliferative activity of propolis samples from Mexico and claimed that the season of propolis collection had an important influence on the antiproliferative activity and all propolis extracts possessed a weak free-radical scavenging activity. In turn, Teixeira et al. (2010) evaluated the effect of seasonality on antioxidant activity of propolis extracts obtained from propolis samples collected from the same apiary over the period of 1 year at monthly intervals and found that the date of harvest had an influence on this parameter.

The aim of this study was to evaluate the effect of seasonality on the chemical profile (contents of flavonoids and phenolic acids) of Polish propolis extracts and their antioxidant potential. The antioxidant properties of extracts were determined by standard assays, namely DPPH$^{\bullet}$ free radical scavenging activity, Fe^{3+} reducing power assay and ferrous ion (Fe^{2+}) chelating activity. The effect of propolis extracts on the morphology of human red blood cells (RBC) and the selective permeability of their membrane was studied to estimate their potential cytotoxicity. To estimate the cytoprotective potential of propolis extracts, their ability to protect RBC against free radical-induced hemolysis was evaluated.

Materials and methods

Propolis samples and ethanolic extacts of propolis (EEP)

Raw propolis was collected three times during 2014, in the spring (from April to June), summer (from June to September) and fall (from September to November). Propolis samples were harvested from the same apiary from fifteen bee colonies using propolis traps and they were stored at −4 °C. The apiary was located in central Poland in the protected area of the Nadwarciański Landscape Park (Trzcianki, 52.09° N, 17.45° E), characterized by a variety of plant species. Numerous poplar (*Populus* sp.), willow (*Salix* sp.), pine (*Pinus sylvestris* L.) and chestnut (*Castanea sativa* Mill.) trees are found in this area. They are a potential source of resinous substances used by bees in propolis production. However, there is no possibility of clearly identifying the botanical origin and possible phenological effects of the propolis harvested for the study except for the occurrence of tree species which are its potential sources within the flight range of bees from the experimental apiary.

Frozen propolis samples were cut into small pieces and extracted with a 10-fold volume of 70% ethanol under shaking (Biosan, Riga, Latvia). The extraction was carried out for 5 days in the dark at ambient temperature. The propolis extracts after filtration were concentrated until constant weight on a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) under reduced pressure at 40 °C. The residues were dissolved in DMSO for antioxidant assays and in methanol for chemical composition analysis.

Reagents

The standards of compounds for chromatographic analyses (apigenin, quercetin, chrysin, myricetin, galangin, kaempferol, rutin, naringenin, pinobanksin, pinocembrin, epicatechin, genistein, pinostrobin, caffeic acid, coumaric acid, sinapinic acid, ferulic acid, *p*-hydoxybenzoic acid, vanillic acid and syringic acid) were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Ethanol, dimethyl sulfoxide (DMSO) were purchased from Avantor Performance Materials Poland SA (Gliwice, Poland). Formic acid, acetonitrile and methanol were of LC–MS grade and purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). The water used in study was of Milli-Q quality (Millipore, Bedford, MA, USA). The standard antioxidants – Trolox, BHT (butylated hydroxyltoluene) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), ferrozine, poly-l-lisine, ethylenedinitrilotetraacetic acid (EDTA), trichloroacetic acid were purchased from Sigma Aldrich Chemie

GmbH (Steinheim, Germany). PBS buffer compounds (NaCl, KCl, Na_2HPO_4, KH_2PO_4), glucose, paraformaldehyde, glutaraldehyde, glycerol, $K_3Fe(CN)_6$, $FeCl_2$ and $FeCl_3$ were purchased from Avantor Performance Materials Poland SA (Gliwice, Poland).

UPLC/PDA/TQD analysis

The UPLC/PDA/TQD analyses were performed on an Aquity UPLC chromatograph (Waters, Manchester, MA, USA) equipped with a photodiode detector (PDA eλ Detector) (Waters, Manchester, MA, USA) and coupled to an electrospray ionization triple quadrupole mass spectrometer (TQD) (Waters, Manchester, MA, USA). All samples of propolis extracts were filtered through a 0.20 μm syringe filter (Chromafil, Macherey-Nagel, Duren, Germany) before analyses. Phenolic compounds were separated at a temperature of 25 °C on an ACQUITY UPLC HSS T3 (Waters, Manchester, MA, USA) 1.8 μm particle size column (2.1 × 150 mm). The injection volume was 3 μl with the flow rate of 0.3 ml/min. Gradient elution was carried out with a binary system consisting of line A – 0.1% aqueous formic acid, and line B – 0.1% formic acid in acetonitrile, which were previously degassed and filtered. The solvent gradient was modified as follows: 0–5 min 25% B, 5–20 min 40% B, 20–30 min 60% B, 30–35 min 90% B, 35–40 min 100% B followed by the return to the initial conditions. Nitrogen above 99% purity was used. The instrument was operated in the negative-ion mode with the full scan in the mass range from m/z 100 to 1000. The molecular ion for each compound was selected by the selective ion mode and then its area was integrated. The MSn data were simultaneously acquired for the selected precursor ion. The collision induced decomposition was run using helium as the collision gas, with a collision energy of 25–40 eV. All samples were injected in triplicate.

Antioxidant assays

DPPH free radical scavenging activity

The solution (0.1 mM) of $DPPH^•$ in ethanol (0.2 ml) was added to 0.2 ml of propolis extracts at two different concentrations (0.01 and 0.1 mg/ml) and vortexed (Bio Vortex V1, Biosan, Riga, Latvia). Trolox and BHT were used as the reference compounds. The samples were incubated in the dark for 30 min at room temperature. Following incubation the absorbance (Abs) was measured at 517 nm in an EPOLL 2000 ECO spectrophotometer (PZ EMCO, Warszawa, Poland). The percentage $DPPH^•$ scavenging effect was calculated using Eq. (1).

$$DPPH^{\bullet} scavenging activity(\%) = [(Abs0 - Absl)/Abs0] \times 100 \qquad (1)$$

where Abs_0 is absorbance of the control sample and Abs_1 is the absorbance in the presence of samples tested. Each sample was made in triplicate and three independent experiments were performed.

Fe^{3+} reducing power assay

Selected concentrations (0.1 and 0.01 mg/ml) of propolis extracts (0.06 ml) were gently mixed with 0.1 ml of 0.20 M PBS (phosphate buffered saline) (6.6 pH) and 0.1 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. Trolox and BHT were used as the reference compounds. The samples were vortexed (Bio Vortex V1, Biosan, Riga, Latvia) and incubated for 20 min at 50 °C. Following incubation 0.1 ml of 10% trichloroacetic acid was added to the samples to acidify the reaction medium. Finally, 0.04 ml of 0.6 M $FeCl_3$ was added to the medium and absorbance (Abs) was measured at 700 nm in an EPOLL 2000 ECO spectrophotometer (PZ EMCO, Warszawa, Poland). Each sample was tested in triplicate and three independent experiments were performed.

Ferrous ion (Fe^{2+}) chelating activity

The propolis extracts (0.2 ml) at 0.01 and 0.1 mg/ml were added to a solution of 0.6 mM $FeCl_2$ (0.05 ml). EDTA was used as the standard metal chelator. The reaction was started by the addition of 5 mM ferrozine (0.05 ml) in ethanol and the mixture was immediately vigorously shaken (Bio Vortex V1, Biosan, Riga, Latvia). The samples were stored for 10 min at room temperature. Following incubation the absorbance (Abs) of the solutions was measured at 562 nm in an EPOLL 2000 ECO spectrophotometer (PZ EMCO, Warszawa, Poland). The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using Eq. (2).

$$Fe^{2+} chelating effect(\%) = [1 - (Abs_1/Abs_0)] \times 100 \qquad (2)$$

where Abs_0 is absorbance of the control sample and Abs_1 is absorbance in the presence of the samples tested. Each sample was tested in triplicate and three independent experiments were performed.

Erythrocyte preparation

Fresh human erythrocyte concentrates (65%) were purchased from the blood bank in Poznań. The erythrocytes were washed three times (960 × g, 10 min, +4 °C) (Sigma 3-30K Sartorious AG, Göttingen, Germany) in 7.4 pH phosphate buffered saline (PBS) supplemented with 10 mM glucose. After washing the cells were suspended in the buffer at 1.65×10^9 cells/ml, stored at +4 °C and used within 5 h.

Hemolysis assay

Erythrocytes (1.65×10^8 cells/ml, ∼1.5% hematocrit) were incubated in PBS (7.4 pH) supplemented with 10 mM glucose and containing propolis extracts tested at the concentration of 0.01 mg/ml for 60 min, 240 min and 24 h at 37 °C in a thermo-shaker (BioSan Thermo-Shaker TS-100C, Biosan, Riga, Latvia). Samples with erythrocytes incubated in PBS without propolis extracts were taken as the controls. Each sample was repeated three times and the experiments were repeated four times with erythrocytes from different donors. After incubation, the erythrocyte suspensions were centrifuged (Sigma 3-30K Sartorious AG, Göttingen, Germany) (960 × g, 10 min, 4 °C) and the degree of hemolysis was estimated by measuring absorbance of the supernatant in an EPOLL 2000 ECO spectrophotometer (PZ EMCO, Warszawa, Poland) at 540 nm. The results were expressed as the percentage (%) of hemolysis. Hemolysis percentage (equal to 0%) was taken as absorbance of the supernatant of erythrocyte suspensions in PBS only, while total hemolysis (100%) was determined when PBS was replaced with cold distilled water.

Microscope studies of erythrocyte shape transformation

Following incubation with propolis extracts at the concentration studied (0.01 mg/ml) cells were fixed in 5% paraformaldehyde (PFA) with 0.01% glutaraldehyde (GA) for 1 h at room temperature (∼22 °C). Fixed cells were gently washed by exchanging the supernatant with PBS. After washing, erythrocytes were settled on poly-l-lysine-treated (0.1 mg/ml, 10 min) cover glasses and mounted on 80% glycerol. The cover slips were sealed with nail polish. A large number of cells in several separate experimental samples was studied under a Zeiss LSM 510 microscope (Axiovert Zoom) (Oberkochen, Germany) (100 × /1.4 aperture immersion oil objective, 10 × ocular). The RBC shape was estimated according to the Bessis classification (Bessis et al., 1973).

Inhibition of free-radical-induced hemolysis

Erythrocytes (1.65×10^8 cells/ml, ~1.5% haematocrit) were pre-incubated in PBS (pH 7.4) supplemented with 10 mM glucose and containing propolis extracts tested at the concentration of 0.01 mg/ml for 20 min at 37 °C in a thermo-shaker (BioSan Thermo-Shaker TS-100C, Biosan, Riga, Latvia). After pre-incubation, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) was added at the final concentration of 60 mM. Samples were incubated for the next 4 h at 37 °C in a shaking water bath. Erythrocytes incubated in PBS and in the presence of AAPH without propolis extracts were taken as the control. After incubation, the erythrocyte suspensions were centrifuged (1280 × g 5 min, +4 °C) (Sigma 3-30K Sartorious AG, Göttingen, Germany) and the degree of hemolysis was determined by measuring absorbance (Abs) of the supernatant at 540 nm in an EPOLL 2000 ECO spectrophotometer (PZ EMCO, Warszawa, Poland). The percentage of hemolysis inhibition was calculated using the following Eq. (3).

$$\text{Inhibition of erythrocytes hemolysis (\%)} = 100 - [(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}/\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}) \times 100] \quad (3)$$

where $\text{Abs}_{\text{sample}}$ is the absorbance value of the supernatant obtained from samples incubated with samples tested, $\text{Abs}_{\text{blank}}$ is the absorbance of supernatant obtained from samples without samples tested and AAPH, and $\text{Abs}_{\text{control}}$ is the absorbance of the supernatant obtained from samples with AAPH and in the absence of the samples tested. Each sample was prepared in triplicate and the results are presented as a mean value (±SD) of ten independent experiments with erythrocytes from different donors.

Statistical analysis

The results were analyzed using the one-way analysis of variance (ANOVA). Groups were compared by Tukey's Honest Significant Differences (THSD) Test. Statistical significance was defined as $p < 0.05$. All of the statistical analyses were performed using Statistica 12.0 software (Tallahassee, USA).

Results and discussion

The seasonal effect on the chemical composition of propolis

The samples of Polish propolis were collected throughout three seasons of the year. The highest amount of material was harvested during the summer (79.92 g) and the lowest amount was sourced in the fall (11.94 g). Also, the yield of extraction varied between propolis samples, amounting to: spring – 41.7%, summer – 66.6% and fall – 46.6%.

Table 1 presents the concentrations of flavonoids and phenolic acids in propolis samples, identified by UPLC analysis with PDA and TQD detection.

The results of the quantitative analysis of propolis samples collected in three seasons during the year showed that the concentrations of most constituents identified in all samples were similar. Pinocembrin was detected in all samples in the largest amounts, with the content of this flavonoid higher in propolis collected in the spring (51.55 ± 3.23 mg/g of EEP) and fall (50.34 ± 0.56 mg/g of EEP) than in propolis harvested in the summer (41.55 ± 0.65 mg/g of EEP). High concentrations among flavonoids identified in propolis samples were also recorded for chrysin and galangin, which together with pinocembrin are common flavonoids identified in Polish propolis (Kedzia, 2009; Szliszka et al., 2013; Socha et al., 2015; Popova et al., 2017). These compounds are also found in propolis samples coming from other regions, including samples from Spain, Australia, Bulgaria, New Zealand or China (Kumazawa et al., 2004, 2013). In EEP1 (spring) and EEP2 (summer) kaempferol

Table 1
The contents of the constituents in ethanolic extacts of propolis (EEP) samples.

Phenols	Concentration [mg/g of EEP]		
	EEP1 (spring)	EEP2 (summer)	EEP3 (fall)
Apigenin	$6.03^c \pm 0.51$	$7.94^b \pm 0.38$	$10.01^a \pm 0.84$
Quercetin	$4.29^a \pm 0.49$	$4.03^a \pm 0.36$	$3.83^a \pm 0.22$
Chrysin	$19.51^a \pm 0.68$	$14.40^b \pm 0.31$	$15.32^b \pm 0.47$
Myricetin	$0.63^a \pm 0.16$	$0.39^a \pm 0.04$	$0.83^a \pm 0.20$
Galangin	$26.89^b \pm 0.90$	$32.53^a \pm 0.32$	$20.14^c \pm 0.83$
Kaempferol	$10.43^a \pm 0.38$	$11.51^a \pm 0.36$	$5.01^b \pm 0.20$
Rutin	$0.72^a \pm 0.12$	$0.56^a \pm 0.12$	$0.49^a \pm 0.05$
Naringenin	$0.88^a \pm 0.26$	$0.83^a \pm 0.06$	$0.83^a \pm 0.16$
Pinobanksin	$4.21^a \pm 0.51$	$3.83^a \pm 0.50$	$3.29^a \pm 0.37$
Pinocembrin	$51.55^a \pm 3.23$	$41.55^b \pm 0.65$	$50.34^a \pm 0.56$
Sum of flavonoids	*125.14*	*117.57*	*110.09*
Caffeic acid	$3.90^a \pm 0.34$	$3.26^a \pm 0.45$	$3.68^a \pm 0.36$
Coumaric acid	$10.00^a \pm 0.59$	$9.17^a \pm 0.52$	$10.04^a \pm 0.52$
Ferulic acid	$3.80^a \pm 0.33$	$2.61^b \pm 0.40$	$3.63^a \pm 0.22$
Vanillic acid	$0.46^a \pm 0.06$	$0.31^b \pm 0.05$	nd
Syringic acid	$1.18^a \pm 0.17$	$0.83^a \pm 0.24$	$1.01^a \pm 0.17$
Sum of phenolic acids	*19.34*	*16.18*	*18.36*

nd, not detected.
Values in the same row followed by the same letter are not significantly different by Tukey's Honest Significant Differences (THSD) Test ($p < 0.05$).

was also determined in relatively large amounts. The content of this compound in propolis collected in the fall was over half lower than in the other samples. The concentrations of quercetin and pinobanksin were similar in all propolis samples, as confirmed by statistical analysis, and they were lower than in propolis samples sourced in Italy, Australia and Bulgaria (Kumazawa et al., 2004; Papotti et al., 2012). Moreover, pinobanksin is one of most abundant flavonoids determined in propolis originating from Europe, including Bulgaria and Hungary (Kumazawa et al., 2004; Ahn et al., 2007a). On the other hand, the content of apigenin was the highest in the fall propolis and the lowest in the spring propolis. The amounts of this constituent in all examined propolis samples were higher than in samples collected in Italy or Ukraine and much lower than in propolis samples from New Zealand, Argentine or Chile (Kumazawa et al., 2004; Papotti et al., 2012). All propolis extracts also contained low and comparable amounts of myricetin, naringenin and rutin. Moreover, in all propolis extracts the concentrations of epicatechin, genistein and pinostrobin were below the UPLC/PDA/TQD detection limits. The significant differences in flavonoids concentrations in propolis depending on the season of its collection were observed in half of the analyzed compounds. The sum of flavonoids in propolis extracts was the highest in the sample collected in the spring (125.14 mg/g of EEP) and it was the lowest in the material harvested in the fall (110.09 mg/g of EEP), but the differences were slightly.

Among the aromatic acids, coumaric acid was found in the largest amounts in all analyzed propolis extracts, ranging from 9.17 ± 0.52 mg/g of EEP1 (summer) to 10.04 ± 0.52 mg/g of EEP3 (fall). The high concentration of this acid was previously reported in other propolis samples collected from Poland (Szliszka et al., 2013; Socha et al., 2015). A significantly greater range of coumaric acid contents in propolis collected in Brazil (27.40 mg/g of EEP) was reported by Kumazawa et al. (2004), while lower contents were reported by Papotti et al. (2012) in propolis samples from Italy (8.31–11.01 mg/g of EEP). Another identified aromatic acid was caffeic acid, which content in Polish propolis was lower than in Italian propolis (5.39–7.85 mg/g of EEP) and higher than in Brazilian (1.6 mg/g of EEP) or Argentinian (0.7 mg/g of EEP) propolis (Kumazawa et al., 2004; Papotti et al., 2012). Ferulic acid was also determined in all propolis extracts, with higher concentrations, recorded in propolis collected in the spring (3.80 ± 0.33 mg/g of EEP) and fall (3.63 ± 0.22 mg/g of EEP) than in the sample from summer (2.61 ± 0.40 mg/g of EEP). Much lower

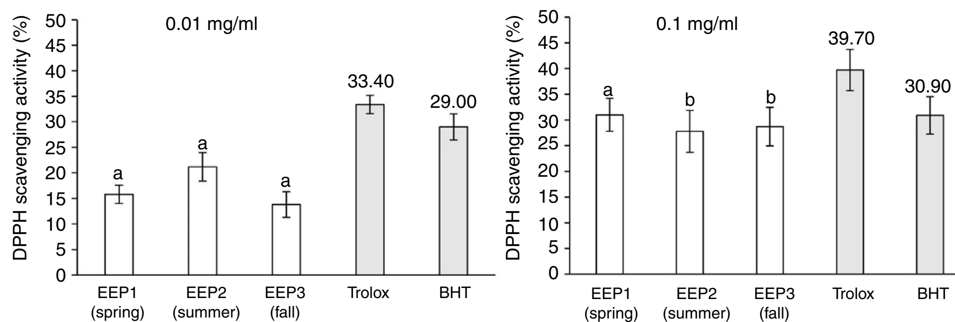

Fig. 1. DPPH• free radical scavenging activity of propolis extracts and standard antioxidants Trolox and BHT. Results are presented as average ± SD (n = 9). Different letters indicate samples that were significantly different (p < 0.05).

amounts of this phenolic acid in propolis samples were reported by Kalogeropoulos et al. (2009) and Papotti et al. (2012). Another phenolic acid identified and quantitatively determined in all propolis extracts was syringic acid, the content of which ranged from 0.83 ± 0.24 mg/g of EEP2 (summer) to 1.18 ± 0.17 mg/g of EEP1 (spring). Syringic acid was previously reported in propolis samples originating *e.g.* from Greece and Cyprus (Kalogeropoulos et al., 2009). Moreover, in propolis extracts obtained from the material collected in the spring and summer vanillic acid was detected in trace amounts. This phenolic acid was previously identified in propolis *e.g.* from Poland and Brazil (Andrade et al., 2017; Popova et al., 2017). All propolis extracts did not contain sinapinic acid and *p*-hydroxybenzoic acid. Among all the examined acids significant differences in concentrations were observed only in two acids (ferulic and vanillic acids) from all examined acids. The highest amount of acids (19.34 mg/g of EEP) was determined in spring propolis, similarly as in the case of flavonoids contents.

Slightly differences in the sum of flavonoids and phenolic acids determined in propolis extracts suggest that the time of harvest of propolis had no significant effect on the relative amount of analyzed constituents in its extracts. Also, Valencia et al. (2012) observed that samples of Mexican propolis collected during four seasons of the year were characterized by similar relative abundance of the main analyzed constituents. On the other hand, the results described by Neto et al. (2017), Simoes-Ambrosio et al. (2010) and Teixeira et al. (2010) indicated that the time of propolis collection has an effect on its chemical composition. Also, Isla et al. (2012) reported that extracts of propolis collected in the summer and spring showed higher concentration of phenolic compounds than the material collected in the other seasons.

The seasonal effect on antioxidant activity of propolis

Biological properties of propolis extracts obtained from the material collected in three seasons during the year were studied using three different cell-free antioxidant assays and *in vitro* RBC-based assays. As demonstrated in Fig. 1, propolis extracts scavenge DPPH• in the dose-dependent manner (0.01 and 0.1 mg/ml). At higher concentration the scavenging activity of all extracts (27.8–31.0%) was similar to the standard antioxidant BHT (30.9%). However, the highest activity was recorded for propolis collected in the spring. The values of antiradical activity of propolis extracts toward DPPH• at a concentration of 0.01 mg/ml ranged from 13.8 to 21.2%. A high DPPH• scavenging activity was also observed in different propolis samples collected in Spain, Argentina, Brazil and China (Ahn et al., 2007a; Lima et al., 2009; Righi et al., 2011; Kumazawa et al., 2013).

The propolis extracts at a lower concentration (0.01 mg/ml) showed no significant differences in DPPH• free radical scavenging activity depending on time of its collection. At a higher concentration (0.1 mg/ml), the propolis extract obtained from the spring material shows a higher activity than samples collected in the other seasons. These differences may be explained by the slightly higher content of some flavonoids and phenolic acids in the spring material (Table 1). There are many studies describing a direct correlation between antioxidant activity of propolis extracts and their contents of phenolic compounds (Nieva Moreno et al., 2000; Ahn et al., 2007a; Kalogeropoulos et al., 2009; Benhanifia et al., 2013; Kumazawa et al., 2013). In our study DPPH• scavenging capacity of propolis extracts at a 0.1 mg/ml concentration showed significant positive correlation with chrysin (r = 0.9943), rutin (r = 0.8378), syringic acid (r = 0.9654) and caffeic acid (r = 0.9101).

The results presented in Fig. 2 indicate that all the propolis extracts exhibited a strong reducing power (reduction Fe^{3+} to Fe^{2+}), comparable to that of the standard antioxidant BHT.

The propolis extracts at the higher concentration (0.1 mg/ml) showed significant differences in their reductive potential. Similarly as in the DPPH• assay, a greater reductive capacity was observed for the extract of spring propolis than in the other samples. The values of reductive power (as the value of absorbance at 700 nm) of propolis extracts at 0.01 mg/ml ranged from 1.12 to 1.25, while at 0.1 mg/ml it was 1.19 to 1.39, respectively. The

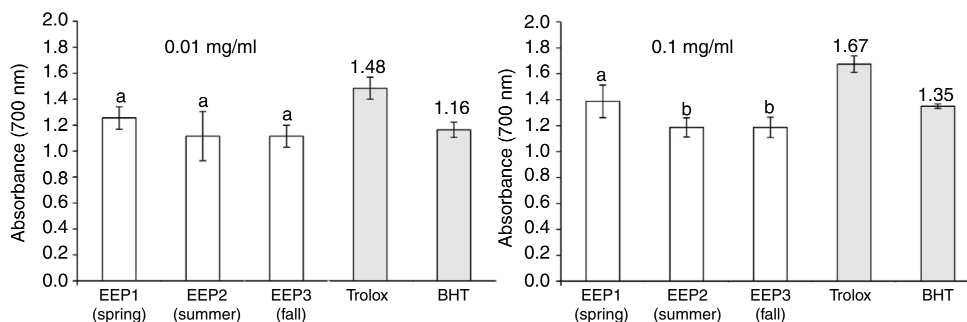

Fig. 2. Fe^{3+}–Fe^{2+} reductive potential of propolis extracts and standard antioxidants Trolox and BHT. Results are presented as average ± SD (n = 9). Different letters indicate samples that were significantly different (p < 0.05).

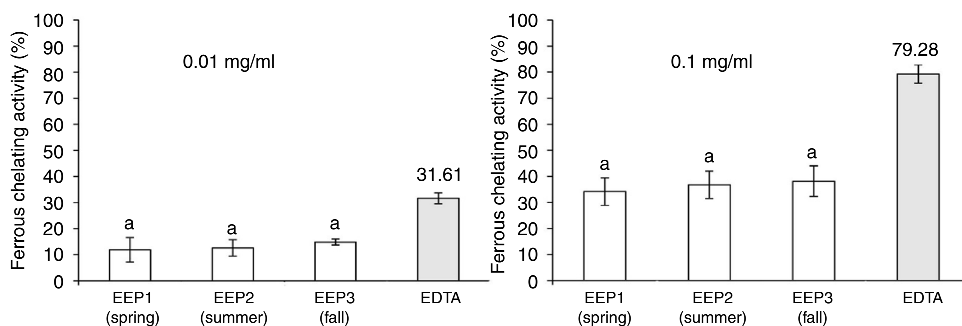

Fig. 3. Ferrous chelating activity of propolis extracts and standard antioxidants Trolox and BHT. Results are presented as average ± SD ($n = 9$). Different letters indicate samples that were significantly different ($p < 0.05$).

results are in line with the data presented in Table 1. High reducing power values were also observed in propolis extracts from Greece, Cyprus, Slovenia, Poland and China (Kalogeropoulos et al., 2009; Yang et al., 2011; Mavri et al., 2012; Socha et al., 2015). The results obtained in the reducing power assay at the propolis concentration of 0.1 mg/ml showed a significant positive correlation with chrysin ($r = 0.9864$), rutin ($r = 0.9536$), quercetin ($r = 0.8993$) and syringic acid ($r = 0.8599$).

As shown in Fig. 3, the propolis extracts at both concentrations also exhibited a significant, dose-dependent Fe^{2+}-chelating activity, equal to ~50% activity of the standard chelator EDTA.

No significant differences were observed in the chelating activity of propolis extracts at the concentrations used. However, the capacity of propolis to chelate Fe^{2+} is significantly lower when compared to standard EDTA. The extracts of Polish propolis at a concentration of 0.1 mg/ml showed a greater chelating activity than methanolic extracts (4.33–29.68%) of Portugal propolis and lower than aqueous extracts (41.11–82.35%) of this propolis (Miguel et al., 2012). Also propolis extracts from Turkey exhibited a stronger chelating effect on ferrous ions (Gulcin et al., 2010). The chelating activity of propolis extracts at a concentration of 0.1 mg/ml showed a significant positive correlation only with apigenin ($r = 0.9816$). Some flavonoids, including apigenin presents good chelating properties in contrast *e.g.* to hesperitin or naringin, which exhibit no chelating activity (Van Acker et al., 1996). Taking together, all studied propolis extracts are very effective as free radicals scavengers and Fe^{3+} reducing agents in a dose-dependent manner. Although the chelating activity on ferrous ions (Fe^{2+}) of the studied propolis extracts seems to be significantly lower when compared to the standard chelator EDTA.

Another set of experiments focused on the estimation of the effects of propolis extracts on RBC shape and their membrane permeability. Erythrocytes play a key role in the oxygen transport in the body, and their changes of shape influence on blood flow and thus in oxygen delivery within the systemic microcirculation (Silva-Herdade et al., 2016). To study the cytotoxic effect of propolis toward RBC the lower concentration (0.01 mg/ml) was selected. Microscopic evaluation of RBC shape after standard incubation (1 h, 37 °C) with propolis extracts showed mainly discocytes for EEP1 (spring), discocytes, discoechinocytes and stomatocytes for EEP2 (summer), and dysocytes and discoechinocytes for EEP3 (fall) (Table 2). Extension of incubation time to 4 h or 24 h did not modify the RBC shape any further. The changes in the RBC shape may be explained by the incorporation of components into the leaflets of the lipid bilayer in the RBC membrane (Jasiewicz et al., 2014). Therefore, it could be concluded that the weak echinocytogenic effect observed after short-term incubation (1 h and 4 h) and stomatocytogenic effects recorded after long-therm incubation (24 h) were induced by the time-dependent incorporation of propolis components into exo- and/or endoplasmic leaflets of the bilayer, respectively. As a consequence of such incorporation, the molecular organization in the lipid bilayer may be stabilized and/or modified.

The propolis extracts at the concentration of 0.01 mg/ml did not enhance the RBC membrane permeability either after 1 and 4 h incubation (hemolysis ≤ 3%), or after 24 h (hemolysis from 2 to 4%).

In line with the results presented above, the lower concentration (0.01 mg/ml) of propolis extracts was selected to study their cytoprotective activity against free radicals generated from AAPH. As shown in Fig. 4, all propolis extracts were found to significantly protect RBC against AAPH-induced oxidative hemolysis by approximately 70%, namely from 70.2 to 73.6%. The protective activity of the reference antioxidant BHT was significantly lower (47.3%) under the same conditions. It should also be noted that no statistically significant differences were observed between the propolis extracts from the different harvesting times. The anti-hemolytic activity of propolis under oxidative stress conditions was also shown by other researches, *e.g.* Bonamigo et al. (2017), Campos et al. (2014), and Dos Santos et al. (2017). The inhibition of AAPH-induced hemolysis may be explained both by the incorporation of propolis components into the RBC lipid bilayer and its stabilization against damage factors, and the antioxidant potential of propolis components, mostly by the high activity against the DPPH free radicals and Fe^{3+} reducing activity. It should be noted that our results obtained with human erythrocytes are different from obtained by others using nucleated cells, *e.g.* neutrophils (Simoes-Ambrosio et al., 2010) or the B-cell lymphoma cancer cell line M12.C3.F6. (Valencia et al., 2012), regarding the seasonal effect on

Table 2
The effect of propolis extracts on human erythrocytes at concentration of 0.01 mg/ml.

Compound	Hemolytic activity (%) 1 h, 37 °C	Dominate RBC shape 1 h, 37 °C	Hemolytic activity (%) 4 h, 37 °C	Dominate RBC shape 4 h, 37 °C	Hemolytic activity (%) 24 h, 37 °C	Dominate RBC shape 24 h, 37 °C
Control/PBS	0–2	D	0–3	D	2–5	D
EEP1 (spring)	0–3	D	0–3	D/DE	2–3	D/DE
EEP2 (summer)	0–2	D/DE,S	0–3	D/DE,S	2–4	D/DE,S
EEP3 (fall)	0–2	D/DE	0–3	D/DE	2–3	D/DE

The predominant erythrocyte shape: D, discocytes; DE, discoechinocytes; S, stomatocytes. Mean values from three independent experiments ($n = 3$) prepared in triplicate are presented.

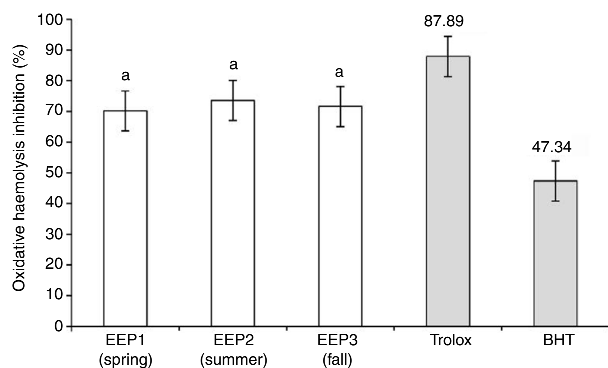

Fig. 4. *In vitro* protective effects of propolis extracts and standard antioxidants Trolox and BHT against 60 mM AAPH-induced hemolysis after 20 min pre-incubation with RBC at concentration 0.01 mg/ml. Results are presented as average ± SD ($n = 9$). Different letters indicate samples that were significantly different ($p < 0.05$).

the antioxidant activity of propolis. Therefore, it can be concluded that the antioxidant activity of propolis is the cell type-dependent, moreover, the seasonal effects is dependent on the place of harvest (climate, type of soil, sun exposure).

It is known that excessive production of reactive oxygen species (ROS) may induce the oxidative stress that may be involved in various ROS-related disorders, including cardiovascular and neurodegenerative diseases, cancer and diabetes (Bonamigo et al., 2017). *In vivo* ROS-induced RBC morphological and functional damage may stimulate hypoxia and the Fenton reaction, therefore the exogenic antioxidants present in the blood stream are important tools for RBC defense. Considering that natural products are intensively studied nowadays as potential antioxidants which may use in various applications, we propose propolis, a bee product, as a source of flavonoids and phenolic acids that can synergically protected cells against ROS detrimental effects.

Conclusions

The results demonstrated that the extracts of Polish propolis collected in three seasons during the year are rich source of phenolic compounds. Pinocembrin, chrysin, galangin and coumaric acid were the main phenols found in all the propolis extracts. The concentrations of examined constituents in all propolis samples were comparable and only seven among fifteen determined components were significantly different (THSD test, $p < 0.05$). The results demonstrate also that the propolis extracts collected in three seasons during the year possess similar and high antioxidant potential, which may be explained by scavenging free radicals and reducing activity of Fe^{2+}. The propolis extract showed high radical scavenging activity and reducing power, whereas the ferrous ion chelating activity was lower. Moreover, the significant cytoprotective activity of all propolis extracts against free radicals induced RBC hemolysis was observed. Based on the obtained results it may be stated, that cytoprotective effects of all propolis extracts are a consequence of both the antioxidant potential and the incorporation of their components into the RBC membrane and its stabilization against ROS injuries.

Author contribution

Conceived and designed the experiments: MW, IR; performed the experiments: MW, LM, AW, TR, IR; analyzed the date: MW, LM, IR; wrote the paper: MW, LM.

Conflicts of interest

The authors declare no conflicts of interest.

References

Ahn, M.R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., Nakayama, T., 2007a. Antioxidant activity and constituents of propolis collected in various areas of China. Food Chem. 101, 1383–1392.

Ahn, M.R., Kunimasa, T., Ohta, T., Kumazawa, S., Kamihira, M., Kaji, K., Uto, Y., Hori, H., Nagasawa, H., Nakayama, T., 2007b. Suppression of tumor-induced angiogenesis by Brazilian propolis: major component artepillin C *in vitro* tube formation and endothelial cell proliferation. Cancer Lett. 252, 235–243.

Andrade, J.K.S., Denadai, M., de Oliveira, C.S., Nunes, M.L., Narain, N., 2017. Evaluation of brow, green and red propolis from Brazilian northeast region. Food Res. Int. 10, 129–138.

Bankova, V., Boundourova-Krasteva, G., Popov, S., Sforcin, J.M., Funari, S.R.C., 1998. Seasonal variations of the chemical composition of Brazilian propolis. Apidologie 29, 361–367.

Banskota, A.H., Tezuka, Y., Kadota, S., 2001. Recent progress in pharmacological research of propolis. Phytother. Res. 15, 561–571.

Benhanifia, M., Mohamed, N.M., Bellik, Y., Benbarek, H., 2013. Antimicrobial and antioxidant activities of different propolis samples from north-western Algeria. Int. J. Food Sci. Technol. 48, 2521–2527.

Bessis, M., Weed, R.I., Lablond, P.F., 1973. Red cell shape. In: Physiology, Pathology, Utrastructure. Springer-Verlag New York, Heidelberg, Berlin.

Bonamigo, T., Campos, J.F., Oliveira, A.S., Torquato, H.F.V., Balestieri, J.B.P., Cardoso, C.A.L., Paredes-Gamero, E.J., de Picoli Souza, K., Dos Santos, E.L., 2017. Antioxidant and cytotoxic activity of propolis of *Plebeia droryana* and *Apis mellifera* (Hymenoptera. Apidae) from the Brazilian Cerrado biome. PLOS ONE 12, e0183983.

Cai, Y., Luo, Q., Sun, M., Corke, M., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74, 2157–2184.

Campos, J.F., Dos Santos, U.P., Macorini, L.F., de Melo, A.M., Balestieri, J.B., Paredes-Gamero, E.J., Cardoso, C.A., de Picoli Souza, K., Dos Santos, E.L., 2014. Antimicrobial, antioxidant and cytotoxic activities of propolis from *Melipona orbignyi* (Hymenoptera, Apidae). Food Chem. Toxicol. 65, 374–380.

Castaldo, S., Capasso, F., 2002. Propolis, an old remedy used in modern medicine. Fitoterapia 73 (Suppl. 1), S1–S6.

Di Dalmazi, G., Hirsherberg, J., Lyle, D., Freij, J.B., Caturegli, P., 2016. Reactive oxygen species in organ-specific autoimmunity. Autoimmun Highlights, http://dx.doi.org/10.1007/s13317-016-0083-0.

Dos Santos, H.F.D., Campos, J.F., Santos, C.M.D., Balestieri, J.B.P., Silva, D.B., Carollo, C.A., de Picoli Souza, K., Estevinho, L.M., Dos Santos, E.L., 2017. Chemical profile and antioxidant, anti-inflammatory, antimutagenic and antimicrobial activities of geopropolis from the stingless bee *Melipona orbignyi*. Int. J. Mol. Sci. 18, 953.

Ghisalberti, E.L., 1979. Propolis: a review. Bee World 60, 59–84.

Gong, S., Luo, L., Gong, W., Gao, Y., Xie, M., 2012. Multivariate analyses of element concentrations revealed the groupings of propolis from different regions in China. Food Chem. 134, 583–588.

Gulcin, I., Bursal, E., Sehitoglu, M.H., Bilsel, M., Goren, A.C., 2010. Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey. Food Chem. Toxicol. 48, 2227–2238.

Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacol. Therapeut. 96, 67–202.

Huang, S., Zhang, C.P., Wang, K., Li, G.Q., Hu, F.L., 2014. Recent advances in the chemical composition of propolis. Molecules 19, 19610–19632.

Isla, M.I., Dantur, Y., Salas, A., Danert, C., Zampini, C., Arias, M., Ordonez, R., Maldonado, L., Bedascarrasbure, E., Moreno, M.I.N., 2012. Effect of seasonality on chemical composition and antibacterial and anticandida activities of Argentine propolis. Design of a topical formulation. Nat. Prod. Commun. 7, 1315–1318.

Jasiewicz, B., Mrówczyńska, L., Malczewska-Jaskola, K., 2014. Synthesis and haemolytic activity of novel salts made of nicotine alkaloids and bile acids. Bioorg. Med. Chem. Lett. 24, 1104–1107.

Kalogeropoulos, N., Konteles, S.J., Troullidou, E., Mourtzinos, I., Karathanos, V.T., 2009. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem. 116, 452–461.

Kasote, D.M., Pawar, M.V., Bhatia, R.S., Nandre, V.S., Gundu, S.S., Jagtap, S.D., Kulkarni, M.V., 2017. HPLC, NMR based chemical profiling and biological characterisation of Indian propolis. Fitoterapia 122, 52–60.

Kedzia, B., 2009. Chemical composition of Polish propolis. Part II. New studies. Post. Fitoter. 2, 122–128.

Kumazawa, S., Bonvehi, J.S., Torres, C., Mok-Ryeon, A., Bermejo, F.J.O., 2013. Chemical and functional characterization of propolis from East Andalusia (Southern Spain). Phytochem. Anal. 24, 608–615.

Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. Food Chem. 84, 329–339.

Kurek-Gorecka, A., Rezpecka-Stojko, A., Gorecki, M., Stojko, J., Sosada, M., Swierczek-Zieba, G., 2014. Structure and antioxidant activity of polyphenols derived from propolis. Molecules 19, 78–101.

Kuropatnicki, A.K., Szliszka, E., Krol, W., 2013. Historical aspects of propolis research in modern times. Evid.-Based Complement. Altern. Med., http://dx.doi.org/10.1155/2013/964149.

Leopoldini, M., Marino, T., Russo, N., Toscano, M., 2004. Antioxidant properties of phenolic compounds: H-atom versus electron transfer mechanism. J. Phys. Chem. A 108, 4916–4922.

Li, F., Awale, S., Zhang, H., Tezuka, Y., Esumi, H., Kadota, S., 2009. Chemical constituents of propolis from Myanmar and their preferential cytotoxicity against a human pancreatic cancer cell line. J. Nat. Prod. 72, 1283–1287.

Lima, B., Tapia, A., Luna, L., Fabani, M.P., Schmeda-Hirschmann, G., Podio, N.S., Wunderlin, D.A., Feresin, G.E., 2009. Main flavonoids, DPPH activity, and metal content allow determination of the geographical origin of propolis from the province of San Juan (Argentina). J. Agric. Food Chem. 57, 2691–2698.

Lobo, V., Patil, A., Phatak, A., Chandra, N., 2010. Free radicals, antioxidants and functional foods: impact on human health. Pharmacogn. Rev. 4, 118–126.

Mavri, A., Abramovic, H., Polak, T., Bertoncelj, J., Jamnik, P., Mozina, S.S., Jersek, B., 2012. Chemical properties and antioxidant and antimicrobial activities of Slovenian propolis. Chem. Biodivers. 9, 1545–1558.

Melliou, E., Stratis, E., Chinou, I., 2007. Volatile constituents of propolis from various regions of Greece – antimicrobial activity. Food Chem. 103, 375–380.

Miguel, M.G., Nunes, S., Dandlen, S.A., Cavaco, A.M., Antunes, M.D., 2012. Phenols, flavonoids and antioxidant activity of aqueous and methanolic extracts of propolis (*Apis mellifera* L.) from Algarve, South Portugal. Food Sci. Technol.-Brazil 34, 16–23.

Neto, M.S.R., Tintino, S.R., da Silva, A.R.P., Costa, M.S., Boligon, A.A., Matias, E.F.F., Balbino, V.Q., Menezes, I.R.A., Coutinho, H.D.M., 2017. Seasonal variation of Brazilian red propolis; antibacterial activity, synergistic effect and phytochemical screening. Food Chem. Toxicol. 107, 572–580.

Nieva Moreno, M.I., Isla, M.I., Sampietro, A.R., Vattuone, M.A., 2000. Comparision of the free radical-scavenging activity of propolis from several regions of Argentina. J. Ethnopharmacol. 71, 109–114.

Papotti, G., Bertelli, D., Bortolotti, L., Plessi, M., 2012. Chemical and functional characterization of Italian propolis obtained by different harvesting methods. J. Agric. Food Chem. 60, 2852–2862.

Phaniendra, A., Jestadi, D.B., Periyasamy, L., 2015. Free radicals: properties, sources, targets, and their implication in various diseases. Ind. J. Clin. Biochem. 30, 11–26.

Popova, M., Giannopoulou, E., Skalicka-Wozniak, K., Graikou, K., Widelski, J., Bankova, V., Kalofonos, H., Sivolapenko, G., Gawel-Beben, K., Antosiewicz, B., Chinou, I., 2017. Characterization and biological evaluation of propolis from Poland. Molecules 22, 1159.Righi, A.A., Alves, T.R., Negri, G., Marques, L.M., Breyer, H., Salatino, A., 2011. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. J. Sci. Food Agric. 91, 2363–2370.

Salas, A.L., Alberto, M.R., Zampini, I.C., Cuello, A.S., Maldonado, L., Rios, J.L., Schmeda-Hirschmann, G., Isla, M.I., 2016. Biological activities of polyphenols-enriched propolis from Argentina arid regions. Phytomedicine 23, 27–31.

Silva-Herdade, A.S., Andolina, G., Faggio, C., Calado, Â., Saldanha, C., 2016. Erythrocyte deformability – a partner of the inflammatory response. Microvasc. Res. 107, 34–38.

Simoes-Ambrosio, L.M.C., Gregorio, L.E., Sousa, J.P.B., Figueiredo-Rinhel, A.S.G., Azzolini, A.E.C.S., Bastos, J.K., Lucisano-Valim, Y.M., 2010. The role of seasonality on the inhibitory effect of Brazilian green propolis on the oxidative metabolism of neutrophils. Fitoterapia 81, 1102–1108.

Socha, R., Galkowska, D., Bugaj, M., Juszczak, L., 2015. Phenolic composition and antioxidant activity of propolis from various regions of Poland. Nat. Prod. Res. 29, 416–422.

Souza, E.A., Zaluski, R., Veiga, N., Orsi, R.O., 2016. Effects of seasonal variations and collection methods on the mineral composition of propolis from *Apis mellifera* Linnaeus Beehives. Braz. J. Biol. 76, 396– 401.

Szliszka, E., Sokol-Letowska, A., Kucharska, A.Z., Jaworska, D., Czuba, Z.P., Krol, W., 2013. Ethanolic extract of polish propolis: chemical composition and TRAIL-R2 death receptor targeting apoptotic activity against prostate cancer cells. Evid.-Based Complement. Altern. Med., http://dx.doi.org/10.1155/2013/757628.

Teixeira, E.W., Message, D., Negri, G., Salatino, A., Stringheta, P.C., 2010. Seasonal variation, chemical composition, antioxidant activity of Brazilian propolis samples. Evid.-Based Complement. Altern. Med. 7, 307–315.

Toreti, V.C., Sato, H.H., Pastore, G.M., Park, Y.K., 2013. Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evid.-Based Complement. Altern. Med., http://dx.doi.org/10.1155/2013/697390.

Valencia, D., Alday, E., Robles-Zepeda, R., Garibay-Escobar, A., Galvez-Ruiz, J.C., Salas-Reyes, M., Jimenez-Estrada, M., Velazquez-Contreras, E., Hernandez, J., Velazquez, C., 2012. Seasonal effect on chemical composition and biological activities of Sonoran propolis. Food Chem. 131, 645–651.

Van Acker, S.A.B.E., Van den Berg, D.J., Tromp, M.N.J.L., Griffioen, D.H., Van Bennekom, W.P., Van der Vijgh, W.J.F., Bast, A., 1996. Structural aspects of antioxidant activity of flavonoids. Free Rad. Biol. Med. 20, 331–342.

Wagh, V.D., 2013. Propolis: a wonder bees product and its pharmacological potentials. Adv. Pharmacol. Sci., http://dx.doi.org/10.1155/2013/308249.

Yang, H., Dong, Y., Du, H., Shi, H., Peng, Y., Li, X., 2011. Antioxidant compounds from propolis collected in Anhui, China. Molecules 16, 3444–3455.