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Electrochemical and Spectrophotometric Characterization of the Propolis Antioxidants Properties

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In this work, ethanol extracts of propolis from Europe, before and after 10 years of storage, were prepared and investigated by electrochemical methods, FTIR, and UV-Vis spectrometry assays. A combination of electrochemistry (cyclic and differential pulse voltammetry) and spectrometry allowed for the establishment of chemical correlation between the composition and the activities of bioactive polyphenols. Based on the content of the individual components and their content groups, the antioxidant properties of propolis precursors were characterized. Extracts of propolis before (ExI) and after 10 years (ExII) of storage were irreversibly oxidized in at least two electrode stages. ExII was oxidized more difficultly than ExI, and revealed better antioxidant properties. The antiradical and overall antioxidant potentials of the substances present in the extract of propolis after 10 years of storage, have been demonstrated in this paper. The storage time (10 years) did not significantly alter the properties of propolis, as evidenced by the high potential for free radical scavenging (ABTS, DPPH) and reduction of metal ions. Some of the materials present in propolis have been oxidized, and as confirmed by the FTIR analysis, this facilitates the passage of polyphenols from the propolis into the anhydrous (ethanol or acetonitrile) environment. Thus, propolis, as a rich source of natural phytocompounds, may be used in the prevention of various free radical-related ailments.

Keywords: propolis; antioxidant; electrochemistry; spectroscopy; phytochemistry

1. INTRODUCTION

The issue of antioxidant properties of plant-derived compounds has been widely reported in numerous scientific papers over the last few years. From the literature it is known that propolis, or bee glue, is a mixture of resin from trees or shrubs and secretions of bee glands, collected by honey bees, Apis mellifera [1-4]. Young tree buds are covered with balm that protects them against bacteria, mites,

fungi and insects. Bees also use propolis to fill any leaks and holes in the hive. A thin layer of propolis covers its internal walls. Propolis is also a natural barrier defending bees against microbial invasion. It is because propolis is a very complex substance rich in biological active chemical compounds, above all, polyphenols that are considered to contribute more to the healing effects than the other propolis constituents [5].

Propolis has a wide and very well-documented therapeutic effect and is used largely in pharmacological and natural medicine [6-8]. Due to the diversity of its chemical composition, propolis exhibits numerous pharmacological activities, such as antioxidant, antimicrobial, antifungal, anti-inflammatory, antiviral and anti-tumour effects [9-11].

Generally, studies show there are numerous, over 300 known substances composing propolis. Most of them are flavonoids, phenolic acids and phenolic acid phenyl esters, as mentioned above. But it also contains lipid-wax substances and terpenes, amino acids, steroids, aldehydes, and ketones, sterols, enzymes, fatty acids as well as proteins, micronutrients and vitamins [12-15]. Other reports indicate the presence of resin (50%), wax (30%), aromatic oils (10%), pollen (5%), and other organic compounds widely used in adult and paediatric medications and in many dietary supplements. Through the synergistic action of these substances, propolis has the ability to destroy bacteria, pathogenic fungi, viruses and protozoa. Its activity against microorganisms, e.g., from the Staphylococcus, Enterococcus faecalis, and Candida utilis groups has been confirmed. Undoubtedly, propolis is one of the most interesting so-called plant-based tar [16-20].

The composition of propolis, primarily based on polyphenols, includes mainly such compounds as caffeic acid, p-coumaric acid, ferulic acid, pinobanksin, quercetin, apigenin, t-cinnamic acid, luteolin, chrysin, pinocembrin, galangin, and kaempferol pinostrobin. It is worth noting that the literature reveals interesting references regarding the influence of latitude on the composition of propolis. Therefore, evidence has been found for the presence of neoflavonoids in Nepalese propolis, as well as an interesting propolin G and flavanone geranyl in Taiwanese propolis [21-30]. Frequently tested and widely available propolis from Brazil is rich in isoflavonoids, while isonymphaeol C, and flavanone geranyl are present in Egyptian propolis [31-32], whereas Mexican propolis contains large amounts of the 8-phenylallyl derivative of galangin. Hence, the geographic origin is a factor determining variations in the chemical composition of propolis and, as a consequence, is associated with the biological activity depending on its type.

Most of the research focuses on studying the antibacterial mechanisms of propolis. Its effect is largely due to the strong antioxidant activity and inhibition of free radicals. In the present paper, we propose a description of the antioxidant properties of the components contained in the material under study. We focused mainly on determining the mechanisms of antioxidant activity by sweeping free radicals and chelating iron ions. The electrolytic reactions of the tested ethanol extracts from propolis have been described [33-36].

The evaluation of the plant material properties of propolis was carried out by combining several research methods, which allowed for the analysis of pro-ecological activity in different environments and by different mechanisms. Electrochemical methods are particularly useful in the study of polyphenols antioxidant properties. In particular, cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques have been successfully used to detect phenolic compounds in a variety

of aqueous and non-aqueous solutions. In the past, some authors have identified an interesting relationship between the electrochemical behaviour of antioxidant compounds and their "antioxidative power" [37].

Because of the high inhibitory activity towards oxidation, propolis can be used medically as a compound for the fight against free radical-induced oxidation, as a free radical scavenger. Literature review from recent years shows a large number of papers regarding composition testing and properties of propolis [38-42]. However, there are few studies on the lifetime of the antioxidant compounds activity and the individual chemical constituents in propolis. In addition, propolis as one of the most interesting plant materials is currently widely studied in many scientific centers [43-46]. However, it should be noted that many studies focus mainly on two of its types: Brazilian green propolis and temperate poplar propolis.

In the present study, the in vitro antioxidant activity of ethanol extracts from propolis, originating from Central Europe and subjected to various ageing times, was examined, and then their individual constituents were analysed. The composition of propolis and its properties before and after 10 years of storage were evaluated. The overriding objective of the study was to assess the lifetime of the active substances in propolis.

2. EXPERIMENTAL

2.1 Reagents

Propolis samples (produced by honey bees, Apis mellifera L., in various regions of Poland) were obtained from a local farmer (Poland, Lodz). All chemicals used were of analytical grade supplied from Fluka and Sigma-Aldrich. Experiments were performed in non-aqueous media. The substrate solutions were prepared by dissolving in 0.1 mol dm^{-3} $(C_4H_9)_4NClO_4$ in acetonitrile. The concentrations of the propolis were 1.995 g dm^{-3} and 4.066 g dm^{-3}. The solutions were thoroughly deoxygenated by purging with purified argon gas (99.99%) for 15 min prior to the electrochemical experiments. An argon blanket was maintained over the solutions to supply an inert atmosphere during voltammetric measurements.

2.2. Preparation of ethanol extracts of propolis

Samples of propolis extracts (ExI) for testing have been prepared using one of the conventional techniques, i.e. the solvent extraction method using ethanol. For this purpose, raw propolis was cut into small pieces and extracted with a 5-fold volume of 70% ethanol under shaking conditions. The extraction was carried out in the darkness and at ambient temperature for 7 days. The final extract of propolis was concentrated on a rotary evaporator under reduced pressure conditions at 30°C to constant weight. The aged samples of propolis (ExII) were stored for 10 years under conditions of low temperature, i.e. in a refrigerator, in a sealed glass container.

2.3. Measurement methods

Cyclic and differential pulse voltammetry

In the electrochemical measurements of the oxidation of the propolis ex-cycle, cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed using the AUTOLAB PGSTA30 electroanalytical kit (Ecochemie, Netherlands). This set was controlled by GPES (version 4.8) and connected to a three-electrode system and a computer for storing and processing data. The indicator electrode (platinum) was tested as the reference electrode with reference to ferrocenium/ferrocene (Fc^{+}/Fc). The third electrode was Pt and used as an auxiliary electrode. The geometrical surface of the test electrode was 0.5 cm^2. CV and DPV were performed from 0 to 2 V potentials at different polarization rates (0.01 to 1 V s^{-1}). DPV was performed with an amplitude modulation of 25 mV and pulse width of 50 ms (scan rate 0.01 V s^{-1}). All the solutions before measurement were deoxygenated for 20 min with argon to remove dissolved oxygen. During measurement the solutions were maintained in an argon atmosphere. The experiments were conducted at room temperature.

DPPH radical-scavenging activity

DPPH (2,2-diphenyl-1- picrylhydrazyl) is a stable free radical due to the delocalization of the spare electron over the whole molecule. Thus, DPPH does not dimerize, as occurs with most free radicals. The radical scavenging activity of the propolis samples was examined by using a DPPH assay [47]. An ethanol solution of DPPH (2.0 mL) with a concentration of 40 mg cm^{-3} (0.1 mM) was added to 0.5 cm^3 of an alcohol solution (90% ethanol) that contained 0.02 mg cm^{-3} antioxidant. Then, 10 minutes after mixing, the absorbance of the solution was determined by UV-Vis spectroscopy at 516 nm. UV-VIS spectra were recorded with a ThermoScientific Evolution 220 spectrophotometer (2015, USA). As a blank, 70% ethanol was used. The capability to scavenge the DPPH radical (AA%) was determined using following equation:

$$\text{Inhibition } (A\%) = [((A_0 - A_1) / A_0)\ 100] \quad (1)$$

where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the sample of antioxidant.

ABTS radical-scavenging activity

The ability of propolis samples to scavenge free radicals was analysed by using an ABTS assay. Potassium persulfate (2.45 mM) and ABTS (6 mM) were mixed in ethanol, and then, the mixture was allowed to stand for 16 h [48]. The ABTS/radical solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. UV-VIS spectra were recorded with a Thermoscientific Evolution 220 spectrophotometer produced in 2015 in the USA. As a blank, 70% ethanol was used. The diluted ABTS solution (6.0 cm^3) was added to 50 mm^3 of each antioxidant solution (6 mg cm^{-3}) or Trolox. The inhibition level was calculated using the standard curve of absorbance at 734 nm. Three parallel measurements were made. The results are given as mean values. The results are presented as Trolox

equivalent antioxidant capacity (TEAC), mmol Trolox/100 g antioxidant. The trolox concentration range was 0 – 20 μmol dm^{-3}.

Ferric reducing antioxidant power assay (FRAP)

The ability of propolis samples to reduce ferric ion (Fe^{3+}-TPTZ complex) under acidic conditions was determined using the method of FRAP [49,50].

The analysis involves the studying the change in absorbance of the blue-coloured ferrous form (Fe^{2+}-TPTZ complex) at 595 nm. The FRAP reagent was obtained by the addition of 25 mL of acetate buffer (0.3 M, pH 3.6), 2.25 cm^3 of a TPTZ solution (10 mM TPTZ in 40 mM HCl) and 2.25 cm^3 of $FeCl_3$ (20 mM) in a water solution. The reaction mixture was held at 37°C for 4 min. As a blank, the reagent mixture without an antioxidant was used.

Cupric ion reducing antioxidant capacity (CUPRAC) assay

0.25 ml of $CuCl_2$ (0.01 M), 0.25 ml of neokuproin ethanol solution ($7.5x10^{-3}$ M) and 0.25 ml of $CH3COONH_4$ (1 M) buffer solution were mixed in a test tube, and then various concentrations of propolis were added. The total volume was then made up to 2 ml with distilled water and mixed. The tubes were closed and left at room temperature. Absorbance at 450 nm was measured against blank reagent (water) after waiting 30 minutes. The Cupric ions (Cu^{2+}) reducing power was calculated as:

$\Delta A = A_{30} - A_0$,

A_0 - absorbance of the reagent test, A_{30} - absorbance after 30 minutes of reaction [49,51,52].

Thermal decomposition

The thermogravimetric (TG) analysis of the propolis was performed by using a Mettler Toledo Thermobalance. Samples of approximately 5 mg were placed in aluminium pans and heated from 20°C to 700°C under a dynamic flow of nitrogen (50 cm^3 min^{-1}). Five heating rates (5°C min^{-1}) were used.

Statistical analysis

Each antioxidant activity assay was done three times from the same extract in order to determine their reproducibility. Pearson's calculation was used to designate a correlation coefficient, with ϱ as the population parameter and r as the sample statistic. This coefficient is affected by extreme values, which can strengthen or dampen the evaluated activity of the tested raw material. In order to correlate between variables x and y, the model for calculation the sample Pearson's correlation coefficient was used.

UV-VIS Spectra

The UV-VIS spectra of the propolis extract solutions were recorded from a mixture of 25 cm^3 of each extract plus 30 cm^3 of 96% ethanol. The mixture was scanned from 190-1100 nm in a UV-spectrophotometer (UV Mini 1240, Shimadzu Co.).

Extracts of propolis in acetonitrile and ethanol at a concentration of 1mg cm^{-3} were diluted one hundred times. The UV-Vis spectrophotometer was calibrated for acetonitrile and ethanol respectively, 2 cm^3 of solutions were taken and absorbance in the 190-1100 nm wavelength range was measured.

FTIR spectroscopy

The BIO-RAD FT-IR 175C spectrophotometer was used for the measurements. The samples of propolis, before and after 10 years of storage, were prepared by washing the material with acetone and then were placed at the output of infrared beams. As the result of the test, oscillating spectra were obtained, the analysis of which allows to determine the functional groups with which the radiation interacted.

3. RESULTS AND DISCUSSION

3.1. FTIR, UV-Vis spectroscopic and thermogravimetric (TG) analyses of propolis extracts

In order to analyze the composition of the test samples, infrared spectra were taken, which is shown in Figure 1 (A, B). The spectra obtained contain so-called “fingerprint” information of the measured phytocompounds and thus reveal the composition of the tested propolis samples.

Evidence of polyphenolic compounds present in the extracts of propolis is clearly visible. Thus, the presence of bands, accordingly, at 3307-3368 cm^{-1} and C-O band at 2879 cm^{-1} accurately confirm the polyphenol and ester moieties,. The bands at 1045 cm^{-1} correspond to the stretch of the aromatic ether C–O bond for flavonoids, as well as the band at 877 cm^{-1}, corresponding to the angular deformation of C-H outside the aromatic plane. The symmetric effects of the N-O and C-N groups present at 1334-1290 cm^{-1} and N-H at 3400-3250 cm^{-1} may indicate the presence of amino acids. C-O and O-H bands at 1200-1000 cm^{-1} can also be seen in the spectra, which can be attributed to the presence of fatty acids, stilbenes, steroids and carboxylic acids. The presence of terpenes is suggested from the weak absorbance of C=C at 1651-1659 cm^{-1}, and C-O at 1320-1000 cm^{-1}. What is worth emphasizing, no spectacular change has been observed in the intensity of absorption bands in the spectra of propolis obtained in the current year and after 10 years of storage.

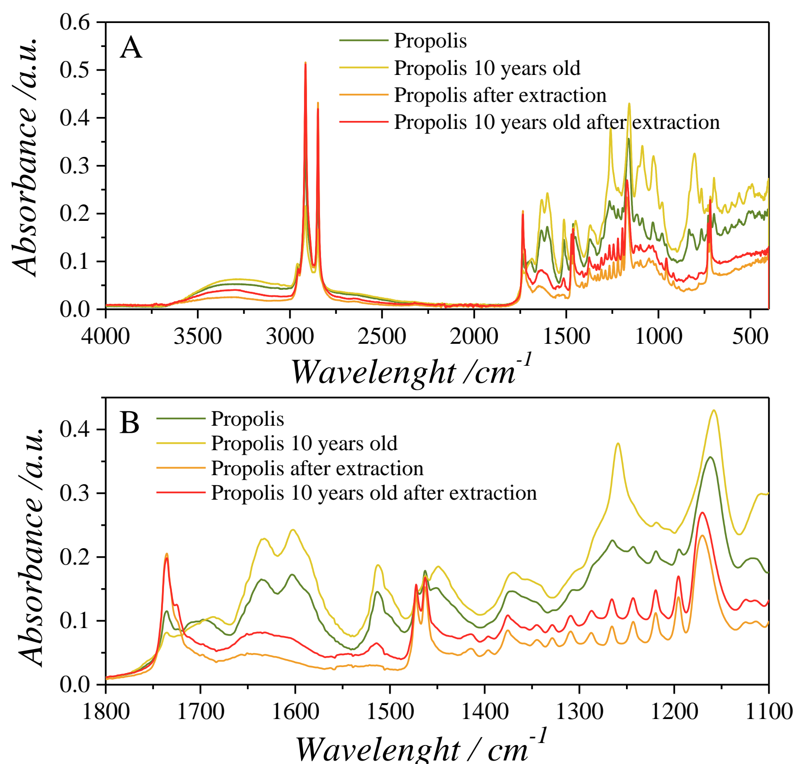

Figure 1. FTIR spectra of propolis extracts under study, before and after 10 years of storage.

Nevertheless, polyphenol compounds contained in propolis after 10 years of storage, dissolved more readily in ethanol, used for extraction. There is a clear increase in absorption in the range attributed to C=C unsaturated bonds, present in the spectrum of propolis after ageing (i.e. 10 years). At the same time, the ageing also affects the intensity of the band from C=O, near 1700 cm^{-1} [53,54]. More hydrophilic groups have better affinity for ethanol, hence, the ethanol extract of 10-year propolis has a slightly higher content of compounds containing carbonyl-unsaturated bonds. In the rest of the FTIR spectrum, it can be seen that the composition of the propolis under study and the resulting properties remain unchanged even after many years.

UV-Vis spectra of the ethanol and acetonitrile extracts of propolis (in anhydrous environments) were obtained (Figure 2). The concentration and content of the polyphenol compounds in the tested extracts were analysed. Spectrophotometric analysis is a cheap and easy way to analyse the content of phytocompounds. From the literature, it is known that phenolic compounds present in plant extracts are visible in UV-Vis spectra in the range of λ = 280-350 nm. The best correlation between the antioxidant capacity and the absorbance of UV–visible spectra was found at 353 nm. Interestingly, Mot et al. [13] claims that the extracts that show a band at 1630 cm^{-1} in the IR region and an absorbance band of 353 nm in the UV-vis area exhibit good antioxidant activity. Often, combining IR and UV-Vis analyses provides information on not only the antioxidant capacity and composition, but also floral origin.

The intensity of the absorbance is higher for the ethanol extract, i.e., corresponding to the aqueous medium. A significantly lower amount of polyphenol compounds passed to the anhydrous environment,

i.e., acetonitrile. Interestingly, the extract from 10-year-old propolis is rich in antioxidants compared with the extract of propolis obtained in the current year. Thus, UV-Vis spectroscopy is a complementary method to FTIR, confirming that the propolis ageing time affects the oxidation of some polyphenolic compounds, which facilitated the extraction of flavonoids, hydroxycynaminic acids and phenolic acid phenyl esters, amino acids into the solution.

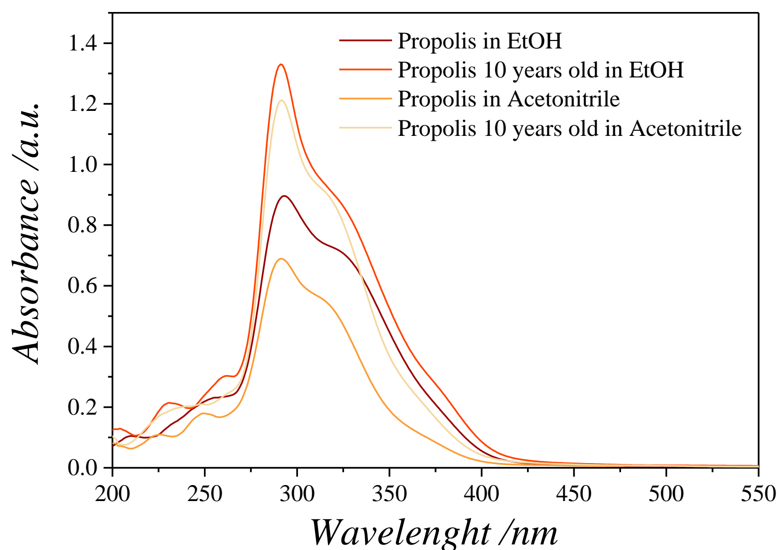

Figure 2. UV-Vis spectra of propolis extracts under study, before and after 10 years of storage.

The thermal stability of propolis samples was investigated by TG-DTG analysis (30–800°C) (Figure 3). The resulting mass change versus temperature curve provides information concerning the thermal stability and composition of the tested plant samples. The obtained results reveal that the decomposition of propolis material is a three-stage process in the range of 27-780°C. The first endothermic transformation is associated with the evaporation of the physically adsorbed water content [55]. The second portion was recorded in the temperature range from 120 to 450°C, with a mass loss of 81.08%. The second transformation is associated with the partial thermal degradation of the polyphenolic constituents, and other material contained in the examined propolis was noted to decompose above 450°C. The loss of weight of the second and subsequent transformations are bound to the disaggregation and the spallation of C–O and C–C bonds in the ring units. There are two transformations in the TG curve of 10-year-old propolis sample. One is similar to the fresh dehydrating of the propolis, while the second transformation, that starts at 267°C, yields a weight loss of 84%. Thus, it can be seen that ageing causes the oxidation of some of the compounds, which showed slightly lower thermal stabilities in this sample of propolis.

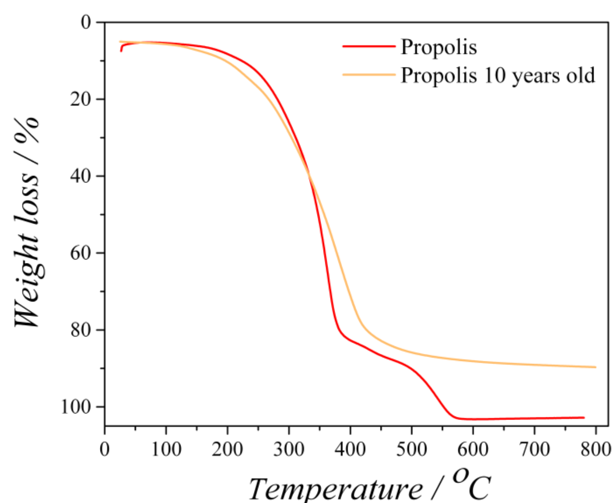

Figure 3. TGA curves of propolis extracts under study, before and after 10 years of storage.

3.2. Evaluation of the antioxidant properties of propolis by electrochemical techniques

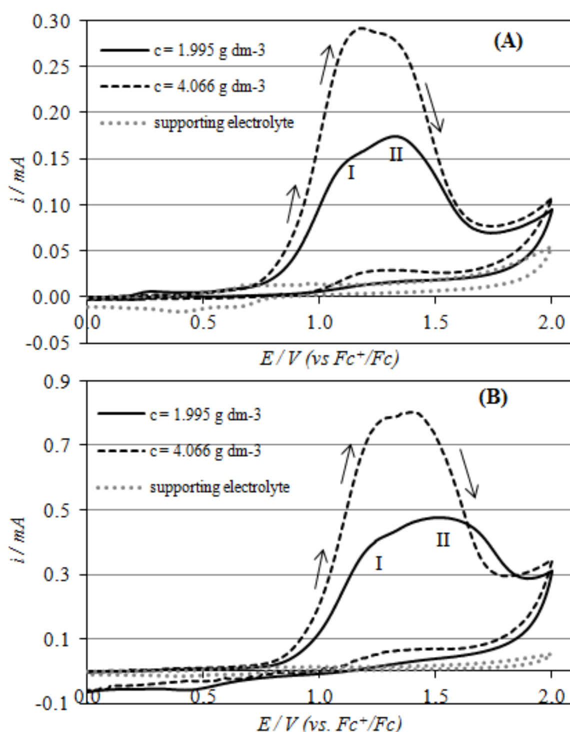

Figure 4. Cyclic voltammograms (CV) of propolis extracts on a platinum electrode with a polarization rate of 0.1 V s^{-1}: (A) – ExI and (B) – ExII.

The relationship between the current (i) and the potential (E) of the test electrode relative to the reference electrode provides basic information about the electrode processes and the antioxidant properties of the test compounds. Electrochemical methods such as cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were used to determine the antioxidant properties of propolis. DPV is characterized by a greater resolution and accuracy than CV, allowing for a better separation of

peaks. DPV involves two measurements of the current intensity for each potential pulse: one measurement before the potential pulse, and the second towards the end of the pulse period. Hence, the double current sampling in DPV allows for quantitative determinations at concentrations as low as 0.05 µM. In addition, with DVP, it is possible to eliminate adsorbing compounds because they are not electro-active in this technique, and there are no visible peaks in the voltammogram. The designated peak potential (E_p) in DPV corresponds to the half-wave potential ($E_{1/2}$) of CV. Two different propolis extracts were tested: ExI (fresh propolis) and ExII (10-year propolis).

Fig. 4 A shows the cyclic voltammograms of ExI oxidation in the 0 to 2.0 V potential range for two concentrations. From the presented current dependency on the potential, ExI is irreversibly oxidized in at least two electrode steps before the potential for electrolyte degradation. The potential of the first oxidation peak is 1.12 V, while that of the second peak is 1.29 V. In the reverse is greater, as evidenced by the increasing peak currents. Within the range of potentials in which the test compounds oxidize, no visible oxidation peaks are present in the supporting electrolyte. From the voltammograms shown in Fig. 4B, ExIIs are irreversibly oxidized with more thermodynamically difficulty compared with ExI, i.e., with more positive potential; The potential of the first peak is 1.25 V, while that of the second peak is 1.39 V. The peak currents for ExII are approximately 2.5 times higher than for ExI, which is indicative of its higher antioxidant power. With the increase in ExI concentration, its antioxidant power differential voltammograms of the electrooxidation of the extracted propolis at the Pt electrode.

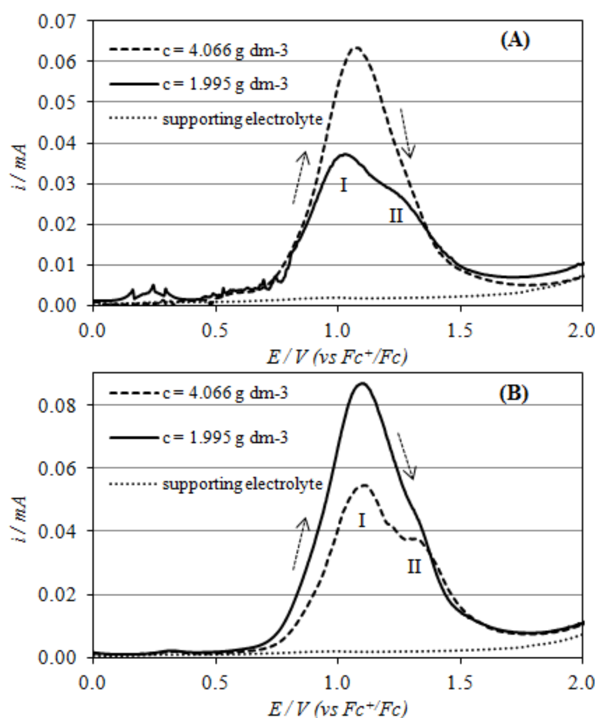

Figure 5. Differential pulse voltammetry (DPV) of propolis extracts in 0.1 mol dm^{-3} $(C_4H_9)_4NClO_4$ in acetonitrile at a Pt electrode; (A) – ExI and (B) – ExII.

Fig. 5 shows the voltammograms recorded by the DPV electrode position method for two concentrations of the propolis extracts. From the relationship shown in Fig. 5A, ExI is oxidized in at

least two electrode stages at 1.02 V and 1.22 V. At lower ExI concentrations there are two electrolytic peaks, while at higher concentrations one broad peak is visible. Fig. 5B shows the voltammograms for ExII, which is oxidized more difficultly than ExI by approximately 80 mV. The potential of the first electrolytic peak for ExII is 1.11 V, while that of the second peak is 1.31 V. Based on the CV and DPV oxidation tests, ExI has better antioxidant properties than does EII in terms of thermodynamics but has less antioxidant power. However, the low electrolysis potentials of ExI and ExII testify to their good antioxidant properties, which were confirmed by other methods.

3.3. Measurement of the radical-scavenging activities of propolis extracts using ABTS, DPPH and FRAP, CUPRAC methods

Examination on antioxidant properties of propolis is one of many areas of intensive research in the field of biology and chemistry. They apply spectrophotometric methods that use stable free radical DPPH (2,2-diphenyl-1- picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) decolorization assays. In the present work, we carried out a study on the efficiency of the scavenging capacity of free radicals, as a function of time in a reaction mixture.

It is known from the literature review that propolis is barely soluble in water and should be purified by extraction with solvents to remove the inert material and preserve the polyphenolic fraction. Thus, it is best to solubilize propolis in hydrophilic solvents, preferably in ethanol or methanol. Polyphenol compounds are one of the best antioxidants of all plant substances. The principal structural skeleton of flavonoid consists of 15 atoms of carbon making up a system of two benzene rings C_6 (A and B) connected with the heterocyclic rings of piron (C) (Fig. 6). Their reactivity depends on their structure; in particular, on the number and position of hydroxyl groups on rings B and C, or at least those on ring A. The compounds may react with free radicals according to two mechanisms: hydrogen atom transfer (HAT) or single electron transfer (SET). The methods used and described in this paper are mainly based on the SET mechanism.

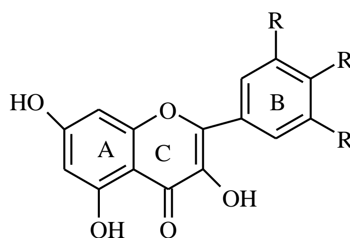

Figure 6. Flavonoid structure.

It is known that during the first extraction, all antioxidants may not pass, so depending on the extraction conditions, the antioxidant activity of the propolis solution may be different. As mentioned above, the most effective extractions for polyphenols are more commonly obtained through conventional techniques, such as ethanolic or aqueous extraction, or by Soxhlet. Moreover, in the last few years, studies on extraction with supercritical fluids have been also reported as a possible alternative method.

However, poor results were achieved in this field, showing low content of Artepillin C and p-coumaric acid, using supercritical carbon dioxide, for example [56].

It is also known that a mixture of antioxidants can give a synergistic or antagonistic effect. Therefore, it is very interesting and necessary to evaluate the properties of the mixture of plant substances in propolis as a whole. In our studies, in addition to electrochemical analysis, in vitro tests were also performed, such as ABTS, DPPH or FRAP and CUPRAC. Our research shows that propolis samples from Central and Eastern Europe exhibits antioxidant properties. Moreover, our research confirms that after 10 years of storage at 50° C, the same propolis samples do not lose their anti-tumour properties. Polyphenols present in propolis undergo strong oxidative processes. They work both through the free radical scavenging mechanism and the reduction of metal ions, such as iron or copper.

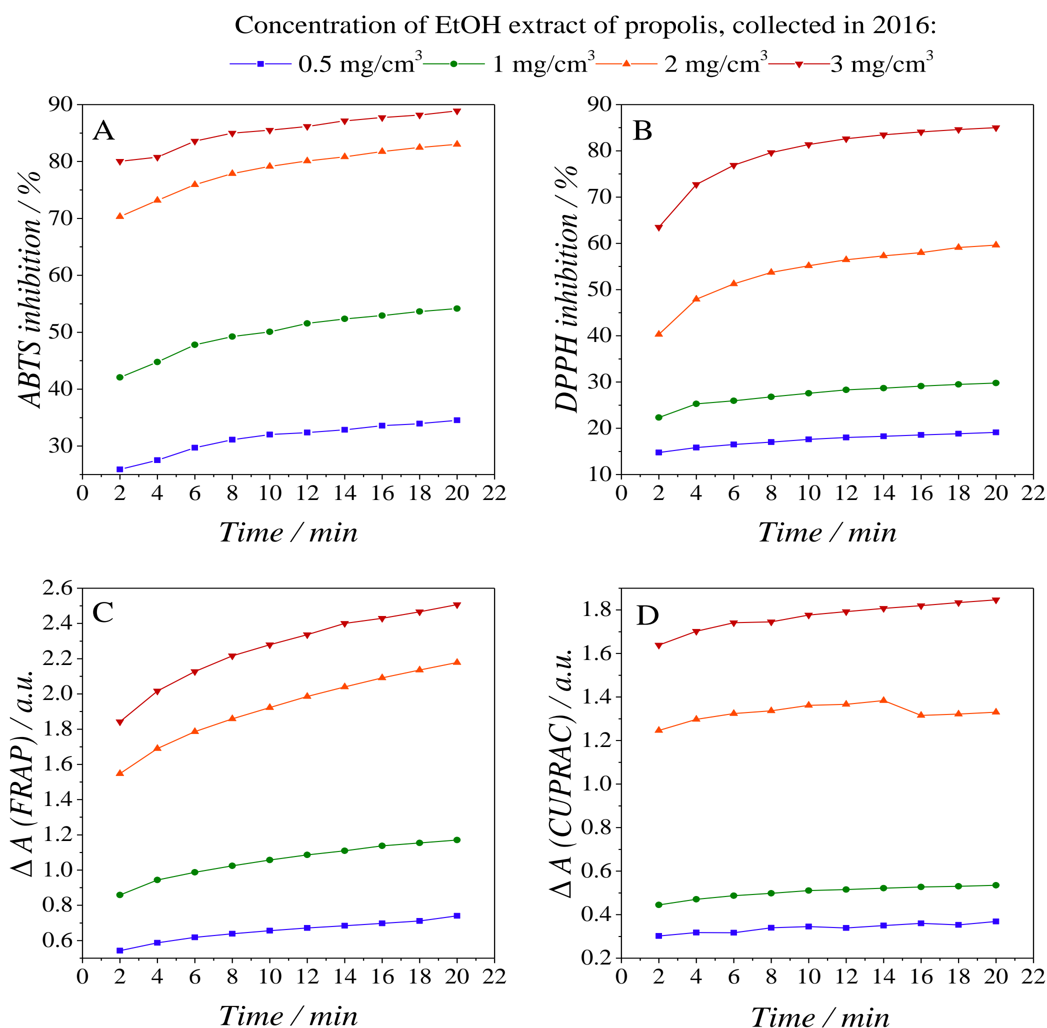

Figure 7. Evaluation of radical scavenging and reduction of metal ion activities of propolis extracts under study, measured by ABTS (A), DPPH (B) and FRAP (C), CUPRAC (D) assays. Corresponds to propolis samples collected in 2016 in Poland. $\Delta A = (A_{ABTS} - A_{propolis})$*; where: A-absorbance.*

The obtained ethanol extracts of propolis show strong properties for scavenging the free radicals ABTS and DPPH by the described SET mechanism (Figures 7 A, B and 8 A, B). The hydrogen-donating

activity of polyphenols from the obtained plant increased as a function of concentration (extraction time) and reaction time with the ABTS and DPPH radicals. DPPH and ABTS assays are widely used for evaluating the antiradical properties of natural phenolic compounds. Polyphenols act by interrupting hydrogen from the phenolic hydroxyl groups break the autoxidation chain.

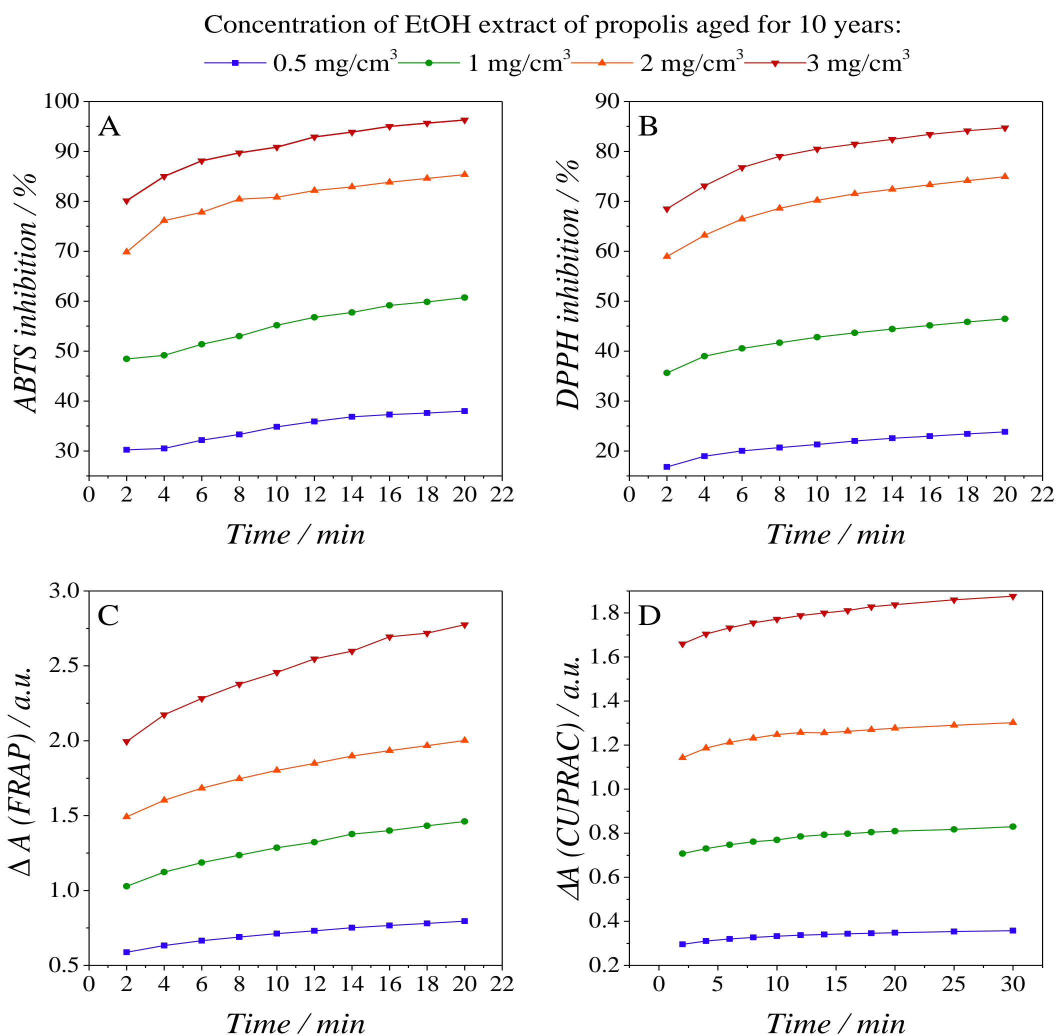

Figure 8. Evaluation of radical scavenging and reduction of metal ion activities of propolis extracts under study, measured by ABTS (A), DPPH (B) and FRAP (C), CUPRAC (D) assays. Corresponds to propolis samples after 10 years of storage, i.e. collected in 2010 in Poland. *ΔA= (A_{ABTS} -$A_{propolis}$); where: A- absorbance.*

From the research it follows that the antioxidant activity of the studied propolis samples increases linearly as a function of the free radical interaction time, measured by both ABTS and DPPH method. A six-fold increase in the extract concentration results in an almost three-fold greater ability to inhibit the oxidation process. At a propolis concentration of 0.5 mg cm^{-3}, Antioxidant Activity was 25.89% and for 3 mg cm^{-3}, AA was 80.03%. The concentration of propolis in the amount of 0.5 mg cm^{-3} in the extract gives an AA value of 25,89%, while an increase in concentration to 3 mg cm^{-3} causes an increase of AA to 80,03%. Within 20 minutes, the activity of the extract was reduced to 88.89%. Similar results are noted for FRAP (Ferric reducing *antioxidant* potential) and CUPRAC (Cupric Reducing Antioxidant

Capacity). However, for both methods, time does not affect the activity to reduce Fe and Cu ions for low concentrations of extract up to 1 mg cm^{-3} (Figure 7, Figure 8).

Interesting results obtained by Jasprica and co-workers come from exploring the propolis properties of Croatia. They introduced an inductive parameter named "antioxidative efficiency" (AOE) based on a mathematical model that explains the effects of propolis [40].

Ramnath, on the other hand, describes the composition and function of polyphenols present in propolis from different geographic regions [57]. Valencia explains that the origin of propolis influences the composition of active substances, and as a result the differences in the rate and effectiveness of the inhibition of cancer cell growth [58]. They also claim that the presence of CAPE and galangin has a determinant effect on the antiproliferative potential on cancer cells. However, there is a lack of correlation between antiproliferative activity, composition and antioxidant activity in the literature [26-29]. They also indicate the influence of seasonality on the composition and thus on the antioxidative activity.

A reduction rate of 18.89 a.u. that quenched for 3 mg cm^{-3} extracts after 20 minutes of reaction is the highest. The same concentration of extract after the same reaction time with free radicals is equal to 2.51 a.u. reducing activity of copper ions. The relationship between the DPPH radical scavenging activity of a propolis solution and the DPPH values of propolis samples was calculated, and the result is surprisingly positive. The iron and copper reducing/antioxidant power of propolis extracts was measured by using FRAP) and CUPRAC methods. The FRAP method is a universal method that can be applied to all antioxidants, has a linear range and is generally well correlated with other in situ methods. Table 1 shows the correlation coefficients for the ABTS and DPPH methods. As a result of research for ethanol extracts from propolis, a significant correlation was found between the results of antiradical activity calculated by ABTS and DPPH, as well as the FRAP and CUPRAC methods, respectively. It is worth adding that the majority of correlation coefficients is at the level above 0.95.

Table 1. Correlation between ABTS and DPPH values.

Propolis concentration (mg ml^{-1})	Correlation between ABTS and DPPH	Correlation between CUPRAC and FRAP
0.5	0.9927	0.9678
1	0.9883	0.9948
2	0.9860	0.6133
3	0.9475	0.9950

4. CONCLUSIONS

In this study, electrochemical techniques, due to their quickness, simplicity and low cost, have been progressively tested and developed, as an alternative tool for the evaluation of antioxidant properties of polyphenols contained in different propolis extracts. For this purpose, and as the research results described in the present paper reveal, cyclic (CV) and differential pulse voltammetry (DPV) are

recommended as they represent a relatively clean chemical system, are easy to control and are not affected by turbid extract solutions.

Based on the analyzes obtained by electrochemical techniques, a strong antioxidant effect of the tested propolis extracts has been demonstrated and expressed in terms of “antioxidant power”. The electro-oxidation of propolis occurs in at least two electrode stages, and the character of the overlapping electrode reactions is a diffusion-adsorption process. The results are also confirmed by the radical scavenging activities, measured by ABTS and DPPH methods.

Up to now, there is a lack, alternatively very few data are available on the lifetime of the antioxidant activity of the compounds contained in propolis. Thus the composition and properties of propolis before (ExI- extract of fresh propolis) and after 10 years (ExII- extract of aged propolis) of storage have been demonstrated and successfully evaluated in the paper. In addition, a linear dependence of the polyphenol content in propolis on the antioxidant activity has been observed. The constructed colorimetric methods for determining the scavenging and propolis reduction properties correlate with each other successfully giving linear results. Eventually, a high content of polyphenols in the tested propolis samples with high reducing activity has been verified in the present paper.

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