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THE INFLUENCE OF STORAGE TEMPERATURE AND UV-IRRADIATION ON FREE RADICAL SCAVENGING PROPERTIES OF ETHANOLIC EXTRACTS OF PROPOLIS

KATARZYNA KOMOSIŃSKA-VASSEV[1]*, PAWEŁ OLCZYK[2], PAWEŁ RAMOS[3], ŁUKASZ MENCNER[1], ALICJA DERKACZ[1], EWA WALUGA[2], KATARZYNA KRYSIK[4], KRYSTYNA OLCZYK[1] and BARBARA PILAWA[3]

[1]Department of Clinical Chemistry and Laboratory Diagnostics, School of Pharmacy and Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia in Katowice, Jedności 8, 41-200 Sosnowiec, Poland
[2]Department of Community Pharmacy, School of Pharmacy and Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia in Katowice, Kasztanowa 3, 41-200 Sosowiec, Poland
[3]Department of Biophysics, School of Pharmacy and Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia in Katowice, Jedności 8, 41-200 Sosnowiec, Poland
[4]ST. Barbara Reginal Specialist Hospital No. 5 in Sosnowiec, Plac Medyków 1, 41-20 Sosnowiec, Poland

Abstract: The free radical scavenging activity of ethanolic extracts of propolis (EEP) at the concentrations of 3%, 7%, and 10% was examined. The impact of storage temperature and exposure to ultraviolet (UV) light on the interactions of extracts of propolis with the model DPPH free radicals was also determined. The quenching of an X-band electron paramagnetic resonance spectra of DPPH free radicals by the extracts stored at room temperature, heated at the temperature of 50°C and exposed to UV-irradiation, were compared. The examined propolis ethanolic extracts revealed an antioxidative character. The storage of the samples at a higher temperature (50°C) caused a decrease of the scavenging activity equaling to 7 and 10% EEP. UV-irradiation of the 3% EEP increased the quenching of DPPH free radical lines. The reverse effect was observed for the 7 and 10% propolis extracts. The 3% ethanolic extract of propolis is more stable for storage at 50°C, and less than other analyzed EEP susceptible for UV-irradiation. Alterations of the antioxidative properties of the analyzed EEP and changes in the kinetics of their interactions with free radicals, indicate that 3, 7, and 10% propolis extracts should not be exposed to the temperature of 50°C and UV-irradiation.

Keywords: ethanolic extracts of propolis; DPPH; UV-irradiation; free radical scavenging; EPR spectroscopy

Propolis and other bee products, due to their safety of use and numerous health-promoting properties are more and more commonly used in the prevention or treatment of many diseases including atherosclerosis, cardiovascular diseases, cancers, diabetes and anti-inflammatory disorders. Different compounds have been identified in propolis extracts, including valuable phenolic compounds such as phenolic acids and flavonoids, as well as terpene and sesquiterpene alcohols and their derivatives, enzymes, vitamins and minerals, aromatic aldehydes, carbohydrates, aminoacids, coumarins, and steroids (1-5). However, the beneficial effects of propolis and its constituents on health result mainly from the presence of phenolic acids and flavonoids, which exert anti-inflammatory, anti-microbial, anti-carcinogenic as well as anti-oxidative activities (6-9).

Among the antioxidants present in propolis the most important are flavonoids, caffeic acid phenethyl ester (CAPE), kaempferol, cinnamyl caffeate, vitamin C and E (10-15).

The majority of therapeutic effects of propolis have been extensively attributed to a wide range of its active constituents. However, a resinous original form of propolis collected and elaborated by honeybee needs to be chemically treated before it can be used as a therapeutic agent. These procedures are a cause of partial loss of biologically active substances responsible for anti-oxidative capacity of propolis. Moreover, propolis solutions do not retain

* Corresponding author: e-mail: e-mail: kvassev@sum.edu.pl; phone/fax.:+48323641157

all the compounds originally contained in native propolis since they are not completely soluble in any known solvent. A number of methods are being developed in order to extract beneficial components from propolis, including water-based extraction techniques, alcohol extraction methods or supercritical fluid extraction procedures (16, 17). Considering different types of processes used to obtain propolis extract, the alcohol is the first choice of solvent for extracting the bioactive substances with high affinity (17, 18).

Thus, the aim of our study was to determine and compare the free radical scavenging activities of ethanolic extracts of propolis (EEP) at different concentrations i.e. 3, 7 and 10%. Anti-oxidative activity of EEP was determined with the use of EPR and their ability to quench DPPH free radicals was assessed. The influence of the environmental conditions of storage of the propolis samples on their interactions with free radicals was also determined. Optimization of the storage conditions of propolis extracts was the practical aspect of this work. The admissibility of the temperature increasing up to 50°C and exposition to UV-irradiation, was tested from the point of view of a free radical. We assumed that the anti-oxidative properties of the propolis extracts should be stable during storage. The performed EPR studies had an innovatory character. In our work we implemented for the first time electron paramagnetic resonance (EPR) spectroscopy as an experimental tool for testing the stability and at the same time evaluating the effect of the environmental factors on the quality of the ethanolic and water Polish propolis formulations. Free radical scavenging activity of 3, 7, and 10% EEP had not been examined by electron paramagnetic resonance spectroscopy before.

EXPERIMENTAL

Characterization and treatment of the ethanolic extracts of propolis

The samples of the study were ethanolic extracts of propolis at three different concentrations, i.e. 3% EEP [propolis content: 3% (25 mg/mL), ethanol content: max 93% (v/v)], 7% EEP [propolis content: 7% (58 mg/mL), ethanol content: max 60% (v/v)] and 10% EEP [propolis content 10%, 70% ethyl alcohol, ethanol content: max 60% (v/v)]. 3% ethanolic propolis extract was obtained from the Apipol-Farma sp. z o.o., Myślenice, Poland and was authorized under the certificate 4482. Ethanolic extracts of propolis at the concentrations of 7 and 10% were obtained from Farmapia, Kraków, Poland and Bartpol s.c., Poznań, Poland and were authorized under the 4433 and 4230 certificates, respectively.

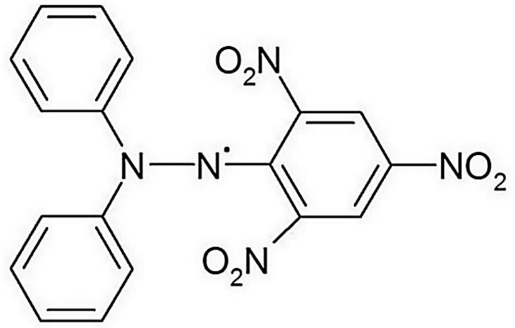


Figure 1. Chemical structure of DPPH free radical, where unpaired electron was presented as (●)

The propolis alcohol extracts stored at room temperature, at the temperature of 50°C, and exposed to UV-irradiation, were studied. The extracts were heated at the temperature of 50°C in the professional hot air oven with air circulation of Memmert (Germany) during 30 min. Time of UV-irradiation was also 30 min. The Medisun 250 lamp (Germany), which produced UVA radiation with the wavelengths (λ) in the range of 315-400 nm, was used. This lamp was equipped with 4 radiators with the power of 20 W. The distance between the lamp and the propolis sample was 30 cm.

EPR analysis

The free radical scavenging activity of propolis ethanolic extracts was examined by the electron paramagnetic resonance spectrometer of Radiopan (Poznań, Poland) and the model DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals. DPPH molecule was paramagnetic and the unpaired electron was located on nitrogen (N) atom, as is shown in Figure 1 (19, 20). DPPH in ethanolic alcohol solution (95%) was the free radical reference. EPR spectra of DPPH in ethanolic alcohol solution and DPPH in contact with the tested propolis samples, were measured. The solutions were located in thin-walled glass tubes with the external diameter of 1 mm. The masses of DPPH were obtained by Sartorius CPT (Germany).

An X-band (9.3 GHz) type of EPR spectrometer with a magnetic modulation of 100 kHz was used in this study. Microwave frequency was measured by MCM101 recorder of EPRAD (Poznań, Poland). Klystron with the total power of 70 mW produced microwaves. The spectra were obtained without a saturation effect by applying the low microwave

power of 2.2 mW. EPR spectra were numerically recorded by the data acquisition system – the Rapid Scan Unit of Jagmar (Kraków, Poland).

For the EPR spectra of DPPH, the amplitudes (A) [±0.02 a.u.], and g-factors [±0.0002], were determined. The free radical scavenging activity of the propolis samples was determined as the amplitudes (A) of the DPPH EPR lines after addition of the propolis extract to the ethanolic alcohol solution decreased (21-26). It reflected the anti-oxidative character of the sample. Kinetic tests were done. The changes of amplitudes (A) of DPPH interacting with the individual propolis sample with an increase of time up to 60 min by 5 min was evaluated. The g-factors were calculated from the resonance condition according to the following formula: $g = h\nu/\mu_B B_r$, where: h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – induction of resonance magnetic field (27-29).

The amplitude (A) of EPR line of DPPH was obtained with the accuracy of [±0.02 a.u.]. The accuracies of the microwave frequency (ν) and the induction of magnetic field (B), were [±0.0002 GHz] and [±0.01 mT], respectively. The g-value was determined with the accuracy of [±0.0002]. The errors for the spectral physical values were determined by the total differential method, which concerned errors of all the factors affecting the determined value. The differential of a function contains its derivatives with respect to all factors. EPR analysis was done with the professional spectroscopic programs of JAGMAR (Kraków, Poland) and LabVIEW 8.5 of National Instruments (USA).

RESULTS AND DISCUSSION

The examined propolis ethanolic extracts: 3, 7, and 10%, showed an anti-oxidative activity, i.e. free

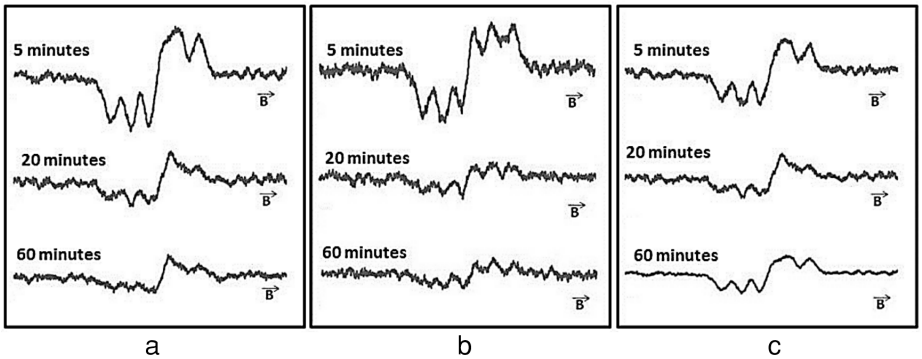


Figure 2. EPR spectra of DPPH interacting with the unheated and non-irradiated a) 3%, b) 7%, and c) 10% ethanol extracts of propolis during 5, 20, and 60 minutes. **B** – magnetic induction

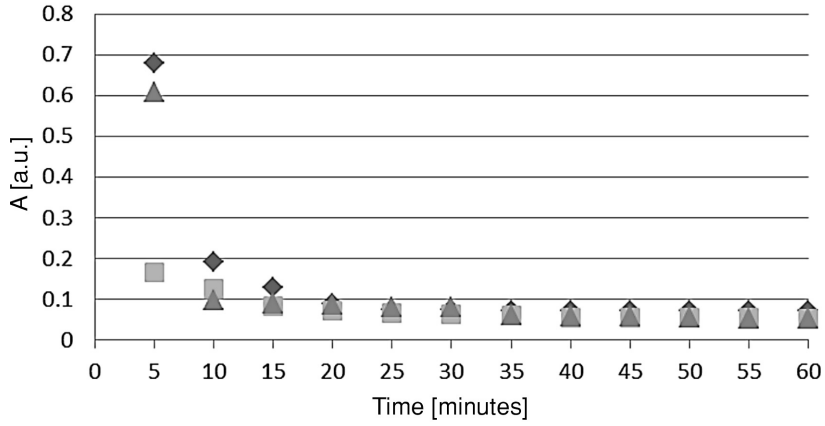


Figure 3. The influence of time (t) of interactions on amplitudes (A) of EPR lines of DPPH in contact with unheated and non-irradiated 3, 7, and 10% ethanol extracts of propolis

radical scavenging activity. They interacted with the model DPPH free radicals and they caused quenching of the DPPH free radical EPR lines. The quenching of the DPPH free radical EPR signals resulted from the quenching of free radicals in the tested solutions by the propolis samples. The quenching of the EPR lines of DPPH increased with increasing time of the interactions. The decrease of the EPR signals of DPPH free radicals caused by contact with the untreated 3, 7, and 10% propolis extracts is visible in Figure 2. The g-factors of the all measured EPR lines of DPPH were 2.0036.

The anti-oxidative capacity of the ethanol-soluble propolis extracts, which has been proven in our study, seems to be associated with the presence of phenolic compounds, especially flavonoids, in the analyzed EEP. It is known that native propolis contains about 25 different flavonoids at significant concentrations (approximately 25–30% of its dry weight) (4). Among them in Polish propolis the following flavonoids have been identified: apigenin, tectochrysin, chrysin, galangin, pinocembrin, genkwanin, kaempferol, and 5-hydroxy-4',7-dimethoxyflavone, pilloin and pinostrobin chalcone (14). Many reports on the relation between the anti-oxidative properties of bee products and their phenolic compounds content have been published so far (10-16), however the free radical-scavenging ability of commercially available ethanolic Polish propolis extracts was assessed for the first time.

High temperature of storage and UV-irradiation could change the anti-oxidative properties of the propolis extracts. The kinetics of interactions of 3, 7, and 10% EEP, for the original samples before heating and UV-irradiation, is shown in Figure 2. The amounts of the scavenged DPPH free radicals increased with increasing time (t) of the interactions with the propolis samples. The decrease of the amplitudes (A) of the DPPH EPR lines with increasing time (t) is presented in Figure 3. After reaching the minimum, the values of amplitudes (A) stabilized.

The ethanolic extract of propolis at the concentrations of 3, 7, and 10%, after both heating at the temperature of 50°C and UV-irradiation, exhibited

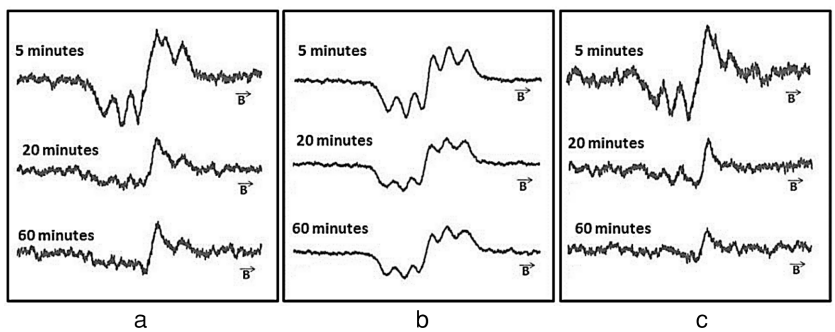


Figure 4. EPR spectra of DPPH interacting with the a) 3%, b) 7%, and c) 10% propolis extracts heated during 5, 20, and 60 minutes. **B** – magnetic induction

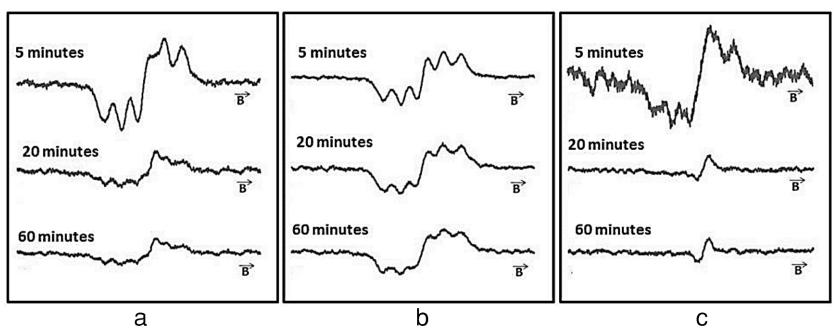
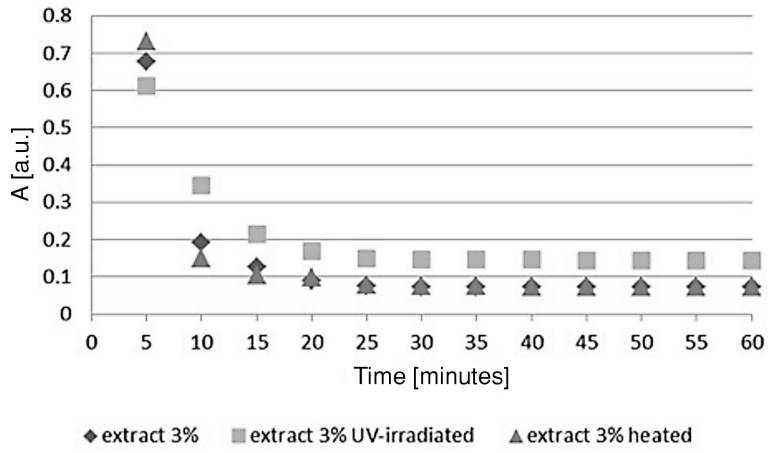
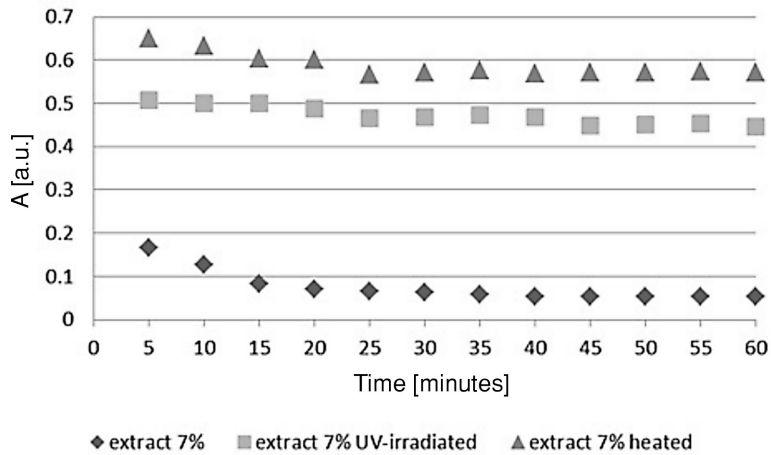


Figure 5. EPR spectra of DPPH interacting with the UV-irradiated a) 3%, b) 7%, and c) 10% propolis extracts during 5 minutes, 20 minutes, and 60 minutes. **B** – magnetic induction



a



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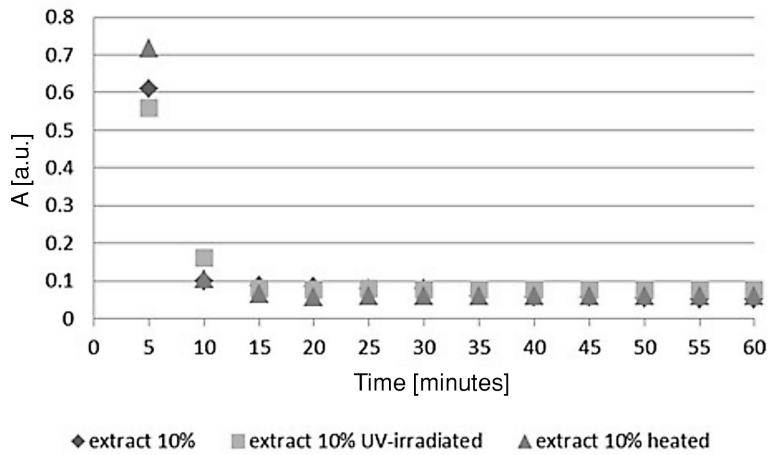

c

Figure 6. The effect of heating at the temperature of 50°C and UV-irradiation on changes of amplitudes (A) of EPR lines of DPPH in contact with a) 3%, b) 7%, and c) 10% propolis extracts with increasing interaction time (t). Times of both heating and UV-irradiation were 30 minutes

the anti-oxidative properties. The heated and UV-irradiated propolis samples 3, 7, and 10%, quenched the EPR lines of DPPH free radicals. The free radical scavenging activity of these samples, stored at the temperature of 50°C is visible in Figure 4. The spectra of DPPH were quenched with increasing time of contact of 3, 7, and 10% samples. The free radical scavenging activity of UV-irradiated 3, 7, and 10% samples is shown in Figure 5. The spectra of DPPH interacting with UV-irradiated propolis

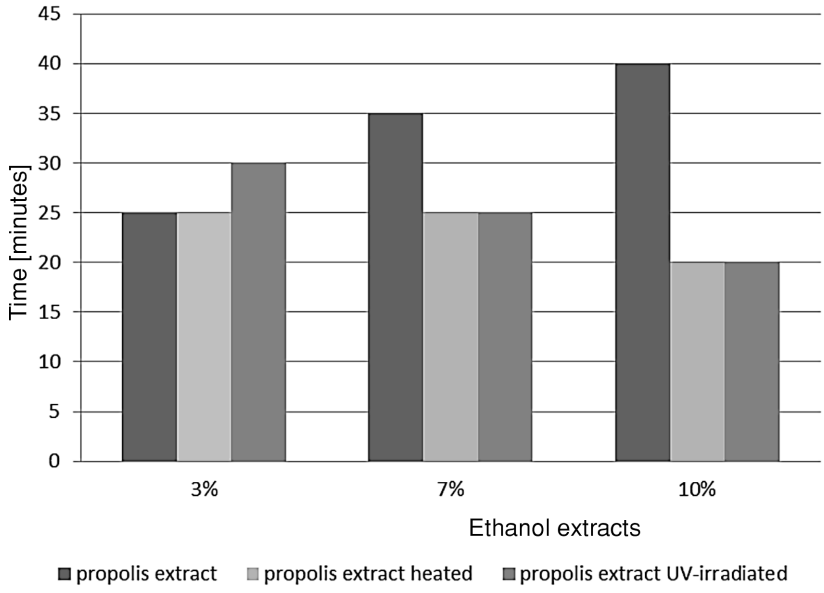

Figure 7. Comparison of the stabilization times (t) of DPPH interactions with propolis extracts (3, 7, 10%) for: the unheated and non-irradiated samples, the heated samples, and the UV-irradiated samples

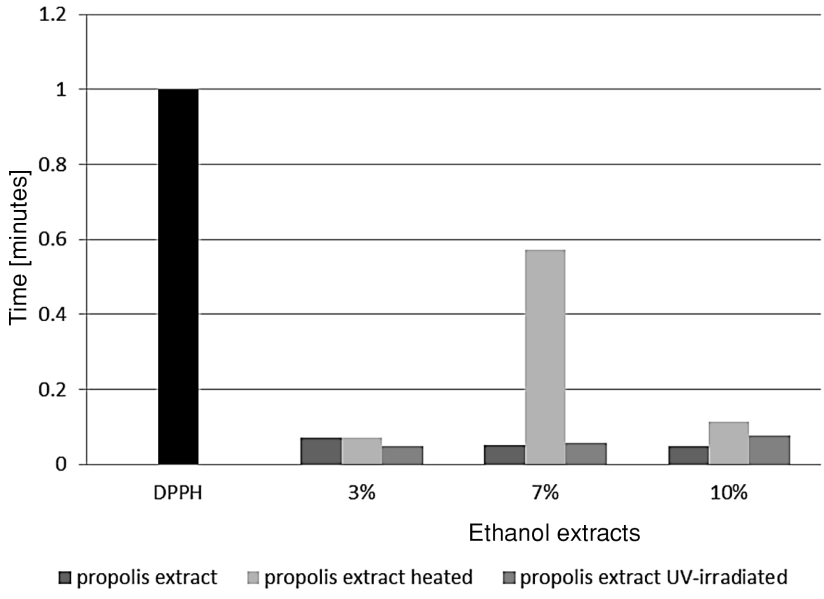

Figure 8. Comparison of the amplitudes (A) of EPR lines of DPPH in contact with propolis extracts (3, 7, 10%) for: the unheated and non-irradiated samples, the heated samples, and the UV-irradiated samples. Data for interaction time of DPPH with the propolis extracts = 60 minutes

extracts were lower for the longer times of contact of 3, 7, and 10% samples.

The effect of heating at the temperature of 50°C and UV-irradiation on the kinetics of the interactions of propolis extracts 3, 7, and 10% samples, with DPPH free radicals was compared in Figure 6. The amplitudes (A) of DPPH lines in contact with the original – the untreated, the heated and the UV-irradiated propolis extracts decreased with increasing time (t) of interactions, reached the minimum, and then stabilized. The kinetics was modified by heating and UV-irradiation of 3, 7, and 10% samples.

The times (t) of stabilization of the amplitudes (A) of DPPH EPR lines for the untreated, heated and UV-irradiated propolis extracts 3, 7 samples, and 10%, were compared in Figure 7. Stabilization time (t) increased for the untreated propolis extracts in the following order: 3% < 7% < 10%. The heating of the propolis samples at the temperature of 50°C decreased the quenching of DPPH free radicals by 7 and 10% (Fig. 7). The fastening of interactions with DPPH free radicals was also observed for UV-irradiated 7, and 10% propolis samples, and they were slower for UV-treated 3% extract.

The minimum amplitudes (A) of DPPH EPR lines for the interactions with the untreated, heated, and UV-irradiated propolis extracts 3%, 7%, and 10% samples, were compared in a diagram in Figure 8. The three propolis ethanolic extracts differed in a scavenging activity of the model DPPH free radicals. The scavenging activity of the unheated and non-irradiated propolis samples decreased in the following order: 10% > 7% > 3%. Storage of the propolis extracts at the temperature of 50°C decreased the scavenging activity of 7, and 10% extracts. This effect was not observed for 3% extract. UV-irradiation increased the quenching of DPPH free radical by the analyzed 3% ethanolic propolis extract. UV-irradiation decreased the scavenging activity of both 7%, and 10% extracts.

This examination confirmed that EPR spectroscopy may be used to determine the best conditions of storage of propolis extracts from the point of view of a free radical. Storage conditions should not considerably change the free radical scavenging activity of the propolis samples. The tested 3, 7, and 10% ethanolic extracts of propolis should not be stored at the temperature of 50°C and under UV-irradiation, because these conditions modified their interactions with free radicals. Due to the changes of the anti-oxidative properties and the kinetics of interactions with free radicals, the 3, 7, and 10% propolis extracts should not be exposed to the temperature of 50°C and UV-irradiation.

The obtained results indicate that the 3% ethanolic extract of propolis is more stable for storage at 50°C, and less than other analyzed EEP susceptible for UV-irradiation. This effect probably results from the percentage concentration of the analyzed ethanolic extracts and is related to the content of the active substances of propolis. However, the observed differences could be also attributed to the different producer of the market products used for the analyses. The achieved EPR results will find important application in medicine and pharmacy. Medicinal application indicated that the ethanolic propolis extracts, exhibited anti-oxidative interactions. The powerful anti-oxidative activity of propolis formulations makes it a useful tool for prevention or treatment of free radical-mediated disorders. The pharmaceutical usefulness of the spectroscopic results was that the higher temperature and UV irradiation are not neutral to the anti-oxidative properties of propolis extracts. Thus, the ERP examination of ethanolic propolis extracts turns out to be a useful analytical tool for defining the recommendations concerning storage conditions of these natural products.

CONCLUSIONS

EPR studies proved the antioxidative character of 3, 7, and 10% ethanolic extracts of propolis: The examined propolis extracts quenched DPPH free radical EPR lines. The free radical scavenging activity of the propolis extracts stored at room temperature decreased in the following order: 10% > 7% > 3% samples. The interactions of the analyzed EEP with DPPH free radicals changed after their heating at the temperature of 50°C and after UV-irradiation. The storage of the samples at the higher temperature decreased the scavenging activity of the 7 and 10% ethanolic extracts of propolis. This effect was not visible for 3% EEP. Moreover, 3% propolis extract exposed to UV-irradiation revealed a higher quenching of DPPH free radicals. UV-irradiation decreased the scavenging activity of both 7%, and 10% extracts. The comparison of the kinetics data for the tested propolis extracts stored at room temperature indicated that the fastest and the slowest interactions with free radicals characterized the 3%, and 10% extracts. Increasing the storage temperature to 50°C or UV-irradiation changed the kinetics of the interactions of 3, 7, and 10% samples, with DPPH free radicals. Anti-oxidative properties and the kinetics of interactions with free radicals were changing at the higher temperature and under UV-irradiation, therefore the 3, 7, and 10% ethanolic propolis

extracts should not be stored at these conditions. However, the 3% extract is more stable for storage at 50°C and less than other analyzed EEP susceptible for UV-irradiation. EPR spectroscopy may be proposed as a helpful method to optimize the storage conditions of the ethanolic extracts of propolis.

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Disclosure of interest

The authors report no conflicts of interest.

All authors have made substantial contributions to the conception and design of the study, acquisition or analysis of data, as well as to drafting the article or revising it critically for important intellectual content. Moreover all authors have approved the final version of the submitted manuscript.

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