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Original Article

KINETIC PROFILE OF THE ANTIOXIDANT ACTIVITY OF PROPOLIS EXTRACT: 2,2-DIPHENYL-1-PICRYLHYDRAZYL RADICAL BLEACHING ASSAY

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Abstract

The objective of this paper was to assess the antiradical effectiveness of propolis extract (PE) based on 2.2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) bleaching assay kinetic profile. The kinetic profile of scavenging DPPH$^{\bullet}$ for PE exhibited one kinetic period characterized by one kinetic constant. The second-order rate constant (k_2) for the oxidation of PE by DPPH$^{\bullet}$, determined for the first time in this study, was 0.17 $dm^3g^{-1}s^{-1}$. The obtained k_2 value was compared to that of synthetic antioxidants and natural extracts used in the food industry. Kinetic analysis of PE antiradical effectiveness showed that the k_2 was within the range values for natural colorants of fruit extracts and should be considered as a fast acting natural antioxidant source. The k_2 parameter indicates the extent of oxidation inhibition that is based on all of the kinetic profiles of DPPH bleaching rather than single point measurements. For this reason, the kinetic analysis should become a necessary step for more precise antioxidative characterization of propolis.

Keywords: antioxidative activity, kinetic analysis, proplis extract, second order rate constant

INTRODUCTION

Propolis, a natural substance collected by honeybees, is often used in the food industry as a supplement as well as in folk medicine to prevent and treat colds, wounds, ulcers, rheumatism, diabetes and dental caries (Castro et al., 2003; Jug, Končić, & Kosalec, 2014; Neves et al., 2016; Prytzyk et al., 2003; Ristivojević et al., 2015). Several pharmacological properties of propolis, antibacterial (Dimkić et al., 2016; Ristivojević et al., 2015a; 2015b), antifungal (Dimkić et al., 2016; Ristivojević et al., 2015b; Stepanović et al., 2003) and antioxidant (Žižić et al., 2013), are attributed to polyphenolic compounds.

The antioxidant properties of propolis are particularly important for food (Viuda-Martos et al., 2013) and biological systems (Dimkić et al., 2016), and therefore its antioxidative activity must be assessed. Among various assays employed to determine propolis antioxidative activity (Kumazawa et al., 2004; Mot et al., 2011; Potkonjak et al., 2012) spectrophotometric assays determining the capacity of free radical scavenging are predominantly used (Da

Silva et al., 2006; Moreira et al., 2008). Free radical scavenging is generally accepted for antioxidants inhibiting lipid oxidation (Rufino et al., 2009). The antioxidative activity of propolis can be quantified through the determination of radical scavenger capacity values in 2.2-diphenyl-1-picrylhydrazyl free radical (DPPH•) scavenging assay systems (Ahn et al., 2017; Gregoris & Stevanato, 2010; Kumazawa et al., 2004; Laskar et al., 2010; Miguel et al., 2010; 2014; Moreira et al., 2008). Traditional DPPH radical scavenging capacity assay is informative for the antioxidative capacity of that sample but does not take into account the whole kinetic profile (Mot et al., 2011). The strong antioxidative activitiy of PE is known from previous works (Potkonjak et al., 2012; Ristivojević et al., 2015; Žižić et al., 2013) but no kinetic characterization has been performed. In this paper we assessed the antiradical effectiveness of propolis extract (PE) using the 2.2-diphenyl-1-picrylhydrazyl radical (DPPH▪) bleaching assay kinetics profile. Afterwards the kinetic analysis of PE studied herein were compared to those of synthetic, natural antioxidants and other natural extracts used in the food industry.

MATERIAL AND METHODS

Chemicals

All chemicals and reagents were of analytical grade and supplied from Sigma (St. Louis, USA) and Merck-Alkaloid (Skopje, FYR Macedonia).

Propolis sample from South Serbia

The propolis sample was collected in southern Serbia. The one representative sample was obtained through the mixing of the propolis from twelve different apiaries. The sample was kept at 0–5°C and in protected room light until analysis. The raw material of the propolis sample was frozen at –20 °C overnight and then rapidly ground in a mortar to obtain a homogeneous powder.

Extract preparation

Extract preparation of propolis was performed using 96% vol. ethanol. The grounded propolis (2.0 g) and solvent (20 cm^3) were put in an Erlenmeyer flask, which was placed in a thermostated water bath. The extraction was performed at the boiling temperature of the solvent (T_k) and it lasted four hours. At the end of the procedure, the liquid extract was separated from the solid residue through vacuum filtration and stored in a freezer for subsequent analysis. The propolis extract (PE) was evaporated in a vacuum for dryness and constant weight.

Characterization of the propolis extract

The yield of extractive substances was calculated as a ratio between the amount of the prepared dry extracts and quantity of extracted raw propolis and expressed as a percentage % (w/w). The total phenolics content in the extracts was determined with a spectrophotometer according to the Folin-Ciocalteu method using Gallic acid as a standard (Kumazawa et al., 2004). Total phenolic content was expressed as Gallic acid equivalents in mg per 1 g of propolis extract (mg GAE g^{-1} of PE). The total flavonoid (TF) content was determined by $AlCl_3$ coloration using Quercetin as a standard (Kumazawa et al., 2004). Total flavonoid content is expressed as Quercetin equivalents in mg per 1 g of propolis extract (mg Q g^{-1} of PE).

DPPH• radical bleaching kinetic study

A dry extract was dissolved in 10 ml of methanol. Through further dissolving a series of solutions with different concentrations were obtained and used for the kinetics study. A solution of $7.9x10^{-5}$ mol·dm^{-3} DPPH• in methanol was prepared for each sample. The studied mixture was obtained from $1x10^{-4}$ dm^3 of dissolved PE and $4x10^{-3}$ dm^3 of DPPH• solution. Bleaching of DPPH• by PE was monitored at 520 nm for 540 s using LKB BIOCHROM ULTROSPEC II UV/VIS Spectrophotometer.

The scavenging reaction between DPPH• and PE can be written as:

$$\left[\mathrm{DPPH}^{\bullet}\right] + \left[\mathrm{PE}\right] \rightarrow \left[\mathrm{DPPH-H}\right] + \left[\mathrm{A}^{\bullet}\right] \quad (1)$$

where A• is a new radical. The rate of reaction (1) is given with the equation:

$$-\frac{d[\mathrm{DPPH}^{\bullet}]}{dt} = k_2[\mathrm{PE}][\mathrm{DPPH}^{\bullet}]_t \quad (2)$$

where t is the time, $[DPPH^{\bullet}]_t$ is the radical concentration at t, and k_2 is the second-order rate constant.

The second-order rate constant (k_2) was determined through the comparison of a large amount of the radical compound [DPPH•] with [PE] (Rufino et al., 2009). Under such conditions, the second-order reaction exhibits first-order characteristics (k_1) described by the equation:

$$-\frac{d[\mathrm{PE}]}{dt} = k_1[\mathrm{PE}]_t \quad (3)$$

where [PE] is considered constant throughout the reaction and $k_1=k_2[DPPH^{\bullet}]$.
The solution of equation (3) is:

$$[\mathrm{PE}]_t = [\mathrm{PE}]_0 \mathrm{e}^{-k_1 t} \quad (4)$$

where $[PE]_0$ is the initial concentration of PE.
The concentration of [DPPH•] was calculated by mass balance with the following equation (5):

$$[\mathrm{DPPH}^{\bullet}]_t = [\mathrm{DPPH}^{\bullet}]_0\left([\mathrm{PE}]_0 \mathrm{e}^{-k_1 t}\right) \quad (5)$$

The [DPPH•] concentration in the reaction medium was calculated from the linear calibration curve given by Brand-Williams, Cuvelier, & Berset (1995):

$$A_{520\mathrm{nm}} = 11592\mathrm{x}[\mathrm{DPPH}^{\bullet}] - 4.9\mathrm{x}10^{-2} \quad (6)$$

where [DPPH•] is expressed in mol dm^{-3} and A_{520nm} is measured absorbance at 520 nm.
The constants, k_1 and k_2 are graphically determined. The first-order rate constant k_1 was obtained from plots of DPPH• concentration dependent on time (Eq. 5) and k_2 was obtained from plots of k_1 dependent on PE concentration. The k_2 represents the rate at which a DPPH• radical can be oxidized by 1 g of PE.

Statistical analysis

Each experiment was performed in triplicate. Results are expressed as the mean ± standard deviation (SD) of three analytical repetitions. Data from spectrophotometric readings of DPPH• disappearance in the presence of various PE were taken. The fittings of the experimental data to Eq.5 were carried out in Origin v. 6.0 programs for Windows. The quality of fit was evaluated based on the coefficient of determination (R^2) and the mean relative percentage deviation (*MRPD*), calculated according to the equation given by Rajković et al. (2016).

RESULTS

Characterization of the propolis extract

The total extract of the south Serbia propolis was 69.8±2.0 %. TP and TF were 155.7±3.5 mg GAE g^{-1} of PE and 68.7±1.3 mg Q g^{-1} of PE, respectively. The values differed beyond the experimental error limits.

DPPH• radical bleaching kinetic study

Fig. 1 illustrates the exponential decrease of the ratio of current and initial DPPH• concentrations with the time of the progress of PE radical scavenge. The reaction's progress was monitored by a color change of the solution from purple to yellow. This occurred because the release of hydrogen leads DPPH• to reduced form DPPH–H (Eq. 1) and as a result the color changed. Experimental bleaching data (Fig. 1) were successfully fitted by Eq. (5) and parameters of this fit, obtained by non-linear regression method, are shown in Tab. 1.

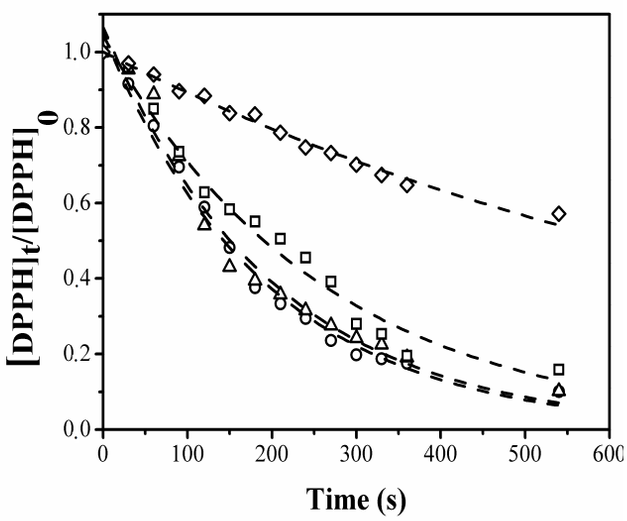

Fig. 1. Kinetic profiles of DPPH consumption by propolis extract for concentrations: 3.5 - □; 2.6 - ○; 1.8 - Δ; ◇ - 0.7 x 10^{-2} g dm^{-3}. Lines represent fits of the experimental data according to Eq. 5 (R^2= 0.990 - 0.974, *MRPD* = 1.7 - 6.7%).

Table 1.
Parameters of DPPH bleaching assays, shown by Eq. (5) and the second-order rate constant (k_2)

Dry weight of PE x 10^2 (g dm^{-3})	k_1 x 10^3* (s^{-1})	R^2	*MRPD* (%)	k_2 * ($dm^3g^{-1}s^{-1}$)
3.5	5.2 ± 0.2	0.990	5.8	0.17 ± 0.01
2.6	4.5 ± 0.2	0.974	6.7	
1.8	3.8 ± 0.1	0.980	5.7	
0.7	1.1 ± 0.1	0.988	1.7	

PE- propolis extract; k_1 - the first-order kinetic rate constant; R^2 - coefficient of determination; *MRPD* - mean relative percentage deviation.
*The mean values± standard deviation

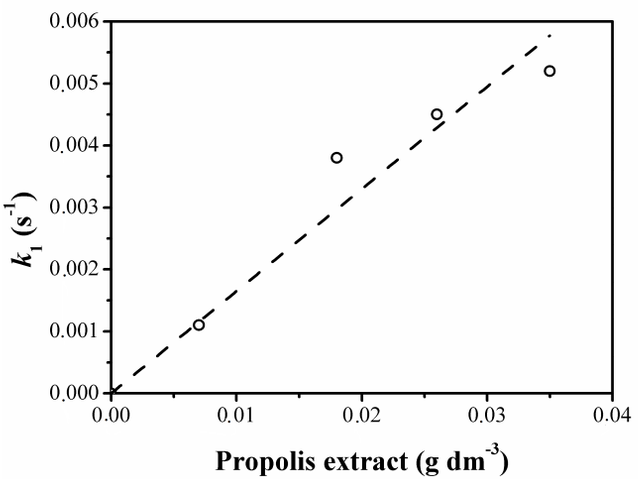

Fig. 2. The first-order rate constant (k_1) dependent on concentration of propolis extract. Lines show linear regression fits (R^2= 0.946).

Parameters of this fit showed that the R^2-values were between 0.974 and 0.990, while *MRPD*-values were between 1.7% and 6.7% for all studied samples. R^2-values close to number one and *MRPD* values less than ± 10% point to good fit quality.
According to our results, the calculated k_1 depends on the PE as shown in Fig. 2. The k_2 value was obtained by linear regression fit of k_1 dependent on the dry weight of PE (Fig. 2). The k_2 value of PE is presented in Tab. 1.

DISCUSSION

Total phenolic and flavonoide compounds, important parameters in assessing the quality of numerous natural products, are related to their health benefits (Potkonjak et al., 2012). The variation in the TP and TF of propolis from different geographic region is mainly attributed to the difference in the preferred regional plants used by honeybees (Cottica et al., 2015; Kumazawa et al., 2004). Geographic origin cause TP and TF of PE to vary from 31 to 299 mg GAE g^{-1} and from 2.5 to 176 mg Q g^{-1}, respectively (Kumazawa et al., 2004). Our values for TP and TF content of propolis from south Serbia were within the range of values from other geographic locations. TP of propolis from south Serbia was extremely close to the results from Brasil, South Africa, Uruguay and Uzbekistan. Our values for the TF content of propolis from south Serbia were close to the results from Ukraine. However, TP and TF content in PE from south Serbia were significantly lower than the values obtained in Australia, China and Hungary (Kumazawa et al., 2004). Besides the origin and source of the propolis, there are other limiting factors affecting the content of TP and TF of PE, such as extraction temperature and solvent (Trusheva, Trunkova, & Bankova, 2007). The higher extraction temperature slightly improved the extraction yield (Jug, Končić, & Kosalec, 2014), but the use of high temperatures degraded some compounds (Bakowska,

Kucharska, & Oszmiański, 2003). However, Trusheva, Trunkova, & Bankova (2007) found that the percentage of total extract varies significantly with the increasing temperature, much than the amount of extracted active compounds (TP and TF). Gonzalez et al. (2009) also found that apigenin, chrysin and galangin, some of the main active flavonoids, were stable from room temperature to 120°C.

Previous studies by Miguel et al. (2010, 2012) and Laskar et al. (2010) showed that solvent selection plays a major role in the content of of TP and TF in PE. Laskar et al. (2010) found that TP content in the water extract of propolis was higher than that in the ethanol extract of propolis, but it was higher in the ethanol extract. Miguel et al. (2012) found that water did not serve as a good solvent to extract TP and TF from propolis because it dissolves only a small amount of propolis constituents. Although toxic, methanol is adequate for extracting polyphenols from propolis samples, but the amounts of compounds extracted with methanol do not justify its utilization for preparing propolis extracts (Miguel et al., 2010). Therefore, ethanol is considered the first choice solvent for propolis extraction since the majority of bioactive compounds in propolis are rather lipophilic and thus soluble in ethanol (Jug, Končić, & Kosalec, 2014) and is not as toxic as methanol. Wide variations of factors affecting the content of TP and TF of PE emphasize the need for the standardization of propolis extraction.

In this work, for the first time the PE from south Serbia was studied using a kinetics analysis based on the model of DPPH$^{\bullet}$ scavenging. A fitting of bleaching data (Fig. 1) with the single-exponential decay function (Eq. (5)) showed that the kinetics profile of scavenging DPPH$^{\bullet}$ of south Serbian PE exhibited one kinetic period characterized by one kinetic constant (Fig. 1). The existence of more than one kinetic period results from the presence of more than one anti-oxidant group in the sample (Mot et al., 2011). It is important to notice that in PE from south Serbia only one possible radical scavenger species is present. However, propolis from Romania has exhibited more than one kinetic period and this propolis may possess several natural radical scavenger species in their composition (Mot et al., 2011). Variation in the kinetics profile of propolis from a different geographic region is mainly attributed to differences in phenolic compounds representing radical scavenger species (Ristivojević et al., 2015a). Variability in the phenolic composition of propolis originating from a different geographical area is a direct consequence of a significant variation in their local flora, and the preferences of bees towards particular plants or plant materials (Bankova, 2009).

The k_2 of south Serbian PE was determined for the first time in this study (Tab. 1). The k_2 indicated the rate at which PE scavenges free radicals. The k_2 value of PE studied herein was compared to values determined in the same test and the same solvent of synthetic, natural antioxidants and natural extracts used in the food industry (Espin, Soler-Rivas, & Wichers, 2000; Rufino et al., 2009). The PE showed slower antioxidant activity than α-tocopherol (1.9 $dm^3g^{-1}s^{-1}$), butylated hydroxyanisole (0.42 $dm^3g^{-1}s^{-1}$) (Espin, Soler-Rivas, & Wichers, 2000) and fruit extract (29.7-38.0 $dm^3g^{-1}s^{-1}$) (Rufino et al., 2009) and when compared to butylated hydroxytoluene (0.051 $dm^3g^{-1}s^{-1}$) (Espin, Soler-Rivas, & Wichers, 2000) was faster. The rate of PE antioxidative activity was within the range of values for natural colorants of fruit extracts (0.0008-0.7 $dm^3g^{-1}s^{-1}$) (Espin, Soler-Rivas, & Wichers, 2000). The k_2 value obtained in this work pointed out that PE could be just as a relevant antioxidative source as natural colorants and synthetic antioxidants (Espin, Soler-Rivas, & Wichers, 2000).

Reports on the antioxidative activity of natural extracts have always been accompanied by a chemical characterization (Espin, Soler-Rivas, & Wichers, 2000; Potkonjak et al., 2012; Suja, Jayalekshmy, & Arumughan, 2004). However, the correlation between individual compound content and the antioxidative activity of Serbian propolis did not give good results (Žižić et al., 2013) due to the fact that each compound possessed different types of activity (Bankova, 2005), and synergistic effects (Boisard et al., 2015; Chen & Shen, 2008). For these reasons, additional kinetic analyses of PE

using k_2 provided new insight into antioxidative activities. The k_2 parameter indicates the extent of oxidation inhibition that is based on the entire kinetic profiles of DPPH• bleaching rather than single point measurements. In this study only the kinetic profile of scavenging DPPH• was determined. However, other free radicals were also used for antioxidant activity assays (Miguel et al, 2010) so it would be necessary to determine the kinetic profiles of their scavenging in future research.

Previous research considered that the k_2 was related to the antioxidative capacity (Espin, Soler-Rivas, & Wichers, 2000; Rufino et al., 2009). However, the inclusion of antioxidative capacity data raised concern. That each antioxidant method measured a different aspect of the sample chemistry and all were non-specific and subject to numerous interferences. However, kinetics analysis is relevant for understanding how pure substances or mixtures could inhibit free radical. The present study is likely to help further research in these directions.

In this paper we reported about the kinetics of free radical scavenging by propolis from south Serbia. Results obtained in this study gave new insight into the antioxidative activities of propolis from south Serbia and indicated that it should be considered as a relatively fast-acting natural antioxidant source. We suggest that kinetic analysis should become a necessary step for more precise antioxidative characterization of propolis.

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