Food and Chemical Toxicology 50 (2012) 3614–3618

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Antioxidant activity of propolis extracts from Serbia: A polarographic approach

Nebojša I. Potkonjak [a,*], Dragan S. Veselinović [b], Miroslav M. Novaković [c], Stanislava Ž. Gorjanović [d], Lato L. Pezo [d], Desanka Ž. Sužnjević [d]

[a] University of Belgrade, Vinča Institute of Nuclear Sciences, Chemical Dynamics Laboratory, P.O. Box 522, 11001 Belgrade, Serbia
[b] Faculty of Physical Chemistry, University of Belgrade, P.O. Box 473, 11001 Belgrade, Serbia
[c] Institute of Chemistry, Technology and Metallurgy, University of Belgrade, P.O. Box 473, 11001 Belgrade, Serbia
[d] Institute of General and Physical Chemistry, P.O. Box 45, 11158 Belgrade 118, Serbia

ARTICLE INFO

Article history:
Received 17 May 2012
Accepted 17 July 2012
Available online 27 July 2012

Keywords:
Propolis extract
Antioxidant activity
Hydrogen peroxide
Polarography

ABSTRACT

Antioxidant activity (AO) of commercial propolis extracts (PEs), available on Serbian market, was determined by direct current (DC) polarography. Polarographic anodic current of 5.0 mmol L^{-1} alkaline solution of H_2O_2 was recorded at potentials of mercury dissolution. Decrease of the current was plotted against the volume of gradually added PEs. The volume of PE causing 20% current decrease was determined from the linear part of the plot. Antioxidant activity was expressed in H_2O_2 equivalent (HPEq), representing the volume of PE that corresponds to 1.0 mmol L^{-1} H_2O_2 decrease. Resulting HPEq ranged between 1.71 ± 0.11 and 8.00 ± 0.18 μL. Range of 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity was from 0.093 ± 0.004% to 0.346 ± 0.006%. Total phenolic content (TCP) of PE with superior AO activity was 5.31 ± 0.05%g GAE, while the extract with the lowest activity contained 1.45 ± 0.02%g GAE. Antioxidant activity, determined by polarographic method, was correlated with DPPH scavenging activity (R^2 = 0.991) and TCP (R^2 = 0.985). Validity of obtained results was further confirmed using ANOVA and post hoc Tukey HSD test.

1. Introduction

Propolis is a complex mixture processed by the honeybees from the resins collected from various plant sources. It has been used in folk medicine since ancient times, dating back to at least 300 BC (Burdock, 1998). More than 300 compounds have been identified in different propolis samples (Bankova et al., 2000). Chemical composition of propolis depends on geographical diversity, the source of plants and local flora (Kujumgiev et al., 1999), as well as seasonal variation (Bankova et al., 1998). Total phenolic content in propolis samples ranged between 10% and 35%, with predominance of flavonoids (Bonvehi et al., 1994). Due to abundant phenolic content, propolis has a broad spectrum of biological and pharmacological activities (Banskota et al., 2001; Sforcin, 2007). Variability in propolis composition makes pharmacological application and quality control challenging (Sforcin and Bankova, 2011). Thus, the standardization of propolis products should be validated throughout series of biological, biochemical and chemical antioxidant (AO) assays (Bankova, 2005).

Biological effects of propolis can be associated with its pronounced AO activity. The general principles, recent applications, as well as strengths and limitations of the methods most widely used to determine AO activity were reviewed recently by Karadag et al. (2009). Various assays have been employed to determine propolis AO activity. Spectrophotometric assays including free radical scavenging (Da Silva et al., 2006; Moreira et al., 2008; Erdogan et al., 2011), ferric reducing antioxidant power (Mohammadzadeh et al., 2007) and cupric reducing antioxidant capacity assay (Gülçin et al., 2010) were used prevalently. Chemiluminiscence assay, based on propolis scavenging activity against superoxide and alkoxy radicals, was applied as well (Pacsual et al., 1994).

Based on electrochemical techniques, such as cyclic voltammetry (Kilmartin et al., 2001), chronoamperometry (Ferreira and Avaco, 2008), flow-injection potentiometry (Shpigun et al., 2006), potentiometric titration (Brainina et al., 2007), potentiometric measurement of Briggs–Rauscher oscillatory reaction (Cervellati et al., 2002) and direct current (DC) polarography (Sužnjević et al., 2011), the broad spectrum of methodologies for AO activity

Abbreviations: ANOVA, analysis of variance; AO, antioxidant activity; BHT, butylated hydroxytoluene; CL, Clarc and Lubs; *i–E*, current–potential; DPPH, 1,1-diphenyl-2-picryl hydrazyl; DC, direct current; DME, dropping mercury electrode; EC_{50}, efficient concentration; EEP, ethanolic extracts of propolis; FC, Folin–Ciocalteu; GEP, glycolic extract of propolis; HSD, honestly significant difference; HPEq, hydrogen peroxide equivalent; $-\Delta_{H_2O_2}$, 1.0 mmol L^{-1} H_2O_2 decrease; i_{p0}, polarographic anodic current; PEs, propolis extracts; Δi_p, relative decrease of polarographic anodic current; SCE, saturated calomel electrode; SD, standard deviation; TPC, total phenolic content.

* Corresponding author. Tel./fax: +381 11 2455654.
E-mail address: npotkonjak@vin.bg.ac.rs (N.I. Potkonjak).

0278-6915/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2012.07.029

determination were developed. However, until now electrochemically based assays were rarely employed in determination of AO activity of propolis extracts (PEs). Amperometric flow injection analysis have been applied by Buratti et al. (2007), in order to evaluate AO power of honeybee products, including PEs, while Laskar et al. (2010) and Rebiai et al. (2011) applied cyclic voltammetry.

In the present study, DC polarography has been used to determine AO activity of commercial ethanolic and glycolic PEs, purchased from Serbian market. Possibility to apply polarographic assay on resinous substances such as PEs has been demonstrated. In parallel, total phenolic content of PEs was determined by Folin–Ciocalteu method and correlated with AO activity determined by polarographic AO method. In order to confirm reliability of polarographic AO method, correlation between AO activity and DPPH free radical scavenging activity has been included. Obtained results have been evaluated statistically using analysis of variance (ANOVA) and also post hoc Tukey HSD test.

2. Material and methods

2.1. Chemicals and propolis extracts

Chemicals used in this study were of p.a. quality: 30% H_2O_2 (POCH), 1,1-diphenyl-2-picrylhydrazyl radical, DPPH (Merck), Folin–Ciocalteu reagent (Merck), boric acid (POCH), potassium chloride (Merck), sodium carbonate (Lach-Ner) and sodium hydroxide (Lach-Ner). Methanol and deionized water with resistivity 18 MΩ cm (Milli-Q purification system, Millipore) were used to prepare experimental solutions.

Five commercial PEs have been purchased from the Serbian market. The investigated samples were ethanolic extracts of propolis obtained from: independent beekeepers (EEP1 and EEP2), pharmaceutical company "Apoteka Beograd" (EEP3) and company for bee products "Kovačević" (EEP4). Glycolic extract of propolis (GEP), advised for the children use, was obtained from the company "Kovačević" as well. All extracts of propolis are made in Serbia.

2.2. Antioxidant activity determination by DC polarographic assay

2.2.1. Instrumentation

The electrochemical measurements were performed using Polarographic Analyzer PAR (Princeton Applied Research) model 174A coupled with X-Y recorder (Houston Instruments, Omnigraphic 2000). A conventional three-electrode cell was used; the cell volume was 30 ml. The working electrode was the dropping mercury electrode (DME). Capillary constant of DME was $m = 2.5$ mg s^{-1} at mercury reservoir high 75 cm. A programmed drop time of DME was 1 s; current oscillations of DME were filtered out with low pass filter of instrument positioned at 3 s. The saturated calomel electrode (SCE) and the platinum foil were used as the reference electrode and the counter electrode, respectively.

2.2.2. Procedure

The supporting electrolyte was Clarc and Lubs (CL) buffer (pH 9.8), prepared by mixing 25 mL of 0.4 M H_3BO_3, 25 mL of 0.4 M KCl and 40.8 mL of 0.2 M NaOH. The volume of the supporting electrolyte in the cell was 20 mL. Hydrogen peroxide was directly added in supporting electrolyte. Initial concentration of H_2O_2 was 5.0 mmol L^{-1}. The samples of PEs, prepared by dilution with methanol (1:10), were gradually added in aliquots of 10 or 20 μL into buffered solution of H_2O_2. The polarographic current–potential (*i*–*E*) curves, with or without presence of PE were recorded, starting from 0.1 V *vs* SCE towards negative potentials, with sweep rate of 10 mV s^{-1}. In order to remove dissolved oxygen, the buffered solution in the electrolytic cell was purged with gaseous nitrogen (>99.995%, Messer, Serbia) for 10 min before H_2O_2 addition, and 30 s after addition of each PE samples. The atmosphere above the cell solution was kept inert during polarographic curve recording by continuous flow of nitrogen.

2.3. Antioxidant activity determination by DPPH radical scavenge

The stable free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) was used for determination of free radical scavenging activity (Blos, 1958). The volume of 1800 μL of a methanol solution of DPPH (0.1 mmol L^{-1}) was mixed with 200 μL of diluted PE. In order to satisfy the criteria for spectrophotometric measurements, e.g. linearity between absorbance and concentration, PEs were diluted in the range 1:300–1:1200. Reaction mixture was kept in dark for 30 min. Four reaction mixtures with different PE dilutions and the control sample (made from methanol and DPPH alone) were used for evaluation of free radical scavenging activity. The absorbance was measured at 517 nm.

2.4. Total phenolic content determination by Folin–Ciocalteu assay

The total phenolic content (TPC) of PEs was determined by Folin–Ciocalteu (FC) assay (Singleton et al., 1999). The chemistry of this assay relies on the electron-transfer reaction from phenolic compound to molybdenum present in FC reagent (phosphomolybdic/phosphotungstic acid) in alkaline media. Phenolic compounds, including phenolic acids and flavonoids, form a blue color complex with FC reagent with maximum absorbance at 740 nm. Gallic acid was used as the reference standard compound; the results were expressed as a gram of gallic acid per 100 mL of PE (%g). The volume of 1000 μL of FC reagent (aqueous diluted 1:10) was mixed with 200 μL of PE (diluted in methanol). Appropriate dilutions of each PE (ranging from 1:300 to 1:900) were experimentally found. The mixtures were kept in the dark for 6 min. After addition of 800 μL of Na_2CO_3 solution (7.5%) they were additionally kept in dark for two hours. The absorbance was measured at 740 nm. Four reaction mixtures with different extract dilutions were used to evaluate TCP. Each absorbance was adjusted to the value of blank probe (distilled water). All samples were done in triplicates.

2.5. Statistical analysis

All methods were carried out in triplicates. The results were expressed as the mean ± standard deviation (SD). Descriptive statistical analyses for calculating the means and the standard error of the mean were performed using Origin Pro 8 and PASS 2008 statistical analysis software package. The evaluation of one-way analysis of variance (ANOVA) and *F*-test of obtained results were performed for comparison of means, and significant differences are calculated according to post hoc Tukey's HSD test at the $p < 0.05$ level, using StatSoft Statistica 10 software.

3. Results and discussion

3.1. Antioxidant activity of propolis extracts determined by DC polarography

Behavior of hydrogen peroxide in alkaline medium was studied by direct current (DC) polarography with dropping mercury electrode (DME) with the aim to apply it in AO activity determination. The formation of $[Hg(O_2H)(OH)]$ complex between perhydroxil ion (HOO^-) and anodically produced Hg^{2+} ion, in alkaline solution, noticed by Morrison et al. (1973) and Kikuchi and Murayama (1976), was unequivocally confirmed by Sužnjević et al. (2011). Sensitivity of polarographic anodic current, originating from mentioned complex formation, to the presence of individual or complex samples with AO activity enabled development of novel AO assay. The relevant experimental conditions, such as temperature, concentration and pH dependence, were assessed. Possibility to determine directly AO activity of colored samples of PEs using DC polarographic assay has been explored. Under optimized working conditions, various propolis extracts, obtained from Serbian market, have been tested for AO activity.

A well defined polarographic anodic current (i_{p0}) of 5 mmol L^{-1} H_2O_2 in alkaline solution (CL buffer, pH 9.8) has been recorded at potentials of mercury dissolution (Fig. 1). Diluted PE (1:10) has been gradually added into the cell solution in aliquots of 10 or 20 μL. Obtained polarograms has been shown on Fig.1. As seen, gradual addition of PEs into buffered H_2O_2 solution causes decrease of i_{p0}. The relative decrease of i_{p0} upon each addition of PE (Δi_p) has been calculated according to the following equation (Sužnjević et al., 2011):

$$\Delta i_p\,(\%) = \left(1 - \frac{i_p}{i_{p0}}\right) \times 100$$

where Δi_p (%) represents relative decrease of i_{p0} upon addition of PE, while i_p remaining part of i_{p0} after sample addition. Finding that the same amount of analyzed PEs resulted in the different Δi_p values indicates possibility to distinguish PE according to AO activity determined using the polarographic assay. Plots of Δi_p (%) against the volume of PE have been shown (Fig. 2). The region of linear decrease of Δi_p is observable. This linearity allows determination of AO activity of the investigated PEs. The volume of the sample capable to decrease i_{p0} to 20% ($\Delta i_{p20} = 20\%$) has been determined. This