Antioxidant Activity of Ethanolic Fractions of Polish Propolis

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There is a great variation in the chemical composition of propolis of different origins. Likewise, the method of its extraction has significant impact on the content of biologically active compounds. Here we compared methods of propolis extraction for optimal antioxidant activities which were measured by means of β-carotene discolouration, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+) radical cation decolouration assays. In the extracts, the contents of polyphenols and flavonoids were measured, and phenolic acids were identified and quantified by HPLC. A three-step extraction allowed obtaining large amounts of phenolic acids from propolis. The propolis fractions obtained had antioxidant properties comparable to those of α-tocopherol and butylated hydroxytoluene. Therefore, they may be used as effective natural antioxidants.

Key words: Propolis, Antioxidant Activity, Extraction

Introduction

Propolis is a resinous substance collected by honey-bees. The composition of propolis is qualitatively and quantitatively variable; it depends primarily on the geographical origin, the time of collection, the breed of bees, and even on its position in the hive (Lofty, 2006; Silici and Kutluca, 2005; Bankova et al., 2000). The chemical profile of poplar propolis is characteristic of the temperate climate zone. This type of propolis originates from Europe, North America, and non-tropical regions of Asia. Studies showed that even in Europe where propolis is believed to be very well studied, there could be surprises concerning the plant origin (Bankova et al., 2000, 2002; Bankova, 2005; Falcao et al., 2010). The aim of the present study was to determine the antioxidant activity of propolis from an ecologically clean area of Poland, where the environment is not polluted.

The antioxidant properties of Polish propolis result mainly from the presence of phenolic acids and flavonoids (Kumazawa et al., 2004; Rice-Evans et al., 1996). Park and Ikegaki (1998) demonstrated that propolis extraction with 70% and 80% ethanol allows obtaining extracts rich in compounds of powerful antioxidant and antibacterial properties. Aqueous extracts also had powerful antioxidant properties (Nagai et al., 2001; Volpert and Elstner, 1993). Ethanol extracts of Turkish propolis displayed higher radical scavenging activity than aqueous extracts (Geckil

Abbreviations: BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; EEP, ethanol extract of propolis after three-step extraction; EEPC, ethanol extract of propolis after single extraction; TEAC, Trolox equivalents antioxidant capacity; TAA, total antioxidant activity.

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et al., 2005). Given the great variety in the chemical composition of propolis, and furthermore, the fact that the method of its extraction has a significant influence on the content of biologically active compounds, an attempt was made at optimizing the extraction of Polish propolis for high antioxidant properties. In order to achieve this aim, the contents of polyphenols and flavonoids were measured in the tested propolis sample. Phenolic acids were identified, and antioxidant activities were measured and compared with those of known antioxidants, i.e. α-tocopherol and butylated hydroxytoluene (BHT).

**Material and Methods**

**Materials**

Propolis was collected once from an apiary in Kamianna, Poland. The village is located in the Beskidy Mountains, far from cities and industrial areas. The sample of propolis was collected in summer 2006 from *Apis mellifera* hives and stored at –20 °C until analysis. Prior to extraction, the propolis was ground and homogenized in an Ultra-Turrax T8 homogenizer (IKA-Labortechnik, Staufen, Germany).

**Reagents**

Solvents were purchased from POCh (Gliwice, Poland). All other chemicals were obtained from Sigma (St. Louis, MO, USA), with the exception of β-carotene which was from Unilever (Katowice, Poland).

**Propolis extraction**

The first method of extraction was a three-step procedure. Ten g of propolis were extracted with 35% (v/v) ethanol, the residue was extracted with 55% ethanol, and the second residue was extracted with 75% ethanol. The proportion of ethanol to propolis was 10:1 (w/w). Maceration was performed over a period of two weeks without shaking. After the removal of waxes, the extract was concentrated in vacuo in a Rotavapor R-3 (Büchi, Flawil, Switzerland) and then reduced to dryness in a vacuum oven OV-12 (Jeio Tech, Gyeonggi-do, Korea) at 40 °C. The solid residue was dissolved in 96% ethanol to a final concentration of 0.2 mg/ml. The 35%, 55%, and 75%, ethanol extracts, respectively, were designated EEP 1, 2, and 3.

For the second method, propolis samples were individually extracted in either 35%, 55% or 75% ethanol and the extracts processed as above, yielding fractions EEPC I, II, and III, respectively. All extraction yields were based on the initial weight of the propolis sample.

**Polyphenol content**

The content of polyphenols was measured with the Folin-Ciocalteau reagent (Singleton et al., 1999) and expressed as gallic acid equivalents.

**Flavonoid content**

The content of flavonoids was determined by the method of Woisky and Salatino (1998) and expressed as quercetin equivalents.

**Analysis of phenolic acids**

Phenolic acids were analysed according to Maciejewicz et al. (2002). For reversed phase high-performance liquid chromatography (HPLC), a Merck (Darmstadt, Germany) LiChrospher column RP-18/5 μm (25 cm x 4 mm) was used. The mobile phase consisted of 1% (v/v) acetic acid in acetonitrile (A) and 1% acetic acid in water (B). The following gradient was applied: 0 – 5 min, 10% A in B; 5 – 15 min, 15% A in B; 15 – 20 min, 30% A in B; 20 – 35 min, 10% A in B. The flow rate was 1 ml/min and detection was at 254 nm. Solutions of authentic compounds in methanol at 0.005%, 0.0025%, 0.00125%, 0.000625% content were injected into the column. Under identical conditions, 1% methanol solutions of propolis fractions were analysed.

**Total antioxidant activity (TAA)**

The measurement was performed according to Emmons et al. (1999) with the following slight modification: 0.2 ml 1% hydrogen peroxide were added per 100 ml distilled H₂O. Hydrogen peroxide causes discoloration by oxidation of linoleic acid and β-carotene. The effect of propolis extracts on the coupled β-carotene/linoleic acid oxidation system was measured.

**Free radical scavenging activity**

Reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by extracts followed the procedure of Brand-Williams et al. (1995). The ability of propolis fractions to reduce DPPH was
measured. EC50 values are defined as amounts of extracts giving 50% DPPH reduction.

**Trolox equivalents antioxidant capacity (TEAC)**

The ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) decolourization assay, using Trolox as standard, was performed according to Erel (2004).

**Statistical treatment**

All measurements were performed five times. The statistical analysis was carried out using the KS and Liliefors test, as well as the Shapiro-Wilk test, the level of significance was \( p < 0.05 \). For comparison of the polyphenol and flavonoid contents, the U Mann-Whitney test was used.

**Results and Discussion**

**Extraction efficiency**

Yields from propolis extraction with 35% ethanol (EEP I and EEPC I) were low (Table I) indicating the presence of only little hydrophilic material. The combined efficiency of the EEP I and EEP II extractions was 35% and was lower than the efficiency of EEPC II. The efficiency of EEP I + EEP II + EEP III was about 66% and was slightly higher than that of EEPC III.

These differences observed in the efficiency of the one- and three-step extractions, respectively, were reflected in the measured content of polyphenols and flavonoids in the particular fractions (Table I), as well as in their antioxidant properties.

**Polyphenol content**

Polyphenols have powerful antioxidant properties. When extracting a 10-g sample of propolis using 35%, 55%, and 75% ethanol in sequence (the EEP I + EEP II+ EEP III fractions), the polyphenol content was 449 mg/g dry extract. The single extraction with 75% ethanol yielded a polyphenol content of 178 mg/g dry extract. This polyphenol content is the same as in propolis from Chilgok in Korea (Ahn et al., 2004), and is less than that of propolis from Cheongju and Muju (228 mg/g EEP), but higher than that from several other regions (85 to 144 mg/g EEP).

Similarly, the single extraction with 55% ethanol (the EEPC II fraction) yielded much less polyphenol than the combined content of EEP I and EEP II in the sequential extraction (Table I).

**Flavonoid content**

The sequential extraction of a sample with 35%, 55%, and 75% ethanol is more efficient concerning the flavonoid extraction than a single extraction with 75% ethanol. As can be calculated from Table I, in the case of the EEP fractions, 216 mg of flavonoids were obtained from 1 g of dry extract. At the same time, a single extraction with 75% ethanol (the EEPC III fraction) yielded only 92 mg of flavonoids. Also, the sum of the EEP I and EEP II fractions contained more flavonoids (about 130 mg) than the EEPC fraction (about 69 mg of flavonoids).

**Analysis of phenolic acids by HPLC**

In the tested fractions of propolis six phenolic acids were identified by HPLC: gallic, ferulic, benzoic, trans-cinnamic, caffeic and trans-p-coumaric acids, respectively. Also, cis-p-coumaric acid was identified, based on the retention time according to Maciejewicz et al. (2002), but it was not measured quantitatively.

The phenolic acid contents in the EEP and EEPC fractions are shown in Table II. In the EEPC fractions, all acids measured (apart from gallic acid) were found in larger amounts than in the EEP fractions. The combined content of the acids measured in the EEP fractions amounted to 1.443 mg/g dry extract. In the EEPC III fraction, the amount of acids was 1.495 mg/g dry extract. In these acids, due to the presence of carboxy and phenolic hydroxy groups, hydrophilic properties prevailed. This may explain the ease of their extraction by more diluted ethanol solutions (Rice-Evans et al., 1996). This was confirmed in the case of the EEPC fractions, as far as benzoic acid

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**Table I. Efficiency of propolis extraction and contents of polyphenols (in gallic acid equivalents) and flavonoids (in quercetin equivalents) per g of propolis dry extract (solid after drying in vacuo). Means ± SD, \( n = 5 \).**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Efficiency of extraction (%)</th>
<th>Content of polyphenols (mg/g)</th>
<th>Content of flavonoids (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEP I</td>
<td>10 ± 1</td>
<td>168 ± 5</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>EEP II</td>
<td>25 ± 4</td>
<td>153 ± 4</td>
<td>93 ± 7</td>
</tr>
<tr>
<td>EEP III</td>
<td>31 ± 1</td>
<td>128 ± 2</td>
<td>86 ± 9</td>
</tr>
<tr>
<td>EEPC I</td>
<td>10 ± 1</td>
<td>172 ± 1</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>EEPC II</td>
<td>51 ± 1</td>
<td>177 ± 2</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>EEPC III</td>
<td>64 ± 3</td>
<td>178 ± 2</td>
<td>92 ± 1</td>
</tr>
</tbody>
</table>
and – partly – ferulic acid were concerned. It has to be noticed that the content of ferulic acid was highest in the EEPC II fraction and lowest in the EEPC III fraction. In short, the propolis extracts obtained with 75% ethanol had a low ferulic acid content.

The content of caffeic acid in the EEPC III fraction was high, but not higher than the sum of its contents in the EEPC I and EEPC II fractions. Thus, a single extraction of propolis with 75% ethanol did extract all caffeic acid present in propolis. The content of caffeic acid in particular fractions of propolis has a significant influence on their antioxidant properties, as the acid has a powerful antioxidant and DPPH radical scavenging potential (Gulcin, 2006; Kumazawa et al., 2004). The content of caffeic acid in the EEP and EEPC fractions of propolis from Kamianna, Poland (Table II) was lower than that in the EEP fraction from Cheongju, Korea (1.7 mg/g EEP), which contained the highest amounts of this compound among Korean propolis from various origins (Ahn et al., 2004).

The antioxidant properties of the EEP and EEPC fractions were assessed by no less than 3 methods, because antioxidants can deactivate radicals by two major mechanisms, i.e. by hydrogen atom transfer (HAT) or by single electron transfer (SET) (Prior et al., 2005). Inhibition of the oxidation of linoleic acid follows the HAT mechanism, while DPPH and ABTS*+ free radical scavenging follows the SET mechanism.

**Total antioxidant activity (TAA)**

Table III presents the TAA of the EEP and EEPC fractions in the \( \beta \)-caroteone/linoleic acid system. The EEP II fraction displayed the highest antioxidant activity. This is probably related to the highest flavonoid content in this fraction of propolis, because flavonoids have powerful antioxidant properties. The results obtained by Pascual et al. (1994) and Krol et al. (1990) confirm this observation. Thus the antioxidative activity of ethanol extracts of propolis may be related to their high flavonoid content.

The EEPC II fraction was most active in inhibiting linoleic acid oxidation. On the other hand, in Brazilian propolis, the highest antioxidant properties were obtained in the 70% and 80% ethanol extracts (Park and Ikegaki, 1998). These differences suggest that the properties of propolis may differ widely depending on its origin. A single extraction with 75% ethanol will not recover the full antioxidant potential of propolis.

The comparison between the antioxidative properties of the propolis fractions with those of well known antioxidants, such as \( \alpha \)-tocopherol and BHT at the same concentration, demonstrated that both the EEP fractions and – to a slightly lesser extent – the EEPC fractions displayed

### Table II. Phenolic acid contents (μg) per g EEP and EEPC propolis dry extract. Means ± SD, \( n = 5 \).

<table>
<thead>
<tr>
<th>Extract</th>
<th>Caffeic acid</th>
<th>Gallic acid</th>
<th>Ferulic acid</th>
<th>Benzoic acid</th>
<th>( \text{trans-Cinnamic acid} )</th>
<th>( \text{trans-p-Coumaric acid} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEP I</td>
<td>116.0 ± 2.4</td>
<td>3.0 ± 0.6</td>
<td>134.0 ± 2.5</td>
<td>31.0 ± 0.0</td>
<td>14.0 ± 0.5</td>
<td>440.0 ± 2.5</td>
</tr>
<tr>
<td>EEP II</td>
<td>64.0 ± 4.6</td>
<td>3.0 ± 0.3</td>
<td>129.0 ± 4.0</td>
<td>45.0 ± 0.0</td>
<td>20.0 ± 0.6</td>
<td>288.0 ± 3.1</td>
</tr>
<tr>
<td>EEP III</td>
<td>17.0 ± 2.9</td>
<td>4.0 ± 0.6</td>
<td>39.0 ± 3.6</td>
<td>14.0 ± 0.8</td>
<td>60.0 ± 0.1</td>
<td>76.0 ± 0.9</td>
</tr>
<tr>
<td>EEPC I</td>
<td>145.0 ± 3.2</td>
<td>3.0 ± 0.1</td>
<td>158.0 ± 2.3</td>
<td>13.3 ± 0.3</td>
<td>15.0 ± 0.1</td>
<td>465.0 ± 2.7</td>
</tr>
<tr>
<td>EEPC II</td>
<td>188.8 ± 4.3</td>
<td>5.0 ± 0.1</td>
<td>369.0 ± 2.7</td>
<td>256.0 ± 0.0</td>
<td>13.0 ± 0.5</td>
<td>482.6 ± 2.6</td>
</tr>
<tr>
<td>EEPC III</td>
<td>238.9 ± 2.5</td>
<td>4.0 ± 0.2</td>
<td>275.0 ± 3.9</td>
<td>109.0 ± 0.0</td>
<td>18.2 ± 0.5</td>
<td>850.0 ± 2.8</td>
</tr>
</tbody>
</table>

### Table III. Antioxidative properties of EEP and EEPC. Means ± SD, \( n = 5 \).

<table>
<thead>
<tr>
<th>Sample</th>
<th>TAA(^a)</th>
<th>EC(_{50})(^b)</th>
<th>TEAC(^c) ([\text{mM}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEP I</td>
<td>69.64 ± 2.94</td>
<td>256.86 ± 4.60</td>
<td>153.52 ± 2.02</td>
</tr>
<tr>
<td>EEP II</td>
<td>77.25 ± 1.55</td>
<td>236.30 ± 6.58</td>
<td>133.99 ± 6.17</td>
</tr>
<tr>
<td>EEP III</td>
<td>72.81 ± 1.31</td>
<td>306.16 ± 5.48</td>
<td>108.41 ± 12.34</td>
</tr>
<tr>
<td>EEPC I</td>
<td>70.3 ± 2.55</td>
<td>268.60 ± 14.53</td>
<td>139.38 ± 2.54</td>
</tr>
<tr>
<td>EEPC II</td>
<td>71.05 ± 1.94</td>
<td>299.80 ± 13.88</td>
<td>125.24 ± 2.06</td>
</tr>
<tr>
<td>EEPC III</td>
<td>68.04 ± 2.53</td>
<td>285.70 ± 8.61</td>
<td>123.22 ± 6.01</td>
</tr>
<tr>
<td>BHT ((\text{in full}))</td>
<td>92.73 ± 0.098</td>
<td>108.33 ± 1.20</td>
<td></td>
</tr>
<tr>
<td>( \alpha )-Tocopherol</td>
<td>95.45 ± 0.32</td>
<td>162.50 ± 0.90</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Average total antioxidant activity expressed as percentage of the inhibition of linoleic acid oxidation in the respective sample versus the control. EEP, EEPC, BHT, VE were used at a concentration of 0.2 mg/ml.

\(^b\) Average quantity of propolis dry extract \((\text{in mg})\) needed to obtain 50% reduction of 1 mol DPPH.

\(^c\) Antioxidant capacity of the tested sample relative to Trolox.
powerful antioxidant activity. When measured by means of inhibiting linoleic acid oxidation, the antioxidative activity of these fractions amounted to about 70% of that of the reference compounds.

Free radical scavenging activity

EC$_{50}$ measurements in the DPPH radical scavenging test confirmed the observations made with the linoleic acid oxidation test. The highest EC$_{50}$ value, and, therefore, the weakest antioxidant properties, was observed in the EEP III fraction. This suggests that there were relatively few compounds with antioxidant properties left in the fraction. The EEP II fraction contained the highest portion of such compounds (see Table III).

Antioxidant activity is generally thought to be correlated with the flavonoid content. The propolis extract from Amaicha del Valle, Tucuman, Argentina, with total flavonoid contents from 37.6 to 42.6 mg/g, had the highest DPPH free radical scavenging activity (Nieva Moreno et al., 2000). Those extracts of Polish propolis from Kamianka (EEP II and EEPC III), which had the highest total flavonoid content, also had the highest DPPH radical scavenging activity (Table I).

The DPPH radical scavenging activity of the EEP fractions was 53–69% that of $\alpha$-tocopherol and 35–46% that of BHT at a concentration of 0.2 mg/ml. EEPC fractions showed slightly weaker activity, i.e., 42–57% that of $\alpha$-tocopherol activity and 28–38% that of BHT activity at 0.2 mg/ml, respectively.

Trolox equivalents antioxidant capacity (TEAC)

The measurement of the TEAC using ABTS$^{•+}$ revealed that the EEP I and EEPC I extracts had the highest antioxidant activity. Therefore, the extraction with 75% ethanol does not allow obtaining extracts with powerful antioxidant properties.

In summary, the differences in the antioxidant activities of propolis fractions indicate that the three-step extraction is more effective. The quantity of extracted polyphenols varies depending on the concentration of ethanol used. Our results show that a propolis extract obtained by means of the three-step extraction of propolis with solutions of increasing ethanol content can be used as a source of effective natural antioxidants. An attempt has already been made to use propolis for pork preservation by Han et al. (2001), with positive results.


