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Antioxidant power of Iranian propolis extract

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Abstract

Propolis is a resinous natural hive product derived from plant exudates collected by honey bees. Due to biological and pharmacological activities, it has been extensively used in folk medicine. The present study was designed to measure the antioxidant power of ethanolic extracts of propolis samples from different parts of Iran with "ferric reducing ability of plasma" (FRAP) assay and compare the results with Trolox at concentrations of 100, 1000 and 2000 μg/ml. FRAP values of propolis ethanolic extracts were in the range of 31.5 ± 14.6 to 1650 ± 72 μM, whereas the values of Trolox ranged from 125.25 ± 9.95 to 3381.64 ± 113.83 μM. The FRAP values of Tehran propolis ethanolic extract and Trolox at concentration of 100 μg/ml did not show any significant difference ($P > 0.05$). Total flavonoid and polyphenol contents of ethanolic extracts of propolis samples, determined by using aluminum nitrate and Folin–Ciocalteu colorimetric methods, were in the range of 1.22 ± 0.33–7.79 ± 0.39 g/100 g and 3.08 ± 0.02–8.46 ± 0.03 g/100 g crude extract of propolis, respectively. The result of this experiment may show that propolis as a natural source of antioxidant compounds may be of use in prevention of free radical-related diseases.

Keywords: Propolis; Antioxidant power; Total flavonoid; Total polyphenol

1. Introduction

Propolis or bee glue is a resinous hive product collected by honey bees from plant exudates and contains more than 160 constituents (Greenaway, May, Scaysbrook, & Whatley, 1991). Historically it has been used for various purposes, especially as a medicine (Ghisalberti, 1979). Flavonoids are thought to be responsible for many of its biological and pharmacological activities including anticancer (Matsuno, 1995), anti-inflammatory (Wang, Mineshita, & Ga, 1993), antimicrobial (Koo et al., 2000; Kujumgiev et al., 1999) and antioxidant effects (Basnet, Matsuno, & Neidlein, 1997; Nieva Moreno, Isla, Sampietro, & Vattuone, 2000).

Active free radicals, together with other factors are responsible for cellular aging and many conditions such as cardiovascular diseases, cancer, diabetes, arthritis, Parkinson disease and Alzheimer. The antioxidant serves as a defensive factor against free radicals in the body. Enzymes such as superoxide dismutase, catalase and glutathione peroxidase are the main system that opposes oxidation. If the free radicals production becomes more than the capacity of enzymatic system, the second line of defense (vitamins) may come to action. Antioxidant such as vitamins C and E quench free radicals and become oxidized and inactive (Halliwell, 1994). Flavonoids and various phenolics are the most important pharmacologically active

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constituents in propolis (Vennat, Arvouet–Grand, Gross, & Pourrat, 1995) and have been shown to be capable of scavenging free radicals and thereby protecting lipids and other compounds such as vitamin C from being oxidized or destroyed during oxidative damage (Popeskovic, Kepcija, Dimitrijevic, & Stojanovic, 1980). Recently, propolis has gained popularity and used extensively in healthy drinks and foods to improve health and prevent diseases such as inflammation, heart disease, diabetes and even cancer (Banskota et al., 2000; Burdock, 1998). Because of such broad spectrum of biological properties and their different uses, there is a renewed interest in its biological activities. Several investigations on propolis in Eastern Europe and South America have indicated that flavonoids concentrated in propolis are powerful antioxidants which are capable to scavenge free radicals (Banskota et al., 2000; Basnet et al., 1997). Due to lack of knowledge about antioxidant activity of Iranian propolis, this study was designed to measure the antioxidant power of Iranian propolis ethanolic extract by using the FRAP assay as an easy to use and inexpensive method, which is based on ferric to ferrous ion reduction at low pH. The FRAP values were compared against Trolox, a water soluble analog of vitamin E, as an antioxidant standard compound.

Flavonoids are thought to account for much of the biological and pharmacological activities in propolis, although other phenolic compounds are also involved (Grange & Davey, 1990). Determination of total flavonoid and polyphenol contents of crude ethanolic extracts of propolis samples were done by using aluminum nitrate and Folin–Ciocalteu colorimetric methods, separately. Estimation of phenolics content can be used for evaluation of propolis quality and its properties.

2. Materials and methods

2.1. Propolis origin

Propolis samples were obtained from colonies of honeybees located in three different geographical parts of Iran in fall 2003. Tehran propolis sample was collected from Tehran to Khojir (nearly north of Iran) and was green propolis; Isfahan propolis sample was gathered from Isfahan to Daran (centre of Iran) and Khorasan sample was gathered from Khorasan to Neishabour (north east of Iran). The last two samples were brown type propolis. Propolis samples were collected by scraping off from the frames of the beehives located in the three regions noted, by Mr. H. Afrouzan from "Animal Science Research Institute of Iran (ASRI)".

2.2. Preparation of propolis ethanolic extract

Raw propolis samples were chopped in to very small pieces and extracted with 25 ml of 95% ethanol/g of propolis with continuous stirring at room temperature for 24 h (single extraction). The suspensions were filtered and concentrated in a rotary evaporator under reduced pressure at 40 °C to obtain the crude extract in paste form and kept in a dry and dark place (Chang, Yang, Wen, & Chern, 2002).

2.3. Measuring the antioxidant power (the FRAP assay)

2.3.1. Reagent preparation

Reagents were prepared according to the method of Benzie and Strains (1996). Working FRAP reagent was prepared as required by mixing 25 ml of 300 mM acetate buffer, pH 3.6 (3.1 g sodium acetate (Merck, Germany) and 16 ml glacial acetic acid per litre of buffer solution) with 2.5 ml of 10 mM 2,4,6-tripyridyl-*S*-triazine (TPTZ) solution (0.031 g of TPTZ (Merck, Darmstadt, Germany) in 10 ml of 40 mM HCl) and 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ solution (3.24 g of ferric chloride (Merck, Darmstadt, Germany) in 1 l of distilled water). Freshly prepared reagent warmed at 37 °C (Benzie & Strains, 1996).

2.3.2. FRAP assay

Propolis crude extracts were separately redissolved in 95% ethanol at a concentration of 50 mg/ml and diluted to 100, 1000 and 2000 μg/ml. Aliquots (100 μl) of each diluted ethanolic extracts were mixed with 3 ml of freshly prepared FRAP reagent. The FRAP values are obtained by comparing the absorbance change of blue coloured ferrous- tripyridyltriazine complex at 593 nm in diluted ethanolic extracts of propolis samples with those containing ferrous ions in known concentrations (Benzie & Strains, 1996). Aqueous solutions of known ferrous sulphate (Merck, Germany) concentrations in the range of 100–1000 μM were used for calibration. In order to make comparison, Trolox (Aldrich, Milwaukee, WI, USA) was also tested under the same conditions as a standard antioxidant compound.

2.4. Estimation of total flavonoid content by aluminum nitrate colorimetric method

Propolis crude extracts were separately redissolved in 95% ethanol at a concentration of 50 mg/ml ethanolic extracts (0.1 ml) were diluted with 80% aqueous ethanol (0.9 ml). Aliquots of diluted extracts (0.5 ml) were added to test tubes and mixed with 0.1 ml of 10% aluminum nitrate, 0.1 ml of 1 M aqueous potassium acetate and 4.3 ml of 80% ethanol. After standing for 40 min at room temperature, the absorbance of the reaction mixtures was measured at 415 nm (Nieva Moreno et al., 2000). Quercetin (Fluka, Switzerland) was used as a standard compound in the range of 5–100 μg/ml concentration to construct a standard curve.

2.5. Estimation of total polyphenol content by Folin–Ciocalteu colorimetric method

Propolis crude extracts were separately redissolved in 95% ethanol at a concentration of 50 mg/ml. Ethanolic

extracts (0.1 ml) were diluted with 95% ethanol (0.9 ml) and mixed with 5 ml of 10-fold diluted solution of 2N Folin–Ciocalteu reagent (Sigma, UK). Four milliliters of saturated sodium carbonate solution were added to the mixtures and then shaken. The absorbance of the reaction mixtures was measured at 765 nm after 2 h (Spanos & Worlstad, 1990). Caffeic acid (Sigma, Steinheim, Germany) was used as a standard compound in the range of 100–500 μg/ml concentration to construct a standard curve.

2.6. Statistical analysis

The results are reported as mean ± SD of three independent replicates. Statistical analysis of data was carried out by computer using SPSS ver. 11.5 software. One-way ANOVA and Tukey post hoc multiple comparison tests were used to analyze data. *P*-values less than 0.05 were considered significant.

3. Results and discussion

Oxidative stress has been defined as an imbalance in the production of free radicals within the body and the biochemical antioxidant defense mechanisms to combat them. Oxidative stress may possibly contribute to the onset of chronic diseases. Oxidative damages may also result in poor liver function. In vitro studies on rat hepatocytes show that propolis extracts protect the liver cells against damages (Basnet et al., 1997). Flavonoids and various phenolics are the most important pharmacologically active constituents in propolis (Vennat et al., 1995) and have been shown to be capable of scavenging free radicals.

The ferric reducing ability of plasma (FRAP) assay was used for assessing "antioxidant power" of propolis samples gathered from different regions of Iran. The antioxidant power of propolis samples were compared with Trolox as a reference antioxidant. In this method the ferric reducing ability of antioxidant compound is measured. At low pH, ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) blue colour complex with an absorption maximum at 593 nm. Test conditions favour reduction of the complex and, thereby, colour development, provided that an antioxidant is present. The FRAP values have been calculated by comparing the absorbance change in 593 nm in test samples with those containing ferrous ions in known concentrations ranging from 100 to 1000 μM. The FRAP values of propolis ethanolic crude extracts ranged from 31.5 ± 14.6 to 1650 ± 72 μM at concentrations of 100, 1000 and 2000 μg/ml against Trolox values ranging from 125.25 ± 9.95 to 3381.64 ± 113.83 μM (Fig. 1). Among the three Iranian propolis samples, Tehran propolis ethanolic extract exhibited the highest FRAP value. Surprisingly the results showed that the FRAP value of Tehran propolis extract was comparable to that of Trolox at a concentration of 100 μg/ml ($P > 0.05$). Antioxidative effect of propolis extracts has been reported in different methods including iodometric method (Yamauchi, Kato, Oida, Kanaeda, & Ueno, 1992), thiobarbituric acid (TBA) method (Isla, Nieva Moreno, Sampietro, & Vattuone, 2001; Yanping, 2002) and free radical scavenging ability with reduction of radical diphenylpicrylhydrazyl (DPPH) (Nieva Moreno et al., 2000; Scheller et al., 1990), but in the FRAP assay the reagents are inexpensive and simple to prepare, results are fast and reproducible and the equipment required is of a type commonly found in biochemical laboratories (Benzie & Strains, 1996).

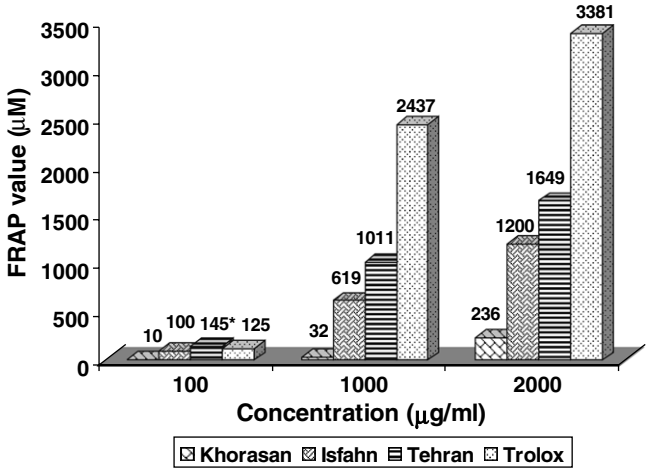

Fig. 1. FRAP value (μM) for propolis ethanolic extracts and Trolox at concentration of 100, 1000 and 2000 μg/ml. *There is no significant differences between Tehran propolis ethanolic extract with Trolox ($P > 0.05$).

The composition of propolis depends upon the vegetation of the area from where it was collected. Due to the difference in geographical location of three propolis samples, estimation of total polyphenol and flavonoid contents of crude ethanolic extracts has been done by colorimetric methods. Although chromatographic techniques in combination with absorption spectrum analysis and mass spectrometry provide definitive information for quantification of phenolics in propolis, these methods usually require advanced instruments, various authentic standards and are time consuming. On the other hand, colorimetric methods are convenient and appropriate for routine analysis of phenolics (Chang et al., 2002). Aluminum nitrate and Folin–Ciocalteu colorimetric methods have been applied to determine total flavonoid and polyphenol contents of crude ethanolic extracts of propolis samples. This may be helpful in evaluation of propolis quality and its properties.

In aluminum nitrate colorimetric method, aluminum nitrate forms acid stable complex with the keto group and either the hydroxyl group in A or C ring of flavonoids, in addition it forms acid labile complexes with ortho-dihydroxyl groups in the A or B ring of flavonoids. The aluminum nitrate complexes of flavonoid compounds show strong absorbance at 415 nm and flavonoids with more functional groups absorb stronger at 415 nm (Chang et al., 2002). We used quercetin as a standard compound because it is one of the widely spread flavonoids in propolis

Table 1
Total flavonoid and polyphenol contents (g/100 g crude extract) determined by aluminium nitrate and Folin–Ciocalteu colorimetric methods

Propolis	Total flavonoid content (g/100 g)[a]	Total polyphenol content(g/100 g)[b]
Tehran	7.79 ± 0.39	8.46 ± 0.03
Isfahan	3.18 ± 0.08	7.11 ± 0.19
Khorasan	1.22 ± 0.33	3.08 ± 0.02

[a] Results are presented as mean ± SD ($n = 3$) and calculated as quercetin equivalents.
[b] Results are presented as mean ± SD ($n = 3$) and calculated as caffeic acid equivalents.

samples and has strong absorbance at concentrations lower than 100 ppm at 415 nm because of its more functional hydroxyl groups. Total flavonoid contents of crude ethanolic extracts of propolis samples varied from 1.22 ± 0.33 to 7.79 ± 0.39 g/100 g crude extract (Table 1) with the lowest amount for Khorasan and the highest for Tehran propolis sample. Total polyphenol contents were estimated with Folin–Ciocalteu colorimetric method. Polyphenols including phenolic acids and flavonoids form a blue colour complex with phosphomolybdic- phosphotungstic acid reagent (Folin–Ciocalteu reagent) with maximum absorbance at 765 nm. Caffeic acid was employed as a standard compound for estimation of total polyphenol contents because it is one of the most abundant phenolic acids found in propolis. Total polyphenol contents of crude ethanolic extracts of propolis samples varied from 3.08 ± 0.02 to 8.46 ± 0.03 g/100 g crude ethanolic extract (Table 1) with the lowest amount of polyphenol content for Khorasan and the highest for Tehran propolis sample.

In conclusion, antioxidative activity was demonstrated in all three propolis samples. In particular, Tehran propolis sample showed the highest antioxidative activity and the lowest activity was showed for Khorasan sample. It is proposed that strong antioxidative activity occurs in propolis with high amounts of phenolic compounds and weak activity with low amounts, but obviously, other non-flavonoid scavenger such as enzymes, antioxidant vitamins in propolis are also involved. Nowadays propolis is used as over the counter dermatological item for wound healing, tissue regeneration, treatment of burns and herpes simplex. It is used in toothpaste and mouthwash preparations in treating gingivitis, cheilitis and stomatitis. It has also found its way into pharmaceutical and cosmetic products such as face creams, ointments, lotions and solutions. It is marketed in tablet, powder and chewing gum (Ayala, Lembo, Nappa, & Balato, 1985; Bankova, Popov, & Marekov, 1983; Bjorkner, 1994; Dobrowolski et al., 1991; Esser, 1986; Ghisalberti, 1979; Hausen, Wollenweber, Senff, & Post, 1987; Marcucci, 1995), but in Iran it has been looked as waste product and does not have its proper applications. Our experiment showed that the antioxidant activity of propolis is extremely dependent to the environment which is obtained showing strongest activity in Tehran sample and lowest in Khorasan sample. Tehran and Isfahan are industrialized areas and some of these flavonoids may enter to the propolis as environmental pollutants. The Khorasan region where the propolis was obtained is not industrialized and propolis by itself shows very low activity. On the other hand the first two areas ecologically have various herbal flora and some of these flavonoids are extracted by bees. By ignorance of source of the additives, if the positive physiological properties and the non-toxicity of the propolis sample is proven it could be suggested as a possible natural antioxidant for incorporation into some food products and supplements to prevent many free radical-mediated diseases and improve the health benefit of consumers.

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