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Antioxidant, Free Radical Scavenging and Metal Chelating Characteristics of Propolis

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Abstract: This study was undertaken to determine the reducing characteristics, metal chelating capability, anti-lipid peroxidative and antiradical properties of propolis compared to two widely used artificial antioxidants, Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT). The water and ethanol extracts of propolis showed significantly a different degree of metal chelating, radical scavenging activity and reducing power. In general, ethanol extracts of propolis showed higher activity regarding these parameters. Synthetic antioxidants showed better activities than both propolis extracts for antioxidant properties, utilizing a -carotene bleaching method. At higher concentrations, the reducing power of ethanol extract of propolis was similar to that of artificial antioxidants. The metal chelating activity of both water and ethanol extracts of propolis was comparable to that of EDTA and significantly higher than both BHA and BHT.

Key words: Propolis, artificial antioxidants, butylated hydroxyanisole, butylated hydroxytoluene; radical scavengers, metal chelating

INTRODUCTION

Propolis, also known as "bee glue" is a resinous hive product collected by honey bees (*Apis mellifica* L.) from various plant sources. Bees collect propolis to seal holes in their hives, smooth out the internal walls and protect the entrance against intruders. Propolis also might serve as an insulation material and draught excluder. The composition of propolis is very complex, varying with the geographic region, with more than 300 constituents having been identified to date. The bulk of propolis is made of resin (50%), wax (30%), aromatic oils (10%), pollen (5%) and other organic compounds[1-3]. The chemical composition of propolis is mainly composed of flavonoids, terpenes, amino acids and caffeic acid phenyl esters. Recently, this nontoxic natural product was reported to possess multiple pharmacological effects[4-7]. In recent years, there has been a growing interest in propolis for its biological activities, especially regarding its antioxidant[4], antibacterial[8], anticancer[9], antifungal[10], anti-inflammatory[11] and antiviral[12] properties. The exact mode of physiological or biochemical mechanisms responsible for these effects, however, is yet to be determined. Most of the therapeutic effect of propolis, however, was suggested to be associated with antimicrobicidal properties and the ability to scavenge free radicals.

Free radicals are compounds that have unpaired electrons with deleterious effects such as harming biological macromolecules (DNA, protein and lipids). Also, they cause deterioration of food components, especially lipids. In food industry generally synthetic antioxidants are used to prevent lipid peroxidation and oxidation of food constituents. However, BHA and BHT are suspected to have some side effects as liver damage and carcinogenesis[13,14]. Hence, there has been an increasing demand for safer natural antioxidants in food industry and other applications. This caused a renewed growing interest in search of natural products that have been used for centuries for a variety of reasons from treatment of acne to zona. Propolis, a material fitting to all these descriptions, is such a product. Thus, we consider that it is worthwhile to study some characteristics of propolis in more detail. Here, the antioxidant activity, reducing power and metal chelating properties of propolis were investigated in *in vitro* settings. Besides using ethanol extracts of propolis, its water extracts were also utilized given that ethanol itself can have differential affects on living organisms. The antioxidant property of propolis water and ethanol extracts compared to two common artificial antioxidant compounds, Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT), was studied.

MATERIAL AND METHODS

Materials: Propolis was collected from the local beehives in Malatya, Turkey and was kept dry and cold

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(-40 °C) until used. Propolis samples were mixed with 10 volume of ethanol or water and stirred for 48 h at ambient temperature and filtered trough Whatman No:1 filter paper. Filtrates were dried under vacuum evaporation and the dried material was stored in refrigerator. The water and ethanol extracts of these propolis extracts were prepared as 1% daily and different aliquots of these extracts were used for the radical scavenging, antioxidant and metal chelating characteristics. All other chemicals used for analytic purposes were obtained from Sigma Chemical Co. unless otherwise specified.

Radical scavenging power: Radical Scavenging Power (RSP) of propolis was assessed by the method of Shimada *et al*. with slight modifications[15]. Three mL reaction mixture contained 2.9 mmol DPPH (2.9 mL $1x10^{-4}$ M DPPH) and 0.1 mL extracted propolis or artificial antioxidant at various concentrations. In control, ethanol or water was used in place of sample depending on the solvent in which extract was prepared. Cuvettes were left in dark at room temperature for 30 min and the resulting color was measured spectrophotometrically at 520 nm against blanks. A decreasing intensity of purple color was related to higher RSP percentage, which was calculated using the following equation;

$$RSP = [1-(\frac{A_{S:30}}{A_{B:30}})]\times 100$$

where, $A_{S:30}$ is absorbance of sample and $A_{B:30}$ is absorbance of blank at 30 min reaction time.

Reducing power: Reducing power of samples was determined according to the method of Oyaizu[16]. Various amount of sample solutions containing 0, 0.2, 0.5, 1.0 and 2.0 mg extracted propolis were placed into tubes and volume was adjusted to 1 mL with water for propolis/water extracts and ethanol for the others. To these tubes 2.5 mL 0.2 M phosphate buffer (pH 6.6) and 2.5 mL 1 % potassium ferricyanide were added and mixed gently. The mixtures were incubated at 50 °C in a water bath for 20 min. A 2.5 mL of 10 % Trichloroacetic Acid (TCA) was added and the mixtures were centrifuged at 6,000 rpm for 10 min. From the top layer of supernatant 2.5 mL was transferred into tubes containing 2.5 mL distilled water and 0.5 mL 0.1 % ferric chloride ($FeCl_3.6H_2O$). After shaking, the mixture was left at room temperature for 5 min and then the absorbance was read at 700 nm against blanks. Two artificial antioxidants, BTH and BHA, were utilized to compare the reducing power. The higher the absorbance was, the better the reducing power of the sample was recognized.

-carotene bleaching: Antioxidant activity of propolis was determined using -carotene bleaching method of Miller[17]. Two mg of crystalline -carotene was dissolved in 10 mL chloroform and to 1 mL of this solution in round-bottom flasks 20 g of linoleic acid and 200 µL of Tween-20 (Merck) were added. Chloroform was removed in rotary evaporator under vacuum at 40°C for 5 min and 50 mL distilled water was added with vigorous stirring to form an emulsion. Five mL of this emulsion was added to each tube containing samples (propolis extracts or artificial antioxidants). Tubes were placed in a water bath at 50°C and absorbance was recorded at 470 nm in 10 min intervals during 2 h incubation.

Metal chelating: Ferrous ions chelating activity of propolis and artificial antioxidants was determined by the method of Dinis *et.al*[18]. Into tubes containing 1.7 mL distilled water and 50 µL of 0.2 mM $FeCl_2{\cdot}4H_2O$, 50 µL of sample solution was added and the mixture was left at room temperature for 1 min. To this mixture 0.2 mL 5 mM ferrozine was added and final color was monitored at 562 nm after 10 min incubation. The metal chelating efficiency of samples was determined by comparing with the chelating activity of Ethylene Diamine Tetraacetic Acid (EDTA, 2 Na-salt). The inhibition percentage of ferrozine-Fe^{2+} complex formation against blanks containing $FeCl_2$ and ferrozine was calculated by the formula;

% Inhibition= $[(A_0-A_1)/A_0] \times 100$

where, A_0 indicates the absorbance of the control and A_1 the absorbance in the presence of the propolis extract, artificial antioxidants or EDTA.

RESULTS

Radical scavenging power: The results for RSP of Propolis/Water Extracts (PWE), Propolis/Ethanol Extracts (PEE), BHA and BHT are summarized in Fig.1. In order to determine the effect of concentration on RSP, we used four different volumes (20, 50, 100 and 200 µL) of samples of same concentration (1 %). Results showed that, PEE and BHA were similar in their RSP characteristics, while BHT and PWE possessed lower RSP values at all concentrations: i.e., 20 L 1 % PEE had 3.6- and 2.5-fold higher RSP than PWE and BHT, respectively. At higher concentrations, however, this difference was less significant. In reactions containing 50 L (0.5 mg) antioxidant samples, above figures were both 1.5-fold and at 100 L (1 mg) and 200 L (2 mg) all four samples showed relatively similar RSP values.

Reducing power: Reducing power characteristics of water and ethanol extracts of propolis and commercial

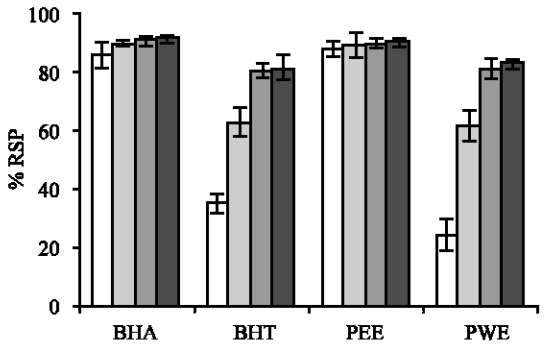

Fig. 1: Radical scavenging power of propolis extract and artificial antioxidants. Extract and antioxidant solutions were prepared at 1% (w/v) concentration and 20 µL (0.2 mg/reaction, □), 50 µL (0.5 mg/reaction, □), 100 µL (1 mg/reaction, ■) and 200 µL (2 mg/reaction, ■) Aliquots of these solutions were used for their RSP characteristics in a total volume of 3 ml reaction mixture. Each value is the average of two experiments in duplicates with error bars indicating stdevs (σ_{n-1})

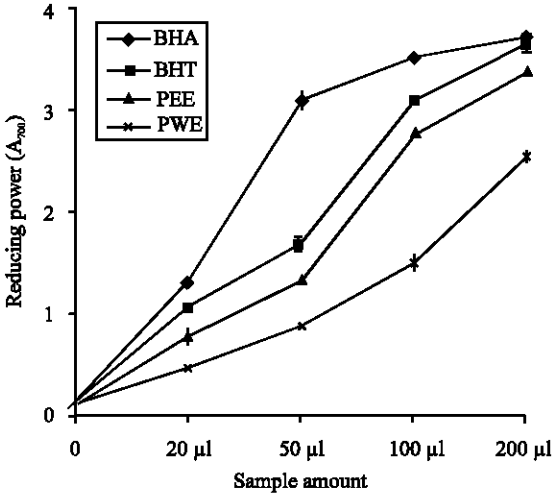

Fig. 2: Reducing power of propolis extracts and artificial antioxidant solutions at different concentrations. Extract and antioxidant solutions were prepared at 1% (w/v) concentration and 20 µL (0.2 mg/reaction), 50 µL (0.5 mg/reaction), 100 µL (1 mg/reaction) and 200 µL (2 mg/reaction) aliquots of these solutions were used for their reducing power characteristics. Each value is the average of two experiments in duplicates with error bars indicating STDEVs (σ_{n-1})

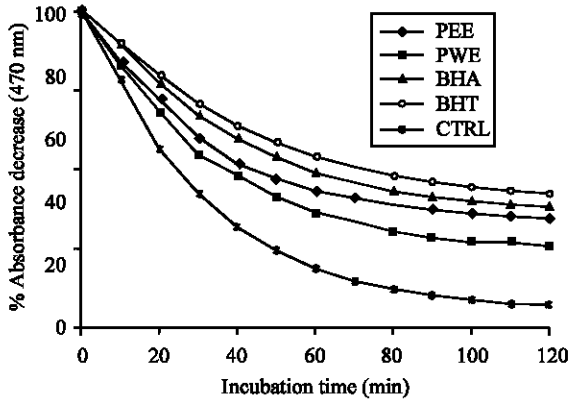

Fig. 3: Antioxidant property of propolis extracts and artificial antioxidants. Relative changes in absorbance of -carotene emulsions containing propolis extracts or artificial antioxidants were measured at 470 nm. Each value is the average of two experiments made in duplicates. For clarity SDEVs are not shown but they are mostly smaller than 5 % of average value

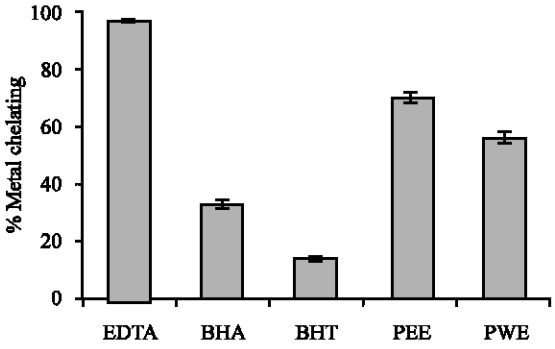

Fig. 4: Metal chelating percentage of propolis extracts and artificial antioxidants compared to ETDA. Each value is the average of two experiments with error bars indicating STDEVs (σ_{n-1})

antioxidants are given in Fig. 2. The concentration dependent reducing power of samples followed the order:BHA>BHT>PEE>PWE. At higher concentrations (1 mg and 2 mg/reaction), however, the reducing power of PEE was similar to that of artificial antioxidants (BHA and BHT). PWE at the same concentrations, however, possessed up to 2-fold weaker reducing power than two artificial samples and PEE. The reducing power of PEE and PWE increased with increasing concentration. At 2 mg/reaction PEE and PWE showed 4.4- and 5.3 -fold higher reducing power, respectively, than that of at 0.2 mg/reaction.

-carotene bleaching: The anti-bleaching activity of samples on -carotene was studied by monitoring the

color intensity of emulsions at 470 nm for every 10 min for 2 h (Fig. 3). This kind of antioxidant activity test was carried out at concentrations 0.2 and 0.5 mg/reaction and the data points are the average values of these two concentrations as percentage of absorbance change from the initial (100 %) reading. All four samples showed a significantly higher activity against lipid peroxidation. In the first 20 min of incubation, samples had 20-30 % higher antiperoxidative effect than the controls. By 1 h of incubation, this figures ranged between 45-80 %. Among the samples BHT had the best lipid peroxidation preventive activity and this was followed by BHA, PEE and PWE, respectively. At the end of the incubation period (2 h), the decrease from initial absorbance was 54, 50, 48, 41% for BHT, BHA, PEE and PWE, respectively, corresponding to about 60 % to 2-fold higher color intensity than the control.

Spectrophotometric determination of iron chelation: The dark color of complex formed by the interaction of ferrozine with Fe^{2+} ions is decreased by the action of metal chelator compounds that exist in the reaction mixtures. Thus, absorbance at 562 nm is inproportinally related to chelating activity of the samples studied. Results here showed that PEE had the highest chelating activity. The chelating activity of PEE was about 70 % that of metal chelating activity of EDTA (Fig. 4). Other samples had metal chelating activity in the order of PWE>BHA>BHT with rates 56, 32 and 13 % compared to that of EDTA.

DISCUSSION

Herbal remedies have been used for centuries for countless treatments from headaches to wound healings. Only in recent years, however, the exact functions of these compounds have been studied in detail. Propolis is a popular ingredient in various consumer health products for both internal and external applications sold in health food stores. There have been reports on broad spectrum of biological activities of propolis as an anticancer, antioxidant, anti-inflammatory, antibacterial and antifungal. The antioxidant and anti-inflamatory effect of propolis have been ascribed to its high flavonoid content. Flavonoids have have been reported to display these e ects by suppressing the formation of free radicals.

The free radical scavenging activity of water and ethanol extracts of propolis and also that of synthetic antioxidants was evaluated through their ability to quench the synthetic DPPH radical. There are numerous methods for evaluating the antioxidant activity of both natural and artificial compounds. The method using stable DPPH radical, however, is a widely used one because of its simplicity and requiring relatively short time compared to other methods[19]. Possible mechanism of DPPH scavenging was suggested to be through reduction (protonation) of this radical by antioxidant compound to a more stable DPPHH form. Because of its unpaired electron, DPPH has an absorption maxima at 520 nm and as it gets reduced (e.g., as this electron becomes paired off) in the presence of a free radical scavenger, the absorbance decreases stoichiometrically with respect to the number of electrons taken up. In our *in vitro* system, PEE was determined to be an excellent compound for this kind of scavenging. The free radical scavenging activity of PWE, however, was relatively weaker than both PEE and BHA, but similar to that of BHT. Furthermore, free radical scavenging capacity of PEE was not effected by the concentrations (0.2-2.0 mg/reaction) used, while PWE showed a concentration dependent DPPH quenching capacity. The higher radical scavenging activity of PEE was probably a function of better solubility of propolis constituents in the ethanol. Related studies have showed that some constituents of propolis can scavenge free radicals both *in vitro* an *in vivo*[19-21], while there are no reports on water extracts of propolis. Although antiradical activity of water extract found to be weaker than the other test compounds, the aspect that propolis is used as food food ingredient makes these findings important.

Reducing power of a compound is also a supporting feature for its antioxidant activity. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation[22]. Being good electron donors, phenolic compounds show the reducing power and have ability to convert Fe^{3+} to Fe^{2+}[23]. In this context, different fractions of propolis reported to possess reducing ability[19]. The results from our *in vitro* setting showed that propolis, especially PEE, had comparable reducing power to synthetic antioxidants. The reducing power (absorbance at 700 nm) increased with increasing concentrations of PEE and PWE, results that are in accordance with that of Wang *et al.*[19], using different fractions of propolis.

There is currently a wealth of experimental evidence suggesting that, being a rich source of polyphenol compounds propolis display potent antioxidant activities. Beta-carotene bleaching is one of the most commonly applied methods for determining the total antioxidant property of extracts and artificial compounds. Our results showed that both PEE and PWE had anti β-carotene bleaching activity similar to BHA and BHT, while in control there was a substantial and rapid oxidation of β-carotene evident from almost complete decolorization of the reaction mixture.

Besides their antioxidant characteristics, flavonoids can chelate trace metals[19]. Here for the first time we have shown that, propolis as an ethanol extract or water extract is an excellent metal chelator. Metal chelation capacity of propolis was well comparable with that of EDTA and substantially (2-5-fold) higher than artificial antioxidants, BHA and BHT. To date, there are no definitive examples in the literature of propolis acting to bind heavy metal ions. On the basis of the results of this study, it is clear that both propolis extracts have substantially higher metal chelating capacity and comparable reducing power and antioxidant activity to two widely used synthetic antioxidants, BHA and BHT. However, there is still a need for further studies regarding the use of propolis extracts or its constituents as the natural antioxidants, possible food supplements and pharmaceutical agents.

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