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Preparation and antioxidant properties of water extract of propolis

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Abstract

A water extract was prepared from fresh propolis from Brazil. Antioxidant activity was measured using a lipid peroxidation model system. The activity was very strong and, at 1 and 5 mg/ml, higher than that of 5 mM ascorbic acid. The scavenging activity against superoxide anion radical of water extract of propolis was high, and the extracts, at 50 and 100 mg/ml, completely inhibited the production of superoxide. The extracts, at 50 and 100 mg/ml, completely inhibited the hydroxyl radical. This suggests that the water extract of propolis has potential as a pharmaceutical for patients with various diseases such as cancer, cardiovascular diseases, and diabetes.

Keywords: Antioxidant properties; Water extract of propolis; Scavenging effects; Active oxygen species

1. Introduction

Propolis is a sticky plant substance that is collected by honey bees which may include different types of secretions or exudates (Walker & Crane, 1987). Propolis has been used as a folk medicine in Europe but, in traditional Chinese medicine, beehives have been used instead of propolis since the Chinese bee produces very little or no propolis at all. In Japan, propolis is used as a health food and the Japanese believe that it can cure inflammation, heart disease, and even diabetes and cancer. Chemical analysis of propolis is still far from satisfactory; however, more than 150 polyphenolic compounds including flavonoids and cinnamic acid derivatives have been reported from propolis using GS–MS analysis (Greenaway, May, Scaysbrook, & Whatley, 1991).

Several biological attributes such as anticancer (Grunberger, Banerjee, Eisinger, Oltz, Efos, Caldwell et al., 1988; Matsuno, 1995; Scheller, Krol, Swiacik, Owczarek, Gabrys, & Shani, 1989), antioxidant (Pascual, Gonzalez, & Torricella, 1994; Scheller, Wilczok, Imielski, Krol, Gabrys, & Shani, 1990), antimicrobial (Bosio, Avanzini, D'Avolio, Ozino, Savoia, 2000; Flegazi, El-Hady, & Abd Allah, 2000; Koo, Gomes, Rosalen, Ambrosano, Park, & Cury, 2000), antiinflammatory and antibiotic (Bianchini & Bedendo, 1998) activities have been reported for propolis and its constituents. These actions have been ascribed to the flavonoid content (Merino, Gonzalez, Gonzalez, & Remirez, 1996).

Although ethanol extract of propolis is the most common (Scheller, Gazda, Gabrys, Szumlas, Eckert, & Shani, 1988), it is known that this extract poses immunological properties in animals and patients (Frankiewicz & Scheller, 1984). Scheller et al. (1990) reported higher survival of mice with Ehrlich ascites carcinoma pre-treated with ethanol extract of propolis and demonstrated its ability to protect mice against gamma irradiation (Scheller et al., 1990). Some of these properties were attributed to the antioxidant effect of the ethanol extract of propolis, that partially stemmed from its high content of flavonoids. The major flavonoids, comprising about 25–30% of the ethanol extract of propolis on a dry weight basis, are galangin, isalpinin, kaempferol, kaemferid, rhamnocitrin, rhamnetin, quercitin, pinocembrin, pinostrobin, and pinobanksin (Havsteen, 1983).

Studies concerning water extract of propolis are increasing (Basnet, Matsushige, Hase, Kadota, &

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Nanba, 1996; Khayyal, El-Ghazaly, & El-Khatib, 1993; Serkedjieva, Manolova, & Bankova, 1992). These have reported that water extract of propolis showed hepatoprotective activity in both chemical and immunological liver injury models (Basnet et al., 1996), antiviral activity, inhibition of platelet aggregation (Serkedjieva et al., 1992), and antiinflammatory activity (Khayyal et al., 1993). However, there are few studies of reactive oxygen species in relation to water extract of propolis. The purpose of the present study is demonstrate the action of water extract of propolis against autoxidation and free radicals, such as superoxide anion radical, DPPH radical, and hydroxyl radical. This work should help to prevent various diseases of increasing interest in human health.

2. Materials and methods

2.1. Materials

Fresh propolis, imported from Brazil, was obtained from Inoue Yohojo Bee Farm Inc. (Hyogo, Japan) and used in this study.

2.2. Chemicals

Linoleic acid, ascorbic acid, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), nitroblue tetrazolium salt (NBT), xanthine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, and 2-thiobarbitric acid (TBA) were purchased from Wako Chemicals Co., Ltd. (Osaka, Japan). Xanthine oxidase from butter milk (XOD; 0.34 U/mg powder) was obtained from Oriental yeast Co., Ltd. (Tokyo, Japan). Other reagents were of analytical grade.

2.3. Preparation of water extract of propolis

Water extract of propolis was obtained as described by Suzuki (1990) with slight modification. In brief, 50.0 g of propolis were suspended and extracted with 5 volumes of distilled water with shaking at 20 °C for 1 day. The extracts were centrifuged at 28,000×*g* for 30 min, and the supernatants were pooled. The residue was re-extracted under the same conditions. The extracts were centrifuged under the same conditions and the supernatants were pooled. Supernatants obtained were combined and dialyzed against distilled water, and then the dialysate was lyophilized. Each solution (1, 5, 10, 50, and 100 mg/ml H_2O) was used as the sample solution for the following tests.

2.4. Determination of total phenolic compounds

The total phenolic compounds were measured spectrophotometrically at 760 nm (Slinkard & Singleton, 1977).

2.5. Effect of autoxidation by the FTC method

The antioxidant activity was assayed by using a linoleic acid model system. A 0.0833 ml of sample solution and 0.208 ml of 0.2 M sodium phosphate buffer (pH 7.0) were mixed with 0.208 ml of 2.5% (w/v) linoleic acid in ethanol. The preoxidation was initiated by the addition of 20.8 μl of 0.1 M AAPH and carried out at 37 °C for 200 min in the dark. The degree of oxidization was measured by the thiocyanate method (Mitsuda, Yasumoto, & Iwai, 1966) for measuring peroxides by reading the absorbance at 500 nm after colouring with $FeCl_2$ and ammonium thiocyanate. A control was performed with linoleic acid but without sample solution. Ascorbic acid (1 and 5 mM) was used as positive control.

2.6. Effect of superoxide anion radical

The effect of superoxide anion radical was evaluated by the method of Nagai, Sakai, Inoue, Inoue, and Suzuki (2001). This system contained 0.48 ml of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 ml of 3 mM xanthine, 0.02 ml of 3 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.02 ml of 0.15% bovine serum albumin, 0.02 ml of 0.75 mM NBT and 0.02 ml of sample solution. After at 25 °C for 10 min, the reaction was started by adding 6 mU XOD and carried out at 25 °C for 20 min. After 20 min, the reaction was stopped by adding 0.02 ml of 6 mM CuCl. The absorbance of the reaction mixture was measured at 560 nm and the inhibition rate was calculated by measuring the amount of the formazan that was reduced from NBT by superoxide. Ascorbic acid (1 and 5 mM) was used as positive control.

2.7. Effect of DPPH

The effect of DPPH radical was evaluated by the method of Okada and Okada (1998) with a slight modification. The assay mixture contained 0.3 ml of 1.0 mM DPPH radical solution, 2.4 ml of 99% ethanol, and 0.3 ml of sample solution. The solution was rapidly mixed and scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. Ascorbic acid (0.1 and 1.0 mM) was used as positive control.

2.8. Effect of hydroxyl radical

The effect of hydroxyl radical was assayed by using the deoxyribose method. The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM $FeSO_4$-EDTA, 0.15 ml of 10 mM H_2O_2, 0.525 ml of H_2O, and 0.075 ml of sample solution in an

Eppendorf tube. The reaction was started by the addition of H_2O_2. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1.0% of TBA in 50 mM NaOH; the solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical (Chung, Osawa, & Kawakishi, 1997). Ascorbic acid (1 and 5 mM) was used as positive control.

3. Results and discussion

3.1. Antioxidant activity of water extract of propolis

The water extract of propolis was effectively extracted and the yield was about 13.0% on a dry weight basis. To evaluate the in vitro effect of it at the initiation stage of lipid peroxidation, the antioxidant effects on the peroxidation of linoleic acid were investigated by the FTC method. These results are shown in Table 1. The antioxidant activity of control was suddenly decreased with passage of the time. The pattern of 1 mM ascorbic acid was the same as that of the control. On the other hand, there were no differences in antioxidant activities among the samples tested. Most active were concentrations higher than 10 mg/ml. The activities of water extract of propolis, at 1 and 5 mg/ml, were higher than that of 5 mM ascorbic acid. Moreover, these activities increased, depending on the concentration of the sample, showing a high antioxidant activity from the initial stage of the peroxidation to 200 min.

3.2. Superoxide-scavenging activity

Superoxide-scavenging activity of water extract of propolis was measured using the xanthine–xanthine oxidase system and these results are indicated as the superoxide productivity. As shown in Table 2, each propolis sample exhibited high superoxide-scavenging activity and these activities tended to increase with increasing degree of the concentration of the sample. These samples, at 50 and 100 mg/ml, completely inhibited the production of superoxide. However, the activities of propolis samples tested were higher than that of 1 mM ascorbic acid.

3.3. DPPH radical scavenging activity

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Hatano, 1995; Hatano, Edamatsu, Hiramatsu, Mori, Fujita, Yasuhara et al., 1989; Hatano, Takagi, Ito, & Yoshida, 1997; Yoshida, Mori, Hatano, Okomura, Vohara, Komagoe et al., 1989). To evaluate the scavenging effect of DPPH on water extract of propolis, DPPH inhibition was investigated and these results are shown as relative activities against control. The activity of 1 mM ascorbic acid was high, followed by propolis samples (Table 3). The activities of water extract of propolis were between those of 0.1 and 1 mM ascorbic acid. The activities of propolis samples tended to decrease with decreasing concentration of the sample. On the other hand, the activity of 0.1 mM ascorbic acid was the same as that of the control.

3.4. Hydroxyl radical scavenging activity

Using Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$), the scavenging effect against hydroxyl radical was investigated and these results are indicated as the inhibition rate. Each propolis sample showed hydroxyl radical scavenging activity and its activity was increased with increasing concentration of the sample (Table 4). Particularly, these samples (50 and 100 mg/ml) completely inhibited the hydroxyl radical. On the other

Table 1
Antioxidant activities of water extract of propolis as measured by the thiocyanate method

Time (min)	O.D. (500 nm)							
	Sample							
	A	B	C	D	E	F	G	H
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
50	0.060	0.038	0.035	0.025	0.007	0.022	0.016	0.176
100	0.071	0.047	0.028	0.022	0.000	0.135	0.032	0.473
200	0.077	0.057	0.009	0.000	0.000	0.469	0.090	1.153

A: 1 mg/ml propolis; B: 5 mg/ml propolis; C: 10 mg/ml propolis; D: 50 mg/ml propolis; E: 100 mg/ml propolis; F: 1 mM ascorbic acid; G: 5 mM ascorbic acid; H: control.

Table 2
Scavenging activities of water extract of propolis on the superoxide anion radical

Sample	O_2^- production (%)
A	13.9
B	1.5
C	1.9
D	0
E	0
F	85.3
G	10.2
H	100

A: 1 mg/ml propolis; B: 5 mg/ml propolis; C: 10 mg/ml propolis; D: 50 mg/ml propolis; E: 100 mg/ml propolis; F: 0.1 mM ascorbic acid; G: 1 mM ascorbic acid; H: control.

Table 3
DPPH radical scavenging activities of water extract of propolis

Time (min)	Relative activity (%)							
	Sample							
	A	B	C	D	E	F	G	H
0	100	100	100	100	100	100	100	100
1	43.5	41.7	30.7	27.9	24.1	94.6	5.3	99.9
2	42.0	41.7	31.0	27.6	24.0	94.4	5.3	98.5
3	41.8	42.3	30.9	27.6	24.1	94.4	5.3	98.8
5	41.7	41.2	30.7	27.9	24.0	94.4	5.3	99.1
10	41.7	40.6	30.9	27.9	23.7	94.4	5.3	98.7

A: 1 mg/ml propolis; B: 5 mg/ml propolis; C: 10 mg/ml propolis; D: 50 mg/ml propolis; E: 100 mg/ml propolis; F: 0.1 mM ascorbic acid; G: 1 mM ascorbic acid; H: control.

hand, the activities of ascorbic acid (1 and 5 mM) were the same as that of the 5 mg/ml propolis sample.

Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from membranes and bring about peroxidic reactions of lipids (Kitada, Igarashi, Hirose, & Kitagawa, 1979). Therefore, it was expected that water extract of propolis would have antioxidant effects against lipid peroxidation on biomembranes and would scavenge hydroxyl radicals and superoxide anions at the stage of initiation and termination of peroxy radicals.

There are many reports concerning ethanol extract of propolis (Krol et al., 1990; Pascual et al., 1994; Scheller et al., 1990; Volpert & Elstner, 1993). It is also reported that some properties, particularly the antioxidant properties, in ethanol extract of propolis, were partly stemmed from its high content of flavonoids. Flavonoids are expected to affect the activity of several systems. They inhibit the activity of enzymes involved in the conversion of membrane polyunsaturated fatty acids to active mediators such as phospholipase A2 (Lee, Matteliano, & Middleton, 1982), cyclooxygenase and lipoxygenase (Baumann, Von-Bruchhausen, & Wurn, 1980a, 1980b) and by scavenging free radicals (Middleton, 1985). From these findings, it seems that flavonoids contained in ethanol extract of propolis behave, at the initiation stage of lipid peroxidation, as scavengers which react with peroxy radicals of polyunsaturated fatty acids, breaking the chain reaction (Cholbi, Paya, & Alcaraz, 1991; Ratty & Das, 1988; Rekka & Koukounakis, 1991).

Table 4
Hydroxyl radical scavenging activities of water extract of propolis

Sample	Inhibition rate (%)
A	0
B	18.2
C	45.8
D	100.0
E	100.0
F	13.2
G	16.1

A: 1 mg/ml propolis; B: 5 mg/ml propolis; C: 10 mg/ml propolis; D: 50 mg/ml propolis; E: 100 mg/ml propolis; F: 0.1 mM ascorbic acid; G: 1 mM ascorbic acid.

On the other hand, there are few reports about water extract of propolis (El-Ghazaly & Khayyal, 1995; Serkedjieva et al., 1992; Volpert & Elstner, 1993). To date it is reported that water extract of propolis has good antioxidant activity, associated with high contents of phenolic compounds. This supports our present findings (quantity of phenolic compounds about 168 μg/mg powder). It is suggested that water extract of propolis contains a mixture of natural substances, such as amino acids, phenolic acids, phenolic acid esters, flavonoids, cinnamic acid, and caffeic acid. The present studies reveal that water extract of propolis is a natural antioxidant and this explains the previously reported beneficial effects. At present, there are few reports about the components of water extract of propolis (Basnet et al., 1996). Basnet et al. (1996) ascribed the high hepatoprotective activity in water extract of propolis to four di-*O*-caffeoyl quinic acid derivatives: methyl 3,4-di-*O*-caffeoyl quinate, 3,4-di-*O*-caffeoyl quinic acid, methyl 4,5-di-*O*-caffeoyl quinate, and 3,5-di-*O*-caffeoyl quinic acid. It was not found whether these four compounds have an effect on the antioxidant activity in water extract of propolis. The water extract of propolis may have further potential value in patients with various diseases, such as cancer, cardiovascular disease, and diabetes. Research is underway to analyze the antioxidant compounds of propolis and to identify differences in chemical composition in relation to different climates and flora.

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