Potent Free Radical Scavenging Activity of Propol Isolated from Brazilian Propolis

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We evaluated free radical scavenging activity of the water, methanol and chloroform extracts of propolis in 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and xanthine-xanthine oxidase (XOD) generated superoxide anion assay systems. The free radical scavenging activity guided fractionation and chemical analysis led to the isolation of a new compound, propol {3-[4-hydroxy-3-(3-oxo-but-1-enyl)-phenyl]-acrylic acid} from the water extract, which was more potent than most common antioxidants such as vitamin C and vitamin E (α-tocopherol) in these assay systems.

Propolis is a sticky substance that is collected from plants by honey bees which may include their own secretions (Walker and Crane, 1987), and it has been considered to protect against the enemies of bees (König, 1985; Ghisalberti, 1979). Propolis has been used as a folk medicine in Europe. In traditional Chinese medicine, bee hives have been widely used rather than propolis since Chinese bees produce a very little or no propolis at all. Several biological activities such as anticancer (Matsuno, 1995; Scheller *et al.*, 1989; Grunberger *et al.*, 1988), antioxidative (Pascual *et al.*, 1994; Yamauchi *et al.*, 1992; Scheller *et al.*, 1990), anti-inflammatory (Wang *et al.*, 1993), and antibiotic (Grange and Davey, 1990; Metzner *et al.*, 1979) activities have been reported on propolis and its constituents. On the other hand, hypersensitivity of propolis and its constituents especially ester derivatives of caffeic acid were reported (Hausen *et al.*, 1987; Hausen *et al.*, 1992). We previously published pancreatic β-cell protective action of the water extract of propolis (PWE) against streptozotocin (STZ)-toxicity in rats (Matsushige *et al.*, 1996) and the isolation of 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 as the active components for the hepatoprotective activity (Basnet *et al.*, 1996a; Basnet *et al.*, 1996b). Continuing studies using, the free radical scavenging activity guided chemical analysis led to the isolation of a new compound, *propol*. In the present paper we report the isolation, structure elucidation and free radical scavenging activity of propol.

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Results

Effect of propolis extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical

The results of free radical scavenging effect of the propolis water extract (PWE), propolis methanol extract (PME), and propolis chloroform extract (PCE) on DPPH free radical system (Hatano *et al.*, 1989) are shown in Fig. 1a. The PWE, PME, and PCE showed a concentration-dependent activity. The free radical scavenging activity of the PWE, PME and PCE was 62, 50 and 42%, respectively at a concentration of 10 μg/ml. The result suggested that the PWE has very strong free radical scavenging activity.

Effect of propolis extracts on superoxide anion radical

We evaluated the free radical scavenging activity of the PWE, PME and PCE on superoxide anion radical generated by an enzymatic method (Imanari *et al.*, 1977) and the results are shown in

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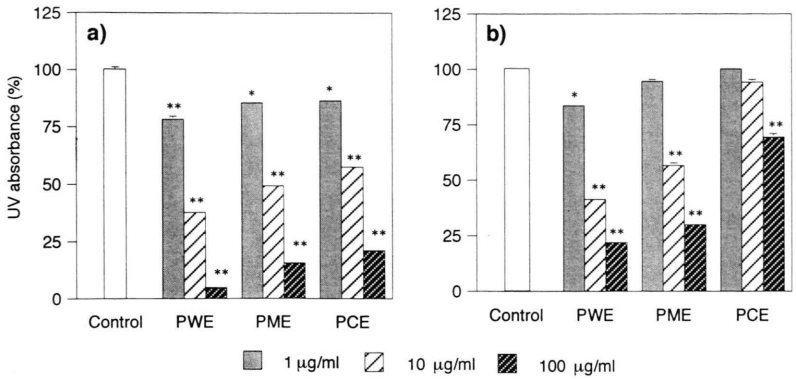

Fig. 1. Free radical scavenging effects of propolis extracts. a) The DPPH free radical scavenging effect was measured by the absorbance at 520 nm in a reaction mixture containing test sample and 30 mM of DPPH. The mean UV absorption (O. D.) for the control was 0.310 which was expressed as 100. b) Superoxide anion radical scavenging effect was measured by the absorbance of NBT at 560 nm in a reaction with superoxide generated in xanthine-XOD system. The mean UV absorption (O. D.) for the control was 0.518 which was expressed as 100. PWE: propolis water extract, PME: propolis MeOH extract and PCE: propolis $CHCl_3$ extract. Results are expressed as mean ± S. D., The value for S. D. were less than 1.5 in both cases. n = 12 (control) and n = 4 (samples), **p < 0.01, *p < 0.05 vs. control.

Fig. 1b. The PWE, PME and PCE showed a concentration-dependent activity as in DPPH system. The PWE, PME and PCE showed 59, 43 and 6% of superoxide anion radical scavenging activity, respectively, at a concentration of 10 μg/ml and the PWE was found to be the most active extract as in DPPH system.

The constituent(s) in the PWE might have very effective free radical scavenging activity. In order to find out the active chemical constituents, the PWE was subjected to Sephadex LH-20 column chromatography as discussed in experimental section to obtain ten fractions. The last fraction was found to be the most active one which decreased the DPPH free radical level by 67.5% compared to the control at a concentration of 10 μg/ml (data not shown). The active fraction (Fr. X) was again subjected to Sephadex LH-20 column chromatography to obtain four fractions (A, B, C and D). Fraction D was most active one (data not shown) and by the RP-18 preparative TLC of fraction D, a new compound propol was obtained.

Structure determination of propol

It is a light green amorphous solid. High-resolution FABMS showed its molecular formula to be $C_{13}H_{12}O_4$ {*m/z*: 233.1349 $[M+H]^+$; Calcd 233.1346}. In the ^{1}H-NMR, two pairs of *trans*-coupling olefinic protons {δ_H 7.91 (1H, d, J = 16.5 Hz, H-10) and 6.94 (1H, d, J = 16.5 Hz, H-11); 7.58 (1H, d, J = 16.0 Hz, H-7) and 6.37 (1H, d, J = 16.0 Hz, H-8)}, three aromatic protons of ABX type {δ_H 7.77 (1H, d, J = 2.0 Hz, H-2), 7.52 (1H, dd, J = 8.5, 2.0 Hz, H-6) and 6.91 (1H, d, J = 8.5 Hz, H-5)} and a methyl group (δ_H 2.38, 3H, s) were observed. It showed 13 signals in its ^{13}C-NMR spectrum. The complete structure and the assignment of all NMR signals were by the analysis of both 1D- and 2D-NMR spectra. In the HMBC spectrum, the aromatic proton at δ_H 7.77 (1H, d, J = 2.0 Hz, H-2)} showing the correlation with the two carbons at δ_C 141.54 (C-10) and 145.88 (C-7) suggested that the proton (δ_H 7.77) lies in between two bulky substituents. Some other important HMBC correlations are shown in Table I. These findings led us to determine the structure of propol as 3-[4-hydroxy-3-(3-oxo-but-1-enyl)-phenyl]-acrylic acid shown in Chart 1.

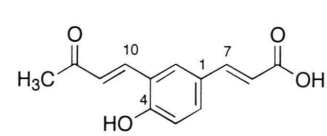

Chart 1. Structure of propol {MW: 232; 3-[4-hydroxy-3-(3-oxo-but-1-enyl)-phenyl]-acrylic acid} isolated from propolis.

Table I. ^{1}H-NMR and ^{13}C-NMR spectral data of propol. All δ-values are in ppm. ^{1}H- and ^{13}C-NMR spectra were measured at 400 and 100 MHz, respectively, in CD_3OD. ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, ^{1}H-^{13}C long-range COSY and HMBC spectra were measured and multiplicities of carbon signals were established by means of the DEPT method.

	^{1}H (δ)	^{13}C (δ)	HMBC-correlation
1	–	128.67 (s)	H-5, H-8
2	7.77 (1H, d, J = 2.0 Hz)	131.25 (d)	H-7, H-10
3	–	123.84 (s)	H-11
4	–	161.09 (s)	H-2, H-6
5	6.91 (1H, d, J = 8.5 Hz)	118.44 (d)	H-6
6	7.52 (1H, dd, J = 8.5, 2.0 Hz)	133.13 (d)	H-2, H-7
7	7.58 (1H, d, J = 16.0 Hz)	145.88 (d)	H-2
8	6.37 (1H, d, J = 16.0 Hz)	118.68 (d)	H-7
9	–	172.17 (s)	H-7
10	7.91 (1H, d, J = 16.5 Hz)	141.54 (d)	H-2
11	6.94 (1H, d, J = 16.5 Hz)	129.16 (d)	H-10
12	–	202.71 (s)	H-10, H-13
13	2.38 (3H, s)	28.06 (q)	–

Antioxidative activity of propol

We measured DPPH free radical scavenging activity of propol and its activity was compared with the most commonly used antioxidants such as vitamin C, vitamin E and caffeic acid. The EC_{50} of propol, vitamin C, vitamin E and caffeic acid was 1.5, 5.8, 12.2 and 5.1 μM, respectively (Fig. 2). Propol was found to be the most potent antioxidant among these compounds in this assay system.

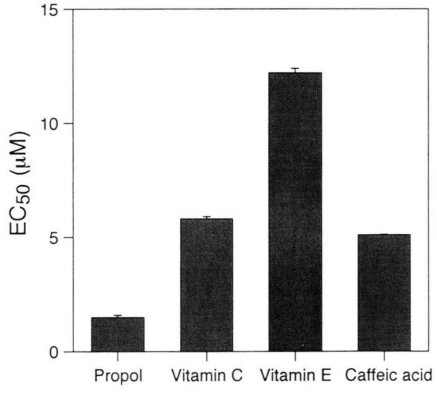

Fig. 2. Free radical scavenging effects of propol, vitamin C, vitamin E and caffeic acid. Experimental condition was same as to that of Fig. 1a. Results are expressed to percent comparing with the control (control: n = 12 and compounds: n = 4). Concentration (mM) giving 50% decrease of DPPH radical is expressed as EC_{50}. The EC_{50} values are the mean ± S. D. of two different experiments.

Discussion

Excess production of superoxide anion and other free radicals cause dysfunction and damage of tissues. The harmful effects of the superoxide anion free radical are reduced by superoxide dismutase (SOD) by converting superoxide to less harmful products in order to provide a protective activity in the animal body. The *in-vitro* quenching of superoxide produced in the xanthine-XOD system can be expected as the SOD-like activity in the living system. DPPH-free radical is not produced in the biological system, however, the scavenging effects of several natural polyphenols on the DPPH radicals, and the correlation of these effects with inhibition of lipid peroxidation in rat liver mitochondria and liver microsomes have been reported (Hanato *et al.*, 1989). In the present paper, to characterize the antioxidative activity of propolis, two simple radical scavenging assays were performed. So free radical scavenging activity guided chemical analysis was carried out and a new and potent antioxidant, propol was isolated. The free radical scavenging activity of propol was more potent than that of vitamin C, vitamin E or caffeic acid (Fig. 2).

We previously reported the β-cell protective action of the PWE against STZ-toxicity (Matsushige *et al.*, 1996) and hepatocytes protective activity against CCl_4 in rats (Basnet *et al.*, 1996a; Basnet *et al.*, 1996b). The diabetogenic action of STZ has been illustrated as free radical-mediated toxicity particularly to β-cells, and nitric oxide free radical play a central role (Kaneto *et al.*, 1995; Rabinovitch, 1994; Turk *et al.*, 1993; Corbett *et al.*, 1992). On the other hand, in CCl_4-induced liver injury, CCl_4 is first metabolized to $\cdot CCl_3$ by cytochrome P450 in the hepatocellular microsome (Schiff and Schiff, 1982). This highly reactive radical injures the hepatocytes and its organelles by peroxidation of the membrane lipids, denaturation of proteins, or other chemical changes that lead to distortion or destruction. The β-cells and hepatocytes protective action of the PWE against STZ and CCl_4, respectively, in rats (Matsushige *et al.*, 1996; Basnet *et al.*, 1996a; Basnet *et al.*, 1996b) might probably due to the reduction of excessively produced toxic free radicals in the animal body. Based on these facts, the components in the PWE having a potent free radical scavenging activity are sup-

posed to be of great interest. It should be noted that propolis constituents especially esters of caffeic acid are regarded to be the allergens in propolis, however compounds with free acid function or substituents to hydroxyl groups in the aromatic ring may contribute to the sensitizing properties to a lesser extent (Hausen *et al.*, 1992). A part of the structure of propol is also similar to caffeic acid, but because of its free acid function and replacement of hydroxyl group in the aromatic moiety it may be less sensitive, however, its skin-sensitizing activity is the matter of further study.

Free radicals mediate several biological functions such as aging, diabetes, hepatitis, inflammation and other chronic diseases and the antioxidants are regarded to be a potential cure for these diseases (Elliott and Chase, 1991; Pryor, 1984). So the potent antioxidant propol from propolis could be a matter of interest related to the clinical hepatitis or preventive action of early diabetes. However, its practical application needs further study.

Experimental Section

General

IR spectrum was recorded on a Hitachi 260–01 spectrometer in KBr discs. UV spectrum was taken on a Shimadzu UV-2200 UV-VIS spectrophotometer. MS and HRFABMS were taken on a JEOL JMS-SX 102A (ionization voltage, 70 eV; accelerating voltage, 5.0 kV) mass spectrometer using a direct inlet system. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with tetramethylsilane as an internal standard. 2D NMR spectra (^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, HMBC) were measured by the use of JEOL standard pulse sequences.

Enzymes

Superoxide dismutase (SOD, Cu, Zn type) and xanthine oxidase (XOD, from butter milk) were purchased from Wako Pure Chemicals Co., Ltd., Osaka, Japan.

Chemicals

Xanthine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), vitamin C, vitamin E and EDTA 2Na were purchased from Wako Pure Chemicals Co., Ltd., Osaka, Japan. Caffeic acid was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Bovine serum albumin (BSA) was purchased from Seikagaku Corporation Tokyo, Japan. Other chemicals were of analytical grades.

Extraction and isolation

Propolis (1.6 kg) was collected in Brazil. It was treated with distilled water (6 l×2) and kept at 95 °C for 2 h and the soluble portion was separated by filtration followed by partial evaporation and lyophilization to obtain the water extract (131 g). The residue was extracted with methanol (6 l×2) under reflux condition for 2 h which gave the methanol extract (331.7 g) after evaporation and lyophilization. The residue was again extracted with chloroform (6 l×2) to obtain the chloroform extract (315.8 g) after evaporation. The water (PWE), methanol (PME), as well as the chloroform (PCE) extracts obtained were used for *in vitro* experiments.

The PWE was found to be the most potent antioxidant among these fractions. So the PWE (81.5 g) was subjected to Sephadex LH-20 column chromatography (7×65 cm) and eluted with the water-methanol gradient to obtain ten fractions. The eluted solvents, volume and yield of each fraction were as follow: Fr-1: water (2000 ml) (0.6 g), Fr-2: 10% methanol in water (1000 ml) (16.9 g), Fr-3: 10% methanol in water (1000 ml) (7.0 g), Fr-4: 20% methanol in water (2000 ml) (6.7 g), Fr-5: 40% methanol in water (2000 ml) (4.2 g), Fr-6: 60% methanol in water (2000 ml) (5 g), Fr-7: 60% methanol in water (1000 ml) (3.4 g), Fr-8: 80% methanol in water (1000 ml) (16.7 g), Fr-9: 80% methanol in water (1000 ml) (22.1 g) and Fr-10: methanol (5000 ml) (3.9 g). The Fr-10 was the most active fraction. A portion of Fr-10 (1.0 g) was again applied to Sephadex LH-20 column chromatography and eluted with 40% MeOH in water to obtain four fractions (A, B, C and D). The last fraction (566 mg) was found to be the main and the most active fraction among four fractions. A portion of fraction D (250 mg) was again purified by the RP-18 preparative TLC with the solvent system methanol-water (1:1, v/v) to isolate propol (9 mg).

Propol

Light green amorphous; UV λ_{max} (MeOH) (log ε) 328.6 (4.20), 239.8 (2.90), 220.8 (3.1); IR ν_{max} (KBr): 3380 (broad, OH), 1735 (CO), 1695 (CO), 1635, 1584, 1508, 1458, 1419, 1339, 1243, 1133, 1000, 635 cm^{-1}; ^{1}H-NMR (methanol-d_4) and ^{13}C-NMR (methanol-d_4) data are shown in Table I; positive ion FABMS *m/z*: 233 $[M+1]^+$; HRFABMS *m/z* 233.1349 (calcd for $C_{13}H_{13}O_4$, 233.1346).

Determination of DPPH radical scavenging activity

The scavenging effect corresponded to the intensity of quenching DPPH, as described by Hatano *et al.,* (1989). The sample solution (500 μl) and equal volume of DPPH solution (6×10^{-5} M) were mixed and allowed to react for 30 min at room temperature with continuous shaking. The absorbance was then measured at 520 nm. Samples and DPPH were dissolved in ethanol or water. The percent scavenging effect was determined by comparing the absorbance of the solution containing the test sample to that of the control solution without the test sample taking the corresponding blanks. The results are the mean of 4 measurements for each sample. The concentration of sample which quenched 50% of DPPH radicals was expressed as EC_{50} and caffeic acid exhibited the EC_{50} as 5.1 μM in this experiment.

Determination of superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity was measured following the methods of Imanari *et al.,* (1991) in xanthine-XOD system. A reaction mixture composed of 700 μl of 0.05 M Na_2CO_3 (pH 10.2), 50 μl of 3 mM xanthine, 50 μl of 3 mM EDTA, 50 μl of 1.5 mg/ml BSA, 50 μl of 0.75 mM NBT and 50 μl of the test sample was mixed thoroughly for 10 min in shaker at room temperature. Then 50 μl of 0.14 mg/ml XOD was added, mixed thoroughly and the solution was incubated for 20 min at 25 °C. The reaction was stopped with the addition of 50 μl of 6 mM $CuCl_2$. The color obtained by the reaction between NBT and superoxide was measured at 560 nm. At this condition, SOD inhibited the activity of superoxide anion by 50% at a concentration of 8.0 ng/ml. The percent scavenging effect was determined by comparing the absorbance of the solution containing test sample to that of the control, solution without the test sample taking the corresponding blanks. Samples were dissolved in ethanol or PBS and the final concentration of ethanol were less than 0.5%. The result is the mean of 4 measurements for each sample. The EC_{50} of caffeic acid was 6.4 μM in this experiment.

Statistical analysis

All value expressed as mean ± S. D. The Student's *t*-test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference and *p*-value of 0.05 or less was considered as statistically significant.

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