Potent free radical scavenging activity of dicaffeoyl quinic acid derivatives from propolis

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Abstract

We evaluated the free radical scavenging activity of the water, methanol and chloroform extracts of propolis in DPPH free radical and xanthine-XOD generated superoxide anion assay systems. The water extract of propolis (PWE) showed a strong free radical scavenging activity. The free radical scavenging activity guided fractionation and chemical analysis led to the isolation of four dicaffeoyl quinic acid derivatives from the PWE. The structures of these isolates were determined to be methyl 3, 4-di-*O*-caffeoyl quinate (**1**), 3,4-di-*O*-caffeoyl quinic acid (**2**), methyl 4,5-di-*O*-caffeoyl quinate (**3**), and 3,5-di-*O*-caffeoyl quinic acid (**4**) by the spectroscopic methods. These compounds showed more potent free radical scavenging activity than the most commonly used antioxidants such as vitamin C, vitamin E and caffeic acid. Quinic acid (**5**) alone did not show free radical scavenging activity. Chlorogenic acid (**6**) or caffeic acid was found to be less potent than dicaffeoyl quinic acid derivatives. Dicaffeoyl quinic acid also showed an inhibitory activity on nitrite formation on lipopolysaccharide (LPS)-induced murine macrophages, J774.1.

Key words propolis, free radical, antioxidant, dicaffeoyl quinic acid, nitric oxide.

Abbreviations BSA, bovine serum albumin; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IC_{50}, 50 % inhibitory concentration; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; XOD, xanthine oxidase; PMS, phenazyne methosulfate; NO, nitric oxide; LPS, lipopolysaccharide; PWE, propolis water extract; PME, propolis methanol extract; PCE, propolis chloroform extract; CA, caffeic acid, QA, quinic acid; CHA, chlorogenic acid; DCQA, dicaffeoyl quinic acid; NAME, L-N^G-nitroarginine methyl ester; STZ, streptozotocin; D-GalN, D-galactosamine.

Introduction

Propolis is a sticky plant substance that is collected from the plant materials by honey bees which may include different types of secretions or exudates.[1] It has been considered that propolis is a protective wall against the enemies of bees.[2] Propolis has been used as a folk medicine in Europe but in traditional Chinese medicine, bee hives have been used instead of propolis since the Chinese bee produces very little propolis or no porpolis at all. Propolis is used as a health food in Japan and people believe that it can cure inflammation, heart diseases and even diabetes and cancer. Chemical analysis of porpolis is still not clear, however, 150 polyphenolic compounds including flavonoids and cinnamic acid derivatives have been reported from propolis by using GC-Mass analysis.[3]

Several biological activities such as anticancer,[4] antioxidant,[5] antiinflammatory[6] and antibiotic[7] activities have been reported on propolis and its constituents. We previously reported the protective action of the water extract of propolis (PWE) against STZ-toxicity in rats.[8] The diabetogenic action of STZ has been illustrated as free radical mediated toxicity particularly to β-cells in the pancreas.[9] It has been reported that the cytokines IL-1 β and nitric oxide play a central role and oxygen free radical

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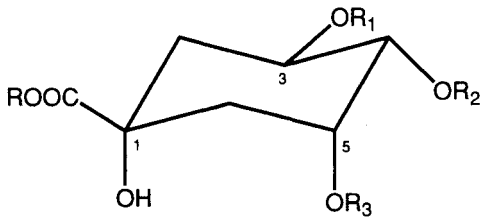

1: R = CH_3, R_1 = R_2 = Caffeoyl, R_3 = H
2: R = H, R_1 = R_2 = Caffeoyl, R_3 = H
3: R = CH_3, R_1 = H, R_2 = R_3 = Caffeoyl
4: R = H, R_1 = R_3 = Caffeoyl, R_2 = H
5: R = R_1 = R_2 = R_3 = H
6: R = H, R_1 = Caffeoyl, R_2 = R_3 = H

Chart 1

scavenger might be expected to partially protect the STZ-toxicity.[10] The PWE also showed a potent hepatoprotective effect on chemically induced hepatitis in rats and immunological liver injury in mice.[11] In CCl_4-induced liver injury, CCl_4 is first metabolized to $\cdot CCl_3$ by such metabolic enzymes as cytochrome P450 in the hepatocellular microsome.[12] This highly reactive radical injures the hepatocytes and its organelles by a direct physicochemical effect, that is, peroxidation of the membrane lipids, denaturation of proteins, or other chemical changes that lead to distortion or destruction. These changes comprise the first stage in the injury that culminates in necrosis and steatosis.[12] We also evaluated the free radical scavenging activities of propolis collected ar different places in Brazil.[13] It has been considered that the free radicals play an important role for such biological activities and some active constituent (s) might be present in PWE to show free radical scavenging and immunomodulating activities. On continuing further, free radical scavenging activity guided chemical analysis which led to the isolation of four active principles for the first time from propolis. In addition to this, we also studied the nitrite formation in LPS-induced murine macrophage, J774.1. In this paper, we wish to present the isolation of methyl 3,4-di-*O*-caffeoyl quinate (**1**), 3,4-di-*O*-caffeoyl quinic acid (**2**), methyl 4,5-di-*O*-caffeoyl quinate (**3**) and 3,5-di-*O*-caffeoyl quinic acid (**4**) from the propolis, thier free radical scavenging activity and nitrite formation in LPS-induced macrophage in relation to hepatoprotective activity.

Materials and Methods

General : Optical rotations were measured on a JASCO DIP-360 digital polarimeter at 25°C. IR spectra were recorded on a Hitachi 260-01 spectrometer in KBr discs. UV spectra were taken on a Shimadzu UV-2200 UV-VIS spectrophotometer. Mass spectra and high-resolution FAB-MS were taken on a JEOL JMS-SX 102A (ionization voltage, 70eV ; accelerating voltage, 5.0 kV) mass spectrometer using a direct inlet system. ^1H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with tetramethylsilane as an internal standard. 2D NMR spectra (^1H-^1H COSY, ^1H-^{13}C COSY, ^1H-^{13}C long-range COSY) 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), sulphanilamide, naphtylethelenediamine dihydrochloride, 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. Lipopolysaccharide (LPS; *E. coli* 055 : B5) was purchased from Difco Laboratories, USA. RPMI (Roswell Park Memorial Institute)-1640 medium was from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was from Sigma, USA. Sephadex LH-20 from Pharmacia, Uppsala, Sweden. Other chemicals were of analytical grades.

Extraction and isolation : Propolis (1.8 kg) collected from Brazil was treated with distilled water (2L ×2) and kept at 95°C for 2 h and the insoluble portion was separated by filtration followed by partial evaporation and lyophilization to obtain the water extract (131.00 g). The residue was extracted with methanol

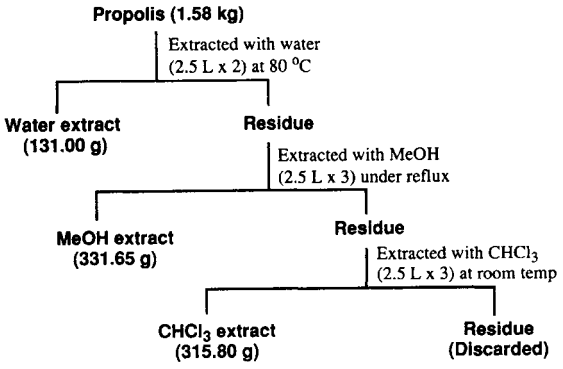

Fig. 1 Extraction flow chart of propolis.

(2L×2) under the reflux condition for 2 h which gave the methanol extract (331.65 g) after evaporation and lyophilization. The residue was again extracted with chloroform (2L × 2) to obtain chloroform extract (315.80 g) after evaporation (Fig. 1). Thus obtained the water, methanol (PME) and chloroform (PCE) extracts were used for *in vitro* experiments.

The PWE showed a very strong free radical scavenging activity both in chemical and enzymatic assay systems. So the PWE (81.45 g) was subjected to Sephadex LH-20 column chromatography (7×65 cm) and eluted with the water-methanol gradient system to obtain ten fractions. The eluted solvents, volume and yield of each fraction were as follows: Fr-1 : water (2000 ml) (0.57 g), Fr-2 : 10 % methanol in water (1000 ml) (16.89 g), Fr-3 : 10 % methanol in water(1000 ml) (7.00 g), Fr-4 : 20 % methanol in water (2000 ml) (6.74 g), Fr-5 : 40 % methanol in water (2000 ml) (4.19 g), Fr-6 : 60 % methanol in water (2000 ml) (4.95 g), Fr-7 : 60 % methanol in water (1000 ml) (3.35 g), Fr-8 : 80 % methanol in water (1000 ml) (16.65 g), Fr-9 : 80 % methanol in water (1000 ml) (22.13 g) and Fr - 10 : methanol (5000 ml) (3.94 g). The Fr-10 showed a very strong free radical scavenging activity in DPPH system. A portion of Fr-10 (1.02 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 (565.6 mg) was found to be the main fraction which showed the DPPH free radical scavenging activity. A portion (250 mg) was again purified by the normal and reverse phase preparative TLC with the solvent systems methanol-chloroform (1 : 9) and methanol water (1 : 1), respectively to obtain methyl 3,4-di-*O*-caffeoyl quinate (**1**) (130 mg), 3,4-di-*O*-caffeoyl quinic acid (**2**) (19 mg), methyl 4,5-di-*O*-caffeoyl quinate (**3**) (12 mg), and 3,5-di-*O*-caffeoyl quinic acid (**4**) (8 mg) (Chart 1).

Compound **1** Light yellow powder, $[\alpha]_D$-210.7°(c= 0.13, MeOH). High-resolution FAB-MS : *m/z* 529.1349 $[M-H]^+$, calcd. for $C_{26}H_{25}O_{12}$ *m/z* 529.1346. ^{1}H-NMR (Methanol-d_4) δ : 7.62 (1H, d, *J* =16.0Hz, 3″-H), 7.52 (1H, d, *J* =16.0 Hz, 3′-H), 7.05 (1H, d, *J* =2.0 Hz, 5″-H), 7.04 (1H, d, *J* =2.0Hz, 5′-H), 6.93 (1H, dd, *J* =8.0, 2.0 Hz, 9″-H), 6.92 (1H, dd, *J* =8.0, 2.0 Hz, 9′-H), 6.77 (2H, d, *J* = 8.0 Hz, 8″-H and 8′-H), 6.31 (1H, d, *J* =16.0 Hz, 2″-H), 6.19 (1H, d, *J* =16.0 Hz, 2′-H), 5.59 (1H, dt, *J* =13.0, 7.0 Hz, 3-H), 5.15 (1H, dd, *J* =9.0, 3.0 Hz, 4-H), 4.38 (1H, dt, *J* =5.5, 3.0 Hz, 5-H), 3.74 (3H, s, OCH_3), 2.32 (1H, dd, *J* = 14.0, 3.0 Hz, 6-Hax), 2.27 (2H, m, 2-H), 2.12 (1H, dd, *J* = 14.0, 6.0 Hz, 6-Heq). ^{13}C-NMR (Methanol-d_4) δ : 175.91 (s, $\underline{C}OOCH_3$), 169.23 (s, C-1″), 168.74 (s. C-1′), 150.35 (s, C-7′ and C-7″), 148.43 (d, C-3′ and C-3″), 147.37 (s, C-6′ and C-6″), 128.40 (s, C-4″), 128.25 (s, C-4′), 123.90 (d, C-9′ and C-9″), 117.23 (d, C-8′ and C-8″), 115.95 (d, C-5′ and C-5″), 115.43 (d, C-2″), 115.28(d, C-2′), 76.64 (s, C-1), 75.76 (d, C-4), 69.69 (d, C-3), 69.42 (d, C-5), 53.96 (q, $O\underline{C}H_3$), 39.48 (t, C-2), 38.99 (t, C-6).

Compound **2** Light yellow powder, $[\alpha]_D$-217.8°(c= 0.13, MeOH). High-resolution FAB-MS : *m/z* 515.1207 $[M-H]^+$, calcd. for $C_{25}H_{23}O_{12}$ *m/z* 515.1190. ^{1}H-NMR (Methanol-d_4) δ : 7.59 (1H, d, *J* = 16.0 Hz, 3″-H), 7.50 (1H, d, *J* = 16.0 Hz, 3′-H), 7.02 (1H, d, *J* = 2.0 Hz, 5″-H), 6.99 (1H, d, *J* =2.0 Hz, 5′-H), 6.90 (1H, dd, *J* =8.0, 2.0 Hz, 9″-H), 6.88 (1H, dd, *J* =8.0, 2.0 Hz, 9′-H), 6.74 (1H, d, *J* = 8.0 Hz, 8″-H), 6.73 (1H, d, *J* = 8.0 Hz, 8′-H), 6.27 (1H, d, *J* = 16.0 Hz, 2″-H), 6.19 (1H, d, *J* = 16.0 Hz, 2′-H), 5.68 (1H, dt, *J* = 10.0, 5.0 Hz, 3-H), 5.12 (1H, dd, *J* = 10.0, 3.0 Hz, 4-H), 4.35 (1H, dt, *J* =3.0, 2.5 Hz, 5-H), 2.29 (1H, dd, *J* =14.0, 3.0 Hz, 6-Hax),2.20 (2H, m, 2-H), 2.02 (1H, dd, *J* = 14.0, 6.0 Hz, 6-Heq). ^{13}C-NMR (Methanol-d_4) δ : 180.10 (s, $\underline{C}OOH$), 169.38 (s, C-1″), 169.26 (s, C-1′), 150.32 (s, C-7′ and C-7″), 148.37 (d, C-3″), 148.22 (d, C-3′), 147.46 (s, C-6′ and C-6″), 128.46 (s, C-4″), 128.40 (s, C-4′), 123.87 (d, C-9′ and C-9″), 117.20 (d, C-8′ and C-8″), 115.89 (d,C-5′ and C-5″), 115.59 (d, C-2″ and C-2′), 77.88 (s, C-1), 77.52 (d, C-4), 71.08 (d, C-5), 70.11 (d, C-3), 40.24 (t, C-2), 39.48 (t, C-6).

Compound **3** Light yellow powder, $[\alpha]_D$-199.5°(c=

0.17, MeOH). High-resolution FAB-MS : m/z 529.1376 $[M-H]^+$, calcd. for $C_{26}H_{25}O_{12}$ m/z 529.1346. ^{1}H-NMR (Methanol-d_4) δ : 7.56 (1H, d, J = 16.0 Hz, 3′-H), 7.54 (1H, d, J = 16.0 Hz, 3″-H), 7.03 (1H, d, J = 2.0 Hz, 5′-H), 7.02 (1H, d, J = 1.8 Hz, 5″-H), 6.91 (1H, dd, J = 8.0, 2.0 Hz, 9′-H), 6.88 (1H, dd, J = 8.0, 2.0 Hz, 9″-H), 6.76 (1H, d, J = 8.0 Hz, 8′-H), 6.73 (1H, d, J = 8.0 Hz, 8″-H), 6.26 (2H, d, J = 16.0 Hz, 2″-H and 2′-H), 5.62 (1H, dt, J = 5.0, 3.0 Hz, 5-H), 5.04 (1H, dd, J = 8.5, 3.5 Hz, 4-H), 4.32 (1H, td, J = 9.0, 8.5 Hz, 3-H), 3.76 (3H, s, $OC\underline{H}_3$), 2.35 (1H, dd, J = 15.0, 4.0 Hz, 6-Heq), 2.15(2H, br d, J = 8.5 Hz, 2-H), 2.12 (1H, dt, J = 15.0, 3.5 Hz, 6-Hax). ^{13}C-NMR (Methanol-d_4) δ : 176.91 (s, $\underline{C}OOCH_3$), 169.32 (s, C-1″), 169.23 (s, C-1′), 150.38 (s, C-7′ and C-7″), 148.19 (d, C-3′ and C-3″), 147.55 (s, C-6′ and C-6″), 128.52 (s, C-4″), 128.46 (s, C-4′), 124.03 (d, C-9″), 123.90 (d, C-9′), 117.26 (d, C-8′ and C-8″), 115.95 (d, C-5″ and C-5′), 115.80 (d, C-2″), 115.60 (d, C-2′), 76.33 (s, C-1), 75.94 (d, C-5), 70.63 (d, C-4), 66.80 (d, C-3), 53.78 (q, OCH_3), 42.12 (t, C-2), 37.57 (t, C-6).

Compound **4** Light yellow powder, $[\alpha]_D$-222.6° (c=0.15, MeOH). High-resolution FAB-MS : m/z 515.1204 $[M-H]^+$, calcd. for $C_{25}H_{23}O_{12}$ m/z 515.1190. ^{1}H-NMR (Methanol-d_4) δ : 7.61 (1H, d, J = 16.0 Hz, 3″-H), 7.58 (1H, d, J = 16.0 Hz, 3′-H), 7.08 (1H, d, J = 1.8 Hz, 5′-H). 7.06 (1H, d, J = 1.8Hz, 5″-H), 6.96 (1H, br d, J = 8.0, 9′-H), 6.95 (1H, br d, J = 8.0 Hz, 9″-H), 6.77 (1H, d, J = 8.0 Hz, 8′-H), 6.78 (1H, d, J = 8.0 Hz, 8″-H), 6.38 (1H, d, J = 16.0 Hz, 2″-H), 6.28 (1H, d, J = 16.0 Hz, 2′-H), 5.47 (1H, td, J = 9.0, 7.0 Hz, 3-H), 5.41 (1H, dt, J = 4.5, 3.5 Hz, 5-H), 3.94 (1H, dd, J = 9.0, 3.5 Hz, 4-H), 2.30 (1H, dd, J = 15.0, 4.0 Hz, 6-Hax), 2.18 (2H, m, 2-H), 2.12 (1H, dd, J = 15.0, 5.5 Hz, 6-Heq). ^{13}C-NMR (Methanol-d_4) δ : 180.37 (s, $\underline{C}OOH$), 169.93 (s, C-1″), 169.47 (s, C-1′), 150.26 (s, C-7′) 150.13 (s, C-7″), 147.83 (d, C-3′), 147.68 (d, C-3″), 147.46 (s, C-6′ and C-6″), 128.76 (s, C-4″), 128.58 (s, C-4′), 123.75 (d, C-9′ and C-9″), 117.23 (d, C-8′ and C-8″), 116.59 (d, C-2″), 116.10 (d, C-2′) 115.92 (d, C-5′ and C-5″), 76.33 (s, C-1), 74.33 (d, C-5), 73.02 (d, C-4), 72.78 (d, C-3), 40.18 (t, C-2), 37.66 (t, C-6).

Determination of DPPH radical scavenging activity : The scavenging effect corresponded to the intensity of quenching DPPH, as described by Hatano *et al.*[14] The sample solution (500 μl) was mixed with the same volume of 6×10^{-5}M DPPH solution and allowed to stand for 30 min. at room temperature. The absorbance was then measured at 520 nm. Sample and DPPH were dissolved in ethanol. 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 result is the mean of 4 measurements for each sample. Caffeic acid was used as a positive control (IC_{50}=5.6 μM).

Determination of superoxide anion radical scavenging activity : The superoxide anion radical scavenging activity was measured following the methods of Imanari *et al.*[15] by generating superoxide anions free radical in xanthine-XOD system. A reaction mixture composed of 700 μl of 0.05M 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 a 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 the 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 was less than 0.5 %. The result is the mean of 4 measurements for each sample. Caffeic acid was used as a positive control (IC_{50}=6.4 μM).

Nitrite formation measurement in LPS-induced murine macrophage as an assay of NO release : The murine monocyte-macrophage cell line J774.1, was obtained from the Japan Cancer Research Bank (JCRB, Tokyo, Japan). Cells were cultured in RPMI-1640 medium supplemented 10 % fetal bovine serum following the method of Sakaguchi *et al.*, 1995.[16] The amount of NO produced by LPS-induced J774.1 cells (5×10^5 cells/ml) was measured in terms of nitrite formation and the amount of LPS was 10 μg/ml. Cells were plated in 24 well cultured plates and allowed to adhere for 24 h. Thereafter, the medium was replaced with the fresh medium containing endotoxin and/or

drug and was incubated at 37°C in 5 % CO_2 for 48 h. The medium (500 μl) was taken out in a plastic tube and mixed with the equal volume of Griess reagent (1 % Sulphanilamide, 0.1 % naphtylethylenediamine dihydrochloride and 2.5 % H_3PO_4) and kept for 10 min. at room temperature. The color reaction was measured at 560 nm expressed the amount of nitrite by 5×10^5 cells/48 h. Cell viability was assessed by a MTT-based colorimetric assay.[17]

Statistical analysis : All values 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.

Results

Effect of propolis extracts on DPPH free radical scavenging activity

The results of the free radical scavenging effect of the PWE, PME and PCE on DPPH free radical system are shown in Fig. 2a. The PWE, PME and PCE showed a concentration-dependent activity. The free radical scavenging activity of PWE, PME and PCE was 62, 51 and 43 %, 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 generated in xanthine-XOD system

We evaluated the free radical scavenging activity on superoxide anion radical generated by an enzymatic method. The results of the free radical scavenging effect of the PWE, PME and PCE on superoxide anion rdical generated in xanthine-XOD system are shown in Fig. 2b. The PWE, PME and PCE showed a concentration-dependent activity as seen in DPPH system. In this system, the PWE, PME and PCE showed 59, 43 and 6 % of free radical scavenging activity, respectively at a concentration of 10 μg/ml and the PWE was found to be the most active extract as seen in DPPH system.

It became clear that the constituent(s) in the PWE might have a very effective free radical scavenging activity. In order to find out the chemical constituents having free radical scavenging activity, the active fraction, the PWE was subjected to the Sephadex LH-20 column chromatography as discussed in experimental section to obtain ten fractions. The results of DPPH free radical scavenging activity of these ten fractions obtained from the Sephadex LH-20 column chromatography are shown in Fig. 3. The activity was

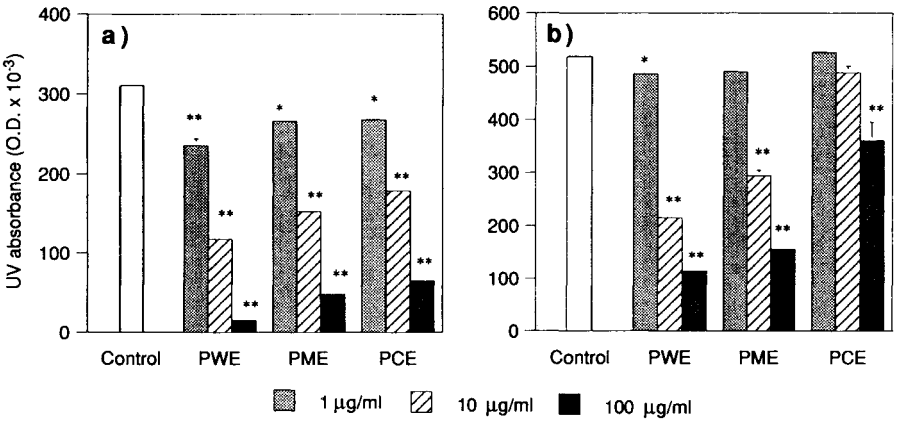

Fig. 2 Free radical scavenging effects of propolis extracts. a) The DPPH free radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing test sample and 30 mM of DPPH. b) Superoxide anion radical scavenging effect was measured by the absorbance of NBT at 560 nm in a reaction with superoxide generated in xanthine-XOD system. PWE : propolis water extract, PME : propolis MeOH extract and PCE : propolis $CHCl_3$ extract. Results are expressed as mean±S.D., n=4, $^{**}p<0.01$, $^{*}p<0.05$ vs. control.

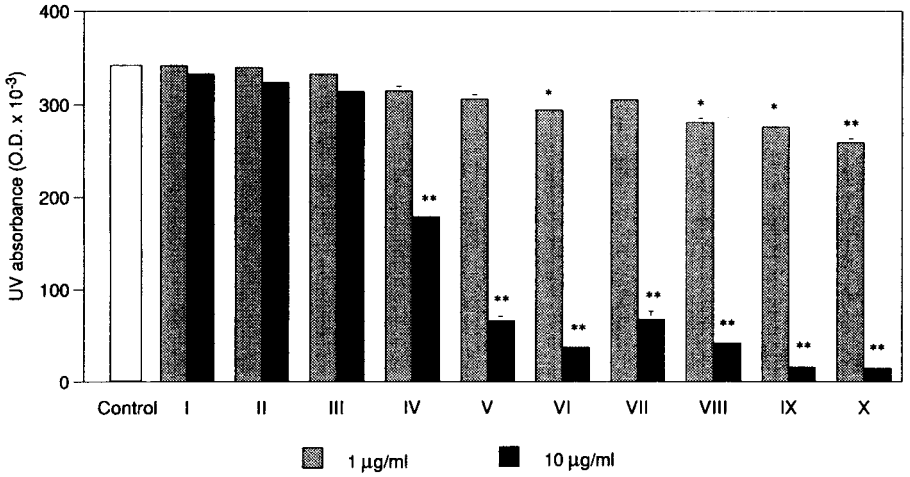

Fig. 3 The DPPH free radical scavenging effects of the fraction obtained from the water extract of propolis. The DPPH free radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing test sample and 30 mM of DPPH. Results are expressed as mean±S.D., n=12 (control) and n=4 (fraction), $^{**}p<0.01$, $^{*}p<0.05$ vs. control.

measured at a concentration of 1 and 10 μg/ml. Fraction X was found to be the most active fraction which decreased the DPPH free radical level by 67.5 % compared to the control. The active fraction (Fr. X) was again subjected to column chromatography to obtain four fractions (A, B, C and D). Fraction D was the most active fraction (data are not shown). Silica gel followed by RP-18 preparative TLC of fraction D led to the isolation of four dicaffeoyl quinic acid derivatives (**1, 2, 3,** and **4**) (Chart 1).

Structure determination

Compound **1** is a light yellow amorphous solid with $[\alpha]_D$ -210.7° (c=0.13, MeOH). High-resolution FAB-MS showed its molecular formula to be $C_{26}H_{26}O_{12}$ {m/z : 529.1349 $[M-H]^+$ Calcd 529.1346}. The ^{1}H-NMR, ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY led us to postulate the presence of two caffeoyl moieties, quinic acid group and an ester methyl group. The ^{1}H-^{13}C long-range COSY indicated the connectivites of these partial structures. The absolute configuration was confirmed by comparing the spectral data and optical rotation with that of previous studies [18] and was determined to be methyl 3,4-di-*O*-caffeoyl quinate (**1**).

Compound **2** is also a light yellow amorphous powder with $[\alpha]_D$-217.8°(c=0.13, MeOH). High resolution FAB-MS showed its molecular formula to be $C_{25}H_{24}O_{12}$ {m/z : 515.1207 $[M-H]^+$; Calcd 515.1190}. The ^{1}H-and ^{13}C-NMR spectra for **2** were identical to that of **1** except for the methyl signal of ester methyl group. Therefore, the structure of **2** was identified to be 3,4-di-*O*-caffeoyl quinic acid.[18]

Compound **3** is also a light yellow amorphous powder with $[\alpha]_D$-199.5° (c=0.17, MeOH). High resolution FAB-MS showed its molecular formula to be $C_{26}H_{26}O_{12}$ {m/z : 529.1376 $[M-H]^+$; Calcd 529.1346}. The ^{1}H-and ^{13}C-NMR spectra for **3** were also of similar pattern to that of **1** with regard to the substituents. The ^{1}H-NMR signals for C-4 and C-5 positions were shifted to the down field, which suggested that the caffeoyl groups were substituted at C-4 and C-5 positions on the quinic acid. Therefore, the structure of **3** was identified to be methyl 4,5-di-*O*-caffeoyl quinate.[18]

Compound **4** is also a light yellow amorphous powder with $[\alpha]_D$-222.6° (c=0.15, MeOH). High resolution FAB-MS showed its molecular formula to be $C_{25}H_{24}O_{12}$ {m/z : 515.1204 $[M-H]^+$; Calcd 515.1190}. The ^{1}H- and ^{13}C-NMR spectra for **4** were of similar pattern to that of **2** with regard to the substituents. The ^{1}H-NMR signals for C-3 and C-5 positions were found to be shifted to the down field suggesting the presence of caffeoyl groups at C-3 and C-5 positions on the quinic acid. Therefore, the structure of **4** was identified to be 3,5-di-*O*-caffeoyl quinic acid.[18]

DPPH Free radical scavenging activity of dicaffeoyl quinic acid derivatives

The results suggested that the PWE has very strong DPPH free radical and superoxide anion free radical scavenging activities. Therefore, DPPH free radical scavenging activity guided chemical analysis led to the isolation of **1, 2, 3** and **4**. The results of DPPH free radical scavenging activity of these compounds are shown in Table I. All these compounds showed a very significant DPPH free radical scavenging activity. It is the first report to find free radical scavenging activity of dicaffeoyl quinic acid derivatives. Among four isolates, **1** was the main compound in propolis and was also found to be the most potent which scavenged 83 % of DPPH free radical at a concentration of 10 μM.

Structure-activity relationship

In order to find structure-activity relationship, we compared the free radical scavenging activity of caffeic acid, quinic acid, chlorogenic acid with those of methyl 3,4-di-*O*-caffeoyl quinate (**1**) in both DPPH (Fig. 4a) and superoxide anion free radicals generated by xanthine-XOD systems (Fig. 4b). In both systems, caffeic acid, chlorogenic acid and methyl 3,4-di-*O*-caffeoyl quinate (**1**) showed very strong free radical scavenging activity, however, quinic acid did not show any significant activity. It is interesting to note that chlorogenic acid was more potent than caffeic acid and methyl 3,4-di-*O*-caffeoyl quinate (**1**) was the most potent in both systems.

Activity of some antioxidants

Next, we measured DPPH free radical of very commonly used antioxidants such as vitamin C, vitamin E and caffeic acid. Two compounds, dexamethasone and glycyrrhizin were also studied (Fig. 5) for comparison since these compounds are very strong hepatoprotective agents. Caffeic acid, vitamin C and vitamin E showed very strong free radical scavenging activity. Caffeic acid was the most potent of all although less than **1** (Fig. 4). The hepatoprotecting agent, dexamethasone and glycyrrhizin did not show free radical scavenging activity.

Nitrite formation in LPS-induced macrophage

The treatment of LPS, which induces acute endotoximia, caused an increase in NO production by the macrophage during the conversion of L-arginine to L-citruline. NO is a highly unstable radical so that it is rapidly converted to NO_2^- or NO_3^-. The amount of NO_2^- measured by Griess reagent is correlated to the NO production by macrophage. We studied the

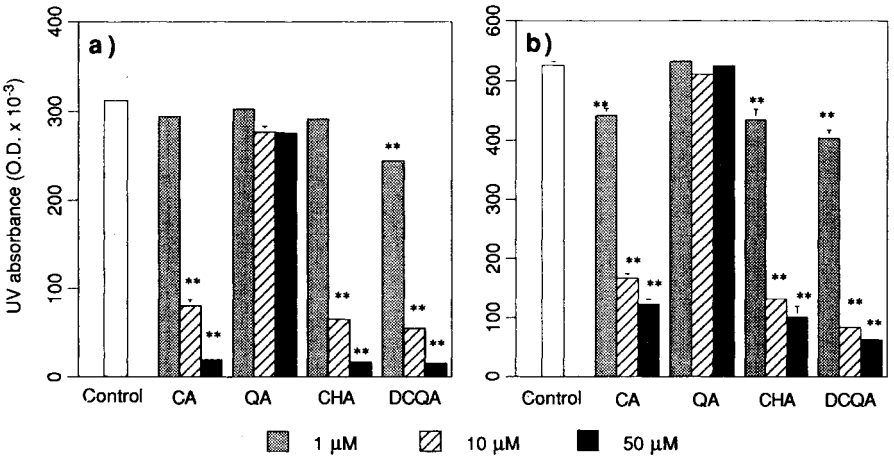

Fig. 4 Free radical scavenging effects of CA, CHA, QA and DCQA. a) The DPPH free radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing test sample and 30 mM of DPPH. b) Superoxide anion radical scavenging effect was measured by the absorbance of NBT at 560 nm in a reaction with superoxide generated in xanthine-XOD system. CA : Caffeic acid, CHA : Chlorogenic acid, QA : Quinic acid and DCQA : 3,4-Di-*O*-caffeoyl quinic acid (**1**), Results are expressed as mean±S.D., n=12 (control) and n=4 (compounds), $^{**}p<0.01$, $^{*}p<0.05$ vs. control.

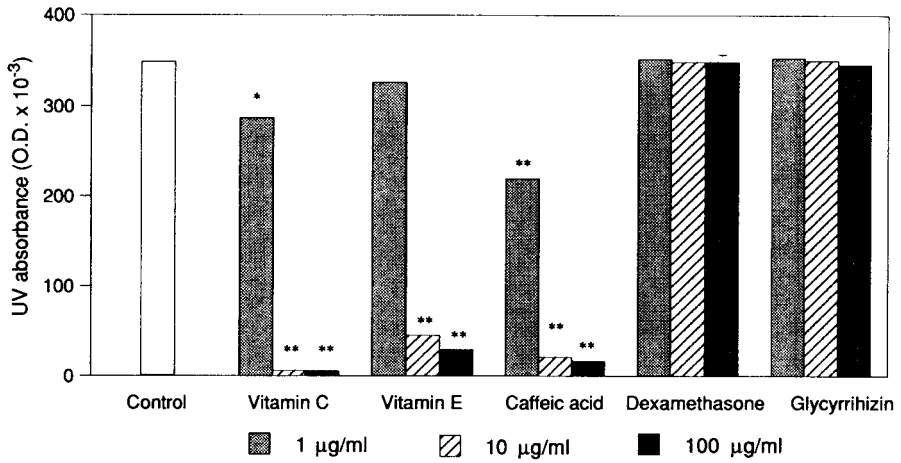

Fig. 5 DPPH free radical scavenging effects of vitamin C, vitamin E, caffeic acid, dexamethasone and glycyrrhizin. The DPPH free radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing test sample and 30 μM of DPPH. Results are expressed as mean±S.D., n=4, $^{**}p<0.01$, $^{*}p<0.05$ vs. control.

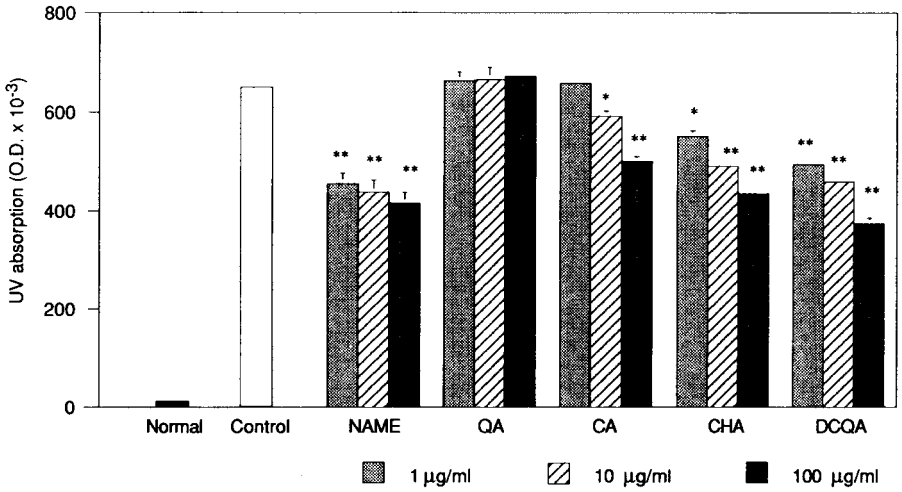

Fig. 6 Effects of NAME, QA, CA, CHA and DQCA on nitrite formation in the LPS-induced murine macrophage cell line J774.1. NAME ; L-N^{G}-Nitroarginine methyl ester, CA:Caffeic acid, CHA :Chlorogenic acid, QA :Quinic acid and DCQA : 3,4-Di-*O*-caffeoyl quinic acid (**1**), Results are expressed as mean±S.D., n=9 (control) and n=4 (compounds), $^{**}p<0.01$, $^{*}p<0.05$ vs. control.

nitrite formation in LPS-induced murine macrophage, J774.1 cells. NAME, caffeic acid, chlorogenic acid and methyl 3,4-di-*O*-caffeoyl quinate (**1**) significantly reduced the formation of nitrite on LPS-induced J774.1 cells in a concentration-dependent manner and **1** was found to be the most active (Fig. 6). In this assay also, quinic acid did not show any inhibitory activity. Cell viability observed by MTT method was more than 90 % at the maximum concentration of the samples.

Discussion

In our previous study,[13] five different samples collected in Brazil were studied in DPPH free radical system and superoxide generated in xanthine-XOD and PMS-NADPH systems. In the DPPH free radical systems, antioxidant directly reacts with DPPH radical. In xanthine-XOD and PMS-NADPH systems, superoxide is generated by enzymatically and nonenzymatically, respectively. The harmful effects of superoxide is reduced by SOD present in the animal body which gives a protective activity in the animal body. Propolis extract also showed similar activity to that of the SOD. The activity of propolis extracts in all these systems were similar trends to each other. So in the present study, DPPH free radical (chemical) and xanthine-XOD (enzymatic) systems were selected to find the active constituents present in the PWE. The PWE, PME and PCE, in both systems, showed a concentration - dependent free radical scavenging activity (Fig. 1). Among these extracts, the PWE showed the most potent activity (Fig. 2). These findings suggested that the PWE contains constituent(s) having very strong free radical scavenging activity.

The diabetogenic action of STZ has been illustrated as free radical mediated toxiciey particularly to β-cells, and cytokines IL-1 β and nitric oxide play a central role.[9,10] On the other hand, in CCl_4-induced liver injury, CCl_4 is first metabolized to $\cdot CCl_3$ by such metabolic enzymes as cytochrome P450 in the hepatocellular microsome.[12] 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-toxicity and CCl_4, respectively, in rats[8,11] might probably via free radical mediated pathway. With regards to these facts, the components in the PWE having free radical scavenging activity are supposed to be of great interest.

The active extract, the PWE was subjected to the Sephadex LH-20 column chromatography to obtain ten fractions and the last fraction was found the be the most active fraction in DPPH system (Fig. 3). The activity guided repeated column chromatography and preparative TLC led to the isolation of four dicaffeoyl quinic acid derivatives, **1**, **2**, **3** and **4** (Chart 1). These compounds were reported from Gradeniae Fructus[18b,c] and coffee beans[18d] but isolated for the first time from the propolis.

The DPPH free radical scavenging activity of

Table I The DPPH free radical scavenging activity of **1**, **2**, **3**, and **4** isolated from the water extract of propolis.

Group	Concentration (μM)	O.D.$\times 10^{-3}$ (mean±S.D.)	n
Control	–	327.7±1.3	12
Methyl 3,4 di-*O*-caffeoyl quinate (**1**)	1	244.3±1.9**	4
	10	55.3±2.8**	4
	50	15.7±3.5**	4
3,4-Di-*O*-caffeoyl quinic acid (**2**)	1	254.6±2.9**	4
	10	75.3±1.8**	4
	50	25.7±3.5**	4
Methyl 4,5-di-*O*-caffeoyl quinate (**3**)	1	251.7±1.6**	4
	10	66.1±2.1**	4
	50	21.7±1.5**	4
3,5-Di-*O*-caffeoyl quinate (**4**)	1	261.9±1.7**	4
	10	82.2±1.6**	4
	50	31.7±4.5**	4

The DPPH free radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing test sample and 30 mM of DPPH. Results are expressed as mean±S.D., $^{**}p<0.01$, vs. control.

these compounds were studied. All these four compounds (**1, 2, 3** and **4**) showed a very strong free radical scavenging activity (Table I). Two of these compounds are the methyl esters (**1** and **3**) and two of them are free acids (**2** and **4**). The position of caffeoyl group in quinic acid on these compounds differ from each other except in **1** and **2**. Methyl esters were found to be more potent than free acids. However, the position of the caffeoyl moiety in the quinic acid does not seem to be specific for the free radical scavenging activity.

In order to find structure-activity relationship, we studied the free radical scavenging activity of caffeic acid, quinic acid, chlorogenic acid and methyl 3,4 - di - *O* - caffeoyl quinate (**1**). These compounds showed similar trends of free radical scavenging activities in DPPH and xanthine - XOD generated superoxide radical systems (Fig. 5). Caffeic acid, chlorogenic acid and methyl 3,4 - di - *O* - caffeoyl quinate (**1**) showed very strong free radical scavenging activity, however, quinic acid alone did not show any effect in both systems. These results show that quinic acid alone does not have free radical scavenging activity but in combination with caffeic acid, the radical scavenging activity was found to be increased, since chlorogenic acid is more potent than caffeic acid. Methyl 3,4-di-*O*-caffeoyl quinate formed by the combination of two caffeoyl moieties with quinic acid showed the most potent activity.

We studied the free radical scavenging activity of vitamin C, vitamin E and caffeic acid which are very commonly used antioxidants. We also studied the free radical scavenging activity of glycyrrhizin and dexamethasone which are regarded as the antihepatotoxic agents. The results showed that vitamin C, vitamin E and caffeic acid were strong DPPH radical scavengers. Caffeic acid was found to be the most active while glycyrrhizin and dexamethasone did not show such activity. Hepatoprotective agents such as glycyrrhizin in CCl_4-induced liver injury were reported to be due to the inhibitory effects on LPO generation.[19] These results clearly show that the mechanism of hepatoprotective activity of caffeic acid and vitamin E must not be like those of glycyrrhizin and dexamethasone. In our experiment caffeic acid showed very strong free radical scavenging activity but was found to be less potent than **1** (Fig. 5).

The mechanism of liver disorder on LPS and its related hepatitis is unclear though a number of the pathophysiology of these immunologically mediated hepatitis have been investigated. The immunological liver injury model specially induced by LPS, several cytokines including IL-β, TNF-α, γ-IFN and NO from activated liver macrophage (Kupffer cells) leads to the death of hepatic parenchymal cells.[20] So immunosuppressive activity might be the cause of the hepatoprotective activity in such models. NO produced by the activated macrophage is involved in hepatitis.[21] In order to clarify the role of dicaffeoyl quinic acid derivative from propolis in the immunosuppressive activity, we studied inhibitory effect of nitrite formation. Methyl 3,4-di-*O*-caffeoyl quinic acid significantly reduced nitrite formation in LPS-induced murine macrophage, J774.1 (Fig. 6). It is interesting to note that dicaffeoyl quinic acid derivative was more potent than caffeic acid or chlorogenic acid in the immunomodulation.

Free radical mediates several biological functions such as aging, cancer, diabetes, hepatitis, inflammation and other chronic diseases and the free radical scavenger are regarded to be the potential cure for these diseases.[22] With this background, these active compounds from PWE could be applicable to clinical hepatitis or preventive action of early diabetes, however, further study is needed.

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和文抄録

プロポリスのクロロホルム，メタノール，水エキスについて，DPPH フリーラジカルと Xanthine-XOD によるスーパーオキシドアニオンの消去作用について検討した。その結果，水エキスに最も強い活性を認め，4 種のキナ酸誘導体（methyl 3,4-di-*O*-caffeoyl quinate (**1**), 3,4-di-*O*-caffeoyl quinic acid (**2**), methyl 4,5-di-*O*-caffeoyl quinate (**3**), 3,5-di-*O*-caffeoyl quinic acid (**4**)）を活性物質として単離，構造解析した。これらのキナ酸誘導体は，抗酸化剤のビタミン C や E，及びカフェイン酸よりもフリーラジカル消去作用が強いことが判っ

た。しかし，キナ酸 (**5**) には，このような消去作用は認められなかった。さらに，化合物 (**1, 2, 3, 4**) は，クロロゲニン酸 (**6**) やカフェイン酸よりもラジカル消去作用が強いことが判った。

また，単離したこれらのジカェイン酸誘導体には，LPSで誘発したマウス由来マクロファージJ774.1のNO産生を著しく抑制することも判明した。

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