Four Di-*O*-caffeoyl Quinic Acid Derivatives from Propolis.[1] Potent Hepatoprotective Activity in Experimental Liver Injury Models

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The water extract of propolis (PWE) showed a strong hepatoprotective activity against CCl_4-toxicity in rats and D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced liver injury in mice. The PWE also showed a significant hepatoprotective activity against CCl_4-induced liver cell injury in cultured rat hepatocytes. The *in vitro* hepatoprotective activity guided fractionation and chemical analysis led to the isolation of four dicaffeoyl quinic acid derivatives from the PWE. The structure of these isolates was 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 spectroscopic methods. These compounds were more potent hepatoprotective agents than glycyrrhizin at a concentration of 10 μg/ml and 1 was the most potent among the four compounds in the cultured hepatocytes. Quinic acid (5) alone did not show hepatoprotective effects in cultured rat hepatocytes against CCl_4-toxicity. On the other hand, chlorogenic acid (6) or caffeic acid alone was found to be less potent than the dicaffeoyl quinic acid derivatives.

Key words propolis; hepatoprotective effect; dicaffeoyl quinic acid; carbon tetrachloride; D-galactosamine-lipopolysaccharide

Propolis is a sticky plant substance that is collected by bees which may include different types of secretions or exudates.[2] 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 people believe that it can cure inflammation, heart diseases and even diabetes and cancer. Chemical analysis of propolis is still far from satisfactory, however, 150 polyphenolic compounds including flavonoids and cinnamic acid derivatives have been reported from propolis using GC-mass analysis.[3]

Several biological attributes such as anticancer,[4-6] antioxidant,[7,8] antiinflammatory,[9] and antibiotic[10,11] activities have been reported for propolis and its constituents. We also evaluated the quality of propolis collected at different places in Brazil based on free radical scavenging activities.[12] The hepatoprotective activity of an alcoholic extract of propolis against chemically induced liver injury in rats has recently been reported.[13] In our study, the water extract of propolis (PWE) showed a significant hepatoprotective activity in both chemical and immunological liver injury models. These findings suggested that PWE contains active constituents to protect against chemical and immunological hepatitis. The *in vitro* hepatoprotective activity guided fractionation and chemical analysis led to the isolation of four dicaffeoyl quinic acid derivatives from the PWE. In this paper, we wish to present full details of 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 and their 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, 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 correlation spectroscopy (COSY), ^{1}H–^{13}C COSY, ^{1}H–^{13}C long-range COSY) were measured using JEOL standard pulse sequences.

Carbon tetrachloride (CCl_4) and D-galactosamine (D-GalN) were obtained from Wako Pure Industry, Osaka, Japan. Lipopolysaccharide (LPS; *E. coli* 055: B5) was purchased from Difco Laboratories, U.S.A. Serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels in rats, blood GPT level in mice and GOT level in the medium of cultured hepatocytes were measured by Reflotron S system (Boeringer Mannheim Co., Ltd., Osaka, Japan). Serum lactate dehydrogenase (LDH) level in rats was measured with LDH monotest (Boeringer Mannheim Co., Ltd., Osaka, Japan) using the UV spectrophotometer. Hanks' balanced salt solution (HBSS), ethylene glycol-*O*,*O*′-bis (2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), trypsin inhibitors and collagenase (Wako Pure Industry, Osaka, Japan) and William's E medium, bovine serum albumin (BSA), insulin, dexamethasone and gentamycin (Sigma, St. Louis, U.S.A.) were used. Hepatocytes cultured in collagen type I-coated 24 well plastic micro plates were from Iwaki Glass, Japan.

Extraction and Isolation Propolis (1.8 kg) collected from Brazil was treated with distilled water (2 l × 2) and kept at 80 °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 (2 l × 2) under the reflux condition for 2 h which gave the methanol extract

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(331.65 g) after evaporation and lyophilization. The residue was again extracted with chloroform (2 l × 2) to obtain chloroform extract (315.80 g) after evaporation. Thus obtained water, methanol (PME) and chloroform (PCE) extracts were used for animal and *in vitro* experiments.

The PWE showed a significant hepatoprotective activity both in *in vivo* and *in vitro*; 81.45 g was then subjected to Sephadex LH-20 column chromatography (7 × 65 cm) and eluted with the water-methanol gradient system to obtain ten fractions. The eluted solvent 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). Fraction 10 showed a very strong hepatoprotective activity against CCl_4 toxicity. 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 one showing the hepatoprotective activity (data not shown). A portion (250 mg) of fraction D was again purified by 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 = 16.0 Hz, 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 = 2.0 Hz, 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-H_{ax}), 2.27 (2H, m, 2-H), 2.12 (1H, dd, J = 14.0, 6.0 Hz, 6-H_{eq}). ^{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, OCH_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-H_{ax}), 2.20 (2H, m, 2-H), 2.02 (1H, dd, J = 14.0, 6.0 Hz, 6-H_{eq}). ^{13}C-NMR (methanol-d_4) δ: 180.10 (s, COOH), 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, OCH_3), 2.35 (1H, dd, J = 15.0, 4.0 Hz, 6-H_{eq}), 2.15 (2H, br d, J = 8.5 Hz, 2-H), 2.12 (1H, dt, J = 15.0, 3.5 Hz, 6-H_{ax}). ^{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 = 1.8 Hz, 5″-H), 6.96 (1H, br d, J = 8.0 Hz, 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-H_{ax}), 2.18 (2H, m, 2-H), 2.12 (1H, dd, J = 15.0, 5.5 Hz, 6-H_{eq}). ^{13}C-NMR (methanol-d_4) δ: 180.37 (s, COOH), 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).

Animals Male Sprague-Dawley (SD) rats, 6 weeks of age, weighing 150—170 g were used for the CCl_4-induced liver injury model. Male ddY mice, 6 weeks of age, weighing 30—32 g were used for the D-GalN/LPS-induced liver injury model. All animals were purchased from Shizuoka Laboratory Animal Center, Japan, and maintained under 12 h light/dark cycle in a temperature and humidity controlled room. The animals were fed with a

laboratory pellet chow (Clea Japan Inc., Tokyo, Japan; protein 24.0%, lipid 3.5%, carbohydrate 60.5%) and given water *ad libitum*.

Hepatocytes Isolation Liver parenchymal cells of SD rat were isolated by collagenase perfusion method according to the procedure of Seglen.[14] The portal vein of rat liver was exposed and canulated with a teflon catheter. The liver was perfused with Ca^{2+}-free HBSS containing 1% BSA and 0.5 mM EGTA which was aerated with 95% O_2/5% CO_2 at 37 °C. Liver was perfused for 10 min with HBSS at a flow rate of 30 ml/min. Then circulation was started with solution containing Ca^{2+}-free HBSS, 0.075% collagenase, 4 mM $CaCl_2$ and 0.005% trypsin inhibitor. Isolated hepatocytes (2×10^5 cells/ml) were cultured with William's E medium supplemented with 10% calf serum, 50 mg/l gentamycin, 1 mM dexamethasone and 10 nM insulin under 5% CO_2 at 37 °C in 24 well collagen I-coated plates.

CCl_4-Induced Hepatocytes Injury *in Vitro* CCl_4-induced hepatocytes injury assay followed the procedure of Kiso *et al.*[15] After pre-culture for 24 h, the hepatocytes were exposed to the fresh medium containing 10 mM CCl_4 and/or various concentrations of drug samples. At 60 min after CCl_4 challenge, GOT concentrations in the medium were measured as an indicator of hepatocytes necrosis.

CCl_4-Induced Liver Injury *in Vivo* Liver injury was induced by CCl_4 in rat according to the literature.[16,17] In each group 7 rats were used. After 12 h fasting a mixture of CCl_4 in olive oil (1 : 1) was injected s.c. at a dose of 4 ml/kg. Propolis extracts were administered at a dose of 200 mg/kg, *p.o.*, three times at 24, 12 and 1 h before CCl_4 challenge. At 24 h after CCl_4 injection, blood samples were collected and serum enzyme levels (GOT, GPT and LDH) were measured which were considered the parameter for the extent of liver damage.

D-GalN/LPS-Induced Liver Injury Liver injury was induced by D-GalN/LPS according to the method described by Tiegs *et al.*[18] with 10 or 11 mice used in each group were used. After 12 h fasting mice were injected i.p. with 700 mg/kg of D-GalN and 10 μg/kg of LPS. The PWE was administered twice at doses of 200 and 100 mg/kg, *p.o.*, 18 and 2 h before D-GalN/LPS injection. The blood GPT level was examined to measure the extent of liver damage at 8 h after D-GalN/LPS injection.

Statistical Analysis All values expressed as mean ± S.E. were obtained from *n* number of experiments. 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 AND DISCUSSION

Effect of Propolis Extracts on CCl_4-Induced Liver Injury in Rats The results of hepatoprotective effect of the PWE, PME and PCE on CCl_4-induced liver injury in rats are shown in Fig. 1. In CCl_4-treated control, serum GOT, GPT, and LDH levels were 1132 ± 140, 395 ± 56, and 353 ± 51 U/l, respectively at 24 h after CCl_4 administration. In contrast, in the PWE pretreated group, GOT (481 ± 88), GPT (157 ± 19) and LDH (247 ± 28 U/l) were found to be significantly decreased, while PME and PCE did not show a significant hepatoprotective activity. Serum parameters suggested that the PWE has a significant protective activity against CCl_4-induced hepatitis.

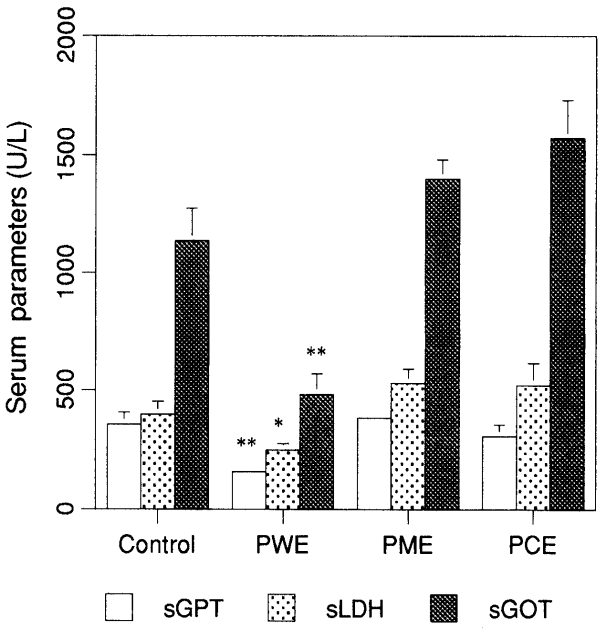


Fig. 1. Hepatoprotective Effect of Propolis Extracts on CCl_4-Induced Liver Injury in Rats

PWE, propolis water extract; PME, propolis MeOH extract; and PCE, propolis $CHCl_3$ extract. Each extract was administered 200 mg/kg, *p.o.*, 3 times. Results are expressed as mean ± S.E., $n=7$, $**p<0.01$, $*p<0.05$ *vs.* control.

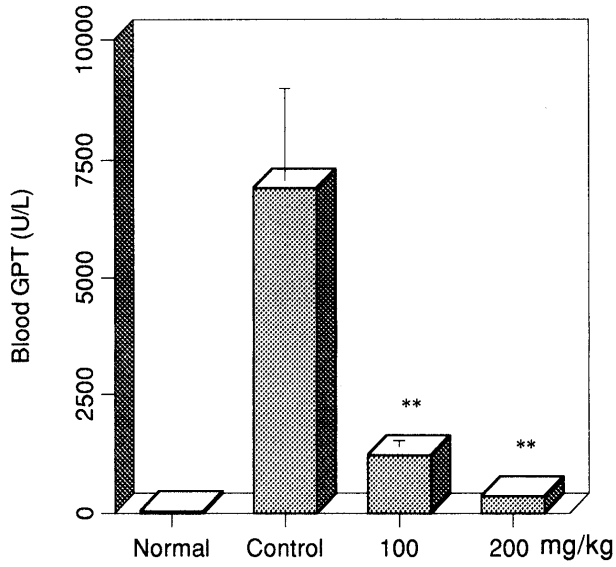


Fig. 2. Hepatoprotective Effect of Propolis Water Extract (PWE) on D-GalN/LPS-Induced Liver Injury in Mice

Drug was administered *p.o.* 2 times at 18 and 2 h before D-GalN/LPS administration. Results are expressed as mean ± S.E., $n=10$ or 11, $**p<0.01$ *vs.* control.

Effect of Propolis Extracts on D-GalN/LPS-Induced Liver Injury in Mice The hepatoprotective effect of PWE was evaluated in immunological liver injury in mice. The extent of liver damage was expressed in terms of GPT level. In the control group, GPT level rapidly increased to 6901 U/l at 8 h after administration of D-GalN/LPS, while GPT levels in 200 and 100 mg/kg, *p.o.* of the PWE-treated group were 355 and 1204 U/l, respectively.

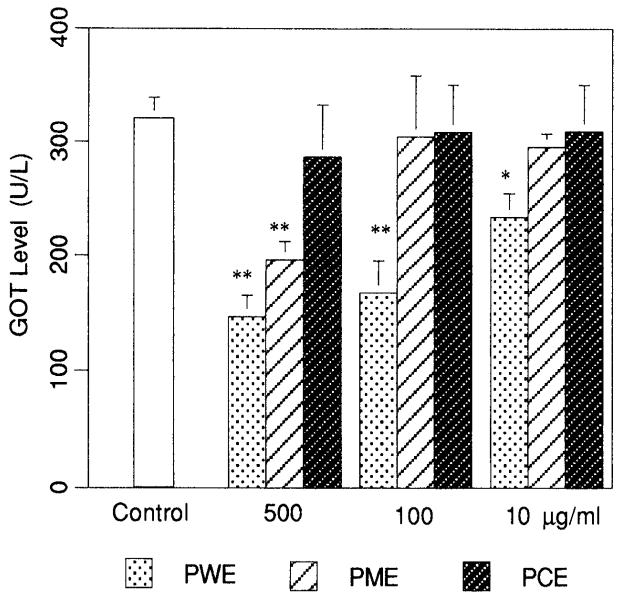


Fig. 3. Protective Effect of Propolis Extracts on CCl_4-Induced Injury on Cultured Rat Hepatocytes

PWE, propolis water extract; PME, propolis MeOH extract; and PCE, propolis $CHCl_3$ extract. Results are expressed as mean ± S.E., $n=4$, $**p<0.01$, $*p<0.05$ *vs.* control.

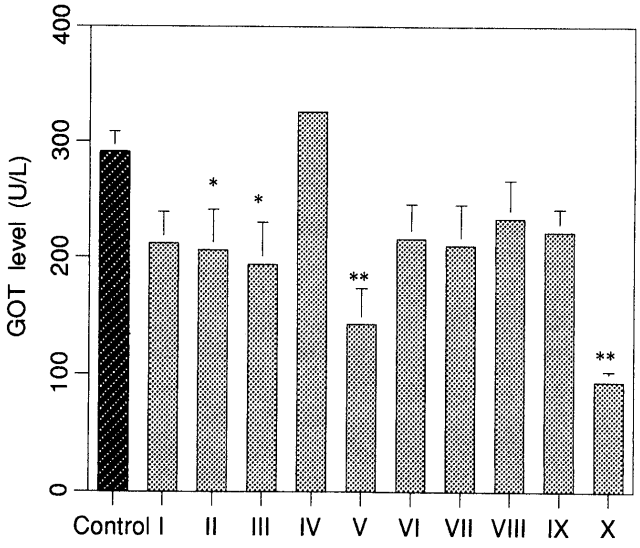


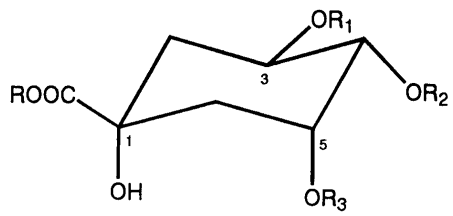
Fig. 4. Protective Effect of Fractions (I—X) from the Water Extract of Propolis (PWE) on CCl_4-Induced Injury in Cultured Rat Hepatocytes

Each fraction was used at a concentration of 100 μg/ml with CCl_4 and the GOT level in the medium was measured at 1 h after drug and/or CCl_4 treatment. Results are expressed as mean ± S.E., $n=4$, $**p<0.01$, $*p<0.05$ *vs.* control.

The results in Fig. 2 indicate that the PWE showed a dose dependent protective activity against D-GalN/LPS-induced liver injury in mice.

Effect of Propolis Extracts on CCl_4-Induced Injury in Cultured Rat Hepatocytes It was recognized that the constituent(s) in the PWE might be effective hepatoprotective agent(s) in both chemically and immunologically induced hepatitis. To identify the hepatoprotective chemical constituents, we performed the *in vitro* assay according to the methods of Kiso *et al.*[15] The results of hepatoprotective activity against CCl_4-induced injury of the PWE, PME and PCE extracts on cultured rat hepatocytes are shown in Fig. 3. The extent of liver cell injury was expressed in terms of GOT levels measured in the medium by the treatment of CCl_4. The GOT level of the control group was 320 ± 19 U/l at 1 h after CCl_4 treatment. The PCE did not show any significant hepatoprotective activity even at a concentration of 500 μg/ml. In the group treated with 500 μg/ml of the PME, GOT level was 196 ± 17 U/l but at the lower concentration of the PME no significant activity was observed. The GOT levels were 146 ± 19, 167 ± 28, 234 ± 21 U/l in the PWE treated group at a concentration of 500, 100 and 10 μg/ml, respectively. These results clearly indicated that PWE was the most active extract and its activity was found to be dose dependent.

Fractionation and *in Vitro* Hepatoprotective Activity PWE showed a significant hepatoprotective activity both in *in vivo* and *in vitro* assays. So it was subjected to Sephadex LH-20 column chromatography as mentioned before to obtain ten fractions. The results of the *in vitro* hepatoprotective activity against CCl_4-toxicity of these ten fractions are shown in the Fig. 4. Fraction X was found to be the most active fraction which decreased the GOT level by 67.5% compared to the control. From the active fraction (fr. X) four dicaffeoyl quinic acid derivatives (**1**, **2**, **3**, and **4**) were isolated (Chart 1). Various dicaffeoyl quinic acid derivatives have been reported but the complete NMR signal assignments of **1**, **2**, **3**, and **4** were not found in the literature so the assignment of these signals was undertaken.



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

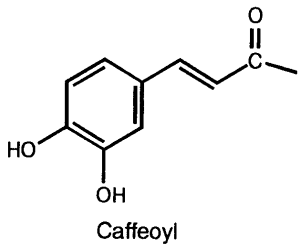


Chart 1

Structure Determination 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 a ester methyl group. The ^{1}H–^{13}C long-range COSY indicated the connectivities of these partial structures. The absolute configuration was confirmed by comparing the spectral

data and optical rotation with that of previous studies[19-23] and was determined to be methyl 3,4-di-*O*-caffeoyl quinate (**1**).

Compound **2** is also a light yellow amorphous powder. 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, **2** was identified as 3,4-di-*O*-caffeoyl quinic acid.[19-23]

Compound **3** is also a light yellow amorphous powder. The ^{1}H- and ^{13}C-NMR spectra for **3** also showed a similar pattern for the substituents to that of **1**. The ^{1}H-NMR signals for C-4 and C-5 positions were shifted to the downfield, which suggested that the caffeoyl groups were substituted at these positions on the quinic acid. Therefore, **3** was identified as methyl 4,5-di-*O*-caffeoyl quinate.[19-23]

Compound **4** is also a light yellow amorphous powder. The ^{1}H- and ^{13}C-NMR spectra for **4** had a 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 these positions on the quinic acid. Therefore, **4** was identified as 3,5-di-*O*-caffeoyl quinic acid.[19-23]

Hepatoprotective Activity of Dicaffeoyl Quinic Acid Derivatives The results suggested that PWE has liver protective action against CCl_4, D-GalN/LPS-induced liver injury animal models. A hepatoprotective activity guided *in vitro* experiment led to the isolation of **1**, **2**, **3**, and **4**. The results of the hepatoprotective activity of these compounds are shown in Table 1; all showed a significant hepatoprotective activity in cultured rat hepatocytes against CCl_4-toxicity. This is the first report to identify the hepatoprotective activity of dicaffeoyl quinic acid, which is more effective than glycyrrhizin. We also studied the hepatoprotective activity of quinic acid, caffeic acid, dihydrocaffeic acid and chlorogenic acid (Table 1). Quinic acid alone did not show any significant hepatoprotective activity; caffeic acid and dihydrocaffeic acid showed a significant activity but both were less active than chlorogenic acid. This study suggests that the caffeoyl group plays an important role in showing hepatoprotective activity and that the presence of quinic acid enhances the activity. It is interesting to note that the activity of dicaffeoyl quinic acid derivatives were more potent than those of caffeic acid, dihydrocaffeic acid or chlorogenic acid.

In CCl_4-induced liver injury, CCl_4 is first metabolized to $\cdot CCl_3$ by cytochrome P450 in the hepatocellular microsome. This highly reactive radical injures the hepatocytes and its organelles by a direct physicochemical effect: 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 an injury that culminates in necrosis and steatosis.[24] Hepatoprotective effect of glycyrrhizin and gomisin A in CCl_4-induced liver injury were due to the inhibitory effects on lipid peroxide (LPO) generation.[25,26] The study of ESR showed that free radicals are generated by the addition of CCl_4 (10 mM) in the cultured medium of rat hepatocytes.[25] PWE showed a very strong free radical scavenging activity which we reported previously[12] so the hepatoprotective activity of propolis is probably due to radical scavenging activity.

Table 1. Protective Effect of the Constituents from Propolis against CCl_4-Induced Injury in Cultured Rat Hepatocytes

Compound	Concentration (μg/ml)	GOT level (%)
Normal	—	6.1±0.2
Control	—	100.0±9.0
Methyl 3,4-di-*O*-caffeoyl quinate (**1**)	100 (188.7 μM)[a]	28.0±4.0**
	10 (18.9 μM)	50.8±5.0**
	1 (1.9 μM)	61.0±7.0**
3,4-Di-*O*-caffeoyl quinic acid (**2**)	100 (193.8 μM)	32.0±4.0**
	10 (19.4 μM)	61.5±7.0**
	1 (1.9 μM)	66.0±5.0**
Methyl 4,5-di-*O*-caffeoyl quinate (**3**)	100 (188.7 μM)	62.4±3.3**
	10 (18.9 μM)	75.6±5.6**
	1 (1.9 μM)	88.3±6.8*
3,5-Di-*O*-caffeoyl quinic acid (**4**)	100 (193.8 μM)	36.6±5.0**
	10 (19.4 μM)	58.6±4.5**
	1 (1.9 μM)	70.2±3.6**
Quinic acid (**5**)	100 (520.8 μM)	97.5±3.5
	10 (52.1 μM)	101.2±4.5
Chlorogenic acid (**6**)	100 (282.5 μM)	36.5±6.2**
	10 (28.3 μM)	59.8±2.1**
Caffeic acid	100 (555.6 μM)	46.5±5.6**
	10 (55.6 μM)	88.9±2.3*
Dihydrocaffeic acid	100 (549.5 μM)	45.3±5.6**
	10 (54.9 μM)	72.5±4.5**
Glycyrrhizin	10 (12.2 μM)	60.3±7.3**
	1 (1.2 μM)	80.9±7.8*

The GOT levels in the medium were measured at 1 h after administration of drugs at various concentrations and/or CCl_4 treatment in the cultured rat hepatocytes (2×10^5 cells/ml). The GOT levels of CCl_4 treated control were 286—315 U/l and the results are expressed as mean±S.E., $n=4$. The GOT levels were expressed as % regarding the control value as 100%. ** $p<0.01$, * $p<0.05$ *vs.* control. *a*) The drug concentration used was calculated in μM and is shown in parenthesis.

The administration of LPS to animals can cause severe metabolic and physiological disturbances leading to death. A common observation in such a septic shock is the acute liver failure. It has been established that macrophage (Kupffer cell) and its secretions such as cytokines, superoxide and nitric oxide (NO) mediate the action of LPS.[27,28] D-GalN is an inhibitor of protein biosynthesis by uridine trapping,[29] and its effect was so specific in liver lesion that sensitivity of hepatocytes for LPS greatly increased because of the inhibition of acute phase protein induction.[30-32] Therefore, administration of both D-GalN and a very small amount of LPS can induce fulminant hepatitis through an immunological pathway mediated by TNF (tumor necrosis factor)-α.[33,34] When the animals are challenged with a normally innocuous amount of endotoxin, huge quantities of TNF-α are produced from these infiltrating mononuclear cells, resulting in liver injury.[35] Increases of serum TNF-α levels have been found in patients suffering from fulminant hepatic failure, chronic hepatitis B virus infection and alcoholic hepatitis.[36-38] Glycyrrhizin has been widely used against viral hepatitis clinically and very effectively lowers the serum enzyme levels. In our previous study,[39] glycyrrhizin at a dose of 100 mg/kg, i.p. lowered the GPT levels by 47% in CCl_4-induced liver injury in rats. It also lowered the GPT levels by 65% at a dose of 200 mg/kg, i.p. administration in D-GalN/LPS-induced liver injury in mice. Comparison of the hepatoprotective effect of PWE

to that of glycyrrhizin in the previous report[39] seems to show PWE as the more effective hepatoprotective agent since it lowered the GPT levels by 55% at a dose of 200 mg/kg, *p.o.* in CCl_4-induced liver injury in rats and 95% at a dose of 200 mg/kg, *p.o.* in D-GalN/LPS-induced liver injury in mice. The results of *in vitro* experiment also clearly showed that compounds **1**, **2**, **3**, and **4** were more potent than glycyrrhizin.

In our investigation, PWE protected chemical and immunological liver injuries induced by CCl_4 and D-GalN/LPS respectively. It could be applicable to clinical hepatitis, although further study is necessary. The active constituents were found to be the dicaffeoyl quinic acid derivatives (**1**, **2**, **3** and **4**). The detailed mechanism of action and study of these compounds in other animal models are in progress in our laboratory.

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