Hepatoprotective Compounds from *Canarium album* and *Euphorbia nematocypha*

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Successive purification of the extract from *Canarium album* and *Euphorbia nematocypha*, guided by antihepatotoxic activity in primary cultured rat hepatocytes, led to the isolation of brevifolin (1), hyperin (2), ellagic acid (3) and 3,3′-di-*O*-methylellagic acid (4) as hepatoprotective compounds. Compounds 1, 3 and 4 also reduced carbon tetrachloride (CCl_4)-induced liver damage in mice. The hepatoprotective activities of 1, 2, 3 and 4 *in vitro* and *in vivo* are apparently due to their antioxidative effects, which were exhibited by further studies using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and CCl_4-induced lipid peroxidation systems.

Keywords brevifolin; hyperin; ellagic acid; 3,3′-di-*O*-methylellagic acid; hepatoprotective activity; hepatotoxicity; antioxidative activity; radical scavenger; lipid peroxidation

Canarium album is widely distributed in southern China. It's fruit and roots have been used as Chinese medicine to treat poisoning, diarrhea and dermatitis.[2)] *Euphorbia nematocypha* also grows in southern China. The roots have been used as Chinese medicine to treat external injury.[3)] Neither plant reportedly has been used to treat hepatitis.

In the course of our screening program for hepatoprotective compounds in crude plant extracts, we found that the ethanol extract of *C. album* and *E. nematocypha* significantly protected primary cultured rat hepatocytes against D-galactosamine (GalN) cytotoxicity. Earlier, we reported the isolation of ursane and oleanane types of hepatoprotective triterpens from the *n*-hexane fraction of *C. album*.[4)]

Here we report the isolation and characterization of the hepatoprotective compounds from *E. nematocypha* and acidic ethylacetate (EtOAc) fraction of *C. album*, as well as the antihepatotoxic activity and antioxidative activity of the purified compounds.

Isolation and Identification of the Active Compounds Antihepatotoxic activity was found to be distributed in *n*-hexane and acidic EtOAc fractions of *C. album*. The EtOAc fraction was separated into MeOH-soluble (fr. A) and MeOH-insoluble (fr. B) parts. Fraction A was chromatographed on a silica gel column, followed by a Sephadex LH-20 column and preparative high performance liquid chromatography (HPLC) to give **1** and **2** with hepatoprotective action.

Compound **1**, $C_{12}H_8O_6$ (*m/z* 248: M^+), ultraviolet (UV) spectrum λ_{max} nm 278, 350, 364, the proton nuclear magnetic resonance (^{1}H-NMR) spectra δ 2.55, 3.20, 7.30, was identified as brevifolin[5)] by direct comparison with an authentic sample.

Compound **2** showed characteristic UV absorption to flavonols. Adding shift reagents[6)] gave bathochromic shifts to 3-*O*-substituted quercetin (see Experimental). Gas chromatography-mass spectrometry (GC/MS) of trimethylsilyl derivatives after hydrolysis of **2** revealed the presence of galactose. In the ^{1}H-NMR spectrum, the anomeric proton signal at δ 5.32 (1H, d, J=7 Hz) led to the assignment of the anomeric configuration of the galactose unit as to be β. So compound **2** was identified as quercetin-3-*O*-β-D-galactoside (hyperin).[7)]

From fr. B, hepatoprotective compound **3** was obtained as a major constituent. Compound **3**, $C_{14}H_6O_8$ (*m/z* 302: M^+), UV spectrum λ_{max} nm 256, 363, ^{1}H-NMR spectra δ 7.18 (2H, s), was identified as an ellagic acid by direct

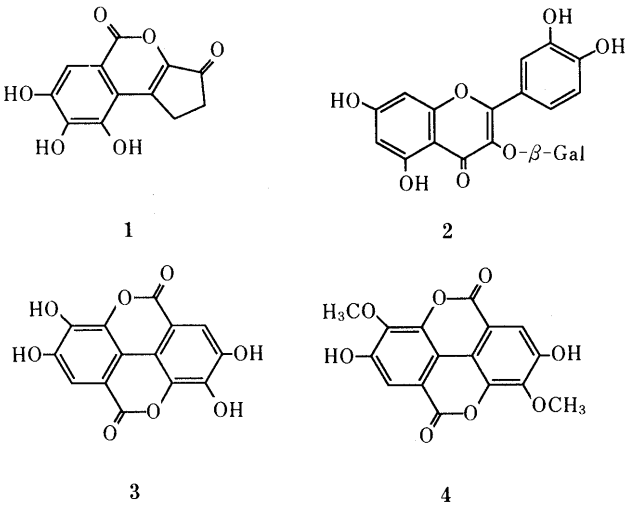

TABLE I. Effect of **1**, **2**, **3** and **4** on GalN-Induced Hepatotoxicity in Primary Cultured Rat Hepatocytes

Compound	Dose (μg/ml)	GPT (%)
Control	—	100.0±0
NKK-105	3	104.7±4.2
	10	93.0±1.2[a)]
	30	84.9±3.1[b)]
DDB	3	87.2±3.1[b)]
	10	60.4±2.0[a)]
	30	53.9±6.0[a)]
1	10	89.5±2.0[a)]
	30	95.4±2.0
	100	84.9±5.1[b)]
2	3	96.9±8.3
	10	79.1±1.2[a)]
	30	62.8±3.5[a)]
3	3	87.2±4.2[b)]
	10	58.9±3.3[a)]
	30	44.6±1.8[a)]
4	3	67.0±0.6[a)]
	10	38.0±1.8[a)]
	30	19.0±1.4[a)]

Significantly different from control (n=3, means±S.D.): *a*) $p<0.001$; *b*) $p<0.01$.

TABLE II. Effect of **1**, **3** and **4** on CCl_4-Induced Liver Damage in Mice

Compound	Dose (mg/kg)	Route	GPT (%)	GOT (%)
Control	—	*p.o.*	100.0±12.1	100.0± 7.8
NKK-105	100	*p.o.*	9.1± 8.2[a]	5.0± 4.0[a]
1	10	*p.o.*	88.4±23.9	74.1±35.1
	30	*p.o.*	86.7±20.9	71.2±23.2[b]
	100	*p.o.*	101.5±26.7	91.9±38.9
3	100	*p.o.*	63.0±23.9[b]	49.1±26.9[a]
4	100	*p.o.*	96.6±39.3	104.9±63.8
Control	—	i.p.	100.0±53.7	100.0±56.4
NKK-105	100	i.p.	8.7± 8.9[a]	7.4± 7.8[a]
1	10	i.p.	68.6±51.6	69.1±53.0
	30	i.p.	32.1±30.9[b]	33.4±36.3
	100	i.p.	1.7± 0.7[a]	3.3± 1.1[a]
3	100	i.p.	5.9± 3.3[a]	4.9± 2.4[a]
4	100	i.p.	32.6±35.8	31.1±33.3

Significantly different from control ($n=8$, means±S.D.): *a*) $p<0.001$; *b*) $p<0.01$.

TABLE III. Antioxidative Activity of **1**, **2**, **3** and **4**

Compound	Radical scavenging effect		Effect on CCl_4-induced lipid peroxidation	
	Compound (M) / DPPH (M)	DPPH (%)	Concentration (M)	MDA formation
Control	—	100.6±1.6	—	100.0±12.0
α-Tocopherol	1	11.0±0.6[a]	10^{-3}	52.0± 7.3[b]
	10	13.0±0.2[a]		
1	1	8.5±0.1[a]	10^{-5}	50.7± 8.8[b]
	10	5.1±0.2[a]	10^{-4}	30.8± 0.9[a]
2	1	28.5±6.4[a]	10^{-5}	73.5± 0.5
	10	56.0±0.6[a]	10^{-4}	37.3± 7.6[b]
3	1	20.3±0.8[a]	10^{-5}	46.7± 6.2[b]
	10	13.6±0.2[a]	10^{-4}	36.7± 1.9[a]
4	1	90.9±0.6[a]	10^{-5}	22.8± 0.6[a]
	10	30.3±1.7[a]	10^{-4}	10.5± 0.6[a]

Significantly different from control ($n=8$, means±S.D.): *a*) $p<0.001$; *b*) $p<0.01$.

comparison with an authentic sample.

The hepatoprotective fraction of *E. nematocypha* was purified (see Experimental) to give compound **4**. Compound **4**, $C_{16}H_{10}O_8$ (*m/z* 330: M^+), showing a UV spectrum similar to that of **3**, was identified as 3,3′-di-*O*-methylellagic acid by comparing their ^1H- and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra.[8]

Hepatoprotective Activity Table I shows antihepatotoxic activity of **1**, **2**, **3** and **4** against GalN-induced cytotoxicity in primary cultured rat hepatocytes. Diisopropyl 1,3-dithiol-2-ylidene malonate (NKK-105) and dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxy-biphenyl-2,2′-dicarboxylate (DDB), which have been used clinically for hepatitis in Japan and China respectively, were used as reference substances.

2, **3** and **4**, as well as reference substances, in doses of 3—30 μg/ml had a dose-related ameliorative effect on the release of alanine aminotransferase (GPT). **1** also showed some effect in doses of 10—100 μg/ml. **4** was the most effective of all the compounds.

1, **3**, **4** and NKK-105 were investigated for *in vivo* hepatoprotective activity. In preliminary experiments, there was no elevation of the level in serum GPT and aspartate aminotransferase (GOT) in the GalN-injected mice. So CCl_4 was used as the hepatotoxin. As shown in Table II, the intraperitoneal administration of **1**, **3** and **4** was protective against liver damage, but in the orally treated groups compound **3** exhibited hepatoprotective activity. Among these compounds intraperitoneally administered **1** significantly reduced the elevation in serum GPT and GOT levels. The hepatoprotective effect of **2** could not be evaluated because it occurred only in limited amounts.

Antioxidative Activity We also investigated a radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH)[9] and an inhibitory effect on microsomal lipid peroxidation induced by CCl_4. The results are summarized in Table III. **1**, **2**, **3** and **4**, as well as α-tocopherol used as a reference, exhibited both a radical scavenging effect and an inhibitory effect on lipid peroxidation. The order was as follows: the radical scavenging effect (**1**>**3**>**2**>**4**), the inhibitory effect on lipid peroxidation (**4**>**1**=**3**>**2**).

Discussion

We first isolated the hepatoprotective compounds, **1**, **2** and **3** from *C. album* and **4** from *E. nematocypha*.

This is the first report of the *in vitro* hepatoprotective activity of **1** and **4** and the *in vivo* activity of **1**, **3** and **4**, while the *in vitro* hepatoprotective activity of **2** and **3** was reported earlier.[10]

CCl_4-induced liver injury is caused by lipid peroxidation of the endoplasmic reticulum membranes initiated by the reactive radicals ($CCl_3\cdot$) of CCl_4.[11] This prompted us to investigate the antioxidative effect of the isolated compounds.

Radical scavenging was much stronger in **1** and **3** than in **4** (Table III). This result was closely associated with the protective effect of these compounds on CCl_4-induced liver damage *in vivo* (Table II). Apparently the phenolic hydroxy groups play an important role in these effects.

The order (**4**≫**3**=**1**) of the inhibitory effect on lipid peroxidation induced by CCl_4 (Table III) was similar to that of the hepatoprotective effects *in vitro* (Table I), in contrast to the radical scavenging effects. This indicates that the process of hepatotoxicity induced by GalN on cultured hepatocytes may be related to lipid peroxidation, while GalN injury reportedly attributes to a deficiency of uridine triphosphate resulting from the trapping of uridylate by the metabolites of GalN.[12] The greater effectiveness of **4** than **3** and **1**, against CCl_4-induced lipid peroxidation as well as GalN-induced hepatotoxicity, must be ascribable to the uncertain mechanism rather than the radical scavenging effect.

Experimental

UV spectra were measured with a Hitachi 220A spectrophotometer. NMR spectra were recorded with a Varian XL-200 (200 MHz for ^1H-NMR) or JEOL JNM-GX400 (400 MHz for ^1H-NMR and 100 MHz for ^{13}C-NMR) and the chemical shifts are given in δ value with tetramethylsilane as an internal standard. The electron impact mass spectra (EIMS) were determined on a Hitachi M-80A mass spectrometer at 70 eV.

Isolation of 1, 2 and 3 from *C. album* The EtOH extract from the dried stem and leaf of *C. album* was partitioned between H_2O and *n*-hexane, as described previously.[4]

The aqueous fraction was extracted with EtOAc at pH 3.0. The EtOAc fraction (21.7 g) was extracted with saturated aqueous $NaHCO_3$ (pH 9.0). The aqueous layer was reextracted with EtOAc after acidification to afford

an acidic fraction (10.0 g).

The acidic fraction was dissolved in hot $CHCl_3$–MeOH–H_2O (30 : 10 : 1, 75 ml) to give the soluble fraction (fr.A) and the insoluble fraction (fr. B). Fraction A was chromatographed on silica gel (Merck, Kieselgel 60, Art 7734, 300 g) and eluted with $CHCl_3$–MeOH–H_2O mixtures, (90 : 10 : 1) to (30 : 15 : 1). The fraction ($CHCl_3$–MeOH–H_2O, 30 : 10 : 1, 680 mg) with hepatoprotective activity was further purified by Sephadex LH-20 (Pharmacia, MeOH) and preparative HPLC (Nucleosil $5C_{18}$, 10 mm i.d. × 250 mm, 17.5% MeCN in 0.1% H_3PO_4, 5 ml/min, 50 °C, 256 nm) to afford **1** (retention time (t_R) = 7 min, 4.7 mg) as yellow flakes and **2** (t_R = 12 min, 4.6 mg) as a yellow powder.

Fraction B was dissolved in MeOH and concentrated to small volume (15 ml) at 50 °C *in vacuo* to give **3** (550 mg) as a yellow powder.

Brevifolin (**1**): UV λ_{max}^{MeOH} nm: 278, 350, 364. EI-MS *m/z*: 248 (M^+), 220, 192, 164. ^{1}H-NMR (DMSO-d_6) δ: 2.55 (2H, m), 3.20 (2H, m), 7.30 (1H, s). ^{13}C-NMR (DMSO-d_6) δ: 23.8 (t), 33.0 (t), 107.9 (d), 112.6 (s), 115.3 (s), 140.9 (s), 141.6 (s), 144.3 (s), 144.9 (s), 149.4 (s), 160.6 (s), 195.5 (s).

Hyperin (**2**): UV λ_{max}^{MeOH} nm 258, 361; +NaOAc, 270, 380; +NaOAc/H_3BO_3, 265, 385; +$AlCl_3$, 274, 305 (sh), 430; +$AlCl_3$/HCl, 268, 305 (sh), 363 (sh), 402; +NaOMe, 275, 411. ^{1}H-NMR (DMSO-d_6) δ: 5.32 (1H, d, J = 7 Hz), 6.11 (1H, d, J = 2 Hz), 6.32 (1H, d, J = 2 Hz), 6.78 (1H, d, J = 8 Hz), 7.52 (1H, d, J = 2 Hz), 7.65 (1H, dd, J = 2, 8 Hz).

Ellagic Acid (**3**): UV λ_{max}^{EtOH} nm: 256, 363. EI-MS *m/z*: 302 (M^+), 246, 190, 162. ^{1}H-NMR (2% NaOD) δ: 7.18 (2H, s). ^{13}C-NMR (2% NaOD) δ: 99.6 (s), 110.2 (d), 114.4 (s), 138.3 (s), 158.9 (s), 162.0 (s), 166.9 (s).

Isolation of 4 from *E. nematocypha* The EtOH extract (1 g) from the dried roots of *E. nematocypha* was suspended in hot MeOH (20 ml). The MeOH-soluble fraction was concentrated to a small volume (5 ml) to give a precipitate (73 mg). The precipitate was suspended in H_2O and washed with *n*-hexane. The aqueous fraction (40 mg) was purified by preparative HPLC (Nucleosil $5C_{18}$ 20 mm i.d. × 250 mm, 40% MeCN, 9.9 ml/min, 50 °C, 215 nm) to afford **4** (t_R = 10—12 min, 3.6 mg) as a yellow powder.

3,3′-Di-*O*-methylellagic Acid (**4**): UV λ_{max}^{EtOH} nm: 247, 286 (sh), 358, 375. EI-MS *m/z*: 330 (M^+), 259, 287, 315. ^{1}H-NMR (DMSO-d_6) δ: 4.04 (6H, s), 7.52 (2H, s). ^{13}C-NMR (DMSO-d_6) δ: 60.9 (q), 111.5 (d), 111.7 (s), 112.1 (s), 140.3 (s), 141.2 (s), 152.6 (s), 158.6 (s).

Assay of Antihepatotoxic Activity (*in Vitro*) Antihepatotoxic activity in primary cultured rat hepatocytes was studied using GalN as a toxin, as described earlier,[4] with little modification. Williams' medium E was used instead of Eagle's MEM and the concentration of GalN was changed to 0.5 mM.

Assay of Antihepatotoxic Activity (*in Vivo*) *In vivo* antihepatotoxicity was evaluated using CCl_4-induced liver damage in mice. The animals used were male ICR mice weighing 25—30 g and they received food and water *ad libitum*. The suspension of test compounds in 5% gum arabic was administered orally or intraperitoneally. CCl_4 (0.03 ml/kg) was administered orally in a volume of 1 ml olive oil per 100 g of body weight 1 h after the drug administration. Blood was obtained from the femoral vein 18 h after CCl_4 administration. Serum GPT and GOT were measured with a Hitachi 7150 automatic analyzer.

Assay of Antioxidative Activity 1) DPPH Method[9]: The radical scavenging effect was determined by adding 5.0×10^{-4} M DPPH dissolved in EtOH (100 μl) to a test compound dissolved in DMSO (100 μl). The mixture was incubated for 10 min at room temperature. The residual DPPH was determined by absorbance at 510 nm.

2) Lipid Peroxidation of Rat Liver Microsome: The microsomal lipid peroxidation induced by CCl_4 was determined by the method of Kornbrust and Mavis.[13] Microsomes were prepared from the liver of Wistar rat according to the method of Kamataki and Kitagawa.[14] The reaction mixture contained 1 ml of the microsomal fraction, 0.1 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 1.5 mM glucose-6-phosphate, 2.5 mM nicotine amide, 1.25 mM $MgCl_2$, phosphate buffered saline, 5 mM CCl_4 (50 μl EtOH) and a test compound (50 μl DMSO) in a final volume of 2 ml.

After incubation at 37 °C for 60 min under N_2, the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid solution. Malondialdehyde (MDA) was measured by the thiobarbituric acid method.[15]

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References and Notes

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