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Antihepatotoxic Zeaxanthins from the Fruits of *Lycium chinense*

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A $CHCl_3$: MeOH extract of the fruit of *Lycium chinense* Mill. (Solanaceae) was found to afford significant protection against carbon tetrachloride-induced toxicity in primary cultures of rat hepatocytes. Subsequent activity-guided fractionation resulted in the isolation of zeaxanthin and zeaxanthin dipalmitate as antihepatotoxic components. Incubation of injured hepatocytes with zeaxanthin dipalmitate reduced the levels of glutamic pyruvic transaminase (GPT) and sorbitol dehydrogenase (SDH) released from damaged cells to 60.5% and 76.3% of those released from untreated controls, respectively. Zeaxanthin also reduced the levels of GPT and SDH to 68.5% and 61.3% of the levels of those released from the untreated control. The results confirm the hepatoprotective activities of zeaxanthins. Antihepatotoxic activities of zeaxanthins are comparable to that of silybin.

Key words : Antihepatotoxic activity, *Lycium chinense* Mill. (Solanaceae), Zeaxanthin, Zeaxanthin dipalmitate

INTRODUCTION

In a contiunation of our research seeking for antihepatotoxic compounds from the fruit of *Lycium chinense* (Solanaceae) which showed significant antihepatotoxic activity against CCl_4-induced toxicity in hepatocytes, the $CHCl_3$:MeOH extract of the fruits of this herb was subjected to fractination to reveal the active components. The dried ripe fruits of *L. chinense* have been used as a tonic in Oriental medicine. A number of neutral volatile, steroidal, alkaloidal compounds and betaine are known as constituents of the fruit of this plant (Sannai *et al.*, 1983; Nishiyama, 1963; Itoh *et al.*, 1978; Maldoni, 1984). This fruit also has been ascribed as having antihypertensive activity, inhibitory effects on the development of fatty liver and the ability to reduce the content of sugar in the blood (Kukokawa, 1962). To date, however, no precise correlation has been made between a particular constituent of this plant and an observed pharmacological activity.

The hexane fraction of the $CHCl_3$:MeOH extract of the fruits was found to contain hepatoprotective components together with EtOAc fraction. Subsequent activity guided fractionation of EtOAc fraction using a screening system consisting of primary cultures of rat hepatocytes damaged by CCl_4 resulted in the isolation of a new cerebroside, 1-O-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-(*N*-palmitoyl)-octadecasphinga-4,8-diene, which showed antihepatotoxic activity and a known cerebroside, 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-(2'-hydroxypalmitoyl)-octadecasphinga-4,8-diene (Kim *et al.*, 1997).

In the present communication, we report the isolation of the polyene alcohols, zeaxanthin and zeaxanthin dipalmitate from the hexane fraction of the $CHCl_3$:MeOH extract of the fruits which possess antihepatotoxic activity in cultured rat hepatocytes exposed to a known hepatotoxin, CCl_4.

MATERIALS AND METHODS

Plant material

Fruits of *L. chinense* were purchased from Chungyang Agricultural Cooperatives Federation in Korea and identified by Dr. Dae S. Han, *Professor Emeritus,* College of Pharmacy, Seoul National University. Voucher specimen documenting this purchase has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

General experimental procedures

^1H- and ^{13}C-NMR spectra were run on a Jeol GSX 400 spectrometer in $CDCl_3$ with TMS as an internal standard at 400 MHz and 100 MHz, respectively. IR spectra were recorded on Perkin Elmer 1710 spectrophotometer. EIMS peaks were obtained on VG Trio-

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2 mass spectrometer. Fabmass peaks were obtained on VG 70-VSEQ mass spectrometer. TLC was carried out on silica gel plates (Merck, Silica gel GF254). Zones were visualized by spraying with anisaldehyde/H_2SO_4 and then heating.

Isolation of compounds 1 and 2

Dried plant material (40 kg) was extracted with a mixture of $CHCl_3$:MeOH (3:1) for 6 hr with refluxing 3 times. An extract (2.8 kg) was yielded upon the removal of the solvent *in vacuo*. The extract was then suspended in water and partitioned with *n*-hexane to yield *n*-hexane extract (30 g) upon evaporation. The *n*-hexane extract was chromatographed on silica gel column (600 g, 230~400 mesh, column size 5.0×100 cm) eluting with a stepwise gradient of petroleum ether:EtOAc (100:1) to (1:1) to give 75 1/fraction. The active fractions from the petroleum ether:EtOAc (30:1) eluates were combined and evaporated to dryness. The residue (500 mg) was further chromatographed on Sephadex LH 20 (30 g, column size 1.5×50 cm) using petroleum ether as an eluent. Compound **1** (40 mg) was purified by repeated recrystallization such as dissolving in benzene and crystallizing with the addition of MeOH. Compound **2** (10 mg) was crystallized from ethanol as luster orange-red plates.

Compound 1: orange colored needle, m.p. 98.5~99.5°C, $[\alpha]^{D}_{25}$-44° (c 0.1, $CHCl_3$), R_f=0.37 (solvent system of petroleum ether:EtOAc (30:1)), $C_{72}H_{116}O_4$; U.V. (petroleum ether) λ_{max} (nm): 452 (log ε 5.10), 480 (log ε 5.04); IR (KBr) ν (cm^{-1}); 1735 (ester bond), 1460, 1438, 1418 (C=C), 1350~1180; Fabmass (*m/z*); 1045 [M+H], 788 [M^+-$C_{16}H_{32}O_2$], 696 [M^+-$C_{16}H_{32}O_2$-C_7H_8]; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.60 (m, H-2, ax), 1.79 (m, H-2, eq), 1.08 (s, H-16, eq), 1.11 (s, H-17, ax), 2.12 (dd, H-4, ax), 2.44 (dd, H-4, eq), 1.72 (s, H-18), 1.97 (s, H-19, 20), 5.10 (m, H-3, ax), 6.0~6.7 (m, vinyl proton), 0.87 (t, *J*=6 Hz, terminal CH_3), 1.26 (s), 2.28 (t, *J*=7.2 Hz), ppm; ^{13}C-NMR ($CDCl_3$, 100MHz) see Table I. Compound **1** was identified as zeaxanthin dipalmitate by physical and spectral data comparison with those of published values (Clccio and Castro, 1984).

Compound 2: m.p. 207~216°C, $[\alpha]^{D}_{25}$ -25° (c 0.1, dioxane), $C_{40}H_{56}O_2$, U.V. (petroleum ether) λ_{max} (nm): 450 (log ε 5.10), 482 (log ε 5.07); IR (KBr) ν (cm^{-1}) 3600 (OH), 1610 (C=C), 965 (trans C=C); EIMS (*m/z*), 568 [M^+, 10%], 550 [M^+-H_2O, <1%], 532 [M^+-$2H_2O$, <1%], 476 [M^+-92, 1%], 440 [M^+-H_2O-92], 119 (8%), 105 (10%), 91 (100%); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.00 (s, 12H), 1.72 (s, 6H), 1.90 (s, 12H), 2.34 (dd, 2H), 3.93 (br.s, OH), 6.0~6.6 (m, vinyl proton), ppm; ^{13}C-NMR ($CDCl_3$, 100 MHz) see Table I. Compound **2** was identified as zeaxanthin by physical and spectral data comparison with those of published values (Bartlett *et al.*, 1969; Bonnett *et al.*, 1969).

Table I. ^{13}C-NMR chemical shifts and assignments for zeaxanthin dipalmitate, zeaxanthin, zeaxanthin diacetate and palmitic acid (solvent; $CDCl_3$).

	zeaxanthin dipalmitate	zeaxanthin	zeaxanthin diacetate	palmitate
1	36.70	37.1	36.73	
2	44.11	48.4	44.24	
3	68.12	65.1	68.47	
4	38.51	42.5	38.57	
5	125.67	126.2	125.66	
6	137.64	137.6	138.03	
7	125.32	125.6	125.36	
8	138.66	138.5	138.73	
9	135.57	135.7	135.55	
10	131.44	131.3	131.52	
11	125.89	124.9	124.96	
12	137.87	137.6	137.74	
13	136.45	136.5	136.46	
14	132.64	132.6	132.68	
15	130.09	130.1	130.18	
16	28.51	28.7	28.58	
17	30.03	30.3	30.07	
18	21.48	21.6	21.40	
19	12.80	12.8	12.75	
20	12.74	12.8	12.75	
α	173.58		170.63	180.50
β	34.72		21.39	34.14
γ	25.04			24.19
	29.69			29.70
	29.46			29.62
	29.36			29.46
	29.27			29.39
	29.13			29.09
	31.92			31.95
	22.69			22.72
	14.11			14.12

Culture of hepatocytes and carbon tetrachloride exposure

Hepatocytes were isolated from Wistar male rats by the method of Berry and Friend (1984) with minor modifications (Berry *et al.*, 1991; Lee *et al.*, 1995).

The cell suspension was diluted to 5.0×10^5 cells/ml in the Waymouth MB 752/l medium supplemented with 5% fetal bovine serum, 2.0 mg/ml bovine serum albumin, 10^{-6} M dexamethasone, 10^{-7} M insulin, 5.32×10^{-2} M L-serine, 4.09×10^{-2} M L-alanine, 2.67×10^{-2} M $NaHCO_3$, 100 IU/ml penicillin, 100 IU/ml streptomycin and 5 μg/ml amphotericin B. Cells were inoculated onto collagen-coated plastic culture dishes and were incubated at 37°C in a humidified incubator gassed 5% CO_2. One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to a medium containing 10 mM CCl_4/ethanol for 1.5 hr to induce hepatic injury (Kiso *et al.*, 1983).

Screening of antihepatotoxic activity in carbon tetrachloride exposure

In order to screen the antihepatotoxic activity of zeaxanthins, they were dissolved in 5% Tween 80. One day after plating, the cultured cells were exposed to culture medium containing 10 mM CCl_4/ethanol with zeaxanthins. One and half hours after the CCl_4 challenge, culture medium was removed and the levels of GPT and SDH were determined. The morphology of the cells was also examined by phase-contrast microscopy.

Measurement of glutamic pyruvic transaminase activity

The activity of GPT in the culture medium was determined by the method of Reitman-Frankel (1957) using an assay kit (YoungDong Pharm. Co., Seoul, Korea).

Measurement of sorbitol dehydrogenase activity

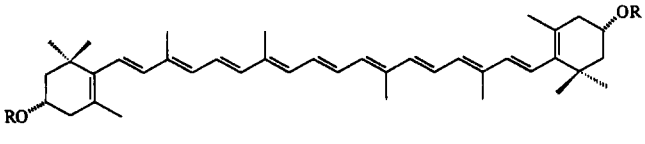
The activity of SDH in the culture medium was determined by the method of Gerlach (1965).

Statistical analysis

The statistical significance was determined by an "ANOVA" test.

RESULTS AND DISCUSSION

A $CHCl_3$:MeOH extract of the fruit of *Lycium chinense* showed significant antihepatotoxic activity in cultured rat hepatocytes intoxicated with CCl_4. This extract was suspended in water and partitioned with *n*-hexane. The *n*-hexane extract was chromatographed on silica gel and then on Sephadex LH 20 yielding compound **1** and **2** (Fig. 1) as active antihepatotoxic ingredients. Compound **1** was obtained as an orange colored needle crystals. The UV spectrum consists of two absorption maxima at 452 nm (log ε 5.10) and 480 nm (log ε 5.04) in petroleum ether. Characteristic features of the UV spectrum presented the conjugated double bond and β-ionone. Its IR spectrum indicated the presence of an ester bond (1735 cm^{-1}), a conjugated double bond (1460, 1438, 1418 cm^{-1}) and long aliphatic chain groups (1350~1180 cm^{-1}).



R : Compound **1** ; $OC_{16}H_{32}$
2 ; H

Fig. 1. Structure of compound **1** and **2**.

The molecular formula was determined to be $C_{72}H_{116}O_4$ by Fabmass (observed *m/z* 1044). Its mass fragment pattern [*m/z* 788, 696] suggested the presence of a palmitic acid ($C_{16}H_{32}O_2$) and a toluene (C_7H_8). The ^{1}H-NMR spectrum exhibited methyl proton signals at δ 1.07 and δ 1.11 (s, C-1, C-1'), δ 1.72 (s, C-5, C-5'), μ 1.96 (s, C-9, C-9', C-13, C-13') and δ 5.07 (m, C-3, C-3'). The resonances of δ 6.1~δ 6.7 (m, C-2, C-2', C-4, C-4') indicated vinyl protons, δ 0.87 (s) terminal methyl protons. It also exhibited the presence of methylene in the palmitate (δ 1.25), and also methylene adjacent to carbonyl carbon (δ 2.28, *J*=7.21 MHz). The ^{13}C-NMR spectrum was compared with that of zeaxanthin diacetate. Compound **1** was identified as zeaxanthin dipalmitate by comparison of its spectral data with those of published values (Moss, 1976; Goowin and Britton, 1988). The UV and MS spectrum of compound **2** also showed a typical pattern of carotenoids. The UV spectrum consists of two absorption maxima in 450 nm (log ε 5.07) and 482 nm (log ε 5.10) in petroleum ether. Its mass fragment pattern [*m/z*, 119, 105, and 91] is typical for most carotenoids (Bonnett *et al.*, 1969). However, its IR spectrum did not show any peak of an ester bond and long alipathic chains that exist in compound **1**. EIMS spectrum indicated the presence of two hydroxyl groups and a toluene (C_7H_8). The ^{13}C-NMR spectrum was compared with that of zeaxanthin diacetate

Table II. Effects of zeaxanthins on the activities of GPT and SDH released from CCl_4-intoxicated primary cultured rat hepatocytes

	μM	GPT[c] (%)	SDH[c] (%)
Control[a]		00.0±2.3	10.0±2.9
Reference[b]		0.0±1.3	0.0±6.9
zeaxanthin-	0.1	5.3±1.1	0.0±2.2
dipalmitate	1.0	34.5±4.3*	57.6±2.6*
	10.0	60.5±4.0*	76.3±1.5*
zeaxanthin	0.1	46.2±3.8*	8.7±4.7
	1.0	53.1±3.1*	57.3±15.3*
	10.0	68.5±3.1*	61.3±3.1*
silybin	10.0	73.8±6.9*	62.7±6.9*

One day after plating, isolated rat hepatocytes were exposed to the medium containing either 10 mM CCl_4/ethanol or 10 mM CCl_4/ethanol+test samples in 5% Tween 80. Ninety minutes after the CCl_4 challenge, culture medium was removed and the levels of GPT and SDH in the medium were determined (n=3).

[a]*Control* is the value for hepatocyte cultures not challenged with CCl_4. Control values for GPT and SDH averaged 10.8± 0.5 IU/L and 2.8±0.1 Unit/ml, respectively.

[b]*Reference* is the value for untreated hepatocytes challenged with CCl_4. Reference values for GPT and SDH averaged 50.8 ±9.2 IU/L and 48.8±0.2 Unit/ml, respectively.

[c]The percent protection is calculated as 100×(GPT or SDH value of reference minus GPT or SDH value of sample)/(GPT or SDH value of reference minus GPT or SDH value of control). Differs significantly from the control; $p<0.01$*

as well. Compound 2 was identified as zeaxanthin on the basis of its spectral data compared with those of published data (Bartlett *et al.*, 1969; Bonnett *et al.*, 1969).

After the isolation of zeaxanthins, their antihepatotoxic activities were assessed by measuring preventive effects on the release of GPT and SDH into the medium of primary cultures of hepatocytes treated with CCl_4. Zeaxanthin as well as zeaxanthin dipalmitate markedly blocked the release of both GPT and SDH from CCl_4-intoxicated primary cultured rat hepatocytes in a dose dependent manner over the concentration ranges from 0.1 μM to 10 μM. (Table II) The results indicated that the presence of palmitic acid did not affect the antihepatotoxic activity of zeaxanthins. The antihepatotoxic activity of zeaxanthins is comparable to that of silybin, a well known hepatoprotective compound (Table II).

Our results suggest that zeaxanthins have significant hepatoprotective activity against CCl_4-induced hepatotoxicity in primary cultured rat hepatocytes. As such, this compound might hold significant therapeutic value in the prevention or treatment of liver disease. Further studies are necessary to evaluate the antihepatotoxic activity of zeaxanthins *in vivo*.

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