Preventive Effect of Taraxasteryl Acetate from *Inula britannica* subsp. *japonica* on Experimental Hepatitis *in vivo*

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Abstract

The survival rate for acute hepatic failure induced by *Propionibacterium acnes* and lipopolysaccharide (LPS) was increased when a hot water extract from the flowers of *Inula britannica* L. subsp. *japonica* Kitam. was injected into the experimental hepatitis mice, and anti-hepatitis substances could be extracted with $CHCl_3$. The $CHCl_3$ extract from *I. britannica* was fractionated and anti-hepatitis fractions IB-3-2 and IB-3-3 were obtained. IB-3-3 had the most potent anti-hepatitis activity among the fractions but further purification of the active compound was not achieved because of the low yield. IB-3-2 contained only one substance which was identified to be taraxasteryl acetate by ^1H- and ^{13}C-NMR and MS. Taraxasteryl acetate showed potent preventive activity against acute hepatic failure induced by *P. acnes* and LPS in a dose-dependent manner, however deacetylation and modification of the olefinic bonds significantly decreased the anti-hepatitis activity of taraxasteryl acetate. Taraxasteryl acetate also inhibited the increment of plasma transaminase on acute hepatic failure induced by carbon tetrachloride (CCl_4) or D-galactosamine. From a histological study it appeared that degeneration and necrosis, which were observed in the liver from CCl_4 mice, were not found in the liver cells from taraxasteryl acetate treated mice. These results indicates that taraxasteryl acetate shows preventive effects on experimental hepatitis caused by either immunologically induced injuries or hepatotoxic chemicals.

Key words

Inula britannica, Compositae, taraxasteryl acetate, anti-hepatitis substance.

Introduction

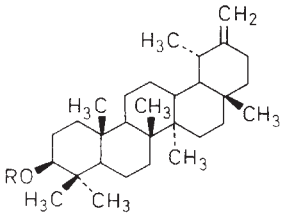
Because effective therapy has not been established for various hepatitis, it has been hoped that the development of new therapeutic medicines for hepatitis will be fruitful. In Japan, most chronic hepatitis has resulted from viral hepatitis and it has been considered that the process and development of these hepatitis are associated with the immunological response. Although hepatitis virus B shows no cytotoxicity to the infected hepatocytes, cytotoxicity to hepatocytes occurs by an autoimmune response against the virus infected-hepatocyte. The dried flowers of *Inula britannica* L. subsp. *japonica* Kitam. (Japanese name; Senpuku-ka) have been used for the treatment of digestive disorders as a component herb of Japanese herbal medicines. We have now found that the hot water extract of *I. britannica* contained anti-hepatitis substances which were screened by using an animal model (1–4) of immunologically induced acute hepatic failure.

The present paper deals with the isolation of active substances from the flowers of *I. britannica* and their chemical and biological characterization.

Materials and Methods

Materials

The dried flowers of *I. britannica* L. subsp. *japonica* Kitam., cultivated in Jiangsu province of China, were obtained from Uchida Wakanyaku (Tokyo, Japan) as a commercial product. A voucher specimen was deposited at the herbarium of Department of Pharmacognosy, School of Pharmaceutical Sci-



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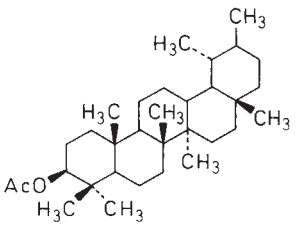

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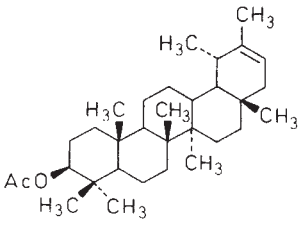

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ences, Kitasato University. *Propionibacterium acnes* (supplied from the Kitasato Institute), was cultured in thioglycollate medium at 37 °C for 48 h, heat-killed, and then used as lyophilizate. Galactosamine (GalN) and LPS (*Escherichia coli* Serotype, 0127: B8) were purchased from Sigma (St Louis, MO).

General methods

Silica gel column chromatography was carried out on Wako gel C-200. Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 (Merck) plates, and spots were visualized by spraying with 10 % sulphuric acid solution followed by heating at 110 °C. Optical rotation was recorded on a JASCO model DIP-370 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Varian XL-400 spectrometer operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. The 2D-NMR experiments were conducted using the standard Varian software for COSY. Low and high resolution mass spectra were recorded on a JEOL DX-300 mass spectrometer.

Isolation of active substances

Flowers of *I. britannica* (3 kg) were refluxed with MeOH (3 l × 5) to obtain the MeOH extract (IB-1), and IB-1 was partitioned with $CHCl_3$-H_2O to give the $CHCl_3$ soluble fraction (IB-3). The active fraction, IB-3 mainly contained 5 substances having R_f = 0.76, 0.47, 0.34, 0.17, and below 0.17 on TLC (silica gel, CH_2Cl_2/*n*-hexane, 1 : 1). IB-3 (57.2 g) was fractionated on a silica gel column (5 × 95 cm) using *n*-hexane-CH_2Cl_2 (1 : 1) (50 ml/fraction). The fractions which were eluted from silica gel column were analyzed by TLC, and the fractions (t_R; 2000–2500 ml) containing an substance having R_f 0.47 were combined to obtain IB-3-2. The fractions which mainly contained substances having R_f 0.76 (t_R; 1000–1500 ml), 0.34 (t_R; 2500–3000 ml), 0.17 (t_R: 3000–3500 ml), and below 0.17 (t_R; 3500–4000 ml), respectively, were each combined to obtain IB-3-1, 3-3, 3-4, and 3-5, respectively. IB-3-1 to IB-3-5 were tested for anti-hepatitis activity, and IB-3-2 and 3-3 were found to contain active substances. Taraxasteryl acetate (**1**) was obtained by recrystallization from IB-3-2 with CH_2Cl_2-acetone (yield: 0.02 %); **1**: colorless crystals, m.p.: 243 °C (decomp.); $[\alpha]_D^{25}$: + 85.0 ($CHCl_3$, *c* 0.3); elemental analysis: C; 82.01 %, H; 11.27 %. $C_{32}H_{52}O_2$ requires C; 81.99 %, H; 11.18 %; high resolution MS: 468.39555. $C_{32}H_{52}O_2$ requires 468.39646; MS: (*m/z*): 468 (M^+), 408 (M^+ – AcOH), 399, 386, 357, 249, 189, 136, 109, 95, 81; ^{1}H-NMR ($CDCl_3$): 0.838–1.020 (7 × CH_3), 2.04 (3H, s, CH_3CO), 2.09 (1H, m, H-19), 2.19 and 2.44 (1H each, m, H-21), 4.48 (1H, m, H-3), 4.60 (2H, dd, H-30).

Chemical modification of taraxasteryl acetate

(a) Deacetylation: **1** (120.4 mg) was refluxed in 5 % ethanolic KOH (10 ml) for 2 h, and the reaction was stopped by addition of H_2O and neutralized with 1 N HCl. The reaction mixture was evaporated to dryness, and partitioned with $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated to obtain taraxasterol (**2**; yield: 98.0 %). High resolution MS: 426.38591. $C_{30}H_{50}O$ requires 426.38869; MS (*m/z*): 426 (M^+), 357, 207, 189, 109.

(b) Reduction with Pd/C: **1** (31.6 mg) was stirred for 4 h at room temperature in dry *n*-hexane containing 5 % Pd/ C (20 mg). The products were purified by HPLC using a column of YMC-pak Silica (10 × 250 mm, YMC, Japan) in *n*-hexane-AcOEt (400 : 1) to give 20,30-dihydrotaraxasteryl acetate (**3**) and ψ-taraxasteryl acetate (**4**); **3**: yield: 7.3 %; high resolution MS: 470.42626; $C_{32}H_{54}O_2$ requires 470.41210; MS (*m/z*): 470 (M^+), 410 (M^+ – AcOH), 249, 191, 189; ^{1}H-NMR ($CDCl_3$): 2.05 (3H, s, CH_3CO), 4.48 (1H, m, H-3); **4**: yield: 38.3 %; high resolution MS: 468.39649; $C_{32}H_{54}O_2$ requires 468.39646; MS (*m/z*): 468 (M^+), 408 (M^+ – AcOH), 249, 189, 136; ^{1}H-NMR ($CDCl_3$): 2.04 (3H, s, CH_3CO), 4.49 (1H, m, H-3), 5.26 (1H, dd, H-21).

Immunologically-induced acute hepatic failure

Immunologically-induced acute hepatic failure was induced according to the modified method of Ferluga and Allison (1). Heat-killed *P. acnes* (160 μg/mouse) were injected intravenously into male ICR mice (Japan SLC Co. Ltd, Hamamatsu, 35–40 g, 7–9 weeks of age) and after 7 days, acute hepatic failure was induced by intraperitoneal injection of LPS (1 μg/mouse). Each test sample suspended in saline containing 10 % Tween 80 was intraperitoneally administered to the mice 90 min before or 30 min after LPS injection. The survival rate of mice was compared until 20 h later with *P. acnes*-LPS-treated mice (control) which were injected with saline containing 10 % Tween 80.

Acute hepatic failure induced by CCl_4

CCl_4-induced acute hepatic failure was performed using the modified method of Hjelle et al. (5). Male ICR mice (35–40 g, 7–9 weeks of age) were fasted for 15 h and were given 12.5 μl/kg of CCl_4 suspended in corn oil orally. Each test sample was injected intraperitoneally to the mice 30 min before CCl_4 administration. After 24 h of CCl_4 administration, blood samples were obtained from the retro-orbital plexus in the treated mice. Blood samples were mixed immediately with phosphate-buffered saline containing 10 U/ml of heparin sodium. GOT and GPT activities in the plasma were measured by the assay kit, GOT and GPT UV-Test Wako (Wako Pure Chemical Indust., Japan), respectively. The liver samples were fixed in 10 % formalin and stained with hematoxylin-eosin for histological analysis.

Acute hepatic failure induced by D-galactosamine (GalN)

GalN-induced acute hepatic failure was performed using the modified method of Keppler et al. (6). Male sprague-Dawley rats (Clea Japan, Tokyo, 250 g, 7 weeks of age) were injected with 800 mg/kg of GalN dissolved in saline. Each test sample was injected intraperitoneally to the rats after 2 h of GalN administration. Then the rats were fasted for 24 h and blood samples were collected from the retro-orbital plexus. Plasma GOT and GPT activities were measured as above.

Statistical analysis

Statistical significance of difference was analyzed by Student's t-test (GOT and GPT) and Wilcoxon test (survival).

Results and Discussion

Effects of I. britannica *extract on immunologically-induced acute hepatic failure*

The survival curves and mortalities of the mice with acute hepatic failure are shown in Fig. **1**. When *P. acnes* and LPS-treated mice were administered only with 10 % Tween 80 saline (solvent control) 90 and 30 min prior to LPS treatment, all the mice died within 4 h after LPS treatment. However, when the hot water extract (130 mg/kg) obtained from *I. britannica* was administered to the mice intraperitoneally, mortality decreased to 40 % at 20 h after LPS injection, and anti-hepatitis substance could be extracted with $CHCl_3$. When anti-hepatitis activity was compared among the subfractions (IB-3-1, 3-2, 3-3, 3-4 and 3-5), which were obtained from the $CHCl_3$-soluble fraction (IB-3), IB-3-3 remarkably inhibited the acute hepatic failure, and IB-3-2 also showed significant inhibitory activity (data not shown).

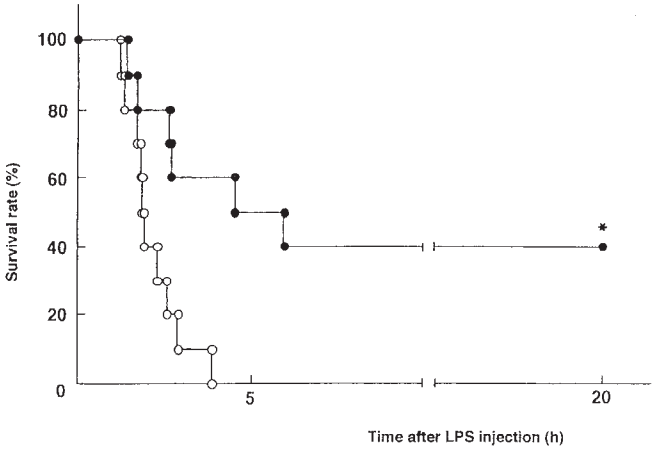

Fig. 1 Effect of hot water extract from *Inula britannica* L. on immunologically-induced acute hepatic failure. Sample was injected to *P. acnes*-treated mice 90 min before LPS injection. ○, control; ●, hot-water extract from *I. britannica*. * $p < 0.05$, compared with the control (Wilcoxon test).

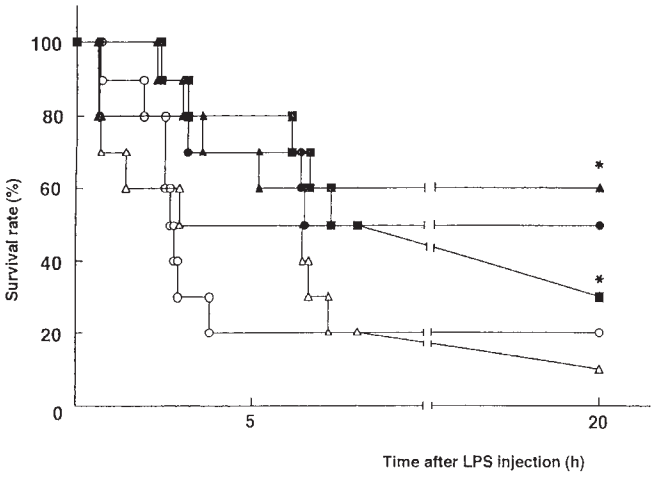

Fig. 2 Effect of taraxasteryl acetate on immunologically-induced acute hepatic failure. Taraxasteryl acetate (suspension in 10% Tween 80 saline) was injected to *P. acnes*-treated mice 2 times 90 min before and 30 min after LPS injection. ○, control; taraxasteryl acetate: ▲, 12 mg/kg × 2; ■, 5.8 mg/kg × 2; ●, 2.9 mg/kg × 2; △, 0.6 mg/kg × 2. * $p < 0.01$, compared with the control (Wilcoxon test).

Table 1 ^{13}C-NMR of taraxasteryl acetate.

C-Atom	Chemical shifts 1[a]	1[b]
C-1	38.4	38.4380
C-2	23.6	23.6911
C-3	80.8	80.9490
C-4	37.7	37.7850
C-5	55.4	55.4373
C-6	18.1	18.1781
C-7	33.9	33.9927
C-8	40.8	40.9075
C-9	50.3	50.3905
C-10	37.0	37.0393
C-11	21.4	21.4586
C-12	25.5	26.1408
C-13	38.8	39.1503
C-14	41.9	42.0253
C-15	26.6	26.6405
C-16	39.1	38.2862
C-17	34.4	34.5167
C-18	48.6	48.6379
C-19	38.3	39.3705
C-20	154.4	154.611
C-21	25.4	25.6093
C-22	39.3	38.8602
C-23	27.8	27.9314
C-24	16.4	16.4938
C-25	15.8	15.8848
C-26	16.2	16.3298
C-27	14.6	14.7184
C-28	26.1	19.4781
C-29	19.4	25.4817
C-30	107.0	107.112
CH_3CO	170.8	170.971
$\underline{CH_3}\bar{C}O$	21.1	21.3113

[a] Values were reported by Patra et al. (5).
[b] Values were obtained in the present study.

Although IB-3-3 seemed to contain several kinds of substances on HPLC, the second active fraction, IB-3-2 revealed a single spot on TLC, and was recrystallized to give a pure compound. When mice were injected the pure compound twice 90 min before and 30 min after LPS injection, anti-hepatitis activity was observed at doses of 12–2.9 mg/kg (Fig. 2).

The pure compound ($C_{32}H_{52}O_2$) gave the same fragment ions due to deacetylation and retro-Diels-Alder fragmentation on EI-MS as taraxasteryl acetate (**1**) which was originally isolated from *Stemmadenia bella* Miers (7). The ^{13}C-NMR spectrum pattern of the active substance also well agreed with that of **1** reported by Patra et al. (5). However, assignments of DEPT spectra for C-13, C-16, C-19, C-21, C-22 and C-28 in the active compound did not agree with those in the literature (5), suggesting that parts of the previous assignments were not correct (Table **1**). The COSY spectrum indicated that the proton at 2.09 was due to H-19, and that protons at 2.19 and 2.44 were due to H-21. The C-H correlation and DEPT analysis indicated that C-19 and C-21 were observed at 39.3705 and 25.6093, respectively. It was also indicated that H-19 coupled with H-29, and C-29 carbon was observed at 25.4817. Therefore the signal at 38.8602 was reassigned to be C-22. The NOE spectrum indicated that C-28 was observed at 19.4781 because H-28 coupled with H-22. Signals at 26.1408, 39.1503, and 38.2862 were also reassigned to C-12, C-13, and C-16, respectively, from the comparison with ^{13}C-NMR of α-lupene (9). Although no pharmacological activity of **1** has been reported, the present study clarified that it has anti-hepatitis activity.

Effect of chemical modification of taraxasteryl acetate on anti-hepatitis activity

During the present study, lupenone was also isolated from IB-3 as an inactive compound against *P. acnes*-LPS induced hepatic failure (data not shown). From the comparison of structures of **1** and lupenone, the acetoxy group at C-3 and the structure of the E-ring were assumed to be important for expression of anti-hepatitis activity. Terapatra et al. have reported that the double bond in the E-ring of **1** was isomerized by Pd/C reduction (7). When **1** was reduced with Pd/C, ψ-taraxasteryl acetate (**4**) was obtained in addition to 20,30-

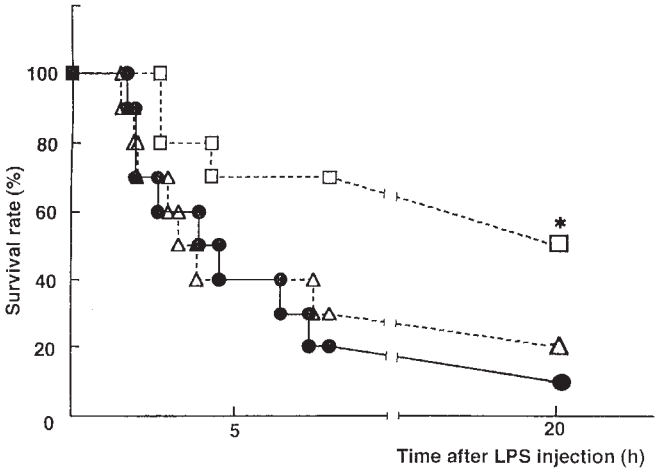

Fig. 3 Effects of taraxasteryl acetate and ψ-taraxasteryl acetate on immunologically-induced acute hepatic failure. The injection of samples was done as in Fig. 1. ●, control; □, taraxasteryl acetate (11.5 mg/kg); △, ψ-taraxasteryl acetate (11.5 mg/kg). *$p < 0.05$, compared with the control (Wilcoxon test).

dihydroxytaraxasteryl acetate (**3**). Although injection of 11.5 mg/kg of **1** decreased the mortality of *P. acnes*-LPS-treated mice, the same dose of **4** did not show anti-hepatitis activity (Fig. 3). Compound **3** (5.75 mg/kg) and taraxasterol (**2**, 23 mg/kg) also did not have anti-hepatitis activity (data not shown). These results indicate that at least the acetoxy group at C-3 and the E-ring-unsaturation in **1** may correspond to its anti-hepatitis activity.

Effects of taraxasteryl acetate on acute hepatic failure induced by CCl_4 or GalN

Treatment of mice with CCl_4 increased plasma transaminase activity significantly, and administration of prednisolone (23 mg/kg) did not affect the level of transaminase activity in CCl_4-treated mice (Table **2**). However, administration of **1** (23 mg/kg) significantly reduced both transaminase activities to the lower level in comparison with CCl_4-treated mice. A histological study indicated that the administration of **1** inhibited degeneration and necrosis of liver cells from CCl_4-treated mice (data not shown).

Although administration of GalN also increased plasma transaminase activity of rats, prednisolone (23 mg/kg) and **1** (5.8 mg/kg and 23 mg/kg) treated rats tended to have reduced transaminase levels in comparison with GalN-treated rats (Table **3**). These results indicated that **1** also showed anti-hepatotoxic activity as well as anti-hepatitis activity, however the mechanism of **1** for the anti-hepatitis activity might be different from that of steroids.

Table 2 Effect of taraxasteryl acetate on acute heptotoxic failure by CCl_4 treatment.

	GOT (U/l)[a]	GPT (U/l)[a]
Vehicle	40 ± 17	21 ± 1
CCl_4	714 ± 488	559 ± 371
CCl_4 + Prednisolone (23 mg/kg)	707 ± 348	715 ± 308
CCl_4 + Taraxasteryl acetate (12 mg/kg)	727 ± 403	922 ± 518
CCl_4 + Taraxasteryl acetate (23 mg/kg)	184 ± 120*	161 ± 94*

[a] Values are mean ± S.D. for five mice.
* $p < 0.05$ (Student's t-test).

Table 3 Effect of taraxasteryl acetate on acute heptotoxic failure by GalN treatment.

	GOT (U/l)[a]	GPT (U/l)[a]
Vehicle	33 ± 3	22 ± 2
GalN	478 ± 633	246 ± 336
GalN + Prednisolone (23 mg/kg)	202 ± 93	120 ± 58
GalN + Taraxasteryl acetate (5.8 mg/kg)[b]	271 ± 360	72 ± 83
GalN + Taraxasteryl acetate (23 mg/kg)[b]	259 ± 237	113 ± 93

[a] Values are mean ± S.D. for five rats.
[b] Administration of taraxasteryl acetate tended to reduce the level of GPT by comparison of control (GalN), however the significances were not observed (Student's t-test) because the standard deviation of control was too large.

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