CHAPTER 29

HEPATO-PROTECTIVE TRITERPENOIDS FROM *GANODERMA TSUGAE* MURRILL

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1. INTRODUCTION

The traditional Chinese herb, Lin-Chi, played an important role in folk legends as a cure for many unspecified illnesses. However, there was no direct evidence to support this role until, in the past decade, over a hundred novel, highly oxygenated, lanosta-type triterpenoids were isolated from basidiocarps or mycelial mats of *Ganoderma lucidum,* and their chemical structure determined. These compounds were mainly ganoderic acids (Hirotani *et al.*,1985; Kohda *et al.*, 1985), ganodermic acids (Lin & Shiao ,1988; Shiao & Lin, 1989), ganoderenic acids (Nishitoba *et al.*, 1987a; Komoda *et al.*, 1985), lucidenic acids, lucidone (Hirotani *et al.*, 1986), ganoderal (Morigiwa *et al.*, 1986) and ganoderols (Nishitoba *et al.*, 1987a). These findings have led us to consider that the abundant triterpenoids might explain the multiple effects of this fungus. However, the physiological activity of these newly isolated triterpenoids remained unclear and less than 20 of these compounds were reported to be physiologically active (Kohda *et al.*, 1985; Morigiwa *et al.*, 1986).

Therefore, it is becoming important to elucidate the triterpenoid pattern and content of each species in the genus *Ganoderma*. *G. tsugae* was used in the present study because it is one of the most widely cultivated species in Taiwan. Morphologically, it resembles *G. lucidum* (Adaskaveg & Gilbertson , 1986) and crude extracts exhibited pronounced hepato-protective activity in our preliminary screening (Su, 1991).

In the present report, four major compounds were isolated from basidiocarps of *G. tsugae* and they were determined as lucidone A(I), lucidenol(II), ganoderic acid B(III) and ganoderic acid C2(IV). Three of the four isolated compounds were previously reported in *G. lucidum* and compound II was a newly isolated triterpenoid. An *in vivo* bioassay was designed to evaluate the hepato-protective activity of each sample.

2. MATERIALS AND METHODS

2.1. Culture Used and Basidiocarp Production

Ganoderma tsugae Murrill CCRC-37063 was purchased from the Culture Collection and Research Center located in Hsinchu, Taiwan, and a sawdust-polypropylene bag system was employed for fruiting body cultivation in Chiayii county, Taiwan. The basidiocarps were collected during a 6-month period with immediate drying at 45°C after harvest. Totally, a 3000g sample was collected for extraction.

2.2. Extraction and Purification of Triterpenoids

The basidiocarps of *G. tsugae* was sliced, powdered and then immersed in 95% EtOH at room temperature. The ethanolic extract was evaporated to dryness in a rotary evaporator. For the partition of triterpenoids, a modified Kohda (1985) method was followed which is summarized in Fig. 1.

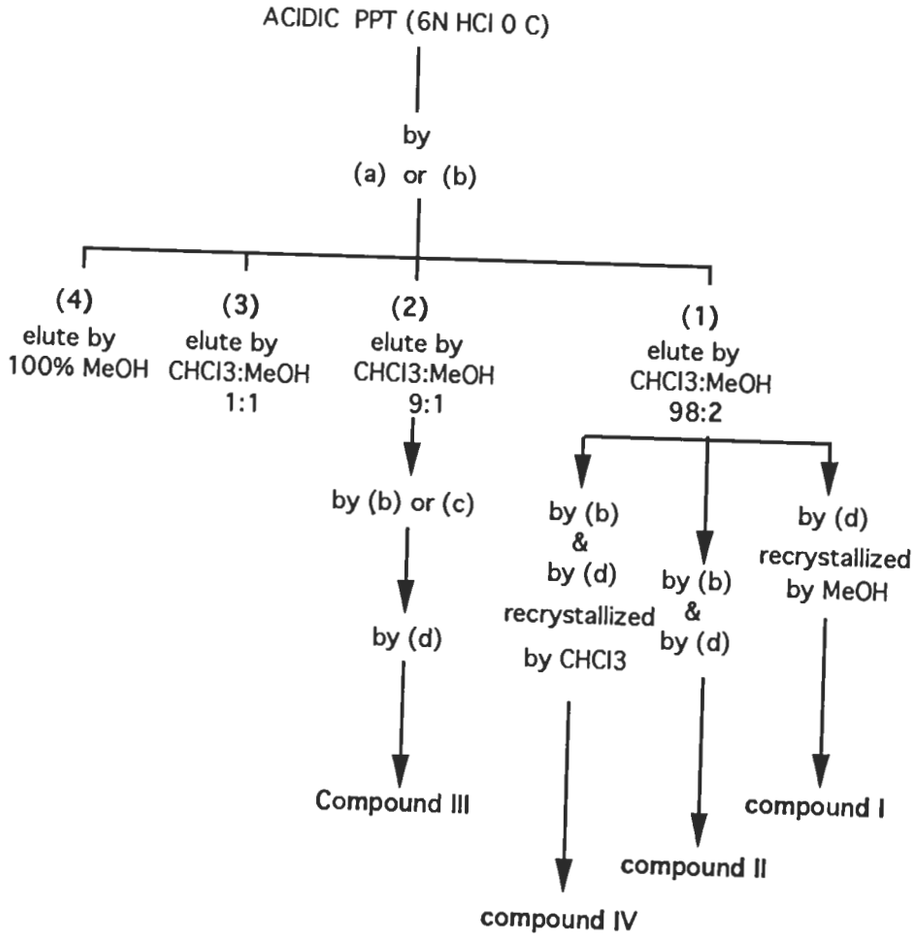

FIGURE 1. Partition of triterpenoids from ethanolic extract of *G. tsugae*. (a) SiO_2 open column chromatography, (b) HPLC, (c) MPLC and (d) TLC.

2.3. Chromatography

For detection of triterpenoids, TLC (Kiesselgel 60 F254, Merck) was used and chromatograms were developed using a $CHCl_3$: MeOH:H_2O (30:4:1) solvent system. The triterpenoids were visualized by UV at 254nm or with 10% H_2SO_4 followed by heating of the TLC plate at 105°C. For a detailed pattern of triterpenoid separation, an HPLC system (LC-4A, Shimadzu) equipped with a UV detector (SPD-6V, Shimadzu) and a column oven set at 45°C was employed. The mobile phase was 40% acetonitrile in 2% methanol with a flow rate of 1 ml/min and a C18 column (30 x 0.5 cm) was used. Similar conditions were used for a medium pressure liquid chromatography system (Bü chi) fitted with a preparative column (60x2.6 cm) packed with C18 gel. However, the flow rate was 5 ml/min. For final purification, a preparative TLC plate (LiChroprep, Merck) was used.

2.4. Structural Analysis

UV (UV-160, Shimadzu), IR (A-100, Jesco), and mass spectrometry (MS, D300, Jeol), and NMR (FT-100, Jeol) were used for spectral analyses.

2.5. Animal Test

Male mice (ICR), 5-6 weeks old, 27± 2 g in weight were subjected to the hepato-protective activity test. CCl_4 (1% in olive oil) was fed to mice at a dose of 2 ml/kg at 0 hr. After 6 hr, the purified triterpenoids were administered at a dose of 0.2 mg/kg (0.1 mg/ml), and a known hepato-protective drug, silymarin, used as a positive control. Blood samples taken from the eye vein was centrifuged for serum separation and then SGOT and SGPT kits (Sigma) were used for analysis at 0, 24 and 48hr, respectively. A dose response test was also carried out in a similar way for compound III with doses of 0.1 mg, 0.4 and 1.0 mg/kg. For each test, 6 mice were used for each group.

3. RESULTS AND DISCUSSION

Around 4.5% (w/w) of crude extract was obtained from the EtOH extraction, but only 27mg, 18mg, 54mg and 28mg of compound I, II, III and IV, respectively, were isolated from each 1000g of *G. tsugae* basidiocarps. The spectra of the four compounds are listed as follows:

Compound I:

UV λ max (nm):	207, 255
IR (cm^{-1}):	2760, 3450-3400, 3960, 1725, 1710, 1660, 1580, 1480, 1360, 1240, 1180, 1090, 1020, 960
Mass spectrum (70ev):	402, 374, 360, 331, 313, 277, 262, 219, 202, 189
^{1}H-NMR (CD30D) (δ) :	0.8342, 0.8794, 1.0223 1.2043, 1.4143, 1.6143, 2.2021, 2.6760 2.7163, 3.1560 3.1963, 4.8317, 4.8746

^{13}C-NMR (CD30D) (δ) : 16.240, 18.800, 25.274,
28.273, 31.327,
36.997, 39.727, 46.196,
54.681, 59.838,
67.794, 78.949,
144.201, 158.338
199.248, 208.374,
216.695

Compound II:

UV λ max (nm): 253
IR (cm^{-1}): 3760, 3500-3300, 1725, 1710
1660, 1580, 1480, 1460, 1440,
1400, 1380, 700
Mass spectrum (70ev): 404, 386, 316, 265, 264, 203,
123, 107, 69, 55
^{1}H-NMR (CD30D) (δ) : 0.7974, 0.8316, 0.9660
1.0136, 1.2457, 1.2982,
1.3018, 1.6084, 2.4805,
2.6934, 3.2915, 3.3085,
3.3122, 3.3391, 3.3427,
4.5153

Compound III:

UV λ max (nm): 206, 253
IR (cm^{-1}): 3760, 1725, 1710, 1690, 1660, 1580,
1480, 1460, 1440, 1420, 1400, 1340,
Mass spectrum (70ev): 516, 501, 500, 482, 402, 389, 374,
359, 344, 279, 262, 207, 189,
167, 149, 139, 121, 105, 95, 83,
69, 57, 55
^{1}H-NMR (CD30D) (δ) : 2.1629, 1.3544, 1.1907,
0.9757, 0.8292
^{13}C-NMR (CD30D) (δ) : 15.972, 17.325, 18.624,
19.794, 24.714, 28.444,
32.888, 35.741, 39.472,
41.615, 46.586, 60.211,
67.709, 78.736, 144,
159.200, 211.218

Compound IV:

UV λ max (nm): 254
IR (cm^{-1}): 3760, 1725, 1710, 1690, 1660,
1580, 1480, 1460, 1440, 1420, 1400
Mass spectrum (70ev): 518, 516, 488, 376, 358, 331, 246,
139, 69, 55
^{1}H-NMR (CD30D) (δ) : 3.6212, 3.3073, 3.2658,
2.6771, 2.3205, 1.7855,

1.5693, 1.2066, 1.1895,
1.0698, 0.9647, 0.9269,
0.8243, 0.8084, 0.7876
^{13}C-NMR (CD30D) (δ) : 16.374, 17.563, 19.995,
8.700, 33.803, 35.906,
39.691, 50.445, 50.847,
53.188, 55.328, 70.159,
73.213, 78.956,
143.055, 161.380,
183.966, 202.400, 212.5

From the spectra obtained, these four compounds were determined as lucidone A (I), lucidenol (II), ganoderic acid B (III) and ganoderic acid C2 (IV). The structures of these four triterpenoids are shown in Fig. 2 and their high performance liquid chromatographs are shown in Fig. 3. Lucidone A (Kohda *et al.*, 1985), ganoderic acid B (Morigiwa *et al.*, 1986) and ganoderic acid C2 (Nishitoba *et al.*, 1987a) are known compounds reported previously in fruitbodies of *Ganoderma lucidum*. However, compound II is a new type of triterpenoid.

The results from animal tests are shown in Figs. 4 to 7. Only compound III showed significant

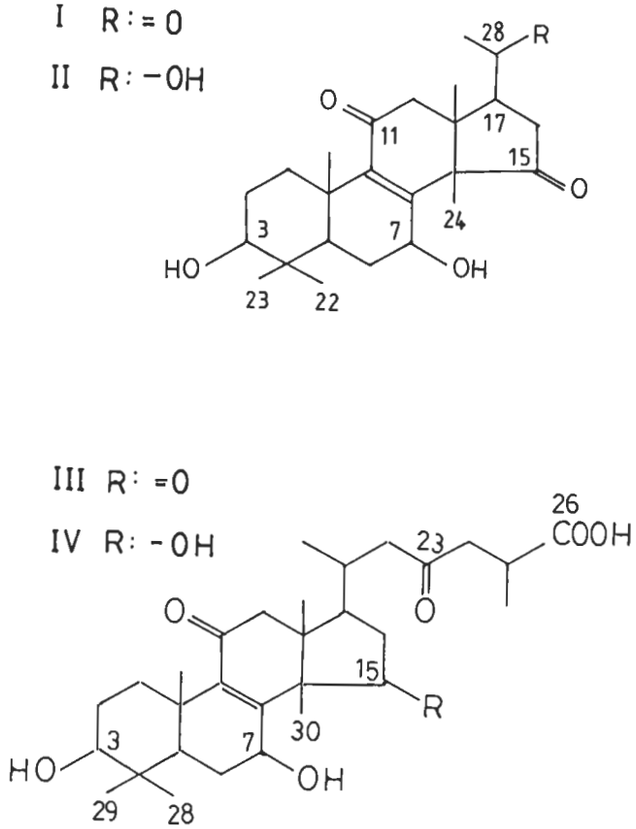

FIGURE 2. Chemical structures of compounds I, II, III and IV isolated from *Ganoderma tsugae*.

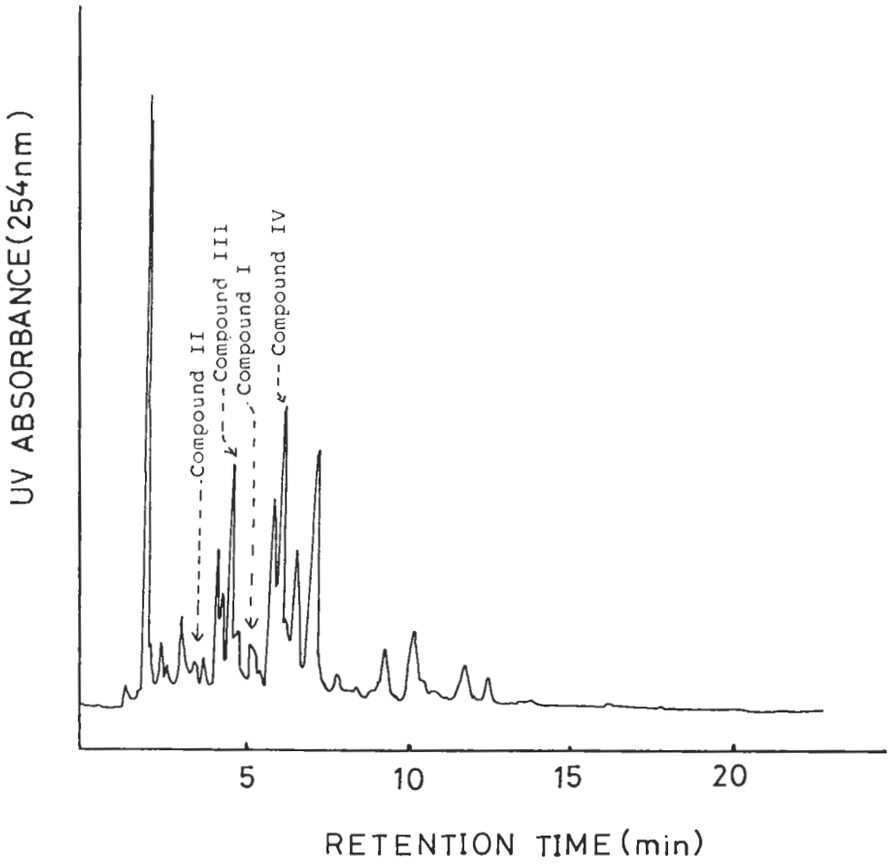

FIGURE 3. Chromatograms of triterpenoids demonstrated by HPLC and the respective retention times of the four isolated compounds.

hepato-protective activity. Only compounds I, II and IV, together with the silymarin group, caused a slight reduction in GPT and GOT levels in serum after 24 hr. The test animals eventually reverted back to the normal condition after 48 hr. However, increased doses of compound III (up to 10 times) did not further reduce GOT/GPT levels in the serum of the mice (Figs. 6 & 7).

Our previous work has demonstrated that a less pure fraction of ganoderic acid B showed a very strong effect in reducing GOT/GPT to almost normal values after 24 hr, but this result was not observed with the pure ganoderic acid B. These controversial results can be explained by the series of triterpenoid analogues functioning as better free radical scavengers than the pure compound in the liver challenged with CCl_4. Further studies on the mechanism of the hepato-protective triterpenoid from *G. tsugae,* and the combined effect of triterpenoid analogues, are now under investigation.

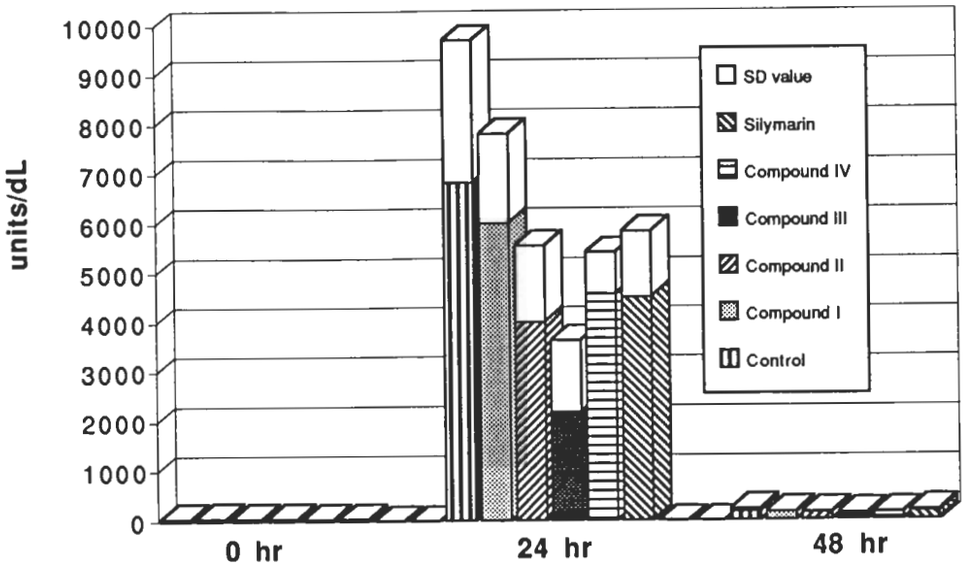

FIGURE 4. The effect of pure compounds isolated from *Ganoderma tsugae* for GPT in mice induced by CCl_4.

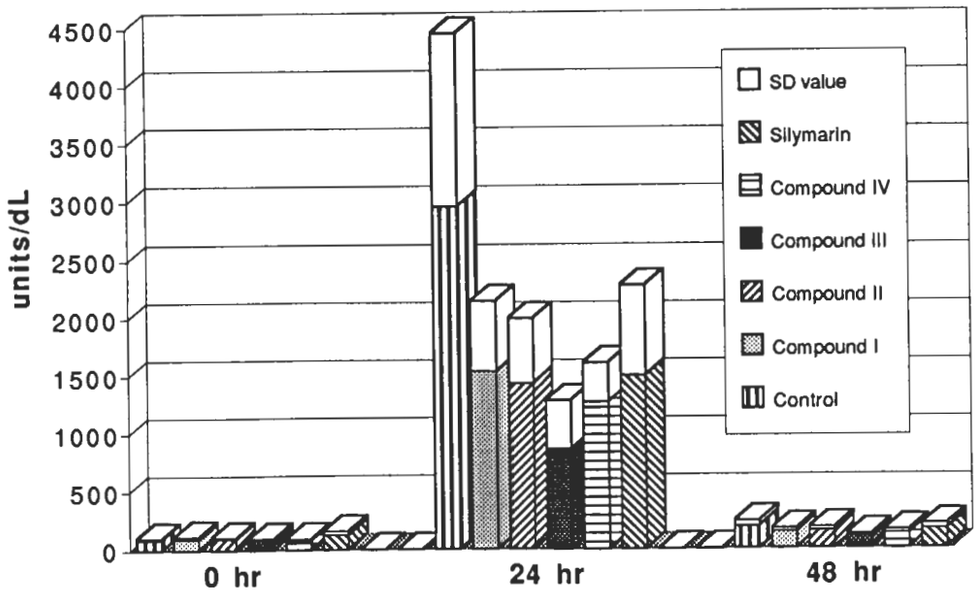

FIGURE 5. The effect of pure compounds isolated from *Ganoderma tsugae* for GOT in mice induced by CCl_4.

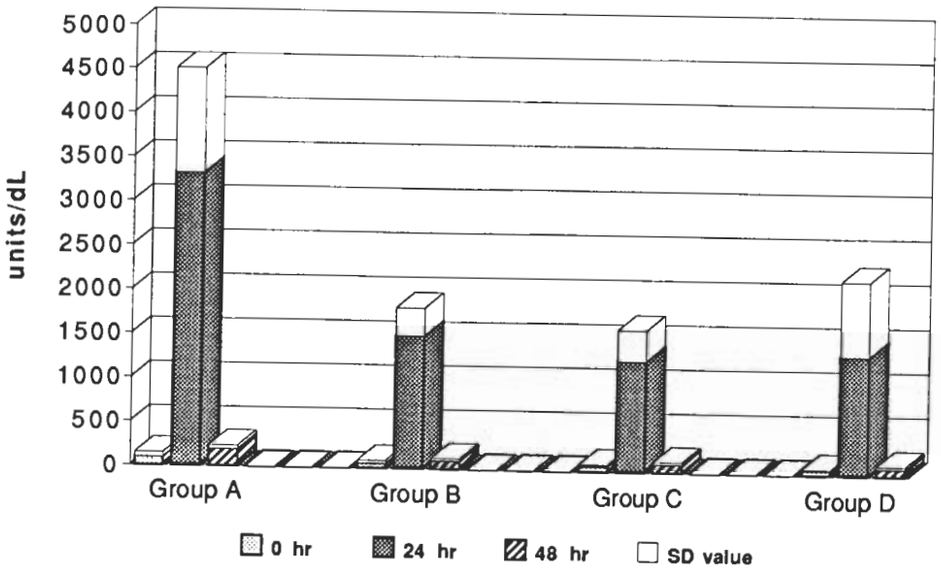

FIGURE 6. Dose response for compound III with CCl_4 induced GPT in mice.

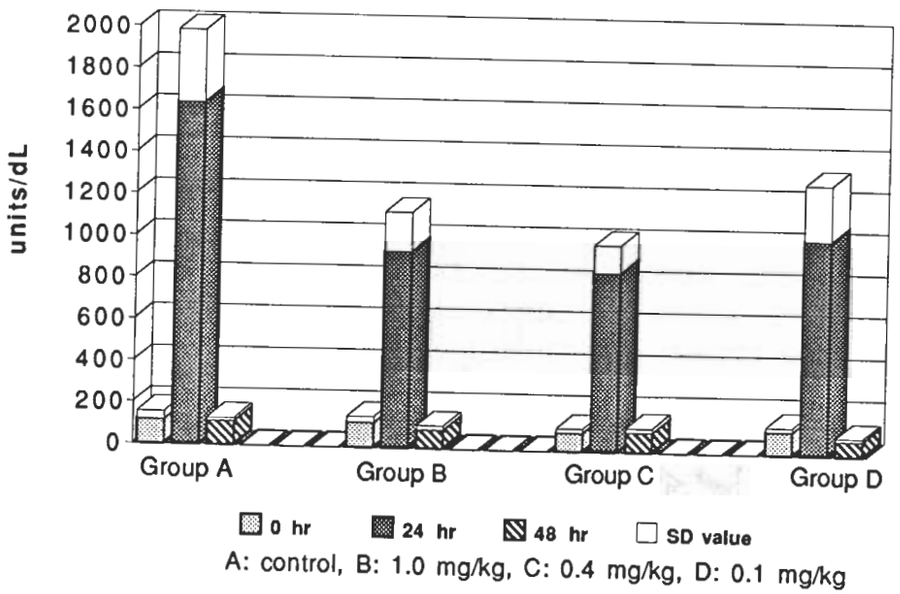

FIGURE 7. Dose response for compound III with CCl_4 induced GOT in mice.

ACKNOWLEDGEMENT

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