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Protective effects of triterpenoids from *Ganoderma resinaceum* on H_2O_2-induced toxicity in HepG2 cells

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ABSTRACT

Ganoderma resinaceum Boud. (Polyporeseae) has long been used for antioxidant, immunoregulation and liver protection. From the fruiting bodies of *G. resinaceum*, eight new lanostanoids, lucidones D–G (**1**–**4**), 7-oxo-ganoderic acid Z_2 (**5**), 7-oxo-ganoderic acid Z_3 (**6**), ganoderesin A (**7**), and ganoderesin B (**8**), together with six known lanostanoids (**9**–**14**) were isolated. The structures of new compounds were elucidated through extensive spectroscopic analysis. In an *in vitro* model, ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) showed inhibitory effects against the increase of ALT and AST levels in HepG2 cells induced by H_2O_2 compared to a control group in the range of their maximum non-toxic concentration (MNTC). However, compounds **8**, **10** and **11** displayed no anti-oxidant activities by DPPH assay. Meanwhile, activation for PXR (Pregnane X Receptor) of ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) was evaluated; ganoderol (**10**) exhibited a vital activation for PXR-induced CYP3A4 expression. These results suggested that GTs (Ganoderma triterpenoids) exhibited hepatoprotective activities by lowering ALT and AST levels.

1. Introduction

The fruiting bodies of *Ganoderma lucidum* Karst. (Polyporaceae), commonly known as *Reishi* mushroom, are widely used as a valuable crude drug and health food in China, Japan, and Korea. Ganoderma triterpenoids (GTs) represent the main bioactive compounds in this mushroom (Fatmawati, Shicizu, & Kondo, 2011). These constituents were claimed to possess cytotoxicity (Gan, Fann, & Lin, 1998), antibacterial (Ofodile, Uma, & Simmonds, 2005), antiviral (Timo, Lindequist, & Michael, 2005), antitumour (Hsu, Ou, & Kao, 2008) and anti-osteoclastic differentiation activities (Liu, Shiono, Shimizu, & Kondo, 2010).

Ganoderma resinaceum has long been used for immunoregulation (Zhu, 1994). Until now, only several common high oxygenated lanostane-type triterpenoids of *G. resinaceum* (Nishitoda, Sato, & Sakamura, 1984) and their cytotoxicity against Hep-2 cells have been reported (Niu, Sheng, & Che, 2007). A systematic research on chemical constituents and their biological activities of *G. resinaceum* has not been carried out.

In this study, eight new lanostanoids (**1**–**8**) and six known lanostanoids (**9**–**14**) were isolated from the fruiting bodies of *G. resinaceum* (Fig. 1). In an *in vitro* HepG2 cells injury model, the hepatoprotective activities of ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) were assessed by testing the levels of ALT and AST in HepG2 cells induced by H_2O_2 in the range of their maximum non-toxic concentration (MNTC). Using the DPPH radical scavenging assay, we measured the antioxidant effects of compounds **8**, **10** and **11**. Meanwhile, activation for PXR of ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) were evaluated.

2. Materials and methods

2.1. General

Optical rotations were obtained with a Jasco P-1020 polarimeter (Jasco, Tokyo, Japan). ^1H and ^{13}C NMR spectra were measured on Bruker AV-400 and DRX-500 instruments (Bruker, Zurich, Switzerland) using transcranial magnetic stimulation (TMS). ESIMS and HRTOF-ESIMS data were recorded on an API QSTAR Pulsar spectrometer and infra-red spectra were recorded on a Bruker Tensor-27 instrument by using KBr pellets. An Agilent 1100 series instrument equipped with Agilent ZORBAX SB-C-18 column (5 μm, 4.6 × 150 mm) was used for high-performance liquid

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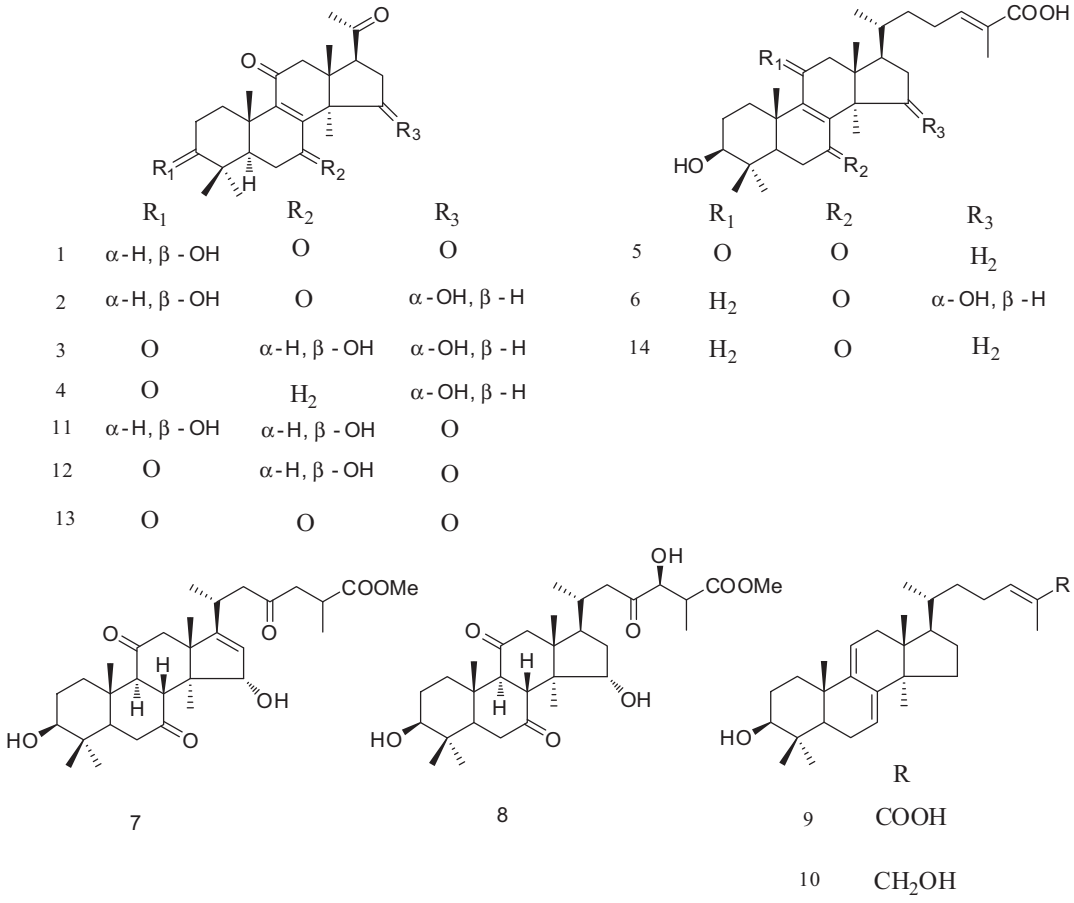

Fig. 1. Structures of isolates from *G. resinaceum*.

chromatography (HPLC) analysis. Biological assay: chemical luminescence enzyme standard instrument (Thermo, Waltham, MA); MEM (modified eagle medium), OPTI-MEM (Gibco, Life Technologies, Grand Rapids, NY) and FBS (foetal bovine serum) (Hyclone, NXB0574 and ASM31.65); tryptase (Colabio, 20111222); biochemical assay kits for measurement of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) contents (Biosino Bio-Technology and Science Inc., Beijing, PR China) cationic liposome, Lipofectamin TM 2000, Cat. No. 11668-019); Double-luciferase reporter gene assay (Promega, Madison, WI).

2.2. Chemical and reagents section

TLC was performed on precoated TLC plates (200–250 μm thickness, F254 Si gel 60, Qingdao Marine Chemical, Inc.) with compounds visualised by spraying the dried plates with 10% aqueous H_2SO_4 followed by heating until dryness. Silica gel (200–300) mesh, Qingdao Marine Chemical, Inc.), Lichroprep RP-18 (40–63 μm; Merck, Darmstadt, Germany) and Sephadex LH-20 (20–150 μm; Pharmacia, Uppsala, Sweden) were used for column chromatography. Methanol, dichloromethane, ethyl acetate, acetone and *n*-butanol were purchased from Tianjing Chemical Reagents Co. (Tianjing, China). H_2O_2 was from Beijing Chemical Works.

2.3. Plant materials

The fruiting bodies of *G. resinaceum* were purchased in April 2010 from Juhuacun Traditional Chinese Medicine Market in Kunming. The mushroom was identified by Professor Liu Peigui, who works at Kunming Institute of Botany, Chinese Academy of Science.

2.4. Extraction and isolation

G. resinaceum (25 kg) mushrooms were chopped and extracted with 95% methanol (MeOH) under reflux at 80 °C three times. The combined MeOH extracts were evaporated under reduced pressure. The 8 kg residue was suspended in H_2O and extracted with petroleum ether (PE), ethyl acetate (EtOAc), and *n*-butanol. The volume of combined EtOAc extracts was reduced to one-third under vacuum. Saturated Na_2CO_3 aqueous solution was then added and extracted by EtOAc. The EtOAc layer (fraction A 500 g) was subjected to silica gel column chromatography with $CHCl_3$/MeOH (100:1, 50:1, 20:1, 5:1) and divided into four fractions.

The 50:1 fraction was applied to a chromatography column for isolation of **13** (5 mg). The 20:1 fraction was treated by silica column chromatography and divided into three sub-fractions (Fr. A, Fr. B and Fr. C). Treatment of Fr. B gave **1** (15 mg), **2** (5 mg) and **12** (20 mg). Fr. C was separated by reverse-phase HPLC (MeOH/H_2O: 45% → 80%, 25 min) to get **2** (20 mg), **3** (25 mg) and **11** (10 mg). The 5:1 fraction was also subjected to silica gel column chromatography and three sub-fractions were obtained (PE/acetone: 5:1, 2:1, 1:2). Compounds **5** (30 mg), **9** (58 mg), **10** (42 mg) and **14** (20 mg) were isolated from the 2:1 fraction by repeated silica gel column chromatography ($CHCl_3$/MeOH). Using reverse-phase silica gel (MeOH/H_2O, step gradient) to treat 1:2 gave **6** (12 mg).

The water layer was extracted with EtOAc again after the pH value was adjusted to 2–3 with 1 N HCl solution. The combined

EtOAc extracts were evaporated to dryness under reduced pressure, and the residue (fraction B 200 g) was divided into three fractions. One-third was taken on esterification reaction. By reverse-phase silica gel (MeOH/H_2O, step gradients), fraction A was divided into three parts (MeOH/H_2O: 50%, 60% and 70%). The 50% fraction was treated by Sephadex LH-20 and subjected to silica gel column chromatography (PE/acetone) to give **8** (5 mg). Compound **7** (20 mg) was obtained from the 60% fraction treated by reverse-phase silica gel chromatography (MeOH/H_2O: 45% → 75%).

2.5. Biological assay

2.5.1. Measurement of maximum nontoxic concentration

HepG2 cells (hepatocellular carcinoma cells) in logarithmic growth phase were seeded in 96-well plates (1×10^4 cells/well, 200 μl/well) in MEM with 10% FBS for 24 h at 37 °C. The MNTC of DMSO in HepG2 cells was determined prior to the cytotoxicity analysis. The MNTC of DMSO was 1% (v/v). Thus, subsequently all compounds were prepared in DMSO at concentrations lower than the DMSO toxic dose. Compounds **1**, **3**, **8**, **9**, **10**, **11**, **13** and **14** were dissolved in 1% DMSO for testing. The concentrated stock of compounds were mixed with MEM of 2% FBS and made to different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 μM) before adding to HepG2 cells in 96-well plates. The MNTC of test compounds was analysed with a colorimetric assay using tetrazolium salt MTT at the end-point of 96 h incubation.

2.5.2. Assessment of hepatoprotective activity

The hepatoprotective activity of isolated compounds was assayed using H_2O_2-treated HepG2 cells. The H_2O_2 concentration used for cell culture treatment was previously determined according to a modified method of Gu et al. HepG2 cells in logarithmic growth phase were made into single cell suspension and seeded in 96-well plates (1×10^4 cells/well, 200 μl/well) for 24 h at 37 °C. 3.2 mM H_2O_2 was added to the cell medium and the mixture was further incubated at 37 °C for 2 h. According to their MNTC, the medium with three different concentrations of test compounds (200 μl) was mixed in cell medium and incubated for 24 h. The obtained reacted supernatant was directly used to detect ALT and AST levels. The control group was a set of cells maintained in culture medium, while the model group was a set of cells maintained in culture medium and treated only with H_2O_2.

2.5.3. Determination of DPPH radical-scavenging ability

The DPPH (α,α-diphenyl-β-picrylhydrazyl) radical scavenging activity was measured using a modified previously established 100 μM DPPH radical was dissolved in 80% aqueous methanol. Compounds **8**, **10** and **11** (150 μl) at different concentrations in methanol were added to 4.35 ml of the methanol DPPH radical solution. The mixture was shaken vigorously and allowed to stand at 23 °C in the dark for 30 min. The decrease in absorbance of the resulting solution was monitored at 517 nm after 30 min. The control consisted of 150 μl of 80% aqueous methanol and 4.35 ml of DPPH radical solution. The scavenging activity was estimated based on the percentage of DPPH radical scavenged using the following equation:

$$\text{DPPH radical scavenging effect}\ (\%) = 100 \times [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}]$$

The EC_{50} value (mg/ml) is the effective concentration at which the DPPH radicals were scavenged by 50%. Vitamin C was used as positive control. Each sample was assayed in triplicate.

2.5.4. Determination of CYP3A4 expression

Plasmids contained tk-(CYP3A4) 3-Luc reporter plasmid, pCMX-hPXR expression plasmid and pRL-TK internal control plasmid. Transfections were performed on 48-well plates. CV-1 and HuH7 cells were transfected using Lipofectamine 2000 and the polyethylenimine polymer. The transfected cells were treated with RIF, DMSO and compounds **8**, **10** and **11** for 24 h before harvesting and assaying for luciferase activity (Hu, Wang, & Zhou, 2007). Luciferase activity was normalised against the co-transfection. All transfections were conducted three times.

2.5.5. Statistical analysis

The percentage of growth inhibition in HepG2 cell was calculated using the following formula:

$$\%\text{Cell inhibition} = 100 \times (OD_{570\text{control}} - OD_{570\text{test compounds}})/OD_{570\text{control}}.$$

Results are expressed as the mean ± SD of at least three independent experiments as indicated. The test for the paired samples was used to determine statistical differences between parameters. These differences were considered significant for $p < 0.05$, 0.01 or 0.001 and non-significant for $p > 0.05$.

3. Results and discussion

3.1. Phytochemical investigation

A molecular formula of **1**, $C_{24}H_{32}O_5$, was established by positive HRESIMS. The ^1H NMR spectrum (Table 1) showed the presence of six tertiary methyl signals at δ 0.73 (s), 0.88 (s), 1.02 (s), 1.26 (s), 1.58 (s), and 2.19 (s). The ^{13}C NMR (Table 2) and DEPT spectra of **1** revealed 24 carbon resonances, including six methyls, four methylenes, three methines, and six quaternary carbons (four carbonyls).

The spectroscopic data suggested that **1** was similar to lucidone A (Nishitoba, Sato, & Sakamura, 1985). However, comparison of ^{13}C NMR data of **1** with those of lucidone A showed the existence of signals at δ 199.0 and 205.6 and a lack of signals at δ 66.6 and 215.8 in **1**. We can assume that **1** was the 7-oxygenated derivative of lucidone A. Since C-7 was substituted by a hydroxyl group, the chemical shift of C-15 was 215.8 ppm (Nishitoba et al., 1985). In contrast, when carbonyl group was connected to C-7, the chemical shift of C-15 was shifted upfield by 10.8 ppm (Nishitoba, Sato, & Sakamura, 1987). Our findings were further confirmed by the HMBC correlations from H-5 and H_2-6 to C-7.

Significant ROESY correlation of H-3/H-5 suggested 3-OH was in β-orientation. Thus, the structure of **1** was determined to be 3β-hydroxy-4,4,14α-trimethyl-7,11,15,20-tetraoxo-5α-pregn-8-ene, named lucidone D (**1**).

HRESIMS and ^{13}C NMR data established the elemental formula of **2** to be $C_{24}H_{34}O_5$. Its ^1H and ^{13}C NMR spectra (Tables 1 and 2) were closely related to those of **1**. Analysis of 1D NMR data of **1** and **2**, the existence of an oxymethine signal at δ 76.8 and the absence of a carbonyl carbon signal at δ 205.6 of C-15 indicated that the carbonyl group of C-15 in **1** was converted into a hydroxyl group in **2**, which was confirmed by the HMBC correlations from H-16, Me-30, and H-17 to the oxymethine carbon (δ 76.8).

The ROESY correlation of H-15/Me-30 suggested 15-OH as α. Therefore, **2** was formulated as 3β,15α-dihydroxy-4,4,14α-trimethyl-7,11,20-trioxo-5α-pregn-8-ene, named lucidone E (**2**).

Compound **3**, called lucidone F, showed a quasi-molecular ion peak at m/z 403 in positive ESIMS. Its molecular formula was assigned as $C_{24}H_{34}O_5$ from HRESIMS. The hydroxyl (3432 cm^{-1}) and α,β-unsaturated carbonyl groups (1695 and 1675 cm^{-1}) were

Table 1
^{1}H NMR spectral data for compounds **1**–**8** [TMS as int. standard].

H	1[a]	2[a]	3[a]	4[b]	5[a]	6[c]	7[a]	8[a]
1α	2.87 m	2.85 m	1.45 m	3.25 m	2.89 m	1.66 m	2.91 m	2.78 m
1β	1.24 m			1.72 m				
2α	1.73 m	1.76 m	2.49 m	2.63 m	1.98 m	2.50 m	1.66 m	1.67 m
2β	1.24 m			2.53 m				
3α	3.25 dd (5.0,20.0)	3.29 dd (5.3, 10.9)	–	–	3.31 m	3.12 m	3.20 m	3.23 m
5α	1.56 d (9.0)	1.56 dd (5.5, 6.4)	1.61 s	1.77 t (13.3,13.3)	1.55 m	1.47 m	1.15 m	1.13 m
6α	2.59 m	2.37 m	1.79 m	1.58 m	2.50 m	1.34 m	2.42 m	2.44 m
6β			1.59 m	1.36 m		2.45 m		
7α	–	–	4.64 m	2.79 m	–	–	–	–
7β	–	–		2.70 m	–	–	–	–
8β	–	–	–	–	–	-	2.92 d (12.2)	2.78 d (16.0)
9α	–	–	–	–	–	-	2.32 d (12.2)	2.25 d (16.0)
11	–	–	–	–	–	1.92 m	–	–
12α	3.05 d (16.0)	2.96 d (14.5)	3.10 d (20.0)	2.76 d (15.0)	2.76 d (15.9)	2.48 d (2.37)	2.75 d (13.3)	2.65 d (16.0)
12β	2.77 d (16.0)	2.71 d (14.5)	2.54 d (20.0)	3.15 d (15.0)	2.65 d (15.9)	2.20 d (2.37)	2.19 d (13.3)	2.34 d (16.0)
15β	–	4.57 d (6.2)	4.84 m	4.72 m	2.10 m	4.52 d (6.8)	4.54 s	4.04 m
16α	2.56 m	2.40 m	2.85 m	3.20 m	1.98 m	1.89 m	5.17 s	2.44 m
16β	2.90 m			1.92 m				
17α	3.34 t (17.5, 17.5)	2.86 m	3.11 m	3.31 ddd (12.1, 21.2,30.1)	1.66 m	3.16 m	–	–
18	0.73 s	1.01 s	0.85 s	0.91 s	0.82 s	0.80 s	0.81 s	0.81 s
19	0.88 s	1.35 s	1.28 s	1.16s	1.27 s	1.30 s	1.23 s	1.26 s
20	–	–	–	–	1.43 m	2.19 m	2.52 m	1.63 m
21	2.19 s	2.17 s	2.12 s	2.10 s	0.95 d (6.5)	0.92 d (6.4)	0.92 d (6.9)	0.86 d (7.1)
22	–	–	–	–	1.25 m	1.42 m	2.65 m	2.46 m
							2.46 m	
23	–	–	–	–	2.26 m	2.10 m	–	–
24	–	–	–	–	6.88 t (7.3, 7.3)	6.68 t (7.1, 7.1)	2.83 m	4.58 m
							2.36 m	
25	–	–	–	–	–	–	2.88 m	2.92 m
27	–	–	–	–	1.86 s	1.71 s	1.11 s	1.02 d (6.5)
28	1.58 s	1.05 s	1.12 s	1.12 s	1.04 s	0.80 s	0.93 s	0.96 s
29	1.02 s	0.92 s	1.10 s	1.10 s	0.82 s	0.92 s	0.81 s	0.84 s
30	1.26 s	1.13 s	1.33 s	1.51 s	1.19 s	0.76 s	1.12 s	1.20 s
COOMe	–	–	–	–	–	–	3.61 s	3.75 s

[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.
[c] Measured in MeOD.

present in the IR spectrum. The ^{1}H and ^{13}C NMR spectra (Table 2) of **3** resembled those of lucidone B (Nishitoba et al., 1985), except for the presence of a hydroxyl carbon signal at δ 72.7 and the absence of carbonyl carbon signal at δ 215.8. In the ^{13}C NMR spectrum of **3**, the upfield shift of C-14 (2.3 ppm) and C-16 (4.6 ppm), and the downfield shift of C-17 (3.7 ppm), indicated that the carbonyl group at C-15 in lucidone B was replaced by a hydroxyl group in **3**. The HMBC correlations of H-15 with C-14, C-16 and C-17 confirmed the above deduction.

In the ROESY spectrum, cross-peak of H-5/H-7 and Me-18/H-15 indicated that 7-OH and 15-OH were β-oriented and α-oriented, respectively. Consequently, the stereochemical structure of lucidone F (**3**) was established to be 7β,15α-dihydroxy-4,4,14α-trimethyl-3,11,20-trioxo-5α-pregn-8-ene.

Compound **4** was accorded the molecular formula of $C_{24}H_{34}O_4$ determined by HRESIMS and ^{13}C NMR data. The resemblance of NMR data of **4** with those of **3** suggested that their chemical structures were similar. However, detailed comparison of NMR data of **3** with those of **4** showed the absence of a hydroxyl carbon signal at δ 69.0 and the presence of a methylene signal at δ 30.0 in the ^{13}C NMR spectrum of **4**. Meanwhile, the HMBC correlations from the methylene hydrogen to C-5, C-6, C-8, C-9 and C-14, along with ^{1}H–^{1}H COSY data, indicated hydrogen at C-7 in **4** instead of the hydroxyl group in **3**. The ROESY correlation of H-15/H_3-30 determined the relative configuration of H-15 to be β. Therefore, the structure of **4** was elucidated as 15α-hydroxy-4,4,14α-trimethyl-3,11,20-trioxo-5α-pregn-8-ene, named lucidone G (**4**).

A molecular formula of $C_{30}H_{44}O_5$ was established for compound **5** from its HRESIMS. The ^{1}H NMR spectrum (Table 1) showed the presence of six singlet methyls at δ 0.82 (s), 0.82 (s), 1.04 (s), 1.19 (s), 1.27 (s), and 1.86 (s), and one doublet methyl at δ 0.95 (d, J = 6.5 Hz). The ^{13}C NMR-DEPT spectra (Table 2) of **5** revealed thirty carbon resonances attributed to seven methyls, eight methylenes, five methines (one oxymethine and one aromatic methine) and ten quaternary carbons (two carbonyl and one carboxyl). 1D NMR characteristics of **5** were similar to those of 7-oxo-ganoderic acid Z (**14**) (Li, Li, & Sun, 2006). However, an additional carbonyl was present in **5**. The HMBC correlation of H_2-12 with this additional carbonyl carbon indicated that the carbonyl group was located at C-11. The relative configuration of 3-OH was assigned as β by the ROESY experiment. Accordingly, the structure of compound **5** was assigned as (24*E*)-3β-hydroxy-7,11-dioxo-lanosta-8,24-dien-26-oic acid, named 7-oxo-ganoderic acid Z_2 (**5**).

Compound **6** was accorded the molecular formula $C_{30}H_{46}O_5$ as established by HRESIMS and ^{13}C NMR data. The IR spectrum of **6** showed the presence of hydroxyl (3424 cm^{-1}) and α,β-unsaturated carbonyl (1684 and 1665 cm^{-1}) groups. 1D NMR data of **6** were closely related to those of 7-oxo-ganoderic acid Z (**14**) (Li et al., 2006) except that a hydroxyl group was connected to C-15 in **6**. An intermolecular hydrogen bond involving the hydroxyl group of C-15 and oxygen atom of carbonyl group at C-7 led to a downfield shift of C-7. The assumption was evidenced by the HMBC correlations of H-5, H-6 and Me-30 with C-7. The ROESY experiment deduced that the relative configurations of 3-OH and 15-OH were β and α, respectively. Therefore, the structure of **6** was (24*E*)-3β,15α-dihydroxy-7-oxo-lanosta-8,24-dien-26-oic acid, named 7-oxo-ganoderic acid Z_3 (**6**).

A molecular formula of $C_{31}H_{45}O_7$ for **7** was established by HRESIMS. The ^{1}H NMR spectrum (Table 1) showed the presence of five tertiary methyl signals at δ 0.81 (s), 0.81 (s), 0.93 (s), 1.11 (s), 1.12

Table 2
^{13}C NMR spectral data for compounds **1–8** [TMS as int. standard].

C	1[a]	2[a]	3[a]	4[b]	5[a]	6[c]	7[a]	8[a]
1	33.5 (t)	34.0 (t)	35.6 (t)	35.5 (t)	33.9 (t)	34.7 (t)	35.9 (t)	35.9 (t)
2	27.3 (t)	27.5 (t)	34.4 (t)	34.4 (t)	27.5 (t)	28.1 (t)	27.1 (t)	27.1 (t)
3	76.9 (d)	77.3 (d)	217.0(s)	216.9 (s)	77.6 (d)	78.7 (d)	77.9 (d)	78.0 (d)
4	39.1 (s)	39.7 (s)	46.8 (s)	47.0 (s)	38.8 (s)	40.2 (s)	39.3 (s)	39.2 (s)
5	50.9 (d)	49.6 (d)	48.8 (d)	51.5 (d)	50.0 (d)	52.4 (d)	52.0 (d)	51.6 (d)
6	36.2 (t)	36.5 (t)	29.3 (t)	18.9 (t)	36.4 (t)	37.6 (t)	38.7 (t)	39.2 (t)
7	199.0 (s)	205.2 (s)	69.0 (d)	30.0 (t)	202.2 (s)	203.5 (s)	213.1 (s)	213.8 (s)
8	146.2 (s)	147.7 (s)	158.5 (s)	164.1 (s)	150.5 (s)	140.1 (s)	53.0 (d)	53.9 (s)
9	151.7 (s)	155.6 (s)	140.5 (s)	138.3 (s)	151.7 (s)	166.1 (s)	61.4 (d)	60.2 (s)
10	40.5 (s)	38.8 (s)	38.2 (s)	37.5 (s)	39.7 (s)	41.4 (s)	37.0 (s)	36.0 (s)
11	198.4 (s)	200.5 (s)	198.4(s)	197.4 (s)	202.4 (s)	29.9 (t)	206.7 (s)	207.7 (s)
12	48.1 (t)	51.4 (s)	50.8 (t)	51.0 (t)	51.5 (t)	43.6 (t)	48.6 (t)	52.5 (s)
13	43.9 (s)	47.2 (s)	46.9 (s)	46.9 (s)	47.3 (t)	44.6 (s)	51.3 (s)	49.0 (s)
14	56.5 (s)	53.7 (s)	54.2 (s)	54.3 (s)	48.9 (s)	50.0 (s)	53.3 (s)	49.2 (s)
15	205.6 (s)	76.8 (s)	72.7 (d)	72.0 (d)	32.1 (t)	67.2 (d)	77.0 (d)	73.9 (d)
16	34.7 (t)	31.1 (t)	30.1 (t)	32.5 (t)	27.3 (q)	33.4 (t)	123.8 (d)	38.4 (t)
17	52.7 (d)	57.3 (d)	56.4 (d)	57.1 (d)	48.9 (d)	49.3 (d)	154.3 (s)	31.7 (s)
18	17.8 (q)	18.5 (q)	19.1 (q)	19.0 (q)	16.7 (q)	28.2 (q)	22.0 (q)	16.3 (q)
19	17.7 (q)	17.5 (q)	19.5 (q)	19.2 (q)	17.5 (q)	25.3 (q)	13.5 (q)	13.5 (q)
20	205.4 (s)	207.6 (s)	207.4 (s)	207.6 (s)	36.0 (d)	37.5 (d)	26.8 (d)	47.4 (d)
21	31.2 (q)	31.1 (q)	31.5 (q)	30.8 (q)	18.3 (q)	19.0 (q)	20.2 (q)	19.4 (q)
22	–	–	–	–	34.5 (t)	36.0 (t)	48.3 (t)	45.1 (t)
23	–	–	–	–	25.7 (t)	26.5 (t)	207.1 (s)	209.7 (s)
24	–	–	–	–	145.1 (d)	144.3 (d)	46.5 (t)	76.8 (t)
25	–	–	–	–	126.6 (s)	128.6 (s)	34.4 (d)	41.9 (d)
26	–	–	–	–	171.7 (s)	171.7 (s)	176.1 (s)	173.8 (s)
27	–	–	–	–	12.0 (q)	12.3 (q)	17.0 (q)	9.5 (q)
28	27.8 (q)	27.5 (q)	27.5 (q)	27.7 (q)	27.8 (q)	16.0 (q)	22.0 (q)	27.5 (q)
29	15.4 (q)	15.3 (q)	20.0 (q)	20.7 (q)	15.4 (q)	17.4 (q)	14.7 (q)	14.8 (q)
30	21.9 (q)	24.9 (q)	20.9 (q)	19.7 (q)	25.8 (q)	19.7 (q)	12.9 (q)	12.4 (q)
COOMe		–		–	–	–	51.8 (q)	53.9(q)

[a] Measured in $CDCl_3$.
[b] Measured in C_5D_5N.
[c] Measured in MeOD.

(s), 1.23 (s), and two doublet methyl signals at δ 0.92 (d, J = 6.9 Hz), and 1.11 (d, J = 7.1 Hz). The ^{13}C NMR-DEPT spectra (Table 2) of **7** revealed thirty-one carbon signals. These NMR characteristics were similar to those of methyl 8β,9α-dihydroganoderate J (Ma, Ye, & Sun, 2002). A sp^2 quaternary carbon signal at δ 154.3 and a sp^2 methine signal at δ 123.8 in the ^{13}C NMR spectrum of **7** indicated the presence of an additional double bond. The HMBC correlations of H-16 with C-15 and C-17, and of H-20, H-21, H-22 with C-17 suggested that the double bond was located at C-16 and C-17. Comparison of the ^{13}C NMR data of **7** with those of methyl 8β,9α-dihydroganoderate J showed that the hydroxyl group (δ 77.9) in **7** replaced the carbonyl group (δ 214.2) in methyl 8β,9α-dihydroganoderate J at C-3, which was confirmed by the HMBC correlations of H-3 with C-1, C-2, and C-4; of Me-28 and Me-29 with C-3.

The ROESY correlations of H-3/H-5; H-3/ Me-29; H-9/H-5; H-9/ H-12α; H-9/ Me-28; H-8/H-6β, H-12β, H-15, Me-18 and Me-19 assigned the relative configuration of H-8 and H-15 as β and H-3, H-9 as α. Thus, the structure of compound **7** was determined.

A molecular formula for **8** was established as $C_{31}H_{48}O_8$ by its HRESIMS and ^{13}C NMR data. Its ^{1}H and ^{13}C NMR data (Tables 1 and 2) were characterised for lanostane triterpenoid and closely related to those of **7**. The group of C(17)=CH(16) in **7** was replaced by the group of CH(17)–CH$_2$(16) in **8**, which was deduced by their different 1D NMR spectra. An additional hydroxyl group signal at δ 76.8 in the ^{13}C NMR data of **8** could be located at C-24, which was confirmed by the HMBC long-range correlations from the signal at δ 76.8 (H-24) to C-23, C-25, C-26 and C-27; from Me-27 to C-24. The further evidence was established from the ^{1}H–^{1}H COSY peak-cross of H-24/H-25/Me-27.

The relative configuration of H-8 and H-15 were assigned as β and H-3, H-9 were assigned as α on the basis of ROESY experiment. The absolute configuration of C-24 was fixed by comparing the ^{13}C NMR values with known compounds having the same chain. C-24 having the *S*-configuration resonated at δ 77.1. In contrast, the atom having the *R*-configuration resonated at δ 80.5 (Hirotani, Zhou, & Furuga, 1994). Signal at δ 76.8 of C-24 in the ^{13}C NMR spectrum of **8** assigned the absolute configuration of C-24 as *S*.

The known isolates were identified by comparing their physical and spectroscopic data with literature data. They were ganoderic acid Y (**9**) (Lee & Ming, 1989), ganoderol B (**10**) (Arisawa, Fujita, & Morita, 1988), lucidone A (**11**) (Nishitoba et al., 1985), lucidone B (**12**) (Nishitoba et al., 1985), lucidone H (**13**) (Nishitoba et al., 1987) and 7-oxo-ganoderic acid Z (**14**) (Li et al., 2006).

3.2. Measurement of maximum nontoxic concentration

The MNTC of eight test compounds in HepG2 cells were 12.88, 137.50, 15.50, 13.70, 33.20, 82.10, 51.10, 371.97 μM, respectively, according to their percentage of growth inhibition in HepG2 cell (<5%).

3.3. Assessment of hepatoprotective activity

The liver is rich in diverse enzymes. Among the most sensitive and widely used of these liver enzymes are the aminotransferases, namely aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT). These enzymes are normally contained within liver cells. If the liver is injured, the liver cells release the enzymes into the blood, raising the enzyme levels in the blood and signalling the liver damage (Nyblom, Berggren, Balldin, & Olsson, 2004). In the present study, ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) at three different doses significantly prevented the elevation of ALT (17.46–22.93 U L^{-1}) and AST

(19.50–29.04 U L^{-1}) levels in HepG2 cells compared to model groups. Meanwhile, compounds **13** and **14** notably inhibited the increase of AST level (23.54–30.09 U L^{-1}) in HepG2 cells induced by H_2O_2. Differences were considered significant for $p < 0.05$, 0.01, as shown in Table 3.

3.4. Assessment of DPPH radical scavenging ability

In the DPPH test, ganoderesin B (**8**), ganoderol B (**10**), and lucidone B (**11**) did not show radical-scavenging effects with scavenging effects (%) of 10.0–14.0% at a concentration of 1.0 mg/ml, compared to 80.77% in vitamin C.

3.5. Determination of CYP3A4 expression

In our study, an *in vitro* system was established to screen inducers of CYP3A4. Ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) were tested for their activation on PXR-mediated CYP3A4 expression. Ganoderol B (**10**) showed significant activation and induction, and its fold induction was stronger than RIF (Fig. 2).

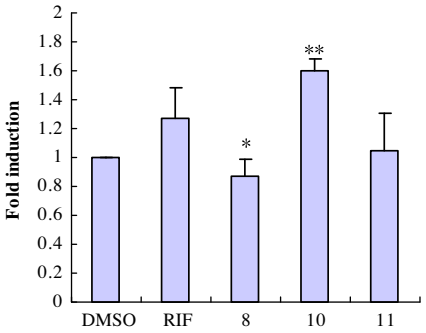

Fig. 2. Compounds **8**, **10**, and **11** (50 μm) induce the expression of CYP3A4 in Huh7 cells. RIF was included as a positive control for inducing CYP3A4 expression. Results are shown as fold induction over vehicle control and represent the average from triplicate assays. Each value represents the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to 50 μm RIF.

4. Discussion

The results showed that ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) could be candidate agents for liver-protection. As far as we know, herbal medicines are always considered to be a safe and useful approach for the treatment of chronic hepatopathy (Gao, Huang, & Zhou, 2003). *Ganoderma* has been widely used for treating and preventing chronic hepatopathy of various etiologies. *G. resinaceum*, as another species of *Ganoderma*, possessed important properties of immunomodulation and liver-protection. Studying the hepatoprotective constituents of *G. resinaceum* provided the chemical basis for researching hepatoprotective activity of *Ganoderma*.

However, the assessment of anti-oxidative activities showed compounds **8**, **10** and **11** did not have DPPH radical scavenging abilities, suggesting that the hepatoprotectve activities of compounds **8**, **10** and **11** were not related to radical scavenging. Because of multiple mechanisms-induced liver injuries, we speculated that compounds **8**, **10** and **11** lowered ALT and AST levels to protect the liver through other pathways.

CYP3A4 was the predominant cytochrome P450 enzyme expressed in the liver and played a critical role in detoxifying drugs. More than 60% of xenobiotics and many other lipophilic compounds increased transcription from target genes such as CYP3A4 by binding and activating PXR (Marko, Andre, & Graham, 2007). An *in vitro* system (PXR-induced CYP3A4 expression) was established to screen inducers of CYP3A4 (Hu et al., 2007). It is reported that two hepatoprotective traditional Chinese medicines (TCM), Wu Wei Zi (*Schisandra chinensis Baill*) and Gan Cao (*Glycyrrhiza*

Table 3
Effects of different concentrations of test compounds on ALT and AST levels in HepG2 cells induced by H_2O_2.

Groups	Concentration (μM)	Number of replications	ALT (U L^{-1})	AST (U L^{-1})
Control	–	3	7.22 ± 1.16	10.53 ± 0.61
Model	–	3	23.22 ± 1.22$^{\#\#}$	32.36 ± 0.79$^{\#\#}$
1	3.23	3	21.14 ± 1.11	30.67 ± 3.73
	6.45	3	20.84 ± 0.86	28.52 ± 2.83
	12.90	3	20.84 ± 1.29	28.29 ± 2.86
3	34.38	3	21.42 ± 1.62	28.05 ± 3.52
	68.75	3	19.90 ± 1.55	31.95 ± 5.84
	137.50	3	20.83 ± 2.63	29.22 ± 7.03
8	3.75	3	22.93 ± 2.67	27.76 ± 1.6*
	7.50	3	19.50 ± 1.40*	26.77 ± 1.69**
	15.00	3	17.46 ± 1.22**	21.18 ± 4.52**
9	3.43	3	22.35 ± 0.93	31.60 ± 1.26
	6.85	3	22.64 ± 1.88	30.27 ± 2.54
	13.70	3	21.65 ± 1.55	27.60 ± 0.78
10	8.30	3	21.19 ± 2.13	29.04 ± 1.56*
	16.60	3	18.51 ± 1.72*	23.98 ± 3.83*
	33.20	3	18.51 ± 1.22**	19.50 ± 1.88**
11	20.53	3	20.87 ± 1.26	28.17 ± 3.47
	41.05	3	20.66 ± 0.86*	27.76 ± 1.20**
	82.10	3	18.97 ± 1.66*	26.42 ± 2.63**
13	12.78	3	23.51 ± 5.80	29.74 ± 5.11
	25.55	3	21.42 ± 0.88	26.83 ± 2.88*
	51.10	3	21.36 ± 1.83	23.54 ± 4.95*
14	93.00	3	21.36 ± 0.71	30.09 ± 1.91
	186.00	3	20.95 ± 1.77	24.27 ± 1.98**
	372.00	3	20.25 ± 2.44	25.63 ± 3.84*

Notes: $n = 3$, mean ± S.D.
$^{\#\#}$ $p < 0.01$, compared to normal group.
* $p < 0.05$.
** $p < 0.01$, compared to model group.

uralensis Fisch) may protect liver by activating PXR and inducing CYP3A4 expression (Mu, Zhang, & Xie, 2006). In our study, activation on PXR-mediated CYP3A4 expression of ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) were tested. Ganoderol B (**10**) showed significant activation and induction activity, which indicated that ganoderol B (**10**) may activate PXR and induce CYP3A4 to decrease ALT and AST levels. The specific mechanism for hepatoprotective effects of ganoderol B (**8**), ganoderol B (**10**) and lucidone A (**11**) should be explored in the future.

5. Conclusion

In summary, this study demonstrated that ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) had significant hepatoprotective activities, due to their remarkable *in vitro* inhibitory activities against the increase of ALT and AST levels in HepG2 cells induced by H_2O_2. Ganoderesin B (**8**), ganoderol B (**10**) and lucidone A (**11**) did not show DPPH scavenging abilities indicating that they did not protect the liver by scavenging radicals. However, ganoderol B (**10**) notably activated PXR-mediated CYP3A4 expression, suggesting that PXR-mediated CYP3A4 expression might be a potential molecular mechanism of ganoderol B (**10**). Future studies are needed to focus on hepatoprotective activities of triterpenoids from *G. resinaceum* and clarify their molecular mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.03.071.

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