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The Triterpenoid High-Performance Liquid Chromatography Analytical Profiles of the Mycelia of *Ganoderma lucidum* (*lingzhi*)

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Abstract: (1) Background: Ganoderic acids (GAs) are specific triterpenes of *Ganoderma lucidum*. The HPLC fingerprint profile of GAs of the fruiting body is well known, but their mycelial fingerprinting remains unclear. (2) Methods: An ethanol extract of the mycelium of *G. lucidum* (*YK-01*) was further purified via preparative HPLC. The triterpenoid compositions for four strains of *G. lucidum* and one strain of *G. formosanum (purple lingzhi)* were analyzed using HPLC. (3) Results: Nineteen lanostane triterpenes, including five new triterpenes, GA-TP (**1**), ganodermic acid J_c (GmA-J_c) (**2**), GmA-J_d (**3**), GA-TQ_1 (**4**), and ganoderal B_1 (**5**), and fourteen known triterpenes **6**–**19** were isolated from the ethanol extract. Their structures were identified by mass and extensive NMR spectroscopy. A green chemical HPLC analytical method was developed using ethanol and acetic acid as a mobile phase, and all isolated compounds can be well separated. These triterpenes comprise a unique HPLC chromatograph of the *G. lucidum* mycelium. All four *G. lucidum* strains showed the same HPLC chromatographic pattern, whereas *G. formosanum* displayed a different pattern. Quantitation methods for ganoderic acid T (**10**) and S (**12**) were also validated. (4) Conclusions: The triterpenoid HPLC analytical method can be used to identify the *G. lucidum* species and to determine the contents of GA-T and GA-S.

Keywords: ganoderic acids; *Ganoderma lucidum*; HPLC fingerprint

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1. Introduction

Ganoderma lucidum, or lingzhi, is a well-known medical fungus. Due to its multiple bioactivities, such as immune modulation, anti-cancer functions, and liver protection [1], many dietary supplements have been developed from lingzhi and are now highly popular on the market. Most of the raw materials are derived from the fruit body and the mycelium (or the fermentation powder) of *G. lucidum* [2], with the same active constituents, polysaccharides, and triterpenes [3]. In our previous studies, nine ganoderic acids [4] and fifteen lucidenic acids (LAs) [5] were isolated and used for the identification of *G. lucidum* fruiting bodies. In contrast to the well-established GA HPLC fingerprint profiles of the fruit body, reports of *G. lucidum* mycelia are rare. In addition, the identification and quality control of the mycelium products of *G. lucidum* are lacking. On the other hand, GAs from the mycelium of *G. lucidum* have attracted much attention due to their significant anti-tumor activities [6]. For example, GA-T (**10**) shows the most prominent effect against LLC metastasis in vitro and in vivo [7] and exerts anti-tumor effects against A549 tumors in xenograft SCID mice [8]. Though GA-T and twenty-four other triterpenes have been used as marker compounds for the HPLC analysis of *G. lucidum* mycelia, the HPLC fingerprint profiles remain to be clarified [9]. Therefore, it is necessary to establish an unambiguous triterpenoid HPLC fingerprint profile for the identification and quality control of *G. lucidum*

mycelium. Here, we isolated nineteen lanostane triterpenes, including five new compounds **1**–**5** and fourteen known compounds, namely, GA-U1 (**6**), ganorbiformin C (**7**), GA-P (**8**), GA-Q (**9**), GA-T (**10**), GA-TS (**11**), GA-S (**12**), GA-R (**13**), GA-Me (**14**), GmA-S (**15**), GmA-T-O (**16**), GA-Mf (**17**), GmA-P2 (**18**), and GA-Y (**19**), from the mycelium of *G. lucidum YK-01*. Their structures were identified via matching with the published literature [10–14] and spectroscopic data (^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC, and mass spectrometry). A novel and green chemical HPLC analytical method for these triterpenes was also established, which used ethanol and acetic acid as a mobile phase. Using this analytical method, a unique HPLC chromatographic profile for the triterpenes of the mycelium of *G. lucidum* was revealed for the first time. Moreover, the HPLC spectral patterns of GA-type mycelia (*YK-01* and *BCRC36065*) were compared with those of the LA-type (*YK-02* and *BCRC36090*) and purple lingzhi (*G. formosanum* and *CCRC37048*). All strains of *G. lucidum* showed the same spectral profile, while *G. formosanum* displayed a varying pattern. The aim of this study was to develop a rapid, inexpensive, and reliable method for routine authentication of *G. lucidum* mycelia. Because GA-T (**10**) and GA-S (**12**) are the major compounds in *G. lucidum* mycelia, the HPLC quantitation methods of **10** and **12** were evaluated.

2. Materials and Methods

2.1. Chemicals and Apparatus

The absolute ethanol (Fisher Chemical, purity 98%) and glacial acetic acid (PanReac AppliChem, purity 99.8%) were HPLC-grade. The cultivation media including glucose (Dongxiao, Zhucheng, China) [15,16], sucrose (Taiwan Sugar Corporation, Kaohsiung, Taiwan), soy peptone (Organotechnie, La Courneuve, France), malt extract (Stbio media, New Taipei City, Taiwan), yeast extract (Guangxi Yipinxian Biotechnology Co., Ltd., Guangxi, China), and potassium phosphate (Lianyungang Hengsheng Food Additive Co., Ltd., Lianyungang, China) were food-grade. MEA (malt extract agar) was purchased from Merck (Darmstadt, Germany), and PDA was purchased from Difco (Becton Dickinson & Co., Sparks, MD, USA). The melting points were determined using a MEL-TEMP II apparatus (Laboratory Device INC., Itasca, IL, USA), and the thermometer (0–300 °C) was calibrated with a standard electronic thermometer (TES 1384, Taiwan). The ^{1}H NMR and ^{13}C NMR spectra were measured using a Bruker AMX-700MHz spectrophotometer (Bruker BioSpin Corporation, Billerica, MA, USA). The mass spectra were measured using an electronic ionization time-of-flight (EI-TOF) mass spectrophotometer (JMS-T200GC AccuTOF GCx-plus, JEOL, Ktoto, Japan) equipped with a DIP sampling device or a high-performance compact mass spectrometer (Expression CMS^{L}, Advion Inc., Ithaca, NY, USA). The measurement conditions were as previously described [7]. Preparative HPLC was performed using a YMC preparative HPLC (DAU-100-700, Kyoto Chromato Co., Ltd., Kyoto, Japan) equipped with a reversed-phase column (ODS-AQ-HG, 15 µm, 100 × 600 mm, YMC, Kyoto, Japan) and a UV-VIS detector. The detective wavelength was set at 243 nm. Semi-preparative HPLC was performed using a SPOT PREP II liquid chromatographer (Armen Instrument, Paris, France). A column of Merck Hibar (25 × 250 mm RP-18e 5 µm) was used to isolate the triterpenes. HPLC analysis was performed using a Hitachi CM 5000 series equipped with a CM5110 pump and CM5340 photo-diode array detector (Hitachi, Tokyo, Japan). The detector wavelength was set at 243 nm. A column of COSMOSIL 5C18-MS-II (4.6 × 250 mm) and a high-speed refrigerated centrifuge were used (Himac CR 22G, Hitachi, Tokyo, Japan).

2.2. Cultivation of Ganoderma Mycelia

The mycelia (in a 7 cm Petri dish of MEA) of five *G. lucidum* strains, *YK-01*, *YK-02*, *BCRC36065*, *BCRC36090*, and *CCRC 37048*, were provided by Lingzhi Agricultural Co., Ltd., Baiho, Tainan City, Taiwan. In order to produce enough mycelia biomass for HPLC analysis, the mycelia with a 1.0 cm edge length were cut from the plates, inoculated into an 18 cm Petri dish containing MEA and 0.1% (weight percentage) plant fiber (Vitacel wheat fiber, Gemfont, Taipei, Taiwan), and cultivated in an incubator at 25 °C for 20 days. The mycelia

were then collected, washed with water, and dried in an oven (50 °C for 48 h) for further HPLC analysis. A seed liquid culture was first prepared for the large-scale cultivation of the *Ganoderma* mycelium *YK-01*. The medium for the seed culture was composed of glucose (30 g/L), sucrose (15 g/L), soy peptone (3 g/L), yeast extract (2 g/L), and potassium phosphate (0.6 g/L) in a 1 L fermentation flask (total volume 400 mL). After sterilization (120 °C, 30 min), three pieces of mycelia with a 0.5 cm edge length were cut from a PDA plate, inoculated into the liquid medium, and cultivated at 28 °C on a rotary shaker at 85 rpm for 10 days. The seed culture was then inoculated into 30 sterilized aluminum containers (60 × 40 × 2 cm) containing 48.0 g of malt extract, 4.8 g of soy peptone, and 1.1 g of plant fiber in 1.1 L of water. The static cultivation of the mycelium was carried out in a clean room at 28 °C with a humidity of 80–100% for 20 days. The harvested mycelia were collected and washed with water to remove the medium. Then, the wet mycelial plates were freeze-dried to yield 1.2 kg of *Ganoderma* mycelium.

2.3. HPLC Analysis of the Triterpenes 1–19

The mobile phase was absolute ethanol (A) and 0.5% aqueous acetic acid (B). The elution program was set as the isocratical mode with 65% A for 40 min. The flow rate was 0.8 mL/min. The injection volume was 10 μL, and detection was set at 243 nm.

*2.4. Isolation of Compounds **1**–**5** from YK-01 Mycelium*

The *YK-01* mycelium powder (1 kg) was grounded and extracted with 20 L of 80% ethanol at 60 °C in a water bath for 3 h. After cooling, the mixture was filtered through a filter paper (Advantech No.1, 110 mm) on a Büchner funnel, and the filtrate was concentrated to 2 L with a rotary evaporator. Then, 10 L of water was added to the concentrate and set aside for precipitation. The precipitate was collected via centrifugation using a freeze centrifuge under 15,100× *g* at 4 °C and then freeze-dried to yield a brown solid (38 g, yield 3.8%). The solid was dissolved in 400 mL of 80% ethanol and subjected to preparative HPLC (YMC) to separate mycelia triterpenes. A gradient elution from 50% to 80% ethanol was used, and the eluents were collected according to their signals. Five fractions were collected and then freeze-dried to yield Fraction-1 (50% ethanol, Fr.1, 2.5 g), Fraction-2 (60% ethanol, Fr.2, 3.3 g), Fraction-3 (65% ethanol, Fr.3, 5.1 g), Fraction-4 (75% ethanol, Fr.4, 15.2 g), and Fraction-5 (80% ethanol, Fr.5, 5.4 g). Fr. 1 was dissolved in 80% ethanol and subjected to semi-preparative HPLC (Armen) for further purification. Four sub-fractions, Fr.1-1, Fr.1-2, Fr.1-3, and Fr.1-4, were collected using 50% ethanol as the mobile phase. The eluents were concentrated and crystallized in situ. From Fr.1-3, compound **2** was obtained as a white powder (4.8 mg). Compound **3** was isolated from Fr. 1-2 as an amorphous powder (3.4 mg). Fr. 1-4 was further purified using the same procedures as Fr. 1-3, and compound **1** (20.4 mg) was obtained as colorless needle crystals. From the remaining mixture of Fr.1-4, compound **4** (2.4 mg) was further isolated as colorless crystals. Fr.4 was further purified using the same conditions as Fr.1, which yielded Fr. 4-1, Fr.4-2, and Fr.4-3. From Fr. 4-3, GA-S (12) was obtained as pale-yellow needle crystals, and compound **5** (2.0 mg) was obtained as colorless needle crystals from the remaining mixture.

*2.5. Determining Ganoderic Acid T (**10**) and S (**12**)*

2.5.1. Sample Pretreatment

A total of 0.5 g of the mycelia powder (100 mesh) in 20 mL of 80% ethanol was sonicated for 3 h at 60 °C. Then, the mixture was centrifuged for 15 min at 6000 rpm. The supernatant (2 mL) was passed through a 45 μM membrane filter (Millex-LCR, Merck, Darmstadt, Germany), and 10 μL of the filtrate was injected into the high-performance liquid chromatographer for analysis.

2.5.2. Calibration Curve

The stock solutions of 10 (4 mg/mL) and 12 (4 mg/mL) were serially diluted with 80% ethanol to the concentrations of 20.0, 100.0, 400.0, 800.0, and 1000.0 μg/mL. Calibration

curves were obtained with five different concentrations in triplicate and were plotted from the linear regression of peak area versus concentration.

2.5.3. Recovery Tests

After 0.5 g of 100 mesh of *G. lucidum* mycelium (*YK-01*) was weighed, 1 mL of 100, 400 and 800 μg/mL solutions of 10 and 12 was added. A blank group was prepared with no external addition and was also used for the determination of 10 and 12 in the mycelium. The mixtures were dried overnight in an oven at 60 °C and then prepared as sample solutions, as mentioned above.

2.5.4. Limit of Detection and Limit of Quantitation

We used diluted solutions of the lowest concentrations of **10** (20 μg/mL) and **12** (20 μg/mL) and performed five injections. The limit of detection (LOD) is the average concentration presented as the signal-to-noise ratio (S/N) $\geq$ 3. The limit of quantitation (LOQ) is the average concentration of S/N $\geq$ 10.

2.5.5. Accuracy and Precision

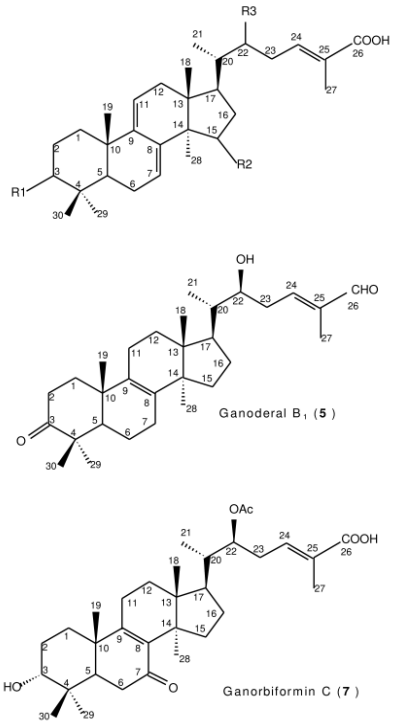
Intraday and interday tests were used to evaluate the accuracy and precision of the analytical methods. Low, medium, and high concentrations in the range of the calibration curve were analyzed five consecutive times for intraday precision. Interday precision was similarly determined on three separate days.

3. Results

3.1. Structural Identification of the New Triterpenes 1~5

The structures of GAs from the mycelia of *G. lucidum* usually have a C30 (carbon numbers, Figure 1) skeleton, with a high number of similarities [3]. Compound **1** was isolated as colorless needle crystals (mp 231~233 °C). The molecular formula was determined as $C_{34}H_{50}O_7$ from its mass ($[M]^+$ *m/z* 570) and DEPT spectra, which were the same for GA-P (**8**) (or GA-Q, **9**), suggesting an isomer of **8** (or **9**). Its ^{13}C NMR (Table 1) spectral data were very similar to those of GA-T (**10**) [10], except for the signal at δ72.5, suggesting a β-hydroxyl group at C-22 instead of an acetyl group in 10. The β-orientation of the 22-hydroxyl group was confirmed by the upfield shift of the C-22 proton (δ3.80, dd, *J* = 8.4, 4.2 Hz, Table 2) compared with its α-hydroxyl stereo-isomer previously isolated from the *G. lucidum* mycelium [11]. These data indicate that compound **1** is a 22β-hydroxyl derivative of GA-T and a positional isomer of GA-P or GA-Q. Assignments of its ^1H NMR and HMBC spectral data were presented in Table 2. Hence, it was assigned as a 3α,15α-diacetoxy-22β-hydroxy-5α-lanosta-7,9(11)-trien-26-oic acid, namely, ganoderic acid TP (GA-TP).

Compound **2** was isolated as a white amorphous powder (mp 175~177 °C). The molecular formula was determined as $C_{30}H_{46}O_4$ from its mass ($[M]^+$ *m/z* 470) and DEPT spectra. Its formula is the same as that of ganodermic acid J_b (GmA-J_b) previously isolated from the *G. lucidum* mycelium [12]. GmA-J_b has two hydroxyl groups at C-3 and C-15; however, a complete assignment of its ^1H NMR is lacking. The ^{13}C NMR spectrum (Table 1) of 2 showed a singlet at δ73.4, corresponding to δ3.80 (dd, *J* = 7.0, 6.3 Hz) in the HSQC spectrum (see GmA-Jc (2) in the Supplementary Materials), indicating a β-hydroxyl group at C-22, the same as compound **1**. Another singlet was observed at δ79.6, corresponding to δ3.16 (dd, *J* = 11.2, 4.2 Hz) in the HSQC spectrum (see GmA-Jc (2) in the Supplementary Materials), suggesting a β-hydroxyl group at C-3 identical to that of the GmA-J_b. Hence, it is a positional isomer of GmA-J_b, assigned as 3β,22β-dihydroxy-5α-lanosta-7,9(11)-trien-26-oic acid, and named as ganodermic acid J_c (GmA-J_c). The assignments of its ^{13}C NMR,^1H NMR, and HMBC spectral data were completed, as shown in Tables 1 and 2.



Compounds	R1	R2	R3
1	α-OAc	α-OAc	β-OH
2	β-OH	H	β-OH
3	α-OH	α-OH	β-OH
4	β-OH	α-OAc	β-OH
6	=O	H	β-OH
8	α-OH	α-OAc	β-OAc
9	α-OAc	α-OH	β-OAc
10	α-OAc	α-OAc	β-OAc
11	β-OAc	α-OAc	β-OAc
12	α-OH	H	β-OAc
13	α-OAc	H	β-OAc
14	α-OAc	α-OAc	H
15	β-OAc	α-OAc	H
16	β-OAc	α-OH	H
17	α-OAc	α-OH	H
18	β-OH	α-OAc	β-OAc
19	β-OH	H	H

Figure 1. Structure of compounds **1~19** isolated from the *G. lucidum* (*YK-01*) mycelium.

Table 1. ^{13}C NMR spectral data of compounds **1–5**. Compounds **1**, **5** and, **2**, **3**, **4** were dissolved in $CDCl_3$ and CD_3OD, respectively.

	1	**2**	**3**	**4**	**5**
No.					
1	30.6 (t)	37.1 (t)	31.2 (t)	37.1 (t)	30.8 (t)
2	23.1 (t)	28.3 (t)	26.7 (t)	28.5 (t)	27.6 (t)
3	78.1 (d)	79.6 (d)	76.8 (d)	79.6 (d)	217.9 (s)
4	36.5 (s)	38.6 (s)	38.4 (s)	38.6 (s)	36.9 (s)
5	44.0 (d)	50.7 (d)	44.3 (d)	50.5 (d)	51.2 (d)
6	22.8 (t)	24.1 (t)	24.0 (t)	24.0 (t)	26.3 (t)
7	121.2 (d)	121.6 (d)	122.6 (d)	122.6 (d)	19.4 (t)
8	140.1 (s)	143.9 (s)	142.4 (s)	141.7 (s)	133.3 (s)
9	145.9 (s)	147.5 (s)	147.9 (s)	147.4 (s)	135.1 (s)
10	37.3 (s)	39.8 (s)	38.5 (s)	39.8 (s)	44.5 (s)
11	115.6 (d)	117.4 (d)	116.7 (d)	117.4 (d)	21.0 (t)
12	38.0 (t)	39.1 (t)	39.8 (t)	39.3 (t)	31.0 (t)
13	43.9 (s)	44.8 (s)	45.4 (s)	45.1 (s)	47.4 (s)
14	51.4 (s)	51.5 (s)	53.4 (s)	52.6 (s)	49.9 (s)
15	77.3 (d)	32.6 (t)	75.2 (d)	78.8 (d)	36.0 (t)
16	36.4 (t)	28.5 (t)	39.9 (t)	37.4 (t)	35.2 (t)
17	45.2 (d)	48.6 (d)	46.4 (d)	46.5 (d)	46.8 (d)
18	15.9 (q)	16.2 (q)	16.5 (q)	16.4 (q)	15.8 (q)
19	22.6 (q)	23.3 (q)	23.3 (q)	23.3 (q)	24.2 (q)
20	40.8 (d)	42.4 (d)	42.3 (d)	42.1 (d)	41.4 (d)
21	11.5 (q)	12.1 (q)	12.2 (q)	12.1 (q)	11.7 (q)
22	72.5 (d)	73.4 (d)	73.4 (d)	73.2 (d)	72.8 (d)
23	35.2 (t)	35.8 (t)	35.8 (t)	35.7 (t)	34.6 (t)
24	141.0 (d)	141.0 (d)	141.0 (d)	140.8 (d)	151.3 (d)
25	128.9 (s)	130.1 (s)	130.1 (s)	130.2 (s)	140.6 (s)
26	171.3 (s)	171.7 (s)	171.5 (s)	173.1 (s)	195.2 (d)
27	12.4 (q)	12.8 (q)	12.7 (q)	12.8 (q)	9.5 (q)
28	18.5 (q)	26.2 (q)	18.0 (q)	18.9 (q)	18.7 (q)
29	27.8 (q)	28.8 (q)	28.9 (q)	28.8 (q)	26.2 (q)
30	22.4 (q)	16.5 (q)	23.4 (q)	16.5 (q)	21.3 (q)
OAc	170.8 (s)			171.6 (s)	
OAc	170.8 (s)				
$OCOCH_3$	21.3 (q)			21.2 (q)	
$OCOCH_3$	21.4 (q)				

Table 2. ^{1}H NMR and HMBC spectral data of compounds **1–5**. Compounds **1**, **5** and, **2**, **3**, **4** were dissolved in $CDCl_3$ and CD_3OD, respectively.

	1		**2**		**3**		**4**		**5**	
	δ_H	HMBC (H to C)	δ_H	HMBC (H to C)	δ_H	HMBC (H to C)	δ_H	HMBC (H to C)	δ_H	HMBC (H to C)
1	1.64 (a, br.t, *J* = 14.0 Hz) 1.75 (b, dt, *J* = 13.3, 3.5 Hz)	10,19 10,19	1.42 (a, m) 2.04 (b, m)	4 3,4,5	1.69 (a, dt, *J* = 13.3, 3.5 Hz) 1.81 (b, br.d. *J* = 10.5, 3.5 Hz)		1.43 (a, ddd, *J* = 14.7, 10.5, 4.2 Hz) 2.04 (b, ddd, *J* = 13.5, 7.7, 3.5 Hz)	 5	1.26 (a, t, *J* = 7.5 Hz) 1.61 (b, m)	 19
2	1.73 (m) 1.95 (br.t, *J* = 12.6, 14.2 Hz)		1.35 (m) 2.09 (m)		2.03 (m)		1.68 (m)	3	1.38 (a, dd, *J* = 7.5, 5.5, 2.0 Hz) 1.98 (b, m)	
3	4.69 (s)	1,2,4,5	3.16 (dd, *J* = 11.2, 4.2 Hz)		3.38 (s)	1,5	3.16 (dd, *J* = 11.2, 4.9 Hz)	29,30		
5	1.50 (dd, *J* = 11.9, 3.56 Hz)	4,6,10	1.10 (dd, *J* = 11.2, 4.2 Hz)	6	1.57 (dd, *J* = 11.2, 4.9 Hz)	9,10	1.08 (dd, *J* = 11.9, 4.2 Hz)	2,4,6,10,29	1.60 (m)	
6	2.03 (m)	7,8,10	2.10 (m)	5	2.08 (m)		2.10 (m)		2.08 (m)	
7	5.50 (d, *J* = 4.9 Hz)	5,6,9,14	5.51 (br.d, *J* = 5.6 Hz)	6,9	5.91 (d, *J* = 5.6 Hz)	8	5.52 (d, *J* = 7.0 Hz)	5,6,9,14	1.64 (m)	
11	5.34 (d, *J* = 5.6 Hz)	8,12,13	5.37 (br.d, *J* = 6.3 Hz)	8,12,13	5.37 (d, *J* = 6.3 Hz)	9,10,13	5.40 (d, *J* = 6.3 Hz)	8,12,13	2.05 (m)	
12	2.05 (a, m) 2.38 (b, d, *J* = 17.5 Hz)	8,9,11,13,18 8,9,11,13,18	2.13 (a,m) 2.27 (b, br.d, *J* = 17.5 Hz)	11,13,14 11,13,18	2.09 (a, m) 2.35 (b, d, *J* = 17.5 Hz)	9,11,14 9,11,13,18	2.13 (a,m) 2.38 (b, d, *J* = 17.5 Hz)	9,11,13,14,18 9,11,13,18	1.70 (a, m) 1.80 (b, m)	
15	5.08 (dd, *J* = 9.1, 5.6 Hz)	28	1.44 (b,m) 1.67 (a, m)	13 28	4.24 (dd, *J* = 9.1, 5.6 Hz)		5.07 (dd, *J* = 9.8, 5.6 Hz)	28	1.63 (a, m) 1.98 (b, m)	
16	1.79 (b, ddd, *J* = 9.8, 5.6, 4.2 Hz) 2.17 (a, m)	13,15 14,15,17,OAc	1.68 (m)		1.88 (m)		1.82 (b, ddd, *J* = 9.8, 8.4, 4.2 Hz) 2.10 (a, m)	13,15 13,17	2.42 (b, m) 2.61 (a, m)	
17	2.08 (m)	13,14,16,20,18	2.03 (m)	16	2.08 (m)		2.11 (m)	16,18	1.87 (dd, *J* = 23.5, 7.0 Hz)	13,18,20
18	0.68 (s)	12,13,14,17	0.61 (s)	8,12,13,14,17	0.66 (s)	12,13,14,17	0.69 (s)	12,13,14,17	0.73 (s)	12,13,14,17
19	0.99 (s)	1,5,9,10	1.01 (s)	1,5,9,10	1.03 (s)	1,5,9,10	1.01 (s)	1,4,5,9,10	0.93 (s)	1,3,9,10
20	1.44 (m)	17,21,22	1.44 (m)		1.38 (m)		1.40 (m)	17	1.48(m)	
21	0.93 (d, *J* = 6.3Hz)	17,20,22	0.92 (d, *J* = 7.0 Hz)	17,20,22	0.91 (d, *J* = 6.3 Hz)	17,20,22	0.92 (d, *J* = 7.0 Hz)	17,20,22	0.96 (d, *J* = 5.0 Hz)	17,20,22
22	3.80 (dd, *J* = 8.4, 4.2 Hz)		3.80 (dd, *J* = 7.0, 6.3 Hz)	23	3.74 (t, *J* = 7.7 Hz)		3.69 (t, *J* = 7.0 Hz)	17,21,24	3.90 (dd, *J* = 6.5 3.5 Hz)	
23	2.24 (m) 2.49 (ddd, *J* = 15.4, 8.4, 7.0 Hz)	20,22,24,25 20,22,24,25	2.29 (ddd, *J* = 14.0, 7.7, 7.0 Hz) 2.43 (ddd, *J* = 14.7, 7.7, 7.0 Hz);	22 22,24,26	2.28(ddd, *J* = 14.0, 7.7, 7.0 Hz) 2.43(ddd, *J* = 14.7, 7.7, 7.0 Hz);	 24	2.29 (ddd, *J* = 14.0, 7.7, 7.0 Hz) 2.43 (ddd, *J* = 14.7, 7.7, 7.0 Hz)	22,25 22,25	2.42 (m) 2.60 (m)	24 22,24,25
24	6.92 (t, *J* = 7.0 Hz)	22,23,25,27	6.83 (dd, *J* = 7.0, 6.3 Hz)	23,26	6.84 (t, *J* = 7.0 Hz)		6.82 (t, *J* = 7.7 Hz)	23,25,27	6.59 (t, *J* = 5.0 Hz)	
26									9.44 (s)	25,27
27	1.87 (s)	24,25,26	1.83 (s)	24,25,26	1.84 (s)	24,25,26	1.83 (s)	24,25	1.78 (s)	24,25,26
28	1.10 (s)	8,13,14,15	0.94 (s)	8,13,14,15	0.98 (s)	7,8,13,14,15	1.07 (s)	8,13,14,15	1.12 (s)	8,15
29	0.90 (s)	3,4,5,30	0.99 (s)	3,5,10,30	0.96 (s)	3,4,5,30	0.99 (s)	3,5,10,30	1.10 (s)	3,5,30
30	1.00 (s)	3,4,5,29	0.88 (s)	3,5,10,29	0.94 (s)	3,4,29	0.87 (s)	3,5,10,29	1.07 (s)	3,5,29
$OCOCH_3$	2.06 (s)						2.09 (s)			
$OCOCH_3$	2.10 (s)									

Compound **3** was isolated as an amorphous powder (mp 197~199 °C). The molecular formula was determined as $C_{30}H_{46}O_4$ from its MS ($[M]^+$ m/z 486) and DEPT spectra, showing the same formula with the 3α,15α, 22α-trihydroxy-lanosta-7,9(11),24-trien-26-oic acid, a GA derivative with three hydroxyl groups in the molecule previously isolated from the mycelium of *G. lucidum* [12]. The ^{13}C NMR (Table 1) of 3 showed three hydroxyl groups at C-3 (δ76.8), C-15 (δ75.2), and C-22 (δ73.4), suggesting an isomer of the trihydroxyl GA [12]. However, a complete assignment of the ^1H NMR of the trihydroxyl GA has not been conducted. The ^1H NMR (Table 2) of 3 showed signals at δ3.38 (s) and δ4.24 (dd, J = 9.1, 5.6 Hz), representing two α-hydroxyl groups at C-3 and C-15, the same as its isomer. But the signal at δ3.74 (t, J = 7.7 Hz) indicated a β-hydroxyl group at C-22, the same as compounds **1** and **2**. Hence, it was assigned as the 3α,15α,22β-trihydroxy-lanosta-7,9(11),24-trien-26-oic acid. We tentatively named it ganodermic acid Jd (GmA-Jd) for systematic consideration. Combining the HSQC (see GmA-Jd (3) in the Supplementary Materials) and HMBC spectral data, the assignments of their ^1H NMR spectral data were completed, as shown in Table 2.

Compound **4** was isolated as colorless needle crystals (mp 227~229 °C). The molecular formula was determined as $C_{32}H_{48}O_6$ from its mass ($[M]^+$ m/z 528) and DEPT spectra. The characteristic signal at δ3.16 (dd, J = 11.2, 4.2 Hz) in its ^1H NMR spectrum (Table 2) represented a β-hydroxyl group at C-3. The ^{13}C NMR (Table 1) spectrum showed a singlet at δ73.2, indicating a β-hydroxyl group on C-22 similar to compound **2**. Another singlet was observed at δ78.8, corresponding to δ5.07 (dd, J = 9.8, 5.6 Hz) in the HSQC spectrum (see GA-TQ_1 (4) in the Supplementary Materials), representing an α-acetoxyl group attached to C-15, similar to those of GA-P (**8**) and GA-T (**10**) [10]. Hence, it was assigned as a 3β,22β-dihydroxy-15α-acetoxy-5α-lanosta-7,9(11)-trien-26-oic acid and tentatively named as ganoderic acid TQ_1. It is a stereo-isomer at C-3 for GA-U2, recently isolated from the mycelia mat of *G. lucidum* [13]. The assignments of its ^{13}C, ^1H NMR, and HMBC spectral data are listed in Tables 1 and 2.

Compound **5** was isolated as colorless needle crystals (mp 168~170 °C). Its molecular formula was determined as C_{30} $H_{46}O_3$ from its MS ($[M]^+$ m/z 454) and DEPT spectra. The formula is the same as that of ganoderal B, originally isolated from the *G. lucidum* fruit body [14]. Its ^{13}C NMR spectrum (Table 1) showed a signal at δ217.9, indicating a carbonyl group at C-3. An aldehyde group was observed at δ195.2, corresponding to δ9.44 (s, 26-H) in its HSQC spectrum (see Ganoderal B_1 (5) in the Supplementary Materials). These data suggested that 5 was a ganoderic aldehyde similar to ganoderal B. However, the ^1H NMR (Table 2) of 5 showed the characteristic signal at δ3.90 (dd, J = 6.5, 3.5 Hz), indicating a β-hydroxy group at C-22. Therefore, it was confirmed as 22β-hydroxy-3-oxo-5-α-lanosta-8,24E-diene-26-al and named as ganoderal B_1, a positional isomer of ganoderal B in which the hydroxyl group is at C-7 [14]. The complete assignments of its ^{13}C, ^1H NMR, and HMBC spectral data are listed in Tables 1 and 2.

3.2. HPLC Fingerprint Profiles of Triterpenes from Ganoderma Mycelia

We found that with aqueous ethanol, we were able to purify GA-T (10) and GA-S (12) from the ethanol extract of the *Ganoderma* mycelium using semi-preparative HPLC. Thus, ethanol and aqueous acetic acid were used as the mobile phase, and the spectrum of the isolated triterpenes is shown in Figure 2a. All compounds were well separated. This is the first green chemical HPLC method for the analysis of *Ganoderma* triterpenes. In the four strains of *G. lucidum*, compounds **9** (GA-Q), **10** (GA-T) and **12** (GA-S) were detected as the major signals (Figure 2b–e). Compounds **1**, **2**, **8**, **11**, and **13**–**17** were detected as minor components, and the rest of the triterpenes were trace compounds. Though the contents of these triterpenes for the four strains were different, they showed an identical HPLC spectral pattern. In *G. lucidum YK-01* (Figure 2b), the first notable peak in the HPLC spectrum was GA-P (**8**), followed by GA-TP (**1**) and GA-Q (**9**), two major signals **10** and **12**, and finally, **13** and **14** as two consecutive signals. The same pattern was observed for the other three *G. lucidum* strains (Figure 2c–e). The strain of *G. formosanum CCRC37048*

displayed a different HPLC spectral pattern from *G. ludcidum* and had an extremely low amount of GAs (Figure 2f).

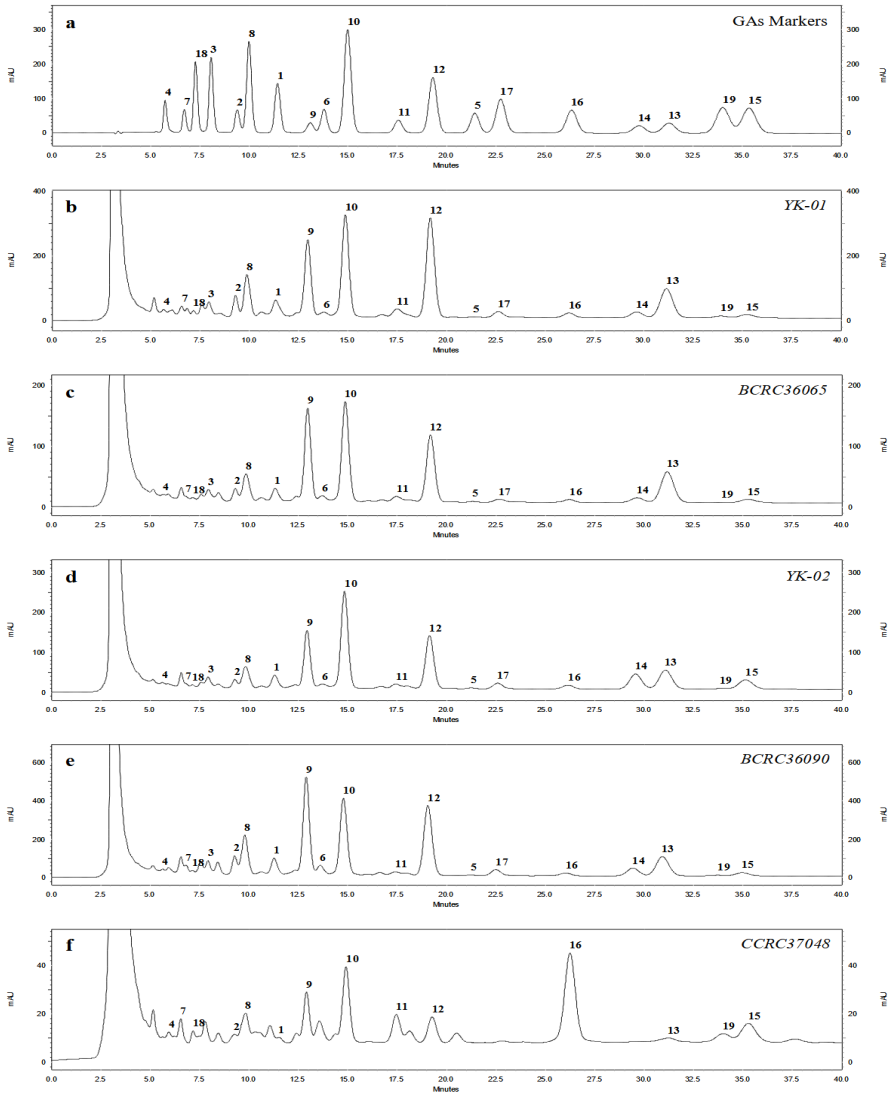


Figure 2. HPLC chromatogram of ganoderic acids **1**–**19** (**a**), *YK-01* (**b**), *BCRC36065* (**c**), *YK-02* (**d**), *BCRC36090* (**e**), and *CCRC37048* (**f**).

3.3. Methods for the Validation and Quantitation of Ganoderic Acid T (10) and S (12)

Because the ganoderic acids T (10) and S (12) are the most abundant compounds with anti-cancer properties in the mycelia of *G. lucidum* and can be well separated from other GAs in HPLC spectra, they were used as the standard compounds for validating the HPLC analytical method. The calibration, precision, and recovery data are shown in Table 3. The correlation coefficients of 10 and 12 were 0.998 and 1.000, respectively, which demonstrate a good linear relationship under this analytical condition. The limits of detection (LOD) were all less than 2.2 μg/mL. The RSD of the interday and intraday precision were between 0.04 and 0.4% for 10 and between 0.11 and 0.44% for 12. Compound **10** was detected with an average amount of 1216.00 ± 9.60 μg/mL (mean ± SD) in the mycelium of *YK-01* (blank), corresponding to 4.86% based on the mycelial dry weight, and compound **12** was 1.47% on average. The recovery percentages for the three concentrations of **10** and **12** were between 96 and 107%, and the RSD results were all under 3%. These data show that this green HPLC analytical method is well suited for determining the contents of **10** and **12** in the mycelium of *G. lucidum*.

Table 3. Calibrations, analytical precision, and recovery data of ganoderic acid T (**10**) and S (**12**).

Linear Regression Calibration Curves						
					10	**12**
R^2					0.998	1.000
Linear range (μg/mL)					25–3000	25–1000
LOD (Limit of Detection, μg/mL)					2.2	2.1
LOQ (Limit of Quantitation, μg/mL)					6.4	6.4
Intraday and interday precision						
Concentration (μg/mL)	Mean ± SD (RSD, %)					
	10				**12**	
	Intraday		Interday		Intraday	Interday
100.0	101.8 ± 0.2 (0.15)		100.7 ± 0.1 (0.06)		101.8 ± 0.4 (0.41)	100.9 ± 0.1 (0.11)
400.0	406.9 ± 1.6 (0.40)		403.6 ± 0.3 (0.06)		407.4 ± 0.5 (0.12)	401.9 ± 0.4 (0.09)
800.0	813.8 ± 0.4 (0.04)		808.0 ± 0.7 (0.09)		814.7 ± 1.7 (0.21)	803.9 ± 3.6 (0.44)
Recovery tests						
Amount added (μg/mL)	Amount measured (μg/mL), mean ± SD		Recovery (%) mean ± SD		RSD (%)	
	10	**12**	**10**	**12**	**10**	**12**
0 (blank)	1216.0 ± 9.60 [a]	368.1 ± 3.6 [a]				
100.0	1322.3 ± 2.39 [b]	473.6 ± 1.6 [b]	106.3 ± 2.4	105.5 ± 1.6	2.25	1.50
400.0	1636.7 ± 6.78 [b]	777.0 ± 11.4 [b]	105.2 ± 1.7	102.2 ± 2.8	1.61	2.78
800.0	2067.3 ± 13.14 [b]	1139.3 ± 5.91 [b]	106.4 ± 1.6	96.4 ± 0.7	1.54	0.77

[a] The average contents (mean ± SD, n = 3) of **10** and **12** in the *YK-01* mycelium. [b] Values including blank.

4. Discussion

Identifying the *Ganoderma* species using DNA analysis is still debated [17]. However, specific GAs have been used to identify the fruiting body of *G. lucidum*, as described in the US Pharmacopeia (USP) [18]. Our previous report also showed that GAs and LAs can be used in species and strain identification for *G. lucidum* [5]. These triterpenes are strain-specific and show diverse bioactivity. Therefore, both GAs and LAs can be used not only for identification but also for quality control for commercial *Ganoderma* products.

In contrast to GAs in the fruiting body, the structures of mycelial GAs usually have the 7,9(11)-diene moiety, as shown in Figure 1, and show lower polarity [19]. Thus, the mobile phases for the HPLC analysis of mycelial GAs are mainly methanol and acetonitrile [9], but they are toxic to human beings. Furthermore, a gradient elution of aqueous methanol fails to separate GA-T (**10**) [9], the most effective anti-cancer compound in the *G. lucidum* mycelium [7]. This study revealed a green HPLC analytical method using ethanol and acetic acid as a mobile phase and successfully separated the isolated triterpenes. Using this method, GA-T and GA-S, the most abundant compounds in the mycelium of *G. lucidum*, could be precisely determined within their linear ranges, as shown in Table 3. The total elution time was completed within 40 min, representing a rapid HPLC analytical method. If the mobile phase used 65% ethanol only, the retention time was prolonged to more than 60 min.; however, contents of GA-T and GA-S remained unchanged (SD < 3.5%). Additionally, if 1% acetic acid was used in the mobile phase, the resolutions and the retention times were unaffected. Thus, 0.5% acetic acid and 65% ethanol were used as an optimized mobile phase. The nineteen triterpenes could be detected in four strains of *G. lucidum* mycelia, and they all displayed the same HPLC chromatographic pattern, indicating that this HPLC method can be used in the species identification of *G. lucidum* mycelia. *YK-02* and *BCRC36090* (Figure 2d,e) are the strains that produce lucidenic acids (LA-type) in their fruit bodies [5]. It was unclear whether GA-type and LA-type fruit bodies

produce the same triterpenoid pattern in their mycelia stages. Our result differs from a previous report in which one strain of LA-type *G. lucidum* produced GA-Ma and GA-Mb as the major signals, with GA-T as a minor component in its mycelium stage [20]. Our results indicate that GA-T is the most abundant compound in the mycelia of both GA and LA-type fruit bodies. This finding is consistent with previous reports in which *G. lucidum* mycelium was cultivated under static conditions [15].

The cultivation media and conditions and the harvest timing for the mycelium of *G. lucidum* may change the relative abundance and pattern of its triterpenes [16]. In addition, a mycelium collected from a liquid fermentation flask will show a lower triterpenoid content than one collected from a static container [16]. Therefore, we used the static cultivation method. Furthermore, to collect more mycelial biomass, plant fiber, a natural nutrient for mushrooms, was added to the media. The mycelia were then collected after 20 days when the color became pale yellow, as shown in Figure 3, indicating that the mycelia were in the mature stages. This cultivation method provides sufficient and stable amounts of mycelial GAs for HPLC analysis. The strain of *G. formosanum CCRC37048* had an extremely low amount of GAs (Figure 2f) and displayed a different HPLC chromatographic pattern from *G. lucidum*, particularly in the retention time between 12 and 30 min. The color of the fruit body of *G. formosanum* is purple, hence its common name, purple lingzhi. It is difficult to differentiate the mycelium of *G. formosanum* from that of *G. lucidum* because of their similarities in appearance (Figure 3). However, the proposed HPLC analytical method can be used to accurately authenticate the species of *G. lucidum*. These results demonstrate that the mycelia of *G lucidum*, either the GA or LA types, show the same triterpenoid HPLC chromatographic pattern. Moreover, compared with the time-consuming (more than 2 months) procedures used to cultivate the fruiting body, the mycelium is easier to produce and can be identified using the same HPLC method for authenticating the *Ganoderma* species. However, for a variety of *Ganoderma* species, continuing efforts are needed to establish more GA fingerprint profiles.

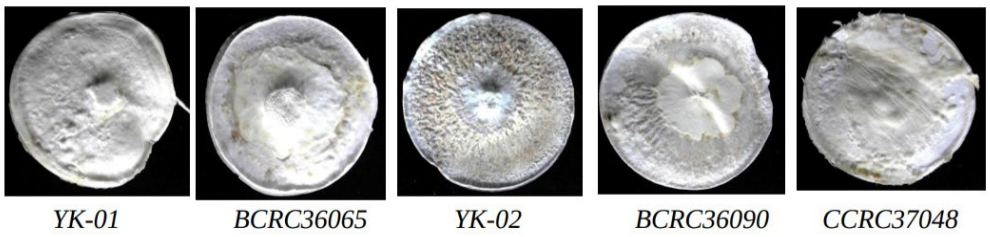


Figure 3. Photo of dry mycelia of *G. lucidum* (*YK-01*, *BCRC36065*, *YK-02*, and *BCRC36090*) and *G. formosanum* (*CCRC37048*).

5. Conclusions

Nineteen triterpenes were isolated from the mycelium of *G. lucidum* and were used as marker compounds in our HPLC analysis. All strains of *G. lucidum* showed the same chromatographic pattern. This HPLC analytical method is rapid, inexpensive, and convenient for identifying G. lucidum species and determining the contents of GA-T and GA-S.

Supplementary Materials: The mass and NMR spectral data can be downloaded at https://www.mdpi.com/article/10.3390/microbiolres14030092/s1.

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