



Development of a rapid and confirmatory method to identify ganoderic acids in *Ganoderma* mushrooms

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To examine the composition of lanostanoids in *Ganoderma lucidum*, we have developed a liquid chromatography–mass spectrometry (LC–MS) method by using the ganoderic acids isolated in our laboratory as reference standards. The identity of 14 peaks in the high performance liquid chromatogram (HPLC) of *G. lucidum* has been confirmed. By using the HPLC retention times of these ganoderic acids and their mass fragmentation patterns established in this paper, one can use LC–MS to analyze *G. lucidum* without requiring the reference standards of these 14 ganoderic acids. Subsequently, only the HPLC–UV method would be needed to analyze routine samples of *G. lucidum*.

Keywords: mushroom, *Ganoderma lucidum*, lanostanoids, triterpenes, ganoderic acids, LC–MS

INTRODUCTION

In our ongoing investigations of Traditional Chinese Medicine (TCM) for dietary supplements we are particularly interested in the medicinal mushroom *Ganoderma lucidum* (W. Curt.:Fr.) Karst because it is a rich producer of lanostanoids. This class of triterpenes has been reported to have distinct pharmacological activities. These include angiotensin-converting-enzyme inhibition, anti-HIV action, antinociceptive action, histamine release inhibition, HMG-CoA reductase inhibition, and liver function stimulation (Mekenna, 1998). The prevalent usage of *G. lucidum* by cancer patients in Asia also persuaded us to further investigate the active components of *G. lucidum*. Indeed, we found that the extract of *G. lucidum* prepared in our labs induced the modulation of secretion from normal human peripheral blood mononuclear leukocytes of cytokines IL-2, IL-4, and IFN- γ (Ma et al., 2002).

Solvent partitioning and repeated chromatography, followed by crystallization resulted in the isolation of 32 lanostanoids from the lipophilic extract of the fruiting body of *G. lucidum*. These include six previously unknown oxygenated lanostanoids: 8 β , 9 α -dihydroganoderic acid J (1), methyl 8 β , 9 α -dihydroganoderate J (2), 20-hydroxyganoderic acid G (3) (Ma et al., 2002), ganoderic acid Sz (4) (Li et al., 2005), 7-oxo-ganoderic acid Z (5), and 15-hydroxy-ganoderic acid S (6) (Figure 1). Compounds 5 and 6 both exhibited inhibitory activities against HMG-CoA reductase and acyl CoA-acyltransferase (Li et al., 2006). We have also isolated 26 known lanostanoids, including ganoderic acids I (7) and C₂ (8), 12-deacetylganoderic acid H (9), ganoderic acid G (10), ganoderenic acid B (11), ganoderic acids B (12), AM₁ (13), A (14),

and H (15), ganoderenic acid D (16), ganoderic acids C (17), F (18), J (19), DM (20), S and Y, methyl ganoderate I, ganoderiols B and F, ganoderol B, ganodermic acid Q, ganodermenonol, ganodermanondiol, ganodermanontriol, lucidone A, and lucidenic A (Ma et al., 2002; Li et al., 2005, 2006; and references cited therein). Their structures were determined by using 1D and 2D NMR and MS spectroscopic methods.

Ganoderma lucidum has usually been taken orally as mushroom powder or in its extract form, whether as prescribed by TCM doctors or in modern pill form. There is a need to understand the composition of the natural material ingested. Liquid chromatography–mass spectrometry (LC–MS) has been used wildly to characterize and standardize triterpenes in mushrooms. However, the availability of only limited number of commercial reference standards of lanostanoids has diminished the number of triterpenes to be analyzed (Adamec et al., 2009; Liu et al., 2011). It is not practical to expect every single lab to obtain reference standards by going through laborious and time-consuming steps of isolating and characterizing each individual lanostanoid. The aim of this work is to develop a rapid and conclusive LC–MS method for identification of major triterpenes in *Ganoderma* mushrooms by using the ganoderic acid samples we isolated.

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES

Melting points were recorded on a Fisher-Johns melting point apparatus. Optical rotations were recorded on a WZZ-1S automatic polarimeter. UV spectra were recorded on a HP-8453 spectrophotometer. IR spectra were recorded on a Nicolet Magna

FTIR-750 spectrometer. One- and two-dimensional NMR spectra were recorded on a Bruker AMX 400 spectrometer. Acetonitrile used in LC-MS analysis was supplied by Sigma-Aldrich Co. LLC. Other reagents were all supplied by Sinopharm Chemical Reagent Co. Ltd (Shanghai).

FUNGAL MATERIAL

The mushroom of *G. lucidum* was identified by Professor Guanyun Gu, School of Pharmacy, Fudan University, Shanghai, People's

Republic of China. A voucher specimen (GL-9807) has been deposited at the Department of Pharmacognosy, Fudan University, Shanghai, People's Republic of China.

IL-2, IL-4, AND IFN- γ ASSAYS

The sample was dissolved in a 0.4% DMSO solution, then incubated with normal human peripheral blood mononuclear leukocytes in a RPMI 1640 buffer containing 10% FBS, 50 unit/mL penicillin, and 50 μ g/mL streptomycin at 37°C for 16 h. Levels of

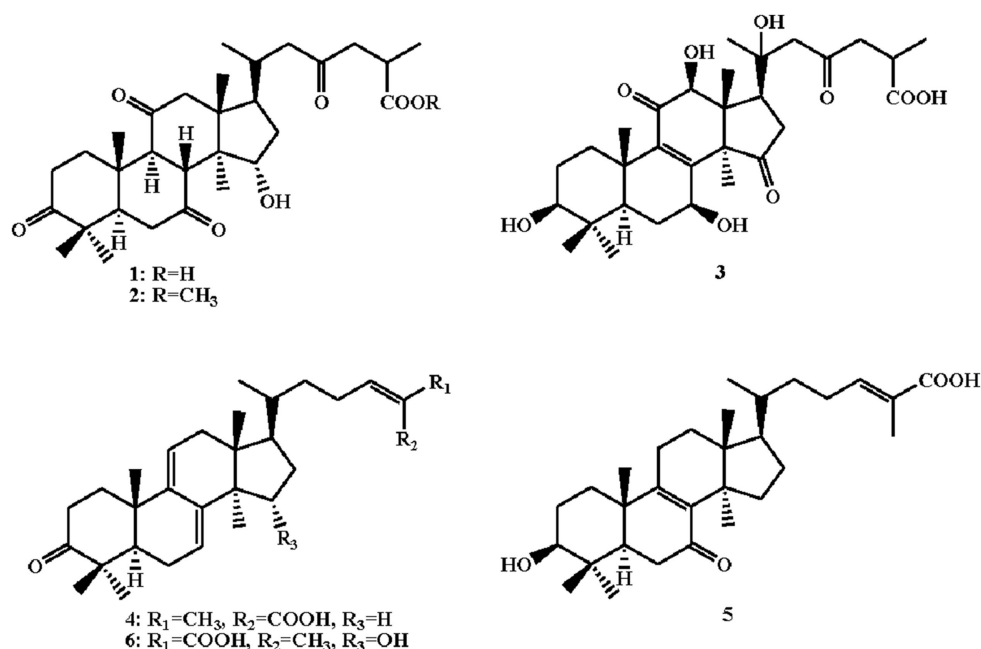


FIGURE 1 | Structures of oxygenated lanostanoids.

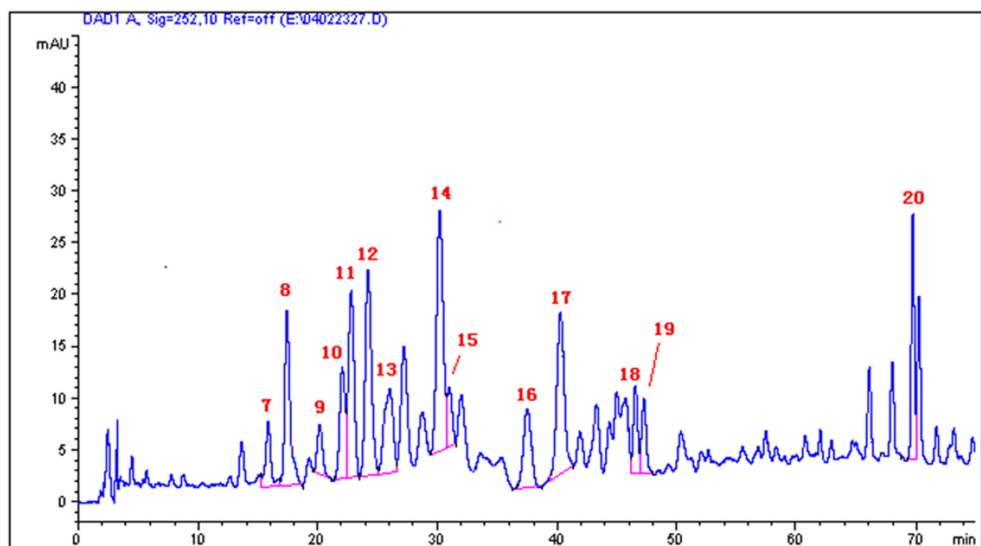
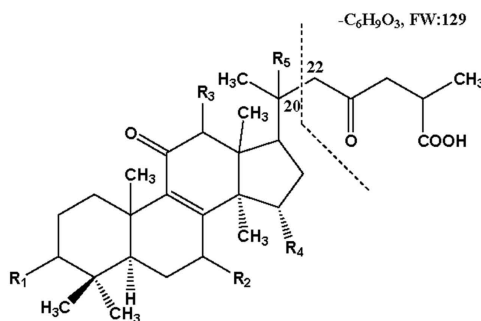
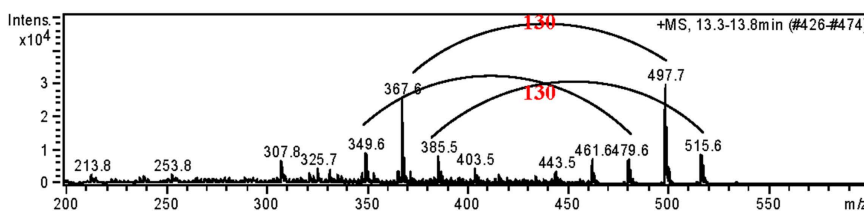


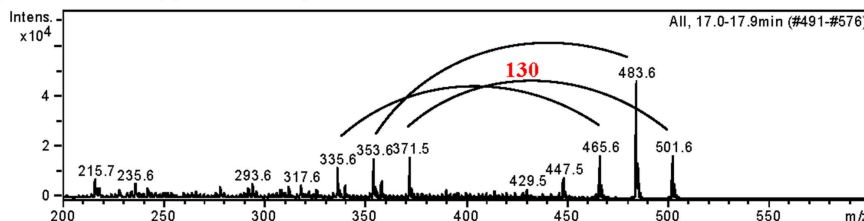
FIGURE 2 | High performance liquid chromatogram of the extract of *G. lucidum*.



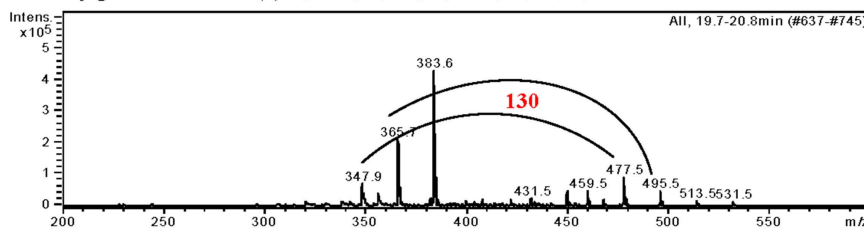
ganoderic acid I (7) R₁=OH, R₂=β-OH, R₃=H, R₄=O, R₅=OH



ganoderic acid C₂ (8) R₁=OH, R₂=β-OH, R₃=H, R₄=OH, R₅=H



12-deacetyl ganoderic acid H (9) R₁=OH, R₂=O, R₃=OH, R₄=O, R₅=H



ganoderic acid G (10) R₁=OH, R₂=β-OH, R₃=OH, R₄=O, R₅=H

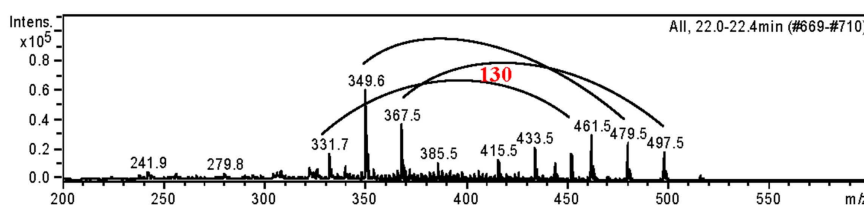


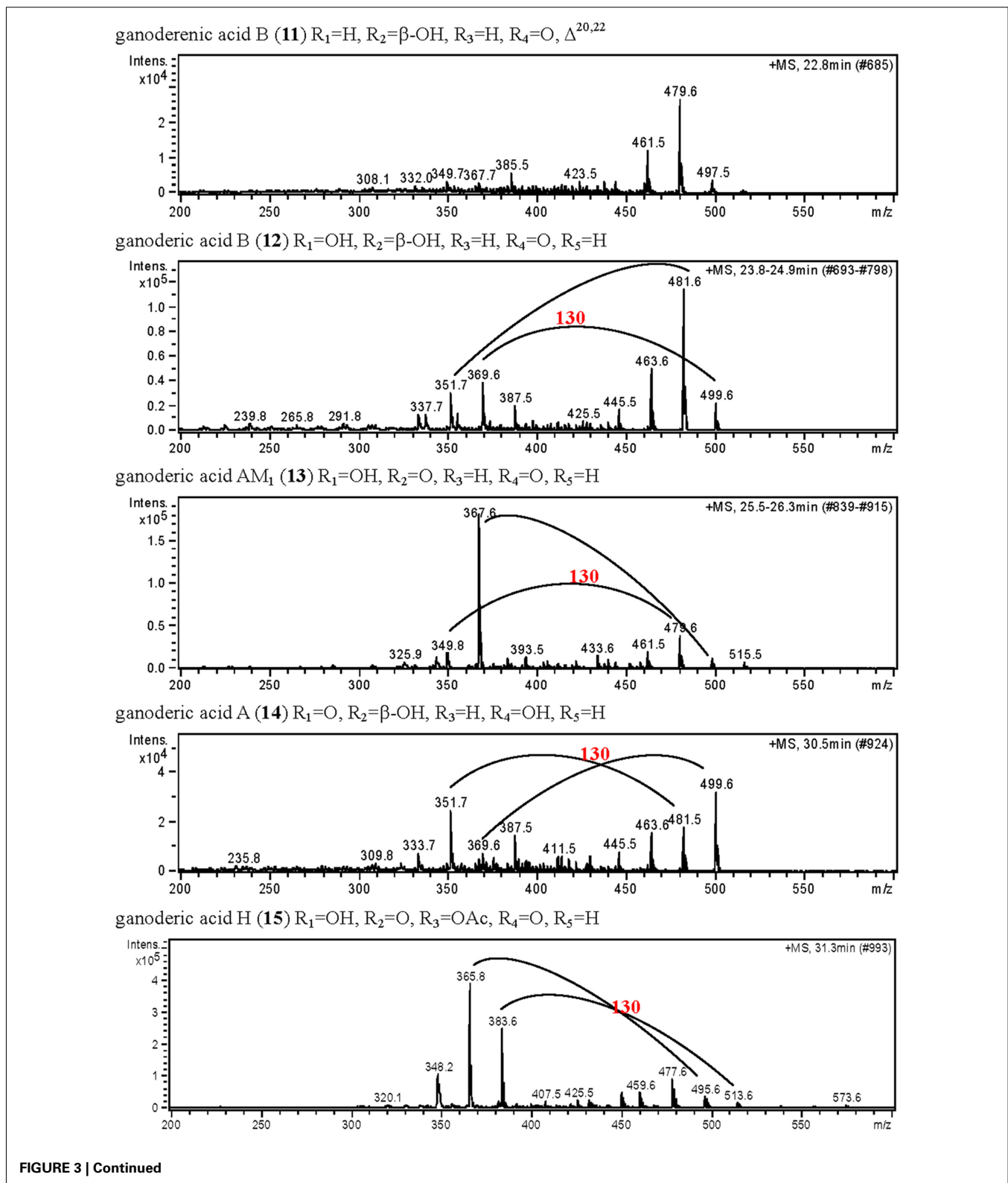
FIGURE 3 | Continued

IL-2, IL-4, and IFN-γ were measured using ELISA (Welker et al., 1996; Ma et al., 2002).

HMG-CoA REDUCTASE ASSAY

A phosphate buffer solution contains 100 mM potassium phosphate (pH 7.5), 8 mM G-6-P, 1 mM NADP, 4 mM EDTA, 2 mM

DTT, and 0.6 U G-6-P-DH was prepared. The compound was dissolved in a 1% DMSO solution and then pre-incubated with microsomal protein in the phosphate buffer solution at 37°C for 15 min. The reaction was started by the addition of 2.5 μM [¹⁴C]HMG-CoA reductase and the reaction was run at 37°C for 15 min. [¹⁴C]mevalonate was then quantified. It was determined



that compounds **1** and **2** inhibited HMG-CoA reductase with $IC_{50} = 22.3$ and $21.7 \mu\text{M}$, respectively (Heller and Gould, 1973; Kubo and Strott, 1987; Li et al., 2006).

ACYL CoA-CHOLESTEROL ACYLTRANSFERASE ASSAY

The compound was dissolved in a 1% DMSO solution and pre-incubated with Wistar rat hepatic microsomes in the 0.2-M

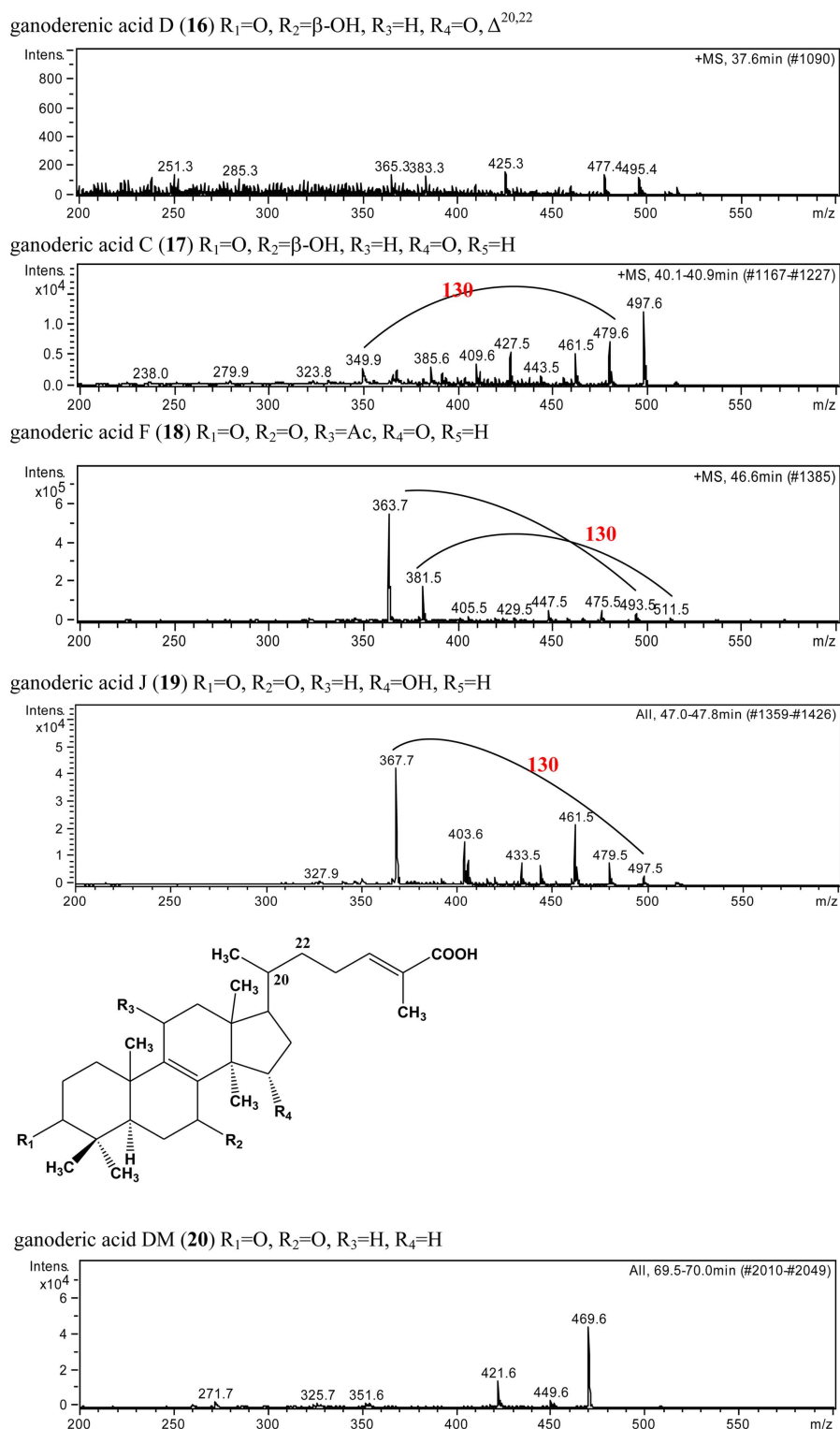


FIGURE 3 | Mass spectra of the major ganoderic acids (7–20) in *G. lucidum*.

phosphate buffer at 37°C for 15 min. The reaction was started by the addition of 18 μM [^{14}C]palmitoyl CoA-acyltransferase and the reaction was run at 37°C for 15 min. [^{14}C]cholesterol ester was

then quantified. The inhibitory activities, IC_{50} , of compounds **1** and **2** against CoA-acyltransferase were determined to be 5.5 and 47.3 μM , respectively (Largis et al., 1989; Li et al., 2006).

GANODERIC ACIDS

Ganoderma lucidum mushroom fruiting bodies (10 kg) were chipped and extracted with 20 L of 95% ethanol at 80°C three times. After filtering and removing of ethanol, the resulting extract was applied onto a chromatographic silica gel column and eluted with a chloroform/acetone gradient system, followed by a reversed-phase C-18 column chromatographic run with water/methanol. The final purification of the ganoderic acids were accomplished by further high performance liquid chromatogram (HPLC) separation and re-crystallization. Their structure were identified and confirmed by NMR and MS analyses (Ma et al., 2002; Li et al., 2005, 2006). The reference standard solution of each ganoderic acid was prepared by dissolving 1 mg of ganoderic acid in 1 mL of methanol.

GANODERMA LUCIDUM EXTRACT

A *G. lucidum* extract (ReishiMax GLP™500 mg) was introduced into 25 mL of ethyl acetate, sonicated for 30 min, and filtered. The remaining insoluble material was extracted two more times as above. Three filtrates were combined and the solvent was removed under vacuum. The resulting residue was dissolved into 10 mL of methanol for LC–MS analysis.

LC–MS ANALYSIS

The HPLC system was controlled by an Agilent Mass-Chrom data system. Chromatographic separation was performed on a Phenomenex luna C-18 column (5 μm, 250 mm × 4.6 mm. I.D.) from Phenomenex Co. Ltd. A step gradient solvent system was used: 0 min CH₃CN/0.1% aqueous CH₃COOH (25/75) → 35 min CH₃CN/0.1% aqueous CH₃COOH (35/65) → 45 min CH₃CN/0.1% aqueous CH₃COOH (45/55) → 90 min CH₃CN with a constant flow rate at 1.0 mL/min. Column temperature was set at 30°C controlled by a column heater-cooler HP 1100. The elution was measured at the wavelength range of 200–500 nm by using a photodiode array detector. Agilent triple-quadrupole ion-trap mass spectrometer was equipped with an atmospheric pressure chemical ionization interface (APCI). The mass spectrometer was mass-calibrated with

a polyglycol solution. In order to achieve maximum sensitivity the mass spectrometer conditions were set at 450°C of APCI temperature, 60 psi of nebulizing gas flow rate, 3500 V of capillary voltage, and 325°C of capillary temperature. Full scan spectra from *m/z* 100 to *m/z* 1000 Da were obtained in the positive ion mode.

RESULTS AND DISCUSSION

An HPLC method was developed to analyze the lipophilic part of the commercial product ReishiMax GLP™. Ganoderic acids were separated by a C-18 column with a step gradient solvent system of CH₃CN/0.1% aqueous CH₃COOH. **Figure 2** showed that the chromatogram of the column eluents measured at the wavelength of 252 nm. Subsequent LC–MS analysis with an ion-trap using APCI ionization technique was followed.

The MS spectra of ganoderic acids 7–20 were established by LC–MS. The APCI mass spectra of these compounds exhibited peaks at *m/z* [M-nH₂O + H]⁺ and [M-nH₂O + H-130]⁺. The loss of 130 Da in the mass spectra represents the characteristic cleavage of the αβ bond (C20–C22) from the C=O in the side chain (**Figure 3**). The absence of these prominent fragments of [M-nH₂O + H-130]⁺ in ganoderic acids B (**11**) and D (**16**), and ganoderic acid DM (**20**) were expected due to the absence of C=O at C23. HPLC retention times (*t_R*) of ganoderic acids 7–20, along with their mass fragments, were established and listed in the **Table 1**.

By comparison of the HPLC retention times and mass spectra of the test sample and reference standards of ganoderic acids (**Table 1**; **Figure 3**), the identities of 14 major peaks in the chromatogram of ReishiMax GLP™ were confirmed. Thus, one can easily use this approach by performing LC–MS analysis on its samples and then comparing with the data in the **Table 1** and mass spectra in **Figure 3** in this paper to establish the identity of each chromatographic peak. Subsequently, only HPLC will be needed for routine analysis of *G. lucidum*. This method has been used in our laboratories and extraction factory to examine the quality and quantity of the triterpene composition in incoming mushrooms, crude extracts, finished goods, and competitor products. Our QC

Table 1 | Mass fragments and HPLC retention times of ganoderic acids (7–20).

Compound	<i>t_R</i>	MW	[M + H] ⁺	[M-nH ₂ O + H] ⁺	[M-nH ₂ O + H-130] ⁺
7	14.3	532	–	515(26) 497(100) 479(25)	385(27) 367(85) 349(30)
8	17.5	518	–	501(35) 483(100) 465(35)	371(35) 353(31) 335 (26)
9	20.1	530	531(4)	495(11) 477(22)	383(100) 365(50) 347(18)
10	22.1	532	–	497(31) 479(40) 461(50)	367(62) 349(100) 331(29)
11	22.8	514	515(2)	479(100) 461(50)	–
12	24.3	516	–	499(20) 481(100) 463(45)	369(35) 351(27)
13	25.8	514	515(4)	497(7) 479(22)	367(100)
14	30.4	516	–	499(100) 481(53) 463(49)	387(47) 351(75)
15	31.1	572	573(4)	495(14) 477(35) 459(20)	383(51) 365(100)
16	37.6	512	–	495(80) 477(100)	–
17	40.4	514	515(4)	497(100) 479(60) 461(45) 427(45)	367(22) 349(22)
18	46.6	570	–	511(3) 493(8) 475(9) 447(11)	381(34) 363(100)
19	47.3	514	515(2)	497(8) 479(18) 461(50)	403(37) 367(100)
20	70.2	468	469(100)	–	–

lab has used the HPLC chromatograms as sample fingerprints for the cGMP compliant purpose. We believe this method could also be an effective tool for researchers in future studies of other related *Ganoderma* species.

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- could be construed as a potential conflict of interest.

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