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Analytical Methods

Qualitative analysis of ganoderic acids in *Ganoderma lucidum* from Iran and China by RP-HPLC and electrospray ionisation-mass spectrometry (ESI-MS)

Somayeh Keypour [a], Hasan Rafati [b,*], Hossain Riahi [a], Fateme Mirzajani [b], Mohammad Fata Moradali [c]

[a] Bioscience Faculty, Shahid Beheshti University, GC, Tehran, Iran
[b] Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, GC, Tehran, Iran
[c] Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology Research Institute of Iran, Karaj, Iran

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ABSTRACT

Ganoderma lucidum is used as a health food and medicine in Far East and produces different ganoderic acids which are well known for their valuable bioactivities. In this study, two different strains of *G. lucidum* from Iran and China were investigated for ganoderic acids, using reversed phase HPLC (RP-HPLC) in combination with UV and electrospray ionisation-mass spectrometry (ESI-MS). The results showed a relatively low intensity ganoderic acid C2 peak in the chromatogram of the Iranian sample. However, three high intensity peaks attributed to the well known ganoderic acids including ganoderic acids T, Me and H from the Chinese strain. These findings clearly indicated that different strains of *G. lucidum* could posses a range of active compounds and hence, bioactivities. Moreover, the variation in the triterpenoid components encourages more studies on the rest of bioactive molecules, and also different strains grown in a variety of climatic and geographical conditions.

1. Introduction

Mushrooms have increasingly being explored as a valuable source of nutrients' and highly bioactive secondary metabolites. *Ganoderma lucidum* (commonly called Lingzhi in China) is used as an elixir of life to promote health and longevity in East Asian countries. It is commercially available in the forms of raw mushrooms, tea, powder and capsules (Bao, Wang, Dong, Fang, & Li, 2002; Di, Chan, Leung, & Huie, 2003). The genus *Ganoderma* (the family: Ganodermataceae) involves different species that most of them are well known for their medicinal properties. *Ganoderma* species are wood decaying fungi, living as parasite or saprophyte on the living or dried trunk of trees, mostly in the temperate forest. Despite the valuable dietary and therapeutic benefits of *G. lucidum*, phytochemical evaluation of the active components have been conducted predominantly in China, Korea, Japan and the United States (Paterson, 2006). However, several experiments demonstrating the medicinal properties of local *Ganoderma* have been performed recently in Europe and India (Joseph, Sabulal, George, Smina, & Janardhanan, 2009; Salteralli et al., 2009). So far, seven species of *Ganoderma* have been reported from Iran (Moradali et al., 2007).

A number of bioactive components have been identified from *G. lucidum* and related species, in which the polysaccharides, triterpenoids, sterols, lectins and some proteins have beneficial properties in treatment and prevention of a variety of ailments. In 2006, researchers reported that the polysaccharides derived from *G. lucidum* had shown inhibitory activity on the malignant human breast cancer cells (Wu et al., 2006). However, the pioneering works about the isolation of triterpens and steroids from *G. applanatum* started by Striginia, Elkin, and Elyakov (1971) and Protiva, Skorkovska, Urban, and Vystrcil (1979). More than 130 ganoderic acid derivatives and other triterpenoids have been isolated and identified from the spores, fruiting bodies and cultured mycelia of the *Ganoderma* spp. in the past two decades (Wang et al., 2006). Ganoderic acids and other triterpenoids have received considerable attention due to their conspicuous pharmacological activities. A number of ganoderic acids including ganoderic acids A, B, H, C1 showed anti-HIV-1 activity (El-Mekkawy et al., 1998; Min, Nakamura, Miyashiro, Bae, & Hattori, 1998). Other possible implications include antihistamine (ganoderic acids C2, D) (Kohda et al., 1985), antinociceptive (ganoderic acids A, B) (Koyama et al., 1997) and anti-hypercholesterolemic activities (ganoderic acids B, C2) (Komoda, Shimizu, Sonoda, & Sato, 1989). Inhibitory activity of angiotensin converting enzyme (ACE; ganoderic acids K, F, S) (Morigiwa, Kitabatake, Fujimoto, & Ikekawa, 1986) and antitumor activity (ganoderic acids T, Me) (Lindequist, Niedermeyer, & Jülich, 2005). For evaluating the chemical profile of ganoderic acids, HPLC procedures have been developed for the isolation and purification of oxygenated triterpenoids from the crude extracts of mushrooms (Chen et al., 1999; Roberts, 2004). RP-HPLC procedure has proved

* Corresponding author. Tel./fax: +98 2122431783.
E-mail address: H_Rafati@sbu.ac.ir (H. Rafati).

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to be a valuable tool in investigating the triterpenoid patterns of *Ganoderma* species (Roberts, 2004). Chen et al. (1999) reported that HPLC analysis of triterpenoids of different *G. lucidum* strains showed different HPLC patterns, indicating the presence of different triterpenoids. Hyphenated chromatographic technique is another valuable, new and effective method for determination of bioactive components (e.g. triterpenes) in *G. lucidum* extract (Tang, Gu, & Zhong, 2006).

In line with a previous study on the antimicrobial activity of *G. lucidum* extract (Keypour, Riahi, Moradali, & Rafati, 2008), the primary objective of the present work was a qualitative evaluation of the presence of ganoderic acids in Iranian samples. Ganoderic acids were identified in the fruit body of the Iranian specimen and compared with the Chinese samples by means of RP-HPLC ultraviolet and electrospray ionisation-mass spectrometry (ESI-MS) detections.

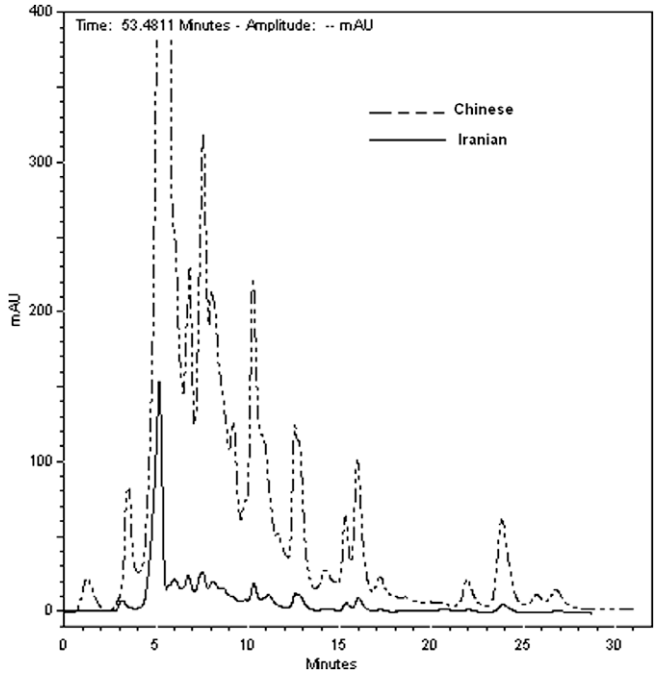

Fig. 1. HPLC chromatogram of mobile phase methanol–water (at ratio of 95:5 mL), for Iranian and Chinese strains.

2. Materials and methods

2.1. Fungal material and extraction

The fruiting body of *G. lucidum* was collected from Shirdare (Mazandaran Province), growing on *Carpinus betulus* L. (Corylaceae). It was characterised and identified as *G. lucidum* according to the key of Iranian *Ganoderma* species (Moradali et al., 2007). Voucher specimen of the sample was deposited at the Herbarium of bioscience Faculty, Shahid Beheshti University, Tehran, Iran. The Chinese samples were obtained from Edible Mushrooms Institute in Shanghai (China).

Dried fruiting bodies of *G. lucidum* samples were powdered to pass a 50-mesh screen using a blender and 2.0 g of powder was extracted with 40 mL $CHCl_3$ in a sonic water bath for 20 min. This extraction was repeated twice. The extracted solution was filtrated through analytical filter paper and the filtrate was evaporated to dryness using a rotary evaporator. The dry extract was dissolved in 5 mL methanol and filtrated through a 0.45 μm membrane filter unit. Then, each sample solution was analysed by HPLC.

2.2. Instrumentation, chromatography and ESI-MS operating conditions

A Knauer HPLC system with a binary pump (model K-1001), and a K-2800 diode array detector (DAD), Knauer Co., model (Berlin, Germany), were used for chromatographic analysis. The column was Eurospher C18 (5 μm, 4.6 × 250 mm) from Knauer Co. (Berlin, Germany). The detection wavelength was set at 254 nm. A 20 μL sample in methanol was injected into the analytical HPLC column for analysis. Literature surveys demonstrated that a mobile phase containing 99.5% methanol (v/v) and 0.5% (v/v) acetic acid (90% (v/v) mobile phase) in addition to 10% (v/v) water had an ability to resolve the ganoderic acids. However, in our experiments, increasing the methanol in the mobile phase to 95% (v/v) and 5% (v/v) water–acetic acid 0.5% improved the resolution and the separation of the peaks. The flow rate of the mobile phase in the HPLC runs was set at 0.5 mL min^{-1}. Since the triterpene acids exhibit a maximum UV absorbance at 254 nm, this wavelength was used for UV detection. The LC-ESI-MS system used in the present study included a microcolumn C18 (5 μm, 150 × 1 mm) from Macherey–Nagel Co. (Duren, Germany) and a ThermoFisher Scientific equipped with ion trap mass spectrometer (model LCQ, mass range *m*/*z* 10–2000) and a nanospray ionisation interface (Bremen, Ger-

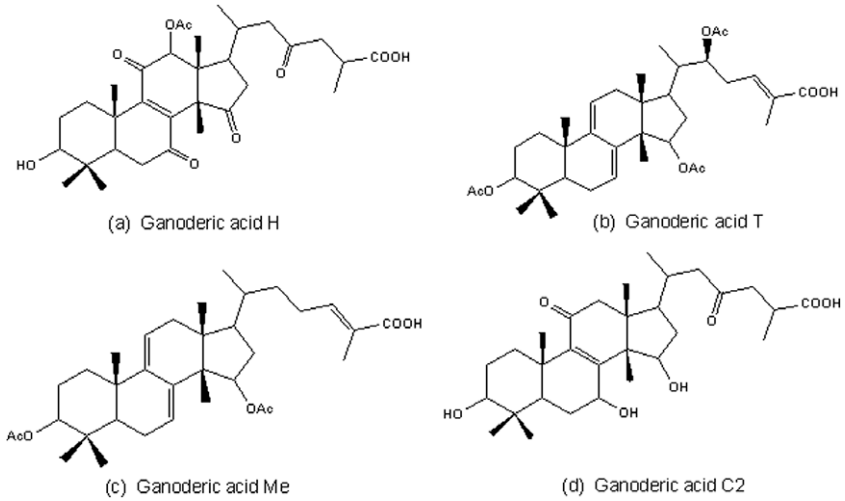

Fig. 2. Chemical structures of the identified ganoderic acids.

many). Instrument control, data acquisition and processing were conducted by the Xcalibur software. Typical negative ESI-MS conditions were: capillary voltage −2.0 kV and skimmer cone voltage −20 V.

3. Results and discussion

3.1. Extraction of ganoderic acids

In order to identify and compare the ganoderic acids in two different samples, a simple and fast two-steps extraction technique by ultrasonic bath and chloroform as a solvent was used. Chloroform was selected for ganoderic acids extraction due to the efficiency and good resolution of chloroform extracts for triterpenoids (Wang et al., 2006). Further extraction did not increase the intensity of the chromatographic peaks. However, Wang et al. (2006) reported an exhaustive and time consuming extraction method from fruit body of *G. lucidum* by solvent extraction and fractionation for semi-preparative and standard development purposes. Moreover, 6 days extraction technique from *G. lucidum* mycelia used by Tang et al. (2006), also used methanol and chloroform to extract and clean the samples for HPLC analysis.

3.2. Separation of ganoderic acids by RP-HPLC

HPLC chromatography of the Iranian and Chinese samples, using 95% (v/v) methanol as the mobile phase are shown in Fig. 1. Comparing the chromatograms of the samples, it can be seen that despite few similarities, the two samples are considerably different with regards to the number and the intensity of the components, separated by the present technique.

3.3. Detection of ganoderic acids in the Chinese and Iranian samples

Electrospray ionisation (ESI) is undoubtedly the softest and the most suitable ionisation technique for MS analysis of extracts, which is constituted from a complex matrix. However, in the present study, we used the routine $[M+NH_4]^+$ and $[M+H]^+$ peaks for interpreting the MS results. Also we investigated the adduct ions associated with alkaline metals, $[M+Na]^+$ and $[M+K]^+$, and losing one or two acetoxy (OAc) groups peaks, $[M-OAc]^+$ and $[M-2OAc]^+$.

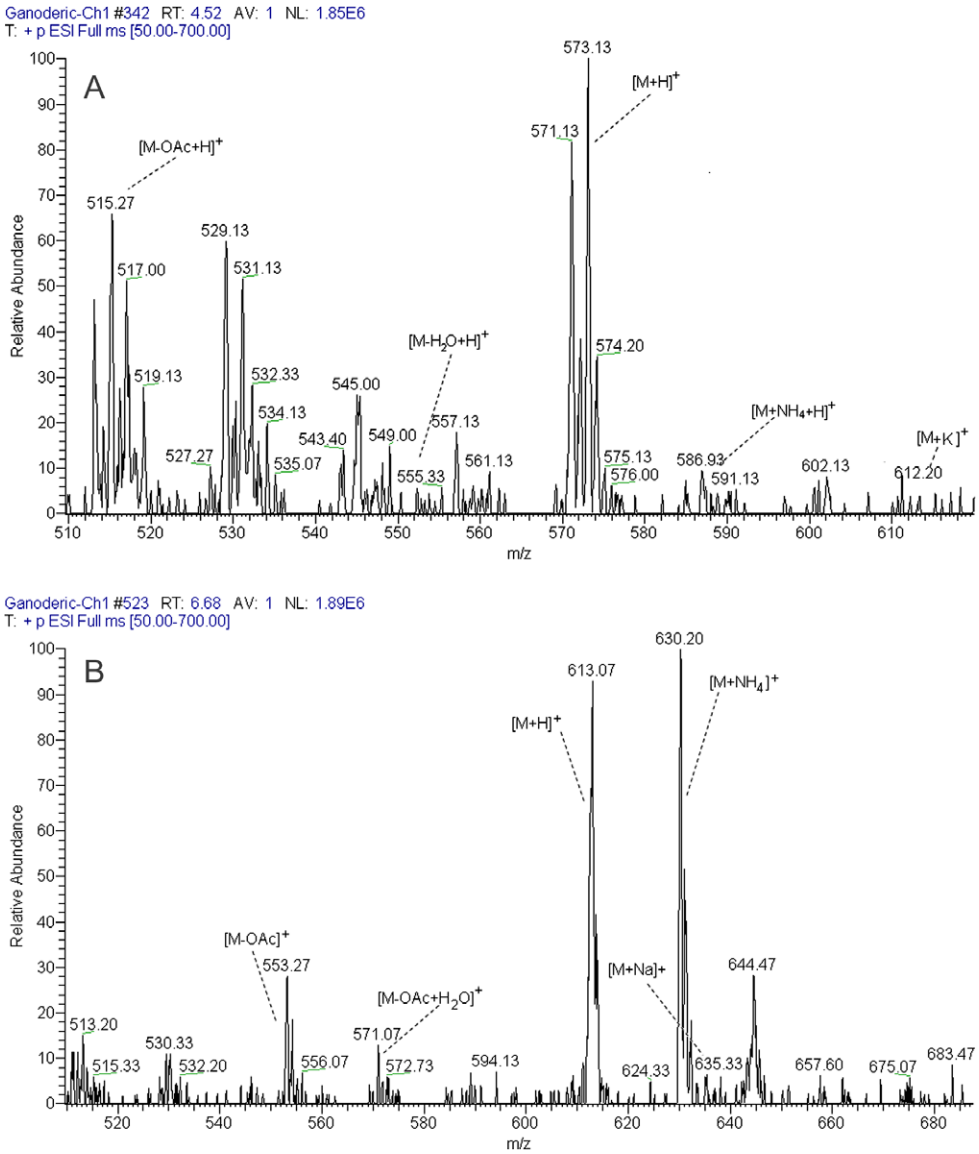

Fig. 3. LC-ESI-MS spectra showing the positive ion ESIMS of ganoderic acid H (A), ganoderic acid T (B), ganoderic acid Me (C) and ganoderic acid C_2 (D).

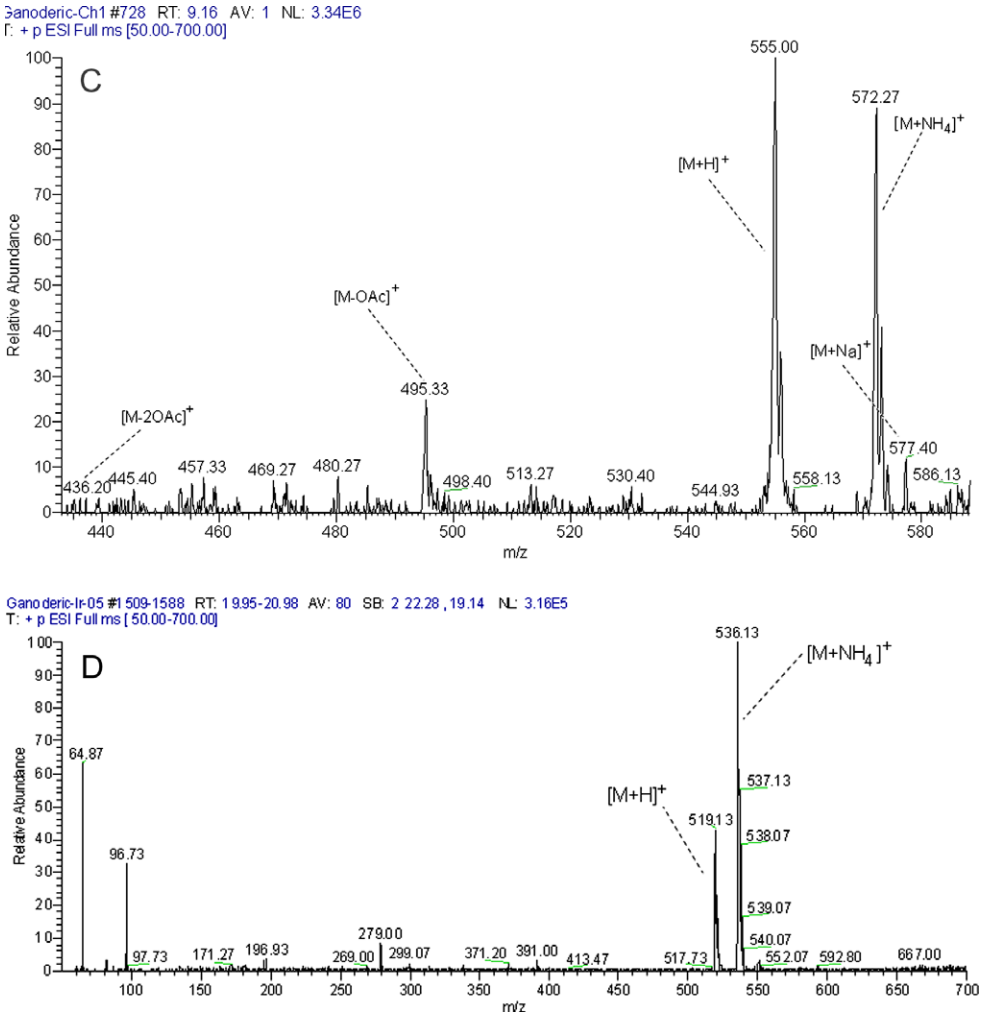

Fig. 3 (*continued*)

The chemical structures of ganoderic acids, detected in the present study are shown in Fig. 2. Three ganoderic acids were detected from the fruiting body of the Chinese sample and one from the Iranian sample. In Chinese sample, ganoderic acid H (chemical structure; 2-a) was identified according to $[M-OAc+H]^+$, 515.27 *m*/*z*, $[M-H_2O]^+$, 555.33 *m*/*z*, $[M+H]^+$, 573.13 *m*/*z*, $[M+NH_4+H]^+$, 591.13 *m*/*z*, and $[M+K]^+$, 612.20 *m*/*z* (Fig. 3A), due to a molecular weight of 572.2 (at 4.52 min in the TIC chromatogram). Ganoderic acid T (chemical structure; 2-b), gave an $[M-OAc]^+$, 553.27 *m*/*z*, $[M-OAc+H_2O]^+$, 571.07 *m*/*z*, $[M+H]^+$,613.07 *m*/*z*, $[M+NH_4]^+$, 630.20 *m*/*z* and $[M+Na]^+$,635.33 *m*/*z* (at 6.68 min in the TIC chromatogram) (Fig. 3B), due to a molecular weight of 612.3 (Tang et al., 2006), and ganoderic acid Me (chemical structure; 2-c), gave an $[M-2OAc]^+$, 436.20 *m*/*z*, $[M-OAc]^+$, 495.33 *m*/*z*, $[M+H]^+$, 555.00 *m*/*z*, $[M+NH_4]^+$, 572.27 *m*/*z*, and $[M+Na]^+$, 577.40 *m*/*z* signal (at 9.16 min in the TIC chromatogram) (Fig. 3C), due to a molecular weight of 554.3 (Tang et al., 2006). Nishitoba, Sato, and Sakamura (1988) suggested a spatial relationship between the hydrophobic methyl groups of different ganoderic acids and hydroxyl or carboxyl oxygen group bonded to C-3, C-7 and C-11. In the Iranian sample, ganoderic acid C2 (chemical structure; 2-d), gave an $[M+H]^+$, 519.13 *m*/*z*, $[M+NH_4]^+$, 536.13 *m*/*z* signal (at 19.95 min in the TIC chromatogram) (Fig. 3D), due to the molecular weight of 518.3.

In the present work, we have reported for the first time the presence of ganoderic acid C2 in the Iranian strain of *G. lucidum*. It can be also concluded that different strains may have different chemical compounds due to differences in the geographical distributions, growth conditions and substrates. In the present study the profile of the ganoderic acids differed significantly in the two different strains. The same result was reported by Roberts (2004) who showed different growth media for *G. lucidum* cultivation and different strains of *G. lucidum*, possess different antibacterial properties (Tang et al., 2006). Differences between the Iranian and Chinese strains and the growth conditions might be the key to differences in producing various ganoderic acids. The result also showed that *G. lucidum* from China has comparatively more ganoderic acids compared to the Iranian sample. Interestingly, Salteralli et al. (2009), also showed that the mycelia of Italian *G. lucidum* was significantly different in protein content, enzymatic activity and water-soluble polysaccharide content compared to the Chinese isolates grown in submerged culture. It can be concluded that despite the fact that *G. lucidum* is currently just available in formulation of nutraceuticals and food supplements in Asia and North America, but much more studies can be done to explore the bioactive components of different strains grown in a variety of climatic and geographical conditions. The immunity enhancing and preventive effect of this mushroom against a wide range of fatal diseases encourages further investigations into the phytochemical profile of samples from different countries and also development of new functional foods and supplements.

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