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Research Article

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Total triterpenoids from the ultrasonic-circulating extract powder of *Ganoderma lucidum* and its antioxidant activity *in vitro*

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ABSTRACT

The constituents of total triterpenoids (TTs) prepared from the ultrasonic-circulating extract powder (UCEP) of Ganoderma lucidum (Lingzhi), were analyzed by liquid chromatography coupled with mass spectrometry (LC-MS) method. Approximately 56 molecular ion peaks were simultaneously acquired from the total ion-current chromatography (MS^+) of TTs. Among these constituents, at least 31 precursor ions for triterpenoids were determined with m/z values of 418.3~678.5, corresponding to the molecular weight of known triterpene acids and triterpenoid saponin. The in vitro assays showed that the TTs from UCEP possessed favorable scavenging capacity for free radicals (DPPH·, $ABTS^{\cdot+}$ and O_2^-) as well as total antioxidant activity (T-AOA) and ferric reducing antioxidant power (FRAP).

Keywords: *Ganoderma lucidum*; ultrasonic-circulating extraction; triterpenoid; antioxidant activity *in vitro*.

INTRODUCTION

Ganoderma lucidum, commonly known as "Lingzhi", is an important medicinal fungus that is well known as a health supplement for promoting longevity and has long been used throughout the world, especially in China and other oriental countries [1]. It has been confirmed that over 150 triterpenoids have been isolated from the fruiting bodies, cultured mycelia and spores of *Ganoderma spp*., and majority of which are lanostane-type tetracyclic triterpenoids [2-4]. Although, High-performance liquid chromatography (HPLC) is commonly used for quantitative determination of triterpenoids in the extract of *G. lucidum* [5-7], but in order to explore how many kinds of triterpenoids in an extract of *G. lucidum*, some known compounds were usually need as the standards. However, we normally don't have the ready-made standard on hand. So that, liquid chromatography coupled with mass spectrometry (LC-MS) was the most selective technique for the rapid qualitative analysis of known compounds as well as the identification of unknown compounds from extracts of *G. lucidum* [8].

These are mounting evidence of antioxidant activities of polysaccharides from *G. lucidum* [9, 10] [11]. But a few studies have suggested that triterpene components from *Ganoderma* also have antioxidant activities, as do polysaccharides. In normally, researcher(s) has a special focus on the monomer compounds of triterpene and its' biological activity, no on the total triterpenoid. Fortunately, there is strong evidence that total triterpenes isolated from *G. lucidum* have a remarkable ability to protect normal cells from radiation-induced damage *in vitro* and did not possess significant toxicity [12].

Recently, a new technique, ultrasonic-circulating extraction (UCE) integrating with superfine-pulverization, was applied to extract the antioxidant polysaccharides and other active ingredients from *G. lucidum* [13]. And the extract powder prepared by UCE was termed UCEP by us. In this work, the total triterpenoids (TTs) isolated from the UCEP were analyzed by LC-MS methods, and its antioxidant properties *in vitro* were evaluated.

EXPERIMENTAL SECTION

2.1 Chemicals

Ganoderic acid A ($C_{30}H_{44}O_7$, m/z 516.3) was provided by Professor Ding Ping (Guangzhou University of Traditional Chinese Medicine, Guangzhou, China) as standard of triterpenoid compound. DPPH (1,1'-diphenyl-2-picrylhydrazyl stable radicals), butylated hydroxyl toluene (BHT) and ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) were purchased from Sigma Chemical Co. NBT (nitroblue tetrazolium), (reduced form of nicotinamide adenine dinucleotide) NADH and PMS (phenazine methosulphate) were obtained from GenView Scientific Inc. All other chemicals and solvents used were of analytical grade, including rutoside (rutin), and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Preparation of total triterpenoids (TTs) from *Lingzhi extract powder*

Log-cultivated fruiting bodies of *Ganoderma lucidum* (Lingzhi), obtained from a cultivation base in Fujian province, China, were finely pulverized into superfine powder for use as a productive material. The extract powder (UCEP) was prepared through ultrasonic-circulating extraction (UCE) integrating with superfine-pulverization that was described previously [13], prior to vacuum concentration and spray drying.

TTs were prepared according to the method with minor modification [7]. Approximately 20.0 g of UCEP was ultrasonically dissolved in 50 mL of 75% ethanol and filtered through analytical filter paper. The filtered solution was evaporated to dryness in vacuum. The residue obtained was dissolved in an appropriate volume of saturated $NaHCO_3$ solution, adjusted to pH 3-4 with 6 mol/L HCl, and then partitioned with $CHCl_3$ (1:1,v/v) three times to obtain the $CHCl_3$ fraction, which was then evaporated to obtain the total triterpenoids (TTs).

2.3 HPLC analysis

0.1 g of TTs was individually dissolved in 5 mL of methanol and filtered through a 0.45 μm membrane filter unit. Next, 10 μL of the sample solution was analyzed by HPLC at a wavelength of 254 nm with ganoderic acid A as standard. Analyses were performed on a Waters 2695 HPLC system (Waters Technologies Corp.) with an Xtimate C18 chromatographic column (4.6 mm × 250 mm, 5 μm; Welch Material, Inc.), according to the conditions described by Ding *et al* [6] with slight modifications. The mobile phase consisted of 1% acetic acid (A) and acetonitrile (B), with a flow rate of 0.9 mL/min and a column temperature of 25 ºC. The gradient elution program was as follows: 70%~65% A from 0 to 10 min, 65%~60% A from 10 min to 58 min, 60%~45% A from 58 min to 60 min, and 45%~30% A from 60 min to 115 min.

2.4 LC-MS analysis

LC-MS analyses were performed on an Agilent 1100 series LC/MSD VL trap system (Agilent Inc., CA, USA) equipped with an electro-spray ionization (ESI) source, auto-sampler and diode-array detector (DAD). The sample preparation, mobile phase and chromatography column were all the same as those used in the HPLC analysis (see *Section 2.3* for details). The DAD detector was monitored at 254 nm. The LC effluent was introduced into the ESI source in a post-column splitting ratio of 5:1 at a flow rate of 0.2 mL/min. The mass spectra were acquired in positive ion mode (MS^+) with an ion spray voltage of 4.0 kV, atomizing gas (He, ultrahigh-purity helium) pressure of 20 psi, capillary temperature of 300 ºC and flow rate of dry gas (N_2, high-purity) of 8 L/min. For full-scan MS analysis, the spectra were recorded in the range of m/z 100 to 1000.

2.5 Evaluation of antioxidant activity in vitro

TT samples from UCEP were dissolved in 80% methanol solution to prepare the tested samples at various concentrations (mg/mL or μg/mL). $DPPH^{\bullet}$ and $ABTS^{\cdot+}$ radical cation and superoxide anion radical (O_2^-) scavenging activity assays were performed according to the methods described by Wang *et al.* [14], Biglari *et al*. [15] and Deliang *et al*. [16], respectively. The total antioxidant activity (T-AOA) and ferric reducing antioxidant power (FRAP) were evaluated according to the methods described by [15] and Prieto *et al*. [17], respectively. All determinations were performed using a Shimadzu UV-1900 spectrophotometer. The antioxidant activities of each sample were assessed using butylhydroxytoluene (BHT, $C_{15}H_{24}O$) or rutin (rutoside, $C_{27}H_{30}O_{16}$) as the positive contrast. The EC_{50} values were calculated as the 50% effective concentration, at which the free radical scavenging ability was 50% or the absorbance was 0.5 for total antioxidant capacity and reducing power. Each value is expressed as the average of three representative samples. Means (n=3) in different letters within a row are significantly different ($P < 0.05$).

2.6 Statistical analysis

All of the experiments were conducted in triplicate, and the data were presented as the mean ± SD. SPSS (Version 16.0; Chicago, IL, USA) and Origin (Version 8.0; Microcal Software Inc., Northampton, MA, USA) were used to

process the results, which were analyzed by one-way ANOVA and Tukey's HSD test. The difference was considered to be statistically significant if the *P*-value was < 0.05.

RESULTS

3.1 Composition of total triterpenoids

The HPLC analyzed results showed that a good separation could be obtained at the maximum UV absorption wavelength (254 nm), showing clear and strong absorption peaks (t_R/min: 4.0~54.0) with a significant baseline separation (**Figure 1**), and the absorption peak (t_R/min: 28.991) was in agreement with the standard of ganoderic acid A (t_R/min: 28.860), implying that there was an abundance of triterpenoid compounds in the UCEP.

Above result was further confirmed by LC-MS analysis. There were 1~3 quasi-molecular ions $[M]^+$ in a discrete subset of each absorption peak position in the total ion chromatogram (t_R/min: 4.1~63.1) obtained by liquid chromatography (**Figure 2**). In addition, 56 molecular ions $[M]^+$ were found by mass spectrometry. Among these ions, the peaks of at least 31 quasi-molecular ions at m/z 418.3~678.5 corresponded to reliable molecular mass information for known triterpenoids from *Ganoderma spp.*, which were summarized in **Table 1**.

Table 1 Molecular mass, molecular ions and related fragment ions of major triterpene acid compounds from the Lingzhi UCE extract powder

Peak No.	t_R/min	quasi-molecular ion (m/z)	MW
1	4.1-4.2	$[M+H]^+$ 679.6, $[M+Na]^+$ 701.5	678.5
2	8.8-8.8	$[M+H]^+$ 433.3, $[M+Na]^+$ 455.3	432.3
6	10.1-10.1	$[M+H]^+$ 531.3, $[M+Na]^+$ 553.3	530.3
9	11.7-11.7	$[M+H]^+$ 419.3, $[M+Na]^+$ 441.3	418.3
10	12.4-12.4	$[M+H]^+$ 423.3, $[M+Na]^+$ 445.3	422.3
11	12.8-12.8	$[M+H]^+$ 517.3, $[M+Na]^+$ 539.3	516.3
15	13.9-13.9	$[M+H]^+$ 479.3, $[M+Na]^+$ 501.4	478
		$[M+H]^+$ 475.3, $[M+Na]^+$ 497.3	474.3
18	14.7-14.8	$[M+H]^+$ 419.3, $[M+Na]^+$ 441.3	418.3
21	16.3-16.3	$[M+H]^+$ 531.3, $[M+Na]^+$ 553.3	530.3
23	17.3-17.3	$[M+H]^+$ 459.3, $[M+Na]^+$ 481.3	458.3
24	17.7-17.7	$[M+H]^+$ 533.3, $[M+Na]^+$ 555.3	532.3
25	18.6-18.6	$[M+H]^+$ 517.3, $[M+Na]^+$ 539.2	516.3
26	19.0-19.0	$[M+H]^+$ 513.3, $[M+Na]^+$ 535.3	512.3
		$[M+H]^+$ 473.3, $[M+Na]^+$ 495.3	472.3
27	19.7-19.8	$[M+H]^+$ 517.3, $[M+Na]^+$ 539.3	516.3
28	20.4-20.4	$[M+H]^+$ 573.3, $[M+Na]^+$ 595.3	572.3
30	21.4-21.4	$[M+H]^+$ 515.3, $[M+Na]^+$ 537.3	514.3
33	22.8-22.9	$[M+H]^+$ 571.3, $[M+Na]^+$ 593.4	570.3
34	23.5-23.6	$[M+H]^+$ 573.3, $[M+Na]^+$ 595.3	572.3
37	24.8-24.8	$[M+H]^+$ 513.3, $[M+Na]^+$ 535.3	512.3
40	28.0-28.0	$[M+H]^+$ 529.3, $[M+Na]^+$ 551.2	528
41	29.1-29.1	$[M+H]^+$ 517.3, $[M+Na]^+$ 539.2	516.3
42	30.7-30.7	$[M+H]^+$ 513.3, $[M+Na]^+$ 535.3	512.3
43	33.0-33.0	$[M+H]^+$ 515.3, $[M+Na]^+$ 537.3	514.3
44	33.8-33.8	$[M+H]^+$ 457.3, $[M+Na]^+$ 479.3	456.3
45	35.1-35.1	$[M+H]^+$ 511.3, $[M+Na]^+$ 533.2	510.3
46	36.0-36.0	$[M+H]^+$ 515.3, $[M+Na]^+$ 537.3	514.3
47	36.8-36.8	$[M+H]^+$ 513.3, $[M+Na]^+$ 535.3	512.3
48	37.4-37.4	$[M+H]^+$ 501.4, $[M+Na]^+$ 523.3	500.4
50	40.4-40.4	$[M+H]^+$ 569.3, $[M+Na]^+$591.2	568
52	42.4-42.4	$[M+H]^+$ 571.3, $[M+Na]^+$ 593.3	570.3
53	43.7-43.7	$[M+H]^+$ 515.3, $[M+Na]^+$ 537.3	514.3
54	63.1-63.1	$[M+H]^+$ 475.4, $[M+Na]^+$ 509.1	474

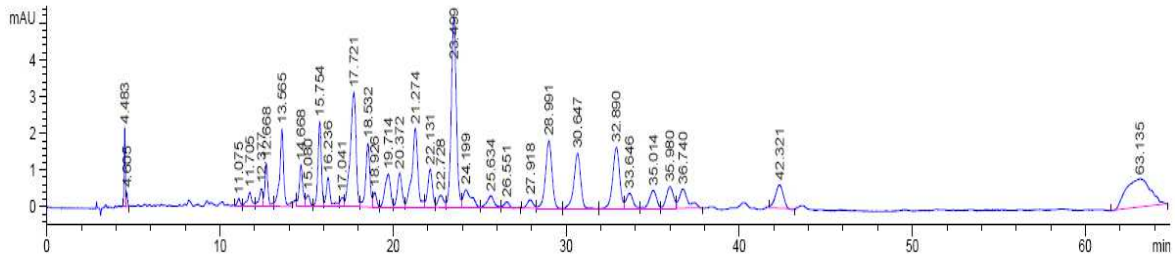

Figure 1 HPLC fragmentation patterns of TTs from the UCEP (calibrated with ganoderma acid A)

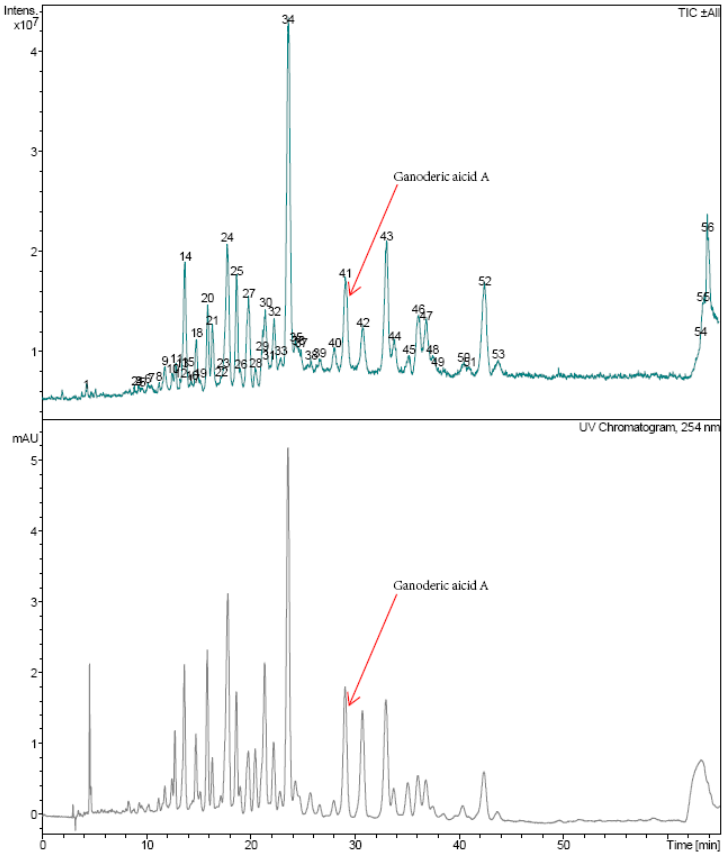

Figure 2 Total ion-current chromatograph (MS^+) and HPLC separation of Lingzhi TTs from UCEP (DAD-UV, Sig=254 nm, Ref=365 nm)

It was suggested that most of these components are lanostane-type tetracyclic triterpenes with C30 skeleton structure, extrapolated as ganoderic acid Z or 2H-ganoderic aldehyde A for $C_{30}H_{48}O_3$ (m/z 456.3); ganolucidic acid A for $C_{30}H_{44}O_6$ (m/z 500.3); ganodermic acid D or ganodermic acid E for $C_{30}H_{40}O_7$ (m/z 512.3); ganoderic acid C1, ganodermic acid A or ganodermic acid B or ganodermic acid C for $C_{30}H_{42}O_7$ (m/z 514.3); ganoderic acid A or ganoderic acid B, ganoderic acid LM2 for formula $C_{30}H_{44}O_7$ (m/z 516.3), 12-hydroxy-3,7,11,15,23-pentaoxo-lanost-8-en-26-oic acid or lucidenic acid D for $C_{30}H_{40}O_8$ (m/z 528.0); ganoderic acid D for $C_{30}H_{42}O_8$ (m/z 530.3); and ganoderic acid G or methyl lucidenate P for $C_{30}H_{40}O_8$ (m/z 532.3).

Other possible C27 or C32 skeleton structures were extrapolated as ganoderic acid H for $C_{32}H_{44}O_9$ (m/z 572.3); ganoderic acid F for $C_{32}H_{42}O_9$ (m/z 570.3) or lanosta-7,9(11),24-trien-15α,22β-diacetoxy-3β-hydroxy-26-oic acid for $C_{34}H_{44}O_6$ (m/z 570.3); lanosta-7,9(11),24-trien-3α,15α-diacetoxy-23-oxo-8-en-26-oic acid for $C_{34}H_{48}O_7$ (m/z 568.0); lucidenic acid D2 for $C_{29}H_{38}O_8$ (m/z 514.3); lucidenic acid B or E1 for $C_{27}H_{38}O_7$ (m/z 474.0), lucidenic acid A for $C_{27}H_{38}O_6$ (m/z 458.3); and 20(21)-dehydro-lucidenic acid A for $C_{27}H_{36}O_6$ (m/z 456.2). Moreover, there may be a triterpene saponin for $C_{30}H_{44}O_7$-$C_6H_{12}O_5$ (m/z 678.5) at t_R/min 4.1-4.2 and methyl lucidenate Q for $C_{28}H_{42}O_6$ (m/z 474.0) at t_R/min 63.1-63.1.

3.2 Antioxidant activity of TTs in vitro

The results of the assays (**Figure 3**) implied that TTs from UCEP had a positive scavenging ability for three free radicals ($DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $O_2^{\cdot-}$, see Figure 3a,3b and 3c) as well as total antioxidant activity (T-AOA, see Figure 3d) and ferric reducing antioxidant power (FRAP, see Figure 3e). The scavenging abilities for $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $O_2^{\cdot-}$ free radicals were 785 μg/mL, 5812 μg/mL and 1524 μg/mL, respectively, and the EC_{50} values of T-AOA and FRAP were 1275 μg/mL and 661 μg/mL, respectively. All antioxidant properties were dependent on the sample concentration, varying within a certain range.

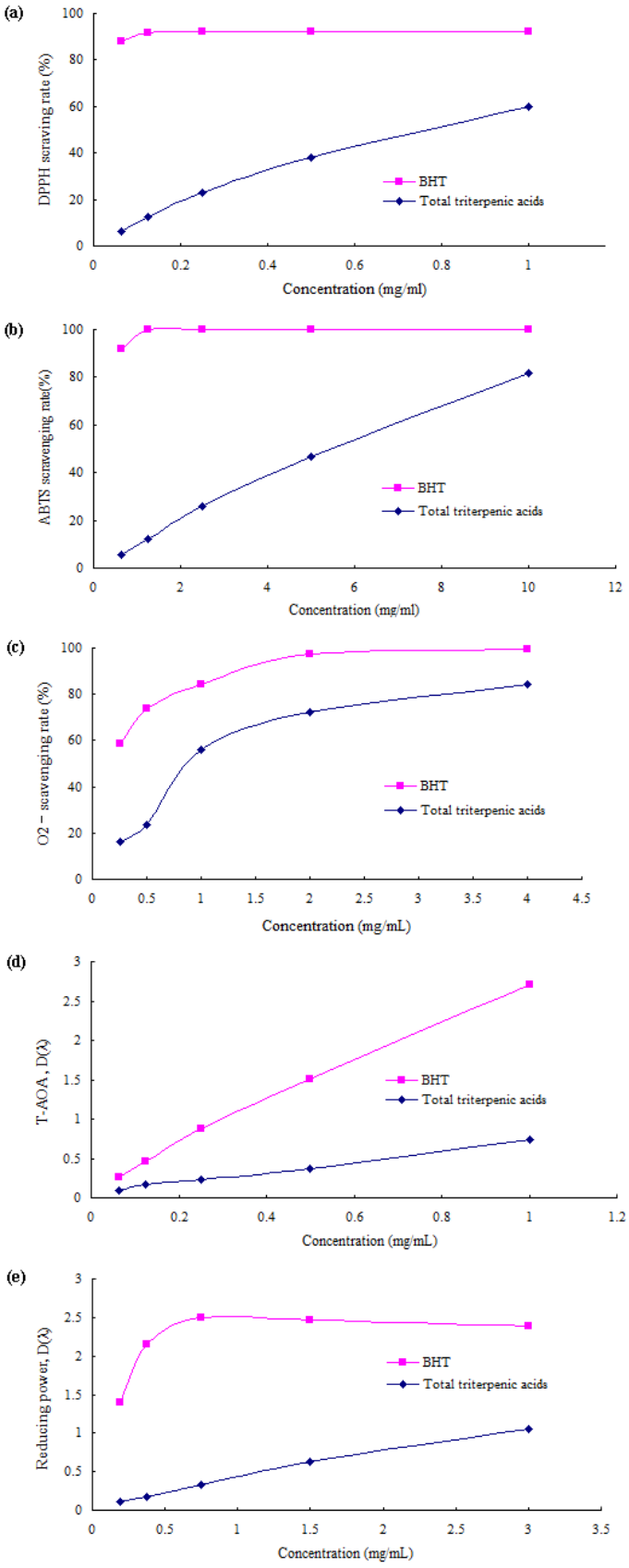

Figure 3 Antioxidant activities of TTs from UCEP

(a) DPPH˙ scavenging ability. (b) $ABTS^{\cdot+}$ scavenging ability.(c) $O_2^{\cdot-}$ scavenging ability. (d) Total antioxidant activity (T-AOA). (e) Ferric reducing antioxidant power (FRAP).

DISCUSSIONS AND CONCLUSION

A few studies have suggested that triterpene components from *Ganoderma* also have strong antioxidant properties, as do polysaccharides. For example, a triterpenoid saponin ($C_{30}H_{44}O_{10}$, m/z 564) from *G. lucidum* showed better antioxidant activity than BHT and vitamin C, and the EC_{50} values for scavenging O_2^- and $DPPH^·$ and achieving 0.5 reducing power were 0.247, 0.09 and 0.425 mg/mL, respectively [18]. The total triterpenoids isolated from *G. lucidum* successfully scavenged $DPPH^·$, $ABTS^{·+}$ and O_2^- radicals; exhibited significant ferric reducing activity; and were highly effective in reducing the *in vitro* lipid peroxidation [12].

There is strong evidence that total triterpenes isolated from *G. lucidum* successfully reduced the formation of intracellular ROS and enhanced endogenous antioxidant enzyme activity in splenic lymphocytes following irradiation, indicate that have a remarkable ability to protect normal cells from radiation-induced damage *in vitro* and did not possess significant toxicity [19]. Thus, further studying the antioxidant activity *in vivo* of TTs from *G. lucidum* and its monomer compounds' biological activity would be of great value.

Prospect

This study revealed that total triterpenoids were one of the most important active ingredients in the ultrasonic-circulating extract powder (UCEP) of *G. lucidum*, with a mean content of 9.91% based on dry weight. Total triterpenoids displayed important antioxidant effects, suggesting their potential use as an antioxidant to enhance the body's antioxidant defense systems.

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Compliance with Ethics Requirements

The authors declare no competing financial interest.

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