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Bioactive Constituent Characterization and Antioxidant Activity of *Ganoderma lucidum* Extract Fractionated by Supercritical Carbon Dioxide

(Pencirian Juzuk Bioaktif dan Aktiviti Antioksidan Ekstrak *Ganoderma lucidum* Terpecah oleh Karbon Dioksida Supergenting)

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ABSTRACT

Ganoderma lucidum *has been recognized as a precious fungus in both Chinese and Japanese traditional medicine for centuries. It contains many bioactive ingredients such as triterpenoids and polysaccharides. The present study used supercritical carbon dioxide (SC-CO_2) fractionation to fractionate* Ganoderma lucidum *extract into four fractions (R, F1, F2, & F3) and evaluate the correlation between the content of functional components and their antioxidant ability. Relatively high concentrations of the three types of bioactive constituents were simultaneously partitioned into different fractionation collecting vessels. The free radical scavenging ability was greatest in F1. The IC_{50} of DPPH scavenging ability was 0.90 mg/mL and that of ABTS radicals scavenging activity was 0.45 mg/mL. The correlation analysis of antioxidant ability with total triterpenoids and total polyphenols showed a positive relationship. In conclusion, this study showed that fractionation of* Ganoderma lucidum *extract using SC-CO_2 fractionation technology was able to effectively partition its bioactive components including triterpenoids, polysaccharides and phenolic compounds and also to increase the antioxidant activities of the fractions.*

Keywords: Antioxidant activities; Ganoderma lucidum*; supercritical carbon dioxide fractionation; total polysaccharides; total triterpenoids*

ABSTRAK

Ganoderma lucidum *telah dikenal pasti sebagai kulat berharga dalam kedua-dua perubatan tradisi Cina dan Jepun selama berabad-abad . Ia mengandungi banyak bahan bioaktif seperti triterpenoids dan polisakarida. Kajian ini menggunakan pemecahan karbon dioksida supergenting (SC-CO_2) untuk ekstrak* Ganoderma lucidum *terpecah kepada empat pecahan (R, F1, F2 , & F3) dan menilai hubung kait antara kandungan komponen berfungsi dan keupayaan antioksidan mereka. Kepekatan yang agak tinggi daripada tiga jenis juzuk bioaktif dibahagikan secara serentak kepada pemeringkatan vesel pengumpulan berbeza. Keupayaan skaveng radikal bebas adalah terbaik dalam F1. IC_{50} keupayaan skaveng DPPH adalah 0.90 mg/mL dan aktiviti skaveng radikal ABTS adalah 0.45 mg/mL. Analisis korelasi kemampuan antioksidan dengan jumlah triterpenoid dan polifenol menunjukkan hubungan yang positif. Kesimpulannya, kajian ini menunjukkan bahawa pemeringkatan ekstrak* Ganoderma lucidum *menggunakan teknologi pemeringkatan SC-CO_2 dapat memisahkan komponen bioaktif dengan berkesan termasuk triterpenoid, polisakarida dan sebatian fenolik serta meningkatkan aktiviti antioksidan pecahan.*

Kata kunci: Aktiviti antioksidan; Ganoderma lucidum*; jumlah polisakarida; jumlah triterpenoid; karbon dioksida superkritikal pemeringkatan*

INTRODUCTION

Ganoderma lucidum (Lingzhi, Reishi) has been recognized as a precious fungus in both Chinese and Japanese traditional medicine for centuries. It contains many bioactive ingredients such as triterpenoids, polysaccharides, proteins (LZ-8), lectins, adenosine and germanium. Of these, triterpenoids and polysaccharides have been extensively studied because of their pharmacological effects (Paterson 2006) and they have been shown to possess bioactivities such as anti-tumor (Harhaji Trajković et al. 2009; Jedinak et al. 2011), immuno-modulating (Lin & Zhang 2004; Xu et al. 2011), hepatoprotective (Jin et al. 2013), antioxidant (Xu et al. 2009), osteoclastogenesis (Miyamoto et al. 2009), antiviral (Eo et al. 1999) and anti-inflammatory (Dudhgaonkar et al. 2009).

G. lucidum is commonly and traditionally extracted using organic solvents or hot water; but these types of extractions are associated with a number of shortcomings such as complexity of extraction, low extraction efficiency, degradation of active components and the presence of toxic organic solvents in extracts (Cui et al. 2012). On the other hand, extraction with supercritical fluids using carbon dioxide as a solvent can provide an excellent alternative instead of chemical solvents. Supercritical

carbon dioxide (SC-CO_2) is a method of physical fractionating and therefore can selectively optimize the final products through modifying the density and solubility of the fluid with different temperatures and pressures. In addition, extraction with SC-CO_2 can be achieved at low temperatures and can avoid the problem of residual organic solvents in the extracts. SC-CO_2 can therefore be regarded as a green process because it does not use chemical solvents and drastically minimize environmental impacts (Hawthorne 1990; Sharif et al. 2014).

Preparative column chromatography involving a series of different columns is generally used to separate each group of functional constituents in *G. lucidum* extract (El-Mekkawy et al. 1998; Zhao et al. 2010). By optimizing the temperature, pressure, and flow rate, the fractions can be partially concentrated in different collecting vessels by SC-CO_2 fractionation technology. Based on the results from our previous studies, the optimal parameters for extracting triterpenoids and polyphenolic compounds from *G. lucidum* using SC-CO_2 was established at 40°C combined with a high pressure of 30 MPa (Lin et al. 2012b). The present study aimed to investigate the concentrations of active components and antioxidant abilities of various fractions obtained from ethanol extracts of *G. lucidum* using SC-CO_2 fractionation technology.

Materials and Methods

Materials

The dried fruiting body of *G. lucidum* was provided by Biotechnology Research Center, Far East University, Tainan, Taiwan. Ursolic acid, vanillin, perchloric acid, phenol, glucose, gallic acid, Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), α-tocopherol, trolox, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), peroxidase, 2,2'-Azobis [2-methylpropionamidine] dihydrochloride (AAPH), fluorescein sodium, phosphoric acid, potassium ferricyanide, trichloroacetic acid and ferric chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sulfuric acid and sodium chloride were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile, methanol and ethanol were obtained from TEDIA Co. (Fairfield, OH, USA).

Preparation and SC-CO_2 fractionation of *G. lucidum* ethanolic extract

The dried *G. lucidum* fruiting body was ground in a Waring blender and passed through a 30-mesh sieve. *G. lucidum* powder was mixed with ethanol at a ratio of 1:10 (v/w) for 24 h to yield the *G. lucidum* ethanolic extract (E). The extract was then filtered and fractionated using a continuous SC-CO_2 apparatus (Zibettia et al. 2013) under the following operational conditions: Temperature at 40°***C***; flow rate of CO_2 at 6 mL/min; and inflow rate of ethanol extract at 3 mL/min. A series of four separation vessels were operated at 30, 15, 10 and 5 MPa to obtain the residues (R) and three fractions (F1, F2 and F3), respectively. The collected samples were then stored at -20°***C*** until use.

Determination of total triterpenoids

The determination of total triterpenoids was performed according to the colorimetric method of Smina et al. (2011). The test samples or ursolic acid standards in tubes were evaporated to dryness in a water bath at 100°C. For each tube, 0.4 mL of 5% vanillin/glacial acetic acid (w/v) and 1.0 mL of perchloric acid solution were added successively. For the reaction, the tubes were then placed in a water bath at 60°C for 15 min. The absorbance of the sample was measured at 548 nm after adding 5.0 mL of glacial acetic acid to the cooled samples.

Determination of total polysaccharides

The total polysaccharides content of extracts was determined using the colorimetric phenol-sulfuric acid method (Albalasmeh et al. 2013) using glucose as the standard. An aliquot of 1 mL of the test samples or glucose standards was mixed with 1 mL of 5% phenol in a test tube followed by the addition of 5 mL of 97% sulfuric acid (v/v). The mixture was allowed to cool to room temperature before reading the absorbance at 490 nm.

Determination of total phenolic compounds

The concentration of total phenolic compounds was determined spectrophotometrically using the Folin-Ciocalteu method (Wang et al. 2005). The samples were oxidized with Folin-Ciocalteu's reagent and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm after 60 min. Total phenolic content, using gallic acid as a calibration standard, was expressed as gallic acid equivalent per gram of dry weight (dw) of the *G. lucidum* sample.

DPPH radicals scavenging activity

The scavenging of DPPH radicals was assayed following the method of Wang et al. (2004). A different concentration of each extract was added at equal volume to methanolic solution of DPPH (0.1 mM). The mixture was incubated at room temperature for 50 min before the absorbance at 517 nm was read.

Trolox equivalent antioxidant capacity

The Trolox equivalent antioxidant capacity (TEAC) was determined according to the method described by Arnao et al. (2001). The radical cation $ABTS^{\cdot+}$ was generated by mixing 1 mL deionized water, 0.2 mL peroxidase (4.4 unit/mL), 0.2 mL of H_2O_2 (50 μM) and 0.2 mL ABTS (100 μM). The mixture was allowed to stand 1 h at room temperature in the dark to form radical cation $ABTS^{\cdot+}$. The *G. lucidum* extract and fractions were added individually and then the absorbance at 734 nm was measured.

OXYGEN RADICAL ABSORBANCE CAPACITY

The oxygen radical absorbance capacity (ORAC) assay was based on the method of Huang et al. (2002). Twenty μL of calibration solutions of Trolox (12.5, 25, 50, 100, and 200 μM) or study samples, followed by the addition of 20 μL of 75 mM potassium phosphate buffer (pH7.4) and 20 μL of 8.16×10^{-5} mM fluorescein sodium were added into individual well of a 96-well microplate. The microplate was then incubated at 37°C for 5 min in a microplate reader. After the incubation, 140 μL of 153 mM 2,2'-Azobis-[2-methylpropionamidine] dihydrochloride (AAPH), as a peroxyl radical generator was rapidly added using a multi-channel pipette to start the reaction. The fluorescence was recorded every two min, until the emitted fluorescence could not be distinguished from the baseline, in a microplate spectrophotometer reader using excitation wavelength of 490 nm and an emission wavelength of 518 nm. The area under the curve (AUC) was calculated to represent the ORAC value. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The relative ORAC value, expressed as Trolox equivalents (TE) was calculated by extrapolation from the Trolox calibration curve.

REDUCING POWER

Extract or fractions of *G. lucidum* in a phosphate buffer (2.5 mL, 0.2 M, pH6.6) were added into potassium ferricyanide (2.5 mL, 10 mg/mL) to determine the reducing power (Shi et al. 1991). After incubation at 50°C for 20 min, trichloroacetic acid (2.5 mL, 100 mg/mL) was added to the mixture and then centrifuged at 650 × g for 10 min. After mixing the supernatant (2.5 mL), distilled water (2.5 mL) and ferric chloride (0.5 mL, 1.0 mg/mL), the absorbance at 700 nm was measured.

STATISTICAL ANALYSIS

All experiments were replicated for three times to obtain mean values. Pearson's product-moment correlation coefficient was used to represent the correlations between antioxidant ability and the contents of total triterpenoids and total polyphenols.

RESULTS AND DISCUSSION

The main bioactive components of *G. lucidum* include triterpenoids, polysaccharides, and polyphenols (Paterson 2006). Table 1 shows the contents of total triterpenoids, total polysaccharides and total polyphenols in the extract, residual and the three fractions obtained from the ethanol extract of *G. lucidum* using SC-CO_2. Fractionation with SC-CO_2 markedly increased all three components from the extract. The content (in mg/g of extract, dw) of total triterpenoids, in the order of decreasing amounts, were F3 (643.06), F2 (425.09), F1 (412.29), E (335.99) and R (196.03). For total polysaccharides (mg glucose equivalent/g of extract, dw), in the order of decreasing amounts, were F1 (156.61), F2 (129.26), E (112.53), R (78.43) and F3 (73.03). For total polyphenols (mg GAE/g of extract, dw), in the order of decreasing amounts, were R (63.76), F1 (50.46), F2 (45.82), F3 (45.40), and E (41.31). The highest concentrations of triterpenoids, polysaccharide and polyphenols were found in F3, F1 and R, respectively. Not only relatively high concentrations of different types of bioactive constituents could be gathered in different collecting vessels, but also the separation could be continuously conducted.

Antioxidant activity is one of the functions provided by various bioactive components of *G. lucidum* (Saltarelli et al. 2009; Smina et al. 2011). Figure 1 shows the relationship of total triterpenoids with DPPH, ORAC, TEAC and reducing power. A clear dose-response relationship could be observed. As the concentrations of total triterpenoids were increased, the antioxidant activities were increased, indicating that triterpenoids could contribute to the antioxidant activity of *G. lucidum*. This finding is consistent with those reported in the literature where ABTS radical cation scavenging activity was demonstrated in triterpenoids isolated from the stems of *Momordica charantiaa* and the seed of *Garcinia subelliptica* (Lin et al. 2012a; Liu et al. 2010).

Polyphenols are a diverse class of phytochemicals and more than 8000 compounds have been identified. Although many of them have no known role in human nutrition, some might reduce the potential cellular damage caused by free radicals (Ferguson 2001). Figure 2 shows

TABLE 1. Contents of total triterpenoids, total polysaccharides, ganoderic acid A, and phenolic compounds of *G. lucidum* extract, residual, and fractions obtained from continuous supercritical fluid extraction

		Operating condition (MPa / °C)	Yield (wt, %)	Total triterpenoids[1]	Total polysaccharides[2]	Total polyphenols[3]
Extract		NA	NA	335.99	112.53	41.31
Residual		30 / 60	46.0	196.03	78.43	63.76
Fraction	F1	15 / 60	45.3	412.29	156.61	50.46
	F2	10 / 60	35.6	425.09	129.26	45.82
	F3	5 / 60	9.8	643.06	73.03	45.40

NA: not applicable
[1] Total triterpenoids expressed as mg/g of extract, dw
[2] Total polysaccharides expressed as mg glucose equivalent/g of extract, dw
[3] Total phenolic compounds expressed as mg gallic acid equivalent/g of extract, dw

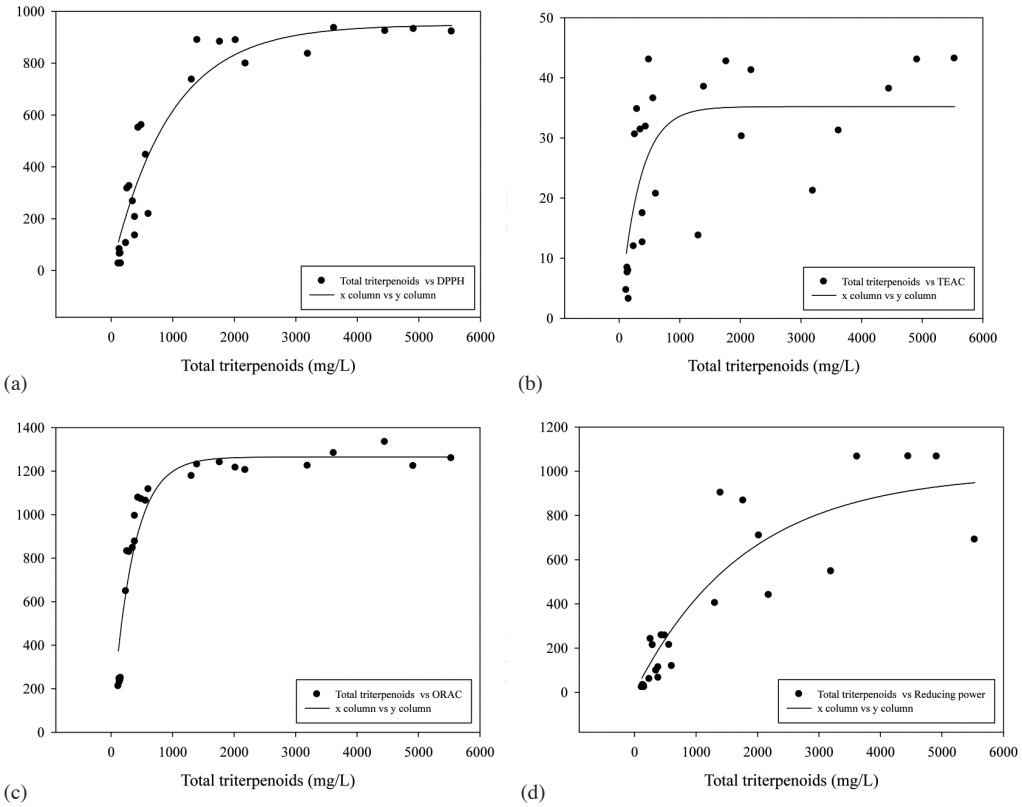

FIGURE 1. Relationship between total triterpenoids contents and (a) DPPH, (b) TEAC, (c) ORAC and (d) reducing power

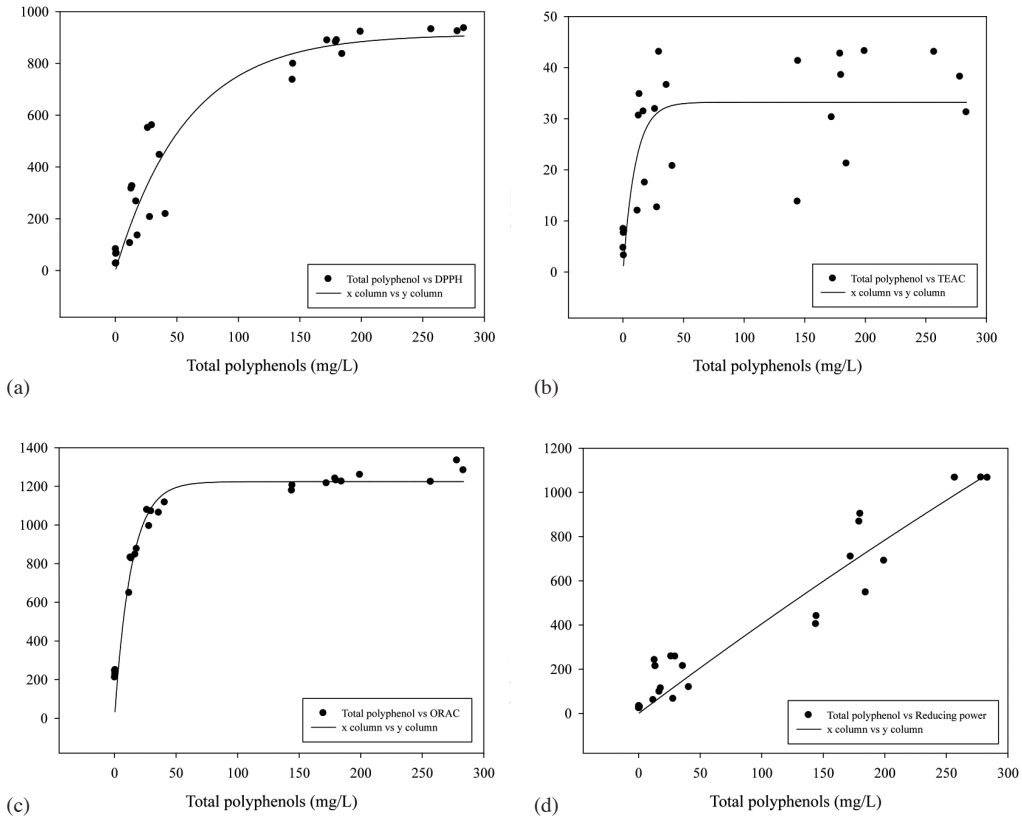

FIGURE 2. Relationship between total polyphenols contents and (a) DPPH, (b) TEAC, (c) ORAC and (d) reducing power

the relationship between contents of total polyphenols and antioxidant activity. The antioxidant activity increased with the increasing contents of polyphenols. This finding is in line with the previous reports showing good antioxidant activity in *G. lucidum* (Chien et al. 2011; Saltarelli et al. 2009).

The triterpenoids and polyphenols present in *G. lucidum* have been reported to show antioxidant activity (Chien et al. 2011; Saltarelli et al. 2009; Smina et al. 2011). Table 2 shows the correlations between antioxidant activity and total triterpenoids and total polyphenols in *G. lucidum*. The correlation coefficients were above 0.8 and 0.9 for DPPH radicals scavenging activity and reducing power in total triterpenoids and total polyphenols, respectively. The strong positive correlations indicated that triterpenoids and polyphenols contributed to the antioxidant activity of *G. lucidum*.

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. Table 3 shows the antioxidant ability of *G. lucidum* extract and fractions. The results from the IC_{50} in DPPH scavenging ability and $ABTS^{\cdot+}$ radical scavenging ability indicated that F1 possessed the best overall activity. The IC_{50} of DPPH scavenging ability was 0.90 mg/mL and that of ABTS radicals scavenging activity was 0.45 mg/mL. For comparisons of reducing power, a lower concentration with absorbance at 700 nm to reach an optical density at 1 represents a better reducing power. The results indicated that F1 possessed the best reducing power (2.05 mg/mL), while R showed the least reducing power (5.79 mg/mL). Since F1 contained relatively higher amount of polysaccharide, our results were in consistent with a previously published study which reported that *G. lucidum* polysaccharides exhibited strong antioxidant effects (Shi et al. 2014).

Conclusion

In conclusion, this study showed that fractionation of *G. lucidum* extract using continuous SC-CO_2 partition technology was able to effectively concentrate bioactive constituents including triterpenoids, polysaccharides and polyphenols. In addition, SC-CO_2 partition technology could increase the antioxidant activities of the fractions, which were highly correlated with the concentrations of bioactive constituents in *G. lucidum*.

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TABLE 2. Correlations between antioxidant ability and contents of total triterpenoids or total polyphenols

Antioxidant assay	Pearson's correlation coefficient (*r*)	
	Total triterpenoids	Total polyphenols
DPPH	0.81	0.92
TEAC	0.56	0.58
ORAC	0.64	0.74
Reducing power	0.84	0.96

DPPH: 1,1-diphenyl-2-picrylhydrazyl
TEAC: Trolox equivalent antioxidant capacity
ORAC: oxygen radical absorbance capacity

TABLE 3. Antioxidant activities of supercritical carbon dioxide partitioning fractions of *G. lucidum* ethanolic extract

	DPPH scavenging ability IC_{50} (mg/mL)	$ABTS^{\cdot+}$ scavenging ability IC_{50} (mg/mL)	ORAC = 1000 μmol Trolox equivalent/L (mg/mL)	Reducing power Optical density = 1 (mg/mL)
Extract (E)	0.92	0.59	0.86	2.64
Residue (R)	3.43	2.34	0.98	5.79
Fraction 1 (F1)	0.90	0.45	0.85	2.05
Fraction 2 (F2)	1.16	0.54	0.85	2.68
Fraction 3 (F3)	3.13	1.14	0.80	4.52
Tocopherol	0.05	ND	ND	ND
Trolox	ND	0.07	0.25	ND
Ascorbic acid	ND	ND	ND	0.03

IC_{50}: half maximal inhibitory concentration
ND: not determined

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