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Extraction and antioxidant activities of broken *Ganoderma lucidum* spore

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Abstract Soxhlet and microwave-assisted extraction (MAE) methods showed the highest extraction yield and total phenolic content of extract from broken spores of *Ganoiderma lucidum* ($p<0.05$). However, the extraction time of MAE method was shorter than the soxhlet extraction method. The broken spores of *G. Lucidum* were extracted using different solvent types with MAE method. Ethanol extraction rendered extract with the highest total phenolic content and ferric reducing antioxidant power (FRAP) value ($p<0.05$). For metal chelating activity, ethanol and hexane extracts were significantly high compared to other extracts of broken *G. lucidum* spores ($p<0.05$). Thus, the use of appropriated extraction method and solvent type rendered to extract with high total phenolic content and antioxidant activities.

Keywords: *Ganoderma lucidum*, Broken spores, Microwave-assisted extraction, Antioxidant activity

Introduction

Ganoderma lucidum (*G. lucidum*) is an edible medicine mushroom and its fruiting body in China is called 'Lingzhi'. Fruiting bodies of *G. lucidum* consisted of polysaccharides, proteins, lipids, phenols, triterpenes, and sterols (Cör *et al.,* 2018). *G. lucidum* extract from fruiting body had various biological activities, such as anticancer activities (Barbieri *et al.,* 2017), antitumor activities (Lin and Zhang, 2004), antimicrobial activities (Cör *et al.,* 2018), anti-inflammatory activities (Barbieri *et al.,* 2017), antioxidant activities (Cör *et al.,* 2018) and immune-stimulation activities (Lin and Zhang, 2004). Moreover, *G. lucidum* spores were found to have many bioactive ingredients like the *G. lucidum* fruit bodies. It has been shown that *G. lucidum* spores exhibited a higher the bioactivity when compared with the *G. lucidum* fruiting bodies (Ma

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et al., 2007). *G. lucidum* spores are spherical to elliptical and tiny particles, 6.5 - 8.0 to 9.6 - 12.6 μm in size, wrapped by outer bilayers of sporoderm. The sporoderm is very hard and acts as a barrier against the release of compounds from the *G. lucidum* spores (Soccol *et al.,* 2016). However, these compounds can also be obtained from spores of *G. lucidum* by broken-sporoderm machine. Intharuksa *et al.* (2010) reported that broken-sporoderm machine could be used for breaking of the spores of *G. lucidum*. Bioactive components were extracted from broken *G. lucidum* spores with a 3 - 4 times higher extract yield than that of non-broken sporoderm.

Yield, total phenolic, and antioxidant properties of extracts from mushroom, plants, herbs, and others are dependent on the extraction methods and extraction solvents used. To extract phenolic compounds, several extraction techniques have been widely used such as conventional soxhlet extraction, maceration extraction, microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UE). All techniques involved organic solvent or water for the extraction. Organic solvents have been used in food industries like ethanol, methanol, hexane, acetyl-acetate, acetone, and isopropanol, etc. However, little information about the effect of the extraction processes on yields, the amount of phenolic compounds, and antioxidant activities of the extracts from broken *G. lucidum* spores. Thus, the research aimed to determine the yield, total phenolic content, and to study the antioxidant properties of the extracts from broken spores of *G. lucidum* which affected by different extraction methods and extraction solvents.

Materials and methods

Collection and chemical composition of G. lucidum spores

The broken spores of *G. lucidum* were obtained from Mae Jo farm inn, San Sai district, Chiang Mai, Thailand. All broken spores of *G. lucidum* were kept in sealed polyethylene bag at -18 ℃ until use within 1 month. Each sample was evaluated for moisture, fat, protein, ash and crude fiber contents using AOAC method (AOAC, 2000). Carbohydrate was calculated as 100% - (% of moisture+fat+protein+ash). The contents were expressed as wet basis of broken *G. lucidum* spore sample.

Extraction for broken spores of G. lucidum

Effect of different extraction methods

The broken spores of *G. lucidum* were obtained using four different extraction methods: 1) soxhlet extraction 2) maceration extraction 3)

microwave-assisted extraction (MAE) and 4) ultrasound-assisted extraction (UE). The extraction conditions used for each method as follows:

Maceration extraction

The broken spores of *G. lucidum* (2 g) were mixed with methanol (60 ml) in an Erlenmayer flask. The mixture was stirred using magnetic stirrer (Heidolph, MR3001, K, Germany) for 24 h at 25±2˚C with a constant stirring.

Soxhlet extraction

The broken spore of *G. lucidum* (2 g) was placed into a thimble and and inserted in the Soxhlet apparatus (Model SOX 416, Gerhardt, Germany) for 30 min at 95 ˚C, using methanol (60 ml) as solvent.

Microwave-assisted extraction (MAE)

The broken spores of *G. lucidum* (2 g) were mixed with 60 ml of methanol for 10 min at 350 Watt using a microwave oven (EME2024, Electrolux, Sweden).

Ultrasound-assisted extraction (UE)

UE was performed in an ultrasonic bath (136H, Ultrasonic, England). The broken spore of *G. lucidum* (2 g) and methanol (60 ml) in a 250-ml beaker were treated with ultrasound at 30 KHz ±50 Hz and 500 W for 30 min.

After extraction, the mixtures of each extraction method were filtered using a filter paper No.4 (Whatman International Ltd., Maidstone, England). The solvent was evaporated at 50 ℃ using a rotary evaporator (VV2000, Heidolph, Schwabach, Germany). The extracts of broken spores of *G. lucidum* were dried in a freeze dryer (STD, US). All extracts of broken spores of *G. lucidum* were preserved in dark brown glass bottles at -18 ℃. The obtained extracts were calculated for extraction yield and were subjected to analyses total phenolic content of extracts from the spores of *G. lucidum*.

Effect of different solvent types

The extraction method rendering the highest extraction yield and total phenolic content was used. The broken spores of *G. lucidum* were extracted using five different solvent types with different polarities: water, ethanol, methanol, hexane, and acetyl-acetate. The obtained extracts were preserved in an amber bottle and kept at -18 ℃ until analysis.

Analyses

% Yield

The yield of extract from broken spores of *G. lucidum* was calculated based on the dry weight of extract using equation 1:

$$\% \text{ Yield crude extract} = \frac{a}{b} \times 100 \qquad (1)$$

a = Weight of extracts (g)
b = Weight of initial broken *G. lucidum* spores (g)

Total phenolic content

Total phenolic content of broken spores of *G. lucidum* extract was evaluated spectrophotometrically using the Folin-Ciocalteu method (Buamard and Benjakul, 2015). The extract (100 µl) was mixed with Folin-Ciocalteu's reagent (0.75 ml and 10-fold dilution). After 5 min, the reaction was mixed with 6% (v/v) sodium carbonate solution (0.75 ml) and allowed to stand at room temperature for 1 h. The absorbance was read at 760 nm using a spectrophotometer (VIS-732G, Rayleigh, China). The phenolic content was calculated from the standard curve of gallic acid (0 - 0.05 mg/ml) and expressed as mg gallic acid equivalents (GAE) / g dry weight of *G. lucidum* spore extract.

Antioxidant activities

DPPH free radical scavenging activity

The scavenging activity of sample extracts on DPPH radicals was determined following the method of Jagtap *et al*. (2010). The standard curve was used by trolox (0-60 µM). The absorbance was evaluated at 517 nm and the activity was calculated as µmol trolox equivalents (TE) / g dry weight of *G. lucidum* spore extract.

Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) of extracts was determined as described by Benzie and Strain (1996). The absorbance was evaluated at 593 nm. The standard curve of trolox (50–600 µM) was prepared. Data were expressed as µmol Trolox equivalents (TE) / g dry weight of *G. lucidum* spore extract.

Metal Chelating activity

Metal Chelating activity of extracts was measured by reported method of Dinis *et al*. (1994). The absorption of the mixture was read at 562 nm. The standard curve was calibrated by EDTA (10 - 60 µm). The metal chelating activity after sample blank substraction was measured and expressed as EDTA equivalents / g dry weight of *G. lucidum* spore extract.

Statistical analysis

All experiments were stuied three times. The data were expressed as mean±standard deviation (SD). A completely randomised design was used for the statistical analysis of the data. The data were subjected to analysis of variance (ANOVA) and the difference of means between treatments were evaluated by Duncan's multiple range test at 95% confidence. Statistical data were performed using computer software.

Results

Chemical composition of broken G. lucidum spores

The broken spores of *G. lucidum* were analyzed for proximate composition, the results indicated that the broken *G. lucidum* spores consist of 58.38 ±0.44% carbohydrate, 22.89 ± 0.57% crude fat, 7.97 ± 0.45% moisture, 7.43 ± 0.34% fiber, 2.00 ± 0.72% crude protein and 1.33 ± 0.08% ash.

Effect of various extraction methods on yield and total phenolic of extracts from broken G. lucidum spores

Table 1. Yields and total phenolic contents of extracts from broken *G. lucidum* spores as affected by various extraction methods

Extraction methods	Yield (%)	Phenolic content (mg GAE/g extract)
Maceration extraction	28.40 ± 2.37[1/b2/]	3.42 ± 0.50[b]
Soxhlet extraction	36.78 ± 0.97[a]	5.13 ± 0.12[a]
Microwave-assisted extraction	36.75 ± 0.35[a]	4.99 ± 0.26[a]
Ultrasound-assisted extraction	31.15 ± 3.43[b]	3.31 ± 0.11[b]

1/: Values are given as mean ±SD (n = 3).
2/: Different small letters in the same column indicate significant differences ($p<0.05$).

Yields and total phenolic contents of methanolic extracts from broken spores of *G. lucidum* extracted using various methods are shown in Table 1. The extraction yields ranged from 28.40% (maceration method) to 36.78% (soxhlet method) by all extraction methods. There was no difference in extraction yields between maceration extraction (28.40%) and UE (31.15%) ($p>0.05$). Soxhlet extraction represented a higher amount of methanolic extract than maceration extraction and UE ($p<0.05$). As the result, soxhlet extraction (36.78%) and MAE (36.75%) provided the highest yields of extraction ($p<0.05$). Total phenolic contents of methanolic spore extracts obtained from different extraction methods vary from 3.31 to 5.13 mg GAE/g extract. As a

result, the highest total phenolic content was achieved in spore extracts from soxhlet and MAE methods ($p<0.05$).

Effect of different solvent types on yields, phenolic contents and antioxidant activities of extracts from broken G. lucidum spores

Table 2. Yields and total phenolic contents of extracts from broken *G. lucidum* spores as affected by various extraction solvents

Extraction Solvents	Yield (%)	Phenolic content (mg GAE/g extract)
Water	$20.41 \pm 0.17^{1/d2/}$	7.41 ± 1.57^{c}
Methanol	27.58 ± 0.83^{c}	4.45 ± 0.73^{d}
Ethanol	33.42 ± 1.45^{b}	17.95 ± 0.07^{a}
Hexane	32.25 ± 1.18^{b}	6.55 ± 0.75^{c}
Acetyl-acetate	42.51 ± 2.24^{a}	9.24 ± 0.99^{b}

1/: Values are given as mean ±SD (n = 3).
2/: Different small letters in the same column indicate significant differences ($p<0.05$).

Five solvents were used for the MAE in this study: (1) water (2) ethanol (3) methanol (4) hexane and (5) acetyl-acetate. The yields and total phenolic contents of various solvent extracts from broken *G. lucidum* spores are shown in Table 2. The water extract had the lowest yield (20.41%), compared with other samples ($p<0.05$). Among organic solvents used, acetyl-acetate rendered extracts with the highest yield (42.51%) ($p<0.05$), followed by ethanol and hexane ($p>0.05$), However, there was no significant different in yields of ethanol extract (33.42%) and hexane extract (32.25%) ($p>0.05$). For methanol solvent, yield of methanol extract (27.58%) was higher than water extract ($p<0.05$). Additionally, the total phenolic content of the extracts ranged from 4.45 mg GAE/g extract for methanol extract to 17.95 mg GAE/g extract for ethanol extract. Ethanol extract had the higher total phenolic content than acetyl-acetate extract ($p<0.05$). The total phenolic content of the water extract (7.41 mg GAE/g extract) was not significantly higher than the hexane extract (6.55 mg GAE/g extract), whereas the total phenolic content of the methanol extract was significantly lower than that of other solvents ($p<0.05$). However, acetyl-acetate extract of broken *G. lucidum* spores showed the highest extraction yield (Table 2).

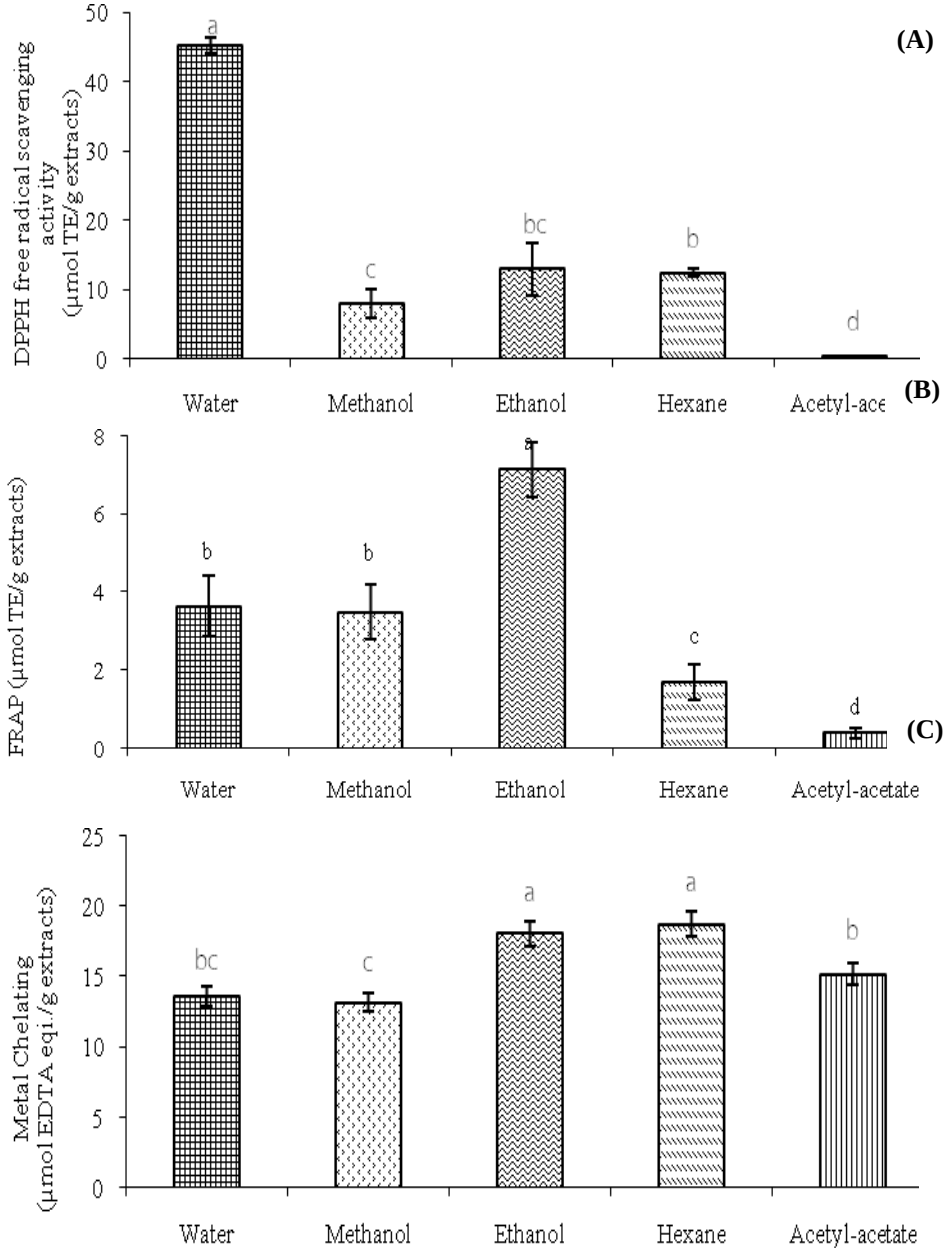

Figure 1. DPPH radical scavenging activity (DPPH) (A), ferric reducing antioxidant power (FRAP) (B) and metal chelating activity (C) of extracts from broken spores of *G. lucidum* prepared using different solvent types. a-d indicated significant differences ($p<0.05$)

Antioxidant activities of extracts from broken *G. lucidum* spores prepared using different solvent types were measured as DPPH free radical scavenging assay (Figure 1 (A)), ferric reducing antioxidant power assay (Figure 1 (B)) and metal chelating assay (Figure 1 (C)). The results found that the highest radical scavenging activity was obtained in water extract ($p<0.05$). Ethanol extract had no difference in DPPH activity, compared to methanol and hexane extracts ($p>0.05$). However, ethanol extract exhibited higher DPPH activity than acetyl-acetate extract. For FRAP assay, ethanol extract had the highest FRAP value ($p<0.05$) followed by water and methanol extracts. However, there was no significant difference of FRAP value between water and methanol extracts ($p<0.05$). The lowest FRAP value was found in acetyl-acetate extract. Metal chelating activity showed that ethanol and hexane extracts were significantly high compared to acetyl-acetate, water, and methanol extracts of broken *G. lucidum* spores ($p<0.05$).

Discussion

Ganaderma lucidum is one of the most highly prized medicinal mushrooms. Orole (2016) showed that *G. lucidum* fruit bodies contained 42.10% carbohydrate, 4.20% fat, 9.90% moisture, 29.30% fiber, 7.73.% protein and 6.33% ash. Carbohydrate and protein have been reported as the major compositions in the fruiting bodies of Ganoderma species (*G. applanatum*, *G. philippii* and *G. lucidum*) (Singh *et al*., 2020). In addition, the amount of the phenolic compounds in *G. lucidum* are linked to polysaccharides (Veljović *et al*., 2017). Based on the results, *G. lucidum* spores revealed the highest carbohydrate content, followed by crude fat, respectively ($p<0.05$). The results indicated that two major compounds in spores of *G. lucidum* were carbohydrate and fat. Thus, spores of *G. lucidum* are an important material of carbohydrate, contain many bioactive components.

When broken spores of *G. lucidum* were extracted using various methods, soxhlet extraction had a higher amount of methanol extract than maceration and UE methods ($p<0.05$). This result could be explained by the different temperatures used in soxhlet extraction. Raising the temperature contributes to increase extraction efficiency (Sharapin, 2000). As the result, soxhlet extraction and MAE provided the highest yields of extraction ($p<0.05$). Similar results were observed in the extraction of phenolic compounds from medicinal plant samples (Nile *et al*., 2017), who suggested that MAE method achieved the highest extraction yield, while maceration extraction showed the lowest extraction yield. The evaluation of extraction methods for *Pinus radiata* bark found extraction yields in the order of soxhlet extraction > MAE > UE > maceration extraction (Aspé and Fernández, 2011). The reason for the high

extraction yield when using MAE method might be because MAE method facilitates faster penetration of solvent into the cell walls (Mandal *et al*., 2007). According to microwave process, the heating up of the water inside the plant cell results in evaporation and generates tremendous pressure on the cell wall of plant. This pressure causes the rupture of the cell wall and enhances the release of the desired intracellular compounds (Gordy *et al*., 1966). Ma *et al*. (2014) showed that cell rupture in the microwave method was much faster than in the ultrasonic method. Many factors including the type of solvent, temperature, extraction time and microwave power have been known to affect the efficiency of MAE method (Osorio-Tobón, 2020). In addition, the yield of extracts obtained in this study was higher than those of extracts from spores of *G. lucidum* (10%) found by Fukuzawa *et al*. (2008), using ethanol maceration extraction method. For fruiting body of *G. lucidum*, the yield of maceration with methanol extract from *G. tsugae* was 30.3% (Hsu *et al*., 2008). Thus, soxhlet extraction and MAE methods showed a high yield of extract from broken spores of *G. lucidum*. However, the time of MAE was shorter than soxhlet extraction (from 30 min with sohxlet method to 10 min with microwave method). Total phenolic contents of methanol extracts from broken *G. lucidum* spores as affected by extraction methods are presented. Different parts of *G. lucidum* had different content of phenolic (Heleno *et al*., 2012). Total phenolic contents of methanol extracts were reported in the literature for fruiting bodies of *Ganoderma* species such as *G. carnosum* (43.28 mg GAE/g of maceration extract) (Yalcin *et al*., 2020), *G. pfeifferi* (8.12 mg GAE/g of maceration extract) (Yalcin *et al*., 2020), *G. lucidum* (39.05 mg GAE/g of soxhlet extract) (Rani *et al*., 2015) and *G. lucidum* (9.87 mg GAE/g of maceration extract) (Mohsin *et al*., 2011). Heleno *et al*. (2012) reported that the methanol extracts of *G. lucidum* fruit body provided the higher total phenolic content than that of *G. lucidum* spore. The amount of the total phenolics extracted from the fruit bodies of *G. lucidum* by the different methods varied from 8.60 to 13.90 g / 100g GAE (Veljović *et al*., 2017). The content of phenolic compound depends on the plant age, plant part used for extraction, extraction method and extraction time (Upadhya *et al*., 2015). As a result, the highest content of total phenolic was achieved in spore extracts from soxhlet and MAE methods ($p<0.05$). This is probably due to the solvent can penetrate the active sites of the matrix which might increase the rate of extractable compounds. Moreover, thermal processing does not affect the phenolic content in these conditions of the tests. Similarly, the extracts obtained from soxhlet extraction (Nantitanon *et al*., 2010) and MAE (Zhao *et al*., 2018) provided higher total phenolic content than that obtained from maceration extraction. However, these results are in contrast with those reported by Salamah *et al*. (2018) who demonstrated that

maceration method gave extracts with higher total phenolic content than soxhlet method. Thus, the extraction methods had a significant impact on the total phenolic contents of methanol extracts from the spores of *G. lucidum*. Soxhlet and MAE methods were the appropriate extraction method to extract total phenolic from the spores of *G. lucidum*. However, the use of MAE is a simple and rapid method suitable to extract total phenolic from the spores of *G. lucidum*.

The yields and total phenolic contents of extracts from broken spores of *G. lucidum* using MAE at different solvent types are demonstrated in Table 2. The extract using water as the extraction solvent had the lowest yield ($p<0.05$). It might be due to the lower solubility of the compositions in water than organic solvents. As a result, the broken spores of *G. lucidum* contained more semi-polar constituents than polar ones. The yield of each extract was different according to types of solvent used (the polarity of the solvent). Polar solvents are highly capable of dissolving polar molecules, while non-polar solvents also extract the non-polar molecules present in the sample (Bernard *et al*., 2012). From the results of Yalcin *et al*. (2020), the extraction solvents (ethyl acetate, methanol, and water) also affect yields of extract from *G. pfeifferi*. Additionally, efficiency of the different solvent extraction strongly depends on the matrix of materials and the type of extractable compounds (Rezaie *et al*., 2015). The compound in broken spores of *G. lucidum* was more dissolved in the organic solvent, and a higher yield was obtained. Additionally, the highest content of total phenolic was obtained when ethanol was used as the solvent. However, acetyl-acetate extract of broken spores of *G. lucidum* showed the highest extraction yield (Table 2). It might be due to the higher solubility of non-phenolic compounds in acetyl-acetate extract rather than in ethanol extract. Rafi *et al*. (2020) demonstrated that the content of total phenolic compounds from ethanol extract gave the highest value due to the possible complex formation of some phenolic components in the extract that dissolved in this solvent. Therefore, ethanol was good solvent for phenolic compounds extraction from broken spores of *G. lucidum*, which generally correlated with the antioxidant activities of broken *G. lucidum* spores.

Antioxidant properties of extracts from broken spores of *G. lucidum* were evaluated as DPPH, FRAP and metal chelating activities (Figure 1). The results showed that antioxidants in the water extract from broken spores of *G. lucidum* possessed the capability of scavenging the radicals (Figure 1 (A)). For FRAP and metal chelating assays, ethanol extract had the higher FRAP and metal chelating activities than other extracts ($p<0.05$), except for metal chelating activity of hexane extract, which had no different from ethanol extract ($p>0.05$) (Figure 1 (B-C)). The results were closely related to the total phenolic content

in ethanol extract from broken spores of *G. lucidum* using MAE (Table 2). It demonstrated that the ethanol extract with the highest total phenolic content exerted the high FRAP and metal chelating activities (Figure 1). Cui *et al*. (2020) reported that the bioactive compounds and antioxidant activities of extracts from medicinal plants were affected by the extraction solvents. The antioxidant capacity of each extract might be related to the content and type of phenolics. Thus, extraction solvent had the effect on the antioxidant activities evaluation. The use of MAE with ethanol or water could improve antioxidant activities of broken spores of *G. lucidum*, particularly DPPH, FRAP and metal chelating activities, where the shorten extraction time could be reached.

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