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Effects of extraction solvents on phytochemicals and bioactivities of *Ganoderma lucidum*

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Abstract

Ganoderma lucidum is a mushroom cultivated in many Asian countries. This work aimed to explore carotenoids and triterpenoids of the mushroom in Vietnam, and to investigate phenolics, antioxidant properties, inhibitory effect on bovine albumin denaturation, inhibition of α-glucosidase and α-amylase of its extracts obtained with methanol, ethyl acetate, acetone and water. The results showed that the mushroom contained average 20.30 μg of carotenoids, 488.62 μg of ergosterol and 532.53 μg of ganoderic acid A per g of dried *G. lucidum* fruiting body. The use of different extraction solvents apparently affected phenolic composition and bioactivities of *G. ludidum*. The methanolic extract had the highest total phenolic content (113.75 ± 5.05 mg GAE/g), followed by the acetonic extract. These two solvents also outperformed the others with respect to the extractability on major phenolics in *G. lucidum*. The methanolic extract generally exhibited the strongest antioxidant activity estimated via ABTS and DPPH assays (33.83 ± 2.40 and 5.36 ± 0.47 mg GAE/g, respectively) while the ethyl acetate extract displayed the most potent activity (IC_{50} = 284.79 μg/mL) to protect bovine albumin from heat-induced denaturation. Extraction with the organic solvents resulted in extracts possessing more potent activity to suppress α-glucosidase and α-amylase in comparison with water. The research provides more data about phytochemicals and additional evidence of potential health endorsing properties of *G. lucidum*, which are useful for applications in food and nutraceutical industries as well as development of new therapeutic agents for disease prevention and treatment.

Keywords: *Ganoderma lucidum; phenolics; bovine albumin; amylase; glucosidase.*.

1. Introduction

Ganoderma lucidum, belonging to the Ganodermataceae family, is an edible mushroom commonly known as lingzhi or reishi. In China and other Asian countries, the mushroom has long been used for prevention or treatment of a variety of diseases, such as hepatitis, nephritis, hypertension and gastric cancer [1,2]. Studies have revealed that *G. lucidum* possessed multiple bioactivities, including antiviral, antibacterial, anti-inflammatory, anticancer, anti-metastatic activities, immune-enhancing and cardioprotective effects [3-7]. Additionally, it was reported to inhibit platelet aggregation and lower blood sugar [8, 9]. Evidence has indicated that these bioactivities have been attributed to chemical components present in *G. lucidum*, such as triterpenoids, polysaccharides and phenolics. For example, ganoderic acid T found in this mushroom exerted an inhibitory effect on expression of matrix metalloproteinase-9, preventing tumor invasion [10]. One in vitro study showed that ganoderiol F exhibited a cytotoxic effect against different cell lines, including Lewis lung carcinoma, T-47D and sarcoma-180 cells [11,12]. In an animal study, polysaccharides in *G. lucidum* had the capacity to increase plasma insulin and reduce plasma sugar levels by simultaneously

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improving the activities of hepatic glucokinase, glucose-6-phosphate dehydrogenase and phosphofructokinase [13,14]. Phenolic-rich extracts of *G. lucidum* was shown to have antibacterial activity by causing protein leakage within bacterial cells [15]. Phenolics, a large group of compounds commonly found in mushrooms [16], are known to have great potentials to quench the formation of free radicals in oxidative stress-related diseases [17].

In Vietnam, lingzhi is cultivated and commercially marketed in different forms, such as dietary supplements or herbal tea. Many studies on taxonomy, distribution and selection of GL strains, as well as determination of *G. lucidum* chemical composition have been performed since the mushroom was first artificially grown in the country in the end of 1970s [18-21]. Most of previous investigations into phytochemicals of *G. lucidum* have been focused on triterpenoids as this class of compounds are believed to account for a variety of bioactivities of the mushroom. In the present study, phenolic compounds and carotenoids which had rarely been explored in *G. lucidum* were assessed. This will hopefully give a better understanding of how these classes of compounds contribute to this mushroom's bioactivities of importance to human health.

2. Experimental

2.1. Sample collection

Ganderma lucidum fruiting body was obtained from a lingzhi farm located in Lac Duong district, Lam Dong province, Vietnam. The dried mushroom was ground using a blender and stored in a refrigerator until analysis. The study was conducted during the period from December 2021 to June 2022.

2.2. Chemicals

Organic solvents (99.5% ACS grade), including methanol, acetone and ethyl acetate, were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phenolic acid analytical standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ganoderic acid A, ergosterol, rutin and quercetin (99%) were purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China).

2.3. Determination of triterpenoids

The dried sample (about 1 g) was mixed with ethanol (10 mL) in an Erlenmeyer flask. The mixture was sonicated at room temperature for 60 min and well-shaken on an orbital shaker. After 24 hours, the mixture was centrifuged at 5500 rpm for 15 min and then the supernatant was collected and filter through a 0.45-micron membrane filter before injection into HPLC system. The HPLC analysis was performed on a Shimadzu LC system equipped with a diode-array detector (HPLC-DAD) and a Zorbax Eclipse XDB C18 column (4.6 × 150 mm, 5 μm, Agilent Technologies, California, USA) following the method previously described by Ha et al. (2015) with minor modifications [20].

2.4. Determination of carotenoids

The estimation of total contents of carotenoid (TCC), lycopene and β-carotene in GL was based on the methods developed by Lichtenthaler and Wellburn (1983) and D'Souza et al. (1992) [22, 23]. The dried sample (1 g) was extracted with 10 mL of acetone in a screw-capped tube. After 24-hour vigorous shaking at room temperature, the mixture was centrifuged at 5500 rpm for 15 min and the supernatant collected was spectrophotometrically determined at 470, 645 and 662 nm. Total carotenoid contents (μg/g) was calculated using the following equation:

$$A = (11.24 \times A_{662} - 2.04 \times A_{645}) \times v/m$$

$$B = (20.13 \times A645 - 4.19 \times A662) \times v/m$$

$$\text{Total carotenoid content} = [1000 \times A_{470} - (2.27 \times A + 81.4 \times B)]/227 \times v/m$$

where v and m are volume of the solvent (mL) and amount of the dried mushroom (g), respectively.

2.5. Preparation of crude extracts for phenolics

2.5.1. Extraction of phenolics

About 10 g of the dried sample were mixed with 100 mL of a solvent (methanol, ethyl acetate, acetone or water). After 24-hour extraction with shaking on an orbital shaker, the mixture was centrifuged at 5500 rpm for 15 min. The supernatant was collected and filtered through a filter paper, resulting in a filtrate which was used for HPLC analysis for phenolics. Additionally, the filtrate was evaporated in a rotary evaporator and the residue obtained was used to estimate total phenolic and flavonoid contents and predict bioactivities. The extraction yield of the methanolic (ME), ethyl

acetate (EA), acetonic (AC) and aqueous (WE) crude extracts were 16.2, 11.3, 9.6 and 26.3%, respectively.

2.5.2. Estimation of phenolic content

Total phenolic content in the crude extracts of GL was determined following the method of Vu and To (2022) [24].

2.5.3. Phenolic composition

Phenolic composition in *G. lucidum was* analyzed on an HPLC-DAD using the method previously described by Vu et al. (2021) [25]. In brief, the diluted extracts obtained in section 2.5 were filtered through 0.45-micron membrane and injected into a Shimadzu HPLC system connected with diode-array detector. The analytes were separated on a VertiSep™ GES C18 reverse-phase column (250 × 4.6 mm, 5.0 µm particle size) with the mobile phase consisting of methanol (A) and 1% formic acid in water (B). Detection wavelengths for phenolic acids and flavonoids were 295 and 340 nm, respectively. The compounds were quantified using their calibration curves with concentrations ranging between 1 and 30 µg/mL.

2.6. Antioxidant activity

ABTS free radical scavenging assay. A mixture containing 7 mM ABTS and 2.45 mM $K_2S_2O_8$) in phosphate buffered saline at a ratio of 1:1 (v/v) underwent a 12 – 16 hour incubation at room temperature in the dark. Afterwards, 3 mL of the mixture was allowed to react with 100 µL of a diluted extract of GL or ascorbic acid solution. The absorbance was spectrophotometrically determined at 734 nm. The activity was estimated using the calibration curve ($y = 0.006x – 0.0035$; $R^2 = 0.999$) plotted for ascorbic acid. The results were expressed as milligram ascorbic acid equivalents per gram of extract (mg AAE/g) [26].

DPPH free radical scavenging assay. To determine antioxidant activity by DPPH assay, 200 µL of a diluted extract of GL or ascorbic acid solution were allowed to react with 300 µL of 40 µg/mL DPPH solution prepared in methanol/water (4:1, v/v), and the mixture was then incubated at 37 °C in the dark. The absorbance was analyzed at 517 nm in a spectrophotometer. The activity was estimated using the calibration curve ($y = 0.0456x + 0.0295$; $R^2 = 0.980$) plotted for ascorbic acid. The results were expressed as milligram ascorbic acid equivalents per gram of extract (mg AAE/g) [26].

2.7. Inhibition of bovine albumin denaturation

A volume (100 µL) of a *G. lucidum* extract in 5% DMSO or diclofenac sodium was mixed with 100 µL of bovine serum albumin solution (0.16%) and 200 µL of sodium acetate buffer (25 mM, pH 5.5). the mixture was incubated at 37 °C for 45 min, and then heated to 67 °C for 3 min. Afterwards, the mixture was allowed to cool to room temperature, and absorbance at 660 nm was measured in a spectrophotometer [27]. The percentage inhibition of albumin denaturation was evaluated as follows:

$$\textbf{Percentage inhibition} = [1 – A/B] \times 100$$

where, A and B represent the absorbance of the sample and blank. IC_{50} values (µg/mL) were used to predict the in vitro anti-inflammatory activity of the extracts.

2.8. Inhibition of α-glucosidase

Fifty microliters of a GL extract diluted in 5% DMSO was mixed with 40 µL of α-glucosidase (0.05 U) dissolved in phosphate buffer (0.1 M, pH 6.8), followed by a 20 min incubation at 37 °C. After adding 4-nitrophenyl-β-D-glucopyranoside (p-NPG) (5 mM, 40 µL), the mixture underwent another 20 min incubation at 37 °C. Sodium carbonate solution (130 µL, 0.2 M) was used to stop the reaction, and the change in absorbance was determined at 405 nm [28]. The activity was predicted using the calibration curve ($y = 0.000059x + 0.001952$; $R^2 = 0.997$) constructed for acarbose. The results were expressed as milligram acarbose equivalents per gram of extract (mg ACAE/g).

2.9. Inhibition of α-amylase

The assay was carried out following the method of Zengin et al. (2020) with minor modification [29]. A diluted extract of *G. lucidum* mixed with 10 µL of α-amylase solution (0.14 U/mL) in phosphate buffer (pH 6.9) was incubated at 37 °C for 15 min. Starch solution (15 µL, 0.25%) was added to initiate the reaction, followed by incubation at 37 °C for 15 min. The blank sample was prepared in similar steps without adding the α-amylase solution. To terminate the reaction, 50 µL of 1 M HCl were added. Afterwards, a volume (100 µL) of KI_3 solution was added. The absorbance was analyzed at 595 nm in a spectrophotometer. The percentage inhibition of the enzymatic activity was calculated as follows:

$$\text{Percentage inhibition} = [1 – A/B] \times 100$$

where, A and B stand for the absorbance of the sample and blank. IC_{50} values (µg/mL) were used to estimate the activity of the extracts.

2.10. Statistical analysis

All the treatments were analyzed using one-way ANOVA with Tukey's HSD test at a significance level of 0.05 to evaluate statistically significant differences in means of TPC, TFC, triterpenoids, phenolics and bioactivities. All the

measurements were carried out in triplicate and the results were presented as means ± standard deviation. XLSTAT 2016 software (Addinsoft, Paris, France) was employed to perform the statistical analyses.

3. Results and discussion

3.1. Carotenoids and triterpenoids

In this study, carotenoids in *G. lucidum* were extracted using acetone and quantified by VIS spectrophotometry. The results were reported as total amount of carotenoids. Table 1 showed that GL contained average 20.30 μg of total carotenoids per gram of dried weight. To our knowledge, no information about carotenoids in GL is available in the literature. This group of lipophilic compounds is widely known as contributors to many aspects of immune functions and detoxification [30].

Ganoderic acid A and ergosterol are two triterpenoids that were quantified in *G. lucidum* using HPLC in this study. The results showed that the average levels of these constituents in *G. lucidum* were 532.53 and 488.62 μg/g, respectively. Previous research revealed that ganoderic acid A in GL fruiting bodies fell within 349 and 1774 μg/g DW (dry weight) [31]. In another study, it was reported that ganoderic acid A and ergosterol ranged from 160.74 to 862.09 μg/g DW [20]. The findings of the present study were comparable with prior research. These studies also indicated that the amounts of triterpenoids in *G. lucidum* may be dependent on postharvest storage, drying methods, sampling sites and collection methods.

Table 1. Carotenoid and triterpenoid contents in *G. lucidum*

	Concentration (μg/g DW)
Total carotenoid content	20.30 ± 2.76
Ergosterol	488.62 ± 42.98
Ganoderic acid A	532.53 ± 32.11

Data are presented as mean ± standard deviation.

3.2. Phenolic composition

The results revealed ME was the extract containing the highest amount of phenolics (113.75 ± 5.05 mg GAE/g), followed by AC and EA. The aqueous extract was composed of the lowest amount of phenolics. As shown in Table 2, TPC of ME was about six times as high as that of WE. Prior research reported that methanolic and aqueous extracts of *G. lucidum* collected in Iran had moderately higher TPC values of 165.58 and 45.58 mg GAE/g, respectively [32]. In other studies, lingzhi samples were shown to have much lower TPC values (28.11 – 55.53 mg GAE/g) [1, 33]. In general, these values are significantly different when compared to the present study, and this discrepancy could be because of extraction methods, geographic variations and/or growing conditions. Nevertheless, the results all proved significantly greater extractability of methanol on *G. lucidum* phenolics compared to water.

Ten phenolic acids and flavonoids were monitored, and the results were displayed in Table 2. All these compounds, except gallic acid, were found to be present in ME. Quercetin was the most abundant constituent in ME and AC, with the concentrations insignificantly different between the two extracts. This flavonoid was also detected in EA and WE, with the concentrations being a fraction. Another major phenolic compound in ME and AC was DHBA, with its levels detected following the order: ME > AC > EA > WE. Similarly, chlorogenic acid, ferulic acid and salicylic acid were found in all the extracts.

It is noted that AC and EA contained the highest amount of these phenolic acids. Rutin was another flavonoid examined in this study. Unlike quercetin, rutin was detected only in ME at a very low level (1.26 μg/g DW). As seen in Table 2, this is comparable to prior research with respect to the level of rutin which fell within the range from 3.31 – 23.02 μg/g DW [33, 34]. Notably, there are remarkable differences in quercetin quantified between the reports. The present study recorded a much higher concentration of quercetin in the methanolic extract compared to the previous findings. This variation could be attributable to sampling location, extraction approach to obtain phenolics (extraction time and/or sample-solvent ratio) and HPLC analysis. Regarding most of the phenolic acids, the present study generally corroborated the previous results as shown in Table 2.

Table 2. Phenolic contents of the *G. lucidum* extracts and comparison of phenolic concentrations among the studies

Phenolics*	Present study (2022)				Gąsecka et al. (2016)	Dong et al. (2019)
	ME	EA	AC	W		
Gallic acid	n.d.	6.80 ± 0.79 b	n.d.	30.97 ± 2.58 a	n.a.	11.12 – 56.00
Chlorogenic acid	29.87 ± 1.69 b	16.98 ± 1.31 c	36.78 ± 3.73 a	22.63 ± 1.86 c	1.99 – 7.64	n.a.
Caffeic acid	14.48 ± 1.76 a	n.d.	17.30 ± 2.44 a	0.54 ± 0.09 b	10.83 – 34.31	≤ 33.69
p-Coumaric acid	3.88 ± 0.27 a	n.d.	n.d.	4.50 ± 0.43 a	≤ 14.87	0.36 – 1.13
Ferulic acid	4.61 ± 0.61 b	4.64 ± 0.60 b	8.53 ± 0.29 a	1.16 ± 0.06 c	≤ 33.61	n.a.
DHBA	120.87 ± 7.11 a	23.78 ± 0.90 c	45.02 ± 6.04 b	8.84 ± 2.45 d	n.a.	n.a.
Salicylic acid	30.68 ± 2.57 b	107.69 ± 6.22 a	24.15 ± 1.54 bc	15.90 ± 1.35 c	n.a.	n.a.
Cinnamic acid	12.06 ± 0.67 a	0.35 ± 0.03 c	2.34 ± 0.09 b	n.d	11.33 – 34.10	1.49 – 5.60
Rutin	1.26 ± 0.24	n.d.	n.d.	n.d.	3.31 – 23.02	9.67 – 17.11
Quercetin	248.13 ± 18.55 a	1.55 ± 0.21 b	234.62 ± 5.41 a	2.83 ± 0.54 b	8.34 – 27.04	9.48 – 12.23
TPC	113.75 ± 5.05 a	50.42 ± 5.67 c	88.75 ± 14.46 b	18.75 ± 3.94 d	n.a.	n.a.

*: μg/g DW for phenolic acids and flavonoids; mg GAE/g for TPC.
Data are presented as mean ± standard deviation. Different lowercased letters for the same phenolic compound indicated significant differences among the extracts ($p < 0.05$).
n.d.: not detected
n.a.: not available

3.3. Antioxidant activity

In the present study, antioxidant activity of the *G. lucidum* extracts (4000 μg/mL) was assessed by measuring the capacity to trap ABTS and DPPH radicals (Table 3). Antioxidant activity of ME measured by ABTS assay was significantly higher than the others, followed by AC and WE. The weakest activity was noted for EA, approximately a half of that of ME. However, the activity determined by DPPH assay showed a different trend in which EA exerted the significantly higher scavenging activity than those of AC and WE. In one study, it was reported that methanolic extract of lingzhi exhibited a significantly higher ABTS and lower DPPH scavenging activities compared to chloroform [32]. It appears to be that extraction with methanol may result in extracts with higher capacity to neutralize ABTS free radicals while a non-polar solvent, such as ethyl acetate or chloroform, could lead to extracts richer in DPPH scavengers.

Table 3. Antioxidant activity of the *G. lucidum* extracts

	ABTS (mg AAE/g)	DPPH (mg AAE/g)
ME	33.83 ± 2.40 a	5.36 ± 0.47 ab
EA	14.91 ± 1.07 c	6.92 ± 0.75 a
AC	20.60 ± 0.29 b	4.01 ± 0.60 b
WE	20.33 ± 0.10 b	4.26 ± 0.97 b

Data are presented as mean ± standard deviation.

3.4. Inhibition of albumin denaturation

Proteins may be denatured under the presence of heat stress or chemicals, leading to alterations in their biological, chemical and physical properties. Thus, tissue protein denaturation may be a marker for inflammatory diseases. In this study, the capacity of the *G. lucidum* extracts to inhibit

bovine albumin denaturation was determined to provide an understanding of anti-inflammatory activity of this mushroom species. As shown in Figure 1, IC_{50} of the extracts was calculated by constructing inhibition curves. With the lowest IC_{50} value (284.79 µg/mL), the ethyl acetate extract was identified as having the highest capacity to inhibit the denaturation of albumin. Not as strong as EA, ME possessed a potential to suppress the albumin denaturation with IC_{50} of 997.53 µg/mL. It also exhibited a greater inhibitory effect than those of AC (1507.44 µg/mL) and W (3027.52 µg/mL). The results also demonstrated that water could be less capable of extracting potential anti-inflammatory chemical constituents in *G. lucidum*. In comparison with diclofenac (IC_{50} = 84.19 µg/mL) which was used as a positive control in the assay, all the extracts may exert lower protective effect on bovine albumin from heat-induced denaturation. Previous studies have been performed to unravel in vitro anti-inflammatory potential of lingzhi extracts. Reportedly, lingzhi extract prepared with 95% ethanol remarkably inhibited the release of tumor necrosis factor-α, interleukin-6, nitric oxide (NO) and prostaglandin E2 from lipopolysaccharide-induced murine RAW264.7 cells [35]. In addition, the lingzhi extract down-regulated lipopolysaccharide-dependent expression of inducible nitric oxide synthase and cyclooxygenase 2 in the cell line. There is also evidence that lingzhi extract rich in polysaccharide decreased interleukin-1β expression in lipopolysaccharide-treated human aortic smooth muscle cells both in vitro and in vivo [36]. These findings suggest that lingzhi possesses anti-inflammatory activities and could play therapeutic roles in inflammatory diseases.

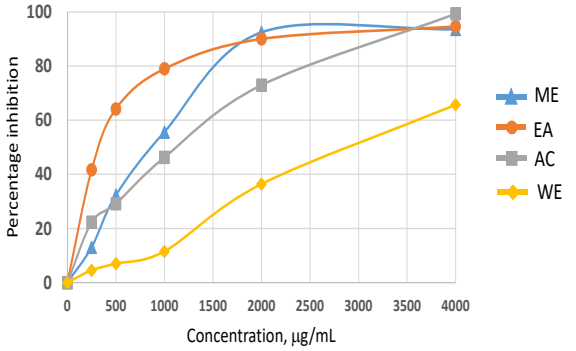

Figure 1: Effectiveness of the extracts in inhibiting bovine albumin denaturation

3.5. Enzymatic inhibitory effects of G. lucidum

Inhibition of α-glucosidase and α-amylase plays important roles in the maintenance of circulating glucose levels by controlling blood sugar absorption. In this study, the *G. lucidum* extracts were tested for ability to inhibit the two enzymes, with acarbose used as a reference standard. As presented in Table 4, the inhibitory effect on α-glucosidase of the extracts followed the order: ME ≈ EA > WE > AC. Furthermore, the activities of ME and EA were about 30% higher than that of AC. Regarding the inhibition of α-amylase, no significant differences in the activity estimated via IC_{50} were observed for ME, EA and AC. These extracts obtained with the organic solvents all exerted more potent activity to suppress the enzyme in comparison with the aqueous extract. Previously, lingzhi was shown to have the greatest concentration-dependent inhibitory activity against α-glycosidase among the examined mushrooms [37]. A recent study demonstrated that a mixture of aqueous extract and alcohol extract of *G. lucidum* fruiting body had hypoglycemic and hypolipidemic effects in streptozotocin-induced diabetic male Wistar rats [38]. Reportedly, *G. lucidum* contained a variety of molecules, such as triterpenoids, polysaccharides and proteins, that possessed potent anti-diabetic properties [39]. As lingzhi is becoming more popular these days, this food material should be recommended to patients during diabetes mellitus treatment.

Table 4. Enzymatic inhibitory effects of the *G. lucidum* extracts.

	α-Glucosidase, mg ACAE/g	**α-Amylase, µg/mL**
ME	1737.49 ± 44.24 a	2268.70 ± 151.66 b
EA	1647.10 ± 33.99 a	2243.94 ± 123.71 b
AC	1145.68 ± 59.37 c	1908.46 ± 40.66 b
W	1474.78 ± 31.99 b	3550.89 ± 192.28 a

IC_{50} (µg/mL) was used to determine the inhibitory effect against α-amylase.
Data are presented as mean ± standard deviation.

Conclusion

The use of various extraction solvents was demonstrated to influence phenolic composition examined in the lingzhi extracts. In addition, their antioxidant activity and inhibition of bovine albumin denaturation, α-glucosidase and α-amylase were affected by the choice of solvents. Methanol and acetone showed much better effectiveness in extracting phenolic compounds in the mushroom compared to ethyl acetate and water. Statistically significant differences were noted for the concentrations of all the phenolics among the

extracts obtained. Generally, the extract prepared with methanol exhibited more potent free radical scavenging activity while the ethyl acetate extract had higher activity to inhibit albumin denaturation. Extraction with the organic solvents resulted in extracts possessing more potent activity to suppress α-glucosidase and α-amylase in comparison with the water. This research provides a better understanding of lingzhi phytochemicals and bioactivities of importance to human health. The findings of the research could be helpful for designing therapeutic agents from the mushroom.

Conflicts of interest

There are no conflicts of interest to declare.

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