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A comparative study of extraction techniques for maximum recovery of bioactive compounds from *Ganoderma lucidum* spores

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Summary

This study aimed at evaluating effective methods for breaking the hard and insoluble spores of *Ganoderma lucidum* to recover functional biomolecules. Rupture techniques were evaluated such as manual maceration (RM), maceration with spheres of various materials (BR), and microwave exposure plus maceration with steel/chrome spheres (MBR1). Spore rupture was evaluated using UV-Vis spectroscopy, which showed vibrations of 2955, 1642, 1240, 1080 and 1746 cm^{-1} corresponding to changes in spore walls. The MBR1 extract contained the largest amounts of carbohydrates (19.80 $mg.g^{-1}$ spores) and polyphenols (2.21 $mg.g^{-1}$ spores), whereas the BR extract had higher antioxidant activity (57.22%Inb DPPH). The MBR1 and BR extracts contained 62.2 and 73.5% glucose, respectively. Both methods also involved significant extraction of carbohydrates and proteins. The best way to extract biomolecules from spore walls is to perform a microwave heat treatment and break the walls with steel/chrome spheres; this produces large quantities of carbohydrates with antioxidant properties.

Key words: Antioxidants, biological activity, spore-breaking techniques, chemical analysis, Fourier-transform infrared spectroscopy, *Ganodermataceae*.

Resumen

Estudio comparativo de técnicas de extracción para la máxima recuperación de compuestos bioactivos a partir esporas de *Ganoderma lucidum*

El objetivo de este estudio fue evaluar varios métodos de ruptura de las esporas de *Ganoderma lucidum* y extraer sus propiedades bioactivas. Para este propósito se evaluaron diferentes técnicas de rompimiento como: la maceración manual (RM), la maceración con esferas de diversos materiales (BR) y la exposición a microondas junto la maceración de las esporas con esferas de acero/cromo (MBR1). La ruptura de las esporas fue evaluada por espectroscopia UV-Vis, la cual mostró que las vibraciones 2955, 1642, 1240, 1080 y 1746 cm^{-1} correspondieron a cambios estructurales en las paredes de las esporas. El extracto MBR1 presento el mayor contenido de carbohidratos (19,80 $mg.g^{-1}$) y polifenoles (2,21 $mg.g^{-1}$), mientras que el extracto BR tuvo una mayor actividad antioxidante (57,22% Inb DPPH). Los extractos MBR1 y BR también presentaron en el análisis de monosacáridos un 62,2 y 73,5% de contenido glucosa. Como conclusión la mejor metodología para extraer biomoléculas de las paredes de las esporas de *G. lucidum* fueron el tratamiento térmico con microondas y la ruptura de las paredes con esferas de acero/cromo, porque este proceso permitió la extracción de una mayor cantidad de carbohidratos con posibles propiedades antioxidantes.

Palabras clave: Antioxidantes, actividad biológica, técnicas de rompimiento, análisis químico, espectroscopia infrarroja por transformada de Fourier, *Ganodermataceae*.

Introduction

Extraction methods for the recovery of polysaccharides and lipids from *Ganoderma lucidum* have been of growing interest because this mushroom contains active biomolecules that treat several diseases, such as arthritis, bronchitis, gastric ulcers, hyperglycemia, insomnia, nephritis, inflammatory diseases, and cancer [1, 2]. In addition, *G. lucidum* is known to have high antioxidant activity [3] and contains triterpenes and ganoderic acids A, B, H, T, C2 and Me, which have hepatoprotective, antihypertensive, hypochondrial, anti-HIV-1, and antihistamine actions [2, 4, 5]. *G. lucidum* is known in traditional Chinese medicine and has been used in the form of extracts and spores for various medical treatments. The spores of this Basidiomycota fungus, which belongs to the Ganodermataceae family [4], have a ductile structure; this property

makes them resistant, and they are considered one of the hardest structures to break in nature [6, 7, 8].

Due to these characteristics, several methodologies have been applied to break mushroom spores, such as micronizers, air currents, knife mills, presses, enzymes, sonication, mechanical chemical extraction by mill, and reagents such as sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), and sodium carbonate (Na_2CO_3) [3, 9]. However, according to several studies on the breaking and description of biomolecules derived from *G. lucidum* spores, mechanical, chemical, and enzymatic breaking can produce variations in the release of compounds. Such variations are not well known in the literature. Therefore, it is important to conduct a study to analyse which alterations can cause the fragmentation of the spore walls and which molecules can be extracted. Thus, the aim of this research was to compare three breaking techniques for *G. lucidum* spores. A manual breaking method, an assisted mechanical breaking method using different types of spheres, and a microwave breaking method were employed. The best isolated and combined spore-breaking techniques were then evaluated by comparing the extracted proteins, carbohydrates, lipids, and antioxidant activity using Fourier-transform infrared spectroscopy (FTIR), light microscopy, scanning electron microscopy (SEM), and chemical characterization techniques.

Material and methods

Breaking of Ganoderma lucidum *spores*

Various spore-breaking approaches were compared using 1 g of spores in each assay. In the first technique, the spores were macerated manually for 20 min. In the second, the spores were macerated with 20 g of spheres of different materials (steel/chrome, polylactic acid, polyvinyl chloride, glass, silica gel, and ceramic) for 20 min. In the third technique, the spores were exposed to the microwaves for 10 min at 1200 W, 127 V, 60 Hz, and 2450 MHz (model HMO22E-13-127V).

An optical microscope and a Neubauer chamber (American Optical Co.) of 0.1-mm depth were used to analyze spore breakage. For each breakage analysis, 0.1050 g of spores were subjected to the breaking method and then, diluted in 30 mL of distilled water. Counting was performed under augmentation of 400× in five small squares, and the samples were evaluated in quadruplicates. The equation 1 was used to calculate the breakage percentage:

$$\frac{\left[\left(\textit{Average unbroken spores} - \textit{Average unbroken spores after treatment}\right)\times 100\right]}{\textit{Average unbroken spores}} \left(\textit{Equation } 1\right)$$

After this analysis, one portion of the sample was analysed by SEM to evaluate the changes occurred in the spore walls. For this process, the *G. lucidum* samples were dried in air stoves at 80 °C for 24 h. The samples were coated superficially with gold for microscopic analysis using a TESCAN VEGA3 LMU.

Preparation of G. lucidum *extracts*

Besides evaluating various spore-breaking techniques for *G. lucidum*, extraction was performed by diluting the spores in 16 mL of water pH 7 and incubating for 24 h at 30 °C and 120 rpm. Table 1 summarizes the breaking and extraction procedures.

Table 1. Methodologies used to prepare the Control, RM, BR and MBR1 *Ganoderma lucidum* extracts.

Extract	Breaking methodology	Extraction technique
Control	Unbroken spores	The spores were diluted in 16 ml of pH 7 water and incubated for 24 h at 30 °C at 120 rpm
RM	Manual maceration 1 g of spores for 20 minutes	
BR	1 g of spores macerated with the aid of spheres, for 20 minutes	
MBR1	1 g of spores exposed to microwave for 10 minutes at 1200 W, 127 V, 60 Hz and 2450 MHz	

Evaluation of functional groups by FTIR

Sample preparation

The functional groups present in the unbroken spores and in the aqueous *G. lucidum* extracts were determined using medium infrared spectroscopy (MIS) with VERTEX 70 (Bruker) equipment and the DRIFT accessory. A total of 64 scans were completed at 4 cm^{-1} resolution, without losing atmospheric compensation in the region between 4000 and 400 cm^{-1}. The samples were crushed, pulverized, and oven dried. Approximately 20 mg of dried samples were mixed and homogenized with 100 mg of spectroscopic potassium bromide (KBr) to conduct the measurements. The FTIR analysis for proteins, carbohydrates, and lipids regions was performed [4, 5].

Spores rupture analysis using FTIR

The extracts of intact spores, manually macerated (RM) spores, steel/chrome sphere macerated (BR) spores and microwave plus steel/chrome sphere macerated (MBR1) spores were analysed with FTIR using stretching bands to describe changes in protein, carbohydrate and lipid extraction [10]. Extracts were evaluated using five regions: 3000 to 2800 cm^{-1} (asymmetric stretching of CH_3 and symmetric stretching of CH_2), 1660 to 1600 cm^{-1} (Amide I), 1400 to 1200 cm^{-1} (α-Helix in Amide III and unordered structures in Amide III), 1100 to 1000 cm^{-1} (C-O stretching if sugars), and 1750 to 1700 cm^{-1} (C= O stretching and α/β unsaturated carboxylic acids).

In each region, a bandwidth peak was analyzed. The first region received contributions of carbohydrates, lipids, and proteins, which appeared in bands at 2925 (asymmetric stretching of CH_2) and 2855 cm^{-1} (symmetric stretching of CH_2). For the second and third regions, the protein structure appeared at 1641 cm^{-1} (Amide I) and 1240 cm^{-1} (unordered structures in Amide III). The fourth sample showed the structure of sugars related to glucopyranose group at 1078 cm^{-1} (polysaccharide band C - O) [11, 12] and the fifth region received fatty acids and lipid contributions at 1746 cm^{-1} (C = O stretching) [10, 13, 14].

Chemical characterization

The total soluble protein (TSP) concentration was determined using the Bradford technique and the reading was conducted in the wavelength (λ) region of 595 nm of UV-VIS spectroscopy (SP2000 Visible SP 1105UV Spectrophotometer) with bovine serum albumin (BSA) as a standard [15].

The total phenolic compound content (TPC) in each extract was determined using the Folin-Ciocalteau method [16]. The results were expressed in mg of gallic acid equivalent per liter of sample (R^2 0.9947).

The carbohydrate content was measured using the Antrone's method [17]. The concentration was determined via UV-VIS spectroscopy with λ = 620 nm (SP2000 Visible SP 1105UV Spectrophotometer). For composition analysis, 2 mg of extract was freeze-dried and hydrolyzed in 2 M of trifluoroacetic acid (TFA) at 110 °C for 2 h [18]. The determination of monosaccharide composition was conducted via reductive hydrolysis [19] using an extra reducing agent (borane 4-methylmorpholine complex or 4-MMB) before and after the pre-hydrolysis and hydrolysis steps [20]. The hydrolytic process was performed [21]. After acetylation [19] the alditol acetate derivatives were analyzed by gas chromatography-mass spectrometry (GC-MS)[21] and identified using their typical electron-impact fragmentation profiles and GC retention times [22]. A double hydrolysis reductive amination method was used to determine the absolute

configuration of the monosaccharide constituents [23]. Chiral 1-amino-2-propanol was used to determine the ratio of d- and l-galactose and their 6-*O*-methyl derivative, whereas the configuration of 2-*O*-methylgalactose, 3,6-anhydrogalactose, and their 2-*O*-methyl derivatives were determined using chiral α-methylbenzylamine. The resulting alditol acetates derivatives were analyzed using GCMS [22].

The determination of antioxidant activity was conducted using 2.2-diphenyl-1-picrylhydrazyl (DPPH). The DPPH method is based on determining the ability to scavenge an antioxidant compound using the stable DPPH free radical in the ethanolic medium. The DPPH method focuses on the UV-VIS region of l = 515 nm [24]. The DPPH solutions (0.004 mg.100 mL^{-1}) were prepared and used immediately. They reflected the standard curve and were prepared with ascorbic acid solution in ethanol, with concentrations ranging from 0 to 200 μM. For each point of the standard curve, triplicate samples of absorbance were determined every 5 min until a regression coefficient (R^2 = 0.991) was obtained. Each sample's radical scavenger activities were calculated according to the inhibition percentage (% Inb) of the DPPH radical, as shown in equation 2:

$$(\%Inb) = \frac{[AC - AR]}{AC} * 100 \left(Equation\ 2\right)$$

Where *AC* represents the absorbance values of the DPPH solution in ethanol, and *AR* represents the absorbance values of ascorbic acid + DPPH (reaction), respectively, at the end of the reaction.

The antioxidant activity value is expressed in μM (equivalent ascorbic acid-AA) from the regression coefficient of the calibration curve (equation 3):

$$DPPH\left(\mu M^{\circ} AA\right) = \left(\%Inb - \frac{intercept}{regression\ coefficient}\right) * dilution \left(Equation\ 3\right)$$

Micro- and macronutrient contents were measured using metallic atomic optical emission in inductively coupled plasma (ICP-OES). The glassware used was placed in a solution of nitric acid (HNO_3) 20% (w/v) for 24 h and subsequently washed with deionized water. All reference solutions were prepared with ultrapure water with a resistivity of 18.2 MΩ cm^{-1} (Millipore, Berford, MA, USA) coupled to a model 534 glass water distiller. One gram of material was transferred to a 250-mL volumetric flask, to which 5 mL of concentrated HNO_3 and 4 mL of H_2O_2 (30%) were added. After reaching the boiling point, the system was kept hot for 40 min. Then, the solution could cool to room temperature, and it was filtered quantitatively with a filter paper. Finally, it was completed to 100 mL in a volumetric flask. The same procedure was

performed for all samples. The three samples from the control group were prepared in the same way. The levels of metals (micro- and macronutrients) were measured using ICP-OES on a VARIAN model 720 ES in conjunction with the axial arrangement and solid-state detector.

To determine total lipid content in *G. lucidum* spores, 1.0 g samples of broken and unbroken spores were assayed in triplicate. In a conical tube, 5 mL of $CHCl_3$, 10 mL of CH_3OH and 4 mL of water were combined. The tube was placed in a shaker for 30 min at 120 rpm. Then, 5 mL of $CHCl_3$, 5 mL of Na_2SO_4 (1.5 %) and the samples were agitated in a vortex for 2 min and centrifuged at 1500 *g* for 2 min. After extraction, the solvent was evaporated in an oven at 50 °C for 8 h [25].

Data analysis

The results were presented as means ± standard deviation (SD). Significance ($p<0.05$) was assessed using one- and two-way analyses of variance (Anova) with the statistical package Statistica.7.0 (2002) for Windows.

Results and discussion

Breaking of Ganoderma lucidum *spores*

Manual maceration of *G. lucidum* spores produced no visible breakage when evaluated by optical microscopy (400×). The maceration with the aid of 1-mm diameter steel/chrome spheres produced a breakage rate of 97.58 ± 1.41%. Other types of spheres (polylactic acid, polyvinyl chloride, glass, ceramic, and silica gel) produced no breakage. After obtaining mechanical breakage with the steel/chrome spheres, three maceration times (5, 10 and 15 min) were tested. For each time, the breakage percentage was 89.10 ± 6.66%, 97.48 ± 3.61%, and 98.58 ± 2.89%, respectively. Ten min of maceration were enough to obtain almost 100% breakage.

Microwave exposure at 1200 W for 10 min produced no ruptures observable with optical microscopy (400×). However, with SEM, it was possible to observe slight fissures in the spore walls (figure 1). Similarly, when images of the maceration techniques with and without the aid of steel/chrome spheres were analysed, the best breakage was obtained using the spheres because they fragmented the entire spore wall.

Ganoderma lucidum spores have two internal caps: the exospoirum and the myxosporium [8, 10]. These structures have high ductility, a property that provides the spore with resistance [7]. For this reason, various methodologies have been developed to break *G. lucidum* spores, including ultrasonication, ultra-fine grinding, high-speed centrifugal grinding, shear grinding, and enzymatic grinding.

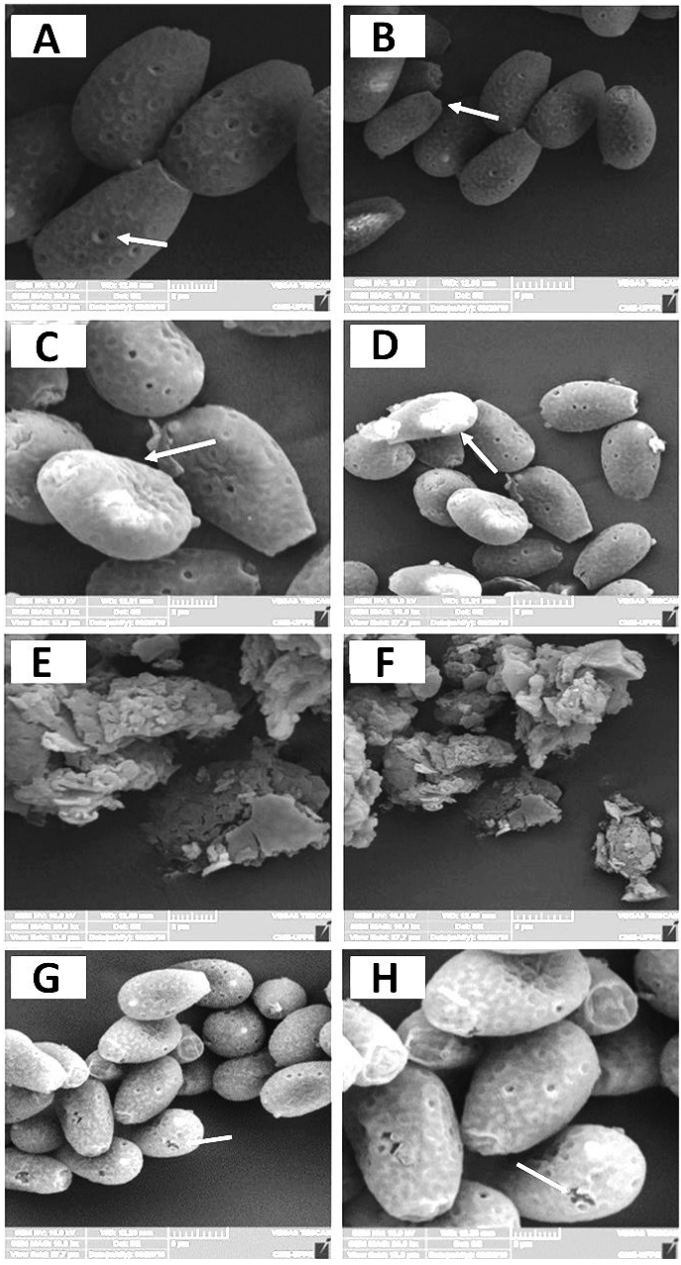

Figure 1. Spore wall breakage analyzed by SEM. A-B) Control spores with no breakage. C-D) Mild fractures and spore wall sinking after using the maceration technique for 20 minutes. E-F) Complete fragmentation of the spore wall when macerated with steel/chrome spheres. G-H) Fissures and sinking all spore walls exposed to microwaves for 10 min.

These have provided between 80 and 100% breaking efficiency [6-8, 26-29]. Nevertheless, they require complex equipment, whereas maceration with steel/chrome spheres provides an easier, more economical way to fragment *G. lucidum* spores, as reported in the present study.

The FITR breakage analyses showed that the BR samples obtained with the aid of steel/chrome spheres underwent changes in the structure of fatty acids, lipids, and proteins.

This occurred due to differences in the asymmetric stretching of CH_3 at 2955 cm^{-1} band found at 2967 cm^{-1}, indicating a difference in the extracts' general structure. The spectral vibration from unordered structures (Amide III at 1240 cm^{-1}) changed to 1231 cm^{-1}. Contributions of C=O spectral vibration from fatty acids and lipids at 1746 cm^{-1} were found at 1738 cm^{-1}. For microwave-exposed spores, the more important changes were observed in the spectral vibration of proteins. This can be attributed to stretching vibration from unordered structures such as Amides I and III, for which spectral vibrations were found at 1630 cm^{-1} and 1629 cm^{-1}, respectively. The change in wavelengths showed that there was a rupture in the chemical structure of the *G. lucidum* spores' walls (table 2).

Table 2. Differences among wavelengths in the walls of unbroken spores, spores broken with steel/chrome spheres, and spores exposed to microwaves.

GLS stretching region	GLS	GLS broken wall	Wave number	Functional groups
3000-2800 cm^{-1}	2955 cm^{-1}	Steel/chrome spheres	2967 cm^{-1}	-Fatty acids, lipids, and proteins -Asymmetric
		Microwaves	2967 cm^{-1}	Stretching of CH_3
1660-1600 cm^{-1}	1642 cm^{-1} Amide I	Steel/chrome spheres	1641 cm^{-1}	-Proteins -Assignment: Amide I
		Microwaves	1629 cm^{-1}	
1400-1200 cm^{-1}	1240 cm^{-1}	Steel/chrome spheres	1231 cm^{-1}	-Proteins -Assignment: Unordered structures
		Microwaves	1230 cm^{-1}	Amide III
1100-1000 cm^{-1}	1080 cm^{-1}	Steel/chrome spheres	1080 cm^{-1}	-Sugars -Assignment: polysaccharide band C-O
		Microwaves	1079 cm^{-1}	
1750-1700 cm^{-1}	1746 cm^{-1}	Steel/chrome spheres	1738 cm^{-1}	-Fatty acids and lipids - Assignment: C = O stretching
		Microwaves	1743 cm^{-1}	

Data compared with Zhu *et al.* [9], Wang *et al.* [13] and Wang *et al.* [14]. GLS *Ganoderma lucidum* spores.

After evaluating the breaking techniques, it was necessary to assess the biomolecule extraction capacity. For the FITR analysis of the control extract, the proteins, fatty acids, and lipids were observed at 2932 cm^{-1}, proteins were observed at 1641 and 1228 cm^{-1}, and sugars were observed at 1079 cm^{-1}. For the RM the extract, proteins were detected at 1635 cm^{-1}, and carbohydrates were detected at 1079 cm^{-1}. For the BR extract, lipids, fatty acids, and proteins were detected at 2929 and 2852 cm^{-1}, proteins were detected at 1641 and 1235 cm^{-1}, and sugars were detected at 1078 cm^{-1}. Finally, for the MBR1 extract, the contribution of fatty acids, proteins, and lipids was 2933 cm^{-1}. For proteins, it was 1647 and 1227 cm^{-1}, and for carbohydrates, it was 1078 cm^{-1} (table 3).

Table 3. Presence of various extracted biomolecules by bands in Control, RM, BR and MBR1 extracts.

GLS stretching region	GLS	GLS broken wall	Wavenumber	Functional groups
3000-2800 cm^{-1}	2925 cm^{-1}	Control	2932 cm^{-1}	-Fatty acids, lipids, and
		RM	-----	proteins
		MBR1	2933 cm^{-1}	-Asymmetric stretching of
		BR	2929 cm^{-1}	CH_2 at 2925 cm^{-1}
				-Symmetric stretching of
	2855 cm^{-1}	Control	------	CH_2 at 2855 cm^{-1}
		RM	------	
		MBR1	------	
		BR	-1	
1660-1600 cm^{-1}	1641 cm^{-1}	Control	1641 cm^{-1}	-Proteins
		RM	1635 cm^{-1}	-Assignment:
		MBR1	1647 cm^{-1}	Amide I
		BR	1641 cm^{-1}	
1400-1200 cm^{-1}	1240 cm^{-1}	Control	1228 cm^{-1}	-Proteins
		RM	-----	-Assignment:
		MBR1	1227 cm^{-1}	Unordered structures,
		BR	1235 cm^{-1}	
				Amide III
1100-1000 cm^{-1}	1078 cm^{-1}	Control	1079 cm^{-1}	-Sugars
		RM	1079 cm^{-1}	-Assignment:
		MBR1	1078 cm^{-1}	Polysaccharide band C-O.
		BR	1078 cm^{-1}	
1750-1700 cm^{-1}	1746 cm^{-1}	Control	------	-Fatty acids and lipids
		RM	------	-Assignment:
		MBR1	------	C = O stretching.
		BR	------	

Data compared with Zhu *et al.* [9], Wang *et al.* [13] and Wang *et al.* [14]. GLS *Ganoderma lucidum* spores.

In addition, by identifying stretching bands for various compounds, the FTIR technique makes it possible to compare the proportions of carbohydrates, lipids, and proteins to better understand which compounds were extracted using each methodology. For this purpose, the band heights (H) of the stretches were analysed at 1748 cm^{-1} for α and β lipid esters (C=O, baseline: 1771-1723 cm^{-1}), at 1642 cm^{-1} for Amide I proteins (baseline: 1700-1581 cm^{-1}), and at 1079 cm^{-1} for carbohydrates (baseline: 1130-951 cm^{-1}) as proposed by Wang and Yano [13, 14, 30], who evaluated the proportions by comparing proteins with lipids (H1642/H1748), carbohydrates in relation to proteins (H1079/H1642), and carbohydrates between lipids (H1079/H1748). We found a significantly higher proportion of proteins than lipids in the control extract, a higher proportion of carbohydrates than proteins in the MBR1 extract, and a higher proportion of carbohydrates than lipids in the control extract (table 4).

Table 4. Mean values of the ratio chains H1642/H1748, H1079/H1642, and H1079/H1748 in *Ganoderma lucidum* spores in various extracts.

Ratios				
	Control	BR	MBR1	RM
H1642/H1748	01.18	n.d.	n.d.	n.d.
H1079/H1642	01.23^{D}	00.83^{C}	40.51^{E}	00.26^{B}
H1079/H1748	01.46	n.d.	n.d.	n.d.

Different letters in rows indicate statistically significant differences at $p<0.05$. **n.d:** not determined. 1748: C=O stretching, 1642: Amide I, 1079: C-O polysaccharide band.

The most soluble compounds under the conditions studied were carbohydrates and some proteins. It is known that small molecules combine with macromolecules to form aquo-complexes with different biological functions [14]. The MBR1 extract contained a major proportion of carbohydrates depending on the regions studied, which indicates that the FTIR approach can be used to study the extracted biomolecules. This result also indicates that various methodologies for breaking *G. lucidum* spores must be studied since different biomolecules are extracted from the spores. The overall process for the extraction of bioactive molecules is represented in figure 2.

Protein, polyphenol, carbohydrate, and antioxidant content

The concentrations of extracted proteins were between 12.24 and 15.55 mg per 1g of spores and showed no statistical difference (table 5). For polyphenols, the MBR1 extract presented the most significant quantity (2.21 ± 0.01 mg per 1g of spores), followed by the BR extract (1.79 ± 0.01 mg per 1g of spores), whereas the control and RM

extracts contained no detectable levels. For polyphenols, the process used in the MBR1 and BR techniques facilitated the recovery of a greater quantity than the processes described in the literature: 0.61 ± 0.05 mg.100g^{-1} of dry weight [3, 26]. Polyphenols are important because they have lanostane-type triterpenes that function against several types of cancerous cells.

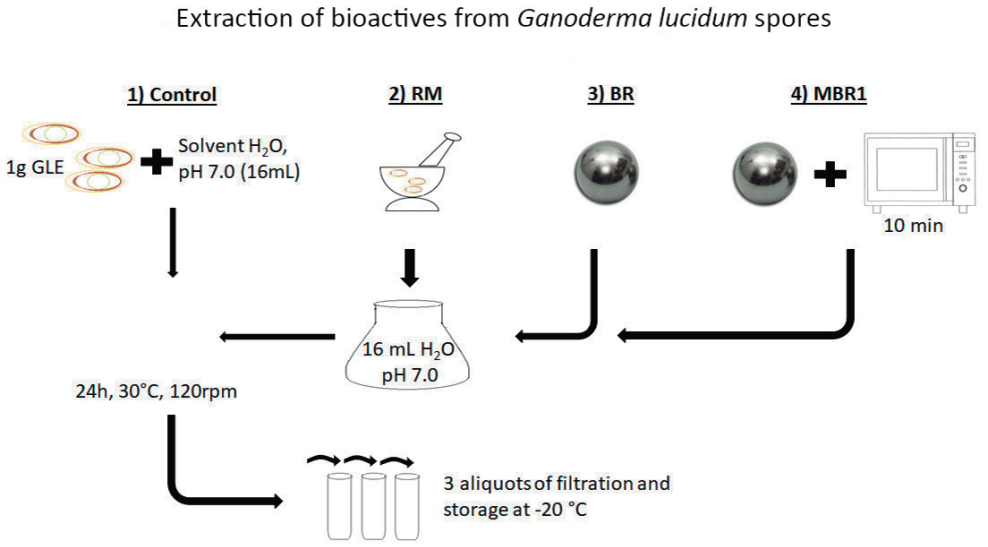

Figure 2. Extraction process of bioactive molecules. GLE: *Ganoderma lucidum* spores; RM: Manual maceration; BR: Maceration with spheres; MBR1: Microwave exposure plus maceration with steel/chrome spheres.

For carbohydrate analysis, the MBR1 process presented 19.80 ± 0.03 mg per 1 g of spores. The extraction of carbohydrates from the MBR1 extract was higher than the reported yield of 8.65 ± 0.46 g per 100 g of dried spores [3]. *Ganoderma lucidum* carbohydrates are important in antitumor therapies due to their polysaccharide content, which includes β-D-glucan chains [18]. For the monosaccharide analysis, the results depended on the methodology used. Glucose was abundant in all extracts, but the BR and MBR1 extracts contained other abundant monosaccharides, such as galactose and mannose, respectively. In the BR extract, galactose was the second most abundant. The MBR1 extract contained the same proportion of arabinose and mannose. Proportions of monosaccharides in RM extract were not equal, but xylose was the second most abundant. These two extraction methods were more effective in isolating a major heterogeneity of monosaccharaides (table 5).

Table 5. Content of proteins, polyphenols, carbohydrates, antioxidant power, and monosaccharides in the Control, RM, BR, and MBR1 extracts.

Extracts	Proteins	Polyphenols	DPPH	Carbohydrates
	mg/1 g GLS	mg/1 g GLS dw	%Inb	mg/1 g GLS dw
Control	12.24 ± 00.09 ^{A}	n.d.	46.83 ± 00.08 ^{AB}	01.24 ± 00.03^{B}
RM	15.55 ± 00.05 ^{A}	n.d.	47.85 ± 00.07 ^{AB}	11.92 ±00.01 ^{A}
BR	13.08 ± 00.00 ^{A}	01.79 ± 00.01^{A}	57.22 ± 00.09 ^{B}	11.26 ±00.06 ^{A}
MBR1	14.03 ± 00.06 ^{A}	02.21 ± 00.01^{B}	45.13 ± 00.03 ^{A}	19.80 ±00.03 ^{C}
Monosaccharides	**BR (%)**	**MBR1 (%)**	**RM (%)**	**Control (%)**
Rhamnose	01.76	n.d.	n.d.	04.91
Fucose	04.19	n.d.	n.d.	n.d.
Arabinose	n.d.	11.72	01.33	n.d.
Xylose	03.90	01.41	09.88	10.92
Mannose	10.15	12.07	04.39	05.83
Glucose	62.19	73.54	77.73	67.74
Galactose	17.21	01.23	06.33	09.36

n.d: not determined; A and B show statistical differences between samples; dw: dry weight. GLS: *Ganoderma lucidum spores.*

The monosaccharides found corresponded to those reported by Yue *et al.* [28] and Soccol *et al.* [29], the majority being identified as glucose, mannose, and galactose. Fucose and arabinose were absent in the control extract. The BR extract contained the most significant antioxidant activity, with 57.22 ± 0.09 % (table 5). The BR extraction process had the best results for antioxidant recovery, as the spores had the highest concentrations of antioxidants compared to other parts of the fungus [3]. Various molecules, such as carbohydrates, phenols, and proteins, also have antioxidant activity. Therefore, breaking the spores increases the release of antioxidant compounds.

The MBR1 and BR extracts' micro- and macronutrients presented less than 0.01 $mg.kg^{-1}$ of V, Se, Pb, Ni, Mo, Cd, Cu, and Co. The MBR1 extract had the lowest B content (9.95 $mg.kg^{-1}$) between the two extracts. High metal values in the broken spores are presented in table 6. Spores broken with steel/chrome spheres had slightly higher Cu (17.34 ± 00.01 $mg.kg^{-1}$) content compared to the unbroken spores

(16.08 ± 00.01 mg.kg^{-1}). Na values (1053.15 ± 00.06 mg.kg^{-1}) were higher for intact spores and lower for MBR1 spores (189.91 ± 00.04 mg.kg^{-1}). The spores' integral and broken walls had less than 0.01 mg.kg^{-1} of Co, Cd, Mo, Ni, Se, and V.

Table 6. Heavy metal content in *G. lucidum* extracts and spores.

Metal	BR * (mg/kg)	MBR1 * (mg/kg)	*Spores of G. lucidum* broken (mg/kg)
Al	23.82 ± 00.01^{A}	30.18 ± 00.02^{A}	1,071.53 ± 00.14^{B}
B	24.92 ± 00.01^{A}	09.45 ± 00.01^{B}	57.75 ± 00.01^{C}
Ba	24.92 ± 00.00^{A}	00.90 ± 00.00^{A}	57.75 ± 00.00^{B}
Ca	270.05 ± 00.03^{A}	162.99 ± 00.01^{A}	3,009.31 ± 00.19^{C}
Co	< 0.01	< 0.01	< 0.01
Cu	< 0.01	< 0.01	17.34 ± 00.01^{A}
Cd	< 0.01	< 0.01	< 0.01
Fe	13.30 ± 00.00^{A}	06.85 ± 00.00^{A}	2,321.24 ± 00.09^{B}
K	152.70 ± 00.04^{A}	114.44 ± 00.03^{A}	2,349.59 ± 00.39^{C}
Mg	42.40 ± ^{AB}	21.32 ±^{A}	899.30 ± ^{C}
Mn	02.79 ± 00.00^{A}	02.16± 00.00^{A}	41.28 ± 00.00^{C}
Mo	< 0.01	< 0.01	< 0.01
Na	233.52 ± 00.04^{A}	189.91 ± 00.04^{A}	780.01 ± 00.05^{B}
Ni	< 0.01	< 0.01	< 0.01
P	199.24 ± 00.03^{A}	153.78 ± 00.03^{A}	1,886.87 ± 00.08^{C}
Pb	< 0.01	< 0.01	< 0.01
Se	< 0.01	< 0.01	< 0.01
V	< 0.01	< 0.01	< 0.01

*Note: a and b show statistical differences between extracts. A and B show statistical differences between samples.

All spore and extract samples had low contents of Co and Pb (< 0.01 mg.kg^{-1}). Spore breakage using the steel/chrome spheres does not present a risk to public health because the metals' concentrations were below the recommended levels. The toxicity

of these elements was described by European Commission 1881/2006 [30-31] where Pb content of edible fungi is set at a minimum of 0.10 $mg.kg^{-1}$, and Cd levels are set at 1.0 $mg.kg^{-1}$. In both extraction processes, the heavy metal quantity was below that of unbroken spores.

Lipid determination was performed for broken and unbroken *G. lucidum* spores; these values were 25.09 ± 3.65% and 7.0 ± 1.41%, respectively. These results indicate a greater release of lipids after spore rupture due to assisted maceration with steel/chrome spheres. This lipid level of 25% is within the range of 26.9% to 23.3% previously reported for *G. lucidum* [32]. Although the rupture process used in this study yielded a high lipid concentration, the extraction process did not allow for their recovery. Other methods can be used to obtain lipids. For example, lipids can be extracted using sonication at a concentration of 23.74% by Soxhlet, and at 29.50% by supercritical CO_2 at 35 MPa [33].

Conclusions

The most effective methods for breaking the walls of *G. lucidum* spores were the BR and the MBR1 processes. The FITR analysis of the chain lengths was an easy tool for the preliminary qualification study and for monitoring the methods used to extract the different chemical compounds present in the *G. lucidum* spores and extracts. This method showed that protein structures were modified during the MBR1 process, whereas they were preserved along the BR process. The MBR1 process showed the highest proportion of carbohydrates and polyphenols, thereby maintaining antioxidant potential. Finally, the rupture and the chemical extraction methods used maintained antioxidant activity and extracted significant amounts of biocompounds.

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Disclosure statement

The authors declare no commercial or financial conflict of interest.

References

1. D. Sliva, Cellular and physiological effects of *Ganoderma lucidum* (*Reishi*), *Mini-Rev. Med. Chem*., **4,** 873-879 (2004).

2. D. Silva, M. Sedlak, V. Slivova, T. Valachovicova, F.P.J. Lloyd, N.W.Y. Ho, Biologic activity of spores and dried powder from human breast and prostate cancer cells, *J. Altern. Complement. Med*., **9**, 491-497(2003).

3. S.A. Heleno, L. Barros, A. Martins, M. João, R.P. Queiroz, C. Santos-Buelga, I.C.F.R. Ferreira, Fruiting body, spores and in vitro produced mycelium of *Ganoderma lucidum* from Northeast Portugal: A comparative study of the antioxidant potential of phenolic and polysaccharidic extracts, *Food Res. Int*., **46**, 135-140 (2012).

4. B. Boh , M. Berovic, J. Zhang, L. Zhi-Bin, *Ganoderma lucidum* and its pharmaceutically active compounds, *Biotechnol. Annu. Rev.*, **13**(7), 265-301 (2007).

5. S. Keypour, H. Rafati, H. Riahi, F. Mirzajani, M.F. Moradali, Qualitative analysis of ganoderic acids in *Ganoderma lucidum* from Iran and China by RP-HPLC and electrospray ionisation-mass spectrometry (ESI-MS), *Food Chem.*, **119**, 1704-1708 (2010).

6. J.E. Adaskaveg, R.L. Gilbertson, Basidiospores, pilocystidia, and other basidiocarp characters in several species of the *Ganoderma lucidum* complex, *Mycologie*, **80**, 493-507 (1988).

7. K.T. Chau, S. Wu, Impact breakage of single particles, double impact test, in: A.D. Salman, M. Ghadiri, M.J. Hounslow, editors, *Handbook of Powder Technology*, Elsevier, Amsterdam, 2007, p. 69-85.

8. F. Hennicke, Z. Cheikh-Ali, T. Liebisch, J.G. MacIá-Vicente, H.B. Bode, M. Piepenbring, Distinguishing commercially grown *Ganoderma lucidum* from *Ganoderma lingzhi* from Europe and East Asia on the basis of morphology, molecular phylogeny, and triterpenic acid profiles, *Phytochem.*, **127**, 29-37 (2016).

9. X. Zhu, X. Chen, J. Xie, P. Wang, W. Su, Mechanochemical-assisted extraction and antioxidant activity of polysaccharides from *Ganoderma lucidum* spores, *Int. J. Food Sci. Technol*., **47**, 927-932 (2012).

10. B. Stuart, FTIR of biomolecules, in: B. Zhang, L. Cheng, editors, *Reviews in cell biology and molecular medicine*, Wiley-VCH, Germany, 2006.

11. X. Bao, C. Liu, J. Fang , X. Li, Structural and immunological studies of a major polysaccharide from spores of *Ganoderma lucidum* (Fr.) Karst, *Carbohydr. Res*., **332**, 67-74 (2001).

12. L. Guo, J. Xie, Y. Ruan, L. Zhou, H. Zhu, X. Yun, Y. Jiang, L. Lü, K. Chen, Z. Min, International immunopharmacology characterization and immunostimulatory activity of a polysaccharide from the spores of *Ganoderma lucidum*, *Int. Immunopharmacol.*, **9,** 1175-1182 (2009).

13. X. Wang, X. Chen, Z. Qi, X. Liu, W. Li, S.Wang, A study of *Ganoderma lucidum* spores by FTIR microspectroscopy. Spectrochim Acta A, *Mol. Biomol. Spectrosc.*, **91**, 285-289 (2012).

14. J. Wang,R. Ke, S. Zhang, Breaking the sporoderm of *Ganoderma lucidum* spores by combining chemical reaction with physical actuation, *Nat. Prod. Res*., **31**, 2428-2434 (2017).

15. M.M. Bradford, A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem*., **72**, 248-254 (1976).

16. V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Viticult*, **16**, 144-158 (1965).

17. F.A. Loewus, Improvement in anthrone method for determination of carbohydrates, *Anal. Chem*., **24**, 219 (1952).

18. Q. Dong, Y. Wang, L. Shi, J. Yao, J. Li, F. Ma, K. Ding, A novel water-soluble β-D-glucan isolated from the spores of *Ganoderma lucidum*, *Carbohydr. Res*, **353**, 100-105 (2012).

19. T. Stevenson, H. Furneaux, Chemical methods from red algae for the analysis of sulphated galactans, *Carbohydr. Res*., **210**, 277-298 (1991).

20. R. Falshaw, R.H. Furneaux, Carrageenan from the tetrasporic stage of *Gigartina decipiens* (*Gigartinaceae, Rhodophyta*), *Carbohydr. Res*., **252**, 171-182 (1994).

21. L.G. Ferreira, M.D. Noseda, A.G. Gonçalves, D.R.B. Ducatti, M.T. Fujii, M.E.R. Duarte, Chemical structure of the complex pyruvylated and sulfated agaran from the red seaweed *Palisada flagellifera* (*Ceramiales, Rhodophyta*), *Carbohydr. Res.*, **347**, 83-94 (2012).

22. P.E. Jansson, L. Kenne, H. Liedgren, *A practical guide to the methylation, analysis of carbohydrates*, Chemical communications/University of Stockholm, Stockholm, 1976.

23. D.A. Navarro, C.A. Stortz, Determination of the configuration of 3,6-anhydrogalactose and cyclizable α-galactose 6-sulfate units in red seaweed galactans, *Carbohydr. Res.*, **338**, 2111-2118 (2003).

24. M.S. Férnandez-Pachón, D.Villaño, A.M. Troncoso, M.C. García-Parrilla, Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with antioxidant activity, *Anal. Chim. Acta*., **563**, 101-108 (2006).

25. E.G. Blight, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol*., **37**, 911-917 (1959).

26. B.S. Min, J.J. Gao, N. Nakamura, M. Hattori, Triterpenes from the spores *Ganoderma lucidum* and their cytotoxicity against meth-A and LLC tumor cells, *Chem. Pharm. Bull*., **48**, 1026-1033 (2000).

27. X. Liu, J.P. Yuan, C.K. Chung, X.J. Chen, Antitumor activity of the sporoderm-broken germinating spores of *Ganoderma lucidum*, *Cancer Lett.*, **182**, 155-161 (2002).

28. G.G.L. Yue, K. Fung, P. Leung, C.B.S. Lau, Comparative studies on the immunomo-dulatory and antitumor activities of the different parts of fruiting body of *Ganoderma lucidum* and *Ganoderma* spores, *Phytother. Res*., **22**, 1282-1291 (2008).

29. C.R. Soccol, L.Y. Bissoqui, C. Rodrigues, R. Rubel, S.R.B.R. Sella, F. Leifa, L.P. Souza Vandenberghe, V. Thomaz-Soccol, Pharmacological properties of biocompounds from spores of the *lingzhi* or *reishi* medicinal mushroom *Ganoderma lucidum* (*Agaricomycetes*), A Review, *Int. J. Med. Mushrooms*, **18**, 757-767 (2016).

30. K. Yano, S. Ohoshima, Y. Gotou, K. Kumaido, Direct measurement of human lung cancerous and noncancerous tissues by fourier transform infrared microscopy, can an infrared microscope be used as a clinical tool?, *Anal. Biochem.*, **287**, 218-225 (2000).

31. M. Jaishankar, T. Tseten, N. Anbalagan, B.B. Mathew, K.N. Beeregowda, Toxicity, mechanism and health effects of some heavy metals, *Interdiscip. Toxicol.*, **7**, 60-72 (2014).

32. L. Li, H.J. Guo, L.Y. Zhu, L. Zheng, X. Liu, A supercritical-CO_2 extract of *Ganoderma lucidum* spores inhibits cholangiocarcinoma cell migration by reversing the epithelial-mesenchymal transition, *Phytomed.*, **23**, 491-497 (2016).

33. X. Liu, S.P. Xu, J.H. Wang, J.P. Yuan, L.X. Guo, X. Li, X.N. Huang, Characterization of *Ganoderma* spore lipid by stable carbon isotope analysis: Implications for authentication, *Anal. Bioanal. Chem.*, **388**, 723-731 (2007).

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