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THE EFFECTS OF DIFFERENT SOLVENTS ON BIOACTIVE METABOLITES AND "*IN VITRO*" ANTIOXIDANT AND ANTI-ACETYLCHOLINESTERASE ACTIVITY OF *GANODERMA LUCIDUM* FRUITING BODY AND PRIMORDIA EXTRACTS

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It is known that the lingzhi mushroom *Ganoderma lucidum* (Fr.) Karst produces various, bioactive metabolites. The objective of this study was to evaluate the antioxidant activity and acetylcholinesterase inhibitory activity of various extracts obtained from *G. lucidum* fruiting body and primordium, which is the initial stage of the mushroom's fruiting body. Classical extraction of *G. lucidum* fruiting bodies and primordia using different solvents (water, methanol, acetone, ethanol and hexane) at two temperatures (at 25 °C and at the boiling point of the solvent) was performed.

The influence of a solvent on the extraction yields and afterwards on the activity of the extract was observed. The extracts are rich in phenols, polysaccharides and proteins. The highest yield of 23.30% was obtained after water extraction. The total phenolic content was between 9 mgGA/g and 74.36 mgGA/g for *G. lucidum* fruiting bodies extracts and from 11.16 mgGA/g to 103.32 mgGA/g for *G. lucidum* primordia extracts. The antioxidant activity using the DPPH* free radical scavenging method was from 0.48% to 23.66% and from 5.32% to 54.57% for fruiting bodies and primordia extracts, respectively.

Assessment of acetylcholinesterase enzyme inhibition was carried out using a colorimetric method based on Ellman's reaction. Acetylcholinesterase enzyme inhibition (AChE inhibition) by *G. lucidum* fruiting bodies extracts was between 18.1% and 32.5%. The highest AChE inhibitory activity of primordia extracts was 29.48%. This is the first report of *G. lucidum* primordia extracts and their biological activity.

Keywords: extraction; antioxidant; phenols; anti-acetylcholinesterase

ВЛИЈАНИЕ НА РАЗЛИЧНИ РАСТВОРУВАЧИ НА БИОАКТИВНИ МЕТАБОЛИТИ И *IN VITRO* АНТИОКСИДАЦИСКА И АНТИАЦЕТИЛХОЛИНЕСТЕРАЗНА АКТИВНОСТ НА ПЛОДНОТО ТЕЛО И ЕКСТРАКТИТЕ ОД ПРИМОРДИУМОТ НА *GANODERMA LUCIDUM*

Добро е познато дека дабовата сјајница *Ganoderma lucidum* (Fr.) Karst произведува разни биоактивни метаболити. Целта на ова истражување беше да се процени антиоксидацискатa активност и ацетилхолинестеразната (AChE) инхибиторна активност на разни екстракти добиени од плодното тело и од примордиумот кој е почетната фаза на плодното тело на *G. lucidum.* Беше извршена класична екстракција од плодното тело и од примордиумот на *G. lucidum* со употреба на различни растворувачи (вода, метанол, ацетон, етанол и хаксан) на две температури (на 25 °C и на температура на вриење на растворувачот).

Беше одредено влијанието на растворувачот врз приносот на екстракцијата, а потоа и врз активноста на екстрактот. Екстрактите беа богати со феноли, полисахариди и протеини. Највисок принос од 23,30% беше добиен при екстракција со вода. Вкупната содржина на фенолите беше

меѓу 9 mgGA/g и 74,36 mgGA/g за екстрактите од плодното тело и од 11,16 mgGA/g до 103,32 mgGA/g за екстрактите од примордиумот. Антиоксидациската активност со методот на прибирање на радикали со употреба на DPPH* се движеше од 0,48% до 23,66% за екстрактите од плодното тело и од 5,32% до 54,57% за екстрактите од примордиумот.

Проценатa на инхибицијата на ензимот AChE беше извршена со колориметриски метод со реакцијата на Ellman. Инхибицијата се движеше од 18,1% до 32,5% за екстрактите од плодното тело. Највисока инхибиција на екстрактите од примордиумот изнесуваше 29,48%. Ова е прво истражување на екстрактите од примордиумот на *G. lucidum* и на нивната биолошка активност.

Клучни зборови: екстракција; антиоксидациско средство; феноли; ацетилхолинестераза

1. INTRODUCTION

Ganoderma lucidum (Fr.) Karst is a member of the mushroom family Polyporaceae and has been used in complementary medicine for over 2000 years [1, 2]. *G. lucidum* develops from a nodule, or pinhead, less than two millimeters in diameter, called a primordium, which is typically found on or near the surface of the substrate. It takes approximately 25 days from primordium formation to the development of a mature fruiting body that is ready for harvest. Spores are the mushroom's reproductive cells formed from hymenium of *G. lucidum* after the fruiting bodies become mature [1].

The mycelia, mature fruiting body and spores of *G. lucidum* contain over 400 different bioactive compounds, including triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins/peptides, and trace elements which have been reported to have a number of medicinal effects [3, 4] such as immunomodulation, antitumor [5, 6] anti-atherosclerotic [7], anti-inflammatory, anti-microbial [8], hypolipidemic [9], hepatoprotective [10], anti-diabetic, anti-androgenic, anti-angiogenic, anti-herpetic, anti-oxidative and radical-scavenging, anti-aging [11], hypoglycemic [12] and anti-ulcer properties [13].

With aging, various pathological conditions and chronic diseases develop as a result of oxidative stress. The attack of free radicals on biomolecules, (lipids, proteins and DNA) eventually leads to many chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans [14]. The use of strong radical scavengers such as antioxidants may potentially delay neurodegeneration in diseases such as Parkinson's, Huntington's, amyotrophic lateral sclerosis and Alzheimer's [15, 16]. One of the most important strategies for the treatment of the neurodegenerative disorder Alzheimer's disease is to control the levels of acetylcholine in the brain through the inhibition of acetylcholinesterase (AChE) [17]. Many synthetic chemicals such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are being used as strong radical scavengers; there is, however, growing interest in dietary antioxidants contained in many foods because of their natural origin and relative safety [14]. Among the sources of natural derived antioxidants, edible mushrooms such as *G. lucidum* are receiving attention as a potential commercial source of antioxidants at present [18, 19].

The antioxidant activity of *G. lucidum* extracts has been found to be correlated to their polysaccharide content as well as to their total phenolic contents [20]. In addition to phenols and polysaccharides, bioactive proteins from *G. lucidum* have been reported as antioxidants [21], [22]. Hasnat demonstrated the potential of *G. lucidum* extract as a valuable source of antioxidants exhibiting anti-acetylcholinesterase activity [23].

In past studies, we observed that extracts obtained from *G. lucidum* fruiting bodies using supercritical carbon dioxide or hot water under different extraction conditions greatly vary in their bioactive capacities [24, 25].

Recently published genomes have revealed the full potential of *G. lucidum* as a source of biologically active compounds [26]. The authors studied variations in gene expression and triterpenoid content across three developmental stages of *G. lucidum:* areal mycelia, primordium and fruiting body. The results of a later study showed that significant numbers of genes (4,668) are up- or down-regulated during at least one of the developing stage transitions. Further, triterpenoid content markedly increased in the primordium; however, their biological activity was not evaluated. New findings demonstrate that the primordium of *G. lucidum* has been overlooked in terms of being a source of bioactive compounds.

Therefore, the aim of our investigation was to provide some insight into the chemical composition as well as the bioactivity (antioxidant and antiAChE) of extracts obtained from *G. lucidum*

primordia and fruiting bodies using various conventional solvents. This is one of the first reports describing *G. lucidum* primordium as a source of biologically active phenols, polysaccharides, fatty acids and proteins.

2. MATERIALS AND METHODS

2.1. *Mushroom material*

G. lucidum fruiting bodies (GL) and its primordium (GL-P) were obtained from MycoMedica d.o.o. (Podkoren, Slovenia). Coomassie brilliant blue (CAS Number: 6104-58-1), bovine serum albumin (⩾96%) (CAS Number: 9048-46-8), phenol (⩾99.9%) (CAS Number: 108-95-2), glucose (⩾98%) and sodium carbonate (⩾99.9%) (CAS Number 497-19-8) were purchased from Sigma–Aldrich (Slovenia). Gallic acid (⩾98%) (CAS Number: 149-91-7) was purchased from Fluka (Germany). Sulfuric acid (99.99%) and Folin–Ciocalteau phenol reagent were purchased from Merck (Germany). Hexane (⩾95%) (CAS Number: 110-54-3) and ethanol (⩾99.9%) (CAS Number: 64-17-5) were purchased from Carlo Erba (Italy) and methanol (⩾99.8%) (CAS Number: 67-56-1) was purchased from J. T. Baker Chemicals (Netherlands).

2.2. *Extraction procedures*

2.2.1. *Extraction of phenolic compounds*

G. lucidum fruiting bodies (GL) and its primordia (GL-P) were lyophilized, crushed using liquid nitrogen, and milled. Two types of extraction, hot (H) (at the boiling point of the solvent) and cold (C) (at 25 °C) were performed using different types of solvents (distilled water (H_2O), ethanol (EtOH), acetone (AcOH), methanol (MeOH) and hexane (Hex)). 5 g of GL or GL-P material was introduced into a flask and 100 ml of solvent was added. Extraction at the boiling temperature of the solvent was performed in a flask with a reflux condenser while extraction at 25 °C was carried out in a closed flask. Extraction time was 3 hours with constant stirring. After that, the extract solution was filtered and the filtrate was evaporated to remove the solvent. Extraction yield (%) was determined as:

$$Y(\%) = \frac{m(GLext/GL-P\text{ext}-\text{H/C}-\text{EtOH/AcOH/MeOH/Hex})}{mGL/GL-P} \cdot 100 \quad (1)$$

where:

Y	– extraction yield (%)
$m\text{GL}_{\text{ekst.}}$/GL-$\text{P}_{\text{ekst.}}$ – H/C – H_2O	– mass of fruiting body/primordia extract using hot(H)/cold(C) water for extraction (g)
$m\text{GL}_{\text{ekst.}}$/GL-$\text{P}_{\text{ekst.}}$ – H/C – MeOH	– mass of fruiting body/primordia extract using hot(H)/cold(C) methanol (g)
$m\text{GL}_{\text{ekst.}}$/GL-$\text{P}_{\text{ekst.}}$ – H/C – EtOH	– mass of fruiting body/primordia extract using hot(H)/cold(C) ethanol (g)
$m\text{GL}_{\text{ekst.}}$/GL-$\text{P}_{\text{ekst.}}$ – H/C – Ac	– mass of fruiting body/primordia extract using hot(H)/cold(C) acetone (g)
$m\text{GL}_{\text{ekst.}}$/GL-$\text{P}_{\text{ekst.}}$ – H/C – Hex	– mass of fruiting body/primordia extract using hot(H)/cold(C) hexane (g)
m (GL/(GL-P))	– mass of fruiting body (GL) and primordia (GL-P) raw material (g)

2.2.2. *Extraction of polysaccharides*

The polysaccharidic extracts were obtained using three different procedures: (1) by conventional extraction and purification of polysaccharides described by Villares et al. [27], (2) by hot water extraction and precipitation with ethanol as described by Skalicka-Woźniak et al. [28], in both cases using 5 g of GL or GL-P, and (3) extraction with methanol (T = 67.7 °C and p = 1 bar), in order to remove phenolic compounds and other related molecules [27]. Then, the methanolic extract was filtered and residue of GL or GL-P was extracted with hot water (T = 100 °C). The water extract was filtered (filtrate 1) and the remaining solid material was extracted with an aqueous basic solution of

2% w/v of NaOH at 100 °C. Again, the extract was filtered (filtrate 2) and GL or GL-P was discharged. Both filtrates were combined and proteins removed by precipitation with trifluoroacetic acid (TFA) (20% w/v). Then, proteins were separated by centrifugation.

Purified polysaccharidic extract of fruiting bodies in addition as - GL-$PS_{ext.}$ – 1 and polysaccharidic extract of primordia in addition as GL-P-PS_{ext} – 1 were finally precipitated from the supernatant by the addition of EtOH in a 2:1 ratio (v/v). In the second procedure, GL or GL-P was extracted with 100 ml of distilled water at 85 °C for 6 hours with stirring. The crude hot water extracts were filtered and polysaccharidic extract of fruiting bodies (GL-$PS_{ext.}$ – 2) and polysaccharidic extract of primordia (GL-P-$PS_{ext.}$ –2) were separated as described by Skalicka-Woźniak. Briefly, cold ethanol in a ratio of 3:1 v/v was added to concentrated hot water extracts and polysaccharides were precipitated overnight at +4 °C. The precipitated polysaccharides were collected after centrifugation (in an Eppendorf 5804 R refrigerated centrifuge) at 3100 rpm for 10 min.

2.2.3. *Extraction of proteins*

Five g of GL or GL-P was extracted with 100 ml of methanol for 3 hours under constant stirring to remove phenolic compounds. After the hot water extraction of residue of GL or GL-P was performed, the extract was filtered and proteins removed from the filtrate using trifluoroacetic acid (20% w/v). Then, protein extract of fruiting bodies, hereafter GL-$P_{ext,}$ and protein extract of primordial, hereafter GL-P-$P_{ext,}$ was separated by centrifugation [27].

2.3. *Chemical content of extracts*

2.3.1. *Total phenol content*

The concentration of total phenols in the extracts was measured by UV spectrophotometry (Varian, USA), based on a colorimetric oxidation/reduction reaction. The total phenols were determined according to the Folin-Ciocalteau method (1927) with some modifications [29]. Briefly, all extracts were diluted in methanol at concentrations of 1 $mg{\cdot}ml^{-1}$. 2.5 ml of Folin-Ciocalteau reagent (diluted with water at a 1:10 ratio) was mixed with 0.5 ml of extract solution and 2.5 ml of Na_2CO_3 (75 $g{\cdot}l^{-1}$). Prepared samples were then thermostated in a water bath at 50 °C for 5 min. After cooling, the absorbance was measured at 760 nm. As a control, 0.5 ml of methanol was used instead of the extract solution.

Quantification was determined based on the standard curve of gallic acid (GA). The amount (%) of phenol content (w_{GA}) was expressed as mg_{GA} per gram (g) of extract.

2.3.2. *Protein content*

The total protein content was measured using the Bradford method. Briefly, 100 mg of Coomassie Brilliant Blue was mixed with 50 ml of 95% ethanol, and 100 ml of 85% (v/v) H_3PO_4 solution was diluted with distilled water to 1 liter. The quantification was determined based on the standard curve of Bovine Serum Albumin (BSA). The calibration curve was in the range of 0.0 $mg{\cdot}ml^{-1}$ to 1 $mg{\cdot}ml^{-1}$ [30].

GL-P_{ext} or GL-P-P_{ext} was diluted with water to a concentration of 1 $mg{\cdot}ml^{-1}$. An aliquot (20 µl) was mixed with 1 ml of Bradford reagent and the absorbance was measured at 595 nm. Total protein content was expressed in mg of BSA per gram (g) of extract (mg $BSA{\cdot}g^{-1}$ GL-P_{ext} or GL-P-P_{ext}).

2.3.3. *Polysaccharide content*

Polysaccharide content was determined using the phenol-sulfuric acid method and D-glucose as standard [31]. Polysaccharidic extract solutions (1 $mg{\cdot}ml^{-1}$) were prepared in distilled water. Then, 0.5 ml of solution was mixed with 0.5 ml of 5% aqueous phenol solution and 2.5 ml of concentrated sulfuric acid. The mixture was stirred for 30 min. The total sugar content was determined on the standard curve for glucose (0.0047 $mg{\cdot}ml^{-1}$ – 0.15 $mg{\cdot}ml^{-1}$) at a wavelength of 490 nm. The results were expressed as mg of glucose equivalents per gram of polysaccharidic extract dry weight.

2.4. *Biological activity of extracts*

2.4.1. *Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals*

For the assay, 3.9 ml of 0.06 mM DPPH* radical (Sigma, CAS Number: 1898-66-4) was added to 0.1 ml of *G. lucidum* fruiting body or primordia extract. The reaction mixture was vortexed and absorbance measured at 515 nm using a spectrophotometer with methanol as a control. The decrease in absorbance was monitored until the reaction reached a plateau. The DPPH* free radical scavenging activity, expressed as a percentage of radical scavenging activity, was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = \frac{A_0 - A_S}{A_0} \cdot 100 \quad (2)$$

where A_0 is the absorbance of 0.06 mM methanolic DPPH and A_s is the absorbance of the reaction mixture after 30 min [32].

2.4.2. *Anti-acetylcholinesterase activity*

The inhibition of acetylcholinesterase (AChE) was measured according to the Ellman method (1961), using acetylthiocholine iodide (1mM) as the substrate in 100 mM potassium phosphate buffer, pH 7.4, at 25 °C, and electric eel AChE as the source of enzyme (6.25 $U \cdot ml^{-1}$, Sigma) (Ellman et al. 1961) [33]. The hydrolysis of acetylthiocholine iodide was measured on a Kinetic Microplate Reader (Varian, USA) at 405 nm. The concentration of the extracts was 1 mg/ml and AChE inhibition was monitored for 5 min. All readings were corrected for their appropriate controls, and a run with only acetylthiocholine chloride served as a positive control assay. Galathamine, a common AChE inhibitor, was used as a control. Every measurement was repeated at least two times.

3. RESULTS AND DISCUSSION

3.1. *Conventional extraction using conventional solvents*

3.1.1. *Yield of extraction using conventional solvents*

Extraction yield (η) using different types of solvents (distilled water (H_2O), methanol (MeOH), ethanol (EtOH), acetone (Ac) and hexane (Hex)) is presented in Table 1.

T a b l e 1

Yield (%) after extraction of G. lucidum fruiting body (GL) and G. lucidum primordia (GL-P.)

Solvent	GL – fruiting body *Y (%)*			GL-P – primordia *Y (%)*		
	Cold (C) T = 25° C	Hot (H) at T_B	T_B (°C)	Cold (C) T = 25° C	Hot (H) at T_B	T_B (°C)
H_2O	19.13	23.30	100 °C	20.10	24.15	100 °C
MeOH	9.20	9.33	64.7 °C	12.05	14.98	64.7 °C
EtOH	6.16	4.22	78.4 °C	4.94	6.15	78.4 °C
Ac	3.58	3.15	56 °C	5.02	6.05	56 °C
Hex	1.42	2.40	69 °C	0.95	3.11	69 °C

*T_B – boiling temperature of a solvent

When water was used as a solvent, the highest yield (23.30%) was obtained. The yield decreased in the order of H_2O > MeOH > EtOH > Ac > Hex. Only slight changes in the total yield were observed in the comparison of hot/cold extraction procedures. Higher extraction yield does not necessarily mean higher medicinal activity of the extracts.

The relative polarity of the solvents used in the present study increases in the order of hexane Hex < Ac < EtOH < MeOH < H_2O. Extraction efficiency also increases in that order. The polarity of the solvent thus greatly influences the extraction yields. Higher temperatures usually lead to higher yields of extraction. However, in our study, the polarity of the solvent shows a greater influence on yield. Solvents such as methanol, ethanol and acetone are often used for the extraction of phenolic compounds, while hexane is more often used for the isolation of fatty acids. The yield in hexane solvent was the lowest. This corresponds to the fact that *G. lucidum* contains very low amounts of fatty acids [34]. In general, the average yields for GL-P are slightly higher than those for GL. Mushrooms store components in the initial phase, which are needed for their further development, and we expected these to show slightly higher extraction efficiency values. We conclude that many of the components such as GL and GL-P are polar and that non-polar components are significantly less common.

3.1.2. *Total phenols in extracts obtained with conventional solvents*

The contents of total phenols in extracts obtained from *G. lucidum* fruiting bodies (GL_{ext}) and *G. lucidum* primordia ($GL\text{-}P_{ext}$) using various conventional solvents are presented in Figure 1 and Figure 2, respectively.

The concentration of total phenolic compounds in $GL_{ext.}$ ranged from 9.01 $mg_{GA} \cdot g_{ext}^{-1}$ to 74.36 $mg_{GA} \cdot g_{ext}^{-1}$, depending on solvent selection and temperature during the extraction process. The

highest concentration of phenolic compounds is 74.36 $mg_{GA} \cdot g_{ext}^{-1}$ EtOH GL_{ext}, decreasing in the order EtOH > MeOH > H_2O > AcOH > Hex. The influence of temperature during the extraction process on the total amount of phenols in GL_{ext} was noticed. Extraction with EtOH at its boiling point resulted in 74.36 $mg_{GA} \cdot g_{ext}^{-1}$ GL_{ext} while the amount was 65.72 $mg_{GA} \cdot g_{ext}^{-1}$ when EtOH at 25 °C was used. The effect of extraction temperature on total phenolic content was also noticed in the case of AcOH (Fig. 1). However, when MeOH was used, a higher content of total phenols, 52.11 mg_{GA}/g_{ext}, was obtained when extraction was performed at 25 °C, while performing extraction using MeOH at boiling point resulted in the production of 32.86 $mg_{GA} \cdot g_{ext}^{-1}$ in GL_{ext}. The effect of solvent type on total phenols in different mushrooms was previously studied by Tsai et al. [35], who noticed that the highest yield of phenolic compounds was in ethanol, which ranked second after water. Orhan et al. (2011) determined the total phenol content of ethanolic extracts in a number of mushroom species growing in Turkey [36]. The phenol content was in the range of 2.5 $mg_{GA} \cdot g_{ext}^{-1}$ to 51.7 $mg_{GA} \cdot g_{ext}^{-1}$.

Heleno et al. extracted *G. lucidum* from fruiting bodies with methanol : water (80:20) at 20 °C for 2 hours [37]. The total phenolic content was 28.64 $mg_{GA} \cdot g_{ext}^{-1}$ in methanol/water extracts of the *G. lucidum* fruiting bodies. In our study, a two-fold higher concentration of total phenolic content at 25 °C was observed in GL_{ext} produced with methanol extraction.

Celik et al. determined a higher total phenol content (49.52 $mg_{GA} \cdot g_{ext}^{-1}$) in *G. lucidum* ethanol extracts in comparison with methanol extracts obtained by Soxhlet extraction [38].

The influence of solvent type as well as temperature during the extraction process has an effect on the total phenol content in the GL_{ext} obtained. There is no report on the influence of different types of solvents at different temperatures on *G. lucidum* phenol content.

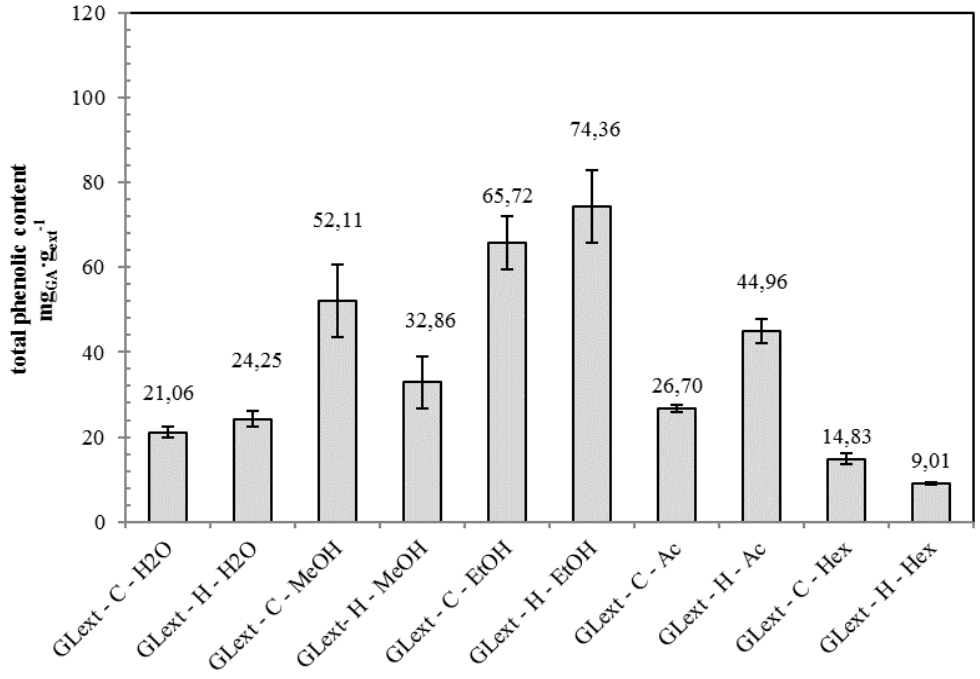

Fig. 1. Total phenol content (w_{GA}) in *G. lucidum* extracts ($GL_{ext.}$) obtained by hot (at boiling point)/cold (at 25 °C) (H/C) extraction with different solvents (H_2O, MeOH, EtOH, AcOH, Hex). The standard deviation is expressed as ± value, from at least two replicates, where two independent experiments were performed.

The concentration of total phenolic compounds in GL-P_{ext} ranged from 11.16 $mg_{GA} \cdot g_{ext}^{-1}$ to 103.32 $mg_{GA} \cdot g_{ext}^{-1}$, depending on solvent type and extraction temperature (Figure 2). The highest yield of GL-P_{ext} phenolic compounds, 103.32 $mg_{GA} \cdot g_{ext}^{-1}$ was obtained with hot Ac. The concentration of phenolic compounds from GL-P_{ext} obtained using hot (H) solvents decreased in the order of Ac > EtOH > H_2O = MeOH. Because of similar polarity, 46 $mg_{GA} \cdot g_{ext}^{-1}$ GL-P_{ext} of phenols were obtained both when (H) MeOH and (H) H_2O were used. The highest influence of extraction temperature on the amount of total phenols from GL-P_{ext} was noticed when Ac was used. GL-P_{ext} obtained

with (H) Ac contained 103.32 $mg_{GA} \cdot g_{ext}^{-1}$, while 64.54 $mg_{GA} \cdot g_{ext}^{-1}$ of phenols were present in extract obtained with cold (C) Ac. (C) extraction generally resulted in a lower amount of phenolic compounds, except for the case of MeOH (Fig. 2). The expected phenolic compounds were not present in the extract obtained using hexane as a solvent.

From Figures 1 and 2, it can be seen that there is similar trend if we compare only temperatures using the same solvent type. In general, the amounts of total phenols in GL–P_{ext} are higher than in GL_{ext}.

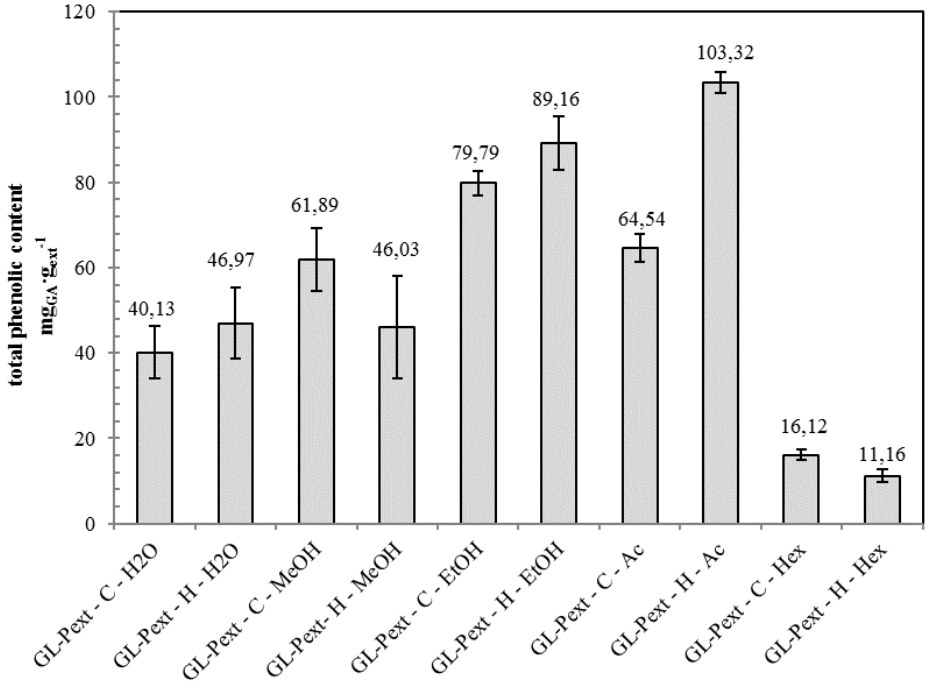

Fig. 2. The content of total phenols in extracts obtained from *G. lucidum* (GL-P_{ext}) primordium. Data are presented as mgGA/g GL-P_{ext} and are expressed as means ± SD from two replicates. Extraction temperature: hot (H) and cold (C); extraction solvent: H_2O, MeOH, EtOH, Ac and Hex.

3.1.3. *DPPH free radical scavenging activity of extracts obtained with conventional solvents*

The results of DPPH* radical scavenging activities of GL_{ext} and GL-P_{ext} obtained using hot or cold extraction are presented in Figure 3 and Figure 4, respectively. The highest DPPH* radical scavenging inhibitory activity was observed for hot AcOH (GL_{ext}–H-Ac) at 23.66%. DPPH* radical scavenging inhibitory activity for cold and hot MeOH and EtOH vary between 12.90% and 16.84% (Fig. 3).

In the present study, the highest total phenol contents did not result in the highest DPPH* radical scavenging activity of the GL_{ext} obtained (Figs. 1 and 2). These results correspond to the observations of Orhan et al. [36].

In general, positive correlations were found between the total phenolic content and the DPPH free radical scavenging activities elicited (Fig. 2 and Fig. 4).

It can be seen from Figure 4 that the maximum inhibition of DPPH radicals occurs when hot acetone solvent is used, followed by hot ethanol, methanol, water and hexane. The values of inhibition of DPPH radicals by GL-P_{ext} were significantly higher than GL_{ext}.

The use of different solvents can result in the extraction of various types of metabolites from *G. lucidum* fruiting bodies and primordia, with varying radical scavenging activities. Furthermore, increased temperatures during the extraction process may result in denaturation and a reduction of the loss of ability to act as an antioxidant.

The lowest radical-scavenging activities were observed for the extracts obtained in Hex, which is mostly used for extraction of lipids; therefore, polar compounds with recognized high antioxidant capacity could not be obtained.

De Bruin et al. investigated the antioxidant properties of extracts obtained from *Grifola gargal* mushrooms, which are of the same order as our results [39].

If we compare the effect of temperature in the same solvent and different material, GL and GL-P can be seen to have similar trends. The reason for the higher values of inhibition of DPPH* radicals in GL-P_{ext} is that in the early stage of fungus growth, it produces substances which are necessary for its growth. Thus, the contents of total phenols, as the values of the inhibition DPPH* radicals for GL-P_{ext}, are higher than in GL_{ext}.

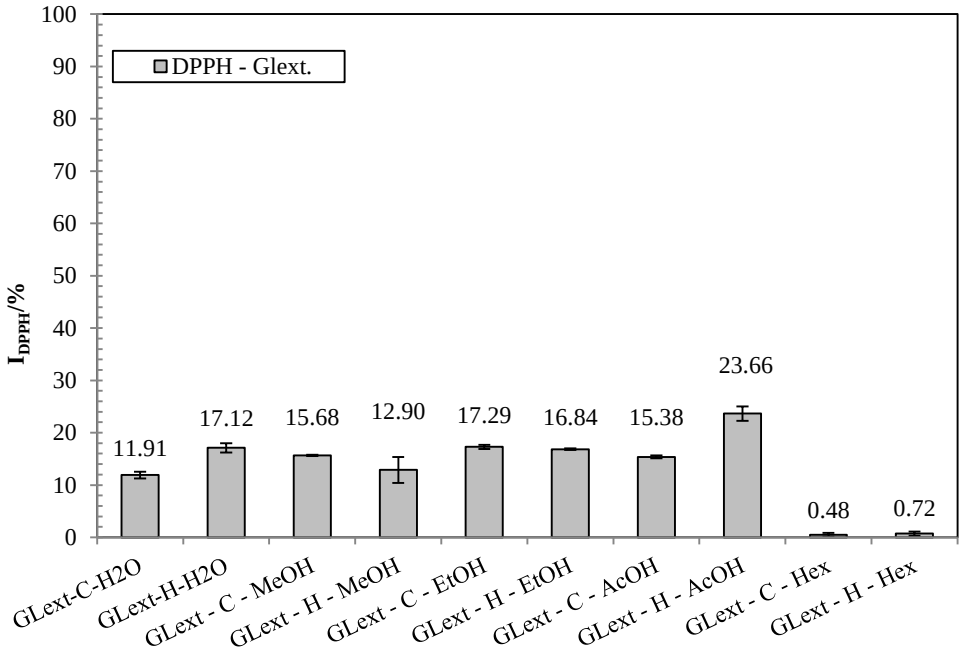

Fig. 3. DPPH radical scavenging activity of extracts obtained from *G. lucidum* fruiting body (GL_{ext}). Data are expressed as inhibition in %. Extraction temperature: hot – at boiling point of the solvent (H), and cold – at 25 °C (C); extraction solvent: H_2O, MeOH, EtOH, Ac and Hex. Data are means ± SD from two replicates, where two independent experiments were performed.

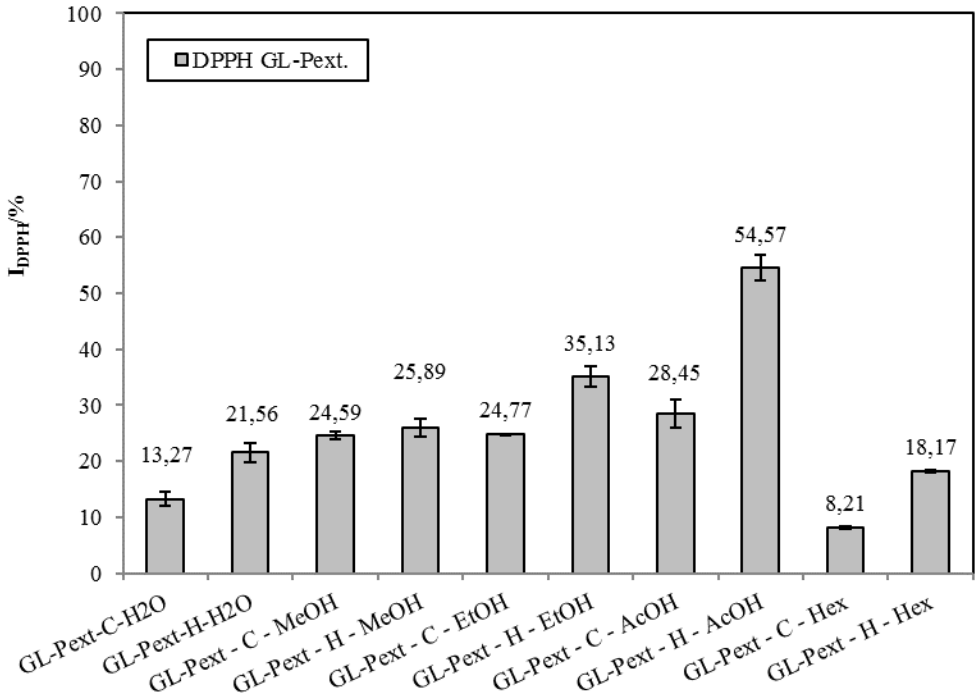

Fig. 4. DPPH radical scavenging activity of extracts obtained from *G. lucidum* primordia (GL-P_{ext}). Data are expressed as inhibition in %. Extraction temperature: hot – at boiling point of the solvent (H) and cold – at 25 °C (C); extraction solvent: H_2O, MeOH, EtOH, Ac and Hex. Data are means ± SD from two replicates, where two independent experiments were performed.

3.1.4. *Inhibition of acetylcholinesterase (AChE) by extracts obtained with conventional solvents*

Since antioxidants play an important role in the protection against aging processes and neuro-degenerative diseases such as Alzheimer's disease (AD), the ability of GL_{ext} and GL-P_{ext} to inhibit the AChE enzyme was determined. Both $GL_{ext.}$ and GL-$P_{ext.}$ contain polar and non-polar components which have the capability to inhibit AChE.

The AChE inhibitory activities of GL_{ext} and GL-P_{ext} were quantified using Ellman's method and the results are summarized in Figure 5 and Figure 6.

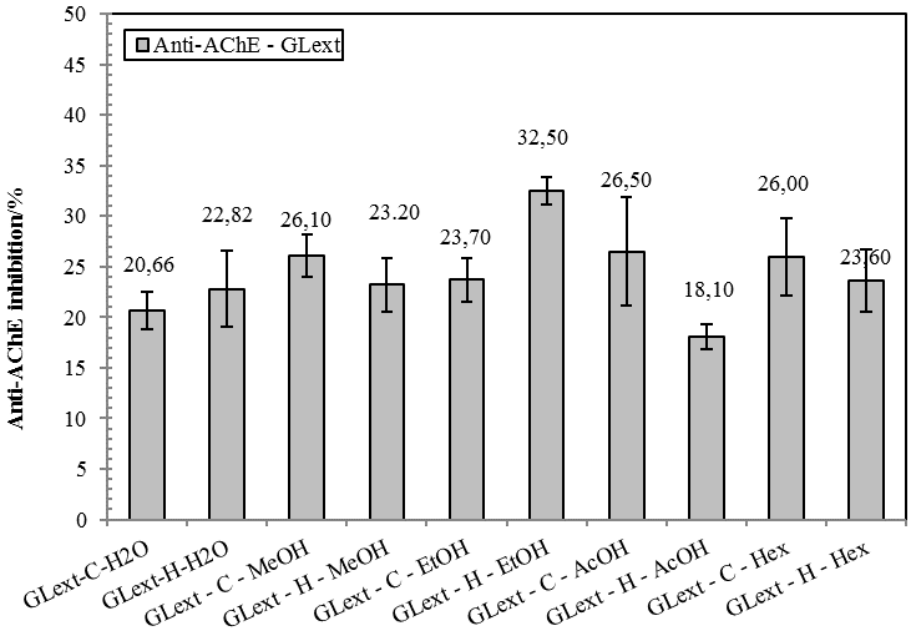

Fig. 5. Acetylcholinesterase (AChE) inhibitory activities of *G. lucidum* extracts ($GL_{ext.}$) obtained using hot (at boiling point of the solvent) / cold (at 25 °C) (H/C) extraction solvent (H_2O, MeOH, EtOH, AcOH, Hex). Data are expressed as percentage (%) of AChE inhibition. Data are means ± SD from two replicates, where two independent experiments were performed. As a control inhibitor, galanthamine, which has an inhibitory activity of 95%, was used.

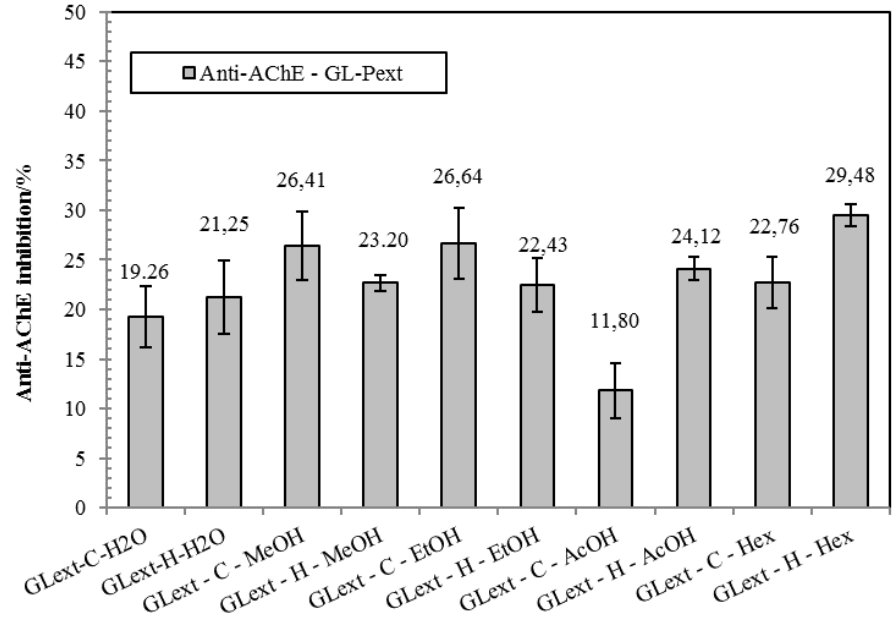

Fig. 6. Acetylcholinesterase (AChE) inhibitory activities of *G. lucidum* primordium extracts (GL-P_{ext}) in *c* = 1mg/ml obtained using hot (at boiling point of the solvent) / cold (at 25 °C) (H/C) extraction solvent (H_2O, MeOH, EtOH, Ac, Hex). Data are expressed as the percentage (%) of AChE inhibition. Data are means ± SD from two replicates, where two independent experiments were performed. As a control, the inhibitor galanthamine was used, with an inhibitory activity of 94%.

The AChE inhibition of $GL_{ext.}$ was between 18.1% for (H) AcOH and 32.5% for (H) EtOH (Fig. 5). A similar result was observed in the case of total phenol content (Fig. 1). Generally, the use of different solvents did not significantly affect AChE inhibitory activity. Furthermore, extraction temperatures did not greatly influence the AChE inhibitory activity of GL_{ext} (Fig. 5).

For primordia, the highest AChE inhibitory activity of 29.48% was obtained when GL-P_{ext}-H-Hex was applied. The effect of solvents and/or extraction temperature is rather small; moreover, there is a correlation between the content of total phenols and AChE inhibitory activities.

There are no reports comparing AChE inhibitory activity of GL_{ext} or GL-P_{ext} obtained with different solvents.

3.1.5. *Bioactivity of polysaccharides*

In both extraction procedures, GL and GL-P material was first pre-extracted with MeOH in order to remove phenolic compounds. Afterwards, the procedures described in section 2.2 were followed.

Total phenols, total polysaccharides, DPPH* free radical scavenging and AChE inhibitory activities were determined for all polysaccharidic extracts. As a control in AChE activity determination, the inhibitor galanthamine, with an inhibitory activity of 93%, was used. The results are summarized in Table 2.

T a b l e 2

Chemical content and bioactivity of polysaccharides obtained from G. lucidum fruiting body (GL-$PS_{ext.}$ – 1/-2) and primordia (GL-P-$PS_{ext.}$ – 1/-2) with two extraction procedures. Data are means ± SD from two replicates, where two independent experiments were performed.

Sample ID	Total phenols ($mg_{GA}\cdot g_{ext.}^{-1}$) ±SD	Total polysaccharides ($mg_{GLC}\cdot g_{Psext}^{-1}$) ±SD	DPPH activity (%)±SD	Anti-AChE activity (%)±SD
GL-$PS_{ext.}$ – 1	8.48 ± 1.67	13.3 ± 4.9	20.46 ± 2.19	22.16 ± 1.9
GL-$PS_{ext.}$ – 2	12.76 ± 1.87	15.9 ± 5.4	2.48 ± 1.20	18.66 ± 1.3
GL-P-$PS_{ext.}$ – 1	7.43 ± 1.65	16.36 ± 1.19	1.94 ± 0.92	14.39 ± 4.1
GL-P-$PS_{ext.}$ – 2	10.44 ± 2.43	17.10 ± 2.16	0.90 ± 0.50	8.24 ± 1.1

Polysaccharide extract obtained using the first described method by Villares et al. had the highest amount of total phenols, 12.76 $mg_{GA}\cdot g_{ext}^{-1}$, as observed by other researchers [27]. Semi-purification can result in a lower polyphenol content in the extracts obtained. An additional purification step involved removal of phenols using MeOH from *G. lucidum*; this resulted in the lowest content of total phenols (GL-$PS_{ext.}$ – 1, Table 2). The polysaccharide content between GL-PS_{ext} – 1/-2 did not change drastically (Table 2), indicating that purification steps did not result in a loss of polysaccharides during the extraction process from the initial *G. lucidum* material. The results of the present study show that the highest DPPH* activity of 20.46% was obtained for GL-PS_{ext} – 1, with the lowest content of total phenols, indicating that purified *G. lucidum* extract has greater antioxidant activity than extract.

All polysaccharidic extracts displayed moderate AChE inhibitory activity between 18.66% and 22.16%.

Both GL-P-PS_{ext} –1/2 contained about 17 mg GLC per g of GL-PS-P_{ext}. The results of chemical analysis showed the presence of phenolic compounds in both extracts.

The purification step using MeOH resulted in the lowest content of total phenols (GL-P-PS_{ext} – 1, Table 2).

In general, the results in Table 2 show that GL-PS_{ext} from *G. lucidum* fruiting bodies have higher DPPH* activity and higher anti-AChE activity compared with GL-P-PS_{ext} from primordia. The amount of total phenols is higher in GL-P-PS_{ext} obtained from primordia.

3.1.6. *Bioactivity of proteins*

Extraction of proteins from *G. lucidum* fruiting bodies and primordia was performed as described by Villares et al. [27] using trifluoroacetic acid (TFA) for precipitation from crude hot water extract, pre-extracted with MeOH. This procedure resulted in protein rich extract (GL-P_{ext}, GL-P-P_{ext}). Total protein and total phenol content, as well as DPPH* and anti-acetylcholinesterase activities, were measured. The total protein content was measured using a method proposed by Bradford. The results of total phenols, total proteins, DPPH and anti-AChE activity for GL-P_{ext}, GL-P-P_{ext} are summarized in Table 3.

T a b l e 3

Chemical content and bioactivity of polysaccharides obtained from G. lucidum fruiting bodies ($GL\text{-}P_{ext}$) and Ganoderma lucidum primordia ($GL\text{-}P\text{-}P_{ext}$). Data are means ± SD from two replicates, where two independent experiments were performed.

Sample ID	Total phenols ($mg_{GA}\cdot g_{ext.}^{-1}$) ±SD	Total proteins ($mg_{BSA}\cdot g_{Pext}^{-1}$) ±SD	DPPH (%)±SD	Inhibition of AChE (%)±SD
$GL\text{-}P_{ext.}$	60.96±5.01	13.3±4.9	17.33±1.14	24.40±1.36
$GL\text{-}P\text{-}P_{ext}$	26.04±4.94	16.3±1.9	3.35±0.55	13.62±5.11

From the results obtained, it can be observed that $GL\text{-}P_{ext}$ contains 13.3% of proteins as well as a surprisingly high content of phenols of 60% (Table 3). Antioxidant and anti-AChE activities of $GL\text{-}P_{ext}$, being 17.33% and 24.40%, respectively, are probably the result of high phenol content.

Chemical analysis showed that $GL\text{-}P\text{-}P_{ext}$ contains 16.3% proteins and 26.04% phenols (Table 3). $GL\text{-}P\text{-}P_{ext}$ shows small DPPH activity, while AChE inhibitory activity was noticed with 13.62% inhibition. As a control inhibitor, galanthamine, which has an inhibitory activity of 88%, was used.

The extraction procedure described resulted in the production of $GL\text{-}P_{ext}$ from *G. lucidum* fruiting bodies. It is well recognized that phenolic compounds present in mushrooms may be complexed to soluble β-D-glucans by weak chemical linkages; therefore, the precipitation of proteins with TFA could result in the breakage of those links and the resulting high content of phenols in $GL\text{-}P_{ext}$, as observed in the present study.

Polysaccharide and protein content from *G. lucidum* fruiting body and primordium also elicited antioxidant and acetylcholinesterase inhibitory activities.

The results indicate that the fruiting body and primordium of *G. lucidum* are rich in higher molecular weight phenolic compounds with strong DPPH-free radical scavenging activity and moderate AChE inhibitory activities. *G. lucidum* primordia and fruiting bodies potentially present a novel source of natural inhibitors of acetylcholinesterase enzyme.

4. CONCLUSION

The aim of our study was to investigate the antioxidant and acetylcholinesterase inhibitory activities of *Ganoderma lucidum* fruiting bodies and primordia extracts. To the best of our knowledge, this is the first report evaluating the biological activity of compounds extracted solely from *G. lucidum* primordia.

Extracts taken from *G. lucidum* fruiting bodies and primordia using various extraction procedures were characterized. Two different procedures were used for polysaccharide extraction and the extraction of proteins.

The extracted components proved to be very effective. In general, we conclude that the components of *G. lucidum* fruiting bodies as well as primordia are more polar than non-polar. The total phenol content is higher in the case of primordia extracts. The results indicate that fruiting body and primordia of *G. lucidum* are rich in higher molecular weight phenolic compounds with strong DPPH free radical scavenging activity and moderate AChE inhibitory activities. *G. lucidum* primordia and fruiting body present a novel potential source of natural inhibitors of acetylcholinesterase enzyme.

There are no reports on AChE inhibitory activity of GL_{ext} or $GL\text{-}P_{ext}$ obtained using other solvents. The ability to inhibit AChE is crucial in the development of Alzheimer's disease (AD). Since antioxidants play an important role in the protection against aging processes and neurodegenerative diseases such as AD, the rate of inhibition of AChE was determined for GL_{ext} and $GL\text{-}P_{ext}$ extracts, which contain both polar and non-polar components with the capability of inhibiting AChE.

According to these results, both types of extracts present a potential source of compounds for slowing the aging and neurodegenerative processes.

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