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DOES THE SOURCE MATTER? PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY FROM MYCELIUM IN LIQUID MEDIUM, WILD AND CULTIVATED FRUITING BODIES OF THE NEOTROPICAL SPECIES *GANODERMA TUBERCULOSUM*

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ABSTRACT

As a continuation of the study of the biologically active compounds of native neotropical *Ganoderma* species in Paraguay, the content of total phenolic compounds, total antioxidants and percentage of antioxidant activity of the fractions extracted with ethanol, hexane, diethyl ether, ethyl acetate and water of mycelium in liquid medium, wild and cultivated fruiting bodies of *G. tuberculosum*, were analyzed. Biologically active compounds were highest in the diethyl ether fractions of wild fruiting bodies, 126.49 ± 4.19 mg. (GAE) g^{-1} (milligrams of Gallic acid equivalent per grams of crude extract), antioxidant compounds content of 130.94 ± 5.302 mg .g^{-1} AAE (milligrams of Ascorbic acid equivalent per grams of crude extract) and activity percentage of 70.93% compared to 67.39 ± 1.5 mg. (GAE) g^{-1}, 48.37 ± 0.73.g^{-1} AAE and 45.63% antioxidant activity. According to the values obtained in the present study, the fruiting body of *Ganoderma tuberculosum* is a promising source of antioxidant compounds for the food industry.

Keywords: antioxidant compounds, biological activity, DPPH radicals, Ganodermataceae, *Ganoderma tuberculosum*

INTRODUCTION

Species of the genus *Ganoderma* P. Karst., known as the 'mushrooms of immortality' are perhaps the most studied taxa in terms of their biological and chemical properties (**Ferreira *et al.*, 2015; Saltarelli *et al.*, 2015; Gong *et al.*, 2019; Zhang *et al.*, 2019**). They have long been recognized to promote health and longevity in Asian countries (**Boh *et al.*, 2007**). So far, more than 20 species of *Ganoderma* have been studied (**Gong *et al.*, 2019**), most of them from Asia and Europe. Molecular phylogenetic analyses have shown that these species differ from the neotropical ones (**Moncalvo & Buchanan, 2008; Costa-Rezende *et al.*, 2017; Costa-Rezende *et al.*, 2020**). Few studies describe the biological and chemical properties of native neotropical *Ganoderma* species, and preliminary studies have shown great potential and the need for research to continue (**Campi *et al.*, 2021**).
Basidiome, hereafter fruiting body, has been the traditional source of fungal metabolites and nutraceuticals, moreover, it has been shown that the production of those compounds depends on the stage of growth, *i.e.* mycelium, primordial stages and mature fruiting body (**Chen *et al.*, 2012; Li *et al.*, 2013**). For example, in *Ganoderma lucidum* (Curtis) P. Karst., the triterpenoid content was shown to be variable: extremely low in cultured mycelia, markedly higher in the fruiting body primordial formation, but with a decrease in the mature fruiting body (**Chen *et al.*, 2012**). These variations in concentrations could be related to the expression of the protein-coding genes as it has been shown that their expression in *G. lucidum* is higher in mycelia or primordial stages compared with those in the fruiting bodies (**Li *et al.*, 2013**). Regarding phenolic and antioxidant compounds, there is inconsistency on which stage of growth has more content of phenolic compounds and/or antioxidant activity. On one hand, a higher concentration in fruiting body than in mycelium was reported (**Reis *et al.*, 2012; Muna *et al.*, 2015; Prasad *et al.*, 2015; Sulistiany *et al.*, 2016**) on the other, it has been shown that mycelium presents a higher concentration of those compounds (**Carvajal *et al.*, 2012; Tešanović *et al.*, 2017**).

In addition to the stage of growth/development of the source, *i.e.* mycelium primordia or fully developed fruiting bodies, and the kind/nature of the compound under study, the extraction method of the compounds plays as the third variable to take into account. The study of the biologically active fungal compounds requires analysis and fine-tuning of the extraction and purification methods for the different types of compounds (**Chen *et al.*, 2007; Heleno *et al.*, 2012; Chen & Kang, 2019; Dong *et al.*, 2019**). Different extraction methods influence the results obtained, as well as the growth conditions of the fruiting body and mycelium (**Heleno *et al.*, 2012**).
Welti *et al.* (**2010**) have isolated ganoderic acid from the fruiting bodies from *Ganoderma tuberculosum* Murril. According to these researchers *G. tuberculosum* extracts might inhibit the growth of cancer cells. **Espinosa-García *et al.*** (**2021**) for their part, studied 14 extracts of mycelium from *Ganoderma* strains from Mexico and concluded that *G. tuberculosum* manifested activity in at least one of the six cancer cell lines tested (HBL-100 and T-47D [breast], HeLa [cervix], A-549 and SW[1]573 [lung], and WiDr [colon]), with a minimum concentration necessary to cause 50% growth inhibition of cancer cells (GI50) < 50 µg/mL−1.
The present study continues with the exploration of the potential of native *Ganoderma* species from Paraguay (**Campi *et al.*, 2021**) and the main objective was to evaluate the concentrations of phenolic compounds and antioxidants in different development stages and growth conditions, *i.e.* cultured mycelium, wild and cultivated fruiting bodies, of *G. tuberculosum*, considering different extraction protocols.

MATERIAL AND METHODS

Identification of species

The wild sample was collected in Central Department, Paraguay, growing on *Tipuana tipu* (Benth.) Kuntze and an herbarium reference specimen is kept at FACEN Herbarium N°4887. The species was identified by its macro and

micromorphological features and confirmed by molecular analyses. For microscopic analysis, free-hand sections of fruiting body were mounted in 3-5% (w/v) aqueous potassium hydroxide (KOH) and 1% (w/v) aqueous phloxine or Melzer's reagent following **Robledo *et al*. (2020)**. Basidiospores were measured in KOH and phloxine mounts under oil immersion at 100X magnification.

Sequence of the ITS marker was obtained from culture. The extraction, amplification and sequencing followed **Robledo *et al*.** (**2020**). The new DNA sequence generated in the present work was combined with sequences retrieved from GenBank (NCBI) to construct a dataset. Scientific names and GenBank Accession Numbers of sequences are listed in Table 1. Dataset was composed of 84 ITS sequences of 18 *Ganoderma* species (Table 1). *Foraminispora rugosa* (Berk.) Costa-Rezende, Drechsler-Santos & Robledo was selected as outgroup (**Costa-Rezende *et al*. 2017, 2020; Morera *et al*., 2021**). ITS region was aligned using ProbCons 1.12 (**Do *et al*., 2005**) and alignments were manually inspected and adjusted using MEGA 6 (**Tamura *et al*., 2013**). ModelFinder (**Kalyaanamoorthy *et al*., 2017**) as implemented in the IQ-Tree software (**Nguyen *et al*., 2015**) was used to estimate the best-fit partitioning strategy and the best-fit model of nucleotide evolution for the dataset using 3 data blocks (ITS1, 5.8S and ITS2). Models were restricted for those implemented in MrBayes 3.2 (**Ronquist *et al*., 2012**). Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses were applied to the concatenated datasets using the partition scheme and evolutionary models defined by ModelFinder. BI was performed following **Robledo *et al.*** (**2020**) in the CIPRES science gateway (**Miller *et al*., 2010**) (http://www.phylo.org/). Maximum likelihood searches were conducted with IQ-Tree, initially involving 100 ML searches, each one starting from one randomized stepwise addition parsimony tree. Branch supports were calculated using the UFBoot (ultrafast bootstrap approximation) (**Hoang *et al*., 2018**) implemented in IQ-TREE with 1000 replications. A node was considered strongly supported with BPP ≥ 0.95 or BS ≥95% (**Hyde *et al*., 2013; Minh *et al*., 2020**).

Table 1 *Ganoderma* species: specimens: origin and ITS Gen Bank accession numbers for sequences used in the phylogenetic analyses

Species **Voucher specimen/culture**	**Origin**	**GenBank Accession number**
Foraminispora rugosa **(Berk.) Costa-Rezende: Drechsler-Santos & Robledo (O)**		
HUEFS DHCR560	Brazil	MF409963
FLOR 52191	Brazil	KU315200
Ganoderma australe **(Fr.) Pat. Clade 1**		
BAFC2411	Argentina	AF169989 / 90*
BAFC2764	Argentina	AF169993 / 4*
BAFC2449	Argentina	AF255187
MVHC 5564	Uruguay	MN191568
MVHC 5620	Uruguay	MN191569
MVHC 5582	Uruguay	MN191570
NC 7177	Uruguay	MN191571
MVHC 5717	Uruguay	MN191572
MVHC 5746	Uruguay	MN191573
Ganoderma australe **Clade 2**		
BAFC2424	Argentina	AF169977 / 8*
BAFC2531	Chile	AF255176
MVHC 5647	Uruguay	MN191549
MVHC 5645	Uruguay	MN191560
MVHC 5587	Uruguay	MN191548
MVHC 5568	Uruguay	MN191559
Ganoderma concinnum **Ryvarden**		
Robledo 3192 (FCOS)	Bolivia	MN077522
Robledo 3235 (FCOS)	Bolivia	MN077523
Ganoderma curtisii **(Berk.) Murrill**		
UMNFL60	USA	MG654105
UMNFL28	USA	MG654097
223FL	USA	MG654167
Ganoderma dorsale **(Lloyd) Torrend**		
MVHC 5588	Uruguay	MN191582
MVHC 5701	Uruguay	MN191581
MVHC 5653	Uruguay	MN191578
MVHC 5648	Uruguay	MN191579
MVHC 5654	Uruguay	MN191580
Ganoderma ecuadoriense **A. Salazar: C.W. Barnes & Ordoñez**		
ASL799	Ecuador	KU128524
PMC126	Ecuador	KU128525
Poly-2-4	Ecuador	KU128526
Ganoderma flexipes **Pat.**		
Wei5491	China	JQ781850
Wei5494	China	JN383979
Ganoderma lucidum **(Curtis) P. Karst.**		
UMNUT1	USA	MG654070
K 175217	England	KJ143911
RYV 33217 T	Norway	Z37096 / Z37073*
CBS 270.81	France	Z37049 / Z37099*
Dai 2272	Sweden	JQ781851
Dai11593	Finland	JQ781852

ATCC 52410	Argentina	X78739 / X78760*
ATCC 52409	Argentina	Z37058 / Z37083*
Ganoderma martinicense **Welti & Courtec.**		
MVHC 5635	Uruguay	MN191574
MVHC 5583	Uruguay	MN191575
MVHC 5684	Uruguay	MN191576
246TX	USA	MG654185
Mart08_55 (**T**)	Martinique	KF963256
Ganoderma mexicanum **Pat.**		
MVHC 5652	Uruguay	MN191583
MUCL 49453 SW17	Martinique	MK531811
MUCL 57308/ BRFM1548	Martinique	MK531818
ATCC 52419	Argentina	X78736 / X78736*
ATCC 52420	Argentina	JQ520205
UMNFL 100	USA	MG654373
XAL D.Jarvio 143	Mexico	MK531823
Ganoderma multipileum **Ding Hou**		
BCRC 37043	China	EU021460
DAI 9447	China	KJ143914
Ganoderma multiplicatum **(Mont.) Pat.**		
SPC9	Brazil	KU569553
CC8	Brazil	KU569515
URM 83346	Brazil	JX310823
Ganoderma platense **Speg.**		
MVHC 5586	Uruguay	MN191585
MVHC 5565	Uruguay	MN191584
BAFC384	Argentina	AH008109
NC 5332	Uruguay	MN191587
NC 7187	Uruguay	MN191588
NC 5104	Uruguay	MN191589
Ganoderma polychromum **(Copel.) Murrill**		
330OR	USA	MG654196
BJ280CA	USA	MG910492
Ganoderma resinaceum **Boud.**		
DP2	Italy	AM906060
CIRM BRFM 753	France	FJ805250
HMAS86599	England	AY884177
BR 4150	France	KJ143915
BCRC 36147	Netherlands	KJ143916
Ganoderma sessile **Murrill**		
111TX	USA	MG654306
103SC	USA	MG654304
113FL	USA	MG654307
228DC	USA	MG654319
117TX	USA	MG654309
Ganoderma tuberculosum **Murrill**		
PLM684	USA	MG654369
UMNFL160	USA	MG654364
LIP SW-Mart08-45		KF963258
FACEN 4887/MC515	**Paraguay**	**OQ092404**
Ganoderma zonatum **Murrill**		
FL-02	USA	KJ143921
179NC	USA	MG654417
UMNFL105	USA	MG654408
UMNSC4	USA	MG654415
123FL	USA	MG654416

Legend: O = outrgoup. **T**= type specimen. ***** = Accession numbers for ITS1 and ITS2. New sequence is highlighted in boldface.

Preparation of samples

From the context of a fresh basidiome, hereafter fruiting body, mycelium (M) was isolated on Potato Dextrose Agar (PDA). The inoculum consisted of a 25-mm^2 surface agar plug from a 10-day-old culture that was transferred to flasks (500 mL) with 200 ml of a liquid medium. The growing medium consisted of (g.L^{-1}): sucrose 35, peptone 5, yeast extract 2.5, KH_2PO_4 x H_2O 1, $MgSO_4 \cdot 7H_2O$ 0.5, and vitamin B1 0.05 (**Tang & Zhong, 2002**). The inoculated flasks were then incubated at 28 °C in an incubator resting for 30 days, the mycelium was filtered through Whatman No. 4 paper.

Cultivation substrates

Spawns were prepared from the PDA isolated strain into heat resistant polypropylene bags with oatmeal (*Avena sativa* L.) that were previously sterilized for 1 hour at 121°C, then inoculated. The mycelium was allowed to grow for two weeks in the dark at 28°C. The formulation for the bag cultivation was sawdust of *Eucalyptus* sp. and *Handroanthus* sp. in a 1:1 ratio. This mixture was hydrated to reach a moisture content of approximately 50% of its total weight, 2% $Ca(OH)_2$ was added to maintain pH. Three kilograms of the mixed substrate were filled into heat-resistant polypropylene bags and sterilized at 121°C for 2 hours. The substrate was inoculated with the spawns and incubated at 28°C for 30 days until the fruiting body were obtained (Figure 3).

Preparation and characterization of phenolic extracts

Solvent extractions with a polarity gradient were carried out following **Heleno *et al.* (2012)** and **Campi *et al.* (2023)** with modifications. Thirty grams of each, the wild fruiting body and the indoor culture were selected for the extraction. The aqueous remnant was submitted to liquid-liquid extraction with n-hexane (3×30 mL), diethyl ether (3×30 mL) and ethyl acetate (3×30 mL). The resulting fractions were re-dissolved in methanol for the antioxidant activity assays after evaporation of the remaining solvents at 40°C. Regarding the mycelium, 4.761 g were macerated with (3×150 mL) of methanol:water (80:20, v/v) and the same process as the fruiting body was performed.
The concentration of total phenolic compounds was measured by the methods described by **Turkoglu *et al.* (2007)** with some modifications by **Campi *et al.* (2023)**. The results were expressed as the mean of the triplicates of the absorbance in milligrams of Gallic acid equivalent per grams of crude extract (mgg^{-1}) ± standard deviation (SD).

Concentration and antioxidant activity

The concentration and oxidant activity were determined with the DPPH• radical (2,2-diphenyl-2-picrylhydrazyl hydrate) absorbance method according to **Campi *et al.* (2021)**. The results were expressed as the mean of the analysis performed in triplicate in milligrams of ascorbic acid equivalent per g of crude extract (mgg^{-1}) ± standard deviation (SD). The percentage of activity (A) was calculated as:

$$A = \frac{\lambda_{DPPH} - \lambda_{Solution}}{\lambda_{DPPH}} \times 100$$

where λ_{DPPH} is the absorbance of DPPH, $\lambda_{Solution}$ is the absorbance of the solution.

Statistical analyses

The data obtained in the trials were analyzed using the two-way ANOVA test (95% confidence interval; with previous verification of assumptions), together with the Tukey post-hoc test (95% confidence interval). One-way ANOVA analysis and Tukey's test were also used to compare the variables evaluated (*i.e.* source, extraction method, polarity of solvent) between the different fractions studied. These analyses and statistical graphs were performed using the statistical programs InfoStat v. 2019e (**Di Rienzo *et al*., 2019**) and Past v. 4.06b (**Hammer *et al*., 2001**).

RESULTS AND DISCUSSION

Morphological and molecular analyses

Main macro and microscopical characters observed in the species under study agree with the reference descriptions (**Welti *et al*., 2010**). Macroscopically *G. tuberculosum* is characterized by large, demediate to commonly substipitate to stipitate basidiomata; pilear surface concentrically sulcate and zonate, dark reddish at the base, yellowish to white at the margin, a homogeneous context with melanoid deposits and 4–5 rounded pores per mm. Microscopically by the ovoid to ellipsoid basidiospores, 9–12 × 5.5–7.8 µm and the tuberculated cutis cells with a weak amyloid reaction (**Loyd *et al*., 2018**). The morphological identification is confirmed with molecular analyses.
Dataset included 84 terminals and 600 characters, of which 126 were parsimony informative, 132 variable and 466 constants. The partitions and evolutionary models selected were: HKY+F+G4 (ITS1 and ITS2) and K2P (5.8S). Bayesian and ML analyses resulted in similar topologies; the ML tree is presented in the Figure 1. The topology recovered is congruent with previous works (**Loyd *et al*., 2018; Morera *et al*., 2021**). The specimen under study groups with *G. tuberculosum* conforming a lineage recovered with maximum support (1/100).

Chemical profile

Our results showed that, in general, there are significant differences in phenolic compounds (TPC), antioxidant concentration and antioxidant activity between the development stages and growth conditions, as well as the extraction technique used (Figure 2A-C, Table 2). The highest concentrations of phenolic compounds were obtained with diethyl ether and ethyl acetate, being significantly higher in wild fruiting bodies than cultivated and mycelium (Figure 2A, Table 2).
The fruiting bodies have a higher concentration of TPC than the cultured mycelia. The difference between the concentrations of TPC in the fruiting bodies and mycelium, have been reported several studies (**Tan *et al*., 2015; Muna *et al*., 2015; Prasad *et al*., 2015; Sulistiany *et al*., 2016**). However, the reverse situation has also been reported, higher concentrations of TPC in the mycelium than in the fruiting body; *v.g. Agaricus blazei* Murrill, *Coprinus comatus* (O.F. Müll.) Pers. and *Coprinellus truncorum* (Scop.) Redhead, Vilgalys & Moncalvo species (**Carvajal *et al*., 2012; Tešanović *et al*., 2017**).

Heleno *et al*. (2012) reported significant differences in TPC of *G. lucidum* between the fruiting body (FB) and the submerged mycelium (M) (28.64±0.28 mg GAE g^{-1} and 5.95±0.09 mg GAE g^{-1}, respectively). **Prasad *et al.* (2015)** reported values of 21.60 ± 2.60 mg GAE g^{-1} (FB) and 10.63 ± 1.00 mg GAE g^{-1} (M) for *G. applanatum* (Pers.) Pat., while for *G. lucidum* values of 16.78 ± 1.46 (FB) and 10.63 ± 1.00 mg GAE g^{-1} (M) were found. On the other hand, **Tan *et al*. (2015)** reported values of 101.01 ± 0.59 (FB) and 20.55 ± 0.53 (M) mg GAE g^{-1} for *G. neojaponicum* Imazeki. These results show similarities to those obtained in our work (Figure 2A-C) where it is seen that in all organic fractions the concentration of phenolic and antioxidant compounds in the fruiting body is higher than in the mycelium. This shows that in the genus *Ganoderma*, phenolic compounds are mainly accumulated in the fruiting body.

Table 2 Phenolic compounds, antioxidant concentration and antioxidant activity values for mycelium, wild and cultivated fruiting bodies of *G. tuberculosum*.

	Phase	Phenolic compounds ($mg\ GAEg^{-1}$)	Antioxidants (mgg^{-1} AAE)	Antioxidant Activity (%)
Wild fruiting bodies	Ethanolic extract	42.96± 1.73	23.14 ± 1.54	18.15
	Hexane	36.69 ± 2.96	48.10 ± 2.41	23.41
	Diethyl ether	126.49 ±4.19	273.13 ± 0.96	70.93
	Ethyl acetate	114.06 ± 4.94	145.00 ± 0.67	53.55
	Aqueous residue	14.07 ± 0.65	13.02 ± 2.17	5.88
Cultivated fruiting bodies	Ethanolic extract	41.24 ±2.34	41.79 ± 0.67	20.44
	Hexane	15.47 ± 0.49	7.77 ± 0.50	6.96
	Diethyl ether	67.39 ± 1.5	48.37 ± 0.73	45.63
	Ethyl acetate	61.50 ± 1.21	38.04 ± 1.33	35.79
	Aqueous residue	13.40 ± 0.62	6.64 ± 0.41	5.88
Mycelia	Ethanolic extract	12.28 ± 0.62	4.95 ± 0.24	4.27
	Hexane	8.62 ± 0.34	3.49 ± 0.05	16.96
	Diethyl ether	20.33 ± 0.34	10.06 ± 0.37	9.03
	Ethyl acetate	70.56 ± 1.51	17.06 ± 0.73	15.80
	Aqueous residue	20.78 ± 0.88	5.84 ±0.50	5.11

Espinosa-García *et al*. (2021) analyzed the mycelial extracts from *G. tuberculosum* obtained with chloroform–methanol (1:1) and they reported promising antioxidant activity, with values of 62.5 ± 3.9 µM TE/mg [micromolar Trolox equivalents (TE) per milligram of extract]. Regarding phenolic compounds, they reported 26.9 ± 1.7 GAE/mg, for mycelia our results show values of 70.56 ± 1.51 GAE/mg for the ethyl acetate fraction from mycelium, this higher concentration could be due to the polarity of the solvent used.
Analyzing the fractions in relation to the concentration of antioxidant compounds and antioxidant capacity, they showed a similar pattern than TPC, the highest concentrations were found in the fruiting body (Figure 2). Regarding the origin, in general, the wild fruiting body had higher concentration of TPC, antioxidant

concentration and antioxidant activity, in accordance with previously observed patterns (**Grangeia *et al*., 2011; Tan *et al*., 2015**).

Another factor influencing the secondary metabolites levels was the extraction solvent, as its polarity has a direct influence on the phenolic content of the resulting extracts (**Tan *et al*., 2015**). A significant difference was found between crude extracts and solvent washes with increasing polarity. Diethyl ether and Ethyl acetate fractions were the ones with the highest concentration in TPC and antioxidant concentration, so a previous washing with non-polar solvents (*e.g.*, petroleum ether, hexane) is recommended to clean the extract lipid components as much as possible.

Figure 1 Phylogenetic tree based on ITS rDNA sequences generated by maximum likelihood showing the position and phylogenetic relationships of *G. tuberculosum* within the genus *Ganoderma*. Posterior probabilities (>0.95) and bootstrap values (>95%) are shown at the branches. Clade names follow **Loyd *et al*. (2018)** and **Morera *et al*. (2021)**. Specimen studied is highlighted in boldface.

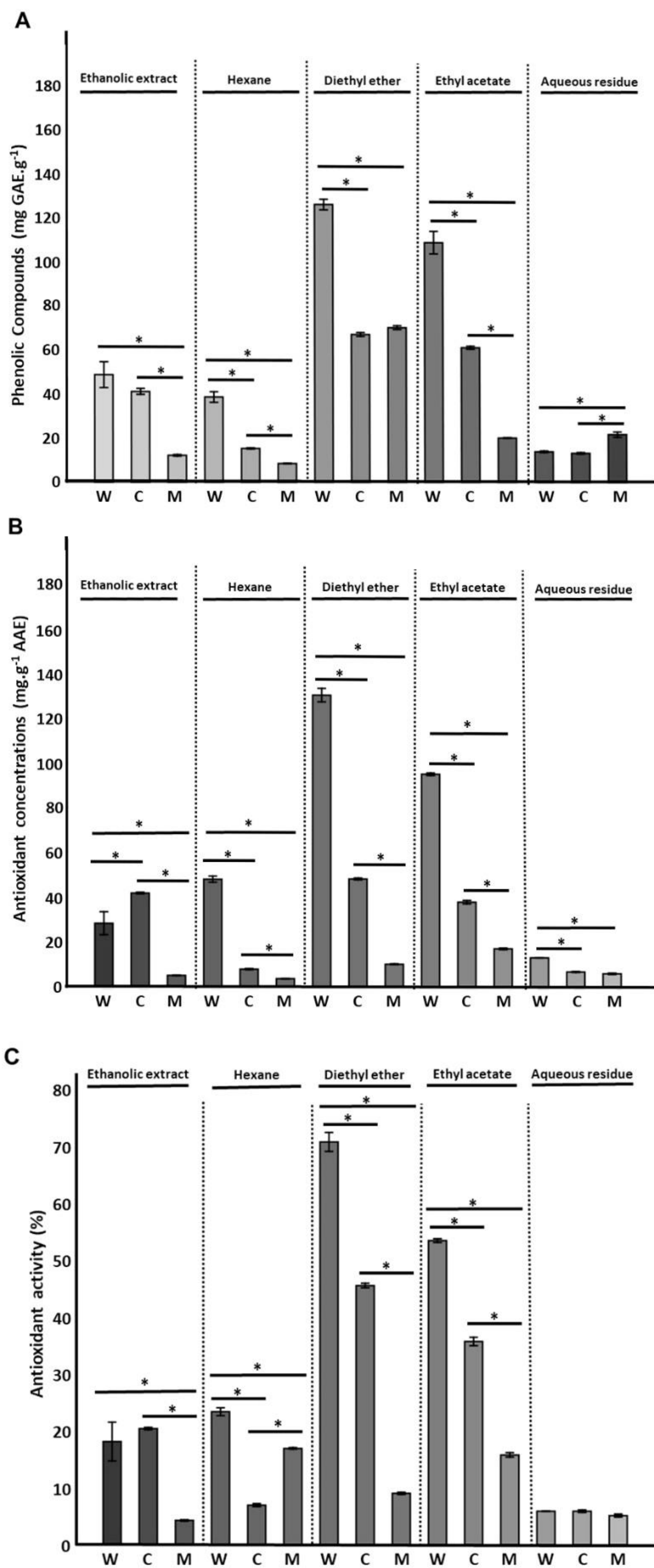

Figure 2 Comparison of source (W: wild, C: cultivated, M: mycelia) and extraction solvent (ethanol, hexane, diethyl ether, ethyl acetate) of: A: Total phenolic compounds, B: Antioxidant concentration, C: Antioxidant activity.

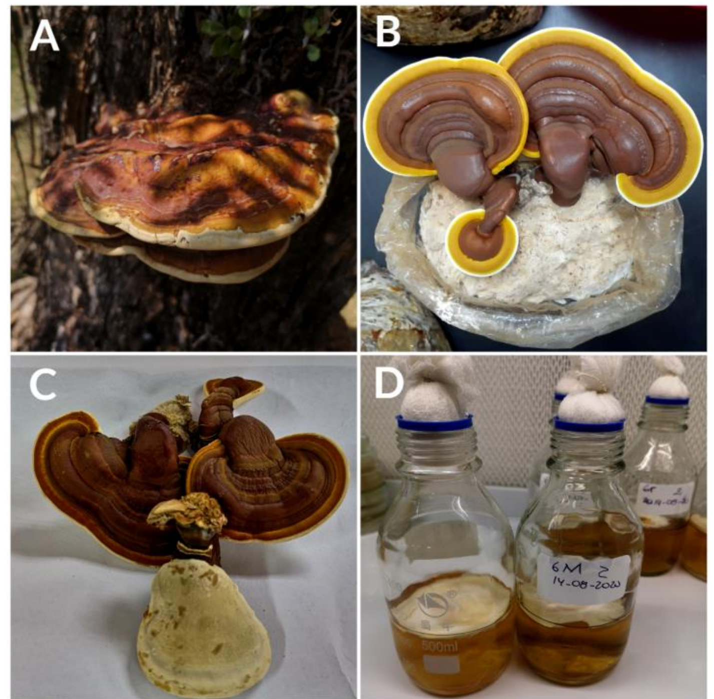

Figure 3 *Ganoderma tuberculosum***:** A wild fruiting body, B-C: fresh and dried fruiting bodies cultivated indoor, D: Mycelium in liquid medium.

CONCLUSION

In conclusion, wild fruiting bodies showed significantly higher concentrations of phenolic compounds than cultivated fruiting bodies and mycelium. Several factors can influence the differences in bioactive compound concentrations: the extraction procedure, the choice of solvents, the culture media for growing mushrooms and the liquid culture of the mycelium. It should also be noted that secondary metabolites are produced in response to environmental stress. Regarding the extraction solvents, it is concluded that the liquid-liquid extraction technique with polarity gradient favors the correct separation of the metabolites to be studied.

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