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Total Polyphenols and Antioxidant Activity of *Ganoderma Curtisii* extracts

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Abstract
Background: Fungi of the *Ganoderma* genus contain bioactive components such as terpenoids, polysaccharides, steroids, phenolic compounds and glycoproteins. Polysaccharides, triterpenes and phenolic compounds have antioxidant properties.
Objective: To determine the antioxidant activity of the hydro alcoholic and the ethanolic extracts, and polysaccharides content of *Ganoderma curtisii* collected in Michoacan, Mexico.
Methods: Hydroalcoholic and ethanolic extracts were obtained by direct heating at 78 °C and 80 °C respectively. Polysaccharides were extracted with water at 85 °C and then ethanol precipitation. The percentage inhibition of the ethanolic extract, hydroalcoholic extract and polysaccharides was evaluated by the DPPH method using ascorbic acid as a reference standard.
Total polyphenol content was determined by the Folin-Ciocalteu method using gallic acid as standard.
Results: Scavenging effects from *Ganoderma* extracts on DPPH radical increased with the concentrations of the extract. The maximum inhibition percentage for ethanol extracts, hydroalcoholic extracts and polysaccharides at a concentration of 1mg/ml, was 90.5%, 89.10% and 83.09%, respectively, while that of ascorbic acid was 96.4%.
Phenolic compounds present in the hydroalcoholic extracts (35.6313 ± 0.1868 mg GAE/g) and ethanol (49.1467±0.1692 mg GAE/g) also contribute to increased antioxidant activity observed in these extracts.
Conclusions: These results suggest that analyzed fungi are of potential interest as sources of strong natural antioxidants that could be used in the food nutrition and industries.

Keywords: Antioxidants, phenolic compounds, hydroalcoholic extract, ethanolic extract, polysaccharides.

Introduction
For its medicinal and nutritional properties, the fungus *Ganoderma* (Lingzhi, Reishi or Mannentake) has been used in traditional Chinese herbal medicine since nearly 2,000 years ago and their use has been extended to worldwide [29]. It is a basidiomycete fungus, presenting a varied polychrome in shades of yellow, ochre, orange, red and even black; outer surface bright and woody texture. It grows in warm latitudes, mainly in dense forests with high humidity and low light intensity, in dead trees: pines and oaks among others [34]. In recent years, the use of fungi of the genus *Ganoderma* has become increasingly important in the human diet for its nutritional and pharmacological characteristics. Its nutritional value and medicinal components have been studied [3], however, little information is available about the antioxidant properties of fungi of the genus *Ganoderma* in Mexico.
Some pharmacological properties of *Ganoderma* have been associated with its ability to reduce the risk of heart disease, cancer and stimulate the immune system [24]. Beneficial properties for the health of *Ganoderma* species are attributed to the bioactive components such as polysaccharides, triterpenes, sterols, lectins and some protein [8]. There are some reports from China, Japan, Poland, India and Portugal, in relation to the antioxidant properties of *G. lucidum* methanol extracts [18] and aqueous extracts [15], as well as their polysaccharides [13] and phenolic compounds [12]. Oxidative DNA damage is able to trigger carcinogenesis [2]. It has been suggested that the antitumor and immunomodulatory activities of *Ganoderma* extracts, are largely related to its antioxidant properties [24].
There are qualitative and quantitative differences in the chemical composition of the fungus depending on the species or strain, the culture conditions and the production method employed for processing [21, 30].

Several extraction methods have been developed in order to obtain extracts with higher yields and lower costs. Such is the case for extraction with organic solvents such as ethanol, chloroform, hexane and acetone [17]. In general, most polysaccharides are extracted with water and alcohol precipitation [35].

The aim of this study is to evaluate the content of phenolic compounds in hydroalcoholic and ethanolic extracts and the antioxidant properties of the hydroalcoholic and ethanolic extracts and polysaccharides obtained from aqueous extraction of mature fruiting bodies of wild strain of the fungus *Ganoderma curtisii*, collected in the locality of La Escalera, at the municipality of Charo in the Michoacán state of México.

Materials and methods

Ganoderma fungus mature fruiting bodies were provided by the company Kamuhro, collected at the community of La Escalera Municipality of Charo, Michoacán. Samples were authenticated by experts of the Mushroom Herbarium of the UMSNH (EBUM) as *Ganoderma curtisii*, a species showing high morphological plasticity that varies depending on the maturity of basidiocarp. This species, however, has two distinctive features such as duplex and resinous layers that do not change with age of the specimen [28] (Figures 1 and 2). Voucher specimens were deposited in EBUM with number 24032.

Fig 1: *Ganoderma curtisii*

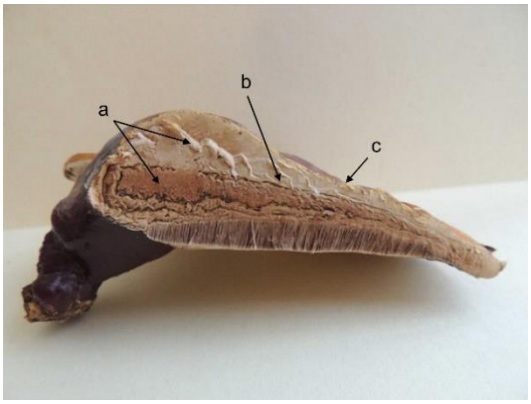

Fig 2: a) Duplex layers, b) Resinous layers y c) Yellow edge.

Ganoderma curtisii Extracts.

Two mushrooms (20 cm of long side) were brush-cleaned, air-dried at 40 °C to constant mass, grounded using hammer mill and were passed through 20 mesh. Three extracts were performed using 5 g of fungus for each one in the following solvents: 1. Ethanol (200 ml) at 78 °C for 120 minutes; 2. Hydroalcoholic (Water: ethanol 120:80 ml) at 80 °C for 120 minutes; and 3. Water (200 ml) at 85 °C for 5 hours. The equipment used for the extractions was a reflux system with direct heating of the fungus with the solvent. Ethanolic and hydroalcoholic extracts were vacuum evaporated to obtain the dry extract powder.

Preparation of polysaccharides from aqueous extracts of *Ganoderma curtisii*

Polysaccharides were isolated from the aqueous extraction by the method proposed by Skalická with some modifications [26]; 5 g of fungal material was extracted three times with 200 ml of hot water (85 °C) for 5 h. Water extracts were filtered, combined and concentrated to 100 ml. Then 300 ml of chilled ethanol was added and left in a cold place (4 °C) for 24 h. The precipitate was collected after centrifugation (3,500 rpm, 15 min), washed with ethanol and dried at 60 °C. Crude polysaccharides were obtained.

Ganoderma curtisii Extracts Antioxidant Activity evaluation.

The ability of *Ganoderma curtisii* extracts to capture free radicals was determined using the reference solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the method reported by Chung-wah Ma *et al*. [5]. For this test, 2 ml of 0.2 mM DPPH ethanol solution was added to 2 ml of each mushroom extract as previously described (ethanol, hydroalcoholic and polysaccharides) at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg / ml).

Reaction mixture was vortexed for 15 seconds and absorbance was measured at 517 nm using a spectrophotometer (Espectro Genesys 10 S UV Vis Thermo Scientific) with methanol as blank. The decrease in absorbance was monitored at 30 min. Ascorbic acid was used as a positive standard at the same concentrations as samples. The experiments were carried out in triplicate.

The DPPH free radical scavenging activity, expressed as percentage of radical scavenging activity, was calculated as follows:

$$Radical\ scavenging\ activity\ (\%) = \left(\frac{A_0 - A_s}{A_0}\right) * 100$$

Where A_0 is the absorbance of 0.2 mM ethanolic DPPH and A_s is the absorbance of the reaction mixture.

The effectiveness of antioxidant capacity was expressed as EC_{50} (mg/ml) value that represented the effective concentration of mushroom extract required to show 50% antioxidant property. Lower EC_{50} value corresponded to higher antioxidant activity of the mushroom extract.

EC_{50} values lower than concentrations of 0.1 mg/ml cannot be calculated from the graph and were expressed as EC_{50} <0.1 mg/ml.

Determination of total polyphenols in the hydroalcoholic and ethanol extracts

For the determination of total polyphenols of *Ganoderma curtisii* extracts, the spectrophotometric method by Nagaraj *et al* [22] with some modifications was followed. Ethanolic and hydroalcoholic extracts were obtained from 5 g of *G. curtisii* in a mixture of 200 ml of water/ethanol (3:2) and ethanol (200 ml) at a concentration of 524.5 mg/100 ml and 344.1 mg/100 ml respectively. Extract samples (0.5 ml) were mixed with Folin-Ciocalteu reagent (2.5 ml, previously diluted in water 1:10 v/v) and sodium carbonate (75 g/l,2 ml). The tubes were mixed by vortexing for 15 s and were left in the dark for 40 min at room temperature (21 °C). Absorbance at 765 nm was

measured. Standard calibration curve of gallic acid in water (0.0094 to 0.15 mg/ml) was performed (Figure 3). For each of the samples gallic acid concentration was calculated using the equation based on the calibration curve: Y = 0.0974x - 0.0047, R^2 = 0.9995; where x was the absorbance and Y was the gallic acid amounth in mg. The content of total phenolics, as gallic acid equivalent (GAE), was calculated using the following equation: T = (C x V) / M, where T is the total phenolic content of the extracts expressed as GAE in mg/g, C is the concentration of gallic acid from the established calibration curve in mg/ml, V is the volume of the extract solution in ml and M is the weight in grams of the extract.

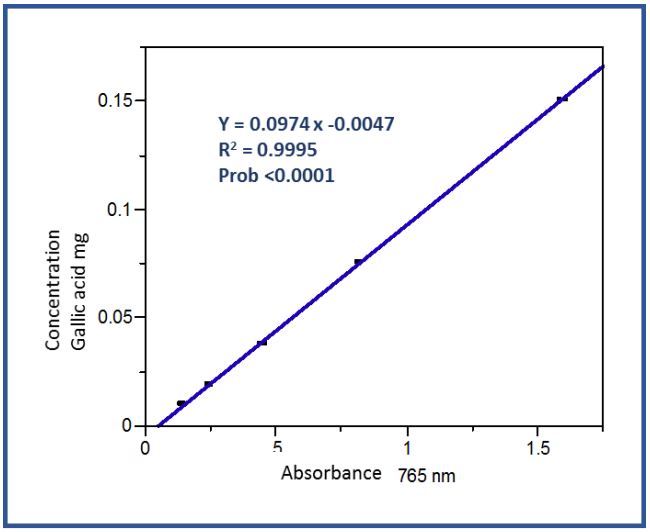

Fig 3: Calibration Curve of Gallic acid mg Statistical Analysis

Data are reported as mean ± standard deviation (SD). All analyzes were performed in triplicate twice. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test α = 0.05. Significant differences between groups were determined at p <0.0001. JMP statistical software version 6 was used.

Results

Antioxidant activity for each type of extract expressed as inhibition percentage is presented in figure 4. Results indicate that the extracts were able to capture DPPH radicals and that such capacity is dependent on the concentration. The hydroalcoholic and ethanolic extracts did not show any statistical differences in the values of percentage inhibition at any concentration.

At concentration of 0.4 mg/ml there are no significant differences in the percentages of inhibition of the hydroalcoholic and ethanol extracts, and polysaccharides, being 74.05% for the ethanol extract, 73.64% for the hydroalcoholic extract and 73.62% for polysaccharides. At concentrations of 0.1, 0.2, 0.6, 0.8 and 1 mg/ml there were significant differences in inhibition percentage values of polysaccharides with respect to the hydroalcoholic and ethanolic extracts of *G. curtisii*.

Highest inhibition percentage obtained for ethanol extracts, hydroalcoholic extracts and polysaccharides at a concentration of 1 mg/ml, was 90.59%, 89.10% and 83.09% respectively, while for ascorbic acid was 96.4%.

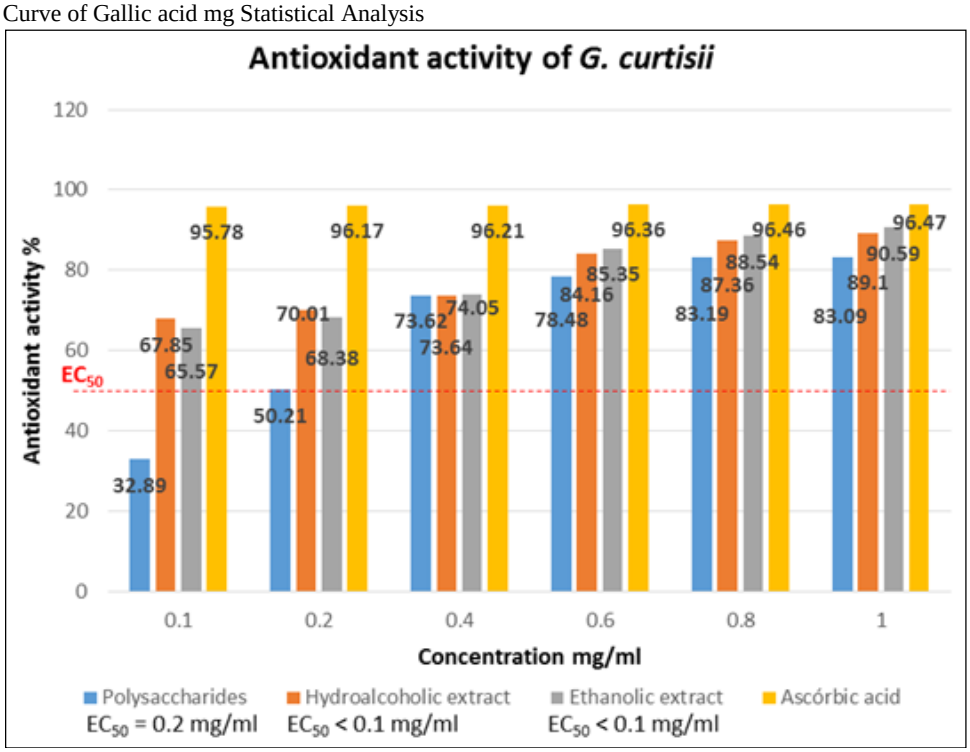

Fig 4: Antioxidant activity of G. curtisii extracts and EC_{50} values

The hydroalcoholic extracts, ethanolic extracts and *G. curtisii* polysaccharides have been shown to reduce the radical DPPH. Percentage inhibition of *Ganoderma curtisii* extracts increases in direct proportion to the concentrations of these. Values obtained agree with those reported by other authors for species of *Ganoderma*. Kozarski *et al.* [13] report values of 77.5-81.9% inhibition for the fungus *G. applanatum* to concentrations ranging from 0.1 to 1 mg/ml. Shi Min *et al.* [19] report inhibition of 88.38% for *G. lucidum* polysaccharides at a concentration of 1.25 mg/ml.

EC_{50} values of antioxidant properties

EC_{50} values for *G. curtisii* hydroalcoholic and ethanolic extracts were less than 0.1 mg/ml, while for the polysaccharides was 0.2 mg/ml. These values indicate that the hydroalcoholic and ethanolic extracts inhibit 50% of the DPPH

radical at concentrations lower than 0.1 mg/ml, while concentrations of 0.2 mg/ml of polysaccharides are required to scavenge DPPH radicals. These results agree with those reported by Kozarski M *et al* [14] and Saltarelli R. *et al* [25] for *G. lucidum* polysaccharides. The comparative values of inhibition percentage and EC_{50} values for *Ganoderma curtisii* extracts evaluated in this study and the results reported by other authors for different species of *Ganoderma* are shown in Table 1, where similar values were observed for *G. curtisii* obtained in the same concentration range.

Table 1: Comparative values of inhibition percentage and EC_{50} values for extracts of *Ganoderma.*

Sample	Concentration (mg/ml)	DPPH Inhibition (%)	EC_{50} Value (mg/ml)	Reference
Polysaccharides *G. curtisii*	0.1-1	32.89-83.09	0.2	The present study
Ethanolic extract from *G. curtisii*	0.1-1	65.57-90.59	<0.1	The present study
Hydroalcoholic extract of *G. curtisii*	0.1-1	67.85-89.10	<0.1	The present study
Polysaccharides from *G. lucidum*	1.5	47.3-63.7	Not reported	Chung-wah *et al*, 2013
Polysaccharides from *G. applanatum*	0.1-1	77.5-81.9	<0.1	Kozarski *et al*, 2012
Polysaccharides from *G. lucidum*	2.5	94.8	0.1	Kozarski *et al*, 2012
Polysaccharides from *G. lucidum*	--	--	0.36	Xingyi Zhu *et al*, 2012
Polysaccharides from *G. lucidum*	0.64	67.6-74.4	Not reported	Jeng-Leun Mau *et al*, 2002
Methanolic extract from *G. tsugae*	0.5	45	Not reported	Mau *et al*, (2002)
Polysaccharides from *G. lucidum* (Italia)	0.5	60	0.23±0.05	Saltarelli R. *et al*, 2009
Polysaccharides from *G. lucidum* (China)	1.2	65	0.69±0.02	Saltarelli R. *et al*, 2009
Aqueous extract from *G. Tsugae*	--	--	0.4	Noorlidah A. *et al*, 2012
Polysaccharides from *G. lucidum*	--	--	2	Ti-Qiang Chen *et al*, 2014
Polysaccharides from *G. lucidum*	1.25	91.48	0.23 mg/ml	Min Shi *et al*, 2014
Polysaccharides from *G. lucidum*	1.25	88.38	Not reported	Min Shi *et al*, 2013
Polysaccharides from *G. lucidum*	1.5	63.7	Not reported	Liuping Fan *et al*, 2012
Polysaccharides from *G. tsugae*	5-20	58.4-93.7	Not reported	Yu-Hsiu Tseng *et al*, 2008

Total Phenolic Compounds

Content of phenolic compounds in extracts of *Ganoderma curtisii* was 49.1467±0.1692 mg GAE/g for the ethanol extract and 35.6313±0.1868 mg GAE/g for the hydroalcoholic extract. These results agree with those reported by other authors for GAE values in *Ganoderma*. Table 2 shows the results of GAE for extracts of *Ganoderma curtisii* and the results reported by other authors for different species of *Ganoderma*. Heleno *et al*. [9] reports a value of 47 mg GAE/g for the hydroalcoholic extract of *G. lucidum* and Cilerdzic *et al*. [6] report values from 33.42 to 52.15 mg GAE/g of *G. lucidum* ethanolic extracts.

Table 2: Comparative GAE values for *Ganoderma* extracts

Sample	Phenolic compounds mg GAE/g	Reference
Ethanolic extract from *G. Curtisii*	49.15±0.17	The present study
Hydroalcoholic extract from *G. Curtisii*	35.63±0.19	The present study
Methanolic extract from *G. applanatum*	71.00	Nagaraj *et al*, 2014
Aqueous extract from *G. applanatum*	47.00	Kozarski *et al*, 2012
Aqueous extract from *G. lucidum*	33.00	Kozarski *et al*, 2012
Hydroalcoholic extract from *G. lucidum*	55.53	Heleno *et al*, 2012
Ethanolic extract from *G. lucidum*	71.43	Imtiyaz *et al*, 2014
Ethanolic extract from *G. lucidum*	52.15	Cilerdzic *et al*, 2014
Ethanolic extract from *G. lucidum*	33.42	Cilerdzic *et al*, 2014
Aqueous extract from *G. lucidum*	43.14	Abul *et al*, 2013
Aqueous extract from *G. lucidum*	63.51	Noorlidah *et al*, 2012

Table 3 shows the correlations for the inhibition percentage of the hydroalcoholic and ethanolic extracts from *G. curtisii* and the amount of phenolics as mg GAE/g. A directly proportional relationship of percent inhibition with the amount of phenolics as mg GAE/g is observed. Several studies have confirmed the relationship between phenolic content and antioxidant capacity [4, 7]. Increased antioxidant capacity of the hydroalcoholic and ethanolic extracts can be attributed to its content of phenolic compounds.

Table 3: Correlation between % DPPH inhibition and phenolic content mg GAE/g in *G. curtisii* extracts

Phenolic compounds	Linear equation	R^2
In the Hydroalcoholic extract mg GAE/g	Y = 0.7197x + 63.6243	0.9600
In the Ethanolic extract mg GAE/g	Y = 0.5811x + 62.8362	0.9605

Discussion

Evaluated *Ganoderma curtisii* extracts showed good inhibition percentages of DPPH radical, 89.10% for the hydroalcoholic extract and 90.59% for the ethanolic extract at concentrations of 1 mg/ml. Inhibition percentages for the hydroalcoholic and ethanolic extracts were observed in the same concentration range, without significant differences. Polysaccharides obtained from the aqueous extract also displayed inhibition percentages over 50.21% at concentrations of 0.2 mg/ml and up to 83.19% for concentrations 0.8 mg/ml. This antioxidant capacity is given by the hydroxyl group of the monosaccharide units that can donate hydrogen to reduce DPPH radicals [32].

The scavenging activity of mushroom extracts towards DPPH free radicals was expressed in terms of IC_{50}. Since a lower IC_{50} value indicates stronger ability of the extracts to act as DPPH radical scavengers, therefore low IC_{50} values obtained in this study indicates good antioxidant activity of *Ganoderma curtisii* extracts.

Phenolics compounds found in hydroalcoholic extracts (35.6313±0.1868 mg GAE/g) and ethanolic extracts (49.1467±0.1692 mg GAE/g) contribute to increased antioxidant capacity observed in these extracts.

Antioxidant effect of *G. curtisii* extracts are not affected by temperatures up to 85 °C used during the extraction process, even for periods of 5 hours.

These results suggest that analyzed fungi are of potential interest as sources of strong natural antioxidants that could be used in the food industries and nutrition.

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Conflict of interest

The authors report no conflict of interest.

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