SHORT COMMUNICATION

PHENOLIC ACIDS, FLAVONOIDS, ASCORBIC ACID, β-GLUCANS AND ANTIOXIDANT ACTIVITY IN MEXICAN WILD EDIBLE MUSHROOMS

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ABSTRACT

Five wild edible mushrooms (*Amanita caesarea*, *Boletus edulis*, *Cantharellus cibarius*, *Lactarius indigo* and *Ramaria* sp.) from Mexico were studied for their total phenolic content, antioxidant activity, flavonoids, ascorbic acid, gallic acid, and cinnamic and chlorogenic acids. *B. edulis* showed the highest contents, expressed in dry weight, of flavonoids (0.92 mg of quercetin equivalents/g), total phenolic acids (8.66 mg of gallic acid equivalents/g), chlorogenic acid (1001.67 μg/g) and cinnamic acid (10.68 μg/g), and it also presented the greatest antioxidant activity (48.6% DPPH scavenging activity and 59.48% reductive power) among the species analyzed. Overall, the Mexican wild edible mushrooms had high concentrations of chlorogenic and ascorbic acids and flavonoids. This study constitutes the first report in Mexico about nutraceuticals from wild edible mushrooms.

Keywords: wild mushrooms, antioxidants, flavonoids, phenolics, β-glucans

1. INTRODUCTION

Some species of mushrooms are appreciated as food not only for their color, texture, flavor and odor but also as valuable sources of nutrients and nutraceuticals (TAOFIQ *et al*., 2015; WANG *et al*., 2014). Several studies of mushrooms from Portugal (HELENO *et al*., 2015a), Italy (MANZI *et al*., 2004), Poland (NOWACKA *et al*., 2014), China (SIU *et al*., 2014), Ethiopia (WOLDEGIORGIS *et al*., 2014) and Turkey (SARIKURCKU *et al*., 2015) have described mushrooms as naturally rich sources of phenolics and flavonoids. These compounds exhibit antioxidant, antibacterial, antiviral, anticarcinogenic and anti-inflammatory activities (HELENO *et al*., 2015b; REIS *et al*., 2011; WANG *et al*., 2014).
In addition, mushrooms are recognized for having other bioactive compounds that are beneficial for human health such as β-glucans and ascorbic acid (KAGIMURA *et al*., 2015; RUTHES *et al*., 2015). β-glucans are polysaccharidic compounds that display anticoagulant, antithrombotic, antioxidant, and anti-inflammatory activities. Therefore, these compounds play an effective role in the prevention of cardiovascular problems, the reduction of blood cholesterol levels and treatment of illnesses such as various cancers and diabetes (ZHU *et al*., 2015). Ascorbic acid is an antioxidant vitamin common in mushrooms (VAZ *et al*., 2011).
In spite of the fact that since precolonial times in Mexico, the fruiting body of mushrooms has been known as nanacatl (mushroom) from Nahuatl, and that a great edible mushroom diversity of approximately 300 species is known in the country (MORENO, 2014), there are no reports with respect to the content of phenolic compounds and phenolics acids in mushrooms. Therefore, thorough studies of the chemical composition of the wild edible mushrooms are needed to identify and quantify the bioactive compounds. The selection of the mushroom species was based on their appreciation as food in Mexico: *A. caesarea*, *B. edulis*, *C. cibarius*, *L. indigo* and *Ramaria* sp. (MORENO, 2014). The aim of this study was to determine the chemical composition of five Mexican wild edible mushrooms in terms of flavonoids, phenolic acids, ascorbic acid, β-glucans and antioxidant activity.

2. MATERIALS AND METHODS

2.1. Samples, standards and reagents

The mushroom samples were collected in different regions of Hidalgo State in Mexico. The regions were Los Reyes, Acaxochitlán (latitude 20.152° and longitude -98.185°, 2199 m a.s.l.), El Susto (latitude 20.074° and longitude -98.509°, 2620 m a.s.l.) and Paxtepec (latitude 20.033° and longitude -98.426°, 2381 m a.s.l.). The sampling was conducted during the rainy season (August-October) of 2014. The taxonomic identification of the sporocarps was made according to mycology keys (LÆSSØE, 2013).
The standards of chlorogenic, cinnamic, gallic, and (L)-ascorbic acid and schizophyllan, the reagents Folin-Ciocalteu (FC) reagent, 2,2-diphenyl-1-picrilhydrazyl radical (DPPH) and 2,6-dichloroindophenol, and the LC solvents methanol 99.8% and acetonitrile 99.9% were all purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). The methanol, hydrochloric acid, phosphoric acid and red Congo dye were purchased from J. T. Baker (Philipsburg, USA). All other chemicals of analytical grade, including sodium hydroxide, metaphosphoric acid, potassium hydroxide, sodium carbonate, citric acid, sodium nitrite, aluminum chloride, ferric chloride, trichloroacetic acid, potassium ferricyanide, sodium phosphate mono and dibasic, were purchased from Meyer Co (Mexico).

The fruiting bodies were cleaned with a brush, cut into slices and dried in a drying oven in a range between 40-60°C until a constant weight was achieved. The dried samples were reduced to fine powder (20 mesh) using an electric mill (IKA A11 basic) and stored in amber plastic bottles until analysis.

2.2. Analysis of antioxidant activity

Preparation of the extracts. The preparation of the extracts was made using the methodologies proposed by PAN *et al*. (2003) and ÖZYUREK *et al*. (2014) with some modifications. Each sample (~200 mg) was extracted with methanol:water (80:20, v/v; 25 mL), using a conventional microwave oven (900 W, 10% power). The mixtures were irradiated as follows: 7 s power on (heating without superboiling of the solution) and 30 s power off (cooling in an ice bath and mixed on a vortex) then 7 s power on and 30 s power off and so on to reach 5 min of heating time. The obtained extracts were filtered through a filter paper (Whatman No. 4), then the filtered volume was adjusted with methanol:water (80:20 v/v) to 25 mL and kept at 4°C in amber bottles until analysis.

DPPH radical scavenging activity assay. Aliquots of 60 μL of extract were mixed with 540 μL of a methanolic DPPH radical solution (0.06 mM). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Genesys 10S VIS, Thermo Scientific, USA). L-ascorbic acid (8 mg/mL) was used as a standard. The radical scavenging activity (RSA) was calculated as a percentage of the DPPH pink discoloration using the following equation:

$$\% \text{ RSA} = [(A_{DPPH} - A_s)/A_{DPPH}] \times 100$$

where A_s is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution (VAZ *et al*., 2011).

Reducing power. The reducing power was determined according to BARROS *et al*. (2011). The methanolic extract (500 μL) was mixed with a sodium phosphate buffer (500 μL, 200 mM, pH 6.6) and potassium ferricyanide (500 μL, 1% w/v). The mixture was incubated at 50-60°C for 20 min, then trichloroacetic acid (500 μL, 10% w/v) was added. An aliquot of the reaction mixture (800 μL) was transferred to vials containing distilled water (800 μL) and ferric chloride (160 μL, 1% w/v). After 90 min, the absorbance was measured at 690 nm (Genesys 10S VIS spectrophotometer, Thermo Scientific). The reducing power was obtained as a percentage of the conversion of an orange Fe^{3+} ferricyanide complex to the Prussian blue Fe^{2+} ferrocyanide form in the presence of reductants (antioxidants from the mushroom extracts). BHT (1 mg/mL) was used as a control by measuring its absorbance in the assay and assigning it 100% of the reductive power.

Total phenolics. The total phenolic content was determined spectrophotometrically with the Folin-Ciocalteu (FC) test, which is an assay based on the capability of the phenolic compounds in an alkaline solution to reduce a colorimetric reagent composed of a mixture of phosphotungstic and phosphomolibdic acids from Mo(VI) to Mo(V) to produce a blue color (HUANG *et al*., 2002). According to HELENO *et al*. (2015a) 100 μL of methanolic extract were mixed with the FC reagent (750 μL, 10% v/v) for 1 min. After 5 min, a solution of Na_2CO_3 (750 μL, 10% w/v) was added to the mixture. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Genesys 10S VIS spectrophotometer, Thermo Scientific, USA). Gallic acid was used to obtain the standard curve (20 - 600 μg/mL). The total phenolic content is expressed as mg of gallic acid equivalents (GAE) per g of dry mushroom.

2.3. Determination of Phenolic Compounds by HPLC

The methanolic extracts were filtered through a 0.45 μm disposable LC filter disk for the HPLC analysis. The phenolic acids determination was performed using an Agilent 1260 CA, USA series liquid chromatograph equipped with a diode array detector. The separation was carried out using 5 μL of extract on a Poroshell 120 EC-C_{18} Agilent CA, USA, column (4.6 x 50 mm, 2.7 μm) thermostated at 25°C. The mobile phase was (A) 0.1% phosphoric acid in water, (B) HPLC-grade acetonitrile and (C) HPLC-grade methanol. The gradient used was 95% A, 5% B, and a flow rate of 1.0 mL/min for 2 min, 50% A, 25% B, 25% C, and a flow rate to 0.5 mL/min for 8 min. The phenolic acids were quantified by comparison of the area of their peaks recorded at 280 nm with the calibration curves obtained from the commercial standards of each acid. The calibration curves were obtained from a concentration range between 20-1000 μg/mL and 2-100 μg/mL for chlorogenic and cynamic acid respectively. The results are expressed in μg per g of dry mushroom.

2.4. Extraction and quantification of β-glucans

Mushroom β-glucans were isolated and quantified according to NITSCHKE *et al*. (2011): 750 mg of dry mushroom powder was heated with 60 mL 1 M KOH during 20 min at 60°C under constant stirring. Then, the suspension was filtered, the filter cake was washed with distilled water and the filtrate was collected and neutralized with 6 M HCl. The neutralized filtrate volume was adjusted to 100 mL with distilled water in a volumetric flask. This fraction was called KOH-fraction. The filter cake was resuspended in 65 mL of 0.58 M HCl and heated in an oil bath at 100°C for 1 h. The suspension was again filtered and the filter cake was washed with distilled water. The collected filtrate was neutralized with 6 M NaOH, transferred to a 100 mL volumetric flask and the volume adjusted with distilled water. This fraction was named HCl-fraction. The filter cake was again resuspended with 60 mL of 1 M NaOH and heated at 60°C for 20 min. The suspension was filtered and the filter cake was washed with distilled water, then the filtrate was neutralized with 6 M HCl. The filtrate was transferred to a 100 mL volumetric flask and the volume was augmented with distilled water. This fraction was called NaOH-fraction. The three fractions were used for the β-glucans determination.
For quantification of the β-glucans, 350 μL of each fraction were mixed with 300 μL of 0.2 M citric acid/sodium hydroxide buffer pH 7 and 50 μL of dye solution (8 mg of Congo red diluted in 10 mL of buffer) was added. The mixture absorbances were read at 523 nm against 350 μL of distilled water, 300 μL of buffer and 50 μL of dye solution as a blank. Because of the light brownish color of some of the fractions, a measurement of the background absorption at 523 nm was necessary. Therefore, 350 μL of the sample was mixed with 350 μL of the buffer, and the absorption was measured at 523 nm. The calibration curve was obtained with stock schizophyllan solutions in the range of 225-600 μg/mL. All analyses were performed in triplicate. The total content of the β-glucan is expressed as mg of β-glucan per g of dry mushroom.

2.5. Ascorbic acid

For the ascorbic acid determination, the mushroom powder (150 mg) was extracted with 10 of 1% (w/v) metaphosphoric acid for 45 min at room temperature under constant stirring and filtered through a Whatman N° 4 filter paper. The filtrate (0.1 mL) was mixed with 0.9 mL of 2,6-dichloroindophenol (0.00125% w/v) and was left to stand 30 min, then the absorbance was measured at 515 nm against a blank (VAZ *et al*., 2011). The ascorbic

acid content was calculated based on the calibration curve of the L-ascorbic acid (0.5-2.01 μg/mL), and the results are expressed as mg of ascorbic acid per g of dry mushroom.

2.6. Flavonoids

The flavonoid quantification was carried out according to PEREIRA *et al.* (2012). A total of 500 μL of methanolic extract was mixed with 150 μL of a 5% sodium nitrite solution and 2 mL of distilled water.
After 5 min, 150 μL of a 10% aluminum chloride solution was added, the reaction was kept for 6 min, then 2 mL of 4% sodium hydroxide solution and 200 μL of distilled water were added to the mixture. The reaction was mixed, and after 15 min the absorbance was measured at 510 nm. Quercetin was used to calculate the calibration curve (1 - 20 μg/mL) and the results are expressed as mg of quercetin equivalents (QE) per g of dry mushroom.

2.7. Statistical analysis

All assays were carried out in triplicate. The results are expressed as the mean values and standard deviation (SD). The results were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. These analyses were carried out using the SPSS v. 22.0 program (IBM Corp., USA).

3. RESULTS AND CONCLUSIONS

3.1. Antioxidant activity and total phenolics

The antioxidant activity of extracts was evaluated through the scavenging activity on the DPPH radicals and the reducing power. The antioxidant activity showed highly significant differences ($p < 0.001$) between the different mushrooms in both assays. The B. edulis extracts had the highest DPPH radical scavenging activity (48.06%) and reducing power (59.48%). These results are consistent with its higher content in phenolic compounds (8.66 GAE mg/g of dry mushroom) compared to the other mushrooms (Table 1).

Table 1. Total phenolics and antioxidant activity of methanolic extracts of wild edible mushrooms from Mexico.

Mushroom	Phenolics (mg of GAE/g)	% DPPH inhibition	% Reducing power
A. caesarea	2.90±0.16^{b}	6.15±1.76^{d}	36.69±2.37^{b}
B. edulis	8.66±0.69^{a}	48.06±6.60^{a}	59.48±0.99^{a}
C. cibarius	1.47±0.10^{c}	30.48±3.94^{b}	23.30±1.97^{c}
L. indigo	1.91±0.23^{c}	31.28±2.42^{b}	18.63±0.55^{c}
Ramaria sp	2.07±0.03^{c}	20.88±3.91^{c}	14.73±5.11^{c}

In each column, the letters imply significant differences ($p < 0.001$). Mean±SD; n = 3.

The *L. indigo*, *C. cibarius* and *Ramaria* sp. presented similar levels of the DPPH scavenging activity, reducing power and total phenolics. Despite the *A. caesarea* extracts showing the lowest (6.15%) DPPH scavenging activity, these extracts registered a high reducing power

(36.69%) and a low phenolic compound content (2.90 GAE mg/g of dry mushroom). The scavenging effect of the L-ascorbic acid was higher with 94.4% inhibition at 8 mg/mL. PALACIOS et al. (2011) reported the phenolics content for the *B. edulis* and *C. cibarius* of 5.5 and 2.5 mg of GAE/g of dry mushroom respectively, while we found 8.66 and 1.47. The differences observed may be due to the nutrimental and environmental conditions that affect the production of these metabolites (HELENO et al., 2015a).

The concentrations of chlorogenic acid range from 108.22 to 1001.67 μg/g of dry mushroom (Table 2), thus this acid constitutes 7.36 to 24.64% of the total phenolics present in the wild edible mushrooms studied. *B. edulis* showed the highest concentration of chlorogenic acid, while *C. cibarius* registered the lowest concentration. Other studies report chlorogenic acid concentrations of 4.55 to 63.73 μg/g (KIM *et al.*, 2008, PALACIOS *et al.*, 2011; WOLDEGIORGIS *et al.*, 2014) in mushrooms. Compared to those reports, the mushrooms evaluated in this study had very high amounts of chlorogenic acid. Our results suggest that chlorogenic acid could make a significant contribution to the DPPH radical scavenging activity of all mushrooms evaluated, except *A. caesarea*.

Cinnamic acid was only detected in *B. edulis* (10.68 μg/g of dry mushroom) and in *A. caesarea* (4.93 μg/g of dry mushroom). While HELENO *et al.* (2015a) report 3.1 μg/g of cinnamic acid in *B. edulis*, TAOFIQ *et al.*, (2015) found 14.2 μg/g. The content of this acid in *A. caesarea* reported by FERNANDES *et al.* (2015) was 24.8 μg/g, and REIS *et al.* (2011) found 0.3 μg/g. Thus, the results obtained in this study are somewhat comparable with those studies.

Table 2. Content of chlorogenic and cinnamic acids (μg/g of dry weight) in wild edible mushrooms from Mexico.

Mushroom species	Chlorogenic acid	Cinnamic acid
A. caesarea	564.45±41.99^{b}	4.93±0.61
B. edulis	1001.67±2.74^{a}	10.68±2.15
C. cibarius	108.22±2.61^{d}	Nd
L. indigo	222.42±1.55^{c}	Nd
Ramaria sp	510.11±8.13^{b}	Nd

In each row, different letters imply significant differences ($p< 0.05$). Mean±SD; n = 3.

3.2. Ascorbic acid

The ascorbic acid concentration ranging from 2.08 to 3.65 mg/g of dry mushroom reported elsewhere for the *L. indigo* reveals highly significant differences ($p< 0.01$) as compared to the mushrooms investigated herein (Table 3). The *L. indigo* registered the highest concentration and the *A. caesarea registered* the lowest. Although BARROS *et al.* (2007) report amounts of ascorbic acid of 0.13 to 0.35 mg/g in mushrooms, REIS *et al.*, (2011) found 1.75 to 8.99 mg/g. Hence, our results are in agreement with the latter study. The presence of ascorbic acid in the mushroom is not surprising as this metabolite is required as part of the defense mechanisms against radicals to avoid oxidative stress; therefore, the concentrations of ascorbic acid may be subject to the effects of geographic locations, maturating stage of the fruiting body, genotype and weather conditions (FERREIRA *et al.*, 2009).

3.3. Flavonoids

The flavonoid content of the mushrooms studied is shown in the Table 3. The concentration varies depending the mushroom species, and the differences are highly significant ($p < 0.001$). The results of the present study are similar to those reported by WOLDEGIORGIS *et al.* (2014) who found concentrations of 0.17 to 1.97 mg catechin equivalent/g. Although KORZASKI *et al.* (2015) report that *C. cibarius* is a rich source of flavonoids (42.9 mg of catechin equivalents/g of dry mushroom), this does not agree with our finding. The results suggest that flavonoids may play an important role in the total antioxidant activity, specifically in reducing power.

Table 3. Concentration of ascorbic acid, flavonoids and β-glucans in wild edible mushrooms from Mexico.

Mushroom	Ascorbic acid (mg/g)	Flavonoids (mg QE/g)	β-glucans (mg/g)
A. caesarea	2.08±0.16^{d}	0.39±0.04^{b}	214.92±2.09^{a}
B. edulis	2.61±0.05^{c}	0.92±0.02^{a}	39.97±0.91^{c}
C. cibarius	2.63±0.09^{c}	0.34±0.02^{b}	nd
L. indigo	3.65±0.00^{a}	0.25±0.00^{c}	88.34±3.62^{b}
Ramaria sp	3.14±0.04^{b}	Nd	nd

nd = not detected. In each row, different letters imply significant differences ($p< 0.001$). Mean±SD; n = 3.

3.4. β-glucans

β-glucans are the most abundant polysaccharide on the fungal cell wall (FESEL and ZUCARO, 2015) and the colorimetric method with Congo red detects β-1,3-1,6 glucans from mushrooms with high precision and without extensive clean-up (ZHU *et al.*, 2015). Thus, the β-glucans content determined by this study showed highly significant differences ($p< 0.01$) between the mushroom species (Table 3). The highest β-glucans content was detected in *A. caesarea* with 214.92 mg/g of dry mushroom, while *Ramaria* sp. and *C. cibarius* did not show detectable β-glucans contents. Despite the fact that there are several reports on β-glucans in mushrooms in other countries (SYNYTSYA and NOVÁK, 2013; RUTHES *et al.*, 2015), the species described here have been not explored yet, except for *B. edulis*. MANZI *et al.* (2004) reported that rehydrated dry samples of *B. edulis* had a β-glucans content of 43.3 mg/g, which is similar to our finding for this mushroom (39.97 mg/g). It seems that *A. caesarea* is a rich source of β-glucans, which will produce health benefits when consumed.

The Mexican wild edible mushrooms studied in this research shown high contents of chlorogenic and ascorbic acids and flavonoids. *B. edulis* has the highest antioxidant activity, which correlates with its contents of chlorogenic and cinnamic acids and flavonoids. *L. indigo* has the highest content of ascorbic acid, and *A. caesarea* registered the highest concentration of β-glucans. The presence of these biologically active molecules in the mushrooms studied reveals the nutraceutical potential of the mushrooms.

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