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Antimicrobial activity, phenolic compounds content, and antioxidant capacity of four edible macromycete fungi from Chihuahua, Mexico

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Abstract

In the present study, antimicrobial activity, phenolic compounds content and antioxidant capacity of four edible fungi (*Amanita rubescens*, *Astraeus hygrometricus*, *Laccaria laccata* and *Lycoperdon perlatum*) were determined. Antimicrobial activities were tested against *Staphylococcus aureus*, *Streptococcus agalactiae* and *Candida albicans*. Phenolic compounds and antioxidant activity were measured by spectrophotometric methods. All mushrooms present high activity against *S. agalactiae.* Phenolics compounds content ranked between 1.54 - 20.93 mg GAE/g DW and the antioxidant activity ranked between 0.0034 – 0.0854 mmol TE/g DW being *A. rubescens* the specie with the highest values. The results obtained for the antimicrobial activity using the disc diffusion method indicated that the extracts exhibited moderated antimicrobial activity. However, the MIC results with both solvents show that all the macromycete species registered inhibition of the microorganisms in different concentration. Generally, the ethanol extracts exerted stronger antimicrobial activity than methanol extracts. Similarly, *S. agalactiae* was the most susceptible microorganism, followed by *C. albicans*. *S. aureus* was the bacteria most resistant. The best antimicrobial activity was found in the ethanolic extracts of *A. hygrometricus* and *L. perlatum* against *S. agalactiae*, with a MIC value of 3.75 mg/mL. In conclusion, it is suggested that these species can be used as a natural source of antimicrobial and antioxidant components.

Keywords: bioactive compounds, microorganisms, edible fungi.

Actividad antimicrobiana, contenido de compuestos fenólicos y capacidad antioxidante de cuatro hongos macromicetos comestibles de Chihuahua, México

Resumen

En el presente estudio se determinó el contenido de compuestos fenólicos y la actividad antimicrobiana y antioxidante en cuatro especies de hongos comestibles (*Amanita rubescens*, *Astraeus hygrometricus*, *Laccaria laccata* y *Lycoperdon perlatum*). Las actividades antimicrobianas se probaron en *Staphylococcus aureus*, *Streptococcus agalactiae* y *Candida albicans*. Los compuestos fenólicos y la actividad antioxidante se midieron mediante métodos espectrofotométricos. Todos los hongos presentan una alta actividad en comparación con *S. agalactiae*. El contenido de compuestos fenólicos se ubicó entre 1.54 - 20.93 mg GAE/g DW y la actividad antioxidante entre 0.0034 - 0.0854 mmol TE / g DW, siendo *A. rubescens* la especie con el valor más alto encontrado. Los resultados obtenidos de la actividad antimicrobiana utilizando el método de difusión en disco indicaron que los extractos exhibieron una actividad moderada. Sin embargo, la Concentración Mínima Inhibitoria (CMI) con ambos disolventes muestra que todas las especies de macromicetos registraron inhibición de los microorganismos en diferentes concentraciones. En general, los extractos etanólicos ejercieron una actividad antimicrobiana mayor a los obtenidos con metanol. La bacteria *S. agalactiae* fue el microorganismo más susceptible y S. *aureus* la más resistente. La mejor actividad antimicrobiana se encontró en los extractos etanólicos de *A. hygrometricus* y *L. perlatum*, principalmente en *S. agalactiae*, con un valor de CMI de 3.75 mg/mL. En conclusión, se sugiere que estas especies de macromicetos se pueden utilizar como fuente natural de componentes antimicrobianos y antioxidantes.

Palabras clave: compuestos bioactivos, microorganismos, hongos comestibles.

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Introduction

Infectious diseases represent one of the major threats worldwide due to the growing prevalence of drug resistance microorganisms (Wright, 2010). Within infectious diseases, the dermatologic infections are a public health problem, since it is estimated that between 30-70% of the world population is affected by at least one type of skin disease (Hay *et al.,* 2014). Bacteria and fungus are considered responsible for dermatologic infections in humans, among the most frequents are *Staphylococcus aureus*, *Streptococcus pyogenes* and the species of the genus *Candida* (Riain, 2013). These microorganisms have been developed resistance to some commercial's antibiotics. For this reason, it is necessary to continue the search for new compounds capable of their inhibition. Whereas the damage derived by the uncontrolled production of free radicals promotes the development of diseases like cancer, rheumatoid arthritis, cirrhosis, arteriosclerosis, and degenerative processes associated with aging (Elmastas, Isildak, Turkekul & Temur, 2007, Ren *et al.,* 2014). Preliminary research has shown that macromycetes fungi produce a wide variety of bioactive compounds like phenolic compounds, which display an important role in the protection against oxidative damage, and the defense against bacteria, viruses, and insects (Leyva, Pérez-Carlón, González-Aguilar, Esqueda & Ayala-Zavala, 2013).

Macromycetes fungi have been recognized worldwide as sources of medicine and food (Quiñónez-Martínez *et al.*, 2014). In Mexico, they have been incorporated into the diet of some ethnic groups and have been used for the treatment of different diseases since prehistoric times (Ruiz, Pérez-Moreno, Almaraz-Suárez & Torres-Aquino, 2013). Specifically, the Sierra Tarahumara of Chihuahua, Mexico has been known to have around 500 species of macromycete fungi (Gómez-Flores *et al.,* 2019). Some of which have been reported to have medicinal and nutritional characteristics, like *A. hygrometricus* and *L. perlatum,* whose carpophores are used for acne problems as well as pain relievers, and burns cuts swelling (Quiñónez-Martínez *et al.,* 2014). Likewise, *L. laccata* and *A. rubescens* show relevance due to their nutritive value, the last one is collected by the inhabitants for consumption and sale. Although there are many studies about the antimicrobial and antioxidant activities of macromycete fungi in the world, there is little information available about the traditional knowledge of mushrooms from the Sierra Tarahumara at Chihuahua, Mexico. The objective of this study was to evaluate the antimicrobial activities, the phenolics compounds and the antioxidant activity of four species of macromycete fungi from Chihuahua.

MATERIALS & METHODS

Samples and samples treatment

The fruiting body of *A. rubescens*, *A. hygrometricus*, *L. laccata,* and *L. perlatum* (Figure 1) were collected in the forest from the municipality of Bocoyna in the State of Chihuahua, Mexico, during August and September of 2017 and 2018. Samples were transported to the Laboratory of Biodiversity at the Institute of Biomedical Sciences (ICB) at the Universidad Autónoma de Ciudad Juárez (UACJ), where the identification and storage of the mushrooms were carried out. The samples were lyophilized (Labconco, Corp, Labconco, Kansas City, MO, EEUU) and grounded before analysis.

Antimicrobial activity

Preparation of extracts

Powdered fruiting bodies (10 g) were extracted with 200 mL of absolute ethanol (Jalmek®) or absolute methanol (CTR scientific) respectively for 24 h. After that, the samples were sonicated (Bransonic® CPX5800H) for 30 min, then the extracts were centrifuged (IEC, HN-SII) for 10 min at 3,000 rpm and the supernatants were collected. The extracts were concentrated using a rotary evaporator (Büchi Rotavapor, R-114) and evaporated to dryness. Finally, the extracts were dissolved in the solvents at a concentration of 50 mg/mL and sterilized through a 0.22-micron membrane filter (Membrane Solutions) (Giri, Biswas, Pradhan, Mandal & Acharya, 2012). The extracts were stored at 4 °C for further use (Barros, Baptista, Estevinho & Ferreira, 2007).

Test microorganisms and growth conditions

For the antimicrobial activity, the microorganisms used were *S. aureus*, *S. agalactiae*, *C. albicans* (for the determination of the Minimum Inhibitory Concentration Test) and *Candida* sp. (for the determination of the diffusion disk test), which were obtained from the Microbiology Department at the university (UACJ).

Bacterial and fungal test organisms were grown in a different medium. *S. aureus* was cultured on Mueller Hilton agar (DIBICO®), *S. agalactiae* was cultured on Brain Heart Infusion Agar (BD Bioxon®). Both were incubated at 37 °C for 24 h. While fungal species were cultured on Potato Dextrose Agar (BD Bioxon®) at 27 °C for 48 h. Then, *S. aureus* and *Candida* sp. were cultured in Trypticase Soy Broth (DIBICO®), while *S. agalactiae* was inoculated in Brain Heart Infusion Broth (DIBICO®) using the conditions before mentioned. Finally, the turbidity for each microorganism cultivated was adjusted to 0.5 McFarland standard which approximated to 1 x 10^6 CFU/mL.

Disk diffusion method

The antimicrobial activity was assessed by the disc diffusion method according to the modified procedure of Kalyoncu, Oskay, Sağlam, Erdoğan & Tamer (2010). Mueller Hinton agar, Brain Heart Infusion agar, and Potato Dextrose agar for *S. aureus, S. agalactiae*, and *Candida* sp., respectively. After 24 h, the turbidity of each microorganism cultivated was adjusted to 0.5 McFarland standard, which approximated to 1 x 10^6 CFU/mL. The surface of the plates was inoculated using 1 µL of the suspension, then was spread using a sterile

Figure 1. Carpophores of *Amanita rubescens* (a), *Astraeus hygrometricus* (b), *Laccaria laccata* (c) and *Lycoperdon perlatum* (d).

cotton swab on Mueller Hinton agar for *S. aureus*, Brain Heart Infusion agar for *S. agalactiae* and Potato Dextrose Agar for *Candida* sp. Each test was carried out twice.

Smalls filter paper discs (6 mm diameter) were impregnated with 10 µL of the extracts and were air-dried until the excess of solvent was removed. After being dried, the discs were placed on the medium. Plates were incubated (at 37 °C for the bacterial and 27 °C for the fungi) and the zones of inhibition were measured after 24 h. Each test was done in duplicate. As positive control, erythromycin (2.5%) was used in case of bacteria and clotrimazole (2%) in case of fungi. As a negative control, methanol and ethanol (10 µL/disc) solvents were used.

Minimum Inhibitory Concentration (MIC)

The MIC of the extracts was determined according to the modified procedure of Padilla (2012). The extracts were incorporated into culture broth a concentration ranging from 1.25 mg/mL to 10 mg/mL, then 1 µL of each microorganism previously adjusted to equal that of 0.5 McFarland standard was inoculated and were incubated during 24 h (at 37 °C for the bacterial and 27 °C for the fungi). After the incubation time, 1 µL of each microorganism was used to inoculate the surface of the plates and were incubated for 24 h. The MIC of the extract for each test microorganism was considered the agar plate with the lower concentration without growth. A control without extract was prepared, and each assay was replicated three times.

Phenolic compounds and antioxidant activity

Preparation of extracts

The crude extracts for the determination of phenolics compounds and antioxidant activity were prepared according to Álvarez-Parrilla, de la Rosa, Martínez & González Aguilar, (2007). Dried samples (0.25 g) were mixed with 25 mL of 80% methanol and sonicated (Bransonic® CPX5800H) for 15 min. Then, the extracts were centrifugated (Eppendorf® 5804r) at 3,000 rpm and the supernatants were filtered using filter paper (Whatman No. 1). The extracting procedure was repeated, the supernatants were collected, and final volume was adjusted to 50 mL with 80% methanol.

Determination of total phenols

Total phenols were determined by the Folin-Ciocalteu method according to the modified procedure reported by Álvarez-Parrilla *et al*. (2007). Extract (250 µL) was mixed with 1,000 µL of 7.5% sodium carbonate (w/v) and 1250 µL of 10% Folin-Ciocalteu reagent (v/v). The mixture was incubated at 50 °C for 30 min, then, the absorbance was measured at 760 nm. A calibration

curve was obtained using gallic acid as standard. Results are shown as mg of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g DW).

In vitro antioxidant assays (ABTS, DPPH and FRAP)

The antioxidant activity was evaluated using the $ABTS^{\bullet+}$ radical (Álvarez-Parrilla, de la Rosa, Amarowicz & Shahidi, 2011). A 7 mM $ABTS^{\bullet+}$ reagent was prepared in a PBS solution (0.1 M, pH 7.4, KCl 0.15 M) mixed with sodium persulfate (2.45 mM) and incubated 16 h in darkness at room temperature. The absorbance of the $ABTS^{\bullet+}$ radical was adjusted to 0.7. Then, 285 µL of $ABTS^{\bullet+}$ solution was mixed with 12 µL of sample and the absorbance was measured at 734 nm for 30 min in a microplate reader (Bio Rad xMark). Inhibition percentage was determined according to equation 1:

$$Inhibition\ \% = \left(\frac{A_b - A_s}{A_b}\right) x\ 100 \qquad (1)$$

Where A_b is the absorbance of the blank and A_s is the absorbance in the presence of the extract. Trolox was used as standard and results were expressed as millimol Trolox equivalents (TE) per gram of dry weight (mmol TE/g DW).

The scavenging activity of the DPPH• radical was measured according to the procedure reported by Álvarez-Parrilla *et al*. (2011). Twenty-five µL of fungi extract was mixed with 200 µL of DPPH• reagent (190 µM in methanol). The absorbance was measured in a microplate reader at 517 nm for 30 min each 30 s at room temperature.

The inhibition percentage was determined using the equation (1). Trolox was used as standard and results were expressed as millimole Trolox equivalents (TE) per gram of dry weight (mmol TE/g DW).

The ferric reducing antioxidant power (FRAP) was measured using the modified methodology by Álvarez-Parrilla *et al.* (2011). The FRAP reagent was prepared by mixed 0.3 M acetate buffer (pH 3.6) with 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3 \bullet H_2O$, at a 10:1:1 ratio. Then, FRAP reagent was incubated at 37 °C for 30 min. Afterwards, 180 µL of FRAP reagent was mixed with 24 µL of each extract, and the absorbance was measured at 595 for 60 min at 37 °C in a microplate reader. Trolox was used as standard and the results were expressed as millimol Trolox equivalents (TE) per gram of dry weight (mmol TE/g DW). All the experiments were done by triplicate.

Statistical analysis

Results of antimicrobial activity, total phenolic content, and antioxidant activity of the four species of macromycete fungi were expressed as mean values ± standard error (SE). The collected data was assumed to follow a normal distribution as determined by the Shapiro-Wilks test for normality ($p > 0.05$). A one-way ANOVA analysis of variance was performed to see if there were significant differences ($p \leq 0.05$), afterwards a Tukey multiple mean comparison test was used. Lastly, a Pearson correlation test (r) was used to correlate the result of phenolic compounds and the antioxidant activity for all species together. Data was statistically analysed using SPSS (IBM, SPSS Statistics 20), Excel (Microsoft® Excel®, version 1903) and GraphPad Prism 8.1.2. software.

Results

Antimicrobial activity

Disk diffusion method

The results for the disk diffusion method are shown in Table I. The extracts obtained had an inhibitory response against *S. agalactiae* and *Candida* sp. to a concentration of 50 mg/mL, being *S. agalactiae* the microorganism most

Table I. Antimicrobial activity of the ethanolic and methanolic extracts of four macromycete fungi from Chihuahua.

Macromycete fungi	Mean inhibition zone (mm)					
	Ethanolic extracts			Methanolic extracts		
	S. aureus	*S. agalactiae*	*Candida* sp.	*S. aureus*	*S. agalactiae*	*Candida* sp.
Amanita rubescens	-	-	-	-	-	-
Astraeus hygrometricus	-	5.79 ± 0.17^{de}	4.5 ± 0.29^{e}	-	6.58 ± 0.31^{c}	-
Laccaria laccata	-	6.43 ± 0.38^{cd}	-	-	6.25 ± 0.47^{c}	-
Lycoperdon perlatum	-	8.25 ± 0.25^{c}	-	-	$5.7\ 5 \pm 0.25^{c}$	-
Erythromycin (2.5%)	13.5 ± 0.20^{b}	19.375 ± 0.47^{a}	-	13.5 ± 0.20^{b}	19.375 ± 0.47^{a}	-
Clotrimazole (2%)	-	-	13.5 ± 0.28^{b}	-	-	13.5 ± 0.28^{b}
Solvent (Ethanol)	-	-	-	-	-	-

Means value ± standard error. Different letters indicate significant differences, according to the Tukey HSD ($p \leq 0.05$). – No inhibition. Extract concentration: 50 mg/mL. Fungal extracts/disc: 10 µL. Solvent/disc: 10 µL.

susceptible to the inhibitory action of macromicetes extracts. On the contrary, *S. aureus* was the most resistant microorganism due to the null activity of the extracts. The highest inhibitory activity was recorded by the ethanolic extract of *L. perlatum* against *S. agalactiae* (8.25 mm) and the ethanolic extract of *A. hygrometricus* showed the lowest activity against *Candida* sp. (4.5 mm). The range of extracts according to their inhibition zones against *S. agalactiae* was as follows: *L. perlatu*m ethanol > *A. hygrometricus* methanol > *L. laccata* ethanol > *L. laccata* methanol > *A. hygrometricus* methanol > *L. perlatum* methanol. In general, the solvent with the best results was ethanol.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration results showed that all the macromycete fungi species registered inhibition of the microorganisms at different concentration (Table II). The ethanol and methanol extracts of the tested mushrooms showed stronger antimicrobial activity when compared with the disc diffusion method. Generally, the ethanol extracts exerted stronger antimicrobial activity than methanol extracts. Similarly, *S. agalactiae* was the most susceptible microorganism, followed by *C. albicans,* and again *S. aureus* was the most resistant bacteria.

The best antimicrobial activity was found in the ethanolic extracts of *A. hygrometricus* and *L. perlatum* against *S. agalactiae*, with a MIC value of 3.75 mg/mL (Table II).

The methanolic extracts of the macromycete fungi evaluated in this study showed complete inhibition of the pathogens at concentrations of 5 to 10 mg/mL (Table II). The lower MIC against the three microorganisms tested was exhibited by *A. hygrometricus* followed by the extracts of *L. perlatum,* and *L. laccata,* which presented similar MIC values. Finally, *A. rubecens* showed slightly weaker activity.

Phenolic compounds and antioxidant activity

The results of the quantification of total phenolic compounds and antioxidant capacity indicated variations in the averages depending on the species and the method used (Table III).

The total phenols content varied between 1.54 and 20.93 mg EAG/g DW. *A. rubescens* showed the highest content, followed by *L. laccata*, *L. perlatum,* and finally *A. hygrometricus* (Table III).

The antioxidant capacity of the four species were evaluated through the FRAP, DPPH and ABTS assays. The results indicate an antioxidant potential ranging from 0.0074 to 0.0854 mmol TE/g DW. The antioxidant capacity varied in a similar pattern to the total phenols content, thus the species with the highest antioxidant activity was *A. rubescens* (0.0854 mmol ET/g DW), while *A. hygrometricus* obtained the lowest activity (0.0034 mmol TE/g DW). In agreement with previously published results, antioxidant capacity methods

Table II. Minimum inhibitory concentration of the ethanolic and methanolic extracts of four macromycete fungi from Chihuahua.

Macromycete fungi	Minimum inhibitory concentration (mg/mL)					
	Ethanolic extracts			Methanolic extracts		
	S. aureus	*S. agalactiae*	*C. albicans*	*S. aureus*	*S. agalactiae*	*C. albicans*
Amanita rubescens	8.75	5	7.5	10	6.25	7.50
Astraeus hygrometricus	7.5	3.75	6.25	8.75	5	6.25
Laccaria laccata	8.75	5	6.25	10	5	8.75
Lycoperdon perlatum	7.5	3.75	7.5	10	5	7.5

Data are presented as the mean of three replicate.

Table III. Content of phenolic compounds and antioxidant capacity of four macromycete fungi from Chihuahua.

Macromycete fungi	Total phenols (mg GAE/g)	ABTS (mmol TE/g)	DPPH (mmol TE/g)	FRAP (mmol TE/g)
Amanita rubescens	20.93 ± 0.90^{a}	0.0854 ± 0.0024^{a}	0.0437 ± 0.0004^{a}	0.0254 ± 0.0006^{b}
Astraeus hygrometricus	1.54 ± 0.07^{c}	0.0074 ± 0.0002^{c}	0.0135 ± 0.0010^{d}	0.0034 ± 0.0002^{d}
Laccaria laccata	8.75 ± 0.54^{b}	0.0293 ± 0.0034^{b}	0.0368 ± 0.0004^{b}	0.0078 ± 0.0009^{c}
Lycoperdon perlatum	6.05 ± 0.29^{b}	0.0240 ± 0.0004^{b}	0.0244 ± 0.0003^{c}	0.0298 ± 0.0013^{a}

Values are presented as the mean of three replicates of analysis performed in triplicate ± standard error. Averages with different letters indicate a significant difference between fungal species due to antioxidant activity, according to the Tukey HSD test ($p \leq 0.05$).

showed different behaviour (Álvarez-Parrilla *et al.,* 2014). In the case of *A. rubescens* and *L. laccata*, the highest averages were observed using the ABTS method, for *A. hygrometricus* it was by the DPPH method and for *L. perlatum* the best averages were found employing the FRAP assay.

Table IV shows the correlation between total phenols content and antioxidant capacity. These results indicate a highly significant correlation between the $ABTS^{\bullet+}$ radical and total phenols (r = 0.9951), there is also a significant correlation between total phenols and $DPPH^{\bullet}$ radical (r = 0.9070), unlike for antioxidant capacity reducing FRAP, no significant correlation with total phenols content was found.

Table IV. Correlation between phenolic compounds (Ft) and antioxidant capacity (ABTS, DPPH, FRAP) of four species of macromycete fungi from Chihuahua.

	Ft	ABTS	DPPH	FRAP
Ft	1			
ABTS	0.9951**	1		
DPPH	0.9070*	0.8612	1	
FRAP	0.5171	0.5415	0.3913	1

*Significant ** Highly significant.

Discussion

Macromycete fungi represent a source of bioactive compounds that can be beneficial to humans (González-Barranco *et al.,* 2009), it is known that their compounds could present different pharmacological activities like antimicrobial and antioxidant activities (Ren *et al.,* 2014). In our study, we found that the four studied mushrooms species presented both activities. The antimicrobial activity of the mushrooms was tested using two different methods, disk diffusion assay and minimum inhibitory concentration (MIC). Only three macromycete fungi (*A. hygrometricus*, *L. laccata* and *L. perlatum*) presented inhibitory action against *S. agalactiae* and *Candida* sp. in the disk diffusion method. Nonetheless, employing the MIC method, all the mushroom extracts shown antimicrobial activity against the three-microorganism tested, which coincides with other studies that report greater effectiveness of the broth dilution method compared to the disk diffusion method when evaluating the antimicrobial activity of fungal extracts (Ren *et al.,* 2014, Hleba *et al.,* 2016). The disk diffusion method, as its name implies, depends primarily on the diffusion capacity of the substance present in the extracts (Ren *et al*., 2014), resulting in little mobility of the compounds in the agar due to its low solubility or high molecular mass.

The solvent with the best results was ethanol. This may be due to the differences in polarity with methanol and dielectric constant, which allows increasing the solubility of compounds with antimicrobial activity, such as polyphenols (Adhikari, Pandey, Agnihotri & Pande, 2018; Do *et al.,* 2014).

The best antimicrobial activity was presented by *A. hygrometricus* and *L. perlatum*, due to the similarity of recorded values. For the disk diffusion method, the high inhibition zones were obtained by the ethanolic extract of *L. perlatum* against *S. agalactiae* (8.25 mm). These results accord with those found by Dulger (2005) who reports the antimicrobial activity of the ethanolic extract of *L. perlatum*, but against *S. pyogenes*. There are several compounds to which antimicrobial activity in *L. perlatum* can be attributed, such as those found in ethanolic and methanolic extracts, mainly phenolic compounds, alkaloids, carbohydrates, tannins, saponins, glycosides, and proteins (Akpi, Odoh, Ideh & Adobu, 2017).

Astraeus hygrometricus obtained the best inhibition values against *Candida* sp. since it was the only one capable of developing inhibition zones (4.5 mm) and the one that reported lower concentrations of the yeast by the MIC method. The antimicrobial activity of *A. hygrometricus* has been demonstrated in previous studies, Giri *et al*. (2012) reported positive zones of inhibition when extracting carpophores with methanol at a concentration of 10 mg/mL. Also, according to Lai *et al*. (2012), the constituents responsible for the inhibition against *C. albicans* in *A. hygrometricus* are two triterpenes known as astrakurkurol and astrakurkurone.

In the case of *L. laccata,* its extracts exhibited similar inhibition actions, this could be attributed to the phenolic compounds found in the carpophores (8.75 mg GAE/g DW), and in addition to the fatty acids found in the petroleum ether and ethyl acetate fractions from the ethanolic extract of *L. laccata* carpophores, such palmitic acid, linoleic acid, and oleic acid (Nieto & Cucaita, 2007), which have been previously reported as compounds with antimicrobial activity.

The extracts of *A. rubescens* did not show inhibition of the microorganisms through the disk diffusion method since this method is not appropriate to test partially or completely hydrophobic compounds (Ren *et al.,* 2014), this specie is rich in compounds with such nature (Kalač, 2013). Among the majority compounds in the species of the *Amanita* genus are some peptides (Li & Oberlies, 2005), which have low solubility in a solid medium such as agar, but they manage to diffuse properly into a liquid (dilution in broth). Although *A. rubescens* was the specie with the highest content of total phenols (20.93 mg GAE/g DW), the low antimicrobial activity may be due to the existence of compounds linked to phenols that make them more hydrophilic, such as sugars, polysaccharides, lignins, amines, long-chain alcohols, and glycerol, as well as long-chain omega fatty acids (Naczk & Shahidi, 2006), making it difficult to diffuse during tests of antimicrobial activity. *A. rubescens* is the species with the least inhibitory effect using the MIC assay,

along with other species of the same genus such as *A. muscaria* and *A. phalloides* which also exhibit a moderate MIC of 6.25 mg/mL against *S. pyogenes* (Chelela, Chacha & Matemu, 2014). *S. agalactiae* was the microorganism most susceptible to the inhibitory action of macromycetes, these results agree with several reports (Canli, Akata & Altuner, 2016; Alves *et al.,* 2013). In general, some studies indicate that fungal extracts have a greater growth inhibitory effect on species belonging to the genus *Streptococcus* than other microorganisms, mainly in the species of *S. pyogenes, S. mutans* and *S. sobrinus* with MIC values ranging from 2.4 to 87.4 µg/mL (Doğan, Duman, Özkalp & Aydin, 2013; Lund *et al*., 2009). Therefore, it is considered the microorganism more susceptible to most antibiotics, since there are few strains with characteristics of resistance to penicillin, ampicillin, and cefotaxime (Torres & Cercenado, 2010), making possible the action of the evaluated extracts against the microorganism in the present study.

Staphylococcus aureus did not show susceptibility since it is considered a microorganism with phenotypes resistant to different antibiotics such as beta-lactams and methicillin (Torres & Cercenado, 2010). However, the phenotype used in these tests of antimicrobial activity is unknown. There are studies about the null activity of fungal extracts against *S. aureus* such as that of Canli *et al*. (2016), who found no inhibition by the ethanolic extracts of *Lycoperdon lividum*; Barros, Venturini, Baptista, Estevinho & Ferreira (2008), whose extracts did not show antimicrobial activity using *L. perlatum,* and Giri *et al.* (2012), reporting no inhibition of the bacteria with methanolic extracts of *A. hygrometricus*.

Phenols compounds are widely distributed in plants and fungal species. Some studies mention that the total phenol content in fungi ranges between 6.08 and 24.85 mg GAE/g (Prasad, Varshney, Harsh & Kumar, 2015), although in the present investigation results are found in a range of 1.54 - 20.93 mg GAE/g DW. According to Mujić, Zeković, Lepojević,Vidović & Živković (2010), the range obtained of total phenolics compounds for mushrooms are in a range of 7.8 - 23.07 mg GAE/g, and for edible mushrooms it goes from 4.94 to 35.56 mg GAE/g (Alispahić *et al.,* 2015). Even though our results are similar and are within the range reported, it is difficult to compare the results due to differences in the mode of expression of the results, extraction methods of the phenolic compounds in the fungi, and environmental conditions from the collection site.

Amanita rubescens (20.93 mg GAE/g DW) presented a high content of total phenols, which differs from that found by Keleş, Koca & Gençcelep (2011), who reported a content of 5.7 mg GAE/g DW. However, there are reports for other species of the genus *Amanita* with similar values than those found in the study, like *A. patherina* (5.27 mg GAE/g), *A. muscaria* (7.59 mg GAE/g), *A. porphyria* (14.53 mg GAE/g) and *A. citrina* (38.44 GAE/g) (Nowacka *et al.,* 2015).

L. laccata and *L. perlatum* presented phenolic content in the range of those reported for this specie (8.75 and 6.05 mg GAE/g DW, respectively). Nowacka *et al.* (2014), found records like those of the study for *L. laccata* (9.38 mg GAE/g dry extract) and different for *L. perlatum* (13.59 mg GAE/g dry extract). According to Heleno, Barros, Sousa, Martins & Ferreira (2010), the variations found are mainly due to the substrate where the macromycetes grow, which causes modifications in the secondary metabolism of the species (shikimic acid and acetate pathway) affecting the production of phenolic compounds. In addition, the area and collection time also causes changes in the composition of total phenols (Smolskaite, Venskutonis & Talou, 2015). *Astraeus hygrometricus* was the species with the lowest content of total phenols (1.54 mg GAE/g DW) compared to the other species analyzed. These differences are mainly attributed to the extraction methods, due to the treatment of the carpophores with boiled water before quantification, maximizing the extraction of the compounds, since cooking results in an increase in total phenols (Pavithra, Sridhar, Greeshma & Tomita-Yokotani, 2016).

In general, fungi have great antioxidant activity (Álvarez-Parrilla *et al.,* 2007; Elmastas *et al.,* 2007), which is in a range that goes from 0.0741 to 7.61 mmol TE /100 g (Srikram & Supapvanich, 2016; Özyürek, Bener, Güçlü & Apak, 2014). However, in the present study lower values were found. The differences in the results of the antioxidant capacity are due to factors such as the analysis of different species, the area in which they are collected or the substrate where they grow (Heleno *et al*., 2010). As well as differences in extraction methods (since that there is still no standardization or consensus on the treatment of the sample before the analysis), among which are the extraction temperature and the relation of the solute with the solvent (Dai & Mumper, 2010). In the present investigation, the extraction temperature to assess antioxidant capacity was 24 °C and according to Özyürek *et al.* (2014), the optimal extraction temperature that improves the performance of antioxidant capacity in mushroom extracts is 80 °C, since an increase in temperature can accelerate cell rupture, generating a raise in internal pressure within the cells of fungi and promoting a higher solubility of the analyte (Dai & Mumper, 2010). Likewise, the use of solvents other than methanol may also show better results, since depending on the polarity of the solvents they will be able to extract various compounds that may have a high antioxidant capacity (Kosanić, Ranković, Rančić & Stanojković, 2016). Kaewnarin, Suwannarach, Kumla & Lumyong (2016), extracting the antioxidant compounds using ethanol, methanol, and water, with water shows highest antioxidant activity efficiency for the same methods evaluated (ABTS, DPPH, and FRAP).

ABTS assay measures the ability of the radical to donate a proton to an unstable cationic radical ($ABTS^{\bullet+}$), it is used to evaluate antioxidant capacity, making possible the analysis of hydrophilic and lipophilic compounds (Kaewnarin *et al.,* 2016). The results obtained by this test showed that *A. rubescens* presents the highest averages of antioxidant capacity (0.0854 mmol TE/g DW), followed by *L. laccata* (0.0293 mmol TE/g DW), *L. perlatum* (0.0240 mmol TE/g DW) and *A. hygrometricus* (0.0074 mmol TE/g DW). This differs from that reported by Iwalokun, Usen, Otunba & Olukoya (2007), whose antioxidant activity ($ABTS^{\bullet+}$ radical) of *Pleurotus ostreatus* extracts was 4.4 millimol. However, they used acetone as a solvent, which can extract another class of antioxidant compound such as carotenoids and tocopherols (Liu, Jia, Kan & Jin, 2013), also the species was different.

DPPH test is considered one of the most widely used methods to evaluate antioxidant capacity because radicals are very stable and easy to use (Acharya, Khatua & Ray, 2017). The antioxidant activity of the extracts showed very similar averages among the species, with *A. rubescens* (0.043 mmol TE/g DW) being the species with the highest radical uptake, followed by *L. laccata* (0.0368 mmol TE/g DW), *L. perlatum* (0.0244 mmol TE/g DW) and *A. hygrometricus* (0.0135 mmol TE/g DW). The same behavior was observed with total phenols content, which coincides with that reported by Alothman, Bhat & Karim (2009), who mentioned that the higher the phenolic content, the higher the DPPH values. Recently Smolskaite *et al*. (2015), evaluated the antioxidant properties of eight macromycete species through different tests (ABTS, DPPH, and FRAP) using sequential extractions with solvents of different polarities (among which was methanol), whose values ranged from 0.12 to 9.62 µM TE/g DW for the DPPH assay is similar to the results obtained by the same method.

FRAP assay is based on measuring the ability of antioxidants to reduce ferric iron to its ferrous form (Kaewnarin *et al*., 2016). In this case, the highest value was presenting in *L. perlatum* (0.0298 mmol TE/g DW), followed by *A. rubescens* (0.0254 mmol TE/g DW), *L. laccata* (0.0078 mmol TE/g DW) and *A. hygrometicus* (0.0034 mmol TE/g DW). This differs from that reported by Srikram & Supapvanich, (2016), who obtained higher results in the species *A. calyptroderma* (1.98 mmol TE/100 g), *A. princeps* (1.14 mmol TE/100 g) and *A. odoratus* (07.61 mmol TE/100 g). In the same way, Yahia, Gutiérrez-Orozco & Moreno-Pérez (2017), reports higher reducing power averages for *L. perlatum* (17 mmol TE/100 g wet weight), *A. flavoconia* (7 mmol TE/100 g), *A. pantherina* (13 mmol TE/100 g) and *A. virosa* (6 mmol TE/100 g), which can be attributed to the same factors previously mentioned (different species, collection site, and extraction methods) (Kosanić *et al*., 2016; Nowacka *et al.,* 2014). In our results, *L. perlatum* and *A. rubescens* were the species with the greatest reducing power, so it is believed that the phenolic compounds present in the species could have more hydroxyl-functional residues in the phenol rings than the other species, which act as electron donors in the reduction reaction (Liu *et al.,* 2013).

Antioxidant capacity varied in a pattern like the phenolic compounds, thus the species with the highest antioxidant activity was *A. rubescens*, while *A. hygrometricus* obtained the lowest activity. According to Ferreira, Barros & Abreu (2009), the antioxidant activity of different species of macromycetes is mainly related to their phenolic content, since these compounds are known to be capable of donating hydrogen to free radicals to stop the lipid chain reaction in the initial stage (due to the presence of their hydroxyl groups) (Kosanić *et al*., 2016).

Some studies demonstrate a significant correlation between total phenols and antioxidant capacity using the radicals $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, as well as those reported by different authors who mentioned correlation coefficients of 0.8 and 0.95 in several species of fungi (Kaewnarin *et al*., 2016; Smolskaite *et al*., 2015); confirming that phenolic compounds are important contributors to the antioxidant characteristics in fungal extracts. In the case of the ferric ion reducing ability (FRAP), there is no significant correlation, which indicates that not only are phenolic compounds responsible for the antioxidant activity of the species but also that other types of compounds with reducing power could be acting as antioxidants such as tocopherols, ascorbic acid, and carotenoids, which after phenolic compounds, are the main antioxidant compounds in macromycetes (Liu *et al.,* 2013; Özyürek *et al.,* 2014).

Finally, there is no direct relation between antimicrobial activity with phenolic compounds and antioxidant activity. Since *A. hygrometricus* and *L. perlatum* showed to be the species with the best antimicrobial capacity, while *A. rubescens* turned out to be the species with the highest total phenol content and antioxidant capacity. These results are similar with some authors reporting that species with high phenol content and antioxidant capacity exhibit better results in tests of antimicrobial activity (Liu *et al.,* 2013; Smolskaite *et al.,* 2015). However, these differences may be due to the extraction methods used for each test, since in most of the studies they used the same extracts for the evaluation of the antimicrobial and antioxidant activity.

Conclusions

The evaluated macromycete fungal species presented antimicrobial activity, which varied depending on the solvent, the method used, and the microorganism tested, highlighting *A. hygrometricus* and *L. perlatum*. This finding promotes these species as an important source for obtaining compounds with pharmacological activities.

The optimal method to evaluate antimicrobial activity was through the minimum inhibitory concentration (MIC) technique since it allowed the correct diffusion of the extracted compounds.

The minimum concentration reached with the ethanolic extracts was of 8.75 mg/mL in *A. hygrometricus* and *L. perlatum* for *S. aureus*; 3.75 mg/mL for *S. agalactiae* with the same fungi previously mentioned, and 6.25 mg/mL for *C. albicans* using *A. hygrometricus* and *L. laccata*. On the other hand, for methanolic extracts the growth of *S. aureus* was inhibited at 8.75 mg/mL using *A. hygrometricus*; for *S. agalactiae* the MIC was 5mg/mL using *A. hygrometricus*, *L. laccata* and *L. perlatum*, finally, *A. hygrometricus* had the best response inhibiting *C. albicans* at a concentration of 6.25 mg/mL. Generally, the ethanol extracts exerted stronger antimicrobial activity than methanol extracts, making it possible to identify the inhibitory capacity of the mushroom species.

On the other hand, it was shown that the four fungi have phenolic compounds and antioxidant properties as measured by several tests, emphasizing *A. rubescens*, which, being an edible species, has the potential to be a source of natural antioxidants and other bioactive compounds for nutrition. Yet, it is important to identify the bioactive compounds responsible for the antimicrobial and antioxidant activities in these species, since they can participate in the production of new antibiotics and cope with diseases caused by oxidative stress.

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