

Original Research Article

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Antioxidant Properties and Antimicrobial Potential of Aqueous Extract of Basidioma from *Lentinus edodes* (Berk.) Sing. (Shiitake)

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ABSTRACT

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Several scientific studies have proven that mushrooms are foods of high nutritional and medicinal value. It is also proven that mushrooms have bioactive compounds with therapeutic properties, such as the ability to produce antioxidant substances. The objective of this study was to evaluate the antioxidant, antimicrobial and chemical composition of the aqueous extract of dried Shiitake mushrooms obtained from local trade. Four methods were used for analysis of antioxidant activity: ABTS, DPPH, reducing power and chelating activity of Fe⁺². The antimicrobial activity was evaluated against bacteria and yeasts. The extraction yield was 25% and the extract presented 5.66 mg/g dry mushroom in total phenolics, 170.96 mg/g in proteins and 380.29 mg/g in total carbohydrates. The lowest EC₅₀ value found was for the ABTS method (0.201 mg/mL), but all methods showed a strong correlation with total phenolics. Some bacteria were sensitive to the extract and the two yeasts tested had a low minimum inhibitory concentration.

Introduction

Reactive species are naturally formed in aerobic metabolism and can cause membrane oxidation and damage to intracellular components such as DNA and proteins. Cells can naturally count on a system of defense against free radicals that protect them from oxidative damage. However, when this system becomes inadequate to protect us efficiently, exogenous antioxidants may be of great importance to prevent or even reduce the damage caused by reactive species (Halliwell, 2012). Several species of mushrooms have been described as a source

of antioxidant compounds and due to their important nutritional value the edible mushrooms are attractive as functional food and still as a source of drug and nutraceutical development (Barros *et al.*, 2007; Kalač, 2009).

The Shiitake mushroom (*Lentinus edodes*) fits taxonomically in the Basidiomycetes class that has approximately 20,300 species, 1,037 genera and 112 families identified. Representatives of this class have been recognized for their therapeutic properties for

millennia in China, Korea and Japan (Kim & Kim, 1999). Extracts have been obtained from Shiitake mushrooms using different extraction methods, using polar and apolar solvents versus extraction time, in order to study the bioactive properties of this mushroom. The antioxidant properties of *L. edodes* have been further studied with methanolic, ethanolic or hydroalcoholic extracts, although the aqueous extract is the closest form of which the use of mushrooms in alternative medicine is recommended. The objective of this study was to perform the aqueous extraction of dry basidiomas of *L. edodes* and evaluate their antioxidant and antimicrobial capacity.

Materials and Methods

Reagents, solvents and standards

All reagents used were of analytical standard and obtained from Sigma-Aldrich (St. Louis, MO, USA). Culture media were purchased from Difco (Detroit, MI, USA) and Himedia (Mumbai, MH, India).

Tools

For the preparation of the extract, an incubator cooled with an orbital shaker (Tecnal Laboratory Equipment, Ltda) was used. All spectrophotometric analyzes were performed using the UV-Visible-UV-1800 (Shimadzu, Corp.) spectrophotometer of 1 nm resolution. For antimicrobial analysis, a SpectraMax M2 Microplate Reader (Molecular Devices, LLC) was utilized.

Preparations of aqueous extract of *L. edodes*

The dried basidiomas of *L. edodes* (Shiitake) were purchased at a local supermarket (Maringá-Paraná). For extraction, 20 g of the ground mushroom were mixed with 200 mL of sterile distilled water. The material was

stirred (130 rpm) for 1 h at room temperature. After vacuum filtration, the extract was stored in a refrigerator. The residual solid was used for two further extractions as described above. The three volumes of extract were then pooled and lyophilized. The lyophilized material (dry extract) was stored at -20 °C. For the analysis, the dry extract was weighed and dissolved in distilled water. All tests were performed in triplicate.

Characterization of the aqueous extract of *L. edodes*

Amino acids

Free amino acids and ninhydrin-reactive amines were determined according to the methodology of Yemm and Cocking (1955), with some modifications. An appropriately diluted aliquot of the sample (1 mL) was added to 0.5 mL of 0.02 M citrate buffer. A solution of 5% ninhydrin (0.2 mL), prepared in ethylene glycol, was added to this mixture followed by 0.1 mL KCN (0.2 mM). The mixture was stirred and left in a boiling heat bath for 20 min. Ethanol 60% (1 mL) was added after cooling. The reading was carried out at 570 nm and alanine was used as standard.

Total Proteins

The determination of the total soluble proteins was performed by the method of Bradford (1976). In 250 µL aliquots of the extract diluted in water, 2.5 mL of the Bradford reagent was added. After 5 min the samples were analyzed at 595 nm. Bovine albumin was used as standard.

Total Sugars

The methodology described by Yemm and Willis (1954) was used to determine the total soluble sugar content. An aliquot of 1.0 mL of

extract diluted in water was added, to which 5 mL of the anthrone reagent was added. After stirring, the tubes were warmed in a heat-bath for 10 min and then placed in an ice bath. The absorbance was determined at 625 nm. Glucose was used as standard.

Reducing sugars

The DNS method (Miller, 1959) was used to determine reducing sugars. In a sample of extract (0.5 mL), adequately diluted, 0.5 mL of DNS reagent (3,5-dinitrosalicylic acid) was added. The sample remained in a boiling heat bath for 5 min. The volume of 5 mL of distilled water was added after the sample was cooled. The reading was at 540 nm. Glucose was used as standard.

Phenolic Compounds

The concentration of total phenolic compounds in the aqueous extract of *L. edodes* was determined by the method of Folin-Ciocalteu (Singleton & Rossi, 1965) where 2.0 mL of properly diluted sample was added 0.3 mL of sodium carbonate Na₂CO₃ 1.9 M and 100 µL of Folin reagent (1 M). The mixture was allowed to stand for 1 h in the dark and the absorbances were determined at 725 nm. Gallic acid was used as standard. The results were expressed as mg/g dry extract of gallic acid equivalents.

Antioxidant activity ABTS Method

The antioxidant activity of the aqueous extract of *L. edodes* was performed according to Carvajal *et al.*, (2013) with some modifications: the ABTS^{•+} radical was obtained by reaction between ABTS (7.4 mM) and potassium persulfate solution (2.6 mM). After 12 h in the dark, stock solution was stored and frozen until use for up to two days. To 1 mL of this solution was added 59 mL of methanol and the absorbance was

corrected to 1.1 ± 0.01. The ability to scavenging the ABTS^{•+} radical was verified by mixing 2850 µL of the ABTS^{•+} solution with 150 µL of the aqueous extract in different concentrations. The mixture remained in the dark for 2 h and absorbance was read at 734 nm (Abs sample). Distilled water was used in place of the sample as control (Abs control). The butylated hydroxytoluene-BHT (0.02%) was used as a positive control. The antioxidant capacity was calculated using the following equation:

$$\text{Scavenging ABTS radical (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

The concentration of extract that promotes 50% reduction (EC₅₀) of the ABTS^{•+} radical was calculated from the ABTS capture activity graph versus extract concentration.

DPPH Method

It was performed as described in Carvajal *et al.*, (2013). Various concentrations of aqueous extract (150 µL) were mixed with 2850 µL of the radical DPPH solution (0.1 mM). The mixture was left for 1 h at room temperature in the dark and subsequent to absorption at 515 nm. Water was used as a negative control in place of the extract and BHT (0.02%) as a positive control. The ability to sequester the DPPH radical was calculated using the following equation:

$$\text{Scavenging DPPH radical (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

The concentration of extract that promotes 50% reduction (EC₅₀) of the DPPH radical was calculated from the ABTS capture activity graph versus extract concentration.

Evaluation of the chelating activity of Fe⁺²

The analysis was performed according to the methodology described by Carvajal *et al.*,

(2013). The chelating activity of the ferrous ion was determined using 0.7 mL extract in different concentrations, diluted in 0.7 mL distilled water and mixed with 0.175 mL FeCl₂ (0.5 mM). Absorbance (Abs₀) was determined at 550 nm. After addition of 0.175 mL ferrozine (0.5 mM) the reaction was started.

The mixture was then stirred vigorously and left at room temperature for 20 min. Absorbance (Abs₁) was determined at the same wavelength. Distilled water was used as control (Abs control). EDTA (0.5 mM) was used as a positive control. The percent inhibition of the Fe⁺²-ferrozine formed was calculated as follows:

$$\text{Activity chelating (\%)} = \frac{\text{Abs control} - (\text{Abs}_1 - \text{Abs}_0)}{\text{Abs control}} \times 100$$

The concentration of extract that promoted 50% reduction (EC₅₀) of the chelating activity of Fe⁺² was calculated from the ABTS capture activity graph versus extract concentration.

Evaluation of the reducing power

The reducing power assay was performed using the method described by Soares *et al.*, (2009), with some modifications. Different concentrations of extract were prepared. For each 1.0 mL of extract 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%) was added.

The mixture was incubated at 50 °C for 20 min. After this time, 2.5 mL of trichloroacetic acid (10%) was added to the mixture. The sample was then centrifuged at 6000 rpm for 10 min; 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.25 mL of FeCl₃ solution (0.1%). The absorbance was measured at 700 nm. A negative control was prepared without extract. The BHT was used as a positive control.

Antimicrobial activity Microorganisms

The microorganisms were kindly provided by Dr. Benício Alves Abreu Filho of the Department of Basic Health Sciences - DBS/UEM. The strains used were *Aeromonas hydrophila* ATCC 7966, *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enterica* ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* (commercial - Sigma). The microorganisms were activated in Müller Hilton agar for bacteria and Sabouraud agar for fungi and incubated at 36 °C for 24 h.

Minimum inhibitory concentration (MIC)

The antimicrobial activity was determined based on the well microdilution methodology described in NCCLS (National Committee for Clinical Laboratory Standards [NCCLS], 2002) and NCCLS (National Committee for Clinical Laboratory Standards [NCCLS], 2003), with some modifications. The test basically consisted in preparing various concentrations of the extract in broth medium of the microorganisms and mixing them with an inoculum of the microorganism of interest, previously diluted in the same broth.

The extracts were prepared taking into account the final dilution in the inoculum, which in turn was adjusted to the final concentration of 1.0 x 10⁵ CFU/mL for bacteria and 2.5 x 10³ CFU/mL for fungi. The plates were incubated for 24 h in an oven at 35 °C and then the reading was carried out at 630 nm.

The MIC was determined as the lowest extract concentration capable of inhibiting the growth of the microorganism.

Minimum bactericidal/fungicidal concentration

The Minimum Bactericidal Concentration (MBC) was determined based on the methodology of Santurio *et al.*, (2007), where the wells that did not have visible bacterial growth, an aliquot of 10 μ L was removed and seeded on the surface of Müller Hilton agar.

The plates were incubated at 36 °C for 24 h. The MBC was defined as the concentration of the extract capable of causing the death of the inoculum.

To determine the Minimum Fungicidal Concentration (MFC), an aliquot of 10 μ L of each well of the microplate was seeded on plates containing Sabouraud dextrose agar, which were incubated for 24 h at 28 °C.

The MFC was defined as the concentration capable of causing the death of the inoculum. Antibiotics (gentamicin and streptomycin) and antifungal (fluconazole) were used as positive controls.

Statistical analysis

Data analysis was performed using GraphPad Prism® software version 5.0 (Graph Pad Software, San Diego, USA). The significance criterion adopted was $P < 0.05$ and all values were expressed as the mean of the independent experiments.

Sample concentrations that resulted in 50% antioxidant activity or absorbance (EC_{50}) were calculated from percentages of antioxidant activity (ABTS, DPPH and ferrous ion chelating activity) or absorbance at 700 nm (reducing power) against sample concentrations, through linear regression.

The Pearson correlation coefficient was obtained using the same program.

Results and Discussion

Productivity and chemical composition of the extract

The aqueous extract obtained from the fruiting body of the *L. edodes* mushroom presented yield of 25%. The foods rich in phenolic compounds exhibit higher yields in the extraction process with polar solvents, due to their solubility (Tsai *et al.*, 2009; Silva and Jorge, 2011). The Table 1 compares extracts obtained from *L. edodes* using solvents other than water. Although similar or even higher yields can be obtained with alcoholic or hydroalcoholic extractions (Silva and Jorge, 2011; Cheung *et al.*, 2003; Yang *et al.*, 2002), water extraction is the closest to the form of consumption, similar to the preparation of medicinal teas. Extraction using nonpolar compounds shows low yields (Table 1) and it is more interesting to use these solvents for serial extractions.

The analysis of the aqueous extract of the basidioma of *L. edodes* showed the presence of amino acids, carbohydrates, proteins and phenolics (Table 2). The concentration of phenolic compounds found in the extract was similar to that found by some researchers using the same extraction method. However, the studies show a great difference in the concentrations obtained. The differences can be due the time of extraction, temperature and composition of the basidiomas, which varies according to some factors, such as age of the fruiting body, substrate for production and cultivation conditions (Mattila *et al.*, 2001). The evaluation of the extract regarding the other components also showed differences between the values found and other findings in the literature. Zhang *et al.*, (2003) found the value of 373.19 ± 8.51 mg protein per gram of dry extract obtained from the hot aqueous extraction of the mushroom residue after ethanol extraction. The amount of total

soluble sugars found in this study was similar to that found in our study, 365.18 ± 39.18 mg/g dry extract.

Antioxidant properties of the aqueous extract of the basidioma of *L. edodes*

ABTS and DPPH

The ability to act as an antioxidant by scavenging free radicals and reactive oxygen species is considered the best properties described for polyphenols (Jung *et al.*, 2003).

The antioxidant properties of the extract were evaluated through four methods (Figure 1). The free radical sequestration, analyzed by the ABTS and DPPH methods, occurred at the different extract concentrations tested (Fig. 1A and Fig. 1B). The antioxidant activity increased with the increase of the sample concentration, totalizing almost 100% of ABTS⁺ radical scavenging at 5 mg/mL extract (Fig. 1A). This same concentration was able to cause approximately 62% of DPPH radical scavenging. In this test, the antioxidants that are able to reduce the DPPH radical (purple colored) for the non-radical form (yellow colored) (Chowdhury *et al.*, 2015).

Ferrous ion chelating activity and reducing power

Radicals can be formed, initially, from the action of transition metals. In stabilizing of transition metals in the living systems, chelating agents are able to inhibit the generation of radicals, also reducing damage to the organism. In the method of the chelating activity of the ferrous ion, ferrozine forms a complex with Fe⁺² resulting in a reddish coloration, being that the presence of chelating agents in the reaction can prevent the formation of ferrozine-Fe⁺² complex resulting in decreased staining. The ability of

the aqueous extract of *L. edodes* to chelate the ferrous ion is shown in Fig. 1C. Low concentrations of the extract were sufficient to cause the chelation of 92% of the ions present in solution.

In the test of reducing power, the yellow coloration of the solution changes to several shades of green and blue, depending on the reducing power of each compound (Barros *et al.*, 2007). The presence of reducers causes the conversion of the Fe⁺³/ferricyanide to the ferrous form; measuring the apparent staining at 700 nm it is possible to determine the concentration of Fe⁺². Reducing power increases with absorbance. The reducing power of the extracts of the basidioma of *L. edodes* is represented by Fig. 1D. In this test, 166 mg/mL extract showed the absorbance of 0.850.

EC50 values and correlation with phenolic compounds

The EC₅₀ values found for the antioxidant activity tests are summarized in Table 3. The use of more than one method to evaluate the antioxidant activity of extracts of different types of foods, takes into account that different antioxidant compounds can act *in vivo* through different mechanisms (Carvajal *et al.*, 2013). The EC₅₀ value obtained by the ABTS method was twice as low as the concentration required to scavenging 50% of the DPPH radical (Table 3). High EC₅₀ values were found for chelating ion activity and reducing power (Table 3).

The antioxidant activity of fruits, juices, wines, mushrooms and other foods can be associated with the presence of polyphenols, vitamins, carotenoids and minerals. In relation with the antioxidant properties of mushroom extracts, compounds such as organic acids (Kayashima and Katayama, 2002), phenolics (Cheung and Cheung, 2005), polysaccharides

(Thetsrimuang *et al.*, 2011) and peptides (Xie *et al.*, 2008) have been reported as antioxidants, but polyphenols are the compounds that have been most associated with these properties (Cheung *et al.*, 2003). In our work a strong correlation was found between the phenolic concentration and the antioxidant activity evaluated by the four study methods employed (Table 3). However, because it is a crude extract, the other

compounds whose concentrations were determined in this study may also present some type of antioxidant activity. As antioxidant activity may be a property found in different types of molecules, the isolation and characterization of the components of the extract would lead to a better understanding of the molecules responsible for the antioxidant activity presented by the aqueous extract of *L. edodes*.

Table.1 Obtaining extracts of basidioma from *L. edodes*

Extraction conditions	Yield (%)	Total phenolics (mg/g)	Extraction time	Reference
Water	25	5.66±0.057	1 h*	This study
Water	29.92	120.44±1.09	30 min.	SILVA & JORGE, 2011
Water	16.2±0.80	1.33±0.04	3 h	CHEUNG; CHEUNG; OOI, 2003
Ethanol/Water (1:1)	23.44	45.33±0.61	30 min.	SILVA & JORGE, 2011
Ethanol	11.46	20.00±0.22	30 min.	SILVA & JORGE, 2011
Methanol/water (1:1)	29.65	43.13±0.66	30 min.	SILVA & JORGE, 2011
Methanol	18.8	6.27 ±0.02	24 h	YANG; LIN; MAU, 2002
Methanol	33.5±1.75	4.79±1.2	3 h	CHEUNG; CHEUNG; OOI, 2003
Petroleum ether	2.4±0.08	0.44±0.07	3 h	CHEUNG; CHEUNG; OOI, 2003
Ethyl acetate	3.65±0.18	0.033±0.01	3h	CHEUNG; CHEUNG; OOI, 2003

*With the residue from the first extraction plus two extraction cycles were performed.

Table.2 Content of the major constituents of the aqueous extract of *L. edodes*

Constituents	Content (mg/g dry extract)*
Amino acids and ninhydrin-reactive amines ^a	28.10 ± 0.86
Total carbohydrates ^b	378.23 ± 8.46
Reducing carbohydrates ^b	89.95 ± 3.58
Non-reducing carbohydrates ^b (by difference)	280.28 ± 4.94
Total phenolics ^c	5.66 ± 0.057
Total proteins ^d	171.65 ± 8.86

*The results were expressed as mean ± standard deviation (n = 3). ^aalanine equivalents; ^bglucose equivalents; ^cgallic acid equivalents; ^dalbumin equivalents.

Table.3 Antioxidant activity of the aqueous extract of *L. edodes* (EC₅₀) and Pearson's correlation coefficient (*r*) between extract concentrations and total phenolics

Methods	EC ₅₀	Pearson <i>r</i>	R ²	Meaningfulness
ABTS	0.191±0.001	0.9801	0.9606	**
DPPH	0.400±0.004	0.9989	0.978	***
Ferrous ion chelating activity	1.488±0.004	0.9552	0.912	*
Reducing power	0.574±0.001	0.9584	0.9185	*

*Significant (P> 0.01 e <0.05); **very significant (P> 0.001 e <0.01); ***extremely significant (P <0.001).

Fig.1 Antioxidant activity of the aqueous extract of the basidioma of *L. edodes*. A: ABTS; B: DPPH; C: chelating activity of Fe⁺²; D: reducing power

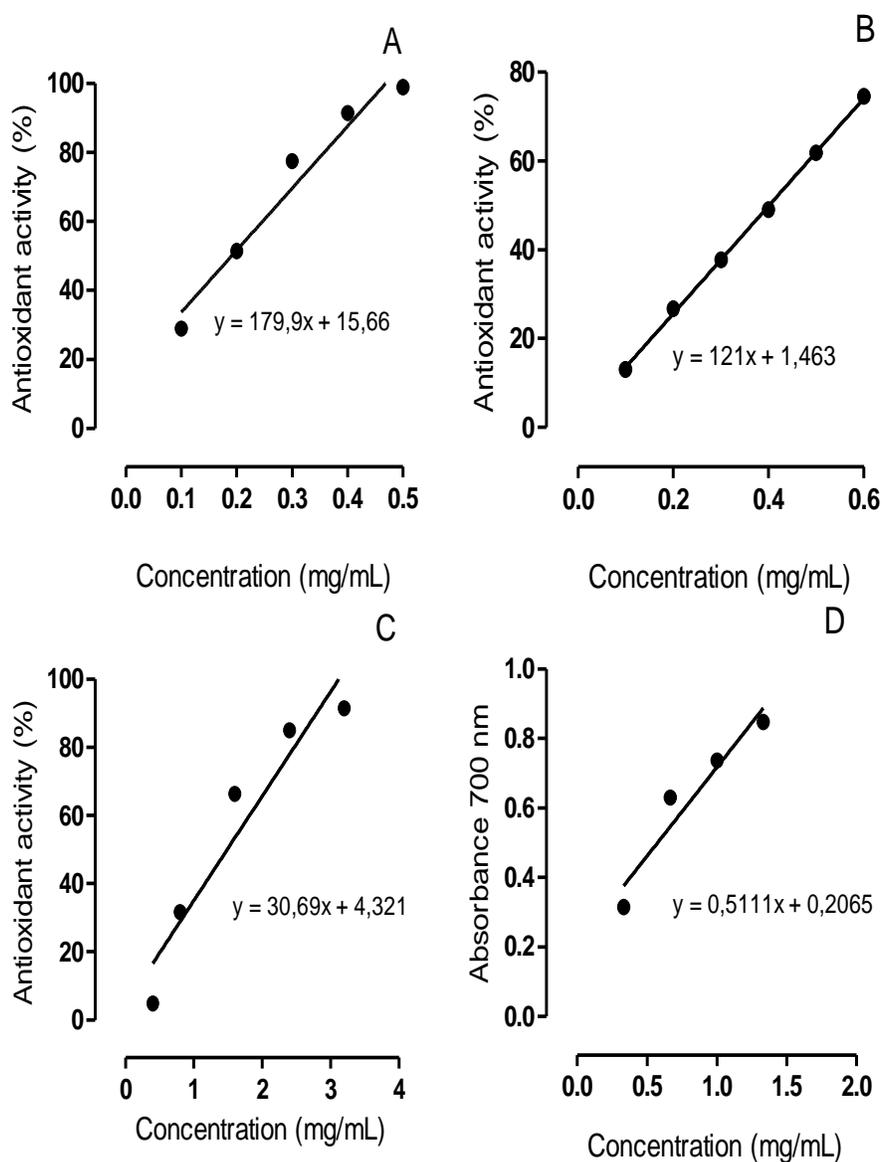
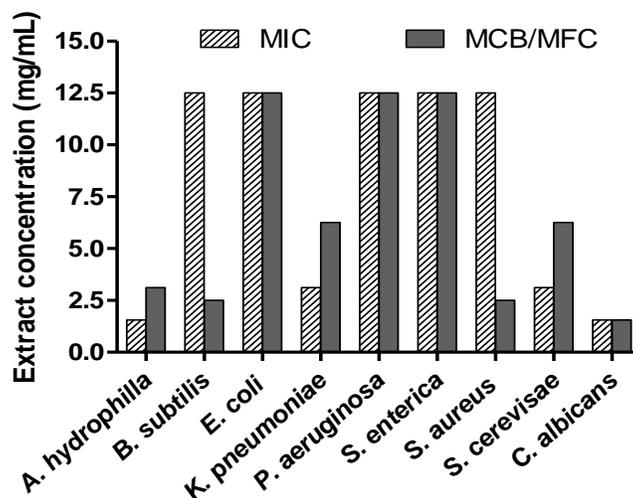


Fig.2 Antimicrobial activity of the aqueous extract of *L. edodes*. MIC (Minimum Inhibitory Concentration); MBC (Minimum Bactericidal Concentration); MFC (Minimum Fungicidal Concentration)



Antimicrobial potential of aqueous extract of basidioma of *L. edodes*

The evaluation of the antimicrobial activity of the extract was performed using the microdilution method in wells. The minimum inhibitory concentration and the minimum bactericidal/fungicidal concentration are showed in the Figure 2. The most sensitive bacteria/ fungi were, in the following order, *A. hydrophilla*, *C. albicans*, *K. pneumoniae* and *S. cerevisiae* with the lowest values of MIC. The other microorganisms tested had the same MIC values (12.5 mg/mL extract). In relation to MCB the most sensitive bacteria were *A. hydrophilla*, *B. subtilis* and *S. aureus* with values of 3.12; 3.0 and 2.50 mg/mL, respectively. Low MFC values (1.56 mg/mL) were found for *C. albicans*.

Extracts from various species of mushrooms have been evaluated for the ability to inhibit the growth of clinically important bacteria (Vamanu, 2012) and different types of extract obtained from basidiomas of *L. edodes* have shown antimicrobial potential. The study conducted by Hirasawa *et al.*, (1999) showed

that three different types of extracts obtained from dry basidiomas of *L. edodes* had action on oral cavity bacteria using the microdilution method in wells. The extracts of chloroform, ethyl acetate and water had their lowest inhibitory concentrations below 50 mg/mL against different species of Gram-positive and Gram-negative bacteria. Contrasting with these results, microorganisms of the most common genera, but also clinically important (*Enterococcus*, *Staphylococcus*, *Escherichia*, *Bacillus* and *Candida*) were resistant. Among the species evaluated in our study, *C. albicans*, *S. aureus*, *S. enterica*, *E. coli* and *B. subtilis* were sensitive to dosages lower than 12.5 mg/mL extract.

The aqueous extract of dried basidiomas of *L. edodes* showed to have antioxidant capacity against different study methods with strong correlation for phenolic compounds. The antimicrobial activity tested against different bacteria and the yeast *C. albicans* resulted in low extract concentration values that inhibited the growth or caused the death of microorganisms of clinical importance, which means that the *L. edodes* bioactives once

isolated can be evaluated for future biotechnological applications.

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