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*Full Length Research Paper*

# Evaluation of antioxidant, immunomodulating, cytotoxic and antimicrobial properties of different strains of Basidiomycetes from Northeastern Mexico

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**A great variety of mushrooms (Basidiomycetes) grow in the Mexican territory, but most of their properties, including pharmacological ones, have been scarcely investigated. Samples of culture broth of ten different strains of Mexican Basidiomycetes cultured in four growing times were taken and lyophilized; then, their antioxidant, immunomodulating, cytotoxic and antimicrobial properties were studied, the antioxidant activity was measured with the dichlorodihydrofluorescein diacetate (DCFDA) assay, the immunomodulating properties were evaluated with the Cunningham´s technique in BALB/c male mice, cytotoxicity was estimated with the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in Chang liver cells and the antimicrobial activity was evaluated with the microdilution method against *Escherichia coli, Staphylococcus aureus, Candida albicans, Mycobacterium smegmatis and Sporothrix schenkii*. Samples of the 1st and 2nd month of growth of *Suillus luteus, Lentinus lepideus,* and *Suillus lakei* demonstrated relevant antioxidant activity (20 - 80%); sample of *S. lakei* showed significant immunostimulation, near 130% with Cunningham´s technique; none of the samples showed cytotoxicity. *Ganoderma applanatum, Armillaria mellea* and *S. lakei* demonstrated antimicrobial activity. The phytochemical screening was assayed for the most active sample (*S. lakei* at two months of growth) and it was positive for: alkaloids and tannins; the content of proteins, carbohydrates and total phenols were calculated. The most active sample was fractionated and the fractions were biologically evaluated. Phytochemical screening, content of proteins, carbohydrates and total phenols were determined, and an IR (infrared) spectra was carried out.**

**Key words:** Basidiomycetes, antioxidant, immunomodulator, antimicrobial.

## INTRODUCTION

Basidiomycetes, also called macromycetes, among the many diverse organisms, are a major source of biologically active natural products (Liu, 2004); they provide a rich variety of active secondary metabolites (Turner, 1971). The number of different mushroom species on earth is estimated at 140000, of which may be only 10% are known, and from those, aproximately 700 species are known to possess significant pharmacological properties (Lull et al., 2005; Chang, 1996).

Higher basidiomycetes mushrooms have been used in folk medicine throughout the world since ancient times (Wasser, 1999; Statements, 2000); they have a wide spectrum of therapeutic and prophylactic properties, among the most important ones are immunomodulating and antitumoral activities of various species (Shamtsyan,

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2004); medicinal mushrooms useful against cancer are known in China, Russia, Japan, Korea, as well as the USA and Canada. Also, there are a large number of mushroom-derived compounds -cellular components and secondary metabolites that could be used to treat a variety of other disease states, such as compounds with potential hypoglycemic, antithrombotic, lowering cholesterol, antimicrobial, cytotoxic, antihypertensive, hepatoprotective properties, among others (Brizuela et al., 1998). Some of those bioactive compounds obtained from different basidiomycetes are a variety of terpenes, sesquiterpenes, steroids and polysaccharides (alone or forming complexes with proteins, like PSK), among others (Lindequist and Niedermeyer, 2005). Most of their main pharmacological properties –like their antitumoral activity- have been atributed to their ability to modulate the immune system. Most of them are immunostimulant but others are immunosuppressive (Daba and Ezeronye, 2003).

On the other hand, the increase on oxidative stress, which particularly occurs with aging, may be one of the contributing factors in the neuronal death occurring after any ischemic/hypoxic insult, and in neuro-degenerative disorders such as Alzheimer's and Parkinson´s diseases. Also, oxidative damage, caused by the action of free radicals may initiate and promote the progression of a number of chronic diseases, including cancer, cardio-vascular diseases and inflammation. Oxidative stress in cells can result from either an increase in the levels of reactive oxygen species, or a reduction of the natural cell antioxidant capacities (Racchi et al., 2002). Antioxidants act as a major defense against radical-mediated toxicity by protecting the damage caused by free radicals (Janardhanan, 2000). Mau et al. (2002) reported a great antioxidant activity in *Ganoderma lucidum*, and found that phenols are the major naturally occurring antioxidant compounds.

Finally, the fact that basidiomycetes have been insufficiently investigated, together with the broad range of structural types of antibiotics which could be produced by these organisms, suggests that they may be a source of new and useful bioactive compounds, without any microbial resistance (Suay et al., 2000; Obuchi et al., 1990; Hirasawa et al., 1999; Atvani 2001). The *in vitro* culture of Basidiomycetes allows controlling growth conditions (pH, humidity, temperature and dark- light cycles) and eliminating contaminants that could interfere with the evaluation of biological activities. Another advantage of growing Basidiomycetes *in vitro* in a liquid medium is that the Basidiomycetes have the ability to produce secondary metabolites and secrete them into the culture broth, so that the compounds can be easily extracted from it. When the Basidiomycetes are grown in liquid medium, the secondary metabolites can be found in the medium as in the mycelium. For the same compound, the distribution between the liquid medium and the mycelium is related to the water solubility and the capability to cross the cell membrane (Turner, 1971; Smith et al., 2002).

## MATERIALS AND METHODS

### Materials

#### *Strains and cell lines*

The carpophores of *Lentinus lepideus, Armillaria tabescens, Calvatia cyathiformis, Coriolus versicolor, Ganoderma applanatum, Ganoderma sp, Suillus luteus, Suillus lakei, Ganoderma lobatum* and *Armillaria mellea* were collected from pine-oak forest in the state of Nuevo Leon, Mexico. These strains are some of the Basidiomycetes most commonly found in the North eastern Mexico. The mushrooms were classified and preserved at the Faculty of Forestry Sciences, UANL (*Armillaria tabescens*: A1, *Armillaria mellea*: A2, *Calvatia cyathiformis*: B1, *Coriolus versicolor*: B2, *Ganoderma applanatum*: C1, *Ganoderma lobatum*: C2, *Ganoderma* sp: C3, *Lentinus lepideus*: D1, *Suillus lakei*: E1, *Suillus luteus*: E2). Strains of *Escherichia coli* 25922, *Candida albicans* 90028 and *Staphylococcus aureus* 25923 were purchased from ATCC and strains of *Candida albicans* Z-83, *Mycobacterium smegmatis* LR-222 and *Sporothrix schenkii* 713 and 1458 were obtained from the collection of University Hospital Jose Eleuterio Gonzalez. The Chang liver cell line was purchased from American Type Culture Collection (ATCC CCL13).

#### *Animals*

Male BALB/c mice between 8 - 12 weeks old were divided into groups (n = 4) and kept in animal facilities at 24 ± 2°C in a 12 h light-dark cycle with free access to standard rodent chow and water *ad libitum*. These experiments were performed in compliance with the appropriate laws and institutional guidelines of the Universidad Autonoma de Nuevo León and the International Guiding Principles for Biomedical Research Involving Animals.

### Growth of tested microorganisms

Pieces from the centre of the carpophores were isolated and placed on solid Melin Norkrans medium. The strains were cultivated for two weeks at 25°C; after that, pieces of the mycelium were removed from the agar plates and transferred into flasks containing Melin Norkrans culture broth at pH 6.5; the flasks were maintained at 25°C until the samples were taken. All bacterial strains were cultured in Saboraud-dextrose agar (Oxoid Ltd), except *Mycobacterium smegmatis*; it as *Sporothrix schenki*i was cultured in Blood agar. The cell line was maintained in minimal essential medium MEM supplemented with 10% of fetal bovine serum.

### Reagents

All the reagents were purchased from Sigma- Aldrich (St Louis, Missouri, USA).

### Pharmacological assays

The samples tested were taken at time: 0, 1 week, 1 and 2 months of growth, At each time, the culture broth was filtered through Whatman No 1 filter paper, in order to separate the liquid from the mycelium, and the liquid samples were freeze dried and stored at 4°C until their evaluation.The antioxidant activity was determined by the 2´-7´-dichlorodihydrofluorescein diacetate (DCFDA) method (Bass et al., 1983). The concentration of the samples was from 10 mg/mL to 0.625 mg/mL, ascorbic acid was used as a positive control for the antioxidant activity. Chang cells at confluence were

exposed to the samples solutions; after the exposure, DCFDA was added to each well and then the plate was incubated at 37°C; fluorescence was measured at excitation and emission wavelengths of 485 and 525 nm, respectively; measurements were taken each hour over a period of 6 h. (Luminiscens spectrophotometer LS50B, Perkin Elmer). For the evaluation of cytotoxicity, Chang liver cells were grown as monolayers in minimal essential medium supplemented with 10% fetal bovine serum and kept at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

Cells were harvested and plated at a density of 5 x $10^5$ cells per well in 96-well plates. Cells were exposed for 72 h to twelve concentrations of each extract (2 mg/mL to 0.098 mg/mL). Three wells were used for each concentration tested. Cytotoxicity was determined by the Microwave Theory and Techniques MTT assay reported by Mossman (1983). The reduction of MTT by mitochondrial dehydrogenase enzymes of viable cells to a blue formazan product was measured spectrophotometrically at 570 nm.

The immunomodulating effect was assessed using the Cunningham hemolytic plate assay, as previously described (Cunningham and Szenberg, 1968). Briefly, aqueous extracts were given by gastric gavage at single dose of 40 mg/Kg to Balb/c mice, after oral administration, mice were immunized intraperitoneally with 0.2 mL of 10% sheep red cells suspension. After 72 h of immunezation the mice were sacrificated by cervical dislocation. The spleen was removed and then the number of plaque forming cells (PFC) was determinated by the method of Jerne modificated by Cunningham (1968). Negative control group was immunized with 0.2 mL of Saline 0.85% and positive control was immunized with 0.2 mL of 10% sheep red cells suspension. These control groups received the extract solvent (water) by gastric gavage.

The antimicrobial activity was tested using the microdilution method. Briefly, Müeller Hinton broth with divalent cations ($MgCl_2$ and $CaCl_2$) was used for all the microorganisms; the inoculums were 0.1 mL from a solution adjusted to 1 in Mc Farland scale, and they were put in a 96-well plates with 0.1 mLof sample solution (the sample solution was previously diluted; the concentration tested were from 16 µg/mL to 8 mg/mL). The plates were incubated at 37°C for 48 h. Turbidity was observed and compared with control (Singh et al., 1994).

The phytochemical screening was developed as follows: the content of proteins was determined by the Lowry technique (1951), carbohydrates by the Sulphuric- phenol method (Dubois et al., 1956) and total phenols by the Folin-Ciocalteau described by Singleton and Rossi (1965). Also, a qualitative phytochemical screening (Dominguez, 1973) was performed; the reactions that were made are: Alkaloids with Dragendorff, Mayer and Wagner; Flavonoids with Shinoda test (Mg y HCl); Tannins with a solution of $FeCl_3$ 10% and the confirmatory test of agar and NaCl; Saponines with the foam formation test; Triterpenes and/or esteroids with Liebermann-Burchard reaction; Volatil coumarines with NaOH and UV light; and Cardiotonic glucosides with Baljet reaction.

The IR spectra were obtained with a Fourier tranform IR spectrometer 1710 Perkin Elmer. ANOVA test were performed as statistic analysis of the corresponding results.

## RESULTS AND DISCUSSION

None of the samples tested had cytotoxic activity. *S. lakei* at 10 mg/mL, was the sample that caused a major reduction of viability with a 20% of reduction at the highest concentration, considering as the 100% of viability, cells grown just with the adequate medium, without any cytotoxic compound (Figure 1). This result was expected because it has been reported for some Basidiomycetes that grow in Asia that their effectiveness against cancer and, even, infections is due to an indirect effect of Basidiomycete´s metabolites in the immune system, those enhanced in different ways in the immune system, but the metabolites do not cause a direct damage over the cancer cells or the pathogen micro-organisms (Statements, 2000; Daba and Ezeronye, 2003; Takimoto et al., 2004; Ramirez et al., 2006). The lack of cytotoxicity of the Basidiomycetes suggest that the secondary metabolites evaluated may show some advan-tages over the actual quimotherapy treatments, because they would not produce the classical toxic effects related with non differentiated cytotoxicity.

Samples from one and two months of growth of *S. luteus, L. lepideus* and *S lakei* had the most important antioxidant property with a 20, 40 and 80% of reduction of oxidative damage caused by Xantine Oxidase, the first month and 50, 60 and 75% of reduction the second month, respectively, compared with the reduction of oxidative damage achieved by the ascorbic acid that was of 50% (Figures 2, 3 and 4). The fact that the culture broth was more active than the vitamin C applied at the same concentration w/w is relevant because the culture broth is not a purified compound. Most of the Basidiomycetes that have been studied in Asia, produce a great variety of antioxidant compounds, most of them phenols, but others, proteins with residues rich of –SH groups (Takimoto et al., 2004; Russell and Paterson, 2006). Liu et al. (1996) reported that samples of mycelium, carpophore and culture broth of *C. versicolor, L. edodes, G. lucidum* and *Tricholoma lobayense,* showed antioxidant activity produced by the capture of hydroxil and superoxide radicals. Also Mau et al. (2002) published that several strains of Basidiomycetes from Taiwan, have an interesting antioxidant activity.

The immunomodulating assay was performed with the most active samples from the antioxidant test: the sample of 2 month-growth of *S. lakei* and L. *lepideus. S. lakei* showed an important immunostimulant activity with an increase of 124% in the number of antibody forming cells, compared with control (Table 1). The immunostimulant effect found, give Mexican Basidiomycetes the capability to prevent and even treat cancer with metabolites that do not show the same adverse effects as many of the traditional drugs that are used in the treatment of different kinds of tumors, because Basidiomycetes do not cause any damage over the patient's cells directly. The antitumoral effect of compounds isolated from culture broth of Basidiomycetes that grow in different countries has been reported (Liu et al., 1995; Ooi and Liu, 2000). As an example, there is a report of the compound called PSPC (polysaccharide-protein complex) isolated from the culture broth of *T. lobayense*.

There are, also, multiple reports of *in vitro* and *in vivo* antitumoral activity produced by compounds isolated from mycelium and carpophores of Basidiomycetes that grown in Asia; antitumoral activity on most cases has been mediated by immunostimulation (Yin et al., 2007;

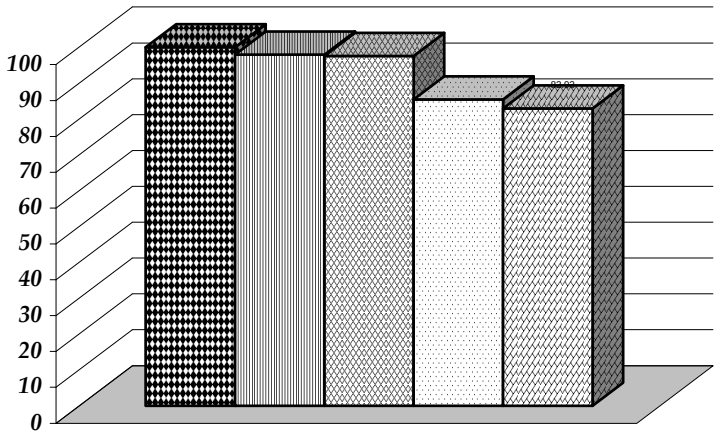


**Figure 1.** Percentage of cell viability after the exposure to a sample of two months growth of *S. lakei* at different concentrations (from left to right: control 100% cell viability; 0.3, 0.6, 1.25, 2.5 mg/mL of *S. lakei* extract) for 48 h.

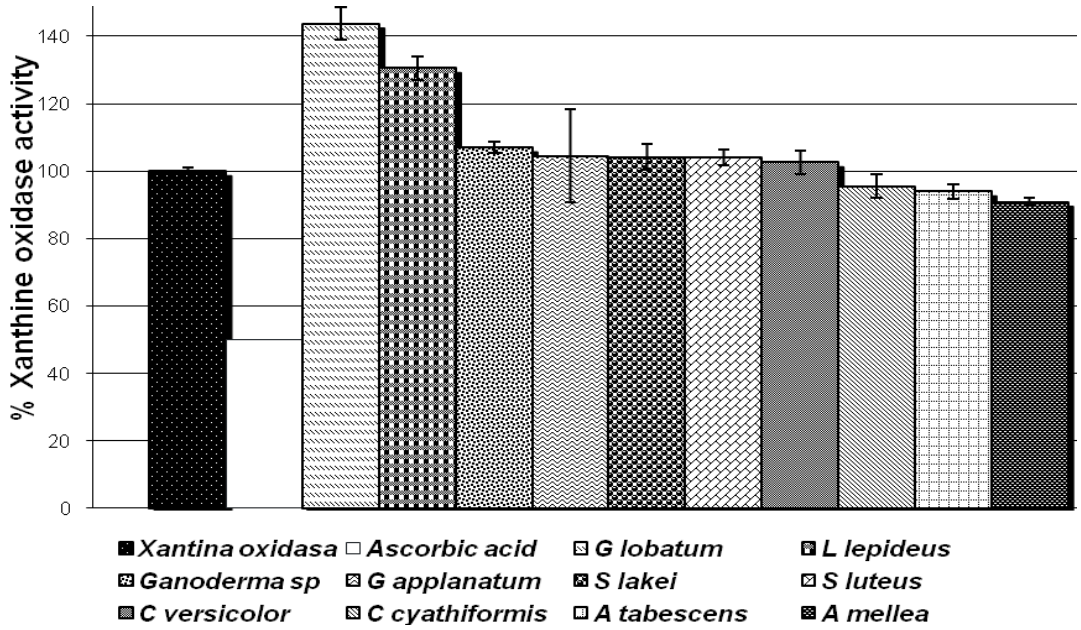


**Figure 2.** Percentage of activity of Xanthine oxidase in absence and presence of the samples of Basidiomycetes of one week of growth.

Carbonero et al., 2006; Daba and Ezeronye, 2003; Takimoto et al., 2004; Tzianabos, 2003; Gomez et al., 2006; Sorimachi et al., 2001). That is the case of the lentinan, compound isolated from the mycelium and carpophore of *L. edodes* that was patented because of its antitumoral activity mediated by its stimulative effect over the immune system (Nanba et al., 1987). Also, it was demonstrated that the β-D- glucanes obtained from *G. lucidum* have an antitumoral activity because of an increase of cytoquines release made by activated macrophages (IL-1β, IL-6, TNF-α) and T-lymphocytes (IFN-γ); this activity is mediated by the transcriptional factor NF-κB activation. This factor is involved in the regulation of the immune response mediators (Kuo et al., 2005).

Samples of *G. applanatum* and *S. lakei* of two-month-growth at concentration of 8 mg/mL showed antimicrobial activity against *S. schenkii 1458* and *A. mellea* showed antimicrobial activity against *E. coli, S. aureus, M. smegmatis* and *S. schenkii 1458* (Table 2). The results

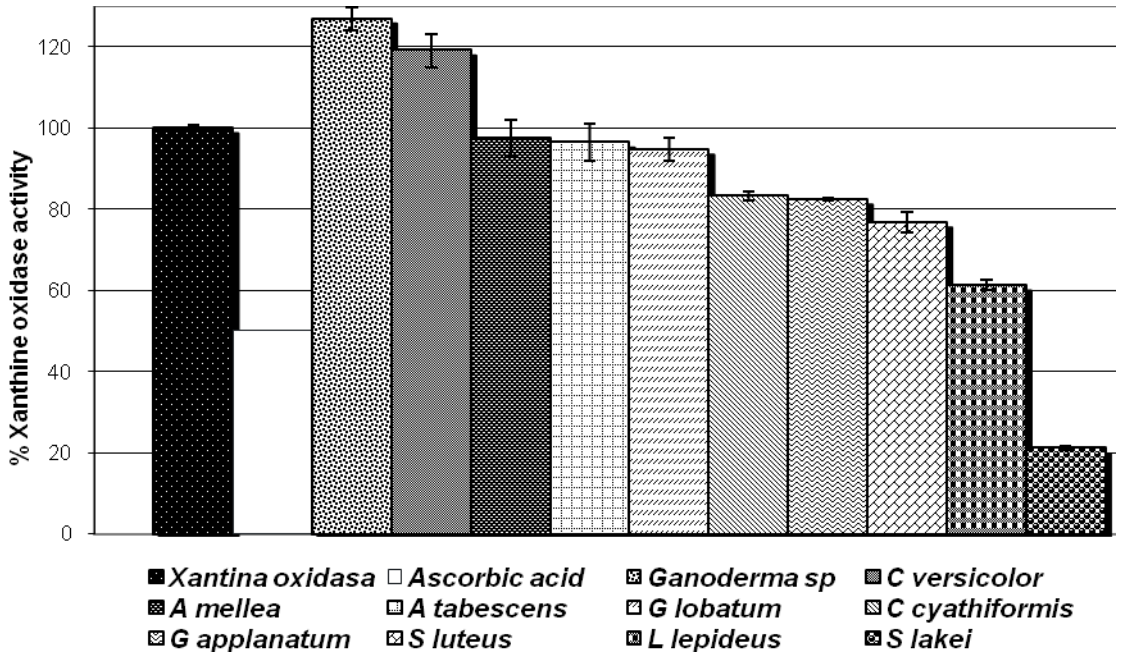


**Figure 3.** Percentage of activity of xanthine oxidase in absence and presence of the samples of Basidiomycetes of one month of growth.

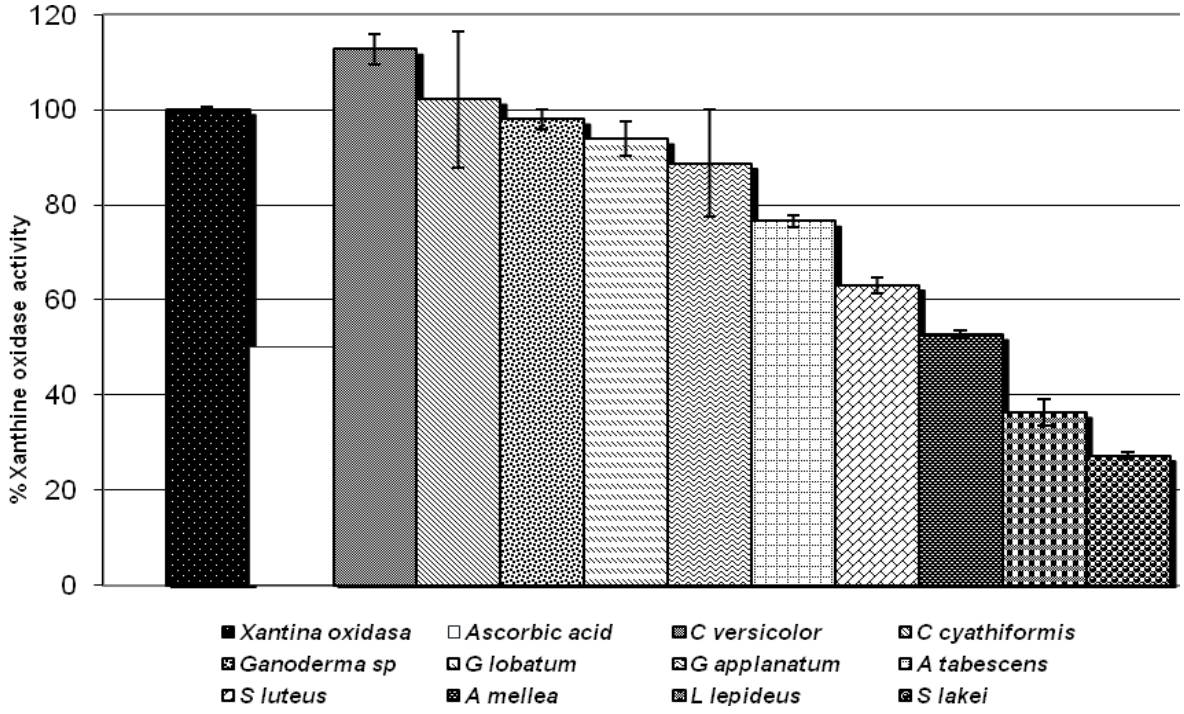


**Figure 4.** Percentage of activity of xanthine oxidase in absence and presence of the samples of Basidiomycetes of two months of growth.

are relevant considering the rising of the incidence of resistance of different microorganisms against known antibiotics. Besides the reports of antimicrobial activity *in vivo* where the main mechanism of action is due to the

**Table 1.** Immunomodulating results of *S. lakei* and *L. lepideus* with the Cunningham´s technique.

| Antigen | PFC/Million |
|---|---|
| NaCl 0.85% (Negative Control ) | 0 |
| Sheep RBC 10% (Positive control) | 54 ± 3 |
| *Lentinus lepideus* | 53 ± 9 |
| *Suillus lakei* | 121 ± 31 |

**Table 2.** Antimicrobial activity results for the ten strains of Basidiomycetes tested against *E. coli, S. aureus, C. albicans, M. smegmatis* and *S. schenkii.* (1. *C. versicolor*; 2. *L. lepideus*; 3.*Ganoderma sp*; 4. *G. applanatum*; 5. *G. lobatum*; 6. *C. cyathiformis*; 7.*S. luteus*; 8. *S. lakei*; 9.*A. mellea,* 10. *A. tabescens* ).

| Microorganisms | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| *E. coli* | - | - | - | - | - | - | - | - | + | - |
| *S. aureus* | - | - | - | - | - | - | - | - | + | - |
| *C. albicans ATCC* | - | - | - | - | - | - | - | - | - | - |
| *C. albicans Z-83* | - | - | - | - | - | - | - | - | - | - |
| *M. smegmatis* | - | - | - | - | - | - | - | - | + | - |
| *S. schenkii 713* | - | - | - | - | - | - | - | - | - | - |
| *S. schenkii 1458* | - | - | - | - | - | - | - | + | + | - |

**Table 3.** Results of the qualitative phytochemical screening of the sample of *S. lakei* of two months of growth.

| Test | Result |
|---|---|
| Alkaloids | + |
| Flavonoid | - |
| Tannins: | + |
| Saponines | - |
| Triterpenes | - |
| Volatil coumarines | - |
| Cardiotonic glucosides | - |

immunostimulation of the host, there has been reported antimicrobial activity of extracts or isolated compounds obtained from mycelium and carpophores against different microorganisms in *in vitro* assays (Rosa et al., 2003; Ofodile et al., 2005; Buenafe et al., 2005; Suay et al., 2000, Park et al., 2009). Anchel et al. (1948) demonstrated that samples of culture broth of *Coprinus similis* and *Lentinus degener* had antimicrobial activity against *S. aureus*.

A qualitative phytochemical screening was performed and the sample of *S. lakeii* of two months of growth was positive for alkaloids and tannins (Table 3). The content of proteins, carbohydrates and total phenols was calculated also for the sample of *S. lakei* of two months of growth; the sample contained 43% of proteins, 1.3% of carbohydrates and 5.6 g of gallic acid per 100 g of freeze dried sample as total phenolic compounds. It suggested an important role of phenols in the antioxidant effect of *S. lakei* and a relevant impact of the complex protein-carbohydrate regulating the immune system.

**Table 4.** Immunomodulating results of fractions of *S. lakei* with the Cunningham´s technique.

| Antigen | CFP/Million |
|---|---|
| NaCl 0.85% (Negative Control ) | 0 |
| Sheep RBC 10% (Positive control) | 29 ± 6 |
| Fraction I | 45 ± 9 |
| Fraction II | 33 ± 9 |
| Fraction III | 18 ± 3 |
| Fraction IV | 87 ± 11 |

**Table 5.** Antimicrobial activity results for fractions of *S. lakei* tested against *E. coli, S. aureus, C. albicans, M. smegmatis* and *S. schenkii.*

| Cepas | FI | FII | FIII | FIV |
|---|---|---|---|---|
| *E. coli* | + | - | + | + |
| *S. aureus* | + | - | + | + |
| *C. albicans ATCC* | + | - | - | + |
| *C. albicans Z-83* | + | - | + | + |
| *M. smegmatis* | + | - | + | - |
| *S. schenkii 713* | + | - | + | - |
| *S. schenkii 1458* | ++ | - | ++ | + |

*S. lakei* was the most active Basidiomycete tested; it demonstrated a relevant antioxidant, immunostimulant and antimicrobial activity, without any cytotoxic effect. Considering the results, a bioassay guided fractionation of samples of *S. lakei* was carried out in order to know which fraction has the active principles. Four fractions were obtained FI (ethanol fraction), FII (hexanic fraction), FIII (ethyl acetate fraction) and FIV (butanolic fraction) and their immunomodulating, antioxidant and antimicrobial activities were evaluated by the methods described previously.

As expected, none of the fractions tested had cytotoxic activity. A reduction of 60% and 45% of oxidative damage caused by xanthine oxidase was observed with FIV and FIII, respectively, at a concentration of 1 mg/mL (Figure 5). Fraction IV was the fraction that showed a major enhancement of the number of antibody forming cells, with an increase of 181% compared with control (Table 4). Fraction FIII (1 mg/mL) showed antimicrobial activity against *E. coli 25922*, *S. aureus 25923, C. albicans Z-83, M. smegmatis LR-222* and *S. schenkii 713* and 1458 and Fraction FIV was active against all the strain with exception of *S. schenkii 713* (Table 5).

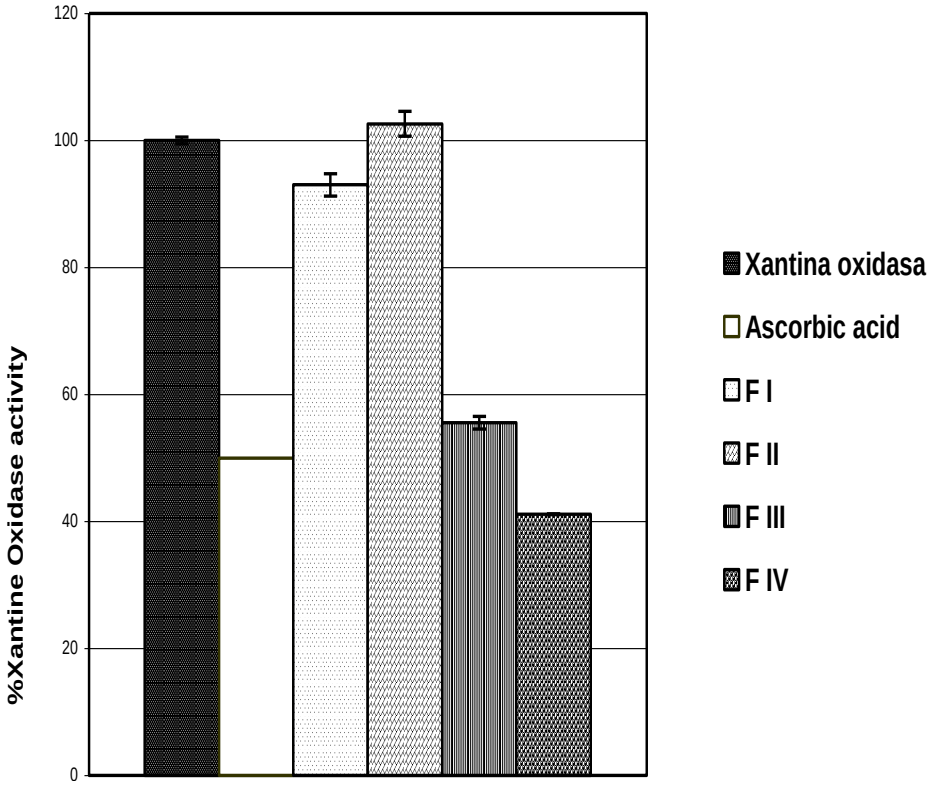


**Figure 5.** Percentage of activity of xanthine oxidase in absence and presence of the samples of fractions of *S. lakei* of two months of growth.

The most active fraction –fraction IV- was then characterized; the fraction IV had 13% of proteins, 57% of carbohydrates and 25 g of gallic acid per 100 g of freeze dried sample as total phenolic compounds. Also, an IR spectrum was carried out, and there was a intense and broad sign at 3449 $cm^{-1}$ which is characteristic of -OH bounds when they are forming part of a glycoside; also, there was a sign of mild intensity at 1719 $cm^{-1}$ that is assigned to functional groups –C=O; the signals at 1653 and 1638 $cm^{-1}$ are because of the presence of –C=C bound of aromatic type, there was a sign at 1348 $cm^{-1}$ indicative of -C-H bounds form alkane groups and finally two bands at 110 and 1200 correspond to –C-O- bounds related with the sign of 3449 $cm^{-1}$.

The results of this study showed that as Oriental's Basidiomycetes species, the Mexican Basidiomycetes are a potential source of non-toxic compounds with immunomodulating, antioxidant and antimicrobial activity. Further studies will be carried out for the characterization of the compounds present in the fractions that are responsible for the pharmacological properties.

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