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Full Length Research Paper

Hypoglycemic and hypocholesterolemic effects of aqueous and methanolic extracts of *Lentinus lepideus*, *Calvatia cyathiformis* and *Ganoderma applanatum*, from Northeastern Mexico, in Wistar rats

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The hypoglycemic and hypocholesterolemic activity of three fungi basidiomycetes from Northeastern Mexico were investigated in this study. Aqueous and methanolic extracts from *Lentinus lepideus*, *Calvatia cyathiformis* and *Ganoderma applanatum* were orally administered to alloxan-induced diabetic rats. Blood glucose and cholesterol was determined using an Accutrend Glucometer (Roche, Germany) at 0, 3 and 6 h after treatment administration. The rats that received the methanolic extract of *L. lepideus* had significantly lower glycemic levels at 3 (30%) and 6 h (35%). In contrast, the methanolic extracts of *C. cyathiformis* and *G. applanatum* had no significant differences when compared with the control group. No statistical difference in cholesterol levels was observed in the experimental groups when compared with the control group with any of the administered extracts.

Key words: Basidiomycetes, hypoglycemic, hypocholesterolemic, glibenclamide.

INTRODUCTION

Plants have always been useful sources of drugs and many of the currently available drugs have been directly or indirectly produced from plants. In accordance with the recommendations of the WHO Expert Committee on diabetes mellitus, it is important to investigate the hypoglycemic action of plants that were originally used in traditional medicine.

Mexico is a country known for its large diversity of fungi (Chalenger, 1998; Dominguez, 1973) and is considered that 200,000 species exist in the country, of which about 7000 are known (Guzman, 1998). In mycological studies, basidiomycetes (macrofungi) are the best known; however, their biological activity has not been investigated. There has been a growing interest in the study of their biological activity since it has been shown that some species have potentially therapeutic antitumoral, immunomodulator, antiinflammatory, antibacterial, hypoglycemic, and cholesterol lowering effects (Ikekawa et al., 1999; Sugiura et al., 1980; Hishida et al., 1988; Misuno et al., 1978; Wasser and Weis, 1999; Williams, 1990; Kinio et al., 1997; Kiho et al., 1994, 1995,

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1996; Hikino et al., 1989; Berger et-al., 2004; Newairy et al., 2002; Chang and But, 1986, 1987; Lindequist., et al., 2005; Sugiyama and Yamakawa, 1989). Studies carried out in Japan, China, Korea, and the United States have demonstrated the medicinal properties of mushrooms, such as Shiitake (*Lentinus edodes*), Reishi (*Ganoderma lucidum*), *Cordyceps*, *Flammulina velutipes*, *Trametes versicolor* and others. Most studies have focused on shiitake (*L. edodes*), from which lentina was isolated (Kenneth, 1995). This substance has immunomodulatory, antitumoral, cholesterol-lowering, and hypoglycemic properties (Harumi et al., 1989).this context, it has been shown that the administration of crude extracts and/or pure products isolated from various species of macrofungi, particularly those known in China and Japan, produce a decrease in glucose levels in animals with experimentally induced diabetes (Kiho et al., 1994, 1995, 1996; Hikino et al., 1989; Yuan et al., 1998; Newairy et al., 2002; Hideyuky et al, 2002; Somani et al., 2006; Brizuela et al, 1998; Alarcon-Aguilara et al., 2002; Chang 1996). Some of the species that have been shown to have this activity are *G. lucidum*, *Cordyceps sinensis*, *Auricularia auricula-judae*, *Agrocybe cylindracea* and *Tremella aurantia*.

In the search for new therapeutic agents, in recent years, the potential of hundreds of Mexican plant extracts have been evaluated. However, the biological activity of multiple species of macrofungi growing in our country is a virtually unexplored field. The Pharmacology and Toxicology Department of the Universidad Autonoma of Nuevo Leon, Mexico, has developed a line of research to systematically study and evaluate the biological activity of species of macrofungi that grow in our country with the purpose of identifying biologically active species as possible sources of substances for the development of new drugs, because of the history of biological activity of macrofungi species that grow in Asian countries.

Previous studies have demonstrated the biological and cytotoxic activity, the production of cellular oxidative stress, the immunomodulating effect (Ramirez et al., 2006), and the antimicrobial activity (Gonzalez-Barranco et al., 2010) of aqueous and methanol extracts of mycelia of *Ganoderma applanatum*, *Calvatia cyathiformis*, *Lentinus lepideus* and *Armillaria tabescens*. The aqueous extracts of all these species, collected in Northeastern Mexico and cultivated *in vitro* in our laboratory, showed a greater antioxidant and immunomodulating effect than methanol extracts.

In this paper, we present as part of the systematic evaluation of the biological activity of the macrofungus species that grow in our country, an evaluation of the hypoglycemic and hypocholesterolemic effects of aqueous and methanolic extracts of *L. lepideus*, *C. cyathiformis* and *G. applanatum* strains of Northeastern Mexico, based on chemotaxonomic and ethnopharmacological criteria. To assess this activity, we established a model of alloxan-induced experimental diabetes in rats (Ramos and Méndez, 1994).

MATERIALS AND METHODS

Plant

The fruitful body of *L. lepideus*, *C. cyathiformis* and *G. applanatum*, was gathered in temperate coniferous forests of the municipality of Galeana, Mexico. The evaluated strains are known for their growth in Mexico and have already been collected and classified and are currently in the culture collection of the Forest Protection Laboratory of the School of Forestry Sciences of the Universidad Autónoma de Nuevo Leon, Mexico, where the samples were obtained for cultivation and breeding. Pure cultures were obtained using Melin-Norkrans medium according to the technical principles for isolation and propagation of fungi (Statements, 1983, 2000; Turner, 1971; Hobbs, 2004). Starting from the isolated stock cultures solid and liquid phase was carried out using a modified Melin-Norkrans medium with and without Agar, respectively. The liquid cultures were incubated in 500 ml flasks at 25°C for two months. The biomass samples of each cultured stock were recovered by filtration and the obtained material was washed with distilled water and was lyophilized. Aqueous and methanolic extractions were obtained from 1 g of dry and milled biomass. The extracts were then lyophilized and stored at 4°C until their use (Statements P, 1983; Lui J, 2004). Based on results of tests performed, the more active extract was selected and initial biodirected fractionation and evaluation of the hypoglycemic and hypocholesterolemic activity of the obtained fractions was carried out (Mc Gowan et al., 1983 Allain et al., 1974; Raabo et al., 1960).).

We assessed the hypoglycemic effects of the four fractions obtained in diabetic Wistar rats, and compared them with a saline control group and a glibenclamide control group. Glibenclamide was purchased in tablet form and was crushed and homogenized, weighed and diluted in saline for administration at the dose in milligrams per kilogram used in humans and extrapolated according to the individual weight of each rat (Goodman, 1996).

Biodirected phytochemical analysis of *L. lepideus* active fractions was carried out, evaluating the protein and carbohydrate content of the fractions F-II and F-III. The design of the study includes prospective, analytic-experimental and longitudinal study.

Animals

Male Wistar rats weighting 100 to 120 g were used. Animals were housed in standard environmental conditions (in an isolation room with a controlled temperature of 23 to 25ºC, a humidity of 40 to 60%, and a 12 h light-dark cycle. They had free access to food and water up to 12 h before and during the period of experiment. These experiments were performed in compliance with the appropriate laws and institutional guidelines of the Universidad Autonoma de Nuevo Leon and International Guiding Principles for Biomedical Research Involving Animals. The Institutional Animals Ethics Committee approved the protocol of the study.

The rats adapted after 7 to 10 days, and fasted for 12 h before an injection of 150 mg/kg ip of alloxan monohydrate. After one week, rats with marked hyperglycemia (blood glucose >200 mg%) were used for the study. The diabetic rats were divided into seven groups of ten each. Animals were treated once a day for five days with saline (0.5 ml) and aqueous or methanolic extract of *L. lepideus*, *C. cyathiformis* and *G. applanatum*, which were solubilized in saline doses of 100 mg/kg and given orally by gavage through an orogastric tube. Blood samples were collected from all animals and blood glucose and cholesterol were determined using an Accutrend Glucometer (Roche Diagnostic, Mannheim, Germany) before, and 3 and 6 h after treatment. For glucose determination, blood was obtained by snipping the tail with a sharp razor.

To determine the action of the fractions, we used six groups of six rats each: saline control, glibenclamide receiving group, FI, FII,

Table 1. Effects of aqueous and methanolic extracts on the plasma glucose levels.

Treatment	Aqueous extracts glucose decrease (%)	Methanolic extracts glucose decrease (%)
Lentinus lepideus		
3 h	16 ± 7	30* ± 4
6 h	25* ± 9	35* ± 7
C. cyathiformis		
3 h	22* ± 8	11 ± 8
6 h	28* ± 10	22 ± 6
G. applanatum		
3 h	14 ± 8	14 ± 8
6 h	22* ± 4	18 ± 11

Data are presented as average ± standard deviation (SD), expressed in percent (%), n=10. *p < 0.05.

FIII, and FIV groups. The preparation and adaptation of rats and treatments were conducted in a manner similar to that used with aqueous and methanolic extracts.

Statistical analysis

Data are shown as means + standard error of mean (SEM). All data were analyzed by a one way analysis of variance, and the differences between means were established by Tukey test. The statistical significance of differences was established as $p < 0.05$. (Woodson RF, 1987)

RESULTS

Blood glucose levels in normal and alloxan-induced diabetic rats control group

There was a transient increase in the blood glucose level at 3 and 6 h after saline administration.

Blood glucose level in alloxan-induced diabetic rats treated with aqueous and methanolic extracts

The aqueous and methanolic extracts of *L. lepideus* (100 mg/kg) caused a significant reduction in blood glucose levels. The reduction rate for aqueous extract was 16, 22, and 14% at 3 h, and 25, 28 and 22% at 6 h for *L. lepideus*, *C. cyathiformis* and *G. applanatum*, respectively (Table 1). The reduction rate for methanolic extract was 30% 11, and 14% at 3 h and 35, 22 and 18% at 6 h for *L. lepideus*, *C. cyathiformis* and *G. applanatum*, respectively (Table 1).

The methanolic extract of *L. lepideus* was significantly lower at 3 (30%) and 6 h (35%). On the other hand, the methanolic extracts of *C. cyathiformis* and *G. applanatum* had no significant difference in comparison with the control group. Changes in 6 h blood glucose concentrations are shown in Figures 1 and 2.

The oral administration of aqueous and methanolic extracts of *L. lepideus*, *C. cyathiformis* and *G. applanatum* caused no changes in the overall behavior and none of the animals died. This rules out the possibility of harmful effects caused by oral administration. Blood cholesterol levels in normal and alloxan-induced diabetic rats treated with aqueous and methanolic extracts. Comparing cholesterol levels of the groups treated with aqueous and methanolic extracts of *L. lepideus*, *C. cyathiformis* and *G. applanatum* with the control group, we observed that none of the extracts produced a decrease in cholesterol levels. Cholesterol levels in the *L. lepideus* control group and extract treated groups are shown in Figure 3.

Biodirected fractionation

Regarding the biological activity of the studied species, *L. lepideus* and *C. cyathiformis* were the species that produced a greater blood glucose lowering effect. In the case of *L. lepideus*, both the aqueous and the methanolic fraction produced a hypoglycaemic effect. For this reason and because the genus *Lentinus* is one of the most studied with regard to its therapeutic potential, as the case of *L. edodes*, *L. lepideus*, was selected for fractionation (Chihara et al., 1970). We selected aqueous extracts of *L. lepideus* from which 4 fractions were obtained: FI, obtained by precipitation with ethanol; FII, obtained by precipitation with ammonium oxalate; FIII, obtained by extraction with sodium hydroxide, and FIV, obtained by extraction with acetic acid.

The amount obtained from 1 g of dry biomass and the

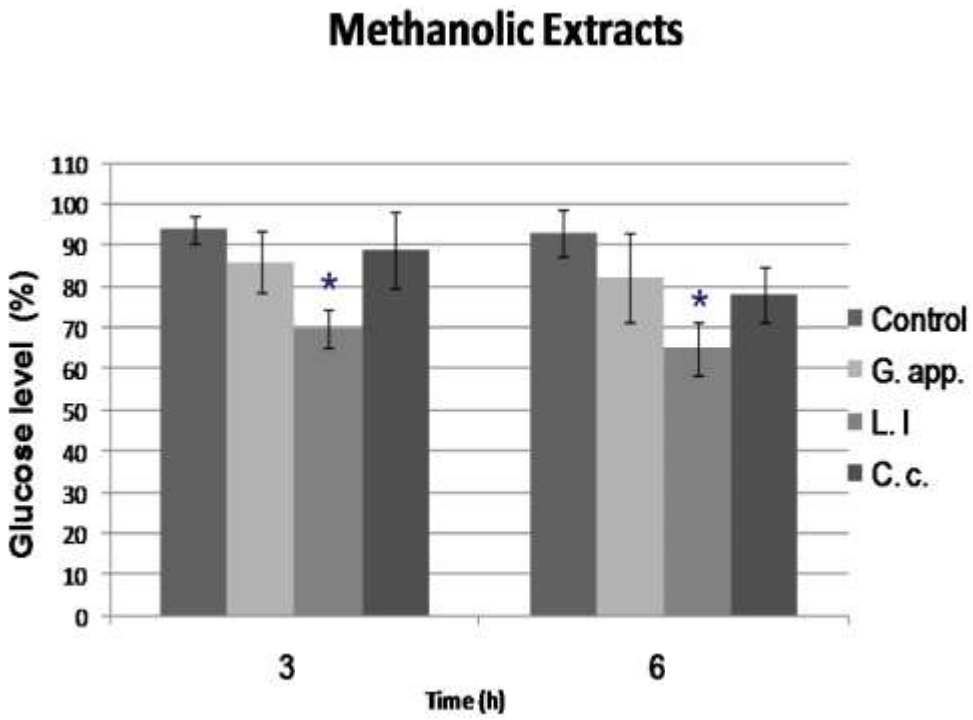

Figure 1. Comparison of the effect of methanolic extracts of *L. lepideus* (L.l), *C. cyathiformis* (C.c) and *G. applanatum* (G.app) on plasma glucose levels. Data are presented as mean ± standard deviation (SD), expressed in percent (%); n= 10, *p < 0.05.

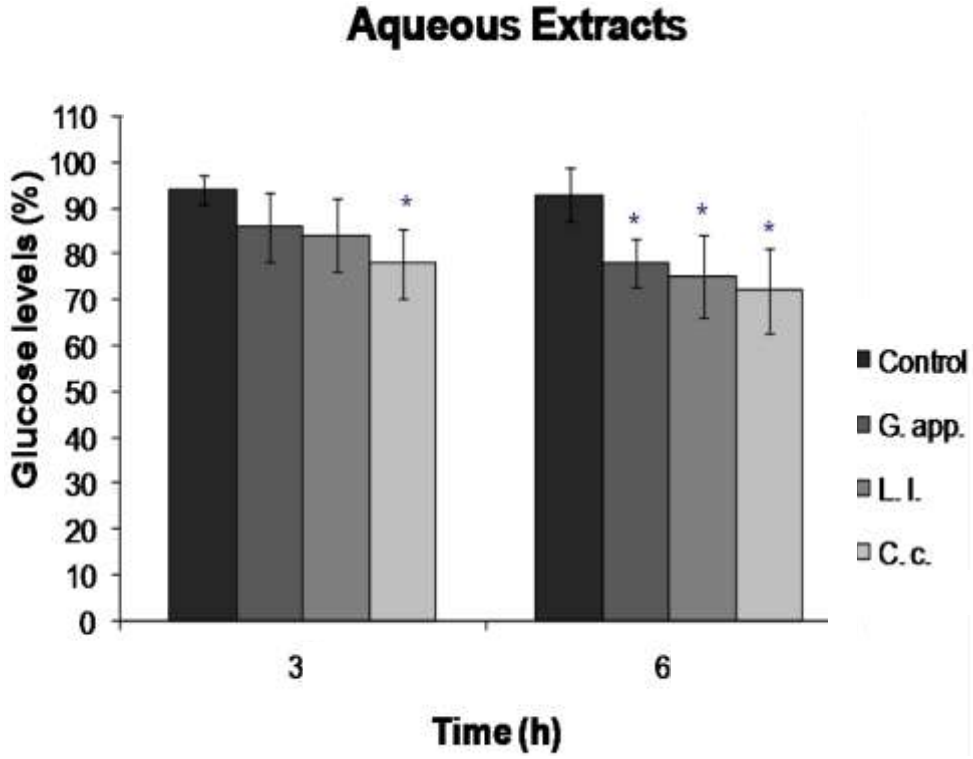

Figure 2. Comparison of the effect of aqueous extracts of *L. lepideus* (L.l), *C. cyathiformis* (C.c) and *G. applanatum* (G.app) on plasma glucose levels. Data are presented as mean ± standard deviation, expressed in percent (%); n= 10. *p < 0.05.

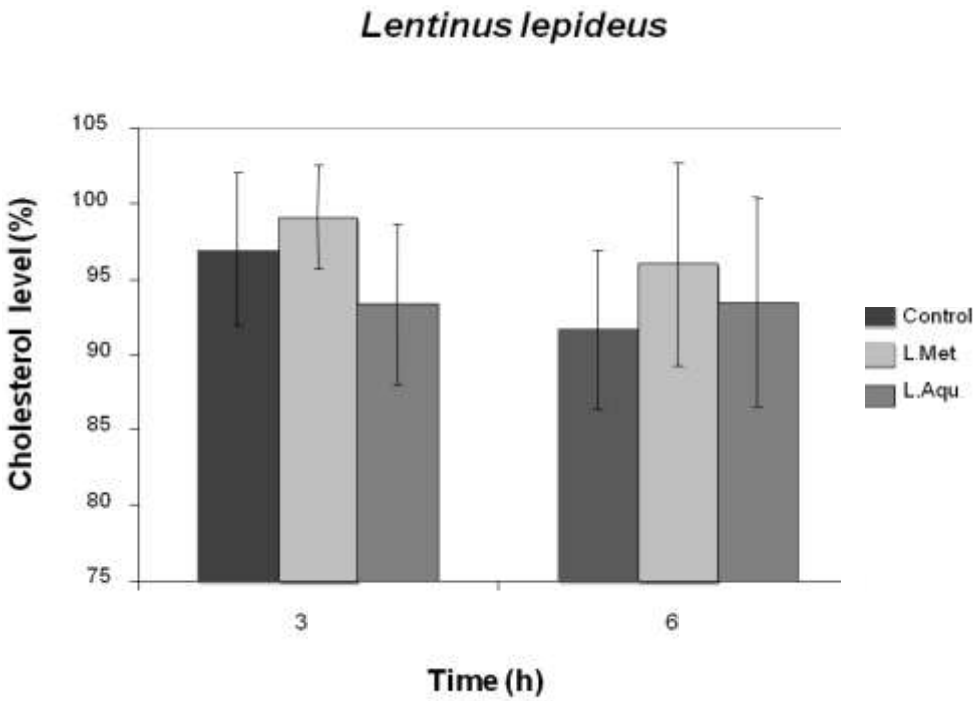

Figure 3. Effect of aqueous and methanolic extracts of *L. lepideus* on cholesterol plasma levels. L.Met: *L. lepideus* methanolic extract. L.Aqu.: *L. lepideus* aqueous extract. Data are presented as mean decrease ± standard deviation, converted to decline percentage (%); (n) = 10. *p < 0.05.

Table 2. Amount obtained and recovery of *L. lepideus* fractions.

Fraction	Amount obtained (mg)	Recovery (%)
FI	14.5 ± 0.05	1.45
FII	31.3 ± 0.08	3.13
FIII	270.0 ± 8.00	27.0
FIV	64.0 ± 6.00	64.0

FI: Fraction 1 (ethanol precipitation); FII: Fraction 2 (ammonium oxalate precipitation); FIII: Fraction 3 (sodium hydroxide extraction); FIV: Fraction 4 (acetic acid extraction).

recovery percentages for each fraction are shown in Table 2.

Effect of *L. lepideus* fractions on glucose levels

The effect of the fractions on glucose levels is as follows: FIV had a statistically significant lowering of blood glucose at 3 h and FII and FIII produced a decrease in glucose at 3 and 6 h. With regard to FI, no hypoglycemic effect was observed. The effect of the fractions of *L. lepideus* on plasma levels of glucose is as shown in Figure 4. Table 3 shows the rates of decrease in glucose levels produced by *L. lepideus* fractions at 3 and 6 h.

FII and FIII showed the highest blood glucose lowering effect. FII produced a statistically significant decrease in glucose levels of 34 and 46% at 3 and 6 h, respectively. Fraction FIII decreased glucose levels by 27 and 38% at 3 and 6 h, respectively. No significant difference was found when comparing the reduction in glucose levels in groups treated with FI, and both fractions FII and FIII, had the same hypoglycemic effect. With the results observed, we proceeded to compare this effect with the effect of glibenclamide (used as standard treatment of diabetes mellitus). No significant difference was observed between the hypoglycemic effect produced by fractions FII and FIII at 3 and 6 h and the effect of glibenclamide in the same time period (Figure 5). Table 3 shows the effect of *L. lepideus* fractions and glibenclamide on plasma glucose levels.

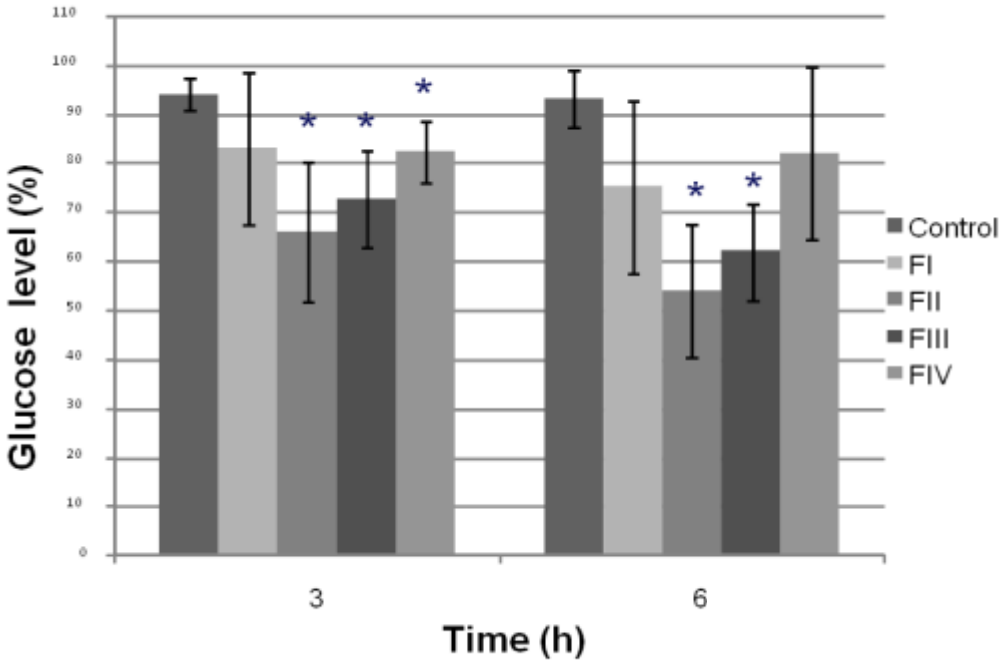

Figure 4. Effect of *L. lepideus* fractions on glucose plasma levels. Data are presented as mean decrease ± standard deviation, converted to decline percentage (%); (n) = 6. *p < 0.05. FI: Fraction 1 (ethanol precipitation); FII: Fraction 2 (ammonium oxalate precipitation); FIII: Fraction 3 (sodium hydroxide extraction); FIV: Fraction 4 (acetic acid extraction).

Table 3. Effect of fractions FI, FII, FIII and FIV of *L. lepideus* on plasma glucose levels in comparison with glibenclamide.

Treatment	Glucose decrease 3 h (%)	Glucose decrease 6 h (%)
Control group	6 ± 3	7 ± 7
FI	16 ± 15	24 ± 18
FII	34* ± 14	46* ± 12
FIII	27* ± 10	38* ± 9
FIV	18* ± 6	18 ± 19
Glibenclamide	20* ± 6	28* ± 5

Data are presented as average ± standard deviation, expressed in (%), n=6. *p < 0.05. FI= Fraction 1 (ethanol precipitation); FII= Fraction 2 (ammonium oxalate precipitation); FIII= Fraction 3 (sodium hydroxide extraction); FIV= Fraction 4 (acetic acid extraction).

Biodirected phytochemical analysis

The phytochemical analysis of fractions FI, FII and FIII of *L. lepideus* focused on the determination of carbohydrates and proteins, because reports of compounds with hypoglycemic activity isolated from species growing in Asia are predominantly glycoproteins (Zhang and Lin, 2004; Byung-Keun et al., 2002; Carbonero et al., 2006; Hikino, 1989; Hikino, 1985; Wasser and Weis, 1999; Lowry et al., 1951). In the evaluation of the protein and carbohydrate content of fractions FI, FII, and FIII, we observed that they comprised of both, with a greater proportion of carbohydrates (Table 4).

DISCUSSION

In this study, the isolation of pure cultures from fruiting bodies of *L. lepideus*, *C. cyathiformis* and *G. applanatum* allowed the removal of contaminants, which assured that the hypoglycemic activity corresponded to bioactive compounds present in the macrofungus and not fungi or bacteria that may be present in the fruiting bodies. Regarding the type of compounds contained in each of

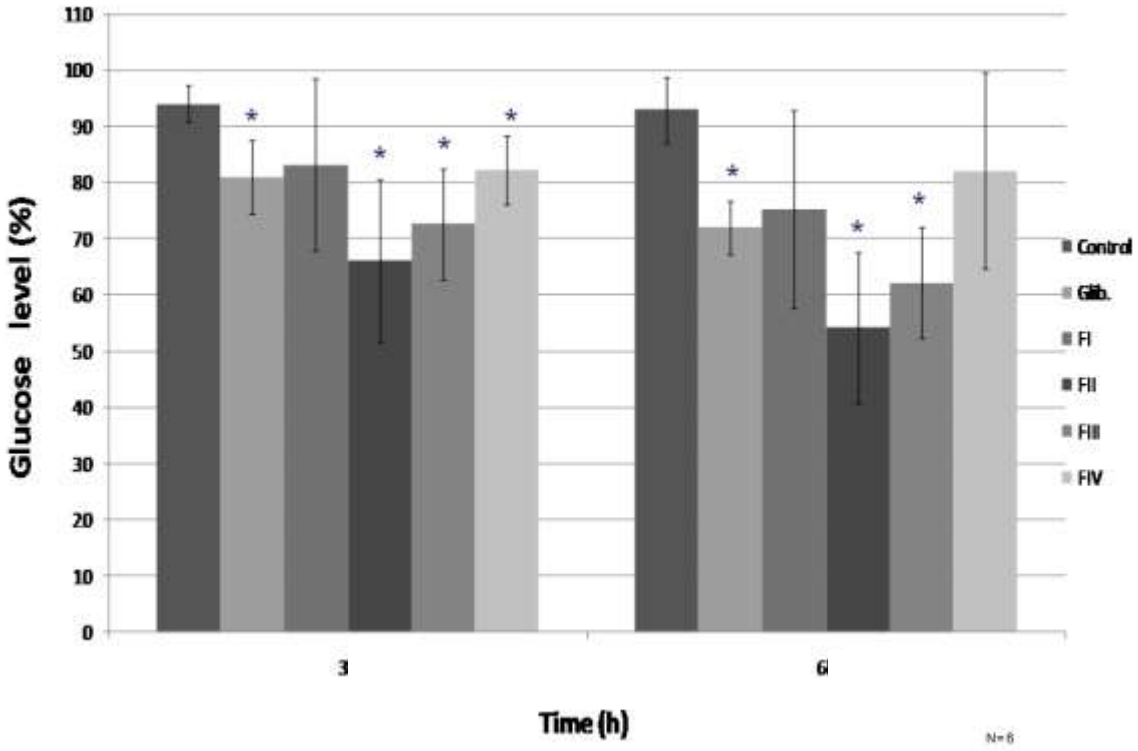

Figure 5. Effect of fractions of *L. lepideus* on glucose plasma levels in comparison with glibenclamide (Glib). Data are presented as mean decrease ± standard deviation (SD), converted to decline percentage (%), (n) = 6, *p <0.05. FI: Fraction 1 (ethanol precipitation); FII: Fraction 2 (ammonium oxalate precipitation); FIII: Fraction 3 (sodium hydroxide extraction); FIV: Fraction 4 (acetic acid extraction).

Table 4. Protein and carbohydrate content of *L. lepideus* fractions FII and FIII.

Fraction	Proteins (%)	Carbohydrates (%)
FII	23.2	30.8
FIII	5	37.3

FII: Fraction 2 (ammonium oxalate precipitation); FIII: Fraction 3 (sodium hydroxide extraction).

the four fractions of *L. lepideus*, the FI fraction, obtained after aqueous extraction and ethanol precipitation corresponds to water soluble proteoglycans. The FII fraction, obtained with 1% ammonium oxalate extractions and ethanol precipitation, corresponds to neutral proteoglycans. FIII, obtained by precipitation with 5% sodium hydroxide, corresponds to acidic proteoglycans, and FIV, obtained with acetic acid, corresponds to alkali proteoglycans.

In the evaluation of the hypoglycemic activity of the fractions obtained, we found that fractions FII (neutral proteoglycans) and FIII (acidic proteoglycans) produced the greatest hypoglycemic effect. Importantly, when compared with glybenclamide, the hypoglycemic action of the fractions was equal to that of the drug.

Therefore, we feel that the compounds present in these fractions are powerful, since even without isolation as pure compounds, the same effect as a pure drug was found.

Regarding the results of the carbohydrate and protein determination of the FI, FII and FIII fractions of *L. lepideus*, the higher carbohydrate content is consistent with that reported by Byung-Keun et al., (2002) in connection with an exopolymer hypoglycemic action, obtained from *L. edodes*, which was described as a glycoprotein composed mostly of carbohydrates.

A similar effect was found by Zhang and Lin. (2004) in their study with *G. lucidum*, Khio et al. (1995) in their studies with *T. aurantia* and in Yuan et al. (1998) with *A. auricula-judae*. None of the species of macrofungi in our study showed a hypocholesterolemic effect, which differs from the results obtained by other researchers with similar species growing in Asia; however, the treatment time in previous studies was greater (weeks or months) than those used in this study. This may explain the lack of a hypocholesterolemic effect, because the time required to observe a response to drug treatments currently used requires weeks or months. It is possible that the administration of treatments used in this study over a longer period of time may produce a hypo-cholesterolemic effect similar to that reported by others.

A greater number of studies are needed to try to solve

this issue, and to guarantee the safety and benefits of the diverse available natural products. This will allow us to have a firm base to offer products with proven effectiveness and safety. Toxicity effects were not observed during this study in any experimental animal, which is in agreement with that observed when it is ingested by humans.

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