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Full Length Research Paper

In vitro cytotoxicity and antioxidant activity of *Agaricus subrufescens* extracts

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***Agaricus blazei* Murill, most recently referred as *Agaricus subrufescens* is a fungus that belongs to the Brazilian diversity and, considered as an important producer of bioactive compounds beneficial to human health. Studies have demonstrated that these compounds present immunomodulatory, antioxidant and antitumor properties. However, there is little information about the appropriate methodology for obtaining extracts with biological activities and their possible citotoxicity. The mushroom extracts were evaluated by cytotoxicity assays using primary cultures of granulocytes and macrophages colony forming cells (CFU-GM) and established V79 cell line. Lipoperoxidation analysis using fibroblast V79 cells were also conducted. The extract fraction that produced a crystalline product was obtained from the alcoholic extraction at 60°C. This fraction exhibited an antioxidant activity on mitochondrial membranes and did not present cytotoxic activity on concentrations lower than 2.5 mg/mL in both cells systems evaluated CFU-GM and V79. Moreover, no significant alterations on the fibroblast V79 adhesion or proliferation capacity was observed. We suggest that the alcoholic fraction obtained from *A. subrufescens* at 60°C has a great potential for biotechnological application since it is easily obtained and presented low cytotoxicity potential.**

Key words: *Agaricus subrufescens*, lipoperoxidation, CFU-GM, cytotoxicity, V79 cells.

INTRODUCTION

In Brazil, among several mesophile mushrooms, those that grow well at sub-tropical temperatures and high humidity, there is *Agaricus blazei* Murill (Wasser et al., 2002). *A. blazei*, identified originally in the town of Piedade, in São Paulo State and commonly found in the mountain regions of Atlantic Forest in this same state (Eira, 2003) is considered a different species from the one originally described by Murill in 1945 (Heinemann, 1993). A recent proposition for name changing to *Agaricus brasiliensis* (Wasser et al., 2002) has been adopted by some authors (Nascimento and Eira, 2003), and rejected by others. Wasser et al. (2002) recognized the latter as a new species and also rejected co-specificity between Brazilian mushrooms and *Agaricus subrufescens.* The statement was based on features of spores and cheilocystidia, although they wrote that the two species seemed to be each other's closest relatives. However, new data presented below indicate that the medicinal mushroom from Brazil and Japan is biologically and phylogenetically the same species as *A. subrufescens* from North America (Kerrigan, 2005).

In Brazil, this fungus is used as a traditional medicine for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis and chronic hepatitis (Kasai et al., 2004). Its popular utilization for therapeutical purposes has led to several researches for confirming its apparent medici-

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nal properties, in particular studies on β-D-glucans from this mushroom, which demonstrated antitumoral effects based on immunostimulation (Fujimiya et al., 1999; Ebina and Fujimija, 1998; Fujimiya et al., 1998; Ito et al., 1997; Itoh et al., 1994; Kawagishi et al., 1989). This effect was also recently reported by Kaneno et al. (2004), who verified the restoration of the activity of natural killer cells, reduced due to subcutaneous inoculation of Ehrlich tumor cells in guinea pigs, by *A. subrufescens* extracts. Despite being found on all cereals, polysaccharides of fungal origin have differentiated biological activity (Park et al., 2003). Among the different biological properties, and one of most important, of *A. subrufescens,* Izawa and Inoue (2004), demonstrated that aqueous extract possessed antioxidant activity and that this property has a thermo-stable characteristic. A similar result from the sub-merge cultured *A. subrufescens* mycelium was recently reported (Ker et al., 2005).

Oxidative stress is a disturbance in the balance between the formation of oxidizing species (reactive oxygen species and other radicals) and their effective removal by protective antioxidants. Overwhelming radicals generated in the bloodstream and tissues can induce oxidative damage to cell membranes, lipoproteins, proteins, and deoxyribonucleic acid. This clinical scenario suggests potential therapeutic strategies involving antioxidants repletion in such patients (Crimi et al., 2004).

In vitro methods have been recommended for evaluating the cytotoxicity of many compounds and several model systems, using established cell lines as well as primary cells, have become available for screening purposes. One of the most used cells in cytotoxicity assays is the established cell line of Chinese Hamister Lung (V79) that has been widely used to study the toxicity, mutagenicity and repair of a wide variety of DNA damaging agents (Chaung et al., 1997). This approach has been shown to be advantageous and more reliable for *in vitro* estimation of toxicity mechanisms of several chemicals (Chiba et al., 1998; Melo et al., 2003).

Whereas most cytotoxicity methods employ permanent cell lines such as V79, murine and human progenitor cells have also been used to assess the cytotoxicity of different chemicals using clonogenic assays (Pessina, 2000). Recently, some authors introduced the clonogenic cultures, using the bone marrow progenitor cells, as a valuable tool in immunotoxicological studies (Parent-Massin, 2001). This organ contains various cell populations including hematopoietic stem cells that give rise to progenitors of different lineages of the lympho-hematopoietic system in vitro in the presence of specific growth factors. For this reason, bone marrow provides a useful cell type to study possible negative effects of compounds on cellular metabolism predicting cell damage when in the presence of various chemicals (Parent-Massin, 2001).

Based on the description above, in this study, the cytotoxiciy of *A. subrufescens* extract was evaluated using a V79 cell line and the murine bone marrow granulocyte-macrophage progenitors cells (CFU-GM). Besides, we evaluated the antioxidant properties of the extracts obtained from *A. subrufescens* and evaluate the non-toxic fraction as a potential antioxidant product by the evaluation of the levels of lipid peroxidation.

MATERIALS AND METHODS

Agaricus subrufescens

Samples from dehydrated mushroom or fresh ones, commercialized in the town of Mogi das Cruzes – SP/Brazil, considered the most important producing region of mushrooms in São Paulo State (Eira, 2003), were used.

Aqueous and ethanolic extracts of *Agaricus subrufescens*

The *A. subrufescens* had been placed in contact with distilled water and ethanol 95°GL [3.0 g (solute) to 100 mL (solvent)]. The aqueous solution was incubated at temperatures of 20 - 25, 40 and 60°C for 2 h. The alcoholic solution was incubated at temperatures of 20 - 25 for 2 h, and for 60°C for 5 min and filtered through a common filter, and then the solution was cooled for 15 min at room temperature. A crystalline precipitate was observed and subsequently separated from the solution and transferred to a desiccator for a complete drying (Figure 1).

Cell culture conditions

Chinese hamster lung (V79) was kindly provided by Prof. Dr. Marcela Haun from the State University of Campinas/SP. These cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM – Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FCS (Gibco, Grand Island, NY, USA), 100 µg/mL streptomycin and 100 IU/mL penicillin in a humidified atmosphere of 5% CO_2 in air at 37°C.

MTT reduction assay

Cells were seeded at a density of 3 x 10^4 cells/ml in 96-well plates and after 48 h incubation time, the medium was replaced by dilution series of each concentration of *A. subrufescens* extract from 0.08 to 2.5 $mg.mL^{-1}$. After treatment, the extracts were removed and cells washed once with PBS. Then, 0.1 ml of a solution containing 0.5 mg 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT – Sigma)/mL serum-free medium was added to each well and cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 4 h. After the incubation period, the supernatant was removed and the dark-blue formazan crystals dissolved in 0.1 mL of ethanol. The plates were shaken for 10 min and the optical densities were read at 570 nm in a multiwell spectrophotometer. Assays were repeated three times to ensure reproducibility.

Clonal culture of hematopoietic granulocyte-macrophage progenitors (CFU-GM) from mouse bone marrow

Assays for growth and differentiation of hematopoietic progenitors (CFU-GM) were performed using bone marrow cells collected from normal male BALB/c mice, 6 - 8 weeks old, bred at the University Central Animal Facilities and raised under specific pathogen-free

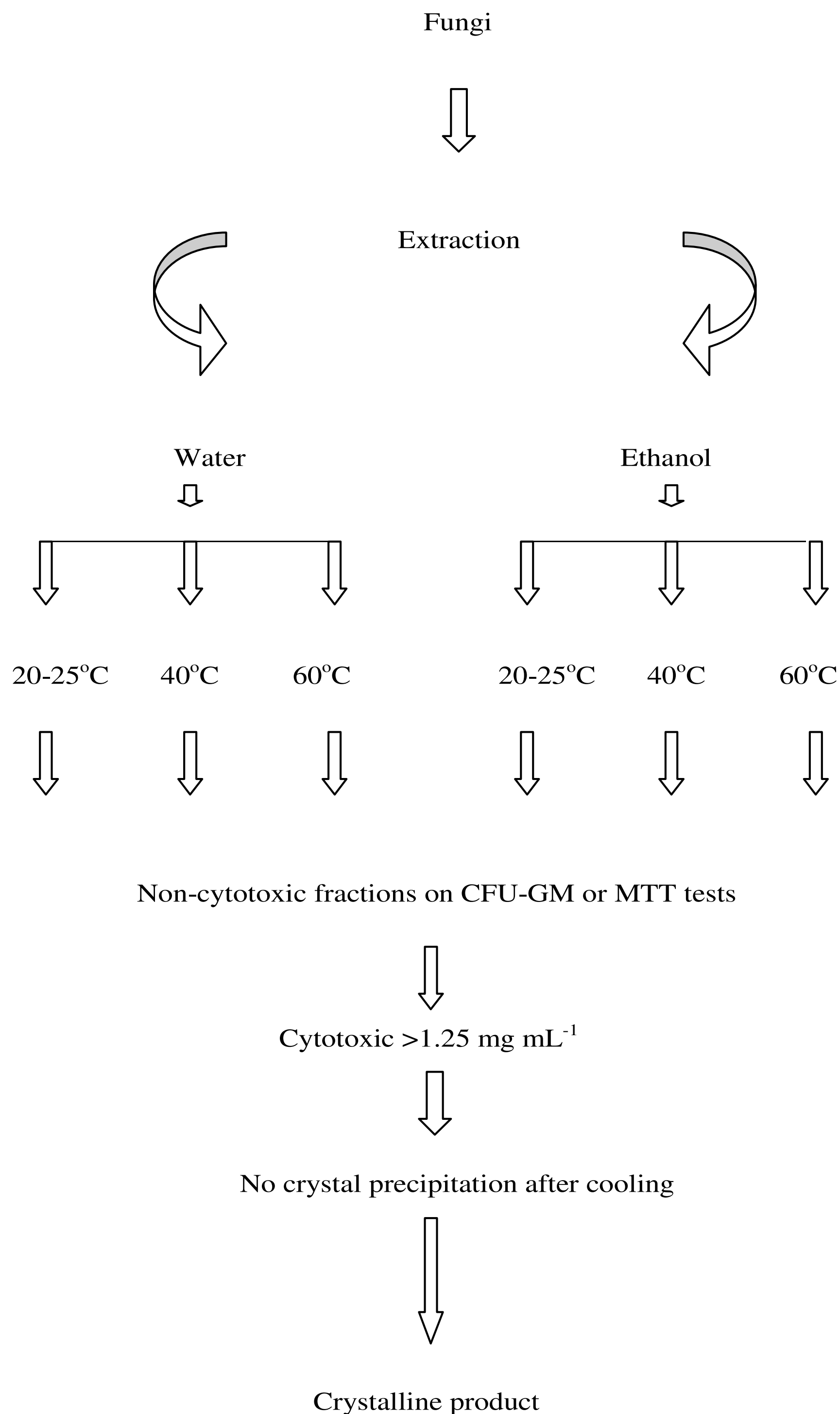

Figure 1. Procedure of fungi extraction.

conditions (Centro de Bioterismo, Universidade de Mogi das Cruzes, Mogi das Cruzes, SP, Brazil). Animal experiments were done in accordance with institutional protocols of the Institutional Animal Care and Use Committee. Cell suspensions of 1 x 10^5 bone marrow cells were cultivated in 1 ml agar cultures in 35 mm Petri dishes. The medium used was DMEM (Sigma) containing 20% FCS (Gibco) and 0.3% agar (Difco – Becton Dickinson, France). Colony formation was stimulated by the addition of recombinant murine macrophage-granulocyte colony-stimulating factor (rmGM-CSF – Sigma) at a final concentration of 0.5 ng/mL. The effect of *A. subrufescens* extract was evaluated adding differents concentrations in the 35 mm Petri dishes (0.08 – 2.5 mg.mL^{-1}). The cultures were incubated for 7 days in a fully humidified atmosphere of 5% CO_2 in air and colony formation (clones > 50 cells) was scored at

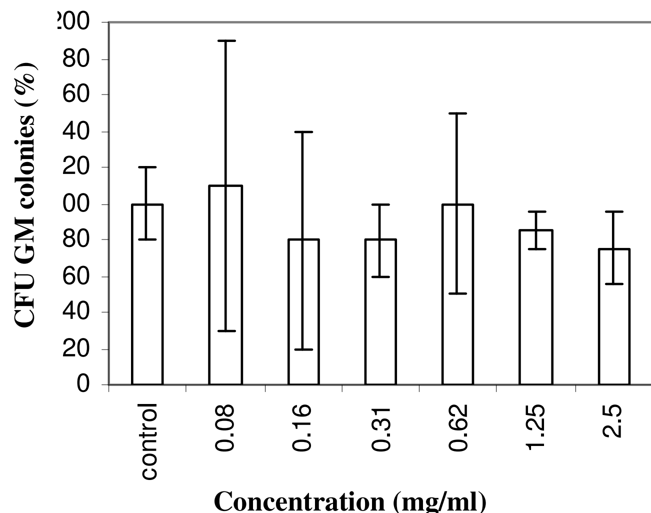

Figure 2. Growth and differentiation of hematopoietic precursors for granulocytes and macrophages (CFU-GM). Different concentrations of crystalline precipitate of warm alcoholic extract obtained at 60°C, from the dehydrated mushroom. The values are expressed in percentage relative to control. The study was performed in two independent experiments. All assays were conducted in duplicate. There was no remarkable difference among the studied groups.

35X magnification using a dissection microscope. Assays were performed in triplicates to ensure reproducibility (Metcalf, 1984).

V79 cells adhesion

In order to verify alterations on the adhesion capacity of fibroblast V79 previously incubated for 48 h with *A. subrufescens* extracts, the cells were detached by trypsinization, centrifuged for 10 min at 1500 rpm and ressuspended in 1 mL culture media for counting in hemocytometer chamber. The resultant cells were incubated in culture plate. After 2 h the wells were gently washed, three times, with the same culture media for the removal of non-adherent cells, then the media containing MTT dye was added and the plate was incubated for 2 more hours, afterward the absorbance reading was conducted following protocol described by Huang et al. (2002).

Lipoperoxidation assay

Adult male Wistar rats 2 months old (180 ± 220 g), bred at the University Central Animal Facilities and raised under specific pathogen-free conditions (Centro de Bioterismo, Universidade de Mogi das Cruzes, Mogi das Cruzes, SP, Brazil). Animal experiments were done in accordance with institutional protocols of the Institutional Animal Care and Use Committee. After the rats were killed, tissues were collected, frozen on dry ice and stored at –80°C until assays were performed. The mitochondrial membrane lipoperoxidation was evaluated based on malondialdehyde (MDA) formation (Buege and Aust, 1978). The mitochondrial suspension (1.0 mg protein) was incubated in standard media composed by 130 mmol L^{-1} KCl and 10 mmol/L HEPES-KOH, pH 7.4, added by 5 mmol/L succinate + 2.5 µmol L^{-1} rotenone, 50 µmol L^{-1} $(NH_4)_2Fe(SO_4)_2$ and 2 mmol L^{-1} sodium citrate for 30 min, at 37°C (1.0 mL of final volume). The amount of mitochondrial protein was determined by the biuret reaction (Cain and Skilleter, 1987). The preparation and isolation of liver mitochondria from rat were performed by differential centrifugation in isosmotic media (Pedersen et al., 1978), frozen at –20°C and defrosted three times, obtaining non-funcional mitochondrial membrane. For MDA determination, 1 mL thiobarbituric acid (TBA) 1% (m/v) (prepared in 50 mmol L^{-1} NaOH), 0.1 mL NaOH 10 mol L^{-1} and 0.5 mL phosphoric acid 20% (v/v) were added, followed by 20 min incubation at 85°C. The complex MDA-TBA was extracted in 2 mL *n*-butanol and absorbance was determined at 532 nm. The results were expressed as percentage of absorbance relative to control absorbance (100%), which means, lower the absorbance percentage, higher the protection against the induced lipoperoxidation for Fe^{2+} in this model.

Statistical analysis

The results were calculated as percentage of controls and the IC_{50} (concentration that produces a 50% inhibitory effect on the evaluated parameter) were obtained from dose-response curves using the computer software package “Origin” (version 6.0). Reported results are expressed as the mean ± standard deviation. Statistical analysis was done by one-way analysis of variance (ANOVA) and in case of significant differences the Tukey’s test was used. Statistical significance was assigned when $P < 0.05$.

RESULTS

MTT reduction and CFU-GM assay

The growth and differentiation of the hematopoietic precursors by macrophages and granulocytes (CFU-GM) showed cytotoxicity for the water extract at 20 - 25°C over 125 mg.mL^{-1} concentration, but no cytotoxicity on V-79 cells by MTT assay (data not shown). All the other extracts, included the ethanolic extract at 60°C, where a crystalline product was formed, exhibited no cytotoxicity either in CFU-GM or MTT assays (Figures 2 and 3).

Test of cell adhesion

The proliferation and adhesion capacity of V79 fibroblast also did not exhibit significant variation relative to control at the studied concentration (data now shown), corroborating with the results from MTT assay.

Lipoperoxidation assay

Since the extract, at the tested concentrations, did not present undesirable effects regarding to metabolism and cell proliferation and differentiation, the antioxidant potential of the crystalline product was investigated. As can be visualized in Figure 4, the crystalline product has an important concentration-dependent antioxidant effect. This effect presented statistical significance in concentrations higher than 1.25 mg mL^{-1}. Even at higher concentrations of the crystalline product e.g 2.5, 5.0 or 10 mg mL^{-1} , a 25, 40 and 80% of the lipoperoxidation inhibition was reached, respectively. This is in agreement with, that the inhibition effect was not correlated with the cell viability

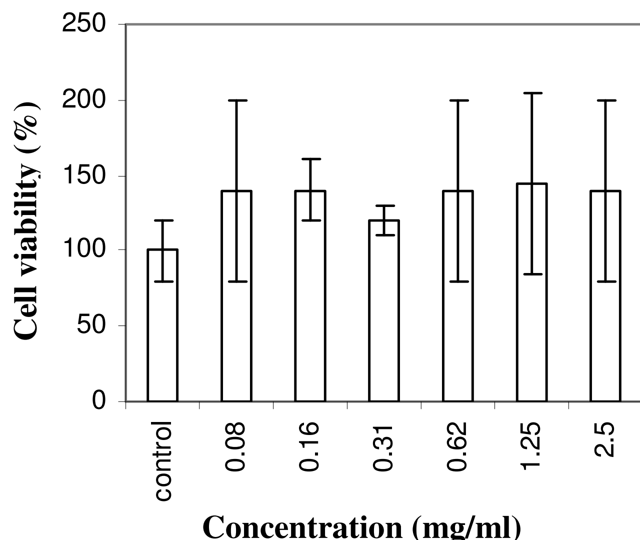

Figure 3. MTT test for cell viability verification of V79 cell line in relation to different concentrations of "crystals" 60°C extract. Two independent experiments in triplicate were performed (**P* < 0.05 ANOVA, Tukey-Kramer).

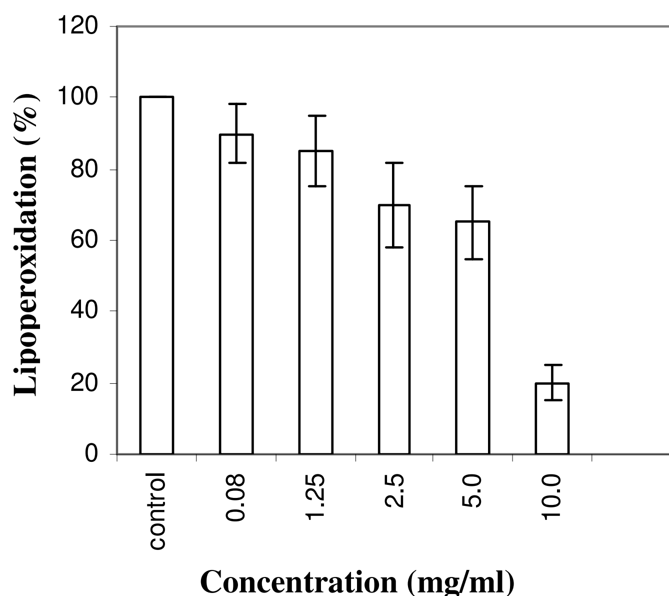

Figure 4. Effect of "crystal" fraction on mitochondria membrane lipoperoxidation of rat liver induced by Fe^{2+}/citrate determined by MDA formation. The control represents the maximum lipoperoxidation (100%). Results obtained from three independent experiments (* *P* < 0.05 ANOVA, Tukey-Kramer).

(Figure 3). The crystalline product under a heat source of undertook a process of caramelization, resulting in a color and odor of caramel. The IR spectrum presented a strong 3283 cm^{-1} band (OH), 2935 cm^{-1} (C- H) band and 1262 cm^{-1} (CH-O-) band.

DISCUSSION

The measurement of cell viability and growth is a valuable tool in a wide range of research areas. In the present study, V79 fibroblasts were used to study the cytotoxicity of the *A. subrufescens* extracts due to their well defined culturing characteristics in experimental conditions and their relevance as an *in vitro* system for screening purposes involving natural products and antimutagenic effects on these cells (Melo et al., 2003; Menoli et al., 2001; Tai et al., 2002).

The reduction of tetrazolium salts (MTT) is now recognized as a safe, accurate alternative to radiometric testing. Among the applications for the method are drug sensitivity, cytotoxicity, response to growth factors, and cell activation (van de Loosdrecht et al., 1994; Ohno and Abe, 1991; Mosmann, 1983).

Our findings shown that the precipitate crystalline product fraction, nearly did not present cytotoxic effects for V79 cell line. The MTT assay, which evaluates the activity of mitochondrial enzyme dehydrogenase succinate, shows that the analyzed fraction of *A. subrufescens* did not trigger any toxic effect on the V79 cell line. On the contrary, a slight influence on the enzyme activity was observed probably by interference in the induction of the dehydrogenase activity and not on the proliferation cells.

The proliferation, survival, metabolism and gene expression are, at least in part, possible due to the cellular adhesion. Cell surface adhesion molecules play vital roles in numerous cellular processes. Some of these include: prolifetation, differentiation, embryogenesis, immune cell transmigration and response, and cancer metastasis. Adhesion molecules are also capable of transmitting information from the extracellular matrix to the cell. There are four major families of cell adhesion molecules. These are the immunoglobulin superfamily, cell adhesion molecules, integrins, cadherins, and selectins (Gonzalez-Amaro, 1999; Rojas, 1999; Hynes, 1999 and Joseph-Silverstein, 1998). In this work, we carried out a cell adhesion assay with V79 cell line; that has as important characteristic of adhesion capacity. Our results shown that the "crystal" fraction was not capable of modifying this important characteristic related with its proliferation, suggesting again, that *A. Subrufescens*t does not present cytotoxicity related with the proliferation of these cells.

Among cytotoxicity tests applied for evaluation of toxicological potential of the *A. subrufescens,* there is the clonogenic assay (Parent-Massin, 2001), which is conducted on semi-solid media and allows the growth and differentiation of hematopoietic precursor cells *in vitro*. Taking into account the high proliferative capacity of hematopoietic tissue, these cells are frequent target of toxic action of many compounds. Therefore, the proliferation and differentiation study of these cells *in vitro*, in the presence of new substances, helps to outline the possible damages on the cellular response.

Although the cell systems used in this study differed

significantly from each other, the higher sensitivity of progenitor cells may be partly explained by the fact that bone marrow undergoes rapid turnover, becoming more susceptible to toxic agents. Moreover, in the CFU-GM assay, cellular toxicity was assessed after a longer exposure time to the *A. Subrufescens* extract. Nevertheless, these results suggest that the clonal culture of hematopoietic progenitors is a punctual indicator of cellular damage, which is becoming a useful tool in cytotoxicity studies.

Since no cytotoxicity in the crystalline product after ethanol extraction at 60°C, this compound was selected to study the antioxidant capacity. Reactive oxygen species (ROS), which include hydrogen peroxide and other chemical forms known as free radicals, such as superoxide anion, hydroxyl, and peroxyl radicals, are produced as part of normal and essential biological processes (Bae Jin et al., 2006).

A cell is generally able to maintain an appropriate balance between oxidants and antioxidants under normal conditions. When this balance is perturbed and shifts toward oxidative stress, either by an increase in oxidants or by a decrease in antioxidants, the cell becomes more susceptible to injuries, such as inflammation (Salvemini et al., 2006), vascular complications in diabetes (Bellin, 2006), and atherosclerosis (Singh, 2006). The oxidative stress established by excessive free radical generation may induce a number of alterations of cell constituents, including inactivation of enzymes, generation of reactive nitrogen species, damage of nucleic acid bases and proteins, and peroxidation of membrane lipids (Halliwell and Gutteridge, 1999).

In this study, lipoperoxidation was used to investigate the antioxidant activity from *A. subrufescens* alcoholic extract that contained the crystallized product. The results obtained in the present paper provide evidence that, crystallized product, presents antioxidant activity *in vitro* and absence of cytotoxic effect for V79 cell line and hematopoietic precursor for granulocytes and macrophages *in vitro*. In relation to ethanol, among the possible compounds obtained, according Luiz et al. (2003) is the linoleic acid, an unsaturated omega-6 fatty acid used in the biosynthesis of prostaglandins and cell membranes and in other natural oils. This compound represents about 70% of the total of lipids on the mushroom body (Luiz et al., 2003). Moreover, Djoussé et al. (2001) shows that linoleic acid was inversely related to the prevalence odds ratio of cardiac arterial disease, and this fatty acid had synergistic effects with linolenic acid on the prevalence odds ratio of this illness. In this sense, Izawa and Inoue (2004), shows a radical-scavenging activity for this mushroom. Finally, Smedman and Vessby (2001) demonstrated that supplementation with linoleic acid may reduce the proportion of body fat in humans and that linoleic acid affects fatty acid metabolism.

Therefore, within this context, new sources of antioxidants, such as *A. subrufescens* extract tested in the present study, have promising applications (Guarente, 2000; Sayre et al., 2001; Serafini et al., 2001; Klein and Ackerman, 2003). Although there are many studies on the promising biological properties of mushroom extracts, there is little information correlating extraction protocols, cytotoxicity and possible biological role. In this sense, new studies on this fraction are necessary for a better characterization of its possible biological application.

Conclusions

It is possible to conclude from the analyses performed here that the extraction condition that allowed obtaining the extract denominated "crystals", in alcoholic solvent at extraction temperature of 60℃, is potentially promising. This extract presented significant antioxidant action *in vitro* and absence of cytotoxic effect for V79 cells and hematopoietic precursor for granulocytes and macrophages *in vitro*. New studies on this fraction are necessary for a better characterization of its possible biological application.

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