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Full Length Research Paper

The acute cytotoxicity and lethal concentration (LC_{50}) of *Agaricus sylvaticus* through hemolytic activity on human erythrocyte

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There is limited information regarding acute toxicity and lethal concentration of edible and medicinal mushrooms. The objective of this paper is to estimate the cytotoxicity of the aqueous extract of *Agaricus sylvaticus* mushroom on human erythrocytes by determining the lethal average concentration (LC_{50}). Six concentrations of the mushroom (17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL) were submitted for evaluation of hemolytic activity *in vitro,* using a suspension of blood. Through the Prism GraphPad Software, using the Tukey test for statistical analysis ($p <0.05$), a curve was constructed with values of *A. sylvaticus* mushroom concentrations versus the values determined by absorbance spectrophotometry at 540 nm. Results of hemolytic activity for the aqueous extract were fitted using nonlinear regression and the equation: $Yi = axi / (b + Xi)$. We used values of y as hemolytic activity and x as log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data. The percentage of haemolysis increased in a concentration-dependent manner of *A. sylvaticus* extract used. The LC_{50} value obtained was 9.213 mg/mL. Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.

Key words: Lethal concentration, *Agaricus sylvaticus,* hemolytic activity, sun mushroom.

INTRODUCTION

Chemicals used in therapy should be effective and provide safety (Goodman and Gilman, 2007). Unfortunately, any substance can be a toxic agent and cause undesirable effects (Goodman and Gilman, 2007; Oga, 2003), depending on the dose administered or absorbed, time and frequency of exposure and routes of administration (Oga, 2003). Highly toxic substances cause death at concentrations equivalent to a fraction of a microgram. In others, low toxicity may be almost harmless in concentrations of several grams or more (Goodman and Gilman, 2007; Oga, 2003).

The toxicity of a substance to an organism refers to its ability to cause serious injury or death. In therapy, the concentration of a substance should be enough to achieve the desired effect and achieve it well with the lowest concentration, and as much as possible, without producing adverse reactions or side effects (Oga, 2003).

The safety of drugs and foods should be determined through the analysis of several factors related not only to the individual characteristics of the organism, but also considering the physic-chemical, pharmacodynamic and pharmacokinetic of each substance, the various routes of exposure and different methods of administration

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(Silva, 2006).

Depending on the cultivation and composting, mushrooms can have varying levels of toxicity and risk to human health, although preliminary studies suggest that experimental use of *Agaricus sylvaticus* may present low toxicity. The use of this mushroom in folk medicine began in ancient peoples and between indigenous communities (Novaes et al., 2007).

The assessment of exposure can be performed by measuring the concentration of a substance administered to a particular organism (Oga, 2003). The study of concentration-response or concentration-effect in toxicology is essential and is used to determine the median lethal concentration (LC_{50}) of drugs and other chemicals (Goodman and Gilman, 2007).

The concentration-response curve is represented by the Gaussian theory, rarely found in practice. This curve is calculated statistically from observations of mortality after exposure related to concentrations of the substance to be tested, and it is widely used to calculate the 50% lethal concentration (LC_{50}). The LC_{50} is thus a statistical index which indicates the concentration of a chemical agent capable of causing death in 50% of organisms in a population with defined experimental conditions (Oga, 2003).

To know the effects of a toxic substance and classify them according to their potential lethality or toxicity and concentration-response curve, one needs to perform toxicological tests (Oga, 2003).

Mushrooms of the genus *Agaricus* have been widely studied for their nutritional characteristics and many medicinal properties they exhibit. The *A. sylvaticus* mushroom (Sun Mushroom) has been reported to have rich nutritional composition, with high protein content (41.16%), carbohydrates (36.21%), low lipid content (6.60%), considerable amounts of fiber (2.34%) and minerals (7.38%), besides having excellent antioxidant activity (Costa et al., 2011).

A. sylvaticus has been widely used as nutritional supplement for cancer patients, with likely effects of growth inhibition, tumor regression and stimulation of the immune system of patients.[4] According to recent studies there seems to be clear evidence of its immunomodulatory activity and efficacy against carcinogenic activity of the drug pristine (Hi et al., 2008).

There is also indication that dietary supplementation with *Agaricus sylvaticus* may reduce total cholesterol, LDL-C and triglycerides, with favorable outcome on lipid metabolism and, consequently, on the prognosis of patients with colorectal cancer in post-operative phase (Fortes et al., 2008). Furthermore, it has contributed to improve the quality of life of these patients by significantly reducing the harmful effects caused by the disease itself (Fortes et al., 2007).

The safety and effectiveness of medicinal plants and fungi are dependent on various factors, of these the quality of the product commercialized can be highlighted. Effectiveness and low toxicity to humans should be verified as well (Arnous et al., 2005).

In this context, the objective of this study is to evaluate the acute toxicity of *A. sylvaticus* mushroom aqueous extract *in vitro,* from the determination of lethal concentration (LC_{50}) through its hemolytic activity on human erythrocytes so as to refer the determination of toxicity parameters for human use.

METHODS

The experiment, in triplicate, was performed at the Nanotechnology Institute Laboratory of Biological Sciences, University of Brasilia, Brazil, in January and February 2011.

Obtaining the sample

The sample of dried *A. sylvaticus* mushroom (Sun Mushroom) was obtained from a producer in Minas Gerais State, Brazil.

Preparation of the solution containing the *A. sylvaticus* mushroom

We weighed 9.0 g of dehydrated *A. sylvaticus* mushroom and added to the sample 105 mL of distilled water. The solution was stirred for 20 min at room temperature, filtered through paper filter, and then 1000 µL of the solution was distributed into previously weighed Eppendorf tubes. The solution was lyophilized and the Eppendorf tubes were then weighed again, in order to obtain the average weight of the mushroom dissolved in water (17 mg/mL).

Serial dilutions were performed resulting in six concentrations for study: 17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL.

Preparation of erythrocyte suspension at 2% (human blood A-)

Erythrocytes were obtained from fresh A Negative type human blood. For erythrocyte suspension, 1 mL of blood was centrifuged for five minutes at 14000 rpm. Next 9.8 mL of saline solution (NaCl 150 mm) and 200 µL of the erythrocytes precipitate were added to the tube. The tube was then centrifuged for ten minutes at 2000 rpm. The supernatant was discarded and the process repeated three more times. Finally, the tube was shaken with the erythrocyte suspension ready for use.

Testing of hemolytic activity - Dose relation/hemolytic activity

Samples with 3 mL of saline solution + 500 µL of erythrocyte suspension + 500 µL of *Agaricus sylvaticus* extract were prepared in six different concentrations. The tubes were stirred manually and incubated at 35°C/60 min. After this interval, the tubes were centrifuged at 2500 rpm for ten minutes. The absorbance of the supernatant was read at 540 nm. The negative control (no haemolysis) was prepared only with saline solution and erythrocyte suspension, and the positive control (100% haemolysis) with 3 mL of distilled water + 500 µL of mushroom extract and a reading taken after 60 min.

We built graphics were built of the kinetics and of the dose-response relationship with mean values and standard deviation (SD). Data were expressed as percentage of viability in control wells, through the GraphPad Prism software, using the Tukey test for statistical analysis ($p < 0.05$).

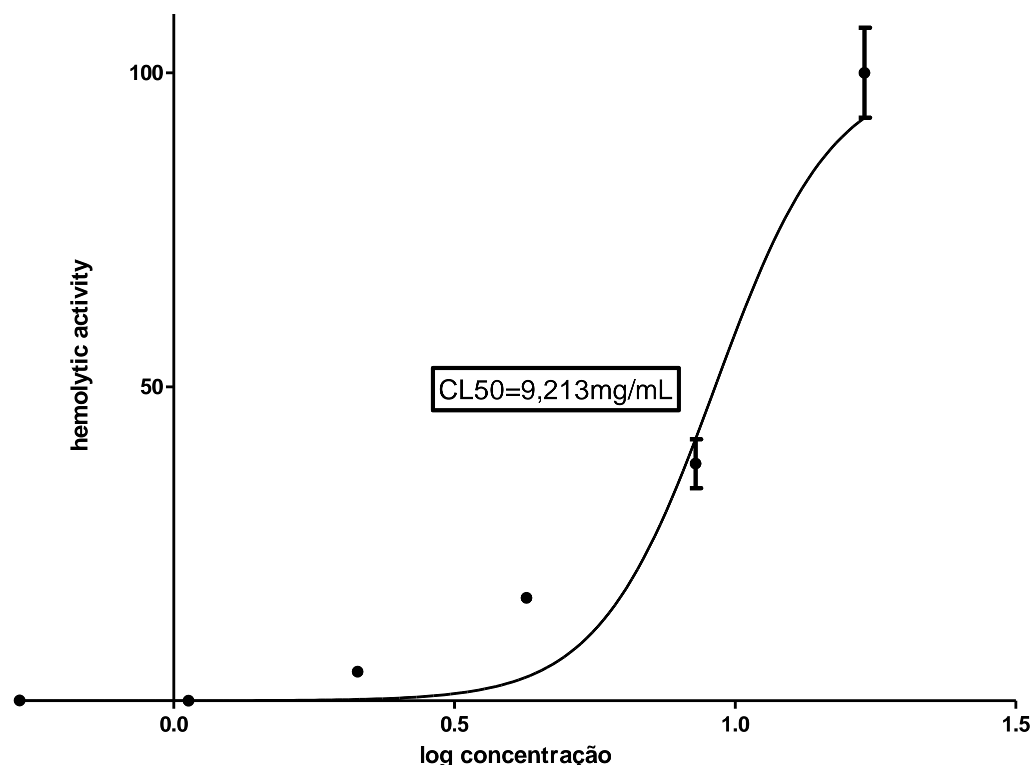

Figure 1. *In vitro* hemolytic activity presented by the aqueous extract of the mushroom *A. sylvaticus* at a 2% suspension of human erythrocytes incubated at 35°C for 60 minutes. The results presented correspond to the average of a test in triplicate.

The assessment of cytotoxicity through hemolytic activity tests has proved to be an alternative screening method for simple toxicity. It is fast, reproducible and inexpensive to evaluate erythrocyte hemolytic activity against concentrations of aqueous extract of *A. sylvaticus,* a fact making it possible to reduce the use of laboratory animals for *in vivo* tests*,* helping reach the goal to decrease, refine and replace studies conducted with animals.

The intent of reducing animals in the research and development of new methodologies in Brazil is timid and will require further discussion with participation of educational institutions and research laboratories together with the industry and regulatory agencies, since this reality affects all those involved in research, registration and approval of new substances.

As the focus of this article is to observe the acute cytotoxicity of mushroom extract, further studies are still necessary to investigate the mechanism of action of this extract and the possible organs or systems sensitive to the same, as well as additional studies on sub-acute and chronic toxicity, mutagenic and teratogenic activity, embriotoxicity and special studies particularly regarding the choice of concentrations of the extract, so as to validate its safety.

RESULTS

Evaluation of toxicity is paramount when considering a safe treatment. Haemolysis is characterized by erythrocytes rupturing with the release of hemoglobin. The *in vitro* haemolysis test is used as a method for substance toxicity screening, estimating any likely *in vivo* damage (Aparício et al., 2005).

Different aqueous extract concentrations of the *A. sylvaticus* mushroom were tested on a suspension of human erythrocytes at 2% and hemolytic activity determined as haemolysis percentage. We built a curve of concentration (µg of *A. sylvaticus* mushroom) versus percentage of haemolysis and concentration of the mushroom aqueous extract required to produce 50% haemolysis, known as 50% hemolytic concentration or 50% effective concentration (EC_{50}).

Test results of the hemolytic activity in tubes for the aqueous extract of *A. sylvaticus* mushroom were then adjusted using nonlinear regression, through the equation:

$$Yi = axi/(b + Xi).$$

The statistical analysis (Tukey test) was defined according to nonlinear fitting model using the Prism Software. To determine the curve we used the values of y as the hemolytic activity and x as the log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data.

The percentage of haemolysis increased in a dependent-concentration manner of the extract of *A. sylvaticus* used. The LC_{50} value obtained in this experiment was 9.213 mg/mL.

The curve obtained (Figure 1) represents the hemolytic

activity of aqueous extract of the *A. sylvaticus* mushroom on the solution of human erythrocytes at 2%.

DISCUSSION

Several authors suggest that the exact calculation of LC_{50} is valid only for substances that pose a lethal concentration of 1 and 5000 mg/kg. However, regulatory international institutions of chemical composition toxicity recommend a limit of 2000 mg/kg for the LC_{50} test (Larini, 1997).

By determining the LC_{50} of aqueous extract from the *A. sylvaticus* mushroom, it was observed that this extract has low toxicity, since many grams are needed to cause cellular damage.

No study has been found in the literature using methods of cytotoxicity *in vitro* so that the extracts of this mushroom could be evaluated and compared. Nevertheless, the present results corroborate the results found by Novaes et al. (2007), where the effects of acute toxicity of the aqueous extract of this mushroom were assessed by clinical, biochemical and histopathological parameters in healthy mice, showing very low toxicity.

The low toxicity of this aqueous extract on erythrocytes may be related to the low toxicity of this extract found in animals, suggesting its potential for therapeutic purposes. But there are few studies in the literature regarding comparative sensitivity between these two methods (Cruz et al., 1998).

In 1927, Trevan suggested that lethal concentration should be considered when it kills 50% of the animals (LC_{50}) since the LC_{50} values vary less than those of LD_1 and LD_{99} (dosage required to kill 1 or 99% respectively of the test population) (Silva, 2006). Many toxicity tests currently used for assessment of toxic agents still employ laboratory animals (Harbell et al., 1997). However, the LC_{50} tests advocated by Trevan have been the subject of several reviews and discussions, especially of ethical nature, owing to the large number of animals sacrificed, the suffering caused during some tests, the imprecision of values obtained and the information it fails to provide (Silva, 2006; Cazarin et al., 2004).

Therefore, the completion of toxicological studies in animals with *in vitro* tests is a global trend (Cazarin et al., 2004). The development of new methods for *in vitro* toxicity testing and its recognition by international organizations such as the FDA (Food and Drug Administration) in 1983 and the OECD (Organization for Economic Cooperation and Development) in 1987 has fostered the replacement of tests using laboratory animals (Cruz et al., 1998; Cazarin et al., 2004).

These two organizations, further to promoting the improvement of toxicity tests, have been engaged in reducing costs and time spent in studies, decreasing and replacing animal use (Cazarin et al., 2004).

In this sense, there has been growing demand for *in vitro* tests, which do not sacrifice animals (13). The evaluation of *in vitro* hemolytic action has been used as screening methodology for various toxic agents (Kublik et al., 1996; Mehta et al., 1984). *In vitro* haemolysis tests have also been employed by several authors for the toxicological evaluation of different plants (Gandhi et al., 2000).

According to Queiroz (2009), laboratory experiments with cells reproduce the conditions and even reactions similar to those occurring in the body, and are thus able to observe and quantify changes undergone by cells from a particular product or medicament, as well as the behavior of each cell component separately, restricting the number of variables.

Ralph et al. (2009) through testing for hemolytic activity rated the degree of *in vitro* toxicity according to the observed mortality rate: 0 to 9% = non-toxic, 10 to 49% = slightly toxic, 50 to 89% = toxic; 90 to 100% = highly toxic. Therefore, for new studies to be conducted, the use of non-toxic concentrations (LC0-9) is suggested.

Arguing that the chemical and the pharmaceutical industry perform the LC_{50} test simply because it is required by authorities, in which case without any scientific justification, some authors propose replacing the LC_{50} with maximum non-lethal concentration (MNLC). The MNLC of a substance is defined as the maximum concentration which does not cause any mortality in a number of animals.

This indicator has been proposed as being more useful than the LC_{50} for evaluating the risk/safety of a product by the fact that it uses the non-occurrence of deaths (most severe of toxic effects) as analytical criterion (Larini, 1997). The maximum concentration is defined as the highest dose tolerated without toxic symptoms. The maximum lethal concentration refers to the smallest amount of drug capable of producing death. The therapeutic dose or effective dose is between the minimum and maximum therapeutic dose (Silva, 2006).

Silva et al. (2009) considering that a safe drug cannot cause injury to the plasma membrane of healthy cells, either by forming pores or breaking down the cell, evaluated the cytotoxic activity of triazoles on human erythrocytes. On the other hand, Ralph et al. (2009) evaluated the cytotoxicity of synthetic naphthoquinones on human erythrocytes, demonstrating the possibility of its use for therapeutic purposes, since it had no cytotoxicity on the human erythrocyte membrane.

The hemolytic activity test was also used by Maia et al. (2009), who evaluated the hemolytic activity of dry extract from the bark of *Maytenus guianensis*, verifying that this species did not cause haemolysis on human erythrocytes and may be used for pharmacological purposes.

Furthermore, Schulz et al. (2005) found positive values of the cytotoxic effect from crude extract of *Bacillus amyloliquefaciens* against sheep erythrocytes.

Vieira et al. (2002) in turn, using the hemolytic activity test to investigate the cytotoxic outcome of chloroform on

human lymphocytes, found results that do not prove the cytotoxic action of chloroform, but its genotoxic consequences, since it is capable of causing DNA damage without affecting the normal activity of cells.

Laranjeira et al. (2010) with the purpose of evaluating the hemolytic activity of ethanol extract from *Croton grewioides* leaves on erythrocytes from mice, found results that prove the absence of hemolytic activity on erythrocytes from these animals, suggesting that the cytotoxicity of the extract under analysis was not related to membrane damage, but rather related to apoptosis.

A study by Pita (2010) evaluated the cytotoxicity of natural products utilized in therapy against cancer, obtained from essential oil of *X. langsdorffiana* leaves (trachylobano-360 and OEX) on erythrocytes from mice. The author found values that show the reduced cytotoxic activity of these products.

Cazarini et al. (2004) points out that the *in vitro* alternative tests validated and accepted with regulatory purposes in substitution to methods performed on animals, are still much more a goal than a reality.

The scarcity of literature data to discuss the results and evaluation of acute cytotoxicity *in vitro,* reasserts the need for scientific research of this nature considering that they contribute greatly towards the safe use of such substances by humans.

Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.

Further study on the safety of using mushroom are needed, since *A. sylvaticus* has now been used for several diseases, including in therapy against cancer.

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