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Influence of *Pleurotus djamor* Bioactive Substances on the Survival Time of Mice Inoculated with Sarcoma 180

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Abstract: Estimates by the National Cancer Institute (INCA), of the Brazilian Ministry of Health, indicate that cancer was responsible for 7.6 million deaths worldwide in 2008. This vast incidence has led to an increase in research for safer and more efficient therapies in preventing and combating cancer. The search for biologically active substances, extracted from plants and fungus has been intensified in the last decades. Various fungi, among which is the *Pleurotus* genus, synthesize polysaccharides that present therapeutic properties, including the antimicrobial, anti-inflammatory and antitumor activity. *In vivo* animal studies have demonstrated that this effect may be attributed to a glucose polymer known as β -glucan found inside the fungus cell wall which is degraded to smaller soluble fragments which activate granulocytes to kill opsonized tumor cells. The objective of this study was to investigate the survival time of mice inoculated with sarcoma 180 (S180) and treated with extracellular polysaccharides from *Pleurotus djamor*. The polysaccharides obtained from liquid cultivation were precipitated with ethanol (PE1) and with acetone (PE2) and administered in mice intraperitoneally. The survival test was carried out for 5 consecutive days commencing 24 h after tumor induction in daily doses of 30, 100 and 300 mg kg⁻¹ of body weight. After 30 days, the evaluation was performed and revealed a 100% survival rate in animals for 30 and 100 mg kg⁻¹ doses of PE1 as well as PE2. PE1 in a dose of 30 mg kg⁻¹ is therefore, suggested for the continuity of studies for providing 100% survival in 30 days with no harmful effect.

Key words: Antitumor activity, intraperitoneal treatment, *in vivo* analysis, polysaccharides

INTRODUCTION

The National Cancer Institute (INCA), of the Brazilian Ministry of Health, reveal that 12.4 million new cases of cancer appeared in 2008 while there were 7.6 million deaths worldwide. In Brazil, since 2003, cancer has been the second cause of death, representing approximately 17% of deaths by known cause in 2007. For 2010, it is estimated that 489.270 new cases of cancer will occur in Brazil (INCA, 2009).

In this sense, the search for new molecules, new targets and new formulations for the treatment of cancer has become a high priority study for governments. The chance of successful treatment has increased based on previous studies that indicate antiproliferative and antitumor activity *in vitro* or *in vivo* (Fortes and Novaes, 2006).

The use of natural products for treating diseases is associated with popular medicine worldwide (Sulaiman *et al.*, 2006; Jeyachandran *et al.*, 2007;

Soeksmanto *et al.*, 2010). Study conducted in Universities and Research Institutes has revealed active substances of a natural origin with potential antitumor, antiviral, analgesic and antioxidant activity, among others (Viegas *et al.*, 2006; Hafidh *et al.*, 2009). Natural products from plants, fungi, bacteria and other organisms continue being used in pharmaceutical preparations in the form of pure compounds or extracts (Koduru *et al.*, 2006; Viegas *et al.*, 2006; Mohan *et al.*, 2008).

Currently, epidemiological studies and pre-clinical tests have revealed a great potential for natural compounds in combating cancer. Among the natural origin substances with potential application in the treatment of cancer are those of fungal origin. Studies have shown that this antitumor activity is related to the polysaccharides present in the fungus cell wall which are degraded to smaller soluble fragments that activate granulocytes to kill opsonized tumor cells (Berovic *et al.*, 2003; Zhang *et al.*, 2007; Chan *et al.*, 2009).

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As reported by Noslova *et al.* (2001) and Paulsen (2002), the biological activity of fungal polysaccharides was characterized by the presence of β -glucans and the first biological test was carried out in 1969. The use of this polysaccharide in transplanted tumors caused the tumors stop growing.

Fungi of *Pleurotus* genus have high contents of protein, essential amino acids, unsaturated fatty acids, vitamins and minerals with high medicinal potential (Silveira *et al.*, 2004). Studies have proven various therapeutic properties of polysaccharides synthesized by *Pleurotus*, as the antimicrobial (Wisbeck *et al.*, 2002), anti-inflammatory (Smiderle *et al.*, 2008) and antitumor activity (Driscoll *et al.*, 2009; Furlan *et al.*, 2009; Dalonso *et al.*, 2010).

Given the above, this study was based on the evaluation of the survival time of mice inoculated with sarcoma 180 (S180) and treated with different doses of extracellular polysaccharides obtained from liquid cultivation of *Pleurotus djamor*.

MATERIALS AND METHODS

This study was carried out from July to December 2009.

Microorganism and maintenance: *Pleurotus djamor* UNIVILLE 001 was isolated by the Biotechnological Processes Research Group of Univille and used for the synthesis and extraction of polysaccharides. The culture was kept in Wheat-Dextrose-Agar (WDA) solid medium, under refrigeration (4°C) (Furlan *et al.*, 1997).

Tumor and maintenance: The Sarcoma 180 (S180) tumor culture was given by courtesy of the Pharmacology Department from UNIVALI (Itajai/SC/Brazil) and maintained through weekly intraperitoneal injections in male Swiss albino mice (Pagno *et al.*, 2006).

Obtaining polysaccharide extracts: The extracellular polysaccharides were produced in *Pleurotus djamor* submerged culture in POL (Polysaccharide) medium (Cavazzoni and Adami, 1992), at 30°C with an air flow of 0.25 L min⁻¹ and 300 min⁻¹ stirring, in 5.0 L bioreactor with 4.0 L work volume. Two methods were used to precipitate the polysaccharides from the culture broth after fungal growth. The first one was based on the method proposed by Pokhrel and Ohga (2007), in which ethanol PA (4:1, v/v) was added to the culture broth and left for 24 h at 4°C with the formed precipitate being separated by centrifugation and lyophilized. This precipitate was denominated PE1. The second method tested was the

addition of PA acetone to the culture broth at a ratio of 3:1, acetone:broth (v/v) and left for 24 h at 4°C with the formed precipitate being separated by centrifugation and lyophilized. This precipitate was denominated PE2.

Evaluation of the animal survival time: For each precipitate tested, the animals were divided into three test groups (n = 5). After tumor induction, the test groups-PET were treated with each of the following precipitate doses: 30, 100 and 300 mg kg⁻¹. For each group tested there was a substance control group-PES (n = 5) (these animals did not receive tumor induction but were treated in the same way as the animals in the test group). One negative control group - NC (without tumor induction and without treatment) and one positive control group-PC (with tumor induction and without treatment), both with 5 animals were also tested. Tumor induction was performed subcutaneously, in the back of each mouse from the test and positive control groups in a concentration of 25×10⁶ cell mL⁻¹, in 0.2 mL volume. The negative control and the substance control groups received 0.2 mL PBS solution (Mizuno *et al.*, 1999). The PE1 and PE2 precipitates were administered in animals from the test and substance control groups, intraperitoneally (i.p.), for 5 consecutive days, commencing 24 h after tumor induction (Kasai *et al.*, 1991) in daily doses of 30, 100 and 300 mg kg⁻¹ of body weight. PBS solution was administered in animals from the positive and negative control groups. After 30 days, the evaluation was performed obtaining a survival rate (number of animals alive in relation to number of animals treated) and the mortality rate (number of animals dead in relation to the number of animals tested) (Mizuno *et al.*, 1999).

Statistical analysis: Data obtained were evaluated through the analysis of variance (ANOVA), using the F Test with a 5% significance level. Multiple comparisons were performed through the Tukey method to identify the significant differences between the means with a 5% significance level.

RESULTS

All animals treated with PE1 and PE2 in the concentration of 30 mg kg⁻¹ had a 100% survival for 30 days while the animals in the Positive Control group began to die after the 12th day, reaching a survival rate of only 20% in 30 days (Fig. 1). Further observing Fig. 1, it can be seen that the survival rate of animals in the precipitate with acetone substance control group (PE2S), reduced to 80% from the 22nd day onwards.

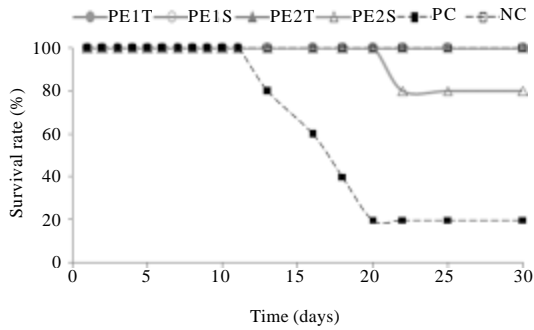


Fig. 1: Effect of precipitate obtained with ethanol (PE1) and precipitate obtained with acetone (PE2) in the dose of 30 mg kg^{-1} on the survival of animals in test groups (PE1T and PE2T), substance control (PE1S and PE2S), Positive Control (PC) and Negative Control (NC)

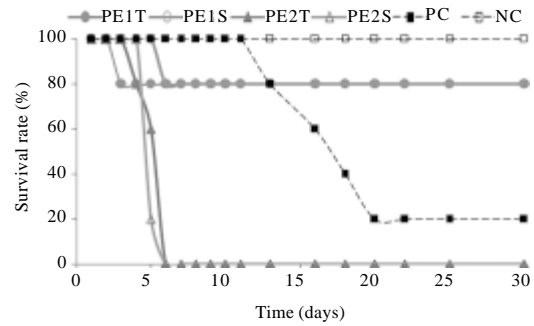


Fig. 3: Effect of precipitate obtained with ethanol (PE1) and precipitate obtained with acetone (PE2) dose of 300 mg kg^{-1} on the survival of animals in test groups (PE1T and PE2T), substance control (PE1S and PE2S), Positive Control (PC) and Negative Control (NC)

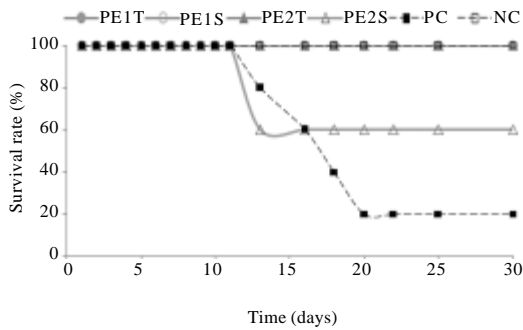


Fig. 2: Effect of precipitate obtained with ethanol (PE1) and precipitate obtained with acetone (PE2) in the dose of 100 mg kg^{-1} on the survival of animals in test groups (PE1T and PE2T), substance control (PE1S and PE2S), Positive Control (PC) and Negative Control (NC)

According to Fig. 2, the treatments with the 100 mg kg^{-1} dose presented a similar profile to that observed for the 30 mg kg^{-1} dose. However, the survival rate of animals in the precipitate with acetone substance control group (PE2S) dropped to 60% on the 13th day, thus remaining until the end of the experiment.

For treatment with 300 mg kg^{-1} ethanolic precipitate (PE1) 20% mortality was observed on the 3rd day while in its substance control (PE1S), this mortality rate was reached on the 6th day (Fig. 3). Treatment with acetone precipitate in this dosage revealed similar profiles for the treated group (PE2) and its substance control group (PE2S), both reaching 100% mortality on the 6th day of the experiment (Fig. 3).

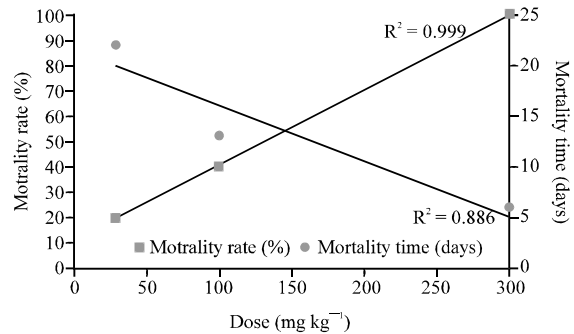


Fig. 4: Relation between the acetone precipitate dose, the mortality rate and time taken to die of animals in the substance control group (SC)

Figure 4 shows that following the increase in doses of the extract obtained with acetone (PE2) in the substance control group there is an increase in the mortality rate and a decrease in mortality time.

Statistical analysis (Fig. 5) showed no significant difference between the treatment using 300 mg kg^{-1} of PE1 and its positive and substance control, suggesting that this dose is harmful to the animals. Significant differences were however observed comparing these groups with all the others ($30, 100 \text{ mg kg}^{-1}$ and NC).

The most harmful effect was observed with 300 mg kg^{-1} of PE2. In this case (Fig. 6), significant differences were observed between the groups treated with this dose (test and substance control) and all the other groups ($30 \text{ mg kg}^{-1}, 100 \text{ mg kg}^{-1}, \text{PC}$ and NC).

No significant difference was observed between the negative control and the groups treated with doses of

DISCUSSION

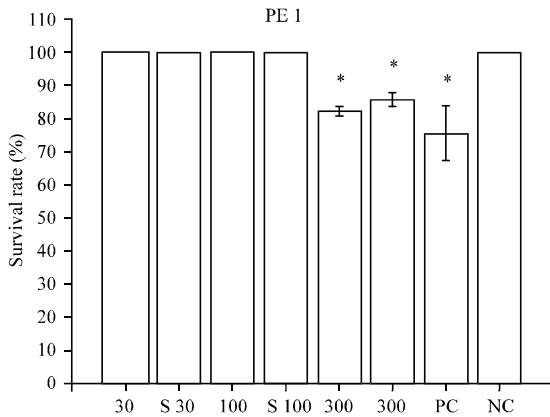


Fig. 5: Survival rates of the groups treated with the precipitate obtained with ethanol (PE1 and PE1S) using the doses of 30, 100 and 300 mg kg⁻¹, Positive Control (PC) and Negative Control (NC). *Means that there is no significant difference between these values but there is significant difference between these values and the others (without*)

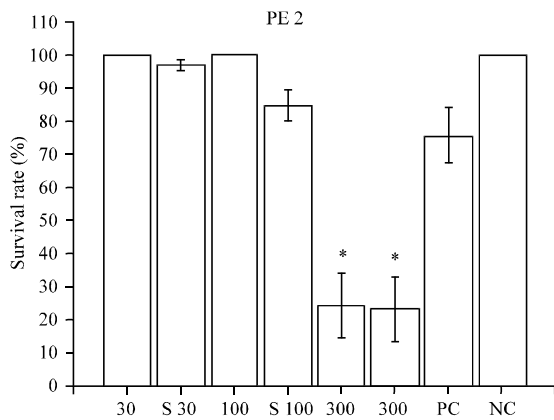


Fig. 6: Survival rates of the groups treated with the precipitate obtained with acetone (PE2 and PE2S) using the doses of 30, 100 and 300 mg kg⁻¹, positive control (PC) and negative control (NC). *Means that there is no significant difference between these values but there is significant difference between these values and the others (without*)

30 and 100 mg kg⁻¹ of PE1 or PE2. However, for PE1 these doses presented significant difference when compared to the positive control, pointing out the effective action of the ethanolic precipitate.

One of the most important scientifically accepted criteria for judging the value of tumor agents is the increase in animal survival time (Clarkson and Burchenal, 1965).

Chang *et al.* (2009) investigated the effects of *Ganoderma lucidum* extracts on the survival rate of BALB/c mice inoculated with WEHI-3 leukemia cells. The negative control group (without WEHI-3 cells) presented 100% survival while the positive control group (with WEHI-3 cells and without treatment) had 30% survival. Animals of the test group (with WEHI-3 cells and treated with 3 and 6 mg/kg/day of extract) had a survival rate of 40 and 50%, respectively. These results cannot be directly compared to those obtained in this work as the tumor investigated is different.

Zhao *et al.* (2003) evaluated Sarcoma 180 inhibition by a lectin obtained from *Agrocybe aegerita*, obtaining an increase in survival rate from 50% (positive control group) to 90% (test group). These results are slightly lower than those obtained in this work where 100% survival was reached.

Gonzaga *et al.* (2009) tested a polysaccharide obtained from *Agaricus blazei* Muril against Sarcoma 180. The dose of 25 mg/kg/day provided a life span of 26.4% when compared with positive control group. The animals survived about 20 days. These results are also lower when compared to those reported in this work, considering that with 30 mg kg⁻¹ of PE1 or PE2, survival increased from 20 to 100% after 30 days.

Shamtsyan *et al.* (2004) tested antitumor activity of *Pleurotus ostreatus* extract against melanoma B16, observing a 60% survival rate.

Wang *et al.* (2000) testing a *Pleurotus ostreatus* extract against Sarcoma 180, obtained 21.9 to 106.7% increase in survival time of tested animals, results similar to those obtained in this study.

Table 1 shows a comparison between the results obtained in this study and those reported in study for the survival rate, revealing that the use of *Pleurotus djamor* extracellular polysaccharide precipitates as antineoplastic agents has a significant impact on the increase in survival of animals treated.

However, as can be observed in Fig. 4, there is a direct relation between doses of the precipitate obtained with acetone (PE2) and the mortality rate in animals from the substance control group. The existence of an inverse relation between the dose and mortality time of the animals in this group was also observed.

Table 1: Survival rates in relation to substance origin and tumor type

| Substance origin | Tumor | Dose | Evaluation period (days) | Survival rate (%) | References |
|-------------------------------|--------------|-------------------------------|-------------------------------|--|--------------------------------|
| <i>Ganoderma lucidum</i> | WEHI-3 | 3 (mg kg ⁻¹) | 28 | 40 | Chang <i>et al.</i> (2009) |
| | Leukemia | 6 (mg kg ⁻¹) | | 50 | |
| <i>Agrocibe aegerita</i> | S180 | 0.1 (mg mouse ⁻¹) | 20 | Increase from 50 to 90% | Zhao <i>et al.</i> (2003) |
| <i>Agaricus blazei</i> Murill | S180 | 25 (mg kg ⁻¹) | Up to all animals died | Increase of 26,4% in relation to PC | Gonzaga <i>et al.</i> (2009) |
| <i>Pleurotus ostreatus</i> | S180 | 1.5 (mg kg ⁻¹) | Up to 50% of the animals died | Increase of 21.9 to 106.7% in relation to PC | Wang <i>et al.</i> (2000) |
| | H22 | | | | |
| <i>Pleurotus ostreatus</i> | Melanoma B16 | 100 (mg kg ⁻¹) | 30 | 60 | Shantsyan <i>et al.</i> (2004) |
| <i>Pleurotus djamor</i> (PE1) | S180 | 30 (mg kg ⁻¹) | 30 | 100 | Present study |
| | | 100 (mg kg ⁻¹) | | 100 | |
| | | 300 (mg kg ⁻¹) | | 80 | |
| <i>Pleurotus djamor</i> (PE2) | S180 | 30 (mg kg ⁻¹) | 30 | 100 | Present study |
| | | 100 (mg kg ⁻¹) | | 100 | |
| | | 300 (mg kg ⁻¹) | | 0 | |

It can therefore, be concluded that the increase in the PE2 precipitate dose has a harmful effect on healthy animals. This effect was not observed in groups of animals inoculated with tumor and treated with 30 and 100 mg kg⁻¹ doses (Fig. 1, 2) but it was observed in animals treated with 300 mg kg⁻¹ dose (Fig. 3).

Kviecinski *et al.* (2008) reveals that adverse effects are associated with the high toxicity presented by substances used in cancer treatment, leading to significant morbidity.

Cheng *et al.* (2003) evaluated the antineoplastic effect of *Bupleurum scorzonerifolium* plant extracts obtained with acetone, methanol and water, against human lung cancer cells A549 *in vitro*. The authors observed that the extract concentration obtained with acetone which was cytotoxic to cells was less than that of extracts obtained with methanol and water. The authors showed that the antitumor effect is due to the performance in mitosis and to the induction of apoptosis of tumor cells and, as the tumor cells tend to divide rapidly, the most notable effect of the extract occurs on these cells, however, as it acts on cellular division, the extract can also act on healthy cells, inducing apoptosis and damaging healthy tissues.

In light of this, it is not recommended to use the precipitate obtained with acetone for this type of treatment. Considering that the results obtained with the 30 and 100 mg kg⁻¹ doses of ethanol precipitate are similar and that the 300 mg kg⁻¹ dose presented a harmful effect on animals, based on the data obtained in this study, the use of this precipitate in a dose of 30 mg kg⁻¹ is suggested for the continuity of studies.

CONCLUSIONS

The survival test revealed a 100% survival rate in animals receiving 30 and 100 mg kg⁻¹ doses of PE1 and also PE2. However, none of the animals that received the 300 mg kg⁻¹ dose of precipitate obtained with acetone (PE2) remained alive. When the PE2 concentration is increased, it has a harmful effect on healthy animals with this effect increasing together with the increased dosage.

PE1 in a dose of 30 mg kg⁻¹ is, therefore, suggested for the continuity of studies for providing 100% survival in 30 days and for having no harmful effect on the animals.

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