

IMMUNOMODULATING PROPERTIES OF *PLEUROTUS* SP. FRUITING BODIES POWDER ON CYCLOPHOSPHAMIDE TREATED MICE.

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ABSTRACT

Since the potentiation of host resistance is one of the most important objectives in cancer and AIDS therapy, the present study examined the immunomodulating effects of *Pleurotus* sp. fruiting bodies powder on cyclophosphamide (CY) treated mice. *Pleurotus* powder was administered during 7 days to Balb/c mice by oral route (1000 mg/kg) and the CY (100 mg/kg) was inoculated intraperitoneally, at the beginning of the experiment or at the fifth day. The influence of the supplement on CY immunosuppression was evaluated on the eighth day. The prophylactic administration of *Pleurotus* powder increased the bone marrow cellularity (9.89×10^6 vs. 4.94×10^6 per femur in therapeutically treated mice, $p=0.035$) and the white blood cell counts (7.8×10^9 vs. 4.7×10^9 cells/L, $p=0.016$). The *Pleurotus* supplement in the prophylactic treatment stimulated the liver protein synthesis; although no significant effects were found in serum protein concentrations. The effect of *Pleurotus* powder on cell-mediated immunity was determined by the delayed-type hypersensitivity reaction (DTH) in mice under the therapeutic schedule. The DTH response measured at 48 and 72 h after antigen challenge was similar that of normal control mice ($p < 0.05$). The induction of DTH reaction was associated with an increase in the mass index of popliteal lymph nodes ($p = 0.044$). An *in vitro* lymphoproliferative-stimulating response evaluated in mice spleen cells was also demonstrated with aqueous and ethanolic extracts obtained from *Pleurotus* powder. These effects suggest that *Pleurotus* supplement could potentiate the host defense mechanisms *in vivo* and should be promising for further pharmacological studies.

Keywords: *Pleurotus*; medicinal mushrooms; fruiting bodies; cyclophosphamide; immunomodulation.

INTRODUCTION

Medicinal mushrooms have an established history of use in traditional oriental therapies and modern clinical practice in several Asian countries continues to rely on mushroom-derived preparations [1, 2]. Medicinal effects have been demonstrated for many traditionally used mushrooms, including extracts of species from genera *Auricularia*, *Flammulina*, *Ganoderma*, *Grifola*, *Hericium*, *Lentinus* (*Lentinula*), *Pleurotus*, *Trametes* (*Coriolus*), *Schizophyllum*, and *Tremella* [3, 4].

The genus *Pleurotus* comprises some of the most popular *Basidiomycetes* edible mushrooms which cultivation has increased greatly throughout the world during the last few decades [5]. Its popularity has been expanded due to its vigorous growth on a variety of

agroforestry substrates and for the production of a high nutritional value-food [6] containing biologically active compounds with therapeutic effects [7].

Recent studies on various *Pleurotus* species have shown a number of pharmacological activities, such as anti-tumour, immunomodulatory, antigenotoxic, antioxidant, anti-inflammatory, hypocholesterolaemic, antihypertensive, antiplatelet-aggregating, antihyperglycaemic, antimicrobial and antiviral activities [8].

With the view of developing new therapeutic agents to potentiate host resistance to cancer and infectious disease, such as AIDS, there has been an upsurge of interest in immunomodulating substances from medicinal mushrooms [9-11]. *Pleurotus* species, like many edible and medicinal mushrooms, are a good source of immunomodulators and substances considered as “host defense potentiators” (HDPs) as judged by their immunostimulating properties. Several molecules able to augment or complement a desired immune response have been isolated from *Pleurotus* spp., such as: water-soluble polysaccharides from *P. citrinopileatus* fermentation broth [12]; glucans from *P. florida* fruiting bodies [13]; proteoglycans, polysaccharides and polysaccharopeptides from *P. ostreatus* mycelia [14-16] and DNA from *P. ostreatus* fruiting bodies [17]. These compounds stimulate different cell populations of the immune system, for instance, macrophages, Natural killer (NK) cells, T cells, and also modulate cytokine system [8].

Much research work has been reported for various extracts and isolated compounds, particularly polysaccharides. Therefore, the study of the synergy exerted by the vast structural diversity of biomolecules found in *Pleurotus* crude extracts, powders and other preparations on immune responses deserves special attention. In this context, dietetic supplements with a high therapeutic potential formulated from refined or partially refined mushroom extracts, or from dried mycelia/fruiting bodies biomass are referred as “mushroom nutraceuticals” [18, 19]. Those supplements with an immunostimulating effect are defined as “immunoceuticals” [20].

In Cuba, the implementation of technologies for the cultivation of *Pleurotus* spp. on agricultural substrates, in addition to food generation for human consumption [21, 22] opened new research activities towards mushroom immunoceuticals. *Pleurotus* fruiting bodies obtained under Good Manufacture Practices can be used in the formulation of biologically active products, such as, powders, capsules and tablets.

The present study examined the immunomodulating effects of *Pleurotus* sp. fruiting bodies powder on the immunosuppression caused by cyclophosphamide in mice. An extended knowledge of the immuno-enhancing activity of *Pleurotus* would be useful in understanding the potential applications of *Pleurotus*-derived preparations for immunotherapy.

MATERIALS AND METHODS

Preparation of *Pleurotus* sp. powder. *Pleurotus* sp. (strain CCEBI-3024) deposited at the Culture Collection of the Center for Studies of Industrial Biotechnology (CEBI) was used in this work. Cultivation was performed by solid-state fermentation of mushroom spawn on pasteurized coffee pulp used as substrate in plastic bags of 2 kg (30x40 cm) [21, 22]. The fruiting bodies were harvested, sliced into small pieces and dried at 45°C for 24 h. The dried material was milled and the resulting powder was preserved from light and humidity in plastic bags for further use.

The sugar and protein contents in the powder were determined by the method of Dubois et al. [23] and by Lowry's method [24] using glucose and bovine serum albumin (BSA) as standards, respectively.

The powder was extracted with hot water and ethanol to obtain both aqueous and ethanolic extracts for assessing the *in vitro* lymphoproliferative activity.

Animals and treatments. Pathogen-free male Balb/c mice were purchased from the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). The 20-25 g mice were fed a standard diet and acidified water *as libitum*. Fifty mice were divided into five groups (n= 10). *Pleurotus* powder was administered during 7 days to Balb/c mice by oral route (1000 mg/kg) and the cyclophosphamide USP 23 for injection (CY, JSLYP, China) (100 mg/kg) was given intraperitoneally (i.p.), at the beginning of the experiment (CY-*Pleurotus* group) or at the fifth day (*Pleurotus*-CY group). CY-Saline and Saline-CY groups were designed as controls replacing the *Pleurotus* powder by physiological saline solution. A non-administered control group of 10 mice (C) was also included in the study. The influence of treatments on CY immunosuppression was evaluated on the eighth day. Blood was collected from the orbital vein of mice and then, the animals were killed.

All experiments were approved by the institutional Ethical Committee (University of Oriente) and have been performed in accordance with Cuban legislation and the National Research Council Guidelines for the Care and Use of Laboratory Animals.

Hematological methods. The blood specimens were analysed for hemoglobin with the Hemotest reagent (HELFA Diagnósticos, EPB Carlos J. Finlay, Havana, Cuba) and for white blood cell counts. Femoral bone marrow cells were withdrawn with Hanks' solution and counted with a Neubauer chamber (Germany) under a binocular microscope. The spleen cell suspension was prepared by gently teasing the tissue with ice-cold Hanks' solution and passing it through antiseptic gauze (Johnson and Johnson Medical, TX, USA). The number was counted with a Neubauer chamber.

Liver and serum protein analysis. Liver samples were homogenized in ice-cold 0.01 mol/L phosphate buffer saline (PBS) pH 7.4 (1:3 w/v). Total protein was measured according to the Lowry's method [24].

Serum was prepared from collected blood and stored at -20°C until required. Total serum proteins were measured by a Biuret colorimetric assay using BSA as standard, albumin by the colorimetric reaction with the bromocresol green reagent and globulins as the difference between total proteins and albumin.

Evaluation of cell immunity. In a parallel-conducted experiment, the cell-mediated immune response was assessed by the delayed-type hypersensitivity reaction (DTH) only in mice under the therapeutic schedule (CY-*Pleurotus* group) and its control (CY-Saline) group.

Animals (n=5) were immunized by an intradermal (i.d.) injection of 50 µL of 5 mg/mL bovine serum albumin (BSA) emulsified in Complete Freund Adjuvant (CFA) (Sigma, St. Louis, MO) at two sites on the abdomen. Eight days after immunization, the mice were rechallenged by injection of 20 µL of 5 mg/mL BSA into one rear foot pad, while the other rear foot pad received a comparable volume of phosphate buffered saline (PBS). Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge by use of micrometer (Mitutoyo, Tokyo, Japan). The magnitude of the DTH response was determined as the differences in foot pad thickness between the antigen and PBS injected foot pads [25].

The popliteal lymph nodes (right and left) of the antigen sensitized and rechallenged animals of DTH experiment were removed and washed with PBS pH 7.4. The excess of humidity was discarded with a filter paper and the lymph nodes were immediately weighed separately in an electronic analytical balance (Sartorius). The mass index was expressed as the relation between the weight of the popliteal node belonging to BSA-injected foot pad with respect to that of PBS-injected pad [26].

In vitro lymphoproliferative test. The assay was carried out according to a modification of the method described by Soto-Velazco et al. [27] using murine spleen lymphocytes instead of human lymphocytes. The suspension of splenocytes was obtained by the gentle teasing of spleens in RPMI-1640 (Sigma, St. Louis, MO) containing 8% fetal calf serum and supplemented with antibiotics. Viable cells estimated by the Trypan Blue exclusion method were counted with a Neubauer chamber and the cell concentration was adjusted to 2×10^6 cells/mL.

Briefly, phytohemagglutinin (PHA) (200 μ L) as a B-cells mitogen was added to conical tubes of 15 mL containing 5 mL of supplemented RPMI-1640 to a final concentration of 5 μ g/tube, followed by the addition of 2×10^6 cells and 100 μ L of powder extracts (aqueous or ethanolic). The tubes were incubated at 37°C for 27 h and then 500 μ L of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H tetrazolium bromide (MTT, 5 mg/mL) was added. After the incubation of the resulting mixture at 37°C for 4 h, the tubes were centrifuged 10 min at 1500 rpm. The supernatants were discarded and 1 mL of isopropanol was added to each tube. The absorbance of the mixture was measured at 570 nm in a Genesys 10 UV/VIS spectrophotometer. The stimulation index was calculated considering the absorbance of control cultures without PHA as the unit.

Statistical analysis. The results were expressed as mean \pm standard deviation (SD). The Kruskal-Wallis rank test followed by the Student-Newman-Keuls test was applied to determine the significance of differences between treatments. The Student's *t*-test was used to compare the two means in the experiment of popliteal lymph nodes mass index. Differences at $p < 0.05$ were accepted as significant. The software Statgraphics Plus version 5.1 (Statistical Graphics Corporation, 1994-2001) was used in all the analysis.

RESULTS AND DISCUSSION

Immune system is a very complex homeostatic system consisting of a network of interacting cells, tissues and organs. It allows the organism to exist within itself and maintains a surveillance to recognize components considered nonself. The body's immunity has been shown to be suppressed in several diseases, like AIDS and cancer. The chemotherapy and radiotherapy in cancer treatment contribute to further depression of the immune system [28]. Use of immunomodulating therapeutic agents can solve these problems largely and efforts to find new immunomodulators are on-going. Among higher fungi investigated for immunomodulating effects, several mushroom species demonstrate great potential and some of them are already commercially developed [29].

Dried *Pleurotus* mushroom would become an attractive alternative for the development of drugs and immunocuticals preparations. The powder evaluated in this work contained 55% (w/w) carbohydrate and 25% (w/w) protein. Traditional preparations of medicinal species, used for thousands of years, might give some support to the idea that a heat-treatment might preserve at least part of the activity [30].

During the experimental period, no environmental factors other than the stated variables were thought to have affected the results of the study. No deaths occurred in either the control or the administered groups.

Cyclophosphamide is probably the most common antineoplastic used in cancer chemotherapy and is an essential component of several effective chemotherapeutic formulas. However, cyclophosphamide shows potent immunosuppressing properties, thus affecting the bone marrow cellular production as well as the B cells and the antibodies responses [31]. As expected, cyclophosphamide severely impaired the mice hematopoietic tissue, but the *Pleurotus* powder was found to have an active protective effect in mice, particularly when administered before cytostatic.

The hematological parameters assayed on the eight day are shown in Fig. 1. The prophylactic administration of *Pleurotus* powder increased the bone marrow cellularity (9.89×10^6 vs. 4.94×10^6 per femur in therapeutically treated mice, $p=0.035$). In mice receiving the therapeutic schedule no significant differences were found compared to CY-saline control groups. A significant contribution to the protective effect of *Pleurotus* product on hemopoiesis, may be in the production and differentiation of auto-regenerative bone marrow hemopoietic cells (precursors of both myeloid and lymphoid lines) and a more rapid recovery of these series of hemopoietic cells in animals protected by the immunocellular supplement. We can infer that one of the mechanisms of increased cyclophosphamide tolerance of animals is the activation effect on hemopoiesis by the evaluated *Pleurotus* powder.

The white blood cell counts in peripheral blood were also higher in the prophylactic administered mice (7.8×10^9 vs. 4.7×10^9 cells/L in therapeutically treated mice, $p=0.016$). The observed significant improved recovery of leukocyte numbers against CY-induced leucopenia could be concerned with enhancement of hematopoietic factors such as colony-stimulating factors. Furthermore, increased white blood cell number would be an important contributing factor to reduce the risk of various infectious diseases in immunocompromised patients.

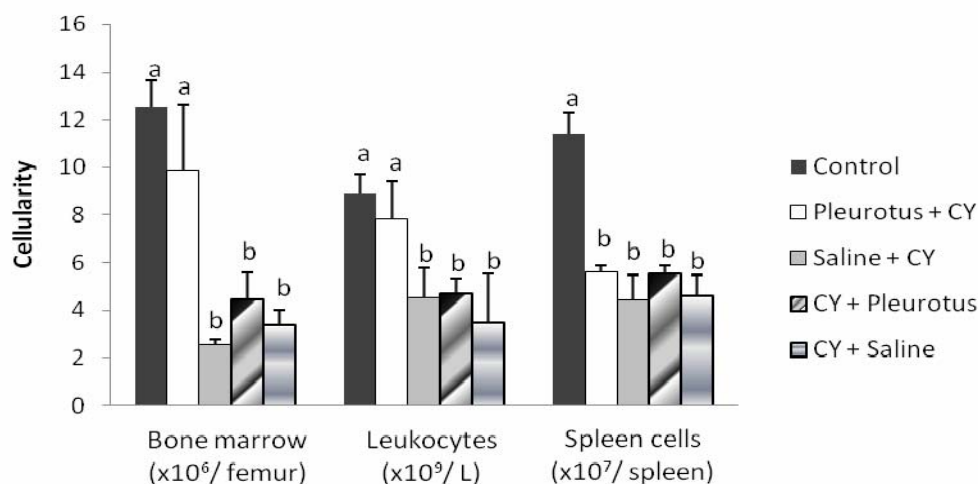


Figure 1: Effect of prophylactic or therapeutic administration of *Pleurotus* powder on the hemopoiesis of cyclophosphamide treated Balb/c mice.

All values are given as the arithmetic mean \pm standard deviation of 10 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls).

The stimulant effect on bone marrow cellularity and leukocyte counts exerted by a hot-water extract from *Pleurotus ostreatus* mycelium (obtained by solid state fermentation) administered in a prophylactic schedule to cyclophosphamide treated mice has been reported by Morris et al. [32]. On the other hand, a *P. ostreatus* mycelial extract inhibited the tumor growth in mice administered alone or concomitant with CY. The extract also decreased the severity of leucopenia caused by the cytostatic [33].

The spleen cellularity of mice treated with *Pleurotus* powder did not differ from their saline control groups. All the experimental treatments showed a significant decreasing in the spleen cell counts compared to the immunocompetent mice ($p= 0.0175$) (Fig. 1). One of the most important functions of this lymphoid organ is the production of antibodies through the antigenic stimulation of B cells [28]. It has been reported that B cells are particularly sensitive to CY (more than T cells) and in some cases, the suppression of antibody response to an antigenic specific stimulus would be lasting [31].

No significant differences were found in hemoglobin levels of mice belonging to both prophylactic or therapeutic administered groups (139±16 vs. 108±9 g/L, respectively). However, only the animals treated with *Pleurotus* powder before CY administration reached hemoglobin concentrations similar to that of the control group (145±3 g/L, p= 0.0231). This favorable effect of *Pleurotus* supplementation would be an important aspect to consider during immunonutritional interventions of patients with cancer or pathologies related with the immune system.

The results of liver and serum protein content in all groups are given in Table 1. The liver total protein concentration was significantly higher in animals administered with *Pleurotus* powder before CY compared to the therapeutic schedule (p= 0,0318), although the levels of control immunocompetent mice were not reached. The partial recovery observed in this parameter could be associated with more efficient nitrogen utilization as judged by the protein content of the powder and therefore, the stimulation of protein anabolism in the liver, including the synthesis of acute phase proteins.

There were non significant differences between groups with respect to the total serum protein concentration, albumin and globulin concentrations (Table 1). However, Llauradó et al. reported an increasing in total serum protein levels in malnourished Balb/c mice supplemented with a cold-water extract from the fruiting bodies of *Pleurotus* sp. [34].

In our study, the prophylactic treated mice showed the lowest values of the albumin-to-globulin (A/G) ratio (p=0.0351). This fact would sustain the stimulation of serum globulins synthesis.

Table 1: Effect of prophylactic or therapeutic administration of *Pleurotus* powder on liver and serum protein content of cyclophosphamide treated Balb/c mice.

	Control	<i>Pleurotus</i> + CY	Saline + CY	CY+ <i>Pleurotus</i>	CY + Saline
Liver protein (mg/g)					69.25 ± 0.35 ^c
(p = 0.0317)	112.04 ± 4.32 ^a	79.9 ± 8.8 ^b	59.2 ± 1.27 ^c	67.5 ± 0.14 ^c	
Serum total protein (g/dL)	6.49 ± 0.05	6.07 ± 0.8	5.29 ± 0.2	5.3 ± 0.35	5.5 ± 0.58
Albumin (g/dL)	3.61 ± 0.11	3.12 ± 0.62	3.99 ± 0.08	3.63 ± 0.37	4.02 ± 0.38
Globulins (g/dL)	2.88 ± 0.48	2.93 ± 1.37	1.8 ± 0.98	1.98 ± 0.57	1.47 ± 0.62
Albumin-to-Globulin ratio (p=0.0351)	1.25 ± 0.43 ^{abc}	1.3 ± 0.76 ^c	3.15 ± 0.74 ^{ab}	2.32 ± 0.74 ^{ab}	3.18 ± 175 ^a

All values are given as the arithmetic mean ± standard deviation of 10 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls).

The immunostimulating properties of *Pleurotus* powder administered therapeutically to cyclophosphamide treated mice on cell-mediated immune response were assessed by the assay of induction of delayed-type hypersensitivity (DTH) response.

Mice supplemented with *Pleurotus* powder showed a higher DTH response as judged by the increasing of foot pad swelling compared to saline control group, particularly at 48 h and 72 h after antigen rechallenge (p <0,05) (Fig. 2). The DTH response mounted at these times by CY-*Pleurotus* group was similar that of control mice. The reconstitution of DTH response reflected the induction of CD4⁺ Th1 cells and the activation of macrophages by cytokines: tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) [25].

Paulik et al [35] evaluated the effects of two glucans obtained from *Pleurotus ostreatus* and yeast in different immunological functions of mice. Both glucans augmented the DTH response compared to control mice, but the induction was higher for *Pleurotus* glucan. A significant increase in the number of T cells (both CD4⁺ and CD8⁺) was found in mice administered with a water soluble polysaccharide extracted from the fermentation broth of *Pleurotus citrinopileatus* [36].

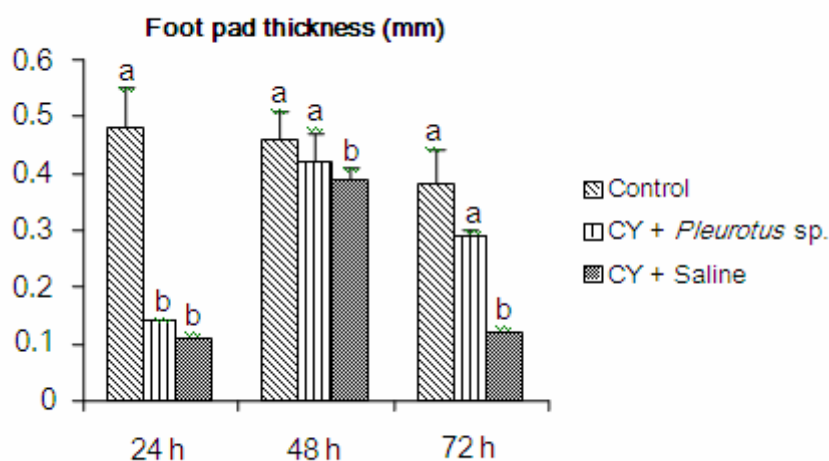


Figure 2: Effect of therapeutic administration of *Pleurotus* powder on the delayed-type hypersensitivity response (foot pad thickness) of cyclophosphamide treated Balb/c mice.

All values are given as the arithmetic mean \pm standard deviation of 5 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls, $p < 0.05$).

These findings suggest that oral administration of edible mushrooms derived products with potential immunostimulating activities would stimulate the immune system after their absorption in the gastrointestinal tract and the activation of gut-associated lymphoid tissues, thus integrating different elements of the immune function.

However, the results of the administration of an aqueous extract of *P. florida* to female Balb/c mice showed that DTH responses were not affected by various doses of the product in different routes. This study indicated that the effects of *P. florida* on cellular responses depends on dose and route of administration [37].

The DTH reconstitution was associated with the increase observed in the mass index of popliteal lymph nodes of the *Pleurotus* supplemented animals (1.87 ± 0.27 vs. 1.34 ± 0.15 in CY-Saline group, $p = 0.044$). Antigens are concentrated in the secondary lymphoid organs, including lymph nodes, where they are presented by mature dendritic cells, the most efficient type of antigen-presenting cell for initiating responses of naive T cells [28].

Cellular immune response was also evaluated by the *in vitro* lymphoproliferative response of murine splenocytes (B cells) through the colorimetric reaction with the MTT reagent. The incubation of splenocytes with aqueous and ethanolic extracts derived from *Pleurotus* powder for 72 h lead to stimulation indexes of 1.90 and 1.28, respectively (Fig. 3). The higher index obtained for aqueous extract could be related with the presence in this fraction of immunostimulating glucans. It has been reported that hydroalcoholic extracts are likely to have less activity, partly because the high-molecular weight compounds are precipitated by alcohol and may not go into solution [30].

Soto-Velazco et al. [27] reported the *in vitro* stimulation of lymphoproliferative response of human mononuclear cells incubated with different extracts from *Ganoderma lucidum*. On the other hand, glucans isolated from *P. florida* fruiting bodies significantly induced the proliferative response as well as phagocytic activity of fish leukocytes (*Catla catla*) *in vitro* [38].

The genotoxic effects of cyclophosphamide and the ability of several- both cold- and hot-water extracts from different mushrooms to protect against damage to cellular DNA [39], suggest that further research on the potential genoprotective activity of *Pleurotus* powder is needed.

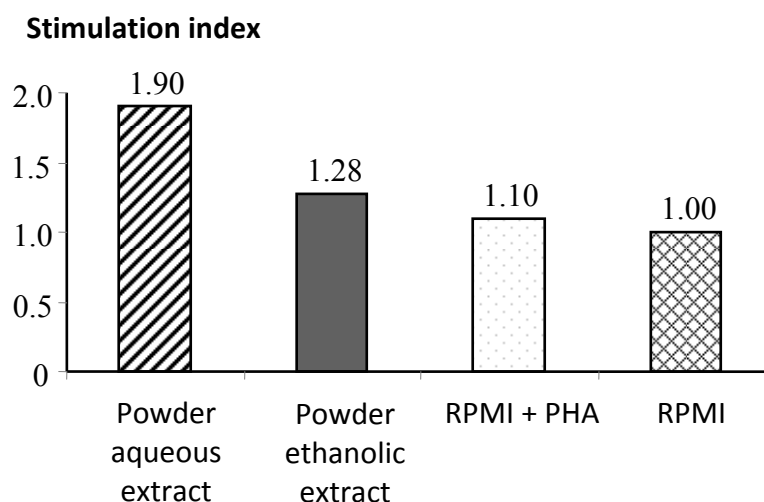


Figure 3: *In vitro* lymphoproliferative-stimulating response of aqueous and ethanolic extracts obtained from *Pleurotus* powder on murine spleen cells.

CONCLUSION

The results of this study evidence that *Pleurotus* supplement, administered orally to cyclophosphamide-treated mice, provide immunological benefits in terms of: (i) the recovery of bone marrow cellularity, (ii) the increase of white blood cell counts, and (iii) the stimulation of cell-mediated immune responses. *Pleurotus* powder could potentiate the host defense mechanisms *in vivo* and should be promising for further pharmacological studies. The effects on cell immunity are especially valuable in the prophylaxis of tumors, immunodeficiencies and as co-adjuvant in chemotherapy. Further studies are needed to address effective phytochemicals of this edible mushroom and their mechanisms.

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