RESEARCH WORK ON THE IMMUNOMODULATING AND ANTITUMOR PROPERTIES OF *PLEUROTUS* SP. IN CUBA

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ABSTRACT

This chapter examined the effects of *Pleurotus* (oyster mushroom) extracts (Myc-E and FB-E) and powders (FB-P) on immunodeficient BALB/c mice. The anti-proliferative effect on NB4 human leukemia cells was measured by flow-cytometry. In addition, the antioxidant activity was investigated by the scavenging of *DPPH* and *ABTS* radicals, the reducing power and the inhibition of lipid peroxidation. *Pleurotus* mycelial extract (Myc-E) exerted a protective effect in both cyclophosphamide and whole-body irradiated mice in terms of bone marrow cellularity, white blood cell counts and the enhancement of monocyte-macrophage system. Cyclophosphamide treated mice also showed a stimulant effect on cell immune response when administered with fruiting bodies powder (FB-P). The fruiting bodies-derived extract (FB-E) stimulated the immunonutritional recovery of malnourished mice after the activation of gut-associated lymphoid tissues. Myc-E reduced the viability of NB4 leukemia cells, particularly at the concentration of 200 µg/ml, by arresting cells in the G_2/M phase. At 10 mg/ml, FB-E showed scavenging effects for *DPPH* and *ABTS* radicals (90.4% and 80%, respectively) and inhibited lipid peroxidation (51.2%), whereas at 5 mg/ml manifested a reducing power of 0.438. *Pleurotus* derived-products could be considered as good candidates for developing nutraceuticals and innovative myco-therapeutics, as judged by their immunomodulating/antitumor and antioxidant effects.

Keywords: antioxidant, antitumor, immunomodulating, mushrooms, myco-therapeutics, nutraceuticals, Pleurotus

INTRODUCTION

Today the well-being of humankind faces unprecedented challenges involving inadequate regional food supplies, deficiency in new insight into healthy eating, diminishing quality of health, and increasing environmental deterioration (Chang and Wasser 2012). In this context, mushrooms are emerging as a vital component of the human diet and have become attractive as a functional food and as a source of drugs and nutraceuticals (Ferreira *et al*. 2009, Patel *et al*. 2012, Gomes Corrêa *et al*. 2016, Morris *et al*. 2017a).

Fruiting bodies as well as mushroom mycelia have a broad range of bioactive properties. Mushrooms are thought to exert approximately 130 pharmacological functions such as antitumor, immunomodulatory,

antigenotoxic, antioxidant, antinflammatory, hypocholesterolemic, antihypertensive, antiplatelet-aggregating, antihyperglycemic, antimicrobial, and antiviral activities (Lindequist 2013, Paterson and Lima 2014, Wasser 2014, Prasad *et al.* 2015). These pharmacological effects have been demonstrated for many traditionally used mushrooms, including species from genera *Ganoderma*, *Lentinus (Lentinula)*, *Agaricus*, *Auricularia*, *Flammulina*, *Grifola*, *Hericium*, *Pleurotus*, *Trametes (Coriolus)*, *Schizophyllum*, *Lactarius*, *Phellinus*, *Cordyceps*, *Inonotus*, *Inocybe*, *Tremella*, and *Russula* (Roupas *et al.* 2012, Vikineswary and Chang 2013, Valverde *et al.* 2015).

Ongoing research projects are aiming to promote mushrooms as a new generation of "biotherapeutics" (Pereira *et al*. 2012, Patel and Goyal 2012). Mycotherapy comprises the use of mushroom-derived extracts and bioactive compounds for their utilization as functional products or drugs with the ability of promoting health. As part of cancer research, mycotherapy is a relatively new and promissory field as a source of agents with immunomodulating and antitumor properties (Popovic *et al.* 2013, Peña-Luna *et al.* 2016). The bioactive molecules comprise high molecular weight compounds, mainly polysaccharides, and low molecular weight secondary metabolites (de Silva *et al*. 2013). Given that only about 10% of mushroom biodiversity has been studied so far, and few of them have been characterized with regard to health benefits, it is likely that new active compounds will be discovered in the future (Hawksworth 2012). Particularly in tropical areas, 22-55% (in some cases up to 73%) of mushroom species have not yet been described (Bass and Richards 2011).

Though *Pleurotus* genus (oyster mushroom) is the second important mushroom of culinary value (Royse 2014), there has been an upsurge in *Pleurotus* research activities in the last two decades in view of its biopotentialities. This genus includes some of the most popular *Basidiomycetes* edible mushrooms which cultivation has increased greatly throughout the world during the last few decades (Sánchez and Royse 2002, Gomes Corrêa *et al*. 2016). 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 containing biologically active compounds with therapeutic effects (Carrasco-González *et al.* 2017).

Pleurotus species have been recognized as mushroom with dual functions to humans both as food and medicine (Khan and Tania 2012, Patel *et al.* 2012). Recent studies on various *Pleurotus* species have shown a number of the pharmacological activities mentioned above (Gregori *et al.* 2007, Deepalakshmi and Mirunalini, 2014, Beltrán *et al.* 2015, Fu *et al.* 2016, Sun *et al*. 2017). In particular, *Pleurotus* spp., is distinguished as important natural resources for immunotherapy, in view of the content of several bioactive compounds able to augment or complement a desired immune response defined as 'host defence potentiators' (HDPs) (El Enshasy *et al.* 2012, Morris *et al.* 2015, Pérez-Martínez *et al.* 2015).

Such bioactive compounds are polysaccharopeptides, polysaccharide-proteins, functional proteins, glucans, proteoglycans and many others. Most of these bioactive compounds follow the immunomodulatory pathway mechanism of mushroom β-glucans by stimulating activities for both innate and adaptive immune systems (El Enshasy *et al.* 2013, Facchini *et al.* 2014). They activate innate immune system components such as natural killer (NK) cells, neutrophils, and macrophages, and stimulate cytokines expression and secretion. These cytokines in turn activate adaptive immunity through the promotion of B cells for antibodies production and stimulation of T-cell differentiation to T helper (Th1 and Th2) cells, which mediate cell and humoral immunities, respectively (Morris *et al*. 2007, Ike *et al*. 2012, Oloke and Adebayo 2015, Tanaka *et al.* 2015).

Both fruiting bodies and mycelia of *Pleurotus* spp. have been studied in search of effector molecules (Kyakulaga *et al.* 2013, Morris *et al.* 2012, 2017b). In the opinion of Chang (2001), mycelial products are the "wave of the future" because they ensure standardized quality and year-round production. Thus, submerged liquid fermentation can provide more uniform and reproducible biomass and may afford valuable medicinal products. However, fruiting bodies obtained under good manufacturing practice

(GMP) can also be used in the formulation of consistent and safe mushroom products such as functional foods, nutraceuticals, and biologically active compounds (Morris *et al.* 2014).

Much research work has been reported for various extracts and isolated compounds, particularly polysaccharides and efforts to find new immunomodulators are ongoing (El Enshasy *et al.* 2013). 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. Better insight into the different roles of multiple active compounds and the mechanisms underlying their biological action will accelerate commercial production of pharmaceuticals for therapeutic applications (Figure 1).

In Cuba, the implementation of technologies for the cultivation of *Pleurotus* spp. on agricultural substrates, in addition to food generation for human consumption opened new research activities towards mushroom immunoceuticals. These immunomodulating therapeutic agents can be used in the management of some immunocompromised patients suffering from different diseases, like cancer, HIV/AIDS, liver cirrhosis, acute respiratory failure, recent bone marrow transplant, in a way of increasing the survival rate.

The present chapter gives, from an experimental perspective, an updated comprehensive account of some medicinal properties of *Pleurotus* sp. involved in anti-cancer mechanism, such as immunomodulating, anti-proliferative and antioxidant, exerted by extracts and powders preparations obtained from both mycelium and fruiting bodies. These results are significant in that they provide a framework for further exploration of the use of *Pleurotus* bioactive preparations for immunotherapy as new life-saving drugs.

MATERIALS AND METHODS

Mushroom material

Pleurotus sp. strain (CCEBI-3024) is deposited at the Culture Collection of the Center for Studies on Industrial Biotechnology (CEBI, Cuba). The strain was maintained on slants with solid medium of potato dextrose agar (PDA) incubated at 5 °C.

Preparation of *Pleurotus*-derived products

Pleurotus sp. cultivation was performed by solid-state fermentation of mushroom spawn on pasteurized coffee pulp used as substrate in plastic bags of 2 kg (30-40 cm) (Bermúdez *et al.* 2001). 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 away from light and humidity in plastic bags for further use (FB-P). The powder contained 55% (w/w) carbohydrate, 20% (w/w) protein and 4% (w/w) lipids.

For obtaining the *Pleurotus* fruiting bodies cold water-extract (FB-E), the collected carpophores were exhaustively washed with distilled water and sliced into 1 cm^2 pieces. They were weighed and 5 ml of distilled water was added per gram of biological material. The extraction was made at 20 °C with continuous stirring at 100 rpm for three hours and the final extracts were collected by centrifugation and filtration. The extracts were stored at -20 °C and freeze dried. They are mainly composed of 43% of carbohydrate and 35% of protein.

The preparation of *Pleurotus* mycelium hot-water extract (Myc-E) started with the inoculation of mycelium in Erlenmeyer flasks, which contained YPG medium (yeast-peptone-glucose). The flasks were incubated at 27 °C with continuous stirring at 100 rpm for 15 days. After the submerged fermentation was carried out, mycelia were collected by centrifugation at 4000 rpm and washed twice with distilled water. Isolated mycelia, suspended in 200 g (wet weight)/l of distilled water, were extracted with boiling water

for 10 h and the final extracts were collected by centrifugation and filtration. The extracts were stored at -20 °C and freeze-dried. The major components of Myc-E were carbohydrate (76.8%) and protein (12%).

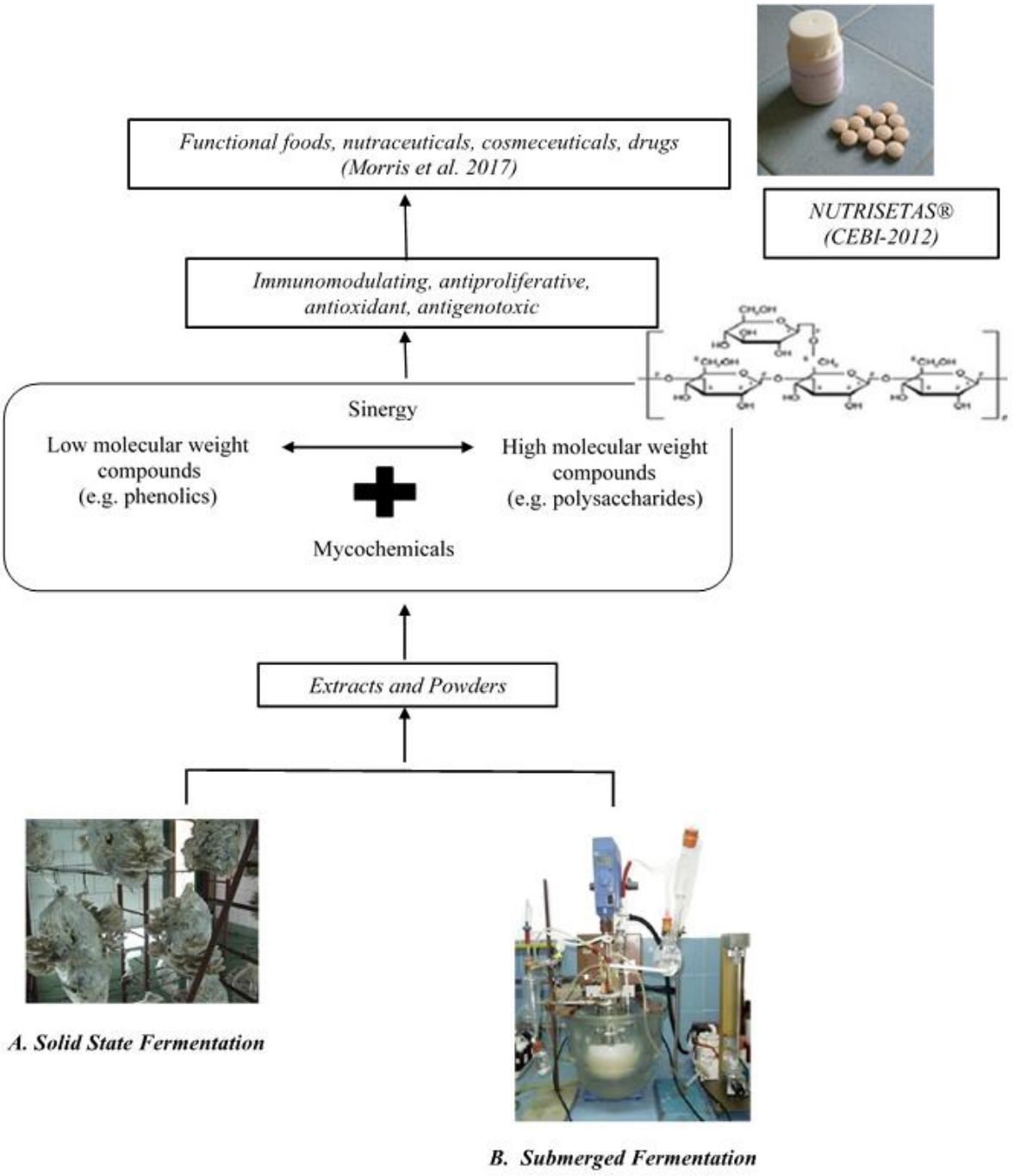


Figure 1. The way for developing nutraceuticals and effector molecules with biological activity from *Pleurotus* mushroom in Cuba.

Laboratory animals

BALB/c mice purchased from the National Center for Production of Laboratory Animals (CENPALAB, Havana) were used. Experiments were done under conventional sanitary conditions and animals were maintained at controlled temperature and humidity throughout the investigation ensuring the optimal interval for the specie. The administration of products was made daily in the morning between 9-10 am. The research was approved by the institutional Ethical Committee (University of Oriente) and has been performed in accordance with Cuban legislation and the National Research Council Guidelines for the Care and Use of Laboratory Animals.

Effect of intraperitoneal (i.p.) administration of *Pleurotus* mycelium hot-water extract (Myc-E) to cyclophosphamide-treated or whole-body irradiated mice

Cyclophosphamide (CY)-treated mice.
Fifteen male mice (20-25 g) were divided into two groups. Myc-E was administered intraperitoneally (i.p.) at 100 mg/kg for 7 days to ten Balb/c mice and cyclophosphamide (CY) USP 23 for injection, obtained from JSLYP (China), at 100 mg/kg was given i.p. on the fifth day. The control group, comprised of five mice, was injected i.p. with physiological saline. On the eighth day, blood was collected from the orbital vein and animals were then bled to death.

Whole-body irradiated mice
Male mice were randomly allocated into two groups (n= 10) for eventual whole-body irradiation with a ^{60}Co source Theratron teletherapy unit (Siemens, Erlanger, Germany) in the Oncological Hospital "Conrado Benítez" (Santiago de Cuba, Cuba) at a dose rate of 0.43 Gy/min for 20 min (date of exposure to be designated day 0). For the analyses of effects of the mushroom-derived materials, one group of mice was administered the extract intraperitoneally (i.p.) at a dose of 100 mg/kg in a volume of 0.2 ml on days -10 to -6 and -2 to +1 with respect to the irradiation. Mice in the control group (n=10) were injected with saline solution in place of the extract; non-irradiated mice were used as negative controls. All mice were euthanized 24 h after the final administration of extract or saline and tissues/bloods isolated for analyses.

In both experiments, blood specimens were analyzed for white blood cell count. Femoral bone marrow cells were withdrawn with Hanks' solution and counted with a Neubauer chamber (Germany). Spleen cell suspensions were prepared by gently teasing the tissue with ice-cold Hanks' solution and passing it through antiseptic gauze (Johnson & Johnson Medical, TX, USA) and counted with a Neubauer chamber. Peritoneal exudate cells were collected from the peritoneal cavity of mice by washing with Hanks' solution and also counted.

The functional activity of the monocyte-macrophage system of each host was evaluated using a carbon clearance test. The clearance rate of carbon was expressed as the ratio of the absorbance for samples from Myc-E treated (or saline-control) mice with respect to values from immunocompetent mice injected with the carbon particles (i.e., received no test substances) (see Morris *et al*. 2008 for details).

Effect of oral administration of *Pleurotus* fruiting bodies powder (FB-P) on cell immune response of cyclophosphamide treated mice

The 20- to 25 g male BALB/c mice were fed a standard diet and acidified water *ad libitum*. Fifteen mice were divided into three groups (n= 5). The two experimental groups were treated intraperitoneally (i.p) with cyclophosphamide (CY) USP 23 for injection obtained from JSLYP (China) at 100 mg/kg on day 0. FB-P was administered by oral route (1000 mg/kg) for 7 days to the 'CY/ FB-P¨ group, whereas physiological saline solution was administered to the 'CY/ Saline' group in a similar schedule. Non-treated mice were used as controls in the experiment.

The effect of FB-P on cell-mediated immunity was determined by the delayed-type hypersensitivity (DTH) reaction (Kim *et al.* 1998). Mice were immunized by an intradermal (i.d) injection of 50 µl of 5 mg/ml BSA emulsified in Complete Freund Adjuvant (CFA; Sigma, St. Louis, MO, USA) 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 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 a 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. A similar immunization protocol was applied to control animals. Histological studies were made with samples taken from the antigen-injection sites with the hematoxylin-eosin staining and observations were performed under an optical microscope (100x).

The popliteal lymph nodes (right and left) of the antigen sensitized and rechallenged animals were removed and washed with PBS pH 7.4. Excess 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 weights of the popliteal node belonging to BSA-injected foot pad with respect to that of PBS-injected pad (Descotes 2006).

Effect of oral administration of *Pleurotus* fruiting bodies cold water-extract (FB-E) to malnourished BALB/c mice

Female BALB/c mice, weighing 20 g, were housed individually at 23 °C with a 12-hour/12-hour light/dark cycle. Thirty mice that were starved for 3 days and had free access to salted water were studied. After this time, blood was collected from the orbital vein of 10 mice and the animals were killed (M group). The others were re-fed *ad libitum* for 8 days with commercial pelleted diet (M-CD group) or with the commercial diet and the *Pleurotus* fruiting bodies cold water-extract (FB-E) administered orally at a dose of 100 mg/kg of body weight per day (M/FB-E group). A control group of 10 mice was fed with commercial diet throughout the study.

After the small intestine was collected, the segment correspondent to jejunum was rinsed thoroughly with ice-cold saline solution, opened, and blotted dry. The mucosa was scraped with a glass slide and weighed separately. Jejunal mucosa was homogenized with ice-cold phosphate-buffered saline with a pH of 6.0 (1:3 w/v). Total protein and DNA were estimated by the methods of Lowry *et al.* (1951) and Burton (1956), respectively.

The functional activity of the monocyte-macrophage system was evaluated as described previously for cyclophosphamide-treated or whole-body irradiated treated with Myc-E.

Humoral immune response was evaluated through an immunization protocol with sheep red blood cells (SRBC) as antigen. Three groups, comprised of five mice, were designed: M-DC, M/FB-E and control as described above. After the starvation (day 0) mice were injected intraperitoneally (i.p.) with 0.2 ml of a 25% SRBC saline solution. After 7 days from the first injection, blood samples of 50 µl were drawn from the orbital plexus to measure antibody titres by a haemagglutination (HA) reaction. The reciprocal serum dilution, which just gave agglutination, was considered the titre. At this time, mice received the second immunization and on day 14, antibody titres were determined.

Cytometric analysis of cell viability of human acute promyelocytic leukemia (NB4) cells

The cells were maintained in RPMI medium (Gibco-Life Technologies) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.02 mg/ml gentamycin. Cells were cultured at 37°C in a humidified air atmosphere with 5% CO_2. Cell viability of NB4 cells was determined by flow cytometry by

measuring the level of impermeability to propidium iodide (PI). Briefly, 5×10^5 cells were seeded in a 12-well culture plate (Falcon, Becton Dickinson, NJ, USA) 24 h prior to treatments. Then, NB4 cells were incubated for 24 h with *Pleurotus* sp. Myc-E extract at doses of 100 and 200 μg/ml; cells incubated with culture medium alone served as controls. After incubation, cells were collected and washed in PBS and centrifuged at 1200 rpm for 5 min. The cells were resuspended in 500 μl of PBS and stained with PI to a final concentration of 50 μg/ml and analyzed in a FACS caliber flow cytometer (Becton Dickinson, San José, CA, USA). Data analysis was performed using WinMDI 2.8 software (Trotter 2011).

For the analysis of cell-cycle distribution of NB4 cells, 5×10^6 treated cells were centrifuged at 1500 rpm for 3 min. Then, they were resuspended in 500 μl PBS containing 0.1% Nonidet P-40 and 0.5 mg/ml DNAse-free RNAse A. The cell suspensions were incubated at room temperature for 30 min in order to extract low molecular weight DNA from cell nuclei. The remnant DNA in cells was stained with 50 μg/ml PI, immediately measured in the cytometer. Cell-cycle progress was expressed as the percentage of cells in G_0/G_1, S, and G_2/M phases. Histograms of the untreated cells were used to define the positions of the different phases of cell cycle.

Assays for *in vitro* antioxidant activity with FB-E extract

DPPH assay

Radical scavenging ability (RSA) of mushroom extract against 1,1-diphenyl-2-picryl-hydrazyl (DPPH, Sigma-Aldrich) was measured according to Cheung *et al.* (2003) using spectrophotometry. FB-E (1 ml) at 10 mg/ml was mixed with 0.5 ml of 0.1 mM DPPH ethanolic solution. Then, the mixture was shaken vigorously and incubated at 25°C for 1 h in the dark. The absorbance of the sample was measured at 520 nm (VIS-723G spectrophotometer, Beijing Rayleigh Analytical Instrument Corporation) and the scavenging ability against DPPH radicals was calculated as a percentage of DPPH discoloration using the equation: % RSA = $[(A_{DPPH} - A_S)/A_{DPPH}]$ x 100, where A_S is the absorbance in the solution when the sample extract has been added, and ADPPH is the absorbance of DPPH solution. L-ascorbic acid was used as a standard.

ABTS assay

Scavenging effect on 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich) radicals was measured according to Choi *et al.* (2006). ABTS radicals were generated by mixing 7 Mm of the ABTS stock solution and 2.45 Mm of potassium persulfate (Sigma-Aldrich) in distilled water, and storing this mixture overnight at room temperature in the dark. The mixture (10 ml) was diluted with 840 ml of distilled water. *Pleurotus* extract (50 μl) at 10 mg/ml was added to 3 ml of ABTS solution and after 90 min, the absorbance was measured at 414 nm (VIS-723G spectrophotometer, Beijing Rayleigh Analytical Instrument Corporation). The scavenging ability against ABTS radicals was calculated using the equation: % RSA = $[(A_{ABTS} - A_S)/A_{ABTS}]$ x 100, where A_S is the absorbance in the solution when the sample extract has been added, and A_{ABTS} is the absorbance of ABTS solution. L-ascorbic acid was used as a standard.

Reducing power

The reducing power was determined according to the method of Lee *et al.* (2007). The mushroom extract FB-E (2.5 ml) at 5 mg/ml was mixed with 2.5 ml of 10 g/l potassium ferricyanide (Sigma-Aldrich) and the mixture was incubated at 50°C for 20 min. Then 2.5 ml of 100 g/l trichloroacetic acid (Merck) was added, and the mixture was centrifuged at 2 000 g for 10 min. A sample of the supernatant (5 ml) was mixed with 5 ml of distilled water and 1 ml of 1 g/l $FeCl_3$ (Merck), and the absorbance was measured at 700 nm (VIS-723G spectrophotometer, Beijing Rayleigh Analytical Instrument Corporation). Butylated hydroxyltoluene (BHT) was used as a standard.

Determination of inhibitory activity on lipid peroxidation.
A reaction mixture containing 8 ml of a suspension of 20% sheep erythrocytes, 7.52 ml of physiological saline, 80 µl of 0.5 mol/l $FeCl_3$, 0.4 ml of ascorbic acid (0.5 mM) and 1 ml of FB-E (10 mg/ml) was incubated at 37 ^oC in a water bath for 120 min. The lipid peroxide formed was estimated by measuring thiobarbituric acid reacting substances (TBARS) with some modifications (Okhawa *et al.* 1979). For this, 2 ml of the incubation mixture was treated with 1 ml of trichloroacetic acid at 10%, the samples were shaken in a vortex for 1 min and centrifuged at 6 000 g for 15 min. Then, 2 ml of the supernatant was transferred to test tubes with 2.5 ml of 0.335% thiobarbituric acid (TBA) and the reaction tube was kept in a water bath at 100^oC for 1 h. After cooling, the absorbance was measured at 532 nm. The percentage of inhibition of lipid peroxidation was determined by comparing the results of the test compounds (treated with mushroom extract) with those of control (not treated with the mushroom extract). The % of lipid peroxide-scavenging ability of the extract was calculated by the formula described in DPPH and ABTS radicals scavenging effect.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). One-way analysis of variance and *post hoc* Tukey's tests or 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 or Mann-Whitney´s *U*-test was used to compare the two means in the experiments related to the effects of extracts in cyclophosphamide-treated or whole-body irradiated mice. Differences at $p< 0.05$ were accepted as significant. The software Statgraphics Plus v. 5.1 (Statistical Graphics Corporation, 1994-2001) was used in the analysis.

RESULTS AND DISCUSSION

Cancer is a worldwide disease, which is causing serious damage to human health, and how to conquer cancer is one of the most important research topics on the medicine. The immune system is the human's ultimate defense against infectious diseases, tumors, and cancer growth (El Enshasy and Hatti-Kaul 2013). The minimal toxicity and potent biopharmacological activities of mushroom metabolites has received increasing attention in cancer therapy. Recently, numerous reports have been published on preclinical studies and clinical trials related to the functionality and bioactive properties of *Pleurotus* mushrooms and their nutraceutical derivatives, including the immune modulatory and antitumor effects (Pérez-Martínez *et al.* 2015, Carrasco-González *et al.* 2017, Xu *et al.* 2016).

Although in our study polysaccharides appear to be the most important bioactive component in *Pleurotus*-derived preparations with respect to immunomodulation, the presence in varying amounts of different secondary metabolites could lead to a synergy in the immune enhancing activity. The result of mycochemical tests showed that both *Pleurotus* fruiting bodies and mycelium extracts contain alkaloids, phenolic compounds like flavonoids and tannins, reducing sugars and amino acids (Morris *et al.* 2014). Moreover, fungal immunomodulatory proteins, purified from medicinal mushrooms comprise a group of novel proteins, possess immunomodulatory properties and have a strong potential of being applied to food or pharmaceutical products for commercial development (Ou *et al.* 2009). Differences in biosynthesis patterns of cell molecular components in distinct stages of the vital cycle would explain the dissimilarities in biochemical composition of fruiting bodies and mycelium extracts.

Immunomodulating effects of Myc-E in cyclophosphamide-treated or whole-body irradiated mice

The chemotherapy and radiotherapy in cancer treatment contribute to further depression of the immune system. Use of immunomodulating therapeutic agents can solve these problems and efforts to find new immunomodulators are on-going (Zhuang 2009). For that reason, we studied the effects of intraperitoneal

administration of *Pleurotus* mycelium hot-water extract (Myc-E) to cyclophosphamide-treated or whole-body irradiated mice

Cyclophosphamide (CY) is currently one of the most widely prescribed alkylating agents in cancer chemotherapy; however, CY treatment often results in potent immunosuppressive and cytotoxic effects (Morris *et al.* 2003). Immunosuppression caused by CY and other anticancer drugs significantly complicates the course of cancer chemotherapy and worsens the condition of the patients (Hou *et al.* 2007). As expected, cyclophosphamide severely impaired the mice hematopoietic tissue, but Myc-E was found to have an active protective effect. Myc-E increased bone marrow cellularity and the white blood cell counts compared to CY-saline control group ($p< 0.05$) (Figure 2); no significant increase was observed in the spleen cellularity. On the other hand, the radioprotective effect exerted by mycelium Myc-E was evident by increases in bone marrow cellularity, leukocyte counts and in spleen cellularity ($p< 0.05$), compared with irradiated control group (Figure 2). The stimulation of production of white blood cells by bone marrow in an immunosuppressed animal model has been classified as an immunomodulatory effect (Gupta *et al.* 2010).

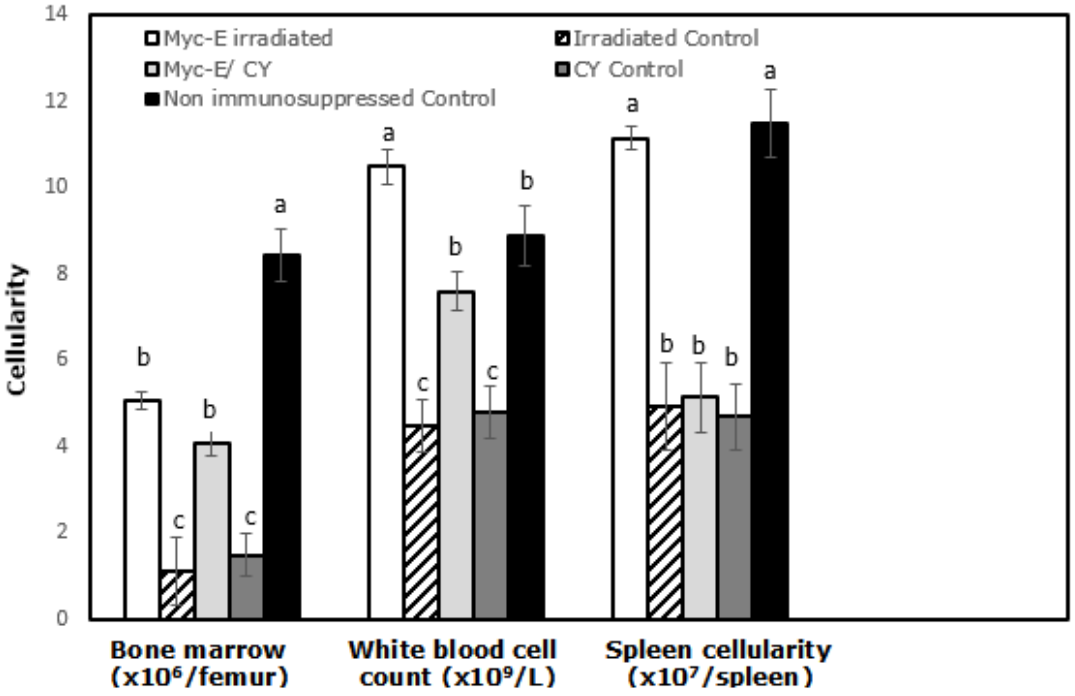


Figure 2. Effects of *Pleurotus* sp. mycelial extract (Myc-E) on haemopoiesis of cyclophosphamide-treated or irradiated BALB/c mice. Values shown are the mean (±SE) of each group (n= 10). Different letters indicate significant differences among groups (Kruskal–Wallis, Student–Newman–Keuls, $p< 0.05$).

Since macrophages have been suggested to play important roles in immunological surveillance, we studied the influence of the administration of Myc-E on the number of peritoneal exudate cells and the phagocytic activity of macrophages (Table 1). In the CY-treated or irradiated mice, immunosuppression manifests as markedly decrease in the numbers and phagocytic activities of macrophages. Myc-E at 100 mg/kg remarkably increased the number of peritoneal exudate cells compared with saline control groups in both immunosuppression models ($p< 0.05$). In the study to evaluate the effects of the extract on *in vivo* phagocytic activity by measuring carbon clearance in peripheral blood (as an index of the phagocytic activity of liver and spleen), a low ratio was deemed to correspond to a high clearance of carbon from the blood. The data show that the treatment with the Myc-E extract potentiated the activity of the host monocyte-macrophage system (relative to that in the CY or irradiated saline treated mice) (Table 1).

Table 1. Effects of *Pleurotus* sp. mycelial extract (Myc-E) in the number of peritoneal exudate cells and macrophage phagocytic activity of cyclophosphamide-treated or irradiated BALB/c mice.

Experimental groups	Number of peritoneal exudate cells (x 10^6/mouse)	Macrophage phagocytic activity (Absorbance ratio at 5 min)
Myc-E/ irradiated mice	4.61 ± 1.43 a	1.62 ± 0.12 b
Irradiated-saline control	1.82 ± 0.65 b	2.01 ± 0.31 a
Myc-E/ CY treated mice	4.9 ± 1.4 a	1.67 ± 0.11 b
CY-saline control	2.9 ± 0.1 b	1.98 ± 0.02 a
Non-immunosuppressed control	3.41 ± 0.57 a	-

Values are means ± SE, n= 10. Different letters indicate significant differences, Student's *t*-test, $p< 0.05$)
For the number of peritoneal exudate cells comparisons were made with respect to non-immunosuppressed mice and for phagocytic activity between both irradiated or CY-treated animals.
(-) The value was used in the estimation of absorbance ratios.

These results were in keeping with the finding of another study wherein water-soluble fractions *of P. ostreatus* mycelium exerted modulating effects on macrophage activation *in vitro* as reflected in enhanced glucose consumption and acid phosphatase activity by the treated cells (Morris *et al.* 2007). The immunomodulatory effects of Myc-E in *in vitro* systems have been investigated (murine macrophages RAW 264.7). The extract can induce the functional activation of macrophages by inducing nitric oxide (NO) release and increasing mRNA expression of inducible nitric oxide synthase (iNOS) (Llauradó *et al.* 2016). These results confirm the vital role of PN-S in triggering immune responses.

The noted increases in macrophage activation might be related to binding of one or more extract components to receptors found on macrophage surface such as glucan receptors (e.g. dectin-1). Polysaccharides appear to be the most important component with respect to antitumor effect and on the average, 1.5% of Myc-E dried mass consists of β-1,3-1,6-glucans (Morris *et al.* 2014).

Pillai and Uma Devi (2013) reported an increase in the survival index (66%) and an improvement in the hepatic function and haematological parameters in bone marrow of irradiated mice (6 and 8 Gy) treated with *Ganoderma* preparations. These findings are in agreement with those of a clinical trial wherein patients with different types of cancer (hepatic, lung, gastric, colorectal and nasopharyngeal) who were undergoing chemotherapy or radiotherapy received a nutritional supplement containing polysaccharides extracted from six different mushrooms (Novaes and Fortes 2005).

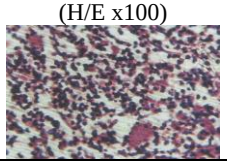
Reversing the function of immune suppressed cells may improve the efficacy of cancer therapy (Cui *et al.* 2015). Hence, Myc-E may be a candidate therapeutic agent with chemo- and radioprotective activity for hematopoiesis damage, particularly to cells involved in immune function. Although other studies described the radioprotector effect on the immune system of mushroom products (Guggenheim *et al.* 2014), application of Myc-E (from *Pleurotus* mycelium) in immune suppression research appears to be new as reflected in the literature. These data could be complemented by further experiments, possibly investigating also the cytotoxicity exerted by the extract on tumor cell lines, as shown later in this chapter.

Immunoenhancing activity of FB-P on cyclophosphamide treated mice

The *in vivo* efficacy of *Pleurotus* preparations in immunological effector cells, which have a key function against tumor growth under immunosuppression, is poorly understood. Most of the *in vivo* results come from studies with a polysaccharide injection, rather than oral administration (Wang *et al*. 2014). Polysaccharides enriched-extracts that elicit effects *in vitro*, or by injection, may be ineffective or may exhibit different effects when taken orally (Boh *et al.* 2007). Evidence of the effectiveness of oral mushroom preparations against immune challenges must be ultimately demonstrated in animals. Moreover, well-characterized formulations must be developed based on a complete understanding of the immunomodulatory effects and specific applications of oral myco-products. Thus, dried and powdered *Pleurotus* mushroom (FB-P) would become an attractive alternative for the development of functional foods and nutraceuticals preparations.

In the present section, the activation of FB-P on cell immune response was evaluated *in vivo* on cyclophosphamide treated mice. The results indicated that none of the FB-P-orally treated mice died, and their body weights showed no significant change during the course of the experiment ($p< 0.05$, data not shown). Mice supplemented with *Pleurotus* powder (FB-P) showed a higher delayed-type hypersensitivity (DTH) response, as judged by the increase of foot pad swelling compared to saline control group, particularly at 48 and 72 h after antigen rechallenge ($p< 0,05$; Table 2). The DTH response mounted at these times by CY-FB-P group was similar to 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) (Janeway *et al.* 2005, Kim *et al.* 1998). Mononuclear cells were shown infiltrating the antigen-injection sites in CY/FB-P treated mice (Figure inserted in Table 2). In addition, the DTH reconstitution was associated with the increase observed in the mass index of popliteal lymph nodes of the FB-P supplemented animals ($p< 0.05$; Table 2). In sum, results evidenced that FB-P reversed the CY-induced damage on adaptive cell immunity and promoted the proliferation of T cells and macrophages.

Table 2. Effect of oral administration of FB-P on cell immune response (DTH response) of cyclophosphamide treated BALB/c mice.

Experimental groups	Foot pad thickness (mm)			Mass index of popliteal lymph nodes	CY/ FB-P 48 h (H/E x100)
	24 h	48 h	72 h		
Control	0.48 ± 0.07^a	0.46 ± 0.04^a	0.38 ± 0.07^a	-	
CY/ FB-P	0.14 ± 0.05^b	0.43 ± 0.02^a	0.29 ± 0.05^a	1.87 ± 0.27*	
CY/ Saline	0.11 ± 0.06^b	0.39 ± 0.01^b	0.12 ± 0.04^b	1.34 ± 0.15	

All values are expressed as the arithmetic mean ± S.D. of five mice. Different letters indicate significant differences among groups (Kruskal-Wallis, Student-Newman-Keuls, $p< 0.05$). The (*) reflects significant differences between the two groups in the Mann-Whitney test ($p< 0.05$).
Mononuclear cells infiltrating the antigen-injection sites in CY/FB-P are shown in the inserted figure (hematoxylin-eosin staining, 100x).

It has been demonstrated that a polysaccharide of *P. citrinopileatus* (PCPS) has the capacity to activate human dendritic cells (DCs) via multiple pathways, leading to the secretion of pro-inflammatory cytokines TNF, IL-1β, IL-6 and IL-12, as well as the anti-inflammatory cytokine IL-10 (Minato *et al.* 2016). As is known, activated macrophages, NK cells, cytotoxic T cells, and their secretory products, such as tumor necrosis factor, reactive nitrogen and oxygen intermediates and interleukins are involved in immunomodulatory responses (Wang *et al.* 2015).

The oral administration of *P. nebrodensis* polysaccharide (PN-S) to CY-immunosuppressed mice increased the thymic and splenic indices, and promoted proliferation of T lymphocyte, B lymphocyte, and macrophages. PN-S also enhanced the activity of natural killer cells and increased the immunoglobulin M (IgM) and immunoglobulin G (IgG) levels in the serum. PN-S also increased the levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), and nitric oxide (NOS) in splenocytes. These results suggest that PN-S treatment enhances the immune function of immunosuppressed mice. This study may provide a basis for the application of this fungus in adjacent immunopotentiating therapy against cancer and in the treatment of chemotherapy- induced immunosuppression (Cui *et al.* 2015).

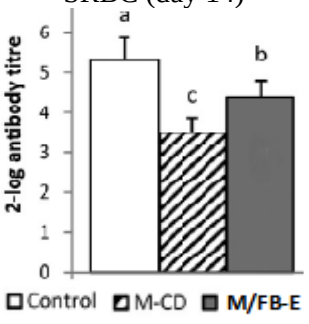
Mariga *et al.* (2014) reported that *P. eryngii* fruiting bodies powder is an active antitumor agent with immunomodulatory activity, where, it targets the lysosomes of cancerous cells concomitantly stimulating macrophage-mediated immune response. Based on the current data, we demonstrated that *Pleurotus* sp. mushrooms might be of potential benefit in anticancer-drug induced immunosuppression. Our findings suggest that oral administration of *Pleurotus* sp. powder 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 (see results on the animal model of malnutrition).

Immunonutritional restoration effects of FB-E on malnourished BALB/c mice

In protein-energy malnutrition (PEM), there are reductions in nutrient absorption and depression of the immune system in gut associated lymphoid tissues (Schaible and Kaufmann 2007). The functionality and integrity of small intestine was also deteriorated. Administration of FB-E to malnourished mice during the refeeding period provided beneficial effects and improved functional alterations in the intestinal tract ($p< 0.05$) (Table 3). The increase of mucosal weight in association with high values of protein and DNA content suggests a higher rate of protein biosynthesis and could be related with the recovery of gastrointestinal tract function. Some components present in *Pleurotus* mushroom could re-establish intestinal architecture after oral consumption.

Table 3. Effect of oral administration of FB-E on immunonutritional restoration of malnourished BALB/c mice with respect to gut mucosa and humoral immune response.

Experimental groups	Gut mucosa parameters		Antibodies titres against SRBC (day 14)
	Protein (μg/ 10 cm)	DNA (μg/ 10 cm)	
Control	352 ± 78^{b}	59 ± 16^{b}	
Malnourished mice (M)	108 ± 38^{c}	45 ± 20^{b}	
M/ CD	277 ± 76^{b}	68 ± 6^{b}	
M/ FB-E	576 ± 87^{a}	98 ± 21^{a}	



Results are expressed per ten centimeters of intestine. All values are reflected as the arithmetic mean ± SD of 10 mice. Different letters indicate significant differences among groups (Kruskal-Wallis, Student-Neuman-Keuls, $p< 0.05$). Subscript legend: (a) ≠(b) ≠ (c).

In general, the enteral nutrition is considered the first method of feeding in critical patients (Botrán and López-Herce 2011) and mushrooms substances might be potential candidates for using in immunonutritional diets. Some studies with mushrooms reported the biological potential of polysaccharides at intestinal level by means of the stimulation of gut associated lymphoid tissues and intestinal macrophages (Bouike *et al.* 2011).

The oral administration of FB-E at a dose of 100 mg/kg stimulated the phagocytic activity in comparison with the standard diet group. The ratio of carbon clearance at 5 min was lower in FB-E group than in the M-CD group (1.4 ± 0.1 *vs*. 1.9 ± 0.1) ($p < 0.05$); a low ratio was deemed to correspond to a high clearance of carbon from the blood. Nevertheless, FB-E does not increase spleen weight and splenic cell counts ($p < 0.05$) (data not shown). The augmentation of phagocytic activity may be owing to the activation of phagocytes and not by an increase in the number of total phagocytes. The effects on intestinal tract linked to macrophage activation might be influenced by a partial absorption of bio-compounds from *Pleurotus* sp., or by the stimulation of the gut associated lymphoid tissues. Nevertheless, mechanism of action of several orally administered bio-substances from mushrooms is still unclear. Among various myco-chemicals, it has been suggested that only fragments of polysaccharides partially hydrolyzed or degraded after ingestion might bind to gut epithelia and exert localized and/or systemic effects on the immune system or the mechanisms could be mediated via a non-specific intestinal absorption (Wasser *et al.* 2014). Although most of the bio-components in FB-E could be probably implicated as immunomodulatory agents, more evidences are required to link the observed actions to any of the identified bio-components.

Although cell-mediated immunity is severely affected in PEM, the atrophy of lymphoid tissues leads to a decrease of circulating and the splenic B cell numbers (Lykke *et al.* 2013). However, the role of humoral immune response in malnourished mice is not well documented. Antibody production by B cells after 14 days of immunization with sheep red blood cells (SRBC) was significantly higher compared with mice refed with commercial diet ($p < 0.05$) (Figure inserted in Table 3). Anti-SRBC antibodies (directed against a T-dependent antigen) titres might also suggest the stimulation of cellular immunity. Other studies indicated that the humoral response might respond to malnutrition, depending on malnutrition type (e.g. acute *vs.* chronic, protein malnutrition *vs.* energy restriction) (Neyestani *et al.* 2004).

The term *immunonutrition* was introduced as emergent subject in last years (Zapatera *et al.* 2015). In this context, FB-E could be used to develop specific enteral formulations with potential applications in the immunotherapy and as immunonutritional support during recovery of the metabolic and immunological disorders associated with malnutrition. This study is a contribution to the knowledge of the immunonutritional properties of *Pleurotus* mushroom and suggests its prospective use in immunocompromised people with special nutritional requirements

Antiproliferative effects of Myc-E on human acute promyelocytic leukemia (NB4) cells

Since research has tended to focus on the dietary value of species of the genus *Pleurotus*, there is relatively little information pertaining to their anticancer mechanisms, particularly in mycelia derived products. The treatment with *Pleurotus* mycelial extract (Myc-E) reduced viability of NB4 human leukemia cells, particularly at the concentration of 200 μg/ml, to 82% with respect to control untreated cells ($p < 0.05$) (data not shown).

Our results presented in Figure 3 indicated that the cytotoxic effect of the hot water extract from *Pleurotus* sp. mycelia on the NB4 cells would be related to its ability to arrest the cell cycle. Independent of the concentration, the extract lowered the leukemic cells in the G_0/G_1 phase compared to untreated cells ($p < 0.05$), but had no significant effect on the S population. Overall, *Pleurotus* sp. mycelial extract significantly increased the number of NB4 cells in G_2/M phase (15.82% and 18.35% for cells treated, respectively, with 100 and 200 μg/ml, *vs* 8.78% in control cells, $p < 0.05$). Thus, *Pleurotus* sp. extract has arrested the NB4 cells in the G_2/M phase supporting a cell-cycle dependent anticancer mechanism.

While a water-soluble non-starch polysaccharide extracted from the mycelium (EDP) of *P. tuber-regium* caused G_2/M arrest in HL-60 cells by lowering the Cdk1 expression, its fruiting-body analog (HWE) caused S arrest in the HL-60 cells by a depletion of Cdk2 and an increase in cyclin E expression (Wong *et al.* 2007). This was in contrast to a previous study of a β-glucan obtained from *Poria cocos* mycelium,

which was found to inhibit the proliferation of MCF-7 cancer cells by G_1 arrest and apoptotic induction via down regulating the anti-apoptotic protein Bcl-2 (Zhang *et al.* 2006).

On the other hand, the effect of several extracts of various polarities obtained from fruiting bodies of a *Pleurotus* sp., was tested on apparent growth of different cell lines (U937, N2A and Caco2 tumoral cells compared to Vero cells). The *in vitro* growth activity of cells with aqueous (CW-P at 4°C and HW-P at 100°C), methanol (MetOH-P) or dichloromethane (DM-E) extracts were estimated through mitochondrial activity using MTT (a Tetrazolium salt) Test and by Neutral Red Uptake assays. Inhibition of cell respiration and cell uptake was observed with CW-P extracts, while HW-P and MetOH-P extracts were less efficient. Compared to HW-P, substance(s) responsible of this effect in CW-P appeared thermo-labile. However, cell proliferation was shown in U937 with intermediate dilutions of HW-P. N2A was specifically sensitive to inhibition by MetOH-P extract (Llauradó *et al.* 2014). The mechanisms by which they drive benefits remain obscure, while the effective doses and their safety need to be evaluated.

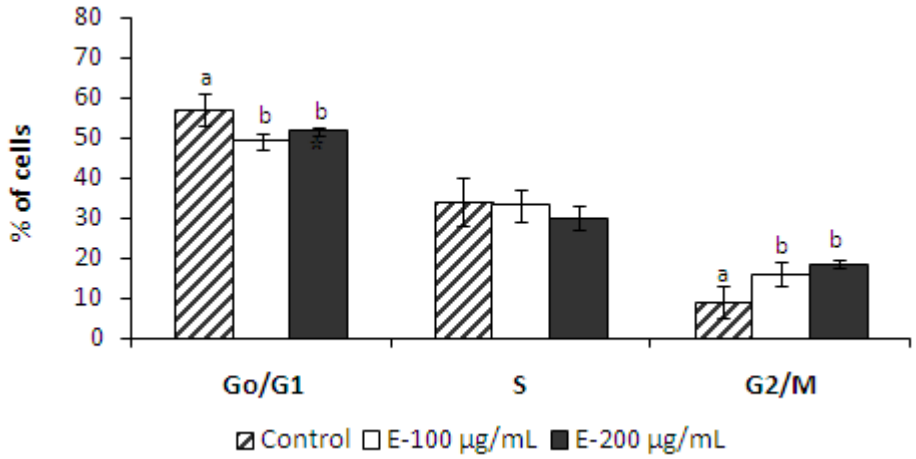


Figure 3. Cell-cycle analysis of human leukemia NB4 cells treated with the *Pleurotus* sp. mycelial extract Myc-E for 24 h at concentrations of 100 and 200 µg/mL. Values represent the mean ± SE of three independent experiments. Significantly differences in Tukey's test ($p< 0.05$) were shown with different letters as compared to control cells.

Dietary supplement of edible *Pleurotus* spp. rich in fungal polysaccharides is associated with anticancer health benefit. Tong *et al.* (2009) isolated a novel water-soluble polysaccharide (POPS-1) from the fruiting bodies of *P. ostreatus* by hot water extraction. Cytotoxicity assay showed that POPS-1 presented significantly higher antitumor activity against Hela tumor cells *in vitro*, in a dose-dependent manner, and exhibited significantly lower cytotoxicity to human embryo kidney 293T cells than Hela tumor cells. These results suggest that *P. ostreatus* water-soluble preparations may be considered as potential candidates for developing new low toxicity antitumor agents.

In vitro antioxidant activities

Free radicals are known to be a major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer. Antioxidant compounds play an important role in preventing and curing chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Sun *et al.* 2017). The antioxidant potential of a compound could be attributed to its various characteristics, the most important of these being the ability to scavenge and reduce free radicals, to chelate transition metal ions, to inhibit lipid peroxidation, among others (Rajeshwar *et al.* 2005).

The antioxidant effects displayed by edible mushrooms, in addition to their immunomodulating properties represent an important contribution to their antitumor activities (Patel and Goyal 2012). The antioxidant properties of *Pleurotus* spp. were of both enzymatical and non-enzymatical nature (Khatun *et al.* 2015). Until now, research has tended to focus on the dietary significance of edible mushrooms; however, there is relatively little information relating to the antioxidant activity and the possible use of such mushrooms to neutralize oxidative stress (Jayakumar *et al.* 2011).

The antioxidant activities of FB-E determined using four *in vitro* assays are presented in Table 4. DPPH is a stable free radical that shows maximum absorbance at 517 nm in methanol; when DPPH encounters a proton-donating substance such as an antioxidant, the radical is scavenged and the absorbance is reduced (Kohen and Nyska 2002). As shown in Table 4, the DPPH radical scavenging activity of the aqueous extract obtained from *Pleurotus* fruiting bodies was of 90.4%, lower than the values achieved with ascorbic acid used as control. In previous studies, a DPPH radical scavenging ability of 96% was reported with a mycelial extract (Morris *et al.* 2017b). Scavenging effects of extracts from several specialty and commercial mushrooms on DPPH radicals augmented with increased extract concentrations. Thus, DPPH radical scavenging activity varied from 9% (*P. nebrodensis*) to 57% (*P. cystidiosus*). Moreover, DPPH scavenging action for *P. citrinopileatus* and some other fungi significantly improved with the gradual elevation of sample concentration from 0.5 to 9.0 mg/ml (Asatiani *et al.* 2010).

Table 4. *In vitro* antioxidant activity of a hot-water fruiting bodies extract of *Pleurotus* sp (FB-E).

Sample	Scavenging of DPPH radicals (%)	Scavenging of ABTS radicals (%)	Reducing power ($A_{700\ nm}$)	Inhibition of lipid peroxidation (%)
Pleurotus extract (FB-E)	90.4 ± 0.8 (10 mg/ml)	80 ± 0.9 (10 mg/ml)	0.438 ± 0.034 (5 mg/ml)	51.2 ± 4.8 (10 mg/ml)
Control	96.3 ± 0.6* (ascorbic acid)	98 ± 0.2* (ascorbic acid)	0.700 ± 0.018* (BHT)	92 ± 1.4* (ascorbic acid)

Results are showed as means ± standard deviation of three replicates; means with an (*) differ when compared with the *Mann–Whitney* test ($p< 0.05$).

With the ABTS radical scavenging test, we can measure the activity of both hydrophilic and lipophilic compounds; therefore, it is useful in the simultaneous study of several natural ingredients (Kuskoski *et al.* 2005). In this study, the ABTS radical scavenging activity of FB-E mycelium at 10 mg/ml was of 80%, higher than a mycelial extract with a value of 55% (Morris *et al.* 2017b). On the other hand, hydro-alcoholic extracts of *Grifola gargal* showed an ABTS radical scavenging ability of 90.9-93.3 ascorbic acid equivalents (mg of ascorbic acid per l of sample) (De Bruijn *et al*. 2009). This method has been also used in the evaluation of the antioxidant activity of neutral polysaccharides from *Auricularia auricular* and their homologues sulfated in the concentrations of 0.2-10 mg/ml, without significant differences (Zhang *et al.* 2011).

Moreover, it has been discussed that the diphenylpropane structure of flavonoids and the aromatic ring structure of phenolics, such as aromatic oxy phenol acids, might contribute to the free radical scavenging ability of these compounds (De Bruijn *et al*. 2008).

The reducing capability of a compound may serve as a significant indicator of its potential antioxidant activity and the efficacy of certain antioxidants is known to be associated with their reducing power (Lü *et al.* 2010). In the present study, the reducing power of a 5 mg/mL concentration of the mushroom extract was found to be 0.438, which was relatively lower than that of BHT ($p< 0.05$) (Table 4). The reducing power of medicinal mushrooms might be due to their hydrogen-donating ability (Jayakumar *et al.* 2011).

Possibly, medicinal mushrooms contain high amounts of reductones, which could react with radicals to stabilize and terminate radical chain reactions. The reducing power of the ethanolic extract of *P. ost*reatus fruiting bodies was found to steadily increase in direct proportion to the increasing concentration of the extract. The reducing power of a 10 mg/ml concentration of the mushroom extract was found to be 1.367, which was relatively higher than that of BHT (1.192) (Jayakumar *et al.* 2009). Further ethanolic extract from the fruiting bodies of the mushroom *Pleurotus citrinopileatus* has been reported to exhibit the reducing power of 1.05 at 10 mg/ml (Lee *et al*. 2007).

Lipid peroxidation (LPO), a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids, inactivates cellular components, and therein plays a key role in oxidative stress in biological systems (Niki 2010). Hence, the inhibitory activity of the mushroom extract on LPO was evaluated. Both the mushroom extract and the ascorbic acid standard inhibited lipid peroxidation (Table 4). At a concentration of 10 mg/ml, the mushroom extract effected 51.2% inhibition of LPO activity and the ascorbic acid standard effected 92%. At least until we know, there is no data available for comparison of our results obtained with FB-E extract on lipid peroxidation in the *in vitro* erythrocyte membrane model estimated by TBARS. Erythrocytes are excellent subjects for studies of biological effects of free radicals, since they are structurally simple, are continuously exposed to high oxygen tensions, the membrane lipids are composed partly of polyunsaturated fatty acid side chains which are vulnerable to peroxidation, and they have antioxidant enzyme systems (Konyalioglu *et al.* 2005).

The *in vitro* antioxidant properties exhibited by the tested *Pleurotus* mushroom preparation may be due to the presence of antioxidant mycochemicals inherent in it, like polyphenols. In addition, the antioxidant activities (chelating ability of ferrous ion, inhibition of LPO and reducing power) found in polysaccharide extracts from the widely used mushrooms *Ganoderma applanatum, Ganoderma lucidum*, *Lentinus edodes, Trametes versicolor* and *Pleurotus eryngii* does not discard the possible contribution of β-1,3-1,6-glucans polysaccharides to the antioxidant effect (Kozarski *et al.* 2012, Fu *et al.* 2016).

In our experiment of LPO inhibition, butylated hydroxyltoluene (BHT) was used as standard. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are amongst the most commonly used synthetic antioxidants that are applied in fats and oily foods to prevent oxidative deterioration. However, BHA and BHT have restricted use in foods as they are suspected to be carcinogenic and to cause liver damage (Botterweck *et al.* 2000). This may explain why there is currently much research on the application of antioxidants from natural products. Although BHT and ascorbic acid, substances used as standards in our work, have significant antioxidant activity, they are additives and are used or are present in mg levels in foods. *Pleurotus* powder could be used in gram levels as functional food or nutraceutical, thus providing health protection to help humans reduce oxidative damage daily.

Research carried out on this way, showed that polysaccharides of *P. tuber-regium* (Fr.) Sing. had strong antioxidant potency and might be exploited as effective natural antioxidant to alleviate oxidative stress. The antioxidant activities of two homogeneous polysaccharides, water-extracted polysaccharide (W-PTR) and alkali-extracted polysaccharide (A-PTR) were evaluated. Results indicated that W-PTR was stronger than A-PTR in superoxide scavenging activity, while A-PTR was stronger than W-PTR in the scavenging activities to hydroxyl, $DPPH^{\bullet}$, inhibition effects on liver lipid peroxidation, liver mitochondria swelling, and red blood cell (RBC) hemolysis (Wu *et al.* 2014).

In the link between antioxidant and antitumor activities, Ren *et al.* (2015) isolated the polysaccharides (PAP) from the fruiting bodies of *P. abalonus*, and evaluated their antiproliferative activity in human colorectal carcinoma LoVo cells. PAP exerted a high antioxidant activity *in vitro* and a dose-dependent antiproliferative effect against LoVo cancer cells. Flow cytometry analysis demonstrated that PAP exhibited a stimulatory effect on apoptosis of LoVo cells, and induced the cell-cycle arrest at the S phase. PAP also increased the generation of intracellular Radical Oxygen Species (ROS), critical mediators in

PAP-induced cell growth inhibition. These findings suggested that PAP may serve as a potential novel dietary agent for human colon cancer chemoprevention.

Additionally, *Pleurotus eryngii* residue polysaccharides obtained by ultrafiltration showed *in vitro* antioxidant properties and cytotoxicities, having CPPS-1 the strongest activity. The expression of tumor suppressor p53 and apoptosis activator Bax were up-regulated by CPPS-1 fraction while the expression of Bcl-2 was down-regulated. The results suggested that the antitumor activity of CPPS-1 may be related to its capability of inducing apoptosis via activation of mitochondria apoptosis pathway (Ma *et al.* 2016).

CONCLUSIONS

Taken together, these observations indicate that *Pleurotus* sp. extracts (Myc-E and FB-E) and powders (FB-P) possess bioactivities involved in antitumor mechanism including immunostimulatory, antiproliferative and antioxidant toward free radicals. Both fruiting bodies and mycelium preparations were well tolerated and can be used in the formulation of consistent and safe mushroom products. Thus, *Pleurotus* sp., a common edible and medicinal mushroom, exerted health promoting benefits to maintain good health by activating the immune system for a multitude of defensive functions. *Pleurotus* sp. may be developed as functional food and potential myco-therapeutic agent for human diseases, especially for enhancing anticancer and immune responses. It appears that more studies are necessary to explore the complete structural characteristics of preparations tested, structure–activity relationship and the molecular signaling pathways of their antitumor activity. As a result, the future research may be oriented in that direction.

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