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REPORT

Evaluation of the immunomodulatory activity of bioproducts obtained from the edible-medicinal mushroom *Pleurotus ostreatus*

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ABSTRACT

Edible mushrooms are an important source for obtaining novel substances of nutritional and biopharmaceutical interest. In this regard, in Cuba, the consumption of mushrooms of the genus *Pleurotus* spp. has been promoted as part of the Urban Agriculture Program. Taking advantage of its availability and considering previous reports on their content of compounds with potential application, in this work, there was investigated the immunomodulatory and immunonutrition properties of biopreparations from this mushroom. New methodologies were developed to generate biopreparations from the mycelium and fruiting bodies of *Pleurotus ostreatus*. The obtained bioproducts were generated by biomass aqueous extraction and/or drying, which led to the registration of the NutriSetas® tradename. The bioproducts contained mainly carbohydrates (including ß-glucans) and proteins, among other secondary metabolites with potential biological activity. They showed M1-polarizing effects on macrophage populations, and the aqueous fraction obtained from the mycelium by submerged fermentation was able to stimulate the complement system alternative pathway. Noteworthy, the aqueous extract displayed radioprotective effects on the hematopoietic and immunological parameters when administered in a mice biomodel of ionizing radiation-induced secondary immunodeficiency. Similar immunostimulatory effects, either in the humoral or cellular immune responses, were found with dry and powdered preparations from fruiting bodies. Their immunonutritional effect in an experimental model of protein-energetic malnutrition in Balb/c mice evidenced nutritional recovery and immunological improvement status. This provided the first report of such an effect for edible mushroom preparations. This work received the Annual Award of the Cuban Academy of Sciences for the year 2017.

Keywords: Pleurotus ostreatus, mycelium, fruting bodies, NutriSetas®, immunomodulation, immunonutrition, immunodeficiencies

RESUMEN

Evaluación de la actividad inmunomoduladora de bioproductos obtenidos de la seta comestible-medicinal *Pleurotus ostreatus*. Los hongos comestibles son una fuente importante para la obtención de nuevas sustancias de interés nutricional y biofarmacéutico. En Cuba se ha promovido el consumo de hongos del género Pleurotus spp. como parte del Programa de Agricultura Urbana. En este trabajo se investigó las propiedades inmunomoduladoras e inmunonutricionales de preparaciones de este hongo, dada su disponibilidad y los informes previos sobre su contenido de compuestos con potencial aplicación para estos fines. Se establecieron nuevas metodologías para obtener biopreparaciones a partir del micelio y de los cuerpos fructíferos de Pleurotus spp., mediante la extracción acuosa de biomasa, el secado, o ambos, lo cual derivó en el registro de la marca comercial NutriSetas®. Los bioproductos contuvieron principalmente carbohidratos (incluidos los ß-glucanos) y proteínas, entre otros metabolitos secundarios con actividad biológica potencial. Las preparaciones tuvieron un efecto polarizador hacia fenotipo M1 en poblaciones de macrófagos, y la fracción acuosa obtenida del micelio mediante fermentación sumergida estimuló la ruta alternativa de activación del sistema del complemento. Notablemente, el extracto acuoso tuvo efecto radioprotector en los parámetros hematopoyéticos e inmunológicos al administrarlo en biomodelos de inmunodeficiencia secundaria inducida por radiación ionizante en ratones. Las preparaciones secas y en polvo de cuerpos fructíferos fueron igualmente inmunoestimuladoras de la respuesta inmune humoral y celular. Se evidenció su efecto inmunonutricional en un modelo de malnutrición proteica-energética en ratones Balb/c. Este fue el primer informe de tales efectos para preparaciones de hongos comestibles. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2017.

Palabras clave: Pleurotus ostreatus, micelio, cuerpos fructíferos, NutriSetas®, immunomodulación, immunonutrición, immunodeficiencias

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*I*ntroduction

Currently, the prevalence of secondary or acquired immunodeficiency states represent a healthcare problem worldwide [1]. Secondary immunodeficiencies are characterized by the deterioration of one or several cellular or molecular components of the system immunity, or both, thereby increasing the susceptibility of the organism to infectious diseases and the development of cancer. Additionally, cancer therapies including chemo- and radiotherapy, malnutrition, HIV-AIDS, surgical procedures, severe burns, stress, among others, are the most frequent causes of immunodeficiencies [1].

For these reasons, there is growing interest in the search for immunomodulators of natural origin, devoid of severe side effects and with application in immunotherapy. In addition to plants, mushrooms are attractive natural sources of compounds with pharmacological potential as immunomodulators [2]. They have been part of the arsenal of traditional medicine throughout the development of the human civilization. Moreover, during the last decade, edible-medicinal mushrooms began to be used for obtaining extracts and biomolecules through biotechnological procedures, bearing with immunomodulatory and antitumor properties. Despite, the field is mostly unexplored, since only 3 % of research on natural products reaching preclinical and clinical phases comprises mushrooms as natural source. Therefore, the so-called 'mycotherapy' is still a promise [3].

In this setting, the *Pleurotus* genus (Pleurotaceae, Basidiomycota) is one of the most frequent mushrooms sources used for identifying bioactive compounds of complementing or stimulatory activity on the immune response [2, 4]. Those compounds comprise substances of high molecular weight, mainly (1,3)-(1,6)-β-glucan polysaccharides, proteins, proteoglycans and polysaccharide-protein complexes, as well as different secondary metabolites of low molecular weight. These substances modulate signaling cascades involved in the immune responses, either innate (activation of natural killer cells (NK), neutrophils, complement, monocyte-macrophage system) or adaptive (stimulation of antibody production by B cells and the differentiation of T helper cells in Th1 and Th2 profiles) [2, 4].

In Cuba, the introduction, production and consumption of mushrooms of the genus *Pleurotus* (fungus oyster) have been promoted in several provinces as part of the National Program of Urban Agriculture due to its high nutritional value [5]. One of the main working strategies at the Center of Studies on Industrial Biotechnology, located in the eastern region of the country, has been the development of projects devoted to *P. ostreatus* cultivation and to obtain new products for nutritional, functional and immunoceutic application. This mushroom has been produced by solid state fermentation on agricultural residual substrates and by submerged culture of mushroom mycelium [6] (Figure 1).

Research advances made in recent years on using *P. ostreatus* as source for medicinal products indicate that there still several aspects remaining to be addressed for the efficient implementation of current technologies. Particularly, the extraction procedures, the establishment of precise characterization assays to elucidate the mechanism of action of the extracted compounds and testing for the possible synergy between some of the extracted bioactive compounds need to get addressed. Noteworthy, the immunomodulatory action over the immune system of the nutritional application of *P. ostreatus* also remained uncovered [7]. Therefore, this work was aimed to evaluate the immunomodulatory action of the *P. ostreatus* mushroom *in vitro* and in experimental animal models of secondary immunodeficiencies.

Figure 1. Production of Pleurotus ostreatus (oyster mushroom) at the Center for Studies on Industrial Biotechnology (CEBI). A) Solid state fermentation in the research-development plant B) Pleurotus fruiting bodies (strain CCEBI-3024). C) Submerged culture of mushroom mycelium.

*M*ain results

A critical path was established with novel theoretical and practical approaches as platform for evaluating the immunomodulatory profile of biopreparations from the mycelium and fruiting bodies of *Pleurotus*. This allowed the development of formulations enriched in bioactive metabolites with potential applications in immunotherapy and immunonutrition: two preparations of fruiting bodies, an aqueous extract named CW-E obtained at low temperatures, and a dry and pulverized biomass as raw material for 500 mg tablets of a nutritional supplement under development. Additionally, an aqueous extract of the mycelium obtained at high temperatures, named Myc-E, was prepared.

These methodologies were completely novel, aimed to obtain preparations from *Pleurotus* spp. which showed immunoceutic activity, with no similar descriptions in the literature. Their novelty allowed issuing a patent of invention and to the registration of the trademark NutriSetas® [8, 9]. The extraction procedure is conducted under controlled conditions and is scalable and technically accomplishable. The resulting immunoceutic preparation can be further fractionated for the aim of increasing its specificity and homogeneity. Studies evaluating the acute oral toxicity and repeated dose tests did not evidenced any alterations in normal clinical signs and biochemical parameters when administered in animals [10]. This indicated that the obtained products were safe and devoid of toxic effects.

Afterwards, the products were characterized for their main components as starting criteria for the preclinical evaluation of their immunomodulatory properties. The biological effects of mushrooms found while conducting this work are summarized in figure 2.

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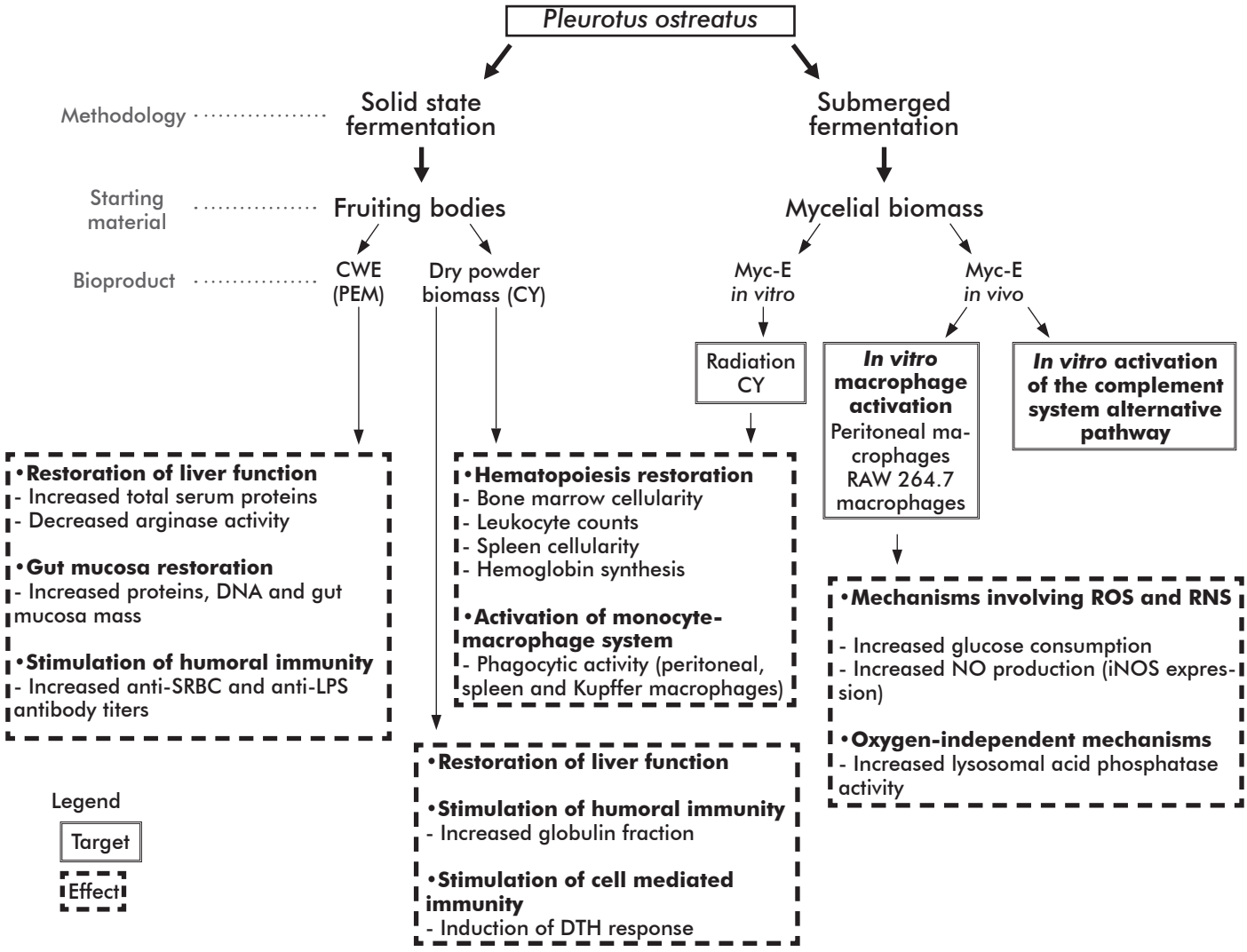

Figure 2. General overview of the *in vitro and in vivo* immunomodulatory effects exerted by *Pleurotus ostreatus* derived bioproducts. CY: cyclophosphamide. PEM: protein-energy malnutrition. Myc-E: *P. ostreatus* mycelial extract obtained at 100 °C; CW-E: *P. ostreatus* fruiting bodies extract obtained at 15-20 °C. ROS: reactive oxygen species. RNS: and reactive nitrogen species. DTH: delayed- type hypersensitivity. NO: nitric oxide, iNOS: inducible NO sintase. SRBC: sheep red blood cells. LPS: lipopolysaccharide.

The aqueous extracts of the mycelium and fruiting bodies, as well as the dry powder preparation, were predominantly composed of carbohydrates (36-70 %) and proteins (15-30 %). The respective values were in the range described for edible and medicinal mushrooms. The potential medicinal applications of the preparations obtained was also supported by the presence in the composition of (1,3)-(1,6)-β-glucan polysaccharides, which are the ones providing most of the biological activity. There were also identified secondary metabolites such as triterpenes, quinones and flavonoids which could display immunomodulatory, antitumor and antioxidant activities [5, 11, 12].

In vitro characterizations showed that the Myc-E mycelial extract assayed at 50-100 μg/well in cell culture plates displayed an activator effect on murine peritoneal macrophages. This was evidenced by the increased glucose consumption (1.8-2.9-fold) by cells when incubated with the extract and a higher lysosomal acid phosphatase activity (133-184 %) as compared to untreated controls. The dose-dependent effect evidenced in the increase of the enzymatic activity suggested the presence of compounds capable of binding receptors on the surface of macrophage, such as β-glucans [13, 14]. Moreover, the expression of the inducible nitric oxide synthase (iNOS) enzyme gene and the production of nitric oxide (ON) were found increased in RAW 264.7 macrophages. These results indicated the polarizing effect of *Pleurotus* extracts towards the M1 pro-inflammatory profile in macrophages, providing the first report on that activity. Macrophage polarization M1 profile plays an important role in host defense and cytotoxic activity against tumor cells [10]. Myc E also stimulated the complement alternative pathway, an activity seldom reported for edible mushrooms [14].

From the clinical perspective, new technologies for obtaining immunoceuticals from edible fungi could

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provide solutions to several issues of immunotherapy. Therefore, this work was completed by evaluating the immunomodulatory effects induced by administering *Pleurotus* biopreparations in three animal models of secondary immunodeficiencies: 1) BALB/c mice immunosuppressed with cyclophosphamide (CY); 2) BALB/c mice immunosuppressed by exposure to ionizing radiation; and 3) malnourished BALB/c mice. Results in these three models provided the first clues on the *Pleurotus*-derived biopreparations mechanism of action *in vivo*.

The Myc-E aqueous extract of *P. ostreatus* mycelium was administered for 7 days (100 mg/kg, intraperitoneally) to BALB/c mice treated with cyclophosphamide (CY). This last is a drug commonly used in cancer therapy and causing secondary immunosuppression. In this model, the treatment with Myc-E was associated with a less pronounced immunosuppression and a faster hematopoietic recovery. This was evidenced by higher white blood cell counts in peripheral blood and stimulation of in vivo activity of the monocyte-macrophage system [15].

Similar results were reported when administering Myc-E in a prophylactic regime to irradiated BALB/c mice, what demonstrated the potential radioprotective effect of Pleurotus spp. bioproducts [16]. This was reflected by an increased maturation, differentiation and proliferation capacity of the different myeloid and lymphoid bone marrow cell populations. The effect was detected as mediated by hematopoietic cytokines as the colony-stimulating factors.

Additionally, the prophylactic oral administration of a dry preparation of *Pleurotus* fruiting bodies favored the synthesis of the globulin fraction and stimulated the cellular immunity in BALB/c mice immunosuppressed with CY. The antigen-specific delayed type hypersensitivity (DTH) reaction measured at 48 and 72 h in treated animals was similar to that of animals without immunosuppressive treatment. It was followed to a higher mass index of popliteal lymph nodes. This result suggested an immunostimulatory effect of the preparation on helper T cell response (CD4+ Th1 lymphocytes). The stimulation of the lymphoproliferative response of splenocytes was also evidenced, as induced by aqueous- and methanol-based extracts obtained from the powder of *Pleurotus* fruiting bodies [11, 17]. Regarding the regenerative properties found at cellular level, which could attenuate the secondary effects derived from conventional treatments against cancer, these natural bioproducts could be applied as alternative treatment against side effects of aggressive chemotherapy, radiotherapy regimens and surgeries.

Another promising line of research comprises immunonutrition as improving gastrointestinal function, cellular immunity and the outcome of inflammatory processes in the gut. In this sense, an animal model of malnutrition was implemented in malnourished BALB/c mice for the evaluation of supplements and medicines [18]. When an aqueaous extract of *P. ostreatus* fruiting bodies (CW-E; 100 mg/kg) was administered in this model, positive immunonutritional effects were detected, involving the recovery of liver function and the induction of cellular synthesis processes in the Intestinal mucosa. CW-E also restored hematopoietic activity, functionality of macrophages and humoral and cellular immune responses, showing increased anti-goat red blood cells antibody titers and DTH responses [7]. These were also the first reports on the application of biopreparations of edible fungi for immunonutrition, paving the way for the obtention of other *Pleurotus* spp.-based products for immunonutrition. Moreover, it was possible to postulate a model of synergistic stimulation of the gut immune system by the compounds present in these bioproducts.

Scientific relevance and impact of the results

Immunoceptive biopreparations were obtained from the aqueous extraction, by drying mycelial biomass and fruiting bodies of *P. ostreatus*, or both, providing the essentials for the formulation of products with immunomodulatory activity. From a theoretical perspective, this work provides new insights on the immunomodulatory profile of compounds found in *P. ostreatus* mushrooms, which could be the source for the development of a new set of biotherapeutic products. The biopreparations obtained could have several potential applications in the food and pharmaceutical industries, to formulate functional foods, dietary supplements and nutraceutics, able to be marketed. They could also contribute to the therapeutic nutritional interventions in cancer, immunodeficiencies and other diseases, aiding to improve the social and economic benefits.

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