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In Vitro Anti-proliferative Effects on NB4 Human Leukemia Cells and Physicochemical Screening of *Pleurotus* sp. (Higher Basidiomycetes) Mycelia from Cuba

Humberto J. Morris,[1,*] Edgar Hernández,[2] Gabriel Llauradó,[1] María Cristina Tejedor,[3] Pilar Sancho,[3] Ángel Herraez,[3] María del Carmen Boyano-Adánez,[3] Ana Isabel García-Pérez,[3] & José Carlos Diez[3]

1Centre of Studies for Industrial Biotechnology (CEBI), Universidad de Oriente, Reparto Jiménez, Santiago de Cuba 5. CP 90 500, Cuba; [2]Department of Biology, Faculty of Natural Sciences, Universidad de Oriente, Santiago de Cuba 5. CP 90 500, Cuba; [3]Department of Biochemistry and Molecular Biology, University of Alcalá de Henares, Madrid, Spain

[*]Address all correspondence to Humberto J. Morris, Centre of Studies for Industrial Biotechnology (CEBI), Universidad de Oriente, Ave. Patricio Lumumba, Reparto Jiménez, Santiago de Cuba 5, CP 90 500, Cuba; Tel.: 53-022-632095; E-mail: hmorris@cebi.uo.edu.cu

ABSTRACT: This study examined the phytochemical profile and the *in vitro* anti-proliferative effects of a hot water mycelial extract from the edible mushroom *Pleurotus* sp. on NB4 human leukemia cells. Flow-cytometry analyses were used to measure cell viability, cell cycle, and apoptosis in cells incubated 24 h with the extract at doses of 100 and 200 μg/mL. *Pleurotus* sp. extract reduced cell viability, particularly at the concentration of 200 μg/mL to 82% compared to control cells, and induced apoptosis demonstrated by an increase in the number of annexin V-FITC$^+$ cells (25% at 200 μg/mL). The NB4 cells were arrested in the G_2/M phase thus supporting a cell-cycle dependent anticancer mechanism. Although carbohydrates (76.8%, w/w) appear to be the most important antitumor compound, secondary metabolites-like phenolics would also contribute to the anti-proliferative activity. The results indicate that *Pleurotus* sp. mycelia obtained by submerged fermentation may be an interesting renewable resource for developing functional foods and new antitumor therapeutic agents.

KEY WORDS: medicinal mushrooms, *Pleurotus*, anti-proliferative, apoptosis, functional foods, leukemia, mycelia

ABBREVIATIONS: CEBI: Centre of Studies for Industrial Biotechnology; **CITMA:** Cuban Ministry of Science, Technology, and Environment; **DNase:** *deoxyribonuclease*; **FBS:** fetal bovine serum; **FITC:** fluorescein isothiocyanate; **NSPs:** non-starch polysaccharides; **PBS:** phosphate-buffered saline; **PDA:** potato dextrose agar; **PI:** propidium iodide; **RNase A:** ribonuclease A;**YPG:** yeast-peptone-glucose medium

I. INTRODUCTION

Oyster mushrooms, species of the genus *Pleurotus* P. Kumm. (Pleurotaceae, higher Basidiomycetes), is an important genus comprising some of the most popular edible mushrooms which cultivation has increased greatly throughout the world during the last few decades. 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.[1]

Species of the genus *Pleurotus,* like many edible and medicinal mushrooms, are a good source of antitumor, immunostimulating, and antioxidant substances.[2,3] Both fruiting bodies and mycelia of *Pleurotus* spp. have been studied in search of biological effector molecules.[4,5] However, between 80% and 85% of all medicinal mushroom products are derived from fruiting bodies; only 15% of all products are based on extracts from mycelia.[6] For that reason, mushroom mycelia may also constitute a good source of healthy compounds, which may be useful in the formulation of nutraceuticals, functional

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foods, and medicinal products.

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. Increasing references confirmed that mushroom polysaccharides or their complexes could have cytotoxic or cytostatic effect on various tumor cell lines *in vitro* mediated by cell-cycle arrest or induction of apoptosis, being less toxic to normal cells.[7,8] In this context, Wong et al.[9] evaluated the *in vitro* anti-proliferative activities of the water-soluble non-starch polysaccharides (NSPs) extracted from different developmental stages of a novel edible mushroom *P. tuber-regium*, focusing on cell-cycle regulation and apoptotic pathway.

In previous papers, we reported the immunomodulating effects of a hot water extract prepared from the mycelium of *P. ostreatus* on the immunosuppression caused by cyclophosphamide in mice[10] as well as the *in vitro* effects of five water-soluble fractions on macrophage activation.[11] In the present work we studied whether a *Pleurotus* sp. mycelial extract possesses tumor cell growth inhibition properties in addition to its immunoceutical properties. To our knowledge, this is the first report describing growth inhibitory properties and cell cycle/apoptosis induction effects of *Pleurotus*-mycelial products on the leukemic human cell line NB4 derived from bone marrow cells of a patient with acute promyelocytic leukemia.

II. MATERIALS AND METHODS

A. Pleurotus sp. Strain and Preparation of Hot Water Mycelia Extract

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

Mycelium was inoculated 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, *Pleurotus* sp. 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 at a yield of 5.5 g/L culture (dried weight) were stored at –20°C and freeze dried. They are composed of 76.8% of carbohydrate, 12% of protein, 0.11% of nucleic acids, and 5% of minerals.

B. Human Acute Promyelocytic Leukemia (NB4) Cells: Cytometric Analysis of Cell Viability

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. 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.[12]

C. Annexin V-FITC Assays: Phosphatidylserine Exposure on the Outer Side of Membrane

For this assay, 5×10^5 treated cells were collected and centrifuged at 1500 rpm for 3 min. Then, they were incubated for 15 min in the dark at room

Running head OK?

temperature in 500 µL PBS containing 10 µL of binding reagent and 1.25 µL Annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit, Calbiochem, EMD Millipore, USA). Subsequently, the cells were centrifuged at 2300 rpm for 5 min and resuspended in 500 µL binding buffer 1× diluted in PBS. After that, 10 µL PI was added and the samples were analyzed in a FACScan cytometer (Becton Dickinson, San José, CA, USA). Data were analyzed using WinMDI 2.8 software. All Annexin V^+/PI^- cells were scored as early apoptotic, while double-stained Annexin V^+/PI^+ cells were considered as late apoptotic.

D. Flow-Cytometric Analysis of Cell-Cycle Distribution of NB4 Cells

For this assay, 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.

AU: Check "DNAse" and "RNAse."

E. Chemical Profile of Hot Water Extract from *Pleurotus* sp. Mycelia

The carbohydrate and protein contents in the extract were determined by the method of Dubois et al.[13] and by Bradford's method.[14] The β-1,3-1,6-glucans were assessed by a new colorimetric method based on the dye congo red, specific for β-glucans with a triple helix. The schizophyllan from *Schizophyllum commune* was used as a calibration standard (Selco Wirkstoffe Vertriebs GmbH; Wald-Michelbach, Germany).[15] Total phenols were quantified with the Folin-Ciocalteu reagent.[16] Also, the chemicals contained in *Pleurotus* sp. mycelia extract were estimated qualitatively.[17]

F. Statistical Analysis

All the experiments were repeated three times and data, expressed as mean±SE, were analyzed using the "Statistical Package for Social Sciences" (SPSS) version 12.0/2003 for Windows (SPSS Inc. 1989–2003). One-way analysis of variance and post hoc Tukey's tests were used to determine mean differences among the groups for the parameters studied. The 95% ($P<0.05$) level was considered as statistically significant.

III. RESULTS AND DISCUSSION

As can be observed in Fig. 1, the treatment with the aqueous extract reduced viability of NB4 human leukemia cells, particularly at the concentration of 200 µg/mL, to 82% with respect to control untreated cells ($p<0.05$).

Apoptosis is the physiological controlled process by which cells actively commit a gene-mediated cell death program essential for cell homeostasis. It is also the key mechanism for activating the chemotherapeutic agents.[18] Figure 1 (middle and lower parts) shows that *Pleurotus* extract induced apoptosis of NB4 cells, which was demonstrated by an increase in the number of annexin V-FITC positive cells, particularly at the dose of 200 µg/mL (25%, $p<0.05$). The apoptosis percentage of NB4 cells induced by *Pleurotus* extract at 100 µg/mL (20%) was intermediate between untreated control cells and NB4 cells treated with 200 µg/mL. The effect on early apoptotic cells (Annexin V^+/PI^-) was concentration dependent (7% and 11% for 100 and 200 µg/mL, respectively) in contrast to late apoptotic cells. Our results presumably would be associated with an increasing in the Bax/Bcl-2 ratio, proteins which are key regulators in apoptosis, particularly the antiapoptotic Bcl-2 and proapoptotic Bax proteins.[19]

AU: Check "activating." "Acting" is not correct.

The apoptosis percentage values obtained in this work were lower compared to those reported for *P. ostreatus* mycelia-derived proteoglycans (5 mg/kg) in the *in vivo* Sarcoma-180-bearing mouse model (45.36%–75.36%).[7] They were also lower than the 31% and 46% obtained after treat-

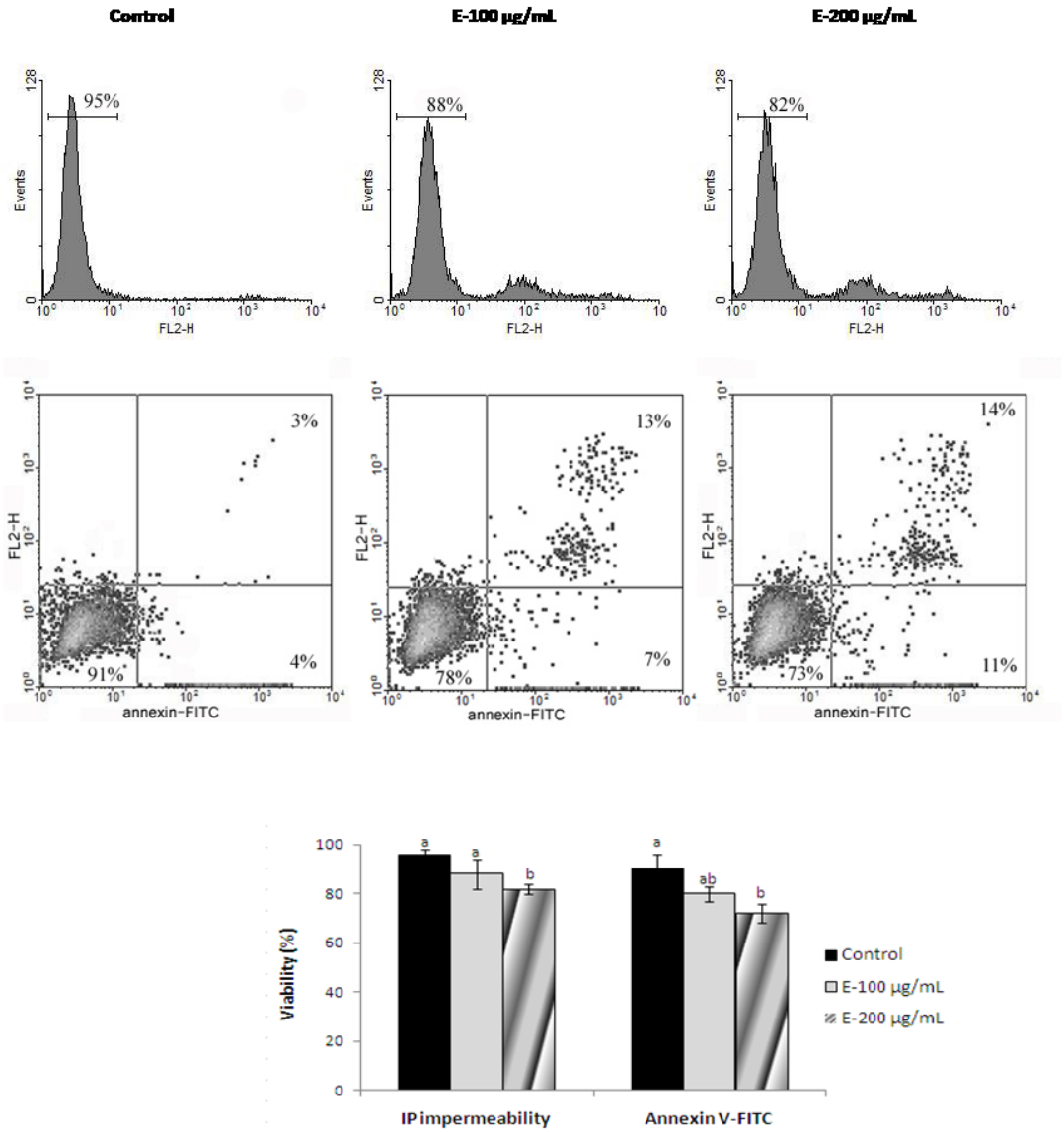

FIGURE 1. Cell viability of human leukemia NB4 cells treated with the *Pleurotus* sp. mycelial extract. NB4 cells were incubated for 24 h with the extract at concentrations of 100 and 200 µg/mL. Cell viability was measured as the preservation of cell impermeability to PI and the counting of cells with low inner propidium fluorescence and low binding to annexin V-FITC. Upper part of the figure shows flow-cytometry profiles of NB4 cells treated as indicated. Middle part of the figure shows flow-cytometry profiles of cell cycle of treated NB4 cells; as shown, the cell populations shown in the right bottom quadrant (PI^-/Annexin V^+) represents early apoptosis cells, whereas the right upper quadrant (PI^+/Annexin V^+) represents late apoptotic cells. Lower part of the figure shows the statistical representation of the results. These values represent the mean of three different experiments. The standard errors of means are shown as errors bars. Different letters indicate significant differences among the groups in Tukey's test (p<0.05).

ments of NB4 cells with *Ganoderma lucidum* E3 fraction diluted at 15% and 40%.[20] On the other hand, the apoptosis ratios achieved in our work were higher than those referenced by Lee et al.[21] on Molt 4 human leukemia cells treated with 1 mg/mL (≈100 µg/mL on the basis of polysaccharide con-

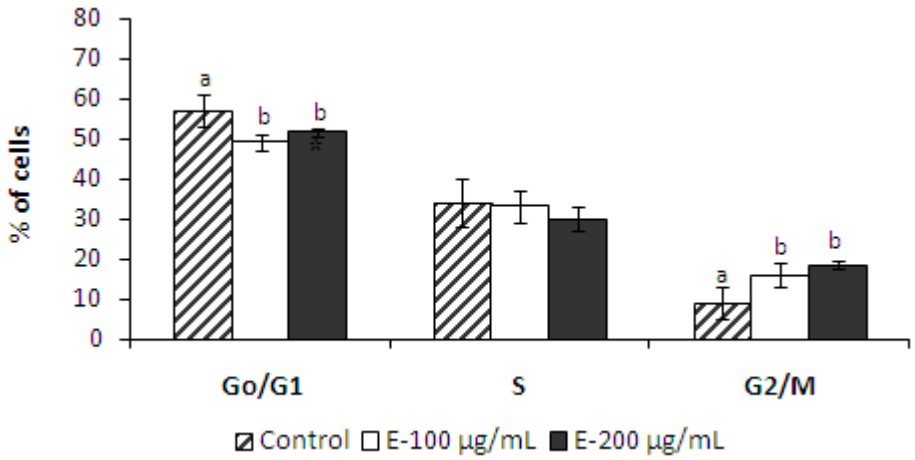

FIGURE 2. Cell-cycle analysis of human leukemia NB4 cells treated with the *Pleurotus* sp. mycelial extract for 24 h at concentrations of 100 and 200 µg/mL. Values represent the mean±SE of three independent experiments. Significantly differences in Tukey's test (p<0.05) were shown with different letters as compared to control cells.

tent) of the polysaccharopeptide PSP isolated from the mycelia of *Trametes* (=*Coriolus*) *versicolor* (5.92±0.7%).

The apoptosis induction observed in this work in NB4 cells contributes to the putative antitumor mechanism of the hot water *Pleurotus* sp. mycelial extract in addition to immunomodulation. Taking into account that the evasion of apoptosis is a hallmark of human cancers (e.g., hematological malignances),[18] products capable of inducing apoptosis deserve special interest.

Our results presented in Fig. 2 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. On the whole, *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.[8] 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 downregulating the anti-apoptotic protein Bcl-2.[22]

The hot water extract from *Pleurotus* sp. mycelia contained 76.8% (w/w) carbohydrate and 12% (w/w) protein. Congo red is used for quantifying glucan tertiary structures because of its interactions with the triple helix of β-1,3-1,6-glucans and because it does not react with other polysaccharides.

On the average, 1.5% of mycelial extract dry mass consists of β-1,3-1,6-glucans. This content was lower compared to that of *P. ostreatus* mycelia (2.97%) but higher than those referred on *P. pulmonarius* (0.41%).[15] The correlation of the β-1,3-1,6-glucan amounts with antitumor effects should be subject of further investigations.

AU: We do not understand what is meant by "referred on". Please rewrite.

Although polysaccharides appear to be the most important bioactive component with respect to antitumor effect, the presence of different secondary metabolites in *Pleurotus* extract could lead

TABLE 1. Chemical Composition of Studied Mycelia of *Pleurotus* sp.

Metabolites	Assays	Aqueous extract[a]
Alkaloids	Dragendorff	++
	Wagner	+
Terpenoids	Solkowski	–
	Lieberman-Burchard	–
Carbohydrates/glycosides	Molisch	++
Reducing sugar	Fehling	+
	Benedict	+
Quinones	Borntrager	–
Phenols and tannins	$FeCl_3$	+
Amino acids	Ninhidrine	++
Flavonoids	Concentrated H_2SO_4	+
	Rosemheim	+

[a]The chemicals contained in the aqueous extract were estimated qualitatively according to Harbourne.[17] Three replicates were used for each assay. (–) none, (+) present, (++) mild, (+++) marked.

AU: Check. Should this be "Rosenheim"?

to a synergy in the anti-proliferative activity. The result of the phytochemical test shows that *Pleurotus* mycelia-derived hot water extract contains alkaloids, phenolic compounds such as flavonoids and tannins, reducing sugars, and amino acids (Table 1).

The total phenolic content of *Pleurotus* mycelial extract tested in the present work was 38±5 mg/g extract (expressed as tannic acid equivalents). The Folin-Ciocalteu assay is highly sensitive for monohydric phenols, polyphenols, flavonoids, and tannins; however, the method could overestimate the total phenolic content since other readily oxidized substances, such as sugars, ascorbic acid, amino acids (tyrosine, tryptophan), etc., could interfere by reacting with the colorimetric reagent.[23]

AU: Check units.

Other authors have reported total phenolic contents of 54.90 mg/g in *P. ostreatus*[2] and 82.5 mg/g in *P. squarrosulus*.[24] These literature values refer to mushroom fruiting bodies; there are no data on phenolic content of *Pleurotus* spp. mycelia aqueous extracts available for comparison.

The expanded knowledge of the molecular basis of tumorigenesis, together with the vast structural diversity of natural compounds found in mushrooms, provide unique opportunities for discovering new drugs that rationally target the abnormal molecular signals leading to cancer. The results obtained in this study demonstrate that not only *Pleurotus* sp. mushrooms but also their mycelia obtained by submerged fermentation may be an interesting renewable resource for developing functional foods and new therapeutic agents with antitumor activity. On these bases, the hot water extract from *Pleurotus* sp. mycelia might have some anti-proliferative effects on NB4 human leukemia cells, probably mediated by apoptosis induction and G_2/M arresting of the cell cycle. However, further studies are needed to characterize effective phytochemicals responsible for the antitumor actions and their mechanisms. The active compounds present in these *Pleurotus* sp. extracts might be particularly useful in human leukemia therapy.

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REFERENCES

1. Gregori A, Švagel M, Pohleven J. Cultivation techniques and medicinal properties of Pleurotus spp. Food Technol Biotechnol. 2007;45:238–49.
2. Jayakumar T, Thomas PA, Sheu JR, Geraldine P. In-vitro and in-vivo antioxidant effects of the oyster mushroom Pleurotus ostreatus. Food Res Int. 2011;44:851–61.
3. Wasser SP. Medicinal mushroom science: history, current status, future trends, and unsolved problems. Int J Med Mushrooms. 2010;12(1):1–16.
4. Llauradó G, Morris HJ, Lebeque Y, Gutiérrez A, Fontaine R, Bermúdez RC, Perraud-Gaime I. Phytochemical screening and effects on cell-mediated immune response of Pleurotus fruiting bodies powder. Food Agric Immunol. 2012;doi:10.1080/09540105.2012.686988.
5. Refaie FM, Esmat AY, Daba AS, Taha SM. Characterization of polysaccharopeptides from Pleurotus ostreatus mycelium: assessment of toxicity and immunomodulation in vivo. Micol Apl Int. 2009;21:67–75.
6. Barros L, Baptista P, Estevinho LM, Ferreira I. Bioactive properties of the medicinal mushroom Leucopaxillus giganteus obtained in the presence of different nitrogen sources. Food Chem. 2007;105:179–86.
7. Sarangi I, Ghosh D, Bhutia SK, Mallick SK, Maiti TK. Anti-tumor and immunomodulating effects of Pleurotus ostreatus mycelia-derived proteoglycans. Int Immunopharmacol. 2006; 6:1287–97.
8. Tong H, Xia F, Feng K, Sun G, Gao X, Sun L, Jiang R, Tian D, Sun X. Structural characterization and in vitro antitumor activity of a novel polysaccharide isolated from the fruiting bodies of Pleurotus ostreatus. Biores Technol. 2009;100:1682–86.
9. Wong SM, Wong KK, Chiu LCM, Cheung PCK. Non-starch polysaccharides from different developmental stages of Pleurotus tuber-regium inhibited the growth of human acute promyelocytic leukemia HL-60 cells by cell-cycle arrest and/or apoptotic induction. Carbohydr Polym. 2007;68:206–17.
10. Morris HJ, Marcos J, Llauradó G, Fontaine R, Tamayo V, García N, Bermúdez RC. Immunomodulating effects of hot-water extract from Pleurotus ostreatus mycelium on cyclophosphamide treated mice. Micol Apl Int. 2003;15:7–13.
11. Morris HJ, Lebeque Y, Fontaine R, Bermúdez RC, Llauradó G, Marcos J. A note on the in vitro macrophage-stimulating activity of water-soluble extracts from mycelium of Pleurotus spp. Food Agric Immunol. 2007;18:31–37.
12. Trotter J. WinMDI (Windows multiple document interface for flow cytometry), version 2.8. 2004. http://facs.scripps.edu/software.html. Accessed 28 October 2011.
13. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956;28:350–56.
14. Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
15. Nitschke J, Modick H, Busch E, Rekowski RW, Altenbach HJ, Mölleken H. A new colorimetric method to quantify β-1,3-1,6-glucans in comparison with total β-1,3-glucans in edible mushrooms. Food Chem. 2011;127:791–96.
16. Slinkard K, Singleton VL. Total phenol analyses: automation and comparison with manual method. Am J Enol Vitic. 1977;28:49–55.
17. Harbourne HB. Phytochemical methods: a guide to modern technique of plant analysis. 2nd ed. London: Chapman and Hall; 1984.
18. Fulda S. Cell death in haematological tumors. Apoptosis. 2009;14:409–23.
19. Raisova M, Hossini AM, Eberle J, Riebeling C, Wieder T, Sturm I. The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. J Invest Dermatol. 2001;117:333–40.
20. Calviño E, Manjón JL, Sancho P, Tejedor MC, Herráez A, Diez JC. Ganoderma lucidum induced apoptosis in NB4 human leukemia cells: involvement of Akt and Erk. J Ethnopharmacol. 2010;128:71–78.
21. Lee ChL, Yang X, Wan JMF. The culture duration affects the immunomodulatory and anticancer effect of polysaccharopeptide derived from Coriolus versicolor. Enzyme Microb Tech. 2006;38:14–21.
22. Zhang M, Chiu LC, Cheung PC, Ooi VE. Growth inhibitory effects of a beta-glucan from the mycelium of Poria cocos on human breast carcinoma MCF-7 cells: cell-cycle arrest and apoptosis induction. Oncol Rep. 2006;15:637–43.
23. Palacios I, Lozano M, Moro C, D'Arrigo M, Rostagno MA, Martínez JA, García-Lafuente A, Guillamón E, Villares A. Antioxidant properties of phenolic compounds occurring in edible mushrooms. Food Chem. 2011;128:674–78.
24. Pal J, Ganguly S, Tahsin KS, Acharya K. In vitro free radical scavenging activity of wild edible mushroom Pleurotus squarrosulys (Mont.) Singer. Indian J Exp Biol. 2010;47:1210–18.