

STUDY OF CYTOTOXIC AND PRO-APOPTOTIC EFFECT OF MEDICINAL MUSHROOM *PLEUROTUS FLORIDA* IN CANCER CELL LINES

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Summary

The use of mushrooms has been recommended in Traditional Medicine meant for treatment of different disease including cancers. In present study, cytotoxic properties of aqueous extract of *Pleurotus florida* body was investigated on AGS, ACHN, Hep-G2, Hela, MCF-7, PC-12 cancer cell lines and normal fibroblast (L929) cells. Also, the pattern of cellular death is evaluated. So, cells were treated with different concentrations of aqueous extract of *P. florida* body for 24 hours. Then, the cell viability was determined using colorimetric MTT assay. Cellular death pattern was evaluated using annexinV-FITC and propidium iodide (PI) staining method (as early apoptosis, late apoptosis and necrosis) using flow cytometry. *P. florida* aqueous extract caused a significant inhibition on the growth of cancer cells in a dose-dependent manner. Fibroblast (L929) cells were less sensitive to it. The extract exhibited the potent growth inhibitory effect on AGS cell line that mostly was induced by early and late apoptotic death. In conclusion, *P. florida* exhibited cytotoxicity on different cancer cell lines and its effect mainly induced by apoptosis. So, *P. florida* could be considered as a potential safe and effective agent for the treatment of different cancers especially gastric cancer.

Key Words: Medical mushroom, MTT assay, Cellular death pattern

Introduction

Cancer is a major public health problem throughout the world (1). Because the treatment of malignant tumors is difficult, so prevention from it is recommended. Modifications of diet and lifestyle could be useful for reducing the risk of cancers. Herbal remedies and alternative medicines have been used throughout the world and a lot of researches have been done to discover new therapeutic compounds from medicinal plants with less toxicity (2-4).

It has been shown that many natural products could selectively eliminate the proliferating cancer cells through various mechanisms including induction of DNA repair systems, activating of immune system, suppression of cell cycle and induction of apoptosis that are critical characteristics of chemoprevention (5-8).

Apoptosis maintains the physiological balance of cells and plays a critical role as protective mechanism against carcinogenesis via eliminating of damaged or abnormal excess proliferating cells (9, 10). Numerous studies reported that many natural products showed anticancer activities throughout the induction of apoptosis in cancer cells (9-11). So, their usage is recommended as a preferred way to manage the cancers.

Mushrooms are nutritional foods and contain nontoxic compounds that have medicinal benefits. Medicinal mushrooms have been used in folk medicine throughout the world for the treatment of human diseases and nowadays, they supposed as effective anticancer agents for clinical uses (12-16). *Pleurotus* species called oyster mushrooms with worldwide distribution (17, 18). Among them, the genus *Pleurotus florida* is an edible mushroom that has antioxidant, anti-inflammatory, antiplatelet aggregating, antitumor, antimutagenic, reducing blood fat and immunoregulation effects and exhibit activity against various chronic diseases (19-25). Its fruit bodies contain 37.19% protein, 3.72% fat and 10.98% ash on a dry weight basis (26, 27). The polysaccharides and protein contents of this mushrooms reported to have different biological activities (28-33). *P. florida* methanol extract exhibited antitumor effect on solid tumor model in Swiss albino mice that induced by Ehrlich's ascites carcinoma (EAC) cell line (23). *P. ostreatus* strain *florida* methanol extract possessed significant antimutagenic activity (34). Ethanol extract of *P. ostreatus* had potent antioxidant activity (35, 36) and its methanol extract showed antiproliferative effect (37). Also, it exhibited significant anti-inflammatory effect on acute and chronic inflammation (38). Aqueous extract of *P. ostreatus* showed host-mediated antitumor activity against sarcoma 180 cells murine model (39). A hot water extract of *P. ostreatus* inhibited the proliferation of HT-29 colon cancer cells and induced apoptosis through the upregulation of pro-apoptotic molecules Bax and cytochrome-c (40).

In the previous study, we studied the effect of *P. florida* extract on the production of nitric oxide (NO) and cell viability of macrophages *in vitro*. We reported that *P. florida* extract increased cell viability of macrophages in almost applied doses but it has not been shown to cause any significant alteration in NO production (41). In another study, Balb/c mice were treated with different doses of *P. florida* aqueous extract and nitric oxide production and cell viability of their intraperitoneal macrophages significantly increased (42).

Crude natural product extracts are generally an extremely complicated mixture of several compounds that possess variable biological properties (43-45). The fundamental strategy for separating these compounds is based on their chemophysical properties that can be cleverly exploited to initially separate them into various chemical groups. However, based on previous literature and reports of the related genera and families, it is possible to predict the types of compounds that might be present in a particular extract. This prediction can be useful for the selection of suitable extraction and partitioning methods and solvents for isolation of desired compounds (46, 47).

This study was done with the aim of identifying the antiproliferative and pro-apoptotic principles present in aqueous extract of *Pleurotus florida*. The human cancer cell lines were studied as

models of tumors. Cellular death pattern was evaluated for the determination of growth inhibitory-mediated of extract in more sensitive cells too.

Materials and methods:

Reagents

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from Gibco Laboratories (Detroit, USA). 3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) was purchased from Sigma chemical Co (St Louis, MO, USA). The annexin V-FITC assay kit was prepared from abcam Co.

Preparation of aqueous extract of *Pleurotus florida*

The fresh mushroom sample was purchased from Boshehr, Iran. For preparation of *P. florida* extract; 42.5 g of its body was mixed with 155cc deionized and distilled water. The solution was centrifuged at 14000g for 20 min and supernatant was collected and store in refrigerator until used (35, 36).

Cell culture and treatment

AGS (human gastric carcinoma cell line), ACHN (human kidney adenocarcinoma cell line), Hep-G2 (human hepatoma cell line), Hela (human cervical adenocarcinoma cell line), MCF-7 (human breast carcinoma cell line), PC-12 (rat adrenal gland pheochromocytoma cell line) and L929 (mouse fibroblast cell line) were obtained from National Cell Bank of Iran (NCBI), Tehran, Iran. Cells were maintained as a monolayer culture in DMEM medium, supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin in a 5% CO₂ humidified atmosphere incubator at 37°C. For the experiments, cells were removed from the flasks using a 0.25% trypsin-EDTA solution.

Cell viability assay

Viability of cancer and normal cells treated with/without aqueous extract of *P. florida* was measured by MTT colorimetric assay (11, 43, 45, 48). The assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells into an insoluble colored Formosan product, which can be measured spectrophotometrically after dissolving in dimethyl sulfoxide (DMSO). Briefly, cells (2×10^4 cells/well) were seeded onto 96-well microculture plates and allowed to adhere overnight. Then, each cell line was exposed to increasing concentrations of aqueous extract (5-2000 µg/ml) for 24 h. The first column of each microplate was assumed as negative control (containing no extract). To assay the cell viability, 25 µl of MTT solution (5 mg/ml in phosphate buffered solution) was added to each well and the plate was incubated for 3 h at 37°C. Then, 200 µl of DMSO was replaced and pipetted to dissolve any Formosan crystals. The optical density (OD) was read on an Elisa reader (Microplate reader MR 600, Dynatech, USA) at a wavelength of 545 nm. The inhibitory rate of cell proliferation was calculated by the following formula: Growth inhibition (%) = $[1 - (OD_{\text{control}} - OD_{\text{treated}}) / OD_{\text{control}}] \times 100$

Determination of cellular death pattern

FACS analysis was used to quantify the death pattern of cells using fluorescein isothiocyanate-labeled Annexin V (annexin V-FITC) and propidium iodide (PI) staining method (11). The combination of annexin V-FITC with PI has been used extensively to distinguish living cells in early and late apoptosis. During early apoptosis, phosphatidylserine, which is usually located in the inner membrane of cells, is transported into the outer portion of the membrane that can be detected by its strong affinity for annexin V-FITC, a phospholipid binding protein. The dead cells can be detected by the binding of PI to the cellular DNA in cells where the cell membrane has been totally compromised. In brief, more sensitive cancer cell (AGS cells) was cultured overnight in 6-well culture plate and then treated with increasing concentrations of extract at 50-2000 µg/ml for 24 h. After incubation, cells were harvested with 0.25% trypsin-EDTA solution, washed twice with Phosphate buffered solution and then resuspended in annexin V-FITC binding buffer and incubated at room temperature for 5 min in the dark with annexin-V FITC and PI. Then, cells were analyzed (10,000 events) by flow cytometry (Ex. = 488nm; Em. = 530nm) using a FACScan flow cytometer (Becton Dickinson, USA). The results showed as percent of cells with (+) Annexin V (early apoptosis), (+) PI (necrosis), both (+/+) (late apoptosis) or none (-/-) indicating non-stained.

Statistical analysis

The significance of difference was evaluated by one way analysis of variance (ANOVA) and Bonferroni's posthoc using SPSS 11.0 software. All results were expressed as mean ± SEM. A probability level of $P < 0.05$ was considered statistically significant.

Results

Cytotoxicity of *Pleurotus florida* aqueous extract

Malignant and normal cell lines were incubated with aqueous extract of *P. florida* for 24 h. The results showed that this extract dose dependently decreased viability of cancer (Figure 1) and normal cells (Figure 2). Its cytotoxic effect started at a concentration as little as 5 µg/mL. Also, the extract showed the most potent cytotoxic activity against AGS and successively Hep-G2 cells. Although aqueous extract at high concentrations (250-2000 µg/ml) had the most cytotoxic activity on AGS cancer cells, but it showed the most cytotoxic activity in Hep-G2 cancer cells at lower concentrations (5-100 µg/ml). So, an AGS cell as the most sensitive cells was chosen for further comparative studies.

Also, the anti-proliferative property of extract against normal, non-tumor L929 cells was the least after 24h. As compared with cancer cell lines analyzed, we found that *P. florida* aqueous extract had some selective anti-tumor effect, because it showed the least cytotoxicity on L929 normal cells.

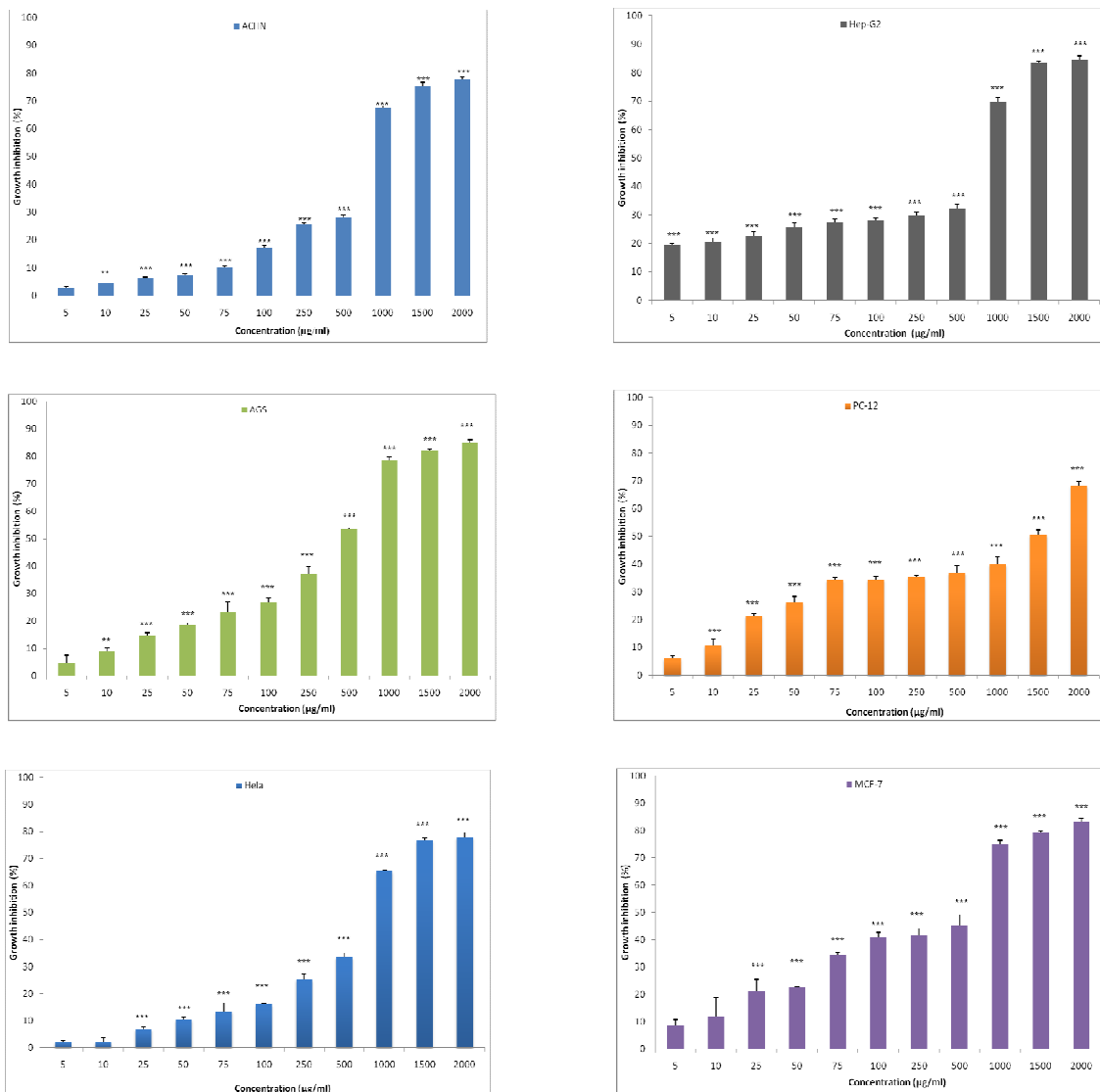


Figure 1. Growth inhibitory effect of aqueous extract of *P. florida* (5-2000 µg/ml) on different cancer cell lines including ACHN, Hep-G2, PC-12, AGS, HeLa and MCF-7 cells was determined using MTT assay after 24 hours of treatment. Results are shown as mean±SEM (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared to untreated control.

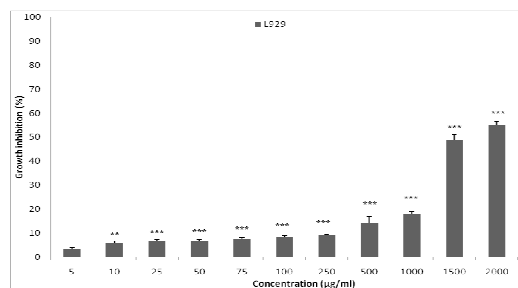


Figure 2. Growth inhibitory effect of aqueous extract of *P. florida* (5-2000 µg/ml) on normal, non-tumor L929 cells was assayed using MTT assay after 24 hours of treatment. Results are shown as mean±SEM (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared to untreated control.

Pattern of cellular death in AGS cells treated with aqueous extract of *Pleurotus florida*

To examine the mechanism responsible for *P. florida* aqueous extract-mediated cell proliferation inhibition, cellular death pattern was evaluated after treatment of AGS cells with extract using flow cytometric analysis. The extract induced apoptosis in AGS cells, which was assessed by annexin V-FITC assay was shown in Figure 3. The annexin V-FITC assay was carried out in conjunction with PI staining.

The results showed that in comparison with untreated control, after 24h of treatment with 50, 100, 250, 500, 1000 and 2000 $\mu\text{g/ml}$ of extract; the early apoptosis (right lower section of fluorocytogram) in AGS cells were increased significantly (2.73% into 9.47%, 13.33%, 15.16%, 52.79%, 27.65%, 16.32%, 8.13%, respectively). Also, the late apoptotic cells (right upper section of fluorocytogram) were increased significantly (1.43% into 0.89%, 6.14%, 7.79%, 23.97%, 66.64%, 81.59%, 90.11%, respectively). But, the percentage of necrotic cells were increased slightly (0.58% into 0.4%, 1.15%, 1.53%, 1.38%, 1.12%, 1.02%, 0.44%, respectively).

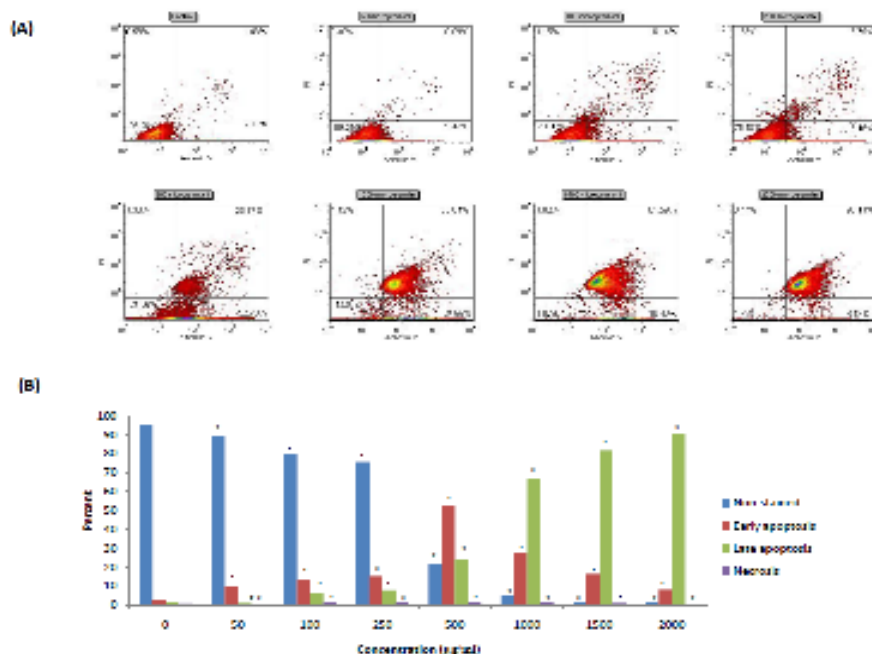


Figure 3. Apoptosis induced by aqueous extract of *P. florida* in AGS cells. (A) AGS cells were treated with different concentration of extract for 24 h. Then cells were collected and stained with annexin V-FITC and PI. In flow cytometric histograms of apoptosis assays, X-axis shows annexin V-FITC positive cells and Y-axis shows PI labeled population. (B) Early apoptotic, late apoptotic and necrotic rates of AGS cells induced by different concentrations of aqueous extract of *P. florida*.

Discussion

Prevention of cancer is one of the most important public health and medicinal purposes throughout the world. In past decades, many natural products have been studied for their chemopreventive effects. Many plants with different pharmacological properties are known to full of chemical compounds that may be potential for the prevention or treatment of malignancies (49, 50).

Plants contain many phytochemicals with various bioactivities including antitumor activity. Some experiments have reported that extracts of natural products showed inhibitory effects against cancers (51). Therefore, many plants have been examined to identify new and effective anticancer compounds (52-54).

One strategy for cancer chemoprevention is the search for nutritional components directed at inducing apoptosis of growth of cancer cells. Edible mushrooms such as *Pleurotus florida* have beneficial effects on health and in the treatment of different diseases (19-25).

The present study was evaluated the growth inhibitory and pro-apoptotic effects of aqueous extract of *P. florida* body. Cytotoxic effect of its extract was studied on different cancer and normal cells. On the basis of obtained results, the extract showed cell growth inhibitory activity against AGS, ACHN, Hep-G2, Hela, MCF-7 and PC12 cell lines which were not previously shown. As shown in Figure 1, extract had remarkable cell growth inhibitory effect on studied cancer cells, especially on AGS cells. It showed inhibitory effects on the proliferation of cancer cells but had less effect on normal cells indicating a degree of specificity for cancer cell lines (Figure 2).

Previous study reported that the concentration of 1000 µg/ml *P. ostreatus* methanol extract inhibited the growth of MCF-7 cells and HT-29 cells with the proliferation index percent of 70% and 17%, respectively (39). On the basis of our obtained results, 1000 µg/ml *P. florida* aqueous extract inhibited the growth of ACHN, AGS, Hela, Hep-G2, MCF-7 and PC-12 cells with the growth inhibition percent of 67.48 ± 0.7 , 78.5 ± 0.48 , 65.44 ± 0.28 , 69.79 ± 1.62 , 75.14 ± 1.2 and 39.91 ± 2.86 percent, respectively. In comparison, *P. florida* aqueous extract had potent inhibitory effect on the growth of different cells.

Because it is important to discover the apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them, in this study *P. florida* extract-induced cell death pattern was investigated in AGS cells. Death pattern of treated AGS cells was evaluated by annexin V-FITC and PI staining method to distinguish living cells in early apoptosis, late apoptosis and necrosis. As shown in Figure 3, extract caused a remarkable early and late apoptosis in AGS cells. *P. florida* extract at concentration of 500 µg/ml induced the highest percent of early apoptosis (52.79%). The extract increased the percent of late apoptosis in AGS cells in a dose-dependently manner and the amount of late apoptosis in the high concentration of 2000 µg/ml of the extract was 90.11%. The portion of necrosis in the cell death-induced of the extract was little.

Our study confirmed that *P. florida* aqueous extract had cytotoxicity against different cancer cell lines, especially on AGS cells and its effect mediated mostly through the induction of early and late apoptotic death.

Taking together, the present study is the first to show growth inhibitory effect of *P. florida* aqueous extract on studied cancer cell lines and here we show that early and late apoptosis (programmed cell death) play critical important role.

Future work will focus on mechanisms involved in this toxicity and animal studies using to examine the effects of this extract and compare it to the current anticancer drugs. This study may provide guidelines for incorporation of mushrooms and/or their extracts into the diet to take advantage of their 'nutraceutical' properties because of their promising chemotherapeutic advantages in cancer treatment.

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