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Cytotoxic Activity of Medicinal Mushroom Extracts on Human Cancer Cells

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Abstract

The aim of this study was to evaluate fruiting bodies and/or mycelial biomasses of Grifolafrondosa, Cordycepsmilitaris, *Ganodermatsugae* var. *jannieae*, Hericiumerinaceus, Trametesversicolor, Coprinuscomatus, and Tremellafuciformis for their anticancer effect against four types of human cancer cell lines: HPAF-II (pancreatic cancer), HCT116 (colon cancer), PC3 (prostate cancer), and T47D (breast cancer). For this reason, ethanol, ethyl acetate, chloroform, and water extracts have been obtained from these mushrooms. Among the extracts examined, those obtained from *C. militaris, T. versicolor* and *H. erinaceus* appeared as the best inhibitors of cell viability in a dose-dependent manner decreasing the cell viability by approximately 40-95%. Ethyl acetate and chloroform extracts appeared to be the most active showing the most profound decrease in cell viability and exhibiting anti-proliferative activity against all cell lines. Low concentrations (25-50 µg/ml) of tested extracts did not show significant effects on viability of all tested cell lines. These results suggest that individual mushroom extracts affect human cancer cells and can be considered as potential natural compounds for cancer prevention and treatment.

Keywords: Apoptosis; Cancer cells; Cytotoxicity; Cell viability; Extracts; Lactate dehydrogenase leakage; Medicinal mushrooms

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Abbreviations

DMSO: Dimethyl Sulfoxide; FBS: Fetal bovine serum; FACS: Fluorescence activated cell sorter; FB: Fruit Bodies; LDH: lactate dehydrogenase; MB: Mycelial Biomass

Introduction

Cancer is one of the most common causes of death worldwide. Depending on the stage of cancer progression, treatments include surgical operation, radiotherapy, chemotherapy and biological treatment. The current anti-cancer chemotherapeutic agents are formulated with toxic solvents and pose several side-effects and complications in clinical management of various forms of cancer. These drugs when administrated, lack specificity and cause significant damage to noncancerous tissues. This often leads to serious, unwanted side effects such as neuro-and/or renal-toxicity, bone marrow suppression, hair loss (alopecia), and the sloughing of the gut epithelial cells [1-3]. This fact highlights the urgent need for novel, effective and less-toxic therapeutic agents. Therefore, the search for new, natural anticancer bioactive compounds has become an immense interest.

Recently, a new direction in cancer treatment has arisen, which is devoted to the adjuvant use of natural bioactive compounds in conventional chemotherapy. The need for this type of research is becoming more apparent. Nowadays, a modern clinical practice in the world continues to rely on mushroom-derived preparations. Medicinal effects have been demonstrated for many traditionally used mushrooms and mushroom-extracted compounds for the prevention and treatment of cancer and numerous clinical trials have been conducted using medicinal mushroom extracts in cancer therapy [4]. Studies have shown that mushroom species have anti-cancer activity through several pathways. Mushroom extracts might be considered alternative and preferable sources for adjuvant

cancer therapy, as they have no adverse effects, activate the cells of the immune system, and reduce free radicals.

Medicinal mushrooms produce a variety of biologically active compounds such as polysaccharides, amino acids, fatty acids, enzymes, lectins, dietary fiber, terpenoids, steroids, statins, phenols, alkaloids, vitamins, minerals etc [5-9]. Many mushroom species belonging to different taxonomical groups, worthily can be considered as pharmaceutical factories producing a wide spectrum of novel biologically active substances with beneficial medicinal properties. This is confirmed by a number of studies (including clinical trials) which undoubtedly demonstrate several therapeutic effects including antitumor, immunomodulating, antioxidant, radical scavenging, anti-inflammatory, anti-hypercholesterolemia, antiviral, antibacterial, anti-parasitic, antifungal, hepato-protective, anti-diabetic, and others [4,10-17]. These studies have led to the creation of new pharmaceutical products that are called "mushroom nutraceuticals" or "fungal dietary supplements". Medicinal effects have been demonstrated for many traditionally used mushrooms, including *Auricularia, Cordyceps, Flammulina, Ganoderma, Grifola, Hericium, Lentinus,Pleurotus, Trametes, Schizophyllum, and Tremella* [18]. Nowadays, mycopharmacology is formed as a new, separate medical direction which has an immense future. However, there is limited information about anticancer properties of extracts prepared from fruiting bodies and mycelial biomasses of many mushroom species and numerous fundamental and clinical studies are needed to fill the gaps in anticancer research of medicinal mushrooms.

The main goal of the present study was to evaluate anticancer properties against human pancreatic, colon, prostate, and breast cancer cells of hot water, chloroform, ethyl acetate, and ethanol extracts from fruit bodies and mycelial biomass of various mushrooms species belonging to different ecological and taxonomical groups.

Materials and Methods

Fungal materials

Fruiting bodies (FB) and/or mycelial biomass (MB) of seven mushroom strains were used in the present study to evaluate their cytotoxic effects: *Grifolafrondosa* FB; *Cordycepsmilitaris* CBS-132098 FB; *Ganodermatsugae* var. *jannieae* CBS-120304MB, FB; *Hericiumerinaceus* MB, FB; *Trametesversicolor* MB, *Coprinuscomatus* FB, and *Tremellafuciformis* FB. Mushroom fruiting bodies and mycelia were kindly provided by Laboratory of Biotechnology and Biodiversity of Fungy (HAI, University of Haifa, Israel). All strains are preserved in the culture collection of Haifa University (Israel).

Extraction

The dried fruiting bodies or mycelial biomasses were homogenized to a fine powder. For extraction, water and three different organic solvents (10mL/g dry weight) were used: chloroform, ethanol, and ethyl acetate (Et-Ac). Water extraction was performed for 3h with distilled H_2O at 80°C (using a water bath), while extraction with ethanol (80%), chloroform and ethyl acetate was carried out on the rotary shaker at 150rpm at 27°C for 3h. After extraction, insoluble compounds were separated by centrifugation at 6000 x g at 4°C for 15min followed by filtration through Whatman® filter paper N1. Filtrates (supernatant) were evaporated. The crude extracts were collected in preliminarily weighed glass tubes, then weighed again in order to determine the exact weight of the obtained fungal extracts [19]. Stock solutions of extracts (50mg/mL) were prepared in 99.9% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and kept at –20°C.

Human cancer cell cultures

Four human cancer cell lines were used for the evaluation of extract effects on cell viability: HPAF-II (pancreatic cancer), HCT116 (colon cancer), PC3 (prostate cancer), and T47D (breast cancer). All human cancer cell lines were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and maintained in a suitable media, supplemented with 1% L-glutamine, 10% fetal bovine serum (FBS), and 1% Pen-Strep (penicillin + streptomycin) (Biological Industries, Kibbutz Beit-Haemek, Israel). Cells were grown in a humidified incubator at 37°C with 5% CO_2 and were fed twice a week with fresh media.

XTT cell proliferation assay

Evaluations of fungal extract effects on cell viability were performed by XTT assay (Biological Industries, Kibbutz Beit Haemek, Israel). Cells (1×104 cells) were seeded in 100µL of medium, using 96-well plates. After 24 hours, the fungal extracts were added in concentration of 25, 50, 100, 250 or 500 µg/ml. Control wells were medium-treated wells. After 24, 48, and 72 h viability levels were determined according to the manufacturer's instructions using an Elisa reader at 450nm and subtracted from the reference absorbance at 620nm (Power Wave Microplate Spectrophotometer, BioTEK, USA). Experiments with at least three replicates were repeated 2-5 times independently. Data were presented as an average proliferation percentage of the respective control.

Lactate dehydrogenase (LDH) leakage assay

LDH is a cytoplasmic enzyme that catalyzes the oxidation of L-lactate to pyruvate with NAD^+ as a hydrogen acceptor, the final step in the metabolic chain of anaerobic glycolysis. LDH leakage into the medium was measured in aliquots of the extracellular fluid of each sample, as described previously [20] using LDH Cytotoxicity Detection Kit (Roche, Mannheim, Germany).

Cell cycle analyses

To analyze the cell cycle, 1×106 cells were treated with selected doses of the extracts for a treatment period that was determined according to XTT results. At the end of that period cells were trypsinized and collected with the growth medium, centrifuged, washed with Phosphate Buffer Solution (PBS), and fixed with 70% ethanol for one hour. This was then followed by incubation with 0.1% NP-40 for 5 minutes in 4°C and then incubated on ice with 100µg/ml RNase for 30 minutes. Finally, 50µg/ml PI was added for 20 minutes. Cell cycle phase distributions were determined by Fluorescence Activated Cell Sorter (FACS) flow cytometry (Becton Dickinson, USA); 10,000 cells were counted for each control and treatment group.

Annexin V-FITC Staining

Cell apoptosis was measured using Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions (MBL, Woburn, MA, USA). HCT116, T47D and PC3 cells were seeded in 6 well plates. HCT116 cells were treated with 0, 100, or 250 µg/ml *C. militaris* extract. PC3 cells were treated with *T. versicolor* or *H. erinaceus* extract and T47D cells were treated with *T. versicolor* or *C. militaris* extracts at the same concentrations as above for 24 hours. At the end of that time, the cells were trypsinized and collected to the tube to which the growth medium was added. Then, cells were centrifuged, washed with PBS and 2x105 cells/ml were counted and collected for the analysis. Cells were re-suspended in 85µl of 1X

binding buffer, 10µl of Annexin V-FITC and 5µl of propidium iodide were added. Cells were incubated for 15 minutes at room temperature in the dark. Cell apoptosis was determined using a BD Facscalibor flow cytometer.

Statistical analysis

Results are displayed as means ± SEM and expressed as percentages of control (XTT, LDH). ONE WAY ANOVA was used for the evaluation of differences between treatment groups and control groups. $P<0.05$ was considered significant, and SPSS software was used for the calculation of differences. All experiments were performed at least three times with at least three replicates (unless indicated otherwise).

Results and Discussion

Cytotoxic activity of fruiting bodies and mycelial biomass

The detection of cell viability is crucial in many biological fields, especially in toxicology, and pharmacology for the assessment of toxic effects elicited by chemicals, drugs or contaminated environmental samples, respectively. For this reason, one of the mostly used XTT cell proliferation assay, which is a quantitative colorimetric method, was used to evaluate cytotoxicity of tested mushroom extracts on cancer cell lines. *Cordyceps militaris* is entomopathogenic fungi widely used in traditional medicine. The literature data confirms that mycelium and fruiting body extracts of *Cordyceps* exhibit direct cytotoxic activity against several kinds of cancer cells, including lung carcinoma, B16 melanoma, lymphocytic, prostate (PC3), breast (MCF7), hepatocellular (HepG2, Hep3B) and colorectal (HT-29 and HCT116) cells [21,22]. Although *Cordyceps* had a cytotoxic effect on cancer cells, it did not show any cytotoxicity against normal cells [23-25]. The majority of these studies have been mainly carried out with isolated compounds (namely cordycepin) [6,26].

C. militaris MB extract effect on the viability of HPAF, HCT116, PC3, and T47D cells by the XTT assay was also evaluated. It is worth noting that among all extracts examined, those from *C. militaris* showed the most profound effect on cell's viability. Treatment of HCT116 and T47D cell lines, led to the most promising effect following 24h, 48h, and 72h treatment by ethanol and ethyl acetate extracts (Figure 1). All extracts treatments using high concentrations (250-500 µg/ml) inhibited PC3 cell viability; a decrease of 60-70% after 24 hours and 90-95% after 48h and 72h of incubation. All extracts at the same dose exhibited 70-80% decrease in HPAF cell viability after 48 and 72 hours. Moreover, inhibition of the cancer cells' viability was found to be dose-dependent.

Anticancer activities of various extracts of the medicinal mushroom *Ganoderma* have been widely demonstrated and are mainly associated with the presence of different bioactive polysaccharides and triterpenoids. In particular, extracts of *Ganoderma* species containing total terpenoids and a purified methanol extract containing mainly acidic terpenoids demonstrated in vitro and in vivo anti-tumor effects [27,28]. In our study, treatment of all cell lines with 250-500 µg/mL of chloroform and ethyl acetate extracts from *Ganodermatsugae* var. *jannieae* FB inhibited cell viability after 48h and 72h treatment. The most profound decrease in cell viability (85-95 %) was shown on HCT116 and PC3 cells. For cell line PC3 the ethanol extract was also promising, inhibiting cell viability by 80 to 92% after 24h, 48h, and 72h. In regard to HPAF-II cell line, ethyl acetate extract demonstrated 75% inhibition of cell viability after the 24h treatment. Unlike *G. tsugae* var. *jannieae* FB extracts, those from MB showed very weak effects for all tested cell lines. Only ethyl acetate extracts demonstrated relatively weak inhibition of T47D cells viability (50%) at the highest concentration (500µg/ml) after the 48h treatment.

C. comatus is considered to have an immense potential as a source of valuable medicinal compounds. It was shown that *C. comatus* ethyl acetate extract inhibited the activity of IKK complex at about 90% when compared to the control of the untreated sample. In addition, *C. comatus* ethyl acetate extract was capable of inhibiting NF-kappaB function and possibly acted as an anti-tumor agent [29]. In the present study, chloroform and ethyl acetate extracts appeared as the most promising for decreasing of the cancer cell viability. Both extracts (250 and 500 µg/ml) were already active after 24h but the highest inhibition (60-90%) of cell viability was observed after 3-days incubation. No effect was revealed at low concentrations of all tested extracts.

Recent studies testify that bioactive compounds from *G. frondosa* exhibit immunomodulatory and anticancer effects in preclinical studies [30,31]. The authors suggest that *G. frondosa* decreases toxicity of some chemotherapeutic agents in cancer treatment. It was showed that polysaccharide from *G. frondosa* significantly inhibited the growth of Heps tumor *in vivo* [30]. In our study, mainly chloroform and ethyl acetate extracts from *G. frondosa* FB inhibited the cell viability at a dose of 250 and 500 µg/ml after 24h, 48h, and 72h treatment. PC3 cells appeared to be the most sensitive as compared with HPAF-II, HCT116, and T47D cells. The ethanol extracts also reduced PC3 cells viability at a dose of 500µg/ml after 24h, 48h, and 72h treatment but no effect was revealed by the water extracts.

It is well known that *H.erinaceus* mycelium (erinacines) or fruit bodies (hericenones) are the source of many bioactive compounds with valuable biological properties including anticancer effect [32]. Studies have shown that *H. erinaceus* extracts strongly suppress the growth of various tumors *in vitro* and *in vivo* [33-35]. In the present study, only chloroform and ethyl acetate extracts of *H. erinaceus* FB demonstrated a biological activity and inhibited the viability of cancer cells in a dose- and time-dependent manner. Among the tested cell lines, the strongest effect was shown on HCT116 and PC3 cancer cells at the concentrations of 250 and 500 µg/ml of ethyl acetate and chloroform extracts after 24h, 48h, and 72h treatment. In addition, the same extracts inhibited cell viability of HPAF-II and T47D cancer cells. By contrast, water and ethanol extracts did not show any effect.

Tremella species are used as a food and as folk medicines and they have been intensively investigated for medicinal effects *in vivo* and *in vitro* model systems [36]. Testing of *T fuciformis* FB showed that chloroform and ethyl acetate extracts inhibited HCT116 and PC3 cancer cells viability in a dose and time-dependent manner. Only the highest concentration of both extracts had a notable effect of reducing HPAF-II cell viability. It is noteworthy that ethanol extracts at concentrations of 250 and 500 µg/ml also appeared to be good inhibitor of PC3 cells viability after 24h, 48h and 72h. Unlike organic solvents, the water extract had no effect on the HCT116, HPAF-II, and T47D cancer cells.

Numerous *in vitro* and *in vivo* studies have shown anti-tumor effects of extracts derived from the medicinal mushroom *T. versicolor* [14]. It was demonstrated that treatment by PSP from *T. versicolor* appears to be associated with slower deterioration in patients with advanced non-small cell lung cancer (NSCLC) [38]. Effect of *T. versicolor* MB extracts on human cancer cell lines' (HPAF-II, HCT116, PC3, and T47D) viability is presented in Figure 2. Treatment

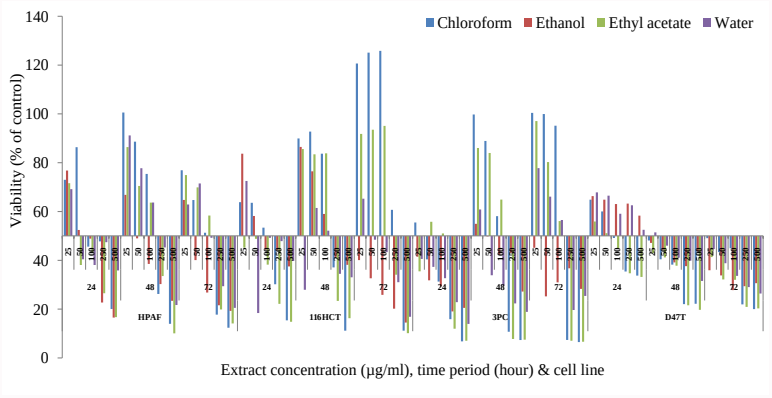

Figure 1: The effect of different *C. militaris* MB extracts on the viability of HPAF, HCT116, PC3, and T47D cells by XTT assay.

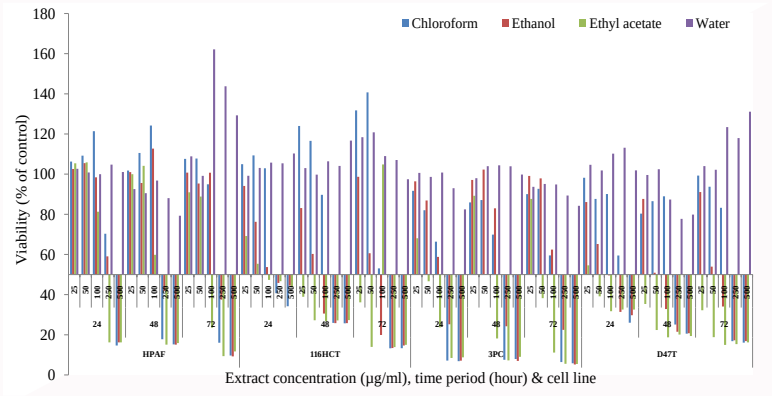

Figure 2: The effect of different *T. versicolor* MB extracts on the viability of HPAF, HCT116, PC3, and T47D cells by XTT assay.

with high concentrations (250-500 µg/ml) of chloroform, ethanol, and ethyl acetate extracts caused a decrease in cell viability of all investigated cell lines by approximately 50-95% in a time-dependent manner. Low concentrations of extracts did not affect the viability of all tested cell lines. Best results were detected in HPAF-II and PC3 cell lines at the extract concentrations of 250-500 µg/ml after 24h, 48h and 72h treatment. It is important to note that after 24h of treatment the effect of ethanol, ethyl acetate, and chloroform extracts achieved 50-70% and it significantly increased after 48h and 72h of treatment. In contrast, no effect was detected by the water extracts.

The effect of selected extracts on the cancer cells viability, ldh leakage, cell cycle and apoptosis

Of all extracts examined for their effect on cell viability, the following extracts showed the most promising results and were chosen for further evaluation: *C. militaris* FB ethyl acetate extract, *H. erinaceus* FB chloroform extract, and *T. versicolor* MB ethyl acetate extract. The strongest effect was observed following treatment with 500µg/ml of the *C. militaris* FB extract for the HPAF-II, HCT116, and PC3 cell lines, which induced more than 80% inhibition of cell growth (Figure 3). The PC3 cells also showed a positive response to the treatment with *T. versicolor* MB and *H. erinaceus* FB extracts. Both induced more than 80% decrease in cell viability following treatment. A dose of 250µg/ml also induced more than 50% inhibition in cell growth for all cell lines treated with *C. militaris* FB extract. *H. erinaceus* FB chloroform extract and *T. versicolor* MB ethyl acetate extract also induced more than an 80% decrease in PC3 cells viability. It is worth noting that the *T. versicolor* MB ethyl acetate extract inhibited cell growth after treatment with a 100 µg/ml dose by more than 65%.

As the effect on cell viability can be the result of apoptotic cell death or necrosis, the LDH assay was performed for all extracts examined by the XTT assay in order to exclude a possible necrotic effect of the extracts on the cells. The results represented in Figure 4 show no cytotoxic effect of the selected extracts on cell viability in comparison to the non-treated controls.

Subsequently, based on the results obtained by the XTT and the LDH assays, in order to establish the reason for the decrease in cell's viability, the most effective extracts were examined for their effect on cell cycle progression. Accumulation of cells in the Sub-G1 phase of the cell cycle served as a marker for the occurrence of the apoptosis process, following the treatment with the extracts.

Figure 5 demonstrates the effect of *T. versicolor* MB and *C. militaris* FB ethyl acetate extracts on the Sub-G1 phase of HCT-116, PC3, and T47D cells following treatment by a dose of 100 and 250 µg/ml for 24h. As seen, both extracts induced more than 70% accumulation of cells in the Sub-G1 phase following treatment with a concentration of 250µg/ml. *H. erinaceus* FB chloroform extract also induced 40% accumulation of cells in the Sub-G1 phase following treatment with a concentration of 250µg/ml. A lower dose (100µg/ml) of the *T. versicolor* MB ethyl acetate extract caused Sub-G1 cell accumulation in more than 50% of PC3 and T47D treated cells. *H. erinaceus* FB chloroform extract also induced 40% accumulation of cells in the Sub-G1 phase following treatment with a concentration of 100µg/ml.

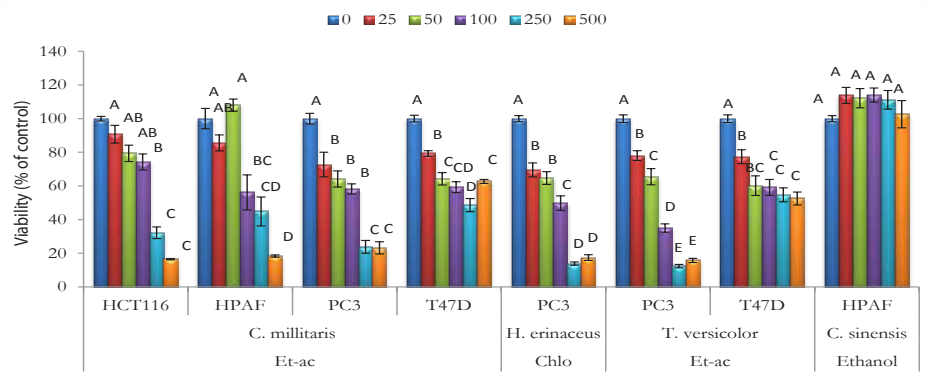

Figure 3: The effect of the selected extracts on the viability of different cancer cell lines by XTT assay. Statistical significance was determined by one way ANOVA, P<0.001.

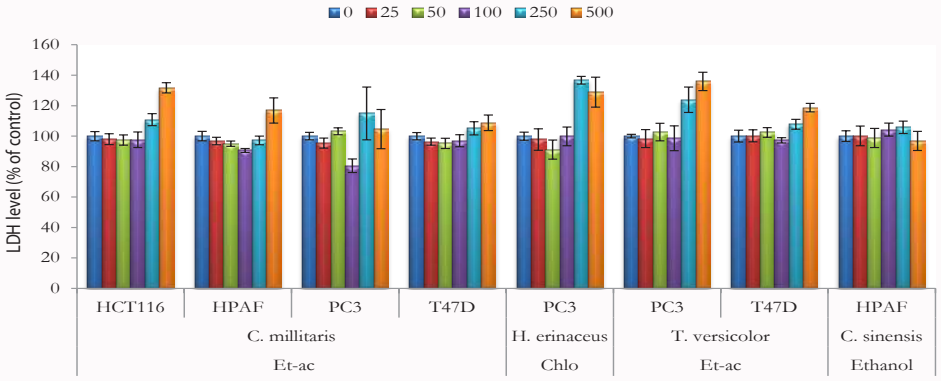

Figure 4: The effect of selected extracts on the cancer cells LDH leakage. Statistical significance was determined by one way ANOVA, P<0.001.

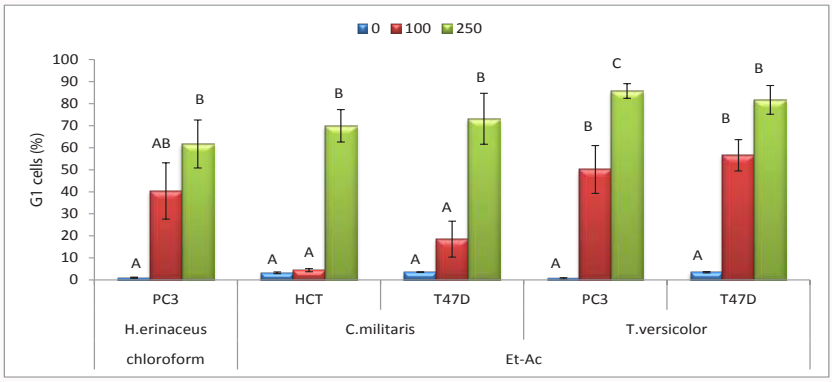

Figure 5: The effect of the selected extracts on Sub-G1 accumulation in cancer cell evaluated by FACS Flow cytometry analysis. Statistical significance was determined by one way ANOVA, P<0.001.

It is known that apoptosis is a programmed physiological cell death that plays a critical role in the healthy survival/death balance in cells, and the induction of apoptosis has been a promising approach by which most anticancer agents exert their effects [39]. The ability to induce tumor cell apoptosis is an important property of a candidate anticancer compound. In the present study, selected cancer cells were treated for 24h with 0, 100 or 250 µg/ml of three mushroom FB/MB chloroform or ethyl acetate extracts and cell apoptosis was measured using Annexin V-FITC Apoptosis Detection Kit. Annexin V-FITC double staining showed the same trend revealed during cell cycle analysis. *H. erinaceus* FB chloroform extract at concentrations of 100 and 250 µg/ml induced apoptosis in 77% and 95% treated PC3 cells (Figure 6). T47D cells treated with *T. versicolor* MB ethyl acetate extract showed almost 60% apoptotic cells following treatment with a dose of 100µg/ml while the HCT116 and T47D cells treated with *C. militaris* FB ethyl acetate extract at a concentration of 250µg/ml showed 77% and 68% apoptotic cells, respectively.

Conclusions

For the screening of anti-cancer activity of selected medicinal mushrooms, ethanol, ethyl acetate, chloroform, and water extracts from fruiting bodies and mycelia were evaluated. The experiments were performed using four types of human cancer cell lines: HPAF-II (pancreatic cancer), HCT116 (colon cancer), PC3 (prostate cancer),

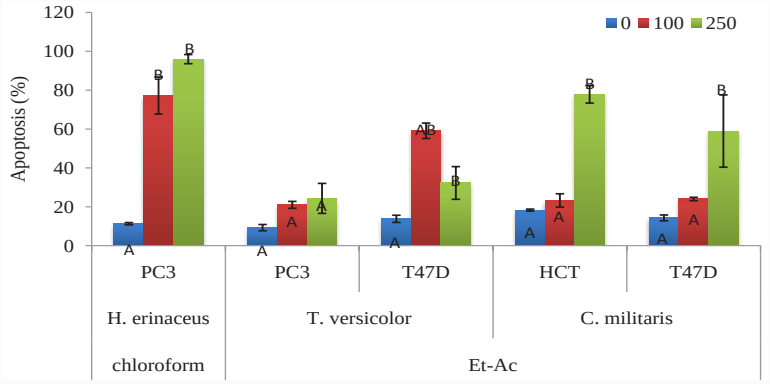

Figure 6: Elucidation of the effect of the selected extracts on apoptosis induction in different cancer cell lines using Annexin V-FITC and propidium iodide staining. Statistical significance was determined by one way ANOVA, P<0.001.

and T47D (breast cancer). Assessment of cell viability revealed that among the extracts examined, ethyl acetate and chloroform extracts are the most active showing the most profound decrease in cell viability and exhibiting anti-proliferative activity against all cell lines used for this research. The most encouraging results were revealed during treatment of cell lines PC3 and HPAF-II by all extracts with exclusion of the water extract. *C. militaris* FB, *H. erinaceus* FB, and *T. versicolor* MB appeared as the best inhibitors of cell viability. The effect observed following treatments for 24, 48, and 72 h by ethanol and ethyl acetate extracts (for cell lines HCT116 and T47D) was most promising. High concentrations (250-500 µg/ml) of all extracts inhibited PC3 cell viability by 60-70% and by 90-95% after 24 h and 48-72 h treatment. HPAF-II cell viability decreased by 70-80% during 48 and 72 h treatments at the same dose of the extracts. Results showed that extracts inhibited cancer cells viability in a dose-dependent manner. The higher concentrations (250-500 µg/ml) of *T. versicolor* MB chloroform, ethanol, and ethyl acetate extracts decreased all investigated cell's viability by approximately 40-95% in a time-dependent manner. Low concentrations of extracts did not affect viability of all tested cell lines. Cell cycle analysis and annexin-V/Pi double staining suggest that apoptosis is the type of death responsible for the decrease in cell's viability and the inhibition of growth. Thus, this study contributes to understanding the biological activity of medicinal mushrooms and provides evidence that the *Cordycepsmilitaris* FB, *H. erinaceus* FB, and *Trametes versicolor* MB extracts have an inhibitory effect on human cancer cells and suggests that they could be potential natural agents for cancer treatment. Further studies are needed to establish the precise mechanisms of action of individual fractions and compounds isolated from promising mushroom extracts.

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