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Original Article

# Bioactive Compounds in *Inocutis levis* and their Cytotoxic Effects on MCF-7 and HT-29 Cancer Cells

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## ABSTRACT

**Background and Objectives:** Fungi are undeniable sources of all types of secondary metabolites and bioactive compounds. *Inocutis levis* is a polypore fungus belonging to Hymenochaetales of basidiomycetes. This study aimed to identify the compounds in the extract and investigate the cytotoxicity effect of *Inocutis levis* on different cell lines (MCF-7, HT-29 and HFF). **Materials and Methods:** The extract was obtained by rotary evaporator and LC/MS was used to identify the compounds in the extract. The cytotoxic effect of *Inocutis levis* against MCF-7, HT-29 and HFF cells was evaluated using MTT assay, 24 and 48 hr after the addition of the extract. Also, flow cytometry analysis was carried out to study the cell cycle arrest and apoptosis.**Results:** The LC/MS assay data showed that the mushroom extract contains antioxidant compounds. Based on the MTT test, *Inocutis levis* extract did not decrease the viability of non-cancerous HFF cells. Still, it was able to inhibit the growth and increase the death in HT-29 cells at a concentration of 100 µg/mL after 24 and 48 hr ($p<0.05$) also, MCF-7 cells at concentrations of 1 and 10 and 100 µg/mL after 24 and 48 hr ($p<0.05$). Evaluation of the cell cycle 48 hr after adding the extract by flow cytometry, showed that the mushroom extract induced apoptosis in MCF-7 cells. **Conclusion:** *Inocutis levis* contains antioxidant compounds and can inhibit the growth of HT-29 and especially MCF-7 cancer cells via the induction of apoptosis.

**Keywords:** Anticancer, Apoptosis, Cell cycle, HT-29, *Inocutis levis*, LC/MS, MCF-7.

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## INTRODUCTION

The search for new natural antioxidants is an active field of investigation. In recent years, Fungi have become attractive as an origin of valuable medicines with few adverse effects. Mushroom usage in therapeutics has a long history of more than 2000 years. Based on traditional medicines, fungi have been developed into anti-cancer remedies. The bioactive compounds have direct cytotoxic effects on tumor cells and indirectly affect cancer treatment by promoting the immune system.[1] Modern scientific screening methods have discovered the anticancer properties of medicinal fungi. Contrary to other diseases traditionally treated with medicinal mushrooms, there is little experience in the treatment of cancer. This may be one reason for little fungi-based anticancer therapeutics currently available.[1]

In searching for new therapeutic alternatives, scientists have studied several kinds of mushrooms and found varying therapeutic activities such as anti-inflammatory, immunosuppression, anticarcinogenic and antibiotic effects. Fungi accumulate various secondary metabolites, such as phenolic compounds, polyketides, terpenes and steroids. Some common edible mushrooms, widely consumed in Asian culture, have been found to possess antioxidant activity, which is well correlated with their total phenolic content. Now, mushrooms are considered to be a good source of protein and phenolic antioxidants, such as variegatic acid and ubiquinone.[2]

Since the effectiveness of a medicinal plant is based on the entire blend of metabolites (synergism) rather than the presence of a single component, developing methods for analyzing the complete extract is a challenge.[3]

There are various techniques for determining the composition of a plant and one of these techniques is High-Performance Liquid Chromatography (HPLC). The combination of mass spectrometry with chromatographic techniques is a powerful tool

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for analyzing organic compounds across various applications. Gas Chromatography-Mass Spectrometry (GC-MS) has been the most popular technique so far, but it is limited to analyzing compounds with sufficient volatility and thermal stability. On the other hand, Liquid Chromatography-Mass Spectrometry (LC-MS) has gained popularity due to advances in MS interface technology. When dealing with natural complex matrices containing compounds with potential biological activity, High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) is a rapid method for obtaining online structural information and molecular weight before isolating and conducting biological assays. In recent years, numerous studies on organic compounds extracted from plant materials have been carried out using HPLC-MS.[4]

Some preclinical and clinical studies suggest the effects of mushrooms on cognition, oral health, weight management and cancer risk. Preliminary evidence indicates that mushrooms may support healthy immune and inflammatory responses through interaction with the gut microbiota, enhancing adaptive immunity and improving immune cell functionality.[5]

*Inocutis levis*, a polypore basidiomycete with sessile and pileate basidiomata and a distinctive granular core at the point of attachment to the plant tissues is a member of the Hymenochaetaceae family. Hymenochaetaceae is a large family of macro basidiomycetous fungi with widespread distribution. Several members of this family, principally those belonging to the genera Phellinus Quél and *Inonotus* P. Karst., have been investigated for their noteworthy medicinal characteristics.[6] *Inocutis levis* (P. Karst.) Y.C. Dai (= *Inonotus levis* P. Karst., *Inonotus pseudohispidus* Kravtzev) with poroid (polypore) fruiting bodies, is predominantly distributed in Asia, grows on living angiosperm trees and its spores are yellowish, ellipsoid and thick-walled.[7,8] This fungus is more or less distributed in Iran, generally found in urban areas on the trunk of plants and elm trees.[9]

Recently, some medicinal properties of *Inocutis levis* comprising anti-diabetic and anti-hypercholesterolemia effects have been reported.[10] Identifying and utilizing new pharmaceutical resources, exploiting natural resources and the potential of the country's genetic reserves and producing new drugs will be an effective step in upgrading and improving current health systems. Since many fungi are naturally occurring in Iran and many species are endemic to the forests of Iran, they can be introduced into the pharmaceutical industry by identifying each of them and extracting their biological constituents. Therefore, in this study, we aimed to identify the constituent compounds of *Inocutis levis* and to assess the antitumor and proapoptotic activity of its extract on MCF-7 and HT29 cells as two cancer cell lines as well as HFF cells as normal cells.

## MATERIALS AND METHODS

### Chemicals

RPMI, Dimethyl Sulfoxide (DMSO) and MTT kit were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH. Sternheim. Germany). Fetal Bovine Serum (FBS), PBS, Trypsin-EDTA and DMEM were purchased from BIO-IDEA (Tehran, Iran). Penicillin/Streptomycin and L-Glutamine were obtained from Gibco (Dublin, A96 K7H7, Ireland). All chemicals and solvents were of analytical grade.

### Cell culture

MCF-7 (Breast Cancer cell line), HT-29 (Colon Cancer cell line) and normal Human Fibroblast (HFF) cells were obtained from Pasteur Institute (Tehran, Iran). The cells were cultured in RPMI or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin and incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C.

### Mushrooms

Fresh specimens of *Inocutis levis* fungus were sampled from plantain and elm trees in the west of Tehran province, Islamshahr region in 2019 and identified by molecular and morphological methods. Specimens were standardized in the Iranian Herbarium Cryptogam's collection with the ICH International Index and their accession number is ICH497F.

### Preparation of Extracts

The fruiting bodies of the mushrooms were dried and stored in an airtight container. The dried mushroom was ground to a fine powder. Then 220 mL of distilled water was added to 36 g of fine powder and the mixture was kept at room temperature for 3 days in a covered container. After three days, the extract was filtered on a Buchner funnel and the solution was concentrated in a vacuum rotary evaporator, the brown gum extract obtained from this solution was dissolved in a little methanol and filtered with filter paper, then the precipitate on the filter paper was placed in a vacuum oven with a temperature of 35-40°C for a day to dry. Finally, the dried powder was prepared from the extract, which was poured into a vial and stored in the refrigerator.

### LC-MS analysis

High-Performance Liquid Chromatography (HPLC) was used to determine the compounds in the extract. The HPLC system (Agilent Chromatography System, Santa Clara, CA, USA) consisted of a G1311A pump and Rheodyne 7725 injection valve equipped with 20, 50 and 100 µL Loops. The system was connected to a G1315D Photodiode Array (PDA) detector controlled by Agilent ChemStation Software. C18 Column (250×4.6 mm, 5 µM) was used for separation. The sample was prepared for injection after passing through a 0.45-micron syringe

filter. Methanol solvents and aqueous TFA (Trifluoroacetic Acid) solution (0.05% v/v) were used as the mobile phase. The column was maintained at 30°C and the elution program according to Table 1 was gradient. Chromatograms were obtained by scanning the sample from 200 to 400 nm and the peaks were monitored at 254 and 305 nm.

Mass spectra were obtained using a mass spectrometer with an ion trap separator manufactured by Thermofisher Scientific (Waltham, MA, United States), FinniganTM LCQTM DECA model. Conditions of the device were positive ESI, spray voltage 5 kV, capillary voltage 46 V, lens voltage - 60 V, capillary temperature 300°C, spray gas 80 mL/min and auxiliary gas 20 mL/min. Xcalibor 2SR.2 software (copyright Thermo Electron Corporation 1998-2006) was used to obtain and analyze the spectra.

## Cytotoxicity assays

The cytotoxicity of *Inocutis levis* extract was tested toward Human Foreskin Fibroblasts (HFF), colon cancer (HT-29) and breast cancer (MCF-7) cell lines, using MTT assay. To prepare cells for treatment, exponentially growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96-well plates at $5\times10^4$ cells/well in RPMI-1640 and DMEM supplemented medium for HT-29, MCF-7 and HFF cell lines, respectively. The cells were incubated for 24 hr to allow adhering before exposure to *Inocutis levis* and after that, various concentrations (1, 10 and 100 µg/mL) of extract were added to wells. After adding the extract, the plates were placed in an incubator and cell viability was evaluated 24 and 48 hr after incubation. Control cells were exposed to the culture medium alone. For the MTT assay, after removal of the culture medium, cells were washed with PBS and incubated with 20 µL/well MTT solution (5 mg/mL in PBS) for 4 hr at 37°C. After the incubation time, the top solution was removed and 150 µL/well of DMSO solution was added to all wells. Cells were shaken at room temperature for 10 min to dissolve formazan crystals. The absorption was measured at 570 nm in an ELISA plate reader. All experiments were conducted in triplicate wells and repeated twice.

## Cell Cycle Analysis by flow cytometry

Flow cytometry was used to measure cellular DNA content and to evaluate the cell cycle of treated cells. HFF, HT-29 and MCF-7 cells were seeded (500000 cells/well) in 6-well plates and after incubation for 24hr, were treated with 100 µg/mL of fungal extract for 48 hr. After 48 hr, cells were collected by trypsinization, washed with cold Phosphate-Buffered Saline (PBS) and counted. Control and treated samples were mixed for 30 min with 20 mg Propodium Iodide and 20 mg RNase (dissolved in 1 mL PBS) and then analyzed by flow cytometry (Becton Dickinson FACS calibometer). The percentages of cells in different cell cycle phases were calculated using the Phoenix statistical software package, advanced DNA cell-cycle software (Phoenix Flow System, San Diego, CA).

## Statistical analysis

The data, presented as mean±SE, were analyzed using one-way Analysis of Variance (ANOVA). If there were significant differences, the groups were compared with the control group using Dunnett *post hoc* tests. $p<0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad InStat software.

# RESULTS

## LC-MASS Results

The Chromatogram of *Inocutis levis* extract is shown in Figure 1. According to the chromatogram and its eluting system, as well as the sample's good solubility in water, the compounds in the sample had high polarity and the least retention time on the C18 column. To identify the compounds in the sample, the mass spectrometer technique was used and the output of the HPLC device was transferred to the ESI-MS mass spectrometer.

According to the mass spectra and their polarity based on the retention time of the compounds in the chromatogram, it appeared that the compounds in the sample were probably from the group of compounds of phenolic acids and histidine derivatives (Table 2).

**Table 1: The HPLC gradient elution program.**

| Time (minutes) | Washing speed (mL/min) | Water solvent % (0/05% TFA) | Methanol Solvent % (0/05% TFA) |
|---|---|---|---|
| 0 | 0.5 | 95 | 5 |
| 5 | 0.5 | 95 | 5 |
| 15 | 0.5 | 75 | 25 |
| 40 | 0.5 | 0 | 100 |
| 50 | 0.5 | 0 | 100 |
| 50.1 | 0.5 | 95 | 5 |
| 55 | 0.5 | 95 | 5 |

The mass shown in Figure 2-a, (m/z =[M + H] $^{+}$ = 155.47), related to the HPLC peak with a retention time of t = 7.56 min, could be attributed to proto-Catechuic acid. Given that the mass spectrum is in positive mode, its mass has been recorded along with the mass of hydrogen.

The mass shown in Figure 2-b, (m/z =[M+ H] $^{+}$ = 198.8), related to the HPLC peak with a retention time of t=7.94 min, could be attributed to phenolic acids such as 2,5 dihydroxyterephthalic acid or syringic acid. Since the mass spectrum is recorded in a positive mode, its mass has appeared along with the mass of hydrogen.

The mass shown in Figure 2-c, (m/z =[M+H]$^{+}$=180.67), which is related to the HPLC peak with a retention time of t = 11.23 min, could be attributed to caffeic acid phenolic acid, given that the mass spectrum is in positive mode its mass is recorded along with the mass of hydrogen.

The mass appeared in Figure 2-d (m/z =[M+H] $^{+}$=365.67), which corresponds to the HPLC peak with retention time t =13.58 min, could be related to chlorogenic acid which is a phenolic acid composition, considering that the mass spectrum is recorded in the positive mode, its mass has appeared along with the mass of hydrogen.

The mass shown in Figure 2-e, (m/z=[M+H] $^{+}$ = 165.53), related to the HPLC peak with a retention time of t = 13.72 min, could be attributed to paracoumaric phenolic acid. Given that the mass spectrum is in positive mode, its mass has been recorded along with the mass of hydrogen (*p*-Coumaric acid [M+H]$^{+}$=165.53).

The mass appeared in Figure 2-f (m/z=[M+H]$^{+}$=179.6), which corresponds to the HPLC peak with retention time t = 35.0 min, could be related to phenolic acid 3,4-dihydroxybenzalactone, considering that the mass spectrum is recorded in the positive mode, its mass has appeared along with the mass of hydrogen.

The mass in Figure 2-g, (m/z=[M+ H]$^{+}$=463.13), which is related to the HPLC peak with retention time t=35.92 min, could be attributed to a histidine derivative called inosanine A, considering that the mass spectrum is recorded in the positive mode, its mass has appeared along with the mass of hydrogen.

## Effect of *Inocutis levis* extract on HFF cells growth

The results of the MTT assay didn't show any significant differences between control and treatment groups (1,10 and 100 µg/mL of the extract of *Inocutis levis*) after 24 and 48 hr, in other words, *Inocutis levis* didn't inhibit the growth of HFF normal cells (Figure 3).

## Effect of *Inocutis levis* extract on HT-29 cell growth

The data of the MTT assay indicated that the treatment with *Inocutis levis* extract at the dose of 100 µg/mL significantly decreased HT 29 cell viability after 24 and 48 hr compared to the control group. It is noteworthy that there weren't any significant differences between treatment groups after 24 and 48 hr, thus, the effect of *Inocutis levis* extract was not in a time-dependent manner (Figure 4).

## Effect of the extract of *Inocutis levis* on MCF-7 cell growth

As shown in Figure 5, the doses of 1, 10 and 100 µg/mL of *Inocutis levis* extract decreased MCF-7 cell viability significantly after 24

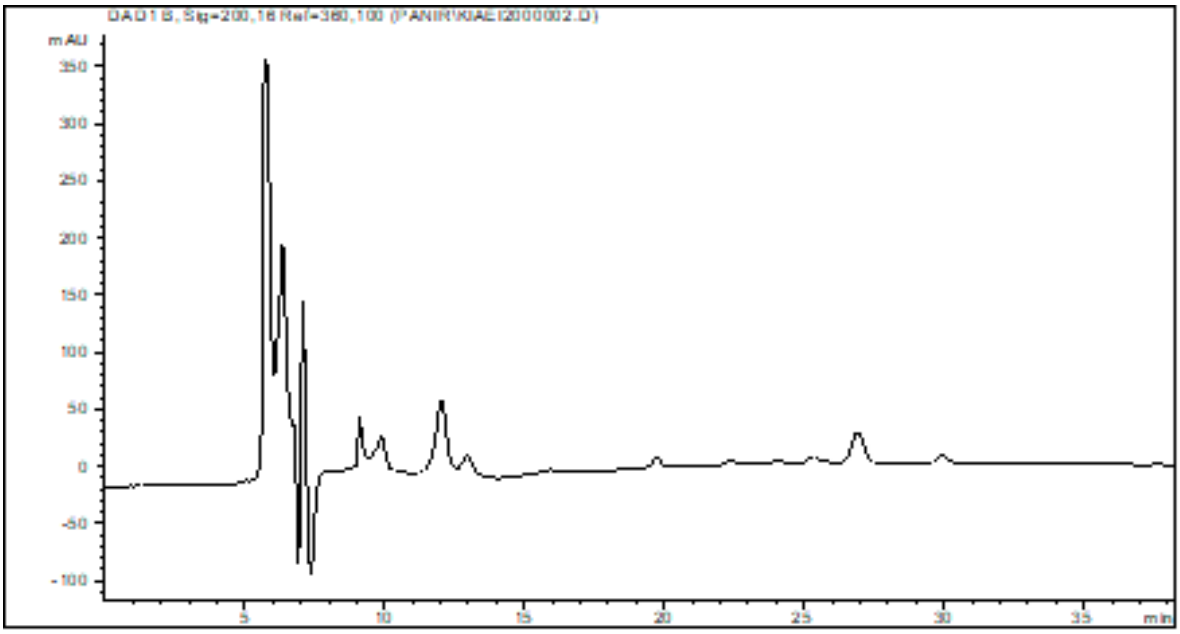



**Figure 1:** HPLC chromatogram related to injecting 20 µL of *Inocutis levis* extract to column C18 and washing it with water and methanol according to the eluting table. HPLC type: Agilent (USA), chromatography C18 column; mobile phase: Methanol solvents and aqueous TFA solution (0.05% v/v); eluting conditions 0-55 min; detected at 254 and 305 nm).

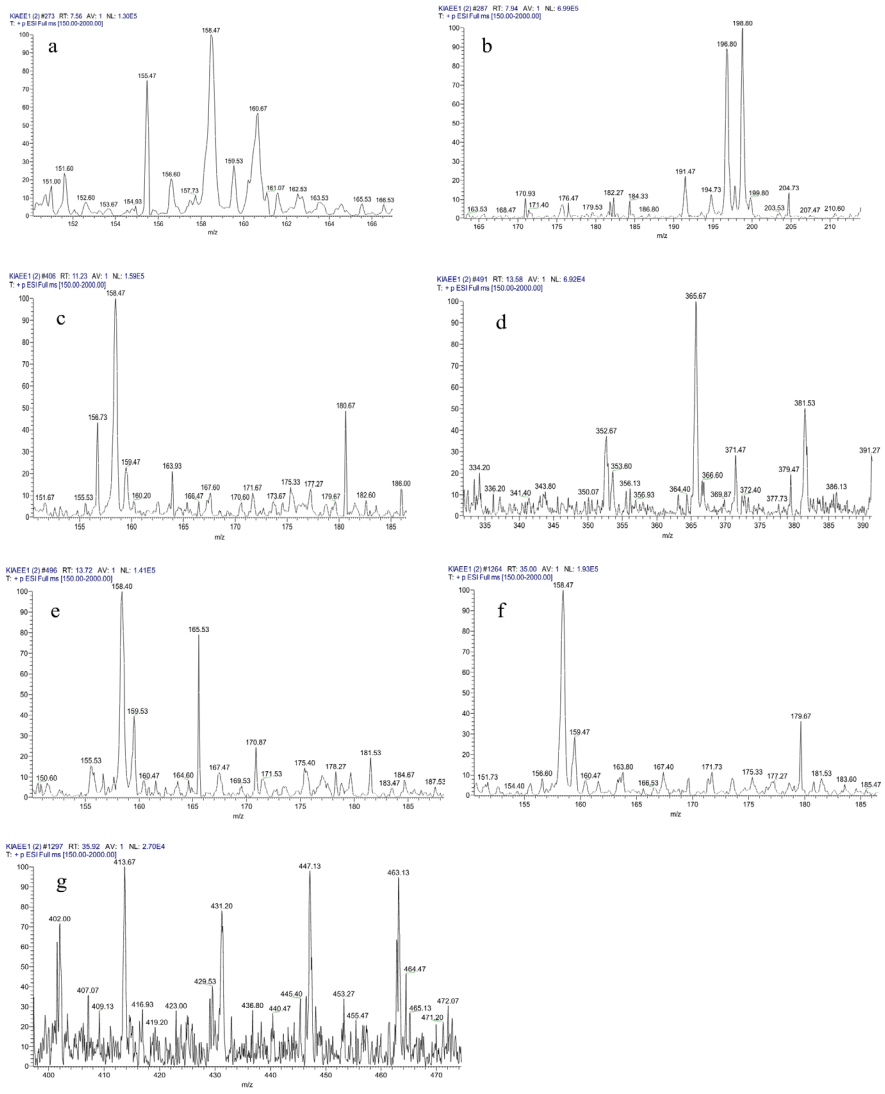



**Figure 2:** Mass spectrums of HPLC peaks at retention time of a) 7.56 min, b) 7.94 min, c) 11.23 min, d) 13.58 min, e) 13.7 min, f) 35 min and g)35.92 min from Figure 1.

and 48 hr. According to these results, the most inhibitory effect on MCF-7 cells was at the concentration of 100 µg/mL after 24 hr.

## Effect of *Inocutis levis* extract on the cell cycle of HFF, HT-29 and MCF-7 cells

The inhibitory effect of *Inocutis levis* extract (100 µg/mL) after 48 hr on cell cycle progression and the presence of the sub-G1 population (apoptotic cells) in HFF, HT-29 and MCF-7 cells were further determined by flow cytometry analysis. According to the results, the extract couldn't induce apoptosis in treated HFF and HT-29 cells compared to non-treated cells (control group). Thus, the cellular population in the sub-G1 stage that shows cell apoptosis didn't alter significantly in treated compared to non-treated cells (Figures 6-a & b). However, Flow cytometry analysis showed that *Inocutis levis* extract at the dose of 100 µg/mL after 48 hr increased the population of sub-G1 apoptotic MCF-7 cells (Figure 6-c). Taken together, these results indicated that *Inocutis levis* extract caused cell cycle arrest in MCF-7 cells, ultimately leading to apoptosis.

## DISCUSSION

Cancer is an unusual state of cells that undergo uncontrolled propagation and produce aggressive malignancies resulting in millions of deaths all over the world every year.[10]

Various treatment strategies including surgery, immunotherapy, radiotherapy and drug therapy are utilized to treat cancer. Unfortunately, the response to treatment is often poor and may come with undesirable side effects in many cases. Resistance to current anticancer drugs has also been observed. Therefore, additional research is needed to develop more effective and less toxic drugs.[11]

There are many natural compounds with diverse biological effects. The exploitation of natural products or synthetic variants using their novel structures, to discover and develop the final drug entity, is still active. For example, in the area of cancer, over the time frame from 1946 to 1980, of the 75 small molecules, 40, or 53.3%, are unchanged natural products or natural product derivatives.[12] Fungi are nutritionally considered valuable sources and one of the most prominent medical effects of fungi and their metabolites is their antitumor effect.[13,14] One strategy to treat cancer is to search for more effective medicinal compounds with less toxicity and side effects that can lead to cancer cell death. In the present study, the effect of *Inocutis levis* extract on cancer cells and normal cells was investigated. The results of this study showed that the fungus extract did not have a toxic effect on normal HFF cells, whereas it could show its inhibitory effect on HT-29 and MCF-7 cells 24 and 48 hr after treatment. The growth of cells was inhibited within the first 24 hr and the inhibitory effect did not increase with increasing treatment time. In MCF-7 cells, the most inhibitory effect of the extract was seen at a concentration of 100 µg/mL after 24, however, it was also effective in inhibiting cell proliferation after 24 and 48 hr at lower concentrations (1 and 10 µg).

Phenolic compounds extracted from fungi have shown the potential of inducing apoptosis in breast and bladder cancer cells.[15] Some proteoglycans present in fungi could inhibit cell proliferation via induction of G2/M phase arrest and apoptosis in SW480 colon cancer cells.[16] They also have anti-inflammatory and anti-angiogenic activities.[17] In a study, to investigate the effects of *Phellinus linteus* on angiogenesis, Lee *et al*. demonstrated that *Phellinus linteus* methanol extract and its subsequent fractions, except for the aqueous fraction, had antitumor effects through inhibiting the proliferation, migration, tube formation and VEGFR-2 phosphorylation of Human Umbilical Vein Endothelial Cells (HUVECs), so in pathological situations involving stimulated angiogenesis, such as inflammation and tumor development, this extract could be considered a potential treatment.[18] In another study, Yu *et al.* examined the effects of *Agaricus blazei* Murill, (a medicinal mushroom that has been conventionally used as a health food for the prevention of cancer) on the growth of human prostate cancer. Their results showed that the broth of *A. blazei* may directly inhibit prostate tumor growth through an apoptotic pathway, antiproliferative and antiangiogenic mechanisms, therefore *A. blazei* potentially might have been used for the preclusion and treatment of human prostate cancer.[19]

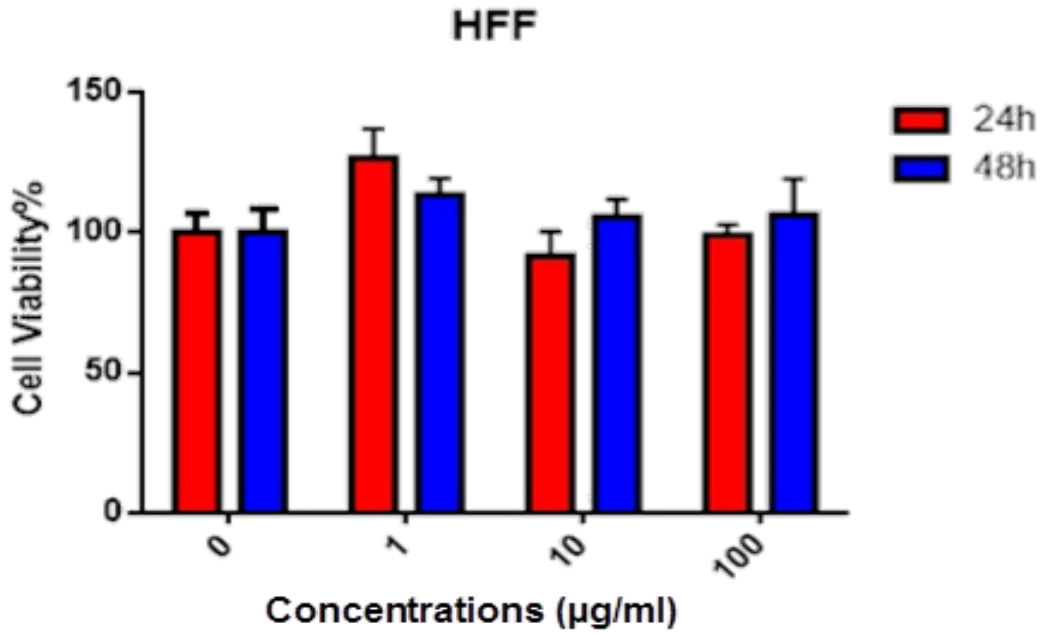
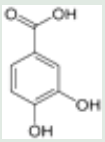
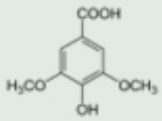
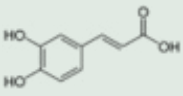
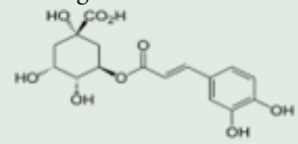
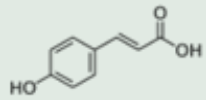
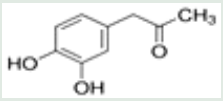
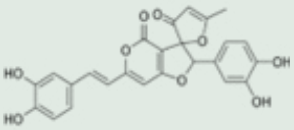



**Figure 3:** The effects of *Inocutis levis* extract treatment on HFF cells After 24 and 48 hr. Cells were treated with 1, 10 and 100 µg/mL of extract and cell viability was evaluated by using the MTT test. The data are presented as three independent tests; Mean±SE, significant differences between treatment groups *vs.* control are reported as *$p$0.05.

**Table 2: List of compounds identified in *Inocutis levis* extract by LC-MS method.**

| Compound | Retention time(min) | m/z positive) $(M+H)^+$ |
|---|---|---|
| **Phenolic compounds** | -- | -- |
| Proto-Catechuic acid  | 7.56 | 155.47 |
| Syringic acid / 2,5-dihydroxyterephetalicAcid  | 7.94 | 198.80 |
| Caffeic acid  | 11.23 | 181.67 |
| Chlorogenic acid  | 13.58 | 365.67 |
| p-Coumaric acid  | 13.72 | 165.53 |
| 3,4-dihydroxybenzalacetone  | 35.00 | 179.67 |
| **Hispidin derivative** | -- | -- |
| Inoscavin A  | 35.92 | 463.13 |

In order to recognize the structures of the main components in the extract of *Inocutis levis*, the sample was analyzed by LC/Mass technique. Numerous peaks were observed in *Inocutis levis* LC/Mass chromatogram, indicating that there are several compounds in the extract of the fungus. According to the mass spectra and the retention time of the compounds in the chromatogram, we concluded that the phenolic acid derivatives including protocatechuic acid, Syringic acid or 2,5-dihydroxyterephetalic acid, Caffeic acid, Chlorogenic acid, p-Coumaric acid, 3,4-dihydroxybenzalacetone and histidine derivatives including Inoscavin A are the most important compounds in *Inocutis levis* fungus. As far as we know, this is the first time that compounds in this mushroom have been identified. Considering that the compounds known in this mushroom mainly have antioxidant effects, the anti-cancer properties of this mushroom can be justified. The antitumor effects of compounds in mushrooms have been investigated in many studies. In a study, Zhang *et al*. isolated a lectin from the mushroom*Russula lepida* and investigated its anti-tumor effect. Their results showed that this proteoglycan had antiproliferative activity toward human breast cancer MCF-7 cells and hepatomaHep G2 cells with an $IC_{50}$ of 0.9 μM and 1.6 μM, respectively. Dailyintraperitoneal injections of lectin (5.0 mg/kg body weight/day for 20 days) caused a 67.6% reduction in the weight of S-180 tumor.[20] In another study, by sequential

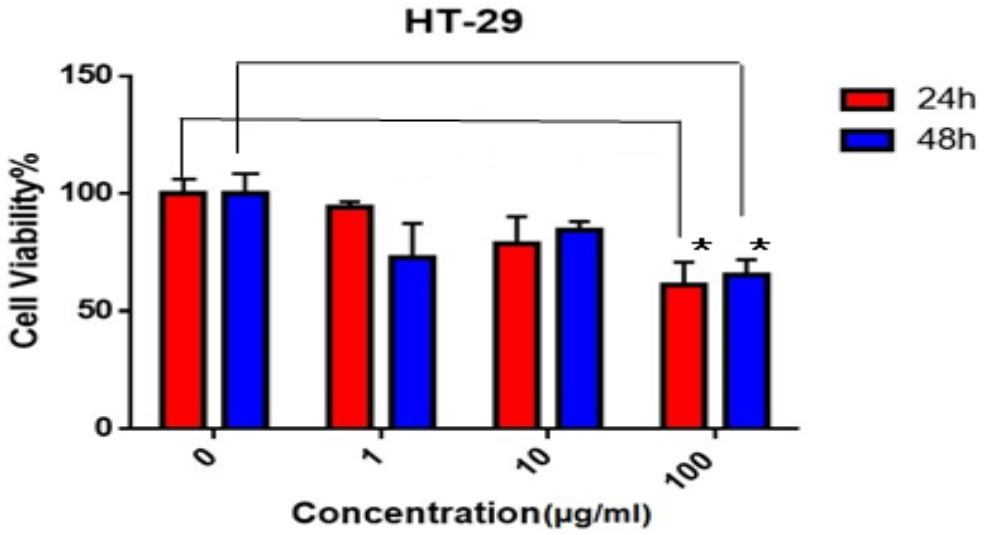



**Figure 4:** The effect of *Inocutis levis* extract at 1,10 and 100 µg/mL doses on HT29 cell proliferation after 24 and 48 hr. The cells were treated with *Inocutis levis* extract then cell viability was evaluated by MTT assay. The data are presented as three independent tests; Mean±SE, significant differences between treatment groups *vs.* control are reported as *$p<0.05$.

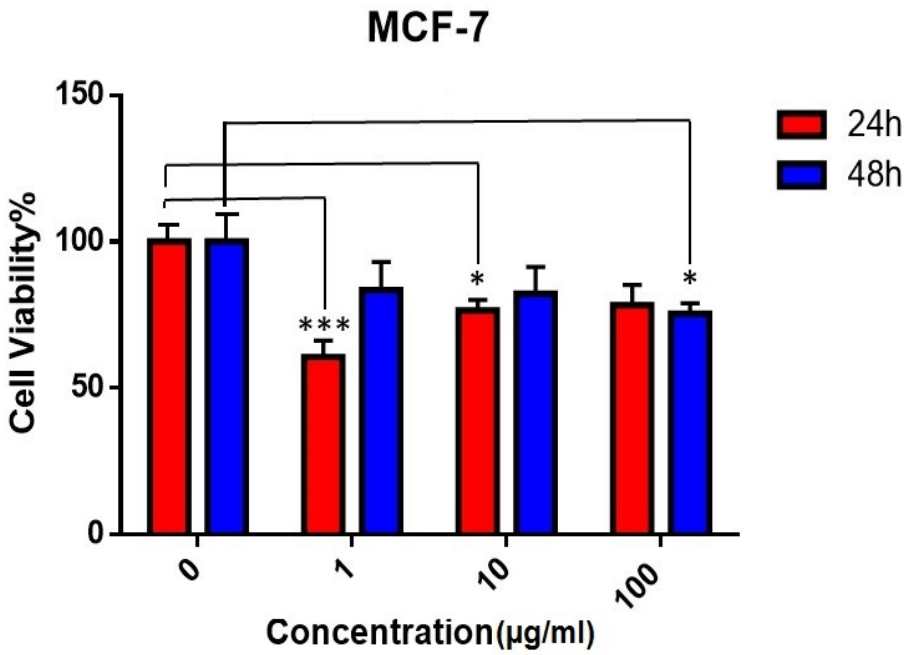



**Figure 5:** Effects of *Inocutis levis* extract on MCF-7 cell growth after 24 and 48 hr. Cells were treated with *Inocutis levis* extract at concentrations of 1, 10 and 100 µg/mL and cell growth was evaluated by MTT assay. The data are presented as three independent tests; Mean±SE, significant differences between treatment groups *vs.* control are reported as * $p<0.05$, ** $p<0.01$.

extraction, polysaccharides of *Agaricus bisporus* were obtained and anti-colon cancer activity of WAAP-1 (a homogeneous polysaccharide) was examined. WAAP-1 was a neutral polysaccharide composed of glucose,mannose andgalactose that could significantly inhibit proliferation of colon cancer cell HT-29 by promoting apoptosis and inhibiting the epithelial mesenchymal transition of HT-29. Their results provided new potential for the development of new functional foods or antitumor drugs.[21]

Cell cycle analysis demonstrated that *Inocutis levis* extract had no significant effect on the cell cycle of HT-29 and HFF cells. However, after 48 hr, the Sub-G1 population was observed in the MCF-7 cells treated with *Inocutis levis* extract, which accounted for about 40% of the total cell population, indicating the occurrence

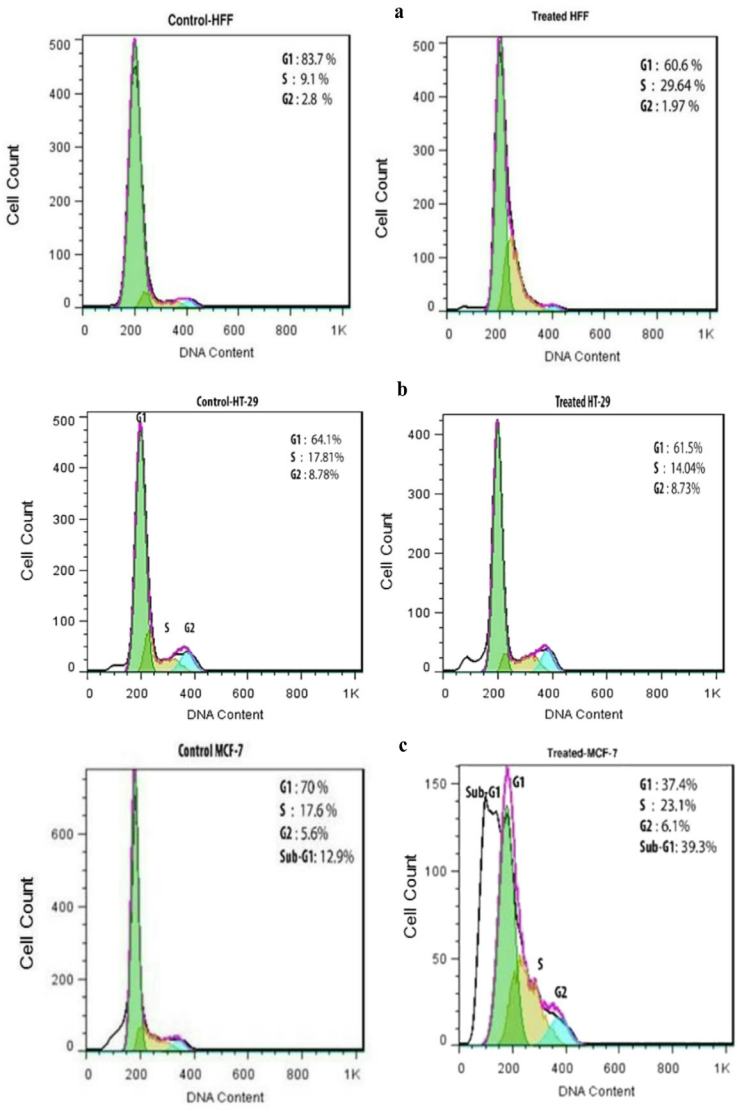



**Figure 6:** The effect of *Inocutis levis* extract on the cell cycle of a) HFF, b) HT-29 and c) MCF-7 cells. The cells were treated with a concentration of 100 µg/mL of the extract for 48 hr and the percentage of cells in each stage of the cell cycle was measured using a flow cytometer in control and treated cells. Histograms show the relative DNA content of nuclei in G1 (green), S (yellow) and G2 (blue) cell cycle phases.

of apoptosis. Thus, the results showed that *Inocutis levis* could induce apoptosis in MCF-7 cells. Some researches indicate the promotion of cell cycle arrest and apoptosis of cancerous cells by mushrooms. Yang *et al.*, showed that non-cytotoxic concentrations (20-80 µg/mL) of *Antrodia camphorata* markedly prevented the invasion/migration of highly metastatic MDA-MB-231 cells through suppression of the MAPK signaling pathway.[22] In another investigation, Yu *et al.* by flow cytometric analysis showed that antroquinonol, a ubiquinone derivative isolated from *A. camphorata*, induced a concentration-dependent inhibition of cell proliferation in Pancreatic Cancer (PANC-1 and AsPC-1) cells via an inhibitory effect on PI3-kinase/Akt/mTOR

pathways. The translational inhibition caused G1 arrest of the cell cycle and an ultimate mitochondria-dependent apoptosis.[23] Also, evaluation of the antiproliferative effects of *Inonotus obliquus* extract on rat melanoma cells (B16-F10) showed cell cycle arrest at G0/G1 stage and induction of apoptosis. These effects were associated with down-regulation of pRb, p53 and p27 expression levels and showed that *I. obliquus* extract resulted in cell cycle arrest at the G0/G1 stage by decreasing Cyclin E/D1 and Cdk 2/4 expression levels.[24]

## CONCLUSION

Taken together, our findings indicate that the extract of the mushroom *Inocutis levis* has an inhibitory effect on the growth of HT-29 and MCF-7 cells. Also, causes apoptosis in MCF-7 cells. These anticancer effects are probably due to the main constituents of *Inocutis levis* with antioxidant effects.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**AKT:** Ak strain transforming (protein kinase B); **DMEM:** Dulbecco's modified Eagle's Medium; **DMSO:** Dimethyl Sulfoxide; **ESI-MS:** Electrospray ionization mass spectrometry; **FBS:** Fetal Bovine Serum**; GC-MS:** Gas chromatography-mass spectrometry; **HFF:** Human foreskin fibroblasts; **HPLC:** High-performance liquid chromatography; **HUVECs:** Human umbilical vein endothelial cells; **LC/MS:** Liquid chromatography-mass spectrometry; **MAPK:** Mitogen-activated protein kinases; **mTOR:** Mammalian target of rapamycin; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PBS:** Phosphate-buffered saline; **PDA:** Photodiode array; **PI3K:** Phosphatidylinositol-3 kinase; **RPMI:** Roswell Park Memorial Institute**; SE:** Standard error; **TFA:** Trifluoroacetic Acid; **VEGFR-2:** Vascular endothelial growth factor receptor.

## ETHICAL APPROVAL

This study was conducted under the consideration of the medical ethics committee of Shahid Sadoughi University of Medical Sciences with the registration number of: IR.SSU.MEDICINE.REC..1395.218.

## SUMMARY

The compounds in the *Inocutis levis* extract was determined by LC/MS. It contains antioxidant compounds. Based on the MTT test, *Inocutis levis* extract did not decrease the viability of non-cancerous HFF cells, but it was able to inhibit the growth and increase the death in HT-29 cells at a concentration of 100 µg/mL after 24 and 48 hr and in MCF-7 cells at concentrations of 1 and 10 and 100 µg/mL after 24 and 48 hr. Evaluation of the cell cycle 48 hr after adding the extract by flow cytometry, showed that the mushroom extract induced apoptosis in MCF-7 cells. Finally, it can be concluded that *Inocutis levis* contains antioxidant compounds and can inhibit the growth of HT-29 and especially MCF-7 cancer cells via the induction of apoptosis.

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