Original Article

Cytotoxicity of Trichoderma *Reesei* Extract in Hepa1-6 Cancer Cells

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Abstract

Background & Objective: In the last decades, many successful anticancer fungal metabolites have been obtained, and fungi have shown great potential to produce beneficial anticancer drug compounds. In this article, the effects of the reference strain of Trichoderma *reesei* ethanolic extract on the Hepa1-6 cell line were studied.

Materials & Methods: The fungus was initially cultured in the potato dextrose agar media, and then, it was transferred to yeast peptone dextrose broth. Afterward, the ethanolic and aquatic extracts were prepared, and were analyzed by chromatography and mass spectrometry (GC-MS). Then, the cell lines were treated with different doses of the extracts. The cytotoxic activity of fungal extracts were evaluated by MTT assay, Ferric reducing ability of plasma (FRAP) assay and Ferric Reducing Antioxidant Power (FRAP) Assay. Alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH) measurement tests were done on the culture and cell fractions. Hematoxylin & Eosin staining was performed for microscopic studies, and an oxidant attack-antioxidant defense test was performed on the cell fraction.

Results: GC-MS determined compounds such as cineal, cyclohexene, hydroxyethyl benzaldehyde, terpinenyl acetate, dihydro esthophyllene, propionic acid, hexadecanoic acid, and octadecanoic acid were extracted from the fungal extract. The half maximal inhibitory concentration (IC50) was between 7-8%, and the highest cytotoxic effectiveness was 10-15%. An increase in the levels of ALP, LDH enzymes, and total protein was observed.

Conclusion: Therefore, the findings suggest that the extract of *T. reesei* has inhibitory effects on Hepa1-6 cancer cell lines.

Keywords: Cytotoxicity, Hepa1-6 cell line, *Trichoderma reesei*, ethanolic extract

Introduction

Among different types of cancer, hepatocellular carcinoma is one of the most common ones which usually develop in association with diabetes mellitus, Hepatitis B virus and Hepatitis C virus and other hepatic diseases like liver steatosis and fibrosis. It rarely occurs in non-cirrhotic livers except in the case of digestion of contaminated foodstuffs with Aflatoxin B1 (1, 2). Unfortunately, hepatocellular carcinoma is usually resistant to radiation and chemotherapy (3). Moreover, hepatic illnesses can affect the whole body system. Hence, treating them correctly is necessary (4). Hepatic cancer afflicts both sexes. However, it is more common in men (5).

One of the most potent anti-cancer drugs, which is approved by The United States Food and Drug Administration (FDA) and is widely used

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is cisplatin(6). In the present time, Platinum compounds like cisplatin are utilized to cure the testicular, ovarian, cervix, bladder, head and neck, esophageal, small and non-small lung, breast, prostate and stomach cancers as well as Hodgkin's and non-Hodgkin's lymphomas, neuroblastoma, sarcomas, multiple myeloma, melanoma and mesotheliomas (7-9). However, tumor cell resistance against cisplatin is possible by intracellular inactivating it with thiol containing molecules, developing new efflux mechanisms or increasing DNA repair mechanisms (10,11). Also, cisplatin has side effects such as: cardio toxicity, ototoxicity, neurotoxicity, nephrotoxicity, immunosuppression, bone marrow damage and hepatotoxicity and unfortunately, there is no specific antidote in the case of an overdose which can result in significant mortality (12). Thus, many attempts to find new drugs and compounds with high anticancer effects and fewer side effects have been made (13-15).

In the recent years, fungal compounds have been tested as treatments in radiotherapy and chemotherapy (16). Some of the fungi tested against cancer cell lines are *Aspergillus fumigatus, Penicillium vulpinum and Trichoderma* species (17).

Among fungi, *Trichoderma* species are terricolous fungi generating compounds such as cyclosporine A which is used as a treatment in transplanted patients (18,19).

The first time a fungus was described as *Trichoderma* dates back to eighteenth century. Because of their efficient utilization of the substrate at hand and their secretion capacity for antibiotic metabolites and enzymes, these species are highly successful colonizers of the environment in which they dwell (20). *Trichoderma* species are ubiquitous colonizers of cellulosic materials (21). To survive in their habitat, *Trichoderma* species have developed special defense mechanisms which make them efficient mycoparasites, antagonists, bio-control agents and the producers of metabolites used as fungicides that can be used to fight the fungal diseases of the plants (22, 23). Until the present time, potential antibiotics (for instance, peptaibols), mycotoxins and more than 100 metabolites which possess antibiotic effects have been detected in *Trichoderma* species. Among the species of the this genus *T. reesei* is the most used species for industrial purposes due to its cellulose, proteins and enzymes such as, calf chymosin production capacity (20, 24). In the present study, the cytotoxic effects of the fungal ethanolic extract and the aquatic extract of T. *reesei* on Hepa1-6 cell lines have been evaluated.

Materials & Methods

Preparation of the Hepa 1-6 cell line

The original sample of the Hepa1-6 (Hepatoma) cell line was obtained from the Pasteur institute of Iran and was cultured in the DMEM (Dulbecco's Modified Eagle Medium) media fortified with 10 percent of the Fetal Bovine Serum (both obtained from Gibco®) in the temperature of 37 centigrade degrees and in the atmosphere containing 5 percent of CO_2.

Preparation of the ethanolic extract of *Trichderma reesei*

T. reesei was acquired from Iranian research organization for science and technology®. *T. reesei* was initially cultured in the Potato Dextrose Agar in order to obtain new fungal spores. To produce enough samples of the fungi, yeast peptone dextrose broth were used which were prepared in 1000 cc Erlenmeyer flasks. Then, they were inoculated and kept in the temperature of 25-27°C for 7-10 days. Afterwards, the fungus was isolated by fungal biomass filter papers and allowed to dry in petri dishes. Then, 10 grams of the dried fungi were wrapped up with a filter paper. Next, the filter paper was put in the Soxhlet extractor; 150 cc of ether was added as the solvent and the device was heated in the boiling temperature. Then, the device was left for three hours so that the additional ether could evaporate. Finally, the ethanolic extract was sent to the nanochemistry lab of Tabriz University for the identification and fraction measurement of the present substances in the extract using Gas chromatography–mass

spectrometry (GC-MS). The aquatic extract was prepared with the same procedure with only one difference that, for the aquatic fungal extract, 150 cc of water was used as the solvent.

Gas chromatography-mass spectrometry (GC-MS) analysis

The fungal extract was analyzed by GC-MS. The chromatograph instrument (Agilent 6890, UK) was equipped with an HP-5ms capillary column (30 × 0.2 mm ID × 0.2 µm film thickness) and the results were taken under the following conditions: initial temperature at 50°C, temperature ramp at 5°C/min, 240°C/min to 300°C (holding for 3 min), and injector temperature at 290°C. The carrier gas was helium and the split ratio was 0.8 mL/min. For confirmation of the analysis results, the fungal extract was also analyzed by GC-MS (Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass-selective detector; Agilent, UK) using the same capillary column and analytical conditions as above. Mass spectrometry was performed in electron-ionization mode with ionization energy of 70 eV.

Treatment

The hepa1-6 cell line cultures were divided into different micro wells of cell culture, and exposed to the concentrations of 1.0, 2.5, 5, 10 and 15 percent ethanolic extract, 1.0, 2.5, 5, 10 and 15 aquatic extract and 1.0 percent cisplatin as a positive control. The exposure time for each micro well of cell culture was 48 hours.

Tests

The hematoxylin & eosin staining was used to stain the treated cells. The treated cells of each well were fixed with methanol for 3 minutes. Then, the alcohol was thrown away and the plate was dried in the room temperature. 3 mL of hematoxylin (Merck Millipore, Germany) was added to the wells. After 1.5 minutes, the hematoxylin was discarded and the plate was washed with water. The plate was checked under a light microscope to check whether the hematoxylin was enough or not. Then, eosin solution 0.5% aqueous (Merck Millipore, Germany) was added with the same amount as the hematoxylin for 3 minutes. Finally, the plate was washed again and dried in the room temperature.

For the analysis of the biochemical factors, the cells of each group were transported into separate falcons and were kept in -80 centigrade degrees. After the mechanical separation of the cells by a sterile spatula and transporting them into separate falcons, the cells were centrifuged for 10 minutes with the speed of 4000 rpm. The supernatant liquid of each sample was collected and, like the cell cultures, was kept in -80 centigrade degrees. Then, the falcons were retrieved from the freezer and were prepared for the tests. To operate the cell fraction process, 1ml of saline was added to the falcons and the falcons were kept in -80 centigrade degrees and after 2 hours, they were retrieved from the freezer.

The measurement of Lactate dehydrogenase (LDH) enzyme which is a mark of cell lysis, was carried out and its results were read in the wavelength of 340 nm for both the isolated supernatant liquid and the cell cultures. After drawing the absorption chart of each group, the amount of the tilt of each chart was divided by 16030 and the result was divided by the total protein amount of each sample in order to calculate the quantity of LDH as unit/L for each gram of protein.

Alkaline phosphatase (ALP) assay was also carried out for both of the cell cultures and the isolated supernatant liquid. The ALP assay results were read in the wavelength of 410 nm. To interpret the results of the biochemical tests, total protein assay was separately performed on the isolated supernatant liquid, cell cultures and

homogenous cell fractions using Lowry method and its results were read in the wavelength of 550 nm. Ferric reducing ability of plasma (FRAP) assay (measures the antioxidant potential) was carried out. In the test, BHT was used as a standard. Then, the results were read in the wavelength of 593 nm. Thiobarbituric acid reactive substance (TBARS) assay was done in order to measure the amount of the lipid peroxidation in cell and tissue extracts, and biological fluids. The standard chart of the Malondialdehyde (MDA) was drawn as nmol/dL. Then, its results were read in the wavelength of 532 nm (25).

To evaluate the cytotoxic effects of the aquatic and ethanolic extract, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay test was carried out. To perform the MTT test, 5 mg/mL concentrated MTT solution was prepared by thoroughly dissolving 0.01 g of 98% MTT powder in 2 mL of sterile PBS in the absence of light. 20 microliters of the MTT solution was added to each microwell. Afterwards, the plate was incubated for 4 hours in the temperature of 37 centigrade degrees. After the incubation, 150 microliters of dimethyl sulfoxide was added to every microwell and the plate was steadily shaken so that the formazan crystals could evenly be dissolved in dimethyl sulfoxide. The Optical density (OD)of the microwells were read in the wavelength of 630 nm (26).

The MTT test for each sample was carried out in two separate microwells to avoid the possible errors of the test. Then, the percentage of the living cells was calculated.

Data analysis

The computing statics were done by Prism Ver.3 software performing T-test and ANOVA for the comparison of the mean and the standard deviation from IC50 and the results of the biochemical tests were analyzed by SPSS software.

Results

Microscopic examination revealed an increase in the number of vacuoles, indicating a more severe degeneration in the cells after exposure with ethanolic and aquatic extract. In addition to the presence of vacuoles in the cells, cytopathic effects (CPE) were also seen and the cell nucleus was pycnosis. At higher concentrations of fungal extract, the number of cells decreased and the cell had cellular swelling and in the final high concentration, severe vacuolation was observed, accompanied by severe cellular swelling and chromatin accumulation (ball-shaped chromatin) and loss of nucleus (Figure 1). The results of GC-MS analysis of the essential oils are presented in detail in Table 1.

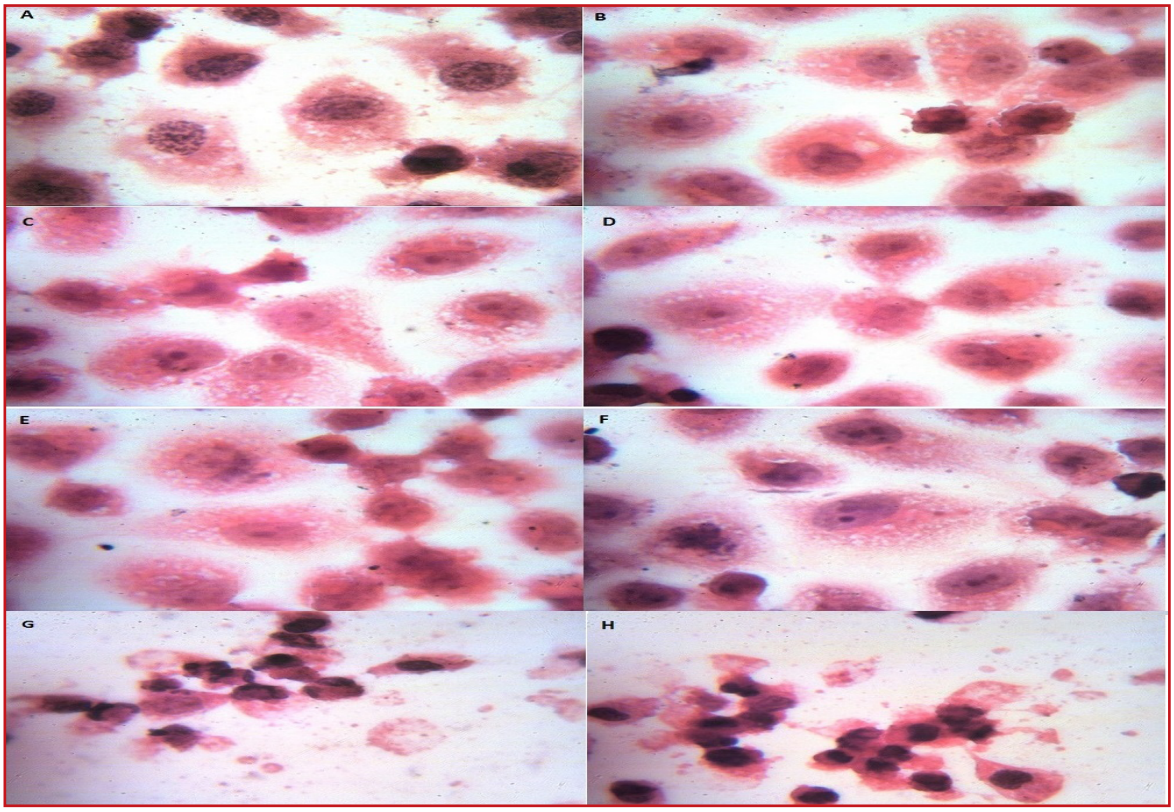

Figure 1-Light microscopy of Hepa1-6 cells in experimental group ×800. A: Small vacuoles are present in the group treated with 5% aquatic extract. B: Vacuoles have grown in size in the group treated with 10% aquatic extract. C: More vacuoles shows more severe degeneration with 15% aquatic extract. D: Beside vacuolation there are some necrotic figures (piknosis of nuclei) present in the group treated with 2.5% ethanolic extract. E: In the group treated with 5% ethanolic extract beside vacuolation decrease in cell number was obvious. F: Severe vacuolation, Clumping of DNA of nuclei in the group treated with 10% ethanolic extract. G: Severe necrosis of the cells in the group treated with 15% ethanolic extract (shrinkage and piknosis and/or loss of nucleus in most of the cells). H: Severe necrosis in the group Cis-pelatin, (Piknosis, Loss of nucleus and fragmentation of cytoplasm).

Table 1. GC mas of Trichodermal reesei extract

NO	Compounds	RT (min)	Abundance%
1	d1-Limonene;Cyclohexene,1-meth	6.257	0.18
2	1,8-Cineole;2-Oxabicyclo[2.2.2]	6.313	2.04
3	Linalool;1,6-Octadien-3-o1,3,7..	7.456	0.46
4	3-Cyclohexene-1-methanol	9.154	0.6

5	Linalyl acetate	10.508	0.54
6	Cinnamaldehyde,(E)-;	11.051	47.5
7	4-(1-Hydroxyethyl)benzaldehyde	11.950	0.69
8	ALPHA.-TERPINENYL ACETATE	12.483	7.45
9	2-Propenoic acid,3-phenyl-	14.070	1.4
10	1,5,7,8-Tetrahydro-2,6-pteridine	18.619	0.75
11	1H-Purin-6-amine,[2-fluorophen]	20.010	0.94
12	Phenanthridine,5-oxide	21.759	0.33
13	1,2-Dihydroacenaphthylen-3-carba	21.504	1.51
14	Bata.N METHYL IONONE;1-Penten	22.581	1.15
15	PYRROLO(3,2,1-JK) CARBAZOLE;	23.402	0.52
16	Cyclodecasiloxane,eicosamethyl-	23.513	0.56
17	Hexadecanoic acid	23.790	2.82
18	9-Octadecenoic acid,(E)-;trans	26.276	3.68
19	Linoleic acid ethyl ester	26.575	0.86
20	Ethyl Oleate	26.653	0.79

After performing the MTT tests, the following results were respectively achieved (Table 2). The IC50 was 8.5 and 7.6 percent for aquatic and Ethanolic extract respectively, and the highest recorded cytotoxic effects were 15 percent.

Table 2. The results of the MTT test and the evaluated amounts of IC50 for the ethanolic and the aquatic extracts
The doses in use for determining IC50 and the rate of the living cells are provided. A: alcoholic, W: aquatic

Time	Negative control	Cisplatin Control	Ethanolic extract %					Aquatic extract %				
			1	2.5	5	10	15	1	2.5	5	10	15
Cell viability (%)after 24h	100	62.9	100	100	74.3	60.2	50.6	100	100	81.4	65.9	60.4
Cell viability (%)after 48h	100	39.6	100	93.5	69.5	42.9	30.3	100	96.2	71.8	44.0	38.4

In the exposure time of 24 hours, the cell lines which were treated with the doses higher than 10 percent had the best cytotoxic effects. Besides, during 24 to 48 hours, the ethanolic extract functioned slightly better than the aquatic extract. The least extract concentration that presented the growth inhibition effect was the 5 percent ethanolic extract which was 20 to 30 percent effective but the result was not significantly high. However, the effectiveness in the 10 and 15 percent-concentrated extract was notable. The inhibitory effect of the 10 percent-concentrated extract was 35 to 45 percent and the effectivenessof the 15 percent extract was 40 to 62 percent. In other words, increasing the dose of the fungal extract scaled up the inhibitory and cytotoxic effects of the extract on the Hepa1-6 cell line.

In addition, increasing the dose of the fungal extract caused a rise in the levels of ALP, LDH and total protein and by scaling up the dose, the statistics became meaningful. As the dose of the fungal extract increased, the amount of lipid and total antioxidant peroxidation increased, which was an increase in total antioxidants due to the antioxidant compounds in the fungal extract. There is very little difference between lipid peroxidation and total antioxidant (Chart 1-4).

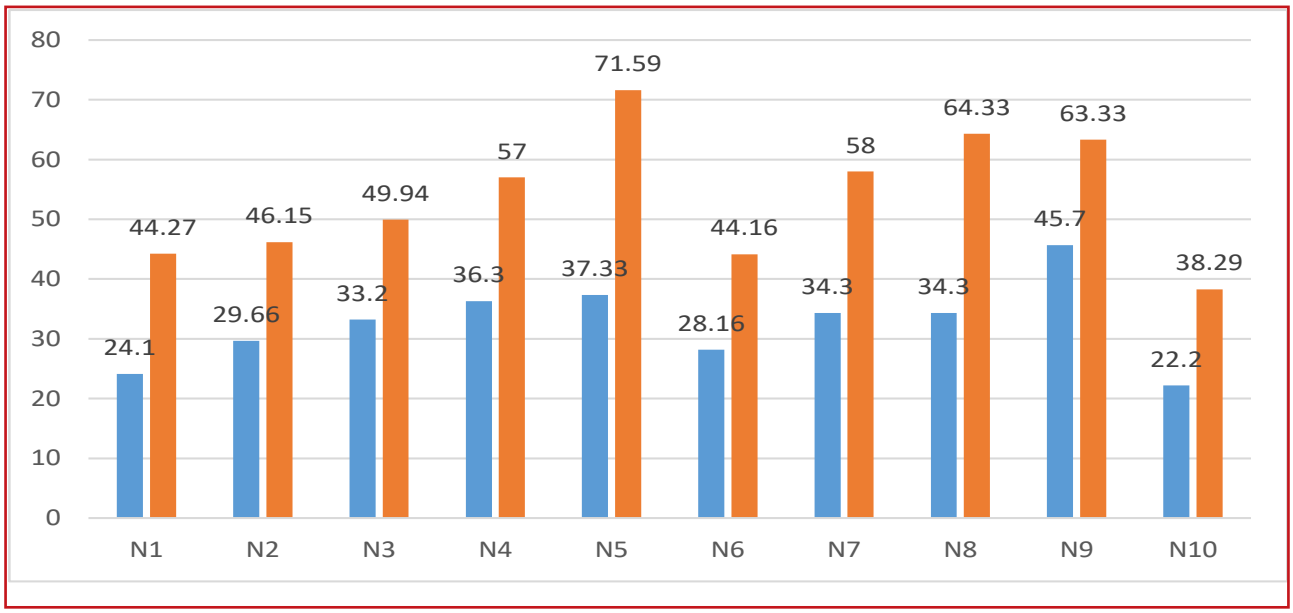

Chart 1. Biochemical results of ALP test for the collected supernatant liquid (Blue) and cell fractions (Green) from each group of Hepa1-6 cells. The columns exhibit the mean of the results of the experimental groups. Vertical axis: ALP U/l, N1=treated with 1% ethanolic extract; N2=treated with 2.5% ethanolic extract; N3=treated with 5% ethanolic extract; N4=treated with 10% ethanolic extract; N5=treated with 15% ethanolic extract; N6=treated with 2.5% aquatic extract; N7=treated with 5% aquatic extract; N8=treated with 10% aquatic extract; N9=treated with 1% cisplatin; N10= negative control

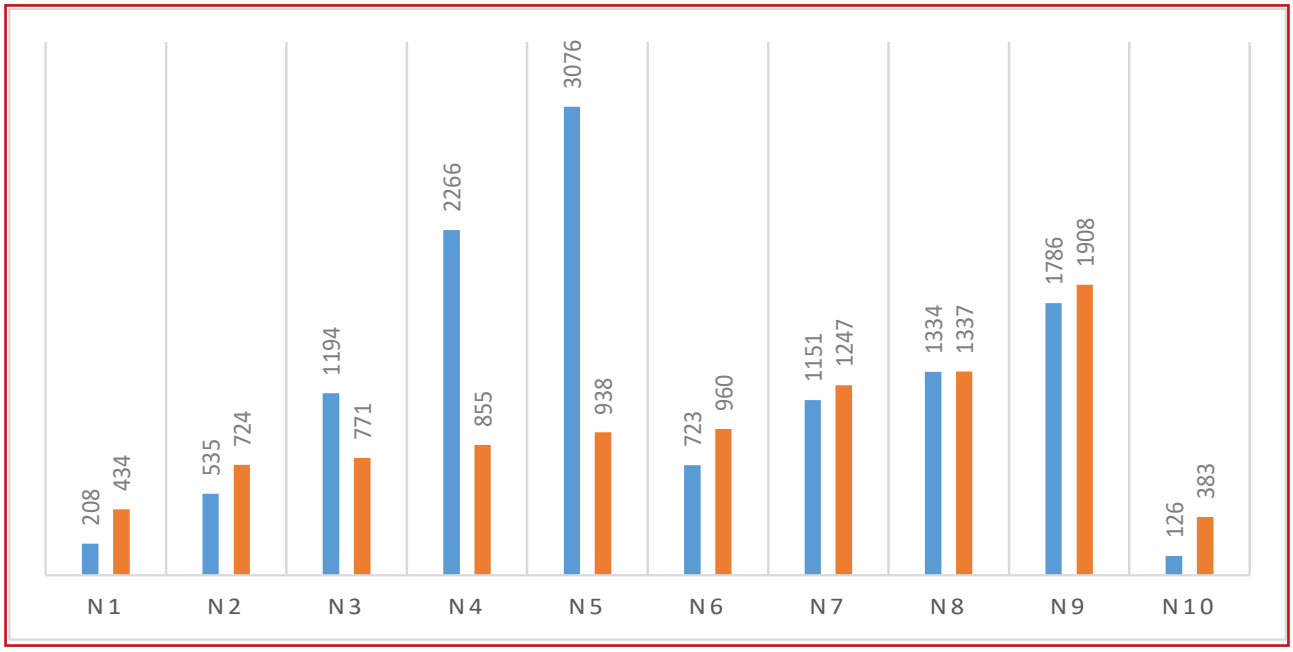

Chart 2. Biochemical results: LDH test results of the supernatant liquid (Blue), the amounts of LDH spread in the cell culture) and for the cell fractions (Green) obtained from each group. Vertical axis: LDH U/l, the mean of results is displayed. N1=treated with 1% ethanolic extract; N2=treated with 2.5% ethanolic extract; N3=treated with 5% ethanolic extract; N4=treated with 10% ethanolic extract; N5=treated with 15% ethanolic extract; N6=treated with 2.5% aquatic extract; N7=treated with 5% aquatic extract; N8=treated with 10% aquatic extract; N9=treated with 1% cisplatin; N10= negative control

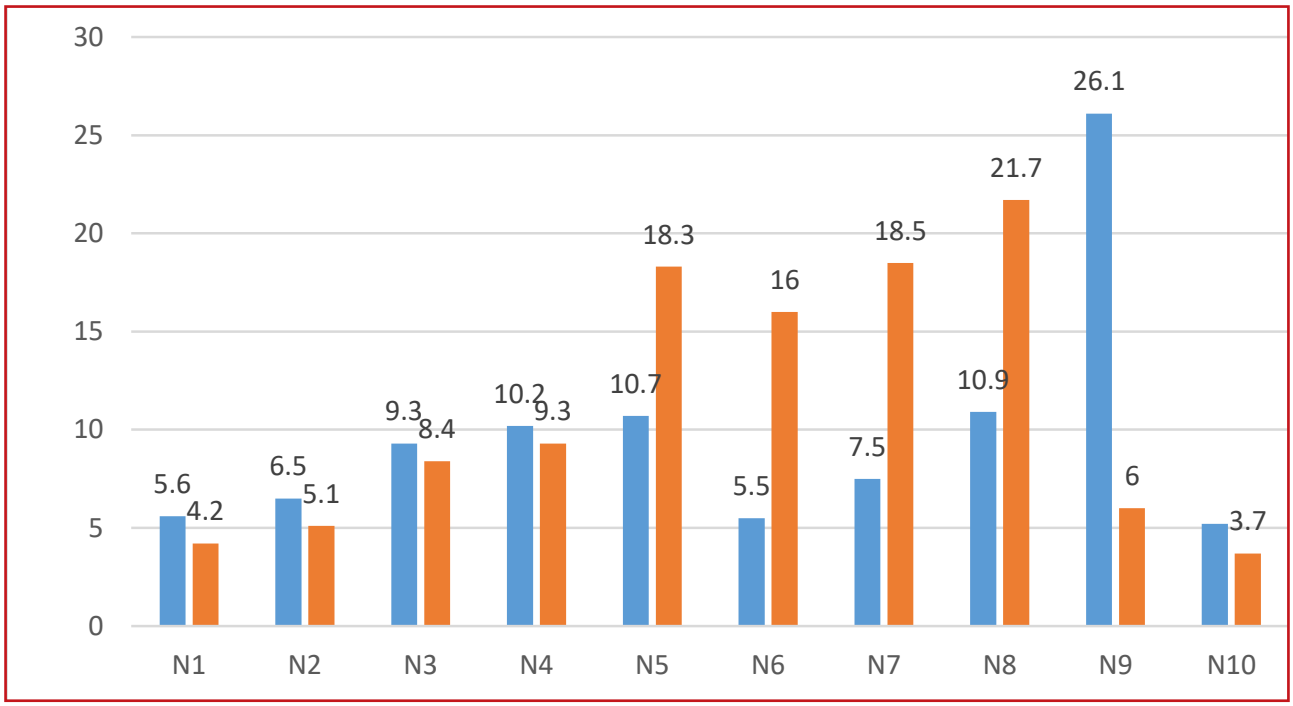

Chart 3. The mean of the results of the TBARS (Blue) and FRAP (Green) test on the cell fractions of each experimental group. Vertical axis: TBARS nmol/dL, The columns present the experimental groups: N1=treated with 1% ethanolic extract; N2=treated with 2.5% ethanolic extract; N3=treated with 5% ethanolic extract; N4=treated with 10% ethanolic extract; N5=treated with 15% ethanolic extract; N6=treated with 2.5% aquatic extract; N7=treated with 5% aquatic extract; N8=treated with 10% aquatic extract; N9=treated with 1% cisplatin; N10= negative control

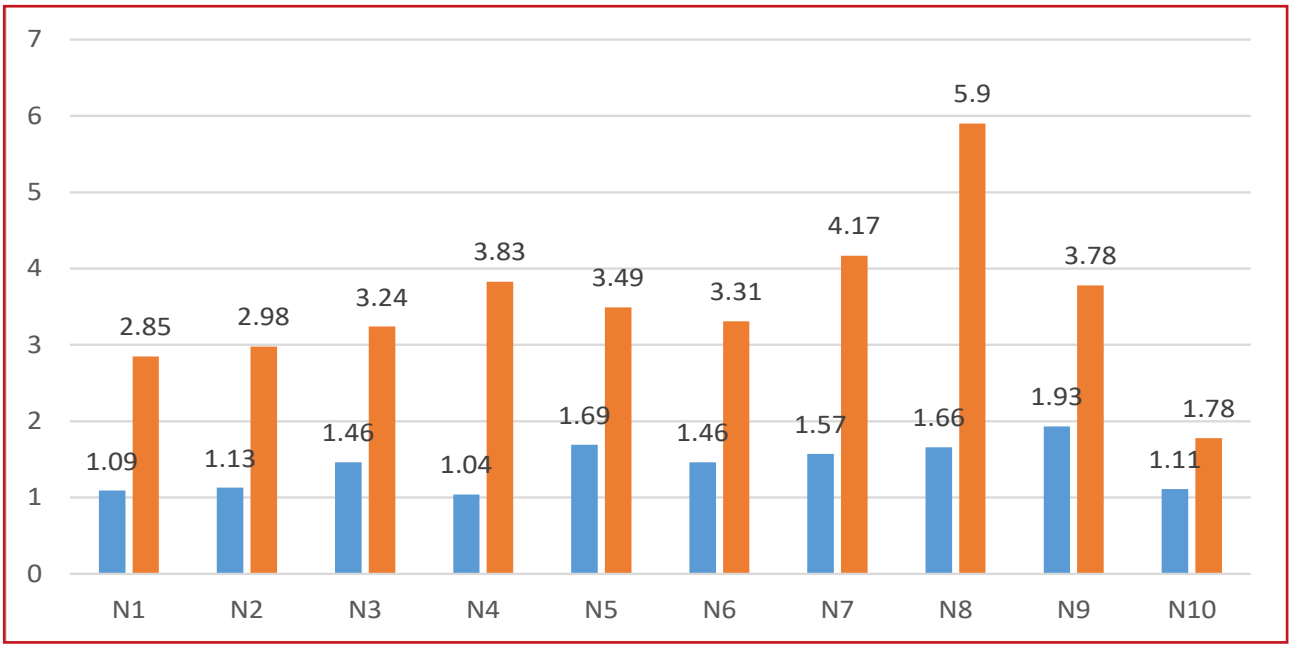

Chart 4. Total Protein test results of the cell fractions (Blue) and the supernatant liquids (Greem). Vertical axis: Total Protein g/dL, the columns present the mean of the results of each group. N1=treated with 1% ethanolic extract; N2=treated with 2.5% ethanolic extract; N3=treated with 5% ethanolic extract; N4=treated with 10% ethanolic extract; N5=treated with 15% ethanolic extract; N6=treated with 2.5% aquatic extract; N7=treated with 5% aquatic extract; N8=treated with 10% aquatic extract; N9=treated with 1% cisplatin; N10= negative control

Discussion

By the technological progresses in bioinformatics and molecular techniques, a lot of information about different types of cancer has been achieved which leads to faster diagnosis of cancer. In the present time, studying the effects of natural agents against cancer has become one of the most important aims of the researchers working on the cancer-related projects because of the less harmful effects that the natural agents possess. So, many researches have been done on the phytogenic agents and other natural and non-chemical agents and their cytotoxic and anticancer effects have been tested.

In the present study, for the first time, the inhibitory effect of the ethanolic and aquatic extracts of *Trichderma reesei* against Hepa1-6 cancer cell line has been investigated. The results of this study are indicative of the inhibitory effects of the fungal extract on the mentioned cell line. The MTT test implicated that the fungal extract is capable of inducing cell death in the tumor cells. According to the MTT test results, after the time of exposure, the least concentration which showed cytotoxic effects were the samples treated with 5 percent- concentrated ethanolic and aquatic extracts. The IC50 was 7.6 percent and the highest recorded cytotoxic effects were 15 percent. Besides, during 24 to 48 hours, the ethanolic extract functioned slightly better than the aquatic extract. Increasing the dose of the fungal extract scaled up the inhibitory and cytotoxic effects of the extract on the Hepa1-6 cell line. Also, increasing the dose of the fungal extract elevated the amounts of lipid peroxidation and total antioxidant because of the antioxidant compounds present in the fungal extract. In the positive control group (cisplatin), a significant difference in lipid peroxidation and total antioxidant could be observed and in the negative control, very little difference was seen. The microscopic examination also indicated that the extract had induced apoptosis in the cancer cells. In addition, increasing the dose of the fungal extract caused a rise in the levels of ALP, LDH and total protein and by scaling up the dose, the statistics became significant. In order to understand the mechanisms by which the *Trihoderma* sp. extracts affect tumor cells, numerous studies have been performed

Li et al. (2017) investigated the antitumor effects of TP1A which they purified from the fermented broth of *Trichoderma* sp. KK19L1 by combination of Q Sepharose fast flow and Sephacryl S-300 chromatography on HeLa and MCF-7 cells. They suggested TP1A could be a potential source for antitumor agents for it could arrest HeLa cells in G2/M phase and it could induce HeLa cell apoptosis in the mentioned research (26).

Moreover, Setyowati et al. (2017) tested the cytotoxic effects of the ethyl acetate extract of *T. reesei* on T47D and Raji cell lines and he fetched up that the extract of *T. reesei* contains terpenoids and phenyl propane derivatives and the ethyl acetate extract was active against the mentioned cell lines with IC50 of 270 and 470 µg/mL (27). You et al. (2010) tested the effects of trichoderone which was obtained from *Trichoderma* sp. on A549, NCI-H460, MCF-7, MDA-MB-435s, HeLA-229, DU-145 and HLF cell lines (28). Then, it was discovered that trichderone had a selective cytotoxicity on cancer cell lines and probably caused apoptosis on the mentioned cancer cells but it did not affect the normal epithelial cell line. Therefore, he suggested trichoderone is a promising anticancer agent which is produced by *Trichoderma* species and exhibits highly selective toxicity to cancer cells. Saravanakumar et al. (2015) measured the cytotoxic and antitumor effects of 16-methylheptadecanoic acid methyl ester (HDA) and 9,12-octadecadienoic acid (ODA) from marine *Trichoderma*, *Hypocrea lixii* TSK8, *Hypocrea rufa* SKS2 on KB oral cancer and skin

carcinoma(A431) cell lines by performing the MTT test and also evaluated the in vivo anti-skin cancer activity of HDA in *Swiss albino* mice induced with skin cancer (29). It concluded that HDA is a highly potent anticancer compound against the skin cancer and provided more support for the successful cytotoxicity of the *Trichoderma* extract against cancer cells by performing the in vivo test.

El-mohsen et al. (2014) studied the effects of *Trichoderma* species culture filtrate against human cervical and breast cancer cell lines (30). During the research, the cells were exposed to 20,40,60,80 and 100 mg/mL of both *Trichoderma harzianum* culture (ThCF) filtrate and *Trichoderma asperellum* culture filtrate (TaCF) for 24 hours and the cell viability and the cytotoxic responses had been assessed by trypan blue and MTT essays. Some intracellular changes such as a formation of vesicles and unusual protoplasmic extension, a decrease in the size of the cells which had been exposed to ThCF and an increase in the nuclear size of the cells exposed to TaCF were observed by him. Also, according to the results of the cell viability percent, ThCF appeared to be more toxic to HeLa cells than TaCF while TaCF appeared to be more toxic to MCF-7 cells than ThCF.

Kummalue et al.(2007) tested and measured the cytotoxicity of 1, 10, 100, 500 mg/mL concentrations of the crude broth extract of *Trichderma ovalisporum* against 4 breast cancer cell lines (SKBR3, MCF7, T47D and MDA-MB435) and 2 human lung cancer cell lines (A549 and SK-LU1) by performing the MTT test but he did not notice any ED50 values and therefore, he concluded that the crude broth extract of *Trichoderma ovalisporum* did not exhibit any anti-proliferative effects on the cancer cell lines he used for the research (31).

In addition, the GC-Mass results of the present study suggested that at least three more compounds with anti-cancer effects were present in *T. reesei* extract. Cinnamaldehyde which has been proved to trigger the mitochondrial depolarization and the inhibition of NF-kB and AP-1 activity, and has also inhibited the PI3K/Akt signaling pathway in colorectal cancer cells, resulting in the induction of apoptosis in both cases (26, 32) 1,8-cineole which can result in the inactivation of survivin and Akt and it can also activate p38, causing apoptosis (33). Also, it has been found that hexadecanoic acid (palmitic acid) targets topoisomerase I and possesses cytotoxic effects (34, 35).Yet, more research may be required to fully discover the mechanisms by which *Trichoderma sp.* and especially *T. reesei* extracts affect tumor cells. To our knowledge, the ethanolic extract of *T. reesei* possesses potential inhibitory effects on the Hepa1-6 cell line.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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