RESEARCH ARTICLE

Cytotoxicity of *Trichoderma* spp. Cultural Filtrate Against Human Cervical and Breast Cancer Cell Lines

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Abstract

***Trichoderma* spp. are known as a rich source of secondary metabolites with biological activity belonging to a variety of classes of chemical compounds. These fungi also are well known for their ability to produce a wide range of antibiotic substances and to parasitize other fungi. In search for new substances, which might act as anticancer agents, the overall objective of this study was to investigate the cytotoxic effects of *Trichoderma harzianum* and *Trichoderma asperellum* cultural filtrates against human cervical and breast cancer cell lines (HeLa and MCF-7 cells respectively). To achieve this objective, cells were exposed to 20, 40, 60, 80 and 100 mg/ml of both *T. harzianum* cultural filtrate (ThCF) and *T. asperellum* cultural filtrate (TaCF) for 24h, then the cell viability and the cytotoxic responses were assessed by using trypan blue and 3-(4,5-dimethylthiazol-2yl)-2,5-biphenyl tetrazolium bromide (MTT) assays. Morphological changes in cells were investigated by phase contrast inverted microscopy. The results showed that ThCF and TaCF significantly reduce the cell viability, have cytotoxic effects and alter the cellular morphology of HeLa and MCF-7 cells in a concentration dependent manner. A concentration of 80 and 100mg/ml of ThCF resulted in a sharp decline in the cell viability percent of HeLa and MCF-7 respectively (25.2%, 26.5%) which was recorded by trypan blue assay. The half-maximal inhibitory concentrations (IC_{50}) of ThCF and TaCF in HeLa and MCF-7 were recorded as 16.6, 12.0, 19.6 and 0.70mg/ml respectively by MTT assay. These results revealed that ThCF and TaCF have a substantial ability to reduce the viability and proliferation of human cervical and breast cancer cells.**

Keywords: Cytotoxicity - *Trichoderma* - HeLa - MCF-7 - cervical and breast cancer cells

Asian Pac J Cancer Prev, **15 (17)**, 7229-7234

Introduction

The genus of *Trichoderma* is an asexual fungi found in the soils and other diverse habitats of all climatic zones (Kubicek and Harman, 2002). It is a secondary opportunistic invader, a fast growing fungus, a strong spore producer, a source of cell wall degrading enzymes and an important antibiotic producer (Vinale et al., 2008). *Trichoderma* species are readily isolated from soil by all available conventional methods, largely because of their rapid growth, abundant condition and present in nearly all soils and other diverse habitats (Kubicek and Harman, 2002; Harman, 2006). *Trichoderma*, in particular, interacts with other microbes in the soil and with plant roots in the rhizosphere (Harman et al., 2004). They have the ability to produce a wide range of secondary metabolites with biological activity (Ghisalberti and Sivasithamparam, 1991; Sivasithamparam and Ghisalberti, 1998). Reino et al. (2008), summarized the most important secondary metabolite types isolated from *Trichoderma* spp. emphasizing their biological activities, especially the role that these metabolites play in biological control mechanisms. It is strain dependent and includes different substances belonging to a variety of classes of chemical compounds such as anthraquinones, daucanes, pyrones, koninginins, trichodermamides, viridins, viridiofungins, nitrogen heterocyclic compounds, trichodenones, cyclopentenone derivatives, azaphilones, harzialactones derivatives, butenolides, trichothecenes, isocyano metabolites, setin-like metabolites, bisorbicillinoids, diketopiperazines, ergosterol derivatives, peptaibols, cyclonerodiol derivatives, statins, heptelidic acid, acoranes, miscelanea and others (Vinale et al., 2008). Furthermore, many cell wall degrading enzymes from different *Trichoderma* strains have been purified and characterized (Lorito, 1998).

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Cancer is a leading cause of death and an emerging public health problem in worldwide due to its disease burden, fatality and tendency for increased incidence (Sahin et al., 2013). Among women breast cancer is the most common type of cancer with the highest fatality rates (Koca et al., 2013; Oztunc et al., 2013). Cervical cancer is a malignant neoplasm arising from cells originating in the cervix. It is the second most prevalent cancer and third most common type of cancer after breast and lung cancers among women (Sogukpinar et al., 2013; Karadag et al., 2014). It is well known that, traditional method to fight the cancer is chemotherapy. The most important effect of chemotherapy is that it kills cancer cells. However, chemotherapy can also affect normal cells that rapidly divide and grow, such as those in the bone marrow, digestive tract, skin, hair and reproductive organs. When normal cells are damaged, this can cause side effects (Monsuez et al., 2010). In the last several years, traditional methods used to fight cancer cells have been largely based on the use of plant natural products. Recent studies have reported that natural products have positive effects against cancer cells compared with chemotherapy (Kim et al., 2012; Wang et al., 2012; Alshatwi et al., 2013; Jung et al., 2014). Therefore, the present study was aimed to investigate the *in vitro* cytotoxic activity of two isolates of *Trichoderma* spp. cultural filtrate against human cervical and breast cancer cell lines.

Materials and Methods

Chemicals and instruments

Dulbecco's Modified Eagle's Medium (DMEM) culture medium, penicillin streptomycin 10.000 units/ml, L-glutamine 200 mM and fetal bovine serum (FBS) were purchased from Gibco, Life Technologies, UK. Trypsin purchased from Serva, Heidelberg, Germany. Tissue culture plates 96 well and serological pipets were purchased from Biofil, Belgium. Ethanol and all other specified reagents and solvents were purchased from Sigma Chemical Company Pvt. Ltd. St. Louis, MO, USA.

Trichoderma isolates and fungal cultural filtrates

T.harzianum isolate selected for this study were generously obtained from the Department of Agricultural microbiology, Faculty of Agriculture, Minia University, El-Minia, Egypt, and the *T.asperellum* isolate was isolated from the soil of Kazan city, Republic of Tatarstan, Russian Federation. Two isolates were cultured in potato-glucose liquid media (28ºC, 140rpm) in an incubator shaker (Innova-43, Hamburg, Germany) for 168h. The culture medium contained potato decoction 200ml, Glucose 20.0g and distilled water 800ml. After 168h of shaking, the culture broth, including *Trichoderma* spores was filtered through Millipore filter paper (0.22µm) to separate the cultural filtrate of *Trichoderma* without spores.

Cell lines and culture conditions

HeLa cervical and MCF-7 breast cancer cell lines were generously provided by Dr Timur Abdullin (Kazan federal university, research and education center pharma 2020) and were grown in DMEM, supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin, Streptomycin and 0.5% L-glutamine 200 mM in a humidified incubator (ESCO, CelCulture, CO_2 incubator) with 5% CO_2 at 37°C. Before beginning the experiments, viability of cells was assessed and the cells have shown more than 95% cell viability.

Experimental design

Cells were seeded at a density of 5,000 cells per well in 96 well plates and after 24h of incubation treated with 20, 40, 60, 80 and 100 mg/ml of both *Trichoderma* cultural filtrates and were incubated for another 24h to assess the cell viability assay by trypan blue dye, and cellular morphology by phase contrast inverted microscope. Cytotoxic responses were determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-biphenyl tetrazolium bromide (MTT) assay after 72h of incubation with both Trichoderma cultural filtrates.

Cell viability assay

To evaluate the viability of treated and untreated cancer cells, the percentage of viable cells was determined, using trypan blue exclusivity stain. Trypan blue is an essential dye, use in estimating the number of viable cells present in a population (Phillips and Terryberry, 1957). Cell viability assay was performed by mixing 50μl of 0.4% trypan blue in 0.9% saline to 50μl of trypsinized cell suspension in a 500μl Eppendorf tube. The final solution was thoroughly and gently mixed and allowed to stand for 2-3 min because longer incubation with the dye may be toxic to viable cells. Cells were counted using the hemocytometer according to the standard procedure (Pienata and Lehr, 1993). A drop of the dye-cell suspension was loaded onto both chambers of the hemocytometer. Cells were examined and counted in duplicates under light microscope at 40x (Zeiss Axio Vert.A1). The percentage of viable cells was calculated by the following formula:

Cell viability(%)=Number of viable cells (unstained cells)÷Total number of cells (stained and unstained)×100

Thus the percentage of viable and non-viable cells (spontaneous and treatment induced death) was determined for each cell line, and the inter treatment results compared with the non-treatment control.

Cytotoxicity Assay

HeLa and MCF-7 cells were seeded at a density of 5,000 cells per well in 96 well plates and incubated for 24 h at 37ºC in 5% CO_2 incubator. After 24h of incubation, when the monolayer formed, the supernatant was removed from each well and 100µl of medium contains a different concentration of *Trichoderma* cultural filtrates were then introduced to the cells in microtitre plates and kept for incubation at 37ºC in 5% CO_2 incubator for 72h. Cells treated with medium only served as a control group. After 72h of incubation, the supernatant was removed and after removing it of each, well and washing twice with PBS, 10µl of MTT solution (5 mg ml^{-1} in PBS) and 90 µl of medium were then introduced. After incubation for another 4h, the resultant formazan crystals were dissolved in dimethyl sulfoxide (100µl) and the absorbance intensity

measured by a microplate reader (TECAN Infinite M200, USA) at 555 nm. Growth inhibition percent was calculated according to Patel et al. (2009) using the following formula:

% Cell inhibition=100-[(At-Ab)÷(Ac-Ab)]×100

Where: At = Absorbance value of test compound; Ab = Absorbance value of blank; Ac = Absorbance value of control

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. The half-maximal inhibitory concentration (IC_{50}) values of test substances were calculated from at least three independent experiments using linear regression of the dose-log response curves by SOFTmaxPro.

Morphological examination

The morphological changes in HeLa and MCF-7 cells exposed to different concentrations of ThCF and TaCF were examined using a Zeiss Axio Vert.A1 microscope at 40 X magnification. The treated cells were compared with untreated cells to can detect the morphological changes.

Statistical analysis

The data were analyzed statistically using SPSS software version 17. The significant level was ascertained by one-way analysis of variance (ANOVA), followed by LSD multiple comparison test. The results were expressed as mean±standard deviation of the means (SD). P value of < 0.05 in the LSD test was considered as significant.

Results

Cell viability assay

The percentage of Hela and MCF-7 viable cells was determined after 24h of incubation with 20 to 100mg/ml of both ThCF and TaCF. Result shows that ThCF and TaCF induced statistically significant ($p<0.05$) decrease in the percentage of Hela and MCF-7 viable cells in a concentration dependent manner. As shown in Figure 1A and B, the HeLa cells exposed to ThCF and TaCF for 24h at 20 mg/ml and above concentrations up to 100 mg/ml were found to be cytotoxic. The cell viability percent of HeLa cells treated with different concentrations of ThCF (20, 40, 60, 80 and 100 mg/ml) for 24h was recorded 50.43%, 48.49%, 51.75%, 25.23% and 50.3% respectively by the trypan blue assay (Figure 1A). Whereas, the cell viability percent of the same cells treated with different concentrations of TaCF was recorded 85.16%, 62.55%, 74.37%, 90.30% and 52.59% respectively (Figure 1B). MCF-7 cells exposed to ThCF and TaCF also show

Table 1. The Cytotoxicity of ThCF and TaCF Against HeLa and MCF-7 Cancer Cell Lines as Determined by the MTT Assay

Cultural filtrate	Concentration (mg/ml)	HeLa % inhibition ± SD	IC_{50}	MCF-7 % inhibition ± SD	IC_{50}
T.harzianum (ThCF)	20	-30.19±4.20	16.64	84.74±3.18	12.02
	40	3.02±0.69		77.13±4.22	
	60	77.95±3.12		77.16±3.12	
	80	85.39±5.44		66.11±5.14	
	100	83.89±3.22		86.46±2.22	
T.asperellum (TaCF)	20	-3.30±0.66	19.64	85.83±2.45	0.7
	40	10.81±2.56		82.15±3.56	
	60	50.10±3.22		56.13±1.22	
	80	62.60±2.89		63.15±4.56	
	100	72.61±3.44		87.24±1.16	

Results represent mean±SD, n=3

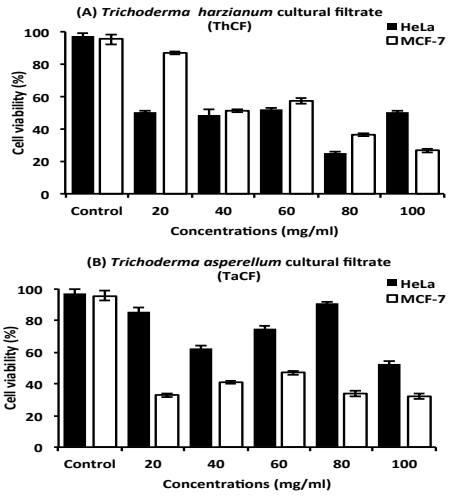


Figure 1. Cell Viability Valuation by Trypan Blue Assay in HeLa and MCF-7 Cells Following the Exposure of Various Concentrations of *Trichoderma harzianum* Cultural Filtrate (ThCF) and *Trichoderma asperellum* Cultural Filtrate (TaCF) for 24h. Values are mean±SD of three replicates. ($p<0.05$ *vs* Control).

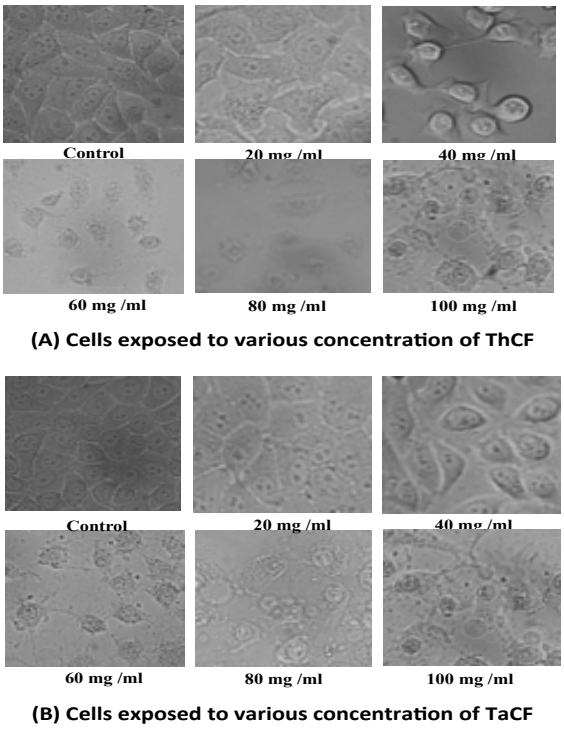


Figure 2. Morphological Changes in HeLa Cells Exposed to Various Concentrations of *Trichoderma harzianum* Cultural Filtrate (ThCF) and *Trichoderma asperellum* Cultural Filtrate (TaCF) for 24h. Images were taken using a Zeiss Axio Vert.A1 microscope at 40 X magnification.

statistically significant ($p<0.05$) decrease in the percentage of MCF-7 viable cells in a concentration dependent manner. The cell viability percent of MCF-7 cells was recorded 86.89%, 50.98%, 57.26%, 36,55% and 26.46% at 20, 40, 60, 80 and 100mg/ml of ThCF respectively by the trypan blue assay (Figure 1A) and 31.46%, 40.64%, 46.83%, 33.50% and 32.24% at 20, 40, 60, 80 and 100 mg/ml of TaCF respectively (Figure 1B).

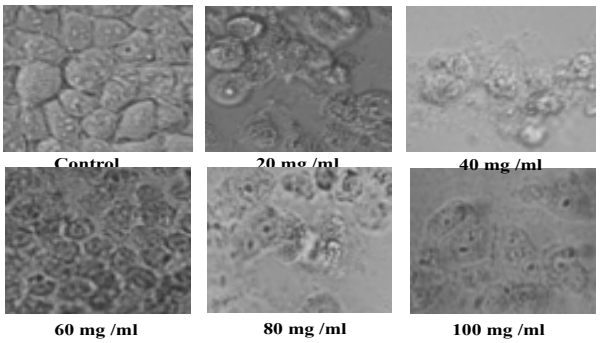
Cytotoxicity by MTT assay

The cytotoxicity assay of Hela and MCF-7 cells was carried out after 72 h of incubation with 20, 40, 60, 80 and 100 mg/ml of both ThCF and TaCF. These cultural filtrates were screened for its cytotoxicity against HeLa and MCF-7 cell lines at different concentrations to determine the IC_{50} by MTT assay. Results indicated that, the maximum percent inhibition of HeLa cells was 85.39% and 72.61% at a concentration of 80 and 100 mg/ml of ThCF and TaCF respectively (Table 1). Whereas, the maximum percent inhibition of MCF-7 cells was 86.46% and 87.24 at a concentration of 100 mg/ml of both ThCF and TaCF respectively (Table 1). Furthermore, as shown in Table 1 the IC_{50} values of ThCF and TaCF on HeLa and MCF-7 cell lines were 16.64, 19.64, 12.02 and 0.70 mg/ml respectively.

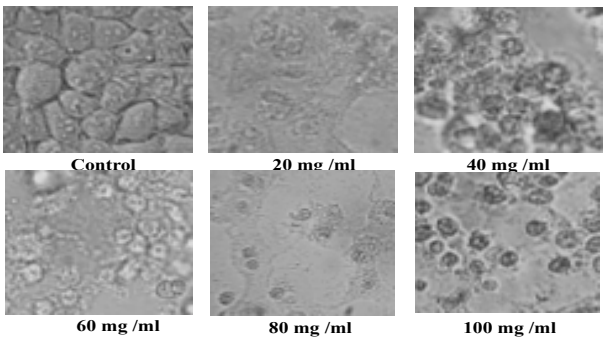
Morphological changes

The morphological changes observed in Hela and MCF-7 cells exposed to different concentrations of ThCF and TaCF were found to be in a concentration dependent manner. Various changes in the morphology of HeLa cells induced by ThCF and TaCF are shown in Figures 2 A and B respectively. It was found that, treatment of the HeLa cells with ThCF and TaCF for 24h caused substantial morphological changes as some cells were shrunken, spaces between cells were increased, intercellular contacts were decreased and many cells were detached from the bottom of the culture plate compared to those in the control group. Some intracellular changes were observed such as formation of vesicles and unusual protoplasmic extension. In addition, it was observed that the size of the cells exposed to ThCF was sharply decreased (Figure 2A) and the nuclear size of the cells exposed to TaCF was increased (Figure 2B) as compared to control group.

Alterations in the morphology of MCF-7 cells due to short-term treatment with ThCF and TaCF are shown in Figures 3 A and B respectively. Morphological examination showed the presence of some morphological changes in the MCF-7 cells treated with ThCF and TaCF for 24h such as weakness of intercellular contacts and decrease in the cell adhesion capacity as compared to control group. Also observed in the MCF-7 cells exposed to ThCF some changes as lost their typical morphology and appeared smaller in size (Figure 3A). Furthermore, in cells treated with TaCF intracellular changes were observed such as enlargement in the nucleus (Figure 3B).



(A) Cells exposed to various concentration of ThCF



(B) Cells exposed to various concentration of TaCF

Figure 3. Morphological Changes in MCF-7 Cells Exposed to Various Concentrations of *Trichoderma harzianum* Cultural Filtrate (ThCF) and *Trichoderma asperellum* Cultural Filtrate (TaCF) for 24h. Images were taken using a Zeiss Axio Vert. A1 microscope at 40 X magnification

Discussion

In Asian women and the other women of the world, breast and cervical cancers are significant causes of mortality and morbidity (Chen and Wang, 2013). In western countries, breast cancer is most common cancer and the second leading cause of cancer death for women (Ries et al., 2005; Lu et al., 2012). The second most common cancer among women is cervical cancer (ACS, 2012). About 80% of cervical cancers occur in developing countries. In addition, mortality rate of breast cancer and cervical cancer among Asian women is similar to that of Caucasian women (Muir and Nectoux, 1996).

It is well known that, secondary metabolites produced by microorganisms are low molecular mass products, not essential for growth of the producing cultures, but very important for human health. They include antibiotics, antitumor agents, cholesterol-lowering drugs, and others (Ruiz et al., 2010). Many *Trichoderma* spp. have been known for decades as agents that biocontrol plant diseases (Gupta et al., 2014), but recent studies have demonstrated that they have many other useful attributes (Keswani et al., 2014). Therefore, the cytotoxic activity of the metabolites isolated from two species of *Trichoderma* have been investigated in this study against human cervical and breast cancer cell lines.

The anticancer activity of plants has been verified to be connected with a variety of phytochemicals, such as polyphenols, flavonoids and catechins. (Uddin et al., 2009). Several recent studies have investigated the cytotoxicity of natural plant extracts against human cervical and breast cancer cell lines (MCF-7 and HeLa) (Hamedeyazdan et al., 2012; Zhao et al., 2014). Proliferation and viability of MCF-7 and HeLa cells were decreased after treatment with

different plant extracts. This decrease in cell proliferation and cell viability can be related not only to the effection of the arresting cell cycle, but also to the induction of apoptosis (Hamedeyazdan et al., 2012). Cytotoxic activities of *Trichoderma* spp. against the human tumor cell lines has not been studied enough. Therefore, the main objective of our study was to investigate the cytotoxic effects of two isolates of *Trichoderma* against MCF-7 and HeLa cells. To our knowledge, this is the first study that has used metabolites of the whole culture filtrate of *T.harzianum* and *T. asperellum* as anticancer agents and aimed to study the cytotoxic effects of *Trichoderma* against human cancer cell lines.

Certain species of *Trichoderma* have been known as a productive producer of important secondary metabolites such as antibiotics, plant growth regulators, and enzymes, which are mainly used to protect plants from pathogens (Singh and Singh, 2009; Respinis et al., 2010).

The mechanism of action of *Trichoderma* as a biocontrol agent is a complex process mediated by the secretion of extracellular enzymes (de la Cruz et al., 1992; Geremia et al., 1993; Lorito et al., 1994). Some enzymes produced by the *Trichoderma* species are known to have antitumor activity (Kusakabe et al., 1979). During the search for new antitumor agents Kusakabe et al. (1980) have found a heat-labile antitumor substance of high molecular weight in the aqueous extract of a wheat bran culture of *T. viride* isolated from soil. The active substance was believed to be L-lysine oxidase. Kusakabe et al. (1980) also provided in his study further evidence that the antineoplastic activity is ascribable to the action of the enzyme.

Traditional methods to treat breast cancer are radiation, chemotherapy and surgery. Radiations cause damage to DNA strand inside the cancer cells, which inhibits its further growth. In the same time, it can also damage the healthy tissues, but the effect is more on cancerous cells. Chemotherapy is a common treatment for a variety of cancers, but is not suitable therapy for all breast cancer patients (Rita, 2012; Majeed et al., 2014). Therefore, in the present study, we evaluated the anticancer effect of ThCF and TaCF in a HeLa and MCF-7 cancer cell lines using *in vitro* techniques. The cytotoxic responses of the cultural filtrates were determined by trypan blue and 3-(4,5-dimethylthiazol-2yl)-2, 5-biphenyl tetrazolium bromide (MTT) assays. In addition, the morphological changes in the treated HeLa and MCF-7 cells were investigated. The results indicate that both ThCF and TaCF appeared to be active in HeLa and MCF-7 cells. From the results of cell viability percent ThCF appeared to be more toxic to HeLa cells than TaCF, whilst TaCF appeared to be more toxic to MCF-7 cells than ThCF. Similarly, the percent inhibition of cell proliferation determined by MTT assay was increased after treatment the HeLa and MCF-7 cells with ThCF and TaCF in a concentration dependent manner. This inhibitory effect of ThCF and TaCF on cervical and breast cancer cells was recorded for the first time in our study. From the other hand, morphological examination of treated HeLa and MCF-7 cancer cells confirmed the previous results and indicated that ThCF and TaCF have a substantial ability to alter the morphological and cytological structure of HeLa and MCF-7 cancer cells.

In a similar study by Lee et al., (2005) harzianum A with a chemical formula of $C_{23}H_{28}O_6$ was isolated from a new *Trichoderma* strain and showed moderate to strong cytotoxicity in human cancer cell lines. Furthermore, a cytotoxic cyclopentenone compound named trichoderone was isolated from *Trichoderma* sp by You et al. (2010) and the cytotoxic activity of this compound was evaluated against six cancer cell lines (A549, NCI-H460, MCF-7, MDA-MB-435 s, Hela and DU-145) and one normal human cell line (HLF) by the MTT assay. Trichoderone displayed cytotoxic activity against cancer cell lines but did not exhibit any cytotoxicity on non-cancer cell lines. The results of these two studies enhance our results and confirm that fungi of the genus *Trichoderma* have potent cytotoxic effects against human cancer cell lines.

Finally, can it be concluded that, our data demonstrate that cultural filtrates of *T.harzianum* and *T. asperellum* significantly reduced the cell viability of human cervical and breast cancer cell lines, and altered the cellular morphology in a concentration dependent manner. The results also revealed that HeLa cells were more sensitive towards ThCF than TaCF while MCF-7 were more sensitive towards TaCF than ThCF. Further studies are required to understand the mechanism(s) of action of these cultural filtrates on HeLa and MCF-7 cells. In addition, the study of *Trichoderma* spp. as a source of biologically active metabolites is especially significant and ensures interest on this subject for years to come.

Acknowledgements

This research was supported by the subsidy of the Russian Government to support the Program of Competitive Growth of Kazan Federal University. The authors earnestly grateful to Dr. Timur Abdullin and Dr. Oksana Bondar, Kazan federal university for their research assistance and support during the accomplishment of this work and also to provide them the cancer cell lines which were used in this study.

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