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ORIGINAL ARTICLE

The assessment of anti-tumoral activity of polysaccharide extracted from terrestrial filamentous fungus

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Abstract Fungal polysaccharides are well-known for the medicinal properties such as antitumor and immunomodulating effects. Hence, this study evaluated antitumor effects of polysaccharide extracted from *Fusarium* sp. isolated from soil samples of Karaj district, Alborz, Iran along with its taxonomic study. The filamentous fungus strain FK1 was isolated from the soil sample of Karaj, Iran. The strain was identified based on cultural, morphological and 18 S rRNA gene parameters as *Fusarium*. Further, the strain *Fusarium* was cultured in fermented broth of modified (PDB) for 10 days at 25 °C. The polysaccharide of strain FK1 was extracted from the mycelium free supernatant by boiling water method and evaluated for antitoxicity effect on two human cancer cell lines: HeLa cell line and Lymphoblastoid cell line (LCL) by MTT method. Findings revealed that water-extracted from mycelia polysaccharide of strain FK1 had the highest cytotoxicity effect against LCL which is the cause of B lymphocyte cancer, at 50 µg/ml concentration dose (114 ± 1.63) followed by 100 µg/ml (105 ± 0.57) and 10 µg/ml (104 ± 0.57), while it did not have a considerable effect on HeLa cell line. *Fusarium* could be alternative sources as an antitumor component.

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1. Introduction

Among microorganisms, fungi have been recognized as the most fruitful sources to produce a varied range of secondary bioactive metabolites such as antimicrobial, antioxidant, antitoxic and anticancer compounds with the various types of pharmaceutical and therapeutic applications (Lavi et al.,

2006; Li et al., 2011; Mizuno et al., 1990; Yu et al., 2010). These bioactive compounds could be mainly classified as terpenoids, alkaloids, quinines, steroid, isocoumarins, ligase, phenols, phenyl propanoids and polysaccharides (Yu et al., 2010; Zhang et al., 2007). Among them, fungal polysaccharides are well known for their nutritional and extracted from medicinal plants. They are long carbohydrate molecules and edible fungi who live extensively in algae, plants, animals, and microorganisms such as fungi and bacteria (Tang et al., 2014; Wang et al., 2014; Zhao et al., 2014; Zhu et al., 2016a). During the past years according to unique biological activities of polysaccharides extracted have attracted increasing attention. The several studies have been reported that the biological actives such as anti-oxidation, anti-inflammatory, anti-tumor, anti-aging, anti-viral, anti-ulcer, neuroprotective and immunological activities (Tang et al., 2014; Wang et al., 2014; Zhao et al., 2014). In recent years, several studies on antitumor activity of polysaccharide extracted from basidiomycete mushrooms such as *Morchella esculenta*, *Agaricus brasiliensis*, *Ganoderma Lucidum* and *Lentinus edodes* have been reported (Chan et al., 2008; Duncan et al., 2002; Hsu et al., 2011; Mizuno et al., 1990; Yap and Ng, 2001; Zhang et al., 2007). Multiple new methods have been used for the extraction of polysaccharides including enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) (Zhu et al., 2016b). The genus *Fusarium* belongs to the *Nectriaceae* family, order *Hypocreales*, *Sordariomycetes* class and Phylum Ascomycota. *Fusarium* species is widely distributed in various habitats. Soil is the most common habitat for this organism. Up to now, not much work on the antitoxicity activities of the polysaccharide extracted from soil *Fusarium* species has been done. The main purpose of this study was to evaluate an antitumor effect of polysaccharide extracted from *Fusarium* spp. isolated from soil samples of Karaj district, Alborz, Iran along with its taxonomic study.

2. Materials and methods

2.1. Isolation of filamentous fungus

Soil samples were collected randomly from the agricultural lands of Karaj district, Alborze province, Iran during the year 2012–2013. Isolation of *Fusarium* species from soil samples were performed by serial dilution method (Leslie and Summerell, 2006) and plated on Sabouraud Dextrose Agar (SDA) medium procured from Merk, Germany. After 7 days, the incubated plates at 25 °C were visualized under the stereo binocular microscope (Mangus MS24) for the presence of *Fusarium* sp. The isolate was subculture on SDA slants supplemented with 50 µg/ml chloramphenicol as antibacterial antibiotic using a single spore method (Leslie and Summerell, 2006). The pure culture of *Fusarium* strain was maintained at 4 °C for further study.

2.2. Taxonomic characterization of the strain FK1

Strain of FK1 was identified based on cultural, morphological and sequence analysis of 18S rRNA gene parameters.

2.3. Cultural and morphological characteristics

Cultural characteristic of the *Fusarium* was studied on SDA medium by visual and stereo binocular microscopic (Mangus MS24) examination. Morphological characters such as macroconidia, microconidia, chlamydospores and conidiogenous cells were also studied by light microscope (Leslie and Summerell, 2006; Gerlach and Nirenberg, 1982).

2.4. DNA extraction

The isolated *Fusarium* was grown in 500 ml flasks containing 100 ml of PDB medium for 5 days at 25 °C by agitation to form pellets of vegetative cells. Total DNA (100 mg was extracted from the mycelium of the isolate using a Fermentase kit (Fermentas Inc., Hanover, MD) according to the manufactures instructions.

2.5. Amplification and sequencing of 18S rRNA Gene

The 18S rRNA gene was amplified using PCR with Taq DNA polymerase and universal fungal primer pairs 0817F (5- TTAGCATGGAATAATRRAATAGGA-3) and 1196 R (5- TCTGGACCTGGTGAGTTTCC-3). The procedure was performed using Thermal Cycler PCR (Eppendorf, Germany), in a total volume of 25 µl containing 50 ng/µl DNA, 10 µmol each primer, 10 mM dNTPs, 2.5 µl 10X PCR buffer and 0.25 Unit *Taq* DNA polymerase Fermentase kit (Fermentas Inc., Hanover, MD). PCR conditions include; initial denaturation at 95 °C for 30 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 30 min and primer extension at 72 °C for 30 s and the final extension at 72 °C for 10 min. The PCR products were detected by 1% (w/v) agarose gel containing DNA safe stain (15 µl) and were visualized by Ultraviolet (UV) fluorescence gel documentation system (UTP-Bio Doc, USA). Sequencing of PCR product was carried out after PCR product clean up. The eluted pure PCR products were, subsequently sequenced by an automated gene sequencer (3730 xl DNA analyzer, Applied Biosystems, U.S.A).

2.6. Fermentation and extraction of polysaccharide compounds from strain Fusarium fungus

The fungus was cultured in SDA medium and incubated at 25 °C for 5 days. Fermentation was carried out in a 1 L Erlenmeyer flask containing 250 ml of modified SDB medium supplemented with 20% of glucose (Shuler and Karagi, 1992). One 6 mm agar disk was used as a seed culture to inoculate the fermented broth and incubated in an orbital shaker (150 rpm) for 10 days at 28 °C. After the incubation period, the mycelium biomass was separated from the culture supernatant through two layer Watchman No. 1 filter paper. Then, the mycelium biomass was used for further analysis. The polysaccharide was extracted from the mycelium free supernatant which was washed by phosphate buffer for three times. Extraction of the polysaccharide was done by boiling water method (Mizuno et al., 1984; Mizuno, 1999).

2.7. Assay of protein and DNA in polysaccharide

The purity of extracted polysaccharide was evaluated by spectrophotometry in 230 and 280 nm for protein assay and 260 nm for assaying of DNA.

2.8. Analysis of water extracted polysaccharide by FT-IR spectroscopy

For survey of lateral groups in extracted polysaccharide FT-IR spectroscopy (shimatzu FT-IR prestige 21) using KBr disk was done. Spectroscopy was used in limitation of 500–4000 cm^{-1}.

2.9. Evaluation of cytotoxicity activity

The cytotoxicity effect of extracted polysaccharide of *Fusarium* was evaluated by MTT assay (Mosmann, 1983). Two human cancer cell lines were employed including HeLa cells and LCLs. These two cell lines were obtained from the cell culture unit of molecular biology laboratory, Karaj Islamic Azad University, Alborz, Iran.

The cells were grown in 96 well plate (Greiner, Germany) in PRMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum. The cell suspension (10^5 cell/ml) was seeded in every well and incubated at 37 °C for 48 h in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells was exposed to various dilutions (10, 50, 100 mg/ml) of a polysaccharide extract for 24 h. The cell viability was measured using the MTT assay. The MTT solution (5 mg/ml) was added to the culture wells and the plates were incubated at 37 °C for 3 h. The optical density (OD) of the well contents was measured at 553 nm with an ELISA plate reader (Voller et al., 1978). ELISA is a simple method which enjoys some advantages such as portability of the equipment, hand-holding validation, and reliability for the analysis of samples (Kamkar et al., 2014; Mozaffari Nejad et al., 2013; 2014; Eslami et al., 2015). Cell control was maintained throughout the experiment and the assay was performed in triplicates.

3. Results

The filamentous fungus designated as strain FK1 isolated from agricultural lands of Karaj district, Alborz, Iran. The isolate FK1 was identified based on cultural, morphological and molecular methods.

The Morphological observation of the 7 days time culturing of fungus grown on SDA medium revealed that the strain formed white floccose that were abundantly uniform mycelium which were initially white to salmon becoming beige with time. Abundant, straight to curved macroconidia usually 4 septa formed from polyphialides. A few 1 to 2-celled microconidia. Chlamydospores formation is variable which distinguished *Fusarium* strain belongs to the family of Poaceae.

The results of the PCR and sequencing of the fungus confirmed that the strain belongs to the genus *Fusarium*. (Fig. 1).

The polysaccharide was extracted from the mycelium free supernatant of *Fusarium* using boiling water method and its purity was evaluated by UV spectrometry. The results showed less than 0.05% of protein and DNA in the extract and the high level of purity of the extract was confirmed.

The structure of the extracted polysaccharide was studied by FT-IR spectroscopy. The Results of FT-IR (KBr) spectrum of extracted polysaccharide showed absorbance in different zones. The band at 2923.07 cm^{-1} region was related to the lateral methylene groups. The bands at 1629.64 cm^{-1}, 1380 cm^{-1} and 1020.61 cm^{-1} were related to C=C, methyl symmetrical C—H, internal C–H groups, respectively. External C–H groups showed an absorption band at 875.12 cm^{-1} (Fig. 2).

The in vitro cytotoxicity effect of a water extracted polysaccharide of strain FK1 against two human cancer cell lines include Hela and LCL cell lines using the MTT method were studied. The polysaccharide extracted considered in different doses of 10, 50 and 100 µg/ml. The cytotoxicity effect was expressed by percentage of cell viability after 24 h exposure to different concentrations of the extracted polysaccharide.

The results of the cytotoxicity assay of water extracted polysaccharide from the *Fusarium* showed different effects on two cell lines. It showed maximum cytotoxicity activity against LCL which is cause of B lymphocyte cancer. The percentage of viability of LCL after treatment with polysaccharide extract of strain FK1 was at 50 µg/ml (114 ± 1.63) followed by 100 µg/ml (105 ± 0.57) and 10 µg/ml (104 ± 0.57), respectively. While it showed less cytotoxicity effects on HeLa cell lines which is cause of carcinoma at 100 µg/ml (100 ± 1.63) followed by 10 µg/ml (76 ± 0.81) and 50 (56.33 ± 0.55), respectively (Figs. 3–5).

Compact microscopy was used to observe the morphological changes of two cancer cell lines (HeLa and LCL). As per the results shown in Figs. 4 and 5, the untreated HeLa and LCL cells displayed a normal shape with no apoptosis, indicating the normal condition of these cells. However, after treatment with polysaccharide extracted from the *Fusarium* at increasing concentrations (10, 50 and 100 µg/µl), the LCL cell lines were found to be damaged or dead with evident cell
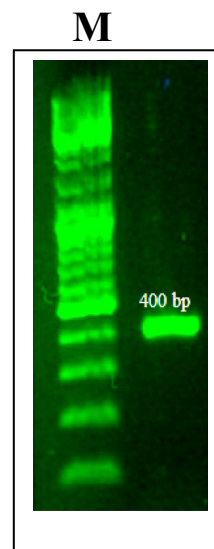

Figure 1   Agarose gel (1%) showing PCR products of *Fusarium* sp., along with Marker DNA (2.0 kb).
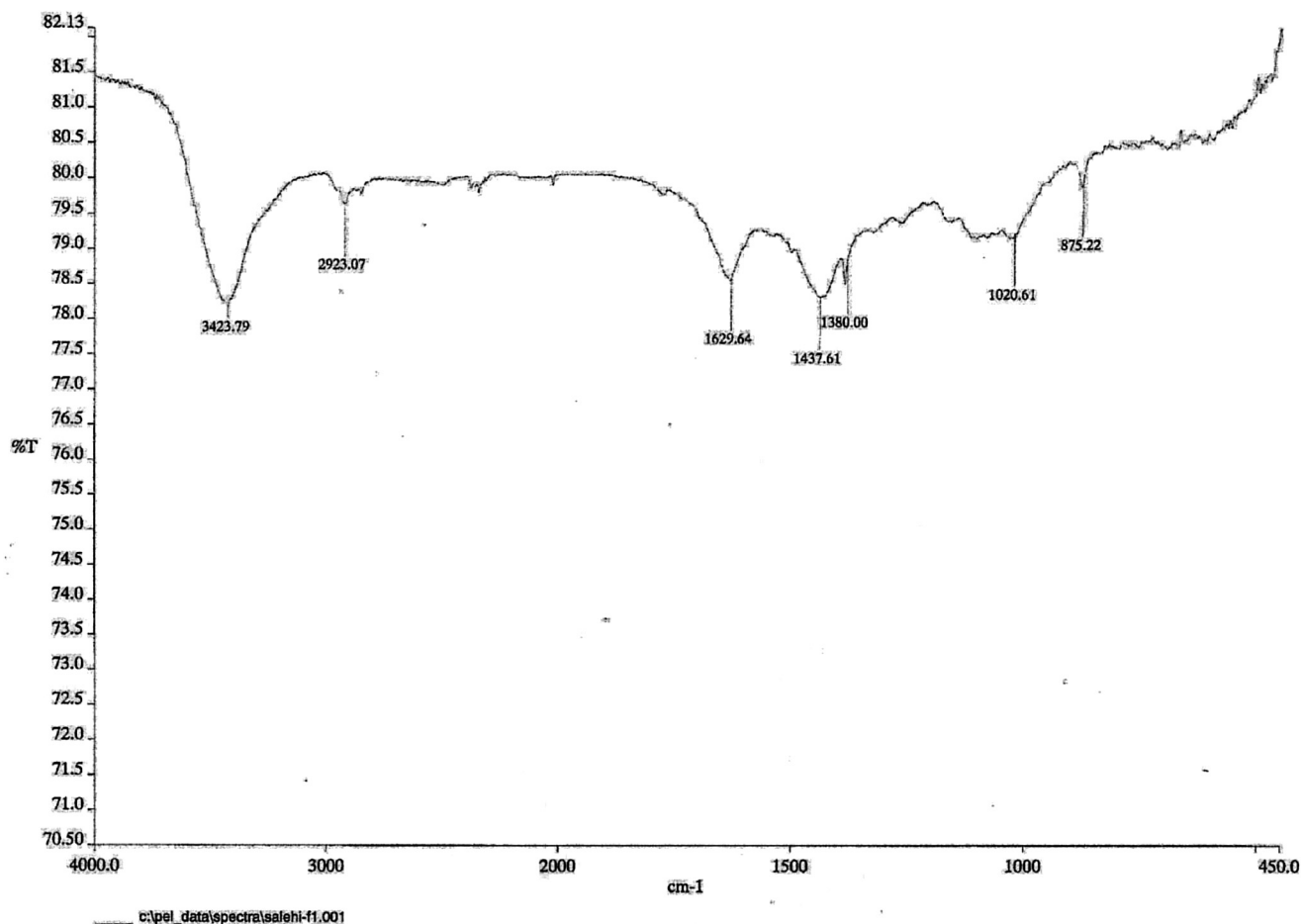
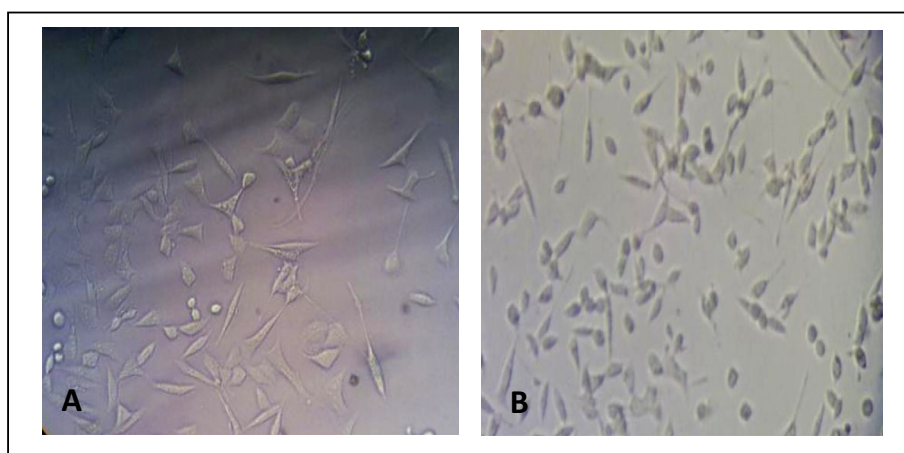**Figure 2** FTIR spectrum of the extracted polysaccharide.

Figure 3 The cytotoxity effect of polysaccharide extracted of strain FK1 on Hela cell line (A) before treatment and (B) after tratment.

morphological abnormalities (Figs. 3 and 4), indicating the apoptosis-inducing effect of the polysaccharides against LCL cell lines.

This difference of activity could be due to the difference in receptors of the cell surface, different signaling pathways and difference in virus that are obtained for carcinogenesis of these cell lines.

4. Discussion

Growth of cancer cells can destroy the surrounding environment and release a danger signal, and the immune system of the body can cause the immune response to this danger signal (Souni and Noel, 2013). These signals can lead to
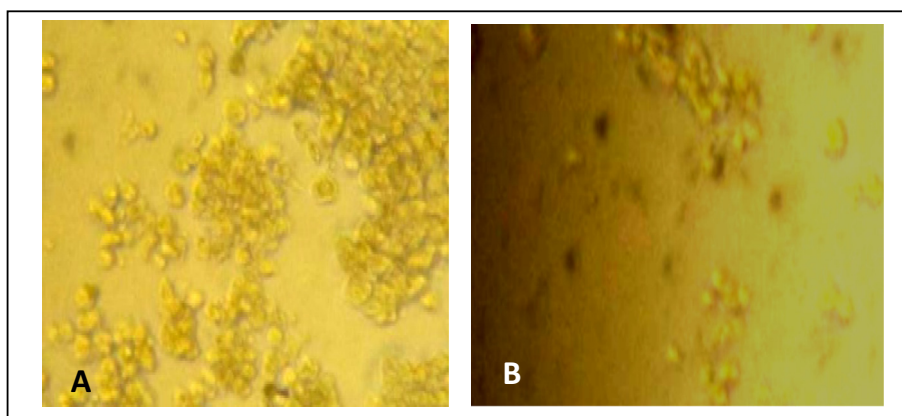
Figure 4 The cytotoxity effect of polysaccharide extracted of strain FK1 on LCL cell line (A) before treatment and (B) after tratment.
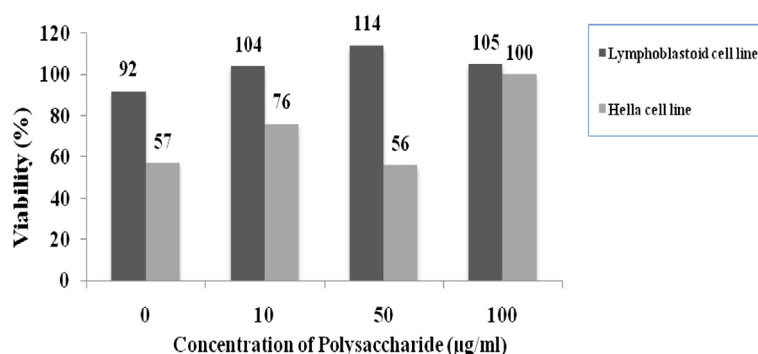

Figure 5 Percentage of cytotoxity effects of different concentration of Water extracted polysaccharide from the strain FK1 against Hella and LCL cell lines.

inflammation, activate the antitumor effector cells and the antigen-presenting cells, triggering the immune response of T cells and B cells (Guo et al., 2015; Trinchieri, 2015). Therefore, increasing the immune system of the body to recognize a dangerous signal or activate the effector cells with antitumor activity, such as, NK cells and LAK cells, is the main target of tumor immunotherapy (West et al., 2011). Polysaccharide drugs are novel antitumor substances, which exhibit a pharmacology role through numerous channels, and for multi –targets, immune regulation is the main pathway (Cheng et al., 2007; Lin et al., 2008). Polysaccharide of fungi can stimulate the immune system by making connection to some receptors of immune system cells such as, TLRs, Dectin, Mannose, CR3, CD4, which can lead to proliferate and differentiate the macrophages and dendritic cells. Therefore, they can activate different immune pathways such as; phagocytosis, complement and respiratory burst (Lull et al., 2005; Schepetkin and Quinn, 2006; Thompson et al., 2010). For example, the antitumor function of lentinan was a way of activated NK cells generation (Huang et al., 2010; Yap and Ng, 2001). Polysaccharide of Morchella esculenta is expressed NF-KB transcriptional factors in THP-1 cell line (Duncan et al., 2002). Polysaccharides of Ganoderma lucidum caused apoptosis of THP-1 (Chan et al., 2008; Hsu et al., 2011). Water-soluble polysaccharide of Agaricus blazei had antitumoral activity (Mizuno et al., 1990). Fungal polysaccharide can promote the phagocytosis of tumor cells by neutrophils (Rubin-

Bejerano et al., 2007) polysaccharide of Phellinus pullus fungus had antitumoral effects on S180 cell line (Yang et al., 2016). Similar research has been carried on polysaccharide extracted from Isaria farinosa B05 and Pleurotus ostreatus which was found to be inhibited the growth of HT-29 and HT-29 colon cancer cell lines (Jiang et al., 2008; Lavi et al., 2006).

5. Conclusion

In the present study, the Fusarium fungus isolated from agricultural soil sample of Karaj district, Alborz and its extracted polysaccharide was evaluated for the antitumor activities against two Human cancer cell lines. The extracted polysaccharide showed anti-tumor effect on LCL cell lines while it did not show considerable activity against HeLa cell lines. The results indicate promising baseline information for the potential uses of the extracted polysaccharide of the indigenous Fusarium isolated from Karaj in the In vivo study for treatment of tumors.

Acknowledgements

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