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Biological Property of *Fritillaria imperialis* L. Extract

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Abstract: A preliminary in vitro screening revealed the therapeutic status of extracts of *Fritillaria imperialis* L. that belongs to the *Liliaceae* family. Its tendrilled bulbs are consumed fresh or prepared in a powdered form and used as a home remedy for cough and phlegm, high fever, hemorrhage, lack of milk, treatment of abscesses, asthma, rheumatism, and eye disease. Herein, we investigated the antiproliferative, cytotoxic effects and antibacterial activities of *Fritillaria imperialis* L. extracts on three cancer cell lines (HeLa, HT29, and C6), and a non-cancer cells (Vero). The potential antiproliferative and cytotoxic impact of *Fritillaria imperialis* L. extracts were investigated *in vitro* through MTT and LDH measurement techniques, and its antimicrobial effects were studied with MIC and disc-zone test. The extracts of *Fritillaria imperialis* L. have been shown to exhibit poor antiproliferative effects and antibacterial activities on some cancer cell lines and bacteria, respectively, at even high concentration. These data suggest that *Fritillaria imperialis* L. extracts are low cytotoxic to cancer cell lines and *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). Our results indicate that clinic consideration of *Fritillaria imperialis* extracts for the treatment of malignant and bacterial disease needs to be re-evaluated due to its different extraction and isolation methods.

Keywords: *Fritillaria imperialis* L., HT29, HeLa, Anticancer activity, Cytotoxic activity.

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INTRODUCTION

Cancer, a common name for many diseases with poor prognosis is a persistent illness and constitutes a significant socioeconomic burden (1). Despite the dizzying advances in cancer studies, tumor treatments still remain far below the desired level with serious side effects together with persistent drug resistance. Therefore, we need to get more effective anticancer agents, natural products may be suitable anticancer drug candidates. From ancient times to present human beings have used plants as medicines for the treatment of different diseases, including cancer, or for the improvement of their health. Currently, human population preferring folk remedies reached approximately 70% of all populations (2, 3). Indeed, nowadays, natural products have received more research attention. This may be one reason that natural products examined so far have exhibited

potent antiproliferative effects adopting a mechanism-based approach. Also, based on their different kinetics, binding to organic molecules such as DNA, RNA or proteins and to be from natural source compared to synthetic drugs, natural products offer a great unexplored pharmacological area concerning the requirements of modern drug design. Today as in the past, the natural compounds and their by-products derived from remedies have gained importance in clinical use including cancer. Therefore, potential anticancer properties of medicinal plant extracts or isolated compounds can be monitored for anticancer drug development. As a result of these studies in the world, nearly 60% of drugs and 50% of all the currently used drugs in the clinic approved by FDA for cancer treatment are naturally occurring agents [4-6]. Several of them, such as taxol, vinblastine, vincristine, topotecan, irinotecan, and etoposide are in clinical use and some of them are about to enter the pipeline. Anatolian plants have marvelous diversity and are still untapped in current pharmacopeia, and they can be evaluated as an invaluable source of 'Hit to Lead' molecule (7, 8).

Medical plants are often consumed by being put into various foods or decocted in herbal tea and they provide significant various products such as alkaloids that have healing activity of folkloric remedy. The topic of this study focused on assessment of the pharmacological activity of extracts from *Fritillaria imperialis* L using cell cytotoxicity assay and visualized morphological alteration. However, to the best of our knowledge, the antiproliferative and cytotoxic features of *Fritillaria imperialis* L. extracts on cancer cells and the mechanisms underlying it have not yet been revealed. The antiproliferative activity for all natural products is performed in compliance with the European Pharmacopoeia 8.0 protocol. According to this protocol, these natural products for *in vitro* antiproliferative activity against HeLa (Human Cervix Carcinoma), HT29 (Human Colorectal Adenocarcinoma), C6 (Rat Brain Tumor Cells), and Vero (African Green Monkey Kidney) cells were conducted using a cell proliferation assay, cytotoxicity assay (LDH), and phase-contrast microscopic image evaluation techniques. In our study, we tried to address the possible anticancer activity of *Fritillaria imperialis* L. extracts.

MATERIALS and METHODS

Preparation of extracts

The stem (MS), leaf (ML), and flower (MF) parts of the *Fritillaria imperialis* L. were dried in the shade for two months at room temperature (each part 50 g ±2.0 g). At the end of this stage, dried *Fritillaria imperi*alis L. parts were thoroughly powdered with the help of a blender. Then, powdered *Fritillaria imperialis* L. parts were placed in an Erlenmeyer flask for extraction. Distilled methanol (250 mL) was added to the samples and was kept for one day at room temperature. At the end of this period, the liquid fraction was filtered through a filter paper to exclude the rough particles. To obtain the methanolic extract, the solvent was removed using a rotary evaporator (45 °C water bath, 700 mmHg). The extracts were stored at +4 °C.

Preparation of cell culture

The anticancer potential of *Fritillaria imperialis* L. extracts was investigated on cancerous HT29 (ATCC® HTB-38™), HeLa (ATCC® CCL-2™), and C6 cells (ATCC® CCL-107™) and nontumorigenic Vero cells (ATCC® CCL-81™). The cell lines were cultured in a cell medium (Dulbecco's modified eagle's medium, DMEM)

enriched with 10% (v/v) fetal bovine serum and 2% (v/v) Penicillin-Streptomycin (10,000 U/mL). First, old medium was removed out of the flask while cells had reached approximately 80% confluence. Next, cells were taken from the flasks surface using 4 mL of 0.5% trypsin-EDTA solution and neutralized by the addition of 15 mL DMEM enriched with 10% (v/v) fetal bovine serum and then subjected to centrifugation. Following, the cell pellet was suspended with 4 mL of DMEM working solution and was counted to obtain a final concentration of 5 × 10^4 cells/mL, and inoculated into wells (100 µL cells/well).

Cell proliferation assay (MTT)

A cell suspension containing approximately 1 × 10^4 cells in 100 µL was seeded into the wells of 96-well culture plates. *Fritillaria imperialis* L. extracts and 5-fluorouracil (5FU) (control drug) were dissolved in sterile DMSO (Dimethyl sulfoxide) (max 0.5% of DMSO) at final concentrations of 25, 50, 100, 150, 200, 250, 375, and 500 µg/mL. The cells were treated with *Fritillaria imperialis* L. extracts and 5FU at 37 °C with 5% CO_2 for overnight. The final volume of the wells was set to 200 µL by medium. Cell proliferation assay was evaluated by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) method. Briefly, An MTT stock solution (5 mg of MTT/mL of distilled water) was filter sterilized and kept for at -20 °C until use. The cells were exposed to MTT reagent for 4 h to form MTT formazan dye followed by the dye dissolved in DMSO with Sorenson's buffer for 30 min at room temperature and then the plate was measured at 560 nm, with 690 nm as a reference interval, using a microplate reader. Each experiment was repeated at least three times for each cell line.

Calculation of IC_{50} and % inhibition

IC_{50} value is a concentration that inhibits half of the cells in vitro. The half maximal inhibitory concentration (IC_{50}) (95% confidence intervals) of the *Fritillaria imperialis* L. *extracts* and control compounds was calculated using XLfit5 or excel spreadsheet. The proliferation assay results were expressed as the percent inhibition according to the following formula: where 'absorbance of treatments' provides information about the absorbance obtained from test compound treated cells and 'absorbance of DMSO' provide information about the absorbance obtained from DMSO treated control cells (maximum final concentration: 0.5 % DMSO).

$$Inhibition\ (\%) = 1 - \left(\frac{Absorbance\ of\ Treatments}{Absorbance\ of\ DMSO}\right) x100 \qquad \text{(Eq.1)}$$

Cytotoxic activity assay

The cytotoxicity of the *Fritillaria imperialis* L. *extracts* and 5-fluorouracil on HeLa, C6, HT29, and Vero cells was determined through a Lactate Dehydrogenase Assay Kit according to the manufacturer's instructions (Roche, LDH Cytotoxicity Detection Kit). Approximately 5 x 10^3 cells in 100 µL were placed into 96-well plates as triplicates and treated with IC_{50} (µg/mL) concentrations of *Fritillaria imperialis* L. extracts at 37 °C with 5% CO_2 for 24 h. LDH activity was obtained by determining absorbances at 492 and 630 nm using a microplate reader. The cytotoxicity assay results were noted as the percent cytotoxicity according to the following formula: where 'low control' provides information about the LDH activity released from the untreated cells, 'high control' is the maximum amount of releasable LDH enzyme activity which is

determined by lysing the cells with Triton X-100 (final concentration: 1% Triton X-100).

$$Cytotoxicity\ (\%) = \left[\frac{(Experimental\ Value - Low\ Control)}{High\ Control - Low\ Control} x100\right] \qquad \text{(Eq. 2)}$$

Cell imaging

Cells were seeded into 96-well plates at a density of 5.000 cells per well and allowed to 24 h. IC_{50} values of the *Fritillaria imperialis* L. extracts were administered and morphology alters of the cells were screened by phase contrast microscopy every 6 h for 24 h. Images of control and *Fritillaria imperialis* L. extracts treated cells were photographed at the end of the process using a digital camera attached to an inverted microscope.

Antimicrobial activity

We investigated the antimicrobial activity of the *Fritillaria imperialis* L. extracts against one gram-positive bacteria (*S. aureus* ATCC25923) and one gram-negative bacteria (*E. coli* ATCC25922) by using disc-diffusion method with reference to EUCAST.

RESULTS AND DISCUSSION

Antiproliferative effect of the *Fritillaria imperialis* L. extracts

Plants generally produce biologically active compounds to protect themselves against a variety of micro- and macro-organisms. For this reason, it is a very realistic approach that the majority of active molecules obtained from natural sources have anticancer and antimicrobial features. Important agents approved by US Food and Drug Administration (FDA) such as anti-cancer (Taxol), topoisomerase inhibitor (Camptothecin), antimicrobial (Erythromycin), antibiotic aminoglycoside (Kanamycin), and antibiotic β-lactam (Cephalosporin C) used in modern medicine are of herbal origin. In addition to the above mentioned medicines, herbal products, which have been known to be effective in the folk cure for many years, are used in various countries according to their pharmacopoeial monographs. The *Fritillaria imperialis* L., which is one of these, is an ornamental plant including various pharmaceutically active components, which have been commonly used as a traditional remedy. Especially, in traditional Chinese medicine, *Fritillaria spp* bulbs are known to have some medicinal features such as treating bronchitis, becoming a diuretic, calming heart spasms (9 – 14). According to traditional descriptions, *Fritillaria spp* bulbs have been added into some special Chinese herbal formulas for cancer patients (9). Here, we aimed to understand the response of cells upon administering *Fritillaria imperialis* L. extract *in vitro*. The antitumor feature of the stem, leaves, and flower extracts of the *Fritillaria imperialis* L. on cancer cells were screened by MTT assay. In this assay, 5-fluorouracil (5-FU) was used as a positive control. As shown in Figure 1, *Fritillaria imperialis* L. extracts did not show any anticancer activity. In addition, none of the concentrations of the *Fritillaria imperialis* L. extracts reached the half maximal inhibitory concentration (IC50). It can be easily seen that stem, leave, and flower part of the

Fritillaria imperialis L. extracts have not shown therapeutic action against the cervix, colon, and brain cancer cells. It is likely that these parts of the plant do not contain a strong metabolite or the extraction method used may be insufficient. Actually, the bulb part of *Fritillaria spp* includes several interesting molecules such as steroidal alkaloids (imperialine and verticine), sesquiterpenes and glycosides as well as many other compounds such as saponins, terpenoids [9 - 14]. To date, stem, leave, and flower part of *Fritillaria spp* were not characterized for their chemical components with potential medicine utility. However, we may speculate that stem, leave, and flower part of *Fritillaria imperialis* L. extracts possibly can be used as an adjuvant agent in treatment along with conventional therapies.

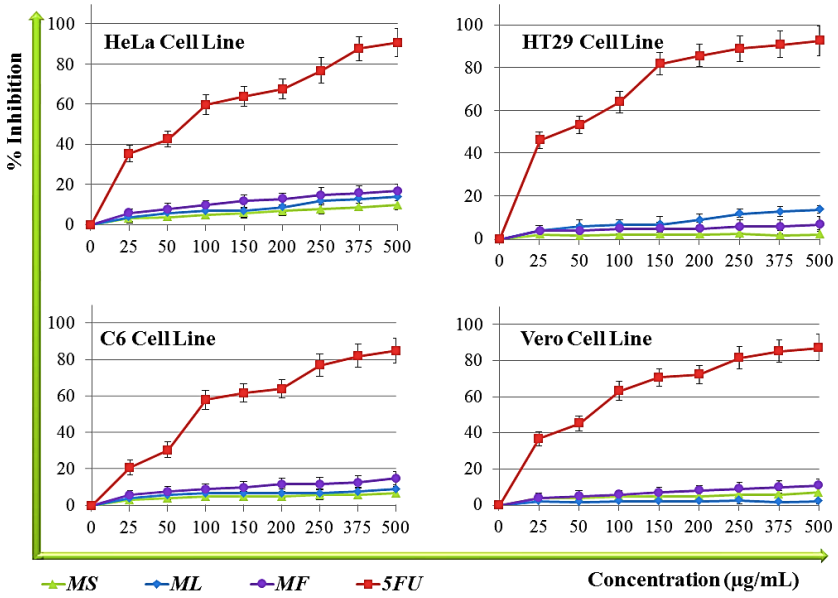

Figure 1. Antiproliferative effects of methanol (MS, ML, and MF) stem, leaves, and flower extracts, respectively, and positive control 5FU on HeLa, HT29, C6, and Vero cell lines. Cell proliferation measurement was carried out with MTT assay. Inhibition percentage was reported as ± SEM value of three independent measurements ($P < 0.05$). Each experiment was triplicated for each cell line.

Cytotoxic activity and morphological assessment of the *Fritillaria imperialis* L. extracts

One of the aims of the present study was to evaluate the effects of *Fritillaria imperialis* L. extracts on the membrane integrity and to determine the cytoprotective activities. Cytotoxic activities of *Fritillaria imperialis* L. extracts on HeLa, HT29, C6, and Vero cell lines was assessed by the LDH cytotoxicity assay kit. HeLa, HT29, C6, and Vero cells were grown in the presence or absence of *Fritillaria imperialis* L. extracts (250 µg/mL) for a period of 24 h.

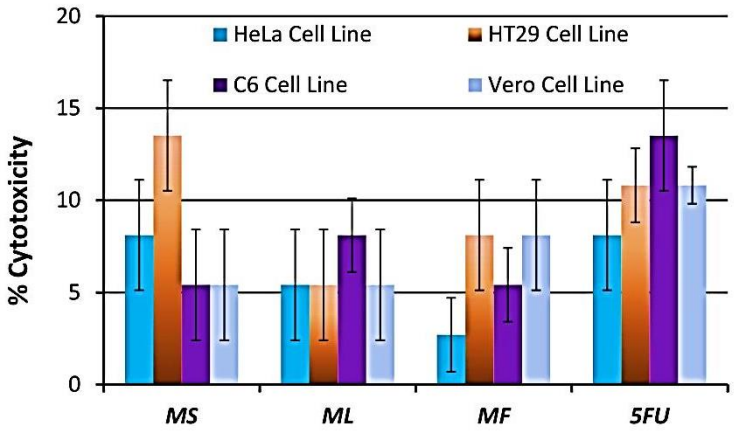

Figure 2. Cytotoxic effects of *Fritillaria imperialis* L. extracts on HeLa, HT29, C6, and Vero cell lines. Cell lines were incubated with extracts of 250 µg/mL for 24 hrs and cytotoxicity was determined using the LDH cytotoxicity kit. Inhibition percentage was reported as ± SEM value of three independent measurements ($P < 0.05$).

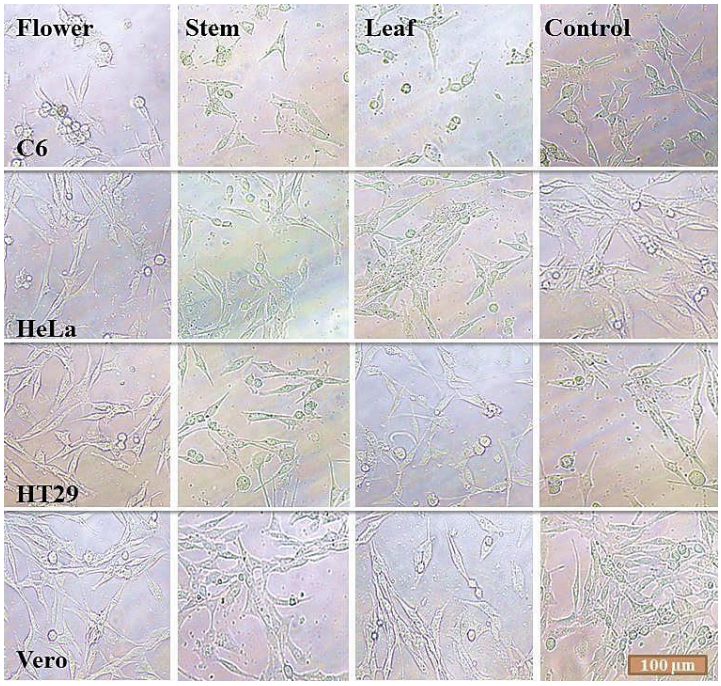

Figure 3. Effects of *Fritillaria imperialis* L. extracts on the morphologies of HeLa, HT29, C6, and Vero cell lines. Exponentially growing cells were incubated overnight with 250 µg/mL concentrations of the extracts at 37 °C. Control cells were treated with only DMSO.

Cytotoxicity results demonstrated that at concentrations 250 µg/mL, *Fritillaria imperialis* L. extracts did not affect LDH leakage from the cells (Figure 2). Therefore, *Fritillaria imperialis* L. extracts protected the cell membrane integrity. This probably means that *Fritillaria imperialis* L. extracts can be used as cytoprotective agents. In order to evaluate the effect of *Fritillaria imperialis* L. extracts on the cell morphology, phase contrast images were visualized by a digital camera attached inverted microscope (Leica IL10, Germany). The picture showed inverted microscope images of the morphology of treated cell lines with 250 µg/mL concentrations of *Fritillaria imperialis* L. extracts as compared to

respective controls. As shown in Figure 3, at 250 µg/mL concentration, treated cells continued to maintain their normal morphology when compared with control cells. Similar to the control cells, all treated cells were astrocyte-like or fibroblast-like appearance and displayed confluent situation in plate surface. Generally, *Fritillaria imperialis* L. extracts treated cells did not show any apoptotic indicator structures such as cell shrinkage, apoptotic bodies, and atypical forms. Thus, microscopic observations may indicate that *Fritillaria imperialis* L. extracts may be associated with cell survival mechanism.

Antimicrobial effect

The screening results revealed that *Fritillaria imperialis* L. extracts showed no antimicrobial activity against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922). In a similar study, it was noted that *Fritillaria imperialis* L. exhibited antifungal activity but not antimicrobial activity (15). However, another study displayed that some *Fritillaria* spp. accomplish strong antimicrobial activity due to different solvents (16).

CONCLUSION

Generally, chemotherapeutic cure resulted in the debilitating effect on cells, and this situation is the most important obstacle for the treatment of cancer. *Fritillaria* spp. bulbs have been long used in traditional medicine for primary health care to possess medicinal features. Today, we know that *Fritillaria* spp. bulbs extracts are found in formulations for treating cancer, Alzheimer's or respiratory system symptoms. However, to the best of our knowledge, the stem, leaf, and flower extracts of *Fritillaria imperialis* L. have not been studied yet. *Fritillaria* spp. bulbs contain various pharmaceutically active constituents that possess anticancer and cytotoxic features by using the cellular stress or immunomodulating mechanisms (17-20). On the other hand, *Fritillaria imperialis* L. extracts (stem, leaf, and flower) may activate survival pathways. The dual effect of *Fritillaria imperialis* L. bulb and the other parts on cells could provide a powerful strategy in cancer cure. Overall, our findings would seem to suggest that further study using both *Fritillaria imperialis* L. bulb extracts and stem, leaf, and flower extracts can be promoted as an efficient way to open desirable treatment options.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AA ED ME. Performed the experiments: AA ED. Analyzed the data: AA ED ME ŞT. Contributed reagents/materials/analysis tools: AA ED ME.

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