Cytotoxic Effects of Ferula Latisecta on Human Glioma U87 Cells

Authors
Mohammad Jalili-Nik1 *, Hamed Sabri1 *, Ehsan Zamiri2, Mohammad Soukhtanloo1, Mostafa Karimi Roshan1, Azar Hosseini2, Hamid Mollazadeh3, Mohammad Mahdi Vahedi4, Amir R. Afshari5, 6, Seyed Hadi Mousavi6

Affiliations
1 Department of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
2 Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran
3 Department of Physiology and Pharmacology, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran
4 Department of Pharmacology, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran
5 Department of Pharmacology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
6 Medical Toxicology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

Key words
glioblastoma multiforme, Ferula latisecta, apoptosis, metastasis

ABSTRACT
Glioblastoma multiforme (GBM) is the fatal type of astrocytic tumors with a survival rate of 12 months. The present study, for the first time, evaluated the cytotoxic impacts of Ferula latisecta (F. latisecta) hydroalcoholic extract on U87 GBM cell line. The MTT assay measured the cellular toxicity following 24- and 48 h treatment with various doses of F. latisecta (0–800 μg/mL). Apoptosis was evaluated by an Annexin V/propidium iodide (PI) staining 24 h after treatment by F. latisecta. Moreover, to determine the cellular metastasis of U87 cells, we used a gelatin zymography assay (matrix metalloproteinase [MMP]-2/-9 enzymatic activity). The outcomes showed that F. latisecta mitigated the viability of U87 cells in a concentration- and time-dependent manner with IC50 values of 145.3 and 192.3 μg/mL obtained for 24- and 48 h treatments, respectively. F. latisecta induced apoptosis in a concentration-dependent manner after 24 h. Also, MMP-9 activity was significantly decreased following 24 h after treatment concentration-dependently with no change in MMP-2 enzymatic activity. This study showed that F. latisecta induced cytotoxicity and apoptosis, and mitigated metastasis of U87 GBM cells. Hence, F. latisecta could be beneficial as a promising natural herb against GBM after further studies.

Introduction
Malignant brain tumors are uncontrolled brain diseases with different clinical features, such as cognition impairment, personality changes, headache, sensory loss, and gait imbalance. The fatal type of brain tumors is glioblastoma multiforme (GBM) and contains some mutations that causing overgrowth and survival of malignant cells, leading to invasion, metastasis, and resistant to apoptosis [1, 2]. Despite the improved therapeutic approach with alkylating agents, particularly temozolomide (TMZ), GBM has high morbidity and
and mortality in patients. Therefore, founding the new medicines is an effective strategy for the treatment of GBM progression [3].

In recent years, the role of medicinal herbs for the treatment of cancer has attracted many scientists [4]. Ferula latisecta (F. latisecta) belonging to the Apiaceae family is widely used in Asian traditional medicine [5]. In natural treatments, the Ferula genus plants are utilized as antispasmodic, antibacterial, anti-fungal, anti-cough, analgesic, and anti-fever agents [6–9]. The genus Ferula is the main source of biologically active compounds, for instance, sesquiterpenes and coumarins. Recent studies have shown potent antioxidant, apoptotic activity, and cytotoxic effects of Ferula genus, as well [10–12]. Also, the cytotoxic impacts of methanolic extract of F. latisecta roots have been reported in preliminary screening assays of Soltani et al. research. It was found that active constituents of F. latisecta and other ferula genus (persicaria-lutea, A. confereone, and feselol) exhibited moderate cytotoxicity against HeLa (cervical), A549 (lung), A2780 (ovarian), and HCT116 (colon) cancer cells [13, 14].

Collectively, these examinations feature the need for an assessment of the cytotoxic effects of F. latisecta in GBM. Hence, the current study was designed to determine the cytotoxic impacts of F. latisecta hydroalcoholic extract against human U87 cells for the first time.

Material and Methods

Cell lines and substances

U87 GBM cells were obtained from the National Cell Bank of Iran (NCBI, Tehran, Iran). The 4, 5-dimethylthiazole-2-yl, 2, 5-diphenyl tetrazolium (MTT) and trypan blue were obtained from Sigma (St. Louis, MO, USA). Annexin V-FITC early apoptosis was purchased from Cell Signaling (MA, USA). Trypsin-EDTA, High glucose Dulbecco’s Modified Eagle’s medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Sodium bicarbonate, sodium hydroxide, dimethyl sulfoxide (DMSO), Triton X-100, and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany).

Preparation of F. latisecta hydroalcoholic extract

Aerial parts and the roots of the plant were gathered and distinguished by botanists in the herbarium (voucher specimen: 477) of Payame Noor University, Dargaz, Iran. The powdered parts (500 g) were dried, weighed, and homogenized in 80 % ethanol at a proportion of 1:10 of the plant to ethanol and left to soak for 3 days at 37 °C with suitable shaking and blending. Then, the mixture was filtered, and the subsequent fluid was concentrated under pressure at 37 °C with suitable shaking and blending. Then, the mixture was poured on 1:10 of the plant to ethanol and left to soak for 3 days at 45 °C in an EYELA rotary evaporator (7 %, w/w). The concentrate was then kept in the incubator at 45 °C for 3 days to evaporate the ethanol residue yielding the crude extract (55.52 % w/w) and then remained at −20 °C until utilize.

Cell proliferation (MTT) assay

10^4 U87 cells (per each well) were seeded, and after overnight, the cells were incubated with various concentrations of F. latisecta hydroalcoholic extract (0–800 μg/mL) for 24 and 48 h. After that, the MTT solution in phosphate-buffered saline (PBS, 5 mg/mL) was added to each well at a final concentration of 0.05 %. Following 3 h, the formazan precipitate was disintegrated in DMSO containing 10 % glycine buffer (pH = 10.5). The microplates were then gently shaken in the dark for 30 min, and the absorbance was measured at 570 and 620 nm (background) using a Stat FAX303 plate reader. All the treatments were done in triplicate.

Determination of cell death by Annexin V/PI staining

Annexin V (AnnV) and PI staining were done by utilizing the Annexin-V-FITC Early Apoptosis Detection Kit (Cell Signaling, USA). After 24 h of cells incubation (7 × 10^4 in 6-well culture plate) with treatments (200 and 800 μg/mL), cells were harvested and washed twice with ice-cold PBS and re-suspended in 200 μL of 1 × binding buffer containing Annexin V. Then, 96 μL of cells was transfer to flowcytometric tube and 1 μL conjugated Annexin V-FITC, and PI (12.5 μL) was added to the cells. The cells were incubated for 10 min at 0 °C in the dark. After that, the final volume was set at 250 μL with 1 × binding buffer containing Annexin V. The quantity of viable, early apoptotic, late apoptotic and necrotic cells was measured by a flow cytometer (Becton-Dickinson, San Diego, CA, USA).

Gelatin zymography activity

The gelatinolytic activity was assayed by gelatin zymography [15]. Briefly, the U87 cells were treated with various concentrations of F. latisecta (200 and 800 μg/mL) for 24 h. Cultured media were centrifuged at 4 °C for 10 min at 15 000 rpm, and 30 μg of total protein from the supernatant was electrophoresed onto a 7.5 % polyacrylamide gel containing 0.1 % sodium dodecyl sulfate (SDS) and 1 mg/mL gelatin as a substrate. After that, the gel was washed three times with washing buffer containing 2 % Triton X-100 (to remove the SDS) every 20 min, and it was then incubated for 24 h at 37 °C in incubation buffer. The gel stained with Coomassie Brilliant Blue R-250 for 30 min to visualize bands of proteolytic activity, and then it was de-stained with the de-staining solution (20 % methanol, 10 % acetic acid in dH₂O) and dried directly. The gelatinolytic activities (zones of gelatin degradation) were detected by utilizing GS-800 calibrated densitometer (Bio-Rad, HC, USA) through bright bands against a background of uniform staining and the analysis was performed by using Image J 1.52a software (NIH, Bethesda, Rockville, MD, USA) compared to the control groups.

Statistical analysis

The obtained data were analyzed utilizing the software GraphPad Prism® 7.01 (GraphPad Software, San Diego, CA, USA) and the values were compared using the one-way analysis of variance (ANOVA) followed by Dunnett test. Furthermore, the study of apoptosis and metastasis was done by FlowJo® xV.0.7 (Tree Star, Ashland, OR, USA) and Image J 1.52a software (NIH, Bethesda, Rockville, MD, USA) software, respectively. Values less than 0.05 were considered to be statistically significant. The results are presented as the mean ± standard error.

Results

The effects of F. latisecta on U87 cells proliferation

U87 cells were treated with various concentrations of hydroalcoholic extract of F. latisecta (0–800 μg/mL) for 24 and 48 h. As shown in Fig. 1, F. latisecta mitigated the U87 cell viability by increasing...
The effects of *F. latisecta* on U87 cells cell death

In 24 h after treatment, apoptotic and necrotic cells at *F. latisecta* concentrations of 200 and 800 μg/mL were reported as 13.25 and 57.51 %, respectively, as shown in [Fig. 2a]. Furthermore, as shown in [Fig. 2b], it is summarized the percentage of apoptosis in each phase and all together. The results revealed that *F. latisecta* induced apoptosis after 24 h of treatment significantly ([Fig. 2c]).

The effect of *F. latisecta* on MMP-2/-9 enzymatic activity

To investigate whether *F. latisecta* (200 and 800 μg/mL) suppresses MMP-2/-9 activity, we performed a gelatin zymography assay. As shown in [Fig. 3], we observed a significant reduction in the level of MMP-9 (p > 0.05 and p < 0.01) enzymatic activity after 24 h. The level of MMP-2 activity 24 h after treatment by *F. latisecta* did not any significant difference compared to the control group.

Discussion

To the best of our knowledge, this examination, for the first time, exhibited that *F. latisecta* might be a useful agent against GBM. The present study showed that *F. latisecta* triggered apoptosis and metastasis and induced cytotoxicity on U87 cell line.

Cancers are some of the disorders that the researchers are interested in them because of their different type and human mortality [16, 17]. GBM is one of the most malignant astrocytic tumors with poor prognosis [18]. Several studies are performing to present some medicines at a lower cost, lower adverse effects, and also with natural sources [19]. Thus, numerous researches have been done to discover a new drug with cytotoxic effects from these sources [20, 21].

*F. latisecta* from Ferula genus is an endemic plant belongs to Iran [22]. Numerous studies have shown various biological activity of Ferula genus [23]. It has been documented that specific constituents of Ferula species include sesquiterpenes, coumarin derivatives, and Sulphur compounds [24]. In the present study, hydroalcoholic extract of *F. latisecta* was evaluated for its cytotoxic properties in human malignant U87 cells, and we have investigated the effect of *F. latisecta* on U87 cells viability, apoptosis, and cell metastasis. The results of the present studies have indicated that *F. latisecta* hydroalcoholic extract has suppressive impacts on the viability of U87 cells. MTT assay revealed that *F. latisecta* extract significantly decreased cell growth dose- and time-dependently. Furthermore, 50 % inhibition of cell proliferation (IC₅₀) values of 145.3 and 192.3 μg/mL, following 24- and 48 h treatment was observed, respectively. Notably, a study showed that *F. latisecta* had no cytotoxic effects in non-malignant fibroblast cells, indicating that *F. latisecta* is more likely to be toxic to cancer cells than non-malignant cells [25]. Also, flow cytometry analysis proved that *F. latisecta* extract remarkably induces apoptosis in GBM cells after 24 h. These outcomes are consistent with previous studies that demonstrate Ferula species have anticancer impacts. The cytotoxic effect of methanolic extract of *F. latisecta* roots has also been shown in some screening assays [26]. In a study, the inhibitory effects of fifteen sesquiterpene coumarins, which were isolated from Ferula species, were evaluated on p-glycoprotein-mediated multidrug resistance in the doxorubicin resistance-breast cancer cell line. It has been concluded that these agents could reverse doxorubicin resistance [27].

Apoptosis is one of the essential approaches in the treatment of cancer [28–32], especially in brain tumors [31–35]. Asemani et al. showed that the anti-cancer potential of Ferula hezarlalehzarica Y. Ajani fraction in Raji lymphoma cell line is through induction of apoptosis [36]. Our results have shown that *F. latisecta* significantly induces apoptosis in a concentration-dependent manner in U87 cells. Although, with increasing concentration, necrosis was the main event in U87 cells. The results of a study displayed Ferula gummosa oleo resin gum induced apoptosis concentration-dependent-
ly by ROS mechanism in (NB4 and HL-60 cells) human leukemic cells [37]. Besides, in a study, Ferula pseudalliacea causes apoptosis in human colorectal cancer HCT-116 cells via a mitochondrial-dependent pathway [38].

Degradation of extracellular matrix (ECM) assumes a significant role for cancers to metastasis. MMPs, particularly for MMP-2 and MMP-9, are the main enzymes for the corruption of ECM. Several studies showed that MMP-9 was detected in 50% of GBM patients.
Also, increased MMP-2 was found in 52% of GBM patients [39]. Xue et al. in their study evaluated the high expression of MMP-9 in glioma, and it is associated with patient survival rates [40]. So, MMP-2 and MMP-9 could be molecular targets in the treatment of GBM. In line with these observations, treatment with F. latisecta significantly decreased the enzymatic activity of MMP-9 in a concentration-dependent manner in GBM cells. We did not see a remarkable difference in MMP-2 enzymatic activity. Therefore, since MMP-9 plays a role in patients’ prognosis, then by inhibiting this enzyme, it can be helpful in the survival of patients.

Taken together, the outcomes of the current study provide the first report that F. latisecta has cytotoxicity and apoptosis effects and inhibits metastasis, possibly through modulation of MMP-9 enzymatic activity in U87 GBM cells. So, F. latisecta could be useful as a promising herbal medicine in treating GBM after further mechanistic studies.

Acknowledgments

This work was supported by Mashhad University of Medical Sciences, Mashhad, Khorasan Razavi, Iran (grant number: 960200). Also, we appreciate all members of the Research Laboratory of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Sciences (MUMS), for their technical support.

We are very thankful to Dr. Sima Khosravi, a kind physician and a great mother for the first corresponding author (ARA) who passed away by GBM disease in fall 2016.

Conflict of Interest

No potential conflict of interest was reported by the authors.

References


[34] Piperigkou Z, Manou D, Karamanou K et al. Strategies to Target Matrix Metalloproteinases as Therapeutic Approach in Cancer, in Proteases and Cancer. 2018: Springer; p 325–348


[40] Xue Q, Cao L, Chen XY et al. High expression of MMP9 in glioma affects cell proliferation and is associated with patient survival rates. Oncology Letters 2017; 13: p 1325–1330