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Antiproliferative Effects of *Ferula assa-foetida's* Extract on PC12 and MCF7 Cancer Cells

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Abstract: Background: *Ferula assa-foetida* is a herbaceous plant of the Umbelliferae family having a broad spectrum of biological activities such as antiseptic, antibacterial, anti-inflammation, and, anti-tumor activity against a wide range of cancers. Among these features, antitumor activity has become more important in recent years and it still demands more investigations to address the underlying mechanisms. Purpose: This current study was conducted to evaluate the anti-proliferative effect of *Ferula assa-foetida* on PC12 and MCF7 cells as well as examining its mechanisms. Materials and Methods: Cells were cultured in DMEM medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells incubated with different concentrations of the ethanolic extract of *Ferula assa-foetida*. Notably, cytotoxicity and apoptosis assay were measured by MTT and PI staining, respectively. Results: The MTT results showed that the ethanolic extract of *Ferula assa-foetida* in concentrations of 10, 7, 5, and 2.5 µM on both PC12 and MCF7 cells had a significant effect in cell viability and apoptosis induction in comparison to control group. Conclusion: In this study, it was determined that *Ferula assa-fetida* through the induction of apoptosis prevented the growth of PC12 and MCF7 cells and made a reduction in cell viability with different concentrations in a time- and dose-dependent manner. However, more studies are needed to reveal the mechanisms of *Ferula assa-foetida's* extract in apoptosis induction.

Keywords: *Ferula assa-foetida*, Apoptosis, Cytotoxicity, Cancer, MCF7, PC12

1. Introduction

Cancer is one of the most important public health problems worldwide, which is the second leading cause of death in the world [1]. In industrialized countries, breast cancer is the most common type of cancer among women [2], and common therapies are including radiotherapy and chemotherapy that stop cell cycle or induce cell death through apoptotic or non-apoptotic mechanisms such as necrosis, mitotic catastrophe, and autophagy [3-5]. These therapies have some disadvantages, including damage to healthy cells [6]. Despite advances in the development of novel drugs, cancer treatment still demands precise and specific drugs in order to reduce the side effects of chemotherapeutics. To date, various natural compounds have shown promising insights on the treatment and prevention of cancer having the advantage to lower the side effects. Natural plant products can play an important role in cancer treatment through blocking tumor and inducing apoptosis [7].

Herbal plants are important sources of bioactive compounds which have shown promising insights in the treatment of various cancers [8]. The advantages of traditional herbs are their lower toxicity, cost and side effects [9, 10]. The efficacy of these chemotherapy drugs is largely related to its antioxidant potential which reduces or inhibits damages of free radicals to DNA, lipids, and proteins as macromolecules [11] likewise antitumor drugs which their mechanism relies on the production of free radicals [12].

However, herbal plants such as *Ferula assa-foetida* has shown apoptosis induction by modulating the expression of genes related to this cellular process [8].

Ferula assa-foetida is found as a natural plant in the central region of Iran. The stems and roots of this plant and other species have clinical applications [13]. The root of this plant is extensively used in traditional medicine including the treatment of influenza [14], and also its dried extract is used in the treatment of hysteria, whooping cough, and ulcers in Afghanistan [15]. The extraction of *Ferula assa-foetida* is utilized as a vermifuge in China [16]. In Egypt, its dried root extract is used as an antispasmodic, analgesic, diuretic, and vermifuge [17]. In Malaysia, it is used as amenorrhea [18], and has applicable as an antiepileptic in Morocco [19]. In Saudi Arabia, the dried plant is used for whooping cough, asthma, and bronchitis [20]. It is used to treat gastrointestinal diseases by removing wind from the stomach and also treats any spasmodic disorders. It is as a nervine stimulant, digestive, and sedative agents [8, 21]. In folk medicine, the roots of this plant are used as perceived anthelmintic, anticarcinogenesis, anti-HIV, antimicrobial, antirheumatic, antispasmodic, diuretic, and emmenagogue [14]. The pharmacological and biological studies of *Ferula assa-foetida have* also reported a large number of drug activities such as antioxidant [22], antileishmanial [23], anticonvulsant [24], anti-diabetic [25], antispasmodic [26], hypotensive [27], and antinociceptive [28]. Recent statistics have shown that cancer rates are higher in countries such as Japan, Russia, China, and Indonesia in comparison to countries using *Ferula assa-foetida*this is common [29]. In current studies, the antioxidant, antimutagenicity, and anticancer activities of this plant have been reported [21].

2. Materials and Methods

2.1. Chemicals

PC12 and MCF7 cell lines were obtained from the Pasteur Institute (Tehran, Iran). Dulbecco's phosphate-buffered saline (PBS) and 4, 5-dimethylimidazole-2-yl, 2, 5-diphenyl tetrazolium (MTT) were purchased from Sigma (St Louis, MO, USA). Glucose-high Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). Dimethyl sulfoxide (DMSO) was bought from Merck (Germany). Propidium iodide (PI), sodium citrate and Triton X-100 were purchased from Sigma (St Louis, MO, USA).

2.2. Cell Culture

Cells were cultured in DMEM with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were plated and incubated with various concentrations of *Ferula assa-foetida* for 24, 48 and 72 h. For MTT assay, cells were seeded at 5000/well on to 96-well culture plates. For each concentration and time course, a control sample which remained untreated and received the equal volume of the medium has been used. For apoptosis assessment, cells were seeded at 1×10^5/well on to a 24-well plate [30]. All different treatments were carried out in triplicate.

2.3. Drug Preparation

The upper part of *Ferula assa-foetida* maintained at room temperature for 10 days. Following being dried, the powder was prepared. The dried powder was extracted by ethanol keeping at room temperature for 7 days. Interestingly, the ethanol extract was dried by evaporation. To prepare concentrations of 10, 5, 2.5, 1.25, 0.62, 0.31, 0.15, 0.07, and 0.03µM, the dried extract was solubilized in culture medium.

2.4. Cell Viability

The cell viability was determined using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay [30]. Briefly, PC12 and MCF7 cells were seeded at 5×10^3/well in flat-bottom 96-well culture plates and allowed to grow for 24, 48 and 72h following treatment with an ethanolic extract of *Ferula assa-foetida* at different concentrations [0.0312 up to 10 µM]. After the medium was removed, cells were incubated with MTT solution (5mg/ml in PBS) for 4h and the resulting formazan was solubilized with DMSO (100 µl). The absorption was measured at 570 nm (620 nm as a reference) using an ELISA reader. Later, IC50s were determined through Prism software. All treatments were carried out in triplicate.

2.5. Cell Apoptosis Assay

Based on PI staining of small DNA fragments, apoptotic cells were detected using flow cytometry. It has been reported that a sub-G1 peak reflects DNA fragmentation observed following the incubation of cells with a hypotonic phosphate-citrate buffer containing a quantitative DNA-binding dye, such as PI. Briefly, PC12 cells were seeded in a 24-well plate and treated with the concentrations of 0.26, 2.63, and 4.69 µM of *Ferula assafoetida*. Also, MCF7 cells were seeded in a 24-well plate and treated with the concentrations of 0.75, 1.30, and 1.28 µM of *Ferula assa-foetida* for 24h, 48h, and 72h, respectively. Floating and adherent cells were then harvested and incubated with 750 µl of a hypotonic buffer (50 mg/ml PI in 0.1% sodium citrate with 0.1% Triton X-100) at 4 °C overnight in the dark. Finally, flow cytometry was carried out using a FACScan flow cytometer (Becton Dickinson). A total of 1×10^4 events were acquired with FACS, and finally, data was analyzed through flowJo-V10 software. All treatments were carried out in triplicate.

2.6. Statistical Analysis

All results were expressed as mean ± SEM. The significance of difference was evaluated with ANOVA and

Bonferroni's test. A probability level of $p<0.05$ was considered statistically significant.

3. Results

3.1. The Effect of Ferula Assa-foetida's Extract on Cell Cytotoxicity

The results of the current study showed that *Ferula assafoetida's* extract had an antiproliferative effect against PC12 and MCF7 cells in a dose-dependent manner.

3.1.1. The Effect of Ferula Assa-foetida's Extract on the Viability of PC12 and MCF7 Cells

The results of the MTT assay showed that the viability of PC12 cells was reduced in time- and dose-dependent manner with higher efficiency in higher concentrations (<5 µM) (Figure 1). At exposure time of 24h, treatment with *Ferula assa-foetida* in the concentrations of 2.5, 5, 7, and 10 µM had a significant effect on cells viability ($p<0.001$) While in exposure time of 48h and 72h, treatment with *Ferula assa-foetida's* extract had a significant effect on cells viability ($p<0.001$) in all concentrations.

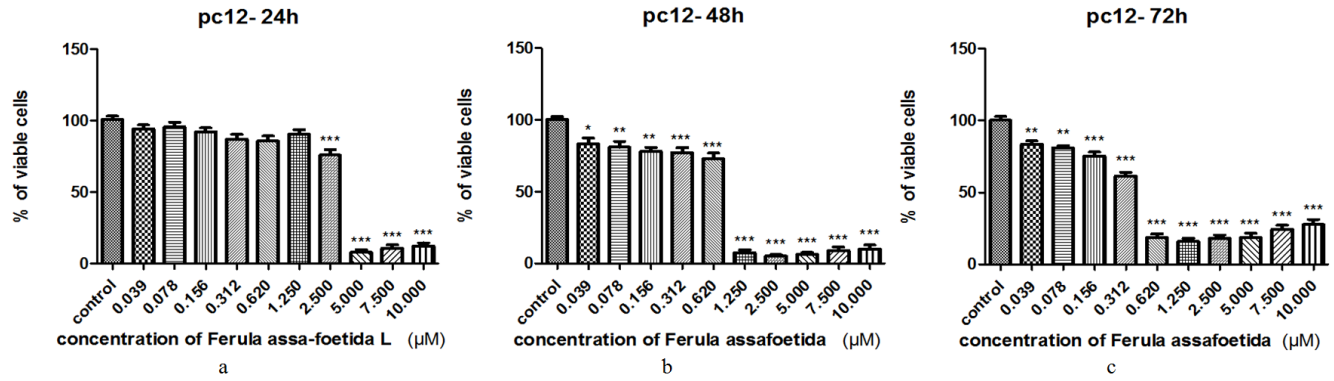

Figure 1. *The effect of Ferula assa-foetida's extract on the viability of PC12 cells. PC12 cells were treated with different concentrations of Ferula assa-foetida (0.0312 -10 µM) for 24h (figure 1a), 48h (figure 1b), and 72h (figure 1c). The highest inhibitory effect was observed at concentration of 5, 7, and 10µM. Time exposure of 48h and 72h were more effective in reducing the viability of PC12 cells (figure 1b and figure 1c). IC50s for 24h, 48h, and 72h treatment were calculated, and were 2.84, 0.8, and 0.4 µM, respectively. Results are expressed as Mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001.*

3.1.2. The Effect of Ferula Assa-foetida's Extract on the Viability of MCF7 Cells

The results showed that the extract of *Ferula assa-foetida* reduced cell viability in a time- and dose-dependent manner (Figure 2). At an exposure time of 24h and 48h, MCF7 cells treated by *Ferula assa-foetida's* extract had a significant effect on cell viability ($p<0.001$) in the concentrations of 10, 7, 5, 2.5, and 1.25 µM. With an exposure time of 72h, in all doses, *Ferula assa-foetida's extract* had a significant effect on cells viability ($p<0.001$).

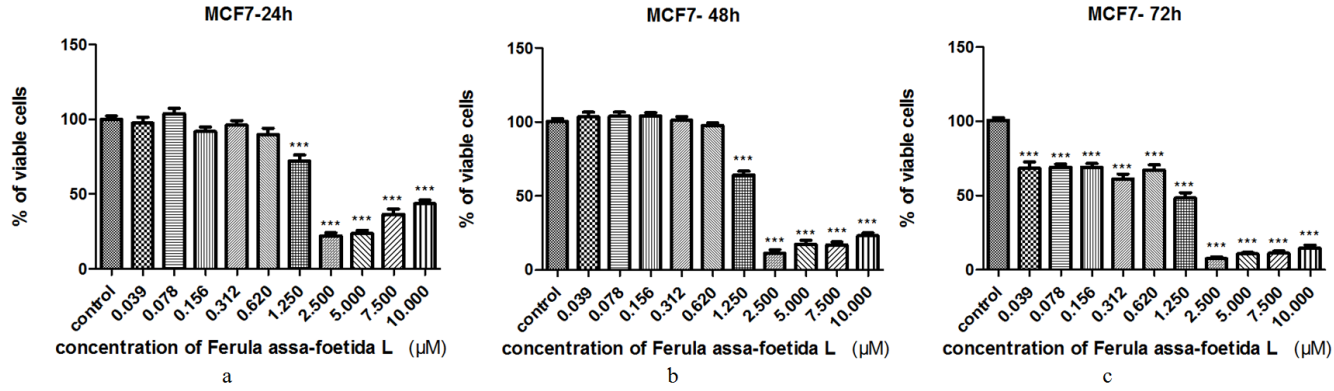

Figure 2. *The effect of Ferula assa-foetida's extract on the viability of MCF7 cells. MCF7 cells were treated with different concentrations of Ferula assa-foetida (0.0312 -10 µM) for 24h (figure 2a), 48h (figure 2b), and 72h (figure 2c). The highest inhibitory effect was observed at concentration of 2.5, 5, 7, 10 µM concentrations. Time exposure of 72h was more effective in reducing the viability of MCF7 cells (figure 2c). IC50s for 24h, 48h, and 72h treatment were calculated, and were 1.30, 1.284, 0.753 µM, respectively. Results are expressed as Mean ± SEM (n=3). ***p<0.0001.*

3.2. Apoptosis Induction Using PI Staining

3.2.1. The Assessment of Apoptotic PC12 Cells Using PI Staining

The toxic effects of *Ferula assa-foetida's* extract were observed on PC12 cells. IC50 concentrations evaluated as 2.84, 0.8, and 0.4 µM for time exposure of 24, 48 and 72h, respectively. The number of apoptotic cells was calculated using PI staining. According to Figure 3, the sub-G1 peak analysis demonstrated the amount of apoptosis induction in PC12 cells treated by *Ferula assafoetida's* extract. Figure 4.

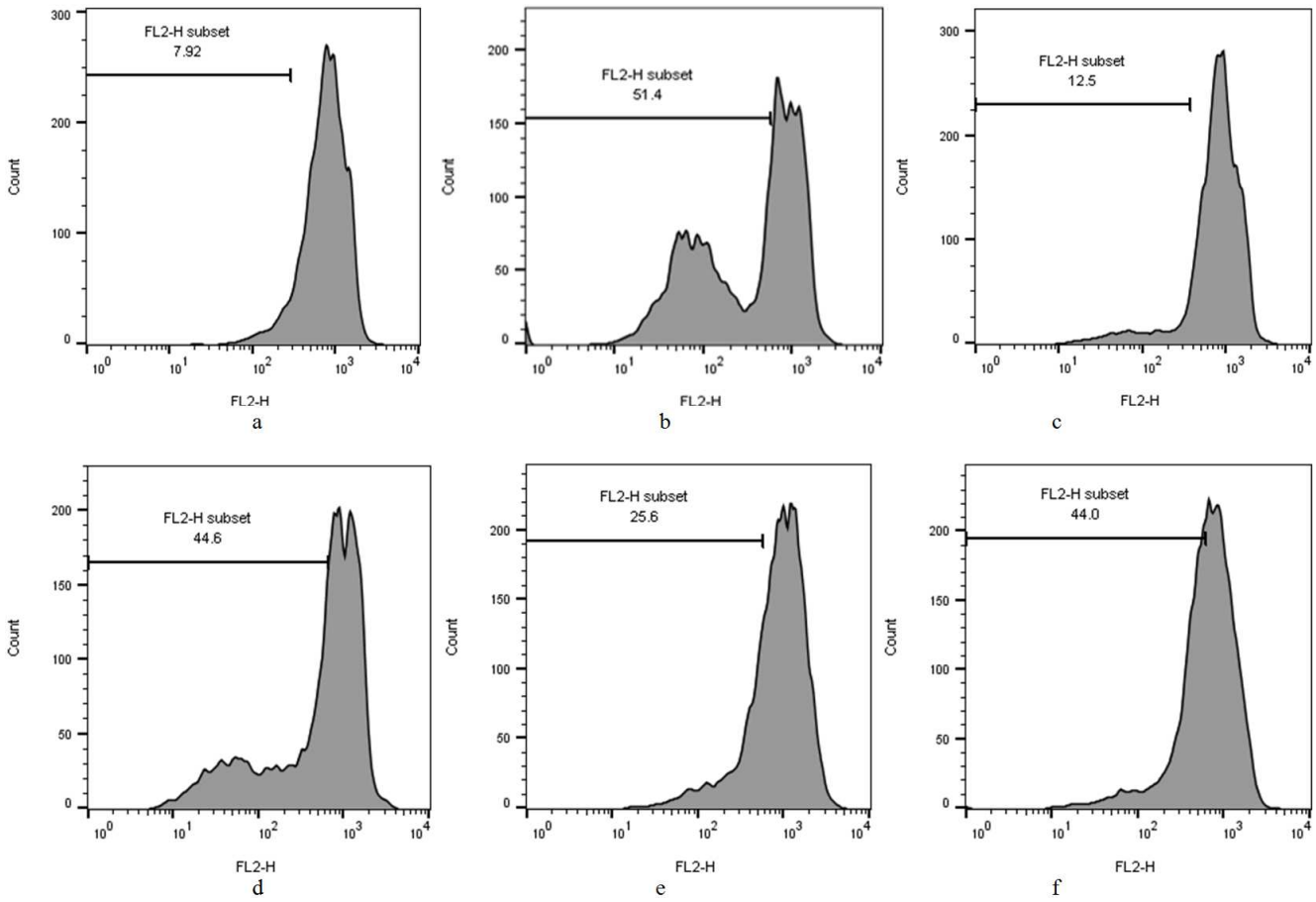

Figure 1. *Ethanolic extract of Ferula assa-foetida induced apoptosis on PC12 cells. Figure 3a, flow cytometry histogram of PI-staining on PC12 cells in the control group for 24h. figure 3b, flow cytometry histogram of PI-staining on PC12 cells that were treated with a concentration of 2.84 µM of Ferula assafoetida's extract for 24 h. figure 3c, flow cytometry histogram of PI-staining on PC12 cells in the control group for 48h. figure 3d, flow cytometry histogram of PI-staining on PC12 cells that were treated with a concentration of 0.8 µM of Ferula assafoetida's extract for 48 h. figure 3e, flow cytometry histogram of PI-staining on PC12 cells in the control group for 72h. figure 3f, flow cytometry histogram of PI-staining on PC12 cells that were treated with a concentration of 0.4 µM of Ferula assa-foetida's extract for 72 h.*

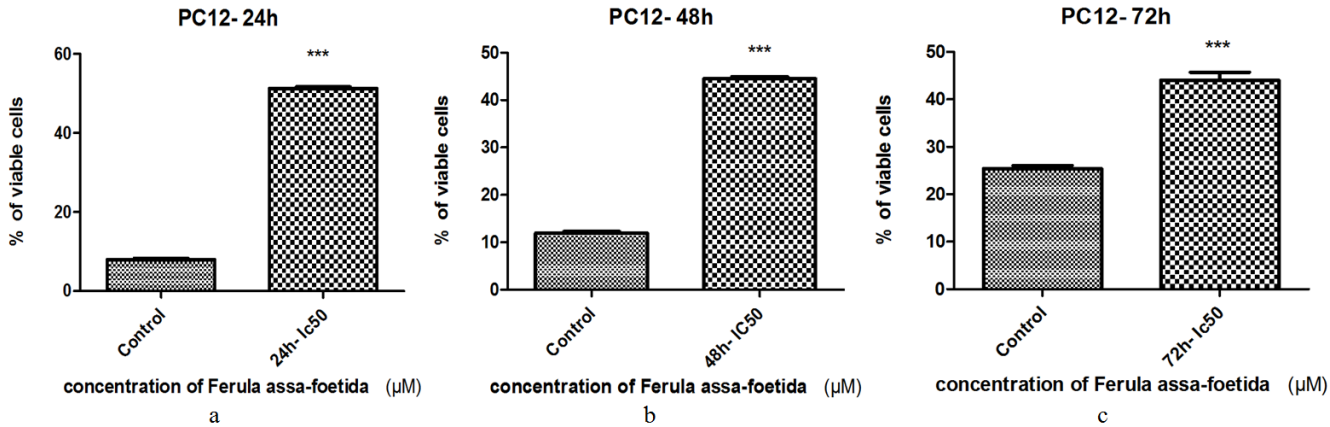

Figure 4. *PI-staining of apoptotic PC12 cells. Figure 4a, PC12 cells were treated with concentration of 2.84 µM of Ferula assa-foetida's extract for 24h. Figure 4b, PC12 cells were treated with concentration of 0.8 µM of Ferula assafoetida's extract for 48h. Figure 4c, PC12 cells were treated with concentration of 0.4 µM of Ferula assafoetida's extract for 72h. It should be noted that these concentrations were IC50s for 24h, 48h and 72h, ***p<0.0001.*

3.2.2. PI Staining on MCF7 Cells

The toxic effects of *Ferula assa-foetida's extract* were observed on MCF7 cells. IC50 concentrations evaluated as 1.30, 1.284, 0.753 µM for time exposure of 24, 48 and 72h, respectively. The number of apoptotic cells was evaluated using PI staining. According to Figure 5, the sub-G1 peak analysis demonstrated the amount of apoptosis induction in MCF7 cells that were treated with *Ferula assa-foetida's* extract. Figure 6.

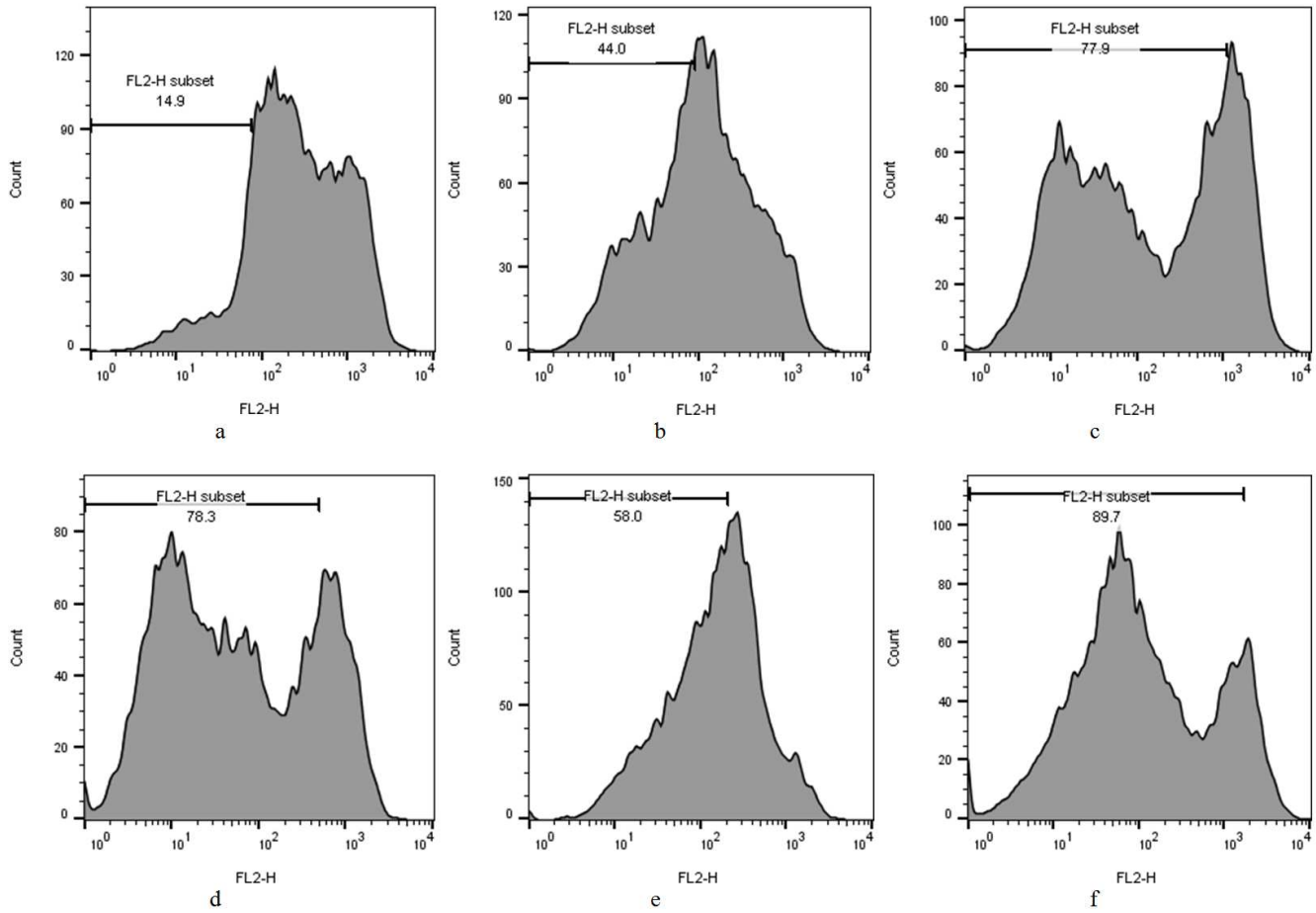

Figure 5. *Ethanolic extract of Ferula assa-foetida induced apoptosis on MCF7 cells. figure 5a, flow cytometry histogram of PI-staining on MCF7 cells in the control group for 24h. figure 5b, flow cytometry histogram of PI-staining on MCF7 cells that were treated with a concentration of 1.30 µM of Ferula assa-foetida's extract for 24 h. figure 5c, flow cytometry histogram of PI-staining on MCF7 cells in the control group for 48h. figure 5d, flow cytometry histogram of PI-staining on MCF7 cells that were treated with a concentration of 1.284 µM of Ferula assa-foetida's extract for 48 h. figure 5e, flow cytometry histogram of PI-staining on MCF7 cells in the control group for 72h. figure 5f, flow cytometry histogram of PI-staining on MCF7 cells that were treated with a concentration of 0.753 µM of Ferula assa-foetida's extract for 72 h.*

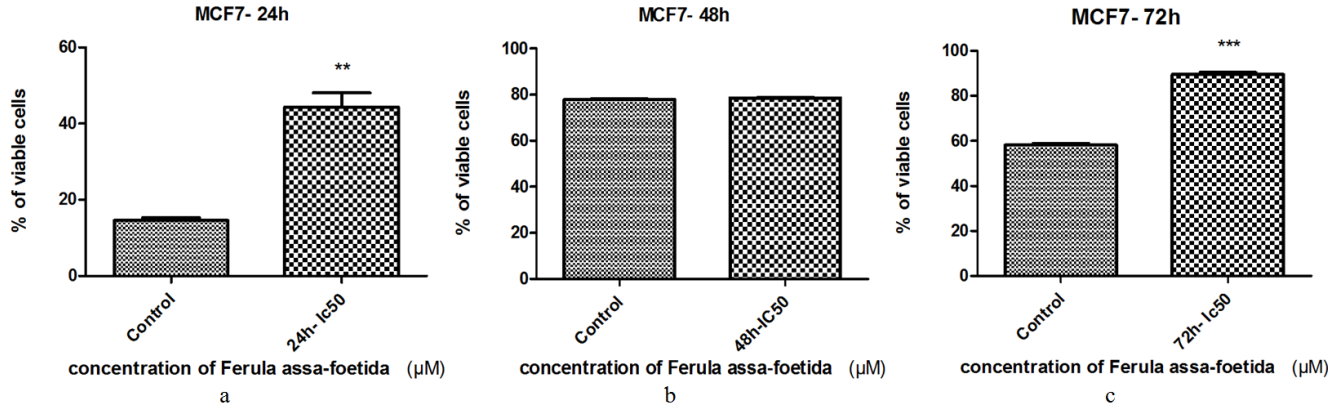

Figure 6. *PI-staining of apoptotic MCF7 cells. Figure 6a, MCF7 cells were treated with concentration of 1.30µM of Ferula assa-foetida's extract for 24h. Figure 6b, MCF7 cells were treated with concentration of 1.28 µM of Ferula assa-foetida's extract for 48h. Figure 6c, MCF7 cells were treated with concentration of 0.75 µM of Ferula assa-foetida's extract for 72h. It should be noted that these concentrations were IC50 for 24h, 48h and 72h, **P<0.01, ***p<0.0001.*

4. Discussion

Our aim was to investigate the anti-proliferative effect of *Ferula assa-foetida* on MCF7 and PC12 cancer cells. In the current study, it was concluded that *Ferula assa-foetida's* extract strongly reduced the viability of PC12 and MCF7 cells in a dose- and time-dependent manner. The results of apoptotic induction showed *Ferula assa-foetida's* extract

causes DNA fragmentation in MCF7 and PC12 cells.

The results of MTT assay on MCF7 and PC12 cell demonstrated *Ferula assa-foetida* had an inhibitory effect. Based on previous results, it seems that this inhibitory effect results from apoptosis initiation by *Ferula assa-foetida's* extract. Gene expression analysis on normal fibroblast has revealed *Ferula assa-foetida*'s extract at high concentrations reduces Bcl-2 while upregulates the expression of *Bax* and *Caspase-3* [*31*]. Our data also partially reflected the matter of concentration, as we observed a higher inhibitory effect at higher concentrations of *Ferula assa-foetida's* extract on MCF7 and PC12 cells which may be due to hormesis [32]. In this regard, our data also showed a sub-G1 peak by flow cytometry and PI staining indicated DNA fragmentation following the initiation of apoptosis. It could be concluded that *Ferula assa-foetida's* extract exerted its antiproliferative effect on cancer cells through apoptosis initiation.

It was shown that lower concentrations of Oleo-gum resin from *Ferula assa-foetida* attenuated cellular senescence in normal human dermal fibroblasts while higher concentrations associated with the apoptosis initiation [31]. Also, *Ferula assa-foetida* extract increased the viability of cerebellar granule neurons of rats in lower concentrations [33].

Ferula assa-foetida has been shown to be one of the main components of the umbelliprenin family that treats asthma through the inhibition of lipoxygenase [34], and also *Ferula assa-foetida* is used in cancer prevention [35].

It has been shown that *Ferula assa-foetida* can reduce the effect of environmental mutagens in food [36]. It is demonstrated that umbelliprenin has a significant role in cancer chemotherapy [37, 38]. Previously, it was suggested that this antitumor activity is the blockage of the enzyme 5-lipoxygenase [27]. Also, the hormesis phenomenon of umbelliprenin has been observed on Jurkat cells [32]. Since *Ferula assa-foetida* is a member of the umbelliprenin family, it seems that the effect of *Ferula assa-foetida* is following hormesis too [32] as our data demonstrated that higher concentrations had higher antitumor activity.

The ability to induce apoptosis instead of necrosis is considered as a key feature of potential anti-tumor medications. Apoptosis induction has been used for the prevention and treatment of cancer. Bcl-2 Proteins are a key factor in the regulation of apoptosis [39]. Bax and Bcl-2 proteins affect the permeability of the mitochondrial membrane. The onset of apoptotic signaling cascades depends on the balance between proapoptotic and antiapoptotic proteins from the Bcl-2 family [40]. The permeability of mitochondrial membrane caused soluble molecules to release from mitochondrial outer space to the cytosol, one of these molecules is Smac/DIABLO which activates the caspase cascade in the cytosol [41]. Caspase activation causes cytochrome C to release which resulted in the inactivation of an apoptosis-inhibiting protein family [42]. The p53, tumor suppressor protein, plays an important role in the onset of apoptosis by intrinsic and extrinsic stresses. Notably, p53 defect leads to failure in cancer chemotherapy [43]. Apoptosis occurs at the end of the phase G1 and G2 with activation of p53 [44]. In the present study, the effect of *Ferula assa-foetida* on the induction of apoptosis was evaluated using flow cytometry. Results showed that *Ferula assa-foetida* could induce apoptosis on MCF7 and PC12 cells. In previous studies, treated with *Ferula assa-foetida*, it has been shown that the *Bax* expression was increased while *Bcl-2* expression decreased, and by inhibiting the cell cycle and activating the mitochondrial pathway, apoptosis was initiated [45]. However, the initiation of apoptosis is similar to what occurred by inducing umbelliprenin's extract on Jurkat cells [32].

To summarize, many reports have illustrated various effects of *Ferula assa-foetida* and its derivatives. Due to hormesis, our results indicated that *Ferula assa-foetida*'s extract at high concentrations inhibited the proliferation of MCF7 and PC12 cells. Also, apoptosis analysis demonstrated that treatment *by Ferula assa-foetida* induced apoptosis in cancer cells. *Ferula assa-foetida*'s extract is a potential herbal product with apoptotic effect even though more investigations are needed to determine the underlying mechanisms.

5. Conclusion

The present study showed that Ferula assa-foetida is a strong inducer of apoptosis that inhibits the growth of PC12 and MCF7 cells so that with the onset of apoptosis it prevents cancer. However, more studies are needed to prove the mechanism of the Ferula assa-foetida extract in inducing apoptosis.

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

All authors declare they have no conflict of interest.

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