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Original Article

Anticancer Potential of *Ferula haussknechtii* Ethyl Acetate Extract against Breast Cancer Cells

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Abstract

Background: The genus Ferula has notable biological features, especially cytotoxic effects. This study examined the cytotoxic effects of isolated extracts from Ferula haussknechtii on breast cancer cells.

Methods: The potential mechanism of action of the compounds was studied by assessing caspase-3 activity and by co-treatment with Z-VAD-FMK, a pan-caspase inhibitor. Cytotoxic effects were evaluated after 48 h of incubation in MCF-7 and MDA-MB-231 breast cancer cells, including determination of IC50 values and selectivity index.

Results: Ethyl acetate extract at 15.34 and 10.5 µg/ml effectively inhibited proliferation of MCF-7 and MDA-MB-231 cells by 50%. The IC50 values for the methanolic extract were 10.53 and 10.56 µg/ml, respectively. Ethyl acetate had the highest selectivity index (SI = 4.04) and the lowest growth inhibitory concentration among the isolated extracts tested on MDA-MB-231 cells. Mechanism-of-action analysis indicated that the ethyl acetate extracts induced cell death in MDA-MB-231 cells through caspase-mediated apoptosis.

Conclusion: The ethyl acetate extract of F. haussknechtii may serve as a promising source of natural compounds for discovering new therapeutic agents targeting triple-negative breast cancer (TNBC).

Keywords: Anticancer, Breast Cancer, *Ferula haussknechtii*

Introduction

Globally, breast cancer ranks as the most commonly diagnosed life-threatening cancer in women, impacting around one in eight women throughout their lifetime [1]. Although there are numerous treatment options available for breast cancer, the effectiveness of these therapies is often hindered by significant side effects and the development of resistance to chemotherapy [2]. Consequently, these limitations have created a demand for research into more effective therapeutic agents.

Plants serve as a vast reservoir of natural compounds, offering novel chemical entities that present a promising avenue for research in cancer therapy [3]. Natural products or compounds originating from natural sources constitute a significant portion of modern therapeutic drugs.

The National Cancer Institute (NCI) has evaluated 35,000 plant species for possible anticancer

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properties, finding that 3,000 exhibit potential for cancer treatment. [4].

The genus *Ferula*, comprising approximately 170 species, is found worldwide. Prior biological studies have demonstrated that species within the *Ferula* genus are abundant in compounds exhibiting antimicrobial characteristics, making them potential candidates for the treatment of infectious diseases, such as methicillin-resistant *Staphylococcus aureus*, diarrhea, and leishmaniasis [5-7]. *Ferula haussknechtii* belongs to the *Ferula* family and has been utilized in Iranian traditional medicine for the treatment of sepsis [8]. To the best of our knowledge, no research has empirically examined the impact of *F. haussknechtii* in the context of cancer treatment.

The MCF-7 cell line is widely used in research on estrogen receptor (ER)-positive breast cancer. Conversely, the MDA-MB-231 cell line represents triple-negative breast cancer (TNBC), a particularly aggressive form of breast cancer characterized by the absence of three key receptors: human epidermal growth factor receptor-2 (HER-2), ER, and progesterone receptors (PR). As a result, conventional targeted therapies are often ineffective for TNBC. In this investigation, given the lack of reports on the antiproliferative effects, we aimed to explore the cytotoxic properties of methanolic, ethyl acetate, hexane, and hydroalcoholic extracts of *F. haussknechtii* against two breast cancer cell lines: MCF-7 and MDA-MB-231, with the HEK-293 cell line serving as a control.

Materials and Methods

Cell lines and culture condition

The MCF7 and MDA-MB-231 cells were cultured in RPMI-1640 (Sigma, USA), while HEK-293 was maintained in Dulbecco's modified Eagle medium (Sigma, USA). All media contained 100 µg/ml streptomycin, 100 units/ml penicillin (Sigma, USA), and 10% heat-inactivated fetal bovine serum (Gibco, BRL). The cells were cultured at 37°C in a 90% humidified incubator with 5% CO2. When the cells reached 80% confluence, they were subcultured into fresh medium.

Plant sample and extract preparation

The root specimens of *F. haussknechtii* were gathered in the Uramanat region of the Kurdistan province in Iran. Voucher samples have been submitted to the Herbarium of Hamadan University of Medical Sciences, Iran, and have been assigned specific voucher numbers. The dry powder roots of *F. haussknechtii* were subjected to extraction using n-hexane, ethyl acetate, and methanol (3×1, at room temperature for 48 h at 25°C) through a maceration technique. The resulting extracts were filtered with Whatman filter paper (Sigma-Aldrich, USA) and subsequently concentrated at 40°C using a vacuum evaporator. The extracts were stored at 4°C in sterile Falcon tubes, shielded from light exposure. To prepare a master stock solution, the isolated extracts were dissolved in DMSO (Sigma, USA) and maintained at −20°C until required. A working solution was freshly formulated in the complete culture medium before use.

Cell viability assay

The cytotoxic effects of the isolated extracts from *F. haussknechtii* were evaluated against the MCF7, MDA-MB-231, and HEK293 cell lines using the MTT assay. The study involved assessing the impact of various extract concentrations, ranging from 5 to 80 µg/ml, over 48 h. Exponentially growing cells were subjected to trypsinization using 0.25% Trypsin-EDTA and subsequently plated in 96-well plates at a density of 7000 cells per well. After allowing the cells to adhere overnight, they were exposed to a series of concentrations of the isolated extracts. After a 48-hour incubation period at 37°C, 20 µl of MTT solution (4 mg/ml in PBS) was added, followed by an additional incubation at 37°C for 4 h. Subsequently, the supernatant was carefully discarded, and 100 µL of DMSO was introduced into each well to dissolve the resulting precipitate. Cell viability was then determined by measuring absorbance at 570 nm using an ELISA plate reader (BioTek, SYNERGY HTX, Vermont, USA). The selectivity index (SI) was calculated by comparing the IC_{50} values of the isolated extract in the HEK-293 cell line with the IC_{50} values obtained in cancer cell lines. It is noteworthy that Doxorubicin was employed as a positive control. Each concentration was tested in triplicate, and the experiments were

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conducted three times; the data are presented as mean±SD of three replicates.

Caspase-3 activity assay

To evaluate the impact of isolated extracts on programmed cell death, the activity of caspase-3 was measured using the caspase-3 DEVD-R110 Fluorometric HTS Assay Kit (Biotium, Hayward, CA) following the manufacturer's guidelines. In summary, cells were seeded in 100 µl of culture medium in each well of a black 96-well plate. Apoptosis was then induced by applying IC_{50} concentrations of the isolated extracts for durations of 4 h and 24 h, as determined by the MTT assay. After the treatment period, 100 µl of assay buffer was combined with 100 µl of the cell culture medium in each well. Following a 60-minute incubation, fluorescence was quantified using 470 nm excitation and 520 nm emission wavelengths. Caspase-3 activity was reported as a fold change relative to untreated cells. Each experimental condition was replicated three times, and the data are presented as mean±SD of the three replicates.

Caspase inhibitor assay

In the inhibitor assay, MDA-MB-231 cells underwent a pre-treatment with 50 µM pan-caspase inhibitor (Z-VAD-FMK) for 30 minutes. Afterward, the cells were exposed to extracts at their IC_{50} concentration. After 48 h of incubation, cell viability was assessed using the MTT assay, as outlined in the cytotoxicity assay section.

Statistical analysis

Data were obtained from three separate experiments and presented as mean±SD. Analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to test significance using the SPSS (version 21.0.0) software. A value of $P<0.05$ was considered statistically significant.

Results

Cytotoxic effects of isolate extract on cancer and normal cell lines

The cytotoxic effects of isolated extracts on MCF-7, MDA-MB-231, and HEK-293 cells were assessed using the MTT assay. As illustrated in Figures 1 and 2, both ethyl acetate and methanolic extracts demonstrated a dose-dependent inhibition of cell proliferation in MCF-7 and MDA-MB-231 cells at concentrations ranging from 5 to 80 µg/ml. After a 48-hour treatment period, the IC_{50} values for the ethyl acetate extract were found to be 15.34±0.2 for MCF-7 and 10.5±0.5 for MDA-MB-231, while the methanolic extract yielded IC_{50} values of 10.53±0.7 and 10.56±0.5, respectively. These IC_{50} values are detailed in Table 1. Among the isolated extracts, ethyl acetate displayed the highest selectivity index (SI=4.04) and the lowest growth inhibitory concentration (IC_{50}=10.5 µg/ml) in MDA-MB-231 cells. These findings indicate a pronounced cytotoxic effect of the ethyl acetate extract against the MDA-MB-231 cell line compared with the methanolic extract, leading to the selection of the ethyl acetate extract for further investigation. In contrast, the hexane and hydroalcoholic extracts demonstrated minimal antiproliferative activity at the specified concentrations against the breast cancer cell lines (data not shown).

Table 1. IC_{50} value and selectivity index of the isolated extract after 48 h and positive control doxorubicin on MCF-7, MDA-MB-231, and HEK-293 (Values are expressed as mean±SD for three independent observations)

Cell line	IC_{50} value (µg/ml) of extract		
	Methanolic	Ethyl acetate	Doxorubicin
MCF-7	10.53±0.7	15.34±0.2	-
MDA-MB-231	10.56±0.5	10.5±0.5	1.22±0.4
HEK-293	28.39±0.8	42.48±0.6	-
Selectivity index (SI) HEK293/MCF-7	2.69	2.76	-
Selectivity index (SI) HEK293/MDA-MB-231	2.67	4.04	-

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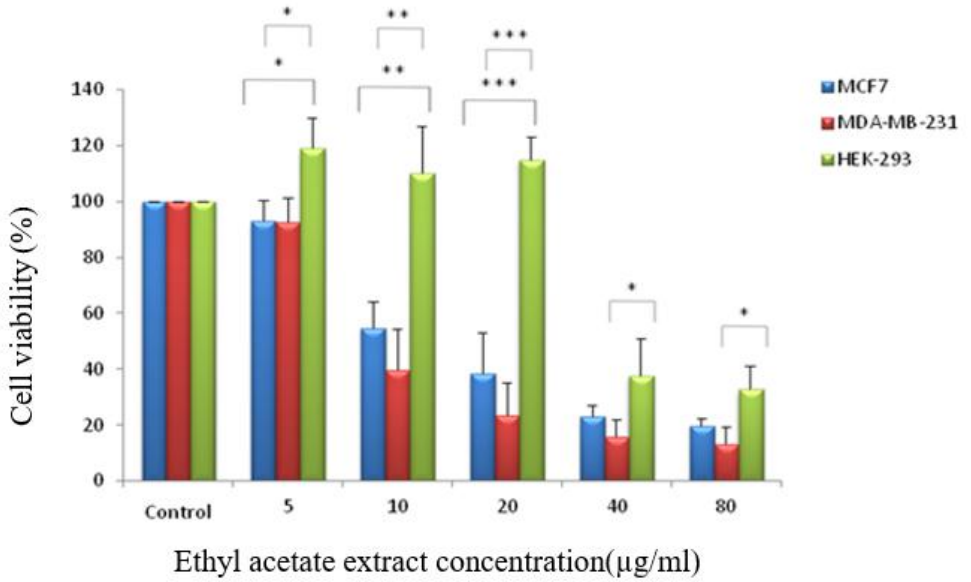


Figure 1. Toxicity effects of ethyl acetate extract on MCF-7, MDA-MB-231, and HEK-293 for 48 h using the MTT assay. Data are expressed as means (n≥3)±SD over controls; *P<0.05, **P<0.01, ***P<0.001.

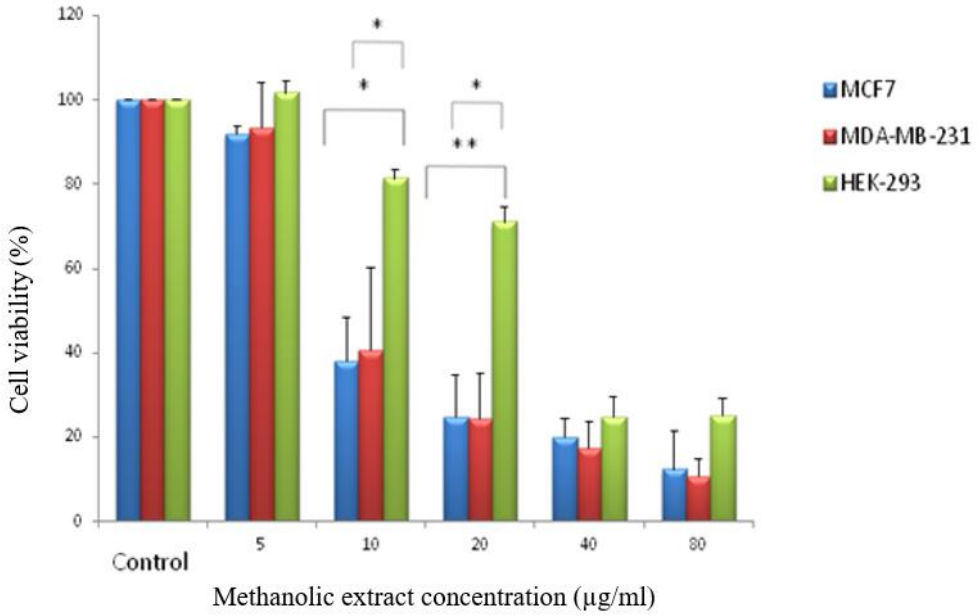


Figure 2. Toxicity effects of methanolic extract on MCF-7, MDA-MB-231, and HEK-293 for 48 h using the MTT assay. Data are expressed as means (n≥3)±SD over controls; *P<0.05, **P<0.01.

Ethyl acetate induces caspase-3-mediated apoptosis in MDA-MB-231

Figure 3 illustrates the activation of caspase-3 activity in MDA-MB-231 cells after exposure to ethyl acetate at a concentration of 10.5 µg/ml. According to this figure, the activity of caspase-3 showed an approximate twofold and threefold increase in induced MDA-MB-231 cells compared to uninduced MDA-MB-231 cells at 4 h and 24 h, respectively. To elucidate whether caspase-3 activation played a pivotal role in cell death, pre-treatment with a pan-caspase inhibitor (Z-VAD-FMK) before induction of cell death with ethyl acetate extract was investigated. The results showed

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that Z-VAD-FMK had a slight effect on ethyl acetate-induced cell death, suggesting that the caspase-dependent mechanism is the main pathway of cytotoxicity.

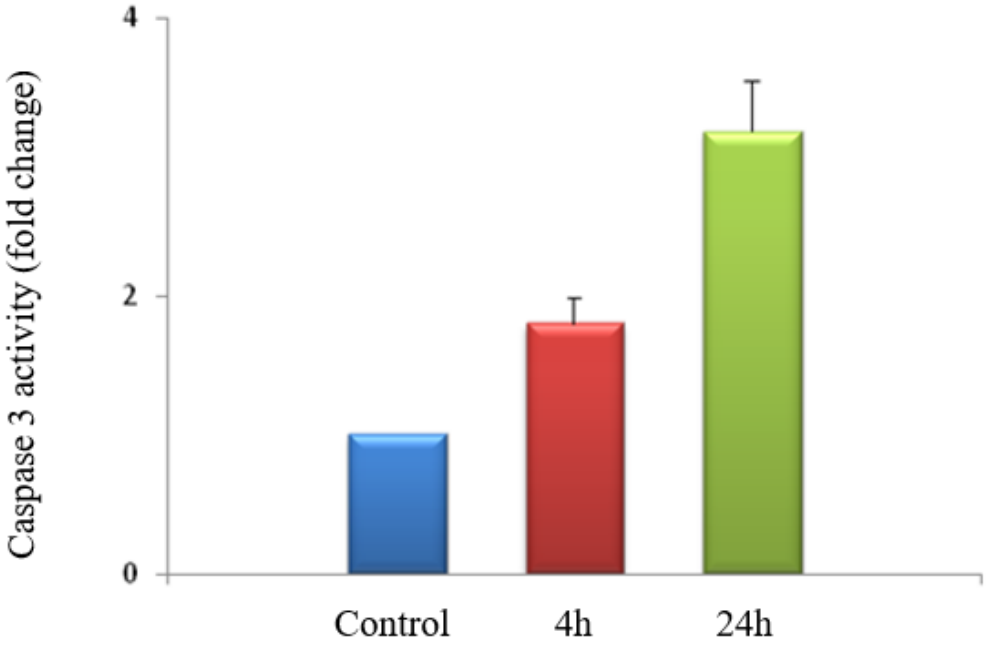


Figure 3. Time-dependent caspase activation by ethyl acetate extract. Caspase activity was measured at 4 h and 24 h following ethyl acetate treatment. Data are presented as fold change in fluorescence as measured relative to untreated cultures. Data are expressed as the mean±SD (n=3).

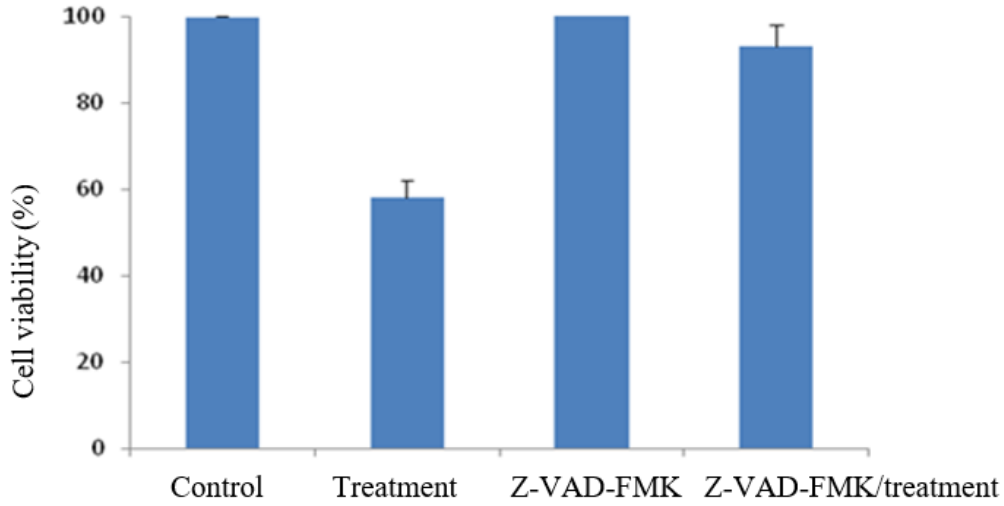


Figure 4. Ethyl acetate mainly induced caspase-dependent cell death. MDA-MB-231 cells were subjected to 1 h treatment with 50 µM caspase inhibitor Z-VAD-FMK and subsequently subjected to 48 h treatment with 10.5 µg/ml ethyl acetate. Values are expressed as mean±SD for three independent observations.

Discussion

Although there are different drugs for breast cancer treatment, some limitations, such as drug resistance and adverse effects, remain pivotal issues [2]. Natural products are a major source of novel therapeutic agents and have been widely investigated as anticancer drugs [9]. Besides, since the chemical diversity of plants is based on

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biological and geographical diversity, the entire globe can be explored by researchers in finding new drugs for cancer therapy [10]. Here, we demonstrated the potential application of the ethyl acetate-isolated extract from *F. haussknechtii* against breast cancer cell lines.

In the present study, ethyl acetate and methanolic extracts effectively inhibited the growth of MCF7 and MDA-MB-231 cells in a dose-dependent manner. The IC_{50} values of the ethyl acetate extracts in MDA-MB-231 and MCF7 were 10.5 and 15.34, while the IC_{50} values for the methanolic extract were 10.53 and 10.56, respectively. According to the National Cancer Institute guidelines, a crude extract with an IC_{50} value of fewer than 20 µg/ml and SI value >3 is considered to have *in vitro* cytotoxicity activity and high selectivity toward cancer cells [11]. This result shows the potential to further explore the promising antitumor effect of the ethyl acetate extract.

As the induction of apoptosis in cancer cells is a promising target for anticancer therapy, many studies have evaluated the effects of plant-derived compounds on the apoptotic pathway [12]. To investigate whether ethyl acetate-induced MDA-MB-231 cell death is mediated via apoptosis, caspase-3 activity assessment, as the central component of the apoptotic cascade, was performed, and toxicity in the presence of a pan-caspase inhibitor was evaluated in the present study. The activity of caspase-3 significantly increased in MDA-MB-231 cells treated with ethyl acetate. These results indicated that activated caspase-3 plays a pivotal role in the ethyl acetate-induced cell death. In addition, whether ethyl acetate-induced MDA-MB-231 cell apoptosis is dependent on the cleavage of procaspases was confirmed by pre-treating MDA-MB-231 cells with Z-VAD-FMK, a pan-caspase inhibitor, before ethyl acetate treatment. Ethyl acetate-induced MDA-MB-231 cell death was significantly inhibited in the presence of Z-VAD-FMK. These results suggest that in MDA-MB-231 cell lines, caspase activation is essential for cell death and most likely caspase-dependent pathways predominate. As plant-derived compounds exhibiting apoptosis-inducing activity are considered potential anticancer agents [13], we concluded that ethyl acetate extract can be explored as a source for new drugs for cancer therapy. However, the mechanisms underlying apoptosis induced by ethyl acetate in MDA-MB-231 cells are incompletely understood. Therefore, the mechanism of cell death induced by ethyl acetate extract requires further elucidation, and its underlying molecular pathways are strongly recommended for further investigation.

Conclusion

The current study confirms the first evidence of the antiproliferative activity of methanolic and ethyl acetate extracts isolated from F. haussknechtii as determined by in vitro MCF-7 and MDA-MB-231 growth inhibition assays. The ethyl acetate extract was considered a potential anticancer agent based on the obtained SI. Furthermore, this extract induced cell death in MDA-MB-231 cell lines through caspase-mediated apoptosis. However, the detailed mechanisms of action remain largely unknown and require further elucidation. These findings show that Ferula haussknechtii can be a potential source for the development of new drugs for breast cancer treatment. Further, isolation of the effective ingredients is underway.

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Competing Interests

The authors declared that they have no conflicts of interest.

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