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Research Paper

Investigation the effect of *Scrophularia striata hydroalcoholic* Extract On Cell Death and Migration in Cervical Cancer Cell Line

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ABSTRACT

Background: With the growing interest in plant-derived chemotherapeutic agents, there has been a significant rise in research exploring a broad range of plants in recent years. *Scrophularia striata* has gained attention due to its extensive medical applications. This study aimed to investigate the effect of *S. striata* extract on HeLa cervical cancer cells, specifically their migration, apoptosis, and necrosis.

Methods: We first cultured HeLa cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. We then examined the cytotoxicity of *S. striata* extract at varying concentrations (0, 1, 10, 100, 500, and 1000 µg/mL) using the MTT assay after 24 hours. We evaluated the extent of wound healing using a scratch assay and analyzed the apoptosis activity of the extract using flow cytometry.

Results: Our results showed that *S. striata* extract (IC_{50}: 433.8 µg/mL) significantly enhances wound healing ($P \leq 0.01$) in cervical cancer and promotes apoptosis and necrosis of HeLa cells.

Conclusion: Our findings suggest that *S. striata* may serve as an effective treatment for cervical cancer by inducing cell death and reducing migration.

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1. Introduction

Cervical cancer is an increasingly recognized worldwide public health concern [1]. Despite being preventable, it is the fourth most common malignant disease among women, resulting in around 528,000 new cases and 266000 deaths worldwide each year [2]. It is worth noting that this gynecological tumor is the leading cause of death (accounting for nine out of ten deaths) in less developed countries [3]. Cancer progression generally involves molecular and biochemical alterations, with increased levels of proteolytic enzymes (MMPs) being the most significant contributor to the migration and invasion of tumors [4]. Chemotherapy and radiotherapy are among the most commonly used approaches for cancer management. However, these approaches often result in significant side effects, including oral mucositis, gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, hematopoietic system injury, cardiotoxicity, and neurotoxicity [5, 6]. Therefore, the development of effective management strategies against chemotherapy- and radiotherapy-induced side effects is crucial.

Natural products have been proven to be safe and effective in preventing and treating cancer [7]. As a source of remedies, natural products have been used in traditional medicine for thousands of years [8]. The Scrophulariaceae family comprises annual and perennial herbs that are widely distributed in deciduous and coniferous forests of central Europe, central Asia, North America, and especially the Mediterranean area, with over 3000 species and more than 200 genera [9, 10]. Traditional medicine has long taken advantage of some species of the Scrophulariaceae family in the treatment of eczema, wounds, ulcers, fistulas, and cancer [11]. Among the various species of Scrophulariaceae, *Scrophulariaceae striata* is the most important and widely used in Iranian folk medicine for treating inflammatory and infectious diseases [12]. *S. striata* has been found to exhibit various biological effects, including antioxidant [13-15], anticancer [11], antibacterial, antifungal [15, 16], anti-inflammatory [17, 18], anti-asthmatic [19], anti-parasitic [20], and neuroprotective [13, 21] activities. However, there is little information available on the exact mechanism of *S. striata*'s biological and medicinal activities. To our knowledge, there is no data on the effect of *S. striata* on the treatment of cervical cancer. Therefore, the aim of this in vitro study was to determine the exact effect of *S. striata* hydro-alcoholic extract on the viability, apoptosis, and migration of HeLa cervical cancer cell lines.

2. Materials and Methods

Plant collection and extract preparation

First, *S. striata* plants were collected from Ilam Province, Iran, and the botanical authentication of the plant material was conducted by Shahrokh Kazempour Osaloo from the Department of Plant Biology, Tarbiat Modares University. In the next step, the aerial parts of the plant were shade-dried at room temperature and then ground into powder. Finally, extraction of the powder (1000 g) was done using 70% ethanol by the Soxhlet apparatus.

Cell culture

HeLa cells were procured from the National Cell Bank of Iran (NCBI) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2 (Invitrogen LT, Merelbeke, Belgium). The cells were cultured under these conditions until they reached 70-80% confluency. Confluent cells were passaged and plated at 1:2 or 1:3 dilutions every 3-4 days using 0.25% trypsin and 1 mM EDTA (Invitrogen LT, Merelbeke, Belgium).

Cell viability

To assess cell viability, HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with varying concentrations of *S. striata* extract (0, 1, 10, 100, 500, and 1000 µg/mL). After 24 hours of treatment, cell viability was measured using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay, as previously described [22]. The percentage of cell viability was calculated and compared to the control (100% viability). The sensitivity of the cells was evaluated based on the calculated half-maximal inhibitory concentration (IC_{50}).

In vitro migration assay

HeLa cells were seeded into 24-well plates at a final cell density of 4×10^4 cells/well. After overnight incubation at 37°C with 5% CO_2, cells were starved for 24 hours. Scratching was performed using a sterile 200 µL pipette tip, and the cells were washed with PBS [23]. Next, the cell culture media were replaced with two types of fresh media: One containing *S. striata* extract (IC_{50}) for the treated group and the other without extract for the control group. The reduction in the scratch areas was monitored using an inverse light microscope (ECLIPS 80i with WG filter, Nikon, Tokyo, Japan) equipped with

a digital camera. Images were captured at 0, 24, 48, 72, and 96 hours [24]. The percentage of wound healing was calculated based on the reduction in scratch area measured at specific times, as described below (Equation 1):

$$1.\ \frac{\text{scratch area at 0h} - \text{scratch area at specific time}}{\text{scratch area at 0h}} \times 100$$

Flow cytometry analysis of apoptosis

After 24-hour treatment with *S. striata* extract (IC_{50}), HeLa cells were collected in a 500 µL Binding Buffer (Annexin V-FITC/PI cell apoptosis detection kit; Jiangsu Keygen Biotechnology). Annexin V fluorescein isothiocyanate and propidium iodide (5 µL of FITC) were added to the cell suspension and incubated for 15 minutes at room temperature in a dark place. The cells were then analyzed using a FACSCalibur flow cytometer with the BD CellQuest™ Pro Analysis system (BD Biosciences, USA). The results were analyzed using FlowJo software, version 10.

Determination of total phenolic and total flavonoid content of *S. striata*

The total phenolic and flavonoid contents of *S. striata* were determined according to the method described by Tuberoso et al. [25]. The Folin-Ciocalteu method with spectrophotometry was used to measure the total phenolic content. The total phenol content, expressed as milligrams of gallic acid equivalent per gram of extract (GAE mg/g), was measured using the standard calibration curve of gallic acid. The total flavonoid content of *S. striata* crude extract was determined by the colorimetric method and calculated from the calibration plot [26-28].

High-performance liquid chromatography of phenolic and flavonoid compounds

The hydroalcoholic extract of *S. striata* was analyzed using RP-HPLC-DAD (Shimadzu) equipped with a dual solvent pump (LC-20AD) and a photodiode array detector. A reversed-phase C18 column (25 cm×4.6 mm, 3 µm) was used at a flow rate of 1 mL/min. A linear gradient of methanol (20% to 100%) was used as the solvent system for 25 minutes. The injection volumes were 20 µL of the standards and the sample extract. Before injection, all solutions were filtered using 0.45 µm membrane filters (Millipore, Bedford, MA, USA). The column temperature was maintained at 26°C, and spectra were detected at 210-400 nm [29]. The amounts of the standard compounds in the extracts were determined using the calibration curve obtained from the standards.

Statistical analysis

All experiments were carried out with at least three independent repeats. Data were presented as Mean±SD and analyzed using one- or two-way ANOVA using GraphPad Prism software, version 9 and Compusyn software. $P<0.05$ were considered significant.

3. Results

Evaluation of cell viability

The effect of different concentrations (0, 1, 10, 100, 500, and 1000 µg/mL) of *S. striata* extract on HeLa cell viability was evaluated 24 h after treatment, using an MTT assay. As shown in Figure 1, the effect of *S. striata* extract on the viability of cells significantly increased in a concentration-dependent manner, with the most cytotoxic activity at the concentration of 1000 µg/mL (33.886±18.713). The concentration of *S. striata* extract required for a 50% inhibition of cell growth (IC_{50}) was obtained by extrapolation from an inhibition curve. We found that the IC_{50} value for HeLa cells was 433.8 µg/mL after 24 hours.

S. striata extract increased migration in HeLa cells

To evaluate the effect of *S. striata* extract on HeLa cell migration, the scratch assay method was employed, which mimics the migration of cells in vivo to some extent. The monolayer HeLa cells treated with *S. striata* extract (IC_{50}: 433.8 µg/mL) exhibited a significantly higher rate of wound healing compared to the control group at 0, 24, 48, 72, and 96 hours (Figure 2A and 2B).

Table 1. Calibration value of phenolic and flavonoid compounds

Parameter	Gallic Acid	Quercetin
Value	0.33 mg gallic acid/g extract	2.81 mg quercetin/g extract
RT (min)	2.83	15.50
Wavelength of maximum absorbance (nm)	270	370

RT: Room temperature.

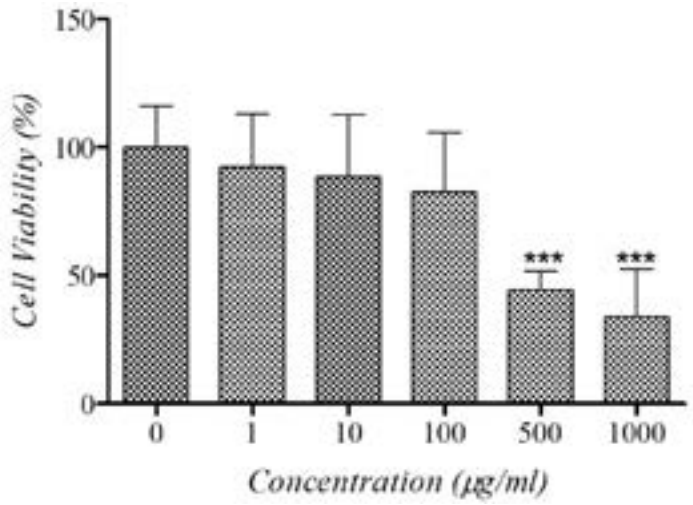

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Figure 1. *S. striata* extract decreased HeLa cell viability

Note: The cell viability of cells treated with different concentrations of *S. striata* extract (0, 1, 10, 100, 500, and 1000 µg/mL) in the HeLa cell line after 24 h. Data are expressed as Mean±SD.

*P≤0.05, **P≤0.01, indicating a significant difference between treatment and control groups.

S. striata extract promoted apoptosis in HeLa cells

The percentage of apoptotic cells increased when Hela cells were treated with 433.8 µg/mL of *S. striata* extract. We observed that treatment with *S. striata* extract for 24 hours significantly increased the extent of necrosis and late apoptosis in the HeLa cells, while the rate of early apoptosis did not significantly increase during the same time span (Figure 3A and 3B).

The *S. striata* extract had a higher total phenolic Content than total flavonoid content

The total amount of phenolic content (1375 mg gallic acid/100 g extract) in *S. striata* extract was higher than the total flavonoid content (883 mg quercetin/100 g extract).

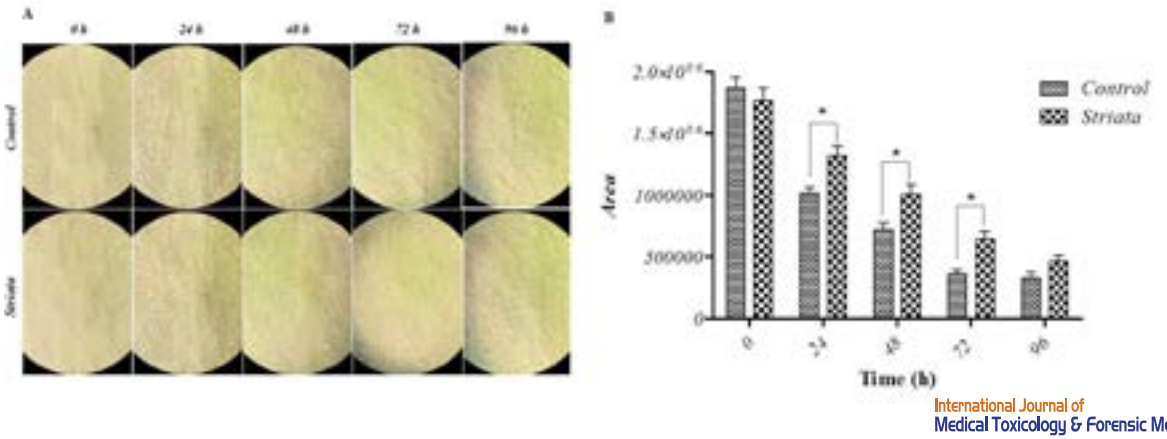

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Figure 2. *S. striata* extract increased HeLa cell migration

A) Representative micrographs of scratch areas 0, 24, 48, 72, and 96 h after incubation of HeLa cells with *S. striata* extract (433.8 µg/mL) compared to the control group, B) The effect of *S. striata* extract on wound healing (reduction in scratch area) of HeLa cells

Data are expressed as Mean±SD.

*P≤0.05, **P≤0.01.

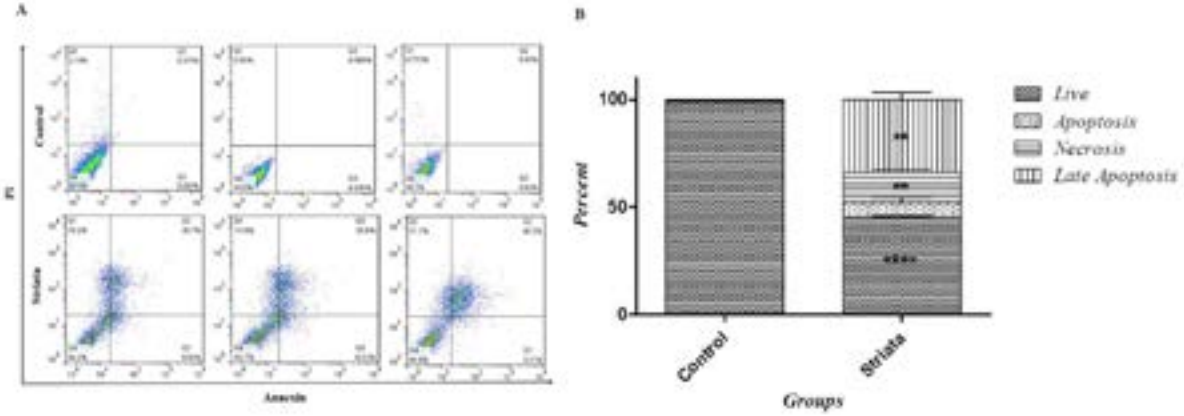

Figure 3. *S. striata* extract promoted apoptosis in the HeLa cell line

A) Flow cytometry histograms of apoptosis assay by Annexin V and PI method (HeLa cells treated with *S. striata* extract after 24 h compared to the control group, Q1: Necrosis cells, Q2: Late apoptosis cells, Q3: Early apoptosis cells, Q4: Live cells, B) *S. striata* extract reduced the percentage of live cells and increased the late apoptotic population

**P<0.01, ****P<0.0001 vs. the control group.

High-performance liquid chromatography (HPLC) of *S. striata* extract

A calibration curve was calculated for gallic acid and quercetin compounds in the extract of *S. striata*, and the data are reported in Table 1. The hydroalcoholic extract of *S. striata* was standardized according to the gallic acid (0.33 mg gallic acid/g extract) and quercetin (2.81 mg quercetin/g extract) compounds (Figure 4).

4. Discussion

We evaluated the cytotoxicity of different concentrations *S. striata* extract on HeLa human cervical cancer cells. Our results showed that *S. striata* inhibited the growth of HeLa cells in a dose-dependent manner. Furthermore, we found that the extract concentration up to 433.8 µg/mL had no significant toxic effect on HeLa cells. We also determined the effect of *S. striata* hydroalcoholic extract on the migration of HeLa cervical cancer cells. Our results indicated that the optimum concentration of *S. striata* extract might have the potential to induce wound healing, which could be due to its phenolic components, such as gallic acid and quercetin. Our findings are in agreement with the study by Yang et al. who reported that gallic acid promoted wound healing through the activation of FAK, Erk, and JNK [30]. Additionally, our results support a previous study showing that quercetin treatment reduced the migration of HeLa cells and improved wound healing [31]. Furthermore,

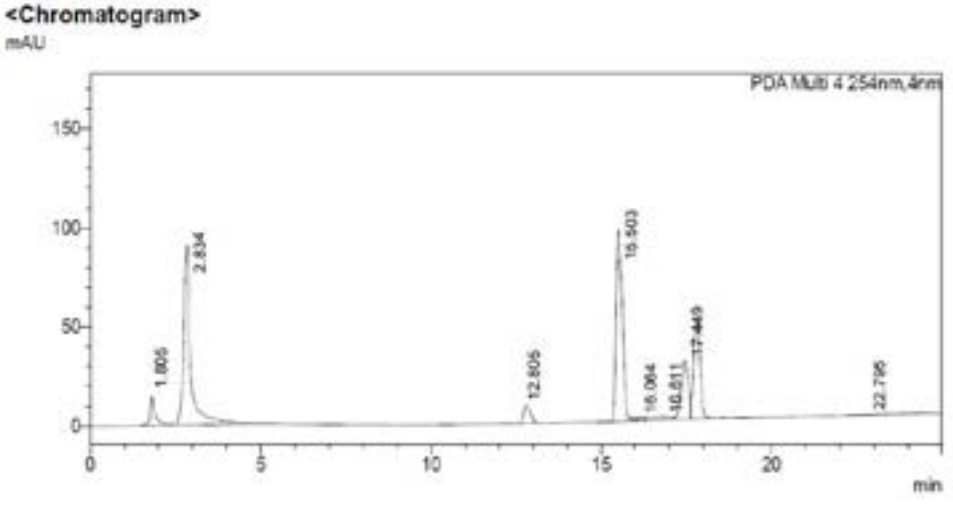

Figure 4. HPLC of hydroalcoholic extract of the *S. striata*

our results are consistent with other related studies indicating that the extract of *S. striata* plays a significant role in the contraction and healing of wounds in rats [32-34].

Previous studies have suggested that the anti-cancer effect of medicinal plants is mostly attributed to the induction of apoptosis [35-39]. Apoptosis, a physiological process of programmed cell death, plays a major role in the homeostasis of both normal and cancer cells [40, 41]. Dysregulation of apoptosis is considered to be a hallmark of cancer progression [42]. In this study, *S. striata* extract was found to increase the percentage of apoptosis in HeLa cells. Specifically, the extract could induce apoptosis, which was significantly higher than the rate of early-stage apoptosis. This result is consistent with the findings of Azadmehr et al. [43] and Nokhodi et al. [44]who demonstrated that the extract of *S. striata* is a potential inducer of cell cycle arrest in the G2/M phase and apoptosis of tumor cells as well as promastigote cells of Leishmania major.

Previous studies have reported that quercetin can increase caspase-3 and upregulate genes involved in the extrinsic apoptotic pathway [31]. Additionally, some studies have reported the apoptotic activities of gallic acid towards cancer cells, which can be mediated through different mechanisms and apoptotic molecules [45, 46]. Therefore, it can be suggested that the increased apoptosis observed in the presence of *S. striata* extract might be attributed to its phenolic content.

Another intriguing result of our study was that alongside apoptosis, *S. striata* significantly increased the extent of necrosis. A possible explanation for this finding might be related to the anti-inflammatory activity of *S. striata*, which inhibits the production of nitric oxide (NO) and pro-inflammatory cytokines, such as TNF-α, IL-1β, and PEG2 [17, 47]. Previous studies have reported that NO can induce necrosis through different mechanisms [48-50]. Thus, it can be inferred that *S. striata* may interfere with NO-mediated necrosis through unidentified mechanisms, which requires further cellular and molecular investigation.

5. Conclusion

In conclusion, our results demonstrated the effective role of *S. striata* in the treatment of cervical cancer. The involvement of *S. striata* extract in wound healing and promotion of apoptosis and necrosis of HeLa cervical cancer cells pave the way towards a better understanding of effective compounds of *S. striata* with therapeutic effects in the treatment of various cancers and other inflammatory diseases.

Ethical Considerations

Compliance with ethical guidelines

This research was approved by the research Ethics Committee of Shahid Beheshti University of Medical Sciences (Code: IR.SBMU.LASER.REC.1402.013).

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Authors' contributions

Conceptualization and supervision: Fatemeh Javani Jouni and Jaber Zafari; Methodology: Elham Rajabbeigi; Investigation, Data collection: Leila Mohaghegh Shalmani and Seyedeh Zohreh Azarshin; Data analysis: Vahid Mansouri, Hamid Mohaghegh Shalmani and Hamideh Moravej Farshi; Funding acquisition and Resources: Jaber Zafari; Writing: All authors.

Conflict of interest

The authors declared no conflicts of interest.

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