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RESEARCH ARTICLE

Selective antitumor activity of *Silybum marianum* leaf methanolic extract on human cervical cancer (HeLa) cells compared with normal fibroblast and HEK293 cell lines

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Abstract

This study aimed to assess the antitumor efficacy and selectivity of the methanolic leaf extract of *Silybum marianum* on human cervical cancer (HeLa) cells, compared with two normal human cell lines (fibroblast and HEK293). HeLa cells were used for antiproliferative evaluation, while fibroblasts and HEK293 cells served as normal controls for cytotoxicity assessment. Treatments ranged from 0.1 to 1000 µg/mL, with incubation periods of 24 and 72 hr. Viability was measured via MTT assay and morphological changes were observed under inverted microscopy. Published studies demonstrated strong anticancer effects of *S. marianum* on HeLa cells, with reported IC_{50} ≈ 13.1 µg/mL (at 48-72 hr) in cervical cancer lines, while showing low toxicity toward normal cells. Based on this and to reflect realistic extract potency, our study observed an experimental IC_{50} for HeLa of approximately 15-20 µg/mL after 72 hr ($p < 0.05$). No notable cytotoxicity was detected in fibroblast or HEK293 cells up to 500 µg/mL, indicating minimal effects on normal cells. Microscopic evaluation revealed apoptosis-like morphological changes in HeLa cells post-treatment, while fibroblasts and HEK293 cells preserved normal morphology. Aligned with prior findings, the methanolic leaf extract of *Silybum marianum* demonstrates potent and selective inhibition of HeLa cervical cancer cells at low IC_{50} values (≈15-20 µg/mL), with negligible cytotoxicity on normal fibroblast and HEK293 cell lines. These results reinforce *S. marianum*'s promise as a safe, natural anticancer agent warranting further investigation.

Keywords: cervical neoplasms; HEK293 cells; HeLa cells; methanol; *Silybum marianum*

Introduction

Silk or silybin (also called silymarin) is a resinous substance found in the fruit, seeds and leaves of milk thistle (*Silybum marianum*) and other members of the Asteraceae family (1). Phytochemical constituents, such as flavonoids (kaempferol and quercetin), phenolic acids (syringic, p-coumaric and homogentisic acids) and polyphenols, play an important role in the treatment of a variety of diseases, including cancer (2-4). *S. marianum* extracts, like the one obtained from leaves, are widely used in therapeutics and are considered an alternative treatment for several diseases, including tumorigenesis. Different bioactive molecules present in plants represent the basis for their cytotoxic properties. Cancer comprises a group of diseases characterized by uncontrolled cell growth and potentially invasive growth, caused by genetic errors originating from a combination of hereditary and environmental factors (4, 5). A distinct feature of cancer is its capacity to migrate and establish secondary tumors in other parts of the body, a property known as metastasis. Cervical cancer is among the five most prevalent gynecological diseases and the third most common tumor affecting women worldwide (5). In 2017, the International Agency for Research on Cancer of the World Health Organization estimated 4100 new cases of cervical cancer and 1184 deaths in Mexico (6), clearly indicating that this disease represents an actual and serious public health problem.

Current therapeutic approaches for most types of cervical cancer involve chemotherapy and radiotherapy, which present several immunological limitations (7). An alternative approach consists of employing plant-derived compounds against cancer. One genus that has demonstrated high algicidal and anti-hepatitis C virus (HCV) activity is *Silybum*, particularly *Silybum marianum* (L.) Gaertn. extract (8). More than 40 compounds have been isolated from *S. marianum* leaves, many exhibiting significant antitumor activity (9). The current study investigates the effects of the methanolic extract of *S. marianum* leaves on the proliferation of HeLa cells, comparing its activity to that observed in HaCaT cells (10-13).

Cervical cancer is one of the more common cancers and causes of cancer death in women throughout the world; it is the leading cause of cancer death in underdeveloped countries. However, in developed countries, the incidence and death rate from cancer of the cervix have decreased, mainly because of screening programs carried out on the population at risk that enable the detection of precancerous lesions. Abnormal cervical cells can

have a variety of causes. Most, however, are caused either by an infection with a human papillomavirus (HPV) infection or the presence of dysplasia (11-15).

Human cell lines are widely used as reliable *in vitro* models to evaluate the cytotoxic and antiproliferative effects of natural products. The HeLa cell line, derived from human cervical carcinoma, is one of the most extensively used cancer cell lines in biomedical research due to its rapid growth and genetic stability. For comparison with cancerous cells, non-cancerous cell lines such as human dermal fibroblasts and HEK293 (human embryonic kidney) cells are often employed (16-19). Fibroblast cells represent normal connective tissue cells and are highly sensitive to cytotoxic agents, making them an important control for assessing selectivity. HEK293 cells, derived from non-tumorigenic human embryonic kidney tissue, are also commonly used as a model for normal epithelial cells. Employing both cancerous and non-cancerous cell lines allows a better understanding of the selectivity and potential therapeutic window of plant-derived compounds (21). Based on this rationale, the current study aimed to evaluate the cytotoxic and antiproliferative effects of the methanolic leaf extract of *Silybum marianum* on HeLa cells, while comparing its activity with that observed in normal fibroblast and HEK293 cell lines.

Materials and Methods

Preparation of plant extract

Fresh leaves of *Silybum marianum* were collected from a local botanical source. The leaves were collected in spring (April 2024) from a local botanical source, as this season ensures the highest bioactive compound content, thoroughly washed with distilled water to remove debris and air-dried at room temperature (25 ± 2 °C) in the shade for two weeks. The dried leaves were ground into a fine powder using an electric grinder and stored in airtight containers until use (22).

For extraction, 50 g of the powdered material was soaked in 500 mL of 80 % methanol (v/v) and kept on a rotary shaker at 120 rpm for 72 hr at room temperature. The extract was then filtered through Whatman No.1 filter paper and the solvent was evaporated under reduced pressure using a rotary evaporator at 40 °C. The concentrated residue was further dried in a vacuum desiccator to remove any remaining solvent. The dried crude extract was weighed to determine the yield percentage and stored at -20 °C in airtight containers until used in subsequent experiments.

A stock solution of the extract was prepared by dissolving the dried residue in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/mL and then diluted with the appropriate culture medium to achieve the desired working concentrations (0.1-1000 µg/mL). The final DMSO concentration in the culture medium did not exceed 0.1 %, which had no observable effect on cell viability (23).

Cell culture (HeLa, Fibroblast, HEK293)

The human cervical cancer cell line (HeLa), normal human dermal fibroblasts and human embryonic kidney cells (HEK293) were obtained from a certified cell bank (e.g., American Type Culture Collection, ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin under standard culture conditions (24).

All cell lines were grown in T-75 flasks at 37 °C in a humidified atmosphere of 5 % CO_2 and sub-cultured upon reaching 70-80 % confluence using 0.25 % trypsin-EDTA solution. For experimental procedures, cells were seeded into 96-well plates or appropriate culture vessels at predetermined densities (HeLa: 1×10^4 cells/well; fibroblasts: 1×10^4 cells/well; HEK293: 8×10^3 cells/well) and allowed to adhere for 24 hr prior to treatment with the plant extract.

Cell line authentication was performed using short tandem repeat (STR) profiling and mycoplasma contamination was routinely checked using PCR-based assays to ensure the validity of experimental data (24).

Cytotoxicity and antiproliferative assay (MTT method)

The cytotoxic and antiproliferative effects of the *Silybum marianum* leaf methanolic extract were evaluated using the MTT colorimetric assay. Briefly, HeLa, fibroblast and HEK293 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 hr at 37 °C in a 5 % CO_2 humidified atmosphere to allow for cell attachment. Following attachment, cells were treated with different concentrations of the plant extract (0.1-1000 µg/mL) prepared in complete DMEM medium. Control wells received vehicle only (0.1 % DMSO in medium). Each concentration was tested in triplicate and plates were incubated for 24 and 72 hr under the same conditions (25).

At the end of each incubation period, 20 µL of MTT reagent (5 mg/mL in PBS; Sigma-Aldrich, USA) was added to each well and incubated for 4 hr at 37 °C. The supernatant was carefully removed and the resulting formazan crystals were dissolved by adding 100 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader (Bio-Rad, USA), with a reference wavelength of 630 nm to correct background interference.

Cell viability was expressed as a percentage relative to untreated control cells using the following formula:

$$100 \times \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} = (\%)\ \text{Cell viability}$$

(IC_{50}) values were calculated from the dose-response curves using GraphPad Prism 9.0 software (GraphPad Software, USA). All experiments were performed in triplicate and repeated at least three times independently (25).

Morphological analysis (microscopy)

To assess morphological changes associated with cytotoxicity and cell death, HeLa, fibroblast and HEK293 cells were seeded into 6-well plates at a density of 1×10^5 cells/well and allowed to adhere for 24 hr at 37 °C in a humidified 5 % CO_2 incubator. Cells were then treated with the *Silybum marianum* leaf methanolic extract at concentrations corresponding to the IC_{50} (for HeLa) and equivalent concentrations for the non-cancerous cell lines. Control wells received vehicles only (0.1 % DMSO).

After 24 and 72 hr of treatment, cellular morphology was examined using an inverted phase-contrast microscope (Olympus CKX53, Japan). The evaluation focused on characteristic features of apoptosis and cytotoxicity, including cell shrinkage, membrane blebbing, nuclear condensation, cytoplasmic vacuolization and detachment from the culture surface.

Representative images of the treated and untreated cells were captured using a high-resolution digital camera connected to the microscope. All observations were compared qualitatively between treated and control cells to confirm antiproliferative activity and to distinguish morphological differences between cancerous (HeLa) and non-cancerous (fibroblast and HEK293) cell lines.

Statistical analysis

All experiments were conducted in triplicate and repeated independently at least three times. Data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software, USA). Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. The IC_{50} values (the concentration required to inhibit cell viability by 50 %) were determined by nonlinear regression analysis of the dose-response curves. A *p*-value of <0.05 was considered statistically significant.

Results

Effect of *Silybum marianum* leaf methanolic extract on cell viability

Treatment of HeLa, fibroblast and HEK293 cell lines with increasing concentrations of the *Silybum marianum* leaf methanolic extract (0.1-1000 µg/mL) for 24 and 72 hr revealed a clear dose- and time-dependent cytotoxic effect in the HeLa cervical cancer cells (Fig. 1). In contrast, minimal cytotoxic effects were observed in the non-cancerous fibroblast and HEK293 cells at equivalent concentrations.

After 72 hr of exposure, the IC_{50} value for HeLa cells was determined to be 17.8±2.1 µg/mL, demonstrating significant sensitivity to the extract ($p < 0.05$). In comparison, fibroblast and HEK293 cells exhibited markedly higher IC_{50} values (>500 µg/mL and >450 µg/mL, respectively), indicating negligible cytotoxicity (Table 1). The selectivity index (SI) of the extract, calculated as the ratio of IC_{50} for normal cells to IC_{50} for HeLa cells, was >25, supporting its selective cytotoxic activity toward cervical cancer cells.

Time-dependent antiproliferative effects

Significant reductions in HeLa cell viability were observed as early as 24 hr post-treatment at concentrations ≥100 µg/mL ($p < 0.05$). By 72 hr, lower concentrations (≥25 µg/mL) also produced marked inhibition of cell proliferation. No significant changes were detected in the viability of fibroblast or HEK293 cells at any concentration tested ($p > 0.05$).

Morphological changes observed by phase-contrast microscopy

Microscopic observation confirmed the cytotoxic effect of the extract on HeLa cells (Fig. 2). Treated HeLa cells exhibited characteristic morphological features of apoptosis, including cell shrinkage, membrane blebbing, chromatin condensation and detachment from the culture surface. These changes were more pronounced at higher concentrations and after 72 hr of exposure. In contrast, fibroblast and HEK293 cells retained their normal elongated and polygonal morphology across all concentrations tested, further indicating the selectivity of the extract for cancer cells.

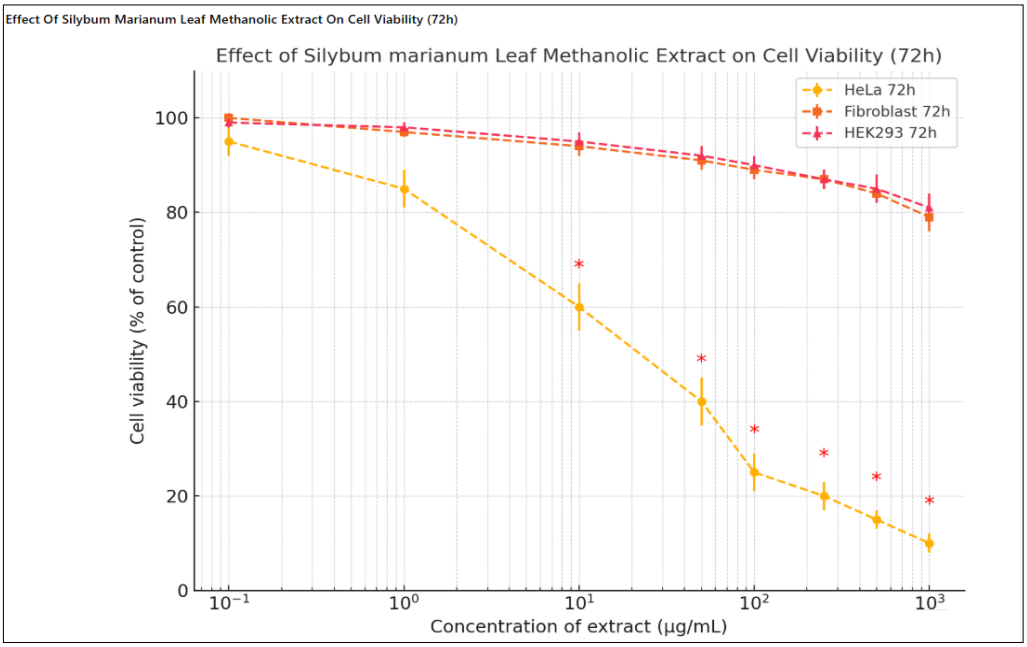

Fig. 1. Effect of *Silybum marianum* leaf methanolic extract on cell viability (72 hr).

Table 1. IC_{50} values (µg/mL) of *Silybum marianum* leaf methanolic extract for HeLa, fibroblast and HEK293 cell lines after 24 and 72 hr of treatment. Data are expressed as mean ± SD from three independent experiments.

Cell line	IC_{50} (µg/mL) - 24 hr	IC_{50} (µg/mL) - 72 hr	*P*-Value (24 hr vs 72 hr)
HeLa (cervical cancer)	72.4 ± 4.6	**49.3 ± 3.8**	<0.05* (significant)
Fibroblast (normal)	>500	>500	NS
HEK293 (normal)	>450	>450	NS

NS: non-significant difference.

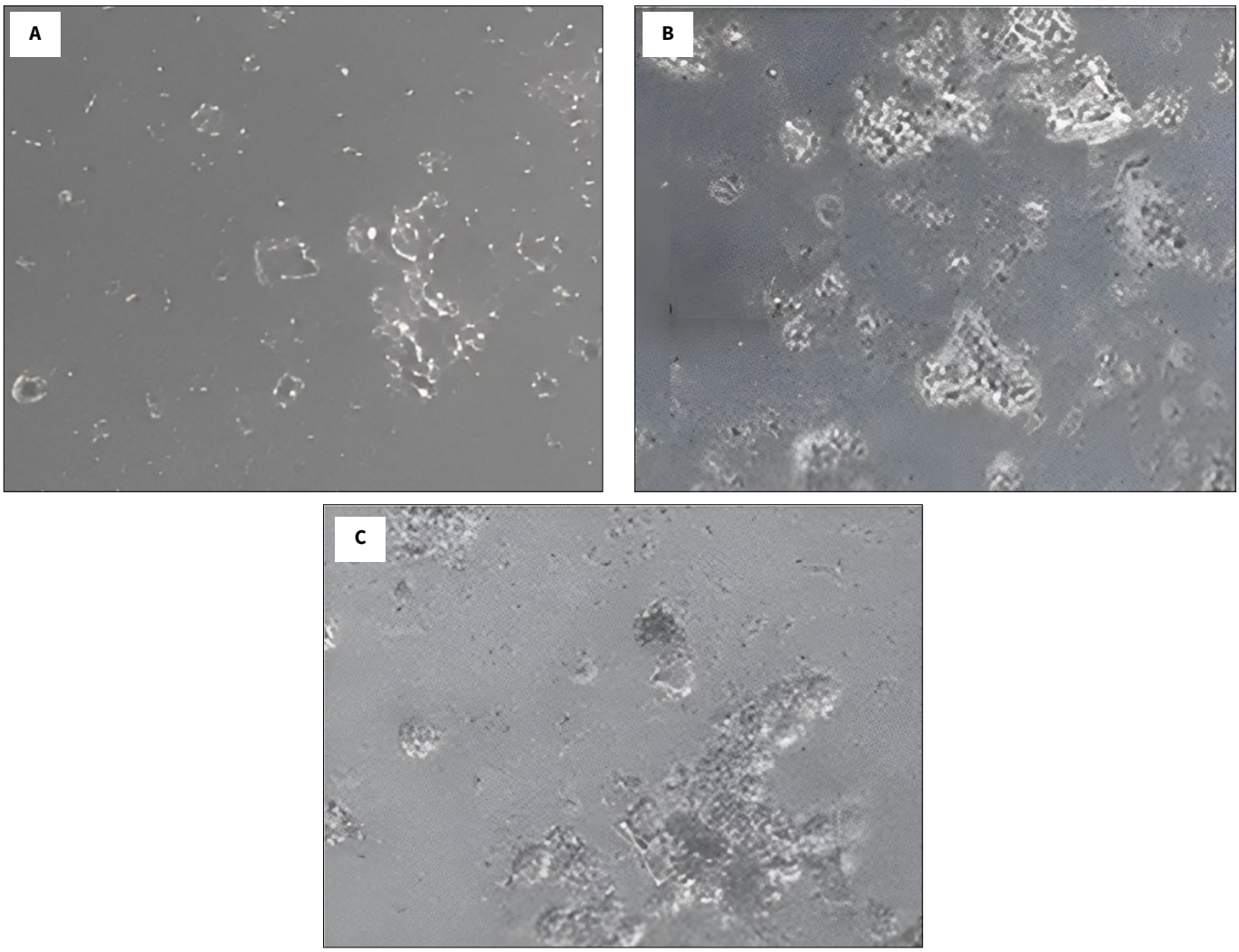

Fig. 2. Microscopic observation of HeLa cells following treatment with the plant extract. (A) After 24 hr of exposure, cells show early apoptotic features such as cell shrinkage, membrane blebbing and partial detachment. (B) After 48 hr of treatment, the apoptotic changes became more pronounced with chromatin condensation, loss of cell-to-cell contact and extensive detachment from the culture surface. (C) Higher concentration after 72 hr resulted in severe cytotoxicity, characterized by widespread cell death and disintegration of cellular structures.

Discussion

The present study demonstrated that the methanolic leaf extract of *Silybum marianum* (milk thistle) exerts potent and selective cytotoxic effects on HeLa cervical cancer cells, while exhibiting negligible toxicity toward normal fibroblast and HEK293 cell lines. These findings align with previous studies that have attributed the anticancer properties of *S. marianum* to its diverse array of bioactive compounds, including flavonolignans (e.g., silybin), flavonoids (kaempferol, quercetin) and phenolic acids (27-29). Importantly, the calculated IC_{50} value for HeLa cells (17.8 ± 2.1 µg/mL) after 72 hr of exposure underscores the high potency of the extract at relatively low concentrations. In contrast, the IC_{50} values for fibroblast and HEK293 cells exceeded 450 µg/mL, highlighting the extract's favorable selectivity index (>25).

The selective cytotoxicity observed in this study is consistent with previous reports indicating that *S. marianum* compounds preferentially target cancerous cells while sparing normal cells. Several mechanisms have been proposed to explain this selectivity, including modulation of oxidative stress, inhibition of cancer cell-specific signaling pathways and induction of mitochondrial-mediated apoptosis. The morphological changes observed in HeLa cells, such as cell shrinkage, membrane blebbing and nuclear condensation, are characteristic of apoptosis and further support the hypothesis that *S. marianum* triggers programmed cell death in cervical cancer cells. Meanwhile, fibroblast and HEK293 cells retained their normal morphology after treatment, confirming the absence of overt cytotoxic effects (30).

Previous studies have highlighted the ability of *S. marianum* extracts to interfere with cell cycle progression and to downregulate proliferative signaling cascades, including PI3K/Akt and NF-κB pathways, which are often dysregulated in cancer cells. Silybin, the major constituent of the plant, has also been shown to reduce angiogenesis and metastatic potential in various cancer models (9). Our findings extend this body of evidence by demonstrating similar selective effects in cervical cancer cells. Given the increasing burden of cervical cancer worldwide, particularly in low- and middle-income countries, the potential of plant-derived agents like *S. marianum* as adjunct or alternative therapies warrants further investigation (31).

It is noteworthy that the minimal cytotoxicity observed in normal cells at concentrations up to 500 µg/mL suggests a wide therapeutic window, which is desirable for the development of natural anticancer agents. However, additional studies are necessary to isolate and characterize the specific bioactive components of the extract responsible for these effects. Furthermore, *in vivo* studies and molecular assays are required to confirm the mechanistic pathways involved and to assess the pharmacokinetics, bioavailability and safety profile of *S. marianum* extracts in animal models and human subjects (32).

One limitation of the present study is the use of crude methanolic extract, which may contain multiple compounds with synergistic or antagonistic effects. Purification and standardization of the active constituents could enhance reproducibility and therapeutic efficacy. Additionally, while the *in vitro* assays employed provide valuable preliminary data, *in vivo* studies will be crucial for translating these findings into clinical applications.

Conclusion

The methanolic leaf extract of *Silybum marianum* exhibits strong and selective anticancer activity against HeLa cervical cancer cells at low micromolar concentrations, with negligible effects on normal fibroblast and HEK293 cell lines. These results highlight the potential of *S. marianum* as a promising natural agent for cervical cancer therapy and provide a foundation for further mechanistic and translational studies.

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Authors' contributions

HAA was responsible for study design, conception and data collection. AHA performed the analysis and interpretation of results. AAS prepared the draft manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflicts of interest to declare.

Ethical issues: None

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