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Evaluation of the Cytotoxic Effects of Plantago lanceolata L. Extract and Its Combinations with Chemotherapeutic Agents on Lung Cancer Cells

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Abstract

Lung cancer remains the leading cause of cancer-related deaths worldwide, underscoring the urgent need for safe and effective therapeutic strategies. In this context, *Plantago lanceolata* L., a medicinal plant traditionally used in the treatment of respiratory disorders, has attracted growing scientific interest due to its rich phytochemical composition and potential anticancer properties. This study investigates the cytotoxic effects of a methanolic extract obtained from *P. lanceolata* L., alone and in combination with conventional chemotherapeutic agents such as Methotrexate and Cytarabine (Alexan), on human lung adenocarcinoma (A549) cells and murine macrophage (J774) cell lines. Previous studies have demonstrated the antiproliferative activity of *Plantago* species and the dose-dependent cytotoxic effects of their phenylethanoid glycosides (e.g., acteoside, plantamajoside) across various cancer cell lines. In the present study, comparable anticancer efficacy was achieved using substantially lower doses of the plant extract than those previously reported. Furthermore, the extract preserved the viability of immune cells, suggesting potential M1 macrophage activation and an immunomodulatory role. The cytotoxic effects were evaluated using the MTT assay. This is the first study to evaluate the synergistic effects of *P. lanceolata* L. in combination with Methotrexate and Alexan against lung cancer cells, offering preliminary evidence supporting its use as an adjunct phytotherapeutic agent. The findings demonstrate selective toxicity of the extract toward cancer cells with minimal effects on healthy immune cells, highlighting its promising therapeutic potential that warrants further validation through in vivo and mechanistic investigations.

Keywords: *Plantago lanceolata L.*, lung cancer, A549 cell line, Methotrexate, Cytarabine, macrophage, phytotherapy

Introduction

Lung cancer is one of the most lethal malignancies worldwide and remains a major public health

problem despite advances in modern therapeutic approaches. Surgery, chemotherapy, radiotherapy and immunotherapy are the primary treatment options for lung cancer, but their severe side effects and limited efficacy have led to the pursuit of new therapeutic strategies (1). Plant-based treatments have gained attention as alternatives that may support conventional therapies due to their lower toxicity and beneficial properties (2). Among these, *Plantago lanceolata L.*, a medicinal plant known for its use in respiratory diseases, has emerged as a promising candidate for investigation against lung cancer due to both its historical applications and pharmacological profile.

Plantago lanceolata L. is a perennial plant native to temperate regions of Europe and Asia and is now widespread globally (3). In Turkey, it is commonly referred to as “sinirli ot” or “sinir otu” and has long been used in folk medicine. It is frequently employed in the treatment of respiratory conditions as a cough suppressant, expectorant and chest soother, particularly in cases such as bronchitis. In Finland and Romania, it is used as a digestive aid and expectorant; in Slovenia and Italy, as a soothing and antimicrobial agent; and in Poland and Belgium, it is consumed as tea, syrup or tablets for various health issues. Herbal tea made from its leaves has been used against cough, whooping cough and dysentery, earning it the reputation of a “breathing herb” in traditional usage (4). Other reported uses include wound healing, alleviating skin inflammations and relieving insect bites. It is listed in several national pharmacopoeias and is officially recognized as a medicinal plant in the British Pharmacopoeia (5).

The therapeutic effects of *P. lanceolata* are associated with its rich phytochemical content. Its leaves and seeds are abundant in polysaccharides, tannins, flavonoids, phenolic acids and iridoid glycosides. Among its major active compounds are the phenylpropanoid derivative verbascoside, and the iridoid glycosides aucubin and catalpol. Flavonoid glycosides such as rutin, myricetin, quercetin and kaempferol have also been identified and quantified by high-performance liquid chromatography (HPLC) analysis (6, 7). Due to these constituents, *P. lanceolata* exhibits antioxidant, anti-inflammatory, antimicrobial and wound-healing properties, which have been confirmed in several studies. The plant also possesses cytotoxic and antiproliferative activities, with demonstrated inhibitory effects on the growth of various cancer cell lines including A549 (lung), MCF-7 (breast), and HeLa (cervical) cells (4). Extracts from the leaves and seeds of *P. lanceolata*, as well as from the related species *Plantago major*, are recognized for their strong antioxidant and anti-inflammatory activities and have been traditionally used as supportive agents in tumor treatment (5).

Recent studies have begun to elucidate the anticancer potential of *Plantago lanceolata*. Leaf extracts of *P. lanceolata* have demonstrated an ability to suppress proliferation in various cancer cell lines under in vitro conditions. A pronounced growth-inhibitory effect was observed particularly in the triple-negative breast cancer cell line CAL51, whereas a more limited impact was detected in other breast cancer cell types. High concentrations of the extract were also reported to induce morphological alterations (e.g., cell shrinkage, membrane blebbing, and cytoplasmic vacuolization) and reduce colony formation in cancer cells. The antiproliferative activity of leaf extracts from species of the *Plantago* genus has also been noted in estrogen receptor-positive MCF7 breast cancer cells and in prostate cancer cell lines (8). Although studies on the effects of *P. lanceolata* in lung cancer remain scarce, initial findings appear promising. A recent case report documented a remarkable 62% reduction in tumor volume following four months of regular administration of *Plantago major* seed extract to a patient with stage IIA lung adenocarcinoma. Significant improvements in inflammatory markers such as ESR and CRP were

observed during this treatment period, which were attributed to the anti-inflammatory and antiproliferative properties of *Plantago* species. The report also referenced previous experimental studies indicating that extracts of *Plantago* exhibited therapeutic effects on lung cancer cell lines (9). These findings suggest that *P. lanceolata* may not only exert direct cytotoxic effects on tumor cells but also modulate the tumor microenvironment and host immune responses to slow cancer progression.

In solid tumors such as lung cancer, immune cells within the tumor microenvironment play a critical role. Macrophages, when polarized into tumor-associated macrophages (TAMs), can exert dual effects in cancer development and immune regulation. TAMs are broadly classified into two phenotypes: M1, which exhibits pro-inflammatory and antitumor activity, and M2, which displays immunosuppressive and tumor-promoting characteristics. In many malignancies, TAMs predominantly exhibit the M2 phenotype, which facilitates tumor growth and metastasis. Redirecting this M2-dominant macrophage population toward an M1 phenotype is considered an important strategy in current cancer immunotherapies (10). The immunomodulatory effects of *Plantago lanceolata*, particularly its ability to influence macrophage functional states, represent a crucial aspect of its anticancer potential. Studies have demonstrated that polysaccharides derived from *Plantago* species can modulate macrophage activation. Acidic polysaccharides isolated from *Plantago major* leaves have activated murine J774 macrophage cells, inducing high levels of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) production (11). These activated M1-like macrophages are capable of eliciting cytotoxic responses against tumor cells and enhancing antitumor immunity. A polysaccharide fraction obtained from *Plantago asiatica* was also shown to polarize macrophages toward an M1 phenotype, thereby indirectly inhibiting the progression of 4T1 breast tumors. In this study, in vivo application of the polysaccharide promoted the accumulation of M1-like macrophages and $CD4^+/CD8^+$ T lymphocytes in the spleen and lymph nodes of tumor-bearing mice, resulting in significantly suppressed tumor growth (12). These data indicate that *P. lanceolata* may inhibit tumor development not only through direct cytotoxicity but also by reprogramming immune cells within the tumor microenvironment.

Plantago lanceolata L., a plant traditionally used in the treatment of respiratory disorders, has attracted attention in recent scientific literature for its multifaceted potential against lung cancer. The antioxidant and anti-inflammatory constituents of the plant demonstrate the ability to directly suppress tumor cell proliferation (e.g., through apoptosis induction, inhibition of cell cycle progression, and modulation of inflammatory pathways). Its capacity to activate immune cells such as macrophages in favor of antitumor responses represents a promising complementary approach for lung cancer treatment. This review will further examine the effects of *P. lanceolata* on lung cancer by integrating historical use and evidence from modern molecular and cellular studies. A comprehensive perspective will be provided regarding the scientific basis of its traditional applications and its potential for future clinical implementation.

2. Materials and Methods

2.1. Preparation of *Plantago lanceolata* L. Extract

Plantago lanceolata L. was collected and mechanically ground. A total of 8 grams of the plant material was weighed and incubated in methanol in the dark for six days. After the incubation period, the extract was filtered using Whatman filter paper. The filtrate was transferred into a beaker, and the methanol was evaporated by air flow and heat exposure (13). The amount of

extract obtained was measured using an analytical balance.

2.2. Cell Culture

Murine macrophage cell line (J774) and human lung cancer cell line (A549) were obtained from the laboratory cryobank. RPMI-1640 medium was prepared for cell propagation. Stock media were supplemented with 1% L-glutamine and penicillin-streptomycin. During experimental procedures, 10% fetal bovine serum (FBS) (Sigma) was added to the culture medium (14). Cells with passage numbers between 10 and 15 were used in this study.

A549 and J774 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS (Sigma). The incubation was carried out at 37°C under 95% humidity and 5% CO_2 atmosphere. A549 cells were harvested enzymatically, while J774 cells were collected by mechanical scraping, followed by centrifugation at 1000 rpm for 5 minutes at 25°C. Cells were seeded into 96-well plates at a density of 1×10^5 cells/mL per well. The seeded cells were incubated for 48 hours (15).

2.3. MTT Assay

The cytotoxic effects of the prepared extract and extract–drug formulations were evaluated using the MTT assay. Cell viability was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Extract and extract–drug combinations were applied to the cells seeded in 96-well plates. Treated cells were incubated for 48 hours at 37°C in a humidified incubator with 5% CO_2. After incubation, 10 µL of MTT solution was added to each well, followed by an additional 3-hour incubation under the same conditions. Subsequently, 100 µL of dimethyl sulfoxide (DMSO) was added to each well. The plates were kept in the dark for 30 minutes (16). Cell viability was measured at a wavelength of 570 nm. The data obtained were analyzed and plotted using Equation 1.

$$\text{Cell viability (\%)} = \frac{\text{OD values in experimental group}}{\text{OD value in control group}} x100 \quad (1)$$

2.4. Statistical Analysis

Data obtained from the study were analyzed using IBM SPSS version 25.0 (IBM Corporation, Armonk, NY, USA). Comparisons between groups were performed using one-way analysis of variance (one-way ANOVA). Results were expressed as mean ± standard deviation (mean ± SD), and statistical significance was accepted at $p < 0.05$.

3. Results

Secondary metabolites found in plants, such as flavonoids, tannins, quinones and phenolic acids, can exert effects in cancer prevention and treatment by suppressing oncogenic pathways (17). In this study, an extract of *Plantago lanceolata* L. was obtained using the maceration method. The extract was applied alone and in combination with chemotherapeutic agents to evaluate its cytotoxic effects on J774 macrophage and A549 lung cancer cell lines. As a result of the experiments, the optimal extract–drug concentrations for the treatment of lung cancer were determined. These formulations are intended for use in subsequent in vivo studies. According to the MTT assay, cell viability was assessed at the end of 48 hours for all groups based on percentage viability values.

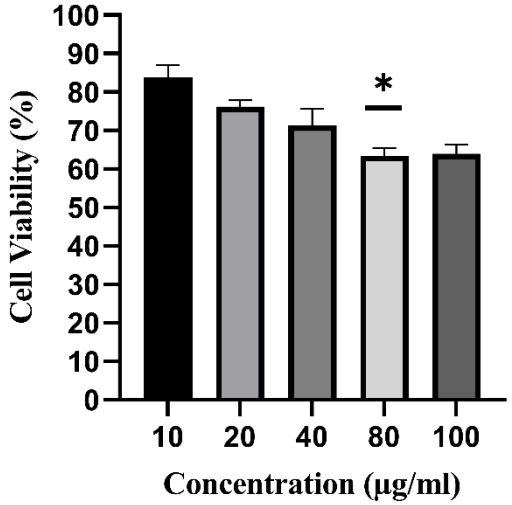

Figure 1. Cytotoxic effect of *Plantago lanceolata* L. extract on A549 lung cancer cell line

Viability analysis of A549 lung cancer cells treated with *Plantago lanceolata* L. extract is presented in Figure 1. Treatment with *Plantago lanceolata* L. extract resulted in a reduction in cell viability. At a concentration of 80 µg/mL, the extract decreased the viability of A549 cells to 63.44% ($p < 0.05$).

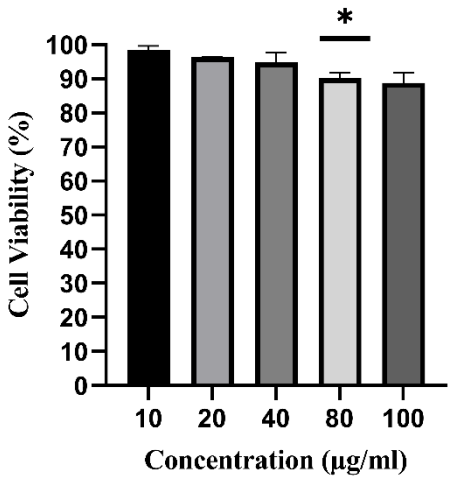

Figure 2. Cytotoxic effect of *Plantago lanceolata* L. extract on J774 macrophage cell line

Viability analysis of J774 murine macrophage cells treated with *Plantago lanceolata* L. extract is shown in Figure 2. At a concentration of 80 µg/mL, the extract alone resulted in 90.12% cell viability in the macrophage line ($p < 0.05$).

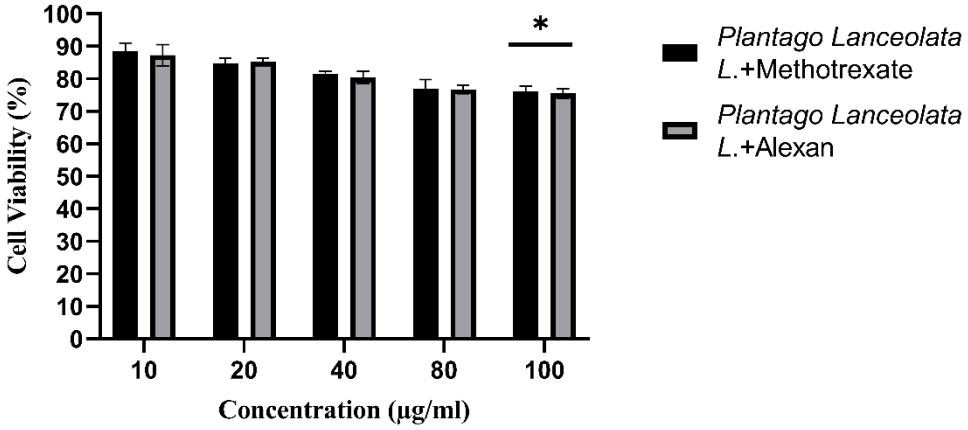

Figure 3. Cytotoxic effect of *Plantago lanceolata* L. extract–drug combinations on J774 macrophage cell line

The cytotoxic analysis of *Plantago lanceolata* L. extract in combination with Methotrexate and Alexan on the J774 macrophage culture system is shown in Figure 3. In the combinations using 80 µg/mL *Plantago lanceolata* L. extract with Methotrexate, the highest cytotoxic effect was observed at 100 µg/mL Methotrexate. Treatment with 80 µg/mL extract + 100 µg/mL Methotrexate resulted in 76.1% cell viability ($p < 0.05$). In the combinations with Alexan, where 80 µg/mL extract was used, the highest cytotoxic effect was also observed at 100 µg/mL Alexan. Treatment with 80 µg/mL extract + 100 µg/mL Alexan resulted in 75.55% cell viability ($p < 0.05$). Compared to Methotrexate or Alexan alone, the extract–drug combinations showed slightly higher cell viability in J774 macrophages, indicating reduced toxicity toward immune cells.

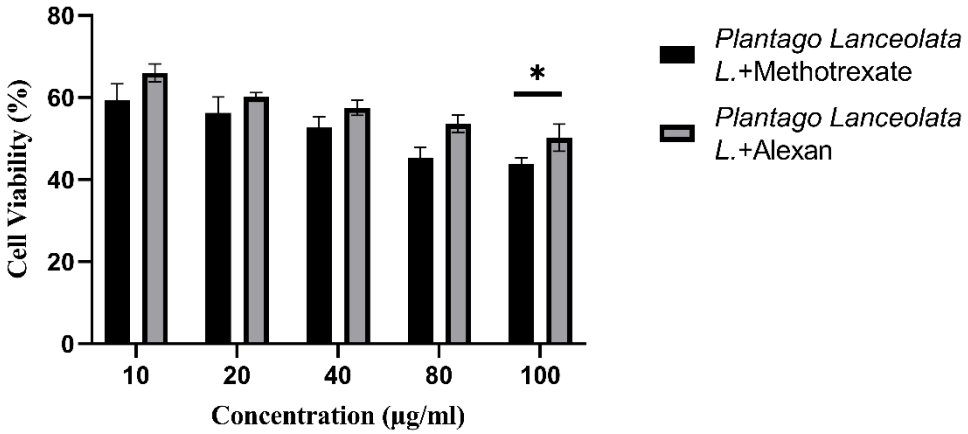

Figure 4. Cytotoxic effect of *Plantago lanceolata* L. extract–drug combinations on A549 lung cancer cell line

The cytotoxic analysis of *Plantago lanceolata* L. extract in combination with Methotrexate and Alexan on the A549 lung cancer cell culture system is shown in Figure 4. In the combinations using 80 µg/mL *Plantago lanceolata* L. extract with Methotrexate, the highest cytotoxic effect was observed at 100 µg/mL Methotrexate. Treatment with 80 µg/mL extract + 100 µg/mL Methotrexate resulted in 43.69% cell viability ($p < 0.05$). In the combinations with Alexan, where 80 µg/mL extract was used, the highest cytotoxic effect was observed at 100 µg/mL Alexan. Treatment with 80 µg/mL extract + 100 µg/mL Alexan resulted in 50.19% cell viability ($p < 0.05$).

4. Discussion and Conclusion

Plantago lanceolata, a stemless medicinal plant belonging to the Plantaginaceae family, possesses a wide range of therapeutic applications due to its rich content of bioactive compounds and minimal adverse effects. It has been used in the treatment of wound healing, digestive and respiratory disorders, infectious diseases, pain, dermatitis, fever and tumors. This broad spectrum of applications suggests that *P. lanceolata* is a potential natural source for the development of novel therapeutics. In 2020, Bahadori et al. identified 28 different phenolic compounds in the methanolic extract of *P. lanceolata* (18). Chemically diverse, this plant contains numerous active compounds such as iridoid glycosides, iridoids, polysaccharides, flavonoids, polyphenols and triterpenic acids. Flavonoids and polyphenols, in particular, exhibit strong antioxidant properties and provide cellular protection (19). Due to these antioxidant characteristics, such phytochemicals are considered to hold potential in the prevention and treatment of diseases in which oxidative damage plays a key role, including cancer. The phenolic compounds of *P. lanceolata* may suppress key mechanisms involved in lung cancer progression such as chronic inflammation, cell proliferation and metastasis. Its bioactive content may exert tumor-suppressive effects on lung cancer cells by reducing oxidative DNA damage or by activating apoptotic pathways. Further in vitro and in vivo studies on *P. lanceolata* may contribute to the development of new phytotherapeutic strategies, particularly for the treatment of lung cancer (18).

Lung cancer is one of the most commonly diagnosed malignancies worldwide and remains the leading cause of cancer-related mortality. According to GLOBOCAN 2022 estimates, 2.48 million new cases of lung cancer (12.4%) and 1.82 million deaths (18.7%) were reported in 2022 (20). These data establish lung cancer as both the most prevalent cancer type and the primary cause of cancer-related deaths. Conventional chemotherapy has long been a cornerstone in cancer treatment, but its lack of selectivity often leads to serious systemic toxicities. These agents target not only malignant cells but also rapidly dividing healthy cells, resulting in adverse effects such as mucositis, alopecia and myelosuppression (21). These complications narrow the therapeutic window, promote drug resistance and contribute to tumor recurrence (22). Methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR), disrupts DNA synthesis and exhibits potent antitumor activity. However, it is associated with dose-limiting toxicities such as hepatotoxicity and myelosuppression, requiring careful clinical management (23). In addition to its DHFR-dependent mechanism, MTX also induces oxidative stress, which contributes to its cytotoxic effects (24). Similarly, cytarabine (Ara-C), a DNA synthesis inhibitor that acts in the S phase of the cell cycle, is a key chemotherapeutic agent in the treatment of hematological malignancies such as acute myeloid leukemia. Its clinical use is limited by a short plasma half-life and severe neurotoxic side effects (25). These limitations highlight the need for more selective and tolerable chemotherapeutic strategies (26). In this context, the use of phytochemicals as adjuvants to chemotherapy has attracted increasing attention (27). Plant-derived compounds with low toxicity profiles and multiple cellular targets, such as resveratrol, curcumin and vinca alkaloids, may enhance tumor cell sensitivity to chemotherapeutic agents (26). These combination strategies aim to achieve therapeutic efficacy at lower doses while minimizing adverse effects (28).

The anticancer effects of plant-derived bioactive compounds, particularly phenylethanoid glycosides, have yielded promising results in recent preclinical studies. In this study, the cytotoxic effects of a methanolic extract obtained from *Plantago lanceolata* L. were evaluated

using the MTT assay on both A549 human lung cancer and J774 murine macrophage cell lines. Additionally, the effects of the extract in combination with chemotherapeutic agents Methotrexate and Cytarabine (Alexan) on cell viability were analyzed. According to the results, treatment with 80 µg/mL of the extract resulted in 63.44% cell viability in the A549 cell line, a statistically significant reduction ($p < 0.05$). This finding indicates that *P. lanceolata* extract possesses the potential to reduce viability in lung cancer cells even when used alone. In contrast, the same concentration of extract preserved 90.12% cell viability in J774 macrophage cells ($p < 0.05$), demonstrating lower toxicity toward healthy immune cells. In combination studies, the extract at 80 µg/mL combined with 100 µg/mL Methotrexate resulted in 43.69% viability in A549 cells, while the combination with 100 µg/mL Alexan resulted in 50.19% viability ($p < 0.05$). These results suggest that *P. lanceolata* extract may enhance the cytotoxic efficacy of chemotherapeutic drugs against cancer cells. In J774 macrophages, the same combinations resulted in 76.1% (with Methotrexate) and 75.55% (with Alexan) viability, respectively ($p < 0.05$). These findings indicate that the extract–drug combinations exert lower toxicity in healthy cells, thereby improving selectivity. This selective cytotoxicity suggests that Plantago lanceolata may potentiate chemotherapeutic efficacy through additive or synergistic mechanisms, possibly involving oxidative stress modulation, apoptosis induction, or immune-mediated tumor suppression. This study represents the first report in the literature to investigate the combination of *Plantago lanceolata* extract with Methotrexate and Alexan. It is a pioneering and original work that explores the synergistic effects of these combinations in A549 human lung cancer cells.

In a 2024 study conducted by El-Emam et al., ethanolic extracts obtained from the seeds and leaves of *Plantago lanceolata* were tested on the A549 lung cancer cell line. Treatment with the seed extract at a concentration of 4 mg/mL induced 78% cell death in A549 cells, whereas the leaf extract did not show a significant cytotoxic effect. Additionally, the seed extract exhibited no toxicity in Beas-2B normal bronchial epithelial cells, supporting its selective cytotoxicity profile (29). In the present study, *P. lanceolata* extract at a considerably lower concentration of 80 µg/mL induced 36.56% cell death in A549 cells and maintained over 90% viability in J774 macrophage cells. Although the effective dose in El-Emam's study was approximately 50 times higher, a similar level of cytotoxicity was achieved. These findings suggest that the extract used in this study may possess a stronger therapeutic potential due to its efficacy at a lower concentration. Furthermore, the observation of low toxicity in normal cells in both studies supports the potential of *P. lanceolata* as a selective and safe anticancer agent.

In a 2024 case report by Jasim et al., oral administration of *P. major* seed extract for approximately four months in a patient diagnosed with non-small cell lung cancer (adenocarcinoma, stage IIA) resulted in a 62% reduction in tumor volume and a significant decrease in inflammatory markers ESR and CRP. This report represents the first clinical case documenting the potential therapeutic effects of *P. major* against lung cancer in humans and highlights the antioxidant, anti-inflammatory and antiproliferative properties of the plant. However, the findings were based on a single patient, and further large-scale, controlled clinical studies are required to confirm and generalize these observations (9).

In a 2023 study conducted by Budzianowska et al., the cytotoxic activities of phenylethanoid glycosides (acteoside and plantamajoside) derived from *Plantago lanceolata* were evaluated on seven human cancer cell lines. These compounds exhibited significant, dose-dependent cytotoxic effects particularly on breast (MCF-7, MDA-MB-231), ovarian (OVCAR-3), glioblastoma

(U138-MG), and liver (HepG2) cancer cells. The reported IC_{50} value for acteoside after 72 hours of treatment in the MCF-7 cell line was 113.1 µM, while plantamajoside exhibited an IC_{50} of 156.1 µM in HepG2 cells. A noteworthy finding in both the Budzianowska study and the current work is the observation of minimal toxicity in normal cell lines. Viability was maintained above 70% in MCF-12A (human mammary epithelial) and J774 (macrophage) cell lines, indicating a potentially high therapeutic index for *P. lanceolata* compounds. Additionally, Budzianowska et al. reported enhanced cytotoxicity when acteoside was encapsulated in liposomal nanoparticles, suggesting that targeted delivery systems could further increase efficacy (30).

In a 2017 study conducted in Indonesia by Kartini et al., the antiproliferative effects of methanolic and aqueous extracts obtained from different parts of *Plantago major*, along with pure compounds such as ursolic acid, oleanolic acid, and aucubin, were evaluated in human cancer cell lines including A549, MCF-7, MDA-MB-231, HeLaS3, and KB. The most pronounced cytotoxic effect was observed in the methanolic seed extract, with an IC_{50} value of 153.38 µg/mL in the A549 cell line. Among the pure compounds, ursolic acid demonstrated the strongest antiproliferative activity (IC_{50}: 6.37 µg/mL in A549). Cytotoxicity assays in THP-1 macrophages revealed that the root extract was non-toxic up to concentrations of 1000 µg/mL, while seed extract and ursolic acid reduced cell viability at higher doses. These findings highlight the selective cytotoxic and potential immunomodulatory properties of *P. major*. In the present study, the extract was prepared from the whole plant material rather than isolated organs. Treatment with 80 µg/mL of this extract resulted in 63.44% viability in A549 cells. The inclusion of leaves, stems, and roots in the extract may have altered the concentration and ratios of active compounds. Furthermore, a different *Plantago* species was used. Despite species variation and the use of total plant material rather than seeds, both studies demonstrated dose-dependent antiproliferative effects, suggesting that species within the *Plantago* genus may share a common cytotoxic potential against lung cancer cells. In both studies, low or moderate doses did not cause significant toxicity in THP-1 macrophages, supporting the safety profile of these extracts for immune cells and indicating possible immunomodulatory effects (31).

The findings from the present study and previous literature suggest that the extract exhibits a high therapeutic index. The observed synergistic effects with chemotherapeutic drugs support the potential use of *P. lanceolata* as an adjuvant therapeutic agent. The development of delivery systems incorporating *P. lanceolata*-derived bioactive compounds may offer a novel strategy for the treatment of epithelial-derived cancers, particularly lung cancer.

- Consent for publication

There is no obstacle for publication approval

- Competing interests

The author declares that she has no conflict of interest.

- Funding

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