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Original Research Article

Glycitin exerts anticancer effect on human lung cancer cells through induction of apoptosis, cell cycle arrest, and inhibition of PI3K/AKT signal pathway

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Abstract

Purpose: *To determine the effect of glycitin on PI3K/AKT signaling, migration, invasion, apoptosis, and cell cycle in A549 lung cancer cells.*
Methods: *3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assays were used to determine the proliferation and colony generation potency of A549 cells, respectively, after treatment with glycitin (0 - 120 µM). Apoptosis in A549 cells was measured using DAPI and Annexin V/PI-FITC assays. Cell cycle arrest was assessed byflow cytometry, while the effect of glycitin on migration and invasion of A549 cells was determined by Transwell assay. The effect of glycitin on expressions of proteins associated with PI3K/AKT signaling in A549 cells was measured using western blotting.*
Results: *Glycitin significantly inhibited the proliferation and colony generation potential of A549 cells ($p < 0.05$). The antiproliferative effects of glycitin on A549 cells were mediated through stimulation of apoptosis and cell cycle arrest at G0/G1-phase. The compound also distorted normal cellular morphology by causing membrane damage and nuclear fragmentation. The proportion of cells in the G0/G1-phase increased after glycitin treatment, when compared to the other two phases, demonstrating cell cycle arrest ($p < 0.05$). Glycitin suppressed the migration and invasion of A549 cells. However, Western blotting results showed that glycitin down-regulated the expressions of PI3K/AKT signaling proteins in A549 cells ($p < 0.05$).*
Conclusion: *Glycitin produced significant anticancer effect on A549 cells via enhanced apoptosis, induction of cell cycle arrest, and inhibition of PI3K/AKT signalling. Moreover, it suppressed the migration and invasion of A549 cells. Therefore, glycitin is a potential lead molecule for the development of a therapeutic agent for invasive lung cancer.*

Keywords: *Lung cancer, Isoflavones, Glycitin, Apoptosis, Cell cycle, PI3K/AKT signaling*

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INTRODUCTION

Worldwide, lung cancer is the most predominant human cancer, and in terms of pervasiveness, it ranks third as most frequently diagnosed cancer in humans [1]. The fatality of this severe disorder is evident in the fact that each year, a large population of humans die from lung cancer-

associated illness globally [2]. In 2018 alone, over two million new cases and about 1.8 million deaths due to lung cancer occurred at the global level [3]. Based on its origin, lung cancer is broadly categorized into non-small cell and small cell lung cancers, the former being the more frequent, accounting for 85% of overall new lung cancer cases diagnosed worldwide [4]. Factors that enhance the lethality of lung cancer include high possibility of disease relapse, metastasis, and poor prognosis, all of which make the existing treatment methodologies ineffective, resulting in unsatisfactory clinical outcomes.

Therefore, there is the need to study in-depth mechanisms that underline lung carcinogenesis so as to discover effective therapeutic targets that will aid in the design of more effective drugs.

Natural products have been very useful in modern drug discovery and design. The medicinal and biological properties of natural products have been extensively studied by scientists. Indeed, several plant-derived phytochemicals and secondary metabolites have been beneficial in modern medicine and drug design. Some of these biologically active compounds were found effective against different human cancers under experimental conditions [5]. Flavonoids are an important class of natural products consisting of over four thousand phenylbenzopyrones mostly found among edible plants. Flavonoids have pharmacological properties: they exert anticancer, anti-inflammatory, and antioxidative effects [6]. Among the flavonoids, isoflavones from soy e.g., glycitein, daidzein, glycitin and genistein have been shown to exert anti-osteoporosis, cardioprotective and chemoprotective effects [7].

Glycitin is an isoflavone found in soy and soy products [8]. This molecule has been shown to exhibit wound-healing, cardioprotective, anti-obesity, and antioxidant properties [9]. Furthermore, glycitin, a major phytochemical constituent of ethanolic extract of *Solanum nigrum* L., exhibited anticancer effects against MCF-7 breast cancer cells via suppression of proliferation, promotion of apoptosis and induction of cell cycle arrest [10].

Several chemotherapy drugs induce anticancer effects via promotion of apoptosis and downregulation of cell cycle and PI3K/AKT signaling pathway. Apoptosis and the cell cycle are the key primary targets of therapeutics in cancer management. In the current study, the anti-lung cancer effect of glycitin molecule was determined, as well as the molecular mechanism involved in the process.

EXPERIMENTAL

Chemicals, cell culture, and conditions

The glycitin molecule (≥ 98 % purity by HPLC) was obtained from Sigma-Aldrich (St. Louis, MO, United States). Propidium iodide (PI), MTT, 4′,6-diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), Annexin V/PI, and phosphate-buffered saline (PBS) were obtained from Sigma (St. Louis, MO, United States). Antibiotics, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were procured from GIBCO (United States). Lung cancer cell line A549 was obtained from ATCC (Rockville, MD, United States). The A549 cells were grown in DMEM containing 10 % FBS and antibiotics in a humidified 5% CO_2 incubator at 37°C.

Proliferation assay

The effect of glycitin on the proliferation of A549 cells was monitored using MTT assay in line with the method of Serpeloni *et al*., with slight modifications [11]. The cells were seeded in 96-well microplates at a density of 1×10^4 per well, and cultured for 24 h to 80 % confluence. Then, the cells were starved by placing them in an incomplete medium at 37 °C for 1 h. This was followed by treatment with different glycitin concentrations viz 0, 15, 30, 60, and 120 µM, for 24 h and 48 h. Thereafter, 25 µL of 5 mg/mL MTT solution was was added to each well and left to incubate for 2.5 h. The resultant formazan crystals were dissolved in DMSO (100 µL), and the solution was placed in the dark for 20 min. Finally, the absorbance of the formazan solution was measured at 570 nm in a multimode plate reader (PerkinElmer, Boston, United States).

Clonogenic assay

To assess the colony formation in A549 cells post-glycitin treatment, clonogenic assay was performed. In essence, the cells were seeded in a 6-well plate at a density of 500 cells/well and subjected to glycitin treatment at different concentrations viz 0, 30, 60, and 120 µM for 48 h. Following the treatment, A549 cells were placed in fresh DMEM and incubated for 12 days at 37°C. After every four days, the medium was replaced with a fresh one. After the incubation period, cell colonies were washed thrice in PBS, fixed in ethanol (70 %), and crystal violet (0.1%) was used to stain the A549 colonies. Finally, the relative number of colonies was determined, and the colonies were photographed under a light microscope.

Cell morphology studies

The nuclear morphology of glycitin-treated A549 cells was evaluated using DAPI staining under fluorescence microscopy. The A549 cells were seeded at a density of 1 × 10^4 cells per well in 24-well plates, followed by treatment with various concentrations of glycitin viz 30, 60, and 120 µM for 24 h. Untreated cells served as control. Then, the cells were washed using PBS, fixed in 70 % ice-cold ethanol, and suspended in DAPI. The stained cells were incubated in the dark for 15 min at 37 ºC, followed by another PBS washing. Finally, the morphology of the glycitin-treated A549 cells was determined under a fluorescence microscope (Nikon Instruments Inc., New York, United States).

Annexin V/PI-FITC assay

The A549 cells were precultured for 48 h. In order to determine populations of apoptotic cells, the glycitin-treated A549 cells were plated at a density of 2 × 10^4 cells per well in 24-well plates containing different glycitin concentrations viz 30, 60, and 120 µM. Thereafter, the glycitin-treated cells were incubated for 24 h at 37ºC, followed by addition of 5 µL each of PI and Annexin-V fluorescein isothiocyanate. The A549 cells were then incubated in the dark for 30 min at room temperature. Finally, apoptotic cells were evaluated via flow cytometry using BD FACSCalibur™ flow cytometer (BD Biosciences, New Jersey, United States).

Flow cytometry

The percentage of A549 cells at each cell cycle checkpoint was estimated using flow cytometry. In essence, A594 cells treated with glycitin at doses of 30, 60, and 120 µM for 48 h were washed in PBS, fixed in 70 % ethanol for 1.5 h, and then rewashed with PBS. The washed A549 cells were incubated with RNase 1 (250 µg/mL) and PI (50 µl/mL) at 25ºC for 30 min. The cell cycle in glycitin-treated A549 cells was monitored using Annexin-V and PI fluorescence via FACS (Beckman Coulter, CA, United States) at emission and excitation wavelengths of 625 and 525 nm, respectively. Ten thousand cells were examined in each group.

Transwell assay

Trans-well chambers containing 8 µm pore-size membranes (Corning Co., Corning, New York, United States) were used to perform migration and invasion analysis of A549 cells. For migration analysis, A549 cells at a density of 4 × 10^4 cells in fresh DMEM were added to the upper compartments of trans-well chambers containing different glycitin concentrations viz 30, 60 and 120 µM. For invasion analysis, Matrigel obtained from BD Biosciences (Franklin Lakes, NJ, United States) was thawed overnight at 4 ºC and diluted with serum-free medium. Then, 30 µL of Matrigel was placed in the upper compartments of the trans-well chamber. Moreover, 1 × 10^4 A549 cells suspended in fresh DMEM (300 µL) were seeded onto the upper trans-well compartments at 37 ºC. For trans-well migration and invasion analysis, the basolateral trans-wells contained FBS (10%) in 600 µL of DMEM only. After incubation period of 24 h, non-migrated and non-invasive glycitin-treated A549 cells were cleaned off by scrubbing, while the migrated and invaded cells were fixed in 4 % paraformaldehyde and sprayed with 10 % crystal violet. The stained cells were viewed and counted under a microscope (Olympus, Tokyo, Japan).

Western blotting assay

The expression levels of PI3K, p-PI3K, AKT, and p-AKT proteins in glycitin-treated A549 cells were assayed with western blotting. In this process, the A549 cells were seeded in 24-well plates containing different glycitin concentrations (30, 60, and 120 µM) and incubated for 24 h. The cells were harvested upon attaining 80 % confluence. Thereafter, the A549 cells were lysed using RIPA lysis buffer. Protein contents of the lysates were quantified using BCA assay, and identical amounts (40 µg) were separated on SDS-PAGE (12%) and transferred onto PVDF membranes. Blocking of membranes was done with bovine serum albumin (BSA, 5%), followed by incubation overnight at 4ºC with corresponding primary antibodies viz PI3K, p-PI3K, AKT, and p-AKT (1:1,000 dilution, Santa Cruz, CA, United States). Then, the membranes were rinsed with Tris-buffered saline Tween-20 (TBST) for 25 min, after which they were incubated with secondary antibodies (1:3,000 dilution; Thermo Scientific) for 1 h in the dark. Thereafter, the membranes were rewashed three times in TBST for 15 min. Finally, the protein bands were visualized with a Bio Rad ECL clarity max kit over X-ray film.

Statistical analysis

The experimental data were statistically processed using GraphPad prism software version 7.0. All experiments were replicated thrice, and results are presented as mean ± SD. Students *t*-test was employed for data analysis. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Antiproliferative and anti-clonogenic effects of glycitin

To evaluate the effect of various concentrations of glycitin (Figure 1 a) on proliferation of A549 cells, MTT assay was employed. Glycitin demonstrated significant concentration- and time-dependent anti-proliferative effects on A549 cells ($p < 0.05$; Figure 1 b). Glycitin produced significant ($p < 0.05$) anti-clonogenic potency against A549 cells (Figure 2 a). The anti-clonogenic effect of glycitin followed a concentration dependent pattern (Figure 2 b).

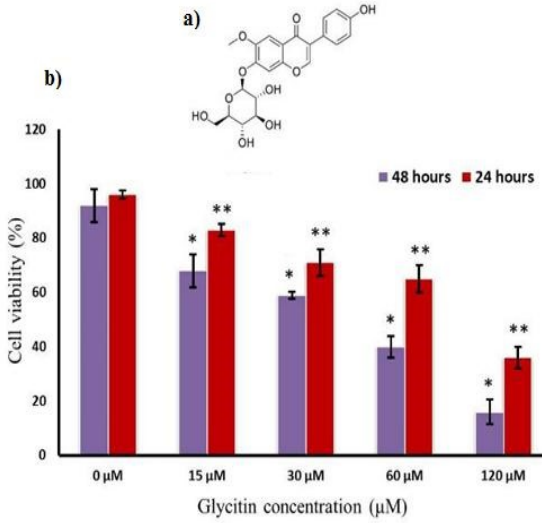

Figure 1: (a) Chemical structure of glycerin molecule. (**b)** Results of MTT assay showing significant inhibition of proliferation of A549 cells by glycitin. A concentration and time-dependent pattern was observed in the proliferation inhibition of A549 cells. *$P < 0.05$; **$p < 0.01$

Pro-apoptotic effects of glycitin molecule

Glycitin showed a high potential of antiproliferative effects by stimulating apoptosis in A549 cells, as indicated by fluorescence investigations of DAPI stained A549 cells. The results showed that glycitin damaged the normal morphology of A549 cells by inducing membrane damage and nuclear fragmentation (Figure 3 a). The degree of proapoptotic effects of glycitin against A549 cells was evaluated using Annexin V/PI staining. The results confirmed that glycitin significantly ($p < 0.05$) increased the population of A549 cells. It was observed that A549 cell population increased from 7 % to almost 65 % when glycitin concentration was increased from 0 in control to 120 µM (Figure 3 b).

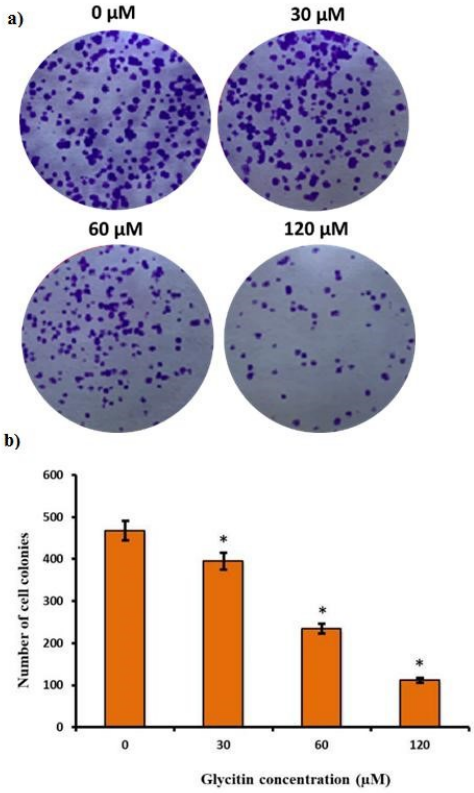

Figure 2: (a) Effect of glycitin on cell colony formation in A549 cells, as was determined using clonogenic assay. (**b)** Effect of glycitin on number of A549 colonies. The results showed a concentration-dependent pattern of colony inhibition of A549 cells by glycitin. *$P < 0.05$

Glycitin induced G0/G1 cell cycle arrest

The effect of glycitin on cell cycle in A549 cells was determined via flow cytometric analysis. After 48 h of glycitin treatment, the percentage of G0/G1phase cells in glycitin-treated A549 cells was markedly increased, when compared to control cells. The effect of glycitin on G0/G1-phase cells followed a concentration-dependent pattern (Figure 4). Nonetheless, the populations of S- and G2/M-phase cells were decreased, when compared to G0/G1-phase cells. Thus, the antiproliferative effects of glycitin in A549 cells was due, not only to apoptosis, but also to cell cycle arrest.

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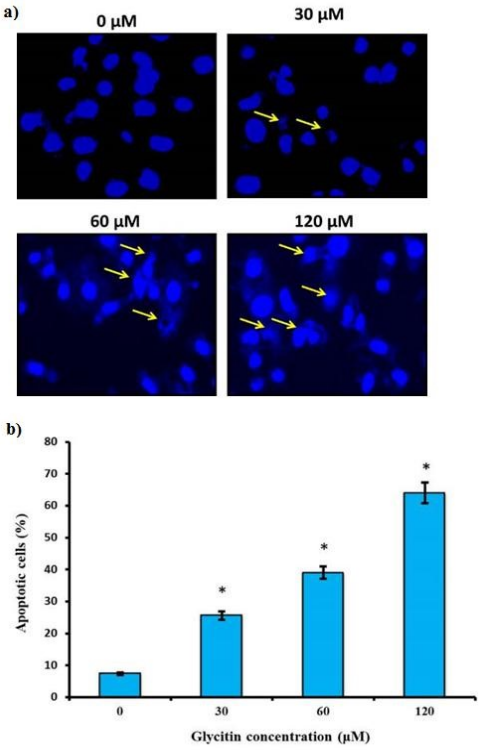

Figure 3: (a) Effect of glycitin on the morphology of A549 cells, as was determined using DAPI staining. The arrows point at changes in the morphology of A549 cells: membrane and nuclear fragmentation, indicating apoptosis, when compared to untreated control group. (**b)** Annexin V/PI-FITC assay indicating the proapoptotic effects of glycitin on A549 cells. *P < 0.05

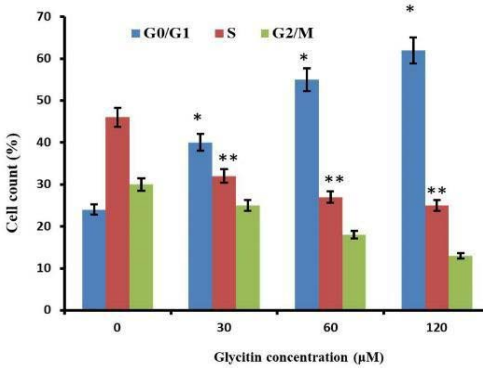

Figure 4: Effect of glycitin on the cell cycle progression of A549 cells. Glycitin induced cell cycle arrest at G0/G1 phase, as shown in increase in cell population at this phase. *P < 0.05; **p < 0.01

Glycitin inhibited migration and invasion of A549 cells

Glycitin exhibited significant suppressive effects on the migration of A549 cells. The anti-migratory effects of glycitin on A549 cells followed a concentration-dependent pattern (Figure 5). The impact of glycitin on invasion of A549 cells showed a pattern similar to that of migration (Figure 6). Thus, glycitin suppressed the migration and invasion of A549 cells, making it an agent with potential for inhibition of invasive lung cancer.

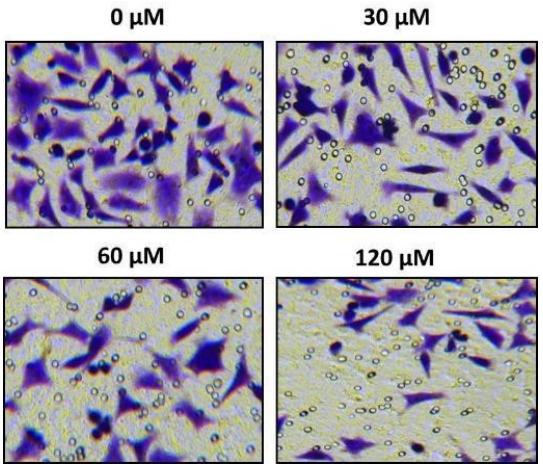

Figure 5: Glycitin significantly inhibited the migration of A549 cells

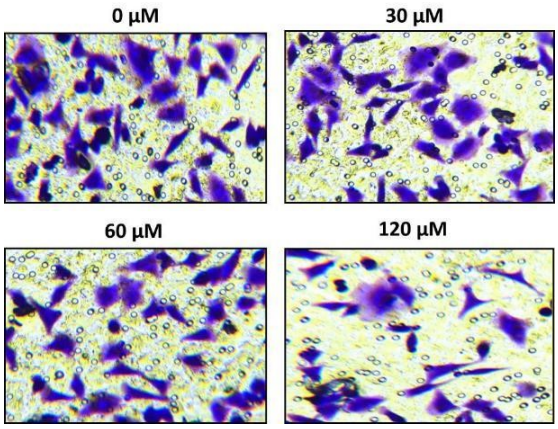

Figure 6: Glycitin significantly inhibited the invasion of A549 cells

Glycitin inhibited PI3K/AKT signaling

The effect of glycitin on PI3K/AKT signaling pathway in A549 cells was determined with western blotting assay. Glycitin treatment produced significant inhibition (p < 0.05) of the expressions of p-PI3K, PI3K, p-AKT in A549 cells, relative to the corresponding expressions in

control cells (Figure 7). The expressions of AKT remained almost unchanged at all glycitin concentrations. Therefore, compared to control groups, glycitin produced marked inhibition of PI3K/AKT signaling in A549 cells.

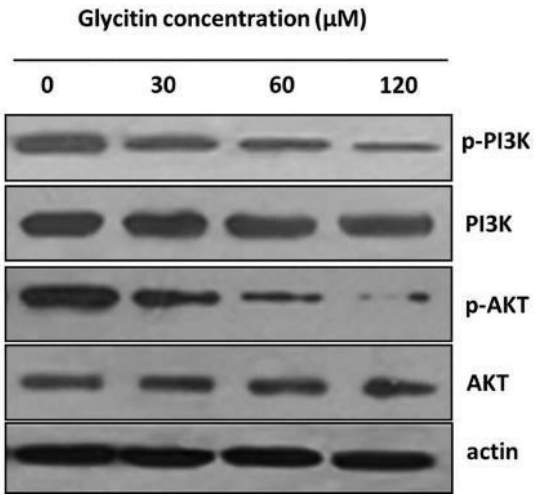

Figure 7: Effect of glycitin on expression levels of PI3K/AKT signaling-allied proteins. Experiments were performed in three independent replicates

DISCUSSION

Lung cancer is one of the frequently diagnosed cancers with high degree of metastasis and high mortality. The overall five-year survival of lung cancer highly depends on histological tumor type and stage, and it varies from 4 to 17 % [12]. Unfortunately, advances in therapeutic and diagnostic strategies such as use of tumor markers, lung biopsy, radiological intervention, chemotherapy and surgery, do not result in desirable clinical outcomes and long-time survival. Therefore, there is need for identification of novel and effective drug candidates for lung cancer treatment which will result in more acceptable clinical outcomes. In this study, for the first time, the anti-lung cancer potential of the natural compound glycitin was investigated, as well as its underlying mechanism. It was found that glycitin significantly targeted proliferation of A549 cells via promotion of apoptosis, inhibition of cell cycle, and suppression of migration, invasion, and PI3K/AKT signaling pathway.

Isoflavones have been reported to exhibit several medicinal and pharmacological effects. They have been shown to exhibit anticancer effects against various rodent and human cancers, with very low IC_{50} values. It has been reported that the cancer growth inhibition by isoflavones proceeded via suppression of activities of ribosomal S6 kinase, tyrosine kinases and DNA topoisomerases, as well as inhibition of phosphatidylinositol breakdown [13]. Some studies have reported that genistein, an isoflavone, showed promising cancer growth-inhibitory effects and stimulated cell differentiation [14]. Previous studies have shown that glycitin was less cytotoxic and proliferation-stimulating against different human cell lines, including fibroblasts and keratinocytes [15]. The inhibition of proliferation by a chemotherapeutic drug is often considered as a first line target in cancer treatment.

Targeting proliferation of cancer cells blocks fast cancer spread and tumor mass formation. In this study, it was found that glycitin significantly inhibited the proliferation of A549 cells.

The survival and death of cancer cells are regulated by several factors such as stress, damage, cell signaling, promotion of apoptosis, suppression of apoptosis, autophagy, and cell cycle progression. [16]. Suppression of apoptosis in a cell often leads to carcinogenesis. Apoptosis is a natural and highly regulated process of cell extermination which is activated by internal or external signals operating during oxidative stress, cell damage, macromolecular aggregation and ageing. Isoflavones have been reported to be highly effective in the activation of apoptosis in cancer cells. A study has reported that glycitin, a key constituent of ethanolic extract of *Solanum nigrum* L. exerted antiproliferative, pro-apoptotic and anti-cell cycle effects [10]. In this study, it was also found that glycitin produced marked induction of apoptosis in A549 cells. Moreover, it inhibited the cell cycle at G0/G1-phase. Therefore, these results are consistent with already reported work in which it was shown that glycitin induced apoptosis and inhibited cell cycle in human breast cancer cells [10].

Metastasis is one of the lethal features of lung cancer. It leads to fast growth and cancer cell proliferation to neighbouring tissues and organs. Isoflavones have shown promising results against metastatic malignancies [17]. In this study, it was found that glycitin targeted and significantly suppressed the invasiveness and migratory potential of A549 cells. Therefore, glycitin may be a leading drug candidate for metastatic lung carcinoma.

The PI3K/AKT signaling pathway is a regulatory route involved in modulating several physiological processes in cells by stimulating downstream effectors. These effectors control key mechanisms involved in cancer cell survival, including growth, cell cycle, and proliferation [18].

The P13K/AKT pathway is overexpressed in cancer cells and other malignancies, making it a leading target for targeted therapy. The pathway plays a key role in lung cancer growth, chemoresistance, and migration [19]. Furthermore, it has been reported that the PI3K/AKT axis enhanced symmetric cell division in lung cancer stem cells, thereby maintaining a pool of stem cells for carcinogenesis [20]. Therefore, targeting PI3K/AKT axis in A549 cells could prove beneficial in overcoming this lethal malignancy. This is the first study on the effect of glycitin on PI3K/AKT axis. The findings showed that glycitin significantly blocked the expressions of PI3K and AKT genes in A549 cells.

CONCLUSION

The results of this study demonstrate the anti-lung cancer effect of the isoflavone molecule, glycitin. Glycitin downregulates cell proliferation and induces apoptosis and cell cycle arrest in A549 cells. Moreover, the antiproliferative, pro-apoptotic, and cell cycle inhibitory effects of glycitin are mediated via blockage of PI3K/AKT axis in A549 cells. Therefore, glycitin is a potential therapeutic agent for the management of lung cancer. However, *in vivo* and clinical studies are required to validate this assertion.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

Yajuan Zhang and Yan Wang have contributed to this work equally. Yajuan Zhang drafted this manuscript. The whole study was designed by Rui Guo. Yan Wang offered assistance during manuscript revision and experiment performance.

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