

Pueraria lobata Leaf Extract Inhibits the P13K/AKT and MAPK/STAT3/NF- κ B Signaling Pathways on Lung Carcinoma Cells

Jingjian Zhang¹, Ying Cui², Ling Yang^{2,*}, Yan Lv^{2,*}

¹Department of Respiratory Medicine, Shanghai Municipal Hospital, Shanghai, CHINA.

²Department of Oncology, Qingdao Municipal Hospital, Qingdao, CHINA.

ABSTRACT

Background: Lung Cancer (LC) is the foremost cause of worldwide cancer-related mortalities, principally, Non-Small Cell Lung Cancer (NSCLC). *Pueraria lobata* (PL) is a prominent traditional Chinese edible remedial plant that exerts anti-tumor and anti-inflammatory activity in many cancer cells. However, its anticancer mechanisms against NSCLC cells remain uncertain. **Objectives:** Hence, this anticancer study targeted the apoptotic activity and its cell signalling regulation of PL leaf extract (PL) on A549 cell proliferation. **Materials and Methods:** The anti-cancer effect of PL leaf extract (100 and 150 μ g/mL) against A549 cells was assessed by the cell proliferation, apoptosis, anti-apoptotic inflammatory mediators, and cell signalling regulatory pathways through MTT, AO/EB, DAPI/Rh-123, RT-PCR, and western blot study. **Results:** Our study exposed that PL leaf extract (100 and 150 μ g/mL) could reduce A549 cell viability and inflammation by the activation of mitochondrial-mediated cell apoptosis in a concentration-related manner. Likewise, PL leaf extract could diminish anti-apoptotic elements and inflammatory cytokines, as well as suppress P13K/AKT and MAPK/STAT3/NF- κ B pathways. PL leaf extract inhibits the p38 and JNK pathways, which further suppresses STAT3 and NF- κ B action, triggering cell apoptosis. Furthermore, PL leaf extract could suppress the P13K/AKT pathway. **Conclusion:** The above findings propose that PL leaf extract may have a potential for NSCLC therapy.

Keywords: Apoptosis, Lung cancer, P13K/AKT signaling, *Pueraria lobata*, ROS.

Correspondence:

Prof. Ling Yang

Department of Oncology, Qingdao Municipal Hospital, Qingdao-266071, CHINA.

Email: qianhe1986@sina.com

Prof. Yan Lv

Department of Oncology, Qingdao Municipal Hospital, Qingdao-266071, CHINA.

Email: lcx20170620@sina.com

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INTRODUCTION

Lung Cancer (LC) remains a worldwide health concern, with a high incidence of malignant tumors.^{1,2} LC cells are described by increased spread and are susceptible to metastasis.^{3,4} Among the various kinds of this disorder, Non-Small-Cell Lung Cancer (NSCLC) stands out as the most predominant and poor prognosis for patients.¹ The existing treatments for this disease are chemotherapy, surgery, and radiotherapy. However, the current treatment is not completely effective and has adverse effects. One risky element contributing to this unfavorable attitude is the insensitivity of NSCLC patients to conventional chemotherapeutic agents.⁵ Natural products unveil numerous favorable actions on human health with fewer toxic properties in cancer treatment,⁶ thereby raising concern about plant derivatives as a substitute for tumor remedies. Finding innovative compounds from natural

resources with chemotherapeutic properties is crucial to prevent and treat NSCLC.

A well-known traditional Chinese medicinal plant, namely *Pueraria lobata* (PL), is a climbing, creeping, and trailing perennial vine.⁷ PL leaf extract was described to reduce obesity and ameliorate glucose metabolism.⁸ Previously, it has been documented that PLR extract has anti-dipsotropic properties.⁹ Besides, daidzein, a major isoflavonoid isolated from PLR, exhibited antioxidative and anti-inflammatory activities, and another compound, lupenone demonstrated cytotoxicity actions.¹⁰ Recently, PLR has been involved in commercially obtainable western dietary supplements for menopausal symptoms. Puerarin is an isoflavone in PLR and has been established to have antitumor and estrogenic effects.^{11,12} Furthermore, numerous reports have exposed that PL has strong anticancer properties. In an earlier study, MTT was used to fix the lethal properties of PL aqueous extracts combined with silver nanoparticles on ovarian carcinoma (SKOV-3) and various breast cancer cells such as MCF-7 and MDA-MB-231; the data revealed that PL had significantly suppressed both cancer cells.¹³ Another investigation established that puerarin-600-O-xyloside had considerable anticancer action against colorectal cancer cells through apoptosis induction.¹⁴ Although PLR and flower extracts



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were presented to have ideal anticancer and pharmacological properties in former studies, the underlying anticancer mechanism of *Pueraria lobata* (PL) leaf extract for the treatment of NSCLC is unknown; thus, the current research intensive search on the anticancer effect of PL extract and elucidation of its precise anti-cancer signaling regulation.

A programmed cell death method of apoptosis signifies cancer treatments' chief cell death mechanism.¹⁵ Induction of malignant cell apoptosis is beneficial for tumor treatments, and several signaling networks are included in this process.¹⁶ An extracellular stimuli trigger the activation of Mitogen-Activated Protein Kinase (MAPK), which controls numerous physiological events that facilitate signal transduction from the cell membrane to the nucleus.¹⁷ MAPK signaling participates in an assortment of the body's pathophysiological processes, and the MAPK followers mainly comprise ERK, JNK, and p38.^{18,19} The MAPK signaling activation can stimulate the release of several types of inflammatory mediators from the body and exacerbate the patient's condition.²⁰ STAT3 signaling plays a crucial part in the control of apoptosis and pathogen resistance.²¹ Many of the phosphorylation sites in STAT3 contain form proteins that connect it to AKT, NF- κ B, and MAPK signaling pathways. The STAT3 and NF- κ B signaling is downstream of the MAPK pathway and together stimulate apoptotic cell death.^{22,23} It is evidenced that the PI3K/Akt pathway plays a key part in the development and existence of tumor cells.^{24,25} Remarkably, a typical Akt initiation is a deprived analytical feature for all phases of NSCLC.²⁶ Furthermore, stimulated AKT guards NSCLC cells from radiation and chemotherapy-prompted cell death.^{27,28} Hence, suppression of the PI3K/Akt pathway is a striking healing approach in NSCLC.

In this study, the anti-proliferative properties of PL leaf extract were examined in NSCLC cells A549. In particular, the underlying anticancer mechanisms with a focus on P13/AKT and MAPK/STAT3/NF- κ B signaling pathways as a potential target that induces apoptosis were determined in NSCLC cells.

MATERIALS AND METHODS

Chemicals and reagents

DMEM, FBS, antibiotics, PBS, MTT, AO/EB, Rh-123, DAPI, DMSO, and SDS were procured from Solarbio (Beijing, China). Antibodies for western blot analysis were obtained from ZSGB-bio (Beijing, China). *P. lobata* leaves were purchased from China.

Preparation of PL leaf extract

PL leaf extract preparation was followed according to the earlier method.²⁹ The PL leaves (100 g) were dried, and aqueous extraction was performed using distilled water. Subsequently, the sample was refluxed at room temperature for 90 min to get the

water extract. Then, suction was filtered over the Whatman No. 41 filter paper, followed by evaporation of the filtered extracts using a rotary vacuum evaporator (Labomiz Scientific, USA) under reduced pressure at 50°C.

Cell culture

Human adenocarcinoma NSCLC cells A549 were procured from GenScript (USA) and were grown in the media, namely DMEM. The culture media was added with FBS (10%), and penicillin/streptomycin (1%). Cells were cultivated in an incubator with 5% CO₂ and humidified air below 95% at 37°C.

Cell proliferation assayed by MTT

The anti-proliferative activity of PL extract against human NSCLC cells A549 was performed according to the MTT experiment.³⁰ A549 cells were cultured into a 96-well dish with a density of 1×10^5 cells/well and maintained at 37°C for 48 hr in a 5% CO₂ incubator. After overnight incubation, these cells were administered with numerous quantities of PL extract (5, 25, 50, 100, 150, 200, and 250 μ g/mL). Later, MTT solution (10 μ L) was supplemented to the preserved cells and permitted for an extra 4h to let the formation of crystals insoluble formazan, it was dissolved with DMSO (150 μ L) addition. The Optical Density (OD) was estimated at 490 nm by a Spectrophotometer. The IC₅₀ was determined as follows:

$$\text{Inhibition of cell viability (\%)} = (\text{Control OD} - \text{Test OD}) \times 100$$

Evaluation of apoptosis by dual staining

The apoptotic effect of PL extract (100 and 150 μ g/mL) against NSCLC cells A549 was examined by AO/EB dual staining.³⁰ A549 cells were added with PL extract (100 and 150 μ g/mL) and incubated for 24 hr. Control and treated groups were administered with a mixture of dyes containing AO/EB (100 μ g/mL per dye). They were kept under the dark; ensuring unbinding dye was eliminated by PBS and observed by a fluorescence microscope (Olympus, Tokyo, Japan).

Assessment of apoptosis in A549 cells

To evaluate the apoptotic effect of PL extract (100 and 150 μ g/mL) on A549, DAPI/Rh-123 staining was conducted. Human NSCLC cells A549 were dispersed in well plates and sustained at 37°C with CO₂ (5%) for 24 hr. Different concentrations of PL extract (100 and 150 μ g/mL) were administered to A549 cells and incubated, the resultant twofold washed with PBS, fixed by paraformaldehyde (4%), rinsed, and DAPI staining, then kept for 20 min in the incubator. Subsequently, DAPI-administered A549 cells exposed to Rh-123 stains were kept at 37°C for 30 min. The stained cells were twofold washed with methanol to eliminate extra dyes and immersed with PBS, and the MMP variant was viewed by a fluorescent microscope (Olympus, Tokyo, Japan).

Western blot study

The A549 (7×10^4 cells/well) cells were in 6-well plates and added with different amounts of PL leaf extract (100 and 150 $\mu\text{g/mL}$) and incubated the whole day in the medium. Cell lysates were prepared by adding lysis buffer in ice-cold, ensuring protease inhibitors and conducting western blot examination. The entire protein was extracted according to the protocol of the BCA Protein Assay Kit (Pierce Chemical Co, USA). Shortly, the extracted proteins were run electrophoretically and transferred to a PVDF film. Successively, the probe was administered to block the film for 60 min, and added primary antibodies were in 1:1,000 dilutions and set apart overnight at 4°C . After that, the secondary antibodies (1:5,000) were added. β -actin was used as an internal control. Then, the protein bands were imagined for protein recognition, and the protein band quantification was assessed by densitometry with Image J software.

Assessment of mRNA expression levels

RNA was isolated from A549 cells as per the kit's procedures by using TRIzol[®] reagent. Isolated RNA was converted into cDNA by employing a High-capacity cDNA Reverse Transcription kit (Abcam, USA) by following the kit's protocols. Later, the Fast Start SYBR Green master mix (Abcam, USA) was used. The concentration of the band was evaluated by using 1.5% agarose gels for electrophoresis and measured with the software Image J 1.48.

Statistical analysis

The analysis of statistics was conducted by using GraphPad Prism software version 8.0.1 and was performed by an Analysis of Variance (ANOVA) followed by Duncan's test. Data were shown as mean $\pm$ SD and considered the statistical significance as $p < 0.05$.

RESULTS

Anti-proliferative effects of PL extract against A549 cells

PL extract (5, 25, 50, 100, 150, 200, 250 $\mu\text{g/mL}$) is assessed for the proliferation of NSCLC cells A549 by the MTT experiment (Figure 1). Data presented that PL extract had decreased ($p < 0.05$) proliferation and cytotoxic activities on A549 cells in a concentration-related way against control cells. On the other hand, high concentrations such as 200 and 250 $\mu\text{g/mL}$ of PL extract, revealed high levels of A549 cell inhibition ($p < 0.05$) and cell destruction. MTT test revealed that the value of IC_{50} in PL extract was 100 $\mu\text{g/mL}$ for A549 cells. The concentration of 100 and 150 $\mu\text{g/mL}$ of PL extract was selected for further studies based on the facts of the inhibitory concentration value.

PL extract-induced A459 cell apoptosis revealed by the staining of AO/EB

The apoptotic activity of PL leaf extracts was studied against A549 cells performed by the two-fold staining of AO/EB (Figure 2). A549 cells displayed homogeneous green live cells. PL extract (100 and 150 $\mu\text{g/mL}$) treated cells developed cell apoptosis in an amount-related mode. PL leaf extracts at a dosage of 100 $\mu\text{g/mL}$ exhibited early apoptosis features such as light greenish with yellow dots, membrane blebbing, and chromatin condensation. A high concentration (150 $\mu\text{g/mL}$) presented late apoptosis with an orange-red color, showing fragmented nuclei, membrane blebbing, and chromatin condensation.

Effect of PL extracts on A459 cell mitochondrial-mediated apoptosis

PL extract (100 and 150 $\mu\text{g/mL}$) evaluated the apoptotic characteristics of A549 cells by DAPI/Rh-123 staining (Figure 3). DAPI staining in the control showed adducts forming over DNA double-stranded. Administration of PL extracts (100 and 150 $\mu\text{g/mL}$) to the A549 cells exhibited higher nuclear body disintegration, the morphology of condensed nuclei, and impaired membrane integrity in a concentration-related manner. These images reveal the apoptotic effect of PL extract against A549 cells.

A mitochondrial membrane depolarization has formed by the Rh-123 staining, which was imagined in primary apoptosis. A fluorescent cationic lipophilic stain, Rh-123 is included functional proximate mitochondria with an intensified MMP and validations mitochondrial regulations in the apoptosis. The higher fluorescence intensity (488/525 nm) strength was detected in the control which has amplified MMP. Administration of PL extract (100 and 150 $\mu\text{g/mL}$) to the A549 cells reduced the MMP in a concentration-related mode, thus stimulating mitochondrial-facilitated apoptosis (Figure 3).

PL extract inhibits anti-apoptotic inflammatory cytokines

To clarify the molecular actions involving the apoptotic activity of PL extract, the anti-apoptotic pro-inflammatory marker protein expression on A549 cells was assessed (Figure 4). The protein expression of COX-2, TNF- α , iNOS, NF- κB , and IL-6 were increased in A549 control cells, whereas PL extract (100 and 150 $\mu\text{g/mL}$) could reduce these protein expressions in a quantity-related way. The above-mentioned inhibitory effects of cytokines designated that suppression of NF- κB is included in the PL extract-generated A549 cell growth inhibition.

Effect of PL extract on the mRNA level of A549 cells

The mRNA intensities of p38, pin-1, STAT-3, JNK, and p65 mRNA levels were decreased on PL leaf extracts-administered A549 cells against control (Figure 5). Supplementation of PL extracts (100

and 150 µg/mL) on A549 cells considerably decreased ($p < 0.05$) the mRNA-associated apoptosis in a dosage-related manner.

PL extract alleviates the P13K/Akt signaling pathway in A549 cells

P13K/Akt signaling is involved in apoptosis and protein level has a very prominent action in the progression of NSCLC (Figure 6). Control cells displayed higher protein expression of P13K, Akt, and its phosphorylated form, while PL extract administration (100 and 150 µg/mL) in the A549 cells decreased the phosphorylated P13K/Akt expression in an amount-related way.

DISCUSSION

Natural products have gained a significant role in the upgrading of anticancer agents. Plant-derived compounds have been explored as lead antitumor candidates. Recently, Chinese herbal medicines and their active ingredients have gained much attention for their anti-cancer and chemo-sensitive improving effects. PL is among the vital medicinal creeper plants innate to South America, Europe, and East Asia.⁷ PL extracts have been known as traditional Chinese medicine for a long time and are even used as food.^{31,32} PL has excellent pharmacological properties in treating cardiovascular diseases, hepatoprotection, and various chronic disorders.³³ Puerarin is an isoflavone isolated from PLR that may also excellently reverse chemically induced liver fibrosis in experimental rats by reinstating hepatic damage and activating apoptosis of activated Hepatic Stellate Cells (HSC). PL has been reported to have antitumor and estrogenic effects.^{11,12} Additionally, isoflavones such as daidzein and genistein isolated from PL have exposed remarkable hepatoprotective effects in mice.³⁴ Furthermore, daidzein, a major isoflavonoid isolated from PLR, unveiled antioxidative and anti-inflammatory activities, and another compound, lupenone, established cytotoxicity actions.¹⁰ Previously, researchers proved that PL exerts anti-tumor action against ovarian carcinoma, breast cancer, and colon cancer.^{13,14} In this report, the antiproliferative and apoptotic effects of PL leaf extracts (100 and 150 µg/mL) against NSCLC cells A549

have been investigated and elucidated the anticancer signaling pathways.

Herein, we explored the efficacy of PL leaf extracts in averting cell viability, induction of apoptosis, and cell signaling pathway regulation in human NSCLC cells A549. MTT assay revealed that PL extracts (100 and 150 µg/mL) could reduce A549 cell proliferation and cytotoxicity in a concentration-related manner. The apoptosis stimulation is a general mechanism projected for the cytotoxic action of antitumor drugs isolated from herbal medicine.^{15,16} Numerous documents have exposed that PL has powerful anticancer and anti-proliferative properties.¹⁰ In a former study, MTT was used to regulate the toxic action of PL aqueous extracts blended with Silver Nanoparticles (AgNP3) on ovarian and breast cancer cells; the data exposed that AgNP3 had expressively suppressed both cancer cells.¹³ Another investigation established that puerarin-600-O-xyloside isolated from PL had considerable anticancer action against Colorectal Cancer Cells

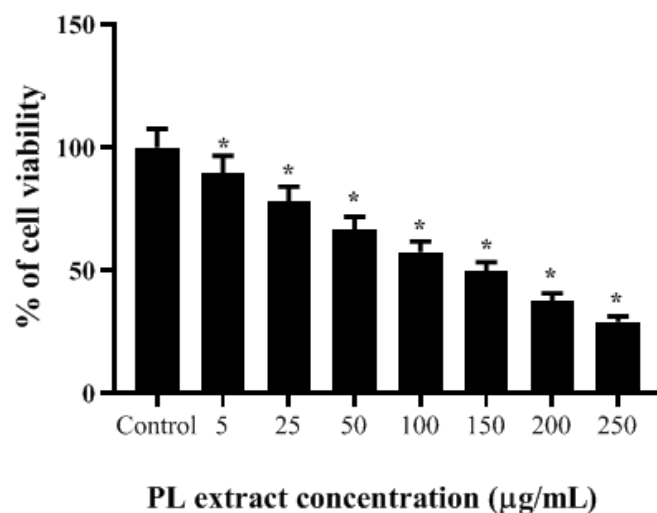


Figure 1: Effect of PL leaf extracts on A549 cell proliferation. NSCLC human cells A549 were incubated for 24 hr with various quantities (5, 25, 50, 100, 150, 200, and 250 µg/mL) of PL leaf extracts. Cytotoxicity was estimated by the MTT analysis. Data were exhibited as mean±SD. The statistical significance is considered as * $p < 0.05$ against the control.

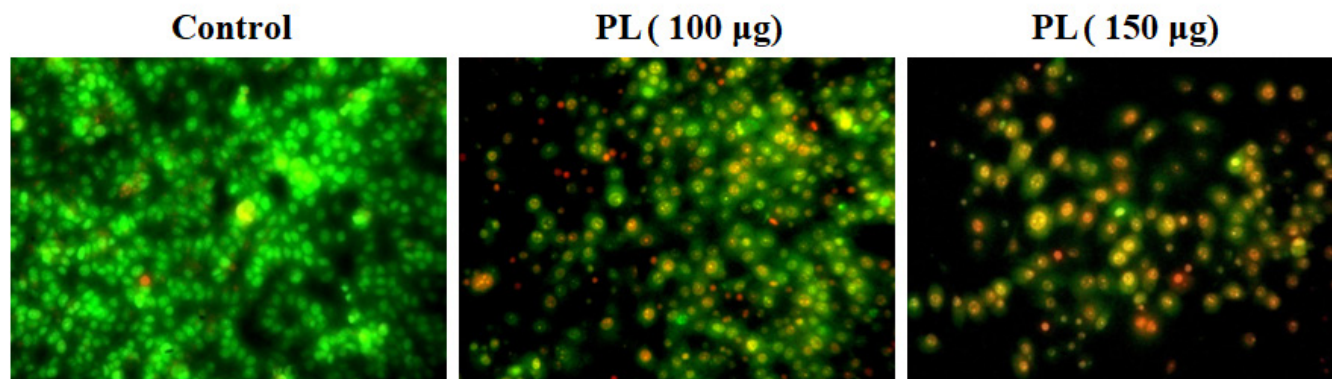


Figure 2: PL leaf extracts ameliorated apoptosis on A549 cells showed by AO/EB dual staining. Administration of PL leaf extracts (100 and 150 µg/mL) to the A549 cells and incubated for 24 hr. The apoptosis was estimated by the dual AO/EB staining.

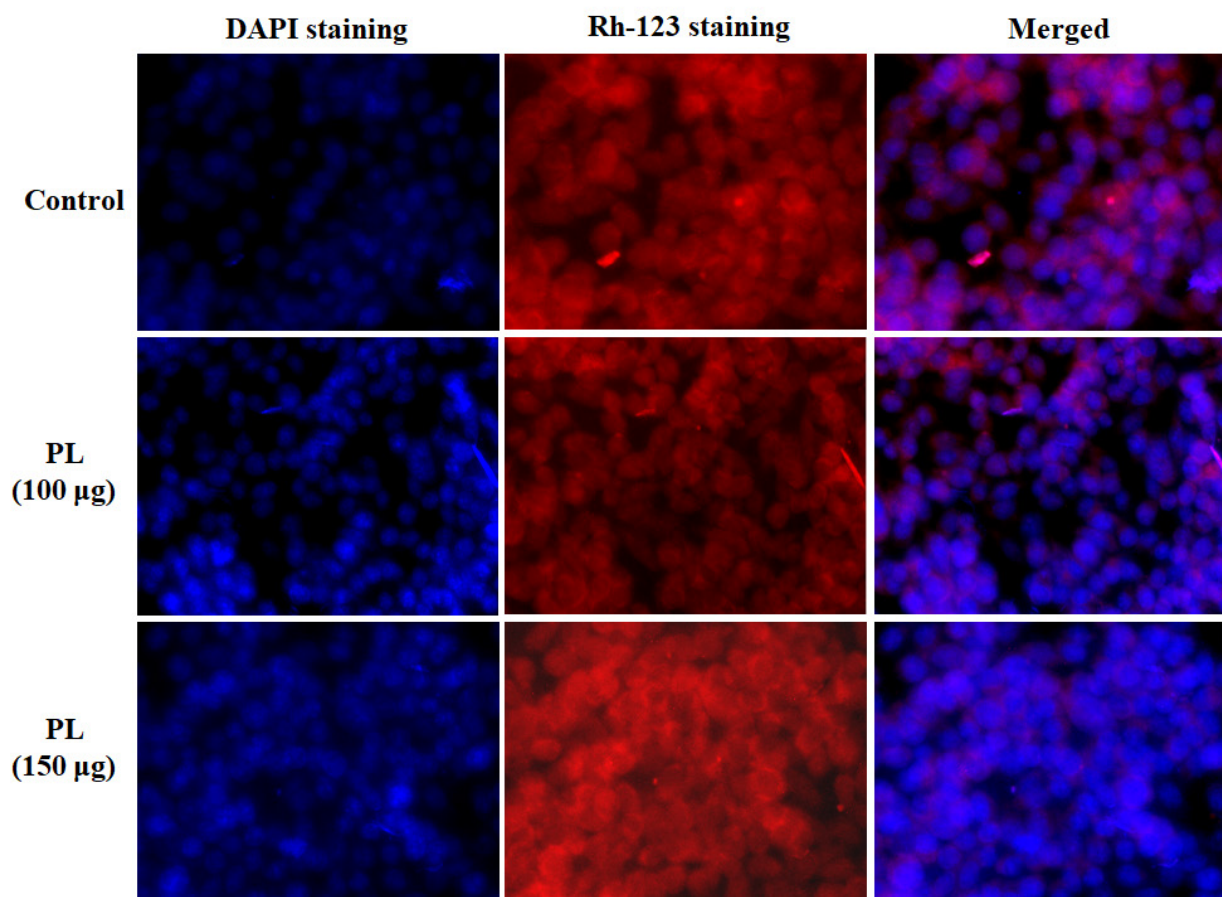


Figure 3: PL leaf extracts promote mitochondrial-mediated apoptosis in A549 cells. Administration of PL leaf extracts (100 and 150 µg/mL) to the A549 cells and incubated for 24 hr. Cell apoptosis was explored by the staining uptake of DAPI and Rh123. Merged DAPI and Rh-123 on the A549 cells kept for 24 hr and imaged by a fluorescence microscope.

(CRC) through apoptosis induction.¹⁴ These data established that PL extract exerts anti-proliferative activity by inducing apoptosis in NSCLC cells A549.

Apoptosis disturbs physiological processes, including progression, disparity, and immune regulation, which is diligently connected to malignant pathological processes.^{15,16} STAT3, MAPK, NF-κB, and various signaling networks can trigger the apoptotic core enzyme, thus stimulating the cleavage of correlated proteins and ultimately completing the apoptotic process.^{22,23} To further explore whether PL leaf extracts promotes A549 cell apoptosis by controlling the STAT3, MAPK, and NF-κB pathways, we evaluated the mRNA expression intensities by RT-PCR and associated protein expression by Western blot. Data presented that PL leaf extracts reduced the mRNA expression of STAT-3, p38, JNK, pin-1, and p65 through the apoptotic induction of A549 cells. Moreover, comparable to the preceding results, the STAT3 pathway is controlled by the MAPK pathway.^{22,23} Several reports demonstrated that there is a comprehensive array of communication between the STAT3, MAPK, and NF-κB signaling pathways.¹⁸⁻²⁰ Here, we exhibited that PL leaf extracts diminished the mRNA expression of p38, JNK, and STAT3 in A549 cells in a quantity-related way. These findings prove that PL leaf extracts inhibit the JNK and p38 pathways and further

suppress NF-κB and STAT3 activity that leads to cell apoptosis. An imperative transcription factor NF-κB, is included in the control of gene expression and apoptosis.^{22,23} NF-κB triggers downstream pathways relating to Bcl-2, TNF-α, and STAT-3 and thus prevents apoptosis.^{35,36} NF-κB expression is upregulated in NSCLC cells and is associated with destructive tumor cell metastasis among NSCLC patients.³⁷ Hence, down-regulation of the NF-κB expression could be an effective remedial method for NSCLC patients.^{38,39} In our work, we have confirmed the dosage-reliant reduction in the protein expression of anti-apoptotic inflammatory cytokines, including NF-κB, TNF-α, COX-2, iNOS, and IL-6 in the A459 cells by PL leaf extracts. Earlier research has documented that PLE has an inhibitory effect against nephritis, and its anti-inflammatory action may be linked to the down-regulation of the NF-κB signaling, which is utilized as a therapeutic agent by suppressing inflammatory cytokines of L-6, TNF-α, and iNOS expression.⁴⁰ This report demonstrates that the anti-cancer mechanism of PL leaf extracts against A549 cells is mitochondrial-mediated apoptosis through down-regulating MAPK, STAT3, and NF-κB signaling pathways.

The PI3K/Akt is the most significant pathway in the regulation of cell growth, proliferation, and apoptosis, and Akt is a main downstream target of PI3K.^{24,25} The PI3K/Akt pathway

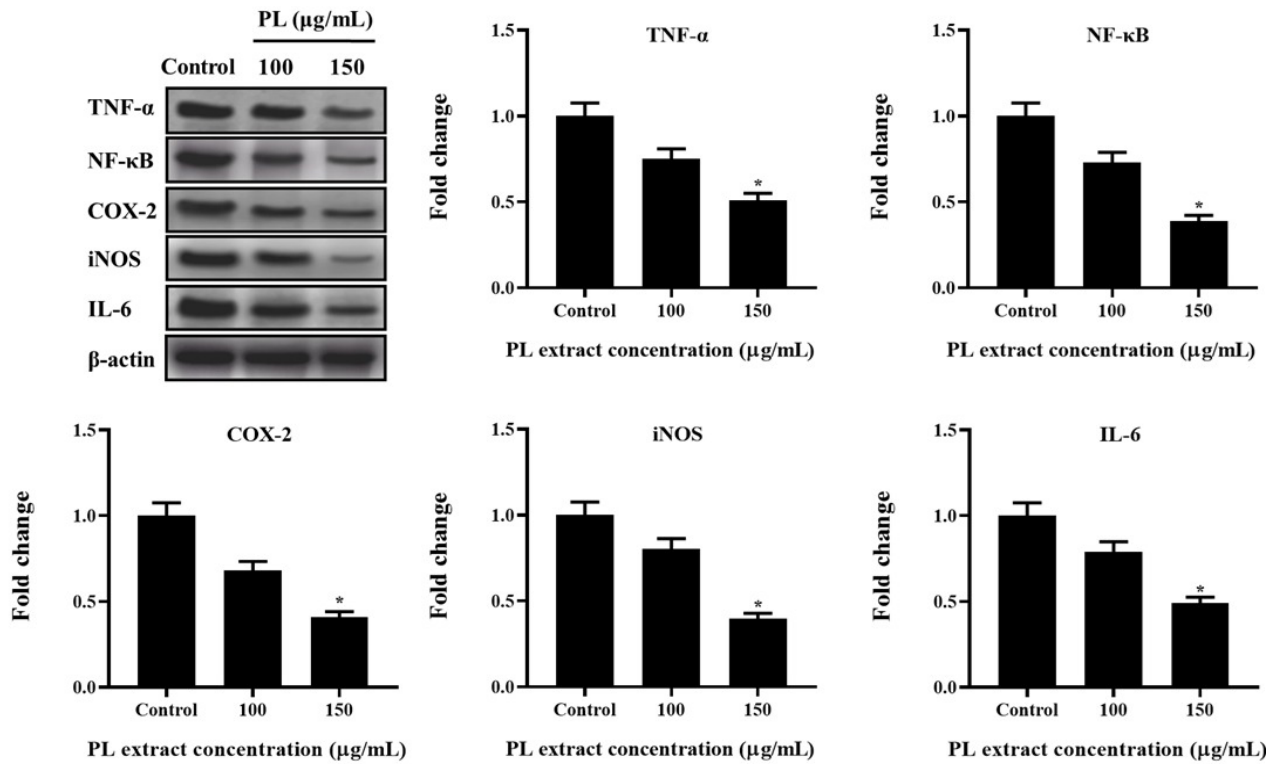


Figure 4: PL leaves extracts inhibited anti-apoptotic cytokines levels. Administration of PL leaf extracts (100 and 150 μ g/mL) to the A549 cells and incubated for 24 hr. iNOS, TNF- α , COX-2, NF- κ B, and IL-6 protein expression in A549 cells were analyzed by Western blot. The statistical significance is considered as $*p < 0.05$ against the control.

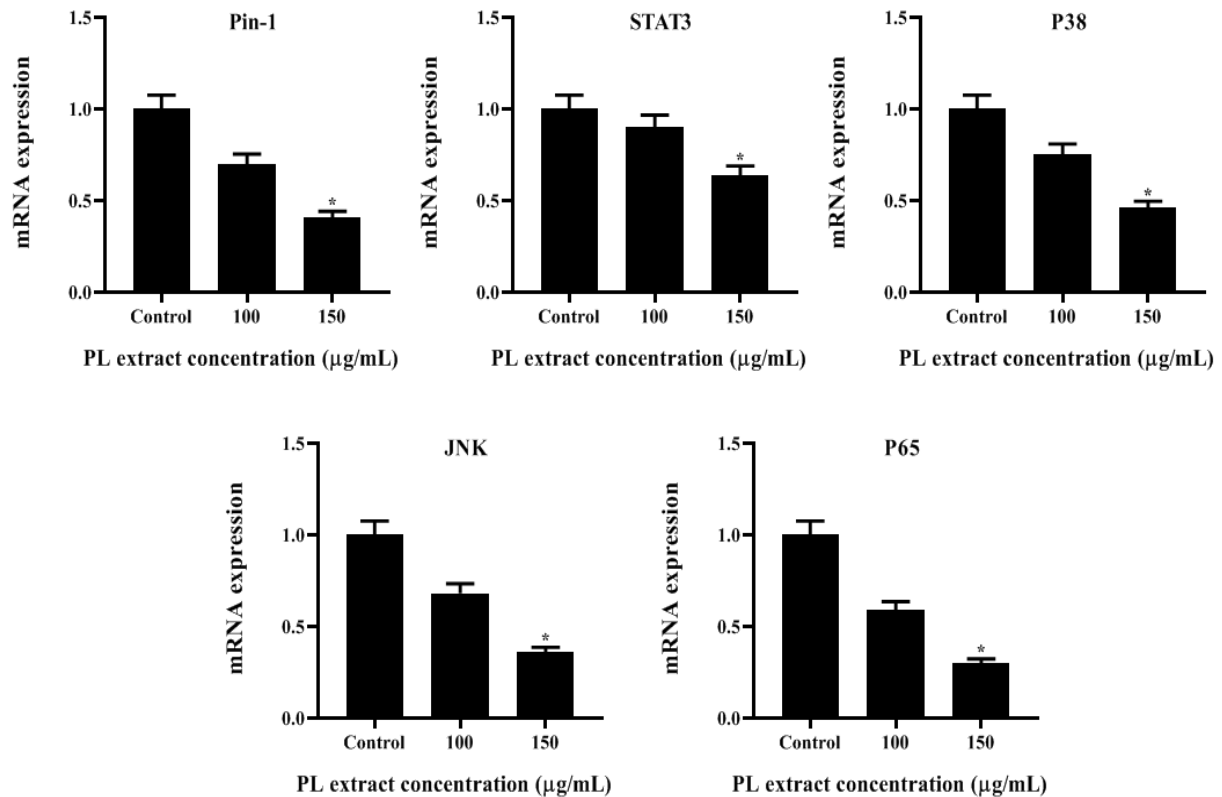


Figure 5: PL leaf extracts suppressed mRNA expression in A549 cells. Administration of PL leaf extracts (100 and 150 μ g/mL) to the A549 cells and incubated for 24 hr. p38, Pin-1, JNK, STAT-3, and p65 mRNA expression were examined by RT-PCR. The statistical significance is considered as $*p < 0.05$ against the control.

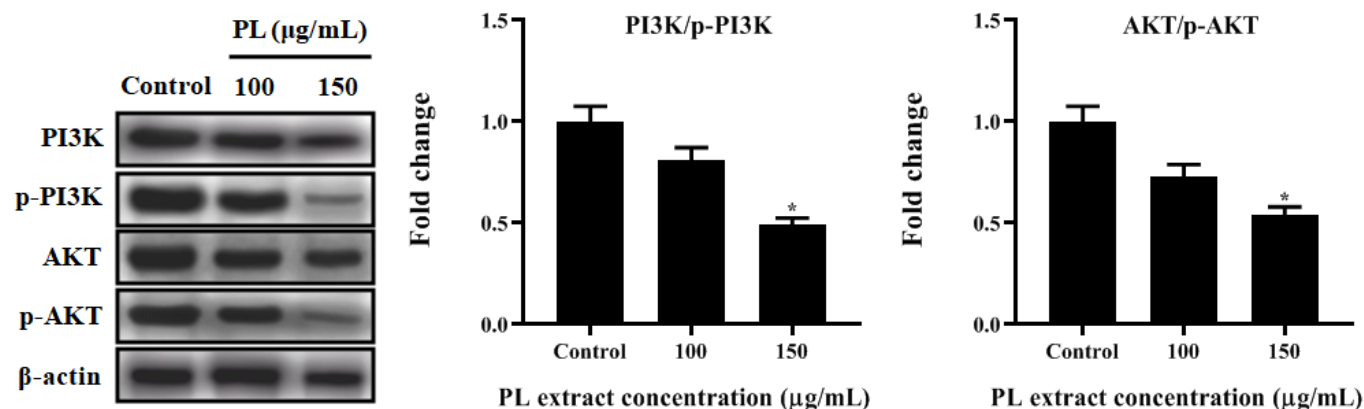


Figure 6: PL leaf extracts suppress the PI3K/AKT signaling pathway. Administration of PL leaf extracts (100 and 150 $\mu\text{g/mL}$) to the A549 cells and incubated for 24 hr. PI3K, p-PI3K, AKT, p-AKT, and β -actin protein expressions were examined by western blot. The data were displayed as mean $\pm$ SD. * $p < 0.05$ against untreated control is deliberated as significant statistically.

controls the growth and development of numerous tumors by enriching the effect of the anti-apoptotic action of Akt, and its phosphorylation is regularly employed for Akt stimulation.²⁶ In this report, we examined the effect of PL leaf extracts on the PI3K/Akt signaling by determining PI3K, p-PI3K, AKT, and p-AKT protein expression. It was established that A549 cells administered with PL leaf extracts (100 and 150 $\mu\text{g/mL}$) decreased the protein expression of PI3K, p-PI3K, AKT, and p-AKT in a concentration-related way. These findings mentioned that the PI3K/AKT signaling might be one of the regulatory mechanisms shown in the antiproliferative and apoptotic activities of PL leaf extracts against NSCLC.

CONCLUSION

In summary, in the current study, we established that PL leaf extracts induce mitochondrial-facilitated apoptosis and anti-proliferation effects in human NSCLC cells A549 by down-regulating the MAPK, STAT3, NF- κ B, and PI3K/AKT signaling pathways. The anticancer property of PL leaf extracts demonstrated *in vivo* should be assessed in future research.

ABBREVIATIONS

LC: Lung cancer; **NSCLC:** Non-small cell lung cancer; **PL:** *Pueraria lobata*; **PLLE:** PL leaf extract; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **AO/EB:** acridine orange/ethidium bromide; **DAPI:** 4',6-diamidino-2-phenylindole; **Rh-123:** Rhodamine 123; **RT-PCR:** Reverse transcription polymerase chain reaction; **PI3K:** Phosphatidylinositol-3-kinase; **AKT:** Protein kinase B; **MAPK:** Mitogen-activated protein kinase; **STAT3:** Signal transducer and activator of transcription 3; **NF- κ B:** Nuclear factor kappa B; **ROS:** Reactive oxygen species.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

SUMMARY

In summary, *Pueraria lobata* (PL) leaf extract demonstrated promising anticancer effects against A549 non-small cell lung cancer (NSCLC) cells. The extract reduced cell viability and inflammation through mitochondrial-mediated cell apoptosis. PL leaf extract suppressed anti-apoptotic elements, inflammatory cytokines, and key cell signalling pathways like PI3K/AKT, MAPK/STAT3/NF- κ B, p38, and JNK, triggering cell apoptosis. These findings highlight the potential of PL leaf extract for NSCLC therapy, emphasizing its role in apoptosis and signalling pathways.

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