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Original Article

# *Plumbago indica* L. Root Extract Induces Anti-proliferation, Anti-migration and Apoptosis on the Human Lung Cancer Cell Line

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## ABSTRACT

**Background:** *Plumbago indica* L. (PI) is a Thai traditional medicine that has been reported to improve health benefit including anticancer property. Nevertheless, the effects of PI and this mechanism are still less data in lung cancer. **Aim:** To investigate and compare the effects of PI root extract with high and low active compound, plumbagin, on the growth inhibition and apoptotic induction in A549 lung cancer cells with underlying mechanism. **Materials and Methods:** Antiproliferative effects of PI were examined by Sulforhodamine B (SRB) and colony formation method. Migratory suppression was studied by Wound healing and Matrigel migration assay. Apoptotic induction, ROS formation, and mitochondrial function were explored by flow cytometric method. Western blotting was used to measure the protein expression. **Results:** PI extract with high plumbagin level reduced cell viability by dose- and time-dependent than low plumbagin extract and the data was correlated with colony formation assay. Further, the results of migration assay indicated that 50 µg/mL of high plumbagin also caused induction of anti-migratory effects than low plumbagin. For apoptosis, both of PI extract activated the late apoptosis, especially at high dose of PI extract (50 µg/mL) along with stimulation of ROS formation and mitochondrial dysfunction. The mechanism of apoptosis was observed through the induction of expression of active-caspase 3 level correlated with reduction of pro-caspase 3 expression. **Conclusion:** Therefore, these anti-cancer activities of the PI extract with high plumbagin could be served as the potential beneficial effects on lung cancer than low plumbagin. Additionally, the study of PI effects *in vivo* study is now required to support this *in vitro* study information.

**Keywords:** *Plumbago indica* (PI), Apoptosis, Anti-proliferation, Human lung cancer cell line, Migration, Mitochondrial function.

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## INTRODUCTION

Alternative therapies for the management of cancer are spreading globally with increased use of herbal medicines including in Thailand. Several anticancer agents of Thai recipes are composed of *Plumbago indica* L. (PI) or *Plumbago rosea* which belongs to the Plumbaginaceae family. PI has several mutual names in many countries and is known as "jetamul- pleung-dang" or "pitpiudang" in Thailand. As traditional use in Thailand, polyherbal recipe named Ben-ja-kul is famous known as commercial product comprising of PI to treat cancer. Focusing on anticancer activity, the evidences indicate that plumbagin is the major component and used as an active compound in PI. From scientific evidences have been suggested that PI is one of important ingredient to treat many diseases and has pharmacological activities such as an antioxidation, anti-inflammation, anti-helminth, anti-bacteria, anti-fungus, and anti-cancer effects.[1] Furthermore, various pharmacological activities of plumbagin have been shown to assist in the treatment of cancer as well.[2] However, the data of PI mechanism on cancer treatment is still less information, therefore, the study of PI on cancer activities are urgently needed to explore.

For anti-cancer activities, ethanolic extract of PI has strong induction the genotoxic and suppression of cell cycle in human lymphocytes.[3] Moreover, the effects of PI root extract against the melanoma cells have been revealed by dose-dependent inhibition with decreasing the expression of Bcl-2 levels.[4] Base on the results obtained, PI root extract demonstrated with several pharmacological activities from high levels of plumbagin. Plumbagin is a natural quinoid constituent that has a various

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effect including anti-bacterial, anti-inflammatory, anti-fungal, and anti-cancer activity as expressed by PI extract.[5,6]

Plumbagin has been shown the growth inhibition or apoptotic induction through several important pathways such as apoptosis, autophagic pathways, cell cycle arrest, anti-angiogenic, anti-metastasis and anti-invasion pathways. Plumbagin controlled key regulatory genes including Nuclear Factor-κB (NF-κB), Signal Transducer and Activator of Transcription 3 (STAT3), and Protein Kinase B (AKT), correlated with showing the powerful stimulator of Reactive Oxygen Species (ROS), potentiator of mitochondrial dysfunction and activator of oxidative stress that lead to DNA damages and further DNA strand break.[7] Therefore, the levels of plumbagin in the PI root extract may have the most important active compound used to be the indicator of pharmacological activities including anti-cancer effects. Consequently, high plumbagin yielding root of PI was interested.[8] However, the suitable period of PI harvesting was recommended after 2 years of cultivation for better alternative source of high plumbagin yielding roots used in the Thai recipe for cancer treatment.

The purpose of this study was to clarify the potency of PI extract with high and low plumbagin responsible for its proliferative suppression on A549 human lung cancer cells and determine the underlying molecular mechanisms. First, to quantify the plumbagin levels in PI extract with High-Performance Liquid Chromatography (HPLC) method and grouping for high and low plumbagin. Second, to explore the actions of PI extract on growth, migration, and apoptosis using Sulforhodamine B (SRB), Matrigel migration, and flow cytometric method. Third, to examine the underlying mechanism using ROS formation and mitochondrial dysfunction by flow cytometry with protein expression by Western blot analysis.

## MATERIALS AND METHODS

### Plant Extractions and High-Performance Liquid Chromatography (HPLC) Method

Roots of PI was obtained from Maha Sarakham Province, Thailand, in November 2020. Proof of identity was made by the Department of Applied Thai Traditional Medicine, Faculty of Medicine, Mahasarakham University (specimen no. 19112020-1) and placed at the Faculty of Science, Mahasarakham University, Thailand. Different batches of PI cultivation within the same area were separately collected. Roots of PI was dried, 20 g of powder macerated with 200 mL methanol (100%) for 3 times, filtered and heated to dryness without methanol solvent. HPLC assay was performed to quantify the plumbagin levels of PI extracts. Briefly, PI extract (100 µg/mL) was loaded onto a Shimadzu HPLC system (Shimadzu, Tokyo, Japan). Mobile phase composed of a flow gradient composition using water (solvent-A) and acetonitrile (solvent-B) (0-16 min, 60% A; 16-20 min, 30% A; 20-35 min, 60% A) at a 1 mL/min of flow rate, the injection was performed using a 20 µL loop at 254 nm with 35 min for total run time.

### Cell and Cell Culture

A549 human lung cancer cells were grown in 10% Fetal Bovine Serum (FBS) of DMEM medium with 1% antibiotics (Streptomycin/Penicillin G) in $CO_2$ incubator as the instruction from ATCC.

### Cytotoxic Assay

A549 cells were seeded in 96-well culture plates at the density of $1\times10^4$ cells/well for 24 hr incubation period and then exposed to 0-100 µg/mL of PI extract with high and low plumbagin for 24-72 hr. End of indicated time, the cells in 96-well plates were added with 10% trichloroacetic acid to fix the cells, 0.4% SRB to stain the cells, 1% acetic acid to wash the cells, and then 10 mM Tris base buffer to solubilize the cells. The OD. was read at 540 nm using a microplate reader.

### Colony Formation Assay

A549 cells were seeded in 6-well culture plates at the density of 500 cells/well for 24 hr and then exposed to 0-100 µg/mL of PI extract with high and low plumbagin for 24 hr, cultured for another 10 days with changing the media for every 2-3 days. End of incubation period, the cancer cells were exposed to 100% methanol to fix the cells, 0.25% crystal violet to stain the cells, and DI water and air dried. The number of colonies were captured and counted.

### Wound Healing Assay

A549 cells were seeded in 24-well cultured plates at the density of $2.5x10^5$ cells/well for 24 hr. Next day, the cells were made a Wound by 200 µL pipette tips and treated with 0-50 µg/mL of PI extract with high and low plumbagin for 24 hr. The Wound images were photographed using a CKX53 inverted microscope (Olympus, USA) at 0 and 24 hr. The percentage of wound healing was measured the denuded area compared between 0 and 24 hr.

### Matrigel Migration Assay

A549 cells were seeded in DMEM medium without FBS in the upper Transwell-well with 0.8 µm at the density of $2x10^4$ cells/well with 0-50 µg/mL of PI extract with high and low plumbagin for 24 hr. The lower chamber was added with complete DMEM medium. End of incubation period, the migrated cells in lower side was fixed with 100% methanol, 0.25% crystal violet was added to stain the cells and air dried. The percentage of migrated cells were counted and compared between control and treatment groups.

### Apoptotic Assay

A549 cells were seeded in 6-well cultured plates at the density of $2x10^5$ cells/well for 24 hr and then exposed to 0-50 µg/mL of PI

extract with high and low plumbagin for 24 hr. End of incubation period, the cells were harvested by 0.25% trypsin-EDTA, PBS buffer was added to wash the cells, and PI and Annexin V-FITC solution (BD Biosciences) to stain the cells in dark at room temperature for 15 min. Intensity of fluorescence was determined by flow cytometric method with BD Accuri C6 Plus software.

### Reactive Oxygen Species Formation Assay

A549 cells were seeded in 6-well culture plates at the density of $2x10^5$ cells/well for 24 hr and then exposed to 0-50 µg/mL of PI extract with high and low plumbagin for 24 hr. End of incubation period, the cells were harvested by 0.25% trypsin-EDTA, added the PBS buffer to wash the cells, and then stained with 25 µM DCF-DA fluorescent solution at 37°C in dark for 30 min. Intensity of fluorescence was determined by flow cytometric method with BD Accuri C6 Plus software.

### Mitochondrial Function Assay

A549 cells were seeded in 6-well cultured plates at the density of $2x10^5$ cells/well for 24 hr and then exposed to 0-50 µg/mL of PI extract with high and low plumbagin for 24 hr. End of incubation period, the cells were harvested by 0.25% trypsin-EDTA, added the PBS buffer to wash the cells, and then stained with JC-1 fluorescent solution (BD Biosciences) at 37°C in dark for 30 min. Intensity of fluorescence was determined by flow cytometric method with BD Accuri C6 Plus software.

### Western Blotting Assay

A549 cells were seeded in 6-well cultured plates at the density of $2x10^5$ cells/well for 24 hr and then exposed to 0-50 µg/mL of PI extract with high and low plumbagin for 24 hr. For each sample, cells were lysed using the RIPA buffer and then measured the protein concentration by Bradford's solution. 20 µg of protein samples were loaded onto a 12% SDS-PAGE gel and further transferred onto a PVDF membrane at room temperature. The PVDF membrane was blocked with 3% bovine serum albumin and exposed to primary antibodies were used at 4°C overnight with shaking: pro-caspase 3, active caspase 3, and beta-actin (ACTB, Cell Signaling Technology). Next day, secondary antibodies with HRP-conjugated were used for 2 hr at room temperature. The band intensity was visualized with ECL reagent (Biorad, USA).

### Statistical Analysis

The results were repeated three separated experiments and the data was tested using GraphPad Prism 5.0 software. The differences between control and treatment groups were determined using Student's *t*-test. The results are represented as the mean±SE. $p<0.05$ was considered to designate a statistically significant difference.

## RESULTS

### Plumbagin Level in PI Extract

Plumbagin is an active compound in PI root extract and it is the active anticancer properties in many cancer cells. Therefore, to confirm the plumbagin content on PI extract in the different anticancer activity was explored including lung cancer A549 cells. First, to examine the plumbagin content in the PI root extract, we used the HPLC method. The presence of plumbagin in different batch of PI extract revealed 2 level of content, to be 23.65±0.03 µg/mL for high plumbagin and 12.36±0.07 µg/mL for low plumbagin (µg/mg crude extract) (Figure 1A) Further study, to explore the plumbagin content with high and low on anticancer effects in lung cancer cells A549.

### The Extracts on Cell Viability and Colony Forming Ability

To determine the actions of PI extract with high and low plumbagin on cell viability in A549 lung cancer cells by using SRB method. Cell viability data demonstrated that the cancer cell viability was strongly inhibited after treatment for 24, 48 and 72 hr, with low $IC_{50}$ values of 17.37±2.01, 16.64±1.66 and 8.76±1.66 µg/mL for PI extract with high plumbagin and 25.50±2.76, 19.52±0.35 and 11.89±1.17 µg/mL for PI extract with low plumbagin, respectively (Figure 1B-C). PI extract with high plumbagin had higher potency to suppress cell viability than low plumbagin levels.

Moreover, the PI extracts inhibited the colony formation of the lung cancer cells, with low very $IC_{50}$ values of 5.42±0.85 µg/mL for high plumbagin and 12.01±0.98 µg/mL for low plumbagin (Figure 1D-E). Interestingly, the both concentration of PI extract desired to suppress the lung cell replication was less than that desired to inhibit the cell viability.

### The Extracts on Cell Migration

The data indicated that PI extracts with high plumbagin strongly suppressed lung cancer cells migration at 24 hr (Figure 2A-B) than that the low plumbagin extract by performing the wound healing method and this effect were companied by reduced the migrated cells (Figure 2C-D) using Matrigel migration assay. At high dose of PI extract with high plumbagin (50 µg/mL) caused antimigratory inhibition and no cells detected.

### The Extracts on Cell Apoptosis

To explore an apoptotic effect of PI extracts on A549 cells and flow cytometry methods was used. From the results obtained that both PI extracts at high dose (50 µg/mL) caused the reduction of viable cells and induction of late apoptosis when compared with control group. High plumbagin extract caused a significant induction of late apoptosis from 0.5%, 1.6%, 2.6%, and 15.9% for 0, 10, 25, 50 µg/mL, respectively (Figure 3A-B). Similarly, low

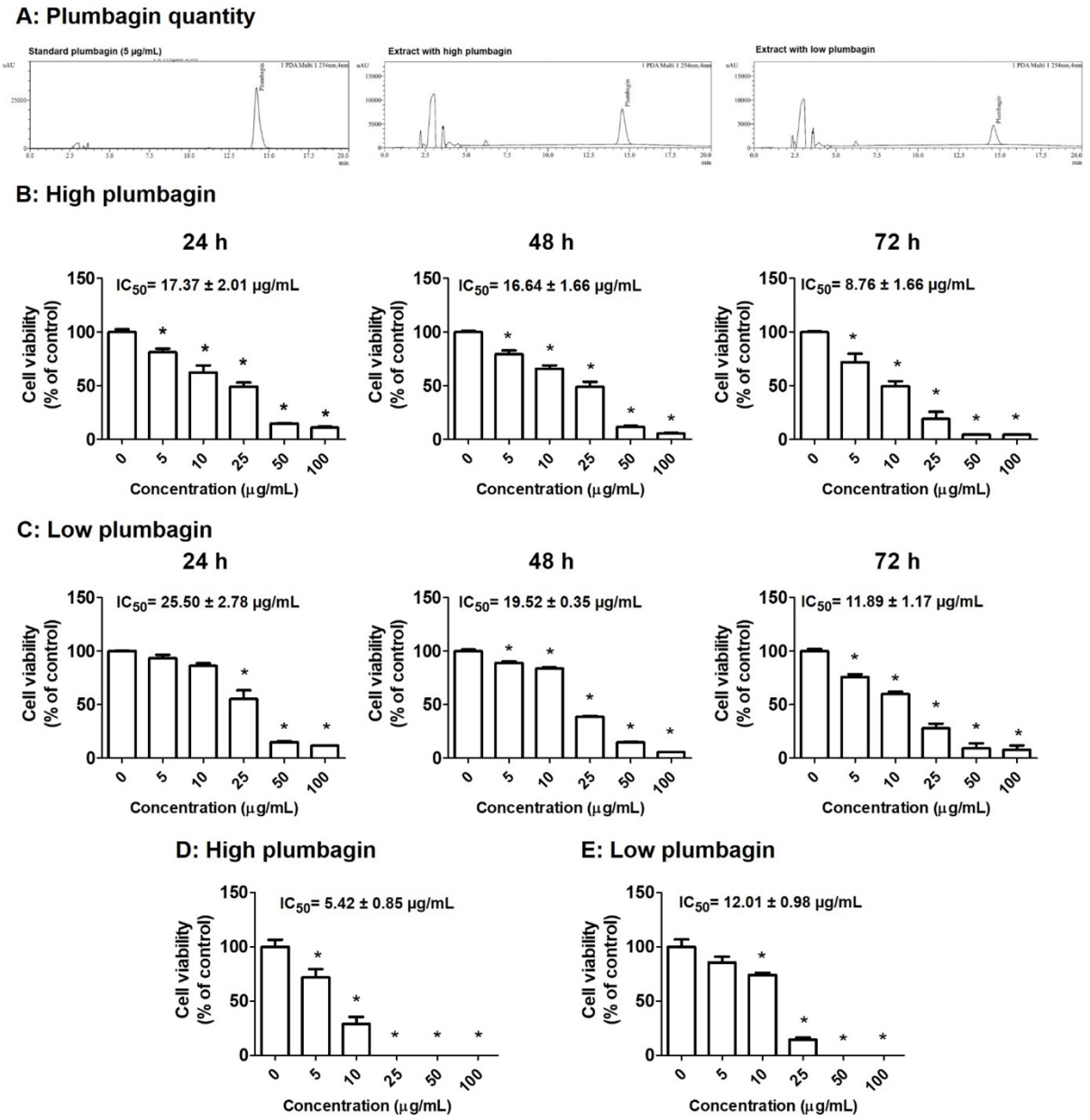


**Figure 1:** High-performance liquid chromatography chromatogram of plumbagin in PI extracts and PI effects on lung cancer cells viability and colony formation. (A) The PI extract was measured the plumbagin quantity by HPLC method. The A549 cells were exposed to PI extracts (B and C) for 24-72 hr and examined the cell viability by SRB method. The colony formation assay, cancer cells were exposed to PI extract for 24 hr, and grown further for 10 days (D and E). The data are presented as mean±SE. $n$=3, * $p$<0.05.

plumbagin extract stimulated the late apoptosis from 0.6%, 1.1%, 2.3%, and 14.3%. PI extract induced lung cancer cells apoptosis.

## The Extracts on ROS Formation and Mitochondrial Function

From the powerful actions of PI extract on A549 cell growth inhibition and apoptosis, the mechanism of action was more interested to explore the production of ROS production and mitochondrial dysfunction. The results demonstrated that PI extract induced ROS in intracellular cells by dose-dependent manner at 24 hr incubation period and the higher concentration of PI extract was increased ROS for 12.9% and 7.3% for high and low plumbagin extract, respectively (Figure 3C-D). From the data obtained, the ROS stimulation was correlated to apoptotic activation from PI extracts when compared with untreated cells and PI-treated cells.

Additionally, PI extract with high plumbagin caused activation of mitochondrial dysfunction by dose-dependent as apoptotic induction and ROS formation. At four levels of treated solution (0, 10, 25, 50 µg/mL) of high and low plumbagin extracts, the reduction of mitochondrial function expressed as induction of JC-1 monomers (unhealthy cells) were 2.2%, 2.8%, 8.3%, 11.5% for high-plumbagin extract and 2.1%, 2.2%, 3.6%, 8.1% for

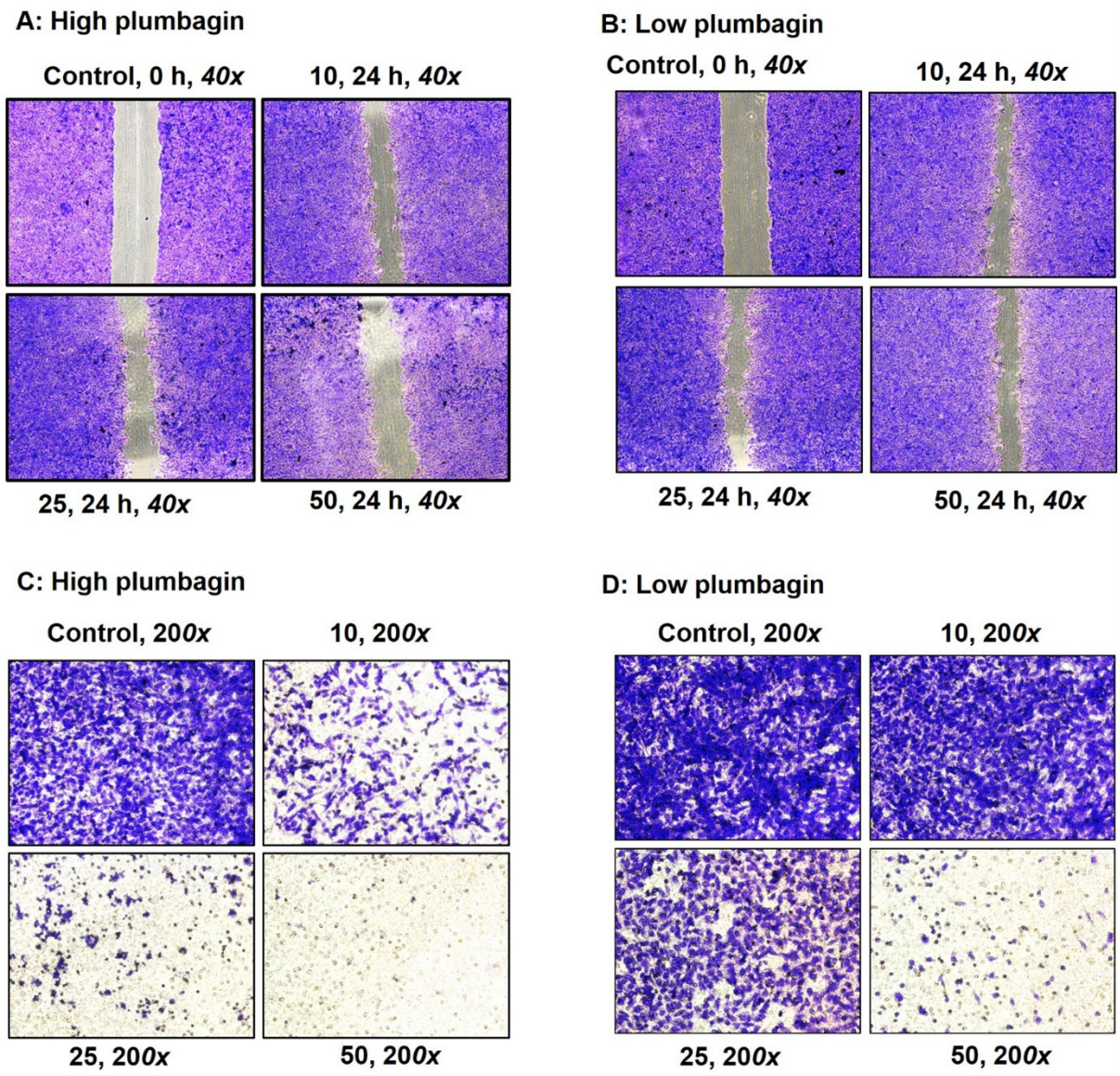


**Figure 2:** PI effects on cell migration. For Wound healing assay, A549 cells were scratched, exposed to PI extracts (A and B) for 24 hr and the distance of wound was measured by Wound healing method. For Matrigel migration, cancer cells were exposed to PI extracts in the upper chamber, and cultured for 24 (C and D), and the cells were stained with crystal violet.

low-plumbagin extract (Figure 3E-F). Apoptosis was induced by observing the ROS formation and mitochondrial dysfunction on lung cancer cells on PI extracts.

### The Extracts on Protein Expression

To investigate how the PI extract, suppress the growth and activate apoptosis in lung cancer cells, we analyzed proteins-related apoptosis, caspase 3 expression levels by Western blotting. Our results indicated that both PI extract induced active-caspase 3 expression levels in A549 cell lines (Figure 4A-B). Especially, high plumbagin extract also significantly decreased pro-caspase 3 correlating with increased active-caspase 3 levels. The groups of PI extract decreased lung cancer cell growth and apoptosis by increasing the active-caspase 3 levels.

## DISCUSSION

The result in this work was indicated that the PI root extracts with high plumbagin has a high powerful growth inhibition on the human A549 lung cancer cells superior to low plumbagin. The strong effects of PI extracts were presented via suppression of cell viability, reduction of colony formation, and further inhibition of migration. Importantly, PI extract significantly stimulated lung cancer cell apoptosis by dose-dependent manner through the induction of ROS formation, reduction of mitochondrial function, and activation of active-caspase 3 expression levels, which may be significant for the anticancer activity detected in A549 lung cancer cells. Finally, the results demonstrated that PI extract with high plumbagin has higher anticancer activity than low plumbagin.

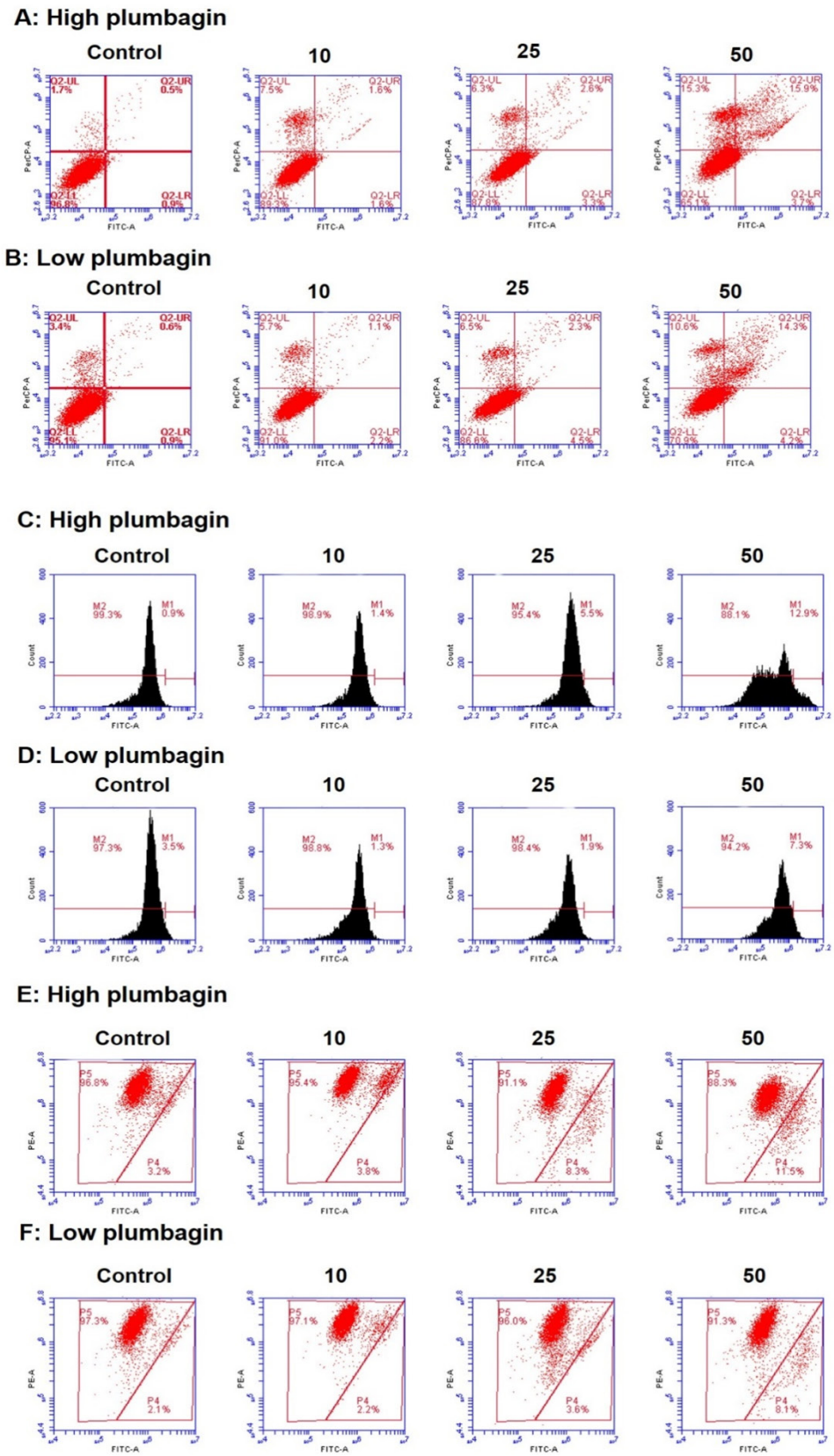


**Figure 3:** Effect of PI on apoptosis, ROS formation and mitochondrial dysfunction. The A549 cells were exposed to PI extracts (A and B) for 24 hr and apoptosis was stained by PI and Annexin V-FTC and determined by flow cytometric method. For ROS formation, A549 cells were exposed to PI extracts (A and B) for 24 hr, stained with DCF-DA fluorescent probe and ROS production was determined by flow cytometric method. For mitochondrial function, cancer cells were treated with PI extracts for 24 hr, stained with JC-1 and mitochondrial function was examined by flow cytometric method (C and D).

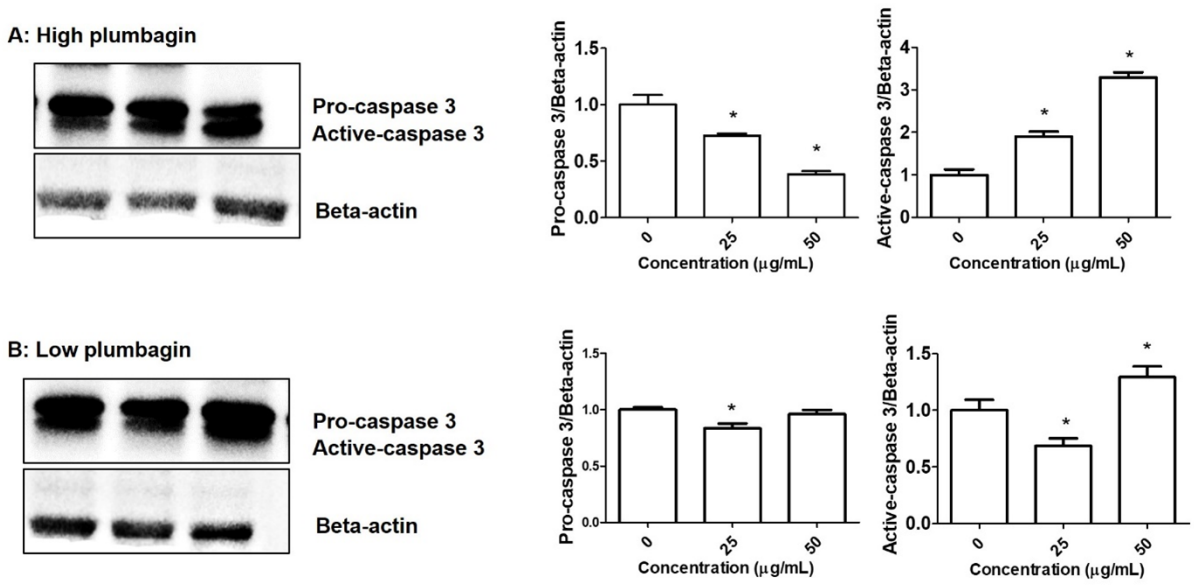


**Figure 4:** Effect of PI on protein expression. A549 cells were exposed to PI extracts (A and B) for 24 hr, separated the protein by SDS-PAGE, incubated with antibodies against pro-caspase 3, active-caspase 3, and beta-actin. Data are represented as mean±SE. *n=3*, * *p*<0.05.

Plumbagin is an active compound that found in PI root and has shown a various pharmacological activity including anticancer activities.[9] The potential role of plumbagin act as an anticancer property has been testified in several cancer cells including ovarian, prostate, cervical, lung, and melanoma.[10] Therefore, PI extract with high plumbagin has a powerful against cancer cells including lung A549 cancer cells act as a model in this study. Plumbagin significantly stopped the cell cycle distribution at S to G2/M phase, activated ROS formation, stimulated the loss of function of Mitochondrial Membrane Potential (MMP) and further lead to activation of intrinsic apoptotic signaling pathway.[11] In this work demonstrated that PI extract with high plumbagin caused induction of lung cancer cells death and reduction of the colony formation than low plumbagin levels. Consistent with the effects of ethanolic extract of PI roots found that these extracts induced melanoma cells death with low $IC_{50}$ values (124 µg/mL) and showed the less effects on normal lymphocyte cells.[4] The mechanisms of plumbagin was proposed with upregulation the protein-related growth inhibition expression, p53 and $p21^{CIP1/WAF1}$, these proteins caused induction of cell cycle arrest in the G2/M phase.[12] Growth suppression of plumbagin was associated with reduction of cyclinB1, Cdc2, and Cdc25C.[13] Therefore, PI extract with high plumbagin caused induction of lung cancer cells death.

Metastasis/migration is the key important point of mortality in cancer patients, which spread the cancer cells to distant organs and secondary tumors are occurred.[14] For migratory effects, this study indicated that PI root extract significantly suppressed the lung cancer cells migration after detecting with Wound healing and Matrigel migration assay, especially in high plumbagin of PI root extract. The migratory suppression of plumbagin effects found that it suppressed migration through inhibition of CXC chemokine receptor-4 expression on both breast and gastric cancer cells.[15] Similar with cholangiocarcinoma cell migration and invasion data, the results were reported a potent effect of plumbagin with low $IC_{50}$ values and complete inhibition at 25 mm.[16] These were indicated that PI extract with high plumbagin may inhibited the cancer cells migration as well. This work observed that PI extract with high plumbagin significantly suppressed lung cancer A549 cells migration by dose-dependent manner than low plumbagin of PI extract when examined by Wound healing method. At the dose of 50 µg/mL of PI extract, it decreased cancer cell migration approximately 56% in high plumbagin group and 78% in low plumbagin group, respectively. Next, the migratory inhibition was examined by Matrigel migration assay was indicated that high plumbagin significantly reduced the cancer cells migration at the dose of 10-25 µg/mL and no cells detected at the dose of 50 µg/mL. For low plumbagin of PI extract showed the migratory suppression less than high plumbagin. Finally, PI extract with high plumbagin has a potency to suppress the lung cancer cells migration.

An apoptotic induction pathway is playing an important role in anticancer process of cell growth, replication and death. Mitochondrial-related apoptosis is an intrinsic pathway to activate apoptosis which correlated the high levels of ROS formation and stimulation of cytochrome C and caspase 3 releasing levels and ultimately leads to apoptosis.[17] Since high levels of ROS are toxic to cancer cells, mitochondria are a promising target for novel anticancer therapeutics.[18] In this study demonstrated that high plumbagin extract caused induction the ROS formation, led to stimulate mitochondrial dysfunction, and finally, the apoptotic induction is occurred. The data of PI effects on lung cancer migration is limited; however, the plumbagin action suppressed lung cancer cells migration has been reported in several

experiments. For example, plumbagin induced lung cancer cells apoptosis via arresting the cell cycle distribution in S to G2/M phase, enhancing ROS generation, and disrupting mitochondrial function resulted in reduction of MMP.[19] Furthermore, plumbagin significantly induced the mitochondrial pathway, caspase 9 and caspase-3, which lead to apoptotic induction of lung cancer cells.[20] Plumbagin induced cancer cells apoptosis has been observed in many cancer cells types such as breast cancer, liver cancer, melanoma, leukemia, and brain cancer.[21] Collectively, material source of PI with high plumbagin was the most important to be chosen as herbal medicine in Thai recipe with proven anticancer activity from this study by defeating cancer growth, proliferative, migratory, antiapoptotic effects. The *in vivo* study is urgently to be explore.

## CONCLUSION

In conclusion, both of PI extract with high and low plumbagin stimulates the lung cancer cell death, activates apoptosis and inhibits migration, especially in high plumbagin extract. For the mechanism of action was observed in induction of ROS formation and inhibition of mitochondrial function. Further, the PI extract activates lung cancer cells death and apoptosis through stimulation of active-caspase 3 expression levels. These results highlight the potential of PI extract with high plumbagin as an effective phytochemical for preventing or treating of lung cancer and the other types of cancer in further. Hence, dosage use of PI desired to be of concern in healthy humans and the study *in vivo* is required to confirm its safety amount in persons.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**AKT:** Protein kinase B; **FBS:** Fetal bovine serum; **HPLC:** High-performance liquid chromatography; **NF-κB:** Nuclear factor-κB; **PI:** *Plumbago indica* L.; **ROS:** Reactive oxygen species; **STAT3:** Signal transducer and activator of transcription 3; **SRB:** Sulforhodamine B.

## SUMMARY

PI extract with high plumbagin level significantly reduced lung cancer cell proliferation with decreasing colony forming ability. High plumbagin of PI extract strongly suppressed the lung cancer cells migration. The mechanism of actions was also through induction of apoptosis, activation of ROS formation, and stimulation of mitochondrial dysfunction and correlated the induction of expression of active-caspase 3 level correlated with reduction of pro-caspase-3 expression.

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