

Evaluation of anticancer activity of *Plumbago indica* root extract on cervical HeLa cancer cells

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Abstract

Plumbago indica L. has a various pharmacological activity; however, the anticancer activity in cervical cancer has less information. To examine the antiproliferative, apoptotic, and antimigratory activity of *Plumbago indica* root extract on cervical cancer cells. The extract of *P. indica* was explored for cell viability, apoptosis, and migration by sulforhodamine B, colony formation, cell cycle distribution, Wound healing, Annexin V-FITC staining apoptotic, mitochondrial function, and reactive oxygen species (ROS) formation. The data indicated that *P. indica* extract suppressed HeLa cervical cells viability by dose- and time-dependent manner along with the reduction of cancer cell number and colony forming ability. After 48 h of incubation period, the PR extract exhibited a significant reduction of migratory ability by dose-dependent manner. Furthermore, these extracts significantly showed a reduction of cell viability along with induction of late apoptosis. The mechanism of action was shown by detecting mitochondrial dysfunction and activating of ROS formation on HeLa cells-treated with *P. indica* extract. The extract of *P. indica* has a growth inhibition, migratory suppression, and apoptotic induction. The extract has an anticancer activity by reducing mitochondrial function and activating ROS formation.

Keywords: apoptosis; cell cycle arrest; cervical cancer cells; migration; mitochondrial dysfunction; *Plumbago indica*

Introduction

The cervical cancer is presently one of the most common tumors and this cancer is the second cause of death in female that reported in worldwide (Siegel *et al.*, 2022). High incidence rate of cervical cancer was reported in developing countries and low incidence rate was found in developed countries. Presently, the standard therapy for cervical cancer includes surgery, radiation and chemotherapy, furthermore, chemotherapeutic agents remain the most common treatment for advanced-stage or progressive cancers (Smith *et al.*, 2000). The effective treatment in an early started with anticancer drugs enable patients to improve

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survival rate in 5 years; however, the data reported that mortality rate has remained relatively high (Canfell *et al.*, 2020). Further, the urgently need to find new agents from natural plants or herbal biomedicines which has a very low toxicity and more high potency for prevention or treatment of cervical cancer.

Thai herbal medicine such as *Plumbago indica* L. which belongs to the Plumbaginaceae family that has been reported in numerous common names in different countries and is known as “jetamul-pleung-dang” or “pitpiudang” in Thailand. It is a one of Thai herbal medicine which uses as an antioxidant, anti-inflammatory, antihelminthic, antibacterial, antifungal, and anticancer activities (Priyanjani *et al.*, 2021). Several active compounds have been reported in many parts of *P. indica*, especially in root, such as plumbagin, sitosterol, stigmasterol, campesterol, naphthaquinone, plumbagic acid, myricetin, roseanoic acid, palmitic acid and β -sitosterol (Kaewbumrungra and Panichayupakarananta, 2012; Jose *et al.*, 2014). Plumbagin is an important naphthoquinone and has more efficacy that used in treatment of cancer (Li *et al.*, 2010; Zhang *et al.*, 2021); however, the scientific evidence of *P. indica* has not been fully understood. In Thai traditional medicine, *P. indica* is generally used for treating cancer and Thai recipe was composed of *P. indica*, such as ben-ja-kul, prabchom-poo-tha-weeb, yha-tri-phi-kad, and yha-phai-pra-lai-kun. Therefore, the study of *P. indica* roots extract was more interested to investigate cancer prevention and treatment, especially in cervical cancer.

P. indica on anticancer effects was reported in melanoma cells with high antiproliferative activity by reducing cell growth, inhibiting colony formation, and detecting apoptotic cells (Anuf *et al.*, 2014). Several information demonstrated that plumbagin is an active content in the root of *P. indica* extract or the extract of Plumbaginaceae family were demonstrated to induction cancer cells death, antimigration, and apoptosis. Potential role of plumbagin, as an anticancer agent has been recognized and its anticancer effects have been reported in diverse cancer models such as prostate, osteosarcoma, lung, esophagus, cervical, colon, ovarian, lung, and melanoma (Yin *et al.*, 2020). Plumbagin-induced cancer cell death with low dose and showed the high efficacy to induction of apoptosis through induction of the formation of reactive oxygen species (ROS), stimulation of caspase 3 and caspase 9 in osteosarcoma cells (Chao *et al.*, 2017). Confirmation with retinoblastoma cells, plumbagin induced apoptosis by increasing the mitochondrial dysfunction and correlating with caspase cascade induction (Gharbaran *et al.*, 2021). Moreover, plumbagin also caused strong activation of apoptosis via inhibition of cell cycle arrest in G2/M and S-G2/M phase in cervical cells (Jaiswal *et al.*, 2018). In conclusion, plumbagin demonstrated act as an active component in *P. indica* extract that strongly induced many cancer cells death and growth inhibition. Consequently, *P. indica* extract may cause induction cervical cancer cells death through inducing apoptosis, activating ROS formation, and decreasing mitochondrial function as information of plumbagin study.

Based on the previous data, we needed to investigate the proliferative inhibition, migratory suppression, and apoptotic induction of *P. indica* root extract on cervical HeLa cells in this study. The *P. indica* root extract was extracted by 95% ethanol and measured the proliferative activation by sulforhodamine B, aridine orange/ethidium bromide staining, cell cycle arrest. Migration suppression was determined by Wound healing and Matrigel migration assay. Apoptosis induction was determined by Annexin V-FITC/Propidium bromide (PI) along with the mechanism of action by mitochondrial function and reactive oxygen species (ROS) formation.

Materials and Methods

Plant extracts

The root of *P. indica* were obtained from Roi-et Province in Thailand in 2019, identified, and collected at the Faculty of Medicine, Mahasarakham University (Herbarium no. MSUT-7286). For extraction, 250 g of root of *P. indica* was dried, sliced into small pieces, soaked with 95% ethanol (1.5 L), macerated for 7 days,

evaporated, and lyophilized. The crude extract was stored at $-20\text{ }^{\circ}\text{C}$ until use. The yield of the extract was 4.56%.

High-performance liquid chromatography method (HPLC)

HPLC assay was used to measure the plumbagin in *P. indica* root extracts. The *P. indica* extract was loaded onto Hypersil gold C18 reverse phase column. The mobile phase composed of a gradient composition using acetonitrile (solvent A) and water (solvent B) (0-16 min, 60% B; 16-20 min, 30% B; 20-35 min, 60% B). The flow rate of injection was 1 mL/min and 20 μl was the injection volume. The total run time was 35 min and the peaks were monitored at a wavelength of 254 nm.

Cell culture and sulforhodamine B (SRB) method

HeLa human cervical cancer cells were cultured in the medium with 10% FBS plus 1% antibiotics in 5% CO_2 at $37\text{ }^{\circ}\text{C}$. The *P. indica* extract on antigrowth effect was examined by SRB method. In brief, the 1×10^4 cells/well were seeded onto 96-well culture plate for overnight and the cells were exposed to various concentrations of *P. indica* extracts (0-250 $\mu\text{g}/\text{mL}$) for 24, 48, and 72 h. Further, HeLa cells were fixed, added with 0.4% SRB solution, solubilized with 10 mM Tris base buffer, and measured the optical density at 540 nm using a spectrophotometer.

Acridine orange/Ethidium bromide (AO/EB) staining method

1×10^4 cells/well of HeLa cells were cultured into 96-well culture plates for overnight, and the cells were exposed to various concentrations of *P. indica* extracts (0-50 $\mu\text{g}/\text{mL}$) for 24 h. Then, cells were incubated with AO/EB solution (1 $\mu\text{g}/\text{mL}$ of each dye) and mixed gently for 15 min at room temperature. HeLa cells in plates were captured by fluorescent inverted microscopy (20x magnification, CKX53, Olympus, USA).

Colony formation method

500 cells/well of HeLa cells were plated into 6-well culture plates for overnight, the cells were exposed to the various concentrations of *P. indica* extracts (0-50 $\mu\text{g}/\text{mL}$) for 24 h, and cultured further for 15 days. HeLa cells were washed, fixed, and stained with 0.25% crystal violet at room temperature for 20 min. Colony forming ability in 6-well plate were captured and counted.

Cell cycle arrest method

2.5×10^5 cells/well of HeLa cells were plated into 6-well culture plates for overnight, the cells were exposed to the various concentrations of *P. indica* extracts (0-50 $\mu\text{g}/\text{mL}$) for 24 h, and then collected the cells pellet. Cells were washed, fixed with 70% ethanol at $-20\text{ }^{\circ}\text{C}$ for 24 h and washed with PBS buffer for several times. The cells were added in propidium iodide (PI) solution (Cat No. 550825, BD Biosciences, CA, USA) at $4\text{ }^{\circ}\text{C}$ for 30 min in the dark and measured the cell cycle distribution by flow cytometry (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Wound healing method

2.5×10^5 cells/well of HeLa cells were plated into 24-well culture plates for overnight, made a wound by 200 μl pipette tips, washed with PBS buffer, and treated with the extracts (0-50 $\mu\text{g}/\text{mL}$) for 48 h. After that, HeLa cells were fixed with absolute methanol for 20 min at $-20\text{ }^{\circ}\text{C}$ and images of the wounds were captured at 0 h and 48 h after scraping at 40x magnification.

Matrigel migration method

2.5×10^4 cells/well of HeLa cells were plated into Inserted-well (upper chamber) of 24-well culture plates with 0% FBS of DMEM medium plus various concentrations of *P. indica* extracts (0-50 $\mu\text{g}/\text{mL}$) for 24 h. The lower chamber was contained with 10% FBS DMEM medium. The cells number in three selected areas of migrated to the lower chamber were captured and then counted.

Apoptosis method

2.5×10^5 cells/well of HeLa cells were plated into 6-well culture plates for overnight, the cells were exposed to the various concentrations of *P. indica* extracts (0-50 $\mu\text{g}/\text{mL}$) for 24 h, and collected the cells. The cells were added with 100 μL of assay buffer with 5 μL Annexin V-FITC and 1.5 μL PI dye (Cat No. 558547, BD Biosciences, CA, USA) at room temperature for 15 min incubation in the dark. The viable cells, early apoptotic, late apoptotic, and necrotic cells were measured by flow cytometric method (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Mitochondrial membrane potentials method

2.5×10^5 cells/well of HeLa cells were plated into 6-well culture plates for overnight, the cells were exposed to the various concentrations of *P. indica* extracts (0-50 $\mu\text{g}/\text{mL}$) for 24 h, and collected the cells. The cells were added with 100 μL of assay buffer with 5 μL JC-1 dye (Cat. No. 1-800-346-9897, Cayman Chemical, Michigan, USA) at room temperature for 15 min incubation in the dark. The mitochondrial function was measured by flow cytometric analysis (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Reactive oxygen species formation (ROS) method

2.5×10^5 cells/well of HeLa cells were plated into 6-well culture plates for overnight, the cells were exposed to the various concentrations of *P. indica* extracts (0-50 $\mu\text{g}/\text{mL}$) for 24 h, and collected the cells pellet. The cells were added with 100 μL of assay buffer with 25 μM DCF-DA dye at room temperature for 30 min incubation in the dark. The ROS formation measured by flow cytometric method (BD Biosciences, San Jose, CA, USA) using BD Accuri C6 Plus software.

Statistical analysis

All the results are presented with the mean \pm SE and Student's *t*-test was employed to analyze the statistical differences by using GraphPad Prism analysis software (version 5.0, GraphPad Software, San Diego, California, USA). The statistically significant set as $p < 0.05$ compared between control and treatment groups.

Results

Plumbagin level in P. indica extract

Plumbagin is an active compound and found the most abundant in *P. indica* root. Interestingly, plumbagin is an active anticancer property with very low dose to induce cells death in several cancer cells. To explore the plumbagin level in the *P. indica* root extract, we used the HPLC method. The data revealed the presence of plumbagin level in the *P. indica* root extract, as shown in Figure 1A-B, to be 43.03 ± 0.03 $\mu\text{g}/\text{mL}$ of plumbagin /dry weight extract. Plumbagin was reported with high content in *P. indica* extract.

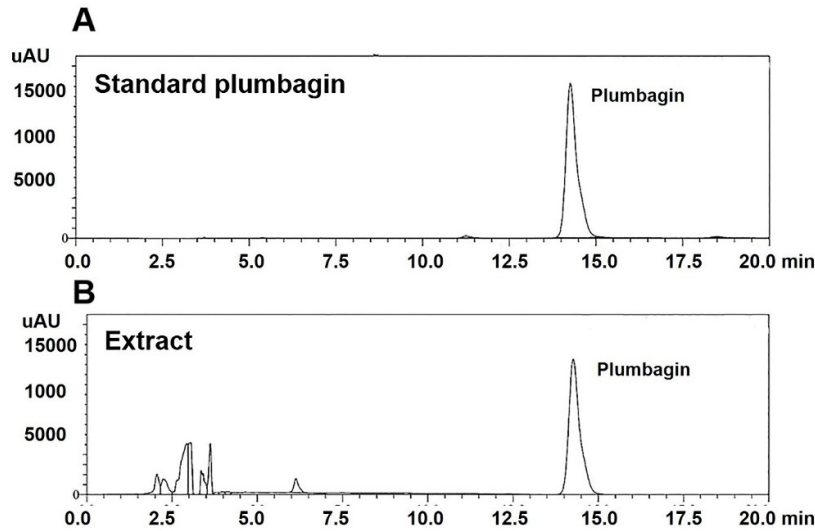


Figure 1. High-performance liquid chromatography chromatogram of plumbagin in *P. indica* extracts, (a) plumbagin standard (b) plumbagin in *P. indica*

P. indica effects on cell viability and morphology

To explore the antiproliferative effect of *P. indica* on cervical cancer cells and SRB method was conducted. From the data demonstrated that the *P. indica* extract inhibited HeLa cell viability by dose- and time-response manner (Figure 2A) with IC_{50} values were 42.03 ± 3.38 , 35.14 ± 0.82 , 22.70 ± 1.41 $\mu\text{g/mL}$ for 24, 48, 72 h, respectively. Next, to evaluate these extract effect on HeLa cell number and morphology by AO/EB double staining method. *P. indica* root extract caused reduction of cancer cells number by dose-dependent manner after treatment with these extract for 24 h. The morphological changes were observed at the high dose of the extract with 25 and 50 $\mu\text{g/mL}$ as apoptotic induction along with showing apoptotic bodies and nuclease condensation, especially 50 $\mu\text{g/mL}$ (Figure 2B).

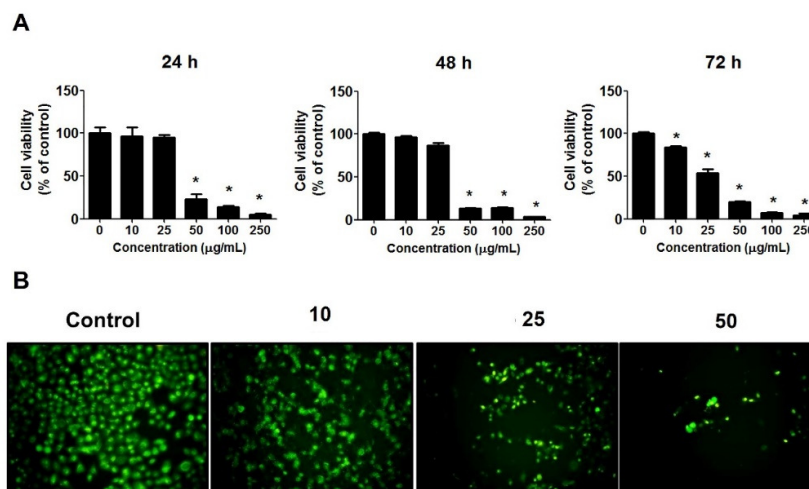


Figure 2. The effects of *P. indica* on cell viability and morphology. The HeLa cells were treated with various concentrations of *P. indica* (0-250 $\mu\text{g/mL}$), fixed, stained with SRB dye, and read O.D. by spectrophotometry (A). The cells were treated with *P. indica* (0-50 $\mu\text{g/mL}$), stained with AO/EB dye and captured by inverted microscopy (200x magnification) (B)

Data represent mean \pm SE from three separated experiments. * $p < 0.05$ vs control.

P. indica effects on colony formation and cell cycle arrest

To evaluate the cell replication effect of *P. indica* extract on cervical cancer HeLa cells and colony formation method was conducted. The data indicated that *P. indica* extract significantly suppressed cell replication after treatment with the root extract. Interestingly, colonies did not detect in all doses of treatment groups when compared with untreated group. Conclusively, *P. indica* extract has a high powerful to suppress the colony formation (Figure 3A). To confirm the cell growth of the extract and cell cycle arrest was performed. The histogram of cell cycle distribution between control, 10 and 25 $\mu\text{g}/\text{mL}$ did not difference; however, at 50 $\mu\text{g}/\text{mL}$ of extract showed reduction of the histogram height and significant different from control group (Figure 3B). From the results demonstrated that *P. indica* extract strongly inhibit HeLa cells growth by evaluating with colony formation and cell cycle arrest.

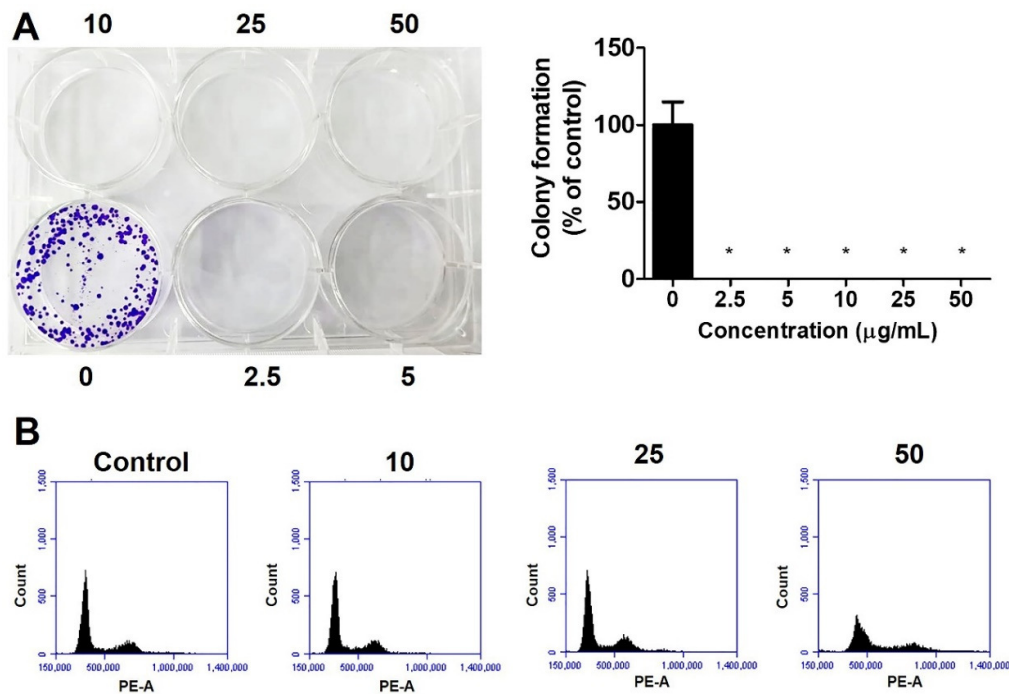


Figure 3. The effects of *P. indica* on colony formation and cell cycle arrest. The cancer cells were plated on 6-well plates for 24 h (500 cells/well), treated with *P. indica* (0-50 $\mu\text{g}/\text{mL}$), cultured for another 15 days, stained and counted (A). Cells were plated on 6-well plates for 24 h (2.5×10^5 cells/well), treated with *P. indica* (0-50 $\mu\text{g}/\text{mL}$) and measured cell cycle arrest by flow cytometry (B)

Data represent mean \pm SE from three separated experiments. * $p < 0.05$ vs. control.

P. indica effects on cell migration

To explore the cell migration effect of *P. indica* extract on cervical cancer HeLa cells and Wound healing method was conducted. After the HeLa cells were exposed with these extracts for 48 h incubation period and the high dose of extract (50 $\mu\text{g}/\text{mL}$) caused suppression of the migration approximately 95% (Figure 4A) when compared with the control group. Confirmation with Matrigel migration assay, this data also confirmed that these extracts significantly decreased the number of HeLa migrated cells to the lower chamber with significant at the dose of 10-50 $\mu\text{g}/\text{mL}$ (Figure 4B). These extracts showed the strong effects on migratory suppression.

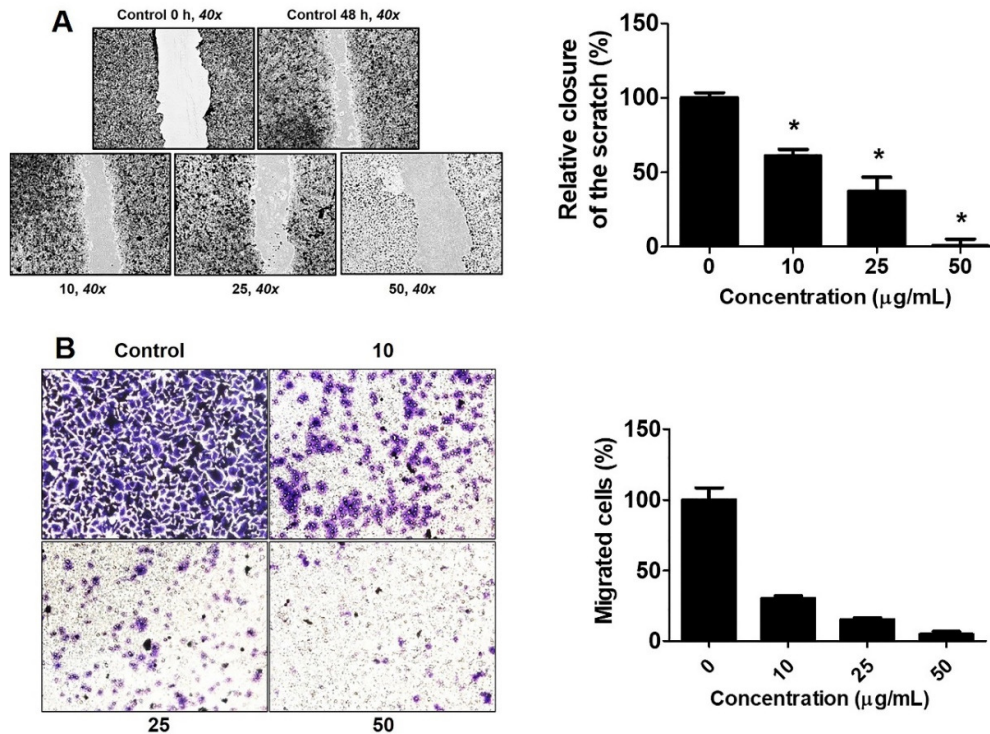


Figure 4. The effects of *P. indica* on wound healing assay and matrigel migration assay. The cancer cells were plated on 24-well plates for 24 h, made a wound, and then treated with *P. indica* (0-50 µg/mL) for 48 h. Cells were captured with inverted microscopy (40x magnification, A). The cancer cells were plated on upper chamber with *P. indica* (0-50 µg/mL) for 24 h. The migrated cells were captured with inverted microscopy (200x magnification, B)

Data represent mean±SE from three separated experiments. **p*<0.05 vs. control.

P. indica effects on cell apoptosis and mitochondrial function

To measure the apoptotic effect of *P. indica* extract on cervical cancer HeLa cells and Annexin V-FITC/PI double staining method was conducted. These extracts significantly decreased percentage of viable cells from 94.9±5.23%, 87.5±3.23%, 35.6±4.92%, 10.9±0.49% for 0, 10, 25, and 50 µg/mL, respectively, along with increased percentage of late apoptotic cells from 3.8±0.25%, 10.2±0.32%, 45.9±3.23%, 47.6±6.65% (Figure 5A). The extract significantly induced the apoptosis in HeLa cancer cells.

Additional, to explore the mechanism of *P. indica* on apoptosis was related with mitochondrial dysfunction and JC-1 staining was conducted. The root extract caused induction the loss of mitochondrial function when compared with control groups which reduction of JC-1 aggregates (healthy cells) from 86.4±5.25%, 87.7±4.52%, 28.6±3.33%, 11.5±1.55% for 0, 10, 25, 50 µg/mL (Figure 5B).

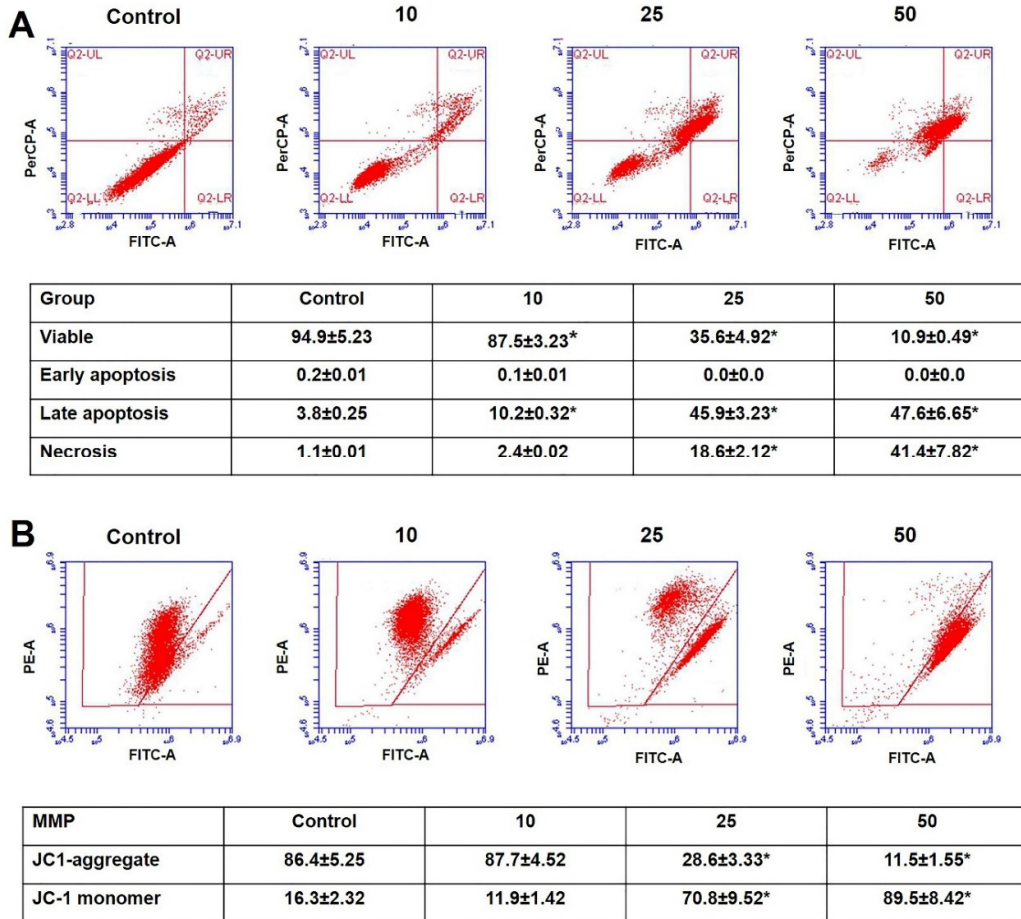


Figure 5. The effects of *P. indica* on apoptosis and mitochondrial function. The cancer cells were treated with *P. indica* (0-50 µg/mL), stained with Annexin V-FITC and PI for apoptosis (A), stained with JC-1 dye for mitochondrial membrane potential (B), and the measured by flow cytometry. Data represent mean±SE from three separated experiments. *p<0.05 vs. control.

P. indica effects on ROS formation

To measure the ROS formation effect of *P. indica* extract on cervical cancer HeLa cells and DCF-DA staining method was conducted. From the results obtained that the extract caused the stimulation of ROS generation starting from 10, 25, and 50 µg/mL extract in HeLa cells (Figure 6). The production of ROS generation maybe activated the cancer cells death and apoptosis.

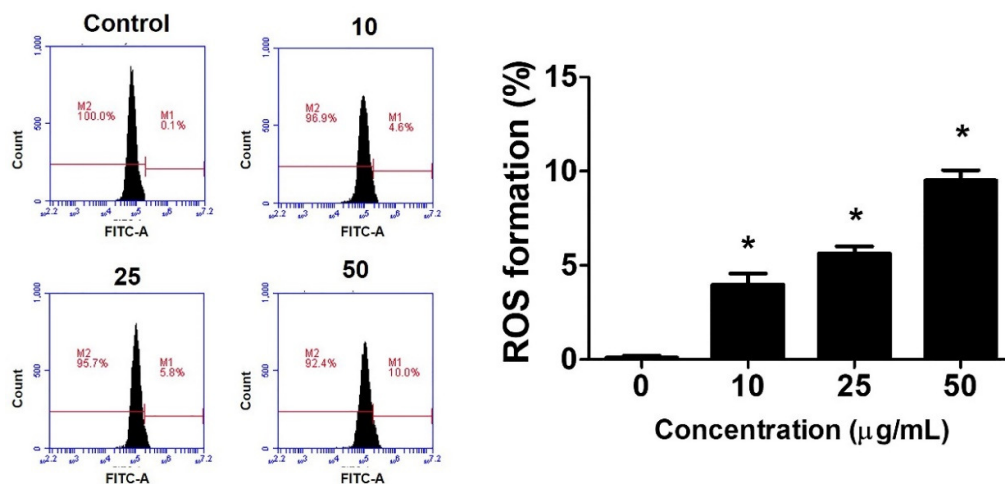


Figure 6. The effects of *P. indica* on ROS formation. The cancer cells were plated on 6-well plates for 24 h and then treated with various concentrations of *P. indica* for 24 h. Cells were trypsinized, incubated with 25 µM DCF-DA-fluorescent probe for 30 min, and the measured by flow cytometry. Data represent mean±SE from three separated experiments. * $p < 0.05$ vs. control.

Discussion

Several natural products have been reported with pharmacological activities including anticancer effects. *P. indica* is one of the Thai herbal medicine/traditional medicine that has been found to be a potent anticancer effects in *in vitro* studies; nevertheless, the information of *P. indica* mechanisms in HeLa cervical cancer is still less information. In this study, we needed to evaluate the *P. indica* root effects on human cervical cancer HeLa cells to suppress the viability, inhibit the migration, and activate the apoptosis. Based on the results obtained, *P. indica* extract has strongly effects on cervical cancer cells growth inhibition, migratory suppression, and apoptotic induction with a significant reduction of mitochondrial membrane potential and acceleration of ROS formation. Finally, *P. indica* may be represented as a novel source of anticancer agents for use in treatment cervical cancer.

In Thailand, *P. indica* is one of the active ingredients of Thai recipe of anticancer agents which showed high efficacy such as Benjakul (Itharat *et al.*, 2021). Therefore, the study of *P. indica* extract effects on anticancer effects were more interested because the data are still very less information in almost types of cancer cells. Confirmation with the other Plumbaginaceae family such as *Plumbago zeylanica* (*P. zeylanica*, known as jetamul-pleung-khao) which has showed the high levels of plumbagin related with high efficacy against cancer cells (Kapare *et al.*, 2020; Raja Sundari *et al.*, 2017). Both of *P. indica* and *P. zeylanica* may be useful for the cancer treatment in the further if they are confirmed by more scientific data support. The results of cytotoxic assay demonstrated that *P. indica* extract decreased melanoma cell lines proliferation by dose-dependent manner through downregulation of BCL-2 expression (Anuf *et al.*, 2014). In addition, these extract activated cell cycle arrest as shown in metaphase phase cells accumulation (Thitiorul *et al.*, 2013). Correlating with *P. zeylanica* results, the root extract of *P. zeylanica* stimulated the lung cancer cells death than stem and leaf extract with showing the low IC₅₀ value from 164.5 µg, 379.5 µg and 274.9 µg, respectively (Raja Sundari *et al.*, 2017). The root extract of Plumbaginaceae family has showed the highest of anticancer activity than the other parts, subsequently, the clinical use to treat the cancer may use the root to prepare the anticancer drugs recipe.

Plumbagin is the main active compound of *P. indica* and *P. zeylanica* and it has the strong anticancer effects in many aspects including growth inhibition, apoptosis induction, and migratory suppression. Several studies have been found that plumbagin suppressed cell migration and invasion of human glioma cells with low dose and the mechanism was reported via the suppression of the matrix metalloproteinase 2 and 9 (MMP 2 and MMP 9) (Chen *et al.*, 2017) activity and protein expression. With less data of *P. indica* on migratory suppression in cervical cancer cells. In this study, our results indicated that *P. indica* extract inhibited the HeLa cells migration by dose-dependent manner. Furthermore, we needed to explore the deeply mechanism of *P. indica* both *in vitro* and *in vivo* study against the cervical cancer cells before studying in clinical study. Metastasis is the major step of a tumor with poor clinical outcome (Fares *et al.*, 2020). Therefore, the natural products or herbal medicines which have the greatest the reduction of migration, may be useful for suppression of secondary tumor occurred including *P. indica*.

The mechanism of action of herbal medicine to inhibit cancer cell death and activate apoptosis were mentioned on ROS formation and mitochondrial dysfunction. ROS formation is a crucial role in activation of DNA damage and may lead to induce the cytotoxic effects in several cancer cells (Navaneetha Krishnan *et al.*, 2019). This study indicated that *P. indica* generated the ROS overproduction and may cause the reduction of mitochondrial function, subsequently, apoptosis induction was occurred. Similarly, plumbagin increased HeLa cells apoptosis via ROS induction and mitochondrial dysfunction, and further, the caspase 3 and caspase 9 were significant detected (Srinivas *et al.*, 2004). To prove the plumbagin-induced cancer cell death via the induction of ROS formation, the human breast cancer cell was pretreated with ROS scavenger, N-acetyl cysteine (NAC), and data demonstrated that plumbagin plus NAC significantly decreased the cancer cells death when compared with plumbagin treatment alone. Upregulation of ROS formation can lead to damage the mitochondria, destroy the mitochondrial DNA, decrease the mitochondrial respiratory chain, increase the membrane permeability, and interruption to Ca²⁺ homeostasis. After the loss of mitochondrial damage and then the cancer cells were activation to death and apoptosis (Guo *et al.*, 2013). In conclusion, *P. indica* root extract with high levels of plumbagin stimulated cervical cancer cells apoptosis via induction of induction of ROS production and mitochondrial dysfunction.

Conclusions

This study demonstrates that the ethanolic root extract of *P. indica* strongly inhibits cervical cancer cell proliferation, suppresses migration, and stimulates apoptosis through inducing mitochondrial dysfunction and activating ROS formation *in vitro* study. Therefore, *P. indica* is worthy to be further investigated and developed as herbal medicine for potential use in cervical cancer therapy.

Authors' Contributions

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by them. B.B. and W.P. conducted the experiments. B.B., P.W., and W.P. designed the experiments, analysed data and wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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