

Anti-migration and Anti-invasion Abilities of Methanolic Leaves Extract of *Clerodendrum Inerme* on Lung Cancer Cells

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ABSTRACT

Background: *Clerodendrum inerme* is a folk medicinal plant used in the treatment of various illnesses such as a cough, scrofulous infection, venereal infection, skin diseases and tumors. It exhibited many pharmacological effects including hepatoprotective, anti-inflammatory, anti-bacterial, anti-oxidant and anticancer properties. **Objective:** The purpose of this study was to investigate the influence of methanolic extract of *C. inerme* leaves on migration, invasion and adhesion activities on human lung adenocarcinoma. **Materials and methods:** Cytotoxicity, cell motility, migration, invasion and adhesion abilities were detected by MTT, wound healing, trans-well mobilization, modified Boyden chamber and cell adhesion assay, respectively. **Results:** The results demonstrated that up to 400 µg/mL methanolic leaves extract has low toxicity on A549 cells showing more than 50% cell viability. At non-cytotoxic and sub-toxic doses (200 and 400 µg/mL) of methanolic leave extract significantly suppressed cell motility, migration, invasion and cell adhesion compared with the untreated control. **Conclusion:** These results suggested that methanolic leaves extract of *C. inerme* inhibited migration, invasion and adhesion of A549 cells. These findings showed new therapeutic potential for *C. inerme* in anti-metastatic therapy.

Key words: *Clerodendrum inerme*, Anti-migration, Anti-invasion, Human lung adenocarcinoma.

INTRODUCTION

Lung cancer is the most common cancer types in men and fourth cancer in women. It is the major cause of cancer-related death in the world. There are about 1,350,000 newly developed cases and 1,180,000 death cases of lung cancer, globally. Lung cancer is divided into two major types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).¹ Non-small cell lung cancer (NSCLC) is the most common type that accounts for 80-90% of lung cancers, which is divided into 3 major pathologic subtypes including adenocarcinoma, squamous cell carcinoma and large cell carcinoma.² About 30-40% of patients with advanced NSCLC will develop metastasis, which is the lung cancer cells migrate to and invade to the distant organs, such as bones, brain and contralateral lung.³ Metastasized lung cancer is difficult for treatment because it is highly resistant to radiation and conventional chemotherapeutic agents. It is the main cause of cancer-related death in lung cancer patients.^{4,5} Thus, the potential therapeutic agent for treating lung cancer metastasis is an urgent therapeutic need.

Clerodendrum inerme is a medicinal plant in the family Verbenaceae. Its common names include glory bower, bag flower, and bleeding-heart.⁶ It is widely distributed in South and South-east Asia, Australia and Pacific islands.⁷ Different parts of *C. inerme* are used as a traditional medicine for the treatment of rheumatism, cough, scrofulous infection, venereal infection, skin diseases, beriberi, and tumors.⁸⁻¹⁰ The leaves of *C. inerme* are used for

treating fever, cough, skin rashes, chronic pyrexia and boils, and are used in conjunction with other plant leaves.¹¹ *C. inerme* exhibited many pharmacological effects including hepatoprotective,¹² anti-inflammatory,¹³ anti-bacterial,¹⁴⁻¹⁶ anti-oxidant,¹⁷ antidiabetic,¹⁸ antiparasitic,¹⁹ antiallergic,²⁰ and anticancer²¹⁻²⁴ activities. Recent studies showed that *C. inerme* leaves extract displays antiproliferation and anticancer activities on lung cancer cell line A549.^{25,26} However, the anti-migration, anti-invasion and anti-adhesion activities of *C. inerme* leaves extract on cancer cells have not been reported. Therefore, the present study investigated the inhibitory effect of methanolic leaves extract of *C. inerme* on cancer migration, invasion and adhesion in A549 cells.

MATERIALS AND METHODS

Chemical reagents

Dimethylsulfoxide (DMSO), trypan blue and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Phosphate buffered saline was purchased from Merck Millipore Corp (Darmstadt, Germany). Powdered Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco/BRL (Gaithersburg, MD, U.S.A). Matrigel matrix was purchased from Corning incorporated (Bedford, MA, U.S.A).

Collection of plant material and extract preparation

C. inerme leaves were collected from Pak-Poon Community, Muang District, Nakhon Si Thammarat

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Province. Fresh leaves were washed under running tap water and then dried for 36 hours using a hot air oven at 60°C. Dried leaves were crushed and subjected to extraction using the maceration process in methanol for 7 days at temperature. The extracts were collected and concentrated at 40°C under reduced pressure using a rotary evaporator. The dried extracts were stored at 4°C until further use.

Cell culture

The human non-small cell lung cancer (NSCLC) cell lines, A549 was obtained from the American Type Culture Collection (Rockville, MD). A549 cells were cultured in DMEM supplemented with 10% FBS (Life Science, Little Chalfont, UK), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Science, Little Chalfont, UK) at 37°C in a humidified atmosphere of 5% CO₂.

Cell viability assays

An MTT assay was carried out to determine the cytotoxic effect of methanolic extract of *C. inerme* leaves on A549 cells. The cells were plated in 96-well plate at a density of 7x10³ cells/well and treated with methanolic extract at different concentrations (0, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL) for 24 hours. After incubation, 0.5 mg/mL of MTT solution was added to each well and the plates were further incubated for 2 hours at 37°C. The supernatant was removed, and 100 µL of DMSO was added to solubilize the water-insoluble purple formazan crystals. The absorbance was measured using a Microplate spectrophotometer at 570 nm and cell viability percentage (%) was calculated relative to the control. IC₅₀ (inhibitory concentration 50%) value was calculated using the software GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA). Cell viability assay was performed with three independent experiments.

Wound healing assay

Wound-healing assay was performed to determine cell motility of A549. Briefly, the cells were seeded a density of 30x10⁴ cells/mL in 6-well plates until 90% confluence. Then the cells were scratched with a sterile 200 µL pipette tip to generate the wound area. Cellular debris was washed with phosphate buffer saline, and cells were treated with non-toxic concentrations of methanolic extract (0, 200 and 400 µg/mL) for 24 hours. The wound closure was observed and photographed at 24 hours with an inverted microscope. The wound area was measured by NIH ImageJ software (NIH, Bethesda, MD). The experiments were repeated three times.

In vitro migration assay

Effect of methanolic extract of *C. inerme* leaves on cell migration was assayed by 24 well transwell chambers. Cells were seeded in 35 mm³ dishes and treated with 0, 200 and 400 µg/mL for 24 hours. After incubation time, cells were harvested and seeded to the upper chamber of the transwell insert (polyethylene terephthalate (PET) filters with 8 µm pore size, corning life sciences) in serum-free medium. FBS medium as a chemoattractant was added to the lower chamber, and thereafter these transwell inserts were incubated for 24 hours at 37°C in 5% (v/v) CO₂. After incubation, the non-migratory cells were removed with a cotton swab and those on the lower surface of the membrane were fixed with methanol and stained with 0.5% crystal violet. Cells that migrate through the membrane were viewed and photographed under an inverted microscope. The percentage of the migratory cells for each treatment was calculated by NIH ImageJ software (NIH, Bethesda, MD).

Cell invasion assay

Effect of methanolic extract of *C. inerme* leaves on cancer cells to invade through membrane coated with 30 µg of Matrigel (Corning

incorporated, Bedford, MA, USA) was measured by Modified Boyden chamber assay.²⁷ A549 cells were pretreated with 0, 200 and 400 µg/mL of the extracts for 24 hours. Cells in serum-free medium were seeded onto the upper chamber and medium containing 10% FBS was added into the lower chamber as a chemoattractant. The Boyden chamber was incubated for 24 hours at 37°C. At the end of incubation, the cells in the upper surface of the membrane were removed with a cotton swab. Cells that invade across the Matrigel to the lower surface of the membrane were fixed with methanol, stained with 0.5% crystal violet and washed with PBS. The invading cells on the lower surface of the membrane filter were viewed and photographed under an inverted microscope. The data are presented as the percentage of cell invasion from five random fields.

Cell adhesion assay

Effect of methanolic extract of *C. inerme* leaves on adhesion ability of A549 cells was determined by 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay.^{28,29} Cells were pretreated with different concentrations of the extracts (0, 200 and 400 µg/mL) for 24 hours, then 1x10⁴ cells/well were seeded into the Matrigel-coated 96-well plate for 1 hour. After incubation time, the non-adherent cells were removed with PBS. The adherent cells were reacted with 0.5 mg/mL of MTT solution at 37°C for 2 hours. After that 100 µL of DMSO was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbance was measured at 570 nm using a microplate reader. The percentage (%) of cell adhesion was calculated relative to the control.

Statistical analysis

All data were edited and entered for the statistical analyses using the SPSS 17.0 software package. Data were reported as mean±standard deviation (±SD) of three independent experiments. Statistical significance was analyzed by the one-way ANOVA test. The differences between treatment groups and the untreated group were considered statistically significant at $p < 0.05$.

RESULTS

Cytotoxic effect of methanolic leaves extract on *C. inerme* on A549 cells

The cytotoxicity of methanolic leaves extract of *C. inerme* on A549 cells was determined by MTT assay and the results are shown in Figure 1. There was no significant effect on cell viability after treatment with methanolic leaves extract of *C. inerme* (6.25-200 µg/mL) but the high concentration, the extract significantly inhibited cell growth of A549 cells. The 20% inhibitory concentration (IC₂₀) and 50% inhibitory concentration (IC₅₀) of the extracts were greater than 400 µg/mL. The non-cytotoxic and sub-cytotoxic concentrations (200 and 400 µg/mL showing >80% cell viability) of methanolic leaves extract of *C. inerme* were used for subsequent experiments.

The effect of methanolic leaves extract on *C. inerme* on cell migration

The anti-migration effects of the methanolic leaves extract of *C. inerme* were determined by using wound healing and transwell chamber assay. The cell monolayers were scratched with a micropipette tip to create the wound area. After treatment with non-toxic concentrations of *C. inerme* leaves extract for 24 hours, cells migrated to the wound area and the wound closure was analyzed. The result showed that the methanolic leaves extract of *C. inerme* could reduce migration of A549 cells to the wound area in a dose-dependent manner when compared with the untreated cells (Figure 2A and 2B). Treatment with methanolic

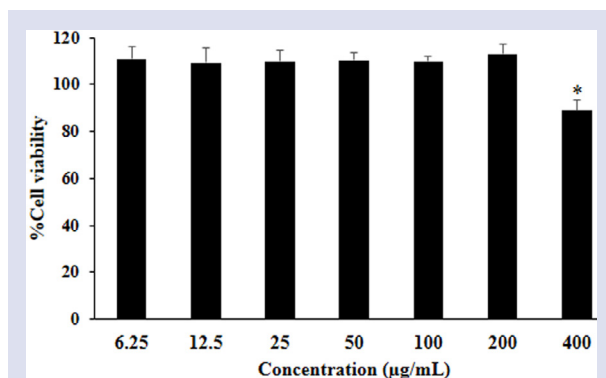


Figure 1: Cytotoxicity of methanolic leaves extract of *C. inerme* on A549 cell lines. A549 cells were treated with methanol leaves extract of *C. inerme* at different concentrations for 24 hours. The non-cytotoxic and sub-cytotoxic concentrations (lower than 400 µg/mL showing >80% cell viability) were selected for the subsequent experiment.

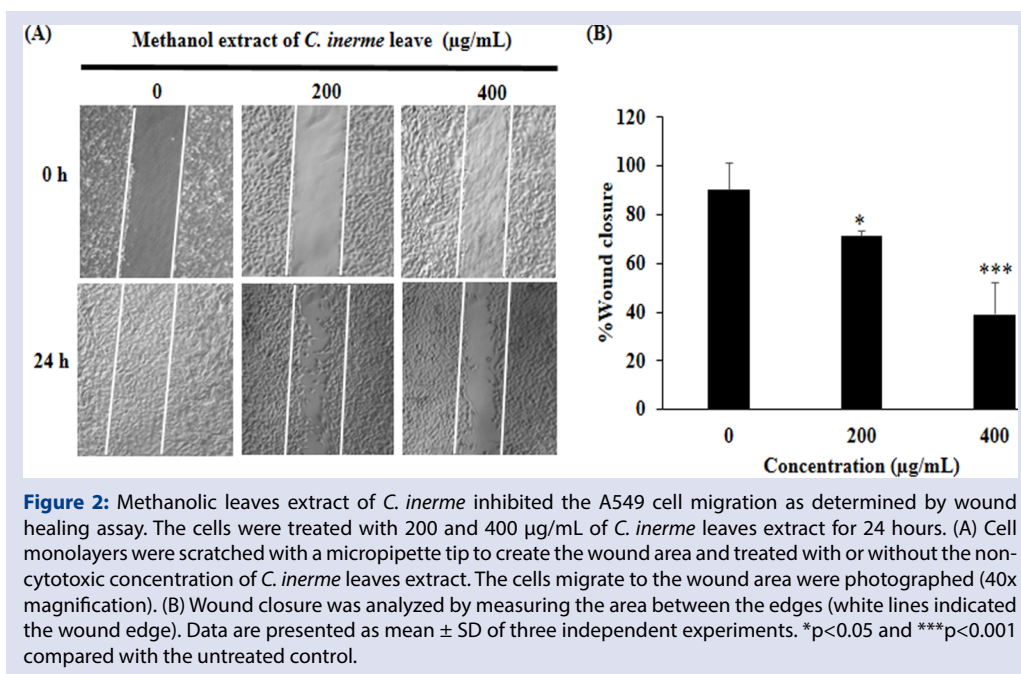


Figure 2: Methanolic leaves extract of *C. inerme* inhibited the A549 cell migration as determined by wound healing assay. The cells were treated with 200 and 400 µg/mL of *C. inerme* leaves extract for 24 hours. (A) Cell monolayers were scratched with a micropipette tip to create the wound area and treated with or without the non-cytotoxic concentration of *C. inerme* leaves extract. The cells migrate to the wound area were photographed (40x magnification). (B) Wound closure was analyzed by measuring the area between the edges (white lines indicated the wound edge). Data are presented as mean \pm SD of three independent experiments. * $p < 0.05$ and *** $p < 0.001$ compared with the untreated control.

leaves extract of *C. inerme* at 200 and 400 µg/mL inhibited $71.1 \pm 1.91\%$ and $39.2 \pm 12.67\%$ of cell migration, respectively. Furthermore, the extract also significantly suppressed cell migration as detected with the transwell chamber as shown in Figure 3A and 3B (** $p < 0.01$). These results indicated that methanolic leaves extract of *C. inerme* significantly inhibited cell migration in A549 cells.

The effect of methanolic leaves extract on *C. inerme* on A549 cell invasion

Matrigel-coated Boyden chamber assay was performed to investigate the anti-invasion of A549 by methanolic leaves extract on *C. inerme*. The invaded cells were stained by crystal violet and the percentage of invaded cells was analyzed by NIH Image]. The result demonstrated that methanolic leaves extract of *C. inerme* significantly suppressed A549 cell invasion compared with the untreated control (** $p < 0.01$) as shown in Figure 4A and 4B. The percentage of invaded cells was $26.20 \pm 4.40\%$ when treated with the extract at the concentration of 400 µg/mL. This result indicated that methanolic leaves extract of *C. inerme* significantly inhibited cell invasion in A549 cells.

Effect of methanolic leaves extract on *C. inerme* on cell adhesion

Effect of methanolic leaves extract on *C. inerme* on cell adhesion was performed by MTT assay. The result demonstrated that methanolic leaves extract of *C. inerme* could inhibit cell adhesion ability of A549 to Matrigel-coated wells at $29.25 \pm 4.17\%$ after treatment with 400 µg/mL of the extract as shown in Figure 5. This result indicated that the methanolic extract of *C. inerme* leaves significantly inhibited the adhesion ability of lung cancer cells

DISCUSSION

Metastasis is the major problem for cancer treatment and is responsible for more than 90% of all cancer-related deaths. Cancer metastasis consists of multi-step and multifunctional biological events which include cell detachment, migration, invasion and adhesion.³⁰ At the earliest stage, cancer cells disseminate from the primary tumor, migrate and invade through the basement membrane, survive in the circulatory system, invade into a secondary site, then adhesion and

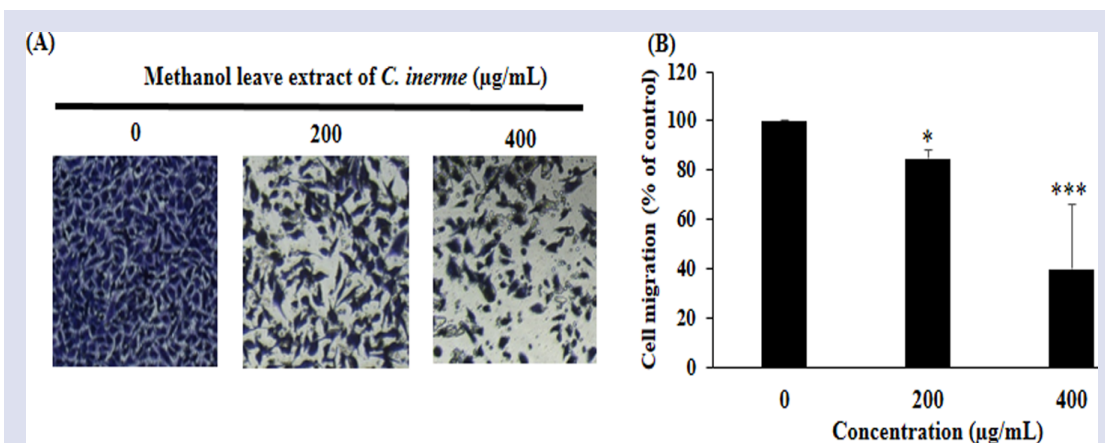


Figure 3: Methanolic leaves extract of *C. inerme* inhibited the A549 cell migration as determined by the transwell chamber assay. The cells were treated with 200 and 400 µg/mL of *C. inerme* leaves extract for 24 hours. (A) Methanolic leaf extract of *C. inerme* also significantly suppressed cell migration as detected with the transwell chamber. The migratory cells were stained with crystal violet and photographed (40x magnification). (B) Quantitative analysis of the migratory cell was calculated by NIH ImageJ. Data are presented as mean ± SD of three independent experiments. *p<0.05 and ***p<0.001 compared with the untreated control.

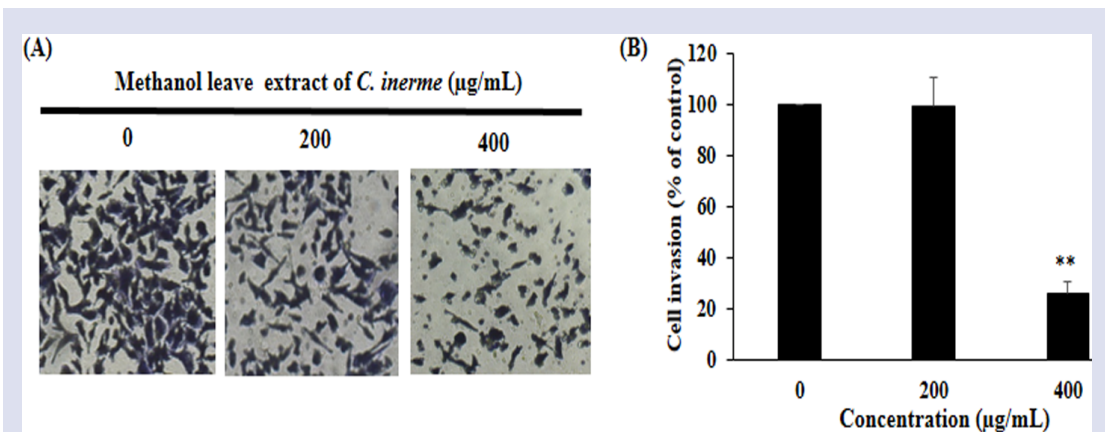


Figure 4: Methanolic leaves extract of *C. inerme* inhibited A549 cell invasion as tested by Matrigel-coated Boyden chamber assay. The cells were treated with 200 and 400 µg/mL *C. inerme* leaves extract for 24 hours. (A) The invaded cells were stained with crystal violet and (B) the percentage of invaded cells showed significantly decreased after treating with methanolic leaves extract of *C. inerme*. Data are presented as mean ± SD of three independent experiments. **p<0.01 compared with the untreated control group.

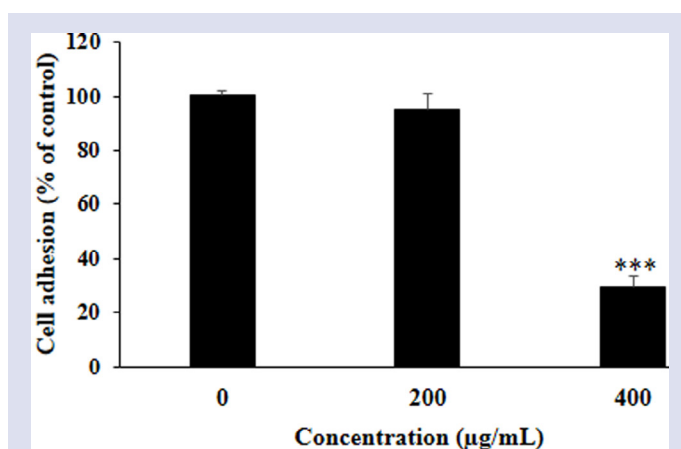


Figure 5: Effect of methanolic leaves extract of *C. inerme* on cell adhesion at 24 hours. The adhesion ability of A549 cells to Matrigel-coated wells was markedly inhibited by methanolic leaves extract of *C. inerme* at a concentration of 400 µg/mL as compared with the untreated control group. Data are presented as mean ± SD of three independent experiments. ***p<0.001 compared with the control group.

start to proliferate.^{31,32} Disruption of any metastatic steps is a target for preventing the development of cancer metastases. Herein, we first demonstrated that the methanolic leaves extract on *C. inerme* inhibited lung cancer cell metastasis by suppressing cell growth, migration, and invasion and adhesion abilities of A549 cells. Before determination of cell migration, the cytotoxicity test was performed to determine a range of crude extract concentrations that provide more than 80% cell viability. We found that 6.25-200 µg/mL methanolic leaves extract of *C. inerme* showed no significant effect on cell viability but the high concentration, the extract significantly inhibited cell growth of A549 cells. The IC_{20} and IC_{50} were greater than 400 µg/mL. This value was higher than the previous studies that showed ethanol leaves extract and hydroalcoholic aerial parts *C. inerme* exhibited anti-proliferation and anti-cancer activities on lung cancer cell line with IC_{50} values at 15.6 µg/mL and 259.5 µg/mL, respectively.^{25,26} To determine the anti-metastatic effects of the extract, the non-toxic concentrations at 200 and 400 µg/mL and allowing over 80% cell survival were selected. Cell migration and invasion are important steps for the metastasis of cancer cells. The ability of cancer cells to migrate and invade other tissues finally lead to metastasis. In this study, we demonstrated methanolic leaves extract of *C. inerme* significantly suppressed cell migration and invasion of A549 cells in a dose-dependent manner when compared with the untreated cells (** $p < 0.001$). Moreover, we found that the methanolic leaves extract of *C. inerme* could inhibit cell adhesion ability of A549 after treatment with 400 µg/mL of the extract as shown in Figure 5. These results indicated that the methanolic left extract of *C. inerme* inhibits cell metastasis of the A549 human lung without apparent cytotoxicity. Several phytochemical constituents have been isolated from *C. inerme* leaves such as alkaloids, flavonoids, terpenes, diterpenes, triterpene

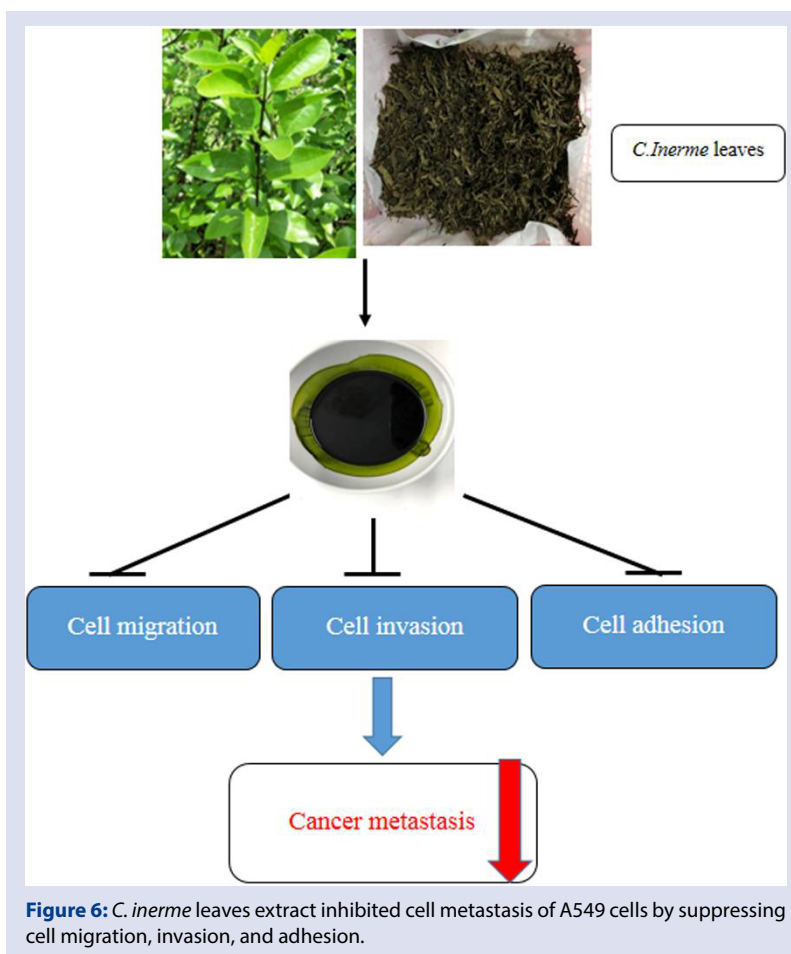
which possess many mechanisms of actions such as anti-cancer, anti-proliferation, anti-metastasis, anti-angiogenesis, anti-oxidation activities, cell cycle arrest and induction of apoptosis.³³ Shen and colleagues demonstrated that acacetin, a flavonoid, inhibits the invasion and migration of human prostate cancer DU145 cells via inactivation of the p38 MAPK signaling pathway.³⁴ Previous reports demonstrated that fatty acids extracted from *C. volubile* leaves could suppress tumor metastasis and invasion in human neuronal glioblastoma cells.³⁵ and breast cancer cells.³⁶ Although the mechanism underlying anti-migration, anti-invasion and anti-adhesion effects are still unclear, the result demonstrated that *C. inerme* methanolic leaves extract could suppress cancer metastasis of lung cancer cell lines. Further study needs to focus on the mechanism involving anti-metastasis and the major active components both in vitro and in vivo for developing a novel chemotherapeutic agent in the future

CONCLUSION

In the present study, we first demonstrated that methanolic leaves extract of *C. inerme* inhibited cell migration, invasion and adhesion of A549 cells (Figure 6). These findings indicated that methanol leave extracts of *C. inerme* may be used as an anti-metastatic agent for lung cancer. However, further biochemical researches are needed to isolate and identify the active compounds responsible for this pharmacological activity.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

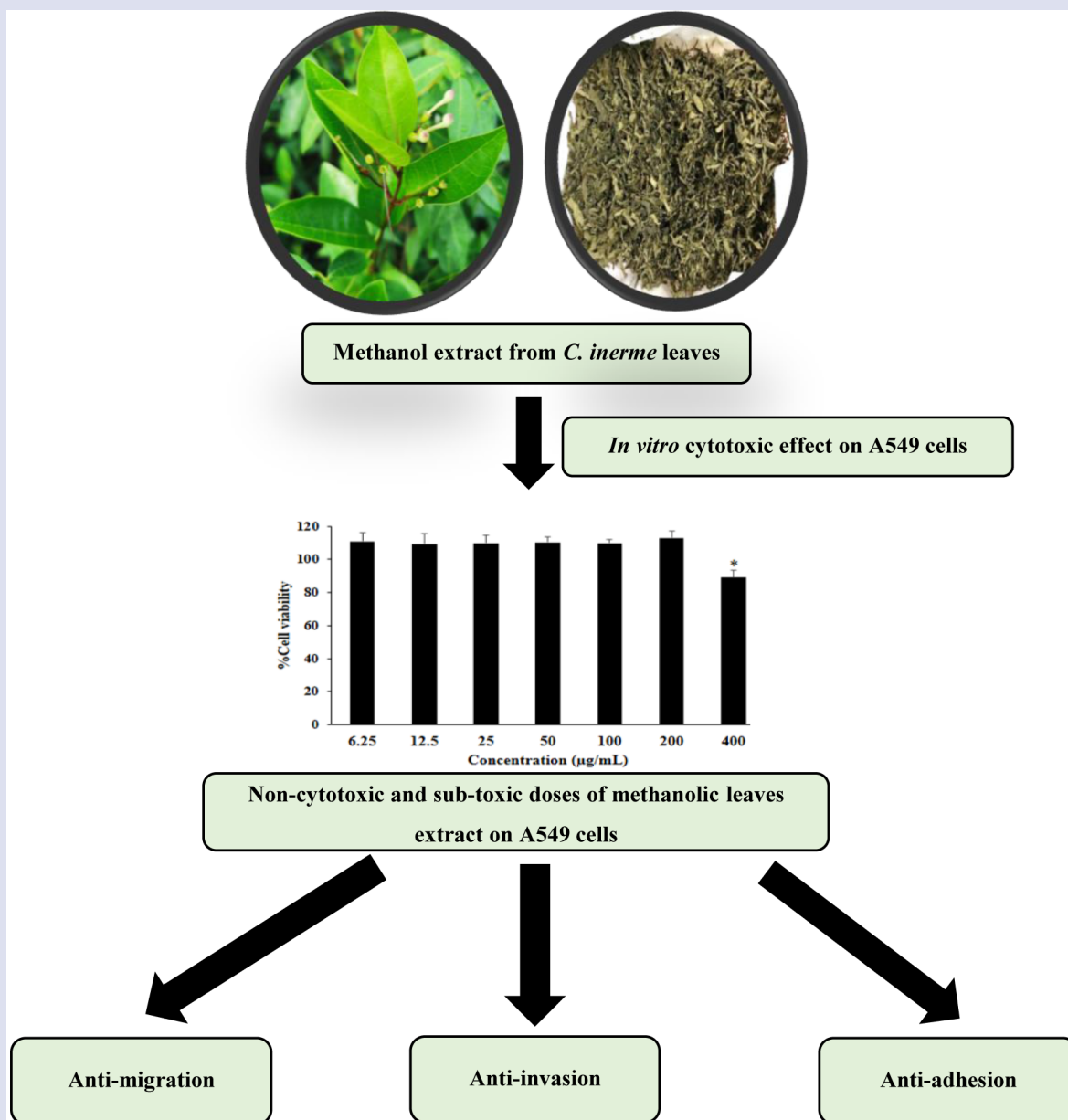
ABBREVIATIONS

MTT: 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyltetrazolium bromide; **IC₂₀**: The 20% inhibitory concentration; **IC₅₀** 50% inhibitory concentration; **CO₂**: Carbon dioxide; **FBS:** Fetal bovine serum.

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GRAPHICAL ABSTRACT



ABOUT AUTHORS



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Dr. Poonsit Hiransai: Assistant Professor in Medical Technology at School of Allied Health Sciences, Walailak University, Thailand, and recognized the Senior Fellowship by Advanced Higher Education. His current research interests focus on issues in immuno-modulation, cell signaling transduction, and natural products.



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