In vitro assessment of *Macleaya cordata* crude extract bioactivity and anticancer properties in normal and cancerous human lung cells

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**A B S T R A C T**

The purpose of this study is to assess the bioactivity and anticancer properties of *Macleaya cordata* crude extract in vitro using normal fetal lung fibroblast MRC5 and adenocarcinomic epithelial cell AS49 as model systems. Treatment of extract induced cell detachment, rounding, and irregularity in shape, in both normal and adenocarcinomic human lung cells, in accompanied of significant reduction in cell proliferation. The data indicated that necrosis appeared to be involved in compromising cell growth in both types of lung cells since membrane permeability and cell granularity were elevated. Although apoptosis was evident, the responses were differential in normal and diseased lung cells. Viability of treated MRC5 cells was reduced in a dose-dependent manner, demonstrating that the normal lung cells are sensitive to the extract. Surprisingly, AS49 viability was slightly elevated in response to extract exposure at low concentration, implying that cells survived were metabolically active; the viability was reduced according to the findings of the study. The present findings demonstrate that the crude extract of *M. cordata* contains agents affecting the functioning of normal and diseased lung cells in vitro. The observed cytotoxic effects against adenocarcinomic lung cells validate the potential of using *M. cordata* as herbal intervention in combined with conventional chemotherapy for lung cancer treatment.

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1. Introduction

*Macleaya cordata* (Willd.) R. Br. is a deciduous perennial plant in the family Papaveraceae, which flowers from July to August. The plant is found almost anywhere in temperate North America east of the Mississippi River at elevations below 1000 m sea level and is native to temperate eastern Asia (e.g. China and Japan). *M. cordata* contains quaternary benz[\(c\)]phenanthridine alkaloids (QBA) and protopine type alkaloids, which are important classes of alkaloids in the isoquinoline alkaloids family. Literature reports that the fraction of QBA and protopine type alkaloids found in *M. cordata* are mainly composed of sanguinarine (SG), chelerythrine (CH), protopine (PR), and allocryptopine (AL) (Kosina et al., 2010). In China, *M. cordata* is a wild plant and commonly distributed in Shanxi, Guizhou, Yunnan provinces. This plant was first described as a medicinal plant in Ben Cao Shi Yi (“A Supplement to Materia Medica”), which is one of the most important Chinese medical material books written by Chen Can-Qi. *M. cordata* was first recorded in this book and prescribed for external use only since the plant was thought to be highly poisonous. Historically, the plant was used as a common medication to temporarily relieve muscle pain and to treat inflamed wounds, as well as bee sting. In the modern days, *M. cordata* is extensively used in traditional Chinese medicine for the treatment of wound, arthritis, rheumatism arthralgia, and trichomonas vaginalis (Jiangsu, 1986). *M. cordata* is also a traditional medicinal plant in North America, Europe to manage insect bites (Grieve, 1984) and ringworm infection (Duke and Ayensu, 1984). As a folk medicine, the roots of *M. cordata* are given to cancer patients as an alternative therapy in China (Xu et al., 1997). Experimental studies show that this plant has a wide spectrum of biological activities such as anti-microbial (Walterova et al., 1995), anti-fungal (Newman et al., 1999), pesticidal (Yang, 2003), anti-inflammatory, as well as anti-tumor properties (Pang et al., 2005). *M. cordata* is on the European Food Safety Authority (EFSA) list of plants exploited as a component in feed additives in animal production (Franz et al., 2005), and the powdered mixture of leaves, capsules, and seeds is the main ingredient of the feed additive Sangrovit for rearing pigs, broilers and dairy cattle (Psotova et al., 2006; Stiborova et al., 2008; Vieira et al., 2008). This commercially available product has been reported to increase intestinal secretion of enzymes in livestock and possess bacteriostatic, mucolytic and anti-inflammatory effects (Lindermayer, 2005). As reported in safety assessment studies, administration of *M. cordata* extract to
experimental animals did not affect animal's body weight, organ weight, organ morphological structure, hematological parameters, as well as oxidative stress parameters (Zdarilova et al., 2008). The extract was also demonstrated to be non-genotoxic in model animals (Psotova et al., 2006; Stiborova et al., 2008). Moreover, rats with acute hepatic injuries caused by tetrachloromethane or galactosamine not only showed improved liver and lymphocyte functions but also reduced mortality following the treatment of M. cordata extract (Yang et al., 1999), in support of the use of Sangrovit in live stock. However, cytotoxicity to human and porcine hepatocytes was reported when cells were exposed to the extract in vitro (Ulrichova et al., 2001). Elevation in the expression of two apoptotic markers, Bcl-2 and Bax proteins was evident in cultures of rodent myocardial cells (Zhang et al., 2009). In China and Russia, M. cordata is widely cultivated and used as a primary source for the production of QBA, such as sanguinarine and chelerythrine. These two alkaloids are most well studied QBAs found in this plant, and the components are reported to be the main active ingredients responsible for the reported pharmacological effects (Franz et al., 2005). A prior study showed that sanguinarine and chelerythrine have inhibitory effect on the activity of rat liver t-alkaline-2-oxoglutarate aminotransferase, which is essential in regulating cellular metabolism (Walterova et al., 1981). Interestingly, these two QBAs impaired the functioning of aminopeptidase N and dipeptidyl peptidase IV, which are enzymologically important in the processes of cell activation and differentiation (Sedo et al., 2002). The observed inhibitory effects were so potent that the efficiency was comparable to that of aminopeptidase N inhibitors Bestatin and Amastatin.

Herbal products and other supplements derived from plants are widely consumed in many countries. The use of herbal intervention is common in all regions of the developing world and rapidly growing in developed countries (Cassileth, 1999; Cassileth and Vickers, 2005). The use of substances with natural origin as medicines, also known as phytotherapy or complementary and alternative therapy (CAM), are sometimes practice along with conventional western medicine as an alternative therapy to treat diseases such as cancers. The most renowned example is the use of taxane, also known as paclitaxel (taxol), which is a mitotic inhibitor used in cancer chemotherapy. The product was discovered in United States in 1967 by Monroe E. Wall and Mansukh C. Wani, and was isolated from the bark of the Pacific yew tree, Taxus brevifolia. With phase II efficacy testing in progress, taxol has been demonstrated clinically to be effective in treating patients with ovarian carcinoma (Burges and Schmalfeldt, 2011) and has some degree of curative activity against non-small cell lung cancer and melanoma (Clegg et al., 2002; Silchenmyer and Von Hoff, 1990).

Cancer is a major worldwide health problem due to the lack of comprehensive early detection methods and effective treatment. Normally, cancer is treated with chemotherapy, radiation therapy and/or surgery. The chances of surviving vary greatly by the type and location of the cancer and the extent of disease at the time of diagnosis. Lung cancer is one of the most lethal cancers in terms of mortality and incidence worldwide and this malignancy accounts for approximately one sixth of all cancer deaths globally. Lung cancer classification can be described into two histological types: non-small cell lung cancer (NSCLC; 80% of diagnosed cases) and small cell lung cancer (SCLC; 20% of diagnosed cases). For most patients with non-small cell lung cancer, surgical resection with curative intent is normally the primary treatment since this type of cancer is relative insensitive to chemotherapy and/or radiation (Kumar et al., 2005). In 2002, greater than 1.3 million individuals were diagnosed with lung cancer worldwide, with the same number of deaths at approximately 1.2 million (Parkin et al., 2005). Recently, research focused on the use of products with natural origin for treating this malignancy has offered possible alternatives for cancer patients; CAM has acquired a growing recognition among scientific field and the acceptance of herbal medicine as an therapeutic option by the West is now emerging. Historically, medicine derived from several herbal plants such as Platycodon grandiflorum (Campanulaceae), Morus alba (Moraceae), Punusarmenica (Rosaceae) and Rhus verniciflua (Anacardiaceae), Perilla frutescens (Labiatae), Stenoma japonica (Stemonaceae), Tussilago farfara (Compositae) and Draba nemorosa (Brassicaceae) have been frequently used for lung diseases, including cancer, as folk remedies and medicines (Jeong et al., 2011). Generally, anti-cancer phytochemicals exhibited their pharmacological effects by inducing apoptosis and/or inhibiting angiogenesis and metastasis by targeting molecules modulating these biological processes. However, a proper in vitro assessment of any potential phytochemical agents using both normal and diseased model systems should be done first to correctly identify any curative properties and possible side-effects of tested substances in initial attempts. Perturbation in cell proliferation and metabolic activities in both normal and malignant tissues caused by the administration of tested anti-cancer agents should also be carefully examined to better predict the therapeutic outcomes.

The development of anti-cancer agents is attractive for lung cancer therapy. Based on the preceding views of application of natural products as alternative therapy, it is to our great interest to discover new plants and of which the extract with pharmacological potential, especially in treating lung cancer. In the present study, normal human fetal lung fibroblast cell line MRC5 and adenocarcinomic human alveolar basal epithelial cell line A549 with NSCLC origin were exposed to the crude extract of M. cordata and the effects on cell proliferation, viability, metabolism, as well as cell death were assessed in vitro.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media, fetal bovine serum (FBS), 0.05% trypsin–EDTA, glutamine, Hank's balanced salt buffer (HBSS), and chemicals used to make all media were purchased from Hyclone (Logan, UT). Trypan blue solution and propidium iodine were purchased from Sigma Chemical Company (St. Louis, MO). 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) was purchased from Biobasic, Inc. (Canada, Ontario).

2.2. Preparation of plant crude extracts

Fresh whole plants were collected in Hsin-Chu county (24°36′39″N, 121°18′1″E, above sea level 1100 m) based on the geographic information provided by voucher specimen (B3382) kept in the Herbarium of Academia Sinica, Taiwan. Specimens were authenticated by Professor Meng-Huai Su, taxonomist at the Department of Forestry and Natural Conservation, Chinese Culture University, Taiwan. All samples were stored at −80 °C prior to plant extraction. To perform crude extraction, plant materials were cleaned with nanopure water to remove adulterant first. Leaves (10 g) were pulverized and mechanically squeezed to collect crude extract (3.43 g) on ice. Collected crude extract was immediately sterilized using 0.2 μm syringe filters for all in vitro experiments. For long-term storage, the filtrate was aliquoted and storage at −80 °C.

2.3. Cell culture

Normal human fetal lung fibroblast cell line MRC5 and adenocarcinomic human alveolar basal epithelial cell line A549 were purchased from Taiwan Bioresource Collection and Research Center. In brief, MRC5 and A549 were passaged in Eagle's Minimum
Essential Medium (MEM) and F-12K medium, respectively. All media were supplemented with 10% (v/v) FBS and 1% glutamine (v/v) and cultures were maintained at 37 °C with 5% CO2 in a humidified atmosphere. To test the effects of crude extract of *M. cordata* on human lung cells, both A549 and MRC-5 cultures were initiated with $5 \times 10^3$ to $1 \times 10^5$ total viable cells and the formations of monolayer cells were visually confirmed using Olympus IX71 inverted phase contrast microscope the next day prior to addition of test solutions. For normal fetal lung fibroblast cell line MRC5, experimental groups were treated with 0.5% and 2.5% of crude extract. For alveolar basal epithelial cell line A549, 0.5%, 2.5%, and 10% of crude extract were added to the cultures. All control groups were not exposed to *M. cordata* extract.

2.4. Cell proliferation assay

After 3 days (72 h) of exposure to *M. cordata* crude extract, cell morphology was examined using inverted microscope and images were recorded. To assess cell proliferation, cultures were trypsinized with 0.05% trypsin–EDTA for 5 min at 37 °C and detached cells were centrifuged at 12,000 $\times$ g for 5 min to collect cell pellets. Cells collected from each culture were enumerated using hemocytometer by trypan blue exclusion assay.

2.5. Assessment of cell death (necrosis and apoptosis)

Cells from control and experimental cultures treated with or without *M. cordata* crude extract were collected and washed in HBSS, supplemented with 10 mM HEPES and 1% FBS (HBSS-H/10%). Washed samples were stained with 0.1 μg/ml propidium iodine (PI), membrane impermeable nucleic acid solution with excitation/emission wavelength at 488 nm/617 nm, for 5 min at room temperature to assess necrosis. Stained samples were analyzed with a BD FACScalibur flow cytometer (Beckton-Dickinson, San Jose, CA, USA). Parameters such as forward scatter (FSC) that correlates with cell size and side scatter (SSC) that correlates with cell granularity were acquired for each sample. Apoptosis was assessed flow cytometrically by the binding of Annexin V to phosphatidylserine on the cell surface in early apoptosis process. TACTM Annexin V kit was purchased from Trevigen (Gaithersburg, MD, USA) to detect apoptotic cells. In brief, collected cells were washed once in cold phosphate buffer saline and reconstituted in same buffer. Samples were then stained using Annexin V–FITC for 15 min at room temperature according to manufacturer’s suggestions. Cells were analyzed using BD FACScalibur flow cytometer.

2.6. 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate (WST-1) assay

Cell viability and metabolic activity was assessed by WST-1 method. In brief, the assay is based on the cleavage of tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenase. Expansion in the number of viable cells, as well as increase in metabolic activity, resulted in an elevation in the activity of this enzyme, which leads to the increase in the amount of formazan dye formed. Human lung cells treated with or without *M. cordata* crude extract were cultured in Thermo Fisher Scientific Nunc 96-well flat bottom plate (Roskilde, Denmark) with 100 μL of culture medium. Three day after exposure, each culture was added 10 μL of WST-1 solution (Biovision, Mountain View, CA, USA) and incubated for 4 h under standard culture conditions. The amount of dye generated in each sample was quantified by multi-well Thermo Scientific spectrophotometer (Waltham, MA, USA) by measuring the absorbance at 440 nm. Viability was defined as the ratio of absorbance of treated cells to untreated cells and data were expressed as percentage.

2.7. Statistical analysis

Results were expressed as median percentage ± SEM. Each experiment was repeated at least three times in duplicates or triplicates. All percentage data were analyzed statistically using a one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test when results of the ANOVA were significant. In both the ANOVA and Dunnett’s test, results were considered significant when $p \leq 0.05$.

3. Results

3.1. Effects of *M. cordata* crude extract on cell proliferation and growth

Recently, isoquinoline alkaloids found in *M. cordata*, such as sanguinarine, chelerythrine, protopine, and allocryptopine, have been implicated for their potential usefulness as complementary and alternative medicine in cancer therapy. Therefore, it was to our great interest to assess bioactivity of *M. cordata* crude extract against human lung cells in vitro. In the initial series of experiments, we used normal human fetal lung fibroblast cell line MRC5 to evaluate whether *M. cordata* crude extract induces any morphological changes. In vitro cultured MRC5 cells were exposed to 0.5% and 2.5% of extract for three days and general cellular morphology were examined. In control groups, MRC5 cells grew in a uniform adherent monolayer with normal fibroblast or epithelial cell shape (Fig. 1A). In contrast, treatment of 0.5% and 2.5% of *M. cordata* crude extract induced cell detachment from the culture dish surfaces and cultures exhibited cell rounding and irregularity in shape. To determine whether the morphological alternation were accompanied by changes in cell proliferation, cells harvested from control and experimental groups were enumerated. All cultures were initiated with $1 \times 10^5$ total cells and an average of 6.3 $\times 10^5$ MRC5 cells were harvested in control groups on day 3 (Fig. 1B). A significant decrease in total cell number was noted in cultures exposed to crude extract when compared to that of control group (0.5%: 4.3 $\times 10^5$ total cell; 2.5%: 1.7 $\times 10^5$ total cell). Our proliferation analysis revealed administration of 0.5% and 2.5% of *M. cordata* crude extract compromised normal human lung cell growth by 32% and 72%, respectively.

To evaluate whether *M. cordata* leave crude extract is cytotoxic to diseased lung cells, adenocarcinomic human alveolar basal epithelial cell line A549 was used for the following experiments. Initially, in vitro cultured A549 cells were exposed to 0.5% and 2.5% of extract for 72 h and general cellular morphology was examined. When cultures were maintained in the absence of *M. cordata* crude extract, a uniform adherent monolayer of A549, with normal fibroblast or epithelial cell shape, was formed (Fig. 2A). In contrast, exposure to 0.5% and 2.5% of extract induced cell detachment from the culture dish surfaces, as well as cell rounding and irregularity in shape. To evaluate whether *M. cordata* crude extract adversely affects cell proliferation of adenocarcinomic A549 cells, total number of cells harvested at the end of experiment was enumerated for both control and treated groups on day 3. All cultures were initiated with $5 \times 10^4$ cells and an average of 4.8 $\times 10^5$ cells were harvested from the control groups (Fig. 2B). A significant decrease in total cell number was evident in cultures exposed to 0.5% and 2.5% of crude extract (0.5%: 3.4 $\times 10^5$ total cell; 2.5%: 1.4 $\times 10^5$ total cell), representing a 30% and 71% of reduction in cell proliferation when compared to that of control group. Interestingly, 98% reduction in cell number was noted in groups exposed to 10% of crude extract (Fig. 3B), which is indicative of a complete retardation in cell growth and an induction of cell death since the final harvest cell number (1.1 $\times 10^4$) was much less than the initial seeding cell.
Severe cell rounding and irregularity in cell shape were also noted in these cultures (Fig. 3A).

3.2. Effects of *M. cordata* crude extract on cell death

Our data showed that the growth of both normal and adenocarcinomic human lung cells were adversely compromised by *M. cordata* crude extract, supported by the observation that less number of cells were harvested from treated cultures than that of control group. We postulate that cell death via apoptosis and/or necrosis, instead of cell proliferation inhibition, is the main mechanism responsible for the finding. Generally, necrotic cells are characterized with the loss of plasma membrane integrity and cell swelling due to inability of regulating ion homeostasis. The process is also accompanied by disintegration of organelles, in result of increase in cellular granularity. Apoptosis, in contrast, is a mode of programmed cell death, which is evident of translocation of phosphatidylserine (PS) from the cytoplasmic to the extracellular side of the membrane. Therefore, necrosis is normally detected using membrane impermeable nucleic acid probe since plasma membrane of necrotic cells is damaged; apoptosis can be readily identified with Annexin V, a phospholipid protein with high affinity for PS exposed on the outer membrane of apoptotic cells. Both assays can be carried out and analyzed using flow cytometric methods. To determine whether *M. cordata* crude extract induces cell death in human lung cells, MRC5 and A549 cells cultured in the absence or presence of crude extract at various final concentrations for 72 h were subject to membrane impermeable nucleic acid stain propidium iodine and Annexin V. Both control and experimental samples were analyzed using BD FACS Calibur flow cytometer.

![Image](https://example.com/image.png)
In addition, cellular status such as cell size and granularity were also assessed by forward scatter (FSC) and side scatter (SSC) parameters. In the initial series of experiments, we noted that administration of 0.5% *M. cordata* crude extract induced an increase in the median intensity of PI stain in normal human MRC5 lung cells (control: 10.2 vs. 0.5%; 36.7), demonstrating the attribution of necrosis in cell growth impairment in treated groups (Fig. 4A and B). This finding was supported by the observation that granularity of cells from cultures treated with crude extract was elevated (control: 403 vs. 0.5%; 532), although cell size (control: 561 vs. 0.5%; 570) was similar (Fig. 4D and E). When cells were exposed to a higher concentration of crude extract (2%), only limited number of cells with high PI stain were available for analysis (25.2; Fig. 4C). Interestingly, cell size and granularity (305/268; Fig. 4F) of cells treated with crude extract at this particular concentration decreased dramatically when compared to that of control, suggesting cells were dead under this condition. Whether cell death is due to necrosis could not be determined due to the limitation of this assay. Next, treatment of 0.5% of *M. cordata* crude extract to adenocarcinomic A549 cells also resulted in an increase in median intensity of PI stain (control: 8.62 vs. 0.5%; 12.3), demonstrating cell proliferation was impeded by necrosis (Fig. 5A and B). This conclusion was supported by the observation that cells were enlarged (control: 366 vs. 0.5%; 463), although cellular granularity (control: 260 vs. 0.5%; 288) was only increased slightly (Fig. 5E and F). Co-cultured with 2.5% of crude extract, A549 cells exhibited higher retention of PI stain and the median PI intensity (control: 8.62 vs. 2.5%; 34.5) increased than 4-fold (Fig. 5A and C). The median forward scatter

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**Fig. 2.** Human adenocarcinomic alveolar basal epithelial cells A549 cultured in the presence or absence of *Macleaya cordata* crude extract. (A) Light micrographs of control and *M. cordata* extract treated A549 cells on day 3. The experimental groups were exposed to 0.5% or 2.5% of extract for total 72 h and cell detachment from the culture dish surfaces, cell rounding, and irregularity in shape were observed (shown with arrowheads). Control groups were not exposed to the crude extract. Images were taken at 100× and 200× magnifications. Scale bar = 100 μm. (B) A549 cultures were initiated with 5 × 10⁴ cells in the absence or present of *M. cordata* extract at a final concentration of 0.5% or 2.5%. The value shown in the parenthesis represented the reduction in total cell number compared to that of control group expressed in percentage. Results were expressed as total cell number (×10⁵) ± SEM on day 3. Statistical differences between control and treated groups were determined using ANOVA followed by Dunnett’s post hoc test when results of the ANOVA were significant (p < 0.05). Experimental data sets that were significantly different from that of control group were labeled with an asterisk (*).
and side scatter values were elevated ~1.3- and 2-fold, respectively (control: 366/281 vs. 2.5%: 469/525), indicating cells were larger in cell size and showed higher degree of cell granularity (Fig. 5E and G). When cultures were administrated with crude extract at the highest concentration (10%), only small number of cells with high PI stain were available for analysis and most harvested cells were high in PI median intensity (18.3; Fig. 5D). Cell size and granularity greatly reduced, suggesting cells were dead under this condition (315/234; Fig. 5H). Whether cell death is due to necrosis could not be determined due to the limitation of this assay.

To determine whether apoptosis is also involved in compromising cell proliferation, Annexin V assay was carried out and analyzed using flow cytometric methods. In control cultures, the percentage of apoptotic MRC5 cells was 2 ± 0.5% (Fig. 6A). Approximately 18.6 ± 9% of cells were apoptotic after exposing to 0.5% of *M. cordata* crude extract. Although the data suggests cytotoxicity in normal human lung cells, the difference in percentage of apoptotic cells was not statistically significant. When MRC5 cells were treated with 2.5% of crude extract, the percentage of cells undergoing apoptosis was significantly higher (80 ± 10.4%) than untreated control cells, indicating that *M. cordata* crude extract...

**Fig. 3.** Human adenocarcinomic alveolar basal epithelial cells A549 cultured in the presence or absence of *Macleaya cordata* crude extract. (A) Light micrographs of control and *M. cordata* extract treated A549 cells on day 3. The experimental groups were exposed to 10% of extract for total 72 h and cell detachment from the culture dish surfaces, cell rounding, and irregularity in shape were observed (shown with arrowheads). Control groups were not exposed to the crude extract. Images were taken at 100× and 200× magnifications. Scale bar = 100 μm. (B) A549 cultures were initiated with 1 × 10^5 cells in the absence or present of *M. cordata* extract at a final concentration of 10%. The value shown in the parenthesis represented the reduction in total cell number compared to that of control group expressed in percentage. Results were expressed as total cell number (× 10^5) ± SEM on day 3. Statistical differences between control and treated groups were determined using ANOVA followed by Dunnett’s post hoc test when results of the ANOVA were significant (p < 0.05). Experimental data sets that were significantly different from that of control group were labeled with an asterisk (*).
is cytotoxic to normal human lung cells when administrated at this concentration. In the study of diseased cells, the percentage of apoptotic A549 cells in control groups was determined to be 1.8 ± 0.3% (Fig. 6B). Exposure to 0.5% and 2.5% of crude extract induced 7.9 ± 3.7% and 30.7 ± 9.1% of programmed cell death in diseased cells A549, respectively. Although higher percentage of apoptotic cells were present under these conditions, the increase was not statistically significant. When cells were exposed to 10% of crude extract, majority of cells underwent apoptosis (76 ± 3.5%), evident that the extract is toxic to human adenocarcinomic lung cells at this dosage. Taken together, our data revealed that both necrosis and apoptosis appeared to be the mechanisms underlying the *M. cordata* crude extract-induced cell growth impairment. Furthermore, normal and diseased cells respond differently to the treatment of crude extract under our experimental condition.

Fig. 4. Analysis of necrosis in human normal fetal lung fibroblast cells MRC5 following exposure to *Macleaya cordata* crude extract by flow cytometry. Flow cytometric plots were obtained and the gating strategy is shown. Cells were first gated on viable fraction using propidium iodide stain and forward scatter, which were expressed in logarithm and linear scales, respectively (A–C). Next, all cells present in the viable gate were shown according to the forward scatter and side scatter values in linear scale (D and E). Median intensity of propidium iodide stain, as well as median values of forward and side scatters are shown in the figure. (A and D) Control groups were not exposed to *M. cordata* extract. Experimental groups were exposed to 0.5% (B and E) and 2.5% of crude extract (C and F).
3.3. Effects of Macleaya cordata crude extract on cell viability

Finally, it is to our great interest to evaluate the physiological status of cells after exposure to Macleaya cordata crude extract and cell viability was monitored spectrophotometrically by WST-1 assay. Generally, this assessment relies on the mitochondrial ability to reduce WST-1 which only occurs in metabolically active cells; therefore, this method has been widely used to measure the viability of the cells, as well as counting of cell number. However, one should keep in mind that results obtained from WST-1 assay directly reflect the mitochondrial activity of the cells and it is possible to detect uncoupled changes in metabolic activity with the

Fig. 5. Analysis of necrosis in human adenocarcinomic alveolar basal epithelial cells A549 following exposure to Macleaya cordata crude extract. Flow cytometric plots were obtained and the gating strategy is shown. Cells were first gated on viable fraction using propidium iodide stain and forward scatter, which were expressed in logarithm and linear scales, respectively (A–D). Next, all cells present in the viable gate were shown according to the forward scatter and side scatter values in linear scale (E–H). Median intensity of propidium iodide stain, as well as median values of forward and side scatters are shown in the figure. (A and E) Control groups were not exposed to M. cordata extract. Experimental groups were exposed to 0.5% (B and F), 2.5% (C and G), and 10% of crude extract (D and H).
sets human an hoc expressed. However, manner, the response when WST-1 and detectable remaining at cytotoxic this cultures adenocarcinomic cells showed that lung M. ascites and crude extracts were tested. As shown in Fig. 7A, the viability of treated MRC5 cells was greatly reduced (0.5% ± 9%: 2.5% ± 6% in a dose-dependent manner, suggesting that the normal human lung cells are sensitive to M. cordata extract. Surprisingly, adenocarcinomic human A549 cells showed a significant elevation in viability (114 ± 3%) in response to the exposure of 0.5% extract (Fig. 7B), indicating that remaining cells survived the treatment were metabolically active. However, a significant reduction in viability was evident (70 ± 2%) when higher concentration of crude extract was administrated to the cultures (2.5%). This finding is inconsistent with the observation that the crude extract compromised cell proliferation when tested at this tested concentration. Finally, only 8 ± 2% of viable cells were detectable in cultures treated with 10% of crude extract.

4. Discussion

The present findings demonstrate that M. cordata crude extract is cytotoxic to normal human fetal lung fibroblast cell line MRC5 and adenocarcinomic human alveolar basal epithelial cell line A549. The crude extract not only adversely affected the cellular morphology but also cell proliferation. Both necrosis and apoptosis appeared to be the mechanisms underlying the cell growth impediment-induced by M. cordata crude extract. The administration of crude extract reduced the viability and mitochondrial activity of normal human lung cells in a dose-dependent manner. The adenocarcinomic human lung cells showed unexpected 14% elevation in metabolic activity when treated at the lowest tested concentration (0.5%) while viability was reduced accordingly when cells were treated with higher concentrations of extract (2.5% and 10%).

M. cordata is a rich source of bioactive compounds, mainly isoquinoline alkaloids. A quantitative study of this plant revealed that sanguinarine and chelerythrine, were two QBA’s mainly found in the capsules while other alkaloids, such as protopine and allocryptopine, were major alkaloids in the aerial part (Kosi et al., 2010). Although a list of other alkaloids in minor quantities has also been reported in M. cordata (Ye et al., 2009; Zdenek et al., 2006), whether some of these alkaloids are natural constituents of the plant or artifacts originated from native alkaloids during the isolation steps has not been confirmed. The present study investigates the bioactivity of M. cordata extract and is to our knowledge the first report of in vitro cytotoxicity of isoquinoline alkaloids with natural origin in human non-small cell lung cancer. Significant amount of sanguinarine, chelerythrine, protoxine, and allocryptopine have been identified in M. cordata, findings described here also provide useful insights into the biological responses of human cancer cells to tested isoquinoline alkaloids.
Cancer is a human disease characterized by uncontrolled cell growth and the field of cancer research has made remarkable advances in better understanding the biology of this human malignancy. Despite decades of efforts, better treatment options are urgently needed to provide more encouraging clinical outcomes for patients with non-small cell lung cancer. Naturally occurring agents often provide opportunities for the management of cancer and plant-derived compounds have played important roles in the development of several clinically useful anticancer agents, such as vinblastine, vincristine, paclitaxel. Recently, isoquinoline alkaloids found in *M. cordata*, such as sanguinarine, chelerythrine, protopine, and allocryptopine, have been implicated for their potential usefulness as complementary and alternative medicine in cancer therapy. Sanguinarine, as one of the major alkaloids, is listed as the Prestwick Chemical Library consisted of 1120 FDA-approved compounds with known safety and bioavailability in humans. This compound has been shown to suppress several type of tumor growth with prostate (Adhami et al., 2004; Sun et al., 2010a), colon (Lee et al., 2012; Matkar et al., 2008a,b), leukemic (Hallock et al., 2007; Han et al., 2008), mammmary (Choi et al., 2008; Matkar et al., 2008a), ovarian (Matkar et al., 2008a), oral squamous cell origins (Tsukamoto et al., 2011). Majority of these studies were in vitro cell line-based screens and the experimental findings demonstrated that sanguinarine is anti-proliferative and induces cell death through apoptosis, except in one study which showed that DNA damage as the cause of compromised cell growth (Matkar et al., 2008b). In the study of prostate cancer, the alkaloid appeared to cause cell cycle blockage in G0-G1 phase via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery irrespective of cellular androgen status (Adhami et al., 2004). Assessment of sanguinarine also revealed that alteration of Bax/Bcl-2 ratio and activation of caspase were responsible for the exhibited anticancer activities (Lee et al., 2012; Tsukamoto et al., 2011). Interestingly, the expression of survivin, an inhibitor of apoptosis protein involved in control of cell division, was inhibited by sanguinarine in prostate cancer cells (Sun et al., 2010a). Since this protein is highly expressed in all human cancers but undetectable in most normal adult tissues (Ambrosini et al., 1997), using sanguinarine to target survivin signaling may be an effective therapeutic approach for treating prostate cancer. Sanguinarine-associated production of reactive oxygen species, in particular hydrogen peroxide, has been noted in breast and colon cancer cells (Choi et al., 2008; Matkar et al., 2008a). This process led to rapid apoptosis in model systems and was restorable by the administration of antioxidant in model systems. Reduction of cellular glutathione content was also observed following addition of sanguinarine; however, this depletion was not reversible by antioxidant treatment shown in PC3 prostate cell line. The discrepancy between these studies implies that cytotoxicity of this isoquinoline alkaloid is tissue specific and the mechanism underlies this antiproliferative effect may not all be identical in different types of cancer. In addition to the apoptotic response, necrosis is evident in epidermoid carcinoma cells exposed to san- guinarine in a dose-dependent manner (Ahmad et al., 2000), similar to the finding of this study. Chelerythrine, another major alkaloid found in *M. cordata*, has been demonstrated to attenuate the growth of breast, colon, and ovarian cancer cells, as well as uveal melanoma, through apoptosis (Kemeny-Beke et al., 2006; Matkar et al., 2008a). Similar to sanguinarine, this molecule is involved in production of reactive oxygen species, which in results of proliferation impairment. Lastly, protopine and allocryptopine, alkaloids present in major quantities in the aerial part of *M. cordata*, has been demonstrated to be bioactive specially against cancer cells. When assessed either in the form of crude extract or single compound, the alkaloids were cytotoxic to human prostate cancer and Jurkat T-lymphoma cells and ultimately hampered cell growth (Chen et al., 2012; Habermehl et al., 2006). In the development of new anticancer agent, tubulin targeting compound(s) have received a great amount of attention since cancer cells are rapidly dividing and tubulin is the core component involves in this process. Interestingly, a recent report documented that protopine promoted tubulin polymerization and that resulted in mitotic arrest of the cell cycle at G2/M phase and apoptosis through cyclin-dependent kinase 1 activation and the alteration of Bcl-2 family of proteins (Chen et al., 2012). The data underscore the novel effect of alkaloids present in *M. cordata* as a microtubule-stabilizing agent. Since our crude extract collected was mainly collected from the aerial part of *M. cordata*, findings present in this study may also assist in fostering novel strategies for cancer therapy.

Lung cancer is a global health problem, which represents one of the most common killing cancers in both men and women in most developed countries. Approximately 80–85% of patients are classified as non-small-cell lung cancer and normally have a five-year survival rate from the time of diagnosis (Jemal et al., 2011). Even in patients with early stage of NSCLC, approximately half will relapse despite surgery/radiation and adjuvant chemotherapy. Therefore, finding better therapeutic agents with enhanced activity against lung cancer is required in the field. Complementary/alternative therapy is convergently becoming a more acceptable way to manage lung cancer. For treatment of NSCLC, cisplatin and carboplatin are often used in combination with other anti–cancer agents such as gemcitabine, paclitaxel, docetaxel, etoposide, or vinorelbine (Clegg et al., 2002). As a new treatment option, herbal medicines have been shown to be promising approach for the practice of lung cancer management. Generally, herbal crude extracts are prepared in water or alcohol (e.g. ethanol or methanol) and in vitro cytotoxicity of extracts are first assessed using model culture systems such as human adenocarcinoma cell line A549. Prior study of water extract of *Brucia javanica* reported growth retardation and apoptosis through caspase 3 activation pathway, in addition to DNA fragmentation, in A549 cells (Lau et al., 2005). Similarly, administration of water extract of *Panax notoginseng* to A549 cells induced apoptosis by activating caspase-3, as well as upregulating pro-apoptotic Bax protein and downregulating anti-apoptotic Bcl-2 expression (Park et al., 2009). Recently, Li et al. revealed that *Rheum officinale* Bail. (Da Huang), one of the common herbs used in traditional Chinese medicine formulae against cancer, has significant growth inhibitory effect on A549 cells in a dose- and time-dependent manner (Li et al., 2009). Moreover, DNA fragmentation and single DNA strand breaks were evident in treated cells. In a small scale screening study of anticancer drug, aqueous extracts of twelve Chinese medicinal herbs, *Anemarrhena asphodeloides*, *Artemisia argyi*, *Commphora myrrha*, *Duchesnea indica*, *Gleditsia sinensis*, *Ligustrum lucidum*, *Rheum palmatum*, *Rubia cordifolia*, *Salvia chinensis*, *Scutellaria barbata*, *Uncaria rhynchophylla* and *Vaccaria segetalis*, were evaluated in vitro for their antiproliferative activity on eight selected cancer cell lines, including A549 (Shoemaker et al., 2005). The experimental data discovered that all the crude extracts, except *g. sinensis*, caused greater than a 50% growth inhibition after three days exposure. *S. baicalensis*, known as Huang Qin or Chinese skullcap, is another frequently used medicinal herb in Asian countries for its broad spectrum of pharmaceutical properties including anti-cancer activity (Bensky et al., 1992; Huang, 1993). Aqueous crude extract of this plant is capable of inhibiting cell cycle progression in G1/S phase by suppressing cyclin D1 expression and significantly compromising cell metastasis by interrupting matrix-metalloproteinase-2 activity in human A549 cells (Park et al., 2011). Similarly, the ethanol extract of this herb induced cell cycle arrest in S phase by reducing the expression of cyclin A, as well as apoptosis by upregulating Bax (Gao et al., 2011). Interestingly, the amount of p53 tumor suppressing molecules increased in treated cells and the altered protein expression was postulated to be responsible in enhancing the cytotoxic effect observed. The ethanol crude extracts...
of Selaginella tamariscina were tumoricidal to human gastric tumor cell lines and the effect was mediated via p53 expression and G1 cell cycle arrest (Lee et al., 1999). When antimitastic activity of S. tamariscina extracts was evaluated in vitro using human A549 cells, reduction of matrix metalloproteinase-2, -9 expression in a dose-dependent manner was evident, while the endogenous tissue inhibitor of metalloproteinase-2 and plasminogen activator inhibitor were elevated (Yang et al., 2007). Similarly, inhibitory effects on the growth and metastasis were confirmed using Lewis lung carcinoma-bearing C57BL/6 mice, which was indicative of the in vivo anticanicancer property. In the study of Olopapax horridus root bark, the growth of non-small cell lung cancer cells A549 was compromised by the administration of the crude extract, and apoptosis was responsible for the observed antiproliferative effect (Sun et al., 2010b). Authors claimed that the active components found in the root bark extract are hydrophilic, which are enriched in 70% and 100% ethanol fractions. Taken together, the preceding experimental findings acknowledge the potentials in using botanical extracts as curative agents for non-small cell lung cancer management. However, one should keep in mind that a proper assessment of any potential phytochemical agents should be done using both normal and diseased model systems to better understand not only the pharmacological properties but also any possible side effects of tested agents.

The Papaveraceae family, which contains various bioactive alkaloids, is widely used as natural sources of potential anticancer agents. Similar to many components with herbal origin, isoquinoline alkaloids exhibit some degree of toxic side effects in addition to their desired pharmacological activity. Although M. cordata crude extract tested in this study is cytotoxic to both normal and adenocarcinomic human lung cells in vitro, one should not rule out the possibility and understand the potential of using this botanical extract as part of the chemotherapy regimen for treating non-small cell lung cancer. Similar to most of existing chemotherapy, the active agents are normally harmful and eradicate both normal and malignant cells by the mean of cell death. As a common dilemma that all oncologists face, finding a perfect balance between chemotherapy effectiveness and patient tolerability to treatment is still an ongoing mission. Therefore, special considerations are required in designing an effective disease management program, and drug dose given to patients should be carefully determined. Since most chemotherapies are not targeted therapy, the side effects associated with the treatment are generally manifested by systematic administration, which allows drug to circulate throughout the entire body. To overcome this shortcoming, anticancer agents for respiratory related diseases can be aerosolized and delivered directly to the malignant tissue to avoid damaging other healthy tissues. Moreover, multiple lines of treatment, in combined with phytochemical therapy, is another option for controlling human cancers. Alternatively, herbal extract may be administrated at low concentration to reduce the size of lung tumor prior to surgical removal for better clinical outcomes. Lastly, drugs specially design to target metabolic active cells, as most of anticancer agents do, can be administrated to eliminate the remaining metabolic active cancer cells that survived the M. cordata extract treatment. Since our crude extract is an unfracticated plant extraction, it is possible that the components mediating cell death in normal and cancerous cells are not identical. Investigations of fractionated crude extract and alkaloids in pure phase form are currently undertaking to better understand the exact mechanisms dictate the observed antiproliferative activity of M. cordata extract. Interestingly, M. cordata has been widely used as medicinal plant in Europe and the powered mixture of leaves, capsules, and seeds is the main ingredient of the feed additive Sangrovit (Psotova et al., 2006; Stiborova et al., 2008; Vieira et al., 2008). Several studies have assessed the safety of using Sangrovit as dietary additive in rearing animal in animal models as well as in vitro system (Stiborova et al., 2008; Ulrichova et al., 2001; Zdarilova et al., 2008). Simanek et al. showed in two independent studies that male Wistar rats fed with various amount of M. cordata extract did not show DNA damage in lymphocytes or hepatocytes (Zdarilova et al., 2008). No changes in general organ morphology and hematological parameters were observed after 90-days oral administration (Stiborova et al., 2008). Only at the highest tested dose (14,000 ppm Sangrovit), the treated animals showed elevation of reduced glutathione level and superoxide dismutase activity in liver. When sanguinarine and chelerythrine, which are two major QBA present in M. cordata extract, were added to cultures of human and porcine hepatocyte, exposed cells showed a time- and dose-dependent cell death as monitored by lactate dehydrogenase leakage, intracellular glutathione depletion, and mitochondrial dehydrogenase activity (Ulrichova et al., 2001). Taken together, the proceeding findings suggest that the toxic effects of M. cordata crude extract to healthy lung cells is manifested only in vitro, and this cytotoxicity may be alleviated under a more physiological condition. To test this hypothesis, antitumor activity of M. cordata extract in disease animal models are currently under evaluation.

Successful management of patients with non-small cell lung cancer continues to pose a considerable challenge to today's oncologist since chemoresistances common in this type of malignancy. While treatment may be curative in the early stages of the disease, most of patients (approximately 77%) are diagnosed at the time when tumor has already progressed beyond the primary site (metastatic stage IV) (Chang, 2011). Patients usually undergo intensive and invasive treatment regimen such as surgery, radiotherapy, and/or chemotherapy depending on disease stage at diagnosis and patient's performance status. Nearly all cases will still require chemotherapy even if their initial surgery is potentially curative and chemotherapy may be the only option for disease management for those at advanced stage. Cancer patients will very likely require multiple lines of therapy as their cancer cells may acquire resistance to the administrated chemotherapeutic agents. Consequently, chemoresistance to currently available chemotherapy for the management of non-small cell lung cancer (NSCLC) represents one of the most significant barriers to improve long-term outcomes and higher survival rate. The present study reveals that the proliferation of human adenocarcinomic A549 cells was attenuated by 30% following three day exposure to 0.5% of M. cordata crude extract even though the metabolic activity of the remaining viable cells was elevated (114 ± 3%) following treatment. Although this observation is indicative of development of chemoresistance in A549 under our experimental condition, these cells are different from most chemoprotective cells, which undergo quiescence until chemotherapy cessation. While the exact mechanisms responsible for this resistance to M. cordata crude extract are elusive, we propose several possible complementary defenses that protect cancer cells under chemotherapy agents. Generally, active efflux of chemotherapeutic agents is achieved via ABC transporters, such as P-glycoprotein (P-gp) and multi-drug resistance proteins (MDRs) (Chang, 2011). This mechanism contributes to the resistance to anthracyclines, taxanes, platinum agents, vinca alkaloids, that are routinely used in treatment of non-small cell lung cancer. Investigation of several in vitro models of human tumor cells suggests that P-gp in the transport and sequestration of drugs represents a protective mechanism of tumor cells against cytotoxic agents (Arancia et al., 2001). Second, studies have revealed that the expression of the DNA repair enzymes excision repair cross-complementation group 1 (ERCC1) has prognostic value for responses to chemotherapy, supported by the observation that NSCLC patients with ERCC1-negative tumors who underwent chemotherapy had significantly longer overall survival (Olausen et al., 2006; Schettino et al., 2008). Since ERCC1 is present in A549


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