Evaluation of the Cytotoxicity of Andrographolide to Human Lung Carcinoma A549 Cells

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Abstract

Andrographolide is a bioactive constituent of *Andrographis paniculata*, a commonly used traditional medicine, which has significant anticancer activity. However, the application of andrographolide as an anticancer agent will depend upon its cytotoxicity. This study therefore aimed to evaluate the cytotoxic effect of andrographolide on the human lung carcinoma A549 cell line. A549 cells were incubated with 25 and 100 µM andrographolide for 1 to 7 days in parallel with cells treated with DMSO at the same concentration as a control. Further negative (complete media only) and positive (10% ethanol) controls were included. At each selected time point, the treated cells were harvested for analysis. The trypan blue dye exclusion method was undertaken to determine the percentages of cell viability, while the induction of apoptosis was determined by detection of the subG1 cell population by flow cytometry. All experiments were undertaken independently in triplicate. A549 cell viability was dramatically decreased by exposure to 100 µM andrographolide in time-dependent manner, whereas 25 µM andrographolide showed little effect. Moreover, 100 µM andrographolide significantly increased the percentage cells in the subG1 population, while there was little effect from exposure to 25 µM andrographolide. These results suggest that the cytotoxic effects of andrographolide are proportional to increased concentration. Andrographolide at a high concentration can cause A549 cell death in a time-dependent manner. The cytotoxic effects are increased with increasing concentration.

Keywords**:** *andrographis paniculata, andrographolide, cell death, A549 cells*

1. Introduction

Andrographis paniculata (Burm. F.) Nees or King of Bitters is an herb that is widely used as a traditional medicine in India, China, Thailand, and many other countries in Asia for the treatment of diseases such as a cold, fever, laryngitis and several infectious diseases. There are several major diterpenoid compounds in *A. paniculata* extracts, including andrographolide, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide and 14-deoxyandrographolide (Suriyo et al., 2014). The main bioactive ingredient of this plant is believed to be andrographolide (Jayakumar, Hsieh, Lee, & Sheu, 2013). Previous studies have reported various biological activities of andrographolide including antibacterial, antiviral, anticancer, anti-inflammation and antioxidant properties (Arifullah et al., 2013; Banerjee, Parai, Chattopadhyay, & Mukherjee, 2017; Chua, 2014; Gupta, Mishra, & Ganju, 2017; Lim et al., 2012). Furthermore, andrographolide have been reported to be potent at inducing autophagic and apoptotic cell death (Chen, Feng, Nie, & Zheng, 2012; Lim et al., 2017).

Cell death is a crucial process for maintaining homeostatic cellular balance in all living organisms. Cell death can be classified into 3 types which are apoptosis (type I), autophagy (type II) and necrosis (type III) (Galluzzi et al., 2007). Apoptosis, or programmed cell death, occurs naturally during cell development and plays a role as a defense mechanism after induction by some chemical agents (Elmore, 2007). The morphology of cells that undergo apoptosis changes by shrinkage, blebbing and condensation. Moreover, the cytoskeleton collapses and chromatin condensation and DNA fragmentation will occur. In the body, the apoptotic bodies that are produced by apoptosis will be rapidly phagocytosed by macrophages or neighboring cells.

There are various methods to detect apoptotic cells. Flow cytometry is an accurate and rapid method to quantitate the number of cells that are undergoing apoptosis. This type of cell death is marked by cell morphological changes, while the plasma membrane can exclude the cationic dyes such as propidium iodide (PI), trypan blue and DAPI (Archana, Bastian, Yogesh, & Kumaraswamy, 2013). The biochemical

hallmark of apoptosis is chromosomal DNA fragmentation in which the DNA will be cleaved in shorter fragments of multiples of approximately 180 bp. After using a DNA-specific fluorochrome such as PI, the lower molecular weight DNA fragments will be stained with less intensity. Thus, after detection by flow cytometry, the population of apoptotic cells will be appeared at a hypodiploid or SubG1 peak in the DNA histogram (Riccardi & Nicoletti, 2006). This study aimed to determine whether two different concentrations of andrographolide exert a cytotoxic effect on A549 cells, human lung carcinoma cell line.

2. Objectives

To investigate the cytotoxic effect of andrographolide on A549 cells by evaluation of cell viability and SubG1 population detection.

3. Materials and Methods

Cell culture

The human lung carcinoma cell line A549 was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen) at 37°C with 5% CO_2 in a humidified incubator. Cells were maintained in T-175 cm^2 tissue culture flasks and subcultured twice per a week. To subculture, cultured media was removed then cells were washed with phosphate buffer saline (PBS). Cells were incubated with 3 ml of 0.25% Trypsin/EDTA at 37°C for 3 minutes. Fresh media was added to inactivate trypsin, and cells were separated to single cells by pipetting through a 10 ml serological pipette. 3-5 ml of cell suspension was sub-cultured to tissue culture flasks. Fresh completed medium was added to a final volume of 25 ml. Cells were incubated under standard conditions.

Andrographolide compound

Andrographolide assessed as ≥ 98% pure by thin layer chromatography was purchased from Sigma-Aldrich (365645, Sigma) and was dissolved in 100% dimethyl sulfoxide (DMSO, Sigma) to a final stock concentration of 100 mM and stored at -30°C. Compound was diluted to various concentrations using complete DMEM media. The final concentration of DMSO in media was less than 0.1%.

Andrographolide treatment of cells

A549 cells were seeded in six wells plates and cultured under standard conditions until the cells reach 90% confluency. The culture medium was removed and cells were incubated with 2 ml of 25 and 100 µM of andrographolide and DMSO at the same concentrations for 1 day post-treatment (d.p.t.) to 7 d.p.t.. Negative control (complete DMEM media) and positive control (10% ethanol) were included.

Cell viability analysis by Trypan blue dye exclusion method

At the indicated time point, the culture supernatant was collected in 15 ml tubes and the cells were trypsinized by addition of 750 µl of 0.25% Trypsin/0.1% EDTA for 3 min. The trypsin activity was stopped by adding 1 ml of the corresponding culture supernatant followed by pipetting approximately 10 times or until cells were separated into a single cell suspension. All collected culture supernatants and cell suspensions were pooled together and centrifuged at 120 g for 3 min. After cells were resuspended in 1 ml of media, cells were diluted to an appropriate dilution and then mixed with 0.4% trypan blue (Invitrogen) in 1X PBS. Subsequently, the number of dead cells (trypan blue positive) and live cells (trypan blue negative) were counted in a hemocytometer under a microscope for determination of the percentage of viable cells. The experiments were undertaken independently in duplicate with triplicate analysis of each sample for each day. Percent viability was calculated by the formation below:

$$\text{Viability (\%)} = \frac{\text{Number of live cells} \times 100}{\text{Number of live cells} + \text{Number of dead cells}}$$

Study of apoptosis of drug treated A549 cells by SubG1 detection by flow cytometry

A549 cells were treated with 2 ml of 25 and 100 µM andrographolide and DMSO using complete media as a negative control whereas 10% ethanol was used as a positive control for 1 to 7 d.p.t. At each time point, cells were harvested and centrifuged at 1,000 g for 5 min. The supernatant was discarded and the cell pellet was washed with 1 ml of iced-cold 1X PBS. After centrifugation at 2,000 g for 5 min, cells were fixed in 1 ml of 70% ethanol and kept at 4°C until the experiment within a week.

For detecting SubG1 by flow cytometry, cell suspensions in 70% ethanol were centrifuged at 6,000g for 5 min, followed by washing with 1 ml of 1X PBS for 2 times. The cell pellet was resuspended in 400 µl of 1X PBS then treated with 10 µl of 10 mg/ml RNase A and incubated for 30 min. Finally, 5 µl of 1 mg/ml of propidium iodide (PI) was added and the fluorescence signal was analyzed by flow cytometry on a BD FACalibur cytometer (Becton Dickinson, BD Biosciences) using the CELLQuest™ software. All experiments were undertaken independently in triplicate.

Statistical analysis

All data were plotted using the GraphPad Prism software. Statistical analysis of significance was undertaken by independent samples t-test using PASW program, with a value of $p < 0.05$ for significance.

4. Results and Discussion

4.1 Results

Cell viability analysis by Trypan blue dye exclusion method

A549 cells were cultured and incubated with 2 ml of 25 and 100 µM andrographolide and DMSO using complete media as a negative control whereas 10% ethanol was used as a positive control for 1 d.p.t. to 7 d.p.t.. At each time point, cells were harvested and trypan blue dye exclusion method was performed to determine cell viability.

The result (Figure 1) showed that the percentages of cell viability were significantly decreased by 100 µM andrographolide in a time-dependent manner. The means of viable cells in 1st to 7th day were decreased from 96.76%, 89.77%, 88.19%, 73.85%, 31.19%, 27.10% and 12.42%, respectively. At the low concentration (25 µM) of andrographolide, the effect on cell viability was lower than the high concentration (100 µM). These results suggested that the cytotoxic effect of andrographolide was proportionally increased with increased concentration.

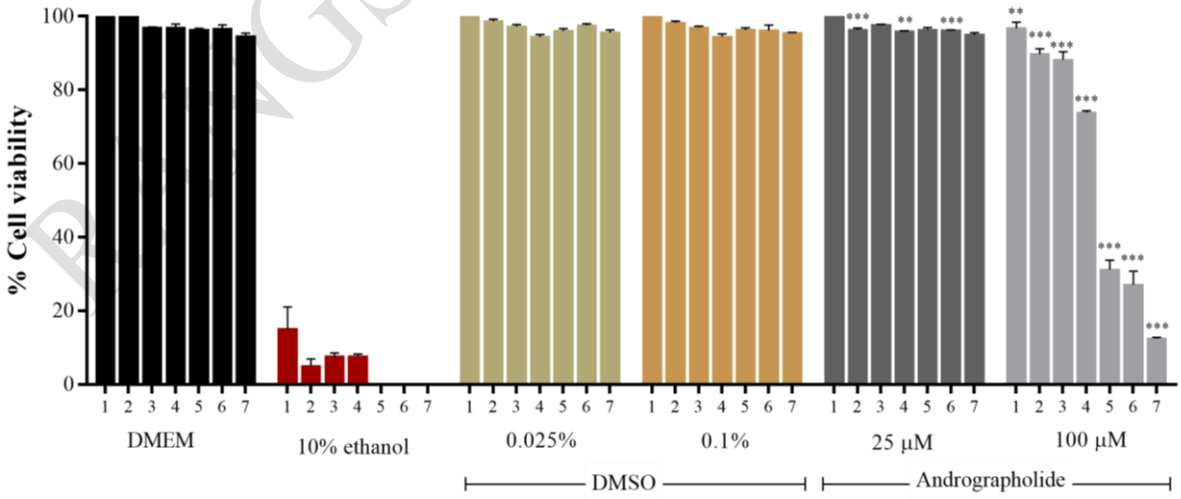

Figure 1 Cell viability test of andrographolide-treated A549 cells

A549 cells were incubated with 25 and 100 µM andrographolide for 1 to 7 d.p.t.. DMSO at the same concentrations was used as a control whereas 10% ethanol was used as a positive control of cell death. Trypan blue dye exclusion method was performed to determine the number of dead cells and live cells. The experiments were undertaken independently in duplicate with triplicate of each sample for each day. The bars show mean ± SD (** is *p* value < 0.01 and *** is *p* value < 0.001)

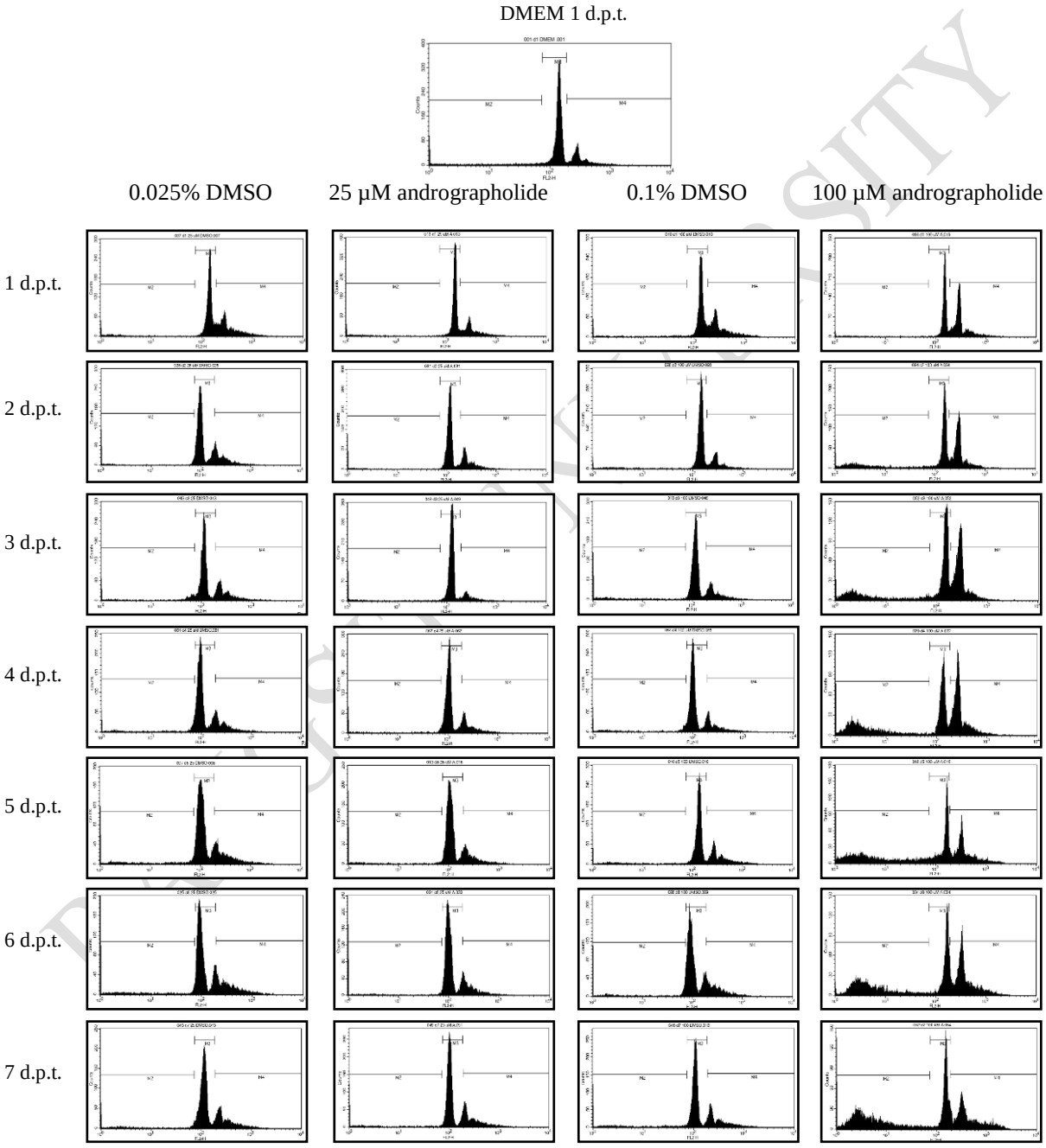

Figure 2 Study of apoptosis of andrographolide-treated A549 cells by SubG1 detection by flow cytometry

A549 cells were incubated with 25 and 100 µM andrographolide for 1 d.p.t. to 7 d.p.t.. DMSO at the same concentrations was used as a control. SubG1 population was carried out by treating cells with RNase A before adding PI and analyzed by flow cytometry. The experiment was undertaken independently in triplicate. Cells populations were divided into 3 parts which are M2, M3 and M4. M2 is an expected SubG1 population.

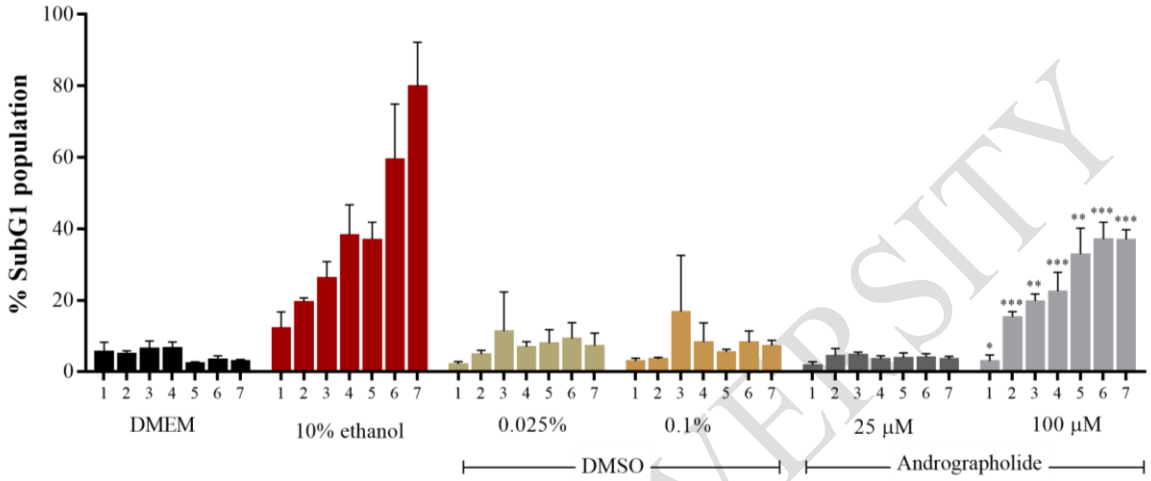

Figure 3 Percentages of SubG1 population in andrographolide-treated A549 cells

M2 population (figure 2) in triplicate were used to quantitate percentage of SubG1 population. The bars show mean ± SD (* is *p* value < 0.05, ** is *p* value < 0.01 and *** is *p* value < 0.001).

Study of apoptosis of andrographolide-treated A549 cells by SubG1 detection by flow cytometry

For detecting SubG1 population, cells were stained with propidium iodine and analyzed by flow cytometry. The histogram results are shown in Figure 2 and the percentages of SubG1 population from each sample are shown in Figure 3.

At low concentration or 25 µM of andrographolide, A549 shows no significant SubG1 population, but the treatment of A549 cells with 100 µM andrographolide resulted in a significantly increased in SubG1 population in a time-dependent manner. The means of the SubG1 population from the 1st to 7th day were increased from 2.95%, 15.33%, 19.75%, 22.51%, 32.91%, 37.09% and 36.98%, respectively.

4.2 Discussion

This study evaluated the cytotoxic effect of andrographolide which is the main bioactive compound of *Andrographis paniculata* on the human lung carcinoma A549 cell line. The results showed that andrographolide at 100 µM can induce cell death in A549 in time-dependent manner. The type of this cell death was suggested to be apoptosis after analysis of the SubG1 cell population by flow cytometry. Unlike necrotic cell death, apoptotic cell death can activate endonucleases to cleave the DNA into fragments which results in decreased in fluorescence intensity and the presence of a SubG1 peak in the histogram.

Andrographolide has been reported to exert cytotoxic activity on different types of cancer cell line, with the cytotoxicity of andrographolide being different in each different cell type. Our laboratory has previously reported the cytotoxicity of andrographolide to the human hepatoma cell line HepG2 and the human cervical cancer cell line HeLa (Panraksa, Ramphan, Khongwichit, & Smith, 2017; Wintachai et al., 2015). Those studies showed that up to 100 µM andrographolide had no significant cytotoxic effect to both HepG2 and HeLa cells after 24 hours of incubation. For this study which was undertaken on human lung A549 cells, the results showed a significant reduction in percentage of cell viability and significant elevation in percentage of SubG1 population by 100 µM andrographolide even at 1 d.p.t..

The previous study has shown that at 24 hours post treatment of andrographolide at the low concentration (0 to 5 µM) had no significant cytotoxicity to A549 cells assessed by MTT assay (Lee et al., 2010). In agreement with that study, our results showed that A549 cells were not significantly affected by 25 µM andrographolide.

5. Conclusion

In conclusion, 100 µM andrographolide can induce A549 cell death in a time-dependent manner. The type of cell death was suggested to be apoptosis. The EC_{50} or half maximal effective concentration should be calculated and reported, so further study of the cytotoxicity mechanism of andrographolide is required.

6. Acknowledgements

This work was supported by a grant from Mahidol University. WK is supported by a Thailand Graduate Institute of Science and Technology (TGIST) M.Sc. Scholarship.

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