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Inhibition in Cell Viability and Cell Migration of Cervical Cancer Cells by Water Extract of *Clinacanthus nutans*

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Abstract

Clinacanthus nutans is a traditional medicinal herb that possesses many biological activities including anti-cancer activity. In this paper, we studied the effects of *Clinacanthus nutans* water extract on HeLa cervical cancer cells. *Clinacanthus nutans* water extract decreased the cell viability and displayed cytotoxicity to HeLa cells concentration-dependently. *Clinacanthus nutans* water extract inhibited cell migration and caused severe morphological change in HeLa cells. Our results suggest that *Clinacanthus nutans* water extract would be promising for the therapy of cervical cancer.

Keywords: water extract of *Clinacanthus nutans*, HeLa cervical cancer cells
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Introduction

Clinacanthus nutans (Burm. f.) Lindau is a perennial herb that belongs to the Acanthaceae family, which family also contains many medicinal plants. *Clinacanthus nutans* is widely distributed in the countries of subtropical Asia including Malaysia, Thailand, Indonesia, China, and even cultivated sporadically in Taiwan. *Clinacanthus nutans* is used as the traditional medicine for the therapy of snake bite, burns/scalds,

and infection from bacteria, fungus, and virus [1]. Recently, its anti-cancer activities gain people's attention. *Clinacanthus nutans* displays anti-cancer activities against lymphoma, leukemia, breast, colorectal, cervical, gastric, liver, lung, head and neck, pancreatic and skin cancers in cells and animal model studies [2]. *Clinacanthus nutans* is rich in phenolic compounds and flavonoids, which are responsible for its health-promoting activities [1]. Different reagents, including organic solvents and water, are used for the extraction and thereafter purification of these useful compounds [1].

Listing after breast, colorectal, and lung cancers, cervical cancer is the fourth most common female cancer in the world and is responsible for 600,000 new cases and 340,000 cancer deaths each year. It is the leading cause of death in the low- and middle-income countries (LMICs) [3]. Cervical cancer also lists among top 10 women cancer deaths in Taiwan in 2020 (Ministry of Health and Welfare, Taiwan). Infection by Human Papillomavirus (HPV) is a high risk for committing cervical cancer [4, 5]. Current therapy for cervical cancer includes surgery, radiation, chemotherapy, immunotherapy, and targeted therapies by interfering with the cell cycle, cell growth and survival, angiogenesis, and DNA repair of cancer cells. Moreover, combination therapy provides more opportunities to tackle with the disease. Despite these efforts, patients of cervical cancer still suffer from severe side effects and drug resistance, pointing out the need for more efficient therapeutic agents.

In this paper, we have obtained water extract from fresh *Clinacanthus nutans* leaves and HeLa cells were treated with *Clinacanthus nutans* water extract in order to know the effects of *Clinacanthus nutans* extract on cervical cancers. Cell viability assay was used to assay the effects of *Clinacanthus nutans* water extract on cell proliferation and cytotoxicity of HeLa cells. Wound-healing assay was used to analyze the effects of extract on cell migration of HeLa cells. Finally, the effects of water extract on cell morphology of treated HeLa cells were examined.

Materials and Methods

Chemicals

MTT (Thiazolyl Blue Tetrazolium Bromide) was from Sigma-Aldrich and prepared in phosphate-buffered saline (PBS) at the concentration of 5 mg/mL.

Preparation of extract

Clinacanthus nutans plant was purchased from the Shulin Horticultural Company in Shulin District, New Taipei City, and verified by Dr. Chi-Ming Chiu, Ming Chuan University. 10.06 grams of fresh leaves of *Clinacanthus nutans* were rinsed thoroughly

in tap water, blot-dried, ground in mortar extensively with 25 mL of sterilized room-temperature water, and passed through two layers of cheese cloth. The filtrate was centrifuged at 3,000 rpm for 20 min, supernatant was collected and passed through two layers of cheese cloth. The filtrate was collected and centrifuged at 12,500 rpm for 30 min. Supernatant was collected, passed through two 0.45 micro-meter of filters successively, and the filtrate was aliquoted and kept at -80 ^{o}C.

Cell culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% of fetal bovine serum (FBS; Hyclone), 1% of penicillin-streptomycin (Gibco), and 1mM of sodium pyruvate (Gibco). Culture chamber was set at 37 ^{o}C, and equipped with 5% of CO_2 in a humidified condition.

Cytotoxicity assay and cell proliferation assay

We used MTT assay to evaluate the effects of water extract of *Clinacanthus nutans* on cytotoxicity and cell proliferation of HeLa cells. For cytotoxicity assay, cells ($5x10^3$/well) were seeded into a 96-well dish for overnight. On the next day, cells were treated with the indicated concentrations of *Clinacanthus nutans* water extract for 48 hours. Medium was removed, MTT solution was then added into cells, and incubated at 37 ^{o}C for 1.5 hours. After a spin at 2,500 rpm for 25 min, medium was completely removed and 100 μl of DMSO was added onto cells. The dish was shacked for an additional 10 min in the dark before reading optical density at 570 nm in an ELISA reader. Data were collected, calculated, and plotted in the Microsoft Excel program. For cell proliferation assay, cells ($2x10^3$/well) were seeded into a 96-well dish and proceeded as the cytotoxicity assay, except that the time for the incubation of cells in drugs was 72 hours.

Cell morphology assay

Cells ($1x10^5$/well) were seeded into a 24-well dish and incubated in culture chamber for overnight. Cells were then treated with the indicated concentrations of *Clinacanthus nutans* water extracts for 72 hours and pictures of cell morphology were taken at 0, 24, 48, and 72 hours respectively.

Migration assay

Cells ($3x10^5$/well) were seeded into a 24-well dish and incubated in culture chamber for overnight. Cells were scratched with a 200 μl of pipet tip, washed with PBS, and then treated with the indicated concentrations of *Clinacanthus nutans* water extracts for 72 hours and pictures of cell morphology were taken at 0, 24, 48, and 72 hours respectively.

Statistics

The T test in Excel program of Microsoft software was used for the statistical analysis between samples and P value less than 0.05 was regarded as significant.

Results and Discussion

1. The effects of *Clinacanthus nutans* water extract on cell viability of HeLa cells

Clinacanthus nutans is known to have anti-cancer activities [2], in order to know the effects of *Clinacanthus nutans* on cervical cancer, we evaluated the effects of *Clinacanthus nutans* water extract on HeLa cervical cancer cells. Water extract was obtained from fresh leaves of *Clinacanthus nutans* to mimic the juice prepared from fresh leaves of *Clinacanthus nutans* by blender, the usual way for people to have this medicine. We first tested the effects of *Clinacanthus nutans* water extract on cell proliferation of HeLa cells. To this purpose, HeLa cells were treated with different concentrations of water extracts for 72 hours and cell proliferation was analyzed by MTT assay. As shown in Figure 1, *Clinacanthus nutans* water extract decreased the cell viability of HeLa cells in a concentration-dependent manner. 9.25 μg/mL of water extract decreased the cell viability more than 50%. The calculated IC_{50} for water extract to HeLa cells at 72 hours was 3.15±0.13 μg/mL.

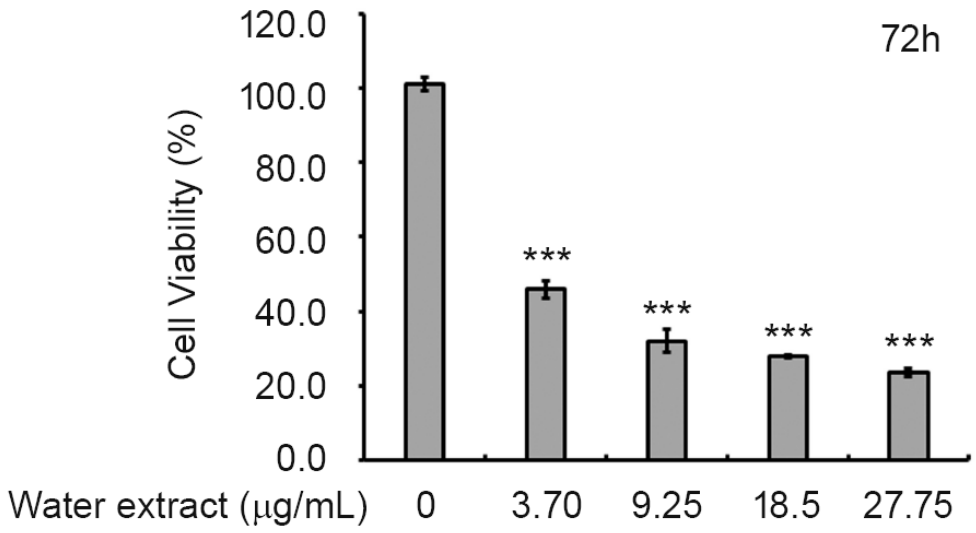

Figure 1. *Clinacanthus nutans* water extract decreased the cell viability of HeLa cells after 72 hours of incubation. HeLa cells ($2X10^3$/well) were seeded into a 96-well dish, treated with different concentrations of *Clinacanthus nutans* water extracts and cell viability was checked 72 hours later by MTT assay. ***, P<0.001. The values from treatments of different concentrations of water extract was compared to treatment without water extract (0).

We then analyzed the effects of *Clinacanthus nutans* water extract on

cytotoxicity of HeLa cells. To this purpose, HeLa cells were treated with different concentrations of water extracts for 48 hours and cytotoxicity was evaluated by MTT assay. As shown in Figure 2, *Clinacanthus nutans* water extract decreased the cell viability of HeLa cells concentration-dependently. 18.50 μg/mL of water extract decreased the cell viability more than 50% for 48 hours. The calculated IC_{50} for water extract to HeLa cells at 48 hours was 15.94±0.29 μg/mL. From the above data, we concluded that *Clinacanthus nutans* water extract was able to decrease the cell viability of HeLa cells.

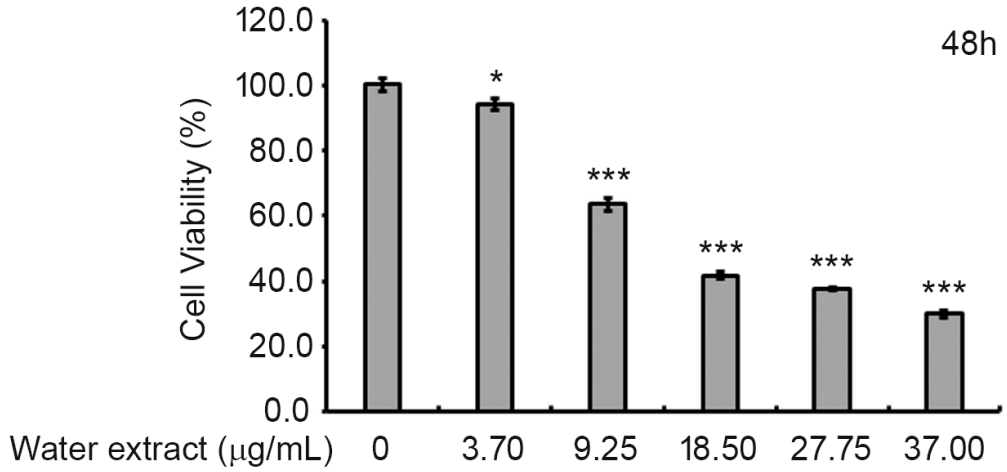

Figure 2. *Clinacanthus nutans* water extract was toxic to HeLa cells after 48 hours of incubation. HeLa cells (5 X 10^3/ well) were seeded into a 96-well dish, treated with different concentrations of *Clinacanthus nutans* water extracts and cell toxicity was assayed 48 hours later by MTT assay. *, P<0.05, ***, P<0.001. The values from treatments of different concentrations of water extract was compared to treatment without water extract (0).

Inhibition of cell proliferation by water extract was observed in HeLa cells and other cancer cells [6, 7]. Yong et al. evaluated the effects of *Clinacanthus nutans* leaves extracts by different reagents, including water, on different cultured human cancer lines, including HeLa cells. Among all extracts obtained by different extraction reagents, chloroform extract displayed the highest anti-proliferative activities against all cell lines excluding IMR-32 cells. Water extract exhibited anti-proliferative activity in a concentration-dependent manner, with 36.31±1.52% of inhibition efficiency at 100 μg/mL for 72 hours [6]. Haron et al. reported the anti-proliferative activities of *Clinacanthus nutans* leaf extracts by 80% methanol on HeLa cells. Methanol extract was further fractionated by hexane, dichloromethane (DCM), and water. All extracts were cytotoxic to cells, while the DCM fraction was the most effective (IC_{50} of 70 μg/mL at 48 hour). In this study, the methanol-water extract was not toxic to HeLa cells [7]. The difference in efficiency of extracts from our results and other laboratories may be due to different method for extract preparation was used or

different geographical condition for cultivation of the plants.

The effects of *Clinacanthus nutans* water extract on cell migration of HeLa cells

Cell migration is one of the key steps in cancer cell metastasis [8]. We then examined the effects of *Clinacanthus nutans* water extract on cell migration of HeLa cells. To this end, cells were scratched and treated with different concentrations of water extracts, and cell migration was analyzed by wound-healing assay for up to 72 hours after wounding. As shown in Figure 3, cells in control treatment closed at 48 hours after wounding (panel 1). However, cell migration was delayed by increasing amount of extracts (panels 2 to 5). Furthermore, there is dramatic morphological change at drug concentration more than 5.55 μg/mL. These data indicated that *Clinacanthus nutans* water extract can inhibit cell migration of HeLa cells.

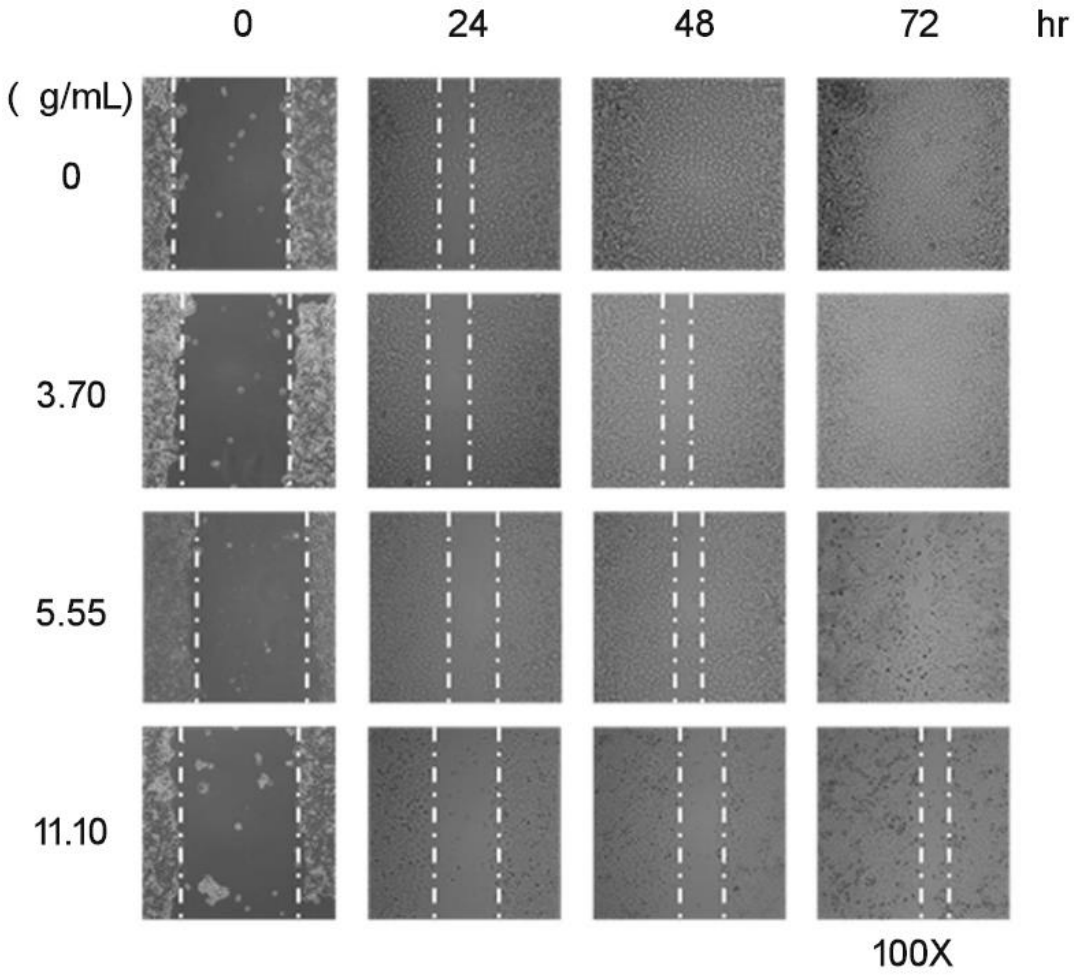

Figure 3. *Clinacanthus nutans* water extract inhibited cell migration of HeLa cells. HeLa cells (3 X 10^5/ well) were seeded into a 24-well dish, scratched with a 200 μl tip, washed, and treated with different concentrations of *Clinacanthus nutans* water extracts for 72 hours. Photos were taken at 0, 24, 48, and 72 hours after drug treatment. Photos were 100X image.

The effects of *Clinacanthus nutans* water extract on cell morphology of HeLa cells

From cell migration assay described above, we observed that there is morphological change after drug treatment. We set up drug treatment by treating cells with different concentrations of water extracts and chased the morphological change in a time-dependent manner for up to 72 hours. Cells in every time point of control treatment grew vigorously and attached well onto bottom of culture dish (panel 1 of Figure 4). Cell debris, probably due to apoptosis of cells, was observed after 48 hours of drug treatment at 3.7 μg/mL of water extract (panel 2 of Figure 4). Cell debris were obvious after 24 hours of incubation in water extract at 7.4 μg/mL (panel 3 of Figure 4), with substantial morphological change after 48 hours of incubation in water extract at 7.4 μg/mL (columns 3 and 4 in panel 3 of Figure 4). These data indicated that *Clinacanthus nutans* water extract caused severe morphological change of HeLa cells after treatment, suggesting that the effect of water extract on cell viability may be due to promotion in cell apoptosis and the effect on cell migration may be due to the inhibition in epithelial-mesenchymal transition [9].

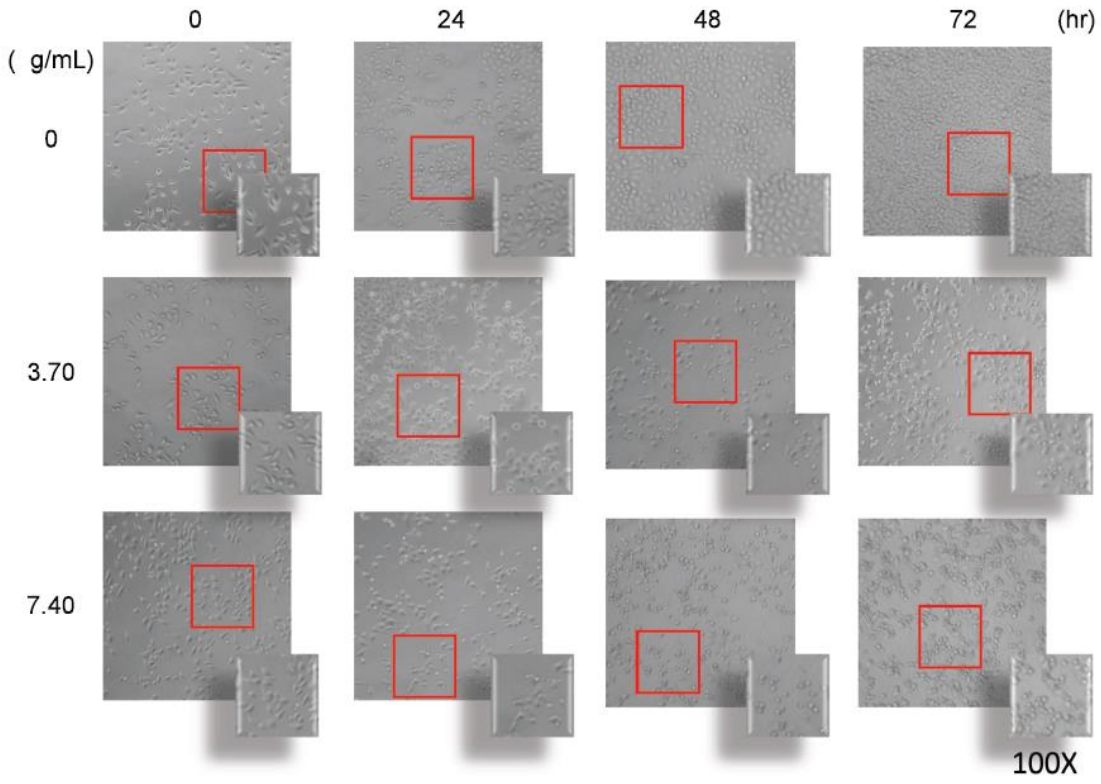

Figure 4. *Clinacanthus nutans* water extract caused morphological change on HeLa cells. HeLa cells (1X 10^5/ well) were seeded into a 24-well dish for overnight, and treated with different concentrations of *Clinacanthus nutans* water extracts for 72 hours. Photos were taken at 0, 24, 48, and 72 hours after drug treatment. Photos were 100X image.

Concluding Remarks

In this paper, we described that *Clinacanthus nutans* water extract decreased cell viability of HeLa cervical cancer cells based on cell toxicity assay and cell proliferation assay (Figures 1 and 2). *Clinacanthus nutans* water extract was able to inhibit cell migration of HeLa cervical cancer cells based on wound healing assay. Substantial morphological change of cells was observed by water extract. We are now perusing the underlying mechanism(s) leading to these biological effects of *Clinacanthus nutans* water extract. *Clinacanthus nutans* would be potential for the use in the therapy of cervical cancer in the future.

Acknowledgement

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