Apoptosis Induction of *Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kurz) Gogel in Resistance Human Hepatocellular Carcinoma (HepG2) Models

การชักนำอะพอพโทซิสของติ้วขน (*Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kurz) Gogel) ในเซลล์โมเดลมะเร็งดับที่ดื้อยา

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ABSTRACT

A resistance mechanism is a major problems of cancer treatment cause decreasing response of cancer cells to chemotherapeutic drug. This study aimed to develop the resistant HepG2 cells by expose to cisplatin for evaluation of anticancer activity of plant extract. The cell viability was tested by using neutral red assay. The resistant index (RI) was calculated from the cytotoxic IC_{50} values of the resistant cells versus the parental cells. Apoptosis induction was analyzed by AV/PI staining. Results showed that the IC_{50} of cisplatin in parental and resistant HepG2 cells were 4.7±0.2 and 16.6±0.4 µg/ml, respectively with approximate 3.5 RI. The IC_{50} of *Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kurz) Gogel in parental and resistant of HepG2 cells were 113.2±11 and 196.7±19 µg/ml, respectively. The extract induced apoptosis in both cells. In conclusion, the induction of resistance was successfully developed for searching the anticancer activity of plant extracts. *Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kurz) Gogel showed anticancer activity via apoptosis induction in both cells.

บทคัดย่อ

กลไกการดื้อยาจัดเป็นปัญหาหลักของการรักษามะเร็งซึ่งเป็นสาเหตุทำให้การตอบสนองของเซลล์มะเร็งต่อยาเคมีบำบัดลดลง การศึกษานี้มีวัตถุประสงค์เพื่อเหนี่ยวนำเซลล์มะเร็งตับ (HepG2) ให้ดื้อยาด้วย cisplatin เพื่อใช้เป็นเซลล์โมเดลในการหาฤทธิ์ต้านมะเร็งของพืชสมุนไพร วิธีการทดสอบการอยู่รอดของเซลล์โดยใช้ neutral red ดัชนีการดื้อยาของเซลล์มะเร็ง (resistant index, RI) คำนวณจากค่า IC_{50} ในเซลล์มะเร็งที่ดื้อยาเทียบกับเซลล์มะเร็งที่ไม่ดื้อยา วิธีการตรวจวัดการชักนำอะพอพโทซิสด้วยการย้อมสี annexin V/propidium iodide จากผลการทดลองพบว่า ค่า IC_{50} ของ cisplatin ในเซลล์ที่ไม่ดื้อยาและเซลล์ดื้อยาคือ 4.7±0.2 และ16.6±0.4 µg/ml ตามลำดับ ซึ่งมีค่าดัชนีการดื้อยาเท่ากับ 3.5 และค่า IC_{50} ของติ้วขน (*Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kurz) Gogel) ในเซลล์ที่ไม่ดื้อยาและเซลล์ดื้อยาคือ 113.2±11 และ 196.7±19 µg/ml ตามลำดับ จากผลการทดสอบฤทธิ์การชักนำอะพอพโทซิสพบว่าติ้วขนสามารถกระตุ้นการตายแบบอะพอพโทซิสได้ทั้งเซลล์มะเร็งที่ไม่ดื้อยาและดื้อยา จากผลการศึกษาสรุปว่าเซลล์โมเดลที่ชักนำให้ดื้อยาสามารถนำมาใช้ในการหาฤทธิ์ต้านมะเร็งของสารสกัดจากพืชสมุนไพรได้ และติ้วขนมีฤทธิ์ต้านมะเร็งโดยผ่านการชักนำให้เซลล์มะเร็งทั้งไม่ดื้อยาและดื้อยาตายผ่านกระบวนการอะพอพโทซิส

Key Words: Resistance mechanism, HepG2 cells, Apoptosis
คำสำคัญ: กลไกการดื้อยา เซลล์ HepG2 อะพอพโทซิส

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Introduction

Cancer is becoming a significant health problem in Thailand as in many other parts of the world (Freeburg et al., 2009). Nowadays, many ways of cancer treatment have been approached, but there is still some failure of the therapy due to high toxicity of chemotherapeutic drug or resistant mechanism developed in the cancer cells. A majority of patients are surgically at the beginning of diagnosis. Even for those underwent resection, the risk of recurrence is extremely high. Consequently, chemotherapy is an important treatment for most cancer patients. However, development of resistance to chemotherapeutic drugs is a major concern in clinical oncology and might be involved in the consequent chemotherapy failure. Thus, the need to discover effective plant extract to suppress the growth of the resistant cancer cells and/or to overcome drug resistance in cancer cells is necessary (Dai et al., 2009; Phadungkit et al., 2010). *Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kurz) Gogel. was found in Southeast Asia including Thailand and belongs to the family of Guttiferae. It was previously reported that *C. formosum* had effect on anticancer activity (Nonpunya et al., 2014). Thus, the study aimed to determine the effect of *C. formosum* on apoptosis induction in resistant HepG2 cells model.

Materials and methods

1 Chemicals

All reagents and media used in cell culture techniques were purchased from GIBCO, Invitrogen Corporation (USA). Neutral red (NR) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Cisplatin was provided by Melatonin Research Group, KKU. Dimethyl sulfoxide (DMSO) was purchased from LAB-SCAN, Analytical Science, Ireland.

2 Plant extraction

The *C. formosum* were macerated with 50% hydro-alcohol and filtered to collect the filtrate. The solvent was removed to get the dry residue by using a rotary evaporator yielding the crude extracts.

3 Cell culture

Human hepatocellular carcinoma (HepG2) cells were cultured with Dulbecco's Modified Eagle Medium (DMEM). The medium was supplemented with 10% fetal bovine serum, penicillin and streptomycin. The cell line was cultured in a humidified 5% CO_2 atmosphere at 37 $^{\circ}$C.

4 Induction of resistant HepG2 cell model

The parental HepG2 cells were induced by cisplatin exposure to lower concentration of drugs for 24 hours. The treated cells were washed with (1X) PBS and cultured in the drug-free medium. After 1-2 days, the dead cells were washed out with (1X) PBS and cells were cultured in the drug-free medium again. The induced cells were subcultured after 70% confluence. Similar step was repeated until the resistant index was achieved (Zhou et al., 2010).

5 Cell viability assay

The cell survivals were tested by neutral red assay. HepG2 cells were seeded into 96-wells plates in the medium, and incubated for 24 h. Various concentrations of sample in medium were added into the HepG2 cells, respectively for 24 h. The untreated cell was a control experiment which only contained the complete medium and cells. The NR solution was added into each well and incubated for 2 hours. Cells were washed and solubilized with 0.33% HCl/ isopropanol. The absorbances of viable cells with NR were detected at dual wavelengths (537 nm and 650 nm).

6 Flow cytometry

Cells were treated with samples for 24 hours. The control experiment was the untreated cells, which only contained the complete medium and cells. After treatment, cells were washed with PBS harvested. The cells were resuspended in binding buffer. Cells were stained with FITC-AV that can stain to the surface of early membrane-intact apoptosis cells. Then PI was added and analyzed by using flow cytometry. PI can bind to DNA in the membrane damaged cells which identifies necrotic cells.

Results and discussions

1 Induction of resistant R-HepG2 cell models

The calculated IC_{50} value of cisplatin treated in parental and resistant HepG2 cells were 4.7±0.2 and 16.6±0.4 µg/ml, respectively with approximate 3.5 RI. This resistant model could be used for screening the anticancer activity of the medicinal plant.

2 Cytotoxicity of *C. formosum* in both cells

The parental and resistant HepG2 cells were treated with various concentrations of *C. formosum* for 24 hours. Cell viability was determined by NR assay. Vero cells were used for normal cells. IC_{50} of *C. formosum* in parental and resistant HepG2 cells were 113.2±11 and 196.7±19µg/ml, respectively. Moreover, IC_{50} of *C. formosum* was 362.39±12 µg/ml in vero cells. *C. formosum* showed selectivity in parental than resistant HepG2 cells. From results, *C. formosum* exhibited cytotoxic activity in both cells. Several of chemotherapeutic drugs can inhibit cancer cells proliferation by induction of apoptosis pathway. Apoptosis is a target of many cancer treatments[1]. Since, the anticancer effect of *C. formosum* was studied on apoptosis induction by flow cytometry.

3 Flow cytometry

C. formosum induced apoptosis in both cells as shown in Table 1. The % average apoptosis of cisplatin was significantly increased in resistant when compare to parental HepG2 cells (P value = 0.01). *C. formosum* induced apoptosis in both cells. While, *C. formosum* showed slightly effect on necrosis in resistant HepG2. Therefore, *C. formosum* can induce apoptosis in both cells. More information about mechanism of cell dead should be investigation.

Table 1 Effect of *C. formosum* on apoptosis induction.

Samples	% Average apoptosis		% Average necrosis	
	Parental HepG2 cells	Resistant HepG2 cells	Parental HepG2 cells	Resistant HepG2 cells
untreated	20	21	7	0
cisplatin	59	48	1	1
C. formosum	39	41	2	20

Conclusion

This resistant model could be used for screening the anticancer activity of the medicinal plants. *C. formosum* showed more cytotoxic activity in parental HepG2 than resistant HepG2 cells. Moreover, *C. formosum* induced apoptosis in parental HepG2 and resistant HepG2 cells. Therefore, *C. formosum* showed anticancer activity in both cell lines.

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Abbreviations

Inhibitory concentration at 50% (IC_{50}), Resistant Index (RI)

References

Nonpunya A, Weerapreeyaku N, Barusrux S. *Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kurz) Gogel. (Hóng yá mù) extract induces apoptosis in human hepatocellular carcinoma HepG2 cells through caspase-dependent pathways. *Chinese Medicine*. 2014; 9: 1-12.

Dai Y, Lawrence T.S, & Xu L. Overcoming cancer therapy resistance by targeting inhibitors of apoptosis proteins and nuclear factor-kappa B. *Am J Transl Res*.2009:1; 1-15.

Freeburg M.E, Alicia A.G, Seidel E.E, Telleria C.M. Resistance to cisplatin does not affect sensitivity of human ovarian cancer cell lines to mifepristone cytotoxicity. *Cancer Cell International*.2009; 9: 1-13.

Phadungkit M, Rattarom R, Rattana S. Phytochemical screening, antioxidant, antibacterial and cytotoxic activities of *Knema angustifolia* extracts. *J Med Plant Res*. 2010;4: 1269-1272.

Zhou Y, Ling XL, Li SW, Li XQ, Yan B. Establishment of a human hepatoma multidrug resistant cell line in vitro. *WJG*. 2010;16:2291-2297.