ความเป็นพิษของกลูโคนาสเตอติอินและสารอนุพันธุ์ต่อเซลล์ MCF-7 และ HepG2

Cytotoxicity of Gluconasturtiin and Its Derivative against MCF-7 and HepG2

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บทคัดย่อ

มีผลงานวิจัยเผยแพร่อย่างกว้างขวางเกี่ยวกับความสามารถในการต้านมะเร็งของสารออกฤทธิ์ทางชีวภาพประเภทไอโซไธโอไซยาเนทในพืชตระกูลกะหล่ำ ในงานวิจัยนี้ได้ทำการศึกษาความสามารถในการเป็นพิษต่อเซลล์มะเร็งเปรียบเทียบระหว่าง gluconasturtiin สารตั้งต้นที่พบตามธรรมชาติใน watercress และสาร phenethyl isothiocyanate (PEITC) ซึ่งเป็นสารอนุพันธุ์ในเซลล์มะเร็งเต้านม (MCF-7) และเซลล์มะเร็งตับ (HepG2) โดยวิธี MTT assay ช่วงความเข้มข้นที่ศึกษาได้แก่ 0-100 µM และผันแปรระยะเวลาในการบ่มสารเป็น 24 48 และ 72 ชั่วโมง โดยมี paclitaxel เป็นตัวอย่างควบคุมที่เป็นบวก ผลการทดลองพบว่า การมีชีวิตของเซลล์ MCF-7 ลดลงเมื่อเลี้ยงด้วยอาหารที่มีสาร PEITC โดยความเข้มข้นที่ทำให้การมีชีวิตของเซลล์ลดลงร้อยละ 50 หรือ IC50 มีค่าเป็น 10.22 3.61 และ 2.68 µM ในขณะที่ค่า IC50 ของ PEITC ใน HepG2 มีค่าเป็น 6.74 4.22 และ 4.19 µM ตามลำดับ นอกจากนี้พบว่า gluconasturtiin ไม่มีความเป็นพิษต่อเซลล์มะเร็งทั้งสองชนิดในช่วงความเข้มข้นที่ทำการศึกษา และเพื่อศึกษากลไกความเป็นพิษของ PEITC จึงได้ทำการทดสอบความสามารถในการทำให้เกิดอะพอพโทสิสในเซลล์ MCF-7 โดยวิธี fluorescence microscopy ด้วยเทคนิคการย้อมด้วย AO/PI ผลการทดลองเลี้ยงเซลล์ MCF-7 ในสภาวะที่มี PEITC ที่ความเข้มข้นเท่ากับ IC50 เป็นเวลา 48 ชั่วโมง พบว่าโครมาตินของเซลล์เกิดการจับกันแน่นและนิวเคลียสเกิดการแตกหัก และพบว่ากลไกการเป็นพิษต่อเซลล์ของ PEITC เกี่ยวข้องกับกระบวนการอะพอพโทสิส แต่อย่างไรก็ตามควรมีการศึกษาความเป็นพิษต่อเซลล์ปกติต่อไป

ABSTRACT

The putative anticancer abilities of isothiocyanates, bioactive compounds in brassica vegetables, have been widely published. In the current study, gluconasturtiin, found naturally in watercress, and its derivative, phenethyl isothiocyanates (PEITC), toxicity against human breast cancer cells MCF-7 and hepatoma cell line HepG2 were determined employing MTT assay. A range of concentrations between 0-100 µM was used to study dose dependent cytotoxicity at 24, 48 and 72 hrs incubation periods. Paclitaxel at the same concentrations was served as a positive control. The results showed that in MCF-7 cell line, PEITC led to a time-dependent decrease in cancer cells viability with IC50 of 10.22, 3.61 and 2.68 µM at 24, 48 and 72 hrs, respectively, whereas those of HepG2 were 6.74, 4.22 and 4.19 µM. Moreover, gluconasturtiin had no effect on both cell lines at the concentration range studied. Apoptogenic effects of PEITC on MCF-7 were studied using fluorescence microscopy (AO/PI double staining). When cells were exposed to PEITC for 48 hrs at IC50 dose chromatin condensation and nuclear fragmentation were noticed. Moreover, apoptosis is one of chemopreventive mechanisms of PEITC which, however, toxicity on normal cells should be further investigated.

คำสำคัญ: พืชตระกูลกะหล่ำ วิธี MTT assay ความเป็นพิษต่อเซลล์ กลูโคซิโนเลท
Keywords: Brassica, MTT assay, Cytotoxicity, Glucosinolates

INTRODUCTION

Cruciferous vegetables, i.e. watercress, Brussels sprouts, broccoli, cabbage, kai choi, kale, horseradish, radish and turnip are categorised within the brassica family (Brassicaceae). Recent scientific research has focused on the chemopreventive effect of cruciferous vegetables due to their high content of beneficial substances, glucosinolates and their metabolites, isothiocyanates (ITCs) (Verhoeven et al., 1997). When the tissue of cruciferous plants is disrupted by mechanical food processing or masticating, glucosinolates are released from the vacuoles and hydrolysed by cytosolic myrosinase, a thioglucoside glucohydrolase enzyme (EC 3.2.3.1) (Nugon-Baudon and Rabot, 1994). Gut flora such as *Escherichia coli and Bacteroides vulgatus*, have also been reported to contribute to dietary glucosinolate hydrolysis (Rabot et al., 1993; Shapiro et al., 1998). Myrosinase breaks the beta-thioglucosidic bond of the glucosinolate molecule to produce glucose, sulphate and unstable aglycone intermediates, which then undergo non-enzymatic intramolecular (Lossen) rearrangement to yield isothiocyanates as shown in Fig. 1 (Fahey and

Talalay, 1999). It has been reported that ITCs exert their chemopreventive activity by enhancing the elimination of carcinogenic substances from the body, inducing apoptosis (programmed cell death) of pre-cancerous cells and by arresting cell proliferation. There is evidence that the intact glucosinolate from broccoli was not active in modulating xenobiotic-metabolising enzymes, and incubation with myrosinase to promote degradation of the glucosinolates to ITCs was necessitated (Vang et al., 2001). Furthermore, some studies demonstrated that some glucosinolates also have adverse effects by inducing DNA damage, which may develop to cancerous cells (Conaway et al., 1999; IARC, 2004; Robbins et al., 2005). There are numbers of research showed cytotoxicity of ITCs in different cell lines. However, the effects are poorly understood in glucosinolates including gluconasturtiin which is the precursor of a potent anticancer compound phenethyl isothiocyanate (PEITC).

Therefore, the current study was aimed to investigate the cytotoxicity property of gluconasturtiin in comparison with phenethyl isothiocyanate. MTT assay was carried out in human breast cancer cells MCF-7 and hepatoma cell line HepG2. Paclitaxel was served as a positive control.

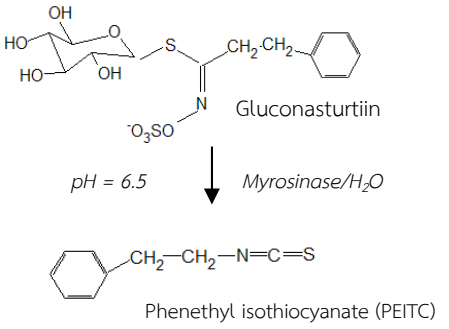

Figure 1 Generation of the breakdown product phenethyl isothiocyanate (PEITC) from myrosinase-dependent gluconasturtiin hydrolysis.

MATERIALS AND METHODS

1. Materials

Glucosaturtiin and thioglucosidase from Sinapsis alba seed were a gift from CRA Agricultural Research Council, Italy. Roswell Park Memorial Institute (RPMI)-1640 medium, DMSO, fecal calf serum (FCS) Trypsin-EDTA solution, penicillin/streptomycin, thiazolyl blue tetrazolium bromide, acridine orange and propidium iodide were purchased from Sigma-aldrich (Singapore).

2. Human cell lines

Human cancer cell lines namely breast (MCF-7) and liver (HepG2) were grown in RPMI-1640 supplemented with 10% FCS and 1% penicillin/streptomycin. The media was stored in refrigerator (2-8°C) and warmed to 37 $^{\circ}$C before use. The cell lines were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity prior to utilization in further steps. The remaining cells were stored

in cryopreservation medium containing 90 % FCS and 10 % DMSO.

3. *In vitro* assay for cytotoxic activity

The cytotoxic potential of the test material in human cancer cell lines were determined employing the method modified from van Meerloo and colleagues (van Meerloo et al., 2011).

3.1. Preparation of cell suspension for assay

Human cancer cell lines were grown in 25 mL conical flasks at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity in complete growth medium to obtain enough number of cells as described above. 1 mL of Trypsin-EDTA was added into 80% confluency in flask in order to harvest cells. The flask was then incubated at 37 °C for 5-7 min and detached cells were collected. Viable cell density at 1 x 10^5 cells were plated in each well. 100 µL of cell suspension in complete growth medium was added into each well. The plates were incubated at 37 °C for 24 hrs in an atmosphere of 5% CO_2 and 95% relative humidity in an incubator. After incubation, the test material and positive control were added.

3.2. Treatment of cells

Serum free RPMI medium containing gluconasturtiin or paclitaxel (positive control) at a range of 0-100 µM was prepared. The 200 µL of RPMI in the presence of test material or positive control was plated in each well. In case of PEITC, 0.3 unit/mL of myrosinase in water was prepared prior to 5 µL was added directly into gluconasturtiin containing medium. Plates were incubated at various incubation periods (24, 48 and 72 hrs) at 37 °C and 5% CO_2 and 95% relative humidity in a CO_2 incubator. The procedure was replicated three times.

3.3. MTT assay

Prior to treatment, the media were discarded and 20 µL of 5 mg/mL MTT prepared freshly in PBS was added. Incubation for 3 hrs at 37 °C in the dark was required. At the end, MTT was discarded and 100 µL DMSO added to start the reaction, absorbance at 570 nm, which indicated viability of cells, was used. Finally, % cells viability and IC50 were calculated.

4. AO/PI staining

AO/PI double staining method was performed according to Guan and colleagues (Guan et al., 2007). Acridine orange (AO) and propidium iodide (PI), nuclear staining dyes, were employed. AO is permeable to live and dead cells while PI enters only dead cells.

MCF-7 was selected to study the apoptotic effect of sample. Firstly, MCF-7 was grown in 25 mL flask for 48 hrs prior to 10 mL of serum free medium containing IC50 of PEITC was added into cells before incubation at 37 °C for 48 hrs. AO and PI stock solutions

at concentration of 1 mg/mL PBS were prepared following with dilution to a working solution at 100 µg/mL. Then equal amount of AO and PI was mixed before use. Attached cells in media were trypsinized and collected. The total cells were spun down at 1,500 rpm for 5 min and washed twice with 10 mL PBS. Immediately, cells were stored on ice and 10 µL was mixed with 10 µL AO/PI solution and placed on a slide prior to viewing under fluorescence microscope to determine the apoptotic cells.

RESULTS AND DISCUSSION

Watercress (Nasturtium officinale) is the most important source of gluconasturtiin, the conversion of which to PEITC following human consumption ranges from 30 to 67% (Chung et al., 1992). It has been reported that PEITC showed high chemoprevention potential. However, such ability in gluconasturtiin, to our knowledge, is yet to be confirmed.

Cytotoxicity of glucosinolate in MCF-7 and HepG2 was assessed employing MTT assay. Figure 2A shows that gluconasturtiin had no effect on viability of MCF-7 at all exposure periods up to the concentration of 100 µM. The effect was, on the other hand, pronounced when PEITC at the same range of concentrations was employed (Figure 2B). Moreover, the higher concentrations led to decreasing in cell viability in an exponential manner. IC50 (concentration causing death of 50% of cells) values of PEITC were 10.22, 3.61 and 2.68 µM at 24, 48 and 72 hrs, respectively (Table 1). These values were only double of those treated with placitaxel, a chemotherapy drug, as can be seen in Figure 2C and Table 1. The IC50 of paclitaxel were 3.50, 1.08 and 0.94 µM at 24, 48 and 72 hrs, respectively. Previous study reported that IC50 of paclitaxel in MCF-7 was 111 nM or 0.11 µM (Liu et al., 2013) which is ten times lower than the findings in the current study. The discrepancy may be due to the nature of cells and laboratory procedures. Nonetheless, the results infer that PEITC is a potent compound that can inhibit breast cancer cells proliferation as strong as modern drug. When compare the effects of glucosinolates and isothiocyanates, it is obvious that the central carbon atom of the isothiocyanate group (–N=C=S) is responsible for such superior potential of isothiocyanates. The thiocyanate group of PEITC is highly electrophilic and the biological activities of ITCs may be primarily mediated through the reaction of this carbon atom with cellular nucleophilic targets (IARC, 2014). However, its mechanisms on proliferation inhibition must be further studied. The results were time dependent where 48 hrs exposure led to a greater inhibition effect compared to 24 hrs. However, similar effect with 48 hrs incubation was seen after 72 hrs.

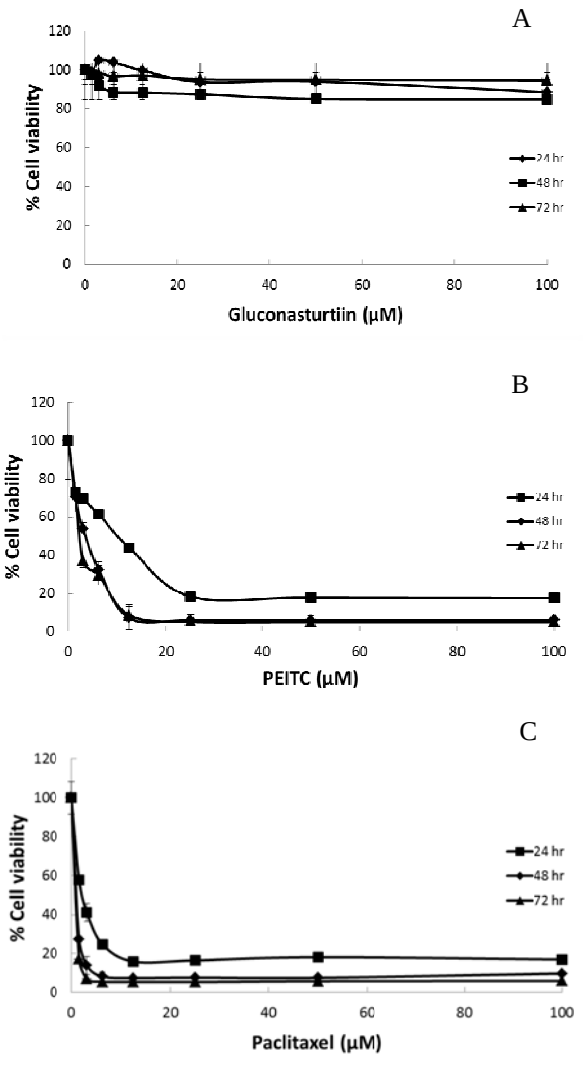

Figure 2 Effects of gluconasturtiin (A), phenethyl isothiocyanate (PEITC) (B) and paclitaxel (C) treatment in MCF-7 twenty four-hrs after seeding of cells in 96 well plates, test compounds and positive control were added to the final concentrations shown in the figure. Incubation periods were 24 hrs (■), 48 hrs (♦) and 72 hrs (▲) of treatment. Effects of gluconasturtiin and PEITC against the viability of MCF-7 cells were evaluated through the MTT assay.

Table 1 IC50 values for MCF-7 and HepG2 cells obtained from MTT assay following exposure to gluconasturtiin and PEITC for 24, 48 and 72 hrs.

Cell lines	Exposure time (hrs)	IC50 (μM)
MCF-7	24	10.22
	48	3.61
	72	2.68
HepG2	24	6.74
	48	4.22
	72	4.19

Likewise, but to a greater extent, PEITC decreased HepG2 cells viability (Figure 3B) whereas that of glucosinolate there was no effect (Figure 3A). IC50 values of PEITC in HepG2 were lower than that of MCF-7 at 24 hrs exposure time being 6.74, 4.22 and 4.19 μM at 24, 48 and 72 hrs, respectively. It can be said that liver cancer cells are more susceptible to PEITC compare to breast cancer cells. Previous study has reported that anticancer ability of PEITC is tissue dependent. Employing *in vivo* study, PEITC modulated biotransformation enzymes with the liver being the most sensitive organ and lung was resistant (Konsue and Ioannides, 2008). Therefore, the different effect of PEITC on antiproliferation of liver and breast cell lines is expected. However, after 48 hrs treatment, the toxicity effect of PEITC in MCF-7 was more pronounced.

Glucosinolate only be converted to isothiocyanate by the enzyme myrosinase after mechanical mastication. As a result, it is important to point out that health benefit effects of watercress, a major source of gluconasturtiin, will be achieved only when consumed raw. The IC50 values in this experiment are achievable by dietary consumption (Konsue et al., 2010). Moreover, a compound with IC50 less than 20 μg/mL or 0.1 mM is considered highly cytotoxic. Regarding to the IC50 of PEITC, it can be considered as bioactive compound (Mahavorasirikul, 2010). Previous studies also reported IC50 of PEITC in MCF-7 following 48 hrs exposure being 7.32 μM (Tseng et al., 2004) and 5.6 μM (Liu et al., 2013). To our knowledge, IC50 of PEITC in HepG2 has never been reported as well as the cytotoxicity of gluconasturtiin.

Apoptosis is one of the mechanisms to inhibit cancer. AO/PI staining results showed nuclear morphology of MCF-7. Figure 4 shows chromatin condensation and nuclear fragmentation of MCF-7 after 48 hrs exposure with PEITC indicating occurrence of apoptotic process. This infers that apoptosis is one of the cytotoxicity mechanisms of PEITC on cancer cell lines.

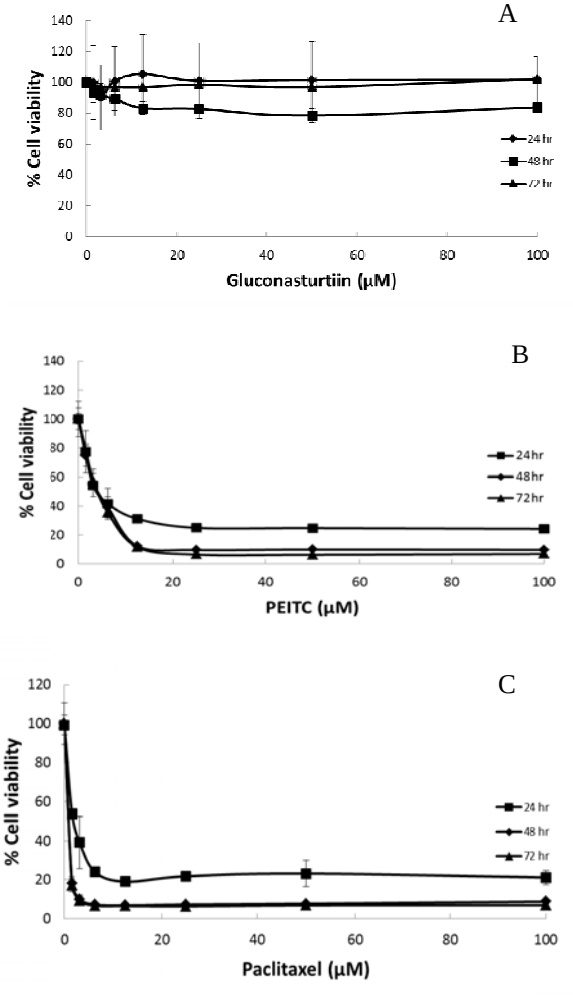

Figure 3 Effects of gluconasturtiin (A), phenethyl isothiocyanate (PEITC) (B) and paclitaxel (C) treatment in HepG2 twenty four-hrs after seeding of cells in 96 well plates, test compounds and positive control were added to the final concentrations shown in the figure. Incubation periods were 24 hrs (■), 48 hrs (♦) and 72 hrs (▲) of treatment. Effects of gluconasturtiin and PEITC against the viability of HepG2 cells were evaluated through the MTT assay.

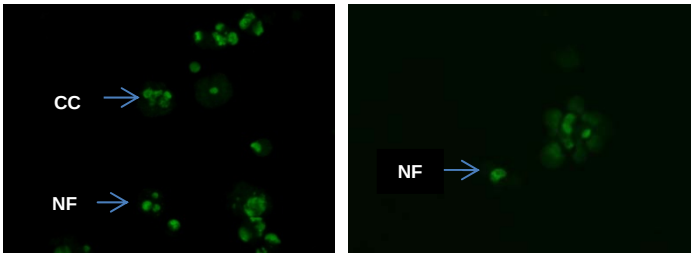

Figure 4 Fluorescent micrograph of AO/PI under darkfield fluorescence microscopy. MCF-7 was treated with IC50 of PEITC for 48 hrs.
CC: chromatin condensation
NF: nuclear fragmentation

CONCLUSIONS

Glucosinolate, a precursor of potent anticancer PEITC, had no effect on cell viability of breast and liver cancer cells. PEITC, on the other hand, inhibited cells proliferation at the dose achievable by human dietary doses. The mechanism of inhibition was enhancement of chromatin condensation and nuclear fragmentation. Lacking of ability to inhibit cancer cell proliferation by gluco-nasturtiin suggested that cruciferous vegetables should be consumed raw to allow conversion of glcosinolate to isothiocyanates, compounds that possesses higher anticancer action.

ACKNOWLEDGEMENTS

The authors would like to thank Mae Fah Luang University and cancer research laboratory, Institute of Bioscience, University Putra Malaysia, Malaysia, for technical and facility support.

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