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Sulforaphene and sulforaphane in commonly consumed cruciferous plants contributed to antiproliferation in HCT116 colon cancer cells

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ABSTRACT

Objective: To analyze two isothiocyanates (sulforaphene and sulforaphane) and their antiproliferative effect of 11 indigenous cruciferous vegetables.

Methods: Phytoconstituents identification was conducted by high performance liquid chromatography and gas chromatography-mass spectrometer techniques. The antiproliferation was evaluated in colon cancer cell line HCT116 by MTT assay.

Results: Isothiocyanate identification by high performance liquid chromatography showed that broccoli, cabbage, "Khi-Hood" (*Raphanus sativus* L. var. *caudatus* Alef) and Chinese radish contained isothiocyanates sulforaphane. Sulforaphene and sulforaphane in broccoli, cabbage and "Khi-Hood" were characterized by the gas chromatography-mass spectrometer analysis. Antiproliferation screening by MTT assay found that the potent plants which possessed IC_{50} below 50 μg/mL were cabbage and "Khi-Hood", while the others had low antiproliferation with IC_{50} higher than 50 μg/mL. Difference in antiproliferation was probably due to difference existed phytochemical constituents in each plant. "Khi-Hood" possessed the highest antiproliferation against HCT116 with the lowest IC_{50} at (9.42 ± 0.46) μg/mL. The IC_{50} of chemotherapeutic drug (mitomycin C) was (19.12 ± 1.00) μg/mL, while both melphalan and 5-fluorouracil possessed the IC_{50} value higher than 50 μg/mL.

Conclusions: Commonly consumed cruciferous vegetables exerted varied antiproliferation and isothiocyanate contents. High isothiocyanate content in "Khi-Hood" was contributed to high antiproliferation. Among 11 plants studied, "Khi-Hood" could be an alternative chemopreventive diet.

1. Introduction

In the present, many epidemiological studies have shown the association between cruciferous vegetables consumption and lower risk of several cancer incidences such as lung and colorectal cancer [1]. Therefore, many researchers have focused on finding an active compound in cruciferous crops, plant of the family Brassicaceae (also called Cruciferae), as a potential chemopreventive source. The bioactive compound in cruciferous plants associated with chemopreventive property is isothiocyanates [2].

Isothiocyanates compounds have been studied intensively for its anticancer property. The compounds have not only shown chemopreventive property but also chemotherapeutic property in both *in vitro* and *in vivo* studies [3]. These properties make isothiocyanates to be a good anticancer candidate. Many studies have been performed to identify and quantify isothiocyanates in cruciferous plants. High amount of isothiocyanates, such as sulforaphane, phenethyl isothiocyanates and allyl isothiocyanate, were found in broccoli, cabbage and

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cauliflower [4]. Hence, these plants have been recognized as a source of isothiocyanates as well as a source of anticancer agent.

Due to the limited information of the isothiocyanates in indigenous Thai cruciferous vegetables, this study aims to identify sulforaphane, as the representative of isothiocyanates, in these plants as well as corresponding antiproliferation indicating anticancer potential of the extracts from the indigenous Thai cruciferous vegetables in colon cancer cell line HCT116. Consequently, this study provides the useful evidence for supporting the use of Thai indigenous cruciferous vegetables as a source of sulforaphane and a source of anticancer agent.

2. Materials and methods

2.1. Materials

D,L-Sulforaphane standard was purchased from Calbiochem (EMD, Darmstadt, Germany). L-Sulforaphene standard was purchased from Enzo Life Science (New York, USA). Dichloromethane (Fisher Scientific, UK) was analytical grade and acetonitrile was high performance liquid chromatography (HPLC) grade (RCI Labscan, Thailand).

2.2. Plant extraction

Cruciferous plants were collected from local market in Khon Kaen and Phayao Province, Thailand, during 2010–2011. The crude extracts were prepared according to the method previously described with slight modification [5]. Each plant was homogenized (50 g fresh weight: 50 mL water) for 30 min and placed at room temperature for 2 h to allow autolysis. The homogenates were extracted with dichloromethane. The dichloromethane layer was collected, filtered and dried by using rotary evaporator. Stock solutions of each plant extracts were prepared in acetonitrile.

2.3. HPLC fingerprints analysis

The cruciferous plant extracts were analyzed by the reversed-phase HPLC (Agilent, 1100 Series G1310A, Germany) to determine sulforaphane content following the methods of Campas-Baypoli *et al.* [6] and Liang *et al.* [5] with minor modifications. The analysis was carried out with acetonitrile: ultrapure water (30:70, v/v) as a mobile phase with isocratic elution on C18 (25 cm × 4.6 mm, 5 μm) column (HiQsil, Japan) at a flow rate of 0.6 mL/min. Detector (Agilent, 1100 Series G1314A, Japan) wavelength was set at 254 nm.

2.4. Gas chromatography-mass spectrometer (GC-MS) operating condition

GC-MS analysis (GC: Agilent, 6890N, China; MS: Agilent, 5973 inert, USA) was performed as previously described to characterize sulforaphane and sulforaphene in cruciferous vegetables [7]. Ultra-high purity helium was used as a carrier gas and the flow rate was set at 40 cm/s. The oven temperature was programmed at 50 °C for 5 min, then increasing to 250 °C in increment of 10 °C/min and remaining steady at 250 °C for 10 min, while injector temperature was steady at 250 °C. Ion source temperature was set at 180 °C and a mass spectrum was obtained by electron ionization at 70 eV.

2.5. Cell culture and antiproliferation test

The human colon cancer (HCT116) cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 °C with 95% air and 5% CO_2. Briefly, cells at a density of 5×10^3 cells/well were plated in 96-well microplates and in 100 μL culture medium. Thereafter, 100 μL of the crude extract in acetonitrile with concentration ranging from 1 to 250 μg/mL was added into each well. The final concentration of acetonitrile in culture medium was maintained to be lesser than 0.2% to avoid solvent toxicity. MTT (Ambresco, USA) solution (in phosphate buffer saline) was added into each wells (final concentration of 0.5 mg/mL) in 22 h and incubated for another 2 h in CO_2 incubator. Then, the medium was discarded and 150 μL of dimethyl sulfoxide was added to solubilize formazan. Absorbance of formazan product was measured at 570 nm wavelength and percentage of antiproliferation was calculated [8]. The chemotherapeutic drugs which were mitomycin C, melphalan and 5-fluorouracil were used as positive control.

3. Results

A total of 11 local cruciferous plants used in this study were "Chun Chai Hang Hong" [*Brassica juncea* var. *sareptana* Sinskaja (*B. juncea* var. *sareptana*)], "San" [*Brassica juncea* var. *gracilis* (*B. juncea* var. *gracilis*)], "Chao Mom" [*Brassica juncea* (*B. juncea*)], "Hin" [*Brassica juncea* L. Czern & Coss (*B. juncea* L. Czern & Coss)], broccoli [*Brassica oleracea* var. *italica* (*B. oleracea* var. *italica*)], cabbage [*Brassica oleracea* var. *capitata* (*B. oleracea* var. *capitata*)], "Khi-Hood" [*Raphanus sativus* L. var. *caudatus* Alef (*R. sativus* L. var. *caudatus* Alef)], Chinese radish [*Raphanus sativus* var. *longipinnatus* (*R. sativus* var. *longipinnatus*)], Chinese cabbage [*Brassica rapa* var. *pekinensis* (*B. rapa* var. *pekinensis*)] and two different varieties of choy sum [*Brassica chinensis* Jusl. var. *parachinensis* (Bailey) Tsen and Lee (*B. chinensis* Jusl. var. *parachinensis* (Bailey) Tsen and Lee)]. The edible parts of all cruciferous vegetables were extracted and analyzed by HPLC-UV. The percentages of yields of the extracts compared to the fresh weight of each vegetable were shown in Table 1. "Khi-Hood" had the highest yield of 0.029% per fresh weight. Since the presence of isothiocyanate compounds such as sulforaphane and sulforaphene were previously reported in various cruciferous plants, sulforaphane and sulforaphene were determined in the plant extracts [9].

The HPLC chromatograms of each vegetable extracts as well as standard sulforaphane and sulforaphene were shown in Figures 1–14. The HPLC peaks of isothiocyanates, sulforaphane and sulforaphene, were presented at the retention time of 11.4 min and 11.1 min, respectively (Figures 1 and 2). When standard sulforaphane and sulforaphene were co-injected, the peaks were merged and detected at retention time 11.1 min (Figure 3). Thus, this merged peak was primarily used to indicate the presence of the combined sulforaphane and sulforaphene. It was interesting that although the extracts of each vegetable had been extracted using the same method and from the same cruciferous family, their HPLC chromatograms were clearly different indicating the existence of different phytochemical compositions in each of the plant extracts (Figures 4–14). The distinct anticancer isothiocyanates (*viz.*, sulforaphane and sulforaphene) in the edible part of

Table 1
IC_{50} values of antiproliferation of cruciferous plants in 24 h exposure time against HCT116 colon cancer cell line.

Groups	Scientific name	General name or Thai name	Part used	Yield (per fresh weight, %)	IC_{50} (μg/mL)
Cruciferae plants	*B. juncea* var. *sareptana*	Chun Chai Hang Hong	Leave	0.020	> 50
	B. juncea var. *gracilis*	San	Leave	0.011	> 50
	B. juncea	Chao Mom	Leave	0.021	> 50
	B. juncea L. Czern & Coss	Hin	Leave	0.014	> 50
	B. oleracea var. *italica*	Broccoli	Flower	0.013	> 50
	B. oleracea var. *capitata*	Cabbage	Leave	0.012	46.03 ± 0.89*
	R. sativus L. var. *caudatus* Alef	Khi-Hood or Thai rat-tailed radish	Pod and flower	0.029	9.42 ± 0.46*
	R. sativus var. *longipinnatus*	Chinese radish	Root	0.009	> 50
	B. rapa var. *pekinensis*	Chinese cabbage	Leave	0.009	> 50
	B. chinensis Jusl. var. *parachinensis* (Bailey) Tsen and Lee (bigger leaves)	Choy sum or Kwang Tung	Leave and flower	0.010	> 50
	B. chinensis Jusl. var. *parachinensis* (Bailey) Tsen and Lee (smaller leaves)	Choy sum or Phak Kad Jon	Leave and flower	0.007	> 50
Chemotherapeutic drugs	Mitomycin C				19.12 ± 1.00
	Melphalan				> 50
	5-Fluorouracil				> 50
Isothiocyanate compounds	Sulforaphane				6.67 ± 0.07*
	Sulforaphene				10.67 ± 2.27*

*Significant difference from positive control (mitomycin C).

"Khi-Hood" were approximated. Only 4 of the 11 cruciferous vegetables tested contained the isothiocyanates (sulforaphane and sulforaphene). The high to low rank of extracted isothiocyanates by comparing peak areas was "Khi-Hood" (pod and flower) > cabbage (leaves) > Chinese radish (root) > broccoli (leaves).

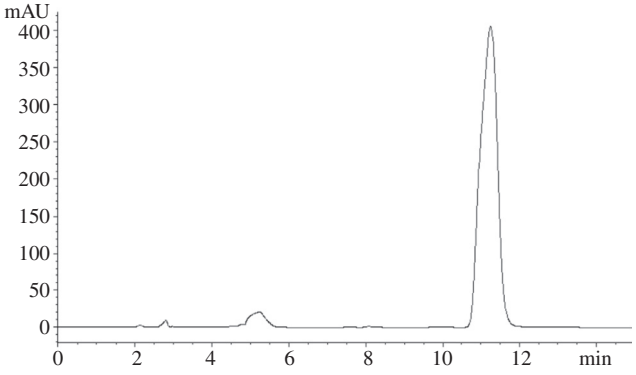

Figure 1. HPLC chromatogram of standard sulforaphane (100 μg/mL) with the retention time at 11.4 min.

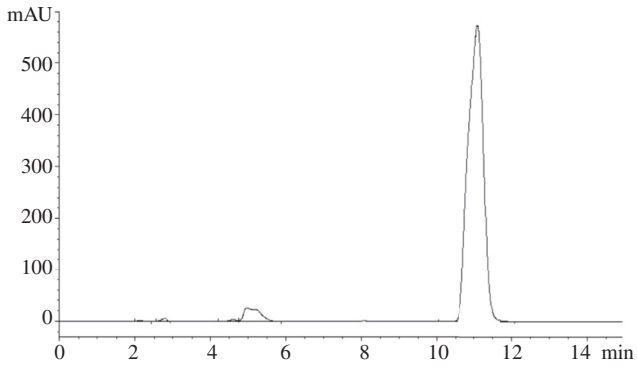

Figure 2. HPLC chromatogram of standard sulforaphene (100 μg/mL) with the retention time at 11.1 min.

These four plants that showed positive HPLC peak for isothiocyanates were further identified by using GC-MS analysis. It was found that some cruciferous plants contained not only sulforaphane but also sulforaphene. GC chromatograms showed the peaks of sulforaphane and sulforaphene at retention time of 19.6 and 19.4 min, respectively (Table 2). The GC-MS profiles illustrated the presence of sulforaphane peaks (m/z = 72, 114 and 160) in three plant extracts including broccoli, cabbage and

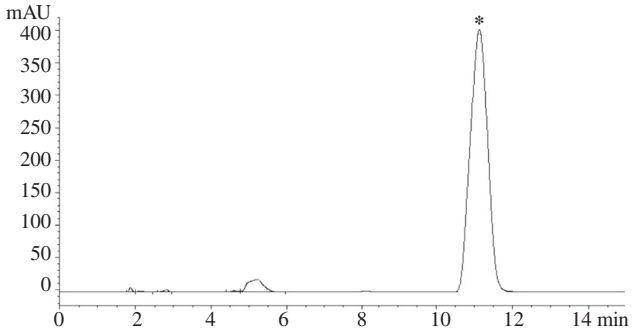

Figure 3. HPLC chromatogram of the co-injection of sulforaphane and sulforaphene (50 μg/mL each).
*: Retention time of sulforaphane and sulforaphene in the extract.

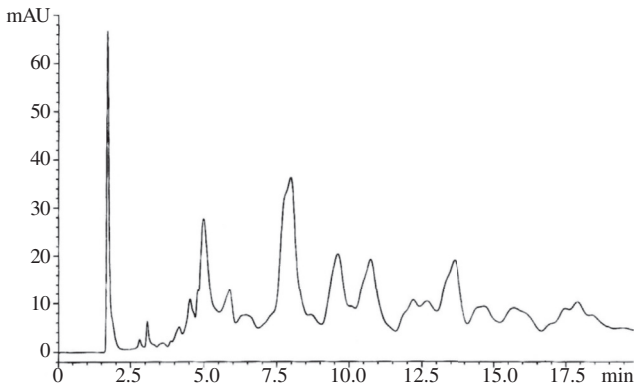

Figure 4. HPLC chromatogram of *B. juncea* var. *sareptana*. (4.2 mg/mL).

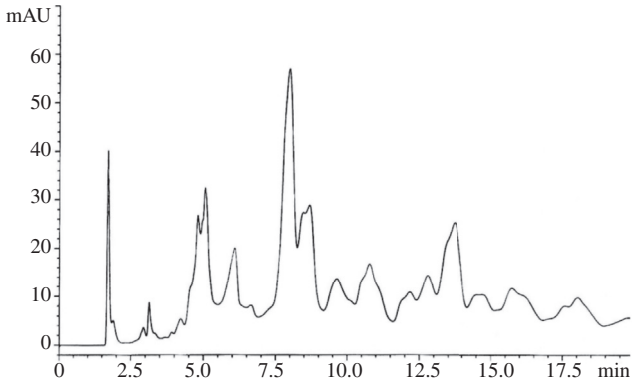

Figure 5. HPLC chromatogram of *B. juncea* var. *gracilis* (2.8 mg/mL).

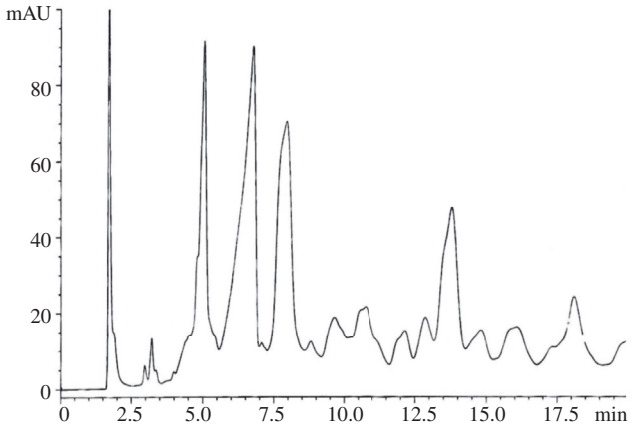

Figure 6. HPLC chromatogram of *B. juncea* (3.9 mg/mL).

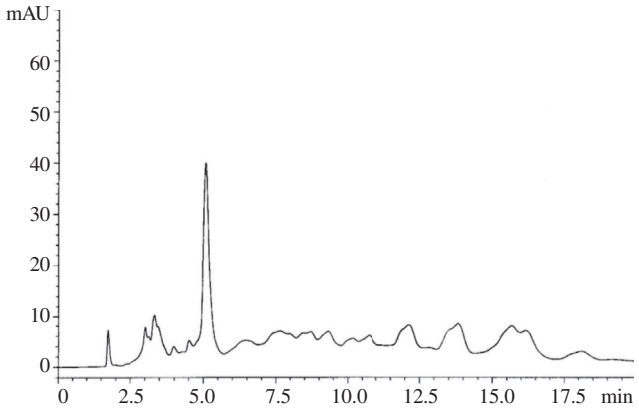

Figure 7. HPLC chromatogram of *B. juncea* L. Czern & Coss (2.3 mg/mL).

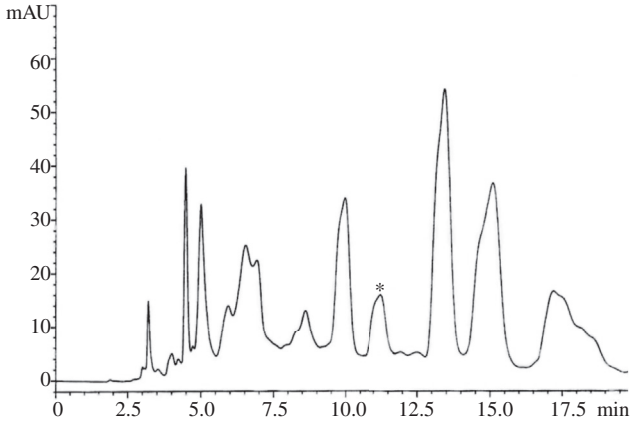

Figure 8. HPLC chromatogram of *B. oleracea* var. *italica* (2.5 mg/mL).
*: Retention times of sulforaphane and sulforaphene in the extract.

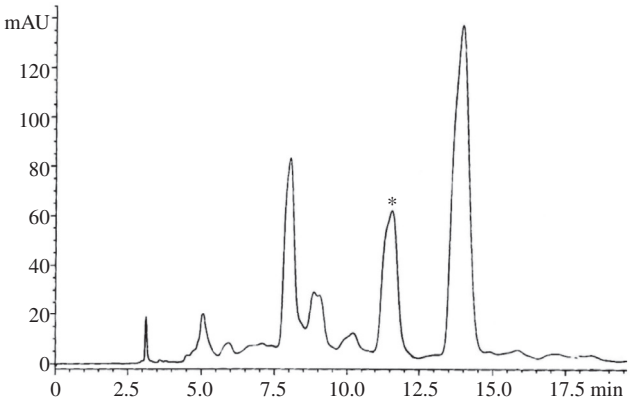

Figure 9. HPLC chromatogram of *B. oleracea* var. *capitata* (1.5 mg/mL).
*: Retention times of sulforaphane and sulforaphene in the extract.

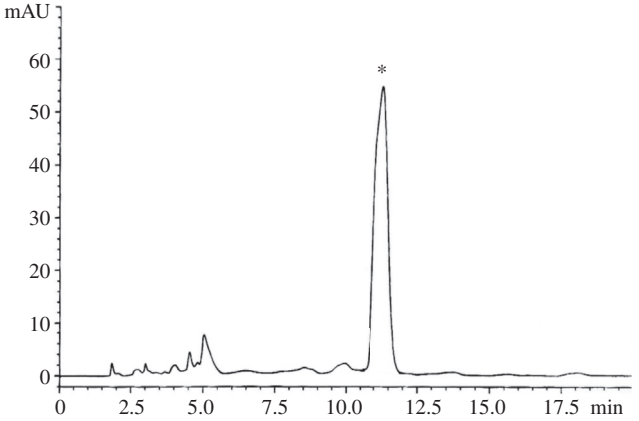

Figure 10. HPLC chromatogram of *R. sativus* L. var. *caudatus* Alef (0.27 mg/mL).
*: Retention times of sulforaphane and sulforaphene in the extract.

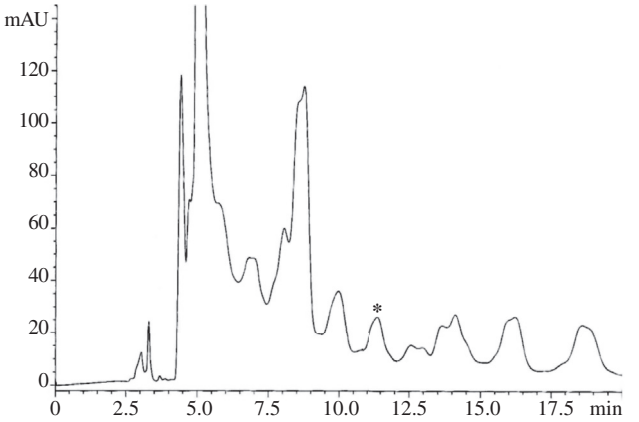

Figure 11. HPLC chromatogram of *R. sativus* var. *longipinnatus* (2.5 mg/mL).
*: Retention times of sulforaphane and sulforaphene in the extract.

"Khi-Hood". The latter also contained high amount of sulforaphene (*m/z* = 72, 112, and 175) (Table 2). Moreover, GC chromatogram of the extracts from broccoli, "Khi-Hood" and cabbage contained the peaks at 8.2 min indicating the presence of 3-butenyl isothiocyanate (*m/z* = 55, 72, 85 and 113), the degraded product of heat-labile sulforaphane from GC-MS analysis. Whilst, Chinese radish did not contain 3-butenyl

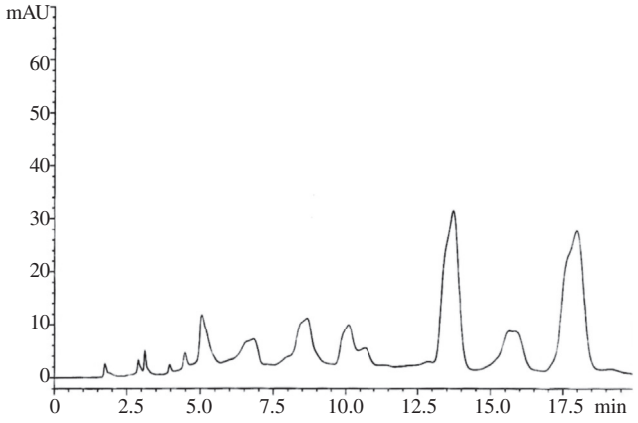

Figure 12. HPLC chromatogram of *B. rapa* var. *pekinensis* (2.3 mg/mL).

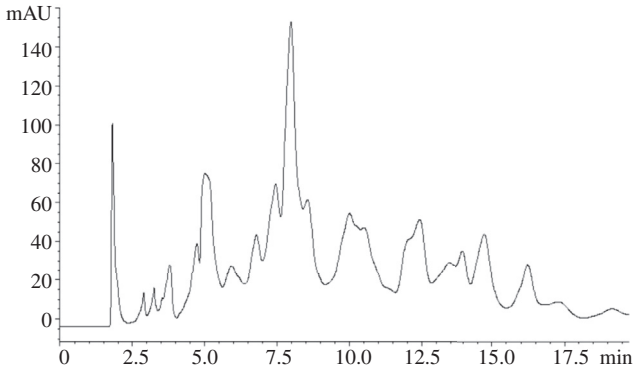

Figure 13. HPLC chromatogram of *B. chinensis* Jusl. var. *parachinensis* (Bailey) Tsen and Lee with bigger leave (8.0 mg/mL).

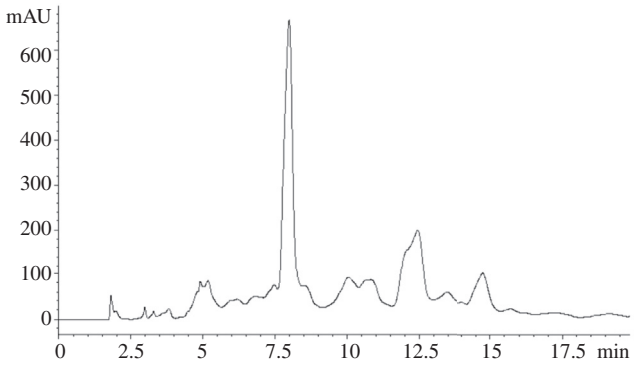

Figure 14. HPLC chromatograms of *B. chinensis* Jusl. var. *parachinensis* (Bailey) Tsen and Lee with smaller leave (2.0 mg/mL).

isothiocyanate indicating no or low sulforaphane content according to GC-MS analysis. Table 3 demonstrates sulforaphane and sulforaphene contents found in these four cruciferous plants.

Due to the diversity of chemical constituents and the variable amounts of sulforaphane and sulforaphene in the cruciferous extracts, the chemopreventive activity was tested. The first chemopreventive test was antiproliferation using the MTT assay. The antiproliferation results of the test compounds against HCT116 colon cancer cell line were presented as IC_{50} (Table 1). Of the 11 cruciferous vegetables, only "Khi-Hood" and cabbage induced HCT116 cell death. The extract from "Khi-Hood" possessed the highest antiproliferation against the HCT116 cell line [IC_{50} = (9.42 ± 0.46) μg/mL] and was classified as an active extract according to National Cancer Research Institute criteria because the IC_{50} value was < 20 μg/mL [9]. The cabbage extract exhibited antiproliferation against the HCT116 with an IC_{50} value of (46.03 ± 0.89) μg/mL, while the IC_{50} value for the other extracts was > 50 μg/mL. In order to avoid the solvent (acetonitrile) cytotoxicity, the highest concentration of the extracts tested was only 50 μg/mL.

The chemotherapeutic drugs (mitomycin C), melphalan and 5-fluorouracil were used as the positive control to compare the sensitivity of the cancer cell line with the clinical and conventional anticancer drugs as well as with the cruciferous plant extracts. Among the chemotherapeutic drugs, only mitomycin C possessed antiproliferation against the HCT116 cells [IC_{50} = (19.12 ± 1.00) μg/mL], whereas the other chemotherapeutic drugs exerted less antiproliferation with IC_{50} > 50 μg/mL. In the current study, sulforaphane and sulforaphene demonstrated a stronger antiproliferation than the chemotherapeutic drugs [IC_{50} = (6.67 ± 0.07) μg/mL and (10.67 ± 2.27) μg/mL, respectively]. "Khi-Hood" extract possessed the lowest IC_{50} among cruciferous plants and chemotherapeutic drugs indicating its greater antiproliferation and suggesting a greater potential chemopreventive activity than the chemotherapeutic drugs.

Table 3
Isothiocyanates contents in cruciferous plants by GC-MS analysis. μg/g.

Cruciferous plants	Yield (fresh weight)	
	Sulforaphane	Sulforaphene
Broccoli	5.80	–
Cabbage	14.69	–
Khi-Hood	19.78	93.12
Chinese radish	–	–

–: Not detected.

4. Discussion

The sulforaphane content in edible part of broccoli and cabbage was previously reported [5]. Sulforaphane content in broccoli ranged from 1.4 to 32.9 μg/g fresh weight, while in cabbage it ranged from 0.7 to 4.7 μg/g fresh weight [5]. The present study on sulforaphane content in broccoli and cabbage is in accordance with the previous reported. Sulforaphene was previously reported to exist in the dichloromethane extracted

Table 2
GC-MS of identified compounds found in cruciferous plants.

Vegetable name	Scientific name	Identified compounds	GC peak (min)	*m/z*
Chinese radish	*R. sativus* L. var. *longipinnatus*	4-(Methylthio)-3-butenyl isothiocyanate	16.60	53, 59, 65, 72, 87, 159
Broccoli	*B. oleracea* var. *italica*	Sulforaphane	19.66	55, 64, 72, 85, 114, 160
Khi-Hood	*R. sativus* L. var. *caudatus* Alef	Sulforaphane	19.41	55, 64, 72, 85, 114, 160
		Sulforaphene	19.60	53, 72, 78, 87, 103, 112, 175
Cabbage	*B. oleracea* var. *capitatata*	Sulforaphane	19.75	55, 64, 72, 85, 114, 160

from seed of "Khi-Hood" [10]. However, the reported amount of sulforaphene was different from that of the present study which might be due to the difference in extracted parts, extraction methods, analytical methods and plant cultivars [10].

Sulforaphane was considered to be the chemopreventive compound in various cruciferous vegetables including broccoli and cabbage. The preventive mechanism of sulforaphane activates mainly via the induction of Phase II metabolism enzymes and induction of apoptosis in cancer cell [3]. Although our study found no sulforaphane and sulforaphene in Chinese radish, another isothiocyanate [4-(methylthio)-3-butenyl isothiocyanate (m/z = 53, 72, 87 and 159)] was detected in this plant at 16.60 min by GC and structure was predicted by MS analysis. Previous studies reported the presence of isothiocyanates in the Chinese radish genus such as phenethyl isothiocyanate, benzyl isothiocyanate, 4-(methylthio) butyl isothiocyanate and 4-(methylthio)-3-butenyl isothiocyanate [11,12]. It was reported that 4-(methylthio)-3-butenyl isothiocyanate was the most abundant isothiocyanates in root of Chinese radish and varied between 70 and 400 μmol/L per 100 g fresh weight depending on plant strains [13]. In addition, this compound was a major contributor for *in vivo* chemopreventive activity of Chinese radish extract [13–15].

The presence of sulforaphane and sulforaphene has already been reported in cruciferous plants as well as their antiproliferative activity [7,10,16–18]. In this study, although the other cruciferous vegetables contained no sulforaphane and sulforaphene or even other isothiocyanates, it did not mean that these vegetables contained no isothiocyanates or other active compounds. It should be noted that the difference in solvents used as well as extraction methods and analytical parts may lead to different amount of isothiocyanates or active compound found in each reports. Thus, the suitable extraction and analytical method is still crucial to track the possible chemopreventive agent presented in these plants.

The different HPLC fingerprints of cruciferous plant extracts indicate the existence of different chemical constituents, which are consequently responsible for their varying antiproliferative activity against the HCT116 cell line. Our HPLC chromatogram displayed the isothiocyanates, as a merged peak from sulforaphane and sulforaphene, from four cruciferous plants including broccoli, cabbage, "Khi-Hood" and Chinese radish. However, the GC-MS analysis on these four plants showed that only broccoli, cabbage, and "Khi-Hood" contained sulforaphane, with the additional sulforaphene in "Khi-Hood". Antiproliferative effect of these 11 cruciferous was also tested using MTT assay. Only "Khi-Hood" and cabbage extract possessed antiproliferative activity against colon cancer cell line HCT116 with IC_{50} lower than 50 μg/mL "Khi-Hood" extract exhibited the highest antiproliferative effect among cruciferous plants, and chemotherapeutic drugs act possibly due to the contribution of both sulforaphane and sulforaphene. Our study showed that some of these cruciferous plants contained isothiocyanates. And the isothiocyanates possibly exhibit their effects via antiproliferative activity observed by MTT assay. This study substantiates the efficacy of cruciferous plant against the colon cancer cell line and suggests the possibility of its use as a potential source of anticancer agent. However, further studies are still required.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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