Asian Pac J Trop Biomed 2017; 7(11): 998–1004

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article https://doi.org/10.1016/j.apjtb.2017.09.022

Sulforaphene in *Raphanus sativus* L. var. *caudatus* Alef increased in late-bolting stage as well as anticancer activity

Piman Pocasap[1], Natthida Weerapreeyakul[2*], Waraporn Tanthanuch[3], Kanjana Thumanu[3]

[1]*Graduate School, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand*

[2]*Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand*

[3]*Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima 30000, Thailand*

ARTICLE INFO

Article history:
Received 2 Sep 2017
Received in revised form 10 Sep 2017
Accepted 28 Sep 2017
Available online 7 Oct 2017

Keywords:
Isothiocyanates
Sulforaphene
Sulforaphane
Thai rat-tailed radish
Anticancer activity
Late-bolting stage

ABSTRACT

Objectives: To evaluate the concentration differences of sulforaphene and sulforaphane at various ages and in different parts of *Raphanus sativus* L. var. *caudatus* with respect to their potential cancer preventive effect on HCT116 colon cancer cells.

Methods: FTIR–ATR and GC–MS were used to characterize the isothiocyanates in the plant extracts followed by HPLC for quantification. Antiproliferation and apoptosis induction were determined by using MTT assay and flow cytometry, respectively.

Results: The respective rank of anticancer activity of *Raphanus sativus* were as follows: vegetative (3 week) < older rosette (4 week) < early-bolting (5 week) < senescence (7 week) < late-bolting (6 week). The low to high concentration of sulforaphene and sulforaphane occurred in the same stage order.

Conclusions: The reproductive parts (flower, pod, and dry seed) of *Raphanus sativus* have the greatest isothiocyanate concentration, evidenced by a sulforaphene concentration higher than the sulforaphane. This result should inform the selection of the most appropriate harvesting stage and plant part for use as a potential chemopreventive agent.

1. Introduction

Isothiocyanates are a group of compounds found mainly in cruciferous plants. Other well-known members of the family are broccoli, cauliflower, kale and cabbage [1]. The presence of the −N═C═S moiety is the unique characteristic of isothiocyanates, which are stored in cruciferous plants in the form of glucosinolates. When the plants are damaged (*i.e.*, from cutting or chewing), the myrosinase enzyme is activated which catalyzes the conversion of glucosinolates to isothiocyanates. The resulting compound is hypothesized to serve as a natural insecticide [2]. The cruciferous family contains several types of isothiocyanates including allyl isothiocyanate, phenethyl isothiocyanate, benzyl isothiocyanate, and sulforaphane. These candidates are being investigated *in vivo* and *in vitro* because of their potent biological activities. The compound also exhibits potent anticancer activities, primarily via induction of phase II metabolism enzymes, which enhance the excretion of carcinogens and induce apoptosis [3,4].

The anticancer mechanisms of isothiocyanates are based on inhibition of cell proliferation, inhibition of tumor invasion, anti-angiogenesis and anti-inflammatory activity. These mechanisms have been observed vis-à-vis many types of cancer *in vitro* including colorectal, breast, lung and prostate cancers [5]. Based on *in vitro* and *in vivo* studies, isothiocyanates induce cancer cell death through an apoptosis induction mechanism [6]. Some mechanisms of clinical agents used for chemotherapeutics (*e.g.*, antiangiogenesis and cancer antibody) do not directly eradicate cancer cells but retard the growth of cancer. By contrast, apoptosis directly causes cancer cell death without an inflammatory response, which is considered the gold standard therapy for chemotherapeutic agents. Both *in vitro* and *in vivo*, isothiocyanates have demonstrated a cancer preventive (blocking) property and a chemotherapeutic (deleting) property.

Raphanus sativus (*R. sativus*), belonging to the Cruciferous family, is used in indigenous Asian herbal medications [7,8] and has dietary nutritional value [9]. Numerous studies of the anticancer effect of the *R. sativus* include indications for colon cancer [10,11]. *R. sativus* is reported to contain isothiocyanates [12], however, there is limited information of its anticancer properties.

*Corresponding author: Natthida Weerapreeyakul, Faculty of Pharmaceutical Sciences, 123 Mittrapap Road, Amphoe Muang, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: +66 43 202 378
Fax: +66 43 202 379
E-mail: natthida@kku.ac.th (N. Weerapreeyakul).

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

R. sativus L. var. *caudatus* Alef (RS) is an edible vegetable found in Thailand. It is called Khi Hood (Thai), Thai rat-tailed radish (in English), and Hatsuka daikon (in Japanese). The potential cancer preventive effect from the edible part (flower and pod) of RS was reported by our lab and found to be correlated with the presence of isothiocyanates [13]. It was unclear, however, whether the cancer preventive effect of RS correlated with the age and/or consumed part of the plant. The anticancer activity of RS was previously evaluated in the colon cancer cell line HCT116 [13], this colon cancer cell line was, therefore, used in the present study. In the current study, the correlation of the isothiocyanate content in RS at different ages and parts was evaluated in order to determine the best harvesting stage and the best plant part with the highest cancer preventive effect. The anticancer effect with respect to apoptosis induction was also determined. The specific objectives of the study were: (1) to identify and quantify isothiocyanates, sulforaphane and sulforaphene, in the plant at difference stages of development and in different plant parts; and (2) to assess the cancer preventive activity of the plant at different ages and plant parts vis-à-vis its anti-proliferative and apoptosis induction characteristics.

2. Materials and methods

2.1. Plant and materials

RS was cultivated in Phayao Province, Thailand, and harvested at different stages of growth: (a) the vegetative phase or young plants having 2–3 leaves (3 week); (b) the older rosette stage where the shoot meristem produces a basal rosette of 6–8 leaves (4 week); (c) the vegetative-reproductive phase transition or an early bolting stage, when the shoot meristem starts to elongate and convert to reproductive development when inflorescences proliferate (5 week); (d) the late bolting stage including flowering and mature pod (fruits) (6 week); and, (e) senescence (7 week). The extracts were prepared from the major plant parts at these various stages of growth. At week 7, each part of the mature plant was collected for study. D, L-sulforaphane (purity > 98%) was purchased from Calbiochem (EMD, Darmstadt, Germant). The L-sulforaphene (purity > 97%) was purchased from Enzo Life Science (NY, USA). The dichloromethane and dimethyl sulfoxide (Fisher Scientific, Loughborough, UK) were analytical grade.

2.2. Plant extraction

RS extracts were prepared as previously reported [13]. Briefly, 50 g of fresh RS at different stages of growth different plant parts were separately homogenized for 30 min. Distilled water (50 mL) was added to the homogenate and left to autolyze at room temperature for 2 h. The homogenates were partitioned 3 times with dichloromethane. The dichloromethane phase was collected, dehydrated by anhydrous sodium sulfate, filtered through Whatman No. 1 paper, and dried using a rotary evaporator. The dry residue was stored at −20 °C.

2.3. Cell culture

The human colon cancer (HCT116) cells-cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) were purchased from Invitrogen and supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37 °C with 95% air and 5% CO_2.

2.4. Antiproliferation based on MTT assay

Exponentially growing cells were plated into 96-well microplates at a density of 5×10^3 cells per well, in 100 μL of culture medium. Thereafter, 100 μL of crude extract in DMSO-with concentrations ranging from 1 to 250 μg/mL were added to each well. The final concentration of DMSO in the culture medium was maintained at 0.6% to avoid solvent toxicity. MTT (Ambresco, OH, USA) in PBS was added to each well at 22 h for a final concentration of 0.5 mg/mL. This was incubated for another 2 h in a CO_2 incubator. The medium was discarded and 150 μL of DMSO added to solubilize the formazan. Absorbance was measured at 570 nm and the cytotoxic was calculated with %.

2.5. FTIR–ATR analysis

The FTIR–ATR spectra of the RS extract were determined using an FTIR spectrometer Tensor 27 (Bruker Optics, Ettlingen, Germany), equipped with a potassium bromide beam splitter and a MCT (HgCdTe) detector cooled with liquid nitrogen with an attached PIKE MIRacle (Madison, WI, USA) ATR accessory equipped with a single bounce Germanium crystal. The scanning range was from (600–4 000) cm^{-1} with a spectral resolution of 4 cm^{-1}. The intense doublet band at (2 100–2 200) cm^{-1} was assigned to the asymmetric stretching of isothiocyanate (–N═C═S) group, while the second band at (1 318–1 347) cm^{-1} was assigned to the bending vibration of the −CH atom contiguous to the −N═C═S group [14]. For each extract sample, at least 16 scans were acquired and the mean was calculated. For each sample, 3 different subsamples were measured and the mean was used for further analyses. The standard deviation of the spectral subsamples was achieved using OPUS 7.0 software (Bruker optic, Ettlingen, Germany).

2.6. GC–MS operating condition

GC–MS analysis (GC: Agilent, 6890 N, Shanghai, China; MS: Agilent, 5973 inert, Little Falls, DE, USA) was performed as previously reported [13], in order to characterize sulforaphane and sulforaphene in the RS extract. By using ultra-high purity helium as a carrier gas, the flow rate was set at 40 cm/s. The oven temperature was programmed to 50 °C for 5 min then it was increased to 250 °C in increments of 10 °C/min until it reached 250 °C for 10 min while the injector temperature was steady at 250 °C. The ion-source temperature was set at 180 °C and the mass spectra obtained by electron ionization at 70 eV.

2.7. HPLC analytical condition

The differences in the stage of plant growth and plant part could affect the production and composition of phytochemical compounds. Many factors including plant age affect the contents of isothiocyanate compounds in cruciferous plants [15]. To determine the effect of stage of plant growth on the content of

the isothiocyanates (sulforaphane and sulforaphene), the isothiocyanate content in the RS was quantified by a reversed-phase HPLC (Agilent, 1100 series G1310A, Wardbronn, Germany) at various plant stages (between 3 and 7 weeks of growth) and in different plant parts following the methods previously reported [12] with minor modifications. The analyses were carried out with 5% tetrahydrofuran in ultrapure water as a mobile phase with isocratic elution on the C18 (25 cm × 4.6 mm, 5 μm) column (HiQsil, Tokyo, Japan) at a flow rate of 1.25 mL/min. The detector (Agilent, 1100 series G1314A, Tokyo, Japan) wavelength was set at 210 nm.

2.8. Apoptosis detection by flow cytometry

The apoptosis assay was performed following the manufacturer's instructions (Bender MedSystem GmbH, Vienna, Austria) and analyzed using a fluorescent activated cell sorter analyzer (BD FACSCanto II, Franklin Lakes, NJ, USA). The effect of stage of growth of RS, and the plant part extract used, on apoptosis induction was investigated using Annexin V/PI staining.

2.9. Statistical analysis

The results-expressed as mean value ± SD were tested for any statistically significant difference between treatments; one-way analysis of variance (ANOVA) was used with Tukey's multiple comparison *post hoc* tests. Differences in P values below 0.05 were considered significant.

3. Results

The different percentages of yield from the extract of each plant part and at each stage are presented in Table 1. Pod and stem, the major parts, produced the highest % yield of crude extract at week 7. Since the dry seed was used for propagation, it was fully dried and as a consequence the obtained % yield of the crude extract was high.

To identify the presence of the isothiocyanates in RS extracts, infrared spectrophotometry was used. The characteristic isothiocyanate band was identified from the IR spectra of the extracts at different stages of growth and in different plant parts (Figure 1). RS older than the early-bolting stage (≥5 week) showed distinct doublet characteristic peaks of isothiocyanate at (2 100–2 200) cm^{-1}. When considering different parts, only the pod and flower contained this doublet characteristic peak. This characteristic isothiocyanate peak was not clearly observed in dry seed (Figure 1). The band of isothiocyanate at (1 318–1 347) cm^{-1} was not clearly observed in our extract samples, so GC–MS analysis was further applied to confirm the presence of isothiocyanates.

The two isothiocyanates (sulforaphane and sulforaphene) were found in the extracts at every growth stage and in plant part (Table 1). The GC chromatogram of the extracts revealed two important peaks. The peak at 19.60 min yielded the characteristic mass spectrum at m/z 160, 114, 72, 64, and 55, which was identical to the sulforaphane standard. In addition, sulforaphene yielded peaks at 19.42 min with characteristic mass spectrum at m/z 175, 112, 103, 87, 78, 72 and 53, which was identical to the authentic standard. HPLC analysis was then conducted to detect the content of sulforaphane and sulforaphene in RS as this technique avoids heat degradation which can occur during GC–MS analysis.

The results indicate that increasing age strongly affects sulforaphene content (Table 1). Cultivation of RS at the late-bolting stage produced the highest concentration of sulforaphane [(13.58 ± 0.51) μg/mg crude extract], and sulforaphene [(194.82 ± 14.78) μg/mg crude extract].

The current study of RS demonstrated the variable sulforaphane and sulforaphene content by plant part (*i.e.*, root, stem, leaf, pod and flower) (Table 1). The flower, pod, and dry seed contained both sulforaphane and sulforaphene. Sulforaphene was detected at a higher concentration than sulforaphane notwithstanding the plant part. The highest concentration of sulforaphane was found in the flower [(18.73 ± 1.33) μg/mg crude extract] as was the greatest concentration of sulforaphene [(371.40 ± 6.53) μg/mg crude extract] (Table 1).

The impact that both a different stage of plant growth and plant part had on the composition of isothiocyanate compounds was also demonstrated (*viz.*, sulforaphane and sulforaphene). The flower, pod, and dry seed possessed potential cytotoxicity at

Table 1

Percentage yield of crude extract, sulforaphane and sulforaphene content (HPLC analysis), antiproliferation (IC_{50} value), SI, and % total apoptosis of the extracts at different stages of growth and different parts (7 week old) of Thai rat-tailed radish.

Extracts		%Yield (per fresh wt)	Sulforaphane (μg/mg crude extract)	Sulforaphene (μg/mg crude extract)	IC_{50} (μg/mL)		SI	% Total apoptosis (100 μg/mL)
					HCT116	Vero		
Different ages	3 week	0.05	3.43 ± 0.24^{a}	21.10 ± 0.41^{a}	62.1 ± 4.1^{f}	135.9 ± 19.9^{ef}	2.2	19.3 ± 3.3^{b}
	4 week	0.02	3.78 ± 0.19^{a}	86.83 ± 1.11 ^{b}	33.9 ± 3.1^{de}	96.3 ± 16.0^{cd}	2.9	26.7 ± 2.8^{c}
	5 week	0.06	9.03 ± 0.32^{b}	105.71 ± 3.21^{c}	32.4 ± 3.6^{de}	161.2 ± 2.2^{f}	5.0	32.3 ± 0.9^{c}
	6 week	0.03	13.58 ± 0.51^{c}	194.82 ± 14.78 ^{d}	19.2 ± 1.3^{bc}	125.6 ± 3.1^{de}	6.5	42.0 ± 9.4^{c}
	7 week	0.03	13.42 ± 0.36^{c}	123.27 ± 2.11^{c}	24.3 ± 0.7^{cd}	97.5 ± 4.5^{cd}	4.0	26.9 ± 9.3^{c}
Different parts (at 7 week)	Leave	0.10	none	none	>250.0	>250.0	–	nd
	Root	0.04	none	none	>250.0	230.0 ± 14.7^{g}	<0.9	nd
	Stem	0.17	none	none	168.6 ± 11.7^{h}	>250.0	>1.5	nd
	Flower	0.07	18.73 ± 1.33^{a}	371.40 ± 6.53^{a}	17.4 ± 1.6^{abc}	87.2 ± 6.1^{bc}	5.0	66.3 ± 10.9^{d}
	Pod	0.15	6.28 ± 0.02^{b}	145.03 ± 6.82^{b}	42.7 ± 5.9^{e}	154.8 ± 11.1^{ef}	3.6	14.8 ± 3.4^{a}
	Dry seed	5.80*	0.89 ± 0.02^{c}	9.26 ± 0.33^{c}	78.7 ± 3.9^{g}	>250.0	>3.2	28.2 ± 0.8^{c}
Isothiocyanates	Sulforaphane	–	–	–	6.7 ± 0.1^{a}	12.5 ± 0.5^{a}	1.9	67.3 ± 7.9^{d}
	Sulforaphene	–	–	–	10.7 ± 2.3^{ab}	57.9 ± 3.8^{b}	5.4	77.0 ± 7.0^{d}

*%Yield was calculated and compared to dry weight; none = could not be detected under the conditions studied; nd = not determined; Means with same superscripts (a, b, c, d, e, f, g and h) between groups in the same column are not significantly different at $P < 0.05$.

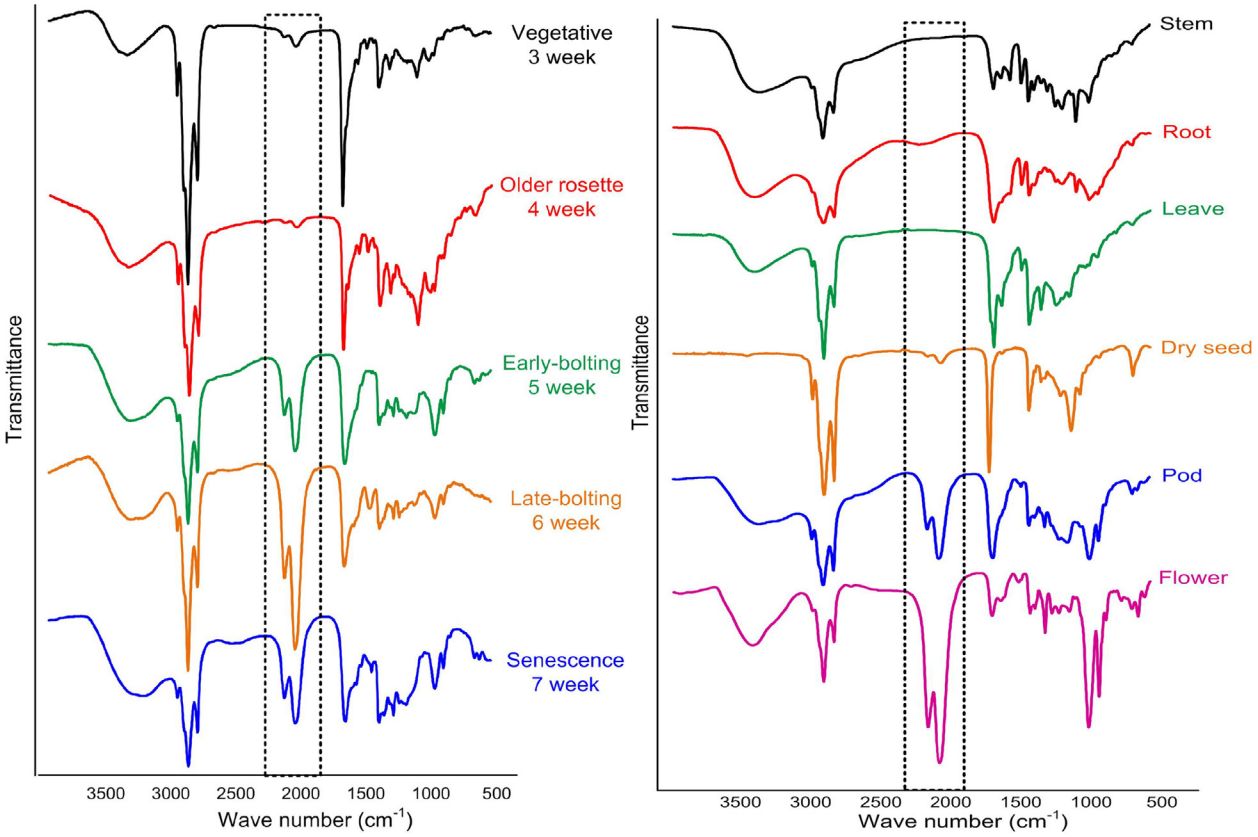

Figure 1. FTIR–ATR spectra of Thai rat-tailed radish extracts at different stages of growth and from different parts. Isothiocyanate bands labeled around 2 200 cm^{-1}.

IC_{50} values < 150 μg/mL. The antiproliferative effect increased with plant age from 3 to 6 week but with lower IC_{50} values [from (62.1 ± 4.1) to (19.2 ± 1.3) μg/mL] and a slightly decreased anti-proliferative effect at week 7 [IC_{50} (24.3 ± 0.7) μg/mL] (Table 1). Interestingly, the IC_{50} values at different stages of growth and plant parts showed an inverse correlation with isothiocyanate content (principally sulforaphene). The results indicate that sulforaphane and sulforaphene contribute to the observed anti-proliferative effect on the HCT116 cell line.

The selectivity index (SI) indicates the safety of the plant extract vis-à-vis noncancerous cells over against cancer cells. The SI of RS extract was compared with the IC_{50} obtained from the Vero cells as compared to the HCT116 cells. Table 1 shows that RS extract at younger plant stage had a low SI that later increased at the early-bolting stage (5 week). At the late-bolting and senescence stages (6 and 7 week), sulforaphane content was not much different, whereas sulforaphene content increased as the plant matured. Of note, sulforaphane showed less selectivity for the HCT116 cell line than sulforaphene. At the late-bolting stage, the RS extract had a relatively higher sulforaphene than sulforaphane content, so it is likely that sulforaphene may be the primary bioactive constituent in RS extract on cancer cell selectivity. The contribution of sulforaphene content with respect to cancer cell selectivity was also evident in the extracts of dry seed [(9.26 ± 0.33) μg/mg crude extract], pod [(145.03 ± 6.82) μg/mg crude extract], and flower [(371.40 ± 6.53) μg/mg crude extract], with SI values of >3.2, 3.6, and 5.0, respectively.

Table 1 shows that % total apoptosis (both early and late-stage) increased with age from the vegetative phase (week 3) to the late-bolting stage (week 6). Sulforaphane and sulforaphene at 100 μg/mL mostly induced early-stage apoptosis 66.8% ± 7.8% and 76.9% ± 6.9%, respectively, with a lesser degree of late-stage apoptosis and necrosis (Tables 1 and 2). The respective extract for the vegetative phase (week 3) and older rosette stage (week 4) RS at 100 μg/mL was found to induce mostly early-stage apoptosis (Table 2). The extract of RS after the early-bolting stage (week ≥ 5) evidently induced less early-stage apoptosis but more late-stage of apoptosis. The highest total apoptosis induction (42.0% ± 9.4%) was found at the late-bolting stage (week 6) (Table 1). Notably, when the concentration of each of the RS extracts reached 100 μg/mL, necrosis-type cell death was observed in a concentration dependent manner.

The RS extract from different plant parts with IC_{50} values <150 μg/mL were chosen for further investigation vis-à-vis their apoptosis induction effect (Table 1). A high to low apoptosis induction effect for the RS extract from the various plant parts was observed from the flower, dry seed, and pod, respectively (Table 1). The extract from the flower at 100 μg/mL induced the highest % total apoptosis (66.3% ± 10.9%), which was not significantly different from either sulforaphane or sulforaphene at the same concentration ($P > 0.05$, one-way ANOVA). The flower extract resulted in 36.8% ± 5.6% at early stage apoptosis and 29.5% ± 7.6% at late stage apoptosis, while sulforaphane and sulforaphene resulted in mostly early stage apoptosis (66.8% ± 7.8% and 76.9% ± 6.9%, respectively) (Table 2). Dry seed also induced mostly early-stage apoptosis (27.9% ± 0.6%). Owing to the higher biomass of the reproductive parts (*i.e.*, flowers and mature pods), they contained more isothiocyanates with increased age, which may explain the higher % apoptosis induction observed at the late-bolting stage.

Table 2
HCT116 cell population at each stage after being treated with RS extracts.

Group		Concentration (μg/mL)	Apoptosis (%)				
			Living	Necrosis	Early apoptosis	Late apoptosis	Total apoptosis
Control	Sulforaphane	0	87.5 ± 2.5[a]	1.2 ± 1.0[a]	10.4 ± 2.9[a]	0.9 ± 0.5[a]	11.3 ± 2.6[a]
		10	67.6 ± 1.6[b]	0.1 ± 0.0[a]	32.2 ± 1.7[b]	0.2 ± 0.1[a]	32.3 ± 1.7[c]
		100	32.7 ± 7.8[c]	0.2 ± 0.1[a]	66.8 ± 7.8[c]	0.5 ± 0.3[a]	67.3 ± 7.9[d]
	Sulforaphene	10	82.2 ± 2.0[a]	0.3 ± 0.4[a]	17.2 ± 1.7[a]	0.3 ± 0.2[a]	17.5 ± 1.9[b]
		100	23.0 ± 7.0[c]	0.0 ± 0.1[a]	76.9 ± 6.9[c]	0.1 ± 0.1[a]	77.0 ± 7.0[d]
Different stage	3 week	10	83.5 ± 4.7[a]	2.0 ± 0.9[a]	12.7 ± 6.4[a]	1.8 ± 1.4[a]	14.4 ± 5.3[a]
		100	77.3 ± 2.6[a]	3.4 ± 1.1[a]	15.6 ± 3.2[a]	3.7 ± 0.2[a]	19.3 ± 3.3[b]
	4 week	10	88.6 ± 0.4[a]	2.6 ± 0.9[a]	7.5 ± 0.7[a]	1.2 ± 0.4[a]	8.8 ± 0.6[a]
		100	67.9 ± 2.5[b]	5.5 ± 0.5[a]	17.9 ± 1.8[a]	8.8 ± 1.0[b]	26.7 ± 2.8[c]
	5 week	10	79.4 ± 5.2[a]	1.7 ± 0.4[a]	18.0 ± 4.8[a]	1.0 ± 0.2[a]	19.0 ± 4.7[b]
		100	28.9 ± 4.3[c]	38.7 ± 4.8[d]	8.3 ± 0.6[a]	24.0 ± 1.6[d]	32.3 ± 0.9[c]
	6 week	10	76.0 ± 10.0[a]	2.8 ± 1.1[a]	18.6 ± 9.9[a]	2.6 ± 0.5[a]	21.2 ± 9.5[b]
		100	29.7 ± 6.2[c]	28.3 ± 3.2[c]	14.1 ± 1.1[a]	27.8 ± 8.6[d]	42.0 ± 9.4[c]
	7 week	10	77.7 ± 6.9[a]	1.8 ± 0.6[a]	18.9 ± 6.1[a]	1.6 ± 0.6[a]	20.5 ± 6.3[b]
		100	26.1 ± 4.6[c]	47.1 ± 5.3[e]	8.6 ± 2.3[a]	18.3 ± 7.1[c]	26.9 ± 9.3[c]
Different part	Flower	10	62.7 ± 3.3[b]	2.4 ± 1.9[a]	32.1 ± 4.6[b]	2.7 ± 1.0[a]	32.8 ± 4.0[c]
		100	23.0 ± 8.9[c]	10.7 ± 2.3[b]	36.8 ± 5.6[b]	29.5 ± 7.6[d]	66.3 ± 10.9[d]
	Dry seed	10	82.4 ± 3.2[a]	0.1 ± 0.0[a]	17.5 ± 3.2[a]	0.1 ± 0.0[a]	17.6 ± 3.2[b]
		100	71.7 ± 0.9[b]	0.2 ± 0.1[a]	27.9 ± 0.6[b]	0.3 ± 0.2[a]	28.2 ± 0.8[c]
	Pod	10	85.6 ± 1.0[a]	0.1 ± 0.0[a]	14.1 ± 1.0[a]	0.2 ± 0.1[a]	14.3 ± 1.0[a]
		100	73.1 ± 5.5[b]	12.1 ± 2.2[b]	12.6 ± 2.5[a]	2.2 ± 1.0[a]	14.8 ± 3.4[a]

Means with same superscripts (a, b, c, d, e, f and g) between groups in the same column are not significantly different at $P < 0.05$.

Our results reveal that plant stage is positively correlated to isothiocyanate content and is also well-correlated to the apoptosis induction effect (Table 1). Apparently, sulforaphene in the extract is the main isothiocyanate contributing to the apoptosis induction effect (Table 1). Considering the different plant part, the anti-proliferation of the flower was higher than the pod and dry seed, respectively. The rank of the % apoptosis induction effect from high to low was flower > dry seed > pod (Table 1), whereas the rank of % necrosis induction from high to low was pod > flower > dry seed. The anti-proliferative effect in the flower > pod > dry seed was attributed to the combination of apoptosis and necrosis induction. These results suggest the existence of other constituents in the flower and pod which might play a role in necrosis induction. Notably, necrotic cell death was significantly observed for the flower and pod at a higher percentage than the other plant parts ($P < 0.05$, one-way ANOVA) and in a concentration dependent manner (Table 2). The other constituents in the RS extracts, which also increased with age and varied with plant part, might be responsible for the necrosis effect, which could explain why there was a poor correlation between isothiocyanate content and apoptotic cell death in the pod.

4. Discussion

Plant age and plant part are major factors affecting the distribution of glucosinolate content. In a study of a model plant, *Arabidopsis thaliana*, a plant in the Brassicaceae family, revealed that total glucosinolate content increased with age [16]. The optimal distribution of defense substances in plants are mainly found in the plant reproductive organs [17], in order to maximize fertilization by making their reproductive organs less palatable to herbivores and less susceptible to pathogens.

Glucosinolates, the precursors of isothiocyanates, are sulfur and nitrogen containing plant metabolites. The distribution of glucosinolate content varies according to the plant organ and is found at the highest concentration in the reproductive organs [*i.e.*, seed, inflorescence (flower) and silique] [16,18]. These compounds possess many functions including: (a) sulfur and nitrogen storage, (b) managing the plant's nutritional status, (c) regulating growth, and (d) defending against herbivores and pathogens [19,20]. Glucosinolates and their hydrolysis products (isothiocyanates) exhibit toxicity on growth inhibition or feeding deterrents to a wide range of potential plant enemies. Isothiocyanates show a higher toxicity on plant enemies than glucosinolate [21]. Our findings confirm that high concentrations of isothiocyanates are detected in the reproductive organs. The results agree with a previous study, which shows that sulforaphane and sulforaphene are the major isothiocyanates in RS detected by UPLC-ESI-QTof-MS/MS, whereas other isothiocyanates such as erysolin, sulforaphane nitrile, and raphasatin are found in minor concentrations [22]. Moreover, the other factor reported to affect the isothiocyanate content is the part of the plant [12,18]. Moreover, the hydrolysis products of glucosinolates, sulforaphane and sulforaphene, varied depending on the stage of plant growth and the plant part extracted, particularly with respect to sulforaphene.

The biomass ratio of reproductive organs of several cruciferous plants (*i.e.*, *Brassica* and *Raphanus genuses*) increases with age and declines in senescence [23,24]. The amount of sulforaphane and sulforaphene in the extracts from flowers, pods and dry seeds, the reproductive organs presenting in the mature growth stage were evaluated. The mass ratio of the reproductive organs increased throughout the vegetative phase (week 3) to the late bolting stage (week 6) when it reached its maximum and declined during senescence (week 7). The isothiocyanates especially sulforaphene in the plant also increased with age until the late-bolting stage then declined. The maximum and low detectable sulforaphene content may therefore be due to the large proportion of reproductive plant parts that come with age and decline toward senescence. The same pattern of reaching a maximum at the late-bolting stage then declining was observed for the anti-proliferative and apoptosis induction effects of RS. Cruciferous plant consumption has been found to reduce the risk of

several types of cancer including colon cancer [25]. Isothiocyanates, the unique compounds found in cruciferous plants displayed various mechanisms of anticancer activity *in vitro*, including: (a) inhibition of carcinogen-activation of P450 enzymes (phase I metabolism); (b) induction of carcinogen-detoxifying enzymes (phase II metabolism); (c) oxidative stress modulation; (d) induction of apoptosis; and, (e) arresting of the cancer cell cycle [6]. These two isothiocyanates exhibit an antiproliferative effect on cancer cells *in vitro* [26–28]. It appears, therefore, that isothiocyanates, especially sulforaphene, potentially contribute to observed anticancer activity, as sulforaphene content correlated well with anticancer activity, as the plants accumulate more in the reproductive organs with age until the late-bolting stage when it declines.

Sulforaphene is sulforaphane derivative with an additional double bond at the C3 alkyl side chain. The central carbon of the isothiocyanate moiety (–N═C═S) displays high electrophilicity and interacts actively with the cellular nucleophile. The interaction of the isothiocyanate central carbon with cysteine residues of Keap1 (cellular nucleophilic target) results in the stabilization of Nrf2 and consequently leads to the higher expression of the phase II metabolism enzyme responsible for antioxidant activity [29]. The central carbon presumably interacts with tubulin resulting in microtubule depolymerization interrupting mitosis cell division and subsequent leading to apoptotic cell death [30,31]. The structural activity relationship of sulforaphane derivatives is possibly affected by its side chain electrophilic when the oxidation stage of methylthio group increases isothiocyanate central carbon reactivity to nucleophilic target, such as GSH, leading to apoptosis induction activity [27]. Our previous work reported that the double bond in the sulforaphene alkyl side chain possibly reduced the electrophilicity of the −N═C═S moiety; thus, minimizing sulforaphene cancer preventive activity [13]. Sulforaphene content in RS was, however, dramatically higher than sulforaphane. Thus, in this study, sulforaphene was the primary constituent responsible for the observed anticancer activity.

Our research underscores the correlation between extractable isothiocyanate and plant growth stage, the various plant parts used, and the anticancer activity via apoptosis induction. This is the first report on isothiocyanate composition of RS-as related to plant stage and plant part-and any cancer preventive effect vis-à-vis the HCT116 colon cancer cell line. Sulforaphene was the predominant chemopreventive compound found in the flower, pod, and dry seed. The optimum harvesting time for RS was at the late bolting stage (6 week) when the reproductive parts (*i.e.*, flower, pod, and seed) predominate and these are also the best source of isothiocyanates (sulforaphene and sulforaphene), which possess both preventive and therapeutic activity against cancer.

Conflicts of interest statement

The authors declare that they have no competing interests.

Acknowledgments

This work was partially supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Food and Functional Food Research Cluster and Research and Development of Herbal Nutraceutics Subcluster of Khon Kaen University (No. F-2553-M-11 and NRU541051) and the Khon Kaen University Research Fund (No. 564301). We thank Mr. Bryan Roderick Hamman for assistance with the English-language presentation.

References

[1] Hanschen FS, Schreiner M. Isothiocyanates, nitriles, and epithionitriles from glucosinolates are affected by genotype and developmental stage in *Brassica oleracea* varieties. *Front Plant Sci* 2017; **8**: 1095.
[2] Angelino D, Dosz EB, Sun J, Hoeflinger JL, Van Tassell ML, Chen P, et al. Myrosinase-dependent and independent formation and control of isothiocyanate products of glucosinolate hydrolysis. *Front Plant Sci* 2015; **6**: 831.
[3] Mahn A, Martin C, Reyes A, Saavedra A. Evolution of sulforaphane content in sulforaphane-enriched broccoli during tray drying. *J Food Eng* 2016; **186**: 27-33.
[4] Abel EL, Boulware S, Fields T, McIvor E, Powell KL, DiGiovanni J, et al. Sulforaphane induces phase II detoxication enzymes in mouse skin and prevents mutagenesis induced by a mustard gas analog. *Toxicol Appl Pharmacol* 2013; **266**: 439-42.
[5] Atwell LL, Beaver LM, Shannon J, Williams DE, Dashwood RH, Ho E. Epigenetic regulation by sulforaphane: opportunities for breast and prostate cancer chemoprevention. *Curr Pharmacol Rep* 2015; **1**: 102-11.
[6] Lenzi M, Fimognari C, Hrelia P. Sulforaphane as a promising molecule for fighting cancer. In: Zappia V, Panico S, Russo GL, Budillon A, Della Ragione F, editors. *Advances in nutrition and cancer*. Berlin, Heidelberg: Springer; 2014, p. 207-23.
[7] Shishu Kaur IP. Inhibition of cooked food-induced mutagenesis by dietary constituents: comparison of two natural isothiocyanates. *Food Chem* 2009; **112**: 977-81.
[8] Shukla S, Chatterji S, Mehta S, Rai PK, Singh RK, Yadav DK, et al. Antidiabetic effect of *Raphanus sativus* root juice. *Pharm Biol* 2011; **49**: 32-7.
[9] Hanlon PR, Barnes DM. Phytochemical composition and biological activity of 8 varieties of radish (*Raphanus sativus* L.) sprouts and mature taproots. *J Food Sci* 2011; **76**: C185-92.
[10] Pocasap P, Weerapreeyakul N. Sulforaphene and sulforaphane in commonly consumed cruciferous plants contributed to antiproliferation in HCT116 colon cancer cells. *Asian Pac J Trop Biomed* 2016; **6**: 119-24.
[11] Beevi SS, Mangamoori LN, Subathra M, Edula JR. Hexane extract of *Raphanus sativus* L. roots inhibits cell proliferation and induces apoptosis in human cancer cells by modulating genes related to apoptotic pathway. *Plant Foods Hum Nutr* 2010; **65**: 200-9.
[12] Sangthong S, Weerapreeyakul N. Simultaneous quantification of sulforaphene and sulforaphane by reverse phase HPLC and their content in *Raphanus sativus* L. var. caudatus Alef extracts. *Food Chem* 2016; **201**: 139-44.
[13] Pocasap P, Weerapreeyakul N, Barusrux S. Cancer preventive effect of Thai rat-tailed radish (*Raphanus sativus* L. var. caudatus Alef). *J Funct Foods* 2013; **5**: 1372-81.
[14] Cerny MS, Taube E, Battaglia R. Identification of bis(4-isothiocyanatobutyl) disulfide and its precursor from rocket salad (*Eruca sativa*). *J Agric Food Chem* 1996; **44**: 3835-9.
[15] De Maria S, Agneta R, Lelario F, Möllers C, Rivelli AR. Influence of nitrogen and sulfur fertilization on glucosinolate content and composition of horseradish plants harvested at different developmental stages. *Acta Physiol Plant* 2016; **38**: 91.
[16] Brown PD, Tokuhisa JG, Reichelt M, Gershenzon J. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry* 2003; **62**: 471-81.
[17] Zangerl AR, Bazzaz FA. Theory and pattern in plant defense allocation. In: Fritz RS, Simms EL, editors. *Plant resistance to herbivores and pathogens*. Chicago: University of Chicago Press; 1992, p. 363-91.

[18] Campas-Baypoli ON, Sanchez-Machado DI, Bueno-Solano C, Ramirez-Wong B, Lopez-Cervantes J. HPLC method validation for measurement of sulforaphane level in broccoli by-products. *Biomed Chromatogr* 2010; **24**: 387-92.
[19] Redovniković IR, Glivetić T, Delonga K, Vorkapić-Furać J. Glucosinolates and their potential role in plant. *Period Biol* 2008; **110**: 297-309.
[20] del Carmen Martínez-Ballesta M, Moreno DA, Carvajal M. The Physiological importance of glucosinolates on plant response to abiotic stress in Brassica. *Int J Mol Sci* 2013; **14**: 11607-25.
[21] Ntalli N, Caboni P. A review of isothiocyanates biofumigation activity on plant parasitic nematodes. *Phytochem Rev* 2017. https://doi.org/10.1007/s11101-017-9491-7.
[22] Sangthong S, Weerapreeyakul N, Lehtonen M, Leppanen J, Rautio J. High-accuracy mass spectrometry for identification of sulphur-containing bioactive constituents and flavonoids in extracts of *Raphanus sativus* var. caudatus Alef (Thai rat-tailed radish). *J Funct Foods* 2017; **31**: 237-47.
[23] Bellostas N, Sørensen JC, Sørensen H. Qualitative and quantitative evaluation of glucosinolates in cruciferous plants during their life cycles. *Agroindustria* 2004; **3**: 5-10.
[24] Malik MS, Riley MB, Norsworthy JK, Bridges W Jr. Glucosinolate profile variation of growth stages of wild radish (*Raphanus raphanistrum*). *J Agric Food Chem* 2010; **58**: 3309-15.
[25] Wu QJ, Yang Y, Vogtmann E, Wang J, Han LH, Li HL, et al. Cruciferous vegetables intake and the risk of colorectal cancer: a meta-analysis of observational studies. *Ann Oncol* 2013; **24**: 1079-87.
[26] Papi A, Orlandi M, Bartolini G, Barillari J, Iori R, Paolini M, et al. Cytotoxic and antioxidant activity of 4-methylthio-3-butenyl isothiocyanate from *Raphanus sativus* L. (Kaiware Daikon) sprouts. *J Agric Food Chem* 2008; **56**: 875-83.
[27] Kim MJ, Kim SH, Lim SJ. Comparison of the apoptosis-inducing capability of sulforaphane analogues in human colon cancer cells. *Anticancer Res* 2010; **30**: 3611-9.
[28] Zeng H, Trujillo ON, Moyer MP, Botnen JH. Prolonged sulforaphane treatment activates survival signaling in nontumorigenic NCM460 colon cells but apoptotic signaling in tumorigenic HCT116 colon cells. *Nutr Cancer* 2011; **63**: 248-55.
[29] Kumar G, Tuli HS, Mittal S, Shandilya JK, Tiwari A, Sandhu SS. Isothiocyanates: a class of bioactive metabolites with chemopreventive potential. *Tumor Biol* 2015; **36**: 4005-16.
[30] Jackson SJ, Singletary KW. Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. *Carcinogenesis* 2004; **25**: 219-27.
[31] Mi L, Xiao Z, Hood BL, Dakshanamurthy S, Wang X, Govind S, et al. Covalent binding to tubulin by isothiocyanates. *J Biol Chem* 2008; **283**: 22136-46.