รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาการต้านการก่อมะเร็ง และกลไกการออกฤทธิ์ของผักขี้หูด

(Study of chemopreventive effect and mechanism of action of Thai rat-tailed radish)

โดย

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ABSTRACT

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Thai rat-tailed radish (*Raphanus sativus* L. var. *caudatus* Alef) is an indigenous cruciferous plant. Various parts of RS were extracted with two extraction techniques—super critical CO_2 fluid extraction (SFE) and dichloromethane (DCM) extraction. The association of solvent and extraction yield of isothiocyanates (ITCs)—sulforaphane (SF) and sulforaphene (SE)—was determined by the FTIR and HPLC analysis. The chemopreventive effect was investigated in cancer cells by determining the apoptosis inducing effect. Results from HPLC analysis showed that DCM extraction yielded higher SF and SE contents than SFE. The characteristic ITCs band was revealed by FTIR at 2,000–2,200 cm^{-1}. SE was quantified at higher content than SF in most RS parts. The order from high to low SE content was stem, mixed arial part, seed, whole pod, mixed pod and flower, flower, root, seedless pod, and leave, respectively. The DCM crude extracts of pod exerted greater anti-proliferation in human colon HCT-116 cancer cell than stem and the other parts. The pod extract showed higher antiproliferation than the stem extract in most of cancer cell lines studied. Therefore, the DCM crude extract from pod was further fractionated by various polar solvents. However, the fractions possessed lesser antiproliferation than the whole DCM crude extract. Hence only the whole DCM crude extract was investigated for apoptosis inducing effect in the HCT-116 cells. The DCM crude extracts from pod and stem induced apoptosis via both extrinsic and intrinsic pathways as evidenced by (i) nuclei morphological changes, (ii) DNA laddering pattern, (iii) increased %apoptotic cells, (iv) increased caspases 3, 8 and 9 activities, and (iv) increased mitochondria membrane potential. Interestingly, only RS pod extract inhibited MRP-1 activity of the efflux protein found in cancer cells similar to SE and SF. The presence of MRP-1 inhibitory action of the RS pod extract may provide benefit in increasing an uptake of the existed active components in the RS pod extract into the HCT-116 cells. The FTIR microspectroscopy of the HCT-116 cells revealed different changes of cellular biochemical compositions—lipid, protein and nucleic acid—between the extracts from pod, stem, SF and SE. It indicated different extracted phytoconstituents between pod and stem parts and different degree of synergy chemopreventive effect. In conclusion, the pod and stem could be an alternative functional food and good source of chemopreventive compounds. However, further studies *in vivo* pharmacodynamics and pharmacokinetics are still required to warrant its clinical relevant action.

Keywords : Thai rat-tailed radish, chemoprevention, cancer, isothiocyanates, FTIR microspectroscopy

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ผักขี้หูด หรือชื่อวิทยาศาสตร์ *Raphanus sativus* L. var. *caudatus* Alef เป็นพืชในตระกูลผักกาดที่นำแต่ละส่วนมาศึกษา โดยสกัดด้วยเทคนิคการสกัด 2 วิธี คือ วิธีการสกัดด้วยคาร์บอนไดออกไซด์ภายใต้สภาวะวิกฤติยวดยิ่งและการสกัดด้วยไดคลอโรมีเทน แล้ววิเคราะห์ความสัมพันธ์ระหว่างชนิดตัวทำละลายและสารกลุ่มไอโซไธโอไซยาเนทที่สกัดได้ ได้แก่ ซัลโฟราเฟนและซัลโฟราฟีนด้วยเทคนิค FTIR และ HPLC และศึกษาฤทธิ์ต้านการก่อมะเร็งจากการชักนำการตายแบบอะพอพโทซิสในเซลล์มะเร็ง ผลจากการวิเคราะห์ด้วย HPLC พบว่าสารสกัดหยาบไดคลอโรมีเทนให้ผลผลิตของซัลโฟราเฟนและซัลโฟราฟีนมากกว่าอีกเทคนิค และวิธี FTIR ตรวจพบลักษณะของแถบสารกลุ่มไอโซไธโอไซยาเนทที่ 2,000–2,200 cm^{-1} โดยพบปริมาณสารซัลโฟราฟีนมากกว่าซัลโฟราเฟน แทบจะทุกส่วนของผักขี้หูด เรียงลำดับจากปริมาณมากไปน้อย คือ ก้าน ส่วนที่เป็นทั้งใบและก้านเหนือดิน เมล็ด ฝัก ส่วนผสมที่มาจากฝักและดอก ดอก ราก ฝักที่แกะเมล็ดออก และใบ ตามลำดับ โดยสารสกัดหยาบไดคลอโรมีเทนจากฝักมีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งลำไส้ใหญ่มนุษย์ HCT-116 ดีกว่าสารสกัดจากก้านหรือส่วนอื่นๆ จึงนำเฉพาะสารสกัดหยาบไดคลอโรมีเทนจากฝักมาแยกต่อด้วยตัวทำละลายขั้วต่างๆ แต่พบว่าสารที่แยกได้มีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งน้อยกว่าสารสกัดหยาบไดคลอโรมีเทนจากฝัก ดังนั้นจึงทำการศึกษาการชักนำการตายแบบอะพอพโทซิสใน HCT-116 ของสารสกัดหยาบไดคลอโรมีเทนจากฝักและก้าน พบว่าสารสกัดหยาบทั้ง 2 ชนิดสามารถชักนำการตายแบบอะพอพโทซิสผ่านทั้งวิถีภายนอกและภายใน ตรวจพบได้จาก (1) การทำให้เกิดการเปลี่ยนรูปสัญฐานของนิวเคลียส (2) เห็นการแยกดีเอ็นเอ แบบขั้นบันได (3) การเพิ่มเปอร์เซ็นการตายแบบอะพอพโทซิส (4) การเพิ่มกิจกรรมของเอนไซม์แคสเปส 3, 8, และ 9 และ (5) การเพิ่มความต่างศักย์ของผนังไมโทคอนเดรีย และที่น่าสนใจคือสารสกัดหยาบจากฝักยับยั้งกิจกรรมของโปรตีนที่ปั๊มยาออกนอกเซลล์มะเร็ง MRP-1 เหมือนที่พบในซัลโฟราฟีนหรือซัลโฟราเฟน ซึ่งเป็นประโยชน์ในการเพิ่มการนำส่งสารประกอบต่างๆที่ออกฤทธิ์ในสารสกัดหยาบให้เข้าเซลล์มะเร็ง HCT-116 เพิ่มขึ้น นอกจากนี้ FTIR ไมโครสเปกโตรสโกปียังตรวจพบความแตกต่างของระดับสารชีวโมเลกุล (ได้แก่ ไขมัน โปรตีน และกรดนิวคลีอิก) ที่เป็นส่วนประกอบในเซลล์ HCT-116 เมื่อได้รับสารสกัดหยาบไดคลอโรมีเทนจากฝัก ก้าน ซัลโฟราฟีน หรือซัลโฟราเฟน ผลดังกล่าวระบุถึงความแตกต่างของสารที่มีอยู่ในสารสกัดหยาบไดคลอโรมีเทนจากฝัก และก้าน ตลอดจนความแตกต่างของการออกฤทธิ์เสริมการต้านการก่อมะเร็ง จากการศึกษานี้สรุปได้ว่าผักขี้หูดมีศักยภาพในการออกฤทธิ์ต้านการก่อมะเร็ง เพราะฝักหรือก้านของผักขี้หูดสามารถนำมาใช้เป็นแหล่งของอาหารเพื่อสุขภาพ และเป็นแหล่งของสารที่ต้านการก่อมะเร็ง อย่างไรก็ตามควรจะมีการศึกษาเพิ่มเติมทางเภสัชพลศาสตร์และเภสัชจลนศาสตร์ ใน *in vivo* ก่อนนำไปใช้เพื่อผลทางคลินิกในคนต่อไป

คำหลัก : ผักขี้หูด, ฤทธิ์ต้านการก่อมะเร็ง, มะเร็ง, ไอโซไธโอไซยาเนท, FTIR ไมโครสเปกโตรสโกปี

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CHAPTER 1
INTRODUCTION

Background and significance

Cancer is one of serious non-communicable disease worldwide which around 30–35% was linked to diet (Anand et al., 2008). International Agency for Research on Cancer (IARC) reported the number of cancer cases in Thailand in 1992-1994 were 150.4 for male and 123.0 for females per 100,000 people. The cancer incidence in Thailand was literally different upon registry. Southern is majority in esophageal cancer, lung cancer predominates in Northern and cholangiocarcinoma liver cancer is significantly high in Northeastern (Vatanasapt et al., 2002). The mentioned reports imply that the variety of cancer type depended on the cultural behavior including great interest in dietary uptake of each registry.

The cancer treatment is mainly by chemotherapy, radiotherapy, surgery, or combination therapy. However, the resistance for the cancer treatment and severe side effects such as myelosuppression, mucositis or alopecia are occurred and consequently leading to patient noncompliance or failure of cancer treatment (Grunberg, 2012). The alternative cancer prevention as well as cancer therapy by using natural compounds or phytotherapy which has long been reputed as remedy since ancient times is thus of interest (Rocha, Lopes & Schwartsmann, 2001). The alternative phytochemicals' mechanisms of action are widely studied based on its cancer prevention that promotes human health benefit without undesirable effects (Reddy, Odhav & Bhoola, 2003). Many epidemiological studies reported the positive association of lowering cancer risk with consuming large quantities of fruits and vegetables (Shapiro et al., 1998; 2001; Potter 1997).

Thailand is the rich source of tropical plants biodiversity. Numerous plants are explored for their diversity and biological compound containing. The cruciferous plants, for example wasabi, mustard, water cress, garden cress, and broccoli sprouts are the rich source of cancer chemopreventive compound, glucosinolate (Zhang et al., 2003; Shapiro et al., 1998; 2001). This plant family provides a unique characteristic of pungent odor whose chemical constituents were described as the secondary metabolites occurred after the cells are disrupted via cutting, chewing or cooking, isothiocyanates (ITCs) were produced as the conversion products from GSL (Vaughn & Berhow, 2005). More than 20 ITCs were reported for their tumor genesis inhibition. ITCs were also found to be effective to even the cell over-expressed multidrug resistance associated protein-1 (MRP-1) or P-glycoprotein-1 (Pgp-1) (Zhang, Tang & Gonzalez, 2003).

Thai rat-tailed radish (*Raphanus sativus* L. var. *caudatus* Alef; **RS**) or “Pak Khi Hood” in Thai is an indigenous vegetable in the northern and northeastern Thailand. It is commonly cooked in the mixed vegetable soup providing the unique pungent odor and taste of the soup or boiled for accompany eaten with other dishes. Few studies have reported the containing of ITCs, mainly as sulforaphane and sulforaphene in Thai rat-tailed radish (Songsak & Lockwood, 2002; Pocasap, Weerapreeyakul & Barusrux, 2013). Moreover, the chemoprotective effect of Thai rat-tailed radish against colon cell line through apoptosis induction was recently published by our group (Pocasap, Weerapreeyakul & Barusrux, 2013). This apoptosis induction effect indicates the successful cancer treatment as it is pharmacodynamic endpoint (Au et al., 1997). However, more scientific information regarding the cellular mechanism of chemoprotective action, other cancer cell type response, and other existed constituents in the Thai rat-tailed radish extract are still lack and worth to be explored. Based on the aforementioned evidences, necessary scientific evidences are still required to affirm the systemic use of Thai rat-tailed radish as an alternative chemopreventive source.

Objectives

This study is aimed to investigate chemopreventive effects and mechanism of Thai native vegetable, Thai rat-tailed radish. The obtained research result may further lead to some guidance of exploiting Thai rat-tailed radish vegetable as the functional food. To achieve the proposed objectives, following specific aims has been conducted.

(1) To determine the optimum extraction technique between super critical CO_2 fluid extraction and conventional solvent extraction.

(2) To qualitative and quantitative analysis of the active component such as ITCs from different parts of RS.

(3) To determine chemopreventive effect of the extracts from each part of RS base on apoptosis induction effect in the sensitive cell line.

- 3.1 To determine antiproliferation of the RS extracts in different cancer cell types in comparison to the normal cell line.
- 3.2 To characterize mechanism of apoptotic cell death from necrosis and define the pathway of apoptosis base on caspases mediates apoptosis, alteration of mitochondria membrane potential and related apoptotic proteins.
- 3.3 To determine the cancer cellular biochemical changes by using FTIR microspectroscopy.

3.4 To determine the inhibition effect of the extracts on efflux pump in resistance cancer cell line.

3.5 To investigate cancer cellular biochemical changes in the treated cancer cells by using FTIR microspectroscopy in the selected cancer cell line.

CHAPTER 2
LITERATURE REVIEW

Cancer is one of serious non-communicable disease worldwide. In 2008, Americans were diagnosed with cancer and 5–10% was caused due to genetic defects, while the remaining was associated with environment and lifestyle which around 30–35% was linked to diet (Anand et al., 2008). International Agency for Research on Cancer (IARC) reported the number of cancer cases in Thailand in 1992–1994 were 150.4 for male and 123.0 for females per 100,000 people. The cancer incidence was literally different upon the registry. Esophageal cancer was majority in Southern, lung cancer was predominant in Northern and cholangiocarcinoma liver cancer was significantly high in Northeastern (Vatanasapt et al., 2002). The mentioned report observable implies that the variety of cancer types depending on the cultural behavior including dietary uptake of each registry.

The main cancer treatments are chemotherapy and radiotherapy. However, the resistance for the cancer treatment and severe side effects such as myelosuppression, mucositis or alopecia are occurred and consequently led to patient noncompliance or failure of cancer treatment (Grunberg, 2012). The alternative cancer prevention as well as cancer therapy by using natural compounds or phytotherapy which has long been reputed as remedy since ancient times is thus of interest (Rocha, Lopes & Schwartsmann, 2001).

The natural compounds are mostly made up for sophisticated plant survival mechanisms. The plant secondary metabolites are synthesized to defense plant from the predators and to prevent of other growth competitive plants. Alkaloids, phenols, and tannin are well-known phytochemicals from the defensive mechanism of plants makes them poisonous and unattractive (Rocha, Lopes & Schwartsmann, 2001). The phytochemicals' mechanisms of cancer prevention action are widely studied because it promotes human health benefit without undesirable effects (Reddy, Odhav & Bhoola, 2003). Phytochemicals were investigated and their mechanism of action against carcinogenesis were reported as follows; (1) inhibit phase 1 enzymes; (2) modify the detoxification through phase 2 metabolic pathways; (3) scavenge DNA reactive agents; (4) suppress the abnormal proliferation; and (5) inhibit certain properties of the cancer cells (Wargovich, 1997). Many epidemiological studies reported the positive association of lowering cancer risk with consuming large quantities of fruits and vegetables (Shapiro et al., 1998; 2001; Potter 1997).

Thailand is the rich source of tropical plants biodiversity. Numerous plants are explored for their diversity and biological compound containing. The cruciferous plants, for example

wasabi, mustard, water cress, garden cress, and broccoli sprouts are the rich source of cancer chemopreventive compound, glucosinolate (Zhang et al., 2003; Shapiro et al., 1998; 2001). This plant family provides a unique characteristic of pungent odor whose chemical constituents were described as the sulfur-containing glucosinolate (GSL). In plant cells, the GSL compounds are separately intact to the cell containing thioglucosidase or myrosinase enzyme. After the cells are disrupted via cutting, chewing or cooking, isothiocyanates (ITC) were produced as conversion products from GSL (Vaughn & Berhow, 2005) which then promote the virtual pungent odor. More than 20 ITCs were reported for their tumor genesis inhibition. ITC were also found to be effective to even the cell overexpressed multidrug resistance associated protein-1 (MRP-1) or P-glycoprotein-1 (Pgp-1) (Zhang, Tang & Gonzalez, 2003).

Glucosinolates—a class of secondary metabolites present primarily in Cruciferae—possess direct biological activity. It is their hydrolysis products—especially indole and isothiocyanates catalyzed by myrosinase activation—that possess biological action including their known anticancer attributes (Holst & Williamson, 2004). ITCs are derived from glucosinolates via specific enzyme-mediated hydrolysis reaction and are classified by the presence of ITC (— N= C= S) moiety. The pharmacological activities of ITSs include anti-inflammatory, antibacterial, antineuronal injury (Kong et al., 2010; Haristoy et al., 2003; Vauzour et al., 2010), and anticancer activities. The reported ITCs possessing anticancer properties are such as sulforaphane, erucin, iberin, phenethyl isothiocyanate, and benzyl isothiocyanates. The ITCs inhibited cytochrome P450 that activates nitrosamine-induced tumorigenesis in lung and esophagus cancer in rodent (Hecht et al., 2000). Glucoraphanin which is a precursor of sulforaphane significantly increased the expression of CYP 1A1, 1A2, 2B1/2, 2C11 and 3A1/2 in lung of Sprague-Drawley rats (Paolini et al., 2004). ITCs induced carcinogen-detoxifying enzymes of phase II metabolism for instance quinone reductase-1 (QR-1), glutathione-s-transferase (GST), UDP-glucuronyltransferase (UGT), γ-glutamylcystein synthetase (GCS), thioredoxin reductase (TR), aldo-keto reductase (AR) and hemeoxygenase (HO-1), in prostate, breast, colorectal, lung, and hepatocyte cell lines (Brooks, Paton & Vidanes, 2001; Kirlin et al., 1999; Petri et al., 2003; Bonnesen et al., 2001; Dahl & Malcahey, 2001; Zhang et al., 2003). Furthermore, the anticancer mechanisms of ITCs were based on inhibition of cell proliferation, inhibition of tumor invasion, anti-angiogenesis and anti-inflammatory activity in several types of cancer *in vitro* including colorectal, breast, lung and prostate (Higdon et al., 2007).

ITCs are reported to inhibit carcinogenesis by various mechanisms. Sulforaphane (SF) displays anticancer activities against several types of cancer *in vitro*, covering the 3 phases of carcinogenesis. In the initiation phase of carcinogenesis, SF decreases carcinogens via both

the phase I and phase II metabolic pathways. SF inhibits some phase I metabolic enzymes (i.e., CYP3A4 and CYP1A1), which activate the transformation of pro-carcinogens to carcinogens (Gross-Steinmeyer et al., 2005; Mahéo et al., 1997). Meanwhile SF increases many phase II metabolic enzymes such as quinone reductase and glutathione S-transferase via Nrf2 leading to reduction of oxidative stress molecules including carcinogens (Zhang et al., 2006). In the promotion phase, SF modulated several processes restraining the development of cancer. These processes include the induction of apoptosis, autophagy and cell-cycle arrest (Clarke, Dashwood & Ho, 2008; Juge, Mithen, & Traka, 2007).

Another mechanism of action of SF is the induction of apoptosis of cancer cells via both intrinsic and extrinsic pathways. In the intrinsic apoptosis pathway, SF modulates the expression of mitochondrial membrane proteins by enhancing pro-apoptotic protein expression and depressing the expression of anti-apoptotic protein, leading to cytochrome c being released and the activation of a caspase cascade (Yeh & Yen, 2009). In the extrinsic apoptosis pathway, SF reportedly enhances TRAIL mediated apoptosis through down-regulation of ERK and Akt in lung adenocarcinomas (Jin et al., 2007). In the last phase, SF interferes with essential steps; such as, the progression from benign to malignant tumors, angiogenesis and metastasis (Bertl, Bartsch & Gerhauser, 2006; Thejass & Kuttan, 2006).

Sulforaphene (SE) is a natural analog of SF. There are few reports on its anticancer activity. The antimutagenesis of SE is stronger than SF via the inhibition of carcinogenic heterocyclic amines (Shishu & Kaur, 2009). SE induces human colon cancer cell death via intrinsic apoptosis induction by increasing pro-apoptotic protein (Bax) expression and decreasing anti-apoptotic protein (Bcl-2) expression (Papi et al., 2008). There is, however, insufficient information to conclude by what mechanism SE induces extrinsic apoptosis.

There are several mechanisms of chemotherapeutic agents using clinically. However, some of these mechanisms, for example antiangiogenesis and cancer antibody, do not directly eradicate cancer cells and just retard the growth of cancer. In contrast, apoptosis directly causes cancer cell death without inflammatory response, thus, it is considered as gold standard therapy for chemotherapeutic agents. Therefore, ITC have not only shown chemopreventive (blocking) property but also chemotherapeutic (deleting) property in both *in vitro* and *in vivo* studies. These properties made ITC a good anticancer candidate. The amounts of ITC were correlated with anticancer activities and could possibly be used to indicate anticancer potential of cruciferous vegetables.

Thai rat-tailed radish (*Raphanus sativus* L. var. *caudatus* Alef) or “Pak Khi Hood” in Thai is an indigenous vegetable in the northern and northeastern Thailand. It is commonly cooked

in the mixed vegetable soup providing the unique pungent odor and taste of the soup or boiled for accompany eaten with other dishes. Few studies reported the containing of ITCs, mainly as SF and SE in Thai rat-tailed radish (Songsak & Lockwood, 2002; Pocasap, Weerapreeyakul & Barusrux, 2013) and others GSL which were glucodehydroerucin, and gluconapin (Songsak & Lockwood, 2002). Moreover, the chemoprotective effect of Thai rat-tailed radish against colon cell line through apoptosis induction was recently published (Pocasap, Weerapreeyakul & Barusrux, 2013). This apoptosis induction effect indicates the successful cancer treatment as it is pharmacodynamic endpoint (Au et al., 1997). However, more scientific information regarding the cellular mechanism of chemoprotective action, other cancer cell type response, and other existed constituents in the Thai rat-tailed radish extract is still lack and worth to be explored. It was anticipated that the useful information for supporting the uses of Thai indigenous cruciferous vegetables as a source of ITCs and a source of anticancer agents could be achieved from this study. Furthermore, the consumption of Thai vegetable for health benefit and well-being could be encouraged.

CHAPTER 3
RESEARCH METHODS

Chemicals and reagents

Tetrahydrofuran (THF) (HPLC grade, Fisher Scientific, UK) and ultrapure water from Milli-Q system (Millipore, USA) were used for the mobile phase preparation. The pure compounds of D,L-sulforaphane (SF), 1-isothiocyanato-4-(methyl sulfinyl) butane (Calbiochem, EMD Millipore, MA, USA) and L-sulforaphene (SE), 1-isothiocyanato-4-(methyl sulfinyl) butane from Santa Cruz Biotechnology (Dallas, TX, USA) were used as standards. Dimethyl sulfoxide (DMSO) was form Sigma (MO, USA). Commercial grade dichloromethane, hexane, and chloroform were purchased for the extraction and distilled before use. Deionized water (ddH_2O) was obtained from a MilliQ system (Millipore, Bedford, MA, USA.). ddH_2O was used for the HPLC analysis. Allyssin and iberin were purchased from Abcam (Cambridge, UK), and erysolin (Calbiochem, EMD Millipore, MA, USA) were also used in the identification. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 4640 medium, and 0.25% trypsin-EDTA (1X) were from Gibco (Barcelona, Spain).

Methods

1. Sample preparation

Thai rat tailed radish (*Raphanus sativus* var. *Alef*; RS) were cultivated in the Northern province, Phayao, Thailand and were harvested at the age of 7 weeks and kept at -20 °C until used. Frozen RS were manually separated into nine parts which were stem, leaf, root, flower, whole pod, seed, seedless pod, mixed of whole pod and flower (mixed of reproductive part), and the mixed of the aerial part excluded root. Samples were blotted with tissue paper to dry out the excess moisture and cut into small pieces immediately before the extraction.

2. Extractions of isothiocyanates

2.1 Conventional extraction method (CE).

RS were extracted as described in Pocasap, Weerapreeyakul & Barusrux (2013). Briefly, fresh RS 50 g was blended with 50 ml deionized water (DW) for 30 min and left for autolyzing at room temperature for another 2 hr. Then the homogenate was filtered through double layers of cheesecloth. The filtrate was continuing extracted by liquid-liquid extraction with 50 ml of dichloromethane ($CHCl_2$) using separatory funnel. This step was done in triplicate. The lower phase of $CHCl_2$ were collected, sodium sulfate anhydrous was added to get rid of the contaminant water. The filtrate was then dried under vacuum rotary evaporator.

2.2 Serial extraction of the whole pod with varied polar solvent

Further extraction method was conducted as serial extraction after knowing that the dichloromethane extract of whole pod exerted high cytotoxicity in the colon cancer cell line. RS pod homogenate was prepared as #2.1. The serial partitions were carried out as in Figure 1. The crude extracts of water layer were collected and lyophilized, called water crude extract. The dichloromethane layer were dried by rotary evaporator and called DCM crude extract. Some of DCM crude extract was re-dissolved with 20% methanol and partitioned with hexane. The hexane layer was collected and dried out to get the hexane crude extract. The methanol layer was dried and added with water for next serial partition. Three different polarity organic solvents; chloroform, ethyl acetate, and dichloromethane were used for partitioning of the water layer, respectively. All solvent was removed to acquire dry residual extracts and used in the next experiments.

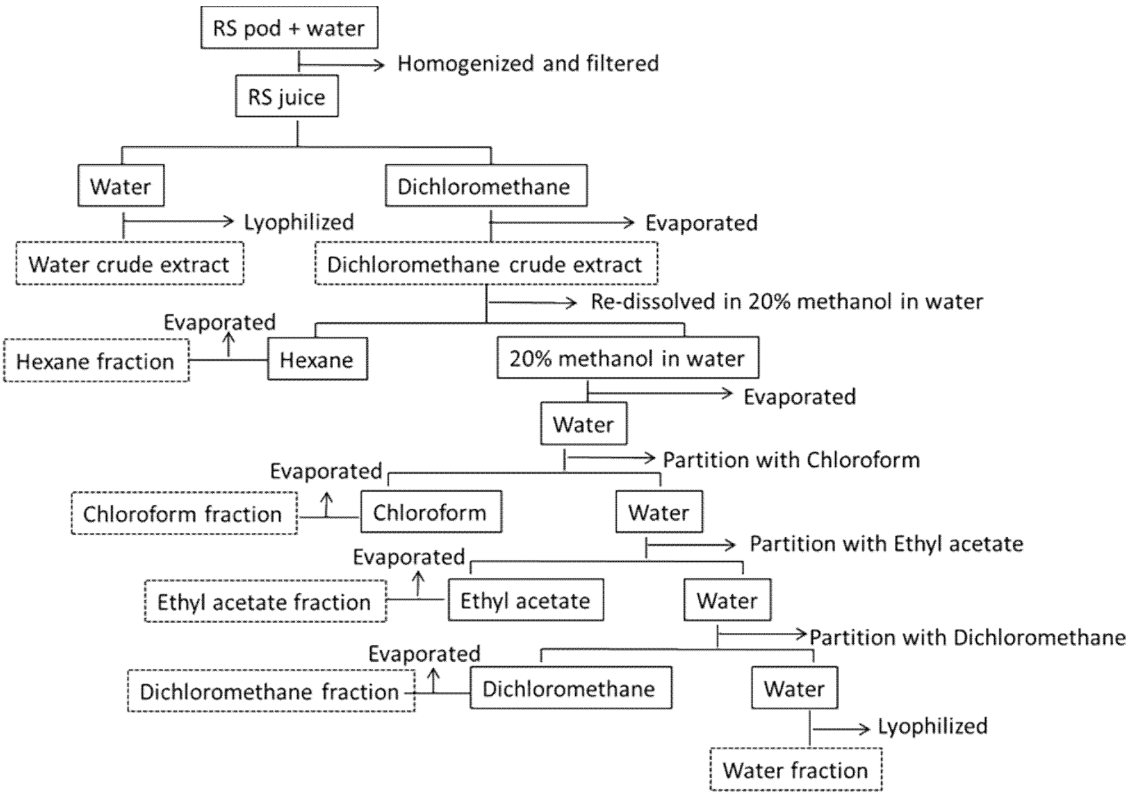


Figure 1-Schematic diagram represented the serial extraction method of the whole pod part of Thai rat-tailed radish (RS).

2.3 Supercritical carbon dioxide extraction method (SCE)

RS were extracted by supercritical fluid extractor (SFX™ 220 extractor, ISCO, Lincoln, USA) using the optimum condition of the previous report of Li et al., (2010). This method was attempted to extract different compounds from the conventional method such as non polar compounds. Small pieces of RS (5 g) was packed in the extraction cartridge, the extraction system was set as 35 °C, 25 MPa with the flow rate of 0.14 ml/min for 3 hr. The residual extract in extraction vessel also vented through empty cartridge for the other hour. The extract was trapped with hexane and then dried under vacuum.

3. Identification of ITCs in RS extracts by FTIR spectroscopy

The obtained RS extracts from those two extraction techniques were subjected to the preliminary structure elucidation by infrared ray (Spectrum One FT-IR spectrometer, PerkinElmer instrument, Germany) with the KBr disc technique. The same amount of extract (1.0 mg) was mixed with KBr and pressed into disc (10 Ton, for 5 min). The transmission results were read from 4,000-400 cm^{-1}.

4. Identification of constituents in RS extracts by HPLC analysis

The previous HPLC analysis (Pocasap, Weerapreeyakul & Barusrux, 2013) to detect ITCs was conducted using isocratic mobile phase. However, the characteristic peak between standard sulforaphane (SF) and sulforaphene (SE) were not well separated. In this study, HPLC elution condition (Agilent 1100 Series HPLC Value System, Agilent Technologies, Hewlett-Packard, Germany) was optimized in order to increase the peak resolution between SF and SE. The stationary phase was C18 reverse phase column (25 cm x 4.6 mm x 5 µm) (HiQsil, Tokyo, Japan). The mobile phase was optimized which was the isocratic of 5% tetrahydrofuran (THF) in ultrapure water with a flow rate of 1 ml/min at 25 °C column thermostat. The UV detector was set wavelength at 210 nm.

5. Determination of the antiproliferative effect of the extract in various human cancer cell lines

5.1 Cell culture

Human cancerous cell lines (i.e., human colon HCT-116, human hepatocellular HepG2, melanoma SK-MEL2, human T lymphocyte Jurkat, human lung SK-LU-1, human breast MCF-7 and human cervical SiHa cancer cells), and normal African green monkey kidney epithelial (Vero) cell lines were cultured with medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were cultured at 37 °C under a humidified atmosphere containing 5% CO_2.

5.2 Antiproliferative effect

The crude extracts were dissolved in dimethyl sulfoxide (DMSO) as stock solutions which were diluted with the media to desired concentrations range. The final concentration of DMSO in each sample did not exceed 1% v/v, to keep the cytotoxicity of DMSO at less than 10%. Cytotoxicity test was performed with a neutral red (NR) method (Machana et al., 2011). Standard anticancer drugs (i.e., melphalan and cisplatin) were used for comparison with the crude extracts. Briefly, the cells were seeded in 96-well plates and treated with various concentrations of the samples for 24 hr. Then, cells were washed with 1x PBS. A total of 100 µl NR solution (50 µg/ml) was added to each well and incubated at 37 °C for another hour. NR was then dissolved by 100 µl of 0.33% HCl. Absorbance of NR dye was detected by a dual-wavelength UV spectrometer at 537 nm with a 650 nm reference wavelength. The percentage of cytotoxicity compared to the untreated cells was determined with the equation given below. A plot of % cytotoxicity versus sample concentrations was used to calculate the concentration which showed 50% cytotoxicity (IC_{50}).

$$\text{Cytotoxicity (\%)} = \frac{[100\times(\text{Absorbance of untreated group} - \text{Absorbance of treated group}]}{\text{Absorbance of untreated group}}$$

The selectivity index (SI), which indicates the cytotoxic selectivity (i.e. safety) of the crude extract against cancer cells versus normal cells, was calculated from the IC_{50} of the crude sample in normal cells versus cancer cells. The most sensitive cancer cell line to the extract was selected for further apoptosis induction study.

6. Determination of the mechanism of cell death

6.1 Nuclei morphological change by DAPI staining

The cells were stained with DAPI according to the method of Pocasap, Weerapreeyakul & Barusrux (2013). The cancer cells were cultured in medium on a chamber slide. After treatment with the extract, the culture medium was discarded and the cells were fixed. The fixed solution was removed and the cells were allowed to dry at room temperature. Then, the cells were stained with DAPI stain solution. In the following step, stain solution was removed and the cells were washed. Then mounting solution was dropped on the cells before covered by a cover slide. Stained nuclei were observed under an inverted fluorescence microscope. The percentage of apoptotic cells (apoptotic index) was calculated.

6.2 DNA fragmentation by using gel electrophoresis

DNA fragmentation was used to determine the induction of apoptosis induction by observing the biochemical change. Briefly, after cancer cells were treated with the test compounds for 24 hr, the cells were collected and washed with media. Then cell suspension was transferred to microcentrifuge tubes (1.5 ml) and centrifuged at 300xg (Wisd Laboratory instrument, Germany) for 5 min to collect the cell pellets. The DNA in the cell pellet was extracted with Flexigene DNA Kit (QIAGEN, Germany). In this study DNA sample was 500 ng/well. DNA content was electrophoresed on 2% agarose gel containing 0.1 mg/ml ethidium bromide. After electrophoresis, DNA fragments were analyzed with a UV-illuminated camera (Syngene, UK).

6.3 Mode of cancer cell death by using flow cytometry

To confirm the occurrence of apoptosis and mode of cell death, Annexin-V FITC/propidium iodide (PI) was performed. The cells were treated with the test extract and positive compounds for 24 hr; then the cells were washed and re-suspended in binding buffer (1X) (BD Biosciences, USA). A saturating concentration of Annexin V and PI was added to the cell samples and incubated for 15 min in the dark at room temperature. The cells were pelleted and analyzed by a fluorescent activated cell sorter (FACS) analyzer (BD FACSCanto II, USA).

6.4 Determination of the apoptosis pathway

6.4.1 Caspase activity assay

The cancer cells were seeded and incubated before being treated with the test compound at various concentrations. The enzyme activity of caspase 3/7, 8 and 9 was determined based on the luminescent technique which detects a substrate of luciferase (aminoluciferin) conjugated with substrate from each of the specified caspase enzymes (Asp-Glu-Val-Asp; DEVD for caspase 3/7, Ile-Glu-Thr-Asp; IETD for caspase 8 and Leu-Glu-His-Asp; LEHD for caspase 9). Aminoluciferin is released after cleaved by the caspase enzyme, resulting in the luciferase reaction and production of light. The caspase activities were expressed as units of relative luminescence (RLU) in direct proportion to the luminescent light and could be detected by the microplate luminometer (Spectramax Gemini XS, RI technology, Singapore). Blank determinations were performed on a test plate only; containing only sample and medium. All of the experiments were performed in triplicate.

6.4.2 Detection of mitochondrial membrane potential

The cells were seeded and incubated at 37 °C. Cells were treated with different concentrations of the extract at various time points. The cells were washed, harvested by trypsinization, and transferred to a microcentrifuge tube followed by staining with lipophillic

fluorogenic dye; 3,3′dihexyloxacarbocyanine iodide (DiOC6) in the dark condition, 37 °C. After incubation with fluorochrome, the cells were immediately analyzed using flow cytometry.

7. Determination of the inhibition effect on efflux pump in the selected cancer cell line (HCT-116 colon cancer cells)

RS pod and stem extracts was determined for their effect on efflux proteins (P-gp and MRP-1) in HCT-116 colon cell line. These two efflux proteins are normally presented in the resistance cancer cells. Cisplatin and melphalan were represented the anti-cancer drugs, while pure ITCs (sulforaphane and sulforaphene) were also used for comparison.

7.1 Inhibition of P-gp activity

The inhibition of P-gp activity in HCT-116 cells was determined using Rhodamine 123 as a fluorescent substrate of P-gp and verapamil was used as a P-gp inhibitor. After reaching 80% cell confluence, cells were seeded into sterile a 24-well plate at 1×10^6 cells/ml and cultured for 24 hr at 37 °C in 5% CO_2 incubator. To determine the uptake of rhodamine 123, the cells were pre-incubated with fresh media in an absence or a presence of P-gp inhibitor, verapamil 30 μM for 30 min at 37°C. After the pre-incubation, the 10 μM of rhodamine 123 was added to each well. Then, cells were incubated for 60 min at 37 °C in a 5% CO_2 incubator. After this final incubation, the cells were washed twice with an ice-cold phosphate-buffered saline (1x PBS), pH 7.4. Cells were re-suspended with 1x PBS. The accumulation fluorescence of rhodamine 123 was detected by using flow cytometry (BD FACSCanto II, Franklin Lakes, NJ, USA) at wavelengths of 488 and 530 nm, respectively.

7.2 Inhibition of MRP-1 activity

The activity of the MRP-1 efflux protein was determined using carboxyfluorescein diacetate (5(6)-CFDA) as a specific substrate for MRP-1 and probenecid acted was used as a MRP-1 inhibitor. Briefly, cells were seeded into a sterile 24-well plate at 1×10^6 cells/ml and cultured for 24 hr at 37 C in a 5% CO_2 incubator. Cells were pre-incubated with fresh media in an absence or presence of 1 mM probenecid for 30 min. After the pre-incubation, 5 μM of CFDA was added into each well. Then, cells were incubated for 60 min at 37 °C in a 5% CO_2 incubator. The cells were washed twice with an ice-cold phosphate-buffered saline (1x PBS), pH 7.4. Cells were re-suspended with 1x PBS. The accumulation fluorescence of CFDA was detected by using flow cytometry (BD FACSCanto II, Franklin Lakes, NJ, USA) at wavelengths of 488 and 530 nm, respectively.

8. Investigation of the cancer cellular biochemical changes in the treated cancer cells by using FTIR microspectroscopy in the selected cancer cell line.

The HCT-11 colon cancer cells were seeded at the concentration of $3x10^5$ cells/ml in 24-wells plate. After incubation, cells were treated with the positive control or the treatments for 24 hr. The control was the untreated cells. The treated cells were washed with PBS and trypsinized out to get the cell. The collected cells were resuspensed with 0.9% normal saline solution. The cell suspension was then dropped onto the Low-E slide and dried under vacuum. Afterward, the cells on slide was gently washed with a drop of deionized water and dried under vacuum. This step was repeated until a thin monolayer of cells (viewable under an inverted microscope) was obtained. The dried cells on Low-E slide were kept in a desiccator until used. Biomolecular changes in the cell samples were determined by using FTIR microspectroscopy—conducted at an offline IR spectroscopy facility, at the Synchrotron Light Research Institute (Public Organization), Thailand. The Bruker Hyperion 2000 microscope (Bruker Optics Inc., Ettlingen, Germany) equipped with a nitrogen cooled MCT (HgCdTe) detector with a 36 IR objective, coupled to a Bruker Vertex 70 spectrometer was used for FTIR data acquisition. The FTIR spectra was obtained in the reflection mode 64 scans, 64 mm aperture size at a resolution of 6 cm^{-1} over a measurement range of 4000-600 cm^{-1}. Spectral acquisition and instrument control were performed using OPUS 6.5 software (Bruker Optics Ltd, Ettlingen, Germany).

CHAPTER 4

RESULTS AND DISCUSSION

Thai rat-tailed radish (RS) samplewas extracted with 2 different tecniques. Percent yield obtained by conventional solvent extraction (CE) or supercritical CO_2 extraction method (SCE) were shown in Table 1. The high extraction yield was obtained from supercritical CO_2 extraction (SCE) leaf extract (0.1220 %w/w, fresh weight) and whole pod mixed with flower (edible part) extracted by conventional solvent extraction (CE) (0.1045 %w/w, fresh weight). Comparing between CE and SCE, there were variations in the extraction efficacy of both techniques. It was observed that the sample which contained rough fiber such as root, stem, and leaf could be extracted easier by SCE and giving higher ITCs yield.

Table 1-RS sample and percent yield obtained by conventional solvent extraction (CE) or supercritical CO_2 extraction method (SCE). **Superscripted numbers indicate high[1] to low[9] %yield.

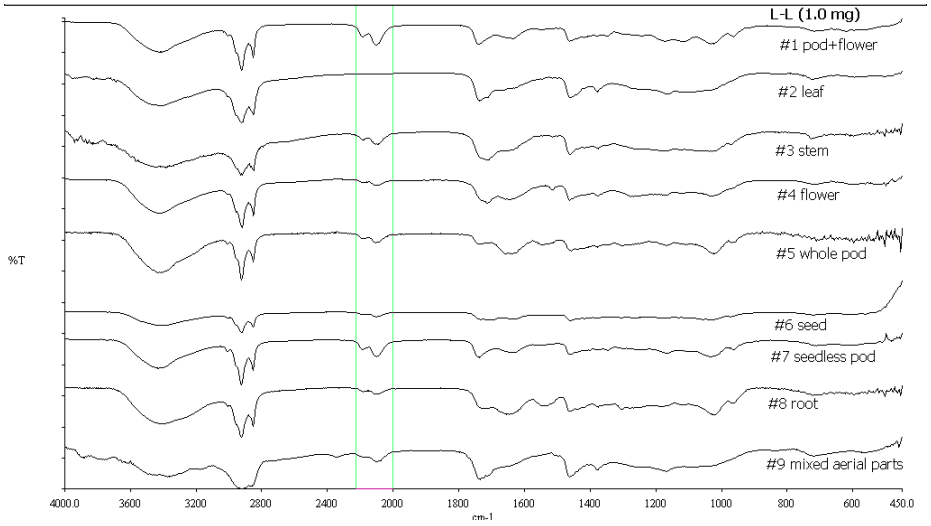
Part		% Yield (w/w fresh weight)	
		CE	SCE
Root		0.0336 [8]	0.0580 [4]
Stem		0.0282 [9]	0.0640 [3]
Leaf		0.0472 [4]	0.1220 [1]
Whole pod and flower		0.1045 [1]	0.0110 [7]
Flower		0.0433 [5]	0.0342 [5]

Part		% Yield (w/w fresh weight)	
		CE	SCE
Whole pod		0.0595 [3]	0.0760 [2]
Seed		0.0981 [2]	0.0064 [9]
Seedless pod		0.0349 [7]	0.0090 [8]
Mix aerial parts		0.0426 [6]	0.0132 [6]

Identification of isothiocyanates constituents in RS extracts by FTIR spectroscopy

FTIR spectra were interpreted into the possible existing compound. Figure 2 showed the characteristic band of –N=C=S functional group of the isothiocyanates at wavenumber 2,000–2,200 cm^{-1} in each extracts obtained from each techniques. It was found that the extraction techniques and parts of the RS plant have different FTIR spectra indicating different constituents in the extracts. The extracts of each part of RS plant from the conventional solvent extraction (CE) reveal characteristic isothiocyanates (ITCs) band at 2,000–2,200 cm^{-1}. Relatively high content of ITCs was found in the seedless pod and reproductive parts such as flower and pod more than the other parts. In consideration of the supercritical CO_2 extraction methods (SCE) techniques, only extract from pod showed minute ITCs band when compared to the other part. Due to the simple and rapid technique, CE was used to prepare the extracts of each RS parts for the next experiment.

(A) CE



(B) SCE

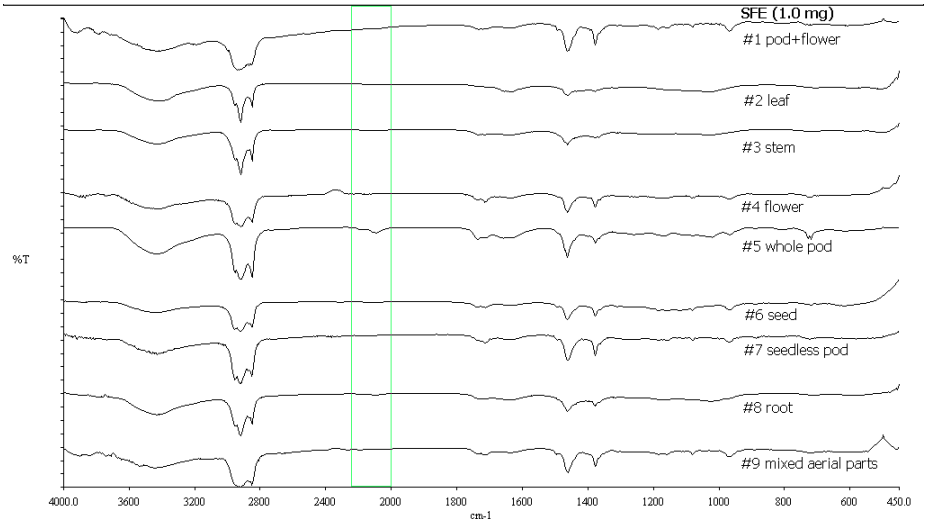


Figure 2-FTIR spectra of RS extracts (1.0 mg) by (A) conventional extraction and from (B) supercritical CO_2 extraction techniques. Isothiocyanate (—N=C=S) was identified at wave number 2,000—2,200 cm^{-1}.

Identification of constituents in RS extracts by HPLC analysis

The HPLC analysis was optimized to detect ITCs using isocratic mobile phase. Figure 3 showed HPLC chromatogram of the extract sample illustrating well separate peaks of sulforaphane (SF) and sulforaphene (SE). The respective retention times of SE and SF were 23.95 min and 25.51 min. The SE and SF contents existed in the extract samples were determined by using the standard curve (Figure 4) of standard SE and SF, respectively.

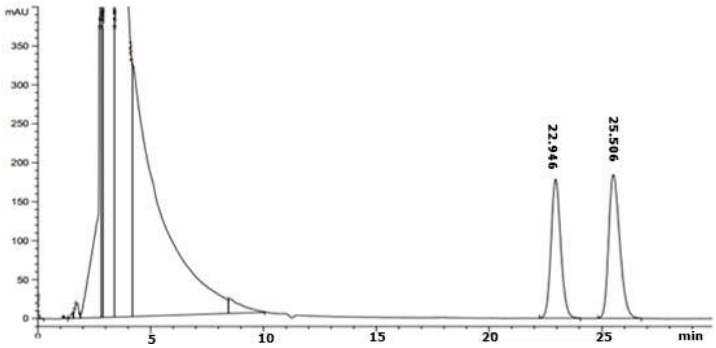


Figure 3-HPLC chromatogram of sulphoraphene (SE) and sulforaphane (SF). The presence of SE and SF were shown at the retention time of 22.946 min and 25.506 min, respectively.

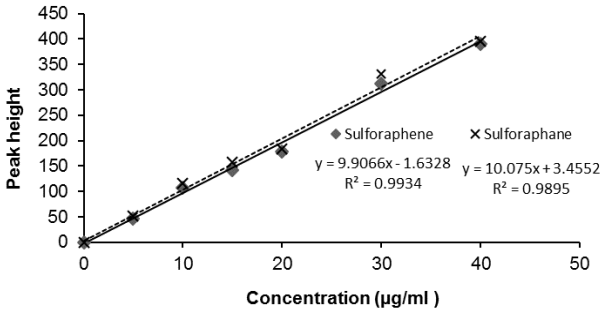
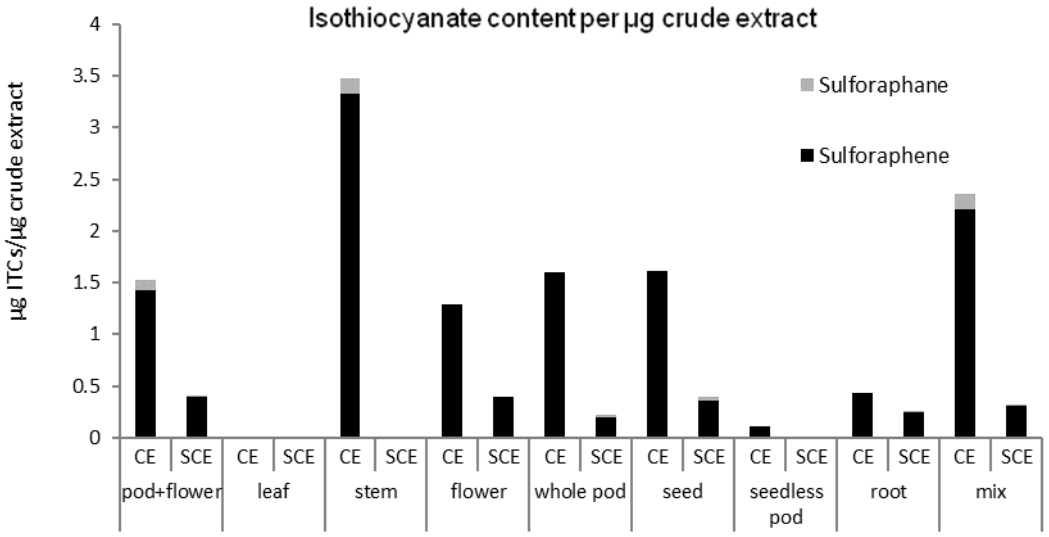


Figure 4-Standard curve obtained from the HPLC analysis of standard sulforaphane and sulforaphene (n=3).

It is evident that the conventional solvent extraction techniques (CE) yielded higher content of sulforaphane and sulforaphene than the supercritical CO_2 extraction methods (SCE) (Figure 5). SE was mainly found in higher amount than SF in most RS parts. The order of high to low SE content in the extracts of the following plant parts are stem > mixed arial part > seed > whole pod > pod & flower > flower > root > seedless pod > leave, respectively. However, when calculated back to the fresh weight, the CE extraction of seed yielded the highest SE content following by pod & flower > stem > whole pod > mixed arial part > flower >

root > seedless pod > leave, respectively. SF content was only found in the extracts of stem > mixed of arial part > pod and flower, respectively.



Extract	Content (µg/g crude extract)	
	SE	SF
Pod & flower	175.14±0.35	15.55±0.29
Leaf	not detected	not detected
Stem	408.30±0.69	23.84±0.23
Flower	158.91±3.24	not detected
Whole pod	195.05±44.66	not detected
Seed	197.62±0.97	not detected
Seedless pod	12.92±0.06	not detected
Root	52.69±2.06	not detected
Mix arial part	270.96±0.45	24.41±0.32

Extract	Content (µg /g fresh weight)	
	SE	SF
Pod & flower	183.03±0.37	16.25±0.30
Leaf	not detected	not detected
Stem	115.14±0.20	6.72±0.06
Flower	68.81±1.40	not detected
Whole pod	116.05±26.57	not detected
Seed	193.87±0.95	not detected
Seedless pod	4.51±0.02	not detected
Root	17.70±0.69	not detected
Mix arial part	115.43±0.19	10.40±0.14

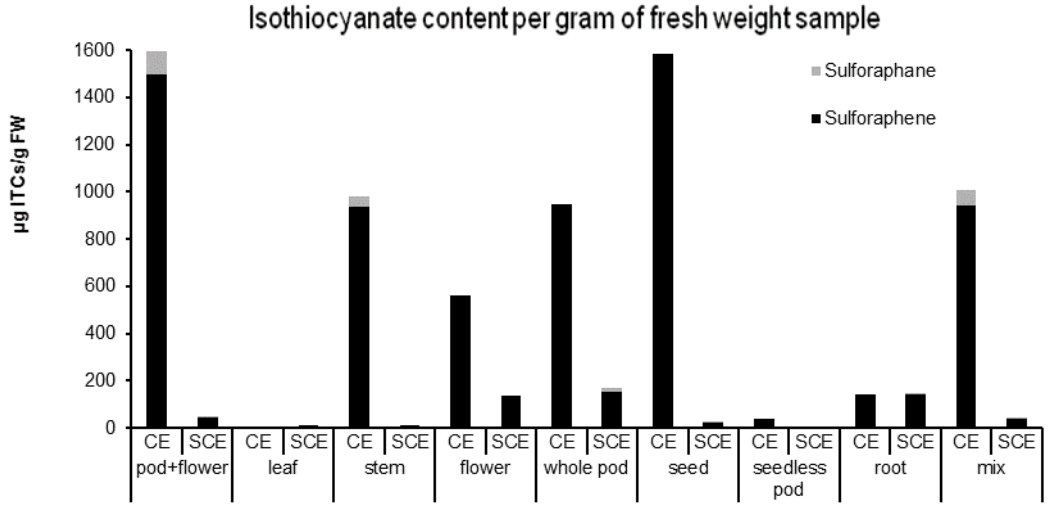


Figure 5-HPLC analysis of isothiocyanates contents (sulforaphane and sulforaphene) in the RS extract.

Determination of the cytotoxic effect of the extracts in various cancer cell lines

The higher cytotoxicity was obtained in the treatment of the extracts from the conventional extraction method (CE) than supercritical CO_2 extraction method (SCE) in all plant parts (Table 2). Different orders from high to low cytotoxicity of each plant parts were observed in each extraction methods. The lowest cytotoxic activity was found from leave part in both extraction methods. The pod part was found to have higher activity than stem from CE but the activity order was reverse in the SCE. From the mentioned results, the RS pod and stem extracts from CE were selected for further experiments.

Table 2-Percentage of cytotoxicity against human colon cancer (HTC-116).

Extract	CE		SCE
	50 µg/ml	500 µg/ml	500 µg/ml
Pods & lowers	inactive	39.70±0.27	16.60±0.86
Leaves	inactive	10.99±0.28	1.19±0.60
Stems	32.90±1.23	95.08±1.34	36.19±0.30
Flowers	inactive	35.28±0.64	20.03±1.26
Pods	69.18±0.87	91.15±0.49	27.58±0.33
Seeds	inactive	17.10±0.39	9.57±0.40
Seedless pods	inactive	39.95±0.69	10.89±0.15
Roots	inactive	19.93±0.53	11.38±0.68
Mixed arial part	inactive	13.71±1.26	7.73±1.20

Note: The cytotoxicity percentages were presented in mean±SD, n=4.

Inactive means no cytotoxicity when treated with the test sample at that concentration.

The study of the most sensitive human cell lines to RS pod and stem extracts obtained from conventional solvent extraction were performed in various human cancerous cell lines, hepatocellular carcinoma (HepG2), lung (SK-LU), breast (MCF-7), cervical (Hela), melanoma (SK-MEL2), colon (HTC-116), T lymphocyte (Jurkat) in comparable with the normal Vero cell line. The lower IC_{50} indicates the higher cytotoxic potential of the tested compounds. Moreover, the commercial chemotherapeutic drugs, cisplatin and melphalan, SF and SE were also tested for a comparison.

Results in Table 3 showed that ITCs—sulforaphane and sulforaphene— were not toxic to the cervical (Hela) cell line. The RS stem extract was toxic to liver (HepG2) and colon (HCT-116) cancer cell lines. Both RS pod and stem were not toxic to Jurkat T lymphoma cell line. The lowest IC_{50} of RS pod and stem were found in the treatment against colon cancer cell (HCT-116) with the IC_{50} of 35.69 ± 0.12 and 128.32 ± 5.36 µg/ml, respectively. IC_{50} value of RS pod extract against HCT-116 is 35.69 ± 0.12 µg/ml or equivalent to 0.084 ± 0.003 µg SE/ml.

Table 3-Cytotoxicity results against human cancer cell lines and normal Vero cell line.

Cell lines	IC_{50} (µg/ml) of test compounds					
	Cisplatin	**Melphalan**	**Sulforaphane**	**Sulforaphene**	**RS pod**	**RS stem**
Vero	121.49±1.60	322.98±2.37	9.68±0.11	9.69±0.10	100.62±1.01	219.60±9.26
HepG2	65.06±0.56	91.28±9.08	13.60±0.90	13.96±0.34	57.26±2.09	172.72±3.19
R-HepG2	101.80±1.72	117.97±1.98	25.42±0.66	16.72±0.09	247.47±1.68	401.69±25.46
SK-LU	211.64±6.03	267.56±6.44	8.10±0.62	17.01±0.17	84.22±2.39	inactive
MCF-7	60.67±1.35	157.10±5.72	73.39±0.56	160.22±2.34	392.23±1.94	inactive
Hela	52.57±2.10	130.53±2.77	inactive	inactive	402.63±9.23	inactive
SK-MEL2	27.66±0.33	52.13±1.31	147.39±0.74	165.33±2.95	421.17±16.16	inactive
HCT-116 ($p53^{+/+}$)	25.85±0.46	116.36±1.27	4.94±0.16	8.60±0.12	35.69±0.12	128.32±5.36
HCT-116 ($p53^{-/-}$)	57.59±0.99	145.63±1.03	7.32±0.09	25.09±1.28	128.23±2.14	308.68±3.13
Jurkat	61.80±1.26	68.07±0.66	11.72±0.16	7.30±0.79	Inactive	Inactive
Molt4	102.39±2.43	77.12±1.82	15.22±0.08	13.41±0.15	323.57±5.94	358.30±3.60
U937	134.27±1.01	174.74±5.69	29.88±3.16	42.12±2.25	60.07±1.70	309.34±12.79
Daudi	111.94±3.23	43.85±1.03	10.39±0.29	13.69±0.23	298.68±11.41	369.41±6.46

The lymphocyte cells; Molt 4, U937, and Daudi were also studied. The cytotoxic effect of RS extracts was studied on the resistance cells of HepG2 and colon cancer cell lines. The results found that when compare IC_{50} value of all treatment against wild type HepG2 cancer cell and resistant HepG2, the wild type cell has higher sensitivity to the toxicity of treatments. As

well as colon cancer cell, the wild type cell possessed lower IC_{50} values than p53 deficient cell line (HCT-116 ($P53^{-/-}$)). The toxicity of treatments against lymphocyte cell lines found that RS extracts were active against MolT4, U937, and Daudi cell lines but not in Jurkat. Especially in U937, RS pod extract give relatively low IC_{50} value of 60.07 ± 1.70 µg/ml.

In order to determine the primary contributed group of compound on cell toxicity effect, the crude extract was also partially separation using liquid extraction. Different polarity organic solvents were used to get the different polarity compounds. The percent yields of each solvent extraction were shown in Table 4. The water crude extract, dichloromethane (DCM) crude extract, and its fractions; hexane, chloroform, ethyl acetate, dichloromethane, and water fractions were tested at final concentration 500 µg/ml against HCT-116, colon cancer cell line. Results in Figure 6 showed that the crude extract from DCM found to have higher cytotoxicity than water extract due to the preferable polarity of ITCs. After partially separation, all fractions gave lower activity than DCM crude extract. It may be suggested that there is synergy of constituents in the DCM crude extract. Therefore, the next apoptosis induction study will be performed by using the DCM crude extract prepared from the conventional solvent extraction (CE).

Table 4-Percent yield of the whole pod extract from various solvent extraction.

Extracts	% Yield (w/w fresh weight)
Water crude extract	3.277
Dichloromethane crude extract	0.068
Hexane fraction	0.021
Chloroform fraction	0.010
Ethyl acetate fraction	0.015
Dichloromethane	0.013
Water fraction	0.012

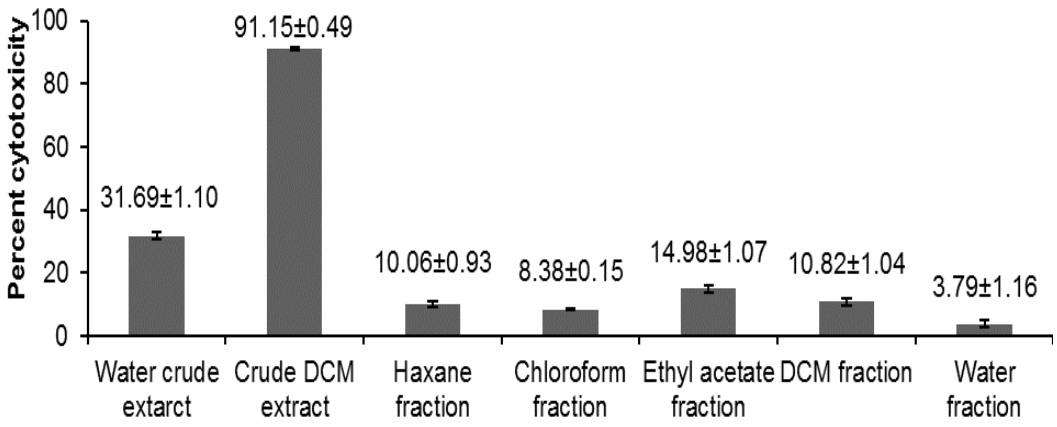


Figure 6-Cytotoxicity of water crude extract, DCM crude extract, and its fractions from whole Pod at 500 µg/ml.

Determination of the mechanism of cell death

Determination of the morphological change of HCT-116 ($p53^{+/+}$) cell line by using fluorescent dye staining

Due to colon HCT-116 ($p53^{+/+}$) cancer cells were most sensitive cell line to the test compounds, apoptosis induction was performed in this HCT-116 cell ($p53^{+/+}$) line. The characteristic of apoptotic cells is the nucleus condensation detected by florescence staining (Figure 7). Cisplatin, melphalan, sulforaphane, sulforaphene, RS pod extract and RS stem extract at $1\times IC_{50}$ concentration induced apoptosis with nuclear morphological changes reminiscent of apoptotic characteristics *i.e.*, chromatin condensation and apoptotic body formation (arrow). The changes of the nuclei in the cells after treated with $1\times IC_{50}$ test compounds can be observed as the vivid heterogeneous staining and apoptotic bodies. The morphological of the untreated cell nuclei or normal nuclei were homogeneously stained.

DNA fragmentation

One of the phenomena that occur with the apoptosis mechanism is DNA fragmentation. After the late stage of apoptosis, the DNA within the cancer cells is fragmented. DNA fragmentation occurs through the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of 180 bp and multiples thereof. The gel electrophoresis will show the ladder pattern of fragmented DNA. The treatments were fixed at 48 hr and the concentrations of each treatment were varied in $1xIC_{50}$ and $2xIC_{50}$ values

to study the concentration dependent manner. Similar to the case for melphalan, the RS pod and stem extracts at $1\times IC_{50}$ and $2\times IC_{50}$ concentrations positively observed exhibited the characteristic DNA ladder formation (Figure 8). Under the condition studied, DNA fragmentation was observed for sulforaphane treatment only at high concentration ($2\times IC_{50}$), but not for sulforaphene. Cells death after treatment with cisplatin, sulforaphane and sulforaphene might be lost during washing step and mounting slide. Thus, additional experiments confirming the apoptosis effect of test compounds were also performed.

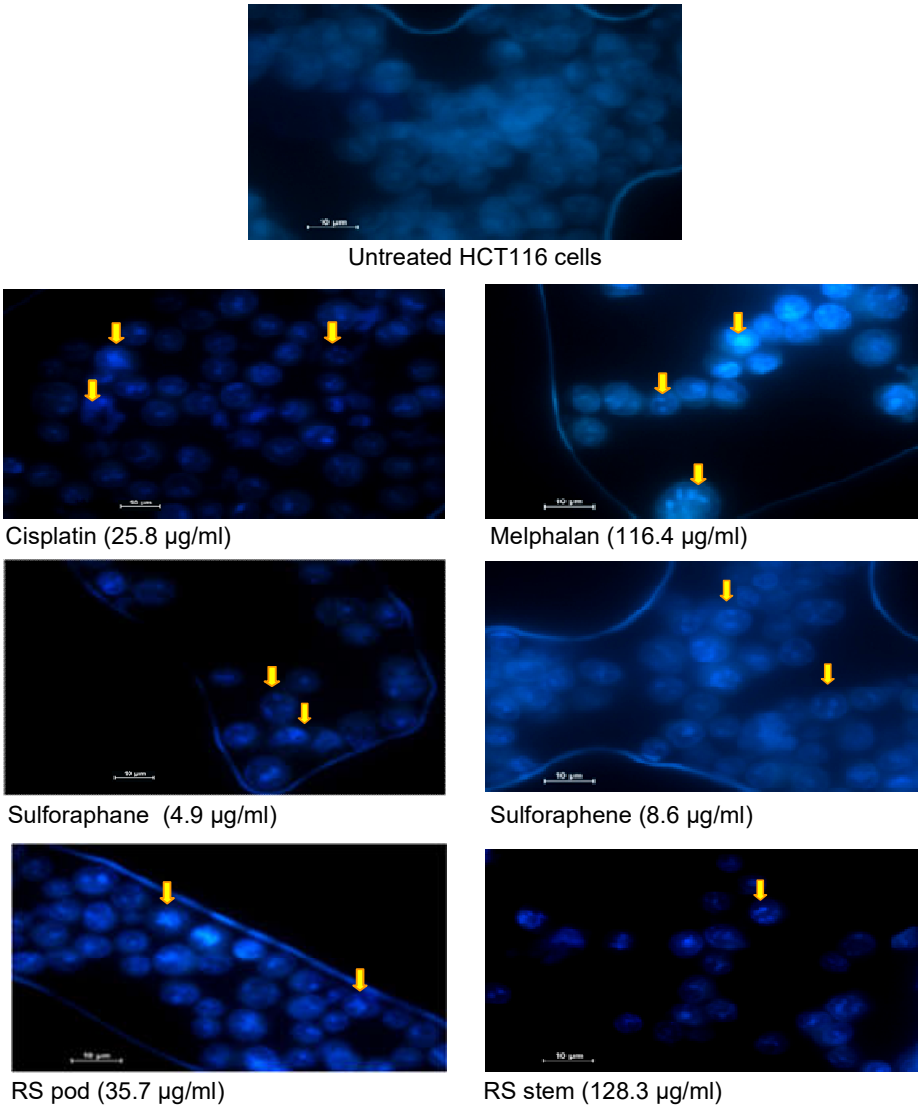


Figure 7-HCT-116 ($p53^{+/+}$) nuclei morphology stained with DAPI. Cells were treated with test compounds at $1\times IC_{50}$ concentrations. Pictures were taken under fluorescent microscope with 400× (objective and eye lens) magnification.

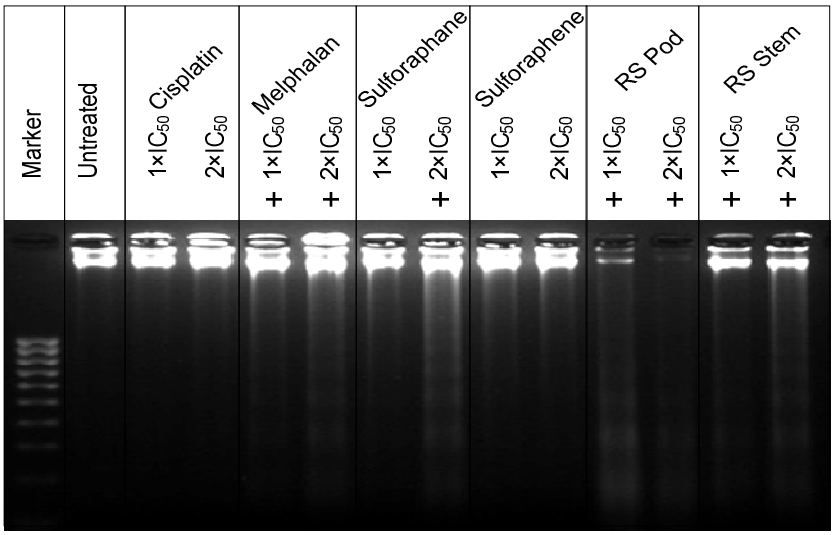


Figure 8-DNA fragmentation of HCT-116 ($p53^{+/+}$) colon cancer cell after treated with the test compounds at 48 hr.

Classification of cell death mode in HCT-116 by using Annexin V-FITC/propidium Iodide

Apoptosis and necrotic cell death were differentiated by flow cytometry using Annexin V-FITC/propidium iodide (PI) staining of cells. Apoptotic colon HCT-116 ($p53^{+/+}$) cells were identified by their externalized phosphatidylserine (PS) bound with Annexin V-FITC, while necrotic cells were identified by their DNA intercalation of PI. During the early stages of apoptosis, the PS comprising the cell membrane flip to the outside and bind with Annexin V-FITC, but not bind with PI (+/-). Damaged or injured cells lose their membrane integrity, including nuclear membrane integrity, such that PI can pass through the nuclear membrane (necrotic cells) (-/+). In the late stages of apoptosis, cells are double-stained and appear (+/+). Normal cells are not stained (-/-).

Figure 9 showed the increment of the treatment concentrations in accordance with the decreasing in viable cell as well as increasing of apoptotic cells. The necrotic cells were also increased along the treatments. Among chemotherapeutic drugs, melphalan possessed higher apoptosis induction against colon HCT-116 cancer cells than cisplatin at 72 hr. And, $2\times IC_{50}$ of RS pod extract possessed higher apoptosis induction than RS stem extract at 24 hr. All treatments were found to induce apoptotic cell death in concentration-dependent manner. The apoptosis induction treated by the positive pure ITC compounds, sulforaphene and sulforaphane were not in concentration-dependent manner at the 24 hr exposure times.

Therefore, the increasing of treatment time was performed with fixed concentration at 1 × IC_{50} and varied 3 different exposure times (24, 48 and 72 hr). The decrements of viable cell as well as the increments of apoptotic cells were found with time-dependent manner (Figure 9).

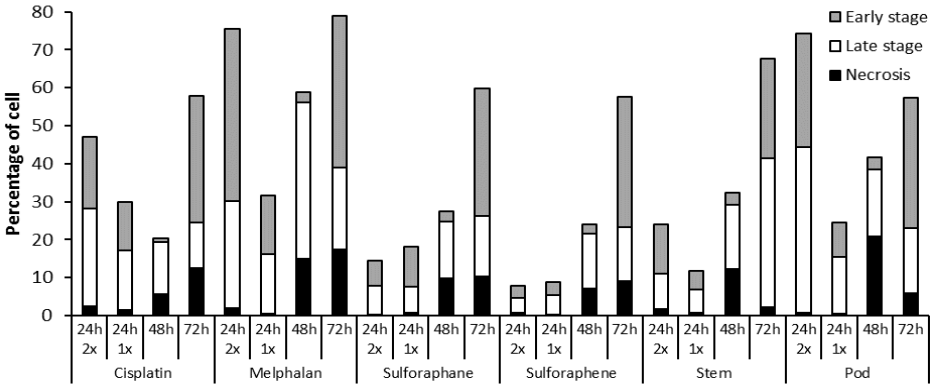


Figure 9-Apoptotic cell death mode in colon HCT-116 cells (wild type). For concentration-dependence, the time was fixed at 24 hr with 1×IC_{50} and 2×IC_{50} concentrations. And the concentration was fixed at 1×IC_{50} at different exposure times (24, 48 and 72 hr) for time-dependence study.

Determination of the apoptosis pathway induced by the extract from Thai rat-tailed radish

Caspases activity assay

The caspase activities were performed on 3 specific caspases which are the initiator caspase 8 and 9 and the effector caspase 3. Caspase 8 is the initiator of extrinsic apoptosis pathway where caspase 9 initiates intrinsic pathway. The results in Figure 10 were presented in the normalized luminescence value of the treated HCT-116 ($p53^{+/+}$) cells by the untreated HCT-116 ($p53^{+/+}$) cells. Caspase 3 activities among all treatments were slightly difference. The highest activity of melphalan was found at 6 hr treatment. While the other test compounds i.e., ITCs, sulforaphane and sulforaphene RS pod and stem extracts reached the highest activity at earlier time point of 3 hr. Caspase 8 representing the extrinsic pathway was gradually increased and maximally observed at 24 hr of all treatments. The caspase 9 activity which mediated the intrinsic apoptosis pathway were different. The maximum caspase 9 activity of melphalan, RS pod, and stem extracts were observed at 24 hr treatment, while the highest caspase 9 activity of SF and SE treatments were found at 12 hr treatment.

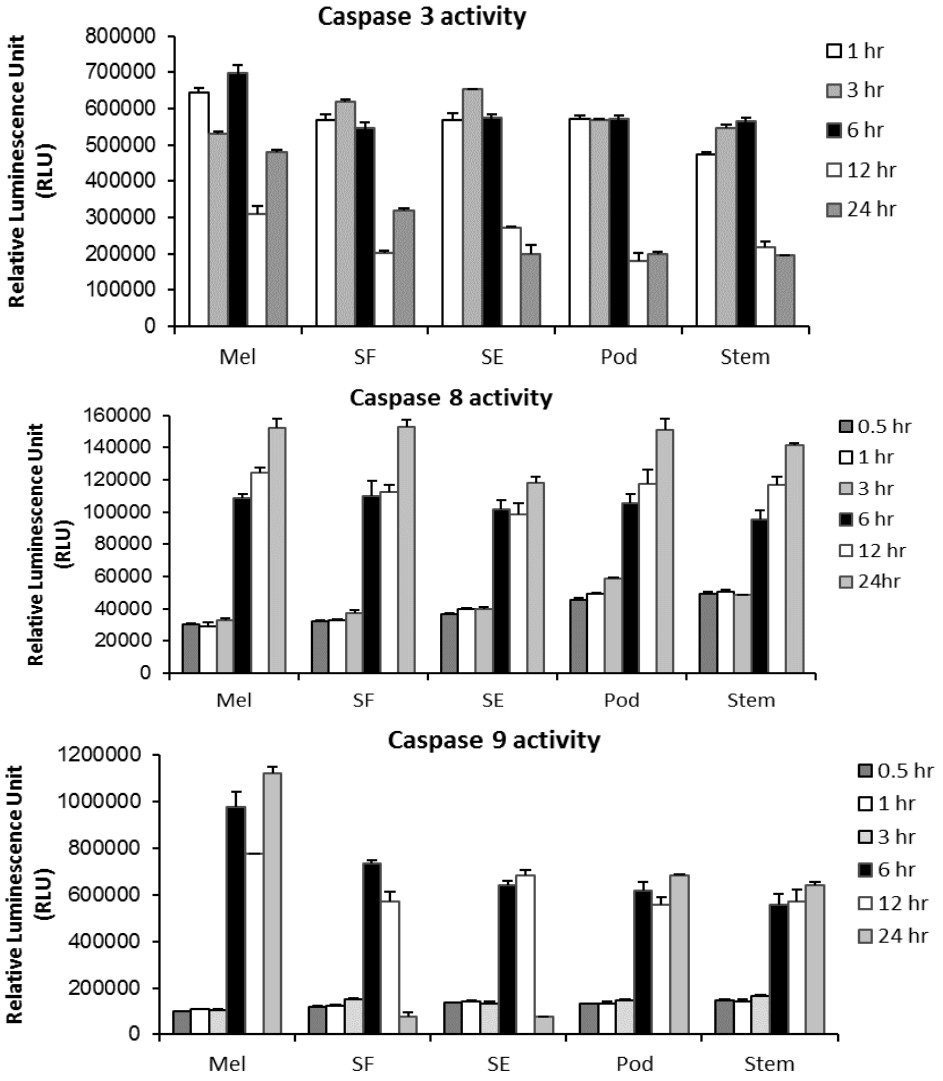


Figure 10-Caspases activities in the colon HCT-116 ($p53^{+/+}$) cells treated with different test compounds at $2\times IC_{50}$ with various times.

Detection of mitochondrial membrane potential in the HCT-116 treated with the test samples

The loss of mitochondrial membrane potential (MMP) is one among many other indications of apoptotic induction via the intrinsic pathway. The cytochrome c is released from the mitochondria then activates the procaspase 9. Figure 11 showed the concentration-dependent manner of almost all treatments. Between the chemotherapeutic drugs, melphalan caused MMP change in colon HCT-116 cell line more than cisplatin. Both positive ITCs did not clearly change the MMP in a concentration-dependent manner. Both RS stem and pod extracts showed MMP alteration in the concentration-dependent manner.

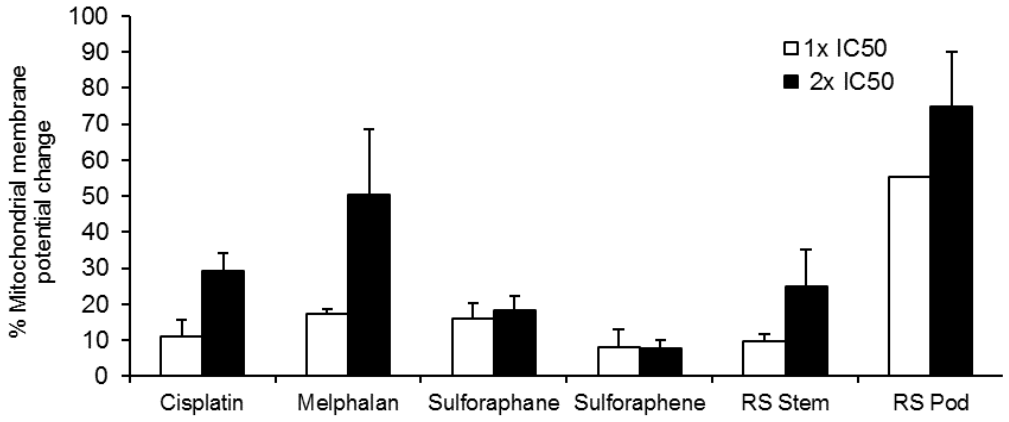


Figure 11-Percentage of mitochondrial membrane potential loss after HCT-116 ($p53^{+/+}$) cell line treated with different concentrations of test compounds.

Inhibition of efflux proteins activity

The efflux proteins are concerned as the factor affected cancer cell resistance and success of chemotherapy. In the present study, the effect of RS pod and stem extracts on inhibition of efflux proteins activity—P-gp and MRP1—were determined. The human HCT-116 ($p53^{+/+}$) cell line was used to study for the possible relationship of RS pod and stem extracts on efflux activity. Two efflux proteins which are P-gp and MRP1 were determined from the intracellular accumulation of specific substrate of each efflux proteins. Cells were divided into control group of untreated cells (untx). The positive treatments were verapamil and probenecid for P-gp and MRP1 protein, respectively. Two concentrations of 1× and 2×IC_{50} values of cisplatin and melphalan anti-cancer drugs, and the treatment of pure ITCs; sulforaphane and sulforaphene were also used for comparing with the RS pod and stem extracts. Result showed

that inhibition of P-gp activity was not significantly observed in all treatments in the HCT-116 cells compared to the control (Figure 12A) except cisplatin at 2×IC_{50} concentrations. Only inhibition of MRP-1 activity was concentration dependently found in HCT-116 treated with SF, SE, RS pod extract, respectively (Figure 12B). Interestingly, RS pod extracts significantly inhibit the MRP-1 pump with an enhanced intracellular uptake of MRP-1 substrate. There might be some constituent(s) in the RS pod extract presented as MRP-1 inhibitor which offered an increasing uptake of their active compound(s) in the RS pod extract into the HCT-116 cells.

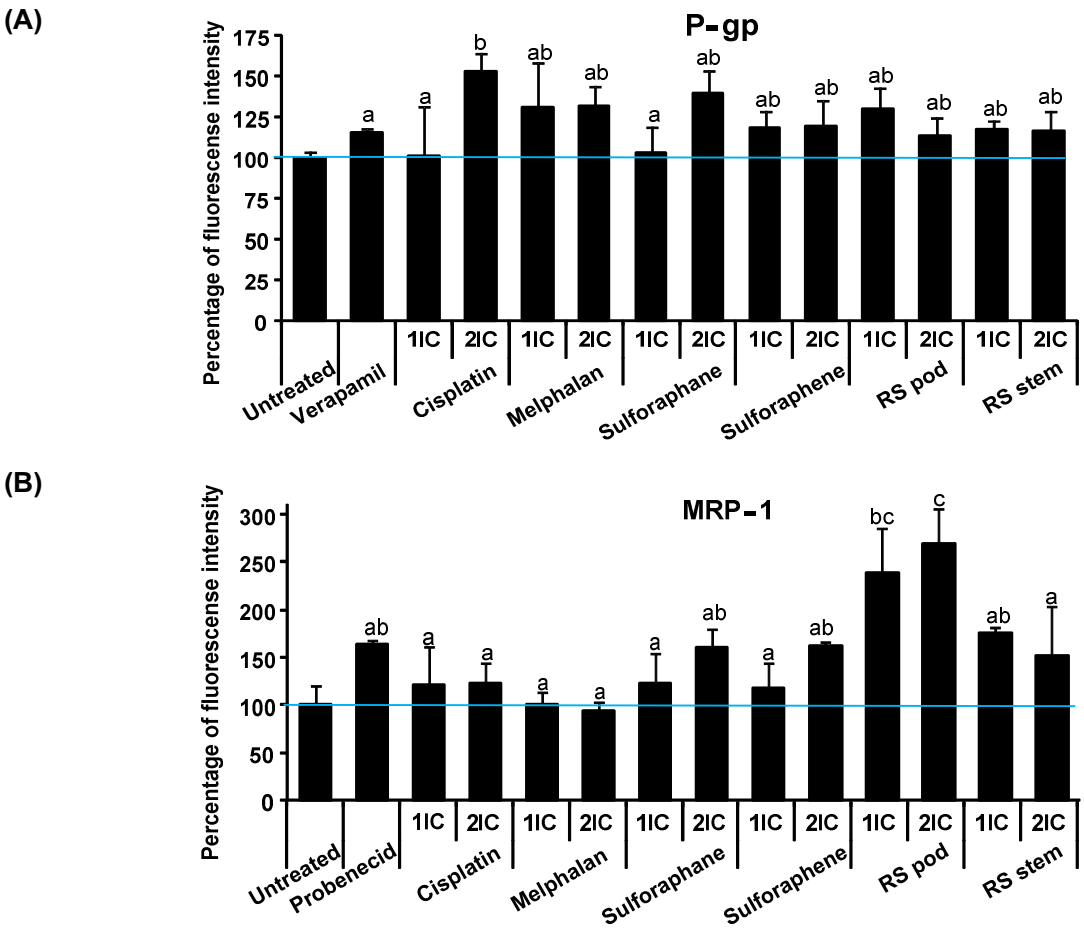


Figure 12-Inhibition of efflux proteins (A) P-gp and (B) MRP-1 activity in HCT-116 ($p53^{+/+}$) colon cancer cell line. Cells were divided into control group of untreated cells, positive treatments which are verapamil and probenecid for P-gp and MRP1 protein, respectively. Cells were treated with 1× and 2×IC_{50} concentrations values of cisplatin and melphalan, pure ITCs; sulforaphane and sulforaphene, and RS pod and stem extracts. Different letters indicate significantly difference between samples ($P<0.05$).

FTIR microspectroscopy defined biomolecular changes in colon cancer cell line

The FTIR spectra show the variation in cellular biochemical changes in various treatments of colon cancer HCT-116 cells. FTIR spectra of HCT-116 cells in the range of 3000–900 cm^{-1} represented the cellular biomolecules (Fig. 13A). The spectra represent 3 major biochemical component regions which are the lipid region (3000–2800 cm^{-1}), the protein region (1700–1300 cm^{-1}), and the carbohydrate and nucleic acid region (1300–900 cm^{-1}).

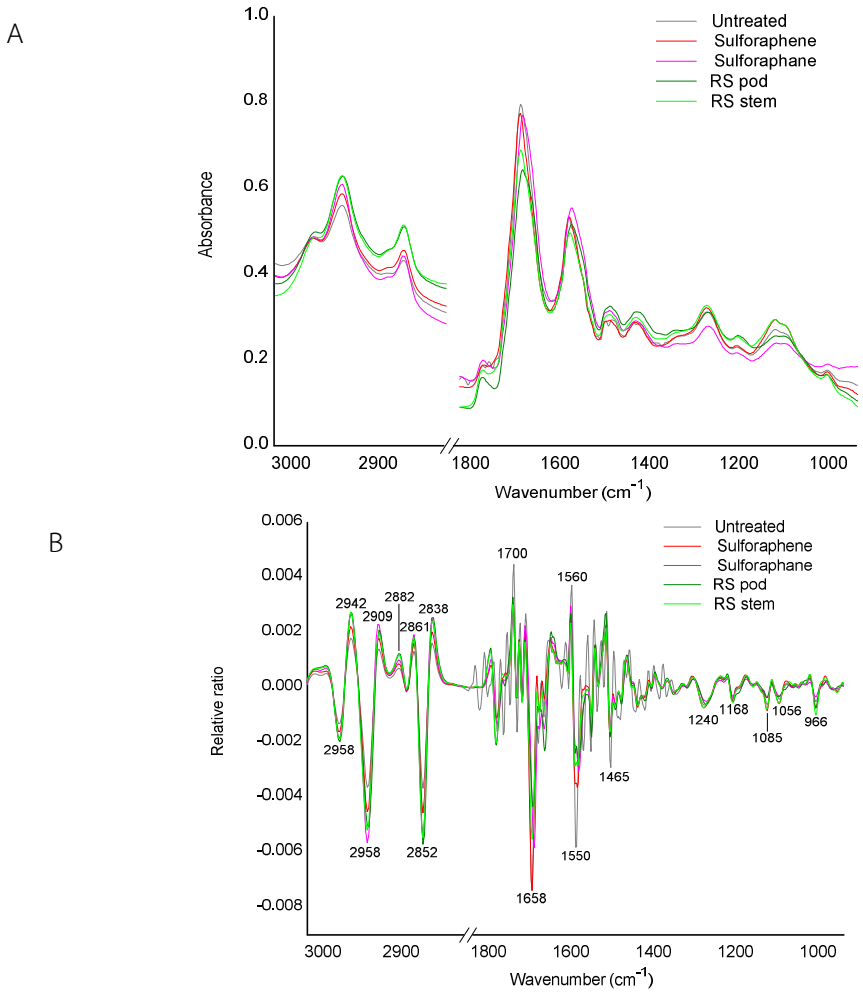


Figure 13-(A) Average primary spectra normalized with extended multiplicative signal correction (EMSC) and (B) average second derivative FTIR spectra processed by taking Savitzky–Golay algorithm and normalizing with EMSC.

To get better determination, the primary spectra were taken for the second derivative using the Savitzky–Golay algorithm (with nine points of smoothing allowing minimization of the effects of variable baselines) and normalized with extended multiplicative signal correction (EMSC). The normalized spectra were accounting for differences in sample thickness and correcting for scattering artifacts into secondary spectra (Figure 13B). The peaks and intensity of secondary spectra were characterized for the changing of cells biomolecules after treatment. Characteristics peaks of cells were identified in (1) the lipid region at 2958, 2942, 2923, 2909, 2882, 2861, 2852, and 2838 cm^{-1}, (2) protein region at 1700, 1658, 1560, 1550, and 1465 cm^{-1}, and (3) the carbohydrates and nucleic acids at 1240, 1168, 1085, 1056, and 966 cm^{-1}.

The lipid region (3000–2800 cm^{-1}) is assigned to the symmetrical and asymmetrical stretching vibrations of the CH_2— and CH_3— groups contained in the fatty acids of the cells (Gasper et al., 2009 & Zelig et al., 2009). The 1800–1700 cm^{-1} region is characteristic of the ester C=O stretching of the lipid head-group (Derenne, Gasper & Goormaghtigh, 2011). The 1478 and 1350 cm^{-1} region is characteristic of the CH_3– and CH_2— bending of the methylene chains in lipids (Gasper et al., 2009).

The protein region (1700–1300 cm^{-1}) is assigned to the amides I and II. The amide I band (1700–1500 cm^{-1}) indicates a secondary structure of protein absorption. The stretching of the carbonyl from the peptide bond was observed at 1650 cm^{-1} (amide I). The deformity of the protein at the amide II of N–H bending and C–N stretching was observed at 1540–1500 cm^{-1}. The bands between 1480 and 1300 cm^{-1} are represented by amino acid side chains and fatty acids (Derenne, Gasper & Goormaghtigh, 2011 & Gasper et al., 2009). The absorption bands at 1245 and 1085 cm^{-1} are characteristic of the asymmetrical and symmetrical phosphodiester vibrations of nucleic acids.

The carbohydrate and nucleic acid region (1300–900 cm^{-1}) is assigned to the absorptions resulting from the carbohydrates and phosphates mainly associated with nucleic acids, *i.e.,* DNA and RNA. The RNA ribose chain and C–C bond of nucleic acids faintly appear at 966–968 cm^{-1} (Gasper et al., 2011a; Gasper et al., 2011b & Tanthanuch et al., 2010).

Principle component analysis

Score plots are used for clustering the biomolecular changes of untreated and all treated cells. Figure 14A shows PC-1, PC-2, and PC-3 which used to discriminate each sample groups. The loading plots express the biomolecular variations in the data set shown in Figure 14B.

Figure 14A (top) shows that the PC-1 can distinguish the untreated cells and cell treated with sulforaphane (SF) from the cells treated with pod and stem extracts and sulforaphene (SE) for 57%. The PC-2 distinguishes cells treated with SF and RS stem extract from the cells treated with SE and RS pod extract for 26%. This data indicated that the cells treated with the RS stem extract seemed to have similar biochemical changes to cells treated with SF, while the cells treated with the RS pod extract exerted similar biochemical changes as the cells treated with SE.

Figure 14B (top) shows that the major peaks found in the untreated cells that make this cell group different from others treated cell groups are lipid peaks at 2944, 2907, and 2840 cm^{-1} and protein peaks at 1658 and 1550 cm^{-1}. And the treated cells have major characteristic peaks of lipids at 2958, 2923, and 2852 cm^{-1} and proteins at 1558, 1540, and 1457 cm^{-1}.

Figure 14B (bottom) shows that the major components commonly found in cells spectra of SE and RS pod extract are lipids at 2917 and 2848 cm^{-1} and protein peaks at 1646, 1629, and 1534 cm^{-1}. The component of SF and RS stem can be distinguished in PC-2 are proteins at 1658, 1556, and 1473 cm^{-1}, and carbohydrates and nucleic acids at 1245, 1174, 1085, 1058, and 968 cm^{-1}.

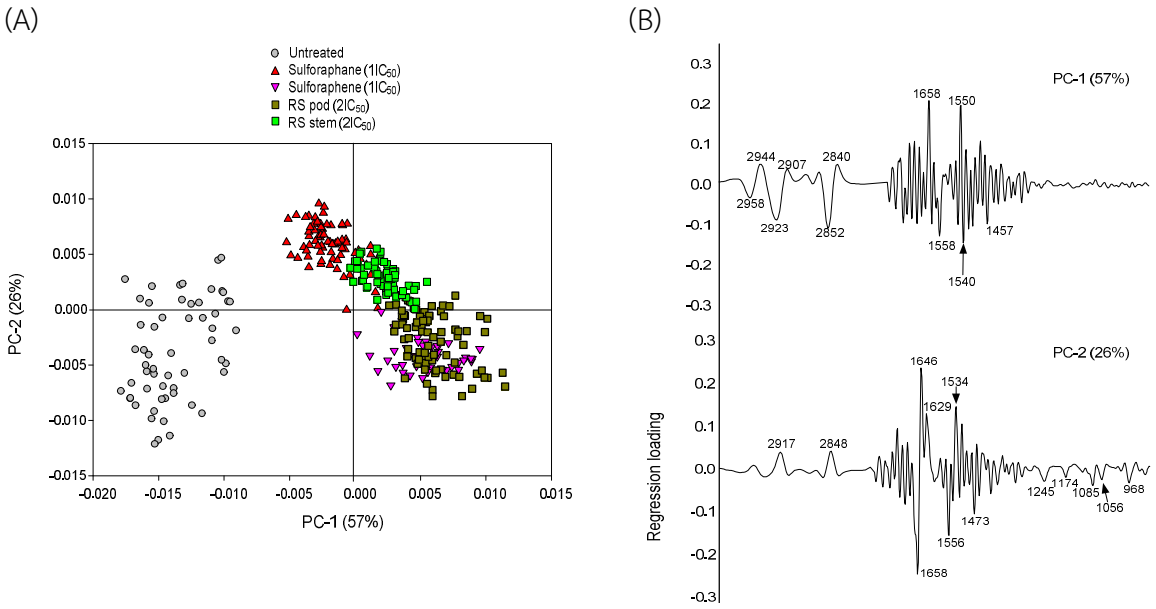


Figure 14-PCA analysis of FTIR spectral range 3000–2800 and 1800–900 cm^{-1} giving PCA score plots (A) and PCA loading plots (B). PCA score plot calculated from second derivative spectra. PCA loading plots indicate biomarker difference by discriminating wave numbers over spectral range of cell samples.

The integration of peak areas at each region was determined using OPUS 7.1 software to indicate the alteration of the biomolecular level after cell treatment. The integrated peak area of lipids (3000–2800 cm^{-1}), amide spectra (1700–1500 cm^{-1}) and nucleic acids (1300–950 cm^{-1}) were presented in Table 6.

The RS pod and stem extracts affect different biochemical contents in the HCT-116 cells from the SF and SE. The lipid contents of all treated cells were significantly increased when compared to the untreated HCT-116 cells or control but not in SF-treated cells. The protein level was decreased in cells treated with SF, RS pod and stem extracts when compared to the control but not in the cells treated with SE. RS stem extract and SE increased carbohydrate and nucleic contents in contrast to SF that decreased carbohydrate and nucleic contents. While the RS pod extract did not significantly increase carbohydrate and nucleic contents when compared to the control.

Table 5-Integral peak area of lipid, proteins, and nucleic acid regions of the FTIR primary spectra.

Cell treatment	Lipid	Protein	Carbohydrates and nucleic acid
Untreated cells	12.92±0.15[a]	44.55±0.25[c]	13.94±0.30[b]
Sulforaphene	17.85±0.40[b]	45.88±0.23[c]	18.85±1.14[c]
Sulforaphane	11.30±0.08[a]	26.83±2.63[a]	4.58±1.13[a]
RS Stem	19.74±1.31[b]	37.15±1.05[b]	17.45±1.07[c]
RS Pod	18.21±1.10[b]	36.55±2.43[b]	14.52±0.91[b]

*The different letters describe the statistically difference ($p<0.05$) between untreated cells and treated cells.

The increased lipid content of the treated cells may be related to cell undergoing apoptosis after treatment. The increment of lipid was previously reported to be affected by the phosphatidylserine exposure on membrane, membrane blebbing and vesicle formation which is contrast to the necrotic cells which lipid membrane decreases due to the cell swelling and loss of membrane integrity (Zelig et al., 2009). The apoptotic cells can be indicated by the alteration of proteins in cell. There are various apoptotic protein factors; the Bcl-2 protein family comprises pro-apoptotic (i.e., Bax and Bid) and antiapoptotic proteins (i.e., Bcl-2), which play important roles in the regulation of apoptosis. The decrement of nucleic acids (DNA, RNA base) regions were caused by apoptosis processes. As a consequence of DNA condensation and despite DNA degradation during apoptosis, DNA absorbs less IR due to DNA opaqueness (Zelig et al., 2009). Our present study showed that the RS pod and stem extracts led to the biochemical change in the HCT-116 cells that evidence from the FTIR spectral difference between the RS and SF and SE treated cells. These biochemical changes may be due to different phytoconstituents extracted from each RS parts. Moreover, the other constituent may also be contributed to different effect.

CHAPTER 5
CONCLUSION

Raphanus sativus var. *caudatus* Alef (Thai rat-tailed radish, RS) was studied whether it can be an alternative source of functional foods focusing on chemopreventive effect. The phytochemicals in Brassica were primarily composed of glucosinolates and their products known as isothiocyanates (ITCs) such as sulforaphene (SE) and sulforaphane (SF). The HPLC analytical assay was developed for the simultaneous detection of both SE and SF in the RS extracts. The successful method was the simple used of isocratic 5% tetrahydrofuran in ultrapure water for 30 min assessment. The RS was extracted by two methods using conventional (dichloromethane extraction) and super critical CO_2 fluid extraction. The higher SE and SF contents and cytotoxicity were obtained from the dichloromethane (DCM) crude extract. It was found that the RS pod has the highest ITCs biomarkers content (SE = 2253.05 µg/g crude extract and S = 111.94 µg/g crude extract) followed by stem extract (SE = 1105.14 µg/g crude extract). The bioassay guided by neutral red assay showed high antiproliferative effect with low IC_{50} of RS pod and stem extract (35.69 and 128.32 µg/ml, respectively). Moreover, the RS pod extract possessed high selective index against colon cancer HCT116 cell line over the normal Vero cell line (SI = 2.8) in comparable to the other cancer cell lines. The RS pod DCM crude extract possessed MRP1 efflux pump protein inhibition determined by the accumulation of fluorescent substrate determined by flow cytometry. The apoptosis induction—the target of chemopreventive compounds of RS pod and stem extracts compared to the chemotherapeutic drugs (cisplatin and melphalan) and ITCs biomarkers using annexin V/propidium iodide (PI) staining and determined by flow cytometry. The hallmark phenomenon of apoptosis such as morphological changes with nuclei condensation was determined by fluorescent staining of DAPI and DNA laddering fragmentation determined by gel electrophoresis were found in HCT116 treated with RS pod and stem extracts. The percent apoptosis was increased with time and concentration dependent manner. The RS pod extract caused apoptotic cell death through the mitochondria-mediated pathway as evident by the mitochondria membrane disruption investigated by $DiOC_6$ fluorescence dye. The apoptosis involving enzyme—caspase activity was investigated by the luminescent technique. The induction of caspase 9 activity was found in HCT116 treated with RS pod and stem extracts indicating the intrinsic apoptosis. In addition, RS stem extract induced apoptosis through the extrinsic pathway by caspase 9 activity. The FTIR microspectroscopy revealed the biomolecular changes of the HCT116 cells by the increasing lipid content affected by the

disruption of lipid membrane bilayer and the secondary protein structure-shifted from α-helix to β-pleated sheet after cells were treated with RS pod and stem extract.

This study presented that Thai rat-tailed radish pod and stem extract from dichloromethane layer induce apoptotic cell death in HCT116 cell line. It suggests that Thai rat-tailed radish pod and stem can be used as the functional food and also be an alternative source of isothiocyanates—chemopreventive compounds. However, the chemopreventive activity of the RS extracts in this study was performed in the cell lines model. Further clinical relevance studies such as the study in an animal model and in clinical trial are still required. The most appropriate RS preparation and food processing is also necessary to obtain the desirable active compounds and chemopreventive effect. It is hoping that our obtained information will lead to the value added to this plant and promote the usage of this vegetable.

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OUTPUT

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1. 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 Chemistry. 201(-): 139-144, **2016**.

1.2. 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). Journal of Functional Foods. 31:237-247, **2017**.

2. อื่น ๆ (Paper presented in the conference as an abstract)

2.1. Sangthong, S, **Weerapreeyakul, N ***, Barusrux, S. Plausible extraction technique for cancer chemopreventive isothiocyanate compounds from the *Raphanus sativus* L. var. *caudatus* Alef. The 23rd Meeting of the European Association for Cancer Research, July 5 – 8, 2014, Munich Germany, European Journal of Cancer 50, Suppl. 5; S192 (2014) (Poster presentation).

2.2. Sangthong, S., **Weerapreeyakul, N.***, Barusrux, S. Antiproliferative effect of *Raphanus sativus* L. var. *caudatus* Alef in human breast MCF-7 and human lung SK-LU1 adenocarcinoma cell lines. The International Conference on Herbal and Traditional Medicine. January 28-30, 2015, Khon Kaen Thailand. p. 75-81 (Poster presentation).

2.3. Sangthong, S., **Weerapreeyakul, N.***, Barusrux, S. Cell survival inhibitory effect of *Raphanus sativus* v. *caudatus* Alef extracts against non-resistant HepG2 and resistant HepG2 hepatocellular carcinoma. The 34th National Graduate Research Conference. March 27, 2015, Khon Kaen Thailand. p. 80-83 (Poster presentation).

2.4. **Weerapreeyakul, N.***, Sangthong, S. Study of Potential Chemopreventive Effect of Thai Rat-Tailed Radish. การประชุมนักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว. ครั้งที่ 15 (The 15th TRF-OHEC Annual Congress 2016, TOAC2016) ในวันที่ 6-8 มกราคม 2559 ณ โรงแรมเดอะ รีเจ้นท์ ชะอำบีช รีสอร์ท หัวหิน ชะอำ จังหวัดเพชรบุรี (Oral presentation).

2.5. **Weerapreeyakul, N.***, Sangthong, S. Apoptosis inducing effect of the extract of *Raphanus sativus* L. var. *caudatus* Alef. การประชุม “นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว.” ครั้งที่ 16 (2017 TRF-OHEC Annual Congress, TOAC2017) ในวันที่ 11-13 มกราคม พ.ศ. 2560 ณ โรงแรมเดอะ รีเจ้นท์ ชะอำบีช รีสอร์ท หัวหิน ชะอำ จังหวัดเพชรบุรี (Oral presentation).

APPENDIXES

Reprints (* corresponding author)

1. 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 Chemistry. 201(-): 139-144, **2016**.
2. 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). Journal of Functional Foods. 31:237-247, **2017**.

Manuscript in preparation

1. "*Raphanus sativus* var. *caudatus* Alef pod extract generates ROS contribute to intrinsic apoptosis in colon cancer cell line HCT116"—Planned to submit to "Planta Medica".

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Analytical Methods

Simultaneous quantification of sulforaphene and sulforaphane by reverse phase HPLC and their content in *Raphanus sativus* L. var. *caudatus* Alef extracts

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ABSTRACT

A simple, rapid and precise HPLC assay was developed for the well-known anti-cancer isothiocyanates—sulforaphene (SE) and sulforaphane (SF). The analytical system comprised RP-C_{18} column with isocratic 5% THF-95% water. High resolution was obtained (and eluted) of two distinct HPLC peaks of similar structures SE and SF analytes (at 23.01 ± 0.02 and 25.65 ± 0.03 min, respectively). The respective LOD vs. LOQ for SE and SF was 0.34 and 0.36 μg/ml vs. 1.02 and 1.08 μg/ml. This assay had the best linearity and accuracy. The recoveries were in the range of 96.83–101.17%. SF and SE were quantified in the pod of *Raphanus sativus* L. var. *caudatus* Alef extracts (2253.05 ± 246.18 and 111.94 ± 16.49 μg/g in the crude extract, respectively), while only SE was detected in the stem (1105.14 ± 243.10 μg/g crude extract), as SF was lower than the detection limit. The validated method thus minimized and expedited simultaneous SE and SF analysis.

1. Introduction

Brassicaceae and related plant families are rich in secondary plant metabolites; including over 120 glucosinolates (GSLs) and various isothiocyanates (ITCs) (De Nicola et al., 2013; Li et al., 2010; Nakamura, 2009; Oerlemans, Barrett, Suades, Verkerk, & Dekker, 2006). GSLs coexist with myrosinase—an endogenous thioglucosidase (EC 3.2.3.1) in adjacent plant cells—and are released and hydrolyzed by it; producing mainly ITCs—sulfur- and nitrogen-containing compounds.

Sulforaphane (SF) is a cancer chemo-preventive agent (Fig. 1) found in broccoli (*Brassica oleracea* L. var. *italica*) (Nakamura, 2009). Clarke, Dashwood, and Ho (2008) reported the anti-cancer effects of SF: (a) blocking the initiation state via inhibiting Phase I enzymes to convert procarcinogens to proximate or ultimate carcinogens; and (b) inducing Phase II enzymes that detoxify carcinogens and facilitate their excretion from the body. A further protective effect associated with oxidative stress was revealed in experimental models (Guerrero-Beltrána et al., 2012).

Sulforaphene (SE) (Fig. 1)—with an additional double bond to SF—has been reported to reduce cancer cell proliferation in a dose-dependent manner and induce apoptosis in: (a) colon carcinoma cell lines (Barillari et al., 2008; Papi et al., 2008); (b) human and murine erythroleukemic cells; (c) human T-lymphoid cells; and, (d) human cervix carcinoma cells (Nastruzzi et al., 2000). SE (at 1 μM) was a potent inducer of hepatic enzymes involved in the detoxification of chemical carcinogens (Razis, Nicola, Pagnotta, Iori, & Ioannides, 2012). Both SF and SE are bioactive constituents in Thai rat-tailed radish (*Raphanus sativus* L. var. *caudatus* Alef, RS or Pak Khi Hood), contributing to an apoptosis induction effect against the HCT116 colon cancer cell line (Pocasap, Weerapreeyakul, & Barusrux, 2013).

Intensive analytical assay techniques have been developed to quantify the bioactive constituents in food, vegetables and herbs. Although present in small amounts, these compounds play critical

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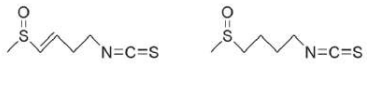

Sulforaphene Sulforaphane

Fig. 1. Chemical structures of sulforaphene (SE) and sulforaphane (SF).

roles in food function and the bioactivity of medicinal plants. Optimum quantitative analysis should lessen the cost of resource consumption—including chemicals, sample used, time and facilities. HPLC has been widely used for quantitative analysis of bioactives for quantitative analysis, as minimum handling is needed. Due to structural similarities between SE and SF (Fig. 1), HPLC results in poor separation. Lim, Lee, and Kim (2009) tried using two different detectors to achieve simultaneous detection but—despite using high specification equipment—low resolution persisted. In the current study, (a) simple, simultaneous HPLC for chemopreventive SF and SE is reported for the first time and (b) quantitative analysis of both biomarkers in extracts of the Thai native vegetable (RS) was successfully achieved.

2. Materials and methods

2.1. Chemicals

Tetrahydrofuran (THF) (HPLC grade, Fisher Scientific, UK) and ultrapure water from Milli-Q system (Millipore, USA) were used for the mobile phase preparation. Pure D,L-sulforaphane, 1-isothiocyanato-4-(methyl sulfinyl) butane (Calbiochem, EMD Millipore, USA) and L-sulforaphene, 1-isothiocyanato-4-(methyl sulfinyl) butane (Enzo Life Sciences, USA) were used as the standards. Dimethyl sulfoxide (DMSO; Sigma, USA) was used as the diluent.

2.2. Standard stock preparations

The respective stock SE, SF and SF–SE mixture was diluted to 5.0, 10.0, 15.0, 30.0 and 40.0 μg/ml in DMSO.

2.3. Chromatographic analytical conditions

The HPLC Value System was used to optimize the elution condition (Agilent, 1100 Series, Waldbronn, Germany). The stationary phase was done in a Reverse Phase-C_{18} column (5 μm particle size, 250 × 4.6 mm) (HiQsil, Tokyo, Japan). The mobile phase was developed in simple filtered and degassed 5% THF in ultrapure water (v/v). The flow rate was stable at 1 ml/min in isocratic elution for 30 min. The photo diode array (PDA) detector (Agilent, 1100 series G1314A, Tokyo, Japan) was set at 254 nm as per Pocasap et al. (2013) and detected at 210 nm. An in-line 0.5-μm filter and a guard column were used to protect the analytical column.

2.4. Validation parameters

The validation parameters included (a) specificity of each compound; (b) selectivity of the elution method between two peaks; (c) limit of detection (LOD); (d) limit of quantification (LOQ); and, (e) linearity of the calibration curves (R^2). The retention times of each standard in the mixture solution were identified and the percentage of relative standard deviation (%RSD) was calculated to confirm the specificity of the peaks.

Evaluation of method repeatability (intra-day precision) and reproducibility (inter-day precision) were performed. Intra-day precision was determined at 5 concentrations in 6 replications. The inter-day precision was determined in 6 replications conducted over 3 days. Precision of the method was expressed as the %RSD for each test. The standard deviation (SD) of the response and slopes of the concentration curves of the calibration curves were used to estimate the limit of detection (LOD) and the limit of quantification (LOQ) with the following formulae: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the residual SD of the regression line and S is the slope of the standard curve. The percentage of recovery of each compound was evaluated. Sulforaphane and sulforaphene were analyzed in triplicate at three different concentrations (5 μg/ml, 15 μg/ml and 40 μg/ml) by using the obtained validated method. The percent recovery was calculated [%Recovery = (recovered conc./injected conc.) × 100] and the results statistically analyzed.

2.5. Sample preparation

RS was grown outdoors on a farm in Phayao province, northern Thailand between December 2014 to January 2015. The plants were harvested after 6 weeks growth. The RS was rinsed with water then patted dry with a paper napkin. RS extracts were prepared from stems and whole pods as these plant parts are reported to contain relatively high amounts of isothiocyanates, according to Pocasap et al. (2013). The fresh RS samples were homogenized with deionized water at a ratio of 1:1 (w/v) for 30 min and the homogenate allowed to stand at room temperature for 2 h. The homogenate was filtered through double layers of cheesecloth. Dichloromethane was added to the aqueous filtrate at a ratio of 1:1 (v/v) and liquid–liquid extraction performed in triplicate. The lower dichloromethane layer was collected and the contaminated water removed by anhydrous sodium sulfate. The organic solvent was removed under vacuum by rotary evaporator yielding dry crude extract.

3. Results and discussion

3.1. Optimization of HPLC analysis

A number of previous studies tried to detect more than one major compound in the crude extract mixture. The HPLC elucidation profile of RS crude extract by Pocasap et al. (2013) suggested the peaks for SE and SF coexisted. The GC analysis moreover supported the co-existence of SE and SF as major compounds in the RS extract. The present study proposes an elution system that affords high resolution between the SE and SF peaks, by the weakly acidic property of THF (Wang, Helliwell, & You, 2000). It was found that the use of tetrahydrofuran in water mobile phases could elute specific derivatives with a THF concentration of 5%. Our present study showed good separation between SE and SF (Fig. 2). The separation of each compound without peak-merging indicates the specificity of the detection method. The respective retention times of SE and SF were 23.01 ± 0.02 min and 25.65 ± 0.03 min with a %RSD of 0.07 and 0.12 (Fig. 2). The selectivity value from this eluting method was 1.11, which is considered high. The individual injection and also the simultaneous mixture of SE and SF show the efficient condition to be used in the quantitative determination in crude extracts. The elution profiles were detected at 254 nm—as per Pocasap et al. (2013). The present study also demonstrated SE and SF signal enhancing by detection at 210 nm.

3.2. Validation of analytical method

The HPLC eluting system—by the simple mobile phase—provides good detection parameters (Table 1). The respective

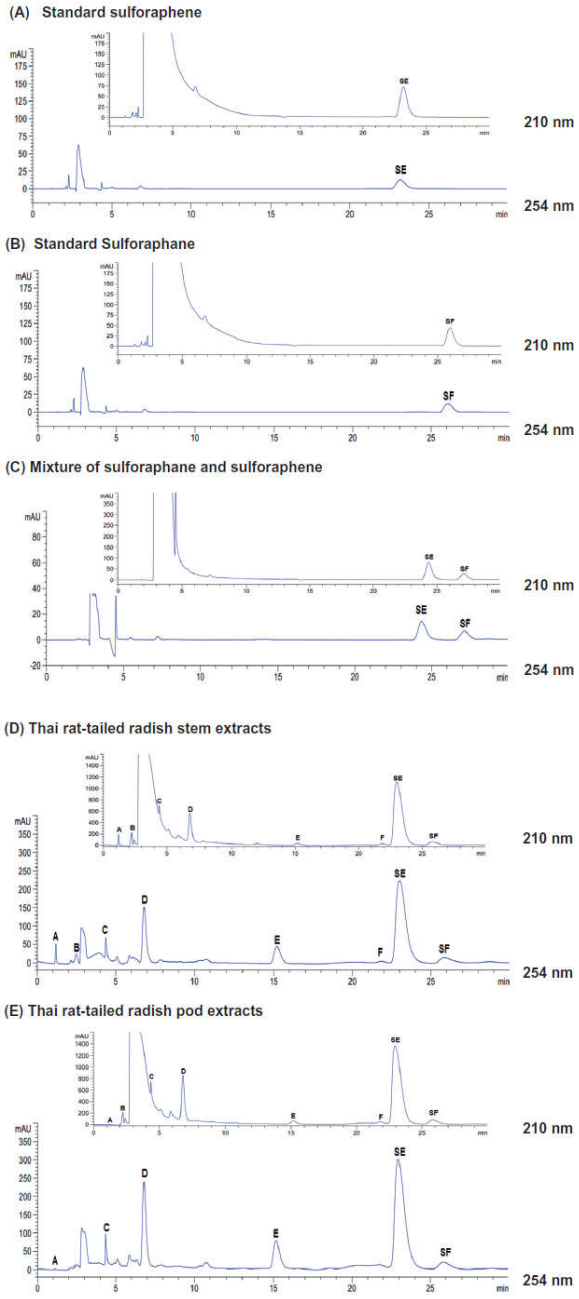

Fig. 2. HPLC chromatograms of analytes detected at 210 and 254 nm. Peaks of (A) standard sulforaphene; SE (23.01 ± 0.02 min), (B) standard sulforaphane; SF (25.65 ± 0.03 min), (C) mixture of sulforaphene and sulforaphane, and Thai rat-tailed radish extracts from (D) pod and (E) stem with the unknown peaks: A (1.17 ± 0.04 min), B (2.29 ± 0.11 min), C (4.37 ± 0.01 min), D (6.80 ± 0.01 min), E (15.15 ± 0.02 min), and F (21.85 ± 0.02 min). The numbers in parentheses are the retention time of each peak.

Table 1
Validation parameters for the liquid chromatographic method; specificity, the selective index, LOD, and LOQ.

Specificity*			Selective index**	LOD	LOQ
Tested compound	Retention time (n = 10)	%RSD		(μg/ml)	
Sulforaphene	23.01 ± 0.02	0.07	1.11	0.34	1.02
Sulforaphane	25.65 ± 0.03	0.12		0.36	1.08

* %RSD of each peak represents high specificity.
** Separation of each compound without peak merging indicates specificity of the detection method. A selectivity value > 1 is considered selective. The higher the value, the higher selectivity.

linearity range (r^2) of SE and SF was 0.99 and 0.99 (Table 3). The respective precision parameter represented by the relative standard deviation (RSD) value for SE and SF was 0.67% and 0.51% while the repeatability was 0.93% to 0.98% (Table 2). The respective limit of detection (LOD) and quantification (LOQ) confirmed the sensitivity of the system (i.e., LOD of SE and SF = 0.34 and 0.36 μg/ml while the LOQ = 1.02 and 1.08 μg/ml). The recovery of SF and SE ranged between 96.83 ± 0.07 and 101.17 ± 0.12% (Table 4): the average 97% recovery underscoring the high accuracy of the protocol.

Table 2
Precision of HPLC method.

Day	Conc. (μg/ml)	No. of injection	Peak area		Average		SD		%RSD	
			SE	SF	SE	SF	SE	SF	SE	SF
Intra-day										
	10	1	3278.61	4098.90	3267.41	4093.53	21.82	21.10	0.67	0.51
		2	3242.28	4070.26						
		3	3281.36	4111.42						
Inter-day										
1	10	1	3278.61	4098.90	3262.60	4084.50	30.39	40.11	0.93	0.98
		2	3242.28	4070.26						
		3	3281.36	4111.42						
2	10	1	3275.40	4097.28						
		2	3188.35	3981.92						
		3	3264.34	4094.02						
3	10	1	3278.87	4099.32						
		2	3276.50	4105.13						
		3	3277.69	4102.23						

Table 3
Linearity of HPLC method.

Linearity									
Conc. (μg/ml)	Replication	Peak area					Slope	Y-intercept	R^2
		Day 1	Day 2	Day 3	Average	SD			
Sulforaphene									
	1	1227.34	1417.52	1418.68					
5	2	1355.54	1415.79	1415.88	1388.30	50.08			
	3	1408.54	1417.06	1418.34					
	1	3278.61	3275.40	3278.87					
10	2	3242.28	3188.35	3276.50	3262.60	17.98			
	3	3281.36	3264.34	3277.69					
	1	4213.01	4243.19	4251.16					
15	2	4268.75	4264.36	4253.57	4253.40	3.36	297.4	−51.59	0.99
	3	4270.31	4263.94	4252.30					
	1	5342.97	5322.68	5318.24					
20	2	5330.26	5317.05	5324.56	5324.626	7.97			
	3	5324.65	5333.93	5307.29					
	1	8792.80	9490.42	9438.62					
30	2	9194.10	9483.93	9439.18	9348.80	199.59			
	3	9373.77	9491.58	9434.80					
	1	10778.1	12006.1	11935.3					
40	2	11423.1	12023.4	11953.1	11754.46	395.08			
	3	11697.1	12001.3	11972.6					
Sulforaphane									
	1	1529.69	1761.96	1766.72					
5	2	1694.53	1772.89	1761.98	1731.80	61.23			
	3	1759.41	1777.08	1761.90					
	1	4098.90	4097.28	4099.32					
10	2	4070.26	3981.92	4105.13	4084.50	23.58			
	3	4111.42	4094.02	4102.23					

Table 3 (*continued*)

Linearity									
Conc. (μg/ml)	Replication	Peak area					Slope	Y-intercept	R^2
		Day 1	Day 2	Day 3	Average	SD			
	1	5262.57	5310.39	5335.87					
15	2	5313.81	5334.17	5335.99	5320.07	15.15	370.6	−87.63	0.99
	3	5332.69	5331.02	5324.13					
	1	6301.11	6310.21	6309.05					
20	2	6313.61	6297.72	6304.47	6305.80	2.38			
	3	6307.44	6301.25	6307.30					
	1	11049.8	11978.4	11981.6					
30	2	11573.2	11977.2	11989.1	11815.47	299.24			
	3	11786.8	12006.3	11996.8					
	1	13296.6	14909.9	14892.3					
40	2	14077.6	14939.7	14,952	14601.84	572.95			
	3	14446.7	14929.2	14972.6					

3.3. Quantification of sulforaphene and sulforaphane in RS extracts based on HPLC determination

The validated HPLC system was then used to determine two biomarkers–SE and SF–in the RS extracts. The extracts obtained from the stem and pod parts yielded 0.0282 and 0.0595 (%w/w of fresh weight), respectively. The standard equivalent quantities were calculated using a linear regression equation obtained from the calibration curve. The RS pod extract had higher respective SE and SF content than the RS stem extract (Table 5). The stem contained 1105.14 ± 243.10 μg SE/g crude extract while the SF content was undetectable. The RS pod extract was 2253.05 ± 246.18 μg SE and 111.94 ± 16.49 μg SE/g in the crude extract.

The present detection agrees Pocasap et al. (2013) who reported that the GC-MS of RS pod and flower extracts contained a higher SE than SF content. The retention time of the major compounds SE and SF were the same for both detected wavelengths. We found that 210 nm was a better signal for analysis of low concentration samples than 254 nm. Interestingly, five unknown peaks were found in the RS extracts with following retention times; A (1.17 ± 0.04 min), B (2.29 ± 0.11 min), C (4.37 ± 0.01 min), D (6.80 ± 0.01 min), E (15.15 ± 0.02 min) and F (21.85 ± 0.02 min) (Fig. 2). The HPLC chromatogram of the extract with the presence of these markers can be used as a fingerprint for standardization or quality control of the extract in future studies of its bioactivity. Identification of these unknown markers is currently under intensive study in our laboratory.

A simultaneous assay for SE and SF would be both convenient and valuable since both compounds are anticancer agents found in edible plants: knowing their respective content would directly indicate the function of RS. To date, simultaneous detection of these two chemo-preventive compounds has been limited by their

Table 5
Quantification of sulforaphene and sulforaphane in *Raphanus sativus* L. var. *caudatus* Alef (RS) extracts.

	Sulforaphene		Sulforaphane	
	μg/g crude extract			
	Mean	SD	Mean	SD
Stem	1105.14	243.10	nd	
Pod	2253.05	246.18	111.94	16.49

*nd means the peak was lower than the detection limit.

Table 4
%Recovery of HPLC method.

%Recovery						
Conc. (μg/ml)	Replication	Peak area	Recovery conc. (μg/ml)	%Recovery	Average	SD
Sulforaphene						
5	1	1418.68	4.9434	98.875	98.80	0.10
	2	1415.88	4.934	98.687		
	3	1418.34	4.9426	98.852		
15	1	4268.75	14.527	96.847	96.83	0.07
	2	4264.36	14.512	96.748		
	3	4270.31	14.532	96.882		
40	1	11935.30	40.306	100.764	100.92	0.16
	2	11953.10	40.365	100.914		
	3	11972.60	40.431	101.078		
Sulforaphane						
5	1	1766.72	5.004	100.073	99.90	0.15
	2	1761.98	4.991	99.817		
	3	1761.90	4.991	99.813		
15	1	5335.87	14.634	97.563	97.55	0.02
	2	5334.17	14.630	97.532		
	3	5335.99	14.635	97.565		
40	1	14909.90	40.468	101.171	101.17	0.12
	2	14892.30	40.421	101.052		
	3	14929.20	40.520	101.301		

structural similarity. The initial approach could not differentiate the SE and SF peaks. The current study instead presents a reliable, rapid, HPLC assay that uses less hazardous solvent while maintaining satisfactory sensitivity and selectivity.

4. Conclusion

We developed a HPCL method for the simultaneous investigation and efficient validation of the chemopreventive compounds—sulforaphene and sulforaphane—found in the extracts of RS. The analytical method is (a) simple (using an isocratic mobile phase of 95% water and 5% THF), (b) commercially available, (c) relatively inexpensive, (d) environmentally safe, (e) had efficient validation parameters, (f) minimized and expedited simultaneous quantification of SE and S and (g) can be used in the standardization and quality control process. Hence the method offers an analytical method for determination of consistent bioactives in each batch. The simple simultaneous and developed HPLC analysis that we had advantages over individual analysis of the respective SE and SF content in samples of plant extracts.

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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)

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ABSTRACT

The bioactive compounds of *Raphanus sativus* var. *caudatus* Alef and their respective cytotoxicity were identified from (a) 2 crude water and dichloromethane extracts and (b) 5 serially partitioned extracts using dichloromethane. Then, using a bioassay-guided cytotoxicity assay, the extracts were tested against the colon cancer cell line HCT116. Among 2 crude extracts and 5 fractions, the dichloromethane crude extract possessed the greatest *in vitro* cytotoxicity against HCT116. The dichloromethane crude extract was subjected to flash column liquid chromatography. Only 4 fractions and unfractionated extract were chosen for further analysis by high accuracy mass spectrometry (UHPLC-QToF-MS/MS). Six glucosinolates, 13 isothiocyanates, 5 indoles, 4 flavonoids, 2 alkaloids, 2 thiocyanates, 1 oxazolidine, and 1 dialkyl disulphide were identified. Two compounds were detected for the first time—isoalliin and butyl 1-(methylthio)propyl disulphide. Several constituents with anti-cancer activity were identified. This information could inform guidelines for quality control of standard plant extracts for further product development.

1. Introduction

Brassicaceae (including *Raphanus sativus* Linn. or radishes) are grown and consumed worldwide (Kim et al., 2014). Vegetables in this family—such as wasabi, mustard, water cress, garden cress, and broccoli sprouts—are a rich source of phytochemicals (phenols, flavonoids, and vitamin C) (Kim et al., 2014; Zhang, Tang, & Gonzalez, 2003). The biological activities of glucosinolates and their enzymatic conversion—sulphur-containing products such as the isothiocyanates in Brassicaceae—have been reported (a) reducing cancer risk and inflammatory response; (b) having antimicrobial, antioxidation, antitumour, and antiviral activity; (c) affecting immunomodulation, and intestine motility stimulation; and, (d) preventing cardiovascular disease (Björkman et al., 2011; Gutierrez & Perez, 2004; Moon & Kim, 2012; Vaughn & Berhow, 2005). Previously identified chemical constituents in *R. sativus* include alkaloids, nitrogen compounds, coumarins, enzymes, gibberellins, glucosinolates, fatty acids, organic acids, phenols, pigments, polysaccharides, sulphur compounds, phytoalexins, β-carotene, and vitamin C.

R. sativus var. caudatus Alef (RS) (Thai rat-tail radish) is an indigenous Thai vegetable cultivated in the north and northeast regions of Thailand. Previous studies of its constituents focused on the identification of the biomarkers sulforaphene and sulforaphane in the RS extract. Sulforaphane, sulforaphene, 3-butenyl isothiocyanate, and 4-methylthio-but-3-enyl isothiocyanate were discovered in the RS seed by GC–MS analysis (Songsak & Lockwood, 2002). Pocasap, Weerapreeyakul, and Barusrux (2013) then used GC–MS analysis to identify sulforaphane, sulforaphene, 3-butenyl isothiocyanate, and dimethyltrisulphide in the edible parts of the RS pod and flower, which were shown to contribute to a chemopreventive effect against the colon cell line HCT116 through induction of apoptosis (Pocasap & Weerapreeyakul, 2016; Pocasap et al., 2013).

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Ultra-high performance liquid chromatography (UHPLC) is a highly selective and sensitive analytical technique that allows physical separation of complex elutes. The speed, resolution, and sensitivity of UHPLC make it ideally suited for use with mass spectroscopy (MS). MS detection is a useful tool for chemical identification because it provides accurate mass and structural information—especially high-resolution mass spectrometric techniques such as quadrupole time-of-flight (QToF). When UHPLC is combined with the mass analysis capabilities of high-resolution mass spectrometry, it provides a powerful technique with very high sensitivity. Its application is for general detection, separation, and potential identification of chemicals of particular masses in the presence of other chemicals (i.e., in complex mixtures, extracts of natural products, and pure substances from mixtures of chemical intermediates) (Khan & Ali, 2015).

Percent cytotoxicity was calculated against the widely reported anti-cancer compounds, sulforaphane and sulforaphene. Sangthong and Weerapreeyakul (2016) reported the quantitative analysis of the latter two compounds: 1 g of dichloromethane crude extract of RS pod contains 0.112 mg sulforaphane and 2.253 mg sulforaphene. The respective percents contribution of sulforaphane and sulforaphene—in the crude extract against the cancer cell line HCT116—were 0.04% and 0.47%. The results suggest the existence of other bioactive components in the RS dichloromethane crude extract.

In the current study, high-accuracy mass spectrometry was used to identify and structurally characterize the presence of chemopreventive compounds in the RS of Thai rat-tailed radish extract, which is reported to have anticancer activity (Guo, Wang, Deng, Zhang, & Wang, 2013; Kim et al., 2016; Munday & Munday, 2004; Reed et al., 2005; Smith, Mithen, & Johnson, 2003; Srivastava et al., 2003). Glucosinolates, isothiocyanates, indoles, alkaloids, thiocyanates, oxazolidine, flavonoids, and dialkyl disulphide were successfully identified in the extract. To the best of our knowledge, this is the first report of the organosulphur compounds—isoalliin and butyl 1-(methylthio)propyl disulphide commonly found in onion and Chinese chives—being detected in the genus Brassica, species *R. sativus* var. *caudatus* Alef.

2. Materials and methods

2.1. Reagent and standards

Commercial grade dichloromethane, hexane, and chloroform were purchased for the extraction and distilled before use. Deionized water (ddH_2O) was obtained from a MilliQ system (Millipore, Bedford, MA, USA). HPLC grade methanol (MeOH), formic acid (Fisher Scientific, Leicestershire, UK) and ddH_2O were used for the HPLC analysis. Allyssin and iberin were purchased from Abcam (Cambridge, UK), erysolin, D,L-sulforaphane from Merck, and L-sulforaphene from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Preparation of RS crude extract

Frozen RS pods were blotted and cut into small pieces and homogenized using a food processer with ddH_2O (1:1, w/v). The homogenate was left for autolysis at 25–27 °C for 2 h and filtered through double-layer cheesecloth. The filtrate was partitioned with dichloromethane. The lower layer of dichloromethane and upper layer of water were collected separately. This partitioning was done in triplicate. Then the serial partitions underwent bioassay-guided chemoprevention testing against colon cancer cell line HCT116 (Pocasap et al., 2013) (Fig. 1). The crude extract of the aqueous layer was collected and dried using lyophilizer (SCANVAC CoolSafe 110-4 Pro, LABOGENE, Lynge, Denmark), yielding a aqueous crude extract (3.277% w/w). The dichloromethane layer

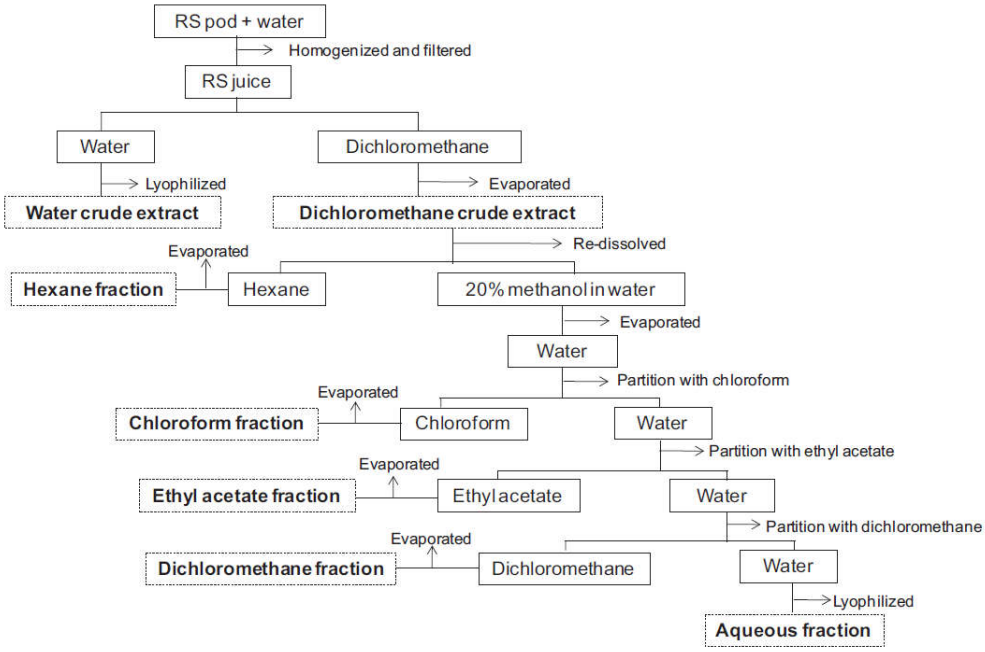


Fig. 1. Schematic diagram represents serial extraction method of whole pod from Thai rat-tailed radish (RS).

was dried by rotary evaporator and all dichloromethane extract fractions were pooled after the dichloromethane was removed using a vacuum rotary evaporator, yielding a dichloromethane crude extract (0.068%w/w). Dichloromethane crude extract was re-dissolved with 20% methanol (2 g/50 ml) and partitioned with 4 organic solvents (100 ml each) with differing polarity (namely, hexane, chloroform, ethyl acetate, and dichloromethane). The organic solvent layer of each partition was collected and dried by rotary evaporator. The percentage yield of dried fractions was calculated as percent weight by weight of the fresh weight: hexane fraction (0.021% w/w), chloroform fraction (0.010%), ethyl acetate fraction (0.013%), dichloromethane fraction (0.013%), and aqueous fraction (0.012%). Seven samples underwent cytotoxicity testing and further identification analysis.

2.3. Cytotoxicity against HCT116 cell lines as bioassay-guided test

Seven samples were dissolved with dimethyl sulphoxide (DMSO) into the final tested concentration of 500 μm/ml. To avoid false cytotoxicity from DMSO, < 1% v/v of DMSO was maintained in the mixture so as to have < 10% cytotoxicity. Neutral red (NR) assay was used to detect the percentage of viable cells or indirectly to determine cytotoxicity as per Machana, Weerapreeyakul, Barusrux, Thumanu, and Tanthanuch (2012). Briefly, the cells (5×10^5 cell/ml) were seeded in 96-well plates and treated with samples for 24 h. Cells were then washed once with phosphate buffer. A total of 100 μl NR solution (50 μg/ml) was added to each well and incubated at 37 °C for another hour. NR was then dissolved by 100 μl of 0.33% hydrochloric acid. Absorbance of the NR dye was detected by a dual-wavelength UV spectrometer at 537 nm with a 650 nm reference wavelength. The percentage of cytotoxicity compared to the untreated cells was determined with the equation given below.

$$\text{Cytotoxicity (\%)} = \frac{[100 \times (\text{Absorbance of untreated group} - \text{Absorbance of treated group})]}{(\text{Absorbance of untreated group})}$$

In addition, the compounds identified in the RS pod extract (D, L-sulforaphane, L-sulforaphene, erysolin, and iberin) were also tested for their cytotoxicity with the same method mentioned above.

2.4. Preparation of flash column chromatography fractions

Dichloromethane crude extract from the RS pod was separated and the fraction collected by Buchi Sepacore® flash systems (BÜCHI Labortechnik, Flawil, Switzerland) equipped with control unit C-620, UV photometer C-640, and fraction collector C-660. RS pod dichloromethane crude extract (3 g) was then loaded into 120 g RediSep® Normal-phase Flash Column (Teledyne Isco, Lincoln, NE, USA). The normal phase eluting was performed with a flow rate of 50 ml/min with eluent A (dichloromethane) and eluent B (methanol). The mobile phase was started with 0% A for 10 min (0–10 min); the linear was then increased to 10% A for 20 min (at 20–40 min); then to 15% A for 5 min (45–50 min); then to 40% A for 10 min (60–80 min); and finally 100% A for 15 min (85–100 min). The fractions were automatically collected every 20 ml. The fraction(s)—which consisted of only one peak as detected by UV detector (254 nm)—were then combined and dried using a rotary evaporator. Finally, 9 fractions were obtained from this step.

2.5. UHPLC-QToF-MS analysis

Four of the nine fractions from the flash column chromatography—the top 4 in yield rank (namely, C, D, E, and F)—were selected. Five samples—including the unfractionated dichloromethane crude extract and the C, D, E, and F fractions were analyzed using an UHPLC-QToF-MS (Agilent Technologies, Waldbronn, Karlsruhe, Germany). The instrument comprised a 1290 LC system, a Jetstream ESI source, and a 6540 UHD accurate-mass quadrupole-time-of-flight (QToF) mass spectrometer. The ionization characteristics were determined with both electrospray ionization polarities (ESI+ and ESI−). Separation was performed on reversed phase analytical column Zorbax RRHD Eclipse XDB-C18, 2.1 × 100 mm, 1.8 μm column (Agilent Technologies, Palo Alto, CA, USA) at 50 °C. The sample injection volume was set at 2 μl. The mobile phases were delivered at 0.4 ml/min, consisting of 0.1% formic acid in water (eluent A) and 0.1% formic acid in methanol (eluent B). The gradient was started with 2–100% B for 10 min (0–10 min) then 100% B for 4.50 min (10–14.50 min), and 2% B for 2 min (14.50–16.50 min). The MS conditions follow: nitrogen as the instrument gas; drying gas temperature 325 °C at 10 L/min; sheath gas temperature 350 °C at 11 L/min; nebulizer pressure 45 psi; capillary voltage 3500 V; nozzle voltage 1000 V; fragmentor voltage 100 V; and, skimmer voltage 45 V. For MS data acquisition, 2 GHz extended dynamic range mode was used in both ion modes from m/z 20–1600. Data were collected in the centroid mode at an acquisition rate of 1.67 spectra/s with an abundance threshold of 150. For automatic data dependent MS/MS analyses, the precursor isolation width was narrow (1.3 Da), the MS/MS scan rate 3.33 spectra/s, and from every precursor scan cycle 4 of the most abundant ions were selected for fragmentation. These ions were excluded after 2 product ion spectra were analyzed and released again for fragmentation after a 0.25 min hold. Precursor scan time was based on ion intensity, ending at 25,000 counts or after 300 ms. Product ion scan time was 300 ms. Collision energies were 10 and 20 V in subsequent runs. The ToF was calibrated on a daily basis and subsequently operated at high accuracy (<2 ppm). Continuous mass axis calibration was performed by monitoring 2 reference ions from an infusion solution throughout the runs. The reference ions were m/z 121.050873 and m/z 922.009798 in the positive mode and m/z 112.985587 and m/z 966.000725 in the negative mode.

2.6. Identification and annotation of compounds

Identification of molecules was generated by Agilent MassHunter™ Qualitative Analysis B.06.00 (MassHunter™ Qual, Agilent Technologies, Santa Clara, CA, USA); based on accurate mass-matching to published databases (namely, Human Metabolome Database (http://www.hmdb.ca/) (Wishart et al., 2013); Metlin (https://metlin.scripps.edu/) (Smith et al., 2005); and SciFinder (https://scifinder.cas.org). The first identification was based on accurate mass and isotopic pattern, matched to the Metlin database using Agilent's Identification Browser software. The results were sorted and an assessment of retention time and a single putative annotation with a matching elemental formula selected. This annotated molecular feature was then compared to other databases. The MS/MS spectra of molecular features were compared and matched to a library of standard spectra in Agilent's MassHunter METLIN Personal Compound Database and Library (PCDL) (Agilent Technologies, Santa Clara, CA). An authenticating reference standard for allyssin, erysolin, iberin, D,L-sulforaphane, and L-sulforaphene were run using identical instrumental conditions, and the detected molecules matched to accurate masses, retention times, and product ion spectra present in the unfractionated and fractionated RS extract. The level of identification (LI) was defined in 4 different levels including; identified metabolites (level 1), putatively annotated compounds (level 2), putatively characterized compound classes (level 3), and unknown compounds (level 4) (Salek, Steinbeck, Viant, Goodacre, & Dunn, 2013).

2.7. Proximate analysis

The RS stems and leaves and RS pods were washed under running water and blotted dry and used for the proximate analysis. Briefly, ash was determined as per AOAC (2012, Method 942.05) using temperature controlled furnace at 600 °C. Crude fiber content was performed using in-house method based on AOAC (2012, Method 978.10). Energy and total carbohydrate content was performed using Compendium of method for food analysis (2003). Moisture content was performed as Loss on Drying Moisture in Plants (AOAC, 2012, Method 930.04). Protein content was performed using in-house method TE-CH-012 based on block digestion method (AOAC, 2012, Method 981.10). Total fat content was performed as per acid hydrolysis method (AOAC 2012, Method 922.06) using Soxhlet apparatus with petroleum ether as the extractant.

3. Results and discussion

3.1. Cytotoxicity bioassay-guided selection

Seven RS pod samples including two crude extracts and five fractions were obtained by liquid-liquid serial partitioning. The cytotoxicity effect of RS extract was preliminarily tested against the HCT116 cell line, using neutral red assay. The results in Fig. 2 show the cytotoxicity of each sample at the same concentration (500 μg/ml). The highest percent cytotoxicity was found in the dichloromethane crude extract (91.1 ± 0.5%). The water crude extract showed 31.7% cytotoxicity followed by the extract fractionated from ethyl acetate, dichloromethane, hexane, chloroform, and water, respectively. The highest cytotoxicity in the dichloromethane crude extract may be due to the preferred partially non-polar isothiocyanates that have been found to play major chemopreventive roles in Brassicaceae plants (Björkman et al., 2011). The water crude extract may consist of polar compounds such as phenolics and flavonoids. The serial partitions possessed a negative effect on cytotoxicity. The complex constituents in the dichloromethane crude extract were assumed to exhibit a synergistic effect, leading to the highest cytotoxicity of the whole crude extract. Our result agrees with previous research on the cytotoxicity of RS pod and flower crude extract against HCT116 using the MTT assay (Pocasap et al., 2013). As dichloromethane crude extract was the most cytotoxic against HCT116, it was selected for partial purification and identification of anticancer biomolecules of RS extract in the next experiment.

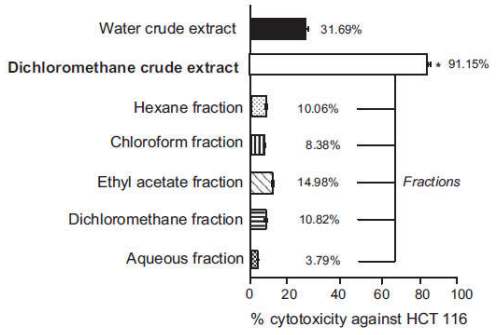


Fig. 2. Cytotoxicity of water crude extract, dichloromethane crude extract, and their respective fractions from Thai rat-tailed radish (RS) pod at the concentration of 500 μg/ml.

Table 1
Percent yield of fractions from dichloromethane crude extract of RS pod from flash column chromatography.

Fraction	Percent yield (w/w)	Characteristic
A	13.8	Yellow wax
B	2.9	Green grease
C	27.4	Dark brownish green
D	43.2	Dark greenish brown
E	3.5	Brown
F	6.2	Greenish brown
G	2.4	Brown
H	4.7	Dark brown
I	3.1	Dark brown

3.2. RS pod fractions from flash column chromatography

RS pod dichloromethane crude extract was partially separated by using flash column chromatography. The yield percentage and physical property of each fraction are presented in Table 1. Four fractions—C, D, E, and F—had high absorbance (range, 0.1–0.9 AU); these then underwent analysis and identification for their respective chemical composition.

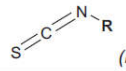
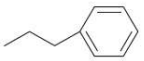
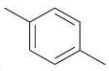
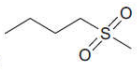
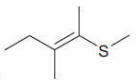
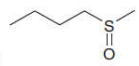
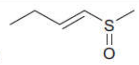
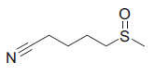
3.3. UHPLC- QToF-MS data of RS pod crude extracts and fractions

RS pod crude extract as well as the fractions obtained from flash column chromatography were analyzed by LC/MS and compared to 5 ITC standard compounds—allyssin, iberin, erysolin, D,L-sulforaphane, and L-sulforaphene. Table 2 presents the results of the unfractionated RS pod (dichloromethane) crude extract and its 4 fractions. In the unfractionated extract, there were 4 glucosinolates, 11 isothiocyanates, 4 indoles, 2 alkaloids, 1 thiocyanates, and 1 oxazolidine as well as a monounsaturated fatty acid, an organic phosphoric acid diamide, a pyranone, a thiocarboxylic acid amide, an alpha amino acid, a benzoic acid ester, and a dialkyl disulphide. In the fractions, there were 3 glucosinolates, 5 isothiocyanates, 1 indole, 4 flavonoids, 2 thiocyanates, and an oxazolidine. The fractions from flash column chromatography showed different detectable compounds from the unfractionated (dichloromethane crude extract) sample. The loss of some compounds might have occurred during elution; however, the compounds detected were annotated and identified along with the level of identification. The level of identification was mostly in level 2 (34%) and level 3 (60%), which were annotated and identified based on accurate mass per charge value. The level 1 identical compounds were sulforaphane (177.029 *m/z*) and sulforaphene (175.0128 *m/z*). The respective fragmentation pattern of the isothiocyanates (SCN = R) and $SCNH_2$ was 60.99 *m/z* (M^+) and 59.99 *m/z* (M^-). The glucosinolate fragmentation pattern was conjugated glucose ($C_6H_{11}O_5$, 163.06 *m/z*, M^-), glucose molecule (180.06 *m/z*, M^-), S-glucose (194.02 *m/z*, $(M\text{-}H)^-$), and NH_2SO_4 (112.98 *m/z*, M^-).

The UHPLC/ESI-QToF-MS/MS technique could not directly provide the quantitative determination of identified chemical substances. Therefore, the content of identified compounds of interest was not determined. However, 1 g of dichloromethane crude extract of RS pod was previously reported to compose of 0.112 mg sulforaphane and 2.253 mg sulforaphene by using HPLC quantitative analysis and standard calibration curve (Sangthong & Weerapreeyakul, 2016).

The existence of these annotated compounds was supported by their biological function to plant protection. Brassicaceae plants have their own defense mechanism against pests and pathogens, attributed to the sulphur-containing compounds such as glucosinolates (Bohinc, Ban, Ban, & Trdan, 2012). These glucosinolates and their generative compounds—isothiocyanates and indoles—are also related to health promotion. The current study identified

Table 2
UPLC-ESI-QToF-MS/MS identified compounds in RS pod unfractionated crude extract and its fractions.

Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
Isothiocyanates  *(m/z= 60)*								
R= 	3-Phenylpropyl	$C_{10}H_{11}NS$	177.0612	3.42 (U)	M + FA-H	222.056	Negative	3
R= 	7-Methylthioheptyl	$C_9H_{17}NS_2$	203.0802	3.5 (U)	M + Cl	238.0511	Negative	3
R= 	1-Pentyl	$C_6H_{11}NS$	129.0612	3.3 (F)	M + Br	207.9799	Negative	3
				4.6 (E)	M + H	129.0607	Positive	3
R= 	3-Butenyl	C_5H_7NS	113.0299	0.62 (U)	M + H	113.0303	Positive	2
R= 	3-Methoxypropyl	C_5H_9NOS	131.0408	2.5 (D)	M + H	131.0409	Positive	2
R= 	4-Hydroxybenzyl	C_8H_7NOS	165.0248	3.08 (U)	2 M + FA-H	375.0484	Negative	3
R= 	Allyl	C_4H_5NS	99.0142	0.93 (U)	M + K-2 H	136.9909	Negative	3
R= 	Butyl	C_5H_9NS	115.0455	1.12 (U)	M + H	115.0637	Positive	2
R= 	4-(Methanesulfonyl) butyl (Erysolin)	$C_6H_{11}NO_2S_2$	193.0231	3.86 (U, F)	M-H	193.0229	Negative	2
				7.56 (C)	M + H	193.0256	Positive	2
				0.74 (U,E)	2 M + H	193.0236	Positive	3
				1.45 (U)	2 M + Na + H	193.0231	Positive	3
R= 	4-Methylthio-3-butenyl (Raphasatin)	$C_6H_9NS_2$	159.0176	4.74 (U)	M + H	159.0176	Positive	3
				4.54 (U)	M + 2Na-H	205.0056	Positive	2
R= 	4-(Methylsulphinyl) butyl (Sulforaphane)	$C_6H_{11}NOS_2$	177.02821	4.74 (U, D)	M + H	177.029	Positive	1
				4.24 (U, D)	2 M + H	177.029	Positive	3
R= 	4-(Methylsulphinyl) butane (Sulforaphene)	$C_6H_9NOS_2$	175.0125	4.54 (U, C, D)	M + H	175.0125	Positive	1
				5.73 (U, C)	M + Na	175.0128	Positive	3
				4.17 (C, D, E, F)	2 M + H + Na	175.0147	Positive	3
R= N 	Sulforaphane nitrile	$C_6H_{11}NOS$	145.0561	4.46 (U)	M + Cl	180.0255	Negative	3

(*continued on next page*)

Table 2 (*continued*)

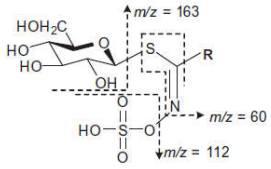
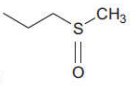
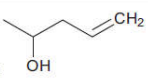
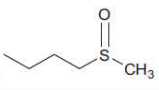
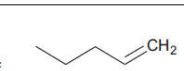
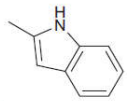
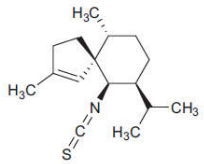
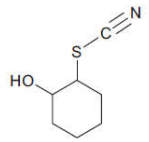
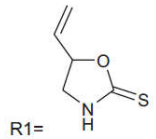
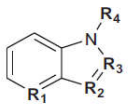
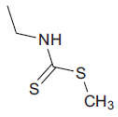
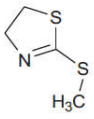
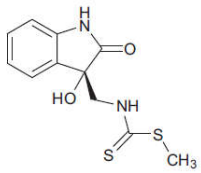
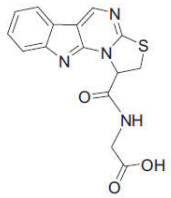
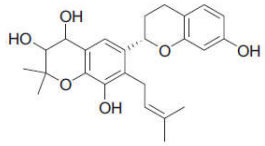
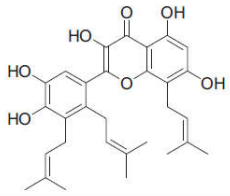
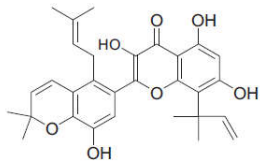
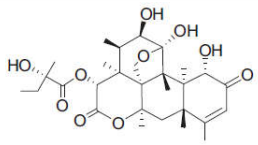
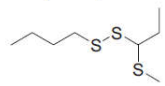
Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
Glucosinolates 								
R= 	Butyl	$C_{11}H_{21}NO_9S_2$	375.0657	0.65 (U)	M + FA-H	420.0625	Negative	3
R= 	3-Methylsulphinylpropyl (Glucoiberin)	$C_{11}H_{21}NO_{10}S_3$	423.0327	0.74 (U)	M + FA-H	468.0313	Negative	3
R= 	2-Hydroxy-4-pentenyl (Gluconapoleiferin)	$C_{12}H_{21}NO_{10}S_2$	403.0606	4.24 (U, F)	M-H	403.0623	Negative	2
R= 	4-Methylsulphinylbutyl (Glucoraphanin)	$C_{12}H_{23}NO_{10}S_3$	437.04841	5.17 (U)	M-H	435.0349	Negative	2
R= 	4-Pentenyl (Glucobrassicanapin)	$C12H_{21}NO_9S_2$	387.0657	7.66 (D, F)	M-H	387.0679	Negative	2
R= 	3-Indolylmethyl (Glucobrassicin)	$C_{16}H_{20}N_2O_9S_2$	448.0610	4.53 (C, D)	2 M-H	223.0164	Negative	3
				7.35 (E)	M + Na-2 H	469.2519	Negative	3
Thiocyanate 	Axisothiocyanate-3	$C_{16}H_{25}NS$	263.1708	3.3 (F)	M-H_2O-H	244.0707	Negative	3
	2-Hydroxycyclohexyl thiocyanate	$C_7H_{11}NOS$	157.0561	2.694 (U, F)	M + H	157.0567	Positive	3

Table 2 (*continued*)

Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
Oxazolidine  R1=	2-Oxazolidinethione (Goitrin)	C_5H_7NOS	129.0248	1.45 (U, E)	M + H	129.0248	Positive	2
Indoles 								
R_2 =  R_4 = H	3-Indolecarbinol	C_9H_9NO	147.0684	3.89 (U)	2 M + K	333.1019	Positive	3
R_2=  R_4 = H	3-Indoleacetonitrile	$C_{10}H_8N_2$	156.0682	3.68 (U)	M + Na-2 H	177.0425	Negative	3
R_3=  R_4 = H	2-Indolecarboxylic acid	$C_9H_7NO_2$	161.0477	2.61 (E)	M + H	161.052	Positive	2
R_2 =OCH_3	Methoxybrassinin	$C_{12}H_{14}N_2OS_2$	266.0547	2.8 (U)	M + Hac-H	325.0663	Negative	3
				3.71 (U)	M + Na-2 H	287.032	Negative	3
R_4= 								
R_2=  R_4 = OCH_3	N-Methoxyspirobrassinol	$C_{12}H_{14}N_2O_2S_2$	282.0496	3.2 (U)	2 M-H	563.0938	Negative	3
Alkaloids 	(-)-Dioxibrassinin	$C_{12}H_{14}N_2O_2S_2$		2.15 (U)	M + Hac-H	327.0453	Negative	3

(*continued on next page*)

Table 2 (*continued*)

Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
	Cabbage identification factor 2	$C_{15}H_{12}N_4O_3S$	328.06301	8.22 (U)	M + 2Na-H	373.0339	Positive	3
Flavonoids 	Broussoflavan A	$C_{25}H_{30}O_6$	426.2042	9.76 (C)	M-H	424.1779	Negative	3
	Broussoflavonol G	$C_{30}H_{34}O_7$	506.2304	7.38 (E)	M + H	504.2147	Positive	3
	Broussoflavonol D	$C_{30}H_{32}O_7$	504.2148	7.75 (E, F)	M-H	504.2171	Negative	3
	Glaucarubinone	$C_{25}H_{34}O_{10}$	494.2152	5.86 (F)	M-H	494.2137	Negative	3
Dialkyl disulphide 	Butyl 1-(methylthio) propyl disulphide	$C_8H_{18}S_3$	210.0571	4.25 (U)	M + $2Na^{2+}$	128.0172	Positive	3

Note: U = unfractionated RS pod extract; C, D, E, F = RS pod fractions.
Adduct type: FA = formic acid; Hac = acetic acid; Na = sodium; Cl = chlorine; K = potassium.

more compounds in *Raphanus sativus* var. *caudatus* Alef than previously detected by GC–MS analysis (Pocasap et al., 2013; Songsak & Lockwood, 2002). Using LC-MS provided detection of thermal degradation compounds undetected in any previous GC–MS analysis. Sulforaphane—the well-known chemopreventive compound and its precursor, glucoraphanin as well as the analogue of erysolin and sulforaphane nitrile—were also detected. Of note, 3 phytoalexins, found in the recent study—(-)-dioxibrassinin and 2 indoles, and a methoxybrassinin have also been previously found in cabbage (*Brassica oleracea* var. *capitata*) (Monde et al., 2003) and 1 phytoalexin stress metabolite N-methoxyspirobrassinol has been found in Japanese radish daikon (*Raphanus sativus* var. *hortensis*) (Monde, Takasugi, & Shirata, 1995). Phytoalexins are produced by plants as stress metabolites as part of the antifungal defensive

mechanism (Monde et al., 2003). In humans, phytoalexins also show anti-proliferative and cancer chemopreventive activity (Mezencev, Mojzis, Pilatova, & Kutschy, 2003). Indole-3-acetronitrile and indole-3-carbinol are the thermal breakdown products of glucobrassicin widely reported as chemopreventive compounds (Chevolleau, Debrauwer, Boyer, & Tulliez, 2002).

Glucosinolates are the bioactive compounds commonly found in crucifers associated with cancer protection. Glucosinolates share a common sulphur-linked β-D-glucopyranose structure with different side chains. The alkylthioalkyl side-chain contains a sulphur group, whereas, the aromatic side-chain contains a phenethyl group. Glucosinolates are not bioactive until they are enzymatically catalyzed to a chemically related isothiocyanates (ITCs) by the endogenous enzyme myrosinase. Myrosinase catalyses hydrolysis of the β-thioglucoside linkage. The resultant aglycones then undergo non-enzymatic, intramolecular (Lossen) rearrangement to yield isothiocyanates, thiocyanates, nitriles or epithionitriles (Burow, Bergner, Gershenzon, & Wittstock, 2007). The structural difference of the glucosinolate is conferred to that of the cognate ITC, e.g., glucoraphanin to sulforaphane, and sinigrin to allyl ITC.

The pharmacophore of isothiocyanates (ITCs) structure was the significance electrophilic property of central carbon atom in the isothiocyanate (–N=C=S) group which attacks to the cellular nucleophilic targets such as DNA bases at nitrogen- and oxygen-based nucleophiles, and GSH at sulphur- (Joozdani, Yari, Joozdan, & Nafisi, 2015; Kolm, Danielson, Zhang, Talalay, & Mannervik, 1995). The conjugation of ITCs (electrophile moiety) with the intracellular antioxidant glutathione (GSH, the nucleophilic target) leads to the reactive oxygen species (ROS)-mediated apoptosis (Sestili & Fimognari, 2015). Moreover, this –N=C=S group acts as a pro-oxidant to generate ROS leading to the ROS-mediated apoptotic cell death. The pro-oxidant effect of ITCs was occurred by the spontaneous hydrolysis of –N=C=S moiety producing hydrogen peroxide or superoxide anion radicals (Sestili & Fimognari, 2015).

ITCs have been shown to block chemical carcinogenesis and are identified as specific inducers of Phase II enzymes such as glutathione S-transferases (GSTs) and quinone reductase (QR; NAD(P)H:(quinone-acceptor) oxidoreductase) in several mouse tissues without the induction of aryl hydrocarbon receptor dependent cytochromes P-450 (phase I enzymes) (Zhang, Talalay, Cho, & Posner, 1992). The chirality, state of oxidation of sulphur of the thiomethyl group, and number of methylene bridging groups in ITCs structure are important for inducer potencies in murine hepatoma cells. The chirality of the sulfoxide (such as sulforaphane; SF) does not affect inducer potency, since isolated (R)-sulforaphane from broccoli and synthetic racemic (R,S)-sulforaphane gave closely similar concentration of a compound required to double the QR specific activity in Hepa lclc7 murine hepatoma cells and were relatively noncytotoxic (Zhang et al., 1992). The presence of oxygen on sulphur enhanced potency. Oxidation of the side-chain sulphide (erucin) to sulfoxide (SF) or to sulfone (erysolin) enhanced inducer potency. The corresponding sulphide (erucin) was about one-third as active as SF on inducing of QR, whereas sulfoxide (SF) and the corresponding sulfone (erysolin) were equipotent (Zhang et al., 1992). Replacement of the S=O by C=O produced an analogue that was equally potent to SF. And compounds with 4 or 5 methylene groups in the bridge linking CH_3S– and –N=C=S were more potent than those with 3 methylene groups (Zhang et al., 1992). However, the rank of biological activities of SF analogues could differ depending on the type of biological activity assessed (Kim, Kim, & Lim, 2010). Oxidative stress may be linked to apoptosis and cell cycle repression in various cell lines. Oxygen attached to sulphur was reported to potentiate the apoptosis-inducing capability of SF analogues by increasing ROS generation. Growth inhibitory effects of SF analogues containing oxidized sulphur in colon cancer cell lines were more potent antiproliferative agents than analogues containing non-oxidized sulphur. SF analogues containing four atoms of carbon between oxidized sulphur and the –N=C=S groups were slightly more potent compared with those containing five atoms of carbon. The number of carbon separating the sulphur atom from the –N=C=S groups was a less important factor determining antiproliferative potency of SF analogues (Kim et al., 2010).

Indoles compounds such as indole-3-carbinol has been undergoing clinical trial for anticancer therapy because it suppressed tumour cell proliferation by targeting a wide spectrum of signaling pathways governing hormonal homeostasis, cell-cycle progression, and cell proliferation and survival (Weng, Tsai, Kulp, & Chen, 2008). Vascular targeting agents which inhibit tubulin-microtubule protein system of the endothelial cells lining tumour microvessels have been a selective therapeutic target for anti-cancer agents. These tubulin polymerization inhibitors were characterized based on the presence of an indole nucleus (Brancale & Silvestri, 2007; Patil, Patil, Beaman, & Patil, 2016). Indole ring acts as a pivotal pharmacophore for being potent tubulin polymerization inhibitors to majorly bind at the same active binding site as colchicine (Brancale & Silvestri, 2007; MacDonough et al., 2013). However, some modification on chemical structure led to alteration of molecular targets. Because some compounds did not bind at either the colchicine or vincristine colchicines-binding site suggesting a different binding site of indole derivatives on tubulin. The rational modifications at indole ring to improved potency have been the focus of many studies with variety information of structure–activity relationship of indole derivatives (Brancale & Silvestri, 2007; MacDonough et al., 2013).

Interestingly, isoalliin and butyl 1-(methylthio)propyl disulphide were identified in the active RS pod DCM crude extract. These sulphur-containing compounds are not commonly found in Brassicaceae. Isoalliin or *S-trans*-prop-1-enyl cysteine sulphoxide causes the characteristic aroma of onion (Jones et al., 2004) and butyl 1-(methylthio)propyl disulphide is found in the onion family, *Allium tuberosum* (Chinese chives) (Yannai, 2012). These organosulphur compounds have a structure related to dimethyl-trisulphide found in the RS pod and flower based on using the GC–MS technique (Pocasap et al., 2013). In addition to the sulphur-containing compounds (e.g., glucosinolates, isothiocyanates), the common plant constituents such as indoles and flavonoids (i.e., Broussoflavonol B) were also found in the RS pod DCM crude extract. Broussoflavonol B is a flavonoid with a reported chemopreventive effect; achieved by inducing cell cycle arrest, apoptosis, and acting as a potent growth inhibitor in breast cancer MDA-MB-231 cells (Guo et al., 2013).

Plant-derived isothiocyanates—allyl isothiocanate, iberverin, iberin, erucin, and sulforaphane were reported to induce phase II detoxification enzymes in rats (Munday & Munday, 2004). Sulforaphane inhibits vestibular schwannoma growth *in vivo* by reducing 27.2% of tumour volume (Kim et al., 2016). Allyl isothiocyanate significantly induces apoptosis and decreases mitosis phase of human prostate cancer PC-3 xenografts *in vivo* (Srivastava et al., 2003). Oral administration of uncooked Brussels sprouts in a juice or powder forms was enhanced levels of apoptosis and aberrant crypt foci in rat colonic mucosal crypts (Smith et al., 2003). For the human clinical trial, broccoli sprout containing

Table 3
Cytotoxicity of four identified compounds in RS pod extract against HCT116 cell lines.

Compounds	IC_{50} value (μg/ml)	IC_{50} value (μM)
D,L-Sulforaphane	4.94 ± 0.16	27.87 ± 0.90
L-Sulforaphene	8.60 ± 0.12	49.06 ± 0.68
Erysolin	5.28 ± 0.02	27.34 ± 0.12
Iberin	6.00 ± 0.07	36.74 ± 0.40

Table 4
The proximate analysis of *Raphanus sativus* var. *caudatus* Alef.

Part	Energy (kcal/100 g)	Protein (g/100 g)	Total carbohydrate (g/100 g)	Total fat (g/100 g)	Crude fiber (g/100 g)	Ash (g/100 g)	Moisture (g/100 g)
Stem and leaf	102.9	4.02	13.73	3.5	0.55	2.21	76.5
Pod	38.9	2.16	0.59	3.1	0.59	0.69	93.5

glucosinolates and isothiocyanates successfully passed through phase I study with no adverse effects in healthy volunteers (Shapiro et al., 2006). Indole-3-carbinol was previously reported to exert chemopreventive effect by changing enzyme activity and steroid metabolism (Reed et al., 2005).

Among compounds identified, no anticancer activity has been reported for oxazolidines, while isothiocyanates, glucosinolates and flavonoids were largely identified in cruciferous vegetables with clinically relevant anticarcinogenic activities. D,L-sulforaphane, L-sulforaphene, erysolin, and iberin (found as glucoiberin in the pod extract) were determined for their cytotoxicity against HCT116 (Table 3). Erysolin exerted the highest cytotoxic (27.34 μM) followed by D,L-sulforaphane (27.87 μM), L-sulforaphene (49.06 μM), and iberin (36.74 μM), respectively. The isothiocyanates (ITCs) were found with more numbers of identified compounds than the other groups. The majority of the anticancer activity of RS pod is likely contributed to a chemical entity, the isothiocyanates.

Thiocyanates and oxazolidine–glucosinolates products, 5-vinyloxazolidine-2-thione (goitrin), were also found in the crude RS extract and in fraction E, which accounted for the lowest % yield (3.50%w/w) among the 4 fractions. The goitrogenic property of the thiocyanate ion occurs via suppression of iodine uptake, however, this effect can be compensated by sufficient iodine intake (Kostogrys, Pisulewski, Pecio, & Filipiak-Florkiewicz, 2010). One possible case of a goitrogenic effect occurred from consumption of thiocyanate and goitrin in 10 kg cauliflower per day reported of a man with extremely low iodine intake (McMillan, Spinks, & Fenwick, 1986). The formation of oxazolidine-2-thione corresponded to hydrolysis of glucosinolates consisting of R chain with hydroxyl at the C-2 position. Oxazolidine-2-thione increases goiter by reducing the production of thyroid hormones (Verhoeven, Verhagen, Goldbohm, Van Den Brandt, & Van Poppel, 1997); however, the goitrogenic effect was inversely affected by Brassica consumption (Mithen, 2001). The conversion of glucosinolates into thiocyanate ion requires two plant enzymes—myrosinase and specific thiocyanate-forming protein (TFP) (Wittstock & Burow, 2007). Since TFP is thermally-degraded, the cooking process could reduce the anti-thyroid effect of reactive thiocyanates in Brussels sprouts. A previous report showed no effect on thyroid function after 4 weeks (150 g daily) of consuming cooked Brussels sprouts with normal diet (McMillan et al., 1986).

3.4. Nutritive composition of RS

Table 4 showed that the percentage of energy, protein, carbohydrate, and ash were higher in RS stem and leaf (102.9 kcal, 4.02, 13.73, and 2.21%, respectively) than RS pods (38.9 kcal, 2.16, 0.59, and 0.69%, respectively). While the percentages of total fat and crude fiber were similar in both parts of samples (stem and leaf—3.5, and 0.55; pod—3.1, and 0.59%). Only moisture content was found to be higher in RS pod (93.5%) than RS stem and leaf (76.5%).

The micronutrients in RS were previously determined by Nutritional Department of Thailand Food and Drug Administration. The vitamin and minerals in RS were 44 mg of calcium, 35 mg of phosphorus, 1.8 mg of iron, 777 IU of vitamin A, 0.11 mg of vitamin B1, 0.05 mg of vitamin B2, and 1.10 mg of vitamin B3 or niacin, and 125 mg vitamin C (Paisukshantiwattana, 2013). The health benefits of RS were reported as the traditional uses by Chiang Mai University, Thailand. The traditional uses of RS pods and leaves for health were for appetite enhancer, anti-flatulence, and believe to clear urethral stone. RS flowers were also used for facilitating biliary excretion (Department of Agriculture Extension., 2005).

4. Conclusion

The current study demonstrated the potential cytotoxic activity of pod DCM crude extract of RS pod against a colon cancer cell line (HCT116). The compounds previously reported as possible cytotoxicity contributors were identified using LC-MS while 2 additional compounds (isoalliin and butyl 1-(methylthio)propyl disulphide) were detected in the genus Brassica for the first time. The information on phytoconstituents in the RS pod extract will be useful for laboratory-scale standardization and botanical authentication of this vegetable in an approach for using it as functional food. The development of dietary supplements containing RS pod could be expanded based on information available on the chemical constituents that can be used for quality control and quality assurance of dietary supplement products containing RS pod extract.

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Analytical Methods

Simultaneous quantification of sulforaphene and sulforaphane by reverse phase HPLC and their content in *Raphanus sativus* L. var. *caudatus* Alef extracts

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ABSTRACT

A simple, rapid and precise HPLC assay was developed for the well-known anti-cancer isothiocyanates—sulforaphene (SE) and sulforaphane (SF). The analytical system comprised RP-C_{18} column with isocratic 5% THF-95% water. High resolution was obtained (and eluted) of two distinct HPLC peaks of similar structures SE and SF analytes (at 23.01 ± 0.02 and 25.65 ± 0.03 min, respectively). The respective LOD vs. LOQ for SE and SF was 0.34 and 0.36 μg/ml vs. 1.02 and 1.08 μg/ml. This assay had the best linearity and accuracy. The recoveries were in the range of 96.83–101.17%. SF and SE were quantified in the pod of *Raphanus sativus* L. var. *caudatus* Alef extracts (2253.05 ± 246.18 and 111.94 ± 16.49 μg/g in the crude extract, respectively), while only SE was detected in the stem (1105.14 ± 243.10 μg/g crude extract), as SF was lower than the detection limit. The validated method thus minimized and expedited simultaneous SE and SF analysis.

1. Introduction

Brassicaceae and related plant families are rich in secondary plant metabolites; including over 120 glucosinolates (GSLs) and various isothiocyanates (ITCs) (De Nicola et al., 2013; Li et al., 2010; Nakamura, 2009; Oerlemans, Barrett, Suades, Verkerk, & Dekker, 2006). GSLs coexist with myrosinase—an endogenous thioglucosidase (EC 3.2.3.1) in adjacent plant cells—and are released and hydrolyzed by it; producing mainly ITCs—sulfur- and nitrogen-containing compounds.

Sulforaphane (SF) is a cancer chemo-preventive agent (Fig. 1) found in broccoli (*Brassica oleracea* L. var. *italica*) (Nakamura, 2009). Clarke, Dashwood, and Ho (2008) reported the anti-cancer effects of SF: (a) blocking the initiation state via inhibiting Phase I enzymes to convert procarcinogens to proximate or ultimate carcinogens; and (b) inducing Phase II enzymes that detoxify carcinogens and facilitate their excretion from the body. A further protective effect associated with oxidative stress was revealed in experimental models (Guerrero-Beltrána et al., 2012).

Sulforaphene (SE) (Fig. 1)—with an additional double bond to SF—has been reported to reduce cancer cell proliferation in a dose-dependent manner and induce apoptosis in: (a) colon carcinoma cell lines (Barillari et al., 2008; Papi et al., 2008); (b) human and murine erythroleukemic cells; (c) human T-lymphoid cells; and, (d) human cervix carcinoma cells (Nastruzzi et al., 2000). SE (at 1 μM) was a potent inducer of hepatic enzymes involved in the detoxification of chemical carcinogens (Razis, Nicola, Pagnotta, Iori, & Ioannides, 2012). Both SF and SE are bioactive constituents in Thai rat-tailed radish (*Raphanus sativus* L. var. *caudatus* Alef, RS or Pak Khi Hood), contributing to an apoptosis induction effect against the HCT116 colon cancer cell line (Pocasap, Weerapreeyakul, & Barusrux, 2013).

Intensive analytical assay techniques have been developed to quantify the bioactive constituents in food, vegetables and herbs. Although present in small amounts, these compounds play critical

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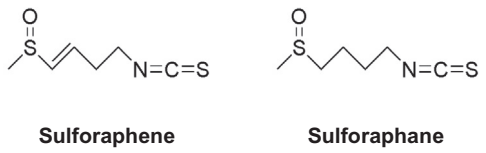

Fig. 1. Chemical structures of sulforaphene (SE) and sulforaphane (SF).

roles in food function and the bioactivity of medicinal plants. Optimum quantitative analysis should lessen the cost of resource consumption—including chemicals, sample used, time and facilities. HPLC has been widely used for quantitative analysis of bioactives for quantitative analysis, as minimum handling is needed. Due to structural similarities between SE and SF (Fig. 1), HPLC results in poor separation. Lim, Lee, and Kim (2009) tried using two different detectors to achieve simultaneous detection but—despite using high specification equipment—low resolution persisted. In the current study, (a) simple, simultaneous HPLC for chemopreventive SF and SE is reported for the first time and (b) quantitative analysis of both biomarkers in extracts of the Thai native vegetable (RS) was successfully achieved.

2. Materials and methods

2.1. Chemicals

Tetrahydrofuran (THF) (HPLC grade, Fisher Scientific, UK) and ultrapure water from Milli-Q system (Millipore, USA) were used for the mobile phase preparation. Pure D,L-sulforaphane, 1-isothiocyanato-4-(methyl sulfinyl) butane (Calbiochem, EMD Millipore, USA) and L-sulforaphene, 1-isothiocyanato-4-(methyl sulfinyl) butane (Enzo Life Sciences, USA) were used as the standards. Dimethyl sulfoxide (DMSO; Sigma, USA) was used as the diluent.

2.2. Standard stock preparations

The respective stock SE, SF and SF–SE mixture was diluted to 5.0, 10.0, 15.0, 30.0 and 40.0 μg/ml in DMSO.

2.3. Chromatographic analytical conditions

The HPLC Value System was used to optimize the elution condition (Agilent, 1100 Series, Waldbronn, Germany). The stationary phase was done in a Reverse Phase-C_{18} column (5 μm particle size, 250 × 4.6 mm) (HiQsil, Tokyo, Japan). The mobile phase was developed in simple filtered and degassed 5% THF in ultrapure water (v/v). The flow rate was stable at 1 ml/min in isocratic elution for 30 min. The photo diode array (PDA) detector (Agilent, 1100 series G1314A, Tokyo, Japan) was set at 254 nm as per Pocasap et al. (2013) and detected at 210 nm. An in-line 0.5-μm filter and a guard column were used to protect the analytical column.

2.4. Validation parameters

The validation parameters included (a) specificity of each compound; (b) selectivity of the elution method between two peaks; (c) limit of detection (LOD); (d) limit of quantification (LOQ); and, (e) linearity of the calibration curves (R^2). The retention times of each standard in the mixture solution were identified and the percentage of relative standard deviation (%RSD) was calculated to confirm the specificity of the peaks.

Evaluation of method repeatability (intra-day precision) and reproducibility (inter-day precision) were performed. Intra-day precision was determined at 5 concentrations in 6 replications. The inter-day precision was determined in 6 replications conducted over 3 days. Precision of the method was expressed as the %RSD for each test. The standard deviation (SD) of the response and slopes of the concentration curves of the calibration curves were used to estimate the limit of detection (LOD) and the limit of quantification (LOQ) with the following formulae: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the residual SD of the regression line and S is the slope of the standard curve. The percentage of recovery of each compound was evaluated. Sulforaphane and sulforaphene were analyzed in triplicate at three different concentrations (5 μg/ml, 15 μg/ml and 40 μg/ml) by using the obtained validated method. The percent recovery was calculated [%Recovery = (recovered conc./injected conc.) × 100] and the results statistically analyzed.

2.5. Sample preparation

RS was grown outdoors on a farm in Phayao province, northern Thailand between December 2014 to January 2015. The plants were harvested after 6 weeks growth. The RS was rinsed with water then patted dry with a paper napkin. RS extracts were prepared from stems and whole pods as these plant parts are reported to contain relatively high amounts of isothiocyanates, according to Pocasap et al. (2013). The fresh RS samples were homogenized with deionized water at a ratio of 1:1 (w/v) for 30 min and the homogenate allowed to stand at room temperature for 2 h. The homogenate was filtered through double layers of cheesecloth. Dichloromethane was added to the aqueous filtrate at a ratio of 1:1 (v/v) and liquid–liquid extraction performed in triplicate. The lower dichloromethane layer was collected and the contaminated water removed by anhydrous sodium sulfate. The organic solvent was removed under vacuum by rotary evaporator yielding dry crude extract.

3. Results and discussion

3.1. Optimization of HPLC analysis

A number of previous studies tried to detect more than one major compound in the crude extract mixture. The HPLC elucidation profile of RS crude extract by Pocasap et al. (2013) suggested the peaks for SE and SF coexisted. The GC analysis moreover supported the co-existence of SE and SF as major compounds in the RS extract. The present study proposes an elution system that affords high resolution between the SE and SF peaks, by the weakly acidic property of THF (Wang, Helliwell, & You, 2000). It was found that the use of tetrahydrofuran in water mobile phases could elute specific derivatives with a THF concentration of 5%. Our present study showed good separation between SE and SF (Fig. 2). The separation of each compound without peak-merging indicates the specificity of the detection method. The respective retention times of SE and SF were 23.01 ± 0.02 min and 25.65 ± 0.03 min with a %RSD of 0.07 and 0.12 (Fig. 2). The selectivity value from this eluting method was 1.11, which is considered high. The individual injection and also the simultaneous mixture of SE and SF show the efficient condition to be used in the quantitative determination in crude extracts. The elution profiles were detected at 254 nm—as per Pocasap et al. (2013). The present study also demonstrated SE and SF signal enhancing by detection at 210 nm.

3.2. Validation of analytical method

The HPLC eluting system—by the simple mobile phase—provides good detection parameters (Table 1). The respective

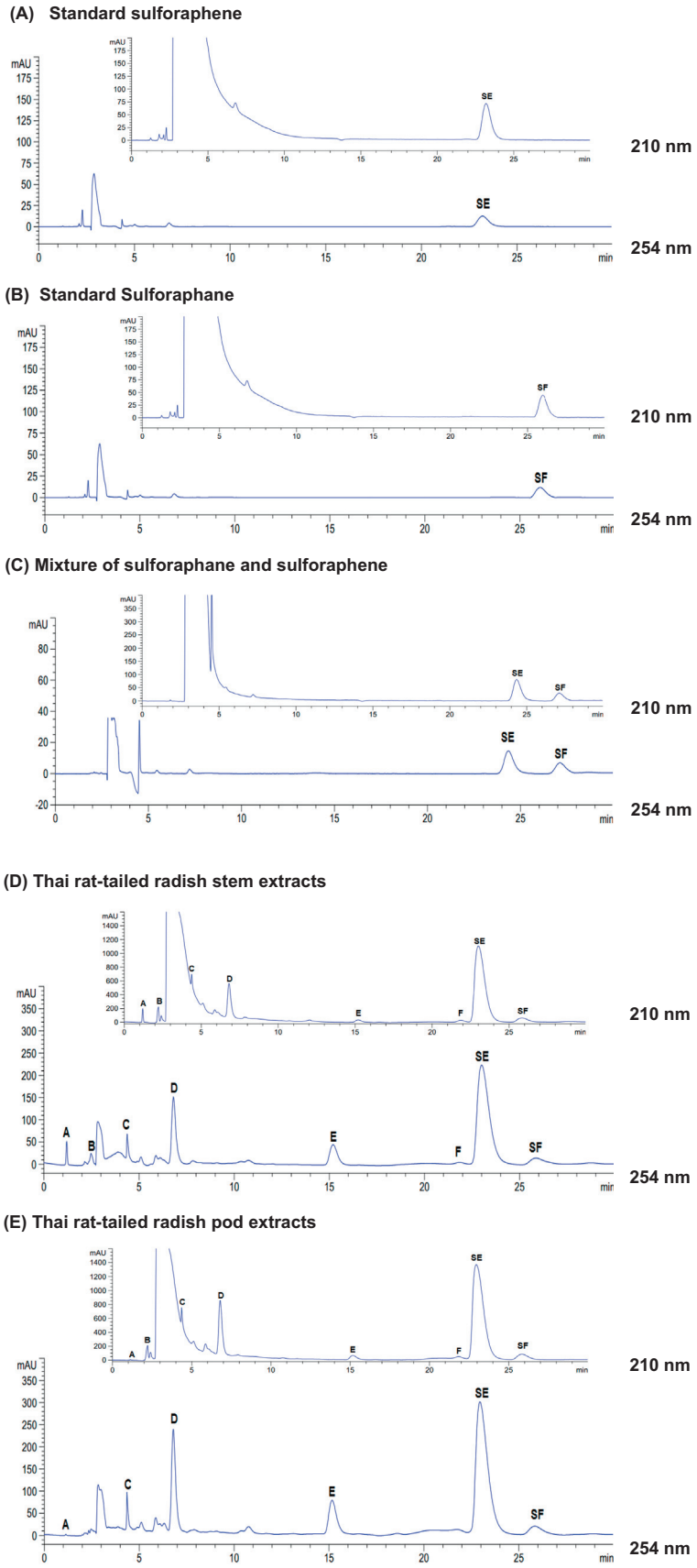

Fig. 2. HPLC chromatograms of analytes detected at 210 and 254 nm. Peaks of (A) standard sulforaphene; SE (23.01 ± 0.02 min), (B) standard sulforaphane; SF (25.65 ± 0.03 min), (C) mixture of sulforaphene and sulforaphane, and Thai rat-tailed radish extracts from (D) pod and (E) stem with the unknown peaks: A (1.17 ± 0.04 min), B (2.29 ± 0.11 min), C (4.37 ± 0.01 min), D (6.80 ± 0.01 min), E (15.15 ± 0.02 min), and F (21.85 ± 0.02 min). The numbers in parentheses are the retention time of each peak.

Table 1
Validation parameters for the liquid chromatographic method; specificity, the selective index, LOD, and LOQ.

Specificity*			Selective index**	LOD	LOQ
Tested compound	Retention time (n = 10)	%RSD		(μg/ml)	
Sulforaphene	23.01 ± 0.02	0.07	1.11	0.34	1.02
Sulforaphane	25.65 ± 0.03	0.12		0.36	1.08

* %RSD of each peak represents high specificity.
** Separation of each compound without peak merging indicates specificity of the detection method. A selectivity value > 1 is considered selective. The higher the value, the higher selectivity.

linearity range (r^2) of SE and SF was 0.99 and 0.99 (Table 3). The respective precision parameter represented by the relative standard deviation (RSD) value for SE and SF was 0.67% and 0.51% while the repeatability was 0.93% to 0.98% (Table 2). The respective limit of detection (LOD) and quantification (LOQ) confirmed the sensitivity of the system (i.e., LOD of SE and SF = 0.34 and 0.36 μg/ml while the LOQ = 1.02 and 1.08 μg/ml). The recovery of SF and SE ranged between 96.83 ± 0.07 and 101.17 ± 0.12% (Table 4): the average 97% recovery underscoring the high accuracy of the protocol.

Table 2
Precision of HPLC method.

Day	Conc. (μg/ml)	No. of injection	Peak area		Average		SD		%RSD	
			SE	SF	SE	SF	SE	SF	SE	SF
Intra-day										
	10	1	3278.61	4098.90	3267.41	4093.53	21.82	21.10	0.67	0.51
		2	3242.28	4070.26						
		3	3281.36	4111.42						
Inter-day										
1	10	1	3278.61	4098.90	3262.60	4084.50	30.39	40.11	0.93	0.98
		2	3242.28	4070.26						
		3	3281.36	4111.42						
2	10	1	3275.40	4097.28						
		2	3188.35	3981.92						
		3	3264.34	4094.02						
3	10	1	3278.87	4099.32						
		2	3276.50	4105.13						
		3	3277.69	4102.23						

Table 3
Linearity of HPLC method.

Linearity									
Conc. (μg/ml)	Replication	Peak area					Slope	Y-intercept	R^2
		Day 1	Day 2	Day 3	Average	SD			
Sulforaphene									
	1	1227.34	1417.52	1418.68					
5	2	1355.54	1415.79	1415.88	1388.30	50.08			
	3	1408.54	1417.06	1418.34					
	1	3278.61	3275.40	3278.87					
10	2	3242.28	3188.35	3276.50	3262.60	17.98			
	3	3281.36	3264.34	3277.69					
	1	4213.01	4243.19	4251.16					
15	2	4268.75	4264.36	4253.57	4253.40	3.36	297.4	−51.59	0.99
	3	4270.31	4263.94	4252.30					
	1	5342.97	5322.68	5318.24					
20	2	5330.26	5317.05	5324.56	5324.626	7.97			
	3	5324.65	5333.93	5307.29					
	1	8792.80	9490.42	9438.62					
30	2	9194.10	9483.93	9439.18	9348.80	199.59			
	3	9373.77	9491.58	9434.80					
	1	10778.1	12006.1	11935.3					
40	2	11423.1	12023.4	11953.1	11754.46	395.08			
	3	11697.1	12001.3	11972.6					
Sulforaphane									
	1	1529.69	1761.96	1766.72					
5	2	1694.53	1772.89	1761.98	1731.80	61.23			
	3	1759.41	1777.08	1761.90					
	1	4098.90	4097.28	4099.32					
10	2	4070.26	3981.92	4105.13	4084.50	23.58			
	3	4111.42	4094.02	4102.23					

Table 3 (*continued*)

Linearity									
Conc. (μg/ml)	Replication	Peak area					Slope	Y-intercept	R^2
		Day 1	Day 2	Day 3	Average	SD			
	1	5262.57	5310.39	5335.87					
15	2	5313.81	5334.17	5335.99	5320.07	15.15	370.6	−87.63	0.99
	3	5332.69	5331.02	5324.13					
	1	6301.11	6310.21	6309.05					
20	2	6313.61	6297.72	6304.47	6305.80	2.38			
	3	6307.44	6301.25	6307.30					
	1	11049.8	11978.4	11981.6					
30	2	11573.2	11977.2	11989.1	11815.47	299.24			
	3	11786.8	12006.3	11996.8					
	1	13296.6	14909.9	14892.3					
40	2	14077.6	14939.7	14,952	14601.84	572.95			
	3	14446.7	14929.2	14972.6					

3.3. Quantification of sulforaphene and sulforaphane in RS extracts based on HPLC determination

The validated HPLC system was then used to determine two biomarkers–SE and SF–in the RS extracts. The extracts obtained from the stem and pod parts yielded 0.0282 and 0.0595 (%w/w of fresh weight), respectively. The standard equivalent quantities were calculated using a linear regression equation obtained from the calibration curve. The RS pod extract had higher respective SE and SF content than the RS stem extract (Table 5). The stem contained 1105.14 ± 243.10 μg SE/g crude extract while the SF content was undetectable. The RS pod extract was 2253.05 ± 246.18 μg SE and 111.94 ± 16.49 μg SE/g in the crude extract.

The present detection agrees Pocasap et al. (2013) who reported that the GC-MS of RS pod and flower extracts contained a higher SE than SF content. The retention time of the major compounds SE and SF were the same for both detected wavelengths. We found that 210 nm was a better signal for analysis of low concentration samples than 254 nm. Interestingly, five unknown peaks were found in the RS extracts with following retention times; A (1.17 ± 0.04 min), B (2.29 ± 0.11 min), C (4.37 ± 0.01 min), D (6.80 ± 0.01 min), E (15.15 ± 0.02 min) and F (21.85 ± 0.02 min) (Fig. 2). The HPLC chromatogram of the extract with the presence of these markers can be used as a fingerprint for standardization or quality control of the extract in future studies of its bioactivity. Identification of these unknown markers is currently under intensive study in our laboratory.

A simultaneous assay for SE and SF would be both convenient and valuable since both compounds are anticancer agents found in edible plants: knowing their respective content would directly indicate the function of RS. To date, simultaneous detection of these two chemo-preventive compounds has been limited by their

Table 5
Quantification of sulforaphene and sulforaphane in *Raphanus sativus* L. var. *caudatus* Alef (RS) extracts.

	Sulforaphene		Sulforaphane	
	μg/g crude extract			
	Mean	SD	Mean	SD
Stem	1105.14	243.10	nd	
Pod	2253.05	246.18	111.94	16.49

*nd means the peak was lower than the detection limit.

Table 4
%Recovery of HPLC method.

%Recovery						
Conc. (μg/ml)	Replication	Peak area	Recovery conc. (μg/ml)	%Recovery	Average	SD
Sulforaphene						
5	1	1418.68	4.9434	98.875	98.80	0.10
	2	1415.88	4.934	98.687		
	3	1418.34	4.9426	98.852		
15	1	4268.75	14.527	96.847	96.83	0.07
	2	4264.36	14.512	96.748		
	3	4270.31	14.532	96.882		
40	1	11935.30	40.306	100.764	100.92	0.16
	2	11953.10	40.365	100.914		
	3	11972.60	40.431	101.078		
Sulforaphane						
5	1	1766.72	5.004	100.073	99.90	0.15
	2	1761.98	4.991	99.817		
	3	1761.90	4.991	99.813		
15	1	5335.87	14.634	97.563	97.55	0.02
	2	5334.17	14.630	97.532		
	3	5335.99	14.635	97.565		
40	1	14909.90	40.468	101.171	101.17	0.12
	2	14892.30	40.421	101.052		
	3	14929.20	40.520	101.301		

structural similarity. The initial approach could not differentiate the SE and SF peaks. The current study instead presents a reliable, rapid, HPLC assay that uses less hazardous solvent while maintaining satisfactory sensitivity and selectivity.

4. Conclusion

We developed a HPCL method for the simultaneous investigation and efficient validation of the chemopreventive compounds—sulforaphene and sulforaphane—found in the extracts of RS. The analytical method is (a) simple (using an isocratic mobile phase of 95% water and 5% THF), (b) commercially available, (c) relatively inexpensive, (d) environmentally safe, (e) had efficient validation parameters, (f) minimized and expedited simultaneous quantification of SE and S and (g) can be used in the standardization and quality control process. Hence the method offers an analytical method for determination of consistent bioactives in each batch. The simple simultaneous and developed HPLC analysis that we had advantages over individual analysis of the respective SE and SF content in samples of plant extracts.

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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)

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ABSTRACT

The bioactive compounds of *Raphanus sativus* var. *caudatus* Alef and their respective cytotoxicity were identified from (a) 2 crude water and dichloromethane extracts and (b) 5 serially partitioned extracts using dichloromethane. Then, using a bioassay-guided cytotoxicity assay, the extracts were tested against the colon cancer cell line HCT116. Among 2 crude extracts and 5 fractions, the dichloromethane crude extract possessed the greatest *in vitro* cytotoxicity against HCT116. The dichloromethane crude extract was subjected to flash column liquid chromatography. Only 4 fractions and unfractionated extract were chosen for further analysis by high accuracy mass spectrometry (UHPLC-QToF-MS/MS). Six glucosinolates, 13 isothiocyanates, 5 indoles, 4 flavonoids, 2 alkaloids, 2 thiocyanates, 1 oxazolidine, and 1 dialkyl disulphide were identified. Two compounds were detected for the first time—isoalliin and butyl 1-(methylthio)propyl disulphide. Several constituents with anti-cancer activity were identified. This information could inform guidelines for quality control of standard plant extracts for further product development.

1. Introduction

Brassicaceae (including *Raphanus sativus* Linn. or radishes) are grown and consumed worldwide (Kim et al., 2014). Vegetables in this family—such as wasabi, mustard, water cress, garden cress, and broccoli sprouts—are a rich source of phytochemicals (phenols, flavonoids, and vitamin C) (Kim et al., 2014; Zhang, Tang, & Gonzalez, 2003). The biological activities of glucosinolates and their enzymatic conversion—sulphur-containing products such as the isothiocyanates in Brassicaceae—have been reported (a) reducing cancer risk and inflammatory response; (b) having antimicrobial, antioxidation, antitumour, and antiviral activity; (c) affecting immunomodulation, and intestine motility stimulation; and, (d) preventing cardiovascular disease (Björkman et al., 2011; Gutierrez & Perez, 2004; Moon & Kim, 2012; Vaughn & Berhow, 2005). Previously identified chemical constituents in *R. sativus* include alkaloids, nitrogen compounds, coumarins, enzymes, gibberellins, glucosinolates, fatty acids, organic acids, phenols, pigments, polysaccharides, sulphur compounds, phytoalexins, β-carotene, and vitamin C.

R. sativus var. caudatus Alef (RS) (Thai rat-tail radish) is an indigenous Thai vegetable cultivated in the north and northeast regions of Thailand. Previous studies of its constituents focused on the identification of the biomarkers sulforaphene and sulforaphane in the RS extract. Sulforaphane, sulforaphene, 3-butenyl isothiocyanate, and 4-methylthio-but-3-enyl isothiocyanate were discovered in the RS seed by GC–MS analysis (Songsak & Lockwood, 2002). Pocasap, Weerapreeyakul, and Barusrux (2013) then used GC–MS analysis to identify sulforaphane, sulforaphene, 3-butenyl isothiocyanate, and dimethyltrisulphide in the edible parts of the RS pod and flower, which were shown to contribute to a chemopreventive effect against the colon cell line HCT116 through induction of apoptosis (Pocasap & Weerapreeyakul, 2016; Pocasap et al., 2013).

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Ultra-high performance liquid chromatography (UHPLC) is a highly selective and sensitive analytical technique that allows physical separation of complex elutes. The speed, resolution, and sensitivity of UHPLC make it ideally suited for use with mass spectroscopy (MS). MS detection is a useful tool for chemical identification because it provides accurate mass and structural information—especially high-resolution mass spectrometric techniques such as quadrupole time-of-flight (QToF). When UHPLC is combined with the mass analysis capabilities of high-resolution mass spectrometry, it provides a powerful technique with very high sensitivity. Its application is for general detection, separation, and potential identification of chemicals of particular masses in the presence of other chemicals (i.e., in complex mixtures, extracts of natural products, and pure substances from mixtures of chemical intermediates) (Khan & Ali, 2015).

Percent cytotoxicity was calculated against the widely reported anti-cancer compounds, sulforaphane and sulforaphene. Sangthong and Weerapreeyakul (2016) reported the quantitative analysis of the latter two compounds: 1 g of dichloromethane crude extract of RS pod contains 0.112 mg sulforaphane and 2.253 mg sulforaphene. The respective percents contribution of sulforaphane and sulforaphene—in the crude extract against the cancer cell line HCT116—were 0.04% and 0.47%. The results suggest the existence of other bioactive components in the RS dichloromethane crude extract.

In the current study, high-accuracy mass spectrometry was used to identify and structurally characterize the presence of chemopreventive compounds in the RS of Thai rat-tailed radish extract, which is reported to have anticancer activity (Guo, Wang, Deng, Zhang, & Wang, 2013; Kim et al., 2016; Munday & Munday, 2004; Reed et al., 2005; Smith, Mithen, & Johnson, 2003; Srivastava et al., 2003). Glucosinolates, isothiocyanates, indoles, alkaloids, thiocyanates, oxazolidine, flavonoids, and dialkyl disulphide were successfully identified in the extract. To the best of our knowledge, this is the first report of the organosulphur compounds—isoalliin and butyl 1-(methylthio)propyl disulphide commonly found in onion and Chinese chives—being detected in the genus Brassica, species *R. sativus* var. *caudatus* Alef.

2. Materials and methods

2.1. Reagent and standards

Commercial grade dichloromethane, hexane, and chloroform were purchased for the extraction and distilled before use. Deionized water (ddH_2O) was obtained from a MilliQ system (Millipore, Bedford, MA, USA). HPLC grade methanol (MeOH), formic acid (Fisher Scientific, Leicestershire, UK) and ddH_2O were used for the HPLC analysis. Allyssin and iberin were purchased from Abcam (Cambridge, UK), erysolin, D,L-sulforaphane from Merck, and L-sulforaphene from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Preparation of RS crude extract

Frozen RS pods were blotted and cut into small pieces and homogenized using a food processer with ddH_2O (1:1, w/v). The homogenate was left for autolysis at 25–27 °C for 2 h and filtered through double-layer cheesecloth. The filtrate was partitioned with dichloromethane. The lower layer of dichloromethane and upper layer of water were collected separately. This partitioning was done in triplicate. Then the serial partitions underwent bioassay-guided chemoprevention testing against colon cancer cell line HCT116 (Pocasap et al., 2013) (Fig. 1). The crude extract of the aqueous layer was collected and dried using lyophilizer (SCANVAC CoolSafe 110-4 Pro, LABOGENE, Lynge, Denmark), yielding a aqueous crude extract (3.277% w/w). The dichloromethane layer

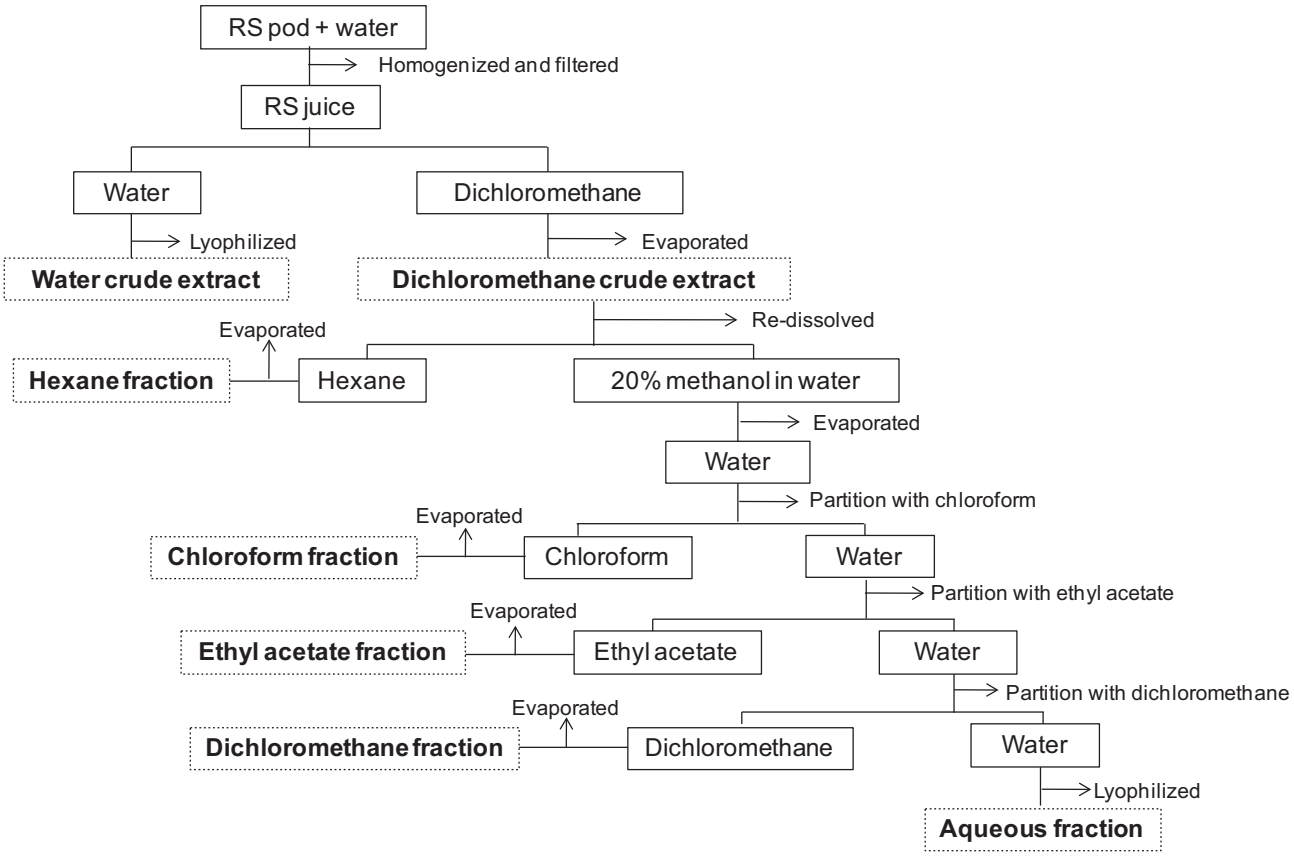


Fig. 1. Schematic diagram represents serial extraction method of whole pod from Thai rat-tailed radish (RS).

was dried by rotary evaporator and all dichloromethane extract fractions were pooled after the dichloromethane was removed using a vacuum rotary evaporator, yielding a dichloromethane crude extract (0.068%w/w). Dichloromethane crude extract was re-dissolved with 20% methanol (2 g/50 ml) and partitioned with 4 organic solvents (100 ml each) with differing polarity (namely, hexane, chloroform, ethyl acetate, and dichloromethane). The organic solvent layer of each partition was collected and dried by rotary evaporator. The percentage yield of dried fractions was calculated as percent weight by weight of the fresh weight: hexane fraction (0.021% w/w), chloroform fraction (0.010%), ethyl acetate fraction (0.013%), dichloromethane fraction (0.013%), and aqueous fraction (0.012%). Seven samples underwent cytotoxicity testing and further identification analysis.

2.3. Cytotoxicity against HCT116 cell lines as bioassay-guided test

Seven samples were dissolved with dimethyl sulphoxide (DMSO) into the final tested concentration of 500 μm/ml. To avoid false cytotoxicity from DMSO, < 1% v/v of DMSO was maintained in the mixture so as to have < 10% cytotoxicity. Neutral red (NR) assay was used to detect the percentage of viable cells or indirectly to determine cytotoxicity as per Machana, Weerapreeyakul, Barusrux, Thumanu, and Tanthanuch (2012). Briefly, the cells (5×10^5 cell/ml) were seeded in 96-well plates and treated with samples for 24 h. Cells were then washed once with phosphate buffer. A total of 100 μl NR solution (50 μg/ml) was added to each well and incubated at 37 °C for another hour. NR was then dissolved by 100 μl of 0.33% hydrochloric acid. Absorbance of the NR dye was detected by a dual-wavelength UV spectrometer at 537 nm with a 650 nm reference wavelength. The percentage of cytotoxicity compared to the untreated cells was determined with the equation given below.

$$\text{Cytotoxicity (\%)} = \frac{[100 \times (\text{Absorbance of untreated group} - \text{Absorbance of treated group})]}{(\text{Absorbance of untreated group})}$$

In addition, the compounds identified in the RS pod extract (D, L-sulforaphane, L-sulforaphene, erysolin, and iberin) were also tested for their cytotoxicity with the same method mentioned above.

2.4. Preparation of flash column chromatography fractions

Dichloromethane crude extract from the RS pod was separated and the fraction collected by Buchi Sepacore® flash systems (BÜCHI Labortechnik, Flawil, Switzerland) equipped with control unit C-620, UV photometer C-640, and fraction collector C-660. RS pod dichloromethane crude extract (3 g) was then loaded into 120 g RediSep® Normal-phase Flash Column (Teledyne Isco, Lincoln, NE, USA). The normal phase eluting was performed with a flow rate of 50 ml/min with eluent A (dichloromethane) and eluent B (methanol). The mobile phase was started with 0% A for 10 min (0–10 min); the linear was then increased to 10% A for 20 min (at 20–40 min); then to 15% A for 5 min (45–50 min); then to 40% A for 10 min (60–80 min); and finally 100% A for 15 min (85–100 min). The fractions were automatically collected every 20 ml. The fraction(s)—which consisted of only one peak as detected by UV detector (254 nm)—were then combined and dried using a rotary evaporator. Finally, 9 fractions were obtained from this step.

2.5. UHPLC-QToF-MS analysis

Four of the nine fractions from the flash column chromatography—the top 4 in yield rank (namely, C, D, E, and F)—were selected. Five samples—including the unfractionated dichloromethane crude extract and the C, D, E, and F fractions were analyzed using an UHPLC-QToF-MS (Agilent Technologies, Waldbronn, Karlsruhe, Germany). The instrument comprised a 1290 LC system, a Jetstream ESI source, and a 6540 UHD accurate-mass quadrupole-time-of-flight (QToF) mass spectrometer. The ionization characteristics were determined with both electrospray ionization polarities (ESI+ and ESI−). Separation was performed on reversed phase analytical column Zorbax RRHD Eclipse XDB-C18, 2.1 × 100 mm, 1.8 μm column (Agilent Technologies, Palo Alto, CA, USA) at 50 °C. The sample injection volume was set at 2 μl. The mobile phases were delivered at 0.4 ml/min, consisting of 0.1% formic acid in water (eluent A) and 0.1% formic acid in methanol (eluent B). The gradient was started with 2–100% B for 10 min (0–10 min) then 100% B for 4.50 min (10–14.50 min), and 2% B for 2 min (14.50–16.50 min). The MS conditions follow: nitrogen as the instrument gas; drying gas temperature 325 °C at 10 L/min; sheath gas temperature 350 °C at 11 L/min; nebulizer pressure 45 psi; capillary voltage 3500 V; nozzle voltage 1000 V; fragmentor voltage 100 V; and, skimmer voltage 45 V. For MS data acquisition, 2 GHz extended dynamic range mode was used in both ion modes from m/z 20–1600. Data were collected in the centroid mode at an acquisition rate of 1.67 spectra/s with an abundance threshold of 150. For automatic data dependent MS/MS analyses, the precursor isolation width was narrow (1.3 Da), the MS/MS scan rate 3.33 spectra/s, and from every precursor scan cycle 4 of the most abundant ions were selected for fragmentation. These ions were excluded after 2 product ion spectra were analyzed and released again for fragmentation after a 0.25 min hold. Precursor scan time was based on ion intensity, ending at 25,000 counts or after 300 ms. Product ion scan time was 300 ms. Collision energies were 10 and 20 V in subsequent runs. The ToF was calibrated on a daily basis and subsequently operated at high accuracy (<2 ppm). Continuous mass axis calibration was performed by monitoring 2 reference ions from an infusion solution throughout the runs. The reference ions were m/z 121.050873 and m/z 922.009798 in the positive mode and m/z 112.985587 and m/z 966.000725 in the negative mode.

2.6. Identification and annotation of compounds

Identification of molecules was generated by Agilent MassHunter™ Qualitative Analysis B.06.00 (MassHunter™ Qual, Agilent Technologies, Santa Clara, CA, USA); based on accurate mass-matching to published databases (namely, Human Metabolome Database (http://www.hmdb.ca/) (Wishart et al., 2013); Metlin (https://metlin.scripps.edu/) (Smith et al., 2005); and SciFinder (https://scifinder.cas.org). The first identification was based on accurate mass and isotopic pattern, matched to the Metlin database using Agilent's Identification Browser software. The results were sorted and an assessment of retention time and a single putative annotation with a matching elemental formula selected. This annotated molecular feature was then compared to other databases. The MS/MS spectra of molecular features were compared and matched to a library of standard spectra in Agilent's MassHunter METLIN Personal Compound Database and Library (PCDL) (Agilent Technologies, Santa Clara, CA). An authenticating reference standard for allyssin, erysolin, iberin, D,L-sulforaphane, and L-sulforaphene were run using identical instrumental conditions, and the detected molecules matched to accurate masses, retention times, and product ion spectra present in the unfractionated and fractionated RS extract. The level of identification (LI) was defined in 4 different levels including; identified metabolites (level 1), putatively annotated compounds (level 2), putatively characterized compound classes (level 3), and unknown compounds (level 4) (Salek, Steinbeck, Viant, Goodacre, & Dunn, 2013).

2.7. Proximate analysis

The RS stems and leaves and RS pods were washed under running water and blotted dry and used for the proximate analysis. Briefly, ash was determined as per AOAC (2012, Method 942.05) using temperature controlled furnace at 600 °C. Crude fiber content was performed using in-house method based on AOAC (2012, Method 978.10). Energy and total carbohydrate content was performed using Compendium of method for food analysis (2003). Moisture content was performed as Loss on Drying Moisture in Plants (AOAC, 2012, Method 930.04). Protein content was performed using in-house method TE-CH-012 based on block digestion method (AOAC, 2012, Method 981.10). Total fat content was performed as per acid hydrolysis method (AOAC 2012, Method 922.06) using Soxhlet apparatus with petroleum ether as the extractant.

3. Results and discussion

3.1. Cytotoxicity bioassay-guided selection

Seven RS pod samples including two crude extracts and five fractions were obtained by liquid-liquid serial partitioning. The cytotoxicity effect of RS extract was preliminarily tested against the HCT116 cell line, using neutral red assay. The results in Fig. 2 show the cytotoxicity of each sample at the same concentration (500 μg/ml). The highest percent cytotoxicity was found in the dichloromethane crude extract (91.1 ± 0.5%). The water crude extract showed 31.7% cytotoxicity followed by the extract fractionated from ethyl acetate, dichloromethane, hexane, chloroform, and water, respectively. The highest cytotoxicity in the dichloromethane crude extract may be due to the preferred partially non-polar isothiocyanates that have been found to play major chemopreventive roles in Brassicaceae plants (Björkman et al., 2011). The water crude extract may consist of polar compounds such as phenolics and flavonoids. The serial partitions possessed a negative effect on cytotoxicity. The complex constituents in the dichloromethane crude extract were assumed to exhibit a synergistic effect, leading to the highest cytotoxicity of the whole crude extract. Our result agrees with previous research on the cytotoxicity of RS pod and flower crude extract against HCT116 using the MTT assay (Pocasap et al., 2013). As dichloromethane crude extract was the most cytotoxic against HCT116, it was selected for partial purification and identification of anticancer biomolecules of RS extract in the next experiment.

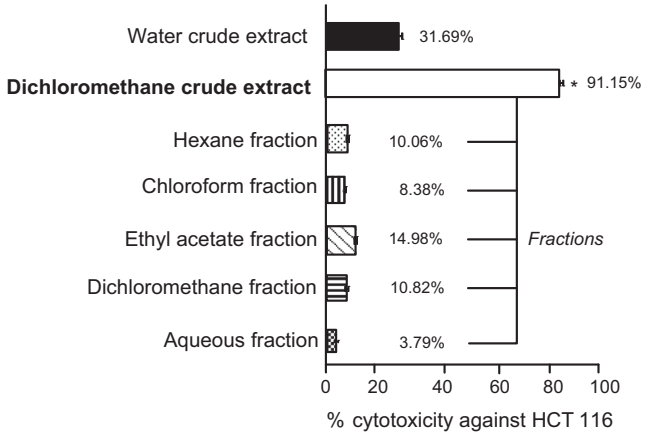


Fig. 2. Cytotoxicity of water crude extract, dichloromethane crude extract, and their respective fractions from Thai rat-tailed radish (RS) pod at the concentration of 500 μg/ml.

Table 1
Percent yield of fractions from dichloromethane crude extract of RS pod from flash column chromatography.

Fraction	Percent yield (w/w)	Characteristic
A	13.8	Yellow wax
B	2.9	Green grease
C	27.4	Dark brownish green
D	43.2	Dark greenish brown
E	3.5	Brown
F	6.2	Greenish brown
G	2.4	Brown
H	4.7	Dark brown
I	3.1	Dark brown

3.2. RS pod fractions from flash column chromatography

RS pod dichloromethane crude extract was partially separated by using flash column chromatography. The yield percentage and physical property of each fraction are presented in Table 1. Four fractions—C, D, E, and F—had high absorbance (range, 0.1–0.9 AU); these then underwent analysis and identification for their respective chemical composition.

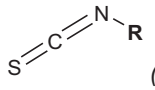
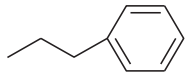
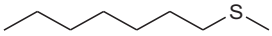
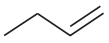
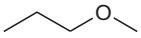
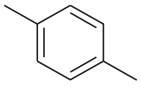
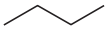
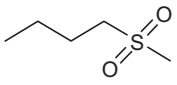
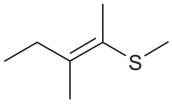
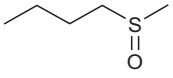
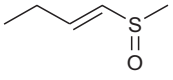
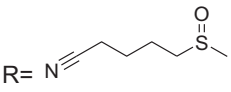
3.3. UHPLC- QToF-MS data of RS pod crude extracts and fractions

RS pod crude extract as well as the fractions obtained from flash column chromatography were analyzed by LC/MS and compared to 5 ITC standard compounds—allyssin, iberin, erysolin, D,L-sulforaphane, and L-sulforaphene. Table 2 presents the results of the unfractionated RS pod (dichloromethane) crude extract and its 4 fractions. In the unfractionated extract, there were 4 glucosinolates, 11 isothiocyanates, 4 indoles, 2 alkaloids, 1 thiocyanates, and 1 oxazolidine as well as a monounsaturated fatty acid, an organic phosphoric acid diamide, a pyranone, a thiocarboxylic acid amide, an alpha amino acid, a benzoic acid ester, and a dialkyl disulphide. In the fractions, there were 3 glucosinolates, 5 isothiocyanates, 1 indole, 4 flavonoids, 2 thiocyanates, and an oxazolidine. The fractions from flash column chromatography showed different detectable compounds from the unfractionated (dichloromethane crude extract) sample. The loss of some compounds might have occurred during elution; however, the compounds detected were annotated and identified along with the level of identification. The level of identification was mostly in level 2 (34%) and level 3 (60%), which were annotated and identified based on accurate mass per charge value. The level 1 identical compounds were sulforaphane (177.029 m/z) and sulforaphene (175.0128 m/z). The respective fragmentation pattern of the isothiocyanates (SCN = R) and $SCNH_2$ was 60.99 m/z (M^+) and 59.99 m/z (M^-). The glucosinolate fragmentation pattern was conjugated glucose ($C_6H_{11}O_5$, 163.06 m/z, M^-), glucose molecule (180.06 m/z, M^-), S-glucose (194.02 m/z, $(M\text{-}H)^-$), and NH_2SO_4 (112.98 m/z, M^-).

The UHPLC/ESI-QToF-MS/MS technique could not directly provide the quantitative determination of identified chemical substances. Therefore, the content of identified compounds of interest was not determined. However, 1 g of dichloromethane crude extract of RS pod was previously reported to compose of 0.112 mg sulforaphane and 2.253 mg sulforaphene by using HPLC quantitative analysis and standard calibration curve (Sangthong & Weerapreeyakul, 2016).

The existence of these annotated compounds was supported by their biological function to plant protection. Brassicaceae plants have their own defense mechanism against pests and pathogens, attributed to the sulphur-containing compounds such as glucosinolates (Bohinc, Ban, Ban, & Trdan, 2012). These glucosinolates and their generative compounds—isothiocyanates and indoles—are also related to health promotion. The current study identified

Table 2
UPLC-ESI-QToF-MS/MS identified compounds in RS pod unfractionated crude extract and its fractions.

Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
Isothiocyanates  *(m/z= 60)*								
R= 	3-Phenylpropyl	$C_{10}H_{11}NS$	177.0612	3.42 (U)	M + FA-H	222.056	Negative	3
R= 	7-Methylthioheptyl	$C_9H_{17}NS_2$	203.0802	3.5 (U)	M + Cl	238.0511	Negative	3
R= 	1-Pentyl	$C_6H_{11}NS$	129.0612	3.3 (F)	M + Br	207.9799	Negative	3
				4.6 (E)	M + H	129.0607	Positive	3
R= 	3-Butenyl	C_5H_7NS	113.0299	0.62 (U)	M + H	113.0303	Positive	2
R= 	3-Methoxypropyl	C_5H_9NOS	131.0408	2.5 (D)	M + H	131.0409	Positive	2
R= 	4-Hydroxybenzyl	C_8H_7NOS	165.0248	3.08 (U)	2 M + FA-H	375.0484	Negative	3
R= 	Allyl	C_4H_5NS	99.0142	0.93 (U)	M + K-2 H	136.9909	Negative	3
R= 	Butyl	C_5H_9NS	115.0455	1.12 (U)	M + H	115.0637	Positive	2
R= 	4-(Methanesulfonyl) butyl (Erysolin)	$C_6H_{11}NO_2S_2$	193.0231	3.86 (U, F)	M-H	193.0229	Negative	2
				7.56 (C)	M + H	193.0256	Positive	2
				0.74 (U,E)	2 M + H	193.0236	Positive	3
				1.45 (U)	2 M + Na + H	193.0231	Positive	3
R= 	4-Methylthio-3-butenyl (Raphasatin)	$C_6H_9NS_2$	159.0176	4.74 (U)	M + H	159.0176	Positive	3
				4.54 (U)	M + 2Na-H	205.0056	Positive	2
R= 	4-(Methylsulphinyl) butyl (Sulforaphane)	$C_6H_{11}NOS_2$	177.02821	4.74 (U, D)	M + H	177.029	Positive	1
				4.24 (U, D)	2 M + H	177.029	Positive	3
R= 	4-(Methylsulphinyl) butane (Sulforaphene)	$C_6H_9NOS_2$	175.0125	4.54 (U, C, D)	M + H	175.0125	Positive	1
				5.73 (U, C)	M + Na	175.0128	Positive	3
				4.17 (C, D, E, F)	2 M + H + Na	175.0147	Positive	3
R= 	Sulforaphane nitrile	$C_6H_{11}NOS$	145.0561	4.46 (U)	M + Cl	180.0255	Negative	3

(*continued on next page*)

Table 2 (*continued*)

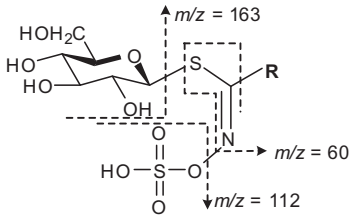
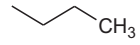
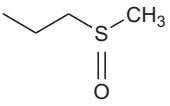
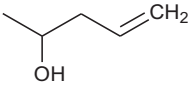
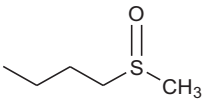
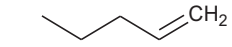
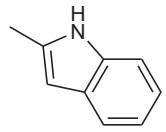
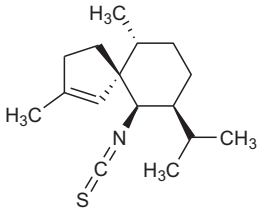
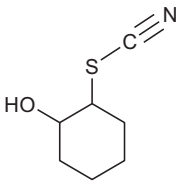
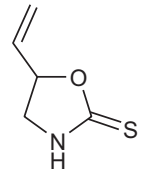
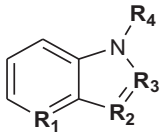
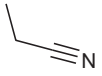
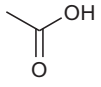
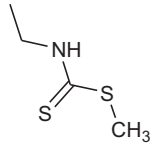
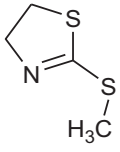
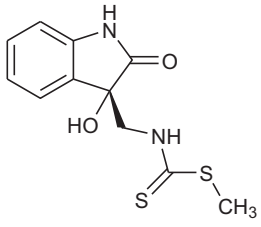
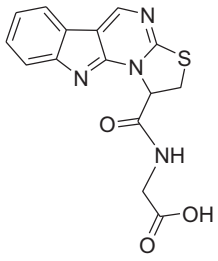
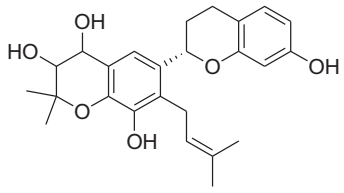
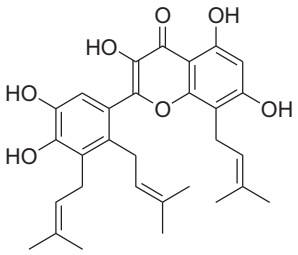
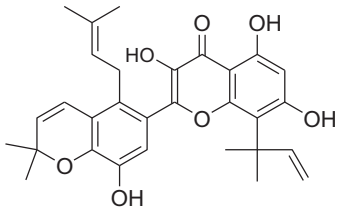
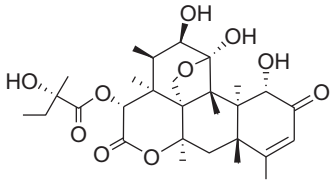
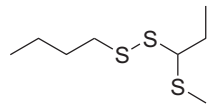
Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
Glucosinolates 								
R= 	Butyl	$C_{11}H_{21}NO_9S_2$	375.0657	0.65 (U)	M + FA-H	420.0625	Negative	3
R= 	3-Methylsulphinylpropyl (Glucoiberin)	$C_{11}H_{21}NO_{10}S_3$	423.0327	0.74 (U)	M + FA-H	468.0313	Negative	3
R= 	2-Hydroxy-4-pentenyl (Gluconapoleiferin)	$C_{12}H_{21}NO_{10}S_2$	403.0606	4.24 (U, F)	M-H	403.0623	Negative	2
R= 	4-Methylsulphinylbutyl (Glucoraphanin)	$C_{12}H_{23}NO_{10}S_3$	437.04841	5.17 (U)	M-H	435.0349	Negative	2
R= 	4-Pentenyl (Glucobrassicanapin)	$C1_2H_{21}NO_9S_2$	387.0657	7.66 (D, F)	M-H	387.0679	Negative	2
R= 	3-Indolylmethyl (Glucobrassicin)	$C_{16}H_{20}N_2O_9S_2$	448.0610	4.53 (C, D)	2 M-H	223.0164	Negative	3
				7.35 (E)	M + Na-2 H	469.2519	Negative	3
Thiocyanate 	Axisothiocyanate-3	$C_{16}H_{25}NS$	263.1708	3.3 (F)	M-H_2O-H	244.0707	Negative	3
	2-Hydroxycyclohexyl thiocyanate	$C_7H_{11}NOS$	157.0561	2.694 (U, F)	M + H	157.0567	Positive	3

Table 2 (*continued*)

Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
Oxazolidine  R1=	2-Oxazolidinethione (Goitrin)	C_5H_7NOS	129.0248	1.45 (U, E)	M + H	129.0248	Positive	2
Indoles 								
R_2 =  R_4 = H	3-Indolecarbinol	C_9H_9NO	147.0684	3.89 (U)	2 M + K	333.1019	Positive	3
R_2=  R_4 = H	3-Indoleacetonitrile	$C_{10}H_8N_2$	156.0682	3.68 (U)	M + Na-2 H	177.0425	Negative	3
R_3=  R_4 = H	2-Indolecarboxylic acid	$C_9H_7NO_2$	161.0477	2.61 (E)	M + H	161.052	Positive	2
R_2 =OCH_3	Methoxybrassinin	$C_{12}H_{14}N_2OS_2$	266.0547	2.8 (U)	M + Hac-H	325.0663	Negative	3
				3.71 (U)	M + Na-2 H	287.032	Negative	3
 R_4=								
 R_2=	N-Methoxyspirobrassinol	$C_{12}H_{14}N_2O_2S_2$	282.0496	3.2 (U)	2 M-H	563.0938	Negative	3
R_4 = OCH_3								
Alkaloids 	(-)-Dioxibrassinin	$C_{12}H_{14}N_2O_2S_2$		2.15 (U)	M + Hac-H	327.0453	Negative	3

(*continued on next page*)

Table 2 (*continued*)

Structure	R-name (Common name)	Formula	Calculated *m/z* (M)	RT (min) (Extract fraction)	Adduct type	m/z value	Ion mode	Level of identification
	Cabbage identification factor 2	$C_{15}H_{12}N_4O_3S$	328.06301	8.22 (U)	M + 2Na-H	373.0339	Positive	3
Flavonoids 	Broussoflavan A	$C_{25}H_{30}O_6$	426.2042	9.76 (C)	M-H	424.1779	Negative	3
	Broussoflavonol G	$C_{30}H_{34}O_7$	506.2304	7.38 (E)	M + H	504.2147	Positive	3
	Broussoflavonol D	$C_{30}H_{32}O_7$	504.2148	7.75 (E, F)	M-H	504.2171	Negative	3
	Glaucarubinone	$C_{25}H_{34}O_{10}$	494.2152	5.86 (F)	M-H	494.2137	Negative	3
Dialkyl disulphide 	Butyl 1-(methylthio) propyl disulphide	$C_8H_{18}S_3$	210.0571	4.25 (U)	M + $2Na^{2+}$	128.0172	Positive	3

Note: U = unfractionated RS pod extract; C, D, E, F = RS pod fractions.
Adduct type: FA = formic acid; Hac = acetic acid; Na = sodium; Cl = chlorine; K = potassium.

more compounds in *Raphanus sativus* var. *caudatus* Alef than previously detected by GC–MS analysis (Pocasap et al., 2013; Songsak & Lockwood, 2002). Using LC-MS provided detection of thermal degradation compounds undetected in any previous GC–MS analysis. Sulforaphane—the well-known chemopreventive compound and its precursor, glucoraphanin as well as the analogue of erysolin and sulforaphane nitrile—were also detected. Of note, 3 phytoalexins, found in the recent study—(-)-dioxibrassinin and 2 indoles, and a methoxybrassinin have also been previously found in cabbage (*Brassica oleracea* var. *capitata*) (Monde et al., 2003) and 1 phytoalexin stress metabolite N-methoxyspirobrassinol has been found in Japanese radish daikon (*Raphanus sativus* var. *hortensis*) (Monde, Takasugi, & Shirata, 1995). Phytoalexins are produced by plants as stress metabolites as part of the antifungal defensive

mechanism (Monde et al., 2003). In humans, phytoalexins also show anti-proliferative and cancer chemopreventive activity (Mezencev, Mojzis, Pilatova, & Kutschy, 2003). Indole-3-acetronitrile and indole-3-carbinol are the thermal breakdown products of glucobrassicin widely reported as chemopreventive compounds (Chevolleau, Debrauwer, Boyer, & Tulliez, 2002).

Glucosinolates are the bioactive compounds commonly found in crucifers associated with cancer protection. Glucosinolates share a common sulphur-linked β-D-glucopyranose structure with different side chains. The alkylthioalkyl side-chain contains a sulphur group, whereas, the aromatic side-chain contains a phenethyl group. Glucosinolates are not bioactive until they are enzymatically catalyzed to a chemically related isothiocyanates (ITCs) by the endogenous enzyme myrosinase. Myrosinase catalyses hydrolysis of the β-thioglucoside linkage. The resultant aglycones then undergo non-enzymatic, intramolecular (Lossen) rearrangement to yield isothiocyanates, thiocyanates, nitriles or epithionitriles (Burow, Bergner, Gershenzon, & Wittstock, 2007). The structural difference of the glucosinolate is conferred to that of the cognate ITC, e.g., glucoraphanin to sulforaphane, and sinigrin to allyl ITC.

The pharmacophore of isothiocyanates (ITCs) structure was the significance electrophilic property of central carbon atom in the isothiocyanate (–N=C=S) group which attacks to the cellular nucleophilic targets such as DNA bases at nitrogen- and oxygen-based nucleophiles, and GSH at sulphur- (Joozdani, Yari, Joozdan, & Nafisi, 2015; Kolm, Danielson, Zhang, Talalay, & Mannervik, 1995). The conjugation of ITCs (electrophile moiety) with the intracellular antioxidant glutathione (GSH, the nucleophilic target) leads to the reactive oxygen species (ROS)-mediated apoptosis (Sestili & Fimognari, 2015). Moreover, this –N=C=S group acts as a pro-oxidant to generate ROS leading to the ROS-mediated apoptotic cell death. The pro-oxidant effect of ITCs was occurred by the spontaneous hydrolysis of –N=C=S moiety producing hydrogen peroxide or superoxide anion radicals (Sestili & Fimognari, 2015).

ITCs have been shown to block chemical carcinogenesis and are identified as specific inducers of Phase II enzymes such as glutathione S-transferases (GSTs) and quinone reductase (QR; NAD(P)H:(quinone-acceptor) oxidoreductase) in several mouse tissues without the induction of aryl hydrocarbon receptor dependent cytochromes P-450 (phase I enzymes) (Zhang, Talalay, Cho, & Posner, 1992). The chirality, state of oxidation of sulphur of the thiomethyl group, and number of methylene bridging groups in ITCs structure are important for inducer potencies in murine hepatoma cells. The chirality of the sulfoxide (such as sulforaphane; SF) does not affect inducer potency, since isolated (R)-sulforaphane from broccoli and synthetic racemic (R,S)-sulforaphane gave closely similar concentration of a compound required to double the QR specific activity in Hepa lclc7 murine hepatoma cells and were relatively noncytotoxic (Zhang et al., 1992). The presence of oxygen on sulphur enhanced potency. Oxidation of the side-chain sulphide (erucin) to sulfoxide (SF) or to sulfone (erysolin) enhanced inducer potency. The corresponding sulphide (erucin) was about one-third as active as SF on inducing of QR, whereas sulfoxide (SF) and the corresponding sulfone (erysolin) were equipotent (Zhang et al., 1992). Replacement of the S=O by C=O produced an analogue that was equally potent to SF. And compounds with 4 or 5 methylene groups in the bridge linking CH_3S– and –N=C=S were more potent than those with 3 methylene groups (Zhang et al., 1992). However, the rank of biological activities of SF analogues could differ depending on the type of biological activity assessed (Kim, Kim, & Lim, 2010). Oxidative stress may be linked to apoptosis and cell cycle repression in various cell lines. Oxygen attached to sulphur was reported to potentiate the apoptosis-inducing capability of SF analogues by increasing ROS generation. Growth inhibitory effects of SF analogues containing oxidized sulphur in colon cancer cell lines were more potent antiproliferative agents than analogues containing non-oxidized sulphur. SF analogues containing four atoms of carbon between oxidized sulphur and the –N=C=S groups were slightly more potent compared with those containing five atoms of carbon. The number of carbon separating the sulphur atom from the –N=C=S groups was a less important factor determining antiproliferative potency of SF analogues (Kim et al., 2010).

Indoles compounds such as indole-3-carbinol has been undergoing clinical trial for anticancer therapy because it suppressed tumour cell proliferation by targeting a wide spectrum of signaling pathways governing hormonal homeostasis, cell-cycle progression, and cell proliferation and survival (Weng, Tsai, Kulp, & Chen, 2008). Vascular targeting agents which inhibit tubulin-microtubule protein system of the endothelial cells lining tumour microvessels have been a selective therapeutic target for anti-cancer agents. These tubulin polymerization inhibitors were characterized based on the presence of an indole nucleus (Brancale & Silvestri, 2007; Patil, Patil, Beaman, & Patil, 2016). Indole ring acts as a pivotal pharmacophore for being potent tubulin polymerization inhibitors to majorly bind at the same active binding site as colchicine (Brancale & Silvestri, 2007; MacDonough et al., 2013). However, some modification on chemical structure led to alteration of molecular targets. Because some compounds did not bind at either the colchicine or vincristine colchicines-binding site suggesting a different binding site of indole derivatives on tubulin. The rational modifications at indole ring to improved potency have been the focus of many studies with variety information of structure–activity relationship of indole derivatives (Brancale & Silvestri, 2007; MacDonough et al., 2013).

Interestingly, isoalliin and butyl 1-(methylthio)propyl disulphide were identified in the active RS pod DCM crude extract. These sulphur-containing compounds are not commonly found in Brassicaceae. Isoalliin or *S-trans*-prop-1-enyl cysteine sulphoxide causes the characteristic aroma of onion (Jones et al., 2004) and butyl 1-(methylthio)propyl disulphide is found in the onion family, *Allium tuberosum* (Chinese chives) (Yannai, 2012). These organosulphur compounds have a structure related to dimethyl-trisulphide found in the RS pod and flower based on using the GC–MS technique (Pocasap et al., 2013). In addition to the sulphur-containing compounds (e.g., glucosinolates, isothiocyanates), the common plant constituents such as indoles and flavonoids (i.e., Broussoflavonol B) were also found in the RS pod DCM crude extract. Broussoflavonol B is a flavonoid with a reported chemopreventive effect; achieved by inducing cell cycle arrest, apoptosis, and acting as a potent growth inhibitor in breast cancer MDA-MB-231 cells (Guo et al., 2013).

Plant-derived isothiocyanates—allyl isothiocanate, iberverin, iberin, erucin, and sulforaphane were reported to induce phase II detoxification enzymes in rats (Munday & Munday, 2004). Sulforaphane inhibits vestibular schwannoma growth *in vivo* by reducing 27.2% of tumour volume (Kim et al., 2016). Allyl isothiocyanate significantly induces apoptosis and decreases mitosis phase of human prostate cancer PC-3 xenografts *in vivo* (Srivastava et al., 2003). Oral administration of uncooked Brussels sprouts in a juice or powder forms was enhanced levels of apoptosis and aberrant crypt foci in rat colonic mucosal crypts (Smith et al., 2003). For the human clinical trial, broccoli sprout containing

Table 3
Cytotoxicity of four identified compounds in RS pod extract against HCT116 cell lines.

Compounds	IC_{50} value (μg/ml)	IC_{50} value (μM)
D,L-Sulforaphane	4.94 ± 0.16	27.87 ± 0.90
L-Sulforaphene	8.60 ± 0.12	49.06 ± 0.68
Erysolin	5.28 ± 0.02	27.34 ± 0.12
Iberin	6.00 ± 0.07	36.74 ± 0.40

Table 4
The proximate analysis of *Raphanus sativus* var. *caudatus* Alef.

Part	Energy (kcal/100 g)	Protein (g/100 g)	Total carbohydrate (g/100 g)	Total fat (g/100 g)	Crude fiber (g/100 g)	Ash (g/100 g)	Moisture (g/100 g)
Stem and leaf	102.9	4.02	13.73	3.5	0.55	2.21	76.5
Pod	38.9	2.16	0.59	3.1	0.59	0.69	93.5

glucosinolates and isothiocyanates successfully passed through phase I study with no adverse effects in healthy volunteers (Shapiro et al., 2006). Indole-3-carbinol was previously reported to exert chemopreventive effect by changing enzyme activity and steroid metabolism (Reed et al., 2005).

Among compounds identified, no anticancer activity has been reported for oxazolidines, while isothiocyanates, glucosinolates and flavonoids were largely identified in cruciferous vegetables with clinically relevant anticarcinogenic activities. D,L-sulforaphane, L-sulforaphene, erysolin, and iberin (found as glucoiberin in the pod extract) were determined for their cytotoxicity against HCT116 (Table 3). Erysolin exerted the highest cytotoxic (27.34 μM) followed by D,L-sulforaphane (27.87 μM), L-sulforaphene (49.06 μM), and iberin (36.74 μM), respectively. The isothiocyanates (ITCs) were found with more numbers of identified compounds than the other groups. The majority of the anticancer activity of RS pod is likely contributed to a chemical entity, the isothiocyanates.

Thiocyanates and oxazolidine–glucosinolates products, 5-vinyloxazolidine-2-thione (goitrin), were also found in the crude RS extract and in fraction E, which accounted for the lowest % yield (3.50%w/w) among the 4 fractions. The goitrogenic property of the thiocyanate ion occurs via suppression of iodine uptake, however, this effect can be compensated by sufficient iodine intake (Kostogrys, Pisulewski, Pecio, & Filipiak-Florkiewicz, 2010). One possible case of a goitrogenic effect occurred from consumption of thiocyanate and goitrin in 10 kg cauliflower per day reported of a man with extremely low iodine intake (McMillan, Spinks, & Fenwick, 1986). The formation of oxazolidine-2-thione corresponded to hydrolysis of glucosinolates consisting of R chain with hydroxyl at the C-2 position. Oxazolidine-2-thione increases goiter by reducing the production of thyroid hormones (Verhoeven, Verhagen, Goldbohm, Van Den Brandt, & Van Poppel, 1997); however, the goitrogenic effect was inversely affected by Brassica consumption (Mithen, 2001). The conversion of glucosinolates into thiocyanate ion requires two plant enzymes—myrosinase and specific thiocyanate-forming protein (TFP) (Wittstock & Burow, 2007). Since TFP is thermally-degraded, the cooking process could reduce the anti-thyroid effect of reactive thiocyanates in Brussels sprouts. A previous report showed no effect on thyroid function after 4 weeks (150 g daily) of consuming cooked Brussels sprouts with normal diet (McMillan et al., 1986).

3.4. Nutritive composition of RS

Table 4 showed that the percentage of energy, protein, carbohydrate, and ash were higher in RS stem and leaf (102.9 kcal, 4.02, 13.73, and 2.21%, respectively) than RS pods (38.9 kcal, 2.16, 0.59, and 0.69%, respectively). While the percentages of total fat and crude fiber were similar in both parts of samples (stem and leaf—3.5, and 0.55; pod—3.1, and 0.59%). Only moisture content was found to be higher in RS pod (93.5%) than RS stem and leaf (76.5%).

The micronutrients in RS were previously determined by Nutritional Department of Thailand Food and Drug Administration. The vitamin and minerals in RS were 44 mg of calcium, 35 mg of phosphorus, 1.8 mg of iron, 777 IU of vitamin A, 0.11 mg of vitamin B1, 0.05 mg of vitamin B2, and 1.10 mg of vitamin B3 or niacin, and 125 mg vitamin C (Paisukshantiwattana, 2013). The health benefits of RS were reported as the traditional uses by Chiang Mai University, Thailand. The traditional uses of RS pods and leaves for health were for appetite enhancer, anti-flatulence, and believe to clear urethral stone. RS flowers were also used for facilitating biliary excretion (Department of Agriculture Extension., 2005).

4. Conclusion

The current study demonstrated the potential cytotoxic activity of pod DCM crude extract of RS pod against a colon cancer cell line (HCT116). The compounds previously reported as possible cytotoxicity contributors were identified using LC-MS while 2 additional compounds (isoalliin and butyl 1-(methylthio)propyl disulphide) were detected in the genus Brassica for the first time. The information on phytoconstituents in the RS pod extract will be useful for laboratory-scale standardization and botanical authentication of this vegetable in an approach for using it as functional food. The development of dietary supplements containing RS pod could be expanded based on information available on the chemical constituents that can be used for quality control and quality assurance of dietary supplement products containing RS pod extract.

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