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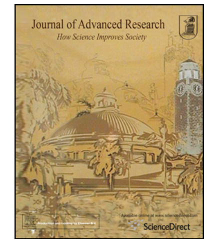

ORIGINAL ARTICLE

Sulforaphane composition, cytotoxic and antioxidant activity of crucifer vegetables

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KEYWORDS

Anticancer;
Antioxidant;
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GC-MS;
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Abstract Sulphur compounds in sulphur rich food have been shown to significantly reduce the risk of cancer development. One such compound is sulforaphane (SF), a cancer chemopreventive agent identified in broccoli (*F. cruciferae*). In this study, SF content was assessed in extracts of several crucifer vegetables including broccoli, brussels sprout, green cabbage, red cabbage, Chinese kale and turnip, in parallel with anticancer and antioxidant activity. Among tested crucifers, cabbage demonstrated a pronounced anticancer effect against A-549 lung cancer cells, with an IC_{50} value of 38 μg mL^{-1}, and correlated with high SF levels at 540 μg g^{-1}. Except for red cabbage and kale, crucifer extracts displayed moderate to weak activity in scavenging 2,2-diphenyl-1-picrylhydrazyl (DDPH) free radicals relative to vitamin E standard.

Introduction

In recent years, a growing body of epidemiological evidence has emerged pointing to the low incidence of some types of tumours in populations, or sections thereof, whose diet includes large quantities of certain vegetables. Following this, interest in food with chemoprevention properties has been steadily increasing. Cruciferous vegetables in particular have attracted a great deal of attention, since they are rich in aromatic and aliphatic isothiocyanates [1,2]. Of these, sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane] (Fig. 1), which has been identified in broccoli as a product of enzymatic or acid hydrolysis of the corresponding ω-(methylsulfinyl)-alkyl-glucosinolate (glucoraphanin), has recently aroused interest as a possible cancer-preventive agent [3,4]. This isothiocyanate can decrease the risk of developing different cancers such as breast cancer [5], gastric cancer [6] and skin cancer [7]. The chemoprotective effect of sulforaphane was thought to be due solely to its ability to behave as an inducer of phase II detoxification enzymes [8]. In subsequent research, however, sulforaphane was also shown to inhibit the CYP2EI isoenzyme of the cytochrome P450, thus emerging as an inhibitor of phase I enzymes [9]. The information in the current literature regarding sulforaphane has focused on its effects in broccoli, with little information on its presence in other cruciferous vegetables [10]. Other common vegetables in this plant family include

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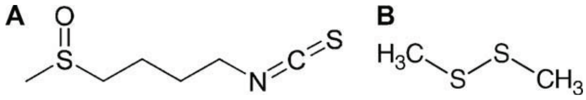


Figure 1 Structural formula of sulforaphane (A) and dimethyldisulphide (B).

brussels sprout, cabbage, radish, Chinese kale, mustard, turnip, and cauliflower. Given the increasing interest in sulforaphane for cancer chemoprevention, we set out to screen other cruciferous vegetables for their sulforaphane content. Further, as there has been little information in the literature on their extract bioactivities relative to that of broccoli, we decided to assess their anticancer effects against human lung cancer cell line A-549 and antioxidant capacity in scavenging 2,2-diphenyl-1-picrylhydrazyl (DDPH) free radicals. Results from this study reveal further evidence for correlation between SF content and the potent cancer-preventive effects of crucifer vegetables.

Material and methods

Chemicals and plant material

Samples of crucifer vegetables including broccoli florets (*Brassica oleracea* var. *italica*), green cabbage (*Brassica oleracea L.* var. *capitata*), brussels sprout (*Brassica oleracea L.* var. *gemmifera*), Chinese kale (*Brassica oleracea* var. *acephala*), turnip leaves (*Brassica rapa* var. *rapa*) and red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*) were bought in a retail outlet in Louisville, KY, USA. After purchasing, vegetables were processed as quickly as possible. All chemical reagents were of analytical grade and all solvents were of GC and HPLC grade. R-sulforaphane was purchased from LKT Laboratories (St. Paul, MN, USA). DDPH and α-tocopherol (vitamin E) standards were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of crucifer aqueous and organic extracts for biological activity

Crucifer aqueous extracts were prepared according to the method of Bertelli et al. [11] with few modifications. Briefly, plant material was ground to a fine homogenous powder and kept at −80 °C with liquid nitrogen. Ten grams of powdered material was left to autolyse in 10 mL de-ionised water at room temperature for 12 h to allow complete hydrolysis of the sulphur glycosides. Aqueous extracts were then centrifuged at 5000*g* for 15 min to discard the leaves' mark. Respective plant extracts were obtained after lyophilisation. Dried aqueous extracts were then dissolved in PBS buffer pH 7.0, filter sterilised through 0.2 μm Milix filter (Millipore, MA, USA) to give stock aqueous solutions of the aqueous extract at a concentration of 50 mg mL^{-1}.

For preparation of organic extracts, 1 mL of plant aqueous extract prepared as above was fractionated with chloroform (2 × 1 mL) at room temperature to extract sulphur aglycone compounds. The chloroform layer was evaporated in a speed-Vac (Vacufuge TM, Eppendorf, Westbury, NY, USA) and residue was resuspended in 1 mL PBS buffer, stored at –20 °C until further testing.

GC-MS quantification of sulforaphane in crucifer extracts

Concentration of sulforaphane in crucifer extracts was determined using the method reported by Matusheski et al. [12] with few modifications. About 1 mL plant aqueous extract prepared as above was extracted with chloroform (2 × 1 mL) twice at room temperature. The chloroform layers were dried over anhydrous sodium sulfate and stored at −20 °C until processed. About 1 μL of the chloroform extract was injected on a GC (Thermoquest) connected to a PolarisQ ion trap MS (ThermoFinnigan, Austin, TX, USA). The column was 0.15 mm i.d. × 50 m fused silica open-tubular, coated with 0.2 μm BPX-5 (5% phenylmethylphenylsiloxane). Injections were made in the splitless mode for 30 s, and the gas chromatograph was operated under the following conditions: injector 220 °C, column oven 40 °C for 3 min, then programmed at a rate of 12 °C/min to 180 °C, kept at 180 °C for 5 min and finally ramped at a rate of 40 °C/min to 250° C, which was then maintained for 2 min. He carrier gas linear flow velocity was maintained at 30 cm s^{-1}. The transfer line and ion-source temperature were adjusted at 230 °C and 190 °C, respectively. The ion trap mass spectrometer was operated in the electron ionisation mode at 70 eV and a source temperature of 180 °C. Volatile components were identified by mass spectrum matching to the EPA/NIH database and with authentic standards. Peaks were quantified by selected abundant fragments (*m*/*z*), calculated using MET-IDEA software to extract peak areas of individual ions characteristic of each component. For SF absolute quantification, an external standard calibration curve of sulforaphane SF (0.1–10 mM) was prepared and extracted under exact conditions.

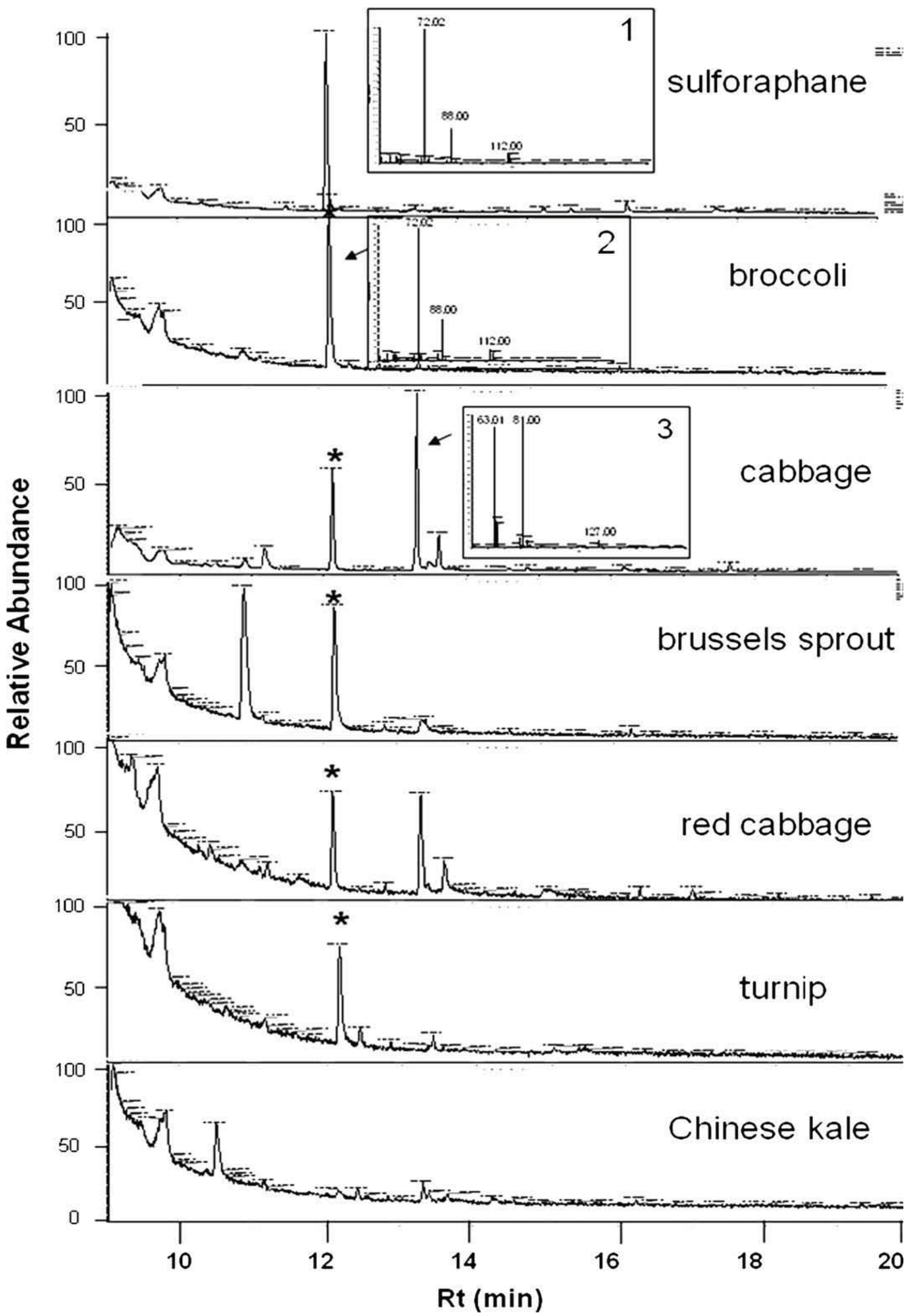
Anticancer activity

The human lung cancer A-549 cell line (ATCC#CCL-185) was obtained from the American type culture collection. Cells were maintained at 37 °C in a 5% CO_2 atmosphere. A-549 cells line were grown in RPMI 1640 medium (MP Biomedicals Inc., Irvine, CA, USA) containing 10% foetal bovine serum (FBS, Hyclone, Logan, UT, USA) as well as 0.2% glucose, 2 mM glutamine, 500 μg mL^{-1} streptomycin, and 500 IU mL^{-1} penicillin.

Exponentially growing cells were plated in 96-well microplates (Costar, Corning Inc., USA) at a density of 3×10^3 cells per well in 100 μL of culture medium, and were allowed to adhere for 16 h before treatment. Increasing concentrations of plant extract in ethanol were then added (100 μL per well). Final concentration of ethanol in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Sulforaphane was used as a positive control at a concentration range from 0.5 to 100 μM. The cells were incubated for 48 h in the presence and absence of the extract. Cytotoxicity was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) according to the vendor's protocol (Promega, Madison, WI, USA). Absorbance was measured on automated 96-well microplate SpectraMax M5 (Molecular devices, CA, USA) at wavelength 570 nm. Cytotoxicity here is expressed as the concentration of plant extract inhibiting cell growth by 50% relative to cells incubated in the presence of 0.5% ethanol (IC_{50} value). Each measurement was performed in triplicate.

Antioxidant activity

Antioxidant activity was assayed using a modified quantitative DDPH assay [13]. The solution of DDPH was prepared with HPLC grade methanol and DDPH (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 0.004%. Lyophilised extracts were dissolved in water at a concentration of 0.1, 1, and 10 mg mL^{-1}, with 5 μL of each test solution added to 100 μL



Figrue 2 GC-MS chromatograms for purified sulforaphane (SF) and sulforaphane peak identified in crucifer extracts. Peaks highlighted with an asterisk represent that of SF. Insets (1) and (2) represent MS spectra of synthetic sulforaphane and isolated sulforaphane in crucifers, whereas (3) illustrates MS spectra of dimethyldisulphide in cabbage. Chromatographic conditions are described under "Materials and methods".

DDPH solution. Blank samples were run using only 99.9% methanol. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 550 nm. Vitamin E (Sigma–Aldrich) was used as positive control at a concentration of 0.1, 1, and 10 mg mL^{-1}. Inhibition of free radicals by DPPH in percent ($I\%$) was calculated according to: $I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the extract), and A_{sample} is the absorbance of the extract. Measurements were carried out in triplicate.

Results and discussion

Sulforaphane content (SF) in crucifer as determined by GC-MS

The GC-MS technique developed by Matusheski et al. [12] was used to quantify sulforaphane in crucifer extracts and to identify other sulphur compounds (Fig. 2). SF (rt 12.15 min) was detected in all tested crucifers except in Chinese kale. The highest level was found in cabbage at a concentration of 540 μg g^{-1} fresh weight, followed by broccoli and brussels sprout at a concentration of 220 and 120 μg g^{-1}, respectively. Lower levels of SF were detected in turnip and red cabbage, at concentrations of 60 and 48 μg g^{-1}, respectively. It should be noted that SF level in broccoli is in agreement with literature data [11]. Green and red cabbage extracts showed, in addition to SF, another major peak (rt 13.31 min) identified as dimethyldisulphide, likely to be an artefact of S-methyl-L-cysteine degradation. Dimethyldisulphide was identified as the predominant volatile compound generated by thermal degradation of both S-methyl cysteine and its sulfoxide in *Brassica* and *Allium* vegetables [14]. Another unknown volatile compound (rt 10.89 min) was detected in brussels sprout's extract (Fig. 2).

Anticancer activity of crucifer aqueous and organic extracts

To correlate between SF composition and cytotoxic effects for crucifer vegetables, anticancer activity was assessed for investigated plant extracts. Anticancer activity of crucifer extracts was assessed against A-549 cell line (human lung carcinoma) along with sulforaphane standard and the antitumour agent sodium selenite as positive control [15]. The cytotoxic activity data are presented in Table 1. Green cabbage extract exhibited a pronounced cytotoxic effect (37% cell survival at 500 μg mL^{-1}) comparable to that of sulforaphane, with 31% cell survival at the same concentration. In contrast, red cabbage extract enriched in anthocyanins exhibited the least cytotoxic activity (73% cell survival) at 500 μg mL^{-1}, implying its weak effect against human A-549 cells. Similarly, anthocyanins present in apple aqueous extracts demonstrated weak cytotoxic effect against human leukemic HL-60 cells [16]. The results in Table 1 indicate that broccoli, Chinese kale and turnip extracts exhibit moderate cell growth inhibition, *ca*. 60–70% cell survival at 500 μg mL^{-1}.

To further confirm whether sulphur compounds in crucifer aqueous extract can account for its cytotoxicity against A-549 cells, organic extracts enriched in SF, as analysed by GC-MS (Fig. 2), were assessed for their cytotoxic effect against A-549 cell line. A positive relationship appears to exist between SF content and cytotoxicity in case of green cabbage and broccoli (Fig. 3). In contrast, turnip and red cabbage extracts exhibiting the lowest SF levels demonstrated the least cytotoxic effects (Fig. 3).

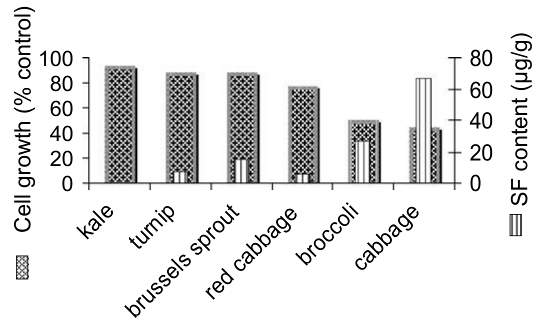


Figure 3 Anticancer activity against A-549 (human lung carcinoma) and sulforaphane (SF) content of crucifer organic extracts. Results are expressed for cytotoxicity as cell growth (% of control) on the Y_1 axis and for SF content in μg g^{-1} as displayed on the Y_2 axis.

Table 1 Cytotoxicity of crucifer vegetables aqueous extracts on A-549 growth.

Extract/compound	Cytotoxicity (cell proliferation as % of control) (μg mL^{-1})			
	0.5	5	50	500
Green cabbage	92	76	65	**37**
Broccoli	76	68	66	61
Brussels sprout	88	75	64	72
Chinese kale	90	71	57	59
Turnip	84	72	68	65
Red cabbage	88	66	74	73
Sulforaphane	99	92	67	**31**

Cells grown to 75% confluence in 96-well plates were incubated in the presence of the extracts for 72 h. Cell viability was measured by the MTT assay and is expressed as a percentage of cell viability without extract. The extracts were tested at 0.5, 5, 50, and 500 μg mL^{-1}. Cytotoxicity values greater than 30% are shown in bold. The results are the average of three independent experiments with less than 10% standard deviation.

Antioxidant activity of crucifer aqueous extracts

Oxidative stress may initiate molecular events in the cancer process, and reduction of oxidative stress may protect against carcinogenesis [17]. Crucifers contain numerous antioxidant substances that could potentially induce antioxidant enzymes, and combinations of these may protect against carcinogenesis [18]. Crucifer aqueous extracts were assessed for their capacity to scavenge DDPH free radicals along with vitamin E as positive control. The antioxidant activity data in terms of free radical inhibition are presented in Table 2. Except for red cabbage and Chinese kale, with an inhibition of 73% and 54%, respectively, other crucifer extracts displayed moderate to weak capacity in scavenging DDPH radicals at a dose of 10 mg mL^{-1}. These results are in agreement with previous reports showing that Chinese kale exhibits the highest antioxidant activity in quenching ABTS free radicals relative to broccoli, brussels sprout and cauliflower. Chinese kale con-

Table 2 Antioxidant activity assayed by DPPH test of crucifer extracts (expressed as % bleaching) in term of free radical inhibition.

Extract/compound	(mg mL^{-1})		
	0.1	1	10
Green cabbage	–	–	3
Broccoli	–	2	21
Brussels sprout	–	1	23
Chinese kale	7	9	**45**
Turnip	1	2	25
Red cabbage	4	20	**73**
Sulforaphane	–	1	2
Vitamin E	17	**82**	**85**

Extracts were tested at 0.1, 1, and 10 mg mL^{-1}. Inhibition values greater than 40% are shown in bold. The results are the average of three independent experiments with less than 10% standard deviation. (–) Indicates inhibition less than 1%.

tains the highest levels of several antioxidising agents, including vitamin C, carotenoids, and polyphenols [19,20]. The pronounced effect in red cabbage could be attributed to the prevalence of anthocyanins in its extract. Several highly conjugated anthocyanins were identified in red cabbage [21], with potential antioxidant activities [22]. Broccoli is reported to provide moderate antioxidant capacity, likely attributed to tocopherols and flavonoids [23]. Results from SF inactivity in scavenging DDPH free radicals (Table 2) suggest that it does not contribute to the antioxidant capacity of broccoli and other crucifers.

Conclusion

This study indicates that green cabbage has potential as a dietary supplement in cancer chemoprevention and helps draw further evidence for the role of SF in cancer prevention in other members of family *Cruciferae*. More research is still needed to help identify other bioactive sulphur compounds in crucifers.

Conflict of interest statement

The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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