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Anticancer, antioxidant activities and GC-MS analysis of glucosinolates in two cultivars of broccoli

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Abstract

In the present study, two cultivars of broccoli i.e. *Palam smridhi* (PS) and *Palam vichitra* (PV) were fingerprinted based upon the presence of glucosinolate (GLS) hydrolytic products by gas chromatography-mass spectroscopy. The extracts were analysed for the antioxidant activity using DPPH, DNA nicking assays and cytotoxic effect using different cell lines *viz.* ovary (OVCAR-5), breast (MCF-7), colon (Colo-205) and prostate (PC-3) by SRB assay. The GC-MS data of two varieties showed a great difference in the profile of GLS. The dichloromethane extract of PS showed the presence of allylisothiocyanates, 3-butenylisothiocyanates, 3-methylthiopropylisothiocyanates, sulforaphane and PV showed the presence allyl isothiocyanates, 3-butenylisothiocyanates, 3-methylthiopropyl-isothiocyanates, iberin, and sulforaphane. It was observed that the sulforaphane was present both in PS and PV. The iberin is present only in PV. The head space analysis of PS showed the presence of 3-butenylisothiocyanates and PV showed the presence of allylisothiocyanates and 3-butenylisothiocyanates.

Keywords: Broccoli; Isothiocyanates; Head space analysis; Gas chromatography; mass spectroscopy; DPPH assay; Plasmid nicking Assay; Sulforhodamine B assay

Introduction

Broccoli is classified under the *italica* cultivar group of *Brassica oleracea* L. The glucosinolates (GLS) are a large group of sulphur-containing secondary plant metabolites, which occur in all the economically important varieties of *Brassica*. The consumption of vegetables especially crucifers, reduces the risk of developing cancer (Zhang, et al., 1992). More than 120 different glucosinolates have been identified till date (Chen and Andreasson, 2001), all of which share a common of β-D-thioglucose, a sulphonated oxime moiety with a variable

side-chain derived from methionine, tryptophan or phenylalanine. Broccoli is very popular due to its high content of dithiolthiones and isothiocyanates; these are organosulfur compounds that have been shown to increase the activity of enzymes involved in the detoxification of carcinogens and other foreign compounds (Talalay and Fahey, 2001; Steinmetz and Potter, 1996). Glucosinolates have been reported to have anti-cancer properties and block the initiation of tumours in a variety of tissues e.g. liver, bladder, pancreas, colon and small intestine (Kim and Milner, 2005). They generally consist of a sugar entity, β-D-thioglucose, with an ester bound to an organic aglycone that is an alkyl group yielding isothiocyanate, nitrile, thiocyanate or a similar compound upon hydrolysis. These compounds often contribute to bitter, hot taste of the condiments (mustard, horseradish).

The crucifer's vegetables reduce the risk of bladder cancer in men (Michaud, et al., 1999). The sulforaphane inhibited prolifer-ation of cultured PC-3 human prostate cancer cells by apoptosis induction (Singh, et al., 2004). SFN-induced apoptosis was associated with up-regulation of Bax, down-regulation of Bcl-2 and activation of caspases-3, -9 and -8. The isothiocyanates (4-methysulfinylbutyl and 7-methylsulphinylheptylisothiocyanates) derived from broccoli and *Rorripa nasturtium aquaticum* (watercress) inhibit metalloproteinase 9 activities and also suppress the invasive potential of human MDA-MB-231 breast cancer cells *in vitro* (Rose, et al., 2005). Keeping this in mind, the fingerprinting of two cultivars of broccoli *viz.* PS and PV on the basis of glucosinolates has been done using GC-MS. The extracts obtained were evaluated for their antioxidative potential by DPPH and DNA nicking methods. Further, cytotoxic studies were performed to determine their effect against different human cancer cell lines employing SRB dye assay.

Materials and Methods

Plant material

The seeds of the two varieties of broccoli *viz.* PS and PS were procured from Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, (CSKHPV) Palampur. Seeds were surface sterilized by rinsing in 70% ethanol for one min (v/v) thereafter the seeds were treated with 0.7% sodium hypochlorite (w/v) for 15 min and allowed to germinate in seed germinator (Narang Scientific work model NSW-191-192) for three days with photoperiod of 16 hours light and 8 hour dark at 22°C temperature. Sprouts were gently collected from the trays and homogenized using grinder.

Extraction procedure

Extraction of glucosinolate hydrolytic products was done using the reported method with slight modifications (Liang, et al., 2006). Freshly harvested broccoli sprouts were homogenized in water. The mixture was left to autolyze at room temperature for 30 min. After autolyzing, the meal was extracted twice with dichloromethane (DCM), which was combined and salted with 2.5g anhydrous sodium sulphate. The DCM fraction was dried at 30°C under vacuum on rotary evaporator. The residue was redissolved in DCM and filtered through a 0.22 µm membrane filter prior to injection into GC-MS**.**

Gas chromatography-mass spectrometry analysis

The GC-MS analysis of isothiocyanates and glucosinolates hydrolytic products was carried out by Shimadzu (QP2010) gas chromatograph mass spectrometer (Tokyo, Japan), with AOC-20i auto-sampler coupled, and a DB-5MS capillary column, (30 m x 0.25 mm i.d., 0.25µm). The initial temperature of column was 70°C held for 4 min and was programmed to 230°C at 4°C/min, then held for 15 min at 230°C; the sample injection volume was 2µl in GC grade DCM. Helium was used as carrier gas at a flow rate of 1.1ml/min on split mode (1:50).

Head space analysis

The Fresh broccoli Plant material of three days old sprouts were collected and sealed in Head space vial and GC-MS analysis was performed.

DPPH assay

The hydrogen donating capacity of the extracts were determined by the DPPH free radical scavenging assay with slight modifications (Blois MS, 1958). It involves the reduction of a purple solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to a stable molecule diphenylpicrylhydrazine (yellow coloured solution) either by hydrogen or electron abstraction mechanism. The DPPH solution with methanol was used as control. The extracts were screened for their antioxidant activity against DPPH free radical. Different concentrations of extract (12.5, 25, 50, 75 and 100µg/ml) were made in methanol. The radical scavenging activity was expressed as the inhibition percentage and monitored as per the equation:

$$\% \text{ DPPH radical scavenging} = (1-A_S/A_C) \times 100$$

Where A_C = absorbance of Control, A_S = absorbance of Sample solution.

Determination of DNA protecting activity

The hydroxyl radical scavenging activities of the extracts were estimated using plasmid nicking assay (Lee, et al., 2002). Briefly, the supercoiled pBR322, plasmid DNA, (0.5 µg) was added to freshly prepared Fenton's reagents (H_2O_2, $FeCl_3$, ascorbic acid), and plant extracts (100µg/ml). The final volume of the mixture was brought up to 20µl, with ddH_2O. The mixture was then incubated for 30 min at 37°C, followed by addition of 2.5 µl of loading buffer (0.25% bromophenol blue, 50% glycerol) and was mixed. Catechin (100 µg/ml), a hydroxyl radical's scavenger, was used as positive control. In agarose gel electrophoresis, using 1% agarose gel, in TBE buffer, at 50V (1.5-2 V/cm) for 4 hours DNA strand breaks in supercoi-led DNA were analyzed using Gel Doc XR system (Bio-Rad, USA).

Cell lines and culture

The human Cancer Cell lines used in the present investigation were ovary (OVCAR-5), breast (MCF-7), colon (Colo-205) and prostate (PC-3) which were grown and maintained in RPMI-1640 medium. The media was supplemented with FCS (10%), penicillin (100 units

/ml), streptomycin (100 µg/ml), glutamine (2mM) and cells were grown in CO_2 incubator at 37°C with 90% humidity and 5% CO_2. Cells were treated with extracts dissolved in DMSO while the untreated control cultures received only DMSO.

SRB assay

The SRB assay was done to assess the cytotoxic effect of the extracts (Skehan, et al., 1990). The 96 well microtiter plate seeded with cells was taken out from the incubator after 48 hours of adding test samples. Then, the reaction was stopped by adding 50µl of chilled 50% TCA (trichloroacetic acid) to each well of the plate, making final concentration of 10%. The plates were incubated at 4°C for one hour to fix the cells attached to bottom of the wells. The cells were washed 5 times with distilled water and plate was then air-dried. 100µl of 0.4% (w/v) sulforhodamine B (SRB) in 1% acetic acid (v/v) solution was added to each well and left at room temperature for 30 min. SRB was removed and the plates were washed 5 times with 1% acetic acid (v/v) before air drying. Then added 100µl of 10.5M Tris buffer to each well and plates were left on a plate shaker for at least 10-15 min. The optical density was recorded with ELISA reader at 540nm wavelength

Statistical analysis

DPPH assay was performed in triplicate and the data presented as mean ± standard error (SE) To compare the difference in means, two way analysis of variance (ANOVA) was performed with Tukey's post hoc test both within the treatments and within the cultivars at $p \leq 0.05$ (95% level of significance) using SigmaStat for Windows® Version 3.5 and Microsoft excel 2007.

Results and Discussion

The individual component (Table 1) was identified by comparison of their mass spectra (MS) with NIST database and Adams libraries (NIST/EPA/NIH, 1998 and Adams RP, 2004).

The extracts were found to have fairly good antioxidant activity. The percentage DPPH radical scavenger activity was found to be concentration dependent. It was increasing significantly (Tukey's post hoc test, $p \leq 0.05$) with the increase of concentration from 12.5 to 100µg/ml (Figure 1). The maximum radical scavenging activity was found at 100µg/ml in both PS (13.92 ± 0.092) and PV (9.33 ± 0.053). The extract of variety PS showed a significantly higher radical scavenging activity at concentration 25, 50, 75 and 100µg/ml than PV cultivar. However, at a lower concentration of 12.5µg/ml, there was no significant difference in DPPH radical scavenging activity of the extracts of both the cultivars.

The results of plasmid nicking assay are shown in Figure 2. It was observed that the extracts were effective hydroxyl radical scavengers, as evident from the results (Figure 2 Lane 4-5). It was seen that although the extract of PS as well as PV were effective in scavenging the hydroxyl radicals generated by Fenton's reagent but the extract of PS seems to be comparatively more effective (Figure 6 Lane 4).

Table 1. GC-MS analysis of isothiocyanates and glucosinolates hydrolytic products.

Isothiocyanates and Glucosinolates hydrolytic product of *Palam vichitra* by GC-MS analysis				
Peak	Retention Time	Area %	Isothiocyanates	Mass fragments
2	4.521	0.14%	Allylisothiocyanates	99 [M^+], 72, 59, 41
5	6.704	0.94%	3-Butenylisothiocyanates	113 [M^+], 85, 72, 60, 55, 53
18	13.043	0.78%	3-Methylthiopropyl isothiocyanates	147 [M^+], 101, 72, 61, 57
22	16.225	0.13%	Sulforaphane	177 [M^+], 160, 114, 85, 72, 64, 55
29	17.522	4.91%	Iberin	163 [M^+], 116, 100, 86, 72, 63, 56
2	4.521	0.14%	Allylisothiocyanates	99 [M^+], 72, 59, 41
5	6.704	0.94%	3-Butenylisothiocyanates	113 [M^+], 85, 72, 60, 55, 53
Isothiocyanates and Glucosinolates hydrolytic product of *Palam smridhi* by GC-MS analysis				
Peak	Retention Time	Area %	Isothiocyanates	Mass fragments
2	4.517	0.14%	Allylisothiocyanates	99 [M^+], 72, 59, 41
5	6.707	0.94%	3-Butenylisothiocyanates	113 [M^+], 85, 72, 60, 55, 53
34	13.043	0.13%	3-Methylthiopropyl isothiocyanates	147 [M^+], 101, 72, 61, 57
67	16.225	4.29%	Sulforaphane	177 [M^+], 160, 114, 85, 72, 64, 55
Isothiocyanates and Glucosinolates hydrolytic product of *Palam vichitra* by Headspace analysis				
Peak	Retention Time	Area %	Isothiocyanates	Mass fragments
2	4.473	0.55%	Allylisothiocyanates	99 [M^+], 72, 59, 41
3	7.198	1.14%	3-Butenylisothiocyanates	113 [M^+], 85, 72, 60, 55, 53
Isothiocyanates and glucosinolates hydrolytic product of *Palam smridhi* by headspace analysis				
Peak	Retention Time	Area %	Isothiocyanates	Mass fragments
2	7.215	1.21%	3-Butenylisothiocyanates	113 [M^+], 85, 72, 60, 55, 53

It was observed that IC_{50} values increased with increase in extract concentration both in PS and PV (Table 2). The minimum IC_{50} value was found in PV at 87.25µg/ml in cell line Colo-205. The PV extract also showed IC_{50} of 131.4µg/ml, 2090µg/ml and 253µg/ml in OVCAR-5, MCF-7 and PC-3 cell lines respectively. The minimum IC_{50} value was found in PS i.e. 221.5µg/ml in cell line Colo-205. The PS extract also showed IC_{50} of 331.9µg/ml, 1657.2µg/ml and 563.5µg/ml in OVCAR-5, MCF-7 and PC-3 cell lines respectively. The positive control adriamycin (1µM) showed % growth inhibition i.e.OVCAR-5(60%), Colo-205(59%), MCF-7(72%) and PC-3(66%).

Table 2. *In vitro* cytotoxicity against different human cancer cell lines by SRB assay.

Broccoli Cultivar	Cell Line			
	OVCAR-5	MCF-7	Colo-205	PC-3
	IC_{50} (µg/ml)			
PS	331.9	1657.2	221.5	563.5
PV	131.4	2090	87.25	253
Standard	% Growth Inhibition			
Adriamycin (1µM)	60	72	59	66

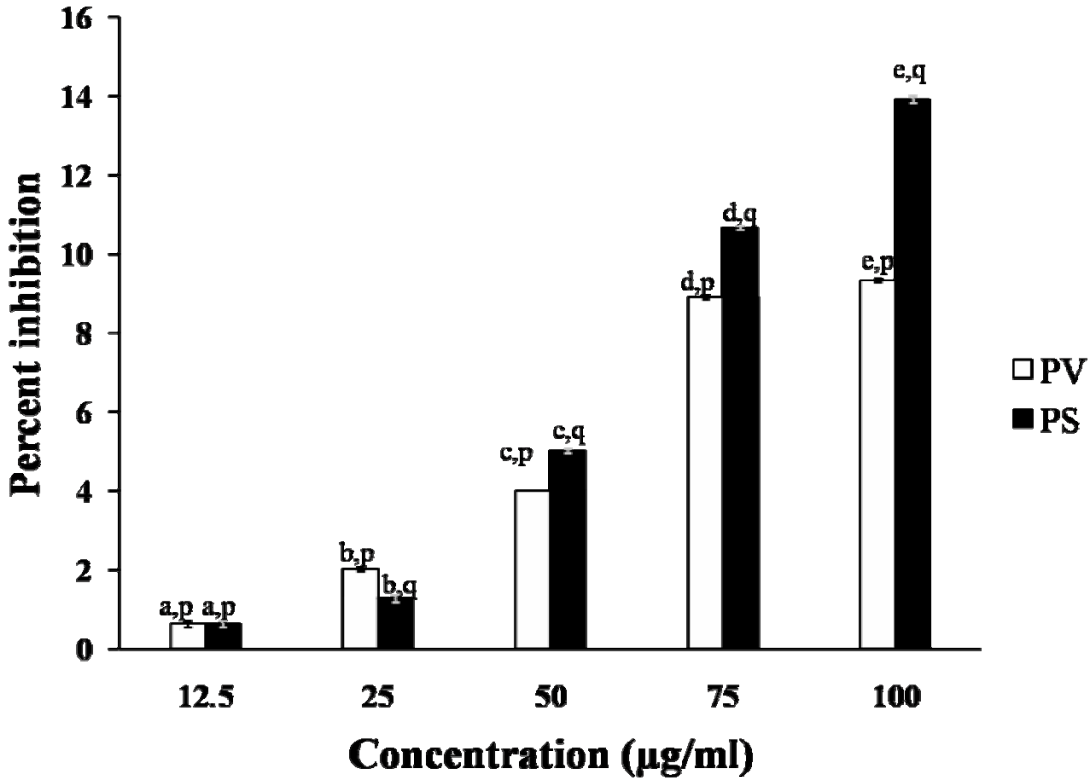

Figure 1. Radical scavenging activities of extracts prepared from broccoli sprouts. Results were expressed as the percent of control. Values are the mean ± SE (n = 3). Different letters (a, b, c, d and e) within different concentrations of the extract are significantly different (Tukey's post hoc test, $p \leq 0.05$) whereas different letters (p and q) within cultivars at a particular concentration are significantly different (Tukey's post hoc test, $p < 0.05$) and signify interactions of different concentrations of cultivar.

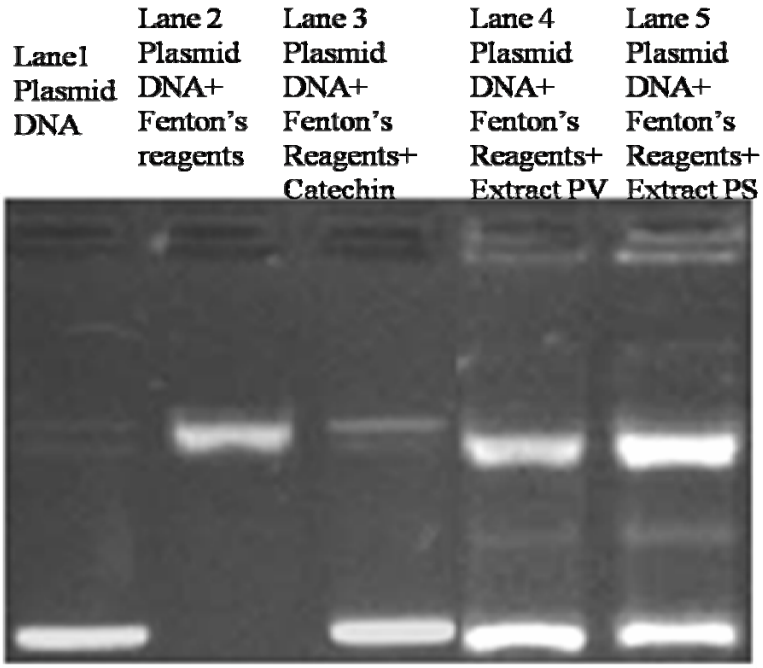

Figure 2. Effects of extracts on radical induced DNA degradation. Plasmid DNA was incubated with different extract concentration in a Fenton's reaction mixture at 37ºC for 30 min. The DNA degradation was analyzed using an agarose-gel electrophoresis as described in the text.

Glucosinolates and their hydrolytic products are known to protect against cancer (Fahey et al., 1997). For example, sulforaphane, the hydrolysis product of the glucosinolate glucoraphanin, is highly potent at upregulating detoxification enzymes and is reported to act against various types of cancers (Vig, et al., 2009). The results of the studies in literature rev-ealed that broccoli have some potent antigenotoxic compounds (Anupama, et al 2008 and Ra-mpal, et al., 2010). In the present study, we found that seeds of PS contained high amount of sulforaphane (4.29%). However, other isothiocyanates were also detected in this variety. Iberin was found to be the major hydrolytic product in PV. The extract of this variety was found to be effective against colon cancer cell line (Colo-205), which can be attributed to the presence of iberin. The results of this study can be correlated with the previous reports (Jakubikova, et al., 2005), according to which the purified iberin was found to inhibit the growth of human colon carcinoma Caco-2 cells by inducing apoptos-is. Iberin is known to increase glutathione S-transferase and quinone reductase activities in the urinary bladder of the rats demonstrating protective effects against chemical carcinogene-sis (Staack, et al., 1998). It is also reported to upregulate the thioredoxin reductase1 expressi-on in human MCF cells suggesting a role in maintenance of redox in cell homeostasis (Wang, et al., 2005). The treatment of neuroblastoma cells with iberin resulted in a dose- and time-dependent inhibition of growth, increased cytotoxicity, and G_1 or G_2 cell cycle arrest depending upon dose and cell type (Jadhav, et al 2007). The results indicated the presence of different glucosinolate hydrolytic products in the two cultivars of broccoli. The extracts of broccoli showed radical scavenging potential and selective *in vitro* cytotoxic effect against human colon cancer cell line (COLO-205). Though, various glucosinolate hydrolytic products were seen, yet their concentration was low. The efforts are being done to develop more effective methods, for their isolation and enrichment. It is pertinent to mention that, the other natural products may be exerting significant role in scavenging activities. Therefore further studies are in progress to identify the active principle.

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References

Adams RP. (2004). Identification of Essential Oil Component by Gas chromatography/ Quadrupole Mass spectroscopy. *Allured Publishing Corporation, Illinois, U.S.A.*

Anupama M, Murgan SS, Murthy PB. (2008). Broccoli flower head extract reduces mitomycin-C induced sister chromatid exchange in cultured human lymphocytes. *Food and Chemical Toxicology* 46, 3351-3353.

Blois MS. (1958). Antioxidant determination by the use of a stable free radical. *Nature* 18, 1119–1120.

Chen S, Andreasson E. (2001). Update on glucosinolate metabolism and transport. *Plant Physiology and Biochemistry* 39, 743–758.

Jadhav U, Ezhilarasan R, Vaughn SF, Berhow MA, Mohanam S. (2007). Iberin induces cell cycle arrest and apoptosis in human neuroblastoma cells. *International Journal of Molecular Medicine*19, 353-361.

Jakubikova J, Bao Y, Sedlak J. (2005). Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines. *Anticancer Research* 25, 3375–3386.

Kim YS, MilnerJA. (2005). Targets for indole-3-carbinol in cancer prevention. *The Journal of Nutritional Biochemistry* 16, 65-73.

Lee JC, Kim HR, Kim J, Jang YS. (2002). Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica* var. saboten. *Journal of Agricultural and Food Chemistry* 50, 6490-6396.

Liang H, Yuan QP, Dong HR, Liu YM. (2006). Determination of sulforaphane in broccoli and cabbage by high-performance liquid Chromatography. *Journal of Food Composition and Analysis* 19, 473–476.

Michaud DS, Spiegelman D, Clinton SK, Rimm EB, Willett WC, Giovannucci EL. (1999). Fruit and vegetable intake and incidence of bladder cancer in a male prospective cohort. *Journal of the National Cancer Institute* 91, 605-613.

NIST/EPA/NIH. (1998). Mass spectral Library database.

Rampal G, Thind TS, Vig AP, Arora S. (2010). Antimutagenic potential of glucosinolate-rich seed extracts of broccoli (*Brassica oleracea* L *var* italic Plenck). *International Journal of Toxicology* 29, 616-624.

Rose P, Huang Q, Ong CN, Whiteman M. (2005). Broccoli and watercress suppress matrix metalloproteinase-9 activity and invasiveness of human MDA-MB-231 breast cancer cells. *Toxicology and Applied Pharmacology* 209, 105-113.

Singh AV, Xiao D, Lew KL, Dhir R, Singh SV. (2004). Sulforaphane inducescaspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. *Carcinogenesis* 25, 83-90.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of the National Cancer Institute* 82, 1107-1112.

Staack R, Kingston S, Wallig MA, Jeffery EH. (1998). A comparison of the individual and collective effects of four glucosinolate breakdown products from Brussels sprouts on induction of detoxification enzymes. *Toxicology and Applied Pharmacology* 149, 17-23.

Steinmetz KA, Potter JD. (1996). Vegetables, fruit, and cancer prevention. a review. *Journal of the American Dietetic Association* 96, 1027-1039.

Talalay P, Fahey JW. (2001). Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *Journal of Nutrition* Suppl.131, 3027-3033.

Vig AP, Rampal G, Thind TS, Arora S. (2009). Bio-protective effects of glucosinolates – A review. *LWT-Food Science and Technology* 42, 1561-1572.

Wang W, Wang S, Howie AF, Beckett GJ, Mithen R, Bao Y. (2005). Sulforaphane, erucin, and iberin up-regulate thioredoxin reductase 1 expression in human MCF-7 cells. *Journal of Agricultural and Food Chemistry* 53, 1417-1421.

Zhang Y, Talalay P, Cho CG, Posner GH. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli isolation and elucidation of structure. *Proceedings of the National Academy of Sciences of the United States of America* 89, 2399-2403.

